British Journal of Pharmacology (1996) 118, 627-634 © 1996 Stockton Press All rights reserved 0007-1188/96 $12.00

The effect of forskolin on 5-HT_1-like and angiotensin II-induced vasoconstriction and cyclic AMP content of the rabbit isolated femoral artery

[3]V.A. Randall, [2]*S.J. MacLennan, [2]*G.R. Martin & [1]V.G. Wilson

Department of Physiology and Pharmacology, Medical School, Queen's Medical Centre, Nottingham NG7 2UH, UK and *Receptor Pharmacology Group, Wellcome Research Laboratories, Langley Court, Beckenham, Kent BR3 3BS

1 A characteristic feature of vasoconstrictor 5-HT_1-like receptors *in vitro* is that responses mediated by these receptors are enhanced by other vasoconstrictor agents. In the present study, we have examined the influence of cellular cyclic AMP on vasoconstrictor responses to activation of 5-HT_1-like receptors in isolated ring segments of the rabbit femoral artery (RbFA), and determined whether modulation of this second messenger underlies the ability of angiotensin II, an endogenous vasoconstrictor, to enhance 5-HT_1-like responses.

2 In the presence of 0.1 μM ketanserin (to antagonize 5-HT_2-receptors) and 0.3 μM prazosin (to antagonize α_1-adrenoceptors), 5-HT produced a concentration-related contraction, which was significantly augmented by pre-contraction of the vessel with 0.1-0.45 nM ([A_{30}]) angiotensin II. Responses to 5-HT in the presence of angiotensin II were inhibited by the 5-HT_1-like/5-HT_2 antagonist, metergoline (1 μM).

3 The directly-acting adenylyl cyclase activator, forskolin (1 μM), abolished responses to angiotensin II and caused a rightward shift and concomitant depression of the 5-HT concentration-effect (E/[A]) curve. Higher concentrations of forskolin (>10 μM) abolished responses to 5-HT and 1 μM sodium nitroprusside abolished responses to 5-HT and angiotensin II ($n=7$).

4 In the presence of angiotensin II (0.1-0.45 nM), however, 1 μM forskolin failed to inhibit 5-HT-induced contractions; the E/A curve for 5-HT (in the presence of forskolin and angiotensin II) was not significantly different from that produced in the presence of angiotensin II alone. Similarly, the presence of angiotensin II (0.1-0.45 nM) was also able to overcome partially the inhibitory effect of 1 μM sodium nitroprusside against 5-HT-induced contractions ($n=7$). In marked contrast, 5-HT failed to elicit a contraction in the presence of angiotensin II and 10 μM forskolin ($n=5$).

5 5-HT (1 μM) significantly reduced basal cyclic AMP accumulation by 35%, whereas angiotensin II (0.45 nM) was without effect. The combination of angiotensin II and 5-HT failed to alter significantly the reduction in cyclic AMP produced by 5-HT alone. Forskolin (1 μM) increased cyclic AMP levels 7 fold above basal, but neither 1 μM 5-HT nor a combination of 1 μM 5-HT and 0.45 nM angiotensin II produced a significant decrease in cyclic AMP content.

6 Whilst moderate concentrations of forskolin can inhibit the reponses to either agent, simultaneous activation of angiotensin II and 5-HT_1-like receptors can overcome the inhibitory effect of elevated levels of cyclic AMP. Since the potentiating effect of angiotensin II, in either the presence or absence of forskolin, occurs without significant alteration of cellular cyclic AMP, it seems likely that a cyclic AMP-independent pathway is implicated in the synergistic interaction between angiotensin II and vasoconstrictor 5-HT_1-like receptors.

Keywords: Vasoconstriction; 5-HT_1-like receptor; angiotensin II; cyclic AMP; forskolin; vascular smooth muscle

Introduction

Vasoconstriction by 5-HT is thought to occur predominantly via 5-HT_{2A} and 5-HT_1-like receptor subtypes (Martin, 1994; Hoyer *et al.*, 1994). 5-HT_{2A} receptors are primarily responsible for contractions to 5-HT in large conduit arteries *in vitro*, e.g. rabbit isolated thoracic aorta (Apperley *et al.*, 1976), rat isolated caudal artery (Van Neuten *et al.*, 1981), bovine pulmonary arteries (MacLean *et al.*, 1994) and pulmonary and coronary arteries from the pig (Glusa, 1992; Cocks & Angus, 1983). In contrast, activation of 5-HT_1-like receptors causes constriction of superficial veins from the dog (Feniuk *et al.*, 1985), rabbit (Martin *et al.*, 1988) and man (Bax *et al.*, 1992). In a number of isolated arteries however, responses mediated via 5-HT_1-like receptors are apparent when 'primed' by another vasoconstrictor agent to either augment small, pre-existing contractions or actually to unmask contractile responses. This type of synergistic interaction between 5-HT and other vasoconstrictor agents appears to be widespread, having been reported in arteries from the rabbit (De la Lande, 1992; MacLennan & Martin, 1992; Choppin & O'Connor, 1993), guinea-pig (Sahin-Erdemli *et al.*, 1991), cow (MacLean *et al.*, 1994) and man (Templeton *et al.*, 1991; Cocks *et al.*, 1993), which raises the possibility that they are of either physiological or pathophysiological significance. To date, however, there has been little information on either the subcellular basis of 5-HT_1-like receptor-mediated contractions or their synergistic interactions with other vasoconstrictor agents.

Evidence from work on the canine saphenous vein (Sumner & Humphrey, 1990) and cultured smooth muscle cells of the bovine basilar artery (Ebersole *et al.*, 1993) indicates that vascular 5-HT_1-like receptors, like those on non-vascular tissue (see Martin, 1994), are negatively coupled to the formation of adenosine 3:5'-cyclic monophosphate (cyclic AMP). However, as demonstrated by Sumner and colleagues (1992), a causal

[1] Author for correspondence.
Present address: [2]Institute of Pharmacology, Roche Bioscience R2-101, 3401 Hillview Avenue, Palo Alto, Ca 94303 USA; [3]Department of Pharmacology, Astra Charnwood, Bakewell Road, Loughborough LE11 0RH, UK.

relationship for this effector mechanism and the associated contractions has yet to be established. Recently, Sweeney *et al.* (1995) showed that 5-HT$_1$-like contractions of the bovine isolated pulmonary artery were enhanced by the thromboxane A_2-mimetic, U46619, and that this synergistic interaction was still evident in the present of forskolin, a direct activator of adenylyl cyclase (Seamon & Daly, 1986). The authors suggested that the known negative coupling of 5-HT$_1$-like receptors to adenylyl cyclase activity might account for the apparent resistance of these contractions to the inhibitory effects of forskolin.

In the present study we have used the rabbit isolated femoral artery (RbFA), a preparation in which 5-HT$_1$-like contractions are greatly potentiated and augmented by the presence of the thromboxane A_2-mimetic, U46619 (MacLennan & Martin, 1992), to examine whether enhancement of 5-HT$_1$-like responses is also observed with angiotensin II. This peptide is of particular interest since it is generated locally within the vasculature (Griendling *et al.*, 1993), and recent evidence suggests that the AT$_1$ receptor subtype has the potential to inhibit adenylyl cyclase (Crawford *et al.*, 1992; Griendling *et al.*, 1993). Thus, we have also conducted complementary contraction-based and biochemical experiments to determine whether the synergistic interation between these two receptors is mediated by a cyclic AMP-dependent or cyclic AMP-independent pathway. Some of these results have been reported in preliminary form to the British Pharmacological Society (Randall *et al.*, 1994).

Methods

Contractile studies

Male New Zealand White rabbits (2.5–3.0 kg) were killed by intravenous administration of pentobarbitone sodium (Sagatal; 80 mg kg^{-1}) and the right and left femoral arteries removed. Each vessel was cleared of adhering connective tissue and fat, and the endothelium removed by gently 'rolling' the tissue against a thin wire; the success of this procedure was assessed in a series of preliminary experiments by the failure of acetylcholine to relax submaximal contractions to the thromboxane-mimetic, U46619. Six 2 mm ring segments were prepared from each artery, and each vascular ring segment was suspended between two wire supports (250 μm thick), the upper support being attached to a force displacement transducer (Grass FTO3c), and the lower support being held stationary by means of a clamped glass rod. This assembly was lowered into a 20 ml organ bath containing modified Krebs-Hensleit buffer solution (pH 7.4) of the following composition (in mM): NaCl 118.41, $NaHCO_3$ 25.00, KCl 4.75, KH_2PO_4 1.19, $MgSO_4$ 1.19, glucose 11.10 and $CaCl_2$ 2.50. This was maintained at 37°C and gassed continuously with 5% CO_2: 95% O_2. In all experiments 0.3 μM prazosin and 0.1 μM ketanserin were included in the Krebs-Henseleit buffer solution to exclude interaction with α_1-adrenoceptors and 5-HT$_{2A}$ receptors, respectively. After a 15 min equilibration period, a 5 g wt force was applied at 1 g wt increments over a 30 min period until a resting tension of approximately 2.5 g wt was achieved. During this period, the tissues were exposed to pargyline (500 μM) to inhibit monoamine oxidase activity. Each tissue was then washed three times to remove excess pargyline and challenged with 80 mM KCl, thereby providing a reference contracture for subsequent responses. The tissues were then exposed to phenoxybenzamine (0.15 μM) to inactivate the intraneuronal transport of 5-HT and to alkylate irreversibly post-junctional α-adrenoceptors, and washed three times after a further 30 min period.

A single cumulative (increasing in 0.5 $\log_{10}$ unit increments until a maximum level was defined) 5-HT concentration-effect curve was constructed in all tissues. The influence of angiotensin II receptor activation on the 5-HT E/[A] curves was examined by a 15 min prior exposure to concentrations of angiotensin II that produced a sustained contraction equal to 30% ([A_{30}]) of its maximal effect (0.1–0.45 nM). The effect of the 5-HT$_1$-like receptor antagonist, metergoline (1 μM) on the 5-HT E/[A] curves in the presence of angiotensin II was investigated by a 60 min incubation with metergoline before the addition of an [A_{30}] concentration of angiotensin II. Forskolin or sodium nitroprusside was incubated with tissues for 15 min prior to the construction of 5-HT E/[A] curves, or 15 min before the addition of an [A_{30}] concentration of angiotensin II (established in paired segments of the artery not exposed to forskolin).

Cyclic AMP studies

Tissues were prepared as described above, and mounted onto single wire hooks (250 μm thick). Each segment was immersed into a glass vial containing 2 ml of pre-gassed modified Krebs-Henseleit buffer solution (containing 0.1 μM ketanserin and 0.3 μM prazosin) and maintained at 37°C. All segments were incubated with phenoxybenzamine (0.15 μM) and pargyline (500 μM) for 30 min, after which three washes ensured removal of excess inhibitors. Tissues taken from a single rabbit were exposed to one of eight conditions in which the period of exposure to 5-HT and forskolin was fixed: basal; angiotensin II (0.45 nM for 15 min); 5-HT (1 μM for 10 min); angiotensin II and 5-HT (angiotensin II 15 min before 5-HT); forskolin (1 μM for 15 min); forskolin and 5-HT (forskolin 5 min before 5-HT); forskolin and angiotensin II (15 min both agents); forskolin, 5-HT and angiotensin II (angiotensin II and forskolin added 15 min and 5 min, respectively, before 5-HT). In all cases the tissues were exposed to the non-selective phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX: 100 μM) during the final 10 min of incubation (see Results).

In order to terminate all reactions and to extract cyclic AMP from the cells, the tissue segments were immersed in 0.5 ml of ice-cold absolute ethanol for 60 min. The ethanol was blown with a stream of N_2 gas, and these vials were stored at −20°C prior to estimation of the cyclic AMP content. The cyclic AMP was resolubilized and tissue content determined by following the non-acetylation protocol of an Amersham Scintillation Proximity assay kit. This method relies on the competition between tissue generated cyclic AMP and [^{125}I]-cyclic AMP for a specific number of binding sites on an antibody. β-electrons emitted from the antibody bound fluomicrospheres activated by the [^{125}I]-cyclic AMP were counted over a 5 min period with a Wallac 1460 scintillation counter.

Data analysis

For the contraction-based studies, all responses have been expressed as a percentage of the contraction to 80 mM KCl determined at the beginning of the experiment, and are shown as the mean ± s.e.mean of a minimum of 5 observations from different animals. Contractions to 5-HT in the presence of angiotensin II have been measured from the level of pre-constrictor rather than the original baseline. Agonist concentration-effect (E/[A]) data were fitted to the function.

$$E = \frac{\alpha[A]^n}{[A]^n + [A_{50}]^n}$$

Where E is the response, α is the asymptote, [A] is the concentration of the agonist, n is the slope of the E/[A] curve and [A_{50}] is the mid-point location of the E/[A] curve. [A_{50}] values are assumed to be log normally distributed and are presented as their negative logarithms, p[A_{50}]. For the experiments involving metergoline, the p[A_{50}] value of 5-HT in the presence and absence of the antagonist was used to estimate the agonist concentration-ratio (CR). The pA_2 value was calculated using the equation of Furchgott (1972).

$$pA_2 = \log(CR - 1) - \log[B]$$

where [B] is the concentration of the antagonist.

For the biochemical studies, cyclic AMP content of each tissue sample was determined from the equation:

$$x = \log_{10} \frac{(\text{d.p.m.} - \text{NSB})}{(B_0 - (\text{d.p.m.} - \text{NSB}))}$$

Where d.p.m. is the disintegration min^{-1} per well determined by a Wallac 1460 scintillation counter, NSB is the non-specific binding of the [^{125}I]-cyclic AMP to a fluomicrosphere and B_0 is the maximum number of binding sites occupied on the antibody by [^{125}I]-cyclic AMP. The x value generated from this equation is subsequently interpolated from a standard curve produced by the inclusion of a range of cyclic AMP concentrations from 12.8 to 0.1 pmol in the assay procedure, to yield the cyclic AMP content of each 2 mm tissue segment.

The conventional way of expressing cyclic nucleotide content of vascular tissue is in pmol mg^{-1} protein (e.g. Sumner *et al.*, 1992; Sweeney *et al.*, 1995). However, in preliminary experiments we found that assessment of protein content did significantly improve the variability of the data. In order to establish whether there was any justification in determining the protein content of individual segments the following experiment was conducted. Fifty seven to sixty 2 mm segments of the femoral artery from 10 rabbits (6 segments from each artery) were gently blotted to remove surface water, weighed on a Mettler College 150 balance with an accuracy of ± 0.1 mg, and placed in cold absolute ethanol for 60 min. The ethanol was then blown off with a stream of N_2 gas and the dried segment reweighed on the balance. Each segment was subsequently dissolved in 0.5 ml 2 M NaOH solution at 55°C (4–6 h) and the protein content of each tissue segment assessed with a Pierce Bio-Rad BCA protein assay kit (sensitivity from 5.0 to 250 μg protein). A similar number of segments of arteries from the same group of rabbits were prepared for isometric tension recording as described above and the force of contraction to 80 mM KCl determined. The frequency distribution of the protein content, wet weight, dry weight and force of contraction to 80 mM KCl for the segments were then compared.

Where necessary, differences between mean values from contraction based studies were determined by Student's paired *t* test, while cyclic AMP values under each condition were compared to the control by a one-way analysis of variance.

Drugs

The following drugs were used (sources in parenthesis): angiotensin II (Sigma Chemical Co Ltd, Poole, Dorset), forskolin (Sigma), 5-hydroxytryptamine creatinine sulphate (Sigma), iso-butyl-1-methylxanthine (IBMX; Sigma), ketanserin (Sigma), metergoline (Farmitalia, Milan, Italy), pargyline hydrochloride (Sigma), phenoxybenzamine hydrochloride (Sigma) and prazosin hydrochloride (Sigma), sodium nitroprusside (Nipride, Roche). All drugs were dissolved in distilled water, with the exception of phenoxybenzamine (0.3 mM, absolute ethanol), prazosin (0.3 mM, 50% ethanol), forskolin (10 mM, 100% dimethylsulphoxide) and metergoline (10 mM, 10% w/v ascorbic acid). All subsequent dilutions were made in distilled water. The Scintillation Proximity assay kit was purchased from Amersham and the Bio-Rad BCA protein determination kit from Pierce (Rockford, IL, U.S.A.).

Results

5-HT E/[A] curves

5-HT (0.1 nM–1 μM) produced small, concentration-dependent contractions of the rabbit isolated femoral artery (Figure 1), with the response to 1 μM 5-HT being equal to 32.6±8.9% of the contraction elicited by 80 mM KCl (8.2±1.2 g wt; $n=5$). This E/[A] curve did not attain a maximum, and thus a p[A_{50}] value could not be accurately determined. However, prior exposure to an [A_{30}] concentration of angiotensin II (47.5±5.8% of the response to 80 mM KCl, $n=5$) caused a significant potentiation of the response to 5-HT at all concentrations; the response to 1 μM 5-HT was increased to 78.6±4.3% of that to 80 mM KCl ($n=5$). Furthermore, in the presence of angiotensin II a p[A_{50}] of 8.10±0.04 ($n=5$) was estimated for 5-HT. Metergoline (1 μM) caused a rightward displacement of the 5-HT E/[A] curve produced in the presence of angiotensin II, which was associated with a 50% reduction of the maxium response (Figure 2). In contrast, metergoline did not affect responses to angiotensin II (data not shown). The p[A_{50}] values for 5-HT in the presence and absence of 1 μM metergoline were 5.78±0.08 and 7.95±0.11, respectively ($n=6$). Schild analysis of the data, based upon the apparent concentration-ratio for 5-HT in the presence and absence metergoline, and disregarding the reduction in the maximum response, generated a pA_2 value of 8.14±0.11 ($n=6$).

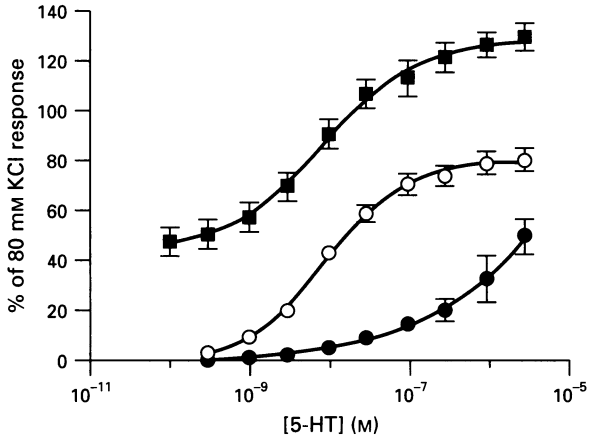

Figure 1 The effect of 5-HT in the absence (●) and presence (○ ■) of 0.1–0.45 nM angiotensin II in the rabbit isolated femoral artery (treated with 0.5 mM pargyline and 0.1 μM phenoxybenzamine and maintained in the presence of 0.1 μM ketanserin and 0.3 μM prazosin): (■) total response to 5-HT and angiotensin II; (○) response to 5-HT minus the angiotensin II-induced tone. Responses have been expressed as a percentage of contraction produced by 80 mM KCl and are shown as the mean±s.e.mean of 5 observations.

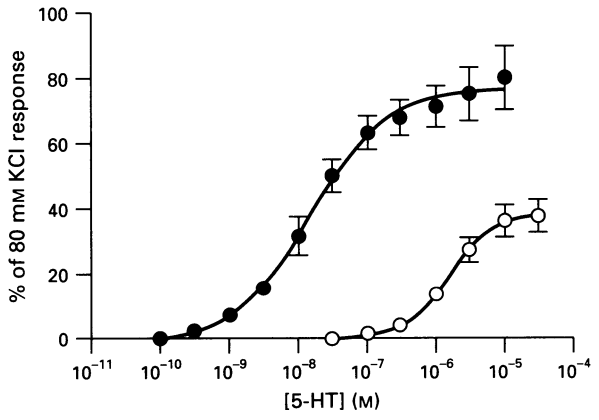

Figure 2 The effect of 5-HT in the absence (○) and presence (●) of 1 μM metergoline in the rabbit femoral artery (treated with 0.5 mM pargyline and 0.1 μM phenoxybenzamine and maintained in the presence of 0.1 μM ketanserin and 0.3 μM prazosin) preconstricted with 0.1–0.45 nM angiotensin II. Responses in the presence of angiotensin II do not include the preconstrictor tone. All responses to 5-HT have been expressed as a percentage of contraction produced by 80 mM KCl and are shown as the mean±s.e.mean of 6 observations.

The effect of forskolin on the 5-HT E/[A] curves

Figure 3 shows the effect of forskolin against 5-HT-induced contractions. Exposure to 1 μM forskolin caused a small relaxation (equivalent to 2.1 $\pm$ 1.0% (n = 5) of the response to 80 mM KCl) of the rabbit isolated femoral artery, and significantly reduced responses to 5-HT; the contraction to 1 μM 5-HT was reduced from 32.6 $\pm$ 8.9% to 12.5 $\pm$ 5.2% (n = 5) of the response to 80 mM KCl (Figure 3). Forskolin (10 μM) also relaxed preparations at rest (by a similar amount to that observed for 1 μM forskolin) and abolished responses to 5-HT (Figure 3). Both concentrations of forskolin abolished the contractions of 0.1 – 0.45 nM angiotensin II. However, in the presence of angiotensin II the inhibitory effect of 1 μM forskolin against contractions to 5-HT was attenuated. Indeed the 5-HT E/[A] was not significantly different from that in the absence of 1 μM forskolin (Figure 4), i.e. the inhibitory effect of 1 μM forskolin against 5-HT$_1$-like contractions was overcome by the presence of angiotensin II. In marked contrast, responses to 5-HT in the presence of angiotensin II were abolished by 10 μM forskolin (Figure 4).

The effect of forskolin, angiotensin II and 5-HT on tissue cyclic AMP accumulation

The above findings indicate that angiotensin II can augment responses mediated by 5-HT$_1$-like receptors, in either the presence or absence of moderate concentrations of forskolin, and raises the possibility that inhibition of cellular cyclic AMP may underlie the synergistic interaction between vasconstrictor agents. We decided, therefore, to determine the levels of cyclic AMP of the rabbit isolated femoral artery under each of these conditions and, as a prelude to these experiments, assess the best way of expressing the data (see Methods).

Figure 5 shows the frequency-distribution for force generation (to 80 mM KCl), protein content and wet weight of 57 – 60 2 mm segments of the isolated femoral artery. The segments were taken from 10 rabbits (6 segments from each artery). The distribution of force generation (3.02 – 6.20 g wt, mean value 4.37 $\pm$ 0.08 g wt, n = 60) and wet weight (0.9 – 3.2 mg, mean value 1.82 $\pm$ 0.07 mg, n = 57) were qualitatively similar with values extending over a 2 – 3 fold range. In contrast, the distribution of values for protein content extended over a 20 fold range (10 – 248 μg protein mean value 81.0 $\pm$ 7.7 μg, n = 57) and was markedly skewed to low values (10 – 39 μg). Following exposure to absolute ethanol, the mean weight of the segments were reduced by approximately 60% to

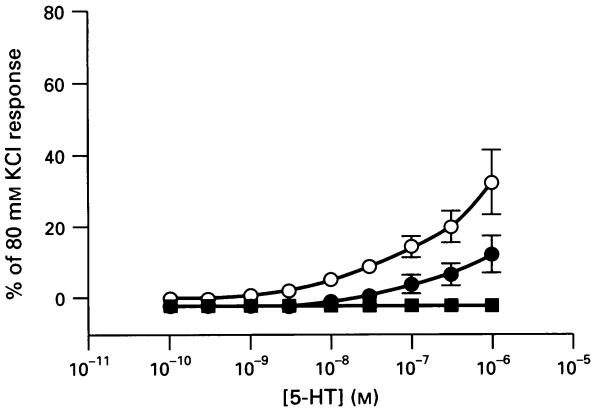

Figure 3 The effect of 5-HT in the absence (○) and presence of 1 μM (●) and 10 μM (■) forskolin in the rabbit isolated femoral artery (treated with 0.5 mM pargyline and 0.1 μM phenoxybenzamine and maintained in the presence of 1.0 μM ketanserin and 0.3 μM prazosin). Responses have been expressed as a percentage of contraction produced by 80 mM KCl and are shown as the mean $\pm$ s.e.mean of 5 observations.

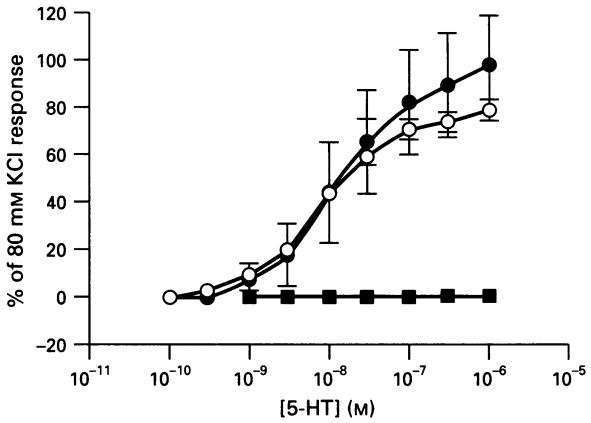

Figure 4 The effect of 5-HT in the absence (○) and presence of 1 μM (●) and 10 μM (■) forskolin in the rabbit isolated femoral artery (treated with 0.5 mM pargyline and 0.1 μM phenoxybenzamine and maintained in the presence of 0.1 μM ketanserin and 0.3 μM prazosin) preconstricted with 0.1 – 0.45 nM angiotensin II. Responses in the presence angiotensin II alone do not include the preconstrictor tone. All responses to 5-HT have been expressed as a percentage of contraction produced by 80 mM KCl and are shown as the mean $\pm$ s.e.mean of 5 observations.

1.68 $\pm$ 0.1 mg (range 0.7 – 2.8 mg wt, n = 28); values which were measured with an accuracy of between 3 – 14%. Thus, neither pmol μg^{-1} protein nor pmol mg^{-1} dry weight were considered to be suitable units for expressing cyclic AMP content. Since the protocol for the measurement of cyclic AMP did not permit routine measurement of wet weight, segments were randomized for each experiment and cyclic AMP content simply expressed as pmol/2 mm segment.

In the absence of 100 μM IBMX, the cyclic AMP content of 2 mm segments of the rabbit femoral artery (approximately 0.15 pmol) was at the very lower limit of detection, 0.1 pmol. Exposure to 100 μM IBMX for 10 min significantly increased basal cyclic AMP levels to 0.60 $\pm$ 0.13 pmol/2 mm segment (n = 6), 6 fold above the lower limit of detection. Similarly, cyclic AMP levels in the presence of 30 μM forskolin (16.5 $\pm$ 4.7 pmol/2 mm segment, n = 6) were increased 3 fold by exposure to 100 μM IBMX (50.3 $\pm$ 5.7 pmol/2 mm segment, n = 6). Hence, all subsequent experiments were conducted in the presence of 100 μM IBMX during the final 10 min of incubation to amplify cyclic AMP levels; those conducted in the absence of forskolin were considered to represent 'basal levels' of cyclic AMP. The use of IBMX to amplify the changes in cyclic AMP is similar that adopted by Sumner *et al.* (1992).

As shown in Table 1, 1 μM 5-HT caused a significant decrease (approximately 35%) in basal cyclic AMP levels, an effect that was slightly less pronounced in the presence of 0.45 nM angiotensin II. Exposure to 1 μM forskolin increased cyclic AMP to approximately 7 fold above basal levels but, under these conditions, neither 1 μM 5-HT nor the combination of 1 μM 5-HT and 0.45 nM angiotensin II was able to reduce cyclic AMP content significantly. Furthermore, 0.45 nM angiotensin alone failed to alter cyclic AMP levels signficantly under basal conditions or in the presence of 1 μM forskolin (data not shown).

A comparison of contractile responses and cyclic AMP levels in the rabbit isolated femoral artery under various conditions is illustrated by Figure 6. Under the basal conditions, the contractile response elicited by 1 μM 5-HT was associated with a significant reduction in cyclic AMP, but the potentiation of contractions produced by angiotensin II was not paralleled by a further inhibition of cyclic AMP levels. Forskolin (1 μM) produced a 7 fold increase in cyclic AMP levels which was associated with a small relaxation of preparations at rest and a reduction in the responses to 1 μM 5-HT. In spite of this large increase in cyclic AMP levels, however, the combination

of angiotensin II and 5-HT was still able to elicit a response comparable to that in the absence 1 μM forskolin (Figure 6). Taken together these results indicate that angiotensin II-mediated facilitation of 5-HT$_1$-like receptor-induced contraction of the rabbit isolated femoral artery does not involve changes in cyclic AMP levels, and is not influence by a 7 fold elevation of tissue cyclic AMP levels.

The effect of sodium nitroprusside on the 5-HT E/[A] curves

In the presence of 1 μM sodium nitroprusside neither 0.1–0.45 nM angiotensin II (data not shown) nor 5-HT (Figure 7) produced contractions. However, concentration-dependent contractions of 5-HT were observed in the presence of 1 μM sodium nitroprusside if angiotensin II (0.1–0.45 nM) was also present (Figure 7).

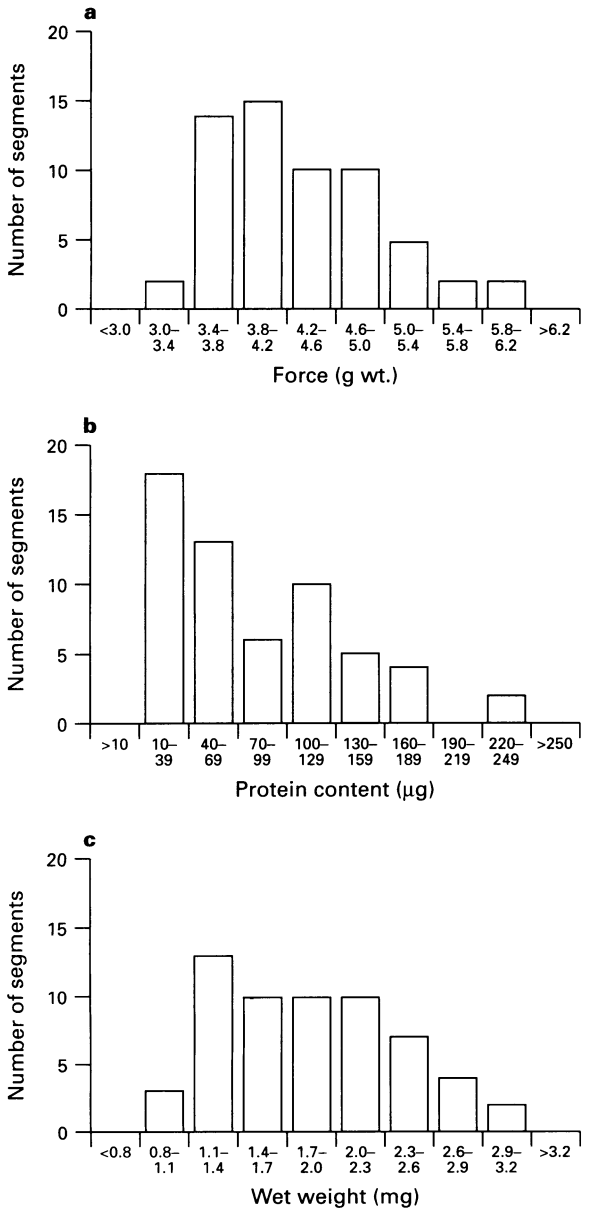

Figure 5 Frequency distribution for (**a**) the force of contraction to 80 mM KCl, (**b**) the protein content and (**c**) the wet weight of 57–60 2 mm segments of the rabbit isolated femoral artery taken from 10 rabbits. Please note that in the interest of clarity the upper limit for each category in (**a**) and (**c**) has been rounded up to the first decimal point.

Table 1 Cyclic AMP content of the rabbit femoral artery in the presence and the absence of a combination of vasoconstrictor and vasodilator agents

	Cyclic AMP content (pmol/2mm segment)
Control	0.60 ± 0.13
5-HT 1 μM	0.38 ± 0.05*
Angiotensin II 0.45 nM	0.81 ± 0.15
Angiotensin II 0.45 nM 5-HT plus 1 μM	0.47 ± 0.08
Forskolin 1 μM	4.2 ± 0.5
Forskolin 1 μM plus 5-HT 1 μM	3.8 ± 0.7
Forskolin 1 μM plus 5-HT 1 μM and Angiotensin II 0.45 nM	3.2 ± 0.4

*Significant difference from control (basal) values ($P<0.05$). All values determined in the presence of IBMX 100 μM and represent the mean ± s.e.mean of 7/8 observation from different animals.

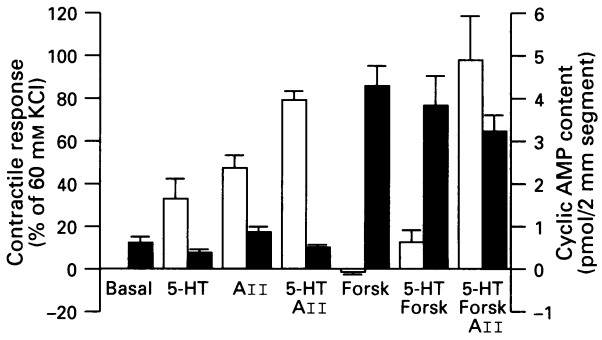

Figure 6 Comparison of the contractile response (expressed as a percentage of response to 80 mM KCl, open column) and associated cyclic AMP content (pmol/2 mm segment, solid column) of the rabbit isolated femoral artery in either the absence of constrictor or dilator agents (basal) or in the presence of 1 μM 5-HT (5-HT), 0.45 nM angiotensin II (AII) and/or 1 μM forskolin (Forsk). The values shown represent the mean ± s.e.mean of 5–8 observations.

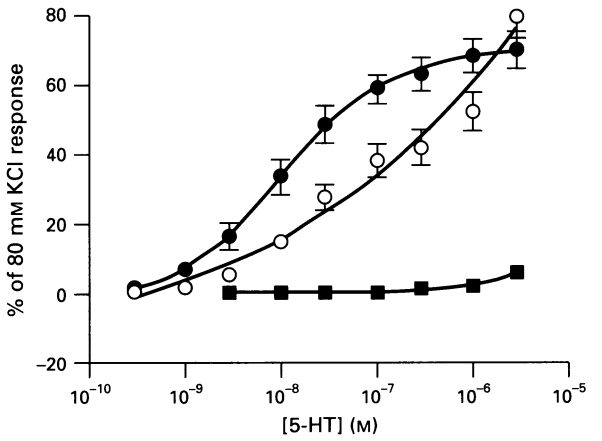

Figure 7 The effect of 5-HT in the presence of 1 μM sodium nitroprusside (■), 0.45 nM angiotensin II (●) and a combination of 1 μM sodium nitroprusside and 0.1–0.45 nM angiotensin II (○) in the rabbit isolated femoral artery (treated with 0.5 mM pargyline and 0.1 μM phenoxybenzamine and maintained in the presence of 0.1 μM ketanserin and 0.3 μM prazosin). Responses in the presence of angiotensin II alone do not include the preconstrictor tone. All responses to 5-HT have been expressed as a percentage of contraction produced by 80 mM KCl and are shown as the mean ± s.e.mean of 7 observations.

Discussion

Previous studies on the rabbit isolated femoral artery have demonstrated that 5-HT produced small, concentration-dependent contractions in the presence of a 5-HT$_2$ receptor antagonist, which were potentiated and augmented by submaximal concentrations of the thromboxane A$_2$-mimetic, U46619 (MacLennan & Martin, 1992). In the present study we have shown that a similar synergistic interaction also occurs between submaximal concentrations of angiotensin II and 5-HT. The potential physiological significance of this interaction is underlined by the evidence that angiotensin II is produced locally within the vasculature (Griendling *et al.*, 1993).

Two observations indicate that the 5-HT receptor implicated in the enhancement of vascoconstrictor responses belongs to the 5-HT$_1$-like subtype. Firstly, 5-HT$_2$ receptors were irreversibly inactivated by prior exposure to phenoxybenzamine and the selective 5-HT$_{2A}$ antagonist ketanserin (0.1 μM) was included in the medium. Secondly, the 5-HT$_1$-like receptor antagonist, metergoline (Martin, 1994) was a potent inhibitor of responses to 5-HT; an effect produced without alteration of the underlying responses to angiotension II. The pA$_2$ for metergoline (8.1) is approximately 10 fold higher than that reported against 5-HT$_1$-like contractions in human isolated pial arteries (6.8; Hamel & Bouchard, 1991) and human isolated saphenous vein (7.3; Bax *et al.*, 1992), but this value may have been overestimated as inhibition by metergoline was associated with a reduction in the maximum response to 5-HT (see Figure 2). Qualitatively similar effects with high concentrations of metergoline have been reported against contractions of the guinea-pig isolated iliac artery produced by the 5-HT$_1$-like selective agonist, sumatriptan (Pertz, 1993).

5-HT produced a significant reduction in the basal levels of cyclic AMP in the rabbit isolated femoral artery, which may indicate that vasconstrictor 5-HT$_1$-like receptors are negatively coupled to adenylyl cyclase activity. Further support for this view is provided by the observation that sumatriptan, a selective agonist for 5-HT$_1$-like receptors (Martin, 1994; Hoyer *et al.*, 1994), produced a similar effect in this preparation (Randall *et al.*, 1994). These findings are comparable to those reported in the dog isolated saphenous vein, where activation of vasoconstrictor 5-HT$_1$-like receptors was associated with a reduction in basal cyclic AMP formation (Sumner *et al.*, 1992). In contrast to 5-HT, neither the direct vasoconstrictor nor the synergistic action of angiotensin II in the rabbit isolated femoral artery appears to involve direct modulation of cyclic AMP metabolism. This is consistent with the known ability of angiotensin II to stimulate phospholipase C activity and enhance phosphoinositide and diacylglycerol production (Griendling *et al.*, 1993), and suggests that angiotensin receptors on the rabbit isolated femoral artery are not negatively coupled to adenylyl cyclase.

The diterpene derivative, forskolin, is a direct activator of adenylyl cyclase (Seamon & Daly, 1986) capable of inhibiting both KCl-induced contractions (Hwang & van Breeman, 1987; Yamagishi *et al.*, 1994) and noradrenaline-induced contractions of vascular smooth muscle (Lincoln & Fisher-Simpson, 1983; McMahon & Paul, 1986; Abe & Karaki, 1989). Abe & Karaki (1989) and McDaniel *et al.* (1991) have shown that forskolin acts at several sites in the excitation-contraction pathway, with low concentrations selectively reducing agonist-induced elevation of intracellular Ca^{2+}, perhaps by increasing Ca^{2+} uptake into the sarcoplasmic reticulum (Ito *et al.*, 1993), while higher concentrations reduce the sensitivity of the contractile proteins to Ca^{2+} ions. In the present study, forskolin produced a concentration-dependent inhibition of responses to 5-HT (in the absence of angiotensin II) and abolished submaximal response to angiotensin II. In the case of 1 μM forskolin, these effects were associated with a 7 fold elevation of cellular cyclic AMP which was not significantly attenuated by 5-HT. The latter observation is an unexpected finding particularly as we have shown that 5-HT can significantly reduce both basal (present study) and 30 μM forskolin-stimulated (100 fold increase; Randall *et al.*, 1994) levels of cyclic AMP in this preparation. At present we have no satisfactory explanation for the failure of 5-HT to reduce modest elevations of cellular cyclic AMP, but it is noteworthy that we have made qualitatively similar observations in another preparation possessing vasoconstrictor 5-HT$_1$-like receptors, the rabbit isolated saphenous vein (unpublished observations). Taken together, our findings clearly indicate that contractile responses to 5-HT in the presence of (1 μM) forskolin are mediated by a cyclic-AMP-independent pathway, rather than by direct modulation of cellular cyclic AMP.

The ability of vasoconstrictor agents to 'bypass' the effects of a modest elevation of cellular cyclic AMP is underlined by experiments involving a combination of angiotensin II and 5-HT in the presence of (1 μM) forskolin. In this instance, the synergistic interaction between these vasoconstrictor agents was still evident in spite of a 7 fold elevation of cellular cyclic AMP (Figure 7) and the abolition of angiotensin II-induced tone. It seems likely, therefore, that the functional synergy described in the rabbit isolated femoral artery involves a cyclic AMP-independent mechanisms, possibly exerted at the level of the contractile proteins. Significantly, angiotensin II has been shown to elevate diacylglycerol levels in cultured vascular smooth muscle cells (Griendling *et al.*, 1986; Lassegue *et al.*, 1993) which, through subsequent activation of protein kinase C (Andrea & Walsh, 1992), has the potential to reduce the threshold sensitivity of contractile proteins for calcium ions. This proposal would also account for two other observations made in this study. First, the ability of high(er) concentrations of forskolin, which have the potential to reduce directly the sensitivity to the contractile proteins to Ca^{2+} (Abe & Karaki, 1989; Yamagishi *et al.*, 1994), to overcome the synergy produced by 5-HT and angiotensin II (see Figure 4). Secondly the finding that the combination of angiotensin II and 5-HT could also overcome the inhibitory effect of sodium nitroprusside, a direct activator of guanylyl cylcase (Lincoln & Fisher-Simpson, 1983). It is noteworthy that a qualitatively similar interaction between 5-HT$_1$-like receptors, sodium nitroprusside and thromboxane receptors has recently been reported in the canine saphenous vein (Kemp & Cocks, 1995). Clearly, elucidation of the cyclic-AMP-independent mechanism underlying synergy between 5-HT$_1$-like receptors and angiotensin II receptors in the rabbit femoral artery is warranted, and this will require simultaneous measurement of intracellular Ca^{2+} and force generation in this vessel.

The finding that vasoconstrictor tone *per se* is not an obligatory requirement for functional synergy between angiotensin II and 5-HT, has implications for other receptor systems where this phenomenon has been observed. Dunn and co-workers (1991a,b) demonstrated that high (30 nM) concentrations of angiotensin II uncovered vasoconstrictor α_2-adrenoceptors in the rabbit isolated saphenous artery which were responsive to both exogenous and endogenous noradrenaline. In these studies the response to angiotensin II was not sustained but subject to desensitization, such that immediately prior to activation of postjunctional α_2-adrenoceptors no vascoconstrictor tone was present. Clearly, if angiotensin II increased the sensitivity of the contractile proteins to Ca^{2+} even in 'desensitized' preparations, subsequent activation of previously quiescent receptors might be sufficient to elicit a contraction. It is noteworthy that angiotensin II-induced enhancement of α_2-adrenoceptor-mediated contractions in the rat isolated tail artery is associated with enhancement of myosin light chain phosphorylation (Triggle *et al.*, 1995). This effect was observed with concentrations of angiotensin II that failed to induce either tone or increase intracellular Ca^{2+} ions.

In conclusion, the data presented indicate that 5-HT can reduced basal levels of cyclic AMP formation in the rabbit isolated femoral artery by activating 5-HT$_1$-like receptors, and that elevation of the intracellular cyclic AMP by forskolin is capable of impairing the associated contractions. The in-

hibitory effect of forskolin was prevented by the inclusion of angiotensin, which alone augmented the 5-HT$_1$-like receptor-induced contractile response, but this effect did not appear to involve an associated reduction in cyclic AMP levels. It seems likely, therefore, that the direct vasoconstriction by 5-HT$_1$-like receptors, and also synergistic interactions with other receptor systems, proceed via a cyclic-AMP independent pathway. In addition, our findings underline the value of complementary contraction-based and biochemical experiments being conducted in the same tissue to provide insights into vasoconstrictor mechanisms.

V.A.R. was supported by a BBSRC/CASE award with The Wellcome Research Laboratories, Beckenham, Kent. The authors would like to thank Richard Wilson for his technical assistance and Dr W.R. Dunn for his advice and comments.

References

ABE, A. & KARAKI, H. (1989). Effect of forskolin on cytosolic Ca^{2+} level and contraction in vascular smooth muscle. *J. Pharmacol. Exp. Ther.*, **249,** 895–900.

ANDREA, J.E. & WALSH, M.P. (1992). Protein kinase C of smooth muscle. *Hypertension*, **20,** 585–595.

APPERLEY, E., HUMPHREY, P.P.A. & LEVY, G.P. (1976). Receptors for 5-hydroxytryptamine and noradrenaline in the rabbit isolated ear artery and aorta. *Br. J. Pharmacol.*, **58,** 211–221.

BAX, W., VAN HEUVEN-NOLSEN, D., BOS, E., SIMOONS, M.L. & SAXENA, P. (1992). 5-Hydroxytryptamine-induced contractions of the human isolated saphenous vein: involvement of 5-HT$_2$- and 5-HT$_{1D}$-like receptors, and a comparison with grafted veins. *Naunyn Schmied. Arc. Pharmacol.*, **345,** 500–508.

CHOPPIN, A. & O'CONNOR, S.E. (1993). Pre-contraction with histamine and U46619 unmasks a 5-HT$_1$-like receptor in rabbit renal artery. *Eur. J. Pharmacol.*, **231,** 469–472.

COCKS, T.M. & ANGUS, J.W. (1983). Endothelium-dependent relaxation of coronary arteries by noradrenaline and serotonin. *Nature*, **305,** 627–630.

COCKS, T.M., KEMP, B.K., PRUNEAU, D. & ANGUS, J.A. (1993). Comparison of contractile responses to 5-hydroxytryptamine and sumatriptan in human isolated coronary artery: synergy with the thromboxane A$_2$-receptor agonist, U46619. *Br. J. Pharmacol.*, **110,** 360–368.

CRAWFORD, K., FREY, E.A. & COTE, T.E. (1992). Angiotensin II receptor recognized by DuP 753 regulates two distinct guanine nucleotide-binding protein signalling pathways. *Mol. Pharmacol.*, **41,** 154–162.

DE LA LANDE, I.S. (1992). Evidence for a 5-HT$_1$-like receptor mediating the amplifying action of 5-HT in the rabbit ear artery. *Br. J. Pharmacol.*, **106,** 550–555.

DUNN, W.R., MCGRATH, J.C. & WILSON, V.G. (1991a). Influence of angiotensin II on the alpha-adrenoceptors involved in mediating the reponse to sympathetic nerve stimluation in rabbit isolated saphenous artery. *Br. J. Pharmacol.*, **102,** 10–12.

DUNN, W.R., MCGRATH, J.C. & WILSON, V.G. (1991b). Postjunctional alpha-adrenoceptors in the rabbit isolated distal saphenous artery: indirect sensitivity to prazosin of responses to noradrenaline mediated via postjunctional alpha$_2$-adrenoceptors. *Br. J. Pharmacol.*, **103,** 1484–1492.

EBERSOLE, B., DIGLIO, C.A., KAUFMAN, D.A. & BERG, K.A. (1993). Hydroxytryptamine-like receptors linked to increases in intracellular calcium concentration and inhibition of cyclic AMP accumulation in cultured vascular smooth muscle cells derived from bovine basilar artery. *J. Pharmacol. Exp. Ther.*, **266,** 692–699.

FENIUK, W., HUMPHREY, P.P.A., PERREN, M.J. & WATTS, A.D. (1985). A comparison of 5-hydroxytryptamine receptors mediating contraction in rabbit aorta and dog saphenous vein: evidence for different receptor types obtained by use of selective agonists and antagonists. *Br. J. Pharmacol.*, **86,** 697–704.

FURCHGOTT, R.F. (1972). The classification of adrenoceptors (adrenergic receptors). An evaluation from the standpoint of receptor theory. In *Handbood of Experimental Pharmacology*, Vol 33, *Catecholamines*, ed. Blaschko, H. & Muscholl, E. pp. 283–335. Berlin: Springer-Verlag.

GRIENDLING, K.K., MURPHY, T.J. & ALEXANDER, W.R. (1993). Molecular biology of the renin-angiotensin system. *Circulation*, **87,** 1816–1828.

GRIENDLING, K.K., RITTENHOUSE, S.E., BROCK, T.A., EKSTEIN, L.S., GIMBRONE, M.A. & ALEXANDER, R.W. (1986). Sustained diacylglycerol formation from inositol phospholipids in angiotensin II-stimulated vascular smooth muscle cells. *J. Biol. Chem.*, **261,** 5901–5906.

GLUSA, E. (1991). Evidence for 5-HT$_{1C}$ receptor-mediated endothelium-dependent relaxations of porcine pulmonary arteries *in vitro*. In *5-Hydroxytryptamine Mechanisms in Primary Headache*. pp. 168–172. ed. Olesen, J. & Saxena, P.R. New York: Raven Press.

HAMEL, E. & BOUCHARD, D. (1991). Contractile 5-HT$_1$-like receptors in human isolated pial arterioles: correlation with 5-HT$_{1D}$ binding sites. *Br. J. Pharmacol.*, **102,** 227–233.

HOYER, D., CLARKE, D.E., FOZARD, J.R., HARTIG, P.R., MARTIN, G.R., MYLECHARANE, E.J., SAXENA, P.R. & HUMPHREYS, P.A. (1994). International Union of Pharmacology classification of receptors for 5-hydroxytyrptamine (serotonin). *Pharmacol. Rev.*, **46,** 157–203.

HWANG, K.S. & VAN BREEMAN, C. (1987). Effect of dibutyryl-cyclic AMP and forskolin on the $^{45}Ca^{2+}$ influx, net Ca uptake and tension in rabbit aortic smooth muscle. *Eur. J. Pharmacol.*, **134,** 155–162.

ITO, S., SUZUKI, S. & ITOH, T. (1993). Effects of a water-soluble forskolin derivative (NKH477) and a membrane permeable cyclic AMP analogue on noradrenaline-induced Ca^{2+} mobilization in smooth muscle of rat mesenteric artery. *Br. J. Pharmacol.*, **110,** 1117–1126.

KEMP, B.K. & COCKS, T.M. (1995). Effects of U46619 on contractions to 5-HT and sumatriptan and methysergide in canine coronary artery and saphenous vein *in vitro*. *Br. J. Pharmacol.*, **116,** 2183–2191.

LASSEGUE, B., ALEXANDER, W., CLARK, M., AKERS, M. & GRIENDLING, K. (1993). Phospatidylcholine is a major source of phosphatidic acid and diacylglycerol in angiotensin II-stimulated vascular smooth muscle. *Biochem. J.*, **292,** 509–517.

LINCOLN, T.M. & FISHER-SIMPSON, V. (1983). A comparison of the effects of forskolin and sodium nitroprusside on cyclic nucleotides and relaxation in the rat aorta. *Eur. J. Pharmacol.*, **101,** 17–27.

MACLEAN, M.R., CLAYTON, R.A., HILLS, S.W., MCINTYRE, P.D., PEACOCKS, A.J., TEMPLETON, A.G.B. (1994). 5-HT$_1$-receptor-mediated vasoconstriction in bovine isolated pulmonary arteries: Influences of vascular endothelium and tone. *Pulm. Pharmacol.*, **7,** 65–72.

MACLENNAN, S.J. & MARTIN, G.R. (1992). Effect of the thromboxane A$_2$-mimetic U46619 on 5-HT$_1$-like and 5-HT$_2$ receptor-mediated contraction of the rabbit isolated femoral artery. *Br. J. Pharmacol.*, **107,** 418–421.

MARTIN, G.R. (1994). Vascular receptors for 5-hydroxytryptamine: distribution, function and classification. *Pharmacol. Ther.*, **62,** 283–324.

MARTIN, G.R., LEFF, P., MACLENNAN, S.J. & DOUGALL, I. (1988). Three types of 5-HT$_1$-like receptor recognised by tryptamine affinity and efficacy 'fingerprints'. *Br. J. Pharmacol.*, **95,** 626P.

MCDANIEL, N.L., REMBOLD, C., RICHARD, H.M. & MURPHY, R.A. (1991). Cyclic AMP relaxes swine smooth muscle predominately by decreasing cell Ca^{2+} concentrations. *J. Physiol.*, **439,** 147–160.

MCMAHON, E.G. & PAUL, R.J. (1986). Effects of forskolin and cyclic nucleotides on isometric force in rat aorta. *Am. J. Physiol.*, **250,** C468–C473.

MULLER, M.J. & BAER, H.P. (1983). Relaxant effects of forskolin in smooth muscle. Role of cyclic AMP. *Naunyn-Schmied. Arch. Pharmacol.*, **332,** 78–82.

PERTZ, H. (1993). 5-Hydroxytryptamine (5-HT) contracts the guinea-pig isolated iliac artery via 5-HT$_1$-like and 5-HT$_2$ receptor. *Naunyn-Schmied. Arch. Pharmacol.*, **348,** 558–565.

RANDALL, V.A., MACLENNAN, S.J. & WILSON, V.G. (1994). 5-HT$_1$-like receptors in rabbit femoral artery and saphenous vein: observations from biochemical studies. *Br. J. Pharmacol.*, **113,** 141P.

SAHIN-ERDEMLI, I., HOYER, D., STOLL, A., SEILER, M.P. & SCHOEFFTER, P. (1991). 5-HT$_1$-like receptors mediate 5-hydroxytryptamine-induced contraction of guinea-pig isolated iliac artery. *Br. J. Pharmacol.*, **102,** 386–390.

SEAMON, K.B. & DALY, J.W. (1986). Forskolin, its biological and chemical properties. *Adv. Cyc. Nucleotide Protein Phosphorylation Res.*, **20,** 1–150.

SUMNER, M.J. & HUMPHREY, P.P.A. (1990). Sumatriptan (GR 43175) inhibits cyclic AMP accumulation in dog isolated saphenous vein. *Br. J. Pharmacol.*, **99,** 219–220.

SUMNER, M.J., FENUIK, W., MCCORMICK, J.D. & HUMPHREY, P.P.A. (1992). Studies on the mechanism of 5-HT$_1$ receptor-induced smooth muscle contraction in dog saphenous vein. *Br. J. Pharmacol.*, **105,** 603–608.

SWEENEY, G., TEMPLETON, A., CLAYTON, R.A., BAIRD, M., SHERIDAN, S., JOHNSTON, E.D. & MACLEAN, M.R. (1995). Contractile responses to sumatriptan in isolated bovine pulmonary artery rings: relationship to tone and cyclic nucleotide levels. *J. Cardiovasc. Pharmacol.*, **26,** 751–760.

TEMPLETON, A.G.B., MCGRATH, J.C. & WHITTLE, M.J. (1991). The role of endogenous thromboxane in contractions to U46619, oxygen, 5-HT and 5-CT in the human isolated umbilical artery. *Br. J. Pharmacol.*, **103,** 1079–1084.

TRIGGLE, C.R., ALLEN, B.G., WALSH, M.P. & LI, X.-F. (1995). Contractile subthreshold concentrations of angiotensin II elevate basal levels of myosine light chain phosphorylation and selectively enhance alpha$_2$-adrenoceptors-mediated responses. *Hypertension*, **25,** 282P.

VAN NEUTEN, J.M., JANSSEN, P.A.J., VAN BEEK, J., XHONNEUX, R., VERBEUREN, T.J. & VANHOUTTE, P.M. (1981). Vascular effects of ketanserin (R 41 468), a novel antagonist of 5-HT$_2$ serotonergic receptors. *J. Pharmacol. Exp. Ther.*, **218,** 217–230.

YAMAGISHI, T., YANAGISAWA, T., SATOH, K. & TAIRA, N. (1994). Relaxant mechanisms of cyclic AMP-increasing agents in porcine coronary artery. *Eur. J. Pharmacol.*, **251,** 253–262.

(Received November 24, 1995
Revised January 25, 1996
Accepted February 16, 1996)