Available online at www.sciencedirect.com
SCIENCE DIRECT®
European Journal of Pharmacology 489 (2004) 93–100
www.elsevier.com/locate/ejphar

Analysis of the effects of phosphodiesterase type 3 and 4 inhibitors in cerebral arteries

Steffen Birk[a,b,*], Lars Edvinsson[b], Jes Olesen[a], Christina Kruuse[a]

[a] Department of Neurology, University of Copenhagen, Glostrup University Hospital, Glostrup, Copenhagen 2600, Denmark
[b] Department of Clinical Experimental Research, University of Copenhagen, Glostrup University Hospital, Glostrup, Copenhagen 2600, Denmark

Received 23 January 2004; received in revised form 17 February 2004; accepted 26 February 2004

Abstract

Inhibitors of phosphodiesterases 3 and 4, the main cyclic AMP (cAMP) degrading enzymes in arteries, may have therapeutic potential in cerebrovascular disorders. We analysed the effects of such phosphodiesterases in guinea pig cerebral arteries with organ bath technique and cyclic nucleotide assays. Guinea pig and human cerebral arteries were used for phosphodiesterase assays. Cilostazol (6-[4-(1-cyclohexyl-1*H*-tetrazol-5-yl)butoxy]-3,4-dihydro-2(1*H*)-quinolinone), a phosphodiesterase 3 inhibitor, was compared to conventional phosphodiesterase 3 and 4 inhibitors.

Phosphodiesterases 3 and 4 were the major contributors to total cAMP hydrolysis in the arteries examined. The phosphodiesterase 3 inhibitors additionally attenuated cyclic GMP (cGMP) hydrolysis, but relaxant responses were not dependent on an intact endothelium or on the nitric oxide–cGMP pathway. Conversely, the phosphodiesterase 4 inhibitor used was endothelium-dependant and affected by cGMP levels.

This suggests that phosphodiesterase 3 inhibitors are still effective under conditions with possible dysfunctional nitric oxide–cGMP pathway, such as in ischemic stroke or cerebral vasospasm.

Keywords: Subarachnoid haemorrhage; Cerebral vasospasm; Phosphodiesterase inhibitor; Cilostazol; Endothelium

1. Introduction

The phosphodiesterases are a family of enzymes responsible for the degradation of cyclic AMP (cAMP) and cyclic GMP (cGMP) (Beavo, 1995). These cyclic nucleotides mediate of a wide range of biological actions, including the regulation of vascular tone. The cyclic nucleotide signal is regulated at several levels; the phosphodiesterases play a pivotal role in hydrolysing the 3′ phosphoester bond thereby terminating the signal. The most important cAMP degrading phosphodiesterases in arterial tissues are phosphodiesterase 3 and phosphodiesterase 4 (Polson and Strada, 1996). Although both hydrolyse cAMP, there are marked differences in substrate affinity and catalytic activity, which may have functional implications (Stoclet et al., 1995). Furthermore, phosphodiesterase 3 is inhibited by cGMP and the level of this molecule may influence the rate of cAMP hydrolysis and the relaxant response to cAMP phosphodiesterase inhibitors (Lugnier and Komas, 1993).

Because of the multiple isoforms, varied tissue distribution and the hitherto successes in developing isozyme-selective inhibitors, phosphodiesterase inhibitors are interesting drug targets in relation to many diseases (Beavo, 1995; Stoclet et al., 1995). In the cerebrovascular system, phosphodiesterases 3 and 4 have may be useful in prevention of ischemic stroke and in the acute treatment of delayed cerebral vasospasm, a frequent and severe complication to subarachnoid haemorrhage (Arakawa et al., 2001; Gotoh et al., 2000). However, these cerebrovascular disorders are believed to be associated with dysfunction of endothelial signalling pathways (Cosentino et al., 2001; Sobey, 2001). Among other vasoactive substances, the normal endothelium synthesizes nitric oxide (NO), which stimulates soluble guanylate cyclase resulting in the synthesis of cGMP in vascular smooth muscle cells (Furchgott and Vanhoutte, 1989). If the NO–cGMP pathway is dysfunctional in stroke and delayed cerebral vasospasm, as previously suggested (Cosentino et al., 2001; Sobey, 2001),

* Corresponding author. Tel.: +45-4323-3060; fax: +45-4323-3960.
E-mail address: birk@dadlnet.dk (S. Birk).

0014-2999/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2004.02.038

this may impair the efficacy of the phosphodiesterase inhibitors as vasodilators.

The present studies aims at investigating the role of phosphodiesterase 3 and 4 inhibitors on cyclic nucleotide turnover and relaxant response in cerebral arteries with an emphasis on the role of the endothelium and the NO–cGMP pathway on the functional response. Among the tested substances was cilostazol (6-[4-(1-cyclohexyl-1*H*-tetrazol-5-yl)butoxy]-3,4-dihydro-2(1*H*)-quinolinone), which besides milrinone is the only currently available phosphodiesterase 3 inhibitor available for use in humans, and which potently dilates the middle cerebral artery in healthy volunteers (unpublished results by the authors, 2003).

2. Materials and methods

Male guinea pigs weighing 350–450 g (Ssc:AL, Statens seruminstitut, Denmark) were decapitated during CO_2 narcosis between 8:00 and 9:00 AM. The brain was removed and the basilar artery carefully dissected free from the brain using a stereomicroscope and kept in cold buffer (composition: Na^+ 135.2, K^+ 4.6, Ca^{2+} 1.5, Mg^{2+} 1.2, HCO_3^- 15.0, Cl^- 129.0, HPO_4^{2-} 1.2, glucose 5.5, mM) aerated with 95% O_2 and 5% CO_2. All experiments involving tissue baths were conducted on the day of sacrifice. The arteries were used either for studies of phosphodiesterase inhibitor effects or for the assessment of cyclic nucleotide metabolism.

2.1. Studies of vasomotor responses

The basilar arteries were cut in annular segments approximately 1 mm long and mounted on a pair of 150-μm-wide teflon-coated steel rods connected to a force displacement transducer (Multi Myograph 610 M, Danish Myo Technology, Aarhus, Denmark) (Mulvany and Halpern, 1976). The mount was placed in a tissue bath of the buffer solution aerated to a pH 7.4 with 95% O_2 and 5% CO_2 and maintained at a temperature of 37 °C. When needed, the endothelium was removed prior to mounting by perfusing the artery with 0.1% Triton X-100 for 10 s followed by a rinse in the buffer solution.

All arteries were distended until a stable resting tension of 2 mN/mm was established. Viability of the arteries was tested by exposing them twice to a buffer with a high potassium concentration (composition: Na^+ 59.5, K^+ 60.0, Ca^{2+} 1.5, Mg^{2+} 1.2, HCO_3^- 15.0, Cl^- 125.3, HPO_4^{2-} 1.2, glucose 5.5, mM). The stability of the pre-contraction for each artery was tested by adding prostaglandin $F_{2\alpha}$ 3 μM for 15–20 min. Experiments were not included in analysis if the period of stable pre-contraction was not sufficient to perform the subsequent experiments. The presence or the absence of a functional endothelium was tested by exposing the pre-contracted arteries to acetylcholine 10 μM. The chosen concentration of prostaglandin $F_{2\alpha}$ has previously been shown to elicit a mean of 80% of maximum contraction in intact and endothelium-denuded arteries alike (Kruuse et al., 2001). For experiments with intact endothelium, $\geq 85\%$ dilatory reaction was accepted, for experiments with endothelium-denuded arteries, arteries with more than 10% dilatation after exposure to acetylcholine were discarded. The arteries were allowed to equilibrate for 45–60 min before starting the experiments. Mean ± S.E.M. contraction after exposing intact arteries to K^+-buffer was 4.93 ± 0.13 mN/mm. In endothelium-denuded arteries, the corresponding value was significantly lower (3.92 ± 0.13 mN/mm, $P < 0.001$).

After pre-contraction with prostaglandin $F_{2\alpha}$ (3 μM), the drugs were added cumulatively in half log steps. The arteries were allowed to fully react before the addition of the succeeding dose, and the interval between doses was approximately 2 min. A maximum of three experiments were performed for each segment, with a wash out period of at least 45 min in between, and only one agent was tested in each segment. $N = 5–12$ for each compound tested. In preliminary experiments ($N = 2–4$), no obvious tachyphylaxis or potentiation after repetitively obtaining dose–response curves for up to three times was detected for any of the tested phosphodiesterase inhibitors. Due to solubility problems and the dilatory effect of the solvent dimethyl sulfoxide itself in high concentrations, drug concentrations above 30 μM could not be tested in some cases. Cilostazol and cilostamide were not tested in concentrations above 10 μM because of lack of phosphodiesterase 3 selectivity (Sudo et al., 2000). Buffer solution was replaced every 15 min, except in experiments using extended pre-treatment.

Vascular relaxation was expressed as percentage of maximum dilatation elicited by exposure to a Ca^+-free buffer (composition: Na^+ 135.2, K^+ 4.6, Mg^{2+} 1.2, HCO_3^- 15.0, Cl^- 126.0, HPO_4^{2-} 1.2, glucose 5.5, mM). This was equal to baseline tension, i.e. no spontaneous muscle tone was observed.

2.2. Cyclic nucleotides measurement in vitro

The basilar arteries were dissected free from connective tissue. The arteries were cut at an approximate length of 2 mm, mounted and tested as described. After standard testing, a single concentration of the tested compound was added. The reaction was stopped after exactly 2 min by swiftly replacing the buffer with ice-cold acidic ethanol and immediately transferring the tissue to an Eppendorf test tube cooled with dry ice. The period of 2 min was chosen since preliminary experiments showed that it was the time necessary to establish a stable relaxant response. Furthermore, published time-effect experiments demonstrated a peak in cyclic nucleotides at 2 min after the application of sodium nitroprusside, isoproterenol and milrinone (Schini et al., 1989; Silver et al., 1988; Vegesna and Diamond, 1986). Samples were stored at −20° until homogenization.

Tissues were homogenized on dry ice and treated as previously described (Kruuse et al., 2001). The amount of cAMP or cGMP in the supernatant was determined with commercially available ^{125}I radioimmunoassays (Amersham, USA) using the sensitive acetylating method. The pellet was re-suspended and analysed spectrophotometrically (wavelength 595, Lambda Bio, Perkin Elmer, USA) for protein content (BioRad, USA) against a bovine serum albumin standard curve. Tension and cyclic nucleotide contents were thus measured in the same arterial rings.

2.3. Tissue preparation for enzyme activity analysis

Guinea pig basilar arteries were harvested as described, and any luminal blood was carefully removed. Three separate experiments with a pool of eight basilar arteries each were performed. Small branches from the human middle cerebral artery were obtained and frozen within 9-h post mortem. The tissue originated from three persons not suffering from cerebrovascular disorders (two female, one male; age 71, 73 and 82 years). The procedure was approved by the ethical committee in Copenhagen County.

The frozen tissue was dissociated mechanically with mortar and pestle. The tissue was transferred to 3 ml homogenization buffer (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, Triton X-100, 0.1%, pH 7.3) and further homogenized on ice with a glass piston. Finally, the homogenate was sonicated approximately three times for 10 s at 20% of maximal output (Branson sonifier, USA). The homogenate was then centrifuged in a two-step procedure (Kruuse et al., 2001). The supernatant was kept at 4 °C during all procedures.

2.4. Phosphodiesterase activity

1 μM cAMP/[^{3}H]cAMP or cGMP/[^{3}H]cGMP (approximately 50.000 cpm) was used as the substrate. Various concentrations of cilostazol, cilostamide and rolipram were added to the homogenate and assay buffer (Kruuse et al., 2001) in the presence of EGTA (1 mM), to investigate the impact of these compounds on total cyclic nucleotide hydrolysis. The reactions were initiated by the addition of the substrate, maintained at 30° C for 10 min and terminated by boiling for 1 min. The assay was subsequently treated with snake venom from Crotalus Atrox at 30° for 5 min, loaded onto DEAE-sephadex A25 columns and eluted using 2 ml of low salt buffer (20 mM Tris−Cl, pH 6.8). The eluate was collected in scintillation vials and was quantified by counting [^{3}H]nucleoside for 10 min.

2.5. Drugs and compounds

Bovine serum albumin, prostaglandin $F_{2\alpha}$, rolipram, forskolin, sodium nitroprusside, 1*H*[1,2,4]oxadiazolo{4,3,-*a*}quinoxalin-1-one (ODQ), 8-4-(chlorophenylthio)-guanosine3′:5′-cyclic monophosphate (pCPT-cGMP), cAMP, cGMP and dimethyl sulfoxide were purchased from Sigma-Aldrich (Vallensbaek Strand, Denmark). Acetylcholine was obtained from RBI (Bie og Berntsen, Roedovre, Denmark), cilostamide from Calbiochem (Sigma-Aldrich, Vallensbaek Strand, Denmark) and [^{3}H]cGMP and [^{3}H]cAMP from NEN Lifescience products (USA). Cilostazol was generously provided by Otsuka America Pharmaceuticals (Maryland, USA).

ODQ, cilostamide, cilostazol, rolipram and forskolin were dissolved in dimethyl sulfoxide 100% for stock solutions. The remaining compounds were dissolved in distilled water to a 0.01 M stock solution stored at −20 °C and further diluted with distilled water immediately before use. The maximal concentration of dimethyl sulfoxide in tissue baths did not exceed 0.4% v/v.

Rolipram is a phosphodiesterase 4 inhibitor widely used for in vitro pharmacology. The IC_{50} for phosphodiesterase 4 from canine basilar artery is 0.76 μM and is generally considered highly selective (Lugnier and Komas, 1993; Willette et al., 1997). Cilostamide is a phosphodiesterase 3 inhibitor likewise used extensively in vitro. IC_{50} for human recombinant phosphodiesterase 3A is 0.027 μM and for 3B 0.050 μM. In the 10-μM range, cilostamide also has phosphodiesterase 2 and 5 activity (Sudo et al., 2000). Cilostazol is less potent with an IC_{50} for phosphodiesterase 3A of 0.20 μM and for 3B 0.38 μM in human recombinant phosphodiesterases. It is less selective than cilostamide in regard to phosphodiesterase 5 with an IC_{50} of 4.4 μM, but with no other relevant effects in human recombinant phosphodiesterase type 1, 2, 4 or 7 (Sudo et al., 2000). Cilostazol is used clinically in the treatment of intermittent claudication in USA and some Asian countries (Sorkin and Markham, 1999). Apart from phosphodiesterase activity, it also inhibits cellular adenosine re-uptake (Liu et al., 2001).

2.6. Statistical methods

Data are given as mean ± S.E.M. unless otherwise indicated. *N* indicates the number of different animals from which the arteries originated. The negative logarithm of EC_{50} values (pD_2) was calculated from a fitted sigmoid concentration−response curve (variable slope). Where the completeness of the sigmoid dose−response curves could not be confirmed due to solubility problems, apparent pD_2 values were calculated from the fitted mean curves. This was the case in all substances tested but sodium nitroprusside and forskolin. Apparent pD_2 values are stated with 95% confidence intervals (CI). (NotePad Prism 3.00®, GraphPad Software, USA) Statistical differences between curves were tested using area under the curve (AUC) as summary measure with Student's *t*-test, paired or unpaired as appropriate. In the single dose

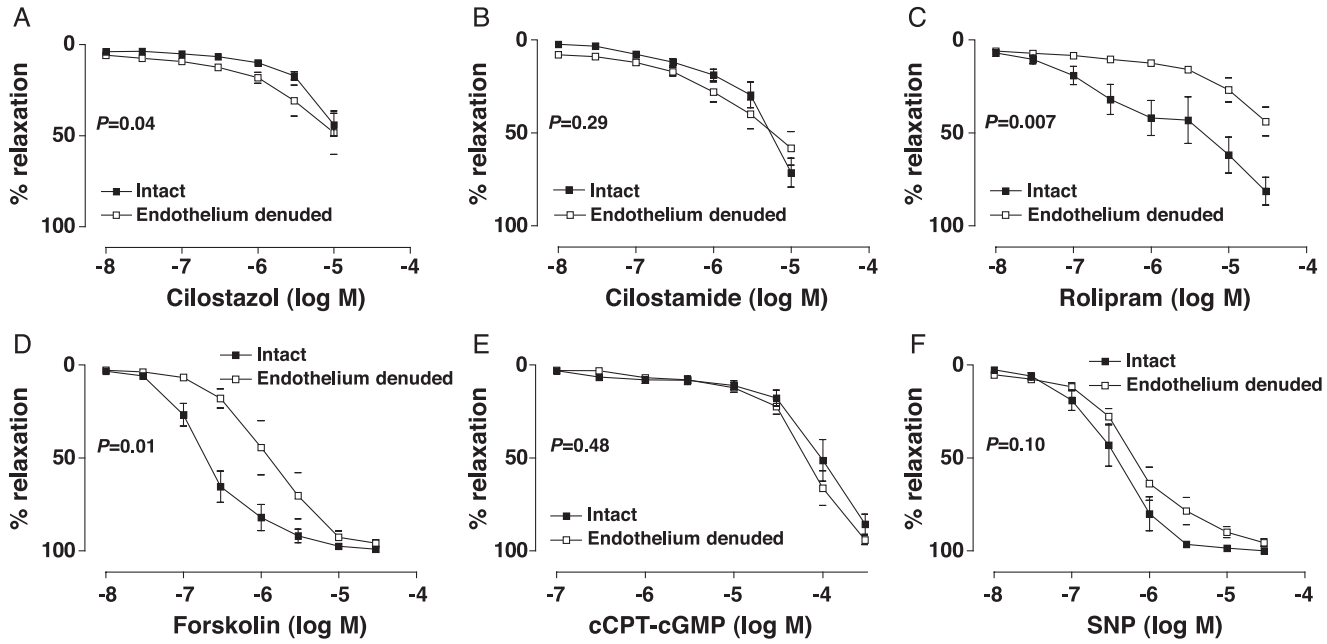

Fig. 1. Concentration–response curves for the phosphodiesterase 3 inhibitors cilostazol (A), cilostamide (B) the phosphodiesterase 4 inhibitor rolipram (C), forskolin (D), sodium nitroprusside (E) and pCPT-cGMP (F) with (■) or without (□) a functional endothelium ($N = 5–12$), using guinea pig basilar artery pre-contracted with prostaglandin $F_{2\alpha}$ 3 μM. Means ± S.E.M. The dilatory response of rolipram is almost completely attenuated upon removal of the endothelium.

experiments, differences in dilatation and cyclic nucleotide content compared to control were calculated with a nonparametric test (Mann–Whitney *U*-test). SPSS for Windows 11.5 (Chicago, IL, USA) was used for all statistical tests. The null hypothesis was rejected at $P < 0.05$.

3. Results

3.1. Arterial vasomotor responses

All phosphodiesterase inhibitors relaxed the arteries concentration-dependently, but failed to show a complete

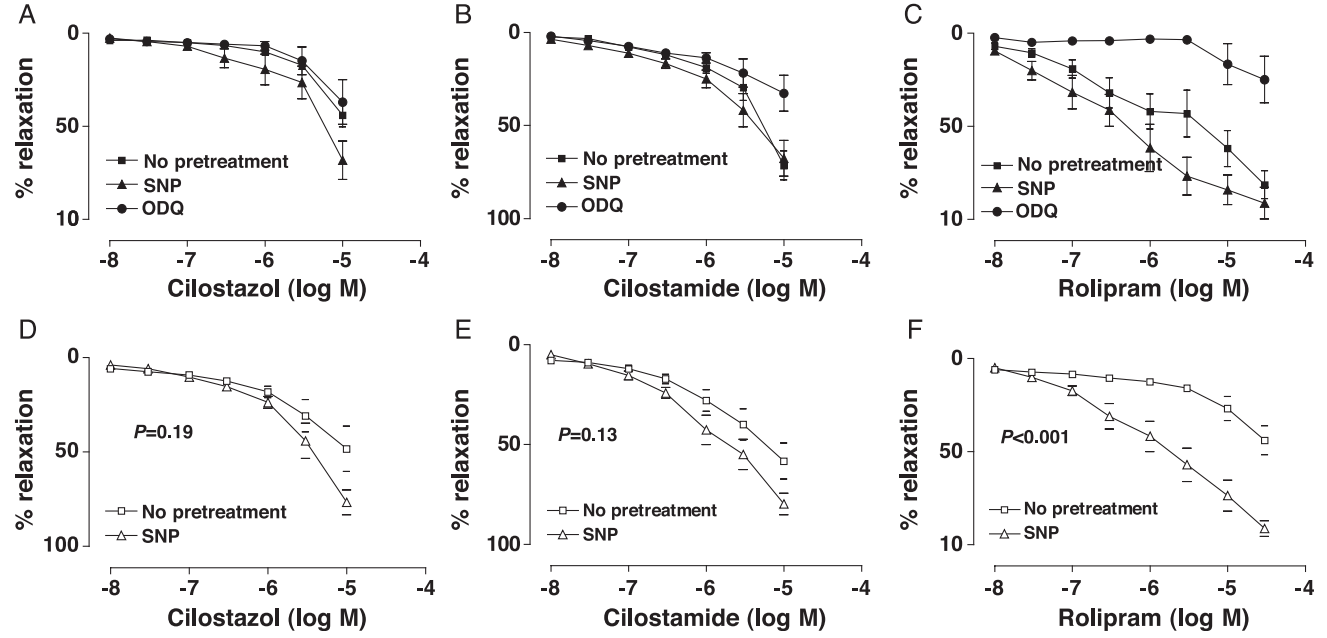

Fig. 2. Concentration–response curves for the phosphodiesterase 3 inhibitors cilostazol (A, C); cilostamide (B, D) and the phosphodiesterase 4 inhibitor rolipram (C, F) pre-treated with sodium nitroprusside 0.1 μM (▲, △) or ODQ 10 μM (■, □) compared to no pre-treatment (●, ○) in guinea pig basilar arteries pre-contracted with prostaglandin $F_{2\alpha}$ 3 μM with (A, B, C) or without endothelium (D, E, F) ($N = 6–12$). Means ± S.E.M. Rolipram is strongly dependent of a functional endothelium and data points to an involvement of NO–cGMP pathway in the dilatory mechanisms.

S-shaped curve. Apparent pD_2 values with 95% CI for cilostazol and cilostamide were 4.83 (5.07–4.60) and 5.22 (5.37–5.06), respectively. Apparent pD_2 for the phosphodiesterase 4 inhibitor rolipram was 5.58 (5.35–5.80). Single-experiment concentration–response curves for all phosphodiesterase inhibitors were sigmoid and did not differ from cumulative concentration–response curves. There was a marked variation in potency from vessel to vessel for all tested phosphodiesterase inhibitors.

3.2. Role of the endothelium

Comparison of relaxant effects in the presence or absence of the endothelium is given in Fig. 1. In endothelium-denuded arteries, dilatations induced by rolipram were markedly attenuated ($P=0.007$), while the response to cilostazol was slightly potentiated ($P=0.04$). Removal of the endothelium did not alter the response to cilostamide ($P=0.23$). The concentration–response curve for forskolin, an activator of adenylate cyclase (Suzuki et al., 1988), was shifted one log unit to the right ($P=0.01$) after removal of the endothelium. The presence or absence of the endothelium had no effect on dilatations induced by sodium nitroprusside ($P=0.10$) or the cell-permeable cGMP analogue pCPT-cGMP ($P=0.48$).

3.3. Signalling pathways

Pre-treating the artery segments with sodium nitroprusside or ODQ did not affect responses to cilostazol or cilostamide in endothelium intact arteries. Conversely, ODQ attenuated the relaxant response to rolipram ($P=0.001$), but there was no significant potentiating effect of sodium nitroprusside ($P=0.13$).

Table 1
Corresponding values of dilatory response of and cyclic nucleotide contents in pre-contracted guinea pig basilar segments after a two-minute incubation with relaxant

	Relaxant (%)	cAMP (pmol/mg) protein	cGMP (pmol/mg protein)
Control	1.8 ± 0.8	0.11 ± 0.02	0.02 ± 0.00
Cilostazol, 1 μM	5.3 ± 1.0[a]	0.18 ± 0.03[a]	0.03 ± 0.01
Cilostazol, 10 μM	23.8 ± 4.4[b]	0.24 ± 0.06[b]	0.04 ± 0.01[a]
Cilostamide, 1 μM	27.8 ± 10.8[b]	0.15 ± 0.02	0.02 ± 0.00
Cilostamide, 10 μM	30.2 ± 10.1[b]	0.12 ± 0.01	0.02 ± 0.00
Rolipram, 1 μM	38.1 ± 14.4[b]	0.20 ± 0.02[b]	0.02 ± 0.00
Rolipram, 10 μM	37.7 ± 11.1[b]	0.16 ± 0.02[a]	0.02 ± 0.00
Forskolin, 0.1 μM	23.2 ± 8.2[b]	0.27 ± 0.07[b]	0.03 ± 0
Sodium nitroprusside pre-treatment (0.1 μM)	− 0.3 ± 1.2	0.25 ± 0.04[b]	0.06 ± 0.01[b]
ODQ pre-treatment (10 μM)	− 0.3 ± 1.2	0.14 ± 0.03	0.00 ± 0.00[b]

Means ± S.E.M. ($N=6–8$).
Significantly different from control with no pre-treatment, Mann–Whitney.
[a] $P<0.05$.
[b] $P\leq 0.01$.

Table 2
Percentage of inhibition of total cGMP and cAMP hydrolysis in human and guinea pig cerebral arteries by phosphodiesterase 3 and phosphodiesterase 4 inhibitors

	Cilostazol			Cilostamide			Rolipram		
Concentration (μM)	0.1	1	10	0.1	1	10	0.1	1	10
% inhibition of total cGMP hydrolysis									
Human	1.4	16.2	30.5	13.9	20.6	40.4	ND	ND	ND
Guinea pig	17.9	20.2	21.9	13.0	13.0	40.7	ND	ND	ND
% inhibition of total cAMP hydrolysis									
Human	9.3	13.6	27.8	19.2	33.2	42.6	24.8	36.8	49.3
Guinea pig	− 5.4	25.4	29.6	19.1	46.0	50.0	16.4	24.7	27.5

Means from three separate experiments.

In endothelium-denuded preparations, sodium nitroprusside did not alter dilatation induced by cilostazol or cilostamide. The removal of the endothelium almost completely blocked the relaxant response to rolipram, and the response was restored by pre-treating the arteries with sodium nitroprusside ($P=0.001$) (Fig. 2).

3.4. Cyclic nucleotide content

Under experimental conditions with an intact endothelium, cilostazol, rolipram and forskolin significantly increased cAMP, but there was no correlation between cAMP level and relaxant response (Table 1). Cilostamide did not alter the cAMP levels in any concentration. Cilostazol (10 μM) significantly increased cGMP about two fold ($P=0.02$, Mann–Whitney), while there was no increase in cGMP after rolipram, cilostamide or forskolin administration. Incubating the arteries with sodium nitroprusside (0.1 μM) for 5 min significantly increased cGMP about three-fold ($P=0.02$, Mann–Whitney) and cAMP two-fold ($P=0.004$). Incubation with ODQ for 15 min significantly decreased cGMP ($P=0.01$), but did not change the cAMP level.

3.5. Phosphodiesterase assay

The phosphodiesterase 3 inhibitors inhibited both cAMP and cGMP hydrolysis in a concentration-dependent manner in human and guinea pig cerebral arteries, cilostamide being generally more potent than cilostazol (Table 2). Maximum inhibition of total cAMP hydrolysis by phosphodiesterase 3 inhibition in both human and guinea pig tissue was ~ 40%. Rolipram (0.1–10 μM) had little effect on cAMP hydrolysis in guinea pig basilar arteries, but was more potent in human cerebral arteries, with a maximum effect of ~ 50% of total cAMP hydrolysis.

4. Discussion

The present findings show that both phosphodiesterase 3 and 4 inhibitors dilate pre-contracted guinea pig cerebral

arteries. The responses to the phosphodiesterase 3 inhibitors were independent of the endothelium and of the NO–cGMP pathway in cerebral arteries. This was in striking contrast to the phosphodiesterase 4 inhibitor, which was ineffective if the endothelium was removed or if the synthesis of cGMP was inhibited.

The apparent pD_2 values in this study for cilostazol and cilostamide were very high compared to reported IC_{50} values (20–175 times higher than the corresponding ED_{50}), but similar to previous findings in other arteries (Nakamura et al., 2001; Schoeffter et al., 1987; Tanaka et al., 1988). Because better correlation has been described for other phosphodiesterase 3 inhibitors (Kauffman et al., 1987), we found it unlikely that the dilator response is not mediated by phosphodiesterase 3 inhibition. A possible explanation is that both substances are difficult to dissolve in the aqueous phase.

The pD_2 of rolipram in the present experiments corresponds well with published IC_{50} values (approximately 1 μM). Regarding relaxant responses, some authors have reported similar potency as we have (Lindgren et al., 1990; Pauvert et al., 2002), while others have reported very low efficacy (ED_{50} 80–250 μM) (Komas et al., 1991; Lindgren et al., 1991; Lugnier and Komas, 1993; Schoeffter et al., 1987; Willette et al., 1997). It is not clear whether this is due to differences in phosphodiesterase 4 activity between the examined arterial tissues or if there are methodological variations.

In the phosphodiesterase assay, phosphodiesterase 3 inhibition maximally inhibited total cAMP hydrolysis ~ 40% in either species examined. A similar contribution to total cAMP hydrolysis has been described in bovine and rat aorta and bovine pulmonary artery (Lindgren et al., 1991; Pauvert et al., 2002; Rabe et al., 1994; Rascon et al., 1992), while the effect is somewhat smaller in canine basilar arteries (Willette et al., 1997) and larger in human pulmonary arteries (Rabe et al., 1994). Presently, the maximal effect of phosphodiesterase 4 inhibition by rolipram was ~ 50% in human cerebral arteries, but only ~ 25% in guinea pig basilar arteries. The contribution of phosphodiesterase 4 to total hydrolysis varies notably from species to species (20–50%) (Lindgren et al., 1991; Rabe et al., 1994; Rascon et al., 1992; Willette et al., 1997), which may underlie the differences in functional response to phosphodiesterase 4 inhibitors.

Cilostazol and cilostamide also attenuated cGMP hydrolysis in human and guinea pig cerebral arteries even at low doses. Further studies with more selective phosphodiesterase 3 inhibitors are necessary to resolve whether this is due to lack of selectivity of the phosphodiesterase 3 inhibitors tested or whether phosphodiesterase 3 contributes to cGMP hydrolysis under the present experimental conditions, i.e. high cGMP concentrations.

As for the role of cyclic nucleotides in the functional response, we could not demonstrate any clear relationship, though significant increases in cAMP or cGMP were seen after some drugs. Others have also failed to demonstrate such a correlation between relaxant response and cyclic nucleotide levels for cilostamide 10 μM (Delpy et al., 1996), cilostazol 10 μM (Tanaka et al., 1988) and rolipram 30 μM (Lindgren et al., 1990). Only when using very high doses of phosphodiesterase inhibitors, some have demonstrated 1.7–2 fold increases in cAMP after rolipram (30–150 μM) (Eckly and Lugnier, 1994; Schoeffter et al., 1987) or cilostamide (35 μM) (Schoeffter et al., 1987). This apparent lack of correlation between vasodilatation and cAMP concentrations has been demonstrated with other cAMP elevating agents such as forskolin, isoproterenol and prostaglandin E_1 (Vegesna and Diamond, 1986). Apart from possible difficulties with assay methodology and the matter of statistical power, it has been proposed that cAMP signalling is sequestered in functional sub-cellular compartments and that significant local increases in cAMP in the cell are immeasurable when the entire tissue is homogenized (Vegesna and Diamond, 1986). If so, we suggest that methods with better resolution in time and space are necessary to establish the role of cAMP the relaxant response. Increasing cAMP tone may also have provided stronger signals and better correlation between cAMP level and relaxant response (Tanaka et al., 1988). However, pretreating the arteries with forskolin 0.1 μM, though itself without dilatory effect, was not feasible since the precontracted state was only stable for 2–3 min.

The endothelium synthesizes a number of vasoactive substances such as NO, which stimulates soluble guanylate cyclase subsequently resulting in an increase in cGMP (Furchgott and Vanhoutte, 1989), which again causes vascular relaxation. In relation to delayed cerebral vasospasm after a subarachnoid haemorrhage, it has been proposed that the sustained contraction seen a few days after the subarachnoid haemorrhage is at least in part caused by decreased levels of cGMP, either due to decreased synthesis or to increased hydrolysis by phosphodiesterase 5 (Inoha et al., 2002; Sobey, 2001). In the present study, there was no significant involvement of the NO–cGMP pathway in the effects of either phosphodiesterase 3 inhibitors tested. This is a consistent finding in previous studies: other phosphodiesterase 3 inhibitors are not potentiated by NO donors, but the effect of compounds that inhibit the formation of NO or cGMP is not clear (Polson and Strada, 1996).

In contrast, ODQ inhibited the effect of rolipram in endothelium intact preparations, while sodium nitroprusside restored the response in endothelium-denuded arteries, which is in concert with others (Komas et al., 1991; Lugnier and Komas, 1993). Lack of phosphodiesterase 4 selectivity is not a likely explanation (Stoclet et al., 1995). The NO–cGMP pathway also potentiates dilator actions of other cAMP-mediated vasodilators such as β-adrenoceptor agonists (Delpy et al., 1996; Jang et al., 1993; Maurice et al., 1991) possibly via inhibition of phosphodiesterase 3, resulting in an increased magnitude and duration of the cAMP signal.

Direct comparison of responses in intact and in endothelium-denuded preparations has to be interpreted with some caution, since the endothelium produces a number of vasoactive substances with possible influence on the responses. Furthermore, the mechanical and chemical process of removing the endothelium may damage the arterial segments. However, identical concentration–response curves for pCPT-cGMP in preparations with and without endothelium were obtained in the present study. This suggests that the relaxant pathway down-stream from cGMP was intact and that there were no gross morphological or functional changes.

Phosphodiesterase 3 inhibitors are generally considered endothelium-independent (Kauffman et al., 1987; Komas et al., 1991; Lugnier and Komas, 1993). In the present study, cilostamide was unaffected by the presence or absence of an endothelium, while cilostazol was slightly *more* effective in endothelium-denuded preparations. The effect was quantitatively small and only barely reached significance ($P=0.04$). Others have described that removing the endothelium may attenuate the response to cilostazol in rat thoracic aorta. (Nakamura et al., 2001), but the effect was likewise small and only apparent in high concentrations where cilostazol also inhibits phosphodiesterase 5 (Sudo et al., 2000). The IC_{50} for cilostazol for the inhibition of phosphodiesterase 5 is 4.4 μM (Sudo et al., 2000) and maximal plasma concentrations during repeated dosing in clinical doses are of the same magnitude (Bramer et al., 1999). Endothelium-dependant relaxation may therefore contribute to a minor extent to the clinical effect of cilostazol.

The dilatory effect of rolipram and other phosphodiesterase 4 inhibitors has been found to be absent in some endothelium-denuded preparations (Komas et al., 1991; Lugnier and Komas, 1993; Rabe et al., 1994), while there was no change in the canine basilar artery (Willette et al., 1997). The response was restored by pre-treatment with a NO-donor in the present study, in agreement with previous reports (Komas et al., 1991). The effect of forskolin was attenuated after removal of the endothelium ($P=0.01$), showing that the influence of the endothelium is not restricted to phosphodiesterase 4 inhibitors. Similar results have been reported by others for isoprenaline, salbutamol, forskolin, the water-soluble forskolin analogue colforcin and the cell-permeable cAMP analogue dibutyryl cAMP (Delpy et al., 1996; Toyoshima et al., 1998). It has been proposed by many researchers that the mechanism involved is the following: (1) endothelial denudation reduces the cGMP levels, (2) low levels of cGMP makes phosphodiesterase 3 more active and, (3) increased cAMP hydrolysis makes rolipram and other cAMP mediated relaxants less effective (Delpy et al., 1996; Jang et al., 1993; Lincoln, 1983; Lindgren et al., 1991; Lugnier and Komas, 1993; Maurice et al., 1991). Because of the lack of effect of cGMP pathway on relaxations caused by cilostazol, we found no direct evidence supporting this hypothesis.

In summary, phosphodiesterase 3 and phosphodiesterase 4 are the major cAMP degrading enzymes in both human and guinea pig cerebral arteries based on studies using phosphodiesterase inhibitors. Both types of inhibitor relaxed guinea pig basilar arteries, but with limited efficacy. Phosphodiesterase 3 inhibitors also attenuated cGMP hydrolysis significantly in the phosphodiesterase assay, but tissue bath studies showed that this was not functionally relevant, because responses were not influenced by the endothelium or NO–cGMP pathway. In contrast, the effect of the phosphodiesterase 4 inhibitor was strongly inhibited if cGMP synthesis was inhibited or the endothelium removed. This is important because the NO–cGMP pathway is believed dysfunctional in stroke and in delayed cerebral vasospasm. Cilostazol is, apart from milrinone, the only phosphodiesterase 3 inhibitor currently available for human studies, and it potently dilates the middle cerebral artery in healthy volunteers (unpublished results by the authors, 2003) without affecting cerebral blood flow or blood pressure. We therefore propose that this compound is an interesting candidate for clinical trials in the treatment of delayed cerebral vasospasm.

Acknowledgements

The authors wish to thank Otsuka America Pharmaceutical and Dr. Junichi Kambayashi MD, PhD for kindly providing cilostazol, Ms. Suzanne Krizmanic Moeller for her dedicated technical assistance, and Dr. Ulla Engel MD, Department of Pathology, Hilleroed Hospital, Hilleroed, for the supply of human pial arteries. The study was supported by the Hoerslev Foundation and The Memorial Foundation for Alice Brenaa, the Lundbaek Foundation and the Danish Medical Research Council. SB is on a scholarship provided by the County of Copenhagen.

References

Arakawa, Y., Kikuta, K., Hojo, M., Goto, Y., Ishii, A., Yamagata, S., 2001. Milrinone for the treatment of cerebral vasospasm after subarachnoid hemorrhage: report of seven cases. Neurosurgery 48, 723–730.

Beavo, J.A., 1995. Cyclic nucleotide phosphodiesterases: functional implications of multiple isoforms. Physiol. Rev. 75, 725–748.

Bramer, S.L., Forbes, W.P., Mallikaarjun, S., 1999. Cilostazol pharmacokinetics after single and multiple oral doses in healthy males and patients with intermittent claudication resulting from peripheral arterial disease. Clin. Pharmacokinet. 37, 1–11.

Cosentino, F., Rubattu, S., Savoia, C., Venturelli, V., Pagannonne, E., Volpe, M., 2001. Endothelial dysfunction and stroke. J. Cardiovasc. Pharmacol. 38 (Suppl. 2), S75–S78.

Delpy, E., Coste, H., Gouville, A.C., 1996. Effects of cyclic GMP elevation on isoprenaline-induced increase in cyclic AMP and relaxation in rat aortic smooth muscle: role of phosphodiesterase 3. Br. J. Pharmacol. 119, 471–478.

Eckly, A.E., Lugnier, C., 1994. Role of phosphodiesterases III and IV in the modulation of vascular cyclic AMP content by the NO/cyclic GMP pathway. Br. J. Pharmacol. 113, 445–450.

Furchgott, R.F., Vanhoutte, P.M., 1989. Endothelium-derived relaxing and contracting factors. FASEB J. 3, 2007–2018.

Gotoh, F., Tohgi, H., Hirai, S., Terashi, A., Fukuuchi, Y., Otomo, E., Shinohara, Y., Itho, E., Matsuda, T., Sawada, T., Yamaguchi, T., Nishimaru, K., Ohashi, Y., 2000. Cilostazol stroke prevention study: A placebo-controlled double-blind trial for secondary prevention of cerebral infarction. J. Stroke Cerebrovasc. Dis. 9, 147–157.

Inoha, S., Inamura, T., Ikezaki, K., Nakamizo, A., Amano, T., Fukui, M., 2002. Type V phosphodiesterase expression in cerebral arteries with vasospasm after subarachnoid hemorrhage in a canine model. Neurol. Res. 24, 607–612.

Jang, E.K., Davidson, M.M., Crankshaw, D., Haslam, R.J., 1993. Synergistic inhibitory effects of atriopeptin II and isoproterenol on contraction of rat aortic smooth muscle: roles of cGMP and cAMP. Eur. J. Pharmacol. 250, 477–481.

Kauffman, R.F., Schenck, K.W., Utterback, B.G., Crowe, V.G., Cohen, M.L., 1987. In vitro vascular relaxation by new inotropic agents: relationship to phosphodiesterase inhibition and cyclic nucleotides. J. Pharmacol. Exp. Ther. 242, 864–872.

Komas, N., Lugnier, C., Stoclet, J.C., 1991. Endothelium-dependent and independent relaxation of the rat aorta by cyclic nucleotide phosphodiesterase inhibitors. Br. J. Pharmacol. 104, 495–503.

Kruuse, C., Rybalkin, S.D., Khurana, T.S., Jansen-Olesen, I., Olesen, J., Edvinsson, L., 2001. The role of cGMP hydrolysing phosphodiesterases 1 and 5 in cerebral artery dilatation. Eur. J. Pharmacol. 420, 55–65.

Lincoln, T.M., 1983. Effects of nitroprusside and 8-bromo-cyclic GMP on the contractile activity of the rat aorta. J. Pharmacol. Exp. Ther. 224, 100–107.

Lindgren, S.H., Andersson, T.L., Vinge, E., Andersson, K.E., 1990. Effects of isozyme-selective phosphodiesterase inhibitors on rat aorta and human platelets: smooth muscle tone, platelet aggregation and cAMP levels. Acta Physiol. Scand. 140, 209–219.

Lindgren, S., Rascon, A., Andersson, K.E., Manganiello, V., Degerman, E., 1991. Selective inhibition of cGMP-inhibited and cGMP-noninhibited cyclic nucleotide phosphodiesterases and relaxation of rat aorta. Biochem. Pharmacol. 42, 545–552.

Liu, Y., Shakur, Y., Yoshitake, M., Kambayashi, J.J., 2001. Cilostazol (pletal): a dual inhibitor of cyclic nucleotide phosphodiesterase type 3 and adenosine uptake. Cardiovasc. Drug Rev. 19, 369–386.

Lugnier, C., Komas, N., 1993. Modulation of vascular cyclic nucleotide phosphodiesterases by cyclic GMP: role in vasodilatation. Eur. Heart J. 14 (Suppl. I), 141–148.

Maurice, D.H., Crankshaw, D., Haslam, R.J., 1991. Synergistic actions of nitrovasodilators and isoprenaline on rat aortic smooth muscle. Eur. J. Pharmacol. 192, 235–242.

Mulvany, M.J., Halpern, W., 1976. Mechanical properties of vascular smooth muscle cells in situ. Nature 260, 617–619.

Nakamura, T., Houchi, H., Minami, A., Sakamoto, S., Tsuchiya, K., Niwa, Y., Minakuchi, K., Nakaya, Y., 2001. Endothelium-dependent relaxation by cilostazol, a phosphodiesterase III inhibitor, on rat thoracic aorta. Life Sci. 69, 1709–1715.

Pauvert, O., Salvail, D., Rousseau, E., Lugnier, C., Marthan, R., Savineau, J.P., 2002. Characterisation of cyclic nucleotide phosphodiesterase isoforms in the media layer of the main pulmonary artery. Biochem. Pharmacol. 63, 1763–1772.

Polson, J.B., Strada, S.J., 1996. Cyclic nucleotide phosphodiesterases and vascular smooth muscle. Annu. Rev. Pharmacol. Toxicol. 36, 403–427.

Rabe, K.F., Tenor, H., Dent, G., Schudt, C., Nakashima, M., Magnussen, H., 1994. Identification of PDE isozymes in human pulmonary artery and effect of selective PDE inhibitors. Am. J. Physiol. 266, L536–L543.

Rascon, A., Lindgren, S., Stavenow, L., Belfrage, P., Andersson, K.E., Manganiello, V.C., Degerman, E., 1992. Purification and properties of the cGMP-inhibited cAMP phosphodiesterase from bovine aortic smooth muscle. Biochim. Biophys. Acta 1134, 149–156.

Schini, V., Schoeffter, P., Miller, R.C., 1989. Effect of endothelium on basal and on stimulated accumulation and efflux of cyclic GMP in rat isolated aorta. Br. J. Pharmacol. 97, 853–865.

Schoeffter, P., Lugnier, C., Demesy-Waeldele, F., Stoclet, J.C., 1987. Role of cyclic AMP- and cyclic GMP-phosphodiesterases in the control of cyclic nucleotide levels and smooth muscle tone in rat isolated aorta, a study with selective inhibitors. Biochem. Pharmacol. 36, 3965–3972.

Silver, P.J., Lepore, R.E., O'Connor, B., Lemp, B.M., Hamel, L.T., Bentley, R.G., Harris, A.L., 1988. Inhibition of the low Km cyclic AMP phosphodiesterase and activation of the cyclic AMP system in vascular smooth muscle by milrinone. J. Pharmacol. Exp. Ther. 247, 34–42.

Sobey, C.G., 2001. Cerebrovascular dysfunction after subarachnoid haemorrhage: novel mechanisms and directions for therapy. Clin. Exp. Pharmacol. Physiol. 28, 926–929.

Sorkin, E.M., Markham, A., 1999. Cilostazol. Drugs Aging 14, 63–71.

Stoclet, J.-C., Keravis, T., Komas, N., Lugnier, C., 1995. Cyclic nucleotide phosphodiesterases as therapeutic targets in cardiovascular diseases. Expert Opin. Investig. Drugs 4, 1081–1100.

Sudo, T., Tachibana, K., Toga, K., Tochizawa, S., Inoue, Y., Kimura, Y., Hidaka, H., 2000. Potent effects of novel anti-platelet aggregatory cilostamide analogues on recombinant cyclic nucleotide phosphodiesterase isozyme activity. Biochem. Pharmacol. 59, 347–356.

Suzuki, Y., Huang, M., Lederis, K., Rorstad, O.P., 1988. The role of adenylate cyclase in relaxation of brain arteries: studies with forskolin. Brain Res. 457, 241–245.

Tanaka, T., Ishikawa, T., Hagiwara, M., Onoda, K., Itoh, H., Hidaka, H., 1988. Effects of cilostazol, a selective cAMP phosphodiesterase inhibitor on the contraction of vascular smooth muscle. Pharmacology 36, 313–320.

Toyoshima, H., Nasa, Y., Hashizume, Y., Koseki, Y., Isayama, Y., Kohsaka, Y., Yamada, T., Takeo, S., 1998. Modulation of cAMP-mediated vasorelaxation by endothelial nitric oxide and basal cGMP in vascular smooth muscle. J. Cardiovasc. Pharmacol. 32, 543–551.

Vegesna, R.V., Diamond, J., 1986. Effects of prostaglandin E1, isoproterenol and forskolin on cyclic AMP levels and tension in rabbit aortic rings. Life Sci. 39, 303–311.

Willette, R.N., Shiloh, A.O., Sauermelch, C.F., Sulpizio, A., Michell, M.P., Cieslinski, L.B., Torphy, T.J., Ohlstein, E.H., 1997. Identification, characterization, and functional role of phosphodiesterase type IV in cerebral vessels: effects of selective phosphodiesterase inhibitors. J. Cereb. Blood Flow Metab. 17, 210–219.