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Role of selective cyclic GMP phosphodiesterase inhibition in the myorelaxant actions of M&B 22,948, MY-5445, vinpocetine and 1-methyl-3-isobutyl-8-(methylamino)xanthine

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1 The mechanism by which M&B 22,948, MY-5445, vinpocetine and 1-methyl-3-isobutyl-8-(methylamino)xanthine (MIMAX), which have been described as selective cyclic GMP phosphodiesterase (PDE) inhibitors, relax rat aorta was investigated.

2 Three cyclic nucleotide PDEs were identified in the soluble fraction of rat aorta; a Ca^{2+}-insensitive form exhibiting substrate selectivity for cyclic GMP (cGMP PDE), a Ca^{2+}/calmodulin-stimulated form which also preferentially hydrolyzed cyclic GMP (Ca^{2+} PDE), and a form demonstrating substrate selectivity for cyclic AMP (cAMP PDE).

3 M&B 22,948 and MIMAX inhibited cGMP PDE ($K_i = 0.16\,\mu M$ and $0.43\,\mu M$, respectively) and Ca^{2+} PDE ($K_i = 9.9\,\mu M$ and $0.55\,\mu M$, respectively), but exhibited weak activity against cAMP PDE ($K_i = 249\,\mu M$ and $42\,\mu M$, respectively). MY-5445 selectivity inhibited cGMP PDE ($K_i = 1.3\,\mu M$) and vinpocetine selectively inhibited Ca^{2+} PDE ($K_i = 14\,\mu M$).

4 M&B 22,948 and MIMAX induced dose-dependent increases in the accumulation of cyclic GMP, but not cyclic AMP, in rat aorta pieces. These effects were greatly reduced by endothelial denudation and by methylene blue ($5\,\mu M$) which blocks the actions of endothelium-derived relaxant factor. MY-5445 and vinpocetine had no effect on rat aorta cyclic GMP or cyclic AMP accumulation.

5 All four compounds caused dose-related relaxation of 5-hydroxytryptamine ($10\,\mu M$) contracted, endothelium-intact rat aorta, the effects of M&B 22,948 and MIMAX being greatly reduced by methylene blue ($5\,\mu M$). Methylene blue also caused 10 fold and 100 fold rightward shifts in the dose-response curves of MY-5445 and vinpocetine, respectively.

6 The results are consistent with the smooth muscle relaxant actions of M&B 22,948 and MIMAX, but not vinpocetine and MY-5445, being mediated through a mechanism involving inhibition of cyclic GMP hydrolysis.

Introduction

Multiple forms of cyclic nucleotide phosphodiesterase (PDE) exist in mammalian tissues (Weishaar *et al.*, 1985). In vascular smooth muscle, several PDE forms have been identified: a Ca^{2+}/calmodulin-insensitive isoform exhibiting substrate selectivity for guanosine 3′:5′-cyclic monophosphate (cyclic GMP) (cGMP PDE), a Ca^{2+}/calmodulin-stimulated activity which also preferentially hydrolyzes cyclic GMP (Ca^{2+} PDE), and an isoform demonstrating substrate selectivity and high affinity for cyclic AMP (cAMP PDE) (Lugnier *et al.*, 1986). Recently, Reeves *et al.* (1987) have indicated that a second cAMP PDE isozyme corresponding to the type IV activity in cardiac muscle is present in smooth muscle from pig aorta and pulmonary artery. It is possible that these PDE isoforms are compartmentalized and each associated with discrete intracellular pools of cyclic AMP or cyclic GMP. The relevance of the different PDE isozymes to the physiological control of cyclic nucleotides remains to be established.

The development of selective inhibitors may provide useful investigative tools for delineating the roles of the PDE isoforms in regulating tissue cyclic AMP and cyclic GMP levels. Several studies (Lorenz & Wells, 1983; Hagiwara *et al.*, 1984b; Schoeffter *et al.*, 1987) have provided evidence that 'second-

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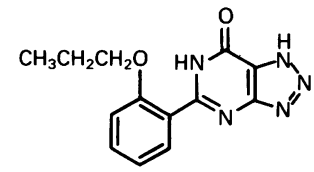
generation' PDE inhibitors exert selective effects on cyclic nucleotide metabolism. One such compound is M&B 22,948 which has proved useful in studying the role of cyclic GMP in regulating smooth muscle contractility. This azapurinone inhibits both cGMP PDE and Ca^{2+} PDE activities while exhibiting weak activity against cAMP PDE (Lugnier *et al.*, 1986). Levels of cyclic GMP, but not cyclic AMP, are elevated in vascular smooth muscle incubated with M&B 22,948 (Lugnier *et al.*, 1986; Martin *et al.*, 1986). Furthermore, M&B 22,948 potentiates the relaxant activities of agents whose actions are mediated by cyclic GMP but not those working via cyclic AMP (Martin *et al.*, 1986).

Relatively little information is available on other selective inhibitors of cyclic GMP hydrolysis. Lorenz & Wells (1983) identified a number of alkylated xanthines which increase bovine coronary smooth muscle cyclic GMP, but not cyclic AMP, and potentiate the relaxant action of sodium nitroprusside (SNP), which is mediated by cyclic GMP, but not that of isoprenaline, which is mediated by cyclic AMP. These xanthines inhibited an activity which probably comprised unresolved cGMP PDE and Ca^{2+} PDE, but only weakly inhibited cAMP PDE. The smooth muscle relaxant actions of vinpocetine have been ascribed to selective inhibition of Ca^{2+} PDE (Hagiwara *et al.*, 1984a). Smooth muscle relaxant activity has also been demonstrated for MY-5445 which is a selective inhibitor of cGMP PDE activity (Hidaka *et al.*, 1984). The structures of these compounds are presented in Figure 1. Theoretically at least, because of their selective inhibitory actions, MY-5445 and vinpocetine could prove useful in elucidating the respective roles of cGMP PDE and Ca^{2+} PDE in regulating cyclic GMP levels in smooth muscle.

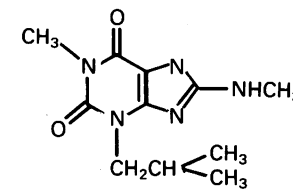
In the present studies, we have compared the effects of M&B 22,948, MY-5445, vinpocetine and 1 - methyl - 3 - isobutyl - 8 - (methylamino)xanthine (MIMAX), one of the more potent and selective inhibitors of cyclic GMP hydrolysis reported by Wells *et al.* (1981), on rat aorta relaxation and cyclic nucleotide levels. Since selective inhibitors of cyclic GMP hydrolysis should potentiate the actions of endothelium-derived relaxing factor (EDRF), which is believed to relax smooth muscle by stimulating guanylate cyclase (Martin *et al.*, 1986), we have investigated whether any endothelium-dependent component is associated with the relaxant effects elicited by the compounds.

Methods

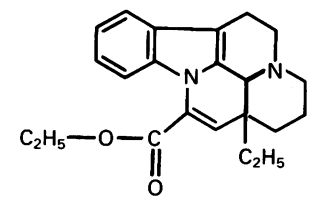
Experiments were conducted on aortae from male Wistar rats (250–400 g) purchased from Charles River U.K. Ltd. (Margate, Kent).



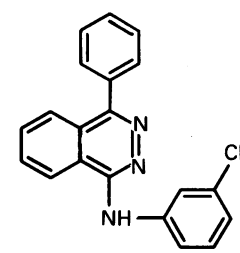
M&B22,948



MIMAX



Vinpocetine



MY-5445

Figure 1 Chemical structures of the phosphodiesterase inhibitors.

Partial purification of rat aorta phosphodiesterase activities

PDE activities were separated from rat aorta smooth muscle essentially according to the method of Lugnier *et al.* (1986). Briefly, aortae were excised from rats and extraneous tissue removed. The blood vessels were then cut open longitudinally and the intimal surface rubbed with a cotton swab to remove the endothelium. The tissue was then minced with

scissors before being homogenized in 6 vol. 20 mM Tris-HCl (pH 7.5), 2 mM magnesium acetate, 1 mM dithiothreitol, 5 mM EDTA, 2000 U ml^{-1} aprotinin, first with an Ultra Turrax (3 × 30 s bursts) and then with a scintered glass pestle homogenizer. The homogenate was then centrifuged at 105,000 *g* for 60 min and the supernatant (20 ml) applied to a DEAE-trisacryl column (IBF, Villeneuve La Garenne, France) pre-equilibrated with column buffer (Tris-HCl 20 mM, magnesium acetate 2 mM, dithiothreitol 1 mM, TLCK (N^{α}-tosyl-L-lysylchloromethane hydrochloride) 20 μM, pH 7.5). The column was washed with 50 ml of column buffer and PDE activities were eluted with 2 successive linear gradients of NaCl (0–150 mM, 210 ml and 150–400 mM, 140 ml) in column buffer; 2 ml fractions were collected and assayed and for long-term storage at −20°C, ethylene glycol was added to a final concentration of 30% (v/v). Activity was stable for several weeks under these conditions.

Measurement of phosphodiesterase activity

PDE activity was determined by the two step, radioisotope method of Thompson *et al.* (1979). The reaction mixture contained 20 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 4 mM 2-mercaptoethanol and 0.05 mg ml^{-1} bovine serum albumin. Unless otherwise stated, the concentration of substrate ([^{3}H]-cyclic AMP or [^{3}H]-cyclic GMP) was 1 μM. [8-^{3}H]-cyclic GMP (13.8 Ci $mmol^{-1}$) and [2, 8-^{3}H] cyclic AMP (41 Ci $mmol^{-1}$) were purchased from Amersham International (Amersham, Bucks).

For the determination of V_{max} and K_m values the concentration of cyclic AMP or cyclic GMP was varied while the amount of ^{3}H-labelled cyclic nucleotide remained constant. The data were analysed by non-linear least squares regression analysis to obtain values of K_m and V_{max}.

Determination of inhibition constants was carried out by selecting a grid of substrate and inhibitor concentrations which spanned the K_m and estimated K_i values of the enzyme. The data were again fitted by non-linear least-squares regression analysis to obtain the K_m and V_{max} values from which the K_i values were calculated.

Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin used as the standard.

Measurement of cyclic GMP and cyclic AMP

Rat aorta cyclic nucleotides were determined by a modification of the method described previously by Bowman & Drummond (1984). Briefly, thoracic aortae were removed from rats, cleaned of extraneous tissue, cut transversely into 4 segments and incubated in 2 ml of Krebs-Henseleit bicarbonate solution (in mM: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.6, $NaHCO_3$ 24.9, KH_2PO_4 1.2, glucose 5.6, pH 7.4) gassed with 95% O_2:5% CO_2.

Incubations were performed with intact rings of arota where the endothelium had been preserved or with aortic strips without endothelium which, prior to segmenting, had been cut open longitudinally and rubbed gently with a cotton swab. Before addition of test reagents, tissue pieces were pre-incubated for 120 min during which time the medium was changed once after 60 min. To terminate incubations, pieces of aorta were quickly removed from the medium and plunged into liquid N_2 before being thawed and homogenized with a glass pestle homogenizer in 1 ml of 5% trichloroacetic acid (TCA).

The homogenate was centrifuged at 2000 *g* for 15 min and the supernatant removed to a clean tube. TCA was removed with 3 washes of H_2O saturated ether (5 vols). The last traces of ether were removed by gassing with N_2, and sodium acetate (pH 6.2) was added to a final concentration of 50 mM. Samples were acetylated and cyclic AMP and cyclic GMP quantified by radioimmunoassay (RIA, NEN Chemicals GmbH). Protein was extracted from the TCA pellets by adding 2 ml 0.1 M NaOH containing 0.1% Triton X-100 (Rohn & Haas) and heating to 100°C in a boiling water bath and was measured in cooled samples by the method of Lowry *et al.* (1951).

Smooth muscle relaxant studies

Male Wistar rats (350–400 g) were killed by stunning and exsanguination. The aortae were carefully removed, cleaned of fat and extraneous tissue and cut transversely (5 mm) and longitudinally. The strips were mounted under 2 g of resting tension in 20 ml muscle chambers and bathed in Krebs Henseleit physiological salt solution (pH 7.4) at 37°C and gassed with 95% O_2:5% CO_2. Tension was measured isometrically using FT03 Grass force-displacement transducers and displayed on a Lectromed recorder. Tissues were equilibrated for 60 min before contraction was induced by 5-hydroxytryptamine (5-HT) (10 μM). Test compounds were added to the organ bath in increasing concentrations, each successive addition being made after maximum relaxation to the previous concentration had been achieved. Relaxation was expressed as percentage of relaxation of 5-HT-induced tone. The concentration of agent giving 50% relaxation (EC_{50}) was estimated by interpolation on the curve drawn through the mean data points for relaxation as a function of log concentration. The presence of an intact endothelium was verified by the ability of acetylcholine (1 μM) to induce relaxation.

Table 1 Kinetic properties of rat aorta smooth muscle phosphodiesterase (PDE) activities

Enzyme	K_m for cyclic GMP (μM)	K_m for cyclic AMP (μM)	Ratio V_{max} for cyclic GMP / V_{max} for cyclic AMP
cGMP PDE	0.24 ± 0.04	10.89 ± 1.38	1.09 ± 0.23
Ca^{2+} PDE − CaM	2.61 ± 0.91	2.81 ± 1.02	1.25 ± 0.33
Ca^{2+} PDE + CaM	2.47 ± 0.40	1.82 ± 0.06	2.19 ± 0.69
cAMP PDE	ND	0.27 ± 0.04	ND

Cyclic nucleotide PDE activities were separated from the cytosolic fraction of rat aorta as shown in Figure 2. The kinetic constants were determined as described in the methods section. Ca^{2+} PDE was assayed in the absence (−) and presence (+) of Ca^{2+} (2 μM) and calmodulin (2.5 u) (CaM). The results represent the means ± s.e.mean of kinetic parameters calculated from data obtained in 4 different experiments with different batches of enzymes. ND = not determined.

Drugs

M&B 22,948 (2-*o*-propoxyphenyl-8-azapurine-6-one), 1-methyl-3-isobutyl-8-(methylamino)xanthine (MIMAX) and MY-5445 [1-(3-chloroanilino)-4-phenylphthalazine] were synthesized by the Department of Pharmaceutical Chemistry, Rhône-Poulenc Ltd. Vinpocetine [ethyl(3α, 16α) eburnamen-14-carboxylate ethyl apovincamine-22-oate] was a gift from Ayerst Laboratories Research, Inc. (Princeton, NJ, U.S.A.). SK&F 94120 {5-(4-acetamido-phenyl) pyrazine-2[1H]-one} was a gift from Smith Kline and French Research Ltd., Welwyn, Herts. and Rolipram (ZK 62711; 4-(3-cyclopentyl-oxy-4-methoxy phenyl)-2-pyrrolidone) was generously provided by Schering A.G. (Hounslow, Middx.). 5-Hydroxytryptamine, methylene blue and acetylcholine were purchased from Sigma Chemical Co. (Poole, Dorset).

Statistical analysis

Data are presented as means ± standard error of the mean (s.e.mean) and analysed by Student's *t* test. Values were considered to be statistically significant at $P < 0.05$.

Results

Resolution of phosphodiesterase activities

In agreement with previous studies (Hagiwara *et al.*, 1984a; Lugnier *et al.*, 1986) three PDE activities could be distinguished in the cytosol from rat aorta following ion-exchange chromatography on DEAE-trisacryl and assaying with 1 μM substrate (Figure 2). For the purposes of this paper, the peaks of activity, in eluting order, have been designated cGMP PDE, Ca^{2+} PDE and cAMP PDE. Interestingly, the elution order of the cGMP PDE and Ca^{2+} PDE was the reverse of that found by Lugnier *et al.* (1986), although the same elution conditions were employed. The reason for this discrepancy is not clear. The total recovery of PDE activity from the column, measured with 1 μM substrate was greater than 90% for cAMP PDE and for cGMP PDE.

The kinetic parameters (K_m, V_{max} for cyclic GMP/V_{max} for cyclic AMP) of the three PDE isoforms are presented in Table 1. cGMP PDE exhibited high affinity and selectivity for cyclic GMP as substrate and was uninfluenced by addition of Ca^{2+}/calmodulin. Ca^{2+} PDE exhibited similar K_ms for the two substrates but the V_{max} for cyclic GMP was over 2 fold greater than against cyclic AMP in the presence of Ca^{2+}/calmodulin. Addition of Ca^{2+}/calmodulin to this isoform increased V_{max} (2 fold) without altering the K_m.

In agreement with previous studies (Lorenz & Wells, 1983; Hagiwara *et al.*, 1984a; Lugnier *et al.*, 1986), only one cytosolic cAMP PDE activity could be distinguished in rat aorta. This contrasts with pig aorta in which two cAMP PDE isoforms have been demonstrated with the same chromatrographic conditions (B.K. Diocee & J.E. Souness, unpublished data). On the basis of their kinetic properties and susceptibility to inhibition by rolipram, SK&F 94120 and cyclic GMP, these pig aorta PDE activities appeared to correspond to the Type III and Type IV PDEs identified in cardiac muscle (Reeves *et al.*, 1987). SK&F 94120 inhibited the cytosolic cAMP PDE in rat aorta but its potency (IC_{50} = 140 μM) was much weaker than against the heart type III enzyme (IC_{50} = 3.7 μM) (Reeves *et al.*, 1987). Rat aorta cAMP PDE was also inhibited by cyclic GMP (IC_{50} = 0.2 μM using 0.25 μM substrate) but activity was only slightly reduced by rolipram (30% inhibition at 1 mM). These data indicate the presence of a PDE activity in rat aorta which resembles the Type III isoform in cardiac muscle. Whether any Type IV enzyme is present as a minor contaminant of the cAMP PDE peak awaits further investigation.

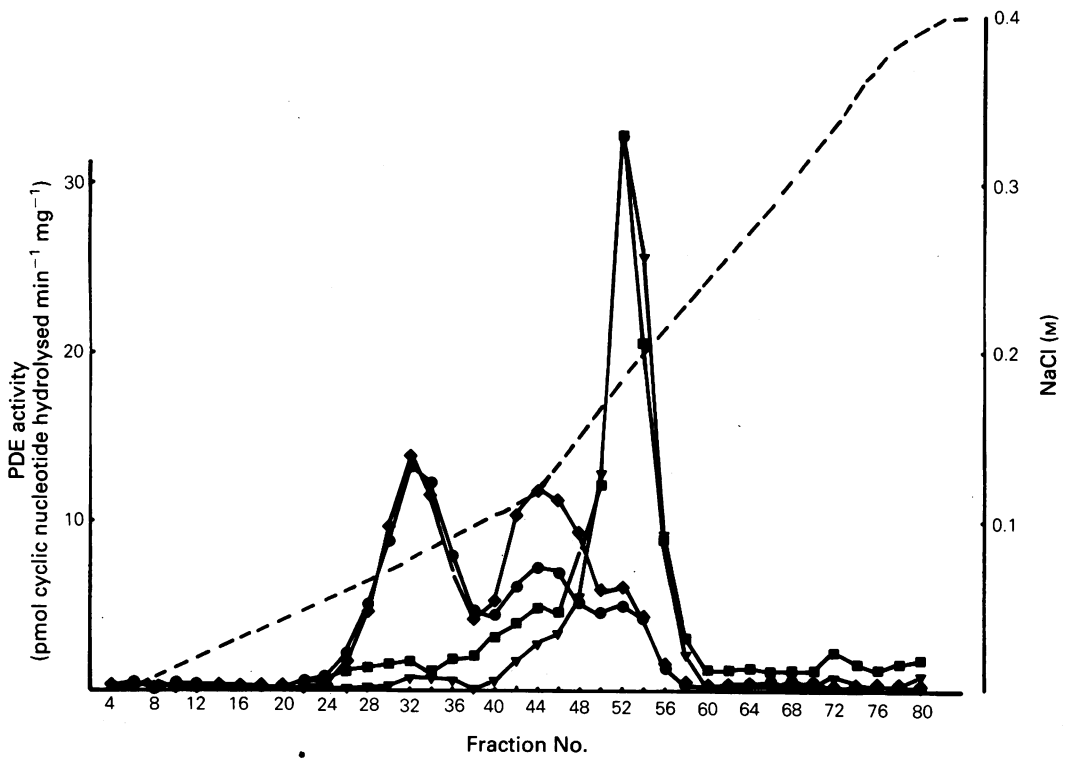


Figure 2 DEAE-trisacryl chromatography of phosphodiesterase (PDE) activity of the 105,000 *g* supernatant fraction from rat aortae. A 100,000 *g* cytosolic fraction from rat aortae was applied to a column of DEAE-trisacryl (0.9 × 7 cm). The column was washed with Tris-HCl 20 mM, magnesium acetate 2 mM, dithiothreitol 1 mM, and TLCK 20 μM (pH 7.5) and the PDE activities were eluted with successive linear gradients of NaCl (0–150 mM; 150–400 mM). PDE activity was determined using 1 μM cyclic AMP (■▼) or 1 μM cyclic GMP (●◆) in the presence of EGTA (100 μM) with (◆■) or without 2 mM $CaCl_2$ + 2.5 u calmodulin (●▼). For further studies, fractions 32–34 were pooled for cGMP PDE activity, fractions 42–46 for Ca^{2+} PDE activity and fractions 51–54 for cAMP PDE activity.

Inhibition of rat aorta phosphodiesterase activities by M&B 22,948, MIMAX, MY-5445 and vinpocetine

The inhibitory activities (K_i values) of M&B 22,948, MIMAX, MY-5445 and vinpocetine against the three resolvable PDE activities in the cytosol of rat aorta are presented in Table 2. M&B 22,948 was a potent competitive inhibitor of cGMP PDE. Ten fold weaker activity was exhibited against the Ca^{2+} PDE and only weak inhibition against cAMP PDE was observed. MIMAX exhibited similar inhibitory potency against cGMP PDE and Ca^{2+} PDE while only weakly inhibiting cAMP PDE. As with M&B 22,948, MIMAX was shown to be a competitive inhibitor of both cGMP PDE and Ca^{2+} PDE. MY-5445 selectively and competitively inhibited cGMP PDE, and vinpocetine selectively and non-competitively inhibited Ca^{2+} PDE.

Actions of phosphodiesterase inhibitors on cyclic nucleotide levels in rat aorta with and without endothelium

The effects of PDE inhibitors on rat aorta cyclic nucleotide levels were studied in rat aortic rings with endothelium intact and endothelium-denuded (rubbed) strips of rat aorta. The effective removal of endothelium by the rubbing technique was confirmed by the abolition of endothelium-dependent stimulation of cyclic GMP levels by acetylcholine without any effect on the direct action of SNP. In intact rings, acetylcholine (0.1 μM) elevated cyclic GMP levels from 1.1 ± 0.5 to 27.2 ± 2.5 pmol mg^{-1} protein (n = 3). Rubbing aortic strips lowered cyclic GMP levels to 0.1 ± 0.1 and acetylcholine only increased levels to 0.4 ± 0.2 pmol mg^{-1} protein (n = 3). SNP (1 μM), on the other hand, elevated

Table 2 Effects of inhibitors on separate phosphodiesterase activities

Compound	*cGMP PDE*	*Ca^{2+} PDE (+)CaM*	*cAMP PDE*
M&B 22,948	0.16 ± 0.04 (C)	9.9 ± 1.4 (C)	249 ± 22 (C)
MIMAX	0.43 ± 0.05 (C)	0.55 ± 0.08 (C)	42 ± 5 (C)
MY-5445	1.3 ± 0.4 (C)	>1000	915 ± 211 (C)
Vinpocetine	>1000	14 ± 2 (NC)	>1000

K_i values (μM) were determined as described in the Methods section. The Ca^{2+} PDE was assayed in the presence of Ca^{2+} (2 mM) + calmodulin (2.5 u) (CaM). The results represent the mean ± s.e.mean of K_i values calculated from the K_m or V_{max} values obtained from 4 experiments using 4 different inhibitor concentrations. The letters in parentheses denote competitive (C) or non-competitive (NC) inhibition.

cyclic GMP to 56.9 ± 7.0 pmol mg^{-1} protein ($n = 3$) in intact tissue and to 52.8 ± 6.4 pmol mg^{-1} protein ($n = 3$) in rubbed tissue.

Both M&B 22,948 (1–100 μM) and MIMAX (1–100 μM) dose-dependently increased cyclic GMP levels, the effect being greatly reduced in endothelium-denuded strips of tissue. Neither vinpocetine nor MY-5445 increased cyclic GMP levels at concentrations up to 100 μM. These data are summarised in Figure 3. None of the four PDE inhibitors influenced aorta cyclic AMP levels over the concentration range tested (basal levels, 11.65 ± 1.41 pmol mg^{-1} protein, $n = 4$, intact; 6.57 ± 1.35 pmol mg^{-1} protein, $n = 4$, rubbed). The effect, or lack of effect, of the 4 PDE inhibitors on both cyclic nucleotides was unaffected by 10 μM 5-HT (Figure 4), 60 mM K^+ or 1 μM noradrenaline (data not shown).

Methylene blue (5 μM) which abolishes EDRF stimulation of soluble guanylate cyclase (Martin *et al.*, 1985) completely blocked the endothelium-dependent elevation of cyclic GMP levels elicited by M&B 22,948 (100 μM) and MIMAX (100 μM) (Figure 4).

Smooth muscle relaxant actions of phosphodiesterase inhibitors

The dose-response relationships for the smooth muscle relaxant actions of the four PDE inhibitors in the presence and absence of methylene blue (5 μM) are presented in Figure 5. All four PDE inhibitors dose-dependently relaxed intact (endothelium-preserved) rat aortic strips contracted with 5-HT (10 μM). In agreement with previous studies (Martin *et al.*, 1986), the relaxant effects of M&B 22,948 ($EC_{50} = 15$ μM; 100% relaxation at 100 μM, $n = 4$) were almost totally abolished by prior addition of methylene blue (5 μM), suggesting that the compound acts by enhancing the relaxant actions of tonically released EDRF.

Similarly, MIMAX induced a relaxation of intact rat aorta ($EC_{50} = 2$ μM; 100% relaxation at 10 μM, $n = 4$) which at concentrations less than 10 μM was abolished by methylene blue; however, at higher concentrations, methylene blue was only partially effective in reducing the action of the xanthine.

MY-5445 also dose-dependently relaxed intact strips of rat aorta ($EC_{50} = 4$ μM; maximum relaxation, 95% at 60 μM, $n = 4$). However, unlike M&B 22,948 and MIMAX, the relaxant effect of MY-5445 was largely maintained in the presence of methylene blue, the dose-response curve only being shifted 10 fold to the right.

Vinpocetine potently inhibited the 5-HT-induced contractions in endothelium-intact rat aorta ($EC_{50} = 1$ μM; maximum relaxation, 100% at 100 μM, $n = 4$). Methylene blue inhibited the relaxant actions of low concentrations (<10 μM) of vinpocetine but was less effective in blocking the relaxant effects of higher concentrations of the drug.

Discussion

The evidence supporting a smooth muscle relaxant role for cyclic GMP is overwhelming (for review, see Murad, 1986); however, much has still to be learnt of the processes regulating the metabolism of this important second messenger. The presence of more than one enzyme capable of selectively hydrolyzing cyclic GMP in vascular smooth muscle (Figure 2) may suggest a complex degradatory interplay between the enzymes. Perhaps Ca^{2+} PDE takes on a dominant role when smooth muscle is exposed to an excitatory stimulus leading to influx of Ca^{2+}. Certainly, increased activity of this enzyme has been reported following exposure of vascular smooth muscle to depolarizing K^+ (Miller & Wells, 1987). Alternatively, Ca^{2+} PDE and cGMP PDE may regulate separate intracellular pools of cyclic GMP. Evidence for compartmentalised cyclic AMP coupled to distinct pools of cAMP-dependent protein kinase in heart has recently emerged (Gristwood *et al.*, 1986; England & Shahid, 1987; Murray *et al.*, 1987). The finding that an inhibitor of Type III cAMP PDE (SK&F 94120) increases contractile force and elevates cyclic AMP content of

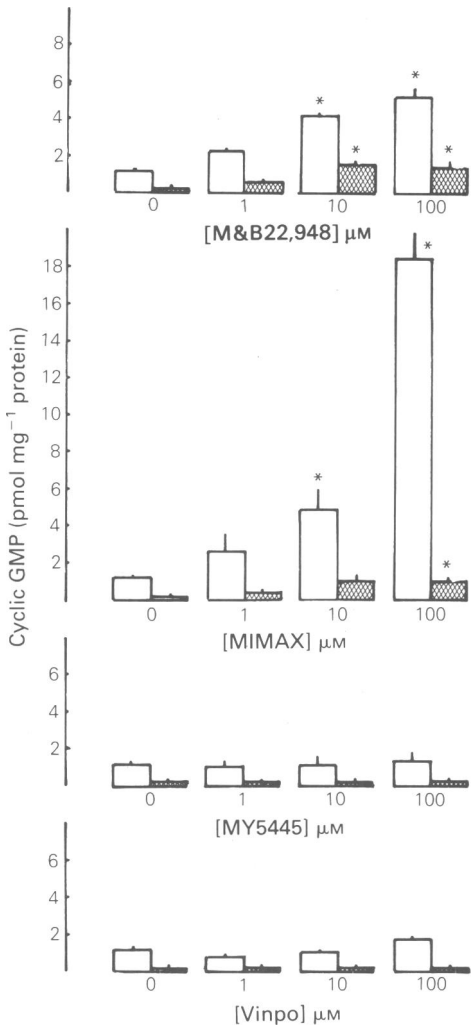


Figure 3 Effects of phosphodiesterase (PDE) inhibitors on the cyclic GMP content of endothelium-intact and endothelium-denuded pieces of rat aorta. Rubbed (endothelium removed) strips (cross hatched columns) and intact (endothelium-preserved) rings (open columns) of rat aorta were incubated with PDE inhibitors for 10 min. Cyclic GMP was extracted and quantified as described in the methods section. The results represent the means with s.e.mean (vertical bars) of 4 separate determinations. * Represents a significant difference from control values (absence of PDE inhibitors) $P < 0.05$.

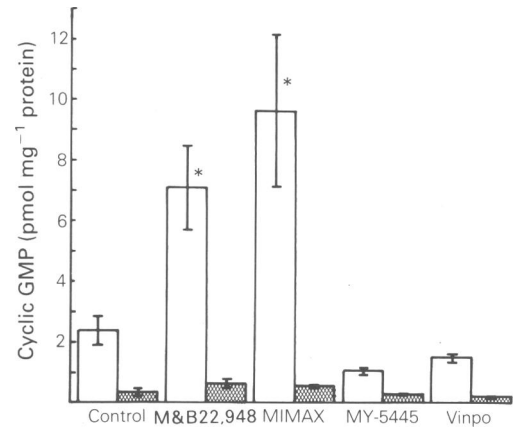


Figure 4 The effect of phosphodiesterase (PDE) inhibitors on cyclic GMP levels in endothelium-intact pieces of rat aorta in the presence and absence of methylene blue. Intact rings of rat aorta were pre-incubated with (cross hatched columns) or without (open columns) methylene blue (5 μM) for 10 min before being contracted by exposure to 5-hydroxytryptamine (5-HT, 10 μM) for 1 min. The tissue was then exposed to PDE inhibitors (100 μM) for a further 10 min in the continued presence of 5-HT. Cyclic GMP was extracted and quantified as described in the methods section. The results represent the means of 3 incubations with s.e.mean shown by vertical bars. * Represents a significant difference from control values (absence of PDE inhibitors) $P < 0.05$.

ventricular muscle strips while a Type IV cAMP PDE inhibitor (Rolipram) raised ventricular cAMP levels without inducing a positive inotropic effect (Gristwood *et al.*, 1986) suggests that different cAMP PDE isozymes are linked to distinct pools of cyclic AMP. Whether discrete pools of cyclic GMP associated with different cGMP PDEs exist in vascular smooth muscle in an analogous manner is a question which, to our knowledge, has not been addressed.

The advent of compounds which selectively inhibit individual PDEs has proved useful for the characterization of the different isozymes present in mammalian tissues. Furthermore, these inhibitors may prove to be useful in understanding the respective roles of the multiple PDEs in regulating cyclic nucleotide metabolism. M&B 22,948 is a selective inhibitor of cyclic GMP hydrolysis and has been an important tool in establishing a causal role for cyclic GMP in a number of biological events. This azapurinone inhibits both cGMP PDE and Ca^{2+} PDE (see Table 2, Lugnier *et al.*, 1986), exhibiting approximately 60 fold greater potency against the former activity. In agreement with previous studies (Martin *et al.*, 1986), exposure of rat aorta to M&B 22,948 resulted in an increase in cyclic GMP, but not cyclic AMP, (Figures 3, 4), and reduction in tone by enhancing the actions of EDRF (Figure 5). MIMAX exhibited similar potency against Ca^{2+} PDE and cGMP PDE, while only weak activity against cAMP PDE was observed (Table 2). It behaved in a similar manner to M&B 22,948 in inducing an elevation of cyclic GMP

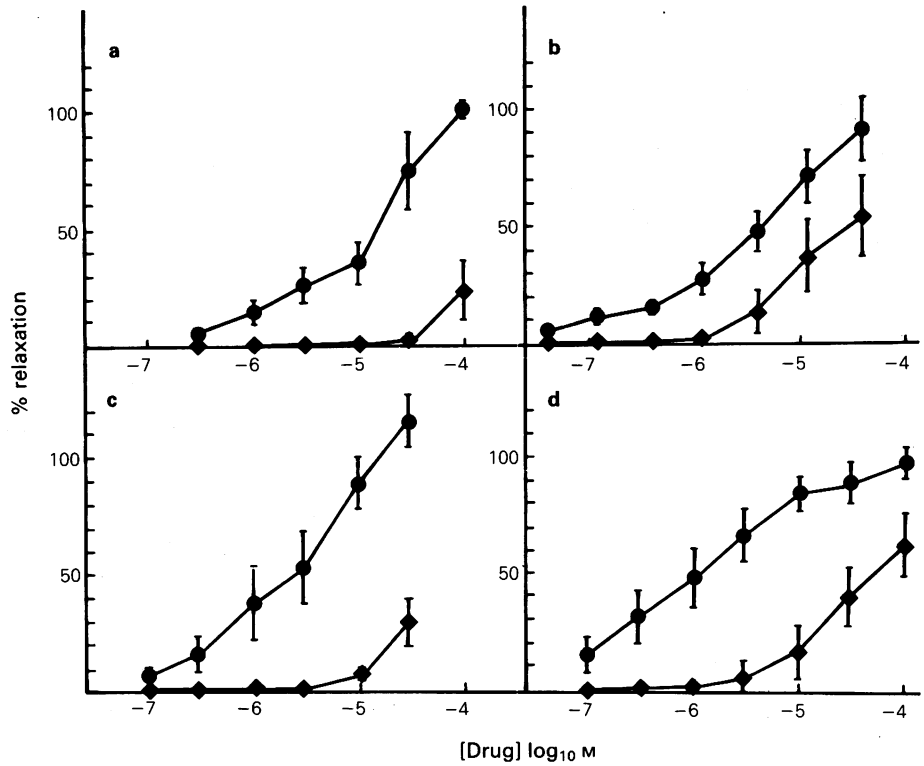


Figure 5 Dose-response curves of the smooth muscle relaxant effects of M&B 22,948, MIMAX, vinpocetine and MY-5445 in endothelium-intact strips of rat aorta in the presence and absence of methylene blue. Aortic strips were contracted with 5-hydroxytryptamine (10 μM). Plotted points show relaxation elicited by M&B 22,948 (a), MY-5445 (b), MIMAX (c) and vinpocetine (d) in aortic strips in the presence (◆) and absence (●) of methylene blue (5 μM). Each point is the mean and vertical bars the s.e.mean of 4 separate experiments.

and a relaxant response which was greatly reduced by abolishing EDRF (Figures 3, 4, 5); however, methylene blue only partially inhibited relaxation induced by 30 μM MIMAX (Figure 5), although the increase in cyclic GMP caused by an even higher concentration (100 μM) of the xanthine was completely abolished (Figure 4). Although this cyclic GMP-dependent component of the myorelaxant actions of MIMAX was observed at a concentration approaching its K_i value against the cAMP PDE ($K_i = 42 \pm 5\,\mu$M, $n = 4$), cyclic AMP involvement would appear to be ruled out as a mediatory mechanism since no increase in the level in rat aorta could be detected at MIMAX concentrations as high as 100 μM (data not shown).

Although M&B 22,948 and MIMAX are of use in studying cyclic GMP-mediated processes, they are of little value in delineating the respective roles of cGMP PDE and Ca^{2+} PDE in regulating cyclic GMP breakdown because of their inability to discriminate sufficiently between these enzymes. The potency of M&B 22,948 required to elevate cyclic GMP and reduce vascular tone ($EC_{50} = 15\,\mu$M) more closely correlated with its inhibitory activity against Ca^{2+} PDE ($K_i = 9.9 \pm 1.4\,\mu$M) than that against cGMP PDE ($K_i = 0.16 \pm 0.04\,\mu$M). From this it has been inferred that the Ca^{2+} PDE is of predominant importance in regulating smooth muscle cyclic GMP hydrolysis (Ahn *et al.*, 1987). However, a number of factors may contribute to discrepancies between the activity of a compound against the isolated enzyme and potency in influencing intracellular cyclic GMP concentrations and the biological response linked to this second messenger. Therefore, ideally, chemical structures which selectively inhibit the activity of one or other of these cyclic GMP hydrolyzing enzymes are required. Very few compounds with such activity have been reported in the literature. The anti-platelet action of MY-5445 has been ascribed to elevation of cyclic GMP levels via a selective inhibition of cGMP PDE (Hagiwara *et al.*, 1984b). This compound has also

been reported to induce relaxation in helical strips of rabbit aorta (Hidaka *et al.*, 1984). It was of interest, therefore, to determine whether this smooth muscle relaxant effect is mediated via a mechanism involving cyclic GMP. The results presented herein (Table 2, Figures 3, 4, 5) indicate, surprisingly, that although MY-5445 selectively inhibited rat aorta cGMP PDE its relaxant action is independent of cyclic GMP-mediated events.

The smooth muscle relaxant action of vinpocetine has also been attributed to blocking cyclic GMP hydrolysis but via inhibition of Ca^{2+} PDE (Hagiwara *et al.*, 1984a). We also demonstrated vinpocetine to be a selective, though relatively weak ($K_i = 14 \pm 2\,\mu M$) inhibitor of Ca^{2+} PDE (Table 2); however, although an endothelium-dependent component of the relaxant action of vinpocetine, especially at low concentrations, was suggested by the inhibition of this effect by methylene blue (Figure 5) our inability to observe an elevation of cyclic GMP (Figures 3, 4) appears to eliminate an involvement of this second messenger in the actions of the drug, at least in rat aorta. Whether methylene blue interferes with the myorelaxant actions of vinpocetine and the other PDE inhibitors through a mechanism independent of EDRF generation and guanylate cyclase activation warrants further investigation. Our results differ from those reported by others (Hagiwara *et al.*, 1984a; Ahn *et al.*, 1987) in which smooth muscle cyclic GMP levels increased following exposure to vinpocetine. Their studies were performed on rabbit aorta, suggesting, perhaps, differences between the contractile processes in vascular smooth muscle from the two species. Possibly the contribution of Ca^{2+} PDE to the regulation of intracellular cyclic GMP may be of less significance in rat aorta. It is possible, as previously mentioned, that the contribution of Ca^{2+} PDE and therefore the effectiveness of vinpocetine in influencing intracellular hydrolysis of cyclic GMP would only become apparent following elevation of cytosolic Ca^{2+} after exposure of the smooth muscle to an excitatory agonist; however, we have been unable to detect any stimulatory effect of vinpocetine on rat aortic cyclic GMP following exposure of the tissue to either 5-HT (Figure 4), depolarizing K^+ (60 mM) or noradrenaline (1 μM).

In conclusion, our results suggest that MIMAX, like M&B 22,948, relaxes rat aorta in an endothelium-dependent manner which is likely to result from the inhibition of the breakdown of smooth muscle cyclic GMP whose accumulation is stimulated by tonically released EDRF. Based on data presented in the present paper, it seems unlikely that such a mechanism can account for the smooth muscle relaxant actions of vinpocetine and MY-5445. The elucidation of the roles of the cGMP PDE and Ca^{2+} in regulating vascular smooth muscle cyclic GMP levels awaits the development of more potent and selective inhibitors. We are at present seeking to identify such compounds to understand better the cyclic GMP degradatory processes which exist in vascular smooth muscle.

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