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Phosphodiesterase 5 and effects of sildenafil on cerebral arteries of man and guinea pig

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Abstract

Sildenafil (Viagra®), a selective inhibitor of phosphodiesterase 5 (PDE5), induces headache and migraine. Although previously supposed to be a "vascular" headache, no significant cerebral artery dilatation was found in vivo. Thus, we hypothesised that PDE5 may not be present or that sildenafil is less effective on the cGMP hydrolysis in cerebral arteries, and that sildenafil may not be an effective dilator of cerebral arteries under baseline conditions.

We evaluated the presence of PDE5 mRNA and protein in human arteries. Furthermore, the effects of two selective PDE5 inhibitors, sildenafil and UK-114,542, and a PDE1 inhibitor UK-90,234 on cGMP hydrolysis were investigated in human and guinea pig cerebral arteries. The vasoactive responses of the compounds were evaluated in guinea pig basilar arteries in vitro, with concomitant measurements of cAMP and cGMP.

PDE5 was found in human middle cerebral arteries. Sildenafil and UK-114,542 inhibited cGMP hydrolysis concentration-dependently in both species. In guinea pig arteries, sildenafil induced an endothelium-dependent vasodilatation only at concentrations above 10 nM, which was augmented by sodium nitroprusside and attenuated by reduction of cGMP, but was cGMP independent at high concentrations. UK-114,542 was more and UK-90,234 was less potent than sildenafil.

In conclusion, PDE5 is present in human and guinea pig cerebral arteries, and is inhibited by sildenafil at micromolar levels. Sildenafil in vitro is a poor dilator of guinea pig cerebral arteries unless a nitric oxide donor is co-administered, corresponding to the previous findings in vivo.

Keywords: Sildenafil; Cerebral artery; Phosphodiesterase 5; Human; Migraine

1. Introduction

The signalling pathway of nitric oxide (NO)—cyclic guanosine monophosphate (cGMP) is of major importance in the regulation of cerebral blood flow (Faraci and Brian, 1994). Dysfunction of the pathway may play a role in various neurological and cerebrovascular diseases such as vascular

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headaches, migraine, stroke and vasospasm occurring after subarachnoid haemorrhage (Pelligrino and Wang, 1998). The cyclic nucleotide phosphodiesterases (PDE) are key modulators of intracellular cyclic nucleotide responses by controlling cGMP and cyclic adenosine monophosphate (cAMP) degradation. So far, 11 different PDE families have been identified with variations in tissue distribution and selectivity towards cAMP or cGMP (Beavo, 1995; Soderling and Beavo, 2000). The cGMP degrading PDEs, such as PDE5, may be central for regulation and cessation of the cGMP response in cerebral arteries (Kruuse et al., 2001; Sobey and Quan, 1999).

Sildenafil (Viagra®) is a potent and selective inhibitor of PDE5. It works by decreasing breakdown of endogenously produced cGMP in smooth muscle cells and nerves (Ballard et al., 1998) and is currently used for treatment of male erectile dysfunction. The most frequent side effect of sildenafil is headache, which was believed to be of vascular origin due to presumed dilatation of cerebral arteries (Morales et al., 1998) as seen with NO donors (Olesen et al., 1994). However, in recent human studies, sildenafil had no significant effects on large cerebral arteries or on cerebral blood flow in humans, although it induced headache and migraine (Arnavaz et al., 2003; Kruuse et al., 2003, 2002). Apart from suggesting a non-vascular origin of the sildenafil induced headache, these findings could also reflect that PDE5 was not present in the cerebral arteries or that the PDE5 present is not affected by sildenafil.

However, to test this hypothesis we needed to establish the presence of PDE5 in human middle cerebral arteries; examine if the PDE5 enzyme is active and inhibited by sildenafil; whether sildenafil is able to dilate cerebral arteries in an in vitro model; and if the effects are unique to sildenafil or applies to another PDE5 inhibitors. We thus evaluated the effect of the PDE5 inhibitors sildenafil and UK-114,542 on cyclic nucleotide degradation in human middle cerebral arteries and guinea pig basilar arteries for comparison. Since sildenafil may affect the Ca^{2+}/CaM stimulated PDE1 at high concentrations, the PDE1 inhibitor UK-90,234 was also included in the investigations. The vasomotor effects and cyclic nucleotide responses of all three compounds were examined in vitro on the guinea pig basilar artery.

2. Methods

2.1. Tissue

Human: Pieces of human middle cerebral artery ($N=3$, one woman and two men: age 79, 74 and 86 years, respectively) and small branches of the middle cerebral artery ($N=3$, two women and one man: age 71,73 and 83 years, respectively) were removed post-mortem and placed in ice-cold buffer (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 mM). In the human middle cerebral artery the smooth muscle layer was separated from adventitia and both used for RT-PCR and Western blot. The smaller middle cerebral artery branches, all retrieved within 9 h post-mortem, were also used for enzyme analysis. Samples were stored at −80 °C until analysis. The tissues originated from deceased with no history of cerebral or cerebrovascular disorders, cause of death varied and was related to bronchitis, cancer (thyroid, intestine and lung) and pulmonary embolism.

Before analysis the samples were mechanically disintegrated and placed in homogenisation 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) for Western blot or enzyme assays. RNA was extracted immediately after mechanical disintegration using the Trizol reagent (Life Technologies, Roskilde, Denmark). The study was approved by the Ethical Committee of Copenhagen County, Denmark.

Guinea pig basilar arteries: Male guinea pigs, 350–450 g, were sacrificed in the morning (8–9 AM.) by decapitation after CO_2 narcosis. The brain was removed and the basilar artery dissected free. After removing luminal blood the arteries were either frozen immediately on dry ice for Western blot and enzyme analysis or placed in a cold buffer solution (NaCl 119, $NaHCO_3$ 15, KCl 4.6, $CaCl_2$ 1.5, $NaHPO_4$ 1.2, $MgCl_2$ 1.2, glucose 5.5, mM, pH 7.4) aerated with 95% O_2 and 5% CO_2 until same day experiments of vasomotor response. For enzyme analysis a pool of eight basilar arteries was used in each experiment. The retrieval of animal tissue followed the ethical guidelines for animal studies in Denmark.

2.2. RT-PCR

Reverse transcription of RNA to cDNA was performed using Superscript II™ kit (Life Technologies, Roskilde, Denmark).

Forward primer for PDE5A was 5′-CCTTGCAGAA-CAGCAGGAGAAG-3′ and backward primer was 5′-GGCATATTGCAGAACACACCATC-3′. The primer design was based on comparison across species of PDE5A. Accession number AJ004865, primer region 2658–2778, size 121 aa. RNA extracted from human platelets was used as control. The PCR products were sequenced using ABI PrismTH (Perkin Elmer, Alleroed, Denmark) for confirmation of products. Furthermore, the presence of PDE5A isoforms, PDE5A1, PDE5A2 and PDE5A3, were investigated using primer pairs given by Lin et al. (2000).

2.3. Western blot

Supernatants from the homogenised tissue for PDE assay analysis, and human middle cerebral artery smooth muscle cells and adventitia homogenised by similar methods were boiled in 2× sample buffer for Western blot. Electrophoresis was performed on an 8% Sodium dodecyl sulfate (SDS)-

polyacrylamide gel and transferred to a nitrocellulose membrane. Polyclonal rabbit antibodies used against PDE1A, PDE1B, PDE1C and PDE5A have been previously characterised (Rybalkin et al., 1997). Protein standards, control tissue (human platelets and guinea pig basilar artery), were processed in parallel. The presence of PDE1 and PDE5 protein in guinea pig basilar arteries has previously been shown (Kruuse et al., 2001).

2.4. Phosphodiesterase activity

Three separate experiments were performed on both guinea pig and human cerebral arteries.

For each experiment the frozen tissue was mechanically dissociated by pestle and transferred to homogenisation buffer. The homogenate was sonicated twice and centrifuged in a two-step procedure (see (Kruuse et al., 2001) for details). The supernatant was kept at 4 °C during all procedures. Less than 1% of hydrolysis was present in the pellet and was not analysed further.

The phosphodiesterase assay was performed according to previously established procedures (Kruuse et al., 2001). 1 μM of either cAMP/[^{3}H]cAMP or cGMP/[^{3}H]cGMP (~50.000 cpm) was used as substrate. 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 different concentrations of the PDE inhibitors with a final assay volume of 125 μl. 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*, loaded onto equilibrated DEAE-sephadex A25 columns and eluted by addition of low salt buffer (20 mM Tris–Cl, pH 6.8) directly into scintillation vials and subsequently quantitated by counting [^{3}H]nucleoside.

2.5. Studies of arterial vasomotor responses

Human cerebral arteries were not used in the functional studies, because of the small amount of tissue available. Thus, studies were performed on both intact and endothelium denuded guinea pig basilar arteries. Endothelium was removed by perfusing the artery with 0.1% Triton-X for 10 s followed by buffer.

The arteries were cut transversally in approximately 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) and aerated with 95% O_2 and 5% CO_2. After 1 h of equilibration the arteries were distended until a stable resting tension of 2 mN/mm was established.

The viability of the arteries was ensured by stimulation with potassium rich buffer (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, pH 7.4) twice. This resulted in a contraction of 2.96±0.24 mN/mm (N=17) in intact arteries and 3.68±0.20 mN/mm (N=17) in endothelial denuded arteries. The presence of a functional endothelium was ascertained after 15–20 min pre-contraction with prostaglandin$F_{2\alpha}$ ($PGF_{2\alpha}$)(3 μM) by application of acetylcholine (10 μM). Only if the pre-contraction was stable and if acetylcholine induced a dilatory response above 80% of pre-contraction in intact arteries and less than 10% in endothelial denuded the arteries were accepted for further analysis. $PGF_{2\alpha}$ (3 μM) was used as pre-contraction in all experiments and after reaching stable tension, cumulative concentrations of compounds were added in half log steps in both intact and endothelial-denuded arteries. When pre-treatment was applied, pre-contraction was induced after addition of pre-treatment.

In intact arteries the potentiating effect of UK-114,542 (0.1 nM for 10 min), UK-90,234 (0.1 μM for 10 min) and sildenafil (1 nM for 10 min) on sodium nitroprusside induced dilatation was investigated. Furthermore, the effects of pre-treatment with the selective soluble guanylate cyclase inhibitor, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) (10 μM for 15 min), were evaluated.

In endothelium denuded arteries the arteries were pre-treated either with sodium nitroprusside (0.1 μM) or with sodium nitroprusside (0.1 μM) plus ODQ (10 μM) before cumulative addition of PDE inhibitors.

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

2.6. Measurements of cyclic nucleotides in vitro

Cyclic nucleotide responses to single concentrations of UK-90,234 (10 μM), UK-114,542 (0.1 μM) and sildenafil (1 μM) were investigated separately on the mounted rings of intact basilar arteries with approximate length of 2 mm. Procedures concerning test of viability, stability and endothelial response were performed as previously described. After a stable pre-contraction a single concentration of PDE inhibitor was added and the dilator 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) (Kruuse et al., 2001). The artery and the ice-cold solution were immediately removed from the organ bath 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 (NEN Lifesciences, USA). The pellet was used for protein analysis (Bradford, Bio-Rad, USA).

2.7. Compounds

Antibodies for use in Western blot were obtained from the laboratory of J.A Beavo, University of Washington, Seattle, USA. Sodium nitroprusside, $PGF_{2\alpha}$ and acetylcholine were purchased from Sigma-Aldrich (Vallensbaek Strand, Denmark). ODQ were purchased from Calbiochem (Sigma-

Aldrich, Vallensbaek Strand, Denmark), while UK-90,234 (5-[[4-(diethylamino)phenyl]methyl]-1,4-dihydro-1-methyl-3-propyl-7H-Pyrazolo[4,3-d]pyrimidin-7-one, for structure see CAS registry number 223430-04-4) and UK-114,542 (5-[2-ethoxy-5(morpholinylacetyl)phenyl]-1,6-dihydro-1-methyl-3-propyl-7H-pyrazolo[4,3-d]pyrimidin-7-one methanesulphanate monohydrate) were generous gifts from Pfizer, Sandwich, Kent, UK. Sildenafil was extracted from commercially available tablets and the purity was ascertained using high pressure liquid chromatography (HPLC). All concentrations are expressed as the final molar concentrations in the tissue bath or phosphodiesterase assay.

Stock solutions were made of all compounds using either 100% dimethyl sulfoxide for dissolving ODQ, UK-90,234 and UK-114,542, and ethylamide for sildenafil. 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 and ethylamide did not exceed 0.1%, where no effect on the dilatory response was detected. Dilutions for determination of the concentration-response curves were made immediately before experiments using distilled water.

2.8. Calculation and statistics

All data are expressed as mean values±S.E.M unless stated otherwise. *N* refers to the number of guinea pigs or human arteries used. Experiments included 1–4 vessel segments from each guinea pig in the tissue baths.

Because of the small sample size of the human material ($N=3$, with 3–4 repeated measurements) the differences in the effect between species of PDE inhibitors on hydrolysis were analysed using a uni-variate analysis of repeated measurements (SPSS Inc., Chicago, IL, U.S.A.) instead of using summary measures as below. Cautions should however be taken when interpreting the data due to the small numbers included.

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 3.0 (GraphPad Software, Inc., San Diego, USA).

Difference in summary measures (pEC_{50} and E_{max}) between treatments and compounds were tested for statistical significance using unpaired Student's *t*-test. $P<0.5$ was considered statistically significant.

3. Results

3.1. Presence of PDE5 in human cerebral arteries

PDE5 mRNA and protein were present in smooth muscle cells and in the remaining adventitia of human middle cerebral arteries as seen by RT-PCR (Fig. 1A) and Western blot (Fig. 1B). The PCR products showed homology to the sequence of the enzyme tested when isolated and sequenced (see Methods). In the smaller branches, RT-PCR using isoform-specific primers revealed the presence of all 3 splice variants of PDE5A (not shown). In the Western blots, we detected three immunoreactive bands of PDE5 in both guinea pig and human cerebral arteries, the top bands being denser (Fig. 1B). In the guinea pig the top band was previously found to correspond to a recombinant PDE5 of approximately 98 kDa (Kruuse et al., 2001; Rybalkin et al., 1997).

In Western blots immunoreactive bands towards PDE1A and PDE1B but not PDE1C were present in the human middle cerebral arteries (not shown).

3.2. Cyclic nucleotide hydrolysis in guinea pig and human cerebral arteries

The effects of the three PDE inhibitors on cGMP and cAMP hydrolysis in crude extracts of guinea pig and human cerebral arteries are listed in Table 1, and were very similar between species.

All three PDE inhibitors significantly attenuated cGMP hydrolysis ($P<0.05$). The potency of the UK-114,542 was slightly lower in the human arteries compared to the IC_{50} of 1.7 nM for PDE5 reported by Pfizer. For sildenafil, approximately 50% inhibition of cGMP hydrolysis was obtained between 0.01 and 0.01 μM in both human and guinea pig arteries. This was about 10–100 fold lower compared to the reported IC_{50} for sildenafil (IC_{50} 3.5 nM) for the isolated PDE5 enzyme (Wallis, 1999) (Table 1). Both UK-114,542 and sildenafil inhibited the Ca^{2+}/CaM stimu-

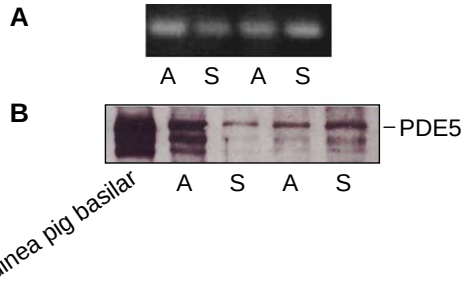

Fig. 1. PCR analysis and Western blot show the presence of PDE5A in human cerebral arteries. Results from reverse transcriptase-polymerase chain reaction (RT-PCR) using human middle cerebral arteries obtained from two different post-mortems and primers for PDE5A are shown above (A). Letter S is mechanically isolated smooth muscle cells from the two subjects, and A is the remaining adventitia. The Western blot analysis on the same tissue shows immunoreactivity towards PDE5A in human smooth muscle cells and adventitia as described above and in guinea pig basilar (far left) arteries used as control tissue (B). At shorter exposition time the guinea pig arteries also showed 3 bands, corresponding to those in human tissue. Three immunoreactive bands are seen in all preparations of human cerebral arteries, with the top bands corresponding to a previously applied recombinant PDE5 of approximately 98 kDa. No exact determination of size was applied due to the low amount of human material. The difference in band intensity reflects variations in protein concentration.

Table 1
Selective inhibition of cGMP and cAMP hydrolysis in human middle cerebral arteries and guinea pig basilar arteries with and without excess Ca^{2+}/CaM in the assay

Concentrations	UK-90,234			UK-114,542				Sildenafil			
	0.1 μM	1 μM	10 μM	0.1 nM	1 nM	10 nM	100 nM	0.01 μM	0.1 μM	1 μM	10 μM
% inhibition of total cGMP hydrolysis											
EGTA											
Human	10.5±3.2	27.8±3.2	58.3±2.7	19.6±1.6	33.1±2.5	44.3±2.1	58.7±6.2[a]	31.4±2.8	59.5±2.3	67.1±3.2	77.1±3.8
Guinea pig	12.1±10.8	36.7±6.9	45.1±8.4	24.7±1.9	47.4±1.6	54.9±4.0	67.0±2.4	40.7±7.1	61.4±2.0	72.1±1.4	75.3±3.5
Ca^{2+}/CaM stimulated											
Human	28.9±8.7	42.9±2.7	74.4±1.8	12.4±1.1	26.2±4.6	32.8±2.9	46.1±4.0	24.7±4.7	39.8±2.9	58.8±3.0	79.3±3.5
Guinea pig	18.1±12.2	48.7±3.4	60.0±3.6	21.0±3.2	30.8±1.1	35.3±2.5	49.8±1.6	18.1±3.1	35.8±7.6	57.8±3.7	76.8±1.9
% inhibition of total cAMP hydrolysis											
EGTA											
Human	1.5±1.1	8.2±1.7	14.2±1.0	3.4±1.4	6.4±2.7	−1.1±3.8	3.7±1.8	5.3±1.7	2.4±3.6	10.5±1.0	26.9±4.2[a]
Guinea pig	−1.6±2.7	0.5±1.5	8.8±4.3	4.1±3.8	0.8±1.3	0.3±4.9	2.3±1.5	1.1±4.5	0.5±2.6	4.1±5.0	15.4±2.0
Ca^{2+}/CaM stimulated											
Human	11.6±5.0	27.5±5.1	40.8±6.8	14.1±6.8	12.3±5.9	13.9±7.3	18.0±3.5	16.0±6.6	13.3±6.9	22.5±3.8	46.2±4.1
Guinea pig	7.6±6.5	16.6±1.8	24.3±3.4	−4.7±1.5	1.9±3.7	0.0±1.5	0.5±5.4	−5.4±3.1	0.0±0.3	12.8±2.0	26.7±1.4

Data are shown as mean±S.E.M and represent percentage inhibition of hydrolysis compared to control hydrolysis. All data are from 3 separate enzyme assay experiments. The human data originated from 3 different patients, guinea pig data represent a pool of basilar arteries from eight different animals each. The assay was performed on crude extract and not purified PDE5 enzyme. There was no significant difference in response between species, except for the general response to UK-114,542 (cGMP hydrolysis with EGTA) ($P=0.04$) and sildenafil (cAMP hydrolysis with EGTA) ($P=0.03$) marked with[a].

lated cGMP hydrolysis at higher concentrations indicating an effect on the PDE1. Approximately 50% inhibition of Ca^{2+}/CaM stimulated cGMP hydrolysis was obtained for 0.1 μM UK-114,542 and between 0.1 and 1 μM for sildenafil (Table 1), which is almost similar to the PDE1 IC_{50} values for sildenafil (IC_{50} 280 nM) (Wallis, 1999) and data given by Pfizer for UK-114,542 (PDE1 IC_{50} 93 nM). The inhibitor UK-90,234 was not PDE1 specific in either human or guinea pig cerebral arteries since it inhibited both EGTA and Ca^{2+}/CaM stimulated cGMP hydrolysis. The IC_{50} of UK-90,234 for PDE5 reported by Pfizer was 2 μM, however, in previous experiments (SD Rybalkin and JA Beavo, personal communication) the IC_{50} was found to be 0.38 μM for recombinant PDE5 and 0.2 μM for bovine lung PDE5. In the same experiments the IC_{50} for PDE1A2 was 0.02 μM, for PDE1B1 0.25 μM and for PDE1C4 0.04 μM.

The rate of cAMP hydrolysis was generally minor compared to cGMP hydrolysis, which is in accordance with a previous report (Kruuse et al., 2001). Sildenafil and UK-90,234 showed an effect on cAMP hydrolysis that did not reach the level of effects seen on the cGMP response. An almost 50% inhibition of Ca^{2+}/CaM stimulated cAMP hydrolysis was obtained using sildenafil (10 μM) in human arteries, indicating a PDE1 effect at higher concentrations (Table 1) UK-114,542 did not affect cAMP hydrolysis dose-dependently.

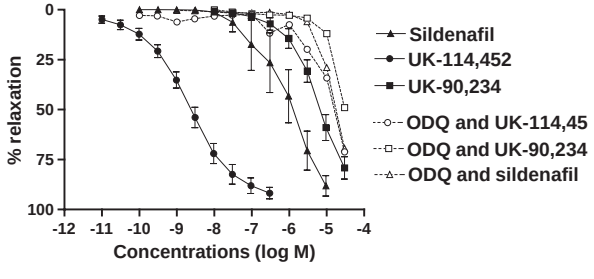

Fig. 2. Concentration-response curve for UK-90,234 (square), UK-114,542 (circle) and sildenafil (triangle) without (filled symbols) and with (open symbols) ODQ (10 μM) pre-treatment in intact guinea pig basilar arteries. Relaxation is given as percent relaxation of pre-contraction and each point shows mean±S.E.M. In high concentrations all compounds elicited a small cGMP-independent dilatory response.

3.3. Vasodilator effects of sildenafil, UK-114,542 and UK-90,234 in guinea pig cerebral arteries

All of the PDE inhibitors relaxed precontracted intact basilar arteries of guinea pig almost completely (Fig. 2). The maximal dilatory responses (E_{max}) and pEC_{50} are given in Table 2. The PDE5 inhibitor UK-114,542 was about 400 times more potent than sildenafil and the PDE1 inhibitor

Table 2
Vasodilator response to sildenafil, UK-114,542 and UK-90,234 in precontracted intact guinea pig basilar arteries

Compound	No pre-treatment			Pre-treatment ODQ (10 μM)		
	pEC_{50}	E_{max} (%)	*N*	pEC_{50}	E_{max} (%)	*N*
UK-90,234	5.2±0.1	78±5	8	a	49±13	8
UK-114,542	8.6±0.1	86±4	11	a	46±13	10
Sildenafil	6.0±0.3	95±2	6	a	69±6	7

Values represent mean±S.E.M. The maximum dilatory effect, E_{max}, of an agonist was calculated as percent of relaxation to pre-contraction.
pEC_{50} is the negative logarithm of the molar concentration that produced half-maximal relaxation. *N*=number of animals (1–4 rings from each animal).
a=pEC_{50} not calculated because a stable plateau was not reached within the highest applicable concentrations of the compound.

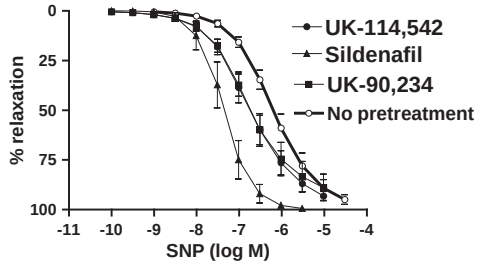

Fig. 3. Concentration-response curve for sodium nitroprusside with no pre-treatment (open circles), with 0.1 μM UK-90,234 (filled squares), 0.1 nM UK-114,542 (filled circles) and 1 nM sildenafil (filled triangles) pre-treatment in intact guinea pig basilar arteries. The dilatory effect of sodium nitroprusside increased 15 fold with sildenafil pre-treatment.

UK-90,234 about six times less potent in intact basilar arteries ($P<0.01$).

Pre-treatment with ODQ, an inhibitor of endogenous cGMP synthesis, reduced the dilatory response of all compounds and they were significantly different from the results without ODQ treatment ($P<0.01$) (Table 2). Since a maximum response of the PDE inhibitors in the presence of ODQ was not obtained the pEC_{50} were not calculated.

The vasodilator response to the NO donor sodium nitroprusside (pEC_{50} 6.2±0.1) was significantly shifted to the left by pre-treatment with both UK-114,542 (pEC_{50} 6.7±0.2, $P<0.05$), sildenafil (pEC_{50} 7.4±0.2, $P<0.01$) and UK-90,234 (pEC_{50} 6.8±0.1)(Fig. 3). Sildenafil thus increased the vessel sensitivity to the NO donor 15-fold.

Removal of the endothelium reduced the vasodilator response to the PDE inhibitors, but did not inhibit vasodilatation completely in high concentrations which parallels with the ODQ pre-treatment in intact arteries (Table 3). The vasodilator responses to all inhibitors were restored in endothelial denuded arteries by sodium nitroprusside pre-treatment (Fig. 4A–C). The sildenafil response was even potentiated approximately 80 fold (pEC_{50} 7.9±0.3), compared to the response in intact endothelium ($P=0.001$). Addition of ODQ returned the response to that seen without pre-treatment in endothelium denuded arteries (Fig. 4A–C) and with ODQ pre-treatment in intact arteries.

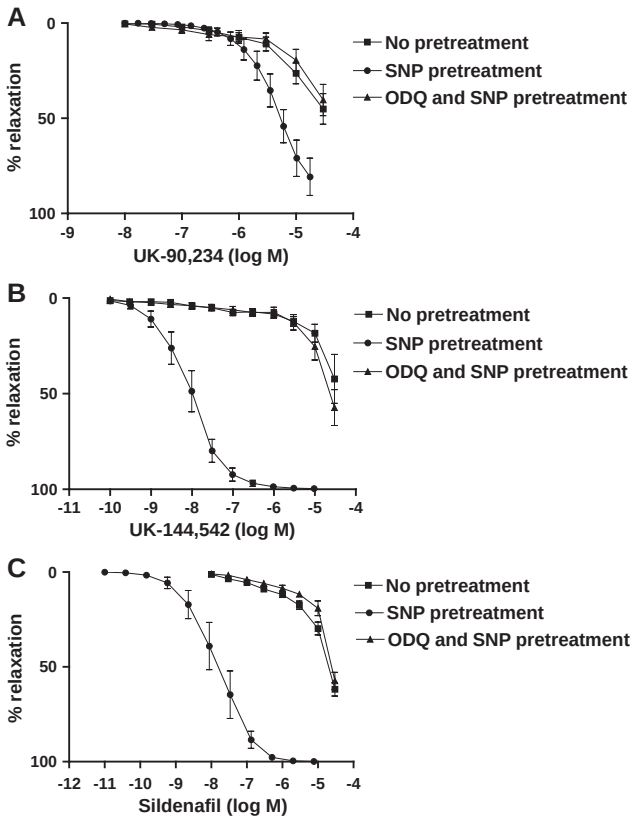

Fig. 4. Concentration-response curve for UK-90,234 (A), UK-114,542 (B) and sildenafil (C) in endothelium denuded guinea pig basilar arteries. With no pre-treatment (filled squares) with sodium nitroprusside (0.1 μM) (filled circles) and sodium nitroprusside (0.1 μM)+ODQ (10 μM) (filled triangles) pre-treatment. Relaxation is given as percent relaxation of pre-contraction and each point shows mean±S.E.M. The dilatory response was greatly attenuated when effects of either endogenous or applied NO was inhibited or blocked. In high concentrations all compounds however elicited a small cGMP-independent dilatory response.

3.4. Effects of selective PDE inhibitors on cyclic nucleotide levels in intact arteries

The concentration of cGMP in basilar arteries increased significantly after administration of all the PDE inhibitors, with UK-114,542 (0.1 μM) being the most potent ($P<0.01$)(Fig. 5A). The cGMP increase was inhibited

Table 3
Vasodilator effect of sildenafil, UK-114,542 and UK-90,234 in endothelium denuded precontracted guinea pig basilar arteries

Compound	No pre-treatment			Pre-treatment sodium nitroprusside (0.1 μM)			Pre-treatment ODQ (10 μM)+sodium nitroprusside (0.1 μM)		
	pEC_{50}	E_{max} (%)	N	pEC_{50}	E_{max} (%)	N	pEC_{50}	E_{max} (%)	N
UK-90,234	a	45±10	7	5.2±0.2	83±11	7	a	41±9	6
UK-114,542	a	32±10	9	8.1±0.2	93±4	7	a	45±9	9
Sildenafil	a	60±4	8	7.9±0.3	99±1	6	a	58±5	7

Endothelium removed by flushing the arteries with 0.1% Triton-X followed by buffer. Values represent mean±S.E.M. The maximum dilatory effect, E_{max}, of an agonist was calculated as percent relaxation to pre-contraction.
pEC_{50} is the negative logarithm of the molar concentration that produced half-maximal relaxation. N=number of animals (1–4 rings from each animal).
a=pEC_{50} not calculated because a stable plateau was not reached within the highest applicable concentrations of the compound.

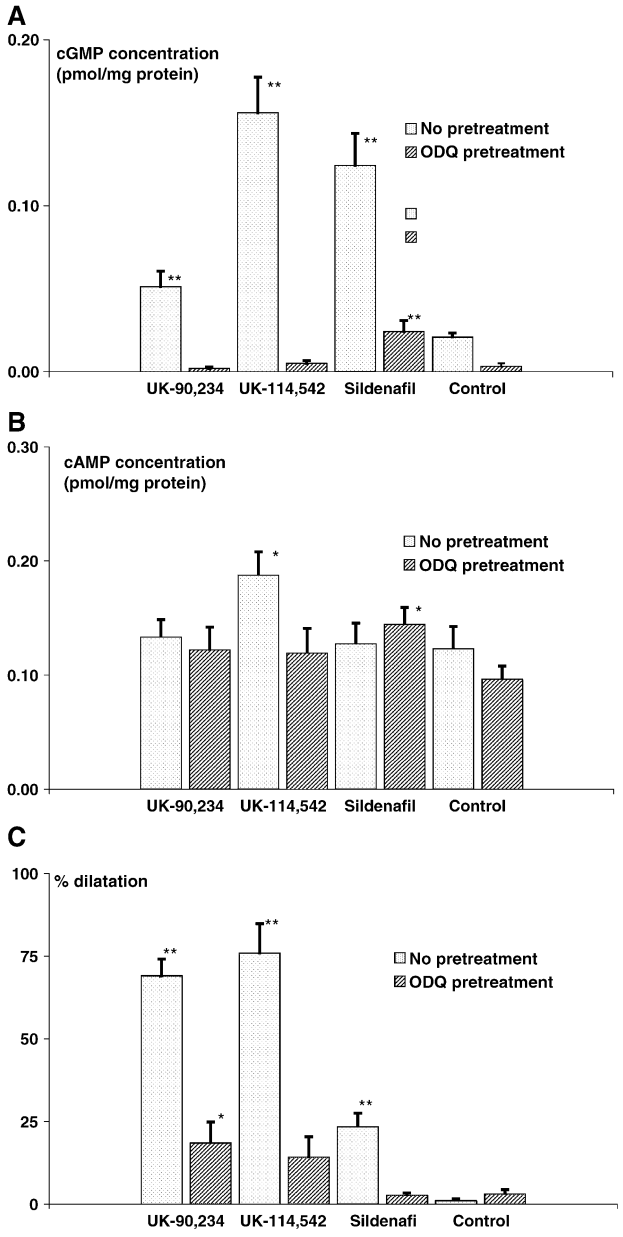

Fig. 5. The levels of cGMP (A), cAMP (B) and the vasodilator response (C) are shown for single concentrations of UK-90,234 (10 μM) UK-114,542 (0.1 μM) and sildenafil (1 μM) in intact guinea pig basilar arteries in the absence (dotted) and presence (lined) of ODQ (10 μM). $N=5–10$ animals 1–2 artery segments each. *$P<0.05$ and **$P<0.01$ compared to control.

significantly by pre-treatment with ODQ. When ODQ was applied, sildenafil (1 μM) induced a small but significant increase in cGMP compared to control values ($P<0.01$) but cGMP did not increase above basal levels. The levels of cAMP (Fig. 5B) were slightly increased by UK-114,542 alone ($P<0.05$) and by sildenafil with ODQ pre-treatment ($P<0.05$).

The dilatory response after single dose applications of PDE inhibitors (Fig. 5C) was similar to that seen above in the cumulative concentration response curves for UK-114,542 and UK-90,542. However, the dilatation to a single dose of sildenafil seemed to be reduced (23±4%) compared to addition of cumulative doses (43±13%). This may be due to the short interval of 1 min from the application of drug to the cessation of the response.

4. Discussion

The PDE isozymes are known to display both tissue and species specific distribution and function (Beavo, 1995). These characteristics make it important to establish their distribution in human tissue as well as the effects of specific PDE inhibitors both in vivo and in vitro.

This study is the first to show the presence of PDE5 mRNA and protein in human middle cerebral arteries. We also for the first time show that the selective PDE5 inhibitor sildenafil inhibits the cGMP hydrolysis in tissue homogenates of both human and guinea pig cerebral arteries, but at higher concentrations than previously reported, and with an almost equal response between species. Furthermore, sildenafil proved to be a poor dilator of guinea pig cerebral arteries, unless stimulation of cGMP production was induced by a nitric oxide donor. Another selective PDE5 inhibitor, UK-114,542 was a more effective inhibitor of cGMP hydrolysis and a more potent vasodilator than sildenafil.

4.1. The possible tissue and species differences

In principle, PDE5 inhibitors need production of cGMP in order to cause accumulation of cGMP and relax smooth muscle cells (Chuang et al., 1998). However, there are reports of a vasorelaxant effect of sildenafil in vitro even without application of a stimulus, showing an EC_{50} of 47 nM (Medina et al., 2000c) in the penile artery and of approximately 0.1 μM in the radial artery (Medina et al., 2000a). In human coronary arteries 30 μM sildenafil was needed to elicit a 50% dilatation (Medina et al., 2000a). The variation in response may reflect a variation in intrinsic cGMP production. An intrinsic cGMP production has been proposed in the cerebral arteries (Faraci and Brian, 1994).

Although the general presence of PDE5 has been established in extra-cerebral smooth muscle cells from animals (Kotera et al., 2000; Lin et al., 2000; Pauvert et al., 2003), there are no previous reports of PDE5 distribution and function in human cerebral arteries. We recently reported on the presence and activity of PDE5, PDE1A and PDE1B in guinea pig basilar arteries, but that may be species dependent, since canine cerebral arteries, for instance, only express PDE5 after application of subarachnoid haemorrhage (Inoha et al., 2002).

Fresh human cerebral arteries are extremely difficult to get and animal tissue with equivalent distribution and function of PDEs has to be used for extensive characterization using the scarce human material for confirmation. In the present case, the occurrence and activity of PDE5 in human cerebral arteries resembled that of the guinea pig cerebral

arteries, so using guinea pig arteries may predict the human responses concerning PDE5 inhibitors. Guinea pigs, compared to rat, have also in other ways been shown to resemble human arteries in cerebral vascular pharmacology and physiological responses (McCulloch and Edvinsson, 1984; Williamson et al., 2001).

The present in vitro method on vasomotor function has previously shown good correspondence with in situ results (McCulloch and Edvinsson, 1984). However, it must be kept in mind that extrapolation of data from animal to man and from in vitro to in vivo has to be made with caution. When looking at the cerebral arteries the blood brain barrier may play a significant role for the cerebrovascular response to drugs in vivo, even for lipophilic compounds such as the PDE5 inhibitors.

4.2. Presence and activity of PDE5 in cerebral arteries

In the present study we show for the first time the occurrence of PDE5A mRNA and protein in human middle cerebral arteries. On the mRNA level we found the presence of all three isoforms of PDE5A; PDE5A1, PDE1A2 and PDE1A3. The Western blots revealed three immunoreactive bands to the PDE5A antibody in both the guinea pig basilar arteries and the human middle cerebral arteries, but the antibody used only detect total PDE5A (Rybalkin et al., 2002). The bands were present in both the immediately frozen arteries and the post mortem arteries, making it less likely to only be due to proteolysis of the samples. Thus, the bands may also represent different isoforms or phosphorylation states of the PDE5 enzyme, as suggested previously in smooth muscle cells (Rybalkin et al., 2002).

We also show a concentration-dependent inhibitory effect of the PDE5 inhibitors sildenafil and UK-114,542, as well as of the supposed PDE1 inhibitor UK-90,345, on cGMP hydrolysis. Especially UK-114,542 showed a specific and potent inhibition of cGMP hydrolysis, whereas the response to sildenafil was less potent than previously reported and with only a ten-fold difference in apparent PDE5 and PDE1 inhibition. Because of the small amount of human tissue available, the enzyme analyses were performed on crude tissue extracts, and not on the purified PDE5 or PDE1 enzyme like in the previous reports (Wallis, 1999). This may explain the discrepancy of our reported potencies compared to that of others.

An effect on cAMP hydrolysis at high concentrations was evident for sildenafil, but not UK-114,542. We found that sildenafil (10 μM) inhibited cAMP hydrolysis up to 50% during Ca^{2+}/CaM stimulation and up to 30% without stimulation in human arteries. A similar cAMP effect of sildenafil (1 μM) has been described in non-cerebral arteries (Pauvert et al., 2003; Wallis et al., 1999). It could either represent a PDE1 inhibitory effect of sildenafil (IC_{50} for PDE1 is 280 nM) (Wallis, 1999) or it could, in the crude extracts with disruption of intra-cellular compartments, be an indirect effect with the increased cGMP modulating the cGMP inhibited PDE3, which is responsible for hydrolysis of mainly cAMP (Beavo, 1995). However, we found no evidence for an effect of sildenafil on cAMP levels in intact arteries through this mechanism.

4.3. Effect of PDE5 inhibitors on cerebral artery dilatation

In the basilar arteries from guinea pig we found that sildenafil and UK-114,542 elicited an endothelium-dependent vasodilatation, indicating a role for an endothelial factor, most likely nitric oxide.

The peak plasma concentration after oral administration of sildenafil 100 mg is reported to be 1 μg/ml which is close to the pEC_{50} we report for sildenafil. But due to the high plasma protein binding, the maximum free plasma concentration of sildenafil rarely exceeds 40 nM (approximately 4–6% of total plasma concentration) with sildenafil tablets of 100 mg (Corbin and Francis, 2002). Sildenafil induced dilatation mainly at concentrations above the free plasma concentrations (40 nM), even though significant inhibition of cGMP hydrolysis was obtained at lower concentration. This may in part reflect a difference between responses in intact tissue and cell lysates, in which the cell membranes are disrupted, and disturb the normal regulation of the cellular signalling pathways.

Although the response to endothelial removal and ODQ pre-treatment was similar to that of sildenafil, the PDE5 inhibitor UK-114,542 (pEC_{50} 8.6) was a far more potent vasodilator than sildenafil (pEC_{50} 6.0). As previously described for PDE5 inhibitors (Kruuse et al., 2001; Sampson et al., 2001; Sobey and Quan, 1999; Willette et al., 1997) an element of cGMP-independent relaxation both in intact and in endothelium denuded arteries at high concentrations (>10 μM) was seen in the present study, which may be due to non-specific effects.

Apart from a possible effect on different PDE5 isoforms, the difference in vascular response to sildenafil and UK-114,542 could be caused by the structural similarity of these compounds to cGMP (Corbin and Francis, 2002) and thus potential effect on downstream cGMP related mechanisms, such as cGMP-dependent protein kinase (PKG) or ion-channels (Lusche et al., 2005; Medina et al., 2000b; Michelakis et al., 2003). A differential effect of UK-114,542 and sildenafil on cyclic nucleotide gated ion channels has recently been described (Lusche et al., 2005). A co-effects on other PDE families than PDE1 seem unlikely for sildenafil (Gbekor et al., 2002), but may be possible for UK-114,452.

UK-90,234 elicited a non-specific response which makes it impossible, based on the present data, to deduce information on the functional role of PDE1 in guinea pig cerebral vasodilatation, although we found the presence of PDE1A and PDE1B but not PDE1C in human cerebral arteries.

In the present study, guinea pig cerebral arteries relaxed in response to sildenafil even without an external NO donor.

The vasodilator response to sildenafil was however greatly enhanced with concomitant application of NO donor just as it is seen in the erectile tissue, showing that sildenafil is more efficient in stimulated guinea pig cerebral arteries.

In conclusion, the present study confirms the presence of the cGMP hydrolysing PDE5 in human middle cerebral arteries. As expected, the activity of PDE5 is inhibited by sildenafil and the other selective PDE5 inhibitor UK-114,542 in both human and guinea pig cerebral arteries. However, higher concentrations were required for inhibition of cGMP hydrolysis than previously reported. The effect of sildenafil on guinea pig basilar artery dilatation is minor, which is compatible with previous findings of no significant cerebrovascular effects of sildenafil in humans. UK-114,542 was more potent and it cannot be excluded that other PDE5 inhibitor may have more pronounced effect on cerebral arteries. The effect of both PDE5 inhibitors were potentiated by sodium nitroprusside, indicating that induction of the cGMP production will make the cerebral arteries more responsive to PDE5 inhibitors. The current data lend support to the hypothesis that sildenafil induced headache and migraine may be due to effects other than dilatation of cerebral arteries. But, further studies are needed to fully determine the in vivo role of PDE5 in regulation of cerebral haemodynamic and headache induction.

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