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www.elsevier.com/locate/ejphar

PII: S0014-2999(15)00371-4
DOI: <http://dx.doi.org/10.1016/j.ejphar.2015.04.027>
Reference: EJP69915

To appear in: *European Journal of Pharmacology*

Received date: 8 September 2014
Revised date: 16 April 2015
Accepted date: 20 April 2015

Cite this article as: Trent F. Reardon, Brid P. Callaghan, James A. Brock, Angiotensin II increases nerve-evoked contractions in mouse tail artery by a T-type Ca^{2+} channel-dependent mechanism, *European Journal of Pharmacology*, <http://dx.doi.org/10.1016/j.ejphar.2015.04.027>

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**Angiotensin II increases nerve-evoked contractions in mouse tail artery by a T-type
Ca²⁺ channel-dependent mechanism**

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Abstract

Angiotensin II (Ang II) increases sympathetic nerve-evoked contractions of arterial vessels. Here the mechanisms underlying this effect were investigated in mouse tail artery.

Isometrically mounted segments of mouse distal tail artery were used to investigate the effects of endothelium denudation, blocking Ca^{2+} channels and inhibiting superoxide signalling on Ang II-induced facilitation of nerve-evoked contractions. In addition, *in situ* amperometry was used to assess effects of Ang II on noradrenaline release. Ang II (0.1–1 nM) increased nerve-evoked contractions but did not change noradrenaline release. Losartan (Ang II type 1 receptor antagonist), but not PD 123319 (Ang II type 2 receptor antagonist), blocked the facilitatory effect of Ang II on nerve-evoked contractions. Ang II increased vascular muscle reactivity to phenylephrine and UK-14304 (α_1 - and α_2 -adrenoceptor agonists, respectively). Endothelial denudation increased nerve-evoked contractions and reduced the facilitatory effect of Ang II on these responses. Efonidipine (L- and T-type Ca^{2+} channel blocker) and NNC 55-0396 (T-type Ca^{2+} channel blocker) also attenuated this effect of Ang II, while nifedipine (L-type Ca^{2+} channel blocker) did not. Blockers of superoxide generation/signalling did not change the facilitatory effect of Ang II on nerve-evoked contractions. The findings indicate that Ang II increases the contribution of T-type Ca^{2+} channels to neural activation of the vascular muscle. In addition, Ang II appears to reduce the inhibitory influence of the endothelium on nerve-evoked contractions.

Keywords: Mouse tail artery, Sympathetic neurovascular transmission, Angiotensin II, T-type Ca^{2+} channel.

1. Introduction

Angiotensin II (Ang II) is a peptide hormone that plays a central role in cardiovascular control and is a major target for drugs that lower blood pressure. One of its main actions is to cause vasoconstriction both by a direct action on vascular smooth muscle (Touyz & Schiffrin, 2000) and by increasing sympathetic neurovascular transmission (Nap et al. 2003). The concentrations of Ang II that increase nerve-evoked constrictions *in vitro* are often lower than those that directly cause vasoconstriction (Hilgers et al. 1993; Kawasaki et al. 1982). Similarly, *in vivo* there are reports that infusion of Ang II increases nerve-evoked vasoconstriction at concentrations that do not themselves produce vasoconstriction (Cline, 1985; Zimmerman, 1967). Furthermore, there is evidence that endogenous Ang II increases sympathetic nerve-mediated vasoconstriction (Cline, 1985; Kaufman and Vollmer, 1985; Moreau et al. 1993). These findings highlight the importance of studying the effects of Ang II on sympathetic neurovascular transmission.

In arterial vessels, Ang II has been demonstrated to act both prejunctionally to increase noradrenaline release from the sympathetic nerve terminals (Balt et al. 2001a) and postjunctionally to increase reactivity of vascular muscle to noradrenaline (Dunn et al. 1991; Thorin and Atkinson, 1994). In rat mesenteric arteries, the facilitatory effect of Ang II on nerve-evoked contractions has been suggested to be mediated by an increase in neurotransmitter release (Balt et al. 2001a). However, Lu et al. (2008) reported that this facilitatory effect of Ang II in mesenteric arteries was mediated by a superoxide-dependent mechanism that increases smooth muscle reactivity to α_1 -adrenoceptor agonists. In addition, Lu et al (2008) provided evidence that Ang II reduced the inhibitory effect of endothelial-derived nitric oxide on nerve-evoked contractions. In rat tail artery, Ang II increases constrictions to both nerve stimulation and exogenously applied noradrenaline at concentrations that do not increase noradrenaline release (Thorin and Atkinson, 1994). The

pre- and postjunctional actions of Ang II are mediated through Ang II type 1 (AT₁) receptors in both rat mesenteric and tail arteries (Balt et al. 2001a; Lu et al. 2008; Pinheiro et al. 2002). Excitation of vascular muscle by AT₁-receptor activation is dependent on intracellular Ca²⁺ release, influx of extracellular Ca²⁺ and Ca²⁺ sensitization (Wynne et al. 2009). However, the mechanisms that underlie the facilitatory action of Ang II on nerve-evoked constrictions have not been fully defined.

The present study investigated the mechanisms that contribute to the marked augmentation of nerve-evoked contractions of the mouse tail artery produced by low concentrations of Ang II. The hypothesis that this action was mediated at a postjunctional site was tested. In addition, the roles of the endothelium, extracellular Ca²⁺ influx and superoxide signalling in mediating the facilitatory effect of Ang II on neurovascular transmission were investigated.

2. Materials and Methods

2.1 Animals and tissue preparation

All procedures conformed to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and were approved by the animal ethics committee at the University of Melbourne. Male C57Bl/6 mice aged 8 - 12 weeks were deeply anaesthetised with isoflurane and then killed by cervical dislocation. Segments of ventral tail artery ~1.75 mm in length were dissected from 60 - 80 mm along the tail. Vessels were maintained in physiological saline solution containing (mM): NaCl, 133; KCl, 4.7; CaCl₂, 2.0; MgCl₂, 1.2; NaH₂PO₄, 1.3; NaHCO₃, 16.3; glucose, 7.8; ethylenediamine tetraacetic acid, 0.02. This solution was bubbled with 95% O₂ / 5% CO₂ and heated to ~36.5°C.

2.2 Mechanical responses

Artery segments were mounted isometrically between two stainless-steel wires (40 μm diameter) in a four-chamber myograph (Multi Myograph Model 610M, Danish Myo Technology, Aarhus, Denmark), with basal conditions normalized as described previously (Reardon and Brock, 2013). All vessels were then stimulated with 2 applications of phenylephrine (2 μM) to confirm viability and when the second contraction had plateaued, carbachol was applied (1 μM) to determine if the endothelium was intact (defined as relaxation to carbachol >70%). In a small number of vessels, the endothelium was denuded by rubbing the lumen with a human hair. Success of this procedure was confirmed by the relaxation to carbachol being <5%. In all experiments, test and control assessments were made in parallel using tissues obtained from the same animal.

2.3 Electrically evoked contractions

Electrical stimuli were applied through platinum plate electrodes mounted on either side of the artery along its length. The stimulus pulse width was 0.2 ms and the voltage was set at 120% of the minimum voltage required for a maximal contraction to 50 pulses at 3 Hz (typically 12 V). At the end of the experiments it was confirmed that α -adrenoceptor blockade (with 0.01 μM prazosin + 0.1 μM idazoxan) or tetrodotoxin (0.5 μM) abolished electrically evoked contractions (establishing that the electrical stimuli did not directly activate the muscle). In rats, postganglionic sympathetic neurons supplying the tail artery typically discharge action potentials at a mean frequency of <1 Hz, with the level of activity increasing maximally up to about 2 Hz when the body core temperature is lowered (Ootsuka et al. 2004). For this reason, we chose to study contractions evoked by trains of stimuli at 2 Hz.

The arteries were stimulated with trains of 50 stimuli at 2 Hz delivered at 8 min intervals. To assess the concentration-dependence of the facilitatory effect of Ang II on nerve-evoked contractions, Ang II was added cumulatively, after 2 control responses, at increasing concentrations (0.1, 0.3 and 1 nM), with each concentration present for 2 contractions. The experiments investigating the effects of drug pre-treatment on the Ang II-induced facilitation of nerve-evoked contractions consisted of a series of 8 or 10 contractions; with 2 under control conditions followed by 2 (or 4 for Ca^{2+} channel blockers) in the presence or in the absence (control) of the drug and then 2 in the presence of each concentration of Ang II studied (0.3 and/or 1 nM). The effect of the drug on nerve-evoked contractions was determined at the contraction just prior to the addition of Ang II and is expressed as a % of the contraction immediately prior to the application of the drug. Comparisons were made with measures made in control tissues from the same animal in which no drug was added. The facilitatory effect of Ang II on nerve-evoked contractions was expressed as a % of the response immediately prior to its addition.

2.4 Alpha-adrenoceptor agonist- and K^+ -evoked contractions

In the experiments with phenylephrine (0.1 - 10 μM , α_1 -adrenoceptor agonist), UK 14,304 (0.001 - 1 μM , α_2 -adrenoceptor agonist) and K^+ (20 – 50 mM, equimolar substitution of KCl for NaCl in the physiological saline), 2 cumulative concentration-response curves were constructed with the concentration increased at 4 min intervals. The first concentration-response curve was done in the absence of Ang II and the second 30 min after the addition of 1 nM Ang II. The curves for K^+ were constructed in the presence of prazosin (0.1 μM , α_1 -adrenoceptor antagonist) and idazoxan (1 μM , α_2 -adrenoceptor antagonist) to block the contractile effects of noradrenaline released from the nerve terminals by K^+ -induced depolarization.

2.5 Amperometry

The release of endogenous noradrenaline was monitored using continuous amperometry as described previously (Brock and Tan, 2004). Briefly, a ~15 mm artery segment was pinned to the Sylgard coated base of a 1 ml recording chamber and electrical stimuli (0.2 ms, 30 V) were applied through a pair of platinum wire electrodes mounted vertically on either side of the artery (~0.5 mm apart). The tissue was superfused with warmed physiological saline (~36.5°C) containing desmethylinipramine (0.1 μ M) to block neuronal noradrenaline re-uptake and prazosin (0.1 μ M) to reduce contractions. A carbon fibre recording electrode (7 μ m diameter) was mounted so that the first 100–200 μ m from the tip of the fibre was in contact with the adventitial surface of the artery in a region ~1 mm distal to the stimulating electrodes. The recording electrode was connected to an AMU130 Nanoamperometer (Radiometer-Analytical SA, Villeurbanne Cedex, France) and a potential difference of +0.3 V was applied between the recording electrode and an Ag/AgCl pellet placed in the recording chamber medium. The current required to maintain this voltage was monitored.

During the experiments, arteries were stimulated at 90 s intervals with trains of 10 stimuli at 10 Hz. Ang II (1 nM) was applied immediately following the 15th train and left in contact with the tissue for a further 15 trains. As electrical stimulation produced an artefact (revealed in tetrodotoxin) that lasted up to 0.3 s following last stimulus in the train, the amplitude of the noradrenaline-induced oxidation currents was measured 0.5 s following the last stimulus. The mean amplitude of oxidation currents evoked by the 11th to 15th trains (S_1 ; control period) and by the 26th to 30th trains (S_2 ; test period) were measured. The S_2/S_1 ratios in Ang II-treated tissues were compared with those determined in the time-matched control tissues. As a positive control, in all experiments it was confirmed that blockade of α_2 -adrenoceptor-mediated autoinhibition with idazoxan (1 μ M) for a further 15 trains increased

the oxidation currents (measured at the last 5 trains in the presence of idazoxan (S_3) and presented as S_3/S_2 ratios). It was also established that the signals were abolished by tetrodotoxin (TTX, 0.5 μ M), confirming they are due to action potential-evoked noradrenaline release.

2.6 Data analysis

The output from the myograph was recorded and analysed using a PowerLab data acquisition system and the program Chart (ADInstruments, Bella Vista, NSW, Australia). For nerve- and K^+ -evoked contractions their peak amplitudes were measured. The α -adrenoceptor agonist-evoked contractions often peaked and then declined slightly to a stable level and in these cases the amplitude of the stable response was measured. The EC_{50} s for phenylephrine were estimated by fitting the data to the Hill equation using nonlinear regression analysis (Igor Pro; Wavemetrics, Lake Oswego, OR, USA) and are presented as their negative logarithm (pEC_{50}). All values are expressed as the mean and S.E.M. Statistical comparisons were made with SPSS 22 (IBM Corporation, NY, USA). Pair-wise comparisons were made with the paired or unpaired t -test. For α -adrenoceptor agonist and K^+ concentration-response curves, the data were compared using repeated measures ANOVA. When multiple pair wise comparisons were made in individual tissues, P values were adjusted using the false discovery rate procedure (Curran-Everett, 2000). Results were considered significant at $P < 0.05$. In all cases, n refers to the number of animals used.

2.7 Drugs

Ang II was from Auspep (Tullamarine, VIC, Australia). L-phenylephrine HCl, carbachol (carbamoylcholine chloride), prazosin HCl, idazoxan HCl, nifedipine, losartan, PD 123319, genistein, tempol and apocynin were from Sigma-Aldrich Chemical Company

(Castle Hill, NSW, Australia). SKF 96365, efonidipine HCl, NNC 55-0396, UK 14,304 tartrate, and NF449 were from Tocris Bioscience (Bristol, UK). Tetrodotoxin was from Alamone (Jerusalem, Israel). Efonidipine was prepared as a 10 mM stock solution in dimethylsulphoxide (DMSO; final working concentration of DMSO 0.01% (v/v)). Prazosin was prepared as a 1 mM stock solution in 10% (v/v) DMSO in water. Nifedipine was prepared as 10 mM stock solutions in ethanol and apocynin was prepared on the day of the experiment at 300 mM in ethanol (final working concentration of ethanol $\leq 0.1\%$ (v/v)). All other drugs were made up as ≥ 1 mM stock solutions in water.

3. Results

3.1 Ang II increases the amplitude of nerve-evoked contractions through AT₁ receptors

Fig. 1A shows a representative trace of nerve-evoked contractions in a tissue treated with increasing concentrations of Ang II (0.1 – 1 nM). Ang II increased the amplitude of nerve-evoked contractions at all concentrations studied (**Fig. 1B**) and caused a small sustained contraction of the arteries (0.03 ± 0.01 mN mm⁻¹ at 1 nM; $P < 0.05$). On their own neither the AT₁ receptor antagonist losartan (0.1 μ M) nor the AT₂ receptor antagonist PD 123319 (1 μ M) changed nerve-evoked contractions (**Table 1**). However, the facilitatory effect of 1 nM Ang II on nerve-evoked contractions was greatly reduced by losartan but unaffected by PD 123319 (**Table 2**).

3.2 Ang II does not increase noradrenaline release

In comparison with time-matched controls, Ang II (1 nM) did not change the amplitude of oxidation currents evoked by trains of 10 pulses at 10 Hz (**Fig. 2A, B**). As a positive control, it was confirmed that blockade of prejunctional α_2 -adrenoceptors with

idazoxan (1 μ M) increased the amplitude of oxidation currents produced by this stimulus (**Fig. 2A, B**). TTX (0.5 μ M) fully blocked the oxidation currents (**Fig. 2A**), confirming they are due to action potential-evoked noradrenaline release. These findings indicate that Ang II mediates its facilitatory effect on nerve-evoked contractions at a postjunctional site.

3.3 Ang II reduces the sensitivity of nerve-evoked contractions to blockade by α -adrenoceptor antagonists

The amplitude of nerve-evoked contractions was reduced by blockade of α_1 - or α_2 -adrenoceptors with prazosin and idazoxan, respectively (**Table 3**). In the presence of 1 nM Ang II, the % blockade of nerve-evoked contractions produced by either prazosin (10 nM) or idazoxan (0.1 μ M) was reduced compared to that in the absence of Ang II (**Table 3**). Increasing the concentration of each antagonist 10-fold in the presence of 1 nM Ang II increased the % blockade they produced to a level similar to that seen with the lower concentration in the absence of Ang II (**Table 3**). In time-matched control tissues treated with Ang II alone, nerve-evoked contractions did not change significantly during the period that the effects of the antagonists were assessed (decreased by $17 \pm 8\%$; $P = 0.18$, $n = 8$). The P2X₁-purinoceptor antagonist NF449 (10 μ M) reduced nerve-evoked contractions (**Table 1**), but did not reduce the facilitation of these responses produced by Ang II (**Table 2**).

3.4 Ang II increases reactivity to α -adrenoceptor agonists and K⁺

The change in effectiveness of the α -adrenoceptor antagonists could potentially be explained by a change in the reactivity of the vascular muscle to α -adrenoceptor stimulation. Ang II (1 nM) increased sensitivity to the α_1 -adrenoceptor agonist phenylephrine (pEC₅₀;

Ang II, 6.21 ± 0.12 ; control, 5.94 ± 0.12 ; $P < 0.01$; $n = 8$), but had no effect on the maximum contraction to this agent (**Fig. 3A**). In the absence of Ang II, the maximum concentration of the α_2 -adrenoceptor agonist UK 14,304 tested ($1 \mu\text{M}$) produced a very small contraction ($< 0.1 \text{ mN mm}^{-1}$; **Fig. 3B**). By contrast, in the presence of Ang II (1 nM), UK 14,304 produced a contraction that plateaued at $0.01 \mu\text{M}$ (**Fig. 3B**). As Ang II also increased sensitivity to K^+ (**Fig. 3C**), there appears to be a generalized increase in reactivity of the vascular muscle.

3.5 Ang II-induced facilitation of nerve-evoked contractions is changed by endothelium denudation

In mouse distal tail artery, the H_2O_2 -induced facilitation of nerve-evoked contractions is dependent on an intact endothelium (Reardon and Brock, 2013). Therefore the effect of endothelium denudation on the Ang II-induced facilitation of nerve-evoked contractions was investigated. In endothelium denuded vessels contracted with phenylephrine ($2 \mu\text{M}$), carbachol ($1 \mu\text{M}$)-induced relaxations were abolished (denuded, $2 \pm 1\%$; intact, $90 \pm 3\%$; $P < 0.001$, $n = 6$ for both). Endothelial denudation also approximately doubled the size of nerve-evoked contractions (**Fig. 4**). In denuded arteries, 0.3 nM Ang II increased the amplitude of nerve-evoked contractions but increasing the concentration to 1 nM did not further increase the size of these responses (**Fig. 4**). By contrast, in intact arteries, 1 nM Ang II produced a greater augmentation of nerve-evoked contractions than did 0.3 nM (**Fig. 4**). However the absolute size of the contractions in the presence of 1 nM Ang II did not differ between intact and endothelium denuded vessels (**Fig. 4**).

3.6 Ang II-induced facilitation of nerve-evoked contractions is, in part, dependent on activity of T-type Ca^{2+} channels

To determine if Ang II mediates its facilitatory effects on nerve-evoked contractions via modulation of Ca^{2+} entry into the vascular muscle, the effects of SKF-96365 (10 μM , store-operated and voltage-gated Ca^{2+} channel blocker), efonidipine (1 μM , L and T-type Ca^{2+} channel blocker), nifedipine (1 μM , L-type Ca^{2+} channel blocker) and NNC 55-0396 (1 μM , T-type Ca^{2+} channel blocker) were investigated. On their own, nifedipine and NNC 55-0396 reduced nerve-evoked contractions while efonidipine and SKF-96365 did not (**Table 1**). The finding that nifedipine reduced nerve-evoked contractions, while efonidipine did not, may be explained by the greater potency of nifedipine at L-type Ca^{2+} channels (Masumiya et al. 1998). NNC 55-0396 at $\geq 10 \mu\text{M}$ abolished nerve-evoked contractions but this effect was accompanied by vasoconstriction, suggesting these high concentrations have non-specific actions on vascular muscle.

Both SKF-96365 and efonidipine on their own reduced the facilitatory effect of Ang II (1 nM), and together their inhibitory action was not greater than that of efonidipine alone (**Fig. 5**). These findings indicate a role for voltage-gated Ca^{2+} channels in the facilitatory effect of Ang II. Nifedipine (1 μM) did not change the facilitatory effect of Ang II but NNC 55-0396 (1 μM) similarly reduced the facilitatory effect of Ang II when applied alone or in combination with nifedipine (**Fig. 5**). These findings suggest a role for T-type Ca^{2+} channels in mediating the facilitatory action of Ang II on nerve-evoked contractions. Consistent with these findings, RT-PCR revealed the presence of both L- and T-type channel isoforms in the mouse distal tail artery (**Supplementary Fig. 1**)

3.7 Ang II-induced facilitation of nerve-evoked contractions is not dependent on superoxide signalling

The role of reactive oxygen species in the facilitation of nerve-evoked contractions produced by Ang II was assessed using the superoxide dismutase mimetic tempol (1 mM) and the inhibitor of NADPH oxidase, apocynin (300 μ M). Nerve-evoked contractions were reduced in amplitude by both tempol and apocynin (**Table 1**). However, neither of these agents changed the facilitation of nerve-evoked contractions produced by Ang II (**Table 2**).

In mesenteric artery, the augmentation of nerve-evoked contractions produced by both Ang II and the superoxide anion generator pyrogallol was reduced by tyrosine kinase inhibition (Lu et al. 2008). In distal tail artery, the tyrosine kinase inhibitor genistein (10 μ M) reduced nerve-evoked contractions (**Table 1**) but did not change the facilitatory effect of Ang II on these responses (**Table 2**).

4. Discussion

This study demonstrates in mouse tail artery that low concentrations of Ang II (0.1 – 1 nM) had no effect on noradrenaline release, but increased both nerve-evoked contractions and the sensitivity of the vascular muscle to α_1 - and α_2 -adrenoceptor agonists. These findings indicate that Ang II increases neurovascular transmission by a postjunctional mechanism. This contrasts with findings in rat mesenteric arteries where the facilitatory effect of Ang II is only seen at concentrations >1 nM and is mediated by a prejunctional action at 10 nM (Balt et al. 2001a). In rat tail artery, Ang II acts at a postjunctional site to increase nerve-evoked contractions (Thorin and Atkinson, 1994) but only at concentrations that are much higher than those in the mouse tail artery (≥ 10 nM; Al Dera and Brock, 2015). While previous findings indicate a role for Ang II stimulated superoxide signalling in mediating the increase

in nerve-evoked contractions produced by a relatively high concentration of Ang II (100 nM; Lu et al. 2008), this mechanism does not explain the effects of physiological relevant concentrations of Ang II in mouse tail artery. Instead, in this vessel, the findings indicate a role for T-type Ca^{2+} channels in mediating the facilitatory action of these low concentrations of Ang II on nerve-evoked contractions.

The facilitatory action of Ang II on nerve-evoked contractions appeared to involve T-type Ca^{2+} channels because it was not affected by the L-type Ca^{2+} channel blocker nifedipine but it was reduced by the L- and T-type Ca^{2+} channel blockers efonidipine (Tanaka and Shigenobu, 2002) and SKF 96365 (Singh et al. 2010) and the T-type Ca^{2+} channel blocker NNC 55-0396 (Huang et al. 2004). SKF 96365 also blocks store-operated Ca^{2+} channels but in combination with efonidipine it did not produce greater inhibition of the facilitatory effects of Ang II than did efonidipine alone, suggesting its actions were entirely due to blockade of voltage-gated Ca^{2+} channels.

There are previous reports that Ang II mediates its direct contractile effect on vascular muscle by increasing the activity of T-type Ca^{2+} channels. In renal afferent and efferent arterioles, Ang II induces constriction through activation of T-type Ca^{2+} channels (Feng and Navar, 2004). The tail artery supplies blood to the skin of the tail and a role for T-type Ca^{2+} channels in mediating the Ang II-evoked contraction has been suggested in human small subcutaneous arteries (Garcha et al. 2001). In these vessels, Ni^{2+} (a blocker of T-type Ca^{2+} channels) strongly reduced both the contraction and the rise in intracellular Ca^{2+} -induced by Ang II acting at AT_1 receptors, while an L-type Ca^{2+} channel blocker (amlodipine) had a much smaller inhibitory effect (Garcha et al. 2001). In contrast, Baan et al. (1999) reported that a high concentration of the L-type Ca^{2+} channel blocker nicardipine almost abolished AT_1 receptor-mediated contractions to Ang II in human subcutaneous arteries. However, while nicardipine is often considered L-type Ca^{2+} channel selective, it has been demonstrated

to block both L- and T-type Ca^{2+} channels with similar potency (Furukawa et al. 2009). In vascular muscle, there have been no direct demonstrations that Ang II increases activity of T-type Ca^{2+} channels, but Ang II has been demonstrated to increase their activity in ventricular myocytes (Bkaily et al. 2005) and adrenal glomerulosa cells (McCarthy et al. 1993).

As observed in the current study, we have previously reported that removing the endothelium increases nerve-evoked contractions of the mouse tail artery (Reardon and Brock, 2013). As blockade of nitric oxide synthase similarly increased nerve-evoked contractions (see Reardon and Brock, 2013), we assume nitric oxide tonically released from endothelium inhibits nerve-evoked contractions. The increment in the size of nerve-evoked contractions produced by 0.3 nM Ang II was similar in vessels with or without an intact endothelium. In contrast, raising the concentration to 1 nM further increased the size of contractions in vessels with an intact endothelium but had no additional effect on these responses in denuded vessels. Denudation of the endothelium has also been reported to reduce the facilitatory action of Ang II on nerve-evoked contractions of rat small mesenteric arteries (Lu et al. 2008). Therefore, while Ang II can potentiate nerve-evoked contractions by a mechanism that does not require an intact endothelium, it is possible that Ang II also mediates part of its effects on these responses by reducing the inhibitory action of nitric oxide released from the endothelium (Lu et al. 2008). Alternatively, as in both endothelium intact and denuded vessels nerve-evoked contractions were of similar size in the presence of 1 nM Ang II, at this concentration the responses may have been maximally facilitated.

In mice, the basal circulating concentration of Ang II is ~0.1 nM (Gupte et al. 2012; Ni et al. 2013) and at this concentration Ang II increased nerve-evoked contractions of mouse tail artery. The concentrations of Ang II that directly constrict arterial vessels *in vitro* are typically much higher than those in plasma. There is a considerable body of evidence that endogenous Ang II has facilitatory actions on neurovascular transmission *in vivo* (Balt et al.

2001a; Cline, 1985; Zimmerman, 1967). *In vivo*, the facilitatory action of Ang II on sympathetic neurotransmission is suggested to be primarily mediated via its prejunctional facilitatory action on noradrenaline release (Balt et al. 2001b). However there are a number of reports that blockade of AT₁ receptors *in vivo* reduces the pressor effects of α -adrenoceptor agonists (e.g. Abdulla et al. 2009; Dendorfer et al. 2002), consistent with endogenous Ang II increasing reactivity of the vasculature to these agents.

Ang II produced a leftward shift in the concentration-response curve for the α_1 -adrenoceptor agonist phenylephrine and revealed contractions to the α_2 -adrenoceptor agonist UK14304. A similar permissive role for Ang II on responses to UK14,304 has been reported in rabbit saphenous artery (Dunn et al. 1989). The mechanisms that underlie these effects of Ang II on adrenoceptor-mediated contractions have not been identified. However, as Ang II also increased sensitivity to elevated levels of K⁺, which triggers contraction by directly depolarizing the smooth muscle, it appears that this peptide produces a generalized increase in reactivity of the mouse tail artery.

Ang II reduced the blockade of nerve-evoked contractions produced by relatively low concentrations of the α -adrenoceptor antagonists prazosin (10 nM) and idazoxan (0.1 μ M). As Ang II did not increase noradrenaline release, the change in blockade produced by prazosin and idazoxan is most likely explained by the increased sensitivity of the vascular muscle to nerve-released noradrenaline. In accord with this suggestion, a 10-fold increase in concentration of the α -adrenoceptor antagonists increased the blockade they produced in the presence of Ang II to that produced by the lower concentration in the absence of Ang II. These findings indicate that Ang II does not change the relative contribution of α_1 - and α_2 -adrenoceptors to nerve-evoked contractions.

AT₁ receptors are known to mediate their cellular effects via a wide range of signalling pathways (Mehta and Griendling, 2007). In rat mesenteric artery, the facilitatory

action of 100 nM Ang II on nerve-evoked contractions was reduced by the NADPH oxidase antagonist apocynin, superoxide dismutase and its mimetic tiron (Lu et al. 2008). Furthermore, the superoxide anion generator pyrogallol mimicked the effects of Ang II on neurovascular transmission in mesenteric arteries and the actions of both Ang II and pyrogallol were reduced by tyrosine kinase and mitogen-activated protein kinase (MAPK) inhibitors. These findings indicate that, in rat mesenteric arteries, superoxide mediates the facilitatory effect of a relatively high concentration of Ang II on neurovascular transmission and that this involves tyrosine kinase-MAPK activation. In mouse tail artery, the marked facilitatory effect of 1 nM Ang II on nerve-evoked contractions involves a different mechanism as it was not reduced by apocynin, the superoxide dismutase mimetic tempol or the tyrosine kinase inhibitor genistein. We also assessed the effects of other potential mediators of Ang II actions in vascular muscle (Mehta and Griendling, 2007) using blockers of Rho kinase, protein kinase C, IP₃ receptors, cyclooxygenase, and HETE-20 synthesis but none of these treatments reduced the facilitatory effect of Ang II on nerve-evoked contractions (**Supplementary Tables 2 and 3**). Therefore the signal transduction mechanisms that mediate the facilitatory action of Ang II on nerve-evoked contractions remain to be completely established.

In conclusion, the augmentation of nerve-evoked contractions produced by physiologically relevant concentrations of Ang II in the mouse tail artery is mediated in part by increasing the contribution of T-type Ca²⁺-channels to neural activation of the vascular muscle. In addition, the findings suggest that Ang II may reduce the tonic inhibitory influence of the endothelium on nerve-evoked contractions.

Acknowledgements: The work was supported by a grant from the National Health and Medical Research Council of Australia (grant number 568850).

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Figure legends

Fig. 1. Ang II increased nerve-evoked contractions of mouse tail artery segments. (A) Representative trace showing contractions evoked by 50 pulses at 2 Hz before and during the sequential application of 0.1, 0.3 and 1 nM Ang II. (B) Peak increases in wall tension evoked by nerve stimulation before and during the sequential addition of these concentrations of Ang II ($n = 6$). Data are presented as mean and S.E.M.. Statistical assessments were made with paired t -tests; * indicates significant difference between contractions in the absence and in the presence of Ang II (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. pre-treatment control).

Fig. 2. Ang II does not increase noradrenaline release. (A) Overlaid traces showing noradrenaline-induced oxidation currents evoked by 10 pulses at 10 Hz in the same tissue before and during the sequential addition of Ang II, idazoxan (Idaz) and tetrodotoxin (TTX). The noradrenaline-induced oxidation current is the slowly decaying signal that follows the stimulation artefact (SA) and is abolished by TTX (0.5 μ M). (B) Mean stimulus period 2 to stimulus period 1 (S_2/S_1) response amplitude ratios in tissues treated with Ang II (1 nM) between the stimulus periods ($n = 6$; *blue bar*) and in time-matched control tissues ($n = 5$; *black bar*). This graph also shows that stimulus period 3 to stimulus period 2 (S_3/S_2) ratios increased significantly in tissues treated with the α_2 -adrenoceptor antagonist idazoxan (0.1 μ M) between the stimulus periods ($n = 6$; *red bar*). Measurements were made 0.5 s after the last stimulus in the train (indicated by the dashed vertical line in A). Data are presented as mean and S.E.M.. Statistical assessments were made with the unpaired t -test; * indicates that idazoxan increased noradrenaline-induced oxidation currents (* $P < 0.05$ vs. time-dependent control).

Fig. 3. Ang II increased sensitivity of mouse distal tail artery to α -adrenoceptor agonists and to K^+ . (A – C) Concentration-response curves for (A) the α_1 -adrenoceptor selective agonist phenylephrine ($n = 8$), (B) the α_2 -adrenoceptor selective agonist UK 14,304 ($n = 6$) and (C) K^+ ($n = 6$) in the absence (*open circles*) or in the presence (*filled circles*) of Ang II (1 nM). Data are presented as mean $\pm$ S.E.M.. The indicated P values are for comparisons between curves in the absence and in the presence of Ang II made with repeated measures ANOVA.

Fig. 4. Denudation of the endothelium increased nerve-evoked contractions and reduced the Ang II-induced increase of nerve-evoked contractions. Nerve-evoked increases in wall tension before (*white bars*) and during the sequential addition of 0.3 nM (*grey bars*) and 1 nM (*black bars*) Ang II in arteries with an intact ($n = 8$) or denuded ($n = 6$) endothelium. Data are presented as mean and S.E.M.. The paired t -test was used to assess if Ang II increased contractions; * indicates that Ang II increased nerve-evoked contractions ($P < 0.01$ vs. pre-treatment control); † indicates that in intact vessels 1 nM Ang II produced a larger increase in nerve-evoked contractions than did 0.3 nM ($P < 0.01$). Statistical comparisons between measures in arteries with an intact or denuded endothelium were made with the unpaired t -test; # indicates that endothelium denudation increased nerve-evoked contractions ($P < 0.05$).

Fig. 5. Ang II-induced facilitation of nerve-evoked contractions is dependent on T-type Ca^{2+} channels. The facilitation of nerve-evoked contractions produced by Ang II (1 nM) in the absence (*white bars*) and in the presence (*grey bars*) of SKF-96365 (SKF; 10 μ M, $n = 8$), efonidipine (Efon; 1 μ M, $n = 6$), efonidipine + SKF-96365 (1 and 10 μ M respectively, $n = 6$), nifedipine (Nif; 1 μ M, $n = 8$), NNC 55-0396 (1 μ M, $n = 10$) and nifedipine + NNC 55-0396 (both at 1 μ M, $n = 9$). Statistical assessments were made with the unpaired t -test; * indicates

that SKF-96365, efonidipine, efonidipine + SKF-9636, NNC 55-0396 and NNC 55-0396 + nifedipine significantly reduced the facilitatory effect of Ang II on nerve-evoked contractions (* $P < 0.05$, ** $P < 0.01$). Nifedipine did not change the facilitatory effect of Ang II.

Table 1. The effects of various drugs on the amplitude of nerve-evoked contractions in the absence of Ang II

Drug name	Concentration	n	% of pre-treatment response		P value
			Control	Drug	
Losartan	0.1 μ M	4	85 $\pm$ 6	79 $\pm$ 15	0.69
PD 123319	1 μ M	3	88 $\pm$ 7	96 $\pm$ 6	0.46
NF 449	10 μ M	4	94 $\pm$ 5	63 $\pm$ 4	<0.01 ^a
SKF 96365	10 μ M	8	96 $\pm$ 5	82 $\pm$ 14	0.38
Efonidipine	1 μ M	6	100 $\pm$ 3	88 $\pm$ 6	0.11
Nifedipine	1 μ M	8	98 $\pm$ 3	63 $\pm$ 8	<0.01 ^a
NNC 55-0396	1 μ M	10	89 $\pm$ 4	72 $\pm$ 6	<0.05 ^a
Tempol	1 mM	4	92 $\pm$ 11	45 $\pm$ 11	<0.05 ^a
Apocynin	300 μ M	4	94 $\pm$ 5	70 $\pm$ 2	<0.01 ^a
Genistein	10 μ M	5	99 $\pm$ 6	78 $\pm$ 7	<0.05 ^a

The peak amplitudes of contractions in the presence of the drug are expressed as a percentage of those immediately prior to its addition. For controls, % change in amplitude of contractions was calculated between the same time points as in drug treated tissues. Data are presented as mean $\pm$ S.E.M.. Statistical assessments were made with the unpaired *t*-test and significant differences between the responses in presence of the drugs from those in time-matched controls are indicated by *a*.

Table 2. The facilitatory effect of 1 nM Ang II in the absence and in the presence of various drugs

Drug name	Concentration	n	% of pre-Ang II treatment response		P value
			Ang II	Ang II + Drug	
Losartan	0.1 μ M	4	547 $\pm$ 87	131 $\pm$ 20	<0.01 ^a
PD 123319	1 μ M	3	472 $\pm$ 65	402 $\pm$ 59	0.47
NF449	10 μ M	4	780 $\pm$ 121	872 $\pm$ 131	0.62
Tempol	1 mM	4	784 $\pm$ 211	563 $\pm$ 117	0.40
Apocynin	300 μ M	4	780 $\pm$ 121	616 $\pm$ 116	0.37
Genistein	10 μ M	5	508 $\pm$ 91	605 $\pm$ 30	0.96

The peak amplitude of contractions in the presence of Ang II is expressed as a percentage of those immediately prior to its addition. Data are presented as mean $\pm$ S.E.M.. Statistical assessments were made with the unpaired *t*-test; only losartan significantly changed the facilitatory effect of Ang II (indicated by *a*).

Table 3. The % blockade of nerve-evoked contractions by prazosin and idazoxan in the absence and the presence of Ang II

Antagonist	<i>n</i>	Concentration	Condition	% blockade
Prazosin	8	10 nM	No Ang II	74 ± 3
	8	10 nM	1 nM Ang II	41 ± 6 ^a
	8	100 nM	1 nM Ang II	68 ± 8
Idazoxan	4	0.1 µM	No Ang II	66 ± 4
	7	0.1 µM	1 nM Ang II	39 ± 8 ^b
	4	1 µM	1 nM Ang II	68 ± 8

Data are presented as mean ± S.E.M.. Statistical comparisons between the % blockade of nerve-evoked contractions produced by prazosin or idazoxan in the absence and in the presence of Ang II were made with the unpaired *t*-test (*a* *P* < 0.05, *b* *P* < 0.01).

Figure 1

Figure 2

Figure 3

Figure 4

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Title:

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Date:

2015-08-15

Citation:

Reardon, T. F., Callaghan, B. P. & Brock, J. A. (2015). Angiotensin II increases nerve-evoked contractions in mouse tail artery by a T-type Ca²⁺ channel-dependent mechanism. EUROPEAN JOURNAL OF PHARMACOLOGY, 761, pp.11-18. <https://doi.org/10.1016/j.ejphar.2015.04.027>.

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