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MRGPRX2 and its role in drug-induced anaphylaxis

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Abstract

Drug-induced anaphylaxis is a growing concern with both the better characterised IgE-dependent mechanism and less well-established IgE-independent mechanisms being implicated. IgE-independent anaphylaxis has recently been suggested to be associated with direct mast cell activation via the newly identified G protein-coupled receptor, MRGPRX2. While the clinical relevance of the receptor is not yet well understood, a range of polybasic agonists, including therapeutic drugs like neuromuscular blocking agents (NMBAs), have been found to be agonists at the receptor. Presumably, mast cells in all humans express MRGPRX2, therefore the reason why only certain individuals are susceptible to sometimes extreme adverse reactions induced by these drugs remains unclear. In this project, we aimed to better characterise the role and activation of MRGPRX2 by:

- 1) Investigating if MRGPRX2-mediated mast cell activation can be halted with chemical antagonists;
- 2) Examining if receptor activation is subject to biased agonism so that some compounds more effectively trigger mast cell activation;
- 3) Determining if MRGPRX2-mediated anaphylaxis can be predicted through the identification of insightful biomarkers.

To achieve this, we quantified mast cell activation through calcium mobilization, degranulation, and cytokine CCL2 release, using an *in vitro* human mast cell model (LAD2). We also examined clinical samples from patients who experienced an anaphylactic reaction during surgery to identify possible predictive biomarkers, whether genomic or serum components.

Firstly, we generated a novel CRISPR-Cas9 MRGPRX2 knock down LAD2 model to verify the activity of known and proposed MRGPRX2 agonists at the receptor. Following this, we observed that chemical antagonism of some MRGPRX2 agonists using sugammadex (counter ligand of rocuronium) and heparin (counter ligand of protamine), when administered after stimulation, does not affect the release of mediators contributing to the immediate symptoms of anaphylaxis. However, these agents were shown to inhibit release of the late

phase mediator, cytokine CCL2, which may perhaps be associated with the more delayed effects of anaphylaxis. Secondly, we demonstrated evidence in LAD2 cells that some NMBAs known to cause perioperative anaphylaxis (rocuronium and atracurium) are paradoxically biased against degranulation and cytokine release compared to calcium mobilization. Another agonist, substance P, was shown to be a full unbiased agonist, while protamine was identified as a partial yet unbiased agonist. Lastly, we have identified a novel double polymorphic form of MRGPRX2 in a patient who experienced anaphylaxis. We have also shown that sera from two anaphylactic patients had the ability to activate resting LAD2 mast cells. Whilst these findings were not broadly predictive of anaphylaxis across the cohort they may contribute in part to susceptibility to anaphylaxis in those individual patients.

The lack of efficacy of post hoc chemical antagonism of MRGPRX2 stimuli on mast cell degranulation is not unexpected given the rapidity of the degranulation process. However, the inhibition of the more slowly released cytokine mediators may have at least some theoretical benefit in patient recovery from anaphylaxis, although further work is needed to establish the role of cytokines in anaphylaxis. In addition, that certain NMBAs were biased against MRGPRX2-induced degranulation suggests against this mechanism underpinning NMBA-induced anaphylaxis. This, however, assumes that the LAD2 mast cell model is broadly representative of human mast cells, but it is possible that patients susceptible to MRGPRX2-dependent anaphylaxis may have 'overreactive' mast cells where NMBAs trigger a full degranulatory response. Our ambitious attempts to identify a genetic or serum biomarker that defined patients who experienced anaphylaxis were not however fruitful. Broader genomic, transcriptomic, and proteomic studies on a larger, more clearly characterised patient cohort are necessary to identify useful biomarkers that help firstly establish and perhaps then predict drug-induced, MRGPRX2-dependent anaphylaxis. In summary, the findings presented in this thesis lay important groundwork for the better understanding of MRGPRX2, and can be effectively utilised in future research to define its role in drug-induced anaphylaxis.

Declaration

I, Nithya Fernandopulle, certify that:

- i. the thesis comprises only my original work towards the PhD, except where indicated in the preface;
- ii. due acknowledgement has been made in the text to all other material used;
- iii. the thesis is fewer than 100,000 words in length, exclusive of tables, bibliographies, and appendices.

Signature: _____

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Communications

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Contribution by others

The analysis of biased agonism was conducted with the assistance of Dr. Lauren May; Laboratory of Cardiac Biology, Monash Institute of Pharmaceutical Sciences, Victoria, Australia.

The stratification of the patient cohort in determining the causative agent of anaphylaxis was conducted by Dr. Jeremy McComish; Clinical Immunologist, Allergist and Immunopathologist; Royal Melbourne Hospital, Victoria, Australia.

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“Great are the works of the Lord; They are studied by all who delight in them.” -Psalms 111:2

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List of Abbreviations

ADGR2	adhesion G protein-coupled receptor E2
ADP	adenosine diphosphate
ANZCA	Australian and New Zealand College of Anaesthetists
AT1R	angiotensin II receptor type I
ATP	adenosine triphosphate
BAT	basophil activation test
BMMC	bone marrow derived mast cell
BSA	bovine serum albumin
Ca ²⁺	calcium ion
CaCl ₂	calcium chloride
cAMP	cyclic adenosine monophosphate
CCL13	chemokine ligand 13
CCL2	monocyte chemoattractant protein 1/ chemokine ligand 2
CCR3	C-C chemokine receptor type 3
CFTR	cystic fibrosis conductance transmembrane regulator
c-Kit	stem cell factor receptor
CPA ₃	carboxypeptidase A3
CPB	cardiopulmonary bypass
CRAC	calcium release-activated channels
CRISPR	clustered regularly interspaced short palindromic repeats
CSP-1	competence-stimulating peptide-1
CSU	chronic spontaneous urticaria
DAG	diacylglycerol
DHT	delayed-type hypersensitivity
DMSO	dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EAACI	European Academy of Allergy and Clinical Immunology
ECP	eosinophil cationic protein
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbent assay
EPX	eosinophil peroxidase
FACS	fluorescence-associated cell sorting
FcεRI	high affinity IgE receptor

FDA	food and drug administrations
fMLP	N-formylmethionine-leucyl-phenylalanine
FPR2	formyl-peptide receptor-2
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GDP	guanosine diphosphate
GPCR	G protein coupled receptor
GTP	guanosine triphosphate
HBSS	Hanks' balanced salt solution
HEK-293	human embryonic kidney 293
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMC-1	human mast cell -1
HPLC	high-performance liquid chromatography
Ig	Immunoglobulin
IL-8	interleukin-8
IMDM	Iscoe's Modified Dulbecco's Media
IP ₃	inositol (1,4,5)-triphosphate
ITAM	immunoreceptor tyrosine-based activation motif
IU	international units
KCL	potassium chloride
KD	knock down
LAD2	laboratory of allergic disease 2
LDH	lactate dehydrogenase
LTC ₄	leukotriene C4
LUVA	laboratory of university of Virginia
MAF	mutated allele frequencies
MAP	mitogen-activated protein kinases
MBP	major basic protein
MC	mast cell
MgSO ₄	magnesium sulphate
MHC	major histocompatibility complex
MIF	macrophage migration inhibitory factor
MRG	Mas-related genes
MRGPR	Mas-related G protein coupled receptor
mRNA	messenger ribonucleic acid
Na ₂ HPO ₄	sodium hydrogen phosphate

NaCl	sodium chloride
NIP	4-hydroxy-3-iodo-5-nitrophenylacetyl
NK-1	neurokinin -1
NMBA	neuromuscular blocking agent
PAMP	pathogen-associated molecular pattern
PAR	protease activated receptors
PBS	phosphate buffered saline
PGD ₂	prostaglandin D ₂
PI3K	phosphoinositide 3-kinases
PIP ₂	phosphatidylinositol (4,5)-bis phosphate
PKA	primarily protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PMA	phorbol 12-myristate 13-acetate
pNAG	p-Nitrophenyl-N-acetyl- β -D-glucosaminide
PRR	pattern recognition receptor
QSM	quorum sensing molecule
RBL-2H3	rat basophilic leukaemia 2H3
RNA	ribonucleic acid
RPMC	rat peritoneal mast cell
RT qPCR	real-time quantitative polymerase chain reaction
Sav-HRP	streptavidin-horseradish peroxidase
SCF	stem cell factor
SDS-PAGE	sodium dodecyl sulphate–polyacrylamide gel electrophoresis
SEM	standard error of mean
SNARE	soluble N-ethylmaleimide-sensitive attachment protein
SPT	skin prick test
STIM1	stromal interaction molecule 1
TLR	toll-like receptor
TMB	3,3',5,5'-tetramethylbenzidine
TNF α	tumour necrosis factor α
UBC	ubiquitin C
WT	wild type

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Chapter 1

General Introduction

1.1.1 Overview of the immune system

The immune system of an organism is composed of a complex array of cells and tissues, communicating via a variety of mediators, both to prevent the intrusion of foreign organisms/substances and to remove dead/damaged/dysfunctional cells ⁽¹⁾. A wide range of cells play a role in this complex system; especially professional immune cells such as T and B lymphocytes, macrophages, eosinophils, neutrophils, basophils, monocytes, and the focus of this thesis, mast cells ^(1, 2). These cells originate from pluripotent hematopoietic stem cells in the bone marrow, where many of them also mature ⁽²⁾. Some cells then migrate into various peripheral tissues via the circulatory system to carry out their specialized roles ⁽²⁾. Neutrophils, eosinophils and basophils remain in the blood circulation unless recruited to a site of tissue insult ⁽²⁾. Monocytes also circulate in the blood until recruited to tissues where they are capable of differentiating into macrophages and dendritic cells ⁽³⁾. Macrophages and lymphocytes, while some remain in circulation, are predominantly localised within lymphoid organs or are tissue resident cells, where they further differentiate into their specialised roles as the front-line innate defence system ^(2, 4). Mast cells, the focus of this thesis, mature in tissues and therefore are rarely found as mature cells in blood circulation ⁽⁴⁾. Although best known for their role in allergy and inflammation, these cells also contribute to both the innate and adaptive host defence systems (discussed further in **section 1.1.3**) ^(1, 2).

There is however, a very fine line distinguishing a controlled immune response against a pathogen, and an uncontrolled immune response against an innocuous substance or drug resulting in undesired hypersensitivity reactions ⁽⁵⁾. Allergic/atopic disorders, deriving their name from the Greek word '*atopos*' which means 'out of place', afflict about 25% of the global population ⁽⁵⁾. Symptoms of allergy range from as mild as an itch or cough, to chest tightness and wheezing, and in extreme circumstances, life-threatening anaphylaxis with cardiovascular collapse ^(5, 6). An allergic response occurs when the immune system becomes hyperresponsive to a substance that could be a common component of food, pollen, dust or even clinically used drugs ⁽⁵⁾. These substances, usually proteins, initiate an abnormally

vigorous/allergic immune reaction (commonly associated with a strong immunoglobulin E (IgE) response) and are thus often called allergens ⁽⁵⁾. Although many allergenic proteins are proteases, other protein allergens have little commonality ⁽⁷⁾.

The reason underpinning why some people react adversely to individual allergens is not well understood ⁽⁷⁾. Generic associations have been made with genetic and environmental factors. Heritability estimates of allergic diseases vary, but meta-analysis of genome-wide association studies have suggested familial links as high as 80% for asthma, atopic dermatitis, and allergic rhinitis ⁽⁸⁾. The rise in allergic diseases over the years has also been attributed to environmental changes such as frequency and diversity of allergen exposure (the so called 'hygiene hypothesis'), diet, physical exercise, and use of antibiotics ⁽⁹⁾. The hygiene hypothesis is commonly discussed as underlying the rise in autoimmune and allergic diseases in developed countries that correlates with the commensurate drop in infectious diseases ⁽¹⁰⁾.

1.1.2 Classifying hypersensitivity reactions

Allergic/hypersensitivity reactions encompass a broad range of symptoms and underlying mechanisms of action. In an attempt to classify hypersensitivity reactions, Gell and Coombs categorised them into 4 broad groups based on the immunological mechanism producing tissue injury ⁽¹¹⁾. It is important to note that this classification was proposed in 1963 and holds significant limitations like the lack of suitability to newly identified mechanisms in the 4 subtypes ^(11, 12). Therefore, the Gell and Coombs' definitions for the 4 subtypes are constantly evolving to include more modern knowledge. For instance, under their initial classification, type I reactions were solely IgE-dependent events that resulted in immediate hypersensitivity reactions (< 30 minutes) such as urticaria and bronchoconstriction (discussed later in **section 1.1.5**) ⁽⁷⁾. However, with the new recognition of reactions that do not conform to the known IgE-dependent pathway, termed 'IgE-independent' reactions, the umbrella of type I hypersensitivity reactions was broadened to include these events ⁽¹³⁾. Further, while the predominant feature of type I hypersensitivities is an acute allergic reaction, there is sometimes a late phase that occurs 4-8 hours following allergen exposure caused by delayed

mediators released by mast cells ⁽¹³⁾. Type II reactions represent cytotoxic effects mediated by either IgG or IgM antibodies targeting cell surface self-antigens, leading to cytolytic destruction ⁽¹³⁾. These type II hypersensitivities encapsulate autoimmune diseases such as haemolytic anaemia ⁽¹³⁾. Type III hypersensitivity reactions are classified as those mediated by immune complexes, facilitated by IgG and IgM binding to soluble antigens (self or foreign antigens) ^(7, 13). These immune complexes then activate the complement cascade resulting in organ damage ⁽¹³⁾. Type IV reactions represent delayed-type hypersensitivity (DHT) effects, generally occurring 24-72 hours post antigen exposure, and unlike type I – III, type IV reactions are not antibody mediated but rather T-cell mediated ⁽⁷⁾. DHTs can be chronic in cases such as tuberculosis and leprosy where the pathogen is unusually resistant to elimination by the immune system ⁽⁷⁾. Some drugs, including antibiotics, are also known to induce type IV reactions with clinical manifestations that range from anaphylaxis (discussed below) to chronic severe reactions such as Steven–Johnson syndrome and its skin manifestations ⁽¹⁴⁾.

1.1.3 Mast cells and allergy

The symptoms of type I hypersensitivity reactions (the focus of this thesis) are primarily attributed to mediators released from activated mast cells. Mast cells are traditionally broadly categorised into 2 groups; connective tissue and mucosal mast cells. The classic connective tissue mast cell was discovered by Paul Ehrlich in 1878, and named ‘mastzellen’, a derivative of the German word ‘mast’, meaning ‘food’ or ‘well-fed’, under the presumption that granules provided cellular nourishment ⁽¹⁵⁾. Almost a century later, connective tissue mast cells were identified as one of 2 major types of mast cells with the primary distinguishing factor being the sensitivity to fixative and staining agents ⁽¹⁶⁾. Connective tissue mast cells could be fixed with any fixative, and stained with the chemical dyes alcian blue or safranin ⁽¹⁵⁾. However, the second group of mast cells termed ‘mucosal mast cells’, could only be fixed with Carnoy’s solution or a weak formaldehyde-acetic acid, and stained with alcian blue only ⁽¹⁵⁾. These differences were later attributed to connective tissue mast cells having much larger quantities of proteoglycans in their granular matrix than mucosal mast cells ⁽¹⁵⁾. Moreover, the two

groups of mast cells had different distribution patterns throughout the body. Mucosal mast cells were localised in the mucosal tissue of the gut, and lung, while connective tissue mast cells were found in tissues such as skin and the submucosa of the gut ^(16, 17).

Aside from these histological differences, mast cells found in connective tissues were later shown to contain tryptase and chymase along with carboxypeptidase and cathepsin G, and so were termed tryptase and chymase-expressing mast cells (MC_{TC}), whereas mucosal mast cells only expressed tryptase, and were termed tryptase only cells (MC_T) ⁽¹⁵⁾. Mast cells are now also characterised by other genomic signatures including carboxypeptidase A3 expression, along with immunohistochemical and cell surface flow cytometry markers such as c-Kit (the stem cell factor (SCF) receptor) and FcεRI (the high-affinity IgE receptor) ^(18, 19).

Whilst mast cells are known to play a protective role in wound healing, maintaining tissue homeostasis and defence against certain pathogens (*e.g.*, flaviviruses, enterobacteria and *Candida albicans* ⁽²⁰⁾), they are more commonly recognized for their role in allergy and inflammatory disease ^(21, 22).

Mast cells contain a range of potent autacoid mediators such as histamine, tryptase and a variety of serine proteases and proteoglycans ^(22, 23). These mediators have actions on a variety of tissues around the body including smooth muscle, the vascular endothelium, mucosal glands, and other inflammatory cells ^(22, 23). Following exposure to allergens or upon tissue insult, mast cells are activated (**Figure 1.1**) resulting in the release their granular content through a process termed "degranulation" that causes the well-known acute symptoms of allergic disease ^(24, 25). The release of these granular contents results in a variety of symptoms depending on the location of the mast cell. For example; in the skin they cause inflammation and itch; in the lungs they cause bronchospasm and mucosal oedema; and in the gastrointestinal tract they increase smooth muscle contraction, fluid secretion and peristalsis producing abdominal discomfort and diarrhoea ^(22, 23, 26).

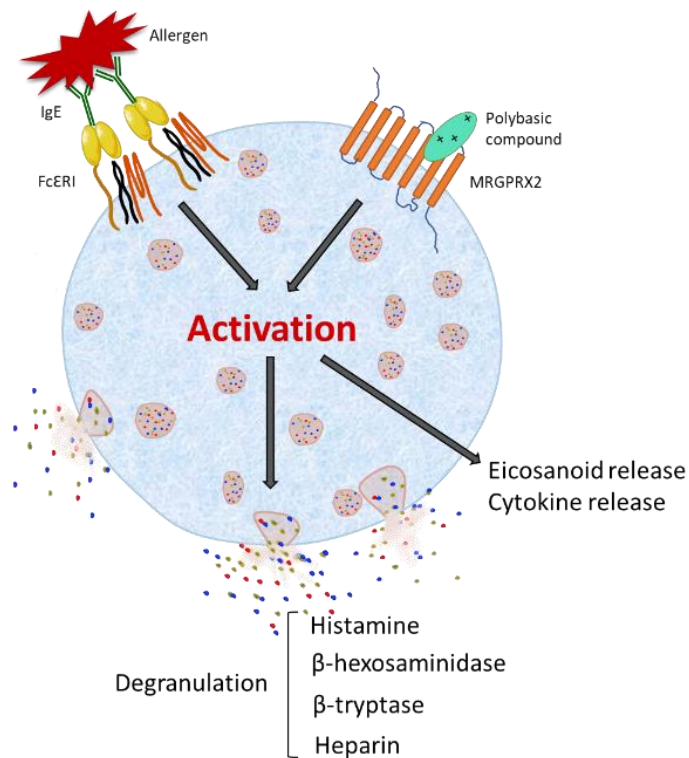


Figure 1.1: Schematic diagram of a typical mast cell activated by the FcεRI and MRGPRX2 receptors to release early stage mediators via degranulation and later stage secretion of eicosanoids and cytokines.

1.1.4 Components released upon mast cell degranulation

Mast cell granules are known to contain biogenic amines (*e.g.*, histamine and serotonin), lysosomal enzymes (*e.g.*, β-hexosaminidase and β-glucuronidase), proteoglycans (*e.g.*, heparin and chondroitin sulphates), proteases (*e.g.*, tryptase and other serine proteases) and some cytokines (*e.g.*, interleukin-8 (IL-8) and tumor necrosis factor α (TNFα)) ⁽²⁷⁾. Upon stimulation, these pre-formed mediators can be released in a matter of 15-90 seconds enabling the possibility of rapid allergic, inflammatory, and anaphylactic responses ⁽²⁷⁾. The cross talk between immune cells, led by the release of these mediators, also enables the infiltration of more immune cells as part of an inflammatory response ⁽²⁷⁾.

These mediators are embedded in a proteoglycan matrix, primarily constituting heparin ⁽²⁸⁾. Heparin is a negatively charged glycosaminoglycan that plays a vital role in the storage of some proteases including tryptase, chymase, and endopeptidase, by forming stable complexes and preventing rapid autolysis ⁽²⁹⁾. Interestingly, heparin has itself been shown to have anti-inflammatory actions, and is also therapeutically utilised for its anti-coagulant properties, especially in cardiac surgery ⁽³⁰⁾.

Mast cell granules are the primary source of histamine. The molar ratio of heparin to histamine in rat serosal mast cells has been estimated at 1: 1,100-6,300 and is a clear indication of the abundance of histamine packed into mast cell granules ⁽²⁸⁾. In the acidic pH of mast cell granules, histamine along with other positively charged molecules, are found complexed with negatively charged proteoglycans ⁽²⁵⁾. Upon degranulation and following the release of granular content into a more neutral pH, histamine dissociates from the proteoglycan matrix and diffuses into surrounding tissues to elicit its effect by binding to histamine receptors, and is later rapidly metabolized by methylation or oxidation ⁽²⁵⁾. Histamine plays a central role in the pathogenesis of allergies and hypersensitivity reactions via the regulation of other cells, modulating a variety of physiological functions including vasodilation and bronchoconstriction ⁽³¹⁾. As a result, anti-histamines, drugs that antagonise the histamine receptor, are some of the first-line therapies for allergic inflammatory reactions. Currently available anti-histamines primarily target H₁ and H₂ receptors (of 4 histamine receptors: H₁₋₄). However, anti-histamines that also target H₄ receptors, in addition to H₁ and H₂, have been proposed to also have utility in controlling allergic reactions ⁽³¹⁾.

β-tryptase is the principle enzyme in mast cell granules and accounts for up to 20% of the granular protein content ⁽²⁵⁾. Broadly, there are 2 types of tryptases (α and β), and are primarily released from mast cells and less abundantly from basophils ⁽²⁵⁾. Unlike most other enzymes, β-tryptase is found active only when complexed with heparin to form a tetramer, and once dissociated from heparin, it immediately and irreversibly dissociates into inactive monomers ⁽³²⁾. β-tryptase has a broad range of roles, these include the cleavage of certain extracellular substrates such as fibronectin, and activation of protease activated receptors (PAR) ⁽³³⁾. Because of its size and association with heparin, it diffuses into tissues more slowly than histamine ⁽³³⁾, and unlike histamine, tryptase is relatively stable in the circulation, and is therefore often used as a marker of mast cell activation, especially in systemic anaphylaxis and in maintaining conditions such as mastocytosis ^(25, 33, 34).

The enzyme β -hexosaminidase makes up about 14% of the granular protein content and is the most commonly used quantitative marker of degranulation in a laboratory setting⁽²⁸⁾. The enzyme is composed of an α - and β - subunit coded by the genes HEXA and HEXB, respectively. It has been identified that different combinations of these subunits form 3 isoenzymes named β -hexosaminidase A, B and S (also termed HexA, HexB, HexS). HexA is a heterodimer with 1 α - and 1 β - subunit while HexB and HexS are homodimers of α - and β - subunits respectively^(35, 36). In mammalian cells, HexA and HexB are the dominant isoforms with HexS being a more labile minor isoform⁽³⁷⁾. The enzyme is now thought have some host defence activity by cleaving peptidoglycan cell walls of certain gram positive bacterial strains such as *B. cereus* and *S. epidermidis* (especially in combination with lysozymes secreted by other immune cells)⁽³⁵⁾. In addition, there is conflicting evidence for the role of the enzyme in asthma. While some studies suggest a positive correlation between enzymatic concentration and progression of asthma, others report a lack of significance in this relationship^(35, 38-40).

The granular mediators mentioned above are released upon mast cell activation and have their respective effects on a wide variety of cells and tissues. Interestingly, there are differences in granular mediator release in response to the variety of agonists that activate mast cells. To explain this, some studies suggest the presence of a variety of granules within mast cells that have different mediator compositions⁽²⁷⁾. One study on bone marrow mast cells categorised mast cell granules into 3 classes (I, II and III), and based on their contents, assigned different granular positionings and maturation stages⁽⁴¹⁾. It is proposed that the diversity in granules is a result of the fusion of immature granules that bud off golgi apparatus, with homotypic fusion to form type III granules, or heterotypic fusion with lysosomes/endosomes (type I granules) to form type II secretory granules⁽²⁷⁾. However, this concept along with its biological relevance is yet to be fully understood. One proposition is the classification of mast cell degranulation into 2 broad groups; piecemeal and anaphylactic⁽⁴²⁾. It is suggested that while anaphylactic degranulation involves a nonselective release of granules, piecemeal degranulation involves the selective release of granules containing

specific mediators ^(41, 42). Both these types of degranulation have been observed in mast cells *in vitro*, *in vivo* and *ex vivo* ⁽⁴²⁾. However, this remains a controversial topic with the pathophysiological role of piecemeal degranulation poorly understood.

1.1.5 Mast cell activation, and its roles in allergy/anaphylaxis

Although the initial response by mast cells to a tissue insult aids recovery, an inappropriate and/or uncontrolled mast cell activation is observed in allergic inflammatory disease. Allergens are often small water soluble proteins of molecular weight between 5 and 50 kDa ⁽⁴³⁾. As mentioned earlier (**section 1.1.1**), allergic inflammatory diseases can be triggered by naturally occurring substances such as pollen, dust or food allergens to result in conditions such as hayfever, asthma, food allergies, and anaphylaxis. Paradoxically, anaphylactic reactions are sometimes iatrogenic in nature, being triggered by drugs, latex and radioiodine contrast dyes.

The activation of mast cells can occur either through an IgE-dependent pathway (the FcεRI pathway) or through a number of IgE-independent pathways ⁽⁴⁴⁾. The IgE-dependent pathway involves the initial production of allergen-specific IgE by B cells with the help of T_H2 cells (**Figure 1.2**) ⁽⁴⁴⁾. Specific IgE generated following first exposure then binds to the high affinity receptor, FcεRI, a heterotrimeric receptor comprised of 1 α, 1 β, and 2 γ chains ⁽⁴⁵⁾. Upon re-exposure, the allergen binds to IgE bound to FcεRI, which causes receptor cross-linking that triggers activation of Lyn kinase which in turn phosphorylates γ and β chain-ITAMs (immunoreceptor tyrosine-based activation motifs) ^(45, 46). This then leads to the recruitment of Syk tyrosine kinase which initiates a signalling cascade involving PLCγ-mediated hydrolysis of PIP₂ (phosphatidylinositol (4,5)-bis phosphate) to IP₃ (inositol (1,4,5)-triphosphate) and DAG (diacylglycerol) ^(45, 46). IP₃ binds to its receptor in the endoplasmic reticulum causing an increase in cytosolic calcium levels ⁽⁴⁵⁾. The depletion of intracellular calcium stores is sensed by STIM1 (stromal interaction molecule 1) which in turn induces the opening of CRAC channels (calcium release-activated channels) in the plasma membrane to compensate for the loss of intracellular calcium, with an influx of extracellular calcium ⁽⁴⁵⁾. DAG, the second product of

PIP₂ hydrolysis, activates protein kinase C (PKC), an additional important signalling molecule in degranulation ⁽⁴⁶⁾. In addition, a parallel pathway is also initiated involving Fyn kinase that causes the phosphoinositide 3-kinases (PI3K) signalling cascade required for optimal degranulation ^(46, 47). The above mentioned calcium mobilization, along with PKC activation, results in granular translocation and docking ⁽⁴⁸⁾. As microtubules translocate the granules closer to the plasma membrane, the fusion of integral membrane proteins called soluble N-ethylmaleimide-sensitive attachment proteins (SNAREs), located on both the plasma membrane and granular membrane, allows the exocytosis of granular contents ⁽⁴⁸⁾. The PKC cascade also causes the phosphorylation of MAPKs (mitogen-activated protein kinases), that together with calcium mobilization leads to PLA₂ (phospholipase A₂) activation resulting in the synthesis and release of cytokines (*e.g.*, monocyte chemoattractant protein-1 (MCP-1/CCL2), IL-8, TNF α , and eicosanoids (*e.g.*, prostaglandin D₂ (PGD₂), leukotriene C₄ (LTC₄)) ^(45, 48-51).

As mentioned above, IgE-dependent allergen recognition is dependent on the initial detection of allergen peptide fragments presented on major histocompatibility complex (MHC) molecules ⁽⁵²⁾. However, most drugs, including NMBAs, are small molecules and are therefore invisible to this part of the immune system ⁽⁵²⁾. The most commonly accepted mechanism of small molecule recognition by the immune system involves them acting as haptens where they bind to host proteins ⁽⁵²⁾. This results in structurally altered self-proteins that are subsequently recognised as non-self, thus generating an immune response ⁽⁵²⁾.

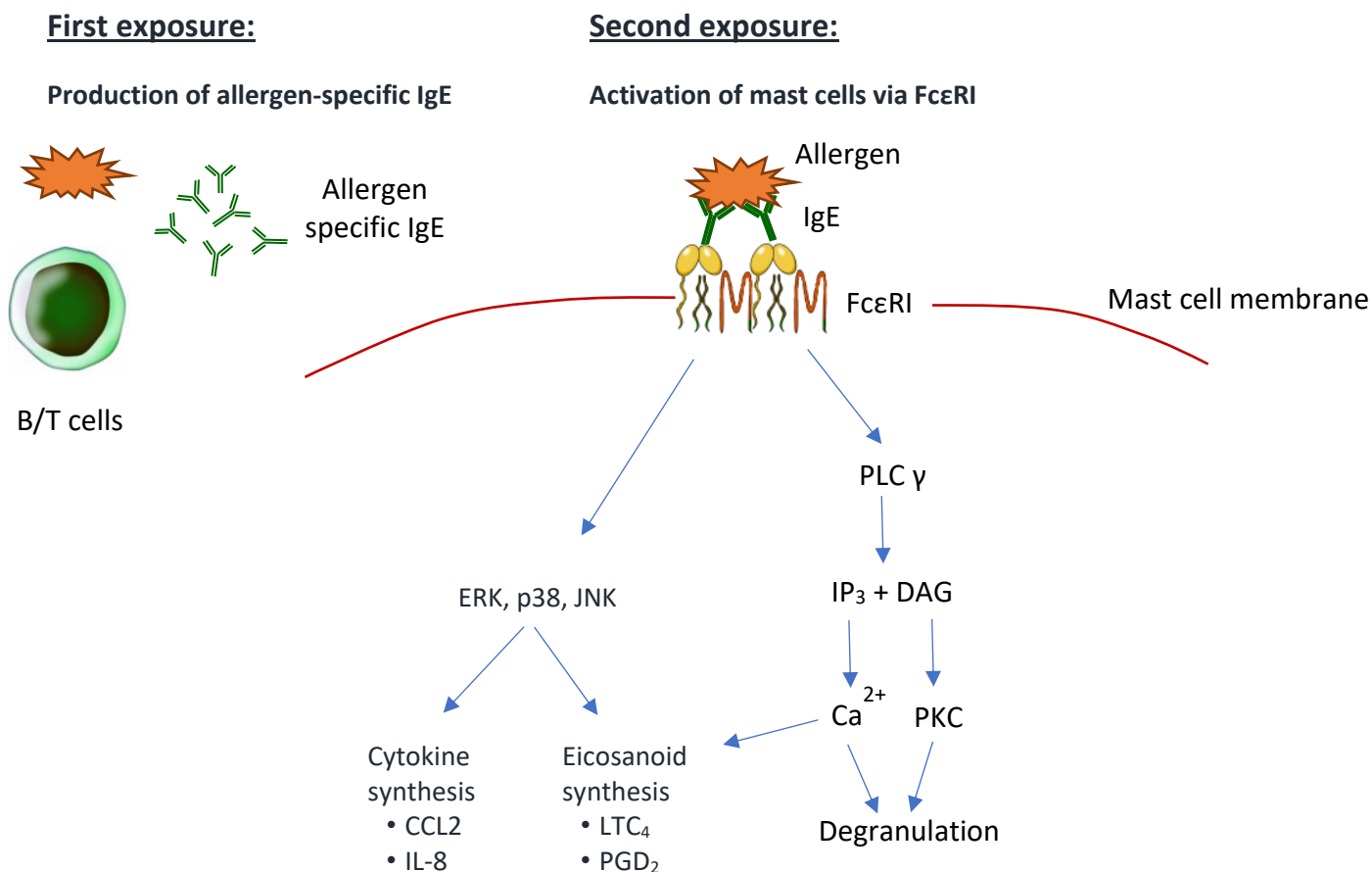


Figure 1.2: Mast cell activation through FcεRI; a simplified schematic of selected signalling molecules involved in the IgE-dependent pathway of mast cell activation. IgE-dependent activation is dependent on at least two allergen exposures. An initial exposure where specific IgE is produced that then binds to FcεRI receptors on the surface of mast cells. Upon re-exposure, the allergen binds to IgE on FcεRI, inducing a signalling array in mast cells that results in multiple outcomes including degranulation, cytokine and eicosanoid synthesis and release.

This IgE-dependent activation of mast cells is the most commonly encountered and studied pathway in allergy. However, it does not always explain the activation of mast cells, especially in an immune system that was not previously exposed to the specific allergen, and hence should not have allergen-specific IgE. Allergic-type inflammatory responses that arise in these conditions may indicate the existence of IgE-independent mechanisms of immune activation.

1.1.6 IgE-independent mechanisms of anaphylaxis

The European Academy of Allergy and Clinical Immunology (EAACI) nomenclature committee termed all IgE-mediated anaphylactic reactions as “IgE-mediated allergic anaphylaxis”. However, as alluded to above, drug-induced anaphylactic reactions are not always IgE-mediated; therefore these non-IgE-mediated anaphylactic events were termed “anaphylactoid” or “pseudo-allergic” reactions, although the term is now discouraged ⁽⁶⁾. These events, now more frequently called ‘non-IgE dependent’ or ‘IgE-independent’ anaphylaxis, are characterised by several factors including the absence of drug-specific IgE and negative skin prick tests, although these methods are now frequently contested as inaccurate depictions (discussed further in Chapter 5) ^(53, 54).

The drugs that elicit an IgE-independent reaction generally contains positively charged groups, typically in the form of a quaternary ammonium ion, and are broadly termed "basic secretagogues" ⁽⁵⁵⁻⁵⁷⁾. The often extreme (*i.e.*, anaphylactic) pathogenic role of these substances drives much research into investigating their mechanisms of action and common structural elements ⁽⁵⁸⁾. Initially, the mechanism by which these basic secretagogues caused mast cell activation was unclear. The theorized mechanisms involved a direct interaction of polybasic secretagogues with cellular G proteins to initiate a calcium influx and degranulation ^(59, 60). Decades of research later, more recent studies have identified the MRGPRX2 (Mas-related G-protein coupled receptor X2) receptor as being associated with these non-IgE-dependent mechanisms of mast cell activation ^(58, 61, 62).

It is important to note that there is no definitive test to clinically identify patients that have had an IgE-independent event of anaphylaxis. Most of the classification of non-IgE dependent reactions rely on the inability to detect direct evidence for an IgE-dependent reaction (such as the presence of drug-specific IgE). In addition to MRGPRX2, alternative pathways for IgE-independent systemic anaphylaxis have also been identified ^(63, 64). Although not well understood, the drug-induced activation of the complement cascade is known to generate small polypeptides (C3a, C5a) that have been correlated with anaphylaxis ⁽⁶⁴⁾. The injection of

these polypeptides, termed anaphylatoxins, into the skin of healthy volunteers produces an immediate wheal and flare ⁽⁶⁴⁾. In addition, a recent study identified a link between certain receptors (CD32A (FcγRIIa) and CD16B (FcγRIIIb)) and NMBA-induced IgG anaphylaxis. While these receptors have previously been associated with IgG-dependent anaphylaxis in animal models ^(65, 66), this study advances this knowledge to propose CD32A as a marker of NMBA-induced IgG-anaphylaxis mediated through neutrophils in humans ⁽⁶³⁾.

Nevertheless, MRGPRX2 remains the most promiscuous IgE-independent pathway identified for the direct activation of mast cells, binding to a wide range of polybasic compounds, although the clinical relevance of this remains unclear.

1.1.7 MRGPRX2 and its role in IgE-independent mast cell activation

The subfamily of G protein-coupled receptors (GPCRs) named Mas-related GPCRs (MRGPRs) originated from the identification of one gene, Mas1, believed to be an oncogenic receptor ⁽⁶⁷⁻⁶⁹⁾. The name Mas was derived from the name, Massey, of the human donor in which the receptor was first identified ⁽⁷⁰⁾. All related genes were classified as the Mas-related genes (MRG) and their receptors as the Mas-related GPCRs (MRGPRs) ⁽¹⁷⁾. Although Mas1 is still classified a proto-oncogene, most of the other related genes are not. The MRGPR family comprises approximately 50 members in total, spread across a variety of mammals including humans, rats and mice ⁽¹⁷⁾.

The human gene family consists of 4 genes, MRGPRX1- MRGPRX4. MRGPRX2 is the most prominent receptor found outside of dorsal root ganglia, and is predominantly located on mast cells ^(17, 68, 71). MRGPRX2 is also thought to be expressed in/on basophils, although this is frequently contested (discussed further in chapter 5) ^(17, 72). Mast cells found within tissues like skin and the peritoneal cavity (connective tissue mast cells) predominantly express the subtype MRGPRX2, with MRGPRX1 expressed at low levels and no expression of MRGPRX3 or MRGPRX4 ⁽⁷¹⁾. In comparison to MRGPRX2, much greater concentrations of basic secretagogues are required to activate MRGPRX1 ⁽⁷¹⁾. In keeping with this, mucosal type mast

cells however (found in alveolar and intestinal tissues), express little MRGPRX2 ⁽⁷¹⁾. Mucosal mast cells are also not known to respond to basic secretagogues ⁽⁷³⁾.

Interestingly, species variation in the activation of MRGPRX2 orthologues in mouse and rat mast cells (MRGPRB2 and MRGPR3 respectively) have been noted ⁽⁵⁸⁾. While some drugs are found to be much more potent at the mouse receptor, other drugs are less potent compared to the human receptor (discussed further in chapter 5) ⁽⁵⁸⁾.

Recently, there has been an increasing recognition of the MRGPRX2 pathway of mast cell activation and its association with IgE-independent reactions ⁽⁷⁴⁻⁷⁶⁾. Agonists that bind to MRGPRX2 elicit activation of a variety of G protein-mediated signalling molecules that influence intracellular mechanisms such as the mobilization of calcium and modulation of adenylate cyclase through receptor coupling to Gq and Gi proteins (**Figure 1.3**) ^(17, 55, 71). The potential coupling to two G proteins opens up the prospect of certain ligands to preferentially activate selected signalling cascades resulting in different cellular outcomes. This notion, often referred to as biased agonism, has been studied both to understand why some ligands of the same receptor may result in adverse outcomes, and to design therapeutic agents that selectively harness beneficial effects while minimising adverse outcomes ⁽⁷⁷⁾. This feature is prevalent in GPCRs, especially class A receptors ⁽⁷⁷⁾ that include opioid and MRGPRX2 receptors (discussed further in chapter 4).

The coupling of Gi-protein is followed by the inhibition of adenylate cyclase that results in a decrease of cyclic adenosine monophosphate (cAMP) ⁽⁷⁸⁾. Subsequently, this results in a decrease in activation of cAMP-associated kinases, primarily protein kinase A (PKA) ⁽⁷⁸⁾. PKA activity is known to be an inhibitory mechanism for MAP kinase phosphorylation (*e.g.*, ERK, JNK and p38) ⁽⁷⁹⁾. Therefore, the reduction of PKA activity results in the enhanced phosphorylation of MAP kinases, leading to cytokine and eicosanoid synthesis and release ⁽⁷⁸⁾. The activation of Gq produces phospholipase C β (PLC β) activation resulting in the break down of PIP₂ to IP₃ and DAG ⁽⁸⁰⁾. This is similar to the activation of the PLC γ -mediated pathway in

IgE-dependent mast cell activation triggering calcium mobilization, PKC activation, and eventually, degranulation ^(24, 46, 80).

Gi and Gq signalling are both found indispensable in MRGPRX2-mediated mast cell activation. For instance, Gq-mediated calcium mobilization is known to increase ERK phosphorylation, and plays a role in the activation of calcium dependent PLA₂, thereby modulating eicosanoid and cytokine release ^(81, 82). Further, Gi signalling is found vital for MRGPRX2-mediated degranulation with a reduction in cAMP and PKA being key contributors ⁽⁸³⁻⁸⁵⁾.

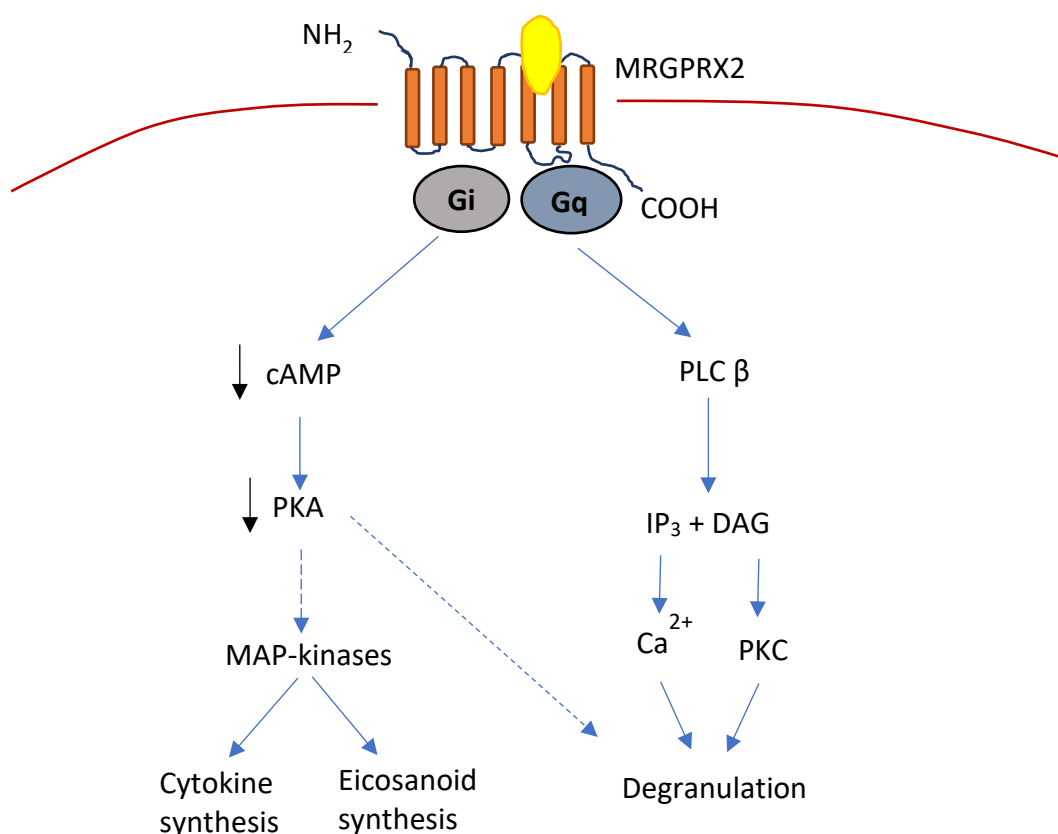


Figure 1.3: Schematic illustrating the IgE-independent MRGPRX2-dependent pathway of mast cell activation. MRGPRX2, couples both Gi and Gq proteins, and elicits a signalling cascade that via the decrease of cAMP and increase in Ca²⁺ mobilization, induces degranulation along with the synthesis and release of cytokines and eicosanoids.

1.1.8 Commonly used models for the study of mast cells

A variety of models have been used to study the role of MRGPRX2 in mast cells, and range from *in vivo* animal models to *in vitro* cell models (both human and non-human) to *in silico* models.

Mast cell deficient mouse models have been used to study the importance of mast cells in health and disease (*e.g.*, the importance of mast cells in regulating gut bacterial levels) ^(26, 86). Recently, MRGPRB2 knock out mice have been established to examine and validate the role of the receptor in both the immune response to pathogens (*e.g.*, bacterial clearance in the skin) and drug-induced IgE-independent anaphylaxis ^(87, 88). However, the presence of species variation between the receptor orthologues (discussed further in chapter 5) means that cell models are widely used for the study of human MRGPRX2.

Mast cells have been isolated from various animals including mouse, rat and human, and can either be used from primary culture or as immortalised cell lines. Commonly used primary cultures include human skin-derived mast cells, human peripheral blood, or cord blood derived mast cells (isolated based on expression of common mast cell progenitor markers CD34⁺ or CD133⁺), bone marrow derived mast cells (BMMCs; both mouse and rat), and peritoneal mast cells (both mouse and rat). Although primary cultures may be a better representation of mast cell biology, they also require laborious and lengthy isolation and maintenance protocols. Therefore, immortalised mast cell lines are frequently used as surrogates to tissue mast cells in order to circumvent these costly isolation procedures ⁽⁸⁹⁾.

An important factor of consideration in determining the relevance of a cell line to *in vivo* human mast cells, is the function of the Kit-receptor. Approximately 90% of adults with mast cell proliferation disorders (*e.g.*, mastocytosis) are known to possess mutations that lead to constitutive activation of the SCF receptor (c-Kit; CD117) ⁽⁹⁰⁾. The activation of this receptor is associated with mast cell proliferation and survival ⁽⁹⁰⁾. Kit receptor activation is also known to synergistically enhance FcεRI-IgE mediated mast cell degranulation, cytokine production

and stimulus-induced chemotactic migration ⁽⁹⁰⁾. For instance, in the body, stromal cells such as fibroblasts produce SCF, which in the presence of other factors like IL-3 and IL-33, increases mast cell production of heparin proteoglycans and induces stable transcripts for a variety of other mediators ⁽¹⁵⁾.

While a range of mast cell cultures, such as BMMCs, are dependent on SCF for survival and are frequently used for the study of mast cell activation via FcεRI ⁽⁹¹⁾, their use in the characterisation of MRGPRX2 activation is limited. The most commonly used BMMCs are isolated from mice, and were not found to express MRGPRB2 ⁽⁹¹⁾. Another commonly used primary cell model are peritoneal mast cells. Rat peritoneal mast cells have been traditionally used for the study of mast cell biology and have been shown to be activated by both the IgE-dependent pathway and the IgE-independent MRGPRB3 (rat orthologue of human MRGPRX2 ^(71, 92)) pathway with agonists such as compound 48/80 and substance P ⁽⁹²⁾.

As an alternative to primary mast cells, transfectable cell lines that do not express constitutive MRGPRX2 such as the rat basophilic leukemia cell line (RBL-2H3), human embryonic kidney cell line (HEK-293) or human osteogenic sarcoma cell line (U2OS) have been used as expression models (by transfection) for the study of MRGPRX2 ^(72, 74, 93-96). RBL-2H3 cells, although a basophilic cell line that expresses MRGPRB3 mRNA, are not found to have functional receptors that are activated by MRGPR agonists (*e.g.*, compound 48/80 and substance P) unless transfected with MRGPRX2 ⁽⁹²⁾. Muhammad Sahid et al., (2020), speculate the unresponsiveness of MRGPRB3 on RBL-2H3 cells to MRGPR agonists to be either due to inadequate expression for mast cell activation or non-functional receptors ⁽⁹²⁾, although the reason underlying the latter is unclear. Alternatively, as mentioned above, the HEK-293 (Human Embryonic Kidney 293) cells are known to be a simple human cell line. Although not a mast cell line, they are known to be easy to transfect and hence modify to express the desired properties ⁽⁹⁷⁾. Most studies note the need for an additional transfection with Gα15 to observe a pronounced MRGPRX2-mediated calcium response in HEK-293 cells ^(58, 96).

The above mentioned cell lines have however, little correlation to human mast cells as they are either not mast cell lines and therefore may have biased signalling cascades, or are from a different species thereby introducing the possibility of species variability. Therefore, a number of human mast cell lines have been used to study the role and function of human mast cells. These primarily include LAD2, HMC-1, and LUVA cells. LUVA (laboratory of university of Virginia) cells were identified in 2011 from a donor with aspirin-exacerbated respiratory disease. These cells have functional Kit and FcεRI receptors, but are not dependent on growth factors (*e.g.*, SCF) for survival compared to normal tissue mast cells ⁽⁹⁸⁾. Further, while preliminary studies suggests the expression of MRGPRX2 on LUVA cells ⁽⁹⁹⁾, its functional response within the model requires further exploration. The HMC-1 (human mast cell -1) cell line is a peripheral blood-derived line isolated from a patient with mast cell leukemia, and was the only human mast cell line from the early 1990s to early 2000s ⁽¹⁰⁰⁾. While HMC-1 cells express a range of known mast cell markers such as histamine, heparin and tryptase, they do not express the high affinity IgE receptor, FcεRI ⁽¹⁰⁰⁾. HMC-1 cells are also immature mast cells with poorly formed granules that degranulate inadequately (if at all) in response to stimuli ^(89, 101). Interestingly, HMC-1 cells were found to have MRGPRX2 mRNA expression, although presumed to be non-functional due to lack of activation by agonists ⁽⁷¹⁾. In addition, while phenotypic characterisation studies detect a high expression of the Kit receptor, 2 Kit mutations have been identified that keep the receptor constitutively active, thereby not requiring the growth factor, SCF, for survival ⁽¹⁰²⁾.

In the early 2000s, another mast cell line was established from the bone marrow of a patient with mastocytosis and termed LAD2 (laboratory of allergic disease-2) ⁽¹⁰³⁾. Wen Guhl et al., 2010, suggested that although cell lines are incapable of being replacements for tissue isolated mast cells, LAD2 mast cells represent a better model of mature skin mast cells when compared to HMC-1. Compared to HMC-1 cells, LAD2 cells expressed higher levels of most markers (except c-kit and histamine), similar to that of mature skin derived mast cells ⁽⁸⁹⁾. Unlike HMC-1s, LAD2 cells have a normal Kit receptor, and thus require SCF for basic survival

and proliferation ⁽¹⁰⁴⁾. In addition, LAD2 cells express FcεRI receptors and therefore, are capable of degranulating in response to an IgE/antigenic stimulation ⁽¹⁰⁴⁾. Moreover, LAD2 cells express the MRGPRX2 receptor and is currently the most commonly used cell line for the study of MRGPRX2 activity ^(58, 105). MRGPRX2 localization in LAD2 cells was found to be similar to that of skin mast cells ⁽¹⁰⁶⁾. Overall, LAD2 cells better represent primary human mast cell cultures because of their functional expression of FcεRI and MRGPRX2 receptors, and their dependence on SCF for survival ⁽¹⁰⁷⁾.

Lastly, *in silico* models based on closely related class A GPCRs (*e.g.*, κ-opioid ⁽⁹⁵⁾ and C5a anaphylatoxin chemotactic receptor 1 ⁽¹⁰⁸⁾) have been used to study MRGPRX2. These models have resulted in the generation of a MRGPRX2-specific agonist (ZINC-3573(R)) from screening thousands of compounds in the ZINC-database ⁽⁹⁵⁾, and allowed the modelling of ligand interactions within the tertiary structure of MRGPRX2 ⁽¹⁰⁸⁾. However, to date there are no crystallography or cryogenic electron microscopy images of MRGPRX2 to define its structure, and thus these models require further validation.

1.1.9 Role of MRGPRX2 in allergic disorders

Mast cells are often found in close proximity to nerve endings, and with the recent identification of a range of neuropeptides and host defence peptides as agonists of the mast cell receptor, MRGPRX2, current interest lies in the association of these peptides with disease propagation. A recent study demonstrated the presence of elevated MRGPRX2 expression levels in asthmatic lungs compared to non-asthmatic patients, both in number of MRGPRX2 expressing mast cells and level of receptor expression ⁽¹⁰⁹⁾. This was then linked with hemokinin-1, a neuropeptide that induces contraction of human bronchi *ex vivo*, and is released by lung macrophages, mast cells and bronchial epithelial cells ⁽¹⁰⁹⁾. Hemokinin-1, also associated with the pathogenesis of asthma, was found to activate MRGPRX2 in *in vitro* experiments ⁽¹⁰⁹⁾. However, further work is necessary to understand the role of MRGPRX2 in the progression of asthma.

The best characterised relationship between MRGPRX2 and atopic disorders are in patients with chronic spontaneous urticaria (CSU) where eosinophil infiltration and co-localization with mast cells were observed in urticarial regions ⁽¹⁰⁶⁾. As mentioned later in **section 1.1.11**, major basic protein and eosinophil peroxidase (released from eosinophil granules) are known to activate MRGPRX2 receptors, thereby suggesting that cross-talk between these immune cells may contribute to the worsening of symptoms ⁽¹⁰⁶⁾.

CSU is characterised by frequent hives (daily or near daily occurrences) that last longer than 6 weeks ⁽¹¹⁰⁾. In a majority of the cases, the underlying cause goes unidentified and hence is classified as spontaneous ⁽¹¹¹⁾. Although poorly understood, some of the identified triggers include physical stimulation such as scratching or vibration, bacterial or viral infections, exercise, stress, along with certain foods, drinks, and medications. While a direct association with an underlying cause remains unclear, studies have demonstrated a significant accumulation of mast cells and mononuclear cells in areas of wheal formation in patients with CSU ^(112, 113). These patients showed significant improvement when treated with the anti-IgE monoclonal antibody, omalizumab, and more recently, ligelizumab, suggesting an association with IgE-dependent hypersensitivities ^(114, 115).

More recently, very rare IgE-independent causes of mast cell involvement in chronic urticaria have been identified. In addition to the above mentioned association with MRGPRX2, a missense mutation in another GPCR, ADGRE2 (adhesion GPCR E2), has been associated with IgE-independent events, where patient's mast cells degranulate in response to a frictional stimulus ⁽¹¹⁶⁾. ADGRE2 is formed of 2 subunits, α and β . In the presence of both dermatan sulphate (predominant glycosaminoglycan in the skin) and mechanical vibration, the α and β subunits detach allowing the signalling to result in mast cell degranulation ⁽¹¹⁷⁾. In a healthy individual, ADGRE2 causes limited degranulation of mast cells and is known to play a role in pain modulation and the innate immune response ⁽¹¹⁷⁾. However, in a person with vibrational urticaria, the mutation in the ADGRE2 gene destabilises the inhibitory interaction between

the 2 subunits allowing uncontrolled degranulation, resulting in both localised hives and systemic manifestations of urticaria in response to a frictional stimulus ^(116, 117).

1.1.10 Mast cells: not all bad

Although mast cells are known for their role in allergy and anaphylaxis, these cells are indispensably important for their multiple protective roles played in wound healing, immune regulation and host defence mechanisms by influencing both the innate and adaptive immune system ^(118, 119).

While the role of mast cells in wound healing remains controversial, their role in multiple stages of the process can not be ignored. Mediators released from mast cells induce collagen production from fibroblasts and aid in wound contraction by stimulating fibroblast conversion to myofibroblasts ⁽¹²⁰⁾. Mast cells also play a role in tissue remodelling by stimulating the migration and proliferation of fibroblasts ⁽¹²⁰⁾. A study using a mouse model associate the absence of mast cells with a significant impairment of wound healing ⁽¹²¹⁾.

Mast cells are also found to be critical in controlling bacterial infections (both gram-negative and gram-positive) that sometimes follow skin wounds ⁽¹²²⁾. Mouse models deficient in mast cells been shown to have a greater bacterial burden than wild type mice ⁽¹²²⁾. In addition to bacterial clearance, the importance of mast cells have also been acknowledged in host-defense against a variety of pathogens including helminths, nematodes, and protozoa ^(118, 119). Mast cells contain a variety of receptors that allow direct recognition of a pathogenic infections via pattern recognition receptors (PRR) including pathogen-associated molecular patterns (PAMPs) and toll-like receptors (TLRs) ⁽¹¹⁹⁾. In addition, recently, bacterial clearance in the skin was associated with MRGPRX2/B2 using a MRGPRX2-deficient murine model ⁽⁸⁷⁾.

Chemical signals released from bacteria (quorum sensing molecules (QSM)) were recently identified as agonists of the MRGPRX2 receptor ⁽¹²³⁾. For maximal immune response against the bacterial infection, MRGPRX2 activation was found necessary in both *in vitro* (human and mouse mast cells) and mouse *in vivo* models ⁽¹²³⁾. This MRGPRX2-QSM interaction also

resulted in the increased infiltration of other immune cells to further facilitate host defense⁽¹²³⁾. Intriguingly, the investigation found that stimulation with the MRGPRX2-agonists, compound 48/80 and competence-stimulating peptide-1 (CSP-1), significantly increased bacterial clearance in mice⁽¹²³⁾. This suggests the potential use of MRGPRX2 stimulation as a therapeutic approach for some bacterial infections⁽¹²³⁾.

Furthermore, compound 48/80, the classic mast cell activator⁽¹²⁴⁾ that was recently identified to be a selective MRGPRX2 agonist, has also been proposed as a safe and effective vaccine adjuvant alongside *bacillus anthracis* protective antigen in mice⁽¹²⁵⁾. Via the activation of mast cells, compound 48/80 increased levels of both serum IgG and lethal toxin neutralising antibodies while also improving migration of inflammatory cells⁽¹²⁵⁾.

In addition the numerous roles in wound healing and bacterial clearance, MRGPRX2 have also been associated with the immune defence against venom components⁽¹²⁶⁾. For instance, mastoporan, an abundant component in venoms, has been identified as an agonist of MRGPRX2⁽¹²⁷⁾. While envenomation is a rare event, the release of proteases such as carboxypeptidase A3 (CPA3) and chemokine ligand 13 (CCL13; MCP4) from activated mast cells aid in the breakdown of these venoms, thereby increasing survival probability in mice injected with venoms⁽¹²⁶⁾.

In summary, mast cells remain indispensable to human health with a fine line separating the beneficial effects from the excessive activation that often results in detrimental effects.

1.1.11 Endogenous agonists of the MRGPRX2 receptor

A variety of molecules are known to act as ligands at the MRGPRX2 receptor, including some endogenous peptides such as neuropeptides, host defence peptides, major basic proteins, and eosinophil peroxidases^(17, 58, 128). However, given that these molecules also act at numerous other receptors, the *in vivo* importance and role of these endogenous compounds as agonists of MRGPRX2 is not well understood.

Substance P, a neuropeptide involved in pain perception by activating neurokinin-1 receptors, is also known for inducing an IgE-independent release of histamine, now identified to be MRGPRX2 dependent ⁽¹²⁹⁾. Therefore, in addition to the neuropeptide's well-characterised role as a neurotransmitter and neuromodulator, it also causes indirect vasodilation and plasma extravasation via the activation of skin mast cells ⁽¹²⁹⁾. Substance P acts in a positive feedback loop like manner where high levels of substance P were associated with increased inflammatory cytokine production by immune cells that in turn regulate substance P production and NK-1 receptor expression (primary substance P receptor) ⁽¹³⁰⁾. These interactions of substance P with NK-1R and MRGPRX2 is presumed to be associated with a variety of neuroinflammatory disorders including multiple sclerosis, Alzheimer's and Parkinson's disease ⁽¹³⁰⁾. Furthermore, substance P-induced inflammation is also associated with rheumatoid arthritis, atopic dermatitis, and the exacerbation of pain in patients with sickle cell anaemia ^(17, 131, 132). In addition, another neuropeptide, cortistatin-14, known to have a role in slow wave sleep induction, was also found to be a potent ligand of MRGPRX2 ⁽¹³³⁾.

Along with neuropeptides, major basic protein (MBP) and eosinophil peroxidase (EPX) are known to contribute to the more chronic symptoms in disorders such as CSU and asthma ^(106, 134). It has long been suggested that cross talk exist between immune cells, including that of eosinophils and mast cells that infiltrate tissues to contribute to late-phase allergic type reactions ⁽¹³⁵⁾. Recently, MBP, ECP (eosinophil cationic protein) have been shown to activate mast cells via the MRGPRX2 receptor ^(106, 128). In addition to these mediators, host defence peptides like LL-37 and β -defensin 3, that both contribute to the clearance of pathogens and also the propagation of disease symptoms like rhinovirus-induced asthma, have been identified to activate mast cells via MRGPRX2 ^(17, 105, 136-138).

As mentioned above, many endogenous ligands have been identified that have affinity towards the MRGPRX2 receptor. However, the biological relevance of these endogenous ligands in a normal healthy individual is not fully understood nor is it well characterised. For

instance, polybasic compounds like the polyamine released from mast cell granules, spermine, has been found to induce histamine release from RPMCs ⁽¹³⁹⁾. However, the mechanism of action and the potential implications remains unclear, thus the more established role of this receptor lies in its capability to activate mast cells in response to a wide variety of therapeutic agents.

1.1.12 Commonly used therapeutic agents as agonists of MRGPRX2

There is increasing clinical interest in hypersensitivity reactions that occur via IgE-independent pathways ^(17, 58, 140). A number of clinically used drugs such as antibiotics (*e.g.*, ciprofloxacin, polymyxin), NMBAs (*e.g.*, rocuronium, atracurium), and peptide drugs (*e.g.*, icatibant) have been associated with hypersensitivity and anaphylactic events that did not align with the then known receptor pathways of mast cell activation ^(17, 141-143).

Given the spectrum of drugs administered, the perioperative period remains one of the primary settings for anaphylaxis, with antibiotics being the most frequently identified causative agent. Whilst cephalosporins and penicillins are the most commonly prescribed antibiotics, the associated anaphylactic events with these drugs are predominantly due to specific IgE ^(144, 145), and there is no evidence for activation of the MRGPRX2 receptor. However, other antibiotics such as ciprofloxacin, vancomycin, polymyxin B and colistin have been found to induce mast cell degranulation via the MRGPRX2 receptor ^(143, 146). Vancomycin, is infamously known for its IgE-independent hypersensitivity reaction termed the 'red-man syndrome' as a result of the pruritic erythematous rashes across the face, neck and upper torso of some patients ⁽¹⁴⁷⁾. These symptoms are sometimes also observed with hypersensitivities associated with other MRGPRX2 agonists like ciprofloxacin and atracurium ⁽¹⁴⁷⁾.

The most commonly used NMBAs rocuronium, vecuronium and atracurium are all known to cause anaphylaxis ^(17, 148, 149). Consistent with a likely MRGPRX2 activating role, they are all bulky positively charged molecules ⁽¹⁵⁰⁾. While the incidences reported vary between studies,

an Australian study showed that the most commonly used NMBAs (rocuronium, vecuronium and atracurium) are all associated with anaphylaxis with rocuronium > vecuronium > atracurium in respective rates of anaphylactic reactions ⁽¹⁴⁹⁾. Similarly, in the UK, rocuronium has an anaphylaxis rate of 5.88 per 100,000 administrations while that of atracurium lies at 4.15 per 100,000 administrations ⁽¹⁵¹⁾.

Recently, there have been a wide range of drugs identified as agonists of MRGPRX2 (a recent study estimated upto 20% of all drug-like compounds) ⁽⁹⁶⁾. In addition to the examples above, other clinically used drugs that activate MRGPRX2 include icatibant (bradykinin B2 receptor antagonist), cetorelix (gonadotropin releasing hormone antagonist), morphine and remifentanyl (narcotic analgesics that shares a quaternary ammonium ion with NMBAs), along with the contrast dyes iopamidol, iomeprol and amidotrizoate ^(94, 146, 152). However, it is important to note that the concentrations used to activate MRGPRX2 in these studies were sometimes higher than accepted normal clinical levels.

1.1.13 Antagonists and inhibitors of MRGPRX2

Given the range of agonists that act through MRGPRX2 to cause inflammatory responses and even anaphylactic reactions (at least in mice), an MRGPRX2 antagonist has been suggested as a novel therapeutic strategy. However, to date, there are no verified, approved, commercially available MRGPRX2 antagonists. There are, however, a range of proposed antagonists that have been identified from currently available drugs, supplements, and plant extracts. However, it is important to note that the postulated antagonists are not well characterised for receptor specificity, and instead display broad inhibitory properties of mast cell activation.

Shikonin, an extract of the dried root of *Lithospermum Erythrorhizon*, a major component of the Chinese herbal medicine, 'Zicao', is used for a variety of biological activities including its anti-inflammatory and immunomodulatory properties, especially in rheumatoid arthritis ^(153, 154). Recently, Shikonin has been shown to inhibit compound 48/80-mediated MRGPRX2 activation in both a human mast cell model (LAD2 cells) and an *in vivo* mouse model ⁽¹⁵⁴⁾.

However, shikonin has also been implicated in the inhibition of IgE-mediated histamine release in basophils ⁽¹⁵⁵⁾ and suppression of allergic inflammation in murine models ⁽¹⁵⁶⁾, thereby suggesting against shikonin as a MRGPRX2-specific antagonist. Another Chinese herbal extract used for its anti-inflammatory and pain-relieving properties is saikosaponin A. Saikosaponin A was shown to inhibit mast cell activation by known MRGPRX2 agonists such as compound 48/80, ciprofloxacin and morphine hydrochloride ⁽¹⁵⁷⁾. Whilst having seemingly comparable potency to Shikonin, more research is required to verify and confirm its mechanism of inhibition and receptor specificity.

Another study screened 12,000 commercially available small molecule compounds to identify MRGPRX2-specific antagonists ⁽¹²⁸⁾. They identified two compounds that displayed properties of competitive antagonism of substance P activation of MRGPRX2: compound 1 (1-(5*H*-10*λ*-phenazin-5-yl)ethan-1-one (MW. 223.26) and compound 2 (3-(pyridine-2-ylmethyl)-2-thioxo-2,3-dihydro-4*H*-1*λ*-quinazolin-4-one (MW. 268.31) ⁽¹⁵⁸⁾. While the potencies of these compounds are comparable with shikonin and saikosaponin A, these compounds were shown to have no effect on the IgE-dependent activation of mast cells.

Genistein is a tyrosine kinase inhibitor that possesses a diverse range of pharmacological activities including protective effects in inflammation, hypersensitivity reactions, cancer and diabetes ⁽¹⁰⁸⁾. Recent studies have looked at the activity of genistein on mast cells to explain some of its anti-inflammatory properties. Genistein was suggested via 3D-modelling to bind non-competitively to MRGPRX2, and inhibited the activation of LAD2 human mast cells by compound 48/80 ⁽¹⁰⁸⁾. In light of their *in vivo* observations in a mouse model, Mukesh Kumar et, al., (2020), proposed the potential use of genistein in drug development against anaphylactic shock.

Quercetin, a plant-based pigment commonly found in red wine, apples and berries, and a known mast cell stabiliser by suppressing IgE-dependent mediator release, was recently found to inhibit MRGPRX2-induced mast cell activation via inhibition of PLC γ -IP $_3$ R associated

calcium fluctuations ⁽¹⁵⁹⁻¹⁶¹⁾. Quercetin, when added less than an hour before the MRGPRX2 agonists, impaired LAD2 human mast cell activation in a concentration dependent manner ⁽¹⁵⁹⁾. Interestingly, results from the RBL-2H3 cell line showed that a longer treatment of cells with quercetin (up to 48 hours) caused an increased degranulatory response to the MRGPRX2 agonist, compound 48/80 ⁽¹⁶²⁾.

While increasing numbers of compounds are being identified as possible antagonists of MRGPRX2, further work is required to validate selectivity at the receptor. In addition, the binding site of MRGPRX2 is seemingly 'fluid', with different agonists selectively interacting with a range of amino acids within the protein's tertiary structure (discussed later in chapter 5). Therefore, the activity of an antagonist would need to be tested against a variety of MRGPRX2 agonists to verify its potential in consistently disabling receptor activation. Nevertheless, given the growing clinical interest in understanding the role of MRGPRX2, in the absence of a convincing antagonists, studies have instead used siRNA knockdown models of MRGPRX2 to verify the role of the receptor in agonist-induced mast cell activation ⁽¹²³⁾.

1.1.14 The clinical problem: drug-induced anaphylaxis

Anaphylaxis by definition refers to a severe, life threatening hypersensitivity reaction, and can sometimes be iatrogenic. Drug-induced anaphylaxis is a growing concern, and as discussed earlier can be caused by IgE-dependent or independent mechanisms. The common culprit agents include certain antibiotics, neuromuscular blocking agents (NMBAs; the focus of this thesis), radio-contrast agents, opioids, non-steroidal anti-inflammatory drugs, and monoclonal antibodies ^(17, 163, 164). The number of anaphylaxis hospital admissions in Australia over the years 1998- 2005 were reported to have risen by ~ 140% and the number of deaths have risen by ~ 300%, with fatalities following anaphylaxis more likely to be drug-related than food-related events ⁽¹⁶³⁾. The incidence of anaphylaxis caused by NMBAs in Australia and New Zealand range from 1 in 1,250 to 13,000 ^(61, 149). The recently conducted national audit project (NAP6) in the UK suggested their NMBA anaphylaxis rate to be 1 in every 19,000 patients (25% of all perioperative anaphylaxis cases), with 32% of the cases leading to cardiac arrest/death

⁽¹⁵¹⁾. The vast majority of fatal hypersensitivity events, over 90%, occur almost immediately in the operating theatre ⁽¹⁶⁵⁾. Around 25% of deaths occur in the operating theatre, ~ 40% of deaths at the intensive care units, and the remainder in holding rooms ⁽¹⁶⁵⁾. The surveillance and monitoring of these adverse reactions is difficult because these occurrences are both rare and random ⁽¹⁶⁶⁾.

For the purpose of developing management and treatment guidelines, anaphylaxis has been categorised into 4 grades based on the severity of symptoms ⁽¹⁶⁷⁾. Grade I or mild anaphylaxis is characterised by mucocutaneous signs such as urticaria and erythema ⁽¹⁶⁷⁾. Grade II or moderate anaphylaxis is characterised by multi-organ manifestations which typically consists of hypotension and/or bronchospasm in addition to mucocutaneous signs ⁽¹⁶⁷⁾. Grade III is considered life threatening anaphylaxis, frequently associated with elevated airway pressure, and immediate treatment is required to avoid progression to cardiac arrest or severe hypoxia ⁽¹⁶⁷⁾. And lastly, grade IV anaphylaxis is when a patient undergoes cardiac arrest ⁽¹⁶⁷⁾.

1.1.15 Neuromuscular blocking agents (NMBAs) and hypersensitivity reactions associated with their use

Besides antibiotics, neuromuscular blocking agents (NMBAs) are the leading cause of anaphylaxis in surgery (being the causative agents in roughly 58% and 25% of perioperative anaphylactic events respectively), with both IgE-dependent and independent mechanisms implicated ^(151, 168). About 50-70% of the documented drug-hypersensitivity reactions arise in response to quaternary ammonium groups on NMBAs in an IgE-dependent fashion, as determined using serology testing for the presence of specific IgE or related IgE to other drugs with the quaternary ammonium group (*e.g.*, morphine) ^(54, 169).

As mentioned earlier, the dogma for an IgE-dependent response is that at least 2 exposures to the same allergen is necessary to induce the sustained IgE-response needed. However, it is known that cross-reactivity can occur where IgE against previously administered structurally similar drugs may cross react with other drugs containing related structural motifs

to trigger an anaphylactic response ^(170, 171). The best known example of this is with the use of pholcodine, a non-prescription antitussive drug containing a tertiary ammonium ion, found to correlate with a 10-fold increase in incidence of NMBA-induced anaphylaxis ^(54, 172). In 2005, a study of two neighbouring countries (with comparable lifestyle and genetic confounders), Norway and Sweden, displayed drastically different rates of anaphylaxis to NMBAs, with these drugs being responsible for over 67% of Norwegian NMBA anaphylactic events (with direct correlation to morphine specific IgE) in contrast to under 10% in Sweden ⁽¹⁷³⁾. Upon investigation, it was found that both countries were exposed to similar compounds apart from pholcodine, a cough medication commonly used in Norway but not Sweden, which was then linked to having contributed to cross-reactive IgE (against a common quaternary ammonium ion) to a majority of NMBA-anaphylactic events in Norway ⁽¹⁷³⁾.

However, these IgE-dependent pathways do not explain approximately 30-40% of hypersensitivity reactions that do not have specific-IgE against NMBAs or structurally similar compounds ^(61, 62). New findings, particularly using mice, suggest that these unexplained responses could be attributed to the recently identified MRGPRX2 on human mast cells ^(58, 61, 62).

NMBAs, sometimes referred to as skeletal muscle relaxants, are commonly used in surgical protocols to reduce muscle tone, thereby inducing a state of temporary paralysis by antagonising acetylcholine nicotinic receptors ⁽¹⁷⁴⁾. NMBAs are of two types; depolarising and non-depolarising. Suxamethonium, the only clinically used depolarising muscle relaxant, is an agonist at nicotinic receptors that, via prolonged binding, extends receptor depolarisation to induce short duration muscle paralysis ⁽¹⁷⁴⁾. Contrastingly, non-depolarising agents, the most commonly used NMBAs, are longer acting, and competitively antagonise the nicotinic receptor to prevent the interaction of acetylcholine with its receptor ⁽¹⁷⁴⁾. The non-depolarising agents are favoured due to the presence of pharmacological reversal or withdrawal agents ^(62, 174). Non-depolarising muscle relaxants can be further classified by chemical composition into steroidal compounds (*e.g.*, rocuronium, vecuronium and

pancuronium) and benzyisoquinolinium substances (*e.g.*, atracurium, mivacurium and cisatracurium) ⁽¹⁷⁴⁾. The choice of non-depolarising muscle relaxant is based largely on the required duration of muscle paralysis required for surgery ⁽⁶²⁾.

Currently, there is no practically feasible test for pre-operative screening for NMBA-related hypersensitivities. Given the rarity of these occurrences, most tests including skin prick tests and anti-IgE ELISAs are conducted a few weeks after an anaphylactic response during follow up testing to ensure the avoidance of the culprit agent in later surgical procedures ⁽⁶²⁾. Rational means to prevent hypersensitivity reactions include pre-treatment with H₁ and H₂ receptor antagonists to interfere with the binding and hence actions of histamine ^(62, 169). However, the blockade of histamine's actions does not prevent the release and actions of other mast cell mediators ⁽⁶²⁾. Therefore, this notion is frequently contested as ineffective ⁽¹⁶⁹⁾. Several authors also suggest the pre-treatment with corticosteroids alongside anti-histamines as an alternative means of preventing adverse reactions ^(165, 175). However, there is a lack of clinical evidence to support the efficacy of such precautionary actions ^(165, 175).

1.1.16 Treating perioperative anaphylaxis

Even though they occur in perhaps the best location to receive treatment, anaphylactic reactions during surgical procedures result in a mortality rate as high as 10% ⁽¹⁷⁶⁾. In the event of anaphylaxis during surgery, the patient is given a cocktail of drugs in an attempt to halt the adverse reactions ⁽¹⁷⁷⁾. These include adrenaline primarily for its systemic role in stabilising blood pressure; bronchodilators to induce smooth muscle relaxation thus counteracting bronchoconstriction; anti-histamines to prevent histamine's actions on its receptors, and corticosteroids to dampen a wide variety of pro-inflammatory markers ^(7, 177). In addition to these, there is conflicting evidence for the use of sugammadex, a drug used to reverse rocuronium-induced neuromuscular blockade, in the treatment of anaphylaxis (discussed further in Chapter 3) ^(178, 179).

1.1.17 Questions posed and project hypothesis

Recent studies have identified MRGPRX2 as a receptor by which a range of drugs used in surgery can directly activate mast cells. However, the clinical implication of MRGPRX2 in drug-induced anaphylaxis is not well defined. As mentioned earlier, although MRGPRX2 is presumably expressed in the mast cells of all humans, the reason underlying why only some individuals might respond so adversely is unclear.

Given the severity and frequent fatality of these reactions, a better understanding of MRGPRX2 activation could lead to categorising individuals who are more likely to suffer from these reaction. In doing this, susceptible patients could be spared from potentially devastating consequences.

Therefore, in this thesis, we hypothesise that **a variability in MRGPRX2 (expression/polymorphisms) and/or function of the receptor is the critical determinant of an individual's sensitivity or hypersensitivity to some drugs used in surgery.**

1.1.18 Aims

Over the course of this project, we sought to address three aims that would contribute to the understanding of MRGPRX2, if its activation can be stopped, is subject to bias, or can be predicted (**Figure 1.4**).

- Firstly, we aimed to investigate the possibility of halting MRGPRX2-mediated mast cell activation using antidotes (sugammadex and heparin) or the commonly used treatment, adrenaline.
- Secondly, we explored if some MRGPRX2-agonists could be more likely to cause MRGPRX2-induced anaphylaxis than others. To achieve this, we aimed to characterize the intracellular signalling cascade utilized by MRGPRX2 in causing mast cell activation in response to MRGPRX2 agonists, and in doing so, establish the likelihood of agonist bias.
- Thirdly, we studied the possibility of a biomarker that could be a defining factor, and thereby increase susceptibility, of patients who had anaphylactic reactions during surgery. To investigate this, we used blood samples from consenting patients who had anaphylactic reactions during surgery and analysed genomics and serum components, comparing results with control patient who had uneventful surgery.

Project aims

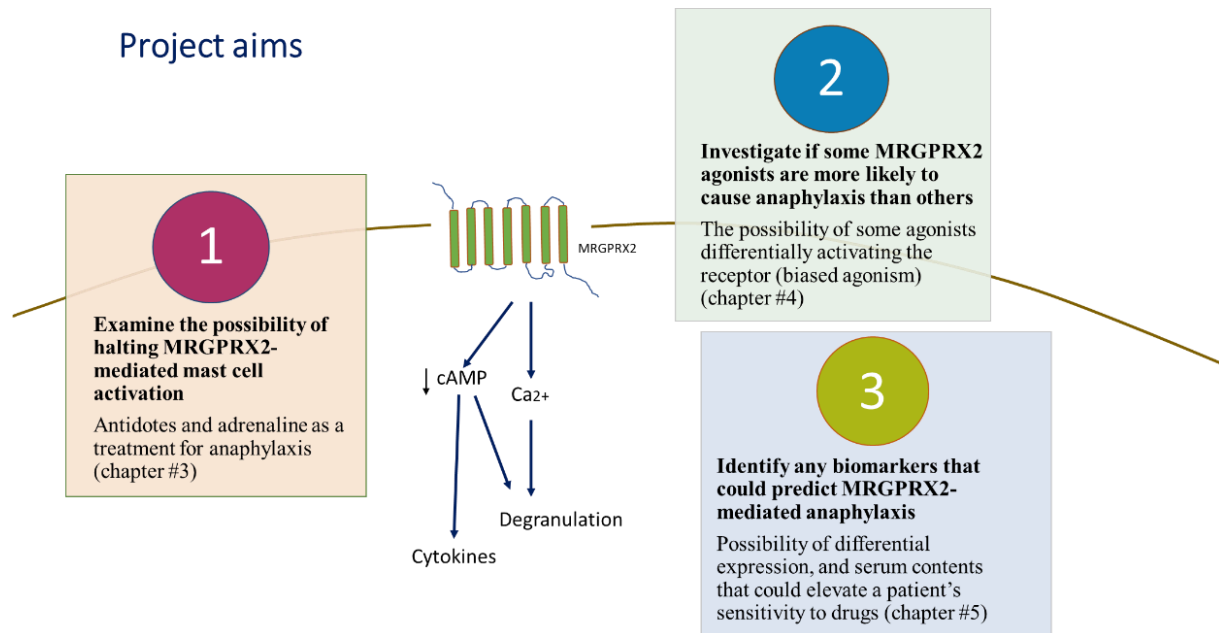


Figure 1.4: Schematic representing the aims addressed in this thesis.

Chapter 2

General materials and methods

This chapter details the methodologies frequently used throughout in this thesis. Additional chapter-specific methods used are described in respective chapters.

2.1.1 Cell culture of LAD2 (Laboratory of Allergic Disease 2) cells

LAD2 cells⁽¹⁸⁰⁾ were kindly provided by Dr. Arnold Kirshenbaum (National Institute of Health, Bethesda, US). The cells were cultured in Stem-pro medium (ThermoFisher Scientific; Scoresby, Victoria, Australia) supplemented with stem cell factor (SCF; 100 ng/mL) (PeproTech; Rocky Hill, New Jersey, US), 1X GlutaMAXTM (20 mM) supplement (ThermoFisher Scientific), penicillin (50 Units/mL) and streptomycin (50 µg/mL) (ThermoFisher Scientific) and maintained at a density between 2×10^5 and 1×10^6 cells/mL in a humidified incubator (5% CO₂ in air, 37°C).

2.1.2 Quantifying calcium mobilization using Fura-2

The Fura2 method was used to quantify calcium mobilization⁽¹⁸¹⁾. Fura-2 is a light-excitabile ratiometric fluorescent dye that binds to free calcium, and the complex is excitable at wavelengths 340 nm and 380 nm with emission at 510 nm. The absorption shift towards 340 nm that follows Fura-2 binding to calcium ions can then be measured ratiometrically⁽¹⁸¹⁾. LAD2 cells (at a density of 5×10^6 cells/mL) were washed and resuspended in HANKS buffered saline solution (HBSS) (1X HANK's salts (NaCl (137 mM), KCL (5.4 mM), Na₂HPO₄ (0.34 mM), KH₂PO₄ (0.44 mM)), sodium bicarbonate (16.7 mM), HEPES (10 mM), glucose (5.5 mM), BSA (0.05%), MgSO₄ (0.73 mM), CaCl₂ (1.8 mM)) supplemented with 2.5 mM probenecid (Sigma-Aldrich; Castle Hill, New South Wales, Australia) solubilized in 0.5 mM sodium hydroxide solution, with final buffer pH of 7.3. Fura-2AM (2 µM; Abcam; Melbourne, Victoria, Australia) made up in DMSO (dimethyl sulfoxide; Sigma-Aldrich) was added and cells were incubated in the dark for 90 minutes in an oscillating incubator (37°C; 70 RPM).

The cells were then washed, resuspended in HBSS (density of 2.5×10^5 cells/mL in 200 µL buffer), and seeded on a flat bottom 96 well plate (Greiner Bio-One, Kremsmünster, Austria). Stimuli/drugs (compound 48/80, ZINC-3573(R), ZINC-3573(S), substance P, rocuronium,

atracurium, protamine, LL-37, cortistatin-14, ionomycin and NIP-BSA; concentrations listed in respective chapters) were prepared and transferred into a round-bottom 96-well plate (Greiner Bio-One). The cell plate, drug plate and tips (Flex station® pipette tips, black; Molecular devices; San Jose, California, US) were then placed in a Flex station III (Molecular Devices; San Jose, California, US) where automated drug addition were performed with real time recordings of calcium mobilisation. Drugs were added after capturing a baseline reading for 30 seconds, and fluorescence readings were taken every 5 second for a total of 6 minutes. Data were extracted from SoftMax® Pro 7 (Molecular Devices) and graphed using PRISM (version 9.1.0 , GraphPad, San Diego, California, US) as a ratio of 340 nm / 380 nm, with or without baseline subtraction (as mentioned in individual figure legends). In some figures, data are represented as peak calcium mobilization responses with the peak response determined using PRISM.

2.1.3 Quantifying LAD2 degranulation using the β -hexosaminidase enzyme assay

β -hexosaminidase, an enzyme found and released from mast cell granules, is a commonly used measure of mast cell degranulation that is quantified via a simple but sensitive colorimetric enzymatic assay. For this assay, LAD2 cells were sensitized with human IgE (1-2 μ g/mL; conditioned media from the NIP (4-hydroxy-3-iodo-5-nitrophenylacetyl)-specific IgE secreting cell line Jw8/5/13 cells) for a period of 24-48 hours in a humidified incubator (37 °C and 5% CO₂). The cells were then washed with Hank's buffered saline (HBSS) and seeded on to flat bottom 96-well plates (2.7 x 10⁵ cells/mL in 200 μ L HBSS). Stimuli (mentioned above in **section 2.1.2**) along with Triton-X-100 (0.1%; Sigma-Aldrich) (to quantify total cellular β -hexosaminidase) were prepared and added to respective wells and placed in an incubator (37 °C) for a period of 45 minutes. Following this, contents of the wells were mixed thoroughly by pipetting up and down and the plates were then centrifuged (233 xg, 5 minutes) to sediment cells.

Cell-free supernatant (25 μ L) along with pNAG (75 μ L of 2 mM PNAG; p-Nitrophenyl-N-acetyl- β -D-glucosaminide solubilised in phosphate-citrate buffer of pH=4.5; Glycosynth; Winwick

Quay, Warrington, United Kingdom), were added to a 96-well flat bottomed plate. The plate was subsequently wrapped in foil and incubated for 120 minutes at 37°C in an oscillating incubator (70 RPM). The reaction was halted and developed by the addition of glycine (100 µL; 0.4 M; pH=10.7) and an absorbance reading was taken at 405 nm (Multiskan Ascent; ThermoFisher Scientific). The absorbance readings were used to calculate β-hexosaminidase release which was expressed as a percentage of the total cellular β-hexosaminidase content (quantified using TritonX-100 (0.1%)-induced cell lysis) (*i.e.*, sample absorbance/ triton-X absorbance *100). The basal (spontaneous) release of β-hexosaminidase (typically around 6% of total enzyme), occurring in the absence of any stimulus, was subtracted from all responses.

2.1.4 Quantification of cytokine levels via ELISA (enzyme linked immunosorbent assay)

NIP-specific IgE sensitized LAD2 cells were seeded into a round-bottom 96-well plate (2.5 x 10⁵ cells/mL in 200 µL buffer) or flat-bottom 24-well plate (1 x 10⁶ cells/mL in 700 µL buffer) in serum free IMDM (Iscove's Modified Dulbecco's; supplemented with 0.1% BSA (bovine serum albumin), 1X glutaMAX™, penicillin (50 Units/mL) and streptomycin (50 µg/mL); all from ThermoFisher Scientific). Drugs/stimuli were prepared, added to cells, and plates incubated overnight in a humidified incubator (37°C and 5% CO₂). The contents of the wells were mixed by gently pipetting up and down, transferred to 1.5 mL microcentrifuge tubes (Axygen; Union City, California, US), and were centrifuged (233 xg, 5 minutes) to sediment cells. Cell-free supernatants were then collected and stored at -20 °C until further use.

Supernatants obtained from cells were quantified for CCL2 levels using ELISA (methodology as per manufacturer's instructions (BD OptEIA™ ELISA kit; BD Biosciences; Scoresby, Victoria, Australia)). Briefly, CCL2 capture antibody (50 µL; 1:250 dilution in ELISA coating buffer) was added in high binding 96 well plates (Greiner Bio-One; Kremsmünster, Austria) and left to coat overnight (4 °C). The plates were then washed in ELISA wash buffer (PBS/0.05% Tween 20), and blocking buffer (PBS containing 5% FBS (fetal bovine serum; 150 µL/well) was added, and incubated at room temperature for 90 minutes. Following this, the plates were washed in

wash buffer and samples (50 μ L; diluted appropriately in blocking buffer) were added along with kit-provided CCL2 for the generation of a standard curve (CCL2 concentrations of 1 – 4,000 pg/mL), and left to incubate overnight at 4 °C. The plates were subsequently washed in wash buffer and then incubated with biotinylated detection antibody and streptavidin-horseradish peroxidase conjugate (Sav-HRP) (dilutions of 1:1000 and 1:250 respectively in blocking buffer). Following an incubation of 90 minutes, the plates were washed and TMB substrate (50 μ L, 3,3',5,5'-tetramethylbenzidine; BD Biosciences) was added. The colour change was assessed visually and upon observing a colorimetric gradient in the standard curve, sulfuric acid (0.2 M; pH=0.4) was added to halt and develop the reaction. Absorbance readings were taken at 450 nm (Multiskan Ascent; ThermoFisher Scientific). Sample data within the linear region of the standard curve were interpolated from the standard curve to quantitate cytokine levels and exported using SkanIt Software (version 6.0.1; ThermoFisher Scientific). Samples that were outside the linear range were re-tested with an adjusted dilution factor. Interpolated data were plotted with PRISM either as raw values or a percentage of basal/maximum CCL2 release observed, as described in individual chapters.

2.1.5 LAD2 RNA isolation

LAD2 cells were seeded into 12 well plates (Greiner Bio-One) at a density of 1.5×10^6 cells/well in serum free IMDM (supplemented with BSA, glutaMAX™ and penicillin/streptomycin as mentioned in **section 2.1.4**; 1.5 mL final volume). Drugs/stimuli were prepared, and the cells were stimulated for a period of 4 hours in a humidified incubator (37°C and 5% CO₂). The cells and supernatant were subsequently transferred to 1.5 mL microcentrifuge tubes, centrifuged (233 xg, 5 minutes) and supernatant discarded. The cells were then washed with ice-cold PBS twice to remove any residual media. Following this, lysis buffer (300 μ L; with 0.1% β -mercaptoethanol; Illustra RNA mini spin kit; GE Healthcare, Marlborough, Massachusetts, US) was added and RNA isolation was performed according to manufacturer's instructions with RNA eluted in 40 μ L RNase free H₂O.

2.1.6 cDNA generation and quantitative PCR

RNA concentration and purity were quantified using a spectrophotometer (Nanodrop 1000; Applied Biosystems, Foster City, California, US). Following this, RNA was diluted (200 ng in a 5 μ L volume), mixed with a kit-provided reverse transcriptase master mix (5 μ L; high-capacity cDNA reverse transcriptase kit; ThermoFisher Scientific), and incubated for 2 hours at 37 °C followed by 5 minutes at 85 °C in a thermocycler (Mastercycler® nexus gradient (Eppendorf, Hamburg, Germany)). Resultant cDNA was diluted 1:10 with RNase free water and 2 μ L was then mixed with SSA Advanced™ universal probes supermix (Bio-Rad; Hercules, California, US) containing TaqMan assay primers (3 μ L; ThermoFisher Scientific) (**Table 2-1**). Real-time quantitative polymerase chain reaction (RT qPCR) was carried out with 40 cycles of 95°C for 15 seconds followed by 60°C for 60 seconds (Quant Studio 6; Applied Biosystems). Δ CT was calculated by the subtraction of the test threshold gene cycle number from the average threshold cycle number of two house-keeper genes (UBC and GAPDH) measured in the same sample. Where an effect of drug addition from basal was quantified, $\Delta\Delta$ CT was calculated by subtraction of untreated gene expression.

TaqMan gene name	Gene ID	TaqMan gene name	Gene ID
GAPDH	Hs02758991_g1	FGD2	Hs00404008_m1
UBC	Hs01871556_s1	FGD3	Hs00382004_m1
CCL2	Hs01871556_s1	HEXA	Hs00942655_m1
FcεRI α	Hs00758600_m1	HEXB	Hs01077594_m1
FcεRI β (MS4A2)	Hs00999628_m1	IL-8 / CXCL8	Hs00174103_m1
FcεRI γ	Hs00175408_m1	GNAQ	Hs00387073_m1
Tryptase (TPSB2T)	Hs02576518_gH	GNAI1	Hs01053355_m1
TNF α	Hs00174128_m1	GNAI2	Hs01064686_m1
MIF	Hs00236988_g10	GNA15	Hs00976150_m1
FcγRIIA	Hs01017702_g1		
MRGPRX2	Hs00365019_s1		

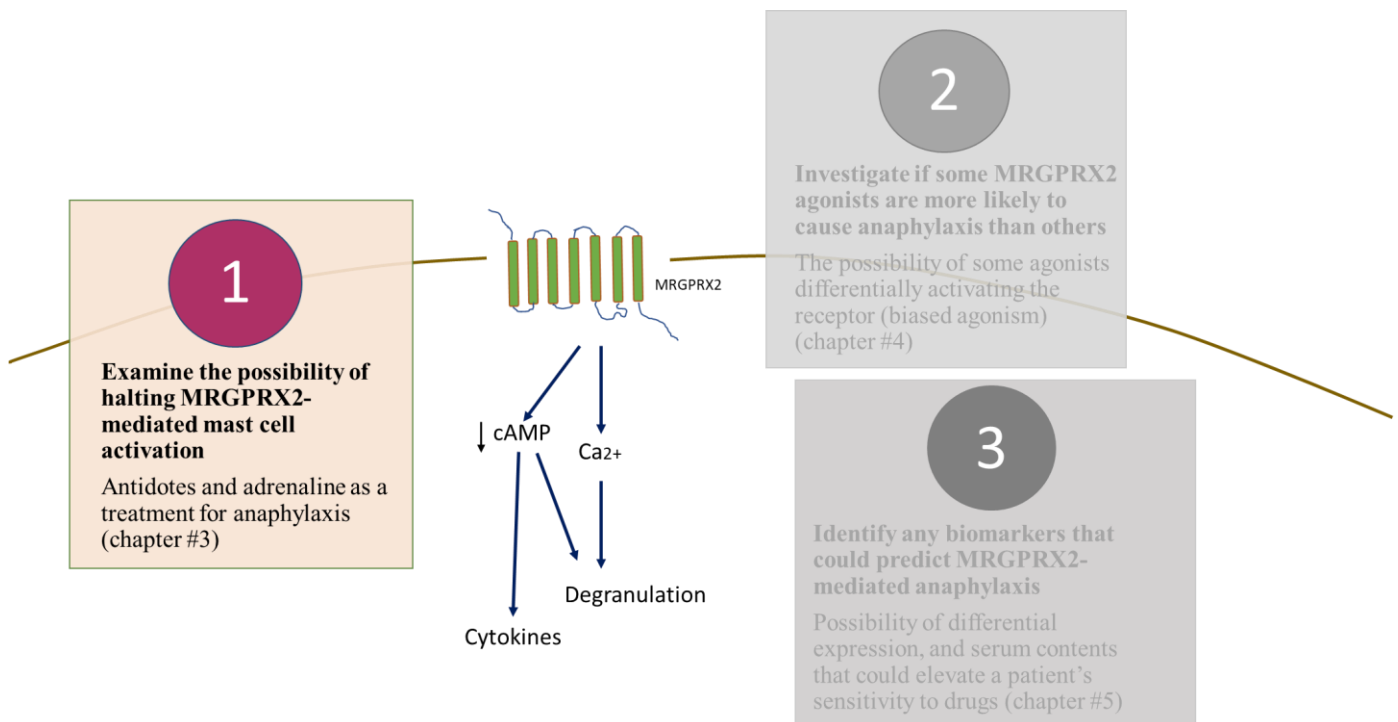
Table 2-1: Taqman primers used for qPCR assays (all from ThermoFisher Scientific)

2.1.7 Statistical analysis

Graphical representations (expressed as mean, $\pm$ SEM, n=x) were drawn using PRISM (version 9.1.0; GraphPad, San Diego, California, US). Where appropriate, statistical significance was determined using either a one-way ANOVA with Dunnett's multiple comparison post hoc test or a two-way ANOVA with Bonferroni multiple comparison post hoc test (version 9.1, GraphPad PRISM) with p values as indicated in figure legends.

Chapter 3

An *in vitro* study investigating sugammadex and heparin as antidotes, and adrenaline as a treatment, for MRGPRX2-mediated mast cell activation



3.1 Chapter overview

With the recent discovery of MRGPRX2 as a candidate receptor responsible for IgE-independent anaphylaxis, there has been a surge of research into characterising clinically used activators of the receptor, along with inhibitors and modulators that could be therapeutically utilised to reduce/prevent adverse outcomes. To date, a wide variety of both clinically used drugs and endogenous compounds have been found to be agonists of the receptor ⁽⁵⁸⁾ (refer to **section 1.1.11 and 1.1.12**). Similarly, a range of antagonists have also been proposed, with varying evidence of efficacy and specificity ^(108, 154, 159) (**section 1.1.13**). In our study, we first investigate and validate the importance of MRGPRX2 in mast cell activation triggered by a range of clinically used drugs (NMBAs (rocuronium, atracurium) and protamine), endogenous agonists (substance P, LL-37, and cortistatin-14), along with some commonly used laboratory tools (compound 48/80 and ZINC-3573(R)). Secondly, we explored the possibility of utilising clinically used specific reversal agents, sugammadex (counter ligand of rocuronium) and heparin (counter ligand of protamine), to determine if these chemical antagonists block MRGPRX2-dependent mast cell activation by their specific counter ligands. Additionally, we examined if these agents also possess more broad activity against other MRGPRX2 agonists.

3.1.1 Anaphylaxis caused by peri-operative drugs

As discussed previously (**section 1.1.15**), NMBAs are one of the leading causes of anaphylaxis during surgery (second only to antibiotics) with a mortality rate ranging from 3.5% in Australia to 10% in UK in those that experience anaphylaxis ⁽¹⁷⁶⁾, although some studies report much lower rates of mortality ⁽¹⁸²⁾. Of the available NMBAs, rocuronium and atracurium are the most commonly used non-depolarising muscle relaxants, and are both leading causes of perioperative anaphylaxis with a prevalence of roughly 5.9 and 4 in every 100,000 rocuronium and atracurium administrations respectively, according to a national audit project conducted in the UK ⁽¹⁵¹⁾. Similarly, in Australia the reported rates lie at 8 and 4 per 100,000 administrations of rocuronium and atracurium, respectively ⁽¹⁴⁹⁾. The overall incidence rate of

perioperative anaphylaxis (common causative agents include antibiotics, NMBAs, chlorhexidine and patent blue dye) was estimated to be 1 case per 10,000 anaesthetic administrations, although this figure is believed to be an underestimate due to underreporting (primarily due to incomplete form submissions and filtering for severe anaphylaxis) ⁽¹⁵¹⁾.

In addition to NMBAs, protamine sulphate, a drug commonly used to reverse the anti-coagulant activity of heparin is also known to cause rare severe anaphylactic responses ⁽¹⁸³⁾. Protamine, a basic peptide made up of poly(arginine) and originally isolated from salmon sperm, is known to be a leading cause of anaphylaxis in cardiac surgery, producing profound cardiac collapse ^(183, 184). In the study conducted by Weiler et al. (1990), of 243 patients, 26 patients (10.6%) had an adverse reaction, with no patients exhibiting positive skin prick tests or measurable IgE against protamine. A significant proportion of protamine-mediated anaphylaxis is thus classified as 'IgE-independent'. Whilst assumed, based on its poly(arginine) structure, whether protamine is an agonist of the MRGPRX2 receptor has not been formally characterised.

In any event of anaphylaxis (especially grades 3 and 4), a range of drugs are given to treat symptoms including adrenaline (epinephrine) along with other β -agonists, anti-histamines and corticosteroids (discussed further in **section 1.1.16**) ⁽¹⁷⁷⁾. However, in surgery where the eliciting agent can be perhaps deduced, more specific approaches are sometimes used. In some cases, although with conflicting evidence of efficacy, neutralising agents such as sugammadex and heparin have been used to counteract anaphylaxis induced by their respective counter ligands ^(178, 179, 185, 186).

3.1.2 Sugammadex: a rocuronium withdrawal agent

Sugammadex is a synthetic γ -cyclodextrin, an encapsulating agent, that was initially discovered while seeking improved solubilizing agents for rocuronium ⁽¹⁸⁷⁾. As a γ -cyclodextrin, sugammadex consists of a large lipophilic cavity that forms stable complexes with bulky amino steroidal NMBAs with a stoichiometry of 1:1 ⁽¹⁸⁸⁾. Upon discovering the stability of the sugammadex-rocuronium complex, this development project changed focus from solubilizing agents to novel drug reversal agents ⁽¹⁸⁷⁾. Sugammadex was subsequently modified to bind more stably to the amino steroidal drug rocuronium, and is now commercially used in over 60 countries for the post-surgical reversal of rocuronium and vecuronium-induced neuromuscular block ^(148, 175, 189). It is important to note that anaphylactic events to sugammadex have also been recorded, with the incidence rates ranging from being as high as that of rocuronium in Japan ⁽¹⁹⁰⁾, to much rarer occurrences (1 in over 64,000 patients) as reported in a recent audit in the UK ^(151, 191). Sugammadex also has no known receptor-mediated activity, thus theoretically having a benefit over the side effects that follow anticholinesterase drugs (the conventional means of reversing non-depolarising neuromuscular block) ⁽¹⁸⁸⁾. Although sugammadex is commonly used to reverse neuromuscular block by certain NMBAs ^(192, 193), its ability to serve as an antidote for amino steroid-induced anaphylaxis (both IgE-dependent and independent) is frequently contested (discussed further in manuscript below (Fernandopulle et al., 2020) ⁽¹⁹⁴⁾). The mechanistic propensity of sugammadex to successfully mask the binding sites required for MRGPRX2 activation or prevent the antigen recognition of small molecules required for IgE-dependent activation is little understood. To address the role of sugammadex on MRGPRX2 activation, in our recent manuscript ⁽¹⁹⁴⁾ we have investigated its ability to interfere with stimulus-induced mast cell activation using an *in vitro* human mast cell model.

3.1.3 Heparin-mediated anti-coagulation and protamine-mediated anaphylaxis

Heparin is a naturally occurring glycosaminoglycan (a primary component of mast cell granules; **section 1.1.4**), commonly known for its frequent clinical use as an anti-coagulant, particularly in cardiac surgery involving cardiopulmonary bypass (CPB) ⁽³⁰⁾. Following surgery, when reduced coagulation is no longer necessary and indeed will lead to an increased risk of bleeding, a heparin-reversal agent, protamine, is given to reverse the drug's anticoagulatory effects ⁽¹⁹⁵⁾. The heparin-protamine interaction is based on the fundamental charge-charge interaction between the acidic heparin and alkaline protamine to form a neutral complex devoid of effect on the blood coagulation pathway ⁽¹⁸⁴⁾. Anaphylaxis to protamine is found to be comprised of both IgE-dependent and independent hypersensitivities ⁽¹⁸³⁾. As described in our submitted manuscript (Fernandopulle et al., 2021), high risk groups for IgE-dependent protamine anaphylaxis include diabetic patients previously exposed to protamine-zinc insulin ^(183, 196), vasectomised or infertile men who have developed anti-sperm antibodies ^(197, 198), and patients with a history of drug hypersensitivities ⁽¹⁹⁹⁾.

The recommended dosage for protamine generally lies between 1 – 1.5 mg per 100 international units (IU) of heparin ^(200, 201). This dosage of protamine to neutralize heparin was established in the mid-20th century being calculated for the initial administered heparin dose ⁽²⁰²⁾. This ratio takes minimal consideration of any interpatient variability in drug sensitivity or the metabolism of heparin over time that directly affects the level of heparin available near the end of surgery when protamine is administered ^(186, 203). Multiple articles suggest protamine overuse that result in significant adverse effects including anaphylaxis but also, and perhaps more commonly, hypotension, increased perioperative bleeding and a paradoxical increase in coagulation time ^(186, 201). In some cases, this increased coagulation time was reversed by the provision of further heparin ⁽¹⁸⁶⁾. In addition, a European survey collating the practices of cardiothoracic surgeons and their use of protamine, suggest a lack of consensus on the standard operating procedure for determination of protamine dosage ⁽²⁰⁰⁾. As mentioned earlier, to date, multiple studies suggest the overdose of protamine in CPB

patients^(186, 201, 203). These studies demonstrated that roughly 2/3rd the recommended dose of protamine resulted in adequate reversal of heparin activity (complementary data presented and discussed further in our manuscript, Fernandopulle et al, 2021)^(186, 201).

In our recent manuscript (Fernandopulle et al., 2021), using an *in vitro* mast cell model, we have investigated whether MRGPRX2 could be involved in the IgE-independent means of mast cell activation by protamine. Additionally, we have studied the possible modulation of this activation by various dose ratios of the counter ligand, heparin.

3.1.4 Adrenaline: the most commonly used treatment for anaphylaxis

Adrenaline is used as the mainstay first-line therapy for anaphylaxis for its predominant systemic role in counteracting the vasodilatation, bronchoconstriction and increased vascular permeability that occurs in anaphylaxis^(6, 204, 205). Adrenaline is a non-specific adrenoceptor agonist, and therefore has a range of effects throughout the body^(206, 207). Stimulation of α_1 adrenoceptors on vascular smooth muscle increase peripheral vascular resistance, thereby restoring blood pressure and reversing mast cell mediator-driven vasodilation in peripheral tissues⁽²⁰⁴⁾. Simultaneously, the stimulation of β_1 adrenoceptors in cardiac tissue both restores heart rate and improves muscle contractility⁽²⁰⁴⁾, and stimulation of β_2 adrenoceptors in lung tissue induces bronchodilation, alleviating the bronchoconstriction that occurs in anaphylaxis⁽²⁰⁴⁾. Adrenaline is also commonly known as a ‘mast cell stabiliser’. Although this activity is found to be primarily driven by the activation of β_2 -adrenoceptors^(204, 208, 209), the presence and function of both α_1 and β_2 adrenoceptors have been well studied in both human and rodent primary tissue mast cell models^(206, 209, 210). The activation of α_1 -adrenoceptors has been associated with mast cell activation and subsequent histamine-induced bronchoconstriction in the pathogenesis of asthma^(209, 211). A recent study demonstrated the potentiation of β_2 -adrenoceptor-mediated mast cell stabilisation by the antagonism of α_1 -adrenoceptors⁽²⁰⁹⁾.

The mechanism of action of β_2 -adrenoceptor-mediated mast cell stabilisation is via a Gs-mediated increase in intracellular cAMP production ^(212, 213). This increase in cAMP was found to decrease IgE-mediated histamine release from stimulated mast cells ^(46, 214, 215). β_2 -adrenoceptor activation has also been associated with a decrease in Fc ϵ RI-mediated PGD₂ and LTC₄ release in human mast cells ⁽²¹⁶⁾. The inhibitory effect of increased cAMP on compound 48/80 (MRGPRX2 agonist)-induced histamine release has also been noted long before the characterisation of MRGPRX2 ⁽²¹³⁾. However, given that in drug-induced anaphylaxis mast cells have been activated prior to adrenaline administration, the clinical significance of mast cell stabilisation in this setting is not well understood.

3.1.5 Chapter Aims

In this chapter, we aim to both validate and characterise the role of MRGPRX2 activation as a possible mechanism of anaphylaxis by certain polybasic drugs. We also examine the effects of known chemical antagonists (sugammadex and heparin) on some of these agents to determine if they attenuate the mast cell activation response. Additionally, we have investigated the role of adrenaline as a mast cell stabiliser, in our *in vitro* model, when added prior to and immediately after MRGPRX2 activation. Responses observed by this established and well known treatment for anaphylaxis (adrenaline) also serves as a useful comparator to the roles played by the lesser understood chemical antagonists in the treatment of anaphylaxis (sugammadex and heparin).

3.2 Materials and methods

Specific methods used in these studies are detailed in the attached manuscripts. A brief summary of the major methods used, are however also shown below.

3.2.1 Generating an MRGPRX2 knock down cell line using CRISPR-Cas9 gene editing

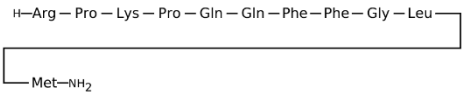
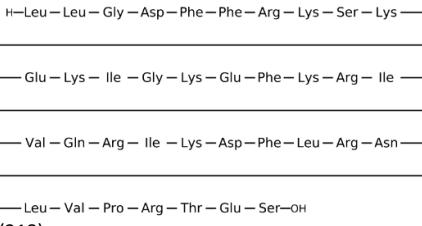
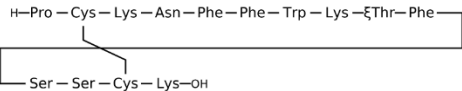
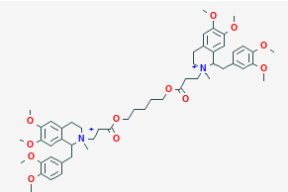
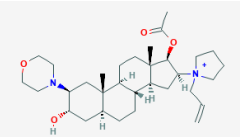
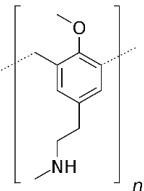
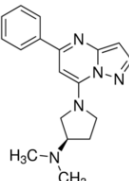
CRISPR cas-9 technology with a guide RNA sequence of *GAGCATGGATCCAACCACC* (targeting the start of the MRGPRX2 coding sequence), was used to interrupt, and thereby prevent expression of MRGPRX2 in LAD2 cells that constitutively express the receptor. These cells were then sorted using fluorescence-associated cell sorting (FACS) using both indirect and direct means of MRGPRX2 expression. An indirect means of sorting was first conducted due to the lack of a verified MRGPRX2 antibody. This involved activation of MRGPRX2 with the agonist compound 48/80 to induce degranulation, followed by cell sorting using an antibody against CD63. CD63 is normally expressed on intracellular mast cell granules, but upon degranulation is detectable on the cell surface. CD63^{low} cells (indicative of MRGPRX2 knock down) were sorted, expanded, and re-tested for reduced activation through MRGPRX2. MRGPRX2 activation was not completely ablated in this mixed CD63^{low} population, and in our hands, LAD2 cells did not expand from single cells, therefore this mixed polyclonal population of cells were termed MRGPRX2 knock down (KD) cells. Following the purchase of a verified MRGPRX2 antibody (clone-K125H4; BioLegend, San Diego, California, US) that binds an extracellular epitope on MRGPRX2, these cells were further sorted and MRGPRX2^{low} cells were then expanded and retested for activation.

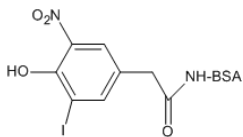
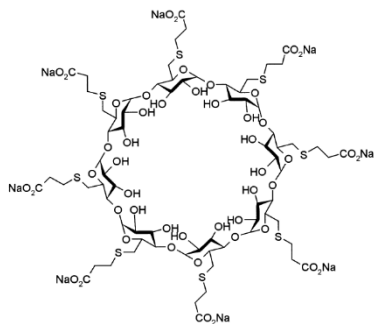
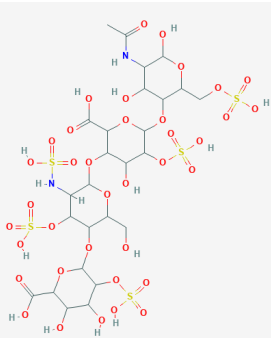
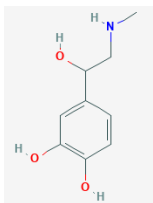
3.2.2 Quantifying LAD2 activation

Activation of LAD2 cells (WT and KD) was quantified using calcium mobilization (Fura-2; refer to **section 2.1.2**), degranulation (β -hexosaminidase enzyme assay; **section 2.1.3**), and CCL2 release (ELISA; **section 2.1.4**). A wide range of drugs and known MRGPRX2 activators were used including clinically used drugs (rocuronium, atracurium, protamine), endogenous peptides (substance P, LL-37, cortistatin-14), other known MRGPRX2 agonists (compound 48/80, ZINC-3573(R)), the inactive enantiomer of ZINC-353(R) (ZINC-3573(S)), and an activator of the IgE-dependent pathway (NIP-BSA). Specific concentrations used are described in respective manuscripts, results subsections, and figure legends. A summary of agonist information (known structure, molecular weight, molecular formulae, and net charge at pH=7) is summarised in **Table 3-1**.

LAD2 stimulation with the above mentioned drugs was also conducted in the presence of prospective reversal agents, sugammadex and heparin (**Table 3-1**), and the most commonly used treatment for anaphylaxis, adrenaline. These agents were either co-treated with the stimulus, or in some cases used to pre-treat the cells (5 minutes prior to stimulus addition), or to better mimic a clinical setting of anaphylaxis, added rapidly (2 minutes) following drug stimulation.

For the study investigating the time-dependent role of sugammadex addition on MRGPRX2 activation (**section 3.3.5**), CCL2 release (y-axis) was normalised as a percentage of maximal stimulus-induced release in the absence of sugammadex. This data curation was done to normalise the evident variability observed in absolute values of CCL2 quantified between the batches of ELISA kits purchased.

Drug/ compound	Structure (2D structure/ amino acid sequence)	Formula	Molecular weight (Da)	Net charge at pH=7
Substance P	 (217)	C ₆₃ H ₉₈ N ₁₈ O ₁₃ S	1347.6	+3 ⁽⁹⁶⁾
LL-37 amide (trifluoroaceta te salt)	 (218)	C ₂₀₅ H ₃₄₀ N ₆₀ O ₅₃	4492.3	+6 ⁽²¹⁹⁾
Cortistatin-14	 (220)	C ₈₁ H ₁₁₃ N ₁₉ O ₁₉ S 2	1721	+3 ⁽⁹⁶⁾
Protamine sulphate	*ND	*ND	Polymer; ~ 4500 ⁽²²¹⁾	Range between +8 and +21 ⁽²²²⁾
Atracurium besylate	 (223)	C ₆₅ H ₈₂ N ₂ O ₁₈ S ₂	929.1	+2 ⁽²²³⁾
Rocuronium bromide	 (224)	C ₃₂ H ₅₃ BrN ₂ O ₄	609.7	+1 ⁽²²⁴⁾
Compound 48/80	 n	C ₃₂ H ₄₆ N ₃ O ₃ . 3HCl	~500 Da Polymer With typically 3 to 6 repeats ⁽⁹⁶⁾	+4.5 ⁽⁹⁶⁾
ZINC-3573(R)	 (95)	C ₁₈ H ₂₁ N ₅	307.39	*ND

Drug/ compound	Structure (2D structure/ amino acid sequence)	Formula	Molecular weight (Da)	Net charge at pH=7
NIP-BSA		*ND	*ND	*ND
Sugammadex	 (225)	C ₇₂ H ₁₀₄ Na ₈ O ₄₈ S ₈	2178	Strongly negatively charged
Heparin	 (226)	C ₂₆ H ₄₂ N ₂ O ₃₇ S ₅	Polymer (*ND)	Strongly negatively charged
Adrenaline (-)	 (227)	C ₉ H ₁₃ NO ₃	183.20	*ND

*ND- Not Determined

Table 3-1: Summary of drug/compound structures, molecular formula, molecular weight and expected net charge at pH=7. Data summarised in this table was sourced from various databases and research publications, and is referenced in text. Due to the lack of data availability, we were unable to determine some pieces of information (structure/formula/ molecular weight/ net charge) of drugs/compounds used (denoted *ND or “not determined”).

3.2.3 Isolating rat peritoneal mast cells and quantifying their activation

Sprague-Dawley rats (male; 9-12 weeks old) were obtained through The University of Melbourne, BRF facility with animal ethics committee approval for secondary use of tissues/cells. Rat peritoneal mast cells (RPMCs) were purified using a Percoll® density gradient technique ⁽²²⁸⁾. Isolated cells (>95% purity) were then either used for live cell imaging or degranulation assays (Fernandopulle et al., 2021, submitted manuscript). Cells were stimulated with a range of polybasic compounds including compound 48/80 (0.08 - 5 µg/mL), protamine (0.75 – 3 µM), ciprofloxacin (100 – 400 µM), rocuronium (800 µM), atracurium (200 µM), ZINC-3573(R) (25 µM), and substance P (6 µM). Live cell images were taken every 2 seconds for up to 60 seconds and compiled using FIJI (National Institute of Health; Bethesda, Maryland, US). Degranulation was quantified using the β-hexosaminidase enzyme assay (*section 2.1.3*).

3.2.4 Statistical analysis

Flow cytometry data were analysed and plotted as overlaid histograms using FlowJo_10.0 (Ashland, Oregon, US). Other graphical representations were drawn and analysed with PRISM (version 9.1, GraphPad) and expressed as mean, ±SEM of n experiments. Statistical significance was determined using either One-way or Two-way ANOVA with either Dunnet's or Bonferroni multiple comparison post hoc tests, with levels of significance as mentioned in respective figure legends.

3.3 Results

3.3.1 Manuscript 1: MRGPRX2 activation in mast cells by neuromuscular blocking agents and other agonists: modulation by sugammadex

As can be seen from the attached manuscript, this body of work, using a novel CRISPR Cas9-mediated MRGPRX2 knockdown LAD2 model verifies the role of MRGPRX2 in mast cell activation by some NMBAs (rocuronium and atracurium) and endogenous agonists (substance P, LL-37, and cortistatin-14). In addition, we have demonstrated the ability of sugammadex, the counter ligand of rocuronium, to form complexes with a range of MRGPRX2 agonists. Our results suggest that, although the addition of sugammadex following a stimulus was too late to impact the effects of degranulation, there was a significant reduction in release of the more delayed mediators such as the cytokine, CCL2.

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MRGPRX2 activation in mast cells by neuromuscular blocking agents and other agonists: modulation by sugammadex.

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Abstract

Background: Neuromuscular blocking agents (NMBAs) can cause both IgE-dependent and independent anaphylactic reactions, with activation of the mast cell receptor MRGPRX2 being important to the latter. Sugammadex, a reversal agent for certain aminosteroid NMBAs, has been proposed as an antidote for these anaphylactic events with conflicting outcomes.

Objective: We further characterise the involvement of MRGPRX2 in NMBA-induced mast cell activation and determine how this is influenced by sugammadex. We then apply these *in vitro* results to infer the possible utility of sugammadex in the acute management of non-IgE dependent anaphylaxis.

Methods: The LAD2 human mast cell line and a MRGPRX2 knock-down derivative were used to validate the involvement of MRGPRX2 and to test the effect of sugammadex on mast cell activation by NMBAs and other MRGPRX2 agonists.

Results: All MRGPRX2 agonists tested were shown to induce MRGPRX2-dependent LAD2 mast cell calcium mobilization and cytokine release and all, apart from rocuronium, induced degranulation. Co-treatment of mast cells with sugammadex and some MRGPRX2 agonists significantly reduced cell activation, but if sugammadex was administered a few minutes following stimulation, degranulation was not attenuated. However, addition of sugammadex up to 180 minutes following LAD2 MRGPRX2 stimulation, significantly reduced CCL2 mRNA and protein induction.

Conclusions and clinical relevance: We show that sugammadex, known to reverse muscle blockade by certain NMBAs, is also able to reduce MRGPRX2 activation by NMBAs and other, but not all, MRGPRX2 agonists. As sugammadex was ineffective in attenuating mast cell degranulation when added rapidly post MRGPRX2 activation, this suggests against the agent having efficacy in controlling acute symptoms of anaphylaxis to NMBAs caused by MRGPRX2 activation. Interestingly however, sugammadex did impair MRGPRX2-induced CCL2 release, suggesting that it may have some benefit in perhaps dampening less well-defined adverse effects of MRGPRX2-dependent anaphylaxis associated with the more slowly elaborated mast cell mediators.

Key words:

MRGPRX2, sugammadex, anaphylaxis, mast cells, drug hypersensitivity, anaphylactoid, IgE-independent drug reactions

1| Introduction

Drug-induced anaphylaxis can have devastating consequences, with anaphylaxis to neuromuscular blocking agents (NMBAs), such as rocuronium and atracurium, being a serious complication during anaesthesia⁽¹⁾. The recorded incidences of perioperative anaphylaxis vary between geographical locations with rates of 1 in 10,000 – 20,000 surgical patients reported in Australia and UK but as high as 1 in 3,500 in France⁽²⁻⁴⁾. Symptoms of anaphylaxis are attributed to the release of mediators from activated mast cells⁽⁵⁾. This activation is generally considered to be antibody-mediated largely by drug-reactive IgE^(5, 6). However, not all events of anaphylaxis following an adverse reaction to NMBAs have been associated with drug-specific IgE⁽⁷⁾. Whilst a role for drug-specific IgG has recently been suggested⁽⁸⁾, new research has also identified that the G protein-coupled receptor (GPCR) MRGPRX2 (Mas-related GPCR X2) can trigger IgE-independent activation of mast cells⁽⁹⁾. Ligands of this receptor are commonly basic molecules including drugs like NMBAs, opioids, neuropeptides such as substance P, antimicrobial peptides such as LL-37, and laboratory research tools such as the long investigated compound 48/80 and the more recently identified ZINC-3573(R)^(10, 11).

Following mast cell activation, pre-formed mediators such as histamine, tryptase and a range of other proteases are released in the process of degranulation that is initiated 1-2 minutes following drug exposure^(12, 13). This almost instantaneous process initiates the immediate symptoms of anaphylaxis including hypotension (caused by vasodilatation), increased heart rate, bronchospasm and urticaria^(14, 15). Following the immediate release of mediators via degranulation, activated mast cells also release other mediators, such as eicosanoids and cytokines, that over a period of minutes-hours can further contribute to the symptoms of anaphylaxis⁽⁵⁾.

In the event of anaphylaxis during surgery, depending on the severity, immediate treatment is necessary to protect the patient and halt potential injury or even death. Adrenaline and resuscitation fluids are used to stabilise blood pressure, and additional supportive treatments may be considered including anti-histamines and steroids^(14, 15). In addition, some case studies have reported the off-label use of sugammadex in the treatment of anaphylaxis induced by certain NMBAs, with conflicting reports of efficacy⁽¹⁶⁻¹⁹⁾.

Sugammadex is a γ -cyclodextrin drug that encapsulates aminosteroid NMBAs such as rocuronium, preventing interaction with the nicotinic receptor, and thereby reversing neuromuscular blockade^(20, 21). However, whether sugammadex is able to shield these NMBAs from activating MRGPRX2 remains unknown.

In this study, we aimed to further investigate the role of MRGPRX2 in NMBA-mediated mast cell activation and determine if the chemical antagonism of NMBAs, and perhaps other MRGPRX2 agonists, by sugammadex can inhibit MRGPRX2-dependent activation of human mast cells.

2| Methods

2.1| Materials

The following products were purchased from Sigma Aldrich (Castle Hill, New South Wales, Australia); rocuronium bromide, ZINC-3573(R), ZINC-3573(S), Triton X, Hank's buffered salts, sodium bicarbonate, HEPES, glucose, bovine serum albumin (BSA), MgSO₄, CaCl₂, probenecid and glycine. Atracurium besylate and Fura-2AM were purchased from Abcam (Melbourne, Australia). Compound 48/80 was obtained from Enzo Life Sciences (Farmingdale, New York, United States). Sugammadex (Bridion®) was obtained from the Royal Melbourne Hospital Pharmacy (Melbourne, Victoria, Australia). NIP-BSA (4-hydroxy-3-iodo-5-nitrophenylacetyl bovine serum albumin) was purchased from Biosearch Technologies (Novato, California, United States). Jw8-IgE with specificity for the hapten NIP was generated by culturing the Jw8/5/13 cell line (ECACC hybridoma collection). Substance P (provided by Ms. Haoyue Zhang and Dr. Susan Northfield (Department of Pharmacology and Therapeutics, University of Melbourne, Victoria, Australia)) was produced by standard solid phase peptide synthesis methods and purified to >95% homogeneity by preparative HPLC⁽²²⁾. The following products were purchased from ThermoFisher Scientific (Scoresby, Victoria, Australia); Iscove's Modified Dulbecco's Media (IMDM), GlutaMAX™ (100X) supplement, phosphate buffered saline (PBS), StemPro™-34 media, high capacity cDNA reverse transcriptase kit, TaqMan™ primers, RNAiMAX lipofectamine, penicillin (5000 unit/mL)/streptomycin (5000 µg/mL), and CyQUANT™ lactate dehydrogenase (LDH) cytotoxicity assay. ELISA high binding 96 well plates were purchased from Greiner Bio-One (Kremsmünster, Austria). Stem cell factor (SCF) was purchased from PeproTech (Rocky Hill, New Jersey, USA). Illustra RNA mini spin kit was purchased from GE Healthcare, Life sciences (Chicago, Illinois, United States). SSA Advanced™ universal probes supermix was obtained from Bio-Rad (Hercules, California, United States). Mouse anti-human CD63-PE, TMB substrate (3,3',5,5'-Tetramethylbenzidine) and CCL2 (MCP-1) cytokine ELISA kit were purchased from BD Biosciences (Scoresby, Victoria, Australia). pNAG (p-nitrophenyl-N-acetyl-β-D-glucosaminide) was purchased from Glycosynth (Winwick

Quay, Warrington, United Kingdom). Alt-R CRISPR-Cas9 kit along with tailor-made guide RNA for the gene interruption of MRGPRX2 was purchased from Integrated DNA Technologies (Singapore Science Park II, Singapore). Human anti-MRGPRX2-PE (clone K125H4), anti-FcεRIα-APC (clone Cra-1), along with appropriate isotype controls, were purchased from BioLegend (San Diego, CA, United States). Anti-CXCR4-PE (clone 12G5) was from R&D systems (Minneapolis, MN, USA). Cortistatin-14 (trifluoroacetate salt) and LL-37 (trifluoroacetate salt) were purchased from Cayman Chemical (Ann Arbor, Michigan, United States).

2.2 | Culture of LAD2 mast cells

LAD2 cells⁽²³⁾ were provided by Dr. Arnold Kirshenbaum (National Institute of Health, Bethesda, United States). The cells were cultured in Stem-pro34 medium supplemented with SCF (100 ng/mL), GlutaMAX™ (1X), penicillin (50 Units/mL) and streptomycin (50 µg/mL) and maintained in a humidified incubator (5% CO₂, 37°C).

2.3 | CRISPR-Cas9 gene disruption of MRGPRX2 in LAD2 cells

CRISPR-Cas9 technology was used to knock out the MRGPRX2 gene from wild type LAD2 cells. CRISPR guide RNA (crRNA) *GAGCATGGATCCAACCACC* was designed (by Dr. Li Eon Kuek, Department of Pharmacology and Therapeutics, University of Melbourne, Victoria, Australia), to specifically target the human MRGPRX2 gene early in the coding region. A transfection control (no CRISPR-Cas9 target sequence) and a scramble control sequence (with no known target in the genome) were used as controls. Transfection was performed in accordance with the manufacturer's protocol. In brief, the crRNA-tracrRNA complex was mixed with lipofectamine RNAiMAX to form a transfection complex. This complex was then mixed with cells and seeded on a 12-well plate at a density of 4.5 x 10⁵ cells/mL in antibiotic free Stem-pro34 growth medium.

48 hours following transfection, MRGPRX2 knock-out cells were selected using a functional screen based on CD63 surface levels. CD63 is a vesicular marker expressed on the mast cell surface upon degranulation and the basis of the screen was that a loss of MRGPRX2 expression would impair CD63 surface expression upon stimulation with receptor agonists. Briefly, cells were stimulated with MRGPRX2 agonist, compound 48/80 (10 $\mu\text{g/mL}$), for 15 minutes in the presence of mouse anti-human-CD63-PE. As is shown in supplementary **Figure S1A**, upon stimulation with compound 48/80, MRGPRX2 gene-targeted cells (red trace) had a much greater CD63^{low} population compared to the control targeted cells (blue and grey traces). CD63^{low} cells were sorted using flow cytometry (BD FACS ARIA III; BD Biosciences), expanded, and then re-stimulated and sorted again as above to enrich further for functional MRGPRX2 knockouts (**Figure S1B**). As a phenotypic screen was used, targeting of pathways unrelated to MRGPRX2 expression might also produce this result. We thus tested if the IgE-pathway of mast cell activation was interrupted in these cells. As shown in **Figure S1C**, IgE-dependent mast cell activation by antigen was little changed by MRGPRX2-targeting. Following two rounds of sorting, there remained a remnant population of MRGPRX2-targeted cells that still responded to compound 48/80, likely reflecting non-targeted cells or single-allelic knockouts. Given that LAD2 cells are slow growing and, in our hands, do not expand readily from clones, this mixed population of cells termed MRGPRX2 knock down (KD) cells were used in all further experiments. The cells were then expanded and tested for surface expression of MRGPRX2 along with another GPCR, CXCR4 and the high-affinity IgE receptor, Fc ϵ RI α , via flow cytometry. Confirming the selectivity of the phenotypic CD63 expression assay, sorted cells had reduced levels of MRGPRX2 whilst, compared to WT cells, expression of Fc ϵ RI α and CXCR4 was essentially unchanged (**Figure S1 D-F**).

2.4 | Quantification of LAD2 cell calcium mobilization

IgE-sensitized cells were incubated with FURA2-AM (2 μ M) in Hank's buffered saline solution (HBSS) (1X Hank's buffered salts, sodium bicarbonate (0.14%), HEPES (10 μ M), glucose (5.5 mM), BSA (0.05%), MgSO_4 (0.7 mM), CaCl_2 (1.8 mM)) supplemented with probenecid (2.5 mM) at a density of 5×10^6 cells/mL for 90 minutes at 37°C. Cells were washed and seeded into 96-well plates (5×10^4 cells/well) and calcium mobilization was measured ratiometrically (excitation 340/380 nm; emission 510 nm) using a Flex station III (Molecular Devices, San Jose, California, USA). LAD2 mast cell stimulating agents (in the absence of or in complex with sugammadex) were added at 30 seconds. In some experiments, sugammadex was added 40 seconds following stimulus addition

2.5 | Quantification of LAD2 mast cell degranulation

β -hexosaminidase release is a commonly used measure of mast cell degranulation. LAD2 cells were sensitized with human IgE (1 μ g/mL; conditioned media from Jw8/5/13 cells) for a period of 24-48 hours. The cells were then washed and resuspended in serum free IMDM (+ 0.1% BSA) or HBSS and seeded (5×10^4 cells/well) into 96-well plates. Stimuli were added to wells and incubated for 45 minutes (37°C). Where applicable, cells were either co-treated with sugammadex and stimulus or sugammadex was added 2 minutes following stimulus addition. Cell supernatant (25 μ L) was then transferred to a fresh plate where pNAG substrate was added (75 μ L; 2 mM pNAG; pH=4.5 in phosphate-citrate buffer). The plate was incubated for 120 minutes at 37°C in an oscillating incubator (70 RPM). The reaction was stopped by the addition of glycine (100 μ L; 0.4 M; pH=10.7), and an absorbance reading was taken at 405 nm (Multiskan Ascent; ThermoFisher Scientific). The absorbance readings were used to calculate β -hexosaminidase release as a percentage of the total cellular β -hexosaminidase content (quantified using TritonX100 (0.1%)-induced cell lysis). The percentage spontaneous release of β -hexosaminidase was subtracted from all responses.

2.6 | Kinetics of CCL2 expression and release by LAD2 mast cells

IgE-sensitized LAD2 cells were activated with various stimuli in serum free IMDM for qPCR measurement (2×10^6 cells/mL; 4-hour stimulation) or CCL2 immunoassay (3.5×10^5 cells/mL; 24-hour stimulation). As above, where applicable, cells were either co-treated with stimuli and sugammadex or sugammadex was added at various time points following stimuli. The plates were then centrifuged (233 xg), supernatants removed and stored at -20°C until further use and cell pellets processed for RNA isolation. CCL2 levels were measured using a sandwich ELISA in accordance with manufacturer's instructions. The plates were developed using TMB substrate the reaction halted using sulfuric acid (0.2 M) and absorbance measured at 450 nm (Multiskan Ascent; ThermoFisher). Absorbance readings were translated to absolute concentrations of CCL2 using a standard curve. Absolute readings were then normalised to respective basal release of CCL2 (percentage of basal CCL2 levels).

RNA concentration and purity were quantified using a nanodrop spectrophotometer (Nanodrop 1000; Applied Biosystems). Diluted RNA (5 μL , containing 200 ng) was mixed with a reverse transcriptase master mix (5 μL) prepared according to manufacturer's instructions (ThermoFisher). The samples were incubated (37°C ; 2 hours) followed by reverse transcriptase denaturation (85°C for 5 minutes; Mastercycler[®] nexus gradient; Eppendorf). The resultant cDNA was further diluted 1:10 with RNase free water, and mixed (2 μL) with SSA Advanced[™] universal probes supermix (3 μL) containing TaqMan assays CCL2 (Hs01871556_s1), GAPDH (Hs02758991_g1) or UBC (Hs01871556_s1) in a 384 well thermocycler plate. Real-time quantitative PCR was carried out with 40 cycles of 95°C for 15 seconds followed by 60°C for 60 seconds (Quant Studio 6; Applied Biosystems). ΔCT was calculated by the subtraction of the sample cycle numbers from the average of the cycle number of two house-keeper genes used (UBC and GAPDH). $\Delta\Delta\text{CT}$ was calculated by the subtraction of resting expression levels of CCL2 from each sample.

2.7| Data Presentation and Statistical analysis

Flow cytometry data was analysed and represented in overlaid histograms using FlowJo_V10.0 . Other graphical representations (expressed as $n=x \pm \text{SEM}$) were plotted using Prism (version 9.1, GraphPad, San Diego, California, USA). Statistical significance was determined using either a one-way ANOVA with Dunnett's multiple comparison post hoc test or a two-way ANOVA with Bonferroni multiple comparison post hoc test (version 9.1, GraphPad PRISM), with levels of significance shown (where relevant) in figure legends.

3 | Results

3.1 | MRGPRX2 is required for NMBA-induced mast cell activation

All drugs produced calcium mobilization in the WT LAD2 cells with varying maximal effects (**Figure 1 A-D**). The calcium mobilization induced by rocuronium, atracurium and compound 48/80 was virtually eliminated in the LAD2 MRGPRX2 KD cells. MRGPRX2-dependency was also observed with cell degranulation where the MRGPRX2 KD cells stimulated with compound 48/80, substance P, ZINC-3573(R), atracurium, LL-37 and cortistatin-14 had significantly reduced degranulation compared to WT cells (**Figure 1E**). However, not all stimuli induced WT LAD2 degranulation. Rocuronium, whilst causing an induction of calcium mobilization, failed to trigger degranulation. Whilst ZINC-3573(R) triggered LAD2 cell degranulation, consistent with previous findings⁽¹¹⁾, its enantiomer ZINC-3573(S) did not (data not shown). As ZINC-3573(R) interfered with the FURA-2AM assay, we were unable to quantify the calcium responses to this ligand.

To investigate the effect of MRGPRX2 KD, if any, on IgE-mediated mast cell activation, the cells were incubated with NIP- specific IgE for 24-48 hours and then challenged with antigen (NIP-BSA). There was no decrease in equivalent IgE-mediated responses in LAD2 MRGPRX2 KD cells (**Figure 1D, E**).

3.2 | Sugammadex-mediated reversal of drug-induced LAD2 activation

Having established MRGPRX2-dependence in NMBA and other agonist-induced mast cell activation, we next aimed to determine if sugammadex could modulate these responses. We examined this by either co-treating cells with sugammadex (500 μ M) and the MRGPRX2 agonists or, to better mimic the possible clinical use of sugammadex in treating NMBA-induced anaphylaxis, adding it to the cells 2 minutes following the MRGPRX2 agonist. Calcium mobilisation was measured and supernatants from other experiments harvested for β -hexosaminidase and CCL2 quantification. In separate studies, sugammadex (500 μ M) was

shown not to mediate any direct cytotoxicity of LAD2 cells as measured by LDH release assays (data not shown).

Compared to agonist alone, co-treatment of cells with sugammadex significantly reduced rocuronium and compound 48/80 calcium mobilization responses (**Figure 2A, C**). Interestingly, although sugammadex is not known to reverse atracurium-induced nicotinic receptor block⁽²⁴⁾, it was able to impair atracurium-induced MRGPRX2- calcium mobilization but not as comprehensively as the rocuronium response (**Figure 2B**). Addition of sugammadex 40 seconds following LAD2 stimulation, also reduced the rocuronium, atracurium and compound 48/80-induced sustained calcium mobilization response (**Figure 2A-C**).

Degranulation induced by atracurium, compound 48/80 and ZINC-3573(R) was significantly diminished when the cells were co-treated with sugammadex (500 μ M) (**Figure 3A-C**). The inhibitory effects of sugammadex on degranulation induced by these stimuli was concentration-dependent with compound 48/80 being particularly sensitive to inhibition by sugammadex (**Figure S2A-C**). However, perhaps unsurprisingly given the rapidity of mast cell degranulation, there was no change in stimulus-induced degranulation when sugammadex was added 2 minutes post drug stimulation (**Figure 3A-C**).

Whilst sugammadex (500 μ M) produced a profound inhibition of atracurium and compound 48/80-induced degranulation, regardless of the MRGPRX2 agonist concentration tested, inhibition of the ZINC-3573(R) response was surmountable by addition of more agonist (**Figure S3A-C**). This suggests a likely lower affinity interaction between sugammadex and ZINC-3573(R). The ability to surmount was harder to test for atracurium, given its lower potency. However, evidence from experiments where the concentration of sugammadex was varied (Fig S2A), also suggest a lower affinity interaction between atracurium and sugammadex.

As cytokines are released from LAD2 cells with slower kinetics, we examined if the addition of sugammadex following addition of stimulus affected CCL2 release. Unlike degranulation,

rocuronium was able to trigger CCL2 release from LAD2 cells (**Figure 4A**). Interestingly, rocuronium, atracurium and compound 48/80-induced CCL2 levels were significantly reduced both when cells were co-treated with agonist and sugammadex, and when sugammadex was added 2 minutes following activation (**Figure 4A-C**). ZINC-3573(R)-mediated CCL2 release was significantly reduced when LAD2 cells were co-treated with sugammadex but whilst inhibited, did not reach statistical significance when sugammadex was added 2 minutes after the stimulus (**Figure 4D**).

Interestingly, while sugammadex significantly impaired MRGPRX2 activation induced by the endogenous peptide agonist LL-37 (**Figure S3G**), it did not impair the actions of substance P and cortistatin-14 (**Figure 2D, 3D, 4E, S2D, S3D, S3F**). This suggests that sugammadex may be capable of selectively influencing the activity of certain endogenous MRGPRX2 peptide agonists.

In calcium mobilisation studies (**Figure 2D**), the co-treatment of cells with sugammadex and substance P appeared to increase responses. However, peak Ca^{2+} levels were not statistically different to substance P alone and this enhancing effect was not seen when examining the actions of sugammadex on substance P-induced degranulation and CCL2 release (**Figure 3D, 4E**).

Throughout all our studies, as expected, sugammadex had no effect on IgE-dependent responses (**Figure 2E, 3E, 4F, S2E, S3E**).

3.3 | Determining the kinetics of the sugammadex-induced reduction in MRGPRX2 agonist-induced CCL2 release.

Having established an effect on cytokine release by the addition of sugammadex 2 minutes post LAD2 cell stimulation, we next aimed to determine how long the addition of sugammadex could be delayed and still produce an inhibition of mast cell CCL2 release.

In these studies, compound 48/80 (10 $\mu\text{g/mL}$) was used as the prototypic MRGPRX2 activator. Even 3 hours following stimulation with compound 48/80, addition of sugammadex (500 μM)

significantly reduced CCL2 levels in LAD2 cell supernatants (**Figure 5**). To better understand the mechanism behind this effect, qPCR studies measuring CCL2 transcript levels were conducted. Stimulation with compound 48/80 resulted in an approximate 5-fold induction of LAD2 cell CCL2 transcript levels. This was significantly reduced by co-treating cells with compound 48/80 and sugammadex or by addition of sugammadex up to 3 hours following cell stimulation (**Figure 5**).

4| Discussion

We have successfully developed a polyclonal LAD2 mast cell model where the MRGPRX2 gene was stably knocked down using CRISPR-Cas9 gene editing. Consistent with, but more pronounced in comparison to existing research using siRNA MRGPRX2 KD^(9,11), the CRISPR-Cas9 knock-down of the receptor significantly reduced the ability of rocuronium, atracurium, compound 48/80, ZINC-3573(R), substance P, LL-37, and cortistatin-14 to activate human LAD2 mast cells across a variety of measures. However, the MRGPRX2 gene disruption had no effect on FcεRI expression, the IgE/FcεRI pathway of mast cell activation nor on expression of another mast cell GPCR, CXCR4. Consistent with previous research^(9,25), the NMBA's rocuronium and atracurium produced MRGPRX2-dependent LAD2 cell calcium mobilisation, although the former NMBA did not trigger degranulation.

It is unclear as to why rocuronium is not degranulation-competent in LAD2 cells, but this observation has been made by several groups^(9,25,26). The calcium flux it induces in LAD2 cells is certainly more transient than is triggered by other MRGPRX2 activators or through the IgE/FcεRI pathway. In addition, calcium-independent contributors to degranulation, albeit with IgE-directed activation, have been identified in mast cells⁽²⁷⁾. Perhaps these additional pathways are poorly activated by rocuronium. Further work on MRGPRX2 signalling in response to rocuronium is required to better understand this. Such efforts might perhaps also provide an explanation as to why some rare individuals respond so dramatically and adversely to NMBAs.

Sugammadex is a negatively charged cyclodextrin drug designed to encapsulate the positively charged aminosteroid NMBAs such as rocuronium, thus preventing it from antagonising nicotinic receptors⁽²⁴⁾. However, whether this interaction prevents drug-induced activation of MRGPRX2 in mast cells has not been examined. This is of potential clinical significance as some case studies report the use of sugammadex in events of anaphylaxis, but with mixed outcomes⁽¹⁶⁻¹⁹⁾. We used an *in vitro* model to gain insight into the possible efficacy of sugammadex in treating MRGPRX2-dependent anaphylaxis. Firstly, the possible interaction of

sugammadex with the known MRGPRX2 agonists (rocuronium, atracurium, compound 48/80, substance P, ZINC-3573(R), LL-37, cortistatin-14) was tested by co-treating the cells with agonist and sugammadex. Secondly, the more clinically relevant model of anaphylaxis treatment during surgery was mimicked by the addition of sugammadex post agonist stimulation.

Sugammadex when co-administered with rocuronium, impaired the ability of the NMBA to induce LAD2 cell calcium mobilisation and CCL2 production. This presumably occurs through sugammadex encapsulating and thus shielding the positive charge on the NMBA that is necessary for MRGPRX2 activation⁽²¹⁾. Interestingly, this action is extended to other known MRGPRX2 agonists including compound 48/80, ZINC-3573(R) and the endogenous peptide agonist LL-37, but not to substance P or cortistatin-14. It is intriguing to consider sugammadex as a potential selective regulator of certain endogenous MRGPRX2 agonists, although the basis for this selectivity remains unclear. As expected, sugammadex had essentially no effect on IgE/FcεRI-dependent responses. Some recent clinical reports have suggested that a complex of rocuronium and sugammadex can itself induce anaphylaxis^(28, 29). Our data indicate that this is unlikely mediated by MRGPRX2 activation and is most likely an IgE-dependent mechanism as has been suggested^(28,29).

Even when given promptly (2 minutes) after MRGPRX2 stimulation, sugammadex was however ineffective in inhibiting mast cell degranulation, the primary cause of acute anaphylactic symptoms. This is not unexpected given the kinetics of degranulation that takes place within a few minutes post stimulus exposure. Therefore, assuming rapid distribution of a MRGPRX2-activating NMBA in the body, with consequent uniform activation of mast cells, it would seem unlikely that sugammadex administration would influence a clinically manifested MRGPRX2-dependent acute anaphylactic response.

Despite this, sustained calcium responses and the more delayed mast cell functional responses (*e.g.*, cytokine release) were inhibited by the later addition of sugammadex. Whilst

most patients suffer a rapid onset “uniphasic” anaphylactic event, others are seen to have a slower or “biphasic” reaction⁽³⁰⁻³²⁾. Biphasic reactions are characterized by symptoms (i.e., worsening of previous symptoms or new symptoms) that recur within 2 – 36 hours after the initial reaction⁽³¹⁾. These later stage symptoms could perhaps be partly mediated by the observed late phase release of mediators from mast cells⁽³⁰⁾. The delayed release of mediators (cytokine CCL2) in response to rocuronium, atracurium and compound 48/80 in LAD2 cells was significantly reduced by the addition of sugammadex following drug stimulation. For compound 48/80, inhibition of CCL2 release by sugammadex was observed even up to 3 hours post MRGPRX2 activation demonstrating that unlike degranulation, CCL2 release requires sustained MRGPRX2 stimulation. Whilst speculative, this suggests that sugammadex administration may indeed have some benefit in reducing more slowly emerging deleterious responses that result from delayed mast cell cytokine release by certain MRGPRX2 agonists.

Although compound 48/80 and ZINC-3573(R) have similar efficacy at the MRGPRX2 receptor, as measured by LAD2 degranulation, sugammadex was less able to inhibit the response of ZINC-3573(R). From this we suggest that the complex formed between sugammadex and ZINC-3573(R), is more transient and as such, may be less able to mediate the sustained block of MRGPRX2 activation necessary for significant CCL2 production. However, binding studies between these drugs are needed to establish this conclusively.

Interestingly, although sugammadex is not clinically used to reverse the neuromuscular block induced by atracurium⁽²⁴⁾, we have shown that it does significantly reduce atracurium-induced MRGPRX2-mediated mast cell activation. This suggests that a molecular interaction takes place between sugammadex and atracurium, that whilst not affecting the NMBA’s ability to block nicotinic receptors, is able to impair its activation of MRGPRX2. Interestingly, compared to degranulation and cytokine release, sugammadex was less able to inhibit the atracurium-induced Ca²⁺ response. The reason for this is as yet unclear, but could relate to the complex of atracurium and sugammadex showing biased ligand activity at MRGPRX2, retaining Ca²⁺ mobilisation activity but being deficient in triggering other signalling pathways

necessary to initiate mast cell functional responses. The concept of biased agonism is now well established with clinically relevant exemplars existing for multiple GPCRs including opioid receptors⁽³³⁾. Interestingly, given their sequence similarity, a molecular model of MRGPRX2 has been derived from crystallography data of the κ opioid receptor⁽¹¹⁾. Signalling studies using atracurium and the putative atracurium/sugammadex complexes would need to be carried out to validate the relevance of biased agonism at MRGPRX2.

The ability of sugammadex to influence ligands beyond its designed aminosteroid NMBA complement, is clearly demonstrated by its ability to neutralise the polybasic research tool compound 48/80. However, there is clear specificity in the actions of sugammadex, as it was inactive in preventing substance P-induced MRGPRX2 activation. Extended modelling, like that carried out for rocuronium⁽¹⁹⁾, on the interaction between sugammadex and these various MRGPRX2 agonists would be useful to help identify the structural basis for the differential effects of the γ -cyclodextrin.

Beyond the clinical implications of this study, the inhibition by sugammadex of ongoing MRGPRX2 responses to some agonists, suggests that it could also be a useful experimental tool. Analogous to the use of free hapten to terminate hapten-specific IgE-mediated Fc ϵ RI responses⁽³⁴⁾, sugammadex, through the rapid neutralisation of some MRGPRX2 agonists, could be used to examine MRGPRX2 receptor activity, signalling and desensitisation.

In summary, we have examined the effect of the aminosteroid NMBA-reversing agent sugammadex on MRGPRX2-dependent activation of mast cells. We have shown that sugammadex has no effect on mast cell degranulation when given after exposure to MRGPRX2 agonists including NMBAs, suggesting against its use in the acute management of non-IgE dependent anaphylaxis. However, sugammadex was able to inhibit release of the more slowly elaborated mast cell mediator CCL2, even with delayed administration. Thus, sugammadex may be of some potential benefit in preventing less well-defined symptoms of

drug hypersensitivity that are associated with the MRGPRX2-driven, delayed release of certain mast cell mediators.

Acknowledgements

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Tables

N/A

Figure Legends

Figure 1: Rocuronium, atracurium, compound 48/80, ZINC-3573(R), LL-37, cortistatin-14, and substance P-induced LAD2 cell stimulation is MRGPRX2 dependent. Calcium mobilization in response to a variety of MRGPRX2 agonists was quantified (Figure 1A-D) using the Ca^{2+} indicator Fura2. Addition of rocuronium (200 μM ; 1A), atracurium (150 μM ; 1B), compound 48/80 (10 $\mu\text{g}/\text{mL}$; 1C) and NIP-BSA (100 ng/mL ; 1D) all produced an increase in intracellular Ca^{2+} levels with all responses (except for NIP-BSA) being significantly reduced in the MRGPRX2 KD cells. A similar effect of MRGPRX2 KD was observed in degranulation studies with compound 48/80 (10 $\mu\text{g}/\text{mL}$), substance P (10 μM), ZINC-3573(R) (10 μM), atracurium (150 μM), LL-37 (5 μM), cortistatin-14 (5 μM) (Figure 1E), although rocuronium lacked a measurable degranulation response. Results shown are $N \geq 5$, means $\pm$ SEM. For statistical analysis (Figure 1E), a two-way ANOVA was conducted with Bonferroni multiple comparison post hoc test. Each MRGPRX2 KD response was compared to the respective response in the LAD2 WT cells. * $p < 0.01$

Figure 2: Sugammadex inhibits calcium mobilization induced by selected MRGPRX2 agonists in LAD2 cells. Calcium mobilization of LAD2 cells was monitored in real time using Fura2. Where relevant, cells were either co-treated with sugammadex and agonist ($t = 30$ seconds) or sugammadex was added 40 seconds following agonist drug addition ($t = 70$ seconds). Sugammadex addition significantly inhibited (* $p < 0.01$) calcium flux induced by rocuronium (100 μM ; 2A), compound 48/80 (1 $\mu\text{g}/\text{mL}$; 2C) with a less pronounced effect on atracurium (150 μM ; 2B). There was no effect of sugammadex on substance P (10 μM ; 2D) or NIP-BSA (100 ng/mL ; 2E) induced responses. Results shown are $N \geq 5$. Mean responses are shown with error bars removed to improve clarity. A two-way ANOVA was conducted, on the peak Ca^{2+} response, with Bonferroni multiple comparison post hoc test.

Figure 3: Sugammadex inhibits degranulation induced by selected MRGPRX2 agonists in LAD2 cells. Mast cell degranulation was quantified by the release of β -hexosaminidase. Where relevant, cells were either co-treated with sugammadex and MRGPRX2 agonist or sugammadex was added 2 minutes following agonist drug addition. Sugammadex, but only when added with agonist, inhibited atracurium (150 μM ; 3A), compound 48/80 (10 $\mu\text{g}/\text{mL}$; 3B) and ZINC-3573(R) (10 μM ; 3C) induced degranulation. The drug had no effect on substance P (10 μM ; 3D) and antigen (100 ng/mL ; 3E) induced degranulation. Results shown are $N \geq 6$, mean $\pm$ SEM. A one-way ANOVA was conducted with Dunnett's multiple comparison post hoc test. Each condition was compared to the respective response in the absence of sugammadex. * $p < 0.01$

Figure 4: Sugammadex inhibits CCL2 release induced by selected MRGPRX2 agonists in LAD2 cells. CCL2 was quantified using a commercial sandwich ELISA. Where relevant, cells were either co-treated with sugammadex and stimuli or sugammadex was added 2 minutes following stimulus addition. Sugammadex addition in both protocols, inhibited rocuronium (200 μ M; 4A), atracurium (150 μ M; 4B) and compound 48/80 (10 μ g/mL; 4C) induced increase in CCL2 levels. The effect on ZINC-3573(R) (10 μ M; 4D) was only observed when cells were co-treated with sugammadex and agonist. The drug had no effect on substance P (10 μ M; 4E) and antigen (100 ng/ml; 4F) -induced degranulation. Results shown are N $\geq$ 6, mean $\pm$ SEM. A One-way ANOVA was conducted with Dunnett's multiple comparison post hoc test. Each condition was compared to the respective response in the absence of sugammadex. * $p < 0.01$

Figure 5. Sugammadex inhibits compound 48/80-induced CCL2 release even when added up to 3 hours following LAD2 cell stimulation. LAD2 cells were stimulated with compound 48/80 (C48/80; 10 μ g/mL) and where applicable, either co-treated with sugammadex or sugammadex was added at various time points following compound 48/80 addition. Supernatant for CCL2 release quantification was obtained 24 hours following stimulation. Cell lysates for CCL2 mRNA expression quantification were harvested 4 hours following compound 48/80 addition. The inhibitory effects of sugammadex were dependent upon its time of addition, but were still manifest when it was added up to 3 hours following compound 48/80 addition. The effect of sugammadex on CCL2 release was paralleled by a concomitant inhibition of CCL2 transcript level. Results shown are N=5, with mean $\pm$ SEM. One-way ANOVA was conducted with Dunnett's multiple comparison post hoc test. Each time point was compared to the compound 48/80 response in the absence of sugammadex. * $p < 0.01$

Figure S1: Fluorescence activated cell sorting (FACS) of CRISPR-Cas9 MRGPRX2 KD LAD2 cells. CRISPR-Cas9 gene editing was used to selectively disrupt the MRGPRX2 gene. Gene targeted cells were stimulated with compound 48/80 (10 μ g/mL) (or NIP-BSA (antigen) (100 ng/mL)) for 15 minutes and MRGPRX2 low responding cells sorted using flow cytometry based on cell surface CD63 expression (Figure S1A). A polyclonal population of MRGPRX2 knock down cells were successfully established (Figure S1B). IgE-mediated responses were minimally affected by MRGPRX2 gene-targeting (Figure S1C). While cell surface expression of MRGPRX2 was significantly reduced in the LAD2 MRGPRX2 KD model (Figure S1D), the expression of Fc ϵ R1 α (Figure S1E) and CXCR4 (Figure S1F) was unaffected.

Figure S2: Concentration-dependent inhibition of atracurium, compound 48/80 and ZINC-3573(R)-induced LAD2 degranulation by sugammadex. LAD2 cells were co-treated with various concentrations of sugammadex and a fixed concentration of stimulus. Degranulation was quantified by the release of β -hexosaminidase. Baseline (BL) degranulation induced by the MRGPRX2 agonist was also measured in the absence of sugammadex. There was a concentration-dependent inhibition by sugammadex of LAD2 mast cell degranulation induced by compound 48/80 (10 μ g/mL; S2A) and to a lesser extent ZINC-3573(R) (10 μ M; S2B) and atracurium (150 μ M; S2C). Sugammadex had no effect on substance P (10 μ M; S2D) or NIP-BSA (100 ng/mL; S2E) -induced degranulation. Results shown are $N \geq 5$, mean $\pm$ SEM. One-way ANOVA was conducted with Dunnett's multiple comparison post hoc test with each measurement compared to that of BL; * $p < 0.05$, ** $p < 0.01$.

Figure S3: Sugammadex has varying activity on mast cell activation induced by different MRGPRX2 agonists. LAD2 degranulation was quantified by the release of β -hexosaminidase. The cells were co-treated with sugammadex (500 μ M) and a concentration range of stimuli. Sugammadex inhibited degranulation induced by atracurium (Figure S3A), compound 48/80 (Figure S3B), ZINC-3573(R) (Figure S3C) and LL-37 (Figure S3G), but with varying potencies. Sugammadex had no effect on substance P (Figure S3D), cortistatin-14 (Figure S3F) or NIP-BSA (Figure S3E) -induced degranulation. Results shown are $N \geq 5$, mean $\pm$ SEM.

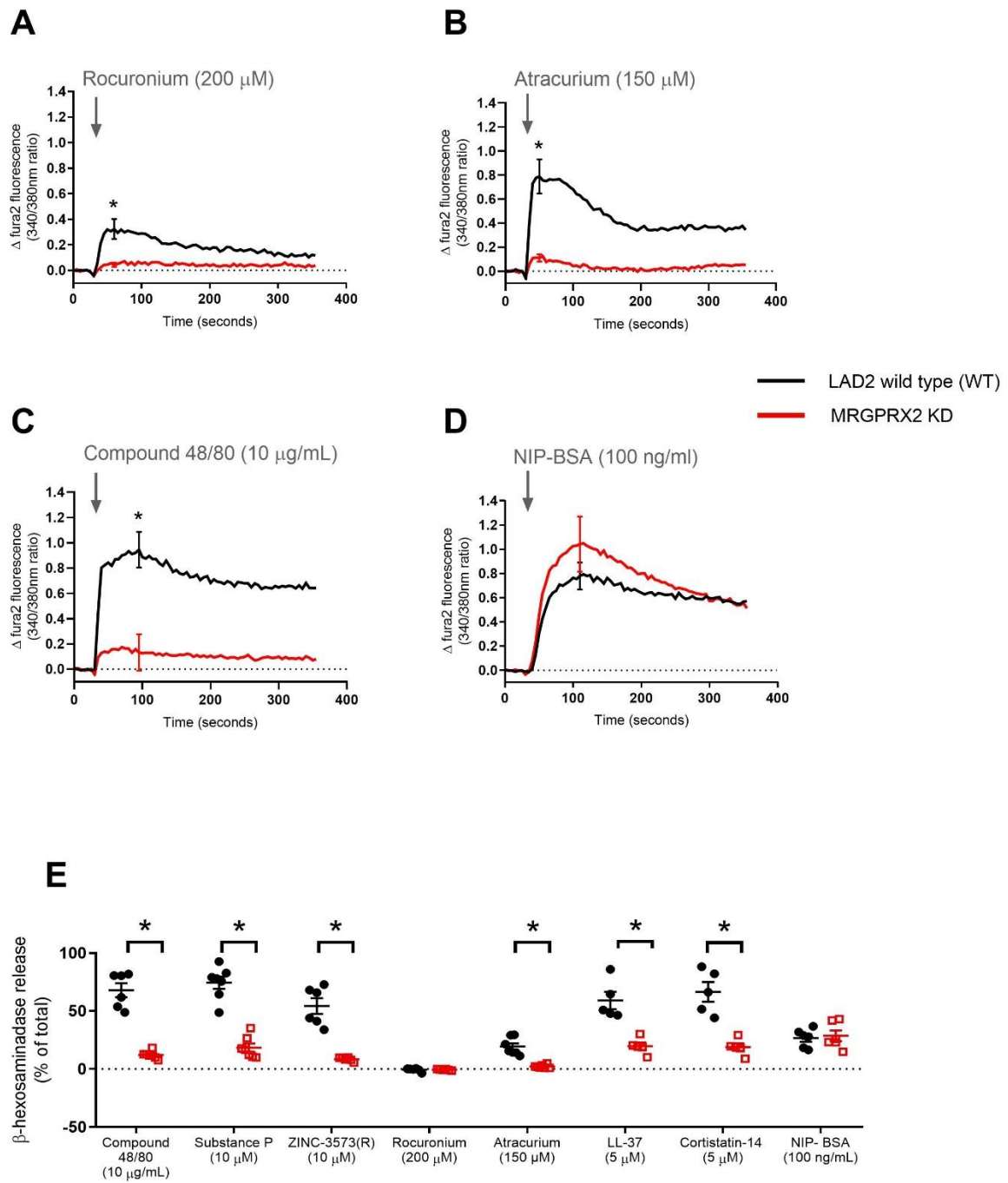
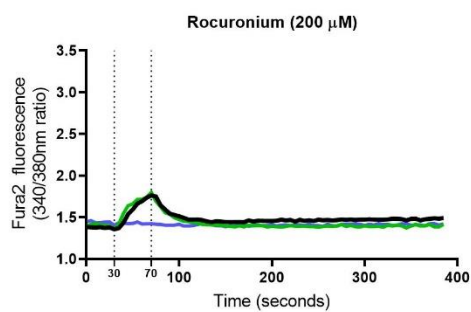
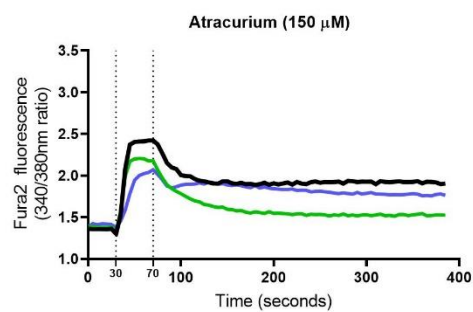
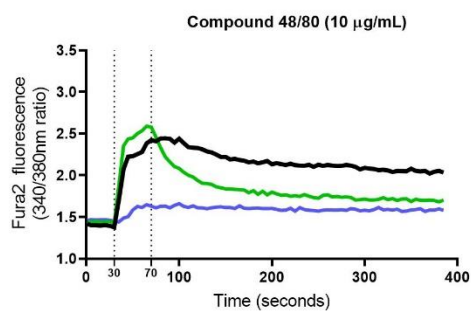
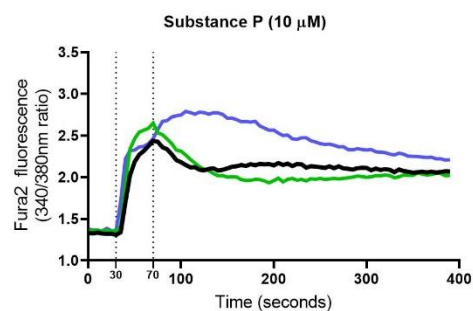
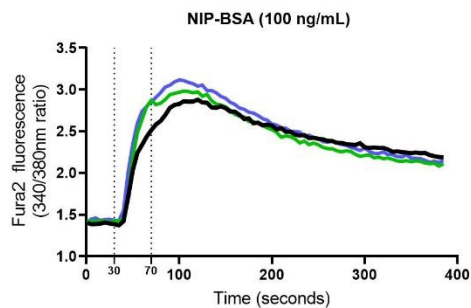
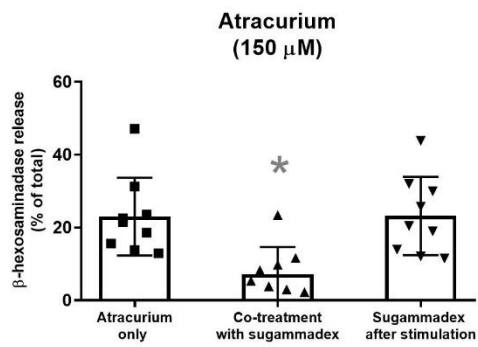
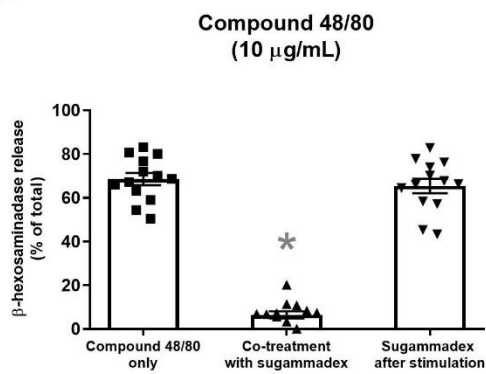
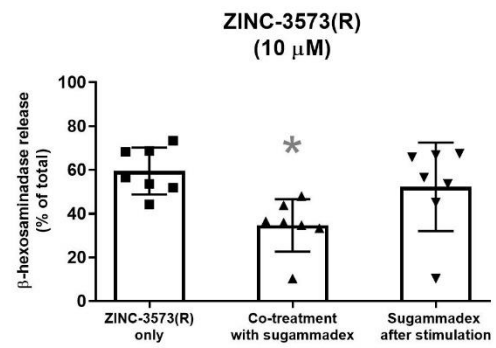
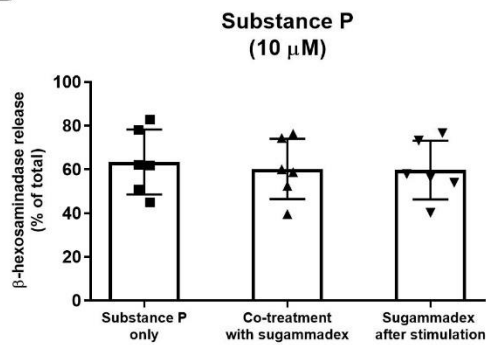
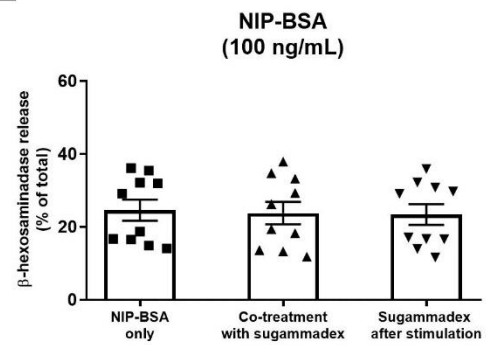


Figure 1

A**B****C****D****E**

— Stimulus only (no sugammadex)
 — Sugammadex added **after** stimulus (post-incubation)
 — Sugammadex **co-treatment** with stimulus

Figure 2

A**B****C****D****E****Figure 3**

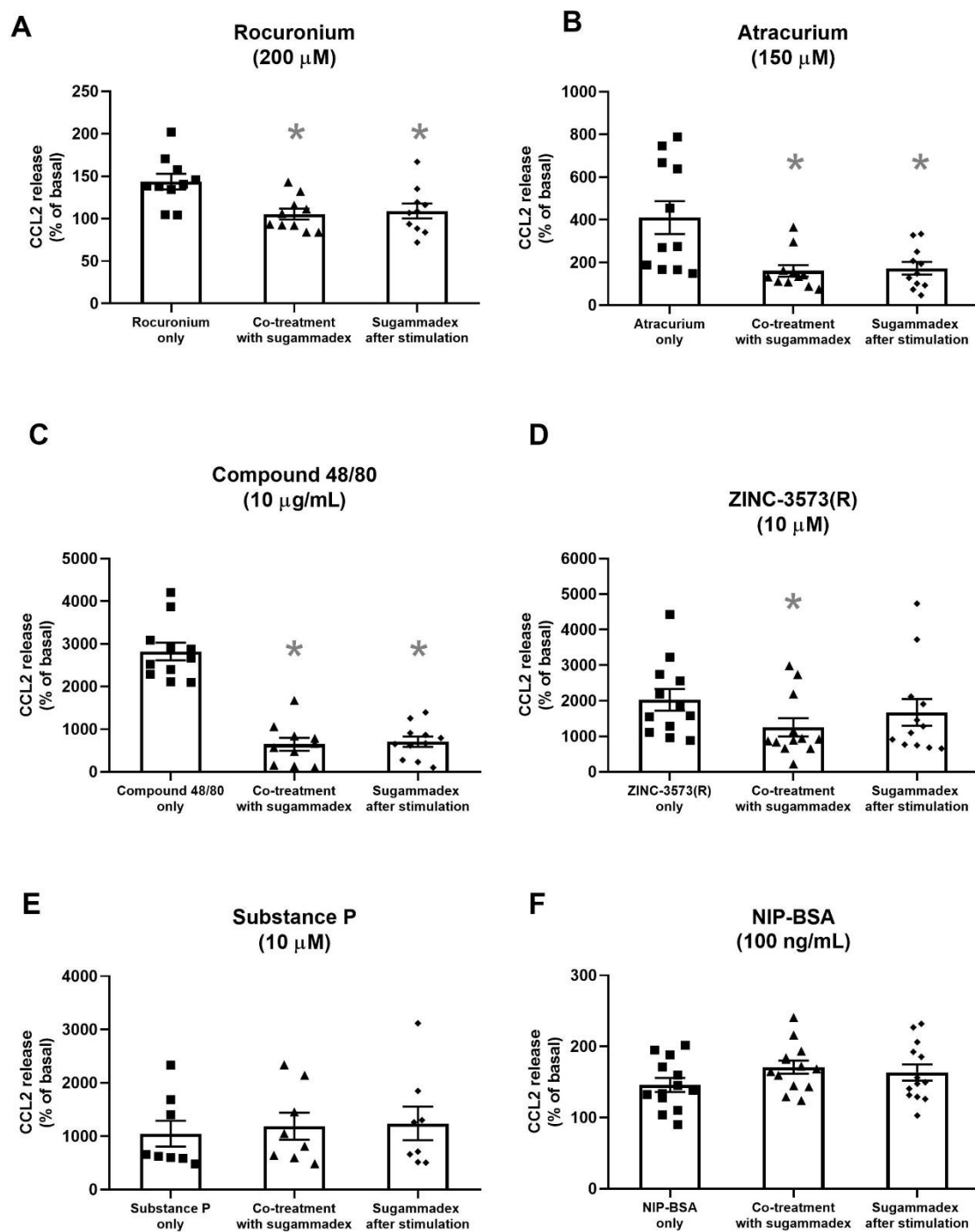


Figure 4

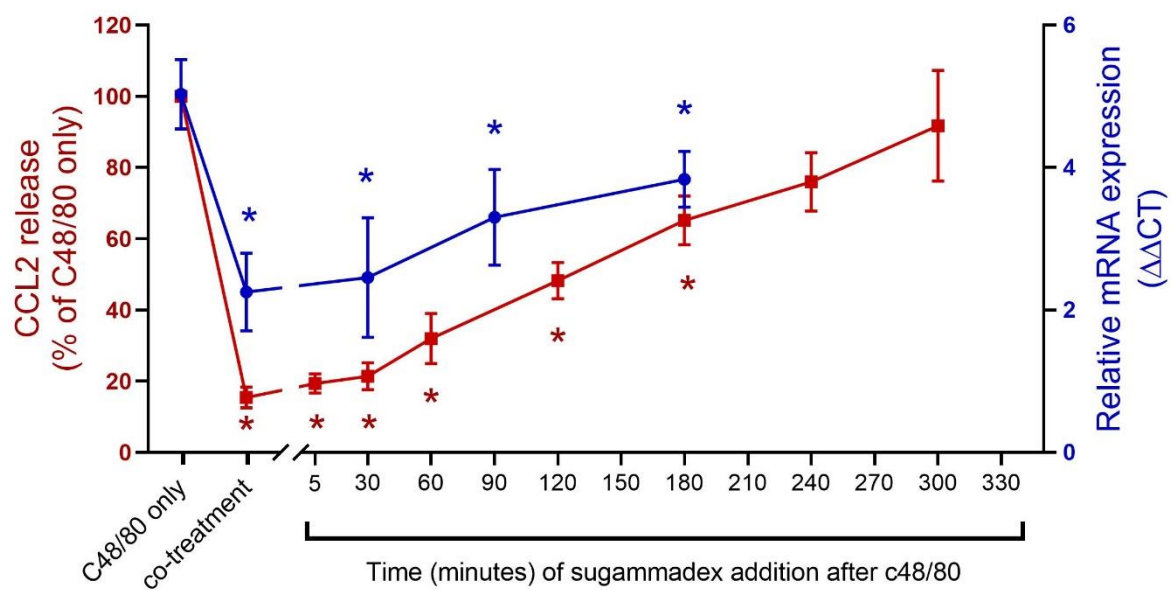


Figure 5

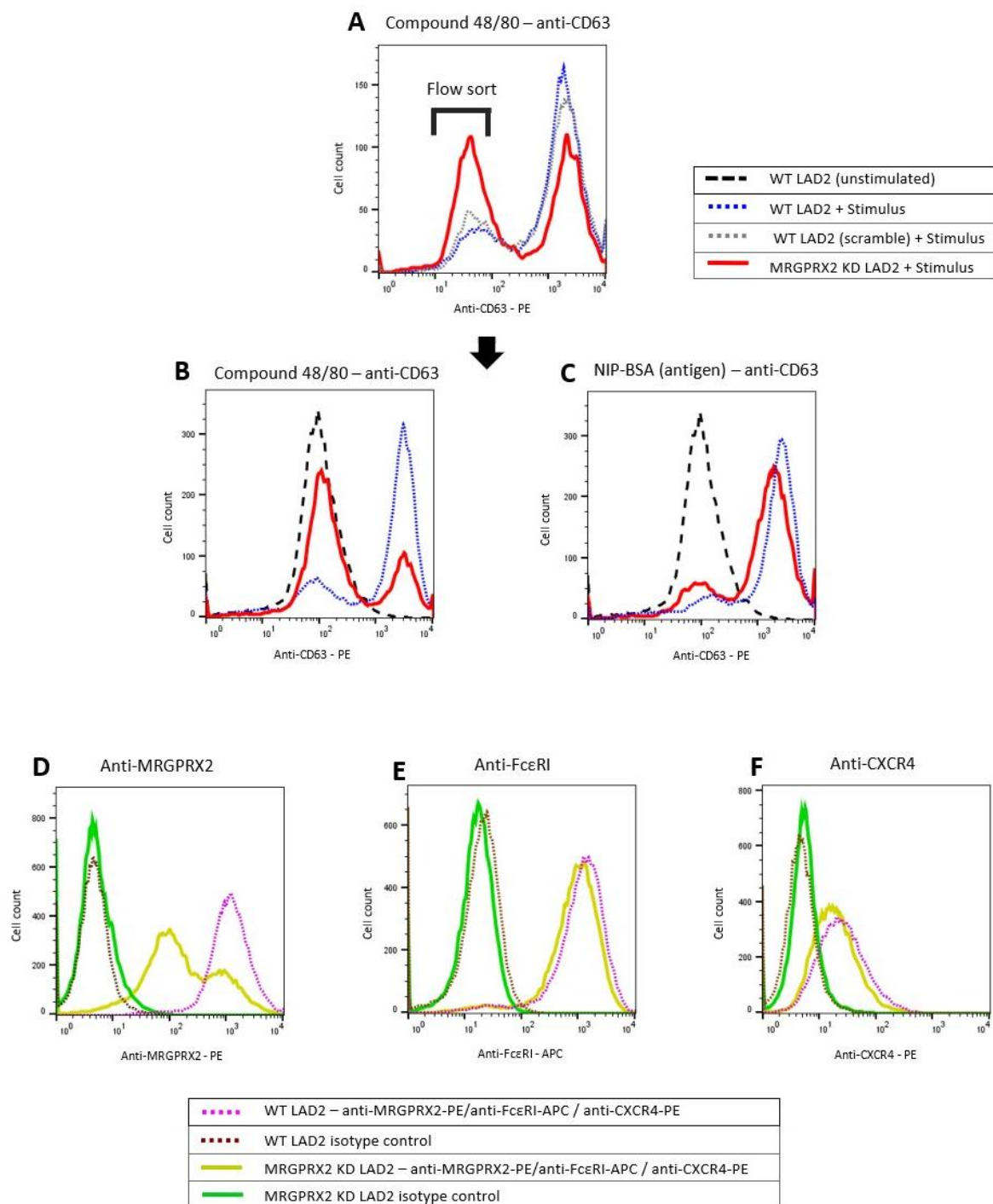


Figure S1

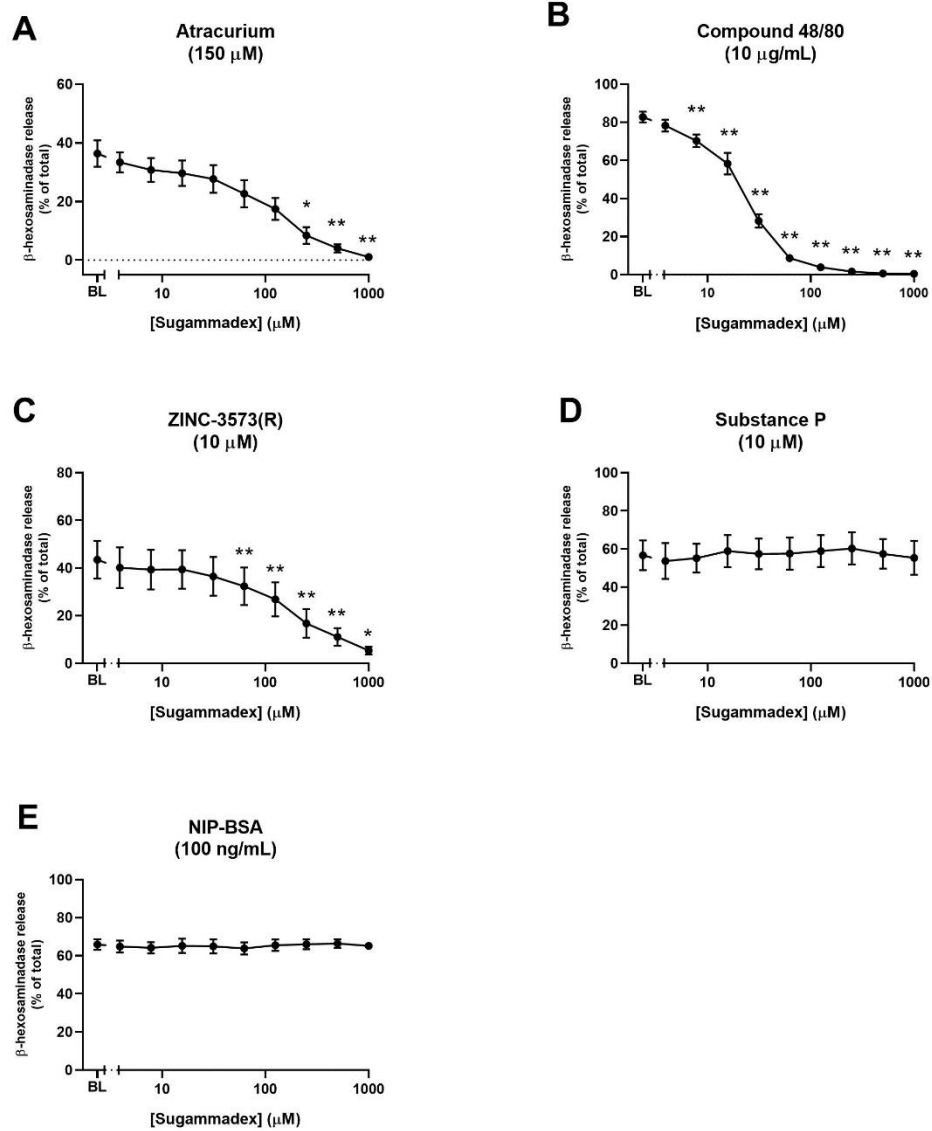


Figure S2

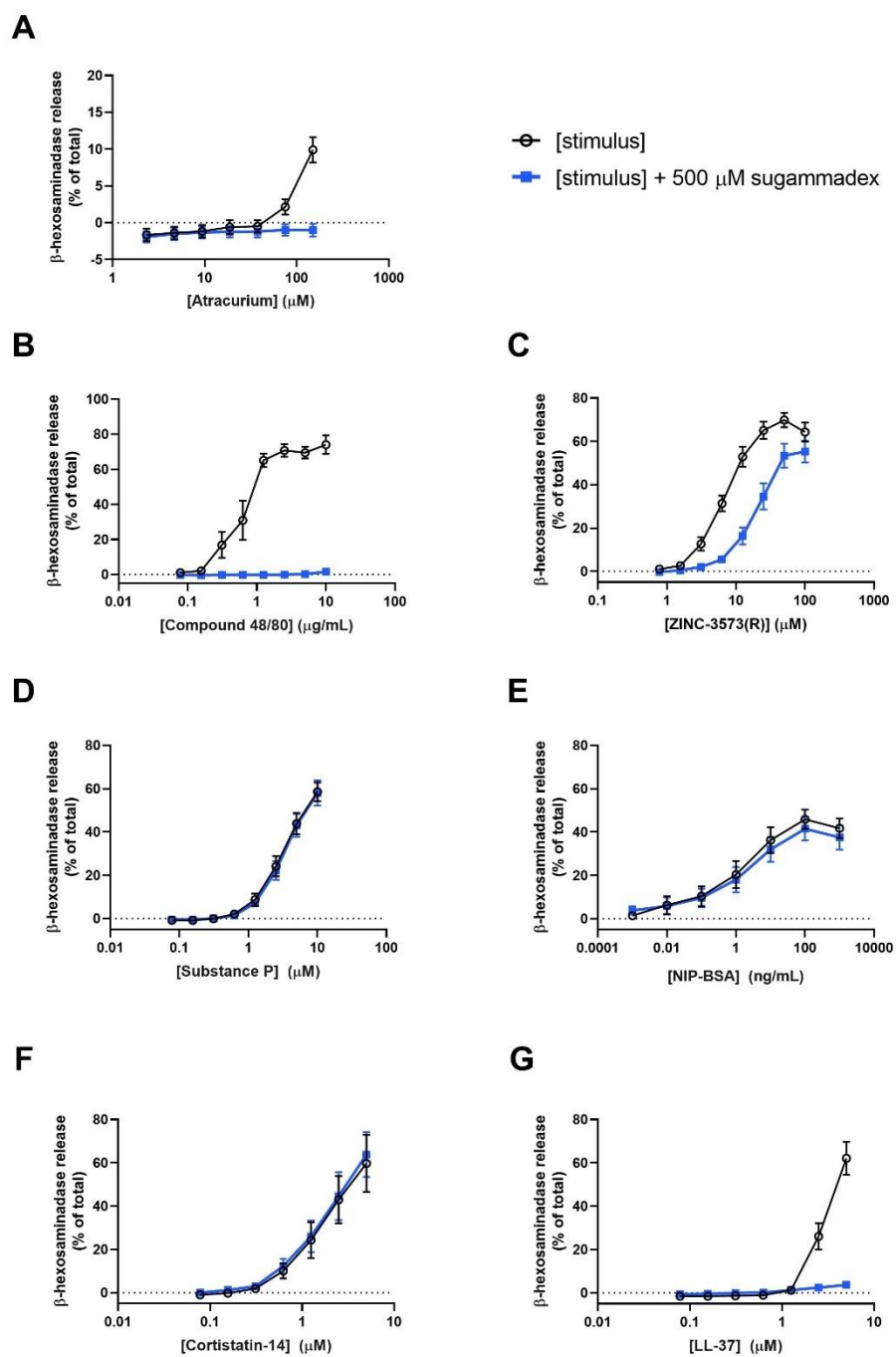


Figure S3

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3.3.2 Manuscript 2: Direct protamine activation of human mast cells is MRGPRX2-dependent and modulated by heparin

As detailed in the manuscript below, this study demonstrates that protamine activates MRGPRX2 to induce direct human mast cell activation, therefore potentially linking MRGPRX2 with protamine-induced non-IgE dependent anaphylaxis. We also demonstrate the ability of heparin (the counter ligand of protamine) to mask this protamine-induced mast cell activation. This study draws cautious attention to the clinically advised dosage ratios of protamine to heparin, along with how excess protamine may evoke adverse reactions.

This manuscript will soon be submitted to European Journal of Pharmacology, and has been cited in this chapter as “Fernandopulle et al., 2021”.

Manuscript 2 (to be submitted for publication):

Direct protamine activation of human mast cells is MRGPRX2-dependent and modulated by heparin.

Article title: Direct protamine activation of human mast cells is MRGPRX2-dependent and modulated by heparin.

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Prior Presentations: NA

Word and Element Counts: 2,234 words; 3 Figures; 3 Supplementary figures with one containing 3 video files

Abbreviated Title (Running Head): Protamine activation of MRGPRX2

Summary Statement (35 words): Protamine-induced anaphylaxis, although rare, is of significant concern. Here we show that protamine can activate mast cells via MRGPRX2, an effect attenuated by heparin. This provides further impetus for consideration of clinically used protamine:heparin ratios.

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Conflicts of Interest: The authors have no conflicts of interest to declare

Abbreviations:

MRGPRX2 – Mas-related G protein coupled receptor X2

WT- wild type

KD- knock down

CRISPR- clustered regularly interspaced short palindromic repeats

IgE- Immunoglobulin E

BSA- bovine serum albumin

NIP-BSA- 4-hydroxy-3-iodo-5-nitrophenylacetyl bovine serum albumin

CPB- cardiopulmonary bypass

FACS- Flow Associated Cell Sorting

Abstract

Background: Protamine is a polybasic drug commonly used during cardiac surgery to chemically antagonise, and thereby reverse, the anti-coagulant effect of heparin. In some cases, protamine can trigger severe anaphylaxis with both IgE and non-IgE dependent mechanisms implicated. New research has identified the receptor MRGPRX2 in IgE-independent mast cell activation by polybasic drugs, but the effect of protamine on this receptor has not been formally investigated.

Aims: To investigate the role of MRGPRX2 in protamine-induced mast cell activation and examine the effect of heparin on this activation and MRGPRX2 activation by other agonists.

Methods: We used an *in vitro* cultured human mast cell line, LAD2, that constitutively expresses the MRGPRX2 receptor along with a MRGPRX2 knock down (KD) LAD2 cell line. As markers of mast cell activation, intracellular calcium mobilisation was measured using FURA-2 and cellular degranulation quantified through β -hexosaminidase release.

Results: Protamine produced a concentration-dependent mast cell activation that was significantly reduced by MRGPRX2 knock-down. Heparin produced a concentration-dependent elimination of protamine's effect and also inhibited compound 48/80 and LL-37-mediated mast cell activation but had no effect on other MRGPRX2 agonists tested. Extrapolating the common ratios of protamine:heparin used clinically, revealed that protamine levels were still sufficient to trigger effective LAD2 mast cell degranulation.

Conclusions: This study demonstrates that protamine can trigger mast cell degranulation by activating the MRGPRX2 receptor, and that this response is attenuated when the drug is complexed with heparin. As a recommended clinically advised ratio of protamine to heparin still triggered MRGPRX2-dependent mast cell activation, this further supports optimising

protamine administration as a means of potentially reducing the risk of protamine-induced adverse effects. Heparin also attenuated the actions of certain endogenous agonists of MRGPRX2 which suggests that this action might, at least in part, contribute to the reported anti-inflammatory effects of the anti-coagulant agent.

1| Introduction

Protamine is primarily used to reverse the anti-coagulant activity of heparin, particularly in cardiac surgery involving cardiopulmonary bypass (CPB) and in clinical settings of heparin overdose^{1,2}. Protamine is a positively charged peptide isolated from salmon sperm that interacts with the negative charge on heparin to form a complex. This polyanionic-cationic binding neutralizes the anti-coagulant activity of heparin enabling rapid and safe separation from CPB¹.

Alongside neuromuscular blocking agents and antibiotics, protamine remains a significant cause of anaphylaxis in cardiac surgery, triggering profound cardiac collapse^{3,4}. Investigations into causative mechanisms of anaphylaxis to protamine has provided evidence for both IgE-dependent and IgE-independent reactions⁵. IgE-dependent anaphylaxis to protamine has been relatively well studied and risk factors have been characterised. High risk individuals include diabetic patients medicated with protamine-zinc-insulin, and infertile or vasectomized men who have developed anti-sperm antibodies^{3,5,6}. However, the mechanism of non-IgE mediated anaphylaxis to protamine is not well characterised, and speculation remains as to whether direct mast cell activation is a primary mechanism⁴.

New research has identified the receptor MRGPRX2 as a possible key player in non-IgE mediated anaphylaxis⁷. Agonists at this receptor are known to have polybasic structures and include clinically used drugs such as certain neuromuscular blocking agents, antibiotics, and opioids as well as endogenous peptides such as substance P, and laboratory tools including compound 48/80 and ZINC-3573(R)^{8,9}. Protamine has been shown to directly activate mast cells^{10,11} and, given its polybasic structure, this response could plausibly be mediated via MRGPRX2 activation.

In this *in vitro* study, we aimed to determine if the non-IgE-dependent mast cell activation by protamine was indeed MRGPRX2-dependent, and also examine if this activation was modulated by heparin.

2| Methods

2.1| Materials

The following products were purchased from Sigma Aldrich (Castle Hill, New South Wales, Australia); ZINC-3573(R), ciprofloxacin, Triton-X100, Hank's buffered salts, sodium bicarbonate, HEPES, glucose, BSA, MgSO₄, CaCl₂, round and flat bottom 96 well plates, probenecid, and glycine. Compound 48/80 was obtained from Enzo Life Sciences (Farmingdale, New York, United States) or Sigma. LL-37 and cortistatin-14 were purchased from Cayman Chemical Company (Ann Arbor, Michigan, United States). Protamine and heparin were obtained from the Royal Melbourne Hospital pharmacy (Melbourne, Victoria, Australia). NIP-BSA (4-hydroxy-3-iodo-5-nitrophenylacetyl bovine serum albumin) was purchased from Biosearch Technologies (Novato, California, United States). Substance P was synthesised and purified (>95% purity) by Ms. Lin Hsin and Dr. Susan Northfield (Department of Biochemistry and Pharmacology, University of Melbourne, Australia). 8-well chamber slides were purchased from IBIDI (Lochhamer Schlag, Gräfelfing, Germany). The following products were purchased from ThermoFisher Scientific (Scoresby, Victoria, Australia); StemPro™-34, glutaMAX™, and penicillin (5000 unit/mL)/streptomycin (5000 µg/mL). Stem cell factor was purchased from Peprotech (Rocky Hill, New Jersey, USA). PNAG (p-nitrophenyl-N-acetyl-β-D-glucosaminide) was purchased from Glycosynth (Warrington, United Kingdom). FURA-2AM was purchased from Abcam (Melbourne, Victoria, Australia). Percoll® was purchased from GE healthcare. Human anti-FcεRIα-APC (clone Cra-1) and anti-MRGPRX2-PE (clone K125H4), along with appropriate isotype controls, were purchased from BioLegend (San Diego, California, United States).

2.2 | Culture of LAD2 mast cells

LAD2 cells¹² were kindly provided by Dr. Arnold Kirshenbaum (National Institute of Health, Bethesda, United States). The cells were cultured in StemPro™-34 medium supplemented with stem cell factor (100 ng/mL), glutaMAX™ (1X) and penicillin (50 Units/mL)/streptomycin (50 µg/mL), and maintained in a humidified incubator (5% CO₂, 37°C).

2.3 | Generating MRGPRX2 Knock-down (KD) LAD2 cells

MRGPRX2 KD LAD2 cells were generated through CRISPR-Cas9 technology and sorted initially on a CD63^{low} population following activation with compound 48/80 (BD FACS ARIA III; BD Biosciences)¹³. With the availability of a MRGPRX2 antibody, we have generated a more refined MRGPRX2 KD cell line via further flow cytometry sorting on a MRGPRX2^{low} population (**Figure S1**). As in our hands, LAD2 cells were not able to be grown clonally, the mixed cell population are termed knock-down (KD) cells. As shown in **Figure S1**, MRGPRX2 expression in the KD cell line was significantly lower than that of wild type LAD2 cells whilst FcεRI expression was unchanged.

2.4 | Quantifying real-time calcium mobilisation in LAD2 cells

LAD2 cells were sensitized with human NIP-specific IgE (1-2 µg/mL; conditioned media from Jw8/5/13; ECACC hybridoma collection) for a period of 24-48 hours. The cells were then incubated with FURA2-AM (2 µM) in Hank's buffered saline solution (HBSS) (1X Hank's buffered salts, sodium bicarbonate (0.14%), HEPES (10 mM), glucose (5.5 mM), BSA (bovine serum albumin) (0.05%), MgSO₄ (0.7 mM), CaCl₂ (1.8 mM)) supplemented with probenecid (2.5 mM) at a density of 5 x 10⁶ cells/mL for 90 minutes at 37°C. Cells were then washed and seeded into 96-well plates and, following a 30 second baseline reading, protamine (3.75-30 µg/mL) (independently or in premixed complexes with heparin (375 - 3000 mUSP units/mL))

was injected into wells and ratiometric recordings of fluorescence emission measurements (excitation 340/380 nm; emission 510 nm) taken every 5 seconds for 6 minutes (Flex station III; Molecular Devices, San Jose, California, USA). Where appropriate, PRISM (version 9.1, GraphPad PRISM) was used to determine peak fluorescence emission values.

2.5 | Quantification of LAD2 degranulation by β -hexosaminidase release

β -hexosaminidase, an enzyme found within intracellular granules of mast cells is a commonly used marker of degranulation. IgE-sensitized LAD2 cells were washed and resuspended in HBSS and seeded onto 96 well plates. Protamine (3.75 - 30 μ g/mL), compound 48/80 (0.5 μ g/mL), ZINC-3573(R) (5 μ M), atracurium (200 μ M), ciprofloxacin (50 μ g/mL), substance P (1 μ g/mL) and NIP-BSA (10 ng/mL) (independently or in premixed complexes with heparin (0.070 – 10 USP units/mL)) were prepared and added to respective wells for 45 minutes. Following this, the plates were centrifuged, cell-free supernatant (25 μ L) removed to a fresh plate and pNAG substrate (75 μ L; 2 mM, in phosphate-citrate buffer pH=4.5) added. The plate was subsequently incubated for 120 minutes at 37°C in an oscillating incubator (70 RPM). The reaction was halted by the addition of glycine (100 μ L; 0.4 M; pH=10.7), and an absorbance reading taken at 405 nm (Multiskan Ascent; ThermoFisher Scientific). The absorbance readings were used to calculate β -hexosaminidase release as a percentage of the total β -hexosaminidase content (quantified using Triton-X (0.1%) induced cell lysis). The percentage spontaneous release of β -hexosaminidase was then subtracted from these values.

2.6 | Isolation of rat peritoneal mast cells (RPMCs) and live cell imaging

Male Sprague-Dawley rats (9-12 weeks old) were obtained through The University of Melbourne, animal ethics committee approved secondary use procedure and peritoneal mast cells isolated using a Percoll® density gradient method as previously described¹⁴. The isolated cells (>95% purity) were then carefully resuspended in HBSS. A portion of these cells were loaded into an 8-well chamber slide for live cell imaging (SP5 microscope; Leica microsystems, Wetzlar, Germany). Images were taken every 2 seconds for up to 60 seconds following stimulus addition and compiled into a video using Image J (FIJI; National Institute of Health, Bethesda, Maryland, USA). Remaining cells (35,000/well) were seeded into round bottom 96 well plates and activated with the various stimuli and degranulation quantified using the β -hexosaminidase assay as above.

2.7 | Statistical analysis

Data are expressed as means $\pm$ SEM of n experiments and are drawn using Prism (version 9.1, GraphPad PRISM). Statistical significance was derived using one-way ANOVA with Dunnett's multiple comparison post hoc test or two-way ANOVA with Bonferroni multiple comparison post-hoc test (version 9.1, GraphPad PRISM). * $p < 0.01$

3| Results

3.1| Role of MRGPRX2 in protamine-mediated mast cell activation

Protamine (30 µg/mL) induced degranulation in LAD2 WT mast cell (**Figure 1A**), which was significantly attenuated by the knockdown of MRGPRX2 in LAD2 cells. Compound 48/80 and ZINC-3573(R), both known agonists of MRGPRX2, displayed a similar trend with significantly reduced activation in MRGPRX2 KD cells (**Figure 1A**). The IgE-dependent pathway of mast cell activation was unchanged in MRGPRX2-KD cells.

Protamine (15 µg/mL) also induced significant degranulation of RPMCs, similar to that of compound 48/80 (5 µg/mL) (**Figure 1B, S2A**).

3.2| Effect of heparin on protamine-induced MRGPRX2 activation

We next examined the actions of heparin (the therapeutic counter-ligand for protamine) on protamine-induced mast cell activation. In both Ca²⁺ mobilisation and degranulation studies in LAD2 cells, heparin produced a concentration-dependent inhibition of protamine's effects visualised by the rightward shift of the protamine concentration-response curve (**Figure 2**). However, at the recommended clinical 1:1 ratio of protamine:heparin, equivalent to 10 µg:1 USP unit (shown by symbols coloured in red), there remained mast cell activation (**Figure 2**). These results are consistent with the visual observation of RPMC degranulation captured in real-time (**Figure S2**) where heparin again inhibits protamine-induced degranulation. Similar to the results using LAD2 WT cells where β-hexosaminidase was measured, there was no observable degranulation following protamine addition when heparin was in excess (**Figure S2C**).

3.3 | Effect of heparin on other exogenous and endogenous MRGPRX2 agonists

In these studies, a submaximal concentration of each stimulus was premixed with a range of heparin concentrations prior to addition to LAD2 cells. As expected, the protamine-induced LAD2 degranulation was significantly, and concentration-dependently, impaired when co-incubated with heparin (**Figure 3**). Degranulation induced by compound 48/80, a known polybasic activator of MRGPRX2, was very effectively inhibited when co-incubated with heparin (**Figure 3**), whilst LAD2 cell degranulation induced by other MRGPRX2 agonists, ZINC-3573(R), and ciprofloxacin, and the FcεRI agonist NIP-BSA, were unaffected when co-treated with heparin (**Figure 3**). Heparin also reduced the activation of MRGPRX2 by the endogenous host defence peptide, LL-37, but not the neuropeptides cortistatin-14 and substance P under the conditions tested (**Figure 3, S3**).

4| Discussion

We have demonstrated that human mast cell degranulation by protamine can occur through direct activation of the MRGPRX2 receptor. In keeping with stoichiometric chemical antagonism between the positively charged protamine and negatively charged heparin, this effect was attenuated in a concentration-dependent manner when protamine was pre-incubated with heparin.

Following CPB, heparin neutralisation requires careful titration since inadequate dosing may result in significant post-operative bleeding and subsequent return to theatre¹. However, excess protamine administration may itself cause coagulopathy and post-operative bleeding^{1,15}. Determining the dose of protamine to best and most safely reverse the anti-coagulant activity of heparin is an area of variability (depending on clinical centre) and continues to be debated¹⁶. Whilst guidelines recommend administration of 1-1.5 mg protamine per 100 USP units of heparin^{1,17,18}, in practice, sequential measurements of activated clotting time further inform protamine dosing^{16,19}. In addition, there has been recent interest in managing cases of protamine anaphylaxis requiring rescue CPB², where the safety of protamine re-exposure following patient stabilisation remains a concern, as outlined recently¹⁵.

Our *in vitro* data indicate that in clinical situations where protamine is in excess, there would be an increase in the likelihood of MRGPRX2 activation, and in susceptible individuals, potentially anaphylaxis. Given that the incidence of protamine anaphylaxis is rare (0.6-1%) and that we currently do not know the factors, pharmacogenomic or other, that render certain individuals susceptible to drug-induced, MRGPRX2-dependent, mast cell activation, the importance of this mechanism to clinically-manifested anaphylaxis to protamine is

speculative. Complexity in the *in vivo* heparin/protamine dose relationship is further compounded by pharmacokinetic considerations of both drugs. Nevertheless, our data support the likely benefit, in terms of reduced risk of IgE-independent anaphylaxis, of the careful use of protamine in neutralising heparin's activity, as has been suggested in other studies^{16,17}. This extends not just to dose but also to the rate of protamine administration, which has additionally been associated with some cases of anaphylaxis ⁴.

A large number of MRGPRX2 agonists have recently been identified which span endogenous peptides like substance P and LL-37, therapeutic drugs such as ciprofloxacin and rocuronium, and laboratory tools such as compound 48/80 and ZINC-3573(R)^{7,9,20,21}. Most MRGPRX2 agonists, like protamine, share a common property of being polybasic compounds. Given our observation of heparin-mediated antagonism of protamine-induced MRGPRX2 activation, we extended our research to investigate the effect of heparin on other MRGPRX2 agonists. Heparin, as mentioned earlier, is a negatively charged glycosaminoglycan, which is an abundant component of the mast cell granular matrix and released upon degranulation²². Heparin is known to have anti-inflammatory properties²³, and so it is plausible that a component of this effect might relate to neutralisation of endogenous MRGPRX2 agonists. Our data shows that heparin inhibits MRGPRX2 activation by certain polybasic compounds, like compound 48/80 and the endogenous host defence peptide, LL-37, but had no effect on other agonists including ZINC-3573(R), ciprofloxacin and the endogenous neuropeptides, substance P and cortistatin-14.

The charge/charge interaction between protamine and heparin is well characterised^{1,18}. However, our results show this is not necessarily a universal simple interaction with other MRGPRX2 agonists, but rather displays selectivity. Although complex formation with heparin

may successfully mask the essential binding sites of protamine and compound 48/80, preventing them from interacting with the specific MRGPRX2 amino acids necessary for receptor activation, the interaction of other basic stimuli with heparin might lack affinity or avidity or may not mask the MRGPRX2-interacting sites of the agonist. Further work using modelling and/or binding studies are required to better understand this observation and determine if indeed neutralization of endogenous polybasic compounds underpins at least part of the observed anti-inflammatory actions of heparin. Intriguingly, heparin's ability to interact with basic residues of the SARSCoV-2 spike protein has been recently examined as a therapy for Covid-19²⁴.

In conclusion, this study has shown that protamine directly activates human mast cells through MRGPRX2. Heparin was found to attenuate this response, highlighting the importance of the protamine/heparin ratio as a potentially important factor in the initiation of anaphylaxis. Furthermore, we show that heparin is capable of inhibiting the action of some MRGPRX2 agonists but not others. Further research is needed to determine if exogenous, or indeed endogenous, heparin might exert some of its actions (*e.g.*, anti-inflammatory) via neutralisation of polybasic activators of MRGPRX2.

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Figure legends

Figure 1: Protamine-induces LAD2 mast cell degranulation via MRGPRX2. **A)** LAD2 wild type (LAD2 WT) and LAD2 MRGPRX2 knock down (MRGPRX2 KD) cells were stimulated with protamine (30 µg/mL), compound 48/80 (10 µg/mL), ZINC-3573(R) (3 µg/mL), and NIP-BSA (100 ng/mL). All stimuli induced significant LAD2 degranulation, with all responses (except NIP-BSA) significantly reduced in the MRGPRX2 KD cell model. $N \geq 5$, mean $\pm$ SEM. For statistical analysis, a two-way ANOVA was conducted with Bonferroni multiple comparison post hoc test. Each MRGPRX2 KD response was compared to the respective response in the LAD2 WT cells. * $p < 0.01$. **B)** Rat peritoneal mast cells (RPMCs) were isolated from male Sprague-Dawley rats using Percoll® gradient separation, and stimulated with protamine (15, 7.5, 3.75 µg/mL) and compound 48/80 (5 µg/mL). $N=5$, mean $\pm$ SEM. For both figures, degranulation was quantified by the release of the enzyme β -hexosaminidase.

Figure 2: Neutralisation of protamine-induced mast cell activation by heparin. WT LAD2 cells were stimulated with premixed complexes of protamine (3.75, 7.5, 15, 30 µg/mL) and heparin (0.375, 0.75, 1.5, 3 USP units/mL). Measurements in the absence of protamine are denoted as baseline (BL). Increasing concentrations of heparin caused a rightward shift in the protamine concentration response curves, both in peak calcium mobilisation (**2A**; quantified using the fluorescence dye, FURA-2) and degranulation (**2B**; quantified by the release of β -hexosaminidase and calculated as a percentage of total cellular β -hexosaminidase). Highlighted in red are the data points that correspond to a protamine-heparin ratio of 1:1. $N \geq 5$; mean $\pm$ SEM.

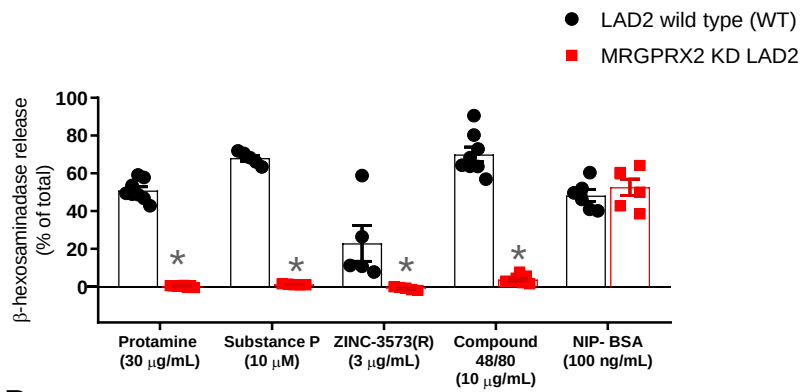
Figure 3: Heparin selectively inhibits MRGPRX2-agonist-induced degranulation. WT LAD2 cells were stimulated with a submaximal concentration of known mast cell and MRGPRX2 activators either independently or in complex with heparin (0.070 – 10 USP units/mL). Responses in the absence of heparin are denoted as baseline (BL). Degranulation was quantified 45 minutes post stimulation by quantification of β -hexosaminidase release. Increasing concentrations of heparin inhibited protamine (5 µg/mL) and compound 48/80 (0.5 µg/mL) -mediated LAD2 degranulation, but had no effect on ZINC-3573(R) (3 µg/mL), substance P (5 µg/mL), ciprofloxacin (200 µg/mL) and NIP-BSA (10 ng/mL)-induced degranulation. $N \geq 5$; mean $\pm$ SEM. One-way ANOVA with Dunnett's multiple comparison post-hoc test was conducted. * $p < 0.01$

Figure S1: MRGPRX2 expression is reduced on LAD MRGPRX2 KD cells. The MRGPRX2 KD cells were sorted using on a MRGPRX2^{low} population. The sorted cells (red traces) were then expanded in culture and re-tested, along with WT LAD2 cells (black traces), for surface receptor expression using flow cytometry. While expression of MRGPRX2 (S1A) was greatly reduced in the MRGPRX2 KD cells, FcεRIα expression (S1B) was unchanged.

Figure S2: Real-time imaging of protamine-induced degranulation in rat peritoneal mast cells (RPMCs). Live cells were imaged every 2 seconds for up to 60 seconds using a Leica SP5 confocal microscope and compiled into a short video using Fiji version 1.42. Protamine (15 µg/mL)-induced RPMC degranulation (**S2A**) was reduced in the presence of heparin at a ratio of 1:1 (15 µg/mL protamine: 1.5 USP units/mL heparin; **S2B**). There was, however, no observed degranulation when the cells were exposed to a protamine:heparin ratio of 1:2 (15 µg/mL protamine: 3 USP units/mL heparin; **S2C**).

Figure S3: Heparin inhibits MRGPRX2-mediated degranulation induced by the endogenous peptide LL-37 but not cortistatin-14. WT LAD2 cells were stimulated with LL-37 (5 µM) or cortistatin-14 (5 µM), either independently or in the presence of heparin (10 USP/mL). Degranulation was quantified by the release of β-hexosaminidase. Heparin significantly inhibited LL-37-induced LAD2 degranulation but had no effect on the cortistatin-14 response. N≥6, mean ±SEM. For statistical analysis, a two-way ANOVA was conducted with Bonferroni multiple comparison post hoc test. * p < 0.01

A



B

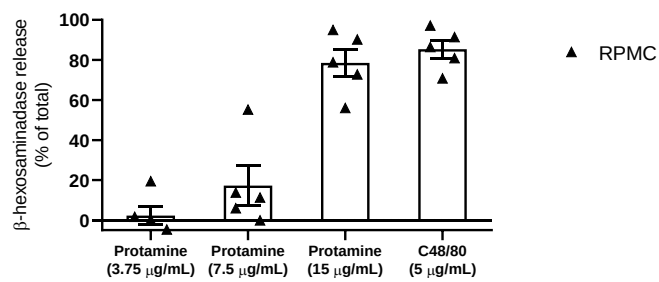
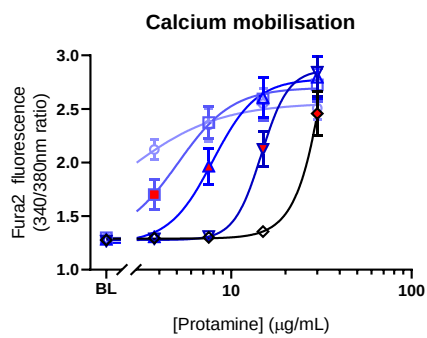


Figure 1

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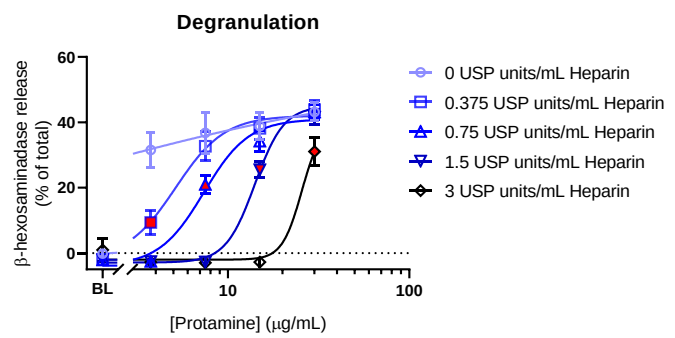


Figure 2

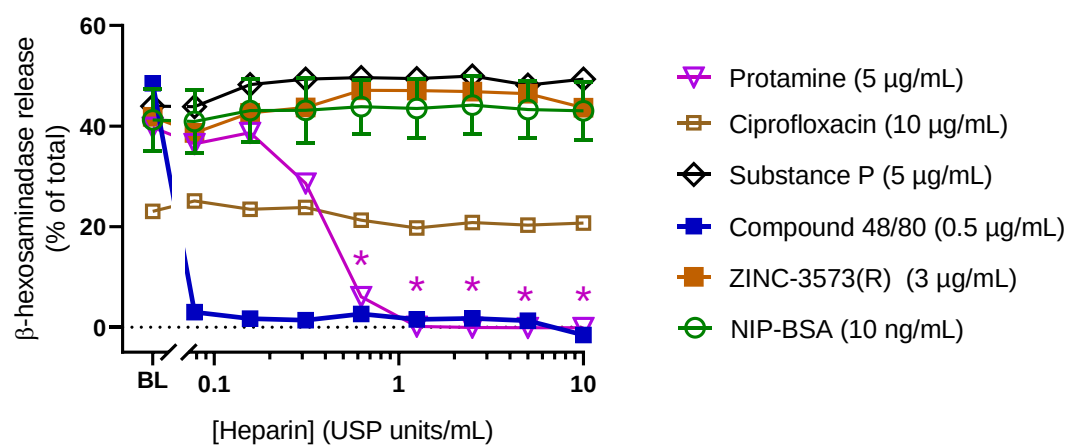


Figure 3

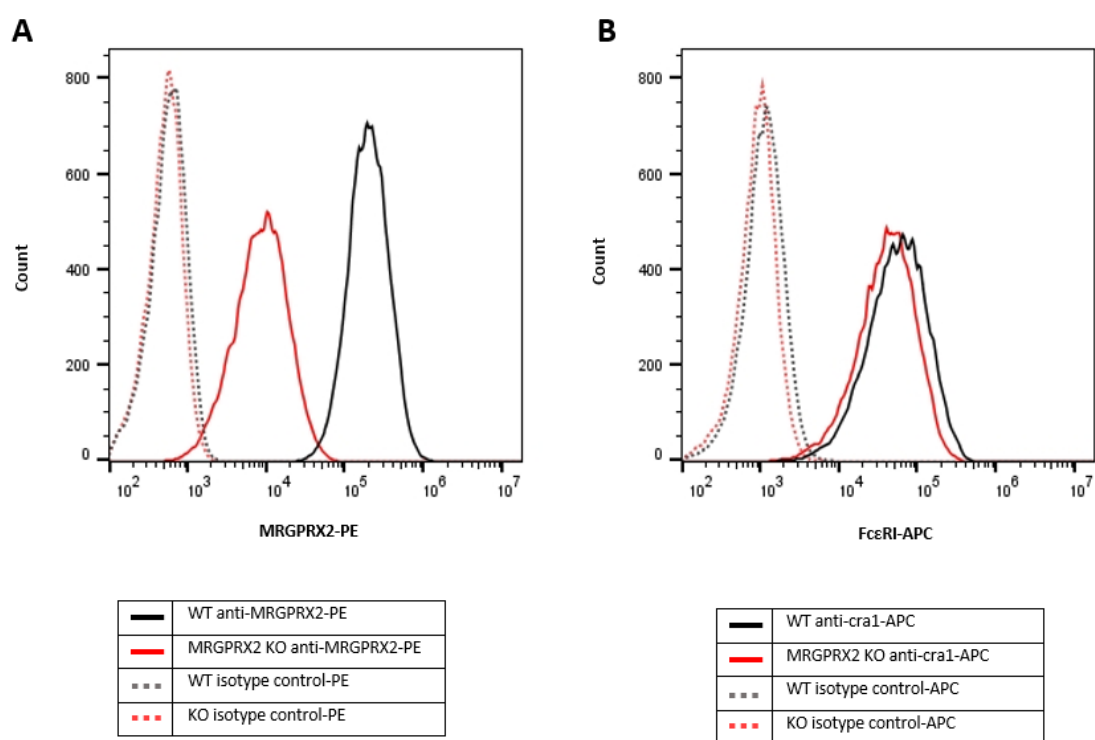


Figure S1

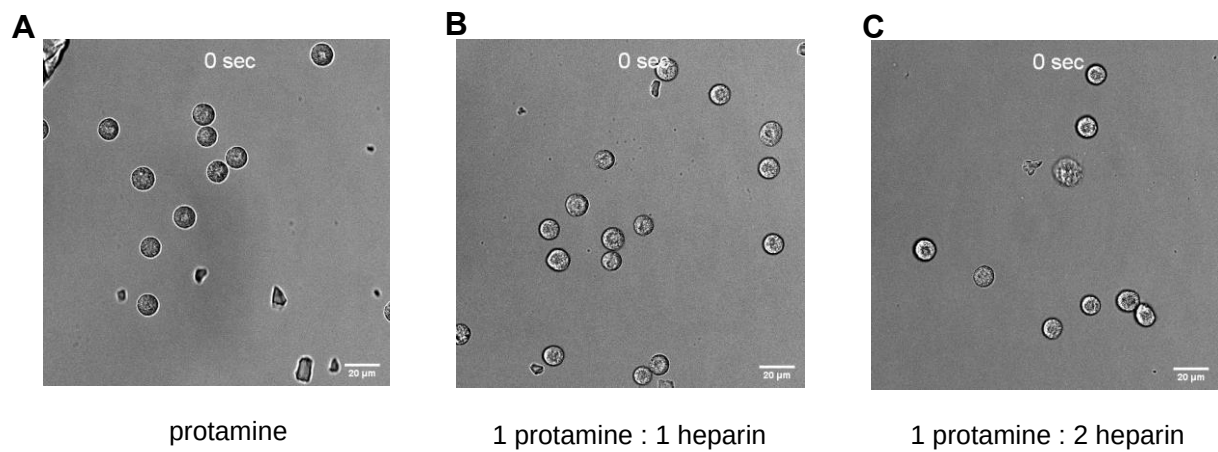


Figure S2

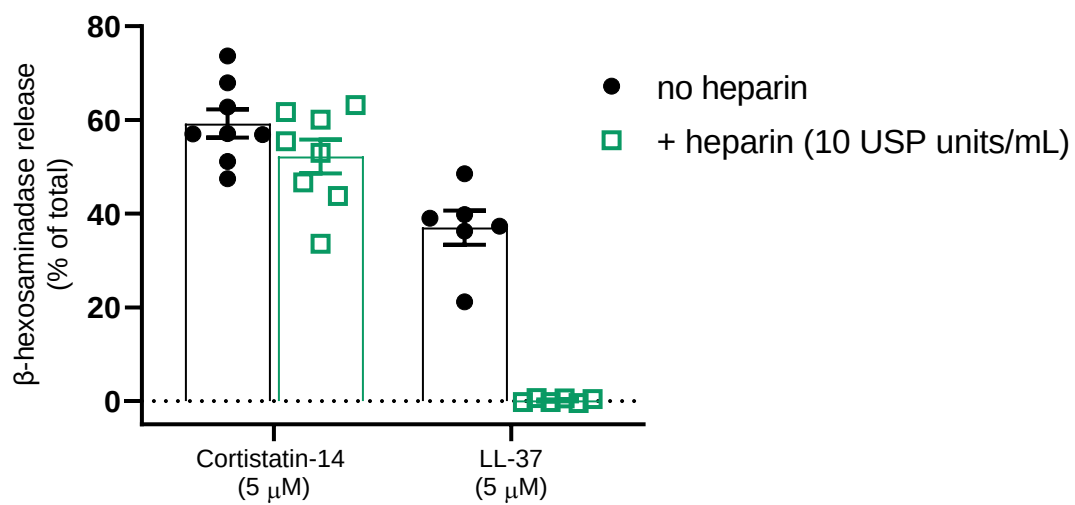


Figure S3

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In addition to the findings described in the above accepted/submitted manuscripts, additional unpublished data presented in this chapter further assesses and validates the role of MRGPRX2 in mast cell activation along with the modulation of this activation by sugammadex and heparin. Moreover, we also present unpublished work on the significance of adrenaline as a mast cell stabiliser in LAD2 mast cells activated by a range of MRGPRX2 agonists.

3.3.3 Comparative analysis of MRGPRX2 knock down cells sorted as CD63^{low} vs MRGPRX2^{low}

LAD2 WT cell degranulation was triggered by compound 48/80 (mean (% degranulation) $\pm$ SEM; 70.1 $\pm$ 3.4), substance P (66.6 $\pm$ 4.5), atracurium (18.9 $\pm$ 2.0), protamine (45.5 $\pm$ 2.9) and cortistatin-14 (60.7 $\pm$ 3.9) (**Figure 3.1**). This response was significantly diminished in both MRGPRX2 KD models, but more so in those sorted as a MRGPRX2^{low} population. Degranulation in these studies was reduced to: compound 48/80 (CD63 sort and MRGPRX2 sort; 12.2 $\pm$ 1.5 and 4.1 $\pm$ 0.8 respectively), substance P (18.4 $\pm$ 3.5 and 0.8 $\pm$ 0.3), atracurium (2.2 $\pm$ 0.5 and -0.1 $\pm$ 0.2), protamine (6.6 $\pm$ 1.2 and 0.3 $\pm$ 0.2), cortistatin-14 (19.0 $\pm$ 3.2 and 0.7 $\pm$ 0.2). These responses are in keeping with stimulus-induced calcium mobilization in WT and KD cells with these agonists (**Figure 3.2**). That is, MRGPRX2^{low} cells were less effectively activated by MRGPRX2 activation compared to cells functionally sorted as CD63^{low}. However, this observation is less pronounced in calcium mobilization responses than degranulation.

WT LAD2 cells also induced significant degranulation when stimulated through the IgE pathway with NIP-BSA (37.5% $\pm$ 3.9). As expected, NIP-BSA-induced degranulation was statistically unaffected in the MRGPRX2 KD model sorted with CD63 (28.7% $\pm$ 4.7). Interestingly, with MRGPRX2^{low} sorted cells, NIP-BSA responses were significantly higher (52% $\pm$ 4.3) than the WT response, with this being observed more prominently in calcium mobilization (**Figure 3.2**) than degranulation experiments (**Figure 3.1**). However, in studies looking at receptor surface expression using flow cytometry, we have shown that FcεRIα levels were unaffected by the knock down of MRGPRX2 (see above submitted manuscript; Fernandopulle et. al., 2021).

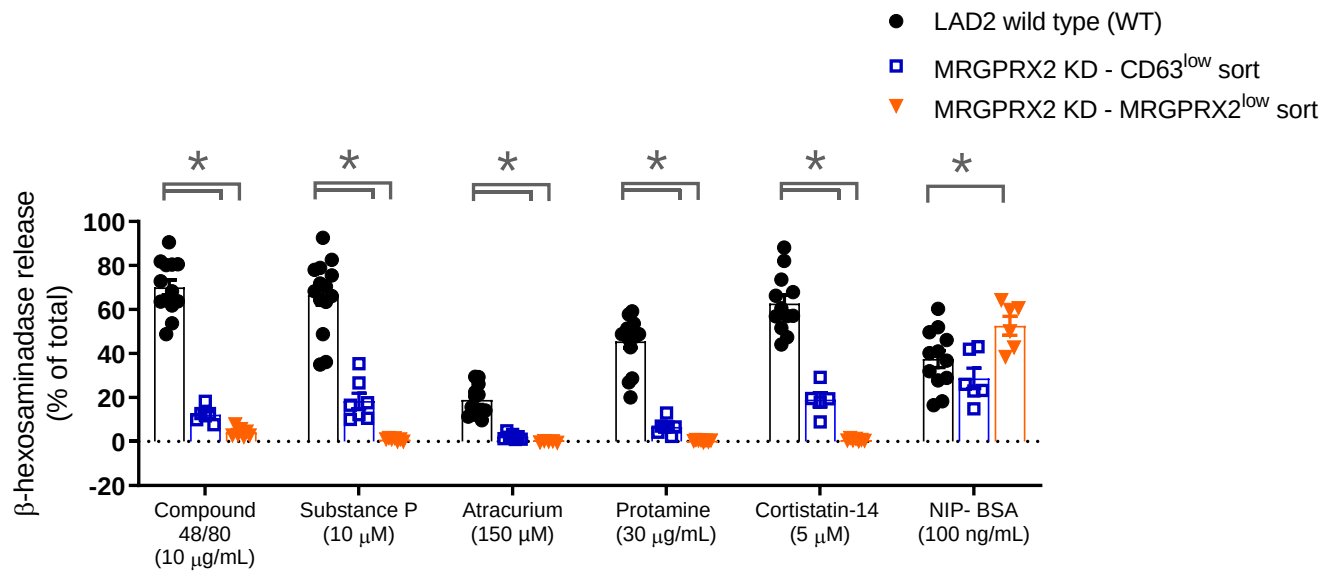


Figure 3.1: Loss of degranulation response to known and potential MRGPRX2 agonists by knockdown of MRGPRX2: comparison of two sorted populations. CRISPR-Cas9 gene editing was used to knock down the MRGPRX2 receptor in WT LAD2 cells. These cells were initially sorted by FACS as a CD63^{low} population (blue). In later experiments these MRGPRX2 KD cells were re-sorted as a MRGPRX2^{low} population (orange). Sorted cells were expanded and stimulated with compound 48/80 (10 µg/mL), substance P (10 µM), atracurium (150 µM), protamine (30 µg/mL), cortistatin-14 (5 µM), or NIP-BSA (100 ng/mL). Degranulation was quantified using released β-hexosaminidase. Data is expressed as mean ± SEM, WT n= 12-15, KD n= 5-8. For statistical analysis, a two-way ANOVA was conducted with Bonferroni multiple comparison post hoc test. *p<0.05

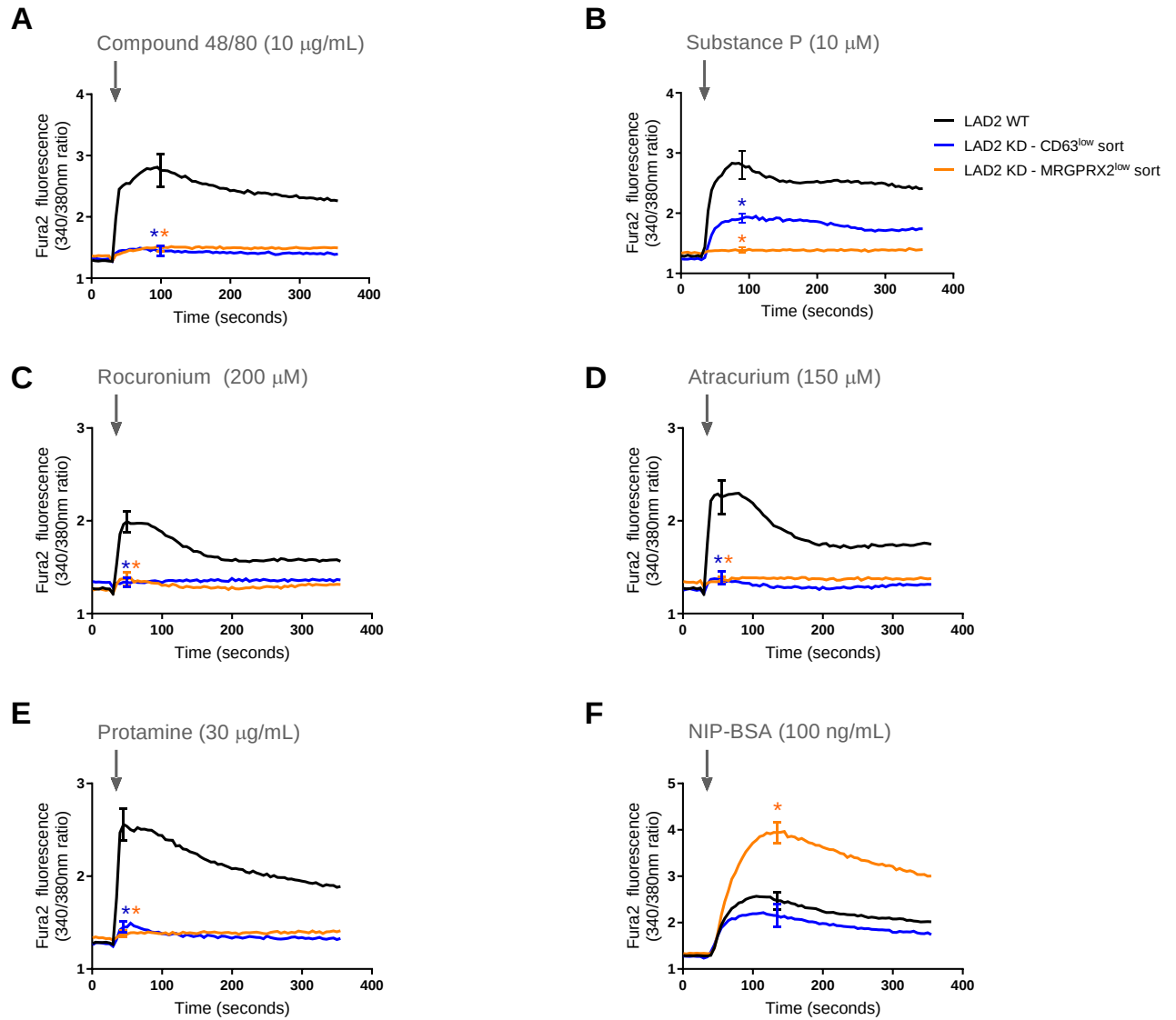


Figure 3.2: Loss of calcium mobilization to known and potential MRGPRX2 agonists by knockdown of MRGPRX2: comparison of two sorted populations. CRISPR-Cas9 gene editing was used to knock down the MRGPRX2 receptor in WT LAD2 cells. These cells were initially sorted by FACS as a CD63^{low} population (blue). In later experiments these MRGPRX2 KD cells were re-sorted as a MRGPRX2^{low} population (orange). Sorted cells were expanded and stimulated with compound 48/80 (10 $\mu\text{g/mL}$; **A**), substance P (10 μM ; **B**), rocuronium (200 μM ; **C**), atracurium (150 μM ; **D**), protamine (30 $\mu\text{g/mL}$; **E**), and NIP-BSA (100 ng/mL; **F**). Calcium mobilization was quantified using Fura-2 fluorescence at excitation wavelengths of 340/380 nm. Data expressed as mean $\pm$ representative SEM, $n=3-8$. For statistical analysis, a two-way ANOVA was conducted on peak calcium mobilization values with Bonferroni multiple comparison post hoc test; * $p < 0.05$ CD63^{low} compared to WT LAD2; * $p < 0.05$ MRGPRX2^{low} compared to WT LAD2.

3.3.4 Purified RPMCs release β -hexosaminidase in response to selected MRGPRX2 agonists

Preclinical drug trials are frequently conducted on rodents such as rats and mice. In this study, we investigated the activation of rat peritoneal mast cells (RPMCs) by agonists of MRGPRX2. RPMCs are known to express MRGPRB3, the rat orthologue of human MRGPRX2 ⁽¹⁷⁾. Protamine (3.75 - 15 $\mu\text{g/mL}$), compound 48/80 (0.08 - 5 $\mu\text{g/mL}$) and ciprofloxacin (100 - 400 μM) induced significant RPMC degranulation (quantified by the release of β -hexosaminidase) (**Figure 3.3**). Interestingly, other known MRGPRX2 agonists which were shown to induce degranulation in LAD2 cells, including atracurium (200 μM), ZINC-3573(R) (25 μM), and substance P (10 μM), did not result in the release of β -hexosaminidase from RPMCs (**Figure 3.3**). Rocuronium (800 μM), a MRGPRX2 agonist that induced calcium mobilization but not degranulation in LAD2 cells (refer to manuscript above; Fernandopulle et. al, 2020), also did not induce β -hexosaminidase release in RPMCs.

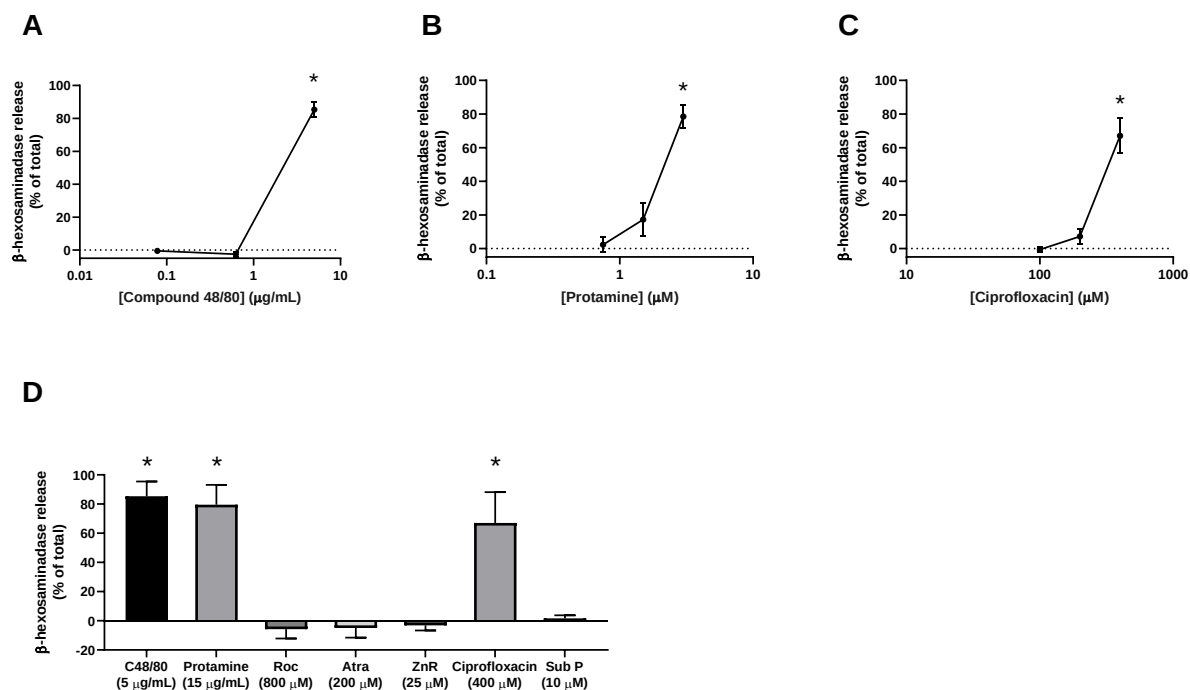


Figure 3.3: Activation of RPMCs by compound 48/80, protamine and ciprofloxacin but not substance P, atracurium or ZINC-3573(R). Rat peritoneal mast cells (RPMCs) were purified using Percoll® density gradient separation. Isolated cells were stimulated with a range of stimuli for 45 minutes and degranulation was quantified by the release of β -hexosaminidase (data expressed as a percentage of total enzyme, with basal release of 9.17 ± 8.9 subtracted). Compound 48/80 (A), protamine (B) and ciprofloxacin (C), induced a concentration-dependent release of β -hexosaminidase, while rocuronium (800 μ M), atracurium (200 μ M), ZINC-3573(R) (25 μ M) and substance P (10 μ M) did not (D). Data presented as mean $\pm$ SEM, n=4-5. For statistical analysis, a two-way ANOVA was conducted on stimulus-induced degranulation responses compared to basal, with Bonferroni multiple comparison post hoc test; * $p < 0.05$.

3.3.5 Effects of sugammadex addition on rocuronium, atracurium, substance P and NIP-BSA-induced LAD2 cytokine release: a kinetic evaluation

We demonstrated the inhibition of compound 48/80-induced LAD2 CCL2 release by sugammadex even when added as long as 3-4 hours (3 hours, $p < 0.01$; 4 hours, $p < 0.05$) following stimulus (Fernandopulle et al., 2020). In this study, we extended this research to investigate the time-dependent role of sugammadex addition on clinically relevant MRGPRX2 agonists, atracurium and rocuronium (**Figure 3.4**). NIP-BSA and substance P were shown to be unaffected by the addition of sugammadex⁽¹⁹⁴⁾, so they were used as negative controls in this time-dependent study. It is important to note that compared to compound 48/80, both rocuronium and atracurium induced significantly less CCL2 release (data shown in **Figure 4.2**). In the presence of sugammadex, there was a significant inhibition of rocuronium-induced release when sugammadex was added up to 30 minutes following stimulus (**Figure 3.4A**). Similarly, with atracurium, there was a significant decrease in CCL2 release when sugammadex was added up to 5 hours (maximum duration of the study) following stimulus addition (**Figure 3.4B**). However, it is interesting to note that the decrease is much more drastic when sugammadex was added less than 60 minutes following atracurium compared to when sugammadex was added after 60 minutes. As also indicated in prior experiments, sugammadex had no effect on substance P (**Figure 3.4C**) and NIP-BSA (antigen; **Figure 3.4D**)-induced CCL2 release from LAD2 mast cell activation, regardless of the time of addition.

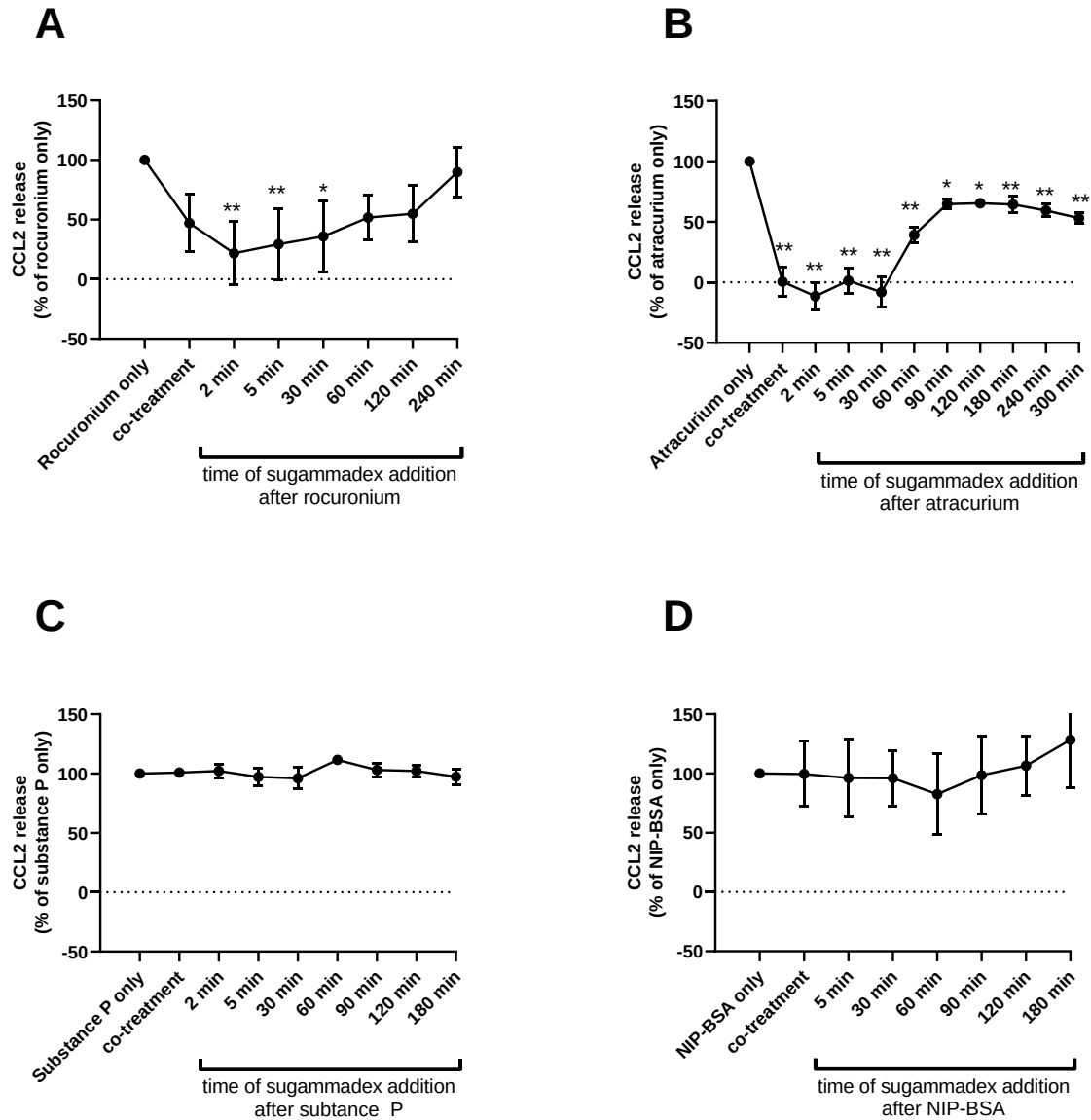


Figure 3.4: Sugammadex inhibits rocuronium and atracurium-induced CCL2 release when added up to 30 minutes and 5 hours following stimulation, respectively. LAD2 cells were stimulated with rocuronium (200 μ M), atracurium (200 μ M), substance P (10 μ M) and NIP-BSA (100 ng/mL), and where applicable, either co-treated with sugammadex, or sugammadex was added at various time points following stimulus addition. Supernatant for CCL2 quantification was obtained 24 hours following stimulation. The inhibitory effects of sugammadex were dependent upon its time of addition, but were still manifest when added up to 30 minutes following rocuronium and 5 hours (300 minutes) following atracurium addition. Results presented as mean $\pm$ SEM, n=3-4. One-way ANOVA was conducted with Dunnett's multiple comparison post hoc test. Each time point was compared to the stimulus response in the absence of sugammadex; *p<0.05, **p<0.01

3.3.6 Modulation of protamine-induced MRGPRX2 activation by sugammadex

In addition to the effect of sugammadex on NMBAs and endogenous agonists mentioned in the above manuscript (Fernandopulle et al., 2020) ⁽¹⁹⁴⁾, we also looked at the possible interaction between sugammadex and the newly identified agonist of MRGPRX2, protamine. Cells were either treated with protamine alone, co-treated with protamine and sugammadex, or treated with sugammadex 2 minutes after protamine addition. Co-treatment of LAD2 cells with protamine and sugammadex significantly reduced calcium mobilization (**Figure 3.5A**), degranulation (**Figure 3.5B**), and cytokine release (**Figure 3.5C**), compared to cells treated only with protamine. When sugammadex was added immediately (40 seconds) after protamine, although too late to have an effect on peak calcium mobilization, it markedly decreased sustained calcium response (**Figure 3.5A**). In studies investigating the effect of sugammadex on degranulation and cytokine release, sugammadex was added 2 minutes after protamine addition. The addition of sugammadex did not alter protamine-induced degranulation (**Figure 3.5B**), however, it did significantly reduce the release of cytokine CCL2 (**Figure 3.5C**). These observations are in keeping with our previous results with compound 48/80 and atracurium (Fernandopulle et al., 2020).

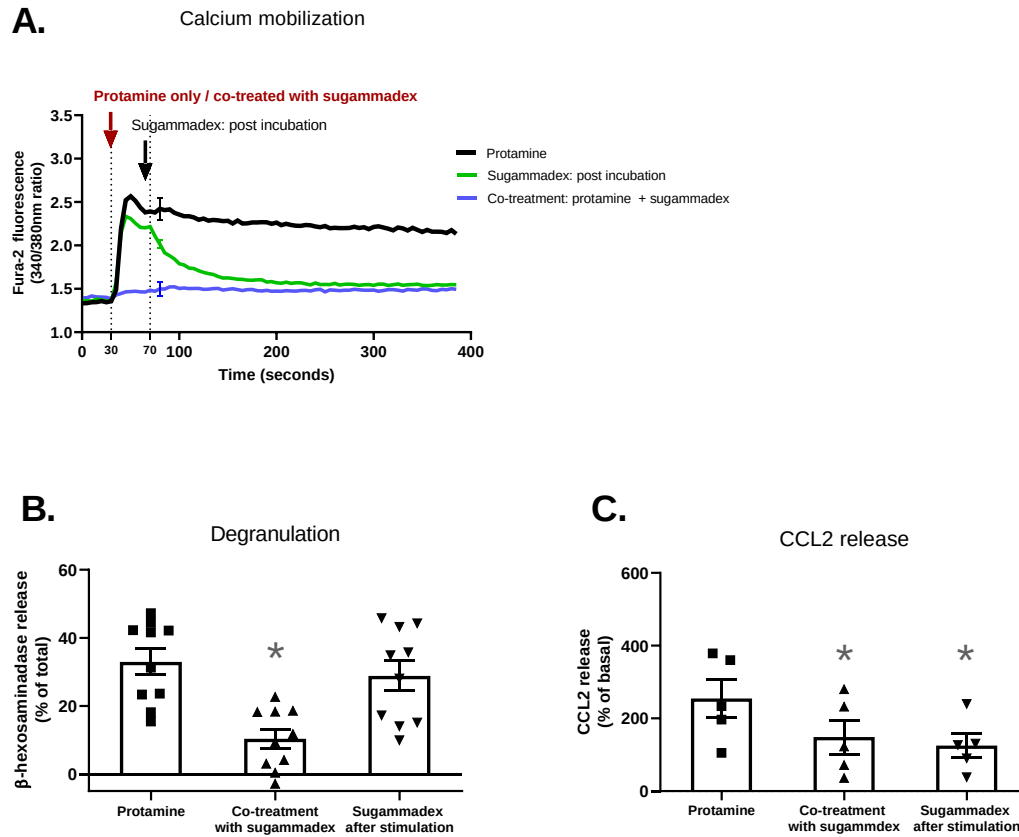


Figure 3.5: Inhibition of protamine-induced LAD2 activation (calcium mobilization, degranulation and CCL2 release) by sugammadex. Protamine (5 $\mu\text{g}/\text{mL}$)-induced calcium mobilization (A), degranulation (B), and CCL2 release (C), were quantified as markers of LAD2 activation using the Fura-2 fluorescence dye, β -hexosaminidase, and CCL2 ELISA, respectively. When co-treated with sugammadex, there was a significant reduction in mast cell activation (across all tested outcomes) induced by protamine. Addition of sugammadex after protamine stimulation significantly decreased CCL2 release but not peak calcium mobilization or degranulation. Data expressed as mean, $\pm$ SEM, $n \geq 5$. Two-way ANOVA was conducted with Bonferroni multiple comparison post hoc test; * $p < 0.05$.

3.3.7 Modulation of LAD2 mast cell activation by adrenaline

In addition to the neutralising agents sugammadex and heparin, we also looked at the most common treatment of anaphylaxis, adrenaline, and its role as an inhibitor of mast cell activation. Although work on adrenaline and its role in anaphylaxis is relatively well established, we investigated its effectiveness when added immediately following a range MRGPRX2 agonists in LAD2 cells. In our studies, this known mast cell ‘stabiliser’ was also utilised as a useful reference tool to compare against the role of the chemical antagonists, sugammadex and heparin.

As seen in **Figure 3.6**, the addition of adrenaline immediately (2 minutes) after stimulus had no significant effect on degranulation. However, pre-treatment of LAD2 mast cells with adrenaline (5 minutes) significantly decreased cell activation by MRGPRX2 agonists (compound 48/80, atracurium, protamine) and antigen (NIP-BSA). As shown earlier, rocuronium does not induce LAD2 degranulation, and the response was not altered by exposure to adrenaline.

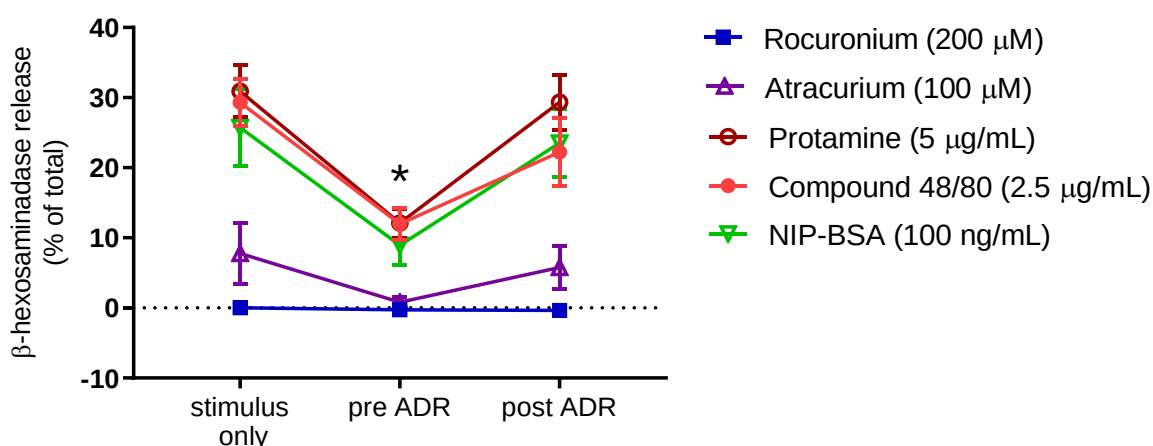


Figure 3.6: Adrenaline significantly inhibits stimulus-induced LAD2 degranulation when pre-incubated but not when added after the stimulus. Degranulation was quantified using released β-hexosaminidase. When adrenaline was added 5 minutes prior to stimulus (pre ADR), protamine, compound 48/80 and NIP-BSA-induced degranulation was significantly (* $p < 0.05$) reduced. However, degranulation was unaffected when adrenaline was added 2 minutes following stimulus (post ADR). Data presented are mean, $\pm$ SEM; $n=4$. Two-way ANOVA was conducted with Bonferroni multiple comparison post hoc test.

3.4 Discussion

We have successfully developed a novel stable CRISPR-Cas9 MRGPRX2 knockdown (KD) LAD2 mast cell model. Following two rounds of sorting, firstly for loss of an activation marker (CD63) and secondly for loss of MRGPRX2 surface expression, the LAD2 MRGPRX2 KD model was shown to be functionally nonresponsive (activation quantified with calcium mobilization and degranulation) to MRGPRX2 agonists. The generation of the stable MRGPRX2 KD model opens up research prospects to examine polymorphic forms of MRGPRX2 by directly transfecting them into a functionally MRGPRX2 null human mast cell model. This is predicted to be a superior model in relatability to human mast cells compared to the widely used cell lines. Current transfection host cell lines, that are used for MRGPRX2 variant modelling, are primarily the rat basophilic cell line, RBL-2H3, and the human embryonic cell line, HEK-293, both likely less translatable to *in vivo* human mast cells than the LAD2 model (refer to **section 1.1.8**).

It is notable that, following the second sort strategy (MRGPRX2^{low}) that virtually ablated the residual response to MRGPRX2 agonists, antigen-induced LAD2 activation (both calcium mobilization and degranulation) was significantly increased, although FcεRI receptor expression remained unaffected. Receptor cross-talk has long been suggested in mast cells, for instance, cross talk between the SCF receptor (Kit) and FcεRI has been associated with an increased acute degranulation response upon co-stimulation ^(229, 230). However, no definitive research is available to suggest crosstalk between MRGPRX2 and FcεRI. Assuming we have diminished constitutive MRGPRX2 activation in our MRGPRX2 KD model, our data suggests a dampening role of MRGPRX2 in the IgE-mediated mast cell activation pathway. This could putatively be a result of constitutive MRGPRX2 activity holding common downstream signalling molecules in a desensitised state. In the MRGPRX2 KD model, these signalling molecules would now be made available for use in the IgE-dependent pathway. This notion is however speculative, and further studies, perhaps following the identification of an inverse agonist to dampen constitutive activity, are needed to add validity to this possibility.

Furthermore, the repeated activation of mast cells with MRGPRX2 agonists followed by an IgE-dependent stimulus (*e.g.*, NIP-BSA) would provide insight to the possibility of cross-pathway desensitization.

In our manuscripts, we have demonstrated the role of MRGPRX2 in the activation of mast cells by a range of polybasic compounds using a LAD2 *in vitro* human mast cell model. However, it is important to highlight that the concentrations of rocuronium (6.3 – 800 μM) and atracurium (4.7 – 150 μM) used in this study are significantly higher than the clinically relevant steady state plasma concentrations (2.3 μM and 1.1 μM respectively) ^(231, 232). At the clinically relevant concentrations, our results show that rocuronium and atracurium would not activate mast cells via MRGPRX2. However, the plasma concentration of protamine is found to sometimes be as high as 5 $\mu\text{g/mL}$ ⁽²³³⁾, a concentration that does induce significant LAD2 mast cell activation (calcium mobilization, degranulation, and cytokine release)).

We have also demonstrated the selective inhibition of this activation by sugammadex and heparin. Our studies show that sugammadex inhibited a broader range of MRGPRX2 agonists than heparin. Sugammadex was able to chemically antagonise, albeit to varying degrees, atracurium, protamine, compound 48/80, ZINC-3573(R) and LL-37, while heparin was only able to antagonise protamine, compound 48/80 and LL-37-induced degranulation. Sugammadex also antagonised rocuronium-induced calcium mobilization (discussed further in Fernandopulle et al, 2020). Additionally, while case studies report the rare occurrence of anaphylaxis against the sugammadex-rocuronium complex ^(234, 235), our data suggests that this is unlikely through the MRGPRX2-mediated pathway of direct mast cell activation as no LAD2 cell activation was observed with the complexes ⁽¹⁹⁴⁾. Since rocuronium did not induce degranulation in LAD2 cells, we did not quantify the effect of heparin on this NMBA.

The interaction between atracurium and sugammadex was unexpected given the preclinical studies that demonstrated no significant effect of sugammadex on atracurium-induced nicotinic block ⁽²³⁶⁾ (discussed further in our manuscript Fernandopulle et al., 2020). In

addition, the MRGPRX2 agonists substance P and cortistatin-14 were neither inhibited by sugammadex nor heparin. The reason underlying this binding disparity of some ligands between sugammadex and heparin is unclear. As outlined in **Table 3-1**, there is no clear link, at least in molecular size and net charge, that distinguishes protamine, compound 48/80 and LL-37 from other MRGPRX2 agonists. Binding affinity of agonists to MRGPRX2 was recently associated with agonist charge ⁽⁹⁶⁾. However, we were unable to associate agonist charge with the selectivity of heparin and sugammadex binding. Although theoretical, perhaps these antagonists do interact with all MRGPRX2 agonists, but only successfully mask the binding sites required to trigger activation in some agonists. Binding studies are needed to confirm this possibility.

Although sugammadex and heparin can antagonise MRGPRX2-mediated degranulation and calcium flux when co-stimulated with the agonists, there was no change in activation when antagonists were added immediately after stimulation. Our study thus supports the body of clinical evidence that suggest a lack of effect by sugammadex on immediate symptoms of NMBA-induced anaphylaxis ⁽²³⁷⁾. As mentioned above, degranulation occurs within seconds to minutes following stimulus addition. We verified this rapidity of degranulation in our time lapse videos of rat peritoneal mast cell degranulation in response to protamine where degranulation takes place within seconds of drug addition (Fernandopulle et al., 2021). Therefore, this lack of effect in antagonising degranulation when the chemical antagonist is added 2 minutes following stimulus is unsurprising. Our data also suggests that an initial calcium mobilization response is adequate to carry out full degranulation as sugammadex inhibited sustained calcium mobilization, but had no effect on the degranulation induced ⁽¹⁹⁴⁾.

In addition, the known mast cell stabiliser, adrenaline, had no effect on stimulus-induced degranulation when added immediately (2 minutes) following a MRGPRX2 or IgE-dependent stimulus. Adrenaline is a non-specific adrenoceptor agonist and is known to inhibit the release of histamine from mast cells by acting as a 'mast cell stabiliser' ^(206, 207). Adrenaline-induced mast cell stabilisation is primarily attributed to β_2 -adrenoceptor activation ^(207, 209, 212). Mast

cells also express α_1 adrenoceptors that have been associated with histamine release, and the inhibition of which was found to potentiate this β_2 -adrenoceptor mediated stabilisation ⁽²⁰⁹⁾. β_2 -adrenoceptors primarily couple to Gs proteins ⁽⁷⁷⁾ that induce cAMP as opposed to MRGPRX2 that couples to Gi proteins that inhibit cAMP production ⁽¹⁷⁾. Although not extensively understood, studies suggest an inhibitory role of elevated cAMP levels on calcium mobilization, perhaps with the involvement of cAMP-dependent protein kinases ^(238, 239). Both MRGPRX2 and Fc ϵ RI pathways are dependent on calcium mobilization for degranulation, therefore, the inhibition of this via activation of β_2 adrenoceptors could effectively attenuate mast cell activation ^(209, 240).

It is important to note that a vast majority of the above mentioned studies were conducted with adrenaline and other β -agonists pre-incubated to stabilise mast cells prior to stimulation. However, in a clinical setting, adrenaline is administered in the event of anaphylaxis, following the stimulus-mediated activation of mast cells. As seen repeatedly in our data, there was no effect of sugammadex/heparin/adrenaline when added 2 minutes following a stimulus. Electron microscopy findings exhibit evidence of compound 48/80-induced degranulation occurring as early as 10 seconds following exposure ⁽²⁴¹⁾. A more recent study, using functional assays, confirmed the speed of degranulation to be within seconds to minutes with both IgE-dependent and independent stimuli ⁽²⁴⁾. Therefore, understandably, the addition of a reversal agent (sugammadex/heparin) or a mast cell stabiliser (adrenaline), would have little effect in halting ongoing degranulation when added 2 minutes following the stimulus. Interestingly, even in a controlled cell culture setting (in a study conducted on rat peritoneal mast cells), not all mast cells were found to be in a similar stage of degranulation ⁽²⁴¹⁾. We verify this observation in our manuscript (Fernandopulle et al., 2021 submitted for publication), where we demonstrate by time-lapse microscopy that RPMC activation by protamine produced varying stages of degranulation per given time. Although intravenously administered drug distribution is likely to be rapid, it would be expected to take longer than that in a cell culture setting where a square wave drug concentration is achieved. Therefore, although speculative,

perhaps *in vivo* treatment with adrenaline/sugammadex/heparin may reach some stimuli before they activate mast cells, thus preventing a proportion of mast cell degranulation, consequently producing some clinical benefit related to mast cell inhibition.

Whilst the post-stimulus addition of sugammadex had no effect on degranulation in our study, it did significantly reduce the release of the proinflammatory cytokine, CCL2. Our findings suggest that cytokine release is dependent on a sustained MRGPRX2 activation. We have also demonstrated the time-dependent role of sugammadex on the release of CCL2 using compound 48/80 as a representative stimulus of an exaggerated mast cell activation response (Fernandopulle et al., 2020). In this chapter, we have extended this study to include the therapeutically relevant drugs, atracurium and rocuronium, that also form complexes with sugammadex. In our manuscript (Fernandopulle et al., 2020) we have shown that sugammadex was able to inhibit compound 48/80-induced CCL2 release even when added 3 hours following the stimulus. Similarly, sugammadex significantly antagonised rocuronium and atracurium-induced CCL2 release when added 30 minutes and up to 5 hours following rocuronium and atracurium stimulation, respectively. As mentioned earlier, the decrease in atracurium-induced cytokine release was much more pronounced when sugammadex was added in a period less than 60 minutes following stimulation. However, the clinical implication of reduced cytokine release and other late-phase mediators in the recovery from anaphylaxis remain undetermined. Further research on the role of mast cell derived cytokines following anaphylaxis and possible correlation in determining if sugammadex would have utility in treating longer term effects of NMBA-induced anaphylaxis.

In addition, in another manuscript (Fernandopulle et al., 2021), we have demonstrated for the first time that protamine induces non-IgE mediated mast cell activation via the MRGPRX2 receptor in the human LAD2 cell model. This activation was antagonised by heparin in a concentration dependent manner. However, with the rarity in protamine-induced anaphylactic events and the absence of a test to distinguish MRGPRX2-dependent anaphylaxis, the clinical relevance of this MRGPRX2-mediated mast cell activation by

protamine is unclear. Interestingly, excess protamine has been associated with anti-coagulant properties that are poorly understood ⁽¹⁸⁴⁾. As mentioned earlier, the receptor MRGPRX2 is presumably found in all human mast cells. Therefore, perhaps excess protamine could cause mast cell activation via MRGPRX2 in all patients that, although insufficient to trigger anaphylaxis, may contribute to the well-known anticoagulant adverse effects of protamine by the release of endogenous heparin from mast cell granules ⁽¹⁸⁴⁾. This aligns with the notion of ‘piecemeal degranulation’ (discussed in **section 1.1.4**) where unlike ‘anaphylactic degranulation’, selected mast cell granules are released that do not initiate anaphylactic symptoms. In theory however, the activation of mast cells that result in the release of adequate heparin to induce its anti-coagulant effects, would also result in the release of histamine to induce at least some symptoms of anaphylaxis. Moreover, this released heparin would be predicted to be neutralised by the excess free protamine, and thereby would have little anticoagulatory effects.

While there is much research on endogenous cationic agents that can act as agonists of MRGPRX2, the possibility of endogenous MRGPRX2 agonist-neutralising agents has been little researched. For instance, an intriguing future study would be to investigate the interaction between MRGPRX2 agonists with endogenous plasma proteins such as alpha-1-acid glycoprotein, a plasma protein known to bind some cationic drugs ⁽²⁴²⁾. It would be interesting to determine if levels of this protein varied in patients who have experienced drug-induced anaphylaxis.

In addition to the data shown in our manuscripts, we demonstrate an inhibitory role of sugammadex on protamine-induced mast cell activation when co-stimulated. Intriguingly, although controversial ^(243, 244), some studies also suggest the administration of sugammadex (sometimes independent of protamine/heparin) increases coagulation time ^(245, 246). Charged molecules are known to play a role in the blood coagulation pathway ⁽²⁴⁷⁾, therefore although speculative, it is plausible that an interaction between sugammadex and an endogenous factor involved in the blood coagulation cascade could result in an increased clotting time.

Alternatively, in the case of cardiac surgery (where protamine, NMBA, heparin and sugammadex are used at different stages), the displacement of heparin due to protamine-sugammadex interactions, may contribute to the anticoagulatory properties of sugammadex. However, binding studies between protamine and heparin with sugammadex are required to verify this. The interaction between the clear liquids, sugammadex and protamine, to form a visible precipitate upon mixing in a laboratory setting has been previously noted, but with limited understanding of its implications ^(248, 249). In light of these observations, the avoidance of simultaneous treatment with sugammadex and protamine has been suggested ^(248, 249).

In the event of protamine-hypersensitivity risk (largely IgE-mediated events with diabetic patients who received protamine-zinc insulin, vasectomised/infertile men, or patients with a history of adverse reactions to protamine), a limited range of alternatives are available. The predominant credible alternative is heparinase I. However, studies confirm the safety profile of protamine to be much better than that of heparinase I ⁽²⁵⁰⁾. Patients were more likely to exhibit more severe anaphylactic events in response to heparinase I and tended to have longer hospital stays compared to those exposed to protamine ⁽²⁵⁰⁾. Another proposed alternative is the use of bivalirudin in place of heparin, thereby removing the need for protamine. Bivalirudin, although a promising alternative, is more costly and has no readily available antidote, thus making it difficult to control (in events of overdose) or reverse (in events of anaphylaxis) ⁽²⁵¹⁾. Therefore, although there are other credible options in case of hypersensitivity reactions, the protamine-heparin drug combination remains the most commonly used in cardiac surgery. Thus, careful attention to the dosing of protamine and heparin is warranted to potentially avoid protamine-induced anaphylactic reactions.

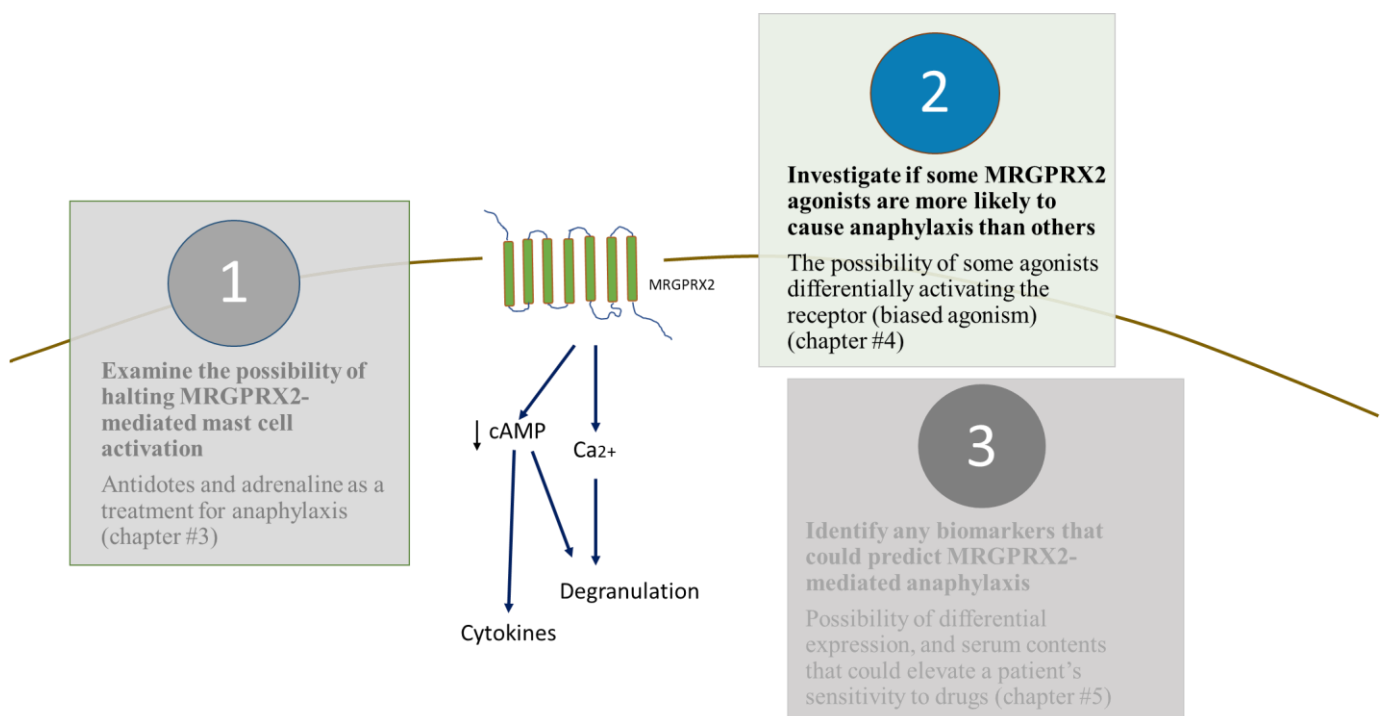
The role of protamine in directly activating mast cells was also demonstrated in rat peritoneal mast cells (RPMCs) where protamine induced significant degranulation, similar to that of compound 48/80. RPMCs are known to have high expression of MRGPRB3, the rat ortholog of the human MRGPRX2 receptor, and other studies have shown sensitivity to stimulation by well-established MRGPRX2 agonists such as substance P and compound 48/80 ⁽⁹²⁾.

Transfected cells (U2OS, RBL-2H3 or HEK-293 depending on the study) expressing the MRGPRB3 receptor have shown receptor activation (quantified through calcium mobilization responses), when stimulated with some MRGPRX2 agonists including compound 48/80, substance P and cortistatin-14 ^(17, 92, 96). Interestingly, our research suggests that MRGPRX2 agonists known to induce LAD2 degranulation used in our study (substance P, ZINC-3573(R) and atracurium), did not induce release of β -hexosaminidase from RPMCs. The reason underlying the substance P-induced histamine release in RPMCs observed by other studies ^(92, 252), and the absence in release of β -hexosaminidase in our hands remain unclear. The functional integrity of substance P in these assays was validated by simultaneous experiments conducted in LAD2 cells. Rocuronium, an agonist of MRGPRX2 that did not induce degranulation in WT LAD2 cells, also failed to induce the release of β -hexosaminidase (degranulation) in RPMCs suggesting that this effect is consistent between species, at least between human and rat.

In conclusion, we have both confirmed and shown for the first time, using a new CRISPR-Cas9 MRGPRX2 KD LAD2 model, the dependence of MRGPRX2 for mast cell activation by a range of polybasic compounds. We have also demonstrated that sugammadex, heparin and adrenaline can all inhibit mast cell activation by selected agonists when added before or co-stimulated with the agonist. However, the provision of the antagonist minutes following a stimulus is too late to modulate degranulation, the primary contributor to the symptoms of anaphylaxis. Nevertheless, there was a significant reduction in release of some late stage mediators, and therefore, provision of antagonists following a stimulus may have a role in controlling the more delayed symptoms of anaphylaxis. In addition, these results add to the knowledge on the mechanism of mast cell activation through MRGPRX2, suggesting that the release of late stage mediators like cytokines are dependent on the sustained activation of the receptor.

Chapter 4

Evidence of biased agonism in MRGPRX2 activation of LAD2 mast cells



4.1 Introduction

MRGPRX2, a class A GPCR (rhodopsin-like) ⁽²⁵³⁾, is known to couple to two G proteins, Gi and Gq, concomitantly resulting in various quantifiable downstream signalling and functional outcomes including calcium mobilization, degranulation and cytokine release (**Figure 1.3**) ^(17, 55, 71). As discussed earlier, although all human mast cells presumably express MRGPRX2, it remains unclear why only a few individuals respond adversely to some MRGPRX2 agonists. It is also unclear whether all agonists are equally likely to induce adverse reactions in patients likely to suffer MRGPRX2-mediated adverse outcomes. If a patient is found to react adversely to one drug-agonist of MRGPRX2, should they also avoid all others? In this chapter, we look at characterising the downstream signalling cascades and functional responses triggered by different MRGPRX2 activators to determine if any agonist bias exists that could make a patient more susceptible to experiencing anaphylaxis with some drugs. Characterising any biased agonism may also point to mechanisms important for degranulation which might logically be overactive in patients susceptible to MRGPRX2-dependent anaphylaxis. In addition, if bias truly exists, this could also be an important tool to be used in drug design, both in investigating agonists and antagonists of MRGPRX2.

4.1.1 Biased agonism

Biased agonism is a phenomenon where ligands activating the same receptor result in dissimilar downstream signalling cascades and consequent cellular outcomes ⁽²⁵⁴⁾. This is presumably a result of variations in receptor conformation and patterns of phosphorylation resulting in different receptor activation states and subsequent variations in recruitment and activation of downstream signalling molecules ⁽²⁵⁵⁾. Better understanding this attribute opens up research into ‘designer drugs’ with increased therapeutic efficacy and reduced side effects ⁽²⁵⁶⁾. The existence of biased agonism is generally observed by quantifying two or more signalling outputs ^(256, 257).

Differences observed in these signalling outputs in response to agonists of the same receptor can be broadly categorised into 3 forms of bias ⁽²⁵⁷⁾. Firstly, system bias (or 'apparent bias') is where the receptor favours a specific signalling cascade based on the availability and proximity of transducer elements ⁽²⁵⁴⁾. System bias is associated with the biased presence of signalling molecules to carry out downstream signaling, depending on cell type and location ⁽²⁵⁷⁾. For instance, dopamine-2 receptors found in the striatum and prefrontal cortex display biased functional responses to the the same agonists, primarily attributed to differential expression of signalling molecules such as β -arrestin-2 between the two locations ⁽²⁵⁸⁾. Therefore, system bias tends to be common for all receptor agonists acting on the same cell, and proves difficult to utilise for therapeutic benefit ⁽²⁵⁷⁾. The second type of bias is observational bias (although sometimes broadly categorised within system bias ⁽²⁵⁹⁾), based on the sensitivity of the assays used to quantify different outcomes ⁽²⁵⁷⁾. Observational bias can be a function of assay conditions including temperature, buffers and reagents, and therefore cannot be utilized as a therapeutic target ⁽²⁵⁷⁾. Finally, if the observed response cannot be understood by system or observational bias, there could be a true ligand/agonist bias, where the ligand favours one signalling pathway over another ⁽²⁵⁷⁾. This is common when differences in receptor-mediated responses to different agonists are quantified in a single cell type. Although the molecular mechanisms underpinning ligand bias are not well characterised, it is presumed to be a result of the ligand's interaction with various binding residues in the receptor's tertiary structure, resulting in a range of active conformational states ⁽²⁶⁰⁾. Ligand bias is associated with the molecular structure of the agonist, which can be designed and therefore can be therapeutically utilized ⁽²⁵⁷⁾. An example of a widely used biased agonist is carvedilol, a medication used to treat heart failure and hypertension, traditionally understood to be a nonselective β -adrenoceptor antagonist, but recently identified as a biased agonist ⁽²⁶¹⁾. Carvedilol, while having negative efficacy at the Gs protein coupled to β_2 - adrenoceptor, has positive efficacy at activating β -arrestin signalling to induce ERK phosphorylation ⁽²⁶²⁾. Thus, while carvedilol antagonises Gs signalling of cardiac β -

adrenoceptors to lower blood pressure ⁽²⁶³⁾, recent research suggests cardioprotective effects of β -arrestin signalling ⁽²⁶⁴⁾. Although the clinical implication of this bias requires further characterisation, it is hypothesised to contribute to carvedilol's unique clinical efficacy in treating a variety of heart conditions ⁽²⁶²⁾.

Recently, the first characterised biased agonist, oliceridine, was approved by the FDA (Food and Drug Administrations, US) ⁽²⁶⁵⁾. Oliceridine is a biased agonist at the μ -opioid receptor and is an intravenous opioid medication used for the management of moderate to severe pain in adults ⁽²⁶⁵⁾. Unlike carvedilol mentioned above, oliceridine preferentially activates G protein signalling over β -arrestin, thus having reduced β -arrestin associated side effects (*e.g.*, nausea and constipation) compared to traditional opioids (*e.g.*, morphine) ⁽²⁶⁵⁾.

While β -arrestin bias is the most common form of biased agonism currently found with clinical relevance, another origin of bias is the biased activation of G-protein subclasses. Angiotensin II receptor type I (AT1R), in addition to the known β -arrestin agonist bias (with angiotensin analogs), has been recently found to exhibit agonist bias selectivity in G protein subtypes ⁽²⁶⁶⁾. Interestingly, studies have suggested that β -arrestin bias ligands at AT1R are frequently biased against Gq signalling ^(266, 267). Notwithstanding the need for further research to understand the underlying mechanism of G protein bias, the clinical utility of rational drug design to harness agonist bias in maximising beneficial effects while minimising adverse effects remains vital.

The most common analysis for quantifying biased agonism is the Black-Leff operational model that compares transduction ratios, a function of affinity and efficacy, between agonists of the same receptor ⁽²⁶⁸⁾. This model, proposed in 1983, still remains the best available model for quantifying bias as it incorporates elements of both ligand-receptor interaction (accounted for with receptor affinity) and the subsequent binding to signalling proteins (accounted for with agonist efficacy) ⁽²⁶⁸⁾.

4.1.2 Mast cell G proteins

Agonists that bind to the MRGPRX2 receptor, activate a variety of signalling molecules that influence intracellular mechanisms such as the mobilization of calcium and modulation of adenylate cyclase through receptor coupling to Gq and Gi proteins (**Figure 1.3**)^(17, 55, 71). G proteins are heterotrimeric proteins composed of an α , β and γ subunit. The human genome encodes for 16 G α , 5 G β and 13 G γ subunits that form 16 heterotrimeric G proteins broadly categorised into 4 groups- the Gs, Gi/0, Gq/11 and G12/13⁽²⁵⁶⁾. The binding of an agonist to the GPCR causes a conformational change in the coupled G protein initiating an exchange of GDP (guanosine diphosphate) to GTP (guanosine triphosphate), resulting in activation followed by the dissociation of the α subunit from the stable $\beta\gamma$ complex⁽²⁶⁹⁾. These effectors in turn regulate the range of intracellular signalling molecules mentioned above⁽²⁶⁹⁾. The α subunit can elicit downstream signalling on its own but the β and γ subunits require to be in complex to function⁽²⁵⁶⁾. The $\beta\gamma$ subunit was once believed to solely function as an inhibitor of G α activity⁽²⁶⁹⁾. However, it is now known that following the uncoupling from G α subunit, the $\beta\gamma$ complex also activates a range of independent effectors and plays a role in the signalling and phosphorylation of some MAP kinases such as ERK^(269, 270).

Additionally, $\beta\gamma$ signalling is known for its association with the arrestin pathway and its regulation in receptor activity via the initiation of receptor internalization^(256, 270). An alteration in the β -arrestin pathway is commonly associated with biased receptor desensitisation⁽²⁷¹⁾. A “barcode hypothesis” has been proposed where certain patterns of receptor phosphorylation results in a variety of arrestin-mediated signalling cascades⁽²⁵⁵⁾. This opens up a therapeutic target window for β -arrestin-biased designer drugs that induce internalisation of an undesired receptor while promoting desired receptor activation⁽²⁵⁶⁾. For instance with NMBAs, perhaps drugs could be designed to retain their ability to block nicotinic receptors but induce internalisation, rather than activation, when bound to MRGPRX2 receptors.

Early studies on MRGPRX2 transfected HEK-293 and RBL-2H3 cells suggested the resistance of the receptor towards phosphorylation and internalisation ⁽¹⁰⁵⁾. However, more recent research conducted using rat peritoneal mast cells and human skin mast cells suggests that MRGPRX2, like most other GPCRs, undergo rapid β -arrestin-mediated receptor internalisation ^(272, 273). The study using skin mast cells suggests the presence of a β -arrestin recruitment bias with some MRGPRX2 agonists (*e.g.*, compound 48/80 and codeine) inducing receptor internalisation faster and more completely than others (*e.g.*, substance P) ⁽²⁷³⁾. This study also proposes the utilisation of ligand-mediated receptor cross-desensitisation by implementing controlled activation/desensitisation of MRGPRX2, to thereby reduce risk/severity of atopic disorders or any condition with undesired MRGPRX2 activity ⁽²⁷³⁾.

4.1.3 Protein kinases

As mentioned above, MRGPRX2 is found to couple to both Gi and Gq. The Gi subunit directly result in the inhibition of adenylate cyclase that inturn results in the inhibition of cAMP formation from ATP. This affects a range of cAMP-dependent kinases, one of which is protein kinase A (PKA). PKA is a tetrameric complex constituting of 2 catalytic units (2 of PRKACA, PRKACB, PRKACC) and 2 regulatory units (2 of PRKAR1A, PRKAR1B, PRKAR2A, PRKAR2B) ^(274, 275). PKA activates a range of signalling molecules that ultimately result in the activation of MAP kinases (mitogen activated protein kinases) such as ERK, JNK and p38 ⁽²⁷⁶⁻²⁷⁸⁾. These MAP kinases, when activated via phosphorylation, regulate cytokine and eicosanoid production ⁽⁷⁸⁾. The importance of Gi signalling to MRGPRX2-mediated effects has been established by studies using the Gi inhibitor, pertussis toxin, that was found to completely abolish compound 48/80-induced mast cell degranulation ^(83, 84).

MRGPRX2 is also coupled to Gq, the activation of which is frequently quantified by an observed calcium mobilization response. As detailed in **section 1.1.7**, Gq is coupled to phospholipase C β that cleaves PIP₂ (phosphatidylinositol 4,5-bisphosphate) into IP₃ (inositol trisphosphate) and DAG (diacylglycerol) ⁽²⁷⁹⁾. IP₃ then induces calcium mobilization, that with DAG, activates protein kinase C (PKC) and initiates a signalling cascade that ultimately results

in degranulation ⁽²⁸⁰⁾. PKC exist in numerous isoforms, each activated by different signalling molecules ⁽²⁸¹⁾. Broadly, PKC isoforms are categorised into 3 groups; calcium dependent (PKC- α , β 1, β 2, γ), calcium independent (PKC- δ , ϵ , θ , η), and atypical isoforms (PKC- ζ and ι/λ) ⁽²⁸²⁾. The former two categories are activated by DAG or phorbol esters such as PMA (phorbol 12-myristate 13-acetate; a synthetic DAG mimetic) while atypical isoforms are unaffected by DAG, PMA or calcium ⁽²⁸³⁾. PKC is known to be associated with the activation of phospholipase D, important in granule movement to the membrane resulting in degranulation ^(284, 285). However, there is little understanding of the role of different PKC isoforms in mast cell activation. While calcium-dependent isoforms are considered essential for signalling via a range of calcium dependent kinases, the calcium-independent isoforms, especially PKC δ and θ , are found to play a defining role in mast cell degranulation as demonstrated by studies using isotype specific inhibitors (*e.g.*, GF109203X) ^(286, 287).

4.1.4 Aim of Study

In this chapter, we investigate the possibility of biased agonism at MRGPRX2 by a range of agonists by characterising different signalling molecules and outcomes of MRGPRX2 activation. We use two models for the analysis of biased agonism. Firstly, we use the Black-Leff operational model that compares transduction ratios of each MRGPRX2 agonist against the assumed full agonist, compound 48/80, for the outcomes measured. Secondly, we normalised stimuli concentrations to a fixed calcium mobilization response and quantified respective responses of other outcomes tested (degranulation, cytokine release and ERK phosphorylation). We next aimed to understand if this bias could be attributed in some way to differential PKC activation.

4.2 Methods:

Common methodologies used are detailed in Chapter 2, however, specific methods used for the results generated in this chapter are shown below.

4.2.1 Quantification of LAD2 mast cell activation

Following treatment with a concentration range of compound 48/80 (0.04 – 20 μ M), substance P (0.1 – 10 μ M), ZINC-3573(R) (0.4 - 200 μ M), rocuronium (6.3 – 800 μ M), atracurium (4.7 – 150 μ M), protamine (0.01 – 12 μ M) and NIP-BSA (0.0001 – 1000 ng/mL), LAD2 activation was quantified via calcium mobilization, ERK phosphorylation, degranulation, and cytokine CCL2 release (*general methods section 2.1.2, 2.1.3, and 2.1.4, respectively*). The average molecular weight of the polymers, compound 48/80 and protamine, were assumed to be 500 and 4500 Da respectively, as described in **Table 3-1**, to calculate molar concentrations.

4.2.2 Quantifying phosphorylation of ERK by western blotting

IgE-sensitized LAD2 cells were washed and incubated for 4 hours in a humidified incubator in serum free IMDM (37 °C). The cells were then washed and resuspended in fresh serum free IMDM (1.5 x 10⁶ cells per condition in 700 μ L). Concentrations of respective stimuli were added and incubated in a humidified incubator for 15 minutes. Cells were washed in ice-cold PBS twice to remove any residual media. Pelleted cells were lysed in sample buffer (Tris-HCl (0.2 M), SDS (277 mM, 8.0% (w/v)), bromophenol blue (6 mM), glycerol (4.3 M)) with DTT (62.5 mM), and stored at -20 °C until further use.

Samples were loaded onto SDS-PAGE gels (12.5% acrylamide gels; Bio-Rad Laboratories; Hercules, California, US) and run at 120 V for ~1.5 hours in running buffer (SDS (0.1%; 3.5 mM), Tris (25 mM), glycine (192 mM)) ⁽²⁸⁸⁾. Gels were then transferred onto nitrocellulose membranes (Bio-Rad Laboratories) using wet transfer (protocol recommended by ThermoFisher Scientific) in transfer buffer (Tris (25 mM), glycine (190 mM), methanol (20%)) at 100 V for 55 minutes. Membranes were subsequently washed in TBST (Tris-Cl (50 mM),

NaCl (150 mM), Tween-20 (1%)), blocked with TBST supplemented with dried milk powder (5% (w/v) milk solubilized in TBST) for 60 minutes and probed with phospho-ERK primary antibody produced in rabbit (Cell Signalling Technologies; Danvers, Massachusetts, US) overnight 4°C. The membranes were then washed thoroughly in TBST, probed with HRP-conjugated anti-rabbit secondary antibody (Bethyl laboratories; Montgomery, Texas, US) for 60 minutes, washed and imaged with ECL luminescent substrate and ChemiDOC™ (Bio-Rad Laboratories).

Following imaging, the blots were stripped to remove phospho-ERK antibody and allow re-probing with a total ERK antibody. For this, mild stripping buffer (glycine (200 mM), SDS (4 mM), Tween-20 (10%); pH=2.2) was used to cover the membranes for 30 minutes and then washed twice with both PBS and TBST. The membranes were blocked again with milk (5% milk solubilised in TBST) for 60 minutes and re-probed with total ERK antibody produced in rabbit (Cell Signalling Technologies). The blots were then washed, incubated with secondary antibody, and imaged as above.

The bands were manually selected and analysed for mean pixel density using Image J (FIJI). Graphical representations were drawn using PRISM (version 9.1; GraphPad PRISM).

4.2.3 Quantifying the role of PMA co-stimulation on stimulus-induced LAD2 degranulation

A concentration range of PMA (0.7 - 50 ng/mL) was pre-mixed with a fixed concentration of stimuli; rocuronium (200 µM), NIP-BSA (100 ng/mL), compound 48/80 (3 µg/mL), atracurium (200 µM), or ionomycin (0.5 µM). These dual stimuli were then used to treat LAD2 cells (2.5 x 10⁵ cells/mL) that were washed, resuspended in HBSS, and seeded in round bottom 96 well plates as per methods **section 2.1.3**. Following a 45 minute stimulation period (at 37 °C in an oscillating incubator), degranulation was quantified using the β-hexosaminidase enzyme assay (refer to **section 2.1.3**).

4.2.4 Quantifying the effect of the calcium-dependent PKC inhibitor, GF109203X, on stimulus-induced LAD2 degranulation

LAD2 cells were treated with a concentration range of GF109203X (0.0001 – 1000 μ M) for 10 minutes prior to stimulation with atracurium (200 μ M), protamine (1 μ M), substance P (5 μ M), compound 48/80 (0.5 μ g/mL), ZINC-3573(R) (5 μ M), NIP-BSA (10 ng/mL), or ionomycin (0.5 μ M). Following a 45 minute stimulation period in an oscillating incubator (37 °C, 70 RPM), cell-free supernatant was harvested to carry out the β -hexosaminidase assay detailed in **section 2.1.3**.

4.2.5 Quantifying ligand bias of MRGPRX2 agonists

The Black-Leff operational model ⁽²⁸⁹⁾ for biased agonism was conducted to analyse and clearly determine any evidence of bias within agonists of MRGPRX2. The analysis was conducted with the generous help of Dr. Lauren May, Laboratory of Cardiac Biology, Monash Institute of Pharmaceutical Sciences, Victoria, Australia. A global analysis was conducted on the group data for each MRGPRX2 agonist using the cellular outcomes of calcium mobilization, degranulation, and cytokine (CCL2) release. Three-parameter log(agonist) vs. response non-linear fit equation was used to fit sigmoidal curves using PRISM version 9.1 (**Figure 4.5**). To quantify signalling bias, Log R values (logarithmic transduction ratios) were calculated as described in the Black-Leff operational model using PRISM version 9.1 ^(268, 289). To offset any system bias, agonist responses were normalised to the maximal system response (*i.e.*, percentage of maximal compound 48/80 response; Δ Log R) (**Figure 4.6**). To determine the existence of bias, the transduction ratios relative to compound 48/80 were normalised to a reference pathway, in this case, calcium mobilization ($\Delta\Delta$ Log R or log(Bias)) (**Figure 4.7**).

4.2.6 Statistical analysis

Data is shown as mean, $\pm$ SEM, and where applicable, non-linear curves were fitted using the three-parameter or four-parameter (variable slope) equation within PRISM (version 9.1; GraphPad, San Diego, California, US). Pixel densities per unit area for quantifying ERK phosphorylation was performed using ImageJ (FIJI; National Institute of Health, Bethesda, Maryland, US). Other graphical representations were drawn and analysed using PRISM (version 9.1, GraphPad) and expressed as mean $\pm$ SEM of n experiments. Statistical significance was determined using either One-way or Two-way ANOVA with either Dunnet's or Bonferroni multiple comparison post hoc tests, with levels of significance as mentioned in respective figure legends.

4.3 Results

4.3.1 MRGPRX2 agonist-induced calcium mobilisation is not always correlated with levels of degranulation, cytokine production, or ERK phosphorylation

Mast cell activation was induced both by the IgE-independent pathway with a range of MRGPRX2 agonists (compound 48/80, substance P, rocuronium, atracurium and protamine), and the IgE-dependent pathway using NIP-BSA. All drugs induced a concentration-dependent calcium mobilization response (**left y-axis**), but with varying maxima (Δ 340/380 nm ratios from basal); compound 48/80 (mean (Δ 340/380 nm ratio) $\pm$ SEM; 0.93, $\pm$ 0.13), substance P (0.78, $\pm$ 0.09), rocuronium (0.90, $\pm$ 0.17), atracurium (0.68, $\pm$ 0.10), and protamine (0.86, $\pm$ 0.14) (**Figure 4.1**).

The concentration-dependent calcium mobilization noted through MRGPRX2 activation was not consistently paralleled by productive degranulation (**right y-axis**). While compound 48/80 (maximal mean (% β -hexosaminidase) $\pm$ SEM; 87 $\pm$ 5), substance P (66 $\pm$ 5), and protamine (44 $\pm$ 6) resulted in concentration-dependent degranulation responses, atracurium (20 $\pm$ 3) induced little degranulation at its highest concentration, and rocuronium induced no degranulation (**Figure 4.1**). Similarly, the observed maximal CCL2 release was significantly varied (**Figure 4.2**). While ZINC-3573(R) (mean (ng/mL) $\pm$ SEM; 20.74 $\pm$ 3.3), compound 48/80 (7.13 $\pm$ 1.02) and substance P (7.78 $\pm$ 1.14) induced much CCL2 release, atracurium (2.39 $\pm$ 0.31), protamine (1.66 $\pm$ 0.28), and rocuronium (0.84 $\pm$ 0.81) induced significantly less release (**Figure 4.2**). IgE-dependent activation (NIP-BSA) is also included for completeness, although not considered in bias analysis (maximal responses of calcium mobilization (mean Δ 340/380 nm ratio $\pm$ SEM) 0.79 $\pm$ 0.1; degranulation (mean % β -hexosaminidase $\pm$ SEM) 62 $\pm$ 5; CCL2 release (mean ng/mL $\pm$ SEM) 0.63 $\pm$ 0.61)

It should be noted that not all concentration-response curves reached maximal responses primarily due to the lack of solubility at higher concentrations of some stimuli. Therefore, some of the values used are approximations of maximal responses observed and correspond to the maximal concentrations used in our study.

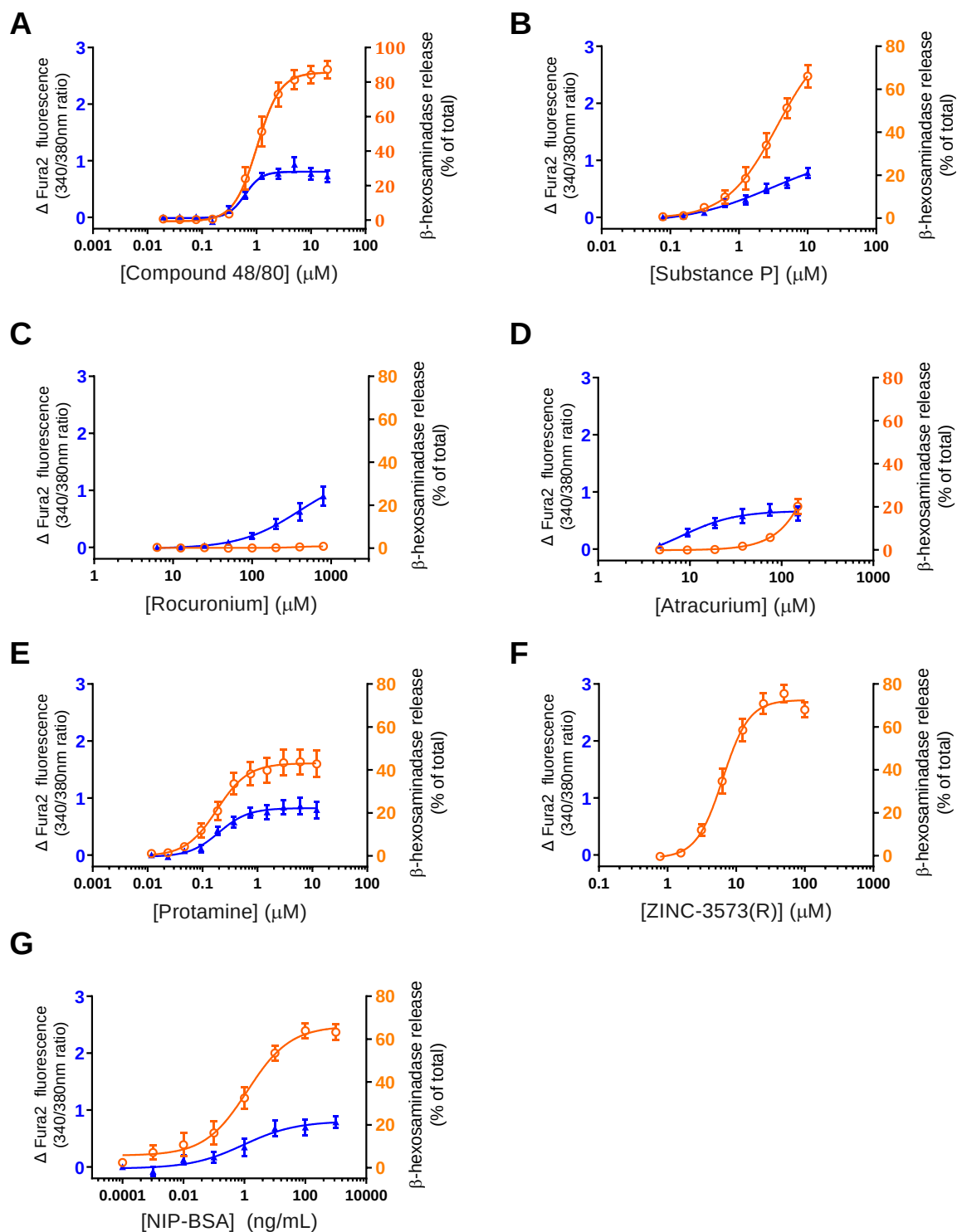


Figure 4.1: LAD2 degranulation does not always correlate to calcium mobilization. Δ peak calcium mobilization (Fura-2 fluorescence with basal levels subtracted; **left y-axis**) and degranulation (% β -hexosaminidase release; **right y-axis**) were quantified from LAD2 human mast cells stimulated with a range of MRGPRX2 agonists, compound 48/80 (**A**), substance P (**B**), rocuronium (**C**), atracurium (**D**), protamine (**E**), ZINC-3573(R) (**F**), and the Fc ϵ RI activator NIP-BSA (**G**). Data presented as mean $\pm$ SEM. N=5-12.

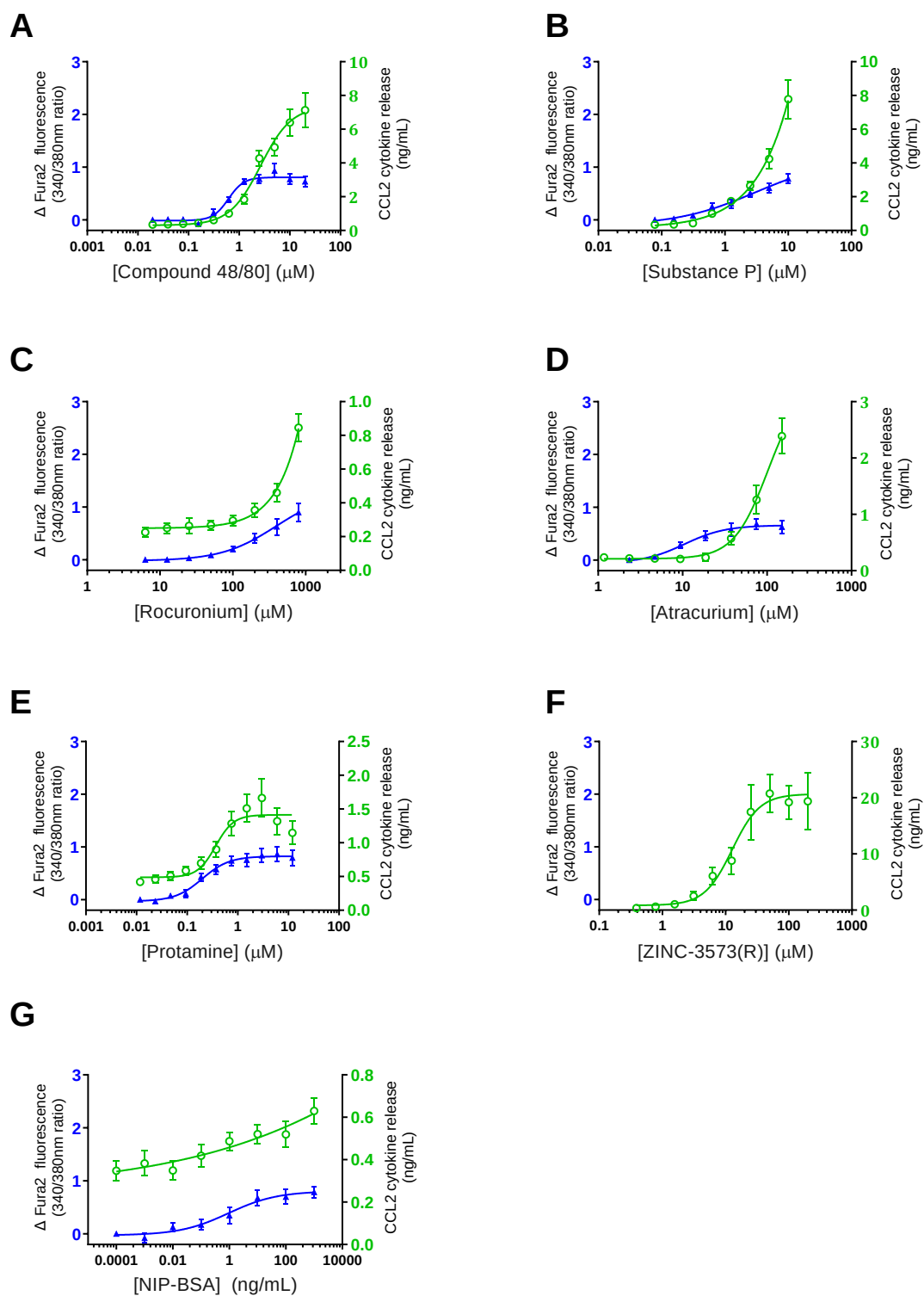


Figure 4.2: LAD2 cytokine CCL2 release does not always correlate to calcium mobilization. Peak calcium mobilization with baseline subtracted (Δ Fura-2 fluorescence; left y-axis) and CCL2 cytokine release (right y-axis) were quantified from LAD2 human mast cells stimulated with a range of MRGRPX2 agonists, compound 48/80 (A), substance P (B), rocuronium (C), atracurium (D), protamine (E), ZINC-3573(R) (F), and the Fc ϵ RI activator NIP-BSA (G). Basal release of CCL2 was 0.35 ± 0.08 ng/mL. Data presented as mean $\pm$ SEM. N=5-12.

4.3.2 Phosphorylation of ERK is not solely dependent on calcium mobilization

In these studies, a single concentration of stimuli was chosen that elicited a Δ calcium mobilization ratio of approximately 0.7 (340/380 nm). Due to interference with Fura2 readings, we were unable to quantify calcium mobilization responses of ZINC-3573(R). Therefore, for ZINC-3573(R), comparative responses in **Table 4-1** were normalised to the concentration that induced approximately 30% degranulation, similar to that induced by the presumed full agonist compound 48/80 when matched to a Δ calcium mobilization ratio of 0.7.

Representative Western blot images with ERK bands (42/44 kD) shown are as in **Figure 4.3**. Western blot images were quantified for pixel density using Image J (FIJI) (described in methods **section 4.2.2** above). As summarised in **Table 4-1**, the normalization of calcium mobilization between stimuli did not result in a consistent ERK phosphorylation response. While compound 48/80 (pixel density $\pm$ SEM; 0.42 ± 0.08), ZINC-3573(R) (0.84 ± 0.19) and NIP-BSA (0.45 ± 0.11) induced significant phosphorylation of ERK when compared to basal, other MRGPRX2 agonists (rocuronium, atracurium, protamine, and substance P) did not (**Figure 4.4**).

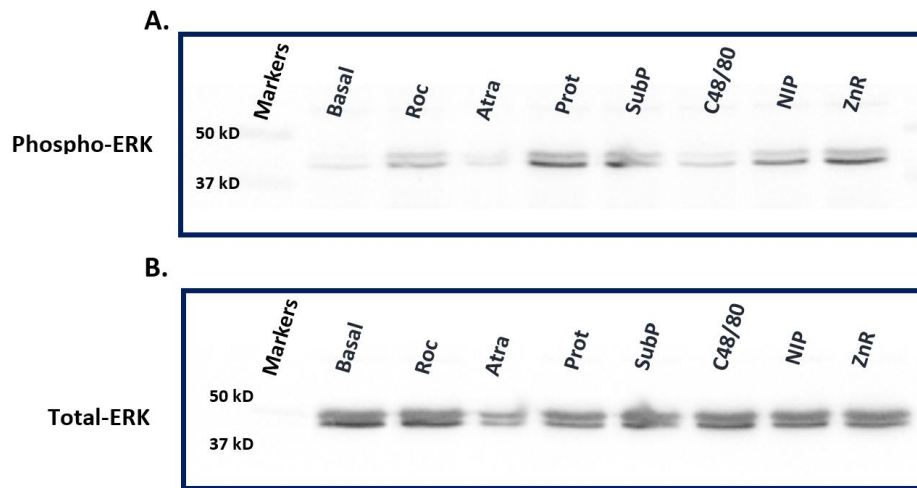


Figure 4.3: Representative Western blots of stimulated LAD2 cells detected with anti-ERK (phospho/total) antibodies. LAD2 cells were stimulated with rocuronium (680 μ M; Roc), atracurium (50 μ M; Atra), protamine (0.3 μ M; Prot), substance P (2.5 μ M; SubP), compound 48/80 (0.4 μ g/mL; C48/80), ZINC-3573(R) (5 μ M; ZnR), and NIP-BSA (5 ng/mL; NIP) for a period of 4 hours. “Basal” represents the basal expression in unstimulated LAD2 cells. Nitrocellulose membranes were stained with phospho-ERK (phosphorylated ERK) antibody, imaged, stripped, re-probed with total-ERK antibody and re-imaged. Blots were imaged using the ChemiDOC™ (Bio-Rad). **A.** phosphorylated ERK. **B.** total ERK.

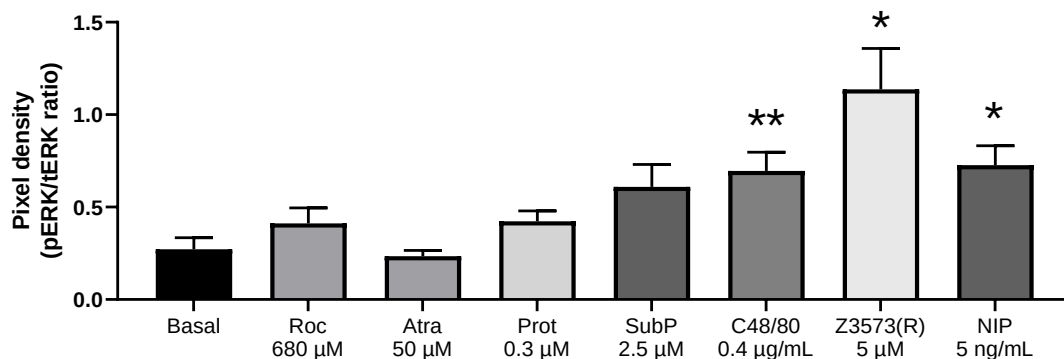


Figure 4.4. Quantification of ERK phosphorylation in LAD2 cells stimulated with a range of agonists normalized to a fixed calcium mobilization ratio. LAD2 cells were stimulated with rocuronium (680 μ M; Roc), atracurium (50 μ M; Atra), protamine (0.3 μ M; Prot), substance P (2.5 μ M; SubP), compound 48/80 (0.4 μ g/mL; C48/80), and NIP-BSA (5 ng/mL; NIP). Concentrations were chosen based on a fixed Δ calcium mobilization ratio of 0.7. ZINC-3573(R) concentration (5 μ M) was chosen based on a ~30% degranulation response induced by the assumed full agonist compound 48/80. “Basal” represents the basal expression in unstimulated LAD2 cells. Band intensities from imaged blots were quantified and expressed as a ratio of pERK/ tERK. Pixel densities from western blot images represented in Figure 4.3 were quantified using Image J (Fiji). Data represented as mean $\pm$ SEM; N $\geq$ 5. One-way ANOVA was conducted with Dunnett’s multiple comparison post hoc test. Each pERK/tERK ratio was compared to that of the basal ratio; * $p < 0.05$; ** $p < 0.001$.

Stimulus	Concentration	Calcium mobilization (FURA2 340/380nm)	Degranulation (% β -hex from total - basal)	CCL2 release minus basal (ng/mL)	ERK phosphorylation (p-ERK/t-ERK minus basal) $\pm$ SEM
Rocuronium	680 μ M	0.7	1	0.72	0.14 $\pm$ 0.08
Atracurium	50 μ M	0.7	2	0.54	-0.04 $\pm$ 0.05
Protamine	0.3 μ M	0.7	29	0.84	0.15 $\pm$ 0.08
Substance P	2.5 μ M	0.7	33.7	2.59	0.35 $\pm$ 0.10
Compound 48/80	0.8 μ M	0.7	30	1.33	0.42 $\pm$ 0.08
ZINC-3573(R)	5 μ M	-	29	3.51	0.84 $\pm$ 0.19
NIP-BSA	5ng/mL	0.7	47	0.49	0.45 $\pm$ 0.11

Table 4-1: Mean degranulation, CCL2 release and ERK phosphorylation response to a matched Δ calcium mobilization ratio. Rocuronium (680 μ M), atracurium (50 μ M), protamine (0.3 μ M), substance P (2.5 μ M), compound 48/80 (0.4 μ g/mL), and NIP-BSA (5 ng/mL) were matched to a fixed Δ calcium mobilization ratio (0.7). ZINC-3573(R) concentration (5 μ M) was chosen based on a ~30% degranulation response induced by the known full agonist compound 48/80. Mean calcium, degranulation and CCL2 readings were interpolated from Figure 4.1 and 4.2.

4.3.3 Analysing evidence of bias within MRGPRX2 agonists

The Black Leff operational model ⁽²⁸⁹⁾ was used to analyse the presence (if any) of ligand bias with MRGPRX2 agonists. The outcomes quantified were calcium mobilization (Fura-2 340/380 nm ratio), degranulation (% β -hexosaminidase release) and cytokine (CCL2) release. As mentioned earlier, Log R values (transduction ratios) were calculated using PRISM 9.1 and each agonist response was normalised to the reference agonist (compound 48/80) and a reference pathway (calcium mobilization).

Compound 48/80 was chosen as the reference full agonist as it induced consistently high responses across all activation markers tested, as required by the model. The agonists analysed in this chapter include substance P, rocuronium, atracurium and protamine. ZINC-3573(R) was not included for the purpose of this analysis for two reasons. Firstly, we were unable to quantify calcium mobilization (a key reference pathway in our analysis) due to interference with the assays used (we unsuccessfully attempted quantification using Fura-2AM, FLUO4 and FLUO8; data not shown). Secondly, although ZINC-3573(R) induced similar degranulation to the reference full agonist compound 48/80, it induced significantly greater CCL2 release. The reason underlying this almost 3-fold greater induction of CCL2 release by ZINC-3573 is unclear. Whilst interesting, due to this discrepancy in activation by ZINC-3573(R), it did not fit the Black-Leff model which requires the reference agonist to elicit maximal responses in all markers quantified.

Using this model, substance P appears to be a full agonist, responding with similar efficacy to compound 48/80 (**Figure 4.5** and **Figure 4.6**). Contrastingly, protamine is a clear partial agonist, eliciting all responses (calcium mobilisation, degranulation, and cytokine release) with a lower maximum efficacy (E_{max}) than that of the full agonist, compound 48/80. Efficacy of rocuronium and atracurium remain indeterminant due to incomplete concentration response curves (primarily due to the lack of solubility at higher concentrations of the agonists).

Inferences on potencies were difficult to make due to the incomplete concentration response curves, especially in degranulation and cytokine release. Nevertheless, it is evident that substance P (pEC_{50} (calcium mobilization) = 5.99) has a greater potency than atracurium (pEC_{50} (calcium mobilization) = 5.12) which shows greater potency than rocuronium (pEC_{50} (calcium mobilization) = 3.28) (**Table 4-2**). This trend is also evident in degranulation and cytokine release quantified (**Table 4-2, Figure 4.6**). Although protamine appears to have marginally higher potency than compound 48/80, it is important to highlight that both protamine and compound 48/80 are polymers, and therefore potency is difficult to establish due to the likely inaccuracies in molecular weight determination, thereby making it difficult to accurately calculate molar concentration.

Interestingly, while the potencies of some agonists (compound 48/80, substance P and protamine) were similar between calcium mobilization, degranulation, and cytokine release, other agonists (rocuronium and atracurium) displayed stark differences (**Table 4-2**). These differences are reflected in log transduction ratios (a function of agonist potency) calculated as log (bias) in **Figure 4.7**. Both rocuronium and atracurium showed significant bias against degranulation and cytokine release in relation to calcium mobilization while substance P and protamine were unbiased agonists at MRGPRX2 when compared to that of compound 48/80 (**Figure 4.7**).

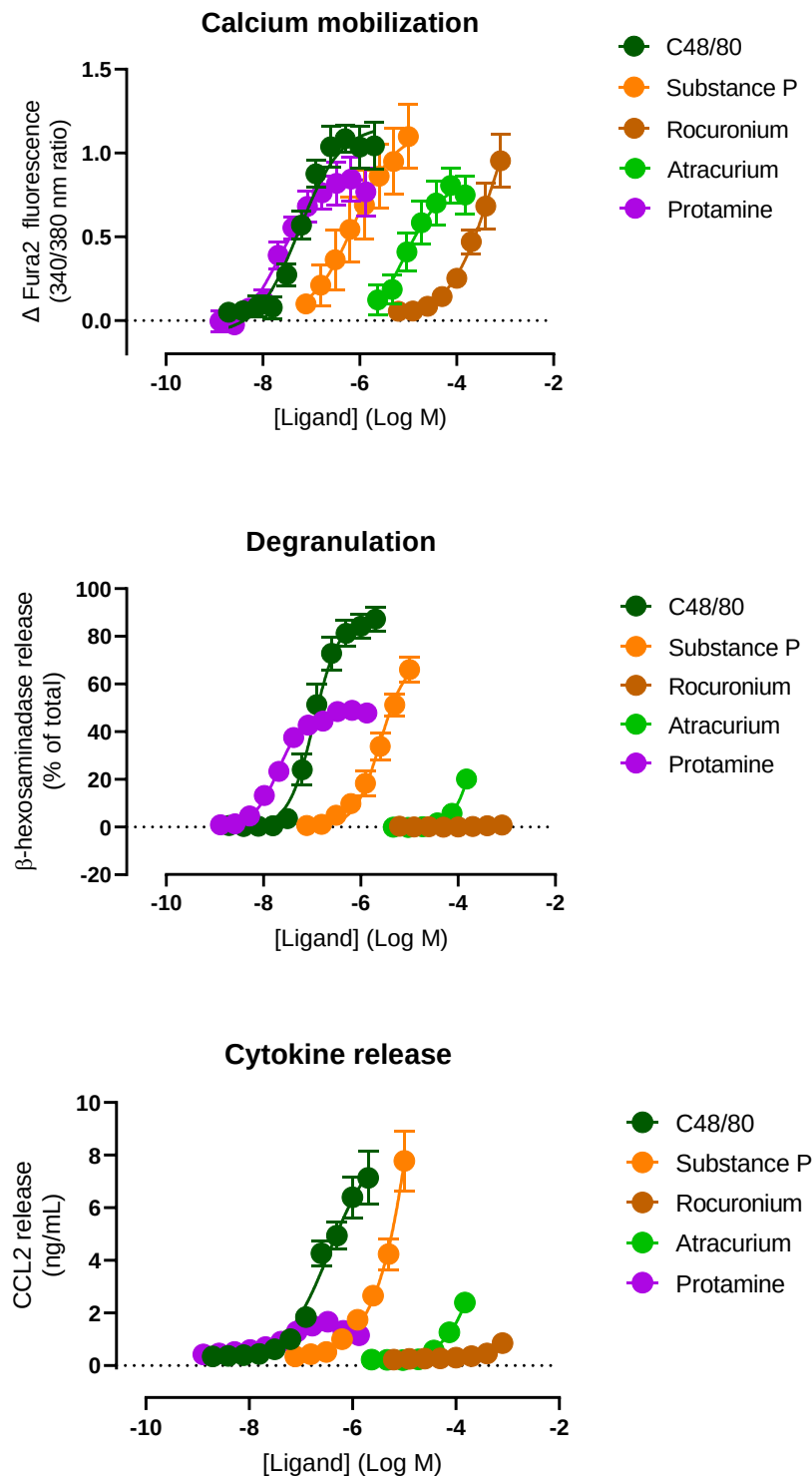


Figure 4.5: Comparison of concentration response curves of MRGPRX2 agonists used in the analysis of agonist bias. LAD2 cells were stimulated with MRGPRX2 agonists compound 48/80 (C48/80), substance P, rocuronium, atracurium and protamine. Three outcomes were quantified for the analysis. Peak calcium mobilization was quantified using Fura-2 fluorescence ratio (represented as change from baseline; Δ 340/380 nm), degranulation was quantified using the release of β -hexosaminidase (% of total cellular enzyme), and CCL2 release was quantified using ELISA. Results shown are mean $\pm$ SEM, $n \geq 5$.

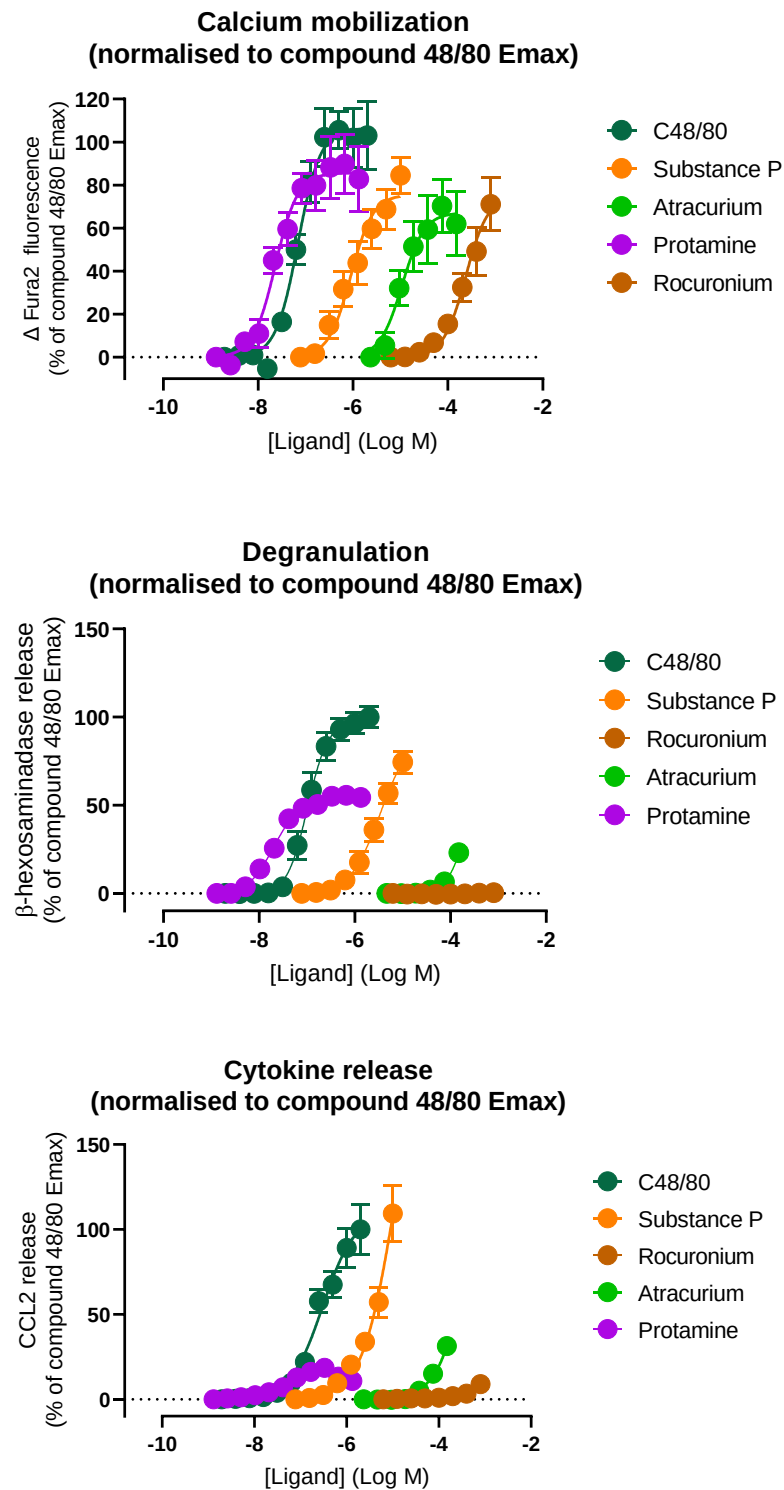


Figure 4.6: Comparison of concentration response curves of MRGPRX2 agonists normalised to compound 48/80 Emax. LAD2 cells were stimulated with MRGPRX2 agonists compound 48/80 (C48/80), substance P, rocuronium, atracurium and protamine. Three outcomes were quantified for the analysis. Peak calcium mobilization was quantified using Fura-2 fluorescence ratio (340/380 nm), degranulation was quantified using the release of β -hexosaminidase (% of total cellular enzyme), and CCL2 release was quantified using ELISA. Each drug response was normalised to that of the assumed full agonist, compound 48/80. Results shown are mean $\pm$ SEM, $n \geq 5$.

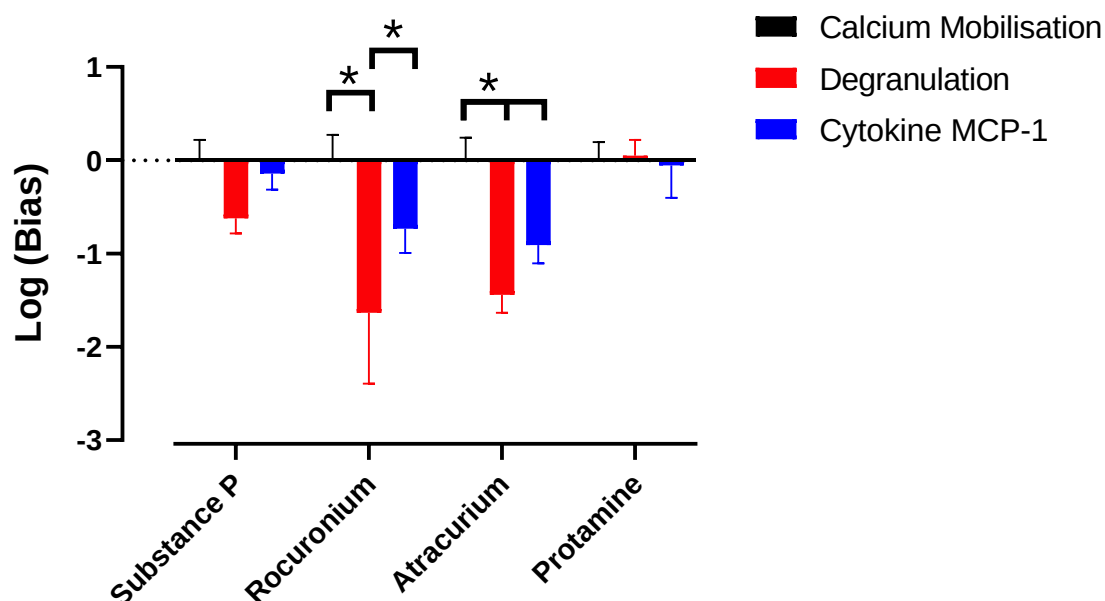


Figure 4.7: Log bias quantified using the Black-Leff operational model of degranulation and cytokine release relative to calcium mobilization. LAD2 cells were stimulated with the MRGPRX2 agonists substance P, rocuronium, atracurium and protamine. Each response was normalised to that of the full agonist, compound 48/80. Log bias ($\Delta\Delta \log R$) was calculated using the Black-Leff operational model on PRISM version 9.1. * $p < 0.05$.

Mast cell activators	Calcium mobilization pEC_{50} (M)	Degranulation pEC_{50} (M)	Cytokine pEC_{50} (M)
Rocuronium	3.28	*ND (>3)	*ND (>3)
Atracurium	5.12	*ND (>4)	*ND (>4)
Protamine	7.73	7.69	7.54
Substance P	5.99	5.47	4.41
Compound 48/80	7.25	6.99	6.45
ZINC-3573(R)	*ND	5.23	4.88

*ND- Not Determined

Table 4-2: Summary of MRGPRX2 agonist potencies in calcium mobilization, degranulation, and cytokine CCL2 release. LAD2 mast cells were stimulated with a range on MRGPRX2 agonists and concentration response curves were fitted according to a three-parameter non-linear fit using PRISM version 9.1. Where agonists did not elicit maximal responses, pEC_{50} values were not established (denoted "ND").

4.3.4 PMA, a protein kinase C activator, has no effect on stimulus-induced LAD2 degranulation

As mentioned earlier, the signalling cascade between calcium mobilization and degranulation is not thoroughly characterised. However, to address why rocuronium is unable to cause degranulation, although it results in significant calcium mobilization, we looked at influencing known factors that affect degranulation. One such factor is protein kinase C (PKC), inducing both calcium-dependent and -independent signalling, depending on the isoform. Although in theory the presence of calcium mobilization via IP₃ should produce equivalent DAG signalling to activate calcium-independent PKC, this correlation has not been proven. Therefore, to investigate the role on PKC in translation of calcium mobilization to degranulation, we co-stimulated LAD2 cells with the PKC activator, phorbol 12-myristate 13-acetate (PMA), and a range of mast cell activators.

Concentrations of stimuli that induced sub-maximal degranulation were chosen to observe an effect, if any, of the non-specific PKC activator, PMA. In our study, we observed that when co-stimulated, there was no effect on rocuronium, or other stimulus-induced degranulation, whether triggered through MRGPRX2 or FcεRI/IgE pathways (**Figure 4.8**).

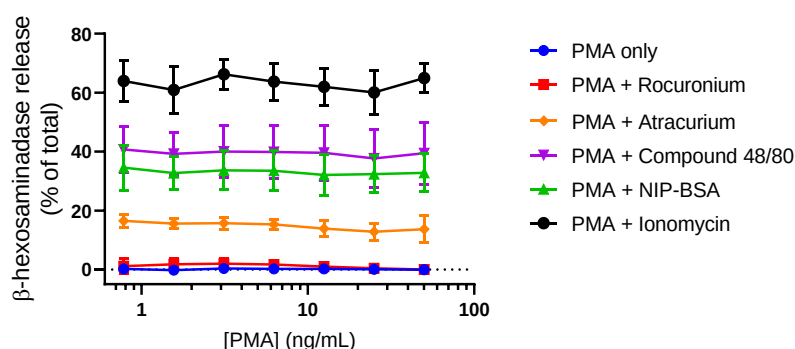


Figure 4.8: Co-stimulation with PMA did not increase stimulus-induced LAD2 degranulation. A concentration range of PMA (0.78- 50 ng/mL) was used to co-stimulate LAD2 mast cells with a fixed submaximal concentration of rocuronium (200 μ M), NIP-BSA (100 ng/mL), compound 48/80 (3 μ g/mL), atracurium (200 μ M), and ionomycin (0.5 μ M). The stimuli were pre-mixed and added to LAD2 cells and incubated for 45 minutes. LAD2 degranulation was quantified using the β -hexosaminidase assay. Triton X was used to lyse cells to quantitate total cellular β -hexosaminidase, from which the percentage of total enzyme was calculated for each stimulus. Data presented as mean $\pm$ SEM, $n \geq 3$. One-way ANOVA was conducted with Dunnett's multiple comparison post hoc test. Each response with PMA was compared to that without.

4.3.5 Effect of calcium-dependent PKC inhibition by GF109203X on stimulus-induced LAD2 activation

In this study, we incubated LAD2 cells with the calcium-dependent PKC inhibitor, GF109203X, for 10 minutes prior to stimulus addition. GF109203X, at low concentrations (<50 μM ; tested in human platelets and Swiss 3T3 fibroblasts), is a selective and potent calcium-dependent PKC inhibitor while at high concentrations, inhibits all PKC isoforms along with other kinases⁽²⁹⁰⁾. Concentrations of GF109203X above 10 nM resulted in a decrease in stimulus-induced degranulation (**Figure 4.9**), with a concentration of 10 μM virtually ablating the response. This effect is consistent between both the IgE-Fc ϵ RI agonist (NIP-BSA; $\log\text{IC}_{50} = -7.05$) and MRGPRX2 agonists; compound 48/80 ($\log\text{IC}_{50} = -6.59$), atracurium ($\log\text{IC}_{50} = -6.73$), protamine ($\log\text{IC}_{50} = -6.54$), ZINC-35673(R) ($\log\text{IC}_{50} = -6.97$), and substance P ($\log\text{IC}_{50} = -6.69$). Ionomycin, a calcium ionophore facilitating direct calcium mobilization, independent of a receptor mediated pathway, also followed a similar trend in GF109203X-induced inhibition with a $\log\text{IC}_{50} -4.40$. The inhibition of rocuronium-induced MRGPRX2 activation was not quantified in this study as rocuronium does not induce LAD2 degranulation.

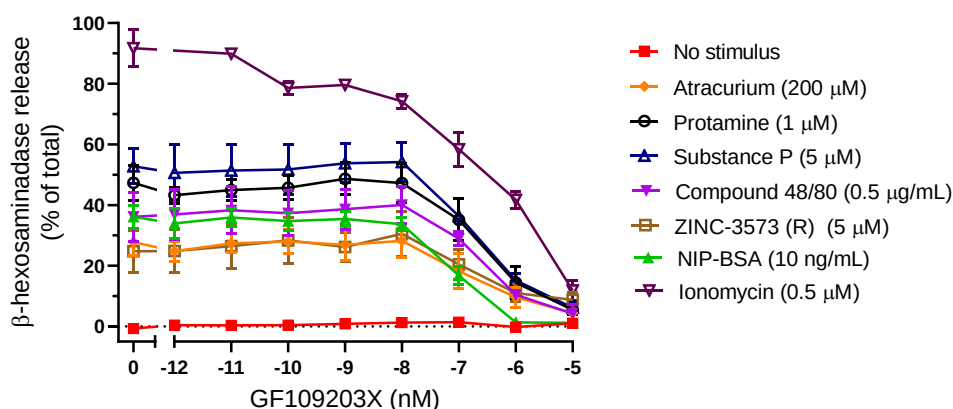


Figure 4.9: Inhibition of calcium-dependent PKC by GF109203X had no differential effect between the mast cell activators. LAD2 cells were incubated for 10 minutes with GF109203X (0.001 – 10,000 nM), and stimulated with atracurium (200 μM), protamine (1 μM), substance P (5 μM), compound 48/90 (0.5 $\mu\text{g/mL}$), ZINC-3573(R) (5 μM), NIP-BSA (10 ng/mL) and ionomycin (0.5 nM). Degranulation was quantified with the enzyme, β -hexosaminidase. Data presented as mean $\pm$ SEM, $n=3-4$. One-way ANOVA was conducted with Dunnet's multiple comparison post-hoc test. Each response in the presence of GF109203X was compared to that without the inhibitor (0 nM GF109203X).

4.4 Discussion

Whilst agonists such as compound 48/80 and substance P induce a concentration-dependent calcium mobilization response that promulgated productive degranulation, the significant calcium mobilisation induced by another MRGPRX2 agonist, rocuronium, did not lead to degranulation (also mentioned in Chapter 3). This led us to explore the likelihood of different agonists inducing different signalling cascades with the possibility that this could trigger functional responses of markedly different magnitudes. In doing so, we aimed to investigate whether a given individual would be equally susceptible to MRGPRX2-dependent adverse reactions (if any) by all agonists of the receptor. In this chapter, we demonstrate preliminary evidence for biased MRGPRX2 signalling in response to various agonists including endogenous compounds, therapeutically used drugs, and laboratory tools.

MRGPRX2 is known to couple to both Gi and Gq proteins. Although calcium mobilization is known to be primarily dependent on Gq coupling, degranulation and cytokine release is found to be influenced by Gi coupling in human LAD2 mast cells ⁽¹⁰⁵⁾. We analysed the possibility of biased agonism by quantifying agonist-induced concentration response curves and measuring calcium mobilization, degranulation, and cytokine (CCL2) release. In this study, we also looked at the phosphorylation of the MAP kinase, ERK, in response to a fixed concentration of agonist that induces a standardised calcium mobilization response.

Our study demonstrates that the endogenous agonist substance P was more potent at MRGPRX2 than the therapeutic drugs, atracurium and rocuronium (order of potency: substance P > atracurium > rocuronium). This observation is consistent with those in other published studies conducted using HEK-293 cells expressing MRGPRX2 ⁽⁹⁵⁾.

While the determination of potencies for the above agonists is reasonable, it remains difficult for polymers where molecular weight is hard to accurately measure and is likely inconsistent. The average molecular weight of protamine isolated from fish sperm is estimated to be 4,500 Da ⁽²²¹⁾. While we used the above for the calculation of molar concentrations in our study,

other studies suggest the molecular weight of protamine to be as high as 10,000 Da ^(291, 292). Therefore, the determination of potency in comparison to the full agonist compound 48/80, also a polymer (with an average molecular weight of 500 Da) and subject to similar considerations, remains a best estimate.

Our findings suggest that compound 48/80 and substance P act as ‘full agonists’ at the MRGPRX2 receptor, eliciting high efficacy responses in calcium mobilization, degranulation and cytokine release. Further, we identified protamine, with submaximal efficacy in comparison to compound 48/80 and substance P, to be a partial agonist at MRGPRX2. Rocuronium and atracurium at the concentrations tested did not induce a plateaued degranulation and cytokine release (higher concentrations were unfeasible given the drugs’ limited solubility in aqueous solution). Therefore, their maximal efficacy, and hence whether they are full or partial agonists, could not be verified. Following this observation, we quantified the bias using the Black-Leff operational model with compound 48/80 used as the reference full agonist. Relative to calcium mobilization, the NMBAs (rocuronium and atracurium) display bias against degranulation and cytokine release when compared with the presumed full agonist compound 48/80.

As mentioned in **section 1.1.12**, studies conducted in both Australia and UK demonstrate that rocuronium causes more frequent events of anaphylaxis than atracurium ^(149, 151). Our observation in potency of NMBAs in activating MRGPRX2 in mast cells, goes against this clinical observation. However, it is important to highlight that the frequency of anaphylaxis has not been stratified to IgE-dependent and MRGPRX2-dependent events. Thus, rocuronium might be more antigenic and thus more likely to trigger IgE-dependent anaphylaxis. We hypothesise that drug-dependent anaphylactic events, would be more prominent with increasing potency at MRGPRX2. However, clinically characterising MRGPRX2-dependent anaphylaxis is currently difficult given the lack of a definitive test that distinguishes MRGPRX2-dependent events from IgE-dependent cases (this is discussed further in chapter 5).

We have also demonstrated a bias in ERK-phosphorylation, recognized to be predominantly dependent on the Gi-coupling of MRGPRX2 ⁽¹⁷⁾. When matched to a similar extent of Gq coupling (quantified by calcium mobilization), ERK-phosphorylation was found to vary significantly between MRGPRX2 agonists with the NMBAs not inducing any ERK phosphorylation above basal levels. Cytokine release is also known to be Gi-dependent in human mast cells and downstream of MAPK pathways (*e.g.*, ERK) phosphorylation ⁽²⁹³⁾. As evident in our analysis using the Black-Leff operational model, rocuronium and atracurium showed significant bias against cytokine release based on their calcium mobilization response.

Although speculative, these observations may suggest a possible bias between Gi and Gq activation by MRGPRX2 upon binding with some agonists, *e.g.*, the NMBAs. Further experiments to quantify second messengers downstream of Gi-activation are needed to validate this possibility. Our attempts to quantify Gi activity through measuring a reduction in cAMP levels upon activation of MRGPRX2 require further optimisation. In these preliminary studies (data not shown) we were unable to induce substantial cAMP (using forskolin) in order to observe an inhibition through Gi signalling. However, with further assay optimisation, quantifying cAMP inhibition by Gi would be important to shed light on any signalling bias at the level of Gq and Gi. Even so, the possibility of an alternative origin of bias (*e.g.*, β -arrestin bias) cannot be discounted, and is worthy of investigation. As mentioned earlier, the agonist substance P was found to be biased against β -arrestin-mediated MRGPRX2 desensitisation in human skin mast cells ⁽²⁷³⁾. However, in the outcomes quantified in this study, substance P was characterised an unbiased agonist. The differences in MRGPRX2 models used between the studies may play a role in this observation; perhaps LAD2 cells differ from skin mast cells in its β -arrestin signalling and thereby masks the presence of β -arrestin bias. Further characterisation of direct β -arrestin signalling, perhaps using the PRESTO-Tango system ⁽²⁷³⁾, is required to analyse the presence of β -arrestin bias in our LAD2 cell model.

Intriguingly, as mentioned above, our studies point to protamine being a clear partial agonist, with its efficacy (as measured by the maximum response achieved) across all parameters evaluated, consistently lower than the full agonist, compound 48/80. This is interesting as protamine is primarily composed of poly(arginine) and so would be expected to perhaps act in a similar manner to the other polymeric activator of MRGPRX2, compound 48/80. That this is not the case, suggests again that there is some level of specific interaction with the receptor needed for maximal downstream activation. More detailed signalling events activated by protamine need to be measured to better identify these pathways that dictate the magnitude of mast cell functional response.

We were unable to quantify calcium mobilization of the newly characterised MRGPRX2 agonist, ZINC-3573(R), due to interference with the Fura-2 assay. We also tried a series of other calcium indicators (*e.g.*, Fluo4 and Fluo8) but similarly ZINC-3573(R) interfered with these assays. The original assay describing ZINC-3573(R) activity used the FLIPR® system, and so in future studies it might be useful to compare our agonists using this system ⁽⁹⁵⁾. Nevertheless, our data show that ZINC-3573(R) induced significantly greater (~ 3-fold) cytokine CCL2 release compared to the known full agonist, compound 48/80. This may indicate biased activation of MRGPRX2 resulting in a much greater activation in the pathway leading to cytokine release. Supporting evidence of this was also seen with ERK phosphorylation by ZINC-3573(R) being greater than compound 48/80 when normalised for magnitude of degranulation. Since both ERK phosphorylation and cytokine release are a known function of Gi signalling, this observation may indicate a G protein subset bias where ZINC-3573(R) favours activation of Gi over Gq. Alternatively, both ERK and cytokine release are also a function of β -arresting signalling ⁽²⁹⁴⁾ and may allude to ZINC-3573(R) being a β -arrestin biased ligand.

As mentioned earlier, a recent study revealed that some MRGPRX2 agonists (*e.g.*, substance P) were β -arrestin biased ligands compared to others (*e.g.*, compound 48/80 and codeine), thereby leading to greater receptor internalisation following activation ⁽²⁷³⁾. However, in the

original study characterising ZINC-3573(R) by Lansu et al., 2017, both calcium mobilization and β -arrestin signalling were quantified, but no bias was evident ⁽⁹⁵⁾. However, an alternative explanation may underlie this effect. Lansu et al., 2017, also note that ZINC-3573(R), although highly specific to MRGPRX2, did induce some non-specific GPCR activation (*e.g.*, α -1A adrenergic receptor, dopamine receptor D4 and protease activated receptor 2) at much higher concentrations ⁽⁹⁵⁾. Follow up experiments with LAD2 MRGPRX2 KD cells and the inactive enantiomer, ZINC-3573(S), would aid in the understanding if possible non-MRGPRX2-specific activation of mast cells by ZINC-3573(R) (whether receptor-dependent or independent) can explain the observed greater cytokine release.

Given our inability to measure a calcium response with ZINC-3573(R) and that the Black-Leff operational model requires a reference agonist that possess highest efficacy in all outcomes tested, we were unable to include ZINC-3573(R) in our biased agonism analysis. Future studies that focus on a single functional output (*e.g.*, cytokine release) and analyse multiple signalling pathways (*e.g.*, β -arrestin, cAMP, IP₁) will help reveal if ZINC-3573(R) is truly biased towards cytokine responses and will reveal the molecular mechanism behind this. This could be of value in identifying novel MRGPRX2 agonist-drugs that may cause adverse effects not through overt degranulation, but rather through perhaps seemingly occult release of cytokines from mast cells.

In this chapter, we also explored the possibility of whether an inadequate or imbalanced activation of PKC isoforms could explain the above observed functional bias between MRGPRX2 agonists, with specifically the inability of rocuronium to induce degranulation being our main question. Specific isoforms of PKC have long been suggested to influence mast cell activation ⁽²⁸⁴⁾. Broadly, the activation of PKC is linked to increased degranulation. Therefore, in our initial study, we investigated if co-stimulation with PMA (a non-specific PKC activator) would increase MRGPRX2 agonist-mediated mast cell activation ⁽¹⁶²⁾, and perhaps facilitate rocuronium-mediated degranulation. However, in our study, we found no change in MRGPRX2 stimulus-induced mast cell activation when co-treated with a range of PMA

concentrations. This suggests either that PKC activation does not act as a limiting factor connecting MRGPRX2-dependent calcium mobilization and degranulation, or that the balanced activation of multiple PKC isoforms by PMA nullifies any enhancing effect as some PKC isoforms (PKC δ) are suggested to be more important in inducing degranulation than others.

In order to examine the latter possibility, we used a selective calcium dependent PKC inhibitor, GF109203X. Studies suggest that the activation and repression of specific PKC isoforms significantly alter degranulation ⁽²⁸⁷⁾. Broadly, there are 3 types of PKC isoforms (**section 4.1.3**); calcium dependent, calcium independent and atypical isoforms ⁽²⁹⁵⁾. Calcium mobilization has consistently been identified as an essential precursor for mast cell degranulation ⁽²⁹⁶⁾. Interestingly, the calcium independent isoforms, PKC δ and θ , have been implicated in the enhancement of mast cell degranulation ^(286, 287). Since rocuronium does induce significant calcium mobilization, we aimed to explore the role of calcium independent PKC activation in this translation to degranulation.

In our study, we intended to investigate if there were differences in the sensitivity of MRGPRX2 agonists to different PKC isoforms, especially the calcium-independent isoforms, thereby suggesting a potential PKC bias in the MRGPRX2 signalling cascade. In this study, we used a specific inhibitor of calcium-dependent PKC (GF109203X) to isolate and observe the role of calcium-independent PKC. We also examined its effect on the IgE-dependent pathway of mast cell activation and the receptor independent activation using the calcium ionophore ionomycin. Traditionally, ionomycin is used in combination with PMA (non-specific PKC activator,) to activate cells and induce release of mediators independent of receptors ⁽²⁹⁷⁾. However, this is not necessarily the case in some mast cells ⁽²⁹⁸⁾. In our hands, LAD2 cells degranulated significantly when stimulated with ionomycin in the absence of PMA.

As mentioned earlier, GF109203X, at low concentrations (<50 nM), is a selective and potent inhibitor of calcium-dependent PKC. However, at high concentrations (>500 nM), inhibits all

PKC isoforms (and other kinases) ^(287, 290). It is important to mention that, although GF109203X has been studied extensively in a rat basophilic line (RBL-2H3) the concentrations required for isoform-specific inhibition in a different species and cell type (human LAD2 mast cells) is unclear. Although GF109203X has not been studied in human mast cells, a change in response between the different activators in the presence of the inhibitor, would highlight evidence for bias in the role of calcium-independent PKC isoforms in MRGPRX2-mediated degranulation. However, we observed a consistent inhibition of all stimulus-induced degranulation by GF109203X suggesting against a defining factor in PKC signalling that introduces biased MRGPRX2 signalling. However, further studies, perhaps using the more precise approach of siRNA knockdown of PKC isoforms, are required to define the role of calcium-independent PKC isoforms in MRGPRX2 agonists that induce equivalent calcium mobilization without degranulation. The observed complete ablation of degranulation from the higher concentrations of the inhibitor (>1 μ M) would likely be a result of non-selective kinase inhibition, including that of myosin light chain kinase (MLCK) ⁽²⁹⁰⁾, an essential component for the translocation of mast cell granules for degranulation ⁽²⁹⁹⁾.

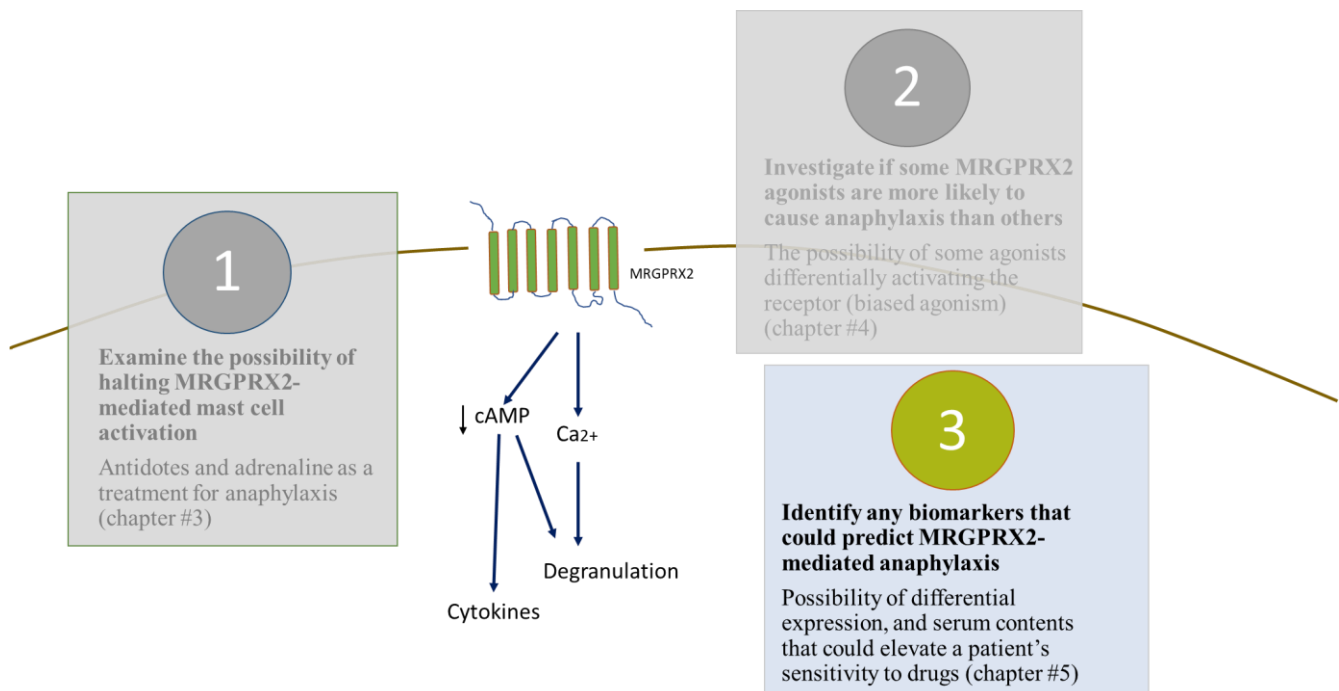
Therefore, based on these observations, despite the difficulties in interpretation of PKC inhibitors, the broad activation or inhibition of PKC subtypes may not be the defining factor that suppresses the degranulatory response of rocuronium and atracurium.

In summary, in this chapter we have outlined some evidence for ligand-induced bias in MRGPRX2 activation. The NMBA agonists, rocuronium and atracurium, are biased against degranulation and cytokine release (compared to their calcium mobilization response) as opposed to the full agonists compound 48/80, substance P, and the partial agonist protamine. We propose that this may be attributable to a G protein bias where although all agonists activate Gq equally, some agonists (*e.g.*, compound 48/80) activate greater Gi signalling, and that both pathways are needed for effective degranulation. To understand the role of Gi signalling in the correlation of calcium mobilization to degranulation, in addition to quantifying cAMP levels, further experiments with an adenylyl cyclase (responsible for the

catalysis of adenosine triphosphate to cAMP) inhibitor (*e.g.*, SQ22536, to mimic some level of G_i signalling) alongside rocuronium stimulation would be insightful. While this study highlights preliminary work in the field of biased agonism at MRGPRX2, the better understanding of this bias may open up the possibility for designer drugs, for instance, NMBAs that effectively antagonise nicotinic receptors while perhaps inducing MRGPRX2 internalisation.

Chapter 5

Investigating the role of MRGPRX2 receptor expression and serum components in patients who experienced anaphylaxis during surgery



5.1 Chapter overview

Although drug-induced anaphylaxis is an established clinical risk, the reasons underlying why only some people react adversely to certain drugs is poorly understood. A wide variety of factors, both intrinsic and extrinsic, may influence the clinical response when a drug interacts with a target receptor ⁽³⁰⁰⁾. In most patients, drug exposure is clinically beneficial without an adverse response, but in susceptible individuals, drug administration may trigger a life-threatening anaphylactic reaction. The risk factors that predispose a patient to anaphylaxis are broad, but current evidence suggests mast cell activation plays a pivotal role in the initiation and evolution of the anaphylactic response ⁽³⁰¹⁾. For IgE-dependent reactions, patient susceptibility relates to complex contributing factors relevant to all allergic disease ⁽⁹⁾. For non-IgE-dependent anaphylaxis, recent interest has centred on genetic variations in mast cell receptor morphology, which may directly influence mast cell reactivity. In particular, we consider polymorphisms of the MRGPRX2 receptor, as well as receptor expression, and the influence of sensitizing serum factors as being possible predisposing factors for some patients to react adversely when exposed to some drugs.

5.1.1 Influence of differential receptor expression on receptor functionality

A wide range of disease states have been identified with differential receptor expression lending support to this hypothesis. For instance, asthma and chronic obstructive pulmonary disorder have been associated with an increased expression of FcεRI receptors in plasmacytoid dendritic cells ⁽³⁰²⁾. In theory, according to the law of mass action, increased receptor expression should result in increased receptor activity, both in the stimulus-mediated response and constitutive activity ^(303, 304). However, this is not always the case. It has been found that the overexpression of one GPCR (even without activating it) can dampen the activity of other receptors ⁽³⁰³⁾. In addition, variations in expression of downstream signalling components or transcriptional factors could also contribute to altered receptor function. For instance, a selective deficiency in the tyrosine kinase, Syk, in basophils has been associated with the 'non-releaser' phenotype ⁽³⁰⁵⁾.

5.1.2 Influence of polymorphisms on receptor functionality

Diseases and disorders are also commonly associated with receptor polymorphisms. Common examples include the inherited BRCA gene associated with breast cancer ⁽³⁰⁶⁾ and the CFTR (cystic fibrosis conductance transmembrane regulator) gene associated with cystic fibrosis ⁽³⁰⁷⁾. More importantly, polymorphisms in mast cell receptors have also been identified as contributors to a variety of immune disorders. For example, vibrational urticaria has been associated with the mutant ADGRE2 gene ⁽¹¹⁶⁾ (refer to **section 1.1.9**), and mastocytosis is frequently linked with mutations in the c-Kit receptor ⁽³⁰⁸⁾. However, little is known on the link between the human mast cell receptor MRGPRX2 with diseases and disorders.

Lately, a significant amount of research has been directed towards the structure of MRGPRX2 and the understanding of amino acid residues essential for agonist binding, receptor activation and desensitisation. MRGPRX2 is classified as a class A GPCR, and due to the absence of an X-ray crystallography image, most structure-based research depend on homology modelling using other closely related class A GPCRs ^(95, 108). Using this approach with reference to the known structure of the κ -opioid receptor, Lansu et. al. (2017) discovered a MRGPRX2 selective agonist (ZINC-3573(R)) from a high-throughput screen of compounds from the ZINC database ⁽⁹⁵⁾. Another group used a different class A GPCR, C5a anaphylatoxin chemotactic receptor 1, to also predict MRGPRX2 structure and derive potential interaction sites of compound 48/80 ⁽¹⁰⁸⁾ (**Table 5-1**). Other groups have conducted point mutations based on conserved sites of class A GPCRs to determine the functionality of the receptor in the presence of specific mutations (**Table 5-1**) ^(94, 138).

Structurally, MRGPRX2 is a 7-transmembrane class A GPCR receptor (**Table 5-1**). Detailed investigation of MRGPRX2 polymorphism has identified important structure–function relationships in ligand interactions with particular residues within the receptor’s tertiary structure. A number of natural variants of MRGPRX2 have been identified with a recorded total of 1,706 single nucleotide variants, 64 copy number variations, 26 deletions and 15 insertions (Variation Viewer, National Institute of Health, Bethesda, US ⁽³⁰⁹⁾). Of these

mutations, 72 have been deemed potentially deleterious in the GPCR database (GPCR.db)⁽³¹⁰⁾. A few of these polymorphisms have been studied in *in vitro* experiments (**Table 5-1**) and were found to alter calcium mobilization and degranulation responses in transfected cells^(94, 311). From these findings, it has now been proposed that the amino acids E164 and D184 are important for opioid-agonists binding to MRGPRX2, with E164 deemed the essential amino acid required for all agonist-MRGPRX2 interactions^(95, 138). The amino acids G165 and D184 have been suggested to be essential in maintaining the agonist binding pocket integrity, hence if disrupted, will prevent agonist-mediated MRGPRX2 activation⁽⁹⁴⁾. Interestingly, disruption of S325 and L329, amino acids in the intracellular C-terminus, significantly increased agonist-mediated MRGPRX2 activation⁽³¹¹⁾. It has been suggested that phosphorylation of these amino acids may be required for receptor internalisation and desensitisation, and therefore by preventing this, may increase activation⁽³¹¹⁾. Although much research is conducted on the ligand interaction sites of MRGPRX2, the clinical consequence of activation of wild type or mutant MRGPRX2 is poorly understood.

It is evident however, that receptor polymorphisms, depending on their location within the protein's tertiary structure, can alter ligand affinity, efficacy, avidity, or even introduce bias in the downstream signalling cascade. Further, polymorphisms near the drug-binding pocket of class A GPCRs can shift the activity of partial agonists and antagonists to full agonists⁽²⁵⁶⁾. Meanwhile, polymorphisms near the G protein binding region have been associated with the transformation of transducer-coupling selectivity profiles leading to biased signalling⁽²⁵⁶⁾. Similarly, polymorphisms that alter β -arrestin binding and signalling could in turn alter receptor desensitisation⁽²⁵⁶⁾.

Polymorphisms in the gene promoter region have also been associated with altered gene expression, thereby altering receptor expression and subsequent activation of downstream signalling. An example of this lies within the polymorphism in the promoter region of Fc ϵ RI β that has been correlated with asthma susceptibility (by modifying the intensity in which mast

cells can be activated)⁽³¹²⁾. Similarly, a polymorphism in the promoter region of FcεRIα have been associated with aspirin-resistant chronic urticaria⁽³¹³⁾.

From a clinical perspective it is evident that variant polymorphism may explain why individual patients respond adversely to drug exposure. Of particular interest is the recent study reporting a causative link between an adverse drug reaction and a mutated MRGPRX2 receptor (reported triple polymorphism: M196P, L237P, L226P)⁽³¹⁴⁾. This described anaphylaxis in a surgical patient, on exposure to the muscle relaxant drug rocuronium. Whilst this single case supports the view that mast cell activation may be influenced by MRGPRX2 polymorphism, we believe further investigation is required to verify this association. In addition, a follow up study on this triple polymorphism using an RBL-2H3 transfection model suggest the lack of a causative link between the polymorphisms and rocuronium-induced mast cell activation⁽³¹⁰⁾. Therefore, further work is warranted to understand the role of the receptor and its polymorphisms in drug-induced adverse reactions.

5.1.3 Allosteric modulation of GPCR activity by serum and dietary factors

GPCR activation is also frequently associated with allosteric modulation⁽³¹⁵⁾. In this study we also hypothesise that serum factors may act as allosteric modulators and alter a patient's response to agonist exposure. Allosteric modulators can either increase the response of an orthosteric ligand by acting as a positive allosteric modulator, or decrease activity by acting as a negative allosteric modulator⁽¹⁶²⁾. There are also reports of 'neutral allosteric ligands' that do not modulate GPCR function but instead compete with positive and negative modulators for the allosteric binding site⁽³¹⁵⁾. In addition, allosteric modulators can alter intrinsic receptor function, for instance, by avoiding receptor desensitization⁽³¹⁵⁾. These ligands can be extremely diverse and include endogenous and exogenous compounds⁽³¹⁵⁾.

As mentioned earlier (**section 1.1.7**), MRGPRX2 is a class A GPCR, coupled to two G-proteins: Gi and Gq. Modulators of class A GPCRs include small molecules, proteins, ions, and lipids^(256, 315). Ions such as sodium, magnesium and manganese are found to play a role in altering GPCR

signalling in response to full agonists, partial agonists, and antagonists ^(300, 316-318). Some studies have reported sodium as a negative allosteric modulator of many class A GPCRs ⁽²⁵⁶⁾. Sodium ions inhibit the G_i/G_o coupled receptor signalling, thus inducing a G-protein signal bias ^(162, 317). G_i signalling is sometimes also associated with spontaneous inverse agonism, predominantly in the absence of sodium ions ^(316, 317). Intriguingly, activation of G_i by the dietary component, quercetin, has been associated with increased mast cell activation ⁽¹⁶²⁾.

Dietary components are frequently interlinked with disease states, for instance, dietary arachidonic and oleic acid has been associated with the development and progression of ulcerative colitis ^(319, 320). In the presence of the dietary component, quercetin, the activation of G_i was linked to an induction of compound 48/80-mediated activation in the otherwise unresponsive RBL-2H3 cells ⁽¹⁶²⁾. Quercetin is a flavonoid, a naturally occurring plant metabolite, found in a variety of common victuals including red wine, onions, and apples. Following consumption of quercetin-rich foods and quercetin supplements, quantifiable blood plasma concentrations have been noted (up to 50 and 430 nmol/L respectively) ^(321, 322). In addition, quercetin has a long half-life in blood plasma (11 – 28 hours) ⁽³²³⁾, suggesting its possible plasma presence even after the standard 2-8 hour preoperative fasting ⁽³²⁴⁾. The study on RBL-2H3 cells demonstrated the increase in compound 48/80-induced degranulation when the cells were pre-conditioned with quercetin for 48 hours ⁽¹⁶²⁾. However, in an acute setting where quercetin is added minutes prior to stimuli (and is not washed off), a decrease in MRGPRX2-mediated degranulation was noted in LAD2 mast cells ⁽¹⁵⁹⁾.

5.1.4 Difficulty in distinguishing IgE-independent anaphylactic events

While research into the possible relationship between MRGPRX2 and anaphylaxis is intriguing, it remains a complex undertaking. Firstly, the symptoms of anaphylaxis itself remain extremely broad, ranging from mild cutaneous signs to cardiac arrest. While extremes can be consistently clinically classified, those in between are often more nuanced. Although clear guidelines are available for the diagnosis of anaphylaxis, in a real-life event, this often proves difficult due to a variety of factors including comorbidities, panic/anxiety attacks and

asthmatic exacerbations⁽³²⁵⁾. This is especially prominent in grades I and II anaphylaxis where rashes and respiratory effects are the primary diagnostic features⁽³²⁵⁾. As a result, it is challenging to classify anaphylaxis according to respective grades, or even distinguish between unrelated conditions that present with related clinical symptoms. Secondly, even if anaphylaxis is clearly identified, there is a lack of clear cut distinguishers between IgE-dependent and independent reactions. At present, although the role of MRGPRX2 in activating mast cells *in vivo* rodent models has been well studied, its role in human anaphylaxis remains speculative.

The identification of anaphylactic events where MRGPRX2 activation is the root cause has proven difficult as there is no definitive test to confirm these speculations. Currently, skin prick tests (SPTs), although somewhat contradictory, are the primary basis to determine IgE-dependent anaphylaxis^(54, 152). In addition to SPTs, morphine-specific IgE is often used as an indicator of IgE against NMBAs^(53, 152). Morphine shares a quaternary ammonium ion (the most commonly identified antigenic epitope) with NMBAs⁽⁵³⁾. Therefore, the presence of IgE against morphine is considered indicative of IgE against NMBAs. The combination of a negative SPT and the absence of morphine specific IgE is the most commonly used gauge of non-IgE dependent anaphylaxis^(53, 54). However, this stance of negative SPTs being indicative of non-IgE mediated anaphylaxis is now contested with the characterisation of MRGPRX2^(152, 326). Human skin tissue is found to be rich in connective tissue type mast cells (MC_{TC}), therefore, would be expected to respond positively to MRGPRX2 agonists^(152, 326). However, the precise dosage required to activate mast cells via the IgE-dependent and independent mechanism by common activators is little understood⁽¹⁵²⁾. MRGPRX2 is considered a low-affinity receptor with an agonist concentration range in µg/mL required for activation, as opposed to FcεRI (IgE receptor) which is activated with agonist concentrations of ng/mL⁽¹⁵²⁾. Therefore, the validity of using SPTs in distinguishing IgE and non-IgE-dependent anaphylaxis is likely strongly concentration dependent and remain contested^(152, 326).

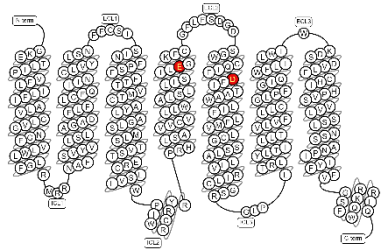
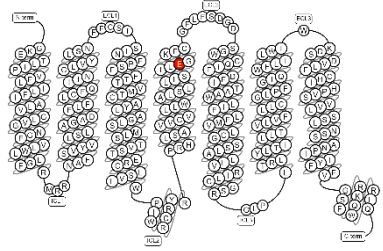
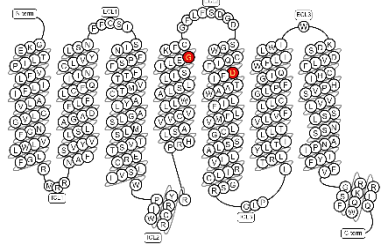
In Australia, after a patient recovers from anaphylaxis, they are requested to attend follow-up testing to gain further understanding of the culprit drug, and in doing so, prevent repeat exposure and recurrence of anaphylaxis ⁽³²⁵⁾. It should however be mentioned that post-operative testing is often ambiguous and an accurate understanding of the mechanism of anaphylaxis is challenging. In addition to the conventional follow up tests (SPTs, total IgE and tryptase levels), although rarely conducted, drug-specific-IgE can be quantified to be used as an alternative indicator of IgE-dependent anaphylaxis ^(327, 328). It is important to note that specific IgE is sometimes associated with a high occurrence of false positives, speculated to be primarily due to cross-reactive, but clinically irrelevant IgE that does not trigger anaphylaxis upon drug exposure ⁽³²⁸⁾.

In addition to the complexity of determining the role of MRGPRX2 in adverse reactions, the use of cellular and animal models display an added difficulty in the translation of pre-clinical studies to humans. MRGPR receptor activation is found to be inconsistently variant between species ⁽⁹⁶⁾. Although some agonists (*e.g.*, compound 48/80) activate orthologues of MRGPRX2 across a variety of species, other agonists are more selective. *In vitro* experiments done on HEK-293 cells expressed with MRGPRX2 and MRGPRB2 (mouse orthologue of human MRGPRX2) demonstrate the variability in MRGPR activation between humans and mice ⁽⁵⁸⁾. While agonists such as compound 48/80, substance P and cortistatin-14 display a significantly higher potency towards human MRGPRX2 activation compared to murine MRGPRB2, rocuronium was almost 12-fold less potent at the human MRGPRX2 receptor ⁽⁵⁸⁾. This introduces an additional hurdle in the clinical correlation of mast cell activation from animal models to humans.

5.1.5 Chapter aims

Whilst the above focus lies on follow-up testing after an event of anaphylaxis, it is important to note that at present there is no test that can be performed to identify patients at risk of anaphylaxis. The risk of an adverse drug reaction is ever present, and clinical vigilance, therefore, remains a vital aspect of management. The current challenge to research is how to identify susceptible patients. One approach is to study differences in mast cell activation between anaphylactic and control patients. This could perhaps explain the mechanism of why only some patients react adversely to drugs. Knowledge of this could also potentially facilitate development of a peri-operative test for screening susceptible individuals.

This chapter therefore focuses on the role of MRGPRX2 expression, and the presence of serum factors in mast cell activation. We have chosen to investigate surgical patients who have experienced anaphylaxis during surgery, and compare them with control patients undergoing similar surgery. We hypothesize that increased MRGPRX2 expression, or distinctive MRGPRX2 polymorphism, and/or the presence of serum factors, would result in increased mast cell sensitivity, and as a consequence, susceptibility to anaphylaxis.

Reference	Brief description of methods	Variants tested	Outcome	Proposed conclusion	Serpentine plot with highlighted amino acids	Additional remarks
(95)	Computer modelling using the κ -opioid receptor and Presto-Tango (MRGPRX2-TANGO) method with HTLA cells (HEK-293 cells stably expressing a β -arrestin2-TEV fusion protein and a tTa-dependent luciferase reporter)	E164Q D184N	Significant decrease in dextromethorphan, ZINC-3573(R), morphine and other opioid-induced calcium mobilization in cells with mutant receptors.	E164 and D184 are amino acids necessary for opioid binding		
(138)	Initial prediction of amino acid significance using CLUSTALW sequence alignment program. Next, substitution testing was conducted on transfected HeLA cells.	E164R	Significant decrease in substance P and compound 48/80-induced calcium mobilization response in cells with mutant receptors. However, there was no observed effect on LL-37 response.	E164 may be an essential amino acid for the signalling of pruritogens like compound 48/80 and substance P.		
(94)	Mutated MRGPRX2 transfected RBL-2H3	G165E D184H	Significant decrease in substance P-induced calcium mobilization and substance P, HK-1, HBD3 and icatibant-induced degranulation in cells with mutant receptors.	Mutations may have disrupted the binding pocket, thus preventing receptor activation		This study also examined W243R and H259Y but the lack of response seems related to a lack of receptor expression than function.

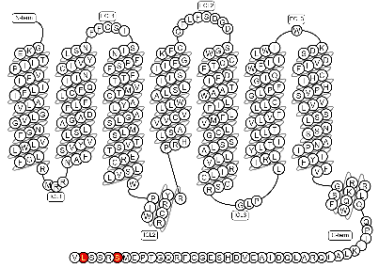
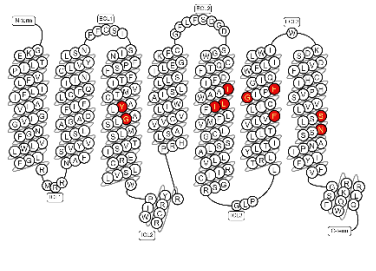
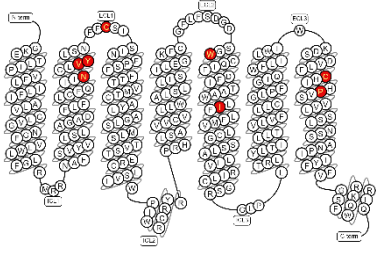
Reference	Brief description of methods	Variants tested	Outcome	Proposed conclusion	Serpentine plot with highlighted amino acids	Additional remarks
(311)	Mutated MRGPRX2 transfected RBL-2H3 cells	S325L L329Q	Variants increased substance P-induced calcium mobilization.	Phosphorylation of these C-terminus residues, especially S325, may be required for receptor internalisation and desensitisation. Preventing this, may increase substance P activation.		This study also examined multiple other amino acid substitutions. However, there seems to be a lack of surface receptor expression.
(108)	Computer modelling using the C5a anaphylatoxin chemotactic receptor 1 model. I-TASSER server was used to identify binding sites of compound 48/80		Proposed sites of compound 48/80 interaction: F239, Y113, T187, S268, N271, Gly236, G116, F232, L191, and I92			
(108)	Computer modelling using the C5a anaphylatoxin chemotactic receptor 1 model. I-TASSER server was used to identify binding sites of genistein. (This paper proposes genistein as an antagonist of MRGPRX2)		Proposed sites of genistein interaction: C258, P262, W179, N85, V88, C95, Y89, and T106.			

Table 5-1: Summary of key published findings reviewing the amino acid interactions of MRGPRX2 with various ligands.

5.2 Methods

5.2.1 Patient cohort and samples collected: isolation of blood components and analyses

Patients within Melbourne, Australia that previously have had an anaphylactic reaction during a surgical procedure were referred to the Department of Clinical Immunology and Allergy, Royal Melbourne Hospital, for follow up testing. Upon receiving signed patient consent, blood samples were collected from consecutive patients for the purposes of this study (**Figure 5.1, Figure 5.2**). It is important to note that in our study, it was not possible to screen for patients with clear NMBA-induced anaphylaxis as these classifications were made accessible in the days after follow-up testing (during which we received blood samples for our study). Therefore, our cohort ranges from patients with defined anaphylaxis to those that may have experienced asthmatic exacerbations.

Samples from control patients were obtained from those undergoing cardiac surgery in the cardiac ward, Royal Melbourne Hospital. Control individuals received a similar drug profile to anaphylactic patients with antibiotics (*e.g.*, ciprofloxacin, cefazolin), standard anaesthetics (*e.g.*, propofol, midazole), pain medication (*e.g.*, fentanyl, morphine) and most importantly, neuromuscular blocking agents (*e.g.*, rocuronium, atracurium), the focus of this study. However, as opposed to anaphylactic patients, control patients had uneventful surgery with no reported adverse effects. Due to the experimental design (as stated above), we were unable to match control patients with anaphylactic patients in regard to age, sex, or type of surgery.

During follow up testing, assessment reports (consisting of a brief description of the anaphylactic event and possible culprit agent) of anaphylactic patients were documented by the attending nurse/doctor. Following this, post-hoc assessments and confirmation of above reports were conducted by Dr Jeremy McComish (Clinical Immunologist, Allergist and Immunopathologist; Royal Melbourne Hospital).

5.2.2 Characterising and stratifying the patient cohort

We received a total of 46 blood samples (**Table 5-2**), of which 18 were control patients with uneventful surgery and 28 had an event of anaphylaxis (**Figure 5.1**). Of the 28 patients that had anaphylaxis, there were 4 grade I (2 females, 2 males) who experienced mucocutaneous signs, 8 grade II (5 female, 3 males) who had multi-organ manifestations, 12 grade III (9 female, 3 male) who experienced life-threatening anaphylaxis, and 4 grade IV (4 female) anaphylactic events who underwent cardiac arrest (**Figure 5.1**). As mentioned earlier, due to the nature of the experimental design and the observation of an unexpected gender bias in the anaphylactic cohort, we were unable to screen for control patients that matched with our anaphylactic cohort. As a result, we have a much greater proportion of females (20 of 28) in the anaphylactic group as opposed to the control patients (4 of 18). The mean ages of anaphylactic and control patients were 48 ± 16 years (mean $\pm$ SEM) and 54 ± 15 years, respectively.

The anaphylactic patients were further categorised to determine causative agents (analysis conducted by Dr. Jeremy McComish). Of the 28 anaphylactic patients, 3 were, beyond reasonable doubt, clearly anaphylactic to NMBAs (**Figure 5.2**). These 3 patients, classified as “Yes” to NMBA as causative agent, were characterised by positive SPTs or relevant sslgE (serum specific IgE) with no clear indication towards an alternative agent, along with the presence of clear anaphylactic symptoms (defined by elevated total IgE and tryptase levels). Of these 3 patients, all had positive skin prick tests, but only 1 had positive morphine specific IgE (**Figure 5.2**). 9 of the 28 patients did not show clear evidence of NMBA-induced anaphylaxis and consisted of patients with a positive SPT to at least one NMBA or positive sslgE, but also had sensitivity to other agents tested, therefore whilst uncertain, these patients may have had alternative culprit agents (*e.g.*, antibiotics) (**Figure 5.2**). 15 of the 28 patients had negative SPTs and sslgE, with either a clear alternative culprit agent or had questionable anaphylaxis, and were categorised as ‘no’ for NMBA as a causative agent (**Figure**

5.2). We were unable to classify 1 patient to a cause of anaphylaxis due to restricted access to the patient's records.

Given the absence of a defining factor that distinguishes MRGPRX2-mediated anaphylaxis, we used the data at hand including that of causative agents, SPTs and sIgE to propose potential MRGPRX2-anaphylactic events (refer to **Figure 5.2**). Firstly, NMBA-s are known to activate mast cells via both IgE-dependent and independent (MRGPRX2) pathways. Therefore, we propose that the sample group with NMBA-induced anaphylaxis would likely encompass both IgE-mediated events and some MRGPRX2-mediated events (if any). Due to the limited size of our cohort, we included reactions that were listed as certainly NMBA-induced ("yes") along with those that were listed as "maybe" NMBA-induced. This totalled 12 of the 28 anaphylactic patients. Next, we stratified the selected patients according to SPT results to NMBA-s. Skin mast cells are known to degranulate in response to both IgE-dependent stimuli and MRGPRX2 agonists ⁽¹⁵²⁾. Although the common agonists were found to be more potent at the FcεRI-IgE receptor than MRGPRX2 ⁽¹⁵²⁾, the implication of this on a single concentration SPT result is unclear. Therefore, for the purpose of this study, we proposed that NMBA-positive SPTs (9 of 12 patients) are indicative of anaphylactic reactions occurring either via IgE-mediated or potentially MRGPRX2-mediated pathways. Finally, we further categorised the 9 patients according to the presence of morphine-specific IgE. As mentioned earlier, morphine-specific IgE is considered indicative of NMBA-specific IgE due to the shared quaternary ammonium ion. Therefore, we proposed that the patients who had positive specific IgE had IgE-dependent anaphylaxis (4 patients), whereas the patients with negative IgE were indicative of potentially MRGPRX2-mediated anaphylaxis (5 patients) .

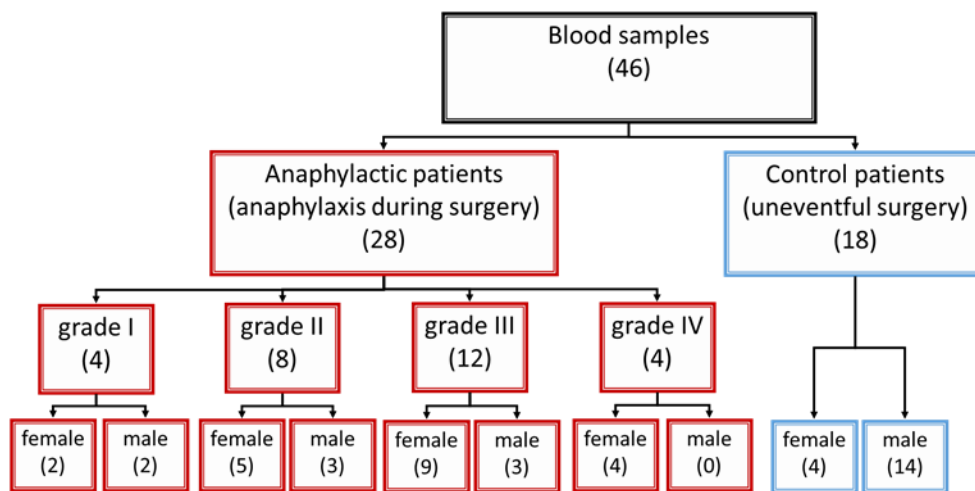
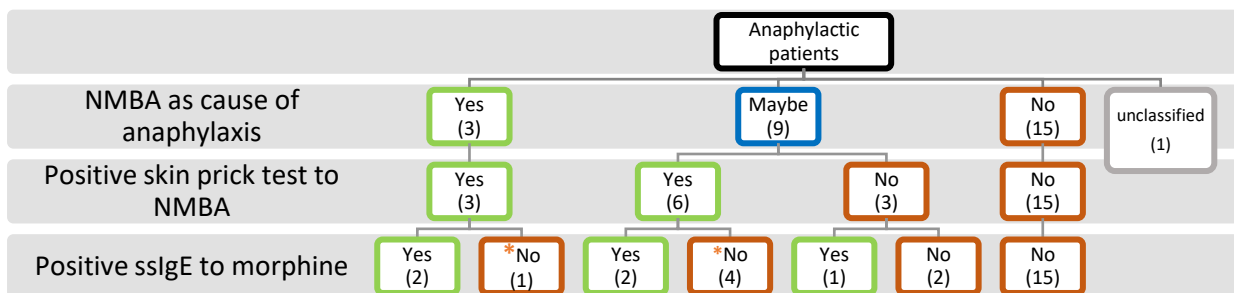


Figure 5.1: Summary of patient cohort characterised by grade of anaphylaxis and patient sex.



*No: Predicted most likely cases of MRGPRX2-dependent anaphylaxis

Figure 5.2: Summary of anaphylactic patients characterised by predicted culprit agent, skin prick test (SPT) and IgE against morphine.

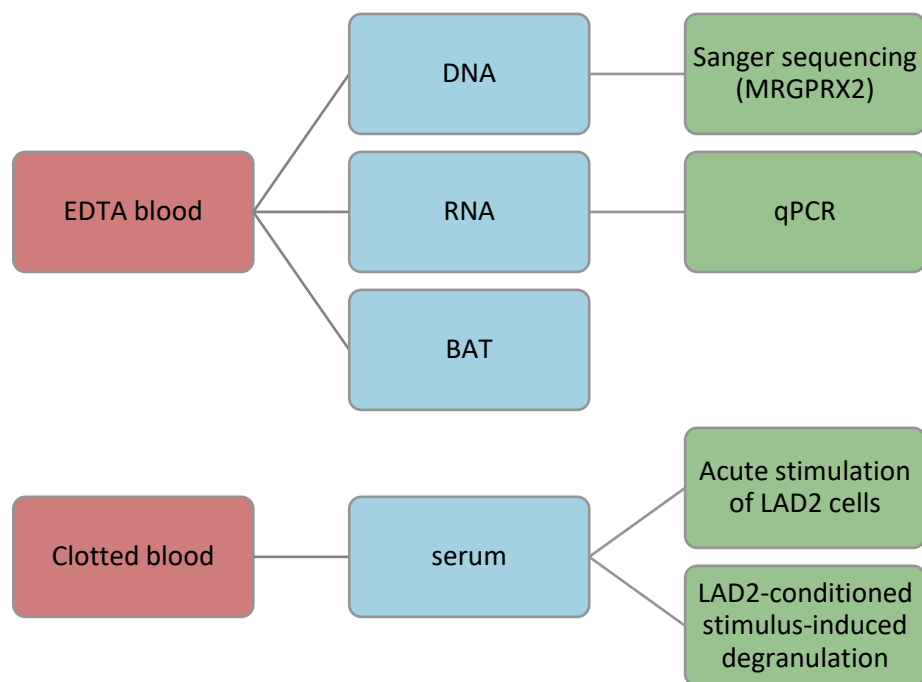


Figure 5.3: Flow chart of experiments conducted on patient blood. Two tubes of blood were collected from the Department of Clinical Immunology and Allergy (Royal Melbourne Hospital): 1 tube with EDTA (used for basophil activation test, plasma, RNA and DNA isolation) and 1 tube without EDTA (clotted blood; used to collect serum).

Anaphylactic (A)/ control (C) patients	Patient code	Age	Sex	NMBA ^{+ve} SPT	Morphine ^{+ve} ssIgE	NMBA as causative agent	Grade of anaphylaxis
A	001 SW	47	F	No	No	No	2
A	002 RJ	54	M	No	No	No	1
A	003 AR	36	F	Yes	No	Maybe	1
A	004 AJ	67	F	No	No	No	3
A	005 GG	54	M	No	No	No	2
A	006 JD	60	F	unclassified	Yes	unclassified	4
A	012 ALF	40	F	Yes	No	Maybe	3
A	013 KM	23	F	No	No	No	2
A	014 JMB	26	M	No	No	No	2
A	015 AG	40	F	No	No	No	3
A	016 DT	62	M	Yes	Yes	Yes	3
A	017 SN	25	M	No	No	No	1
A	018 DS	85	F	No	No	Maybe	3
A	019 SJ	35	M	No	No	No	3
A	022 JC	55	F	No	No	No	1
A	023 CF	22	F	No	No	No	3
A	027 CMH	59	F	Yes	No	Maybe	2
A	028 OHO	76	F	Yes	Yes	Yes	3
A	029 JW	42	F	No	No	No	2
A	030 NJT	53	M	Yes	No	Yes	3
A	036 JH	68	F	Yes	Yes	Maybe	4
A	037 BN	50	F	Yes	Yes	Maybe	3
A	038 AB	44	F	No	No	No	4
A	042 NM	36	F	No	No	No	3
A	043 AM	40	F	No	No	Maybe	4
A	049 DF	26	M	Yes	No	Maybe	2
A	050 EM	45	F	No	Yes	Maybe	2
A	051 VM	64	F	No	No	No	3
C	007 NR	35	M	-	-	-	-
C	009 GB	66	M	-	-	-	-
C	010 MV	53	F	-	-	-	-
C	011 SH	63	M	-	-	-	-
C	020 AHA	69	M	-	-	-	-
C	021 GTC	61	M	-	-	-	-
C	024 CAB	54	M	-	-	-	-
C	025 EAG	82	M	-	-	-	-
C	026 LCS	28	F	-	-	-	-
C	031 PMG	56	M	-	-	-	-
C	039 TYT	64	F	-	-	-	-
C	040 SSH	30	F	-	-	-	-
C	041 KJM	56	M	-	-	-	-
C	044 ABS	58	M	-	-	-	-
C	045 ST	42	M	-	-	-	-
C	046 RSB	49	M	-	-	-	-
C	047 CBM	46	M	-	-	-	-
C	048 ISS	52	M	-	-	-	-

Table 5-2: Summary of patient cohort with age, sex, NMBA^{+ve} SPT results, morphine^{+ve} ssIgE, and whether NMBA is the presumed causative agent.

5.2.3 Basophil activation test (BAT) studies using patient whole blood

BAT was conducted according to manufacturer's instructions (BÜHLMANN Laboratories AG; Baselstrasse, Schönenbuch, Switzerland). Briefly, 100 μ L of whole blood (collected in EDTA to prevent coagulation) was added to a mixture of stimulation buffer with the antibodies CCR3-PE (a marker of basophils and mast cells) and CD63-APC (a marker of degranulation as described in **chapter 3**). A fixed concentration of stimuli that induced significant activation of the human mast cell line, LAD2, without inducing cell death (as demonstrated in **chapter 3 and 4**) was then added and incubated for 15 minutes at 37 °C (MRGPRX2 agonists compound 48/80 (10 μ g/mL), rocuronium (200 μ M), protamine (6 μ M), polymyxin (25 μ M), and the calcium ionophore, ionomycin (0.5 μ M)). Following this, the samples were mixed with pre-warmed lysis buffer to lyse red blood cells, and were then centrifuged and washed in ice cold FACS buffer (PBS supplemented with FCS (2%) and EDTA (2 mM)). Flow cytometry (BD LSRFortessa™ Flow Cytometer; BD Biosciences) was used to quantify activated basophils. Basophils were first gated as cells positive for CCR3, with low side scatter (in selecting for low side scatter, eosinophils, having high side scatter, were excluded). Activated basophils were quantified by gating for cells that were both positive to CCR3 and CD63 receptor expression. Where possible, at least 500 basophils were counted for each patient sample. Positive control stimuli of FMLP (activates basophils via the FPR2 receptor) and an anti-Fc ϵ RI antibody (activates basophils by initiating Fc ϵ RI cross-linking) were used to determine evaluability of each sample. As recommended by the kit, if one or both of the positive controls activated at least 10% of gated cells, the sample is evaluable. Percentage of activated basophils were then calculated from the total basophil count using FlowJo version 8.0 (Ashland, Oregon, US).

5.2.4 Quantifying relative gene expression

RNA was isolated from whole blood leukocytes in accordance with the manufacturer's instructions (QIAamp DNA mini spin kit; Qiagen; Hilden, Germany). cDNA conversion was also conducted according to manufacturer's instructions (ThermoFisher Scientific; refer to **section 2.1.5**). qPCR was carried out using TaqMan expression assays according to Chapter 2, **section 2.1.5**. The sample group constituted of 28 anaphylactic patients and 18 control patients. Gene expression measured include the primary receptor of interest, MRGPRX2, along with the 3 subunits of the FcεRI receptor (FcεRI α, β, γ), FGD2 and FGD3, FcγRIIA, GNAq, GNA15, GNAi1, GNAi2, tryptase, interleukin-8 (IL-8), macrophage inhibitory factor (MIF) and tumour necrosis factor- α (TNF-α) (refer to **Table 2-1** for the list of TaqMan assays used). ΔCT was calculated by subtracting the gene cycle number from the housekeeper cycle number (average of GAPDH and UBC). A lower cycle number (CT) is indicative of higher expression. Therefore, negative ΔCT values indicate lower expression than the housekeeper while positive ΔCT values indicate higher expression.

5.2.5 Sanger sequencing of amplified genomic MRGPRX2

Genomic DNA was isolated from whole blood leukocytes using QiaAMP DNA mini kit (Qiagen) in accordance with the manufacturer's instructions. Primers were designed and synthesised (Integrated DNA technologies; Newark, New Jersey, US) that spanned the MRGPRX2 gene (**Table 5-3, Figure 5.4**) and amplified using a Phusion high fidelity PCR kit (New England BioLabs; Ipswich, Massachusetts, US). Three sets of primers were designed so that in the event where primer 1 (spanning the full coding region; **Table 5-3, Figure 5.4**) failed to amplify, primer sets 2 and 3 with different annealing regions could be used to sequence the coding region. Primers were custom designed to be between 18 -26 bases with a G-C content of 30-60%, and melting temperatures between 50- 60 °C, and within 5°C between each forward and reverse primer (**Table 5-3**).

Samples were prepared by mixing DNA (200 ng in a 13 µL volume of water) with master-mix (7 µL) as per manufacturer's recommended protocol. These samples were then incubated for

1 minute at 98 °C for initial denaturation (Mastercycler® nexus gradient; Eppendorf). This was followed by 40 cycles of denaturation (98°C, 10 seconds), annealing (60 °C, 30 seconds), extension (72 °C, 180 seconds), and lastly, a final extension at 72 °C for 10 minutes. The resultant amplicons were stored at -20 °C.

DNA amplification was verified using agarose gel electrophoresis. The samples along with forward and reverse primers were then submitted to AGRF (Australian Genome Research Facility; Melbourne, Victoria, Australia) for dual direction un-purified PCR product (PD+) Sanger sequencing. The sequences received were aligned against the MRGPRX2 cDNA sequence published on NCBI using the online software, CodonCompiler aligner (Massachusetts, US). Serpentine plots of MRGPRX2 with any identified polymorphisms were created using the online platform and database, GPCRdb (<https://gpcrdb.org/>). Other schematics were plotted using Microsoft Office 365 (Washington, US). Percentage mutated allele frequencies (MAF%) of each identified polymorphism were sourced from the following databases: ALFA project, TOPMED, GO-ESP and 1000 genome project.

Primer set # and direction	Sequence	Length (bp)	Melting temperature (°C)	GC %	PCR product length
Set 1 Forward primer	TGTA AACAGGGGCGGTAAT	20	57.12	45	1166
Set 1 Reverse primer	TTTGAGAGGCAACTTTGCC	19	56.33	47.37	1166
Set 2 Forward primer	AGCATGGATCCAACCACC	18	56.90	55.56	698
Set 2 Reverse primer	TGCTCACAGTGCTGGTGTT	20	60.82	55	698
Set 3 Forward primer	CTGCTGGTCAGGATCCTCTG	20	59.54	60	481
Set 3 Reverse primer	TGAAC TTTCTCAGTCCTGATTTTAAA	26	57.25	30.77	481

Table 5-3: Primers designed and synthesised (Integrated DNA technologies) for dual direction sanger sequencing of patient DNA.



Figure 5.4: Schematic of primer-spanning in the MRGPRX2 coding region. Exon coding region (coloured black) spans between the start and stop codon. Forward and reverse primer sequences are detailed in *Table 5-3*. While primer set 1 (green) spans the whole MRGPRX2 coding region, primer sets 2 (blue) and 3 (orange) spans shorter lengths.

5.2.6 Quantification of acute serum-induced LAD2 degranulation via flow cytometry

LAD2 cells (2×10^5 cells/sample) were washed, resuspended in FACS buffer (PBS supplemented with FCS (1%) and EDTA (2 mM)), and treated with patient sera (overall a 1/5 dilution of sera) for 30 minutes (37 °C) in the presence of mouse anti-human CD63-PE (BD Biosciences). Following this, the cells were washed twice in ice-cold FACS buffer and resuspended in FACS buffer. Flow cytometry was performed to quantify cell surface CD63. LAD2 cells were considered activated with a CD63-PE signal of $> 10^2$ as determined through control stimulation with compound 48/80.

5.2.7 Quantifying stimulus-induced degranulation of serum/quercetin-conditioned LAD2 cells

LAD2 cells were seeded on a 24 well plate and incubated with patient sera (1:20 in serum free IMDM) for 18 hours, or quercetin (3, 10, 100 μ M) for 24 or 48 hours, in a humidified incubator (37 °C). The cells were then washed and seeded in 96 well round bottom plates (2.5×10^5 cells/mL) in HBSS and stimulated with a range of stimuli (MRGPRX2 agonists compound 48/80 (10 μ g/mL), rocuronium (200 μ M), atracurium (200 μ M), protamine (1 μ M), ZINC-3573(R) (10 μ M); IgE-Fc ϵ RI agonists 3B4 (2 μ g/mL), anti-IgE (2 μ g/mL; Maravai Lifesciences, San Diego, California, US), NIP-BSA (100, 1 ng/mL); and the calcium ionophore ionomycin (0.5 μ M)). To investigate any acute effects of quercetin, LAD2 cells were incubated with quercetin (3, 10, 100 μ M) 5 minutes prior to stimulus addition (quercetin was not washed off). Degranulation was quantified using the β -hexosaminidase-PNAG assay as detailed in **General methods, section 2.1.3**.

5.3 Results

5.3.1 Detection of a double mutant form of the MRGPRX2 receptor using Sanger sequencing

MRGPRX2 was amplified and sequenced using dual direction Sanger sequencing. Point mutations observed in only one sequencing direction (forward or reverse) were considered likely sequencing errors and excluded. Misaligned base pairs (point mutations) are denoted in red.

Of the 46 patient samples, we successfully amplified 43 samples for sequencing. Patient samples 046, 047 and 048 (all control patients) could not be amplified with any of the primer sets. The reason for this is unclear, but as they are consecutive patients, it may be an error made during DNA isolation. Interestingly, the 5 patients with anaphylactic events that were categorised as potentially MRGPRX2-mediated (**section 5.2.2**), all had wild type MRGPRX2 coding region (**Figure 5.5**).

Of these 43 samples, all samples, except two, were amplified using primer set 1 that spanned the whole MRGPRX2 exon (**Figure 5.4, Table 5-3**). However, these two samples were successfully amplified using primer sets 2 and 3.

Our results show that virtually all (39 of 43) genomic MRGPRX2 sequences were identical to that of the published WT cDNA sequence, suggesting against a defining polymorphism that distinguishes all anaphylactic patients from control patients. However, four individuals (one control and three anaphylactic) showed single nucleotide polymorphisms (SNPs) that resulted in amino acid changes. The control patient coded 025 EAG, and anaphylactic patients coded 005 GG and 019 SJ, had one polymorphism that resulted in an amino acid change from an asparagine to serine (N62S) (**Figure 5.5**). Interestingly, the anaphylactic patient coded 004 AJ, had two polymorphisms (**Figure 5.5**); one of which is identical to that of the previous patients (N62S), and the other resulted in a change of an asparagine to histidine (N16H) in the N-terminal, predicted extracellular region (**Figure 5.5**). It is however important to highlight that

none of the anaphylactic patients mentioned above had definitive cases of NMBA anaphylaxis. The expected frequency (mutated allele frequency; MAF) of N62S and N16H is 31% and 8% respectively (**Table 5-4**). The probability of the amino acid substitution having damaging consequences were predicted using the polyphen-2 score (polymorphism phenotype score), with '0' being benign and values closer to 1 being more likely deleterious. Both variants identified in our study were rated 'probably damaging' on the polyphen-2 score (**Table 5-4**). In our limited sample size of 43 sequenced DNA from patients carefully selected under restricted criteria, we observed fewer than expected occurrences with N62S appearing four times (4/43; 9.3%) and N16H appearing once (1/43; 2.3%) in our cohort.

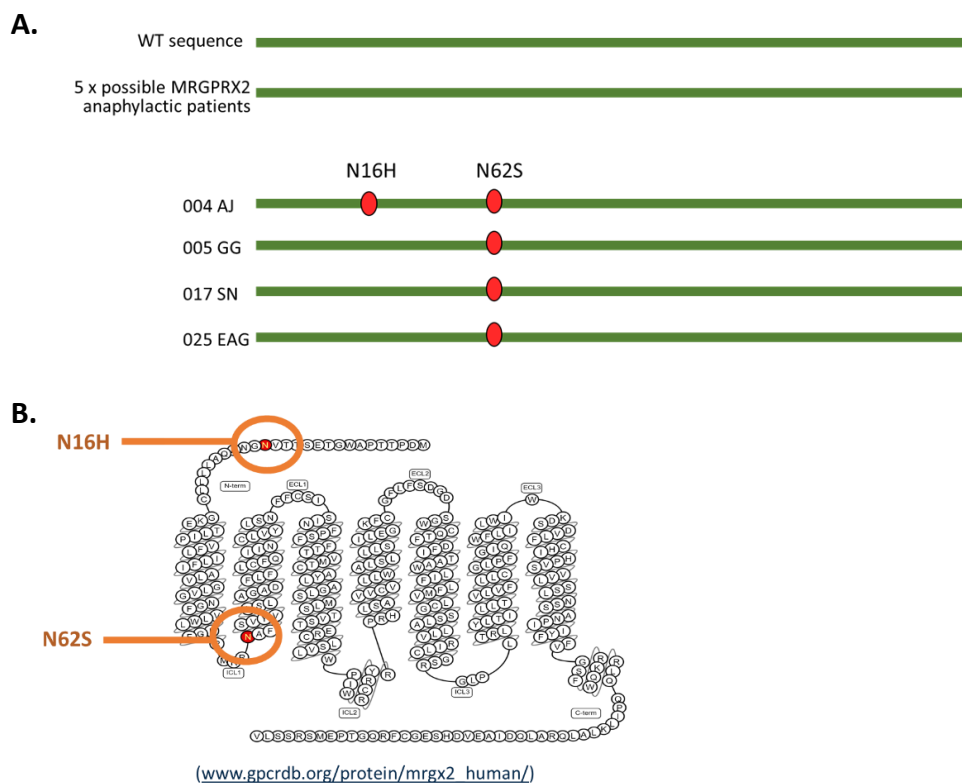


Figure 5.5: Schematics of MRGPRX2 protein sequence with identified polymorphisms highlighted.

A. MRGPRX2 was sequenced using dual direction Sanger sequencing. Sequenced DNA was aligned and translated to amino acid sequences using CodonCode aligner (Massachusetts, US) and schematic was drawn using Microsoft Office 365 (Washington, US). Displayed DNA strands: reference sequence obtained from UniProtKB (Washington, US), 004AF, 005GG, 017SN, 025EAG. Two anaphylactic patients (005 GG, 017 SN) and one control patient (025 EAG) displayed the N62S polymorphism while one anaphylactic patient (004 AJ) possessed a double polymorphism (N16H and N62S). **B.** The above identified polymorphisms were annotated on a MRGPRX2 serpentine plot (GPCRdb.org).

Rs ID	Protein change	Polyphen-2 score	MAF% ALFA project	MAF% TOPMED	MAF % GO-ESP	MAF% 1000G	Average MAF
Rs11024970	N16H	0.937 (possibly damaging)	5.32	9.09	8.70	11.30	8.60
Rs10833049	N62S	0.976 (probably damaging)	26.97	33.18	33.56	31.85	31.31

Table 5-4: N16H and N62S MRGPRX2 variants as identified on ALFA project, TOPMED, NCBI-GO-ESP, and 1000 genome project databases. Rs ID corresponding to the known SNPs are summarised in this table along with the percentage frequency of occurrence in each data base (mutates allele frequency; MAF%) and polyphen-2 score (probability of the substitution being deleterious).

5.3.2 Gene expression of anaphylactic and control patients

mRNA was isolated from whole blood leukocytes cells obtained from the complete cohort of 46 patients, both anaphylactic and control. In addition to investigating a possible alteration in the expression of MRGPRX2, we also investigated 16 other genes related to the MRGPRX2 pathway or other pathways of mast cell activation. Further characterisation of the patient cohort was conducted for a detailed analysis that accounts for the presumed underlying pathway of anaphylaxis, sex, grade of anaphylaxis, and cause of anaphylaxis. Genes investigated included the FcεRIα, FcεRIβ, and FcεRIγ genes that encode the FcεRI receptor responsible for the IgE-dependent pathway of mast cell activation. We also looked at the signalling proteins FGD2 and FGD3, both proposed to be negative regulators of mast cell activation (Mackay et al., unpublished). HEXA and HEXB are genes encoding for the enzyme β-hexosaminidase, a commonly used marker of degranulation. Other mast cell activation markers were also investigated including tryptase, MIF (macrophage migration inhibitory factor), IL-8 and TNF-α. A change in expression of these activation markers may signify an altered sensitivity of the patient's mast cells. The most common genes coding for Gi (GNAi1 and GNAi2) and Gq (GNAq and GNA15) proteins, both coupling to MRGPRX2 were also examined for any evidence of expression differences in some patients. We also tested the

gene FcγRIIA, recently proposed as marker of NMBA-induced IgG-mediated neutrophil anaphylaxis ⁽⁶³⁾.

Although not surprising given the rarity of mast cells in blood, we observed a very low (bordering on the range of detection) MRGPRX2 expression in all patients, with levels being undetectable in one anaphylactic and one control patient. There was no observed statistically significant difference in MRGPRX2 expression between the unstratified anaphylactic and control patient groups (**Figure 5.6**). There was also no difference in the expression of other genes explored in this study between the unstratified anaphylactic and control groups. The 5 patients who experienced anaphylactic reactions categorised as potentially MRGPRX2-mediated, also had no differential gene expression from those categorised as IgE-dependent anaphylaxis or control patients (**Figure 5.7**). When stratified according to sex, there was similarly no observable difference in MRGPRX2 and other gene expression (**Figure 5.8**). Subcategorization into grade or cause of anaphylaxis also did not result in any differential gene expression in our patient cohort (**Figure 5.9, Figure 5.10**).

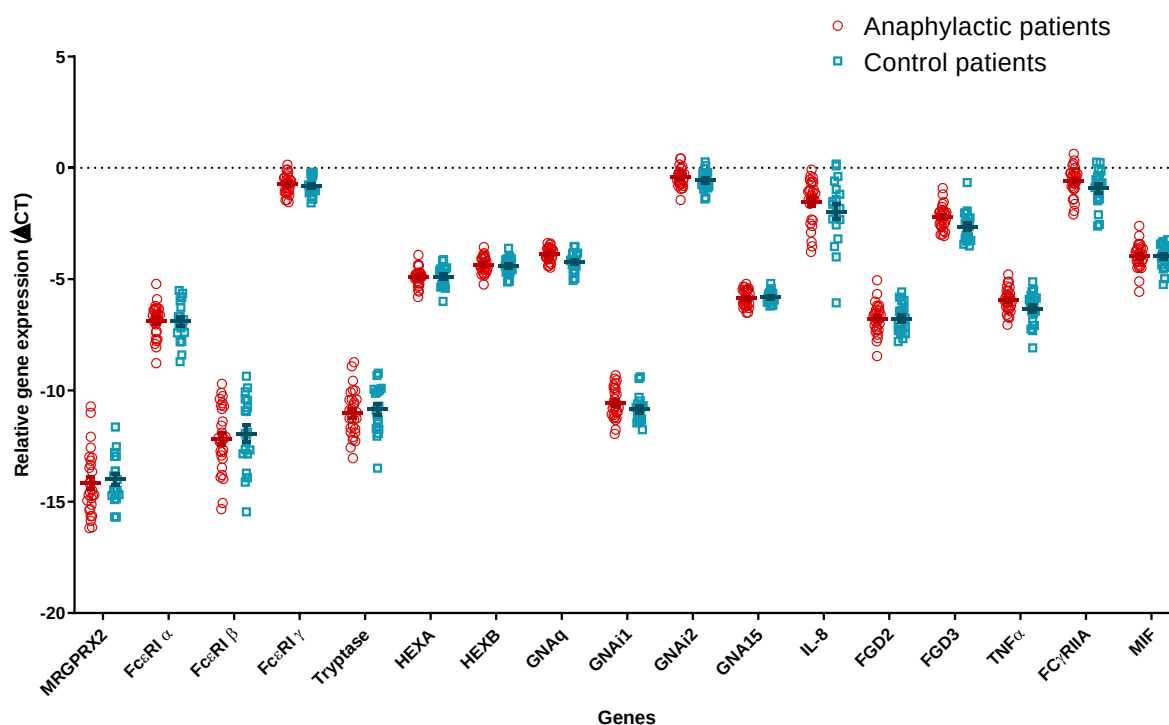


Figure 5.6: No difference was observed in the expression of a range of genes between control and unstratified anaphylactic patients. mRNA expression levels were quantified using RTqPCR. TaqMan probes were used to quantify expression of each gene which was then subtracted from the average of 2 housekeeper genes (GAPDH and UBC) to obtain ΔCT . 27 anaphylactic patients and 19 control patients were used in this study. Genes examined in this study include MRGPRX2, FcεRIα, FcεRIβ, FcεRIγ, tryptase, HEXA, HEXB, GNAq, GNAi1, GNAi2, GNA15, IL-8, FGD2, FGD3, TNFα, FcγRIIA, MIF. There was no significant difference in gene expression observed between the unstratified anaphylactic and control patients. Data expressed as mean, $\pm$ SEM. One-way ANOVA was conducted with Dunnett's multiple comparison post hoc test with anaphylactic patients compared against respective control patients.

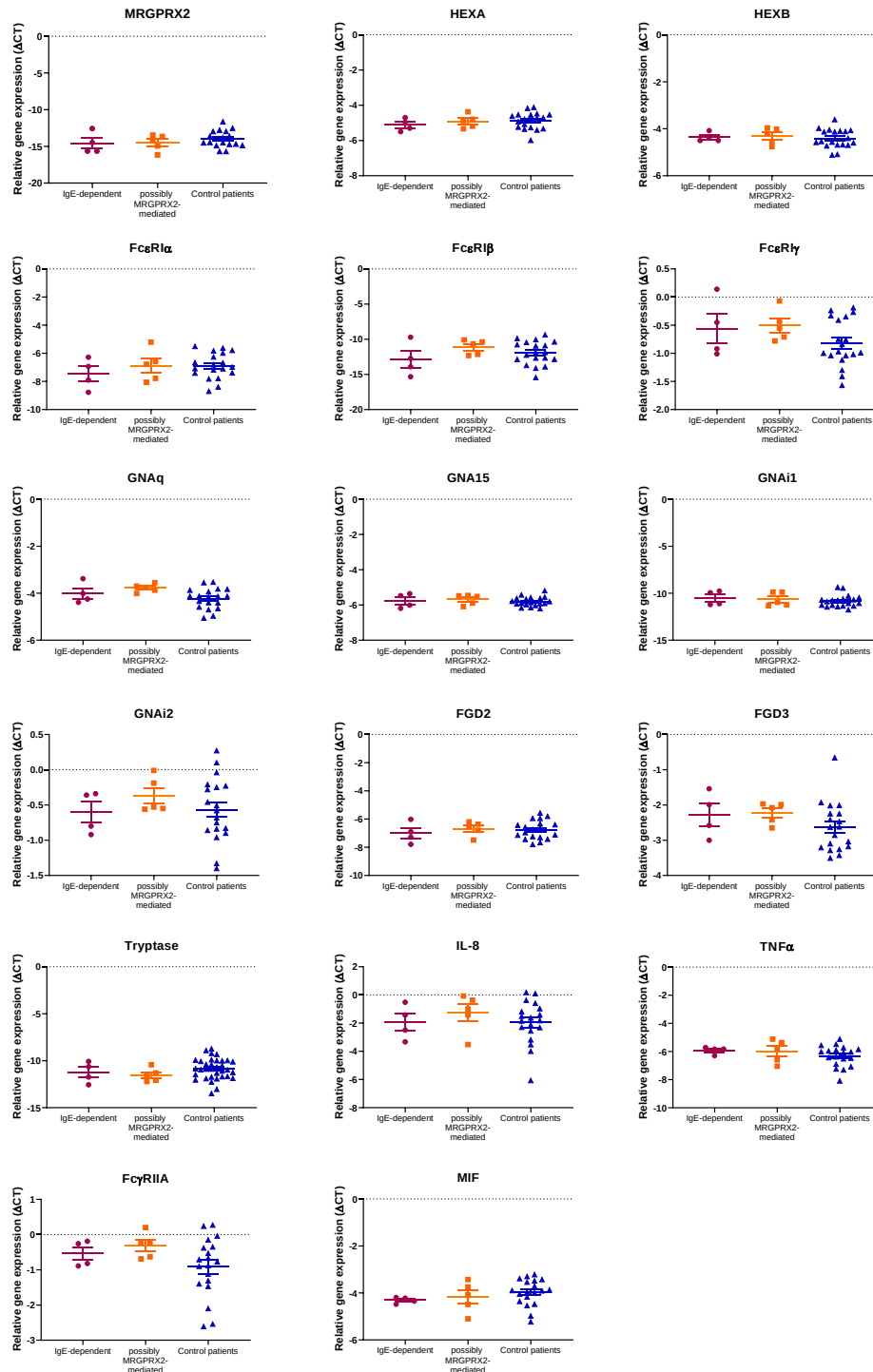


Figure 5.7: No distinguishable difference was observed in gene expression when stratified according to proposed underlying pathway of anaphylaxis. A total of 9 anaphylactic patients who presumably experienced NMBA-induced anaphylaxis, and had positive skin prick test to NMBAs, were stratified according to the presence of morphine specific serum IgE (ssIgE). Patients who were ssIgE positive (4 patients) were categorised as having IgE-dependent anaphylaxis, whereas patients with negative ssIgE (5 patients) were considered potentially MRGPRX2-mediated anaphylaxis. The 2 subgroups of anaphylactic patients were compared against control patients (18). One-way ANOVA was conducted with Dunnett's multiple comparison post hoc test. Each pathway of anaphylaxis was compared against the control patients.

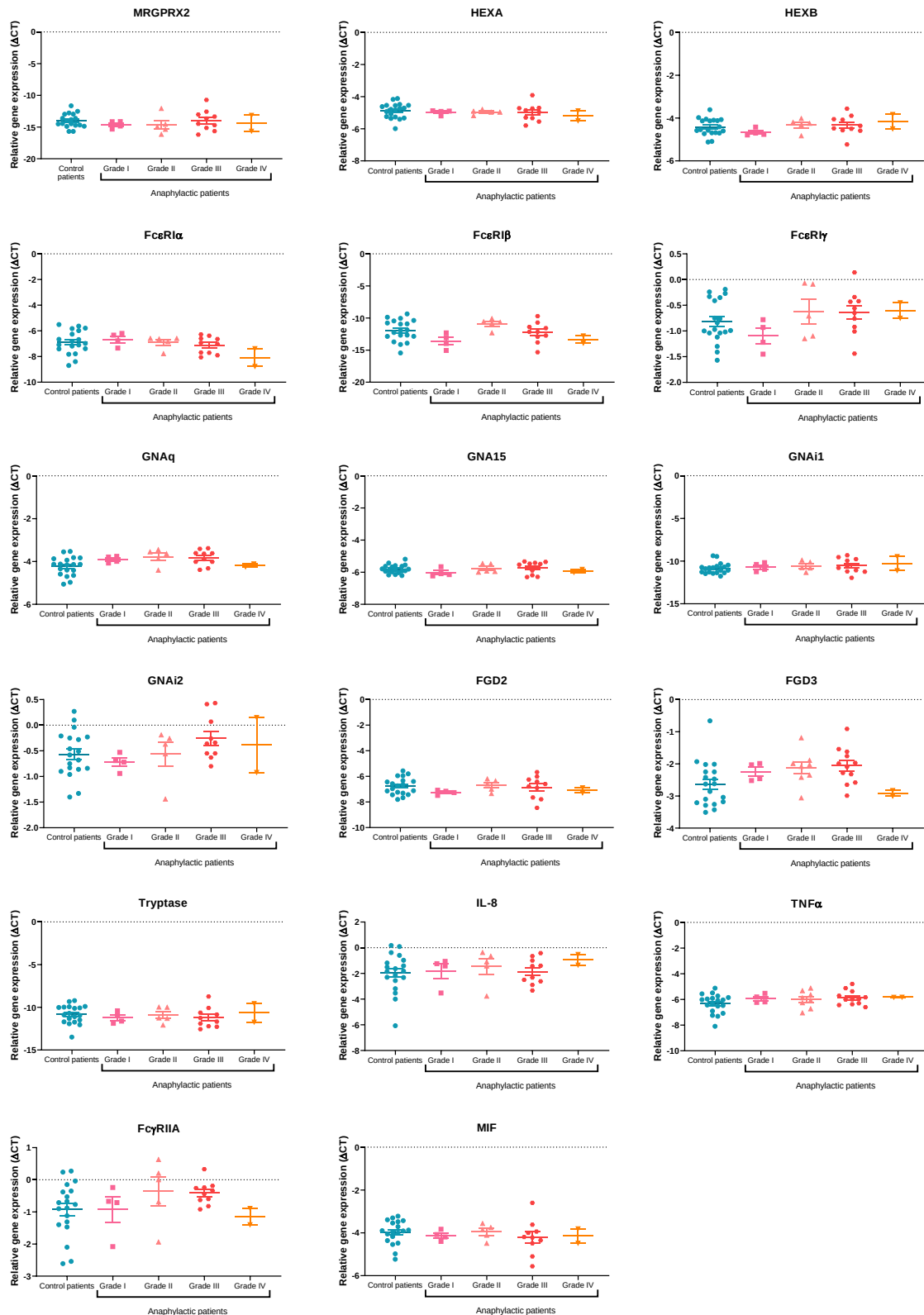


Figure 5.8: No distinguishable difference was observed in gene expression of anaphylactic and control patients when stratified according to grade of anaphylaxis. The patient cohort (27 anaphylactic, 18 control) was characterised according to grade of anaphylaxis: grade I (4 patients), grade II (8), grade III (12), grade IV (3). One-way ANOVA was conducted with Dunnett's multiple comparison post hoc test with each grade of anaphylaxis compared against that of control patients.

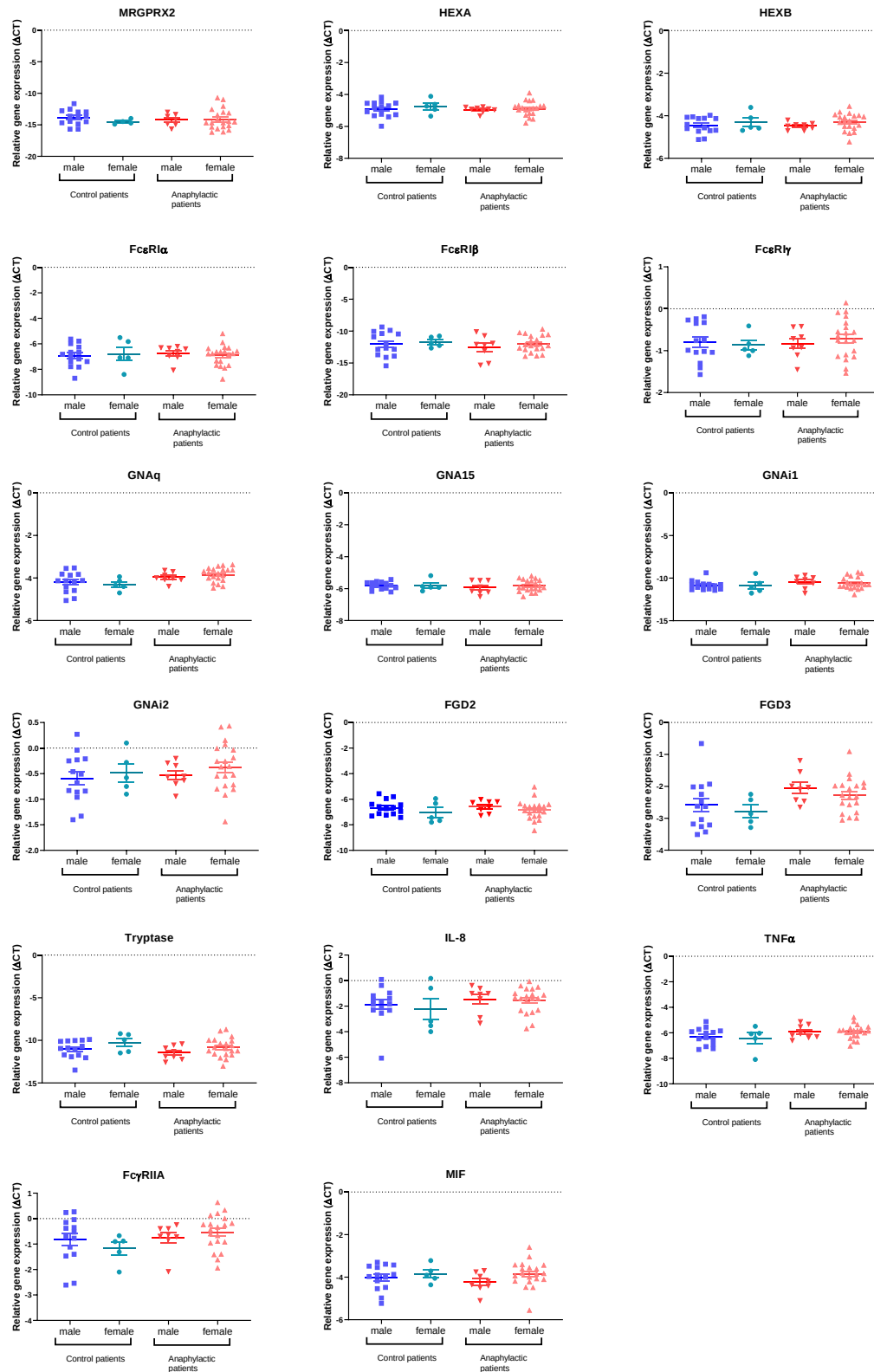


Figure 5.9: No distinguishable difference was observed in gene expression of anaphylactic and control patients when stratified according to sex. Patient cohort of 18 control patients (4 female, 14 male) and 27 anaphylactic patients (19 female, 8 male) were categorised according to sex. One-way ANOVA was conducted with Dunnett's multiple comparison post hoc test with anaphylactic patients compared against respective control patients.

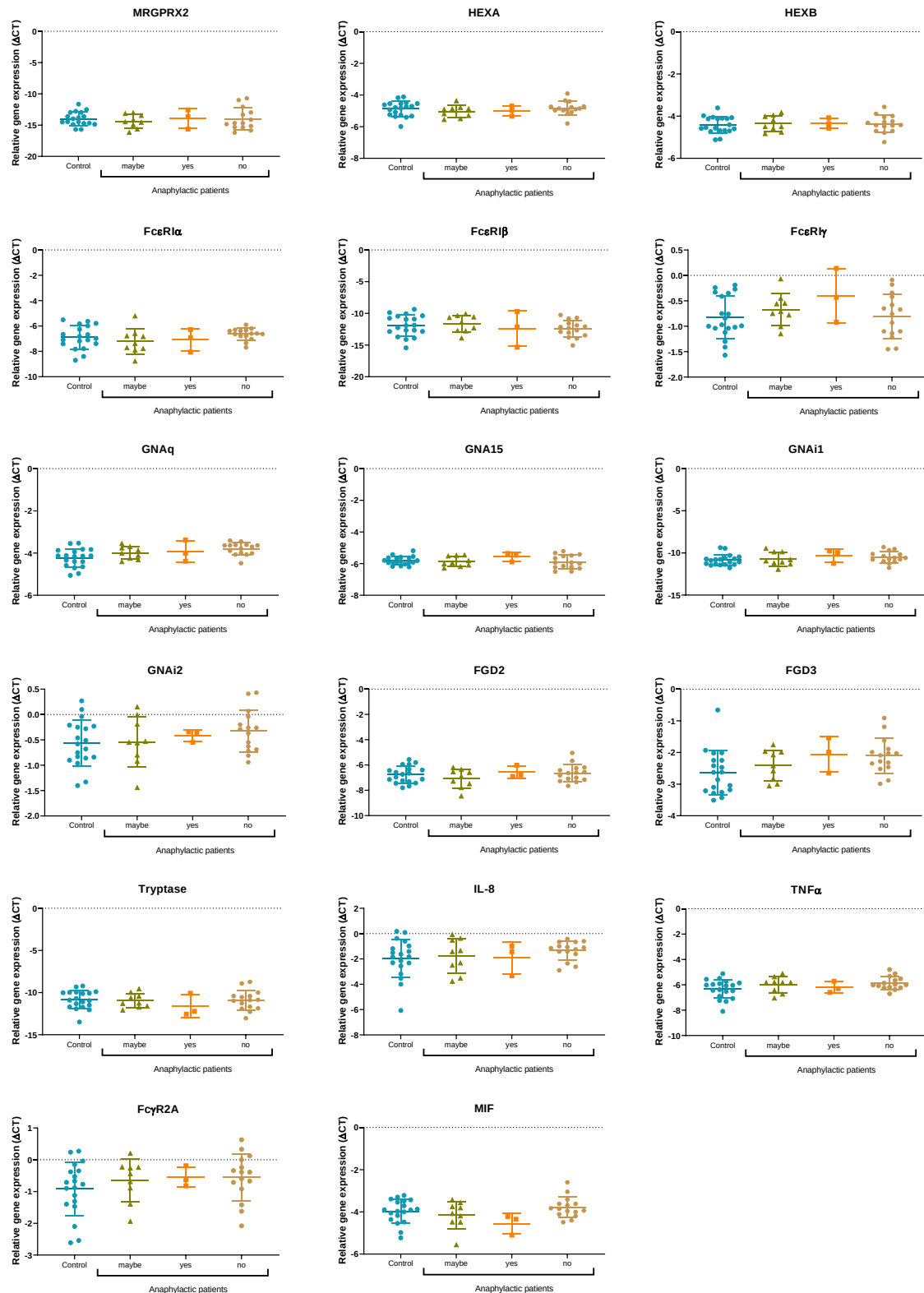


Figure 5.10: No distinguishable difference was observed in gene expression when stratified according to whether NMBA was the causative agent to anaphylaxis. Patient cohort of 12-15 control patients and 15-19 anaphylactic patients were sub-categorised on NMBA as the probable cause of anaphylaxis (maybe, yes, and no). One-way ANOVA was conducted with Dunnett's multiple comparison post hoc test. Each cause was compared against the control patients.

5.3.3 The effect of acute treatment with patient sera on LAD2 degranulation.

A recent study demonstrated an increased MRGPRX2-dependent, LAD2 mast cell activation in response to sera of patients anaphylactic to NMBA^s (152). In light of this publication, we used serum from our patient cohort to treat LAD2 cells and quantify acute degranulation as measured by surface CD63 expression. In our cohort, whilst most patient sera did not activate LAD2 cells (representative traces shown with 006JD, 016DT and 004AJ), 2 patients (coded 012ALF and 019SJ), showed a significant population of activated LAD2 cells (**Figure 5.11**). The experiment was repeated twice to confirm this positive activation.

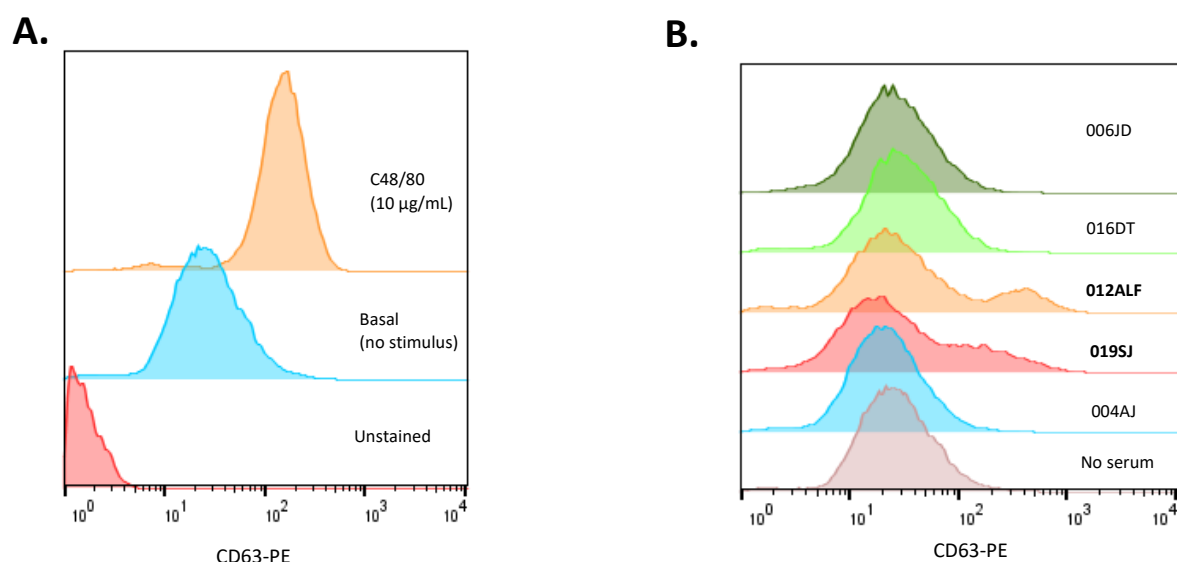


Figure 5.11: Some anaphylactic patient sera induced LAD2 degranulation. Patient sera (1:5 in FACS buffer) was used to acutely stimulate LAD2 cells for 15 minutes in the presence of anti-CD63-PE. Population within 10^2 and 10^3 (x-axis) represents resting/non-degranulating cells while the population within 10^2 and 10^3 represents CD63⁺/^{ve}/degranulating cells. Degranulation was quantified using cell surface CD63 levels with flow cytometry. **A.** Assay controls with compound 48/80 (C48/80; 10 µg/mL) showing mast cell activation, ‘basal’ representing resting CD63 expression levels, and an unstained sample in the absence of antibody or stimulus. **B.** All 46 patient sera were tested for serum-induced LAD2 activation. Most sera did not activate LAD2 cells (representative traces are displayed in patient sera coded 006JD, 016DT and 004AJ). Sera from anaphylactic patients 012ALF and 019SJ activated a population of LAD2 cells.

5.3.4 Effect of serum-conditioning, on stimulus-induced LAD2 degranulation

To investigate the effect of the long term presence of serum factors that could perhaps modulate agonist-mediated cell signalling, LAD2 cells were conditioned with patient sera (1:20 dilution in HBSS buffer; 18 hours), washed, and then stimulated with a fixed concentration of known mast cell activators. The MRGPRX2 agonists compound 48/80, rocuronium, atracurium and protamine were tested along with 3B4 (anti-human FcεRI-α monoclonal antibody), anti-IgE and ionomycin (calcium ionophore; a receptor-independent stimulus). In addition to the effect on stimulus-induced activation, the effect of serum-conditioning on the basal/spontaneous levels of LAD2 cell degranulation was also quantified and graphed (**Figure 5.13**).

In our preliminary experiments we conditioned LAD2 cells with sera or BSA (to mimic approximate equivalent protein concentration) for 3 hours and 18 hours (**Figure 5.12**). There was an intriguing significant increase in degranulation observed in anaphylactic patients compared to that of control patient sera when incubated for 18 hours (**Figure 5.12**). Although this underlies the rationale for choosing an 18 hour conditioning, this trend was not reproducible when more patient sera were analysed (**Figure 5.13**). In this preliminary experiment, BSA (n=1) was underpowered, therefore it was not possible to reliably analyse comparative trends.

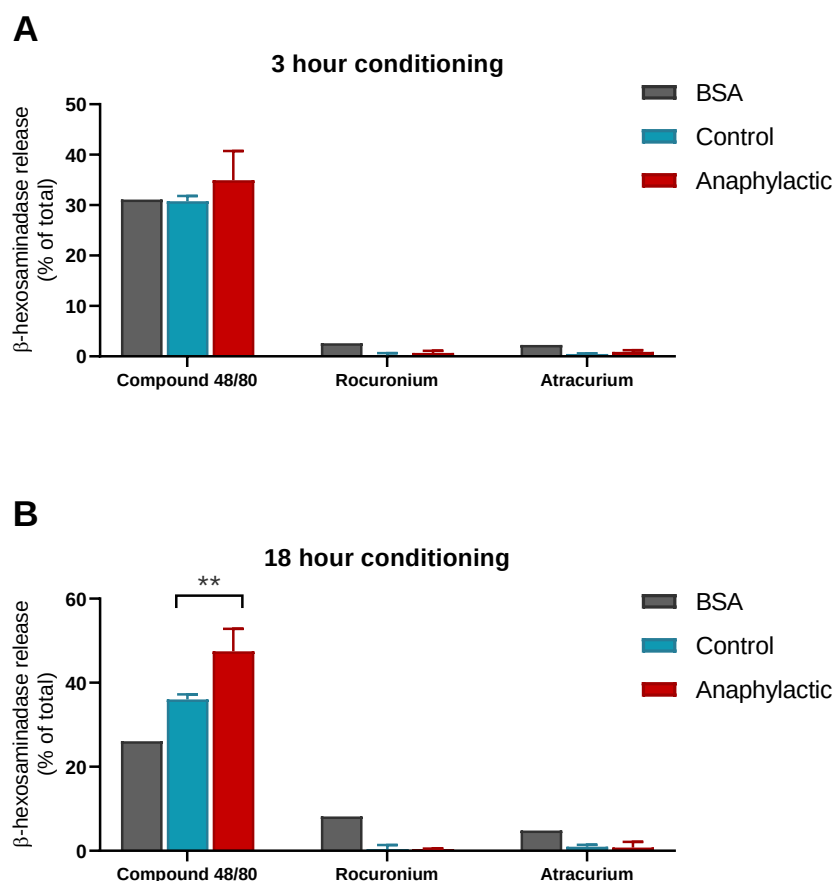


Figure 5.12: Preliminary experiments used to determine the time period for serum conditioning of LAD2 cells. LAD2 cells were incubated with patient sera or BSA with approximately equal protein concentration for 3 hours or 18 hours. Serum-free cells were stimulated with compound 48/80 (10 $\mu\text{g}/\text{mL}$), rocuronium (200 μM), and atracurium (150 μM). Degranulation was quantified using β -hexosaminidase. Data presented as mean, $\pm$ SEM. $N_{(\text{BSA})}=1$, $N_{(\text{control})}=3$, $N_{(\text{anaphylactic})}=4$. One-way ANOVA was conducted with Dunnett's multiple comparison post hoc test. The results of anaphylactic patients were compared to respective control patients. Data from BSA conditioning was not included in statistical analysis as $n<3$. ** $p<0.001$

As observed in LAD2 cells not conditioned with patient sera, all stimuli, with the exception of rocuronium, induced significant degranulation (**Figure 5.13 B**). Interestingly, there was an overall increase in 3B4 and anti-IgE-induced degranulation (**Figure 5.13 E, F**) in anaphylactic patients compared to that of control patients. There was an expected positive correlation in LAD2 degranulation between activators of the $\text{Fc}\epsilon\text{RI}$ -IgE pathway, anti-IgE (αIgE) and 3B4 ($R^2 = 0.71$). In addition, there was also a positive correlation between the activation of the IgE pathway (quantified by 3B4 and anti-IgE) with total serum IgE levels ($R^2 = 0.73$ and 0.72 respectively) (**Figure 5.13, Figure 5.18**).

However, there was no change in MRGPRX2 agonist-induced degranulation between the 2 groups. While compound 48/80 and ionomycin activated all conditioned mast cells (**Figure 5.13 A, G**), in some conditions, atracurium, protamine, 3B4, and anti-IgE (**Figure 5.13 C, D, E, F**) resulted in almost no degranulation above basal release. This observation was non-discriminatory between control and anaphylactic patients.

When these observations were further stratified according to proposed underlying pathway of anaphylaxis (**Figure 5.14**), patient sex (**Figure 5.15**), grade (**Figure 5.16**), and cause of anaphylaxis (**Figure 5.17**), there was still no discriminating factor between groups in LAD2 degranulation responses.

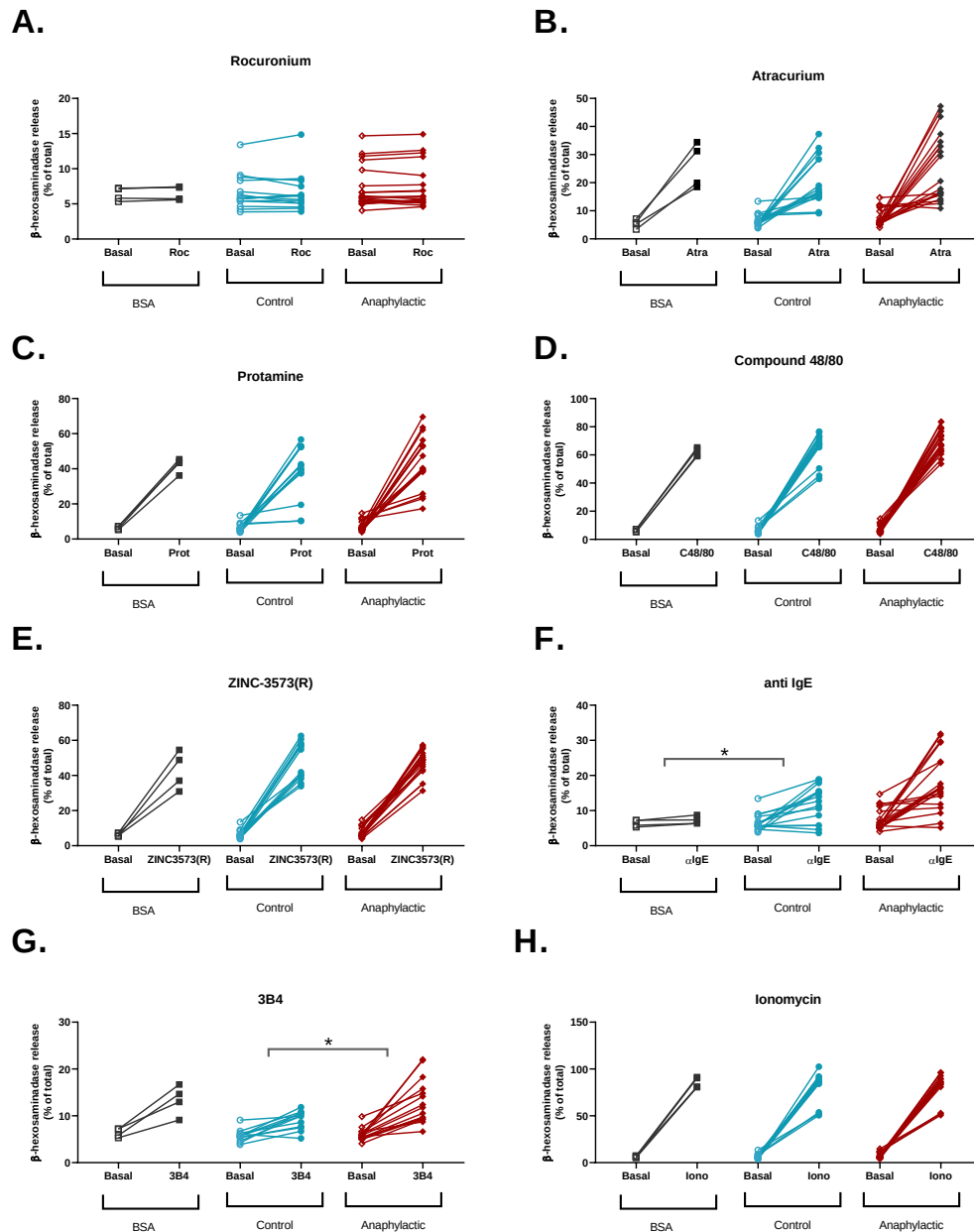


Figure 5.13: LAD2 cells conditioned to patient sera (anaphylactic vs control) showed no difference in response to MRGPRX2-dependent stimuli, but serum from anaphylactic patients enhanced FcεRI-dependent activation. LAD2 cells were incubated with patient sera diluted 1/20 in serum-free media or BSA (0.4%) for 18 hours. The serum conditioned cells were washed and stimulated with a range of drugs (**A.** rocuronium (200 μM), **B.** atracurium (200 μM), **C.** protamine (1 μM), **D.** compound 48/80 (10 μg/mL), **E.** ZINC-3573(R) (10 μM), **F.** anti-IgE (2 μg/mL), **G.** 3B4 (2 μg/mL), **H.** ionomycin (0.5 μM)) and degranulation was quantified using the β-hexosaminidase enzyme assay. LAD2 degranulation was not differentially affected by the conditioning of control vs anaphylactic patient sera except when stimulated with 3B4 and anti-IgE. A proportion of anaphylactic patients showed elevated degranulation in response to 3B4 and anti-IgE. N=15-19 anaphylactic patients, 12-15 control patients. Individual levels of degranulation were plotted for each patient. One-way ANOVA was conducted with Dunnett's multiple comparison post hoc test. The change in degranulation (stimulus – basal) was compared between BSA, control and anaphylactic patients; *p<0.05.

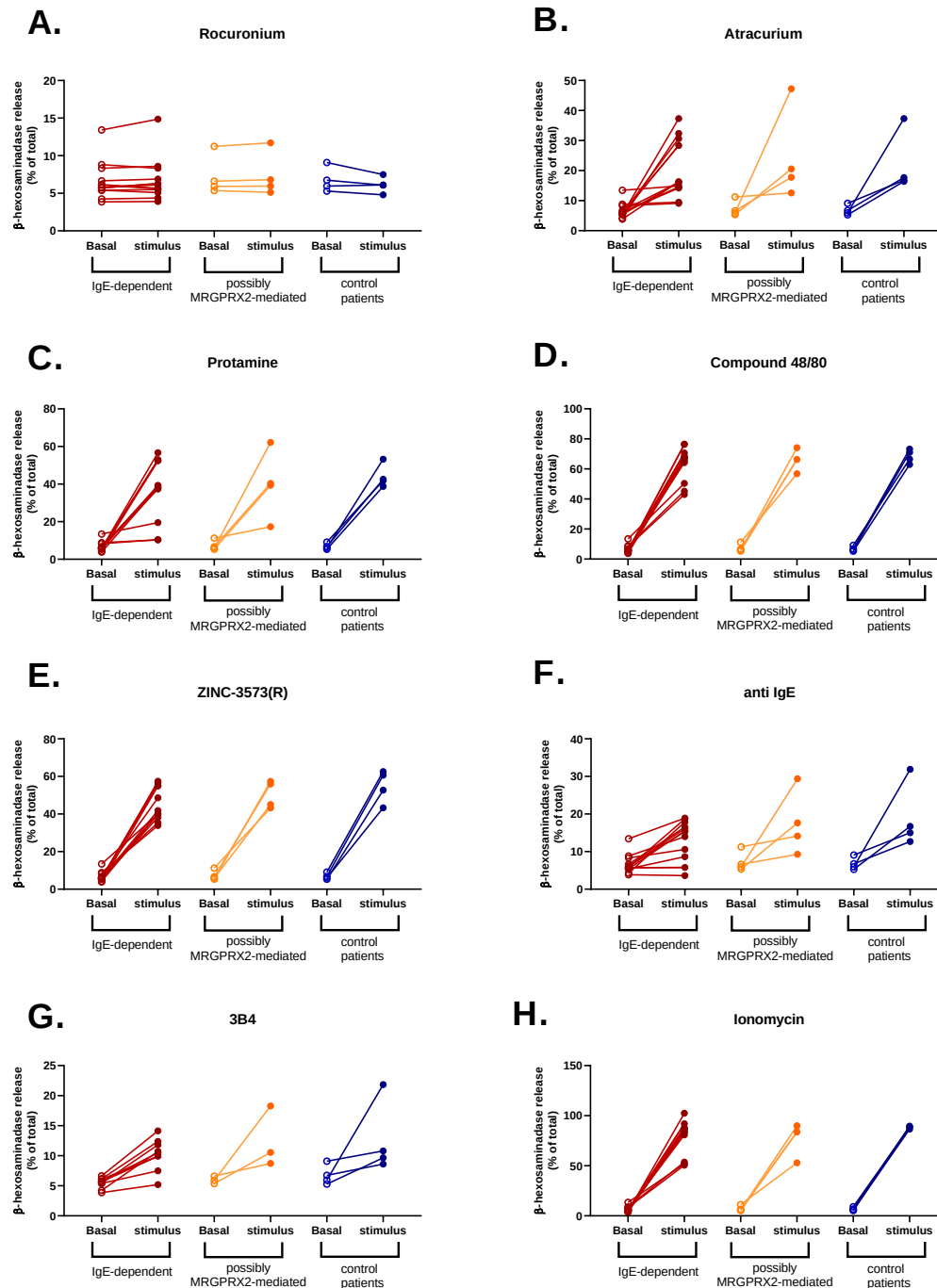


Figure 5.14: Degranulation response of serum-conditioned-LAD2 cells in response to stimuli: stratified according to proposed pathway of anaphylaxis. Patient sera or BSA were diluted 1/20 in serum-free media and incubated with LAD2 cells for 18 hours. The serum conditioned cells were washed and stimulated with a range of drugs (**A.** rocuronium (200 μ M), **B.** atracurium (200 μ M), **C.** protamine (1 μ M), **D.** compound 48/80 (10 μ g/mL), **E.** ZINC-3573(R) (10 μ M), **F.** anti-IgE (2 μ g/mL), **G.** 3B4 (2 μ g/mL), **H.** ionomycin (0.5 μ M)) and degranulation was quantified using the β -hexosaminidase enzyme assay. N= 15-19 anaphylactic patients, 12-15 control patients. Individual levels of degranulation were plotted for each patient. One-way ANOVA was conducted with Dunnett's multiple comparison post hoc test. The change in degranulation (stimulus – basal) of anaphylactic patients were compared against control patients.

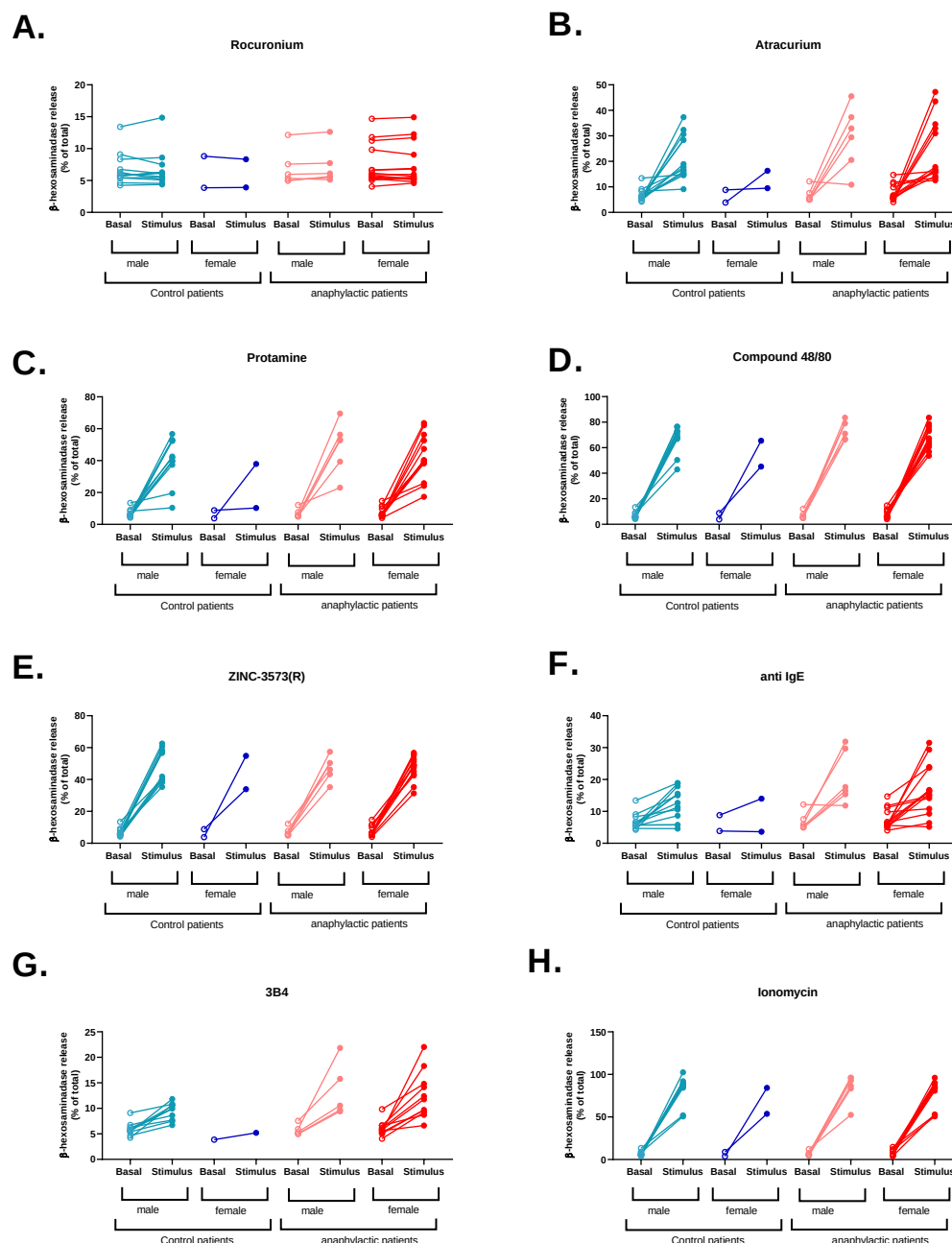


Figure 5.15: Degranulation response of serum-conditioned-LAD2 cells in response to stimuli: characterised according to sex. Following 18 hour serum conditioning, LAD2 cells were stimulated with **A.** rocuronium (200 μ M), **B.** atracurium (200 μ M), **C.** protamine (1 μ M), **D.** compound 48/80 (10 μ g/mL), **E.** ZINC-3573(R) (10 μ M), **F.** anti-IgE (2 μ g/mL), **G.** 3B4 (2 μ g/mL), **H.** ionomycin (0.5 μ M)) and degranulation was quantified using the β -hexosaminidase enzyme assay. Patient cohort of 12-15 control patients and 15-19 anaphylactic patients were sub-categorised into sex. One-way ANOVA was conducted with Dunnett's multiple comparison post hoc test. The change in degranulation (stimulus – basal) of each anaphylactic patient group were compared against respectively control patients.

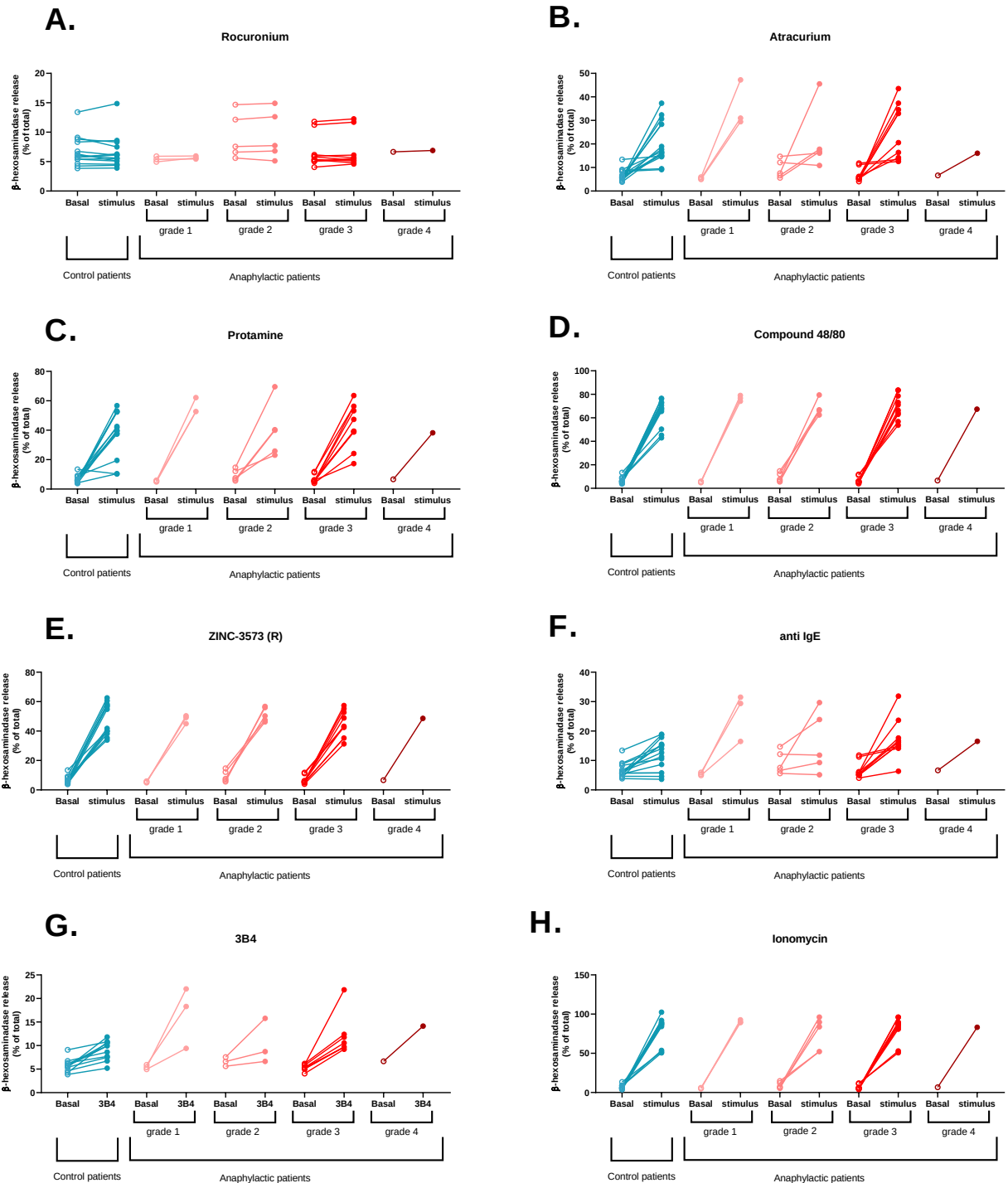


Figure 5.16: Degranulation response of serum-conditioned-LAD2 cells in response to stimuli: characterised according to grade of anaphylaxis. Following 18 hour serum conditioning, LAD2 cells were stimulated with **A.** rocuronium (200 μ M), **B.** atracurium (200 μ M), **C.** protamine (1 μ M), **D.** compound 48/80 (10 μ g/mL), **E.** ZINC-3573(R) (10 μ M), **F.** anti-IgE (2 μ g/mL), **G.** 3B4 (2 μ g/mL), **H.** ionomycin (0.5 μ M)) and degranulation was quantified using the β -hexosaminidase enzyme assay. Patient cohort of 12-15 control patients and 15-19 anaphylactic patients were sub-categorised into grade of anaphylaxis (grade I – IV). One-way ANOVA was conducted with Dunnett's multiple comparison post hoc test. The change in degranulation (stimulus – basal) was compared between control and anaphylactic patients.

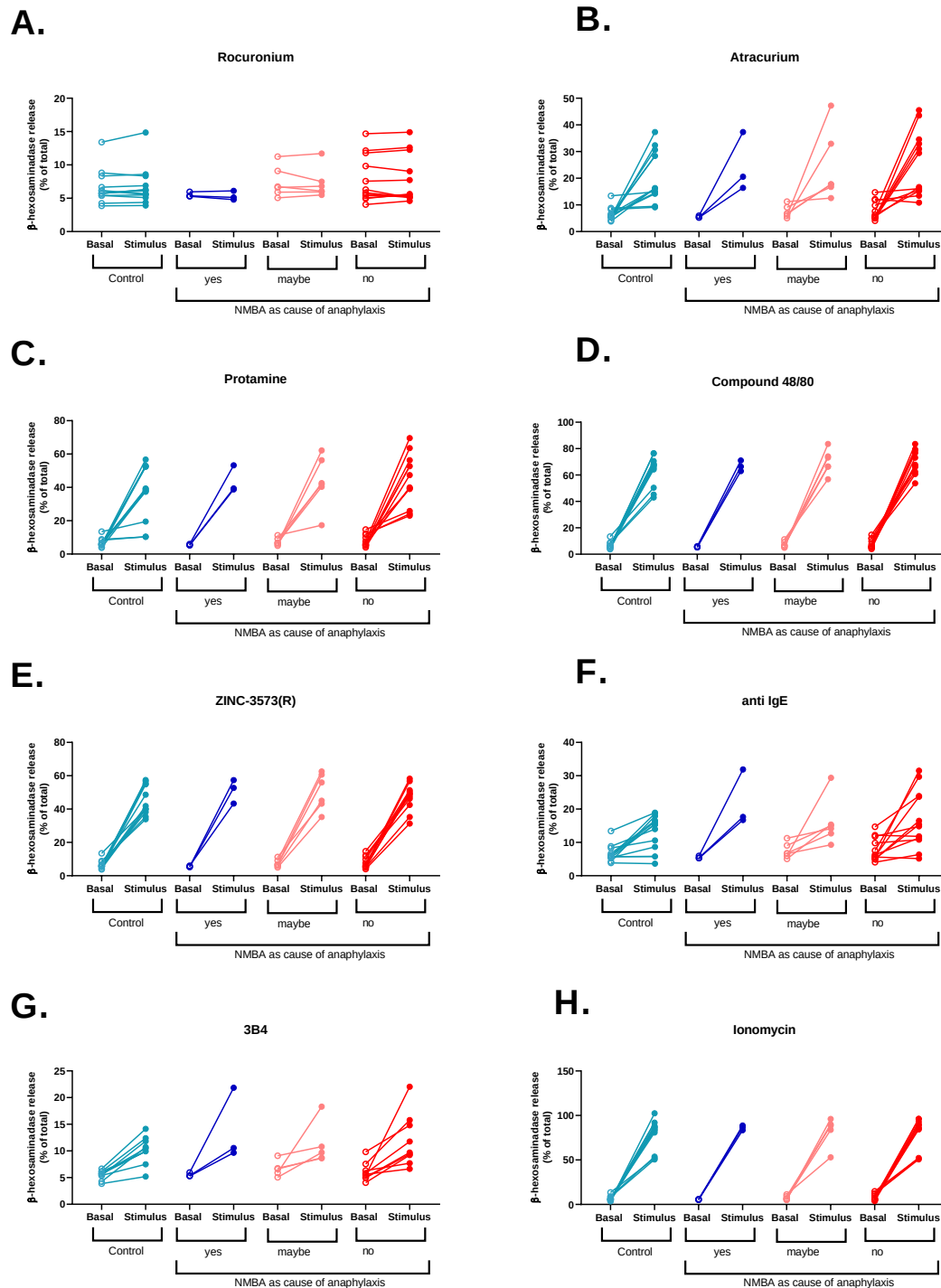


Figure 5.17: Degranulation response of serum-conditioned-LAD2 cells in response to stimuli: characterised according to cause of anaphylaxis. Following 18 hour serum conditioning, LAD2 cells were stimulated with **A.** rocuronium (200 μ M), **B.** atracurium (200 μ M), **C.** protamine (1 μ M), **D.** compound 48/80 (10 μ g/mL), **E.** ZINC3573(R) (10 μ M), **F.** anti-IgE (2 μ g/mL), **G.** 3B4 (2 μ g/mL), **H.** ionomycin (0.5 μ M)) and degranulation was quantified using the β -hexosaminidase enzyme assay. Patient cohort of 12-15 control patients and 15-19 anaphylactic patients were sub-categorised on NMBA as the probable cause of anaphylaxis (yes, maybe, and no). One-way ANOVA was conducted with Dunnett's multiple comparison post hoc test. The change in degranulation (stimulus – basal) of each cause of anaphylaxis was compared against control patients.

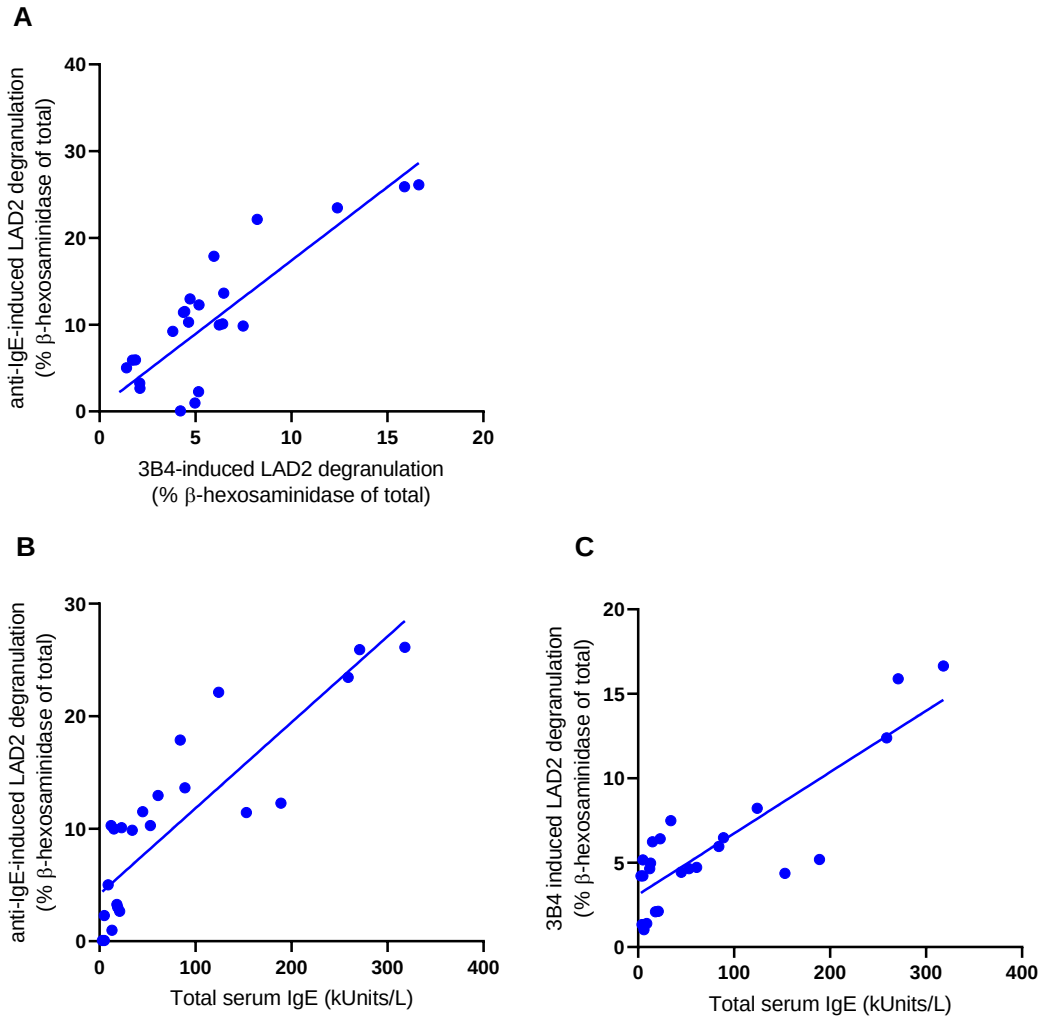


Figure 5.18: Correlation between the IgE-dependent activation of serum conditioned LAD2 cells and total serum IgE levels. LAD2 cells were conditioned with sera for 18 hours and stimulated with 3B4 (2 μ g/mL) and anti-IgE (2 μ g/mL). Degranulation was quantified using the enzyme β -hexosaminidase and calculated as a percentage of total enzyme content, from which the basal release was subtracted. Total serum IgE was quantified by the Department of Pathology, Royal Melbourne Hospital. **A.** LAD2 degranulation induced by α IgE vs 3B4 ($R^2=0.72$) **B.** LAD2 degranulation induced by α IgE vs total serum IgE ($R^2=0.73$) **C.** LAD2 degranulation induced by 3B4 vs total serum IgE ($R^2=0.71$). $N \geq 26$.

5.3.5 MRGPRX2 agonists did not induce degranulation in blood basophils

Basophil activation tests (BAT) are increasingly proposed as an *ex vivo* diagnostic tool for anaphylaxis, with it also being specifically recommended for NMBA-induced IgE-dependent anaphylaxis^(329, 330). In doing so, perhaps BAT could aid in the identification of IgE-dependent reactions from IgE-independent events. However, it is important to highlight that there is also conflicting evidence for the expression of MRGPRX2 in basophils^(72, 331). Basophils were gated from blood leukocytes using the cell surface marker CCR3. The possible activation of MRGPRX2 expressed on basophils, were examined using 11 anaphylactic patients (patient numbers 1-6, 12, 13, 19, 22, 23) and 4 control patients (patient numbers 7-11). All, except 1 control patient, had basophils that were activated (at least 10%) by one or both of the positive controls (fMLP and anti-FcεRI Mab; data not shown), bringing the number of control patients down to 3 (patient numbers 7, 8, 10). Of the 11 anaphylactic patients, 1 had grade IV anaphylaxis, 4 grade III, 3 grade II, and 3 grade I anaphylaxis. Although unclear, 2 of the 11 patients may have had anaphylaxis with NMBAs as the causative agent (**Table 5-1**). In the cohort tested, while all anaphylactic patient basophils were deemed evaluable by their response to positive controls, there were no measurable responses to any of the known MRGPRX2 agonists tested.

Additionally, although high total serum IgE levels would be expected to increase basophil activation likely indicating IgE-dependent anaphylaxis, in this study there appears no correlation ($R^2 = 0.1258$) between total serum IgE and the IgE-dependent activation when stimulated with the anti-FcεRI mAb (**Figure 5.20**).

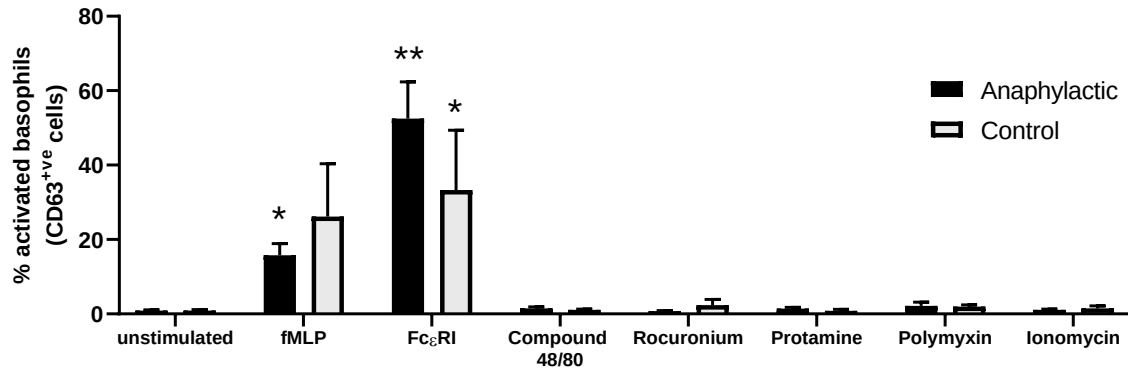


Figure 5.19: MRGPRX2 agonists had no effect on CD63 surface expression of whole blood basophils. Percentage of activated basophils in response to a range of stimuli were quantified from whole blood samples of 11 anaphylactic patients and 3 control patients by flow cytometry. Patient blood was incubated with a range of stimuli for 15 minutes; fMLP (kit provided positive control), anti- FcεRI Mab (kit provided positive control), compound 48/80 (10 µg/mL), rocuronium (200 µM), protamine (6 µM), polymyxin (25 µM), ionomycin (0.5 µM). Data was analysed using Flowjo_V10. Mean, ±SEM. One-way ANOVA was conducted with Dunnett's multiple comparison post hoc test. Each stimulus was compared to its respective unstimulated percentage (anaphylactic or control). *p<0.05, **p<0.01

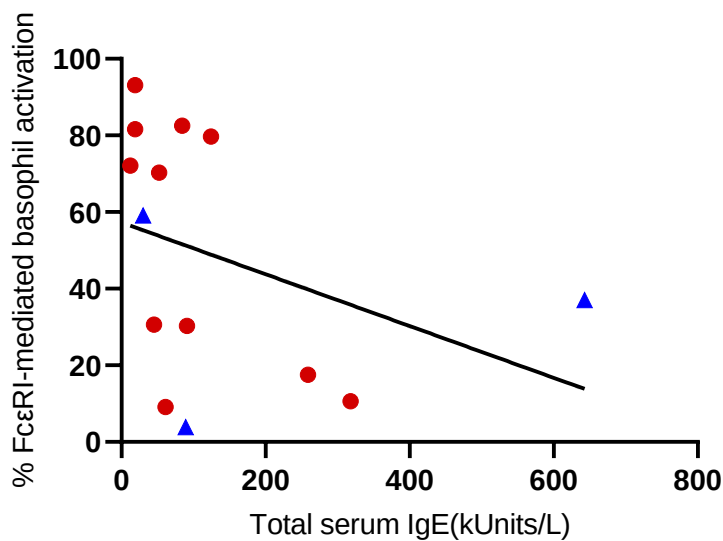


Figure 5.20: There was no correlation between the IgE-dependent activation of BAT and total serum IgE levels. Activation of FcεRI was quantified on whole blood using BAT (11 anaphylactic patients (red circles), 3 control patients (blue triangles)), and plotted against respective total serum IgE. There was no correlation observed ($R^2 = 0.1258$).

5.3.6 Quercetin-mediated inhibition of stimulus-induced LAD2 degranulation

Although the effects of quercetin remain broad, we wished to explore its effect on drug-induced mast cell degranulation when incubated for varying periods of time (acute, 24 hours and 48 hours). In light of existing research, we examined 3 concentrations of quercetin (3, 10, 100 μ M), when either incubated acutely (5 minutes prior to drug stimulation and remains over the course of stimulation) or 24 hours and 48 hours prior to MRGPRX2 agonist addition (with quercetin removal prior to drug addition). In an acute setting, there was a visible inhibitory trend, although our study was underpowered to show significant differences in LAD2 activation (**Figure 5.21**). Interestingly, we observed a significant decrease in compound 48/80-induced LAD2 degranulation when quercetin (100 μ M) was incubated for 24 hours and washed off prior to stimulus (**Figure 5.21D**).

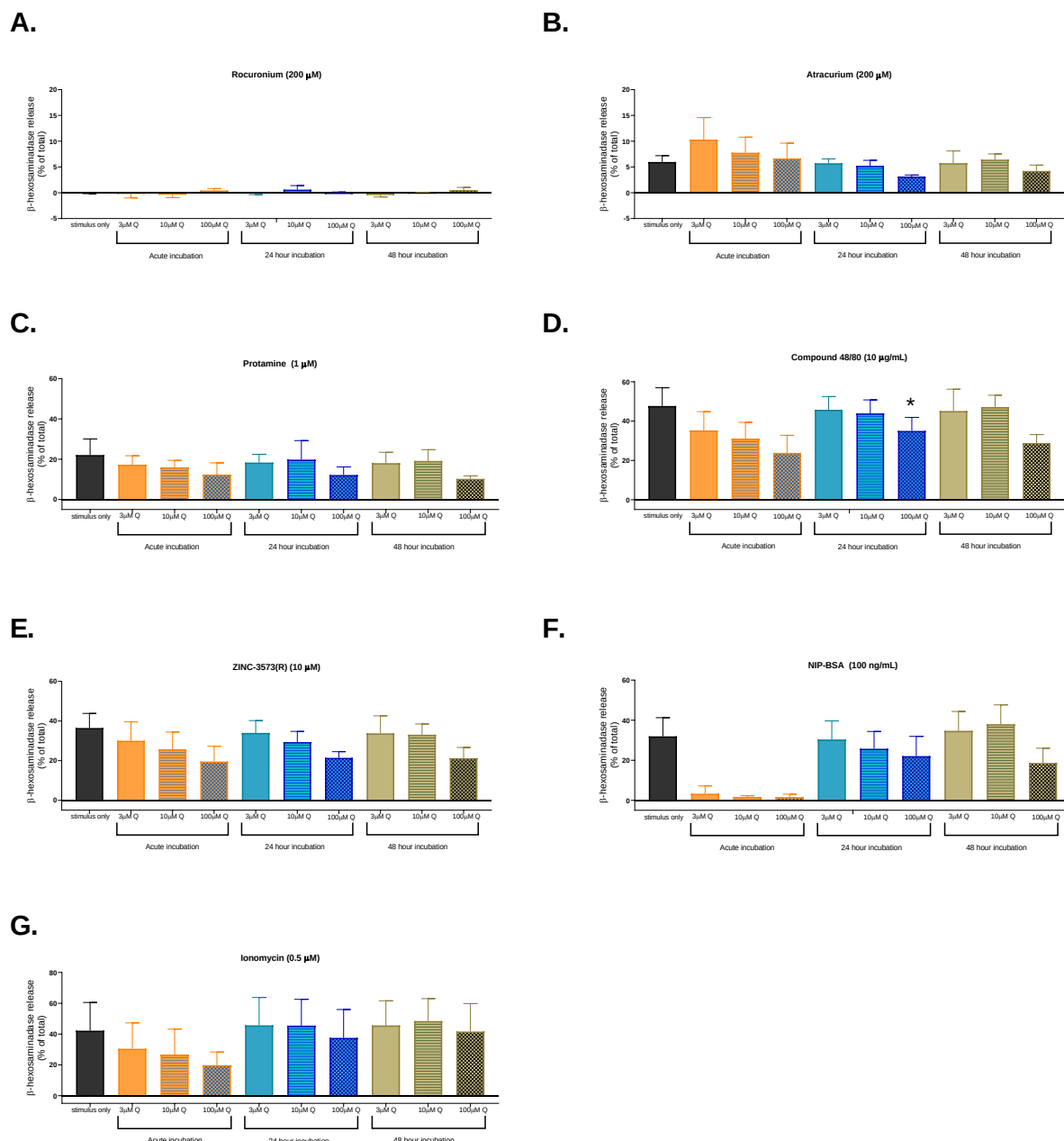


Figure 5.21: The effect of quercetin-conditioning on stimulus-induced LAD2 degranulation. Quercetin conditioned (acute, 24 hours and 48 hours) LAD2 cells were stimulated with **A.** rocuronium (200 μ M), **B.** atracurium (150 μ M), **C.** protamine (1 μ M), **D.** compound 48/80 (10 μ g/mL), **E.** ZINC-3573(R) (10 μ M), **F.** NIP-BSA (100 ng/mL) and **G.** ionomycin (0.5 μ M). 45 minutes following stimulus, degranulation was quantified using the β -hexosaminidase assay. Enzyme release was then calculated as a percentage of total cellular β -hexosaminidase (Triton X-100 induced cell lysis). Conditioning of quercetin (acute, 24 hour and 48 hour), decreased stimulus-induced LAD2 degranulation. N=3-4; mean $\pm$ SEM. One-way ANOVA was conducted with Dunnett's multiple comparison post hoc test. Each response in the presence of quercetin was compared to that in the absence of quercetin. * $p < 0.05$.

5.4 Discussion

We have successfully conducted a small study comparing a range of factors between patients who experienced adverse reactions during surgery and control patients. We categorised the cohort to identify the patients that potentially had an event of MRGPRX2-mediated anaphylaxis (patients who had NMBA-induced anaphylaxis with positive SPT and negative specific IgE). However, none of these patients showed a defining biomarker that distinguished them from IgE-mediated anaphylactic events or control patients. Despite the lack of a specific defining marker that distinguishes anaphylactic patients, we analyse and propose potential factors that may predispose some patients to NMBA-induced non-IgE mediated anaphylaxis.

It is important to highlight some limitations and challenges faced in this study. Firstly, experiments on serum-conditioning and stimulation used the *in vitro* human mast cell line LAD2, on the basis that it represents normal human mast cells. Although the MRGPRX2 receptor sequence is identical to normal human mast cells, we have not carried out a full genome sequencing. This is also true for DNA isolated from our patient cohort. A full genome sequence would inform of any differences in factors that remain unconsidered in our study. However, these assays remain costly and the analysis laborious. A more useful study would include experiments carried out using patient peripheral blood or skin/fat derived mast cells^(332, 333) to assess the sensitivity of each patient's mast cells to MRGPRX2 agonists. This, however, requires a retrospective study that is designed to source samples from patients that have had an anaphylactic reaction, along with the collection of much larger volumes of blood than we received (we collected approximately 20 mL) or a punch-biopsy. Secondly, our study had unmatched control and anaphylactic patients with many more females in the anaphylactic patient cohort while having more males in the control group. Furthering this study to include more control patients that matched (in sex, age and surgeries received) existing anaphylactic patients would rectify this. Moreover, our patient cohort only consists of 3 patients that have been classified as certainly NMBA-anaphylaxis. For future studies, a

more refined approach for selecting patients with severe NMBA anaphylaxis should be applied.

Despite this, we have made a few interesting observations in this study. Our key findings include four individuals (one control and three anaphylactic) with single nucleotide polymorphisms (SNPs) that resulted in amino acid changes. The control patient coded 025EAG, and anaphylactic patients coded 005GG and 019SJ, had one polymorphism that resulted in an amino acid change from an asparagine to serine (N62S). It is important to note that none of the above mentioned anaphylactic patients had confirmed anaphylaxis to NMBAs. Therefore, our results were unable to identify a specific polymorphism or serum factor that distinguished anaphylactic patients from control patients.

The prevalence of MRGPRX2 polymorphisms in our cohort was 10.7% (3 of 28) and 5.5% (1 of 18) in anaphylactic control patients, respectively. As mentioned earlier in our results, the occurrence frequency of polymorphisms (both N16H and N62S) in our study was less than that reported in genomic databases (ALFA project, TOPMED, NCBI-GO-ESP, and 1000 genome project). Further, to the best of our knowledge, the double mutation observed (patient 004 AJ), although recorded individually in the SNP database, have not been reported in tandem. While one study investigated the N16H mutation and found it did not survive the standard process for stable transfection on RBL-2H3 cells ⁽⁹⁴⁾, the N62S SNP has not yet been investigated. Transient transfections demonstrated that the N16S variant induced similar degranulation to WT MRGPRX2 when stimulated with the agonists substance P and icatibant ⁽⁹⁴⁾. It is important to note that these were experiments conducted on a rat basophilic cell line, and the differences in downstream signalling cascades in human mast cells may affect the translation of a polymorphism into cellular outcomes. In addition, as mentioned earlier, it has also not been studied in tandem with the N62S mutation. Intriguingly, the N62S mutation also occurs in a control patient who was not reported to have any adverse reactions to the NMBAs administered. This indicates that perhaps, by itself, the N62S mutation, although having a high probability of being deleterious (polyphen2 factor of 0.976; **Table 5-4**), may not alter the

individual's susceptibility to NMBA-induced anaphylaxis. However, although no correlations can be drawn from our study, the presence of both mutations in tandem may alter receptor functionality, shifting the susceptibility of an individual to anaphylaxis. We intend to carry out further work on the effect of the double polymorphic form of MRGPRX2 to determine if these polymorphisms change receptor activation/function.

In our DNA sequencing studies, we focused on the possibility of polymorphisms solely in the coding region of MRGPRX2, however other mutations, especially in the promoter region, can alter the receptor expression, and thereby, alter susceptibility to adverse outcomes. To investigate this, we analysed the expression of MRGPRX2 along with a range of genes associated with mast cell activation on leukocyte mRNA isolated from whole blood buffy coat. In our studies we found that MRGPRX2 expression was unchanged between the control and anaphylactic patient cohort, negating our initial hypothesis. It is important to note that MRGPRX2 was very difficult to detect, and as a result, the data obtained were bordering on the margins of detection levels, thus lowering the experimental certainty. This is unsurprising due to the low proportion of mast cells in peripheral blood circulation ($<0.1\%$)⁽³³⁴⁾. Therefore, any subtle differences in receptor expression would be difficult to distinguish. Further, mast cells found in blood, in addition to being extremely rare, are almost exclusively immature as they primarily mature in connective and mucosal tissue. Therefore, findings of peripheral blood mast cells may not be representative of mast cells found in connective tissues, the primary contributor to the acute symptoms of anaphylaxis. To address this, a retrospective study with skin/fat specimens⁽³³³⁾ from patients who experienced potentially MRGPRX2-mediated anaphylaxis, could be used to isolate, and culture connective tissue mast cells. These samples, compared against mast cells from control individuals, could be used to explore the possibility of a biomarker in mature mast cells that alters susceptibility of individuals to anaphylaxis. Further, instead of a singular focus on polymorphisms within the MRGPRX2 coding region, future studies could investigate whole genome sequencing to identify the

presence (if any) of mutant or differentially expressed modulators of MRGPRX2 activation that could bias signalling in susceptible individuals.

We also investigated the expression of a range of other receptors and signalling molecules including the high affinity IgE receptor, FcεRI, Gi and Gq proteins, along with pro-inflammatory genes such as IL-8, TNFα and MIF. Similar to MRGPRX2, these genes, although detected at much higher levels, were unchanged between control and anaphylactic patient groups. However, as mentioned earlier, mRNA was isolated from buffy coat that primarily constitutes of neutrophils and lymphocytes ⁽³³⁴⁾, and as observed in MRGPRX2 expression, mast cell mRNA is difficult to detect. Most other genes assessed in this study, especially that of G-proteins and pro-inflammatory genes, are abundantly expressed in all white blood cells. Therefore, given the overwhelming proportion of non-mast cells in the buffy coat, observing a difference in specific signalling molecules or receptors in mast cells is improbable. To address this, future studies could involve isolation of mast cells from peripheral blood (using markers such as CD34⁺ or CD133⁺) prior to analysis.

We further stratified our results according to sex, grade of anaphylaxis and cause of anaphylaxis, for identification of other discriminatory factors between anaphylactic and control patients. Sex has been associated with differential immune activity, making females more susceptible to diseases such as asthma, but with limited supporting studies ^(335, 336). While some reports, including a recent publication from Western Australia, have observed that female patients were more likely to suffer drug-induced anaphylactic reactions ^(182, 337, 338), there are other reports where males seem more susceptible ^(339, 340). Given the conflicting and contradictory studies available, no firm association can be made between sex and anaphylaxis. Nevertheless, in receiving consecutive patients without screening for sex, in our cohort, we have over twice as many females who experienced an anaphylactic reaction during surgery compared to males (20 females and 8 males). However, no clear distinguishing factor was identified that would aid in the recognition of anaphylactic patients based on sex. This

was also true for when the data were stratified according to the grade/intensity or cause of anaphylaxis.

The study also focussed on whether serum plasma factor(s) from patients could perhaps directly activate MRGPRX2. A recently published study suggested that sera of patients anaphylactic to NMBAs activated LAD2 mast cells via MRGPRX2 ⁽¹⁵²⁾. Although our study was an extension of this research, two key alterations in our protocol need to be noted. Firstly, in the study conducted by Navines-Ferrer et al., (2018), the significant increase in MRGPRX2 activation was observed from patient sera that were collected within 24 hours of anaphylaxis ⁽¹⁵²⁾. When repeated on two sets of sera collected >24 hours from event of anaphylaxis, there was no significant mast cell activation observed ⁽¹⁵²⁾. In our study, patient sera were obtained during follow up testing conducted 4-6 weeks following an anaphylactic event. This factor of time from the event of anaphylaxis may significantly alter serum components (as a result of natural metabolism, diet, medication, or lifestyle), that may have contributed to an altered susceptibility to anaphylaxis. Navines-Ferrer et al., (2018), also highlight that the possibility of mast cell activation being a result of residual drug metabolites or other active substances present as a result of anaphylaxis. Secondly, an alteration was made in the assay protocol where we used a serum dilution of 1:5 instead of 1:2 used by the group led by Navines-Ferrer et. al., (2018). This was primarily due to the limited volume of patient sera available in our study. However, in our study, while most patient sera did not activate LAD2 mast cells, two anaphylactic patients significantly increased LAD2 degranulation. Although speculative, while not enough to trigger anaphylaxis in all patients, the presence of this additional serum factor may increase constitutive mast cell activity and tip the balance towards an uncontrolled anaphylactic reaction in response to some drugs used in surgery (*e.g.*, NMBAs). Further experiments using a concentration range of agonists to stimulate peripheral blood derived mast cells from anaphylactic and control patients would add weight to these claims. In addition, further validation is required, perhaps using mass spectrometry, to identify if there is a defining factor between the sera that activated LAD2 mast cells and other patient sera.

Interestingly, although there was no significant difference between anaphylactic and control patients when stimulated with most activators, there was an increase in IgE-dependent LAD2 activation when conditioned with some patient sera. When investigated further, an association between total serum IgE and IgE-dependent mast cell activation of serum conditioned LAD2 cells was evident, and likely indicates patients who had IgE-dependent anaphylaxis. In addition, some patient sera (both anaphylactic and control) seemed to inhibit mast cell activation induced by MRGPRX2-agonists protamine and atracurium but not that of compound 48/80 or ZINC-3573(R). We suggest a presence of a serum factor that may perhaps stabilise mast cells, and in doing this, prevent activation by some MRGPRX2 agonists. Further studies would include obtaining concentration response curves of serum conditioned mast cell degranulation in response to a range of MRGPRX2 agonists to determine the presence, if any, of dampening factors in sera. Additionally, proteome profiles of patient sera could be analysed to further investigate potential biomarkers in patients who experienced anaphylaxis during surgery.

In addition, we conducted basophil activation tests (BATs) as these assays have been linked to identify patients that experienced IgE-dependent NMBA anaphylaxis. Although controversial, BAT has been suggested to be a more reliable means of distinguishing IgE-dependent reactions from IgE-independent anaphylactic events ⁽⁵⁴⁾. BAT uses flow cytometry to detect cell surface markers and because of its sensitivity, is increasingly used as an *ex vivo* diagnostic measure for anaphylaxis ⁽³⁴¹⁾. In addition, the assay can be conducted directly on whole blood, and is utilised clinically alongside skin prick tests and ELISAs to understand the immune response to an allergen ⁽³⁴¹⁾. FMLP (N-formylmethionine-leucyl-phenylalanine), an independent GPCR activator of basophils (acting at the FPR2 receptor) ⁽³⁴²⁾, was used as a positive control along with anti-FcεRI (initiates cross-linking of FcεRI, thereby inducing IgE-dependent activation of basophils and mast cells) ⁽³⁴³⁾. Our data shows no significant basophil activation observed in response to any of the drugs used (with the exception of positive controls). However, none of the patient samples tested had confirmed clinical diagnosis of

NMBA anaphylaxis (although 3 were listed as 'maybe' NMBA-induced). This may explain the lack in IgE-dependent basophil activation observed in response to NMBA.

It is important to highlight that there is also ambiguity in the expression of MRGPRX2 on/in basophils, and as a consequence, their response to NMBA in an IgE-independent manner ^(72, 341, 344, 345). There is further conflict on whether EDTA or heparinised tubes are more effective in quantifying basophil activation ^(341, 346, 347). While there are multiple ways of conducting basophil activation tests, we used the Bühlmann basophil activation kit that recommends the use of EDTA blood and uses the antibodies CCR3 (basophil identification marker) and CD63 (activation marker) to gate activated basophils ⁽³⁴⁶⁾. We have shown that basophil activation was significantly increased in response to the positive controls fMLP and anti-FcεRI, thus validating assay functionality. While elevated serum IgE levels have been correlated with increased FcεRI-mediated basophil activation ⁽³⁴⁸⁾, our results do not confirm this, and may perhaps point towards the presence of clinically irrelevant IgE. Further, as mentioned above, we did not observe basophil activation to any MRGPRX2 agonists used. Intriguingly, several studies have reported the presence of MRGPRX2 on basophils ^(72, 344). However, it is possible that basophil-MRGPRX2 is predominantly found intracellularly ⁽³⁴⁴⁾, therefore, extracellular agonist activation, unless extremely lipophilic to activate intracellular MRGPRX2 (if any), seems an unlikely outcome. Therefore, given the uncertainty, the use of BAT to understand MRGPRX2 mediated non-IgE anaphylaxis remains controversial ^(344, 349, 350).

Lastly, we investigated the effect of the dietary component, quercetin, in altering LAD2 activation. As mentioned earlier, quercetin is a plant pigment (flavonoid) found in a variety of food and drinks including wine, onions, and berries. Intriguingly, France, known to be one of the highest consumers of wine ⁽³⁵¹⁾, reports a much higher rate of drug-induced anaphylaxis ⁽³⁵²⁾ compared to the UK ⁽¹⁵¹⁾ or Australia. Among its countless effects, quercetin was reported to increase stimulus-induced degranulation in the rat mast cell line RBL-2H3 by enhancing expression of the α subunit (the effector subunit) of Gi ⁽¹⁶²⁾. This led us to hypothesise that the exposure of mast cells to quercetin may alter its stimulus-dependent downstream

signalling cascade leading to an altered degranulation response. It is important to note that in a clinical setting, patients are required to go without food and drink prior to surgery, generally for a period of 2-8 hours ⁽³²⁴⁾. However, the half-life of quercetin in blood plasma is between 11 and 28 hours – much longer than the duration of the preoperative fast ⁽³²³⁾. In this study, we were unable to survey patients on their intake of red wine or other quercetin-rich diets, therefore no correlations could be drawn in our clinical cohort. Alternatively, we used our LAD2 mast cell model to investigate the effect of quercetin on MRGPRX2 stimuli. We have demonstrated earlier (chapter 3) that although rocuronium induces calcium mobilization through the MRGPRX2 receptor, it does not induce LAD2 degranulation. In this study, our focus lay upon the possible induction of degranulation by rocuronium in the presence of quercetin. This is based on the hypothesis that if LAD2 mast cells are presumed “normal” mast cells that do not respond adversely to rocuronium, perhaps an external modulating factor like quercetin, by elevating Gi expression, may shift this response to induce degranulation. However, our data suggests that quercetin, and by extension the increase of Gi expression, does not transform calcium mobilization into degranulation when stimulated with rocuronium.

Interestingly, quercetin was also proposed to be a MRGPRX2 receptor antagonist, inhibiting both agonist (compound 48/80 and substance P) -induced degranulation in LAD2 cells and histamine-mediated flaring in rat hind paws ⁽¹⁵⁹⁾. This was associated with inhibition of the PLCγ-IP₃ pathway, thus inhibiting calcium mobilization required for mast cell activation ⁽¹⁵⁹⁾. While our data lack significance to affirm these observations, the inhibitory trend on LAD2 activation appear consistent. Interestingly, we also observed a decrease in compound 48/80-induced degranulation even when quercetin was washed off prior to drug stimulation. One proposed mechanism of action is via the inhibition of Lyn-kinase, essential for the IgE-dependent pathway of mast cell activation ⁽³⁵³⁾. Although speculative, this data suggests the inhibitory effect of quercetin is not restricted to specific MRGPRX2 antagonism, but also involves other signalling cascades that broadly inhibit mast cell degranulation.

In conclusion, in our small study of 28 anaphylactic patients and 18 controls patients, only 3 patients had confirmed cases of NMBA-induced anaphylaxis. We were unable to identify any singular factor that differentiated anaphylactic patients from control patients. However, we have identified a double mutant form of MRGPRX2 in one anaphylactic patient, along with sera of 2 anaphylactic patients that activated resting LAD2 mast cells, that may perhaps predispose those patients to adverse reactions in response to certain drugs. In addition, we have also not observed basophil activation when stimulated with a range of MRGPRX2 agonists, suggesting against the existence of functional surface MRGPRX2. Further studies on a refined cohort of patients stratified more precisely to those most likely to have had MRGPRX2-mediated anaphylaxis, with a matched control cohort, along with experiments conducted on peripheral blood derived or skin/fat-derived mast cells, would greatly enhance and complement this research.

Chapter 6

General discussion

Drug-induced IgE-independent anaphylaxis, although strongly suspected to occur, is poorly characterised in comparison to that mediated through drug-reactive IgE. Recent studies propose a role for the newly identified receptor, MRGPRX2, in causing non-IgE-mediated anaphylaxis via direct mast cell activation ⁽⁵⁸⁾. However, with the lack of a definitive clinical test to identify MRGPRX2-dependent anaphylaxis, the receptor's importance in drug-induced anaphylaxis in humans is unclear. Given that MRGPRX2 is presumably expressed in mast cells of all humans, current research interest centres around the better understanding of MRGPRX2 activation in an attempt to understand why only some people respond adversely to known MRGPRX2 agonist drugs.

Over the course of this project, we sought to characterise if MRGPRX2 activation can be stopped, is subject to ligand-induced bias, or can be predicted. The receptor is found to be promiscuously activated by a range of positively charged compounds ⁽⁹⁶⁾, both endogenous peptides and clinically used drugs. In undertaking this project, we aimed to add to the existing knowledge base of MRGPRX2, and in doing so, be one step closer to understanding its role in anaphylaxis (if any), along with potential means of treatment, or better yet, prevention.

Firstly, we investigated if mast cell activation via MRGPRX2 can be halted with chemical antagonists (of MRGPRX2 stimuli), or the mainstay treatment for anaphylaxis, adrenaline. Secondly, we explored if there was any ligand bias induced in MRGPRX2 activation, thereby highlighting if some drugs are more likely to cause anaphylaxis than others. Lastly, we investigated the possibility of a biomarker that could predict MRGPRX2-mediated drug-induced anaphylaxis and thereby prevent some patients from experiencing detrimental consequences.

This final chapter aims to consolidate and integrate the findings detailed in this thesis with our current understanding of MRGPRX2. In addition, this chapter outlines future directions and limitations that can be addressed in additional studies.

6.1.1 Models used to study MRGPRX2

The activation of MRGPRX2 has been studied in a variety of models, both *in vivo* and *in vitro*, ranging from human skin specimens ⁽³⁵⁴⁾, to rodent models ⁽³⁵⁵⁾, and cultured cell models ⁽⁹⁴⁾. Although skin mast cells are likely the most accurate model from which to extrapolate clinical value, access to primary human specimens remains challenging, and so most studies are designed within the confines of *in vivo* rodent and *in vitro* cell line models. The most widely used models are MRGPRX2 expressed within *in vitro* cell lines that are either not mast cells (*e.g.*, HEK-293 cells), or are non-human (*e.g.*, RBL-2H3), neither of which are accurate representations of human connective tissue type mast cells. In this project, we have generated a CRISPR-Cas9 MRGPRX2 knock down (KD) human mast cell (LAD2) model to validate the role of MRGPRX2 in mast cell activation across a range of stimuli. The generation of this stable knock down model not only provides a more physiologically representative model to study MRGPRX2, but also will permit the transfection and analysis of MRGPRX2 variants into a functionally MRGPRX2 null human mast cell. The efficiency of CRISPR-Cas9-mediated gene editing in LAD2 cells was surprising as LAD2 cells have previously been difficult to genetically modify. Perhaps this relates to our use of recombinant Cas9 rather than plasmid encoded enzyme that might be more challenging to transfect to effective levels than enable guide RNA-mediated gene disruption. This suggests that this approach could have broad utility in gene deletion in LAD2 cells. However, the compromise to this is that LAD2 cells grow poorly from single cell clones and thus polyclonal populations would result unless a very clear selection strategy was feasible.

Interestingly, CRISPR-Cas9 technology has been harnessed to investigate activation and signalling of endogenous receptors, not simply through gene disruption, but also through gene modification. Through designed excision and replacement, GPCRs such as CXCR4 (CXC chemokine receptor 4) have been modified by tagging with a genetically coded luminescent biosensor ⁽³⁵⁶⁾. Because this technology can be utilised in the same cell model where the receptor is constitutively expressed, it removes confounding variables (*e.g.*, system bias) that appear in transfection expression models. Moreover, as expression is driven by the natural

promoter, receptors are expressed at native levels without the usual over expression and resultant possible signalling and functional artefacts. In our study, given that LAD2 cells were found CRISPR Cas-9 sensitive, this technology could be utilised to generate luminescently tagged MRGPRX2 to directly quantify its activation, observe conformational changes, and investigate receptor internalisation ⁽³⁵⁶⁾. This further understanding of MRGPRX2 activation would contribute significantly to the understanding of agonist bias observed in our studies, all within a human mast cell system.

The most frequently used model for the screening of MRGPRX2 activating drugs commonly involves high receptor expression in HEK-293 cells (or other cell line such as U2OS) with the quantification of signalling molecules such as calcium mobilisation and β -arrestin recruitment ⁽⁹⁶⁾. However, we have demonstrated in our studies that MRGPRX2 activation, that results in a significant calcium mobilization response, does not always induce the functional response of mast cell degranulation, the primary contributor to the symptoms of acute anaphylaxis. For the screening of drugs that may potentially induce detrimental MRGPRX2-mediated mast cell activation, the quantification of signalling molecules could therefore be misleading as the drugs may not necessarily induce productive degranulation. Therefore, the use of LAD2 WT cells that possess the ability to degranulate in response to stimuli, along with the MRGPRX2 KD model, could avoid the 'screening out' of potentially useful compounds in drug discovery that are unlikely to induce productive degranulation in humans.

6.1.2 Stimulation of MRGPRX2 and the associated release of mediators

Considering the signalling of MRGPRX2, the coupling of the receptor to Gi and Gq proteins has been well established along with the necessity for the activation of both G proteins to induce degranulation ^(17, 84). Mast cell activation results in the release of a range of potent mediators, with some released essentially instantaneously (*e.g.*, histamine) that contribute to the acute symptoms of anaphylaxis, while others are more slowly elaborated (*e.g.*, cytokines) and potentially contribute to longer term symptoms associated with recovery from anaphylaxis. In our studies with LAD2 mast cells, we have quantified MRGPRX2 activation with

calcium mobilization (that occurs within seconds following stimulus exposure), degranulation (that occurs within seconds to minutes following stimulus), and cytokine CCL2 (released over the timeframe of hours). In our study with sugammadex, we demonstrated that the chemical antagonist had no effect on degranulation (the primary contributor to acute anaphylactic symptoms) after MRGPRX2 had been activated. As mentioned earlier, this aligns with what is expected given the rapidity of degranulation and clinical reports that suggest the lack of efficacy of sugammadex in managing acute episodes of anaphylaxis ⁽²³⁷⁾.

Interestingly, we have shown that the more slowly elaborated mediator release (cytokine CCL2) depends on sustained activation of MRGPRX2 that increases cytokine mRNA expression. In this setting, chemical antagonism of MRGPRX2 agonists significantly reduced CCL2 release that correlated to a reduction in CCL2 mRNA. However, the underlying mast cell signalling mechanism that correlates sustained activation with increased mRNA expression is unclear.

Furthermore, the role of mast cell cytokine release in the recovery from anaphylaxis is poorly understood. It is known that cytokines play a mixed role in post-surgical recovery with both beneficial (*e.g.*, wound healing) and adverse roles (*e.g.*, neuroinflammation) implicated ⁽³⁵⁷⁾. Cytokines are largely released as a consequence of tissue damage during surgery that initiates an innate immune response. However, we have shown in our studies that the NMBA's rocuronium and atracurium can also directly induce CCL2 release from mast cells via MRGPRX2 activation. Although the proportion of CCL2 release from mast cells compared to that resulting from tissue damage is uncertain, it is possible that chemical antagonism of NMBA's may reduce some cytokine-mediated harmful inflammatory effects. A suitable area to study the correlation between recovery and cytokine levels would be to compare patients who receive rocuronium followed by sugammadex to those that receive rocuronium but not sugammadex (*e.g.*, in cardiac surgery where rocuronium is allowed to wear off naturally or an anti-cholinesterase is used). However, matching the surgical procedures to permit this comparison would be challenging.

Aside from the clinical implication of sugammadex in dampening MRGPRX2 activation, the chemical antagonist, through the rapid neutralisation of selected MRGPRX2 agonists, could be used as an experimental tool to understand and further investigate MRGPRX2 activation, signalling, and desensitisation. While MRGPRX2 is most frequently studied as a potential cause of detrimental effects, multiple studies have proposed beneficial roles for MRGPRX2 agonists ranging from heightening innate defence to use as a vaccine adjuvant ^(87, 125). However, given the receptor's role in anaphylaxis is still unclear, sugammadex could be used to explore the kinetics of MRGPRX2 activation in *in vivo* humanised animal models by enabling controlled stimulus-dependent activation progressively neutralised by sugammadex. In doing this, the optimal duration of MRGPRX2 activation necessary to promote beneficial effects, while preventing potential adverse effects, could be investigated.

6.1.3 Is MRGPRX2 involved in drug-induced acute anaphylaxis?

Currently, there is no definitive test to clinically identify IgE-independent anaphylaxis, with the available diagnostic measures being dependent largely on the absence of IgE-dependent markers (*i.e.*, presence of drug-specific/cross-reactive serum IgE). Although frequently proposed as a possible mechanism for non-IgE mediated anaphylaxis, the role of the newly identified MRGPRX2 receptor in this field still remains unclear and further research that explains the clinical implication is warranted. Indeed, some studies argue that MRGPRX2-agonists/drugs like rocuronium almost solely induce IgE-dependent anaphylaxis, and therefore MRGPRX2-mediated activation by these drugs may be irrelevant to clinical anaphylaxis ⁽³⁵⁸⁾. This of great significance to resolve as a recent study concluded that roughly 20% of drug-like compounds act as agonists at MRGPRX2 ⁽⁹⁶⁾.

In our studies, interestingly, the NMBAs, rocuronium and atracurium were found biased against degranulation and cytokine release. This observation was unexpected as although rare, both rocuronium and atracurium are known causes of perioperative anaphylaxis ⁽¹⁴⁹⁾. This may support the above claims that perhaps MRGPRX2 activation may not be an important component in rocuronium/atracurium-induced anaphylaxis. Alternatively, it could

be hypothesised that the LAD2 cell model is representative of 'normal/healthy' mast cells in patients that do not respond adversely to these drugs. This suggests that patients who do respond adversely to these drugs might have 'overactive' mast cells, whether through MRGPRX2 or other signalling pathway polymorphisms or protein expression, have altered the sensitivity to drug stimuli. In these patients, perhaps rocuronium and atracurium act as complete agonists, thereby inducing degranulation, and in extension, anaphylaxis.

A link between receptor mutations and agonist bias have been identified in some GPCRs. For instance, naturally occurring variants of the angiotensin II type I receptor demonstrate bias in coupling to Gi and Gq proteins when stimulated with angiotensin II analogues in a HEK-293 expression system, although the clinical consequence of this is not well understood ⁽²⁶⁶⁾. In light of these associations, we intended to identify potential polymorphisms or differences in MRGPRX2 expression, or serum components that could perhaps be linked to increased anaphylaxis susceptibility in some patients. In our small patient cohort, we have identified 5 individuals who potentially experienced MRGPRX2-mediated anaphylaxis, although this classification of MRGPRX2-mediated anaphylaxis is challenging (as described earlier) and based often on clinical best guesses. None of the patients identified however, had polymorphisms in the MRGPRX2 coding region or differences in MRGPRX2 expression levels suggesting against heightened MRGPRX2-mediated mast cell activation underpinning the anaphylactic episode. Although it was our intention to expand this patient cohort, and thereby increase the analysis of samples from patient who likely experienced MRGPRX2-mediated anaphylaxis, we were unable to do so due to Covid-19 enforced restrictions. We have however identified a novel natural double mutant form of MRGPRX2, and work is currently underway to examine the effect of these polymorphisms on MRGPRX2 activity.

Moreover, our MRGPRX2 expression studies were conducted on peripheral blood leucocytes in which mast cells are a rare find. Peripheral blood mast cells are also almost exclusively immature/progenitor mast cells, and therefore do not accurately represent mature connective tissue mast cells that express MRGPRX2. Thus, detecting differences in MRGPRX2

between patient groups using blood would likely be a poor correlator of what might be occurring in tissue mast cells. Indeed, MRGPRX2 expression levels measured by qPCR were generally very low, commonly bordering on the limits of detection. Thus, the possibility of MRGPRX2 overexpression in mast cells of those who experience anaphylaxis may not be observable with the examination of a total leukocyte preparation. To address this in the future, perhaps a larger retrospective study with patients who were characterised as having had ‘potentially MRGPRX2-mediated’ anaphylaxis, could be recruited with analysis of skin-derived mast cells isolated from punch biopsies (with informed consent). Whilst blood-derived mast cells could be grown from such patients, this involves a long processes of likely non-physiological culture conditions where resultant cells again might not reflect an *in vivo* differentiated skin mast cell. Analysis of skin mast cells could be achieved through focussed single cell transcriptomics approaches or through immunohistochemical staining of MRGPRX2 as has been conducted in studies linking enhanced MRGPRX2 expression to asthma and some skin disorders ^(106, 109).

In addition, instead of focusing solely on MRGPRX2 polymorphisms and expression, a broader search using whole genomic/transcriptomic analysis could shed light to the presence (if any) of a genomic/expression marker that may predict drug-induced anaphylaxis and in particular within pathways known to be connected to MRGPRX2. Given the limited number of mast cells that can be isolated from skin biopsies, single cell genomics and transcriptomics ⁽³⁵⁹⁾ could be utilised to profile a repertoire of mast cell activation markers, receptors, and signalling molecules. Although a more costly process, single cell genomics would provide a broader, unconstrained analysis of connective tissue type mast cells. Interestingly, a recent study identified the high expression of MRGPRX2, similar to skin-derived mast cells, in human fat-derived mast cells ⁽³³³⁾. Moreover, methods for effective expansion of mast cells from fat have been recently published ⁽³³²⁾ and thus the use of fat-derived mast cells may also be a viable alternative.

6.2 Conclusions

Considering the number of clinically used drugs that were found to activate MRGPRX2, understanding the receptor's role in drug-induced anaphylaxis remains vital. The findings in this thesis act as an important steppingstone in the understanding of MRGPRX2 activation and signalling, and by extension, its role in anaphylaxis.

In this body of work, I have detailed our findings on MRGPRX2-mediated mast cell activation and the possibility of its association with anaphylaxis. Due to the rapidity of MRGPRX2 activation and degranulation, neutralising agents or mast cell stabilising agents have no effect on the release of mediators that contribute to the acute symptoms of anaphylaxis. However, some beneficial effect of halting sustained MRGPRX2 activation is found in the delayed release of mediators that may contribute to the recovery outcomes from anaphylaxis. I have also highlighted that some MRGPRX2 agonists were unexpectedly biased against degranulation and cytokine release. Lastly, I have conducted a small clinical study assessing MRGPRX2 expression and serum components of patients that potentially experienced MRGPRX2-mediated anaphylaxis, although in this study, no defining predictive signature was identified. Further clinical studies with clearer patient categorisation along with the analysis of more relevant mast cells (*e.g.*, skin derived mast cells) would greatly enhance the strength of this research.

In summary, the findings of this thesis have provided new insight into drug-induced MRGPRX2-dependent mast cell activation and how it might be treated. Further analysis of agonist bias in MRGPRX2 and a more comprehensive collection and categorisation of patient samples obtained from those who experienced drug-induced anaphylaxis, will better define the importance of MRGPRX2 in this damaging event. Such knowledge could assist with the ultimate goal of predicting patients who are in danger of experiencing drug-induced anaphylaxis and thus personalising therapeutic approaches accordingly.

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