cAMP phosphodiesterase inhibitors potentiate effects of prostacyclin analogs in hypoxic pulmonary vascular remodeling

Peter G. Phillips,1 Lu Long,2 Martin R. Wilkins,1 and Nicholas W. Morrell2

1Section on Clinical Pharmacology, Faculty of Medicine, Imperial College London, Hammersmith Hospital, London; and 2Department of Medicine, University of Cambridge, Cambridge, United Kingdom

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The development of chronic pulmonary hypertension involves pulmonary vascular remodeling. The effects of prostacyclin analogs and isoform-selective phosphodiesterase (PDE) inhibitors, alone and in combination, on pulmonary vascular remodeling in vitro and in vivo. Vascular smooth muscle cells (VSMC) isolated from pulmonary (proximal and distal) and systemic circulations demonstrated subtle variations in expression of PDE isoform mRNA. However, using biochemical assays, we found PDE3 and PDE4 isoforms to be responsible for the majority of cAMP hydrolysis in all VSMC. In growth assays, the prostacyclin analogs cicaprost and iloprost inhibited mitogen-induced proliferation of VSMC in a cAMP-dependent manner. In addition, isoform-selective antagonists of PDEs 1, 3, or 4 inhibited VSMC proliferation, an effect that synergized with the effect of prostacyclin analogs. The inhibitory effects were greater in cells isolated from pulmonary circulation. In an in situ perfused rat lung preparation, administration of prostacyclin analogs or the PDE inhibitors vinpocetine (PDE1), cilostamide (PDE3), or rolipram (PDE4), but not EHNA (PDE2), attenuated acute hypoxic vasoconstriction (HPV). Combinations of agents led to a greater reduction in HPV. Furthermore, during exposure to hypoxia for 13 days, Wistar rats were treated with iloprost, rolipram, cilostamide, or combinations of these agents. Compared with normoxic controls, hypoxic animals developed pulmonary hypertension and distal pulmonary artery muscularization. These parameters were attenuated by iloprost+cilostamide, iloprost+rolipram, and cilostamide+rolipram but were not significantly affected by single agents. Together, these findings provide a greater understanding of the role of cAMP PDEs in VSMC proliferation and provide rationale for combined use of prostacyclin analogs plus PDE3/4 inhibitors in treatment of pulmonary vascular remodeling.

Smooth muscle cells; cyclic nucleotides; heterogeneity

The development of chronic pulmonary hypertension involves a complex interplay between altered endothelial function, dysregulation in vasoactive mediators (e.g., prostanoids) (1, 6, 10, 11, 33), pulmonary vasoconstriction (5), and in situ thrombosis. These processes lead to distinct pathological changes within the pulmonary vasculature, including neointimal formation and increased muscularization of small arteries, termed collectively “vascular remodeling” (15).

Prostacyclin therapy has been shown to improve morbidity and mortality in primary pulmonary hypertension (PPH) (2). This is probably attributable to prostaglandin receptor-mediated elevation of cAMP levels in pulmonary artery smooth muscle cells (PASMC) leading to acute pulmonary vasodilatation (10) and long-term growth-inhibitory effects (19, 22). Although effective in PPH, prostacyclin therapy has major cost implications and is not without significant side effects (e.g., systemic hypotension). Alternative or additional therapeutic strategies to effect elevation in intracellular cyclic nucleotide levels have been considered, including inhibition of cyclic nucleotide hydrolysis. Metabolism of cAMP and cGMP is regulated by the phosphodiesterase (PDE) enzyme superfamily. Eleven kinetically distinct PDE isoform families have so far been identified in humans (3, 31). Previous evidence suggests that activity of PDEs 1, 3, 4, and 5 predominates in pulmonary arterial tissue (25), although the profile of PDE subtypes in PASMC and the presence/absence of more recently identified isoforms is unknown. In animal models there is evidence that isoform-selective PDE inhibitors can mimic and augment the effects of cAMP agonists on acute pulmonary vasoconstriction induced by the thromboxane mimetic U-46619 (26–29, 35). PDE inhibitors have been shown to inhibit proliferation of systemic vascular smooth muscle cells (VSMC) in vitro (18), but the effects of these agents on PASMC proliferation in vitro and the in vivo effect of chronic administration of these agents on pulmonary vascular remodeling have not, however, been determined.

We sought to investigate the PDE isoform and subtype profile in cultured pulmonary (main pulmonary artery and distal pulmonary artery) and systemic (coronary artery, aorta, and renal artery) arteries. Furthermore, we established the effects of isoform-selective PDE inhibitors and the prostacyclin analog cicaprost on acute hypoxia-induced pulmonary vasoconstriction using an in situ perfused rat lung preparation, and we determined the effect of the prostacyclin analog iloprost alone and in combination with PDE inhibitors on the hemodynamic and structural changes of pulmonary hypertension in the chronically hypoxic rat model.

Methods

Drugs and Analytical Reagents

Erythro-9-(2-hydroxy-3-nonyl)-adenine (EHNA) was purchased from BIOLOG Life Science Institute (Bremen, Germany). Cilostamide was purchased from Calbiochem-Novabiochem (Nottingham, UK). Cicaprost and iloprost were donated by Schering (Berlin, Germany). The Nucleon HT DNA extraction kit was from Scotlab (Coatbridge, UK). Access RT-PCR systems were purchased from Promega (Madison, WI). [3H]methyl thymidine and [3H]methyl cAMP were from Amersham Pharmacia Biotech (Little Chalfont, Bucks, UK). Gold-Star scintillation fluid was from Meridian (Epsom, Surrey, UK). The cAMP RIA kit was from NEF Life Science

Address for reprint requests and other correspondence: Nicholas W. Morrell, Dept. of Medicine, Univ. of Cambridge, Box 157, Addenbrooke’s Hospital, Hills Road, Cambridge CB2 2QQ, United Kingdom (E-mail: nwm23@cam.ac.uk). The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Products (Boston, MA). The cGMP RIA kit was purchased from Amersham Pharmacia Biotech, Pentobarbital sodium and isoflurane were from Rhône Mérieux (Harlow, Essex, UK). Miniosmotic pumps (2ML2) were purchased from Charles River UK (Margate, Kent, UK). Ribolyzer tubes were from Hybaid (Teddington, UK). All other drugs and reagents were from Sigma-Aldrich (Poole, Dorset, UK).

Isolation and Culture of VSMC

VSMC were isolated from male Wistar-Kyoto rats weighting ~300 g. Animals were killed using intraperitoneal (ip) injection of 100 mg/kg of pentobarbital sodium. Individual animals were used either for isolation of peripheral and main PASMC or, alternatively, for isolation of abdominal aortic, renal, and coronary arterial smooth muscle cells. Home Office approval was given for these experiments under project licence PPL/30/1567.

Proximal and distal PASMC. Distal PASMC were isolated using a method similar to that described by Johnson et al. (17), with minor modifications. To obtain proximal PASMC, the main pulmonary artery was dissected free from lung and cardiac tissue, and a single full-length incision was made. Intimal and adventitial layers were carefully removed by being rubbed with a sterile scalpel blade. Main pulmonary artery tissue was then digested in type II collagenase as described above. Cells were resuspended in 1 ml of culture medium containing 20% FCS for subsequent culture in 25-cm² flasks.

Aortic, coronary, and pulmonary artery smooth muscle cells. Aortic, coronary, and renal artery smooth muscle cells were isolated from the same individual rats. Aortic VSMC were isolated using a digest technique previously described by Wang et al. (36). Renal artery smooth muscle cells were isolated using iron oxide and magnetic separation, as previously described by Dubey et al. (9). The latter methodology was adapted for isolation of coronary artery smooth muscle cells.

Characterization of all rat pulmonary and systemic VSMC was undertaken at passage 2 using fluorescent immunocytochemical staining for α-smooth muscle actin and smooth muscle myosin.

Analysis of PDE Isoform Expression By RT-PCR

Confluent VSMC were detached with trypsin/EDTA and harvested at passage 2 in TRizol reagent (1 ml per 5–10 × 10⁶ cells). RNA was extracted (as per manufacturer’s instructions) and stored at −70°C until it was used for RT-PCR. RNA was also extracted from a single Wistar-Kyoto rat brain to provide a positive control for subsequent RT-PCR detection of mRNA for PDE10.

Immediately before RT-PCR experiments, RNA concentration and purity were determined by spectrophotometry. RT-PCR was performed using Access RT-PCR System (Promega) in accordance with the manufacturer’s instructions. Primer pairs for genes of interest were all manufacturer’sinstructions. Primer pairs for genes of interest were all manufacturer’sinstructions. Primer pairs for genes of interest were all manufacturer’sinstructions. Primer pairs for genes of interest were all previously described primer pairs, product size was compared with figures quoted in the literature. The identity of PCR products not previously characterized in the literature were confirmed by direct sequencing.

Assays of PDE Activity

PDE assay for isoforms catalyzing cAMP hydrolysis was performed in rat distal PASMC, main PASMC, and aortic, renal artery, and coronary artery smooth muscle cells. Smooth muscle cells from each vascular bed were seeded at an initial plating density of 40,000 cells/well into separate six-well plates (2 plates/cell line). Cells were grown in culture medium with 10% FCS until subconfluent (covering ~80% of the well). Medium was then removed, and cells were washed twice with PBS (at room temperature). Cell counts were performed in two representative wells/plate, and a mean cell count/well was calculated. Cells from the four remaining wells were scraped into 1-ml of ice-cold lysis TE buffer (20 mM triethylamine, pH 8, and 1 mM EDTA) containing the proteinase inhibitors benzamidine (2 mM), leupeptin (50 µM), PMSF (100 µM), bacitracin (100 µg/ml), and soybean trypsin inhibitor (20 µg/ml). Individual samples were centrifuged at 12,000 rpm for 20 min. Supernatant was removed and stored at −20°C until subsequent assay. PDE assay was performed as previously described (30). Isoform-selective PDE inhibitors included vinpocetine (100 µM), EHNA (50 µM), cilostamide (5 µM), and rolipram (50 µM). One pair of assay samples contained 30 µl of lyase plus vehicle plus reaction mixture (270 µl) but no PDE inhibitor (this sample was used to estimate total PDE activity). One further pair of assay samples contained reaction mixture (270 µl) plus vehicle only (this sample was subsequently used to determine [3H]cAMP radioactive count in the presence of zero PDE activity). Individual isoform activity was expressed as picomoles of cAMP hydrolyzed/min · 10⁶ cells. Isoform activity could be measured directly for cAMP PDE isoforms 1–4 because of the availability of selective inhibitors. The sum of activity of the remaining PDEs 7, 8, 10 and 11 was estimated by subtracting the sum of activity of PDEs 1–4 from total cAMP PDE activity.

Growth Assays

Pulmonary artery and systemic VSMC growth was assessed using [3H]thymidine incorporation assays, representing DNA synthesis, or cell counting using a hemocytometer to assess proliferation, as previously described (24). Cell viability was assessed by trypan blue exclusion. Assays were undertaken in the presence of 0.1% FCS, 10% FCS, or platelet-derived growth factor-BB (PDGF-BB, 10 ng/ml). To study the effect of specific agents on cell proliferation, assays were also conducted in the presence of ciprofloxacin and iloprost (prostacyclin analogs), vinpocetine (PDE1 inhibitor), EHNA (PDE2 inhibitor), cilostamide (PDE3 inhibitor), rolipram (PDE4 inhibitor), sildenafil (PDE5 inhibitor), IBMX (nonselective PDE inhibitor), dibutyl cAMP (stable cAMP analog), L-arginine (cGMP precursor), forskolin (adenylate cyclase activator), 2’5’-dideoxyadenosine (8) (adenylate cyclase inhibitor), and Rp-cAMPs (protein kinase A inhibitor).

RIA for cAMP

The effect of treatments used in growth assays on intracellular cAMP levels was investigated using a commercially available kit (NE-N Life Science Products). At selected time points (0, 1, 2, 4, 6, and 24 h), all treatments were terminated by aspiration of medium, and cells were lysed in 250 µl/well of acid/ethanol (750 ml of ethanol:248.5 ml of double-distilled H₂O:1.5 ml of concentrated HCl) containing IBMX (500 µM). Plates were sealed with cellophane and kept at −20°C overnight to complete cell lysis. One hundred twenty-five microliters were then removed from each well and vacuum-dried overnight. RIA for cAMP was performed according to the manufacturer’s instructions, and the mean cAMP concentration/well was calculated. Results were expressed as pmol/10⁶ cells for each treatment time point.
In Situ Perfused Rat Lung

The effect of agents on mean pulmonary artery pressure (MPAP) during acute hypoxic pulmonary vasoconstriction was investigated using a previously described in situ perfused rat lung preparation (40) using adult male Wistar rats (300 g). On establishing the perfusion circuit, we allowed MPAP to equilibrate for 20 min before bolus injection of angiotensin II (0.1 μg/l) into the main pulmonary artery (to augment subsequent acute hypoxia-induced pressor responses). After 10 min of equilibration, hypoxic pulmonary vasoconstriction (HPV1) was induced (FIO2 2%). Treatments were administered by bolus (10 μl) injection into the pulmonary artery with these initial concentrations: cicaprost (0.1 mM), vinpocetine (40 mM), EHNA (10 mM), cilostamide (10 mM), rolipram (10 mM), and IBMX (500 mM). Individual treatments were interspersed by 10-min equilibration periods. For combinations of agents, treatments were not washed out before administration of the second agent. On completion of treatment administration, normoxic ventilation was resumed before repeat hypoxic challenge (HPV2).

In a subset of experiments, 100-μl samples of left atrial effluent were collected in tubes containing 15 μl of ice-cold EDTA before and 10 min after peak in HPV1, as well as immediately before and 2 min after peak response to bolus injection of cicaprost, cilostamide, or rolipram. Animals treated with cicaprost subsequently received a bolus injection of rolipram, with repeat sampling 2 min after peak response. All samples were kept on ice until the end of each individual experiment and were then centrifuged at 2,500 g (15 min, 4°C) to separate plasma. Plasma samples underwent cyclic nucleotide RIA.

Chronic Hypoxia Studies

Adult male Wistar rats (~300 g) were anesthetized (isoflurane, 0.6 l/min medical oxygen) for insertion of intraperitoneal osmotic minipumps (Charles River UK; 2ML2; capacity 2 ml, infusion rate 5 μl/h) as previously described (23). Treatments (n = 6 per group) included vehicle (10% DMSO), iloprost (0.2 mg·kg⁻¹·day⁻¹), cilostamide (2.92 mg·kg⁻¹·day⁻¹), rolipram (2.92 mg·kg⁻¹·day⁻¹), and ibuprofen (3 mg·kg⁻¹·day⁻¹). Treatments were administered subcutaneously at 24 h intervals. On completion of treatment administration, animals were returned to individual enriched cages and allowed 7 days of recovery. Sequential hypoxic challenges were performed on the seventh day of treatment administration, followed by 7 days of recovery before repeat hypoxic challenge (HPV2).
Table 2. RT-PCR expression profile of PDE isozyme and subtype mRNA in rat pulmonary and systemic VSMC

<table>
<thead>
<tr>
<th>PDE</th>
<th>Pulmonary</th>
<th>Systemic</th>
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<tr>
<td></td>
<td>Peripheral PASMC</td>
<td>Main PASMC</td>
</tr>
<tr>
<td>1AB</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>1C</td>
<td>+</td>
<td>+</td>
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<td>2</td>
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<td>+</td>
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<tr>
<td>3A</td>
<td>+</td>
<td>+</td>
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<tr>
<td>3B</td>
<td>+</td>
<td>+</td>
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<td>4A</td>
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<td>4C</td>
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<td>9</td>
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<td>10A</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

VSMC, vascular smooth muscle cells; PASMC, pulmonary artery smooth muscle cells.

In rats, iloprost plus cilostamide, iloprost plus rolipram, and rolipram plus cilostamide.

Animals were housed for 13 days under hypoxic (10% FIO2) conditions, as previously described (40). A further vehicle group (n = 6) was maintained under normoxic conditions for 13 days.

On day 13, MPAP and systemic blood pressure were measured in the anesthetized animals (pentobarbital sodium, 60 mg/kg ip) as previously described. The right ventricle (RV), left ventricle (LV), and septum (S) were each weighed, and an index of right ventricular mass (RV/LV + S) was calculated. The right lung was rapidly frozen and septum (S) were each weighed, and an index of right ventricular mass (RV/LV + S) was calculated. The right lung was rapidly frozen and septum (S) were each weighed, and an index of right ventricular mass (RV/LV + S) was calculated. The right lung was rapidly frozen and septum (S) were each weighed, and an index of right ventricular mass (RV/LV + S) was calculated. The right lung was rapidly frozen and septum (S) were each weighed, and an index of right ventricular mass (RV/LV + S) was calculated. The right lung was rapidly frozen and septum (S) were each weighed, and an index of right ventricular mass (RV/LV + S) was calculated. The right lung was rapidly frozen and septum (S) were each weighed, and an index of right ventricular mass (RV/LV + S) was calculated.

Assessment of PDE Isoform Expression By RT-PCR

In rat distal PASMC, mRNA species were detected for PDEs 1, 2, 3, 4, 5, 7, and 9. RT-PCR products for PDE4C, PDE8, and PDE10 were not detected. PDE8 and PDE10 were detectable, however, in samples of rat brain RNA. Isoform 4C was absent in distal PASMC but was detectable in cells from systemic arteries (Table 2). We also compared PDE mRNA expression in cultured cells isolated from main pulmonary artery, aorta, coronary arteries, and renal arteries of the rat. Although most isoforms were similarly expressed in cells, there was subtle variation in PDE mRNA expression between cells from different vascular beds and between cells isolated from proximal and distal pulmonary artery (summarized in Table 2).
The predominant isoforms responsible for cAMP hydrolysis were PDE3 and PDE4.

**Effects of Manipulation of cAMP on Cell Growth**

Treatment of rat distal PASMC with cicaprost, iloprost, dibutyryl cAMP, or forskolin resulted in a concentration-dependent attenuation of PDGF-BB-induced proliferation (Fig. 2). Similar responses were observed with isoproterenol (data not shown). The similarity of response to these agents, all of which elevate intracellular cAMP, suggested that these effects were likely to be cAMP dependent. Treatment of distal PASMC with PDE 1–4 inhibitors and the nonselective inhibitor IBMX also inhibited PDGF-BB-induced proliferation (Fig. 3). However, the PDE2 inhibitor EHNA only reduced[^1H]thymidine incorporation at a concentration (0.1 mM) associated with markedly reduced cell viability as assessed by trypan blue exclusion. Thus pharmacological concentrations of EHNA had no significant effect on[^1H]thymidine incorporation. A similar degree of inhibition was also observed with the cGMP PDE inhibitor sildenafil, which was included in these experiments for comparison. A comparison was then made of the effects of cicaprost or isoform-selective PDE inhibition in cells from distal pulmonary arteries, main pulmonary arteries, and systemic VSMC. In distal and main PASMC, all agents except EHNA (at nontoxic concentrations) inhibited PDGF-BB-induced DNA synthesis (Fig. 4, A and B). Similar results were observed in cells from the aorta and renal arteries (data not shown). However, some variation in the magnitude of these responses was evident between vascular beds. For example, at individual treatment concentrations, the anti proliferogenic effect of cicaprost on coronary artery smooth muscle cells was of lesser magnitude than for proximal and distal PASMC (Fig. 4C).

The combination of cyclic nucleotide agonists with isoform-selective or nonselective PDE inhibitors elicited a synergistic effect on inhibition of cellular proliferation (Fig. 5A). A similar effect was evident on coadministration of cilostamide with rolipram. We tested the combined effects of cicaprost and rolipram at concentrations at which, individually, no effect on PDGF-BB-induced DNA synthesis was seen. The combination of “subthreshold” concentrations of these agents was able to
inhibit PASMC DNA synthesis (Fig. 5B). Similar effects were observed with the appropriate concentrations of cilostamide and rolipram (not shown). Cell counting studies confirmed that the observed effects on \[^3H\]thymidine incorporation were a reflection of cell proliferation (Fig. 6). Again, the combination of cicaprost with either rolipram or cilostamide had a more profound effect on proliferation than either agent alone. The antimitogenic effect of prostacyclin analogs and PDE inhibitors on PDGF-induced \[^3H\]thymidine incorporation could be partially or wholly reversed by coadministration with the adenylyl cyclase inhibitor 2',5'-dideoxyadenosine (DDA; 1 \(\mu M\)) and the protein kinase A antagonist Rp-cAMPS (50 \(\mu M\)) (Fig. 7), suggesting this effect was dependent on cAMP.

Effects of Prostacyclin Analogs and cAMP PDE Inhibitors on Intracellular cAMP

Treatment of rat distal PASMC with cicaprost (1 \(\mu M\)) resulted in a rapid and marked elevation in intracellular cAMP concentration evident at 1 h with subsequent reduction to control levels by 4–6 h (Fig. 8). Although significant elevation in intracellular cAMP compared with controls was also demonstrated in aortic and coronary artery cells following treatment with cicaprost, peak elevation was of lesser magnitude than in PASMC (not shown). Treatment of rat distal PASMC with cilostamide or rolipram resulted in modest, although more sustained, elevation in intracellular cAMP (Fig. 8). Peak levels of cAMP after treatment with cilostamide or rolipram occurred at later time points (2–6 h) than for cicaprost. Elevation in cAMP was not evident following treatment with vinpocetine or EHNA (data not shown). Combination of cicaprost with cilostamide or combination of cilostamide with rolipram resulted in an at least additive effect on peak cAMP (Fig. 8). The combination of cicaprost with cilostamide or combination of cilostamide with rolipram also seemed to change the dynamics of the rise in cAMP compared with rolipram or cilostamide alone. Thus the peak cAMP level was of greater magnitude, but of lesser duration, with combinations of PDE inhibitors (Fig. 8).

In Situ Perfused Rat Lung

To study the acute vasodilator effects of cAMP, experiments were conducted in the isolated perfused rat lung. Ventilation with hypoxic gas mixture (\(F_{\text{IO}_2}\) 2%) led to a prompt and sustained rise in MPAP (Fig. 9A). Addition of cicaprost (10 \(\mu l\) of 0.1 mM) elicited a rapid and sustained attenuation of HPV1 (Fig. 9B). Subsequent injection of rolipram after 10 min caused a further reduction in MPAP (Fig. 9B). Changing the order of administration of rolipram and cicaprost achieved the same effect, and the combination resulted in a greater fall in MPAP than either agent alone (Fig. 9C). Pulmonary artery pressure was reduced to the normoxic baseline by sequential administration of these agents. Further hypoxic challenge was thereafter ineffective in eliciting a significant pulmonary pressor response (HPV2; Fig. 9C). Bolus injection of DMSO (10%), used as the solvent for some compounds, or PBS did not significantly change pulmonary artery pressure during HPV1 (Fig. 9C). The combination of rolipram followed by cilostamide also resulted in a greater fall in MPAP than either agent alone (Fig. 9D). The mean changes in MPAP for all agents and combinations of agents used are presented in Table 3. EHNA demonstrated no effect on acute HPV1 on bolus administration.
Fig. 4. Effect of 24-h treatment with cicaprost and isoform-selective PDE inhibitors on PDGF-induced [³H]thymidine incorporation in Wistar-Kyoto rat cultured distal PASMC (n = 8 cell lines) (A), main PASMC (n = 4) (B), and coronary artery SMC (n = 4) (C). *P < 0.05, **P < 0.01, ***P < 0.001.
Fig. 5. A: effect of cicaprost, cilostamide (PDE3 inhibitor), and rolipram (PDE4 inhibitor) on PDGF-induced [3H]thymidine incorporation in Wistar-Kyoto rat cultured distal PASMC. Combinations of these agents elicited a synergistic effect on attenuation of proliferation. B: combining subthreshold concentrations of these agents also elicited inhibition of proliferation. These are mean data from 3 experiments in distinct rat distal PASMC lines; each experiment involved quadruplicate measurements for individual data points. *P < 0.05, **P < 0.01, ***P < 0.001.

Fig. 6. Growth curves demonstrating effect of cicaprost, cilostamide (PDE3 inhibitor), rolipram (PDE4 inhibitor), and combinations of cicaprost plus PDE inhibitors on mitogen (10% FCS)-induced Wistar-Kyoto rat distal PASMC proliferation. Data are from 3 experiments in distinct rat distal PASMC lines; each experiment involved quadruplicate measurements for individual data points. *P < 0.05, **P < 0.01 vs. vehicle-treated cells.
at a pharmacological concentration (10 mM), but at a higher concentration (100 mM), it resulted in vasorelaxation of similar magnitude to IBMX.

HPV1 was associated with a modest nonsignificant reduction in left atrial effluent cAMP and cGMP concentrations (Table 3). Individual treatments were associated with elevation in effluent concentrations of cAMP (cicaprost and rolipram), cGMP (vinpocetine), or both cyclic nucleotides (cilostamide and IBMX). Sequential administration of cicaprost and rolipram led to additive elevation in effluent cAMP concentration. Correlation between MPAP and cAMP or cGMP levels per se was poor ($r = 0.503$, $P < 0.05$ and $r = 0.294$, $P < 0.05$, respectively).

Chronic Hypoxia Studies

We next determined the effects of manipulation of cAMP on chronic hypoxia-induced pulmonary hypertension and vascular remodeling in vivo. Exposure of animals to chronic hypoxia was associated with significant elevation in MPAP, RV mass index (RV/LV+S), and pulmonary vascular muscularization ($P < 0.001$, $P < 0.01$, and $P < 0.01$, respectively) compared with normoxic controls (Table 4). Treatment with iloprost plus cilostamide, iloprost plus rolipram, and cilostamide plus rolipram resulted in synergistic attenuation in MPAP, RV mass index, and vessel muscularization compared with hypoxic controls, whereas these parameters were not significantly affected by single-agent treatment. There was no significant difference in mean systemic blood pressure between individual groups.

Lung homogenate cyclic nucleotide levels were measured in normoxic and chronically hypoxic animals and in groups of animals exposed to hypoxia plus treatments. There was no statistically significant difference in lung homogenate cAMP concentrations between animals exposed to normoxia or hypoxia or any of the chronic treatment groups under hypoxic conditions. Only chronic treatment with cilostamide plus rolipram resulted in a small, but statistically significant, elevation in whole lung cGMP compared with hypoxic controls ($437 ± 26$ pmol/mg of protein vs. $391 ± 15$ pmol/mg of protein, $P < 0.05$).

DISCUSSION

This study has produced novel data regarding the PDE isoform and subtype expression profile in cultured PASMC. Furthermore, this study has clearly demonstrated the potential role for combining prostacyclin analogs with PDE inhibitors in the treatment of pulmonary hypertension.

RT-PCR was used to characterize PDE mRNA expression in cultured rat pulmonary and systemic VSMC. These experiments revealed that the majority of PDE isoforms and subtypes are expressed in rat VSMC. PDE10 was the only isoform not detected in any VSMC line. The PDE mRNA expression profile in VSMC showed variation between vascular beds that did not appear to clearly delineate by organ of origin or the relative caliber of vessels investigated. For example, mRNA for PDE1AB and PDE9 were detected in distal PASMC but not in cells from the larger caliber main pulmonary arteries, and comparison of PDE profile in macrovascular smooth muscle cells revealed that mRNA for PDE4C was expressed in main PASMC but not in aortic smooth muscle cells.
The biological relevance of observations regarding PDE expression profiles generated by RT-PCR may be viewed as somewhat limited without concurrent experimental data interrogating functional implications of mRNA expression (i.e., translation to active enzyme). In the current study, quantitation of functional enzyme activity was performed using PDE assay for cAMP hydrolysis. These experiments suggested that the PDE isoform activity profile is broadly similar in all rat pulmonary and systemic VSMC. In these cells, cAMP hydrolysis appeared to be predominantly catalyzed by PDEs 3 and 4.

In DNA incorporation studies, prostacyclin analogs, dibutyryl cAMP, isoproterenol, forskolin, L-arginine, and isoform-
Table 4. Effect of chronic treatment with iloprost, rolipram, cilostamide, and combinations on indexes of chronic hypoxia-induced pulmonary hypertension

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MPAP, mmHg</th>
<th>Mean Systemic BP, mmHg</th>
<th>RV/LV+S</th>
<th>Distal Vessel Muscularization, %</th>
</tr>
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<tbody>
<tr>
<td>Normoxia</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Vehicle</td>
<td>14.2±1.1‡</td>
<td>105.4±3.0</td>
<td>0.22±0.01†</td>
<td>8.51±0.26‡</td>
</tr>
<tr>
<td>Hyoxia</td>
<td></td>
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<tr>
<td>Vehicle, (10% DMSO)</td>
<td>30.0±2.1</td>
<td>100.8±4.3</td>
<td>0.39±0.02</td>
<td>12.79±1.08</td>
</tr>
<tr>
<td>Rolipram (2.92 mg·kg⁻¹·day⁻¹)</td>
<td>26.1±1.6</td>
<td>103.0±5.6</td>
<td>0.32±0.02</td>
<td>12.12±2.22</td>
</tr>
<tr>
<td>Cilostamide (2.92 mg·kg⁻¹·day⁻¹)</td>
<td>26.7±1.6</td>
<td>99.7±4.3</td>
<td>0.32±0.02</td>
<td>12.38±6.95</td>
</tr>
<tr>
<td>Iloprost (0.2 mg·kg⁻¹·day⁻¹)</td>
<td>24.7±0.7</td>
<td>101.0±5.1</td>
<td>0.34±0.02</td>
<td>14.19±1.15</td>
</tr>
<tr>
<td>Iloprost + rolipram</td>
<td>16.3±0.7†</td>
<td>99.0±3.2</td>
<td>0.27±0.01†</td>
<td>9.92±3.88†</td>
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<tr>
<td>Iloprost + cilostamide</td>
<td>16.4±1.3†</td>
<td>98.8±4.6</td>
<td>0.26±0.01†</td>
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<td>Cilostamide + rolipram</td>
<td>19.7±2.0*</td>
<td>101.0±4.0</td>
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<td>9.94±3.57*</td>
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*P < 0.05, †P < 0.01, ‡P < 0.001 compared with hypoxic control group. N = 6 animals/treatment group. RV/LV+S, right ventricle/left ventricle + septum; BP, blood pressure.
pressor response to acute hypoxia (HPV2). Such augmentation occurred regardless of whether cicaprost preceded or followed PDE inhibition. The fact that bolus administration of EHNA at 10 mM concentration did not affect HPV1 and was not associated with change in effluent cyclic nucleotide levels, whereas bolus administration at 10⁻¹ M concentration caused very significant attenuation of HPV1 and elevation in both effluent cAMP and cGMP to similar magnitude as the nonselective PDE inhibitor IBMX, suggests that the higher concentration may have been associated with loss of isoform selectivity. This may indicate that PDE2 activity in rat pulmonary arteries is low or negligible (as is the case in humans) and/or does not significantly contribute to cyclic nucleotide metabolism in PASMC. Our findings regarding the vasorelaxant effects of prostacyclin analogs and PDE inhibitors in the isolated perfused hypoxic lung are supported by work from other groups using isolated arterial ring preparations (7, 13, 16, 35, 37) or acute pulmonary hypertension induced by thromboxane analogs (e.g., U-46619) (27–29).

Few previous studies have investigated the potential role of cyclic nucleotides in mediating such acute changes in pulmonary vascular tone. In our study, acute HPV was associated with modest, statistically insignificant diminution in pulmonary vascular effluent cAMP and cGMP levels. At the doses selected, acute administration of cicaprost, cilostamide, rolipram, and IBMX caused significant elevation in effluent plasma levels of cAMP compared with pretreatment (HPV1) levels (Table 3). Vinpocetine, cilostamide, and IBMX elicited significant elevation in cGMP. These findings are consistent with the probable mechanism of action of these agents and the RIA findings in cultured VSMC. PDE1 is known to preferentially hydrolyze cGMP, PDE4 preferentially metabolizes cAMP, and PDE3 shows similar Kᵣ for cGMP and cAMP. The nonselective agent IBMX is known to inhibit both cAMP (e.g., PDE3 and PDE4)-metabolizing and cGMP (e.g., PDE1 and PDE2)-metabolizing isoforms.

In the current study using a hypoxic rat model of chronic pulmonary hypertension, treatment with single-agent iloprost, cilostamide, or rolipram did not significantly attenuate the development of pulmonary hypertension. There may be several potential explanations for this. It is possible that the dose of single agents was subtherapeutic and insufficient to elicit adequate cyclic nucleotide elevation to attenuate remodeling. Alternatively, if active drug plasma concentrations were actually achieved, it may be that the level and/or duration of cyclic nucleotide elevation with single agents was insufficient to attenuate the vasoconstrictive and promitogenic stimulus of chronic hypoxia. One potential mechanism for the lack of therapeutic effect seen with single agents may have been PDE upregulation. Isoform upregulation (either of targeted or alternative isoforms) may have compensated for initial elevation in intracellular cyclic nucleotides. Previous studies in alternative cell types (e.g., monocytes) have reported upregulation of specific PDE isoforms and subtypes in response to cyclic nucleotide agonists (32). Furthermore, the development of chronic hypoxia-induced pulmonary hypertension per se has been shown to result in isoform upregulation in rat pulmonary arteries (21). It may be that combination treatments used in the current study were the only treatments to successfully elevate cyclic nucleotides for sufficient duration and/or to sufficient levels to attenuate pulmonary vascular remodeling.

As with acute HPV, the development of chronic hypoxia-induced pulmonary hypertension did not result in significant changes in whole lung cGMP or cAMP compared with normoxic controls. With the exception of combined treatment with cilostamide and rolipram, whole lung cyclic nucleotide levels were not significantly influenced by single-agent or combination therapy. The reason for the modest elevation in cGMP in the cilostamide plus rolipram group is unclear, although this presumably reflects the cGMP PDE activity of cilostamide.

The lack of change in whole lung cyclic nucleotide levels with chronic hypoxia (regardless of treatment) may have several potential explanations. First, it may be that whole lung analysis is insufficiently sensitive in representing concurrent changes in pulmonary vascular cyclic nucleotide levels. Second, therapeutic elevation in cyclic nucleotide levels may have occurred earlier than the 13-day time point (potentially preceding metabolic compensation). It is unlikely that rapid metabolism of cyclic nucleotides occurred in the period between removal of animals from the hypoxic chamber to completion in measurement of hemodynamic data, since these measurements were made during continuing drug administration via osmotic pump.

In conclusion, the current study supports the use of isoform-selective PDE inhibitors as a potentially novel therapeutic approach in the treatment of pulmonary hypertension. Our data suggest that the combination of subtherapeutic concentrations of existing therapies (e.g., prostacyclin or iloprost) with these agents, or indeed the combination of individual PDE isoform inhibitors (e.g., PDE3 and PDE4), may lead to a therapeutic effect on acute and chronic pulmonary vascular response to hypoxia without significant systemic hypotension. Whether such an approach in clinical cases will result in a reduction in the side effect profile associated with full-dose individual drug therapy remains to be established.

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