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Mechanisms regulating cAMP-mediated growth of bovine neonatal pulmonary artery smooth muscle cells

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Guldemeester, Alexandra, Kurt R. Stenmark, George H. Brough, and Troy Stevens. Mechanisms regulating cAMP-mediated growth of bovine neonatal pulmonary artery smooth muscle cells. *Am. J. Physiol.* 276 (*Lung Cell. Mol. Physiol.* 20): L1010–L1017, 1999.—Neonatal pulmonary artery smooth muscle cells (PASMCs) exhibit enhanced growth capacity and increased growth responses to mitogenic stimuli compared with adult PASMCs. Because intracellular signals mediating enhanced growth responses in neonatal PASMCs are incompletely understood, we questioned whether *1*) G_q agonists increase cAMP content and *2*) increased cAMP is proproliferative. Endothelin-1 and angiotensin II increased both cAMP content and proliferation in neonatal but not in adult PASMCs. Inhibition of protein kinase C and protein kinase A activity nearly eliminated the endothelin-1- and angiotensin II-induced growth of neonatal PASMCs. Moreover, cAMP increased proliferation in neonatal but not in adult cells. Protein kinase C-stimulated adenylyl cyclase was expressed in both cell types, suggesting that insensitivity to stimulation of cAMP in adult cells was not due to decreased enzyme expression. Our data collectively indicate that protein kinase C stimulation of cAMP is a critical signal mediating proliferation of neonatal PASMCs that is absent in adult PASMCs and therefore may contribute to the unique proproliferative phenotype of these neonatal cells.

adenosine 3′,5′-cyclic monophosphate; lung; development; endothelin-1; angiotensin II; signal transduction

ADAPTATION OF THE PULMONARY CIRCULATION to postnatal life is a process that requires both growth and differentiation of vascular wall cells. In smooth muscle cells (SMCs), there is a transition from a fetal to a more adultlike phenotype (27). Several studies (15, 26) have demonstrated that when the normal transition to postnatal life is interrupted by hypoxia or increased pulmonary blood flow, marked proliferative changes in pulmonary artery (PA) SMCs (PASMCs) are observed that exceed those observed when adult animals are exposed to these stimuli. Similarly, SMCs derived from neonatal pulmonary arteries are less differentiated and exhibit enhanced growth responses to mitogenic stimuli compared with the relatively differentiated and quiescent SMCs derived from the adult pulmonary artery (6, 30). Thus the increased growth capacity of neonatal PASMCs likely contributes to both normal pulmonary vascular development and the predisposition to develop exorbitant pulmonary vascular remodeling in response to injury in the neonatal period.

Although it is generally accepted that neonatal PASMCs possess increased growth responses to mitogenic stimuli, the unique intracellular signaling mechanism(s) that account for the enhanced growth responsiveness are incompletely understood. Ligands such as insulin-like growth factor-I and platelet-derived growth factor are coupled to tyrosine kinase signal transduction pathways that activate extracellular signal-regulated kinases (ERKs) and potently increase PASMC growth (2). Dempsey and colleagues (6, 8) demonstrated that receptor tyrosine kinase-dependent agonists induced fourfold greater increases in neonatal than in adult PASMC growth, suggesting that ERK-dependent proliferation is developmentally controlled. It has additionally been shown that constitutive and phorbol 12-myristate 13-acetate-sensitive protein kinase (PK) C activity is increased in neonatal compared with adult PASMCs and that increased PKC activity promotes neonatal PASMC growth and also synergistically promotes ERK-dependent PASMC proliferation (6, 7). However, how PKC synergistically interacts with ERK to enhance neonatal PASMC growth and what accounts for enhanced PKC-dependent proliferation in neonatal compared with adult SMCs is not clear at the present time.

Emerging data indicate that in some cell systems PKC may synergistically promote ERK-dependent proliferation by elevating cAMP. Faure and colleagues (11, 12) demonstrated that either G_q or $G_q\alpha$ activation of PKC or direct activation of PKC with phorbol esters stimulated ERK activity, and, similarly, activation of G_s or direct activation of adenylyl cyclase elevated cAMP and stimulated ERK activity. Although these data implicate either G_q- or G_s-coupled mechanisms in regulation of ERK activity, they do not clearly demonstrate how G_q-coupled agonists may elevate cAMP. However, recent elucidation of the molecular complexity of adenylyl cyclases revealed type II adenylyl cyclase is activated by PKC (32). These data suggest the possibility that a linkage between PKC and ERK activation is PKC stimulation of type II adenylyl cyclase and elevation of cAMP. Thus, as suggested by Faure and colleagues (11, 12), G_q-coupled signal transduction pathways activate PKC, which may promote adenylyl cyclase II synthesis of cAMP that, in turn, regulates ERK. It is equally clear that cAMP can have opposite effects on ERK activity in other cell systems (11). Thus second messenger regulation of ERK and proliferation may be unique in phenotypically distinct cell types.

Because neonatal SMCs demonstrate unique PKC-associated growth properties, it is possible that PKC regulation of cAMP may play an important role in the

increased growth responses in neonatal compared with adult PASMCs. Therefore, the goal of the present study was to test the hypothesis that G_q-coupled agonists promote PKC-dependent stimulation of cAMP in neonatal PASMCs and that such elevation of cAMP would be proproliferative. To test our hypothesis, we utilized two endogenous polypeptides, endothelin (ET)-1 and angiotensin II (ANG II), widely recognized as G_q-coupled PKC agonists that control SMC growth and differentiation (1, 9, 10, 13, 14, 16, 24, 25, 27). Both cAMP responses and indexes of proliferation were measured in neonatal and adult PASMCs.

METHODS

Isolation and culture of neonatal and adult PASMCs. SMCs were obtained from the main PAs of neonatal (14-day-old) calves and adult (2-yr-old) cows. Neonatal and adult PASMCs were considered matched because they were derived from the middle media at the same vascular site with previously described techniques (6–8). Briefly, main PAs were dissected from calves and cows immediately after death and transported to the laboratory immersed in MEM (pH 7.4) containing 200 U/ml of penicillin, 0.2 mg/ml of streptomycin, and 5 mg/ml of amphotericin B at 25°C. The PAs were opened, and the endothelium was scraped off. Explants of smooth muscle tissue (2×3 mm) were dissected from the middle media of PA strips. They were plated in petri dishes containing MEM with 10% serum, 100 U/ml of penicillin, and 0.1 mg/ml of streptomycin. The PASMCs at confluence exhibited characteristic "hill-and-valley" (adult) and "swirllike" (neonatal) morphologies by phase-contrast microscopy and stained in a homogeneous fibrillar pattern with smooth muscle-specific monoclonal anti-α-smooth muscle actin antibody (Sigma, St. Louis, MO) (6–8). Cell cultures were maintained in MEM (pH 7.4) containing 1% L-glutamine, 200 U/ml of penicillin, 0.2 mg/ml of streptomycin, and 0.5% MEM-nonessential amino acid solution (all from Sigma) with 10% bovine calf serum (BCS; Hyclone Laboratories, Logan, UT) and incubated in a humidified atmosphere with 5% CO_2 at 37°C. The medium was changed biweekly. To ensure that any differences seen between the cell populations were due to intrinsic differences and not induced in vitro, we controlled identical sites of harvest, time in culture, passage number, and growth conditions of the neonatal and adult PASMCs. The cells were studied between primary culture and third passage. Cells were grown to confluence in T75 flasks in the presence of 10% BCS, removed from the tissue culture flasks by trypsinization (0.2 g/l of trypsin-0.5 g/l of EDTA; Sigma), and then seeded at equal density into 24-well tissue culture plates (50×10^3 cells/well). Cells were grown to confluence in the presence of 10% serum in 2–3 days and incubated for 72 h in serum-deprived medium (0.1% BCS) to achieve a quiescent state.

Measurement of [³H]thymidine incorporation into DNA. DNA synthesis was measured as previously described (6–8). For these experiments, neonatal and adult PASMCs were grown to confluence, and a quiescent state was achieved after 72 h in 0.1% BCS-MEM. [³H]thymidine (0.5 µCi/well; ICN Biochemicals, Irvine, CA) was added together with ET-1, ANG II, forskolin, or 8-bromo-cAMP (Sigma) for 24 h. In studies of [³H]thymidine incorporation during PKC or cAMP blockade, the cells were pretreated with the specific PKC inhibitors chelerythrine chloride and Ro 31-8220 or the cAMP antagonist *Rp* diasteromer of adenosine 3′,5′-cyclic monophosphothioate (*Rp*-cAMPS; all Alexis, San Diego, CA) for 15 min, followed by application of [³H]thymidine (0.5 µCi/well) together with ET-1, ANG II, forskolin, or 8-bromo-cAMP for 24

h. Cell counts were obtained at the end of the incubation with a hemocytometer. After the cells were washed with phosphate-buffered saline (PBS) and 0.2 M perchloric acid (0.5 ml/well) was added for 2–3 min, the cells were again washed with PBS (1 ml/well), and then 1.0% SDS-0.01 N NaOH (0.3 ml/well) was added. The contents of each well were added to 4 ml of Ecoscint H scintillation cocktail (National Diagnostics, Atlanta, GA), and the radioactivity was measured with a Beckman LS 7500 beta-scintillation counter (Irvine, CA). Incorporation of [³H]thymidine into DNA is expressed as counts per minute (cpm) per cell.

Measurement of change in cell number. Cells were trypsinized for 10 min, gently triturated four times after the addition of an equal volume of MEM-10% serum, and counted with a standard hemocytometer.

Measurement of cAMP accumulation. cAMP measurements were made with confluent, quiescent neonatal and adult PASMCs grown to confluence in 24-well plates (plated at 50×10^3 cells/well) with a standard radioimmunoassay (Biomedical Technologies, Stoughton, MA). Studies were conducted with MEM at pH 7.35–7.45. In studies of cAMP accumulation, either vehicle control, ET-1, or ANG II was added to the cells, and the cells were incubated at 37°C for 90 min. In selected experiments, the cAMP signal was amplified with the α-adrenergic agonist isoproterenol (Sigma) along with the vehicle control, ET-1, or ANG II. In all studies, the solutions contained the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX; Sigma) to inhibit cAMP breakdown. In studies of cAMP accumulation during PKC blockade, the cells were pretreated with the specific PKC inhibitors chelerythrine chloride or Ro 31-8220 for 15 min, followed by application of the vehicle control, ET-1, or ANG II. After incubation, the cells were washed with PBS, and the reactions were stopped with 1 M NaOH and then neutralized to pH $\approx$ 7.0 with 1 M HCl. The solutions were acetylated, the tubes were centrifuged, and the supernatant was decanted. Radioactivity of the precipitate was counted, and sample cAMP was calculated from a standard curve. cAMP was standardized to cell counts obtained from untreated wells at the end of the incubation.

RT-PCR detection of adenylyl cyclase. Expression of PKC-stimulated adenylyl cyclase (type II) was tested generally as previously described (4). Briefly, total RNA was extracted from cell monolayers with Qiagen RNeasy (Qiagen, Santa Clarita, CA) and subjected to first-strand synthesis with reverse transcriptase (GIBCO BRL) and oligo(dT) primer after treatment of RNA with DNase I (GIBCO BRL). Degenerate primers directed to the highly conserved C_{2A} region of adenylyl cyclase were used for PCR amplification (28). PCR products were ligated into a cloning vector (TOPO TA cloning kit, Invitrogen, San Diego, CA) and transformed into competent cells. After PCR screening of clones for proper inserts, bacterial cultures were made and grown for 16–18 h. Plasmids were purified by QIAprep Spin Plasmid kit (Qiagen). Sequencing was performed by automated fluorescence sequencing (ABI370A DNA Sequencer).

Lung fixation. Neonatal calves were killed by an intravenous overdose of barbiturate and exanguinated before removal of the lungs. The superior lobe of the left lung was fixed in Formalin and cut, and lung blocks were embedded in paraffin. Sections of paraffin-embedded tissue were cut with a microtome at 4 µm. Before the slides were stained, the paraffin was removed from the slides with xylene, and the tissue was rehydraded in a graded alcohol series.

Immunohistochemistry. Immunostaining was generally as previously described (4, 17). To test for adenylyl cyclase expression, the slides were incubated in 0.3% H_2O_2 in metha-

nol for 30 min to decrease endogenous peroxidase activity. The slides were incubated with blocking solution (1% BSA and 0.05% Triton X-100 in PBS) to reduce nonspecific binding of antibodies. Primary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) for type II adenylyl cyclase were diluted 1:250 in PBS with 0.1% BSA and 0.05% Triton X-100 and incubated overnight at room temperature. After the slides were washed, biotinylated goat anti-rabbit IgG antibody diluted 1:250 in PBS with 0.05% Tween was added for 2 h. The slides were washed and incubated in an avidin-biotin-horseradish peroxidase complex (Vectastain ABC Kit, Vector Laboratories, Burlingame, CA) diluted 1:250 in PBS with 0.05% Tween for 1.5 h. The slides were again washed and developed in 5 mg of diaminobenzidine, 10 ml of 50 mM Tris, pH 7.4, and 10 µl of 30% H_2O_2 for 1 min, rinsed with tap water, and counterstained briefly with hematoxylin before dehydration and mounting. In control experiments, blocking peptide (Santa Cruz Biotechnology) was diluted 1:250 and coincubated with the type II adenylyl cyclase polyclonal antibody.

The antibody for type II adenylyl cyclase was generated against the sequence KTYFVNTEMSRSLSQSNVAS of the type II enzyme. Specificity of the antibody for type II adenylyl cyclase has been confirmed by Santa Cruz Biotechnology with Western blotting and immunocytochemistry.

Statistical analysis. Data are reported as means ± SE. One-way ANOVA with multiple comparisons was used to compare means between groups. A *P* value < 0.05 was used to indicate significance.

RESULTS

Stimulation of cAMP. To address whether ET-1 and ANG II could increase proliferation in neonatal PASMCs through a cAMP-mediated pathway, we first measured cAMP levels in PASMCs in the presence of the phosphodiesterase inhibitor IBMX (500 µM) and in response to ET-1 and ANG II. Baseline cAMP was higher in neonatal PASMCs than in adult cells (Fig. 1*A*). Both ET-1 (10 nM) and ANG II (10 nM) stimulated cAMP synthesis (≈12- and 4-fold, respectively; *P* < 0.05; *n* = 6 cells) in neonatal PASMCs over 90 min but did not change cAMP levels in adult cells (Fig. 1*A*). Similar results were observed in response to ET-1 and ANG II in the presence of isoproterenol (25 µM) and IBMX over a 5-min time course (data not shown).

We investigated the possibility that ET-1 and ANG II increased cAMP in neonatal PASMCs by PKC-mediated stimulation of adenylyl cyclase. Pretreatment of these cells with the PKC inhibitor chelerythrine (1 µM) reduced baseline cAMP (30%) and eliminated ET-1 and ANG II stimulation of cAMP (Fig. 1*B*). Similar results were obtained with the specific PKC blocker Ro 31-8220 (5 µM; data not shown). These data are consistent with the idea that basal cAMP levels and elevation of cAMP after application of ET-1 and ANG II are regulated through PKC stimulation of adenylyl cyclase.

Expression of type II (PKC-stimulated) adenylyl cyclase in vitro and in vivo. The activity of type II adenylyl cyclase is stimulated by PKC (32). We next sought to identify whether the type II enzyme is expressed in neonatal PASMCs using RT-PCR cloning. Sequence analysis of a 261-nucleotide product revealed 94 and 90% homology between the bovine product and
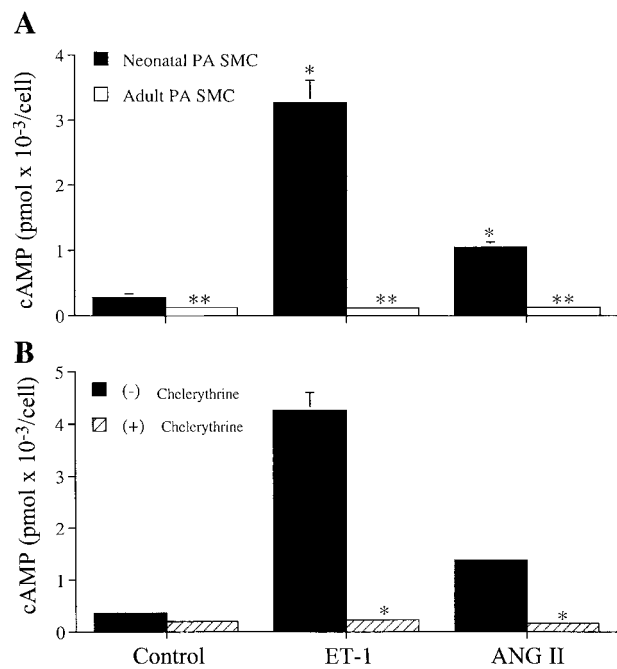

Fig. 1. Endothelin (ET)-1 and ANG II activate protein kinase (PK) C and increase cAMP in neonatal pulmonary artery (PA) smooth muscle cells (SMCs). Cells were plated at 50,000 cells/well and studied in a confluent and quiescent state. After application of vehicle or treatment conditions, cAMP was measured with radioimmunoassay and standardized to cell counts. Values are means ± SE. *A*: baseline cAMP in presence of IBMX was higher in neonatal than in adult PASMCs. ET-1 (10 nM) and ANG II (10 nM) increased baseline cAMP over a 90-min time course in neonatal but not in adult cells (*n* = 6/group). Significantly different (*P* < 0.05) from: *control PASMCs; **neonatal PASMCs. *B*: pretreatment with (+) chelerythrine (1 µM) reduced baseline cAMP and eliminated ET-1- and ANG II-induced rise in cAMP in neonatal PASMCs (*n* = 6/group). −, Without. *Significantly different from without chelerythrine, *P* < 0.05.

the respective human and rat species at the nucleotide level. Deduced amino acid alignments demonstrated 97 and 95% homology between the presently cloned bovine and respective human and rat sequences (Table 1).

Although type II adenylyl cyclase is expressed in cells in culture, isolation and culture per se may induce phenotypic changes in the population of PASMCs. Thus we determined whether PKC-stimulated adenylyl cyclase was evident in neonatal and adult PASMCs in vivo using established immunohistochemical techniques. A recently developed adenylyl cyclase type II-specific polyclonal antibody was utilized. We found expression of type II adenylyl cyclase throughout the pulmonary vasculature. Especially important is detection of the type II PKC-stimulated adenylyl cyclase in the medial layer at the site where excessive proliferation of PASMCs in response to mitogenic stimuli occurs (Fig. 2). Immunoreactivity was eliminated by coincubation of the PKC-stimulated adenylyl cyclase type II antibody with a blocking peptide, suggesting antibody specificity to type II adenylyl cyclase.

SMC proliferation. To assess the contribution of cAMP to growth in neonatal and adult PASMCs, we measured the effects of the direct adenylyl cyclase agonist forskolin (10 µM) and the cAMP analog 8-bromo-cAMP (1 µM) on [³H]thymidine incorporation. Basal

Table 1. *Comparison of rat, human, and bovine amino acid sequences of type II adenylyl cyclase*

Type II Isoform	Sequence
	1 10
Rat	KTIGSTYMAA
Human	KTIGSTYMAA
Bovine	KITG**C**TYMAA
	11 20
Rat	TGLSAIPSQE
Human	TGLSAVPSQE
Bovine	TGLSAVPSQE
	21 30
Rat	HSQEPERQYM
Human	HSQEPERQYM
Bovine	H**A**QEPERQYM
	31 40
Rat	HIGTMVEFAY
Human	HIGTMVEFAF
Bovine	HIGTMVEFAF
	41 50
Rat	ALVGKLDAIN
Human	ALVGKLDAIN
Bovine	ALVGKLDAIN
	51 60
Rat	KHSFNDFKLR
Human	KHSFNDFKLR
Bovine	KHSFNDFKLR
	61 70
Rat	VGINHGPVIA
Human	VGINHGPVIA
Bovine	VGINHGPVIA
	71 80
Rat	GVIGAQKPQY
Human	GVIGAQKPQY
Bovine	GVIGA**H**KPQY
	81 87
Rat	DIWGNTV
Human	DIWGNTV
Bovine	DIWGNTV

Sequences are within C_{2A} region of 2nd cytosolic loop of adenylyl cyclase. Nos. on *top,* amino acid no. Boldface type, nonhomologous amino acids. Bovine sequence is 97 and 95% similar to human and rat sequences, respectively, within this region.

[³H]thymidine incorporation was threefold higher in neonatal than in adult cells. Forskolin (10 μM) and 8-bromo-cAMP (1 μM) induced a twofold increase in [³H]thymidine incorporation in neonatal PASMCs but did not affect [³H]thymidine incorporation in adult PASMCs ($P < 0.05$; $n = 4$ cells; Fig. 3). Cell counts after forskolin and 8-bromo-cAMP application were higher in neonatal but not in adult PASMCs (data not shown), consistent with a proproliferative effect of cAMP in these neonatal cells (30).

Our next studies investigated whether neonatal PASMCs exhibit greater growth responses than adult cells to the $G_q\alpha$ agonists ET-1 (10 nM) and ANG II (10 nM). ET-1 and ANG II increased [³H]thymidine incorporation three- and twofold, respectively, in neonatal PASMCs but did not increase [³H]thymidine incorporation in adult PASMCs ($P < 0.05$; $n = 4$ cells; Fig. 4). Cell counts after ET-1 and ANG II application were also higher in neonatal but not in adult PASMCs (data not shown).

We investigated the role of PKC in the proliferative response to ET-1 and ANG II in neonatal PASMCs using chelerythrine. Figure 4*B* shows that PKC inhibition attenuated the basal and ET-1- and ANG II-mediated increase in [³H]thymidine incorporation (6, 75, and 75%, respectively; $P < 0.05$; $n = 4$ cells). Identical results were achieved with the PKC blocker Ro 31-8220 (5 μM; data not shown). A previous report from our laboratory (30) has shown that PKC inhibitors at concentrations presently reported do not cause significant cell death, confirming that inhibition of PKC decreased proliferation rather than induced apoptosis or necrosis. We next tested the role of adenylyl cyclase and cAMP in increased proliferation by blocking cAMP-dependent protein kinase activity with *Rp*-cAMPS (1 mM). *Rp*-cAMPS attenuated basal and ET-1- and ANG II-mediated increases in [³H]thymidine incorporation (6, 85, and 78%, respectively; $P < 0.05$; $n = 4$ cells; Fig. 5), confirming a proproliferative action of cAMP in neonatal PASMCs. Altogether, these data suggest that stimulation of proliferation in quiescent neonatal PASMCs is at least partly regulated through PKC stimulation of adenylyl cyclase and cAMP.

DISCUSSION

Vascular SMCs derived from neonatal PAs exhibit enhanced growth capacities to growth-promoting stimuli compared with SMCs derived from adult pulmonary arteries (6, 30). The reason for this unique phenomenon is unclear, although enhanced growth capacity may contribute to normal adaptive mechanisms after birth as well as the need for continued pulmonary vascular growth. Recent evidence (2, 6–8) indicated that compared with adult PASMCs, neonatal PASMCs exhibit enhanced growth responses to activation of ERK and PKC. Moreover, stimulation of ERK occurred after elevation of cAMP, suggesting that PKC stimulation of cAMP may be a critical link to ERK-dependent proliferation (2, 6, 8, 11, 12, 22). However, it was unclear whether activation of PKC influences PASMC cAMP content and whether cAMP is proproliferative in PASMCs. Novel findings from our study are that *1*) G_q agonists ET-1 and ANG II elevate neonatal but not adult PASMC cAMP, *2*) both neonatal and adult PASMCs express a PKC-stimulated adenylyl cyclase, and *3*) ET-1, ANG II, and direct elevation of cAMP is proproliferative in neonatal but not in adult PASMCs. These data suggest that PKC stimulation of cAMP is a critical signal mediating proliferation of neonatal PASMCs that is absent in adult PASMCs and therefore may contribute to the unique proproliferative phenotype of neonatal PASMCs.

Our initial studies sought to determine whether G_q agonists ET-1 and ANG II promote cAMP synthesis. Activation of PKC in diverse cell types, including bronchial SMCs, increases cAMP content (22). Both ET-1 and ANG II increased cAMP content in PASMCs over a 90-min time course, and inhibition of PKC prevented the ET-1- and ANG II-induced rise in cAMP. Interestingly, neither G_q agonist tested elevated cAMP content in adult PASMCs. Thus these data are the first to demonstrate that PKC stimulation of cAMP is developmentally controlled.
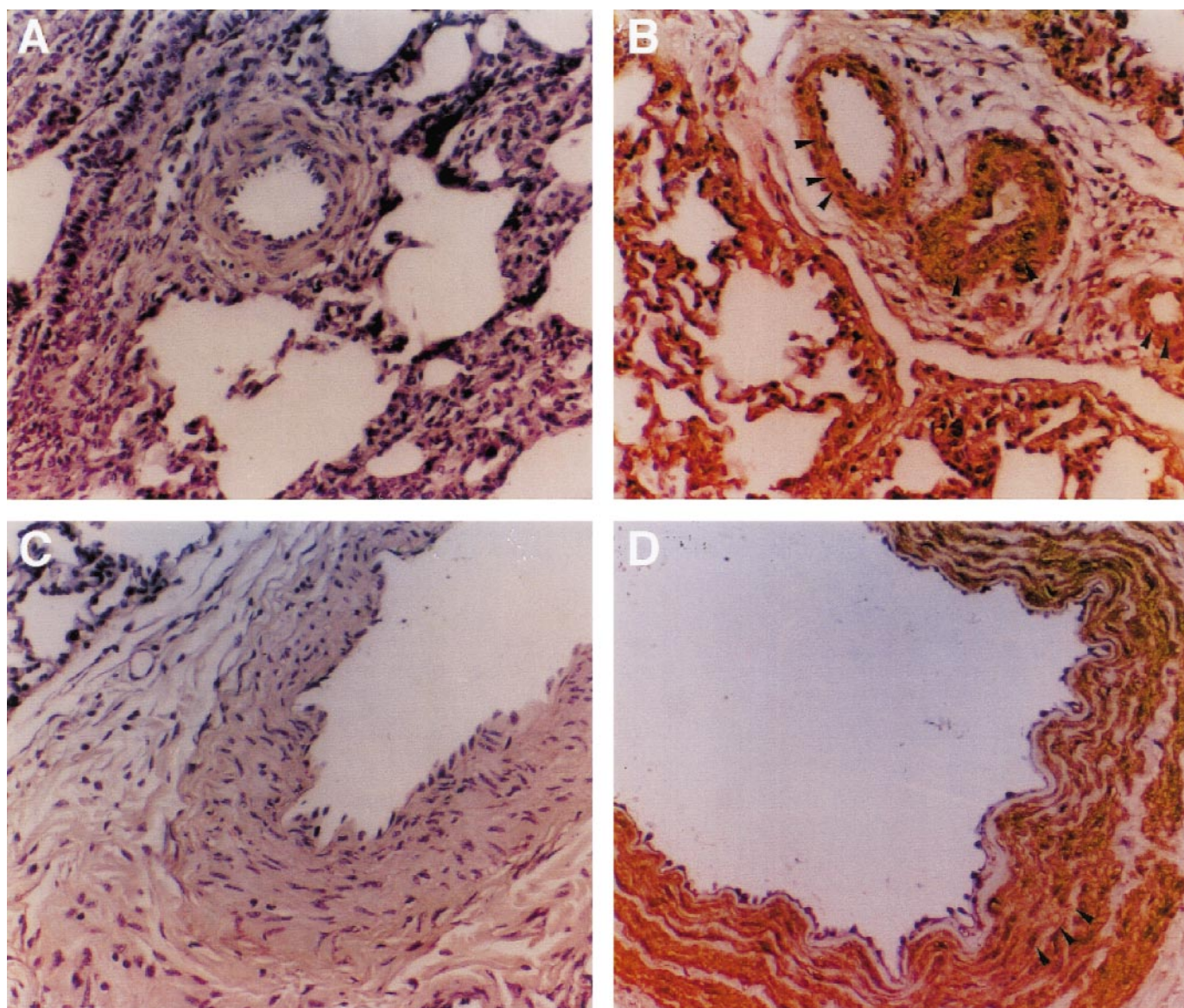
Fig. 2. PKC-stimulated adenylyl cyclase (type II) is expressed in pulmonary arteries and arterioles of neonatal and adult animals. Diaminobenzadine staining (brown) was used to determine expression of type II adenylyl cyclase in intact pulmonary circulation. Antibody specificity was tested by blocking diaminobenzadine staining with peptide inhibitors against primary antibody (*A* and *C*). Medial sections of small (*B*) and large (*D*) neonatal pulmonary arteries stained positive with adenylyl cyclase type II-specific polyclonal antibody. Most SMCs in medial layer of small and large pulmonary arteries stained brown (*B* and *D*, arrowheads), demonstrating expression of adenylyl cyclase type II. Data with pulmonary vessels from adult animals are not shown. Hematoxylin was used to counterstain cell nuclei (purple).

Recent elucidation of multiple adenylyl cyclase species revealed that certain isoforms (e.g., type II) are stimulated by PKC (32), providing a putative mechanism through which PKC may increase cAMP content. Our next studies therefore determined whether PKC-stimulated adenylyl cyclase was selectively expressed in neonatal PASMCs. We tested expression of the type II isoform by RT-PCR cloning using sequence-specific oligonucleotide primers. Sequence analysis revealed that type II adenylyl cyclase is expressed in both neonatal and adult PASMCs. To confirm that expression of this enzyme was not an artifact of cell culture per se, immunostaining was performed on sections from intact neonatal and adult bovine lungs. Positive staining was observed in the medial layers of large and small vessels from animals of both developmental stages. Thus these data indicate that the expression of type II adenylyl cyclase is not developmentally controlled and does not account for the distinct ET-1 and ANG II responses in neonatal versus adult PASMCs.

Although PKC-stimulated adenylyl cyclase is expressed in both neonatal and adult cells, our data indicated that PKC only stimulated the type II enzyme in neonatal PASMCs, supporting the idea that mechanisms controlling adenylyl cyclase activation are developmentally regulated. Dempsey et al. (6) previously
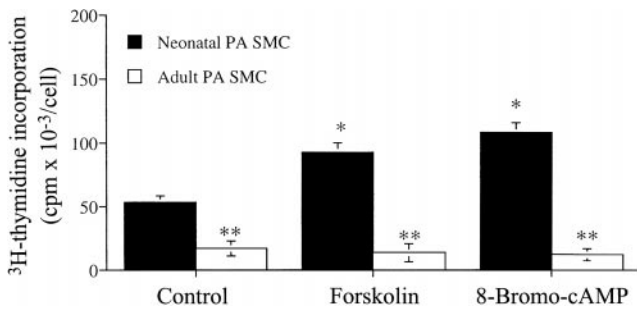
Fig. 3. Elevated cAMP promotes growth in neonatal PASMCs. Cells were plated at 50,000 cells/well and studied in a confluent and quiescent state. After application of treatment conditions, incorporation of [³H]thymidine into DNA was measured. cpm, Counts/min. Values are means $\pm$ SE; $n = 4$ cells/group. Basal [³H]thymidine incorporation in neonatal PASMC was threefold higher than in adult cells. Forskolin (10 μM) and 8-bromo-cAMP (1 μM) increased [³H]thymidine incorporation after 24 h in neonatal but not in adult cells. Significantly different ($P < 0.05$) from: *control PASMCs; **neonatal PASMCs.

demonstrated that relative to adult cells, neonatal PASMCs exhibit increased PKC activity under basal conditions and increased sensitivity to the direct PKC activator phorbol 12-myristate 13-acetate. It is therefore reasonable that increased PKC activity in neonatal PASMCs stimulates type II adenylyl cyclase, whereas lower PKC activity in adult cells does not stimulate type II adenylyl cyclase. Multiple isoforms of PKC are present in neonatal PASMCs, but the α-isozyme has
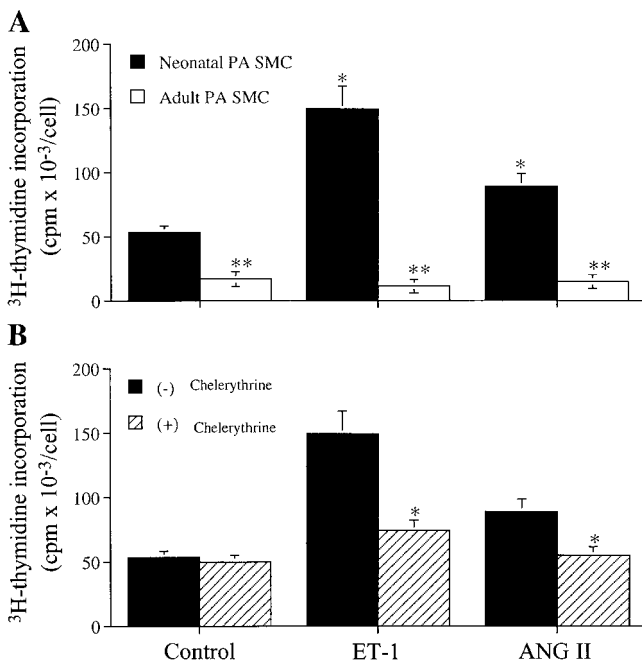

Fig. 4. ET-1 and ANG II activate PKC and stimulate growth of neonatal PASMCs. Cells were plated at 50,000 cells/well and studied in a confluent and quiescent state. After application of treatment conditions, incorporation of [³H]thymidine into DNA was measured. Values are means $\pm$ SE; $n = 4$ cells/group. *A*: ET-1 (10 nM) and ANG II (10 nM) increased [³H]thymidine incorporation after 24 h in neonatal but not in adult cells. Significantly different ($P < 0.05$) from: *control PASMCs; **neonatal PASMCs. *B*: pretreatment with chelerythrine (1 μM) inhibited ET-1- and ANG II-stimulated neonatal PASMC proliferation. *Significantly different from without chelerythrine, $P < 0.05$.
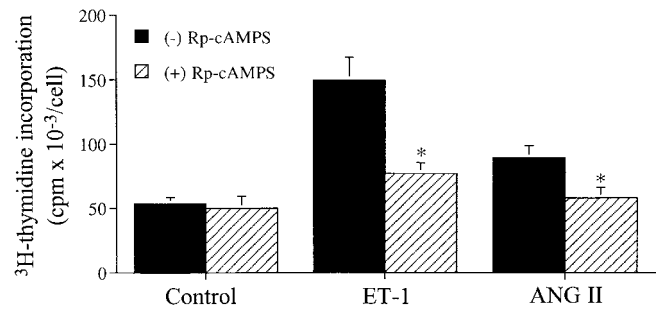

Fig. 5. Inhibition of PKA prevents ET-1 and ANG II stimulation of neonatal PASMC growth. After pretreatment with *Rp* diasteromer of adenosine 3′,5′-cyclic monophosphothioate (*Rp*-cAMPS; 1 mM) and application of ET-1 or ANG II, incorporation of [³H]thymidine into DNA was measured. Values are means $\pm$ SE; $n = 4$ cells/group. PKA blockade attenuated basal and ET-1- and ANG II-induced increase in [³H]thymidine incorporation in neonatal PASMCs. *Significantly different from neonatal PASMCs, $P < 0.05$.

been implicated in increased growth responses (30). Interestingly, the α-isozyme of PKC activates type II adenylyl cyclase in Sf9 cells (34). Future studies will be required to directly test the nature of PKC stimulation of adenylyl cyclase activity in neonatal PASMCs, e.g., which PKC isoforms account for increased whole cellular PKC activity and activation of type II adenylyl cyclase.

Our next studies were designed to address whether a link exists between PKC stimulation of cAMP and neonatal PASMC proliferation by determining whether *1*) elevated cAMP is proproliferative, *2*) PKC activation is proproliferative, and *3*) PKC stimulation of proliferation depends on cAMP. The role of cAMP on SMC proliferation is controversial. Previous reports suggested that cAMP may have either a negative or positive influence on proliferation (23), with the effect of cAMP depending on cell type (23), state of cell differentiation (3), and stage of cell cycle (21). Neonatal and adult PASMCs were "growth arrested" to mimic the in vivo environment. Although previous studies (6, 30) showed that neonatal PASMCs exhibit enhanced growth capabilities, our present studies demonstrated that these cells also exhibit higher basal cAMP levels, consistent with the possibility that cAMP may function as a positive stimulus for proliferation. We found that two agents that increase cAMP (8-bromo-cAMP and forskolin) also stimulate [³H]thymidine incorporation and cell proliferation in neonatal but not in adult PASMCs. Furthermore, direct inhibition of the cAMP-dependent PK lowered basal [³H]thymidine incorporation in neonatal PASMCs. These data suggest that cAMP is proproliferative in neonatal PASMCs and that the action of cAMP-induced growth is developmentally regulated as recently suggested in Schwann cells (31).

We next evaluated the influence of PKC on growth in neonatal PASMCs. PKC activity is increased in neonatal versus adult PASMCs, and ET-1 and ANG II activate PKC. Furthermore, activation of PKC is generally found to stimulate proliferation (6, 16, 20, 25, 34). In our present studies, inhibition of PKC with chelerythrine and Ro 31-8220 decreased basal and ET-1- and ANG II-stimulated [³H]thymidine incorporation, sug-

gesting that increased growth in neonatal PASMCs depends at least partly on PKC activity. However, PKC inhibitors did not influence proliferation in adult PASMCs. Both ET-1 and ANG II are generally believed to stimulate growth in adult SMCs derived from the systemic circulation (1, 5, 10, 27). Indirect evidence for the involvement of ET-1 and ANG II in medial thickening of pulmonary arteries has also been shown in adult rats (18, 33), but the direct effects of the polypeptides on PASMC proliferation are less clear. For example, ET-1 was previously reported (16) to increase growth in adult swine PASMCs in the presence of 0.5% serum, whereas in the present study, ET-1 and ANG II were not proproliferative in adult bovine PASMCs in the presence of 0.1% serum. The reason for this discrepancy is unclear, although it is possible that these agents act as comitogenic stimuli, requiring other growth factors to stimulate proliferation. Independent support for this idea comes from the work of Morrell and Stenmark (19), who observed that ANG II stimulated proliferation of adult rat PASMCs only when the cells were primed by preincubation with 10% serum but not under serum-deprived conditions (0.1%). Thus our data are consistent with the idea that ET-1 and ANG II alone are insufficient to promote proliferation in adult PASMCs and suggest that PASMCs possess a developmentally regulated sensitivity to these vasoconstrictors (29).

Our final series of experiments tested whether inhibition of PKA blocks ET-1- and ANG-II-stimulated increase in neonatal PASMC proliferation. Indeed, the PKA inhibitor Rp-cAMPS prevented G_q activation from stimulating neonatal PASMC proliferation but did not affect proliferation of adult PASMCs. Our data therefore demonstrate that ET-1 and ANG-II stimulate PKC-dependent production of cAMP that is proproliferative in neonatal PASMCs; inhibitors of either PKC or PKA prevent this stimulation of proliferation.

In summary, ET-1 and ANG-II activation of G_q activates PKC, which increases cAMP and promotes proliferation of neonatal PASMCs. In contrast, ET-1 and ANG II activation of G_q neither increases cAMP nor promotes proliferation of adult PASMCs. The explanation for this apparent developmental distinction is not yet fully determined but is not due to altered expression of PKC-stimulated (type II) adenylyl cyclase. Based on earlier work from our laboratory (6, 8), a likely explanation is that increased constitutive PKC activity and enhanced PKC responsiveness to activation accounts for PKC stimulation of cAMP in neonatal versus adult PASMCs. Now that a key link between PKC and cAMP production has been established in neonatal PASMCs, future studies may address the regulation of ERK-dependent proliferation by cAMP.

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