Effects of cyclic AMP and analogues on neurogenic transmission in the rat tail artery

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1 The effects of two 8-substituted analogues of adenosine 3':5'-cyclic monophosphate (cyclic AMP) were compared with those of forskolin and isoprenaline on [³H]-noradrenaline release and vasoconstriction induced by electrical field stimulation (24 pulses at 0.4 Hz, 200 mA, 0.3 ms duration) in the rat tail artery, in the absence and in the presence of protein kinase inhibitors.

2 8-Bromo-adenosine 3':5'-cyclic monophosphate (8-bromo-cyclic AMP, 10–300 µM), 8-(4-chlorophenylthio)-adenosine 3':5' cyclic monophosphate (8-pCPT-cyclic AMP, 3–300 µM), forskolin (0.3–10 µM) and isoprenaline (1 nM–1 µM) all concentration-dependently enhanced stimulation-induced [³H]-noradrenaline release. The effect of cyclic AMP analogues was larger (2.5 fold at 300 µM) than those of cyclic AMP elevating drugs (1.6 fold at 10 µM for forskolin and 1.5 fold at 30 nM for isoprenaline).

3 At concentrations active at the prejunctional level, the four drugs had differential effects on stimulation-induced vasoconstriction, which was enhanced by the two cyclic AMP analogues, decreased by forskolin and not significantly altered by isoprenaline.

4 The [³H]-noradrenaline release-enhancing effects of 8-bromo-cyclic AMP, forskolin and isoprenaline were significantly decreased by the cyclic AMP-dependent protein kinase (PKA) inhibitor {N-[2-((3-(4-bromophenyl)-2-propenyl)-amino)-ethyl]-5-isoquinolinesulphonamide, di-hydrochloride} (H-89; 100 nM). By contrast they were unaffected by the cyclic GMP-dependent protein kinase (PKG) inhibitor, 8-bromo-guanosine 3':5'-cyclic monophosphorothioate, Rp-isomer (Rp-8-bromo-cyclic GMPS; 10 µM). At the same concentrations the PKA inhibitor attenuated only the nerve-induced vasoconstrictor responses obtained in the presence of 8-bromo-cyclic AMP, whereas the PKG inhibitor did not modify that obtained in the presence of 8-bromo-cyclic AMP or forskolin.

5 Exposure to the protein kinase C (PKC) activator, phorbol 12-myristate 13-acetate (1 µM) enhanced nerve-evoked [³H]-noradrenaline release, and this effect was decreased by the PKC inhibitor, 2-[1-(3-dimethylaminopropyl)-indol-3-yl]-3-(indol-3-yl)-maleimide (GF 109203X; 100 nM). However, the latter drug did not modify the enhancing effect of 8-bromo-cyclic AMP on [³H]-noradrenaline release.

6 It is concluded that activation of cyclic AMP-dependent protein kinase is involved in the enhancing effect of cyclic AMP-elevating compounds on prejunctional release of noradrenaline. In addition the results provide no clear-cut evidence for a vasodilator role of PKA.

Keywords: Neurogenic vasoconstriction; noradrenaline release; cyclic AMP; forskolin; isoprenaline; cyclic AMP-dependent protein kinase; rat tail artery

Introduction

Adenosine 3':5'-cyclic monophosphate (cyclic AMP) is an ubiquitous second messenger which is involved in the mediation of functional responses to a variety of hormones and neurotransmitters (reviewed by Beebe & Corbin, 1986). In vascular smooth muscle cells, β -adrenoceptor stimulation or forskolin-induced activation by adenylyl cyclase are associated with an increase in the intracellular level of cyclic AMP (Kramer & Hardman, 1980) and with relaxation, but the precise mechanism by which the cyclic nucleotide causes relaxation is not clear. Cyclic AMP is also operative in perivascular sympathetic nerve endings where it modulates neurotransmitter release. In support of this, we and others have reported that drugs which increase intracellular level of cyclic AMP facilitate electrically-induced noradrenaline release (Göthert & Hentrich, 1984; Hentrich *et al.*, 1985; Johnston & Majewski, 1986; Costa & Majewski, 1988; Bucher *et al.*, 1990). However, although cyclic AMP is able to modulate noradrenaline release, it does not appear to play a role in the initiation of the release process itself (Bucher *et al.*, 1990).

Until recently, all known actions of cyclic AMP or its derivatives were assumed to operate via activation of cyclic AMP-dependent protein kinase (PKA) (reviewed by Beebe & Corbin, 1986). However, several recent studies addressed the

question whether cyclic AMP activation of PKA is the sole mechanism of cyclic AMP-dependent relaxation in smooth muscle. It has been shown by use of dibutyryl cyclic AMP and 8-bromo-cyclic AMP that a dissociation is observed between PKA activation and inhibition of contractions of rat vas deferens, suggesting that PKA is not responsible for the relaxant effects of these cyclic nucleotide analogues in this tissue (Hei *et al.*, 1991). Other investigators reported that activation of the cyclic GMP-dependent protein kinase (PKG) rather than PKA was responsible for smooth muscle relaxation elicited by cyclic nucleotide analogues (Francis *et al.*, 1988). In addition, recent data support the view that activation of PKG by endogenously formed cyclic AMP may lead to a reduction of intracellular calcium and subsequent relaxation (Lincoln *et al.*, 1990; Lincoln & Cornwell, 1991). Interestingly, there is also evidence for the existence of other mechanisms involving a non-ATP-sensitive K⁺ channel and possibly PKG in the mediation of smooth muscle relaxation elicited by cyclic AMP (Haynes *et al.*, 1992).

The possible role for activation of either cyclic AMP-dependent protein or cyclic GMP-dependent protein kinases in the regulation of both adrenergic neurotransmitter release and pressor responses has not been documented as yet in a vascular preparation. Therefore, the present study was designed to examine further the effects of cyclic AMP on vasoconstriction and noradrenaline release evoked by electrical field

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stimulation of the rat tail artery. Specifically, we investigated the influence of selective cyclic AMP- and cyclic GMP-dependent protein kinase inhibitors on cyclic AMP-mediated effects.

Methods

Measurement of [^3H]-noradrenaline release and vasoconstriction

Male Wistar rats (12 weeks old) were killed by cervical dislocation and exsanguinated. A segment of about 2–2.5 cm of the proximal part of the ventral tail artery was dissected out as previously described (Illes *et al.*, 1987; Bucher *et al.*, 1992) and kept in oxygenated (95% O_2 ; 5% CO_2) physiological saline solution which contained (mM): NaCl 118, KCl 4.8, CaCl_2 2.5, KH_2PO_4 0.9, NaHCO_3 25, glucose 11, ascorbic acid 0.3 and disodium EDTA 0.03. The two latter compounds were added to prevent the spontaneous oxidation of catecholamines. The arteries were cannulated at one end and preincubated for 1 h at 37°C in 1.5 ml of medium of the same composition as above containing in addition 2.2 μM (–)-[^3H]-noradrenaline (specific activity 4.4 Ci mmol $^{-1}$). The vessels were then washed three times with 20 ml of [^3H]-noradrenaline-free medium, and suspended vertically, distal end uppermost, between two platinum wire electrodes. The gaps between the arteries and the electrodes were wide enough to allow undisturbed contraction or relaxation, yet sufficiently narrow to stimulate intramural nerve terminals effectively. The arteries were perfused via their proximal ends with medium by means of a roller pump with physiological saline solution containing 10 μM cocaine in order to block the re-uptake of released [^3H]-noradrenaline. The perfusion rate was gradually increased from 0 to 2.2 ml min $^{-1}$ during the first 10 min after suspension of the arterial segment between the two electrodes, and kept constant thereafter. The intraluminal perfusion pressure was determined with a pressure transducer and recorded on a pen recorder. Changes in perfusion pressure reflected changes in the resistance to flow, i.e. the degree of vasoconstriction. After having passed through the lumen, the perfusate was allowed to superfuse the adventitial surface of the vessel.

Experiments were done with arteries with functional endothelium unless otherwise stated. We have previously shown that electrical field stimulation did not produce endothelial damage (Bucher *et al.*, 1992). In some arteries the endothelium was removed by passing a stream of 95% O_2 :5% CO_2 through the lumen of the arterial segment at a pressure of 40–50 mmHg. The presence or absence of functional endothelium was verified by the presence or absence of relaxant responses to 10 μM acetylcholine in noradrenaline (1 μM) precontracted arteries (Bucher *et al.*, 1992).

Each artery was subjected to six stimulation periods (24 pulses at 0.4 Hz; 0.3 ms; 200 mA). The first stimulation period was applied after 96 min of perfusion and others followed at intervals of 16 min. Collection of the perfusate/superfusate started after 124 min of perfusion in 1, 2 or 6 min fractions. The stimulation period beginning at 128 min was termed S_1 and subsequent ones S_2 – S_n . S_2 served as a control of stimulation-evoked tritium overflow (since the response was stable thereafter). The compounds were infused with a syringe pump into the perfusion stream at a rate of 17 μl min $^{-1}$ for 8 min before either S_1 or S_3 and this was maintained until the end of the experiment when the arteries were solubilized in 1 ml Soluene 100 (Packard Instrument, Paris, France). Tritium in the superfusate samples and arteries was measured by liquid scintillation spectrometry.

Tritium overflow was calculated as a fraction of the amount of tritium present in the tissue at the start of the respective collection period (fractional rate of outflow). For evaluation of stimulation-evoked tritium overflow, the difference between the overall tritium outflow during stimulation

plus the following 4 min and the estimated basal outflow was calculated. The latter was assumed to change linearly from the 1 min collection period before the beginning of stimulation to that 5 to 6 min after the start of stimulation. The evoked tritium overflow was calculated as a percentage of the amount of tissue tritium at the beginning of the respective stimulation period.

Data and statistical analysis

In order to quantify drug effects on the evoked overflow or vasoconstriction, the ratio of the overflow or vasoconstriction evoked by the stimulation period in the presence of the drug at the fourth stimulation period (S_4) over the last evoked overflow or vasoconstriction preceding application of the drug (S_2) was determined. S_4 was chosen because in preliminary experiments the maximal effect of each drug concentration was reached in these conditions. Drug effects on basal outflow were quantified in the following way: ratios of the fractional rate of outflow (b_n) in the fraction collected immediately before the onset of the stimulation periods in the presence of drugs (S_3 – S_4) over the fractional rate of outflow in the fraction immediately before the onset of the last stimulation period preceding the application of any drug (S_2) were calculated (b_n/b_2).

Results are given as mean $\pm$ s.e.mean where n is the number of experiments. Comparisons were made by the Mann-Whitney test if the Kruskal-Wallis analysis indicated a significant difference between multiple groups. A probability level of 0.05 or less was considered significant. For multiple comparisons with the same control group, the limit of significance was divided by the number of comparisons according to Bonferroni (Wallenstein *et al.*, 1980).

Drugs

The following compounds were used: (–)-noradrenaline hydrochloride, 8-bromo-adenosine 3':5'-cyclic monophosphate (sodium salt; 8-bromo-cyclic AMP), 8-(4-chlorophenylthio)-adenosine 3':5'-cyclic monophosphate (sodium salt; 8-pCPT-cyclic AMP), ($\pm$)-isoprenaline hydrochloride, forskolin, phorbol 12-myristate 13-acetate (PMA) (Sigma, L'Isle d'Abbeau Chesnes, France); 8-bromo-guanosine 3':5'-cyclic monophosphorothioate, Rp-isomer (sodium salt; Rp-8-bromo-cyclic GMPS; Biolog, Bremen, Germany); {N-[2-((3-(4-bromophenyl)-2-propenyl)-amino)-ethyl]-5-isoquinolinesulphonamide, di-hydrochloride} (H-89), (Calbiochem, Meudon, France); 2-[1-(3-dimethylaminopropyl)-indol-3-yl]-3-(indol-3-yl)maleimide (GF 109203X; kindly donated by Glaxo, France). Stock solutions of all other substances were prepared with Milli-Q water (Millipore) and diluted as required with the exception of forskolin which was dissolved in 5% ethanol and of PMA and GF109203X which were dissolved in 10% dimethylsulphoxide (DMSO). Preliminary experiments showed that at the concentration used, ethanol and DMSO did not alter basal or electrically-evoked overflow of tritium or vasoconstriction (see also control values in the figures and table). (–)-[Ring-2,5,6- ^3H]-noradrenaline, specific activity 43.7–56.9 Ci mmol $^{-1}$ (New England Nuclear, Dreieich, Germany) was diluted with unlabelled (–)-noradrenaline hydrochloride in order to obtain a specific activity of 4.4 Ci mmol $^{-1}$.

Results

Effects of 8-substituted analogues of cyclic AMP, forskolin and isoprenaline

In control as well as in treated arteries, tritium overflow and peak vasoconstriction evoked by the stimulation period S_2 amounted respectively to $0.191 \pm 0.005\%$ of tissue tritium and 100.7 ± 2.4 mmHg ($n = 158$; all appropriate experiments

pooled). Under control conditions, stimulation-evoked overflow of tritium and vasoconstriction did not change upon subsequent stimulations, resulting thus in S_4/S_2 ratio which were not different from the unity but the fractional rate of basal tritium outflow declined with time (not shown). 8-Bromo-cyclic AMP (300 μM), increased the basal rate of tritium outflow compared to control experiments (b_4/b_2 : 1.07 ± 0.02 ; $n = 15$ and 0.98 ± 0.02 ; $n = 18$, respectively; $P < 0.01$). Infusion of the solvent or of the drugs did not cause any change in basal perfusion pressure (not shown).

8-Bromo-cyclic AMP (10–300 μM), 8-pCPT-cyclic AMP (3–300 μM), isoprenaline (0.001–1 μM) and forskolin (0.3–10 μM) produced concentration-dependent increases in electrically-evoked tritium overflow (Figures 1 and 2). However, although forskolin and isoprenaline were more potent than the 8-substituted cyclic AMP analogues, the maximal effect obtained with either 10 μM forskolin (S_4/S_2 : 1.62 ± 0.06 ; $n = 8$; $P < 0.01$ vs control) or isoprenaline (S_4/S_2 : 1.54 ± 0.03 ; $n = 6$; $P < 0.05$ vs control) was significantly lower ($P < 0.01$) than the maximal effect obtained with either 300 μM 8-bromo-cyclic AMP (S_4/S_2 : 2.31 ± 0.07 ; $n = 15$; $P < 0.01$ vs control) or 300 μM 8-pCPT-cyclic AMP (S_4/S_2 : 2.38 ± 0.17 ; $n = 5$; $P < 0.05$ vs control). In addition, when isoprenaline (30 nM) was added in combination with propranolol (30 nM), the facilitatory effect of the β -adrenoceptor agonist on the electrically-evoked release of tritium was significantly reduced ($P < 0.01$), the respective S_4/S_2 ratios in the absence and

presence of the β -antagonist being 1.54 ± 0.03 ; $n = 6$ and 1.17 ± 0.03 ; $n = 6$, respectively.

The stimulation-induced vasoconstriction was slightly increased by 8-bromo-cyclic AMP (Figure 1). However, the maximal effect of 8-bromo-cyclic AMP was less pronounced than that observed with 8-pCPT-cyclic AMP which reached its maximum at 30 μM (S_4/S_2 : 1.66 ± 0.05 ; $n = 7$; $P < 0.05$ vs control) and decreased thereafter. In contrast, forskolin (3 μM) induced a significant decrease in the stimulation-induced vasoconstriction (Figure 4) whereas isoprenaline had no effect. The S_4/S_2 ratio for 30 nM isoprenaline was not significantly different from the corresponding control values (1.18 ± 0.07 ; $n = 6$ and 1.07 ± 0.05 ; $n = 6$; respectively; $P > 0.05$).

In arteries without functional endothelium, forskolin (1 μM), produced the same relative enhancement of the tritium overflow (S_4/S_2 : 1.20 ± 0.05 ; $n = 5$) as it did in the presence of functional endothelium (S_4/S_2 : 1.25 ± 0.03 ; $n = 7$). The forskolin-mediated vasodilatation was slightly decreased in arteries without endothelium compared to the response observed in the presence of endothelium (S_4/S_2 : 0.62 ± 0.08 ; $n = 5$ and 0.47 ± 0.05 ; $n = 7$; respectively; $P > 0.05$).

Influence of H-89

The enhancing effect of 8-bromo-cyclic AMP (100 μM) on both electrically-induced vasoconstriction and tritium overflow (S_4/S_2 : 1.42 ± 0.07 and 1.63 ± 0.06 ; $n = 6$; respectively) was not significantly inhibited by H-89 10 nM (S_4/S_2 : 1.39 ± 0.07 and 1.56 ± 0.03 ; $n = 6$; respectively) and 30 nM (S_4/S_2 : 1.38 ± 0.12 and 1.50 ± 0.06 ; $n = 6$; respectively). However, a concentration of 100 nM of the protein kinase A inhibitor maximally inhibited the 8-bromo-cyclic AMP-mediated effects on both electrically-evoked noradrenaline overflow and vasoconstriction (Figure 3). A concentration of 300 nM of

Figure 1 Effects of 8-bromo-cyclic AMP (○, ●) and of 8-pCPT-cyclic AMP (□, ■) on electrically-evoked overflow of tritium (open symbols; a) and change in perfusion pressure (solid symbols; b) in rat tail arteries with functional endothelium preincubated with [^3H]-noradrenaline. Four periods (S_1 – S_4) of field stimulation were delivered at intervals of 16 min (24 pulses at 0.4 Hz, 200 mA, 0.3 ms duration). Each concentration of 8-bromo-cyclic AMP and of 8-pCPT-cyclic AMP or the solvent (control group) was added 8 min before S_3 and maintained in the medium for the duration of the experiment. The effect of the drug is presented as the ratio of tritium overflow or change in perfusion pressure evoked by S_4 over that evoked by S_2 . Each point represents the means $\pm$ s.e. mean from 5–15 arteries. Significant difference from control: * $P < 0.05$, ** $P < 0.01$.

Figure 2 Concentration-dependent effects of isoprenaline (●) and forskolin (○) on electrically-evoked overflow of tritium in rat tail arteries preincubated with [^3H]-noradrenaline. Four periods (S_1 – S_4) of field stimulation were delivered at intervals of 16 min (24 pulses at 0.4 Hz, 200 mA, 0.3 ms duration). Each concentration of isoprenaline or its solvent and forskolin or its solvent was added 8 min before S_3 and maintained in the medium for the duration of the experiment. The effects of the drugs are presented as the ratio of tritium overflow evoked by S_4 over that evoked by S_2 . Each point represents the means $\pm$ s.e. mean from six arteries. Significant difference from control: * $P < 0.05$, ** $P < 0.01$.

H-89 produced a non-specific increase of the basal outflow of tritiated noradrenaline and the stimulation-induced overflow could not be satisfactorily estimated.

Figure 3 Effects of H-89 and 8-bromo-cyclic AMP alone or in combination on electrically-evoked tritium overflow (open columns) and change in perfusion pressure (hatched columns) in rat tail arteries preincubated with [³H]-noradrenaline. Four periods (S₁–S₄) of field stimulation were delivered at intervals of 16 min (24 pulses at 0.4 Hz, 200 mA, 0.3 ms duration). H-89, 8-bromo-cyclic AMP or their solvents (control group) were added 8 min before S₃. All compounds were maintained in the medium for the duration of the experiment. The effect of the drugs are presented as the ratio of tritium overflow or change in perfusion pressure evoked by S₄ over that evoked by S₂. Each column represents the mean with s.e.mean from 6–7 arteries. The statistically significant differences between some values are indicated.

Figure 4 Effects of H-89 and forskolin alone or in combination on electrically-evoked tritium overflow (open columns) and change in perfusion pressure (hatched columns) in rat tail arteries preincubated with [³H]-noradrenaline. Four periods (S₁–S₄) of field stimulation were delivered at intervals of 16 min (24 pulses at 0.4 Hz, 200 mA, 0.3 ms duration). H-89 or its solvent (control group) was added 8 min before S₁. Forskolin or its solvent was added 8 min before S₃. All compounds were maintained in the medium for the duration of the experiment. The effects of the drugs are presented as the ratio of tritium overflow or change in perfusion pressure evoked by S₄ over that evoked by S₂. Each column represents the mean with s.e.mean from 6–8 arteries. The statistically significant differences between some values are indicated.

By itself, 100 nM of the cyclic AMP-dependent protein kinase inhibitor, H-89, added to the perfusion/superfusion medium before S₁ or S₃ did not induce any modification in tritium overflow and vasoconstrictor response to periarterial nerve stimulation (Figures 3, 4 and 5). As shown in Figure 4, the enhancing effect of 3 μM forskolin on electrical field-elicited tritium overflow was significantly decreased in the presence of 0.1 μM H-89 whereas stimulation-induced vasoconstriction was unchanged. Also, H-89 (100 nM) significantly attenuated the facilitatory effect of isoprenaline (30 nM) on tritium overflow without effecting the stimulation-induced vasoconstriction (Figure 5).

Influence of Rp-8-bromo-cyclic GMPS

A stable membrane permeant competitive inhibitor of cyclic GMP-dependent protein kinase, Rp-8-bromo-cyclic GMPS, was used in order to determine whether a PKG is involved in the effects of the cell permeant analogue 8-bromo-cyclic AMP or the adenylyl cyclase activator forskolin. Rp-8-bromo-cyclic GMPS (10 μM) added before S₁ or S₃ had no significant effect by itself or on the electrically-evoked tritium overflow and vasoconstriction (Figures 6 and 7). Furthermore, it did not modify the effects of 8-bromo-cyclic AMP (100 μM) (Figure 6) and forskolin (1 μM) (Figure 7) on the electrically-evoked tritium overflow and vasoconstriction.

Influence of protein kinase C activators and inhibitors

The addition of PMA (1 μM) before S₃ resulted in an increase in both the stimulation-induced overflow of tritium and resulting vasoconstriction (Table 1). A potent protein kinase C inhibitor, GF 109203X (100 nM), produced no significant effect by itself but it significantly attenuated the enhancing effects of PMA (1 μM) on the stimulation-evoked noradrenaline release (Table 1). By contrast, the solvent of both GF 109203X and PMA (10% DMSO) was without effect by

Figure 5 Effects of H-89 and isoprenaline alone or in combination on electrically-evoked tritium overflow (open columns) and change in perfusion pressure (hatched columns) in rat tail arteries preincubated with [³H]-noradrenaline. Four periods (S₁–S₄) of field stimulation were delivered at intervals of 16 min (24 pulses at 0.4 Hz, 200 mA, 0.3 ms duration). H-89 or its solvent (control group) was added 8 min before S₁. Isoprenaline or its solvent was added 8 min before S₃. All compounds were maintained in the medium for the duration of the experiment. The effects of the drugs are presented as the ratio of tritium overflow or change in perfusion pressure evoked by S₄ over that evoked by S₂. Each column represents the mean with s.e.mean from 6 arteries. The statistically significant differences between some values are indicated.

itself. GF 109203X (100 nM) had no effect on the facilitatory effect of 8-bromo-cyclic AMP (100 μ M) on electrical field-evoked tritium overflow and vasoconstriction (Table 1), showing no evidence for the involvement of PKC in the effects of cyclic AMP.

Discussion

The results of the present investigation provide additional evidence supporting the view that cyclic AMP increases the electrically-evoked overflow of [3 H]-noradrenaline from the rat tail artery. In addition, they clearly show that the pre-junctional enhancing effects of cyclic AMP-elevating drugs

and cyclic AMP analogue on electrical-field induced [3 H]-noradrenaline release were reduced by a PKA inhibitor, but were unaffected by either a PKG or a PKC inhibitor.

The cyclic AMP-mediated enhancement of electrically-stimulated neurotransmitter release observed here is in good agreement with previous results obtained in the same vessel (Bucher *et al.*, 1990) and in other vascular preparations (Göthert & Hentrich, 1984; Hentrich *et al.*, 1985; Johnston & Majewski, 1986; Costa & Majewski, 1988). The two adenylyl cyclase stimulating drugs, isoprenaline and forskolin, acted with the potency that could be expected from their effects on cyclic AMP production (Seamon & Daly, 1986). Their maximal effects (1.6 fold stimulation) were not different from each other, but were lower than the effects of high concentra-

Figure 6 Effects of Rp-8-bromo-cyclic GMPS and 8-bromo-cyclic AMP alone or in combination on electrically-evoked tritium overflow (open columns) and change in perfusion pressure (hatched columns) in rat tail arteries preincubated with [3 H]-noradrenaline. Four periods (S_1 – S_4) of field stimulation were delivered at intervals of 16 min (24 pulses at 0.4 Hz, 200 mA, 0.3 ms duration). Rp-8-bromo-cyclic GMPS, 8-bromo-cyclic AMP or their solvents (control group) were added 8 min before S_3 . All compounds were maintained in the medium for the duration of the experiment. The effect of the drugs are presented as the ratio of tritium overflow or change in perfusion pressure evoked by S_4 over that evoked by S_2 . Each column represents the mean with s.e.mean from 4–7 arteries. The statistically significant differences between some values are indicated.

Figure 7 Effects of Rp-8-bromo-cyclic GMPS and forskolin alone or in combination on electrically-evoked tritium overflow (open columns) and change in perfusion pressure (hatched columns) in rat tail arteries preincubated with [3 H]-noradrenaline. Four periods (S_1 – S_4) of field stimulation were delivered at intervals of 16 min (24 pulses at 0.4 Hz, 200 mA, 0.3 ms duration). Rp-8-bromo-cyclic GMPS, or its solvent (control group) was added 8 min before S_3 . Forskolin or its solvent was added 8 min before S_3 . All compounds were maintained in the medium for the duration of the experiment. The effects of the drugs are presented as the ratio of tritium overflow or change in perfusion pressure evoked by S_4 over that evoked by S_2 . Each column represents the mean with s.e.mean from 4–7 arteries. The statistically significant differences between some values are indicated.

Table 1 Effects of phorbol 12-myristate 13-acetate (PMA) and 8-bromo-cyclic AMP alone or in combination with GF 109203X on electrically-evoked tritium overflow and change in perfusion pressure in rat tail arteries

Treatment	n	S_4/S_2 ratio	
		Tritium overflow	Vasoconstriction
H ₂ O	6	1.01 $\pm$ 0.03	1.03 $\pm$ 0.01
DMSO 10%	9	1.02 $\pm$ 0.02	1.06 $\pm$ 0.02
GF 109203X 100 nM	6	1.06 $\pm$ 0.04	1.05 $\pm$ 0.04
PMA 1 μ M	7	1.67 $\pm$ 0.09 ^b	1.23 $\pm$ 0.08 ^a
PMA 1 μ M + GF 109203X 100 nM	6	1.28 $\pm$ 0.02 ^{b,c}	1.17 $\pm$ 0.07 ^a
8-bromo-cyclic AMP 100 μ M	4	1.57 $\pm$ 0.04 ^b	1.31 $\pm$ 0.05 ^b
8-bromo-cyclic AMP 100 μ M + GF 109203X 100 nM	4	1.44 $\pm$ 0.09 ^b	1.15 $\pm$ 0.02 ^a

Rat tail arteries were preincubated with [3 H]-noradrenaline. Four periods (S_1 – S_4) of field stimulation were delivered at intervals of 16 min (24 pulses at 0.4 Hz, 200 mA, 0.3 ms duration). PMA, 8-bromo-cyclic AMP and GF 109203X or their solvents (control group) were added 8 min before S_3 . All compounds were maintained in the medium for the duration of the experiment. The effects of the drugs are presented as the ratio of tritium overflow or change in perfusion pressure evoked by S_4 over that evoked by S_2 . Mean $\pm$ s.e.mean from n arteries.

^a $P < 0.05$; significant differences from drug-free control experiments.

^b $P < 0.01$; significant differences from drug-free control experiments.

^c $P < 0.01$; significant differences from experiments in the absence of GF 109203X.

tions (300 μM) of the two cyclic AMP analogues (2.5 fold stimulation), suggesting that maximal stimulation of adenylyl cyclase by either isoprenaline or forskolin could not produce maximal enhancement of noradrenaline release. As expected from its higher lipophilicity resulting in good membrane permeability (Sandberg *et al.*, 1991), 8-pCPT-cyclic AMP acted at lower concentrations than 8-bromo-cyclic AMP.

In the current study, H-89, a selective PKA inhibitor (Chijiwa *et al.*, 1990) by itself did not alter spontaneous and electrical stimulation-induced release of neurotransmitter. However, it reduced the enhancement of [^3H]-noradrenaline release by forskolin, isoprenaline and 8-bromo-cyclic AMP. Taken together these results indicate that following stimulation by exogenous agents, PKA enhances noradrenaline release, but this pathway appears not to be endogenously active in the absence of such agents. Furthermore, neither Rp-8-bromo-cyclic GMPS, an 8-bromo derivative of Rp-cyclic GMPS, which is a membrane permeant and metabolically stable selective inhibitor of PKG (Butt *et al.*, 1990), nor GF 109203X, a potent and selective inhibitor of PKC (Toullec *et al.*, 1991), had any effect on stimulation-induced noradrenaline release or vasoconstriction. Neither poor membrane permeability nor the use of inadequate concentrations of these drugs could explain their lack of effects: in the same experimental conditions GF 109203X was able to antagonize PMA (this study) and Rp-8-bromo-cyclic GMPS was able to attenuate significantly the 8-bromo-cyclic GMP-induced relaxation (data not shown).

As previously discussed (Bucher *et al.*, 1990), the effects of drugs on vasoconstriction elicited by periarterial nerve stimulation are more difficult to interpret, since they may represent the net result of pre- and post-junctional actions. Although the rat tail artery is endowed with postjunctional β -adrenoceptors (Bao *et al.*, 1990; Rajanayagam *et al.*, 1990), isoprenaline had no significant effect on neurogenic vasoconstriction. Although there was a prejunctional β -adrenoceptor-mediated increase in noradrenaline release from nerve terminals, any postjunctional α -adrenoceptor-mediated response was probably inhibited via the postjunctional β -adrenoceptor-mediated relaxant effect of isoprenaline. In spite of increasing neurotransmitter release, forskolin produced a concentration-dependent vasodilatation, whereas both of the cyclic AMP analogues potentiated nerve-induced vasoconstriction. These differences may reflect differences in the sensitivity of the pre- and postjunctional target systems and/or differences in the ability of the drugs to diffuse into the respective cellular compartments. Additionally, 8-pCPT-cyclic AMP, although increasing stimulation-induced vasoconstriction at concentrations up to 30 μM , decreased it at higher concentrations. It has been shown recently that 8-pCPT-cyclic AMP shows little selectivity for the activation of PKA over PKG (Sandberg *et al.*, 1991) and that it is also a potent inhibitor of the cyclic GMP-specific phosphodiesterase (PDE V_A) (Connolly *et al.*, 1992). This provides further reasons to interpret with caution the biphasic effect on stimulation-induced vasoconstriction observed in the present experiments. It is therefore possible that the observed effects with higher concentrations of this cyclic AMP derivative might be secondary to PDE V_A inhibition which in turn increases the intracellular level of cyclic GMP and induces vasorelaxation. In these conditions, due to the complex pharmacological effects of this derivative and its poor selectivity for PKA the effects of protein kinase inhibitors were only investigated with the 8-bromo-analogue.

The attenuation by H-89 of the enhancing effect of 8-

bromo-cyclic AMP on stimulation-induced vasoconstriction could result from the above discussed inhibition of the pre-junctional effect of the cyclic AMP analogue on noradrenaline release. The findings that, in spite of antagonizing the cyclic AMP-induced increase in neurotransmitter release, H-89 did not modify vasoconstrictor responses obtained in the presence of isoprenaline and forskolin, would imply that both pre- and postjunctional effects of these drugs were decreased by the PKA inhibitor. However, the persistence of a marked depressor effect of forskolin on vasoconstriction, in the presence of H-89, suggests that a mechanism other than PKA activation may be involved. It has previously been shown that a non-cyclic AMP-mediated relaxant effect of forskolin due to a direct effect on K^+ channels (Hoshi *et al.*, 1988; Ertl & Nawrath, 1989) is not likely since 1,9-dideoxy-forskolin was unable to decrease the stimulation-elicited vasoconstriction (Bucher *et al.*, 1990). Other mechanisms, perhaps involving adenylyl cyclase activation or not, cannot be excluded. The results obtained here with forskolin are consistent with recent observations showing that Rp-cyclic AMPS (Rp diastereoisomer of adenosine 3':5'-cyclic monophosphorothioate), a PKA inhibitor had no effect on forskolin-mediated vasodilatation (Haynes *et al.*, 1992). In addition, the present finding showing that the PKG inhibitor, Rp-8-bromo-cyclic GMPS, did not alter forskolin-induced inhibition of the vessel contraction and had no effect on vasoconstriction elicited in the presence of 8-bromo-cyclic AMP do not support a role of PKG in these drugs actions.

It is clear from our previous research that neither the endothelium nor the L-arginine-NO pathway is implicated in the modulation of stimulation-induced noradrenaline release from postganglionic nerve endings in the rat tail artery (Bucher *et al.*, 1992). However, an important question that arises from the present study relates to the possible influence of the endothelium on the cyclic AMP-dependent effects on vasoconstriction. In the present study and in previous work (Bucher *et al.*, 1990) only forskolin produced a clear-cut concentration-dependent decrease in the stimulation-induced vasoconstriction. This inhibitory effect is considered to occur via an increase in cyclic AMP within the smooth muscle cells (Kukovetz *et al.*, 1981). The fact that in the present study endothelium removal failed to alter significantly forskolin-induced relaxation suggests that the vascular effects of the adenylyl cyclase activator occurred independently of the endothelium. A similar conclusion has been reached by others (Kamata *et al.*, 1989; Graves & Poston, 1993).

Reciprocal interactions between the cyclic AMP/PKA pathway and PKC had been suggested in a variety of tissues (Rozengurt *et al.*, 1987; Naghshineh *et al.*, 1989; Gorin *et al.*, 1990; Anderson *et al.*, 1991; Mazancourt *et al.*, 1991). In the rat tail artery, a role for PKC in the prejunctional release of noradrenaline has recently been suggested (Bucher *et al.*, 1991). Consistent with this suggestion, it was found here that PMA enhanced electrical field-induced noradrenaline release. However, inhibition of PKC did not modify the effect of 8-bromo-cyclic AMP at this level, providing no evidence for a role of PKC in cyclic AMP-mediated modulation of neurotransmitter release.

Altogether, the current study shows that, in the rat tail artery, the prejunctional cyclic AMP-mediated facilitation of stimulated noradrenaline release involves the activation of cyclic AMP-dependent protein kinase but provides no substantial evidence for a postjunctional vasodilator role of the kinase.

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