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ROLE OF CYCLIC AMP- AND CYCLIC GMP-PHOSPHODIESTERASES IN THE CONTROL OF CYCLIC NUCLEOTIDE LEVELS AND SMOOTH MUSCLE TONE IN RAT ISOLATED AORTA

A STUDY WITH SELECTIVE INHIBITORS

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Abstract—Three isoforms of cyclic nucleotide phosphodiesterase (PDE) have been recently isolated from aortic tissue and two of them specifically hydrolyzed adenosine 3',5'-cyclic monophosphate (cAMP) and guanosine 3':5'-cyclic monophosphate (cGMP), respectively (Lugnier *et al.*, *Biochem. Pharmac.* **35**, 1743, 1986). The role of these forms in controlling cyclic nucleotide levels and smooth muscle tone was investigated by the use of PDE inhibitors. The effects of selective inhibitors of the two forms specifically hydrolyzing cAMP or cGMP (cAMP–PDE and cGMP–PDE, respectively) were compared to those of non-selective inhibitors of the three aortic PDE forms, including the calmodulin-sensitive one (CaM–PDE). Relaxation responses and accumulation of tissue cAMP and cGMP induced by these drugs were studied in precontracted rat isolated aorta, and compared to the effects of isoprenaline and forskolin (stimulants of adenylate cyclase) or sodium nitroprusside (SNP) and sodium azide (stimulants of guanylate cyclase). The eight PDE inhibitors tested all relaxed aorta with potencies that correlated with their potencies as inhibitors of cAMP–PDE, but not of cGMP–PDE. At a concentration producing half-maximal relaxation, all PDE inhibitors induced a moderate but significant accumulation of cAMP, which was comparable to the accumulation of cAMP elicited by half-maximally relaxing concentrations of adenylate cyclase stimulating agents. At this concentration, some PDE inhibitors (M&B 22,948, dipyridamole and to a lesser extent, trequinsin) also induced a significant increase in cGMP levels, of the same order of magnitude as that caused by agents stimulating guanylate cyclase. However, the cGMP-increasing effect of these inhibitors was dissociated from their relaxing effect. In particular, the relaxing concentrations of M&B 22,948 (a selective inhibitor of cGMP–PDE) were clearly higher than the cGMP-increasing concentrations of the compound. At a concentration at which they elicited 10% relaxation by themselves, the selective cAMP–PDE inhibitor, rolipram, as well as the mixed inhibitor of cAMP- and cGMP–PDE, AAL 05 (a cilostamide analogue) enhanced both the cAMP-increasing and the relaxing effect of isoprenaline. Under the same conditions, no clear enhancement of the relaxation induced by SNP was observed. Only M&B 22,948 showed a slight potentiating effect on SNP-induced relaxation, but this effect was limited to low concentrations of SNP (< 10 nM). Conversely, M&B 22,948 inhibited the relaxation elicited by higher concentrations of SNP ($\geq 0.1\ \mu$M), and some of the PDE inhibitors completely displaced the concentration–relaxation curve of SNP to the right. One of them, cilostamide, also inhibited the cGMP-increasing effect of SNP, but the other PDE inhibitors did not. The results strongly suggest that inhibition of cAMP–PDE produces relaxation of rat aorta, as a consequence of cAMP accumulation. They do not provide evidence that inhibition of aortic cGMP–PDE can lead by itself to relaxation via cGMP accumulation in this tissue.

Adenosine and guanosine 3':5'-cyclic monophosphates (cAMP and cGMP, respectively)† can both produce smooth muscle relaxation. There is evidence suggesting that the two cyclic nucleotides exert an inhibitory control of contractile activity in smooth muscle cells, but their precise roles and the regulation of their levels are still not well understood in these cells (for reviews see [1–3]). In fact, discrepancies have been repeatedly reported between relaxation and cAMP or cGMP accumulation induced in smooth muscles by drugs with either stimulate the production or inhibit the degradation of cAMP or cGMP in the same tissues [4–22].

Hydrolysis catalyzed by cyclic nucleotide phosphodiesterase (PDE) is the only known pathway for biochemical degradation of cyclic nucleotides in eukaryotic cells and several PDE isoforms have been isolated from a variety of tissues (for reviews see [23–25]). In particular, three PDE forms which can be inhibited by different drugs have been isolated

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† Abbreviations used: cAMP, adenosine 3':5'-cyclic monophosphate; cGMP, gaunosine 3':5'-cyclic monophosphate, PDE(s), 3':5' cyclic nucleotide phosphodiesterase(s); CaM, calmodulin; EC_{50}, concentration eliciting a half-maximal effect; IC_{50}, concentration inhibiting the effect by 50%; SNP, sodium nitroprusside; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$.

from aorta [26]. The aim of the present work was to study the roles of the aortic PDE forms that specifically hydrolyze cAMP or cGMP in controlling cAMP and cGMP levels and contractile function in aortic smooth muscle. The ability of selective inhibitors of these PDE forms, as well as that of non-selective PDE inhibitors, to produce cyclic nucleotide accumulation and relaxation by themselves, and to modify the effects of agents that stimulate adenylate cyclase (isoprenaline) or guanylate cyclase (sodium nitroprusside or SNP), was investigated on rat isolated thoracic aortas, precontracted by serotonin or prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). The PDE inhibitors used belong to different chemical families and included: rolipram [27] and Ro 20-1724 [28], both specific inhibitors of the aortic PDE form (cAMP–PDE) selectively hydrolyzing cAMP; M&B 22,948 [29], a selective inhibitor of the aortic PDE form hydrolyzing cGMP (cGMP–PDE, insensitive to calcium and calmodulin); cilostamide [30], its analogue AAL 05 [31] and dipyridamole [32], mixed inhibitors of both cAMP– and cGMP–PDE, but not of the calmodulin-sensitive–PDE form (CaM–PDE) from aorta; IBMX (3-isobutyl-1-methylxanthine [33]) and trequinsin (or compound HL 725; [34]) which non-selectively inhibit the three PDE forms isolated from aorta.

The results show that the relaxing effect of the PDE inhibitors can be correlated with their ability to inhibit the enzyme form (cAMP–PDE) that specifically hydrolyzes cAMP. They do not offer evidence that inhibition of aortic cGMP–PDE can lead by itself to relaxation.

MATERIALS AND METHODS

Aortic relaxation

Seven-week-old male Wistar rats bred in the laboratory were used throughout this study. The rats were killed by a blow on the neck and the thoracic aorta quickly removed and cleaned of adherent tissue.

For the study of PDE inhibitors alone, helical strips were generally prepared according to Furchgott and Bhadrakom [35], using a metal probe introduced into the lumen of the vessel, thus destroying the endothelium [36]. They were then suspended under a basal tension of 1 g in a modified Krebs solution of the following composition: (in mM) NaCl 122, KCl 4.73, $CaCl_2$ 1.25, $MgCl_2$ 1.19, $NaHCO_3$ 15.5, KH_2PO_4 1.19, glucose 11.5 and ascorbic acid 1.2, adjusted to pH 7.4 by bubbling with a mixture of 95% O_2–5% CO_2 at 37°. Isometric changes in tension were recorded on a Beckman R511A dynograph using an isometric transducer (Statham UC 2). After equilibration for 120 min, during which time tissues were periodically washed, contractions were induced by serotonin (10 μM) in the presence of the alpha-adrenergic blocking agent prazosin (0.1 μM), added 15 min prior to serotonin. When the contraction had plateaued (about 15 min after addition of serotonin), one or other of the PDE inhibitors was added cumulatively, the maximal relaxing effect at each concentration being allowed to develop (generally for 5 min). Only one concentration–relaxation curve was obtained in each preparation, because the serotonin response was not sustained on a second exposure. Control measurements (decline of contraction plateau in the presence of solvent during the time of the experiment) were performed on separate aortic strips. In each test strip, the effect of the PDE inhibitor was calculated by correcting for tension changes observed at corresponding time intervals in a control strip which developed a similar degree of serotonin-induced tension. The relaxing effects of isoprenaline and sodium azide were studied using the same protocol. SNP- and forskolin-induced relaxation of aortic rings was studied after precontraction by $PGF_{2\alpha}$ (2 μM) for 30 min, and in the case of forskolin, which elicited very slow responses, the effect of a unique concentration (0.1 μM) was tested and the time necessary to decrease maximal tension by 50% was estimated. In other cases relaxation was expressed as percent of the maximal possible relaxation (i.e. 100% = return to baseline) and the concentration producing a mean decrease by 50% in the serotonin- or $PGF_{2\alpha}$-induced elevation of tension (IC_{50}) was determined graphically from the mean log concentration–response curve for each compound.

For the study of PDE inhibitors in combination with isoprenaline or SNP, aortae were cut in four rings 2 mm long, which were denuded of the endothelium by gently rubbing the interior of the vessel with a wooden matchstick. Rings were allowed to equilibrate in the physiological solution under a basal tension of 19.6 mN for 60 min with periodical washings. Contraction was elicited by $PGF_{2\alpha}$ (2 μM) in the presence of prazosin (0.1 μM) added 15 min prior to $PGF_{2\alpha}$. When contraction had stabilized (after 30 min), isoprenaline or SNP was added in a cumulative manner, each addition being allowed to fully develop its effect, in order to obtain a complete concentration–effect curve of relaxation. When required, dimethylsulfoxide (DMSO), the vehicle of some of the PDE inhibitors, was present in the solution at the same final concentration as that present with PDE inhibitors (0.02% v/v). After washings and resting for 60 min, a second contraction was elicited by $PGF_{2\alpha}$ (30 μM), still in the presence of prazosin. After 30 min, the contraction stabilized to a plateau about 10% higher than that obtained during the first contraction. An appropriate concentration of one or the other of the PDE inhibitors was then added (for 5–10 min) to relax the preparation by about 10%, i.e. to stabilize the tension at the level of this first contraction. A second concentration–effect curve to isoprenaline or SNP was then obtained. The net relaxation to isoprenaline or SNP was expressed as percent decrease in the tension existing at the moment before their administration (100% = return to baseline). EC_{50} values (concentrations producing a half-maximal effect of the given agents) were determined graphically from the log concentration–response curves for each preparation. The experimental design of this part of the study was adopted because two successive control curves thus obtained for isoprenaline or SNP were similar.

Measurement of cyclic nucleotide levels. Aortic cyclic nucleotide levels were determined as previously described [37]. Briefly, the rat thoracic aorta

was removed, cleaned of adjacent tissues, cut transversely into 4–6 rings and incubated in 1 or 2 ml of the above mentioned Krebs solution (37°, pH 7.4, bubbled with 95% O_2–5% CO_2). Rings were distributed among the different experimental groups and incubated for a 120-min period with several changes of the incubation solution. The experiments were planned so as to reproduce the same conditions as for relaxation experiments. Therefore, the effects of PDE inhibitors or activators of cyclases were tested in the presence of prazosin and contractile agents (serotonin or $PGF_{2\alpha}$). In some experiments the effect of isoprenaline and SNP on cAMP or cGMP levels, respectively, was investigated in the absence or in the presence of PDE inhibitors. In these experiments the inhibitors were present under the same conditions as in relaxation experiments, isoprenaline and SNP being added for the last 1 min of incubation at concentrations close to the respective mean EC_{50} values obtained as described above. Control tissues were always exposed to solvents at the same final concentrations as present in the final drug solutions used.

At the end of the treatment, tissues were quickly frozen using an aluminium clamp precooled in liquid N_2, then stored at −80° for a few days, before being thawed and homogenized in 1 N $HClO_4$. Cyclic nucleotides were assayed in the 10,000 *g*–5 min perchlorate supernatants by radioimmunological methods including a succinylation step [38, 39]. Cyclic nucleotide levels were expressed with respect to the DNA content, which was extracted from the 10,000 *g*–5 min pellet and assayed by a previously described adaptation [37] of fluorometric procedures [40, 41].

Drugs. 3-Isobutyl-1-methylxanthine (IBMX), serotonin hydrochloride and (−)-isoproterenol hydrochloride (isoprenaline) were purchased from the Sigma Chemical Co. (St Louis, MO). Forskolin, sodium nitroprusside and sodium azide were purchased from Calbiochem–Behring (France), Merck Darmstadt, F.R.G. and Prolabo (France), respectively. Prazosin hydrochloride and prostaglandin $F_{2\alpha}$ (dinolytic) were gifts from Pfizer (Sandwich, U.K.) and the Upjohn Co. (Kalamazoo, MI), respectively. Forskolin (1 mM) was dissolved in ethanol to provide a stock solution.

4-(3-Butoxy-4-methoxybenzyl)-2-imidazolidinone (Ro 20-1724, a gift from Hoffman–LaRoche), 4-(3-cyclopentyloxy-4-methoxyphenyl)-2-pyrrolidone (rolipram or ZK 62711, a gift from Schering), *N*-cyclohexyl-*N*-methyl-4-(1,2-dihydro-2-oxo-6-quinolyloxy) butyramide (cilostamide, synthesized by C. Lugnier; [31]) and *N*-cyclohexyl-*N*-methyl-4-(1,2-dihydro-2-oxo-6-quinolyloxy) valeramide (AAL 05; [31]) were dissolved in DMSO or ethanol. The final concentration of these solvents never exceeded 0.5% (v/v). 9,10-dimethoxy-2-mesitylimino-3-methyl-3,4,6,7-tetrahydro-2*H*-pyrimido(6,1-*a*)isoquinolin-4-one hydrochloride (trequinsin, a gift from Hoechst) were dissolved in water or physiological solution. 2-*O*-propoxylphenyl-8-azapurin-6-one (M&B 22,948, a gift from May & Baker, Dagenham, U.K.) and dipyridamole (a gift from Boehringer–Ingelheim, London, U.K.) were dissolved in 1 N NaOH and 1 N HCl, respectively, then neutralized and diluted 25 times in water to provide stock solutions of 30 and 4 mM, respectively.

Standard DNA, cAMP and cGMP were from the Sigma Chemical Co. (St Louis, MO), as were the succinyltyrosylmethyl esters of cyclic nucelotides, iodinated to provide the [^{125}I]-radiolabelled derivatives used in the assay of cAMP and cGMP. Anti-cAMP and anti-cGMP antibodies were the gift of Drs Cailla and Delaage (Centre d'Immunologie, Marseille-Luminy, France).

Statistical treatment of data. The Student's *t*-test was used for statistical comparisons of relaxant effects and of cyclic nucleotide levels. Relaxant EC_{50} values of isoprenaline and SNP in the absence and presence of PDE inhibitors were compared by means of the Wilcoxon test for paired observations. The coefficients of the increases in cyclic nucleotide levels were analysed by the Mann and Whitney's *U* test. Attempts were made to correlate the relaxant IC_{50} values from the present study with PDE inhibitory IC_{50} values previously obtained [26]. In this case the significance of the correlation was estimated by the *t* test for the correlation coefficient. Results are given as means ± SE. P values less than 5% were considered significant.

RESULTS

Relaxation of rat aorta by PDE inhibitors

The eight PDE inhibitors tested were able to relax the rat isolated aorta precontracted by serotonin in a concentration-dependent manner. Figure 1 shows the concentration–response relationships obtained with these compounds. Estimation of the maximal effect could not be made in all cases because of the poor solubility of some of the substances. Nevertheless, the concentration required to inhibit by 50% serotonin-induced contractions (IC_{50}) gives an indication of the relative potency of the compounds as relaxing agents. Trequinsin appeared to be by far the most potent (IC_{50} 0.25 μM). Compounds AAL 05 and IBMX were approximately equipotent (respective IC_{50} values 8.3 and 10 μM). Cilostamide and dipyridamole relaxed rat aorta with IC_{50} values ranging

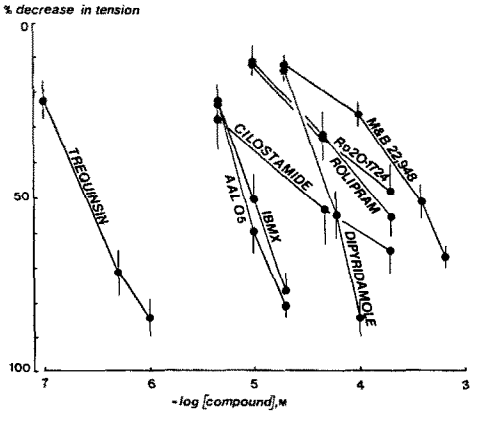

Fig. 1. Relationships between concentration and relaxing effect of PDE inhibitors in rat aorta. The aorta strips were precontracted by serotonin (10 μM) in presence of prazosin (0.1 μM). The points are the means of 7–12 determinations. The vertical bars represent SE.

between 10 and 100 μM (36 and 52 μM, respectively), whereas the IC_{50} values of rolipram (150 μM, Ro 20-1724 (240 μM) and M&B 22,948 (350 μM) exceeded 100 μM. It is noteworthy that the apparent slopes of the concentration–response curves of the PDE inhibitors were similar for trequinsin, AAL 05, IBMX and dipyridamole. In the case of the other compounds the slope seemed to be less steep, perhaps suggesting an impaired access of the latter to their target sites in smooth muscle cells or the existence of several mechanisms of action.

Effects of PDE inhibitors on the levels of cAMP and cGMP

Table 1 shows the levels of cAMP and cGMP in rat isolated aorta incubated with PDE inhibitors or with agents stimulating the production of cAMP or cGMP. All these drugs were used under conditions inducing 50% relaxation of the aorta precontracted by serotonin. All the PDE inhibitors tested were found to increase significantly the cAMP levels. This increase was modest and ranged from 1.2–2.2-fold, depending on the compound. Amongst inhibitors, only M&B 22,948, dipyridamole, and to a lesser extent trequinsin, significantly elevated the cGMP levels as well (by 2.6-, 3.3- and 1.4-fold, respectively). Isoprenaline, a relaxing agent known to stimulate vascular adenylate cyclase [42], significantly increased cAMP levels (by 2.0-fold) but not cGMP levels. Conversely, sodium azide, a relaxing agent known to stimulate vascular guanylate cyclase [43], produced a significant rise in cGMP levels (by 1.6-fold) without affecting cAMP levels.

In separate experiments, the effects of forskolin and sodium nitroprusside (SNP), other relaxing agents able to stimulate vascular adenylate cyclase [44] and guanylate cyclase [45], respectively, were investigated under conditions inducing 50% relaxation of the aorta precontracted by $PGF_{2\alpha}$ (2 μM). Under such conditions, forskolin (0.1 μM, 25 min) significantly increased cAMP levels by 1.5-fold (from 58.5 ± 3.2 to 85.0 ± 5.4 fmol/μg DNA, N = 5, $P < 0.01$) without altering cGMP levels, whereas SNP (20 nM, 1 min) significantly increased cGMP levels by 1.7-fold (from 23.1 ± 2.1 to 38.7 ± 2.7 fmol/μg DNA, N = 4, $P < 0.01$) but not cAMP levels.

As a whole, these results show that half-maximal relaxing concentrations of either PDE inhibitors or cyclase stimulants induced a similar moderate increase in cAMP and/or cGMP levels.

Since the levels of cGMP, unlike those of cAMP, were not systematically increased by the different PDE inhibitors, a more detailed study was made of the effects of M&B 22,948, dipyridamole and trequinsin on cGMP levels. M&B 22,948 increased tissue levels of cGMP in a concentration-dependent manner (Fig. 2), a significant 1.7-fold increase being evoked by 5 μM of the inhibitor. The maximal cGMP-increasing effect (3.3-fold) of M&B 22,948 was reached with 50 μM, no further increase being seen with 500 μM. Under the same conditions, M&B 22,948 5, 50 and 500 μM induced mean relaxations of about 10, 20 and 60%, respectively.

The half maximal relaxing concentration of dipyridamole (50 μM) evoked the maximal cGMP-increasing effect of the PDE inhibitor, since dipyridamole 500 μM did not further increase cGMP levels. Yet the tissue could be relaxed by more than 80% in the presence of dipyridamole 100 μM. Similar observations were made with trequinsin, which slightly increased cGMP levels at 0.3 μM (i.e. about the half-maximal relaxing concentration), but did not evoke any further increase at 1 μM, a concentration relaxing the aorta by 85%.

To summarise, the concentration–effect curves of relaxation and increase in cGMP levels were clearly not superimposable in the case of three PDE inhibitors. In particular, the cGMP-increasing effect of M&B 22,948 was clearly dissociated from its relaxing effect, the latter requiring higher concentrations than the former.

Effects of PDE inhibitors in combination with isoprenaline or SNP

The influence of pre-treatment by PDE inhibitors on relaxation responses and on tissue cyclic nucleotide levels elicited by isoprenaline or SNP were investigated. The effects of the most selective inhibitors of cAMP–PDE and cGMP–PDE, respectively rolipram and M&B 22,948, as well as of cilostamide and its analogue AAL 05, two compounds recently described as inhibitors of aortic cAMP–PDE [31], were studied in this regard. They were compared to those of the non-selective PDE inhibitor IBMX. A relatively low concentration (relaxing the precontracted aorta by about 10%, see Materials and

Table 1. Effect of PDE inhibitors and activators of adenylate and guanylate cyclase on cyclic nucelotide levels in rat isolated aorta under conditions inducing 50% relaxation of the precontracted tissue

	cAMP	cGMP	N
Control	100	100	
IBMX	180 ± 34*	146 ± 23	5
Rolipram	166 ± 19*	132 ± 39	6
Ro 20-1724	217 ± 20***	135 ± 27	6
M&B 22,948	134 ± 13*	263 ± 34**	6
Cilostamide	203 ± 17***	119 ± 5	6
AAL 05	122 ± 8*	158 ± 25	4
Trequinsin	158 ± 16*	136 ± 11*	6
Dipyridamole	194 ± 15***	330 ± 24**	6
Isoprenaline	203 ± 22**	129 ± 17	6
Sodium azide	127 ± 9	165 ± 5***	6

Tissues were preincubated in the presence of prazosin (0.1 μM) and serotonin (10 μM), as in relaxation experiments, before the addition of vehicle (control) or one of the relaxant drugs. IBMX (10 μM), rolipram (150 μM), Ro 20-1724 (250 μM), M&B 22,948 (350 μM), cilostamide (35 μM), AAL 05 (8 μM), trequinsin (0.3 μM) and dipyridamole (50 μM) were left in contact for 10 or 15 min; isoprenaline (0.1 μM) for 1 min; sodium azide (80 nM) for 30 sec. Results are the means ± SE of 4–6 determinations made in duplicate and expressed in percentages of their control values. Absolute control values of cAMP and cGMP were: 39.7 ± 2.7 (N = 15) and 13.6 ± 1.8 fmol/μg DNA (N = 14), respectively. Statistical significance: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ versus respective control values.

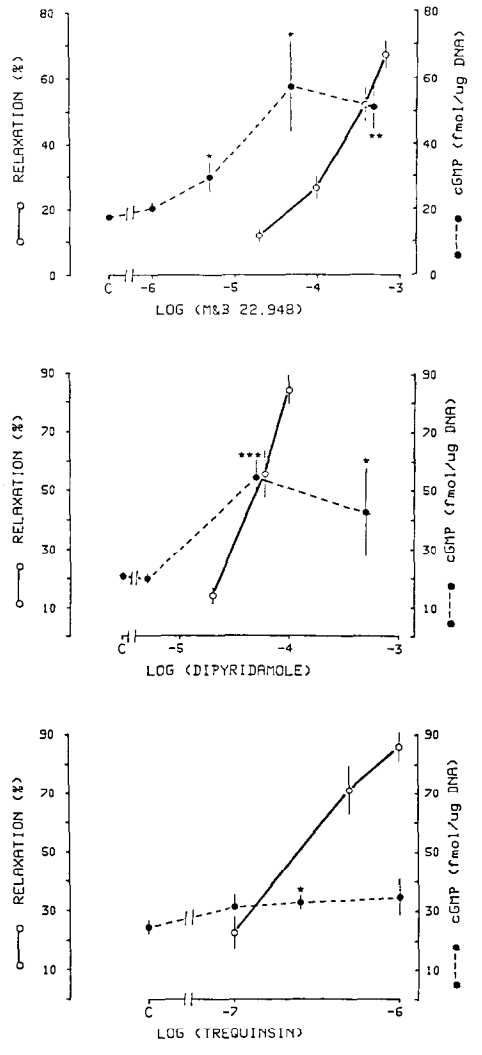

Fig. 2. Relationships between concentration and both relaxing effect and cGMP-increasing effect of M&B 22,948, dipyridamole and trequinsin in rat aorta. Relaxation data are taken from Fig. 1. Cyclic GMP levels were measured under the same conditions as for relaxation experiments and mean values ± SE (vertical bars) of 4–11 determinations are represented. Cyclic GMP levels significantly different from control (C) value are denoted by asterisks: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Methods) of each inhibitor was used, in order to look for potentiation of the effects of the cyclase activators by PDE inhibitors.

Under these conditions, only rolipram (1 μM) and compound AAL 05 (1 μM) significantly ($P < 0.01$ and $P < 0.05$, respectively) enhanced the cAMP-increasing effect of applying a concentration of isoprenaline close to the EC_{50} of the beta-adrenergic agonist (70 nM, 1 min), tested in pairs of aortic rings. In the absence of PDE inhibitor, isoprenaline stimulated an increase in cAMP levels by 1.6 ± 0.1-fold (from 55.7 ± 3.9 to 90.1 ± 6.6 fmol/μg DNA, N = 8). In the presence of rolipram, the stimulation by isoprenaline amounted to 2.1 ± 0.1-fold (from 87.2 ± 7.8 to 182 ± 23 fmol/μg DNA, N = 4). In the presence of compound AAL 05, isoprenaline increased aortic cAMP levels by 2.1 ± 0.2-fold (from 50.0 ± 5.4 to 99.1 ± 7.3 fmol/μg DNA, N = 7).

Using the same concentrations of inhibitors, only rolipram and compound AAL 05 were capable of shifting the concentration–relaxation curve elicited by isoprenaline significantly to the left (Fig. 3). The EC_{50} value of isoprenaline was lowered from 84 ± 17 to 41 ± 18 nM (N = 7, $P < 0.05$) in the presence of rolipram; and from 73 ± 17 to 24 ± 5 nM (N = 10, $P < 0.01$) in the presence of compound AAL 05. IBMX (2.5 μM), the PDE inhibitor most widely used to potentiate the effect of cAMP-generating hormones, did not significantly affect the concentration–response curve to isoprenaline (Fig. 3). Neither did cilostamide (1 μM) or M&B 22,948 (5 μM).

The cGMP-increasing effect of a half-maximal relaxing concentration of SNP (20 nM, 1 min)

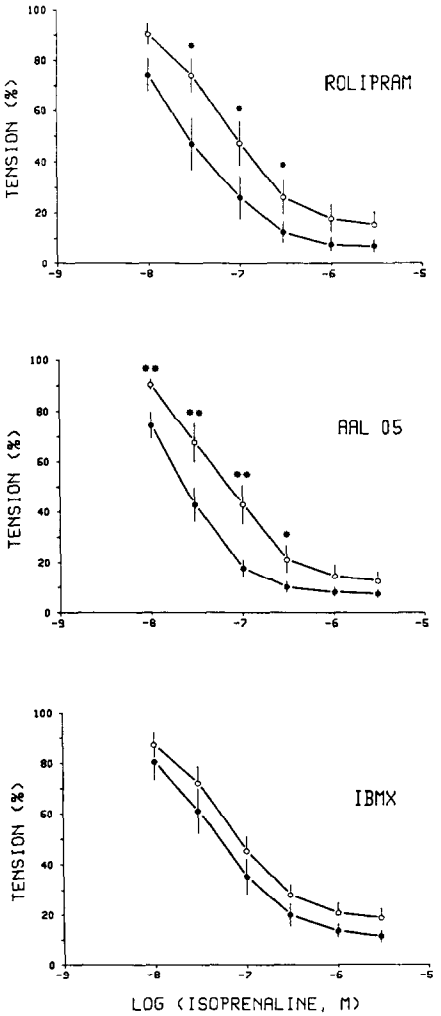

Fig. 3. Concentration–effect curves of the relaxation induced by isoprenaline in $PGF_{2\alpha}$-precontracted rat aorta, in the absence (open symbols) and in the presence (closed symbols) of the indicated PDE inhibitors. Rolipram (1 μM), compound AAL 05 (1 μM) and IBMX (2.5 μM) were left in contact for 10 min before the first addition of isoprenaline. Statistical significance of differences between corresponding values: * $P < 0.05$; ** $P < 0.01$. Vertical bars represent SE of the means of 7–10 values.

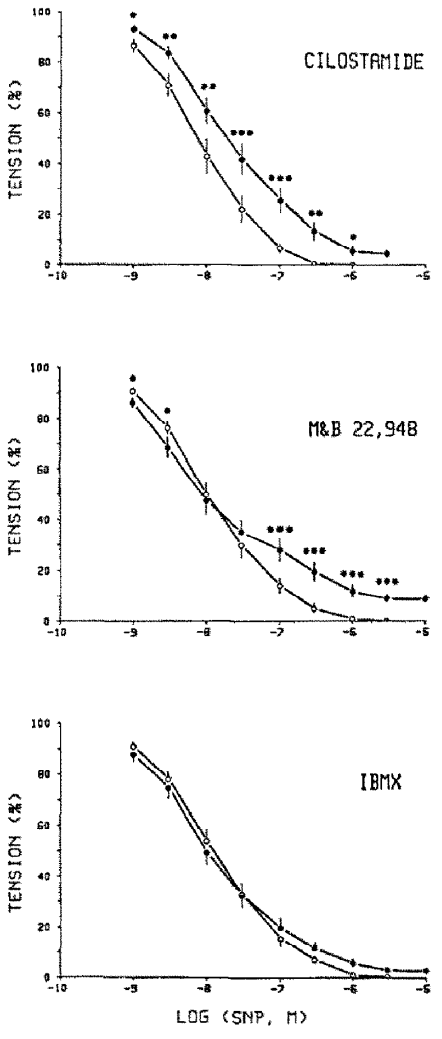

Fig. 4. Concentration–effect curves of the relaxation induced by SNP in $PGF_{2\alpha}$-precontracted rat aorta, in the absence (open symbols) and in the presence (closed symbols) of the indicated PDE inhibitors. Cilostamide (1 μM), M&B 22,948 (5 μM) and IBMX (2.5 μM) were left in contact for 10 min before the first addition of SNP. Statistical significance of differences between corresponding values: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Vertical bars represent SE of the means of 7–9 values.

amounted to 1.7 ± 0.1-fold (from 23.7 ± 1.3 to 39.2 ± 2.0 fmol/μg DNA, N = 7). None of the PDE inhibitors, at a concentration producing 10% relaxation by themselves, was able to enhance this effect of SNP. Under these conditions, cilostamide (1 μM) even significantly ($P < 0.01$) decreased the effect of SNP to 1.3 ± 0.1-fold (from 25.2 ± 2.6 to 33.5 ± 3.2 fmol/μg DNA, N = 4).

The ability of the same concentrations of PDE inhibitors to modify the relaxant effect of SNP was also tested. As illustrated in Fig. 4, in the presence of cilostamide (1 μM) the concentration–relaxation curve elicited by SNP was significantly displaced to the right, the EC_{50} value of SNP being increased from 11 ± 3 to 28 ± 10 nM (N = 9, $P < 0.01$). In addition cilostamide slightly depressed the maximal effect of SNP. Similar, although less marked alterations in the concentration–relaxation curves of SNP were seen with compound AAL 05 (1 μM). M&B 22,948 (5 μM) produced a biphasic alteration in the concentration–relaxation curve of SNP (Fig. 4), slightly but significantly enhancing the effect of lower (< 10 nM) concentrations of SNP and inhibiting the effect of higher concentrations (≥ 0.1 μM). IBMX (2.5 μM) did not significantly modify SNP-induced relaxation responses (Fig. 4).

DISCUSSION

In the recent work, we confirm, using aorta exposed to a contracting stimulus, that PDE inhibitors can increase cAMP and/or cGMP levels according to their inhibitory effects on aortic isolated PDE forms, an observation that has been already made in quiescent tissues [26]. It appears therefore that increase in intracellular Ca^{2+} and subsequent activation of CaM–PDE, reportedly occurring after stimulation of intact vascular smooth muscle [46], did not greatly modify the effect of PDE inhibitors on cyclic nucleotide levels in the rat aorta.

The eight structurally different PDE inhibitors used in the present study were all able to relax precontracted rat isolated aorta with varying potencies. These potencies were correlated ($r = 0.81$, $P < 0.02$) with the potencies of the same compounds as inhibitors of the PDE form specifically hydrolyzing cAMP (cAMP–PDE) in aorta [26]. Inhibition of cAMP hydrolysis by crude PDE preparations has been correlated with vascular relaxation in some [47–50] but not all [5] reports. This apparent discrepancy may be explained by the use of unpurified enzyme preparations. The correlation reported here between relaxing activity and inhibition of isolated cAMP–PDE is consistent with a regulatory role of this enzyme form in aortic contractility. However, such a correlation between results obtained in acellular preparations and those from studies in intact isolated tissues must be taken with caution. Some compounds might penetrate and be distributed within the cell differently from others, as suggested by the observation that the slopes of relaxation curves in rat isolated aorta were not identical for all PDE inhibitors. Of importance therefore is the demonstration that the eight inhibitors tested did actually increase cAMP levels in the intact aorta at concentrations that induced 50% relaxation. This increase was moderate (about two-fold) but quite similar to that produced by agents activating cAMP biosynthesis either by a receptor-mediated mechanism (isoprenaline) or by direct stimulation of the catalytic site of adenylate cyclase (forskolin [51]), and whose effect on cAMP levels temporally and quantitatively paralleled their relaxing effect in rat aorta [37, 52, 53]. As a whole, these results strongly suggest that selective inhibition of cAMP–PDE can lead to relaxation subsequently to cAMP accumulation in aorta. Furthermore, potentiation of the cAMP-increasing effect of isoprenaline by some PDE inhibitors (rolipram, the most selective inhibitor of aortic cAMP–PDE, and AAL 05, a compound active at inhibiting this PDE form [26]) was accompanied by potentiation of the relaxing effect of the beta-adrenergic agent. This observation strengthens the conclusion that cAMP–

PDE have a regulatory role in smooth muscle function.

The relaxing potency of the PDE inhibitors found in the present study was not significantly correlated ($r = 0.19$, $P > 0.10$) with their ability to inhibit the aortic PDE form selectively hydrolyzing cGMP (cGMP–PDE [26]). Moreover, only some of these inhibitors were able to increase cGMP levels (in addition to increasing cAMP levels) in rat aorta under conditions of about half-maximal relaxation. The concentration–effect curves of relaxation and cGMP-increasing effect induced by these inhibitors were not superimposable. Half-maximal relaxing concentrations of dipyridamole and trequinsin significantly increased cGMP levels but no further increase were observed with concentration eliciting a relaxation of 80–85%. In the case of M&B 22,948, the most selective cGMP–PDE inhibitor amongst the eight compounds tested, there was a clear-cut dissociation between relaxation and increase in cGMP levels. A concentration of M&B 22,948 (5 μM) which induced a significant rise in cGMP levels (of the same order of magnitude as that induced by half-maximal relaxing concentrations of SNP and sodium azide) only weakly relaxed the aorta (by 10%). Furthermore, the maximal effect of M&B 22,948 on cGMP levels was reached with 50 μM, a concentration of the drug producing only 20% relaxation. Thus our results do not support the view that selective inhibition of cGMP–PDE can elicit relaxation *per se* in rat aorta. At variance with cGMP accumulation due to cGMP–PDE inhibition, rises in cGMP levels elicited by SNP in rat aorta seem to be well correlated with relaxation [54, 55]. Such a dissociation could find an explanation in a compartmentalization of cGMP within smooth muscle cells, as has been proposed for cAMP in bovine coronary artery [21]. A slight but significant potentiation of the relaxant responses elicited by lower SNP concentrations ($\leq$ 10 nM) could nevertheless be seen here with M&B 22,948 (5 μM). This result is in accordance with observations made by others who observed a more marked potentiating action of the drug on SNP [32] or nitroglycerin [56] relaxant responses. In these studies, however, M&B 22,948 was used at concentrations (185 and 30 μM, respectively) which are able to inhibit the caldomulin-dependent PDE form (CaM–PDE) from aorta in addition to cGMP–PDE [26]. That M&B 22,948 (5 μM) could not potentiate the cGMP-increasing effect of SNP (20 nM) may be due to a counteracting inhibitory action of the former compound on the effect of SNP. In fact, M&B 22,948 (5 μM) inhibited the relaxant responses to higher concentrations of SNP ($\geq$ 0.1 μM), and cilostamide, another PDE inhibitor which antagonized the relaxant responses to SNP, also inhibited the cGMP-increasing effect of the nitro-compound. Alternatively, a compartmentalization of cGMP within smooth muscle cells, as mentioned above, might explain the lack of potentiating effect of M&B 22,948 on SNP-induced cGMP-increasing effect.

The ineffectiveness of IBMX (2.5 μM, a concentration able to relax aorta by about 10% by itself), the unselective and most widely used PDE inhibitor, to potentiate the effects of agents stimulating adenylate or guanylate cyclase, can be variously explained. The concentration of IBMX used could be subthreshold for inhibition of PDE but sufficient to interfere with other mechanisms possibly involved in smooth muscle relaxation. In bovine coronary artery, Lorenz and Wells [57] could not see any potentiating effect of IBMX (at concentrations less than 15 μM) on SNP relaxant responses. Alternatively, IBMX could have inhibitory effects on adenylate and guanylate cyclases, as has been reported for methylxanthines in other tissues [58, 59].

In conclusion, our results strongly support the view that aortic cAMP–PDE regulates a cAMP pool associated with relaxation and hence may control aortic smooth muscle tone. They suggest that inhibition of aortic cGMP–PDE cannot induce relaxation by itself, but may in some instances potentiate the relaxation induced by agents stimulating guanylate cyclase.

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