Role of phosphodiesterase isoenzymes in regulating intracellular cyclic AMP in adenosine-stimulated smooth muscle cells

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Three phosphodiesterase (PDE) isoenzymes were separated by Mono Q h.p.l.c. column chromatography from the soluble fraction of a homogenate of pig aortic smooth muscle cells. The first peak of PDE activity was stimulated by calmodulin in the presence of calcium. The second broad peak contained at least two activities, which were sensitive to inhibition by CI-930 or rolipram respectively. The distribution of total cellular enzyme activity in different subcellular fractions was also determined. The majority (78 %) of the total activity was present in the cytosolic fraction, 18 % of activity was in a membrane-bound form and 4 % of activity was associated with the cytoskeleton. Rolipram-sensitive PDE was present predominantly in the cytosolic fraction, whereas cyclic GMP-inhibited, CI-930-sensitive PDE was evenly distributed between the cytosolic and particulate fractions. All of the calmodulin-dependent PDE activity was

found in the soluble fraction. CI-930 and rolipram enhanced, by 2-fold and 3–4-fold respectively, the adenosine-stimulated rise in cellular cyclic AMP level. The increase in cyclic AMP levels due to CI-930 or rolipram was dose-dependent. Removal of adenosine once cyclic AMP had risen resulted in a rapid fall in cyclic AMP levels even in the presence of rolipram and CI-930. M&B 22,948, the calmodulin-dependent PDE inhibitor, caused less than a 25 % increase of the adenosine-stimulated cyclic AMP levels by itself, but it contributed substantially to controlling the cyclic AMP levels after the removal of adenosine when used together with CI-930 and rolipram. These phenomena suggested that all three PDE isoenzymes participated in modulating cellular cyclic AMP levels after adenosine stimulation, and that differential importance of the individual isoenzymes depends on cellular cyclic AMP levels.

INTRODUCTION

Since the discovery of multiple molecular forms of cyclic nucleotide phosphodiesterase (PDE) in 1971 (Thompson and Appleman, 1971), a number of studies have focused on the purification, characterization and roles of the different PDE isoenzymes in modulating cyclic nucleotide metabolism and signal transduction in response to extracellular stimulation. The isoenzymes of PDE display different substrate specificities, kinetic properties and sensitivity to calmodulin (CaM) and to selective PDE inhibitors (Weishaar et al., 1987; Beavo, 1988). Selective PDE inhibitors have been important tools for characterization and study of the roles of PDE isoenzymes in different cellular functions. In pig aortic endothelial cells, using selective inhibitors, Souness et al. (1990) showed that cyclic GMP-stimulated cyclic AMP PDE controls cellular cyclic AMP in the absence of stimulants other than the PDE inhibitor. Rolipram-sensitive cyclic AMP PDE played a role in forskolin-stimulated but not in unstimulated cells. The presence of two isoforms of PDE, CaM-dependent and low- K_m cyclic AMP PDEs, in smooth muscle was reported by Lugnier, Silver and Hagiwara (Hagiwara et al., 1984; Lugnier et al., 1986; Silver et al., 1988a,b). The role of these forms of enzyme activity in controlling cyclic AMP levels and smooth muscle tone was investigated by the use of selective and non-selective PDE inhibitors. In Schoeffter's work, eight PDE inhibitors tested were able to relax the isolated rat aorta precontracted by 5-hydroxytryptamine in a concentration-dependent manner which correlated with their potencies as inhibitors

of cyclic AMP PDE, but not of cyclic GMP PDE (Schoeffter et al., 1987). Many other functional studies have shown good correlation between the effectiveness of selective PDE isoenzyme inhibitors on cyclic AMP PDE activity and their effect on contractile activity in cardiac muscle or relaxation of smooth muscle (Polson et al., 1982; Weishaar et al., 1985b; Kishi and Numano, 1986; Kauffman et al., 1987; Miller and Wells, 1987; Marsh et al., 1988; Fujimoto and Matsuda, 1990; Torphy and Cieslinski, 1990; Cheng and Dage, 1991).

Previous work in our laboratory showed that cultured smooth muscle cells from pig aorta respond to stimulation with extracellular adenosine by increasing their cellular cyclic AMP level (Goldman et al., 1983). The extent of this response varied among the primary cultures as well as under different culture conditions (Xiong et al., 1991). We have also observed that the elevated level of cyclic AMP induced by adenosine falls very rapidly after adenosine is removed, even in the presence of enough PDE inhibitor (Ro 20-1724) to sustain a 4-fold increase in the magnitude of the initial response to adenosine (Fehr et al., 1990). These data suggest that the rate of cyclic AMP turnover plays a major role in regulating cellular cyclic AMP levels in this cell type.

In this study, we determine the components of cyclic AMP PDE activity in pig aortic smooth muscle cells, and identify the isoenzymes using selective PDE inhibitors. M&B 22,948 was used to identify CaM-dependent PDE activity (Lugnier et al., 1986; Silver et al., 1988b); CI-930 was used as a cyclic GMP-inhibited PDE-selective inhibitor (Weishaar et al., 1985a; Silver

Abbreviations used; CaM, calmodulin; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; PDE, phosphodiesterase; PMSF, phenylmethanesulphonyl fluoride.

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et al., 1988b); and rolipram was used to identify PDE sensitive to that compound (Weishaar et al., 1985a; Lugnier et al., 1986). Each of these PDE isoenzymes has a role in modulating cellular cyclic AMP levels after adenosine stimulation in pig aortic smooth muscle cells.

MATERIALS AND METHODS

Materials

[³H]Cyclic AMP was purchased from New England Nuclear. AG-1-X2 was purchased from Bio-Rad (Richmond, CA, U.S.A.). The Mono Q HR 5/5 h.p.l.c. column was obtained from Pharmacia LKB Biotechnology, Uppsala, Sweden. ¹²⁵I-labelled cyclic AMP (1.5 µCi/0.15 ml) was obtained from Biomedical Technologies Inc., Stoughton, MA, U.S.A. Leupeptin, aprotinin, snake venom 5'-nucleotidase (*Crotalus adamanteus*, fraction VT), phenylmethanesulphonyl fluoride (PMSF), dithiothreitol (DTT), Triton X-100, trypsin inhibitor and cyclic AMP were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. CI-930 was a gift from Parke-Davis Pharmaceutical Research Division, Warner-Lambert Company, Ann Arbor, MI, U.S.A. Rolipram was a gift from Berlex Laboratories, Cedar Knolls, NJ, U.S.A. M&B 22,948 was a gift from Rhone-Poulenc Rorer, Dagenham Research Centre, Dagenham, Essex RM10 7XS, U.K. Collagenase was obtained from Worthington Biochemicals, Freehold, NJ, U.S.A. All other tissue-culture materials were obtained from Gibco, Grand Island, NY, U.S.A.

Cell preparation

Smooth muscle cells were isolated from pig aortas and cultured as described by Goldman et al. (1983). Briefly, medial tissue was minced into 1 mm³ pieces and incubated overnight at 37 °C in Dulbecco's modified Eagle's medium (DMEM) with 0.3% collagenase. The mixture was centrifuged and the pellet was resuspended in DMEM containing 10% (w/v) fetal bovine serum and distributed in a T-25 flask. After the culture became confluent in the T-25 flask, the cells were transferred into a T-75 flask by trypsinization. Subsequent passages were divided 1:4. Cells were used between the third and ninth passages. Some cultures used were stored frozen at the third passage. For experiments, cells were plated in 35 mm-diam. dishes and grown to confluence. They were fed every other day, and always 24 h before an experiment.

Cellular cyclic AMP in response to adenosine

Cells were incubated with 50 µM adenosine as described by Fehr et al. (1990). For experiments, medium was removed and the cells were rinsed with 1 ml of serum-free DMEM containing 20 mM Hepes (pH 7.4). Cells were preincubated for 5 min with 0.5 ml of serum-free DMEM containing 20 mM Hepes (pH 7.4) and various pharmacological agents as noted. Agonist was added in a volume less than 1% of the total volume and the incubation continued until the appropriate time. Medium was removed and the cells were rinsed with 1.0 ml of Krebs-Ringer solution (pH 7.4) and then lysed with 1.0 ml of 0.1 M HCl to retrieve the intracellular cyclic AMP. The effects of PDE inhibitors on adenosine-induced cellular cyclic AMP concentrations were determined by preincubation of PDE inhibitors with the cells for 10 min at 37 °C before adenosine was added. Intracellular samples were assayed for cyclic AMP according to the procedure of Brooker et al. (1979). Briefly, samples in 0.5 ml of 0.1 M HCl were acetylated using 15 µl of triethylamine followed by 5 µl of acetic anhydride. An aliquot (50 µl) of acetylated sample was placed in a 12 mm × 75 mm glass tube to which 50 µl [(8–10) × 10³

c.p.m.] of ¹²⁵I-cyclic AMP in 50 mM sodium acetate, pH 5.5, was added. Next 200 µl of a 1:200000 dilution of cyclic AMP antiserum in 50 mM sodium acetate containing 5 mg/ml BSA was added. Samples were mixed and incubated overnight at 4 °C. After incubation, 1.0 ml of charcoal-BSA suspension was added. The samples were mixed, incubated at 4 °C for 15 min, and centrifuged at 500 g for 10 min. Supernatants were decanted and counted directly. The antiserum used was prepared as described by Dickinson et al. (1987).

Subcellular fractionation

Cells were collected by trypsinization and homogenized in a Dounce homogenizer in approx. 10 vol. of cold homogenizing buffer (10 mM Tris/HCl, pH 7.5, containing 0.15 M KCl, 10 µM leupeptin, 1350 units/ml aprotinin, 2.9 mM PMSF and 0.1 mM DTT). The homogenate was centrifuged at 1500 g for 5 min and the resulting supernatant was then centrifuged at 100000 g (Beckman 50 Ti) for 1 h at 4 °C. The supernatant from the 100000 g centrifugation was termed the 'cytosolic fraction'. The pellet was suspended in homogenizing buffer containing 1% Triton X-100 for 30 min on ice, with occasional inversion of the centrifuge tube, then homogenized and centrifuged at 1000 g for 15 min. The pellet (cytoskeleton fraction; Patton et al., 1989) was resuspended in homogenizing buffer and sonicated in an ice-cold water bath for 5 min. The Triton X-100 supernatant from the 1000 g centrifugation contained the membrane-bound fraction. For Mono Q chromatography, the soluble fraction was adjusted to pH 6.5 by addition of Bis-Tris buffer at pH 5.5.

Assay of PDE activity

The enzyme activity was measured according to the one-step procedure described by Thompson and Appleman (1971) with minor modifications. The assay was initiated by the addition of substrate, 1 µM [³H]cyclic AMP (50000 c.p.m.) to an incubation mixture containing, in a total final volume of 100 µl, 40 mM Tris/HCl buffer, pH 8.0, 5 mM MgCl₂, 0.25 mg/ml 5'-nucleotidase, with or without 15 nM CaM and 1 mM calcium, and enzyme preparation. According to the nature of the sample used, protein concentrations in enzyme assays were from 0.005 mg/ml to 0.17 mg/ml. After 10 min incubation at 30 °C, the reaction was terminated by heating for 3 min in a water bath at 90 °C. The final product, [³H]adenosine, was isolated by chromatography of the entire reaction mixture over an AG-1-X2 column (Cl⁻ form, 0.1 ml of bed volume), eluting with 1.5 ml of 0.1 M HCl. Aliquots (0.5 ml) of the samples eluted from the AG-1-2X columns were counted in the liquid-scintillation counter. For assessing PDE inhibition, the inhibitors were added into the reaction tubes before the substrate. The vehicle for CI-930 was distilled water and for rolipram, 1% dimethyl sulphoxide in water. The vehicle for M&B 22,948 was 50 mM triethanolamine. None of the vehicles affected PDE activity significantly in the concentrations added.

PDE activity was linear during the 10 min incubation and within 15% of substrate consumption. PDE activity was linear within that range for dilutions of total cell homogenate from 1:2 to 1:10, at either 5 min or 10 min reaction time (results not shown).

RESULTS

Characterization of smooth-muscle-cell cyclic AMP PDEs

PDE activity from cultured smooth muscle cells was separated into cytosolic, membrane-bound and cytoskeletal fractions. At a 1 µM cyclic AMP substrate concentration, 78 ± 2% (n = 6) of

Figure 1 Resolution of PDE activities by Mono Q h.p.l.c.

A sample (2 ml; 1 mg of protein) of the soluble fraction from a pig aortic smooth muscle cell homogenate was applied to a Mono Q h.p.l.c. column (Code No. 17-0546-01, bed volume 1.0 ml) equilibrated with 25 mM Bis-Tris/HCl, 0.1 mM DTT, pH 6.5, at a flow rate of 0.8 ml/min. PDE activity was eluted with a linear gradient of NaCl from 75 mM to 0.5 M in the equilibrating buffer. Fractions of 0.8 ml were collected. The total run time was 40 min. The enzyme activity in 40 μ l of every other fraction was measured using 1 μ M cyclic AMP as substrate, in the presence of 15 nM CaM and 1 mM Ca^{2+} (○), 1 μ M CI-930 plus CaM and Ca^{2+} (△), or 1 μ M rolipram plus CaM and Ca^{2+} (□). Enzyme activity is expressed as a percentage of substrate hydrolysed in 10 min. Hatched areas show fractions combined for subsequent experiments. Similar separations were obtained in three other preparations.

the cyclic AMP-metabolizing activity was contributed by cytosolic enzymes. $18 \pm 1\%$ was membrane-associated and $4 \pm 0.6\%$ was found associated with the cytoskeleton.

At least three cyclic AMP-hydrolysing PDE isoenzymes could be demonstrated by Mono Q h.p.l.c., coupled with characterization by selective PDE inhibitors. The 100000 *g* supernatant fraction was resolved into two major peaks (Figure 1). The first peak of activity but not any part of the second broad peak was stimulated by 15 nM CaM in the presence of 100 μ M calcium (results not shown). For consistency, buffer containing calcium and CaM was used throughout the column fractions to establish basal conditions against which inhibitors were measured. The left-hand side of the second peak was more sensitive to CI-930, while activity on the right-hand side of the second peak was sensitive to rolipram (Figure 1). Activity in the first peak was not sensitive to either CI-930 or rolipram (Figure 1). Similar separations of activity were observed in three preparations of whole-cell detergent-treated lysates chromatographed on DEAE-cellulose, except that the two activities in the second peak were less well resolved. There was no evidence for stimulation of activity by cyclic GMP in any fraction. Activity in the region inhibited by CI-930 was also inhibited by cyclic GMP (results not shown).

H.p.l.c. fractions were combined as shown by the hatched areas in Figure 1. In the presence of calcium and CaM, activity from the first peak was inhibited similarly by 1 mM EGTA (59%) or 100 μ M M&B 22,948 (57%). Neither of the pooled activities in the second peak were inhibited by EGTA or M&B 22,948.

To assess the contributions of each of the three activities displayed on the Mono Q column to control of cellular cyclic AMP, the effects of the three inhibitors, CI-930, rolipram and

Figure 2 Dependence of the inhibition of PDE activity in cell lysate and the accumulation of cyclic AMP after 50 μ M adenosine stimulation in intact cells on the concentrations of (a) CI-930, and (b) rolipram

Cellular cyclic AMP concentrations (○), and inhibition of PDE activity (●) in detergent-treated lysates of whole cells were determined as described in the Materials and methods section. (a) Values of cyclic AMP levels are the mean of three experiments. In each experiment, duplicate assays were made of the response in three separate dishes for each dose of inhibitor. Bars indicate S.D. For the inhibition of PDE activities, the mean and range of two experiments are shown. (b) Values of the mean and S.D. of the inhibition of PDE activity were obtained from six independent experiments. For cyclic AMP, the mean and range of two experiments are shown. In each experiment, duplicate assays were made from three separate dishes for each dose of inhibitor.

M&B 22,948, were examined using intact cells, cell lysates and subcellular fractions. Figure 2(a) shows the concentration dependence for enhancement by CI-930 of cyclic AMP accumulation in smooth muscle cells stimulated with 50 μ M adenosine. The concentration dependence for inhibition of hydrolysis of 1 μ M cyclic AMP in a detergent-treated lysate of whole cells is also shown. A similar comparison of the effects of rolipram is shown in Figure 2(b). The total enhancement by optimal concentrations of rolipram was substantially greater than that by CI-930 (3.8 ± 0.9 , $n = 5$ for rolipram, compared with 2.1 ± 0.4 , $n = 5$ for CI-930). M&B 22,948 had only a modest effect on cellular cyclic AMP accumulation (22% increase in cyclic AMP when used at 100 μ M with 50 μ M adenosine, compared with adenosine alone). The concentration dependence of this effect was not determined.

It is apparent from Figure 2 that CI-930 is more potent in inhibiting PDE in the cell lysate than it appears to be in enhancing a cellular rise in cyclic AMP, while rolipram is more potent with whole cells. It is possible that transport barriers

Figure 3 Dependence of the inhibition of PDE activity in h.p.l.c.-separated fractions (■), the total soluble fraction (●) and particulate fraction (▲) on the concentrations of CI-930 (a), rolipram (b) and M&B 22,948 (c)

The inhibitory effects of rolipram and M&B 22,948 were determined in the presence of 100 μM CI-930. The maximum inhibitions observed were as follows. CI-930: h.p.l.c. fraction, 47%; cytosol, 48%; membrane, 73%. Rolipram: h.p.l.c. fraction, 88%; cytosol, 53%; membrane, 19%. M&B 22,948: h.p.l.c. fraction, 87%; cytosol, 21%. The values for h.p.l.c.-separated fractions were obtained from a single preparation. Each point represents the average of three determinations. For cytosolic and membrane fractions, data were obtained from two separate experiments. Each point represents the mean of six determinations. Bars indicate S.D.

prevent the full effect of CI-930. The greater potency of rolipram with intact cells suggests that its principal effect in intact cells may be exerted by a subset of the activities measured in cell lysates at 1 μM cyclic AMP. It is also possible that the inherent

Table 1 Percentage distribution of PDE isoenzymes: relative capacity to hydrolyse 1 μM cyclic AMP

Data were calculated based on the observation that enzyme activity in the soluble fraction was 78% of total and in the particulate fraction, 22% of total. Percentage activity sensitive to each inhibitor in each fraction was that observed in the concentration-dependence studies at 10 μM CI-930, and at 100 μM rolipram (ROL), or 1 mM M&B 22,948, each in the presence of 100 μM CI-930, as given in Figure 3 and its legend.

Isoenzyme	Fraction		Whole cell
	Cytosolic	Membrane	
Cyclic CI-PDE	20	16	36
ROL-PDE	40	4	44
CaM-PDE	16	1	17

maximum effect of rolipram on whole cells, and thus its IC_{50} , may be underestimated; we observed a small but reproducible inhibition of the total response to adenosine at the highest doses of rolipram tested (Figure 2b).

The concentration dependence of inhibition for each inhibitor was also determined on each of three different preparations: pooled fractions from h.p.l.c. separation of cytosol, as marked in Figure 1; whole cytosol (100000 g supernatant); and membrane fraction (100000 g pellet). The behaviours of the three inhibitors are shown in Figure 3. The h.p.l.c.-separated fraction and the membrane-bound activity showed IC_{50} values of less than 1 μM for CI-930, and about half of the cytosolic activity affected by CI-930 was also inhibited with similar sensitivity (Figure 3a). An IC_{50} less than 1 μM agrees well with the concentration dependence observed for the whole-cell detergent-treated lysate (Figure 2a).

As can be seen in Figure 3(a), CI-930 appears to be able to inhibit more than one activity in the cytosolic fraction. A similarly broad curve was observed for rolipram in experiments not shown. The measurements shown in Figures 3(b) and 3(c) for rolipram and M&B 22,948 respectively were made in the presence of 100 μM CI-930. This permitted a clear discrimination of the sensitivity of the principal activities affected by rolipram and M&B 22,948. Under these conditions, sensitivity of the three fractions to rolipram (IC_{50} about 0.2 μM , Figure 3b) agrees well with the concentration dependence of whole-cell accumulation of cyclic AMP on rolipram (Figure 2b).

M&B 22,948 inhibited both the h.p.l.c.-separated and cytosolic fractions with similar sensitivities at 1 μM cyclic AMP (Figure 3c). The membrane-bound fraction contained very little activity sensitive to M&B 22,948.

Effects of PDE isoenzymes on the adenosine-stimulated cellular cyclic AMP level

The relative contributions of the three PDE isoenzymes in hydrolysing 1 μM cyclic AMP, and their distributions in the membrane and soluble fractions were estimated from the experiments shown in Figure 3; the distributions are shown in Table 1. The percentages of total activity inhibitable by rolipram and M&B 22,948 were each determined in the presence of 100 μM CI-930 and thus represent minimal estimates. Because rolipram had a greater impact on the cyclic AMP level in adenosine-stimulated cells than the distribution shown in Table 1 suggested it would, and M&B 22,948 had less, the role of each PDE isoenzyme in modulating the cellular cyclic AMP level after adenosine stimulation was further assessed. The effects of the

Figure 4 Effect of CI-930, rolipram and M&B 22,948 on the breakdown of cellular cyclic AMP

Smooth muscle cells in 35-mm-diam. dishes were preincubated with 10 μ M CI-930 plus 3 μ M rolipram ($\blacktriangle$), 100 μ M CI-930 plus 100 μ M rolipram ($\triangle$), 200 μ M M&B 22,948 alone ($\diamond$) or 200 μ M M&B 22,948 together with 100 μ M CI-930 and 100 μ M rolipram ($\square$) for 10 min followed by 6 min adenosine stimulation in the preincubation medium ($\blacktriangle$, 50 μ M; $\triangle$, 30 μ M; $\square$, $\diamond$, 20 μ M adenosine). After 6 min stimulation, adenosine was removed by replacing the adenosine-containing medium with fresh preincubation medium. Cellular cyclic AMP levels were determined at the indicated time after removal of adenosine. Samples preincubated with DMEM/Hepes without PDE inhibitors and stimulated with 20 μ M adenosine were used as the control ($\circ$). The number of determinations of cyclic AMP for each point is given next to the symbols. Within experiments, two or three dishes were used for each point. Each point was tested in at least two separate experiments using separate cell batches.

inhibitors on the levels of cellular cyclic AMP were determined, using the inhibitors alone and in combinations, in the presence of adenosine and after removal of adenosine from stimulated cells.

Neither CI-930 nor rolipram altered the time course of change in cyclic AMP level in response to adenosine, but each elevated the stimulated cellular cyclic AMP level. The new cyclic AMP level was achieved within 2 min and elevation persisted for at least 12 min in the presence of adenosine (results not shown). In subsequent experiments, inhibitors were preincubated with cells for 10 min before stimulation with adenosine.

Cells were preincubated with medium that contained CI-930, rolipram, or CI-930 plus rolipram for 10 min followed by 50 μ M adenosine stimulation. Adenosine was then removed, after 6 min stimulation, by replacing the adenosine-containing medium with fresh preincubation medium. Cellular cyclic AMP levels were assayed in the samples collected at 0 and 6 min after adenosine stimulation, as well as 30 and 90 s after adenosine was removed. As was found in other experiments, the basal cellular cyclic AMP

level was not significantly affected by PDE inhibitors, while the adenosine-stimulated cyclic AMP levels were enhanced by either CI-930 or rolipram at 6 min stimulation. When adenosine was not removed after 6 min, cyclic AMP levels did not decline between 6 and 9 min of stimulation (results not shown). However, 30 s after the removal of adenosine, cellular cyclic AMP levels in the cells treated with either CI-930 or rolipram alone decreased 80 % from the adenosine-stimulated level, and cyclic AMP came back to the basal level in 90 s despite the presence of the inhibitors (results not shown).

Figure 4 compares the effects of CI-930 and rolipram used together at different doses and in the presence and absence of M&B 22,948. Various doses of adenosine were used in order to achieve similar cellular levels of cyclic AMP under the different PDE inhibitor conditions. In the cells treated with both CI-930 and rolipram, the cyclic AMP levels also declined very quickly after the removal of adenosine, but were still at 20–60 pmol/ 1×10^6 cells after 90 s or 3 min depending on the concentrations of the inhibitors used in the experiments. Although treatment with M&B 22,948 alone had little effect in maintaining the cellular cyclic AMP level, cellular cyclic AMP barely decreased in the presence of all three inhibitors after the removal of adenosine (Figure 4).

In contrast with their effects on the adenosine-stimulated cellular cyclic AMP level, PDE inhibitors did not affect basal cyclic AMP. Because the PDE inhibitors had so little effect on basal cellular cyclic AMP, we examined basal PDE activity, and whether pretreatment of cells with adenosine altered PDE activity. Total PDE activity was measured in detergent-treated lysates of cells pretreated with 50 μ M adenosine in DMEM/Hepes for 6 min, and compared with activity in cells treated with DMEM/Hepes alone. This comparison was done on cells from 11 different smooth muscle preparations. Several findings emerged from the multiple comparisons. First, unstimulated cells have very substantial PDE activity. Secondly, total activity varied over a 6-fold range, from 28 to 168 pmol/min per mg, among the cell batches examined. Finally, adenosine treatment enhanced activity in eight of the preparations examined, but only slightly, the greatest enhancement being 70 %.

DISCUSSION

The present study demonstrates that at least three PDE isoenzymes play an important role in modulating the adenosine-induced rise in cellular cyclic AMP level in pig aortic smooth muscle cells. Characterization of h.p.l.c.-separated PDE activities from the cytosolic fraction by PDE inhibitors showed the presence of three cyclic AMP-hydrolysing PDE isoenzymes; CaM-dependent PDE, cyclic GMP-inhibited PDE (CI-930-sensitive) and rolipram-sensitive PDE. Use of the three PDE inhibitors with adenosine-stimulated cells showed that each of the three isoforms of PDE participates actively in the modulation of the cyclic AMP level and hence is important in regulating the cyclic AMP signal.

Others have shown that the three PDE isoenzymes have different kinetic properties. Both cyclic GMP-inhibited and rolipram-sensitive PDEs belong to the low- K_m cyclic AMP PDEs, with K_m values for hydrolysis of cyclic AMP at 0.2 μ M (Grant and Colman, 1984; Beavo, 1988) and 2 μ M respectively (Epstein et al., 1982; Reeves et al., 1987), while in most species and organs the CaM-dependent PDE has a K_m in the range of 10–70 μ M for hydrolysing cyclic AMP (Keravis et al., 1980; Sharma and Wang, 1986; Beavo, 1988). A few reports showed lower K_m values (0.8–1 μ M) for hydrolysing cyclic AMP by CaM-dependent PDE (Reeves et al., 1987). We found that the relative

importance of different isoenzymes in modulating the cellular response depended on cellular cyclic AMP concentrations. When cyclic AMP formation is stopped by removal of adenosine, the rate of decrease in cyclic AMP level reflects PDE activity. As shown in Table 1, 80% of total cellular PDE activity was inhibited by CI-930 and rolipram when the substrate concentration was 1 μ M. But the presence of maximally effective concentrations of CI-930 and rolipram in intact cells did not prevent a rapid decline in the cyclic AMP level until it reached 20–50 pmol/ 1×10^6 cells (Figure 4). On the other hand, M&B 22,948 alone did not cause significant enhancement of adenosine-induced cyclic AMP and did not prevent the decrease of the cyclic AMP level after removal of adenosine when the cellular cyclic AMP level was low. At higher cellular cyclic AMP levels, the same amount of M&B 22,948 did control cellular cyclic AMP (Figure 4). In the experiments in which the CaM-sensitive PDE activity was not inhibited, there was a rapid decline of cyclic AMP during the first 30 s and a subsequent slow change over 2.5 min, suggesting high activity of this isoenzyme when substrate concentration was high. As the cyclic AMP level decreased, the contribution of the CaM-sensitive PDE activity in hydrolysing cyclic AMP, and thus the velocity of cyclic AMP clearance, decreased. CI-930 and rolipram inhibited almost the same fraction of total cellular PDE activity at 1 μ M substrate (Table 1), but preincubation with rolipram alone more effectively enhanced the adenosine-induced cyclic AMP level. This is also consistent with the difference in their K_m values.

Our interpretation is that at low cellular cyclic AMP concentrations, the low- K_m PDEs play the major role in controlling the cellular cyclic AMP level. At high cyclic AMP concentrations, as might occur with multiple hormonal stimulation and as we observed in the presence of CI-930 and rolipram after adenosine stimulation (Figure 4), the low- K_m cyclic AMP PDEs reach their V_{max} . The velocity of hydrolysis of cyclic AMP by the high- K_m cyclic AMP PDE increases with the increase of substrate concentration, and this enzyme becomes more important in modulating cellular cyclic AMP.

Enhancement by PDE inhibitors of the cyclic AMP level was found only in adenosine-stimulated cells, and not in resting cells. However, substantial PDE activity is present in unstimulated cells. It is possible that in the intact cells, basal cyclic AMP is in a bound form, or is sequestered in some way, and is therefore not hydrolysable by PDEs.

Total PDE and PDE isoenzyme activities in pig aortic smooth muscle cells are differentially distributed in subcellular fractions. The subcellular location of PDE activity varies in different systems (Flawn and Loten, 1990) or under different homogenizing conditions. We used isotonic conditions for homogenization to prevent breakage of lysosomes and movement of enzyme activity between soluble and membrane-bound fractions. A level of 78% of cyclic AMP PDE was found in the soluble fraction. This result is in good agreement with those previously obtained in aortic smooth muscle and other systems (Bergstrand and Lundquist, 1978; Leroy et al., 1985; Hoey and Houslay, 1990; Nicholson et al., 1991; Kishi et al., 1992). We further separated cytoskeleton-associated PDE activity from the 100000 g particulate fraction (Patton et al., 1989). A small fraction of the total activity was reproducibly found associated with the cytoskeleton.

CaM-dependent PDE activity is sensitive to protease (Silver et al., 1988b). We also found that protease inhibitors were essential for maintaining the level of this isoenzyme activity (results not shown). This isoenzyme was found entirely in the cytosolic fraction (Figure 3). A similar result was reported using human heart by Masuoka et al. (1990). M&B 22,948 is also a cyclic GMP PDE inhibitor (Schoeffter et al., 1987; Silver et al., 1988b).

In rat vascular smooth muscle cells, the cyclic GMP level was shown to increase after adenosine stimulation, and the effect of adenosine on intracellular cyclic GMP was potentiated by M&B 22,948 (Kurtz, 1987). Since cyclic GMP is a regulator of cyclic GMP-stimulated and cyclic GMP-inhibited cyclic AMP PDEs, the very small effect of M&B 22,948 alone on the adenosine-induced cellular cyclic AMP level suggests that cyclic GMP is not a major player in modulating the adenosine-stimulated cyclic AMP signal in pig aortic smooth muscle cells. Consistent with other studies in smooth muscle (Keravis et al., 1980; Polson et al., 1982; Leroy et al., 1985; Lugnier et al., 1986; Silver et al., 1988a,b), no cyclic GMP-stimulated cyclic AMP PDE activity was found in pig aortic smooth muscle cells.

In conclusion, by using the selective PDE inhibitors, M&B 22,948, CI-930 and rolipram, three PDE isoenzymes and their functions in modulating cellular cyclic AMP level in response to adenosine stimulation in pig aortic smooth muscle cells were characterized. It is quite striking that in the presence of all three PDE inhibitors, the cellular cyclic AMP level in adenosine-stimulated cells rises to more than ten times the amount observed when PDE activity is not inhibited, suggesting that ordinarily the turnover of cyclic AMP in stimulated cells must be very rapid, as has been reported in other cells and tissues (Rudolph et al., 1971; Barber et al., 1980; Goldberg et al., 1983). The presence of three isoenzymes with a range of K_m values permits cells to return cyclic AMP to the basal level quickly from a wide range of stimulated levels. Presumably this rapid turnover serves to shorten the response time upon agonist binding and clearance.

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