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Involvement of cyclic nucleotide-dependent protein kinases in cyclic AMP-mediated vasorelaxation

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1 The involvement of cyclic AMP-dependent protein kinase (PKA) and cyclic GMP-dependent protein kinase (PKG) in the effects of cyclic AMP-elevating agents on vascular smooth muscle relaxation, cyclic nucleotide dependent-protein kinase activities and ATP-induced calcium signalling ($[Ca^{2+}]_i$) was studied in rat aorta. Cyclic AMP-elevating agents used were a β-adrenoceptor agonist (isoprenaline), a phosphodiesterase 3 (PDE3) inhibitor (SK&F 94120) and a PDE4 inhibitor (rolipram).

2 In rat intact aorta, the relaxant effect induced by isoprenaline (0.01–0.3 μM) was decreased by a specific inhibitor of PKA, H-89, whereas a specific inhibitor of PKG, Rp-8-Br-cyclic GMPS, was without effect. No significant difference in PKA and PKG activity ratios was detected in aortic rings when isoprenaline 10 μM was used. At the same concentration, isoprenaline did not modify ATP-induced changes in $[Ca^{2+}]_i$ in smooth muscle cells. Neither H-89 nor Rp-8-Br-cyclic GMPS modified this response. These findings suggest that PKA is only involved in the relaxant effect induced by low concentrations of isoprenaline (0.01–0.3 μM), whereas for higher concentrations, other mechanisms independent of PKA and PKG are involved.

3 The relaxant effects induced by SK&F 94120 and rolipram were inhibited by Rp-8-Br-cyclic GMPS with no significant effect of H-89. Neither SK&F 94120, nor rolipram at 30 μM significantly modified the activity ratios of PKA and PKG. Rolipram inhibited the ATP-induced transient increase in $[Ca^{2+}]_i$. This decrease was abolished by Rp-8-Br-cyclic GMPS whereas H-89 had no significant effect. These results suggest that PKG is involved in the vascular effects induced by the inhibitors of PDE3 and PDE4. Moreover, since it was previously shown that PDE3 and PDE4 inhibitors only increased cyclic AMP levels with no change in cyclic GMP level, these data also suggest a cross-activation of PKG by cyclic AMP in rat aorta.

4 The combination of 5 μM SK&F 94120 with rolipram markedly potentiated the relaxant effect of rolipram. This relaxation was decreased by H-89 and not significantly modified by Rp-8-Br-cyclic GMPS. Moreover, the association of the two PDE inhibitors significantly increased the activity ratio of PKA without changing the PKG ratio. The present findings show that PKA rather than PKG is involved in this type of vasorelaxation. The differences in the participation of PKA vs PKG observed when inhibitors of PDE3 and PDE4 were used alone or together could be due to differences in the degree of accumulation of cyclic AMP, resulting in the activation of PKA or PKG which are differently localized in the cell.

5 These findings support a role for both PKA and PKG in cyclic AMP-mediated relaxation in rat aorta. Their involvement depends on the cellular pathway used to increase the cyclic AMP level.

Keywords: Cyclic AMP; cyclic GMP; vasorelaxation; cyclic AMP-dependent protein kinase; cyclic GMP-dependent protein kinase; calcium; phosphodiesterase; isoprenaline

Introduction

It is well known that many agents able to elevate intracellular adenosine 3′:5′-cyclic monophosphate (cyclic AMP) content can induce smooth muscle relaxation (Murray, 1990). Intracellular accumulation of cyclic AMP can be achieved by the use of drugs which either stimulate adenylyl cyclase or inhibit cyclic nucleotide phosphodiesterases (PDEs). Among the various PDEs, PDE3 and PDE4 are of most interest since they preferentially hydrolyse cyclic AMP (Beavo *et al*., 1994). In previous studies, relaxant responses and cyclic nucleotide contents were investigated in rat aortic rings after activation of adenylyl cyclase by isoprenaline or after inhibition of PDE3 and PDE4 by SK&F 94120 and rolipram, respectively (Komas *et al*., 1991; Eckly *et al*., 1994, Eckly & Lugnier, 1994). Interestingly, the responses produced by these stimuli were differentially modulated in the presence or absence of functional endothelium. Neither the relaxant effect of isoprenaline nor the accumulation of cyclic nucleotides were strictly dependent on the presence of endothelium (Eckly *et al*., 1994). By contrast, the presence of endothelium was necessary for the relaxation elicited by the PDE4 inhibitor. Moreover, the accumulation of cyclic AMP in the presence of the PDE4 inhibitor was markedly enhanced by addition of a low concentration of a PDE3 inhibitor or by cyclic GMP elevating agents (Komas *et al*., 1991; Eckly & Lugnier, 1994). Since it is known that the presence of endothelium increases intracellular guanosine 3′:5′-cyclic monophosphate (cyclic GMP) content by the L-arginine/nitric oxyde (NO) pathway (Rubanyi & Vanhoutte, 1985) and that PDE3 is inhibited by cyclic GMP (Beavo *et al*., 1994), these results indicate that inhibition of PDE3 by cyclic GMP is necessary to enhance the relaxing effect of PDE4 inhibition (Eckly & Lugnier, 1994).

The mechanism whereby an increase in cyclic AMP content could induce vasorelaxation remains unclear. It has been assumed that activation of cyclic AMP-dependent protein kinase (PKA) could represent one of the mechanisms by which these drugs could induce vasorelaxation (Haynes *et al*., 1992). However, several studies have suggested that PKA is not the sole mediator of cyclic AMP-dependent vasorelaxation and an important role has been attributed to cyclic GMP-dependent protein kinase (PKG) in this vasorelaxation (Francis *et al*., 1988; Lincoln *et al*., 1990; Komalavilas & Lincoln 1996). Al-

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though the involvement of PKA and/or PKG in β-adrenoceptor-mediated effects has been largely studied, less is known about the participation of cyclic nucleotide-dependent protein kinases in tissues treated with PDE inhibitors. At present, it is not clear whether the vasorelaxant effects mediated by adenylyl cyclase stimulation or by PDE inhibition involve PKA and/or PKG activation.

The aim of the present study was to investigate the possible involvement of cyclic nucleotide-dependent protein kinases in vasorelaxation induced either by β-adrenoceptor agonist (isoprenaline), or by inhibitors of PDE3 and PDE4 (SK&F 94120 and rolipram, respectively). Three different experimental approaches were used: (1) the effects of specific inhibitors of PKA and PKG (H-89 and Rp-8-Br-cyclic GMPS, respectively) on the relaxation elicited by cyclic AMP-elevating agents in rat intact aortic rings; (2) the effects of cyclic AMP-elevating agents on PKA and PKG activitites in rat intact aortic rings; (3) the effects of H-89 and Rp-8-Br-cyclic GMPS on the modulation of ATP-induced calcium signalling by cyclic AMP-elevating agents in aortic smooth muscle cells were studied. Since it has been shown that the relaxation induced by rolipram was dependent on the presence of a functional endothelium (Komas *et al*., 1991), we used intact rings with endothelium. A preliminary account of parts of this work has been published in an abstract form (Eckly *et al*., 1995).

Methods

Tissue preparation and tension studies in rat intact aortic rings

Male Wistar rats (400–450 g) were killed by cervical dislocation and the thoracic aorta was quickly dissected, cleaned of fat and connective tissues and cut into rings of about 3 mm in length. Care was taken to avoid abrasion of the intimal surface of the rings to maintain the integrity of the endothelial layer. The rings were mounted under 2 g of resting tension in an organ bath containing Krebs solution of the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 1.25, KH_2PO_4 1.14, $MgSo_4$ 1.19, glucose 10, $NaHCO_3$ 25, at 37°C and gassed with 95% O_2 and 5% CO_2, pH = 7.4. All experiments were carried out in the presence of 10 μM indomethacin in order to inhibit cyclo-oxygenase activity. Tissues were allowed to equilibrate for 75 min with periodical washing before the experiments were started. Tension was measured with an isometric force transducer. The presence of functional endothelium was checked by the presence of at least 50% relaxation in response to acetylcholine (1 μM) in rings precontracted with noradrenaline (1 μM).

The relaxant effect of cyclic AMP-elevating agents was then determined. The tissue was allowed to relax and equilibrate before treatment with protein kinase inhibitors. The role of PKG and PKA was studied by use of specific inhibitors, Rp-8-Br-cyclic GMPS and H-89, respectively. In treated rings, Rp-8-Br-cyclic GMPS at 10 μM and H-89 at 1 μM were added 30 min before precontraction by noradrenaline. When the contractile response reached a steady state, cumulative concentration-tension dependencies of isoprenaline, SK&F 94120 or rolipram were measured. In some experiments, SK&F 94120 5 μM (which has been shown to increase PDE4 potency on relaxation; Komas *et al*., 1991) was added 5 min before the cumulative concentration-tension curve to rolipram was obtained. In control rings, the same protocol was used, but addition of protein kinase inhibitors before contraction was omitted. Relaxation produced by each concentration of drug was measured after 5 min incubation and values are expressed as the percentage decrease in tension of the contractile force induced by noradrenaline. During these experiments, the final concentration of the solvent for PDE inhibitors, i.e. dimethylsulphoxide (DMSO), never exceeded 0.5%.

For direct comparison to be carried out, the concentration of noradrenaline used to precontract the tissues was guided by earlier studies in which cyclic nucleotide levels were measured in this preparation. The concentrations of 0.3 μM and 1 μM noradrenaline were used in the relaxant studies induced by isoprenaline and PDE inhibitors, respectively. The choice of the concentration of protein kinase inhibitor was determined by previous work performed in this laboratory (Ouedraogo *et al*., 1994) and by the K_i values (Butt *et al*., 1990; Chijiwa *et al*., 1990). During the 30 min incubation, neither H-89 (1 μM) nor Rp-8-Br-cyclic GMPS (10 μM) significantly altered the tension of the rings precontracted with noradrenaline.

Tissue extraction and PKA/PKG activities determination

To allow accurate measurement of protein kinase activities, rat thoracic aortae were divided in two halves. They were first incubated for 5 min in tissue bath (Krebs composition was the same as in tension measurement) with 1 μM noradrenaline and treated as follows: (1) drug vehicle (0.1% DMSO); (2) isoprenaline (10 μM); (3) SK&F 94120 (30 μM); (4) rolipram (30 μM); (5) combination of SK&F 94120 (5 μM) with rolipram (30 μM); (6) 8-Br-cyclic GMP (30 μM). The concentrations of cyclic AMP-elevating agents were chosen from previous studies performed on cyclic nucleotide levels (Eckly & Lugnier, 1994). 8-Br-cyclic GMP at 30 μM was used as a positive control of PKG activation. After exposure to the different agents, the tissues were quickly removed from the organ bath and frozen with a clamp precooled in liquid N_2. The preparations were stored at −80°C until determination of protein kinase activity and protein content. The frozen tissues were then homogenized by a glass pestle homogenizer in ice-cold buffer (20 volumes) consisting of (mM): potassium phosphate buffer 20, EDTA 10, dithiothreitol 6 and NaCl 150, pH 7.0. Homogenates were centrifuged at $12{,}000 \times g$ for 5 min at 4°C.

PKA and PKG activities were determined in the same sample as described by Giembycz and Diamond (1990) and Colbran *et al*. (1992) with some modifications. To initiate the assay, aliquots (25 μl) of supernatant were added to reaction buffer (65 μl) consisting of potassium phosphate buffer (20 mM), magnesium acetate (10 mM), specific substrate (Kemptide at 71 μM or peptide G at 0.1 mM for PKA and PKG activities, respectively), PKI (0.5 μM) and [γ-^{32}P]-ATP (0.1 mM, 100 c.p.m. pmol^{-1}), pH 6.8. Tween 20 (0.01%) was present in the reaction buffer for the determination of PKA activity in order to stabilize the catalytic subunit. The mixture was incubated for 30 min at 30°C. Blank samples contained 25 μl homogenization buffer instead of homogenized aorta supernatant. The reaction was stopped by spotting a 72 μl aliquot on phosphocellulose paper (2×2 cm, Whatman P81) and immersion in phosphoric acid (0.5%). The papers were washed in the acid 3 times for 5 min. Bound radioactivity was determined by placing each paper in 7 ml liquid scintillation cocktail and counting. The activities of protein are expressed as fmol phosphate incorporated into substrate min^{-1} mg^{-1} protein. The kinase activity of each sample was expressed as the ratio of PKA and PKG activities in the presence and the absence of a maximally activating concentration of cyclic AMP (100 μM) or cyclic GMP (100 μM). Protein content was determined according to Bradford (1976).

Preparation of rat aortic smooth muscle cells and calcium measurements

Rat aortic smooth muscle cells were prepared by the method of Travo *et al*. (1980) as modified by Corriu *et al*. (1994).

For calcium measurements, the experiments were carried out on confluent quiescent cells. Cells were washed three times with balanced salt solution (BSS in mM: NaCl 135, KCl 5, $MgCl_2$ 1, $CaCl_2$ 1.25, glucose 10 and HEPES 20), pH 7.5. The cells were then incubated in the dark with 5 μM fura-2/acetoxymethylester (fura-2/AM), for 75 min at room temperature. After being loaded, the cells were washed twice in BSS, suspended by means of brief trypsin treatment (0.05%) and prepared to a density of 10^6 cells ml^{-1}. The cells were kept at 37°C and used within 2 h,

during which time they remained viable and responsive. The fura-2 fluorescence of the cells (excitation wavelengths: 340 and 380 nm; emission wavelength 510 nm) was measured at 37°C with a Hitachi Spectrometer (F-2000). Each cuvette contained 1.5 ml of magnetically stirred cell suspension and the different agents were added in a volume of 15 μl. Cyclic AMP-elevating agents (isoprenaline at 10 μM and rolipram at 30 μM) were added 2 min before ATP at 100 μM. The specific inhibitors of PKA and PKG (H-89 at 300 nM and Rp-8-Br-cyclic GMPS at 10 μM respectively) were incubated in the cell suspension 20 min before the start of intracellular calcium measurements. The $[Ca^{2+}]_i$ was calculated every 0.2 s according to Grynkiewicz *et al*. (1985). At the end of the experiment the maximal fluorescence ration (R_{max}) was determined after the addition of ionomycin (10 μM) and the minimal fluorescence ration (R_{min}) was determined by addition of 5 mM EGTA.

Materials

Noradrenaline bitartrate, indomethacin, isoprenaline, ATP, 8-Br-cyclic GMP, protein kinase inhibitor (PKI), Kemptide, bovine serum albumin (BSA) were from Sigma Chemical Co. Rp-8-Br-cyclic GMPS and H-89 (N-[2-p-bromocinnamylamino)ethyl]-5-isoquinoline-sulphonamide) were from Biolog and Calbiochem, respectively. Peptide G was from Peninsula. [γ-^{32}P]-ATP was a Dupont-NEN product. Fura-2 acetoxymethyl ester was from Molecular Probes. Rolipram and 5-(4-acetamidophenyl)pyrazin-2(1H)-one (SK&F 94120) were obtained as a kind gift from Schering (AG) and Smith Kline & Beecham Research. Cilostamide was synthesized as previously described (Lugnier *et al*., 1985).

Statistical analysis

Statistical comparisons were performed according to analysis of variance (ANOVA) and Student's *t* test. Results are expressed as the mean ± s.e.mean. In figures, vertical lines indicate s.e.mean. Differences were considered significant when $*P<0.05$, $**P<0.01$, $***P<0.001$.

Results

Effects of H-89 and Rp-8-Br-cyclic GMPS on the relaxation induced by cyclic AMP-elevating agents in rat intact aortic rings

Isoprenaline (0.01 – 100 μM) caused concentration-dependent relaxation in control aortic rings precontracted with noradrenaline with an EC_{50} value (concentration of drug giving 50% of maximal effect) of 0.13 ± 0.04 μM and a E_{max} value (maximal relaxant response) of 86 ± 3% (Figure 1a). Treatment with

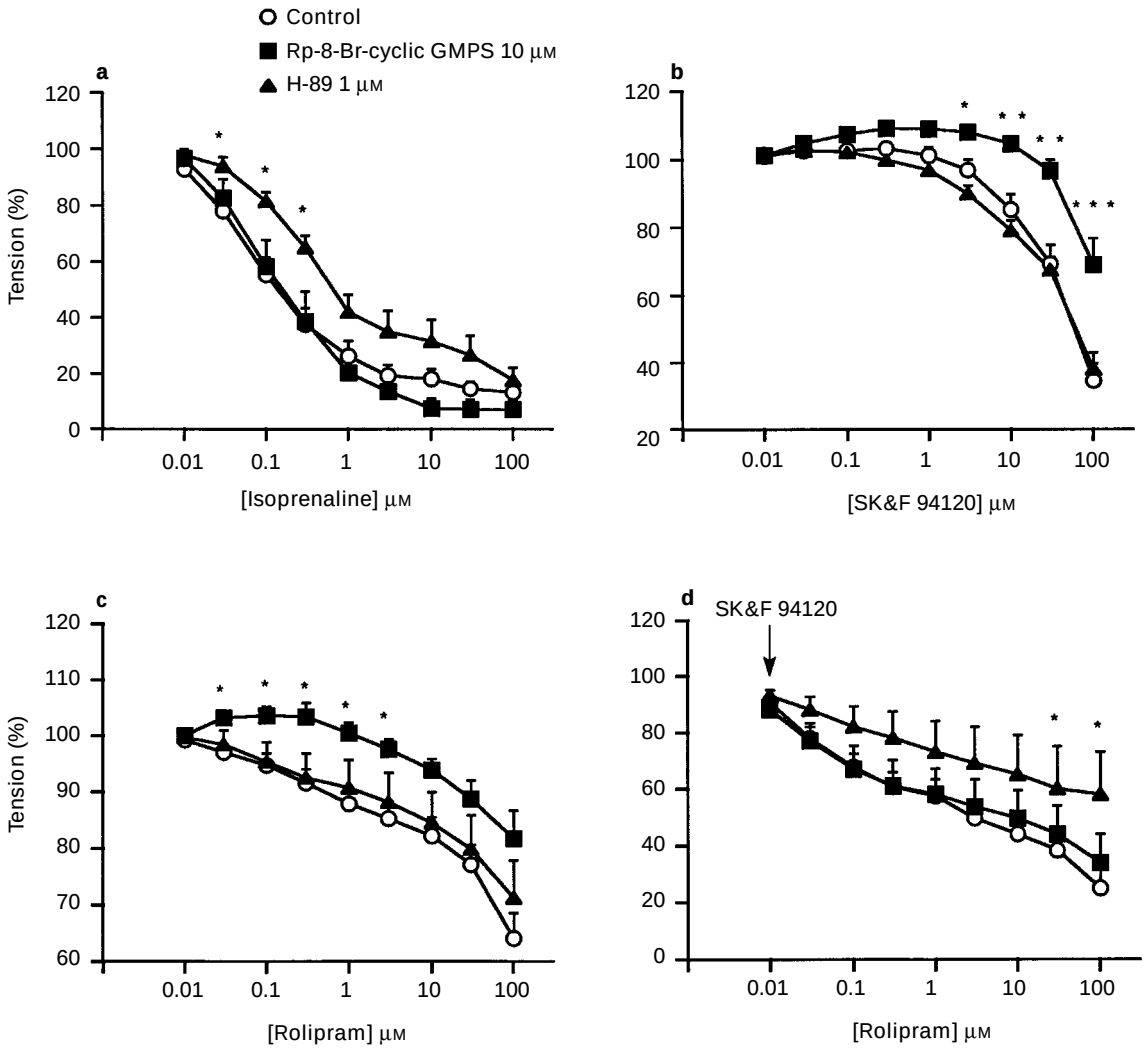

Figure 1 Relaxation induced by isoprenaline (a), SK&F 94120 (b), rolipram (c) and a combination of 5 μM SK&F 94120 and rolipram (d) in rat aortic rings with endothelium. The results are expressed as the mean of 6 to 10 experiments and vertical lines show s.e.mean. Significant differences were determined by ANOVA ($*P<0.05$, between rings treated or not with protein kinase inhibitors).

H-89 significantly decreased the relaxant effect of the lower concentrations of isoprenaline used (0.01 to 0.3 μM), whereas the relaxant effects of isoprenaline was not significantly modified by Rp-8-Br-cyclic GMPS.

Neither SK&F 94120 nor rolipram produced maximal relaxant effects at the concentrations used. H-89 did not significantly alter concentration-response curves to SK&F 94120 (Figure 1b). On the contrary, Rp-8-Br-cyclic GMPS induced a significant inhibition of the response elicited by SK&F 94120 (3 to 100 μM). The relaxation obtained with rolipram 100 μM (34.4 ± 4.4% of the initial tension) was less than that obtained with SK&F 94120 100 μM (Figure 1c). H-89 did not significantly affect concentration-response curves to rolipram whereas Rp-8-Br-cyclic GMPS decreased the relaxation induced by the lowest concentrations of rolipram (from 0.03 to 3 μM).

Results from studies of combinations of a low concentration of SK&F 94120 (5 μM) with rolipram are shown in Figure 1d. SK&F 94120 (5 μM) induced by itself a relaxation of 10 ± 1.3% and significantly potentiated the relaxant effect of rolipram (100 μM) from 34.4 to 75% relaxation. H-89 significantly decreased the maximal relaxant effect induced by the combination of both PDE inhibitors by 33% in comparison to control, whereas Rp-8-Br-cyclic GMPS was without effect.

For further experiments (protein kinase activities and calcium measurements), we chose the concentrations of drugs according to previous studies where cyclic nucleotide contents had been measured under the same experimental conditions (Schoeffter *et al*., 1987; Eckly *et al*., 1994): i.e. 10 μM isoprenaline and 30 μM PDE3 and PDE4 inhibitors.

Effects of cyclic nucleotide-elevating agents on protein kinase activities in rat intact aortic rings

Table 1 shows the effects of cyclic nucleotide-elevating agents on PKA and PKG activities. In rat thoracic aorta, basal PKA activity (activity in absence of cyclic AMP) was 4.5 fold higher than basal PKG activity (see legend of Table 1). At the concentrations used, neither isoprenaline (10 μM), nor PDE3 and PDE4 inhibitors (30 μM) had a significant effect on the PKA and PKG activity ratio.

Association of SK&F 94120 (5 μM) to rolipram (30 μM) significantly increased the activity ratio of PKA from 0.46 ± 0.05 to 0.84 ± 0.1 whereas this treatment did not significantly modify the activity ratio of PKG.

As expected, treatment with 8-Br-cyclic GMP (30 μM) maximally increased the activity ratio of PKG which was 3.4 fold higher than in control rings. Interestingly, an increase in the activity ratio of PKA has also been detected after exposure to 8-Br-cyclic GMP, but this was smaller than that seen with PKG (increase of 1.7 fold as compared to control).

Involvement of protein kinase in the effects of cyclic AMP-elevating agents on ATP-induced Ca^{2+} signalling in rat aortic smooth muscle cells

Isoprenaline (10 μM) and rolipram (30 μM) did not significantly modify basal $[Ca^{2+}]_i$ levels, whereas PDE3 inhibitors (SK&F 94120 and cilostamide at 10 μM) decreased the basal level of $[Ca^{2+}]_i$ (by about 75 and 67%, respectively). Therefore, we only studied the effects of isoprenaline and rolipram on peak rises in $[Ca^{2+}]_i$ for 100 μM ATP (Figure 2a). Exposure of the cells to ATP (100 μM) induced a rapid and transient rise in $[Ca^{2+}]_i$, reaching a peak after 0.5–1 s followed by a small sustained rise in $[Ca^{2+}]_i$ which rapidly (8–10 s) returned to basal levels. Treatment with rolipram (30 μM) caused a marked attenuation (by 40 ± 6.2%) of the transient rise induced by ATP, whereas it was not significantly affected by isoprenaline at 10 μM.

Incubation of aortic smooth muscle cells with H-89 (300 nM) did not significantly change the effects of isoprenaline and rolipram on ATP-induced Ca^{2+} transient rise (Figure 2b). On the contrary, Figure 2c shows that the decrease in ATP-induced calcium signalling due to rolipram addition was totally inhibited by Rp-8-Br-cyclic GMPS (10 μM) and the transient rise in $[Ca^{2+}]_i$ induced by ATP was not significantly different from the control cells. Rp-8-Br-cyclic GMPS did not modify the calcium response induced by ATP after isoprenaline treatment.

Discussion

The aim of the present study was to examine the participation of cyclic nucleotide-dependent protein kinases in the relaxation induced by drugs known to increase cyclic AMP levels by different molecular mechanisms in rat aorta. The study of the involvement of cyclic nucleotide dependent-protein kinases in vasorelaxation was made possible by the use of three different experimental approaches performed either with intact tissue or with smooth muscle cells.

Considering the relaxation induced by the β-adrenoceptor agonist, we found that H-89 decreased the relaxant response elicited by low concentrations of isoprenaline, whereas Rp-8-Br-cyclic GMPS had no effect suggesting an involvement of PKA in this relaxant effect. In agreement with these results, it has been shown that isoprenaline-induced relaxation of coronary arteries correlated well with activation of PKA (Silver *et al*., 1983; Vesgesna & Diamond, 1986). In a previous study, we found that, under the same experimental conditions, isoprenaline increases cyclic AMP levels in rat aorta (Schoeffter *et al*., 1987; Eckly *et al*., 1994). Thus, it is likely that the increase in cyclic AMP level induced by low concentrations of isoprenaline activated PKA rather than PKG in rat aorta with endothelium. Moreover, our results on protein kinase activities show that a high concentration of isoprenaline (10 μM) neither

Table 1 Effects of cyclic nucleotide-elevating agents on PKA and PKG activities in rat intact aorta

Treatment	*PKA activity ratio* (−cyclic AMP/+cyclic AMP)	*PKG activity ratio* (−cyclic GMP/+cyclic GMP)	n
Control	0.46 ± 0.05	0.28 ± 0.04	4
Isoprenaline 10 μM	0.60 ± 0.04	0.23 ± 0.05	4
SK&F 94120 30 μM	0.62 ± 0.07	0.20 ± 0.06	4
Rolipram 30 μM	0.56 ± 0.01	0.27 ± 0.08	4
Rolipram 30 μM + SK&F 94120 5 μM	0.84 ± 0.14*	0.29 ± 0.10	3
8-Br-cyclic GMP 30 μM	0.82 ± 0.09*	0.97 ± 0.04**	4

Rat aortae were treated with noradrenaline for 5 min and cyclic nucleotide-elevating agents were then added as a single dose. Tissues were frozen for determination of protein kinase activities. Activity in the absence of cyclic nucleotide (−cyclic AMP or −cyclic GMP) or in the presence (+cyclic AMP or +cyclic GMP) is expressed as fmol phosphate incorporated into specific substrate min^{-1} mg^{-1} protein. In control rings, PKA activities in the absence and presence of cyclic AMP were 0.8 ± 0.1 and 2.1 ± 0.6 fmol min^{-1} mg^{-1} protein, respectively. PKG activities in the absence and presence of cyclic GMP were 0.18 ± 0.03 and 0.61 ± 0.03 fmol min^{-1} mg^{-1} protein, respectively. *$P<0.05$, **$P<0.01$ significantly different from control.

modified PKA nor PKG activities which is in accordance with functional results. Interestingly, it has been proposed recently that accumulation of cyclic AMP induced by low concentrations of isoprenaline preferentially activates PKA whereas higher concentrations of the agonist activate both PKA and PKG (Murphy & Makhlouf, 1995). By Fura-2 miocrofluorometry, our findings show that isoprenaline did not modify the calcium signal induced by ATP. Controversial studies have investigated the relationship of cyclic AMP pathway to Ca^{2+} (Ushio-Fukai *et al*., 1993; Hoiting *et al*., 1996). Although tissue diversity may be partially responsible for some of the differences in β-adrenoceptor-mediated modulation of smooth muscle $[Ca^{2+}]_i$, some of these differences may be related to experimental conditions. For example, it has been shown that a low concentration of isoprenaline (0.1 μM) produces modest increases in Ca^{2+}-channel activity, whereas higher concentrations (10 μM) result in inhibition of Ca^{2+}-channel activity in vascular smooth muscle cells (Ishikawa *et al*., 1993). Since the inhibitory effect of higher concentrations of cyclic AMP was mimicked by cyclic GMP, these authors suggest mediation by a common mechanism, involving activation of PKG. In our experiments, no involvement of PKA and PKG was observed when the effect of isoprenaline at 10 μM was studied on ATP-induced rise in $[Ca^{2+}]_i$.

In summary, the present findings suggest that at low concentrations, isoprenaline activates PKA rather than PKG whereas at higher concentrations neither PKA nor PKG seem to be involved in the relaxant effect (Figure 3). The question remains as to how a larger concentration of isoprenaline can relax smooth muscle cells without activating cyclic nucleotide dependent protein kinases? One possibility which could be considered is that isoprenaline-induced relaxation in smooth muscle is mediated by a protein kinase-independent mechanism, for example via an action on ion-channels. It has been recently demonstrated that isoprenaline activates Ca^{2+}-activated K^+ channels in the absence of ATP, in isolated airway smooth muscle cells (Kume *et al*., 1994). Under these conditions protein phosphorylation does not take place suggesting that PKA/PKG, or other kinases, are not functionally active. Moreover, it is interesting to note that a cyclic nucleotide-gated ion channel has been characterized in rabbit aorta (Biel *et al*., 1993). Although the functional result of the stimulation of this channel by cyclic nucleotides is as yet unknown, it is likely that this channel could also play a role in the regulation of vascular tone.

The participation of PKA and PKG was studied in PDE inhibitor-induced relaxation. We found that Rp-8-Br-cyclic GMPS inhibited the relaxation mediated by SK&F 94120 and rolipram in aorta with endothelium. H-89 did not affect the functional responses elicited by these PDE inhibitors. At the present time, little is known about the participation of protein kinase in the response mediated by PDE inhibitors. In oesophageal sphincter smooth muscle, it has been shown that

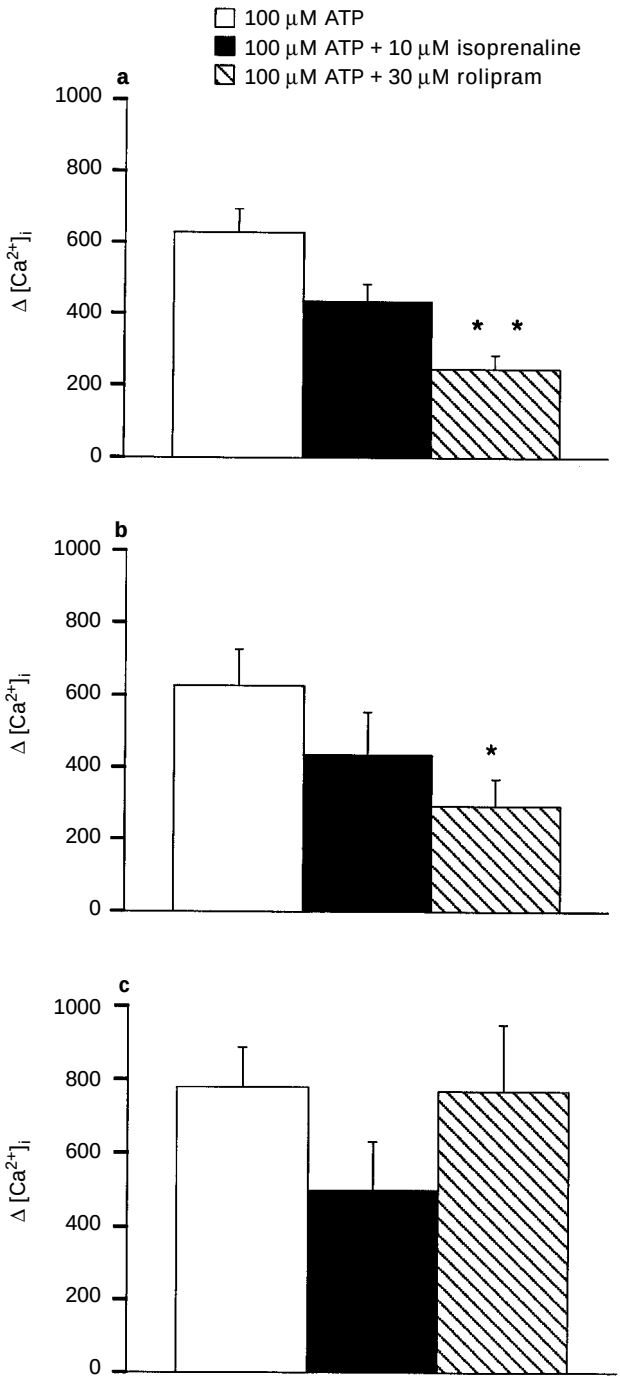

Figure 2 Effects of isoprenaline and rolipram on the increase in $[Ca^{2+}]_i$ (peak – basal) induced by ATP in rat aortic smooth muscle cells. Cells were treated for 2 min in absence, or in presence of 10 μM isoprenaline, or in presence of 30 μM rolipram, followed by addition of 100 μM ATP. Cells were pretreated for 20 min in absence (a) or presence of 300 nM H-89 (b), or in presence of 10 μM Rp-8-Br-cyclic GMPS (c). The basal $[Ca^{2+}]_i$ values of 245 ± 24 nM ($n = 18$) were not significantly modified by treatment with the protein kinase inhibitors. The autofluorescence was 180 ± 7 nM ($n = 3$). The results are expressed as the mean of 6–12 experiments ± s.e.mean. Significant differences were determined by Student's t test. $^*P < 0.05$, $^{**}P < 0.01$ compared to controls.

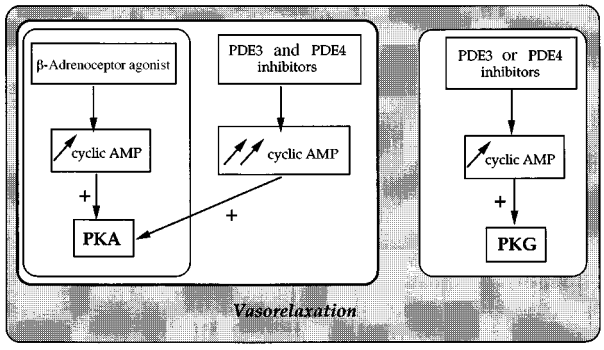

Figure 3 Scheme of hypothetical compartments of cyclic AMP pathway in vascular smooth muscle cells. Although, a β-adrenoceptor agonist and PDE3/PDE4 inhibitors increase cyclic AMP content to a similar extent, they differ in their ability to activate PKA or PKG, respectively. This suggests that functional compartmentalization exists within the cell. Moreover, the association of PDE3 and PDE4 inhibitors potentiates the increase in cyclic AMP content and subsequently activates PKA. This result shows that the degree of accumulation of cyclic AMP diffusing in the cell plays an important role in the involvement of PKA or PKG.

SK&F 94120 produced concentration-dependent increases in the activity of PKA (Barnette *et al*., 1990). The apparent discrepancy between this finding and ours might be attributed to the differences in tissue as well as the differences in the stimulation used to precontract the preparations. In accordance with our functional results, the calcium inhibitory effect of rolipram was totally abolished by preincubation of these cells with Rp-8-Br-cyclic GMPS. The abilities of Rp-8-Br-cyclic GMPS to reverse the effects of PDE inhibitors suggest that PKG rather than PKA is involved in the relaxation induced by PDE3 and PDE4 inhibitors in rat aorta with endothelium. However, measurement of the protein kinase activity ratio showed that neither PKA, nor PKG, are activated by the PDE3 and PDE4 inhibitors. This apparent discrepancy could be explained by our findings showing that PKG activity is lower than PKA activity in rat aorta. Thus, it is possible that activation of PKG by rolipram is not important enough to be detected in our experimental conditions. Additionally, the possibility cannot be excluded that an eventual local PKG activation could not be detected in the whole aorta. The fact that 8-Br-cyclic GMP at 30 μM activated PKG demonstrates that PKG is present and functional in rat aorta. Interestingly, our results showed that 8-Br-cyclic GMP, at 30 μM, is also able to activate PKA. This is in accordance with the findings that PKA activation is involved in the noradrenaline release-effect induced by the 8-substituted cyclic GMP analogues (Ouedraogo *et al*., 1994). In a previous study performed under the same experimental conditions, we have shown that PDE3/PDE4 inhibitors increased cyclic AMP level to a similar extent as isoprenaline, with no changes in cyclic GMP levels (Eckly & Lugnier, 1994). Our present results imply that PKG is activated by cyclic AMP. It has been found that cyclic AMP can cross-activate PKG in intact cells (Murphy & Makhlouf, 1995). The affinity of PKG for cyclic AMP is 50–100 fold lower than the affinity of this enzyme for cyclic GMP (Landgraf *et al*., 1986), but cyclic AMP levels are about 5 fold higher than cyclic GMP levels in rat aorta treated with SK&F 94120 or rolipram (Eckly & Lugnier, 1994). Moreover, autophosphorylation of PKG which is known to occur *in vivo* increases the affinity for cyclic AMP activation about 2–6 fold (Landgraf *et al*., 1986). In the present studies, we worked on rat aortic rings with endothelium. Since the presence of endothelium has been shown to increase cyclic nucleotide contents (Eckly & Lugnier, 1994) and since an increase in cyclic nucleotide levels is crucial for autophosphorylation (Foster *et al*., 1981), it might be possible that the endothelium plays an important role in the autophosphorylation and subsequent activation of PKG by cyclic AMP. In summary, the present findings suggest that PKG rather than PKA is involved in the relaxant effects of PDE3 and PDE4 inhibitors.

Interestingly, we found that H-89, in contrast to Rp-8-Br-cyclic GMPS, inhibited the relaxation mediated by the association of PDE3 and PDE4 inhibitors. In the same way, addition of both inhibitors activated PKA with no change in PKG activity. Some explanations must be provided for the fact that PDE inhibitors alone or combined differ in their ability to activate PKA or PKG. One possible explanation is that the amount of cyclic AMP diffusing in the cell plays an important role in the involvement of cyclic nucleotide dependent-protein kinases. We previously demonstrated that a combination of both PDE inhibitors potentiates the increase in cyclic AMP (Eckly & Lugnier, 1994). Therefore, the degree of accumulation in cyclic AMP by PDE inhibitors alone or associated may produce different intracellular cyclic AMP distribution resulting in activation of PKG or PKA (Figure 3). This observation suggests that some functional compartmentalization of the cyclic AMP-triggered protein phosphorylation cascade exists within the cell. Little is known about the exact subcellular compartmentalization of the different components of the cyclic AMP cascade system in smooth muscle. However, there are several pieces of evidence indicating that access of the protein kinase to cyclic nucleotides and to potential substrates is a factor determining substrate phosphorylation and subsequent relaxation (Lincoln & Cornwell, 1993; Jurevicius & Fischmeister, 1996).

In conclusion, our results show that the involvement of PKA and PKG may differ within the same tissue depending on the pathway used to increase cyclic AMP level. Participation of PKA was demonstrated for low concentrations of a β-adrenoceptor agonist and association of PDE3 and PDE4 inhibitors. The involvement of PKG was obvious for PDE3 and PDE4 inhibitors alone. The differences in the degree of cyclic AMP accumulation and the functional compartmentalization of the cyclic AMP cascade may be responsible for the differences in the involvement of PKA and PKG.

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