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SELECTIVE INHIBITION OF cGMP-INHIBITED AND cGMP-NONINHIBITED CYCLIC NUCLEOTIDE PHOSPHODIESTERASES AND RELAXATION OF RAT AORTA

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Abstract—In the supernatant (50,000 *g*, 1 hr) fraction from rat aortic smooth muscle homogenates, approximately 50% of total cAMPE PDE activity was inhibited by OPC 3911 (3 μM), while approximately 20% was inhibited by rolipram (30 μM). A cGMP-inhibited cyclic nucleotide phosphodiesterase (cGI-PDE) was further purified using DEAE chromatography followed by affinity chromatography on the *N*-(2-isothiocyanato)ethyl derivative of cilostamide conjugated to aminoethyl agarose (CIT-agarose). OPC 3911, CI-930, and milrinone, but not rolipram, were potent and selective inhibitors of this enzyme. The PDE-activity in the CIT-agarose flow through fraction (RI-PDE), however, was inhibited potently by rolipram, but not by cGMP, OPC 3911, CI-930 or milrinone. Functional studies showed that OPC 3911, CI-930, and milrinone were potent relaxants of contracted rat aorta. Rolipram had little relaxant effect. When OPC 3911 or milrinone was combined with rolipram more than additive effects on aortic relaxation and cAMP content were obtained. OPC 3911 combined with milrinone had only additive effects. These results demonstrate the presence of a cGI-PDE in rat aortic smooth muscle, and that inhibition of this isozyme may be of primary importance for the relaxant effects of OPC 3911, CI-930, and milrinone. A RI-PDE activity was also found, but it appeared to be less important for modulation of vascular tone unless the cGI-PDE was already inhibited. This may explain the synergistic relaxant effects observed when both PDE-isozymes were inhibited.

Cyclic AMP (cAMP) is believed to be involved in the regulation of vascular smooth muscle tone [1]. The hydrolysis of cAMP is catalysed by cyclic nucleotide phosphodiesterases (PDEs) which are known to exist in multiple molecular forms in most mammalian tissues [2].

A cyclic GMP (cGMP) inhibited PDE (cGI-PDE) has been identified and purified from bovine cardiac muscle [3]. New cardiotonic drugs, assumed to be selective cGI-PDE inhibitors, are currently being investigated in clinical trials as therapeutic agents in the treatment of congestive heart failure [4, 5]. A considerable part of the hemodynamic effects of these drugs is probably due to their actions on the peripheral vasculature. *In vivo* it has been shown that the bipyridine milrinone, one of the newer cardiotonic agents, has direct vasodilator effects [6, 7]. Vasorelaxant actions of milrinone and other presumed cGI-PDE inhibitors, which include the cilostamide derivative OPC 3911 and the dihydropyridazinone derivative CI-930, have been demonstrated in several *in vitro* studies [8–12].

The relaxant effects of the presumed cGI-PDE inhibitors have been correlated with a partially purified PDE located in sarcoplasmic reticulum of cardiac muscle [9] and a partially purified low K_m cAMP PDE from aortic smooth muscle [10]. Thus, the presence of a cGI-PDE in vascular smooth muscle has been suggested. However, another cAMP PDE activity appears to exist in vascular smooth muscle [13, 14]. This PDE (hereafter denoted as RI-PDE) is inhibited by rolipram but not by cGMP [2]. In these earlier studies no attempts were made to distinguish between the contributions of cGI-PDE and RI-PDE activities. Moreover, the functional role for the RI-PDE as a modulator of vascular tone is not clear [15].

In view of the issues outlined above, the objectives of this study were to: (1) separate cGI-PDE and RI-PDE in rat aortic smooth muscle, (2) determine if inhibition of these PDEs is associated with vasorelaxation and (3) assess the relative functional importance of cGI-PDE and RI-PDE by using selective inhibitors.

MATERIALS AND METHODS

Drugs

DEAE-Sephacel was from Pharmacia (Uppsala, Sweden). The heterogeneous non-ionic alkyl polyoxyethylene glycol detergent $C_{13}E_{12}$ (abbreviated as C_nE_x, from the general formula $C_nH_{2n+1}(OCH_2CH_2)_xOH$) was obtained from Berol Kemi AB (Stenungsund, Sweden). *Crotalus atrox* venom, cAMP, cGMP, NaBr, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes), dithioerythriol,

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aminoethylagarose, acetylcholine chloride, phenylephrine and serotonin were from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Leupeptin and antipain were from the Peptide Institute (London, U.K.). [2,8-^{3}H]cAMP (36.4 Ci/mmol) was from New England Nuclear (Dreilich, F.R.G.) and purified by thin-layer chromatography on cellulose with 0.5 M ammonium acetate:ethanol 2.5 (v/v) and on columns of DEAE-Sephadex (from Pharmacia P-L Biochemicals) before use. OPC-3911 (*N*-cyclohexyl-*N*-2-hydroxyethyl-4(6-(1,2-dihydro-2-oxoquinolyloxy)) butyramide was supplied by Dr H. Hidaka of Mie University and Otsuka Pharmaceuticals, Japan; CI-930 (4,5-dihydro-6-[4-(1*H*-imidazol-1-yl)phenyl]-5-methyl-3(2*H*)-pyridazone) by Dr R. Weishaar of Warner Lambert Co. (Ann Arbor, U.S.A.); and the starting compound for the affinity ligand, 4-(1,2-dihydro-2-oxo-6-quinolyloxy)-butyric acid by Drs Akio Sonoda and S. Ayukawa from Otsuka Pharmaceuticals (Osaka, Japan and Rockville, U.S.A.). The *N*-(2-isothiocyanato)ethyl derivative of cilostamide (CIT) was synthesized and coupled to aminoethylagarose as previously described [16]. Milrinone (1,6-dihydro-2-methyl-6-oxo-[3,4'-bipyridine]-5-carbonitrile) and rolipram (4-[3-cyclopentyloxy-4-methoxyphenyl)-2-pyrrolidone) were gifts from Sterling Winthrop Research Institute (Rensselaer, NY) and Schering AG (Berlin, F.R.G.), respectively.

Separation of cGI-PDE and RI-PDE from rat aortic smooth muscle

Sprague–Dawley rats were killed by cervical dislocation. The aortas were quickly excised and after removal of adhering fat, the media and adventitia tissue (from 50 rats) were cut into small pieces and immediately frozen in liquid nitrogen and stored at −70° until further use. The tissue was homogenized in 20 mM Tris, pH 7.5, containing 1 mM EDTA, 250 mM sucrose, 10 μg/mL leupeptin, 10 μg/mL antipain and 10 μg/mL pepstatin using a glass–glass homogenizer. The homogenate (10 mL) was centrifuged (4000 *g* for 10 min) and the resultant supernatant centrifuged at 50,000 *g* for 1 hr. The (50,000 *g*, 1 hr) supernatant was loaded onto a 1 × 1 cm DEAE-Sephacel column previously equilibrated with 100 mM sodium acetate buffer pH 7.0 containing 10% glycerol (w/v), 1 mM EDTA, 1 mM dithioerythriol, 1 μg/mL leupeptin, 1 μg/mL pepstatin and 10 μg/mL antipain (buffer *A*). The column was washed with 5 mL of buffer *A*, whereafter cGI-PDE activity was eluted with 4 mL of buffer *A* containing 250 mM sodium acetate.

The eluate was dialysed for 24 hr with 2 changes of 50 mM Tris, pH 7.5 containing 5 mM $MgCl_2$, 1 mM EDTA, 10% (w/v) glycerol, 1 μg/mL leupeptin and 1 μg/mL pepstatin (buffer *B*). The dialysed material was applied to a 0.5 mL CIT-agarose affinity column previously equilibrated with buffer *B* containing 0.03% $C_{13}E_{12}$ (buffer *C*). The column was washed with 4 mL of 2 M NaBr in buffer *C* and then with 6 mL of 100 mM NaBr in buffer *C*. To elute the enzyme, the gel was incubated for 4 hr with 1 mL of buffer *C* containing 100 mM NaBr and 50 mM cAMP. The CIT-agarose eluate was collected and the gel was washed with 3 mL of 100 mM NaBr in buffer *C*. To remove the cAMP, the CIT-agarose eluated was applied to a small (0.3 mL) DEAE Sephacel column, equilibrated with 2.5 mL of 100 mM NaBr in buffer *C*. After extensive washing the enzyme activity was eluted with 1 mL of 200 mM NaBr in buffer *C*.

Phosphodiesterase assay

Activity was assayed by a modification of a previously described procedure [17]. Samples were incubated at 30° for 20 min in total volume of 0.3 mL containing 50 mM Hepes, pH 7.40, 0.1 mM EDTA, 8.3 mM $MgCl_2$, 0.5 μM [^{3}H]cAMP (≈50,000 cpm). Hydrolysis of substrate did not exceed 20%; under these conditions phosphodiesterase activity was proportional to time and enzyme concentration. For inhibition studies the assay was performed in presence of OPC 3911, CI 930, milrinone, cGMP or rolipram.

Cyclic nucleotide assay

Frozen tissues were stored at −70° until assayed for cAMP and protein content as previously described [15]. Briefly, frozen aortic tissue was homogenized at 4° in 1.2 mL of 10% trichloroacetic acid (TCA) and centrifuged at 2000 *g* (4°) for 15 min. The pellets were reconstituted in 0.5 M NaOH and tested for protein content. The supernatants were extracted with water-saturated diethyl ether, the aqueous phase was evaporated and the residue stored at −20°. Residues were dissolved in 0.05 M sodium acetate, pH 6.2, and the amount of cAMP was quantitated using [^{125}I]cAMP RIA kits (Rianen®, Du Pont). Cyclic nucleotides were acetylated with acetic anhydride to increase the sensitivity of the assay. The final values of cAMP were corrected for recoveries.

Protein determination

Proteins were determined using the Bradford assay with bovine serum albumin as a reference protein [18].

Measurement of aortic tension

Preparation. After the aortas had been cleaned of connective tissues, in some tissues a stream of carbogen was directly insufflated through the lumen from both ends of the vessels, thus destroying the endothelium [19]. Aortas were cut into ring segments 2 mm in length. Tension studies were performed as previously described [20]. Briefly, tissues were mounted between two L-shaped metal holders immersed in temperature-controlled (37°) organ baths containing 5 mL of gassed (95% O_2, 5% CO_2) Krebs solution of the following composition (mM concentration); NaCl 119, $NaHCO_3$ 15, KCl 4.6, $CaCl_2$ 1.5, NaH_2PO_4 1.2, $MgCl_2$ 1.2 and glucose 11 (pH ≈ 7.4). Isometric tension was measured by a Grass Instrument FT 03C transducer connected to a Grass Instrument model 7D polygraph. The preparations were allowed to equilibrate during a period of 1 hr with repeated washings and adjustments of initial mounting tension until a stable tension of 10 mN was maintained.

Procedure. All ring segments were initially contracted by exposure to 124 mM K^+ solution which

was prepared by substituting all NaCl in the standard Krebs solution with equimolar concentrations of KCl. The 124 mM K^+ contractions were repeated and only vessel segments with reproducible contractions (variation less than 10%) were accepted. After a wash-out period (30 min), contractions were induced by 1 μM phenylephrine or 30 μM serotonin (in standard Krebs solution), which in separate experiments had been shown to produce stable contractions (approximately 90% of maximum). A successful de-endotheliazation was confirmed in tissues where no relaxant effect was observed after addition of 1 μM acetylcholine. Following a new wash-out period the contractile agonists were added once again; when the contractions had stabilized (15 min) the relaxant effects were studied by cumulative addition of the various PDE inhibitors. A new concentration was added only when a stable effect had been observed and the time elapsing between each concentrations differed between 10 and 30 min. The concentration response curves for the different PDE inhibitors thus obtained were used for determination of the EC_{50} values. In separate experiments with phenylephrine-contracted aortic rings, cumulative concentration–response curves were obtained for OPC 3911, CI-930 and milrinone in the presence of 10 μM rolipram (added 5 min before the induced contraction).

In a different series of experiments the interactions between OPC 3911, milrinone and rolipram were examined by studying the effects on aortic relaxation and cAMP levels. The chosen concentrations of the PDE inhibitors were such that approximately 10–15% relaxation of the phenylephrine-contracted aorta was obtained. Six segments from one aorta were used for each experiment. After the phenylephrine-induced contraction had stabilized, OPC 3911 and milrinone were each added to two aortic rings. After 10 min rolipram was added to three segments; one treated earlier with OPC 3911, one treated with milrinone and one untreated. The last segment served as control. At the time when maximum relaxation was observed (after an additional 15 min) all tissues were quickly frozen by immersion in liquid nitrogen. In separate experiments on aortic relaxation the interaction between OPC 3911 and milrinone was studied, but the cAMP content was not measured. In additional experiments interactions between rolipram and OPC 3911 or milrinone were examined also in 60 mM K^+-contracted rat aorta (cAMP levels were not measured).

Statistics

The IC_{50} value represents the concentration of the drug that causes 50% inhibition of the enzyme. The EC_{50} value represents the concentration of the agent that produces a 50% relaxation of the previously contracted aorta. The unpaired Student's *t*-test (two-tailed) was used to detect significant differences between groups. Changes in cAMP levels were compared using Student's *t*-test (two-tailed) for paired data. Data are given as the mean ± SEM or means with associated 95% confidence intervals. A P-value less than 0.05 was considered significant.

RESULTS

Separation of cGI-PDE and RI-PDE

Supernatants (50,000 *g*, 1 hr) from homogenates of smooth muscle tissue of 50 rat aortas contained > 90% of total cAMP-PDE activity at 0.5 μM cAMP (negligible cAMP-PDE activity was lost in the initial low-speed centrifugation) and were used as starting material for the separation of cGI-PDE and RI-PDE. Approximately 50% of the enzyme activity in the supernatant was inhibited by OPC 3911 (3 μM), and about 20% was inhibited by rolipram (30 μM), thus indicating the relative amounts of the two enzymes in this fraction. When both OPC 3911 and rolipram (3 and 30 μM, respectively) were present, approximately 90% of the supernatant PDE activity was inhibited. The separation of the two PDE activities by DEAE-Sephacel chromatography,

Table 1. Separation of the cGI-PDE and the RI-PDE from rat aortic smooth muscle

Fraction	Total activity* (pmol/min)	OPC-inhibited activity† (pmol/min)	Total protein (mg)	Specific activity‡ (pmol/min/mg)
50,000 *g* Supernatant	4520	2170	17.9	121
DEAE-Sephacel eluate	2490	1480	3.1	448
CIT-agarose eluate	100	100	ND§	—
CIT-agarose flow-through‖	670	<10	2.8	<4

* Substrate concentration 0.5 μM [^{3}H]cAMP.

† Part of total activity that was inhibited by 3 μM OPC 3911; calculated as the difference of (activity in absence of OPC 3911) − (activity in presence of OPC 3911). Reflects cGI-PDE activity.

‡ Based on OPC 3911 inhibited activity.

§ Not detectable.

‖ The rolipram-inhibited and cGMP-insensitive DPE activity (RI-PDE) was found in this fraction (see also Table 3).

Table 2. Inhibitor values for the cGI-PDE in the CIT-agarose eluate

Inhibitor	IC_{50}^{*} (μM) (95% confidence intervals)
OPC 3911	0.03 (0.02–0.05)
CI-930	0.23 (0.07–0.76)
cGMP	0.30 (0.26–0.40)
Milrinone	0.49 (0.23–1.0)
Rolipram	1200†

The cAMP PDE activity was assayed in the presence or absence of five concentrations of the indicated inhibitors (substrate concentration 0.5 μM [^{3}H]cAMP). IC_{50} values were obtained graphically.

* Values are given as geometric means of duplicate or triplicate determinations and are representative of two to three experiments.

† The value is approximative, not determined accurately because of the weak inhibition observed.

Table 3. Inhibitor values for the RI-PDE in the CIT-agarose flow-through fraction

Inhibitor	IC_{50}^{*} (μM) (95% confidence intervals)
Rolipram	3.5 (1.1–5.1)
Milrinone	22 (14–32)
OPC 3911	130 (75–220)
CI-930	270 (160–460)
cGMP	<15% at 30 μM

The cAMP PDE activity was assayed in the presence or absence of five concentrations of the indicated inhibitors (substrate concentration 0.5 μM [^{3}H]cAMP). IC_{50} values were obtained graphically.

* Values are given as geometric means of duplicate or triplicate determinations of three experiments.

dialysis, and CIT-agarose affinity chromatography is shown in Table 1. The cAMP PDE activity in the CIT eluate was nearly abolished by OPC (1 μM), CI-930 (10 μM), cGMP (10 μM), and milrinone (10 μM) with IC_{50} values as shown in Table 2. The concentration–inhibition curves for these agents were sigmoidal and monophasic (data not shown). Rolipram, however, had little inhibitory effect on the cAMP PDE-activity present in this fraction. These findings demonstrate that the rat aortic smooth muscle cGI-PDE accounted for virtually all the PDE activity measured in the CIT-eluate. Due to the low tissue concentration of the cGI-PDE, the amount of cGI-PDE protein obtained after affinity chromatography on CIT-agarose was too low to be used for protein determinations.

In the CIT-agarose flow-through fraction, rolipram readily inhibited the cAMP PDE-activity, whereas GMP (30 μM) did not affect the rate of hydrolysis of cAMP (Table 3). Hence, this fraction contained the RI-PDE. No attempts to purify the RI-PDE was made. Therefore some low degree of non-specific inhibition of other types of PDEs at the highest concentrations of inhibitor cannot be excluded.

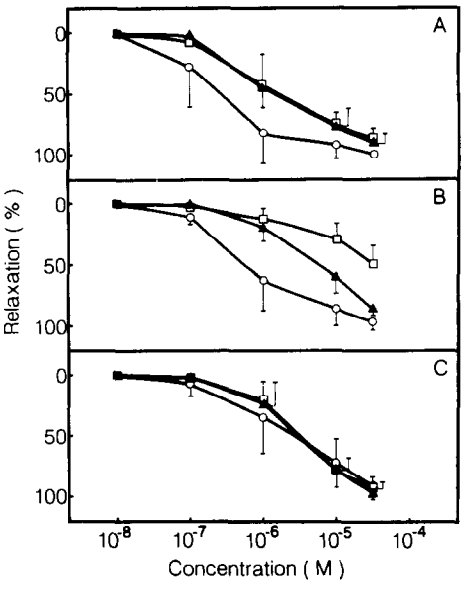

Fig. 1. Relaxant concentration–response curves for (A) OPC 3911, (B) CI-930 and (C) milrinone in rat aortic rings contracted by 1 μM phenylephrine (intact endothelium, ▲; de-endothelialized, □) or 30 μM serotonin (de-endothelialized, ○). Data are given as the mean ± SEM; N = 6–7.

Contractile responses

When 124 mM K^+ was added to rat aortic rings with intact endothelium, a contraction of 13.0 ± 0.5 mN (N = 21) was obtained; in aortic rings with removed endothelium, a plateau of 14.9 ± 0.6 mN (N = 20) was reached. The subsequent addition of 1 μM phenylephrine caused a contraction amounting to (in per cent of the previous 124 K^+-contraction) 92.5 ± 2.7% in aortic segments with intact endothelium and 107.9 ± 2.1% in aortic rings with removed endothelium ($P < 0.001$). Correspondingly, in aortic segments with removed endothelium 30 μM serotonin produced a contraction which was 115.7 ± 2.0% of the preceding 124 mM K^+-contraction.

Relaxant effects

Cumulative concentration–response curves. OPC 3911, CI-930, and milrinone caused concentration-dependent relaxation of aortic rings contracted with either phenylephrine or serotonin (Fig. 1). In contrast, rolipram had slight relaxant effects (Table 4). Relaxation of phenylephrine-contracted aortic rings produced by OPC 3911 and milrinone was not affected by the presence or absence of intact endothelium (Fig. 1; Table 4). However, the relaxant effects of CI-930 were significantly reduced when the endothelium had been removed (Fig. 1; Table 4). The milrinone-induced relaxation was independent of the contractile agonist, whereas both OPC 3911 and CI-930 were significantly more potent in serotonin-contracted aortic rings compared with phenylephrine-contracted aortic rings (Fig. 1; Table 4).

Table 4. Comparison of the relaxant effects of the PDE inhibitors on 1 μM phenylephrine- or 30 μM serotonin-contracted rat aortic rings

	EC_{50} (95% of confidence intervals)* (μM)							
	Phenylephrine†					Serotonin†		
	Intact endothelium	N	Removed endothelium	N	Removed endothelium (10 μM rolipram present)	N	Removed endothelium	N
OPC 3911	1.5 (0.81–2.8)	7	1.6 (0.83–3.5)	7	0.09 (0.04–0.17)¶	8	0.20 (0.06–0.68)‡	6
CI-930	6.0 (3.5–10)	7	34 (19–66)§	7	0.28 (0.19–0.42)¶	9	0.71 (0.32–1.7)‖	6
Milrinone	2.8 (1.7–4.9)	7	3.2 (2.0–5.1)	7	0.56 (0.38–0.83)¶	8	2.8 (0.9–8.7)	6
Rolipram	19% at 30 μM**	7	2% at 30 μM**	7	—		20% at 30 μM**	6

* EC_{50}, concentrations causing 50% relaxation.
† Contractile agonist.
‡ Significantly different ($P < 0.05$) as compared with EC_{50} values in aortic rings with removed endothelium contracted by other agent.
§ Significantly different ($P < 0.01$) as com pared with EC_{50} values in aortic rings with intact endothelium contracted by same agent.
‖ Significantly different ($P < 0.001$) as compared with EC_{50} values in aortic rings with removed endothelium contracted by other agent.
¶ Significantly different ($P < 0.001$) as compared with EC_{50} values in aortic rings with removed endothelium contracted by same agent but in the absence of rolipram.
** Agent failed to produce 50% relaxation.

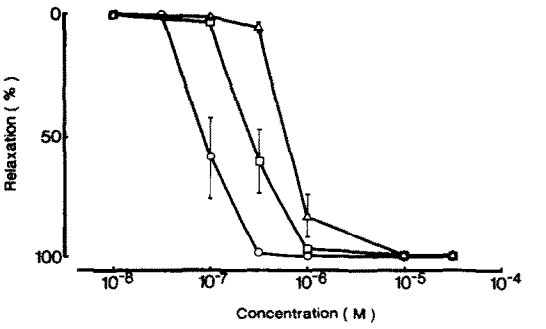

Fig. 2. Relaxant concentration–response curves in the presence of 10 μM rolipram for OPC 3911 (○), CI-930 (□) and milrinone (△) in de-endothelialized rat aortic rings contracted by 1 μM phenylephrine. Data are given as the mean ± SEM; N = 8–9.

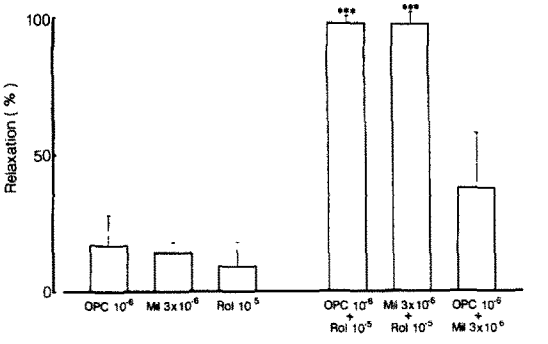

Fig. 3. Relaxation of de-endothelialized rat aortic rings contracted by 1 μM phenylephrine produced by addition of OPC 3911 (OPC), milrinone (Mil) or rolipram (Rol). Relaxation was measured as described in methods following the addition of 1 μM OPC 3911, 3 μM milrinone or 10 μM rolipram alone (left side) or in the indicated combinations (right side). ***$P < 0.001$ as compared with the values calculated by assuming the additivity of the drug-induced relaxation. Data are given as the mean ± SEM; N = 7–8.

To eliminate the influence of the RI-PDE on the vasorelaxation produced, another set of experiments was performed with rolipram present when the aortic rings were contracted by 1 μM phenylephrine. The presence of 10 μM rolipram, which had negligible relaxant effect by itself, produced a significant leftward shift of the concentration–response curves for OPC 3911, CI-930 and milrinone (Fig. 2; Table 4).

Interactions between OPC 3911, milrinone and rolipram added via single dose fashion. Aortic relaxation: OPC 3911, milrinone and rolipram were added at concentrations which resulted in 10–15% relaxation of phenylephrine-contracted aortic rings. When rolipram was combined with either OPC 3911 or milrinone almost complete relaxation, i.e., back to baseline, was observed (Fig. 3). This amount of relaxation was significantly greater than that predicted from the additive effects of the drugs. Similar synergistic effects were also obtained when

Table 5. Effects of OPC 3911, milrinone, and rolipram on cAMP levels in rat aortic rings

Inhibitor	Concn (μM)	cAMP (pmol/mg protein)*	N†
Control	—	8.4 ± 1.5	8
OPC 3911	1	11.9 ± 1.5	7
Milrinone	3	11.8 ± 1.7	8
Rolipram	10	10.3 ± 1.1	8
OPC 3911 + rolipram	1 10	17.3 ± 2.4‡	7
Milrinone + rolipram	3 10	16.7 ± 2.6‡	8

* Mean values ± SEM.

† Number of experiments using paired tissues.

‡ $P < 0.05$ compared with the values calculated assuming that changes produced in paired tissues by rolipram and by OPC 3911 or milrinone were additive.

the aortic rings were contracted by 60 mM K^+-depolarization (data not shown). OPC 3911 combined with milrinone had only additive effects (Fig. 2).

cAMP measurements: At the concentrations used, none of the PDE-inhibitors caused increases in cAMP levels large enough to become statistically significant (Table 5). The combination of OPC 3911 and rolipram as well as the combination of milrinone and rolipram, produced increases in cAMP levels that were significantly greater than those predicted from the additive effects of the drugs (Table 5).

DISCUSSION

These results demonstrate in rat aortic smooth muscle the presence of a cGI-PDE activity, which was potently and completely inhibited by drugs such as OPC 3911 > CI-930 > milrinone (order of potency), but not by rolipram. A RI-PDE activity was demonstrated in the CIT-agarose flow-through fraction. The inhibitory properties of the rat aortic cGI-PDE were similar to those of the bovine heart [3], bovine and human platelets [21, 22], rat and bovine adipose tissue [16, 23]. Characterization of the rat aortic cGI-PDE protein was not possible due to the low tissue concentration of the enzyme. Using the same purification procedure, but in a larger scale, the bovine aortic smooth muscle cGI-PDE has been purified to apparent homogeneity.* Therefore, inhibition studies on the CIT-agarose eluate represent inhibition of the cGI-PDE with no contamination by other types of PDEs.

The cGI-PDE inhibitors caused concentration-dependent relaxation of contracted rat aortic rings. OPC 3911, which was the most potent cGI-PDE inhibitor, was consistently found to be the most potent relaxant of rat aorta. In contrast, rolipram had little relaxant effects. The differences in vasorelaxant effects of the two drugs may reflect the relative contributions of cGI-PDE and RI-PDE. OPC 3911 inhibited approximately 50% of total smooth muscle cAMP-PDE activity present in the aorta supernatant (50,000 g, 1 hr), whereas rolipram inhibited approximately 20%.

Inhibition of cGI-PDE seems to be closely linked to vasorelaxation, at least in aortic smooth muscle [present study, 9, 10, 15]. OPC 3911, CI-930, and milrinone were much more potent inhibitors (50–4000-fold) of the cGI-PDE than of the RI-PDE. OPC 3911 and CI-930 had little or no inhibitory effects on the RI-PDE at the concentrations used in the functional studies. On the other hand, the IC_{50} value for inhibition of the RI-PDE by milrinone ($\approx$22 μM) was approximately the same as the highest concentration of milrinone (30 μM) used in the experiments with aortic relaxation. Thus, inhibition of the RI-PDE, in addition to inhibition of the cGI-PDE, may have enhanced the relaxation caused by milrinone (see later), whereas this effect probably was of little importance for both OPC 3911 and CI-930. This suggestion is strengthened by the observation that in the presence of rolipram, there was a leftward shift of the concentration–response curves which was more pronounced for OPC 3911 and CI-930 than for milrinone. This may at least partly explain why there was not a perfect correlation between functional effects of these agents and their potency for inhibition of the cGI-PDE. In rat aorta contracted by serotonin, but not by phenylephrine, there was a rather good agreement between vasorelaxation and cGI-PDE inhibition. However, when the influence of the RI-PDE was eliminated by the presence of rolipram during phenylephrine-induced contractions the relaxing activity of the compounds corresponded well with their inhibitory effects on the cGI-PDE.

The vasorelaxation produced by milrinone was independent of both the contractile agonist used (phenylephrine or serotonin) and of an intact endothelium. This finding is in agreement with the ones previously reported by Kauffman et al. [9]. Milrinone at a concentration of 100 μM inhibits a low K_m cGMP PDE and significantly increases cGMP levels in aortic smooth muscle [9, 10]. An inhibitor of such a low K_m cGMP PDE has been shown to have reduced relaxant effects after removal of the endothelium-dependent stimulation of cGMP production [24, 25]. In the present study, however,

* Rascón A, Lindgren S, Stavenow L, Belfrage P, Andersson K-E, Manganiello VC and Degerman E, unpublished observations.

it is less likely that inhibition of the cGMP-PDE was involved in the vasorelaxant actions of milrinone, since the concentration–response curve was not affected by de-endotheliazation. Furthermore, at a concentration of 30 μM none of the presently investigated cGI-PDE inhibitors had any significant effects on cGMP levels (unpublished observations). The vasodilator actions of OPC 3911 were also independent of intact endothelium, whereas the relaxant effects of CI-930 were significantly reduced after removal of the endothelium. These somewhat inconsistent data are difficult to explain and further studies appear to be justified.

Both OPC 3911 and CI-930 more effectively relaxed contractions induced by serotonin than by phenylephrine. Others have also observed that the contractile agonist used may modulate the relaxant effect obtained by a PDE inhibitor. Silver and Harris [26] found that the relaxant potency of zaprinast, a cGMP-PDE inhibitor, was greatly enhanced when guinea pig aorta was contracted by an equieffective concentration of $PGF_{2\alpha}$ instead of phenylephrine. As suggested by these investigators, it is possible that the vasorelaxant importance of PDE inhibitors is related to the different signalling mechanisms that operate for different vasoconstrictors. When compared with OPC 3911 and CI-930, the somewhat dissimilar selectivity of milrinone for the RI-PDE may explain the differences in effect of these drugs on contractions induced by phenylephrine or serotonin in the present study.

Experiments were also performed where the agents were added in a single-dose fashion. It was found that the induced relaxation was less than expected when compared with the relaxant response obtained by cumulative addition of the drugs. Similar observations have been made by Kauffman *et al.* [9], who suggested that a relatively slow passage of the PDE inhibitors across the cell membrane may be involved.

The synergistic effects observed on both aortic relaxation and increases in cAMP levels when OPC 3911 or milrinone was combined with rolipram are noteworthy. OPC 3911 combined with milrinone had only additive relaxant effects (cAMP content was not measured). Our interpretation of the data is that hydrolysis of cAMP in the rat aorta is mainly regulated by the cGI-PDE activity. When this PDE activity is inhibited by either OPC 3911 or milrinone, the otherwise less prominent RI-PDE becomes apparent. This may be caused by cAMP-dependent phosphorylation and activation of the RI-PDE [27] or by increased 'shunting' of cAMP to the RI-PDE. Thus, when both PDE activities are inhibited accumulation of cAMP is enhanced. Indeed, approximately 90% of the cAMP hydrolysis in the aorta supernatant was inhibited when both OPC 3911 and rolipram were present. In comparison, the value calculated by assuming the additivity of the drug-induced inhibition would be 70% (see above). At the chosen concentrations of the drugs, none of the agents added separately caused a significant increase in cAMP levels even though significant relaxant effects were observed. Similar observations have been made previously in airway and vascular smooth muscle, where dissociations between functional effects and changes in cAMP levels were noted [10, 16, 28]. Measuring activation of cAMP-dependent protein kinase may be a more sensitive indicator of activation of the cAMP system [29].

Recently, it was shown that milrinone, but not OPC 3911, at a concentration of 30 μM produced a significant increase in cAMP levels in rat aorta [15]. Furthermore, it has been reported that milrinone (100 μM) caused a dramatic increase in cAMP content as compared with some other presumed cGI-PDE inhibitors [9]. One explanation for the finding that at high concentrations of the agents, milrinone has more pronounced effects on cAMP levels than OPC 3911, may be that milrinone also inhibits the RI-PDE.

Synergistic effects have been observed between OPC 3911 and rolipram (unpublished observations) and between SKF 94120 and rolipram [30] in increasing guinea pig cardiac papillary muscle contractility. The possibility of synergism between milrinone and rolipram has not been investigated. It has been shown that milrinone produces a biphasic concentration–response curve for increase in isometric force in cardiac preparations [31]. It was recently suggested that the biphasic contractile response to milrinone is caused by inhibition of a peak III PDE at low concentrations and a peak I PDE at high concentrations [29]. The so-called peak III PDE would correspond to the cGI-PDE in the present study, even though contamination by RI-PDE was not excluded, whereas peak I PDE represents still another PDE activity. However, the increase in force noted at high concentrations of milrinone may also be due to inhibition of RI-PDE in addition to inhibition of cGI-PDE which occurs at low concentrations of milrinone. Thus, if milrinone was combined with rolipram, synergistic effects would be expected at low concentrations of milrinone, but not at high concentrations. Preliminary results imply that this assumption may be correct [32].

In summary, the results demonstrate the existence of cGI-PDE and RI-PDE isozymes in rat aortic smooth muscle. It is suggested that the hydrolysis of cAMP is regulated mainly by the cGI-PDE activity and that the RI-PDE is of less importance unless the cGI-PDE is inhibited. This may explain the synergistic effects on tension and cAMP content observed when both PDE activities were inhibited.

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