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The role of cGMP hydrolysing phosphodiesterases 1 and 5 in cerebral artery dilatation

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Abstract

The aim was to investigate the presence and activity of cGMP hydrolysing phosphodiesterases in guinea pig basilar arteries and the effect of selective and non-selective phosphodiesterase inhibitors on cerebral artery dilatation involving the nitric oxide (NO)–guanosine cyclic 3′5-monophosphate (cGMP) pathway. Immunoreactivity to phosphodiesterases 1A, 1B and 5, but not phosphodiesterase 1C was found in fractions of homogenised cerebral arteries eluted by high-pressure liquid chromatography (HPLC). Both the phosphodiesterase 1 inhibitor 8-methoxymethyl-1methyl-3-(2methylpropyl)-xanthine (8-MM-IBMX) and the phosphodiesterase 5 inhibitors zaprinast and dipyridamole induced dilatation of cerebral arteries. The dilatory response to 8-MM-IBMX was reduced by 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ) (10 μM) and endothelial removal and restored by sodium nitroprusside (0.1 μM) pretreatment, indicating a close relation to the nitric oxide–cGMP pathway. The responses to zaprinast and dipyridamole, however, were not only moderately affected, but also restored by sodium nitroprusside (0.1 μM) pretreatment. At high concentrations, the dilatory effects of zaprinast and dipyridamole were partly caused by cGMP-independent mechanisms. Targeting the phosphodiesterases present in cerebral arteries, with selective inhibitors or activators of phosphodiesterase, may be a possible new way of treating cerebrovascular disease. © 2001 Published by Elsevier Science B.V.

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1. Introduction

The regulation of cerebral artery diameter and cerebral blood flow is complex. Induction of an arterial dilatory response in general includes activation of either adenylate cyclases or guanylate cyclases, which increases the intracellular levels of the cyclic nucleotides, guanosine cyclic 3′5-monophosphate (cGMP) and adenosine cyclic 3′5-monophosphate (cAMP).

In cerebral arteries, nitric oxide (NO) seems to be a very important modulator of cerebral vascular tone in both normal and pathological conditions (Faraci and Brian, 1994; Iadecola et al., 1994) through its induction of cGMP production (Schmidt et al., 1993).

The levels of cGMP and cAMP are regulated in three ways; through production via the cyclases in the cell, by efflux from the cell, or following degradation by the intracellular enzymes cyclic nucleotide phosphodiesterases (Beavo, 1995; Schini et al., 1989).

To date, 11 different phosphodiesterase families have been described. Each phosphodiesterase family differs in the affinity to cAMP or cGMP, kinetic properties, mode of regulation, cellular and tissue distribution and sensitivity to phosphodiesterase inhibitors (Beavo, 1995; Fawcett et al., 2000; Fisher et al., 1998; Fujishige et al., 1999; Soderling et al., 1998).

This variation in distribution and physiological function of phosphodiesterases not only between tissues in general, but also between vessel types and vascular beds in particular (Beavo, 1995; Schmidt et al., 1993) makes them excellent pharmacological targets. Modulation of phosphodiesterase activity and thus cyclic nucleotide levels through specific inhibitors or activators of phosphodiesterases seem

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to be relevant in the treatment of diseases involving different vascular tissue (Beavo and Reifsnyder, 1990; Murray and England, 1992).

Much is known about the distribution and function of phosphodiesterases in smooth muscle cells in general (Lindgren and Andersson, 1991; Lugnier and Komas, 1993; Wallis et al., 1999), but the distribution of phosphodiesterases in cerebral arteries is not fully elucidated. Previous research has described the distribution of phosphodiesterases in vascular smooth muscle cells from other tissue than the brain as being phosphodiesterases 1, 3, 4 and 5, and in endothelial cells phosphodiesterases 2 and 4 (Stoclet et al., 1995). The few studies investigating on the distribution of the different phosphodiesterase families in cerebral arteries have mainly been concerned with the cAMP signaling pathway (Willette et al., 1997), phosphodiesterase in brain slices (Adachi and Numano, 1977; Wei et al., 1996) or in cerebrospinal fluid (Parfenova et al., 1993).

Several studies have investigated the dilatory effects of more or less selective phosphodiesterase inhibitors on either intact cerebral arteries (Cosentino et al., 1992; Harris et al., 1989a; Parfenova et al., 1993; Rosenblum et al., 1993; Willette et al., 1997) or arteries with induced vasospasm (Kim et al., 1992; Sobey and Quan, 1999). But only a few have measured the effects of phosphodiesterase inhibitors on cyclic nucleotide levels concomitantly (Kim et al., 1992; Parfenova et al., 1993).

The aim of the present study was therefore to examine the presence and activity phosphodiesterase enzymes in guinea pig cerebral arteries. Furthermore, we wished to examine the relation between the phosphodiesterases and the NO–cGMP pathway and evaluate the ability of selective and non-selective phosphodiesterase inhibitors to dilate cerebral arteries and modulate arterial cAMP and cGMP concentrations in vitro.

2. Materials and methods

2.1. Guinea pig basilar arteries

Male guinea pigs, 350–450 g, were sacrificed in the morning by decapitation after sedation with CO_2 inhalation. The basilar artery was dissected free, frozen immediately on dry ice for enzyme analysis after removal of luminal blood or placed in a modified buffer solution of the following composition (mM): pH 7.4, NaCl 119, $NaHCO_3$ 15, KCl 4.6, $CaCl_2$ 1.5, $NaHPO_4$ 1.2, $MgCl_2$ 1.2, glucose 5.5.

2.2. Tissue preparation for enzyme activity analysis

The basilar arteries were stored at −80°C until analysis. Basilar arteries from eight guinea pigs equal were pooled for each high-pressure liquid chromatography (HPLC) analysis. The frozen tissue was mechanically dissociated by pestle and transferred to 3 ml of homogenisation buffer (pH 7.3, β-glycerophosphate 50 mM, EGTA 1.5 mM, Na_3VO_4 0.1 mM, dithiothreitol 1 mM, aprotinin 10 μg/ml, pepstatin 5 μg/ml, leupeptin 20 μg/ml, benzamidine 1 mM). The homogenate was sonicated twice for 5 s at 50% output (Branson Sonifier, USA) and centrifuged in a two-step procedure first at $5000 \times g$ for 5 min, then the supernatant at $100.000 \times g$ for 20 min, at 4°C. The supernatant was kept at 4°C during all procedures. The pellet was re-suspended in homogenisation buffer. Over 99% of the phosphodiesterase activity was present in the supernatant after centrifugation.

2.3. High pressure anion-exchange chromatography

A MonoQ anion exchange column HR5/5 was equilibrated with buffer A (50 mM Tris–HCl, pH 7.5, 2 mM dithiothreitol, 1 mM benzamidine). The filtered (0.2 μm micropore) supernatant was loaded onto the column at 0.5 ml/min. The bound phosphodiesterase was eluted using a linear gradient of high salt buffer B (Buffer A + 0.8 M NaCl) over 40 min. Fractions of 0.25 ml were collected at 4°C and assayed for phosphodiesterase activity.

2.4. Phosphodiesterase activity

The phosphodiesterase assay was performed according to previously established procedures (Beltman et al., 1995). A 1-μM of either cAMP/[^{3}H]cAMP or cGMP/[^{3}H]cGMP (~ 50.000 cpm) was used as substrate. Assay contents were 40 mM 3-[*N*-Morpholino]propanesulfonic acid (MOPS), pH 7,5, 20 mM imidazole, 15 mM MgAcetate, 3 mM $MgCl_2$ and 0.2 mg/ml bovine serum albumine. Assays were carried out at 30°C in the presence of either 1 mM EGTA or 4 μg/ml Calmodulin and 200 μM $CaCl_2$ and the final volume of assay was 125 μl. The concentration of zaprinast used in the phosphodiesterase assay was 10 μM.

The reaction was initiated by addition of cyclic nucleotides and terminated by boiling for 1 min. The assay was treated with snake venom from *Crotalus atrox*, and loaded onto equilibrated DEAE-sephadex A25 columns. A 20-ml of low salt buffer (20 mM Tris–Cl, pH 6.8) was used to elute the neutrally charged nucleoside, which was collected directly into scintillation vials and quantitated by counting [^{3}H]nucleoside.

The HPLC run and the phosphodiesterase assay were performed as a 1-day procedure, since phosphodiesterase 1 activity was found to decrease over days. Four experiments on eight basilar arteries each were performed. Values from representative samples are illustrated in results.

2.5. Western blot

Aliquots of supernatant and fractions were boiled in 2× sample buffer. Electrophoresis was performed on an 8% Sodium dodecyl sulfate (SDS)-polyacrylamide gel and transferred to a nitrocellulose membrane. Only antibodies for phosphodiesterases 1A, 1B, 1C and 5 were available and hence only the presence of these phosphodiesterases could be investigated by Western blot. All antibodies were polyclonal rabbit antibodies, their selectivity has been described in Rybalkin et al. (1997). Protein standards and standards for the selected phosphodiesterases were used in each blot. Because of the small amount of aliquots available, only one Western blot analysis was possible for each enzyme and the fractions were thus analysed on three consecutive blots, which did not always respect the peak enzyme activities.

2.6. Studies of arterial vasomotor responses

Studies were performed on both intact and endothelium-denuded guinea pig basilar arteries. Results are given as a means ± S.E.M for the number of guinea pigs. Endothelium was removed by perfusing the artery with 0.1% Triton-X for 20 s followed by buffer. The arteries were cut transversally in 0.6- to 1-mm pieces and each ring was mounted on two Teflon-coated L-shaped prongs (0.15 mm). They were immersed under isometric conditions in temperature-controlled tissue-baths (37°C) (Multimyograph 610, J.P Trading, Denmark) containing 5 ml of buffer and aerated with 95% O_2 and 5% CO_2. After 1 h of equilibration, each ring of artery was normalised to the optimum diameter determined in a passive-tension curve (Mulvany and Halpern, 1977).

The viability of the arteries was ensured by stimulation with 60 mM KCl. This resulted in a contraction of 3.6 ± 0.09 mN/mm ($N = 36$) in intact arteries and 3.41 ± 0.12 mN/mm ($N = 16$) in endothelial-denuded arteries. The presence of a functional endothelium was ascertained by application of acetylcholine (10 μM) and only a dilatory response above 80% of precontraction was accepted in intact arteries.

Prostaglandin$F_{2\alpha}$ in a concentration (3×10^{-6} M) showing 80% contraction in both intact (3.9 ± 0.09 mN/mm) ($N = 29$) and endothelial-denuded arteries (4.02 ± 0.10 mN/mm) ($N = 16$) was used as precontraction. In intact arteries, histamine (10^{-5} M) (3.37 ± 0.21 mN/mm) was used in a few cases ($N = 7$) and no significant difference in response was seen between these two pre-constrictions (data not shown). After a stable contraction was reached, cumulative concentrations of compounds were added.

Concentration–response curves were performed by cumulative addition of phosphodiesterase inhibitors, sodium nitroprusside or atrial natriuretic peptide in both intact and endothelial-denuded arteries. When pretreatment was used, precontraction was induced after addition of pretreatment.

In intact arteries, the potentiating effect of zaprinast (1 μM for 10 min) on sodium nitroprusside-induced dilatation was investigated. Furthermore, the selective soluble guanylate cyclase inhibitor, 1*H*-[1,2,4]oxadiazolo[4,3-*a*]-quinoxalin-1-one (ODQ) (10 μM for 15 min) (Garthwaite et al., 1995) was used as pretreatment before adding cumulative concentrations of phosphodiesterase inhibitors, sodium nitroprusside or atrial natriuretic peptide to elucidate the role of the endogenously produced NO in the isolated arteries.

In endothelium-denuded arteries where endogenous NO production and other effects of the endothelium were removed, the arteries were pretreated either with sodium nitroprusside (0.1 μM) or with sodium nitroprusside (0.1 μM) plus ODQ (10 μM) before cumulative concentrations of the phosphodiesterase inhibitors were given.

The phosphodiesterase inhibitors used were the phosphodiesterase 1 inhibitor 8-methoxymethyl-1methyl-3-(2methylpropyl)-xanthine (8-MM-IBMX) and the phosphodiesterase 5 inhibitors, zaprinast and dipyridamole. Moreover, the dilatory effect of the selective inhibitor milrinone (phosphodiesterase 3) and the non-selective inhibitors 1-methyl-3-isobutylxanthine (IBMX) and pentoxifylline were tested for comparison. Sodium nitroprusside was used as a donor of nitric oxide, stimulating the soluble guanylate cyclase, while atrial natriuretic peptide was a stimulator of the particulate guanylate cyclase.

The identity of the compounds was blinded during data analysis, which was performed using Myodata, Myonic Software (J.P Trading, Denmark).

Stock solutions were made of all compounds using either 100% dimethyl sulfoxide for dissolving ODQ, 8-MM-IBMX, rolipram, zaprinast, dipyridamole, pentoxifylline and IBMX or distilled water was used for atrial natriuretic peptide and sodium nitroprusside. All drugs were kept at −20°C until use. No compounds were used in concentrations above 30 μM because of solubility problems and to ensure the concentrations of dimethyl sulfoxide did not exceed 0.1%. Dilutions for determination of the concentration–response curves were made immediately before experiments using buffer.

2.7. Measurements of cyclic nucleotides in vitro

Cyclic nucleotide responses to single concentrations of sodium nitroprusside, zaprinast, dipyridamole, milrinone and pentoxifylline were investigated separately on the mounted rings of intact basilar arteries. Endothelial func-

tion was ascertained. After a stable pre-contraction, a single concentration of dilator was added and the dilatory response was terminated after 60 s by quickly exchanging the buffer with ice-cold acidic ethanol (1 ml 1 M HCl in 100 ml ethanol) (Gray and Marshall, 1992).

The artery and the ice-cold solution were immediately removed and frozen at −20°C before homogenisation, which was performed on dry ice using a glass homogeniser.

Each sample was assayed for both cAMP and cGMP concentrations using commercially available radioimmunoassay kits (Amersham Pharmacia Biotech, UK). The pellet was used for protein analysis (Bradford, Bio-Rad).

2.8. Compounds

[^{3}H]cGMP and [^{3}H]cAMP were obtained from NEN Lifescience Products, USA. Antibodies for use in Western blot were obtained from the laboratory of J.A Beavo, University of Washington, Seattle, USA. cGMP and cAMP, prostaglandin$F_{2\alpha}$, acetylcholine, IBMX, pentoxifylline, zaprinast, rolipram, dipyridamole, sodium nitroprusside and atrial natriuretic peptide were purchased from Sigma–Aldrich, USA. 8-MM-IBMX and ODQ were purchased from Calbiochem, USA, while milrinone was a generous gift from Boehringer-Ingelheim, Germany. All concentrations are expressed as the final molar concentrations in the tissue bath or phosphodiesterase assay.

2.9. Calculation and statistics

All data are expressed as mean values ± S.E.M. N refers to the number of guinea pigs used including one to two vessel segments from each guinea pig in the tissue baths. The maximum dilatory effect of an agonist was calculated as percentage of the pre-contraction and expressed as E_{max}. pEC_{50}, the negative logarithm of the molar concentration that produced half-maximal relaxation, was calculated using best-fit sigmoidal curve of the concentration–response curve using GraphPad Prism 2.01 (GraphPad Software, San Diego, USA).

Difference between treatments and compounds were tested for statistical significance using paired and unpaired Student's t-test where appropriate. $P < 0.5$ was considered statistically significant.

3. Results

3.1. Separation of phosphodiesterase iso-enzymes and detection of phosphodiesterases 1 and 5 activity

Superior hydrolysis of cGMP over cAMP was found both in the supernatant and in the fractions of the homogenised guinea pig basilar arteries. However, because of the small amount of tissue available, only low activity was found in the fractions. Hydrolysis of cAMP was only seen as a small peak stimulated by Ca^{2+}/Calmodulin and was concurrent to the first cGMP hydrolysing phosphodiesterase 1A and 1B peaks. A second smaller peak eluted late in the gradient run (fractions 71–75). It did not elicit stimulation by Ca^{2+}/Calmodulin and may represent either phosphodiesterase 3 or 4.

A representative curve of cGMP hydrolysis in the eluted fractions, with and without calcium and calmodulin (Ca^{2+}/Calmodulin) stimulation, is shown in Fig. 1. Two initially eluted major peaks of cGMP hydrolysing enzyme followed by a smaller peak were found. Immunoreactivity to phosphodiesterase 1A, usually as a single confluent peak, was found around fractions 53–56, with a molar weight slightly below the standard protein marker 62 kDa and equal to the control used for phosphodiesterase 1A (Rybalkin et al., 1997) (Fig. 1). Furthermore, phosphodiesterase 1B showed immunoreactivity with two peak intensities, the first around fraction 52, and the second around fraction 58 separated by very week immunoreactive bands, all with similar molar weight just below 62 kDa. On a few occasions, week immunoreactivity towards phosphodiesterases 1B and 1A was seen in the fractions 62–65. The lower enzyme activity and thus a protein concentration close to detection level by Western blot, could explain the apparent lack of consistency in these findings. The later eluted part of cGMP hydrolysing activity not stimulated by Ca^{2+}/Calmodulin, showed phosphodiesterase 5 immunoreactivity as shown by Western blot analysis (peak between fraction 56–58). No phosphodiesterase 1C reactivity was found in any of the fractions or supernatants.

Both the phosphodiesterase 1 and 5 peaks were inhibited by the addition of zaprinast (10 μM) to the phosphodiesterase assay (data not shown). This suggests a corresponding inhibition of phosphodiesterases 1 and 5 at a concentration, which elicited 50% relaxation in isolated basilar arteries in vitro.

3.2. Studies of vasodilator responses in intact arteries

3.2.1. Response to phosphodiesterase inhibitors and stimulators of guanylate cyclases

In intact arteries, all phosphodiesterase inhibitors induced a dilatory response after precontraction (Table 1) although with slightly different potencies. Atrial natriuretic peptide dilated the arteries at lower concentrations than sodium nitroprusside, but with a similar maximal dilatation (Table 1).

3.2.2. The effect of inhibition of cGMP production on response to phosphodiesterase inhibitors and stimulators of guanylate cyclases

ODQ did not influence the basal tone of the arteries. When cGMP production was inhibited by the soluble guanylate cyclase inhibitor ODQ, it significantly decreased

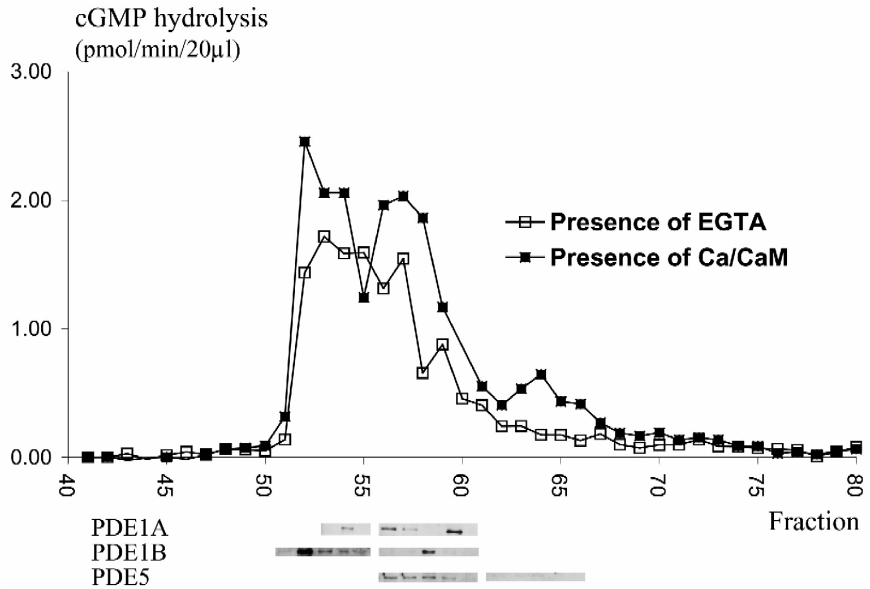

Fig. 1. This figure shows cGMP hydrolytic activity of phosphodiesterase iso-enzymes eluted in 0.25 ml fractions by using a NaCl gradient during HPLC. Filled squares (■) are the Ca^{2+}/Calmodulin stimulated hydrolysis and open squares (□) are hydrolysis when EGTA is added to the assay. Because only small amounts of fractions were available, immunostaining were performed in three Western blots including fractions 51–55, fractions 56–60 and fractions 61–65, respectively. Each blot also included standard and controls (not shown). Inserts are the blots showing reactivity to phosphodiesterases 1A, 1B and 5. In this case, phosphodiesterase 1A did not show a single intensity peak since fraction 55 seems to lack phosphodiesterase 1A, however, most common immunoreactivity to phosphodiesterase 1A was found as a confluent single peak from 53 to 56.

the E_{max} of 8-MM-IBMX ($P < 0.001$) and of soluble guanylate cyclase activator sodium nitroprusside ($P < 0.001$) (Table 1). It shifted the concentration–response curve for zaprinast (Fig. 2A) ($P = 0.003$) and dipyridamole (Fig. 2B) ($P < 0.003$) significantly to the right with no significant change in E_{max} ($P = 0.21$ and $P = 0.56$, respectively) (Table 1). The response to IBMX was likewise shifted to the right ($P = 0.01$) with no difference in E_{max} (Fig. 2D).

The dilatory response to the particulate guanylate cyclase activator atrial natriuretic peptide (not shown) and milrinone (Fig. 2F) was not significantly affected by ODQ pretreatment.

3.2.3. The possible potentiating effect of zaprinast on sodium nitroprusside-induced dilatation

The potentiating effect of zaprinast on sodium nitroprusside-induced dilatation previously found in rat intact aorta was investigated in intact cerebral arteries. However, zaprinast (1 μM) was not found to potentiate sodium nitroprusside-induced dilatation in intact guinea pig cerebral arteries ($N = 7$) ($P = 0.78$). Using higher concentra-

Table 1
Relaxant response to phosphodiesterase inhibitors, sodium nitroprusside and atrial natriuretic peptide in guinea pig cerebral arteries with intact endothelium

Compound	No pretreatment			Pretreatment ODQ 10 μM		
	pEC_{50}	E_{max} (%)	N	pEC_{50}	E_{max} (%)	N
Sodium nitroprusside	6.98 ± 0.13	96 ± 2	8	Nob	23 ± 8[a]	8
Atrial natriuretic peptide	9.60 ± 0.08	96 ± 1	9	9.30 ± 0.13	92 ± 3	7
8-MM-IBMX	5.38 ± 0.07	89 ± 2	7	Nob	20 ± 8[a]	7
Dipyridamole	5.94 ± 0.20	76 ± 6	8	5.07 ± 0.10[a]	81 ± 4	7
Zaprinast	5.18 ± 0.21	83 ± 7	9	4.40 ± 0.21[a]	66 ± 12	8
Milrinone	6.00 ± 0.16	90 ± 5	7	5.80 ± 0.24	91 ± 4	7
Pentoxifylline	4.73 ± 0.19	69 ± 13	7	Nob	26 ± 11[a]	6
IBMX	5.92 ± 0.12	98 ± 1	7	5.41 ± 0.09[a]	94 ± 2	7

Values represent means ± S.E.M. N = number of animals, from each animal one to two vessel segments were used. Nob = not obtainable. The maximum dilatory effect, E_{max}, of an agonist was calculated as % relaxation of the pre-contraction. pEC_{50} is the negative logarithm of the molar concentration that produced half-maximal relaxation.

[a] $P < 0.05$ and refers to significant difference between responses with and without inhibition of soluble guanylate cyclase by ODQ pretreatment.

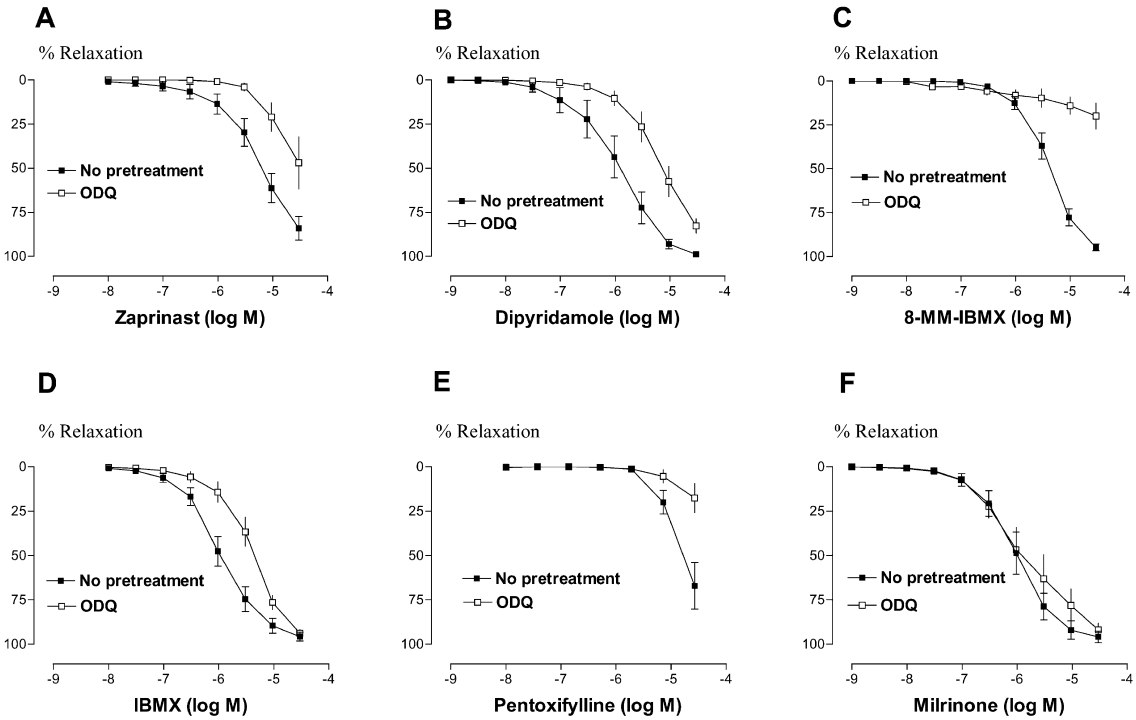

Fig. 2. Concentration–response curve for the selective phosphodiesterase 5 inhibitors zaprinast (A) and dipyridamole (B), the selective phosphodiesterase 1 inhibitor 8-MM-IBMX (C), the non-selective phosphodiesterase inhibitors IBMX (D) and pentoxifylline (E), and the selective inhibitor of the cGMP inhibited cAMP degrading phosphodiesterase 3, milrinone (F) with (□) and without (■) ODQ (10 μM) pretreatment in intact arteries. Relaxation is given as % relaxation of pre-contraction and each point shows mean ± S.E.M. Only the concentration–response curve for 8-MM-IBMX and pentoxifylline was almost completely inhibited by ODQ, while zaprinast and dipyridamole still was able to induce relaxation. $N = 6–9$.

tions of zaprinast dilated the arteries before addition of sodium nitroprusside in intact arteries and was thus not possible. Since the intention was to study the cerebral arteries in set-up close to the in vivo conditions, arteries without endothelium were not investigated.

3.3. Studies of vasodilator effects in endothelial-denuded arteries

3.3.1. Response to phosphodiesterase inhibitors and stimulators of guanylate cyclase when activating factors from the endothelium were not present

The E_{max} to most of the inhibitors were significantly decreased after endothelial removal (Table 2), the exceptions being dipyridamole (Fig. 3B) and milrinone (Fig. 3F). The concentration–response curve for dipyridamole was however, significantly shifted to the right (Table 2). The pEC_{50} was not obtainable for 8-MM-IBMX, zaprinast and pentoxifylline (Table 2) since higher concentrations could not be used because of solubility problems. The response to atrial natriuretic peptide was not significantly affected by endothelium removal (Table 2) but the response to sodium nitroprusside was slightly shifted towards higher concentrations ($P = 0.02$) and E_{max} was decreased by 13% ($P = 0.04$) (Table 2). This could be caused by the lack of endogenously produced endothelial NO.

3.3.2. Response to phosphodiesterase inhibitors when a possible NO production from the endothelium was replaced by the NO donor sodium nitroprusside

In the presence of sodium nitroprusside (0.1 μM), added just before precontraction, the dilatory responses to 8-MM-IBMX, zaprinast, dipyridamole and IBMX in arteries without endothelium, were restored or even potentiated, as compared to the values seen in intact arteries. The pentoxifylline response was only marginally affected in the presence of sodium nitroprusside and did not return completely to the response seen with intact endothelium (Figs. 2E and 3E).

3.3.3. Response to phosphodiesterase inhibitors after pretreatment of low concentration sodium nitroprusside and inhibition of the soluble guanylate cyclase

After the addition of ODQ (10 μM) as well as sodium nitroprusside (0.1 μM), the relaxant effects of the phosphodiesterase compounds returned to the values seen in intact arteries with ODQ pretreatment (Tables 1 and 2). The E_{max} of zaprinast was slightly but significantly increased in the presence of both sodium nitroprusside and ODQ compared to no pretreatment (Table 2, Fig. 3A).

3.4. Cyclic nucleotide concentration

The basal concentration of cGMP was 1.15 ± 0.12 pmol/mg protein, which was significantly reduced to 0.17

Table 2
Relaxant response to phosphodiesterase inhibitors, sodium nitroprusside and atrial natriuretic peptide in guinea pig cerebral arteries where endothelium is removed

Compound	No pretreatment			Pretreatment sodium nitroprusside 0.1 μM			Pretreatment sodium nitroprusside 0.1 μM + ODQ 10 μM		
	pEC_{50}	E_{max} (%)	N	pEC_{50}	E_{max} (%)	N	pEC_{50}	E_{max} (%)	N
Sodium nitroprusside	6.19 ± 0.28[a]	83 ± 6[a]	6	–	–	–	–	–	–
Atrial natriuretic peptide	9.73 ± 0.07	96 ± 1	7	–	–	–	–	–	–
8-MM-IBMX	Nob	47 ± 14[a]	7	6.18 ± 0.21	96 ± 2[b]	6	Nob	34 ± 1	6
Dipyridamole	4.64 ± 0.23[a]	73 ± 10	6	6.50 ± 0.29[b]	92 ± 3[b]	6	5.04 ± 0.08	92 ± 3	6
Zaprinast	Nob	39 ± 10[a]	6	5.74 ± 0.21	91 ± 3[b]	7	4.90 ± 0.27	77 ± 13	6
Milrinone	5.47 ± 0.28	84 ± 7	6	5.20 ± 0.15	84 ± 5	7	5.28 ± 0.25	82 ± 9	6
Pentoxifylline	Nob	10 ± 1[a]	6	Nob	49 ± 11[b]	6	Nob	33 ± 16	7
IBMX	5.36 ± 0.14[a]	93 ± 1[a]	7	6.44 ± 0.30[b]	96 ± 1[b]	6	5.33 ± 0.16	92 ± 7	6

Values represent mean ± S.E.M. Nob = not obtainable. N = number of animals. The maximum dilatory effect, E_{max}, of an agonist was calculated as % relaxation of the pre-contraction. pEC_{50} is the negative logarithm of the molar concentration that produced half-maximal relaxation.

[a] $P < 0.05$ refers to the significant difference between reactions in arteries with (Table 1) and without endothelium.

[b] $P < 0.05$ refers to significant difference between responses with and without sodium nitroprusside pretreatment (5 min) in arteries without endothelium.

± 0.10 pmol/mg protein after addition of ODQ (10 μM) ($P < 0.001$). Sodium nitroprusside (1 μM) caused a fourfold increase in cGMP concentration that was reduced to 0.2 ± 0.07 pmol/mg after ODQ pretreatment (Fig. 4). The cGMP concentration increased threefold compared to control after addition of zaprinast (30 μM) (Fig. 4) and dipyridamole (30 μM). These concentrations were significantly reduced four- and fivefold after pretreatment with ODQ. Despite the minor cGMP concentration in the presence of ODQ, both dipyridamole and zaprinast induced fairly strong dilatory responses amounting to $49.8 \pm 9.4\%$ and $57.4 \pm 7.5\%$, respectively.

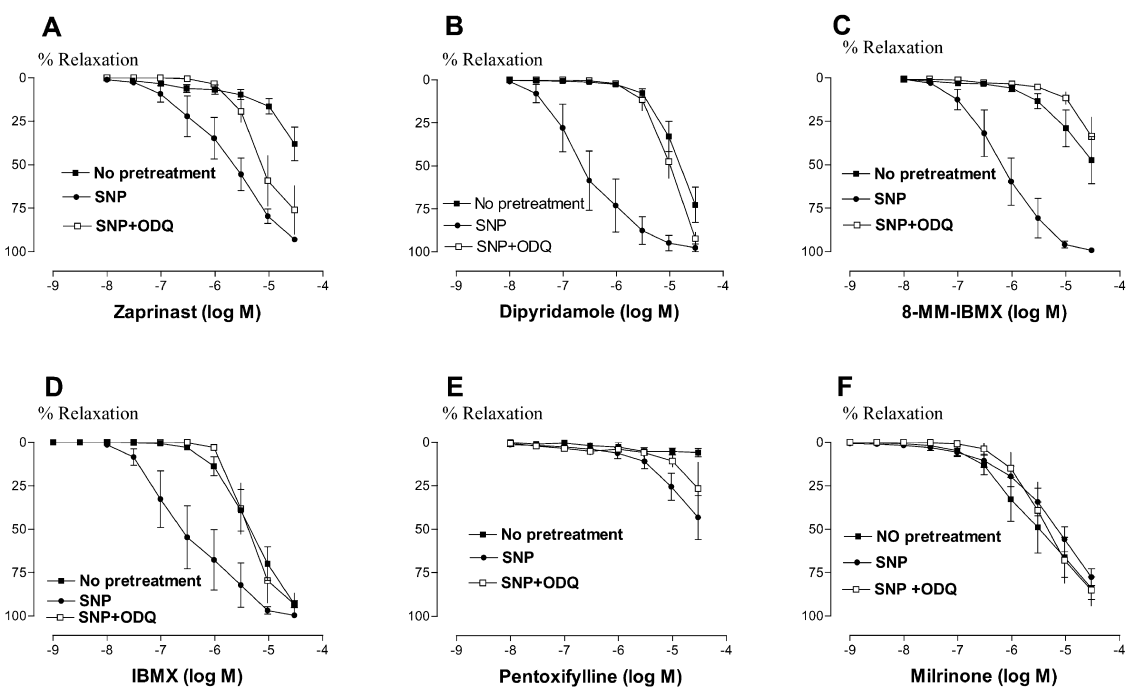

Fig. 3. Concentration–response curve for the inhibitors of the cGMP related phosphodiesterases, phosphodiesterases 1 (8-MM-IBMX) and 5 (zaprinast and dipyridamole), the non-selective phosphodiesterase inhibitors (IBMX and pentoxifylline) and the selective phosphodiesterase 3 inhibitor milrinone in guinea pig basilar arteries where the endothelium has been removed. The response is expressed as % relaxation of pre-contraction and each point shows mean ± S.E.M. The curves for zaprinast (A), dipyridamole (B), 8-MM-IBMX (C), IBMX (D), pentoxifylline (E) and milrinone (F) are shown without (■) and with sodium nitroprusside (0.1 μM) (●) and sodium nitroprusside (0.1 μM) + ODQ (1 μM) (□) pretreatment. Removing the endothelium significantly decreases the dilatory response of these phosphodiesterase inhibitors, however, addition of sodium nitroprusside restored or even potentiated (8-MM-IBMX, $P = 0.045$) the response to the selective phosphodiesterase inhibitors and reduced when ODQ was applied. $N = 6–7$.

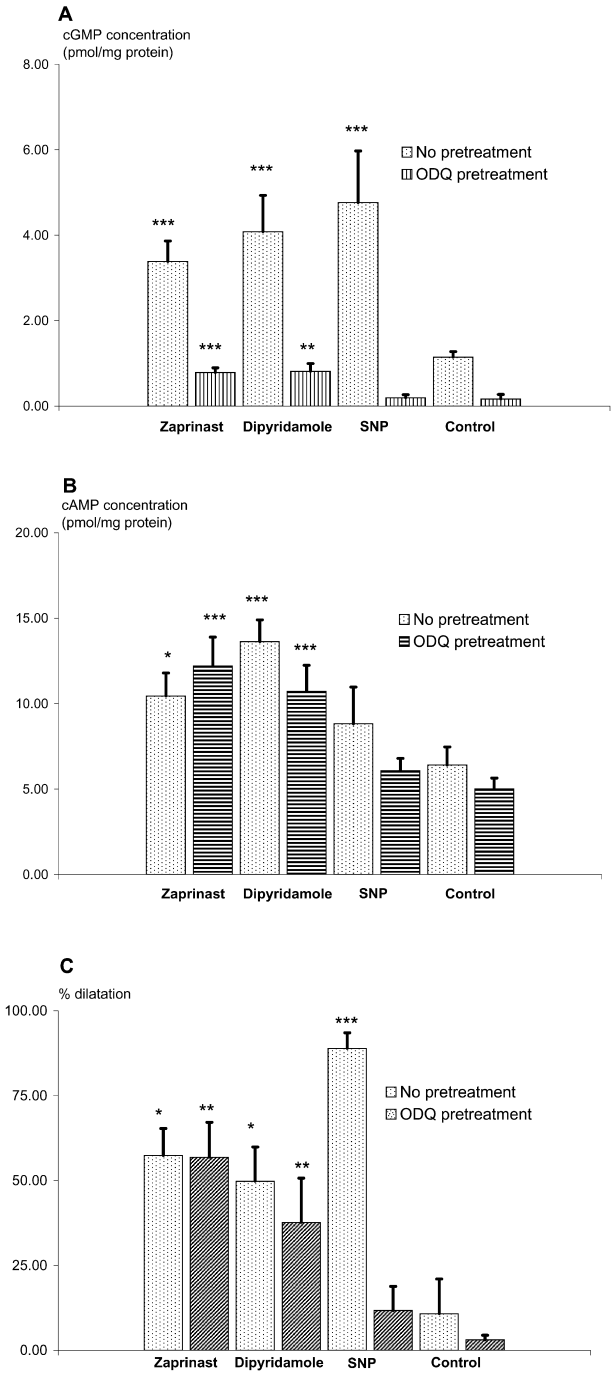

Fig. 4. The concentrations of cGMP (A), cAMP (B) and the relaxant response (C) are shown for single concentrations of zaprinast (30 μM), dipyridamole (30 μM) and sodium nitroprusside (1 μM) in intact arteries in the absence and presence of ODQ (10 μM). Values are given as means ± S.E.M, $N = 7$. Statistical analysis was performed using an unpaired t-test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Generally, a higher basal level ($P < 0.001$) of cAMP than cGMP was found in the arteries with concentrations of 6.40 ± 1.01 pmol/mg protein without ODQ and 5.02 ± 0.60 with ODQ pretreatment ($P = 0.17$). No significant increase in cAMP was seen after addition of sodium nitroprusside. However, zaprinast ($P = 0.02$), dipyridamole ($P < 0.01$), milrinone ($P = 0.02$) and pentoxifylline ($P = 0.002$) all increased cAMP concentrations compared to that seen in control vessels. Concentrations of cAMP were 10.39 ± 0.99 pmol/mg protein for milrinone and 15.54 ± 2.36 pmol/mg protein for pentoxifylline. ODQ pretreatment did not significantly change the cAMP response to zaprinast, dipyridamole and milrinone.

4. Discussion

Phosphodiesterases are key molecules in the process of modulating arterial diameter, since they show variations in tissue distribution (Beavo, 1995; Rybalkin et al., 1997), specialised functions in different vascular beds (Boolell et al., 1996; Sandner et al., 1999; Torphy et al., 1993) and are easily targeted using selective inhibitors (Beavo and Reifsnyder, 1990; Stoclet et al., 1995).

In the cerebral circulation, the possibility of modulating the blood flow using selective phosphodiesterase inhibitors or maybe activators, may thus turn out to be effective the treatment of conditions such as cerebral vasospasms after subarachnoid haemorrhage, stroke and migraine. However, in order to use the most selective inhibitors and minimising unwanted side effects, it is essential to know the exact distribution of the different phosphodiesterase families in cerebral arteries, and the role and importance of the different phosphodiesterase families in the physiological response of the cerebral arteries.

The presence of phosphodiesterases in cerebral arteries has mainly been shown indirectly through studies on the physiological effects of various phosphodiesterase inhibitors (Cosentino et al., 1992; Harris et al., 1989a; Kim et al., 1992; Parfenova et al., 1993; Rosenblum et al., 1993; Sobey and Quan, 1999; Willette et al., 1997). In canine basilar arteries zaprinast induced dilatation with an increase in cGMP (Kim et al., 1992), whereas milrinone was found to be less effective in dilating basilar arteries compared to renal and coronary arteries (Harris et al., 1989a). In porcine pial arteries, Parfenova et al. (1993) suggested the presence of phosphodiesterases 1, 4 and 5, using selective inhibitors to induce vasodilatation and measuring concomitant increases in cGMP and cAMP. However, actual phosphodiesterase activity was not investigated in these arteries.

In the present study, the cGMP hydrolysing phosphodiesterases, phosphodiesterases 1A and 1B, stimulated by Ca^{2+}/Calmodulin, and phosphodiesterase 5, specific for cGMP, displayed the most prominent hydrolysing activity. It is, however, not possible to exclude the presence of other cGMP hydrolysing phosphodiesterases, like phos-

phodiesterase 9, since the Western blot profile did not entirely match the cGMP hydrolysis profile in all fractions and only antibodies for phosphodiesterases 1 and 5 were available. A prominent cGMP hydrolysis has been observed before in bovine (Lugnier and Komas, 1993), human, monkey and rat (Rybalkin et al., 1997) aortic smooth muscle cells.

Phosphodiesterases 3 and 4 may also be present, but only minor cAMP hydrolysing activity was seen in the soluble fraction of the tissue. The lower cAMP hydrolysing activity in the arteries is somehow striking because of a higher absolute level of cAMP both reported earlier (Gray and Marshall, 1992; Parfenova et al., 1993) and seen in the present study. It may be due to the cAMP hydrolysing enzymes being membrane-bound and activity was measured on the soluble fraction. However, it could be speculated that the actual activity and effects of cAMP hydrolysing phosphodiesterases might be independent of the total cAMP concentration. Thus, cAMP and cAMP-related phosphodiesterase enzymes may be located in distinct compartments of the cerebral smooth muscle cells where it exerts its actions, like it is reported for cardiac tissue (Stoclet et al., 1995).

Zaprinast is a phosphodiesterase 5 inhibitor previously found to show approximately 40 times higher potency for phosphodiesterase 5 than phosphodiesterase 1 (Lugnier and Komas, 1993). In the present study, both zaprinast and dipyridamole relaxed cerebral arteries in a concentration-dependent manner and they both showed endothelial-dependent relaxation, which could be restored by application of the NO donor sodium nitroprusside. However, their function did not seem to be entirely due to phosphodiesterase 5 inhibition. First of all, zaprinast inhibited the cGMP hydrolysing activity of phosphodiesterases 1A and 1B as well as of phosphodiesterase 5 when administered in concentrations eliciting 50% relaxation of isolated basilar arteries. This suggests that the dilatory effects may be mediated through inhibition of both phosphodiesterases 1 and 5, as suggested for other tissues earlier (Pelligrino and Wang, 1998). Furthermore, we found that the dilatation induced by both phosphodiesterase 5 inhibitors did not fully correlate to an increase in cGMP concentration, since dilatation occurred with only minor increase in cGMP production in the presence of ODQ (Fig. 4). Neither did we see a complete inhibition of the dilatory response to zaprinast after endothelial removal like previously reported in aortic rings by Harris et al. (1989b).

The mechanism behind the apparent cGMP independent dilatory effect of the phosphodiesterase 5 inhibitors in cerebral arteries remains obscure. In studies by Souness et al. (1989), Parfenova et al. (1993) and by Ahn et al. (1989), cGMP concentrations correlated to the increase in zaprinast concentration, however, in the latter not to an increase in dipyridamole concentrations. The significant increase in cAMP concentrations after zaprinast and dipyridamole application could support the suggestion of a lack of specificity of zaprinast and dipyridamole at high concentrations and the cAMP increase may moreover play an active role in the dilatation seen. The equivocal effect of the used phosphodiesterase 5 inhibitors may also be caused by a lack of selectivity towards the phosphodiesterase 5 present in cerebral arteries, which could be another iso-form of phosphodiesterase 5, however with a similar molar weight based on Western blot.

Thus, the exact physiological role of phosphodiesterase 5 in cerebral artery dilatation is difficult to establish from the present results using zaprinast and dipyridamole. These results should perhaps be kept in mind when reviewing previous results elucidating the function of phosphodiesterase 5 in cerebral arteries using high concentrations of these less selective phosphodiesterase 5 inhibitors, in studies where no concomitant cyclic nucleotide measurements were performed (Sobey and Quan, 1999). A more selective inhibitor would have been preferred in the present study and should be used in future studies. However, for the present study, it was not possible to obtain the more selective phosphodiesterase 5 inhibitor sildenafil.

In contrast, more consistent results were seen with the phosphodiesterase 1 inhibitor 8-MM-IBMX. The dilatation induced by 8-MM-IBMX was absent when the soluble guanylate cyclase was inhibited by ODQ or after removal of the endothelium. It was restored following addition of sodium nitroprusside to the endothelium-denuded arteries and inhibited again with ODQ, implying a close correlation between cGMP production and the dilatory effect of 8-MM-IBMX, putatively via phosphodiesterase 1.

Zaprinast has previously been reported to potentiate the effect of NO-induced dilatation in some tissues like rat and rabbit aorta (Harris et al., 1989b; Martin et al., 1986) but not in guinea pig aorta (Harris et al., 1989b) or canine basilar arteries (Katusic et al., 1989). In consistence with the latter, we found no potentiating effect of zaprinast on sodium nitroprusside-induced dilatation in intact guinea pig cerebral arteries. This may be related to specificity in response between vascular beds, a species difference or a variation in experimental design. However, we did find a potentiating effect of sodium nitroprusside pretreatment on dilatation induced by several phosphodiesterase inhibitors in arteries without endothelium, which was most likely caused by the increased production of cGMP induced by sodium nitroprusside compared to the basal production of cGMP in arteries with intact endothelium. Since addition of both selective and non-selective phosphodiesterase inhibitors relaxed guinea pig basilar arteries in vitro, it supports that there is a basal production of cGMP and cAMP even in isolated cerebral arteries (Laing et al., 1995). This basic production was inhibited when inhibiting soluble guanylate cyclase activity by ODQ although basal arterial tone was unaffected. ODQ also inhibited the dilatory response to sodium nitroprusside, but not that of atrial natriuretic peptide, demonstrating the selective inhibition by ODQ of the soluble guanylate cyclase, but not the

particulate guanylate cyclase. This inhibition of NO-induced vasodilatation supports the hypothesis that NO predominantly dilates cerebral arteries through activation of soluble guanylate cyclase (Faraci and Sobey, 1999) and not as it has been suggested previously, through direct effects on potassium channels (Bolotina et al., 1994).

The reported decrease of sensitivity towards sodium nitroprusside in arteries without endothelium compared to intact arteries, is in contrast to previous reports on rat aortas (Harris et al., 1989b), but it could be caused by a difference in species or in vascular beds used.

Although we observed only minor cAMP hydrolysing activity in the soluble fraction, milrinone induced cerebral artery dilatation with a pEC_{50} similar to that of dipyridamole but higher than zaprinast and with a slightly higher E_{max}. The increase in cAMP induced by the selective phosphodiesterase 3 inhibitor milrinone was similar to the increase seen after the phosphodiesterase 5 inhibitors. A compartmentalisation of cAMP pools or phosphodiesterase 3 may play a role in the dilatation induced. The dilatory response of milrinone was largely unaffected by ODQ pretreatment or by endothelial removal indicating no effects of cGMP on the milrinone response. Using milrinone in specific modulation of cerebral artery diameter does, however, not seem appropriate in light of the more pronounced effect the compound seem to have on the coronary and renal arteries (Harris et al., 1989a).

Although there may be species difference, and the phosphodiesterase distribution could be different in human cerebral arteries, studies of cerebral artery dilatation in healthy subjects have shown that pentoxifylline does not induce artery dilatation (Kruuse et al., 2000a), whereas dipyridamole selectively dilates large cerebral arteries (Kruuse et al., 2000b). This is in agreement with the present findings of pentoxifylline being a poor vasodilator and dipyridamole, although not a very selective phosphodiesterase 5 inhibitor, being effective as a vasodilator of cerebral arteries in vitro.

Thus, the use of selective phosphodiesterase inhibitors could be excellent tools to investigate the role of phosphodiesterases in cerebral circulation also in vivo. A characterisation of the cAMP and cGMP responses in combination with a thorough investigation of the effects of the selective phosphodiesterase inhibitors in vitro should ideally be performed prior to the in vivo studies.

Furthermore, the present study clearly shows that more selective phosphodiesterase 5 inhibitors than zaprinast and dipyridamole should be used in the future when they become available.

If the distribution and functions of phosphodiesterases are similar in humans, selective inhibitors of the cGMP hydrolysing phosphodiesterases 1A, 1B, 5 and perhaps the cAMP degrading phosphodiesterase 3 could prove to be useful tools in selective modulation of the cerebral blood flow and may thus be useful in treatment of cerebrovascular disease. Studies using selective inhibitors both in vitro and in vivo in human tissue, healthy subjects and patients may be able to reveal this interesting possibility.

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