

Investigating ligand- and cell-specific signal transduction at relaxin family peptide receptor 1

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Abstract

Relaxin, a peptide hormone and the endogenous agonist of the relaxin family peptide receptor 1 (RXFP1), has shown substantial promise for the therapeutic treatment of cardiovascular disorders and fibrosis. RXFP1 has received considerable therapeutic interest as a drug target over the years, and a lot of effort has been put into the development of novel ligands targeting this receptor. However, we do not understand how novel RXFP1 agonists work because we do not understand RXFP1 signalling in enough detail, and do not fully understand which pathways are important for the therapeutic actions, where they are activated in the signal transduction cascade, and in what cell types. Furthermore, development of novel ligands raises the question of biased signalling, which is the ability of different ligands for the same receptor to preferentially activate certain signal transduction pathways relative to one another. It may be possible to utilise bias to create effective drugs that have fewer side effects, but this requires us to first understand which effectors and signal transduction pathways are important for the therapeutic versus harmful actions. Furthermore, recent research has highlighted the importance of measuring the temporal aspects of signalling in order to understand bias, as signalling and bias can change over time, and using end point assays can produce a misleading picture of efficacy and bias depending on which time point was chosen. Additionally, validating findings from recombinant cells in physiologically-relevant cells is also important to understand how ligands signal across cell types, and to distinguish biased signalling from cell-specific signalling. The development of sensitive, real-time assays that can be used across cell types will aid our understanding of the molecular mechanisms underlying the actions of relaxin and other ligands targeting this promising receptor. The general aim of this thesis was to apply and develop bioluminescence resonance energy transfer (BRET)-based methods in conjunction with human native and primary cells in order to examine real-time signalling and bias at RXFP1.

First, the functionality and versatility of the CAMYEL (cAMP sensor using YFP-Epac-Rluc) real-time BRET-based cAMP biosensor was demonstrated for RXFP1 and the related GPCRs RXFP2, RXFP3, and RXFP4. CAMYEL was a sensitive alternative to end point assays, as it detected concentration-dependent changes in cAMP activity at all receptors in recombinant cell lines, was dynamic and reversible, detected kinetic differences between different ligands for the same receptor, and showed potencies comparable to those seen in end point assays. CAMYEL was cloned

into a lentiviral vector, and lentivirus was used to transduce THP-1 cells, which endogenously express low levels of RXFP1. THP-1 CAMYEL cells showed robust cAMP activation after relaxin stimulation and will therefore streamline the process of screening novel RXFP1 ligands. The lentiviral vector will also allow for the transduction of many mammalian cell types for real-time analysis of cAMP activity at various GPCRs, including in primary cells. However, it appeared that the CAMYEL assay was unable to detect a delayed, $G\alpha_{i3}$ -mediated phase of RXFP1 cAMP activity that has been demonstrated using other assays, suggesting that CAMYEL might not detect cAMP generated in specific compartments of the cell.

Second, we developed, validated, and characterised a BRET-based biosensor for cGMP activity, known as CYGYEL (cyclic GMP sensor using YFP-PDE5-Rluc8), based on the Förster/fluorescence resonance energy transfer (FRET) biosensor cGES-DE5. CYGYEL was cloned into a lentiviral vector, enabling its use across different mammalian cell types. CYGYEL was initially characterised in HEK293T cells, where it was shown to be sensitive, dynamic, reversible, and also very selective for the detection of cGMP over cAMP. CYGYEL was then used to detect cGMP after transduction of human primary vascular cells, namely endothelial and smooth muscle cells. CYGYEL detected differences in cGMP signalling kinetics both between cell types, and also between ligands that increased cGMP production via soluble versus membrane guanylate cyclases. So far we have been unsuccessful at detecting GPCR-mediated cGMP using CYGYEL, but further work is required in this area. Regardless, CYGYEL still has many uses for drug discovery.

Finally, we used a variety of BRET-based assays for G protein association, second messenger activity, and ERK1/2 activity, as well as physiologically-relevant primary cells, in order to understand the mechanisms of action underlying the beneficial actions of the relaxin peptide analogue B7-33. According to previous work, B7-33 appeared to show cell-specific signalling and biological responses, whereby it had weak activity in recombinant and cancer cells, but potent activity in fibroblasts and vascular cells, as well as *in vivo*. Our results across several cell types indicated that B7-33 is a biased agonist that favours signalling via $G\alpha_{i3}$ /cGMP over $G\alpha_s$ /cAMP, relative to relaxin which signals potently via both pathways. These findings are consistent with B7-33's actions as a potent vasodilator and anti-fibrotic, which depend on cGMP rather than cAMP. Relatedly, we demonstrated that B7-33 shows transient cAMP activity relative to relaxin in all cell types tested, and that in a real-time cAMP assay involving ligand washout, the cAMP response from B7-33 dropped drastically relative to relaxin, suggesting that B7-33 dissociated from RXFP1 far more readily than relaxin. We thus hypothesised that the bias shown by B7-33 is related to kinetics, whereby the relaxin/RXFP1 complex catalyses more cycles of $G\alpha_s$ activation due to the sustained duration of the active receptor conformation, relative to B7-33 which has a faster off-rate

associated with its weaker activation of $G\alpha_s$. However, both agonists equally activate $G\alpha_{i3}$ suggesting that the relative rates of activation and deactivation of the different G proteins may also be important. Finally, it was also observed that ERK1/2 is activated by its upstream effectors in a cell type-specific manner. Specifically, whereas previous findings have shown that ERK1/2 is primarily downstream of $G\alpha_{i/o}$ in native cells, our findings show that ERK1/2 is activated downstream of $G\alpha_s$ in HEK-RXFP1 cells, which explains B7-33's weak ERK1/2 activation in HEK-RXFP1 cells but potent activation in native cells. These findings have implications for the development of novel biased drugs targeting RXFP1, as it is believed that the negative actions of exogenously-administered relaxin, including for example its ability to promote tumour growth in mouse models *in vivo*, are related to its potent cAMP activity. Conversely, equi-molar doses of B7-33 do not promote tumour growth but do retain the beneficial actions of relaxin which occur via $G\alpha_i$. Thus, we could potentially aim to retain the kinetic bias to maintain potent cGMP signalling, while minimising cAMP activity, and at the same time aim to develop compounds that are more drug-like with longer half-lives.

Declaration

This is to certify that

- i. This thesis comprises only my original towards the degree of Doctor of Philosophy, except where indicated in the Preface
- ii. Due acknowledgement has been made in the text to all other material used
- iii. The thesis is less than 100,000 words in length, exclusive of tables, bibliographies, and appendices

Adam Valkovic

Preface

- i. Chapter 3 was accepted for publication by Pharmacology Research & Perspectives on 29 August, 2018. Work conducted by or in collaboration with others includes the following:
 - a. Molecular cloning of the CAMYEL biosensor was conducted in collaboration with Sharon Layfield and Alice Whitehead
 - b. The THP-1 CAMYEL cell line was generated by Sharon Layfield
 - c. The TAMRA-relaxin ligand was generated in the laboratory of A/Prof. Akhter Hossain
 - d. Signalling data in Figure 3D-G and Figure 4 was generated by Miranda Leckey
- ii. Some work in Chapter 5 was conducted by or in collaboration with others:
 - a. The cyclic nucleotide accumulation assays in Figure 9 were conducted by Dr. Martina Kocan
 - b. The G protein association data in Figure 10 was conducted in collaboration with Jenny Lu and Dr. Martina Kocan
- iii. Appendix A was accepted for publication by Molecular and Cellular Endocrinology on 22 December, 2018

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Abbreviations

5-HT	5-hydroxytryptamine
AC	adenylate cyclase
AHF	acute heart failure
AKAP	A-kinase anchoring protein
ANOVA	analysis of variance
AR	adrenoceptor
AT	angiotensin II
ATCC	American Type Culture Collection
AVP	arginine vasopressin
β ARK	β -adrenergic receptor kinase
BLAST-AHF	biased ligand of the angiotensin receptor study in acute heart failure
BODIPY	4,4-difluoro-4-bora-3a,4a-diaza-s-indacene
BRET	bioluminescence resonance energy transfer
BSA	bovine serum albumin
CaM	calmodulin
cAMP	cyclic adenosine monophosphate
CAMYEL	<u>c</u> AMP sensor using <u>Y</u> FP- <u>E</u> pac- <u>R</u> luc
CaS	calcium-sensing
cDNA	complementary deoxyribonucleic acid
CFP	cyan fluorescent protein
cGES-DE5	cGMP energy transfer sensor derived from PDE5A
cGMP	cyclic guanosine monophosphate
CHO	Chinese hamster ovary
CMV	cytomegalovirus
CNP	C-type natriuretic peptide
CO	cardiac output
cPPT	central polypurine tract

CRE	cAMP response element
CREB	cAMP response element binding protein
CRF	corticotropin-releasing factor
CRISPR	clustered regularly interspaced short palindromic repeats
CT	calcitonin
cygnet	<u>c</u> yclic <u>G</u> MP <u>i</u> ndicator using <u>e</u> nergy <u>t</u> ransfer
CYGYEL	<u>c</u> yclic <u>G</u> MP sensor using <u>Y</u> FP-PDE <u>5</u> -R <u>l</u> uc8
DAG	diacylglycerol
DAPI	4',6-diamidino-2-phenylindole
DEA	diethylamine NONOate diethylammonium
DMEM	Dulbecco's modified Eagle medium
DNA	deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
dNTP	deoxynucleotide triphosphate
DPBS	Dulbecco's phosphate buffered saline
EBM-2	endothelial cell growth basal medium-2
eBRET	extended BRET
EC ₅₀	half maximal effective concentration
ECFP	enhanced cyan fluorescent protein
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
Ef1 α	elongation factor 1-alpha
EGM-2	endothelial cell growth medium-2
EKAR	extracellular signal-regulated kinase activity reporter
eNOS	endothelial nitric oxide synthase
Epac	exchange protein activated by cAMP
ERK	extracellular signal-regulated kinase
ESC	endometrial stromal cell
EST	expressed sequence tag
EYFP	enhanced yellow fluorescent protein
FACS	fluorescence-activated cell sorting

FBS	fetal bovine serum
FRET	Förster/fluorescence resonance energy transfer
FSC-A	forward scatter area
FSC-H	forward scatter height
FSC-W	forward scatter width
FSH	follicle-stimulating hormone
FZD	frizzled
GABA	gamma-Aminobutyric acid
GC	guanylate cyclase
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
GFR	glomerular filtration rate
GHRH	growth hormone-releasing hormone
GIP	gastric inhibitory polypeptide
GLP	glucagon-like peptide
GPCR	G protein-coupled receptor
GREAT	G protein-coupled receptor affecting testis descent
GRK	GPCR kinase
GTP	guanosine triphosphate
HCF	human cardiac fibroblast
HEK	human embryonic kidney
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	human immunodeficiency virus
HSC	hepatic stellate cells
HTRF	homogenous time-resolved fluorescence
HUASMC	human umbilical artery smooth muscle cell
HUVEC	human umbilical vein endothelial cell
HUVSMC	human umbilical vein smooth muscle cell
IBMX	3-isobutyl-1-methylxanthine
ICL	intracellular loop

IGF	insulin-like growth factor
iNOS	inducible nitric oxide synthase
INSL	insulin-like peptide
IP ₃	inositol 1,4,5-trisphosphate
IPF	idiopathic pulmonary fibrosis
IRES	internal ribosome entry site
JNK	c-Jun N-terminal kinase
LB	lysogeny broth
LB2	second ligand binding domain of the LDL receptor
LDLa	low-density lipoprotein class A
LGR	leucine-rich repeat-containing GPCR
LH	luteinising hormone
LRR	leucine-rich repeat
LSD	lysergic acid diethylamide
MAPK	mitogen-activated protein kinase
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
mTORC2	mammalian target of rapamycin complex 2
NF-κB	nuclear factor-κB
NHE	normal human endometrial
Nluc	NanoLuc
NMR	nuclear magnetic resonance
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NOS	nitric oxide synthase
ODQ	1H-1,2,4Oxadiazolo4,3-aquinoxalin-1-one
PCR	polymerase chain reaction
PDE	phosphodiesterase
PKD1	phosphoinositide-dependent protein kinase 1
pGC	particulate guanylate cyclase
PH	pleckstrin homology

PI3K	phosphoinositide 3-kinase
PIP ₂	phosphatidylinositol 4,5-bisphosphate
PIP ₃	phosphatidylinositol (3,4,5)-trisphosphate
PKA	protein kinase A
PKC	protein kinase C
PKG	protein kinase G
PLC	phospholipase C
Pre-RELAX-AHF	preliminary study of relaxin in acute heart failure
PTH	parathyroid hormone
PTX	pertussis toxin
RAMP	receptor activity-modifying protein
RELAX-AHF	relaxin in acute heart failure
RET	resonance energy transfer
REV	Rluc8-ERKsubstrate-Venus
Rluc	<i>Renilla</i> luciferase
RNA	ribonucleic acid
RPF	renal plasma flow
RPM	rotations per minute
RPMI 1640	Roswell Park Memorial Institute 1640 medium
RRE	Rev response element
RXFP1	relaxin family peptide receptor 1
RXFP2	relaxin family peptide receptor 2
RXFP3	relaxin family peptide receptor 3
RXFP4	relaxin family peptide receptor 4
S1P	sphingosine-1-phosphate
SEM	standard error of the mean
sGC	soluble guanylate cyclase
siRNA	small interfering RNA
SMO	smoothened
SSC-A	side scatter area
SSC-H	side scatter height

SSC-W	side scatter width
SVR	systemic vascular resistance
TAMRA	tetramethylrhodamine
TamRLX	TAMRA-relaxin
TGF- β	transforming growth factor beta
TIMP	tissue inhibitor of metalloproteinase
TMD	transmembrane domain
TNF- α	tumour necrosis factor alpha
TR-FRET	time-resolved FRET
TSH	thyroid-stimulating hormone
VEGF	vascular endothelial growth factor
VSV-G	vesicular stomatitis virus glycoprotein
WPRE	woodchuck post-transcriptional regulatory element
YFP	yellow fluorescent protein
Zeo	zeocin

1 Introduction

1.1 G protein-coupled receptors

Cells require the ability to transduce signals from the extracellular environment into the cytosol. One way of accomplishing this is via membrane-spanning cell surface proteins known as G protein-coupled receptors (GPCRs). After binding of an extracellular ligand, GPCRs transduce a signal across the membrane by coupling to intracellular effectors that activate networks of signalling pathways. Ligands for GPCRs are extremely diverse and can include ions, amino acids, peptides, proteins, hormones, neurotransmitters, nucleotides, lipids, odorants, tastants, and photons, for example (Wettschureck & Offermanns, 2005). GPCRs for which the endogenous ligand is unknown are called “orphan receptors”. In humans, there are over 800 genes encoding GPCRs, which is about 2% of the genome (Fredriksson et al., 2003) and over 50% of the “receptorome” (Strachan et al., 2006). GPCRs are expressed in the majority of human tissues and are involved in the regulation of most physiological processes. Dysfunction of GPCR signalling can contribute to psychiatric disorders (Catapano & Manji, 2007), metabolic diseases (Blad et al., 2012), endocrine diseases (Vassart & Costagliola, 2011), and cancer (Dorsam & Gutkind, 2007), for example.

1.1.1 Properties of GPCRs

All GPCRs contain an extracellular N-terminus, seven transmembrane α -helices, and an intracellular C-terminus. The transmembrane domains are connected by three intracellular loops (ICLs) and three extracellular loops. Some GPCRs have multiple splice variants, which can change the pharmacology of the receptor (Markovic & Challiss, 2009). GPCRs also have sites for post-translational modifications that affect their function and regulation (Qiu & Law, 2011). For example, extracellular domains contain sites for glycosylation, which is important for receptor trafficking, and the ICLs and C-terminal tails contain sites for phosphorylation, which is important for desensitisation and internalisation of the receptor. GPCRs can also form complexes, which can alter the pharmacology, signalling, and trafficking of the receptor (Gomes et al., 2016).

1.1.2 Classification of GPCRs

In 1994, a GPCR classification system was introduced that divided GPCRs into six classes (A, B, C, D, E, and F), which was intended to cover all GPCRs in vertebrates and invertebrates (Kolakowski, 1994). In the early 2000s, sequencing of the human genome allowed a comprehensive analysis of GPCR phylogeny, which revealed five main families of human GPCRs: glutamate, rhodopsin, adhesion, frizzled/taste2, and secretin (Fredriksson et al., 2003). In this phylogenetic system, class B GPCRs are divided into two families (adhesion and secretin), and the taste2 receptors, which had recently been discovered, are also included.

1.1.2.1 Rhodopsin (class A)

Rhodopsin, which is located in the retina and is activated by light, was the first GPCR to have a high-resolution crystal structure published (Palczewski et al., 2000). The rhodopsin receptor family is by far the largest GPCR family, containing over 700 members, of which more than 400 are olfactory receptors (Fredriksson et al., 2003). The rhodopsin class is divided into α , β , γ , and δ subgroups, which have 13 branches (Fredriksson et al., 2003). Although rhodopsin-like receptors tend to contain short N termini and bind their ligand within the transmembrane domain (TMD), there are many exceptions to this rule, such as the leucine-rich repeat (LRR)-containing GPCRs which bind their endogenous ligands in the extracellular domain (Fredriksson et al., 2003). The rhodopsin class is targeted by many notable therapeutic drugs, such as anti-cholinergics (muscarinic acetylcholine receptor antagonists), anti-histamines (histamine receptor antagonists), anti-psychotics (dopamine D_2 receptor antagonists), anti-asthmatics (β_2 -adrenergic receptor agonists), opioid analgesics (opioid receptor agonists), and anti-hypertensives (angiotensin II type 1 receptor (AT_1 receptor) and α_1 -adrenergic receptor antagonists) (Sriram & Insel, 2018).

1.1.2.2 Secretin (class B)

The secretin receptor family consists of 15 receptors that have endogenous peptide ligands, and is so named because the secretin receptor was the first receptor in this family to be cloned (Fredriksson et al., 2003). The secretin-like receptors include the calcitonin receptor (CT receptor), calcitonin receptor-like receptor, corticotropin-releasing factor receptors (CRF_1 and CRF_2 receptors), growth-hormone-releasing hormone receptor (GHRH receptor), gastric inhibitory polypeptide receptor (GIP receptor), glucagon receptor, glucagon-like peptide receptors (GLP-1 and GLP-2 receptors), secretin receptor, parathyroid hormone receptors (PTH1 and PTH2

receptors), pituitary adenylate cyclase-activated polypeptide type 1 receptor (PAC₁ receptor), and the vasointestinal peptide receptors (VPAC₁ and VPAC₂ receptors). These receptors have large N-termini important in binding their endogenous ligands (Fredriksson et al., 2003; Hoare, 2005). Some secretin-like receptors also interact with receptor activity-modifying proteins (RAMPs), which are membrane-spanning proteins that can change the pharmacology, signalling, and trafficking of the receptor (Hay & Pioszak, 2016). Some receptors in this class are targeted by approved therapeutic drugs. For example, salmon calcitonin is used as a treatment for osteoporosis, and various GLP-1 receptor agonists are used to treat type 2 diabetes (Bortolato et al., 2014).

1.1.2.3 Adhesion (class B)

Adhesion receptors possess long, diverse N-termini that contain varying numbers of protein domains such as cadherin, immunoglobulin, LRR, and lectin domains (Basilana et al., 2019; Hayflick, 2000; Yona et al., 2008). These domains often have roles in cell adhesion, reflecting the naming of this group of receptors (Fredriksson et al., 2003; Hamann et al., 2015), but adhesion receptors also have numerous other biological roles such as cytoskeletal organisation, regulation of the cell cycle, cell migration, neural development, and immune function (Basilana et al., 2019; Hamann et al., 2015; Yona et al., 2008). Ligands for adhesion receptors mainly include cellular and extracellular matrix molecules (Yona et al., 2008), but the majority of adhesion receptors remain orphanised (Hamann et al., 2015). Adhesion receptors are not currently targeted by therapeutic drugs, but are thought to have potential as therapeutic targets (Bondarev *et al.*, 2020).

1.1.2.4 Glutamate (class C)

The glutamate receptor class consists of 22 GPCRs in humans, including eight glutamate receptors (mGlu₁₋₈ receptors), two GABA receptors (GABA_{B1} and GABA_{B2} receptors), the calcium-sensing receptor (CaS receptor), sweet and umami taste receptors (TAS1R1-3), the GPRC6A receptor, and seven orphan receptors (Fredriksson et al., 2003). These receptors typically contain a large extracellular domain called the Venus fly trap domain, which is the site of ligand binding (Kniazeff et al., 2011). They also typically form constitutive dimers, which is important for their activation (Kniazeff *et al.*, 2011). The GABA_B receptor, which is a dimer of GABA_{B1} and GABA_{B2}, is targeted by baclofen for treatment of muscle spasticity, and the CaS receptor is targeted by etelcalcetide for treatment of patients undergoing haemodialysis who have secondary hyperparathyroidism (Sriram & Insel, 2018). Artificial sweeteners are agonists or allosteric

modulators of the sweet receptor, which is a TAS1R2/TAS1R3 heterodimer (Zhang *et al.*, 2010), and enhancers of umami taste are positive allosteric modulators of the umami receptor, which is a TAS1R1/TAS1R3 heterodimer (Zhang *et al.*, 2008). The mGlu receptors are considered to have therapeutic potential for treatment of psychiatric and neurological disorders (Niswender & Conn, 2010).

1.1.2.5 Frizzled/taste2 (class F)

The frizzled group of this class consists of 10 frizzled receptors (FZD₁₋₁₀) and the smoothened receptor (SMO) (Fredriksson *et al.*, 2003). The extracellular domain of frizzled receptors contains a conserved cysteine-rich domain for ligand (Wnt) binding (Dann *et al.*, 2001). FZDs signal via β -catenin-dependent or -independent pathways (Schulte, 2010). Wnt/FZD signalling is involved in development and cell differentiation (Logan & Nusse, 2004). FZDs are potential targets for anti-cancer drugs (Gurney *et al.*, 2012; Nambotin *et al.*, 2011).

SMO forms a complex with the transmembrane protein patched, and this complex forms the sonic hedgehog receptor (Murone *et al.*, 1999). SMO is targeted by antagonists such as sonidegib for the treatment of basal cell carcinoma (Burness, 2015). The 25 taste2 receptors are bitter taste receptors that are expressed in taste receptor cells on the tongue and palate epithelia (Adler *et al.*, 2000). Some bitter-masking molecules used by food and drug industries target these receptors (Ley, 2008).

1.2 GPCR-mediated signal transduction

1.2.1 The G protein cycle

GPCRs canonically transduce an external signal into an intracellular response by coupling to heterotrimeric G proteins, which consist of an α subunit and a $\beta\gamma$ dimer. Activation of G proteins occurs in a cycle (Oldham & Hamm, 2008) (**Figure 1.1**). In their inactive state, guanosine diphosphate (GDP)-bound $G\alpha$ and the $G\beta\gamma$ dimer are bound together and cannot interact with downstream effectors. When an agonist binds to a GPCR and stabilises the active state, the receptor can couple to the heterotrimeric G protein. The receptor acts as a guanine nucleotide exchange factor (GEF) that facilitates the exchange of GDP for guanosine triphosphate (GTP) in the $G\alpha$ subunit, leading to dissociation of the heterotrimeric G protein into the active, GTP-bound $G\alpha$

subunit and the G $\beta\gamma$ dimer, which each activate downstream effectors leading to intracellular signalling. Hydrolysis of GTP to GDP by the GTPase activity of the G α subunit, initiated after binding to the effector, terminates G α activation, after which re-association of G α with G $\beta\gamma$ occurs.

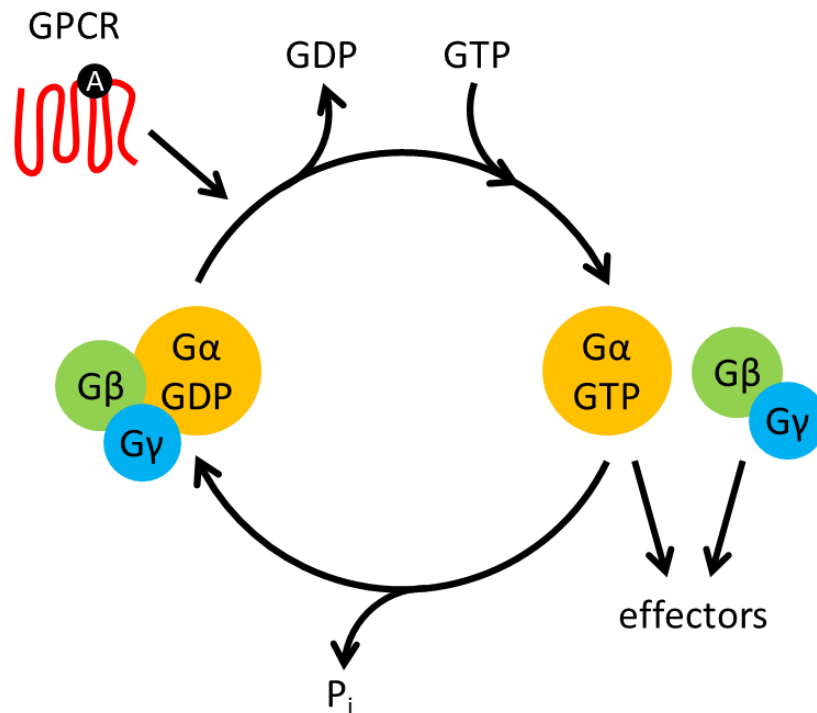


Figure 1.1. The G protein cycle. The inactive form of the G protein consists of G α -GDP bound to G $\beta\gamma$. The agonist (A)-bound, active GPCR is a guanine nucleotide exchange factor that facilitates the exchange of GDP for GTP, leading to dissociation of G α from G $\beta\gamma$. Active G proteins couple to their effectors to activate networks of intracellular signalling. Hydrolysis of GTP to GDP terminates G protein activity, leading to re-association of the heterotrimer. Adapted from Syrovatkina *et al.* (2016).

1.2.2 G protein families

In humans, there are 16 genes for G α , five genes for G β , and 12 genes for G γ (Downes & Gautam, 1999). G α subunits are divided into four main subfamilies – G α_s , G α_i , G α_q , and G α_{12} – which each activate different signal transduction cascades. Many GPCRs can also couple to multiple G proteins, thereby activating a variety of signalling pathways (Hermans, 2003).

1.2.2.1 The $G\alpha_s$ family

The $G\alpha_s$ family consists of $G\alpha_s$ and $G\alpha_{olf}$. $G\alpha_s$ has two splice variants that are widely expressed, whereas $G\alpha_{olf}$ is primarily expressed in neural tissue including olfactory sensory neurons (Belluscio et al., 1998). Active $G\alpha_s$ and $G\alpha_{olf}$ proteins bind to and activate the membrane-associated enzyme adenylate cyclase (AC), which synthesises the second messenger cyclic adenosine monophosphate (cAMP). Effectors of cAMP include protein kinase A (PKA), GEFs known as exchange proteins activated by cAMP (Epacs), and cyclic nucleotide-gated ion channels (Cheng et al., 2008; Kaupp & Seifert, 2002). PKA affects gene transcription by phosphorylating the cAMP response element binding protein (CREB) transcription factor (Mayr & Montminy, 2001). cAMP is inactivated by phosphodiesterase (PDE)-mediated hydrolysis (Francis et al., 2011).

1.2.2.2 The $G\alpha_i$ family

The $G\alpha_i$ family is the largest family of $G\alpha$ proteins, consisting of $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_o$ (which has two splice variants: $G\alpha_{oA}$ and $G\alpha_{oB}$), $G\alpha_t$, $G\alpha_g$, and $G\alpha_z$. Active $G\alpha_{i/o}$ proteins inhibit ACs, thereby inhibiting the production of cAMP. $G\alpha_z$ can also inhibit some ACs (Wong et al., 1992). $G\alpha_t$ (transducin) is expressed in the rod and cone photoreceptors of the retina, where it couples to rhodopsin and is involved in detection of light (Fung et al., 1981; Lerea et al., 1986). $G\alpha_g$ (gustducin), which is closely related to transducin, is found on taste receptor cells on the tongue and is involved in detection of some tastes (McLaughlin et al., 1992; Wong et al., 1996). Transducin and gustducin can couple to PDEs, leading to decreases in cyclic nucleotide levels (Kinnamon, 2009).

1.2.2.3 The $G\alpha_q$ family

The $G\alpha_q$ family consists of $G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14}$, and $G\alpha_{16}$. $G\alpha_q$ members activate phospholipase C (PLC)- β , a membrane-associated enzyme that cleaves the membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P₂ or PIP₂) into the second messengers diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP₃) (Litosch, 2016). DAG then activates protein kinase C (PKC), whereas IP₃ binds to IP₃ receptors on Ca²⁺ channels located on the endoplasmic reticulum or sarcoplasmic reticulum membrane, thereby leading to release of Ca²⁺.

1.2.2.4 The $G\alpha_{12}$ family

The $G\alpha_{12}$ family consists of $G\alpha_{12}$ and $G\alpha_{13}$, which activate some RhoGEFs such as p115RhoGEF (Siehler, 2009). RhoGEFs subsequently activate Rho proteins by facilitating exchange

of GDP for GTP on the Rho protein. Rho proteins then activate downstream effectors, the most well-known of which are Rho kinases (Sah et al., 2000). $G\alpha_{12/13}$ -mediated Rho activation is involved in a range of cellular processes, including cell migration, adhesion, and proliferation (Siehler, 2009).

1.2.2.5 The $G\beta\gamma$ dimer

The $G\beta\gamma$ dimer remains associated after activation, acting as a complex to activate a variety of effectors to increase intracellular signalling, such as ion channels, ACs, phosphoinositide 3-kinases (PI3Ks), and mitogen-activated protein kinases (MAPKs) such as extracellular signal-regulated kinase 1/2 (ERK1/2) and c-Jun N-terminal kinases (JNKs) (Khan et al., 2013). Knockout and knockdown studies have suggested that different isoforms of $G\beta$ and $G\gamma$ have unique physiological roles (Khan et al., 2013).

1.2.3 GPCR kinases and arrestins

Many GPCRs show reduced signalling after extended agonist stimulation, known as desensitisation (Ferguson, 2001). Desensitisation of GPCRs is mediated primarily by GPCR kinases (GRKs) and scaffold proteins known as arrestins. In mammals, there are seven GRKs, which includes GRK1 (rhodopsin kinase), GRK2 (β -adrenergic receptor kinase 1 or β ARK1), GRK3 (β -adrenergic receptor kinase 2 or β ARK2), and GRK4-7 (Ferguson, 2001), and four arrestins, which includes arrestin-1 (visual arrestin), arrestin-2 (β -arrestin1), arrestin-3 (β -arrestin2), and arrestin-4 (cone arrestin) (Peterson & Luttrell, 2017). GRKs phosphorylate agonist-bound receptors at the ICLs or C-terminal tail, which leads to the recruitment of arrestins. The visual and cone arrestins bind to opsins, whereas the β -arrestins can bind to most GPCRs. Arrestins block the binding of G proteins to the receptor, thereby terminating G protein signalling. Following this, the β -arrestins can mediate internalisation of the receptor by functioning as adapters that target the receptor for endocytosis into clathrin-coated vesicles (Luttrell & Lefkowitz, 2002). After internalisation, the GPCR can be returned to the cell membrane (resensitisation) or can be targeted to lysosomes for degradation. In addition to their role in terminating G protein signalling, β -arrestins can also act as scaffolds for a variety of other proteins, leading to “non-canonical” intracellular signal transduction (Peterson & Luttrell, 2017) (**section 1.2.4.1**). However, not all GPCRs couple to arrestins. For example, relaxin family peptide receptor 1 (RXFP1) (**section 1.9.1.1**), RXFP2 (Callander *et al.*, 2009), and the bile acid receptor TGR5 (Jensen *et al.*, 2013) do not couple to β -arrestin and thus show sustained signalling from the plasma membrane.

1.2.4 Spatiotemporal organisation of GPCR signalling

There is a large body of literature demonstrating that second messenger activity can be compartmentalised within the cell (McCormick & Baillie, 2014). cAMP activity, for example, can be localised to “hotspots” or microdomains at particular subcellular locations (Lefkimmatis & Zaccolo, 2014), which can be accomplished via scaffolding proteins such as A-kinase anchoring proteins (AKAPs) that bring cAMP, PKA, and other signalling proteins into proximity (Wong & Scott, 2004), as well as through localised PDE-mediated cAMP degradation (Stangherlin & Zaccolo, 2012). As described below, recent research has highlighted that GPCRs also contribute to compartmentalised signalling. Specifically, rather than only occurring at the plasma membrane, GPCR-mediated signalling can occur in spatially and temporally distinct “waves” from several intracellular locations, which can be mediated by either G proteins or β -arrestins.

1.2.4.1 β -arrestin signalling at endosomes and the plasma membrane

β -arrestins can bind MAPKs such as ERK1/2 and JNK, leading to intracellular MAPK signalling that is spatially and temporally distinct from G protein-mediated MAPK signalling (Peterson & Luttrell, 2017). However, recent approaches have suggested that it is likely that G protein activation does need to occur in order for β -arrestin-mediated signalling to occur (Grundmann et al., 2018; Luttrell et al., 2018). Early research suggested that β -arrestin-mediated MAPK activity occurs from receptors in endosomes, as seen for the β_2 -adrenoceptor (β_2 -AR) (Daaka et al., 1998; Luttrell et al., 1999) and angiotensin II type 1A receptor (Luttrell et al., 2001). However, for some receptors, β -arrestin-mediated signalling can occur at the plasma membrane. For example, the β_1 -adrenoceptor (β_1 -AR), which shows little or no clathrin-mediated endocytosis, is proposed to signal through β -arrestin via an “activation at a distance” mechanism whereby the receptor couples to β -arrestin2 at the plasma membrane, and the arrestin then dissociates and activates MAPK signalling after trafficking to clathrin-coated structures (Eichel et al., 2016). In addition to being spatially separated from G protein-mediated MAPK activity, β -arrestin-mediated MAPK activity also tends to show a different temporal profile. Specifically, whereas G protein-mediated ERK1/2 activity tends to be rapid and transient, β -arrestin-mediated ERK1/2 activity tends to be delayed and sustained (Ahn et al., 2004; Ren et al., 2005; Shenoy et al., 2006).

1.2.4.2 G protein signalling at subcellular locations

G protein activation can also occur away from the plasma membrane. For example, a delayed phase of sustained cAMP activity from internalised receptors has been suggested for some rhodopsin- and secretin-like receptors, including the luteinising hormone receptor (LH receptor) (Lyga et al., 2016), thyroid-stimulating hormone receptor (TSH receptor) (Calebiro et al., 2009; Godbole et al., 2017; Werthmann et al., 2012), PTH1 receptor (Feinstein et al., 2011; Ferrandon et al., 2009; White et al., 2020a), and V₂ vasopressin receptor (Feinstein et al., 2013). Additionally, sustained endosomal inhibition of cAMP activity has been reported for the sphingosine 1-phosphate receptor (S1P₁ receptor) (Mullershausen et al., 2009). Furthermore, acute cAMP activity, which is less temporally separated from plasma membrane signalling, occurs from internalised D₁ dopamine receptors (Kotowski et al., 2011), and acute endosomal signalling was also observed for the β_2 -AR (Irannejad et al., 2013). However, cAMP is not the only pathway that internalised receptors can signal from. For example, internalised tachykinin receptor 1 (NK₁ receptor) can signal via PKC and ERK1/2, in addition to cAMP (Jensen et al., 2017), and internalised calcitonin receptor-like receptors signal via PKC and ERK1/2 (Yarwood et al., 2017). Importantly, these unique spatiotemporal profiles can depend on the type of cell that the receptor is expressed in. For example, whereas signalling from internalised TSH receptors occurs in thyroid cells, it did not occur when the TSH receptor is expressed in HEK293 cells, even when internalisation was enhanced by overexpression of β -arrestin2 (Werthmann et al., 2012). Signalling from GPCRs at subcellular locations can occur via several mechanisms.

A mechanism by which G protein-mediated cAMP activity indirectly occurs from endosomes was discovered for the PTH1 receptor, whereby receptor-arrestin-G $\beta\gamma$ complexes caused an increased rate of activation of G α_s , leading to sustained production of cAMP (Wehbi et al., 2013). It was later determined that G $\alpha_{q/11}$ is also involved in mediating the endosomal response of the PTH1 receptor, as PI3K and phosphatidylinositol (3,4,5)-trisphosphate (PIP₃) activity mediated downstream of the G_q $\beta\gamma$ subunits is necessary for β -arrestin coupling to occur (White et al., 2020a). Another mechanism for sustained endosomal signalling, involving receptor crosstalk, was also seen for the PTH1 receptor. Specifically, activated β_2 -AR potentiated endosomal PTH1 receptor signalling by releasing G $\beta\gamma$ subunits that formed complexes with PTH receptor, β -arrestin, and G α_s , leading to activation of AC2 by G $\beta\gamma$ (Jean-Alphonse et al., 2017). Similarly, at the V₂ receptor, “megaplexes” of a GPCR, G protein, and β -arrestin were identified, which may provide a mechanism for sustained G protein signalling from internalised receptors (Thomsen et al., 2016).

If β -arrestin binding and internalisation can promote this type of signalling, then it raises the question of how it is terminated. For the V₂ receptor, it has been suggested that retromer,

which is a complex involved in recycling proteins, binds to the receptor at endosomes, leading to desensitisation (Feinstein et al., 2013). It was also suggested that binding of retromer terminates cAMP signalling from internalised PTH receptors (Feinstein et al., 2011). Additionally, signalling from internalised PTH receptors is thought to be terminated when endosomal acidification leads to dissociation of PTH and coupling of the receptor to retromer (Gidon et al., 2014).

Interestingly, acute G protein signalling has also been identified from internalised receptors at endosomes, which is similar to the canonical G protein signalling that occurs from the plasma membrane. The most direct evidence for this type of G protein-mediated signalling from internalised receptors involved the use of nanobody-green fluorescent protein (GFP) fusion proteins as genetically-encoded conformational biosensors that recognised either the active state of the β_2 -AR or an intermediate G protein activation state (Irannejad et al., 2013). These biosensors revealed that the β_2 -AR becomes active at the plasma membrane after agonist addition, which is followed by activation at the endosome after internalisation and dissociation of β -arrestin (Irannejad et al., 2013).

Following on from this, it was shown that β_2 -ARs localised to the Golgi apparatus can also signal via G proteins (Irannejad et al., 2017). As this pool of receptors arises via the biosynthetic pathway rather than the endocytic pathway, agonists must either diffuse through the membrane or be transported across the membrane in order to activate these receptors (Irannejad et al., 2017). This phenomenon was also demonstrated at the μ - and δ -opioid receptors (ORs), whereby only membrane-permeable ligands were able to reach Golgi-localised receptors, whereas endogenous OR peptide agonists, as well as peptide antagonists, cannot bind to this pool of receptors (Stoeber et al., 2018). The potential implications of signalling from intracellular GPCRs for drug discovery are discussed below (**section 1.4.4**).

1.3 GPCRs as targets for therapeutic drugs

As GPCRs are involved in the regulation of most physiological processes, it is not surprising that they are a major target of therapeutic drugs. In fact, GPCRs are the largest family of proteins to be exploited for drug discovery (Hopkins & Groom, 2002; Russ & Lampel, 2005). Drugs targeting GPCRs account for about 30% of therapeutic drugs on the market, and drugs targeting GPCRs are continuing to be approved for therapeutic use (Hauser et al., 2017; Rask-Andersen et al., 2011; Santos et al., 2017). However, only a small subset of GPCRs is actually targeted by therapeutic drugs. Most notably, aminergic rhodopsin-like GPCRs such as the adrenergic receptors, dopamine

receptors, histamine receptors, and muscarinic acetylcholine receptors, are overrepresented as drug targets (Garland, 2013; Sriram & Insel, 2018). However, due to technological advances and an increased understanding of receptor pharmacology, there is still large scope for drug development targeting GPCRs (Garland, 2013). For example, even GPCRs that were thought to be “forbidden” drug targets due to dangerous side effects now have the potential to be targeted by therapeutic drugs due to our increased understanding of biased signalling (Kenakin, 2019).

1.4 Biased signalling

1.4.1 Efficacy and bias

“Efficacy” is the ability of a drug to produce a biological response (Stephenson, 1956). Historically, ligands for a receptor were classified either as agonists, which have efficacy, or antagonists, which do not have efficacy. It was also recognised that efficacy could vary in strength, such as in the case of partial agonists, which have a lower efficacy than full agonists. This view of efficacy was a consequence of pharmacological bioassays that involved whole animals or isolated tissue and generated a single output of receptor activation, such as contraction of ileum (Kenakin, 2009). However, with the continued development of *in vitro* assays, it became clear that efficacy is more complex than this. For example, the identification of receptors that show constitutive activity led to the discovery of inverse agonists, which have negative efficacy as they reduce the activity of constitutively-active receptors (Costa & Herz, 1989). Furthermore, the discovery that GPCRs can show pleiotropic coupling to multiple G proteins and effectors means that a ligand-GPCR pair can show varying efficacy toward a range of processes, such as G protein coupling, β -arrestin coupling, signalling at multiple pathways, and internalisation (Kenakin, 2002). Following on from this, it became clear that different ligands for the same receptor in the same cell background can favour activation of certain G proteins and effectors relative to one another (**Figure 1.2**). This phenomenon is referred to as biased agonism or biased signalling, and is a current focus in GPCR drug discovery (Kenakin, 2019). A variety of examples of biased signalling from endogenous ligands have been identified. For example, at the V_2 receptor, arginine vasopressin (AVP) and oxytocin both activate cAMP, whereas AVP induces strong β -arrestin recruitment and internalisation but oxytocin induces very weak and transient β -arrestin recruitment and does not induce internalisation (Feinstein et al., 2013).

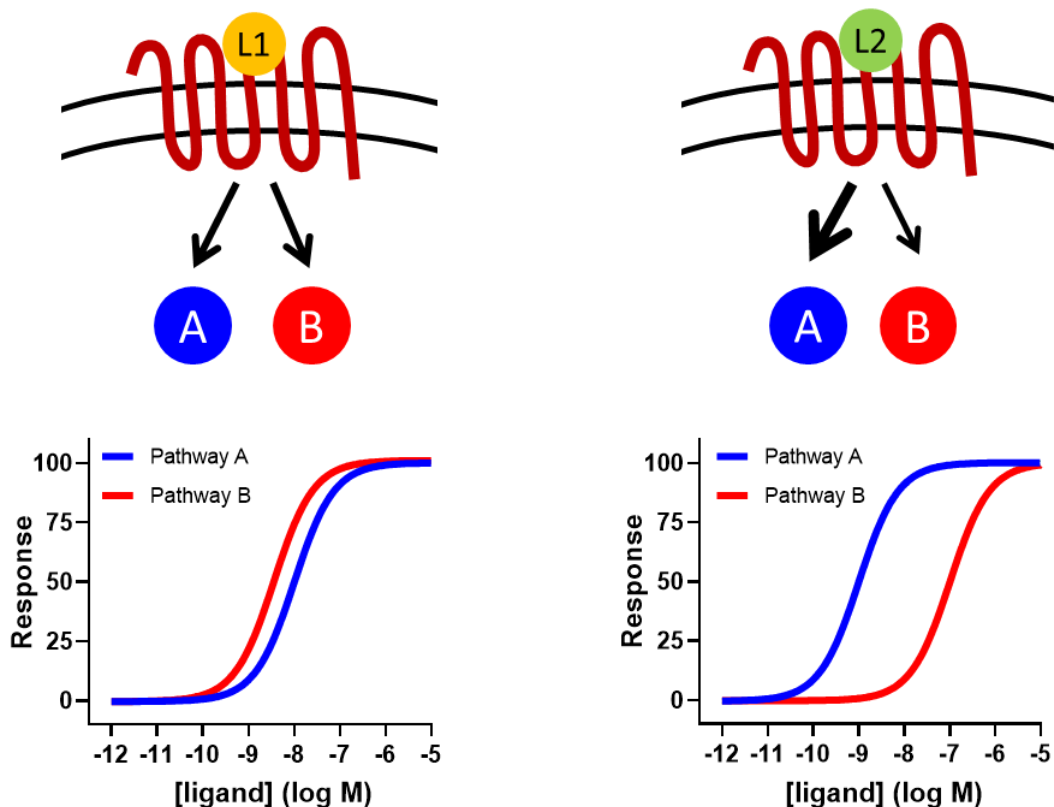


Figure 1.2. An example of biased signalling. Ligand 1 (L1) signals through pathway A and pathway B with approximately equal potency. Ligand 2 (L2), acting at the same receptor in the same cell type, however, shows a reversal of potency and strongly favours signalling via pathway A while signalling through pathway B only weakly. The two ligands are thus biased in relation to one another. Adapted from Evans *et al.* (2010).

1.4.2 Bias and drug development

In the context of drug development, it is anticipated that biased signalling can be exploited to develop better therapeutic drugs by (1) selectively activating beneficial signalling pathways, (2) not activating harmful signalling pathways, and (3) blocking the ability of the endogenous agonist to activate harmful signalling pathways (Kenakin, 2017; Violin *et al.*, 2014). In other words, it may be possible to utilise bias to create effective drugs with fewer side effects. Biased ligands have therefore been developed for certain GPCRs with the aim of being used as therapeutic drugs. For example, agonists for the AT₁ receptor have been developed for conditions such as heart failure that are biased towards β -arrestin (which mediates therapeutic effects such as improvement of cardiac performance) over G protein signalling (which mediates negative effects such as vasoconstriction) (Violin *et al.*, 2010), which are expected to have advantages over currently-

available AT₁ receptor antagonists, which block signalling at both pathways. The β -arrestin-biased agonist TRV027 was the first drug developed for its ability to selectively activate signalling pathways to be tested in humans. Unfortunately, in the BLAST-AHF phase IIb clinical trial for acute heart failure (AHF), TRV027 failed to meet the primary or secondary endpoints, highlighting the difficulty of translating *in vitro* bias into therapeutic benefits (Pang et al., 2017). Similarly, agonists for the μ -opioid receptor that are biased towards G protein signalling (which is thought to mediate the analgesic effect of opioids) over β -arrestin recruitment (which is thought to mediate side effects such as respiratory depression) have also been developed, such as oliceridine (TRV130), which is currently in clinical trials (Madariaga-Mazon et al., 2017). However, recent research has cast doubt on the idea that β -arrestin mediates the respiratory depression effect of opioid receptor activation, which brings this strategy of developing G protein-biased opioid receptor agonists into question (Kliwer et al., 2020).

Notably, some drugs that have been approved for therapeutic use in humans have subsequently been shown to be biased. For example, the β -AR ligand carvedilol, which is used as a treatment for high blood pressure and heart failure, is an inverse agonist for cAMP activity and an agonist for β -arrestin recruitment, whereas other ligands tend to act as either agonists or antagonists for both pathways (Wisler et al., 2007). This bias is thought to be important for the therapeutic actions of carvedilol (Wisler et al., 2007).

1.4.3 Conformational bias

GPCRs in their basal state do not exist in a single conformation. Rather, they are dynamic and sample a variety of conformations, referred to as an ensemble (Kenakin, 2002). Biased signalling can therefore be explained by the observation that different ligands for the same receptor stabilise distinct conformations of that receptor, thereby shifting the equilibrium in favour of these conformations at the expense of others. These distinct conformations subsequently show preferential coupling to a subset of that receptor's downstream effectors, thereby producing unique signalling profiles. This ability of different ligands to stabilise different active states of a GPCR has been supported by a variety of methods such as nuclear magnetic resonance (NMR) spectroscopy (Liu et al., 2012) and conformational biosensors (Devost et al., 2017; Zörn et al., 2009). Additionally, the conformation adopted by the GPCR may not only affect which effectors are recruited, but may also influence the downstream activity of that effector. For example, distinct GPCR conformations can be phosphorylated at different sites by GRKs and other kinases, producing a "phosphorylation barcode" that in turn promotes coupling of β -arrestins in distinct

conformations, with potential to produce discrete downstream signalling or receptor trafficking outcomes (Lee et al., 2016a; Nobles et al., 2011).

1.4.4 Location bias

Although GPCR conformation has typically been considered the main driver of biased signalling, it is becoming increasingly recognised that other factors, such as location of signalling within the cell, can also drive ligand-specific signalling patterns. As described above (**section 1.2.4.2**), some GPCRs can signal after internalisation, and some ligands can enter the cell and activate subcellular GPCRs. The potential implications of subcellular GPCR signalling were demonstrated by a recent study showing that the location of cAMP produced within the cell has an impact on downstream gene transcription (Tsvetanova & von Zastrow, 2014). First, inhibition of internalisation of the β_2 -AR substantially reduced downstream β_2 -AR-mediated gene transcription, including abolishing transcription of some genes. Second, an optogenetic approach was used to show that photoactivatable AC localised to endosomes produced more efficient cAMP-dependent gene transcription compared to plasma membrane-localised AC, even when similar total levels of cAMP were produced, which was likely due to proximity of cAMP to downstream effectors and transcription factors (Tsvetanova & von Zastrow, 2014). Similarly, another study showed that signalling from internalised TSH receptors is important for TSH-mediated CREB phosphorylation and gene transcription to occur in primary thyroid cells (Godbole et al., 2017). TSH receptors were trafficked to the *trans*-Golgi network after internalisation, leading to activation of cAMP/PKA that was proximal to the nucleus (Godbole et al., 2017).

The ability of signalling at particular subcellular locations to favour gene transcription compared to other locations was named “location bias” (Tsvetanova & von Zastrow, 2014). Importantly, location bias only becomes apparent when different ligands for the same receptor show differential abilities to cross the cell membrane, be transported across the cell membrane, or induce internalisation of the receptor. Additionally, location bias highlights the fact that some drug effects that were previously thought to be mediated by stabilisation of a particular receptor conformation might actually arise due to location of signalling within the cell (Tsvetanova & von Zastrow, 2014).

Notably, endosomal signalling can be physiologically important or involved in human disease. For example, endosomal signalling from the δ -opioid receptor is suggested to be involved in endogenous inhibition of inflammatory pain (Jimenez-Vargas *et al.*, 2020). Additionally, as outlined earlier, μ - and δ -opioid receptors located at the Golgi apparatus can also be activated by

membrane-permeant agonists, but the implications of opioid receptor signalling from the Golgi are not yet understood (Stoeber *et al.*, 2018). However, given that endosomal GPCR signalling has physiological relevance, targeting of subcellular receptors for therapeutic benefit is a current area of research (Gottesman-Katz *et al.*, 2020; Retamal *et al.*, 2019). In fact, several approaches to exploit location bias for therapeutic benefit have been undertaken, including conjugation of ligands to the transmembrane lipid cholesterol to facilitate delivery to the endosome (Jimenez-Vargas *et al.*, 2018; Yarwood *et al.*, 2017), as well as drug delivery via pH-responsive nanoparticles that selectively lead to activation or inhibition of endosomal receptors (Jimenez-Vargas *et al.*, 2020; Ramirez-Garcia *et al.*, 2019).

1.4.5 Kinetic and observational biases

The kinetics of binding and signalling have become increasingly recognised as important for agonist efficacy and biased signalling (Lane *et al.*, 2017). For example, a correlation between ligand off rate and efficacy was observed at the M₃ muscarinic receptor, whereby the ligands with the highest efficacy for GTPγS binding and Ca²⁺ mobilisation also showed the slowest rates of dissociation (Sykes *et al.*, 2009), however this relationship between efficacy and binding kinetics is not universal for all GPCRs (Lane *et al.*, 2017). Notably, the kinetics of ligand binding and signalling influenced observations of biased signalling at the D₂ dopamine receptor, showing that observed bias can reverse over time (Klein Herenbrink *et al.*, 2016). Kinetic bias is linked to conformational bias in the sense that ligand residence time can influence which effectors are recruited to the receptor over time (Lane *et al.*, 2017). For example, the prolonged residence time of lysergic acid diethylamide (LSD) at the 5-HT_{2B} receptor is associated with bias towards β-arrestin2 recruitment (Wacker *et al.*, 2017). These studies highlight the importance of using assays that detect the kinetic aspects of signalling, as measuring signalling at single time points and assuming equilibrium can produce a misleading picture of efficacy and bias depending on which time point was chosen. This contributes to “observational bias”, or bias that is observed only due to aspects of the assays used, such as temporal factors, buffers, and temperature (Kenakin & Christopoulos, 2013; Klein Herenbrink *et al.*, 2016).

1.4.6 System bias

Different cell types show preferential coupling to particular effectors and pathways, which is known as “system bias” (Kenakin & Christopoulos, 2013). *In vitro*, cell lines recombinantly

expressing a GPCR of interest are commonly used to characterise biased signalling, as they are easy to grow and maintain and are usually transfectable. However, analysis of mRNA expression has shown markedly different expression and stoichiometry of endogenous receptors and their downstream effectors among different cell lines (Atwood et al., 2011), and altering the stoichiometry of receptors and their effectors can also change the efficacy and relative potency of ligands for different signalling pathways (Newman-Tancredi et al., 2000; Newman-Tancredi et al., 1997; Onfroy et al., 2017; Thompson et al., 2016; Watson et al., 2000). Furthermore, expression of accessory proteins such as RAMPs and other GPCRs that form complexes with the receptor of interest vary among cell types, which will also affect the observed pharmacology. Expression levels of cell components including GPCRs and their effectors can also change under certain physiological or pathological conditions, which is an alteration to system bias that has been named “dynamic bias” (Michel et al., 2014). For example, some $G\alpha_i$ isoforms are upregulated in failing myocardium (Böhm et al., 1994; Dschietzig et al., 2011), and there may therefore be an emphasis on $G\alpha_i$ signalling under pathological conditions that is not seen under certain assay conditions. Several other factors also differ among cell types. For example, conformational biosensors have revealed that different cell backgrounds can influence the conformation of a receptor (Devost et al., 2017). Additionally, cell membrane composition also differs between cell types and disease states, which can in turn affect receptor conformations, leading to alterations in ligand binding, signalling, and trafficking (Desai & Miller, 2018). These findings highlight the importance of using appropriate, physiologically-relevant cell types when detecting signal transduction and bias, as biased signalling profiles may look different across cell types, and drugs could have disease-specific actions not seen in recombinant cell lines (Michel et al., 2014).

1.5 The peptide hormone relaxin

1.5.1 Hisaw’s observations and the discovery of relaxin

The hormone relaxin was discovered in the mid-1920s by Dr Frederick Hisaw, who was studying the pelvic modifications that occur alongside parturition in mammals. Hisaw observed that the pubic bones of the pocket gopher meet to form a pubic symphysis, yielding a constricted pelvis that would be advantageous for moving around in burrows, but not wide enough for the gopher to give birth (Hisaw, 1925). However, in sexually mature female gophers, Hisaw observed resorption of the pubic bones, which was particularly pronounced during pregnancy and allowed

parturition to occur (Hisaw, 1925). Upon injection of sow ovarian extracts into castrated male pocket gophers, Hisaw noted that the symphysis was also usually lost (Hisaw, 1925). Hisaw subsequently directed his attention towards the guinea pig, which is a species that shows relaxation of the pubic ligament leading to separation of the pubic symphysis to allow parturition, rather than resorption of the pubic bones. Hisaw conducted his most well-known experiment, in which he injected serum from pregnant guinea pigs or rabbits into virgin guinea pigs soon after oestrus, and observed sustained relaxation of the pubic ligament, thereby mimicking the effect seen in pregnancy and suggesting that it was under hormonal control (Hisaw, 1926). Hisaw soon reported that the substance responsible for this effect was also present in the serum of pregnant cats, dogs, and mare, as well as in rabbit placenta and sow corpus luteum (Hisaw, 1927; Hisaw, 1929; Hisaw et al., 1930; Hisaw et al., 1928). Hisaw and colleagues purified an acid alcohol extract of this substance from sow corpus luteum and named it “relaxin” (Fevold et al., 1930). Although other researchers initially questioned the existence of relaxin due to the ability of other sex hormones such as oestrogen or progesterone to produce pelvic relaxation, Hisaw and colleagues showed that injection of these hormones took days to induce relaxation, whereas relaxin caused relaxation within hours (Hisaw et al., 1944). Additionally, it was noted that relaxin was peptide-like and shared several properties with insulin, thereby making it distinct from steroid hormones (Fevold et al., 1930). However, it also became clear that the relaxative effect of relaxin in the guinea pig did depend on the presence of oestrogen (Zarrow, 1948).

In the 1940s and 1950s, more actions of relaxin were discovered (Hisaw & Zarrow, 1950). For example, it was shown that administration of relaxin alongside oestradiol and progesterone caused growth of the mammary glands of immature ovariectomised female rats (Hamolsky & Sparrow, 1945). Relaxin administration also caused opening of the pubic symphysis of oestrone-primed mice (Hall & Newton, 1947) and relaxation of the cervix in estrogen-primed cows (Graham & Dracy, 1953). Furthermore, as described below (**section 1.6.1**), purification of relaxin from various animals in the 1970s and beyond led to determination of the structure of relaxin, and cloning of the gene for relaxin led to the discovery that there are actually two genes for relaxin in humans: *RLN1*, which encodes relaxin-1 (H1 relaxin), and *RLN2*, which encodes relaxin-2 (H2 relaxin). Relaxin-2 is the equivalent peptide to the relaxin peptide that circulates in other mammals, so is usually just referred to as “relaxin”.

1.5.2 Physiological roles and actions of relaxin

Relaxin research in the 1980s and beyond demonstrated that relaxin is a pleiotropic hormone with a variety of physiological roles across reproductive and non-reproductive tissues in both males and females, although these vary among species (Bathgate et al., 2006a; Sherwood, 2004). The physiological roles of endogenous relaxin were mostly determined by ovariectomy (e.g., Hurley *et al.*, 1991; Min *et al.*, 1997; Novak *et al.*, 2001; Zaleski *et al.*, 1996), neutralisation of circulating relaxin using a monoclonal antibody (Hwang *et al.*, 1989; Hwang & Sherwood, 1988; Summerlee *et al.*, 1998), or the use of mice with knockout of the gene for either relaxin (Zhao et al., 1999) or the relaxin receptor (Kamat et al., 2004; Krajnc-Franken et al., 2004).

1.5.2.1 Female reproductive system

Relaxin circulates during pregnancy in many mammalian species and has numerous physiological roles in female reproduction, many of which are influenced by the activity of other hormones such as oestrogen and progesterone (Sherwood, 2004). In humans and most other mammals, the corpora lutea are the primary source of relaxin production during pregnancy, although in some mammals the placenta or the uterus is the primary source of relaxin (Bathgate et al., 2006a). In human pregnancy, relaxin levels are highest during the first trimester, where they rise to about 1 ng/ml before decreasing (Bell et al., 1987; Eddie et al., 1986; Szlachter et al., 1982). However, this contrasts with other species such as rodents, in which relaxin levels are highest during the third trimester and can increase to over 100 ng/ml (O'Byrne & Steinetz, 1976; Sherwood et al., 1980), which suggests that the roles of relaxin in pregnancy will vary between humans and other species. In fact, the reproductive role of relaxin in humans is not well understood, although it appears that in humans and other primates relaxin may aid implantation, which is consistent with the higher levels of relaxin seen during the first trimester (Bathgate et al., 2006a; Sherwood, 2004).

Endogenous relaxin influences development of the pubic symphysis and relaxation of the pubic ligament in pregnant mice (Zhao et al., 1999; Zhao et al., 2000). Relaxin is also involved in the growth of the cervix that occurs during pregnancy in pigs (Min et al., 1997), rats (Burger & Sherwood, 1995; Burger & Sherwood, 1998; Hwang et al., 1989; Hwang & Sherwood, 1988), and mice (Zhao et al., 2000). Similarly, relaxin has a role in cervical ripening in pregnant pigs (O'Day et al., 1989) and rats (Hwang et al., 1989). However, in humans, cervical ripening was reported to occur even when no serum relaxin was detectable (Eddie et al., 1990), and in a phase II clinical trial, infusion of recombinant human relaxin failed to increase cervical ripening in humans (Weiss *et al.*, 2016). Relaxin is also involved in growth of the uterus in pigs (Huang et al., 1997; Min et al., 1997),

monkeys (Goldsmith et al., 2004), and rats (Vasilenko et al., 1986), and influences uterine relaxation in rats (Cheah & Sherwood, 1981; Sanborn et al., 1980) and pigs (MacLennan et al., 1986; Porter & Watts, 1986). Conversely, *in vitro* stimulation of human uterine tissue with relaxin had little or no effect on contractility (MacLennan et al., 1995; MacLennan et al., 1986). Relaxin also increases growth of the vagina in pregnant mice (Zhao et al., 2000), rats (Burger & Sherwood, 1995), and pigs (Min et al., 1997), and also has a role in growth or differentiation of the mammary gland of mice (Bani & Bigazzi, 1984; Bani et al., 1985), rats (Wahab & Anderson, 1989), and pigs (Hurley et al., 1991; Zaleski et al., 1996). Additionally, mice that do not express relaxin or the relaxin receptor show impaired development of the nipples and cannot deliver milk to their young (Kamat et al., 2004; Krajnc-Franken et al., 2004; Zhao et al., 1999).

1.5.2.2 Male reproductive system

Relaxin is also present in the male reproductive tract, although its roles here are less well defined. In rodents, relaxin is expressed in the testes and prostate (Gunnarsen et al., 1995; Samuel et al., 2003a). In humans, relaxin-1 and relaxin-2 are both expressed in the prostate (Garibay-Tupas et al., 2000; Gunnarsen et al., 1996; Hansell et al., 1991), and relaxin immunoreactivity has also been detected in the prostate, seminal vesicle, and vas deferens (Yki-Jarvinen et al., 1983), as well as in boar seminal vesicle (Kohsaka et al., 1992). Relaxin is also present in the semen of humans, boars, bulls, and goats (Kohsaka et al., 2003), and it has been confirmed that human seminal relaxin is a product of the *RLN2* gene and is therefore the same peptide that is produced in the corpus luteum in females (Winslow et al., 1992). Seminal relaxin may have a role in sperm motility, as treatment of human sperm with relaxin increases motility (Carrell et al., 1995). Relaxin knockout mice have smaller testes, prostate, epididymis, and seminal vesicles, and show decreased sperm maturation and decreased fertility, as well as increased collagen in the prostate, testes, and epididymis (Samuel et al., 2003a). Similarly, relaxin receptor knockout mice have smaller epididymis and show impaired spermatogenesis and reduced fertility (Krajnc-Franken et al., 2004). However, not all strains of relaxin receptor knockout mice show decreased fertility (Kamat et al., 2004).

1.5.2.3 Fibrosis and connective tissue

Hisaw's original observations implied that relaxin administration has effects on the breakdown of collagen in reproductive tissues (Hisaw, 1926). Relaxin stimulation was subsequently demonstrated to regulate collagen turnover *in vitro* in human dermal fibroblasts (Unemori &

Amento, 1990), human scleroderma fibroblasts (Unemori *et al.*, 1992), human pulmonary fibroblasts (Unemori *et al.*, 1996), and rat hepatic stellate cells (Williams *et al.*, 2001). At the same time, it was shown that relaxin administration also inhibited collagen accumulation *in vivo* in rodent models of fibrosis (Unemori *et al.*, 1993; Unemori *et al.*, 1996). In fact, it is now recognised that treatment with relaxin can decrease collagen accumulation in a variety of animal models of fibrosis, such as hypertension, myocardial infarction, cardiomyopathy, atrial fibrillation, and diabetes (Samuel *et al.*, 2017). A role for endogenous relaxin in collagen turnover was confirmed when relaxin knockout mice showed increased collagen content in the vagina and nipple (Zhao *et al.*, 2000), which may be responsible for impaired nipple development (Zhao *et al.*, 1999). Male relaxin knockout mouse showed increased collagen levels in the epididymis, prostate, and testis, which may be linked to the underdevelopment of the reproductive tract in these animals (Samuel & Amento, 2001). Furthermore, relaxin knockout mice develop pulmonary fibrosis (Samuel *et al.*, 2003b), renal fibrosis (Samuel *et al.*, 2004b), cardiac fibrosis (Samuel *et al.*, 2004a), and dermal fibrosis (Samuel *et al.*, 2005), which can be reversed by treatment with relaxin. Similarly, relaxin receptor knockout mice also showed increased collagen and fibrosis in the lungs, particularly surrounding the bronchioles and blood vessels (Kamat *et al.*, 2004).

1.5.2.4 Cardiovascular and renal systems

In the 1960s, Hisaw and colleagues observed that relaxin treatment can augment the hypertrophy and hyperplasia of blood vessels in the endometrium that occurs in pregnant monkeys, and that this effect can also be induced in non-pregnant and castrated monkeys (Hisaw *et al.*, 1967). Since then, it has been shown that a variety of the adaptive cardiovascular changes that occur during pregnancy are associated with endogenous relaxin activity. For example, neutralisation of circulating relaxin in pregnant rats blocked increases in stroke volume and cardiac output (CO), and blocked decreases in systemic vascular resistance (SVR) (Debrah *et al.*, 2006). Furthermore, administration of relaxin to rats induces cardiovascular changes similar to those caused by endogenous relaxin during pregnancy. For example, treatment of female rats with relaxin increased vasodilation, CO, glomerular filtration rate (GFR), and renal plasma flow (RPF), and decreased SVR and plasma osmolality (Danielson *et al.*, 1999; Novak *et al.*, 2001), and administration of relaxin to male rats increased GFR and RPF and decreased plasma osmolality (Danielson *et al.*, 2000). Additionally, aged pregnant relaxin knockout mice showed stiffer uterine arteries, which was reversed by treatment with relaxin (Gooi *et al.*, 2013), and pregnant relaxin knockout mice also lacked the attenuation of angiotensin II-mediated constriction of mesenteric arteries that is seen in normal pregnant mice (Marshall *et al.*, 2016). Endogenous relaxin also has

effects in the renal system of pregnant rats, as ovariectomy or neutralisation of circulating relaxin using a monoclonal antibody abolished the increases in GFR and RPF and decreases in renal vascular resistance that are normally seen during pregnancy (Novak et al., 2001).

Relaxin is also produced locally in the vasculature of male and female rats (Novak et al., 2006), and studies using relaxin knockout mice have demonstrated that relaxin has physiological roles in the cardiovascular system outside of pregnancy. For example, male relaxin knockout mice show some dysfunction of arterial structure and function (Jelinic et al., 2015; Leo et al., 2014; Ng et al., 2015), as well as diastolic dysfunction and increased collagen in the left ventricle (Du et al., 2003).

The physiological roles of relaxin in the human cardiovascular and renal systems are not well understood. However, relaxin has shown cardiovascular and renal effects when administered to humans in several clinical trials. For example, in healthy human volunteers treatment with relaxin increased RPF, but did not change GFR (Smith et al., 2006). Relaxin treatment also decreased blood pressure in a clinical trial for scleroderma (Khanna et al., 2009) and increased vasodilation and decreased SVR in a clinical trial for compensated heart failure (Dschietzig et al., 2009). A meta-analysis of six clinical trials involving infusion of relaxin for treatment of AHF showed that relaxin administration was associated with reduced worsening of heart failure, improvements in markers of renal function, and decreases in blood pressure (Teerlink *et al.*, 2020).

1.5.2.5 Cancer

Relaxin is expressed in tumours from human breast (Tashima et al., 1994), prostate (Feng et al., 2007; Thompson et al., 2006), thyroid (Hombach-Klonisch et al., 2006), and endometrium (Kamat et al., 2006). Increased levels of circulating relaxin have also been observed in women with breast cancer (Binder et al., 2004). Several *in vivo* studies have demonstrated a role for relaxin in cancer. For example, in a mouse model of spontaneous breast cancer, relaxin administration increased tumour growth (Binder et al., 2014). Additionally, overexpression of relaxin in a mouse model of human prostate cancer led to increased tumour size (Silvertown et al., 2006), whereas downregulation or inhibition of the relaxin receptor using small interfering RNA (siRNA) or a relaxin receptor antagonist inhibited tumour growth (Feng et al., 2010; Silvertown et al., 2007). Exogenous administration of the relaxin receptor antagonist also suppressed prostate cancer growth in a mouse model of human prostate cancer (Neschadim et al., 2014). *In vitro*, stimulation with relaxin increased invasiveness of breast cancer cells (Binder et al., 2002), thyroid cancer cells (Radestock et al., 2010), prostate cancer cells (Feng et al., 2007), endometrial cancer cells (Kamat et al., 2006),

and glioblastoma cells (Glogowska et al., 2013). Similarly, overexpression of relaxin in breast cancer cells is associated with increased cell proliferation and migration (Silvertown et al., 2003).

1.5.2.6 Central nervous system

Relaxin mRNA has been detected in regions including the neocortex, piriform cortex, hippocampus, tenia tecta, and anterior olfactory nucleus of the rat brain (Osheroff & Ho, 1993), and cerebellum, cortex, hippocampus, thalamus, pons, and medulla of the mouse brain (Ma et al., 2006). Immunohistochemistry has also revealed the presence of the relaxin peptide in the arcuate nucleus of the hypothalamus, anterior olfactory nucleus, piriform cortex, and tenia tecta of the rat brain (Gunnarsen et al., 1995; Ma et al., 2005). Relaxin may act as a neuropeptide as its receptor is expressed in regions that receive projections from relaxin-expressing neurons (Ma et al., 2005), however relaxin in the circulation may also act on the brain (Mumford et al., 1989; O'Byrne et al., 1987; Summerlee et al., 1987; Sunn et al., 2002). There are currently no studies examining the expression or physiological roles of relaxin in the human brain, however intravenous or ventricular administration of relaxin to rodents has suggested that relaxin is implicated in central processes such as inhibition of milk ejection (Summerlee et al., 1984; Way & Leng, 1992), control of blood pressure (Mumford et al., 1989; Parry & Summerlee, 1991), and water consumption and the changes in plasma osmolality that occur during pregnancy (Summerlee et al., 1998; Sunn et al., 2002). Additionally, infusion of relaxin into the basolateral amygdala (where its receptor is expressed) impaired memory consolidation after an aversive event (Ma et al., 2005), and intravenous administration of relaxin also had neuroprotective effects in rat models of stroke (Bergeron et al., 2015; Wilson et al., 2006). Some central actions of relaxin appear to involve crosstalk with the activity of a variety of other neuropeptides, including oxytocin, vasopressin, angiotensin II, and opioids (Mumford et al., 1989; O'Byrne et al., 1986; Parry & Summerlee, 1991; Summerlee et al., 1984).

1.6 The relaxin family peptides

Relaxin is now classified as part of a larger group of relaxin family peptides, which includes relaxin-1, relaxin-2, and relaxin-3, as well as insulin-like peptide 3 (INSL3), INSL4, INSL5, and INSL6. Due to structural similarities, the seven relaxin family peptides are categorised as part of the larger insulin superfamily, which also includes insulin and the insulin-like growth factors (IGFs) I and II (Shabanpoor et al., 2009) (**Figure 1.3**).

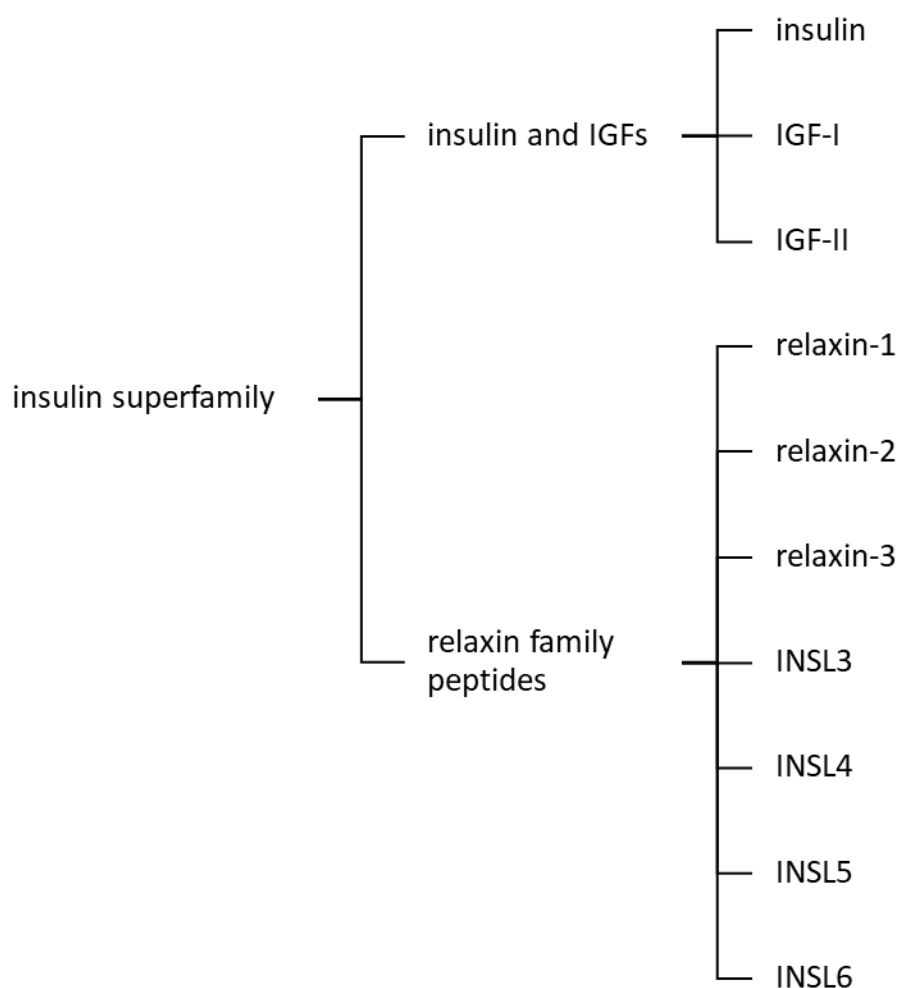


Figure 1.3. The human insulin superfamily of peptides. The insulin superfamily of peptides is divided into two branches: (i) insulin and insulin-like growth factors (IGFs) I and II, and (ii) the relaxin family peptides (relaxin-1, relaxin-2, relaxin-3) and insulin-like peptides (INSLs) 3-6.

1.6.1 Relaxin-1 and relaxin-2

Isolation of pure relaxin in the late 1970s and after led to the determination of the primary amino acid sequence of relaxin from a range of mammals such as the pig (James et al., 1977), rat (John et al., 1981), and human (Hudson et al., 1983), as well as non-mammals such as the spiny dogfish (Bullesbach et al., 1986) and skate (Bullesbach et al., 1987). Relaxin was demonstrated to be a 6 kDa peptide that has a similar structure to insulin, in that it consists of two chains, known as the A and B chains, which are linked by two disulfide bonds, with a third disulfide bond within the A chain (Schwabe & McDonald, 1977; Sherwood & O'Byrne, 1974).

The genes for rat (Hudson et al., 1981) and porcine (Haley et al., 1982) relaxin were discovered and cloned in the early 1980s by screening corpus luteum cDNA libraries. This revealed that relaxin, like insulin, is synthesised as a prepropeptide that is divided into four segments: a signal peptide, the B chain, a large C chain, and the A chain (Haley et al., 1982). As with insulin, the relaxin prepropeptide is subsequently processed into its mature form. Around this time, it was also discovered that there are two homologous relaxin genes in humans. A gene for human relaxin-1 or H1 relaxin (*RLN1*) was identified by screening porcine relaxin cDNA against a human genomic library (Hudson et al., 1983), and one year later a gene for human relaxin-2 or H2 relaxin (*RLN2*) was identified by screening a human corpus luteum cDNA library (Hudson et al., 1984). These homologous genes are also found in other primates and occur as a result of gene duplication, whereas other mammals only contain one relaxin gene (*RLN1*) (Evans et al., 1994a; Evans et al., 1994b; Wilkinson et al., 2005). Relaxin-2 is produced in the corpus luteum of the ovary and is released into circulation during pregnancy (Hudson et al., 1984; Khan-Dawood et al., 1989), and is therefore the equivalent of the circulating peptide discovered by Hisaw, and is usually just referred to as “relaxin”. In other words, human *RLN2* is the equivalent of *RLN1* in non-primates. And as noted above (**section 1.5.2.2**), human seminal relaxin is also relaxin-2, and is thus same peptide as that produced in the corpus luteum (Winslow *et al.*, 1992). The purpose of human relaxin-1, encoded by the *RLN1* gene, is currently unknown, although it is known to be expressed in some tissues including the decidua, placental trophoblast, and prostate, but *not* the corpus luteum of the ovary (Hansell et al., 1991). The gene for relaxin contains an intron within the region encoding the C chain (Hudson et al., 1983), which is in a similar position to an intron found in the gene for insulin (Bell et al., 1980).

In the early 1990s, the X-ray crystal structure of relaxin was solved, confirming that relaxin has a similar fold to insulin, although the two peptides have different mechanisms of binding to their receptors (Eigenbrot et al., 1991). One notable feature of the relaxin B chain is the presence of a conserved RXXRXXI/V motif, referred to as the arginine binding cassette, which is important for binding of relaxin to its receptor (Bullesbach & Schwabe, 1991; Bullesbach & Schwabe, 2000; Bullesbach & Schwabe, 2005; Bullesbach et al., 1992). The A chain of relaxin, on the other hand, is less conserved between species, but appears important for providing the correct structure for the B chain to bind to the receptor (Bullesbach & Schwabe, 1994; Hossain *et al.*, 2008b; Sethi *et al.*, 2016). The solution NMR structure of relaxin (Haugaard-Kedstrom *et al.*, 2015) is shown in **Figure 1.4**.

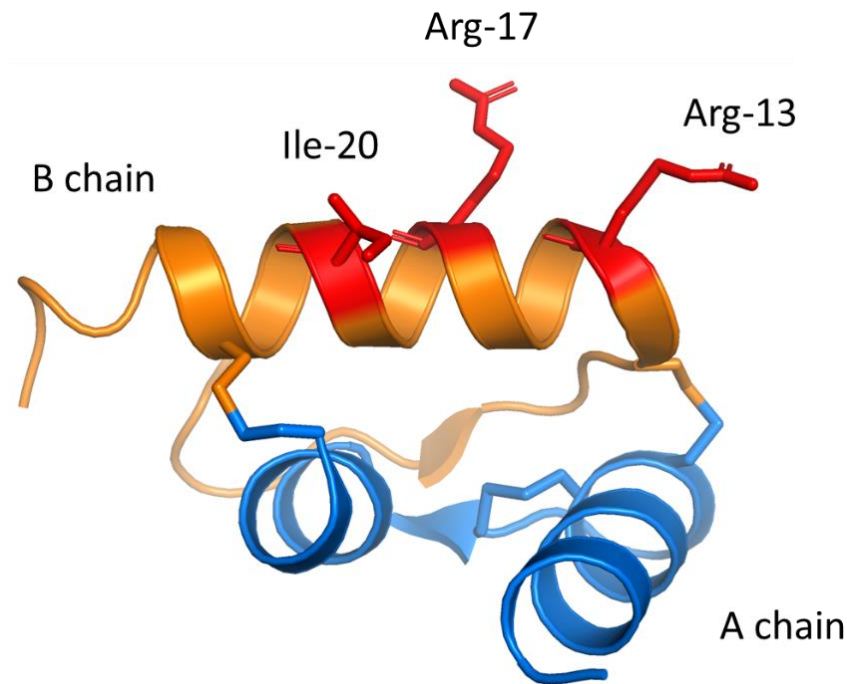


Figure 1.4. Solution NMR structure of relaxin-2 (PDB: 2MV1). The human relaxin-2 B chain is coloured orange, and the A chain is coloured blue. The arginine binding cassette of the B chain is highlighted in red. Two inter-chain and one intra-A-chain disulfide bonds are also shown.

1.6.2 Insulin-like peptide 3

The gene for INSL3, also known as Leydig insulin-like peptide or relaxin-like factor, was discovered in the 1990s by screening a cDNA library from boar testis (Adham et al., 1993). INSL3 was suggested to be part of the insulin superfamily due to similarities in organisation of the peptide (Adham et al., 1993). INSL3 was soon shown to be present in Leydig cells of the mouse (Pusch et al., 1996) and human testis (Ivell et al., 1997). INSL3 is also expressed in the female, including in the corpus luteum of the human ovary (Tashima et al., 1995). In the male, INSL3 is important for testis descent, as INSL3 knockout mice displayed cryptorchidism (impaired descent of the testes) due to abnormalities in the development of the gubernaculum, a ligament involved in gonadal descent (Nef & Parada, 1999; Zimmermann et al., 1999). INSL3 is produced at lower levels in the female, but is also present in reproductive tissues, including the ovary of the mouse, bovine, and human (Balvers et al., 1998; Bamberger et al., 1999; Bathgate et al., 1996). In females, INSL3 also has important roles in reproduction, as INSL3 knockout mice have impaired fertility associated with a longer oestrus cycle (Nef & Parada, 1999) and accelerated decay of the corpus luteum (Spaniel-Borowski et al., 2001).

1.6.3 Insulin-like peptide 4

Insulin-like peptide 4, originally known as early placenta insulin-like peptide or placentin, was discovered by two groups in the mid-1990s, by screening cDNA libraries from first trimester human placenta (Chassin et al., 1995; Koman et al., 1996). INSL4 is highly expressed in early placenta (Chassin et al., 1995), but apart from that not much is known.

1.6.4 Insulin-like peptide 5

Insulin-like peptide 5 was discovered in 1999 by two groups searching expressed sequence tag (EST) databases for conserved relaxin/insulin motifs (Conklin et al., 1999; Hsu, 1999). INSL5 was shown to be expressed in human rectum, colon, and uterus (Conklin et al., 1999), and is now recognised as a gastrointestinal hormone expressed by colonic enteroendocrine L-cells (Grosse et al., 2014; Thanasupawat et al., 2013). Studies on INSL5 knockout mice or INSL5 receptor knockout mice have revealed that INSL5 is involved in glucose homeostasis and promotion of appetite (Burnicka-Turek et al., 2012; Grosse et al., 2014; Lee et al., 2016b; Luo et al., 2015). Additionally, administration of an INSL5 analogue increased colon motility in mouse models of constipation (Diwakarla *et al.*, 2020).

1.6.5 Insulin-like peptide 6

Insulin-like peptide 6 was discovered by three groups in 1999 and 2000, by searching the EST database for conserved relaxin/insulin motifs in the mouse and human (Hsu, 1999; Kasik et al., 2000; Lok et al., 2000). In the human, INSL6 was shown to be expressed in the testis (Hsu, 1999; Lok et al., 2000). Male INSL6 knockout mice showed impaired spermatogenesis and therefore reduced fertility, whereas females remained fertile (Burnicka-Turek et al., 2009). Similarly, a human male with a missense mutation in the *INSL6* gene, which likely disrupted processing of the prohormone, had spermatogenic failure (Chen et al., 2011).

1.6.6 Relaxin-3

In the early 2000s, a novel relaxin gene was discovered after screening the human genome database for relaxin homologs (Bathgate et al., 2002). Although the peptide had the highest homology with INSL5, it was classified as a relaxin peptide rather than an insulin-like peptide due

to the presence of the RXXXXXXI motif on the B chain (Bathgate et al., 2002). The peptide was therefore named relaxin-3 (H3 relaxin), as it was the third human relaxin gene to be discovered (Bathgate et al., 2002). Although relaxin-3 is expressed across a number of tissues it is primarily located in the brain, in particular the nucleus incertus of the pons (Bathgate et al., 2002; Burazin et al., 2002; Ma et al., 2007). Relaxin-3 functions as a neuropeptide, and is involved in a range of physiological processes including feeding, metabolism, motivated behaviours, stress, and anxiety (Ma et al., 2017).

1.7 The relaxin family peptide receptors

1.7.1 Discovery of relaxin and insulin-like peptide receptors

Due to the structural similarities between relaxin and insulin, it was initially thought that the relaxin receptor would be a tyrosine kinase, as with the insulin receptor. In fact, tyrosine phosphorylation of a 220 kDa protein was observed following treatment of human lower uterine segment fibroblasts with relaxin, and this protein was proposed to be the relaxin receptor (Palejwala et al., 1998). However, when it was shown that INSL3 knockout mice show cryptorchidism, which also occurs as a result of mutation to the gene for the G protein-coupled receptor affecting testis descent (GREAT) in mice, it was suggested that relaxin-related peptides may activate GPCRs instead (Gorlov et al., 2002; Zimmermann et al., 1999). In the early 2000s, the receptors for relaxin and INSL3 were deorphanised. Leucine-rich repeat-containing GPCR 7 (LGR7) and LGR8 were expressed in HEK293T cells and stimulated with porcine relaxin, which produced concentration-dependent increases in cAMP activity, suggesting that these GPCRs are cognate relaxin receptors and likely couple to $G\alpha_s$ (Hsu et al., 2002). However, it was later shown that LGR8, the human equivalent of GREAT, is activated by INSL3, consistent with the previous finding on cryptorchidism (Kumagai et al., 2002). In fact, LGR8 is the only receptor for INSL3, and relaxin does not bind to LGR8 in all species (Scott et al., 2005), demonstrating that LGR8 is not a true relaxin receptor. Soon after deorphanisation of LGR7 and LGR8, the orphan receptors GPCR135 and GPCR142 were demonstrated to be the endogenous receptors for relaxin-3 and INSL5, respectively (Liu et al., 2003b; Liu et al., 2005b).

1.7.2 Classification of relaxin and insulin-like peptide receptors

The four identified relaxin family peptide receptors have now been re-classified as RXFP1 (LGR7), RXFP2 (LGR8), RXFP3 (GPCR135), and RXFP4 (GPCR142) (Bathgate et al., 2006b). The receptors for INSL4 and INSL6 have not yet been identified, and these peptides do not interact with any of the identified relaxin family peptide receptors. In contrast, there is overlap of binding and activation of relaxin family peptide receptors by their ligands. As noted, relaxin from some species can bind to and activate RXFP2, albeit with lower affinity. Relaxin can also bind to RXFP3, and is a biased ligand at this receptor compared to relaxin-3 (**section 1.10.2**) (Kocan et al., 2014; van der Westhuizen et al., 2010). Relaxin-3 can also bind to and activate RXFP1 (Bathgate et al., 2002), rat RXFP2 (Scott et al., 2005), and RXFP4 (Liu et al., 2003a). INSL5 can bind to RXFP3, but is a weak antagonist (Liu *et al.*, 2005b). However, as these peptides and receptors show different expression patterns, this overlap in binding and activation does not necessarily occur endogenously.

The four relaxin family peptide receptors are rhodopsin-like GPCRs (**Figure 1.5**). RXFP1 and RXFP2 belong to the δ subclass of rhodopsin class receptors, and are further grouped into the LGR family (Fredriksson et al., 2003). The LGR subfamily of GPCRs is characterised by a large extracellular domain composed of repeating leucine-rich motifs and a hinge region that connects the extracellular domain to the TMD (Van Loy et al., 2008). The LGRs are divided into three classes: type A LGRs are the glycoprotein hormone receptors, namely follicle-stimulating hormone receptor (FSH receptor), LH receptor, and TSH receptor; type B LGRs are the three R-spondin receptors (LGR4-6); and type C LGRs are RXFP1 and RXFP2. RXFP1 and RXFP2 are notable members of the LGR family because they are the only two mammalian GPCRs known to contain a low-density lipoprotein class A (LDLa) module at the C-terminus (Petrie et al., 2015). RXFP3 and RXFP4, on the other hand, belong to the γ subclass of rhodopsin receptors, do not have large ectodomains, and are more similar to small peptide GPCRs. RXFP3 shares relatively high structural similarity with the somatostatin and angiotensin peptide receptors (Matsumoto et al., 2000), whereas RXFP4 was initially thought to be a receptor for bradykinin and shows high homology to RXFP3 (Boels & Schaller, 2003).

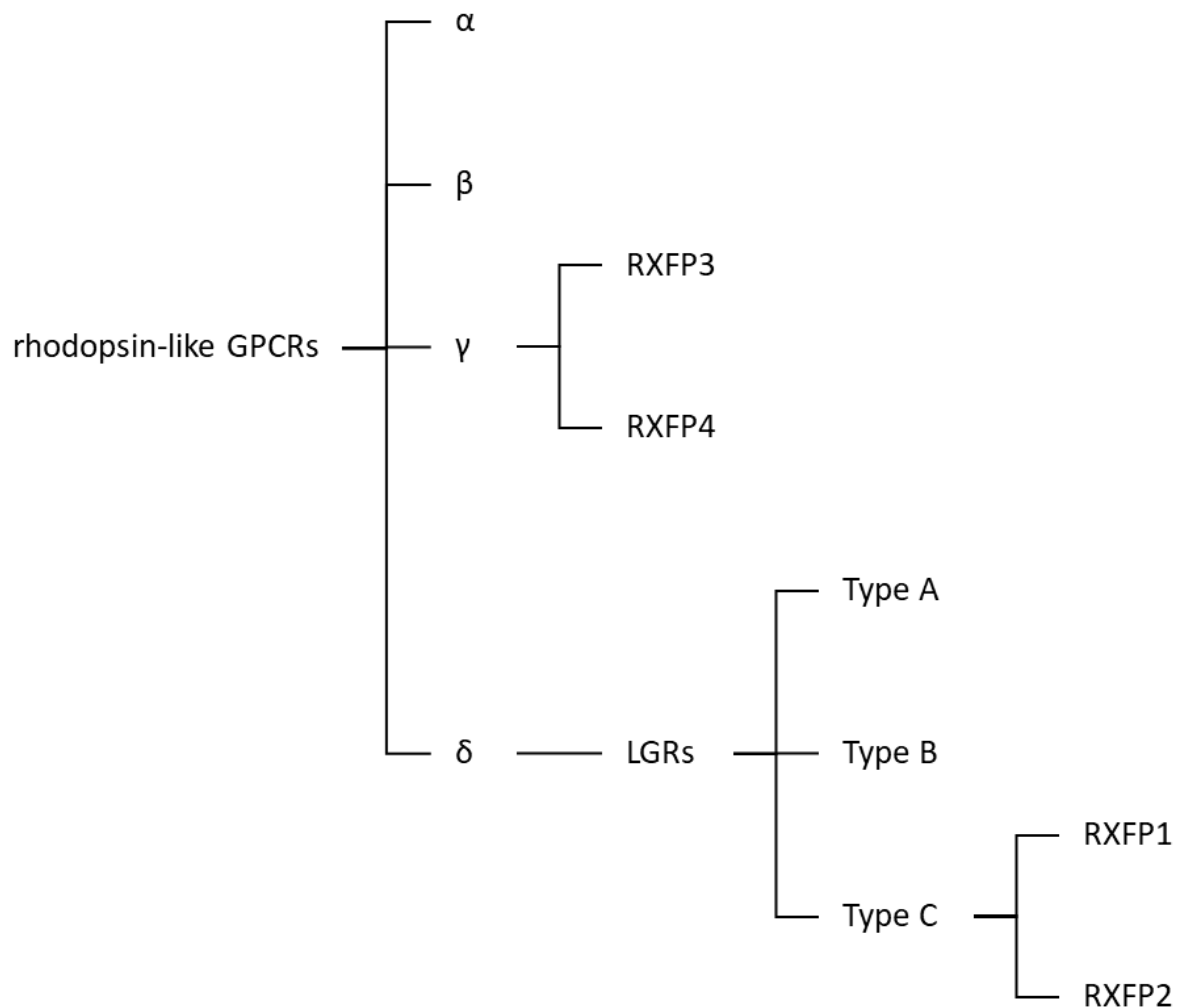


Figure 1.5. Classification of the relaxin family peptide receptors. RXFP1 and RXFP2 are in the Type C group of the leucine-rich repeat-containing GPCR (LGR) subfamily of the δ branch of rhodopsin-like GPCRs, whereas RXFP3 and RXFP4 are classified as part of the γ group of rhodopsin-like GPCRs.

1.8 Structure and activation of RXFP1

As a Type C LGR, RXFP1 contains a large extracellular domain consisting of ten LRRs and an N-terminal LDLa module (**Figure 1.6**). The LDLa module of RXFP1 is tethered to the LRR region by an unstructured, 32-residue linker. There are also a variety of splice variants of RXFP1, but the physiological implications of these are unknown (Kern et al., 2008; Lowndes et al., 2006; Muda et al., 2005; Scott et al., 2006).

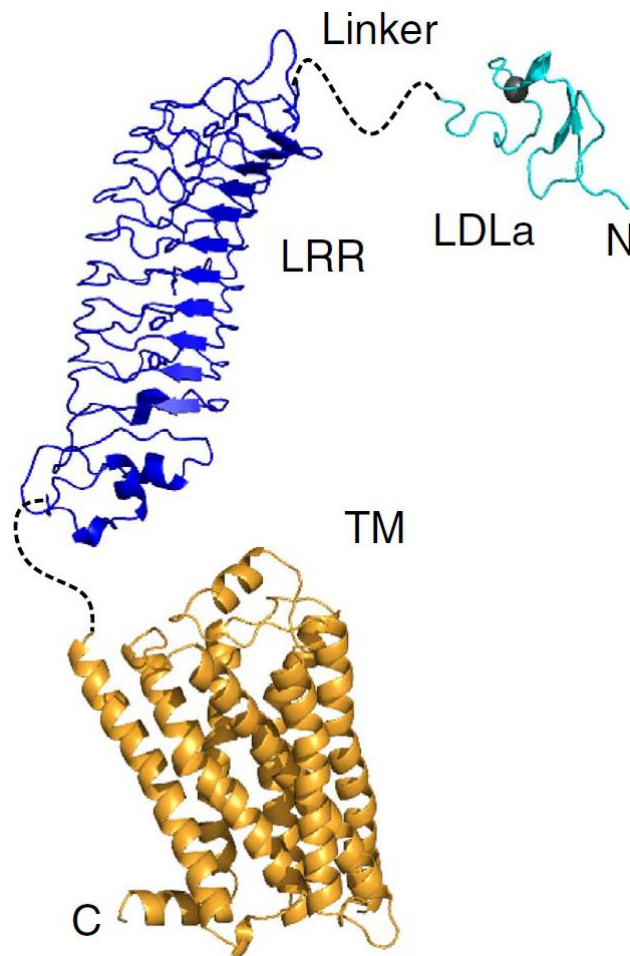


Figure 1.6. Model of the structure of RXFP1. The C terminus (C), transmembrane (TM) domains, leucine-rich repeats (LRR), linker, low-density lipoprotein class A module (LDLa), and N terminus (N) are labelled. Figure from Sethi *et al.* (2016).

1.8.1 Intracellular loops and C-terminal tail

As with many other GPCRs, ICL3 is important for RXFP1 coupling to $G\alpha_s$ (Shpakov et al., 2007), whereas the C-terminal tail of RXFP1 is important for RXFP1 coupling to $G\alpha_{i3}$ (Halls et al., 2009). Unlike most GPCRs, RXFP1 is only weakly phosphorylated after agonist stimulation (Callander et al., 2009).

1.8.2 Leucine-rich repeats

The LRRs contain five N-linked glycosylation sites (Yan et al., 2008), and also contain the high-affinity binding site for relaxin. In particular, the arginine binding cassette of the relaxin B

chain binds to LRR4-8 of RXFP1 (Bullesbach & Schwabe, 2005). However, as described below, binding of relaxin to the LRRs is not in itself sufficient for activation of the receptor.

1.8.3 Transmembrane domain

Chimeric receptors of RXFP1 and RXFP2 have been engineered to understand the mechanism of activation of RXFP1 (Sudo et al., 2003). Using relaxin-3 (which is selective for RXFP1 over RXFP2) at a chimeric receptor containing the ectodomain from RXFP1 and TMD from RXFP2 showed that ligand binding and signalling still occurs, but with lower potency than at wild type RXFP1, suggesting that the RXFP1 TMD is also involved in binding of relaxin (Sudo et al., 2003). Similarly, re-introducing exoloop 2 of the RXFP1 TMD, but not exoloop 1 or 3, into this RXFP1/2 chimera restored binding and cAMP activity (Sudo et al., 2003). It was therefore believed that relaxin also interacts with exoloop 2 of the TMD of RXFP1. NMR studies using a soluble scaffold consisting of exoloop 1 and exoloop 2 of RXFP1 also suggested that interactions occur between relaxin and the RXFP1 TMD (Diepenhorst et al., 2014).

1.8.4 Low-density lipoprotein class A module and linker

The LDLa module of RXFP1 contains three disulfide bonds, ligates Ca^{2+} to maintain its structure (Hopkins et al., 2005), and contains one N-linked glycosylation site (Yan et al., 2008). The structure of the LDLa module has been solved by solution NMR spectroscopy, and this is currently the only part of RXFP1 to have a solved structure (Hopkins et al., 2007). The LDLa module is essential for relaxin-mediated activation of RXFP1 to occur, as receptors lacking the LDLa module are unable to signal in response to relaxin via any pathway tested, even though the truncated receptor binds relaxin with comparable affinity to wild type RXFP1 and is expressed on the cell surface to the same degree (Kong et al., 2013; Scott et al., 2006). Substitution of the LDLa module with the structurally-similar second ligand binding domain of the LDL receptor (LB2), to create an RXFP1-LB2 chimera, produced the same effect as truncation (Hopkins et al., 2007). As the structurally-similar domain was not interchangeable, this suggested that there are key residues within the RXFP1 LDLa module that are important for activation of the receptor (Hopkins et al., 2007). Site-directed mutagenesis of the LDLa module was therefore conducted to identify key residues involved in RXFP1 activation. Gain-of-function mutations were introduced into the RXFP1-LB2 chimera. In particular, the conserved stretches of residues SLGYFP and NITK were introduced to create an SLGYFP-NITK RXFP1-LB2 chimera, which partially restored cAMP activity (Kong et al.,

2013). Complementary loss-of-function mutations introduced into wild type RXFP1 revealed that Leu-7, Tyr-9, and Lys-17 form a binding surface that contributes to the activation of RXFP1 by hydrophobic protein-protein interactions with the exoloops of the RXFP1 TMD after relaxin is bound to the receptor (Diepenhorst et al., 2014; Kong et al., 2013).

The 32-residue linker that connects the LDLa module to the LRRs has recently been shown to be important for binding and activation of RXFP1. Chimeras that were created by swapping the LDLa modules of RXFP1 and RXFP2 showed that maintaining the length of the RXFP1 linker was important for activation of the receptor (Bruell et al., 2013). The role of the linker was investigated further by mutagenesis, which showed that mutating conserved RXFP1/RXFP2 linker residues in RXFP1 resulted in a substantial reduction in relaxin binding and activation of RXFP1 (Sethi et al., 2016). Site-directed mutagenesis and NMR spectroscopy suggested that the linker has two regions: one region involved in binding relaxin, and one region involved in receptor activation (Sethi et al., 2016).

1.8.5 Mechanism of RXFP1 activation

The current model of relaxin-mediated RXFP1 activation suggests that relaxin binds to the LRR region and linker, which stabilises a helical conformation of the linker resulting in the LDLa-linker interacting with the TMD to drive receptor activation (Sethi et al., 2016) (**Figure 1.7**). In this way, the LDLa-linker is considered to be the “true ligand” for RXFP1, as it is responsible for inducing the conformational changes required for G protein coupling (Sethi et al., 2016). The most recent unpublished data suggest that the B chain alone interacts with the LRRs and the linker domain but that the A chain is required to provide the appropriate conformation of the B chain to bind to RXFP1.

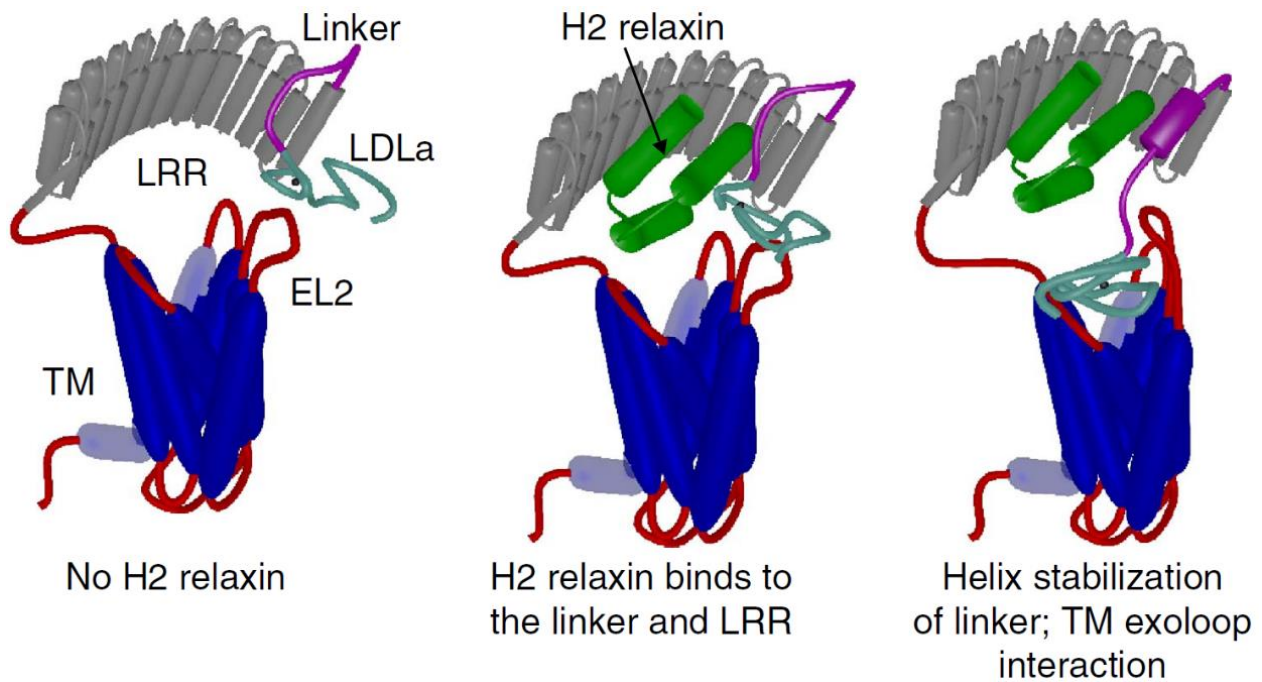


Figure 1.7. Model of relaxin-mediated activation of RXFP1. Relaxin binds to the leucine-rich repeat (LRR)-linker region, which stabilises a helix within the linker. The LDLa-linker interacts with the transmembrane (TM) domain, leading to receptor activation. Figure from Sethi *et al.* (2016).

1.9 RXFP1 signal transduction

Relaxin binding to RXFP1 induces interactions between RXFP1 and $G\alpha_s$, $G\alpha_i$, and $G\alpha_o$, activating networks of signal transduction pathways including cAMP, nitric oxide (NO)/cyclic guanosine monophosphate (cGMP), PI3K/Akt, MAPKs, and PKC. Furthermore, signalling downstream of RXFP1 shows functional crosstalk with signalling from other receptors, most notably the angiotensin II receptors and transforming growth factor beta 1 (TGF- β_1) receptor. Importantly, the signalling pathways activated and crosstalk depend on the type of cell being stimulated, and whether the relaxin stimulation is acute (up to one hour) or chronic (24-96 hours). A more detailed and complete description of the intracellular signal transduction pathways activated by relaxin/RXFP1 can be found in **Appendix A**.

1.9.1 Cyclic adenosine monophosphate

Acute relaxin stimulation increases cAMP activity in several cell types, including cells from human reproductive tissue such as normal human endometrial (NHE) cells (Fei et al., 1990), endometrial stromal cells (ESCs) (Bartsch et al., 2001), and myometrial cells (Heng et al., 2008). Relaxin also acutely increases cAMP activity in some human cancer cell types such as THP-1 monocytic leukaemia cells (Parsell et al., 1996), MCF-7 breast cancer cells (Bigazzi et al., 1992), and some glioblastoma cells (Glogowska et al., 2013). Furthermore, relaxin acutely raises cAMP levels in some primary vascular cell types including human umbilical vein endothelial cells (HUVECs), human umbilical vein smooth muscle cells (HUVSMCs), and human umbilical artery smooth muscle cells (HUASMCs) (Sarwar et al., 2015). Notably, although relaxin/RXFP1 activates other signalling pathways in fibroblasts, most fibroblasts tested show little or no cAMP activity after stimulation with relaxin (Huang et al., 2011; Mookerjee et al., 2009; Palejwala et al., 1998; Samuel et al., 2004a; Sarwar et al., 2015). However, there are a few exceptions, as human hepatic stellate cells (HSCs) and LX-2 human hepatic stellate cells are reported to show increases in cAMP activity after acute relaxin stimulation (Fallowfield et al., 2014; Singh et al., 2015).

RXFP1 increases cAMP activity via coupling to $G\alpha_s$ in several cell types, which is negatively modulated by coupling to $G\alpha_{oB}$ (Halls et al., 2006; Sarwar et al., 2015). Interestingly, in some cell types there is also a delayed increase in cAMP mediated via $G\alpha_{i3}$ - $\beta\gamma$ -PI3K, followed by translocation of PKC- ζ and activation of AC5, which occurs 15-20 minutes after stimulation (Halls et al., 2006; Nguyen & Dessauer, 2005a; Nguyen & Dessauer, 2005b; Nguyen et al., 2003). This delayed phase of cAMP may be linked to the ability of RXFP1 to localise into lipid rafts after relaxin stimulation, as seen when RXFP1 is expressed in HEK293T cells (Halls *et al.*, 2009). However, the influence of $G\alpha_s$, $G\alpha_i$, and $G\alpha_o$ on the cAMP response differ depending on cell type. For example, cAMP concentration-response curves for relaxin in HUVECs and HUVSMCs were bell-shaped and sensitive to pharmacological inhibition of $G\alpha_{i/o}$ or PI3K, whereas in HUASMCs the cAMP concentration-response curve was sigmoidal and not affected by pharmacological inhibition of $G\alpha_{i/o}$ or PI3K (Sarwar et al., 2015). Additionally, in THP-1 cells, pharmacological inhibition of phosphotyrosine phosphatases led to increased cAMP activity after relaxin stimulation, suggesting some involvement of tyrosine kinases in the cAMP response (Bartsch *et al.*, 2001). It was suggested that tyrosine kinases regulate PDE activity leading to increased cAMP production after stimulation with relaxin (Bartsch *et al.*, 2001).

1.9.1.1 Biological effects

Relaxin-induced cAMP activity has been linked to increases in markers of decidualisation in ESCs, consistent with a role for relaxin in the maintenance of pregnancy (Bartsch et al., 2004; Huang et al., 1987; Telgmann et al., 1997). The ability of relaxin to induce proliferation of cancer cells may also be related to cAMP activity (Bigazzi et al., 1992; Vinall et al., 2011). Upregulation of vascular endothelial growth factor (VEGF) after relaxin treatment is also thought to be related to increases in cAMP activity in some cell types such as NHE cells (Unemori et al., 1999), and might be involved in wound healing (Unemori et al., 2000) and chronic vasodilation (McGuane et al., 2011a), depending on cell type. However, it should be noted that relaxin treatment also upregulates VEGF in cell types that do not signal via cAMP, such as human cardiac fibroblasts (HCFs) (Sarwar et al., 2015). Finally, the $G\alpha_i$ -mediated phase of cAMP is thought to be physiologically relevant, playing a role in chronotropic and inotropic effects of relaxin in rat atria (Kompa et al., 2002), and inotropic effects in human atrial preparations, particularly in failing atria where $G\alpha_{i2}$ and $G\alpha_{i3}$ were upregulated (Dschietzig et al., 2011).

1.9.1.2 Sustained cAMP activity and regulation of RXFP1

In the absence of PDE inhibitors, acute relaxin treatment leads to cAMP activity that is sustained for at least three hours in ESCs (Bartsch et al., 2001), again consistent with a role for relaxin in maintenance of pregnancy. Similarly, relaxin signals via cAMP for at least six hours in HEK293T cells overexpressing RXFP1 (HEK-RXFP1 cells) (Callander et al., 2009). This sustained cAMP activity is consistent with the finding that RXFP1 does not couple to β -arrestin and shows weak or no agonist-mediated desensitisation and internalisation (Callander et al., 2009; Kern & Bryant-Greenwood, 2009).

1.9.2 Phosphatidylinositol-4,5-bisphosphate 3-kinase and Akt

PI3K and Akt are kinases that are primarily involved in cell survival, growth, and proliferation, as well as metabolism and protein synthesis (Manning & Toker, 2017). PI3K can be activated by $G\beta\gamma$, receptor tyrosine kinases, or Ras GTPases (Vanhaesebroeck et al., 2010). Once activated, class I PI3Ks phosphorylate the membrane lipid PIP_2 , yielding PIP_3 , which subsequently binds to the pleckstrin homology (PH) domain of Akt. Akt is then phosphorylated at Thr-308 by phosphoinositide-dependent protein kinase 1 (PDK1) and at Ser-473 by mammalian target of rapamycin complex 2 (mTORC2) or DNA-dependent protein kinase (DNA-PK), after which Akt

becomes fully active (Manning & Toker, 2017). PI3K/Akt signalling is negatively modulated by phosphatases for PIP₃ and Akt.

Relaxin stimulation acutely increases PI3K and/or Akt activity in cancer cells such as THP-1 cells (Nguyen et al., 2003) and prostate cancer cells (Liu et al., 2008), as well as in aortic endothelial cells (McGuane et al., 2011b), neonatal rat cardiomyocytes (Aragon-Herrera et al., 2018), and rat Sertoli cells (Nascimento et al., 2012). Chronic relaxin treatment increases PI3K/Akt activity in human glioblastoma cells (Glogowska et al., 2013), mouse embryonic fibroblasts, rat cardiomyoblasts (Boccalini et al., 2018), and rat aortic endothelial cells (Dschiezig et al., 2012).

Pharmacological inhibition of PI3K has confirmed that acute relaxin-mediated Akt activation is dependent on PI3K, as expected (McGuane et al., 2011b). Furthermore, studies in HEK-RXFP1 cells showed that PI3K/Akt activity is downstream of G α_{i3} - $\beta\gamma$ (Halls et al., 2006). However, another study using mesenteric arteries from G α_{i2} and G α_{i3} knockout mice showed that G α_{i2} rather than G α_{i3} is involved in the biological effects of relaxin (Lian et al., 2018), thus the G protein isoforms involved in activation of the PI3K/Akt pathway may be cell- and/or species-specific. Furthermore, crosstalk between Akt and ERK1/2 can occur in some cell types, such as rat Sertoli cells (Nascimento et al., 2012).

1.9.2.1 Biological effects

Relaxin is thought to have cytoprotective actions against injury via PI3K/Akt across several cell types and tissues, including rat cardiomyocytes (Moore et al., 2007), cytotrophoblasts (Ogunleye et al., 2017), proximal tubule epithelial cells (Xie et al., 2017), and rat aortic rings (Dschiezig et al., 2012). PI3K/Akt might also be involved in renoprotective actions of relaxin (Collino et al., 2013) and migration of glioblastoma cells (Glogowska et al., 2013). Additionally, as described below (**section 1.9.2.1**), the vasodilatory actions of relaxin depend on PI3K/Akt activity.

1.9.3 Nitric oxide and cyclic guanosine monophosphate

Nitric oxide is a signalling molecule synthesised from L-Arginine by any of the three isoforms of nitric oxide synthase (NOS): endothelial NOS (eNOS), inducible NOS (iNOS), and neuronal NOS (nNOS). NOS can be activated downstream of PI3K/Akt. eNOS, for example, is activated after phosphorylation at Ser-1177 (in humans) by Akt (Fulton et al., 1999). Nitric oxide can then be used for post-translational modifications (Ischiropoulos & Gow, 2005), but also produces effects via activation of soluble guanylate cyclase (sGC), leading to the synthesis of cGMP

from GTP (Derbyshire & Marletta, 2012). cGMP activates protein kinase G (PKG) and some cyclic nucleotide-gated ion channels, and is hydrolysed by PDEs (Francis et al., 2010). NO/cGMP activity is of particular importance in the physiology of the cardiovascular and nervous systems (Feil et al., 2005; Hofmann et al., 2004; Tsai & Kass, 2009). For example, in the vascular system NO/cGMP activity produces acute vasodilation via PKG phosphorylation of several targets leading to decreased mobilisation of calcium (Francis et al., 2010).

Relaxin stimulation acutely increases the production of NO in some cell types, but can also induce upregulation and chronic activation of NOS. Acute relaxin stimulation increases production of NO in human bone marrow-derived endothelial cells (Segal et al., 2012) and human coronary artery endothelial cells (McGuane et al., 2011b). Relatedly, acute relaxin stimulation increases production of cGMP in rat renal myofibroblasts (Wang et al., 2016), human HSCs (Fallowfield et al., 2014), HUVECs, HUVSMCs, HUASMCs, and HCFs (Sarwar et al., 2015). Concentration-response curves for cGMP were bell-shaped in HUVECs, HUVSMCs, and HCFs, but became sigmoidal after pharmacological inhibition of $G\alpha_{i/o}$ or lipid rafts, again suggesting a role for lipid rafts in RXFP1 signalling downstream of $G\alpha_i$ (Sarwar et al., 2015).

Chronic relaxin treatment increases chronic eNOS phosphorylation in rat aortic rings (Dschiezig et al., 2012) and tissue from fibrotic rat liver (Fallowfield et al., 2014), upregulates nNOS in rat renal myofibroblasts (Chow et al., 2012; Chow et al., 2014; Mookerjee et al., 2009), HUVECs, HUVSMCs, HUASMCs, and HCFs (Sarwar et al., 2015), induces chronic nNOS phosphorylation in HCFs (Wang et al., 2020), and upregulates iNOS in bovine artery smooth muscle cells (Bani et al., 1998) and rat coronary endothelial cells (Failli et al., 2002). Similarly, relaxin produces chronic increases in cGMP production in bovine artery smooth muscle cells (Bani et al., 1998), idiopathic pulmonary fibrosis (IPF)-derived lung myofibroblasts (Huang et al., 2011), tissue from fibrotic mouse kidney (Wetzel et al., 2016), and tissue from fibrotic rat liver (Fallowfield et al., 2014). Relatedly, relaxin was confirmed to lead to activation of PKG in IPF-derived lung myofibroblasts (Huang et al., 2011) and healthy or fibrotic mouse kidney tissue (Wetzel et al., 2016).

1.9.3.1 Biological effects

Pharmacological inhibition of NOS, sGC, and other pathways outlined above has revealed biological actions of relaxin-mediated NO/cGMP activity. Importantly, the ability of relaxin to cause acute vasodilation occurs via a $G\alpha_i$ - $\beta\gamma$ -PI3K-Akt-eNOS-NO-dependent pathway (Lian et al., 2018; McGuane et al., 2011b). Similarly, the ability of relaxin to cause sustained vasodilation also occurs via NO (McGuane et al., 2011a; Novak et al., 2002), but involves crosstalk with other receptors and

proteins including VEGF, gelatinases, and the endothelin B receptor (Leo *et al.*, 2017; Sarwar *et al.*, 2016a).

Relaxin also has chronic vasoprotective effects associated with eNOS/NO activity. For example, relaxin abated vascular dysfunction induced by tumour necrosis factor alpha (TNF- α) in rat aortic rings, which was partly related to eNOS activity (Dschietzig *et al.*, 2012). Similarly, upregulation and activation of eNOS might also contribute towards the vasoprotective actions of relaxin against cigarette smoke observed in guinea pigs (Pini *et al.*, 2016). Similarly, relaxin has protective actions against ischemia/reperfusion injury in rat lung, which is dependent on iNOS upregulation downstream of PI3K and ERK1/2 (Alexiou *et al.*, 2013).

NO/cGMP activity is also key for the actions of relaxin in fibroblasts, which is described in more detail below (**section 1.9.6**). Importantly, as pharmacological inhibition of sGC blocks the actions of relaxin in fibroblasts, this suggests that NO has its full effect via activation of sGC (Chow *et al.*, 2012; Mookerjee *et al.*, 2009; Wang *et al.*, 2016).

1.9.4 Mitogen-activated protein kinases

The MAPK family of Ser/Thr kinases includes ERK1/2, JNK, p38 MAPK, and ERK3-8 (Pearson *et al.*, 2001). MAPKs are involved in cell survival and apoptosis, cell proliferation, immune cell function, synaptic plasticity, memory, and learning (Chang & Karin, 2001; Rincon & Davis, 2009; Thomas & Huganir, 2004). MAPK activation occurs as part of a signalling cascade involving phosphorylation of successive kinases, ultimately leading to phosphorylation and activation of the MAPK itself (Pearson *et al.*, 2001). MAPK activation can occur at various parts of signalling cascades, including downstream of receptor tyrosine kinases, G α , G $\beta\gamma$, and β -arrestins (Goldsmith & Dhanasekaran, 2007). MAPKs are inactivated by phosphatases.

1.9.4.1 Extracellular signal-regulated kinases 1 and 2 (ERK1/2)

Relaxin stimulation leads to acute ERK1/2 phosphorylation that returns to baseline by 30 minutes in NHE cells, human coronary artery and pulmonary artery smooth muscle cells, THP-1 cells (Zhang *et al.*, 2002), HUVMCs, HUASMCs, HCFs (Sarwar *et al.*, 2015), and rat Sertoli cells (Nascimento *et al.*, 2012). Additionally, relaxin produces acute but sustained (more than 60 minutes) ERK1/2 phosphorylation in HeLa cervical cancer cells and HUVECs (Dschietzig *et al.*, 2012; Sarwar *et al.*, 2015). In rat renal myofibroblasts, relaxin has shown both transient ERK1/2 phosphorylation (Hossain *et al.*, 2016), as well as ERK1/2 phosphorylation that was sustained for

at least one hour (Mookerjee et al., 2009). Chronic ERK1/2 phosphorylation also occurs after chronic treatment with relaxin. For example, increased ERK1/2 phosphorylation occurred after 72 hours of relaxin treatment in rat renal myofibroblasts (Chow et al., 2012; Chow et al., 2014) and human cardiac fibroblasts (Wang et al., 2020).

Notably, although acute sustained ERK1/2 activity is usually associated with β -arrestin-mediated signalling, relaxin binding does not promote interactions between RXFP1 and β -arrestins (Callander et al., 2009). Use of pharmacological inhibitors have shown that for RXFP1, $G\alpha_{i/o}$ and PI3K have a large role in mediating ERK1/2 phosphorylation in HUVECs, HUVSMCs, HUASMCs, HCFs (Sarwar et al., 2015), and rat Sertoli cells (Nascimento et al., 2012). However, in rat renal myofibroblasts, inhibition of $G\alpha_{i/o}$ actually enhanced ERK1/2 phosphorylation at two minutes (Mookerjee et al., 2009).

1.9.4.1.1 Biological effects

Pharmacological inhibition of ERK1/2 phosphorylation demonstrated that acute relaxin-mediated ERK1/2 activity is involved in the upregulation of VEGF in NHE cells (Zhang & Liu, 2002). Additionally, acute relaxin-mediated ERK1/2 activity is implicated in upregulation of the endothelin B receptor, which also has a role in the chronic vascular actions of relaxin (Dschietzig et al., 2003). As described below (**section 1.9.6**), chronic relaxin-mediated ERK1/2 activity has also been implicated in the anti-fibrotic roles of relaxin.

1.9.4.2 p38 MAPK

Stimulation with relaxin increases phosphorylation of p38 MAPK in HUVSMCs, HUASMCs (Kocan et al., 2017), LX-2 cells (Singh et al., 2015), and human vascular smooth muscle cells from mammary arteries (Dschietzig et al., 2003). The biological effects of this p38 MAPK activity are not yet understood.

1.9.5 Protein kinase C

PKC is a family of Ser/Thr kinases involved many processes including cell growth, proliferation, differentiation, carcinogenesis, immune cell function, and regulation of smooth muscle tone (Koivunen *et al.*, 2006; Tan & Parker, 2003; Webb *et al.*, 2000). As noted above, relaxin mediates a delayed phase of cAMP activity in some cell types, which occurs via activation of $G\alpha_i$ - $\beta\gamma$ -PI3K, followed by translocation of PKC- ζ to the plasma membrane (Halls et al., 2006; Nguyen &

Dessauer, 2005a; Nguyen & Dessauer, 2005b; Nguyen et al., 2003). Increased phosphorylation of PKC- ζ and PKC- δ was also observed after acute relaxin stimulation of glioblastoma cells, which was inhibited by pharmacological inhibition of PI3K (Glogowska et al., 2013). Although PKC activity is often associated with $G\alpha_q$ coupling, PKC- ζ is an atypical isoform that does not require DAG or Ca^{2+} to become activated (Hirai & Chida, 2003). Rather, PKC- ζ can be activated by lipids such as PIP_3 , which for RXFP1 is consistent with the activation of PKC- ζ downstream of PI3K. PKC- δ , on the other hand, is classified as a “novel” PKC isoform, which requires DAG but not Ca^{2+} (Webb *et al.*, 2000).

1.9.5.1 Biological effects

Pharmacological inhibition of PKC, PKC- ζ , and PKC- δ suppressed the migration of glioblastoma cells after relaxin stimulation (Glogowska *et al.*, 2013). PKC- δ is also involved in the ability of relaxin to increase murine cardiac myofilament activation, consistent with the inotropic effect of relaxin (Shaw et al., 2009).

1.9.6 Signalling pathways involved in the anti-fibrotic actions of relaxin

Fibrosis refers to a process whereby wound repair becomes pathogenic, involving excess build-up of extracellular matrix (ECM) components (Wynn, 2008; Wynn & Ramalingam, 2012). Fibrosis can be severe to the point of causing organ failure and death (Rockey et al., 2015). A key aspect of fibrosis is the activation of fibroblasts to ECM-secreting myofibroblasts, which is triggered by cytokines such as TGF- β_1 that are secreted by immune cells after injury. TGF- β_1 binds to a Ser/Thr kinase receptor on the plasma membrane, leading to phosphorylation of Smad protein effectors. Smad proteins function via modulation of gene transcription, which can lead to myofibroblast differentiation, downregulation of anti-fibrotic matrix metalloproteinases (MMPs) that normally break down collagen, and upregulation of pro-fibrotic tissue inhibitors of metalloproteinases (TIMPs) (Biernacka et al., 2011).

Chronic relaxin administration produces signalling via nNOS-NO-sGC-cGMP to inhibit the pro-fibrotic actions of TGF- β_1 both *in vitro* and *in vivo* (reviewed in Samuel et al., 2017; Samuel et al., 2016). It is also evident that iNOS is involved in the chronic anti-fibrotic actions of relaxin in some fibroblasts (Alexiou et al., 2013; Chow et al., 2012). Additionally, although ERK1/2 has also been implicated in the chronic anti-fibrotic actions of relaxin and is thought to be upstream of nNOS activation in fibroblasts (Chow et al., 2012), the small molecule RXFP1 agonist ML290 does

not acutely increase ERK1/2 phosphorylation, yet still demonstrates anti-fibrotic effects with a similar efficacy to relaxin in fibroblasts (Kocan et al., 2017). Notably, ML290 has not yet been tested for chronic activation of ERK1/2 phosphorylation or upregulation of ERK1/2 in fibroblasts.

RXFP1 signalling produces anti-fibrotic actions in several related ways. Specifically, chronic relaxin treatment inhibits differentiation of fibroblasts into myofibroblasts, inhibits collagen expression (Bennett et al., 2003; Mookerjee et al., 2009; Samuel et al., 2004a; Unemori & Amento, 1990; Wang et al., 2020), inhibits the actions of the pro-fibrotic Smad2 and Smad3 (Bennett et al., 2014; Heeg et al., 2005; Mookerjee et al., 2009; Sassoli et al., 2013; Wang et al., 2016), upregulates MMPs (Bennett et al., 2014; Bennett et al., 2003; Chow et al., 2012; Chow et al., 2019; Heeg et al., 2005; Unemori & Amento, 1990; Unemori et al., 1996; Wang et al., 2016), and downregulates TIMPs (Bennett et al., 2014; Bennett et al., 2003; Chen et al., 2014; Unemori & Amento, 1990). In general, cGMP/PKG can inhibit TGF- β signalling, leading to anti-fibrotic actions including inhibition of myofibroblast differentiation and inhibition of pSmad3 translocation (Schinner et al., 2017), which is consistent with the signalling pathways activated by chronic relaxin treatment in fibroblasts. It was also demonstrated in neonatal murine cardiac fibroblasts that anti-fibrotic actions of relaxin occur through crosstalk between RXFP1 and the single transmembrane receptor Notch-1 (Sassoli et al., 2013).

Additionally, it is clear that the anti-fibrotic actions of relaxin are mediated through functional crosstalk between RXFP1 and the angiotensin II type 1 and 2 receptors (AT₁ and AT₂ receptors) (Chow et al., 2014; Chow et al., 2019). Relaxin itself does not bind to angiotensin II receptors, but anti-fibrotic actions of relaxin were blocked by an AT₁ receptor antagonist in rat renal myofibroblasts, HCFs, and in rats *in vivo* (Chow et al., 2019; Wang et al., 2020), and by an AT₂ receptor antagonist in rat renal myofibroblasts and in mice *in vivo* (Chow et al., 2014). Functional crosstalk between all three receptors was demonstrated when antagonists for each receptor blocked the anti-fibrotic actions of relaxin or the AT₂ receptor agonist compound 21 in rat renal myofibroblasts or HCFs (Chow et al., 2019). Protein-protein interaction studies have also suggested that there are interactions between RXFP1 and the AT₁ (Chow et al., 2019) or AT₂ (Chow et al., 2014) receptors when expressed in HEK293 cells.

1.9.7 Other RXFP1 agonists

1.9.7.1 ML290

ML290 is a small molecule allosteric agonist of RXFP1 that was identified in a high-throughput screen of human RXFP1 (Chen *et al.*, 2013; Xiao *et al.*, 2013). ML290 binds to the TMD of RXFP1 rather than to the LRRs like relaxin, and shows no activation of RXFP2 (Huang *et al.*, 2015; Xiao *et al.*, 2013). Furthermore, ML290 acts as a biased agonist for RXFP1, relative to relaxin (Kocan *et al.*, 2017). ML290 is a full but low potency agonist for cAMP activity in HEK-RXFP1 and THP-1 cells, and like relaxin signals via cAMP and cGMP in HUVECs, HUVSMCs, and HUASMCs, albeit with weaker potency than relaxin, and also signals via cGMP but not cAMP in HCFs (Kocan *et al.*, 2017; Xiao *et al.*, 2013). However, although relaxin is equipotent for cAMP and cGMP activity in vascular cells, ML290 favours signalling via cGMP over cAMP by about ten-fold (Kocan *et al.*, 2017). Furthermore, whereas relaxin induces acute ERK1/2 phosphorylation in HEK-RXFP1 cells, HUVECs, HUVSMCs, HUASMCs, and HCFs, ML290 does not acutely increase ERK1/2 phosphorylation in any cell type tested (Kocan *et al.*, 2017). However, although ML290 does not acutely signal via ERK1/2, it has not yet been tested for ERK1/2 upregulation or chronic activation.

Like relaxin, ML290 produces a chronic anti-fibrotic response in fibroblasts such as HCFs (Kocan *et al.*, 2017) and HSCs (Kaftanovskaya *et al.*, 2019). ML290 does not activate rodent RXFP1, but transgenic mice expressing human RXFP1 have been developed, allowing ML290 to be tested in mouse models of human disease (Kaftanovskaya *et al.*, 2017). In mice expressing human RXFP1, ML290 showed anti-fibrotic actions in a mouse model of human liver fibrosis (Kaftanovskaya *et al.*, 2019).

1.9.7.2 B7-33

Due to their two-chain, three disulfide bond structure, relaxin family peptides are costly and laborious to produce. Furthermore, as noted above, the relaxin family peptides show cross-reactivity among the four receptors. Therefore, much effort has been put into the generation of simplified relaxin family peptides which are easier to synthesise but are specific for their receptor targets (Praveen *et al.*, 2019). B7-33 is a single-chain analogue of relaxin that binds to and activates RXFP1 but not RXFP2 (Hossain *et al.*, 2016). B7-33 appears to show cell-specific actions that are not yet understood, as it has similar activity to relaxin in some cell types such as fibroblasts and vascular cells, but appears to have weaker activity than relaxin in cancer cells and cells recombinantly

expressing RXFP1. Specifically, B7-33 is a full but low potency agonist for cAMP activity in HEK-RXFP1 and THP-1 cells relative to relaxin, and signals via ERK1/2 with low efficacy and potency in HEK-RXFP1 cells compared to relaxin (Hossain et al., 2016). Interestingly, however, in rat renal myofibroblasts relaxin signals via ERK1/2 with approximately equal potency and efficacy to relaxin, and also has chronic anti-fibrotic actions *in vitro* and *in vivo* (Hossain et al., 2016). Additionally, B7-33 has potent actions in arteries, where it mimics the actions of relaxin (Marshall et al., 2017). Unlike relaxin, B7-33 did not promote prostate tumour growth in mice, which may be related to its weak ability to increase cAMP levels (Hossain et al., 2016). The actions of B7-33 are studied in Chapter 5 of this thesis.

1.10 RXFP2, RXFP3, and RXFP4 signal transduction

1.10.1 RXFP2

INSL3 increases cAMP activity in HEK293T cells overexpressing RXFP2 (HEK-RXFP2 cells) (Halls et al., 2006), MG63 human osteoblast cells (Ferlin et al., 2008), and rat gubernacular cells (Kumagai et al., 2002). However, in oocytes and seminiferous tubular cells, INSL3 stimulation inhibited the production of cAMP (Kawamura et al., 2004). Relaxin also activates RXFP2 in some species (Scott *et al.*, 2005). For example, relaxin stimulation increases cAMP activity via RXFP2 in HEK cells expressing RXFP2, albeit with a lower potency than INSL3 (Halls *et al.*, 2009; Hsu *et al.*, 2002).

In HEK-RXFP2 cells, it was shown that RXFP2 increases cAMP activity via $G\alpha_s$, which is negatively modulated by $G\alpha_{oB}$ and $G\beta\gamma$ (Halls et al., 2006). RXFP2 does not couple to $G\alpha_{i3}$ and does not show the biphasic cAMP response seen at RXFP1 (Halls et al., 2006). INSL3 also increases ERK1/2 phosphorylation in osteoblasts (Ferlin et al., 2011). RXFP2 shows only weak internalisation and does not couple to β -arrestin in HEK293T or COS-7 cells expressing RXFP2, which, like RXFP1, provides an explanation for its sustained cAMP activity (Callander et al., 2009).

1.10.2 RXFP3

Relaxin-3 inhibits cAMP activity in CHO-K1 cells overexpressing RXFP3 (CHO-RXFP3 cells), HEK293T cells overexpressing RXFP3 (HEK-RXFP3 cells), and murine septal neuronal SN56 cells (Liu

et al., 2003b; van der Westhuizen et al., 2007). Protein-protein interaction studies confirmed that RXFP3 can couple to $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_{oA}$, and $G\alpha_{oB}$ when expressed in CHO-K1 cells (Kocan et al., 2014). Relaxin-3 also increases phosphorylation of ERK1/2 in HEK-RXFP3, CHO-RXFP3, and SN56 cells, and increases phosphorylation of p38 MAPK and JNK1/2 in CHO-RXFP3 cells (Kocan et al., 2014; van der Westhuizen et al., 2010; van der Westhuizen et al., 2007). Pharmacological inhibition of $G\alpha_{i/o}$ completely abolished ERK1/2 phosphorylation in HEK-RXFP3 cells, and almost completely abolished ERK1/2 phosphorylation in CHO-RXFP3 cells and SN56 cells (van der Westhuizen et al., 2007). Similarly, inhibition of PI3K and PKC inhibited ERK1/2 phosphorylation in CHO-RXFP3 and HEK-RXFP3 cells, suggesting these pathways are downstream of $G\alpha_{i/o}$ coupling (van der Westhuizen et al., 2007). RXFP3 also couples to β -arrestin1 and β -arrestin2 when expressed in CHO-K1 cells (Kocan et al., 2014), which is consistent with internalisation of RXFP3 after a 10-minute stimulation with relaxin-3 in CHO-RXFP3, HEK-RXFP3, and SN56 cells (van der Westhuizen et al., 2010). Coupling of RXFP3 to β -arrestins is also partially dependent on $G\alpha_{i/o}$ (Kocan et al., 2014).

Interestingly, relaxin is a biased agonist for RXFP3, relative to relaxin-3 (Kocan et al., 2014). Specifically, relaxin only induces coupling of RXFP3 to $G\alpha_{i2}$ and $G\alpha_{oB}$, does not induce coupling to β -arrestin when constructs are expressed in CHO cells (Kocan et al., 2014), and unlike relaxin does not lead to internalisation of RXFP3 in CHO-RXFP3, HEK-RXFP3, or SN56 cells (van der Westhuizen et al., 2010). Relaxin and relaxin-3 also produce different patterns of kinase phosphorylation and activation of downstream reporter genes (Kocan et al., 2014).

1.10.3 RXFP4

INSL5 inhibits cAMP activity in CHO-K1 cells overexpressing RXFP4 (CHO-RXFP4 cells) (Belgi et al., 2013), SK-N-MC human neuroblastoma cells stably expressing RXFP4 (Liu *et al.*, 2003a; Liu *et al.*, 2005b), and NCI-H716 colorectal adenocarcinoma cells (Ang et al., 2016). Studies using pertussis toxin (PTX)-sensitive G protein mutants in CHO-RXFP4 cells showed that RXFP4 can couple to $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_{oA}$, and $G\alpha_{oB}$ (Ang et al., 2016). INSL5 also induces ERK1/2 phosphorylation in CHO-RXFP4 cells (Ang et al., 2016; Belgi et al., 2013), HEK293 cells expressing RXFP4, and MIN6 mouse pancreatic beta cells (Luo et al., 2015). INSL5 also increases phosphorylation of Akt, p38 MAPK, and S6 ribosomal protein in CHO-RXFP4 cells (Ang et al., 2016) and NCI-H716 cells (Ang et al., 2018). INSL5-mediated kinase phosphorylation was shown to be downstream of $G\alpha_{i/o}$ via pharmacological inhibition of $G\alpha_{i/o}$ in CHO-RXFP4 cells, and is suggested to be responsible for the ability of INSL5 to stimulate proliferation of CHO-RXFP4 cells (Ang et al., 2016). Protein-protein interaction studies in CHO-K1 cells have shown interactions between RXFP4 and GRK2, β -arrestin1,

and β -arrestin2, suggestive of desensitisation and internalisation (Ang et al., 2016). This is consistent with the decrease in interactions between RXFP4 and membrane-localised KRas, and increase in interactions between RXFP4 and endosome-localised Rab5a, that occur after INSL5 stimulation (Ang et al., 2016).

1.11 RXFP1 as a therapeutic target

As outlined above (**section 1.5.2**), treatment with relaxin has beneficial cardiovascular and anti-fibrotic actions. Therefore, there has been a lot of interest in the use of relaxin as a therapeutic drug for cardiovascular and fibrotic diseases, as well as for some reproductive disorders (Unemori, 2017). Cloning of the *RLN2* gene allowed the production of recombinant human relaxin (often referred to as serelaxin), which enabled the use of human relaxin in clinical trials. After preclinical development and phase I clinical trials, relaxin has progressed to phase II and III clinical trials as a proposed treatment for several conditions. For example, intravenous relaxin was tested in a phase II clinical trial for cervical ripening in pregnant women who were to undergo induction of labour (Weiss et al., 2016). Relaxin failed to increase cervical ripening in these patients, which may be due to differences in relaxin physiology between humans and the animal models used (Jelinic et al., 2018). Another phase II clinical trial was the use of relaxin to treat moderate to severe systemic sclerosis (scleroderma), a chronic condition involving fibrosis of the skin and internal organs (Seibold et al., 2000). In this trial, a 24-week infusion of relaxin led to reduced skin thickness and increased mobility (Seibold et al., 2000). However, in a larger phase III trial, relaxin failed to improve scleroderma, as measured by skin thickness (Khanna et al., 2009).

Relaxin has also been under investigation as a treatment for heart failure. The first clinical trial in this area was for treatment of compensated heart failure, in which a range of doses of relaxin were infused over 24 hours to observe safety and tolerability (Dschiertzig et al., 2009). In this trial, relaxin caused vasodilation, improved renal function at lower doses, and was safe (Dschiertzig et al., 2009). Similarly, the preliminary study of relaxin in acute heart failure (Pre-RELAX-AHF) phase II clinical trial identified an appropriate dose of relaxin to use in further trials, and demonstrated that relaxin improved several clinical outcomes (Teerlink et al., 2009). Following on from this, the large, international relaxin in acute heart failure (RELAX-AHF) phase III clinical trial was initiated, which involved over 1100 AHF patients. A 48-hour infusion of relaxin was associated with reduced mortality at 180 days and improvement in clinical outcomes such as dyspnoea relief, but did not affect readmission to hospital (Teerlink et al., 2013). A *post hoc* analysis of the trial data

revealed that relaxin treatment was also associated with a reduction in markers of damage to the heart, liver, and kidneys, as well as improved relief from congestion (Metra et al., 2013). As reduced mortality was not a primary endpoint of the RELAX-AHF trial, a phase IIIb RELAX-AHF-2 trial involving over 6000 AHF patients was designed, in order to further investigate this beneficial action of relaxin (Teerlink et al., 2017). Unfortunately, the RELAX-AHF-2 trial did not meet its primary endpoints of reduced cardiovascular mortality and reduced worsening of heart failure (Metra et al., 2019). The initial positive results of the RELAX-AHF trial may have been due to chance, as the trial was not designed to discover mortality, and this was only discovered after exploration of the data (Metra et al., 2019). It has also been argued that AHF is not just a 48-hour illness, and therefore a 48-hour infusion of relaxin is insufficient to achieve a long-term therapeutic benefit anyway (McCullough, 2017; Metra et al., 2019). Regardless, a meta-analysis of six clinical studies involving treatment with intravenous relaxin administered within 16 hours of admission for AHF concluded that relaxin was safe and associated with reduction in five-day worsening of heart failure and a reduction in all-cause mortality, but did not reduce length of stay, cardiovascular death, or rehospitalisation for heart or renal failure (Teerlink et al., 2020).

Ever since relaxin was first administered to humans, it has been demonstrated to be safe and well tolerated, and relaxin is still generating therapeutic interest for a range of cardiovascular and fibrotic illnesses. For example, relaxin has been proposed as a potential therapeutic drug for heart failure with preserved ejection fraction (a subset of chronic heart failure) (Dschiezig, 2019), cardiovascular complications of diabetes (Ng et al., 2018), arthofibrosis (Blessing et al., 2019), preeclampsia (Conrad, 2016), renal dysfunction in cirrhosis (Snowdon et al., 2017), and fibrosis of the urinary bladder and ligaments (Kanai et al., 2019). Additionally, the use of an RXFP1 antagonist may also be of value for cancer treatment (Thanasupawat et al., 2019). However, there are several unresolved issues that must be addressed. One remaining issue for the use of relaxin as a therapeutic is its short half-life in circulation, which limits its potential for chronic administration (Chen et al., 1993). Development of relaxin analogues with improved half-lives, or development of sustained-release formulations, may improve drug delivery. Similarly, as relaxin is a peptide, oral administration is not possible, but further development of small molecule RXFP1 agonists that can be taken orally may be of use. Another issue is the lack of animal models for some diseases (Jelinic et al., 2018). For example, although relaxin may have potential as a treatment for preeclampsia, animal models do not well reproduce the human disease (McCarthy et al., 2011). Furthermore, the clinical trials of relaxin for treatment of AHF may also have been complicated by the possible use of angiotensin receptor agonists by some patients, which block some of the actions of relaxin (Chow et al., 2014; Chow et al., 2019). A final remaining issue hindering the advancement of RXFP1

as a therapeutic target is our limited understanding of the molecular mechanisms underlying the biological effects of RXFP1 activation. The actions of relaxin/RXFP1 across cell types depend on differential activation of signalling pathways and functional crosstalk between activity from other receptors, which also depends on cell type. Furthermore, the relaxin peptide analogue B7-33 appears to show cell-specific actions that we do not understand. In order to understand the molecular mechanisms underlying cell- and ligand-specific bias at RXFP1, signalling assays that improve upon the shortcomings of traditional end point assays are required.

1.12 Resonance energy transfer approaches to understanding GPCR function

Resonance energy transfer (RET) refers to the transfer of energy from an excited donor chromophore to a suitable acceptor chromophore by non-radiative dipole-dipole coupling (Forster, 1948), leading to a decrease in emission intensity from the donor and an increase in emission intensity from the acceptor. The efficiency (E) of RET depends on the distance between the two chromophores (r), according to the following equation:

$$E = \left[1 + \left(\frac{r}{R_0} \right)^6 \right]^{-1}$$

Where R_0 refers to the Förster distance, which is the distance between donor and acceptor at which 50% of the maximum energy transfer occurs. R_0 depends on the spectral characteristics of the particular donor and acceptor involved, but is usually less than 10 nm (Dacres et al., 2012; Dacres et al., 2010; Patterson et al., 2000; Wu & Brand, 1994). As the efficiency of energy transfer is inversely proportional to the distance between donor and acceptor to the power of six, this means that small changes in distance between donor and acceptor can produce large increases or decreases in RET.

There are three criteria that must be fulfilled for RET to occur: (1) the donor and acceptor must be in close proximity, (2) the donor emission spectrum must overlap substantially with the acceptor excitation spectrum, and (3) the donor and acceptor must be favourably oriented in relation to one another (Boute et al., 2002; Pflieger & Eidne, 2003). RET can occur between a fluorescent donor and acceptor (Förster/fluorescence resonance energy transfer or FRET), or between a luminescent donor and a fluorescent acceptor (bioluminescence resonance energy transfer or BRET).

1.12.1 Fluorescence and Förster/fluorescence resonance energy transfer

For fluorescence to occur, an external light source must excite a fluorophore at an appropriate wavelength. If another fluorophore with a compatible excitation wavelength is in proximity before the electron returns to ground state, energy transfer takes place via the Förster mechanism. Variants of GFP from the jellyfish *Aequorea victoria* with shifted excitation and emission wavelengths are commonly used as FRET donors and acceptors. For example, the most common and most effective fluorescent protein FRET pair uses a variant of cyan fluorescent protein (CFP) as the donor ($\lambda_{\text{max}} \approx 475 \text{ nm}$), and a variant of yellow fluorescent protein (YFP) as the acceptor ($\lambda_{\text{max}} \approx 530 \text{ nm}$) (Piston & Kremers, 2007) (**Figure 1.8**). However, FRET also works with fluorophores that are not proteins, such as fluorescent dyes, which can be conjugated to ligands, receptors, intracellular proteins, or antibodies.

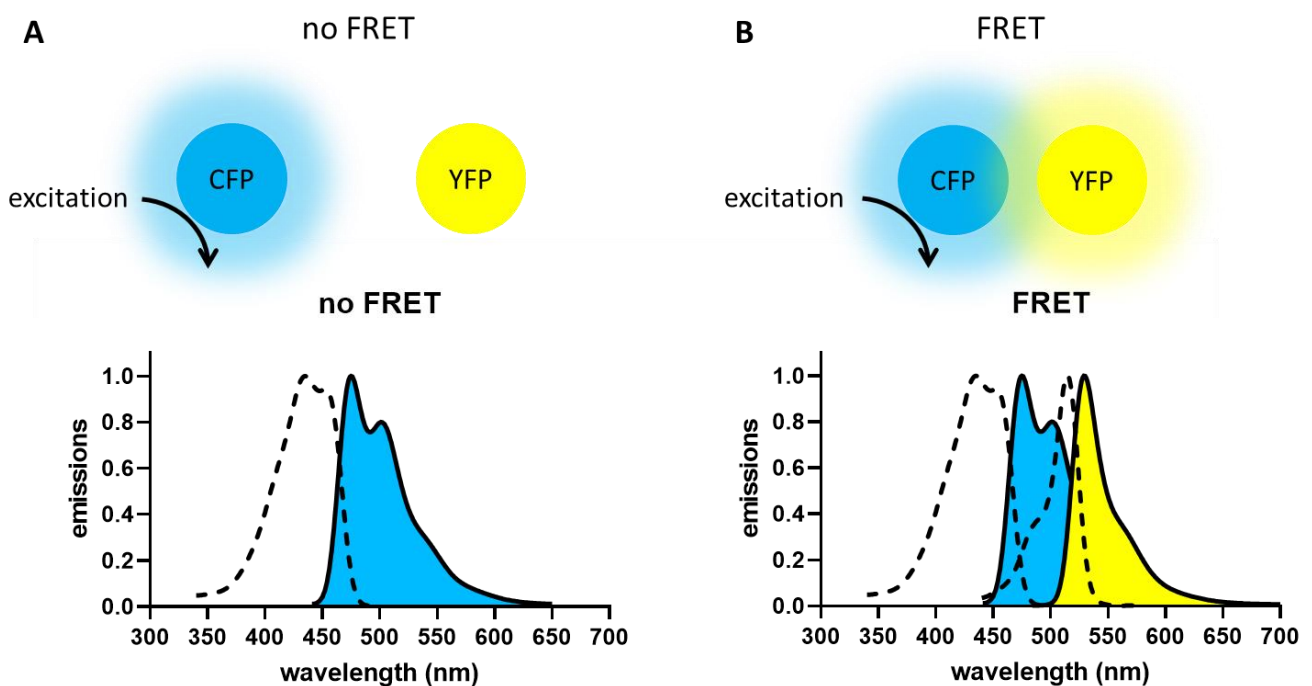


Figure 1.8. The principle of fluorescence resonance energy transfer (FRET). (A) Laser excitation of a donor (cyan fluorescent protein, CFP) at an appropriate excitation wavelength (dotted line) produces emissions with a peak at about 480 nm. (B) When the donor is in proximity to an acceptor that has an excitation spectra (second dotted line) overlapping with the donor's emission spectra (yellow fluorescent protein, YFP), energy transfer occurs, producing emissions from the acceptor. Adapted from Pflieger and Eidne (2003); Pflieger and Eidne (2005).

1.12.2 Bioluminescence and bioluminescence resonance energy transfer

Bioluminescence is a naturally-occurring form of chemiluminescence, whereby visible light is generated in a living organism due to a chemical reaction (Wilson & Hastings, 1998). Specifically, an enzyme (a photoprotein or a luciferase) catalyses the oxidation of a chemical substrate (luciferin), producing an unstable excited state that emits light when decaying to ground state, generating an oxyluciferin in the process. Bioluminescence occurs in various animals including the jellyfish *Aequorea victoria*, the sea pansy *Renilla reniformis*, and the deep-sea shrimp *Oplophorus gracilirostris* (Haddock et al., 2010). BRET also naturally occurs in *A. victoria* and *R. reniformis* when the luminescent protein is co-localised with a fluorescent protein such as a GFP (Morin & Hastings, 1971). For example, in *R. reniformis*, oxidation of coelenterazine (a membrane permeable small molecule present in bioluminescent marine organisms) by *Renilla* luciferase (Rluc) produces blue light ($\lambda_{\text{max}} \approx 480 \text{ nm}$) (DeLuca et al., 1971; Morin & Hastings, 1971), which overlaps with the excitation spectrum of *Renilla* GFP ($\lambda_{\text{max}} \approx 500 \text{ nm}$), and therefore energy transfer occurs via the Förster mechanism when Rluc and GFP are in proximity, producing green emissions ($\lambda_{\text{max}} \approx 510 \text{ nm}$) (Morin & Hastings, 1971; Ward & Cormier, 1979). However, for reasons described below, YFP is more commonly used as an acceptor for Rluc in the laboratory (**Figure 1.9**).

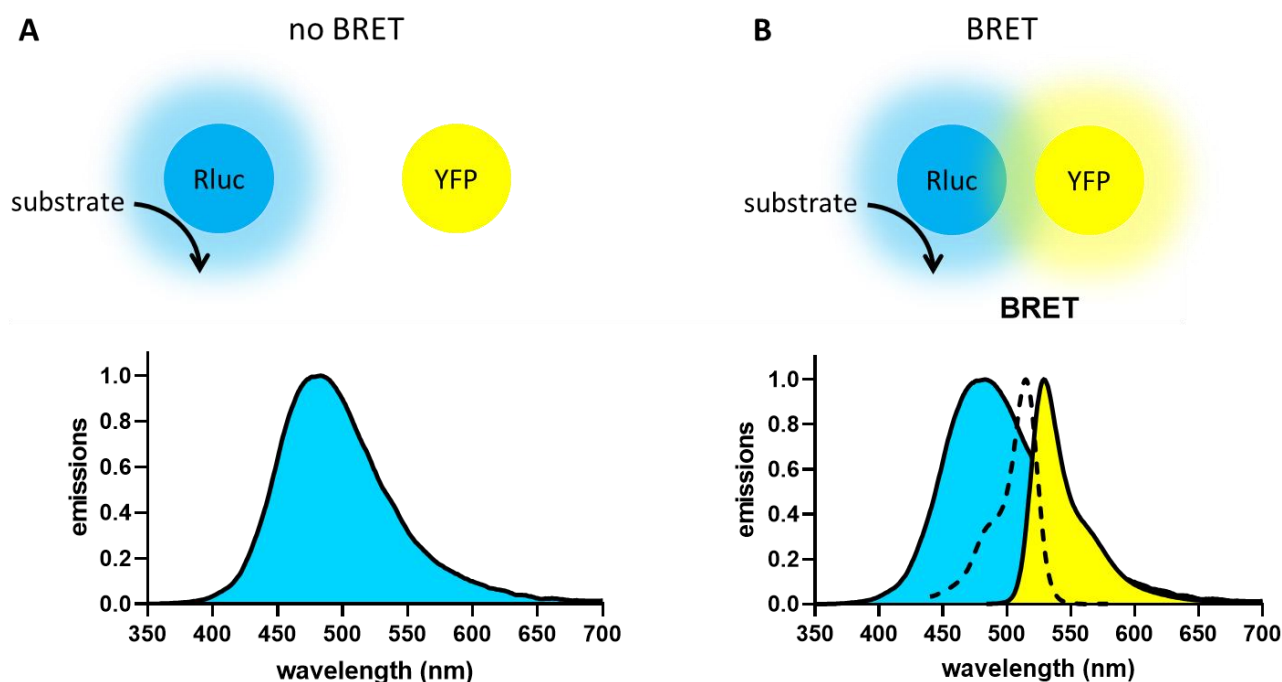


Figure 1.9. The principle of bioluminescence resonance energy transfer (BRET).

(A) The donor protein (*Renilla* luciferase, Rluc) oxidises a substrate to produce luminescent emissions. (B) When the donor is in close proximity to an acceptor that has an excitation spectrum (dotted line) overlapping with the donor's emission spectrum (yellow fluorescent protein, YFP), energy transfer occurs, producing emissions from the acceptor. Adapted from Pflieger and Eidne (2003); Pflieger and Eidne (2005).

1.12.3 FRET and BRET as tools in the laboratory

As the Förster distance is usually less than 10 nm, FRET and BRET are suitable for detecting proximity within or between biological molecules (Clegg, 1995; Stryer & Haugland, 1967). FRET and BRET have been adapted for molecular and cell biology by generating fusion proteins with appropriate donor/acceptor pairs as tags, to conduct assays in live cells or on purified proteins, for example (Pflieger & Eidne, 2006). These assays have been used for real-time detection of (i) intramolecular RET, where the donor and acceptor are tagged to the same protein and conformational changes within the protein produce changes in RET, and (ii) intermolecular RET, where the donor and acceptor are tagged to different proteins and changes in proximity of those molecules produce changes in RET. Assays then involve detection of emissions from donor and acceptor after addition of substrate, using a plate reader with appropriate bandpass filters (Pflieger & Eidne, 2006). The RET signal can be quantified ratiometrically, whereby emissions from the donor and acceptor are each detected and the ratio of emission intensity at the two wavelengths is calculated.

1.12.3.1 FRET versus BRET

Due to brightness of the fluorophores, FRET is a valuable technique for applications such as cellular microscopy involving imaging individual cells with subcellular resolution (Sekar & Periasamy, 2003). However, FRET has several limitations. For example, laser excitation of the donor can directly excite the acceptor (known as crosstalk), can damage cells, and can lead to photobleaching, whereby the FRET signal is lost over time (Pfleger & Eidne, 2003). Additionally, autofluorescence of cell components can occur after laser excitation, which produces high background noise (Boute et al., 2002). Although this background noise can be eliminated when using single-cell imaging techniques by selecting an unlabelled region of the cell as a control (Tsien, 1998), this is not possible for populations of cells in plate-based assays.

BRET, on the other hand, is valuable for plate-based assays. As excitation of the donor with an external light source is not required, the problems of cellular autofluorescence, photobleaching, crosstalk, and cell damage that occur using FRET are not a concern for BRET assays (Boute et al., 2002; Pfleger & Eidne, 2003). Therefore, BRET has lower background noise and higher sensitivity compared to FRET for population-based signalling assays (Arai et al., 2001; Boute et al., 2002). That is, FRET is valuable for live imaging of single cells, whereas BRET is valuable for population-based assays that generate an aggregate response.

1.12.4 BRET variants

The gene for Rluc was cloned in the 1990s (Lorenz et al., 1991), allowing its expression in mammalian cells (Lorenz et al., 1996). The first BRET assay was published in the late 1990s, demonstrating protein-protein interactions between circadian clock proteins in *E. coli* (Xu et al., 1999). Since then, BRET technology and applications have dramatically progressed. For example, brighter and more stable mutants of Rluc, including Rluc2 (De et al., 2007) and Rluc8 (Loening et al., 2006), have been generated and are now widely used. BRET-based approaches for understanding GPCR function now include assays for ligand binding, G protein coupling, β -arrestin recruitment, GPCR oligomerisation, GPCR internalisation, and detection of second messenger and kinase activity (Ayoub, 2016; Lohse et al., 2012). Additionally, there are currently several variants of BRET used in the laboratory, which differ based on their combination of donor, acceptor, and substrate, and are used for different applications (**Table 1.1**).

1.12.4.1 BRET¹

The combination of an Rluc variant as the donor, a YFP variant as the acceptor, and coelenterazine *h* as the substrate, is sometimes referred to as BRET¹. One common donor/acceptor pair is that of Rluc8 and Venus (Nagai et al., 2002), which has high sensitivity due to increased brightness (Kocan et al., 2010; Kocan et al., 2008). Although BRET can occur between Rluc and GFP, YFP is used as the acceptor in BRET¹ assays due to the greater spectral separation. That is, if spectra overlap too much as in the case of Rluc/GFP, a low signal-to-noise ratio occurs (Pfleger & Eidne, 2003).

A variant of BRET¹, known as extended BRET (eBRET), allows monitoring of BRET over an extended period of time (Pfleger *et al.*, 2006). eBRET uses a caged form of coelenterazine *h* known as EnduRen, which is metabolised to coelenterazine *h* inside cells by esterases (Pfleger *et al.*, 2006). Whereas BRET¹ remains sensitive over 1-2 hours, eBRET can be used for at least five or six hours (Kocan *et al.*, 2008; Pfleger *et al.*, 2006).

1.12.4.2 BRET²

BRET² was developed to increase the spectral separation between the emissions from the donor and acceptor, thus decreasing the bleedthrough from the donor into the acceptor channel (Bertrand et al., 2002). BRET² uses the combination of an Rluc variant as the donor, GFP² or GFP10 as the acceptor, and a modified form of coelenterazine known as DeepBlueC (coelenterazine 400a) as the substrate (Bertrand et al., 2002). Upon oxidation of DeepBlueC, Rluc produces emissions with a peak at ≈ 395 nm. Additionally, GFP² and GFP10 have large Stokes shifts with excitation peaks at ≈ 395 nm and emission peaks at ≈ 510 nm (Pfleger & Eidne, 2006). Therefore, BRET² has a spectral separation of ≈ 115 nm, compared to ≈ 55 nm for BRET¹. However, some publications have reported Rluc emissions at ≈ 420 nm in the presence of DeepBlueC rather than the ≈ 395 nm originally reported (Dacres et al., 2012; Kobayashi et al., 2019; Kocan et al., 2008).

Although there is less background noise in BRET² assays than BRET¹ assays (Hamdan et al., 2005), the light output of Rluc is substantially lower when using DeepBlueC compared to coelenterazine *h*, and the decay in luminescence is also much faster with DeepBlueC (Hamdan et al., 2005). Therefore, BRET² does not typically retain the sensitivity of BRET¹ and can only detect changes in BRET rapidly after substrate addition rather than over a longer time-course. Consequently, the advantage of increased spectral separation is somewhat negated. The use of Rluc2 or Rluc8 rather than Rluc does improve sensitivity of a BRET² assay over a longer period of

time (more than one hour), but the luminescence intensity is still substantially lower than that of BRET¹ (Kocan et al., 2008). BRET¹, rather than BRET², is the most commonly used variant of BRET.

1.12.4.3 NanoBRET

A recent advance in luminescence and BRET technology is the development of NanoLuc (Nluc), which was engineered from the native luciferase from *O. gracilirostris* (Hall et al., 2012). Nluc is about half the size of Rluc (19 kDa versus 36 kDa) and oxidises the novel coelenterazine analogue furimazine to produce blue emissions ($\lambda_{\text{max}} \approx 460$ nm) that are substantially brighter than emissions from all other available luciferases (Hall et al., 2012). Additionally, the use of DeepBlueC with Nluc has also been reported to produce very bright emissions with the same peak wavelength (Kobayashi et al., 2019).

The use of Nluc in BRET assays is referred to as NanoBRET (Machleidt et al., 2015). Due to its brightness, Nluc can be used as a donor for a greater variety of acceptors, including when there is a large spectral separation (Stoddart et al., 2015; White et al., 2017) (**Figure 1.10**). NanoBRET is gaining traction as a technique for understanding some aspects of GPCR function. In particular, NanoBRET ligand binding assays that detect interactions between receptors tagged with Nluc at the N-terminus and fluorescent ligand acceptors are very sensitive (Stoddart et al., 2015; Stoddart et al., 2018). This improved sensitivity is related to the ability of Nluc-tagged receptors to traffic to the cell surface better than Rluc8-tagged receptors, as well as the fact that a signal is only detected when the fluorescent ligand is in close proximity to the tagged receptor, in comparison with traditional assays that detect higher background noise as the ligand can show “non-specific” binding to the membrane, for instance (Stoddart et al., 2015; Stoddart et al., 2018). NanoBRET may also become a preferred option in instances where emissions are too low using BRET¹, such as when it is desired to express proteins at endogenous levels. In fact, genome editing using CRISPR/Cas9 has been recently used to introduce Nluc or NanoBRET constructs into cells under endogenous promotion (White et al., 2020b; White et al., 2018; White et al., 2017).

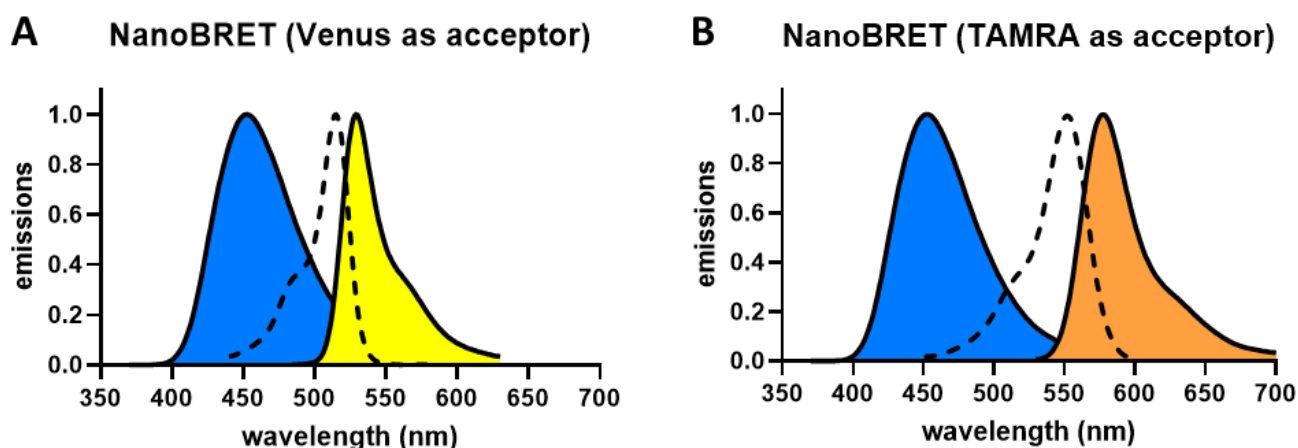


Figure 1.10. NanoBRET can use various acceptors. (A) The emission spectra of Nanoluc (Nluc, blue) overlaps with the excitation spectrum (dotted line) of the yellow fluorescent protein Venus, leading to emissions from Venus (yellow). (B) The emission spectra of Nluc overlaps with the excitation spectrum (dotted line) of the fluorescent dye tetramethylrhodamine (TAMRA), leading to emissions from TAMRA (orange).

Table 1.1. BRET assay variants.

	BRET ¹	BRET ²	NanoBRET
Substrate	Coelenterazine <i>h</i> EnduRen (eBRET)	DeepBlueC (coelenterazine 400a)	Furimazine
Donor	Rluc2 Rluc8	Rluc2 Rluc8	Nluc
Acceptor	YFP	GFP ² GFP10	Various, including YFP, TAMRA, BODIPY 630/650
Donor peak emissions (nm)	≈480	≈395	≈460
Acceptor peak excitation (nm)	≈515	≈395	YFP: ≈515 TAMRA: ≈550 BODIPY 630/650: ≈625
Acceptor peak emissions (nm)	≈530	≈510	YFP ≈ 530 TAMRA: ≈580 BODIPY 630/650: ≈640
Separation between peak emissions (nm)	≈55	≈115	YFP: ≈ 70 TAMRA: ≈ 120 BODIPY 630/650: ≈ 180

1.13 BRET-based biosensors for G protein coupling and signal transduction

BRET-based methods are proving to be valuable for detecting G protein coupling, signalling, and bias in plate-based assays. Importantly, BRET-based techniques can be used to measure real-time activity in live cells at 37°C. They therefore have high temporal resolution, which is important for detection of kinetic bias and elimination of observational bias. As described below, BRET-based methods can also have high spatial resolution when required. In contrast, traditional end point assays involve lysing cells at individual points in time before analysing the contents of the lysates, and therefore show low temporal resolution and no spatial resolution.

1.13.1 Intramolecular biosensors for second messenger activity

BRET-based biosensors for second messengers are single proteins that usually consist of a “sensor” domain typically derived from an endogenous effector of the particular second messenger, which is sandwiched between an appropriate BRET pair. When the second messenger binds to the sensor, a conformational change occurs that alters the distance between the BRET pair, thereby increasing or decreasing BRET efficiency (**Figure 1.11**). For example, CAMYEL (cAMP sensor using YFP-Epac-Rluc) is a BRET-based biosensor for cAMP activity that uses a truncated and catalytically inactive form of human Epac1 as the “sensor”, sandwiched between Rluc as the donor and a monomeric and circularly permuted variant of the YFP citrine as the acceptor (Jiang *et al.*, 2007). When cAMP binds to the “sensor” domain, the donor and acceptor move farther apart, causing a decrease in BRET. CAMYEL has been validated for the detection of cAMP downstream of several GPCRs, such as the cannabinoid 1 receptor (Hunter *et al.*, 2017) and mu-opioid receptor (Thompson *et al.*, 2016). Validation of CAMYEL for detection of signalling at relaxin family peptide receptors is the focus of Chapter 3 of this thesis.

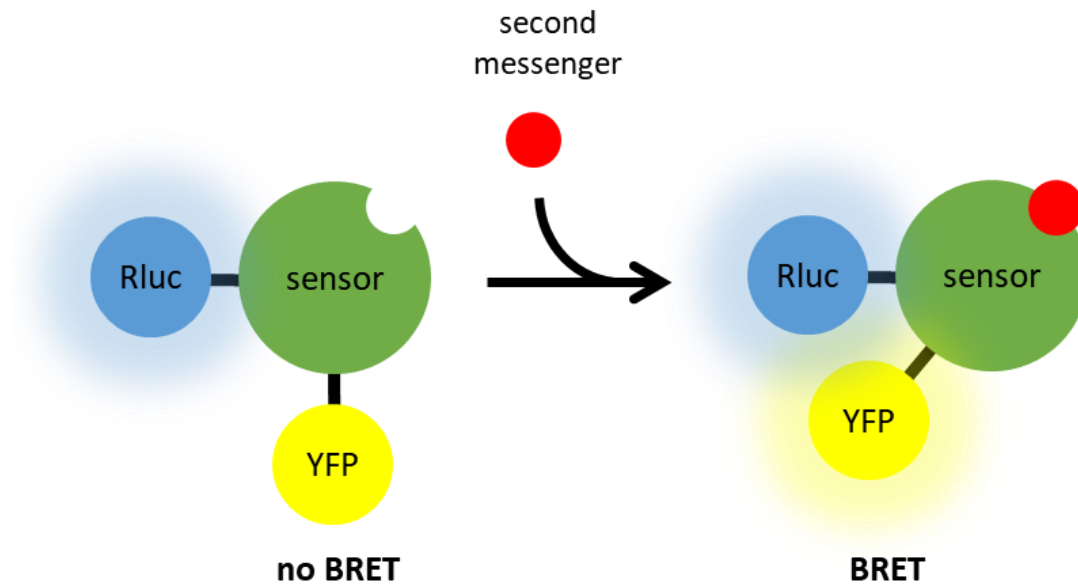


Figure 1.11. Generic structure of a BRET-based sensor for second messenger activity. Binding of a second messenger of interest to the sensor domain produces a conformational change that brings the donor and acceptor (in this case Rluc and YFP, respectively) into close enough proximity for BRET to occur. Adapted from Salahpour *et al.* (2012).

1.13.2 Intramolecular biosensors for kinase activity

The generic structure of a BRET-based “kinase activity reporter” includes a substrate peptide specific for the kinase of interest, a phospho-amino binding domain, and a long linker region, which are flanked by the BRET pair (Ni *et al.*, 2006). When the substrate sequence is phosphorylated by the kinase of interest, it binds to the phospho-amino binding domain, which brings the BRET pair together. Substrate sequences can be either a consensus sequence or a sequence from a protein known to be phosphorylated by the kinase of interest and the phospho-amino binding domains can be derived from existing domains such as WW domains or forkhead-associated domains (Ni *et al.*, 2006). A kinase sensor may also include a docking domain selective for the kinase of interest to provide selectivity of the sensor, as different kinases can share common substrates (Fosbrink *et al.*, 2010) (**Figure 1.12**).

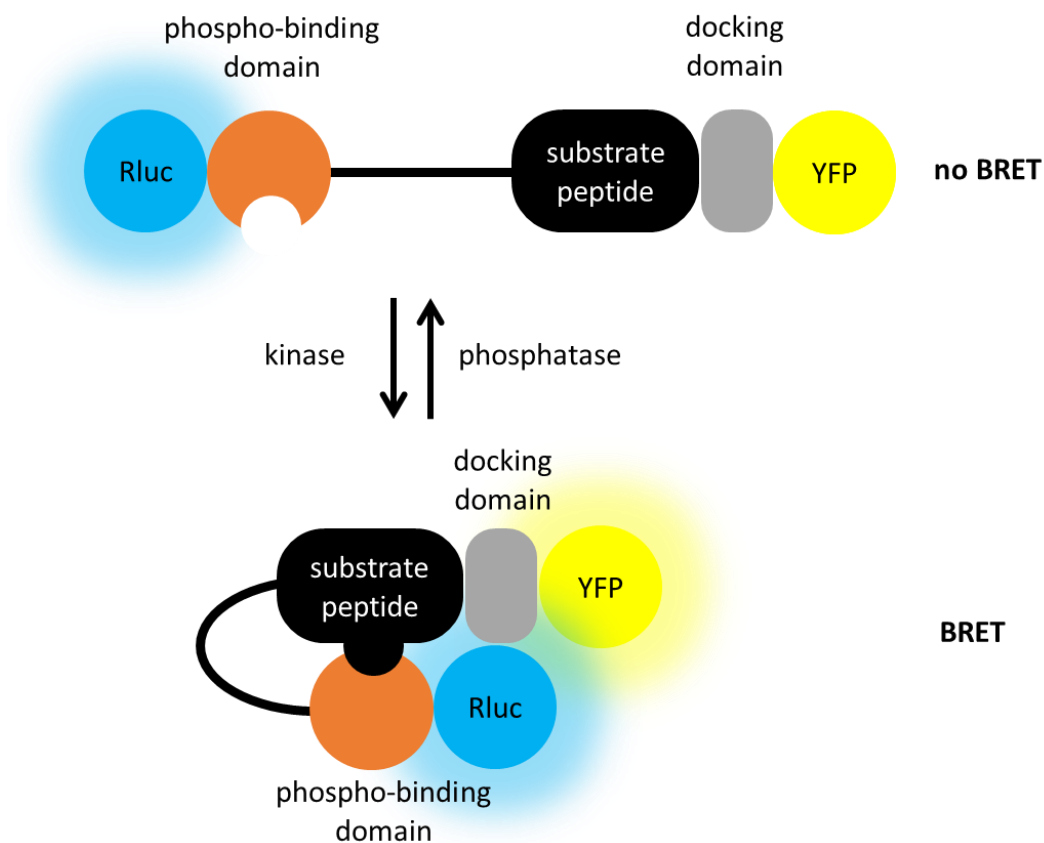


Figure 1.12. Generic structure of a BRET-based sensor for kinase activity. Phosphorylation of a substrate sequence by the kinase of interest produces a conformational change leading to binding of the substrate sequence to the phospho-amino binding domain, which brings the donor and acceptor (in this case Rluc and YFP, respectively) into close enough proximity for BRET to occur. A docking domain specific for the kinase of interest is sometimes included to increase specificity of the biosensor for the desired kinase. Adapted from Xu *et al.* (2013).

1.13.3 Intermolecular biosensors for G protein association

There are several methods for detecting G protein association using BRET, including detecting interactions between two different G protein subunits (usually $G\alpha$ and $G\gamma$) tagged with a donor and acceptor, or interactions between a tagged receptor and a G protein subunit (usually $G\alpha$) (Ayoub, 2016). However, BRET¹- and BRET²-based assays for G protein association or dissociation are not always sensitive enough to detect concentration-response relationships, such as in the case of RXFP1 (Kocan *et al.*, 2017). Novel NanoBRET assays for G protein coupling have been used for detection of concentration-dependent G protein activation (Zhao *et al.*, 2020). Relatedly, NanoBRET has also been used to detect interactions between GPCRs and β -arrestin (White *et al.*, 2017).

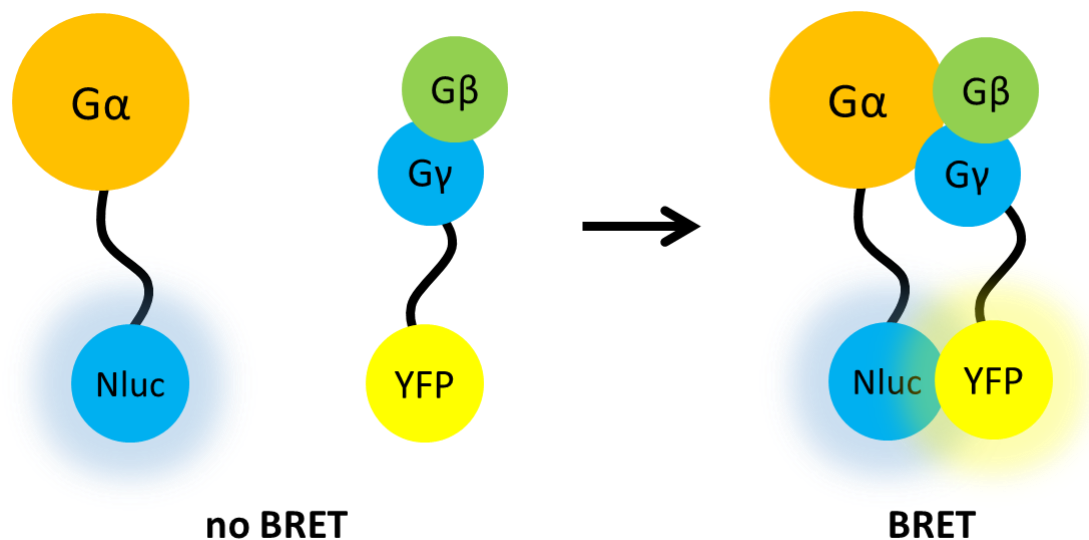


Figure 1.13. Generic design of a NanoBRET-based G protein association assay. In this example, changes in proximity between a donor (NLuc)-tagged G α subunit and acceptor (YFP)-tagged G γ subunit lead to changes in the BRET ratio.

1.13.4 Localisation of biosensors to detect compartmentalised signalling

Spatial resolution of biosensors can be achieved by the introduction of sequences that target the biosensor to particular subcellular locations, such as the plasma membrane (Matthiesen & Nielsen, 2011), cytosol, nucleus (Harvey et al., 2008), mitochondria (DiPilato et al., 2004), or to enzymes such as ACs (Wachten et al., 2010). Given the prevalence and physiological relevance of compartmentalised signalling, this approach may prove valuable, including for the study of GPCRs. In fact, targeted FRET-based biosensors in conjunction with confocal microscopy have been used to examine localised GPCR signalling for the TSH receptor, for example (Godbole et al., 2017).

1.14 End point signalling assays

In contrast to real-time BRET-based assays, traditional *in vitro* signalling assays involve incubating populations of cells with ligands, then lysing the cells and detecting second messenger or kinase activity within the lysates at individual points in time (Hill et al., 2010; Williams, 2004). Some common end point assays are reporter gene assays and time-resolved FRET (TR-FRET)-based assays.

1.14.1 Reporter gene assays

Reporter gene assays exploit the ability of signalling molecules to affect downstream gene transcription. In these assays, regulatory elements that are normally activated or inhibited by signalling at chosen pathways are positioned upstream of the coding sequence for a reporter such as an enzyme or fluorescent protein that can be detected later via colorimetric, fluorescent, or luminescent readouts after ligand-induced reporter gene expression (Hill et al., 2001). For example, the cAMP response element (CRE) reporter gene assay, which measures activity downstream of cAMP (Chen et al., 1995), has been used extensively for characterisation of relaxin/RXFP1 signalling (Halls et al., 2007). However, reporter gene assays have several disadvantages. For example, in the case of the CRE assay, CRE is not only activated downstream of cAMP/PKA. Rather, this assay integrates signals from other pathways that lead to CREB phosphorylation, including ERK1/2, PI3K, and Ca^{2+} (Lonze & Ginty, 2002). Therefore, the readout from this assay is a mixture of activity from a variety of pathways and cannot easily be used to detect biased signalling. Furthermore, reporter gene assays only measure total downstream activity with no temporal resolution due to the long incubation time required for the gene expression to occur.

1.14.2 Time-resolved FRET

Plate-based FRET assays show high background noise due to autofluorescence from cell components and certain chemicals and buffers used in the assay. However, this autofluorescence is relatively short-lived, and therefore emissions from fluorophores with long lifetimes can be distinguished from background fluorescence if the detection of emissions after excitation is delayed. Assays based on TR-FRET exploit this principle. For example, homogenous time-resolved fluorescence (HTRF; Cisbio) technology is a TR-FRET-based method that utilises rare earth chelates, which have long-lived fluorescence, as FRET donors (Degorce, 2006; Degorce et al., 2009; Mathis, 1999). The current generation of HTRF technology uses europium cryptate (Eu^{3+}) as the donor and the organic dye d2 as the acceptor, which are both conjugated to the detection reagents. In the cAMP HTRF assay, for example, cells are incubated with ligands in the presence of a PDE inhibitor to stop the breakdown of cAMP, before being lysed at chosen time points. Cell lysates are then combined with detection reagents, including d2-labelled cAMP and a Eu^{3+} cryptate-labelled anti-cAMP antibody. The labelled cAMP competes with cAMP present in the cell lysates for binding to the labelled antibody. The long-lived emissions from the donor lead to long-lived emissions from

the acceptor if they are in close proximity, and the TR-FRET signal is detected as the ratio of emissions at 665 nm divided by emission at 620 nm (multiplied by 10,000). However, as with all end point assays involving cell lysis, it is laborious to generate temporal data, and there is also no spatial resolution in these assays.

1.15 Aims

Despite showing promising therapeutic actions for cardiovascular and fibrotic diseases, relaxin has failed several clinical trials, and it is clear that we do not well understand the molecular mechanisms underlying the beneficial actions of relaxin and other ligands targeting RXFP1. Much research into relaxin/RXFP1 signalling has been conducted using end point assays in recombinant cells. However, recent research has started to elucidate the significant role of spatiotemporal regulation on GPCR function and its connection to biased signalling. The importance of using assays that take the kinetic aspects of signalling into account is now clear, as end point assays do not necessarily allow an accurate comparison of ligand activity and can introduce an observational bias. Similarly, assays that can detect signalling in physiologically-relevant native cell types are of importance given that signalling and bias can change across cell types and disease conditions. Unfortunately, RXFP1 signalling in human primary cells has been hard to study due to the lack of such appropriate assays. Development of sensitive, real-time assays that can be used in primary cells will aid in the understanding of the molecular mechanisms underlying RXFP1 signalling. Specifically, novel, genetically-encoded BRET-based biosensors provide the means to detect G protein coupling, second messenger activity, and kinase activity with temporal resolution, therefore aiding in the detection of cell- and ligand-specific signalling patterns. Given that primary cells are usually difficult to transfect, expression of genetically-encoded biosensors can instead be achieved using lentiviral-mediated gene delivery. Specifically, lentiviral vectors with broad tropism can achieve stable and long-lasting expression of transgenes across many different types of cells (Ramezani & Hawley, 2002), thus enabling the use of BRET-based biosensors across these cell types.

Therefore, the general aim of this thesis is to apply and develop BRET-based methods in conjunction with human native and primary cells in order to examine real-time signalling and bias at RXFP1. Specifically, we first aim to demonstrate the use and versatility of the CAMYEL (cAMP sensor using YFP-Epac-Rluc) BRET-based biosensor (Jiang *et al.*, 2007) for detection of real-time cAMP activity at the four relaxin family peptide receptors, to clone the biosensor into a lentiviral

vector, and to demonstrate the utility of the lentiviral vector for detection of cAMP in natively-expressing RXFP1 cells. Next, we aim to develop, validate, and characterise a BRET-based biosensor for cGMP activity based on the cGES-DE5 FRET-based biosensor (Nikolaev *et al.*, 2006), to clone it into a lentiviral vector, and to investigate its utility for detection of cGMP in human primary vascular cells for the purpose of drug discovery. Finally, we aim to use a variety of BRET-based assays for G protein association, second messenger activity, and kinase activity, as well as physiologically-relevant human native and primary cells, in order to understand the mechanism of action underlying the beneficial actions of the relaxin peptidomimetic B7-33.

2 Methods

2.1 Introduction

This chapter describes general methods and reagents used for cell culture, signalling assays, molecular cloning, lentivirus, and flow cytometry throughout this thesis. Methods specific to individual chapters are described in more detail within those chapters.

2.2 Cell culture

Mammalian cell lines and primary cells were cultured in an incubator (BINDER) at 37°C with 5% CO₂.

2.2.1 Products and reagents

Products and reagents used to culture and transfect mammalian cells are listed in **Table 2.1**.

Table 2.1. Products, reagents, and buffers used in the culturing and transfection of mammalian cells.

Product	Source	Catalogue number
Dulbecco's modified Eagle medium (DMEM)	Gibco	10313-021
Dulbecco's modified Eagle medium, no phenol red	Gibco	31053-028
Dulbecco's modified Eagle medium/Nutrient mixture F-12 (DMEM/F-12)	Gibco	11320-033
Dulbecco's modified Eagle medium/Nutrient mixture F-12, no phenol red	Gibco	21041-025
Dulbecco's phosphate-buffered saline (DPBS)	Gibco	14190-136
Endothelial cell growth basal medium-2 (EBM-2)	Lonza	CC-3156
Endothelial cell growth medium (EGM-2) SingleQuots	Lonza	CC-4176
Fetal bovine serum (FBS)	Scientifix	FFBS-500
Geneticin selective antibiotic (G418 sulfate)	Gibco	10131-035
HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)	Sigma-Aldrich	H3375
L-Glutamine	Gibco	25030-081
Lipofectamine 2000	Gibco	11668-019
Medium 199, Earle's salts	Gibco	11150-059
Medium 199, no phenol red	Gibco	11043-023
Opti-MEM reduced serum media	Gibco	31985-070
Penicillin-streptomycin	Gibco	15070-063
Roswell Park Memorial Institute 1640 medium (RPMI 1640) (ATCC modification)	Gibco	A10491-01
Roswell Park Memorial Institute 1640 medium, no phenol red	Gibco	11835-030
Smooth muscle cell growth supplement	ScienCell	1152
Trypsin-EDTA, 0.25%	Gibco	25200-056
Zeocin selection antibiotic	InvivoGen	ant-zn

2.2.2 Cell culture media

Table 2.2 describes the media used for culturing each cell type used in this thesis. Media containing all supplements is referred to as “complete media” from now on.

Table 2.2. List of media used for culturing mammalian cells.

Cell type	Basal media	Supplements
BJ3 human dermal fibroblasts	DMEM	15% heat-inactivated FBS 17% Medium 199 1% penicillin/streptomycin 1% L-glutamine
Chinese hamster ovary K1 (CHO-K1)	DMEM/F-12	10% FBS 1% penicillin/streptomycin 1% L-glutamine
Flp-In-CHO		
Human embryonic kidney 293A (HEK293A)	DMEM	10% FBS 1% penicillin/streptomycin 1% L-glutamine
Human embryonic kidney 293T (HEK293T)		
Human umbilical vein endothelial cells (HUVECs)	EBM-2	EGM-2 endothelial SingleQuots kit 1% penicillin/streptomycin
Human umbilical vein smooth muscle cells (HUVSMCs)	M199	5% heat-inactivated FBS 1% penicillin/streptomycin Smooth muscle cell growth supplement
THP-1 human monocytic leukaemia cells	RPMI 1640 (ATCC modification)	10% FBS 1% penicillin/streptomycin 1% L-glutamine

2.2.3 Cell lines

Table 2.3 lists all cell lines used throughout this thesis. Most stable cell lines were generated by lentiviral transduction. Cell lines that were previously generated by stable transfection are noted.

Table 2.3. Cell lines used throughout this thesis.

Cell type	Stable gene expression	Source	Chapters
BJ3 human dermal fibroblasts	Parental cells	Hahn <i>et al.</i> (1999)	5
	CAMYEL	This thesis	5
Chinese hamster ovary K1 (CHO-K1)	RXFP4	Belgi <i>et al.</i> (2013)	3
Flp-In CHO	RXFP3 ¹	van der Westhuizen <i>et al.</i> (2007)	3
Human embryonic kidney 293T (HEK293T)	Parental cells	ATCC CRL-3216	3,4,5
	RXFP1 ²	Bathgate lab	3,5
	RXFP1 REV _{Cyto} ²	Bathgate lab	5
	RXFP1 REV _{Nuc} ²	Bathgate lab	5
	RXFP2	Bathgate lab	3
	RXFP3	Bathgate lab	3
	CAMYEL	This thesis	4,5
Human embryonic kidney 293A (HEK293A)	Parental cells	A/Prof Asuka Inoue ³	3
	$\Delta G\alpha_s$	A/Prof Asuka Inoue ³ Stallaert <i>et al.</i> (2017)	3
Human umbilical vein endothelial cells (HUVECs)	Parental cells	Dr. Sarah Marshall ⁴	4
	RXFP1 (low, high)	This thesis	4,5
	RXFP1 CAMYEL	This thesis	5
	RXFP1 CYGYEL	This thesis	4
Human umbilical vein smooth muscle cells (HUVSMCs)	Parental cells	ScienCell (#8020)	4
	CYGYEL	This thesis	4
THP-1 human monocytic leukaemia	Parental cells	Tsuchiya <i>et al.</i> (1980)	3
	CAMYEL (low, medium, high)	This thesis	3

¹ CHO-RXFP3 cells were generated by stable transfection of Flp-In CHO cells and were maintained by selection in geneticin (200 µg/ml)

² HEK-RXFP1 cells and their derivatives were generated by stable transfection and maintained by selection in zeocin (200 µg/ml)

³ Graduate School of Pharmaceutical Sciences, Tohoku University, Aoba, Miyagi, Japan

⁴ Department of Obstetrics and Gynaecology, School of Clinical Sciences, Faculty of Medicine Nursing and Health Sciences, Monash University, Australia

2.2.4 Plasmids and transfections

In Chapters 3 and 5, pcDNA3.1/Zeo(+) or pcDNA3.1/His mammalian expression vectors containing a transgene of interest were transfected into HEK293T, HEK293A, or CHO-K1 cells, for the purpose of BRET-based assays. These vectors contain the transgene under the control of the constitutive cytomegalovirus (CMV) promoter. In Chapters 3, 4, and 5, HEK293T cells were transfected with lentiviral packaging and envelope plasmids, as described in **section 2.7**. HEK293A and HEK293T cells were transfected using Lipofectamine 2000, whereas CHO-K1 and Flp-In-CHO cells were transfected using Viafect. Opti-MEM reduced serum media was used for incubation of transfection mixtures.

2.3 Ligands, inhibitors, and other compounds

A variety of agonists, antagonists, pharmacological inhibitors, and other compounds were used in assays throughout this thesis, which are listed in **Table 2.4**.

Table 2.4. List of ligands, inhibitors, and other compounds

Ligand or compound	Description	Source	Catalogue number	Chapters
1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ)	sGC inhibitor	Sigma-Aldrich	O3636	4
3-isobutyl-1-methylxanthine (IBMX)	Non-selective PDE inhibitor	Sigma-Aldrich	I5879	4,5
Analogue 2	Minimised relaxin-3 analogue	Shabanpoor <i>et al.</i> (2012)	-	3
Analogue 13	Minimised INSL5 analogue	Patil <i>et al.</i> (2016)	-	3

B7-33	Single-chain RXFP1 agonist	Hossain <i>et al.</i> (2016)	-	5
Bovine serum albumin (BSA)	Protein used for non-specific binding sites	Sigma-Aldrich	A7906	3,5
C-type natriuretic peptide (CNP)	Guanylate cyclase B agonist	Sigma-Aldrich	N8768	4
Carbachol	Cholinergic agonist	Sigma-Aldrich	C4382	4
Diethylamine NONOate diethylammonium (DEA)	NO donor	Sigma-Aldrich	D5431	4,5
Forskolin	AC activator	Sigma-Aldrich	F6886	3,4,5
INSL3	Cognate RXFP2 ligand	A/Prof. Akhter Hossain ¹	-	3
INSL3 B-chain dimer	RXFP2 antagonist	Shabanpoor <i>et al.</i> (2011)	-	3
INSL5	Cognate RXFP4 ligand	A/Prof. Akhter Hossain ¹		3
Isoprenaline	β -AR agonist	Sigma-Aldrich	I5627	3,4,5
ML290	Small molecule RXFP1 agonist	Xiao <i>et al.</i> (2013)	-	3
Peptide 5	Single-chain RXFP3 agonist	Hojo <i>et al.</i> (2016)	-	3
Pertussis toxin (PTX)	G $\alpha_{i/o}$ inhibitor	Gibco	PHZ1174	3,5
R3/I5	RXFP3/RXFP4 agonist	Liu <i>et al.</i> (2005a)	-	3
R3 B1-22R	Single-chain RXFP3 antagonist	Haugaard-Kedstrom <i>et al.</i> (2011)	-	3
Relaxin (relaxin-2) (recombinant)	Cognate RXFP1 ligand	Corthera	-	3,4,5
Rolipram	PDE4 inhibitor	Sigma-Aldrich	R6520	4
TAMRA-relaxin	Fluorescent relaxin-2	A/Prof. Akhter Hossain ¹	-	3,5
Vardenafil	PDE5 inhibitor	Sigma-Aldrich	SML2103	4

¹ Florey Institute of Neuroscience and Mental Health, Melbourne, Australia

2.4 Bioluminescence resonance energy transfer assays

Bioluminescence resonance energy transfer was the main technique used for signalling assays throughout this thesis.

2.4.1 Products and reagents

General cell culture reagents used for BRET assays are listed in **Table 2.1**. Additional reagents specific to BRET-based assays are listed in **Table 2.5**.

Table 2.5. List of additional reagents used in BRET-based assays.

Product	Source	Catalogue number
Coelenterazine 400a (DeepBlueC)	Nanolight	301
Coelenterazine <i>h</i>	Nanolight	340
Nano-Glo luciferase assay system (furimazine)	Promega	N1110

2.4.2 Real-time assays

Assays were conducted in cells either transiently transfected or stably transduced with BRET constructs. Cells that were stably expressing BRET constructs were plated out directly onto a white, opaque, 96-well CulturPlate and were assayed either the same day (THP-1 suspension cells) or the next day (adherent cells). For experiments involving transient transfections, cells were plated out on six-well plates and were transfected the following day. The day after transfection, cells were resuspended in complete media with 25 mM HEPES but no phenol red, and were re-seeded into 96-well CulturPlates. On the day of the BRET assay, cells were pre-incubated with phenol-red-free complete media (unless otherwise noted) containing 5 μ M coelenterazine *h* to establish a baseline, which was followed by addition of vehicle or ligand. Specific details for real-time NanoBRET assays are described in Chapter 5.

Emissions were detected simultaneously at 475/30 nm (donor emission channel for Rluc/Rluc8) or 450/40 (donor emission channel for Nluc) and 535/30 nm (acceptor emission channel) using a POLARstar Omega or PHERAstar FSX microplate reader (BMG Labtech) at 37°C. For luminescence only assays (Nluc spectral scans below), the luminescence module on the PHERAstar FSX microplate reader was used.

2.4.3 Spectral scans

Below, spectral scans were generated from live cells in order to compare luciferase substrates. HEK293T cells were plated out and transfected as described above. On the day of the assay, cell culture media was replaced with fresh complete media with 25 mM HEPES, no phenol red, and containing the relevant substrate for the luciferase. Emission spectra were then generated using the spectral scan mode on a CLARIOstar plate reader (BMG Labtech) with a resolution of 2 nm.

2.4.4 Choice of substrate for NanoBRET assays

NanoBRET assays, which use Nanoluc (Nluc) as the donor luciferase rather than an Rluc variant, were conducted in HEK293T cells. Although furimazine is usually used as the substrate for Nluc (Hall *et al.*, 2012), it was recently reported that coelenterazine 400a (DeepBlueC), the substrate used for BRET² assays (Bertrand *et al.*, 2002), produces bright emissions when used as a substrate for Nluc (Kobayashi *et al.*, 2019). As use of the Nluc/coelenterazine 400a pair had only been reported in one source, furimazine, coelenterazine 400a, and coelenterazine *h* were compared here as substrates for Nluc after transfection with 20 ng of G α_s -Nluc per well of a six-well plate (the same conditions used for the NanoBRET assays in Chapter 5). A spectral scan revealed that all three substrates produced luminescence that peaked at about 450 nm (**Figure 2.1A**). However, it was clear from a luminescence time-course that the brightness of the emissions was highly variable among substrates, as expected (**Figure 2.1B**). Specifically, coelenterazine *h* produced very dim emissions when used as a substrate for Nluc, consistent with the original Nluc report (Hall *et al.*, 2012), and is therefore not an optimal substrate for Nluc. In contrast, coelenterazine 400a produced emissions that were closer in brightness to those produced by Nluc, as previously reported (Kobayashi *et al.*, 2019). It was also evident that emissions decayed faster when using coelenterazine 400a compared to furimazine. However, this was not of great concern for conducting assays, and coelenterazine 400a (5 μ M) was thus used as the substrate for NanoBRET assays due to its brightness.

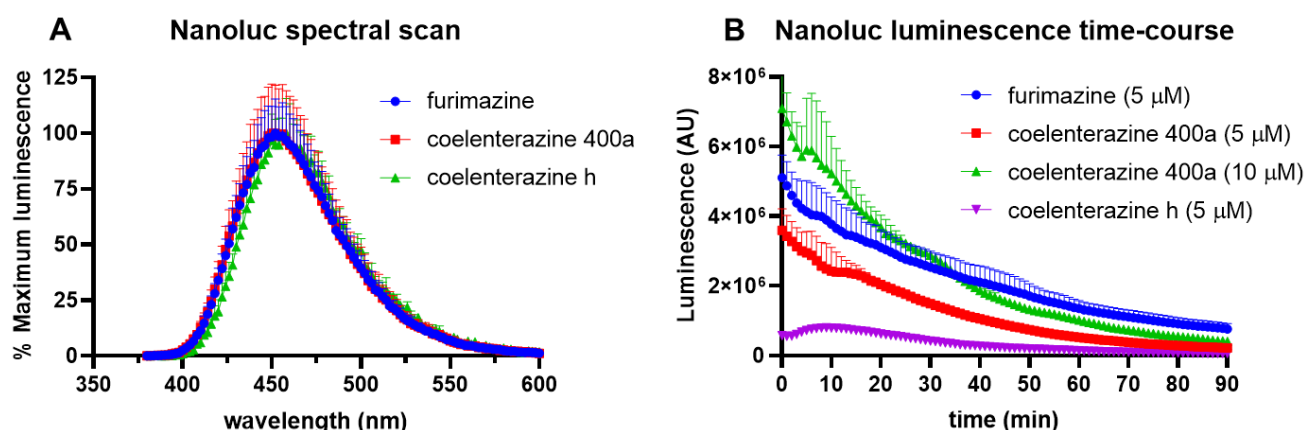


Figure 2.1. Comparison of Nluc luminescence profiles using different substrates.

HEK293T cells were transfected with $G\alpha_s$ -Nluc. (A) Spectral scans were generated after addition of substrate or vehicle and normalised as a percentage of maximum luminescence. (B) Time-courses were generated immediately after addition of substrate or vehicle and were plotted as raw luminescence values against time. One representative example with standard deviation is shown.

2.4.5 Data analysis and presentation

For signalling assays, the ligand-induced BRET ratio was calculated by subtracting the ratio of donor channel emissions over acceptor channel emissions for wells containing ligands, from wells containing vehicle:

$$\text{ligand-induced BRET ratio} = \frac{\text{acceptor channel (ligand)}}{\text{donor channel (ligand)}} - \frac{\text{acceptor channel (vehicle)}}{\text{donor channel (vehicle)}}$$

For CAMYEL assays only, decreases in ligand-induced BRET ratio represent increases in cAMP, so all values were subtracted from zero.

For time-course data, BRET response was plotted against time, with the final pre-incubation reading at the zero time point. Concentration-response curves were generated by fitting a non-linear regression to either the ligand-induced BRET ratio taken from individual time points, or the area under the curve, as noted. Data are presented as mean and standard error of the mean (SEM), unless otherwise noted.

For spectral scans, wavelength (nm) was plotted against emissions.

2.5 Homogenous time-resolved fluorescence assays

In Chapter 5, accumulation of cAMP and cGMP was detected using HTRF technology (Cisbio). A two-plate protocol was followed, whereby cells were stimulated with ligands on 48-well plates, and lysates were later transferred to a white 384-well plate along with HTRF detection reagents.

2.5.1 Products and reagents

Products, reagents, and buffers used in cell culture are listed in **Table 2.2**. Additional products and reagents used specifically for HTRF assays are listed in **Table 2.6**.

Table 2.6. Additional products and reagents used in HTRF assays.

Product	Source	Catalogue number
cAMP Gs dynamic kit	Cisbio	62AM4PEB
cGMP kit	Cisbio	62GM2PEG
Polysorbate 20 (Tween 20)	Sigma-Aldrich	P1379

2.5.2 Stimulation and lysis

HUVEC-RXFP1 cells were plated on 48-well plates in complete media and incubated until a confluent monolayer was formed for assaying. On the day of the assay, cell culture media was removed and replaced with EGM-2 without FBS, containing BSA (1 mg/ml) and IBMX (500 μ M), before stimulation with ligands. After a 40-minute stimulation at 37°C, stimulation buffer was removed and cells were lysed in 70 μ l lysis buffer containing BSA (1 mg/ml), HEPES (5 mM), and Tween 20 (0.3%), and stored at -20°C

2.5.3 Detection

Reagents were reconstituted and assay performed according to the manufacturer's instructions. Lysates were transferred into a white 384-well plate, along with cAMP-d2 or cGMP-d2 and anti-cAMP cryptate or anti-cGMP cryptate solutions. A standard curve for cAMP or cGMP was generated by serial dilution, which was also transferred to the 384-well plate along with HTRF reagents, to determine the dynamic range of the assay. Samples were incubated at room

temperature for one hour, after which TR-FRET was detected on a PHERAstar FSX microplate reader. Samples were excited at 337 nm and emissions were detected at 620 nm (europium cryptate donor) and 660 nm (d2 acceptor) using the dedicated HTRF optic module.

2.5.4 Data analysis and presentation

The HTRF signal is determined ratiometrically as acceptor channel emissions divided by donor channel emissions. The HTRF ratio is also multiplied by 10,000:

$$\text{HTRF ratio} = \frac{\text{emissions at 665 nm}}{\text{emissions at 620 nm}} \times 10,000$$

Concentration-response curves were generated by fitting either a three-parameter or bell-shaped non-linear regression to the data. HTRF data are represented as a percentage of positive control.

2.6 Molecular cloning using Gateway technology

In Chapters 3 and 4, genes for BRET-based biosensors were cloned into lentiviral vectors using MultiSite Gateway Pro (Invitrogen) technology. Gateway technology is based on site-specific recombination reactions between DNA fragments flanked with appropriate recombination sequences and target vectors with complementary recombination sequences (Hartley *et al.*, 2000; Katzen, 2007). In short, polymerase chain reaction (PCR) fragments flanked with *att* sites are generated and then recombined into donor vectors with complementary *att* sites to generate entry clones, and up to four entry clones can then be recombined into a destination vector to generate expression clones.

2.6.1 Products and reagents

Products and reagents used for molecular cloning are listed in **Table 2.7**.

Table 2.7. List of products and reagents used for molecular cloning.

Product	Source	Catalogue number
1 Kb Plus DNA ladder	Invitrogen	10787-018
Agar (bacteriological)	Oxoid	LP0011
Ampicillin	GoldBio	A-301
BP Clonase II enzyme mix	Invitrogen	11789-020
Deoxynucleotide triphosphate (dNTP) mix	Promega	U1515
DH5 α chemically competent <i>E. coli</i>	Bathgate lab	-
ISOLATE II PCR and gel kit	Bioline	BIO-52060
ISOLATE II plasmid mini kit	Bioline	BIO-52057
Kanamycin	Sigma-Aldrich	K1377
LR Clonase II Plus enzyme mix	Invitrogen	12538-120
One Shot Stbl3 chemically competent <i>E. coli</i>	Invitrogen	C7373-03
Phusion high-fidelity DNA polymerase	New England BioLabs	M0530S
PureLink HiPure plasmid maxiprep kit	Invitrogen	K2100-07
PureLink HiPure plasmid midiprep kit	Invitrogen	K2100-14
Restriction enzymes	Promega	-
Sodium chloride	Chem-Supply	SA046
SYBR safe DNA gel stain	Invitrogen	S33102
Tryptone	Oxoid	LP0042
Vent DNA polymerase	New England BioLabs	M0254S
Yeast extract	Oxoid	LP0021
Zeocin selection antibiotic	InvivoGen	ant-zn

2.6.2 Choice of vector and promoter

The pLenti X1 Zeo DEST vector was used as a backbone to clone lentiviral constructs (Campeau *et al.*, 2009). This is a promoter-less lentiviral vector based on Gateway technology that was chosen due to its ability to aid in the production of high-titre lentivirus. The constitutive human elongation factor 1- α (Ef1 α) promoter was chosen as the promoter for lentiviral constructs due to its ability to promote long-lasting transgene expression across a range of cell types, whereas the

constitutive CMV promoter was avoided as it can show higher levels of inactivation of transgenes after lentiviral transduction of some human cell types (Ramezani *et al.*, 2000; Xia *et al.*, 2007).

2.6.3 Generation of *att*-flanked PCR products

Two-fragment recombination was used to clone the Ef1 α promoter and a biosensor gene into the pLenti X1 plasmid via two-fragment recombination. The Ef1 α promoter was previously cloned into the pDONR L1-R5 vector to create the pENTR L1-R5 Ef1 α entry clone, which was the first fragment. The relevant gene for the second fragment was flanked with *attB5* and *attB2* sites and amplified by PCR using Phusion high-fidelity DNA polymerase (Chapter 3) or Vent DNA polymerase (Chapter 4), using forward and reverse primers listed in those chapters. The PCR product was then run on an agarose gel, and the band of the correct size was excised and purified.

2.6.4 Generation of entry clones

The purified *attB*-flanked PCR product was cloned into the pDONR 221 P5-P2 vector using BP Clonase II, according to the manufacturer's instructions, to create the L5-L2 entry clone. DH5 α cells were transformed with the entire reaction and grown on lysogeny broth (LB) agar plates with kanamycin (50 μ g/ml). Plasmid DNA was isolated by miniprep and analysed using restriction digest in order to identify a clone that had correctly recombined. One of the correct entry clones was sequenced using Sanger sequencing (Australian Genome Research Facility) to ensure the sequence was correct. This clone was then used for a midiprep, after which an analytical restriction digest was conducted to confirm it was the correct product.

2.6.5 Generation of expression clones

The correct L5-L2 entry clone was cloned along with the L1-R5 Ef1 α entry clone into the pLenti X1 Zeo DEST vector using LR Clonase II, according to the manufacturer's instructions, to generate the lentiviral expression clone. Stbl3 cells were transformed with the reaction, according to the manufacturer's instructions, and grown on low-salt LB agar plates with zeocin (50 μ g/ml). Plasmid DNA was isolated by miniprep and analysed using restriction digest in order to identify a clone that had correctly recombined. Correct clones were transfected into HEK293T cells to ensure they could be expressed, which was checked by using a fluorescence microscope. A correct clone

was used for a maxiprep, after which an analytical restriction digest was conducted to confirm it was the correct product. The maxiprep was used to produce lentivirus.

2.7 Lentivirus

In Chapters 3, 4, and 5, lentivirus was used to stably introduce transgenes into various cell types. In some cases, this involved crude lentivirus, whereas in other cases purified lentivirus was used, which is noted in each chapter. The third-generation lentiviral vector method was used to produce lentivirus. In this system, three human immunodeficiency virus type 1 (HIV-1) genes (*gag*, *pol*, and *rev*) are contained across the two packaging plasmids, pMDL/pRRE and pRSV-Rev (Dull *et al.*, 1998). The *gag* and *pol* genes code for polypeptides that are cleaved into various virus structural proteins and retroviral enzymes, whereas *rev* codes for a regulatory protein allowing export of mRNA from the nucleus (Ramezani & Hawley, 2002). The lentivirus is pseudotyped with the envelope protein from another virus, in this case the vesicular stomatitis virus glycoprotein (VSV-G), to broaden the tropism compared to HIV-1, which is encoded on a third plasmid, pCMV-VSV-G (Yee *et al.*, 1994). Finally, a transfer plasmid is also used, which is cloned to contain the transgene or transgenes to be introduced into target cells under the control of a desired promoter. In this thesis, the transfer plasmid used was the pLenti X1 Zeo DEST vector (Campeau *et al.*, 2009), which contains *cis*-acting elements required for transgene expression, as well as a central polypurine tract (cPPT) and Woodchuck post-transcriptional regulatory element (WPRE) that increase transduction efficiency (Barry *et al.*, 2001).

2.7.1 Products and reagents

Most reagents used for lentiviral methods are general tissue culture reagents listed in **Table 2.1**. Additional reagents specific to lentiviral methods are listed in **Table 2.8**.

Table 2.8. List of additional products and reagents used for lentivirus.

Product	Source	Catalogue number
Hexadimethrine bromide (Polybrene)	Sigma-Aldrich	H9268

2.7.2 Generation of stable cell lines using crude lentivirus

In Chapter 4, crude lentivirus was used to transduce HEK293T cells for stable expression. The method for doing so is described in that chapter.

2.7.3 Production and purification of recombinant lentivirus

In Chapters 3, 4, and 5, lentivirus was produced and purified to allow quick and easy generation of stable cell lines. HEK293T cells were plated out on five 20 cm dishes at 11 million cells per dish. The next day, fresh cell culture media was added onto cells before transfecting them with a total of 39 µg pMDL/pRRE, 15 µg pRSV-Rev, 21 µg pCMV-VSV-G, and 60 µg of a pLenti X1 plasmid containing the promoter and gene of interest, using 375 µl of Lipofectamine 2000. Two days later, media containing secreted lentivirus was harvested and spun at 2,000 RPM for five minutes to pellet any cellular debris. The supernatant was then filtered through a 0.45 µm filter and spun in a Hitachi CP100MX ultracentrifuge at approximately $72,000 \times g$ at 4°C for two and a half hours to pellet the virus. The supernatant was then discarded, and 50 µl of cold DPBS was added per tube (for a total of 150 µl per viral preparation), which was stored at 4°C overnight. Some of the crude lentivirus-containing media was also stored at 4°C overnight. The next day, the virus was resuspended in the DPBS, pooled, and stored at -80°C in 10 µl aliquots. 1 µl of the virus was also used for a visual titration, along with some of the crude lentivirus, to confirm that the virus was of high enough titre to transduce cells.

2.7.4 Visual titration of purified lentivirus

The same day the lentivirus was harvested, HEK293T cells were plated out on a 96-well plate at 2,000 cells per well. The next day, when the lentivirus was aliquoted, a serial dilution of lentivirus was also created, for a final volume of 1 µl to 0.0001 µl of lentivirus in complete DMEM. Each dilution of lentivirus was added to one well of cells in 90 µl of media. One well also contained a 1/10 dilution of the crude lentivirus. An extra 100 µl of media was added on top after about eight hours. Cells were visualised on a fluorescence microscope for fluorophore expression after three days, to ensure virus was of high enough titre to transduce cells (indicated by strong fluorescence).

2.7.5 Generation of stable cell lines using purified lentivirus

Depending on cell type, cells to be transduced were either plated out on a dish 24-48 hours in advance of transduction, transduced in a T175 flasks, or transduced via “spinoculation” for non-adherent cells. The spinoculation protocol is described in Chapter 3. For adherent cells, on the day of transduction, lentivirus was resuspended in cell culture media along with 4 µg/ml hexadimethrine bromide. Cell culture media was removed from cells to be transduced, and was replaced with media containing lentivirus. The next day, media containing lentivirus was removed and replaced with fresh cell culture media. Cells plated on a dish were transferred to a T175 flask when they reached confluency. In some cases, cells in the T175 flasks were subsequently sorted via fluorescence-activated cell sorting (FACS), as noted in individual chapters.

2.8 Flow cytometry

FACS was conducted in Chapters 3, 4, and 5 using a Becton Dickinson FACS Aria III cell sorter. Analytical flow cytometry was conducted in Chapters 3, 4, and 5 using a Beckman Coulter CytoFLEX S benchtop flow cytometer. Methods specific to individual chapters are described within those chapters.

2.8.1 Products and reagents

General tissue culture reagents are listed in **Table 2.1**, whereas additional reagents specific to flow cytometry are listed in **Table 2.9**.

Table 2.9. List of additional reagents used for flow cytometry.

Product	Source	Catalogue number
DAPI (4',6-diamidino-2-phenylindole)	Sigma-Aldrich	D9542
TrypLE Express enzyme	Gibco	12604-013

2.8.2 Lasers and filters

Several fluorophores were detected using lasers and emission filters listed in **Table 2.10**.

Table 2.10. List of excitation lasers and emission filters used in flow cytometry.

Fluorophore	Excitation laser	FACSAria III emission filter	CytoFLEX S emission filter
DAPI	Violet (405 nm)	450/40	450/45
GFP	Blue (488 nm)	510/20 ¹ 530/30	-
YFP	Blue (488 nm)	530/30	525/40
TAMRA	Yellow/Green (561 nm)	-	585/42

¹ Specifically when sorting cells expressing both GFP and YFP, in addition to applying compensation (Chapter 4)

2.8.3 Analytical flow cytometry

In Chapters 3 and 5, relaxin labelled with the fluorescent dye tetramethylrhodamine (TAMRA) was used for flow cytometry-based fluorescent ligand binding assays to show expression of RXFP1 across various cell types. Cells were plated out for binding 24-48 hours in advance. On the day of the assay, cells were washed with DPBS and adherent cells were detached using TrypLE Express rather than trypsin in order to avoid cleavage of cell surface receptors, except when only HEK cells were being used, in which case they were detached with DPBS. Cells were resuspended in 1% BSA DPBS and were spun down at 1,500 RPM for 2-3 minutes. Cells were then resuspended in 1% BSA DPBS and incubated either alone, with a saturating concentration of TAMRA-relaxin (20 nM), or with TAMRA-relaxin (20 nM) and an excess of unlabelled relaxin (2 μ M) for 30-60 minutes. Cells were then resuspended in cold 1% BSA DPBS containing DAPI (for a final concentration of 1X) in order to stain dead cells.

Cells were isolated by gating after plotting forward scatter area (FSC-A) versus side scatter area (SSC-A). Single cells were then isolated by gating after plotting FSC-A versus forward scatter height (FSC-H). Dead cells were removed by gating after laser excitation at 405 nm and plotting FSC-A against DAPI emissions (450/45 nm). From the population of live, single cells, TAMRA emissions were detected by laser excitation at 561 nm and emission at 585/42 nm.

For flow cytometry assays involving only detection of YFP expression, cells were prepared as above, but without incubating cells in the presence of ligands. Single, live cells were isolated as above, and YFP was detected by laser excitation at 488 nm and emission at 525/40 nm.

2.8.4 Fluorescence-activated cell sorting

For FACS, cells were either plated out on a dish 24-48 hours in advance (most cell types) or were detached from a confluent flask on the day of the sort (HUVECs and HUVSMCs). On the day of the sort, adherent cells were washed with DPBS, detached using trypsin-EDTA, TrypLE, or PBS, resuspended in 1% FBS DPBS, and spun down for two minutes at 1,500 RPM. Supernatant was then aspirated, and cells were washed in 1% FBS DPBS and spun down again. Supernatant was aspirated, and cells were then resuspended in ice cold 1% FBS DPBS containing 1X DAPI and filtered through a 40 μ m sieve before being sorted.

Cells were isolated by gating after plotting FSC-A versus SSC-A. Single cells were then isolated by gating after plotting side scatter width (SSC-W) against side scatter height (SSC-H) and again after plotting forward scatter width (FSC-W) against FSC-H. Dead cells were removed by gating after laser excitation at 405 nm and plotting FSC-A against DAPI emissions (450/40 nm). From the population of live, single cells, expression of the desired fluorophore was detected and gated for sorting, as noted in individual chapters.

2.8.5 Analysis and presentation of flow cytometry data

Flow cytometry data was analysed using FlowJo 10. For fluorescent ligand binding data, TAMRA emissions were plotted as a histogram against number of cells. Mean TAMRA emissions for each population of cells were extracted to obtain total and non-specific binding. Non-specific binding was subtracted from total binding to obtain a value for specific binding. For YFP expression data, YFP emissions were plotted as a histogram against number of cells. For representations of FACS, desired emissions (see individual chapters) were plot against number of cells.

3 Real-time examination of cAMP activity at relaxin family peptide receptors using a BRET-based biosensor

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3.1 Abstract

Relaxin family peptide (RXFPs) 1-4 receptors modulate the activity of cyclic adenosine monophosphate (cAMP) to produce a range of physiological functions. RXFP1 and RXFP2 increase cAMP via $G\alpha_s$, whereas RXFP3 and RXFP4 inhibit cAMP via $G\alpha_{i/o}$. RXFP1 also shows a delayed increase in cAMP downstream of $G\alpha_{i3}$. In this study we have assessed whether the bioluminescence resonance energy transfer (BRET)-based biosensor CAMYEL (cAMP sensor using YFP-Epac-Rluc), which allows real-time measurement of cAMP activity in live cells, will aid in understanding ligand- and cell-specific RXFP signalling. CAMYEL detected concentration-dependent changes in cAMP activity at RXFP1-4 in recombinant cell lines, using a variety of ligands with potencies comparable to those seen in conventional cAMP assays. We used RXFP2 and RXFP3 antagonists to demonstrate that CAMYEL detects dynamic changes in cAMP by reversing cAMP activation or inhibition respectively, with real-time addition of antagonist after agonist stimulation. To demonstrate the utility of CAMYEL to detect cAMP activation in native cells expressing low levels of RXFP receptor, we cloned CAMYEL into a lentiviral vector and transduced THP-1 cells, which express low levels of RXFP1. THP-1 CAMYEL cells demonstrated robust cAMP activation in response to relaxin. However, the CAMYEL assay was unable to detect the $G\alpha_{i3}$ -mediated phase of RXFP1 cAMP activation in PTX-treated THP-1 cells or HEK293A cells with knockout of $G\alpha_s$. Our data demonstrate that cytoplasmically-expressed CAMYEL efficiently detects real-time cAMP activation by $G\alpha_s$ or inhibition by $G\alpha_{i/o}$ but may not detect cAMP generated in specific intracellular compartments such as that generated by $G\alpha_{i3}$ upon RXFP1 activation.

3.2 Introduction

G protein-coupled receptors (GPCRs) are one of the largest families of proteins in the human genome, and regulate most aspects of human physiology. GPCRs initiate a variety of intracellular signalling cascades in response to a diverse range of ligands, by coupling to effectors including G proteins and β -arrestins. Biased signalling is a key research topic in GPCR drug development, referring to the ability of different ligands to favour coupling of the receptor to particular effectors, leading to activation of only a subset of the receptor's signalling pathways (Rajagopal et al., 2010). A related concept is system bias, whereby different cell types can show preferential coupling to particular downstream signalling pathways (Smith et al., 2018).

The relaxin family of peptides target GPCRs to produce a broad array of physiological functions across a range of tissues including the reproductive system, cardiovascular system, connective tissue, gastrointestinal tract, and brain (reviewed in Bathgate et al., 2013; Bathgate et al., 2018). In humans, the relaxin family of peptides contains seven members, including H1 relaxin (relaxin-1), H2 relaxin (relaxin-2), H3 relaxin (relaxin-3), insulin-like peptide (INSL) 3, INSL4, INSL5, and INSL6. The actions of relaxin peptides are mediated by relaxin family peptide receptors (RXFPs) 1-4 (Bathgate *et al.*, 2006b). H2 relaxin and INSL3 are the cognate ligands for RXFP1 (Hsu et al., 2002) and RXFP2 (Kumagai et al., 2002), and relaxin-3 and INSL5 are the cognate ligands for RXFP3 (Liu *et al.*, 2003b) and RXFP4 (Liu et al., 2005b), respectively. The receptors for INSL4 and INSL6 have not been identified.

One of the primary second messengers involved in relaxin family peptide receptor signalling is 3',5'-cyclic adenosine monophosphate (cAMP). Effectors of cAMP include protein kinase A (PKA), exchange protein directly activated by cAMP (Epac), and cyclic nucleotide-gated ion channels. Activation of a $G\alpha_s$ -coupled GPCR leads to synthesis of cAMP via adenylate cyclases (ACs), whereas activation of a $G\alpha_{i/o}$ -coupled receptor leads to inhibition of ACs. RXFP1 and RXFP2 couple to $G\alpha_s$ to increase cAMP activity, which is negatively modulated by $G\alpha_{oB}$ (Halls et al., 2006; Hsu et al., 2002). Unusually, RXFP1 also couples to $G\alpha_{i3}$ to increase cAMP downstream of $G\beta\gamma$, PI3K, PKC- ζ , and AC5 in a number of cell types (Halls et al., 2006; Nguyen & Dessauer, 2005a; Nguyen & Dessauer, 2005b; Nguyen et al., 2003). In contrast, RXFP3 and RXFP4 couple to $G\alpha_{i/o}$ proteins to inhibit the production of cAMP (Ang et al., 2016; Kocan et al., 2014). However, patterns of cAMP activity also depend on the cell type being stimulated. For example, fibroblasts that natively express RXFP1 show little or no increases in cAMP activity when stimulated with an RXFP1 agonist (Kocan *et al.*, 2017; Mookerjee *et al.*, 2009; Samuel *et al.*, 2004a).

Traditional assays for detecting cAMP activity do not easily measure the temporal aspects of signalling, but measuring the kinetics of signalling aids accurate detection of biased signalling (Klein Herenbrink et al., 2016). A real-time assay for cAMP activity that can be used in native cells will therefore be valuable for understanding ligand- and cell-specific effects of RXFP signalling, as well as for screening novel ligands acting at these receptors. Fortunately, there is a real-time, genetically-encoded bioluminescence resonance energy transfer (BRET)-based biosensor for cAMP activity. BRET is the transfer of energy from an excited luciferase donor to a fluorophore acceptor, when they are in close proximity. CAMYEL (cAMP sensor using YFP-Epac-Rluc) is a unimolecular BRET-based biosensor for cAMP activity, consisting of truncated and catalytically inactive human Epac1 sandwiched between Rluc (the donor) and a monomeric and circularly permuted form of the YFP citrine (the acceptor) (Jiang et al., 2007). When cAMP is not present, Epac adopts a “closed” conformation, where the donor and acceptor are in close proximity, producing a BRET signal. When cAMP binds to Epac, the donor and acceptor move farther apart, reducing BRET. These changes in BRET can be monitored in real time in live cells at 37°C.

In this study, we demonstrated the use and versatility of CAMYEL for detecting real-time cAMP activity at RXFP1-4 and developed and validated a CAMYEL lentiviral vector for the transduction of native cells expressing RXFP1. However, we were unable to detect the delayed phase of cAMP activity at RXFP1 mediated by $G\alpha_{i3}$ suggesting that the CAMYEL sensor, when expressed in the cytoplasm, may not detect cAMP generated in specific intracellular compartments.

3.3 Materials and methods

3.3.1 Reagents and materials

DMEM, DMEM/F12, RPMI 1640, trypsin-EDTA, L-glutamine, penicillin/streptomycin, DPBS, Gateway enzymes, Lipofectamine 2000 (Invitrogen); FBS (Scientifix); BSA, forskolin, HEPES, PTX (Sigma-Aldrich); Viafect (Promega); coelenterazine *h* (Nanolight); CulturPlate96 (PerkinElmer); RXFP1 agonists: H2 relaxin and the small molecule ML290 (Xiao et al., 2013); RXFP2 agonists: INSL3 and H2 relaxin; antagonist: INSL3 B-chain dimer (Shabanpoor et al., 2011); RXFP3 agonists: analogue 2 (H3 relaxin analogue) (Shabanpoor et al., 2012), peptide 5 (single-chain H3 relaxin analogue) (Hojo et al., 2016), R3/I5 (Liu et al., 2005a); antagonist: R3 B1-22R (Haugaard-Kedstrom et al., 2011); RXFP4 agonists: INSL5 and analogue 13 (INSL5 analogue) (Patil et al., 2016).

3.3.2 Synthesis of H2 relaxin labelled with TAMRA

Individual A- and B-chains of H2 relaxin with appropriate regioselective S-protection were synthesised using a CEM Liberty peptide synthesiser (Hossain *et al.*, 2008a). Upon complete coupling and Fmoc deprotection of the final amino acid, glutamine (Q), at the N-terminus of the A-chain sequence, the amine-reactive fluorophore 5(6)-TAMRA succinimidyl ester (Anaspec) was attached using manual coupling procedure. Following final TFA cleavage and purification of the crude peptides (TAMRA A-chain and B-chain), stepwise formation of the three disulfide bonds was conducted via oxidation, thiolysis and iodolysis consecutively (Hossain *et al.*, 2008a). The final TAMRA-labelled H2 relaxin was subjected to comprehensive characterization by RP-HPLC and MALDI-TOF mass spectrometry to confirm its high purity and correct molecular mass (m/z calculated for TamRLX $[M+Na]^+$, 6415.456, found 6419.70).

3.3.3 Cell culture

Cell culture media was supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% L-glutamine (complete media). Human embryonic kidney (HEK) 293T and 293A cells were grown in complete Dulbecco's modified Eagle medium (DMEM), Chinese hamster ovary (CHO-K1) cells were grown in complete DMEM/F12, and THP-1 cells were grown in complete RPMI 1640 medium (ATCC modification). All cells were cultured at 37°C with 5% CO₂. Experiments were conducted in HEK293T cells stably expressing RXFP1 (HEK-RXFP1), RXFP2 (HEK-RXFP2), or RXFP3 (HEK-RXFP3), THP-1 cells endogenously expressing RXFP1, CHO-K1 (FlpIn) cells stably expressing RXFP3 (CHO-RXFP3) (van der Westhuizen et al., 2007), and CHO-K1 cells stably expressing RXFP4 (CHO-RXFP4) (Belgi et al., 2013). Experiments were also conducted in HEK293A parental cells and HEK293A cells that do not express the G α_s subunit (Δ G α_s) (Stallaert et al., 2017).

3.3.4 Real-time BRET assays for cAMP activity in live cells

Adherent cells were seeded on six-well plates and were transfected the following day with CAMYEL in the pcDNA3.1/His vector (Jiang et al., 2007), using Lipofectamine 2000 (HEK) or Viafect (CHO), according to the manufacturers' instructions. HEK293A cells were also transfected with human RXFP1 or the G α_s subunit in the pcDNA3.1/Zeo vector. In the plasmid containing RXFP1, a prolactin signal peptide and FLAG-tag were located at the N-terminus of RXFP1 (Hsu *et al.*, 2000). The next day, cells were detached resuspended in complete media containing 25 mM HEPES but no phenol red, re-seeded into opaque white 96-well CulturPlates and grown overnight. Non-adherent THP-1 cells stably expressing CAMYEL (see below) were plated directly onto CulturPlates at 80,000 (PTX assays) or 120,000 cells per well in complete RPMI 1640 containing 25 mM HEPES but no phenol red. For all G $\alpha_{i/o}$ inhibition assays, PTX was added to wells at a final concentration of 100 ng/ml at least 16 hours before ligand stimulations. Peptides were diluted in complete phenol red free media and ML290 was diluted in DMSO. Media for cells stimulated with ML290 was also replaced with 0.5% FBS media before the assay.

Light emissions from the luciferase (Rluc) and YFP (citrine) were measured using a POLARstar Omega plate reader (BMG Labtech) at 37°C. Cells were pre-incubated with the luciferase substrate coelenterazine *h* (Nanolight) at a final concentration of 5 μ M for five minutes to establish a baseline, followed by addition of vehicle or ligand. For G $\alpha_{i/o}$ assays, indicated concentrations of forskolin were added after a pre-incubation of at least four minutes with agonist. Emissions were measured simultaneously at the wavelength windows 475/30 nm (Rluc) and

535/30 nm (citrine). Ligand-induced BRET ratio was calculated by subtracting the ratio of citrine to Rluc emissions for vehicle-treated wells from the same ratio for ligand-treated cells (Kocan et al., 2008). For CAMYEL, a decrease in ligand-induced BRET ratio corresponds to an increase in cAMP, so negative values were converted to positive values and vice versa.

3.3.5 Analysis of BRET data

cAMP data were analysed using GraphPad Prism 7. Data represent the mean $\pm$ standard error of the mean (SEM) of at least three independent experiments. For time-courses, data were normalised as a percentage of the maximum forskolin response, and were plotted against time, with the final pre-incubation reading displayed at the zero-time point (time of vehicle/ligand addition). Concentration-response curves were generated by fitting non-linear regressions to data from indicated time points, which generated pEC_{50} and E_{max} values. For $G\alpha_s$ data, the curves were expressed as a percentage of maximum forskolin response, whereas for $G\alpha_{i/o}$ data, the curves were expressed as a percentage of the forskolin response at the same time point. Statistical significance was determined using an unpaired *t*-test or one-way ANOVA.

3.3.6 Molecular cloning of the CAMYEL biosensor into lentiviral vector

A lentiviral vector containing the CAMYEL sequence under the control of the Ef1 α promoter was cloned using Gateway technology (Invitrogen). The CAMYEL-coding sequence from the pcDNA3.1/His vector was flanked with Gateway-compatible *attB5* and *attB2* sites and amplified by polymerase chain reaction (PCR) using the forward primer 5' GGGG ACA ACT TTG TAT ACA AAA GTT GAC CAT GAT CAC TCT CGG CAT GGA C 3' and reverse primer 5' GGGG ACC ACT TTG TAC AAG AAA GCT GGG TAT TAC TGC TCG TTC TTC AGC ACT C 3'. The PCR product was gel purified and cloned into the pDONR 221 P5-P2 vector using BP Clonase II, to create the pENTR L5-L2 CAMYEL entry clone. The entry clone was sequenced using Sanger sequencing to confirm the sequence was correct. Our pENTR L1-R5 Ef1 α entry clone was cloned along with the pENTR L5-L2 CAMYEL entry clone into the pLenti X1 Zeo DEST vector (Campeau et al., 2009) using LR Clonase II, to create the pLenti X1 Ef1 α CAMYEL expression clone.

3.3.7 Production of the THP-1 CAMYEL stable cell lines

For transduction of THP-1 suspension cells with CAMYEL lentivirus, a spinoculation protocol using purified lentivirus was followed. HEK293T cells were seeded on five 20 cm² dishes at 12 million cells per dish. The following day, cells were transfected with the pLenti X1 Ef1 α CAMYEL lentiviral vector and the lentiviral packaging and envelope plasmids pMDL, pRSV-Rev, and pCMV-VSV-G, using Lipofectamine 2000. After two days, media containing secreted lentivirus was harvested and spun at 2,000 RPM for five minutes, and supernatant containing lentivirus was filtered through a 0.45 μ m filter and spun at 20,000 RPM at 4°C for 2.5 hours to concentrate the virus. Supernatant was removed and virus was resuspended in 50 μ l of cold PBS per tube and stored at -80°C. 400,000 THP-1 cells in 2 ml of cell culture medium were transduced with 2 μ l of purified lentivirus and 4 μ g/ml hexadimethrine bromide (Polybrene) for 20 minutes at room temperature, then centrifuged for 30 minutes at 800 RPM. The cell pellet was resuspended in media and cultured for three days before Fluorescence-activated cell sorting (FACS) analysis using a Becton Dickinson FACSaria III to select viable cells that expressed CAMYEL (based on fluorescence of the YFP) and sort them into three expression levels (low, medium, and high). DAPI was used to stain dead cells, which were detected by laser excitation at 405 nm and emission at 450/40 nm, and were removed by gating. From the population of live single cells, YFP fluorescence was measured by laser excitation at 488 nm and emission at 530/30 nm.

3.3.8 Flow cytometry analysis and fluorescent ligand binding assay

A sample of the same HEK293A parental and Δ G α_s cells that were co-transfected with CAMYEL and RXFP1 and used for BRET assays, along with untransfected control cells, were detached from a plate and analysed using flow cytometry to confirm co-expression of CAMYEL (by YFP expression) and RXFP1 (by fluorescent ligand binding), using a Beckman Coulter CytoFLEX S. To show specific binding at RXFP1, cells were washed and resuspended in DPBS containing 1% bovine serum albumin (BSA) and incubated either alone, with TAMRA-labelled H2 relaxin (20 nM), or with TAMRA-labelled H2 relaxin and an excess of unlabelled H2 relaxin (2 μ M), for 30 minutes at room temperature. Unbound relaxin was removed by centrifugation, and cells were resuspended in cold 1% BSA DPBS containing DAPI, and used for flow cytometry. Live cells expressing YFP and TAMRA were isolated as above, but with a few differences. DAPI emission was measured at 450/45 nm, YFP emission was measured at 525/40 nm, and TAMRA was measured by laser excitation at 561 nm and emission at 585/42. A quadrant gate was used to show there was a subpopulation of cells expressing YFP and binding TAMRA-labelled relaxin. Data were analysed using FlowJo 10.

3.4 Results

3.4.1 Detection of cAMP activity at RXFP1

To establish the range of intracellular cAMP activity that can be detected using CAMYEL, HEK-RXFP1 cells transiently transfected with CAMYEL were stimulated with a range of concentrations of forskolin, a diterpene that directly activates adenylate cyclase (Seamon et al., 1981). HEK-RXFP1 cells showed fast, concentration-dependent increases in cAMP activity that peaked at about 5-10 minutes and were sustained over 45 minutes (**Figure 3.1A**). A concentration-response curve generated for 45 minutes showed a potency (pEC_{50}) of 6.77 ± 0.07 (**Figure 3.1B**) and a maximal response at 10 μ M forskolin.

After establishing that CAMYEL can detect concentration-dependent increases in cAMP activity in HEK-RXFP1 cells, we investigated agonist activation. Stimulation with the native ligand H2 relaxin or ML290 (Xiao et al., 2013) showed concentration-dependent cAMP increases that also plateaued by 5-10 minutes and were maintained for 45 minutes (**Figure 3.1C, D**). The responses approached or reached the maximum forskolin response, suggesting they are approaching the detection limit of the sensor. Notably, sub-maximal concentrations of ML290 showed slow decreases in cAMP activity after peaking at about five minutes. Concentration-response curves were generated for five and 45 minutes to compare ligand efficacy and potency values over time (**Figure 3.1E, Table 3.1**). Ligand efficacy was greater at 45 minutes compared to five minutes and was similar for both ligands. However, the potency of H2 relaxin was more than 10,000 times higher than that of ML290 at both time points ($p < 0.01$).

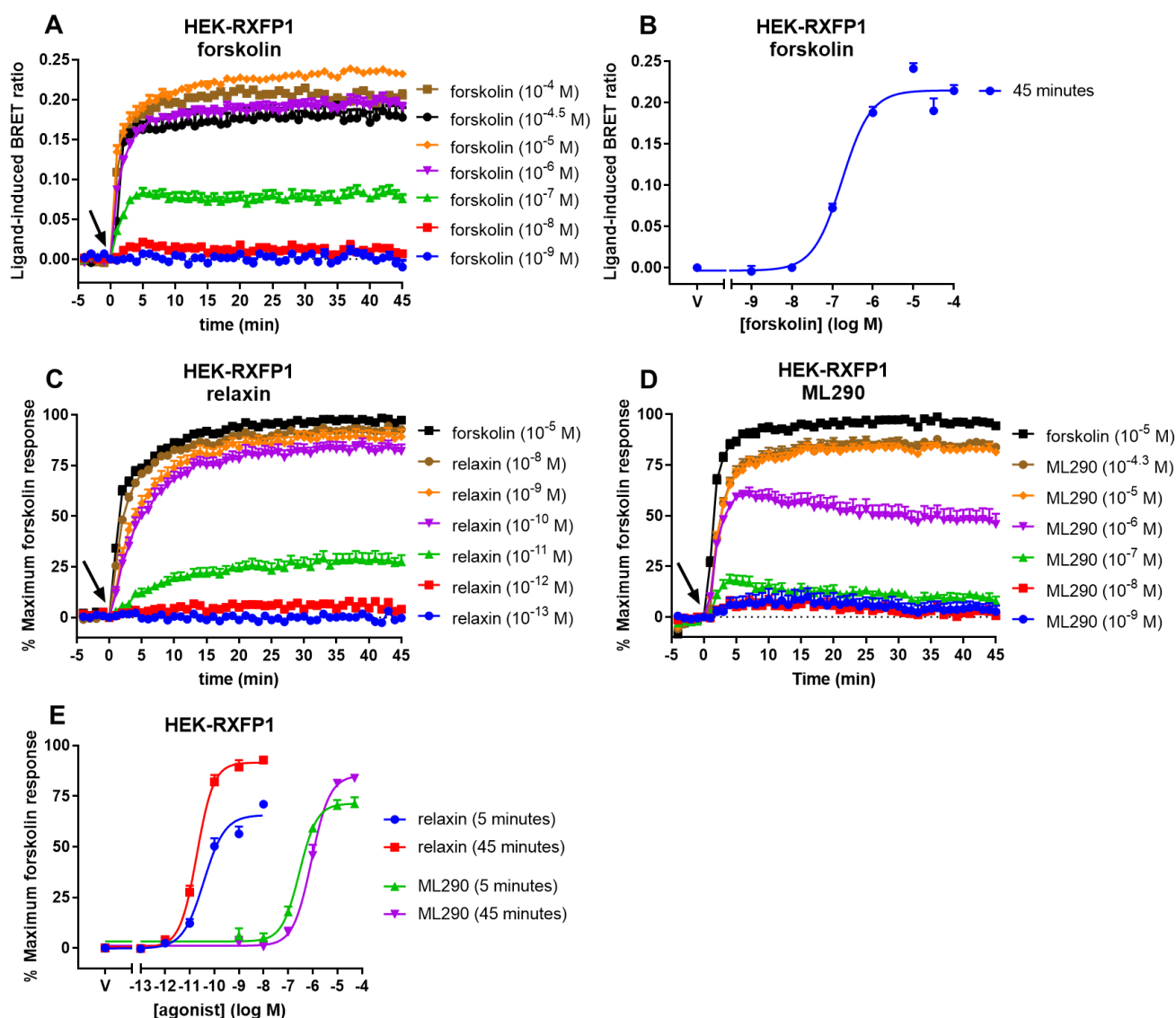


Figure 3.1. Ligand-induced cAMP activity in HEK-RXFP1 cells. HEK293T cells stably expressing RXFP1 were stimulated with vehicle, forskolin, or an RXFP1 agonist for 45 minutes. (A) Time-course of forskolin-induced cAMP activity. (B) Concentration-response curve for forskolin at 45 minutes. (C) Time-course for H2 relaxin. (D) Time-course for ML290. (E) Concentration-response curves for H2 relaxin and ML290 at five and 45 minutes. Time of ligand or vehicle addition is represented by an arrow. Data represent mean $\pm$ SEM of three independent experiments.

Table 3.1. Efficacy and potency of agonists acting at RXFP1. HEK-RXFP1 cells were stimulated for 45 minutes with ML290 or H2 relaxin. The efficacy ($E_{\max}$) and potency (pEC_{50}) were determined at five and 45 minutes. Efficacy is expressed as a percentage of the maximum response to forskolin (10 μ M). Data represent mean $\pm$ SEM of three independent experiments. * $p < 0.01$ compared to five minutes; ** $p < 0.05$ compared to five minutes; *** $p < 0.01$ compared to H2 relaxin (unpaired t -tests).

	Time point	H2 relaxin	ML290
$E_{\max}$ (% maximum forskolin)	5 minutes	65.65 $\pm$ 2.21	71.32 $\pm$ 2.02
	45 minutes	91.58 $\pm$ 1.84*	85.22 $\pm$ 2.72**
pEC_{50}	5 minutes	10.43 $\pm$ 0.08	6.54 $\pm$ 0.08***
	45 minutes	10.71 $\pm$ 0.05	6.05 $\pm$ 0.06***

3.4.2 Detection of cAMP activity at RXFP2

A forskolin time-course in HEK-RXFP2 cells showed a similar profile to that seen in HEK-RXFP1 cells, with rapid cAMP activity increases that became stable after 5-10 minutes and were sustained for 45 minutes (**Figure 3.2A**). A concentration-response curve for forskolin at 45 minutes showed a potency (pEC_{50}) of 5.92 $\pm$ 0.08 (**Figure 3.2B**), which was nearly ten times lower than that seen in the HEK-RXFP1 cells ($p < 0.01$), despite being isolated from the same parental cell line.

Stimulation of HEK-RXFP2 cells with the native ligand INSL3 or H2 relaxin (which also activates RXFP2) induced concentration-dependent increases in cAMP activity which approached the forskolin maximum response more slowly than agonists in HEK-RXFP1 cells (**Figure 3.2C, D**). The two ligands showed different temporal patterns of cAMP activity which was most evident at sub-maximal concentrations where INSL3 showed slow, sustained increases, whereas equivalent concentrations of H2 relaxin produced fast cAMP increases to five minutes which were then sustained. These differences were evident in concentration-response curves generated at five and 45 minutes whereby the potency of INSL3 increased nearly ten-fold between five and 45 minutes ($p < 0.01$), whereas the potency of relaxin stayed relatively stable for the duration of the time-course (**Figure 3.2E, Table 3.2**).

As stimulation of HEK-RXFP1 and HEK-RXFP2 both demonstrate rapid and sustained increases in cAMP, it is possible that the CAMYEL sensor is not dynamic and does not easily reverse the conformational change that occurs when cAMP binds. To simply assess whether the sensor

was able to respond to rapid changes in cAMP we utilised a specific receptor antagonist. As there is no relevant RXFP1 antagonist, we utilised a well-characterised RXFP2 antagonist INSL3 B-chain dimer (Shabanpoor et al., 2011). Blockade of RXFP2 by addition of the antagonist 10 minutes before adding the agonist INSL3 abolished the ability of INSL3 to increase cAMP activity (**Figure 3.2F**). Importantly, addition of antagonist 10 minutes *after* addition of INSL3 resulted in real-time reversal of the BRET signal highlighting the CAMYEL sensor is able to respond to dynamic changes in cAMP activity.

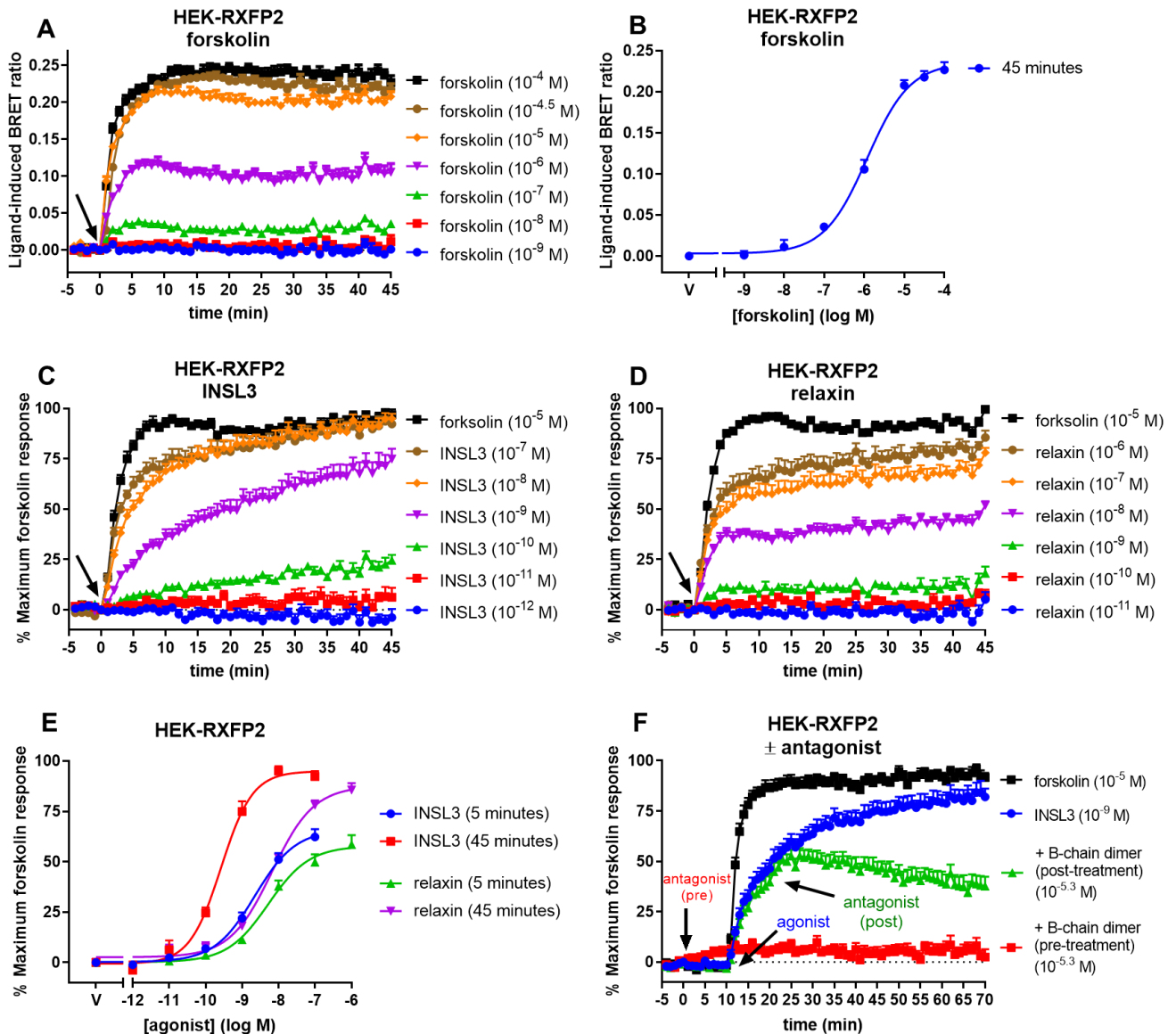


Figure 3.2. Ligand-induced cAMP activity in HEK-RXFP2 cells. HEK293T cells stably expressing RXFP2 were stimulated with ligands for 45 minutes. (A) Time-course of forskolin-induced cAMP activity. (B) Concentration-response curve for forskolin at 45 minutes. (C) Time-course for INSL3. (D) Time-course for H2 relaxin. (E) Concentration-response curves for INSL3 and H2 relaxin at five and 45 minutes. (F) Blocking and reversibility of the CAMYEL signal was demonstrated by adding an RXFP2 agonist (INSL3 B-chain dimer) 10 minutes before or after agonist addition. Time of ligand or vehicle addition is represented by an arrow. Data represent mean $\pm$ SEM of three independent experiments.

Table 3.2. Efficacy and potency of agonists acting at RXFP2. HEK-RXFP2 cells were stimulated for 45 minutes with INSL3 or H2 relaxin. The efficacy ($E_{\max}$) and potency (pEC_{50}) were determined at five and 45 minutes. Efficacy is expressed as a percentage of the maximum response to forskolin (10 μ M). Data represent mean $\pm$ SEM of three independent experiments. * $p < 0.01$ compared to five minutes; ** $p < 0.01$ compared to INSL3 (unpaired t -tests).

	Time point	INSL3	H2 relaxin
$E_{\max}$ (% maximum forskolin)	5 minutes	65.92 $\pm$ 3.94	57.79 $\pm$ 3.02
	45 minutes	94.84 $\pm$ 2.89	87.53 $\pm$ 3.25
pEC_{50}	5 minutes	8.65 $\pm$ 0.12	8.28 $\pm$ 0.13
	45 minutes	9.58 $\pm$ 0.08*	8.18 $\pm$ 0.09**

3.4.3 Inhibition of cAMP activity at RXFP3

We further validated the use of the CAMYEL assay for the $G\alpha_{i/o}$ -coupled RXFP receptors, starting with RXFP3. As $G\alpha_{i/o}$ -coupled receptors inhibit adenylate cyclase to reduce cAMP activity, we stimulated cells with forskolin and measured inhibition of forskolin-stimulated cAMP. CHO-RXFP3 cells transiently expressing CAMYEL were stimulated with either analogue 2 or peptide 5 for four minutes, before forskolin was added. 10 μ M forskolin was used, as it is the approximate EC_{50} for cAMP response in these cells (**Figure S3.1**), and has been used previously for measuring cAMP inhibition using CAMYEL in CHO cells at other GPCRs (Klein Herenbrink et al., 2016; Thompson et al., 2016). The two agonists showed similar, concentration-dependent cAMP inhibition profiles (**Figure 3.3A, B**). Complete or near-complete inhibition was reached by higher concentrations of agonist (100 nM-10 μ M) at about 20-25 minutes, before cAMP activity started to slowly increase again. Inhibitory concentration-response curves were generated for 10 and 45 minutes, expressed as a percentage of forskolin at each time point (**Figure 3.3C, Table 3.3**). Notably, ligand efficacy was not different at 10 or 45 minutes however peptide 5 demonstrated greater potency at 45 minutes than at 10 minutes and was more potent than analogue 2 only at 45 minutes ($p < 0.05$).

As our lab also had HEK-RXFP3 cells, they were also tested for agonist-induced cAMP inhibition together with another agonist, R3/I5. Forskolin is more potent in HEK293T than CHO-K1 cells, so we used a lower concentration (500 nM, $\sim EC_{50}$) to allow detection of agonist-induced inhibition (**Figure S3.2**). All agonists showed concentration-dependent inhibition of forskolin-stimulated cAMP, which reached maximum inhibition after about 15-20 minutes and, unlike in

CHO-RXFP3 cells, were sustained for the duration of the time-course (**Figure 3.3D-F**). All the ligands demonstrated lower efficacy than in CHO-RXFP3 cells, especially R3/I5 which had significantly lower efficacy than the other ligands but only at 10 minutes (**Figure 3.3G, Table 3.4**). Importantly this ligand is not a partial agonist of cAMP activation (Liu et al., 2005a) and the data may reflect the strong potency of forskolin in HEK cells and the R3/I5 data stimulations being performed at a later time to the other agonists. Notably R3/I5 potency was the same as the other ligands as expected from previous data although the data from analogue 2 at 45 minutes was not of sufficient quality to generate accurate curve fits in Prism.

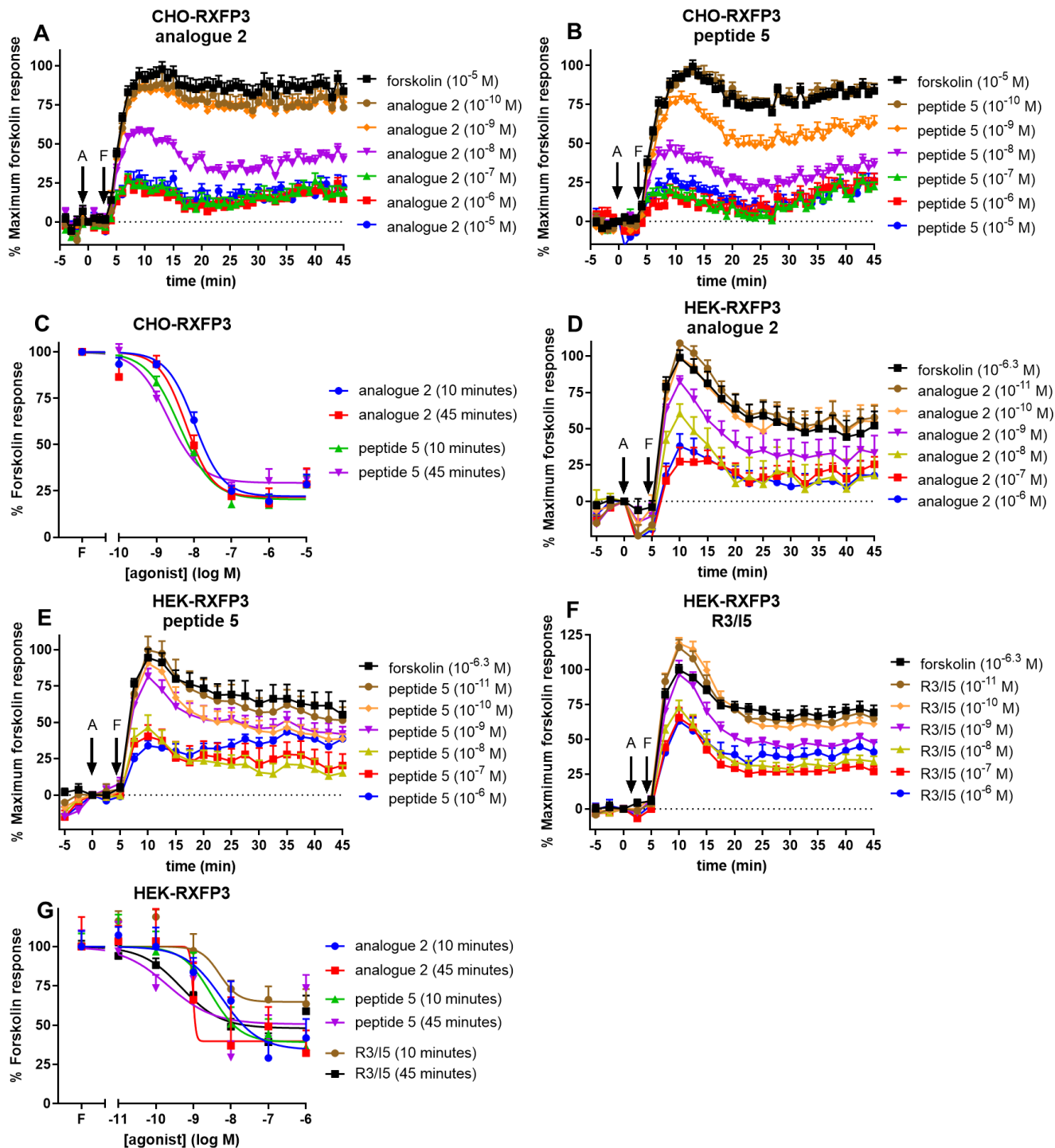


Figure 3.3. Ligand-induced cAMP activity in cells expressing RXFP3. CHO-K1 and HEK293T cells stably expressing RXFP3 (CHO-RXFP3 and HEK-RXFP3) were stimulated with vehicle, forskolin, and an RXFP3 agonist for 45 minutes. CHO-RXFP3 cells were stimulated with (A) analogue 2 or (B) peptide 5, with addition of forskolin (10 μ M) after four minutes. (C) Concentration-response curves were generated for CHO-RXFP3 at 10 and 45 minutes. HEK-RXFP3 cells were stimulated with (D) analogue 2, (E) peptide 5, or (F) R3/I5, with addition of forskolin (500 nM) after five minutes. (G) Concentration-response curves were generated for HEK-RXFP3 cells at 10 and 45 minutes. Time of agonist or vehicle (A) and forskolin or vehicle (F) addition are represented by arrows. Concentration-response data is expressed as a percentage of forskolin for the indicated time point. Data represent mean $\pm$ SEM of three independent experiments.

Table 3.3 Efficacy and potency of agonists acting at RXFP3 in CHO-K1 cells. CHO-RXFP3 cells were stimulated with agonist for 45 minutes, with addition of forskolin four minutes after agonist addition. The efficacy ($E_{\max}$) and potency (pEC_{50}) were determined at five and 45 minutes. Efficacy is expressed as a percentage of the response to forskolin (10 μ M). Data represent mean $\pm$ SEM of three independent experiments. * $p < 0.05$ compared to analogue 2 (unpaired t -test).

	Time point	Analogue 2	Peptide 5
$E_{\max}$ (% forskolin) (bottom)	10 minutes	22.06 $\pm$ 2.54	20.42 $\pm$ 2.79
	45 minutes	21.58 $\pm$ 3.93	29.4 $\pm$ 2.68
pEC_{50}	10 minutes	7.98 $\pm$ 0.07	8.39 $\pm$ 1.00
	45 minutes	8.21 $\pm$ 0.12	8.70 $\pm$ 0.11*

Table 3.4. Efficacy and potency of agonists acting at RXFP3 in HEK293T cells. HEK-RXFP3 cells were stimulated with agonist for 45 minutes, with addition of forskolin four minutes after agonist addition. The efficacy ($E_{\max}$) and potency (pEC_{50}) were determined at five and 45 minutes. Efficacy is expressed as a percentage of the response to forskolin (10 μ M). Data represent mean $\pm$ SEM of three independent experiments.

	Time point	Analogue 2	Peptide 5	R3/I5
$E_{\max}$ (% forskolin) (bottom)	10 minutes	34.27 $\pm$ 9.834	39.18 $\pm$ 7.80	64.77 $\pm$ 6.23
	45 minutes	39.72 $\pm$ 10.82	50.57 $\pm$ 9.44	47.97 $\pm$ 4.61
pEC_{50}	10 minutes	8.24 $\pm$ 0.36	8.52 $\pm$ 0.32	8.30 $\pm$ 0.43
	45 minutes	Ambiguous	9.68 $\pm$ 0.65	9.32 $\pm$ 0.26

3.4.4 Modulation of CAMYEL signal using $G\alpha_{i/o}$ inhibition and antagonist

We also demonstrated the ability to inhibit or reverse the BRET signal at a $G\alpha_{i/o}$ receptor, in HEK-RXFP3 cells. Treatment with the $G\alpha_{i/o}$ inhibitor pertussis toxin (PTX) (100 ng/ml; 20 hours) abolished the ability of R3/I5 to inhibit forskolin-induced cAMP activity at RXFP3 (**Figure 3.4A**). Treatment with the antagonist R3 B1-22R (10 μ M) after stimulation with agonist (30 minutes) rapidly reversed the BRET signal (**Figure 3.4B**), again demonstrating that we are able to measure dynamic cAMP changes using the CAMYEL sensor.

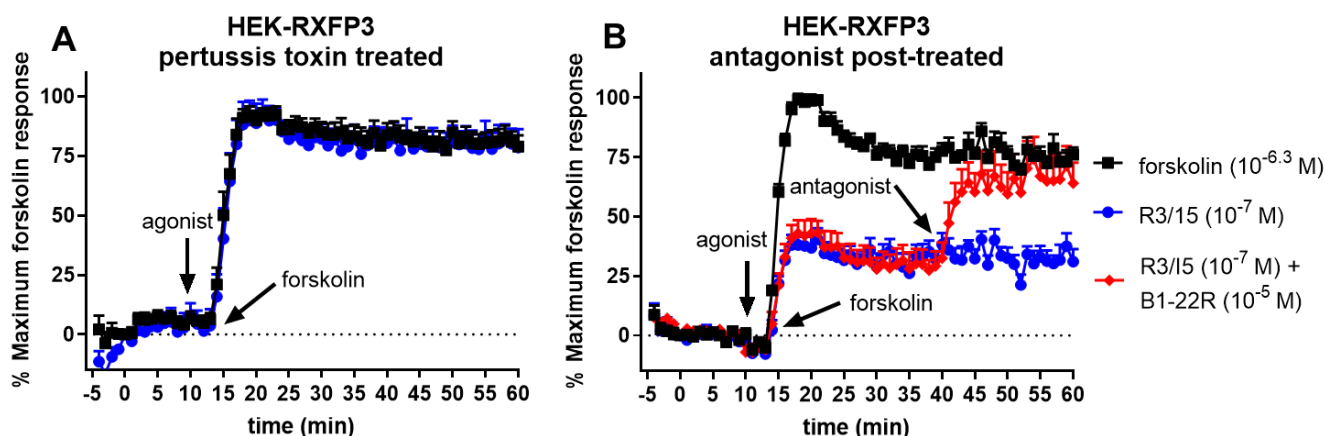


Figure 3.4. Blocking and reversing the CAMYEL signal in HEK-RXFP3 cells. (A) Pre-treatment of HEK-RXFP3 cells with the $G\alpha_{i/o}$ inhibitor pertussis toxin (100 ng/ml; 20 h) abolished R3/I5-induced inhibition of cAMP activity. (B) Treatment with the RXFP3 antagonist R3 B1-22R after stimulation with R3/I5 (30 min) and forskolin showed reversal of the R3/I5 response in real time. Time of ligand or vehicle addition is represented by an arrow. Data represent the mean $\pm$ SEM of three independent experiments.

3.4.5 Inhibition of cAMP activity at RXFP4

Finally, in our initial characterisation of CAMYEL in recombinant cells, we tested the ability of agonists to inhibit cAMP activity at RXFP4. We only had CHO-K1 cells stably expressing RXFP4 (CHO-RXFP4) available, and tested the native ligand INSL5 and analogue 13 (Patil et al., 2016). As with CHO-RXFP3, cells were pre-incubated for four minutes with agonist, before adding forskolin at a concentration of 10 μ M, which is the approximate EC_{50} for forskolin in these cells (Figure S3.3). The two agonists showed concentration-dependent decreases in cAMP activity, which plateaued after about 30 minutes (Figure 3.5A, B). Concentration-response curves were generated for 10 and 45 minutes, expressed as a percentage of forskolin activity at the same time point (Figure 3.5C). At 45 minutes, the efficacy of INSL5 was greater than that of analogue 13 but this was not statistically significant ($p > 0.05$) (Table 3.5). The lower efficacy of the ligands seen in these cells compared to ligands in CHO-RXFP3 cells may be related to the lower expression of RXFP4 in the CHO-RXFP4 cells. The potency of INSL5 (8.58 ± 0.16) was not significantly higher than analogue 13 (7.75 ± 0.49) ($p > 0.05$).

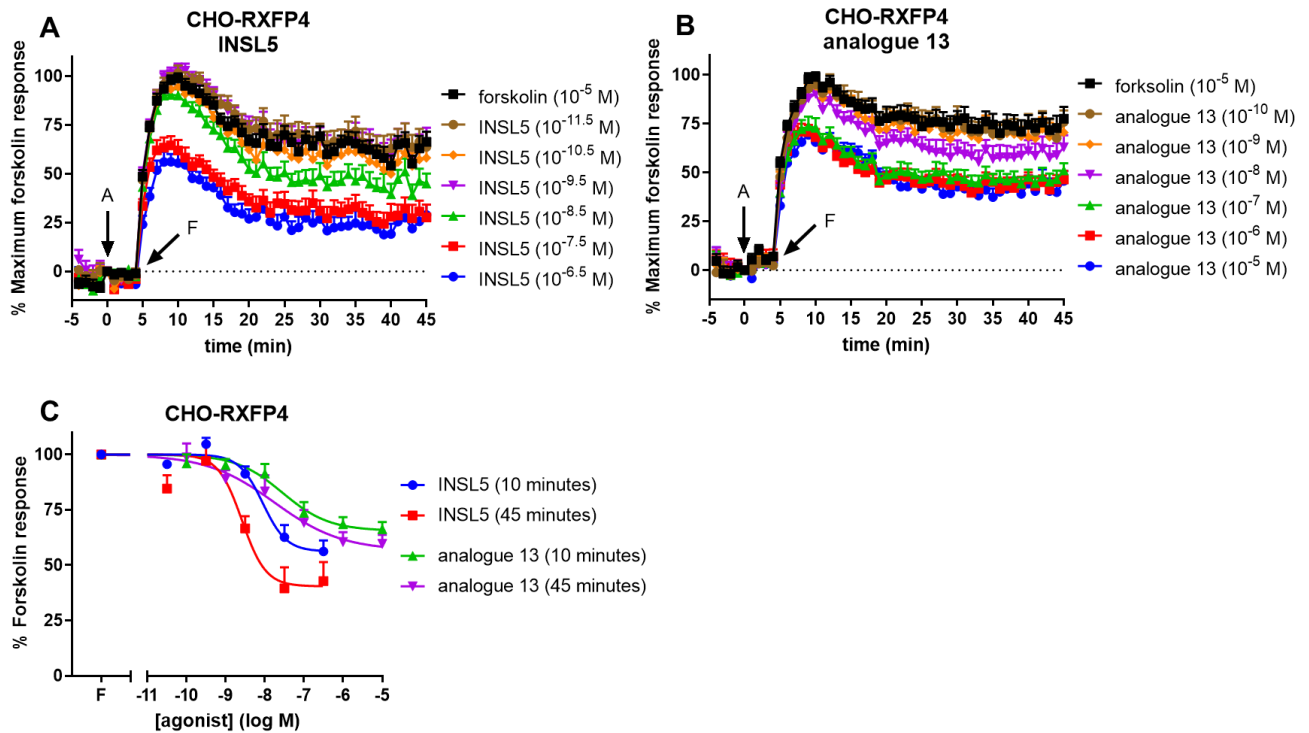


Figure 3.5. Ligand-induced cAMP activity in CHO-RXFP4 cells. CHO-K1 cells stably expressing RXFP4 (CHO-RXFP4) were stimulated with vehicle, forskolin, and an RXFP4 agonist for 45 minutes, with addition of forskolin ($10 \mu\text{M}$) four minutes after agonist addition. Time-courses were generated for (A) INSL5 and (B) analogue 13. (C) Concentration-response curves were generated for both agonists at 10 and 45 minutes. Time of agonist or vehicle (A) and forskolin or vehicle (F) addition are represented by arrows. Concentration-response data is expressed as a percentage of forskolin for the indicated time point. Data represent mean $\pm$ SEM of three independent experiments.

Table 3.5. Efficacy and potency of agonists acting at RXFP4. CHO-RXFP4 cells were stimulated with agonist for 45 minutes, with addition of forskolin four minutes after agonist addition. The efficacy ($E_{\max}$) and potency (pEC_{50}) were determined at five and 45 minutes. Efficacy is expressed as a percentage of the response to forskolin (10 μ M). Data represent mean $\pm$ SEM of three independent experiments.

	Time point	INSL5	Analogue 13
$E_{\max}$ (% forskolin) (bottom)	10 minutes	56.16 $\pm$ 3.92	65.51 $\pm$ 4.01
	45 minutes	40.42 $\pm$ 5.91	56.52 $\pm$ 7.49
pEC_{50}	10 minutes	8.05 $\pm$ 0.17	7.55 $\pm$ 0.28
	45 minutes	8.58 $\pm$ 0.16	7.75 $\pm$ 0.49

3.4.6 Investigation of RXFP1 signalling in a native expressing cell line using CAMYEL

So far, we have shown that CAMYEL can detect cAMP activity with high sensitivity in cells that recombinantly overexpress the receptor of interest. However, we have not demonstrated its use in cells that natively express the receptor at lower levels. To enable the use of CAMYEL in all types of mammalian cells, we cloned CAMYEL into the pLenti X1 Zeo DEST vector under control of the constitutive Ef1 α promoter using Gateway cloning, to create the pLenti X1 Ef1 α CAMYEL expression clone (see Methods). We showed that transfection of this vector allows robust expression of CAMYEL (data not shown) and therefore produced lentivirus for the transduction of cells.

Human monocytic leukaemia THP-1 cells have commonly been used for detecting relaxin induced cAMP activity even though they have low endogenous expression of RXFP1 (~275 receptors per cell (Parsell et al., 1996)), in contrast to HEK-RXFP1 cells, which have ~50,000-100,000 receptors per cell (Sudo et al., 2003). As THP-1 cells are a non-adherent cell line we used a spinoculation protocol involving purified lentivirus to transduce the cells, followed by sorting using FACS into populations expressing low, medium, or high levels of CAMYEL (**Figure 3.6**). The “high” CAMYEL population was initially tested for concentration-dependent increases in forskolin-induced cAMP activity (**Figure 3.7A**). Forskolin produced rapid dose-dependent increases in cAMP with a maximum response at 100 μ M and a higher potency at five minutes (5.29 $\pm$ 0.03) than at 45 minutes (4.93 $\pm$ 0.05) ($p < 0.01$) (**Figure 3.7B**).

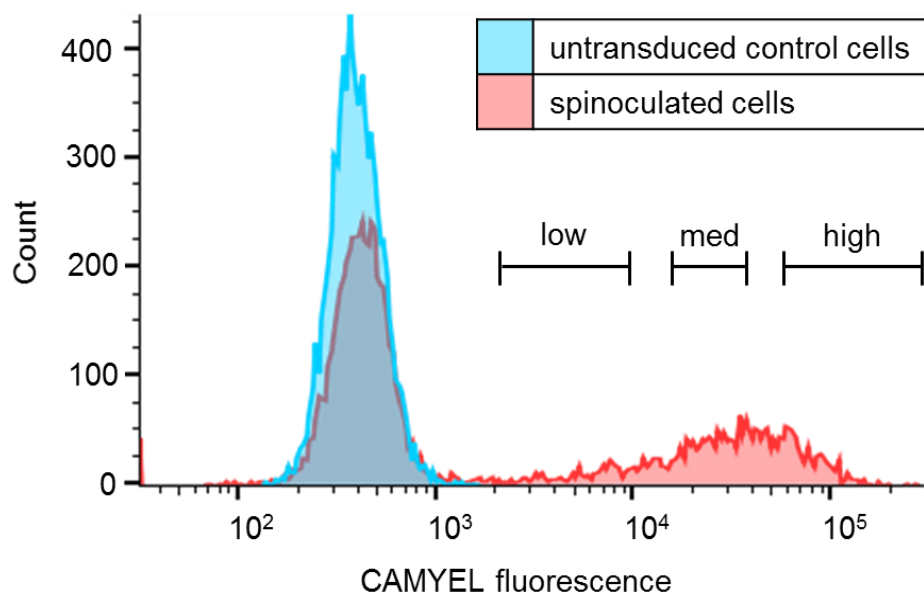


Figure 3.6. Representation of fluorescence-activated cell sorting (FACS) of transduced THP-1 CAMYEL cells. THP-1 cells were transduced by spinoculation with CAMYEL lentivirus and were sorted to remove untransduced cells. Single, live cells were isolated, and sorted by expression of YFP into low, medium, and high levels of CAMYEL expression, based on YFP fluorescence. Cells with YFP expression that was no greater than control THP-1 cells were removed.

Stimulation of the three sorted THP-1 CAMYEL populations with H2 relaxin for 45 minutes revealed robust concentration-dependent increases in cAMP activity (**Figure 3.7C, E, G**). The higher concentrations of H2 relaxin (10 and 100 nM) showed more transient responses than seen in HEK-RXFP1 cells, as cAMP reached a maximum by five minutes before declining to less than half of the maximum forskolin response. Notably, the response to H2 relaxin did not approach or reach the maximal detectable response by the CAMYEL in THP-1 cells (**Figure 3.7A**). Concentration-response curves were generated for five and 45 minutes to compare the three populations (**Figure 3.7D, F, H**). Notably, there was no difference in H2 relaxin efficacy or potency between the cells, indicating the level of CAMYEL had no effect on the response to H2 relaxin (**Table 3.6**). Ligand efficacy was greater at five minutes as expected from the shape of the response curves, but ligand potency was higher at 45 minutes ($p < 0.05$).

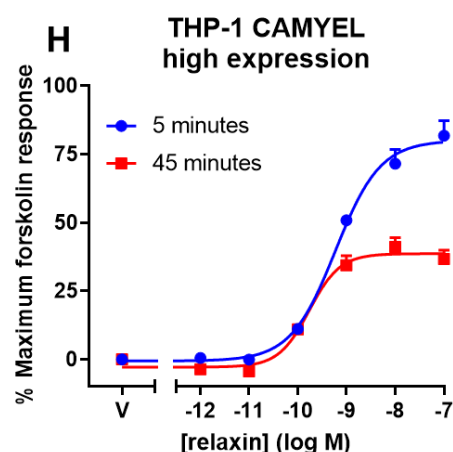
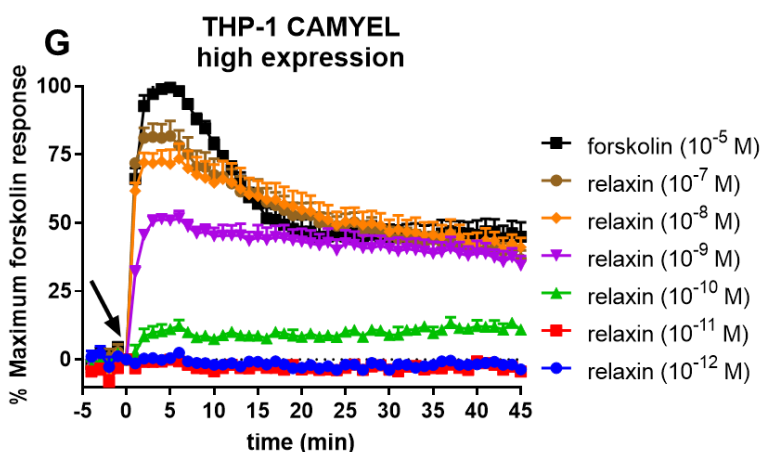
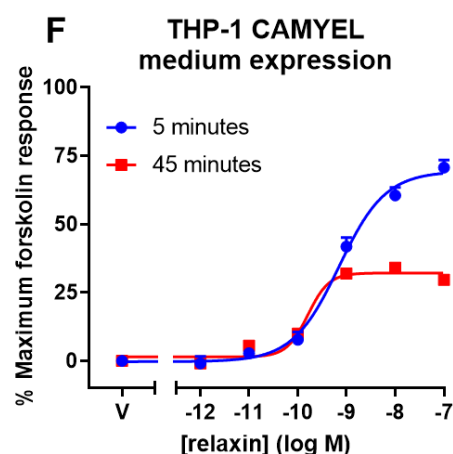
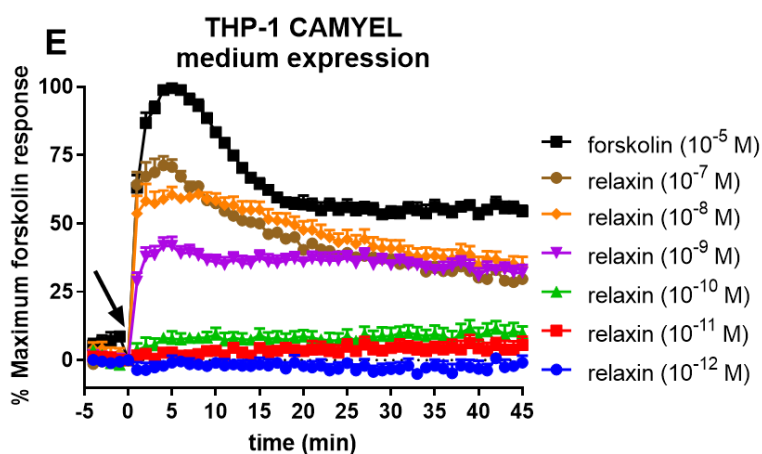
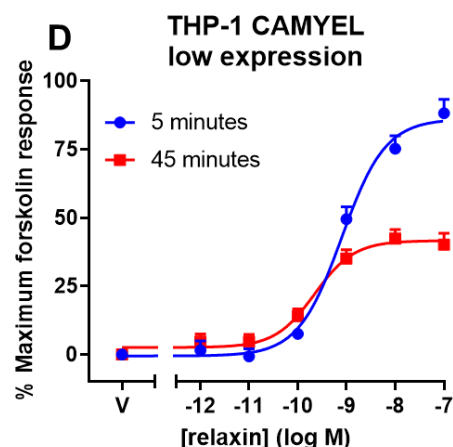
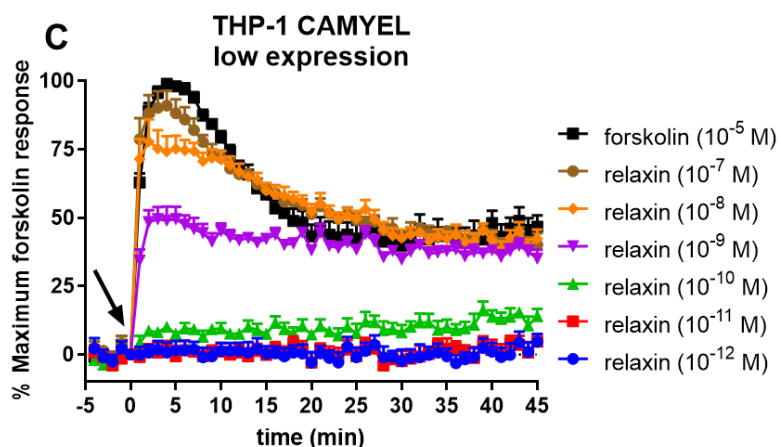
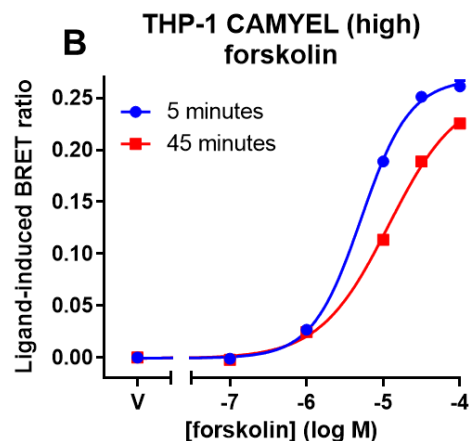
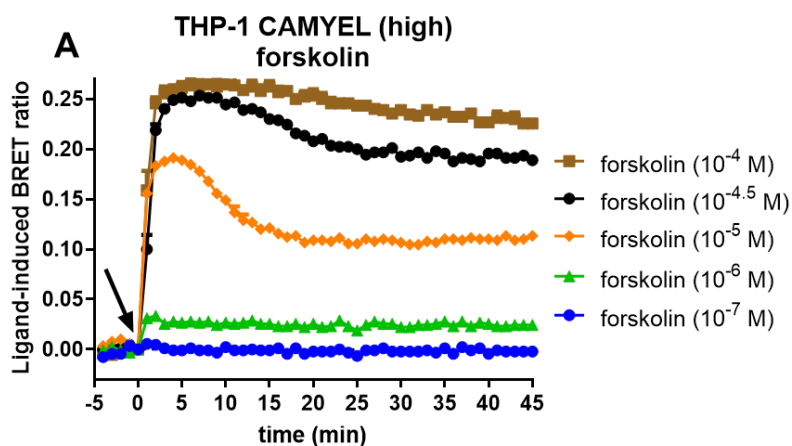


Figure 3.7. Ligand-induced cAMP activity in THP-1 CAMYEL cells. THP-1 cells that endogenously express RXFP1 and stably express high, medium, or low levels of CAMYEL (THP-1 CAMYEL) were stimulated with vehicle, forskolin, or H2 relaxin for 45 minutes. (A) Time-course for forskolin activity in high expression cells. (B) Concentration-response curves for forskolin at five and 45 minutes. (C, E, G) Time-courses for H2 relaxin in the low-, medium-, and high-expressing cells. (D, F, H) Concentration-response curves for H2 relaxin at five and 45 minutes in the low-, medium-, and high-expressing cells. Time of ligand or vehicle addition is represented by an arrow. Data represent mean $\pm$ SEM of three independent experiments.

Table 3.6. Efficacy and potency of H2 relaxin in the THP-1 CAMYEL stable cell line.

THP-1 CAMYEL cells sorted into three levels (high, medium, low) of CAMYEL expression were stimulated with H2 relaxin for 45 minutes. The efficacy ($E_{\max}$) and potency (pEC_{50}) was determined for each expression level at five and 45 minutes. Efficacy is expressed as a percentage of the maximum response to forskolin (10 μ M), with vehicle expressed as 0%. Data represent mean $\pm$ SEM of three independent experiments. * $p < 0.05$ compared to five minutes (unpaired t -test).

	Time point	Low CAMYEL	Medium CAMYEL	High CAMYEL
$E_{\max}$ (% maximum forskolin)	5 minutes	86.11 $\pm$ 2.23	69.29 $\pm$ 2.52	80.02 $\pm$ 2.93
	45 minutes	41.61 $\pm$ 2.29*	32.15 $\pm$ 1.87*	38.64 $\pm$ 1.77*
pEC_{50}	5 minutes	9.10 $\pm$ 0.09	9.15 $\pm$ 0.08	9.23 $\pm$ 0.08
	45 minutes	9.67 $\pm$ 0.15*	9.80 $\pm$ 0.21*	9.77 $\pm$ 0.10*

Relaxin-stimulated intracellular cAMP accumulation through RXFP1 has been shown to be modulated by three G proteins. Immediate increases in cAMP are mediated by $G\alpha_s$ and negatively modulated by $G\alpha_{oB}$ (Halls et al., 2006), whereas there is a delayed increase in cAMP activity after ~15 minutes mediated by $G\alpha_{i3}$ in a number of cell types, including HEK-RXFP1 and THP-1 (Halls et al., 2006; Nguyen & Dessauer, 2005b; Nguyen et al., 2003). However, using the CAMYEL assay we have not detected a biphasic increase in cAMP activity at RXFP1. To investigate further, we tested the effect of treatment with PTX (100 ng/ml; 16-18 hours) on H2 relaxin-induced cAMP activity over time in THP-1 CAMYEL cells (**Figure 3.8A, B**). Comparing the H2 relaxin responses as a percentage of the forskolin response at five minutes showed a trend towards higher cAMP activity in PTX-treated cells at higher concentrations of H2 relaxin, suggesting that PTX is inhibiting the $G\alpha_{oB}$ inhibition, but this was not significant ($p > 0.05$) (**Figure 3.8C**). At 45 minutes, there was no difference between treated and untreated cells (**Figure 3.8D**), suggesting we are not seeing a delayed $G\alpha_{i3}$ -mediated enhancement of cAMP.

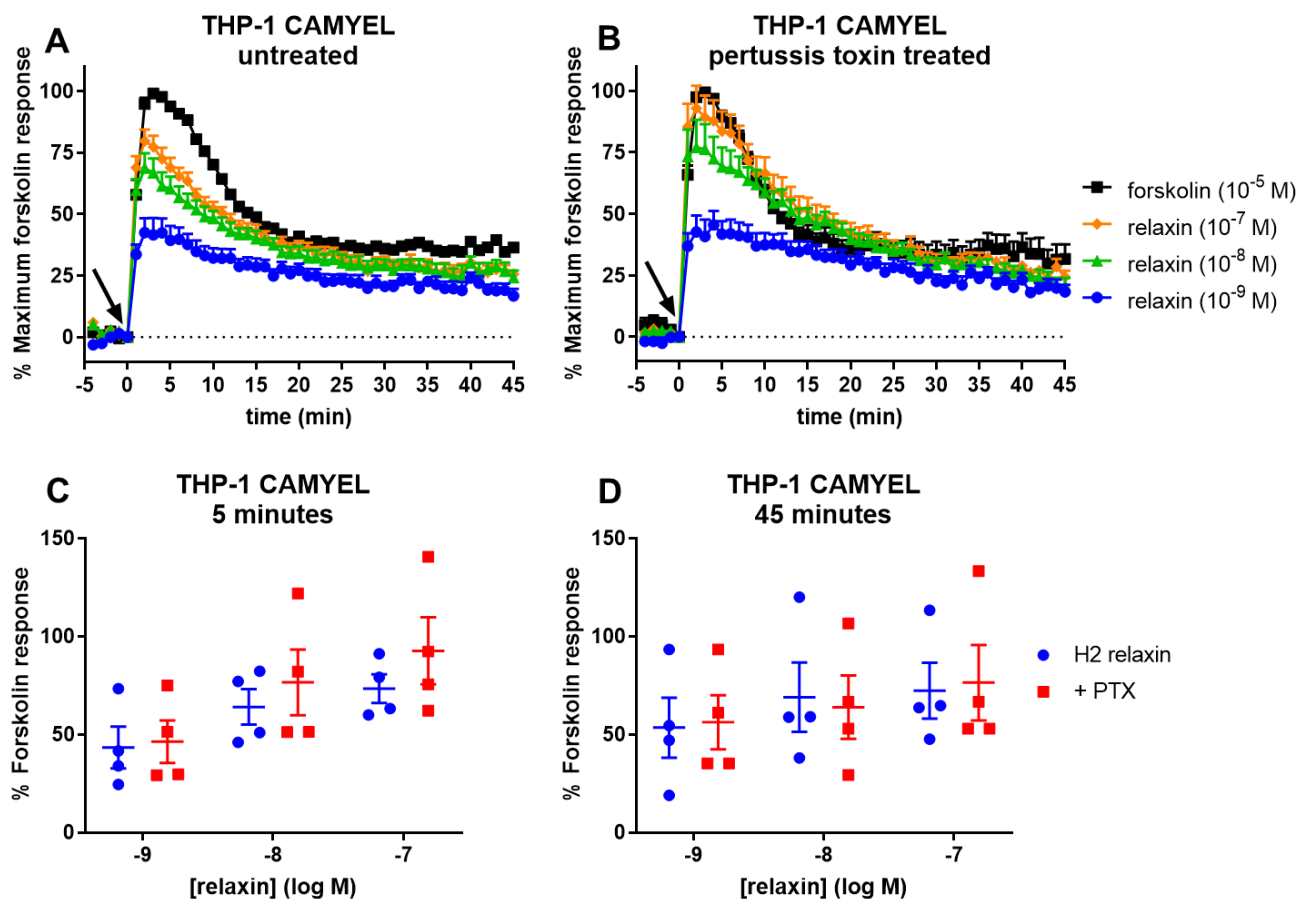


Figure 3.8. Effect of pertussis toxin on cAMP activity in THP-1 CAMYEL cells. THP-1 CAMYEL (high expression) were either (A) untreated or (B) treated with pertussis toxin (100 ng/ml; 16-18 h), and stimulated with vehicle, forskolin, or H2 relaxin for 45 minutes. Treated and untreated cells were compared at (C) five and (D) 45 minutes. Time of ligand or vehicle addition is expressed by an arrow. Data in (C) and (D) are expressed as a percentage of the forskolin response at the same time point. Data represent the mean $\pm$ SEM of four independent experiments.

3.4.7 Dissecting RXFP1 cAMP activity using HEK293A $\Delta G\alpha_s$ cells

To further investigate this potential lack of $G\alpha_{i3}$ cAMP effect, we used novel HEK293A cells with CRISPR/Cas9-mediated knockout of $G\alpha_s$ members (*GNAS* and *GNAL* genes) (Stallaert et al., 2017). We first produced forskolin concentration-response data for the HEK293A parental and $G\alpha_s$ knockout ($\Delta G\alpha_s$) cells. HEK293A parental cells showed robust increases in cAMP activity that were very similar to those seen earlier in HEK293T cells expressing RXFP1 or RXFP2 (**Figure 3.9A**). However, the $\Delta G\alpha_s$ cells showed very weak cAMP activity, requiring relatively high concentrations of forskolin ($>10 \mu\text{M}$) to detect a cAMP increase, which was only transient (**Figure 3.9B**). As forskolin has a lower affinity for adenylate cyclase in the absence of $G\alpha_s$ (Dessauer et al., 1997), we transfected $G\alpha_s$ into these cells to show that forskolin potency could be rescued by re-expression of $G\alpha_s$ (**Figure 3.9C**). At 45 minutes, the potency of forskolin in HEK293A parental cells was 5.91 ± 0.04 . In $\Delta G\alpha_s$ cells with re-expression of $G\alpha_s$, the potency was 7.1 ± 1.29 (**Figure 3.9D**).

Parental HEK293A and $\Delta G\alpha_s$ cells were then transiently co-transfected with CAMYEL and RXFP1 to detect the $G\alpha_{i3}$ -mediated phase of cAMP activity. Isoprenaline ($10 \mu\text{M}$), which targets $G\alpha_s$ -coupled β -adrenoceptors endogenous to HEK293 cells, was used as a control for these experiments to confirm lack of $G\alpha_s$ activity. As expected, the response to isoprenaline was robust in the parental cells (**Figure 3.9E**), but was abolished in the $\Delta G\alpha_s$ cells (**Figure 3.9F**). Similarly, the response to H2 relaxin (100 pM - 100 nM) was robust in parental cells, but was completely abolished in the $\Delta G\alpha_s$ cells. To test whether pre-activation of adenylate cyclase is required to detect the delayed $G\alpha_{i3}$ -mediated phase of cAMP activity, we primed the $\Delta G\alpha_s$ cells with forskolin ($33 \mu\text{M}$) to compensate for the loss of $G\alpha_s$, before adding the ligands (**Figure 3.9G**). However, no response to H2 relaxin was detectable. Additionally, we used flow cytometry to confirm that a subpopulation of cells from the same transfected population used in the assay were co-expressing CAMYEL (by YFP expression of the sensor) and RXFP1 (by binding of fluorescent TAMRA-H2 relaxin) (**Figure S3.4**), demonstrating that the lack of response is not due to CAMYEL and RXFP1 not being present in the same cells.

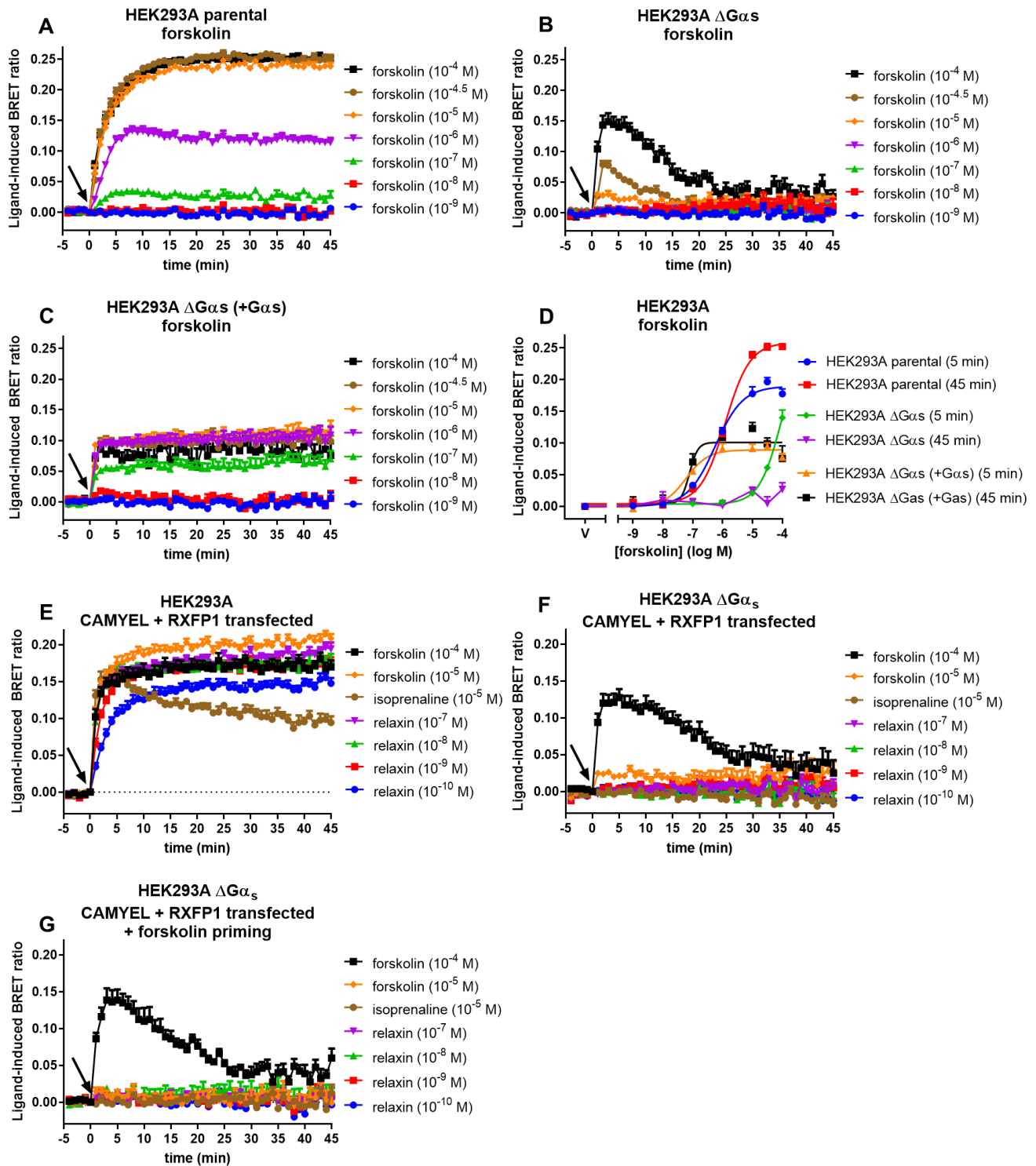


Figure 3.9. cAMP activity in HEK293A parental and $G\alpha_s$ knockout ($\Delta G\alpha_s$) cells. (A) HEK293A parental cells, (B) HEK293A $\Delta G\alpha_s$ cells, and (C) HEK293A $\Delta G\alpha_s$ cells with $G\alpha_s$ transfected in, were transfected with CAMYEL and stimulated with vehicle or forskolin for 45 minutes. (D) Concentration-response curves were generated for forskolin at five and 45 minutes. (E) HEK293A parental and (F) HEK293A $\Delta G\alpha_s$ cells were co-transfected with CAMYEL and RXFP1, and were stimulated with vehicle, forskolin, isoprenaline, or H2 relaxin for 45 minutes. (G) HEK293A $\Delta G\alpha_s$ cells were primed with forskolin (33 μ M) to compensate for the loss of $G\alpha_s$, before adding forskolin, isoprenaline, or H2 relaxin. Time of ligand or vehicle addition is represented by an arrow. Data represent mean $\pm$ SEM of three independent experiments.

3.5 Discussion

Biased signalling is the ability of a ligand to selectively activate particular signalling pathways downstream of a GPCR in the same cell type, compared to the endogenous ligand. Biased signalling has been shown to occur at relaxin family peptide receptors. For example, at RXFP1, H2 relaxin promotes ERK1/2 activation but ML290 does not (Kocan et al., 2017), whereas both agonists signal via other pathways such as cAMP. Relatedly, system bias has been shown, particularly for RXFP1. For example, HEK-RXFP1 cells signal via cAMP but not cGMP, whereas fibroblasts that natively express RXFP1 signal via cGMP but not cAMP, and human vascular cells that express RXFP1 signal through both (Kocan et al., 2017). Measuring the temporal aspects of signalling facilitates accurate detection of biased ligands (Klein Herenbrink et al., 2016), and the use of native cells will facilitate understanding of how relaxin family peptide receptors signal in different cell backgrounds. Therefore, in this study, we set out to demonstrate the use of the BRET-based biosensor CAMYEL for real-time cAMP signalling at relaxin family peptide receptors, initially in recombinant cells, but with the goal of detecting cAMP in native cells.

RXFP receptor signalling has typically been measured in HEK293T or CHO-K1 cells expressing the receptor of interest, using traditional end point cAMP assays, such as a cAMP response element (CRE) reporter gene assay, or accumulation kits including AlphaScreen and homogenous time resolved fluorescence (HTRF). However, the CRE reporter gene assay provides a readout of total downstream activity from a combination of signalling pathways, including cAMP/PKA, Ca^{2+} /CaM, Ras/ERK, and PI3K/Akt (Lonze & Ginty, 2002), and is therefore inappropriate for detecting bias. Although kit-based assays directly measure cAMP, they are laborious and involve stimulations in the presence of phosphodiesterase inhibitors to stop the degradation of cAMP, and therefore do not reveal the kinetics of cAMP activity. We have demonstrated that CAMYEL is a sensitive and robust alternative for detecting real-time cAMP activity in cells in the absence of phosphodiesterase inhibitors. Notably, we were able to detect both $\text{G}\alpha_s$ -mediated cAMP activation and $\text{G}\alpha_{i/o}$ -mediated cAMP inhibition for all RXFP receptors and multiple cell types, including recombinant and native cells. At the $\text{G}\alpha_s$ -coupled RXFP1 and RXFP2 stably expressed in HEK293T cells, we showed sustained concentration-dependent increases in cAMP activity that differed over time between the agonists. At RXFP3 and RXFP4 expressed in CHO-K1 or HEK293T cells, we showed sustained concentration-dependent inhibition of forskolin-stimulated cAMP activity, with more similar patterns of cAMP inhibition induced by agonists. Agonists at the four receptors also showed similar potencies to those already seen using traditional cAMP end point assays.

The sustained cAMP activity seen at RXFP1 and RXFP2 is consistent with the finding that these receptors lack β -arrestin-mediated desensitisation and show poor internalisation in response to ligand stimulation (Callander et al., 2009). However, we also saw sustained cAMP inhibition at RXFP3 (particularly in HEK293T cells) and RXFP4, which have been shown to interact with β -arrestins and internalise after stimulation with their endogenous ligands (Ang et al., 2016; Kocan et al., 2014). Although the original characterisation of CAMYEL in RAW 264.7 murine macrophage cells showed that CAMYEL can report substantial and rapid decreases in BRET signal (Jiang et al., 2007), studies on some GPCRs, including the cannabinoid 1 (Cawston et al., 2013), dopamine D₂L (Klein Herenbrink et al., 2016), and mu-opioid (Thompson et al., 2016) receptors, have also shown relatively sustained changes in CAMYEL signal over extended periods of time. However, most studies using CAMYEL do not show time-course data and some include phosphodiesterase inhibitors in the assay to stop the breakdown of cAMP. As sustained changes in the BRET signal have been observed in the present study and other studies, it was thought possible that cAMP does not easily dissociate from the sensor, or that the sensor does not easily reverse its conformation and therefore does not precisely detect real-time fluctuations in cAMP levels. Therefore, we demonstrated reversibility of the BRET signal at RXFP2 and RXFP3 by adding an antagonist after pre-incubation with agonist, which both showed real-time reversal of the signal. These experiments demonstrated that the sensor is dynamic and able to detect changes in BRET signal reflective of changes in cAMP levels, over extended periods of time.

Targeting RXFP receptors with drugs has potential for a range of therapeutic applications (reviewed in Bathgate et al., 2013). Much effort has therefore been put into designing analogues of relaxin family peptides, including agonists and antagonists used in the present study (reviewed in Hossain & Bathgate, 2018). In particular, RXFP1 is a promising therapeutic target for cardiovascular diseases and fibrosis. Novel ligands acting at RXFP1, including peptide analogues (e.g., Hossain et al., 2016) and small molecules (Xiao et al., 2013), have been screened in THP-1 cells due to the robust cAMP activation by relaxin in these cells and their low native RXFP1 receptor expression level. Our newly-developed THP-1 CAMYEL cell line will be a valuable tool for drug discovery targeting RXFP1, as it is sensitive and able to detect concentration-dependent increases in cAMP activity over time in the absence of phosphodiesterase inhibitors. Importantly, the assay showed a similar potency of H2 relaxin to that observed using traditional cAMP assays such as enzyme immunoassay (Bathgate et al., 2002) and HTRF cAMP assays (Hossain et al., 2016). The THP-1 CAMYEL assay also streamlines the process of screening ligands, as these suspension cells can be plated out on the day of the assay, and no extra steps for lysis or addition of detection reagents are required. Additionally, while recombinant HEK-RXFP1 cells showed cAMP responses

that reached or closely approached the ceiling set by forskolin, the native THP-1 CAMYEL cells showed that the level of agonist-induced cAMP activity was clearly within the dynamic range of the assay. The sorted THP-1 CAMYEL cells also showed that the level of biosensor present in the cells did not affect the observed potency of H2 relaxin, suggesting that the expression level of CAMYEL is of little importance and does not need to be tightly controlled. The successful use of CAMYEL in a cell line that has low RXFP1 expression also suggests that the lentiviral CAMYEL construct can be utilised to transduce other native cells, including human primary cells, in order to understand RXFP1 signalling in the context of human diseases.

RXFP1 has been shown to couple to at least three G proteins to modulate cAMP activity in some human cells. $G\alpha_s$ is the primary mediator of cAMP, and its actions are negatively modulated by $G\alpha_{oB}$ (Halls et al., 2006). Further increases in cAMP activity have also been shown to occur in a number of cell types, including THP-1 (Nguyen et al., 2003), HEK-RXFP1 (Halls et al., 2006), and some human primary vascular cell types (Sarwar et al., 2015) downstream of $G\alpha_{i3}$, $G\beta\gamma$, PI3K, PKC ζ , and adenylate cyclase 5 (Halls et al., 2006; Nguyen & Dessauer, 2005b; Nguyen et al., 2003). Coupling of RXFP1 to $G\alpha_{i3}$ has also been confirmed by BRET protein-protein interaction studies (Kocan et al., 2017), and is dependent on localisation of RXFP1 and $G\alpha_{i/o}$ in membrane raft microdomains (Halls et al., 2009). This finding suggests that cAMP activity at RXFP1 is compartmentalised, which is consistent with the finding that at RXFP1, $G\alpha_s$ -induced cAMP activity affects CRE transcription, whereas $G\alpha_{i3}$ -induced cAMP activity affects NF- κ B transcription (Halls et al., 2007). In the present study, PTX inhibition in THP-1 CAMYEL cells demonstrated a potential inhibition by $G\alpha_{oB}$ at five minutes which was not significant but no effect at any other time point indicated a complete absence of a $G\alpha_{i3}$ effect. We then used HEK293A cells that do not express the $G\alpha_s$ subunit to further investigate. Although the potency of forskolin was diminished in these cells, re-expression of $G\alpha_s$ restored the potency of forskolin, showing that the diminished forskolin response was not an off-target effect of the gene editing in these cells. However, stimulation of these cells with H2 relaxin when RXFP1 and CAMYEL were co-expressed did not show any increases in cAMP activity, including when adenylate cyclase was primed using forskolin to compensate for the loss of $G\alpha_s$. Therefore, we have been unable to reproduce the $G\alpha_{i3}$ -mediated increase in cAMP activity at RXFP1 using the CAMYEL assay. It is therefore possible that there is a localised pool of cAMP that CAMYEL is unable to detect. The original paper that described CAMYEL (Jiang et al., 2007) suggested that CAMYEL is expressed throughout the cytoplasm, as it uses a cytosolic mutant of Epac1 (Ponsioen et al., 2004). Further studies have shown cytosolic distribution of CAMYEL via confocal microscopy and western blot (Matthiesen & Nielsen, 2011). We have confirmed this using confocal microscopy in HEK293T cells stably expressing CAMYEL (data not shown). Importantly,

there is precedent for the inability of cytosolic biosensors to detect localised hotspots of cAMP activity. For example, in GH₃B₆ cells treated with vasoactive intestinal peptide, FRET-based biosensors for cAMP activity located around the cytosol or localised to the plasma membrane reported robust cAMP increases that were not detected by an AC8-localised sensor (Wachten et al., 2010). In contrast, increases in cAMP activity after stimulation with thyrotropin-releasing hormone were detected by the AC8-localised sensor but not the cytosolic or membrane forms (Wachten et al., 2010).

In summary, CAMYEL is a valuable tool for the direct detection of real-time cAMP activity at the relaxin family peptide receptors 1-4. We have shown robust cAMP activation and inhibition at G α_s - and G $\alpha_{i/o}$ -coupled receptors when CAMYEL was transfected into recombinant cell lines, and have also shown robust cAMP activity in a cell line that expresses low native levels of RXFP1, when CAMYEL was delivered virally. We have also detected differences in cAMP activity between different ligands and cell types, which can be modulated by antagonists and an inhibitor. However, we could not replicate the biphasic cAMP response at RXFP1 using this assay, which may suggest compartmentalisation of cAMP activity at RXFP1, consistent with previous findings. The development of a lentiviral CAMYEL construct will allow the transduction of any mammalian cell for the real-time analysis of cAMP signalling.

3.6 Acknowledgements

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3.7 Conflicts of interest

The authors declare that they have no conflicts of interest with the contents of this article.

3.8 Supplementary data

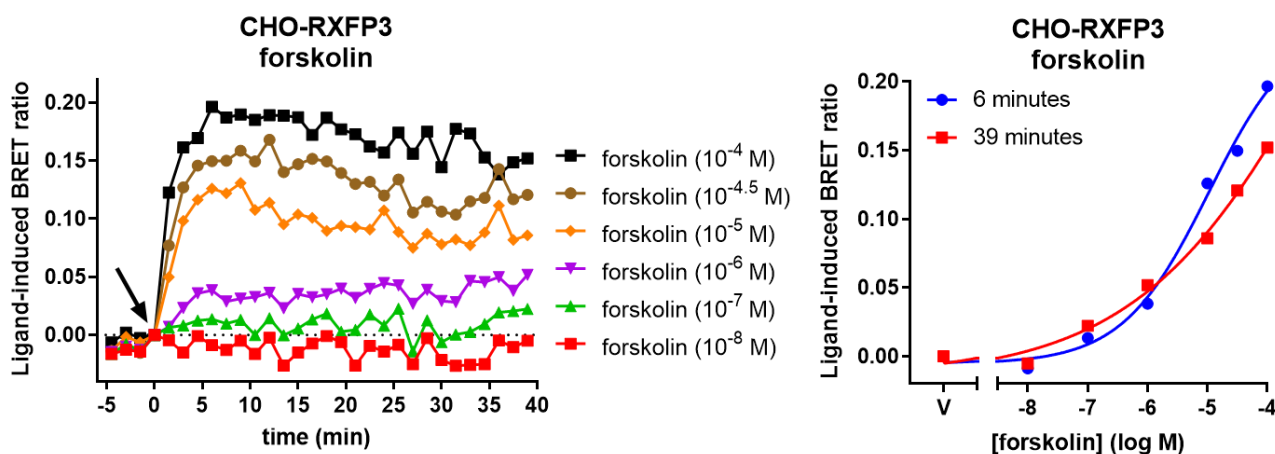


Figure S3.1 Forskolin-induced cAMP activity in CHO-RXFP3 cells. CHO-K1 (FlpIn) cells stably expressing RXFP3 and transiently expressing CAMYEL were stimulated with a range of concentrations of forskolin. (A) Time-course of forskolin-induced cAMP activity. (B) Concentration-response curves for forskolin at six and 39 minutes. Time of forskolin or vehicle addition is represented by an arrow. Data represent mean of one experiment.

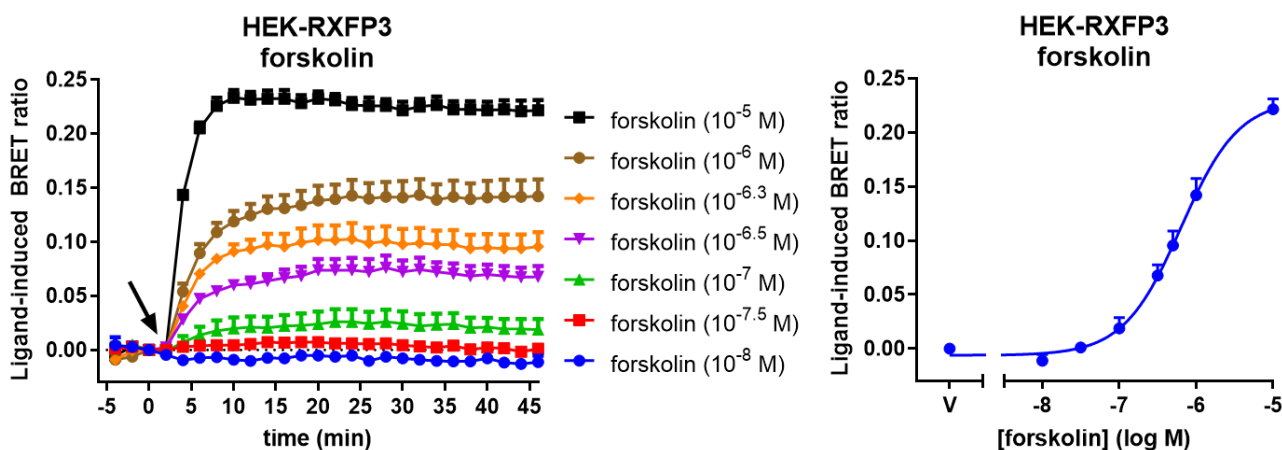


Figure S3.2 Forskolin-induced cAMP activity in HEK-RXFP3 cells. HEK293T cells stably expressing RXFP3 and transiently expressing CAMYEL were stimulated with a range of concentrations of forskolin. (A) Time-course of forskolin-induced cAMP activity. (B) Concentration-response curve for forskolin at 45 minutes. Time of forskolin or vehicle addition is represented by an arrow. Data represent mean and SEM of two or three independent experiments.

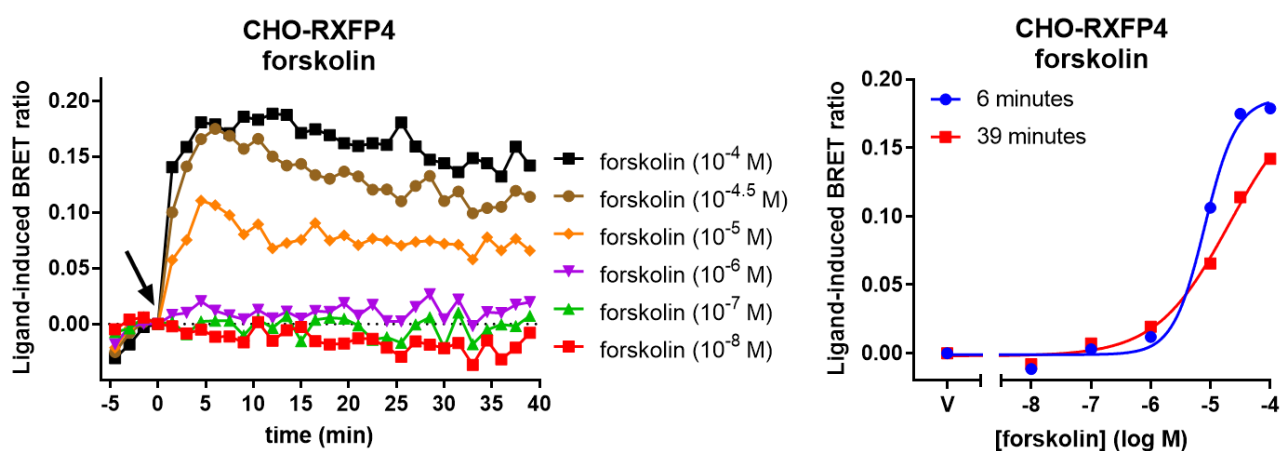


Figure S3.3 Forskolin-induced cAMP activity in CHO-RXFP4 cells. CHO-K1 cells stably expressing RXFP4 and transiently expressing CAMYEL were stimulated with a range of concentrations of forskolin. (A) Time-course of forskolin-induced cAMP activity. (B) Concentration-response curves for forskolin at six and 39 minutes. Time of forskolin or vehicle addition is represented by an arrow. Data represent mean of one experiment.

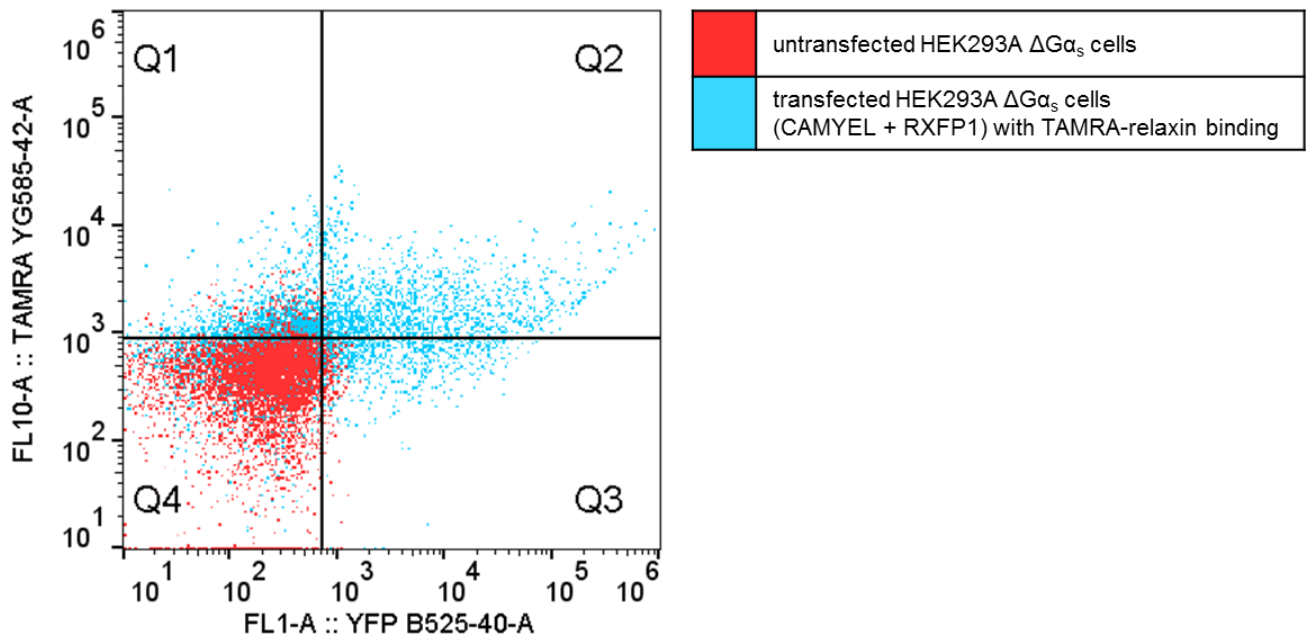


Figure S3.4 Co-expression of transfected CAMYEL and RXFP1 in HEK293A $\Delta G\alpha_s$ cells. HEK293A $\Delta G\alpha_s$ that were co-transfected with CAMYEL and RXFP1 were incubated either alone or with TAMRA-labelled H2 relaxin (20 nM). Flow cytometry was used to isolate single, live cells. Emissions from YFP (showing expression of CAMYEL) were plot against emissions from TAMRA (showing expression of RXFP1 by binding of TAMRA-relaxin). Q2 represents a population of cells that bind TAMRA-relaxin and show YFP expression above baseline, demonstrating co-expressing of CAMYEL and RXFP1. It was also possible to show RXFP1-specific binding by incubating cells with unlabelled H2 relaxin (2 μ M) in addition to TAMRA-relaxin.

4 A real-time, plate-based BRET assay for detection of cGMP in primary cells

4.1 Introduction

Cyclic guanosine monophosphate (cGMP) is a second messenger involved in the regulation of many physiological processes including cardiovascular homeostasis, smooth muscle tone, blood pressure, platelet aggregation, memory, learning, and sensory transduction (Feil *et al.*, 2005; Hofmann *et al.*, 2004; Tsai & Kass, 2009). cGMP is synthesised from GTP by two types of guanylate cyclases (GCs): soluble GCs (sGCs), which are cytosolic enzymes activated by nitric oxide (NO) and carbon monoxide (Derbyshire & Marletta, 2012), and particulate GCs (pGCs), which are transmembrane receptors activated by natriuretic peptides and some intestinal peptides (Kuhn, 2016). cGMP is inactivated by PDE-mediated hydrolysis (Francis *et al.*, 2010). cGMP signals via PKG and cyclic nucleotide-gated ion channels, and also modulates the activity of some PDEs (Francis *et al.*, 2010).

Modulation of cGMP activity has therapeutic value for several disorders. For example, NO donors such as glyceryl trinitrate are used to treat angina (Miller & Megson, 2007), the sGC stimulator riociguat is used for treatment of pulmonary arterial hypertension and chronic thromboembolic pulmonary hypertension (Conole & Scott, 2013), PDE5 inhibitors such as sildenafil and vardenafil are used to treat erectile dysfunction (Lasker *et al.*, 2013), and GC-C (a form of pGC) agonists such as linacotide and plecanatide are used to treat irritable bowel syndrome with constipation (Waldman & Camilleri, 2018). Natriuretic peptides are also under investigation for treatment of cardiovascular disorders such as heart failure (Buglioni & Burnett, 2016), and drug discovery targeting cGMP signalling cascades is under continued investigation for the treatment of a variety of other disorders and diseases (Ben Aissa *et al.*, 2016; Greene *et al.*, 2013; Lukowski *et al.*, 2014; Shim *et al.*, 2016; Windham & Tinsley, 2015). Additionally, as NO/sGC signalling occurs downstream of GPCRs and receptor tyrosine kinases, drugs targeting these types of receptors may also produce therapeutic actions via cGMP. For example, NO/cGMP is involved in the potential therapeutic actions of relaxin/RXFP1 signalling, such as vasoprotective effects (Dschietzig *et al.*, 2012) and anti-fibrotic actions (Samuel *et al.*, 2016).

However, detection of the kinetics of cGMP signalling in plate-based cellular assays for the purposes of drug discovery can be challenging. Typically, traditional end-point signalling assays such as the HTRF assay have been used to detect cGMP in populations of cells in a plate-based format. Although such assays may be sensitive, they are laborious as they involve lysing cells at individual time points. Furthermore, these assays tend to include PDE inhibitors to stop the breakdown of cGMP, leading to accumulation and measurement of total cGMP. Consequently, end point signalling assays can produce a type of “observational bias” whereby the kinetics of signalling are ignored, leading to an incomplete or misleading idea of signalling patterns induced by different ligands (Klein Herenbrink et al., 2016).

Conversely, assays that detect signalling in real time will help remove the confounding effects of observational bias and will also allow more efficient examination of the kinetic aspects of signalling. Genetically-encoded biosensors based on FRET are already used to image cGMP activity in real-time in single cells, for instance (Honda *et al.*, 2001; Nikolaev *et al.*, 2006; Sato *et al.*, 2000). FRET is the transfer of energy from an excited donor fluorophore to an appropriate acceptor fluorophore when they are in close proximity. FRET-based biosensors for cGMP consist of a “sensor” domain that binds cGMP, which is sandwiched by an appropriate FRET donor/acceptor fluorophore pair. When cGMP binds to the sensor, the FRET pair moves either closer together or farther apart, producing a change in FRET that can be monitored in real time. However, although FRET-based biosensors are valuable for microscopy and imaging single cells, FRET is unsuitable for detecting activity from populations of cells in plate-based assays for several reasons, but primarily because autofluorescence produced by laser excitation leads to high background noise (Boute *et al.*, 2002). On the other hand, BRET, which uses a luciferase as the donor protein, is a more sensitive technique for plate-based assays because it does not involve laser excitation, leading to lower background noise (Boute *et al.*, 2002).

A BRET-based assay for cGMP activity will allow for real-time, plate-based cGMP assays in populations of cells. Here, we report the conversion of the cGES-DE5 FRET-based cGMP biosensor (Nikolaev *et al.*, 2006) to a BRET-based biosensor (CYGYEL; cyclic GMP sensor using YFP-PDE5-Rluc8), cloning of the novel biosensor into a lentiviral vector for stable expression in mammalian cells, validation and characterisation of its use in live cells in a real-time multi-well plate-based format, and investigation of the applicability of the sensor for the detection of both sGC- and pGC-mediated cGMP activity in human primary vascular cells with the goal of use for drug discovery.

4.2 Methods

4.2.1 Reagents

Ligands, inhibitors, and other compounds used were carbachol, CNP, coelenterazine *h*, forskolin, H2 relaxin IBMX, isoprenaline, ODQ, Polybrene, rolipram, and vardenafil. Sources of reagents are listed in Chapter 2.

4.2.2 Cell culture

Cell lines and culture methods are described in Chapter 2. Cell types used include HEK293T, HUVECs, and HUVSMCs.

4.2.3 Design of a BRET-based biosensor for cGMP activity

The BRET-based biosensor for cGMP activity, CYGYEL, was created based on the cGES-DE5 FRET-based biosensor (Nikolaev et al., 2006). cGES-DE5 consists of the cGMP-binding domain (Glu154 to Ala308) from human PDE5A1 (GenBank accession number NM_001083) sandwiched between enhanced yellow fluorescent protein (EYFP) and enhanced cyan fluorescent protein (ECFP) using *EcoRI* and *XbaI* restriction sites, respectively. The sequence for EYFP was replaced with the sequence for Venus, and the sequence for ECFP was replaced with the sequence for Rluc8. A Kozak sequence (GCCACC) was placed before the start codon, and the gene for the biosensor was synthesised by GenScript into pcDNA3.1/Zeo(+) between *NheI* and *Apal* restriction sites.

4.2.4 Cloning CYGYEL into a lentiviral vector

CYGYEL was cloned into a lentiviral vector under the control of the constitutive human Ef1 α promoter using MultiSite Gateway Pro technology (Invitrogen), using methods described in detail in Chapter 2. Briefly, the CYGYEL coding sequence from the pcDNA3.1/Zeo (+) vector was flanked with appropriate *attB5* and *attB2* sequences and amplified by polymerase chain reaction (PCR) using the forward primer 5' GGGG ACA ACT TTG TAT ACA AAA GTT GGC CAC CAT GGT GAG CAA GGG C 3' and reverse primer 5' GGGG ACC ACT TTG TAC AAG AAA GCT GGG TAT TA CTG CTC GTT CTT CAG CAC 3'. The PCR product of the correct size was extracted, gel purified, and cloned

into the pDONR 221 P5-P2 vector using BP Clonase II. The resulting pENTR L5-L2 entry clone was confirmed to have the correct sequence using Sanger sequencing. Our pENTR L1-R5 Ef1 α entry clone was cloned along with the pENTR L5-L2 CYGYEL entry clone into the pLenti X1 Zeo DEST vector (Campeau *et al.*, 2009) using LR Clonase II, to generate the pLenti X1 Ef1 α CYGYEL expression clone.

4.2.5 Generation of HEK-CAMYEL and HEK-CYGYEL cell lines

HEK293T cells were transduced with crude CAMYEL or CYGYEL lentivirus. HEK293T cells were plated at 3.5 million cells on a 10 cm dish. The next day, cells were transfected with 8 μ g of either pLenti X1 Ef1 α CAMYEL (Chapter 3) or pLenti X1 Ef1 α CYGYEL, along with 5.2 μ g pMDL, 2 μ g pRSV-Rev, and 2.8 μ g pCMV-VSV-G, using 50 μ l Lipofectamine 2000. The following day, HEK293T cells to be transduced were plated on a 10 cm dish at 400,000 cells. The next day, media containing lentivirus from transfected cells was harvested and filtered through a 0.45 μ m syringe filter. The newly-plated HEK293T cells were transduced by replacing the cell culture media with the media containing lentivirus, mixed with 4 μ g/ml hexadimethrine bromide (Polybrene) to increase transduction efficiency. Three rounds of transduction were performed over 32 hours, before allowing cells to recover for three days in complete DMEM.

4.2.6 Purification of lentivirus and transduction of primary vascular cells

CYGYEL lentivirus was produced in HEK293T cells and purified via ultracentrifugation for transduction of primary cells (HUVECs and HUVSMCs), as described in detail in Chapter 2. After production and purification of lentivirus, HUVECs and HUVSMCs were transduced with the purified virus in a T175 flask at approximately 70% confluency. 5 μ l of purified lentivirus was resuspended in cell culture media along with 4 μ g/ml hexadimethrine bromide (Polybrene). Cell culture media in the flask was replaced with virus-containing media, which was left on cells overnight before replacing it with fresh cell culture media. In the case of HUVECs, for unrelated reasons (see Chapter 5), cells were originally transduced with an RXFP1/internal ribosome entry site (IRES)/GFP construct and sorted into “low” and “high” expression before transduction of the “low” RXFP1 cells with CYGYEL lentivirus. RXFP1 lentivirus and transduction were conducted previously as described above and in Chapter 2, using a pLenti X1 Ef1 α RXFP1 IRES GFP expression construct.

4.2.7 Flow cytometry and fluorescence-activated cell sorting

Transduced cells were sorted from non-transduced cells using FACS, as described in Chapter 2. After gating, from the population of single, live cells, cells showing higher YFP (or GFP in the case of HUVEC-RXFP1 cells) emissions than control cells, as detected after laser excitation at 488 nm and emissions at 530/30 nm, were sorted into separate populations as noted. For the HUVEC-RXFP1 cells that were subsequently transduced with YFP after already expressing GFP (as described above), emissions were separated from YFP emissions by using a separate emission filter and applying compensation. For analytical flow cytometry, cells were prepared, gated, and analysed as described in Chapter 2.

4.2.8 BRET assays in live cells

Real-time BRET assays were conducted in cells stably expressing CAMYEL or CYGYEL, as described in Chapter 2, using a PHERAstar *FSX* plate reader, but with a few modifications. At the time of the assay, HEK293T or HUVSMC cell culture media was replaced with phenol red free complete media containing 25 mM HEPES and 5 μ M coelenterazine *h* (with PDE inhibitors when indicated), whereas HUVEC cell culture media, EGM-2, was replaced with complete EGM-2 containing 25 mM HEPES and 5 μ M coelenterazine *h*. In the case of “post-addition” assays, the assays were briefly paused for addition of vardenafil or ODQ as indicated. Data were analysed and presented as described in Chapter 2.

4.2.9 BRET assays in cell lysates

HEK-CYGYEL cells were washed and resuspended in DPBS, and then centrifuged in order to pellet the cells. DPBS was aspirated and cells were resuspended in buffer containing 5 mM Tris and 2 mM EDTA (pH 7.3), then sonicated on low power for three 10-second cycles at 4°C. Lysates were then spun down at $21,000 \times g$ for 20 minutes at 4°C, after which the supernatant was obtained and stored at -80°C or used for BRET assays. During the assay, lysates were mixed with coelenterazine *h* (5 μ M), distributed into a white, 384-well OptiPlate (PerkinElmer) with lysates corresponding to the equivalent of approximately 5,000 cells per well, and then assayed as above, at room temperature, using a PHERAstar *FSX* plate reader.

4.3 Results

4.3.1 Design of a BRET-based biosensor for cGMP activity and generation of a HEK293T stable cell line

Several FRET-based biosensors have been engineered and used for imaging cGMP activity in single cells, including CGY (Sato *et al.*, 2000) and cygnet (Honda *et al.*, 2001), which are based on PKG, and cGES-DE5, which is based on PDE5 (Nikolaev *et al.*, 2006). cGES-DE5 was chosen as the basis to generate a BRET-based biosensor due to its superior temporal resolution and high selectivity for cGMP over cAMP, compared to other FRET-based sensors (Nikolaev *et al.*, 2006). cGES-DE5 consists of the cGMP-binding domain from human PDE5A as the “sensor”, sandwiched between an EYFP acceptor and ECFP donor. To convert cGES-DE5 to BRET, the sequence for ECFP was replaced with the sequence for the luciferase Rluc8 (Loening *et al.*, 2006). Additionally, the sequence for EYFP was replaced with the sequence for the YFP variant Venus, due to its increased brightness (Nagai *et al.*, 2002) (**Figure 4.1**). Upon binding of cGMP to the cGMP-binding domain, the sensor, known as CYGYEL, responds by decreasing the distance between donor and acceptor, leading to an increase in BRET ratio.

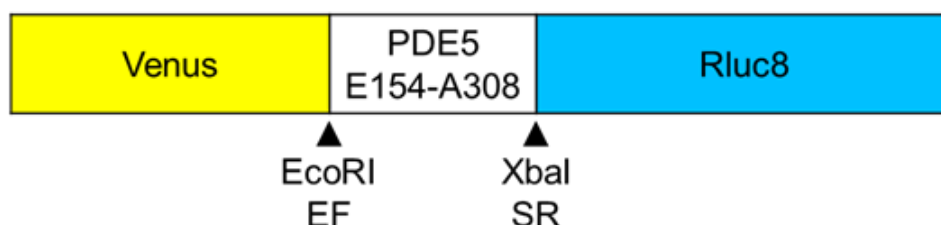


Figure 4.1. Schematic diagram showing the structure of CYGYEL, a BRET-based biosensor for cGMP activity. The isolated cGMP-binding domain from human PDE5 is sandwiched between Venus and Rluc8.

4.3.2 Characterisation of real-time cGMP activity in live cells

As the biosensor was intended for use in human primary cells that are difficult to transfect, it was cloned into a lentiviral vector under the control of the constitutive human Ef1 α promoter, to enable viral delivery of the sensor. Transfection of the lentiviral vector into HEK293T cells led to expression of YFP as visualised using a fluorescent microscope, demonstrating that the vector works (not shown). Before moving on to primary cells, CYGYEL was characterised as a real-time signalling assay in live HEK293T cells. Lentivirus was produced and used to transduce HEK293T cells, which were sorted by FACS into populations that expressed low, medium, or high levels of the biosensor (**Figure 4.2**).

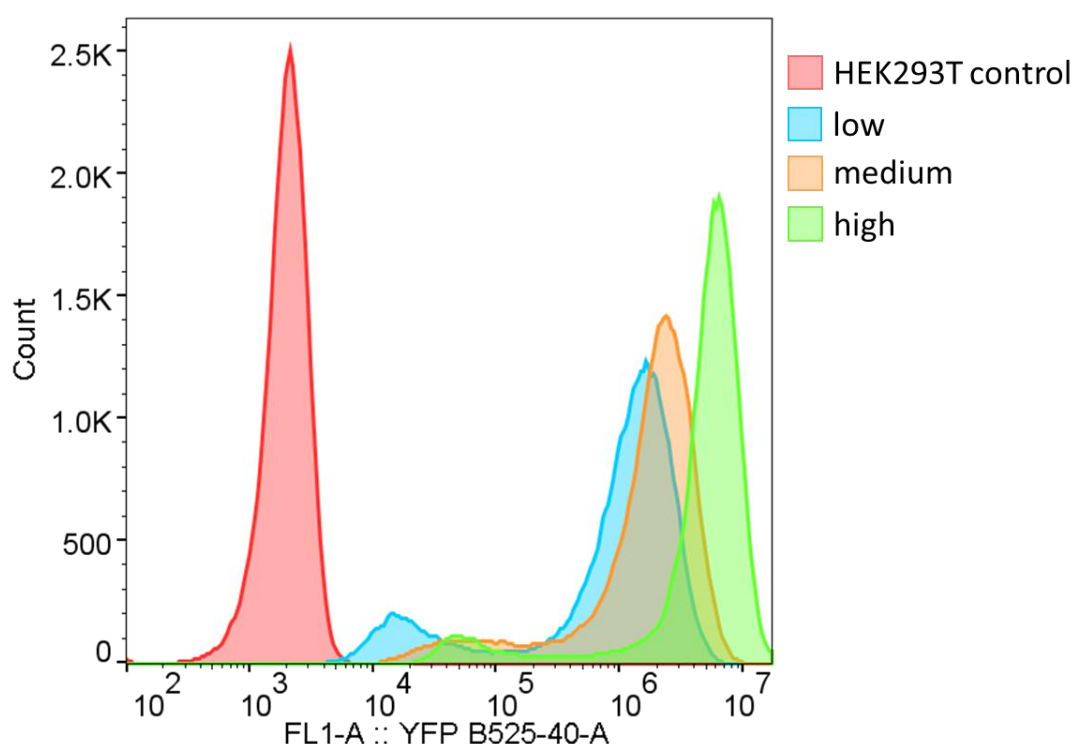


Figure 4.2. Analytical flow cytometry of HEK-CYGYEL stable cells. HEK293T cells were transduced with CYGYEL lentivirus and were sorted into populations expressing “low”, “medium”, or “high” levels of the sensor. Cells were later checked for YFP expression using analytical flow cytometry. Single, live cells were isolated by gating, after which histograms showing mean YFP emissions were generated (excitation: 488 nm; emission: 525/40 nm).

HEK-CYGYEL (high) cells were stimulated for 90 minutes with the NO donor diethylamine NONOate (DEA), which releases NO via a pH-dependent mechanism (Ramamurthi & Lewis, 1997) (**Figure 4.3**). In the absence of PDE inhibitors, there were steep increases in the BRET ratio for at least 25 minutes following the treatment with DEA, representative of increases in cGMP (**Figure 4.3A**). The response then either remained relatively steady before showing a slow decline (DEA 100 μ M), or declined for the duration of the time-course (DEA 10 μ M). As expected, pre-incubation with the cGMP-specific PDE5 inhibitor vardenafil (100 nM; approximately 10 minutes) led to more sustained increases in BRET ratio for both concentrations of DEA as well as higher maximum responses that peaked at approximately 40 minutes, which was much later than in the absence of vardenafil (**Figure 4.3B**), suggesting that the more transient responses seen without vardenafil were at least partly due to PDE5-mediated degradation of cGMP. Similarly, addition of vardenafil *after* an extended incubation with DEA (40.5 minutes) led to further increases in cGMP for both tested concentrations of DEA, although the effect was more pronounced for 100 μ M DEA and was sustained for the duration of the time-course, in contrast to 10 μ M which started to decline after a brief increase (**Figure 4.3C**). These results demonstrate that the biosensor is dynamic and able to detect robust increases and decreases in cGMP over an extended period of time (at least 90 minutes). To demonstrate the full dynamic range of the biosensor, cells were stimulated with DEA after pre-treatment (approximately 10 minutes) with the non-selective PDE inhibitor IBMX (500 μ M) (**Figure 4.3D**). In comparison to the response in the presence of vardenafil, the response for 10 μ M DEA in the presence of IBMX was more sustained. In the presence of IBMX, the maximal response of the sensor to cGMP (DEA 100 μ M; 87 minutes) corresponded to an increase of $39.90 \pm 0.68\%$ in the BRET ratio over vehicle.

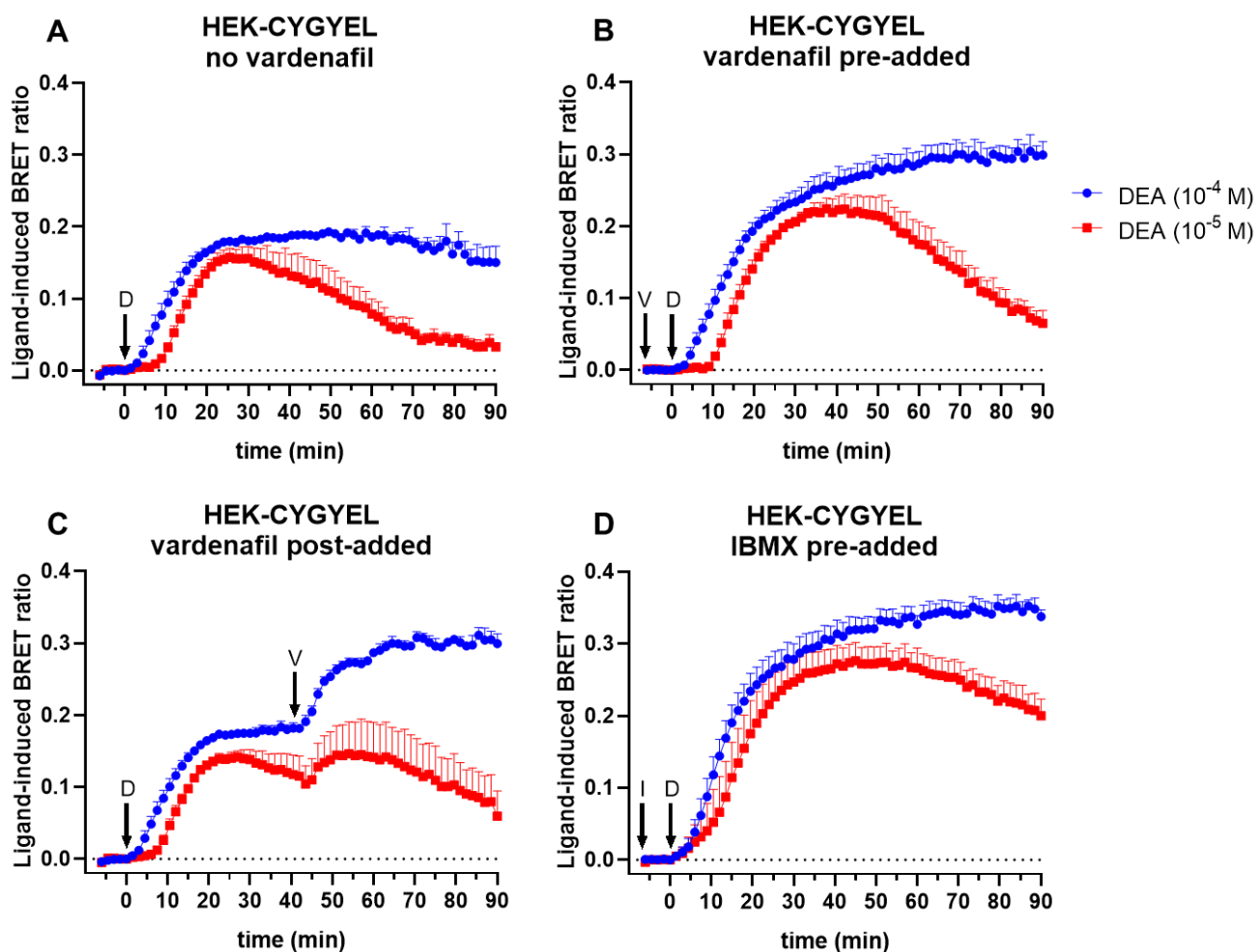


Figure 4.3. Characterisation of the CYGYEL BRET-based biosensor for cGMP activity. Intracellular cGMP was detected in real time after stimulation of HEK293T cells stably expressing CYGYEL with the NO donor DEA. Cells were stimulated with DEA (D; 10 or 100 μ M) for 90 minutes either (A) without any PDE inhibitors, (B) with the cGMP-specific PDE5 inhibitor vardenafil (V; 100 nM) pre-added (approximately 10 minutes), (C) with vardenafil added after 40.5 minutes, or (D) with the non-selective PDE inhibitor IBMX (I; 500 μ M) pre-added (approximately 10 minutes). Time of ligand or vehicle addition is represented by arrows. Data represent mean and SEM of three independent experiments.

To further demonstrate reversibility of the sensor, and blocking of the cGMP response, cells were stimulated with DEA in the absence or presence of the sGC inhibitor ODQ (100 μ M) (**Figure 4.4**). Blockade of sGC with ODQ for approximately 10 minutes before addition of DEA either abolished the DEA response (10 μ M DEA) or almost abolished it while also delaying the peak response until about 40 minutes rather than about 25 minutes (100 μ M DEA) (**Figure 4.4B**). Furthermore, addition of ODQ *after* a 20.5-minute stimulation with DEA led to near-immediate decreases in the cGMP response (**Figure 4.4C**). These results again show that the sensor is specific for cGMP, dynamic, and reversible.

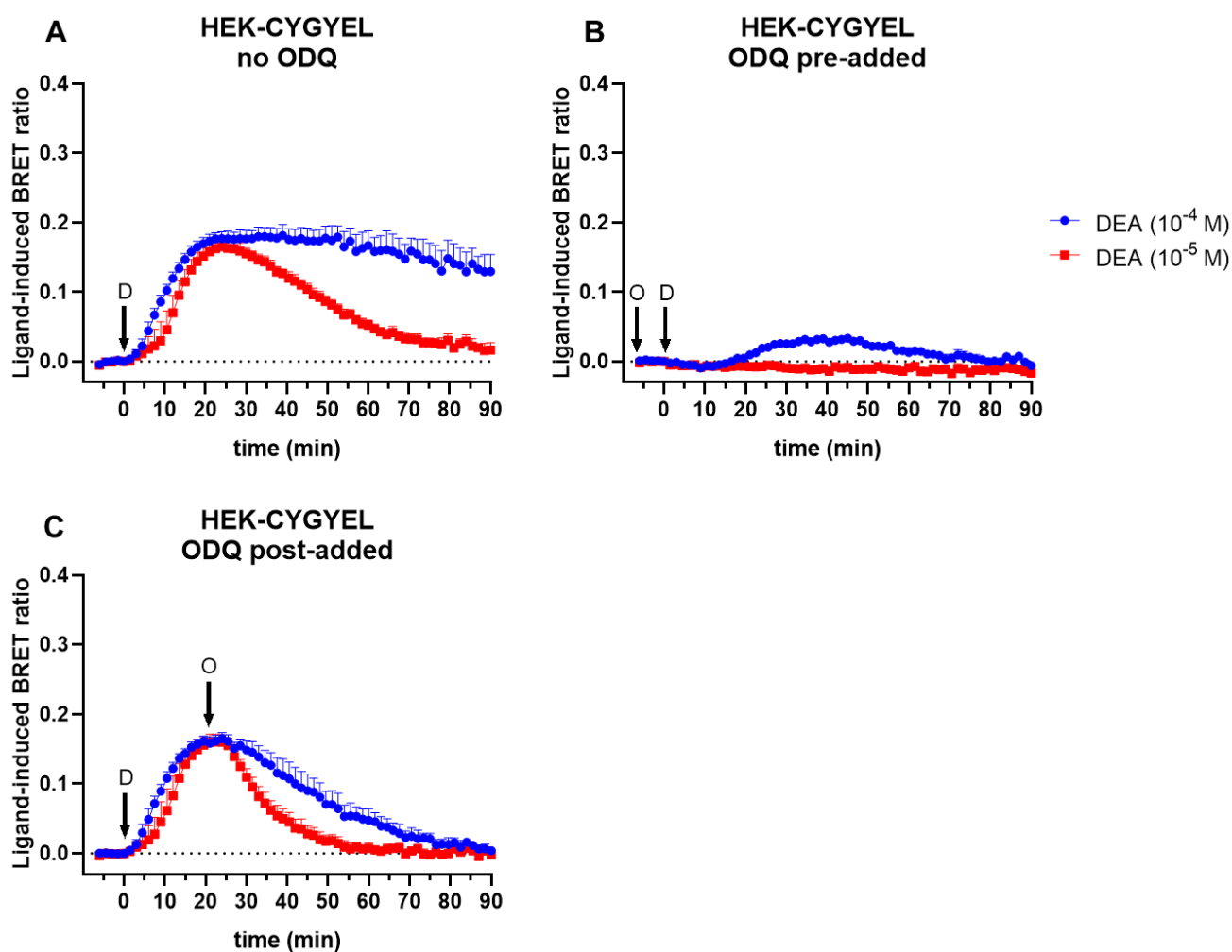


Figure 4.4. Blocking and reversing the cGMP sensor signal in HEK-CYGYEL cells.

Intracellular cGMP was detected in real time after stimulation of HEK293T cells stably expressing CYGYEL with the NO donor DEA (D; 10 or 100 μ M) in the absence or presence of the sGC inhibitor ODQ (O; 100 μ M). (A) Cells were stimulated with DEA without ODQ. (B) Cells were stimulated with DEA after pre-incubation (approximately 10 minutes) with ODQ. (C) Cells were stimulated with DEA for 20.5 minutes before addition of ODQ. Time of ligand or vehicle addition is represented by arrows. Data represent mean and SEM of three independent experiments.

4.3.3 Selectivity of CYGYEL for cGMP over cAMP

One major concern for genetically-encoded biosensors for cGMP activity is that cAMP can still bind to and “activate” the sensor, albeit with a lower potency than that of cGMP. This crosstalk might pose a problem when detecting signalling from ligands or receptors that increase activity of both cAMP and cGMP, or when the basal concentration of cAMP inside the cell is high, leading to high background activation of the sensor. In the original study, the FRET biosensor cGES-DE5 was activated by cGMP with a potency of $\sim 1.5 \mu\text{M}$ and by cAMP with a potency of $\sim 630 \mu\text{M}$, making it ~ 420 times more selective for cGMP than cAMP (Nikolaev *et al.*, 2006). To compare these results to our BRET variant of the sensor, HEK-CYGYEL lysates were directly stimulated with a range of concentrations of cGMP or cAMP (**Figure 4.5**). cGMP (**Figure 4.5A**) and cAMP (**Figure 4.5B**) both increased the BRET ratio in a concentration-dependent manner, however higher concentrations of cAMP were required to activate the sensor relative to cGMP, and cAMP also induced slower activation of the sensor than cGMP, particularly at the higher concentrations of cyclic nucleotide (100 μM , 1 mM cAMP). Concentration-response curves (generated by taking the area under the curve due to the different kinetics of the two cyclic nucleotides) showed that cGMP activated CYGYEL with a potency of $1.32 \pm 0.43 \mu\text{M}$, whereas cAMP activated CYGYEL with a potency of $42.15 \pm 15.44 \mu\text{M}$ (**Figure 4.5C; Table 4.1**). Thus, cGMP activated CYGYEL with an approximately 32-fold higher potency relative to cAMP, which was a lower selectivity compared to cGES-DE5.

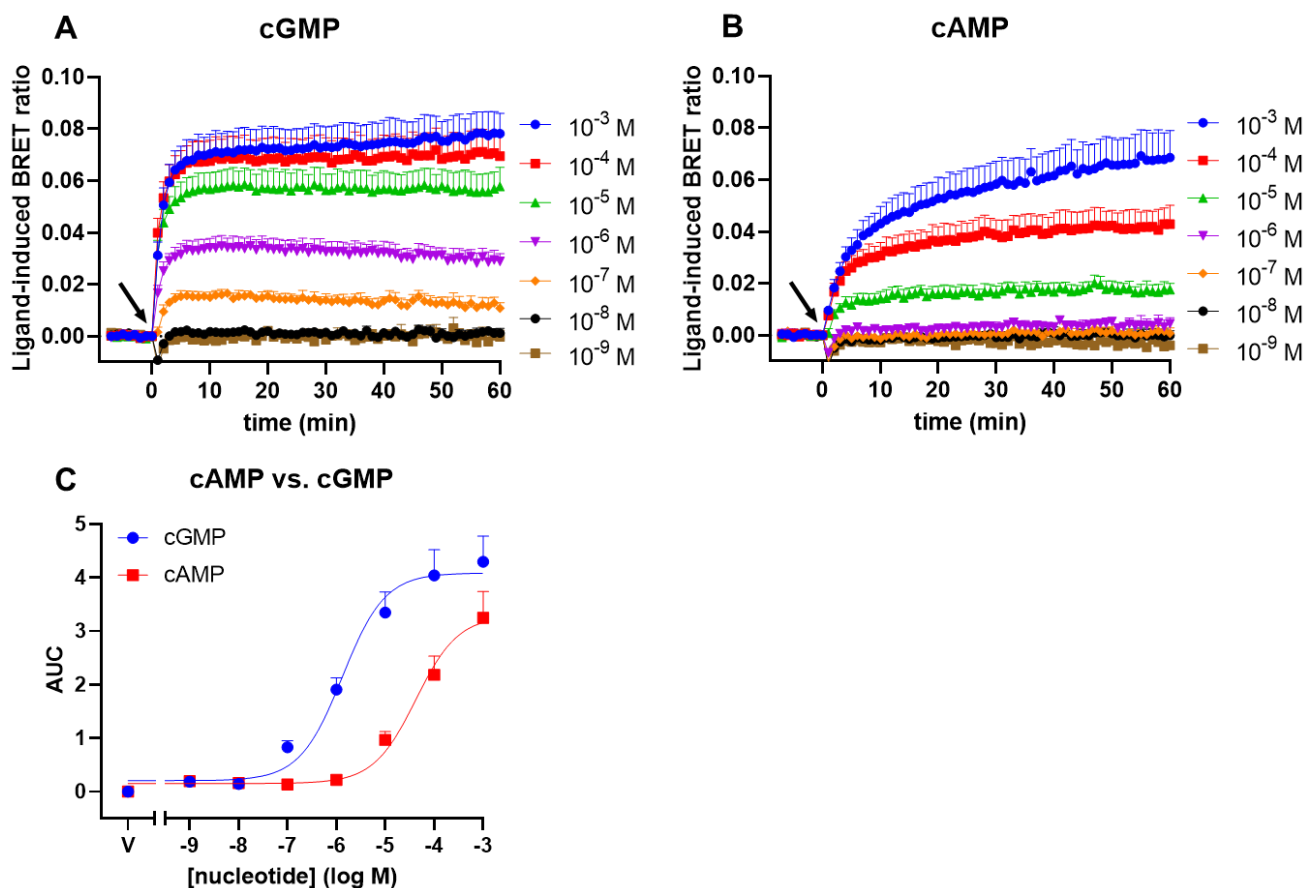


Figure 4.5. Activation of CYGYEL by cGMP and cAMP. Lysates from HEK-CYGYEL cells were stimulated with a range of concentrations of (A) cGMP or (B) cAMP for 60 minutes. (C) Concentration-response curves were generated by taking the area under the curve (AUC) from time-course data. Time of vehicle or ligand addition is represented by an arrow. Data are the mean and SEM of four independent experiments.

Table 4.1. Potency of cyclic nucleotides for activation of CYGYEL. Lysates from HEK-CYGYEL cells were stimulated with a range of concentrations of cGMP or cAMP for 60 minutes. Potencies were generated by taking the area under the curve from time-course data. Selectivity was determined by dividing the EC_{50} for cAMP by the EC_{50} for cGMP. Data are the mean and SEM of four independent experiments. * $p < 0.01$ compared to cGMP in an unpaired t -test.

	cGMP	cAMP	cAMP/cGMP
EC_{50} (μ M)	1.32 ± 0.43	42.15 ± 15.44	32
pEC_{50}	5.88 ± 0.14	$4.38 \pm 0.16^*$	

Although these results reflect the biochemistry seen in cell lysates, they do not necessarily reflect what may occur in live cells, as cell lysates may not reflect localisation of second messengers or localised PDE activity, for example. Therefore, we also compared cAMP-mediated activation of CYGYEL to cAMP-mediated activation of the cAMP sensor CAMYEL (Jiang *et al.*, 2007), which was characterised extensively in Chapter 3. The CAMYEL lentiviral vector developed in Chapter 3 was used to generate a HEK-CAMYEL cell line using crude lentivirus according to the same method described above for CYGYEL. Live HEK-CAMYEL and HEK-CYGYEL cells were stimulated with a range of concentrations of forskolin, which increases intracellular cAMP levels by activating AC (**Figure 4.6**). Time-courses showed sustained increases in BRET ratio for the duration of a 60-minute stimulation for both CAMYEL (**Figure 4.6A**) and CYGYEL (**Figure 4.6B**). Concentration-response curves revealed that the forskolin response was detected with a potency of $0.62 \pm 0.06 \mu\text{M}$ in CAMYEL cells and $100.5 \pm 11.8 \mu\text{M}$ in CYGYEL cells (**Figure 4.6C**; **Table 4.2**). Thus, when expressed in live HEK293T cells, cAMP activated CYGYEL with approximately 160-fold lower potency than it activated CAMYEL.

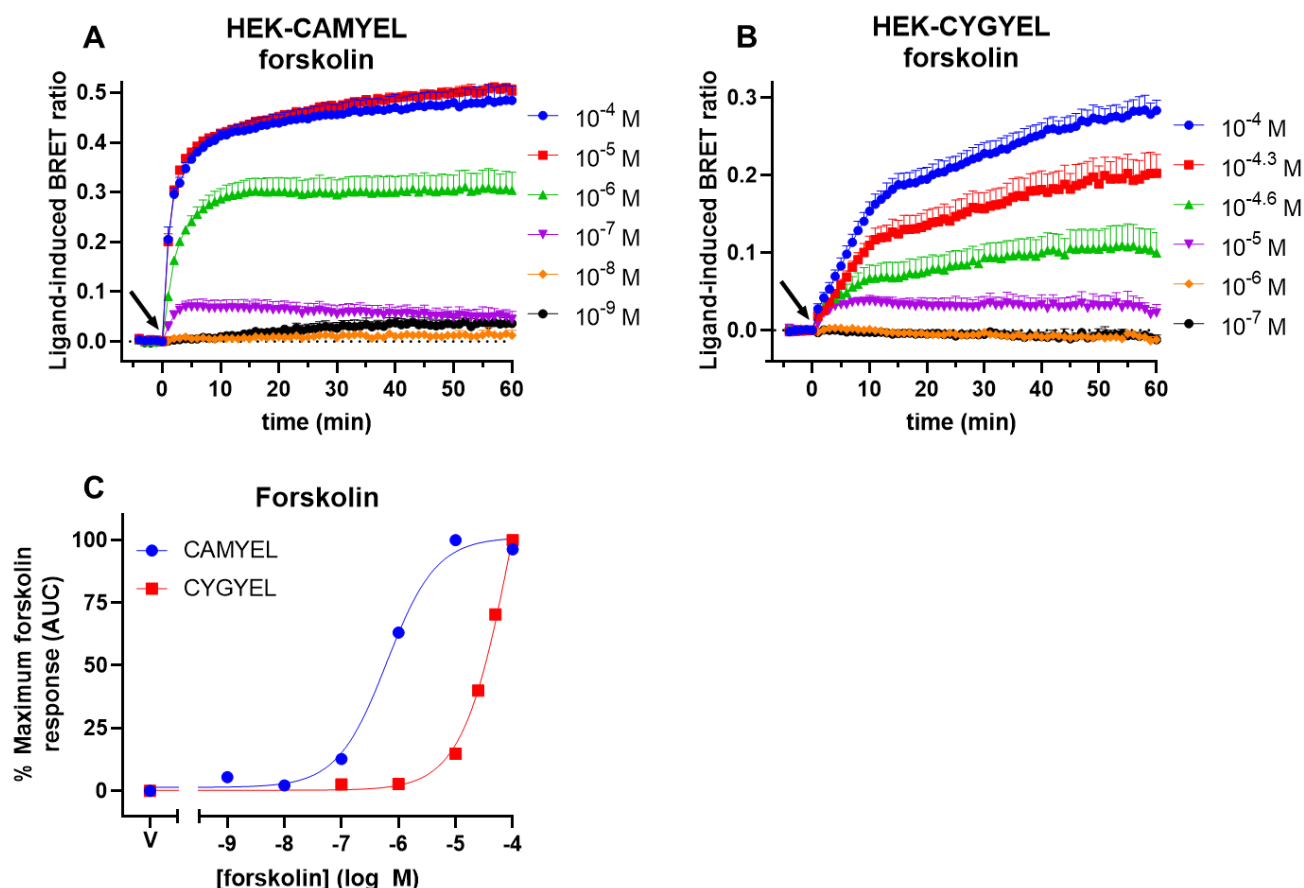


Figure 4.6. Forskolin-mediated cAMP activation of the CAMYEL and CYGYEL biosensors expressed in HEK293T cells. HEK293T cells stably expressing (A) the CAMYEL BRET-based cAMP sensor, or (B) the CYGYEL BRET-based cGMP sensor, were stimulated with a range of concentrations of forskolin for 60 minutes. (C) Concentration-response curves were generated by taking area under the curve (AUC) normalised as a percentage of maximum forskolin response. Time of vehicle or ligand addition is represented by an arrow. Data represent the mean and SEM of three independent experiments.

Table 4.2. Forskolin-mediated cAMP activation of the CAMYEL and CYGYEL biosensors expressed in HEK293T cells. HEK293T cells stably expressed with either the CAMYEL cAMP sensor or the CYGYEL were stimulated with a range of concentrations of forskolin. Concentration-response curves were generated by taking area under the curve normalised to maximum forskolin response in order to compare the potencies (EC_{50} and pEC_{50}) of forskolin for downstream activation of each biosensor. Data represent mean $\pm$ SEM of three independent experiments. * $p < 0.01$ compared to HEK-CAMYEL in an unpaired t -test.

Forskolin	HEK-CAMYEL	HEK-CYGYEL
EC_{50} (μ M)	0.62 ± 0.06	100.5 ± 11.8
pEC_{50}	6.21 ± 0.04	$3.99 \pm 0.05^*$

Next, assays were conducted to compare the DEA-mediated cGMP response to membrane receptor-mediated cAMP responses in HEK-CYGYEL cells. First, HEK-CAMYEL cells were stimulated with a range of concentrations of isoprenaline, which targets cAMP-linked β -adrenoceptors endogenous to HEK293T cells, as well as the positive control forskolin (10 μ M), to determine a concentration of isoprenaline that produces a maximal cAMP response. Isoprenaline and forskolin both induced robust increases in cAMP activity in the first few minutes of stimulation (**Figure 4.7A**). Forskolin responses were sustained for the duration of the time-course, whereas isoprenaline responses started to decrease after a few minutes. The maximum isoprenaline responses occurred at 1-10 μ M. As expected, when cells were pre-incubated (approximately 10 minutes) with the cAMP-specific PDE4 inhibitor rolipram (10 μ M) in order to stop the breakdown of cAMP, isoprenaline induced a more sustained response (**Figure 4.7B**). To determine whether this robust adrenoceptor-mediated cAMP increase would activate CYGYEL in live cell assays when the sensor was expressed in the same cell background, and to compare it to the DEA response, HEK-CYGYEL

cells were stimulated with isoprenaline (10 μ M), forskolin (10 μ M), and the positive control DEA (10 μ M). As before, the DEA response peaked at about 25 minutes, whereas forskolin produced a lower but sustained response, and, notably, isoprenaline (10 μ M) did not produce a detectable response (**Figure 4.7C**). When cells were pre-incubated with rolipram (10 μ M; approximately 10 minutes), however, the forskolin response became more robust, peaking at an approximately equivalent BRET ratio as DEA, and the isoprenaline response was also slightly above baseline for the duration of the time-course, which an area under the curve analysis showed was statistically significant ($p < 0.05$) (**Figure 4.7D**). Therefore, although relatively high levels of cAMP induced by the positive control forskolin markedly activated the sensor in signalling assays, endogenous adrenoceptor-mediated cAMP showed minimal activation of the sensor.

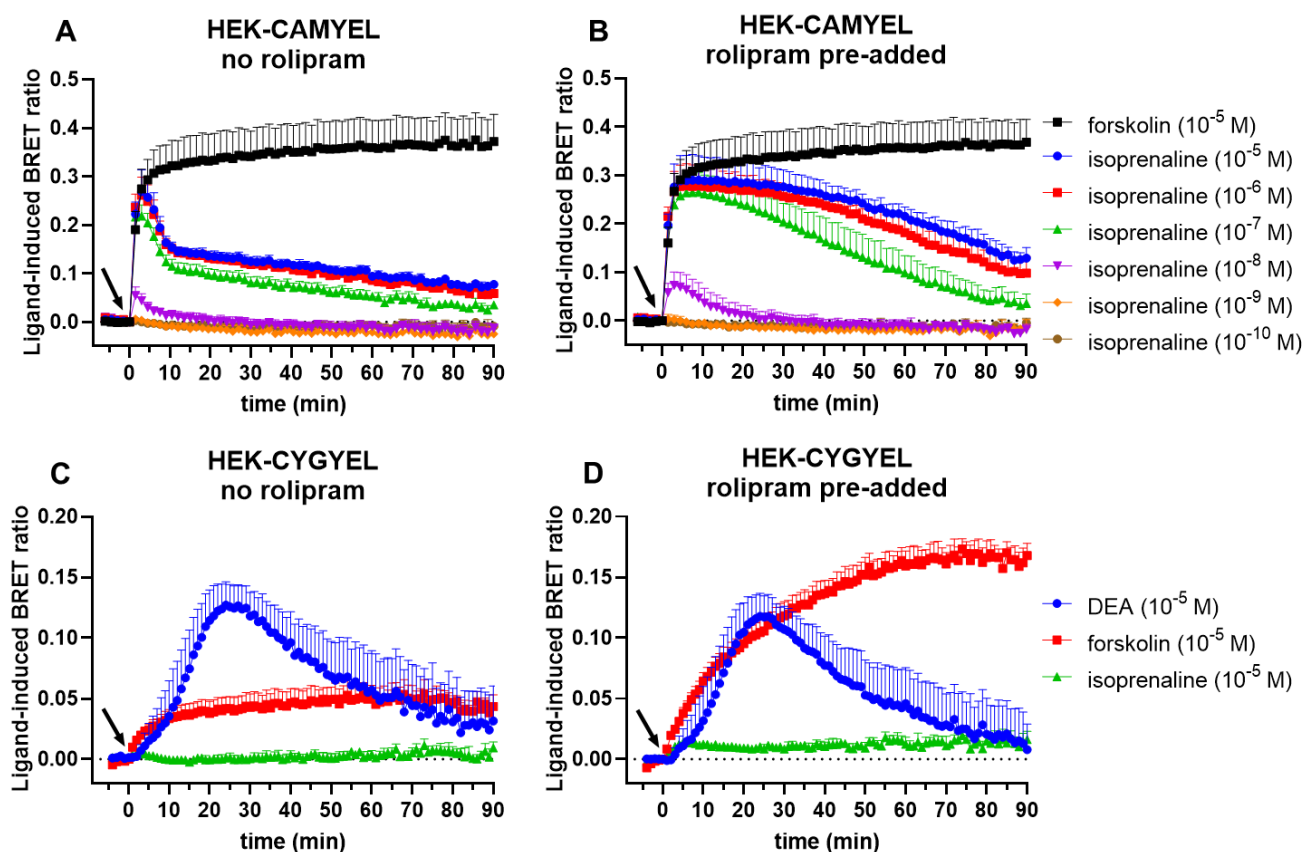


Figure 4.7. cAMP-mediated activation of CAMYEL and CYGYEL expressed in HEK293T cells. HEK-CAMYEL cells were stimulated with isoprenaline or forskolin (10 μ M) in the (A) absence or (B) presence of rolipram (10 μ M; approximately 10-minute pre-treatment). HEK-CYGYEL cells were stimulated with DEA (10 μ M), forskolin (10 μ M), or isoprenaline (10 μ M) in the (C) absence or (D) presence of rolipram (10 μ M; approximately 10-minute pre-treatment). Time or vehicle or ligand addition is represented by an arrow. Data represent the mean and SEM of 3-4 independent experiments.

4.3.4 Detection of cGMP in human primary vascular cells

cGMP is a key signalling pathway for the regulation of cardiovascular physiology. cGMP is synthesised via both sGC and pGC (natriuretic peptide receptors) in vascular cells, and has a variety of roles including regulation of vascular tone, angiogenesis, and apoptosis (Tsai & Kass, 2009). To demonstrate the use of the biosensor for real-time detection of cGMP activity in human primary vascular cells, CYGYEL lentivirus was produced, purified, and used to transduce two types of human primary vascular cells: (i) human umbilical vein smooth muscle cells (HUVSMCs); and (ii) human umbilical vein endothelial cells (HUVECs) that had previously been transduced with lentivirus to overexpress the relaxin receptor RXFP1 (HUVEC-RXFP1) for unrelated reasons and had been sorted for RXFP1-positive cells via FACS (**Figure 4.8**). The transduced HUVSMCs and HUVEC-RXFP1 cells were subsequently sorted via FACS to remove cells that were not successfully transduced with CYGYEL, resulting in HUVSMC-CYGYEL (**Figure 4.9**) and HUVEC-RXFP1-CYGYEL cell lines (**Figure 4.10**).

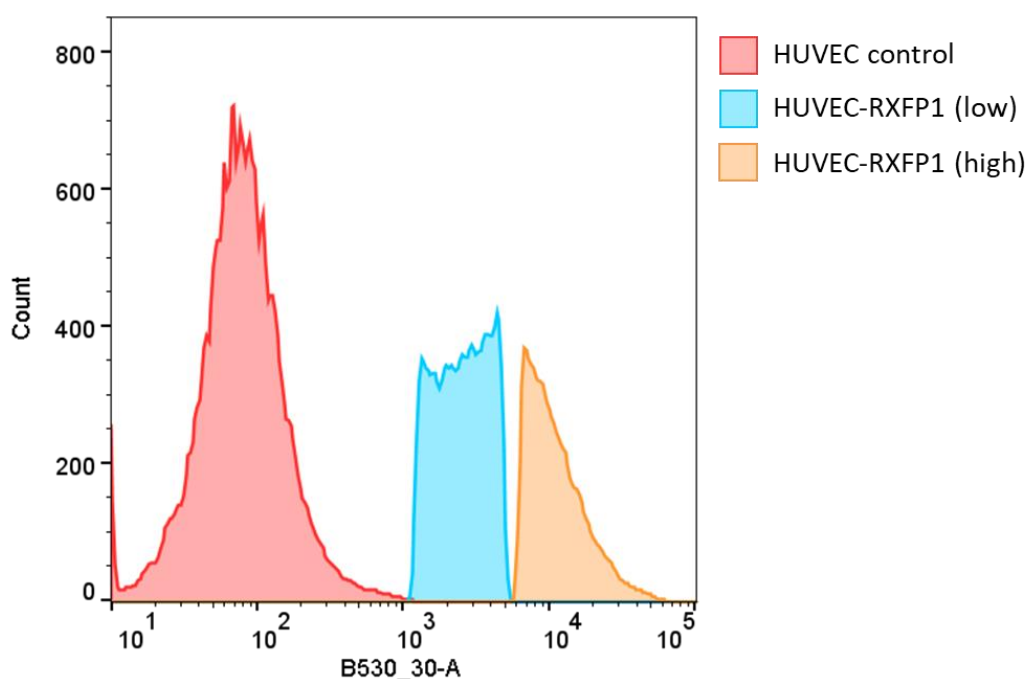


Figure 4.8. Representation of fluorescence-activated cell sorting of HUVEC-RXFP1 cells. Human umbilical vein endothelial cells (HUVECs) were transduced with RXFP1 IRES GFP lentivirus and then sorted by FACS to remove non-transduced cells and to sort transduced cells into “low” (blue) and “high” (orange) populations. Single, live cells were isolated by gating, and cells that expressed RXFP1 (based on GFP emissions that were higher than control cells) were sorted into separate populations (excitation: 488 nm; emission 530/30 nm). Non-transduced control cells are shown in red.

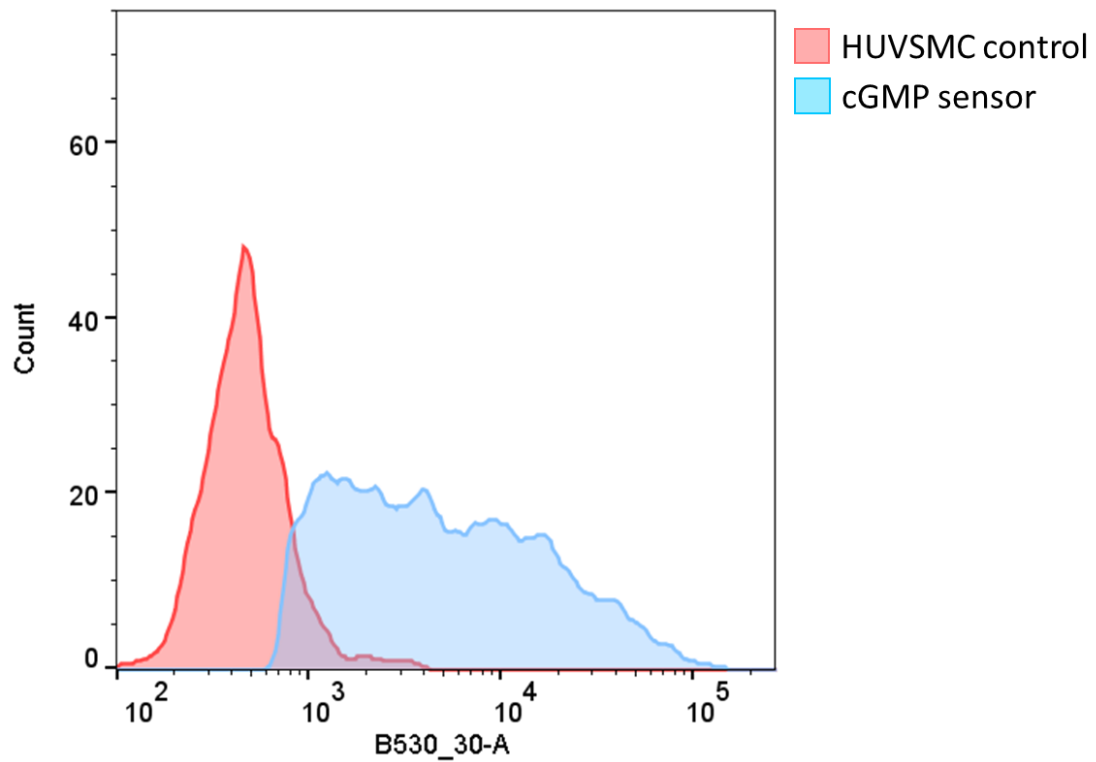


Figure 4.9. Representation of fluorescence-activated cell sorting (FACS) for HUVSMC-CYGYEL cells. Human umbilical vein smooth muscle cells (HUVSMCs) were transduced with CYGYEL lentivirus and then sorted by FACS to remove non-transduced cells. Single, live cells were isolated by gating, and cells that expressed CYGYEL (based on YFP emissions that were higher than control cells) were sorted into a separate population (excitation: 488 nm; emission: 530/30 nm).

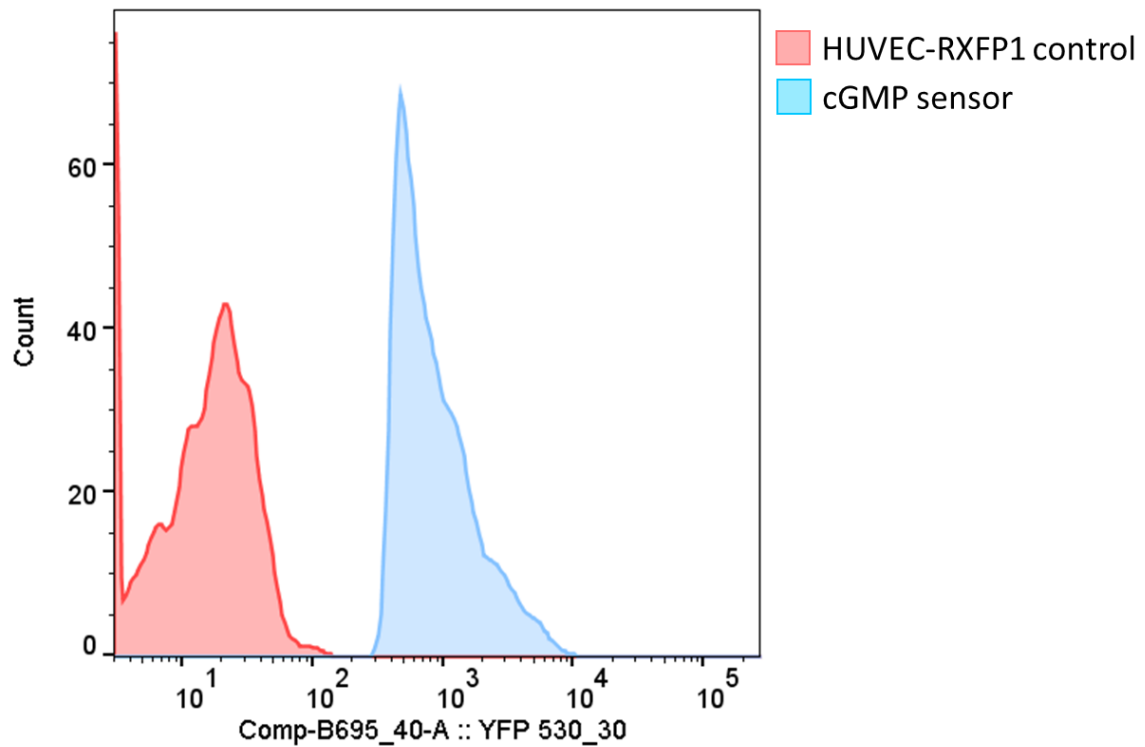


Figure 4.10. Representation of fluorescence-activated cell sorting (FACS) for HUVEC-RXFP1-CYGYEL cells. Human umbilical vein endothelial cells (HUVECs) expressing RXFP1 IRES GFP were transduced with CYGYEL lentivirus and then sorted by FACS to remove non-transduced cells. Single, live cells were isolated by gating, and cells that expressed CYGYEL (based on YFP emissions that were higher than control cells) were sorted into a separate population (excitation: 488 nm; emission: 530/30 nm). As cells already expressed GFP, separate filters were used and compensation was applied to separate emissions from the two fluorophores. The histogram here represents only YFP emissions of the two populations.

To detect sGC-mediated cGMP activity, HUVSMC-CYGYEL and HUVEC-RXFP1-CYGYEL cells were stimulated with the NO donor DEA, in the absence of any PDE inhibitors. Time-courses for HUVSMCs showed robust, concentration-dependent increases in cGMP activity (**Figure 4.11A**), which were transient at sub-maximal concentrations of DEA. In contrast, concentration-dependent data was unable to be generated for HUVECs, as responses were difficult to detect (**Figure 4.11B**). Concentration-response curves were generated for HUVSMCs in response to DEA at 10, 20, and 60 minutes (**Figure 4.11C**), demonstrating EC_{50} values in the micromolar range with highest potency at 20 mins (**Table 4.3**).

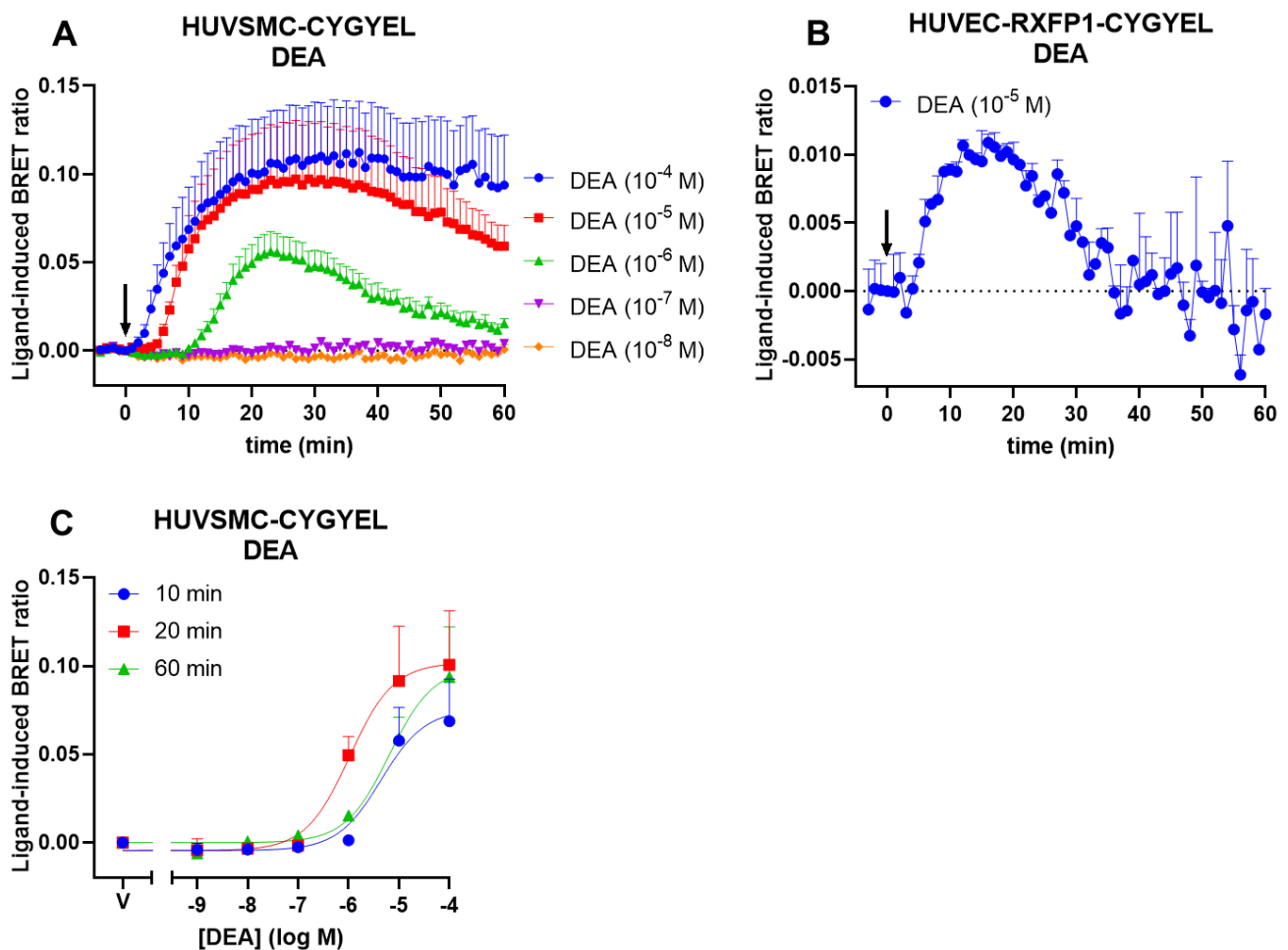


Figure 4.11. DEA-induced cGMP activity in HUVSMCs and HUVECs. HUVSMCs and HUVECs stably expressing CYGYEL were stimulated with vehicle or the nitric oxide donor DEA for 60 minutes. Time-courses were generated for (A) HUVSMCs and (B) HUVECs. (C) Concentration-responses curves were generated at 10, 20, and 60 minutes for HUVSMCs. Time of vehicle or DEA addition is represented by an arrow. Data represent the mean and SEM for two (panel B) or three independent experiments.

Table 4.3. Potency of DEA for cGMP activity in HUVSMCs. HUVSMCs stably expressing CYGYEL were stimulated with the nitric oxide donor DEA for 60 minutes. The potencies (EC_{50} and pEC_{50}) were extracted from concentration-response curves at 10 and 60 minutes. Data represent the mean $\pm$ SEM for three independent experiments.

	HUVSMC		
DEA	10 min	20 min	60 min
EC_{50} (μ M)	4.44 ± 3.42	1.05 ± 7.43	6.44 ± 3.95
pEC_{50}	5.35 ± 0.33	5.98 ± 0.31	5.19 ± 0.27

HUVSMCs were then used to try to detect GPCR/sGC-mediated cGMP activity. HUVECs were not tested here as the DEA response in these cells was very weak, implying sGC-mediated cGMP activity would be difficult to detect, whereas the HUVSMCs had robust DEA responses. HUVSMCs were therefore stimulated with relaxin (100 nM), which has previously been reported to increase cGMP activity in HUVSMCs (Kocan *et al.*, 2017; Sarwar *et al.*, 2015), and carbachol (100 μ M), which has been reported to increase cGMP in some cell types (Castoldi *et al.*, 1993). Unfortunately, no response from either ligand was detected, despite the robust DEA response (**Figure 4.12**). It is too early to tell whether this relates to a lack of sensitivity of the sensor, as the cGMP responses from these receptors may be weak, or whether it relates to poor expression of relevant proteins in these cells. For example, we did not test cell surface expression of the relevant receptors in the HUVSMCs.

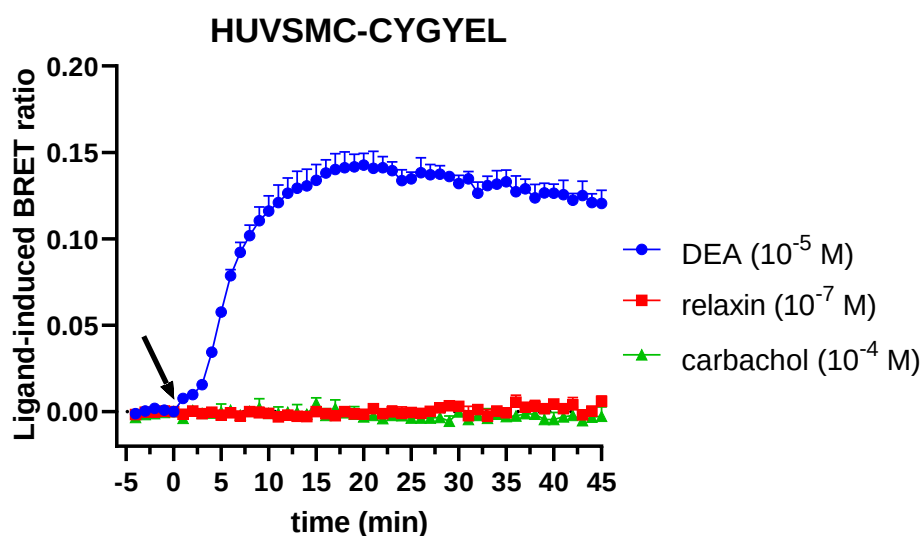


Figure 4.12. cGMP activity in HUVSMC-CYGYEL cells. HUVSMCs stably expressing CYGYEL were stimulated with vehicle, DEA ($10 \mu\text{M}$), relaxin (100 nM), and carbachol ($100 \mu\text{M}$) for 45 minutes. Time of vehicle or ligand addition is represented by an arrow. Data represent the mean and standard deviation of one assay.

In addition to sGC-mediated cGMP production, CYGYEL was tested for its ability to detect pGC-mediated cGMP activity in human vascular cells. HUVSMCs and HUVECs were stimulated with a range of concentrations of C-type natriuretic peptide (CNP), which activates the GC-B form of pGC, for 60 minutes. Time-courses generated for HUVSMCs (**Figure 4.13A**) and HUVECs (**Figure 4.13B**) showed concentration-dependent increases in cGMP activity after CNP stimulation, which reached maximum activity at $1\text{-}5 \mu\text{M}$ for both cell types. However, whereas the cGMP responses in HUVSMCs were relatively sustained for the duration of the time-course, the cGMP responses in HUVECs peaked at about 10 minutes before showing a slow decline that started to stabilise by the end of the time-course. Concentration-response curves were generated for CNP in HUVSMCs (**Figure 4.13C**) and HUVECs (**Figure 4.13D**) at 10 and 60 minutes. Despite the difference in kinetics of signalling, CNP had almost identical potencies between the two cell types at both time points (**Table 4.4**).

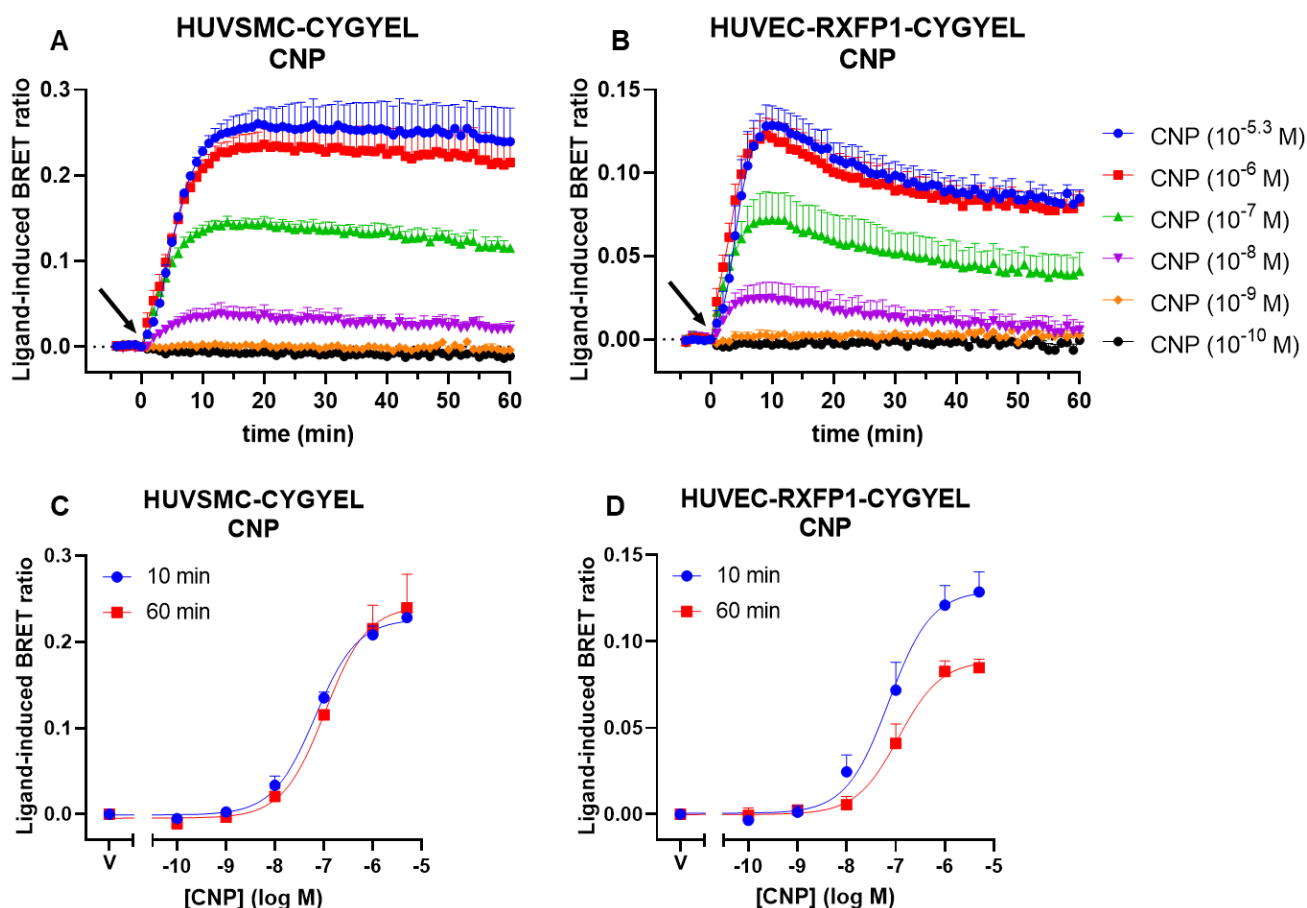


Figure 4.13. CNP-induced cGMP activity in HUVMSCs and HUVECs. HUVMSCs and HUVECs stably expressing CYGYEL were stimulated with vehicle or CNP for 60 minutes. Time-courses were generated for (A) HUVMSCs and (B) HUVECs. Concentration-responses curves were generated at 10 and 60 minutes for (C) HUVMSCs and (D) HUVECs. Time of vehicle or CNP addition is represented by an arrow. Data represent the mean and SEM for three independent experiments.

Table 4.4. Potency of CNP for cGMP activity in HUVMSCs and HUVECs. HUVMSCs and HUVECs stably expressing CYGYEL were stimulated with the GC-B agonist CNP for 60 minutes. The potencies (EC_{50} and pEC_{50}) were extracted from concentration-response curves at 10 and 60 minutes. Data represent the mean $\pm$ SEM for three independent experiments.

	HUVMSC		HUVEC	
CNP	10 min	60 min	10 min	60 min
EC_{50} (nM)	65.91 ± 8.70	105.20 ± 33.37	71.83 ± 23.12	114.30 ± 31.61
pEC_{50}	7.18 ± 0.06	6.98 ± 0.14	7.14 ± 0.14	6.94 ± 0.12

4.4 Discussion

Traditional end point assays for cGMP do not easily measure the temporal aspects of signalling, as they involve lysing cells at individual time points. The ability of genetically-encoded, BRET-based biosensors to detect signalling in real time in plate-based cellular assays led us to develop a BRET-based biosensor for cGMP activity. The cGES-DE5 FRET-based biosensor was converted to BRET due to its high selectivity for cGMP over cAMP and high temporal resolution in comparison to other available biosensors (Nikolaev *et al.*, 2006). This was accomplished by swapping the region coding for ECFP to Rluc8, as well as changing the region coding for EYFP to Venus. The resulting biosensor, which was named CYGYEL, was cloned into a lentiviral vector that can be used to transduce different types of mammalian cells. In all cell types tested, CYGYEL was able to detect real-time changes in cGMP activity, and in some cases showed differences in temporal patterns between ligands and cell types.

HEK293T cells were first transduced and sorted by FACS for basic characterisation of the sensor. Real-time assays in live HEK293T cells were conducted, which showed that CYGYEL can detect robust increases and decreases in sGC-mediated cGMP activity for extended periods of time (at least 90 minutes) after stimulation with a NO donor. The assay was sensitive enough to detect cGMP activity in the absence of any PDE inhibitor, but the addition of the cGMP-specific PDE5 inhibitor vardenafil or the non-specific PDE inhibitor IBMX did increase the BRET ratio and prolong the response. Furthermore, pre-incubation with the sGC inhibitor ODQ diminished or abolished the increases in BRET ratio, further confirming the assay was specific for cGMP activity.

Following on from this, it was demonstrated that CYGYEL can detect cGMP activity mediated through both sGC and pGC in human primary vascular cells, specifically HUVECs and HUVSMCs. Cells were transduced with purified CYGYEL lentivirus and sorted via FACS, before being stimulated with either DEA to produce activation of sGC or CNP to activate pGC. Both cell types showed robust, concentration-dependent responses to CNP, but increases were larger and mostly sustained for the duration of the time-course in HUVSMCs, whereas the increases in BRET ratio were lower and more transient in HUVECs. Notably, however, potency of CNP was almost identical between cell types at multiple time points. In contrast, DEA responses were very weak and difficult to detect in HUVECs, whereas there was robust, concentration-dependent cGMP activity in HUVSMCs after addition of DEA. The temporal profile of DEA also differed markedly from that of CNP, showing more transient responses that rise and fall over the time-course, much like the DEA responses seen in HEK293T cells. Additionally, the weaker DEA responses compared to natriuretic peptide responses in HUVECs are consistent with studies showing more substantial cGMP

responses in HUVECs after stimulation with natriuretic peptides compared to NO donors (Chen *et al.*, 2008; Rivero-Vilches *et al.*, 2003). Finally, as robust cGMP activity was detected in both types of vascular cells tested, it is expected that the lentiviral vector will also aid in the detection of cGMP across other cell types.

Due to the weak response to DEA stimulation in HUVECs, HUVSMCs were used to test for GPCR-mediated cGMP activity by stimulation with relaxin and carbachol. However, no response was detected from either agonist. There may be several explanations for this. The first explanation suggests that these are not the most appropriate cell types for detecting cGMP in the first place. Regarding the physiology of a blood vessel, smooth muscle cell-mediated cGMP activity is generally dependent on NO produced in endothelial cells that diffuses into smooth muscle cells where it activates sGC to synthesise cGMP (Furchgott & Vanhoutte, 1989). Thus, it is not necessarily expected that direct stimulation of smooth muscle cells (or endothelial cells) alone would lead to robust cGMP activity. Furthermore, cell surface expression of the relevant GPCRs in the HUVSMCs was not checked. One possible next step would be to try detection of cGMP in other cell types that have been characterised to generate robust cGMP activity after GPCR activation, such as some types of neuroblastoma cells (Matsuzawa & Nirenberg, 1975). Another explanation for the lack of GPCR-mediated activity so far is that CYGYEL is not sensitive enough to detect GPCR-mediated cGMP, especially as these pools of cGMP activity might be weak. Regardless, even if CYGYEL proves to be inappropriate for detecting GPCR signalling, it is clear that it can detect robust activity from both pools of GC in human primary cells, and thus CYGYEL has many applications related to drug discovery.

Low selectivity for cGMP over cAMP is in some instances an obstacle for the use of genetically-encoded biosensors for cGMP activity. Therefore, low crosstalk from cAMP was one of the primary reasons that cGES-DE5 was chosen as the basis for a BRET-based biosensor. cGES-DE5 is activated by cGMP with a potency of about 1.5 μ M and was over 400 times more selective for cGMP over cAMP when cyclic nucleotides were added to lysates from TsA201 cells that expressed the sensor (Nikolaev *et al.*, 2006). Interestingly, a variant of cGES-DE5 that swapped the CFP/YFP FRET pair to a green/red T-sapphire/dimer2 FRET pair showed substantial increases in both affinity for cGMP (~40 nM) as well as selectivity for cGMP over cAMP (Niino *et al.*, 2009). This allowed single-cell detection of cGMP in adult cardiomyocytes, which the original cGES-DE5 was unable to do, as cGMP levels are very low in adult cardiomyocytes (Gotz *et al.*, 2014). Here, we demonstrated that CYGYEL was activated by cGMP with a potency of about 1.3 μ M and by cAMP with a potency of about 42 μ M, which is a selectivity of about 32-fold. Thus, although the potency of cGMP is comparable between cGES-DE5 and CYGYEL, the potency of cAMP is considerably lower for

activation of CYGYEL than cGES-DE5. As noted, however, results from biochemical assays in cell lysates will not always reflect what occurs in live cells, due to a variety of factors such as localised cyclic nucleotide and PDE activity. Therefore, we also demonstrated selectivity by stimulating live HEK293T cells stably expressing either CYGYEL or the commonly-used CAMYEL cAMP sensor with the adenylate cyclase activator forskolin, which showed that CAMYEL detects forskolin-mediated cAMP with approximately 160-fold higher potency than the cGMP sensor does. It should be noted, however, that a maximal concentration of forskolin produced considerable activation of CYGYEL relative to DEA, and a concentration of isoprenaline that produced a maximal response in a cAMP assay produced a minimal response in the cGMP assay but only when rolipram was used to stop the breakdown of cAMP. Using biosensors for cGMP activity might become problematic when a ligand signals via both cAMP *and* cGMP, such as in the case of agonists for some GPCRs including RXFP1, or when there is high basal cAMP activity that produces basal activation of the sensor. In some cases, CYGYEL may not be sensitive enough to detect robust cGMP activity, such as in specific cell types or receptors where only weak activity is expected. Furthermore, although generation of a BRET-based cGMP sensor with higher affinity for cGMP may be of great value, this might come at the cost of also increasing the affinity for cAMP.

Compartmentalisation of cyclic nucleotides and other second messengers is a current research topic in cell biology (McCormick & Baillie, 2014). cAMP activity, for example, is well known to occur in microdomains, rather than being homogenously distributed throughout the cell (Lefkimmatis & Zaccolo, 2014). cGMP compartmentalisation is less well understood, but given that sGC and pGC are already spatially separated, it is not surprising that NO and natriuretic peptides have been shown to stimulate the synthesis of spatially-segregated pools of cGMP, which can also be modulated by specific PDE isoforms (Castro *et al.*, 2006; Piggott *et al.*, 2006). Importantly, microdomains of cGMP have different functional roles in cardiovascular physiology, which is still under investigation (Bork *et al.*, 2019). Therefore, understanding the biological roles of cGMP signalling from microdomains may in turn aid drug development favouring activation of particular microdomains (Bork *et al.*, 2019). CYGYEL successfully detected cGMP regardless of whether it was mediated by sGC or pGC, which suggests the sensor is relatively widely expressed in the cell, however high-resolution microscopy was not conducted to confirm expression at specific locations. One of the benefits of genetically-encoded biosensors is that targeting sequences can be introduced to the gene in order to detect signalling localised to different subcellular locations, compared to traditional end point assays that detect total concentration of the second messenger in cell lysates and therefore have no spatial resolution. In fact, targeted biosensors have been used to detect cAMP activity from specific regions in single cells via FRET (DiPilato *et al.*, 2004), and in

populations of cells via BRET (Matthiesen & Nielsen, 2011). Targeting of CYGYEL may therefore allow detection of specific microdomains of cGMP.

In summary, we have generated a genetically-encoded, BRET-based biosensor for cGMP activity that can detect changes in sGC- and pGC-mediated cGMP activity in primary cells over extended periods of time in a plate-based format. The lentiviral vector of the biosensor allows for generation of stable cell lines, aiding the process of drug discovery by allowing the straightforward detection of cGMP activity in live cells without requiring additional lysis and detection steps.

5 Characterising ligand- and cell-specific signalling at RXFP1

5.1 Introduction

Relaxin acts on several types of cells and tissue to mediate a variety of physiological roles. For example, relaxin is well-known as a pregnancy hormone in mammals, where it is involved in processes such as implantation, pubic relaxation, and cervical ripening, depending on the species (Sherwood, 2004). Circulating relaxin also has several adaptive roles in the cardiovascular and renal systems of pregnant rats, including increases in cardiac output, stroke volume, systemic and renal vasodilation, glomerular filtration rate, and renal plasma flow (Debrah *et al.*, 2006; Novak *et al.*, 2001). Relaxin is also produced locally in the vasculature of rats (Novak *et al.*, 2006), and is involved in vascular homeostasis in male rats (Jelinic *et al.*, 2015; Leo *et al.*, 2014; Ng *et al.*, 2015). Additionally, endogenous relaxin has a role in the breakdown and regulation of collagen across the reproductive, pulmonary, renal, cardiac, and integumentary systems of mice (Samuel *et al.*, 2007).

Administration of exogenous relaxin has several promising actions that are consistent with these physiological roles. For example, when administered to rats, relaxin mimics some of the cardiovascular changes caused by endogenous relaxin during pregnancy (Danielson *et al.*, 1999; Novak *et al.*, 2001). Relaxin also regulates collagen turnover *in vitro* upon stimulation of human fibroblasts (Unemori & Amento, 1990; Unemori *et al.*, 1992; Unemori *et al.*, 1996), decreases collagen accumulation in several animal models of fibrosis such as myocardial infarction, hypertension, cardiomyopathy, and diabetes (Samuel *et al.*, 2017), and was also a more effective anti-fibrotic than the clinically-used angiotensin-converting enzyme inhibitor enalapril in a mouse model of cardiac fibrosis (Samuel *et al.*, 2014). It is thus not surprising that RXFP1 has generated substantial interest as a therapeutic target for the treatment of cardiovascular disorders and fibrosis in humans (Unemori, 2017). In fact, relaxin has progressed to clinical trials for several cardiovascular and fibrotic conditions. In clinical trials for AHF, for example, short-term relaxin administration had several cardiovascular and renal effects, including systemic and renal vasodilation, in addition to a reduction in the worsening of heart failure and improvements in markers of renal function (Teerlink *et al.*, 2020). Importantly, relaxin was safe and well tolerated in

humans, and was associated with a reduction in all-cause mortality (Teerlink *et al.*, 2020). However, despite relaxin showing promising therapeutic potential, clinical trials have unfortunately not met their end points. Regardless, relaxin is still proving to be of great therapeutic interest for various cardiovascular and fibrotic conditions (Dschiezig, 2019; Kanai *et al.*, 2019; Ng *et al.*, 2018).

There are several unresolved issues hindering the advancement of relaxin or other ligands targeting RXFP1 for use as therapeutic drugs. For example, although chronic administration of relaxin would be necessary for the treatment of conditions such as fibrosis, the two-chain, three-disulfide-bond structure of relaxin is complicated and expensive to produce either chemically or recombinantly (Hossain & Wade, 2014). This, coupled with the short half-life of relaxin in serum (Chen *et al.*, 1993), precludes the chronic, systemic administration of relaxin. Furthermore, in addition to binding to RXFP1, relaxin can also bind to and activate RXFP2 (Hsu *et al.*, 2002) and RXFP3 (van der Westhuizen *et al.*, 2010), which might produce undesired actions when administered systemically. Thus, the development of single-chain relaxin analogues that are easy to synthesise and are specific for RXFP1 is highly desirable, as these analogues would not activate the other relaxin family peptide receptors, would be easier to functionalise into long-acting variants by improving their pharmacokinetic properties, and would allow chronic systemic administration. However, the development of novel RXFP1 agonists raises the issue of biased signalling. RXFP1 couples to multiple effectors to increase activity of several different second messengers and kinases, but we do not understand how novel RXFP1 agonists work, because we do not have a detailed enough understanding of the relative importance of different effectors for the therapeutic actions, which signal transduction are downstream of which effector, and in which cell types. Thus, although biased signalling provides us with the opportunity to develop more effective therapeutic drugs that have fewer side effects, exploiting biased signalling requires us to first understand the molecular mechanisms underlying the biological effects of receptor activation by different ligands in greater detail.

For example, a minimised relaxin B-chain analogue known as B7-33 has been developed, but we do not understand the molecular mechanisms underlying its actions (Hossain *et al.*, 2016). B7-33 was created by truncating the 29-residue B chain of relaxin by six residues at the N terminus and adding four residues to the C terminus in order to increase cationic charges for improved solubility (Hossain *et al.*, 2016). B7-33 is cheaper and less demanding to produce than the two-chain relaxin peptide, activates RXFP1 albeit with low affinity, and does not activate RXFP2 (Hossain *et al.*, 2016). However, studies so far have suggested that B7-33 has cell-specific effects whereby it is potent in some cell types but has a low potency in others, which we do not understand. In HEK-RXFP1 cells and THP-1 cells which natively express RXFP1, B7-33 was a full but low potency agonist

for cAMP activity (Hossain *et al.*, 2016). Additionally, when tested for ERK1/2 phosphorylation in HEK-RXFP1 cells, B7-33 had much weaker potency and efficacy compared to relaxin (Hossain *et al.*, 2016). Interestingly, however, when tested for ERK1/2 phosphorylation in rat renal myofibroblasts, which natively express RXFP1, B7-33 showed approximately equal potency and efficacy to relaxin. Importantly, B7-33 also showed potent anti-fibrotic actions via RXFP1 *in vitro* in human cardiac fibroblasts and rat renal myofibroblasts, and was an anti-fibrotic *in vivo* in rodent models of cardiac and lung fibrosis (Hossain *et al.*, 2016). Furthermore, B7-33 had potent protective effects against simulated ischemia reoxygenation in mouse primary cardiomyocytes, which was dependent on ERK1/2, and protected against ischemia-reperfusion in mice *in vivo* (Devarakonda *et al.*, 2020). Additionally, B7-33 potentiated bradykinin-mediated relaxation of rat mesenteric artery at an equivalent dose to relaxin, and chronic (24-hour) treatment with B7-33 also potently mimicked the vasoprotective actions of relaxin in mouse mesenteric artery in an *ex vivo* model of endothelial dysfunction (Marshall *et al.*, 2017). B7-33 was also a full but low potency agonist for cAMP activity in HEK293T cells expressing rat or mouse RXFP1, demonstrating that the potent actions of B7-33 are not specific to rodents (Hossain *et al.*, 2016). Rather, these findings appear to suggest that B7-33 is a cell-specific agonist that has weak activity in recombinant and cancer cells, yet potent activity in fibroblasts and vascular cells. However, we cannot yet rule out the idea that B7-33 is a biased agonist.

In order to elucidate the molecular mechanisms underlying the biological effects of B7-33, and RXFP1 activation in general, better *in vitro* assays that improve upon the shortcomings of traditional end point assays are required. The assays should measure signalling in real time to detect kinetic effects and avoid observational bias, and should be able to be used in native and primary cells in order to distinguish biased signalling from system bias and understand how signalling differs across cell types. With the development of appropriate cell lines and real-time BRET-based assays, we can now begin to understand the mechanisms underlying the actions of B7-33 in more detail. Here, we use real-time BRET-based assays for cAMP, ERK1/2, and G protein association, in addition to end point assays for cAMP and cGMP, across several cell types, in order to understand the mechanisms of action underlying the beneficial actions of B7-33.

5.2 Methods

5.2.1 Reagents

Ligands, inhibitors, and other compounds used were B7-33, coelenterazine 400a, coelenterazine *h*, DEA, forskolin, IBMX, isoprenaline, pertussis toxin, relaxin, and TAMRA-relaxin. Sources of reagents are listed in Chapter 2.

5.2.2 Cell culture

Cell lines and culture methods are described in Chapter 2. Cell types used include BJ3 human dermal fibroblasts, HEK293T, and HUVECs.

5.2.3 Generation of stable cell lines using lentivirus

Lentiviral methods were conducted as described in Chapter 2. pLenti X1 Ef1 α CAMYEL and pLenti X1 Ef1 α RXFP1 IRES GFP lentiviruses were produced and purified and used to generate stable cell lines. HUVECs were transduced in T175 flasks, whereas BJ3 cells were transduced on 20 cm dishes.

5.2.4 Flow cytometry-based ligand binding assay and fluorescence-activated cell sorting

The flow cytometry-based fluorescent ligand binding assay using TAMRA-relaxin was conducted as described in Chapter 2. FACS was also conducted as described in Chapter 2.

5.2.5 Cyclic nucleotide accumulation assays

cAMP and cGMP accumulation assays in HUVEC lysates were conducted using HTRF technology as described in Chapter 2.

5.2.6 BRET assays for cAMP and ERK1/2 activity

Real-time BRET assays were conducted as described in Chapter 2, using a POLARstar Omega plate reader (for data in Figure 5.2 only) or PHERAstar FSX plate reader, but with some modifications. For HUVECs, BRET assays were conducted in EGM-2 containing 25 mM HEPES, as in Chapter 4. For the ligand washout assay, cells were stimulated with ligands as normal, but after 25 minutes the assay was paused and cell culture media in some wells, as indicated, was removed and replaced with fresh cell culture media containing coelenterazine *h* (5 μ M), after which the time-course was resumed.

ERK1/2 activity assays were conducted using the Rluc8-ERKsubstrate-Venus (REV) BRET-based biosensors for cytosolic (REV_{Cyto}) and nuclear (REV_{Nuc}) activity (Xu *et al.*, 2013). Assays were conducted in HEK-RXFP1-REV_{Cyto} and HEK-RXFP1-REV_{Nuc} cells. Cells were serum starved in phenol red free DMEM containing 0.5% FBS, 1% penicillin-streptomycin, 1% L-Glutamine, and 25 mM HEPES, for approximately three hours before conducting the assay.

5.2.7 NanoBRET assays for G protein association

G α -Nluc and G β_1 constructs were provided by A/Prof. Denise Wootten (Monash Institute of Pharmaceutical Sciences, Melbourne, Australia). Nluc was inserted into the helical domain of the G α_s or G α_{i3} subunit. The Venus-G γ_2 construct was provided by Prof. Kevin Pflieger (Harry Perkins Institute of Medical Research, Perth, Australia). Venus was fused to the N-terminal of G γ_2 .

HEK-RXFP1 cells were plated out on a six-well plate, and were transfected the following day with 20 ng G α_s -Nluc or G α_{i3} -Nluc, 200 ng G β_1 , and 200 ng Venus-G γ_2 in the pcDNA3.1 vector per well using Lipofectamine 2000. The next day, cells were resuspended in complete media containing 25 mM HEPES but no phenol red, and were re-plated on a 96-well CulturPlate.

NanoBRET assays were then conducted the next day similarly to BRET assays, but with a few modifications. NanoBRET assays were conducted using coelenterazine 400a as the substrate for Nluc, as described in Chapter 2. Cell culture media was replaced with 0% FBS phenol red free media containing 1 mg/ml BSA and 5 μ M coelenterazine 400a. Cells were pre-incubated for approximately 50 minutes before conducting the assay to allow luminescence to stabilise.

5.3 Results

5.3.1 Human dermal fibroblasts do not signal via cAMP after relaxin stimulation

Although relaxin stimulation acutely increases cGMP activity in several types of fibroblasts such as rat renal myofibroblasts (Wang *et al.*, 2016) and human cardiac fibroblasts (Kocan *et al.*, 2017; Sarwar *et al.*, 2015), most types of fibroblast tested show little or no cAMP activity after relaxin stimulation, including rat ventricular fibroblasts (Samuel *et al.*, 2004a), rat renal myofibroblasts (Mookerjee *et al.*, 2009), and human cardiac fibroblasts (Sarwar *et al.*, 2015). It is not known whether this relates to the low expression of RXFP1 in these cell types, or whether it is due to other, cell-specific factors.

BJ3 human dermal fibroblasts, which have been used to demonstrate the anti-fibrotic actions of relaxin *in vitro* (Chan *et al.*, 2012; Chow *et al.*, 2012; Hossain *et al.*, 2011; Shabanpoor *et al.*, 2012), were used to investigate further and to determine whether they may be valuable for understanding the actions of B7-33. To establish whether BJ3 cells signal via cAMP after relaxin stimulation, CAMYEL lentivirus was produced, purified, and used to transduce BJ3 cells. FACS was used to isolate single, live cells, and cells that expressed YFP (from the sensor) at higher levels than non-transduced BJ3 cells were sorted into a separate population (**Figure 5.1**).

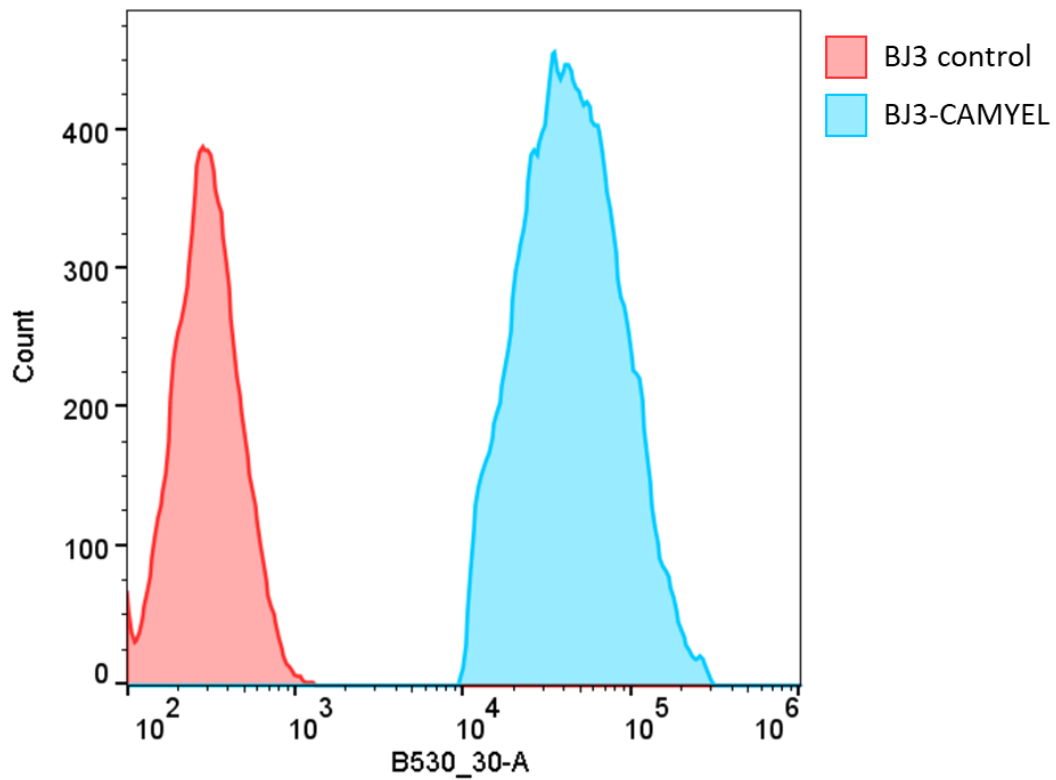


Figure 5.1. Representation of fluorescence-activated cell sorting of BJ3-CAMYEL cells. BJ3 human dermal fibroblasts were transduced with CAMYEL lentivirus and then sorted by FACS to remove non-transduced cells and sort the transduced cells into a separate population. Single, live cells were isolated by gating, and cells that expressed CAMYEL (based on the intrinsic YFP) were sorted (excitation: 488 nm; emission: 530/30 nm).

To test whether RXFP1 or other GPCR-mediated cAMP activity occurs in these cells, BJ3-CAMYEL cells were stimulated with isoprenaline or relaxin, and the positive control forskolin (10 μ M), in the absence or presence of the non-selective PDE inhibitor IBMX (500 μ M), where isoprenaline is an agonist for the β -adrenoceptors endogenous to many cell types. These assays confirmed that, whereas isoprenaline induced robust, concentration-dependent increases in cAMP levels in the absence (**Figure 5.2A**) or presence (**Figure 5.2B**) of IBMX, relaxin did not detectably increase cAMP activity in BJ3 cells (**Figure 5.2C**), even in the presence of IBMX (**Figure 5.2D**), despite its chronic actions in these cells, consistent with other types of fibroblasts.

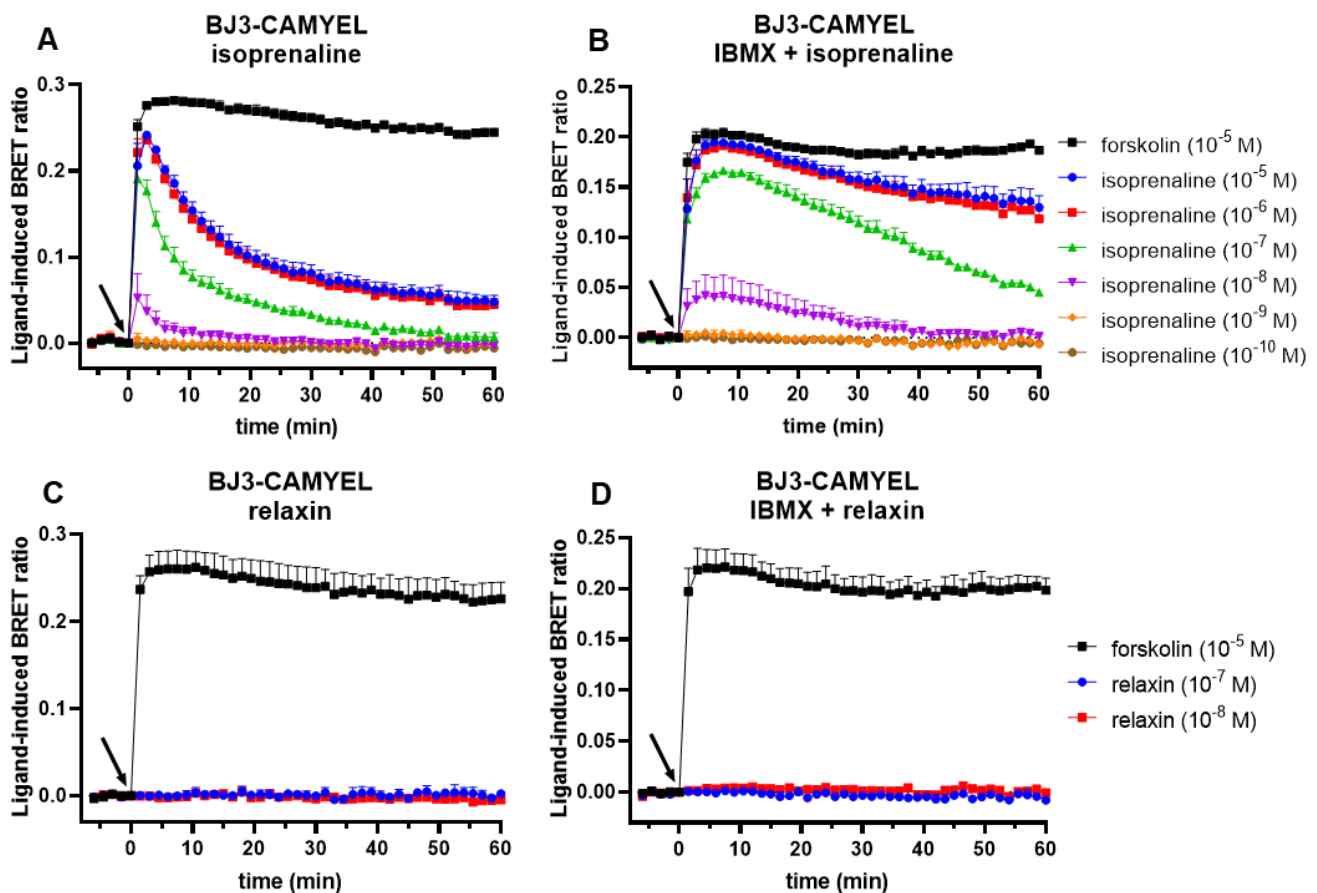


Figure 5.2. Ligand-induced cAMP activity in BJ3-CAMYEL cells. BJ3 human dermal fibroblasts expressing CAMYEL were stimulated with forskolin (10 μ M) and either (A-B) isoprenaline or (C-D) relaxin, in the (A, C) absence, or (B, D) presence of IBMX (500 μ M). Time of ligand or vehicle addition is represented by an arrow. Data represent the mean and SEM for three independent experiments.

However, we investigated the effect of expressing higher levels of RXFP1 in fibroblasts on the cAMP response after RXFP1 activation, as it may provide valuable information on cell- or ligand-specific actions of RXFP1 activation. To accomplish this, RXFP1 IRES GFP lentivirus was produced, purified, and used to transduce the BJ3-CAMYEL cells, thus generating the BJ3-CAMYEL-RXFP1 cell line, which was not further sorted. To compare the expression levels of RXFP1 among cell types, a flow cytometry-based fluorescent ligand binding assay using TAMRA-relaxin was conducted for HEK-RXFP1 positive control cells (**Figure 5.3A**), BJ3-CAMYEL cells (**Figure 5.3B**), and BJ3-CAMYEL-RXFP1 cells (**Figure 5.3C**). Specific binding was extracted by subtracting mean TAMRA emissions for non-specific binding from total binding (**Figure 5.3D**), which confirmed that BJ3-CAMYEL-RXFP1 cells showed substantially higher specific binding than BJ3-CAMYEL cells, which were also confirmed to natively express RXFP1 despite their lack of cAMP response. Additionally, HEK-RXFP1 cells, which were previously shown to have 50,000-100,000 receptors per cell (Sudo *et al.*, 2003), showed substantially higher specific binding than BJ3-CAMYEL-RXFP1 cells.

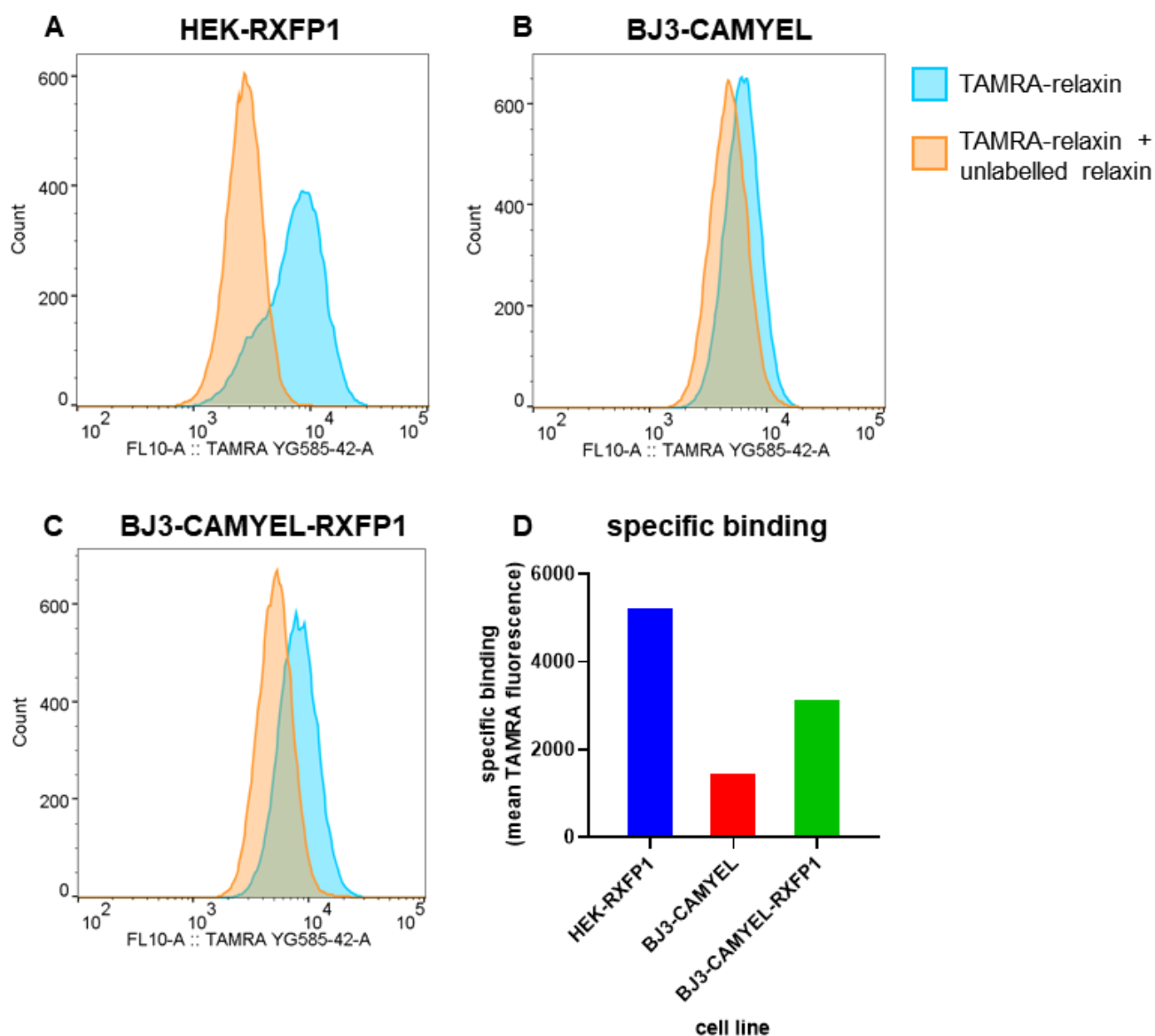


Figure 5.3. Fluorescent ligand binding assay using fluorescent relaxin. A flow cytometry-based ligand binding assay using TAMRA-relaxin was conducted. Single, live cells were first isolated by gating, after which histograms showing mean TAMRA fluorescence (excitation: Y/G 561 nm; emission: 585/42) were generated. TAMRA-relaxin (20 nM) was incubated with (A) HEK-RXFP1, (B) BJ3-CAMYEL, or (C) BJ3-CAMYEL-RXFP1 cells in the absence or presence of an excess of unlabelled relaxin (2 μ M). (D) Specific binding was extracted from flow cytometry histograms by subtracting mean emissions for non-specific binding from total binding. Data represents one flow cytometry assay.

5.3.2 Human dermal fibroblasts cells overexpressing RXFP1 signal via cAMP after relaxin and B7-33 stimulation

BJ3-CAMYEL-RXFP1 cells were subsequently stimulated with a range of concentrations of relaxin, as well as forskolin (10 μ M). Interestingly, in contrast to the BJ3-CAMYEL cells, there were robust increases in cAMP activity after relaxin stimulation, which were sustained for the duration of a 60-minute time-course, in the absence of IBMX (**Figure 5.4A**). Therefore, to investigate the cell-specific actions of B7-33 in fibroblasts, BJ3-CAMYEL-RXFP1 cells were also stimulated with B7-33. Notably, B7-33 also activated cAMP as a full agonist (**Figure 5.4B**), but with two key differences compared to the relaxin response. First, unlike relaxin the responses induced by B7-33 were not sustained for the duration of the time-course, except for the maximal concentration (10 μ M). Second, B7-33 was not potent for cAMP activity compared to relaxin. Over the time-course relaxin remained at least 1,000 times more potent than B7-33 (**Table 5.1**), consistent with the cAMP activity seen in HEK-RXFP1 and THP-1 cells (Hossain *et al.*, 2016). These data thus gave the first hint that B7-33 could be a biased agonist, as it appears to only be potent for *some* outputs in fibroblasts (such as the anti-fibrotic actions). Further assays in other cell types were thus conducted to directly compare signalling pathways.

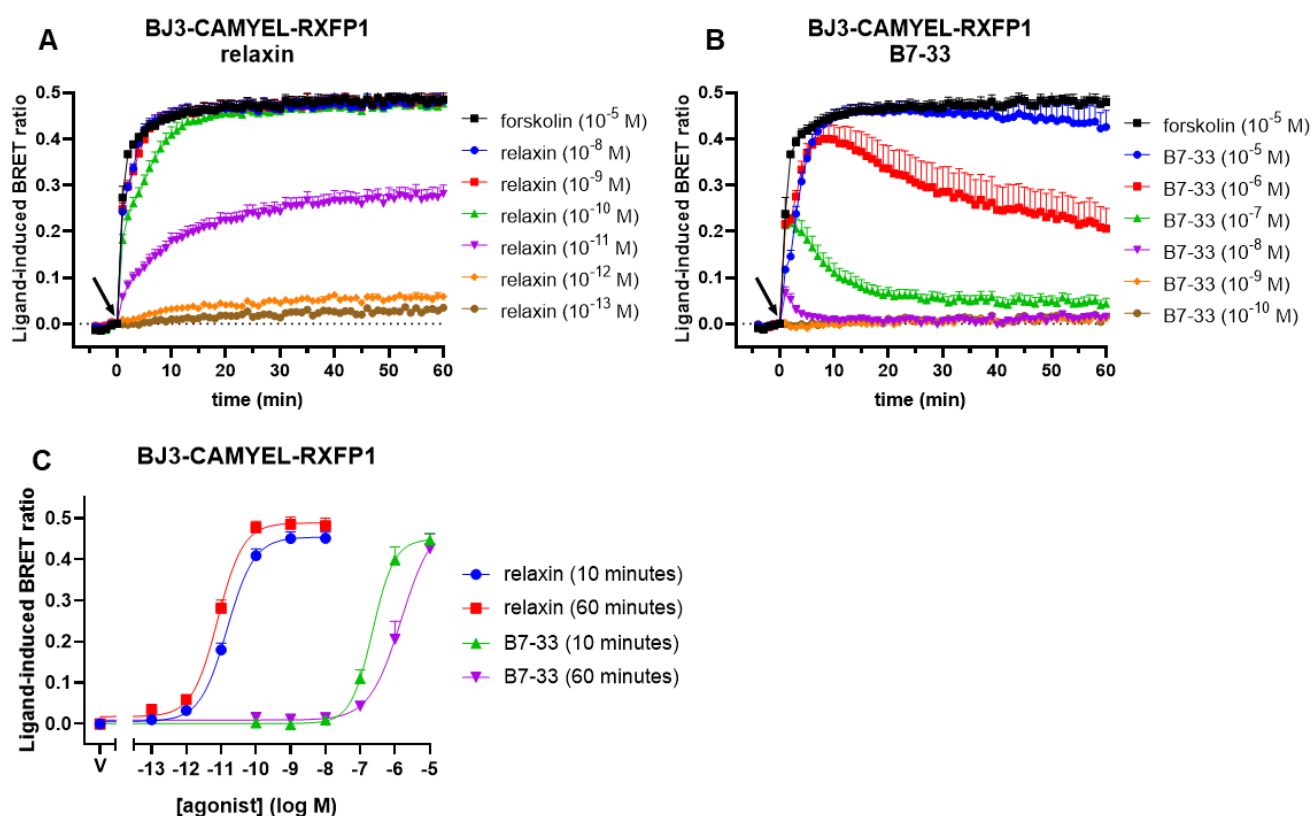


Figure 5.4. Ligand-induced cAMP activity in BJ3-CAMYEL-RXFP1 cells. BJ3 human dermal fibroblasts expressing CAMYEL and higher levels of RXFP1 were stimulated for 60 minutes with forskolin ($10 \mu\text{M}$), and either (A) relaxin or (B) B7-33. (C) Concentration-response curves were generated at 10 and 60 minutes. Time of ligand or vehicle addition is represented by an arrow. Data represent mean and SEM for three independent experiments.

Table 5.1. Potency of RXFP1 agonists in BJ3-CAMYEL-RXFP1 cells. BJ3-CAMYEL-RXFP1 cells were stimulated for 60 minutes with relaxin or B7-33. The potencies (pEC_{50} s) were determined at 10 and 60 minutes. Data represent mean $\pm$ SEM of three independent experiments.

pEC_{50}	Relaxin	B7-33
10 minutes	10.83 ± 0.05	6.65 ± 0.07
60 minutes	11.10 ± 0.05	5.82 ± 0.25

5.3.3 Cyclic nucleotide signalling profiles in vascular cells suggest B7-33 is a biased agonist

The biased or cell-specific actions of B7-33 were investigated further by comparing cyclic nucleotide signalling in vascular cells, specifically in HUVECs, which express RXFP1 and have previously shown cAMP and cGMP accumulation after relaxin stimulation (Sarwar *et al.*, 2015). However, due to difficulties in the ability to repeatedly detect cAMP and cGMP activity in these cells using end point assays after multiple passages, which was likely due to an apparent loss of cell surface RXFP1 expression over several passages (not shown), HUVECs were also transduced with the purified RXFP1 IRES GFP lentivirus and sorted into “low” and “high” populations via FACS based on expression of the GFP, which was described in Chapter 4.

To compare RXFP1 expression levels, a flow cytometry-based fluorescent ligand binding assay using TAMRA-relaxin was conducted using HEK-RXFP1 (**Figure 5.5A**), non-transduced HUVEC (**Figure 5.5B**), HUVEC-RXFP1 (low) (**Figure 5.5C**), and HUVEC-RXFP1 (high) (**Figure 5.5D**) populations. As expected, non-transduced HUVECs showed the lowest level of specific binding (**Figure 5.5E**), whereas the “low” population showed higher specific binding compared to non-transduced cells, the “high” population showed increased specific binding compared to the “low” population, and HEK-RXFP1 cells showed the highest levels of specific binding.

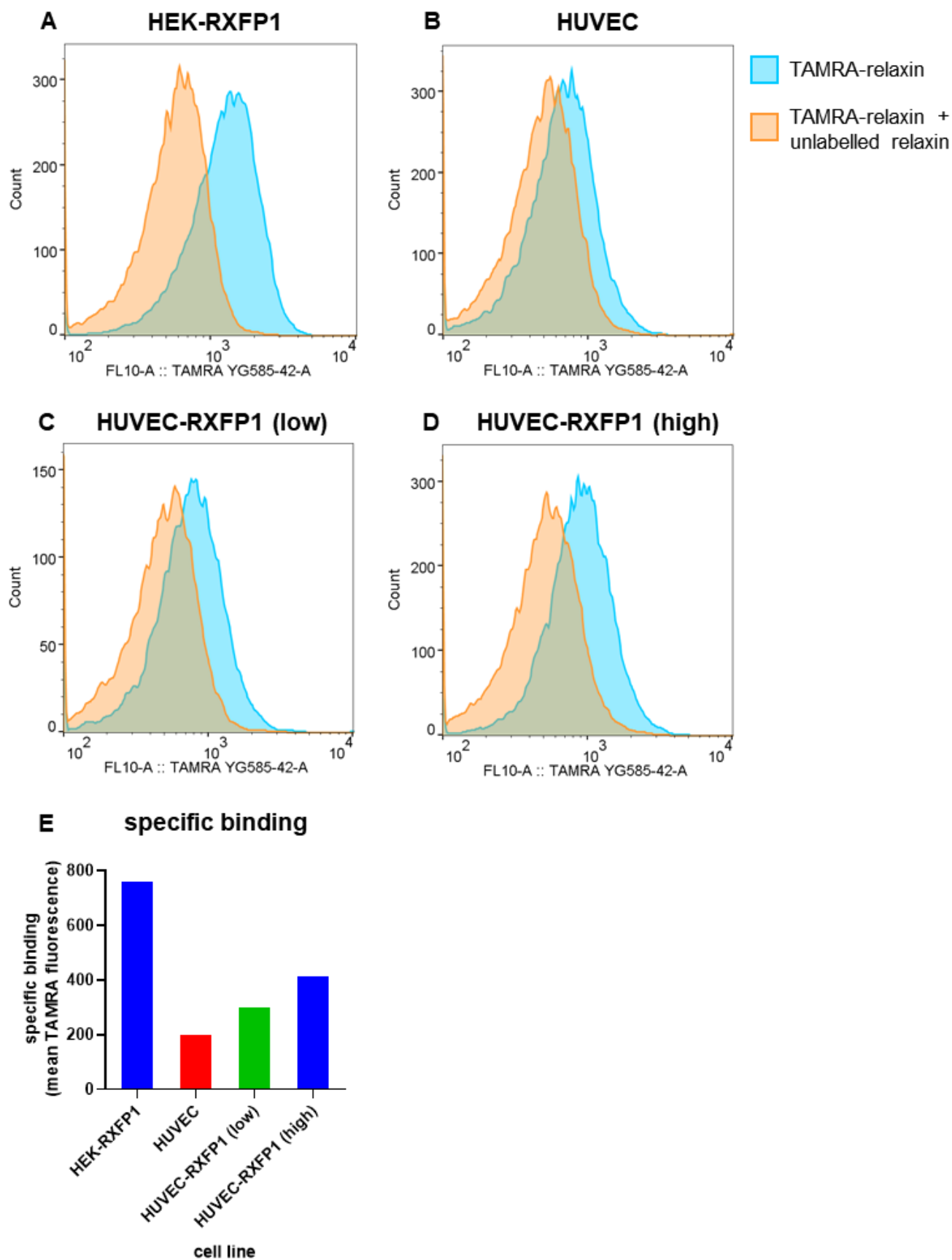


Figure 5.5. Fluorescent ligand binding assay using fluorescent relaxin. A flow cytometry-based ligand binding assay using TAMRA-relaxin was conducted. Single, live cells were first isolated by gating, after which histograms showing mean TAMRA fluorescence (excitation: Y/G 561 nm; emission: 585/42) were generated. TAMRA-relaxin (20 nM) was incubated with (A) HEK-RXFP1, (B) HUVEC, (C) HUVEC-RXFP1 (low), or (D) HUVEC-RXFP1 (high) cells in the absence or presence of an excess of unlabelled relaxin (2 μ M). (E) Specific binding was extracted from flow cytometry histograms by subtracting mean emissions for non-specific binding from total binding. Data represents one flow cytometry assay.

To detect cAMP activity in real time, HUVEC-RXFP1 (low) cells were transduced with purified CAMYEL lentivirus, and were sorted based on the YFP on the sensor (which was separated from GFP the emissions by using a separate filter in addition to applying compensation), thus generating the HUVEC-RXFP1-CAMYEL cell line (**Figure 5.6**).

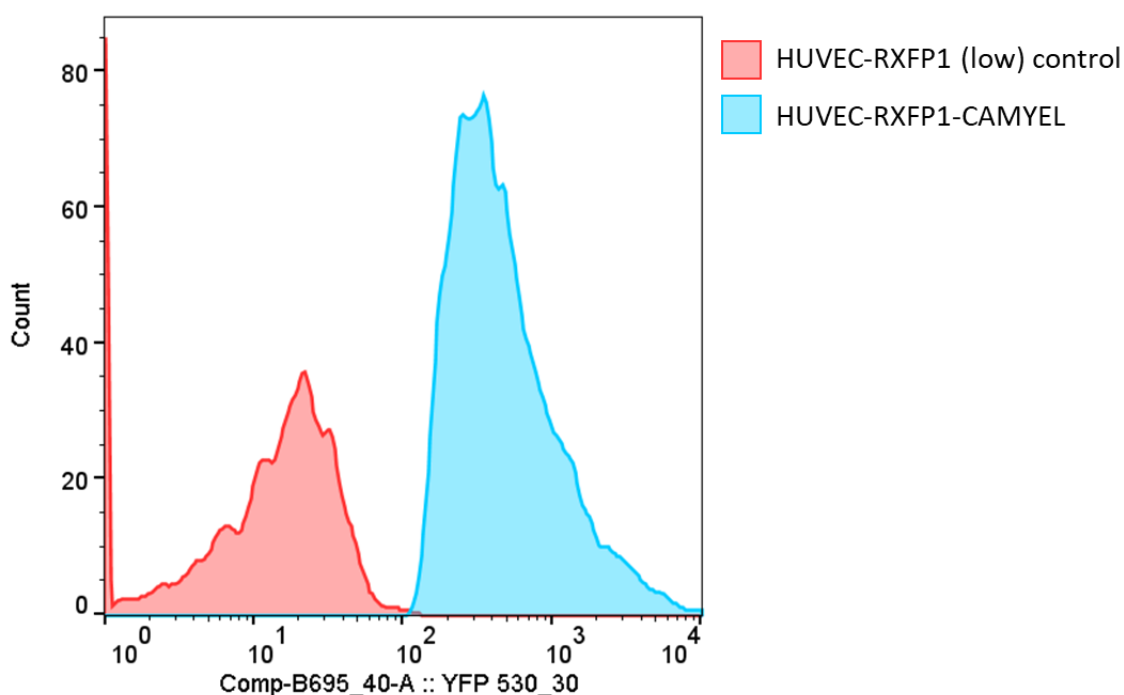


Figure 5.6. Representation of fluorescence-activated cell sorting (FACS) of HUVEC-RXFP1-CAMYEL cells. Human umbilical vein endothelial cells expressing RXFP1 (HUVEC-RXFP1) were transduced with CAMYEL lentivirus and then sorted by FACS to remove non-transduced cells and sort the transduced cells into a separate population. Single, live cells were isolated by gating, and cells that expressed CAMYEL (based on the intrinsic YFP) were sorted (excitation: 488 nm; emission: 530/30 nm). Emissions from cells expressing RXFP1 (which were previously sorted for GFP) were separated from YFP emissions using compensation and a separate emission filter.

Cells were subsequently stimulated with a range of concentrations of relaxin or B7-33, in addition to forskolin (10 μ M) (**Figure 5.7**). Relaxin again induced robust cAMP increases for the duration of the 60-minute time-course, although these were somewhat transient at the maximal concentrations (10-100 nM) compared to the BJ3 cells, peaking at about six minutes before showing a slow decline (**Figure 5.7A**). As with BJ3 cells, B7-33 showed more transient activity than relaxin, which peaked at 10 minutes for the maximal concentration (10 μ M), or earlier for the sub-maximal concentrations, before declining to baseline or near baseline by the end of the time-course (**Figure 5.7B**). Generation of concentration-response curves for relaxin and B7-33 indicated that relaxin was at least 10,000 times more potent for generation of cAMP activity than B7-33 over the time-course (**Table 5.2**).

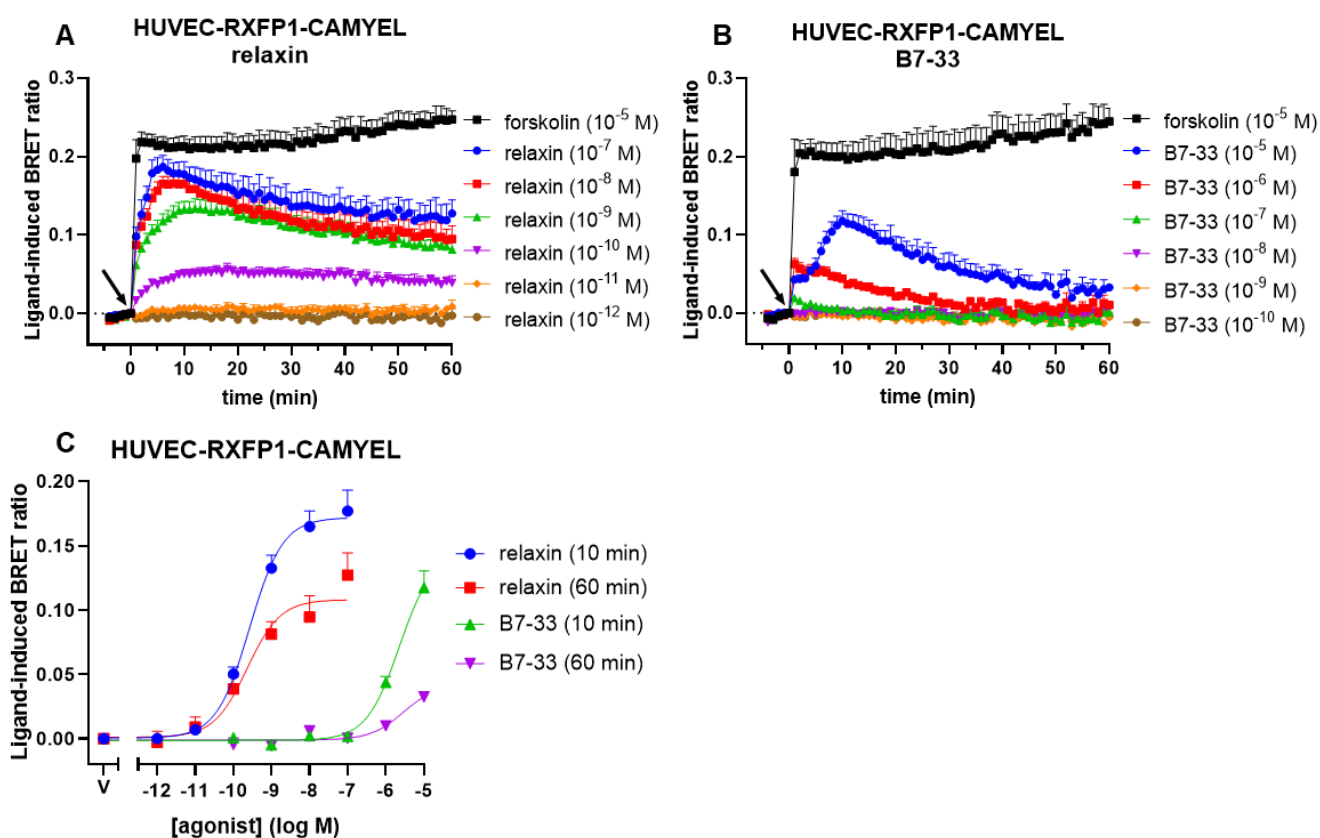


Figure 5.7. Ligand-induced cAMP activity in HUVEC-RXFP1-CAMYEL cells. Human umbilical vein endothelial cells (HUVECs) expressing RXFP1 and CAMYEL were stimulated with forskolin (10 μ M) and either (A) relaxin or (B) B7-33. (C) Concentration-response curves were generated at 10 and 60 minutes. Time of ligand or vehicle addition is represented by an arrow. Data represent mean and SEM for three independent experiments.

Table 5.2. Potency of RXFP1 agonists in HUVEC-RXFP1-CAMYEL cells. HUVEC-RXFP1-CAMYEL cells were stimulated for 60 minutes with relaxin or B7-33. The potencies (pEC₅₀s) were determined at 10 and 60 minutes. Data represent the mean ± SEM of three independent experiments.

	Time point	Relaxin	B7-33
$E_{\max}$ (ligand-induced BRET ratio)	10 minutes	0.17 ± 0.01	0.14 ± 0.01
	60 minutes	0.11 ± 0.01	0.04 ± 0.01
pEC ₅₀	10 minutes	9.58 ± 0.09	5.64 ± 0.10
	60 minutes	9.64 ± 0.21	5.54 ± 0.27

Based on the finding that B7-33 potently mimics the actions of relaxin in the vasculature (Marshall *et al.*, 2017), and as the vascular actions of relaxin depend on NO activity (Lian *et al.*, 2018; McGuane *et al.*, 2011b), which is upstream of cGMP, it could be reasonably expected that B7-33 would potently signal via cGMP in HUVECs. As we did not have an appropriate real-time assay for RXFP1-mediated cGMP activity, end point assays were used to detect cGMP and cAMP accumulation in cell lysates after stimulation and lysis of HUVEC-RXFP1 (high) cells. Cells were stimulated for 40 minutes with relaxin, B7-33, forskolin (10 µM), or the cGMP positive control DEA (10 µM), in the presence of IBMX (500 µM) to stop the breakdown of cyclic nucleotides. Relaxin potently increased cAMP accumulation, whereas B7-33 showed little or no increases in cAMP activity up to 1 µM (**Figure 5.8A**), which was consistent with the CAMYEL assay. Importantly, however, both ligands signalled via cGMP with approximately equal potency and efficacy (**Figure 5.8B**; **Table 5.3**), suggesting that B7-33 is a biased agonist. Incidentally, concentration-response curves for cGMP accumulation were bell-shaped for both ligands.

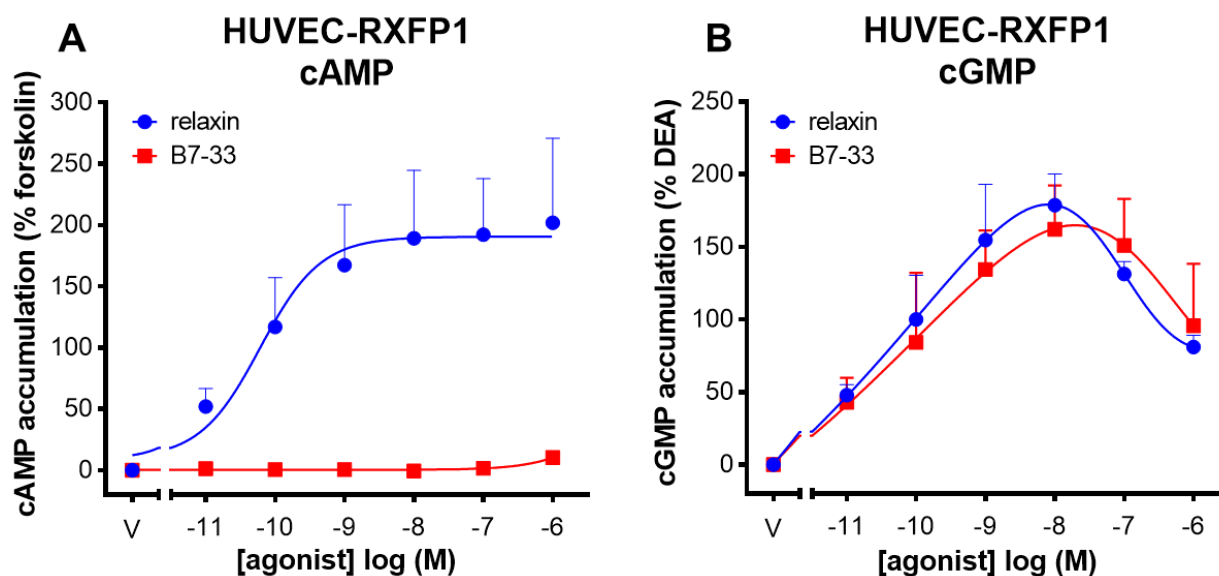


Figure 5.8. Cyclic nucleotide accumulation in HUVEC-RXFP1 cells. Human umbilical vein endothelial cells expressing RXFP1 (HUVEC-RXFP1) were stimulated with relaxin, B7-33, the cAMP positive control forskolin (10 μ M), or the cGMP positive control DEA (10 μ M) for 40 minutes, in the presence of IBMX (500 μ M). (A) cAMP or (B) cGMP accumulation was detected in cell lysates. Data represent the mean and SEM of three independent experiments.

Table 5.3 Potency of RXFP1 agonists for cAMP and cGMP accumulation in HUVEC-RXFP1 cells. HUVEC-RXFP1 (high) cells were stimulated for 40 minutes with relaxin or B7-33 in the presence of IBMX (500 μ M). The potencies (pEC_{50} s) were extracted from the concentration-response curves. Data represent the mean $\pm$ SEM of three independent experiments.

pEC_{50}	Relaxin	B7-33
cAMP	10.22 ± 0.47	-
cGMP	10.15 ± 1.49	10.17 ± 2.88

5.3.4 G protein association profiles confirm biased signalling

At RXFP1, cAMP activity is mediated primarily via $G\alpha_s$ (Halls *et al.*, 2006; Sarwar *et al.*, 2015). In contrast, cGMP activity is mediated via $G\alpha_i$. Specifically, NO, which activates sGC to increase cGMP production, was shown to be increased via a $G\alpha_i$ - $\beta\gamma$ -PI3K-Akt-eNOS-dependent pathway in blood vessels (Lian *et al.*, 2018; McGuane *et al.*, 2011b), and we have recently confirmed that inhibition of $G\alpha_{i/o}$ using PTX in HUVEC-RXFP1 cells blocked relaxin- and B7-33-mediated cGMP activity (unpublished data, Bathgate lab). However, it should be noted that the pathways activated after RXFP1 activation are cell-specific, as, for example, acute RXFP1 activation does not increase cGMP levels in HEK-RXFP1 cells (Kocan *et al.*, 2017). Regardless, it is clear that in HEK-RXFP1 cells, RXFP1 activation still induces signalling via $G\alpha_{i3}$ - $G\beta\gamma$ -PI3K (Halls *et al.*, 2006), which is upstream of NO/cGMP in other cell types. Thus, comparing the relaxin- and B7-33-mediated activation profiles of $G\alpha_s$ and $G\alpha_{i3}$ might allow direct confirmation that B7-33 is a biased agonist that favours coupling of RXFP1 to $G\alpha_i$ over $G\alpha_s$ relative to relaxin, as implied by its cGMP and cAMP signalling profiles.

Although BRET-based G protein association assays have not been sensitive enough to generate concentration-dependent data for RXFP1 (Kocan *et al.*, 2017), it was hoped that a novel NanoBRET G protein association assay would show some improvements in this regard. Therefore, a NanoBRET assay for G protein subunit interaction was conducted for interactions between either $G\alpha_s$ -Nluc or $G\alpha_{i3}$ -Nluc and Venus-G γ_2 , with untagged $G\beta_1$ also transfected. HEK-RXFP1 cells were used due to their ability to be transfected, which was essential as this assay involved transfection with three plasmids, and the $G\alpha_{i3}$ isoform was chosen for the assay as it is the specific isoform involved in RXFP1 signalling in HEK-RXFP1 cells (Halls *et al.*, 2006; Halls *et al.*, 2009).

HEK-RXFP1 cells transiently transfected with $G\alpha_s$ -Nluc, $G\beta_1$, and Venus-G γ_2 were stimulated with a range of concentrations of agonist for 30 minutes. Relaxin and B7-33 both increased $G\alpha_s$ association, but it was clear that the two agonists had distinct kinetic profiles. Specifically, relaxin appeared to induce faster increases in $G\alpha_s$ association that started to plateau by the end of the time-course (**Figure 5.9A**), whereas the highest concentration of B7-33 showed a slower increase in $G\alpha_s$ association than relaxin and sub-maximal concentrations led to a plateau within minutes of stimulation (**Figure 5.9B**). Attempts were made to quantify the kinetics of G protein activation using established curve-fitting methods, as has been done previously using this assay for the GLP-1 receptor in cell membranes for example (Zhao *et al.*, 2020), and attempts were also made to quantify initial rates of signalling as a measure of efficacy, according to a published method (Hoare *et al.*, 2020). However, due to poor curve fits for the B7-33 time-course, we were

unable to use this approach for quantify and compare the rates of G protein activation here, although a one-phase association curve did fit the relaxin time-course well. Potencies were generated by taking the area under the curve. Relaxin was substantially more potent than B7-33 for activation of $G\alpha_s$, consistent with the cAMP activity seen in all cell types tested here and elsewhere (Hossain *et al.*, 2016) (**Table 5.4**).

Next, HEK-RXFP1 cells transiently transfected with $G\alpha_{i3}$ -Nluc, $G\beta_1$, and Venus- $G\gamma_2$ were stimulated with a range of concentrations of agonist for 30 minutes. Both agonists induced concentration-dependent increases in $G\alpha_{i3}$ association for the duration of the time-course (**Figure 5.9C-D**). Interestingly, maximal or near-maximal concentrations of relaxin (10 nM) and B7-33 (100 nM) showed a kind of biphasic increase in $G\alpha_{i3}$ association whereby there were initial fast increases within five minutes after ligand addition, followed by either plateau (relaxin) or slower increases (B7-33) thereafter. Notably, despite small differences, the kinetics of $G\alpha_{i3}$ association were also much more similar between the two agonists compared to $G\alpha_s$. For example, responses to 1 nM of agonist were near-identical between the two agonists for $G\alpha_{i3}$ activation. Again, rates of G protein activation were difficult to quantify, as the time-courses from the two agonists did not resemble typical signalling profiles and we could not accurately fit curves to these data. However, both agonists increased $G\alpha_{i3}$ activation with approximately equal potency (**Table 5.4**), thus confirming B7-33 as a biased agonist that favours activation of $G\alpha_{i3}$ /cGMP over $G\alpha_s$ /cAMP. Despite the initial appearance of a bell-shaped curve for relaxin due to the decreased response at 100 nM, only a sigmoidal curve was able to be fit to the data.

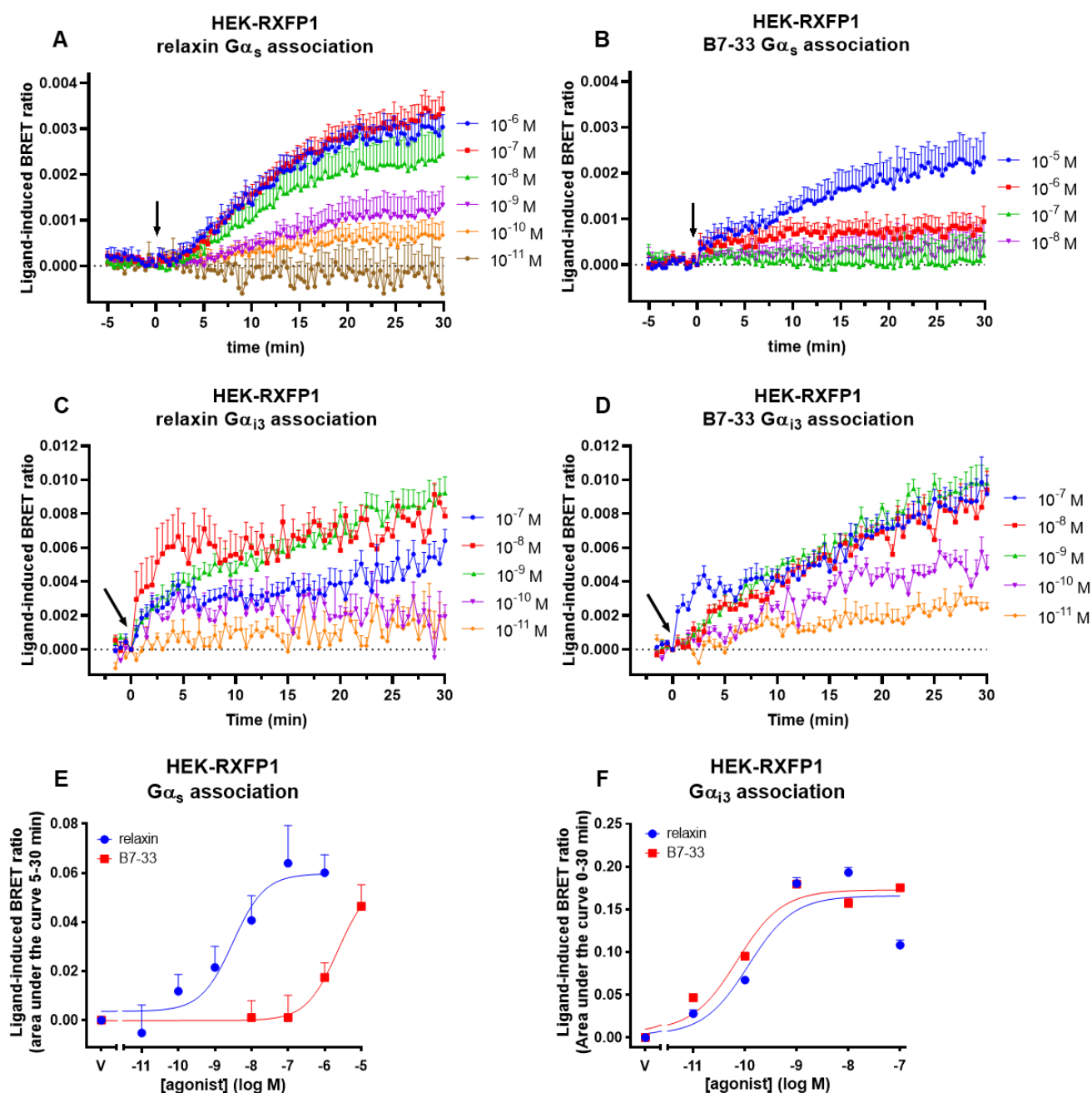


Figure 5.9. G protein association in HEK-RXFP1 cells. HEK-RXFP1 cells transiently transfected with $G\alpha_s$ -Nluc, $G\beta_1$, and Venus- $G\gamma_2$ were stimulated for 30 minutes with vehicle and (A) relaxin or (B) B7-33. HEK-RXFP1 cells transiently transfected with $G\alpha_{i3}$ -Nluc, $G\beta_1$, and Venus- $G\gamma_2$ were also stimulated for 30 minutes with vehicle and (C) relaxin or (D) B7-33. Concentration-response curves were generated for (E) $G\alpha_s$ and (F) $G\alpha_{i3}$ association by taking the area under the curve of the time-courses. Time of ligand or vehicle addition is represented by an arrow. Data represent the mean and SEM of at least three independent experiments.

Table 5.4. Potency of RXFP1 agonists for G protein association in HEK-RXFP1 cells.

HEK-RXFP1 transiently transfected with $G\alpha_s$ -Nluc or $G\alpha_{i3}$ -Nluc in addition to $G\beta_1$ and Venus- $G\gamma_2$ were stimulated for 30 minutes with relaxin or B7-33. Concentration-response curves were generated by taking the area under the curve from time-course data. Data represent the mean and SEM of at least three independent experiments.

pEC ₅₀	Relaxin	B7-33
$G\alpha_s$	8.51 ± 0.31	5.64 ± 0.38
$G\alpha_{i3}$	9.93 ± 0.17	10.15 ± 0.09

5.3.5 ERK1/2 activation after relaxin stimulation is cell-specific

Previously, pharmacological inhibition of $G\alpha_{i/o}$ using PTX demonstrated that relaxin-induced ERK1/2 phosphorylation is primarily mediated via $G\alpha_{i/o}$ in some human vascular cells, human cardiac fibroblasts (Sarwar *et al.*, 2015), and rat Sertoli cells (Nascimento *et al.*, 2012). Additionally, as noted above, B7-33 activates ERK1/2 weakly in HEK-RXFP1 cells, but potently in fibroblasts (Hossain *et al.*, 2016). However, if B7-33 is potent for $G\alpha_{i3}$ association in HEK-RXFP1 cells, and ERK1/2 phosphorylation is downstream of $G\alpha_i$ activation, this appears inconsistent with the ability of B7-33 to only weakly activate ERK1/2 in HEK-RXFP1 cells. We therefore questioned whether RXFP1-mediated ERK1/2 phosphorylation might occur at different locations in the signal transduction cascade depending on cell background.

The Rluc8-ERKsubstrate-Venus (REV) BRET-based biosensors for cytosolic (REV_{Cyto}) and nuclear (REV_{Nuc}) ERK1/2 activity (Xu *et al.*, 2013) were used to further investigate ERK1/2 activity downstream of RXFP1 in HEK-RXFP1 cells. REV is based on the FRET-based extracellular signal-regulated kinase activity reporter (EKAR) (Harvey *et al.*, 2008), and consists of a substrate peptide for phosphorylation by ERK1/2, a phospho-binding domain, a docking site specific for ERK1/2, and a flexible linker that separates the substrate peptide from the phospho-binding domain, all sandwiched by the Rluc8/Venus BRET pair (Xu *et al.*, 2013). In its basal state, REV adopts an “open” conformation, but when ERK1/2 is activated it phosphorylates the substrate peptide, leading to a conformational change in the sensor that leads to binding of the substrate peptide and phospho-binding domain, thus inducing a “closed” conformation that brings Rluc8 and Venus into close enough proximity for BRET to occur (Xu *et al.*, 2013).

To determine which G proteins are involved in the ERK1/2 response downstream of RXFP1 in HEK-RXFP1 cells, HEK-RXFP1 cells stably expressing cytosolic or nuclear REV (HEK-RXFP1-REV_{Cyto} and HEK-RXFP1-REV_{Nuc}) were stimulated with relaxin with or without pre-treatment with the G $\alpha_{i/o}$ inhibitor PTX (100 ng/ml; at least 16 hours) (**Figure 5.10**). Without PTX treatment, relaxin induced increases in cytosolic ERK1/2 activity that peaked at six minutes after stimulation (**Figure 5.10A**) and nuclear ERK1/2 activity that peaked at seven minutes after stimulation (**Figure 5.10B**) before declining to baseline or near baseline. Interestingly, G $\alpha_{i/o}$ inhibition did not affect either cytosolic or nuclear ERK1/2 activity after relaxin stimulation in these cells. Specifically, whereas the FBS time-courses had a different shape after PTX pre-treatment, the relaxin time-courses appeared the same (**Figure 5.10C-D**), peaking at approximately the same time points and the same percentage of the maximum FBS response ($p > 0.05$ in unpaired t -tests for peak responses for treated versus untreated cells) (**Table 5.5**). Thus, we have shown for the first time that the ERK1/2 response after RXFP1 activation is activated by its upstream effectors in a cell-specific manner. This is thus consistent with the ability of B7-33 to potently increase G α_{i3} association but only weakly increase ERK1/2 activity in HEK-RXFP1 cells. Due to a lack of sensitivity of the assay, however, B7-33 stimulation did not produce a detectable ERK1/2 response in this assay (not shown), although this is again consistent with its very low efficacy for activation of ERK1/2 compared to relaxin seen in the end point assay (Hossain *et al.*, 2016).

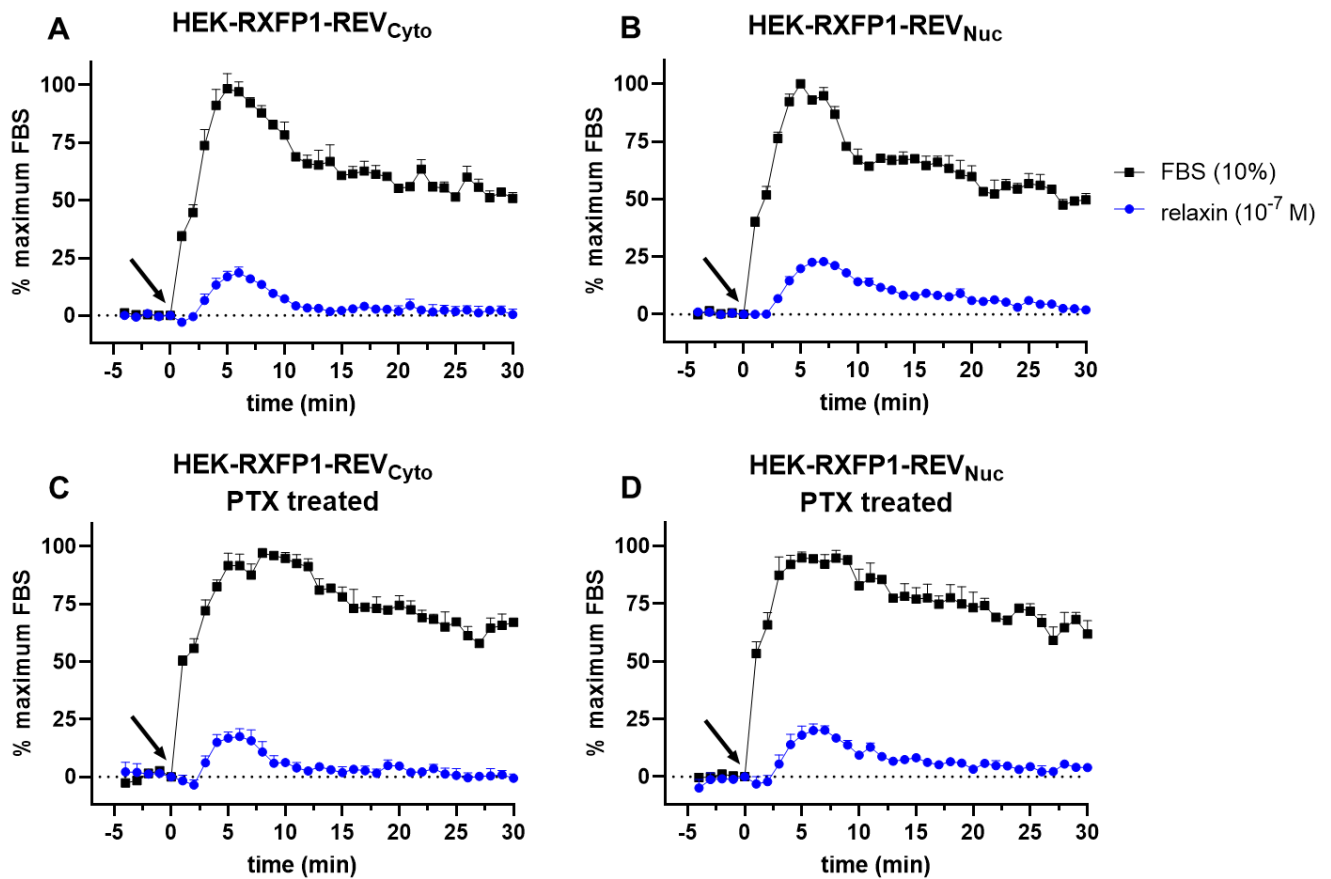


Figure 5.10. Relaxin-induced ERK1/2 activity in HEK293T cells expressing RXFP1 and ERK biosensors. HEK293T cells stably expressing RXFP1 and either (A, C) cytosolic or (B, D) nuclear Rluc8-ERKs substrate-Venus (REV_{Cyto} or REV_{Nuc}) ERK1/2 biosensors were stimulated with relaxin (100 nM) and the positive control fetal bovine serum (FBS; 10%) for 30 minutes. For (C, D), this was done after pre-treatment with the $G\alpha_{i/o}$ inhibitor pertussis toxin (PTX; 100 ng/ml; at least 16 hours). Responses are normalised as a percentage of the maximum FBS response. Data represent mean and SEM of three independent experiments.

Table 5.5 Relaxin-induced ERK1/2 activity in HEK293T cells expressing RXFP1 and ERK biosensors. HEK293T cells stably expressing RXFP1 and either the cytosolic or nuclear Rluc8-ERKsubstrate-Venus biosensor were stimulated with relaxin (100 nM) or the positive control fetal bovine serum (FBS; 10%), either with or without pre-treatment with the $G\alpha_{i/o}$ inhibitor pertussis toxin (PTX; 100 ng/ml; at least 16 hours). Maximum relaxin response as a percentage of maximum FBS response and time point of maximum response are listed. Data represent mean $\pm$ SEM of three independent experiments. Unpaired *t*-tests showed no statistically significant differences between peak responses for treated versus untreated cells.

	Pertussis toxin	
	–	+
% maximum FBS (time)		
Cytosolic ERK1/2	18.56 $\pm$ 2.47 (6 min)	17.51 $\pm$ 3.34 (6 min)
Nuclear ERK1/2	22.96 $\pm$ 1.17 (7 min)	20.20 $\pm$ 1.87 (7 min)

5.3.6 Transient cAMP activity may be related to dissociation kinetics

Previously, competition binding assays in HEK-RXFP1 cells showed that B7-33 bound to RXFP1 with substantially lower affinity than relaxin did (B7-33 pK_i : 5.54 $\pm$ 0.13; relaxin pK_i : 8.96 $\pm$ 0.03) (Hossain *et al.*, 2016). Similarly, B7-33 has also shown low affinity for binding to RXFP1 in BJ3-RXFP1 and HUVEC-RXFP1 cells (unpublished data, Bathgate lab), whereas relaxin binds to RXFP1 with high affinity in every cell type tested. The weak binding affinity of B7-33 is consistent with the slow $G\alpha_s$ association, transient cAMP activity, and weak potencies for these outputs observed here. We thus wondered whether there is a link between the binding kinetics of B7-33 and its weak activation of $G\alpha_s$. Specifically, it was hypothesised that B7-33 dissociates from RXFP1 faster than relaxin does, which in turn leads to less favourable activation of $G\alpha_s$ compared to $G\alpha_{i3}$. As the affinity of B7-33 is too low to conduct kinetic binding experiments with labelled ligand in order to determine whether B7-33 rapidly dissociates, a CAMYEL assay was instead used to examine duration of cAMP signalling after removal of unbound ligand. First, however, as only end point cAMP assays had been conducted for B7-33 in HEK-RXFP1 cells so far, HEK-RXFP1 cells transiently expressing CAMYEL were stimulated with a range of concentrations of B7-33, as well as forskolin (10 μ M), in order to determine an approximately EC_{80} concentration of B7-33 (**Figure 5.11**). In HEK-RXFP1 cells, B7-33 induced sustained signalling only at higher concentrations (3.2, 10 μ M), and the potency of B7-33 decreased over the duration of the time-course (**Table 5.6**).

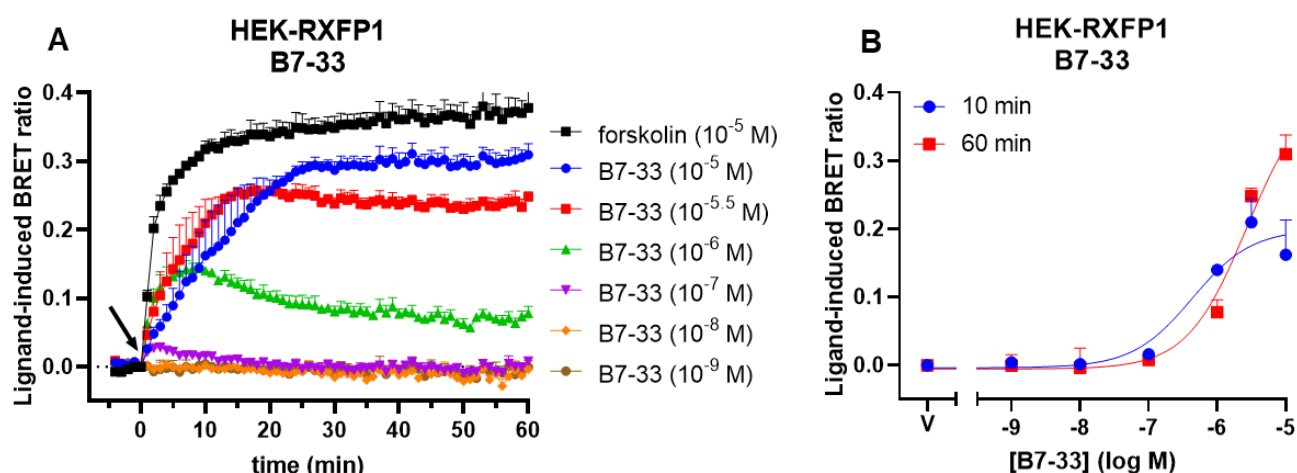


Figure 5.11. B7-33-mediated cAMP activity in HEK-RXFP1 cells. (A) HEK-RXFP1 cells transiently expressing CAMYEL were stimulated with vehicle, forskolin (10 μ M), or a range of concentrations of B7-33 for 60 minutes. (B) Concentration-response curves were generated at 10 and 60 minutes. Time of ligand or vehicle addition is represented by an arrow. Data represent the mean and SEM of three independent experiments.

Table 5.6. Potency of B7-33 in HEK-RXFP1 cells. HEK-RXFP1 cells transiently expressing CAMYEL were stimulated with B7-33 for 60 minutes. The potencies (pEC_{50} s) were determined at 10 and 60 minutes. Data represent the mean $\pm$ SEM of three independent experiments.

pEC_{50}	10 min	60 min
B7-33	6.36 ± 0.28	5.57 ± 0.10

To assess the duration of signalling for relaxin and B7-33 after removal of unbound ligand, HEK-RXFP1 cells transiently expressing CAMYEL were stimulated with relaxin, B7-33 (3.2 μ M), or forskolin (10 μ M). Several concentrations of relaxin were used to ensure that a concentration of equivalent efficacy and receptor occupancy to that of B7-33 was included. cAMP activity was recorded for 25 minutes before the assay was paused (for approximately 2-3 minutes), at which time cells were either left alone, or cell culture media was removed and replaced with fresh media (still containing coelenterazine *h*). cAMP responses were then monitored for a further 35 minutes. As before, relaxin responses were relatively sustained for the duration of the time-course, regardless of which concentration of relaxin was used (**Figure 5.12A**), and showed only a small and slow decline in cAMP activity after removal of media containing unbound ligand (**Figure 5.12B**). In contrast, whereas the B7-33 response was sustained when media was not replaced (**Figure 5.12C**),

removal of unbound ligand led to a precipitous drop in the B7-33 response, which returned approximately to baseline (**Figure 5.12D**). A likely explanation for these findings is that relaxin shows slow dissociation kinetics and a long ligand residence time that contributes to a sustained cAMP response after removal of unbound ligand because active, ligand-bound receptors are still present, whereas B7-33 shows fast dissociation kinetics that contributes to a more transient response as few active, ligand-bound receptors remain after removal of media containing ligand. This finding therefore supports the idea that the slow $G\alpha_s$ activation and transient cAMP activity may be related to the fast dissociation kinetics of B7-33.

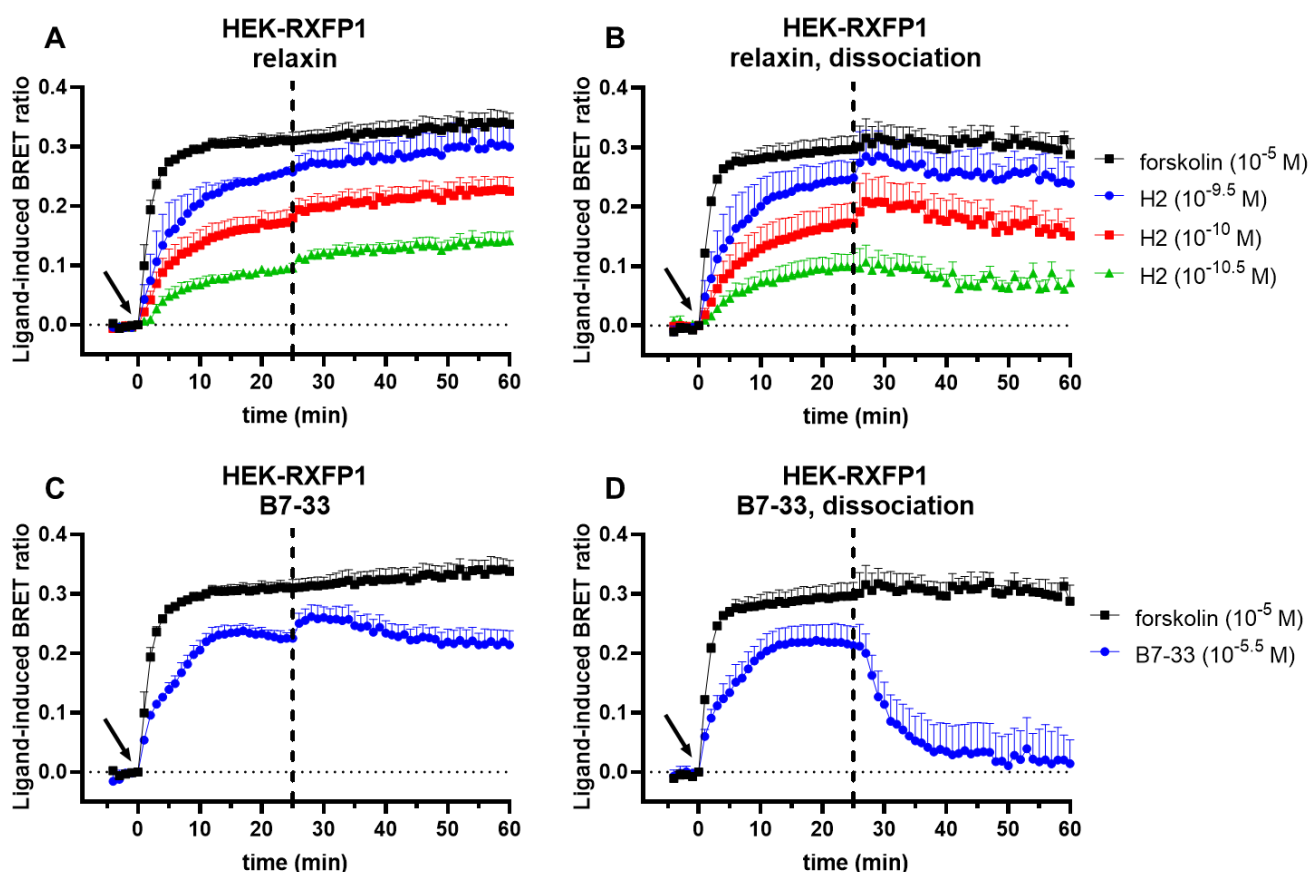


Figure 5.12. cAMP activity at RXFP1 after removal of unbound ligand. HEK-RXFP1 cells transiently expressing CAMYEL were stimulated with forskolin (10 μ M), relaxin, or B7-33 for 25 minutes, before the assay was paused for approximately 2-3 minutes (vertical dotted line). At this time, either (A, C) cells were left alone, or (B, D) unbound ligand was removed by removal of cell culture media and replacement with fresh media. The assay was then continued for another 35 minutes. Time of vehicle or ligand addition is represented by an arrow. Data represent the mean and SEM of three independent experiments.

5.4 Discussion

One of several issues hindering the advancement of RXFP1 as a therapeutic target is our limited understanding of the molecular mechanisms that underlie the biological actions of relaxin and newly-developed peptide and small molecule agonists targeting RXFP1. According to previous work, the single-chain relaxin analogue B7-33 appeared to show cell-specific signalling and biological responses, whereby it had weak activity in recombinant and cancer cells, but potent activity in fibroblasts and vascular cells, as well as potent activity *in vivo* (Hossain *et al.*, 2016; Marshall *et al.*, 2017). Here, we aimed to understand the molecular mechanisms underlying these biological effects. To do this, real-time assays for cAMP, ERK1/2, and G protein association, in addition to end point assays for cAMP and cGMP, were implemented across several cell types including primary cells and cell lines. The results suggested, first, that B7-33 is a biased agonist that favours signalling via $G\alpha_{i3}$ over $G\alpha_s$, relative to relaxin; second, that the biased agonism may be related to binding kinetics; and finally, that RXFP1 signals via ERK1/2 in a cell-specific manner.

As RXFP1 is a proposed target for the treatment of fibrosis and cardiovascular diseases, the signalling patterns of relaxin and B7-33 were first compared in fibroblasts and vascular cells. Stimulation of BJ3 human dermal fibroblasts expressing CAMYEL with relaxin did not lead to any detectable increases in cAMP activity, consistent with most other types of fibroblasts that have been tested for cAMP activity after relaxin stimulation (Huang *et al.*, 2011; Mookerjee *et al.*, 2009; Palejwala *et al.*, 1998; Samuel *et al.*, 2004b; Sarwar *et al.*, 2015). Notably, this was despite cell surface expression of RXFP1 and despite endogenous β -adrenoceptors producing a robust cAMP response after isoprenaline addition which demonstrated that other GPCRs can signal via cAMP in these cells. BJ3 cells transduced with additional RXFP1, however, signalled robustly via cAMP after stimulation with either relaxin or B7-33, but with two key differences between the two ligands. First, the cAMP response from all concentrations of relaxin was sustained for the duration the time-course, whereas the B7-33 response was transient at sub-maximal concentrations. Second, relaxin signalled via cAMP with a potency at least three orders of magnitude higher than that of B7-33. Given that that B7-33 has potent anti-fibrotic actions in fibroblasts and that the anti-fibrotic actions of relaxin are cGMP-dependent, these results hinted that B7-33 might be biased in favour of cGMP over cAMP.

To further investigate, HUVECs were transduced with additional RXFP1 and sorted into “low” and “high” populations (see Chapter 4). The “low” RXFP1 population was transduced with CAMYEL for real-time cAMP assays. Consistent with the BJ3 cell assays, relaxin and B7-33 both signalled via cAMP, but B7-33 had a substantially lower potency and a more transient response

than relaxin. Due to the unavailability of a real-time assay for RXFP1-mediated cGMP activity (see Chapter 4), end point assays for both cAMP and cGMP were conducted in the “high” RXFP1 population to directly compare potencies. These assays suggested that B7-33 is a biased agonist, as it increased cAMP accumulation with substantially lower potency than relaxin, yet was approximately equipotent to relaxin at inducing accumulation of cGMP. Although these assays and the BJ3 assays involved transduction of cells with higher levels of RXFP1, as our primary goal was to compare potencies of the two ligands we did not believe that the receptor expression level was detrimental to detection of bias. This was confirmed by recent unpublished end point assay data from the laboratory, which demonstrated that HUVECs without additional RXFP1 expression still signal via cAMP and cGMP after relaxin or B7-33 stimulation with comparable potencies to those presented here, but efficacy was low and the response was difficult to detect, particularly after additional passages. Similarly, relaxin and B7-33 were both shown to signal via cGMP with equal potency in BJ3 cells (unpublished data, Bathgate lab). Interestingly, additional RXFP1 expression was only required to detect cAMP activity, not cGMP activity, in BJ3 cells.

The above data provides evidence that B7-33 is biased in favour of cGMP activation relative to relaxin. Several studies have shown that NO/cGMP activity downstream of RXFP1 is dependent on $G\alpha_i$. Specifically, NO is increased via a $G\alpha_i$ - $\beta\gamma$ -PI3K-Akt-eNOS-dependent pathway in blood vessels (Lian *et al.*, 2018; McGuane *et al.*, 2011b), and pharmacological inhibition of $G\alpha_{i/o}$ using PTX also blocked cGMP activity in HUVEC-RXFP1 cells (unpublished data, Bathgate lab), confirming the involvement of $G\alpha_{i/o}$ proteins in the same cells that were used for cGMP assays here. These findings, in addition to the understanding that RXFP1-mediated cAMP activity occurs primarily via $G\alpha_s$ (Halls *et al.*, 2006; Sarwar *et al.*, 2015), suggest that B7-33 is strongly linked to $G\alpha_i$ activation, but weakly linked to $G\alpha_s$ activation. Proximal assays at the level of G protein or effector association can show this directly and give unambiguous evidence of biased activation of effectors. NanoBRET G protein association assays showed concentration-dependent increases in proximity between Nluc-tagged $G\alpha_s$ or $G\alpha_{i3}$ subunits and Venus-tagged $G\gamma_2$ subunits after addition of either agonist, suggestive of association of G protein heterotrimers and recruitment to RXFP1. Consistent with the cAMP assays, B7-33 induced association of $G\alpha_s$ heterotrimers with a substantially lower potency than relaxin did, and notably, B7-33 induced the activation of $G\alpha_s$ at a slower rate than relaxin. In contrast to $G\alpha_s$, B7-33 increased $G\alpha_{i3}$ association with a much more similar kinetic profile to relaxin. Another key finding was that relaxin and B7-33 increased $G\alpha_{i3}$ association with approximately equal potency, thus confirming that B7-33 is a biased agonist relative to relaxin, favouring the activation of $G\alpha_{i3}$ over $G\alpha_s$. Additionally, as with the sustained cAMP activity observed here and elsewhere, the sustained G protein activation is consistent with the finding that RXFP1 does not

couple to β -arrestin and shows weak or no relaxin-mediated desensitisation and internalisation (Callander *et al.*, 2009; Kern & Bryant-Greenwood, 2009). However, given that B7-33 also showed sustained increases in $G\alpha_s$ despite its transient cAMP activity, the sustained responses seen in this assay may be at least partly related to the overexpression of G proteins in the transfected cells, rather than simply being due to the lack of RXFP1 desensitisation. Incidentally, the concentration-response curves for $G\alpha_{i3}$ association and cGMP activity were bell-shaped, in contrast to the typical sigmoidal curves seen for $G\alpha_s$ association and cAMP activity. This is consistent with previous studies, including research using HUVECs that demonstrated bell-shaped cGMP accumulation curves for relaxin (Sarwar *et al.*, 2015). Bell-shaped concentration-response curves are thought to be related to localisation of $G\alpha_{i/o}$ proteins into membrane rafts (Sarwar *et al.*, 2015).

The transient cAMP activity observed for B7-33 is a new finding, as until now cAMP activity had only been detected for B7-33 using end point assays that included PDE inhibitors to stop the breakdown of cAMP, and thus the kinetics of signalling were not detected. However, it should be noted that these “transient” B7-33 cAMP responses observed using the CAMYEL assay were still more prolonged than cAMP activity mediated via β -arrestin-coupled receptors. For example, in HEK-CAMYEL cells (Chapter 4) and BJ3-CAMYEL cells, even maximal concentrations of isoprenaline, which is an agonist for the β -arrestin-coupled β -adrenoceptors, led to rapid decreases in cAMP activity (due to desensitisation and internalisation), in contrast to the B7-33 responses which were sustained at higher concentrations and decayed much more slowly than isoprenaline at lower concentrations. Thus, the lack of RXFP1 desensitisation does still appear to be important for the B7-33 responses, but these findings also suggest that the lack of desensitisation may only partially explain the sustained cAMP activity seen for relaxin. That is, other factors such as binding kinetics and ligand residence time may also be important for prolonging the cAMP response. Real-time cAMP assays involving the removal of unbound ligand after an initial stimulation were therefore conducted to compare the loss of the signalling response for relaxin and B7-33 to correlate with ligand off-rates. Several concentrations of relaxin were used to ensure that a concentration of approximately equal receptor occupancy and efficacy to that of B7-33 was included, enabling direct comparison of the loss of signal between the two ligands. Importantly, the cAMP responses from the two ligands after removal of unbound ligand were easily distinguished. Specifically, the relaxin response showed a slow and partial decline in activity, regardless of the concentration of relaxin, whereas B7-33 showed an abrupt drop in cAMP activity after removal of unbound ligand. The most likely explanation for this is that relaxin has a long residence time (in addition to the lack of RXFP1 desensitisation) that continues to drive signalling after removal of unbound ligand, whereas B7-33 likely dissociates faster than relaxin, leading to termination of cAMP activity once unbound ligand

is removed. In fact, kinetic NanoBRET binding assays have previously been conducted, demonstrating that TAMRA-relaxin had an off-rate (k_{off}) of $0.054 \pm 0.009 \text{ min}^{-1}$ at Nluc-RXFP1 (Hoare *et al.*, 2019), suggesting persistent interactions between relaxin and RXFP1 consistent with sustained signalling. As noted, however, the affinity of B7-33 was too low to be used in this kind of assay, but the above data suggests that the k_{off} for B7-33 would be considerably faster than that observed for relaxin.

These differences in off-rates between relaxin and B7-33 may in fact be important for driving the biased signalling observed here. The most common explanation for biased signalling relates to the ability of different ligands to stabilise unique conformations of the GPCR, shifting the equilibrium in favour of these conformations at the expense of others, which subsequently favours the activation of certain effectors (Kenakin, 2002). However, it is becoming clear that this explanation does not provide the full picture, and other factors such as the kinetics of binding and signalling are becoming increasingly recognised as important for bias (Lane *et al.*, 2017). For example, the prolonged ligand residence time of LSD at the 5-HT_{2B} receptor was shown to be associated with bias towards recruitment of β -arrestin2 (Wacker *et al.*, 2017). Similarly, our results demonstrate an association between shorter ligand residence time and bias away from $G\alpha_s$, which does not exclude conformational selection but suggests that the duration of the conformational change may be an important factor that drives biased signalling. Specifically, we hypothesise that the relaxin/RXFP1 complex catalyses more cycles of $G\alpha_s$ activation (GDP-GTP exchange) due to the sustained duration of the active receptor conformation, whereas for B7-33 the rate of $G\alpha_s$ activation is slower due to unbinding of B7-33 that leads to a shorter duration of active receptor. Interestingly, however, B7-33 remained potent for activation of $G\alpha_{i3}$, which suggests that the relative rates of nucleotide exchange and/or GTPase activity intrinsic to $G\alpha_s$ versus $G\alpha_{i3}$ may also be important. In fact, these data are remarkably similar to recent observations at the GLP-1 receptor, where GLP-1 and the biased agonist TT-OAD2 activated $G\alpha_{i2}$ at approximately the same rate, but GLP-1 activated $G\alpha_s$ at a much faster rate than TT-OAD2 (Zhao *et al.*, 2020). This slower activation of $G\alpha_s$ after stimulation with TT-OAD2 was also consistent with slower increases in cAMP activity (Zhao *et al.*, 2020). Further studies should clarify the link between ligand residence time and bias towards the activation of specific GPCR effectors.

The above data left one apparent inconsistency regarding our understanding of how ERK1/2 is activated by relaxin/RXFP1. Previous studies have demonstrated that $G\alpha_{i/o}$ has a large role in mediating the ERK1/2 phosphorylation that occurs after relaxin stimulation in some types of native cells (Nascimento *et al.*, 2012; Sarwar *et al.*, 2015). Additionally, B7-33 increases ERK1/2 phosphorylation potently in rat renal myofibroblasts, but with only a low potency in HEK-RXFP1

cells (Hossain *et al.*, 2016). However, the weak ERK1/2 phosphorylation in HEK-RXFP1 cells appears to be inconsistent with the potent $G\alpha_{i3}$ activation in these cells. We therefore investigated whether ERK1/2 activation occurs at different locations of the signal transduction cascade depending on cell type, by using the REV BRET-based biosensors for cytosolic and nuclear ERK1/2 activity stably expressed in HEK-RXFP1 cells. These assays showed that relaxin induced activation of ERK1/2 that peaked at 6-7 minutes after stimulation, but unlike in native cells, pharmacological inhibition of $G\alpha_{i/o}$ using PTX did *not* disrupt relaxin-mediated ERK1/2 activity in HEK-RXFP1 cells, thus suggesting that in these cells the ERK1/2 activity is *not* downstream of $G\alpha_{i3}$. Thus, ERK1/2 is suggested for the first time to be activated downstream of RXFP1 in a cell-specific manner. In general, ERK1/2 phosphorylation can occur at various locations throughout the signal transduction cascade, including downstream of $G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_q$, $G\alpha_{12}$, $G\beta\gamma$, or β -arrestin (Jain *et al.*, 2018). Based on our results, however, as there is no evidence that RXFP1 couples to $G\alpha_q$, $G\alpha_{12}$, or β -arrestin, and as the ERK1/2 activity was not downstream of $G\alpha_{i/o}$, it is almost certain that RXFP1-mediated ERK1/2 phosphorylation in HEK-RXFP1 cells is $G\alpha_s$ -dependent. cAMP, for example, can modulate ERK activity via activation of PKA (Jain *et al.*, 2018). Given that B7-33 has a much weaker potency for activation of $G\alpha_s$ and cAMP than relaxin, this would explain why B7-33 also has a very weak potency for ERK1/2 phosphorylation in HEK-RXFP1 cells (Hossain *et al.*, 2016), whereas B7-33 being potent for $G\alpha_{i3}$ activation is consistent with the potent ERK1/2 activation in fibroblasts being downstream of $G\alpha_{i3}$. Relatedly, other recent data has demonstrated that a potent relaxin-mediated ERK1/2 response in HUVEC-RXFP1 cells is only partially inhibited by PTX, whereas the weak B7-33 response is completely inhibited by PTX, until 1 μ M B7-33 is added at which point the response appears to occur via a different G protein (unpublished data, Bathgate lab), which is consistent with $G\alpha_s$ -mediated activity occurring once a high enough concentration of B7-33 is added. This finding also stresses the importance of using physiologically-relevant cell types in detection of signalling and bias, as cell-specific signalling will affect all agonists acting at the receptor in that cell type and must be distinguished from biased signalling for the purposes of drug targeting.

The signalling data described above finally explains the mechanisms underlying the *in vitro* and *in vivo* actions of B7-33. But what is the relevance of biased signalling at RXFP1? The beneficial vascular and anti-fibrotic actions of relaxin are well known. However, potentially harmful actions resulting from the administration of relaxin have also been noted, in particular the ability of relaxin to promote tumour growth. *In vitro*, relaxin promotes the proliferation or invasion of several types of cancer cells, including breast cancer cells (Binder *et al.*, 2002), endometrial cancer cells (Kamat *et al.*, 2006), glioblastoma cells (Glogowska *et al.*, 2013), prostate cancer cells (Feng *et al.*, 2007), and thyroid cancer cells (Radestock *et al.*, 2010). Further studies have also suggested that relaxin

plays a role in cancer cell proliferation *in vivo*. For example, relaxin administration increased tumour growth in a mouse model of spontaneous breast cancer (Binder *et al.*, 2014), and overexpression of relaxin in a mouse model of human prostate cancer also led to increased tumour growth (Silvertown *et al.*, 2006). Additionally, inhibition of RXFP1 using small interfering RNA suppressed prostate tumour growth in a mouse model of human prostate cancer (Feng *et al.*, 2010), as did expression or exogenous administration of an RXFP1 antagonist (Neschadim *et al.*, 2014; Silvertown *et al.*, 2007). Interestingly, unlike relaxin, an equimolar dose or higher of B7-33 did not promote tumour growth in a mouse model of prostate cancer (Hossain *et al.*, 2016). Originally, this was thought to be linked to cell-specific actions whereby B7-33 has weak activity in cancer cells. However, it now makes more sense in the context of biased signalling. The ability of relaxin to induce proliferation of MCF-7 breast cancer cells, for example, is thought to be at least partly related to cAMP activity, as addition of a cAMP analogue produced the same effect (Bigazzi *et al.*, 1992). Similarly, inhibition of PKA suppressed the growth of human prostate cancer cells stably expressing relaxin, further suggesting a role for cAMP in cancer cell proliferation (Vinall *et al.*, 2011). Thus, given that the cancer-promoting actions of RXFP1 activation appear to be related to cAMP activity, and that B7-33 has a substantially lower potency than relaxin for activation of $G\alpha_s$ /cAMP, it makes sense that B7-33 does not promote tumour growth at an equimolar dose to relaxin. In contrast, beneficial actions of relaxin in vascular cells and fibroblasts occur primarily via the $G\alpha_{i3}$ /cGMP pathway, so it makes sense that B7-33, which is potent for $G\alpha_{i3}$ activation, maintains the beneficial actions of RXFP1 activation. B7-33 was thus a valuable tool for confirming the beneficial and harmful signalling pathways related to RXFP1 activation by exogenous ligands. Of course, cAMP is at least partly responsible for several other actions of relaxin, but these appear to be less crucial for the beneficial actions of exogenously administered relaxin and may be more related to the physiological roles of endogenous relaxin, such as maintenance of pregnancy (Bartsch *et al.*, 2004; Huang *et al.*, 1987; Telgmann *et al.*, 1997).

In conclusion, these data demonstrate that B7-33 is a biased agonist relative to relaxin, preferentially inducing activation of $G\alpha_{i3}$ and signalling via cGMP over activation of $G\alpha_s$ and signalling via cAMP. This is consistent with the chronic *in vitro* and *in vivo* actions of B7-33, and notably is of clinical importance as the beneficial actions of relaxin administration appear to occur primarily via $G\alpha_{i3}$ and cGMP, whereas negative effects appear to occur primarily via $G\alpha_s$ and cAMP. Given that relaxin has a short half-life in circulation, cannot be administered orally, and may require sustained administration for treatment of chronic illnesses, these findings will aid in the further development of small molecule agonists or relaxin analogues with long half-lives. Finally, these findings suggest that kinetics, not simply stabilisation of unique receptor conformations, may play

a role in the biased signalling observed for B7-33 acting at RXFP1, which also has implications for the further development of agonists targeting this receptor.

6 General discussion

6.1 Introduction

Drugs that target GPCRs account for approximately 30% of drugs approved by the United States Food and Drug Administration (Hauser *et al.*, 2017). However, at the same time, only a minority of GPCRs are actually targeted by therapeutic drugs (Hauser *et al.*, 2017). This is explained by the fact that some GPCRs, particularly the rhodopsin-like aminergic receptors, are vastly overrepresented as drug targets (Sriram & Insel, 2018). Yet many other GPCRs are thought to have therapeutic potential, including the relaxin receptor, RXFP1. The beneficial cardiovascular and anti-fibrotic actions of relaxin are well known, and there has thus been a great deal of therapeutic interest in the use of relaxin or relaxin analogues as therapeutic drugs. However, despite its promising actions, relaxin has unfortunately failed several clinical trials (Unemori, 2017). Increasing our understanding of the molecular mechanisms underlying the actions of relaxin and other RXFP1 ligands may aid in the successful targeting of this promising receptor, including by biased agonists.

It is hoped that the development of biased ligands will help us therapeutically target receptors that until now have not been successfully drugged. However, detection of bias and translation of bias into *in vivo* benefits is complicated, and the limited success in developing biased agonists so far has highlighted the fact that improved *in vitro* assays are needed in order to accurately detect biased signalling. In recent years, it has become clear that some signalling assays, particularly end point assays, can introduce “observational bias”, which is bias that is only apparent due to the conditions of the assays used, such as temporal factors and sensitivities of the assays (Kenakin & Christopoulos, 2013). Observational bias can have confounding effects and has been suggested to lead to inconsistent conclusions about bias (Klein Herenbrink *et al.*, 2016). Furthermore, we are also starting to appreciate the importance of the kinetics of binding and signalling (Lane *et al.*, 2017), location of signalling (Irannejad *et al.*, 2017), and their relationship to biased signalling. Additionally, signalling can also be influenced by system bias or the differential expression of proteins associated with signalling pathways across cell types (Kenakin & Christopoulos, 2013).

Improved *in vitro* signalling assays should measure signalling in real time to detect any kinetic effects and avoid observational bias. Additionally, although recombinant expression of GPCRs in common cell lines such as HEK293T may be a valuable initial tool for drug discovery and

detection of biased signalling, it is important to validate the findings in native or primary cells in order to show whether bias occurs in physiologically-relevant cells and to predict how a drug might act *in vivo* (Smith *et al.*, 2018), as well as to distinguish between biased signalling and system bias, as system bias is a property of the cell type and cannot be exploited for drug discovery (Kenakin & Christopoulos, 2013). Taking these factors into account, the general aim of this thesis was to apply and develop BRET-based methods in conjunction with human cells that natively express RXFP1 in order to examine real-time signalling and understand ligand- and cell-specific signalling at RXFP1. To do this, several approaches were taken. First, in Chapter 3, we aimed to demonstrate the use and versatility of CAMYEL for detection of cAMP at the four relaxin family peptide receptors, to clone CAMYEL into a lentiviral vector, and to demonstrate its use in cells that endogenously express RXFP1. Second, in Chapter 4, we aimed to develop, validate, and characterise a BRET-based biosensor for cGMP activity, clone it into a lentiviral vector, and investigate its utility for detection of cGMP activity in primary human vascular cells. Finally, in Chapter 5, we aimed to use a variety of BRET-based and end point assays, in addition to human native and primary cells, in order to understand the molecular mechanisms underlying the biological effects of the relaxin analogue B7-33.

6.2 Detecting cAMP activity at the relaxin family peptide receptors

cAMP is one of the primary second messengers involved in signalling of the relaxin family peptide receptors, RXFP1-4. Until now, signalling at RXFP1 had primarily been conducted using end point assays such as CRE reporter gene assays in HEK-RXFP1 cells (e.g., Kong *et al.*, 2013) and HTRF assays in some types of native or primary cells (e.g., Kocan *et al.*, 2017). The availability of a sensitive, BRET-based biosensor for the detection of cAMP, CAMYEL, prompted us to investigate whether this would be a viable alternative method for detecting signalling downstream of these receptors, particularly RXFP1, including in native cells.

In Chapter 3, CAMYEL was successfully used for the detection of cAMP activity downstream of RXFP1 and RXFP2, and inhibition of cAMP activity downstream of RXFP3 and RXFP4, in cell lines recombinantly overexpressing the receptor, using a combination of endogenous ligands, peptide analogues, and the small molecule RXFP1 agonist ML290. These assays showed that CAMYEL was sensitive for the detection of real-time cAMP activity at all four relaxin family peptide receptors, in addition to detecting differences in signalling patterns between ligands, which may be a reflection

of the binding kinetics of ligands at these receptors (for example, increases in potency of cAMP activity over time after INSL3 stimulation compared to relaxin stimulation of RXFP2 may be associated with a slower off-rate of INSL3), although this was not specifically investigated in this chapter. In addition to detecting robust cAMP activity, potencies for every ligand tested were comparable to those seen using end point assays in previous research. Furthermore, although the cAMP activity was generally sustained at these receptors, CAMYEL was demonstrated to be reversible by using antagonists for RXFP2 and RXFP3, which both showed real-time reversals of signalling. Furthermore, reversibility was also subsequently demonstrated by stimulation of cells with ligands that led to transient responses, such as forskolin acting at ACs in HEK293A $\Delta G\alpha_s$ cells (Chapter 3), isoprenaline acting at endogenous β -adrenoceptors in HEK293T cells (Chapter 4), and B7-33 acting at RXFP1 expressed in HEK293T, BJ3, and endothelial cells (Chapter 5).

CAMYEL was subsequently cloned into a lentiviral vector to enable stable expression across a range of mammalian cell types. THP-1 cells were successfully transduced and tested for relaxin-mediated cAMP activity from endogenous RXFP1. CAMYEL lentivirus was later used to transduce other cell types including HEK93T cells (Chapter 4), BJ3 cells, and HUVECs (Chapter 5), which were used to detect cAMP activity from endogenous β -adrenoceptors as well stably-expressed RXFP1. These CAMYEL assays were clearly a valuable alternative to typical end point assays for several reasons. First, they were able to detect kinetic differences in signalling between different agonists of the same receptor, which as described below helped advance our understanding of the actions of B7-33. Second, the lentiviral vector allowed cAMP detection across all cell types tested, including primary cells, with potencies similar to those observed using other assays in the same types. Finally, the CAMYEL assay streamlined the process of screening ligands for cAMP activity, as the lentiviral transduction is permanent and additional steps involved in end point assays such as lysis and detection are not required.

As CAMYEL proved valuable for detecting RXFP1 signalling, we hoped to develop a complementary BRET-based biosensor for cGMP activity, particularly as the detection of biased signalling requires characterisation of at least two signalling pathways.

6.3 Developing a BRET-based sensor for cGMP activity

Drug discovery targeting the cGMP signalling cascade is ongoing for the therapeutic treatment of a variety of diseases and conditions, such as cardiovascular diseases, heart failure, fibrosis, and central nervous system disorders (Friebe *et al.*, 2020; Sandner & Stasch, 2017; Sandner

et al., 2021). Relatedly, the potentially therapeutic effects of relaxin such as vasodilation (Lian *et al.*, 2018; McGuane *et al.*, 2011b), vasoprotection (Dschiertzig *et al.*, 2012; Pini *et al.*, 2016), and anti-fibrotic actions (Samuel *et al.*, 2016) have been shown to be downstream of NO/cGMP. Although Chapter 4 did not end up focusing on RXFP1 signalling for reasons outlined in that chapter and below, the connection between cGMP and the beneficial actions of relaxin was the original impetus for this chapter. Thus, in Chapter 4 we engineered a BRET-based biosensor for cGMP activity based on the FRET biosensor cGES-DE5, with the hope of using it in plate-based assays in a similar fashion to CAMYEL. The lentiviral delivery method was again successful and allowed detection of cGMP in cell lines and primary cells, and detected activity of both the sGC- and pGC-mediated pools of cGMP.

It was hoped that CYGYEL would be suitable for detecting GPCR-mediated signalling in live cells in real-time, particularly for RXFP1, but unfortunately this has not yet been successful. As described in Chapter 4, as HUVECs had a very low DEA response in the CYGYEL assay, we instead stimulated HUVSMCs with relaxin and carbachol, but did not detect any response from either agonist. One explanation for this is that stimulation of smooth muscle cells alone is not expected to produce robust cGMP activity, as cGMP activity in smooth muscle cells is dependent on NO produced in endothelial cells that diffuses into smooth muscle cells where it activates sGC (Furchgott & Vanhoutte, 1989). Likewise, this suggests that endothelial cells would also not be an ideal cell type for detection of GPCR-mediated cGMP activity. Furthermore, we did not test HUVSMCs for expression of the relevant GPCRs. On the other hand, RXFP1-mediated cGMP activity has previously been detected in HUVSMCs using an end point assay (Sarwar *et al.*, 2015), however the responses were more robust after co-culturing the HUVSMCs with HUVECs (Sarwar *et al.*, 2016b), consistent with our understanding of blood vessel physiology. Additionally, it was later observed during our Chapter 5 work that we could successfully detect relaxin-mediated cGMP in HUVECs (without additional RXFP1 transduction) using an end point assay, but importantly these responses were difficult to detect repeatedly without additional expression of RXFP1, which was likely due to a loss in cell surface expression of RXFP1 with additional passages of the cells (unpublished data, Bathgate lab). This highlights the primary disadvantage of conducting BRET assays in human primary cells, which is that the lentiviral transduction method (and FACS) requires additional passages of cells in comparison to end point assays, which in some cases could lead to loss of expression of certain proteins over time. Another possible explanation for the lack of GPCR-mediated cGMP activity observed in these cells is that the sensor is not sensitive enough to detect cGMP, particularly if the activity is weak, which is again described in Chapter 4.

Low selectivity for cGMP over cAMP has been an issue for some cGMP sensors. The high selectivity of the FRET biosensor cGES-DE5 for cGMP over cAMP was the primary reason we chose to convert this particular FRET biosensor to BRET, because RXFP1 signals via both cyclic nucleotides. Selectivity of CYGYEL was determined by stimulating lysates from HEK-CYGYEL cells with cGMP and cAMP, and comparing their potencies; and was also determined in live HEK-CAMYEL and HEK-CYGYEL cells by comparing potency for activation by forskolin-mediated cAMP. Fortunately, we know from live cell assays that endogenous β -adrenoceptor-mediated cAMP activity showed little or no activation of CYGYEL, however it is still possible that GPCRs could produce a cAMP response that is detectable to CYGYEL, particularly if the receptor is overexpressed. On the other hand, it should also be noted that cAMP activity will generally not occur from most ligands or receptors, particularly when GPCRs are not involved, and thus selectivity will not often be an issue. For example, we do not expect cAMP activity from pGCs.

Due to the lack of appropriate real-time cGMP assay for RXFP1, in Chapter 5 an end point HTRF assay was used to detect cGMP after relaxin or B7-33 stimulation of HUVEC-RXFP1 cells. This assay included the non-selective PDE inhibitor IBMX to stop the breakdown of second messenger, so we did not detect any kinetic effects the way we were able to for cAMP activity by using CAMYEL. It would be valuable to have a sensitive, real-time cGMP assay in the future, although we fortunately had a complementary real-time G protein association assay that aided in our understanding of the kinetic aspects of RXFP1 signalling upstream of cGMP. However, further efforts to design a highly-sensitive cGMP sensor may be beneficial for many GPCRs, not just RXFP1. Alternatively, there are other parts of the signal transduction cascade that might provide complementary data, and sensors for these pathways might prove to be valuable alternatives to a cGMP sensor. For example, Akt is known to phosphorylate eNOS (Dimmeler *et al.*, 1999), and at RXFP1 Akt has been identified as being upstream of NO/cGMP (Lian *et al.*, 2018; McGuane *et al.*, 2011b). Thus, concentration-response data for Akt phosphorylation or activity could be a substitute for cGMP activity data, particularly for cell types where cGMP activity might not be expected such as some types of cancer cells. Fortunately, several FRET-based sensors for Akt already exist (e.g., Komatsu *et al.*, 2011), which could potentially be converted to BRET.

6.4 B7-33 is a biased agonist

Previous work suggested that the minimised relaxin analogue B7-33 had cell-specific actions, whereby it appeared to be potent in some cell types and *in vivo*, but weak in other cell

types, particularly cancer cells and recombinant cells (Hossain *et al.*, 2016; Marshall *et al.*, 2017). Using a combination of assays for cAMP, cGMP, ERK1/2, and G protein association, and several recombinant and relevant primary cell types, Chapter 5 demonstrated for the first time that B7-33 is actually a biased agonist that favours signalling via $G\alpha_{i3}$ /cGMP over $G\alpha_s$ /cAMP, relative to relaxin. Two key pieces of evidence underlie this idea. First, whereas relaxin potently signals through both cAMP and cGMP, B7-33 signals weakly via cAMP but potently via cGMP in the same cell backgrounds (and using the same lysates to detect cAMP and cGMP). Second, whereas relaxin potently increases activation of both $G\alpha_s$ and $G\alpha_{i3}$, B7-33 only weakly activates $G\alpha_s$ but potently activates $G\alpha_{i3}$ in the same cell background. Given that $G\alpha_s$ is upstream of cAMP and $G\alpha_{i3}$ is upstream of cGMP, these findings made sense and were consistent with B7-33's actions as a potent vasodilator and anti-fibrotic, which depend on cGMP activity.

Interestingly, B7-33 showed transient cAMP activity relative to relaxin in all cell types tested. This difference in the kinetics of signalling between the two agonists had not previously been observed, as only end point assays using IBMX had been conducted so far. Similarly, the kinetics of $G\alpha_s$ activation were also distinct between the two agonists; specifically, relaxin increased $G\alpha_s$ activation at a faster and more sustained rate than B7-33 did. These findings suggested that differences in binding kinetics between the two ligands may play a role in the differences observed between the two agonists. A CAMYEL assay involving dissociation of unbound ligand demonstrated that whereas the relaxin response showed only a slow and small decline in cAMP activity after removal of unbound ligand, the B7-33 response dropped precipitously. This finding suggested that relaxin has a relatively long residence time compared to B7-33 and that B7-33 dissociates relatively quickly. We hypothesised that these differences in off-rates (and thus residence time) may be important for driving the biased signalling pattern of B7-33.

6.5 Ligand binding kinetics and biased signalling

As described in Chapter 1, the primary explanation for biased signalling relates to stabilisation of specific GPCR conformations. Yet it is becoming increasingly clear that this explanation alone does not always fully explain the mechanism underlying biased signalling. In some cases, there is a kinetic component to biased signalling and an interplay between kinetics and conformational change, whereby rates of ligand dissociation and G protein turnover can affect biased signalling.

Ligand residence time, which is the reciprocal of the dissociation rate (i.e., $1/k_{\text{off}}$), is an important factor for drug action (Copeland, 2016; Copeland *et al.*, 2006; Hothersall *et al.*, 2016; Schuetz *et al.*, 2017) and potentially even biased signalling. At the PTH1 receptor, for example, it has been hypothesised that a combination of prolonged residence time and rebinding may promote and maintain location bias, whereby slowly-dissociating agonists promote sustained signalling from internalised receptors, in contrast to faster-dissociating agonists which do not (Hothersall *et al.*, 2016). Similarly, slow off-rate at the S1P₁ receptor was linked to efficient β -arrestin recruitment and internalisation (Sykes *et al.*, 2014), and at the 5-HT_{2B} receptor sustained residence time of LSD was associated with bias towards β -arrestin2 recruitment (Wacker *et al.*, 2017). Furthermore, studies on the D₂ dopamine receptor have shown that kinetics can influence observed signalling bias and lead to reversals in bias over time as, for example, slower dissociation kinetics can lead to increases in potency over time relative to faster dissociating ligands (Klein Herenbrink *et al.*, 2016). This may explain discrepancies observed between previous studies and highlights the importance of measuring signalling over time in order to eliminate observational bias (Klein Herenbrink *et al.*, 2016).

To these findings, we can potentially add the importance of residence time for influencing the rate of activation of different G proteins. As noted in Chapter 5, it was recently observed that at the GLP-1 receptor, GLP-1 and the biased agonist TT-OAD2 activated $G\alpha_{i2}$ at approximately the same rate, whereas GLP-1 activated $G\alpha_s$ much faster than TT-OAD2 did (Zhao *et al.*, 2020). This was associated with the ability of TT-OAD2 to displace an orthosteric ligand only weakly and partially compared to GLP-1 (Zhao *et al.*, 2020). These observations were similar to our findings for RXFP1, where relaxin appeared to activate $G\alpha_s$ at a faster rate than the weakly-binding B7-33 (and was more potent for cAMP activity), whereas both agonists activated $G\alpha_{i3}$ at approximately the same rate (and were approximately equi-potent for cGMP activity). However, unfortunately we were unable to apply kinetic models to the RXFP1 G protein activation data, such as association exponential curves as has been done previously for the GLP-1 receptor (Zhao *et al.*, 2020), and could also not quantify the initial rate of signalling as has been suggested as a measure of efficacy (Hoare *et al.*, 2020), due to poor or ambiguous curve fits for the $G\alpha_s$ time-course for B7-33 and the $G\alpha_{i3}$ time-courses for both agonists. Furthermore, it should be noted that the differences in rates of G protein activation for the GLP-1 receptor, a secretin-like receptor, were much starker than those seen at RXFP1, a rhodopsin-like receptor. But it does appear that there is an interplay between residence time and biased signalling for at least some GPCRs. This does not exclude conformational selection, but demonstrates that the duration of the conformational change, which is affected by ligand residence time, may be an important factor in selective activation of

downstream effectors as, for example, it may influence rates of nucleotide exchange. We suggest that relaxin and B7-33 stabilise a similar receptor active state, but the different ligand off-rates are key for the differences in biological effects between the two agonists. However, there are limited published findings describing a link between binding kinetics and activation or deactivation of specific G proteins. Future studies could measure the dissociation of a range of agonists at several receptors to correlate with activation of the receptors' G proteins.

This kinetics-driven bias would have implications for the development of novel drugs targeting RXFP1. For instance, we could retain the kinetic bias to maintain potent signalling via $G\alpha_{i3}$ and minimise signalling at $G\alpha_s$ due to the potentially harmful side effects (see below), but at the same time aim towards developing more drug-like compounds with long half-lives. Relatedly, it would be interesting to see how many examples of bias that were thought to be primarily conformation-driven are actually driven by kinetics and residence time (or, relatedly, by location of signalling within the cell).

6.6 Compartmentalisation of signalling

Compartmentalisation of second messenger activity has been recognised for many years (McCormick & Baillie, 2014) and is noteworthy because, for example, location of signalling within the cell can affect downstream gene transcription (Tsvetanova & von Zastrow, 2014), and as with the temporal aspects of signalling, the spatial aspects of signalling help explain how different ligands and receptors that activate the same signalling pathways can lead to such different biological outcomes. In fact, detection of GPCR signalling with spatial resolution is a current focus in GPCR drug discovery (Eichel & von Zastrow, 2018). Much of the current work on compartmentalisation of GPCR signalling relates to sustained signalling from internalised receptors, but given that RXFP1 does not couple to β -arrestin and shows weak or no internalisation (Callander et al., 2009; Kern & Bryant-Greenwood, 2009), this type of signalling does not relate to RXFP1, and we did not set out to examine the spatial aspects of signalling in detail. However, several observations related to localisation of signalling throughout this thesis are noteworthy.

First, as described in detail in Chapter 3, it appeared that CAMYEL was unable to detect the delayed $G\alpha_{i3}$ -mediated phase of cAMP activity at RXFP1 that has previously been observed using other assays. Interestingly, however, cAMP data generated using CAMYEL in HEK-RXFP1 cells (Chapter 3), THP-1 cells (Chapter 3), and HUVEC-RXFP1 cells (Chapter 5) showed relaxin potencies very similar to those observed using end point assays (e.g., Bathgate *et al.*, 2002; Hossain *et al.*,

2016). Furthermore, end point assays and CAMYEL even showed similar cAMP potencies for B7-33, which is biased in favour of $G\alpha_{i3}$ but shows only weak cAMP activity (Chapter 5). Thus, although it seems that there is a pool of cAMP that was not detected by CAMYEL, this pool appears minimal relative to the pools of cAMP that were detected (downstream of $G\alpha_s$). Interestingly, it has been suggested that for RXFP1, cAMP activity mediated by $G\alpha_s$ versus $G\alpha_{i3}$ has different transcriptional outcomes (Halls *et al.*, 2007), but this has not been investigated further other than the finding that the $G\alpha_{i3}$ -dependent cAMP appears to be segregated in the cell as it occurs after localisation of RXFP1 into lipid rafts (Halls *et al.*, 2009), which was consistent with the inability of CAMYEL to detect specific pools of activity, as described in Chapter 3.

Second, recent findings that membrane-permeable agonists can activate receptors arising from the biosynthetic pathway located at the Golgi apparatus (Irannejad *et al.*, 2017; Stoeber *et al.*, 2018), raise the question as to whether the small molecule RXFP1 agonist ML290 is membrane permeable, whether it would be able to activate receptors located at particular regions such as the Golgi in a way that peptide agonists cannot, and how this type of signalling might produce alternative outcomes to signalling from the membrane only.

Third, in Chapter 4 we demonstrated that CYGYEL was able to detect cGMP that was mediated via both sGC and pGC (specifically GC-B, the receptor for CNP). Compartmentalisation of other second messengers, particularly cAMP and Ca^{2+} , has been studied in more detail than cGMP (McCormick & Baillie, 2014), but it does appear that there are distinct pools of cGMP that are regulated separately by PDEs (Bork *et al.*, 2019), which is not surprising as pGCs are membrane enzymes whereas sGC is a soluble enzyme, so they are already segregated in the cell. One of the benefits of genetically-encoded biosensors is that targeting sequences can be introduced to the biosensor gene in order to detect signalling localised to different subcellular locations such as the nucleus or cytosol, whereas end point assays typically detect total activity in cell lysates and therefore do not normally have spatial resolution. For example, targeted FRET biosensors have been used to detect localised GPCR signalling (Godbole *et al.*, 2017; Jensen *et al.*, 2017). BRET, on the other hand, has rarely been used for detection of signalling at specific subcellular locations, although there are some examples of this in the literature. For example, a membrane-targeted variant of CAMYEL (PDE2-CAMYEL) was generated by tagging CAMYEL with the membrane-targeting sequence from PDE2A3 (Matthiesen & Nielsen, 2011). Going forward, it would be interesting to see whether targeting CYGYEL or other future cGMP sensors to specific locations would aid in the detection of microdomains of cGMP in the plate-based format, which could potentially be accomplished by targeting the sensor to specific PDEs or GCs. However, it should be noted that targeting a cGMP sensor to or near the membrane for detection of pGC-mediated

activity may also increase the detection of cAMP, so caution should be exercised if taking this approach.

Finally, in Chapter 5, the REV ERK1/2 biosensors that were localised to either the nucleus or cytosol were used to detect ERK1/2 activity in HEK-RXFP1-REV cells after stimulation with a maximal concentration of relaxin. For both variants of the sensor, the ERK1/2 response was transient, and the nuclear and cytosolic responses were almost identical to each other. As described below, the implications of the location and duration of relaxin-mediated ERK1/2 activity have not yet been studied, but it is known that in general ERK1/2 has different substrates in the cytosol versus nucleus, and ERK1/2 activity is regulated in space and time to control its biological actions (Shaul & Seger, 2007).

6.7 Further investigation of the role of different signalling pathways is required

As described in Chapter 5, the finding that B7-33 only weakly activates $G\alpha_s$ /cAMP is important because the ability of relaxin to signal via cAMP is associated with its ability to promote the proliferation of cancer cells *in vitro* and promote tumour growth in mouse models *in vivo* (Hossain *et al.*, 2016). Furthermore, another potentially negative role of cAMP activity is that cAMP may be pro-arrhythmic and harmful for treatment of heart failure, however this is not fully understood, and the production of some specific pools of cAMP may actually be beneficial in mice after myocardial infarction (Leineweber *et al.*, 2006). We do not fully understand the role of cAMP for the biological effects of relaxin, and do not understand the role and importance of cAMP for treatment of heart failure, so this will be important to study going forward. Although cAMP can have harmful effects after relaxin administration, cAMP is also at least partly responsible for several other roles and actions of relaxin, as would be expected, although these appear to be more related to the physiological roles of endogenous relaxin rather than the beneficial effects of pharmacological relaxin (or other ligand) administration, given that B7-33 maintains the beneficial actions of relaxin when given as an equimolar dose. For example, relaxin-mediated cAMP activity has been linked to decidualisation, consistent with the role of relaxin as a pregnancy hormone (Bartsch *et al.*, 2004; Huang *et al.*, 1987; Telgmann *et al.*, 1997). Additionally, although cAMP has been shown to play a role in chronotropic and inotropic actions of relaxin, this is thought to be related to the $G\alpha_i$ -mediated phase of cAMP activity rather than $G\alpha_s$ (Dschietzig *et al.*, 2011; Kompa *et al.*, 2002). Additionally, in some cell types relaxin-mediated cAMP activity has been linked to the

upregulation of VEGF (Unemori *et al.*, 1999), but in some cell types it is clear that other signalling pathways are involved (Sarwar *et al.*, 2015). Relaxin-mediated upregulation of VEGF activity is thought to be involved in angiogenesis and wound healing (Unemori *et al.*, 2000), and there is also some evidence that VEGF is involved in sustained vasodilation after relaxin treatment (McGuane *et al.*, 2011a), but this is not settled (Bathgate *et al.*, 2013). Further studies should thus continue to delineate the different pathways responsible for specific actions of relaxin, and in this respect biased agonists such as B7-33 are useful tools.

Relatedly, another pathway whose role needs to be clarified is ERK1/2. Previous studies suggested that chronic administration of relaxin resulted in anti-fibrotic actions via an ERK1/2-nNOS-NO-sGC-cGMP-dependent pathway in myofibroblasts (Chow *et al.*, 2012), yet a key finding of another study was that ML290 is a biased agonist that does not acutely increase ERK1/2 phosphorylation in any cell type tested, but still has chronic anti-fibrotic actions *in vitro* (Kocan *et al.*, 2017). Furthermore, ML290 was also demonstrated to be anti-fibrotic *in vivo* in mice expressing human RXFP1 (Kaftanovskaya *et al.*, 2019). On the other hand, ML290 has not been tested for chronic activation of ERK1/2 or upregulation of ERK1/2 in fibroblasts *in vitro*, so it is still possible that ML290 will induce chronic ERK1/2 activation which would be consistent with its chronic *in vitro* and *in vivo* actions. The role and importance of ERK1/2 activation for the beneficial actions of relaxin in different cell types is not well understood, but the finding that ML290 does not acutely activate ERK1/2 makes ML290 a potentially useful tool compound to help distinguish the biological roles of ERK1/2 activity after RXFP1 activation.

Furthermore, the importance of location and duration of RXFP1-mediated ERK1/2 activity is not understood. In Chapter 5, there was little or no difference between cytosolic and nuclear ERK1/2 activity in HEK-RXFP1-REV cells after relaxin stimulation, in terms of both peak response and duration of activity. This transient ERK1/2 activity is consistent with the transient ERK1/2 phosphorylation observed in end point assays in HEK-RXFP1 cells (Hossain *et al.*, 2016), but it should be noted that in other cell types, such as HUVECs, relaxin has been reported to induce sustained ERK1/2 phosphorylation (Dschietzig *et al.*, 2012; Sarwar *et al.*, 2015). Interestingly, sustained ERK1/2 activity is usually associated with β -arrestin activity (Ahn *et al.*, 2004; Ren *et al.*, 2005; Shenoy *et al.*, 2006), but this clearly does not apply to RXFP1. Thus, further studies could elucidate the role and importance of different locations and durations of ERK1/2 activity.

6.8 Conclusion

Here, we have examined biased and cell-specific signalling in real-time at RXFP1 using a range of tools, including BRET-based biosensors for cAMP activity, ERK1/2 activity, and effector recruitment, end point second messenger assays, primary cells, cell lines, lentiviral-mediated gene delivery, pharmacological inhibitors, and CRISPR/Cas9-mediated G protein knockout cells. We demonstrated that BRET-based biosensors are valuable alternatives to end point assays for RXFP1 signalling as they provide additional kinetic information that is hard to detect using end point assays. We also showed that the relaxin peptide analogue B7-33 is a biased agonist that favours activation of $G\alpha_{i3}$ /cGMP over $G\alpha_s$ /cAMP, consistent with its *in vivo* actions. We demonstrated that this biased signalling may have been related to binding kinetics, whereby the faster off-rate of B7-33 is associated with reduced activation of $G\alpha_s$ /cAMP compared to relaxin. We also showed that RXFP1 activates ERK1/2 signalling in a cell-specific manner. Finally, we developed a BRET-based biosensor for cGMP activity that may prove valuable for drug discovery, even if it might not be used for GPCR signalling.

Of course, even though the mechanism of action for B7-33 is now better understood, it must be noted that B7-33 is still a peptide, which makes chronic administration difficult. However, the data gathered for B7-33 may aid in the development of other novel RXFP1 agonists. Our data has supported the idea that cGMP activity is important for therapeutic actions at RXFP1, and we note that other research has demonstrated potentially harmful actions of signalling via cAMP. Thus, future RXFP1 ligands could potentially maintain the faster dissociation and “kinetic bias” of B7-33, but should also be more drug-like and have potential for chronic administration. Meanwhile, we should continue to study the roles of the different signalling pathways activated by relaxin or other RXFP1 agonists, as well as the importance of location and duration of signalling at different pathways. Biased agonists such as B7-33 and ML290 may act as useful tool compounds for distinguishing the roles of different signal transduction pathways across cell types.

References

- Adham IM, Burkhardt E, Benahmed M, & Engel W (1993). Cloning of a cDNA for a novel insulin-like peptide of the testicular Leydig cells. *J Biol Chem* 268: 26668-26672.
- Adler E, Hoon MA, Mueller KL, Chandrashekar J, Ryba NJ, & Zuker CS (2000). A novel family of mammalian taste receptors. *Cell* 100: 693-702.
- Ahn S, Shenoy SK, Wei H, & Lefkowitz RJ (2004). Differential kinetic and spatial patterns of beta-arrestin and G protein-mediated ERK activation by the angiotensin II receptor. *J Biol Chem* 279: 35518-35525.
- Alexiou K, Wilbring M, Matschke K, & Dschietzig T (2013). Relaxin protects rat lungs from ischemia-reperfusion injury via inducible NO synthase: role of ERK-1/2, PI3K, and forkhead transcription factor FKHRL1. *PLoS One* 8: e75592.
- Ang SY, Evans BA, Poole DP, Bron R, DiCello JJ, Bathgate RAD, Kocan M, Hutchinson DS, & Summers RJ (2018). INSL5 activates multiple signalling pathways and regulates GLP-1 secretion in NCI-H716 cells. *J Mol Endocrinol* 60: 213-224.
- Ang SY, Hutchinson DS, Patil N, Evans BA, Bathgate RAD, Halls ML, Hossain MA, Summers RJ, & Kocan M (2016). Signal transduction pathways activated by insulin-like peptide 5 at the relaxin family peptide RXFP4 receptor. *Br J Pharmacol* 174: 1077-1089.
- Aragon-Herrera A, Feijoo-Bandin S, Rodriguez-Penas D, Rosello-Lleti E, Portoles M, Rivera M, Bigazzi M, Bani D, Gualillo O, Gonzalez-Juanatey JR, & Lago F (2018). Relaxin activates AMPK-AKT signaling and increases glucose uptake by cultured cardiomyocytes. *Endocrine* 60: 103-111.
- Arai R, Nakagawa H, Tsumoto K, Mahoney W, Kumagai I, Ueda H, & Nagamune T (2001). Demonstration of a homogeneous noncompetitive immunoassay based on bioluminescence resonance energy transfer. *Anal Biochem* 289: 77-81.
- Atwood BK, Lopez J, Wager-Miller J, Mackie K, & Straiker A (2011). Expression of G protein-coupled receptors and related proteins in HEK293, AtT20, BV2, and N18 cell lines as revealed by microarray analysis. *BMC Genomics* 12: 14.
- Ayoub MA (2016). Resonance energy transfer-based approaches to study GPCRs. In *Methods Cell Biol.* Elsevier Ltd, pp 255-292.
- Balvers M, Spiess AN, Domagalski R, Hunt N, Kilic E, Mukhopadhyay AK, Hanks E, Charlton HM, & Ivell R (1998). Relaxin-like factor expression as a marker of differentiation in the mouse testis and ovary. *Endocrinology* 139: 2960-2970.
- Bamberger AM, Ivell R, Balvers M, Kelp B, Bamberger CM, Riethdorf L, & Loning T (1999). Relaxin-like factor (RLF): a new specific marker for Leydig cells in the ovary. *Int J Gynecol Pathol* 18: 163-168.
- Bani D, Failli P, Bello MG, Thiemermann C, Sacchi TB, Bigazzi M, & Masini E (1998). Relaxin activates the L-Arginine–nitric oxide pathway in vascular smooth muscle cells in culture. *Hypertension* 31: 1240-1247.
- Bani G, & Bigazzi M (1984). Morphological changes induced in mouse mammary gland by porcine and human relaxin. *Acta Anat (Basel)* 119: 149-154.
- Bani G, Bigazzi M, & Bani D (1985). Effects of relaxin on the mouse mammary gland. I. The myoepithelial cells. *J Endocrinol Invest* 8: 207-215.
- Barry SC, Harder B, Brzezinski M, Flint LY, Seppen J, & Osborne WR (2001). Lentivirus vectors encoding both central polypurine tract and posttranscriptional regulatory element provide enhanced transduction and transgene expression. *Hum Gene Ther* 12: 1103-1108.

- Bartsch O, Bartlick B, & Ivell R (2001). Relaxin signalling links tyrosine phosphorylation to phosphodiesterase and adenylyl cyclase activity. *Mol Hum Reprod* 7: 799-809.
- Bartsch O, Bartlick B, & Ivell R (2004). Phosphodiesterase 4 inhibition synergizes with relaxin signaling to promote decidualization of human endometrial stromal cells. *J Clin Endocrinol Metab* 89: 324-334.
- Bassilana F, Nash M, & Ludwig MG (2019). Adhesion G protein-coupled receptors: opportunities for drug discovery. *Nat Rev Drug Discov* 18: 869-884.
- Bathgate R, Balvers M, Hunt N, & Ivell R (1996). Relaxin-like factor gene is highly expressed in the bovine ovary of the cycle and pregnancy: sequence and messenger ribonucleic acid analysis. *Biol Reprod* 55: 1452-1457.
- Bathgate RA, Halls ML, van der Westhuizen ET, Callander GE, Kocan M, & Summers RJ (2013). Relaxin family peptides and their receptors. *Physiol Rev* 93: 405-480.
- Bathgate RA, Hsueh AJ, & Sherwood OD (2006a). Physiology and molecular biology of the relaxin peptide family. In Knobil and Neill's physiology of reproduction. Elsevier, pp 679-768.
- Bathgate RA, Ivell R, Sanborn BM, Sherwood OD, & Summers RJ (2006b). International Union of Pharmacology LVII: recommendations for the nomenclature of receptors for relaxin family peptides. *Pharmacol Rev* 58: 7-31.
- Bathgate RA, Samuel CS, Burazin TC, Layfield S, Claasz AA, Reytomas IG, Dawson NF, Zhao C, Bond C, Summers RJ, Parry LJ, Wade JD, & Tregear GW (2002). Human relaxin gene 3 (H3) and the equivalent mouse relaxin (M3) gene. Novel members of the relaxin peptide family. *J Biol Chem* 277: 1148-1157.
- Bathgate RAD, Kocan M, Scott DJ, Hossain MA, Good SV, Yegorov S, Bogerd J, & Gooley PR (2018). The relaxin receptor as a therapeutic target - perspectives from evolution and drug targeting. *Pharmacol Ther* 187: 114-132.
- Belgi A, Bathgate RA, Kocan M, Patil N, Zhang S, Tregear GW, Wade JD, & Hossain MA (2013). Minimum active structure of insulin-like peptide 5. *J Med Chem* 56: 9509-9516.
- Bell GI, Pictet RL, Rutter WJ, Cordell B, Tischer E, & Goodman HM (1980). Sequence of the human insulin gene. *Nature* 284: 26.
- Bell RJ, Eddie LW, Lester AR, Wood EC, Johnston PD, & Niall HD (1987). Relaxin in human pregnancy serum measured with an homologous radioimmunoassay. *Obstet Gynecol* 69: 585-589.
- Belluscio L, Gold GH, Nemes A, & Axel R (1998). Mice deficient in Golf are anosmic. *Neuron* 20: 69-81.
- Ben Aissa M, Lee SH, Bennett BM, & Thatcher GR (2016). Targeting NO/cGMP Signaling in the CNS for neurodegeneration and Alzheimer's disease. *Curr Med Chem* 23: 2770-2788.
- Bennett RG, Heimann DG, Singh S, Simpson RL, & Tuma DJ (2014). Relaxin decreases the severity of established hepatic fibrosis in mice. *Liver Int* 34: 416-426.
- Bennett RG, Kharbanda KK, & Tuma DJ (2003). Inhibition of markers of hepatic stellate cell activation by the hormone relaxin. *Biochem Pharmacol* 66: 867-874.
- Bergeron LH, Willcox JM, Alibhai FJ, Connell BJ, Saleh TM, Wilson BC, & Summerlee AJ (2015). Relaxin peptide hormones are protective during the early stages of ischemic stroke in male rats. *Endocrinology* 156: 638-646.
- Bertrand L, Parent S, Caron M, Legault M, Joly E, Angers S, Bouvier M, Brown M, Houle B, & Ménard L (2002). The BRET2/arrestin assay in stable recombinant cells: a platform to screen for compounds that interact with G protein-coupled receptors (GPCRs). *J Recept Signal Transduct* 22: 533-541.
- Biernacka A, Dobaczewski M, & Frangogiannis NG (2011). TGF-beta signaling in fibrosis. *Growth Factors* 29: 196-202.
- Bigazzi M, Brandi ML, Bani G, & Sacchi TB (1992). Relaxin influences the growth of MCF-7 breast cancer cells. Mitogenic and antimitogenic action depends on peptide concentration. *Cancer* 70: 639-643.

- Binder C, Chuang E, Habla C, Bleckmann A, Schulz M, Bathgate R, & Einspanier A (2014). Relaxins enhance growth of spontaneous murine breast cancers as well as metastatic colonization of the brain. *Clin Exp Metastasis* 31: 57-65.
- Binder C, Hagemann T, Husen B, Schulz M, & Einspanier A (2002). Relaxin enhances in-vitro invasiveness of breast cancer cell lines by up-regulation of matrix metalloproteases. *Mol Hum Reprod* 8: 789-796.
- Binder C, Simon A, Binder L, Hagemann T, Schulz M, Emons G, Trumper L, & Einspanier A (2004). Elevated concentrations of serum relaxin are associated with metastatic disease in breast cancer patients. *Breast Cancer Res Treat* 87: 157-166.
- Blad CC, Tang C, & Offermanns S (2012). G protein-coupled receptors for energy metabolites as new therapeutic targets. *Nature reviews Drug discovery* 11: 603.
- Blessing WA, Okajima SM, Cubria MB, Villa-Camacho JC, Perez-Viloria M, Williamson PM, Sabogal AN, Suarez S, Ang LH, White S, Flynn E, Rodriguez EK, Grinstaff MW, & Nazarian A (2019). Intraarticular injection of relaxin-2 alleviates shoulder arthrofibrosis. *Proc Natl Acad Sci U S A*: 201900355.
- Boccalini G, Sassoli C, Bani D, & Nistri S (2018). Relaxin induces up-regulation of ADAM10 metalloprotease in RXFP1-expressing cells by PI3K/AKT signaling. *Mol Cell Endocrinol* 472: 80-86.
- Boels K, & Schaller HC (2003). Identification and characterisation of GPR100 as a novel human G-protein-coupled bradykinin receptor. *Br J Pharmacol* 140: 932-938.
- Böhm M, Eschenhagen T, Gierschik P, Larisch K, Lensche H, Mende U, Schmitz W, Schnabel P, Scholz H, & Steinfath M (1994). Radioimmunochemical Quantification of Gi α in Right and Left Ventricles from Patients with Ischaemic and Dilated Cardiomyopathy and Predominant Left Ventricular Failure. *J Mol Cell Cardiol* 26: 133-149.
- Bondarev AD, Attwood MM, Jonsson J, Chubarev VN, Tarasov VV, & Schioth HB (2020). Opportunities and challenges for drug discovery in modulating Adhesion G protein-coupled receptor (GPCR) functions. *Expert Opin Drug Discov* 15: 1291-1307.
- Bork NI, Molina CE, & Nikolaev VO (2019). cGMP signalling in cardiomyocyte microdomains. *Biochem Soc Trans* 47: 1327-1339.
- Bortolato A, Dore AS, Hollenstein K, Tehan BG, Mason JS, & Marshall FH (2014). Structure of Class B GPCRs: new horizons for drug discovery. *Br J Pharmacol* 171: 3132-3145.
- Boute N, Jockers R, & Issad T (2002). The use of resonance energy transfer in high-throughput screening: BRET versus FRET. *Trends Pharmacol Sci* 23: 351-354.
- Bruell S, Kong RC, Petrie EJ, Hoare B, Wade JD, Scott DJ, Gooley PR, & Bathgate RA (2013). Chimeric RXFP1 and RXFP2 receptors highlight the similar mechanism of activation utilizing Their N-terminal low-density lipoprotein class A modules. *Front Endocrinol (Lausanne)* 4: 171.
- Buglioni A, & Burnett JC, Jr. (2016). New pharmacological strategies to increase cGMP. *Annu Rev Med* 67: 229-243.
- Bullesbach EE, Gowan LK, Schwabe C, Steinetz BG, O'Byrne E, & Callard IP (1986). Isolation, purification, and the sequence of relaxin from spiny dogfish (*Squalus acanthias*). *Eur J Biochem* 161: 335-341.
- Bullesbach EE, & Schwabe C (1991). Total synthesis of human relaxin and human relaxin derivatives by solid-phase peptide synthesis and site-directed chain combination. *J Biol Chem* 266: 10754-10761.
- Bullesbach EE, & Schwabe C (1994). Functional importance of the A chain loop in relaxin and insulin. *J Biol Chem* 269: 13124-13128.
- Bullesbach EE, & Schwabe C (2000). The relaxin receptor-binding site geometry suggests a novel gripping mode of interaction. *J Biol Chem* 275: 35276-35280.
- Bullesbach EE, & Schwabe C (2005). The trap-like relaxin-binding site of the leucine-rich G-protein-coupled receptor 7. *J Biol Chem* 280: 14051-14056.

- Bullesbach EE, Schwabe C, & Callard IP (1987). Relaxin from an oviparous species, the skate (*Raja erinacea*). *Biochem Biophys Res Commun* 143: 273-280.
- Bullesbach EE, Yang S, & Schwabe C (1992). The receptor-binding site of human relaxin II. A dual prong-binding mechanism. *J Biol Chem* 267: 22957-22960.
- Burazin TC, Bathgate RA, Macris M, Layfield S, Gundlach AL, & Tregear GW (2002). Restricted, but abundant, expression of the novel rat gene-3 (R3) relaxin in the dorsal tegmental region of brain. *J Neurochem* 82: 1553-1557.
- Burger LL, & Sherwood OD (1995). Evidence that cellular proliferation contributes to relaxin-induced growth of both the vagina and the cervix in the pregnant rat. *Endocrinology* 136: 4820-4826.
- Burger LL, & Sherwood OD (1998). Relaxin increases the accumulation of new epithelial and stromal cells in the rat cervix during the second half of pregnancy. *Endocrinology* 139: 3984-3995.
- Burness CB (2015). Sonidegib: first global approval. *Drugs* 75: 1559-1566.
- Burnicka-Turek O, Mohamed BA, Shirneshan K, Thanasupawat T, Hombach-Klonisch S, Klonisch T, & Adham IM (2012). INSL5-deficient mice display an alteration in glucose homeostasis and an impaired fertility. *Endocrinology* 153: 4655-4665.
- Burnicka-Turek O, Shirneshan K, Paprotta I, Grzmil P, Meinhardt A, Engel W, & Adham IM (2009). Inactivation of insulin-like factor 6 disrupts the progression of spermatogenesis at late meiotic prophase. *Endocrinology* 150: 4348-4357.
- Calebiro D, Nikolaev VO, Gagliani MC, de Filippis T, Dees C, Tacchetti C, Persani L, & Lohse MJ (2009). Persistent cAMP-signals triggered by internalized G-protein-coupled receptors. *PLoS Biol* 7: e1000172.
- Callander GE, Thomas WG, & Bathgate RA (2009). Prolonged RXFP1 and RXFP2 signaling can be explained by poor internalization and a lack of beta-arrestin recruitment. *Am J Physiol Cell Physiol* 296: C1058-1066.
- Campeau E, Ruhl VE, Rodier F, Smith CL, Rahmberg BL, Fuss JO, Campisi J, Yaswen P, Cooper PK, & Kaufman PD (2009). A versatile viral system for expression and depletion of proteins in mammalian cells. *PLoS One* 4: e6529.
- Carrell DT, Peterson CM, & Urry RL (1995). The binding of recombinant human relaxin to human spermatozoa. *Endocr Res* 21: 697-707.
- Castoldi AF, Manzo L, & Costa LG (1993). Cyclic GMP formation induced by muscarinic receptors is mediated by nitric oxide synthesis in rat cortical primary cultures. *Brain Res* 610: 57-61.
- Castro LR, Verde I, Cooper DM, & Fischmeister R (2006). Cyclic guanosine monophosphate compartmentation in rat cardiac myocytes. *Circulation* 113: 2221-2228.
- Catapano LA, & Manji HK (2007). G protein-coupled receptors in major psychiatric disorders. *Biochimica et Biophysica Acta (BBA)-Biomembranes* 1768: 976-993.
- Cawston EE, Redmond WJ, Breen CM, Grimsey NL, Connor M, & Glass M (2013). Real-time characterization of cannabinoid receptor 1 (CB1) allosteric modulators reveals novel mechanism of action. *Br J Pharmacol* 170: 893-907.
- Chan LJ, Rosengren KJ, Layfield SL, Bathgate RA, Separovic F, Samuel CS, Hossain MA, & Wade JD (2012). Identification of key residues essential for the structural fold and receptor selectivity within the A-chain of human gene-2 (H2) relaxin. *J Biol Chem* 287: 41152-41164.
- Chang L, & Karin M (2001). Mammalian MAP kinase signalling cascades. *Nature* 410: 37-40.
- Chassin D, Laurent A, Janneau JL, Berger R, & Bellet D (1995). Cloning of a new member of the insulin gene superfamily (INSL4) expressed in human placenta. *Genomics* 29: 465-470.
- Cheah SH, & Sherwood OD (1981). Effects of relaxin on in vivo uterine contractions in conscious and unrestrained estrogen-treated and steroid-untreated ovariectomized rats. *Endocrinology* 109: 2076-2083.
- Chen CZ, Southall N, Xiao J, Marugan JJ, Ferrer M, Hu X, Jones RE, Feng S, AgoulNIK IU, Zheng W, & AgoulNIK AI (2013). Identification of small-molecule agonists of human relaxin family

- receptor 1 (RXFP1) by using a homogenous cell-based cAMP assay. *J Biomol Screen* 18: 670-677.
- Chen GW, Luo X, Liu YL, Jiang Q, Qian XM, & Guo ZY (2011). R171H missense mutation of INSL6 in a patient with spermatogenic failure. *Eur J Med Genet* 54: e455-457.
- Chen H, Levine YC, Golan DE, Michel T, & Lin AJ (2008). Atrial natriuretic peptide-initiated cGMP pathways regulate vasodilator-stimulated phosphoprotein phosphorylation and angiogenesis in vascular endothelium. *J Biol Chem* 283: 4439-4447.
- Chen SA, Perlman AJ, Spanski N, Peterson CM, Sanders SW, Jaffe R, Martin M, Yalcinkaya T, Cefalo RC, Chescheir NC, & et al. (1993). The pharmacokinetics of recombinant human relaxin in nonpregnant women after intravenous, intravaginal, and intracervical administration. *Pharm Res* 10: 834-838.
- Chen W, Shields TS, Stork PJ, & Cone RD (1995). A colorimetric assay for measuring activation of Gs- and Gq-coupled signaling pathways. *Anal Biochem* 226: 349-354.
- Chen X, Bai B, Tian Y, Du H, & Chen J (2014). Identification of serine 348 on the apelin receptor as a novel regulatory phosphorylation site in apelin-13-induced G protein-independent biased signaling. *J Biol Chem* 289: 31173-31187.
- Cheng X, Ji Z, Tsalkova T, & Mei F (2008). Epac and PKA: a tale of two intracellular cAMP receptors. *Acta Biochim Biophys Sin (Shanghai)* 40: 651-662.
- Chow BS, Chew EG, Zhao C, Bathgate RA, Hewitson TD, & Samuel CS (2012). Relaxin signals through a RXFP1-pERK-nNOS-NO-cGMP-dependent pathway to up-regulate matrix metalloproteinases: the additional involvement of iNOS. *PLoS One* 7: e42714.
- Chow BS, Kocan M, Bosnyak S, Sarwar M, Wigg B, Jones ES, Widdop RE, Summers RJ, Bathgate RA, Hewitson TD, & Samuel CS (2014). Relaxin requires the angiotensin II type 2 receptor to abrogate renal interstitial fibrosis. *Kidney Int* 86: 75-85.
- Chow BSM, Kocan M, Shen M, Wang Y, Han L, Chew JY, Wang C, Bosnyak S, Mirabito-Colafella KM, Barsha G, Wigg B, Johnstone EKM, Hossain MA, Pflieger KDG, Denton KM, Widdop RE, Summers RJ, Bathgate RAD, Hewitson TD, & Samuel CS (2019). AT1R-AT2R-RXFP1 functional crosstalk in myofibroblasts: impact on the therapeutic targeting of renal and cardiac fibrosis. *J Am Soc Nephrol*.
- Clegg RM (1995). Fluorescence resonance energy transfer. *Curr Opin Biotechnol* 6: 103-110.
- Collino M, Rogazzo M, Pini A, Benetti E, Rosa AC, Chiazza F, Fantozzi R, Bani D, & Masini E (2013). Acute treatment with relaxin protects the kidney against ischaemia/reperfusion injury. *J Cell Mol Med* 17: 1494-1505.
- Conklin D, Lofton-Day CE, Haldeman BA, Ching A, Whitmore TE, Lok S, & Jaspers S (1999). Identification of INSL5, a new member of the insulin superfamily. *Genomics* 60: 50-56.
- Conole D, & Scott LJ (2013). Riociguat: first global approval. *Drugs* 73: 1967-1975.
- Conrad KP (2016). G-Protein-coupled receptors as potential drug candidates in preeclampsia: targeting the relaxin/insulin-like family peptide receptor 1 for treatment and prevention. *Hum Reprod Update* 22: 647-664.
- Copeland RA (2016). The drug-target residence time model: a 10-year retrospective. *Nat Rev Drug Discov* 15: 87-95.
- Copeland RA, Pompliano DL, & Meek TD (2006). Drug-target residence time and its implications for lead optimization. *Nat Rev Drug Discov* 5: 730-739.
- Costa T, & Herz A (1989). Antagonists with negative intrinsic activity at delta opioid receptors coupled to GTP-binding proteins. *Proc Natl Acad Sci U S A* 86: 7321-7325.
- Daaka Y, Luttrell LM, Ahn S, Della Rocca GJ, Ferguson SS, Caron MG, & Lefkowitz RJ (1998). Essential role for G protein-coupled receptor endocytosis in the activation of mitogen-activated protein kinase. *J Biol Chem* 273: 685-688.
- Dacres H, Michie M, Wang J, Pflieger KD, & Trowell SC (2012). Effect of enhanced Renilla luciferase and fluorescent protein variants on the Förster distance of Bioluminescence resonance energy transfer (BRET). *Biochem Biophys Res Commun* 425: 625-629.

- Dacres H, Wang J, Dumancic MM, & Trowell SC (2010). Experimental determination of the Forster distance for two commonly used bioluminescent resonance energy transfer pairs. *Anal Chem* 82: 432-435.
- Danielson LA, Kercher LJ, & Conrad KP (2000). Impact of gender and endothelin on renal vasodilation and hyperfiltration induced by relaxin in conscious rats. *Am J Physiol Regul Integr Comp Physiol* 279: R1298-1304.
- Danielson LA, Sherwood OD, & Conrad KP (1999). Relaxin is a potent renal vasodilator in conscious rats. *J Clin Invest* 103: 525-533.
- Dann CE, Hsieh JC, Rattner A, Sharma D, Nathans J, & Leahy DJ (2001). Insights into Wnt binding and signalling from the structures of two Frizzled cysteine-rich domains. *Nature* 412: 86-90.
- De A, Loening AM, & Gambhir SS (2007). An improved bioluminescence resonance energy transfer strategy for imaging intracellular events in single cells and living subjects. *Cancer Res* 67: 7175-7183.
- Debrah DO, Novak J, Matthews JE, Ramirez RJ, Shroff SG, & Conrad KP (2006). Relaxin is essential for systemic vasodilation and increased global arterial compliance during early pregnancy in conscious rats. *Endocrinology* 147: 5126-5131.
- Degorce F (2006). HTRF((R)): pioneering technology for high-throughput screening. *Expert Opin Drug Discov* 1: 753-764.
- Degorce F, Card A, Soh S, Trincet E, Knapik GP, & Xie B (2009). HTRF: a technology tailored for drug discovery—a review of theoretical aspects and recent applications. *Curr Genom* 3: 22-32.
- DeLuca M, Dempsey ME, Hori K, Wampler JE, & Cormier MJ (1971). Mechanism of oxidative carbon dioxide production during *Renilla reniformis* bioluminescence. *Proc Natl Acad Sci U S A* 68: 1658-1660.
- Derbyshire ER, & Marletta MA (2012). Structure and regulation of soluble guanylate cyclase. *Annu Rev Biochem* 81: 533-559.
- Desai AJ, & Miller LJ (2018). Changes in the plasma membrane in metabolic disease: impact of the membrane environment on G protein-coupled receptor structure and function. *Br J Pharmacol* 175: 4009-4025.
- Dessauer CW, Scully TT, & Gilman AG (1997). Interactions of forskolin and ATP with the cytosolic domains of mammalian adenylyl cyclase. *J Biol Chem* 272: 22272-22277.
- Devarakonda T, Mauro AG, Guzman G, Hovsepian S, Cain C, Das A, Praveen P, Hossain MA, & Salloum FN (2020). B7-33, a functionally selective relaxin receptor 1 agonist, attenuates myocardial infarction-related adverse cardiac remodeling in mice. *J Am Heart Assoc* 9: e015748.
- Devost D, Sleno R, Petrin D, Zhang A, Shinjo Y, Okde R, Aoki J, Inoue A, & Hebert TE (2017). Conformational Profiling of the AT1 Angiotensin II Receptor Reflects Biased Agonism, G Protein Coupling, and Cellular Context. *J Biol Chem* 292: 5443-5456.
- Diepenhorst NA, Petrie EJ, Chen CZ, Wang A, Hossain MA, Bathgate RA, & Gooley PR (2014). Investigation of interactions at the extracellular loops of the relaxin family peptide receptor 1 (RXFP1). *J Biol Chem* 289: 34938-34952.
- Dimmeler S, Fleming I, Fisslthaler B, Hermann C, Busse R, & Zeiher AM (1999). Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature* 399: 601-605.
- DiPilato LM, Cheng X, & Zhang J (2004). Fluorescent indicators of cAMP and Epac activation reveal differential dynamics of cAMP signaling within discrete subcellular compartments. *Proc Natl Acad Sci U S A* 101: 16513-16518.
- Diwakarla S, Bathgate RAD, Zhang X, Hossain MA, & Furness JB (2020). Colokinetic effect of an insulin-like peptide 5-related agonist of the RXFP4 receptor. *Neurogastroenterol Motil* 32: e13796.

- Dorsam RT, & Gutkind JS (2007). G-protein-coupled receptors and cancer. *Nature reviews cancer* 7: 79.
- Downes GB, & Gautam N (1999). The G protein subunit gene families. *Genomics* 62: 544-552.
- Dschietzig T, Alexiou K, Kinkel HT, Baumann G, Matschke K, & Stangl K (2011). The positive inotropic effect of relaxin-2 in human atrial myocardium is preserved in end-stage heart failure: role of G(i)-phosphoinositide-3 kinase signaling. *J Card Fail* 17: 158-166.
- Dschietzig T, Bartsch C, Richter C, Laule M, Baumann G, & Stangl K (2003). Relaxin, a pregnancy hormone, is a functional endothelin-1 antagonist: attenuation of endothelin-1-mediated vasoconstriction by stimulation of endothelin type-B receptor expression via ERK-1/2 and nuclear factor-kappaB. *Circ Res* 92: 32-40.
- Dschietzig T, Brecht A, Bartsch C, Baumann G, Stangl K, & Alexiou K (2012). Relaxin improves TNF-alpha-induced endothelial dysfunction: the role of glucocorticoid receptor and phosphatidylinositol 3-kinase signalling. *Cardiovasc Res* 95: 97-107.
- Dschietzig T, Teichman S, Unemori E, Wood S, Boehmer J, Richter C, Baumann G, & Stangl K (2009). Intravenous recombinant human relaxin in compensated heart failure: a safety, tolerability, and pharmacodynamic trial. *J Card Fail* 15: 182-190.
- Dschietzig TB (2019). Relaxin-2 for heart failure with preserved ejection fraction (HFpEF): Rationale for future clinical trials. *Mol Cell Endocrinol* 487: 54-58.
- Du XJ, Samuel CS, Gao XM, Zhao L, Parry LJ, & Tregear GW (2003). Increased myocardial collagen and ventricular diastolic dysfunction in relaxin deficient mice: a gender-specific phenotype. *Cardiovasc Res* 57: 395-404.
- Dull T, Zufferey R, Kelly M, Mandel RJ, Nguyen M, Trono D, & Naldini L (1998). A third-generation lentivirus vector with a conditional packaging system. *J Virol* 72: 8463-8471.
- Eddie L, Cameron I, Leeton J, Healy D, & Renou P (1990). Ovarian relaxin is not essential for dilatation of cervix. *Lancet* 336: 243.
- Eddie LW, Bell RJ, Lester A, Geier M, Bennett G, Johnston PD, & Niall HD (1986). Radioimmunoassay of relaxin in pregnancy with an analogue of human relaxin. *Lancet* 1: 1344-1346.
- Eichel K, Jullie D, & von Zastrow M (2016). beta-Arrestin drives MAP kinase signalling from clathrin-coated structures after GPCR dissociation. *Nat Cell Biol* 18: 303-310.
- Eichel K, & von Zastrow M (2018). Subcellular organization of GPCR signaling. *Trends Pharmacol Sci* 39: 200-208.
- Eigenbrot C, Randal M, Quan C, Burnier J, O'Connell L, Rinderknecht E, & Kossiakoff AA (1991). X-ray structure of human relaxin at 1.5Å. *J Mol Biol* 221: 15-21.
- Evans BA, Fu P, & Tregear GW (1994a). Characterization of primate relaxin genes. *Endocrine* 2: 81-86.
- Evans BA, Fu P, & Tregear GW (1994b). Characterization of two relaxin genes in the chimpanzee. *J Endocrinol* 140: 385-392.
- Evans BA, Sato M, Sarwar M, Hutchinson DS, & Summers RJ (2010). Ligand-directed signalling at beta-adrenoceptors. *Br J Pharmacol* 159: 1022-1038.
- Failli P, Nistri S, Quattrone S, Mazzetti L, Bigazzi M, Sacchi TB, & Bani D (2002). Relaxin up-regulates inducible nitric oxide synthase expression and nitric oxide generation in rat coronary endothelial cells. *FASEB J* 16: 252-254.
- Fallowfield JA, Hayden AL, Snowdon VK, Aucott RL, Stutchfield BM, Mole DJ, Pellicoro A, Gordon-Walker TT, Henke A, Schrader J, Trivedi PJ, Princivalle M, Forbes SJ, Collins JE, & Iredale JP (2014). Relaxin modulates human and rat hepatic myofibroblast function and ameliorates portal hypertension in vivo. *Hepatology* 59: 1492-1504.
- Fei DTW, Gross MC, Lofgren JL, Mora-Worms M, & Chen AB (1990). Cyclic AMP response to recombinant human relaxin by cultured human endometrial cells—a specific and high throughput in vitro bioassay. *Biochem Biophys Res Commun* 170: 214-222.
- Feil R, Hofmann F, & Kleppisch T (2005). Function of cGMP-dependent protein kinases in the nervous system. *Rev Neurosci* 16: 23-41.

- Feinstein TN, Wehbi VL, Ardura JA, Wheeler DS, Ferrandon S, Gardella TJ, & Vilardaga JP (2011). Retromer terminates the generation of cAMP by internalized PTH receptors. *Nat Chem Biol* 7: 278-284.
- Feinstein TN, Yui N, Webber MJ, Wehbi VL, Stevenson HP, King JD, Jr., Hallows KR, Brown D, Bouley R, & Vilardaga JP (2013). Noncanonical control of vasopressin receptor type 2 signaling by retromer and arrestin. *J Biol Chem* 288: 27849-27860.
- Feng S, AgoulNIK IU, Bogatcheva NV, Kamat AA, Kwabi-Addo B, Li R, Ayala G, Ittmann MM, & AgoulNIK AI (2007). Relaxin promotes prostate cancer progression. *Clin Cancer Res* 13: 1695-1702.
- Feng S, AgoulNIK IU, Truong A, Li Z, Creighton CJ, Kaftanovskaya EM, Pereira R, Han HD, Lopez-Berestein G, Klonisch T, Ittmann MM, Sood AK, & AgoulNIK AI (2010). Suppression of relaxin receptor RXFP1 decreases prostate cancer growth and metastasis. *Endocr Relat Cancer* 17: 1021-1033.
- Ferguson SS (2001). Evolving concepts in G protein-coupled receptor endocytosis: the role in receptor desensitization and signaling. *Pharmacol Rev* 53: 1-24.
- Ferlin A, Pepe A, Ganesello L, Garolla A, Feng S, Giannini S, Zaccolo M, Faccioli A, Morello R, AgoulNIK AI, & Foresta C (2008). Mutations in the insulin-like factor 3 receptor are associated with osteoporosis. *J Bone Miner Res* 23: 683-693.
- Ferlin A, Perilli L, Ganesello L, Tagliavero G, & Foresta C (2011). Profiling insulin like factor 3 (INSL3) signaling in human osteoblasts. *PLoS One* 6: e29733.
- Ferrandon S, Feinstein TN, Castro M, Wang B, Bouley R, Potts JT, Gardella TJ, & Vilardaga JP (2009). Sustained cyclic AMP production by parathyroid hormone receptor endocytosis. *Nat Chem Biol* 5: 734-742.
- Fevold HL, Hisaw FL, & Meyer RK (1930). The relaxative hormone of the corpus luteum. Its purification and concentration. *Journal of the American Chemical Society* 52: 3340-3348.
- Forster T (1948). Zwischenmolekulare Energiewanderung Und Fluoreszenz. *Annalen Der Physik* 2: 55-75.
- Fosbrink M, Aye-Han NN, Cheong R, Levchenko A, & Zhang J (2010). Visualization of JNK activity dynamics with a genetically encoded fluorescent biosensor. *Proc Natl Acad Sci U S A* 107: 5459-5464.
- Francis SH, Blount MA, & Corbin JD (2011). Mammalian cyclic nucleotide phosphodiesterases: molecular mechanisms and physiological functions. *Physiol Rev* 91: 651-690.
- Francis SH, Busch JL, Corbin JD, & Sibley D (2010). cGMP-dependent protein kinases and cGMP phosphodiesterases in nitric oxide and cGMP action. *Pharmacol Rev* 62: 525-563.
- Fredriksson R, Lagerstrom MC, Lundin LG, & Schioth HB (2003). The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints. *Mol Pharmacol* 63: 1256-1272.
- Friebe A, Sandner P, & Schmidtko A (2020). cGMP: a unique 2nd messenger molecule - recent developments in cGMP research and development. *Naunyn Schmiedebergs Arch Pharmacol* 393: 287-302.
- Fulton D, Gratton JP, McCabe TJ, Fontana J, Fujio Y, Walsh K, Franke TF, Papapetropoulos A, & Sessa WC (1999). Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. *Nature* 399: 597-601.
- Fung BK, Hurley JB, & Stryer L (1981). Flow of information in the light-triggered cyclic nucleotide cascade of vision. *Proc Natl Acad Sci U S A* 78: 152-156.
- Furchgott RF, & Vanhoutte PM (1989). Endothelium-derived relaxing and contracting factors. *The FASEB Journal* 3: 2007-2018.
- Garibay-Tupas JL, Bao S, Kim MT, Tashima LS, & Bryant-Greenwood GD (2000). Isolation and analysis of the 3'-untranslated regions of the human relaxin H1 and H2 genes. *J Mol Endocrinol* 24: 241-252.
- Garland SL (2013). Are GPCRs still a source of new targets? *J Biomol Screen* 18: 947-966.

- Gidon A, Al-Bataineh MM, Jean-Alphonse FG, Stevenson HP, Watanabe T, Louet C, Khatri A, Calero G, Pastor-Soler NM, Gardella TJ, & Vilardaga JP (2014). Endosomal GPCR signaling turned off by negative feedback actions of PKA and v-ATPase. *Nat Chem Biol* 10: 707-709.
- Glogowska A, Kunanuvat U, Stetefeld J, Patel TR, Thanasupawat T, Krcek J, Weber E, Wong GW, Del Bigio MR, Hoang-Vu C, Hombach-Klonisch S, & Klonisch T (2013). C1q-tumour necrosis factor-related protein 8 (CTRP8) is a novel interaction partner of relaxin receptor RXFP1 in human brain cancer cells. *J Pathol* 231: 466-479.
- Godbole A, Lyga S, Lohse MJ, & Calebiro D (2017). Internalized TSH receptors en route to the TGN induce local Gs-protein signaling and gene transcription. *Nat Commun* 8: 443.
- Goldsmith LT, Weiss G, Palejwala S, Plant TM, Wojtczuk A, Lambert WC, Ammur N, Heller D, Skurnick JH, Edwards D, & Cole DM (2004). Relaxin regulation of endometrial structure and function in the rhesus monkey. *Proc Natl Acad Sci U S A* 101: 4685-4689.
- Goldsmith ZG, & Dhanasekaran DN (2007). G protein regulation of MAPK networks. *Oncogene* 26: 3122-3142.
- Gomes I, Ayoub MA, Fujita W, Jaeger WC, Pflieger KD, & Devi LA (2016). G protein-coupled receptor heteromers. *Annu Rev Pharmacol Toxicol* 56: 403-425.
- Gooi JH, Richardson ML, Jelinic M, Girling JE, Wlodek ME, Tare M, & Parry LJ (2013). Enhanced uterine artery stiffness in aged pregnant relaxin mutant mice is reversed with exogenous relaxin treatment. *Biol Reprod* 89: 18.
- Gorlov IP, Kamat A, Bogatcheva NV, Jones E, Lamb DJ, Truong A, Bishop CE, McElreavey K, & AgoulNIK AI (2002). Mutations of the GREAT gene cause cryptorchidism. *Hum Mol Genet* 11: 2309-2318.
- Gottesman-Katz L, Latorre R, Vanner S, Schmidt BL, & Bunnett NW (2020). Targeting G protein-coupled receptors for the treatment of chronic pain in the digestive system. *Gut*.
- Gotz KR, Sprenger JU, Perera RK, Steinbrecher JH, Lehnart SE, Kuhn M, Gorelik J, Balligand JL, & Nikolaev VO (2014). Transgenic mice for real-time visualization of cGMP in intact adult cardiomyocytes. *Circ Res* 114: 1235-1245.
- Graham EF, & Dracy AE (1953). The effect of relaxin and mechanical dilation on the bovine cervix. *J Dairy Sci* 36: 772-777.
- Greene SJ, Gheorghiade M, Borlaug BA, Pieske B, Vaduganathan M, Burnett JC, Jr., Roessig L, Stasch JP, Solomon SD, Paulus WJ, & Butler J (2013). The cGMP signaling pathway as a therapeutic target in heart failure with preserved ejection fraction. *J Am Heart Assoc* 2: e000536.
- Grosse J, Heffron H, Burling K, Akhter Hossain M, Habib AM, Rogers GJ, Richards P, Larder R, Rimmington D, Adriaenssens AA, Parton L, Powell J, Binda M, Colledge WH, Doran J, Toyoda Y, Wade JD, Aparicio S, Carlton MB, Coll AP, Reimann F, O'Rahilly S, & Gribble FM (2014). Insulin-like peptide 5 is an orexigenic gastrointestinal hormone. *Proc Natl Acad Sci U S A* 111: 11133-11138.
- Grundmann M, Merten N, Malfacini D, Inoue A, Preis P, Simon K, Ruttiger N, Ziegler N, Benkel T, Schmitt NK, Ishida S, Muller I, Reher R, Kawakami K, Inoue A, Rick U, Kuhl T, Imhof D, Aoki J, Konig GM, Hoffmann C, Gomeza J, Wess J, & Kostenis E (2018). Lack of beta-arrestin signaling in the absence of active G proteins. *Nat Commun* 9: 341.
- Gunnersen JM, Crawford RJ, & Tregear GW (1995). Expression of the relaxin gene in rat tissues. *Mol Cell Endocrinol* 110: 55-64.
- Gunnersen JM, Fu P, Roche PJ, & Tregear GW (1996). Expression of human relaxin genes: characterization of a novel alternatively-spliced human relaxin mRNA species. *Mol Cell Endocrinol* 118: 85-94.
- Gurney A, Axelrod F, Bond CJ, Cain J, Chartier C, Donigan L, Fischer M, Chaudhari A, Ji M, Kapoun AM, Lam A, Lazetic S, Ma S, Mitra S, Park IK, Pickell K, Sato A, Satyal S, Stroud M, Tran H, Yen WC, Lewicki J, & Hoey T (2012). Wnt pathway inhibition via the targeting of Frizzled receptors results in decreased growth and tumorigenicity of human tumors. *Proc Natl Acad Sci U S A* 109: 11717-11722.

- Haddock SH, Moline MA, & Case JF (2010). Bioluminescence in the sea. *Ann Rev Mar Sci* 2: 443-493.
- Hahn WC, Counter CM, Lundberg AS, Beijersbergen RL, Brooks MW, & Weinberg RA (1999). Creation of human tumour cells with defined genetic elements. *Nature* 400: 464-468.
- Haley J, Hudson P, Scanlon D, John M, Cronk M, Shine J, Tregear G, & Niall H (1982). Porcine relaxin: molecular cloning and cDNA structure. *DNA* 1: 155-162.
- Hall K, & Newton WH (1947). The effect of oestrone and relaxin on the x-ray appearance of the pelvis of the mouse. *J Physiol* 106: 18-27.
- Hall MP, Unch J, Binkowski BF, Valley MP, Butler BL, Wood MG, Otto P, Zimmerman K, Vidugiris G, Machleidt T, Robers MB, Benink HA, Eggers CT, Slater MR, Meisenheimer PL, Klaubert DH, Fan F, Encell LP, & Wood KV (2012). Engineered luciferase reporter from a deep sea shrimp utilizing a novel imidazopyrazinone substrate. *ACS Chem Biol* 7: 1848-1857.
- Halls ML, Bathgate RA, & Summers RJ (2006). Relaxin family peptide receptors RXFP1 and RXFP2 modulate cAMP signaling by distinct mechanisms. *Mol Pharmacol* 70: 214-226.
- Halls ML, Bathgate RA, & Summers RJ (2007). Comparison of signaling pathways activated by the relaxin family peptide receptors, RXFP1 and RXFP2, using reporter genes. *J Pharmacol Exp Ther* 320: 281-290.
- Halls ML, van der Westhuizen ET, Wade JD, Evans BA, Bathgate RA, & Summers RJ (2009). Relaxin family peptide receptor (RXFP1) coupling to G(α)i3 involves the C-terminal Arg752 and localization within membrane Raft Microdomains. *Mol Pharmacol* 75: 415-428.
- Hamann J, Aust G, Arac D, Engel FB, Formstone C, Fredriksson R, Hall RA, Harty BL, Kirchhoff C, Knapp B, Krishnan A, Liebscher I, Lin HH, Martinelli DC, Monk KR, Peeters MC, Piao X, Promel S, Schoneberg T, Schwartz TW, Singer K, Stacey M, Ushkaryov YA, Vallon M, Wolfrum U, Wright MW, Xu L, Langenhan T, & Schioth HB (2015). International Union of Basic and Clinical Pharmacology. XCIV. Adhesion G protein-coupled receptors. *Pharmacol Rev* 67: 338-367.
- Hamdan FF, Audet M, Garneau P, Pelletier J, & Bouvier M (2005). High-throughput screening of G protein-coupled receptor antagonists using a bioluminescence resonance energy transfer 1-based β -arrestin2 recruitment assay. *J Biomol Screen* 10: 463-475.
- Hamolsky M, & Sparrow RC (1945). Influence of relaxin on mammary development in sexually immature female rats. *Proc Soc Exp Biol Med* 60: 8.
- Hansell DJ, Bryant-Greenwood GD, & Greenwood FC (1991). Expression of the human relaxin H1 gene in the decidua, trophoblast, and prostate. *J Clin Endocrinol Metab* 72: 899-904.
- Hartley JL, Temple GF, & Brasch MA (2000). DNA cloning using in vitro site-specific recombination. *Genome Res* 10: 1788-1795.
- Harvey CD, Ehrhardt AG, Cellurale C, Zhong H, Yasuda R, Davis RJ, & Svoboda K (2008). A genetically encoded fluorescent sensor of ERK activity. *Proc Natl Acad Sci U S A* 105: 19264-19269.
- Haugaard-Kedstrom LM, Hossain MA, Daly NL, Bathgate RA, Rinderknecht E, Wade JD, Craik DJ, & Rosengren KJ (2015). Solution structure, aggregation behavior, and flexibility of human relaxin-2. *ACS Chem Biol* 10: 891-900.
- Haugaard-Kedstrom LM, Shabanpoor F, Hossain MA, Clark RJ, Ryan PJ, Craik DJ, Gundlach AL, Wade JD, Bathgate RA, & Rosengren KJ (2011). Design, synthesis, and characterization of a single-chain peptide antagonist for the relaxin-3 receptor RXFP3. *J Am Chem Soc* 133: 4965-4974.
- Hauser AS, Attwood MM, Rask-Andersen M, Schioth HB, & Gloriam DE (2017). Trends in GPCR drug discovery: new agents, targets and indications. *Nat Rev Drug Discov* 16: 829-842.
- Hay DL, & Pioszak AA (2016). Receptor activity-modifying proteins (RAMPs): new insights and roles. *Annu Rev Pharmacol Toxicol* 56: 469-487.
- Hayflick JS (2000). A family of heptahelical receptors with adhesion-like domains: a marriage between two super families. *J Recept Signal Transduct* 20: 119-131.

- Heeg MH, Koziolk MJ, Vasko R, Schaefer L, Sharma K, Muller GA, & Strutz F (2005). The antifibrotic effects of relaxin in human renal fibroblasts are mediated in part by inhibition of the Smad2 pathway. *Kidney Int* 68: 96-109.
- Heng K, Ivell R, Wagaarachchi P, & Anand-Ivell R (2008). Relaxin signalling in primary cultures of human myometrial cells. *Mol Hum Reprod* 14: 603-611.
- Hermans E (2003). Biochemical and pharmacological control of the multiplicity of coupling at G-protein-coupled receptors. *Pharmacol Ther* 99: 25-44.
- Hill SJ, Baker JG, & Rees S (2001). Reporter-gene systems for the study of G-protein-coupled receptors. *Curr Opin Pharmacol* 1: 526-532.
- Hill SJ, Williams C, & May LT (2010). Insights into GPCR pharmacology from the measurement of changes in intracellular cyclic AMP; advantages and pitfalls of differing methodologies. *Br J Pharmacol* 161: 1266-1275.
- Hirai T, & Chida K (2003). Protein kinase C ζ (PKC ζ): activation mechanisms and cellular functions. *J Biochem* 133: 1-7.
- Hisaw FL (1925). The influence of the ovary on the resorption of the pubic bones of the pocket gopher, *Geomys bursarius* (Shaw). *J Exp Zool* 42: 411-441.
- Hisaw FL (1926). Experimental relaxation of the pubic ligament of the guinea pig. *P Soc Exp Biol Med* 23: 661-663.
- Hisaw FL (1927). Experimental relaxation of the symphysis pubis of the guinea pig. *Anat Rec* 37: 126.
- Hisaw FL (1929). The corpus luteum hormone. I. Experimental relaxation of the pelvic ligaments of the guinea pig. *Physiol Zool* 2: 59-79.
- Hisaw FL, Fevold HL, & Meyer RK (1930). The corpus luteum hormone. II. Methods of extraction. *Physiol Zool* 3: 135-144.
- Hisaw FL, Hisaw Jr FL, & Dawson AB (1967). Effects of relaxin on the endothelium of endometrial blood vessels in monkeys (*Macaca mulatto*). *Endocrinology* 81: 375-385.
- Hisaw FL, Meyer RK, & Weichert CK (1928). Inhibition of ovulation and associated histological changes. *P Soc Exp Biol Med* 25: 754-756.
- Hisaw FL, & Zarrow MX (1950). The physiology of relaxin. In *Vitam Horm*. Elsevier, pp 151-178.
- Hisaw FL, Zarrow MX, Money WL, Talmage RVN, & Abramowitz AA (1944). Importance of the female reproductive tract in the formation of relaxin. *Endocrinology* 34: 122-134.
- Hoare BL, Bruell S, Sethi A, Gooley PR, Lew MJ, Hossain MA, Inoue A, Scott DJ, & Bathgate RAD (2019). Multi-component mechanism of H2 relaxin binding to RXFP1 through NanoBRET kinetic analysis. *iScience* 11: 93-113.
- Hoare SR (2005). Mechanisms of peptide and nonpeptide ligand binding to Class B G-protein-coupled receptors. *Drug Discov Today* 10: 417-427.
- Hoare SRJ, Tewson PH, Quinn AM, Hughes TE, & Bridge LJ (2020). Analyzing kinetic signaling data for G-protein-coupled receptors. *Sci Rep* 10: 12263.
- Hofmann F, Biel M, Feil R, & Kleppisch T (2004). Mouse models of NO/natriuretic peptide/cGMP kinase signaling. In *Transgenic Models in Pharmacology*. Springer, pp 95-130.
- Hojo K, Hossain MA, Tailhades J, Shabanpoor F, Wong LL, Ong-Palsson EE, Kastman HE, Ma S, Gundlach AL, Rosengren KJ, Wade JD, & Bathgate RA (2016). Development of a single-chain peptide agonist of the relaxin-3 receptor using hydrocarbon stapling. *J Med Chem* 59: 7445-7456.
- Hombach-Klonisch S, Bialek J, Trojanowicz B, Weber E, Holzhausen HJ, Silvertown JD, Summerlee AJ, Dralle H, Hoang-Vu C, & Klonisch T (2006). Relaxin enhances the oncogenic potential of human thyroid carcinoma cells. *Am J Pathol* 169: 617-632.
- Honda A, Adams SR, Sawyer CL, Lev-Ram V, Tsien RY, & Dostmann WR (2001). Spatiotemporal dynamics of guanosine 3',5'-cyclic monophosphate revealed by a genetically encoded, fluorescent indicator. *Proc Natl Acad Sci U S A* 98: 2437-2442.
- Hopkins AL, & Groom CR (2002). The druggable genome. *Nat Rev Drug Discov* 1: 727-730.

- Hopkins EJ, Bathgate RA, & Gooley PR (2005). The human LGR7 low-density lipoprotein class A module requires calcium for structure. *Ann N Y Acad Sci* 1041: 27-34.
- Hopkins EJ, Layfield S, Ferraro T, Bathgate RA, & Gooley PR (2007). The NMR solution structure of the relaxin (RXFP1) receptor lipoprotein receptor class A module and identification of key residues in the N-terminal region of the module that mediate receptor activation. *J Biol Chem* 282: 4172-4184.
- Hossain MA, Bathgate RA, Kong CK, Shabanpoor F, Zhang S, Haugaard-Jonsson LM, Rosengren KJ, Tregear GW, & Wade JD (2008a). Synthesis, conformation, and activity of human insulin-like peptide 5 (INSL5). *Chembiochem* 9: 1816-1822.
- Hossain MA, & Bathgate RAD (2018). Challenges in the design of insulin and relaxin/insulin-like peptide mimetics. *Bioorg Med Chem* 26: 2827-2841.
- Hossain MA, Kocan M, Yao ST, Royce SG, Nair VB, Siwek C, Patil NA, Harrison IP, Rosengren KJ, Selemidis S, Summers RJ, Wade JD, Bathgate RAD, & Samuel CS (2016). A single-chain derivative of the relaxin hormone is a functionally selective agonist of the G protein-coupled receptor, RXFP1. *Chem Sci* 7: 3805-3819.
- Hossain MA, Rosengren KJ, Haugaard-Jonsson LM, Zhang S, Layfield S, Ferraro T, Daly NL, Tregear GW, Wade JD, & Bathgate RA (2008b). The A-chain of human relaxin family peptides has distinct roles in the binding and activation of the different relaxin family peptide receptors. *J Biol Chem* 283: 17287-17297.
- Hossain MA, Rosengren KJ, Samuel CS, Shabanpoor F, Chan LJ, Bathgate RA, & Wade JD (2011). The minimal active structure of human relaxin-2. *J Biol Chem* 286: 37555-37565.
- Hossain MA, & Wade JD (2014). Synthetic relaxins. *Curr Opin Chem Biol* 22: 47-55.
- Hothersall JD, Brown AJ, Dale I, & Rawlins P (2016). Can residence time offer a useful strategy to target agonist drugs for sustained GPCR responses? *Drug Discov Today* 21: 90-96.
- Hsu SY (1999). Cloning of two novel mammalian paralogs of relaxin/insulin family proteins and their expression in testis and kidney. *Mol Endocrinol* 13: 2163-2174.
- Hsu SY, Kudo M, Chen T, Nakabayashi K, Bhalla A, van der Spek PJ, van Duin M, & Hsueh AJ (2000). The three subfamilies of leucine-rich repeat-containing G protein-coupled receptors (LGR): identification of LGR6 and LGR7 and the signaling mechanism for LGR7. *Mol Endocrinol* 14: 1257-1271.
- Hsu SY, Nakabayashi K, Nishi S, Kumagai J, Kudo M, Sherwood OD, & Hsueh AJ (2002). Activation of orphan receptors by the hormone relaxin. *Science* 295: 671-674.
- Huang CJ, Li Y, & Anderson LL (1997). Relaxin and estrogen synergistically accelerate growth and development in the uterine cervix of prepubertal pigs. *Anim Reprod Sci* 46: 149-158.
- Huang JR, Tseng L, Bischof P, & Janne OA (1987). Regulation of prolactin production by progestin, estrogen, and relaxin in human endometrial stromal cells. *Endocrinology* 121: 2011-2017.
- Huang X, Gai Y, Yang N, Lu B, Samuel CS, Thannickal VJ, & Zhou Y (2011). Relaxin regulates myofibroblast contractility and protects against lung fibrosis. *Am J Pathol* 179: 2751-2765.
- Huang Z, Myhr C, Bathgate RA, Ho BA, Bueno A, Hu X, Xiao J, Southall N, Barnaeva E, AgoulNIK IU, Marugan JJ, Ferrer M, & AgoulNIK AI (2015). Activation of relaxin family receptor 1 from different mammalian species by relaxin peptide and small-molecule agonist ML290. *Front Endocrinol (Lausanne)* 6: 128.
- Hudson P, Haley J, Cronk M, Shine J, & Niall H (1981). Molecular cloning and characterization of cDNA sequences coding for rat relaxin. *Nature* 291: 127-131.
- Hudson P, Haley J, John M, Cronk M, Crawford R, Haralambidis J, Tregear G, Shine J, & Niall H (1983). Structure of a genomic clone encoding biologically active human relaxin. *Nature* 301: 628-631.
- Hudson P, John M, Crawford R, Haralambidis J, Scanlon D, Gorman J, Tregear G, Shine J, & Niall H (1984). Relaxin gene expression in human ovaries and the predicted structure of a human preprorelaxin by analysis of cDNA clones. *EMBO J* 3: 2333-2339.

- Hunter MR, Finlay DB, Macdonald CE, Cawston EE, Grimsey NL, & Glass M (2017). Real-time measurement of cannabinoid receptor-mediated cAMP signaling. In *Methods Enzymol.* pp 43-59.
- Hurley WL, Doane RM, O'Day-Bowman MB, Winn RJ, Mojonier LE, & Sherwood OD (1991). Effect of relaxin on mammary development in ovariectomized pregnant gilts. *Endocrinology* 128: 1285-1290.
- Hwang JJ, Shanks RD, & Sherwood OD (1989). Monoclonal antibodies specific for rat relaxin. IV. Passive immunization with monoclonal antibodies during the antepartum period reduces cervical growth and extensibility, disrupts birth, and reduces pup survival in intact rats. *Endocrinology* 125: 260-266.
- Hwang JJ, & Sherwood OD (1988). Monoclonal antibodies specific for rat relaxin. III. Passive immunization with monoclonal antibodies throughout the second half of pregnancy reduces cervical growth and extensibility in intact rats. *Endocrinology* 123: 2486-2490.
- Irannejad R, Pessino V, Mika D, Huang B, Wedegaertner PB, Conti M, & von Zastrow M (2017). Functional selectivity of GPCR-directed drug action through location bias. *Nat Chem Biol* 13: 799.
- Irannejad R, Tomshine JC, Tomshine JR, Chevalier M, Mahoney JP, Steyaert J, Rasmussen SG, Sunahara RK, El-Samad H, Huang B, & von Zastrow M (2013). Conformational biosensors reveal GPCR signalling from endosomes. *Nature* 495: 534-538.
- Ischiropoulos H, & Gow A (2005). Pathophysiological functions of nitric oxide-mediated protein modifications. *Toxicology* 208: 299-303.
- Ivell R, Balvers M, Domagalski R, Ungefroren H, Hunt N, & Schulze W (1997). Relaxin-like factor: a highly specific and constitutive new marker for Leydig cells in the human testis. *Mol Hum Reprod* 3: 459-466.
- Jain R, Watson U, Vasudevan L, & Saini DK (2018). ERK activation pathways downstream of GPCRs. *Int Rev Cell Mol Biol* 338: 79-109.
- James R, Niall H, Kwok S, & Bryand-Greenwood G (1977). Primary structure of porcine relaxin: homology with insulin and related growth factors. *Nature* 267: 544-546.
- Jean-Alphonse FG, Wehbi VL, Chen J, Noda M, Taboas JM, Xiao K, & Vilardaga JP (2017). beta2-adrenergic receptor control of endosomal PTH receptor signaling via Gbetagamma. *Nat Chem Biol* 13: 259-261.
- Jelinic M, Marshall SA, Stewart D, Unemori E, Parry LJ, & Leo CH (2018). Peptide hormone relaxin: from bench to bedside. *Am J Physiol Regul Integr Comp Physiol* 314: R753-R760.
- Jelinic M, Tare M, Conrad KP, & Parry LJ (2015). Differential effects of relaxin deficiency on vascular aging in arteries of male mice. *Age (Dordr)* 37: 9803.
- Jensen DD, Godfrey CB, Niklas C, Canals M, Kocan M, Poole DP, Murphy JE, Alemi F, Cottrell GS, Korbmacher C, Lambert NA, Bunnett NW, & Corvera CU (2013). The bile acid receptor TGR5 does not interact with beta-arrestins or traffic to endosomes but transmits sustained signals from plasma membrane rafts. *J Biol Chem* 288: 22942-22960.
- Jensen DD, Lieu T, Halls ML, Veldhuis NA, Imlach WL, Mai QN, Poole DP, Quach T, Aurelio L, Conner J, Herenbrink CK, Barlow N, Simpson JS, Scanlon MJ, Graham B, McCluskey A, Robinson PJ, Escriou V, Nassini R, Materazzi S, Geppetti P, Hicks GA, Christie MJ, Porter CJH, Canals M, & Bunnett NW (2017). Neurokinin 1 receptor signaling in endosomes mediates sustained nociception and is a viable therapeutic target for prolonged pain relief. *Sci Transl Med* 9.
- Jiang LI, Collins J, Davis R, Lin KM, DeCamp D, Roach T, Hsueh R, Rebres RA, Ross EM, Taussig R, Fraser I, & Sternweis PC (2007). Use of a cAMP BRET sensor to characterize a novel regulation of cAMP by the sphingosine 1-phosphate/G13 pathway. *J Biol Chem* 282: 10576-10584.
- Jimenez-Vargas NN, Gong J, Wisdom MJ, Jensen DD, Latorre R, Hegron A, Teng S, DiCello JJ, Rajasekhar P, Veldhuis NA, Carbone SE, Yu Y, Lopez-Lopez C, Jaramillo-Polanco J, Canals M, Reed DE, Lomax AE, Schmidt BL, Leong KW, Vanner SJ, Halls ML, Bunnett NW, & Poole DP

- (2020). Endosomal signaling of delta opioid receptors is an endogenous mechanism and therapeutic target for relief from inflammatory pain. *Proc Natl Acad Sci U S A*.
- Jimenez-Vargas NN, Pattison LA, Zhao P, Lieu T, Latorre R, Jensen DD, Castro J, Aurelio L, Le GT, Flynn B, Herenbrink CK, Yeatman HR, Edgington-Mitchell L, Porter CJH, Halls ML, Canals M, Veldhuis NA, Poole DP, McLean P, Hicks GA, Scheff N, Chen E, Bhattacharya A, Schmidt BL, Brierley SM, Vanner SJ, & Bunnett NW (2018). Protease-activated receptor-2 in endosomes signals persistent pain of irritable bowel syndrome. *Proc Natl Acad Sci U S A* 115: E7438-E7447.
- John MJ, Borjesson BW, Walsh JR, & Niall HD (1981). Limited sequence homology between porcine and rat relaxins: implications for physiological studies. *Endocrinology* 108: 726-729.
- Kaftanovskaya EM, Ng HH, Soula M, Rivas B, Myhr C, Ho BA, Cervantes BA, Shupe TD, Devarasetty M, Hu X, Xu X, Patnaik S, Wilson KJ, Barnaeva E, Ferrer M, Southall NT, Marugan JJ, Bishop CE, AgoulNIK IU, & AgoulNIK AI (2019). Therapeutic effects of a small molecule agonist of the relaxin receptor ML290 in liver fibrosis. *FASEB J*: fj201901046R.
- Kaftanovskaya EM, Soula M, Myhr C, Ho BA, Moore SN, Yoo C, Cervantes B, How J, Marugan J, AgoulNIK IU, & AgoulNIK AI (2017). Human relaxin receptor is fully functional in humanized mice and is activated by small molecule agonist ML290. *J Endocr Soc* 1: 712-725.
- Kamat AA, Feng S, AgoulNIK IU, Kheradmand F, Bogatcheva NV, Coffey D, Sood AK, & AgoulNIK AI (2006). The role of relaxin in endometrial cancer. *Cancer Biol Ther* 5: 71-77.
- Kamat AA, Feng S, Bogatcheva NV, Truong A, Bishop CE, & AgoulNIK AI (2004). Genetic targeting of relaxin and insulin-like factor 3 receptors in mice. *Endocrinology* 145: 4712-4720.
- Kanai AJ, Konieczko EM, Bennett RG, Samuel CS, & Royce SG (2019). Relaxin and fibrosis: Emerging targets, challenges, and future directions. *Mol Cell Endocrinol* 487: 66-74.
- Kasik J, Muglia L, Stephan DA, & Menon RK (2000). Identification, chromosomal mapping, and partial characterization of mouse *InsI6*: a new member of the insulin family. *Endocrinology* 141: 458-461.
- Katzen F (2007). Gateway((R)) recombinational cloning: a biological operating system. *Expert Opin Drug Discov* 2: 571-589.
- Kaupp UB, & Seifert R (2002). Cyclic nucleotide-gated ion channels. *Physiol Rev* 82: 769-824.
- Kawamura K, Kumagai J, Sudo S, Chun SY, Pisarska M, Morita H, Toppari J, Fu P, Wade JD, Bathgate RA, & Hsueh AJ (2004). Paracrine regulation of mammalian oocyte maturation and male germ cell survival. *Proc Natl Acad Sci U S A* 101: 7323-7328.
- Kenakin T (2002). Efficacy at G-protein-coupled receptors. *Nat Rev Drug Discov* 1: 103-110.
- Kenakin T (2017). Signaling bias in drug discovery. *Expert Opin Drug Discov* 12: 321-333.
- Kenakin T (2019). Biased receptor signaling in drug discovery. *Pharmacol Rev* 71: 267-315.
- Kenakin T, & Christopoulos A (2013). Signalling bias in new drug discovery: detection, quantification and therapeutic impact. *Nat Rev Drug Discov* 12: 205-216.
- Kenakin TP (2009). Cellular assays as portals to seven-transmembrane receptor-based drug discovery. *Nat Rev Drug Discov* 8: 617-626.
- Kern A, & Bryant-Greenwood GD (2009). Characterization of relaxin receptor (RXFP1) desensitization and internalization in primary human decidual cells and RXFP1-transfected HEK293 cells. *Endocrinology* 150: 2419-2428.
- Kern A, Hubbard D, Amano A, & Bryant-Greenwood GD (2008). Cloning, expression, and functional characterization of relaxin receptor (leucine-rich repeat-containing g protein-coupled receptor 7) splice variants from human fetal membranes. *Endocrinology* 149: 1277-1294.
- Khan-Dawood FS, Goldsmith LT, Weiss G, & Dawood MY (1989). Human corpus luteum secretion of relaxin, oxytocin, and progesterone. *J Clin Endocrinol Metab* 68: 627-631.
- Khan SM, Sleno R, Gora S, Zylbergold P, Laverdure JP, Labbe JC, Miller GJ, & Hebert TE (2013). The expanding roles of Gbetagamma subunits in G protein-coupled receptor signaling and drug action. *Pharmacol Rev* 65: 545-577.

- Khanna D, Clements PJ, Furst DE, Korn JH, Ellman M, Rothfield N, Wigley FM, Moreland LW, Silver R, Kim YH, Steen VD, Firestein GS, Kavanaugh AF, Weisman M, Mayes MD, Collier D, Csuka ME, Simms R, Merkel PA, Medsger TA, Jr., Sanders ME, Maranian P, Seibold JR, Relaxin I, & the Scleroderma Clinical Trials C (2009). Recombinant human relaxin in the treatment of systemic sclerosis with diffuse cutaneous involvement: a randomized, double-blind, placebo-controlled trial. *Arthritis Rheum* 60: 1102-1111.
- Kinnamon SC (2009). Umami taste transduction mechanisms. *Am J Clin Nutr* 90: 753S-755S.
- Klein Herenbrink C, Sykes DA, Donthamsetti P, Canals M, Coudrat T, Shonberg J, Scammells PJ, Capuano B, Sexton PM, Charlton SJ, Javitch JA, Christopoulos A, & Lane JR (2016). The role of kinetic context in apparent biased agonism at GPCRs. *Nat Commun* 7: 10842.
- Kliwer A, Gillis A, Hill R, Schmidel F, Bailey C, Kelly E, Henderson G, Christie MJ, & Schulz S (2020). Morphine-induced respiratory depression is independent of beta-arrestin2 signalling. *Br J Pharmacol*.
- Kniazeff J, Prezeau L, Rondard P, Pin JP, & Goudet C (2011). Dimers and beyond: The functional puzzles of class C GPCRs. *Pharmacol Ther* 130: 9-25.
- Kobayashi H, Picard LP, Schonegge AM, & Bouvier M (2019). Bioluminescence resonance energy transfer-based imaging of protein-protein interactions in living cells. *Nat Protoc* 14: 1084-1107.
- Kocan M, Dalrymple MB, Seeber RM, Feldman BJ, & Pflieger KD (2010). Enhanced BRET technology for the monitoring of agonist-induced and agonist-independent interactions between GPCRs and beta-arrestins. *Front Endocrinol (Lausanne)* 1: 12.
- Kocan M, Sarwar M, Ang SY, Xiao J, Marugan JJ, Hossain MA, Wang C, Hutchinson DS, Samuel CS, AgoulNIK AI, Bathgate RAD, & Summers RJ (2017). ML290 is a biased allosteric agonist at the relaxin receptor RXFP1. *Sci Rep* 7: 2968.
- Kocan M, Sarwar M, Hossain MA, Wade JD, & Summers RJ (2014). Signalling profiles of H3 relaxin, H2 relaxin and R3(BD Δ 23-27)R/I5 acting at the relaxin family peptide receptor 3 (RXFP3). *Br J Pharmacol* 171: 2827-2841.
- Kocan M, See HB, Seeber RM, Eidne KA, & Pflieger KD (2008). Demonstration of improvements to the bioluminescence resonance energy transfer (BRET) technology for the monitoring of G protein-coupled receptors in live cells. *J Biomol Screen* 13: 888-898.
- Kohsaka T, Hamano K, Sasada H, Watanabe S, Ogine T, Suzuki E, Nishida S, Takahara H, & Sato E (2003). Seminal immunoreactive relaxin in domestic animals and its relationship to sperm motility as a possible index for predicting the fertilizing ability of sires. *Int J Androl* 26: 115-120.
- Kohsaka T, Takahara H, Sasada H, Kawarasaki T, Bamba K, Masaki J, & Tagami S (1992). Evidence for immunoreactive relaxin in boar seminal vesicles using combined light and electron microscope immunocytochemistry. *J Reprod Fertil* 95: 397-408.
- Koivunen J, Aaltonen V, & Peltonen J (2006). Protein kinase C (PKC) family in cancer progression. *Cancer Lett* 235: 1-10.
- Kolakowski LF, Jr. (1994). GCRDb: a G-protein-coupled receptor database. *Recept Channels* 2: 1-7.
- Koman A, Cazaubon S, Couraud PO, Ullrich A, & Strosberg AD (1996). Molecular characterization and in vitro biological activity of placentin, a new member of the insulin gene family. *J Biol Chem* 271: 20238-20241.
- Komatsu N, Aoki K, Yamada M, Yukinaga H, Fujita Y, Kamioka Y, & Matsuda M (2011). Development of an optimized backbone of FRET biosensors for kinases and GTPases. *Mol Biol Cell* 22: 4647-4656.
- Kompa AR, Samuel CS, & Summers RJ (2002). Inotropic responses to human gene 2 (B29) relaxin in a rat model of myocardial infarction (MI): effect of pertussis toxin. *Br J Pharmacol* 137: 710-718.

- Kong RC, Petrie EJ, Mohanty B, Ling J, Lee JC, Gooley PR, & Bathgate RA (2013). The relaxin receptor (RXFP1) utilizes hydrophobic moieties on a signaling surface of its N-terminal low density lipoprotein class A module to mediate receptor activation. *J Biol Chem* 288: 28138-28151.
- Kotowski SJ, Hopf FW, Seif T, Bonci A, & von Zastrow M (2011). Endocytosis promotes rapid dopaminergic signaling. *Neuron* 71: 278-290.
- Krajnc-Franken MA, van Disseldorp AJ, Koenders JE, Mosselman S, van Duin M, & Gossen JA (2004). Impaired nipple development and parturition in LGR7 knockout mice. *Mol Cell Biol* 24: 687-696.
- Kuhn M (2016). Molecular physiology of membrane guanylyl cyclase receptors. *Physiol Rev* 96: 751-804.
- Kumagai J, Hsu SY, Matsumi H, Roh JS, Fu P, Wade JD, Bathgate RA, & Hsueh AJ (2002). INSL3/Leydig insulin-like peptide activates the LGR8 receptor important in testis descent. *J Biol Chem* 277: 31283-31286.
- Lane JR, May LT, Parton RG, Sexton PM, & Christopoulos A (2017). A kinetic view of GPCR allostery and biased agonism. *Nat Chem Biol* 13: 929-937.
- Lasker GF, Pankey EA, & Kadowitz PJ (2013). Modulation of soluble guanylate cyclase for the treatment of erectile dysfunction. *Physiology (Bethesda)* 28: 262-269.
- Lee M-H, Appleton KM, Strungs EG, Kwon JY, Morinelli TA, Peterson YK, Laporte SA, & Luttrell LM (2016a). The conformational signature of β -arrestin2 predicts its trafficking and signalling functions. *Nature* 531: 665.
- Lee YS, De Vadder F, Tremaroli V, Wichmann A, Mithieux G, & Backhed F (2016b). Insulin-like peptide 5 is a microbially regulated peptide that promotes hepatic glucose production. *Mol Metab* 5: 263-270.
- Lefkimmatis K, & Zaccolo M (2014). cAMP signaling in subcellular compartments. *Pharmacol Ther* 143: 295-304.
- Leineweber K, Bohm M, & Heusch G (2006). Cyclic adenosine monophosphate in acute myocardial infarction with heart failure: slayer or savior? *Circulation* 114: 365-367.
- Leo CH, Jelinic M, Gooi JH, Tare M, & Parry LJ (2014). A vasoactive role for endogenous relaxin in mesenteric arteries of male mice. *PLoS One* 9: e107382.
- Leo CH, Jelinic M, Ng HH, Marshall SA, Novak J, Tare M, Conrad KP, & Parry LJ (2017). Vascular actions of relaxin: nitric oxide and beyond. *Br J Pharmacol* 174: 1002-1014.
- Lerea CL, Somers DE, Hurley JB, Klock IB, & Bunt-Milam AH (1986). Identification of specific transducin alpha subunits in retinal rod and cone photoreceptors. *Science* 234: 77-80.
- Ley JP (2008). Masking bitter taste by molecules. *Chemosens Percept* 1: 58-77.
- Lian X, Beer-Hammer S, Konig GM, Kostenis E, Nurnberg B, & Gollasch M (2018). RXFP1 receptor activation by relaxin-2 induces vascular relaxation in mice via a Galphai2-protein/PI3Kbeta/gamma/nitric oxide-coupled pathway. *Front Physiol* 9: 1234.
- Litosch I (2016). Decoding G α q signaling. *Life Sci* 152: 99-106.
- Liu C, Chen J, Kuei C, Sutton S, Nepomuceno D, Bonaventure P, & Lovenberg TW (2005a). Relaxin-3/insulin-like peptide 5 chimeric peptide, a selective ligand for G protein-coupled receptor (GPCR)135 and GPCR142 over leucine-rich repeat-containing G protein-coupled receptor 7. *Mol Pharmacol* 67: 231-240.
- Liu C, Chen J, Sutton S, Roland B, Kuei C, Farmer N, Sillard R, & Lovenberg TW (2003a). Identification of relaxin-3/INSL7 as a ligand for GPCR142. *J Biol Chem* 278: 50765-50770.
- Liu C, Eriste E, Sutton S, Chen J, Roland B, Kuei C, Farmer N, Jornvall H, Sillard R, & Lovenberg TW (2003b). Identification of relaxin-3/INSL7 as an endogenous ligand for the orphan G-protein-coupled receptor GPCR135. *J Biol Chem* 278: 50754-50764.
- Liu C, Kuei C, Sutton S, Chen J, Bonaventure P, Wu J, Nepomuceno D, Kamme F, Tran DT, Zhu J, Wilkinson T, Bathgate R, Eriste E, Sillard R, & Lovenberg TW (2005b). INSL5 is a high affinity specific agonist for GPCR142 (GPR100). *J Biol Chem* 280: 292-300.

- Liu JJ, Horst R, Katritch V, Stevens RC, & Wuthrich K (2012). Biased signaling pathways in beta2-adrenergic receptor characterized by 19F-NMR. *Science* 335: 1106-1110.
- Liu S, Vinall RL, Tepper C, Shi XB, Xue LR, Ma AH, Wang LY, Fitzgerald LD, Wu Z, Gandour-Edwards R, deVere White RW, & Kung HJ (2008). Inappropriate activation of androgen receptor by relaxin via beta-catenin pathway. *Oncogene* 27: 499-505.
- Loening AM, Fenn TD, Wu AM, & Gambhir SS (2006). Consensus guided mutagenesis of Renilla luciferase yields enhanced stability and light output. *Protein Eng Des Sel* 19: 391-400.
- Logan CY, & Nusse R (2004). The Wnt signaling pathway in development and disease. *Annu Rev Cell Dev Biol* 20: 781-810.
- Lohse MJ, Nuber S, & Hoffmann C (2012). Fluorescence/bioluminescence resonance energy transfer techniques to study G-protein-coupled receptor activation and signaling. *Pharmacol Rev* 64: 299-336.
- Lok S, Johnston DS, Conklin D, Lofton-Day CE, Adams RL, Jelmsberg AC, Whitmore TE, Schrader S, Griswold MD, & Jaspers SR (2000). Identification of INSL6, a new member of the insulin family that is expressed in the testis of the human and rat. *Biol Reprod* 62: 1593-1599.
- Lonze BE, & Ginty DD (2002). Function and regulation of CREB family transcription factors in the nervous system. *Neuron* 35: 605-623.
- Lorenz W, Cormier M, O'Kane D, Hua D, Escher A, & Szalay A (1996). Expression of the Renilla reniformis luciferase gene in mammalian cells. *J Biolumin Chemilumin* 11: 31-37.
- Lorenz WW, McCann RO, Longiaru M, & Cormier MJ (1991). Isolation and expression of a cDNA encoding Renilla reniformis luciferase. *Proc Natl Acad Sci U S A* 88: 4438-4442.
- Lowndes K, Amano A, Yamamoto SY, & Bryant-Greenwood GD (2006). The human relaxin receptor (LGR7): expression in the fetal membranes and placenta. *Placenta* 27: 610-618.
- Lukowski R, Krieg T, Rybalkin SD, Beavo J, & Hofmann F (2014). Turning on cGMP-dependent pathways to treat cardiac dysfunctions: boom, bust, and beyond. *Trends Pharmacol Sci* 35: 404-413.
- Luo X, Li T, Zhu Y, Dai Y, Zhao J, Guo ZY, & Wang MW (2015). The insulinotrophic effect of insulin-like peptide 5 in vitro and in vivo. *Biochem J* 466: 467-473.
- Luttrell L, Ferguson S, Daaka Y, Miller W, Maudsley S, Della Rocca G, Lin F-T, Kawakatsu H, Owada K, & Luttrell D (1999). β -Arrestin-dependent formation of β 2 adrenergic receptor-Src protein kinase complexes. *Science* 283: 655-661.
- Luttrell LM, & Lefkowitz RJ (2002). The role of beta-arrestins in the termination and transduction of G-protein-coupled receptor signals. *J Cell Sci* 115: 455-465.
- Luttrell LM, Roudabush FL, Choy EW, Miller WE, Field ME, Pierce KL, & Lefkowitz RJ (2001). Activation and targeting of extracellular signal-regulated kinases by beta-arrestin scaffolds. *Proc Natl Acad Sci U S A* 98: 2449-2454.
- Luttrell LM, Wang J, Plouffe B, Smith JS, Yamani L, Kaur S, Jean-Charles PY, Gauthier C, Lee MH, Pani B, Kim J, Ahn S, Rajagopal S, Reiter E, Bouvier M, Shenoy SK, Laporte SA, Rockman HA, & Lefkowitz RJ (2018). Manifold roles of beta-arrestins in GPCR signaling elucidated with siRNA and CRISPR/Cas9. *Sci Signal* 11.
- Lyga S, Volpe S, Werthmann RC, Gotz K, Sungkaworn T, Lohse MJ, & Calebiro D (2016). Persistent cAMP signaling by internalized LH receptors in ovarian follicles. *Endocrinology* 157: 1613-1621.
- Ma S, Bonaventure P, Ferraro T, Shen PJ, Burazin TC, Bathgate RA, Liu C, Tregear GW, Sutton SW, & Gundlach AL (2007). Relaxin-3 in GABA projection neurons of nucleus incertus suggests widespread influence on forebrain circuits via G-protein-coupled receptor-135 in the rat. *Neuroscience* 144: 165-190.
- Ma S, Roozendaal B, Burazin TC, Tregear GW, McGaugh JL, & Gundlach AL (2005). Relaxin receptor activation in the basolateral amygdala impairs memory consolidation. *Eur J Neurosci* 22: 2117-2122.

- Ma S, Shen PJ, Burazin TC, Tregear GW, & Gundlach AL (2006). Comparative localization of leucine-rich repeat-containing G-protein-coupled receptor-7 (RXFP1) mRNA and [33P]-relaxin binding sites in rat brain: restricted somatic co-expression a clue to relaxin action? *Neuroscience* 141: 329-344.
- Ma S, Smith CM, Blasiak A, & Gundlach AL (2017). Distribution, physiology and pharmacology of relaxin-3/RXFP3 systems in brain. *Br J Pharmacol* 174: 1034-1048.
- Machleidt T, Woodroffe CC, Schwinn MK, Méndez J, Robers MB, Zimmerman K, Otto P, Daniels DL, Kirkland TA, & Wood KV (2015). NanoBRET: a novel BRET platform for the analysis of protein–protein Interactions. *ACS Chem Biol* 10: 1797-1804.
- MacLennan AH, Grant P, & Bryant-Greenwood G (1995). hRLX-1. In vitro response of human and pig myometrium. *J Reprod Med* 40: 703-706.
- MacLennan AH, Grant P, Ness D, & Down A (1986). Effect of porcine relaxin and progesterone on rat, pig and human myometrial activity in vitro. *J Reprod Med* 31: 43-49.
- Madariaga-Mazon A, Marmolejo-Valencia AF, Li Y, Toll L, Houghten RA, & Martinez-Mayorga K (2017). Mu-Opioid receptor biased ligands: A safer and painless discovery of analgesics? *Drug Discov Today* 22: 1719-1729.
- Manning BD, & Toker A (2017). AKT/PKB signaling: navigating the network. *Cell* 169: 381-405.
- Markovic D, & Challiss RA (2009). Alternative splicing of G protein-coupled receptors: physiology and pathophysiology. *Cell Mol Life Sci* 66: 3337-3352.
- Marshall SA, Leo CH, Senadheera SN, Girling JE, Tare M, & Parry LJ (2016). Relaxin deficiency attenuates pregnancy-induced adaptation of the mesenteric artery to angiotensin II in mice. *Am J Physiol Regul Integr Comp Physiol* 310: R847-857.
- Marshall SA, O'Sullivan K, Ng HH, Bathgate RAD, Parry LJ, Hossain MA, & Leo CH (2017). B7-33 replicates the vasoprotective functions of human relaxin-2 (serelaxin). *Eur J Pharmacol* 807: 190-197.
- Mathis G (1999). HTRF® technology. *J Biomol Screen* 4: 309-313.
- Matsumoto M, Kamohara M, Sugimoto T, Hidaka K, Takasaki J, Saito T, Okada M, Yamaguchi T, & Furuichi K (2000). The novel G-protein coupled receptor SALPR shares sequence similarity with somatostatin and angiotensin receptors. *Gene* 248: 183-189.
- Matsuzawa H, & Nirenberg M (1975). Receptor-mediated shifts in cGMP and cAMP levels in neuroblastoma cells. *Proc Natl Acad Sci U S A* 72: 3472-3476.
- Matthiesen K, & Nielsen J (2011). Cyclic AMP control measured in two compartments in HEK293 cells: phosphodiesterase K(M) is more important than phosphodiesterase localization. *PLoS One* 6: e24392.
- Mayr B, & Montminy M (2001). Transcriptional regulation by the phosphorylation-dependent factor CREB. *Nat Rev Mol Cell Biol* 2: 599-609.
- McCarthy FP, Kingdom JC, Kenny LC, & Walsh SK (2011). Animal models of preeclampsia; uses and limitations. *Placenta* 32: 413-419.
- McCormick K, & Baillie GS (2014). Compartmentalisation of second messenger signalling pathways. *Curr Opin Genet Dev* 27: 20-25.
- McCullough PA (2017). How trialists and pharmaceutical sponsors have failed us by thinking that acute heart failure is a 48-hour illness. *Am J Cardiol* 120: 505-508.
- McGuane JT, Danielson LA, Debrah JE, Rubin JP, Novak J, & Conrad KP (2011a). Angiogenic growth factors are new and essential players in the sustained relaxin vasodilatory pathway in rodents and humans. *Hypertension* 57: 1151-1160.
- McGuane JT, Debrah JE, Sautina L, Jarajapu YP, Novak J, Rubin JP, Grant MB, Segal M, & Conrad KP (2011b). Relaxin induces rapid dilation of rodent small renal and human subcutaneous arteries via PI3 kinase and nitric oxide. *Endocrinology* 152: 2786-2796.
- McLaughlin SK, McKinnon PJ, & Margolskee RF (1992). Gustducin is a taste-cell-specific G protein closely related to the transducins. *Nature* 357: 563.

- Metra M, Cotter G, Davison BA, Felker GM, Filippatos G, Greenberg BH, Ponikowski P, Unemori E, Voors AA, Adams KF, Jr., Dorobantu MI, Grinfeld L, Jondeau G, Marmor A, Masip J, Pang PS, Werdan K, Prescott MF, Edwards C, Teichman SL, Trapani A, Bush CA, Saini R, Schumacher C, Severin T, Teerlink JR, & Investigators R-A (2013). Effect of serelaxin on cardiac, renal, and hepatic biomarkers in the Relaxin in Acute Heart Failure (RELAX-AHF) development program: correlation with outcomes. *J Am Coll Cardiol* 61: 196-206.
- Metra M, Teerlink JR, Cotter G, Davison BA, Felker GM, Filippatos G, Greenberg BH, Pang PS, Ponikowski P, Voors AA, Adams KF, Anker SD, Arias-Mendoza A, Avendano P, Bacal F, Bohm M, Bortman G, Cleland JGF, Cohen-Solal A, Crespo-Leiro MG, Dorobantu M, Echeverria LE, Ferrari R, Golland S, Goncalvesova E, Goudev A, Kober L, Lema-Osores J, Levy PD, McDonald K, Manga P, Merkely B, Mueller C, Pieske B, Silva-Cardoso J, Spinar J, Squire I, Stepinska J, Van Mieghem W, von Lewinski D, Wikstrom G, Yilmaz MB, Hagner N, Holbro T, Hua TA, Sabarwal SV, Severin T, Szecsy P, Gimpelewicz C, & Investigators R-A-C (2019). Effects of serelaxin in patients with acute heart failure. *N Engl J Med* 381: 716-726.
- Michel MC, Seifert R, & Bond RA (2014). Dynamic bias and its implications for GPCR drug discovery. *Nat Rev Drug Discov* 13: 869.
- Miller MR, & Megson IL (2007). Recent developments in nitric oxide donor drugs. *Br J Pharmacol* 151: 305-321.
- Min G, Hartzog MG, Jennings RL, Winn RJ, & Sherwood OD (1997). Evidence that endogenous relaxin promotes growth of the vagina and uterus during pregnancy in gilts. *Endocrinology* 138: 560-565.
- Mookerjee I, Hewitson TD, Halls ML, Summers RJ, Mathai ML, Bathgate RA, Tregear GW, & Samuel CS (2009). Relaxin inhibits renal myofibroblast differentiation via RXFP1, the nitric oxide pathway, and Smad2. *FASEB J* 23: 1219-1229.
- Moore XL, Tan SL, Lo CY, Fang L, Su YD, Gao XM, Woodcock EA, Summers RJ, Tregear GW, Bathgate RA, & Du XJ (2007). Relaxin antagonizes hypertrophy and apoptosis in neonatal rat cardiomyocytes. *Endocrinology* 148: 1582-1589.
- Morin JG, & Hastings JW (1971). Energy transfer in a bioluminescent system. *J Cell Physiol* 77: 313-318.
- Muda M, He C, Martini PG, Ferraro T, Layfield S, Taylor D, Chevrier C, Schweickhardt R, Kelton C, Ryan PL, & Bathgate RA (2005). Splice variants of the relaxin and INSL3 receptors reveal unanticipated molecular complexity. *Mol Hum Reprod* 11: 591-600.
- Mullershausen F, Zecri F, Cetin C, Billich A, Guerini D, & Seuwen K (2009). Persistent signaling induced by FTY720-phosphate is mediated by internalized S1P1 receptors. *Nat Chem Biol* 5: 428-434.
- Mumford AD, Parry LJ, & Summerlee AJ (1989). Lesion of the subfornical organ affects the haemotensive response to centrally administered relaxin in anaesthetized rats. *J Endocrinol* 122: 747-755.
- Murone M, Rosenthal A, & de Sauvage FJ (1999). Sonic hedgehog signaling by the patched-smoothed receptor complex. *Curr Biol* 9: 76-84.
- Nagai T, Ibata K, Park ES, Kubota M, Mikoshiba K, & Miyawaki A (2002). A variant of yellow fluorescent protein with fast and efficient maturation for cell-biological applications. *Nat Biotechnol* 20: 87-90.
- Nambotin SB, Lefrancois L, Sainsily X, Berthillon P, Kim M, Wands JR, Chevallier M, Jalinot P, Scoazec JY, Trepo C, Zoulim F, & Merle P (2011). Pharmacological inhibition of Frizzled-7 displays anti-tumor properties in hepatocellular carcinoma. *J Hepatol* 54: 288-299.
- Nascimento AR, Pimenta MT, Lucas TF, Royer C, Porto CS, & Lazari MF (2012). Intracellular signaling pathways involved in the relaxin-induced proliferation of rat Sertoli cells. *Eur J Pharmacol* 691: 283-291.
- Nef S, & Parada LF (1999). Cryptorchidism in mice mutant for *Ins13*. *Nat Genet* 22: 295-299.

- Neschadim A, Pritzker LB, Pritzker KP, Branch DR, Summerlee AJ, Trachtenberg J, & Silvertown JD (2014). Relaxin receptor antagonist AT-001 synergizes with docetaxel in androgen-independent prostate xenografts. *Endocr Relat Cancer* 21: 459-471.
- Newman-Tancredi A, Audinot V, Moreira C, Verri  le L, & Millan MJ (2000). Inverse agonism and constitutive activity as functional correlates of serotonin h5-HT1B receptor/G-protein stoichiometry. *Mol Pharmacol* 58: 1042-1049.
- Newman-Tancredi A, Conte C, Chaput C, Verri  le L, & Millan M (1997). Agonist and inverse agonist efficacy at human recombinant serotonin 5-HT1A receptors as a function of receptor: G-protein stoichiometry. *Neuropharmacology* 36: 451-459.
- Ng HH, Jelinic M, Parry LJ, & Leo CH (2015). Increased superoxide production and altered nitric oxide-mediated relaxation in the aorta of young but not old male relaxin-deficient mice. *Am J Physiol Heart Circ Physiol* 309: H285-296.
- Ng HH, Leo CH, Parry LJ, & Ritchie RH (2018). Relaxin as a therapeutic target for the cardiovascular complications of diabetes. *Front Pharmacol* 9: 501.
- Nguyen BT, & Dessauer CW (2005a). Relaxin stimulates cAMP production in MCF-7 cells upon overexpression of type V adenylyl cyclase. *Ann N Y Acad Sci* 1041: 296-299.
- Nguyen BT, & Dessauer CW (2005b). Relaxin stimulates protein kinase C zeta translocation: requirement for cyclic adenosine 3',5'-monophosphate production. *Mol Endocrinol* 19: 1012-1023.
- Nguyen BT, Yang L, Sanborn BM, & Dessauer CW (2003). Phosphoinositide 3-kinase activity is required for biphasic stimulation of cyclic adenosine 3',5'-monophosphate by relaxin. *Mol Endocrinol* 17: 1075-1084.
- Ni Q, Titov DV, & Zhang J (2006). Analyzing protein kinase dynamics in living cells with FRET reporters. *Methods* 40: 279-286.
- Niino Y, Hotta K, & Oka K (2009). Simultaneous live cell imaging using dual FRET sensors with a single excitation light. *PLoS One* 4: e6036.
- Nikolaev VO, Gambaryan S, & Lohse MJ (2006). Fluorescent sensors for rapid monitoring of intracellular cGMP. *Nat Methods* 3: 23-25.
- Niswender CM, & Conn PJ (2010). Metabotropic glutamate receptors: physiology, pharmacology, and disease. *Annu Rev Pharmacol Toxicol* 50: 295-322.
- Nobles KN, Xiao K, Ahn S, Shukla AK, Lam CM, Rajagopal S, Strachan RT, Huang T-Y, Bressler EA, & Hara MR (2011). Distinct phosphorylation sites on the β 2-adrenergic receptor establish a barcode that encodes differential functions of β -arrestin. *Sci Signal* 4: ra51-ra51.
- Novak J, Danielson LA, Kerchner LJ, Sherwood OD, Ramirez RJ, Moalli PA, & Conrad KP (2001). Relaxin is essential for renal vasodilation during pregnancy in conscious rats. *J Clin Invest* 107: 1469-1475.
- Novak J, Parry LJ, Matthews JE, Kerchner LJ, Indovina K, Hanley-Yanez K, Doty KD, Debrah DO, Shroff SG, & Conrad KP (2006). Evidence for local relaxin ligand-receptor expression and function in arteries. *FASEB J* 20: 2352-2362.
- Novak J, Ramirez RJ, Gandley RE, Sherwood OD, & Conrad KP (2002). Myogenic reactivity is reduced in small renal arteries isolated from relaxin-treated rats. *Am J Physiol Regul Integr Comp Physiol* 283: R349-355.
- O'Byrne EM, & Steinetz BG (1976). Radioimmunoassay (RIA) of relaxin in sera of various species using an antiserum to porcine relaxin. *Proc Soc Exp Biol Med* 152: 272-276.
- O'Byrne K, Eltringham L, & Summerlee A (1987). Central inhibitory effects of relaxin on the milk ejection reflex of the rat depends upon the site of injection into the cerebroventricular system. *Brain Res* 405: 80-83.
- O'Byrne KT, Eltringham L, Clarke G, & Summerlee AJ (1986). Effects of porcine relaxin on oxytocin release from the neurohypophysis in the anaesthetized lactating rat. *J Endocrinol* 109: 393-397.

- O'Day MB, Winn RJ, Easter RA, Dziuk PJ, & Sherwood OD (1989). Hormonal control of the cervix in pregnant gilts. II. Relaxin promotes changes in the physical properties of the cervix in ovariectomized hormone-treated pregnant gilts. *Endocrinology* 125: 3004-3010.
- Ogunleye O, Campo B, Herrera D, Post Uiterweer ED, & Conrad KP (2017). Relaxin confers cytotrophoblast protection from hypoxia-reoxygenation injury through the phosphatidylinositol 3-kinase-Akt/protein kinase B cell survival pathway. *Am J Physiol Regul Integr Comp Physiol* 312: R559-R568.
- Oldham WM, & Hamm HE (2008). Heterotrimeric G protein activation by G-protein-coupled receptors. *Nat Rev Mol Cell Biol* 9: 60-71.
- Onfroy L, Galandrin S, Pontier SM, Seguelas MH, N'Guyen D, Senard JM, & Gales C (2017). G protein stoichiometry dictates biased agonism through distinct receptor-G protein partitioning. *Sci Rep* 7: 7885.
- Osheroff PL, & Ho WH (1993). Expression of relaxin mRNA and relaxin receptors in postnatal and adult rat brains and hearts. Localization and developmental patterns. *J Biol Chem* 268: 15193-15199.
- Palczewski K, Kumasaka T, Hori T, Behnke CA, Motoshima H, Fox BA, Le Trong I, Teller DC, Okada T, Stenkamp RE, Yamamoto M, & Miyano M (2000). Crystal structure of rhodopsin: A G protein-coupled receptor. *Science* 289: 739-745.
- Palejwala S, Stein D, Wojtczuk A, Weiss G, & Goldsmith LT (1998). Demonstration of a relaxin receptor and relaxin-stimulated tyrosine phosphorylation in human lower uterine segment fibroblasts. *Endocrinology* 139: 1208-1212.
- Pang PS, Butler J, Collins SP, Cotter G, Davison BA, Ezekowitz JA, Filippatos G, Levy PD, Metra M, Ponikowski P, Teerlink JR, Voors AA, Bharucha D, Goin K, Soergel DG, & Felker GM (2017). Biased ligand of the angiotensin II type 1 receptor in patients with acute heart failure: a randomized, double-blind, placebo-controlled, phase IIB, dose ranging trial (BLAST-AHF). *Eur Heart J* 38: 2364-2373.
- Parry LJ, & Summerlee AJ (1991). Central angiotensin partially mediates the pressor action of relaxin in anesthetized rats. *Endocrinology* 129: 47-52.
- Parsell DA, Mak JY, Amento EP, & Unemori EN (1996). Relaxin binds to and elicits a response from cells of the human monocytic cell line, THP-1. *J Biol Chem* 271: 27936-27941.
- Patil NA, Hughes RA, Rosengren KJ, Kocan M, Ang SY, Tailhades J, Separovic F, Summers RJ, Grosse J, Wade JD, Bathgate RA, & Hossain MA (2016). Engineering of a novel simplified human insulin-like peptide 5 agonist. *J Med Chem* 59: 2118-2125.
- Patterson GH, Piston DW, & Barisas BG (2000). Förster distances between green fluorescent protein pairs. *Anal Biochem* 284: 438-440.
- Pearson G, Robinson F, Beers Gibson T, Xu BE, Karandikar M, Berman K, & Cobb MH (2001). Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. *Endocr Rev* 22: 153-183.
- Peterson YK, & Luttrell LM (2017). The diverse roles of arrestin scaffolds in G protein-coupled receptor signaling. *Pharmacol Rev* 69: 256-297.
- Petrie EJ, Lagaida S, Sethi A, Bathgate RA, & Gooley PR (2015). In a Class of Their Own - RXFP1 and RXFP2 are Unique Members of the LGR Family. *Front Endocrinol (Lausanne)* 6: 137.
- Pfleger KD, Dromey JR, Dalrymple MB, Lim EM, Thomas WG, & Eidne KA (2006). Extended bioluminescence resonance energy transfer (eBRET) for monitoring prolonged protein-protein interactions in live cells. *Cell Signal* 18: 1664-1670.
- Pfleger KD, & Eidne KA (2003). New technologies: bioluminescence resonance energy transfer (BRET) for the detection of real time interactions involving G-protein coupled receptors. *Pituitary* 6: 141-151.
- Pfleger KD, & Eidne KA (2005). Monitoring the formation of dynamic G-protein-coupled receptor-protein complexes in living cells. *Biochem J* 385: 625-637.

- Pfleger KD, & Eidne KA (2006). Illuminating insights into protein-protein interactions using bioluminescence resonance energy transfer (BRET). *Nat Methods* 3: 165-174.
- Piggott LA, Hassell KA, Berkova Z, Morris AP, Silberbach M, & Rich TC (2006). Natriuretic peptides and nitric oxide stimulate cGMP synthesis in different cellular compartments. *J Gen Physiol* 128: 3-14.
- Pini A, Boccalini G, Baccari MC, Becatti M, Garella R, Fiorillo C, Calosi L, Bani D, & Nistri S (2016). Protection from cigarette smoke-induced vascular injury by recombinant human relaxin-2 (serelaxin). *J Cell Mol Med* 20: 891-902.
- Piston DW, & Kremers GJ (2007). Fluorescent protein FRET: the good, the bad and the ugly. *Trends Biochem Sci* 32: 407-414.
- Ponsioen B, Zhao J, Riedl J, Zwartkruis F, van der Krogt G, Zacco M, Moolenaar WH, Bos JL, & Jalink K (2004). Detecting cAMP-induced Epac activation by fluorescence resonance energy transfer: Epac as a novel cAMP indicator. *EMBO Rep* 5: 1176-1180.
- Porter DG, & Watts AD (1986). Relaxin and progesterone are myometrial inhibitors in the ovariectomized non-pregnant mini-pig. *J Reprod Fertil* 76: 205-213.
- Praveen P, Kocan M, Valkovic A, Bathgate R, & Hossain MA (2019). Single chain peptide agonists of relaxin receptors. *Mol Cell Endocrinol* 487: 34-39.
- Pusch W, Balvers M, & Ivell R (1996). Molecular cloning and expression of the relaxin-like factor from the mouse testis. *Endocrinology* 137: 3009-3013.
- Qiu Y, & Law P-Y (2011). Posttranslational Regulation of G Protein-Coupled Receptors. In *Methods for the discovery and characterization of G protein-coupled receptors*. pp 133-152.
- Radestock Y, Willing C, Kehlen A, Hoang-Vu C, & Hombach-Klonisch S (2010). Relaxin enhances S100A4 and promotes growth of human thyroid carcinoma cell xenografts. *Mol Cancer Res* 8: 494-506.
- Rajagopal S, Rajagopal K, & Lefkowitz RJ (2010). Teaching old receptors new tricks: biasing seven-transmembrane receptors. *Nat Rev Drug Discov* 9: 373-386.
- Ramamurthi A, & Lewis RS (1997). Measurement and modeling of nitric oxide release rates for nitric oxide donors. *Chem Res Toxicol* 10: 408-413.
- Ramezani A, & Hawley RG (2002). Overview of the HIV-1 lentiviral vector system. *Curr Protoc Mol Biol* Chapter 16: Unit 16 21.
- Ramezani A, Hawley TS, & Hawley RG (2000). Lentiviral vectors for enhanced gene expression in human hematopoietic cells. *Mol Ther* 2: 458-469.
- Ramirez-Garcia PD, Retamal JS, Shenoy P, Imlach W, Sykes M, Truong N, Constandil L, Pelissier T, Nowell CJ, Khor SY, Layani LM, Lumb C, Poole DP, Lieu T, Stewart GD, Mai QN, Jensen DD, Latorre R, Scheff NN, Schmidt BL, Quinn JF, Whittaker MR, Veldhuis NA, Davis TP, & Bunnett NW (2019). A pH-responsive nanoparticle targets the neurokinin 1 receptor in endosomes to prevent chronic pain. *Nat Nanotechnol* 14: 1150-1159.
- Rask-Andersen M, Almen MS, & Schioth HB (2011). Trends in the exploitation of novel drug targets. *Nat Rev Drug Discov* 10: 579-590.
- Ren XR, Reiter E, Ahn S, Kim J, Chen W, & Lefkowitz RJ (2005). Different G protein-coupled receptor kinases govern G protein and beta-arrestin-mediated signaling of V2 vasopressin receptor. *Proc Natl Acad Sci U S A* 102: 1448-1453.
- Retamal JS, Ramirez-Garcia PD, Shenoy PA, Poole DP, & Veldhuis NA (2019). Internalized GPCRs as potential therapeutic targets for the management of pain. *Front Mol Neurosci* 12: 273.
- Rincon M, & Davis RJ (2009). Regulation of the immune response by stress-activated protein kinases. *Immunol Rev* 228: 212-224.
- Rivero-Vilches FJ, de Frutos S, Saura M, Rodriguez-Puyol D, & Rodriguez-Puyol M (2003). Differential relaxing responses to particulate or soluble guanylyl cyclase activation on endothelial cells: a mechanism dependent on PKG-I alpha activation by NO/cGMP. *Am J Physiol Cell Physiol* 285: C891-898.

- Rockey DC, Bell PD, & Hill JA (2015). Fibrosis--a common pathway to organ injury and failure. *N Engl J Med* 372: 1138-1149.
- Russ AP, & Lampel S (2005). The druggable genome: an update. *Drug Discov Today* 10: 1607-1610.
- Sah VP, Seasholtz TM, Sagi SA, & Brown JH (2000). The role of Rho in G protein-coupled receptor signal transduction. *Annu Rev Pharmacol Toxicol* 40: 459-489.
- Salahpour A, Espinoza S, Masri B, Lam V, Barak LS, & Gainetdinov RR (2012). BRET biosensors to study GPCR biology, pharmacology, and signal transduction. *Front Endocrinol (Lausanne)* 3: 105.
- Samuel CS, & Amento EP (2001). Relaxin knockout mice demonstrate delayed growth and have underdeveloped male reproductive tracts. 221-223.
- Samuel CS, Bodaragama H, Chew JY, Widdop RE, Royce SG, & Hewitson TD (2014). Serelaxin is a more efficacious antifibrotic than enalapril in an experimental model of heart disease. *Hypertension* 64: 315-322.
- Samuel CS, Hewitson TD, Unemori EN, & Tang ML (2007). Drugs of the future: the hormone relaxin. *Cell Mol Life Sci* 64: 1539-1557.
- Samuel CS, Royce SG, Hewitson TD, Denton KM, Cooney TE, & Bennett RG (2017). Anti-fibrotic actions of relaxin. *Br J Pharmacol* 174: 962-976.
- Samuel CS, Summers RJ, & Hewitson TD (2016). Antifibrotic actions of serelaxin - new roles for an old player. *Trends Pharmacol Sci* 37: 485-497.
- Samuel CS, Tian H, Zhao L, & Amento EP (2003a). Relaxin is a key mediator of prostate growth and male reproductive tract development. *Lab Invest* 83: 1055-1067.
- Samuel CS, Unemori EN, Mookerjee I, Bathgate RA, Layfield SL, Mak J, Tregear GW, & Du XJ (2004a). Relaxin modulates cardiac fibroblast proliferation, differentiation, and collagen production and reverses cardiac fibrosis in vivo. *Endocrinology* 145: 4125-4133.
- Samuel CS, Zhao C, Bathgate RA, Bond CP, Burton MD, Parry LJ, Summers RJ, Tang ML, Amento EP, & Tregear GW (2003b). Relaxin deficiency in mice is associated with an age-related progression of pulmonary fibrosis. *FASEB J* 17: 121-123.
- Samuel CS, Zhao C, Bond CP, Hewitson TD, Amento EP, & Summers RJ (2004b). Relaxin-1-deficient mice develop an age-related progression of renal fibrosis. *Kidney Int* 65: 2054-2064.
- Samuel CS, Zhao C, Yang Q, Wang H, Tian H, Tregear GW, & Amento EP (2005). The relaxin gene knockout mouse: a model of progressive scleroderma. *J Invest Dermatol* 125: 692-699.
- Sanborn BM, Kuo HS, Weisbrodt NW, & Sherwood OD (1980). The interaction of relaxin with the rat uterus. I. Effect on cyclic nucleotide levels and spontaneous contractile activity. *Endocrinology* 106: 1210-1215.
- Sandner P, & Stasch JP (2017). Anti-fibrotic effects of soluble guanylate cyclase stimulators and activators: A review of the preclinical evidence. *Respir Med* 122 Suppl 1: S1-S9.
- Sandner P, Vakalopoulos A, Hahn MG, Stasch JP, & Follmann M (2021). Soluble guanylate cyclase stimulators and their potential use: a patent review. *Expert Opin Ther Pat* 31: 203-222.
- Santos R, Ursu O, Gaulton A, Bento AP, Donadi RS, Bologa CG, Karlsson A, Al-Lazikani B, Hersey A, Oprea TI, & Overington JP (2017). A comprehensive map of molecular drug targets. *Nat Rev Drug Discov* 16: 19-34.
- Sarwar M, Du XJ, Dschietzig TB, & Summers RJ (2016a). The actions of relaxin on the human cardiovascular system. *Br J Pharmacol* 174: 933-949.
- Sarwar M, Samuel CS, Bathgate RA, Stewart DR, & Summers RJ (2015). Serelaxin-mediated signal transduction in human vascular cells: bell-shaped concentration-response curves reflect differential coupling to G proteins. *Br J Pharmacol* 172: 1005-1019.
- Sarwar M, Samuel CS, Bathgate RA, Stewart DR, & Summers RJ (2016b). Enhanced serelaxin signalling in co-cultures of human primary endothelial and smooth muscle cells. *Br J Pharmacol* 173: 484-496.

- Sassoli C, Chellini F, Pini A, Tani A, Nistri S, Nosi D, Zecchi-Orlandini S, Bani D, & Formigli L (2013). Relaxin prevents cardiac fibroblast-myofibroblast transition via notch-1-mediated inhibition of TGF-beta/Smad3 signaling. *PLoS One* 8: e63896.
- Sato M, Hida N, Ozawa T, & Umezawa Y (2000). Fluorescent indicators for cyclic GMP based on cyclic GMP-dependent protein kinase α and green fluorescent proteins. *Anal Chem* 72: 5918-5924.
- Schinner E, Wetzl V, Schramm A, Kees F, Sandner P, Stasch JP, Hofmann F, & Schlossmann J (2017). Inhibition of the TGFbeta signalling pathway by cGMP and cGMP-dependent kinase I in renal fibrosis. *FEBS Open Bio* 7: 550-561.
- Schuetz DA, de Witte WEA, Wong YC, Knasmueller B, Richter L, Kokh DB, Sadiq SK, Bosma R, Nederpelt I, Heitman LH, Segala E, Amaral M, Guo D, Andres D, Georgi V, Stoddart LA, Hill S, Cooke RM, De Graaf C, Leurs R, Frech M, Wade RC, de Lange ECM, AP II, Muller-Fahrnow A, & Ecker GF (2017). Kinetics for drug discovery: an industry-driven effort to target drug residence time. *Drug Discov Today* 22: 896-911.
- Schulte G (2010). International Union of Basic and Clinical Pharmacology. LXXX. The class Frizzled receptors. *Pharmacol Rev* 62: 632-667.
- Schwabe C, & McDonald JK (1977). Relaxin: a disulfide homolog of insulin. *Science* 197: 914-915.
- Scott DJ, Fu P, Shen PJ, Gundlach A, Layfield S, Riesewijk A, Tomiyama H, Hutson JM, Tregear GW, & Bathgate RA (2005). Characterization of the rat INSL3 receptor. *Ann N Y Acad Sci* 1041: 13-16.
- Scott DJ, Layfield S, Yan Y, Sudo S, Hsueh AJ, Tregear GW, & Bathgate RA (2006). Characterization of novel splice variants of LGR7 and LGR8 reveals that receptor signaling is mediated by their unique low density lipoprotein class A modules. *J Biol Chem* 281: 34942-34954.
- Seamon KB, Padgett W, & Daly JW (1981). Forskolin: unique diterpene activator of adenylate cyclase in membranes and in intact cells. *Proc Natl Acad Sci U S A* 78: 3363-3367.
- Segal MS, Sautina L, Li S, Diao Y, AgoulNIK AI, Kielczewski J, McGuane JT, Grant MB, & Conrad KP (2012). Relaxin increases human endothelial progenitor cell NO and migration and vasculogenesis in mice. *Blood* 119: 629-636.
- Seibold JR, Korn JH, Simms R, Clements PJ, Moreland LW, Mayes MD, Furst DE, Rothfield N, Steen V, Weisman M, Collier D, Wigley FM, Merkel PA, Csuka ME, Hsu V, Rocco S, Erikson M, Hannigan J, Harkonen WS, & Sanders ME (2000). Recombinant human relaxin in the treatment of scleroderma. *Ann Intern Med* 132: 871-879.
- Sekar RB, & Periasamy A (2003). Fluorescence resonance energy transfer (FRET) microscopy imaging of live cell protein localizations. *J Cell Biol* 160: 629-633.
- Sethi A, Bruell S, Patil N, Hossain MA, Scott DJ, Petrie EJ, Bathgate RA, & Gooley PR (2016). The complex binding mode of the peptide hormone H2 relaxin to its receptor RXFP1. *Nat Commun* 7: 11344.
- Shabanpoor F, Hossain MA, Ryan PJ, Belgi A, Layfield S, Kocan M, Zhang S, Samuel CS, Gundlach AL, Bathgate RA, Separovic F, & Wade JD (2012). Minimization of human relaxin-3 leading to high-affinity analogues with increased selectivity for relaxin-family peptide 3 receptor (RXFP3) over RXFP1. *J Med Chem* 55: 1671-1681.
- Shabanpoor F, Separovic F, & Wade JD (2009). The human insulin superfamily of polypeptide hormones. In *Vitam Horm*. pp 1-31.
- Shabanpoor F, Zhang S, Hughes RA, Hossain MA, Layfield S, Ferraro T, Bathgate RA, Separovic F, & Wade JD (2011). Design and development of analogues of dimers of insulin-like peptide 3 B-chain as high-affinity antagonists of the RXFP2 receptor. *Biopolymers* 96: 81-87.
- Shaul YD, & Seger R (2007). The MEK/ERK cascade: from signaling specificity to diverse functions. *Biochim Biophys Acta* 1773: 1213-1226.
- Shaw EE, Wood P, Kulpa J, Yang FH, Summerlee AJ, & Pyle WG (2009). Relaxin alters cardiac myofilament function through a PKC-dependent pathway. *Am J Physiol Heart Circ Physiol* 297: H29-36.

- Shenoy SK, Drake MT, Nelson CD, Houtz DA, Xiao K, Madabushi S, Reiter E, Premont RT, Lichtarge O, & Lefkowitz RJ (2006). beta-arrestin-dependent, G protein-independent ERK1/2 activation by the beta2 adrenergic receptor. *J Biol Chem* 281: 1261-1273.
- Sherwood CD, & O'Byrne EM (1974). Purification and characterization of porcine relaxin. *Arch Biochem Biophys* 160: 185-196.
- Sherwood O, Crnekovic V, Gordon W, & Rutherford J (1980). Radioimmunoassay of relaxin throughout pregnancy and during parturition in the rat. *Endocrinology* 107: 691-698.
- Sherwood OD (2004). Relaxin's physiological roles and other diverse actions. *Endocr Rev* 25: 205-234.
- Shim S, Shuman M, & Duncan E (2016). An emerging role of cGMP in the treatment of schizophrenia: A review. *Schizophr Res* 170: 226-231.
- Shpakov AO, Gur'yanov IA, Kuznetsova LA, Plesneva SA, Shpakova EA, Vlasov GP, & Pertseva MN (2007). Studies of the molecular mechanisms of action of relaxin on the adenylyl cyclase signaling system using synthetic peptides derived from the LGR7 relaxin receptor. *Neurosci Behav Physiol* 37: 705-714.
- Siehler S (2009). Regulation of RhoGEF proteins by G12/13-coupled receptors. *Br J Pharmacol* 158: 41-49.
- Silvertown JD, Geddes BJ, & Summerlee AJ (2003). Adenovirus-mediated expression of human prorelaxin promotes the invasive potential of canine mammary cancer cells. *Endocrinology* 144: 3683-3691.
- Silvertown JD, Ng J, Sato T, Summerlee AJ, & Medin JA (2006). H2 relaxin overexpression increases in vivo prostate xenograft tumor growth and angiogenesis. *Int J Cancer* 118: 62-73.
- Silvertown JD, Symes JC, Neschadim A, Nonaka T, Kao JC, Summerlee AJ, & Medin JA (2007). Analog of H2 relaxin exhibits antagonistic properties and impairs prostate tumor growth. *FASEB J* 21: 754-765.
- Singh S, Simpson RL, & Bennett RG (2015). Relaxin activates peroxisome proliferator-activated receptor gamma (PPARgamma) through a pathway involving PPARgamma coactivator 1alpha (PGC1alpha). *J Biol Chem* 290: 950-959.
- Smith JS, Lefkowitz RJ, & Rajagopal S (2018). Biased signalling: from simple switches to allosteric microprocessors. *Nat Rev Drug Discov* 17: 243-260.
- Smith MC, Danielson LA, Conrad KP, & Davison JM (2006). Influence of recombinant human relaxin on renal hemodynamics in healthy volunteers. *J Am Soc Nephrol* 17: 3192-3197.
- Snowdon VK, Lachlan NJ, Hoy AM, Hadoke PW, Semple SI, Patel D, Mungall W, Kendall TJ, Thomson A, Lennen RJ, Jansen MA, Moran CM, Pellicoro A, Ramachandran P, Shaw I, Aucott RL, Severin T, Saini R, Pak J, Yates D, Dongre N, Duffield JS, Webb DJ, Iredale JP, Hayes PC, & Fallowfield JA (2017). Serelaxin as a potential treatment for renal dysfunction in cirrhosis: Preclinical evaluation and results of a randomized phase 2 trial. *PLoS Med* 14: e1002248.
- Spaniel-Borowski K, Schafer I, Zimmermann S, Engel W, & Adham IM (2001). Increase in final stages of follicular atresia and premature decay of corpora lutea in *Ins13*-deficient mice. *Mol Reprod Dev* 58: 281-286.
- Sriram K, & Insel PA (2018). GPCRs as targets for approved drugs: How many targets and how many drugs? *Mol Pharmacol*: 251-258.
- Stallaert W, van der Westhuizen ET, Schonegge AM, Plouffe B, Hogue M, Lukashova V, Inoue A, Ishida S, Aoki J, Le Gouill C, & Bouvier M (2017). Purinergic receptor transactivation by the beta2-adrenergic receptor increases intracellular Ca(2+) in nonexcitable cells. *Mol Pharmacol* 91: 533-544.
- Stangherlin A, & Zaccolo M (2012). Phosphodiesterases and subcellular compartmentalized cAMP signaling in the cardiovascular system. *Am J Physiol Heart Circ Physiol* 302: H379-390.
- Stephenson RP (1956). A modification of receptor theory. *Br J Pharmacol Chemother* 11: 379-393.

- Stoddart LA, Johnstone EKM, Wheal AJ, Goulding J, Robers MB, Machleidt T, Wood KV, Hill SJ, & Pflieger KDG (2015). Application of BRET to monitor ligand binding to GPCRs. *Nat Methods* 12: 661-663.
- Stoddart LA, Kilpatrick LE, & Hill SJ (2018). NanoBRET approaches to study ligand binding to GPCRs and RTKs. *Trends Pharmacol Sci* 39: 136-147.
- Stoeber M, Jullie D, Lobingier BT, Laeremans T, Steyaert J, Schiller PW, Manglik A, & von Zastrow M (2018). A genetically encoded biosensor reveals location bias of opioid drug action. *Neuron* 98: 963-976 e965.
- Strachan RT, Ferrara G, & Roth BL (2006). Screening the receptorome: an efficient approach for drug discovery and target validation. *Drug Discov Today* 11: 708-716.
- Stryer L, & Haugland RP (1967). Energy transfer: a spectroscopic ruler. *Proc Natl Acad Sci U S A* 58: 719-726.
- Sudo S, Kumagai J, Nishi S, Layfield S, Ferraro T, Bathgate RA, & Hsueh AJ (2003). H3 relaxin is a specific ligand for LGR7 and activates the receptor by interacting with both the ectodomain and the exoloop 2. *J Biol Chem* 278: 7855-7862.
- Summerlee AJ, Hornsby DJ, & Ramsey DG (1998). The dipsogenic effects of rat relaxin: The effect of photoperiod and the potential role of relaxin on drinking in pregnancy. *Endocrinology* 139: 2322-2328.
- Summerlee AJ, O'Byrne KT, Jones SA, & Eltringham L (1987). The subfornical organ and relaxin-induced inhibition of reflex milk ejection in lactating rats. *J Endocrinol* 115: 347-353.
- Summerlee AJ, O'Byrne KT, Paisley AC, Breeze MF, & Porter DG (1984). Relaxin affects the central control of oxytocin release. *Nature* 309: 372-374.
- Sunn N, Egli M, Burazin TC, Burns P, Colvill L, Davern P, Denton DA, Oldfield BJ, Weisinger RS, Rauch M, Schmid HA, & McKinley MJ (2002). Circulating relaxin acts on subfornical organ neurons to stimulate water drinking in the rat. *Proc Natl Acad Sci U S A* 99: 1701-1706.
- Sykes DA, Dowling MR, & Charlton SJ (2009). Exploring the mechanism of agonist efficacy: a relationship between efficacy and agonist dissociation rate at the muscarinic M3 receptor. *Mol Pharmacol* 76: 543-551.
- Sykes DA, Riddy DM, Stamp C, Bradley ME, McGuinness N, Sattikar A, Guerini D, Rodrigues I, Glaenzel A, Dowling MR, Mullershausen F, & Charlton SJ (2014). Investigating the molecular mechanisms through which FTY720-P causes persistent S1P1 receptor internalization. *Br J Pharmacol* 171: 4797-4807.
- Syrovatkina V, Alegre KO, Dey R, & Huang XY (2016). Regulation, signaling, and physiological functions of G-proteins. *J Mol Biol* 428: 3850-3868.
- Szlachter BN, Quagliarello J, Jewelewicz R, Osathanondh R, Spellacy WN, & Weiss G (1982). Relaxin in normal and pathogenic pregnancies. *Obstet Gynecol* 59: 167-170.
- Tan SL, & Parker PJ (2003). Emerging and diverse roles of protein kinase C in immune cell signalling. *Biochem J* 376: 545-552.
- Tashima LS, Hieber AD, Greenwood FC, & Bryant-Greenwood GD (1995). The human Leydig insulin-like (hLEY I-L) gene is expressed in the corpus luteum and trophoblast. *J Clin Endocrinol Metab* 80: 707-710.
- Tashima LS, Mazoujian G, & Bryant-Greenwood GD (1994). Human relaxins in normal, benign and neoplastic breast tissue. *J Mol Endocrinol* 12: 351-364.
- Teerlink JR, Cotter G, Davison BA, Felker GM, Filippatos G, Greenberg BH, Ponikowski P, Unemori E, Voors AA, Adams KF, Jr., Dorobantu MI, Grinfeld LR, Jondeau G, Marmor A, Masip J, Pang PS, Werdan K, Teichman SL, Trapani A, Bush CA, Saini R, Schumacher C, Severin TM, Metra M, & Investigators REIAHF (2013). Serelaxin, recombinant human relaxin-2, for treatment of acute heart failure (RELAX-AHF): a randomised, placebo-controlled trial. *Lancet* 381: 29-39.
- Teerlink JR, Davison BA, Cotter G, Maggioni AP, Sato N, Chioncel O, Ertl G, Felker GM, Filippatos G, Greenberg BH, Pang PS, Ponikowski P, Edwards C, Senger S, Teichman SL, Nielsen OW,

- Voors AA, & Metra M (2020). Effects of serelaxin in patients admitted for acute heart failure: a meta-analysis. *Eur J Heart Fail* 22: 315-329.
- Teerlink JR, Metra M, Felker GM, Ponikowski P, Voors AA, Weatherley BD, Marmor A, Katz A, Grzybowski J, Unemori E, Teichman SL, & Cotter G (2009). Relaxin for the treatment of patients with acute heart failure (Pre-RELAX-AHF): a multicentre, randomised, placebo-controlled, parallel-group, dose-finding phase IIb study. *Lancet* 373: 1429-1439.
- Teerlink JR, Voors AA, Ponikowski P, Pang PS, Greenberg BH, Filippatos G, Felker GM, Davison BA, Cotter G, Gimpelewicz C, Boer-Martins L, Wernsing M, Hua TA, Severin T, & Metra M (2017). Serelaxin in addition to standard therapy in acute heart failure: rationale and design of the RELAX-AHF-2 study. *Eur J Heart Fail* 19: 800-809.
- Telgmann R, Maronde E, Tasken K, & Gellersen B (1997). Activated protein kinase A is required for differentiation-dependent transcription of the decidual prolactin gene in human endometrial stromal cells. *Endocrinology* 138: 929-937.
- Thanasupawat T, Glogowska A, Nivedita-Krishnan S, Wilson B, Klonisch T, & Hombach-Klonisch S (2019). Emerging roles for the relaxin/RXFP1 system in cancer therapy. *Mol Cell Endocrinol* 487: 85-93.
- Thanasupawat T, Hammje K, Adham I, Ghia JE, Del Bigio MR, Krcek J, Hoang-Vu C, Klonisch T, & Hombach-Klonisch S (2013). INSL5 is a novel marker for human enteroendocrine cells of the large intestine and neuroendocrine tumours. *Oncol Rep* 29: 149-154.
- Thomas GM, & Huganir RL (2004). MAPK cascade signalling and synaptic plasticity. *Nat Rev Neurosci* 5: 173-183.
- Thompson GL, Lane JR, Coudrat T, Sexton PM, Christopoulos A, & Canals M (2016). Systematic analysis of factors influencing observations of biased agonism at the mu-opioid receptor. *Biochem Pharmacol* 113: 70-87.
- Thompson VC, Morris TG, Cochrane DR, Cavanagh J, Wafa LA, Hamilton T, Wang S, Fazli L, Gleave ME, & Nelson CC (2006). Relaxin becomes upregulated during prostate cancer progression to androgen independence and is negatively regulated by androgens. *Prostate* 66: 1698-1709.
- Thomsen ARB, Plouffe B, Cahill TJ, 3rd, Shukla AK, Tarrasch JT, Dosey AM, Kahsai AW, Strachan RT, Pani B, Mahoney JP, Huang L, Breton B, Heydenreich FM, Sunahara RK, Skiniotis G, Bouvier M, & Lefkowitz RJ (2016). GPCR-G protein-beta-arrestin super-complex mediates sustained G protein signaling. *Cell* 166: 907-919.
- Tsai EJ, & Kass DA (2009). Cyclic GMP signaling in cardiovascular pathophysiology and therapeutics. *Pharmacol Ther* 122: 216-238.
- Tsien RY (1998). The green fluorescent protein. *Annu Rev Biochem* 67: 509-544.
- Tsuchiya S, Yamabe M, Yamaguchi Y, Kobayashi Y, Konno T, & Tada K (1980). Establishment and characterization of a human acute monocytic leukemia cell line (THP-1). *Int J Cancer* 26: 171-176.
- Tsvetanova NG, & von Zastrow M (2014). Spatial encoding of cyclic AMP signaling specificity by GPCR endocytosis. *Nat Chem Biol* 10: 1061-1065.
- Unemori E (2017). Serelaxin in clinical development: past, present and future. *Br J Pharmacol* 174: 921-932.
- Unemori EN, & Amento EP (1990). Relaxin modulates synthesis and secretion of procollagenase and collagen by human dermal fibroblasts. *J Biol Chem* 265: 10681-10685.
- Unemori EN, Bauer EA, & Amento EP (1992). Relaxin alone and in conjunction with interferon-gamma decreases collagen synthesis by cultured human scleroderma fibroblasts. *J Invest Dermatol* 99: 337-342.
- Unemori EN, Beck LS, Lee WP, Xu Y, Siegel M, Keller G, Liggitt HD, Bauer EA, & Amento EP (1993). Human relaxin decreases collagen accumulation in vivo in two rodent models of fibrosis. *J Invest Dermatol* 101: 280-285.

- Unemori EN, Erikson ME, Rocco SE, Sutherland KM, Parsell DA, Mak J, & Grove BH (1999). Relaxin stimulates expression of vascular endothelial growth factor in normal human endometrial cells in vitro and is associated with menometrorrhagia in women. *Hum Reprod* 14: 800-806.
- Unemori EN, Lewis M, Constant J, Arnold G, Grove BH, Normand J, Deshpande U, Salles A, Pickford LB, Erikson ME, Hunt TK, & Huang XF (2000). Relaxin induces vascular endothelial growth factor expression and angiogenesis selectively at wound sites. *Wound Repair Regen* 8: 361-370.
- Unemori EN, Pickford LB, Salles AL, Piercy CE, Grove BH, Erikson ME, & Amento EP (1996). Relaxin induces an extracellular matrix-degrading phenotype in human lung fibroblasts in vitro and inhibits lung fibrosis in a murine model in vivo. *J Clin Invest* 98: 2739-2745.
- van der Westhuizen ET, Christopoulos A, Sexton PM, Wade JD, & Summers RJ (2010). H2 relaxin is a biased ligand relative to H3 relaxin at the relaxin family peptide receptor 3 (RXFP3). *Mol Pharmacol* 77: 759-772.
- van der Westhuizen ET, Werry TD, Sexton PM, & Summers RJ (2007). The relaxin family peptide receptor 3 activates extracellular signal-regulated kinase 1/2 through a protein kinase C-dependent mechanism. *Mol Pharmacol* 71: 1618-1629.
- Van Loy T, Vandersmissen HP, Van Hiel MB, Poels J, Verlinden H, Badisco L, Vassart G, & Vanden Broeck J (2008). Comparative genomics of leucine-rich repeats containing G protein-coupled receptors and their ligands. *Gen Comp Endocrinol* 155: 14-21.
- Vanhaesebroeck B, Guillermet-Guibert J, Graupera M, & Bilanges B (2010). The emerging mechanisms of isoform-specific PI3K signalling. *Nat Rev Mol Cell Biol* 11: 329-341.
- Vasilenko P, Mead JP, & Weidmann JE (1986). Uterine growth-promoting effects of relaxin: a morphometric and histological analysis. *Biol Reprod* 35: 987-995.
- Vassart G, & Costagliola S (2011). G protein-coupled receptors: mutations and endocrine diseases. *Nature Reviews Endocrinology* 7: 362.
- Vinall RL, Mahaffey CM, Davis RR, Luo Z, Gandour-Edwards R, Ghosh PM, Tepper CG, & de Vere White RW (2011). Dual blockade of PKA and NF-kappaB inhibits H2 relaxin-mediated castrate-resistant growth of prostate cancer sublines and induces apoptosis. *Horm Cancer* 2: 224-238.
- Violin JD, Crombie AL, Soergel DG, & Lark MW (2014). Biased ligands at G-protein-coupled receptors: promise and progress. *Trends Pharmacol Sci* 35: 308-316.
- Violin JD, DeWire SM, Yamashita D, Rominger DH, Nguyen L, Schiller K, Whalen EJ, Gowen M, & Lark MW (2010). Selectively engaging β -arrestins at the angiotensin II type 1 receptor reduces blood pressure and increases cardiac performance. *JPET* 335: 572-579.
- Wachten S, Masada N, Ayling LJ, Ciruela A, Nikolaev VO, Lohse MJ, & Cooper DM (2010). Distinct pools of cAMP centre on different isoforms of adenylyl cyclase in pituitary-derived GH3B6 cells. *J Cell Sci* 123: 95-106.
- Wacker D, Wang S, McCorvy JD, Betz RM, Venkatakrisnan AJ, Levit A, Lansu K, Schools ZL, Che T, Nichols DE, Shoichet BK, Dror RO, & Roth BL (2017). Crystal structure of an LSD-bound human serotonin receptor. *Cell* 168: 377-389 e312.
- Wahab IM, & Anderson RR (1989). Physiologic role of relaxin on mammary gland growth in rats. *Proc Soc Exp Biol Med* 192: 285-289.
- Waldman SA, & Camilleri M (2018). Guanylate cyclase-C as a therapeutic target in gastrointestinal disorders. *Gut* 67: 1543-1552.
- Wang C, Kemp-Harper BK, Kocan M, Ang SY, Hewitson TD, & Samuel CS (2016). The anti-fibrotic actions of relaxin are mediated through a NO-sGC-cGMP-dependent pathway in renal myofibroblasts in vitro and enhanced by the NO donor, diethylamine NONOate. *Front Pharmacol* 7: 91.
- Wang C, Pinar AA, Widdop RE, Hossain MA, Bathgate RAD, Denton KM, Kemp-Harper BK, & Samuel CS (2020). The anti-fibrotic actions of relaxin are mediated through AT2R-associated

- protein phosphatases via RXFP1-AT2R functional crosstalk in human cardiac myofibroblasts. *FASEB J* 34: 8217-8233.
- Ward WW, & Cormier MJ (1979). An energy transfer protein in coelenterate bioluminescence. Characterization of the Renilla green-fluorescent protein. *J Biol Chem* 254: 781-788.
- Watson C, Chen G, Irving P, Way J, Chen W-J, & Kenakin T (2000). The use of stimulus-biased assay systems to detect agonist-specific receptor active states: implications for the trafficking of receptor stimulus by agonists. *Mol Pharmacol* 58: 1230-1238.
- Way SA, & Leng G (1992). Relaxin increases the firing rate of supraoptic neurones and increases oxytocin secretion in the rat. *J Endocrinol* 132: 149-158.
- Webb BL, Hirst SJ, & Giembycz MA (2000). Protein kinase C isoenzymes: a review of their structure, regulation and role in regulating airways smooth muscle tone and mitogenesis. *Br J Pharmacol* 130: 1433-1452.
- Wehbi VL, Stevenson HP, Feinstein TN, Calero G, Romero G, & Vilardaga JP (2013). Noncanonical GPCR signaling arising from a PTH receptor-arrestin-Gbetagamma complex. *Proc Natl Acad Sci U S A* 110: 1530-1535.
- Weiss G, Teichman S, Stewart D, Nader D, Wood S, Breining P, & Unemori E (2016). Recombinant human relaxin versus placebo for cervical ripening: a double-blind randomised trial in pregnant women scheduled for induction of labour. *BMC Pregnancy Childbirth* 16: 260.
- Werthmann RC, Volpe S, Lohse MJ, & Calebiro D (2012). Persistent cAMP signaling by internalized TSH receptors occurs in thyroid but not in HEK293 cells. *FASEB J* 26: 2043-2048.
- Wettschureck N, & Offermanns S (2005). Mammalian G proteins and their cell type specific functions. *Physiol Rev* 85: 1159-1204.
- Wetzel V, Schinner E, Kees F, Hofmann F, Faerber L, & Schlossmann J (2016). Involvement of cyclic guanosine monophosphate-dependent protein kinase I in renal antifibrotic effects of serelaxin. *Front Pharmacol* 7: 195.
- White AD, Jean-Alphonse FG, Fang F, Pena KA, Liu S, Konig GM, Inoue A, Aslanoglou D, Gellman SH, Kostenis E, Xiao K, & Vilardaga JP (2020a). Gq/11-dependent regulation of endosomal cAMP generation by parathyroid hormone class B GPCR. *Proc Natl Acad Sci U S A*.
- White CW, Caspar B, Vanyai HK, Pflieger KDG, & Hill SJ (2020b). CRISPR-mediated protein tagging with nanoluciferase to investigate native chemokine receptor function and conformational changes. *Cell Chem Biol* 27: 499-510 e497.
- White CW, Johnstone EKM, See HB, & Pflieger KDG (2018). NanoBRET ligand binding at a GPCR under endogenous promotion facilitated by CRISPR/Cas9 genome editing. *Cell Signal* 54: 27-34.
- White CW, Vanyai HK, See HB, Johnstone EKM, & Pflieger KDG (2017). Using nanoBRET and CRISPR/Cas9 to monitor proximity to a genome-edited protein in real-time. *Sci Rep* 7: 3187.
- Wilkinson TN, Speed TP, Tregear GW, & Bathgate RA (2005). Evolution of the relaxin-like peptide family. *BMC Evol Biol* 5: 14.
- Williams C (2004). cAMP detection methods in HTS: selecting the best from the rest. *Nat Rev Drug Discov* 3: 125-135.
- Williams EJ, Benyon RC, Trim N, Hadwin R, Grove BH, Arthur MJ, Unemori EN, & Iredale JP (2001). Relaxin inhibits effective collagen deposition by cultured hepatic stellate cells and decreases rat liver fibrosis in vivo. *Gut* 49: 577-583.
- Wilson BC, Connell B, & Saleh TM (2006). Relaxin-induced reduction of infarct size in male rats receiving MCAO is dependent on nitric oxide synthesis and not estrogenic mechanisms. *Neurosci Lett* 393: 160-164.
- Wilson T, & Hastings JW (1998). Bioluminescence. *Annu Rev Cell Dev Biol* 14: 197-230.
- Windham PF, & Tinsley HN (2015). cGMP signaling as a target for the prevention and treatment of breast cancer. *Semin Cancer Biol* 31: 106-110.

- Winslow JW, Shih A, Bourell JH, Weiss G, Reed B, Stults JT, & Goldsmith LT (1992). Human seminal relaxin is a product of the same gene as human luteal relaxin. *Endocrinology* 130: 2660-2668.
- Wisler JW, DeWire SM, Whalen EJ, Violin JD, Drake MT, Ahn S, Shenoy SK, & Lefkowitz RJ (2007). A unique mechanism of beta-blocker action: carvedilol stimulates beta-arrestin signaling. *Proc Natl Acad Sci U S A* 104: 16657-16662.
- Wong GT, Gannon KS, & Margolskee RF (1996). Transduction of bitter and sweet taste by gustducin. *Nature* 381: 796.
- Wong W, & Scott JD (2004). AKAP signalling complexes: focal points in space and time. *Nat Rev Mol Cell Biol* 5: 959-970.
- Wong YH, Conklin BR, & Bourne HR (1992). Gz-mediated hormonal inhibition of cyclic AMP accumulation. *Science* 255: 339-342.
- Wu P, & Brand L (1994). Resonance energy transfer: methods and applications. *Anal Biochem* 218: 1-13.
- Wynn TA (2008). Cellular and molecular mechanisms of fibrosis. *J Pathol* 214: 199-210.
- Wynn TA, & Ramalingam TR (2012). Mechanisms of fibrosis: therapeutic translation for fibrotic disease. *Nat Med* 18: 1028-1040.
- Xia X, Zhang Y, Zieth CR, & Zhang SC (2007). Transgenes delivered by lentiviral vector are suppressed in human embryonic stem cells in a promoter-dependent manner. *Stem Cells Dev* 16: 167-176.
- Xiao J, Huang Z, Chen CZ, AgoulNIK IU, Southall N, Hu X, Jones RE, Ferrer M, Zheng W, AgoulNIK AI, & Marugan JJ (2013). Identification and optimization of small-molecule agonists of the human relaxin hormone receptor RXFP1. *Nat Commun* 4: 1953.
- Xie XC, Zhao N, Xu QH, Yang X, Xia WK, Chen Q, Wang M, & Fei X (2017). Relaxin attenuates aristolochic acid induced human tubular epithelial cell apoptosis in vitro by activation of the PI3K/Akt signaling pathway. *Apoptosis* 22: 769-776.
- Xu C, Peter M, Bouquier N, Ollendorff V, Villamil I, Liu J, Fagni L, & Perroy J (2013). REV, A BRET-based sensor of ERK activity. *Front Endocrinol (Lausanne)* 4: 95.
- Xu Y, Piston DW, & Johnson CH (1999). A bioluminescence resonance energy transfer (BRET) system: application to interacting circadian clock proteins. *Proc Natl Acad Sci U S A* 96: 151-156.
- Yan Y, Scott DJ, Wilkinson TN, Ji J, Tregear GW, & Bathgate RA (2008). Identification of the N-linked glycosylation sites of the human relaxin receptor and effect of glycosylation on receptor function. *Biochemistry* 47: 6953-6968.
- Yarwood RE, Imlach WL, Lieu T, Veldhuis NA, Jensen DD, Klein Herenbrink C, Aurelio L, Cai Z, Christie MJ, Poole DP, Porter CJH, McLean P, Hicks GA, Geppetti P, Halls ML, Canals M, & Bunnett NW (2017). Endosomal signaling of the receptor for calcitonin gene-related peptide mediates pain transmission. *Proc Natl Acad Sci U S A* 114: 12309-12314.
- Yee JK, Miyanohara A, LaPorte P, Bouic K, Burns JC, & Friedmann T (1994). A general method for the generation of high-titer, pantropic retroviral vectors: highly efficient infection of primary hepatocytes. *Proc Natl Acad Sci U S A* 91: 9564-9568.
- Yki-Jarvinen H, Wahlstrom T, & Seppala M (1983). Immunohistochemical demonstration of relaxin in the genital tract of men. *J Reprod Fertil* 69: 693-695.
- Yona S, Lin HH, Siu WO, Gordon S, & Stacey M (2008). Adhesion-GPCRs: emerging roles for novel receptors. *Trends Biochem Sci* 33: 491-500.
- Zaleski HM, Winn RJ, Jennings RL, & Sherwood OD (1996). Effects of relaxin on lactational performance in ovariectomized gilts. *Biol Reprod* 55: 671-675.
- Zarrow MX (1948). The role of the steroid hormones in the relaxation of the symphysis pubis of the guinea pig. *Endocrinology* 42: 129-140.

- Zhang F, Klebansky B, Fine RM, Liu H, Xu H, Servant G, Zoller M, Tachdjian C, & Li X (2010). Molecular mechanism of the sweet taste enhancers. *Proc Natl Acad Sci U S A* 107: 4752-4757.
- Zhang F, Klebansky B, Fine RM, Xu H, Pronin A, Liu H, Tachdjian C, & Li X (2008). Molecular mechanism for the umami taste synergism. *Proc Natl Acad Sci U S A* 105: 20930-20934.
- Zhang Q, Liu SH, Erikson M, Lewis M, & Unemori E (2002). Relaxin activates the MAP kinase pathway in human endometrial stromal cells. *J Cell Biochem* 85: 536-544.
- Zhang W, & Liu HT (2002). MAPK signal pathways in the regulation of cell proliferation in mammalian cells. *Cell Res* 12: 9-18.
- Zhao L, Roche PJ, Gunnersen JM, Hammond VE, Tregear GW, Wintour EM, & Beck F (1999). Mice without a functional relaxin gene are unable to deliver milk to their pups. *Endocrinology* 140: 445-453.
- Zhao L, Samuel CS, Tregear GW, Beck F, & Wintour EM (2000). Collagen studies in late pregnant relaxin null mice. *Biol Reprod* 63: 697-703.
- Zhao P, Liang YL, Belousoff MJ, Deganutti G, Fletcher MM, Willard FS, Bell MG, Christie ME, Sloop KW, Inoue A, Truong TT, Clydesdale L, Furness SGB, Christopoulos A, Wang MW, Miller LJ, Reynolds CA, Danev R, Sexton PM, & Wootten D (2020). Activation of the GLP-1 receptor by a non-peptidic agonist. *Nature* 577: 432-436.
- Zimmermann S, Steding G, Emmen JM, Brinkmann AO, Nayernia K, Holstein AF, Engel W, & Adham IM (1999). Targeted disruption of the *Ins13* gene causes bilateral cryptorchidism. *Mol Endocrinol* 13: 681-691.
- Zürn A, Zabel U, Vilardaga JP, Schindelin H, Lohse MJ, & Hoffmann C (2009). Fluorescence resonance energy transfer analysis of α_2 -adrenergic receptor activation reveals distinct agonist-specific conformational changes. *Mol Pharmacol* 75: 534-541.

Appendix A



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Understanding relaxin signalling at the cellular level

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ABSTRACT

The peptide hormone relaxin mediates many biological actions including anti-fibrotic, vasodilatory, angiogenic, anti-inflammatory, anti-apoptotic, and organ protective effects across a range of tissues. At the cellular level, relaxin binds to the G protein-coupled receptor relaxin family peptide receptor 1 (RXFP1) to activate a variety of downstream signal transduction pathways. This signalling cascade is complex and also varies in diverse cellular backgrounds. Moreover, RXFP1 signalling shows crosstalk with other receptors to mediate some of its physiological functions. This review summarises known signalling pathways induced by acute versus chronic treatment with relaxin across a range of cell types, it describes RXFP1 crosstalk with other receptors, signalling pathways activated by other ligands targeting RXFP1, and it also outlines physiological relevance of RXFP1 signalling outputs. Comprehensive understanding of the mechanism of relaxin actions in fibrosis, vasodilation, as well as organ protection, will further support relaxin's clinical potential.

1. Introduction

The peptide hormone relaxin activates the G protein-coupled receptor (GPCR) relaxin family peptide receptor 1 (RXFP1) to mediate physiological functions across a variety of tissues, including reproductive, cardiovascular, renal, hepatic, and connective tissues. Acute or chronic treatment with human relaxin (referred to here as relaxin but also known as serelaxin or H2 relaxin) leads to activation of intracellular signalling pathways such as cyclic adenosine monophosphate (cAMP), phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K), Akt (PKB), nitric oxide (NO), cyclic guanosine monophosphate (cGMP), mitogen-activated protein kinases (MAPKs), and protein kinase C (PKC), depending on the cell type studied. Although relaxin signalling has been extensively studied using HEK293T cells over-expressing RXFP1 (HEK-RXFP1 cells), it is now clear that there are substantial differences in relaxin signalling between recombinant and native RXFP1-expressing cells, as well as amongst different types of native cells. This includes differences in G protein coupling, signalling pathways activated, duration of signalling, and downstream physiological effects. Furthermore, recent studies have highlighted that crosstalk between RXFP1 signalling and other receptors may influence the signalling pathways mediating some of the actions of relaxin, particularly its anti-fibrotic actions. For example, relaxin/RXFP1 signalling shows

crosstalk with transforming growth factor β 1 (TGF- β 1) (Heeg et al., 2005), Notch-1 (Sassoli et al., 2013), the angiotensin II type 2 receptor (AT₂R) (Chow et al., 2014), peroxisome proliferator-activated receptor gamma (PPAR γ) (Singh et al., 2015), the endothelin B receptor (ET_B) (Dschietzig et al., 2003), and the glucocorticoid receptor (Dschietzig et al., 2004), depending on the cell type involved. Other recent developments in RXFP1 pharmacology include the discovery of a biased small molecule agonist (Xiao et al., 2013), development of a relaxin analogue that shows cell-specific actions (Hossain et al., 2016), and discovery of other ligands that can activate RXFP1 (Glogowska et al., 2013; Shemesh et al., 2008, 2009).

Relaxin has demonstrated potential for use as a therapeutic drug for cardiovascular and fibrotic diseases [reviewed in Bathgate et al. (2018)], but there is still a lot to learn about its downstream signalling from binding and activating RXFP1, the contribution and importance of different signalling pathways and interactions with other receptors towards its physiological outcomes, and why in some cases there is cell-specific activity. Understanding relaxin signalling in detail may help understand why it has failed clinical trials despite showing promising actions experimentally *in vitro* and *in vivo*, and will assist in understanding the signalling pathways that are important for its therapeutic actions. In this review, we will outline the signalling pathways activated by acute (up to 1 h) or chronic (24–96 h) treatment of native cells

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with relaxin, examine the crosstalk between relaxin/RXFP1 signalling and other ligands and receptors, and discuss how these signalling pathways mediate the short- and long-term effects of RXFP1 activation.

2. Signal transduction pathways activated by relaxin

2.1. Cyclic adenosine monophosphate (cAMP)

Cyclic adenosine monophosphate (cAMP) is a ubiquitous second messenger that is synthesised from adenosine triphosphate by adenylate cyclases (ACs), and is degraded by phosphodiesterases (PDEs) (Beavo and Brunton, 2002). cAMP signals by activating protein kinase A (PKA), Epac, and cyclic nucleotide-gated ion channels. Relaxin administration acutely increases cAMP activity across a range of cell types, including normal human endometrial (NHE) cells (Fei et al., 1990), endometrial stromal cells (Bartsch et al., 2004), human myometrial cells (Heng et al., 2008), human decidual cells from fetal membranes (Kern and Bryant-Greenwood, 2009), and human decidual macrophages (Horton et al., 2011). Relaxin also increases cAMP activity in rat anterior pituitary cells (Cronin et al., 1987) and various human cancer cell types including MCF-7 breast cancer cells (Bigazzi et al., 1992), monocytic leukaemia THP-1 cells (Parsek et al., 1996), prostate cancer cells (Silvertown et al., 2007; Vinall et al., 2011), and some glioblastoma cells (Glogowska et al., 2013). Additionally, relaxin stimulates cAMP activity in primary human hepatic stellate cells (Fallowfield et al., 2014; McBride et al., 2017), LX-2 human hepatic stellate cells (Singh et al., 2015; McBride et al., 2017), and some primary vascular cell types including human umbilical vein endothelial cells (HUVECs), human umbilical vein smooth muscle cells (HUVSMCs), and human umbilical artery smooth muscle cells (HUASMCs) (Sarwar et al., 2015). However, some cell types that express RXFP1 show either weak and transient or no cAMP activity when stimulated with relaxin, including human lower uterine segment fibroblasts (Palejwala et al., 1998), rat ventricular fibroblasts (Samuel et al., 2004), rat renal myofibroblasts (Mookerjee et al., 2009), idiopathic pulmonary fibrosis-derived human lung fibroblasts (Huang et al., 2011), and human cardiac fibroblasts (Sarwar et al., 2015).

2.1.1. G proteins associated with cAMP activity

GPCRs initiate signalling by coupling to heterotrimeric G proteins, which consist of a $G\alpha$ subunit and a $G\beta\gamma$ dimer. Upon activation of the GPCR, the G proteins dissociate from the GPCR and interact with downstream effectors. RXFP1 couples to $G\alpha_s$ to mediate cAMP increases in various cell types (Sarwar et al., 2015; Halls et al., 2006; Valkovic et al., 2018), which is negatively modulated by coupling to $G\alpha_{oB}$ (Halls et al., 2006). In some cell types there is also a delayed increase in cAMP mediated by $G\alpha_i$, $\beta\gamma$ subunits, PI3K, translocation of PKC- ζ to the plasma membrane, and activation of AC5 (Halls et al., 2006; Nguyen and Dessauer, 2005a, 2005b; Nguyen et al., 2003). However, the contributions of different G proteins towards the cAMP response differ among cell types. For example, the relaxin-induced cAMP concentration-response curves in HUVECs and HUVSMCs were sigmoidal and were sensitive to inhibition of $G\alpha_{i/o}$ using pertussis toxin (PTX) or PI3K using wortmannin, whereas the concentration-response curve for HUASMCs was bell-shaped and was not sensitive to inhibition of $G\alpha_{i/o}$ or PI3K (Sarwar et al., 2015). The relaxin-stimulated biphasic cAMP response was shown to be physiologically relevant. For example, treatment of rat atria with PTX inhibited relaxin-induced chronotropic and inotropic responses, as well as reducing cAMP activity, suggesting a role for cAMP in this effect (Kompa et al., 2002). Additionally in human atrial preparations, positive inotropic effects of relaxin were associated with $G\alpha_{i/o}$ -PI3K-PKA signalling, particularly for failing atria, where expression of $G\alpha_{i2}$ and $G\alpha_{i3}$ were also upregulated by about 2-fold compared to non-failing tissue (Dschiez et al., 2011). Fig. 1 summarises acute signal transduction pathways activated by relaxin in vascular cells.

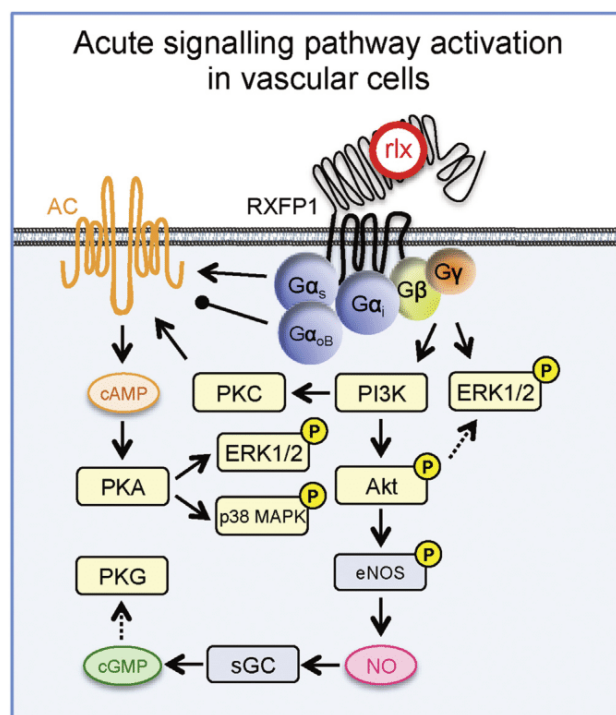


Fig. 1. Acute signal transduction mechanism activated by relaxin in vascular cells. Relaxin activates variety of acute (up to 1 h) signal transduction pathways in diverse vascular cells via RXFP1. These relaxin-induced signalling mechanisms are described in the text and summarized in Table 1. Note: arrows with dotted lines, indicate indirect or postulated pathways and ↑, indicates upregulated pathways.

In some cell types, relaxin-mediated cAMP activity is also downstream of tyrosine kinase activation. For example, in THP-1 cells, inhibition of phosphotyrosine phosphatases potentiated porcine relaxin-mediated cAMP activity, and inhibition of tyrosine kinases reduced the cAMP response in THP-1 and endometrial stromal cells (Bartsch et al., 2001). This potentiation of cAMP activity might be a result of inhibition of PDEs via tyrosine kinases (Bartsch et al., 2001; Anand-Ivell et al., 2007).

2.1.2. Sustained cAMP activity

Relaxin stimulation produces sustained activation of cAMP in some cell types. For example, treatment of endometrial stromal cells with relaxin produces sustained increases in cAMP activity for at least 3 h, which is associated with decidualisation and is consistent with the role of relaxin in maintaining pregnancy (Bartsch et al., 2001). Typically, GPCR signalling is terminated when the receptor is phosphorylated by GPCR kinases (GRKs), which is followed by binding of β -arrestins that impede G protein binding and promote internalisation of the receptor (Ferguson, 2001). Interestingly, RXFP1 shows weak or no agonist-induced desensitisation and internalisation, providing a potential mechanism for the sustained actions of relaxin (Kern and Bryant-Greenwood, 2009; Callander et al., 2009).

2.1.3. Biological effects of cAMP activity

In endometrial stromal cells (ESCs), relaxin-induced cAMP activity is associated with increases in markers of decidualisation such as prolactin (Bartsch et al., 2004; Huang et al., 1987; Telgmann et al., 1997). The cAMP pathway was also suggested to be involved in anti-inflammatory and wound healing actions of relaxin. In bovine bronchial epithelial cells, relaxin increased PKA activity (but not PKC or PKG), which was followed by an increase in the rate of wound healing in a

model of wound repair (Wyatt et al., 2002). In decidual macrophages, relaxin treatment for 24 h led to interleukin 6 (IL-6) secretion, which was also associated with cAMP signalling (Horton et al., 2011). Additionally, it has been suggested porcine relaxin or relaxin increases proliferation of cancer cells via a cAMP-dependent mechanism (Bigazzi et al., 1992; Vinall et al., 2011).

2.2. Vascular endothelial growth factor (VEGF)

Relaxin promotes the upregulation of vascular endothelial growth factor (VEGF) in a variety of cell types, which is partially linked to cAMP activity. This includes NHE cells (Unemori et al., 1999), stromal cells and glandular epithelial cells from secretory endometrium (Palejwala et al., 2002), THP-1 cells (Unemori et al., 2000), HUVECs, HUASMCs, HCFs (Sarwar et al., 2015), and also in the cortex of female spontaneously hypertensive rats (SHRs) (Chan et al., 2013). Notably, VEGF was not upregulated in HUASMCs despite increased cAMP accumulation, yet VEGF was upregulated in HCFs despite the lack of cAMP activity, suggesting that VEGF can also be regulated independently of cAMP activation (Sarwar et al., 2015). Physiologically relevant functions of relaxin-mediated VEGF activity may include angiogenesis, wound healing (Unemori et al., 2000), and chronic vasodilation (McGuane et al., 2011a).

2.3. Phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) and protein kinase B (Akt)

The PI3K/Akt pathway is involved in regulation of the cell cycle, including mediation of cell survival, apoptosis, and proliferation. PI3K can be activated by G $\beta\gamma$ dimers, Ras, and receptor tyrosine kinases. Class I PI3Ks can phosphorylate PtdIns(4,5)P₂ to produce PtdIns(3,4,5)P₃, which can bind to the pleckstrin homology (PH) domain of Akt, a Ser/Thr kinase that has a range of substrates (Manning and Toker, 2017). Subsequent phosphorylation of Akt at Thr-308 and Ser-473 increases its kinase activity. Relaxin enhances PI3K/Akt activity both acutely and chronically. For example, relaxin treatment induces acute increases in PI3K activity in THP-1 cells (Nguyen et al., 2003) and acute phosphorylation of Akt in LNCaP prostate cancer cells (Thr-308 phosphorylation peaked at 30 min after treatment; Ser-473 phosphorylation occurred 30 min after treatment and peaked at 180–360 min) (Liu et al., 2008), aortic endothelial cells (Ser-473) (McGuane et al., 2011b), mouse fibrochondrocytes (Ahmad et al., 2012), rat Sertoli cells (Ser-473) (Nascimento et al., 2012) and neonatal rat cardiomyocytes (Ser-473) (Aragon-Herrera et al., 2018). Chronic relaxin treatment (24 h or more) increased PI3K or Akt activity in human glioblastoma cells (Glogowska et al., 2013), rat aortic endothelial cells (Dschietszig et al., 2012), NIH3T3 mouse embryonic fibroblasts, and H9c2 rat cardiomyoblasts (Boccalini et al., 2018). *In vivo*, fibrotic rats showed increased hepatic Akt (Ser-473) phosphorylation after relaxin treatment for 72 h (Fallowfield et al., 2014).

As expected, acute activation of Akt via RXFP1 occurs downstream of PI3K, which has been shown numerous times by using pharmacological inhibitors of PI3K (such as LY294002 and/or wortmannin (McGuane et al., 2011b)). In HEK-RXFP1 cells, it was demonstrated that PI3K/Akt signalling occurs downstream of G α_{i3} $\beta\gamma$ subunits (Halls et al., 2006). Studies on rat Sertoli cells support involvement of G α_i (Nascimento et al., 2012), however G α_i isoform involved in relaxin signalling depends on the cell type studied. A recent study using G α_{i2} and G α_{i3} knockout mice and PI3K inhibitors demonstrated that G α_{i2} , not G α_{i3} , was involved in the actions of relaxin on mesenteric arteries, and that PI3K β and PI3K γ isoforms in particular were involved (Lian et al., 2018). There also appears to be crosstalk between the ERK1/2 and PI3K/Akt pathways. For example, in rat Sertoli cells, the MEK inhibitor U0126 blocked the ability of relaxin to cause phosphorylation of Akt, whereas inhibition of PI3K impaired the ability of relaxin treatment to increase phosphorylation of ERK1/2 (Nascimento et al., 2012).

2.3.1. Biological effects of PI3K/Akt activation

Relaxin has important vasodilatory actions via PI3K/Akt and nitric oxide (described in the nitric oxide section below). Relaxin also demonstrates anti-apoptotic effects via the PI3K/Akt pathway across several cell types. For example, relaxin inhibited oxidative-stress-induced apoptosis in rat cardiomyocytes, which occurred alongside increased phosphorylation of Akt and ERK1/2 (Moore et al., 2007), and produced anti-apoptotic effects via PI3K (but not ERK1/2) in rat aortic rings with TNF- α -induced endothelial dysfunction (Dschietszig et al., 2012). Similarly, relaxin produced cytoprotection against hypoxia/reoxygenation injury in HTR-8/SVneo trophoblast cells via PI3K/Akt (Ogunleye et al., 2017) and reduced aristolochic acid-induced apoptosis in HK2 proximal tubule epithelial cells via PI3K/Akt (Xie et al., 2017). Additionally, rats that underwent ischemia/reperfusion (I/R) injury showed decreased phosphorylation of Akt (Ser-473) in kidney samples, but this decrease was inhibited by relaxin treatment during a 6-h reperfusion, which may potentially be associated with the renoprotective actions of relaxin (Collino et al., 2013). PI3K or Akt activity has also been implicated in other chronic actions of relaxin, including migration of glioblastoma cells (Glogowska et al., 2013), and its anti-fibrotic actions (see below).

2.4. Nitric oxide synthase (NOS) and nitric oxide (NO)

Nitric oxide (NO) is a gaseous signalling molecule involved in a range of processes across the cardiovascular system, immune system, and nervous system (Forstermann and Sessa, 2012). NO is synthesised from L-arginine by nitric oxide synthase (NOS), which has three isoforms: neuronal NOS (nNOS or NOS1), inducible NOS (iNOS or NOS2), and endothelial NOS (eNOS or NOS3). Relaxin promotes NO production by at least two mechanisms: (1) acute activation of NOS and (2) up-regulation and chronic activation of NOS. The isoform of NOS that is activated by relaxin is also cell-dependent. Relaxin treatment induces rapid increases in NO production (measured at 30 min or earlier) in rat serosal mast cells (Masini et al., 1994), human coronary artery endothelial cells (HCAECs) (McGuane et al., 2011b), and human bone marrow-derived endothelial cells (Segal et al., 2012). Long-term relaxin treatment increases the expression of nNOS in rat renal myofibroblasts (Chow et al., 2012, 2014; Mookerjee et al., 2009), HUVECs (Sarwar et al., 2015; Quattrone et al., 2004), HUASMCs and HCFs (Sarwar et al., 2015). Additionally, long-term porcine relaxin or relaxin stimulation also upregulates iNOS in bovine artery smooth muscle cells (Bani et al., 1998), guinea pig hearts challenged with ovalbumin (Masini et al., 2002), rat coronary endothelial cells (Failli et al., 2002), kidney tissue of rats that underwent I/R injury (Collino et al., 2013), and lung from rats that underwent I/R injury (Alexiou et al., 2013).

eNOS is activated after phosphorylation at Ser-1177 (or the equivalent Ser in other species), which is predominantly mediated by Akt (Dimmeler et al., 1999). Acute increases in eNOS phosphorylation occur after relaxin stimulation of human aortic endothelial cells (McGuane et al., 2011b), and chronic increases in eNOS phosphorylation after relaxin treatment were observed in rat aortic rings (Dschietszig et al., 2012), rat kidney after ischemia/reperfusion injury (Collino et al., 2013), and fibrotic rat liver (Fallowfield et al., 2014), for example. This is consistent with the idea that after relaxin stimulation, eNOS is upregulated and then activated via phosphorylation in some cell types. Porcine relaxin treatment also upregulated eNOS in uterine tissue of oestrogenised mice (Bani et al., 1999).

2.4.1. Biological effects of nitric oxide activity

NOS inhibitors such as nitro-L-arginine (L-NNA), nitro-L-arginine methyl ester (L-NAME), and L-N^G-monomethyl arginine (L-NMMA) have been used to determine the biological effects of relaxin-mediated NO activity. Importantly, relaxin-mediated NO activity has a key role in its vascular actions, most notably its vasodilator actions which occur via a G α_i - $\beta\gamma$ -PI3K-Akt-eNOS-NO pathway (McGuane et al., 2011b; Lian

et al., 2018). Acute treatment with porcine relaxin or relaxin increased coronary flow via NO in isolated guinea pig and rat hearts (Bani-Sacchi et al., 1995), inhibited thrombin-induced rises in intracellular calcium in BASMCs, which is associated with NO activity and suggestive of vasodilation (Bani et al., 1998), and also increased vasodilation via NOS in human subcutaneous arteries, rat small renal arteries (McGuane et al., 2011b), and mouse mesenteric arteries (Lian et al., 2018). Similarly, a 3-h relaxin treatment reduced myogenic constriction of rat small renal arteries and human subcutaneous arteries via NOS (McGuane et al., 2011b), and administration of relaxin for five days produced vasodilation in isolated mouse small renal arteries (McGuane et al., 2011a) and rat small renal arteries (Novak et al., 2002), via NOS/NO. Sustained relaxin-mediated vasodilation also appears to involve VEGF, gelatinases (MMP-2 and MMP-9), and the endothelin B receptor, which also contribute towards eNOS/NO activity in some cell types [reviewed in Leo et al. (2017) and Sarwar et al. (2016a)]. Relaxin treatment also produces vasoprotective actions via NO. For example, relaxin stimulation for 48 h mitigated TNF- α -induced vascular dysfunction of rat aortic rings partly by promoting eNOS activity (Dschiertzig et al., 2012). Similarly, chronic relaxin-mediated upregulation of eNOS may also contribute towards its vasoprotective actions in guinea pigs exposed to cigarette smoke (Pini et al., 2016). Fig. 2 summarises chronic signal transduction pathways activated by relaxin in vascular cells.

Additionally, relaxin also has a role in organ protection via NO activity. For example, relaxin showed protective effects against I/R injury in rat lung when administered during the entire I/R cycle, which was dependent on a balance of PI3K and ERK1/2 activity leading to upregulation of iNOS (Alexiou et al., 2013). The ability of chronic relaxin treatment to reduce portal pressure in fibrotic rat liver is also dependent on NOS (Fallowfield et al., 2014).

Relaxin was also suggested to have anti-inflammatory actions via

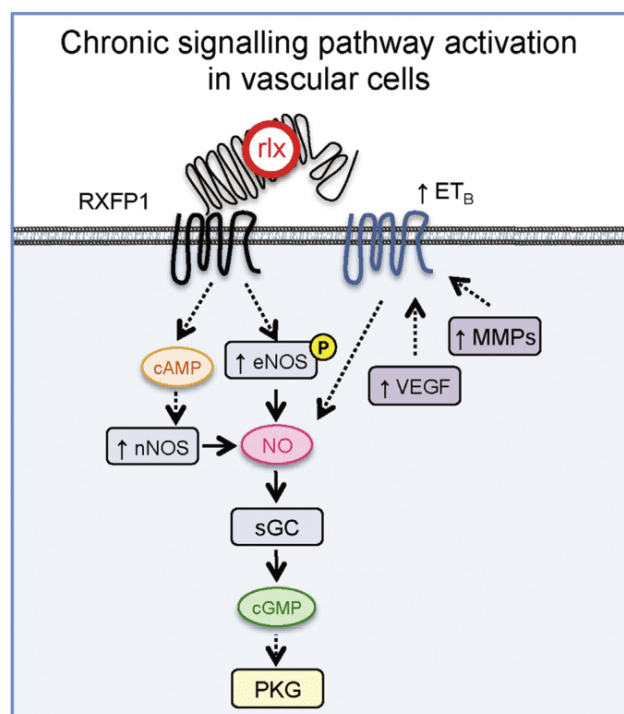


Fig. 2. Chronic signal transduction mechanism activated by relaxin in vascular cells. Relaxin activates variety of chronic (24–96 h) signal transduction pathways in diverse vascular cells via RXFP1 also involving cross talk with endothelin B receptor (ET_B). These relaxin-induced signalling pathways are described in the text and summarized in Table 2. Note: arrows with dotted lines, indicate indirect or postulated pathways and †, indicates upregulated pathways.

NO. For example, in co-cultures of rat coronary endothelial cells primed with LPS and neutrophils, stimulation with porcine relaxin for 24 h reduced adhesion of neutrophils via NOS (Nistri et al., 2003). Porcine relaxin also acutely inhibited histamine release by mast cells (Masini et al., 1994) and activation of human basophils via NO (Bani et al., 2002). Moreover, inhibition of human platelet aggregation after relaxin treatment was also likely associated with NO activity (Bani et al., 2007). Furthermore, NO is also involved in the anti-fibrotic actions of relaxin (see below).

Finally, relaxin-induced NO activity has roles in reproductive tissues. For example, acute porcine relaxin treatment caused sustained relaxation of pre-contracted mouse uterus (Bani et al., 1999) and was involved in growth of the cervix after an 8-h treatment (Sherwood et al., 2000), via NO.

2.5. Cyclic guanosine monophosphate (cGMP)

NO binds to and activates soluble guanylate cyclase (sGC), an intracellular enzyme that synthesizes cGMP from GTP. cGMP activates protein kinase G (PKG) and cyclic nucleotide-gated ion channels, and is broken down by PDEs (Lucas et al., 2000). Relaxin stimulation produces acute increases in cGMP in human platelets (Bani et al., 2007), human hepatic stellate cells (Fallowfield et al., 2014), rat renal myofibroblasts (Wang et al., 2016), HUVECs, HUVSMCs, HUASMCs, and HCFs (Sarwar et al., 2015). The relaxin-induced cGMP concentration-response curves in HUVECs, HUVSMCs, and HCFs cells were bell-shaped. This was altered to a sigmoidal curve in HUVSMCs by the G $\alpha_{i/o}$ inhibitor NF023 or the lipid raft disruptor filipin III, suggesting that G $\alpha_{i/o}$ localised to lipid rafts determines the shape of the concentration-response curve (Sarwar et al., 2015).

Moreover, relaxin treatment produced long-term increases in cGMP levels (detected at 24–96 h) in MCF-7 cells (Bani et al., 1995), bovine artery smooth muscle cells (Bani et al., 1998), and idiopathic pulmonary fibrosis (IPF)-derived lung myofibroblasts (Huang et al., 2011). cGMP has also been confirmed to activate PKG following relaxin treatment (Huang et al., 2011; Wetzl et al., 2016). For example, mice treated with relaxin for a week after unilateral ureteral obstruction (a model for renal fibrosis) showed increases in cGMP levels in fibrotic kidney tissue and increases in PKG1 activity in fibrotic and non-fibrotic tissues (Wetzl et al., 2016). Similarly in fibrotic rats, intrahepatic levels of cGMP were increased after relaxin treatment for 72 h (Fallowfield et al., 2014).

2.5.1. Biological effects of cGMP activity

cGMP has been measured for relaxin activity less frequently than NO, but as the use of the sGC inhibitor ODQ in fibroblasts blocked the downstream effects of relaxin, it is believed that NO is having its full effect via activation of sGC (Mookerjee et al., 2009; Chow et al., 2012; Wang et al., 2016). NO-cGMP-PKG signalling is implicated in the ability of chronic relaxin treatment to inhibit phosphorylation of myosin light chain and contraction of IPF-derived lung myofibroblasts (Huang et al., 2011), and has key roles in the anti-fibrotic actions of relaxin (see below).

2.6. Mitogen-activated protein kinases (MAPKs)

The mitogen-activated protein kinases (MAPKs) are a family of Ser/Thr kinases that includes ERK1/2 (also known as p44/42 MAPK), c-Jun N-terminal kinases (JNKs), p38 MAPKs, and ERKs 3–8 (Zhang and Liu, 2002). MAPK signalling can occur after GPCR activation, including downstream of G α or G $\beta\gamma$ subunits, and also downstream of scaffold proteins β -arrestins (DeWire et al., 2007; Goldsmith and Dhanasekaran, 2007).

2.6.1. Extracellular signal-regulated kinases 1 and 2 (ERK1/2)

ERK1/2 are some of the more well-characterised MAPKs that are

involved in the regulation of a range of cellular processes such as cell survival, proliferation, and differentiation. Relaxin induces rapid increases in ERK1/2 phosphorylation (pERK1/2) that peak by 5–10 min, and return to basal by 30 min in NHE cells, THP-1 cells, human primary coronary artery smooth muscle cells, pulmonary artery smooth muscle cells (Zhang et al., 2002), primary rat Sertoli cells (Nascimento et al., 2012), HUVMCs, HUASMCs, and HCFs (Sarwar et al., 2015). Relaxin-induced concentration-response curves for pERK1/2 in HUVECs, HUVMCs, and HCFs were bell-shaped, whereas HUASMCs showed a sigmoidal curve (Sarwar et al., 2015).

Some cell types exhibit sustained ERK1/2 activity after relaxin stimulation. For example, in HeLa cells and HUVECs, relaxin was reported to produce increased ERK1/2 activation up to 90 min after stimulation (Dschietszig et al., 2003). Another study showed sustained, but weak, ERK1/2 activity in HUVECs for at least 60 min following relaxin treatment (Sarwar et al., 2015). In neonatal rat cardiomyocytes, relaxin increased ERK1/2 activity at 5 min, which was still above basal ERK1/2 activity at 30 min (Aragon-Herrera et al., 2018). Likewise, relaxin-treatment of rat renal myofibroblasts induced a large, rapid increase in ERK1/2 phosphorylation within a few minutes of stimulation, which declined (from 8-fold to 3-fold above baseline) but was sustained (at 3-fold above baseline) for at least an hour (Mookerjee et al., 2009). Another study using rat renal myofibroblasts showed relaxin-induced ERK1/2 activity that appeared more transient, however was still higher than the baseline over its 20-min time-course (Hossain et al., 2016). In mouse fibrochondrocytes, relaxin stimulation produced ERK1/2 phosphorylation at 30 min (Ahmad et al., 2012). Sustained ERK1/2 activation at GPCRs is typically associated with β -arrestin-mediated signalling rather than G protein-mediated signalling (Shenoy et al., 2006; Ren et al., 2005; Ahn et al., 2004). However, as relaxin does not promote β -arrestin recruitment, this is unlikely to be the case for its signal transduction mechanisms.

Prolonged relaxin treatment can also produce chronic increases in ERK1/2 phosphorylation. For example, relaxin stimulation of rat renal myofibroblasts for 72 h increased ERK1/2 phosphorylation (Chow et al., 2012, 2014). Similarly, inhibition of ERK using a MEK inhibitor, PD98059, inhibited nNOS expression, suggesting that signalling via an ERK/nNOS pathway occurred after chronic relaxin stimulation of these myofibroblasts (Chow et al., 2012). Treatment of rats that had undergone I/R injury with relaxin during a 6-h reperfusion also led to increased phosphorylation of ERK1/2 (Collino et al., 2013).

2.6.1.1. Signalling pathways involved in ERK1/2 activation. In NHE stromal cells, ERK1/2 activation was shown to be downstream of MEK by using a MEK inhibitor (Zhang et al., 2002). In vascular cells and cardiac fibroblasts, ERK1/2 was shown to be downstream of $G\alpha_{i/o}$ and PI3K, as responses were inhibited by treatment with PTX or wortmannin (Sarwar et al., 2015). However, ERK1/2 phosphorylation at 2 min in rat renal myofibroblasts was enhanced by inhibition of $G\alpha_{i/o}$, and unaffected by inhibition of PI3K, adenylate cyclase, or PKA, suggesting ERK1/2 activity can occur at multiple different points in the RXFP1 signalling cascade (Mookerjee et al., 2009). In rat Sertoli cells, the use of pharmacological inhibitors also showed that a number of pathways can contribute to activation of ERK1/2, including MEK, $G\alpha_{i/o}$, PKA, Src, and PI3K (Nascimento et al., 2012). As noted above, there was also crosstalk between ERK1/2 and PI3K/Akt in Sertoli cells (Nascimento et al., 2012).

2.6.1.2. Biological effects of ERK1/2 activation. Relaxin upregulated VEGF expression in NHE cells, which was partially blocked by use of a MEK inhibitor, demonstrating that, like cAMP, the MAPK pathway is also involved in relaxin-mediated upregulation of VEGF (Zhang et al., 2002). Relaxin-mediated ERK1/2 activity in vascular cells also mediates upregulation of the endothelin B receptor, which is implicated in vascular actions of relaxin (Dschietszig et al., 2003). ERK1/2 is also involved in the anti-fibrotic actions of relaxin (see

below).

2.6.2. p38 mitogen-activated protein kinase (p38 MAPK)

Relaxin stimulation acutely increased p38 MAPK phosphorylation (p-p38 MAPK), but not ERK1/2 phosphorylation, in human VSMCs from mammary arteries (Dschietszig et al., 2003), and also increased p-p38 MAPK in HUASMCs, HUVMCs (Kocan et al., 2017), and LX-2 cells (Singh et al., 2015). However, our understanding of the role of p38 MAPK in the actions of relaxin is currently limited.

2.7. Protein kinase C (PKC)

The protein kinase C (PKC) family comprises Ser/Thr kinases involved in regulation of the cell cycle (Griner and Kazanietz, 2007). Although PKC activity is typically associated with $G\alpha_q$ signalling, there is no evidence that RXFP1 couples to this G protein. Rather, as outlined in the cAMP signalling section above, relaxin-mediated PKC- ζ translocation has been shown to occur as part of a $G\alpha_i$ - $\beta\gamma$ -PI3K pathway in some cell types. Relaxin treatment activates translocation of PKC- ζ to the plasma membrane in human secretory endometrial stromal cells (Kalbag et al., 1991), THP-1 cells, MCF-7 cells, pregnant human myometrial (PHM1-31) cells, and mouse mesangial cells (Nguyen and Dessauer, 2005b). In glioblastoma cells, increased phosphorylation of PKC- ζ and PKC- δ via PI3K were detected after stimulation with relaxin, and inhibition of PKC- δ with rottlerin or PKC- ζ with the myristoylated peptide inhibited relaxin-induced cell migration (Glogowska et al., 2013). Relaxin treatment also led to increased phosphorylation of cardiac myofilaments in mouse heart via PKC (but not PKA), consistent with its inotropic effects (Shaw et al., 2009). In particular, myofilament-associated PKC- δ was increased after stimulation with relaxin, whereas PKC- α and PKC- β_{II} were decreased, and inhibition of PKC- δ using rottlerin blocked this action of relaxin (Shaw et al., 2009). Moreover, PKC activation is also involved in anti-fibrotic actions of relaxin (see below).

3. Crosstalk with other receptors and signalling pathways

Recent studies have demonstrated that some of physiological actions of relaxin/RXFP1 signalling are mediated by crosstalk with signalling pathways activated by other receptors including TGF- β receptors, AT $_2$ R, Notch-1, and PPAR γ , which are outlined below. As noted above, there is also crosstalk with the ET $_B$ receptor. Interestingly, relaxin also binds to and activates the glucocorticoid receptor (Dschietszig et al., 2004) to produce anti-inflammatory and vasoprotective actions that have been reviewed elsewhere (Halls et al., 2015).

3.1. Transforming growth factor beta (TGF- β) and matrix metalloproteinases (MMPs)

Upon binding to RXFP1 in human and rat myofibroblasts (key fibrosis-producing cells), relaxin activates RXFP1 signalling to mediate its anti-fibrotic actions [reviewed in Samuel et al. (2017) and Samuel et al. (2016)]. Briefly, chronic relaxin administration to myofibroblasts results in pERK1/2-nNOS-NO-sGC-cGMP signalling to inhibit intracellular Smad2 phosphorylation, as a means of disrupting TGF- β 1 signal transduction and its pro-fibrotic actions on myofibroblast differentiation and myofibroblast-mediated extracellular matrix (ECM)/collagen deposition *in vitro* (Heeg et al., 2005; Chow et al., 2014; Sarwar et al., 2015; Mookerjee et al., 2009; Wang et al., 2016; Kocan et al., 2017; Hossain et al., 2011) and *in vivo* (Chow et al., 2014; Royce et al., 2014; Samuel et al., 2014; Bennett et al., 2014). In cardiac myofibroblasts, relaxin also activates a Notch-1-dependent pathway to inhibit intracellular Smad3 phosphorylation (Sassoli et al., 2013; Kocan et al., 2017) to suppress the pro-fibrotic influence of TGF- β 1 (see below). As relaxin stimulates cAMP either weakly or not at all in myofibroblasts (Sarwar et al., 2015; Samuel et al., 2004; Mookerjee

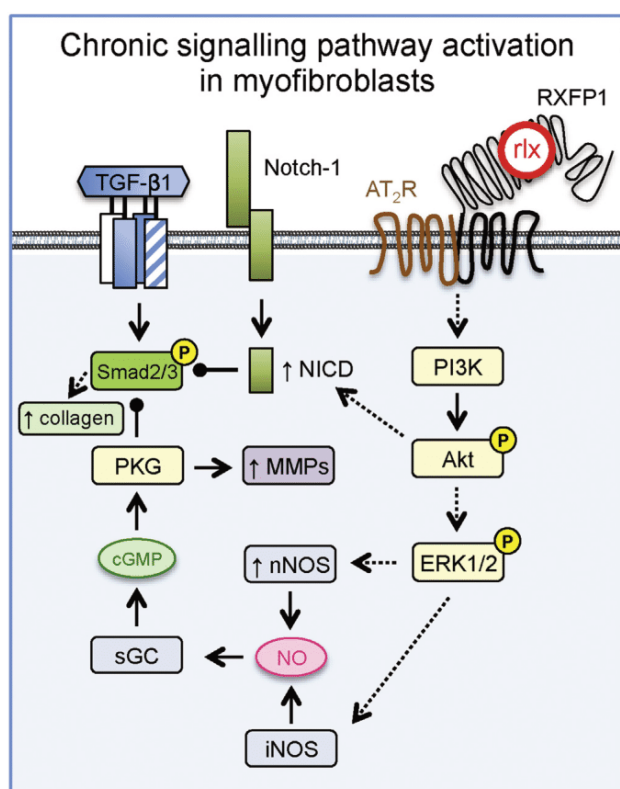


Fig. 3. Chronic signal transduction mechanism activated by relaxin in myofibroblasts. Relaxin activates variety of acute (up to 1 h) and chronic (24–96 h) signal transduction pathways in diverse myofibroblasts via RXFP1 involving cross talk with Angiotensin II type 2 receptor (AT₂R). These relaxin-induced signalling pathways are described in the text and summarized in Table 2. Note: arrows with dotted lines, indicate indirect or postulated pathways and ↑, indicates upregulated pathways.

et al., 2009), its anti-fibrotic actions appear to be largely independent of cAMP.

Relaxin has also been shown to signal via RXFP1-pERK1/2-nNOS-NO-sGC-cGMP in myofibroblasts to promote the expression and activity of various ECM/collagen-degrading matrix metalloproteinases (MMPs; MMP-1/-13, MMP-2, MMP-9) (Chow et al., 2012; Wang et al., 2016). Interestingly, although ERK1/2 is suggested to be important for cGMP production and the anti-fibrotic actions of relaxin, the small molecule biased agonist ML290 does not activate ERK1/2, yet still produces cGMP accumulation and anti-fibrotic actions in myofibroblasts (see below for further details). In addition to pERK1/2, roles for PI3K/Akt and PKC-ζ have also been identified in relaxin-mediated upregulation of MMPs in mouse fibrochondrocytes (Ahmad et al., 2012). Additionally, the ability of relaxin to inhibit TGF-β1 signal transduction and its profibrotic actions, which is a known suppressor of iNOS activity (Chow et al., 2012; Zhang and Phan, 1999), allows iNOS to be released and be involved in the relaxin-induced up-regulation of MMPs (Chow et al., 2012). Recent studies have confirmed that cGMP activates PKG to mediate the anti-fibrotic actions of relaxin, including its ability to reduce pSmad2 and myofibroblast differentiation (Wetzel et al., 2016). Fig. 3 summarises chronic signal transduction pathways activated by relaxin in myofibroblasts.

3.2. Notch-1

Relaxin activates Notch-1 signalling to mediate its anti-fibrotic and other actions. Notch-1 is a single transmembrane receptor that is activated by ligand binding (including Jagged-1) followed by proteolytic

cleavage of the receptor by ADAM (a disintegrin and metalloprotease) proteases and γ-secretase. This releases Notch intracellular domain (NICD), which can translocate to the nucleus to regulate target genes such as Hes-1. In NIH3T3 and H9c2 cells, upregulation of ADAM10 and NICD occurred via PI3K/Akt after a 24-h treatment with relaxin (Boccalini et al., 2018). Relaxin treatment prevented the TGF-β1-induced differentiation of neonatal murine cardiac fibroblasts to myofibroblasts and decreased Smad3 phosphorylation via Notch-1 (Sassoli et al., 2013), and also activated Notch signalling to attenuate the endothelial-mesenchymal transition in HUVECs (Zhou et al., 2015). Relaxin also produced cardioprotective effects after hypoxia/reoxygenation injury in H9c2 cells, when cells were treated with relaxin before hypoxia or at reoxygenation (Boccalini et al., 2015), and inhibited high glucose-induced oxidative stress, hypertrophy, and apoptosis of H9c2 cells (Wei et al., 2018), via Notch-1. Moreover, relaxin-induced Notch-1 signalling may have contributed towards its hepatoprotective actions in a mouse model of liver ischemia/reperfusion injury/orthotopic liver transplantation (Kageyama et al., 2018).

3.3. Angiotensin II type 2 receptor (AT₂R)

The AT₂ receptor was shown to be essential for relaxin-induced anti-fibrotic effects (Chow et al., 2014). Use of an AT₂R antagonist, PD123319, blocked the anti-fibrotic actions of relaxin *in vitro* in rat renal myofibroblasts, and *in vivo* in mice (Chow et al., 2014). Moreover, the effects of relaxin were also abolished in AT₂R knockout mice, further supporting the role of AT₂R in the relaxin-stimulated signal transduction mechanism (Chow et al., 2014).

3.4. Peroxisome proliferator-activated receptor gamma (PPARγ)

Peroxisome proliferator-activated receptor gamma (PPARγ) is a nuclear receptor and transcription factor that is expressed across a range of tissues, including adipose tissue, vascular cells, fibroblasts, and immune cells, where it mediates a range of processes, including adipogenesis, glucose homeostasis, cell proliferation and differentiation, as well as anti-inflammatory and anti-fibrotic actions (Lehrke and Lazar, 2005; Tontonoz and Spiegelman, 2008). Upon ligand binding to PPARγ, a heterodimer of PPARγ and the retinoid X receptor binds to peroxisome proliferator response elements (PPREs) in the promoter of target genes to increase transcription. PPARγ can also be activated by coactivators such as PPARγ coactivator 1α (PGC-1α) independent of ligand binding. Like relaxin, PPARγ agonists disrupt TGF-β1 signalling to abrogate fibrosis (Burgess et al., 2005; Kawai et al., 2009).

Treatment of HEK-RXFP1 cells with relaxin increased PPRE-mediated transcriptional activity of PPARγ via RXFP1 independently of PPAR ligand binding (Singh and Bennett, 2010). Inhibition of PKA completely blocked relaxin-mediated PPRE transcription, whereas inhibition of Gα_{i/o}, PI3K, or p38 MAPK partially blocked it (Singh et al., 2015). p38 MAPK was also downstream of PKA, and phosphorylation of CREB (a target of both PKA and p38 MAPK) mediated the activation of PPAR (Singh et al., 2015). Upregulation of PGC-1α via CREB is believed to mediate activation of PPARγ by relaxin (Singh et al., 2015). Importantly, relaxin-mediated, ligand-independent activation of PPARγ was confirmed in LX-2 cells (Fig. 4), which natively express RXFP1 (Singh et al., 2015), and another study showed that the small molecule agonist ML290 also increases mRNA levels of PGC-1α at 72 h in activated human hepatic stellate cells (Wilson et al., 2018). Similarly, relaxin treatment for two weeks restored PGC-1α levels (which had been decreased) *in vivo* in mice with hepatic fibrosis (Bennett et al., 2017). Relaxin-mediated activation of PPARγ is also involved in its ability to induce hypertrophic remodelling of rat brain parenchymal arterioles (Chan and Cipolla, 2011).

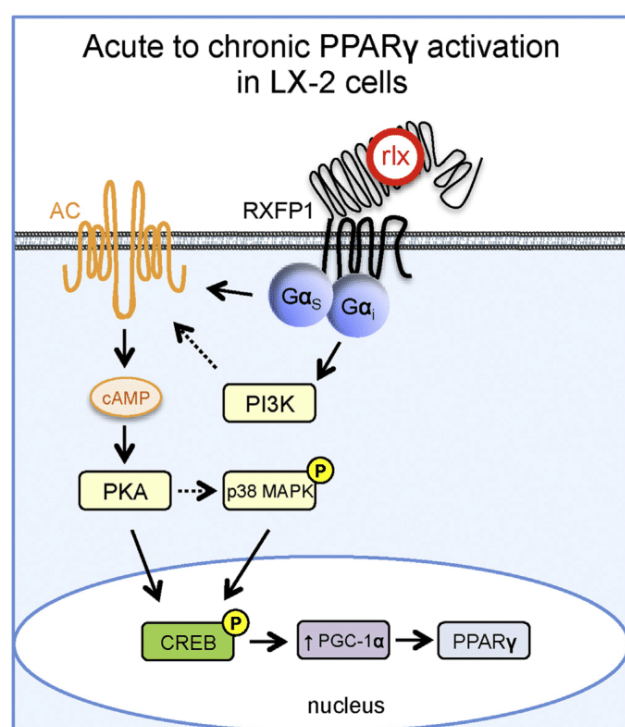


Fig. 4. Signal transduction mechanism of PPAR γ activation by relaxin in LX-2 cells. Relaxin activates peroxisome proliferator-activated receptor gamma (PPAR γ) chronically in LX-2 cells via cAMP dependent mechanism. The signal transduction pathways involved in PPAR γ activation are described in the text. Note: arrows with dotted lines, indicate indirect or postulated pathways and $\uparrow$, indicates upregulated pathways.

4. Signal transduction pathways activated by other ligands through RXFP1

There is now a biased small molecule agonist for RXFP1 (Xiao et al., 2013), and a relaxin peptide analogue that has cell-specific actions (Hossain et al., 2016). It is also known that the C1q/tumour necrosis factor-related protein 8 (CTRP8), and fragments of it, can also activate RXFP1. These are all valuable tools that are currently helping to advance our understanding of the relaxin/RXFP1 signalling mechanism. The signalling profiles of these ligands are outlined below.

4.1. Small molecule agonist ML290

ML290 is a novel small molecule agonist for RXFP1 that shows good pharmacokinetic properties *in vivo*, and is selective for RXFP1 over RXFP2 (Xiao et al., 2013). ML290 was identified in a high-throughput screen of human RXFP1 and does not activate rodent RXFP1 receptors (Huang et al., 2015). Like most GPCR agonists, ML290 binds directly in the transmembrane bundle and therefore in a different site to relaxin (Xiao et al., 2013). ML290 has recently been demonstrated to be a biased agonist of RXFP1 (Kocan et al., 2017). In THP-1 cells, ML290 is a full agonist for cAMP activity, but has a substantially lower potency than relaxin (Xiao et al., 2013). Similarly, ML290 acutely increased cAMP and cGMP activity in HCAECs, HUVECs, HUASMCs, and HUVSMCs, but with lower potency than relaxin, and also increased cGMP but not cAMP activity in HCFs (Kocan et al., 2017). Notably, whereas relaxin is equipotent for cAMP and cGMP activity, ML290 is about 10 times more potent in activating cGMP over cAMP (Kocan et al., 2017). However, ML290 did not acutely activate ERK1/2 activity in any cell type tested, but did activate p38 MAPK activity in HUASMCs and HUVSMCs, but not HUVECs or HCAECs (Kocan et al., 2017).

ML290 also upregulated VEGF mRNA after a 2-h treatment period (Xiao et al., 2013).

As with relaxin, ML290 has anti-fibrotic actions. Chronic treatment of activated HCFs with relaxin or ML290 (for 72 h) upregulated MMP-2 and inhibited TGF- β 1-induced Smad2 and Smad3 phosphorylation (Kocan et al., 2017). In activated hepatic stellate cells, ML290 upregulated MMP-1 (but not MMP-2) and PGC-1 α mRNA after 72 h (Wilson et al., 2018). Relaxin-mediated ERK1/2 activity in fibroblasts has typically been measured after chronic treatment, but to our knowledge ML290 has not been tested for chronic ERK1/2 activity. Therefore, it is unknown whether ML290 signals via ERK1/2-nNOS after chronic treatment to produce its anti-fibrotic actions. Additionally, transgenic mice expressing human RXFP1 have recently been developed, providing the ability to test ML290 in mouse models of human disease (Kaftanovskaya et al., 2017).

4.2. Single chain relaxin analogue B7-33

As the primary residues for binding of relaxin to RXFP1 are located on the relaxin B-chain, a B-chain analogue of relaxin, known as B7-33, has been developed (Hossain et al., 2016). B7-33 has shown cell-specific actions, as it has similar activity to relaxin in some cell types, but weaker activity in other cells. B7-33 is a full agonist for cAMP activity in HEK-RXFP1 and THP-1 cells, but has a substantially lower potency than relaxin (Hossain et al., 2016). Acute treatment with B7-33 also induced ERK1/2 activity with much weaker efficacy and potency than relaxin in HEK-RXFP1 cells, yet B7-33 signals via ERK1/2 with nearly identical efficacy and potency to relaxin in rat renal myofibroblasts, and upregulated MMP-2 to a similar degree as relaxin in rat renal myofibroblasts and HCFs after 72 h, via RXFP1 (Hossain et al., 2016). As with relaxin, B7-33 is anti-fibrotic in rodent models of heart and lung fibrosis, and upregulated MMP-2 via RXFP1-AT $_2$ R complexes (Hossain et al., 2016). Unlike relaxin, however, B7-33 did not promote prostate tumour growth *in vivo* in mice, which may be a result of its reduced potency for cAMP activity (Hossain et al., 2016). B7-33 also has similar actions to relaxin in rat mesenteric arteries, but the signal transduction mechanisms involved remain to be examined (Marshall et al., 2017).

4.3. C1q/tumour necrosis factor-related protein 8 (CTRP8)

CTRP8 is a secreted protein that forms complexes with C1q-related factor (CRF) but has been studied little due to the lack of an ortholog in mice (Peterson et al., 2009). The small linear peptides P59 (CGEN25010) and P74 (CGEN25009), which were discovered by computational screening and are derived from the precursor for CTRP8, were shown to activate RXFP1 and RXFP2 expressed in CHO-K1 cells (Shemesh et al., 2008, 2009). CTRP8 itself also interacts with RXFP1 in some primary glioblastoma (GB) cells and GB cell lines that did not show expression of relaxin-1, -2, or -3, or the CTRP8 partner C1q, but did express RXFP1 and CTRP8 (Glogowska et al., 2013). Like relaxin, recombinant CTRP8 increased cAMP activity in primary GB cells that expressed RXFP1, as well as in HEK-RXFP1 cells but not parental HEK293T cells, demonstrating that the effect was RXFP1-specific (Glogowska et al., 2013). Treatment of RXFP1-positive GB cells with relaxin, CTRP8, P59, or P74 increased cell migration and increased the production of cathepsin B via PI3K, PKC- δ , and PKC- ζ which led to increased invasion of GB cells via laminin (Glogowska et al., 2013). CTRP8 treatment of GB cells also led to phosphorylation of the STAT3 transcription factor in GB cells by 5 min after stimulation, and showed DNA protective and cytoprotective effects against temozolomide (a GB chemotherapeutic drug that alkylates DNA) via RXFP1 and STAT3 (Thanasupawat et al., 2018).

5. Conclusions and future directions

As summarized above, RXFP1 activates a variety of signalling

Table 1

Summary of acute relaxin signalling in diverse cell types (up to 1 h).

	cAMP	NO/cGMP	ERK1/2	PI3K/Akt
Reproductive				
Human endometrial cells	+		+	
Human myometrial cells	+			
Human decidual cells from fetal membranes	+			
Human decidual macrophages	+			
Rat Sertoli cells			+	+
Fibroblasts				
Human lower uterine segment fibroblasts	–			
Human cardiac fibroblasts	–	+	+	
Rat ventricular fibroblasts	– (or weak)			
Rat renal myofibroblasts	–	+	+/+ (sustained)	
Human primary hepatic stellate cells	+	+		
Human LX-2 hepatic stellate cells	+			
Mouse fibrochondrocytes			+	+
Cardiac and vascular				
HUVECs	+	+	+	
HUVSMCs	+	+	+	
HUASMCs	+	+	+	
Human pulmonary artery smooth muscle cells			+	
Human coronary artery smooth muscle cells			+	
Human aortic endothelial cells		+		+
Human coronary artery endothelial cells		+		
Rat neonatal cardiomyocytes			+	+
Bovine artery smooth muscle cells		+		
Cancer				
Human monocytic leukaemia THP-1 cells	+	+	+	+
Human glioblastoma cells	+			
Human MCF-7 breast cancer cells	+	+		
Human prostate cancer cells	+			+
Human cervical cancer HeLa cells			+	
Other				
HEK-RXFP1	+	–	+	
Rat anterior pituitary cells	+			
Rat serosal mast cells		+		
Human bone marrow-derived endothelial cells		+		
Human platelets		+		

Note: +, indicates activated pathways and –, not activated pathways following acute treatment with relaxin. Blank spaces represent pathways that have not been determined.

Table 2

Summary of chronic relaxin signalling in diverse cell types (24–96 h).

	NO/cGMP	ERK1/2	PI3K/Akt
Fibroblasts			
Idiopathic pulmonary fibrosis (IPF) lung myofibroblasts	+		
Rat renal myofibroblasts	+	+	
NIH3T3 mouse embryonic fibroblasts			+
Human cardiac fibroblasts	+		
Cardiac and vascular			
HUVECs	+		
HUVSMCs	+		
HUASMCs	+		
Bovine artery smooth muscle cells	+		
Rat coronary endothelial cells	+		
Rat aortic endothelial cells			+
H9c2 rat cardiomyoblasts			+
Cancer			
Human glioblastoma cells			+
Human MCF-7 breast cancer cells	+		

Note: +, indicates activated pathways following chronic treatment with relaxin. Blank spaces represent pathways that have not been determined.

cascades to produce its pleiotropic actions, including anti-apoptotic, anti-inflammatory, anti-fibrotic, and vasodilatory actions. Tables 1 and 2 summarise key pathways activated by relaxin in RXFP1-expressing cells, but also highlight the gaps in our knowledge of relaxin-induced signalling mechanisms in diverse cells. Recent studies have also demonstrated that relaxin signalling shows substantial crosstalk with signalling cascades downstream of other receptors, which explains some of its physiological actions and may also offer a rationale behind

its cell-specific signalling outputs (Figs. 1–4). Furthermore, there is also emerging research demonstrating crosstalk between relaxin and prostaglandin signalling (Leo et al., 2016; Ng et al., 2016; Sarwar et al., 2016b). In summary, further studies are required to help us better understand the pleiotropic actions of relaxin. Detailed investigation of biased signalling of a small molecule agonist ML290 and cell specific signalling of a peptide mimetic B7-33 will also aid our understanding of RXFP1 signal transduction mechanisms. Such a comparative study of relaxin and other ligands targeting RXFP1 in diverse pathophysiological models will determine the therapeutic importance of different signalling pathways among cell types and will also highlight the clinical potential for ML290 and B7-33.

Conflicts of interest

RADB is inventor on the patent application PCT/AU 2015/050184, Modified Relaxin B chain peptides. All other authors declare that there are no conflicts of interest.

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References

- Ahmad, N., et al., 2012. Relaxin induces matrix-metalloproteinases-9 and -13 via RXFP1: induction of MMP-9 involves the PI3K, ERK, Akt and PKC-zeta pathways. *Mol. Cell. Endocrinol.* 363 (1–2), 46–61.

- Ahn, S., et al., 2004. Differential kinetic and spatial patterns of beta-arrestin and G protein-mediated ERK activation by the angiotensin II receptor. *J. Biol. Chem.* 279 (34), 35518–35525.
- Alexiou, K., et al., 2013. Relaxin protects rat lungs from ischemia-reperfusion injury via inducible NO synthase: role of ERK-1/2, PI3K, and forkhead transcription factor FKHL1. *PLoS One* 8 (9), e75592.
- Anand-Ivell, R., et al., 2007. Relaxin signalling in THP-1 cells uses a novel phosphotyrosine-dependent pathway. *Mol. Cell. Endocrinol.* 272 (1–2), 1–13.
- Aragon-Herrera, A., et al., 2018. Relaxin activates AMPK-AKT signaling and increases glucose uptake by cultured cardiomyocytes. *Endocrine* 60 (1), 103–111.
- Bani, D., et al., 1995. Relaxin activates the L-arginine-nitric oxide pathway in human breast cancer cells. *Cancer Res.* 55 (22), 5272–5275.
- Bani, D., et al., 1998. Relaxin activates the L-arginine-nitric oxide pathway in vascular smooth muscle cells in culture. *Hypertension* 31 (6), 1240–1247.
- Bani, D., et al., 1999. Relaxin up-regulates the nitric oxide biosynthetic pathway in the mouse uterus: involvement in the inhibition of myometrial contractility. *Endocrinology* 140 (10), 4434–4441.
- Bani, D., et al., 2002. Inhibitory effects of relaxin on human basophils activated by stimulation of the Fc epsilon receptor. The role of nitric oxide. *Int. Immunopharm.* 2 (8), 1195–1204.
- Bani, D., et al., 2007. A novel, simple bioactivity assay for relaxin based on inhibition of platelet aggregation. *Regul. Pept.* 144 (1–3), 10–16.
- Bani-Sacchi, T., et al., 1995. Relaxin-induced increased coronary flow through stimulation of nitric oxide production. *Br. J. Pharmacol.* 116 (1), 1589–1594.
- Bartsch, O., Bartlick, B., Ivell, R., 2001. Relaxin signalling links tyrosine phosphorylation to phosphodiesterase and adenyl cyclase activity. *Mol. Hum. Reprod.* 7 (9), 799–809.
- Bartsch, O., Bartlick, B., Ivell, R., 2004. Phosphodiesterase 4 inhibition synergizes with relaxin signaling to promote decidualization of human endometrial stromal cells. *J. Clin. Endocrinol. Metab.* 89 (1), 324–334.
- Bathgate, R.A.D., et al., 2018. The relaxin receptor as a therapeutic target - perspectives from evolution and drug targeting. *Pharmacol. Ther.* 187, 114–132.
- Beavo, J.A., Brunton, L.L., 2002. Cyclic nucleotide research – still expanding after half a century. *Nat. Rev. Mol. Cell Biol.* 3 (9), 710–718.
- Bennett, R.G., et al., 2014. Relaxin decreases the severity of established hepatic fibrosis in mice. *Liver Int.* 34 (3), 416–426.
- Bennett, R.G., Simpson, R.L., Hamel, F.G., 2017. Serelaxin increases the antifibrotic action of rosiglitazone in a model of hepatic fibrosis. *World J. Gastroenterol.* 23 (22), 3999–4006.
- Bigazzi, M., et al., 1992. Relaxin influences the growth of MCF-7 breast cancer cells. Mitogenic and antimetastatic action depends on peptide concentration. *Cancer* 70 (3), 639–643.
- Boccalini, G., et al., 2015. Relaxin protects cardiac muscle cells from hypoxia/reoxygenation injury: involvement of the Notch-1 pathway. *Faseb. J.* 29 (1), 239–249.
- Boccalini, G., et al., 2018. Relaxin induces up-regulation of ADAM10 metalloprotease in RXFP1-expressing cells by PI3K/AKT signaling. *Mol. Cell. Endocrinol.* 472, 80–86.
- Burgess, H.A., et al., 2005. PPARgamma agonists inhibit TGF-beta induced pulmonary myofibroblast differentiation and collagen production: implications for therapy of lung fibrosis. *Am. J. Physiol. Lung Cell Mol. Physiol.* 288 (6), L1146–L1153.
- Callander, G.E., Thomas, W.G., Bathgate, R.A., 2009. Prolonged RXFP1 and RXFP2 signaling can be explained by poor internalization and a lack of beta-arrestin recruitment. *Am. J. Physiol. Cell Physiol.* 296 (5), C1058–C1066.
- Chan, S.L., Cipolla, M.J., 2011. Relaxin causes selective outward remodeling of brain parenchymal arterioles via activation of peroxisome proliferator-activated receptor-gamma. *Faseb. J.* 25 (9), 3229–3239.
- Chan, S.L., Sweet, J.G., Cipolla, M.J., 2013. Treatment for cerebral small vessel disease: effect of relaxin on the function and structure of cerebral parenchymal arterioles during hypertension. *Faseb. J.* 27 (10), 3917–3927.
- Chow, B.S., et al., 2012. Relaxin signals through a RXFP1-pERK-nNOS-NO-cGMP-dependent pathway to up-regulate matrix metalloproteinases: the additional involvement of iNOS. *PLoS One* 7 (8), e42714.
- Chow, B.S., et al., 2014. Relaxin requires the angiotensin II type 2 receptor to abrogate renal interstitial fibrosis. *Kidney Int.* 86 (1), 75–85.
- Collino, M., et al., 2013. Acute treatment with relaxin protects the kidney against ischaemia/reperfusion injury. *J. Cell Mol. Med.* 17 (11), 1494–1505.
- Cronin, M.J., Malaska, T., Bakht, C., 1987. Human relaxin increases cyclic AMP levels in cultured anterior pituitary cells. *Biochem. Biophys. Res. Commun.* 148 (3), 1246–1251.
- DeWire, S.M., et al., 2007. Beta-arrestins and cell signaling. *Annu. Rev. Physiol.* 69 (1), 483–510.
- Dimmeler, S., et al., 1999. Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature* 399 (6736), 601–605.
- Dschietzig, T., et al., 2003. Relaxin, a pregnancy hormone, is a functional endothelin-1 antagonist: attenuation of endothelin-1-mediated vasoconstriction by stimulation of endothelin type-B receptor expression via ERK-1/2 and nuclear factor-kappaB. *Circ. Res.* 92 (1), 32–40.
- Dschietzig, T., et al., 2004. Identification of the pregnancy hormone relaxin as glucocorticoid receptor agonist. *Faseb. J.* 18 (13), 1536–1538.
- Dschietzig, T., et al., 2011. The positive inotropic effect of relaxin-2 in human atrial myocardium is preserved in end-stage heart failure: role of G(i)-phosphoinositide-3 kinase signaling. *J. Card. Fail.* 17 (2), 158–166.
- Dschietzig, T., et al., 2012. Relaxin improves TNF-alpha-induced endothelial dysfunction: the role of glucocorticoid receptor and phosphatidylinositol 3-kinase signalling. *Cardiovasc. Res.* 95 (1), 97–107.
- Failli, P., et al., 2002. Relaxin up-regulates inducible nitric oxide synthase expression and nitric oxide generation in rat coronary endothelial cells. *Faseb. J.* 16 (2), 252–254.
- Fallowfield, J.A., et al., 2014. Relaxin modulates human and rat hepatic myofibroblast function and ameliorates portal hypertension in vivo. *Hepatology* 59 (4), 1492–1504.
- Fei, D.T.W., et al., 1990. Cyclic AMP response to recombinant human relaxin by cultured human endometrial cells—a specific and high throughput in vitro bioassay. *Biochem. Biophys. Res. Commun.* 170 (1), 214–222.
- Ferguson, S.S., 2001. Evolving concepts in G protein-coupled receptor endocytosis: the role in receptor desensitization and signaling. *Pharmacol. Rev.* 53 (1), 1–24.
- Forstermann, U., Sessa, W.C., 2012. Nitric oxide synthases: regulation and function. *Eur. Heart J.* 33 (7), 829–837 837a–837d.
- Glogowska, A., et al., 2013. C1q-tumour necrosis factor-related protein 8 (CTRP8) is a novel interaction partner of relaxin receptor RXFP1 in human brain cancer cells. *J. Pathol.* 231 (4), 466–479.
- Goldsmith, Z.G., Dhanasekaran, D.N., 2007. G protein regulation of MAPK networks. *Oncogene* 26 (22), 3122–3142.
- Griner, E.M., Kazanietz, M.G., 2007. Protein kinase C and other diacylglycerol effectors in cancer. *Nat. Rev. Canc.* 7 (4), 281–294.
- Halls, M.L., Bathgate, R.A., Summers, R.J., 2006. Relaxin family peptide receptors RXFP1 and RXFP2 modulate cAMP signaling by distinct mechanisms. *Mol. Pharmacol.* 70 (1), 214–226.
- Halls, M.L., et al., 2015. International Union of Basic and Clinical Pharmacology. XCV. Recent advances in the understanding of the pharmacology and biological roles of relaxin family peptide receptors 1–4, the receptors for relaxin family peptides. *Pharmacol. Rev.* 67 (2), 389–440.
- Heeg, M.H., et al., 2005. The antifibrotic effects of relaxin in human renal fibroblasts are mediated in part by inhibition of the Smad2 pathway. *Kidney Int.* 68 (1), 96–109.
- Heng, K., et al., 2008. Relaxin signalling in primary cultures of human myometrial cells. *Mol. Hum. Reprod.* 14 (10), 603–611.
- Horton, J.S., Yamamoto, S.Y., Bryant-Greenwood, G.D., 2011. Relaxin modulates proinflammatory cytokine secretion from human decidual macrophages. *Biol. Reprod.* 85 (4), 788–797.
- Hossain, M.A., et al., 2011. H3 relaxin demonstrates antifibrotic properties via the RXFP1 receptor. *Biochemistry* 50 (8), 1368–1375.
- Hossain, M.A., et al., 2016. A single-chain derivative of the relaxin hormone is a functionally selective agonist of the G protein-coupled receptor. *RXFP1. Chem Sci* 7 (6), 3805–3819.
- Huang, J.R., et al., 1987. Regulation of prolactin production by progesterone, estrogen, and relaxin in human endometrial stromal cells. *Endocrinology* 121 (6), 2011–2017.
- Huang, X., et al., 2011. Relaxin regulates myofibroblast contractility and protects against lung fibrosis. *Am. J. Pathol.* 179 (6), 2751–2765.
- Huang, Z., et al., 2015. Activation of relaxin family receptor 1 from different mammalian species by relaxin peptide and small-molecule agonist ML290. *Front. Endocrinol.* 6 (JUL), 128.
- Kaftanovskaya, E.M., et al., 2017. Human relaxin receptor is fully functional in humanized mice and is activated by small molecule agonist ML290. *J. Endocr Soc* 1 (6), 712–725.
- Kageyama, S., et al., 2018. Serelaxin induces Notch1 signaling and alleviates hepatocellular damage in orthotopic liver transplantation. *Am. J. Transplant.* 18 (7), 1755–1763.
- Kalbag, S.S., et al., 1991. Phorbol ester, prolactin, and relaxin cause translocation of protein kinase C from cytosol to membranes in human endometrial cells. *Biochim. Biophys. Acta* 1094 (1), 85–91.
- Kawai, T., et al., 2009. PPAR-gamma agonist attenuates renal interstitial fibrosis and inflammation through reduction of TGF-beta. *Lab. Invest.* 89 (1), 47–58.
- Kern, A., Bryant-Greenwood, G.D., 2009. Characterization of relaxin receptor (RXFP1) desensitization and internalization in primary human decidual cells and RXFP1-transfected HEK293 cells. *Endocrinology* 150 (5), 2419–2428.
- Kocan, M., et al., 2017. ML290 is a biased allosteric agonist at the relaxin receptor RXFP1. *Sci. Rep.* 7 (1), 2968.
- Kompa, A.R., Samuel, C.S., Summers, R.J., 2002. Inotropic responses to human gene 2 (B29) relaxin in a rat model of myocardial infarction (MI): effect of pertussis toxin. *Br. J. Pharmacol.* 137 (5), 710–718.
- Lehrke, M., Lazar, M.A., 2005. The many faces of PPARgamma. *Cell* 123 (6), 993–999.
- Leo, C.H., et al., 2016. Time-dependent activation of prostacyclin and nitric oxide pathways during continuous i.v. infusion of serelaxin (recombinant human H2 relaxin). *Br. J. Pharmacol.* 173 (6), 1005–1017.
- Leo, C.H., et al., 2017. Vascular actions of relaxin: nitric oxide and beyond. *Br. J. Pharmacol.* 174 (10), 1002–1014.
- Lian, X., et al., 2018. RXFP1 receptor activation by relaxin-2 induces vascular relaxation in mice via a Galphai2-protein/PI3Ks/gamma/Nitric oxide-coupled pathway. *Front. Physiol.* 9 (September), 1234.
- Liu, S., et al., 2008. Inappropriate activation of androgen receptor by relaxin via beta-catenin pathway. *Oncogene* 27 (4), 499–505.
- Lucas, K.A., et al., 2000. Guanylyl cyclases and signaling by cyclic GMP. *Pharmacol. Rev.* 52 (3), 375–414.
- Manning, B.D., Tokar, A., 2017. AKT/PKB signaling: navigating the network. *Cell* 169 (3), 381–405.
- Marshall, S.A., et al., 2017. B7-33 replicates the vasoprotective functions of human relaxin-2 (serelaxin). *Eur. J. Pharmacol.* 807 (April), 190–197.
- Masini, E., et al., 1994. Effects of relaxin on mast cells. In vitro and in vivo studies in rats and Guinea pigs. *J. Clin. Invest.* 94 (5), 1974–1980.
- Masini, E., et al., 2002. Protective effect of relaxin in cardiac anaphylaxis: involvement of the nitric oxide pathway. *Br. J. Pharmacol.* 137 (3), 337–344.
- McBride, A., et al., 2017. In search of a small molecule agonist of the relaxin receptor RXFP1 for the treatment of liver fibrosis. *Sci. Rep.* 7 (1), 10806.
- McGuane, J.T., et al., 2011a. Angiogenic growth factors are new and essential players in the sustained relaxin vasodilatory pathway in rodents and humans. *Hypertension* 57

- (6), 1151–1160.
- McGuane, J.T., et al., 2011b. Relaxin induces rapid dilation of rodent small renal and human subcutaneous arteries via PI3 kinase and nitric oxide. *Endocrinology* 152 (7), 2786–2796.
- Mookerjee, I., et al., 2009. Relaxin inhibits renal myofibroblast differentiation via RXFP1, the nitric oxide pathway, and Smad2. *Faseb. J.* 23 (4), 1219–1229.
- Moore, X.L., et al., 2007. Relaxin antagonizes hypertrophy and apoptosis in neonatal rat cardiomyocytes. *Endocrinology* 148 (4), 1582–1589.
- Nascimento, A.R., et al., 2012. Intracellular signaling pathways involved in the relaxin-induced proliferation of rat Sertoli cells. *Eur. J. Pharmacol.* 691 (1–3), 283–291.
- Ng, H.H., Leo, C.H., Parry, L.J., 2016. Serelaxin (recombinant human relaxin-2) prevents high glucose-induced endothelial dysfunction by ameliorating prostacyclin production in the mouse aorta. *Pharmacol. Res.* 107, 220–228.
- Nguyen, B.T., Dessauer, C.W., 2005a. Relaxin stimulates cAMP production in MCF-7 cells upon overexpression of type V adenylyl cyclase. *Ann. N. Y. Acad. Sci.* 1041, 296–299.
- Nguyen, B.T., Dessauer, C.W., 2005b. Relaxin stimulates protein kinase C zeta translocation: requirement for cyclic adenosine 3',5'-monophosphate production. *Mol. Endocrinol.* 19 (4), 1012–1023.
- Nguyen, B.T., et al., 2003. Phosphoinositide 3-kinase activity is required for biphasic stimulation of cyclic adenosine 3',5'-monophosphate by relaxin. *Mol. Endocrinol.* 17 (6), 1075–1084.
- Nistri, S., et al., 2003. Relaxin inhibits lipopolysaccharide-induced adhesion of neutrophils to coronary endothelial cells by a nitric oxide-mediated mechanism. *Faseb. J.* 17 (14), 2109–2111.
- Novak, J., et al., 2002. Myogenic reactivity is reduced in small renal arteries isolated from relaxin-treated rats. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 283 (2), R349–R355.
- Ogunleye, O., et al., 2017. Relaxin confers cytotrophoblast protection from hypoxia-reoxygenation injury through the phosphatidylinositol 3-kinase-Akt/protein kinase B cell survival pathway. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 312 (4), R559–R568.
- Palejwala, S., et al., 1998. Demonstration of a relaxin receptor and relaxin-stimulated tyrosine phosphorylation in human lower uterine segment fibroblasts. *Endocrinology* 139 (3), 1208–1212.
- Palejwala, S., et al., 2002. Relaxin gene and protein expression and its regulation of procollagenase and vascular endothelial growth factor in human endometrial cells. *Biol. Reprod.* 66 (6), 1743–1748.
- Parsell, D.A., et al., 1996. Relaxin binds to and elicits a response from cells of the human monocytic cell line, THP-1. *J. Biol. Chem.* 271 (44), 27936–27941.
- Peterson, J.M., Wei, Z., Wong, G.W., 2009. CTRP8 and CTRP9B are novel proteins that hetero-oligomerize with C1q/TNF family members. *Biochem. Biophys. Res. Commun.* 388 (2), 360–365.
- Pini, A., et al., 2016. Protection from cigarette smoke-induced vascular injury by recombinant human relaxin-2 (serelaxin). *J. Cell Mol. Med.* 20 (5), 891–902.
- Quattrone, S., et al., 2004. Relaxin potentiates the expression of inducible nitric oxide synthase by endothelial cells from human umbilical vein in vitro culture. *Mol. Hum. Reprod.* 10 (5), 325–330.
- Ren, X.R., et al., 2005. Different G protein-coupled receptor kinases govern G protein and beta-arrestin-mediated signaling of V2 vasopressin receptor. *Proc. Natl. Acad. Sci. U. S. A.* 102 (5), 1448–1453.
- Royce, S.G., et al., 2014. Intranasally administered serelaxin abrogates airway remodeling and attenuates airway hyperresponsiveness in allergic airways disease. *Clin. Exp. Allergy* 44 (11), 1399–1408.
- Samuel, C.S., et al., 2004. Relaxin modulates cardiac fibroblast proliferation, differentiation, and collagen production and reverses cardiac fibrosis in vivo. *Endocrinology* 145 (9), 4125–4133.
- Samuel, C.S., et al., 2014. Serelaxin is a more efficacious antifibrotic than enalapril in an experimental model of heart disease. *Hypertension* 64 (2), 315–322.
- Samuel, C.S., Summers, R.J., Hewitson, T.D., 2016. Antifibrotic actions of serelaxin - new roles for an old player. *Trends Pharmacol. Sci.* 37 (6), 485–497.
- Samuel, C.S., et al., 2017. Anti-fibrotic actions of relaxin. *Br. J. Pharmacol.* 174 (10), 962–976.
- Sarwar, M., et al., 2015. Serelaxin-mediated signal transduction in human vascular cells: bell-shaped concentration-response curves reflect differential coupling to G proteins. *Br. J. Pharmacol.* 172 (4), 1005–1019.
- Sarwar, M., et al., 2016a. The actions of relaxin on the human cardiovascular system. *Br. J. Pharmacol.* 174 (10), 933–949.
- Sarwar, M., et al., 2016b. Enhanced serelaxin signalling in co-cultures of human primary endothelial and smooth muscle cells. *Br. J. Pharmacol.* 173 (3), 484–496.
- Sassoli, C., et al., 2013. Relaxin prevents cardiac fibroblast-myofibroblast transition via notch-1-mediated inhibition of TGF-beta/Smad3 signaling. *PLoS One* 8 (5), e63896.
- Segal, M.S., et al., 2012. Relaxin increases human endothelial progenitor cell NO and migration and vasculogenesis in mice. *Blood* 119 (2), 629–636.
- Shaw, E.E., et al., 2009. Relaxin alters cardiac myofilament function through a PKC-dependent pathway. *Am. J. Physiol. Heart Circ. Physiol.* 297 (1), H29–H36.
- Shemesh, R., et al., 2008. Discovery and validation of novel peptide agonists for G-protein-coupled receptors. *J. Biol. Chem.* 283 (50), 34643–34649.
- Shemesh, R., et al., 2009. Activation of relaxin-related receptors by short, linear peptides derived from a collagen-containing precursor. *Ann. N. Y. Acad. Sci.* 1160, 78–86.
- Shenoy, S.K., et al., 2006. beta-arrestin-dependent, G protein-independent ERK1/2 activation by the beta2 adrenergic receptor. *J. Biol. Chem.* 281 (2), 1261–1273.
- Sherwood, O.D., et al., 2000. Inhibition of nitric oxide synthase activity diminishes the acute effects of relaxin on growth, but not softening, of the cervix in the rat. *Endocrinology* 141 (7), 2458–2464.
- Silvertown, J.D., et al., 2007. Analog of H2 relaxin exhibits antagonistic properties and impairs prostate tumor growth. *Faseb. J.* 21 (3), 754–765.
- Singh, S., Bennett, R.G., 2010. Relaxin signaling activates peroxisome proliferator-activated receptor gamma. *Mol. Cell. Endocrinol.* 315 (1–2), 239–245.
- Singh, S., Simpson, R.L., Bennett, R.G., 2015. Relaxin activates peroxisome proliferator-activated receptor gamma (PPARGgamma) through a pathway involving PPARGgamma coactivator 1alpha (PGC1alpha). *J. Biol. Chem.* 290 (2), 950–959.
- Telgmann, R., et al., 1997. Activated protein kinase A is required for differentiation-dependent transcription of the decidual prolactin gene in human endometrial stromal cells. *Endocrinology* 138 (3), 929–937.
- Thanasupawat, T., et al., 2018. C1q/TNF-related peptide 8 (CTRP8) promotes temozolomide resistance in human glioblastoma. *Mol. Oncol.* 12 (9), 1464–1479.
- Tontonoz, P., Spiegelman, B.M., 2008. Fat and beyond: the diverse biology of PPARGgamma. *Annu. Rev. Biochem.* 77 (1), 289–312.
- Unemori, E.N., et al., 1999. Relaxin stimulates expression of vascular endothelial growth factor in normal human endometrial cells in vitro and is associated with menorrhagia in women. *Hum. Reprod.* 14 (3), 800–806.
- Unemori, E.N., et al., 2000. Relaxin induces vascular endothelial growth factor expression and angiogenesis selectively at wound sites. *Wound Repair Regen.* 8 (5), 361–370.
- Valkovic, A.L., et al., 2018. Real-time examination of cAMP activity at relaxin family peptide receptors using a BRET-based biosensor. *Pharmacol. Res. Perspect.* 6 (5), e00432.
- Vinall, R.L., et al., 2011. Dual blockade of PKA and NF-kappaB inhibits H2 relaxin-mediated castrate-resistant growth of prostate cancer sublines and induces apoptosis. *Horm. Cancer* 2 (4), 224–238.
- Wang, C., et al., 2016. The anti-fibrotic actions of relaxin are mediated through a NO-SGC-GMP-Dependent pathway in renal myofibroblasts in vitro and enhanced by the NO donor, diethylamine NONOate. *Front. Pharmacol.* 7 (MAR), 91.
- Wei, X., et al., 2018. Relaxin ameliorates high glucose-induced cardiomyocyte hypertrophy and apoptosis via the Notch1 pathway. *Exp. Ther. Med.* 15 (1), 691–698.
- Wetzel, V., et al., 2016. Involvement of cyclic guanosine monophosphate-dependent protein kinase I in renal antifibrotic effects of serelaxin. *Front. Pharmacol.* 7 (JUL), 195.
- Wilson, K.J., et al., 2018. Optimization of the first small-molecule relaxin/insulin-like family peptide receptor (RXFP1) agonists: activation results in an antifibrotic gene expression profile. *Eur. J. Med. Chem.* 156, 79–92.
- Wyatt, T.A., et al., 2002. Relaxin stimulates bronchial epithelial cell PKA activation, migration, and ciliary beating. *Exp. Biol. Med.* 227 (11), 1047–1053.
- Xiao, J., et al., 2013. Identification and optimization of small-molecule agonists of the human relaxin hormone receptor RXFP1. *Nat. Commun.* 4, 1953.
- Xie, X.C., et al., 2017. Relaxin attenuates aristolochic acid induced human tubular epithelial cell apoptosis in vitro by activation of the PI3K/Akt signaling pathway. *Apoptosis* 22 (6), 769–776.
- Zhang, W., Liu, H.T., 2002. MAPK signal pathways in the regulation of cell proliferation in mammalian cells. *Cell Res.* 12 (1), 9–18.
- Zhang, H.Y., Phan, S.H., 1999. Inhibition of myofibroblast apoptosis by transforming growth factor beta(1). *Am. J. Respir. Cell Mol. Biol.* 21 (6), 658–665.
- Zhang, Q., et al., 2002. Relaxin activates the MAP kinase pathway in human endometrial stromal cells. *J. Cell. Biochem.* 85 (3), 536–544.
- Zhou, X., et al., 2015. Relaxin inhibits cardiac fibrosis and endothelial-mesenchymal transition via the Notch pathway. *Drug Des. Dev. Ther.* 9, 4599–4611.