Pergamon

*Biochemical Pharmacology*, Vol. 48, No. 4, pp. 827–835, 1994.

0006-2952/94 $7.00 + 0.00

0006-2952(94)E0210-X

# SYNERGISTIC INTERACTIONS BETWEEN SELECTIVE PHARMACOLOGICAL INHIBITORS OF PHOSPHODIESTERASE ISOZYME FAMILIES PDE III AND PDE IV TO ATTENUATE PROLIFERATION OF RAT VASCULAR SMOOTH MUSCLE CELLS

XIAOLEI PAN,* ELIZABETH ARAUZ, JOSEPH J. KRZANOWSKI, DAVID F. FITZPATRICK and JAMES B. POLSON†

Department of Pharmacology and Therapeutics, College of Medicine, University of South Florida, Tampa, FL 33612-4799, U.S.A.

(*Received* 29 *June* 1993; *accepted* 23 *March* 1994)

**Abstract**—The interaction between selective inhibitors of 3′,5′-cyclic-nucleotide phosphodiesterase (PDE) III (cyclic GMP inhibited phosphodiesterase) and selective inhibitors of PDE IV (Ro 20-1724 inhibited phosphodiesterase) to attenuate fetal bovine serum-stimulated incorporation of [$^3$H]thymidine into DNA and cell proliferation was studied in a line (A10) of vascular smooth muscle cells (VSMC). The nonselective PDE inhibitors 3-isobutyl-1-methylxanthine (IBMX) and papaverine attenuated DNA synthesis with $EC_{50}$ values (16 and 18 $\mu$M, respectively) in the same range as their published $IC_{50}$ values (2–50 and 2–25 $\mu$M, respectively) as PDE inhibitors. The selective PDE III inhibitors CI-930 and cilostamide used alone attenuated DNA synthesis with $EC_{50}$ values (>300 and 5.3 $\mu$M, respectively) that were much higher than published $IC_{50}$ values (0.15–0.46 and 0.005–0.064 $\mu$M, respectively) for inhibition of PDE III. In the presence of the PDE IV inhibitor rolipram (10 $\mu$M), their $EC_{50}$ values were shifted (0.66 and 0.16 $\mu$M, respectively) much closer to their respective $IC_{50}$ values. When the selective PDE IV inhibitors rolipram and Ro 20-1724 were used alone, they attenuated DNA synthesis with $EC_{50}$ values (111 and >100 $\mu$M, respectively) much higher than their $IC_{50}$ values (0.6–2.6 and 2–13 $\mu$M, respectively) as inhibitors of PDE IV, but 10 $\mu$M CI-930 (PDE III inhibitor) shifted their $EC_{50}$ values (0.56 and 1.5 $\mu$M, respectively) much closer to their $IC_{50}$ values. In experiments that assessed VSMC proliferation using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] method, IBMX and papaverine attenuated proliferation with $EC_{50}$ values (27 and 58 $\mu$M, respectively) close to their $IC_{50}$ values. CI-930 and cilostamide used alone did not cause 50% attenuation of proliferation at the highest concentrations tested (100 and 10 $\mu$M, respectively). In the presence of 5 $\mu$M rolipram, however, their effects were enhanced greatly with $EC_{50}$ values (0.86 and 0.23 $\mu$M, respectively) that were close to their $IC_{50}$ values as PDE III inhibitors. Similarly, rolipram and Ro 20-1724 attenuated VSMC proliferation with $EC_{50}$ values close to their $IC_{50}$ values in the presence (2.1 and 4.6 $\mu$M, respectively) but not in the absence (>100 and >10 $\mu$M, respectively) of 2 $\mu$M CI-930. The interactions between PDE III inhibitors and PDE IV inhibitors to attenuate DNA synthesis and VSMC proliferation were synergistic as determined by the combination index. The data demonstrate that the synergistic interactions that attenuate incorporation of [$^3$H]thymidine into DNA are accompanied by synergistic attenuations of VSMC division. The closeness of the $EC_{50}$ values of PDE III inhibitors when PDE IV is blocked, and of the PDE IV inhibitors when PDE III is blocked, to their respective $IC_{50}$ values as selective PDE inhibitors supports the view that the effects on DNA synthesis and cell division are caused by inhibition of the respective PDE isozymes. A hypothesis is proposed to explain the synergistic interactions.

*Key words:* cyclic AMP; 3′,5′-cyclic-nucleotide phosphodiesterase; phosphodiesterase inhibitors; vascular smooth muscle cells; cell division; synergy

Proliferation of VSMC‡ is an essential feature of the pathogenesis of atherosclerosis [1]. In addition, VSMC proliferation is a well-documented cause of the restenosis that occurs in a large percentage of patients following coronary angioplasty [2]. Rational development of pharmacological agents to impede these very significant pathogenic processes depends on a clear understanding of the mechanisms that control VSMC proliferation.

Substances that stimulate adenylyl cyclase activity

* This work was presented by Xiaolei Pan in partial fulfilment of requirements for the Ph.D. degree.

† Corresponding author: James B. Polson, Ph.D., Department of Pharmacology and Therapeutics, Box 9, College of Medicine, University of South Florida, 12901 Bruce B. Downs Boulevard, Tampa, FL 33612-4799. Tel. (813) 974-2543. FAX (813) 974-3081.

‡ Abbreviations: CI-930, 3-(2*H*)-pyridazinone-4,5-dihydro-6-[4-(1*H*-imidazol-1yl) phenyl]-5-methyl-monohydrochloride; cAMP, adenosine-3′,5′-cyclic monophosphate; cilostamide, *N*-cyclohexyl-*N*-methyl-4-(1,2-dihydro-2-oxo-6-quinolyloxy) butyramide; IBMX, 3-isobutyl-1-methylxanthine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PDE, 3′,5′-cyclic-nucleotide phosphodiesterase (EC 3.1.4.17); Ro 20-1724, *d,l*-1,4-[3-butoxy-4-methoxybenzyl]-2-imidazolidinone; rolipram, 4-[3-(cyclopentyloxy)-4-methoxyphenyl]-2-pyrrolidinone: and VSMC, vascular smooth muscle cells.

attenuate DNA synthesis in VSMC as a result of cAMP accumulation [3–8]. Similar effects can be produced by theophylline and IBMX, which raise cAMP levels by nonselective inhibition of PDEs [7–9].

More than 25 isozymes of PDE have been reported and have been classified according to their kinetic characteristics, responses to modulators, and primary amino acid sequences [10–12]. The classification proposed by Beavo and Reifsnyder [10] is used to designate isozymes in this paper.

Little is known about the contribution of individual PDE isozymes to the regulation of cAMP levels in cells [13] or about the capacity of individual isozymes to modulate cell proliferation. Development of pharmacological inhibitors with remarkable selectivity for individual isozyme families has provided probes to search for answers to these questions [14]. Robicsek *et al.* [15] recently reported that CI-930, a selective inhibitor of PDE III, interacted synergistically with Ro 20-1724, a selective inhibitor of PDE IV, to attenuate mitogen-stimulated incorporation of [$^3$H]thymidine into DNA in human T lymphocytes. More recently, Souness *et al.* [7] reported a similar synergistic interaction between SK&F 94836 (PDE III inhibitor) and rolipram (PDE IV inhibitor) to attenuate incorporation of [$^3$H]-thymidine in VSMC cultured from pig aorta.

The purpose of the present investigation was to further elucidate the synergistic interactions between inhibitors of PDE III and PDE IV in VSMC. Specific goals were: (1) to determine whether there is a synergistic effect to attenuate mitogen-induced increases in cell numbers, (2) to compare the $EC_{50}$ values for effects on DNA synthesis and cell replication with known $IC_{50}$ values for the same compounds as PDE inhibitors, and (3) to determine whether the synergistic interactions could be reproduced by combinations of nonselective PDE inhibitors, or combinations of PDE inhibitors selective for the same isozyme family. In addition, a hypothesis is presented to explain the synergism.

A line of VSMC (A10) derived from embryonic rat aorta was used for this purpose. These cells possess morphological and biochemical characteristics similar to VSMC of the synthetic phenotype, and exhibit stable pharmacological responsiveness between passages 16 and 150 [16, 17]. Some of the results of this study were published previously in abstract form [18, 19].

## MATERIALS AND METHODS

*Materials.* A10 VSMC were purchased from the American Type Culture Collection (Rockville, MD). [$^3$H]Thymidine (6.7 Ci/mmol) was purchased from NEN Research Products (Boston, MA). Fetal bovine serum (FBS) was purchased from HyClone Laboratories, Inc. (Logan, UT). Trypsin/EDTA, penicillin/streptomycin, papaverine, IBMX and MTT were purchased from the Sigma Chemical Co. (St. Louis, MO). CI-930 was provided by Parke-Davis, Pharmaceutical Research Division of Warner-Lambert Co. (Ann Arbor, MI). Rolipram and Ro 20-1724 were gifts from Berlex Laboratories, Inc. (Cedar Knolls, NJ), and Hoffmann-LaRoche, Inc. (Nutley, NJ), respectively. Cilostamide was provided by the Otsuka Pharmaceutical Co., Ltd. (Tokushima, Japan). All other chemicals and reagents were purchased from Sigma.

*VSMC culture.* A10 VSMC were cultured in 75 cm$^2$ flasks in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% (v/v) FBS, 25 U penicillin G and 25 $\mu$g streptomycin. All cell cultures were incubated at 37° under a humidified atmosphere of 95% air and 5% $CO_2$. The presence of smooth muscle $\alpha$-actin in the cultured cells was confirmed using a specific monoclonal antibody (63-793-1, ICN Biomedical, Irvine, CA), an FITC-labeled anti-mouse IgG secondary antibody, and fluorescence microscopy. Cells between passages 16 and 50 were used in all experiments.

*PDE assay.* Confluent A10 cells in 75 cm$^2$ flasks were detached by scraping, centrifuged (100 *g*, 5 min), resuspended in 1 mL of assay buffer (50 mM Tris–HCl, pH 8.0; 0.05% BSA; 10 $\mu$M $CaCl_2$; 10 mM $MgCl_2$) containing 20 $\mu$g/mL of leupeptin and 100 kallikrein U/mL of aprotinin, and sonicated to disrupt the cells. The sonicate was centrifuged at 23,600 *g* for 30 min at 4°, and the pellet was washed once and resuspended in 0.5 mL of the same buffer.

The assay described by Thompson and Appleman [20] as modified in this laboratory [15] was used. Briefly, dilutions of soluble and particulate cell extracts were incubated for 10 min at 37° with 0.2 $\mu$M [$^3$H]cAMP (40,000 cpm/assay) in 20 $\mu$L volumes of assay buffer containing 3.75 mM $\beta$-mercaptoethanol and 0.02 U/$\mu$L of alkaline phosphatase. Reactions were started by addition of cell extracts and stopped by addition of 0.5 mL volumes of a 1:3 (v:v) slurry of AG 1-X8 anion exchange resin in a mixture of equal volumes of water and isopropanol. After centrifugation (550 *g*, 15 min) to remove resin-bound [$^3$H]cAMP, the labeled reaction product was counted using liquid scintillation spectrometry.

*Assay of DNA synthesis.* Optimal cell and FBS concentrations and incubation times were determined in preliminary studies. Confluent VSMC (A10) in 75 cm$^2$ flasks were detached with trypsin-EDTA (1) and plated in 96-well plates at concentrations of 5000 cells/well in 200 $\mu$L DMEM containing 10% FBS. The cells were allowed to adhere for 24 hr, and then brought to quiescence by changing to DMEM containing no serum. Twenty-four hours later, cells were stimulated by addition of fresh medium containing 0.5% (v/v) FBS. PDE inhibitors were added at the same time. After incubution for 20 hr, [$^3$H]thymidine was added. After an additional 4-hr incubation, VSMC were harvested as described by Sauro and Zorn [21] using a SKATRON (Lier, Norway) cell harvester, and radioactivity in the DNA that was collected on filters was counted by liquid scintillation spectrometry. For a typical experiment, addition of 0.5 $\mu$Ci/well resulted in 14,000–15,000 cpm counted on filters for control wells (no PDE inhibitors). More than 90% of the cells were alive at the time of harvesting, as determined by Trypan blue exclusion.

*Assay of cell proliferation.* Cell numbers were assayed by the MTT method [22]. Optimal cell and FBS concentrations and incubation times were

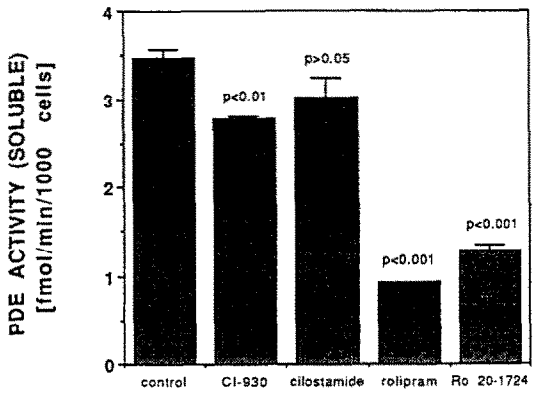


Fig. 1. Effect of selective PDE inhibitors on soluble PDE activity from A10 cell extracts. Inhibitors were used at 10 $\mu$M concentrations except for cilostamide, which was used at 0.1 $\mu$M. Enzyme activity was assayed as described in Materials and Methods. Means ± SEM from 3 assays are shown.

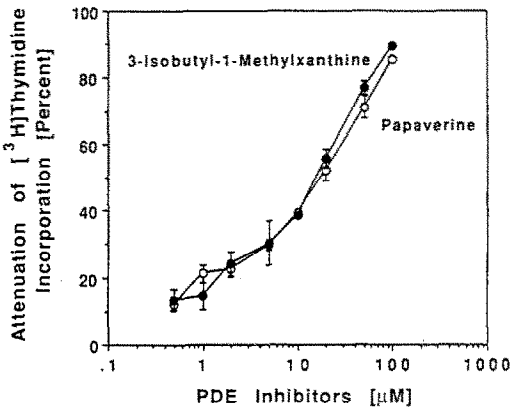


Fig. 3. Effects of IBMX and papaverine on the incorporation of [$^3$H]thymidine into VSMC DNA. FBS was used to stimulate VSMC proliferation for 24 hr in the presence of the concentrations of PDE inhibitors indicated. Incorporation of [$^3$H]thymidine was measured during the last 4 hr of the incubation period as described in Materials and Methods. Means ± SEM from 4 (IBMX) or 6 (papaverine) experiments are shown.

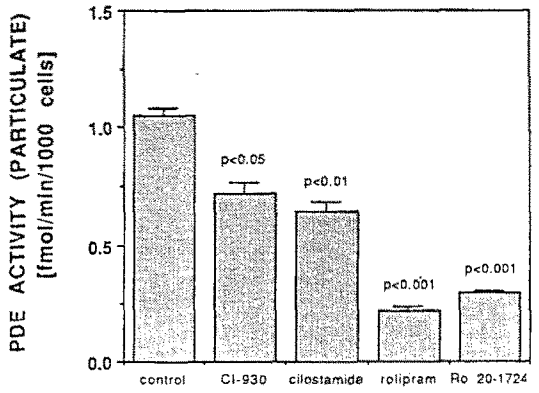


Fig. 2. Effect of selective PDE inhibitors on particulate PDE activity from A10 cell extracts. Inhibitors were used at 10 $\mu$M concentrations except for cilostamide, which was used at 0.1 $\mu$M. Means ± SEM from 3 assays are shown.

determined in preliminary studies. Absorbance was found to be linearly correlated (not shown) with the number of VSMC in the culture system used in these studies, and the presence of PDE inhibitors had no effect on the MTT assay.

Confluent VSMC (A10) in 75 cm$^2$ flasks were detached with trypsin/EDTA and plated in 96-well plates at concentrations of 10,000 cells well in 200 $\mu$L volumes of DMEM containing 10% FBS. The cells were allowed to adhere for 48 hr, washed twice with serum-free DMEM, and then brought to quiescence by incubation in serum-free medium for 48 hr. The cells were then stimulated to proliferate by addition of DMEM containing 5% (v/v) FBS and allowed to grow in the absence or presence of PDE inhibitors for an additional 48 hr. The number of live cells was assayed by adding 10 $\mu$L of MTT solution (10 mg/mL in PBS) to each well for the final 2.5 hr of the incubation, and then adding 50 $\mu$L of 20% SDS to dissolve the formazan crystals that were formed. Color development was read at 570 nm in an ELISA plate reader with a reference at 630 nm. Absorbance for control wells (no PDE inhibitors added) typically ranged from 0.29 to 0.32 O.D. units. Cell viability was >95% in all experiments.

*Statistical analysis.* Interactions between PDE inhibitors were analyzed by calculating the combination index [23] based on $EC_{40}$ or $EC_{30}$ values as indicated. The $EC_{50}$, $EC_{40}$ and $EC_{30}$ values were determined from concentration–response curves by computer analysis (InPlot program from GraphPad, San Diego). Data are expressed as means ± SEM and were analyzed by one-way analysis of variance (ANOVA) or Student's *t*-test. $P < 0.05$ was considered statistically significant.

## RESULTS

*PDE activity.* The activities measured in soluble and particulate cell extracts are shown in Figs. 1 and 2, respectively. Soluble activity represented about three-fourths and particulate activity about one-fourth of the total activity measured. CI-930 and cilostamide were used to detect the presence of PDE III, whereas rolipram and Ro 20-1724 were used to detect PDE IV. By these criteria, PDE III represented 13–20% and PDE IV represented 63–73% of the soluble activity. PDE III represented about 30% and PDE IV about 70% of the particulate activity.

*Effect of nonselective PDE inhibitors on* [$^3$H]*-thymidine incorporation into DNA.* To evaluate the effects of nonselective inhibition of PDE isozymes, IBMX and papaverine were used. Figure 3 shows that both agents produced concentration-dependent attenuation of the incorporation of [$^3$H]thymidine

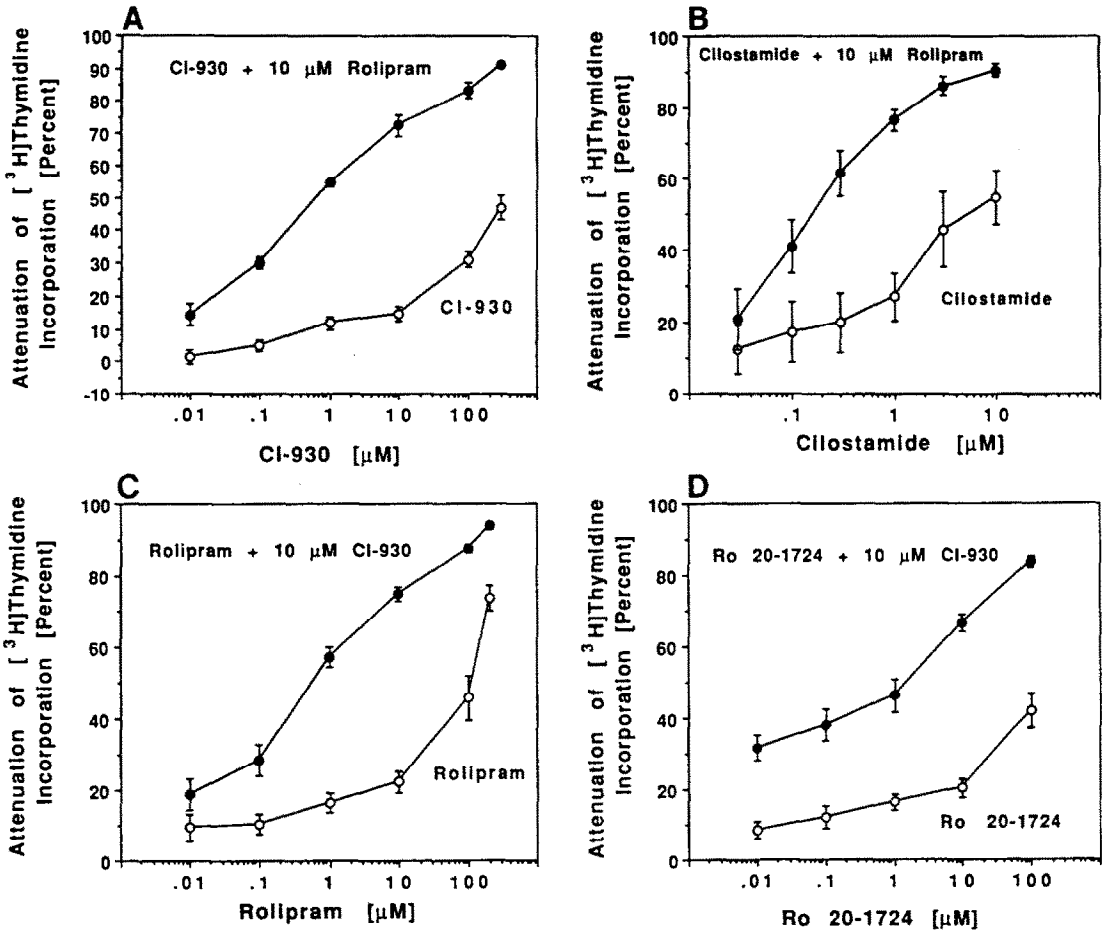


Fig. 4. Effect of selective PDE inhibitors on the incorporation of [$^3$H]thymidine into VSMC DNA. The experimental protocols were the same as for Fig. 3. Means ± SEM are shown. The number of experiments represented by each curve ranged from 4 to 12.

into VSMC DNA. The $EC_{50}$ values for IBMX and papaverine were 16 and 18 μM. Both agents produced more than 80% attenuation at 100 μM concentrations.

*Effect of PDE III inhibitors on [$^3$H]thymidine incorporation into DNA.* CI-930 and cilostamide were used as selective inhibitors of PDE III. Both caused weak concentration-dependent attenuation of [$^3$H]thymidine incorporation when used alone (Fig. 4, A and B). However, when either agent was used in combination with 10 μM rolipram, a selective PDE IV inhibitor, greater attenuation was observed. CI-930 alone produced less than 50% attenuation at the highest concentration tested (300 μM), and therefore the combination indexes shown in Table 1 were calculated using $EC_{40}$ values. Addition of 10 μM rolipram shifted the $EC_{40}$ values of CI-930 about 714-fold, from 186 to 0.26 μM, and the combination index was less than 1.0, indicating a synergistic interaction. Cilostamide interacted synergistically with 10 μM rolipram (Table 1) to cause a 24-fold shift in its $EC_{40}$ from 2.14 to 0.09 μM.

*Effect of PDE IV inhibitors on [$^3$H]thymidine incorporation into DNA.* Rolipram and Ro 20-1724 were used as selective inhibitors of PDE IV. Rolipram alone produced more than 70% attenuation of [$^3$H]thymidine incorporation (Fig. 4C), but 200 μM concentrations were required. Addition of 10 μM CI-930 interacted synergistically (Table 1) with rolipram to shift the $EC_{40}$ 225-fold from 56 to 0.25 μM. CI-930 (10 μM) also interacted synergistically with Ro 20-1724 (Fig. 4D, Table 1), shifting the $EC_{40}$ 471-fold from 80 to 0.17 μM.

*Effect of nonselective PDE inhibitors on VSMC proliferation assessed by MTT assay.* Both IBMX and papaverine caused concentration-dependent attenuation of VSMC proliferation, as illustrated in Fig. 5. The $EC_{50}$ values were 27 and 58 μM, respectively. Although 10 and 20 μM papaverine shifted the $EC_{50}$ of IBMX 8.6- and 8.0-fold, respectively, the combination indexes indicated antagonistic interactions (Table 2).

*Effect of PDE III inhibitors on VSMC proliferation.* Both CI-930 and cilostamide caused only slight concentration-dependent attenuation of VSMC proliferation, but these effects were enhanced by addition of 5 μM rolipram (Fig. 6, A and B). Because the PDE III inhibitors alone did not cause 40% attenuation in the concentrations tested, $EC_{30}$ values

Table 1. Combination indexes for attenuation of VSMC DNA synthesis

| PDE inhibitors | Combination index* | Probability† | Type of interaction | $EC_{40}$ alone / $EC_{40}$ in combination |
|---|---|---|---|---|
| Cl-930 + 10 μM rolipram | 0.18 ± 0.0005 (5) | P < 0.0001 | Synergism | 714 |
| Cilostamide + 10 μM rolipram | 0.21 ± 0.0004 (4) | P < 0.0001 | Synergism | 24 |
| Rolipram + 10 μM Cl-930 | 0.061 ± 0.0004 (6) | P < 0.0001 | Synergism | 225 |
| Ro 20-1724 + 10 μM Cl-930 | 0.056 ± 0.0009 (4) | P < 0.0001 | Synergism | 471 |

* The experimental protocols were the same as in Fig. 3. Each experiment was carried out using a 96-well plate that contained various concentrations of one drug (drug *a*) in the absence and presence of the indicated concentration of a second drug (drug *b*). The combination index [23] was determined for each experiment and means ± SEM are shown for the number of experiments indicated in parentheses. Combination index = $(d_a/D_a) + (d_b/D_b)$, where $d_a$ represents the $EC_{40}$ for drug *a* used in combination with the $d_b$ concentration of drug *b*. $D_a$ and $D_b$ represent $EC_{40}$ values for drug *a* and drug *b* used alone, respectively. A combination index >1.0 indicates an antagonist interaction, <1.0 indicates synergism, and equal to 1.0 indicates zero interaction.

† P was calculated using Student's *t*-test for comparison of the combination index with 1.0. Degrees of freedom = n − 1.

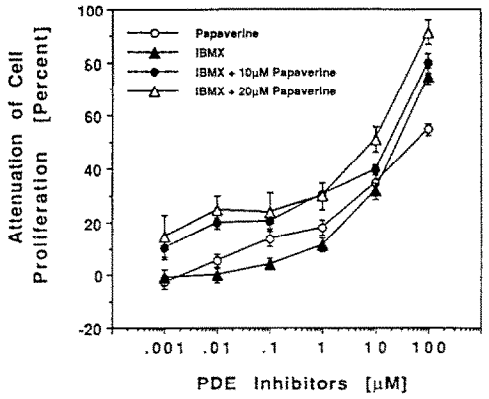


Fig. 5. Attenuation of VSMC proliferation by IBMX and papaverine. FBS was used to stimulate VSMC proliferation for 48 hr in the presence of the concentrations of PDE inhibitors indicated. At the end of the incubation period, the number of cells was determined using the MTT method as described in Materials and Methods. Means ± SEM are shown. Each curve represents data from 8 experiments.

were chosen as the equi-effective concentrations for calculation of combination indexes, as shown in Table 2. Rolipram (5 μM) shifted the $EC_{30}$ for CI-930 250-fold from 32.5 to 0.13 μM, and the combination index was <1.0 indicating synergism. Although cilostamide alone did not produce 30% attenuation, the combination index was estimated using an $EC_{30}$ > 10 μM. This index was <1.0 (Table 2), suggesting a synergistic interaction. It appears that 5 μM rolipram would have shifted the $EC_{30}$ for cilostamide >588-fold from >10 to 0.017 μM. When concentration–response curves for cilostamide were constructed substituting 2 μM CI-930 for 5 μM rolipram, the combination did not produce 30% inhibition at the highest concentrations tested (Fig. 6B), so the combination index based on $EC_{30}$ values could not be calculated.

*Effect of PDE IV inhibitors on VSMC proliferation.* Figure 6 (C and D) shows that rolipram and Ro 20-1724 used alone caused only slight concentration-dependent attenuations of VSMC proliferation, but that their effects were greatly enhanced by 2 μM CI-930. This concentration of the PDE III inhibitor shifted the $EC_{30}$ of rolipram 306-fold from 33.7 to 0.11 μM. The combination index was <1.0 indicating

Table 2. Combination indexes for attenuation of VSMC proliferation assessed using the MTT assay

| PDE inhibitors | Combination index | Probability† | Type of interaction | $EC_{30}$ alone / $EC_{30}$ in combination |
|---|---|---|---|---|
| IBMX + 10 μM papaverine | 2.01 ± 0.039 (5) | P < 0.0001 | Antagonism | 8.6 |
| IBMX + 20 μM papaverine | 3.94 + 0.077 (4) | P < 0.0001 | Antagonism | 8.0 |
| Cl-930 + 5 μM rolipram | 0.27 ± 0.11 (5) | P < 0.005 | Synergism | 250 |
| Cilostamide + 5 μM rolipram | <0.15 (9) | | Synergism | >588 |
| Rolipram + 2 μM Cl-930 | 0.065 ± 0.00 (6) | P < 0.0001 | Synergism | 306 |
| Ro 20-1724 + 2 μM Cl-930 | <0.099 (8) | | Synergism | >26 |

The experimental protocols were the same as in Fig. 5. Combination indexes [23] were calculated as indicated in Table 1 except that $EC_{30}$ values were substituted for $EC_{40}$ values, and are presented as means ± SEM for the number of experiments indicated in parentheses.

† P was calculated using Student's *t*-test for comparison of the combination index with 1.0. Degrees of freedom = n − 1.

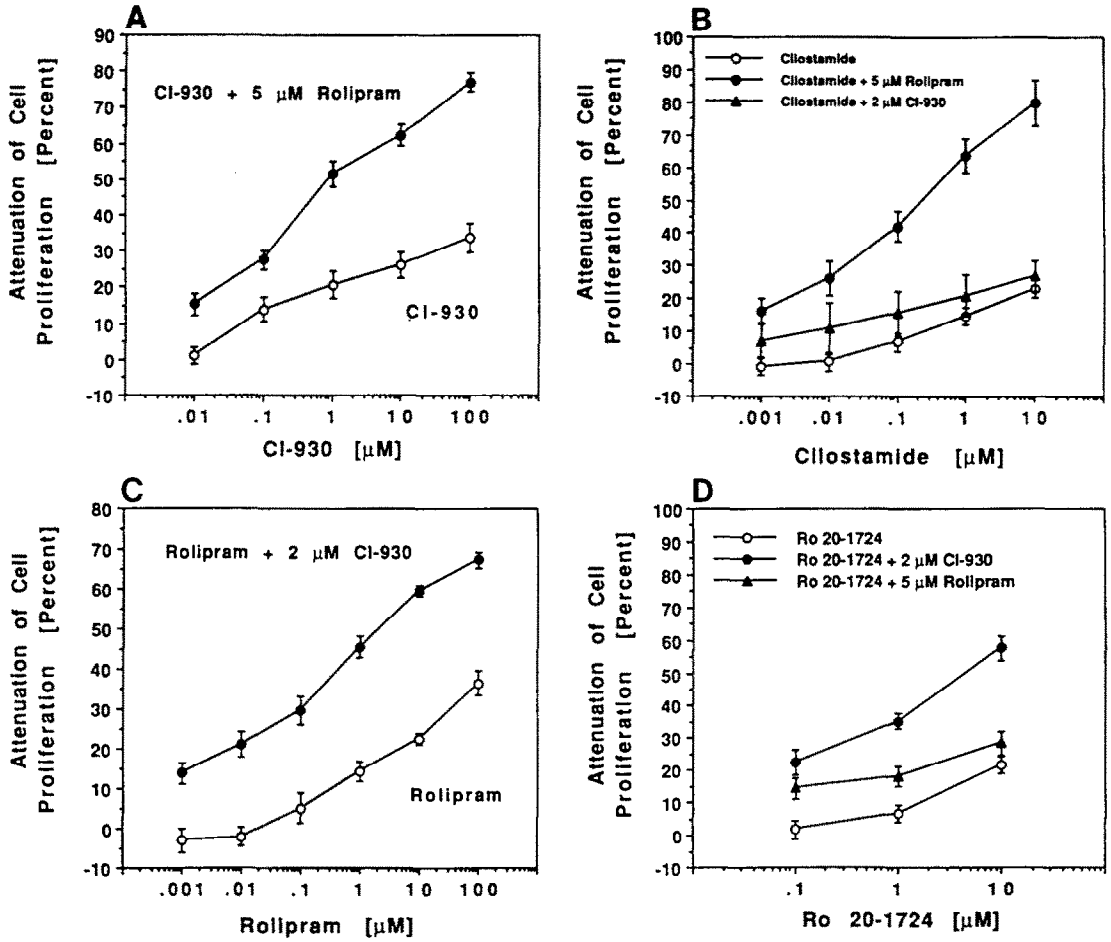


Fig. 6. Attenuation of VSMC proliferation by selective PDE inhibitors. Experimental protocols were the same as for Fig. 5. Means ± SEM are shown. The number of experiments represented by each curve ranged from 8 to 19.

synergism (Table 2). Because Ro 20-1724 alone did not cause 30% attenuation at the highest concentration tested (10 $\mu$M), the combination index was estimated using $EC_{30} > 10$. This index was <1.0 suggesting synergism (Table 2). It appears that 10 $\mu$M CI-930 would have shifted the $EC_{30}$ for Ro 20-1724 > 26-fold from > 10 to 0.38 $\mu$M. When 5 $\mu$M rolipram was substituted for 2 $\mu$M CI-930, the combination did not produce 30% inhibition at the highest concentration of Ro 20-1724 tested (Fig. 6D), so the combination index based on $EC_{30}$ values could not be calculated.

## DISCUSSION

The A10 VSMC used in this investigation contained both PDE III and PDE IV activities (Figs. 1 and 2). Schoeffter *et al.* [24] had shown previously that rat aortic myocytes in primary culture contain PDE I, and Souness *et al.* [7] have demonstrated the presence of PDE I, PDE III, PDE IV and PDE V in cultured VSMC from porcine aortic explants.

The nonselective PDE inhibitors IBMX and papaverine attenuated DNA synthesis with $EC_{50}$ values in the same range as their reported $IC_{50}$ values as PDE inhibitors (Table 3). By contrast, individual selective inhibitors of PDE III or PDE IV only weakly attenuated DNA synthesis with $EC_{50}$ values considerably higher than their $IC_{50}$ values as inhibitors of their target isozymes. However, by adding an agent that blocked PDE IV (10 $\mu$M rolipram), 50% attenuation of DNA synthesis was produced by concentrations of PDE III inhibitors much closer to their respective $IC_{50}$ values. Similarly, by adding an agent that blocked PDE III (10 $\mu$M CI-930), 50% attenuation of DNA synthesis was produced by concentrations of PDE IV inhibitors much closer to their respective $IC_{50}$ values. No agents were added to stimulate cAMP synthesis in our experiments, but the synergistic interactions appeared similar to those reported by Souness *et al.* [7] using forskolin as an agonist.

Although [$^3$H]thymidine incorporation into DNA is often used as an indicator of cell division, it does not always correlate with the number of cells that

Table 3. Comparison of $EC_{50}$ values for attenuation of DNA synthesis and VSMC proliferation with published $IC_{50}$ values for inhibition of PDE

| | $EC_{50}$ for effects on VSMC ($\mu$M) | | | | |
|---|---|---|---|---|---|
| | Alone | | In combination | | |
| Inhibitor | DNA synthesis | Cell proliferation | DNA synthesis | Cell proliferation | $IC_{50}$ for PDE inhibition ($\mu$M) |
| IBMX | 16 | 27 | | | 2–50 [10] |
| Papaverine | 18 | 58 | | | 2–25 [10] |
| CI-930 | >300 | >100 | 0.66* | 0.86† | 0.15–0.46 (PDE III) [25, 26] |
| Cilostamide | 5.3 | >10 | 0.16* | 0.23† | 0.005–0.064 (PDE III) [10, 27] |
| Rolipram | 111 | >100 | 0.56‡ | 2.1§ | 0.6–2.6 (PDE IV) [10, 25, 28] |
| Ro 20–1724 | >100 | >10 | 1.5‡ | 4.6§ | 2–13 (PDE IV) [10, 25, 28] |

The $EC_{50}$ values for attenuation of DNA synthesis were derived from experiments illustrated in Figs. 3 and 4. The $EC_{50}$ values for attenuation of cell proliferation were derived from experiments illustrated in Figs. 5 and 6. The $IC_{50}$ values are from the references indicated.

* Combined with 10 $\mu$M rolipram.

† Combined with 5 $\mu$M rolipram.

‡ Combined with 10 $\mu$M CI-930.

§ Combined with 2 $\mu$M CI-930.

progress through the cell cycle [29]. Because a major objective of this study was to examine the effects of PDE inhibitors on cell division, rather than on DNA synthesis *per se*, experiments were carried out using the MTT method to assess numbers of cells. The data indicated that PDE III and PDE IV inhibitors interacted synergistically to attenuate FBS-induced increases in the numbers of VSMC in culture. Furthermore, when PDE IV was blocked by 5 $\mu$M rolipram, the $EC_{50}$ values for the PDE III inhibitors were shifted to the same range as the $IC_{50}$ values for inhibition of their target isozymes (Table 3). Similarly, when PDE III was blocked by 2 $\mu$M CI-930, the $EC_{50}$ values for PDE IV inhibitors were shifted to the same range as their $IC_{50}$ values. Comparable synergistic interactions were not observed when the combinations contained two nonselective PDE inhibitors (IBMX + papaverine), two PDE III inhibitors (cilostamide + CI-930) or two PDE IV inhibitors (Ro 20-1724 + rolipram). It should be noted that these studies were carried out under conditions of presumably non-stimulated or basal adenylyl cyclase activity. The data do not show whether the synergy would be unchanged, enhanced, or abrogated if adenylyl cyclase were stimulated, for example, by hormones or forskolin.

The cause of the synergistic interactions is not known. We hypothesize that it is caused by the mechanisms illustrated in Fig. 7. In this model, when PDE III is blocked, the hydrolysis of cAMP catalyzed by PDE IV is increased (panel B). Conversely, when PDE IV is blocked, the hydrolysis of cAMP catalyzed by PDE III is increased (panel C). The most effective rise in cAMP levels is caused by the simultaneous inhibition of both PDE III and PDE IV such as is produced by the combination of selective PDE III and PDE IV inhibitors (panel D). In addition to the effects on cell proliferation, this mechanism might explain similar synergisms that have been reported for PDE inhibitors as relaxants of smooth muscle [25, 30, 31], positive inotropic agents [32] and inhibitors of mediator release from basophils [33].

The mechanisms by which the rate of hydrolysis catalyzed by these isozymes is increased are not known. Conti and coworkers [34–36] have demonstrated that rising cAMP levels in Sertoli cells and transfected MA-10 Leydig tumor cells can increase PDE IV activity by increasing the transcription of mRNA coding for the enzyme. Also, recent evidence from several laboratories has demonstrated that rising cAMP levels in rat adipocytes [37–39], liver [40] and hepatocytes [41, 42] can cause activation of PDE III by phosphorylation. Therefore, it seems possible that elevation of cAMP caused by an inhibitor of PDE III induces the expression of PDE IV in VSMC, and that cAMP elevation caused by inhibition of PDE IV promotes activation of PDE III, thereby contributing to the synergism. In any case, if PDE III and PDE IV are unsaturated, which seems likely [43], the kinetic consequences of rising cAMP levels may be sufficient to increase cAMP hydrolysis by the non-inhibited isozyme even in the absence of $K_m$ or maximum velocity changes.

The model assumes that the synergistic interactions are produced by increases in intracellular cAMP levels. In their studies on pig aortic smooth muscle cells, Souness *et al.* [7] showed that, in the presence of 0.5, 5 or 10 $\mu$M forskolin, the combination of SK&F 94836 (PDE III inhibitor) and Ro 20-1724 (PDE IV inhibitor) causes a significantly greater rise in cAMP levels than either PDE inhibitor alone. Enhancement of forskolin-induced elevations of cAMP levels by individual PDE inhibitors diminished with time, but was still detected after 24 hr. Although these investigators did not find cAMP elevations unless forskolin was present, they did show that combined PDE III and PDE IV inhibitors greatly enhance cAMP-dependent protein kinase activity whether forskolin is present or not, suggesting that

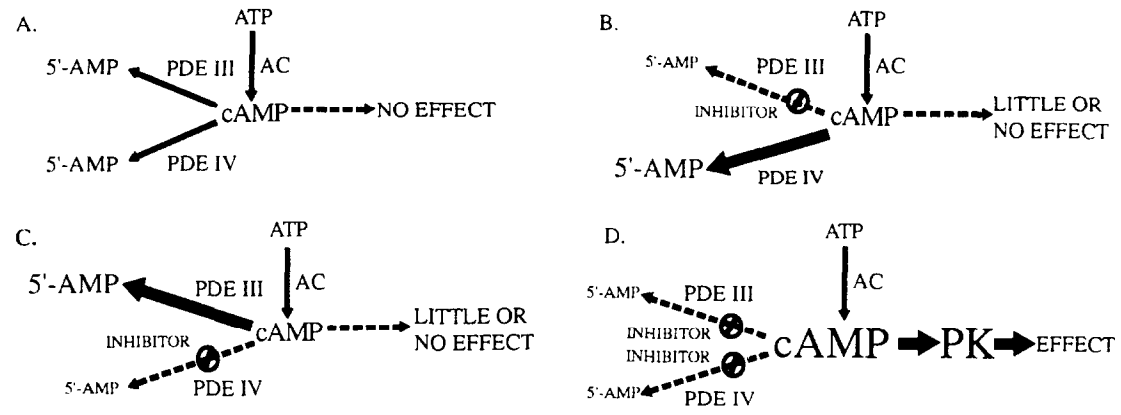


Fig. 7. A simple model showing how inhibitors of PDE III may interact with inhibitors of PDE IV to produce synergistic effects. Cyclic AMP is depicted as the product of adenylyl cyclase (AC), and it is shown to be hydrolyzed by two alternative pathways, one catalyzed by PDE III and the other by PDE IV (panel A). Inhibition of PDE III is compensated for by increased hydrolysis of cAMP through the pathway catalyzed by PDE IV, resulting in little or no elevation of cAMP, and little or no effect on cell proliferation (panel B). Inhibition of PDE IV is compensated for by increased hydrolysis of cAMP through the pathway catalyzed by PDE III, resulting in little or no elevation of cAMP, and little or no effect on proliferation (panel C). Concomitant inhibition of both PDE III and PDE IV markedly increases cAMP levels causing an attenuation of cell proliferation (panel D), probably by activation of cAMP-dependent protein kinase (PK). Possible changes in cAMP hydrolysis catalyzed by other PDE isozymes or changes in the extrusion of cAMP from cells are thought to be insignificant as causes of the synergistic interactions, and are not illustrated.

even in the absence of adenylyl cyclase stimulation, simultaneous inhibition of PDE III and PDE IV raises endogenous cAMP levels sufficiently to activate cAMP-dependent protein kinase.

*Acknowledgements*—The authors are grateful to Karl E. Muffly, Ph.D., for demonstrating the presence of smooth muscle $\alpha$-actin in the A10 cells used in this study. They also thank Paul E. Gottschall, Ph.D., and Marzenna Wiranowska, Ph.D., for their expert advice concerning the MTT assay. This work was supported by the American Heart Association Florida Affiliate.

## 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. Ip JH, Fuster V, Badimon L, Badimon J, Taubman MB and Chesebro JH, Syndromes of accelerated atherosclerosis: Role of vascular injury and smooth muscle cell proliferation. *J Am Coll Cardiol* **15**: 1667–1687, 1990.
3. 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.
4. 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.
5. 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 $E_1$. *Exp Cell Res* **160**: 117–125, 1985.
6. Nakaki T, Nakayama M, Yamamoto S and Kato R, $\alpha_1$-Adrenergic stimulation and $\beta_2$-adrenergic inhibition of DNA synthesis in vascular smooth muscle cells. *Mol Pharmacol* **37**: 30–36, 1990.
7. Souness JE, Hassall GA and Parrott DP, Inhibition of pig aortic smooth muscle cell DNA synthesis by selective type III and type IV cyclic AMP phosphodiesterase inhibitors. *Biochem Pharmacol* **44**: 857–866, 1992.
8. Uehara Y, Ishimitsu T, Kimura K, Ishii M, Ikeda T and Sugimoto T, Regulatory effects of eicosanoids on thymidine uptake by vascular smooth muscle cells of rats. *Prostaglandins* **36**: 847–857, 1988.
9. Chamley JH and Campbell GR, Trophic influences of sympathetic nerves and cyclic AMP on differentiation and proliferation of isolated smooth muscle cells in culture. *Cell Tissue Res* **161**: 497–510, 1975.
10. Beavo JA and Reifsnyder DH, Primary sequence of cyclic nucleotide phosphodiesterase isozymes and the design of selective inhibitors. *Trends Pharmacol Sci* **11**: 150–155, 1990.
11. Thompson WJ, Cyclic nucleotide phosphodiesterases: Pharmacology, biochemistry and function. *Pharmacol Ther* **51**: 13–33, 1991.
12. Hall IP, Isoenzyme selective phosphodiesterase inhibitors: Potential clinical uses. *Br J Clin Pharmacol* **35**: 1–7, 1993.
13. Bentley JK and Beavo JA, Regulation and function of cyclic nucleotides. *Curr Opin Cell Biol* **4**: 233–240, 1992.
14. Torphy TJ and Undem BJ, Phosphodiesterase inhibitors: New opportunities for the treatment of asthma. *Thorax* **46**: 512–523, 1991.
15. Robicsek SA, Blanchard DK, Djeu JY, Krzanowski JJ, Szentivanyi A and Polson JB, Multiple high-affinity cAMP-phosphodiesterases in human T-lymphocytes. *Biochem Pharmacol* **42**: 869–877, 1991.
16. Cascieri MA, Chicchi CG, Hayes NS and Slater EE,

Stimulation of DNA synthesis in rat A10 vascular smooth muscle cells by threonine-59 insulin-like growth factor I. *Circ Res* **59**: 171–177, 1986.
17. Kimes BW and Brandt BL, Characterization of two putative smooth muscle cell lines from rat thoracic aorta. *Exp Cell Res* **98**: 349–366, 1976.
18. Pan X, Sauro MD, Coffey RG, Fitzpatrick DF, Krzanowski JJ and Polson JB, Effect of selective inhibitors of isozymes of cyclic nucleotide phosphodiesterase on proliferation of A10 cells. *Pharmacologist* **33**: 189, 1991.
19. Pan X, Arauz E, Krzanowski JJ and Polson JB, Synergistic interaction of selective pharmacological inhibitors of the phosphodiesterase isozymes PDE III and PDE IV to attenuate proliferation of A10 vascular smooth muscle cells. *Pharmacologist* **35**: 170, 1993.
20. Thompson WJ and Appleman MM, Multiple cyclic nucleotide phosphodiesterase activities from rat brain. *Biochemistry* **10**: 311–316, 1971.
21. Sauro MD and Zorn NE, Prolactin induces proliferation of vascular smooth muscle cells through a protein kinase C-dependent mechanism. *J Cell Physiol* **148**: 133–138, 1991.
22. Green LM, Reade JL and Ware CF, Rapid colorimetric assay for cell viability: Application to the quantitation of cytotoxic and growth inhibitory lymphokines. *J Immunol Methods* **70**: 257–268, 1984.
23. Berenbaum MC, What is synergy? *Pharmacol Rev* **41**: 93–141, 1989.
24. Scoeffter P, Lugnier C, Travo C and Stoclet J-C, A comparison of cyclic AMP signaling system in rat aortic myocytes in primary culture and aorta. *Lab Invest* **61**: 177–182, 1989.
25. Harris AL, Connell MJ, Ferguson EW, Wallace AM, Gordon RJ, Pagani ED and Silver PJ, Role of low $K_m$ cyclic AMP phosphodiesterase inhibition in tracheal relaxation and bronchodilation in guinea pig. *J Pharmacol Exp Ther* **251**: 199–206, 1989.
26. Silver PJ, Hames LT, Perrone MH, Bentley RG, Bushover CR and Evans DB, Differential pharmacologic sensitivity of cyclic nucleotide phosphodiesterase isozymes isolated from cardiac muscle, arterial and airway smooth muscle. *Eur J Pharmacol* **150**: 85–94, 1988.
27. Elks ML and Manganiello VC, Selective effects of phosphodiesterase inhibitors on different phosphodiesterases, adenosine 3′,5′-monophosphate metabolism, and lipolysis in 3T3-L1 adipocytes. *Endocrinology* **115**: 1262–1268, 1984.
28. Torphy TJ and Cieslinski LB, Characterization and selective inhibition of cyclic nucleotide phosphodiesterase isozymes in canine tracheal smooth muscle. *Mol Pharmacol* **37**: 206–214, 1990.
29. Murat JC, 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.
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. Rabe KF, Tenor H, Dent G, Schundt C, Liebig S and Magnussen H, Phosphodiesterase isozymes modulating inherent tone in human airways: Identification and characterization. *Am J Physiol* **264**: L458–L464, 1993.
32. 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.
33. Peachell PT, Undem BJ, Schleimer RP, MacGlashan DW Jr, Lichtenstein LM, Cieslinski LB and Torphy TJ, Preliminary identification and role of phosphodiesterase isozymes in human basophils. *J Immunol* **148**: 2503–2510, 1992.
34. Swinnen JV, Joseph DR and Conti M, The mRNA encoding a high-affinity cAMP phosphodiesterase is regulated by hormones and cAMP. *Proc Natl Acad Sci USA* **86**: 8197–8201, 1989.
35. Swinnen JV, D'Souza B, Conti M and Ascoli M, Attenuation of cAMP-mediated responses in MA-10 Leydig tumor cells by genetic manipulation of a cAMP-phosphodiesterase. *J Biol Chem* **266**: 14383–14389, 1991.
36. Swinnen JV, Tsikalas KE and Conti M, Properties and hormonal regulation of two structurally related cAMP phosphodiesterases from the rat Sertoli cell. *J Biol Chem* **266**: 18370–18377, 1991.
37. Smith CJ and Manganiello VC, Role of hormone-sensitive low $K_m$ cAMP phosphodiesterase in regulation of cAMP-dependent protein kinase and lipolysis in rat adipocytes. *Mol Pharmacol* **35**: 381–386, 1989.
38. Degerman E, Smith CJ, Tornqvist H, Vasta V, Belfrage P and Manganiello V, Evidence that insulin and isoprenaline activate the cGMP-inhibited low-$K_m$cAMP phosphodiesterase in rat fat cells by phosphorylation. *Proc Natl Acad Sci USA* **87**: 533–537, 1990.
39. Gettys TW, Vine AJ, Simonds MF and Corbin JD, Activation of the particulate low $K_m$ phosphodiesterase of adipocytes by addition of cAMP-dependent protein kinase. *J Biol Chem* **263**: 10359–10363, 1988.
40. Shibata H and Kono T, Cell-free stimulation of the insulin-sensitive cAMP phosphodiesterase by the joint actions of ATP and the soluble fraction from insulin-treated rat liver. *Biochem Biophys Res Commun* **170**: 533–539, 1990.
41. Kilgour E, Anderson NG and Houslay MD, Activation and phosphorylation of the "dense-vesicle" high affinity cyclic AMP phosphodiesterase by cyclic AMP-dependent protein kinase. *Biochem J* **260**: 27–36, 1989.
42. Houslay MD, Griffiths SL, Horton YM, Livingstone C, Lobban M, Macdonald F, Morris N, Pryde J, Scotland G, Shakur Y, Sweeney G and Tang EKY, Regulation of intracellular cyclic AMP concentrations in hepatocytes involves the integrated activation and desensitization of adenylyl cylase coupled with the action and activation of specific isoforms of cyclic AMP phosphodiesterase. *Biochem Soc Trans* **20**: 140–146, 1992.
43. Barber R, Goka TJ and Butcher RW, Cyclic AMP turnover in intact tissue. *Adv Second Messenger Phosphoprotein Res* **25**: 1–11, 1992.