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Effect of CI-930 [3-(2*H*)-Pyridazinone-4,5-dihydro-6-[4-(1*H*-imidazolyl) phenyl]-5-methyl-monohydrochloride] and Rolipram on Human Coronary Artery Smooth Muscle Cell Proliferation

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ABSTRACT. Experiments were conducted to determine how selective inhibitors of certain cyclic nucleotide phosphodiesterase (PDE) families, namely CI-930 (PDE3 inhibitor; 3-(2*H*)-pyridazinone-4,5-dihydro-6-[4-(1*H*-imidazolyl) phenyl]-5-methyl monohydro chloride) and rolipram (PDE4 inhibitor), may affect human coronary artery smooth muscle cell (HCASMC) proliferation. CI-930- and rolipram-inhibitable PDEs accounted for most of the cyclic AMP hydrolyzing activity in HCASMC. Twenty micromolar CI-930 and 20 μM rolipram used individually attenuated proliferation of HCASMC from some, but not all donors, as measured by flow cytometry. The simultaneous addition of 10 μM CI-930 plus 10 μM rolipram caused greater attenuation. This attenuation represented a reduction of the number of cells entering the S phase of the cell cycle and not merely a delay in cell cycle traverse. No statistically significant elevation of cyclic AMP was detected following the addition of either PDE inhibitor individually, but the combination produced significant elevations. It is concluded that CI-930- and rolipram-inhibitable PDE isozymes are expressed in HCASMC and that selective inhibitors of these isozymes can attenuate HCASMC proliferation. The data suggest that selective PDE inhibitors may prevent restenosis in patients following percutaneous transluminal coronary angioplasty because of their effect on HCASMC proliferation, and they may also be useful in retarding the progression of atherosclerosis in individuals at risk. PDE3 and PDE4 inhibitors in combination are more effective than the inhibitors used individually. BIOCHEM PHARMACOL **56**;8:1065–1073, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. cyclic AMP; 3′,5′-cyclic-nucleotide phosphodiesterase; human coronary artery smooth muscle cells; phosphodiesterase inhibitors; vascular smooth muscle cells

VSMC† proliferation is a major factor contributing to the pathogenesis of atherosclerosis and to restenosis following coronary angioplasty [1–3]. Hence, pharmacological attenuation of coronary artery VSMC proliferation provides a rational approach to preventing and/or treating these conditions. cAMP is known to attenuate the proliferation of a variety of cell types [4, 5], including smooth muscle cells [6–11]. PDEs hydrolyze cAMP in cells [12], and over 30 isoforms of PDE have been identified and classified as seven families [13], based on their sequence homology, biochemical characteristics, and sensitivity to modulators. The diversity among PDEs raised the question as to whether selective inhibition of one or more of these isoforms can attenuate VSMC proliferation. Previously published reports showed that selective inhibitors of PDE3 and PDE4 can attenuate or delay proliferation of aortic smooth muscle cells from rats [14] and pigs [15]. Kunishima *et al.* [16] reported recently that the PDE3 inhibitor cilostazol reduced the incidence of restenosis in patients after successful coronary stent implantation. However, at the present time there are no reports showing direct evidence that PDE3 or PDE4 inhibitors can affect the proliferation of HCASMC. Such information is crucial because models based on animal cells may not accurately reflect the responses of HCASMC to drugs. The present study was designed to determine whether the PDE3 inhibitor CI-930 [3-(2*H*)-pyridazinone-4,5-dihydro-6-[4-(1*H*-imidazolyl) phenyl]-5-methyl monohydrochloride] or the PDE4 inhibitor rolipram used alone or in combination can affect HCASMC proliferation. Some of the data from this study have been reported previously in abstract form [17, 18].

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† *Abbreviations:* cAMP (cyclic AMP), adenosine 3′,5′-cyclic monophosphate; HCASMC, human coronary artery smooth muscle cell(s); hEGF, human recombinant epidermal growth factor; hFGF, human recombinant fibroblast growth factor; PDE, 3′,5′-cyclic nucleotide phosphodiesterase; rolipram, 4-[3-(cyclopentyloxy)-4-methoxyphenyl]-2-pyrrolidinone; SMBM, smooth muscle basal medium; SMGM2, smooth muscle growth medium-2; and VSMC, vascular smooth muscle cell(s).

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MATERIALS AND METHODS

Materials

CI-930 was provided by Parke Davis, Pharmaceutical Research Division of the Warner-Lambert Co., and rolipram was provided by Berlex Laboratories, Inc. FBS was purchased from HyClone Laboratories, Inc.; Dulbecco's PBS was purchased from Life Technologies; SMBM was purchased from the Clonetics Co.; [^{3}H]cAMP and ^{125}I were obtained from DuPont/NEN; neutral alumina (Art. 1077) was from Merck; and trypsin/EDTA, propidium iodide, ribonuclease A, and other reagents were purchased from the Sigma Chemical Co. Polyclonal antibody against cAMP was provided by Akira Arimura, M.D., Ph.D. (Department of Medicine, Tulane University School of Medicine).

Cell Culture

Cryopreserved primary cultures of HCASMC from deceased donors were obtained from the Clonetics Co. The cellular purity of the preparations was 100% based on smooth muscle α-actin content and cell morphology. The cells were cultured in 75 cm^2 flasks in SMGM2 medium (SMBM medium supplemented with 5% FBS, 10 ng/mL of hEGF, 2 ng/mL of hFGF, 50 μg/mL of gentamicin, 50 ng/mL of amphotericin-B, and 5 μg/mL of insulin). Cells were incubated at 37° in a humidified atmosphere of 95% air and 5% CO_2. For experiments, cells were used in the fifth passage.

Proliferation of HCASMC

At 60–70% confluence, growth of HCASMC was arrested by changing the medium to SMBM medium without serum or growth factors for 48 hr. Cell proliferation was then stimulated by the addition of SMGM2 or SMBM supplemented with 10% FBS and 2 ng/mL of hFGF, with or without addition of PDE inhibitors. After the cells were incubated for predetermined periods of time, they were detached using trypsin/EDTA, centrifuged for 10 min at 150 *g*, resuspended in cold PBS containing glucose, and recentrifuged for 10 min at 4° to pellet the cells. Cell viability was determined by trypan blue exclusion methodology. Cell counts were determined using a hemacytometer or a spectrophotometric method [19], and the cells were fixed in sufficient volumes of cold 70% ethanol to provide 1×10^6 cells/mL and stored overnight for cell cycle analysis.

Cell Cycle Analysis

To determine the percentage of cells in the various phases of the cell cycle, DNA content was analyzed by flow cytometry. Ethanol-fixed cells were centrifuged at 240 *g* for 5 min at 4°. Ethanol was removed, and the cells were resuspended in 1 mL PBS containing 50 μg/mL of propidium iodide and 100 U/mL of RNAse A. After incubation in the dark for 1 hr at room temperature, the cells were analyzed using a Facscan cytofluorometer (Becton–Dickinson) using an excitation wavelength of 488 nm and a detection wavelength of 585 nm. The resulting DNA histograms were analyzed using ModFit, a Microsoft Windows application that determines the percentage of cells in the various phases of the cell cycle ($G_0 + G_1$, S, $G_2 + M$) based on fluorescence.

Cyclic Nucleotide Phosphodiesterase Assay

Confluent HCASMC were detached from 75 cm^2 flasks using a cell scraper, and suspended in 50 mL of SMBM medium. Cells were counted, centrifuged (100 *g*, 7 min, 4°), resuspended in 2 mL of homogenization buffer (50 mM Tris–HCl, pH 8.0, 0.05% BSA, 10 mM $MgCl_2$, 10 μM $CaCl_2$) containing 20 μg/mL of leupeptin and 100 kallikrein U/mL of aprotinin, and then sonicated. The sonicates were centrifuged at 23,600 *g* for 30 min at 4°, and the supernatant fluids were collected and stored frozen until assayed. The pellets were washed once and resuspended in 1 mL vol. of homogenization buffer and stored frozen.

PDE activity was measured using the method of Thompson and Appleman [20], as modified by Robicsek *et al.* [21]. Briefly, dilutions of soluble and particulate extracts were incubated for 10 min at 37° with 0.2 μM [^{3}H]cAMP (40,000 cpm/assay) in 20 μL vol. of assay buffer containing 3.73 μM β-mercaptoethanol and 0.02 U/μL of alkaline phosphatase, with or without PDE inhibitors. Reactions were started by the addition of cell extract and stopped 10 min later by the addition of 0.5 mL vol. of a 1:2 (v:v) slurry of Bio-Rad resin (AG 1-X8) in a 1:1 mixture of isopropanol and water. The mixture was then centrifuged at 550 *g* for 20 min, and the radiolabeled product in the supernatant fractions was counted using liquid scintillation spectrometry.

cAMP Levels

cAMP was assayed according to the procedure described by Coffey *et al.* [22]. Briefly, HCASMC were incubated in the presence or absence of PDE inhibitors in 6-well plates. At the end of the predetermined incubation times, perchloric acid was added to each well to provide a final concentration of 0.5 M, and the plates were centrifuged at 1900 *g* for 1 hr. The supernatant fractions were purified using 0.22 g neutral alumina columns in Pasteur pipets. The columns were washed with 0.5 M $HClO_4$ followed by water, and then eluted with 1 mL vol. of 0.2 M sodium acetate (pH 6.2).

Column eluates were assayed for cAMP content using radioimmunoassay [22]. Briefly, 20- or 50-μL aliquots of column eluates were diluted with 50 mM sodium acetate (pH 6.2) to provide final volumes of 100 μL. To these samples were added 10 μL vol. of a 1:2 (v:v) mixture of acetic anhydride plus triethylamine with immediate mixing. [^{125}I]Succinyl cAMP tyrosine methyl ester and antibody were added to each reaction tube, and the mixtures were stored overnight at 4°. The next day, ethanol (200

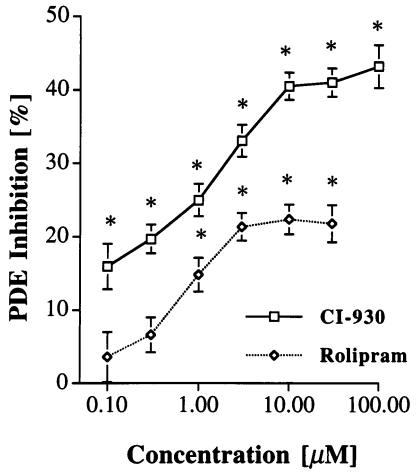

FIG. 1. PDE inhibition by CI-930 (PDE3 inhibitor) and rolipram (PDE4 inhibitor). Soluble HCASMC PDE activity from a 32-year-old male donor was assayed as described in Materials and Methods. Data represent means ± SEM calculated from eight reactions run in the presence of each concentration of inhibitor using combined extracts of HCASMC from 5 to 6 culture flasks. Activity in the absence of inhibitors was 24.5 ± 1.3 pmol/min/million cells. *Significant inhibition ($P < 0.05$).

proof) was added to each reaction tube to provide a final concentration of 75–85% ethanol. The tubes were then centrifuged at 1900 *g* for 30 min. The supernatant fluids were aspirated, and the radioactivity in each pellet was counted using a Beckman Gamma Counter 5500. cAMP levels were determined by comparison to a standard curve that was constructed using 10, 30, 100, 300, 1000, and 3000 fmol of cAMP.

Statistical Analysis

Data were analyzed using one-way ANOVA followed by the Student–Newman–Keuls test. $P < 0.05$ was considered statistically significant.

RESULTS

PDE Inhibition by CI-930 and Rolipram

Inhibition of soluble HCASMC PDE activity by various concentrations of the PDE3 inhibitor CI-930 and the PDE4 inhibitor rolipram was measured in order to find concentrations of these selective inhibitors that could be used to assay the total CI-930-inhibitable and rolipram-inhibitable PDE activities in cell extracts. The results, presented in Fig. 1, show plateaus of maximum inhibition by 10–100 μM CI-930 and 3–30 μM rolipram. It was considered that these plateaus corresponded to the concentrations at which selective inhibition of the respective isozymes was maximal. Ten micromolar concentrations of the selective PDE inhibitors were chosen for subsequent assay of the CI-930- and rolipram-inhibitable PDEs in cells from other donors.

CI-930- and Rolipram-inhibitable PDE Activities in HCASMC

CI-930- and rolipram-inhibitable PDE activities in soluble and particulate extracts of quiescent (non-proliferating) and proliferating HCASMC were determined by assaying the amount of PDE activity that was inhibitable by 10 μM CI-930 (PDE3 inhibitor) and 10 μM rolipram (PDE4 inhibitor), respectively. This approach is similar to the method used by Rabe *et al.* [23] to determine PDE3 and PDE4 activities in the human pulmonary artery.

cAMP hydrolyzing activity is shown in Table 1 for quiescent HCASMC from 48-year-old and 58-year-old deceased male donors. CI-930-inhibitable PDE was found in both the soluble and particulate fractions, but the soluble activity was the greater of the two. Substantial rolipram-inhibitable activity was found in the soluble fractions, but the particulate rolipram-inhibitable activity was barely detectable. Total CI-930-inhibitable activity (soluble plus particulate) was 2.2- and 1.8-fold greater than total rolipram-inhibitable activity in HCASMC from the 48-year-old and 58-year-old donors, respectively. Furthermore, the sum

TABLE 1. cAMP-hydrolyzing activity in quiescent (non-proliferating) HCASMC from 48-year-old and 58-year-old male deceased donors

Fraction	Total PDE activity		CI-930-inhibitable PDE		Rolipram-inhibitable PDE	
	pmol/min/10^6 cells	% of *a*	pmol/min/10^6 cells	% of *a*	pmol/min/10^6 cells	% of *a*
48-Year-old donor						
Soluble	10.6 ± 0.1	79	4.3 ± 0.10	32	2.3 ± 0.10*	17
Particulate	2.8 ± 0.1	21	1.4 ± 0.04	10	0.3 ± 0.03*	2
Sol. + Part.	13.4 = *a*	100	5.7	42	2.6	19
	pmol/min/10^6 cells	% of *b*	pmol/min/10^6 cells	% of *b*	pmol/min/10^6 cells	% of *b*
58-Year-old donor						
Soluble	15.6 ± 0.4	85	6.9 ± 0.30	38	4.3 ± 0.20*	23
Particulate	2.7 ± 0.2	15	1.1 ± 0.03	6	0.3 ± 0.06*	2
Sol. + Part.	18.3 = *b*	100	8.0	44	4.6	25

Each data point represents the mean ± SEM calculated from 8 reactions run on extracts of HCASMC from 5 to 6 culture flasks.
*Significantly different from CI-930-inhibitable PDE activity ($P < 0.05$).

TABLE 2. cAMP-hydrolyzing activity in randomly proliferating HCASMC from 32-year-old and 58-year-old male deceased donors

Fraction	Total PDE activity		CI-930-inhibitable PDE		Rolipram-inhibitable PDE	
	pmol/min/ 10^6 cells	% of *a*	pmol/min/ 10^6 cells	% of *a*	pmol/min/ 10^6 cells	% of *a*
32-Year-old donor						
Soluble	23.8 ± 0.7	76	8.9 ± 0.61	28	5.1 ± 0.57*	16
Particulate	7.6 ± 0.37	24	5.4 ± 0.22	17	0.5 ± 0.34*	2
Sol. + Part.	31.4 = *a*	100	14.3	45	5.6	18
	pmol/min/ 10^6 cells	% of *b*	pmol/min/ 10^6 cells	% of *b*	pmol/min/ 10^6 cells	% of *b*
58-Year-old donor						
Soluble	16.5 ± 0.29	81	6.7 ± 0.32	33	5.5 ± 0.24*	27
Particulate	3.8 ± 0.08	19	2.3 ± 0.05	11	0.7 ± 0.06*	3
Sol. + Part.	20.3 = *b*	100	9.0	44	6.2	30

Each data point represents the mean ± SEM calculated from 8 reactions run on extracts of HCASMC from 5 to 6 culture flasks.
*Significantly different from CI-930-inhibitable PDE activity ($P < 0.05$).

of the CI-930-inhibitable PDE plus the rolipram-inhibitable PDE activities accounted for 61 and 69% of the total PDE in cells from the 48-year-old and 58-year-old donors, respectively.

cAMP hydrolyzing activity was determined for randomly proliferating HCASMC (in SMGM2) obtained from 32-year-old and 58-year-old deceased male donors (Table 2). CI-930-inhibitable PDE and rolipram-inhibitable PDE were found in both the soluble and particulate fractions with most of the activity in the soluble fractions. Total CI-930-inhibitable activity was 2.5-fold and 1.5-fold greater than total rolipram-inhibitable activity in HCASMC from the 32-year-old and 58-year-old donors, respectively. Also, the sum of CI-930-inhibitable plus rolipram-inhibitable activities accounted for 63 and 74% of the total PDE activity in cells from the 32-year-old and the 58-year-old donors, respectively.

Effects of CI-930 and Rolipram on Proliferation

The PDE inhibitors were added to HCASMC growing in cell culture to determine effects on proliferation. For this purpose, the growth of HCASMC was arrested by placing them in serum-free/growth factor-free medium for 48 hr. Then HCASMC were stimulated to proliferate synchronously by addition of 5% FBS and growth factors with and without PDE inhibitors, and the cells were harvested at the time of maximum appearance of cells in the S phase of the cell cycle based on preliminary flow cytometry studies (data not shown). This time varied among HCASMC from different donors, but was in the range of 24–30 hr after the addition of FBS and growth factors for all cells. Figure 2 shows typical flow cytometry fluorescence histograms for HCASMC from a 48-year-old male donor. Twenty micromolar concentrations of either inhibitor used alone reduced the percentage of cells appearing in the S phase at the time point (27 hr) selected for this donor. The combination of 10 μM CI-930 plus 10 μM rolipram produced an even greater reduction.

Figure 3 shows data from flow cytometry analyses of multiple flasks of cultured cells obtained from four donors. Twenty micromolar CI-930 appeared to slightly reduce (11–18%) the number of HCASMC in the S phase in cultures from all three of the male donors studied (Fig. 3, A–C), but the reduction was statistically significant ($P < 0.05$) for only one of the three donors (Fig. 3C). HCASMC from a 61-year-old female donor were more sensitive to the effects of CI-930, and 10 μM concentrations produced a 33% ($P < 0.05$) reduction (Fig. 3D). Twenty micromolar rolipram significantly ($P < 0.05$) reduced (26–28%) HCASMC in the S phase in cultures from two (Fig. 3, A and B) of the three (Fig. 3, A–C) male donors. In HCASMC from the 61-year-old female donor, 10 μM rolipram produced a 24% ($P < 0.05$) reduction (Fig. 3D).

Ten micromolar CI-930 plus 10 μM rolipram produced a significantly greater effect ($P < 0.05$) than 20 μM concentrations of either PDE inhibitor used alone (Fig. 3, A–C), suggesting that the two inhibitors interacted synergistically.

The question was raised as to whether the reduced number of HCASMC appearing in the S phase at selected times after stimulation of proliferation was due to attenuation of the number of cells entering the S phase or due merely to a delay in the progression of cells through the cell cycle. To answer this question, HCASMC from the three male donors were examined at various times after the peak appearance of cells in the S phase. The results, shown in Fig. 4, indicate that the entry of cells into the S phase was not merely delayed, but that the number of cells entering the S phase was actually decreased.

If the effects on proliferation were due to inhibition of PDE, then it would be expected that cAMP levels would be increased. To examine this possibility, cAMP levels were measured in the presence and absence of the PDE inhibitors. In preliminary studies, we found that the activities of PDE3 and PDE4 isozymes increased during the G_1 phase of the cell cycle and peaked at slightly different times for each donor (data not shown). We chose to harvest the cells for cAMP assay at times that corresponded to the time of the

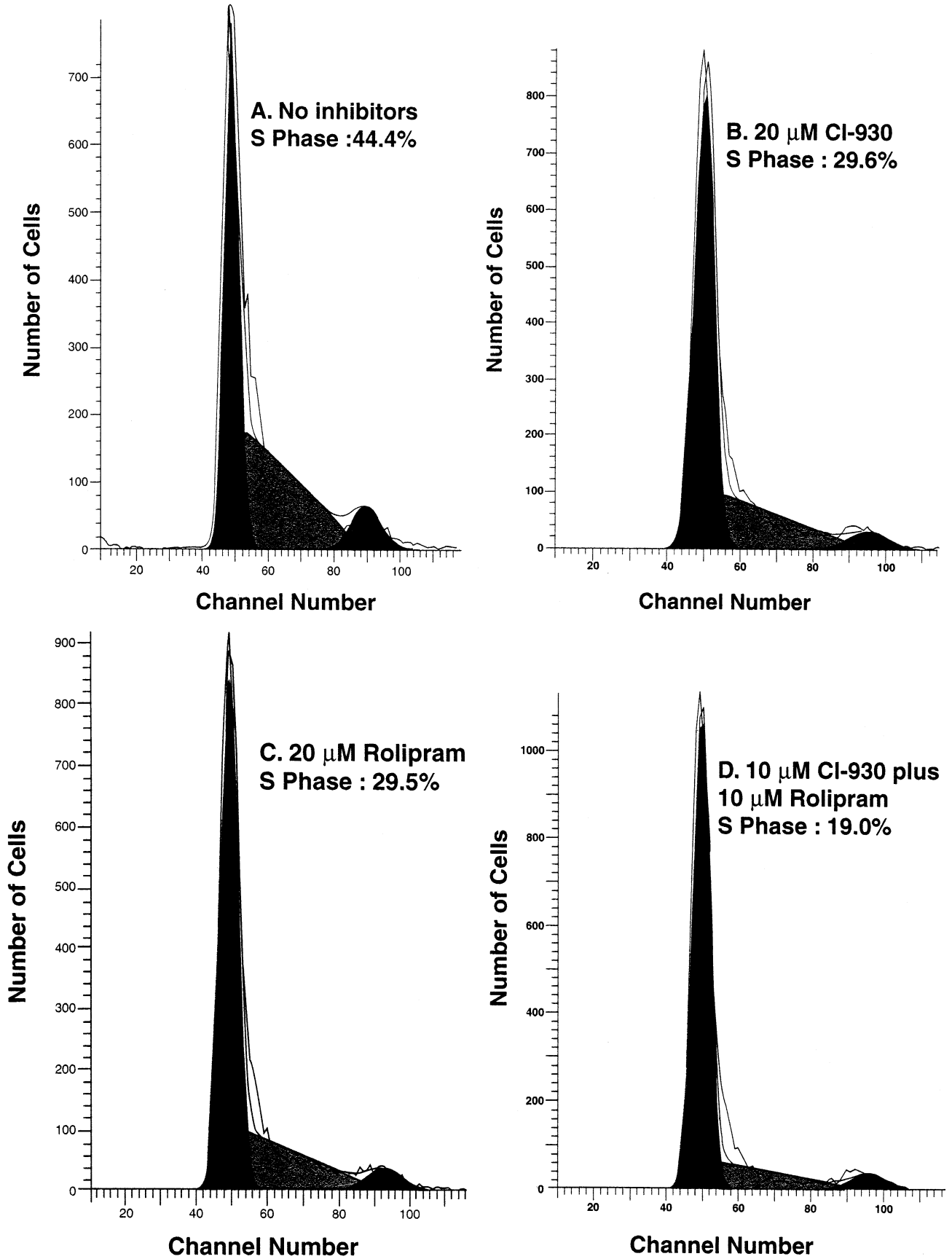

FIG. 2. Effects of CI-930 and rolipram on flow cytometry fluorescence histograms. The solid black peak on the left of each histogram represents HCASMC in G_0 and G_1 phases. The smaller black peak on the right represents HCASMC in G_2 and M phases. The area under the curve between the solid black peaks represents the HCASMC in the S phase.

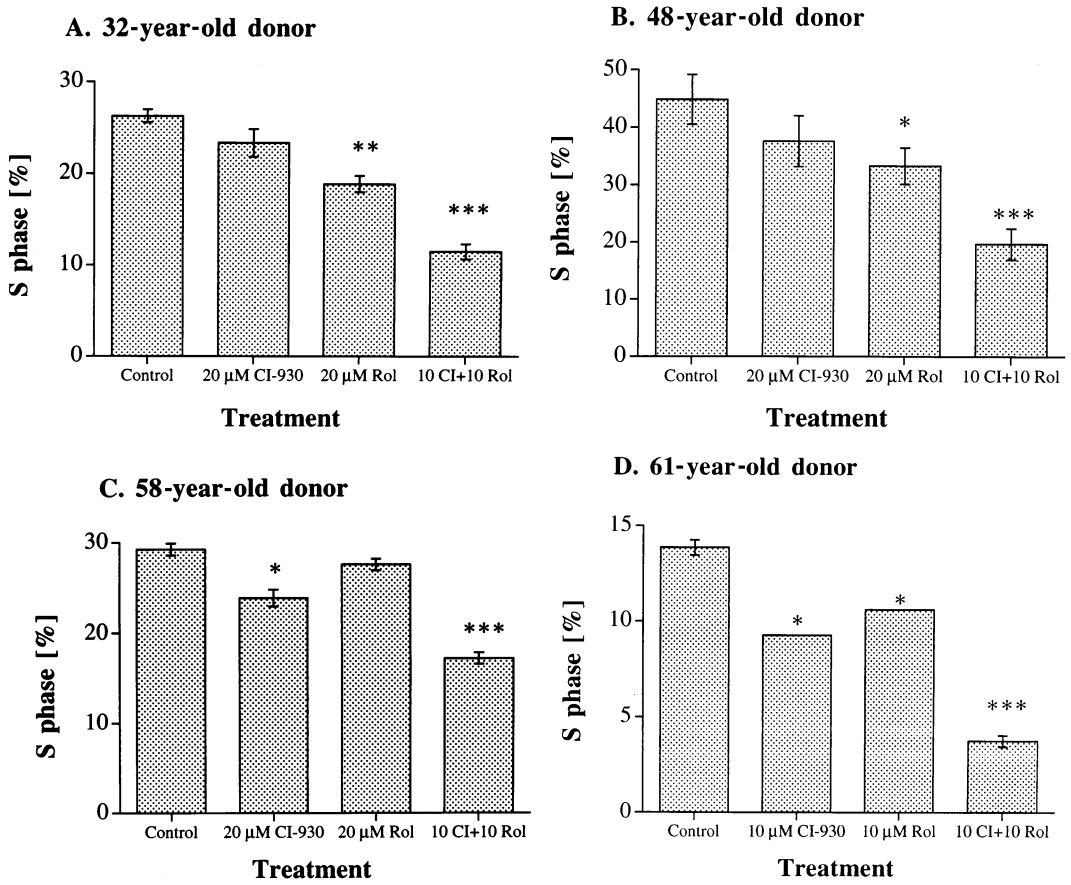

FIG. 3. Effects of CI-930 and rolipram on the proliferation of HCASMC from four donors. Proliferation is indicated by the percentage of cells in S phase as determined by flow cytometry. Each data point represents the mean ± SEM of flow cytometry determinations run on HCASMC from each of 5–6 (A and B), 9 (C), or 2 (D, CI-930 and rolipram used individually) or 3 (D, control and drug combination) individual culture flasks. Key: (*) significantly different from control ($P < 0.05$); (**) significantly different from control and CI-930 alone ($P < 0.05$); and (***) significantly different from control, CI-930 alone, and rolipram alone ($P < 0.05$).

peak rise of PDE activity for each donor. These times were 25 hr for the 32-year-old donor, 20 hr for the 48-year-old donor, and 15 hr for the 58-year-old donor. The cAMP levels are shown in Fig. 5. In the absence of PDE inhibitors, cAMP levels ranged from 1.5 to 3.7 pmol/million cells. Although cAMP levels in the presence of 20 μM CI-930 and 20 μM rolipram used individually were higher than in control cells for two (Fig. 5, B and C) of the three donors studied, these elevations did not attain statistical significance. However, the combination of 10 μM CI-930 plus 10 μM rolipram produced statistically significant cAMP elevations in cells from all three of the donors studied ($P < 0.05$). The variation in cAMP elevations produced by the PDE inhibitors was large among donors and may have been due to individual variations or to the fact that the cAMP levels were measured at different times corresponding to the times of peak PDE3 and PDE4 activities for each donor following addition of FBS, hFGF, and PDE inhibitors to the cells.

DISCUSSION

The presence of PDE3 and PDE4 has been demonstrated previously in non-primate mammalian vascular smooth muscle cells [14, 15, 24]. However, these studies did not answer the question of whether CI-930- or rolipram-inhibitable isozymes are expressed in the smooth muscle cells of human coronary arteries. In the present study, we answered this question by demonstrating that CI-930 and rolipram each inhibited significant amounts of PDE activity in HCASMC extracts (Tables 1 and 2). Because these inhibitors are selective for PDE3 and PDE4, respectively, the results suggest that HCASMC express PDE3 and PDE4.

Proliferation of HCASMC contributes significantly to the development of atherosclerotic plaques and accounts for most of the restenosis that occurs following coronary angioplasty [25–27]. Pharmacological agents that can attenuate HCASMC proliferation may be useful for preventing restenosis and possibly also for decreasing the progression of atherosclerosis in individuals at risk. Studies on animal models indicated that PDE3 and/or PDE4 inhibitors can attenuate or delay aortic [14, 15] and pulmonary artery [28] smooth muscle cell proliferation in cell cultures and attenuate or delay neointima formation in injured rat carotid artery *in vivo* [28]. Significantly, Kunishima *et al.* [16] recently reported that 35 patients receiving the PDE3 inhibitor cilostazol experienced only about one-third the incidence of restenosis as patients who received aspirin for

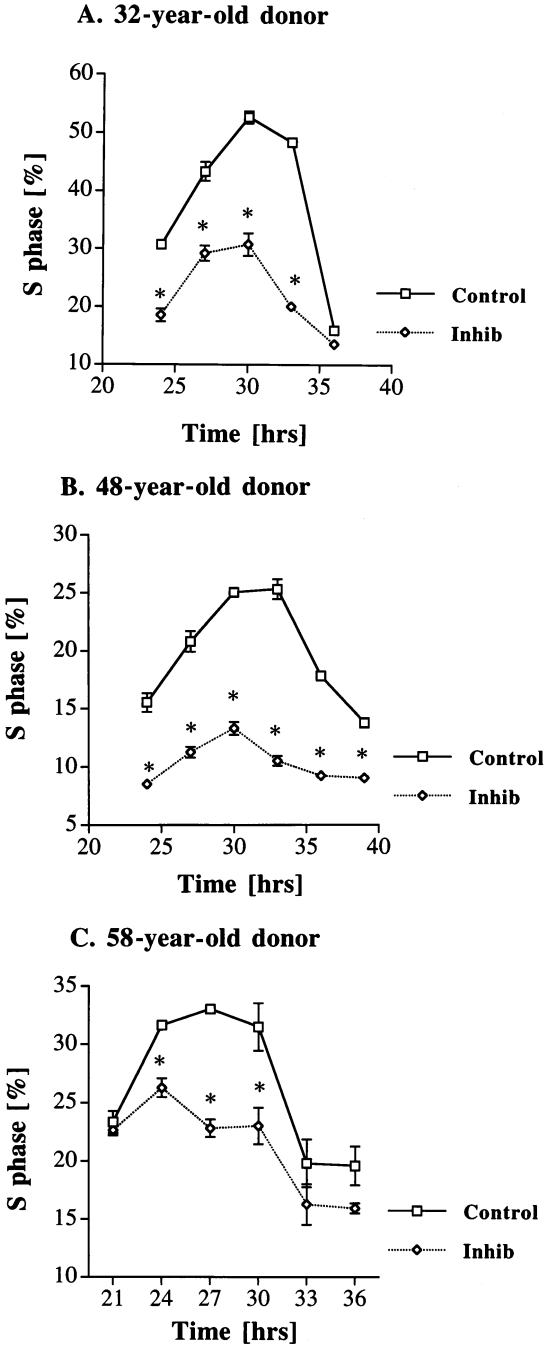

FIG. 4. Effect of 10 μM CI-930 plus 10 μM rolipram on the appearance of HCASMC in the S phase at various times after stimulation of cell proliferation. After growth of HCASMC was arrested, cells were stimulated by the addition of 10% FBS and 2 ng/mL of hFGF in the presence or absence of PDE inhibitors. Each data point represents the mean ± SEM of flow cytometry determinations run on HCASMC from each of three culture flasks. *Significantly different from control ($P < 0.05$).

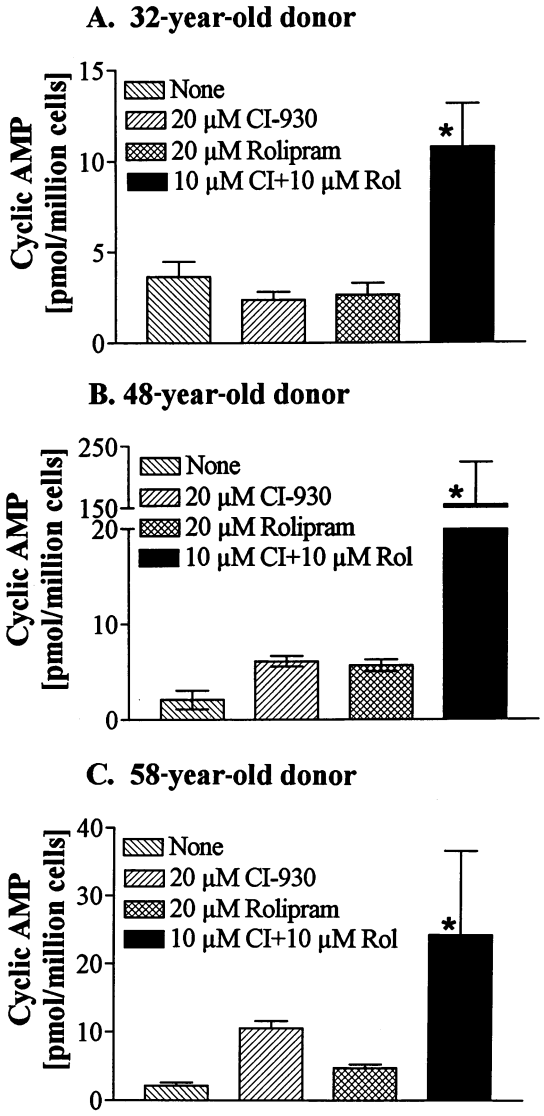

FIG. 5. Effect of CI-930 and rolipram on cAMP levels. cAMP levels were assayed by radioimmunoassay as described in Materials and Methods. Each data point represents the mean ± SEM of duplicate determinations made on HCASMC from each of three to six different culture plate wells. *Significantly different from control ($P < 0.05$).

about 5 months after coronary stent implantation. Taken together, these studies suggest, but do not prove, that PDE3 and PDE4 inhibitors attenuate HCASMC proliferation and that they can prevent restenosis in patients by that mechanism. The major purpose of the present study was to determine the effects of CI-930 (PDE3 inhibitor) and rolipram (PDE4 inhibitor) on HCASMC proliferation.

The data show that CI-930 and rolipram used individually attenuated or delayed proliferation of HCASMC from some but not all of the donors. However, the two agents in combination produced a greater effect and modulated the proliferation of HCASMC from all four donors studied. Previous studies using animal cell models did not rule out the possibility that progression through the cell cycle may have been merely delayed rather than attenuated. In the experiments on HCASMC reported here, we observed the number of cells in S phase at several time points after stimulation, which showed that progression through the cell cycle was actually attenuated and not merely delayed. Because the effect of the combination was to attenuate

proliferation, it seems likely that the effect of the PDE inhibitors used individually was also to attenuate and not merely delay proliferation. However, this latter question was not answered directly by the data in this study.

The combination of CI-930 plus rolipram elevated cAMP levels in cells from all three of the donors studied. This finding appears to support the hypothesis that the antiproliferative effects of the combination are produced by cAMP accumulations (except as noted in the paragraph below). Souness *et al.* [15] had measured cAMP levels in porcine aortic smooth muscle cells in the presence of PDE3 and PDE4 inhibitors, but did not detect any significant increase unless forskolin was added to stimulate adenylyl cyclase. In our experiments, none of the reagents added is known to stimulate adenylyl cyclase. The divergent results may be explained by species variations, differences between aorta and coronary artery smooth muscle cells, differences in the technical procedures used, or by the times that were selected for cAMP measurements after PDE inhibitors were added.

The cAMP elevations produced by the combination of inhibitors in HCASMC from the 48-year-old donor were very large compared with those from the 32- and 58-year-old donors (Fig. 5), but the effects on proliferation were comparable among these three donors (Fig. 3, A–C). This finding would seem to detract from the hypothesis that the antiproliferative effects are mediated by cAMP. However, it may be speculated that the cAMP elevations in cells from the 48-year-old donor exceeded the levels necessary to maximally activate cAMP-dependent protein kinase, and therefore the higher elevations produced no greater antiproliferative effect.

Although two of the three donors studied showed elevated cAMP levels in the presence of either CI-930 or rolipram used individually, none of these elevations was statistically significant using the ANOVA tests chosen for this study. The failure to find statistically significant cAMP elevations in the presence of the PDE inhibitors used alone was unexpected inasmuch as the individual agents affected proliferation of HCASMC from some donors. However, similar results are not unusual for this type of data, and examples of PDE inhibitors producing biological effects in the absence of significant cAMP elevations are common [15, 29]. Although these findings may seem to call into question the hypothesis that PDE inhibitors produce their biological effects by raising cAMP, it has been pointed out that small increases in cAMP levels may occur, which are large enough to produce a biological effect, but too small to be detected by radioimmunoassay [15].

From the data presented in this report, the following conclusions may be drawn. CI-930- and rolipram-inhibitable PDEs are expressed in HCASMC. PDE3 and PDE4 inhibitors used alone can attenuate or delay HCASMC proliferation, but some individuals respond more than others. The combination of a PDE3 inhibitor (CI-930) with a PDE4 inhibitor (rolipram) produces an enhanced effect, which is an attenuation of proliferation and not merely a delay of cell cycle traverse. The enhanced effect is associated with significant increases in cAMP accumulation. The data suggest that selective PDE inhibitors may prevent restenosis in patients following percutaneous transluminal coronary angioplasty because of their effect on HCASMC proliferation, and may also be useful in retarding the progression of atherosclerosis in individuals at risk. Combinations of PDE3 and PDE4 inhibitors may be more effective than the inhibitors used individually.

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References

1. 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.
2. Campbell GR and Campbell JH, Recent advances in molecular pathology. Smooth muscle phenotypic changes in arterial wall homeostasis: Implications for the pathogenesis of atherosclerosis. *Exp Mol Pathol* **42:** 139–162, 1985.
3. Ross R, The pathogenesis of atherosclerosis—An update. *N Engl J Med* **314:** 488–500, 1986.
4. Abell CW and Monohan TM, The role of adenosine 3′,5′-cyclic monophosphate in the regulation of mammalian cell division. *J Cell Biol* **59:** 549–558, 1973.
5. Berridge MJ, The interaction of cyclic nucleotides and calcium in the control of cellular activity. *Adv Cyclic Nucleotide Res* **6:** 1–98, 1975.
6. Coffino P, Gray JW and Tomkins GM, Cyclic AMP, a nonessential regulator of the cell cycle. *Proc Natl Acad Sci USA* **72:** 878–882, 1975.
7. Pardee AB, Dubrow R, Hamlin JL and Kletzien RF, Animal cell cycle. *Annu Rev Biochem* **47:** 715–750, 1978.
8. 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.
9. Loesburg C, Van Wijk R, Zandbergen J, van Aken WG, van Mourik JA and deGroot G, Cell cycle dependent inhibition of human vascular smooth muscle cell proliferation by prostaglandin E_1. *Exp Cell Res* **160:** 117–125, 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. 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.
12. Appleman MM and Terasaki WL, Regulation of cyclic nucleotide phosphodiesterase. *Adv Cyclic Nucleotide Res* **5:** 153–162, 1975.
13. Beavo JA, Conti M and Heaslip RJ, Multiple cyclic nucleotide phosphodiesterases. *Mol Pharmacol* **46:** 399–405, 1994.
14. Pan X, Arauz E, Krzanowski JJ, Fitzpatrick DF and Polson JB, Synergistic interaction between selective pharmacological

inhibitors of phosphodiesterase isozyme families PDE III and PDE IV to attenuate proliferation of rat vascular smooth muscle cells. *Biochem Pharmacol* **48:** 827–835, 1994.
15. Souness JE, Hassal 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.
16. Kunishima T, Musha H, Eto F, Iwasaki T, Nagashima J, Masui Y, So T, Nakamura T, Oohama N and Murayama M, A randomized trial of aspirin versus cilostazol therapy after successful coronary stent implantation. *Clin Ther* **19:** 1058–1066, 1997.
17. Johnson K, Arauz E, Krzanowski JJ and Polson JB, Effect of PDE3 and PDE4 inhibitors on the proliferation of human coronary artery smooth muscle cells. *FASEB J* **11:** A151, 1997.
18. Johnson-Mills K, Coffey RG, Arauz E, Krzanowski JJ and Polson JB, Effects of CI-930 and rolipram on cyclic AMP levels in human coronary artery smooth muscle cells. *FASEB J* **12:** A155, 1998.
19. Mohler WA, Charlton CA and Blau HM, Spectrometric quantitation of tissue culture cell number in any medium. *Biotechniques* **21:** 260–266, 1996.
20. Thompson WJ and Appleman MM, Multiple cyclic nucleotide phosphodiesterase activities from rat brain. *Biochemistry* **10:** 311–316, 1971.
21. Robicsek SA, Krzanowski JJ, Szenitvanyi A and Polson JB, High pressure liquid chromatography of cyclic nucleotide phosphodiesterase from purified human lymphocytes. *Biochem Biophys Res Commun* **163:** 554–560, 1989.
22. Coffey RG, Hadden EM and Hadden JW, Phytohemagglutinin stimulation of guanylate cyclase in human lymphocytes. *J Biol Chem* **256:** 4418–4424, 1981.
23. Rabe KF, Tenor H, Dent G, Schudt C, Nakashima M and Magnussen H, Identification of PDE isozymes in human pulmonary artery and effect of selective PDE inhibitors. *Am J Physiol* **266:** L536–L543, 1994.
24. Xiong Y, Westhead EW and Slakey LL, Role of phosphodiesterase isoenzyme in regulating intracellular cyclic AMP in adenosine-stimulated smooth muscle cells. *Biochem J* **305:** 627–633, 1995.
25. Ross R and Glomset JA, The pathogenesis of atherosclerosis. *N Engl J Med* **295:** 369–377, 1976.
26. Ross R, Cell biology of atherosclerosis. *Annu Rev Physiol* **57:** 791–804, 1995.
27. Polson JB and Strada SJ, Cyclic nucleotide phosphodiesterases and vascular smooth muscle. *Annu Rev Pharmacol Toxicol* **36:** 403–427, 1996.
28. Indolfi C, Vittorio E, Di Lorenzo E, Esposito G, Rapacciulo A, Guiliano P, Grieco D, Cavuto L, Stingone AM, Ciullo I, Condorelli G and Chiariello M, Activation of cAMP-PKA signaling *in vivo* inhibits smooth muscle cell proliferation induced by vascular injury. *Nat Med* **3:** 775–779, 1997.
29. Silver PJ, Lepore RE, O'Connor B, Lemp BM, Hamel LT, Bentley RG and Harris AL, Inhibition of low K_m cyclic AMP phosphodiesterase and activation of the cyclic AMP system in vascular smooth muscle by milrinone. *J Pharmacol Exp Ther* **247:** 34–42, 1988.