Platelet-Activating Factor Modulates Activity of Cyclic Nucleotides in Fetal Ovine Pulmonary Vascular Smooth Muscle

Basil O. Ibe, Adnan Ameer, Ada Mae Portugal, Lissette Renteria, and J. Usha Raj

Division of Neonatology, Department of Pediatrics, Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center, Torrance, California

Received August 2, 2006; accepted November 2, 2006

ABSTRACT

At birth, release of endogenous vasodilators such as nitric oxide and prostacyclin facilitate pulmonary vasodilation via the cyclic nucleotides, cGMP and cAMP. Interaction of cyclic nucleotides and platelet-activating factor (PAF)-mediated responses in pulmonary vascular smooth muscle is not known. We studied the effects of cGMP and cAMP on PAF-mediated responses in ovine fetal intrapulmonary venous smooth muscle cells. Studies were done in hypoxia or normoxia with buffer with 8-Br-cGMP (BGMP) and 8-Br-cAMP (BAMP), as well as cGMP-dependent protein kinase (PKG) and a cAMP-dependent protein kinase (PKA) inhibitors. All groups were treated with 1 nM PAF and incubated for 30 min for the binding assay or 20 min for measurement of inositol 1,4,5-phosphate (IP3) production. BGMP and BAMP decreased PAF binding in normoxia by 63 and 14%, respectively. Incubations with the PKG inhibitor Rp-8-(4-chlorophenylthio)-guanosine-3',5'-cyclic monophosphorothioate sodium and the PKA inhibitor Rp-adenosine-3',5'-cyclic monophosphorothioate abrogated the inhibitory effects of BGMP and BAMP. PAF-stimulated IP3 production was 8565 ± 314 dpm/106 cells in hypoxia and 5418 ± 118 dpm/106 cells in normoxia, a 40% decrease. BGMP attenuated IP3 production by 67 and 37% in hypoxia and normoxia, respectively; the value for BAMP was 44% under both conditions. Pretreatment with PKG or PKA inhibitor abrogated BGMP and BAMP inhibition of IP3 release. PAF receptor (PAFr) protein expression decreased in normoxia, but pretreatment with 10 nM PAF up-regulated PAFr expression. Pretreatment with PAF decreased expression and activities of PKG or PKA proteins in normoxia and hypoxia. Our data demonstrate the existence of cGMP/cAMP-PAF cross-talk in pulmonary vascular smooth muscle cells, which may be one mechanism by which PAF-mediated vasoconstriction is down-regulated at birth.

Platelet-activating factor (PAF) evokes a wide range of biological actions (Stafforini et al., 2003). In lungs, PAF is a potent vasoactive mediator with different sites of action in different species (Toga et al., 1992; Argiola et al., 1995; Ibe et al., 1998). In the fetus, PAF plays an important physiological role in maintaining a high vasomotor tone in the pulmonary circulation (Ibe et al., 1998). In fetal lungs, PAFr binding and PAFr mRNA expression are high, so that a high level of pulmonary vasomotor tone can be maintained in utero (Ibe et al., 2000), whereas in lungs of the newly born lamb, PAFr binding and PAFr mRNA expression are low, suggesting a down-regulation of PAFr-mediated effects in vivo (Ibe et al., 1998, 2000). In addition, the effects of PAF can be completely abrogated by the enzymatic degradation of PAF by PAF acetylhydrolase (Kim et al., 2000), by specific PAFr antagonists (Argiolas et al., 1995; Ibe et al., 1998), or by agents that decrease PAFr protein expression (Ibe et al., 2000).

PAF evokes its effects by binding to its G protein-coupled receptor, which is a seven-transmembrane receptor (Carlson et al., 1996; Parent et al., 1996). Activation of G protein-coupled receptor by an agonist results in activation of signal transduction pathways (Ferguson et al., 1996), which may involve recruitment of intracellular second messengers such as cAMP, cGMP, inositol 1,4,5-triphosphate (IP3), and calcium (Lin and Rui, 1994; Rehring et al., 1996; Lee et al., 2001). cGMP and cAMP act via their endogenous receptors, respectively. cAMP and cGMP stimulate protein kinase A (PKA) and cGMP-dependent protein kinase (PKG), which is involved in the regulation of smooth muscle tone (Carlson et al., 1996; Parent et al., 1996).

This work was supported in part by Grant HL-077819 from the National Heart Lung and Blood Institute and Grant R25 GM56902 from the National Institute of General Medical Sciences, National Institutes of Health, Bethesda, MD.

Article, publication date, and citation information can be found at http://jpet.aspetjournals.org. doi:10.1124/jpet.106.111914.

ABBREVIATIONS: PAF, platelet-activating factor; PAFr, PAF receptor; IP3, inositol 1,4,5-triphosphate; PKG, cGMP-dependent protein kinase; PKA, cAMP-dependent protein kinase; PPHN, persistent pulmonary hypertension of the newborn; SMC-PV, intrapulmonary venous smooth muscle cells; 8-Br-cGMP, 8-bromoguanosine-3',5'-cyclic monophosphate; Rp-8-cGPT-cGMP, Rp-8-(4-Chlorophenylthio)-guanosine-3',5'-cyclic monophosphorothioate sodium; 8-Br-cAMP, 8-bromoadenosine-3',5'-cyclic monophosphate; Rp-cAMPS, Rp-adenosine-3',5'-cyclic monophosphorothioate; PMSF, phenylmethylsulfonyl fluoride; BSA, bovine serum albumin; PBS, phosphate-buffered saline; IBMX, 3-isobutyl-1-methyl-xanthine; SNP, sodium nitroprusside; PAGE, polyacrylamide gel electrophoresis, NO, nitric oxide.
cGMP-dependent protein kinase (PKG) and cAMP-dependent protein kinase (PKA), respectively, to elicit smooth muscle relaxation (Abdel-Latif, 2001). Both cGMP and cAMP mediate relaxation of pulmonary vessels, but cGMP has been shown to be more effective than cAMP in producing relaxation of perinatal ovine pulmonary vessels (Dhanakoti et al., 2000). We recently showed that acute hypoxia up-regulates PAFr-mediated intracellular signaling in ovine fetal pulmonary vascular smooth muscle (Ibe et al., 2005). Therefore, chronic hypoxia in the perinatal period may result in abnormal up-regulation of PAFr protein expression, PAFr binding, and PAFr-mediated cell signaling, leading to increased pulmonary vasomotor tone and vascular remodeling, a key event in the onset of clinical disorders such as persistent pulmonary hypertension of the newborn (PPHN) (Caplan et al., 1990).

Pulmonary vascular resistance in utero is high, such that pulmonary blood flow is only 8 to 10% of total cardiac output (Heymann et al., 1990). At birth, with oxygenation, pulmonary vascular resistance falls dramatically, and blood flow to the lung increases to accommodate the total cardiac output (Fineman et al., 1995). We are interested in understanding the mechanisms of pulmonary vascular relaxation at birth. Our primary hypothesis is that with oxygenation at birth and the increased production of cGMP and cAMP in pulmonary vascular smooth muscle, PAFr-mediated cell signaling is down-regulated via cross-talk between the cyclic nucleotides and PAF-PAF receptor complex. We used ovine fetal intrapulmonary venous smooth muscle cells (SMC-PV) to study the effects of cGMP and cAMP on PAFr binding and PAFr-mediated cell signaling in both normoxia and hypoxia. We also studied the effects of cGMP and cAMP on PAFr expression and the effects of PAF on expression and activities of PKG and PKA proteins in these venous smooth muscle cells.

Materials and Methods
Materials
Pregnant ewes (146–148 days of gestation (term 150 days)) were purchased from Nebekar Farms (Santa Monica, CA). Authentic PAF standards 1-O-hexadecyl-2-O-acetyl-sn-glycerol-3-phosphocholine (C16:0-PAF) and 1-O-hexadecyl-sn-glycerol-3-phosphocholine (lyso-C16:0-PAF) as well as cGMP, cAMP, 8-bromoguanosine-3′-5′-cyclic monophosphate (8-Br-cGMP), Rp-8-cPT-cGMP, 8-bromoadenosine-3′,5′-cyclic monophosphate (8-Br-cAMP), and Rp-cAMPS were purchased from Biomol (Plymouth Meeting, PA). Radiolabeled PAF standards and substrates, hexadecyl-2-acetyl-sn-glycero-3-phosphorylcholine and 1-O-[acetate-13C]-glycerol-3-phosphorylcholine and O-acetyl-1H-Ni, (1H)acetyl-C16-PAF, 21.5 Ci/mmol (370 GBq/mmol), were purchased from PerkinElmer Life Sciences (Boston, MA). Phenylmethylsulfonyl fluoride (PMSF), leupeptin, pepstatin, bovine serum albumin (BSA), and antibody to actin were purchased from Sigma-Aldrich (St. Louis, MO). Antibodies to PKA and PKG were purchased from Cell Signaling Technology Inc. (Beverly, MA), and PAF receptor antibody was purchased from Cayman Chemical (Ann Arbor, MI). Studies were done with freshly made reagents. Ecolite+ liquid scintillation cocktail was purchased from MP Biochemicals (Irvine, CA).

Preparation of Pulmonary Vascular Smooth Muscle Cells
Intrapulmonary vessels were isolated from freshly killed term fetal lambs, and then smooth muscle cells were harvested from the freshly excised arteries and veins under sterile conditions as previously reported (Ibe et al., 2005). Cells were used at the fourth to sixth passage and identity of the smooth muscle cells at each passage was characterized with a smooth muscle cell-specific monoclonal antibody (Sigma Chemical). The smooth muscle cells were devoid of endothelial cells and fibroblasts. The cell phenotype did not change from the fourth to sixth passages as determined by the expression of α-smooth muscle actin and myosin light chain kinase proteins.

Studies with Smooth Muscle Cells.
All studies were done with smooth muscle cells harvested from intrapulmonary veins of term fetal lambs, because they exhibit more PAF binding than cells from arteries (Ibe et al., 2005). Also, we have previously reported that in the fetus, pulmonary veins often demonstrate significantly greater vasoactivity than pulmonary arteries, and veins contribute a significant fraction to pulmonary vascular resistance in utero (Kääpä et al., 1991; Toga et al., 1992).

Study Conditions. All studies were done with adherent cells in normoxia or in hypoxia.

Normoxia. Cells were studied in a humidified incubator at 37°C aerated with 5% CO₂ in air. The oxygen concentration was monitored with a TEO 60T percent oxygen sensor (Teledyne Analytical Instruments, City of Industry, CA). The incubator oxygen concentration was 21% and pO₂ determined as in the hypoxia condition was normoxic at 80 to 100 torr.

Hypoxia. An incubator set at 37°C was first equilibrated for at least 1 h with a gas mixture of 2% O₂, 10% CO₂, and balance N₂ to maintain incubator pO₂ <40 torr, as determined on a Nova Stat 3 blood-gas instrument (Nova Biomedical Corp., Waltham, MA) (Ibe et al., 2002). Cells were then placed in this incubator for experimentation and were continuously aerated with the hypoxia gas mixture throughout the duration of the study.

Study of PAF Receptor Binding
General Protocol. Receptor binding assays were done during hypoxia and normoxia as we reported previously (Ibe et al., 2005). Brieﬂy, cells were washed with calcium and magnesium-free PBS before use according to the specific study protocol. After a 30-min incubation in normoxia or hypoxia, unbound [3H]PAF was washed off with ice-cold PBS and then incubated on ice for 30 to 45 min in a saline-EDTA mixture containing 154 mM saline and 5 mM EDTA (Korth et al., 1995; Ibe et al., 2005). Receptor bound [3H]PAF was extracted on Whatman GF/C membrane filters using an in-line vacuum system as we reported previously (Ibe et al., 2005). Cell-bound PAF radioactivity was quantified by scintillation spectrometry (Beckman Coulter, Inc., Fullerton, CA). In studies probing the interaction of PAF with its receptors in the presence of other agonist or antagonists, cells were preincubated with the agent before the addition of [3H]PAF and then incubated further according to the specific experimental protocol.

Specific Protocols. Effect of cGMP/cAMP on PAFr binding. Cells were preincubated for 30 min, in hypoxia or normoxia, with buffer for controls or 10 μM concentrations of each of the cell-permeable cyclic nucleotide analogs, 8-Br-cGMP or 8-Br-cAMP. Then 1 nM [3H]PAF was added, and the incubation was continued for 30 min more. In preliminary studies, we found no significant difference between inhibition of PAF binding produced by 10 or 100 μM concentrations of the cell-permeable cAMP and cGMP analogs. To study the effects of cGMP and cAMP on PAF receptor protein expression, cells were incubated for 3 h in normoxia only with 10 μM concentrations each of 8-Br-cGMP and 8-Br-cAMP or with 0.1% BSA for a baseline, and then protein was prepared for Western blotting of PAF receptor protein.

Effect of PAF and PKG inhibitors on PAFr binding. To study the effect of endogenous cyclic nucleotides and their receptor interaction on PAF binding, cells were preincubated in hypoxia or normoxia for 30 min with 10 μM concentrations each of the cGMP-dependent PKG antagonist Rp-8-Br-PET-cGMP and the cAMP-dependent PKA antagonist Rp-cAMPS, and then 1 nM [3H]PAF was added and the incubation was continued for 30 min more. PAF bound to receptor was extracted and quantified.

Downloaded from jpet.aspetjournals.org at ASPET Journals on October 15, 2017
Study of cGMP and cAMP Release by the Cells

Cells were seeded at $5 \times 10^5$ cells in 100-mm Petri dishes and allowed to attain confluence. The confluent cells were washed twice with protein-free PBS and preincubated for 15 min in normoxia or hypoxia at 37°C in Krebs bicarbonate buffer, pH 7.4, containing 119 mM NaCl, 4.6 mM KCl, 1.5 mM CaCl$_2$, 1.2 mM MgCl$_2$, 15 mM NaHCO$_3$, 1.2 mM NaH$_2$PO$_4$, and 5.5 mM glucose and 0.1 mM 3-isobutyl-1-methyl-xanthine (IBMX) to inhibit phosphodiesterase-catalyzed breakdown of cAMP and cGMP produced during experimentation. After preincubation, cells were treated with 10 nM PAF and 0.1 mM concentrations each of forskolin and sodium nitroprusside (SNP) and incubated for 15 min more in normoxia and hypoxia. After incubation, the media were acidified with 0.1 ml of 10% trichloroacetic acid and aspirated into borosilicate glass tubes. The cells were treated with 0.5 ml of ice-cold ethanol, and the ethanolic cell suspension was combined with its incubation media. Ethanol content was evaporated with nitrogen in ambient temperature. The aqueous fraction was spun for 10 min at 2000 rpm to pellet cell debris. The resulting aqueous supernatant containing the cAMP and cGMP produced were extracted with diethyl ether saturated with water. The water fraction containing the cyclic nucleotides was lyophilized and then redissolved in 0.05 M sodium acetate. The amounts of cAMP and cGMP measured are presented as picomoles per milliliter.

Release of Inositol Phosphates. Labeling of cells with [3H]myo-inositol. Cells were washed with protein-free, inositol-free medium and then fed with a culture medium containing 10% fetal bovine serum and 5 µCi/ml [3H]myo-inositol suspended in inositol-free medium and incubated for 16 to 24 h in 5% CO$_2$ in air as we reported previously (Ibe et al., 2005).

Stimulation of [3H]inositol phosphate release by cells. Each test stimulus was prepared in freshly made 10 mM LiCl buffer. Labeled cells were stimulated with PAF or other agents and incubated in hypoxia or normoxia for 10 min at 37°C to induce inositol phosphate production. Reactions were quenched by addition of 10 mM formic acid and incubation in an ice bath for 30 min, followed with neutralization with 15 mM ammonium hydroxide. The cell suspension was loaded on pre-equilibrated AG 1-X8 columns and washed with 2 ml of distilled water. The tritium-labeled inositol phosphate accumulated was eluted from the column with a mixture of 1.0 M ammonium formate and 0.1 M formic acid. [3H]Inositol phosphate radioactivity was quantified by scintillation spectrometry.

Specific Protocols. Effect of cGMP and cAMP on PAF-stimulated IP$_3$ release. Cells prelabeled with [3H]inositol were preincubated for 20 min with buffer alone for control or 10 µM 8-Br-cGMP or 8-Br-cAMP after which 1 nM nonradiolabeled PAF was added, and the incubation was continued for 20 min more. Effect of PKG and PKA inhibitors on PAF-stimulated IP$_3$ release. To study the effect of endogenous cyclic nucleotides on PAF-stimulated inositol phosphate release, cells were preincubated with buffer alone or with 10 µM concentrations each of cGMP and cAMP receptor antagonists Rp-8-cPT-cGMPs and Rp-cAMPS. Then nonradiolabeled PAF was added, and the incubation was continued for 20 min more. The amount of inositol phosphate released was extracted and quantified.

Western Blotting. Preparation of proteins for Western blot analysis. Proteins were prepared from stimulated and unstimulated cells that were studied in normoxia or hypoxia as described above. Briefly, after incubation in hypoxia or normoxia, cells were washed with PBS and lysed with a modified 40 mM HEPES hypotonic lysis buffer, pH 7.4, containing the following: 1 mM EGTA, 4 mM EDTA, 2 mM MgCl$_2$, 10 mM KCl, 1 mM dithiothreitol, 0.1 mM PMSF, 5 µg/ml leupeptin, 1 µg/ml pepstatin, 1 µM 4-(2-aminoethyl) benzene sulfonil fluoride, 200 mM sodium fluoride, 20 mM sodium pyrophosphate, 0.2 mM sodium vanadate, and 0.1 mg/ml trypsin inhibitor. Proteins were recovered from lysed cells by centrifugation at 1500g for 15 min in a refrigerated centrifuge, stored in 0.2-ml aliquots at −80°C, and used for Western blotting.

SDS-PAGE Electrophoresis. Proteins were subjected to Coomassie Blue quantitation before use in Western blotting with some modification of published methods (Ali et al., 1994; Ibe et al., 2005). The signals were developed for 1 min using an Amersham ECL Western Blot detection kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK) and then exposed to X-ray film. A band corresponding to PAFr protein was scanned with an EagleEye densitometer (Stratagene, La Jolla, CA) to quantify blot density.

PKG and PKA Activity in Extracts of Pulmonary Vascular Smooth Muscle Cells

Incubations with or without PAF. We are interested in understanding the postnatal effects of PAF on the pulmonary circulation. Therefore, we studied the effect of PAF on activities of PKG and PKA during normoxia only. Subconfluent cells were washed with PBS at 37°C and then incubated at 37°C with 0.1% BSA in Dulbecco’s modified Eagle’s medium for 210 min. Then, cells were stimulated with 1.0 or 10.0 nM PAF and incubated for 30 min more in normoxia. After incubation, media were removed, and cells were washed twice with ice-cold phosphate-buffered saline; then cells were detached and centrifuged at 400g for 5 min. The cell pellet was homogenized on ice in 50 mM Tris buffer, pH 7.4, containing 10 mM EDTA, 2 mM dithiothreitol, 1 mM IBMX, 0.1 mM nitro-l-arginine, 0.01 mM indomethacin, and 0.5 mM PMSF. After homogenization, the homogenate was sonicated for 30 s and recentrifuged at 14,000g for 10 min at 4°C. The protein concentration of the extracts was determined by the Bradford method using BSA (fraction V) as the standard. The supernatant was assayed for PKG and PKA activities as described previously (Dhanakoti et al., 2000) after assay standardization.

Assay of PKG and PKA Activities. Our preliminary data showed that the activity of PKG (picomoles per minute per milligram of protein) in the absence or presence of 5 µM cGMP was similar (34.1 ± 4.1 and 105.4 ± 7.3 versus 32.7 ± 1.8 and 100.9 ± 5.1) whether in fresh cell extract or in frozen extracts stored at −80°C. Also, in preliminary experiments, we found that PKG activity was linear up to 15 µg of protein extract and up to 15 min of incubation, and the activity was saturated with 5 µM exogenous cGMP. Similar results were obtained for PKA, except that the activity was saturated with 2 µM cAMP. Inclusion of a variety of protease inhibitors (leupeptin, pepstatin, antipain, and aprotinin) and phosphatase inhibitors (calyculin A, cypermethrin, and sodium vanadate) did not significantly enhance the activities of PKG (123 ± 10.3 versus 129 ± 8.4 pmol/mg/mg protein with 5 µM cGMP) or PKA (1063 ± 35 versus 1143 ± 86 pmol/mg/mg protein with 2 µM cAMP) and thus were excluded in our subsequent assays. PKG and PKA assays were done essentially as reported previously (Dhanakoti et al., 2000).

Data Analysis

All numerical data are means ± S.E.M. In all instances in which radioisotope was used, background radioactivity was subtracted before radioactivity was quantified. Data were analyzed with a two-tailed t test followed by analysis of variance (GraphPad Prism; GraphPad, San Diego, CA). Results were considered significant at $p < 0.05$.

Results

cGMP and cAMP decrease PAF receptor binding through their respective kinases, PKG and PKA. We used the cell-permeable cyclic nucleotide analogs, 8-Br-cGMP and 8-Br-
cAMP, to study the effect of vasodilators on PAF receptor binding.

Figure 1 shows the effect of cGMP and the PKG inhibitor, Rp-8-pCPT-cGMPS, on PAF receptor binding. PAF receptor binding in hypoxia was 22 fmol/10^6 cells. Treatment with 10 μM 8-Br-cGMP in hypoxia inhibited PAF binding by 20%. When endogenous cGMP activity was inhibited with the cGMP/PKG receptor antagonist Rp-8-pCPT-cGMPS, PAF binding was restored to the level of binding by PAF alone. During normoxia, PAF binding was 15 fmol/10^6 cells. Treatment with 8-Br-cGMP decreased binding by 65% compared with PAF alone in normoxia. When endogenous cGMP activity was inhibited with the cGMP/PKG receptor antagonist Rp-8-pCPT-cGMPS, PAF receptor binding increased compared to effect of 8-Br-cGMP alone and then was comparable with binding by PAF alone in hypoxia. PAF binding to its receptors during normoxia, in the absence or presence of 8-Br-cGMP, was lower than binding during hypoxia, and the

cGMP/PKG receptor antagonist increased PAF receptor binding to comparable levels during hypoxia and normoxia.

Figure 2 shows the effect of cGMP and the PKG inhibitor, Rp-8-pCPT-cGMPS, on PAF receptor binding. PAF receptor binding in hypoxia was 22 fmol/10^6 cells. Treatment with 10 μM 8-Br-cGMP in hypoxia inhibited PAF binding by 20%. When endogenous cGMP activity was inhibited with the cGMP/PKG receptor antagonist Rp-8-pCPT-cGMPS, PAF binding was restored to the level of binding by PAF alone. During normoxia, PAF binding was 15 fmol/10^6 cells. Treatment with 8-Br-cGMP decreased binding by 65% compared with PAF alone in normoxia. When endogenous cGMP activity was inhibited with the cGMP/PKG receptor antagonist Rp-8-pCPT-cGMPS, PAF receptor binding increased compared to effect of 8-Br-cGMP alone and then was comparable with binding by PAF alone in hypoxia. PAF binding to its receptors during normoxia, in the absence or presence of 8-Br-cGMP, was lower than binding during hypoxia, and the

Because PAF stimulates release of inositol phosphates downstream from the PAF receptor, we investigated the possibility that cGMP and cAMP will attenuate PAF receptor-mediated inositol phosphate release via PKG and PKA. Figure 3 shows the effects of PAF, 8-Br-cGMP, and the cGMP/PKG antagonist, Rp-8-pCPT-cGMPS, on PAF-stimulated IP₃ production. Under baseline conditions in hypoxia, IP₃ release by cells (means ± S.E.M.) was 1150 ± 33 dpm/10⁶ cells. During normoxia, baseline release of IP₃ decreased by 22% (896 ± 38 dpm/10⁶ cells). Thus, IP₃ release by the cells was significantly reduced by normoxic conditions. Treatment of cells with 5 nM PAF increased IP₃ release 7-fold during hypoxia to 8565 ± 314 dpm/10⁶ cells and 6-fold during normoxia to 5418 ± 118 dpm/10⁶ cells. PAF-stimulated inositol phosphate release was attenuated by normoxia.

 Pretreatment of the cells with 8-Br-cGMP decreased PAF-stimulated IP₃ release during hypoxia by 65% and during normoxia by 37%, compared with PAF treatment alone under the two conditions. When the cells were pretreated with Rp-8-pCPT-cGMPS to inhibit activity of endogenous cGMP,
PAF-stimulated IP$_3$ release increased 2-fold during hypoxia (19,172 ± 159 dpm/10$^6$ cells) and normoxia (10,392 ± 216 dpm/10$^6$ cells), and PAF-stimulated IP$_3$ release was still down-regulated by conditions of normoxia. Thus, whereas the cell-permeable cGMP analog attenuated PAF-stimulated IP$_3$ release, inhibition of endogenous cGMP activity with cGMP/PKG antagonist augmented PAF-stimulated IP$_3$ release. The increase in IP$_3$ release after inhibition of PKG activity suggests that the PKG effect on PAF-stimulated IP$_3$ release occurred downstream from the PAF-PAF receptor interaction.

Figure 4 shows the effect of PAF, 8-Br-cAMP, and the cAMP/PKA antagonist, Rp-cAMPS, on PAF-stimulated IP$_3$ release. Under baseline conditions in hypoxia, IP$_3$ release (means ± S.E.M.) was 1150 ± 33 dpm/10$^6$ cells. During normoxia, baseline release of IP$_3$ decreased by 22% (896 ± 36 dpm/10$^6$ cells). Treatment of cells with 5 nM PAF increased IP$_3$ release 7-fold during hypoxia to 8565 ± 314 dpm/10$^6$ cells and 6-fold during normoxia to 5418 ± 118 dpm/10$^6$ cells. As with baseline conditions, normoxia down-regulated PAF-stimulated IP$_3$ release by the cells. Pretreatment of cells with 8-Br-cAMP decreased PAF-stimulated IP$_3$ release by 45%, both during hypoxia and normoxia: 4748 ± 298 and 3403 ± 112 dpm/10$^6$ cells, in hypoxia and normoxia, respectively, compared with PAF treatment alone under the two conditions. When the cells were pretreated with Rp-cAMPS to inhibit activity of endogenous cAMP at its receptor protein, PAF-stimulated IP$_3$ release increased by 50% during hypoxia (13,104 ± 1480 dpm/10$^6$ cells) and by 60% during normoxia (8669 ± 169 dpm/10$^6$ cells), and PAF-stimulated IP$_3$ release was still down-regulated by conditions of normoxia. Thus, whereas the cell-permeable cAMP analog attenuated PAF-stimulated IP$_3$ release, inhibition of endogenous cAMP activity with cAMP/PKA antagonist augmented PAF-stimulated IP$_3$ release. As with the PKG effect, the increase in IP$_3$ release after inhibition of PKA activity suggests that the PKA effect on PAF-stimulated IP$_3$ release occurred downstream from PAF-PAF receptor interaction.

We investigated cGMP and cAMP production to show that the cells can synthesize these cyclic nucleotides in situ, thereby facilitating postnatal pulmonary vascular relaxation. Figure 5 shows cGMP (Fig. 5A) and cAMP (Fig. 5B) levels measured in the smooth muscle cells under unstimulated (baseline) and stimulated conditions. Baseline production of cGMP in hypoxia was 1.13 ± 0.06 pmol/ml and was not different from production in normoxia, which was 1.13 ± 0.04 pmol/ml. Cyclic GMP production by cells on treatment with PAF was 1.23 ± 0.05 pmol/ml, which decreased significantly to 0.90 ± 0.11 pmol/ml in normoxic conditions. Production of cGMP after forskolin treatment was not different from baseline values in hypoxia or normoxia. However, SNP treatment increased cGMP levels to 3.44 ± 0.05 pmol/ml in hypoxia, which increased to 4.75 ± 0.12 pmol/ml in normoxic conditions. Thus, PAF, the pulmonary vasoconstrictor, decreased cGMP synthesis during normoxia, whereas SNP, the nitric oxide-generating agent, increased synthesis of cGMP.

Baseline values for production of cAMP (Fig. 5B) were 2.06 ± 0.21 and 2.06 ± 0.03 pmol/ml in hypoxia and normoxia, respectively. There was no difference in production between hypoxia and normoxia. cAMP production by cells

![Fig. 4](image_url)

**Fig. 4.** Effect of cAMP and PKA on PAF-stimulated IP$_3$ production by stimulated cells. Data are means ± S.E.M.; n = 5. Cells were preincubated for 15 min in hypoxia or normoxia with buffer alone, with 10 μM 8-Br-cAMP, or with 10 μM Rp-cAMPS; then 5 nM PAF was added as needed and incubated for 20 min more. PAF stimulated the release of inositol phosphate. 8-Br-cAMP inhibited PAF-stimulated inositol phosphate release. cAMP inhibitory effect was abrogated by Rp-cAMPS. *, p < 0.05, different from hypoxia; **, p < 0.05, different from baseline; #, p < 0.05, different from effect of PAF alone in hypoxia or normoxia.

![Fig. 5](image_url)

**Fig. 5.** Stimulation of cGMP production by the cells. Data are means ± S.E.M.; n = 5. Cells were preincubated for 15 min in hypoxia or normoxia with 0.1 mM IBMX; then each stimulus was added as needed and incubated for 15 min more in hypoxia or normoxia. PAF inhibited cGMP production in normoxia (A). cGMP production with forskolin stimulation was not different from baseline; however, SNP stimulated cGMP production during hypoxia and normoxia. B, forskolin stimulated the production of cAMP during hypoxia and normoxia. However, PAF and SNP did not stimulate cAMP production above baseline conditions. *, p < 0.05, different from hypoxia; **, p < 0.05, different from baseline, PAF and forskolin treatments (cGMP production) or SNP treatment (cAMP production) in hypoxia and normoxia.
after treatment with PAF was $2.31 \pm 0.06$ pmol/ml, which decreased, but nonsignificantly ($p = 0.07$), to $1.96 \pm 0.07$ pmol/ml in normoxic conditions. Production of cAMP after SNP treatment was not different from baseline values in hypoxia or normoxia. However, forskolin treatment increased cAMP levels to $8.99 \pm 0.01$ pmol/ml in hypoxia, which further increased to $10.17 \pm 0.01$ pmol/ml in normoxic conditions. Thus, forskolin, a cAMP-elevating agent, increased cAMP production above baseline conditions in hypoxia and normoxia.

**PAF Attenuates PKG and PKA Protein Expression.** We studied the effect of PAF on PKG and PKA protein expression to demonstrate that one mechanism by which PAF sustains high pulmonary venous vasomotor tone in utero is by down-regulation of expression of PKG and PKA proteins. Figure 6 shows representative Western blots of expression of PAFr, PKA and PKG proteins, and actin internal standard by the cells at baseline and after treatment with $10 \text{nM PAF}$ for a total incubation time of 120 min in normoxia or hypoxia (Fig. 6A) and the densitometry of the respective protein expression (Fig. 6, B, PAFr; C, PKA; and D, PKG). Hypoxia increased PAF receptor protein expression by 40%. Treatment with $10 \text{nM PAF}$ increased PAF receptor protein expression in both normoxia and hypoxia, but more so in hypoxia than normoxia.

PKG and PKA protein expressions increased by 30 and 35%, respectively, during normoxia. Treatment of cells with $10 \text{nM PAF}$ decreased PKA protein expression by 15% during hypoxia and 60% during normoxia. The corresponding values for PAF-stimulated decreases in PKG protein expression are 55% in hypoxia and 65% in normoxia.

**Cyclic Nucleotides cGMP and cAMP Attenuate PAF Receptor Protein Expression during Normoxia.** We studied the effects of cGMP and cAMP on PAF receptor expression to demonstrate that one mechanism by which cGMP and cAMP facilitate postnatal pulmonary adaptation is via down-regulation of PAF receptor protein. Figure 7 shows a representative Western blot (inset) and densitometry of the effects of cGMP and cAMP on PAF receptor protein expression in normoxia. Treatment of cells with $10 \text{mM 8-Br-cGMP}$ attenuated PAF receptor protein expression by 80% compared with baseline conditions. Treatment of cells with $10 \text{mM 8-Br-cAMP}$ also decreased PAF receptor protein expression by 41%. Thus, both cGMP and cAMP attenuated PAF receptor protein expression during normoxia.

Because the greatest effects of cGMP and cAMP on PAF receptor-mediated responses were observed during normoxia, we then investigated the effect of PAF on PKG and PKA activities in extracts of cells preincubated with PAF during normoxia only. Figure 8A shows PKG activity in extracts of cells studied in normoxia only. In the absence of PAF pretreatment (0 nM PAF concentration), exogenous

![Fig. 6. Effect of PAF and hypoxia on expression of PAFr and PKA and PKG proteins. The plots are shown as the ratio of density of the specific protein to the blot density of the actin standard. Numerical data are means ± S.E.M., n = 3. Cells were incubated for 3 h in hypoxia or normoxia; then proteins were prepared for Western blotting. A, expression of the proteins measured by SDS-PAGE. Lanes 1 and 2 are protein bands from studies in normoxia with (+) and without (−) PAF treatment. Lanes 3 and 4 are protein bands from studies in hypoxia with (+) and without (−) PAF treatment. B, densitometry of Western blots of the protein expression: B, PAFr; C, PKA; D, PKG. PAF treatment and hypoxia led to increased PAFr protein expression. With PKA and PKG proteins, PAF treatment decreased protein expression in hypoxia and normoxia. *, $p < 0.05$, different from hypoxia; **, $p < 0.05$, different from baseline.](https://jpet.aspetjournals.org/content/733/4/733/F1)
cGMP enhanced PKG activity (means ± S.E.M.) more than 3-fold from 25 ± 5 to 86 ± 16 pmol/min/mg protein. Pretreatment of cells with 1 nM PAF abolished the cGMP-stimulated PKG activity. Pretreatment of cells with 10 nM PAF did not produce any further decrease in cGMP-stimulated PKG activity. With respect to PKA activity, exogenous cAMP stimulated PKA activity 4-fold from 290 ± 15 to 1190 ± 50 pmol/min/mg of protein (Fig. 8B). Pretreatment of cells with 1 nM PAF decreased cAMP/PKA activity by 30%. With 10 nM PAF treatment, cAMP/PKA activity decreased by 50% compared with no PAF treatment. In general, pretreatment of cells with PAF significantly decreased cGMP-dependent PKG and cAMP-dependent PKA activities in the cells.

Discussion

In fetal pulmonary circulation, vasoconstrictors such as PAF and thromboxane A2 (Toga et al., 1992; Argiolas et al., 1995; Ibe et al., 1998) maintain high vasomotor tone by increasing intracellular levels of IP3 and calcium. At birth, release of vasodilators such as cGMP and cAMP induce smooth muscle relaxation by acting through their respective protein kinases, PKG and PKA (Dhanakoti et al., 2000; Abdel-Latif, 2001). In ovine fetal SMC-PV, hypoxia up-regulates PAFr binding and PAFr-mediated intracellular IP3 and calcium release (Ibe et al., 2005); therefore, the fetal hypoxic environment facilitates PAFr binding, PAFr-mediated signaling, and maintenance of high pulmonary vasomotor tone in utero. The possibility that PAF may actively down-regulate vasodilator pathways in the pulmonary circulation of the fetal hypoxic environment has not been previously explored. Likewise, the decreased PAFr-mediated activity in the higher oxygen environment of the postnatal lung may involve down-regulation of PAFr-mediated cell signaling by other endogenous mediators such as cGMP and cAMP (Dhanakoti et al., 2000; Gao et al., 2004). In the present report, we investigated the interactions between cGMP/cAMP and PAF signaling pathways in fetal pulmonary vascular smooth muscle. We found that both cGMP and cAMP decrease PAFr binding in normoxia through their respective protein kinases, cGMP/PKG and cAMP/PKA. Cell-permeable analogs of cGMP and cAMP significantly attenuated PAFr protein expression, suggesting a translational regulation of PAFr protein expression. PAFr protein expression can be down-regulated by the protein synthesis inhibitor cycloheximide (Ibe et al., 2005). Thus, cGMP and cAMP, acting through their respective receptors PKG and PKA, do down-regulate PAF receptor protein expression by translational mechanisms. During normoxia, a physiological concentration of PAF in culture decreased PKG and PKA protein expression and activity. These results suggest that in the fetal hypoxic environment, PAF may be actively down-regulating cGMP- and cAMP-dependent signaling pathways and that postnatally cGMP and cAMP actively inhibit PAFr binding, PKG expression, and PAFr-mediated signaling. This cross-talk between the two pathways will effectively maintain a high pulmonary vasomotor tone in utero and facilitate vasorelaxation at birth via down-regulation of PAFr-mediated signaling by the cyclic nucleotides. Scheme 1 explains our hypothesis for the interaction of PAF and the cyclic nucle-
Cyclic Nucleotides and PAFr-Mediated Effects

mediated intracellular signaling. Specifically, the cGMP and cAMP produced bind to their receptors in perinatal pulmonary adaptation. During hypoxia, PAFr binding activates intracellular signaling pathways that decrease PKG and PKA activities, resulting in pulmonary vascular constriction. During normoxia, stimulation of guanylyl cyclase (GC) and adenylate cyclase (AC) leads to cGMP and cAMP production increases. cGMP and cAMP bind to PKG and PKA, respectively, leading to down-regulation of PAFr protein expression and ultimately resulting in relaxation of pulmonary vascular smooth muscle.

otides in perinatal pulmonary adaptation. During hypoxia in utero, PAFr binding activates intracellular signaling pathways that decrease PKG and PKA activities, resulting in pulmonary vascular constriction. During normoxia postnatally, the cGMP and cAMP produced bind to their receptors PKG and PKA to inhibit PAFr expression and PAFr-mediated intracellular signaling.

Cyclic Nucleotides and PAFr-Mediated Effects

Role of cGMP. Endothelium-derived nitric oxide produced under basal conditions or by a stimulus readily diffuses into the contiguous smooth muscle to activate soluble guanylyl cyclase, resulting in increased cGMP synthesis and smooth muscle relaxation (Ignarro, 1990; Nakahara et al., 2002; Gao et al., 2004). Nitric oxide (NO), a potent vasodilator in the pulmonary circulation, is important in the transition of the pulmonary circulation from fetal to postnatal life. Activation of soluble guanylyl cyclase activity by NO increases cGMP synthesis and relaxation of the pulmonary vasculature (Block et al., 1997). In this study, oxygen in physiological levels down-regulated PAFr binding. Also, 8-Br-cGMP, decreased PAFr binding in hypoxia and normoxia, suggesting that cGMP produced in vivo will counteract the vasoconstricting properties of PAF. Interestingly, when activity of endogenous cGMP was inhibited with an inhibitor of cGMP-dependent PKG, the endogenous cGMP receptor, the ability of cGMP to inhibit PAFr binding was obliterated, showing that cGMP acts via its receptor to inhibit PAFr binding. The exact mechanism of this inhibition is not yet clear; however, our data show that the adverse effects of PAF can be ameliorated postnatally by the down-regulation of PAFr protein expression.

PAF-stimulated inositol phosphate release is augmented in hypoxia (Ibe et al., 2005), and the profile of release is similar to the effect of phorbol myristate acetate, a specific activator of PKC in the pulmonary circulation (Orton et al., 1990). This indicates that PKC/phospholipase B activation is one mechanism of PAF-stimulated inositol phosphate release in SMC-PV. Activation of PKC increases its affinity for calcium under physiological conditions (Standaert et al., 1997). Also activation of PKC inhibits cGMP production in vascular smooth muscle cells by inhibiting NO release (Nambi et al., 1987). Pretreatment of SMC-PV with the PKG antagonist, Rp-8-pCPT-cGMPS, increased PAF-stimulated inositol phosphate release. We can infer that in vivo, cGMP acting via its receptor, PKG, will inhibit PAF-stimulated inositol phosphate synthesis and decrease calcium release coupled with vasodilation. On the other hand, inhibition of cGMP release with a concomitant increase in the calcium level will result in increased vasoconstriction. The inhibitory effect of cGMP on PAF-stimulated inositol phosphate release was more pronounced under hypoxic conditions. In line with our findings, cGMP has been shown to inhibit endothelin-stimulated inositol phosphate release in pulmonary arteries of fetal lambs studied in an organ bath (Millard et al., 1998). Endothelin and PAF are potent endogenous vasoconstrictors in the pulmonary circulation. PAF inhibited cGMP production in normoxia by 26%, whereas SNP, an NO donor, augmented cGMP production, especially in normoxia. The physiological relevance of PAF inhibition of cGMP production is presently unclear, but we can surmise that increased levels of endogenous PAF postnatally can inhibit cGMP release, leading to elevated intracellular calcium levels and increased vasoconstriction.

Role of cAMP. cAMP production is linked to β-adrenergic receptor-mediated activation of adenyl cyclase (Gilman, 1995; Milligan and Kostenis, 2006). Inhibition of cGMP- and cAMP-dependent phosphodiesterases can also result in high cellular levels of cGMP and cAMP (Nakahara et al., 2002; Hicks et al., 2005). Although 8-Br-cAMP did not significantly alter PAF binding in hypoxia, inhibition of cAMP/PKA with Rp-cAMPS, obliterated the endogenous cAMP effect and significantly increased PAF binding in hypoxia, suggesting that cGMP and cAMP interact with PAFr after activation of their own receptors.

Similar to our observations on cGMP/PKG activity, inhibition of PKA with Rp-cAMPS led to increased inositol phosphate release, suggesting that the influence of PKG and PKA on PAF-mediated responses may be occurring downstream from the PAFr. This means that other endogenous mediators of inositol phosphate production such as diacyl glycerol may have been activated after inhibition of PKG and PKA activities.

Forskolin, a cAMP-elevating agent, increased cAMP production, suggesting that in vivo increased production of cAMP may activate PKA, resulting in inhibition of PAFr-mediated effects. This inhibition may constitute one mechanism whereby the vasodilator properties of cAMP are maintained postnatally.

Previous reports have shown that cGMP and cAMP signaling pathways may cooperate under certain conditions to regulate tissue physiological end points, for instance, decreases of cAMP and cGMP release by hypoxia (Taylor et al., 1998) and stimulation of NO release by cAMP in coronary microvessels of conscious pigs (Kudej et al., 2000) and by rat aortic endothelium (Ray and Marshall, 2006). The mechanisms of these stimulus-induced cAMP/cGMP interactions are not well understood, but may involve phosphorylation of endothelial nitric-oxide synthase by PKA (Ray and Marshall, 2006). Although cAMP and cGMP were not measured in the reports cited above, other studies (Gao et al., 1995) have shown that administration of NO or SNP does increase cGMP release. In this study, we measured...
cGMP release in forskolin-stimulated cells and cAMP in SNP-stimulated cells. Neither of the two stimuli produced a crossover effect on cyclic nucleotide release. Therefore, it is possible that cAMP activation of NO production does not occur in SMC-PV in culture. However, more studies are needed to address this paradigm.

**PAF and Regulation of PKG and PKA Activities in Perinatal Pulmonary Adaptation**

In normoxia, activities of PKG and PKA are high (Dhanakoti et al., 2000; Gao et al., 2004). The physiological implications of these findings are that up-regulation of PPKG and PKA activities postnatally is one mechanism by which fetal high pulmonary vasomotor tone is down-regulated to facilitate postnatal pulmonary adaptation. Thus, favorable perinatal pulmonary vascular adaptation can be achieved by both down-regulation of mediators of pulmonary vascular constriction, such as PAF, and by up-regulation of mediators of pulmonary vasodilation, such as cGMP/PKG and cAMP/PKA. Endothelin (Fratz et al., 2004) and PKC (Johnson and Barman, 2004) are two other mediators that have been reported to evoke vasoconstriction in the perinatal pulmonary circulation. We report here that during normoxia PAF significantly down-regulates expression and activities of both cGMP/PK and cAMP/ PK. Exposure of SMC-PV to PAF resulted in significant down-regulation of expression of PKG and PKA proteins with attendant up-regulation of PAFr protein. These data strongly indicate that congenital perinatal pulmonary adaptation entails a combination of down-regulation of PAFr-mediated effects via cyclic nucleotide-mediated pathways and up-regulation of cyclic nucleotide production as we have shown in this article. This is accomplished postnatally by down-regulation of translation of PAFr protein by cyclic nucleotides as well as by up-regulation of cGMP- and cAMP-mediated pathways as has been previously reported (Dhanakoti et al., 2000; Egenov et al., 2004).

PPHN is a pathological condition with different etiologies. Neonates with PPHN have high PAF levels (Caplan et al., 2004) and the inability of cyclic nucleotides to down-regulate PAFr protein is due to down-regulation and up-regulation of PKG and PKA activities by high PAF levels and the inability of cyclic nucleotides to down-regulate PAFr-mediated effects postnatally will also contribute to development of PPHN.

**Acknowledgments**

We thank Amy McPeak and Stephen Douglass for help with the assays.

**References**

Abdel-Latif AA (2001) Cross talk between cyclic nucleotides and polyphosphoino- 

Ali H, Richardson RM, Tomhaye ED, Dubose RA, Haribabu B, and Snyderman R (1994) Regulation of stably transfected platelet activating factor receptor in RBL- 

tion and bronchoconstriction produced by PAF in guinea-pig: role of platelets and 

Block KD, Filippov G, Sanchez LS, Nakane M, and de la Monte SM (1997) Pulmo-
nary soluble guanylate cyclase, a nitric oxide receptor, is increased during the perinatal period. Am J Physiol 272:H90–H96.

Respir Dis 142:1258–1262.


Address correspondence to: Dr. Basil O. Ibe, Division of Neonatology, Los Angeles Biomedical Research Institute, 1124 W. Carson St., RB-1, Torrance, CA 90502. E-mail: ibe@labiomed.org