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INHIBITION OF PIG AORTIC SMOOTH MUSCLE CELL DNA SYNTHESIS BY SELECTIVE TYPE III AND TYPE IV CYCLIC AMP PHOSPHODIESTERASE INHIBITORS

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Abstract—Foetal calf serum (FCS) and platelet-derived growth factor (PDGF)-stimulated incorporation of [^{3}H]thymidine into pig aortic smooth muscle cell (ASMC) DNA was decreased by agents that either stimulated the synthesis (forskolin) or inhibited the breakdown (3-isobutyl-1-methylxanthine, IBMX) of cAMP. FCS-stimulated incorporation of [^{3}H]thymidine into DNA was also reduced by selective inhibitors of cAMP-specific phosphodiesterase (PDE IV) (Ro-20-1724, rolipram) and cGMP-inhibited cAMP PDE (PDE III) (SK&F 94836). IBMX, Ro-20-1724, rolipram and SK&F 94836 enhanced forskolin inhibition of DNA synthesis. Alone, rolipram was a relatively weak inhibitor of FCS-induced ASMC DNA synthesis ($IC_{25} > 20\ \mu M$); however, in the presence of a threshold concentration of SK&F 94836 (20 μM), the potency of rolipram increased ($IC_{25} = 4\ \mu M$), suggesting synergy in the actions of PDE III and PDE IV inhibitors. SK&F 94836 and rolipram elicited 30% and 37%, respectively, reductions in FCS-induced ASMC proliferation and potentiated the inhibitory actions of forskolin. PDE III and PDE IV inhibitors alone, exerted minimal effects on ASMC cAMP levels after a short term (10 min) or long-term (2 or 24 hr) exposure, but enhanced forskolin-induced accumulation of cAMP. ASMC spontaneously released cAMP into the extracellular medium, a process that was increased by forskolin. PDE III and PDE IV inhibitors had no effect alone on cAMP extrusion but enhanced the effect of forskolin. Exposure of ASMC to forskolin or SK&F 94836 for 15 min increased the activity ratio (AR) of cAMP-dependent protein kinase from 0.05 to 0.17 and 0.23, respectively. Ro-20-1724, alone, did not affect cAMP-dependent protein kinase but enhanced the stimulatory effect of forskolin (AR = 0.37) and SK&F 94836 (AR = 0.27). Agents that increased cGMP synthesis (glycerol trinitrate, atrial natriuretic factor) or decreased its hydrolysis by selectively inhibiting cGMP-specific PDE (PDE V) (zaprinast) exerted no effects on FCS- or PDGF-stimulated [^{3}H]thymidine incorporation into DNA either alone or in combination. The cytosolic fraction of pig ASMC contained four cyclic nucleotide PDEs which were categorized as PDE V, Ca^{2+}/calmodulin-stimulated PDE (PDE I), PDE III and PDE IV. PDE I and III activities were also associated with the particulate fraction. The results demonstrate that inhibitors of PDEs III and IV alone or in combination with forskolin, reduce ASMC DNA synthesis and proliferation, through an action likely to involve elevation of intracellular cAMP. In contrast, inhibition of cGMP hydrolysing PDE subtypes (I and V) exerted no effect on DNA synthesis in this cell type.

Migration of aortic smooth muscle cells (ASMC†) from the tunica media into the intima and the subsequent proliferation of these cells is a prominent feature in the pathogenesis of atherosclerosis [1]. Much research has therefore been directed towards understanding the mechanisms by which ASMC mitogenesis is controlled with the aim of developing therapeutic agents to alleviate these disease processes.

Intracellular signalling events which regulate vascular smooth muscle cell proliferation are beginning to be elucidated. A number of second messenger pathways implicated in the stimulation of mitogenesis are also responsible for increases in tone in contractile smooth muscle [2, 3]. Furthermore, a number of spasmogenic agonists exhibit mitogenic or co-mitogenic activity [4–7]. Conversly, several smooth muscle relaxants may also inhibit DNA synthesis in ASMC both *in vitro* and *in vivo* [8]. Amongst these are substances which increase cyclic nucleotide levels. Several studies have demonstrated that agents which increase accumulation of cAMP, such as adenosine [9], prostacyclin [10], prostaglandin E_1 [11] and β_2-agonists [12], inhibit ASMC DNA synthesis. Inhibitory actions of substances which stimulate cGMP synthesis on ASMC mitogenesis have also been observed [13–16]. Causal roles for cyclic nucleotides in the actions of these agents are supported by the inhibition of ASMC DNA synthesis elicited by membrane-permeable analogues of cAMP and cGMP [16, 17].

Although an inhibitory action of the non-selective cyclic nucleotide phosphodiesterase (PDE) inhibitor, 3-isobutyl-1-methylxanthine (IBMX) on [^{3}H]thymidine incorporation into the DNA of proliferating

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† Abbreviations: PDE, cyclic nucleotide phosphodiesterase (EC 3.1.4.17); IBMX, 3-isobutyl-1-methylxanthine; ASMC, aortic smooth muscle cell(s); PBS, phosphate-buffered saline; FCS, foetal calf serum; PDGF, platelet-derived growth factor; PKA, cAMP-dependent protein kinase; AR, activity ratio; DMEM, Dulbecco's Modified Eagle medium; TCA, trichloroacetic acid; TLCK, *p*-tosyl-L-lysine-chloromethyl ketone; GTN, glycerol trinitrate; ANF, atrial natriuretic factor; cGPK, cGMP-dependent protein kinase.

rat ASMC has been reported [18], little is known about the respective roles of the individual PDE isozymes in regulating ASMC DNA synthesis. Five major families of cyclic nucleotide PDEs have been characterized in mammalian tissues. With the advent of "second-generation", selective inhibitors, their specific roles in regulating cAMP or cGMP levels and, consequently, cell/tissue function is emerging [19]. PDE types have been designated as follows: Ca^{2+}/calmodulin-dependent family (PDE I), selectively inhibited by vinpocetine; cGMP-stimulated family (PDE II), for which there are no selective inhibitors; cGMP-inhibited family (PDE III), selectively inhibited by a large number of positive inotropic agents such as SK&F 94836; cAMP-specific family (PDE IV) selectively inhibited by rolipram and Ro-20-1724; cGMP-specific family (PDE V), selectively inhibited by zaprinast [19].

In the present studies, we have investigated the contributions of individual PDE isozymes in regulating [^{3}H]thymidine incorporation into the DNA of ASMC grown from explants of pig aorta by employing isozyme selective and non-selective inhibitors. The results demonstrate that inhibition of PDEs III and IV, but not PDE V, results in a reduction of [^{3}H]thymidine incorporation into DNA and ASMC proliferation.

MATERIALS AND METHODS

Materials. [6-^{3}H]Thymidine (28 Ci/mmol), cyclic [2,8-^{3}H]AMP (41 Ci/mmol), cyclic [8-^{3}H]GMP (13.8 Ci/mmol), [γ-^{32}P]ATP (30 Ci/mmol) and genetically engineered platelet-derived growth factor (PDGF) (β–β chain) were purchased from Amersham International (Amersham, U.K.). The cAMP radioimmunoassay kit was from NEN Chemicals GmbH. Zaprinast (M&B 22948, 2-*O*-propoxyphenyl-8-azapurin-6-one) and rolipram [4-(3-cyclopentyloxy-4-methoxyphenyl)-2-pyrrolidone] were synthesized by the Department of Discovery Chemistry, Rhône-Poulenc Rorer Ltd (Dagenham, U.K.). SK&F 94836 {2-cyano-1-methyl-3-[4-(methyl-6-oxo-1,4,5,6-tetrahydropyridazin-3-yl)-phenyl]guanidine} was from SmithKline and French Research Ltd (Welwyn, U.K.). Denbufylline (BRL 30892; 1,3-di-*n*-butyl-7-[2′-oxopropyl]-xanthine) was a gift from Beecham Pharmaceuticals (Epsom, U.K.). Trequinsin (HL-725; 9,10-dimethoxy-2-mesitylimino-3-methyl-3,4,6,7-tetrahydro-2*H*-pyrimido[6,1-*a*]isoquinolin-4-one) was supplied by Hoechst Pharmaceuticals (Hounslow, U.K.). Ro-20-1724 [1-4-(3-butoxy-4-methoxybenzyl)-2-imidozolidinone] was obtained from Roche Products Ltd (Welwyn Garden City, U.K.). ITS + (TM)PREMIX (consisting of: 12.5 mg insulin, transferrin and selenium; 2.5 g bovine serum albumin in 20 mL of aqueous solution) was purchased from Flow Laboratories (Rickmansworth, U.K.). Other cell culture reagents and the cAMP-dependent protein kinase (PKA) assay system were purchased from Gibco BRL (Paisley, U.K.). All other chemicals were obtained from the Sigma Chemical Co., BDH Chemicals (both of Poole, U.K.) or Rhône-Poulenc Ltd (Eccles, Manchester, U.K.).

Pig ASMC culture. ASMC cultures were established from medial smooth muscle explants of porcine thoracic aortae, based on a methodology described previously [20]. All cell cultures were incubated at 37° in a humidified atmosphere of 95% air/5% CO_2. Cells (previously passaged between 7 and 15 times) were plated out in 35 mm dishes containing 2 mL Dulbecco's Modified Eagle medium (DMEM) supplemented with 10% (v/v) foetal calf serum (FCS) and penicillin/streptomycin antibiotics. After 3 days, the culture medium was replaced with 2 mL DMEM containing 0.1% (v/v) FCS and the cells were incubated for a further 3 days so that they became arrested at the Go phase of the cell cycle.

Assay of ASMC DNA synthesis. Growth-arrested ASMC were stimulated to undergo DNA synthesis by replacing the medium with 2 mL Medium 199 supplemented with 1% (v/v) ITS + (TM) Premix, FCS (or PDGF), penicillin/streptomycin antibiotics and appropriate test substance or vehicle control. After 20 hr incubation, 2 μCi [6-^{3}H]thymidine were added per dish of cells. A further 4 hr after addition of [6-^{3}H]thymidine, the medium was removed and the cell layer washed in phosphate-buffered saline (PBS), pH 7.4 at 4°. The cells were then detached from the dish by addition of 0.5 mL typsin solution [(0.05% (w/v) in PBS]. Samples of cell suspension (200 μL) were taken for cell number determination (using a Coulter counter) and for determination of DNA synthesis. DNA synthesis was measured as the incorporation of [6-^{3}H]thymidine into trichloroacetic acid (TCA) [10% (w/v) at 4°] precipitable DNA. After washing [10% (w/v) TCA at 4°], the precipitate was dissolved in sodium hydroxide (1 M) and the radioactivity measured by lipid scintillation counting.

Assay of ASMC proliferation. Growth arrested ASMC were stimulated to undergo DNA synthesis as described above. However, the stimulated ASMC cultures were incubated for 6 days with a change to fresh medium and supplements after 3 days. Cell number determinations were carried out (as indicated above) on growth-arrested ASMC (immediately prior to stimulation) and following 6 days incubation. Percentage inhibition was calculated as:

$$\% \text{ inhibition } (I) = 100 - [(T - B) \times 100/(C - B)]$$

where B = mean number of cells per dish prior to stimulation, C = mean number of cells per dish with vehicle control and T = mean number of cells per dish with test compound.

Measurement of [^{3}H]*thymidine uptake into ASMC.* Confluent growth-arrested cells in 35 mm dishes were incubated in Medium 199 for varying time periods up to 1 hr with 2 μCi [^{3}H]thymidine. At the end of the incubation, cells were washed three times with PBS before addition of 2 mL of 5% (w/v) TCA. The disrupted cells were scraped from the dishes, briefly sonicated (10 sec), transferred to polypropylene tubes and centrifuged (2000 g, 5 min). Radioactivity in the soluble fraction was then determined. The DNA content of cell pellets was measured by an automated spectrofluorometric procedure employing Adriamycin® [21].

Measurement of cAMP. Cyclic nucleotide studies were performed on confluent, growth-arrested ASMC grown in 35 mm multiwell dishes as described

previously for endothelial cells [22]. Drugs were added to the cells at the concentrations and times indicated in the Results section. For long-term studies, cells were incubated in Medium 199 supplemented with 1% (v/v) ITS (TM) + Premix, penicillin/streptomycin antibiotics and 3% FCS. In these experiments cAMP was measured in cells and medium.

Measurement of PKA. PKA was measured in confluent growth-arrested cells incubated in 2 mL Hank's balanced saline solution in 50 mm dishes. Cells were incubated with test substances for 15 min before medium was removed and 0.5 mL ice-cold homogenization buffer (50 mM Tris–HCl, 5 mM EDTA, 0.2 mM dithiothreitol, 125 mM NaCl, 0.5 mM IBMX, 0.2% Triton X-100, pH 7.4) was added to the dishes. The cells were scraped from the surface of the dish, transferred to a Dounce homogenizer and homogenized (10 strokes).

To assess the long-term effects of forskolin and/or PDE inhibitors, cells were incubated with test substances for 20 hr in Medium 199 supplemented with 1% (v/v) ITS (TM) + Premix, penicillin/streptomycin antibiotics and 3% FCS. At the end of this period, cells were washed with PBS and then treated as above.

The PKA activity of ASMC Triton X-100 extracts was determined with an assay kit (Gibco) in which incorporation of ^{32}P from [γ-^{32}P]ATP into the synthetic specific substrate, kemptide, was measured. Briefly, 10 μL of the ASMC extract was added to an assay mixture (total volume 40 μL)containing 50 μM kemptide, 0.1 mM [γ-^{32}P]ATP (20 μCi/mL), 10 mM $MgCl_2$, 0.25 mg/mL bovine serum albumin, 50 mM Tris–HCl (pH 7.5) in the presence and absence of 10 μM cAMP and/or synthetic PKA inhibitor (Gibco) (1 μM). Incubations were allowed to proceed for 15 min before the reaction was stopped by pipetting 20 μL of the assay mixture onto phosphocellulose disc ion-exchange papers, which were then washed twice, extensively in 1% (v/v) phosphoric acid and then twice with water. Individual discs were placed in scintillation vials and ^{32}P measured by liquid scintillation spectrometry after addition of scintillation cocktail (Lumagel, May & Baker Laboratory Chemicals). The extent of PKA activation was expressed as the activity ratio (AR), which is the ratio of specific activity in the absence of added cAMP to that in the presence of sufficient cAMP to fully activate the enzyme (−cAMP/+cAMP activity ratio).

Partial purification of pig ASMC PDE activities. Cells (2–3 × 10^8) grown in 250-mL culture flasks (Nunclon) were washed three times with PBS before being scraped from the surface and transferred in PBS to a 50 mL polypropylene centrifuge tube. The cells were then centrifuged (2000 *g*, 5 min) and, after the PBS had been removed, the pellet was stored at −70° until required.

The cell pellet was homogenized in 6 vol. of Tris–HCl (20 mM, pH 7.5) containing 2 mM $MgCl_2$, 1 mM diothiothreitol, 5 mM EDTA, 20 μM *p*-tosyl-L-lysine-chloromethyl ketone (TLCK) and aprotinin (1 mg/mL) with a Dounce homogenizer. The homogenate was then centrifuged at 100,000 *g* for 60 min and the supernatant (8 mL) applied to a DEAE-trisacryl column (7 cm × 0.9 cm), pre-equilibrated with column buffer (20 mM Tris–HCl, 2 mM $MgCl_2$, 1 mM dithiothreitol, 20 μM TLCK, pH 7.5). The column was washed with 50 mL of column buffer, and PDE activities were eluted with two successive linear gradients of NaCl (0–200 mM, 80 mL and 200–300 mM, 70 mL) in column buffer.The flow rate throughout was 0.75 mL/min and 2-mL fractions were collected and assayed. For storage at −20°, ethylene glycol was added to a final concentration of 30% (v/v).

Measurement of PDE activity. PDE activity was determined by the two-step radioisotope method of Thompson *et al.* [23]. The reaction mixture contained 20 mM Tris–HCl (pH 8.0), 10 mM $MgCl_2$, 4 mM 2-mercaptoethanol, 0.2 mM EGTA and 0.05 mg/mL bovine serum albumin. Unless otherwise stated, the concentration of substrate was 1 μM for [^{3}H]cAMP and [^{3}H]cGMP.

The IC_{50} values (concentration which produced 50% inhibition of substrate hydrolysis) for the compounds examined were determined from cor centration–response curves in which concentrations ranged from 0.1 μM to 1 mM. At least three concentration–response curves were generated for each agent.

Protein was determined as desribed by Lowry *et al.* [24] with bovine serum albumin as the standard.

Categorization of PDE isozymes. The nomenclature for the different cyclic nucleotide PDEs adopted in this paper is based on that of Beavo and Reifsnyder [19].

Statistical analysis. Data are presented as means ± SEM and analysed by one-way analysis of variance (ANOVA). To assess whether a positive interaction resulted from a combination of treatments (PDE III, PDE IV inhibitors) data were analysed by two-way ANOVA. Values are considered to be statistically significant when P is less than 0.05.

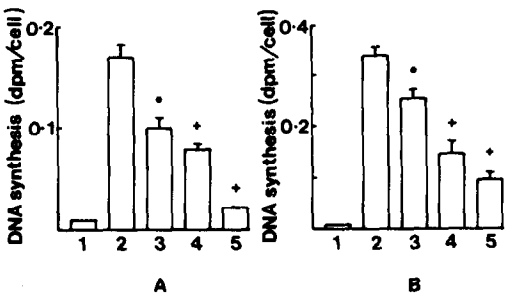

Fig. 1. Effects of forskolin and IBMX on ASMC DNA synthesis. DNA synthesis was stimulated in quiescent ASMC by addition of 10 ng/mL PDGF (A) or 10% (v/v) FCS (B), in the absence (2) or presence of 10 μM forskolin (3), 100 μM IBMX (4) and forskolin + IBMX (5). In the absence of growth stimulus ASMC DNA synthesis was 0.008 ± 0.0004 dpm/cell (bar 1). Results represent means ± SEM (N = 3). *P < 0.05, †P < 0.01, denote significant effect compared to control.

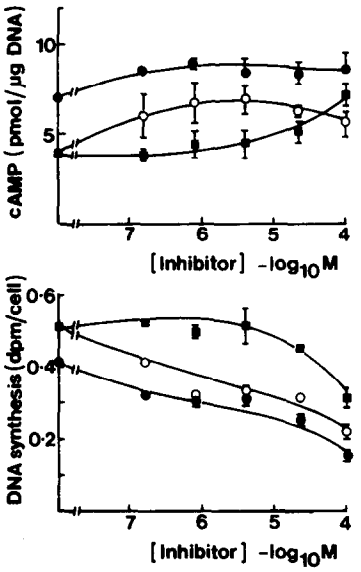

Fig. 2. Effects of SK&F 94836 and rolipram on forskolin-induced inhibition of ASMC DNA synthesis and stimulation of cAMP accumulation. DNA synthesis (lower panel) was stimulated in quiescent ASMC by addition of culture medium containing 3% (v/v) FCS in the presence of forskolin (10 μM) with the indicated concentrations of rolipram (○), SK&F 94836 (■) or rolipram in the presence of 50 μM SK&F 94836 (●). In the absence of growth stimulus, ASMC DNA synthesis was 0.025 ± 0.003 dpm/cell and FCS-stimulated ASMC DNA synthesis in the absence of forskolin was 0.858 ± 0.006 dpm/cell. cAMP (upper panel) was measured as described in the legend to Table 3. Symbols are as in the upper panel. cAMP levels in cells in the absence of forskolin were 0.63 ± 0.04 pmol/μg DNA. Results represent means ± SEM (N = 3).

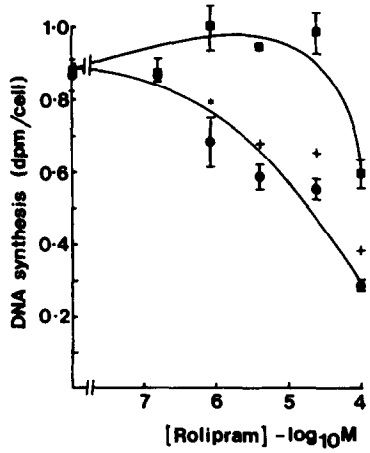

Fig. 3. Effect of a threshold concentration of SK&F 94836 on the inhibition of ASMC DNA synthesis by rolipram. DNA synthesis was stimulated in quiescent ASMC by addition of culture medium containing 3% (v/v) FCS in the absence (■) and presence (●) of 20 μM SK&F 94836 and the indicated concentrations of rolipram. In the absence of growth stimulus ASMC DNA synthesis was 0.025 ± 0.003 dpm/cell. Results represent means ± SEM (N = 3). *P < 0.0.5, †P < 0.01, denote significant effects of SK&F 94836.

RESULTS

Effects of forskolin and PDE inhibitors on [^{3}H]-*thymidine incorporation into ASMC DNA*

FCS-induced [^{3}H]thymidine incorporation into ASMC DNA was inhibited by agents that increased cAMP accumulation either by stimulating its synthesis (forskolin) or by blocking intracellular hydrolysis. The non-selective PDE inhibitor, IBMX, as well as selective inhibitors of PDEs III (SK&F 94836) and IV (Ro-20-1724, rolipram) reduced DNA synthesis and enhanced the actions of forskolin (Figs 1–3, Table 1). PDGF-induced [^{3}H]thymidine incorporation into DNA was also inhibited by forskolin and IBMX (Fig. 1).

Ro-20-1724 (50 μM) and SK&F 94836 (50 μM) individually inhibited DNA synthesis by 30% and 41%, respectively, and in combination by 68% (Table 1). The inhibition elicited by a combination of these two compounds was significantly greater ($P < 0.05$) than that achieved by either individually, but no evidence for a positive interaction (> additive) between the two treatments at these concentrations was obtained (two-way ANOVA).

PDE III and PDE IV inhibitors, only weakly ($IC_{25} > 20$ μM) reduced ASMC DNA synthesis when tested alone (Fig. 2); however, the potency of rolipram was increased markedly in the presence of forskolin ($IC_{25} = 1$ μM) (Fig. 2) or a threshold concentration (20 μM) of SK&F 94836 ($IC_{25} = 4$ μM) (Fig. 3). Two-way ANOVA indicated a positive interaction ($P < 0.01$) between this threshold concentration of SK&F 94836 and rolipram (0.8–100 μM).

Neither glycerol trinitrate (GTN) (1 μM), which increases cGMP by activating soluble guanylate cyclase, nor ANF (atrial natriuretic factor) (0.1 μM), which stimulates particulate guanylate cyclase, influenced [^{3}H]thymidine incorporation into DNA. Zaprinast (50 μM), a potent inhibitor of PDE V [25], either alone or in combination with ANF or GTN, was also without effect (Table 2).

Inhibition of ASMC proliferation by forskolin and PDE inhibitors

To test whether the inhibition of [^{3}H]thymidine incorporation into DNA by agents which elevate cAMP levels translated into a reduction in cell number, forskolin and PDE inhibitors were tested for their effects on FCS-induced ASMC proliferation (Table 3). Forskolin (5 μM) alone, inhibited the increase in cell numbers induced by 10% FCS by 11%. PDE inhibitors also reduced ASMC proliferation, SK&F 94836 (50 μM), rolipram (50 μM) and the non-selective inhibitor, trequinsin (5 μM), eliciting 30%, 31% and 54% reductions in cell numbers, respectively. SK&F 94836, rolipram and trequinsin enhanced the inhibitory actions of forskolin by 31%, 43% and 62%, respectively.

Table 1. Effects of forskolin on ASMC DNA synthesis in the absence and presence of selective PDE inhibitors

Inhibitor	DNA synthesis (dpm/cell) Forskolin (μM) 0	0.4	2	10	50
None	0.391 ± 0.038	0.266 ± 0.043	0.242 ± 0.014	0.188 ± 0.027	0.116 ± 0.007
Ro-20-1724 (50 μM)	0.275 ± 0.027*	0.195 ± 0.010	0.139 ± 0.008†	0.111 ± 0.006*	0.051 ± 0.004†
SK&F 94836 (50 μM)	0.232 ± 0.025*	0.179 ± 0.007	0.165 ± 0.009†	0.151 ± 0.010	0.082 ± 0.007*
Ro-20-1724 + SK&F 94836	0.126 ± 0.008†	0.138 ± 0.018*	0.101 ± 0.004†	0.076 ± 0.007†	0.055 ± 0.009†

DNA synthesis was stimulated in quiescent ASMC by addition of culture medium containing 3% (v/v) FCS in the absence and presence of the various additions. In the absence of growth stimulus ASMC DNA synthesis was 0.036 ± 0.006 dpm/cell.

Results represent means ± SEM (N = 3).

*$P < 0.05$, †$P < 0.01$, denote significant effects of PDE inhibitor(s) alone or plus forskolin from control or the effect of an equivalent concentration of forskolin in the absence of a PDE inhibitor.

Table 2. Effects of agents that stimulate cGMP accumulation on FCS- and PDGF-induced ASMC DNA synthesis

Addition	DNA synthesis (dpm/cell) FCS	PDGF
None	1.05 ± 0.07	0.31 ± 0.02
Zaprinast (50 μM)	0.88 ± 0.05	0.36 ± 0.02
GTN (1 μM)	1.13 ± 0.08	0.47 ± 0.01
ANF (0.1 μM)	1.11 ± 0.05	0.39 ± 0.04
Zaprinast + GTN	1.04 ± 0.02	0.35 ± 0.05
Zaprinast + ANF	0.96 ± 0.09	0.40 ± 0.06

DNA synthesis was stimulated in quiescent ASMC in the absence and presence of various compounds by addition of culture medium containing 10% (v/v) FCS or 10 ng/mL PDGF. In the absence of growth stimulus ASMC DNA synthesis was 0.18 ± 0.002 dpm/cell.

Results represent means ± SEM (N = 3).

Table 3. Effects of forskolin and PDE inhibitors on FCS-induced ASMC proliferation

Addition	Cells/dish (×10⁶) None	+Forskolin
None	1.32 ± 0.03	1.18 ± 0.01
SK&F 94836 (50 μM)	0.92 ± 0.04*	0.82 ± 0.02*
Rolipram (50 μM)	0.83 ± 0.02*	0.68 ± 0.02*
Trequinsin (5 μM)	0.58 ± 0.03*	0.46 ± 0.01*

Growth-arrested ASMC were stimulated to proliferate with 10% FCS in the absence and presence of forskolin (5 μM) plus and minus the indicated concentrations of PDE inhibitors. Cell number determinations were made as described in Materials and Methods. Cell number at the start of the experiment was 0.057×10^6.

The results represent means ± SEM (N = 4).

*$P < 0.01$, denotes significant effects of PDE inhibitor compared to control or forskolin alone.

Effect of forskolin with and without PDE inhibitors on ASMC cAMP accumulation

A 10 min incubation of ASMC with forskolin (0.5–50 μM) induced a dose-dependent elevation in cAMP levels, with a 24-fold increase being observed at the highest concentration (Table 4). PDE inhibitors, when added alone, exerted either a minimal effect on cAMP accumulation or were completely without effect; however, SK&F 94836 and Ro-20-1724 enhanced the stimulatory effect of forskolin. A combination of SK&F 94836 with either Ro-20-1724 or rolipram elicited a greater enhancement of forskolin (0.5–10 μM) stimulated cAMP accumulation compared to that observed when either the PDE III or PDE IV inhibitors were tested individually (Table 4, Fig. 2). At the highest forskolin concentration (50 μM) the enhancement of cAMP accumulation caused by a combination of SK&F 94836 and Ro-20-1724, was not significantly greater than that induced by the PDE inhibitors individually (Table 4). The concentrations of SK&F 94836 and rolipram required to enhance forskolin (10 μM)-induced inhibition of ASMC DNA synthesis were similar to those which enhanced forksolin-induced cAMP accumulation (Fig. 2).

As in the short-term (10 min) incubations, no significant effect of either Ro-20-1724 (50 μM) or SK&F 94836 (50 μM) either alone or in combination on cAMP accumulation was detected after 2 or 24 hr (Table 5). The cellular content of cAMP in cells stimulated with forskolin (10 μM) either in the absence or presence of PDE III and/or PDE IV inhibitors had declined by 2 hr and had almost reached basal values by 24 hr. In untreated cells, considerable cAMP was detected in the incubation medium after 24 hr. Neither PDE III nor PDE IV inhibitors influenced basal extrusion of the second messenger; however, forskolin greatly increased medium cAMP levels and this effect was enhanced by both SK&F 94836 and Ro-20-1724 (Table 5).

Effects of forskolin and PDE inhibitors on PKA

The PKA AR in control cells was very low (AR = 0.05). A 15 min incubation with forskolin (10 μM) increased the PKA AR to 0.23. SK&F 94836 (50 μM) alone, increased PKA activity (AR = 0.17) and enhanced the stimulation elicited by forskolin (AR = 0.33). No effect of Ro-20-1724 (50 μM) alone, was detected, but the PDE IV inhibitor significantly

Table 4. Effects of forskolin on ASMC cAMP accumulation in the absence and presence of selective PDE inhibitors

Inhibitor	cAMP (pmol/μg of DNA) Forskolin (μM) 0	0.5	5	50
None	1.60 ± 0.14	3.30 ± 0.55	12.70 ± 2.31	38.15 ± 18.88
Ro-20-1724 (50 μM)	2.16 ± 0.53	7.29 ± 1.33*	26.22 ± 4.91*	61.31 ± 16.79
SK&F 94836 (50 μM)	1.52 ± 0.16	6.06 ± 1.39	23.59 ± 8.95*	59.00 ± 22.07
Ro-20-1724 + SK&F 94836	2.24 ± 0.15	11.72 ± 1.56†	33.87 ± 8.86*	58.35 ± 17.01

Quiescent cells grown to confluence were exposed to PDE inhibitors and forskolin for 10 min.
The results represent means ± SEM (N = 5–6).
*$P < 0.05$, †$P < 0.01$, denote significant effects of PDE inhibitor(s) plus forskolin compared to the effect of an equivalent concentration of forskolin in the absence of PDE inhibitor.

Table 5. Effects of prolonged exposure to forskolin and PDE inhibitors on cAMP accumulation in ASMC and release into the medium

Addition	cAMP (pmol/μg DNA) 10 min Cells	10 min Medium	2 hr Cells	2 hr Medium	24 hr Cells	24 hr Medium
None	2.2 ± 0.2	—	1.7 ± 0.1	1.5 ± 0.5	1.4 ± 0.03	6.6 ± 0.4
SK&F 94836 (50 μM)	3.2 ± 0.7	—	2.4 ± 0.1	1.9 ± 0.4	1.3 ± 0.1	7.3 ± 0.4
Ro-20-1724 (50 μM)	2.8 ± 0.1	—	2.3 ± 0.3	1.9 ± 0.8	1.3 ± 0.1	7.7 ± 0.9
Forskolin (10 μM)	9.7 ± 1.3*	—	6.0 ± 0.4*	17.3 ± 6.1*	3.1 ± 0.2†	46.5 ± 12.3*
Forskolin + SK&F 94836	11.3 ± 0.9*	—	6.6 ± 0.4*	22.8 ± 5.0*	3.0 ± 0.8*	77.4 ± 28.4*
Forskolin + Ro-20-1724	12.3 ± 1.5*	—	9.8 ± 0.01*	34.7 ± 10.1*	4.0 ± 0.8*	71.8 ± 17.0*

Cells were incubated in Medium 199 supplemented with 1% (v/v) ITS + (TM) Premix with 3% FCS in the absence and presence of forskolin and/or PDE inhibitors. At the end of the indicated incubation periods medium was removed, cells washed with PBS and intracellular as well as extruded cAMP extracted and measured as described in Materials and Methods. Little or no extruded cAMP was detected in the medium after 10 min.
The results represent means ± SEM (N = 3).
*$P < 0.01$, denotes significant effects of treatments from control values.

enhanced forskolin (AR = 0.37) and SK&F 94836 (AR = 0.27)-induced activation of PKA. Incubation of ASMC with a combination of forskolin, Ro-20-1724 and SK&F 94836 increased the activity ratio to 0.45. Two-way ANOVA indicated a positive interaction (> additive) ($P < 0.05$) between Ro-20-1724 and SK&F 94836 on the PKA activity ratio in the absence, but not in the presence, of forskolin. These results are summarized in Table 6. No effect of any of these treatments on the ASMC PKA AR was detected after a 24 hr incubation (data not shown).

Effects of PDE inhibitors and forskolin on [^{3}H]-*thymidine uptake into ASMCs*

Forskolin (50 μM), Ro-20-1724 (50 μM) and SK&F 94836 (100 μM) had no effect on [^{3}H]thymidine incorporation into ASMC; however, uptake was inhibited by trequinsin (10 μM) by greater than 60% and almost abolished by the known nucleoside transport inhibitor dipyridamole (50 μM) (data not shown).

ASMC cyclic nucleotide PDEs

Smooth muscle cells grown from explants of pig aorta contained both cAMP PDE and cGMP PDE activities (Table 7). Over 80% of the cGMP PDE and greater than 70% of the cAMP PDE was located in the cytosolic fraction. Addition of calcium plus calmodulin stimulated both cytosolic and particulate cGMP PDE by 66% and 100%, respectively. cAMP PDE (2 μM substrate) was also increased by calcium plus calmodulin in the cytosolic (34%) and particulate (21%) fractions (data not shown). cAMP PDE (2 μM substrate) was inhibited by cGMP (1 μM) in the cytosolic (20%) and membrane fractions (25%) (Table 7). At lower substrate concentration (0.25 μM cAMP), cGMP (1 μM) inhibited cAMP hydrolysis

Table 6. Effects of PDE inhibitors on ASMC PKA in the absence and presence of forskolin

	PKA AR	
	− Forskolin	+ Forskolin
None	0.06 ± 0.01	0.27 ± 0.05
Ro-20-1724 (50 μM)	0.05 ± 0.02	0.37 ± 0.02*
SK&F 94836 (50 μM)	0.17 ± 0.03*	0.33 ± 0.03
Ro-20-1724 + SK&F 94836	0.27 ± 0.02†	0.45 ± 0.02†

Cells were incubated with PDE inhibitors for 15 min in the absence and presence of forskolin (10 μM). Incubations were terminated, cell-free extracts prepared and PKA measured as described in Materials and Methods. PKA activity (−cAMP) in control cells was 0.16 ± 0.2 pmol/min/mg protein and total PKA (+cAMP) was 2.34 ± 0.12 pmol/min/mg.

The results represent means ± SEM (N = 3).

*$P < 0.05$, †$P < 0.01$, denote significant effects of PDE inhibitor(s) compared to control or forskolin alone.

Table 7. Effect of Ca^{2+} + calmodulin on cGMP PDE and cGMP on cAMP PDE in the cytosolic and particulate fractions of ASMC

	Cyclic nucleotide hydrolysis (pmol/min/mg)			
	cGMP		cAMP	
Fraction	No addition	+ Calmodulin	No addition	+ cGMP
Homogenate	110 ± 29	163 ± 32	344 ± 48	278 ± 65
Cytosol	254 ± 54 (83%)	422 ± 54 (75%)	785 ± 71 (71%)	634 ± 73 (72%)
Pellet	29 ± 6 (17%)	58 ± 23 (25%)	129 ± 38 (29%)	98 ± 26 (28%)

Cells were homogenized and subcellular fractions prepared as described in Materials and Methods. Assays were performed at 30° in the presence of 200 μM EGTA with or without $CaCl_2$ (2 μM) plus calmodulin (2.5 U/mL) (in the case of cGMP PDE) or with and without cGMP (1 μM) (in the case of cAMP PDE). The substrate concentration was 1 μM for cGMP and 2 μM for cAMP.

Results represent means ± SEM (N = 2). Values in parentheses show percentage of total cellular activity.

by 30% in both the soluble and particulate fractions (data not shown).

Partial purification of the soluble nucleotide PDE activity by DEAE trisacryl ion-exchange chromatography revealed two peaks of cGMP PDE activity (Fig. 4). The first peak, which eluted at 100 mM NaCl, was only slightly stimulated by addition of calcium plus calmodulin, while the second peak, eluting at 135 mM NaCl, was stimulated at least 4-fold. Both peaks were almost completely inhibited by zaprinast (20 μM). cAMP PDE was also resolved, albeit poorly, into two distinct activities (Fig. 4). A shoulder of activity eluted from the column at about 170 mM NaCl prior to a major peak eluting at 190 mM NaCl. The first shoulder of cAMP PDE activity was completely inhibited by SK&F 94836 (20 μM), but not by rolipram (20 μM) while the reverse was the case for the second peak.

Particulate cAMP PDE was potently inhibited by SK&F 94836 (IC_{50} = 5 μM) but only weakly inhibited by rolipram (IC_{50} = 270 μM). Particulate cGMP PDE was inhibited by IBMX (IC_{50} = 6 μM) and relatively weakly by zaprinast (IC_{50} = 15 μM).

DISCUSSION

In agreement with previous studies [9–12], agents that stimulate cAMP accumulation reduce ASMC DNA synthesis regardless of the mitogenic stimulus, as demonstrated by forskolin and/or IBMX inhibition of either FCS—or PDGF—stimulated [^{3}H]thymidine incorporation into DNA. The effect of forskolin on FCS-stimulated DNA synthesis was enhanced by non-selective PDE inhibitors such as IBMX or selective inhibitors of PDE III (SK&F 94836) and PDE IV (Ro-20-1724, rolipram). Synergy between the inhibitory activities of PDE III and PDE IV inhibitors on DNA synthesis is suggested by the demonstration that, in the absence of forskolin, a concentration of SK&F 94836 (20 μM), which alone had no effect on DNA synthesis, greatly increased the inhibitory potency of rolipram. Previous studies have demonstrated that PDE III and PDE IV inhibitors exert a more pronounced effect on T-lymphocyte blastogenesis when used in combination [26].

PDE III and PDE IV inhibitors enhanced the

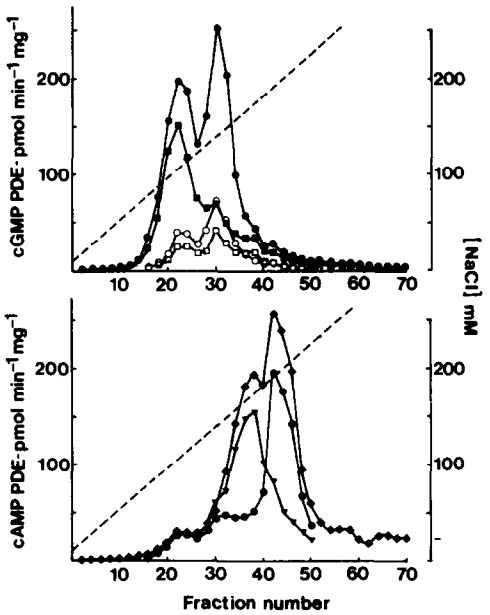

Fig. 4. DEAE-triacryl anion exchange chromatography of PDE activity of the 100,000 *g* supernatant fraction from ASMC. A 100,000 *g* cytosolic fraction from ASMC was prepared and chromatographed as described in Materials and Methods: cGMP PDE activity (upper panel) was determined with 1 μM substrate concentration in the absence (●■) and presence (○□) of M&B 22948 (20 μM) with (○●) and without (□■) 2 mM $CaCl_2$ + 2.5 U/mL calmodulin. cAMP PDE activity (lower panel) was determined with 1 μM substrate concentration in the absence of PDE inhibitors (◆), with 20 μM rolipram (▼) or with 20 μM SK&F 94836 (●).

actions of forskolin on DNA synthesis, cell proliferation and cAMP accumulation. Furthermore, in the presence of forskolin, PDE III and PDE IV dose–response curves for inhibition of DNA synthesis closely mirrored those for enhancement of cAMP accumulation. Given that PDEs III and IV exist in pig ASMC, these data lend support to a causal link between elevation of cAMP and decreased DNA synthesis; however, in the absence of forskolin, PDE III and PDE IV inhibitors significantly reduced DNA synthesis at concentrations which exerted minimal effects on short-term cAMP accumulation. This raises questions regarding the role of cAMP in the inhibition of DNA synthesis by PDE inhibitors. It is possible that increased cAMP levels below the detection limit of the assay method may be sufficient to inhibit DNA synthesis. Examples of cAMP PDE inhibitors influencing biological responses in the absence of detectable changes in cAMP are common [27, 28]. SK&F 94836, increased the ASMC PKA AR 3-fold in the absence of forskolin. Curiously, Ro-20-1724 alone exerted no effect on PKA, although it did enhance the activations elicited by SK&F 94836 and (like the PDE III inhibitor) forskolin. These results, although lending further weight to causal roles of cAMP and PKA in the actions of the PDE III inhibitor, raise further questions about the mechanism of action of Ro-20-1724. They do not, however, eliminate the possibility that PDE IV inhibitors, alone, can exert longer-term PKA-mediated inhibition of DNA synthesis by potentiating the actions of an adenylate cyclase activator released at a defined point in the cell cycle following addition of the mitogenic stimulus. In this regard, it is of interest that FCS-treated cells, in the absence of agents known to influence the cAMP cascade, produced cAMP which was extruded into the medium; however, Ro-20-1724 did not influence levels of the released second messenger nor did any of the above treatments influence the PKA AR after prolonged (24 hr) incubations.

Although the bulk of the data presented herein and elsewhere generally support an inhibitory action of cAMP on ASMC DNA synthesis, absolute levels of the cyclic nucleotide are difficult to relate to decreased mitogenesis. As stated above, SK&F 94836, alone or in combination with Ro-20-1724, activated PKA and inhibited DNA synthesis with minimal increases in cAMP. Indeed, the inhibition of thymidine incorporation and activation of PKA elicited by a combination of these PDE III and PDE IV inhibitors were equal to that caused by forskolin even though the latter induced an 8-fold increase in cellular cAMP. It is noteworthy that even though very large increases in cAMP were observed in reponse to forskolin, without or with PDE inhibitors, the maximal PKA AR induced by any of the treatments was less than 0.5. Perhaps, the majority of cAMP produced by forskolin, alone or in combination with PDE inhibitors, occurs in a pool inconsequential to DNA synthesis from where it is extruded.

As mentioned in the introduction, signalling systems causing increased smooth muscle tone have been implicated in the stimulation of ASMC DNA synthesis whereas second messengers which relax smooth muscle inhibit ASMC DNA synthesis. The results reported herein demonstrate further parallels between smooth muscle contractility and ASMC DNA synthesis. As with ASMC DNA synthesis, marked synergy between the relaxant actions of PDE III and PDE IV inhibitors on both vascular and bronchial smooth muscle has been reported [29–31]; however, little effect of agents that elevate cGMP levels, including zaprinast (PDE V), on DNA synthesis in pig ASMC was observed, highlighting perhaps a major difference in the regulation of contractile smooth muscle tone and synthetic smooth muscle cell DNA synthesis. cGMP is a powerful relaxant of both bronchial and vascular smooth muscle and zaprinast potentiates the actions of activators of both the soluble and particulate guanylate cyclases [30, 32, 33]. In contrast, neither GTN nor ANF inhibited [^{3}H]thymidine incorporation into pig ASMC DNA either in the absence or presence of zaprinast, although these agents elevate cGMP accumulation in these cells (data not shown). These results contrast with previous reports demonstrating inhibitory effects of both NO-generating substances and ANF on DNA synthesis in rat ASMC [14, 15]. This may be suggestive of species differences in the susceptibility of ASMC mitogenesis to inhibition by cGMP; however, other

explanations may account for these discrepancies—Cornwell and Lincoln [34] demonstrated that cGMP-dependent protein kinase (cGPK) activity is lost from smooth muscle cells after several passages which results in an abolition of the ANF inhibition of the K^+-induced Ca^{2+} signal. Incorporation of cGPK into cells by osmotic lysis restored the ability of ANF to lower intracellular Ca^{2+}. Thus, the lack of effect of agents which elevate cGMP on DNA synthesis in pig ASMC may be due to the absence of the enzyme through which the actions of this second messenger are mediated; however, in experiments where inhibitory effects of cGMP were reported [14, 15], the cells had undergone a similar number of passages to those employed in our studies.

The limitations of using [^{3}H]thymidine incorporation into DNA as a measure of mitogenesis have been highlighted previously [35]. Employing this technique, apparent inhibition of DNA synthesis may be observed with agents that inhibit [^{3}H]-thymidine transport into cells. Dipyridamole, a non-selective PDE inhibitor, is a well known inhibitor of nucleoside uptake [36]. We have shown that trequinsin, a very potent but non-selective cAMP PDE inhibitor [37] reduces [^{3}H]thymidine uptake into ASMC. Both of these compounds produced an apparent profound inhibition of [^{3}H]thymidine incorporation into ASMC DNA (data not shown); however, neither Ro-20-1724 nor SK&F 94836 influenced thymidine transport at the concentrations employed to measure DNA synthesis. That the effects of these compounds on [^{3}H]thymidine incorporation are due to inhibition of DNA synthesis is sustained by their abilities to reduce the increase in cell numbers induced by FCS.

The profile of PDE activities in pig ASMC is similar to that of the contractile smooth muscle from which these cells were derived (data not shown) and of smooth muscle from other sources, although in bronchial tissue substantial cGMP-stimulated activity (Type II) is present [38]. It is of interest that the PDE profile in the proliferative (synthetic phenotype) pig ASMC is similar to that in the contractile tissue since in certain cells differentiation or dedifferentiation processes are associated with changes in the PDE complement [39]. As in contractile smooth muscle [38], it is probable that the cGMP-inhibited (Type III) and cAMP-specific (Type IV) PDEs are responsible for cAMP hydrolysis, whereas the cGMP-specific PDE (Type V) and, perhaps, the Ca^{2+}/calmodulin-dependent PDE (Type I) hydrolyse cGMP.

In summary, although anomalies are apparent, the results suggest that both PDE III and PDE IV are involved in regulating pig ASMC DNA synthesis and proliferation. In contrast, no role for the cGMP-hydrolysing PDE V could be demonstrated even when cGMP synthesis was stimulated by activation of the soluble or particulate guanylate cyclases. Similarities between the regulation of ASMC DNA synthesis and smooth muscle contractility by PDE III and PDE IV inhibitors are indicated by the data.

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REFERENCES

1. Ross R, Masuda H and Raines EW, Cellular interactions, growth factors and smooth muscle cell proliferation in atherogenesis. *Ann NY Acad Sci* **598**: 102–112, 1990.
2. Kariya K, Kawahara Y, Fukuzaki H, Hagiwara M, Hidaka H, Fukumoto Y and Takai Y, Two types of protein kinase C with different functions in cultured rabbit aortic smooth muscle cells. *Biochem Biophys Res Commun* **161**: 1020–1027, 1989.
3. Thyberg J, Hedin U, Sjolund M, Palmberg L and Bottger BA, Regulation of differentiated properties and proliferation of arterial smooth muscle cells. *Arteriosclerosis* **10**: 966–990, 1990.
4. Berk BC and Alexander RW, Vasoactive effects of growth factors. *Biochem Pharmacol* **38**: 219–225, 1989.
5. Nemecek GM, Coughlin SR, Handley DA and Moskowitz MA, Stimulation of aortic smooth muscle cell mitogenesis by serotonin. *Proc Natl Acad Sci USA* **83**: 674–678, 1986.
6. Nakaki T, Nakayama M, Yamamoto S and Kato R, Endothelin-mediated stimulation of DNA synthesis in vascular smooth muscle cells. *Biochem Biophys Res Commun* **158**: 880–883, 1989.
7. Araki S, Kawahara Y, Kariya K, Sunako M, Tsudo T, Fukuzaki H and Yoshimi T, Stimulation of platelet-derived growth factor-induced DNA synthesis by angiotensin II in rabbit vascular-smooth muscle cells. *Biochem Biophys Res Commun* **168**: 350, 1990.
8. Nilsson J, Sjölund M, Palmberg L, Von Euler AM, Jonzon B and Thyberg J, The calcium antagonist nifedipine inhibits arterial smooth muscle cell proliferation. *Atherosclerosis* **58**: 109–122, 1985.
9. Jonzon B, Nilsson J and Fredholm BB, Adenosine receptor-mediated changes in cyclic AMP production and DNA synthesis in cultured arterial smooth muscle cells. *J Cell Physiol* **124**: 451–456, 1985.
10. Morisaki N, Kanzaki T, Motoyama N, Saito Y and Yoshida S, Cell cycle-dependent inhibition of DNA synthesis by prostaglandin I_2 in cultured rabbit aortic smooth muscle cells. *Atherosclerosis* **71**: 165–171 1988.
11. Loesberg C, Van Wijk R, Zandbergen J, Van Aken WG, Van Mourik JA and De Groot PhG, Cell cycle dependent inhibition of human vascular smooth muscle cell proliferation by prostaglandin E. *Exp Cell Res* **160**: 117–125, 1985.
12. Nakaki T, Nakayama M, Yamamoto S and Kato R, α_1-Adrenergic stimulation and β_2-adrenergic inhibition of DNA synthesis in vascular smooth muscle cells. *Mol Pharmacol* **37**: 30–36, 1990.
13. Abell TJ, Richards AM, Ikram H, Espiner EA and Yandle T, Atrial natriuretic factor inhibits proliferation of vascular smooth muscle cells stimulated by platelet-derived growth factor. *Biochem Biophys Res Commun* **160**: 1391–1396, 1989.
14. Garg UC and Hassid A, Nitric oxide-generating vasodilators and 8-bromo-cyclic guanosine monophosphate inhibit mitogenesis and proliferation of cultured rat vascular smooth muscle cells. *Clin Invest* **86**: 1774–1777, 1989.
15. Itoh H, Pratt RE and Dzau VJ, Atrial natriuretic polypeptide inhibits hypertrophy of vascular smooth muscle cells. *Clin Invest* **86**: 1690–1697, 1990.
16. Kariya K, Kawahara Y, Araki S, Fukuzaki H and Takai Y, Antiproliferation action of cyclic GMP-elevating vasodilators in cultured rabbit aortic smooth muscle cells. *Atherosclerosis* **80**: 143–147, 1989.
17. Southgate K and Newby AC, Serum-induced proliferation of rabbit aortic smooth muscle cells from the

contractile state is inhibited by 8-Br-cAMP but not 8-Br-cGMP. *Atherosclerosis* **82**: 113–127, 1989.

18. Orekhov AN, Tertov VV, Kudryashov SA, Khashimov KhA and Smirnov VN, Primary culture of human aortic intima cells as a model for testing antiatherosclerosis drugs. Effects of cAMP, prostaglandins, calcium antagonists, antioxidant and lipid lowering agents. *Atherosclerosis* **60**: 101–110, 1986.
19. Beavo JA and Reifsnyder DH, Multiple isozymes of cyclic nucleotide phosphodiesterase. *Trends Pharmacol Sci* **11**: 150–155, 1990.
20. Ross R, The smooth muscle cell. II. Growth of smooth muscle in culture and formation of elastic fibres. *J Cell Biol* **50**: 172–186, 1971.
21. Kennedy DG, Nelson J, Van den Berg HW and Murphy RF, A modified spectrofluorometric assay for DNA using adriamycin. *Anal Biochem* **167**: 124–127, 1987.
22. Souness JE, Diocee BK, Martin W and Moodie SA, Pig aortic endothelial-cell cyclic nucleotide phosphodiesterases. Use of phosphodiesterase inhibitors to evaluate their roles in regulating cyclic nucleotide levels in intact cells. *Biochem J* **266**: 127–132, 1990.
23. Thompson WJ, Terasaki W, Epstein PM and Strada SJ, Assay of cyclic nucleotide phosphodiesterase and resolution of multiple molecular forms of the enzyme. *Adv Cyclic Nucl Res* **10**: 69–92, 1979.
24. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
25. Nicholson CD, Challiss RAJ and Shahid M, Differential modulation of tissue function and therapeutic potential of selective inhibitors of cyclic nucleotide phosphodiesterase isoenzymes. *Trends Pharmacol Sci* **12**: 19–27, 1991.
26. Robicsek SA, Blanchard DK, Djeu JY, Krzanowski JJ, Szentivanyi A and Polson JB, Multiple high-affinity cAMP-phosphodiesterase in human T-lymphocytes. *Biochem Pharmacol* **42**: 869–877, 1991.
27. Silver PJ, Lepore RE, O'Connor B, Lemp BM, Hamel LT, Bentley RG and Harris AL, Inhibition of the low Km cAMP phosphodiesterase and activation of the cyclic AMP system in vascular smooth muscle by milrinone. *J Pharmacol Exp Ther* **247**: 34–42, 1988.
28. Souness JE, Carter CM, Diocee BK, Hassall GA, Wood LJ and Turner NC, Characterization of guinea-pig eosinophil phosphodiesterase activity. *Biochem Pharmacol* **42**: 937–945, 1991.
29. Lindgren S, Rascon A, Anderson K-E, Manganiello V and Degerman E, Selective inhibition of cGMP-inhibited and cGMP-non inhibited cyclic nucleotide phosphodiesterase and relaxation of rat aorta. *Biochem Pharmacol* **42**: 545–552, 1991.
30. Torphy TJ, Zhou H-L, Burman M and Huang LBF, Role of cyclic nucleotide phosphodiesterase isozymes in intact canine trachealis. *Mol Pharmacol* **39**: 376–384, 1991.
31. Harris AL, Connell MJ, Ferguson EW, Wallace AM, Gordon RJ, Pagani ED and Silver PJ, Role of low K_m cAMP phosphodiesterase inhibition in tracheal relaxation and bronchodilation in the guinea pig. *J Pharmacol Exp Ther* **251**: 199–206, 1989.
32. Martin W, Morgan RO, Smith JA and White DG, Atriopeptin II-induced relaxation of rabbit aorta is potentiated by M&B 22948 but not blocked by haemaglobin. *Br J Pharmacol* **89**: 557–561, 1986.
33. Souness JE, Brazdil R, Diocee BK and Jordan R, Role of selective cGMP phosphodiesterase inhibition in the myorelaxant actions of M&B 22948, MY-3445, vinpocetine and 1-methyl-3-isobutyl-8-(methylamino) xanthine. *Br J Pharmacol* **98**: 725–734, 1989.
34. Cornwell TL and Lincoln TM, Regulation of intracellular Ca^{2+} levels in cultured vascular smooth muscle cells. Reduction of Ca^{2+} by atriopeptin and 8-bromo-cGMP is mediated by cGMP dependent protein kinase. *J Biol Chem* **264**: 1146–1155, 1989.
35. Murat J-C, Gamet L, Cazenave Y and Trocheris V, Questions about the use of [^{3}H]thymidine incorporation as a reliable method to estimate cell proliferation rate. *Biochem J* **270**: 563–564, 1990.
36. Belt JA, Heterogeneity of nucleoside transport in mammalian cells. Two types of transport activity in L1210 and other cultured neoplastic cells. *Mol Pharmacol* **24**: 479–484, 1983.
37. Ruppert D and Weithmann KU, HL 725, an extremely potent inhibitor of platelet phosphodiesterase and induced platelet aggregation *in vitro*. *Life Sci* **31**: 2037–2043, 1982.
38. Torphy TJ and Cieslinski LB, Characterisation and selective inhibition of cyclic nucleotide phosphodiesterase isozymes in canine trachael smooth muscle. *Mol Pharmacol* **37**: 206–214, 1990.
39. Souness JE, Pratt ML, Shen C-C, Thompson WJ and Strada SJ, Comparison of immunological, kinetic and hormone sensitive properties of Type IV cAMP phosphodiesterase during differentiation of 3T3-L1 cells. *Fed Proc* **43**: 990, 1984.