Characterization of adenylyl cyclase isoforms in rat peripheral pulmonary arteries

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Characterization of adenylyl cyclase isoforms in rat peripheral pulmonary arteries. Am J Physiol Lung Cell Mol Physiol 280: L1359–L1369, 2001.—Activation of adenylyl cyclase (AC), of which there are 10 diversely regulated isoforms, is important in regulating pulmonary vascular tone and remodeling. Immunohistochemistry in rat lungs demonstrated that AC2, AC3, and AC5/6 predominated in vascular and bronchial smooth muscle. Isoforms 1, 4, 7, and 8 localized to the bronchial epithelium. Exposure of animals to hypoxia did not change the pattern of isoform expression. RT-PCR confirmed the presence of AC2, AC3, and AC5/6 transcripts in smooth muscle. Western blotting confirmed the presence of AC2, AC3, and AC5/6 proteins. Functional studies provided evidence of cAMP regulation by Ca2+ and protein kinase C-activated but not Gi-inhibited pathways, supporting a role for AC2 and a Ca2+-stimulated isoform, AC8. However, NKH-477, an AC5-selective activator, was more potent than forskolin in elevating cAMP and inhibiting serum-stimulated [3H]thymidine incorporation, supporting the presence of AC5. These studies demonstrate differential expression of AC isoforms in rat lungs and provide evidence that AC2, AC5, and AC8 are functionally important in cAMP regulation and growth pathways in pulmonary artery myocytes.

vascular smooth muscle; pulmonary hypertension; hypoxia; adenosine 3',5'-cyclic monophosphate; proliferation

SIGNALING OF VASOCITIVE MEDIATORS via G protein-coupled receptors (GPCRs) has been shown to regulate pulmonary vascular tone both under basal conditions and in the setting of acute and chronic pulmonary hypertension (35, 37). Activation of Gα-coupled receptors leads to activation of the enzyme adenylyl cyclase (AC), which converts ATP to cAMP, leading to vascular smooth muscle relaxation and inhibition of mitogenic pathways (15, 28, 38). Conversely, Gi agonist-receptor coupling leads to inhibition of AC activity (2, 26, 29, 30). Thus AC plays a pivotal role in the integration of tone or growth signals via cell surface GPCRs. In addition, in most systems, elevated intracellular cAMP antagonizes the effects of other vasoconstrictor or growth signals acting via Gq-coupled receptors and receptor tyrosine kinases (14, 25). In human airway smooth muscle, there is evidence that AC is the rate-limiting component in the Gq-AC signaling pathway (2). Despite the potentially critical role of AC in coordinating these diverse signals, little is known regarding the pattern of expression of AC isoforms present in the pulmonary circulation.

To date, 10 isoforms of AC have been cloned in mammals, each with a distinct set of regulatory elements and varying degrees of amino acid homology. Nine of these are membrane-bound proteins with 12 hydrophobic membrane-spanning domains. Based on their regulatory properties, particularly Ca2+ sensitivity (12) and amino acid homology, the AC isoforms have been classified into five subclasses (18). All isoforms are activated by the Gα subunit of the heterotrimeric G protein and by the diterpine forskolin (with possible exception of AC9) (47). Notably AC5 and AC6 are inhibited by Gα subunits. Ca2+/calmodulin can regulate AC activity in a positive (AC1 and AC8) (7, 12) or negative (AC3, AC5 and AC6) (36, 44, 45, 49) manner. Stimulation or inhibition of AC isoforms by protein kinase (PK) C-mediated phosphorylation is another tier in the complex regulation of these enzymes. AC2 has consistently been shown to be activated by PKC (20, 24, 50, 52). Thus PKC can modulate the responsiveness of AC and alter the ability of the enzyme to integrate signals derived from multiple hormonal inputs.

During the development of pulmonary hypertension, the pulmonary circulation becomes less responsive to vasodilators and structural remodeling of pulmonary arteries occurs, involving vascular cell proliferation and hypertrophy. Numerous studies have demonstrated a role for cAMP in the control of pulmonary vascular tone (28) and remodeling (5, 34). Of note, prostacyclin binds to a GPCR, the prostacyclin receptor, which stimulates AC via Gα and causes pulmonary vasodilatation and inhibition of smooth muscle...
proliferation by the elevation of cAMP. To begin to understand the key pathways regulating cAMP levels in the pulmonary vasculature, we characterized the mRNA and protein expression of AC isoforms and determined the predominant functional isoforms in pulmonary artery smooth muscle cells (PASMCs) isolated from the peripheral pulmonary circulation. This is the first study to systematically examine the cellular distribution of AC isoforms in the lung, the mRNA expression profile of multiple isoforms, and the functional contribution of PKC-stimulated, Gαi-inhibited, and Ca²⁺-sensitive isoforms to the regulation of cAMP in these cells. Our results demonstrate cell-specific localization of AC isoforms and provide evidence of a role for AC2, AC5, and AC8 in the regulation of cAMP in pulmonary artery myocytes.

METHODS

Materials. All chemicals were purchased from Sigma–Aldrich unless otherwise stated. NKH-477 was a gift from Makoto Hosono (Nippon Kayaku, Tokyo, Japan). Polyclonal antibodies to AC1, AC2, AC3, AC4, AC5/6, AC7, and AC8 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary antisera and ABC kits were purchased from Vector Laboratories. Tissue-Tek OCT compound was supplied by Raymond A. Lamb (Eastbourne, UK).

Animals. Adult male Wistar-Kyoto rats were obtained from Charles River (Margate, UK). The animals were fed standard rat chow, allowed free access to food and water, and studied at 10–14 wk of age. To induce pulmonary hypertension, groups of animals (n = 8/group) were exposed to hypoxia (10% inspired O2 fraction) for 7 or 21 days in a specially constructed environmental chamber previously described (1). Humidity and temperature were controlled and matched to normal laboratory conditions. Control animals were housed in the same room but outside the chamber.

Preparation of lung tissue. At the specified time point, the rats were killed with an overdose of ketamine (150 mg/kg im) and midazolam (3 mg/kg im). The lungs were harvested, and the right cardiac lobe was inflated with a solution containing 50% embedding medium for frozen tissue specimens (TissueTek OCT compound) in PBS. The tissue was placed in a cryomold, covered with embedding medium, and immediately frozen in isopentane cooled by liquid nitrogen.

Immunohistochemistry. Polyclonal antibodies specific for AC1, AC2, AC3, AC4, AC5/6, AC7, and AC8, combined with a horseradish peroxidase detection method, were used to investigate the distribution of AC isoforms in rat lung. AC isoforms 5 and 6 have a high degree of amino acid homology, and the polyclonal antibody cross-reacts with both enzymes. The specificity of these antibodies for AC isoforms has been previously demonstrated (29). Cryostat sections of OCT-embedded tissue were cut (8 μm thick), air-dried, and fixed in ice-cold acetone for 20 min. The sections were then washed with 0.1 M phosphate-buffered saline (PBS) for 5 min. To block endogenous peroxidase activity, the sections were immersed in 0.3% (vol/vol) hydrogen peroxide in methanol for 20 min. After three washes with PBS for 5 min each, the sections were incubated with nonimmune or normal serum from the animal (goat or horse) in which the secondary antibody was raised to block nonspecific binding of the secondary antibody for 30 min at a dilution of 1:30 [in PBS with 0.1% (wt/vol) BSA and 0.1% (wt/vol) sodium azide]. The sections were incubated overnight at 4°C with the primary antibody and washed three times with PBS for 5 min each. The sections were then incubated for 30 min with a rat-absorbed biotinylated secondary antibody (Vector Laboratories) against rabbit or goat as appropriate (1:100 in PBS and 0.1% BSA). The sections were then incubated with an avidin-biotin-peroxidase complex for 1 h (ABC Elite, Vector Laboratories) followed by three washes with PBS for 5 min each. The ABC complex was visualized with the 3,3'-diaminobenzidine (0.25%) and hydrogen peroxide method, which results in the formation of a brown reaction product. The sections were then washed with PBS (5 min) and under running tap water before being counterstained with hematoxylin and sequential dehydration in increasing alcohol concentrations to xylene. Finally, the sections were mounted with DePex mounting medium (BDH Laboratory Supplies).

Isolation of peripheral PASMCs. Rat PASMCs were isolated from precapillary pulmonary arteries with a modification of a previously described method (21). Briefly, the rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (60 mg/kg), and midline and lateral thoracotomies were performed to expose the trachea. The pulmonary artery was then cannulated through the right ventricle, the left atrium was incised, and the lungs were flushed with 10 ml of PBS at 37°C over 30 s. Fifteen milliliters of 0.5% iron oxide-0.5% agarose in Dulbecco’s modified Eagle’s medium (DMEM) at 45°C were then immediately infused into the pulmonary artery over 30 s, and the pulmonary artery was clamped. The trachea was cannulated, and the entire lung block was removed. The lungs were inflated by tracheal instillation of 1% (wt/vol) agarose in DMEM (40 ml/kg). The preparation was then transferred to ice-cold DMEM for 10 min until the agarose had set. Subpleural sections (1–2 mm thick) were sliced from the outer surface of the lungs and minced with a razor blade. The tissue was then partially digested with collagenase (80 U/ml of culture medium) for 60 min at 37°C and sheared by five strokes through progressively smaller gauge (18–25) needles to remove the surrounding parenchyma. The peripheral arteries were isolated with a magnetic separator (Promega), rinsed with 4°C DMEM, resuspended in 1 ml of DMEM-20% fetal bovine serum (FBS), and plated in 25-cm² tissue culture flasks. The flasks were incubated in humidified air with 5% CO₂ at 37°C. After adherence, 4 ml of culture medium were added to the flask 24 h later. After 10–14 days, a confluent layer of cells had grown from explanted arteries. The cells were trypsinized, passed into 75-cm² flasks (passage 1), and grown to confluence in DMEM-10% FBS. Subsequent passages were carried out by splitting the flasks 1:3. Cells were used for experiments between passages 2 and 6.

The phenotype of isolated cells was investigated with antibodies to smooth muscle-specific antigens: monoclonal anti–α-smooth muscle actin (IA4) and anti-smooth muscle-specific myosin (hsm-v). For immunostaining, cells were grown to subconfluence in eight-well slide chambers. Cells were fixed in acetone at −20°C for 10 min and then washed three times with PBS for 5 min each. The cells were incubated with primary antibody for 1 h at room temperature and then with anti-mouse FITC-conjugated secondary antibody for 1 h,
after transfer to nitrocellulose, the membranes were incubated with isofrom-specific primary antibodies to AC (Santa Cruz Biotechnology) overnight. The blots were washed with PBS containing 0.1% Tween 20 and incubated with the corresponding secondary antibody conjugated to horseradish peroxidase and developed by enhanced chemiluminescence (Amersham Pharmacia Biotech). Kaleidoscope prestaed standards (Bio-Rad) were used for molecular mass determinations.

Measurement of cAMP production. Rat PASMCS were plated at 20,000 cells/well into 24-well plates for the determination of cAMP synthesis. After 2 h of incubation with serum-free DMEM alone, the cells were pretreated for 1 h with the nonselective inhibitor of cyclic nucleotide phosphodiesterases, 3-isobutyl-1-methylxanthine (IBMX, 50 μM). The presence of PKC-activated AC isoforms was studied by incubating the cells (15 min) with phorbol 12-myristate 13-acetate (PMA; 10 μM) in the presence and absence of bisindolmaleimide (Bis), a potent, specific inhibitor of PRC (100 nM). The cells were preincubated with Bis for 1 h before the addition of PMA. The cells were incubated with the direct activator of ACs, forskolin, the forskolin derivative NKK-477 (6-[3-(dimethylamino)propionyl]forskolin), an AC5-selective forskolin derivative, or the Goα-coupled receptor agonist ciceprost (all 10 μM) for 15 min to determine the stimulated level of cAMP. The contribution of forskolin-stimulated AC isoforms sensitive to Giα inhibition (AC5 and AC6) was determined by coinubation for 15 min with the Goα-coupled receptor agonists carbachol (muscarinic 2 receptor agonist; 1 mM) or clonidine (α2-adrenergic receptor agonist; 1 mM). The contribution of the Ca2+-sensitive isoforms was determined by depletion of intracellular Ca2+ for 1 h with 1,2-bis(o-amiphenyloxy)ethane-N,N,N,N'-tetraacetic acid tetra(acetoxyethyl) ester (BAPTA-AM; 10–8 to 10–4 M; Calbiochem) in Ca2+-free medium containing 500 μM EGTA before stimulation with and without forskolin and by elevation of intracellular Ca2+ by 15 min of treatment with A-23187 or thapsigargin (10–8 to 10–4 M; Calbiochem). After the incubations, the medium was removed, 250 μl of acid-ethanol (0.15% HCl and 75% ethanol) were added, and the plates were placed at −20°C for 24 h before the lysates were dried in a rotary dryer. The samples were rehydrated with assay buffer, and

Table 1. Source of primer sequences and conditions used for RT-PCR

<table>
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<th>AC Isoform</th>
<th>Reference GenBank Accession No.</th>
<th>Primers</th>
<th>Denaturation Temperature, °C</th>
<th>Time, s</th>
<th>Annealing Temperature, °C</th>
<th>Time, s</th>
<th>Extension Temperature, °C</th>
<th>Time, s</th>
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<td>45</td>
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<td>70</td>
<td>60</td>
<td>35</td>
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<td>5/6</td>
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<td>60</td>
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<td>60</td>
<td>72</td>
<td>60</td>
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<tr>
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<td>180</td>
<td>35</td>
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AC, adenylyl cyclase.
cAMP levels were measured with a radioimmunoassay kit purchased from NEN Life Science Products.

Cell proliferation assays. The growth responses of rat PASMCs were determined by incorporation of \[^3H\]thymidine and by cell counts. For \[^3H\]thymidine incorporation, the cells were quiesced by incubation with DMEM-0.1% FBS for 72 h and then stimulated for 24 h with DMEM-10% FBS with and without PMA (10 \(\mu\)M); pertussis toxin (PTX; 200 ng/ml), which uncouples G\(_{\alpha}\) from its associated receptors; forskolin; or NKH-477 (10\(^{-9}\) to 10\(^{-4}\) M). The cells were then pulsed with [methyl-\(^3H\)]thymidine (AP Biotech) for 4 h, after which they were washed with PBS and incubated with 10% trichloroacetic acid for 30 min to precipitate the nucleic acid. The DNA was then dissolved in 0.2 M sodium hydroxide overnight, the samples were transferred into vials, and liquid scintillant was added. Incorporated thymidine was determined in a liquid scintillation counter (Canberra Packard Tri-Carb 1900CA).

For cell proliferation experiments, the cells were plated in 24-well plates (15,000/well) and serum deprived for 72 h before the addition of DMEM-10% FBS with and without forskolin or NKH-477 (both 10 \(\mu\)M). Medium and agonists were refreshed every 2 days. On days 1, 4, and 7, the cells were trypsinized and counted with a hemacytometer. Cell viability was confirmed by trypan blue exclusion.

Statistical analysis. Data points are means \(\pm\) SE. Significant differences between groups were assessed by one-way or two-way ANOVA as appropriate, with values of \(P < 0.05\) sufficient to reject the null hypothesis.

RESULTS

Distribution of AC isoforms in normoxic and hypoxic rat lungs. All AC isoforms studied were detected in rat lungs but exhibited an isoform-specific pattern of distribution (Table 2). AC isoforms AC2, AC5/6, and, to a lesser extent, AC3 were the predominant isoforms detected in pulmonary arterial smooth muscle (Fig. 1). AC7 and AC8 were present, albeit at low levels, in arterial smooth muscle. Bronchial smooth muscle also expressed AC2 and AC5/6, but AC3 was not detected (Fig. 1). Isoforms AC1, AC4, AC7, and AC8 were demonstrated in airway epithelium (Fig. 1). In contrast to the arterial media, venous smooth muscle showed prominent staining not only for AC4 and AC7 but also for AC5/6 (Fig. 1).

During exposure to chronic hypoxia, rats developed an increased density of muscularized arteries associated with alveolar ducts and alveolar walls in the lung periphery (Fig. 2). Immunostaining for AC isoforms showed a similar distribution in normoxic and hypoxic animals. However, newly muscularized arteries also demonstrated immunostaining for AC2 and AC5/6.

AC mRNA expression in peripheral rat PASMCs. Cells isolated from peripheral pulmonary arteries demonstrated the typical morphology of vascular smooth muscle cells. The smooth muscle cell phenotype of these cells was confirmed by positive immunofluorescence with anti-smooth muscle myosin and anti-\(\alpha\)-smooth muscle actin (data not shown). We employed RT-PCR to further characterize expression of the AC isoforms in peripheral rat PASMCs. RNA transcripts for AC2, AC3, AC5, AC6, AC7, and AC8 were present in the total RNA isolated from PASMCs (Fig. 4). Specificity of the reactions for RNA was confirmed by the absence of PCR products when the samples were run without RT. Electrophoresis of PCR products demonstrated bands of the predicted size for each AC isoform. Where we used primers based on sequence homology in other species, the identity of the PCR products was confirmed by direct sequencing. Consistent with the immunohistochemistry, RNA transcripts corresponding to AC1 and AC4 were not detected in rat PASMCs. As a positive control, AC1 transcripts were readily demonstrated in RNA isolated from the rat brain.

AC protein expression in rat PASMCs. To demonstrate the presence of AC isoform protein in rat PASMCs, membrane preparations of AC isoform-selective antibodies were used for immunoblotting. Immunoblotting confirmed the presence of isoforms AC2, AC3, and AC5/6 (Fig. 5). Specificity of the antibodies was confirmed by preadsorption of the primary antibody with the peptide to which the antibody was raised. No specific bands were identified with antibodies to isoforms AC1, AC4, AC7, and AC8. The molecular masses of the visualized bands were \(\sim 175\) kDa for all three isoforms. The sizes of the proteins detected were greater than predicted (120–140 kDa) from their amino acid sequences, and the bands were diffuse. However, it is likely that this is due to glycosylation of the AC proteins (see Discussion).

AC isoform identification by manipulation of regulatory pathways. To provide supporting enzymatic evidence for the importance of AC2, the Ca\(^{2+}\)-sensitive isoforms, and AC5/6 in rat PASMCs, we aimed to take advantage of the distinct mechanisms involved in the regulation of these isoforms. Stimulation of PASMCs with the PKC activator PMA in the presence of IBMX led to a fourfold increase in cAMP level (Fig. 6A). This PMA-induced increase in cAMP could be inhibited by the selective PKC inhibitor Bis. Activation of AC by PKC suggests the presence of AC2.

Isoforms AC5 and AC6 are the only isoforms known to be inhibited by the G protein G\(_{\alpha}\). Stimulation of the cells with forskolin or cicaprost, the stable prostacyclin mimetic, consistently increased cAMP levels (Fig. 6, B and C). To demonstrate the presence of G\(_{\alpha}\)-inhibitable AC isoforms, PASMCs were coincubated with the G\(_{\alpha}\)-coupled receptor agonists carbachol or clonidine (26, 29) and forskolin or cicaprost (Fig. 6, B and C). How-

<table>
<thead>
<tr>
<th>AC Isoform</th>
<th>Airway</th>
<th>Smooth Muscle</th>
<th>Vascular</th>
<th>Endothelium</th>
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<tr>
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Staining was graded from weak (+) to strong (+++++).
ever, neither carbachol nor clonidine resulted in inhibition of cAMP compared with forskolin or cicaprost alone. Further experiments with other G\(\alpha\)-coupled receptor agonists, oxymetazoline and uridine triphosphate, also failed to demonstrate significant inhibition of forskolin- or cicaprost-induced cAMP (data not shown).

At a concentration of 10 \(\mu\)M, NKH-477 induced a significantly greater increase in cAMP than forskolin, indicating the presence of AC5 in PASMCs (Fig. 6D).

A-23187, a Ca\(^{2+}\) ionophore, concentration dependently increased cAMP in the presence of IBMX (Fig. 7A). Furthermore, thapsigargin treatment, which blocks Ca\(^{2+}\) reuptake into the sarcoplasmic reticulum leading to increased intracellular Ca\(^{2+}\), caused a concentration-dependent increase in cAMP (Fig. 7B). Moreover, 1 h of preincubation of cells in Ca\(^{2+}\)-free conditions with BAPTA-AM, a cell-permeable Ca\(^{2+}\) chelator, inhibited basal cAMP (Fig. 7C). BAPTA-AM also caused an inhibition of forskolin-stimulated cAMP (data not shown).

**Effect of AC activation on PASMC growth.** Functional evidence for the presence of AC2 and AC5 was provided by growth experiments. Incubation of PASMCs with PMA resulted in inhibition of [\(^3\)H]thy-
midine uptake (Fig. 8A), consistent with a stimulatory role of PKC in cAMP production in these cells, likely to be an effect on AC2.

Consistent with the cAMP data, a 24-h exposure to carbachol had no effect on serum-stimulated thymidine uptake (Fig. 8A). However, inhibition of G\(_\text{i}\) by 6 h of pretreatment with PTX resulted in a decrease in \([^3H]\)thymidine uptake into cells stimulated for 24 h with 10% FBS (Fig. 8A), suggesting the presence of G\(_\text{i}\) receptors, possibly coupled to growth. (Trypan blue exclusion assay was unaffected by 24 h of PTX; data not shown.)

Treatment of serum-stimulated PASMCs with forskolin led to a concentration-dependent inhibition of \([^3H]\)thymidine incorporation (Fig. 8B). The forskolin derivative NKH-477, which selectively activates AC5, inhibited \([^3H]\)thymidine incorporation with a potency that was 10-fold greater than that for forskolin (log EC50: forskolin, -4.975; NKH-477, -5.981; Fig. 8B). In cell proliferation assays, both forskolin and NKH-477 inhibited serum-stimulated cell proliferation at 4 and 7 days, although NKH-477 produced significantly more inhibition of cell proliferation than forskolin (Fig. 8C). These data provide evidence...
for the involvement of AC isoforms in the regulation of PASMC growth and support a specific role for AC5 in this response.

**DISCUSSION**

These results demonstrate the cell-specific localization of AC isoforms within the lung. Immunohistochemical studies indicated that the AC isoforms AC2, AC3, AC5, and AC6 are present in the media of rat pulmonary arteries, whereas AC1, AC4, AC7, and AC8 predominate in the epithelium of airways. Studies in PASMCs isolated from the peripheral pulmonary circulation demonstrated mRNA expression of AC2, AC3, AC5, AC6, AC7, and AC8, although Western blotting for AC isoform proteins revealed that AC2, AC3, AC5, and AC6 predominate at the level of protein expression. Functional studies utilizing the known sensitivities of specific isoforms to regulation by PKC (AC2), G_i (AC5 and AC6), and a selective activator of AC5 (NKH-477) provided further evidence of a major role for AC2 and AC5 in the regulation of intracellular cAMP in these cells. The results also demonstrate the presence of a Ca^{2+}-stimulated AC isoform in PASMCs, which is likely to be AC8 on the basis of the recognized Ca^{2+}-stimulated activity of this isoform (7).

Although previous studies have investigated the role of specific AC isoforms in systemic (AC3) (51) and pulmonary (AC2) (13) vascular smooth muscle cells, in the present study, we determined the cellular localization of multiple isoforms and examined their integrated function in cells isolated from the peripheral pulmonary vasculature.
AC2 (10, 33), AC3 (46, 48), AC4 (11), AC5 (33), AC6 (33), and AC7 (43) have been detected in whole lung RNA preparations by either Northern blot or RT-PCR in various animals; however, few previous studies have addressed the localization of AC isoforms within the lung. Guldemeester et al. (13) previously showed medial staining of AC2 in small and large neonatal and adult bovine pulmonary arteries. AC2, AC4, AC6, and AC7 mRNAs were detected in bovine pulmonary artery endothelial cells (39). Billington et al. (2) identified AC2, AC6, AC7, and AC9 in human airway smooth muscle, with AC6 and AC9 the predominant isoforms, although a later report (6) with a different RT-PCR system detected all isoforms except AC2 and AC8.

During chronic hypoxia, we observed an increase in the density of muscularized peripheral pulmonary arteries as described in RESULTS and in Fig. 2. Immunostaining did not reveal any changes in the level of expression or cellular localization of AC isoforms during chronic hypoxia. However, all newly muscular arteries exhibited the same pattern of AC isoform expression as normoxic muscular arteries.

Isoforms AC2, AC3, and AC5/6 observed by Western blots in arterial smooth muscle are ~40 kDa heavier.
than expected and appear as diffuse bands of protein. However, the absence of bands when the primary antibody was preadsorbed by coincubation with the blocking peptide confirmed the specificity of the antibody for AC isoforms. From an initial cloning study and sequence analysis, AC2 has a predicted size of 1,090 amino acids or 123 kDa (10). AC3 is expected to have 1,144 amino acids (~129 kDa; 48). AC5/6 is predicted to have 1,184 (19) or 1,165 (49) amino acids, whereas AC6 is anticipated to have 1,166 amino acids (23), which summates to ~139 kDa. The discrepancies between observed and predicted sizes and diffuse as opposed to tight bands are probably accounted for by N-linked glycosylation at consensus sites present in all AC isoforms in the second hydrophobic domain (M2) (38). Deglycosylation of AC8 reduced the molecular mass of the enzyme by 40 kDa as determined by Western blot (3). Furthermore, glycosylation of a protein results in diffuse bands due to uneven migration of the protein through the gel, partially because SDS does not evenly coat sugar residues, resulting in unevenly charged proteins. Thus the diffuse and the ~40-kDa heavier than expected protein bands obtained with AC-selective antibodies are likely to be glycosylated forms of the protein.

The results of immunohistochemistry in lung sections and analysis of PASMC mRNA and protein expression were broadly concordant in our study. However, we were unable to convincingly detect AC7 and AC8 protein expression by Western blotting despite the low-level expression of the isoforms observed in the arterial media by immunohistochemistry and the presence of specific mRNA transcripts by RT-PCR. This presumably reflects the low abundance of AC7 and AC8 protein in cultured PASMCs.

Having identified the expression pattern of AC isoforms in rat peripheral PASMCs, we sought to provide functional evidence for the involvement of specific isoforms in the regulation of cAMP in these cells. AC2 has consistently been shown to be stimulated by PKC (20, 24, 50, 52), and isoforms 5 and 6 are the only 2 isoforms to be inhibited by Gα proteins as well as stimulated by Gα protein (41). PMA stimulation and Bis inhibition of PMA-stimulated cAMP production indicate the presence of an AC isoform in which activity is upregulated by PKC. Thus we may conclude that AC2 is functionally important in rat PASMCs, supporting the mRNA and protein expression data. We were unable to demonstrate Gα inhibition of AC activity in our cells. There are a number of possible explanations for this. One possibility is loss of Gα-coupled receptors in culture, although this does not appear to have been a significant problem in a previous study (2). In addition, we used a range of Gα-coupled receptor agonists without response, and it seems unlikely that all Gα receptors would be lost in culture. Moreover, we were able to demonstrate that PTX inhibited serum-stimulated growth, suggesting the presence of functional Gα receptors. A more likely explanation is the predominance of AC2 in these cells. Our demonstration of AC2 as a dominant isoform in peripheral rat PASMCs is in contrast to findings in human airway smooth muscle where AC2 was one of two isoforms not detected by RT-PCR (6). However, this disparity may explain why in human airway smooth muscle cells, stimulation of forskolin-treated cells with a Gα agonist, carbachol, led to a reduction in cAMP (2), but in rat PASMCs, we did not observe this effect due to the presence of AC2, which is activated by the Gbg subunit (9, 32, 40) released concomitantly with Gα. Thus any inhibition of AC5 or AC6 resulting from Gα activation is masked by simultaneous activation of AC2 by Gbg. Nevertheless, we were able to provide evidence for the potential involvement of AC5 in the growth responses of PASMCs; NKH-477, a forskolin derivative that is selective for AC5 (42), stimulated cAMP to a greater extent than forskolin and was a more potent inhibitor of rat peripheral PASMC growth than forskolin as demonstrated by inhibition of cell proliferation and thymidine uptake.

Although there has been uncertainty in the past regarding the Ca2+ sensitivity of AC isoforms, there is now a consensus that AC1 and AC8 are both stimulated by low Ca2+ concentrations (7, 12). AC3 activity in vivo is inhibited by an elevation in intracellular Ca2+ (7, 45). PASMCs responded to increased intracellular Ca2+ from both extracellular and intracellular stores with elevated cAMP synthesis, consistent with the presence of AC8, the expression of which was also demonstrated by RT-PCR. AC3 and low-level AC8 have previously been reported in rat systemic vascular smooth muscle cells (51). The authors demonstrated Ca2+-dependent stimulation of AC in these cells and concluded that the results were consistent with AC3 activity. However, based on recent observations (7, 44, 45), Ca2+-dependent stimulation is attributable to the presence of AC8 in that study.

It is well documented that elevation of cAMP through either AC activation or phosphodiesterase inhibition results in decreased proliferation of vascular smooth muscle cells (16, 17, 22). The pathway involves PKA activation preceding phosphorylation of the transcription factor cAMP response element binding protein (5, 27). Interestingly, activation of PKC-stimulated AC2 and cAMP accumulation stimulates growth of neonatal but not of adult bovine PASMCs (13), possibly through activation of extracellular signal-regulated kinase by cAMP (8). These results suggest developmental differences in the regulation of growth by cAMP.

Is there potential for AC isoform-selective therapies in pulmonary hypertension? Development of isoform-selective stimulator compounds for AC would allow selective manipulation of cAMP in the smooth muscle or endothelium of the pulmonary vasculature. NKH-477 is an example of an AC5-selective forskolin derivative (42). Further development of compounds such as this may produce agonists with relative selectivity for vascular smooth muscle. Although our in vitro data are encouraging, it remains to be seen whether chronic treatment over a longer period of time may inhibit and possibly reverse vascular remodeling.
In summary, this study has defined the cell-specific localization of AC isoforms in the rat lung and has characterized in detail AC isoform expression in PASMCs derived from the peripheral pulmonary circulation of the rat. In addition, we have provided evidence that AC2, AC5, and AC8 play key roles in the regulation of cAMP in these cells. Finally, our results suggest that specific AC isoforms are involved in the regulation of growth-inhibitory pathways in pulmonary vascular smooth muscle.

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