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α_2-Adrenoceptor-mediated contractions of the porcine isolated ear artery: evidence for a cyclic AMP-dependent and a cyclic AMP-independent mechanism

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1 The aim of this study was to determine the conditions under which the α_2-adrenoceptor agonist UK14304 produces vasoconstriction in the porcine isolated ear artery.

2 UK14304 (0.3 μM) produced a small contraction of porcine isolated ear arteries which was $7.8 \pm 3.3\%$ of the response to 60 mM KC1. Similar sized contractions were obtained after precontraction with either 30 nM angiotensin II, or 0.1 μM U46619 ($8.2 \pm 1.8\%$ and $10.2 \pm 2.6\%$ of 60 mM KC1 response, respectively). However, an enhanced α_2-adrenoceptor response was uncovered if the tissue was precontracted with U46619, and relaxed back to baseline with 1–2 μM forskolin before the addition of UK14304 ($46.9 \pm 9.6\%$ of 60 mM KC1 response).

3 The enhanced responses to UK14304 in the presence of U46619 and forskolin were not inhibited by the α_1-adrenoceptor antagonist prazosin (0.1 μM), but were inhibited by the α_2-adrenoceptor antagonist rauwolscine (1 μM), indicating that the enhanced responses were mediated via postjunctional α_2-adrenoceptors.

4 In the presence of 0.1 μM U46619 and 1 mM isobutylmethylxanthine (IBMX), 1 μM forskolin produced an increase in [^{3}H]-cyclic AMP levels in porcine isolated ear arteries. Addition of 0.3 μM UK14304 prevented this increase.

5 The enhanced UK14304 response was dependent upon the agent used to relax the tissue. After relaxation of ear arteries precontracted with 10 nM U46619 and relaxed with forskolin the UK14304 response was $46.9 \pm 9.6\%$ of the 60 mM KC1 response, and after relaxation with sodium nitroprusside (SNP) the response was $24.8 \pm 3.3\%$. However, after relaxation of the tissue with levcromakalim the UK14304 response was only $8.2 \pm 1.7\%$, which was not different from the control response in the same tissues ($12.2 \pm 5.6\%$). An enhanced contraction was also obtained after relaxation of the tissue with the cyclic AMP analogue dibutyryl cyclic AMP ($23.2 \pm 1.3\%$) indicating that at least part of the enhanced response to UK14304 is independent of the ability of the agonist to inhibit cyclic AMP production.

6 Relaxation of U46619 contracted ear arteries with SNP could be inhibited by the NO-sensitive guanylyl-cyclase inhibitor 1H-[1,2,4] oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) indicating that production of cyclic GMP is necessary for the relaxant effect of SNP. However, ODQ had no effect on the relaxation of tissue by forskolin, suggesting that this compound does not act via production of cyclic GMP. Biochemical studies showed that while forskolin increases the levels of cyclic AMP in the tissues, SNP had no effect on the levels of this cyclic nucleotide.

7 In conclusion, enhanced contractions to the α_2-adrenoceptor agonist UK14304 can be uncovered in porcine isolated ear arteries by precontracting the tissue with U46619, followed by relaxation back to baseline with forskolin, SNP or dibutyryl cyclic AMP before addition of UK14304. There was a greater contractile response to UK14304 after relaxation with forskolin than with SNP or dibutyryl cyclic AMP, suggesting that cyclic AMP-dependent and- independent mechanisms are involved in the enhancement of the UK14304 response.

Keywords: α_2-adrenoceptors; porcine ear artery; vasoconstriction; cyclic nucleotides; UK14304; sodium nitroprusside; ODQ; U46619

Introduction

The existence in the vasculature of vasopressor α_2-adrenoceptors has been known for almost twenty years (see McGrath, 1982). However, in that time, the subcellular mechanism(s) underlying vasoconstriction by this receptor subtype has proved difficult to elucidate because most of the large, isolated blood vessels suitable for biochemical studies (e.g. conduit arteries) simply do not respond to selective α_2-adrenoceptor agonists (McGrath *et al*., 1989; 1991). Rather, vasoconstrictor α_2-adrenoceptors appear to exist on either arteriolar blood vessels (Neilsen *et al*., 1989; Faber, 1988) or on cutaneous arteries and veins. Presently, there is evidence for vasoconstrictor α_2-adrenoceptors on veins from the rabbit (Schumann & Lues, 1983; Daly *et al*., 1988) the dog (Constantine *et al*., 1982; MacLennan *et al*., 1997), man (Smith *et al*., 1992) and, more recently, the pig (Blaylock & Wilson, 1995; Wright *et al*., 1995c). However, demonstration of vasoconstrictor α_2-adrenoceptors on large arteries often requires experimental manipulation, involving elevation of vascular tone (Templeton *et al*., 1989) or the presence of an ancillary vasoconstrictor (Sulpizio & Hieble, 1987; Dunn *et al*., 1989; Aidulis *et al*., 1991), which raises the possibility that cross-talk between receptor systems is an obligatory requirement for the functional expression of arterial α_2-adrenoceptors.

In most cells α_2-adrenoceptors couple negatively to adenylyl cyclase (Bylund *et al*., 1994) and this may be the mechanism by

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which the receptor produces vasoconstriction- by reducing levels of adenosine 3′: 5′-cyclic monophosphate (cyclic AMP). However, observations on both the rabbit isolated saphenous vein (Daly *et al*., 1988; Aburto *et al*., 1995) and the porcine isolated palmar lateral vein (Wright *et al*., 1995c) indicate that any modification of cellular cyclic AMP in venous smooth muscle is likely to be of secondary importance and suggest that contraction proceeds by a cyclic AMP-independent mechanism. The latter notion is consistent with findings that vascular α_2-adrenoceptors can release intracellular calcium in porcine thoracic aorta cells (Erdbrugger *et al*., 1993), stimulate phosphoinositide metabolism in the porcine isolated palmar lateral vein (Wright *et al*., 1994), and elicit transient contractions of the isolated veins in the absence of extracellular calcium (Guan *et al*., 1989; Daly *et al*., 1990).

Studies performed in this laboratory have demonstrated that the density of α_2-adrenoceptor binding sites on isolated veins usually exceeds that of the corresponding artery (Wright *et al*., 1995a). In addition, α_1-adrenoceptor binding sites are present at a higher density on porcine arteries compared to α_2-adrenoceptor binding sites. An exception to the latter rule was the porcine isolated ear artery, where α_2-adrenoceptor binding sites (260 fmol mg^{-1}) exceeded α_1-adrenoceptors (90 fmol mg^{-1}) 3-fold. Since an arterial density of α_2-adrenoceptors of 160 fmol mg^{-1} protein on the isolated common digital artery was associated with small, but overt, α_2-adrenoceptor-mediated contractions to noradrenaline (Blaylock & Wilson, 1995), we have examined the effect of a selective α_2-adrenoceptor agonist, UK14304 (Cambridge, 1981), on the porcine isolated ear artery. Our results indicate that arterial α_2-adrenoceptors in the pig can utilize cyclic AMP-dependent and cyclic AMP-independent pathways to mediate contractions. Some of these results were presented in a preliminary form to the British Pharmacological Society (Roberts *et al*., 1997).

Methods

Functional studies

Isometric tension recordings Porcine ears were obtained from a local abattoir and transported to the laboratory on ice. Ear arteries were dissected out and placed in Krebs-Henseleit buffer containing 2% Ficoll which had been pre-gassed with 95% O_2/5% CO_2, and stored overnight at 4°C. The following day ear arteries were dissected into 5 mm ring segments and suspended in a 5 ml isolated organ bath containing Krebs-Henseleit buffer maintained at 37°C and constantly gassed with 95% O_2/5% CO_2. The lower support was fixed and the upper support was connected to a force transducer (World Precision Instruments, Sarasota, Florida, U.S.A.) linked to a MacLab data acquisition system (AD Instruments Ltd., Hastings, U.K.) via an amplifier. After a 20 min equilibration period, tension was applied to the tissue which was allowed to relax to a final resting tension of between 1–1.5 g wt. Before each experiment the tissues were contracted 3 times with 60 mM KCl. Between each separate study, tissues were washed 3 times with Krebs-Henseleit buffer and allowed to recover for 20 min.

In a separate series of experiments the endothelium was removed from the ear artery segments by rubbing the lumen with a fine pair of forceps. The segments were set up in the organ bath as usual. Removal of the endothelium was confirmed by the inability of the tissue to relax to 30 nM substance P after the tissue had been contracted with 0.1 μM U46619.

Responses to UK14304 in the presence of angiotensin II Ear arteries were contracted with 30 nM angiotensin II (AII) which gave a transient contraction. After the tension had returned to baseline, 0.3 μM UK14304 was added. Responses to UK14304 after the addition of AII were compared to those obtained in the absence of AII in the same tissues.

Responses to UK14304 in the presence of U46619 All tissues were exposed to the thromboxane mimetic U46619 (0.1 μM) and relaxed with forskolin (1–2 μM) back to $<10\%$ of the 60 mM KCl response before exposure to UK14304. Cumulative concentration-response curves were constructed for UK14304 (1 nM to 3 μM). In some experiments the combination of U46619 and forskolin was then removed by washing 3 times with Krebs-Henseleit buffer. The tissue was allowed to recover for 20 min before a concentration-response curve to UK14304 was constructed. The antagonists rauwolscine (1 μM) or prazosin (0.1 μM) were added after relaxation with forskolin, and 45 min before the addition of UK14304 where appropriate.

In a separate set of experiments, tissues were contracted with 0.1 μM U46619 and the response allowed to reach a plateau before the addition of a single concentration of UK14304 (0.3 μM).

Effect of relaxing agents on subsequent UK14304 response Ear arteries were contracted with U46619 (0.1 μM or 10 nM – see Results section) and relaxed with forskolin (1–2 μM), sodium nitroprusside (SNP; 10 μM to 100 μM), levcromakalim (1–5 μM), or dibutyryl cyclic AMP (3–5 mM). Tissues were relaxed to $<10\%$ of the 60 mM KCl response before 0.3 μM UK14304 was added. Control responses to UK14304 (i.e 0.3 μM UK14304 on its own) were also performed in the same tissues and compared to the responses obtained after precontraction and relaxation.

Effect of a guanylyl cyclase inhibitor on relaxations to forskolin or SNP The appropriate concentration of 1H-[1,2,4] oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) in dimethylsuphoxide (DMSO; 0.1% final concentration of DMSO in the organ bath) was added to the water bath 45 min before the addition of agonists. DMSO (0.1%) was added to the control tissue (no ODQ). The tissues were contracted with 10 nM U46619, and the response allowed to reach a plateau. Cumulative concentration-response curves were constructed for forskolin (10 nM to 1 μM) and SNP (0.1 μM to 300 μM).

Cyclic AMP measurements

Ear arteries were cut into 5 mm lengths and then incubated in Krebs-Henseleit buffer for 60 min at 37°C in a shaking water bath. After this period of time the tissue segments were incubated with 37 kBq ml^{-1} [^{3}H]-adenine (specific activity = 851 GBq $mmol^{-1}$) in Krebs-Henseleit buffer for a further 60 min at 37°C in a shaking water bath. Tissue segments were then washed 3 times with Krebs-Henseleit buffer before being transferred into a flat-bottomed incubation vial (2 tissue segments per vial) containing Krebs-Henseleit buffer in a final volume of 300 μl. Each experiment was performed in quadruplicate. Vials were placed in a shaking water bath at 37°C and allowed to equilibrate for 10 min. All vials contained 0.1 μM U46619 in the absence or presence of either 1 μM forskolin or 100 μM SNP. U46619 was added 10 min before forskolin or SNP addition. In experiments performed in the presence of phosphodiesterase (PDE) inhibitors (either 1 mM

isobutylmethylxanthine (IBMX) or 10 μM rolipram), the inhibitors were added 5 min before U46619. In experiments in which the effect of UK14304 on the production of cyclic AMP by forskolin was studied, 0.3 μM UK14304 was added 5 min before the addition of forskolin, in the presence of 1 mM IBMX. After a 10 min incubation period, reactions were terminated by addition of 200 μl 1 M HCl and 750 μl distilled water; 100 μl of buffer was removed for total ^{3}H counts. [^{3}H]-cyclic AMP was separated from [^{3}H]-adenine and other ^{3}-H products by sequential Dowex/alumina column chromatography. Briefly, 1 ml of the reaction buffer was added to 100 μl of [^{14}C]-cyclic AMP (30 Bq/tube) and applied to Dowex columns. The eluate was discarded, and then 2 ml of distilled water was added to the columns. The eluate was again discarded. A further 4 ml of distilled water was added to the columns, and the eluate was dripped onto alumina columns. Imidazole (5 ml, 0.1 M) was added to the alumina columns, and the eluate collected. Levels of [^{3}H]-cyclic AMP and [^{14}C]-cyclic AMP in the eluate were measured by liquid scintillation counting. [^{3}H]-cyclic AMP levels were adjusted for the recovery from the dowex/alumina column chromatography (using the [^{14}C]-cyclic AMP as a standard) and also for the amount of total ^{3}H taken up into the tissue.

Drugs

1H-[1,2,4] oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), (Tocris); rauwolscine HCl (Roth); prazosin (RBI); 5-bromo-6-[2-imidazolin-2-ylamine]-quinoxaline bitartrate (UK14304), (Pfizer); (5Z, 9α, 11α, 13E, 15 (S))-15-hydroxy-9 (11) methanoepoxyprosta-5,13-dien-1 oic acid (U46619), (Cascade Biochem Ltd); 3-isobutyl-1-methylxanthine (IBMX), (Sigma); rolipram (Schering); Forskolin (Sigma); [^{3}H]-adenine (Amersham); [^{14}C]-cyclic AMP (NEN-DuPont); sodium nitroprusside (SNP; David Bull Labs); angiotensin II (Ciba). All other compounds were obtained from Sigma (Poole, U.K.).

Statistics

For single comparisons, an F test for equal variances was performed on all the data to test for normality. Normally distributed data were then subjected to Student's 2-tailed, unpaired t test. Data which were not normally distributed were subjected to a non-parametric 2-tailed, Mann-Whitney U-test. Multiple comparisons were performed using analysis of the variance (ANOVA) followed by a Bonferroni test. Results are expressed as mean$\pm$s.e.mean. Statistical significance was assumed when $P<0.05$.

Results

UK14304 responses in the absence or presence of angiotensin II or U46619

The integrity of the endothelium in rubbed and unrubbed segments of the ear artery was assessed by the effect of 30 nM substance P, a known endothelium-dependent relaxant (Moncada *et al*., 1992), against U46619-induced contractions. Substance P (30 nM) produced a short-lived relaxation of unrubbed segments but failed to affect U46619-induced tone in rubbed segments. The maximum relaxation produced by substance P was 32.2$\pm$7.4 % ($n=5$) of U46619-induced tone. The magnitude of the contraction to 60 mM KCl was significantly less in rubbed segments compared to unrubbed segments (1.6$\pm$0.4 g. wt in rubbed segments compared to 3.2$\pm$0.7 in unrubbed segments, $P<0.05$, unpaired t test: $n=5$). However, the contraction to 0.1 μM U46619 expressed as a percentage of the 60 mM KCl response was similar in rubbed (77.5$\pm$11.8%, $n=5$) and unrubbed segments (82.3$\pm$9.4%, $n=5$). Unless otherwise indicated, all further experiments were conducted on unrubbed segments of the ear artery.

The α_2-adrenoceptor agonist UK14304 (0.3 μM) produced a small, maintained contraction in the porcine isolated ear artery which was 7.8$\pm$3.3% (mean$\pm$s. e.mean, $n=6$) of the response to 60 mM KCl (60 mM KCl response was 3.15$\pm$0.18 g.wt, $n=28$). Angiotensin II (AII) (30 nM) produced a transient contraction (maximum 75.5$\pm$14.7% of the response to 60 mM KCl, duration 10–15 min, $n=5$) which relaxed to baseline. Subsequent addition of 0.3 μM UK14304 produced a small contraction of the tissue (8.2$\pm$1.8% of 60 mM KCl response, $n=5$) which was not significantly different from that seen in the absence of AII in the same tissues. In a separate set of experiments, tissues were precontracted with the thromboxane-mimetic U46619. U46619 (0.1 μM) produced a sustained contraction (86$\pm$5.9% of 60 nM KCl response, $n=7$). The responses were allowed to reach a plateau before the addition

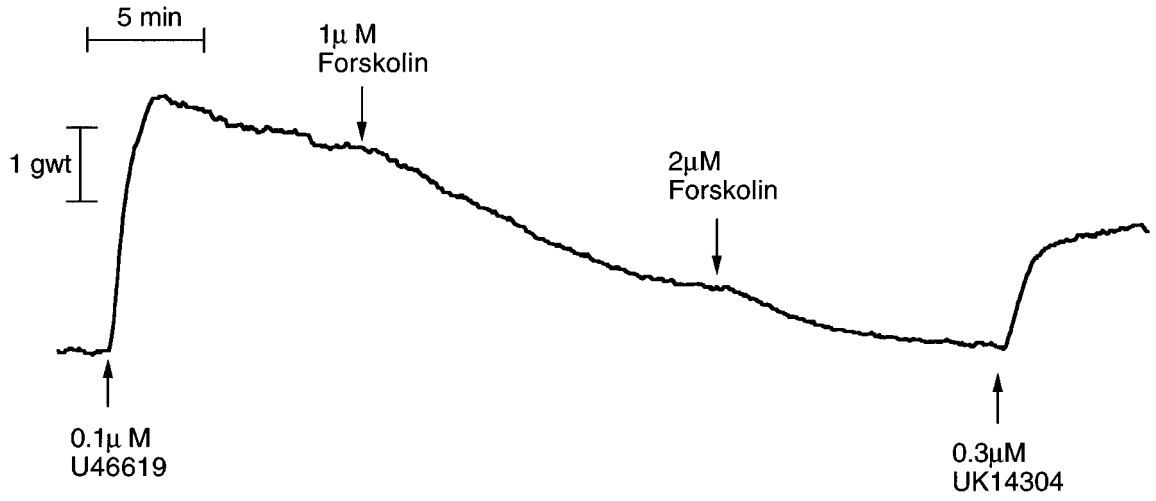

Figure 1 A typical trace recording showing the effect of 0.3 μM UK14304 on porcine isolated ear artery ring segments suspended in an isolated organ bath in the presence of 0.1 μM U46619 and forskolin.

of 0.3 μM UK14304. There was a further contraction of the tissue, although the increase in contraction induced by UK14304 (10.2$\pm$2.6%, $n=6$; measured from the maximum of the U46619 response to the maximum of the U46619/UK14304 response) was comparable to that seen with UK14304 on its own.

UK14304 responses after precontraction with U46619, and relaxation with forskolin

UK14304 (0.3 μM) alone produced a slight contraction of the ear artery. However, following exposure to 0.1 μM U46619 and subsequent relaxation by forskolin (back to $<10\%$ of the response to 60 mM KCl), UK14304 produced a large contraction (46.9$\pm$9.6% 60 mM KCl response, $n=6$). Figure 1 shows a typical trace recording of the effect of UK14304 in the presence of U46619 and forskolin. In the presence of forskolin alone, UK14304 failed to elicit an enhanced contraction (data not shown). In a separate series of experiments, the response to 0.3 μM UK14304 alone was not significantly affected by removal of the endothelium (9.2$\pm$3.5% in the presence of the endothelium compared to 4.4$\pm$2.6% in the absence of the endothelium, $n=5$). The response to 0.3 μM UK14304 in the presence of U46619 and forskolin was also unaffected by removal of the endothelium (45.2$\pm$14.1% in the presence of the endothelium compared to 45.4$\pm$9.6% in endothelium denuded vessels, $n=5$).

Concentration-response curves to UK14304 were obtained in the absence or presence of the combination of U46619 and forskolin. In the absence of U46619 and forskolin, UK14304 (1 nM to 3 μM) produced a concentration-dependent contraction of the ear artery (maximum 22.0$\pm$10.8% of the response to 60 mM KCl; $n=7$; Figure 2a). However, the UK14304 response was greatly enhanced in tissues which had been precontracted with U46619 and relaxed with forskolin (maximum 49.6$\pm$10.9%; $n=7$) (see Figure 2a). The UK14304 response was abolished by rauwolscine (1 μM), but not by 0.1 μM prazosin (7.8$\pm$0.24 (pD_2 for UK14304 in the presence of prazosin) compared to 7.51$\pm$0.24 in the absence of prazosin, $n=7$) (Figure 2b). Rauwolscine was also able to inhibit the control response to UK14304 (the response to UK14304 in the absence of U46619 and forskolin). In the absence of 1 μM rauwolscine 0.3 μM UK14304 produced a contraction which was 7.9$\pm$3.5% of the 60 mM KCl response. The response was completely abolished by rauwolscine.

Effect of different relaxing agents on the response to UK14304

In a separate series of experiments, contractions to U46619 were relaxed back to baseline with either forskolin (1–2 μM), SNP (10 μM to 100 μM), dibutyryl cyclic AMP (3–5 mM), or levcromakalim (1–5 μM). UK14304 (0.3 μM) produced a contraction in each instance, but only in the presence of forskolin (46.9$\pm$9.6% of 60 mM KCl response), SNP (24.8$\pm$3.3%), or dibutyryl cyclic AMP (23.2$\pm$1.3%) was the response significantly greater than control (7.6$\pm$3.3%, $n=6$) (Figure 3). The contractions to UK14304 in the presence of the combination of dibutyryl cyclic AMP and U46619, and the combination of SNP and U46619, were comparable in

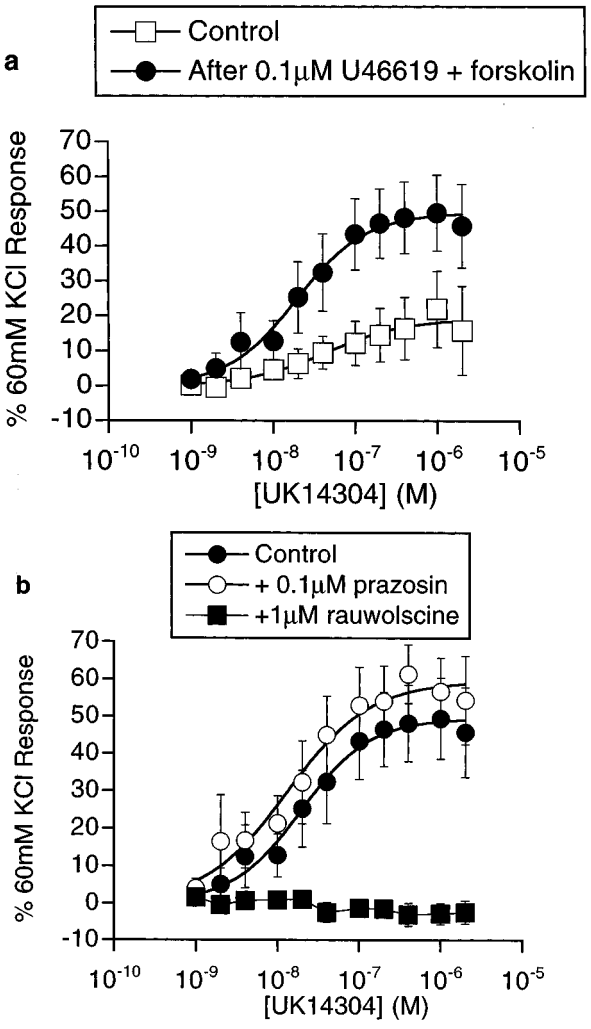

Figure 2 Concentration-response curves for UK14304 after various treatments in porcine isolated ear artery ring segments expressed as % 60 mM KCl response in the same tissues. (**a**) Responses to UK14304 alone (control) and after pre-contraction with 0.1 μM U46619 and relaxation with forskolin. (**b**) Responses to UK14304 after pre-contraction with 0.1 μM U46619 and relaxation with forskolin in the absence or presence of 0.1 μM prazosin or 1 μM rauwolscine.

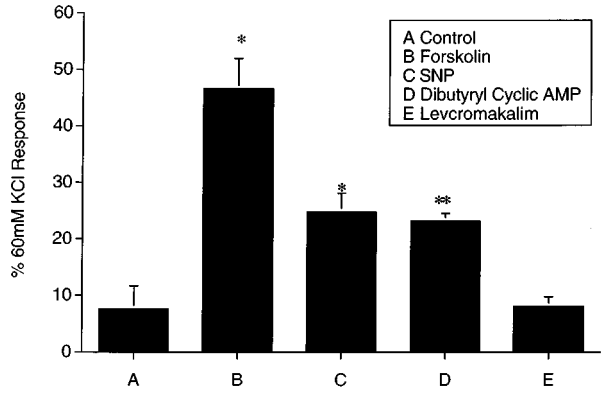

Figure 3 Bar charts showing contractile responses to 0.3 μM UK14304 in porcine isolated ear artery ring segments expressed as % 60 mM KCl response. (**A**) Control; (**B**) after contraction with 0.1 μM U46619 and relaxation with 1–2 μM forskolin ($n=6$); (**C**) after contraction with 10 nM U46619 and relaxation with 10 μM to 100 μM SNP ($n=6$); (**D**) after contraction with 0.1 μM U46619 and relaxation with 1–5 mM dibutyryl cyclic AMP ($n=10$); (**E**) after contraction with 0.1 μM U46619 and relaxation with 1–5 μM levcromakalim ($n=6$). N.B. 10 nM U46619 was used for (**C**) as SNP was unable to relax the tissue to $<10\%$ of the 60 mM KCl response in the presence of 0.1 μM U46619. *Indicates $P<0.05$ 2-tailed, Mann-Whitney U-test *vs* control; **indicates $P<0.0001$ 2-tailed, unpaired *t* test *vs* control.

magnitude, and both were significantly less than that observed in the presence of forskolin and U46619.

Thus, responses to UK14304 are only uncovered in the presence of agonists that either elevate cyclic nucleotide levels or that activate the corresponding protein kinases. In the latter instance (in the presence of dibutyryl cyclic AMP) the involvement of a cyclic AMP independent mechanism is indicated.

Comparison of the actions of forskolin and SNP

It is possible that the enhanced response to UK14304 after relaxation with forskolin or SNP occurs through a common pathway. To determine whether this is indeed the case, the following experiments were performed.

Comparison of tissue relaxation The effects of forskolin and SNP on U46619-induced contractions were examined in the presence and absence of 1H-[1,2,4] oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), an inhibitor of soluble guanylyl cyclase (Schrammel *et al*., 1996). Forskolin (10 μM to 1 μM) produced a concentration-dependent relaxation of U46619-induced contractions which was unaffected by the presence of 1 μM ODQ (Figure 4a). In contrast, 1–3 μM ODQ caused a rightward displacement of the SNP concentration-response curve (Figure 4b). There was no further rightward displacement of the SNP curve in the presence of 5 μM ODQ (data not shown). Thus SNP, unlike forskolin, causes relaxation by a mechanism involving cyclic GMP.

Effect of forskolin and SNP on intracellular cyclic AMP levels As shown in Table 1, while neither 1 μM forskolin nor 100 μM SNP caused a significant elevation of [^{3}H]-cyclic AMP accumulation, 30 μM forskolin produced a 3-fold increase. SNP (100 μM) failed to elevate [^{3}H]-cyclic AMP accumulation in the presence of either 1 mM IBMX or 10 μM rolipram. In marked contrast, 1 μM forskolin produced a 2-fold and a 5-fold increase in [^{3}H]-cyclic AMP accumulation in the presence of 10 μM rolipram and 1 mM IBMX respectively (Table 1).

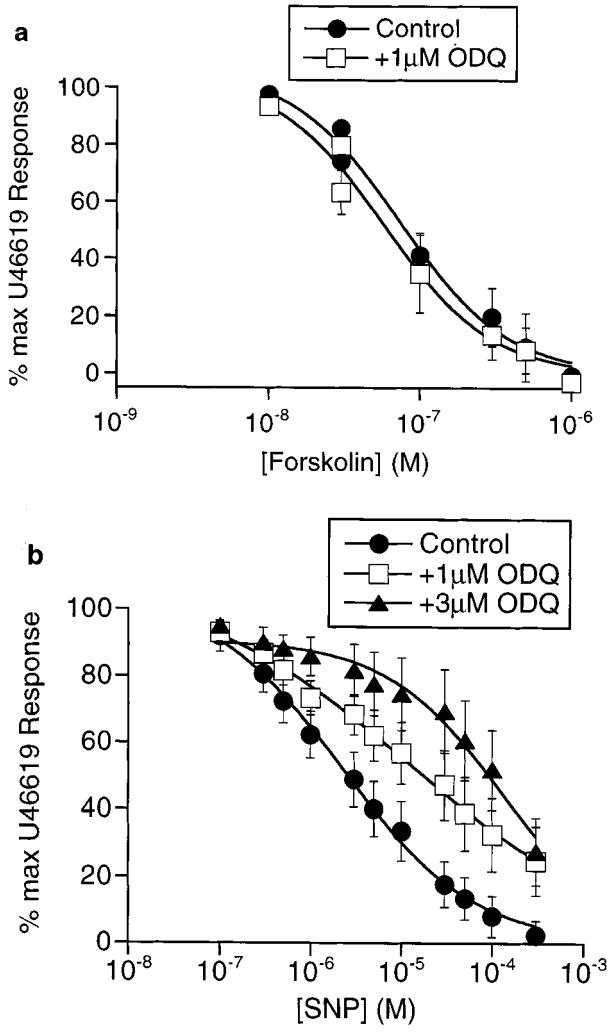

Figure 4 (**a**) Concentration-response curves to forskolin in porcine isolated ear artery ring segments after contraction with 10 nM U46619 in the absence (control) or presence of 1 μM ODQ. Data are expressed as % maximum U46619 response. (**b**) Concentration-response curves to SNP in porcine isolated ear artery ring segments after contraction with 10 nM U46619 in the absence (control) or presence of 1 μM or 3 μM ODQ. Data are expressed as % maximum U46619 response.

Table 1 The effect of forskolin and sodium nitroprusside on [^{3}H]-cyclic AMP accumulation in the porcine isolated ear artery in the presence or absence of IBMX and rolipram

	[^{3}H]-cyclic AMP production (% conversion)		
	Control	*IBMX 1 mM*	*Rolipram 10 μM*
Basal	0.50±0.06	0.81±0.17 (6)	0.56±0.11 (6)
SNP 100 μM	0.54±0.07	1.49±0.33 (6)	0.62±0.14 (6)
Forskolin 1 μM	0.65±0.1	4.69±0.61* (6)	1.36±0.19 (6)*
Forskolin 30 μM	1.83±0.15*	6.03±0.71* (8)	

Values shown are the mean±s.e.mean of 6–8 experiments (each performed in quadruplicate). *Denotes a significant elevation of [^{3}H]-cyclic AMP above basal, Bonferonni test.

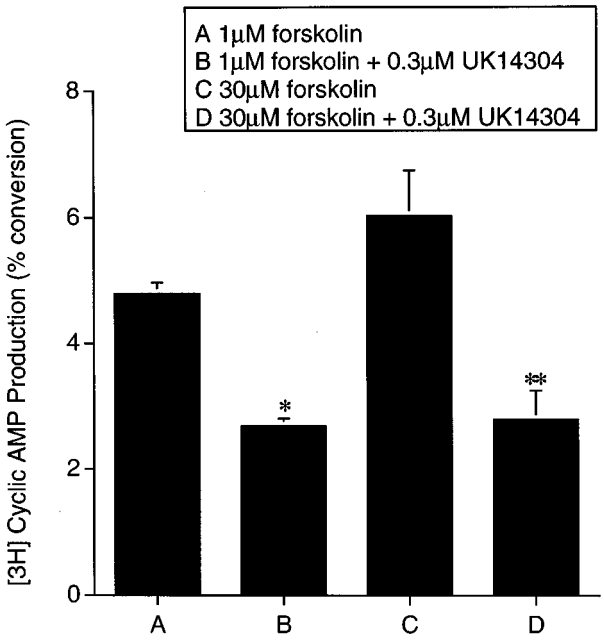

Figure 5 [^{3}H]-cyclic AMP production (% conversion/2×5 mm tissue segments) in porcine isolated ear artery segment in the presence of 0.1 μM U46619 and 1 mM IBMX. *Indicates statistical significance *vs* (**A**) Bonferroni test; **indicates statistical significance *vs* (**C**) Bonferroni test.

Effect of UK14304 on forskolin stimulated cyclic AMP

In a separate series of experiments, basal [^{3}H]-cyclic AMP accumulation in the presence of 0.1 μM U46619 and 1 mM IBMX (0.12 $\pm$ 0.04%, $n=6$) was increased in the presence of 0.3 μM UK14304 (0.22 $\pm$ 0.05%, $n=6$), but this was not statistically significant. In contrast, 0.3 μM UK14304 significantly reduced forskolin-stimulated [^{3}H]-cyclic AMP accumulation in the presence of 0.1 μM U46619 and 1 mM IBMX by approximately 50% (Figure 5).

Discussion

The data presented in this study show that contractile responses to the α_2-adrenoceptor agonist UK14304 in the porcine isolated ear artery are altered under different experimental conditions. UK14304 alone produces a small contraction, as does addition of UK14304 in the presence of either AII or the thromboxane-mimetic U46619. However, if the tissue is precontracted with U46619 and then relaxed back to baseline with forskolin, UK14304 gives a much larger response. This uncovered response exhibits the properties of an α_2-adrenoceptor. The potency of UK14304 agrees with earlier studies on α_2-adrenoceptors (Daly *et al*., 1988; Wright *et al*., 1995a). Also, the relative effects of prazosin and rauwolscine on the enhanced UK14304 response indicate an α_2-adrenoceptor-mediated response. Rauwolscine is a specific α_2-adrenoceptor antagonist, having a pK_B of 5.38 at α_1-adrenoceptors (rabbit ear artery; Hieble & Woodward, 1984) compared to 8.63 at α_2-adrenoceptors (dog saphenous vein; MacLennan *et al*., 1997). Prazosin, on the other hand, is an α_1-adrenoceptor antagonist, having a pK_B of 8.19 at α_1-adrenoceptors (rabbit ear artery; Hieble & Woodward, 1984), compared to 5.19 at α_2-adrenoceptors (dog saphenous vein; MacLennan *et al*., 1997).

Previous studies in this laboratory have shown that the density of α_2-adrenoceptor binding sites on the porcine ear artery is 60% greater than that on the porcine common digital artery (Wright *et al*., 1995). However, in the present study, the magnitude of the α_2-adrenoceptor mediated contraction of the ear artery in the absence of U46619 and forskolin is significantly smaller than that noted for the digital artery (see Blaylock & Wilson, 1995). This indicates that receptor density is not the primary factor determining the ability of α_2-adrenoceptors to produce vasoconstriction. The possibility exists, therefore, that other cellular factors and/or experimental conditions may exert on overriding influence on α_2-adrenoceptor function. Interestingly, observations in rabbit saphenous artery (Dunn *et al*., 1989) and rat skeletal muscle arterioles (Ikeoka & Faber, 1993) lend support to the view that the presence of AII may be important. However, AII failed to uncover/enhance α_2-adrenoceptor-mediated vasoconstriction in the ear artery in the present study indicating that a different mechanism is involved. Also there was no large UK14304 response in the presence of U46619 alone, demonstrating that raising vascular tone alone does not induce an enhanced α_2-adrenoceptor-mediated response. The combination of a precontracting agent and forskolin to uncover a large UK14304 response is similar to studies performed in the rat tail artery in which enhanced responses to UK14304 were seen in the presence of one of a variety of contracting agents, and a relaxing agent which increased the intracellular concentration of cyclic nucleotides (Aidulis *et al*., 1991; Aidulis & Pollock, 1992). In this latter study it was suggested that the addition of UK14304 inhibited the relaxation induced by forskolin and SNP, and the resulting contraction merely represents a recovery of the response to the precontracting agent, rather than a contraction to UK14304 *per se* (Aidulis & Pollock, 1992). However, the contraction to UK14304 in the porcine isolated ear artery does not seem to involve a simple reversal of the inhibitory action of forskolin, as the response was less than the pre-forskolin tone induced by U46619.

The enhanced response to UK14304 in the porcine isolated ear artery does seem to be dependent upon the agent used to relax the tissue. Although there was a contraction to UK14304 after relaxation of the tissue with the K_{ATP} channel agonist levcromakalim (Quast & Cook, 1989), the response was no greater than that seen with UK14304 on its own. However, the same concentration of UK14304 did produce an enhanced response after relaxation with either forskolin, SNP or dibutyryl cyclic AMP, a finding which is also similar to that found in previous studies in the rat tail artery (Aidulis *et al*., 1992). These results suggest that the ability of UK14304 to produce an enhanced response may relate to changes in cyclic nucleotide levels.

Evidence for a cyclic AMP-dependent mechanism

The response to UK14304 after relaxation of the U46619 contraction with forskolin in the porcine isolated ear artery was greater than that seen after the tissue has been relaxed with either SNP or dibutyryl cyclic AMP. Forskolin directly activates adenylyl cyclase (Seamon & Daly, 1986). At a concentration similar to that used to relax the tissue in the functional studies (1 μM), it was able to increase levels of cyclic AMP, although a significant increase was only detectable in the presence of the general phosphodiesterase (PDE) inhibitor IBMX, or the PDE IV selective inhibitor rolipram. α_2-Adrenoceptors are negatively coupled to adenylyl cyclase and, in the present study, we have demonstrated that, under similar conditions to those used in the contraction-based studies, UK14304 can inhibit cyclic AMP production induced by 1 μM forskolin (in the presence of IBMX) in the porcine isolated ear artery. These data clearly support the possibility that UK14304 produces a response as a result of a reduction in cyclic AMP levels.

Evidence for a cyclic AMP-independent mechanism

The simplest explanation for the ability of UK14304 to produce an enhanced response after the addition of U46619/forskolin is inhibition of adenylyl cyclase leading to a reduction in forskolin-stimulated cyclic AMP production as stated above. However, enhanced responses to UK14304 were also obtained in the porcine isolated ear artery after the tissue had been relaxed with dibutyryl cyclic AMP, a cell permeable, protein kinase A activator (Hei *et al*., 1991), although the responses were only 50% of those obtained in the presence of forskolin. This suggests that at least part of the α_2-adrenoceptor-mediated response is due to an action distal to the changes in cyclic AMP i.e. it is cyclic AMP independent.

The contractile response to UK14304 after relaxation with SNP also favours this cyclic AMP-independent mechanism. The UK14304 response after relaxation with SNP is less than that with forskolin, but comparable to that obtained with dibutyryl cyclic AMP. SNP increases cyclic GMP levels through activation of soluble guanylyl cyclase (Southam *et al*., 1996), and this appears to be the mechanism through which it causes relaxation of the porcine ear artery, as evidenced from the inhibition of the SNP-mediated relaxation by ODQ, an inhibitor of NO-sensitive guanylyl cyclase (Schrammel *et al*.,

1996). Further evidence that UK14304 acts through a cyclic AMP-independent mechanism to cause an enhanced response after relaxation with SNP comes from the fact that SNP was unable to increase cyclic AMP levels either in the presence of IBMX, or rolipram. Vascular smooth muscle cells contain the cyclic GMP-inhibited phosphodiesterase III which breaks down cyclic AMP (Komas *et al*., 1991). Therefore it is possible that the increase in cyclic GMP induced by SNP leads to an increase in cyclic AMP through inhibition of this phosphodiesterase. Delpy and co-workers showed that in rat aortic rings and cultured aortic smooth muscle cells, isoprenaline causes an increase in cyclic AMP production which is potentiated by cyclic GMP-elevating agents (Delpy *et al*., 1996), indicating that an increase in cyclic GMP can increase cyclic AMP levels. In human vascular smooth muscle cells, the insulin stimulated increase in cyclic AMP is dependent upon cyclic GMP formation (Trovati *et al*., 1996), possibly through an interaction at the level of the PDEs. The failure of SNP to increase cyclic AMP levels discounts the possibility that SNP increases cyclic AMP levels in the porcine isolated ear artery through inhibition of PDE III. However, it is possible that our assay system for measuring changes in [^{3}H]-cyclic AMP may not be sufficiently sensitive to detect biologically-significant changes in this cyclic nucleotide.

The cyclic AMP-independent mechanism for α_2-adrenoceptors could involve sensitization of the contractile proteins, allowing an α_2-adrenoceptor agonist which would not normally cause a response in the tissue, to cause a contraction. For example, it has been shown that in rat tail arteries a concentration of AII which does not increase tone or Ca^{2+} levels enhances the basal level of myosin light chain phosphorylation and also enhances the level of myosin light chain phosphorylation resulting from α_2-adrenoceptor activation, possibly through activation of myosin light chain kinase (Triggle *et al*., 1995). Further studies have produced evidence that protein kinase C is involved in α_2-adrenoceptor-mediated contractions (Aburto *et al*., 1995; Shimamoto *et al*., 1995), and could be part of the cyclic AMP-independent pathway. There is also evidence that α_2-adrenoceptors increase Ca^{2+} entry through dihydropyrodine-sensitive Ca^{2+} channels and it is possible that activation of α_2-adrenoceptors can also increase release of intracellular Ca^{2+} (Daly *et al*., 1990; Dunn *et al*., 1991). A preliminary study by Wright and co-workers suggested that α_2-adrenoceptor activation increases inositol 1,4,5-trisphosphate production in the porcine palmar lateral vein (Wright *et al*., 1994). Work is currently underway to determine whether UK14304 increases intracellular Ca^{2+} levels in the porcine isolated ear artery, and to investigate the changes in intracellular Ca^{2+} levels associated with the enhanced contractile response to UK14304.

Recent studies in our laboratory have indicated that other compounds which can inhibit cyclic AMP accumulation may also produce vasoconstriction through a cyclic AMP-independent mechanism, for example 5-HT acting through 5-HT_1-like receptors (Randall *et al*., 1996), and neuropeptide Y (Wright *et al*., 1995b). Future studies will determine whether there is a similarity between α_2-adrenoceptor-mediated contractions, and contractions mediated via other receptors. In conclusion, enhanced contractions to the α_2-adrenoceptor agonist UK14304 can be uncovered in porcine isolated ear arteries by pre-contracting the tissue with the thromboxane-mimetic U46619, followed by relaxation back to baseline with forskolin, SNP or dibutyryl cyclic AMP before addition of UK14304. There was a greater contractile response to UK14304 after relaxation with forskolin than with SNP or dibutyryl cyclic AMP suggesting that there is more than one mechanism involved in the enhancement of the UK14304 response.

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References

ABURTO, T., JINSI, A., QINGBING, Z. & DETH, R.C. (1995). Involvement of protein kinase C activation in α_2-adrenoceptor-mediated contractions of rabbit saphenous vein. *Eur. J. Pharmacol.,* **277,** 35 – 44.

AIDULIS, D.M., FUGLSANG, A., SAVAGE, S.A.H. & POLLOCK, D. (1991). Drug-induced enhancement of contractions to clonidine in rat tail artery rings *in vitro*. *Br. J. Pharmacol.,* **104,** 51P.

AIDULIS, D.M. & POLLOCK, D. (1992). Inhibition of enhanced contractions to UK-14304 by yohimbine, prazosin or a vasopressin antagonist in rat tail artery rings *in vitro*. *Br. J. Pharmacol.,* **107,** 183P.

BLAYLOCK, N.A. & WILSON, V.G. (1995). Pharmacological characterization of noradrenaline-induced contractions of the porcine isolated palmar lateral vein and palmar common digital artery. *Br. J. Pharmacol.,* **114,** 694 – 702.

BYLUND, D.B., EIKENBURG, D.C., HIEBLE, J.P., LANGER, S.Z., LEFKOWITZ, R.J., MINNEMAN, K.P., MOLINOFF, P.B., RUFFOLO, R.R. & TRENDELENBURG, U. (1994). International Union of Pharmacology nomenclature on adrenoceptors. *Pharmacol. Rev.,* **46,** 121 – 136.

CAMBRIDGE, D. (1981). UK-14304, a potent and selective α_2-agonist for the characterisation of α-adrenoceptor subtypes. *Eur. J. Pharmacol.,* **72,** 413 – 415.

CONSTANTINE, J.W., LEBEL, W. & ARCHER, R. (1982). Functional post-synaptic α_2- but not α_1-adrenoceptors in dog saphenous vein exposed to phenoxybenzamine. *Eur. J. Pharmacol.,* **85,** 325 – 329.

DALY C.J., DUNN W.R., MCGRATH J.C., MILLER D.J. & WILSON V.G. (1990). An examination of the sources of calcium for contractions mediated by post-junctional α_1- and α_2-adrenoceptors in several isolated blood vessels from the rabbit. *Br. J. Pharmacol.,* **99,** 253 – 260.

DALY, C.J., McGRATH, J.C. & WILSON, V.G. (1988). Pharmacolgical analysis of post-junctional alpha-adrenoceptors mediating contractions to (−)-noradrenaline in the rabbit isolated saphenous vein can be explained by interacting responses to simultaneous activation of α_1- and α_2-adrenoceptors. *Br. J. Pharmacol.,* **95,** 485 – 500.

DELPY, E., COSTE, H. & LE MONNIER DE GOUVILLE, A.C. (1996). Effects of cyclic GMP elevation on isoprenaline-induced increase in cyclic AMP and relaxation in rat aortic smooth muscle: role of phosphodiesterase 3. *Br. J. Pharmacol.,* **119,** 471 – 478.

DUNN, W.R., DALY, C.J., McGRATH, J.C. & WILSON, V.G. (1991). The effects of nifedipine on α_2-adrenoceptor -mediated contractions in several isolated blood vessels from the rabbit. *Br. J. Pharmacol.,* **103,** 1493 – 1499.

DUNN, W.R., McGRATH, J.C. & WILSON, V.G. (1989). Expression of functional postjunctional α_2-adrenoceptors in rabbit isolated distal saphenous artery – a permissive role for angiotensin II? *Br. J. Pharmacol.,* **96,** 259 – 261.

ERDBRUGGER, W., VISCHER, P., BAUCH, H-J. & MICHEL, M.C. (1993). Norepinephrine and neuropeptide Y increase intracellular Ca^{2+} in cultured porcine aortic smooth muscle cells. *J. Cardiovasc. Pharmacol.,* **22,** 97 – 103.

FABER, J.E. (1988). In situ analysis of α-adrenoceptors on arteriolar and venular smooth muscle in rat skeletal muscle microcirculation. *Circ. Res.,* **62,** 37 – 50.

GUAN, Y.Y., KWAN, C.Y. & DANIEL, E.E. (1989). Does *in vitro* activation of post-synaptic α_2-adrenoceptors utilize intracellular Ca^{2+} for contraction in dog saphenous vein? *Can. J. Physiol.,* **67,** 1086 – 1091.

HEI, Y-J., MACDONNELL, K.L., MCNEILL, J.H. & DIAMOND, J. (1991). Lack of correlation between activation of cyclic AMP-dependent protein kinase and inhibition of contraction of rat vas deferens by cyclic AMP analogs. *Mol. Pharmacol.,* **39,** 233 – 238.

HIEBLE, J.P. & WOODWARD, D.F. (1984). Different characteristics of post-junctional alpha-adrenoceptors on arterial and venous smooth muscle. *Naunyn-Schmiedeberg's Arch. Pharmacol.,* **328,** 44 – 50.

IKEOKA, K. & FABER, J.E. (1993). ANG II reverses selective inhibition of α_2-adrenoceptor sensitivity after in vitro isolation of arterioles. *Am. J. Physiol.,* **265,** H1988 – H1995.

KOMAS, N., LUGNIER, C. & STOCLET, J-C. (1991). Endothelium-dependent and independent relaxation of the rat aorta by cyclic nucleotide phosphodiesterase inhibitors. *Br. J. Pharmacol.,* **104,** 495 – 503.

MACLENNAN, S.J., LUONG, L.A., JASPER, J.R., TO, Z.P. & EGLEN, R.M. (1997). Characterization of α_2-adrenoceptor mediating contraction of dog saphenous vein: identity with the human α_{2A} subtype. *Br. J. Pharmacol.,* **121,** 1721 – 1729.

MCGRATH, J.C. (1982). Evidence for more than one subtype of post-junctional alpha-adrenoceptor. *Biochem. Pharmacol.,* **31,** 467 – 484.

MCGRATH, J.C., BROWN, C.M. & WILSON, V.G. (1989). α-Adrenoceptors: a critical review. *Med. Res. Rev,* **9,** 407 – 535.

MCGRATH J.C., WILSON V.G., DUNN, W.R. & TEMPLETON, A.G.B. (1991). The interaction between α_1- and α_2-adrenoceptors – the search for vascular α_2- adenoceptors *in vitro*. In *Adrenoceptors, Structure, Mechanisms and Function – Advances in Pharmacological Sciences*. ed. Szabadi, E. & Bradshaw, P. M., Basal: Birkhauser Verlag pp. 211 – 220.

MONCADA, S., PALMER, M.J. & HIGGS, E.A. (1991). Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol. Rev.,* **43,** 109 – 142.

NIELSEN, H., THOM, S. McG., HUGHES, A.D., MARTIN, G.N., MULVANY, M.J. & SEVER, P.S. (1989). Postjunctional α_2-adrenoceptors mediate vasoconstriction in human subcutaneous resistance vessels. *Br. J. Pharmacol.,* **97,** 829 – 834.

QUAST, U. & COOK, N.S. (1989). Moving together: K^+ channel openers and ATP-sensitive K^+ channels. *Trends Pharmacol. Sci.,* **10,** 431 – 435.

RANDALL, V.A., MACLENNAN, S.J., MARTIN, G.R. & WILSON, V.G. (1996). The effect of forskolin on 5-HT_1-like and angiotensin II-induced vasoconstriction and cyclic AMP content of the rabbit isolated femoral artery. *Br. J. Pharmacol.,* **118,** 627 – 634.

ROBERTS, R.E., KENDALL, D.A. & WILSON, V.G. (1997). Evidence for α_2-adrenoceptor-mediated vasoconstriction in porcine isolated ear artery. *Br. J. Pharmacol.,* **122,** 126P.

SCHUMANN, H-J. & LUES, I. (1983). Postjunctional α-adrenoceptors in isolated saphenous vein of the rabbit. *Naunyn-Schmiedeberg's Arch. Pharmacol.,* **323,** 328 – 334.

SCHRAMMEL, A., BEHRENDS, S., SCHMIDT, K., KOESLING, D. & MAYER, B. (1996). Characterization of 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one as a heme site inhibitor of nitric oxide-sensitive guanylyl cyclase. *Mol. Pharmacol.,* **50,** 1 – 5.

SEAMON, K.B. & DALY, J.W. (1986). Forskolin: Its biological and chemical properties. *Adv. Cyclic Nucleotide Res.,* **20,** 1 – 150.

SHIMAMOTO, H., SHIMAMOTO, Y., KWAN, C-Y. & DANIEL, E.E. (1995). Activation of protein kinase C as a modulator of potentiated UK-14304-induced contractions in dog mesenteric artery and vein. *J. Cardiovasc. Pharmacol.,* **26,** 923 – 931.

SMITH, K., CONNAUGHTON, S. & DOCHERTY, J.R. (1992). Investigation of the subtype of α_2-adrenoceptor mediating contraction of human saphenous vein. *Br. J. Pharmacol.,* **106,** 447 – 451.

SOUTHAM, E., CHARLES, S.L. & GARTHWAITE, J. (1996). The nitric oxide-cyclic GMP pathway and synaptic plasticity in the rat superior cervical ganglion. *Br. J. Pharmacol.,* **119,** 527 – 532.

SULPIZIO, A. & HIEBLE, J.P. (1987). Demonstration of α_2-adrenoceptor-mediated contraction in the isolate canine saphenous artery treated with Bay K8644. *Eur. J. Pharmacol.,* **135,** 107 – 110.

TEMPLETON, A.G.B., MACMILLAN, J., MCGRATH, J.C. STOREY, N.D. & WILSON, V.G. (1989). Evidence for a population of prazosin-resistant, rauwolscine-sensitive α-adrenoceptors mediating contractions in the isolated vascular bed of the rat tail. *Br. J. Pharmacol.,* **97,** 563 – 571.

TRIGGLE, C.R., ALLEN, B.G., WALSH, M.P. & LI, X-F. (1995). Contractile subthreshold concentrations of angiotensin II elevate basal levels of myosin light chain phosphorylation and selectively enhance α_2-adrenoceptor mediated responses. *Hypertension,* **25,** 1424.

TROVATI, M., MASSUCCO, P., MATTIELLO, L., CAVALOT, F., MULARONI, E.M., HAHN, A.W. & ANFOSSI, G. (1996). Studies on the influence of insulin on cyclic adenosine monophosphate in human vascular smooth muscle cells: Dependence on cyclic guanosine monophosphate and modulation of catecholamine effects. *Diabetologia,* **39,** 1156 – 1164.

WRIGHT, I.K., BLAYLOCK, N.A., KENDALL, D.A. & WILSON, V.G. (1995a). The relationship between density of α-adrenoceptor binding sites and contractile responses in several porcine isolated blood vessels. *Br. J. Pharmacol.,* **114,** 678 – 688.

WRIGHT, I.K., BLAYLOCK, N.A., STARK, G., KENDALL, D.A. & WILSON, V.G. (1995b). Neuropeptide Y-induced modulation of cyclic adenosine monophosphate and contractile responses in porcine isolated blood vessels. *J. Physiol.,* **483,** 111P.

WRIGHT, I.K., HARLING, R., KENDALL, D.A. & WILSON, V.G. (1995c). Examination of the role of inhibition of cyclic AMP in α_2-adrenoceptor mediated contractions of the porcine isolated palmar lateral vein. *Br. J. Pharmacol.,* **114,** 157 – 165.

WRIGHT, I.K., KENDALL, D.A. & WILSON, V.G. (1994). α_2-adrenoceptor-mediated stimulation of inositol 1,4,5-trisphosphate accumulation in the porcine isolated palmar lateral vein. *Br. J. Pharmacol.,* **113,** 21P.

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