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

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monophosphate, cGMP; intrapulmonary venous smooth muscle cells, SMC-PV; platelet activating factor, PAF; pulmonary vascular smooth muscle cells, PVSMC; protein kinase A, PKA; protein kinase G, PKG; 8-bromoguanosine-3',5'-cyclic monophosphate, 8-Br-cGMP; 8-bromoadenosine-3',5'-cyclic monophosphate, 8-Br-cAMP; Rp-Adenosine-3',5'-cyclic monophosphorothioate, Rp-cAMPS; Rp-8-(4-Chlorophenylthio)-guanosine-3',5'-cyclic monophosphorothioate.sodium, Rp-8-pCPT-cGMPS; persistent pulmonary hypertension of the newborn, PPHN.

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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 effect 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), 8-Br-cAMP (BAMP) as well as PKG and PKA inhibitors. All groups were treated with 1nM PAF and incubated for 30 min for binding assay or 20 min for measurement of inositol phosphate (IP_3) production. BGMP and BAMP decreased PAF binding in normoxia by 63% and 14% respectively. Incubations with PKG inhibitor Rp-8-pCPT-cGMPS and PKA inhibitor Rp-cAMPS abrogated the inhibitory effects of BGMP and BAMP. PAF-stimulated IP_3 production (DPM/10^6 cells) was 8565±314 in hypoxia and 5418±118 in normoxia, a 40% decrease. BGMP attenuated PAF-stimulated IP_3 production by 67% and 37% in hypoxia and normoxia, the value for BAMP was 44% under both conditions. Pre-treatment with PKG or PKA inhibitor abrogated BGMP and BAMP inhibition of IP_3 release. PAF receptor (PAFr) protein expression decreased in normoxia, but pretreatment with 10nM PAF up-regulated PAFr expression. Pretreatment with PAF decreased expression and activities of PKG or PKA proteins in normoxia and hypoxia. Our data demonstrate existence of cGMP/cAMP-PAF cross-talk in PVSMC, which may be one mechanism by which PAFr mediated vasoconstriction is down-regulated at birth.

INTRODUCTION

Platelet activating factor (PAF) evokes a wide range of biological activities (Stafforini et al., 2003). In lungs, PAF is a potent vasoactive mediator with different sites of action in different species (Toga et al., 1992; Ibe et al., 1998; Argiola et al., 1995). 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; Ibe et al., 2000). In addition, effects of PAF can be completely abrogated by the enzymatic degradation of PAF by PAF acetylhydrolase (Kim et al., 2000), by specific PAFr antagonists (Ibe et al., 1998; Argiolas et al., 1995), or by agents that decrease PAFr protein expression (Ibe et al., 2000).

PAF evokes its effects by binding to its G protein-coupled receptor (GPCR), which is a seven trans-membrane receptor (Parent et al., 1996; Carlson et al., 1996). Activation of GPCR 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 (IP_3), and calcium (Rehring TF et al., 1996; Lee et al., 2001; Lin and Rui, 1994). cGMP and cAMP act via their endogenous receptors, 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 8-10% of total cardiac output (Hayman 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 effect of cGMP and cAMP on PAFr expression and the effect of PAF on expression and activities of PKG and PKA proteins in these venous smooth muscle cells.

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MATERIALS AND METHODS

Materials

Pregnant ewes (146-148 d gestation, term being 150 d) were purchased from Nebekar Farms, Santa Monica, CA. Authentic standards of 1-O-hexadecyl-2-O-acetyl-sn-glycero-3-phosphorylcholine ,PAF, (C_{16}-PAF) and 1-O-hexadecyl-sn-glycero-3-phosphorylcholine (lyso-C_{16}-PAF) standards as well as cyclic adenosine monophosphate (cAMP), cyclic guanosine monophosphate (cGMP), 8-bromoguanosine-3',5'-cyclic monophosphate (8-Br-cGMP), Rp-8-pCPT-cGMPS, 8-bromoadenosine-3',5'-cyclic monophosphate (8-Br-cAMP), Rp-cAMPS were purchased from Biomol, Plymouth Meeting, PA. Radiolabeled PAF standards and substrates; hexadecyl-2-acetyl-sn-glyceryl-3-phosphorylcholine, 1-O-[acetyl-^{3}H-(N)]-, (^{3}H-acetyl-C_{16}-PAF), 21.5 Ci/mmol (370 GBq/mmol) were purchased from Perkin Elmer Life Sciences (Boston, MA). Phenylmethysulfonyl fluoride (PMSF), leupeptin, pepstatin, bovine serum albumin (BSA), as well as antibody to actin were purchased from Sigma Chemical Company (St. Louis, MO). Antibody to PKA and PKG were purchased from Cell Signaling, while PAF receptor antibody was purchased from Cayman Chemical Company (Ann Arbor, MI). Studies were done with freshly made reagents. Ecolite(+) liquid scintillation cocktail was purchased from ICN Biochemicals (Irvine, CA).

Methods

Preparation of pulmonary vascular smooth muscle cells (PVSMC).

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 4^{th} to 6^{th} passage and identity of the smooth muscle cells at each passage was characterized with a smooth muscle cell-specific monoclonal antibody (SIGMA, St. Louis, MO). The SMC were devoid of endothelial cells and fibroblasts. Cell phenotype did not change from 4^{th} to 6^{th} 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, since 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 significant vasoactivity greater 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 humidified incubator at 37 ^{O}C aerated with 5% CO_2 in air. Oxygen concentration was monitored with TED 60T percent oxygen sensor, Teledyne Analytical Instruments (City of Industry, CA). The incubator oxygen concentration was 21% and pO_2 determined as in hypxia condition was normoxic at 80-100 torr.

Hypoxia: An incubator set at 37 ^{O}C was first equilibrated for at least 1 h with a gas mixture of 2% O_2, 10% CO_2, and balance N_2 to maintain incubator pO_2 <40 torr, determined on Nova Stat Profile 3 blood-gas instrument, Nova Biomedical, Waltham, MA

(Ibe et al., 2002). Cells were then placed in this incubator for experimentation and the cells 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 previously reported (Ibe et al., 2005). Briefly, cells were washed with calcium and magnesium free PBS before use according to the specific study protocol. After 30 min incubation in normoxia or hypoxia, unbound ^{3}H-PAF was washed off with ice-cold PBS, and then incubated on ice for 30-45 min in saline/EDTA mixture containing 154 mM saline and 5 mM EDTA (Korth et al., 1995; Ibe et al., 2005). Receptor bound ^{3}H-PAF was extracted on Whatman GF/C membrane filters using in-line vacuum system as we previously reported (Ibe, et al, 2005). Cell-bound PAF radioactivity was quantified by scintillation spectrometry (Beckman Instruments, Fullerton, CA). In studies probing the interaction of PAF with its receptors in the presence of other agonist or antagonists, cells were pre-incubated with the agent before the addition of ^{3}H-PAF, and then incubated further according to the specific experimental protocol.

Specific Protocols.

Effect of cGMP/cAMP on PAF-r binding. Cells were pre-incubated for 30 min, in hypoxia or normoxia, with buffer for controls or 10 μM each of cell permeable cyclic nucleotide analogs, 8-Br-cGMP or 8-Br-cAMP. Then 1nM [^{3}H]-PAF was added and incubated for 30 min more. In preliminary studies, we found no significant difference

between inhibition of PAF binding produced by 10μM or 100μM of the cell permeable cAMP and cGMP analogs. To study effect of cGMP and cAMP on PAF recptor protein expression, cells were incubated for 3 hr in normoxia only with 10μM each of 8-Br-cGMP and 8-Br-cAMP or with 0.1% BSA for baseline and then protein was prepared for Western blotting of PAF receptor protein.

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

Study of cGMP and cAMP release by the cells: Cells were seeded at 5 x 10^5 cells in 100 mm Petri dishes and allowed to attain confluence. The confluent cells were washed twice with protein-free PBS and pre-incubated for 15 min in normoxia or hypoxia at 37^{O}C in Krebs bicarbonate buffer, pH 7.4, containing the following reagents in mM: NaCl, 119; KCl, 4.6; $CaCl_2$, 1.5; $MgCl_2$, 1.2; $NaHCO_3$, 15; NaH_2PO_4, 1.2; glucose, 5.5; and 0.1mM of 3-isobutyl-1-methyl-xanthine (IBMX), to inhibit phosphodiesterase catalyzed breakdown of cAMP and cGMP produced during experimentation. After pre-incubation, cells were treated with the following agents; 10nM PAF, and 0.1mM 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

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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 re-dissolved in 0.05 M sodium acetate. The amounts of cAMP and cGMP produced were determined by radioimmunoassay with a commercial kit (Biomedical Technologies Inc.) following the instructions provided by the vendor. Amounts of cAMP and cGMP measured are presented as pmol/ml.

Release of inositol phosphates

Labeling of cells with [^{3}H]-myo-inositol. Cells were washed with protein-free, inositol-free medium and then fed with a culture medium containing 10% FBS and 5 μCi/ml of [^{3}H]-myo-inositol suspended in inositol-free media, and incubated for 16-24 h in 5% CO_2 in air as we previously reported (Ibe et al, 2005).

Stimulation of ^{3}H-inositol phosphate release by cells. Each test stimulus was prepared in freshly prepared 10 mM LiCl buffer. Labeled cells were stimulated with PAF or other agents and incubated in hypoxia or normoxia for 20 min at 37^{O}C to induce inositol phosphate production. Reactions were quenched by adding 10 mM formic acid, incubating on ice bath for 30 min, followed with neutralization with 15 mM ammonium hydroxide. Cell suspension was loaded on pre-equilibrated AG 1-X8 columns and washed with 2 ml distilled water. 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. [^{3}H]-inositol phosphate radioactivity was quantified by scintillation spectrometry.

Specific protocols.

Effect of cGMP and cAMP on PAF-stimulated IP_3 release. Cells pre-labeled with [^{3}H]-inositol were pre-incubated for 20 min with buffer alone for control or 10 μM of 8-Br-cGMP or 8-Br-cAMP after which 1nM of non-radiolabeled PAF was added and incubated for 20 min more.

Effect of PKG and PKA inhibitors on PAF-stimulated IP_3 release: To study effect of endogenous cyclic nucleotides on PAF-stimulated inositol phosphate release, cells were pre-incubated with buffer alone or with 10 μM each of cGMP and cAMP receptor antagonists Rp-pCPT-cGMPS and Rp-cAMPS, and then non-radiolabeled PAF was added and incubated for 20 min more. Amount of inositol phosphate released was extracted and quantified.

Western blotting

Preparation of proteins for Western 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 (DTT), 0.1 mM PMSF, 5 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μM 4-(2-aminoethyl) benzene sulfonyl 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 refrigerated centrifuge and stored in 0.2 ml aliquots at -80^{o}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 (Ibe et al., 2005; Ali et al., 1994). The signals were developed for 1 min using Amersham ECL Western Blot detection kit and then exposed to X-ray film. Band corresponding to PAF-R protein was scanned with an EagleEye densitometer (Stratagene) to quantify blot density.

PKG and PKA activity in extracts of PVSMC

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^{O}C and then incubated at 37 ^{O}C with 0.1% BSA in DMEM 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 PBS, then cells were detached and centrifuged at 400g for 5 min. Cell pellet was homogenized on ice in 50 mM Tris buffer, pH 7.4, containing 10 mM EDTA, 2 mM DTT, 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 seconds and re-centrifuged at 4 ^{O}C at 14,000g for 10 min. 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 activity of PKG (pmol/min/mg protein) in the absence or presence of 5 μM cGMP was similar (34.1 ± 4.1 and 108.4 ± 7.3 vs. 32.7 ± 1.8 and 100.9 ± 5.1) whether in fresh cell extract or in frozen extracts stored at -80^{O}C. Also, in preliminary experiments, we found that PKG activity was linear up to 15 μg of protein extract, up to 15 minutes 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, aprotinin) and phosphatase inhibitors (calyculin A, cypermethrin, sodium vanadate), did not significantly enhance the activities of PKG (123 ± 10.3 vs 129 ± 8.4 with 5 μM cGMP) or PKA (1063 ± 35 vs. 1143 ± 86 with 2 μM cAMP) and thus were excluded in our subsequent assays. PKG and PKA assays were done essentially as previously reported (Dhanakoti et al., 2000).

Data analysis. All numerical data are means ± SEM. In all instances where radioisotope was used, background radioactivity was subtracted before quantifying radioactivity. Data were analyzed with two-tailed t-test followed with ANOVA (GraphPad Prism, San Diego, CA). Results were considered significant at $p < 0.05$.

RESULTS

Cyclic GMP and cAMP decrease PAF receptor binding through their respective kinases cGMP-dependent protein kinase (PKG) and cAMP-dependent protein kinase (PKA). We used the cell permeable cyclic nucleotide analogs, 8-Br-cGMP and 8-Br-cAMP to study 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 10μM 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 to PAF alone in normoxia. When endogenous cGMP activity was inhibited with the cGMP/PKG receptor antagonist Rp-8-pCPT-cGMPS, PAF receptor increased and then was comparable to 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 cAMP and cAMP-dependent PKA inhibitor on PAF receptor binding. During hypoxia the cell permeable cAMP analog, 8-Br-cAMP, had no effect on PAF receptor binding. Pretreatment of cells with the PKA inhibitor, Rp-cAMPS in hypoxia to inhibit endogenous cAMP activity led to a 30% increase in PAF binding

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compared to PAF alone, and to PAF in the presence of 8-Br-cAMP. During normoxia, treatment with 8-Br-cAMP attenuated PAF receptor binding by 26% to 11.4 ± 0.5 fmol/10^6 cells compared to PAF alone in normoxia, 15.5± 0.6. Treatment of cells with Rp-cAMPS to inhibit endogenous cAMP activity led to an augmentation of PAF binding by 65%, 25.9± 0.5, compared to PAF alone in normoxia, and to a level comparable to PAF alone in hypoxia. In general, normoxic conditions attenuated PAF receptor binding.

Since PAF stimulates release of inositol phosphates down-stream from 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 effect of PAF, 8-Br-cGMP and the cGMP/PKG antagonist, Rp-8-pCPT-cGMPS, on PAF-stimulated inositol phosphate (IP_3) production. Under baseline conditions in hypoxia, IP_3 released by cells (DPM/10^6 cells, means ± SEM) was 1150± 33. During normoxia, baseline release of IP_3 decreased by 22% (896 ± 38). Thus IP_3 release by the cells was significantly reduced by normoxic conditions. Treatment of cells with 5 nM PAF increased IP_3 release 7-fold during hypoxia to 8565 ± 314 and 6-fold during normoxia to 5418± 118. PAF-stimulated inositol phosphates release was attenuated by normoxia.

Pretreatment of the cells with 8-Br-cGMP decreased PAF-stimulated IP_3 release during hypoxia by 65% and by 37% during normoxia, compared to PAF treatment alone under the two conditions. When the cells were pre-treated with Rp-8-pCPT-cGMPS, to inhibit activity of endogenous cGMP, PAF stimulated IP_3 release increased 2-fold during

hypoxia (19172± 159) and normoxia (10392± 216), and then 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 following inhibition of PKG activity suggests that PKG effect on PAF-stimulated IP_3 release occurred downstream from 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 (DPM/10^6 cells, means ± SEM) was 1150± 33. During normoxia, baseline release of IP_3 decreased by 22% (896± 38). Treatment of cells with 5 nM PAF increased IP_3 release 7-fold during hypoxia to 8565 ± 314 and 6-fold during normoxia to 5418± 118. 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 3043± 112, in hypoxia and normoxia respectively; compared to PAF treatment alone under the two conditions. When the cells were pre-treated with Rp-cAMPS, to inhibit activity of endogenous cAMP at its receptor protein, PAF stimulated IP_3 release increased by 50% during hypoxia (13104± 1480) and by 60% during normoxia (8669± 169), and then 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 PKG effect, the increase in IP_3 release following inhibition of PKA activity suggest that 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 (figure 5a) and cAMP (figure 5b) levels measured in the smooth muscle cells under unstimulated (baseline) and stimulated conditions. Baseline production of cGMP (pmol/ml) in hypoxia was 1.13 ± 0.06 and was not different from production in normoxia which was 1.13 ± 0.04. Cyclic GMP production by cells on treatment with PAF was 1.23 ±0.05, which decreased significantly to 0.90 ± 0.11 in normoxic conditions. Production of cGMP on forskolin treatment was not different than baseline values in hypoxia or normoxia. However, SNP treatment increased cGMP levels to 3.44 ± 0.05 in hypoxia, which increased to 4.75 ± 0.12 in normoxic conditions. Thus PAF, the pulmonary vasoconstrictor, decreased cGMP synthesis during normoxia while SNP, the nitric oxide generating agent increased synthesis of cGMP.

Baseline production of cAMP (figure 5b) was 2.06 ± 0.21 and 2.06 ± 0.03 in hypoxia and normoxia respectively. There was no difference in production between hypoxia and normoxia. Cyclic AMP production by cells on treatment with PAF was 2.31 ± 0.06, which decreased, but non-significantly ($p = 0.07$) to 1.96 ± 0.07 in normoxic conditions. Production of cAMP on SNP treatment was not different than baseline values in hypoxia or normoxia. However, forskolin treatment increased cAMP levels to 8.99 ±

0.01 in hypoxia, which further increased to10.17 ± 0.01 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 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 PAF receptor (PAFr), PKA, PKG proteins and actin internal standard, by the cells in baseline and on treatment with 10nM PAF for a total incubation time of 120 min in normoxia or hypoxia (figure 6a) and the densitometry of the respective protein expression (figure 6b: PAFr, 6b(i); PKA, 6b(ii); and PKG, 6b(iii)). Hypoxia increased PAF receptor protein expression by 40%. Treatment with 10nM PAF increased PAF receptor protein expression in both normoxia and hypoxia, but more so in hypoxia than normoxia.

PKG and PKA protein expression increased by 30% and 35% respectively during normoxia. Treatment of cells with 10nM PAF decreased PKA protein expression by 15% during hypoxia and 60% during normoxia. The corresponding values for PAF-stimulated decrease 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 effect 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 representative Western blot (inset) and densitometry of effect of cGMP and cAMP on PAF receptor protein expression in normoxia. Treatment of cells with 10μM of 8-Br-cGMP attenuated PAF receptor protein expression by 80% compared to baseline conditions. Treatment of cells with 10μM 8-Br-cAMP also decreased PAF receptor protein expression 41%. Thus both cGMP and cAMP attenuated PAF receptor protein expression during normoxia.

Since the greatest effect 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 pre-incubated with PAF during normoxia only. Figure 8a, shows PKG activity (pmol/min/mg protein, means ± SEM) in extract of cells studied in normoxia only. In the absence of PAF pretreatment (0nM PAF concentration), exogenous cGMP enhanced PKG activity more than 3-fold from 25± 5 to 86± 16. Pretreatment of cells with 1nM 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 (pmol/min/mg protein, means ± SEM), exogenous cAMP stimulated PKA activity 4-fold from 290± 15 to 1190± 50, figure 8b. Pretreatment of cells with 1nM PAF decreased cAMP/PKA activity by 30%. With 10nM PAF treatment, cAMP/PKA activity decreased by 50% compared to 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 A_2 (Ibe et al., 1998; Toga et al., 1992; Argiolas et al., 1995) maintain high vasomotor tone by increasing intracellular levels of IP_3 and calcium. At birth, release of vasodilators such as cGMP and CAMP induce smooth muscle relaxation by acting through their respective protein kinases (PK), PKG and PKA (Abdel-Latif, 2001; Dhanakoti et al., 2000). In ovine fetal SMC-PV, hypoxia up-regulates PAFr binding and PAFr-mediated intracellular IP_3 and calcium release (Ibe et al., 2005), therefore fetal hypoxic environment facilitates PAFr binding, PAFr-mediated signaling and maintenance of high pulmonary vasomotor tone *in utero*. The possibility that PAF may actively downregulate vasodilator pathways in pulmonary circulation of fetal hypoxic environment has not been previously explored. Likewise, the decreased PAFr-mediated activity in higher oxygen environment of the post-natal lung may involve down-regulation of PAFr-mediated cell signaling by other endogenous mediators such as cGMP and cAMP (Dhanakotu et al., 2000; Gao et al., 2004). The present report 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 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, physiological concentration of PAF, in culture, decreased PKG and PKA protein expression and activity. These results suggest that in 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, PAFr expression and PAFr-mediated signaling. This cross-talk between the two pathways will effectively maintain a high pulmonary vasomotor tone *in utero* and facilitate vaso-relaxation 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 nucleotides in perinatal pulmonary adaptation. During hypoxia, *in utero*, PAFr binding activates intracellular signaling pathways that decrease PKG and PKA activity resulting in pulmonary vascular constriction. During normaxia, postnatally, 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 (EDNO) 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 (Gao et al., 2004; Ignarro, 1990; Nakahara et al., 2002). 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 pulmonary vasculature (Block et al., 1997). In this study, oxygen in physiologic 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 PAF 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 (PMA), 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 physiologic 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). Pre-treatment 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, decrease calcium release coupled with vasodilation. On the other hand, inhibition of cGMP release with concomitant increase in 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 artery of fetal lambs studied in 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. Cyclic AMP production is linked to β-adrenergic receptor-mediated activation of adenylyl cyclase (Gilman AG, 1995; Milligam et al., 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 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 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 following 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 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 physiologic endpoints. For instance decrease of cAMP and cGMP release by hypoxia (Taylor et al., 1998), 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 eNOS 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. None of the two stimuli produced a cross-over 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 activity in perinatal pulmonary adaptation

In normoxia, activities of PKG and PKA are high (Dhanakoti, et al., 2000; Gao et al., 2004). The physiologic implications of these findings are that up-regulation of PKG 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 et al., 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/PKG and cAMP/PKA. 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 congenial perinatal pulmonary adaptation entails combination of down-regulation of PAFr-mediated effects via cyclic nucleotide mediated pathways as we have shown in this report. 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 el., 2000; Evgenov et al., 2004).

Persistent pulmonary hypertension of the newborn (PPHN) is a pathological condition with different etiologies. Neonates with PPHN have high PAF levels (Caplan et al., 1990), showing that persistence of high PAF levels postnatally leads to abnormal perinatal pulmonary adaptation. We speculate that inhibition of PKG and PKA activities by high PAF levels and inability of cyclic nucleotides to down-regulate PAFr-mediated effects postnatally will also contribute to development of PPHN.

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FOOTNOTES

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LEGENDS FOR FIGURES

Scheme1. Interaction of PAFr-mediated intracellular signaling in hypoxia and cGMP- and cAMP-mediated responses in normoxia. During hypoxia, PAFr binding activates a series of intracellular signals which include increased intracellular calcium mobilization and the inhibition of PKG and PKA protein expression (broken line), and ultimately resulting in pulmonary vascular smooth muscle constriction. During normoxia, cGMP and cAMP production increases. cGMP and cAMP bind to PKG and PKA respectively leading to down- regulation PAFr protein expression (solid line) and PAFr-mediated signaling pathway, and ultimately resulting in relaxation of pulmonary vascular smooth muscle.

Figure 1. Effect of cGMP and PKG inhibitor Rp-8-pCPT-cGMPS on PAF receptor binding. Data are means ± SEM, n = 6. Cells were pre-incubated for 30 min in normoxia or hypoxia with buffer alone, or 10μM of 8-Br-cGMP or with 10μM of cGMP-dependent protein kinase (PKG) inhibitor Rp-8-pCPT-cGMPS, then PAF was added and incubated for 30 min more as described in the methods section. PAF receptor binding was inhibited by 8-Br-cGMP. The PKG antagonist, Rp-8-pCPT-cGMPS, reversed the inhibitory effect of cGMP on PAF receptor binding. *$p < 0.05$, different from hypoxia; **$p < 0.05$, different from PAF binding in hypoxia or normoxia.

Figure 2. Effect of cAMP and cAMP-dependent protein kinase (PKA) inhibitor Rp-cAMPS on PAF receptor binding. Data are means ± SEM, n = 6. Cells were pre-incubated for 30 min with buffer alone, or with 10μM 8-Br-cAMP, or with 10μM of cAMP-dependent protein kinase (PKA) inhibitor, Rp-cAMPS, then PAF was added and incubated for 30 min more as described in

methods section. PAF receptor binding in normoxia was inhibited by 8-Br-cGMP. The PKA antagonist, Rp-cAMPS, reversed the inhibitory effect of cAMP on PAF receptor binding. *p <0.05, different from hypoxia; **p <0.05, different from PAF binding in hypoxia or normoxa.

Figure 3. Effect of cGMP and PKG on PAF-stimulated inositol phosphate (IP_3) production by stimulated the cells. Data are means ± SEM, n= 5. Cells were preincubated for 20 min during hypoxia or normoxia with buffer alone, or with 10μM of 8-Br-cGMP, or with 10μM Rp-8-pCPT-cGMPS, then 5nM PAF was added as needed and incubated for 20 min more. PAF stimulated the release of inositol phosphate. PAF-stimulated inositol phosphate production was inhibited by 8-Br-cGMP. The inhibitory effect of cGMP was abrogated by Rp-8-pCPT-cGMPS. *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.

Figure 4. Effect of cAMP and PKA on PAF-stimulated inositol phosphate (IP_3) production by stimulated cells. Data are means ± SEM, n= 5. Cells were preincubated for 20 min in hypoxia or normoxia with buffer alone, or with 10μM of 8-Br-cAMP, or with 10μM Rp-cAMPS then 5nM 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.

Figure 5. Stimulation of cGMP production by the cells. Data are means ± SEM, n = 5. Cells

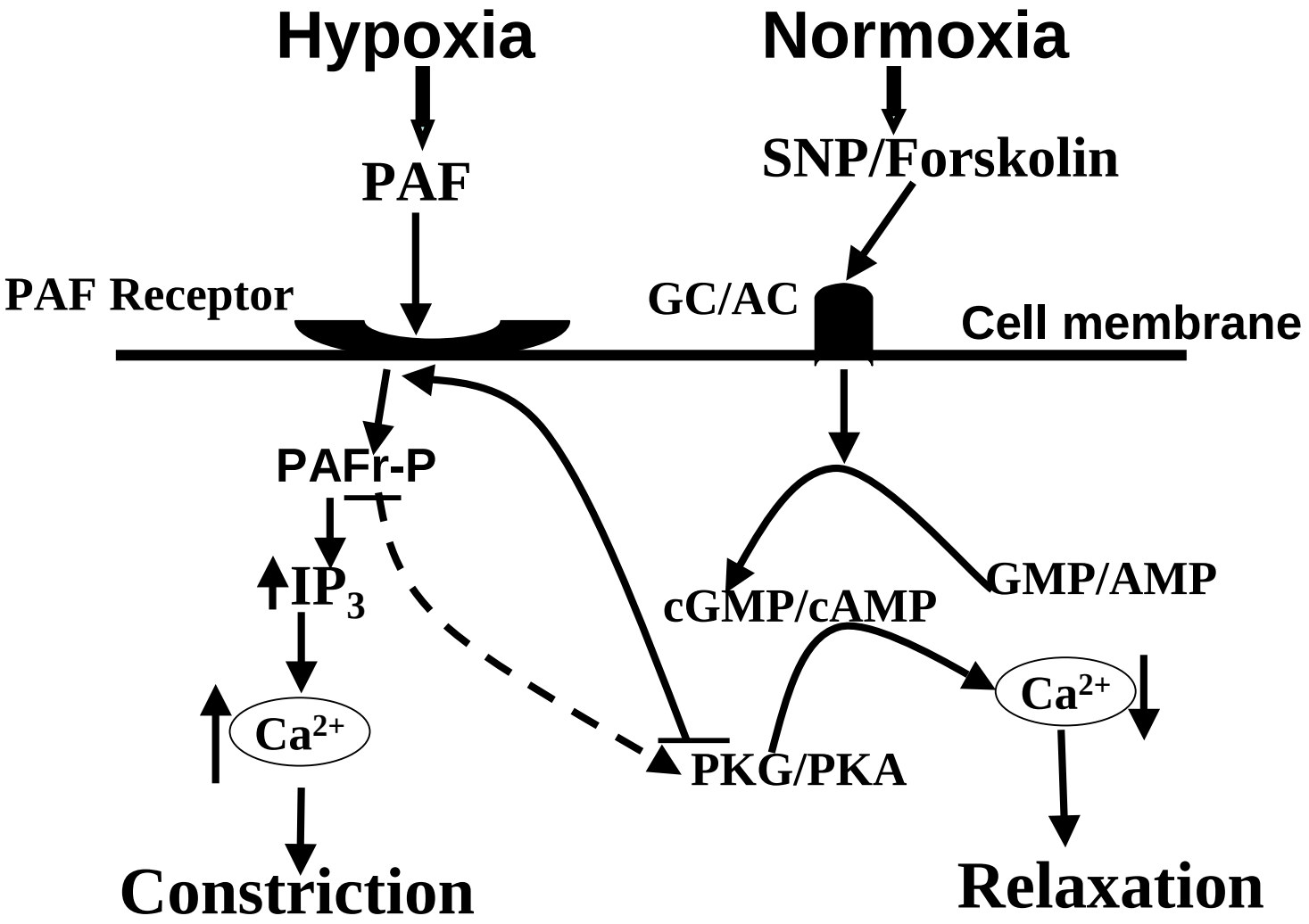
were pre-incubated for 15 min in hypoxia or normoxia with 0.1mM IBMX then each stimulus was added as needed and incubated for 15 min more in hypoxia or normoxia. PAF inhibited cGMP production in normoxia (figure 5a). cGMP production with forskolin stimulation was not different from baseline, however sodium nitroprusside (SNP) stimulated cGMP production during hypoxia and normoxia. In figure 5b, 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.

Figure 6. Effect of PAF and hypoxia on expression of PAFr, 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 ± SEM, n=3. Cells were incubated for 3 hr in hypoxia or normoxia and then proteins were prepared for Western blotting. Figure 6a shows 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. Figure 6b shows the densitometry of the Western blots of the protein expression. 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.

Figure 7. Effect of 8-Br-cGMP and 8-Br-cAMP on PAFr protein expression. Numerical data are means ± SEM, n=4. Cells were incubated for 3 hr in normoxia and then proteins were prepared

for Western blotting. Figure 7 inset shows expression of PAFr protein measured by SDS-PAGE. Lanes 1 and 3 show PAFr expression during baseline conditions. Lanes 2 and 4 are PAFr protein bands from studies with cGMP treatment, lane 2 and cAMP treatment, lane 4. Treatment of cells with the cyclic nucleotides decreased PAFr protein expression. *p <0.05, different from baseline conditions.

Figure 8. Effect of PAF on PKG and PKA activities during normoxia. Data are means ± SEM, n=5. Cells were pre-incubated for 210 min in normoxia only and then PAF was added and incubated for 30 min more in normoxia. Activities of PKG (figure 8a) and PKA (figure 8b) were assayed from cell lysates. PAF treatment inhibited both cGMP-dependent PKG activity and cAMP-dependent PKA activity. *p <0.05, different from PKG/PKA activity without PAF treatment.



Scheme 1

Figure 1.

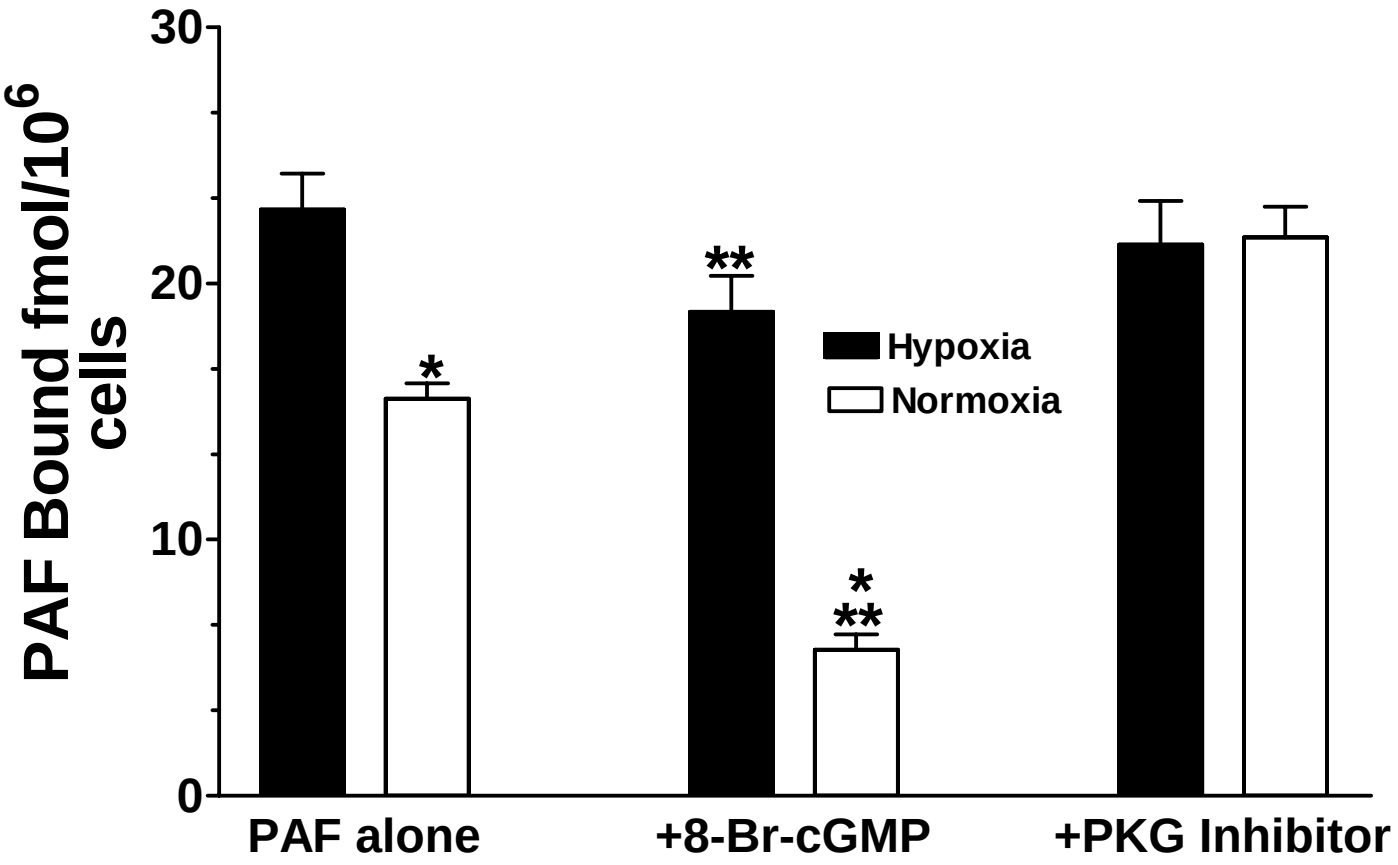


Figure 2.

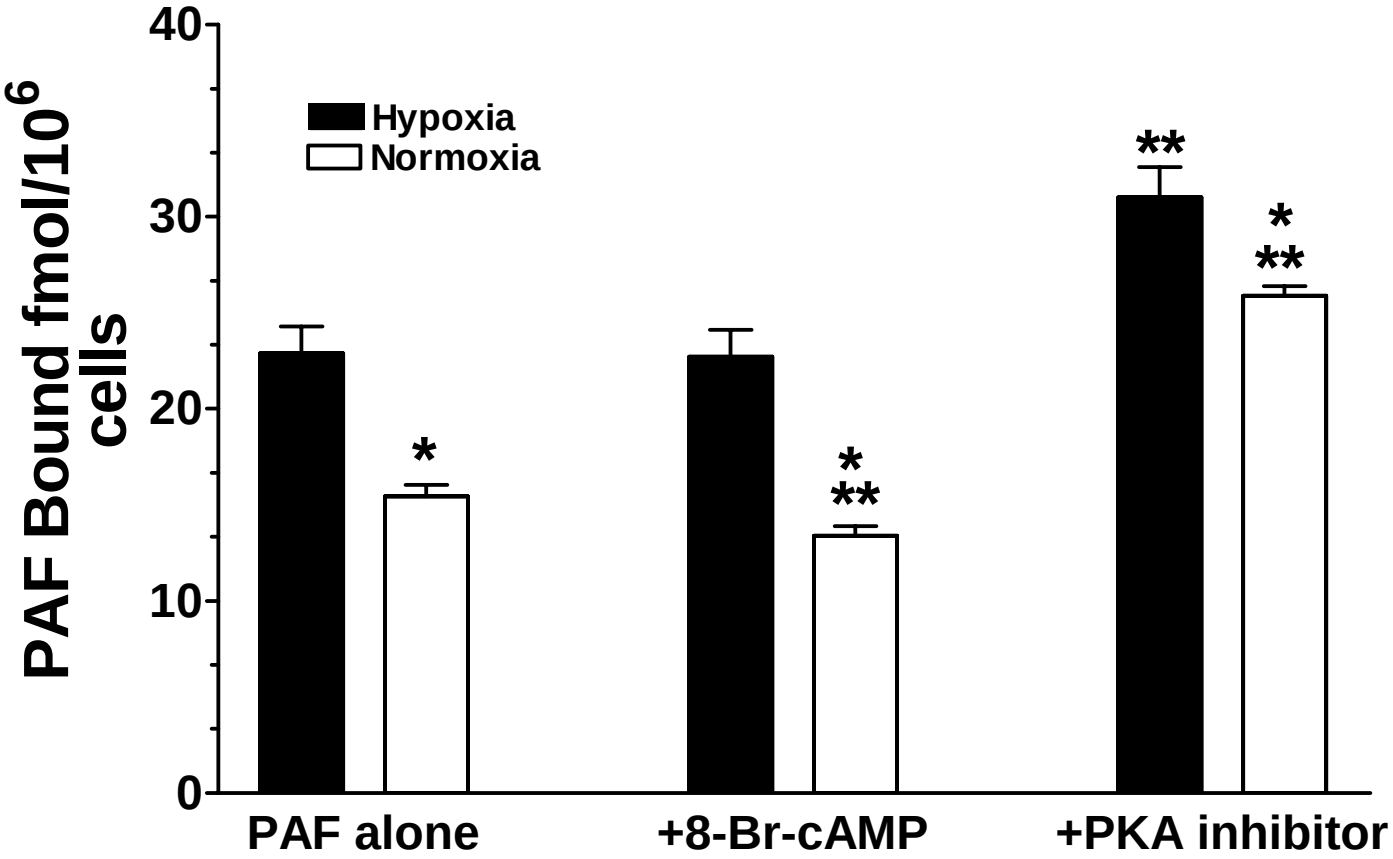


Figure 3.

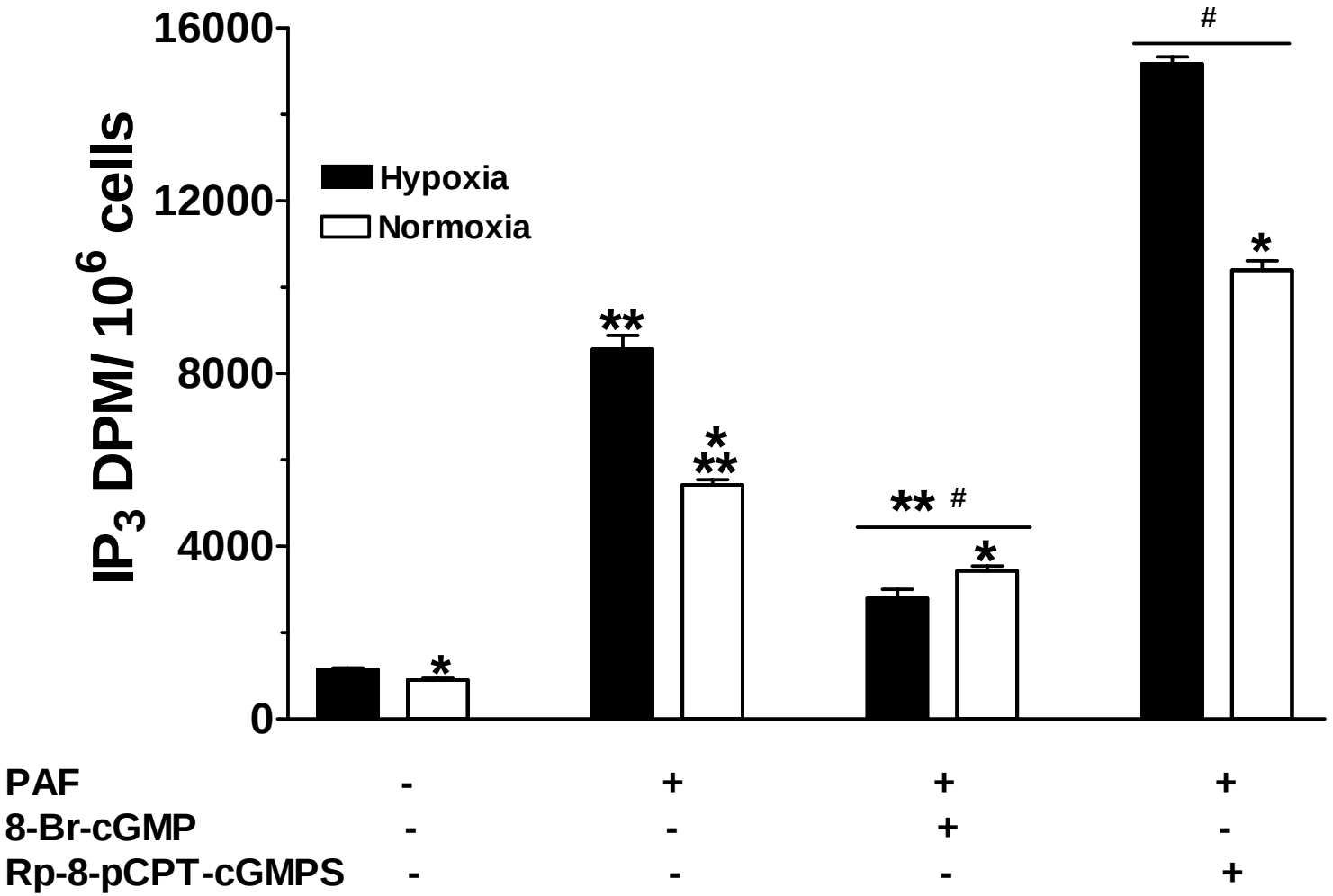


Figure 4.

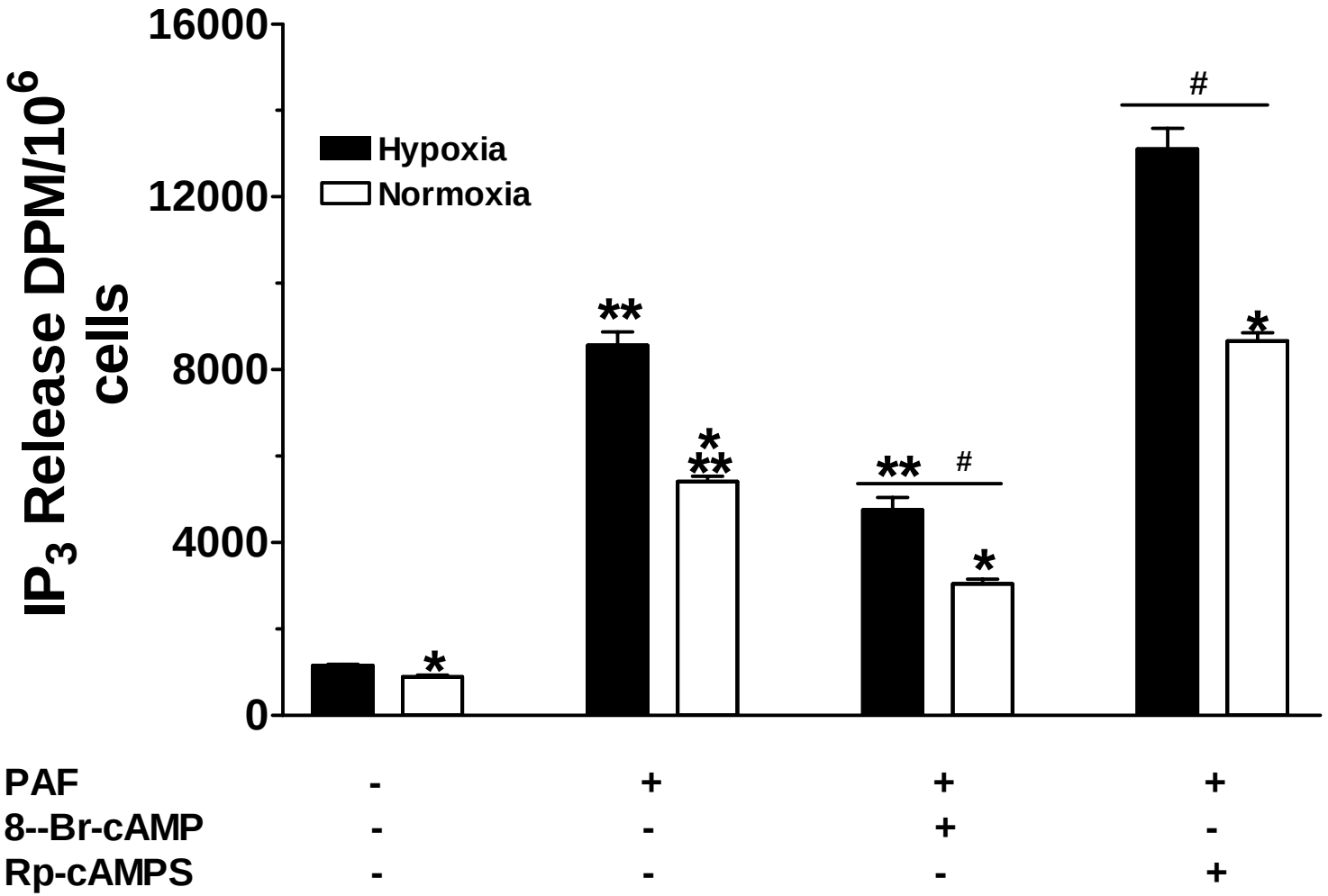


Figure 5a.

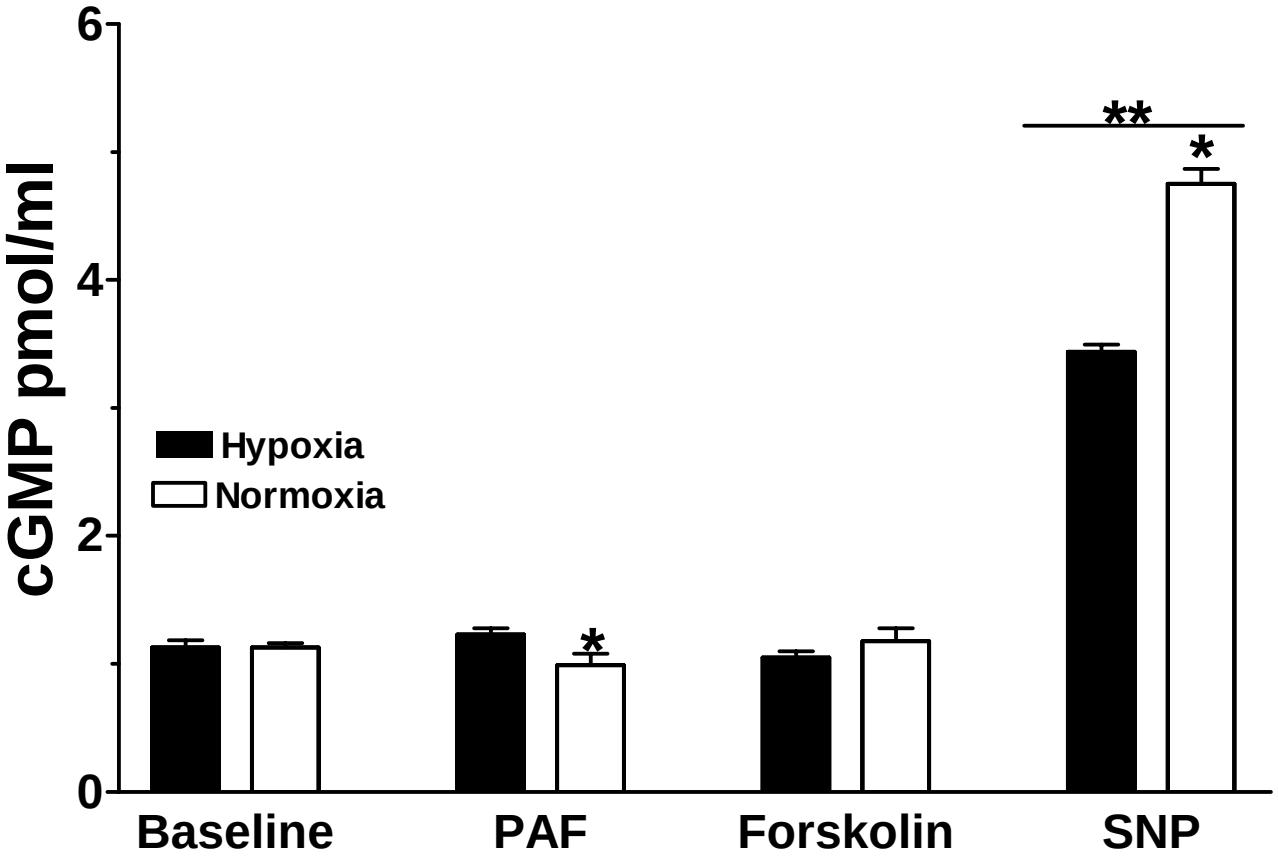


Figure 5b.

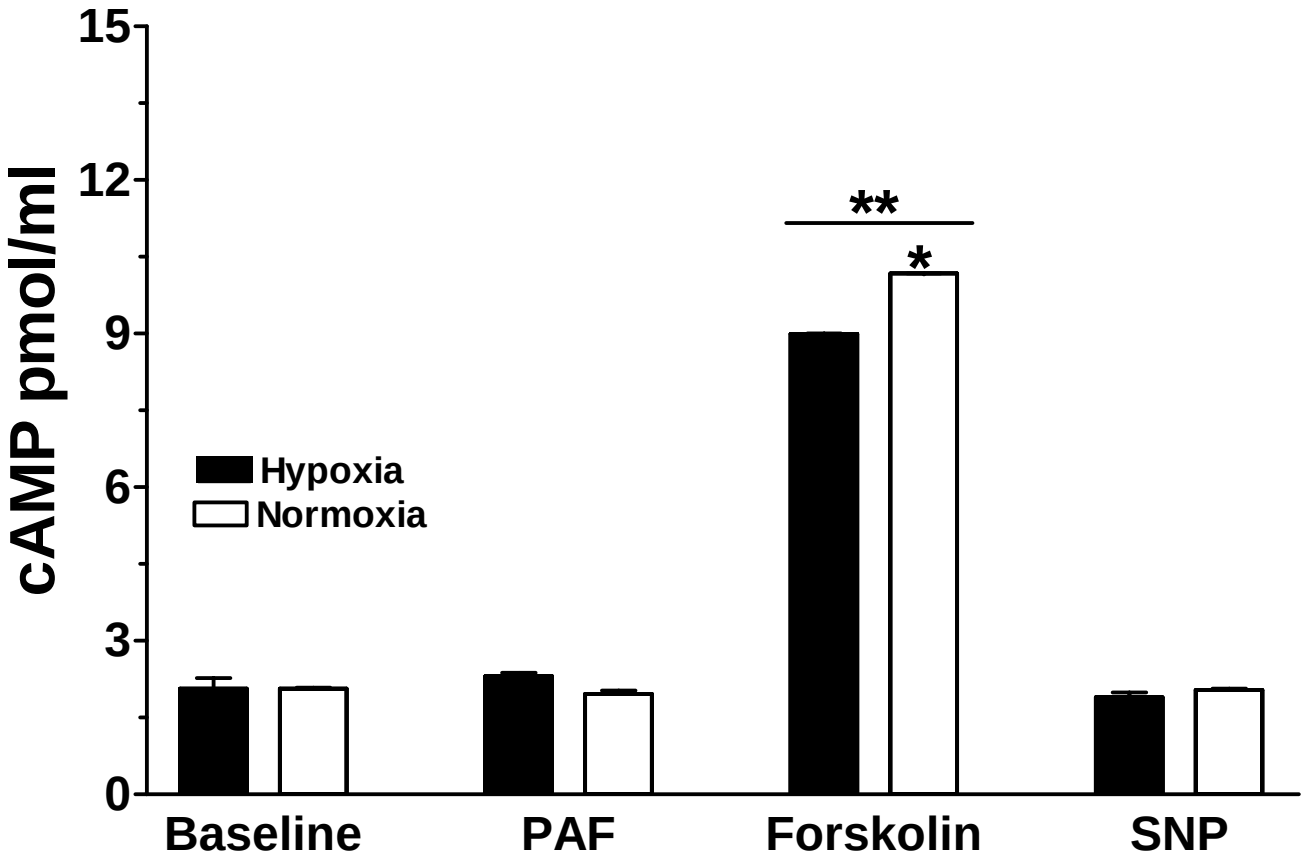


Figure 6a.

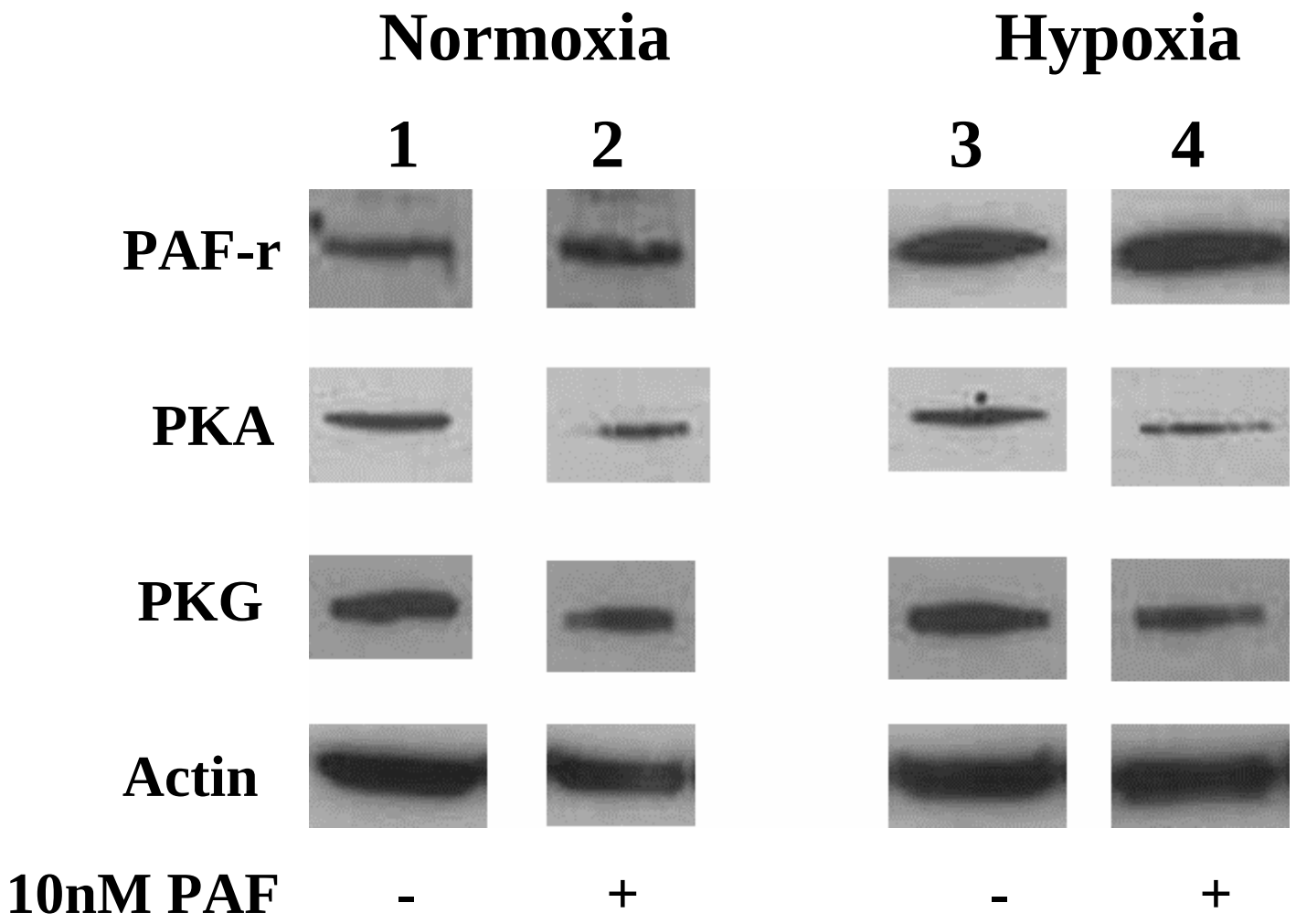


Figure 6b(i).

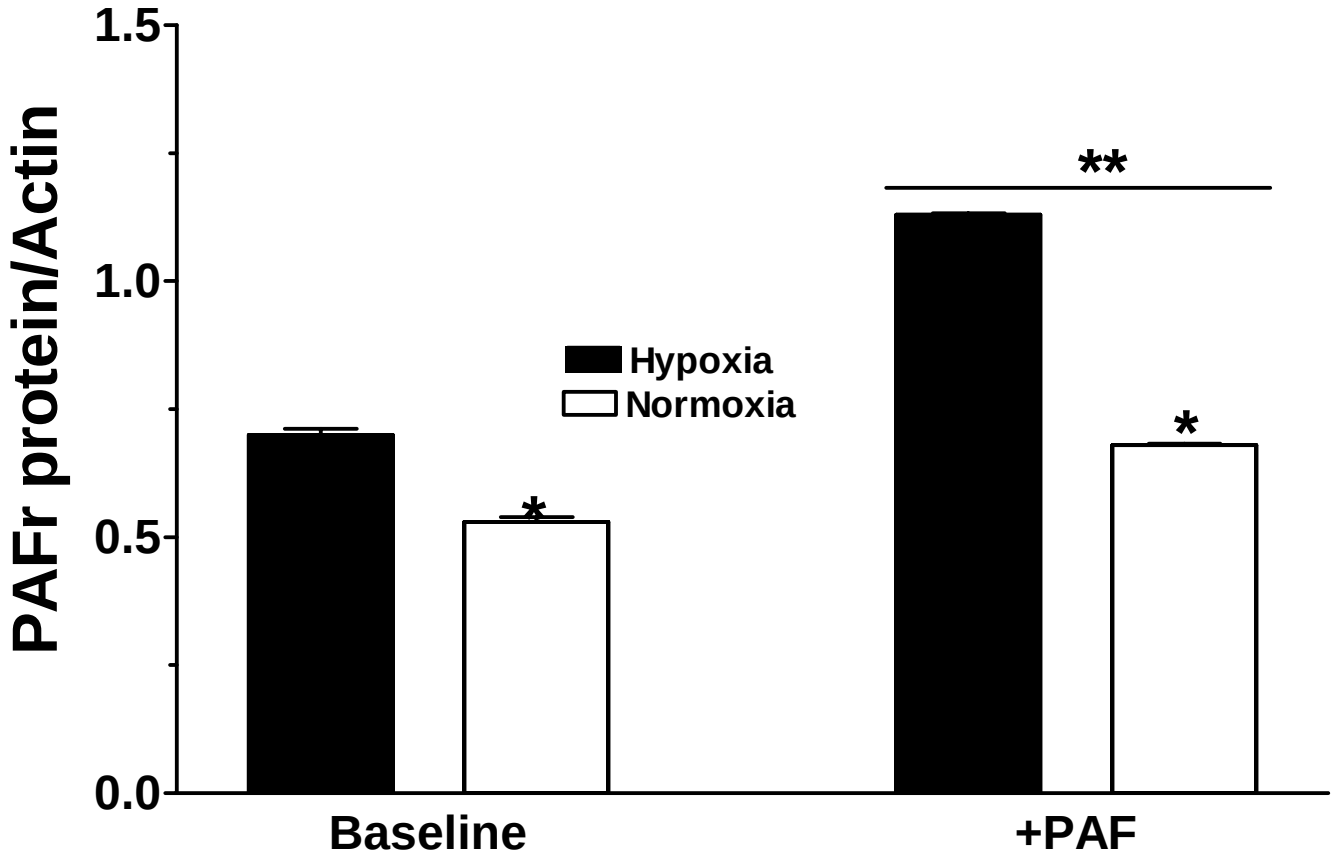


Figure 6b(ii).

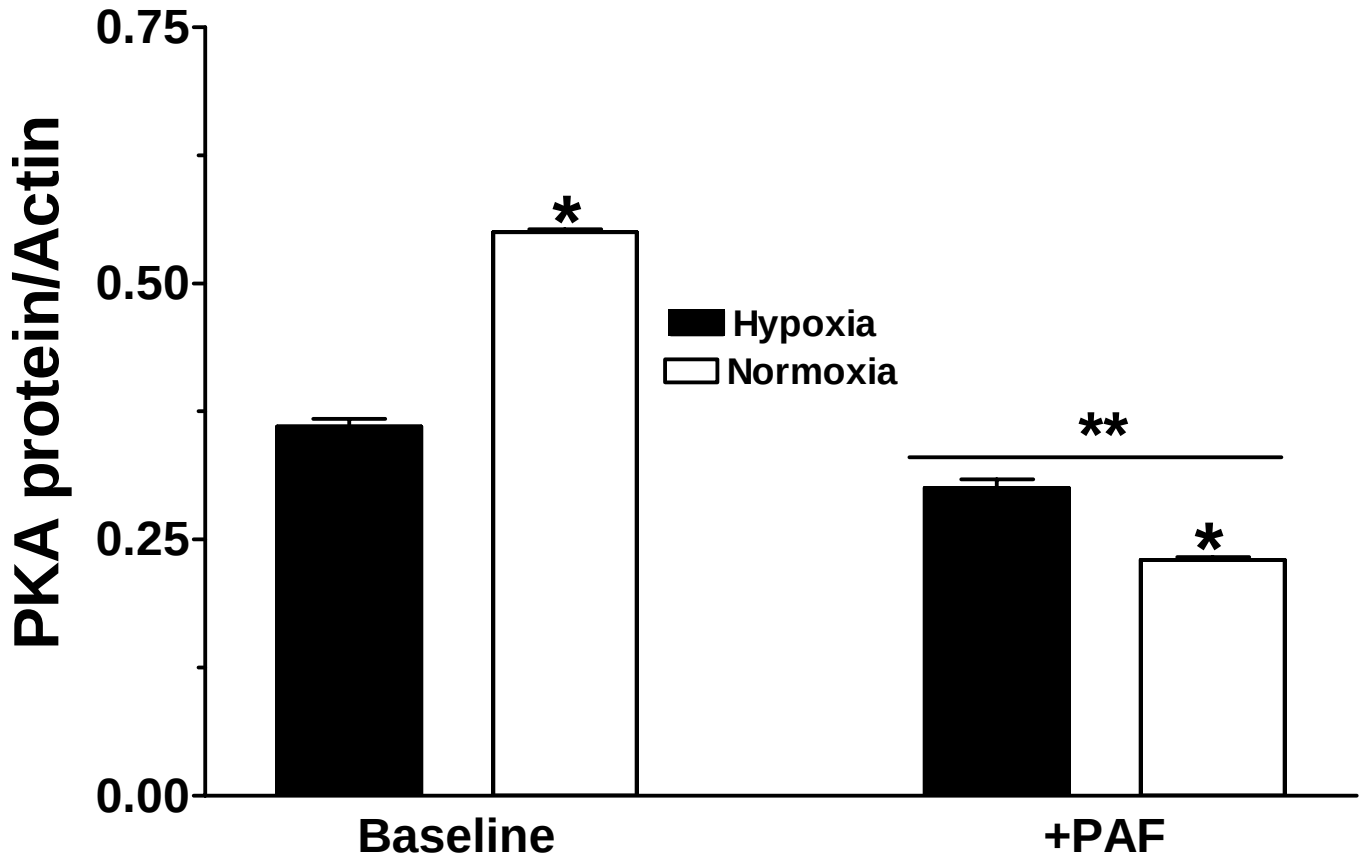


Figure 6b(iii).

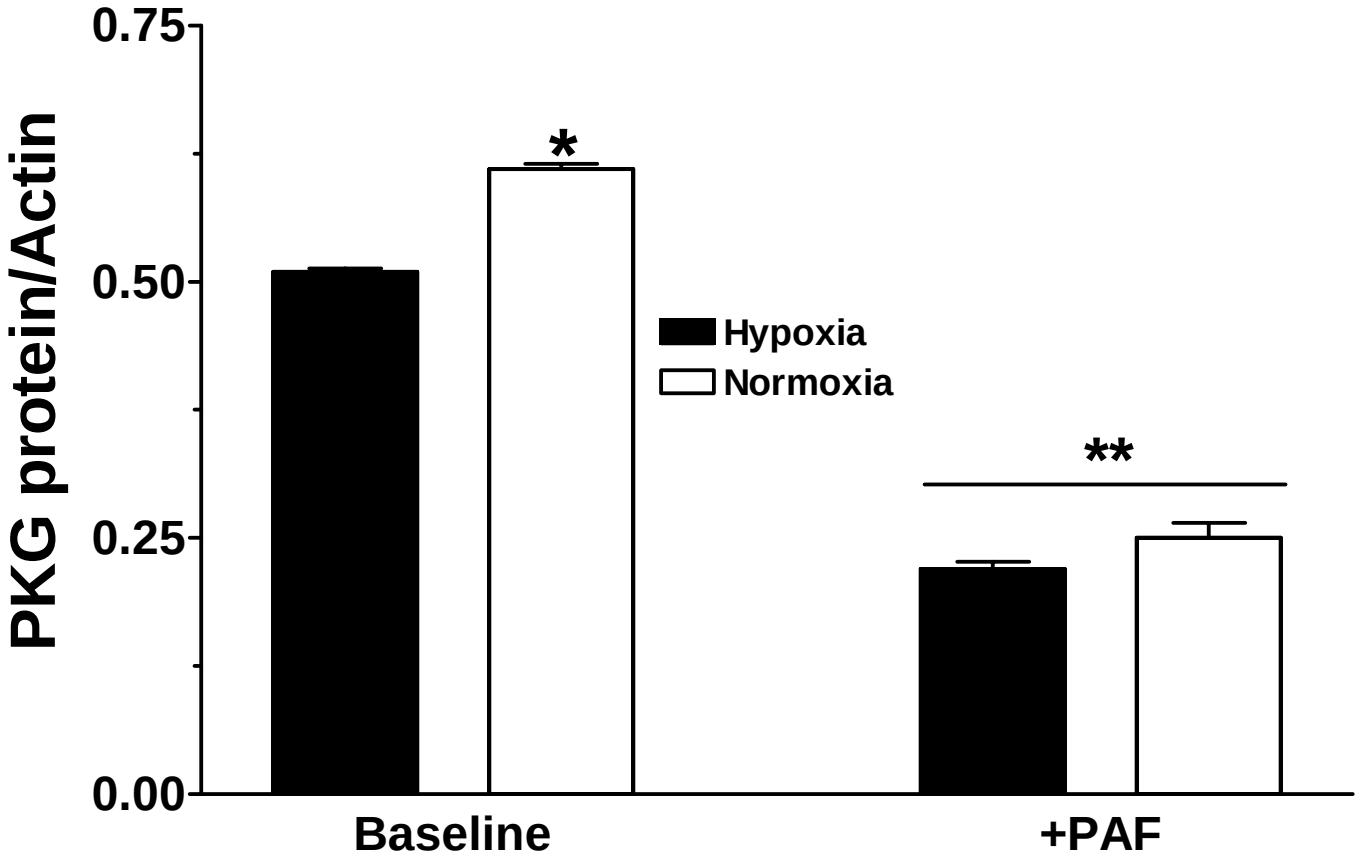


Figure7.

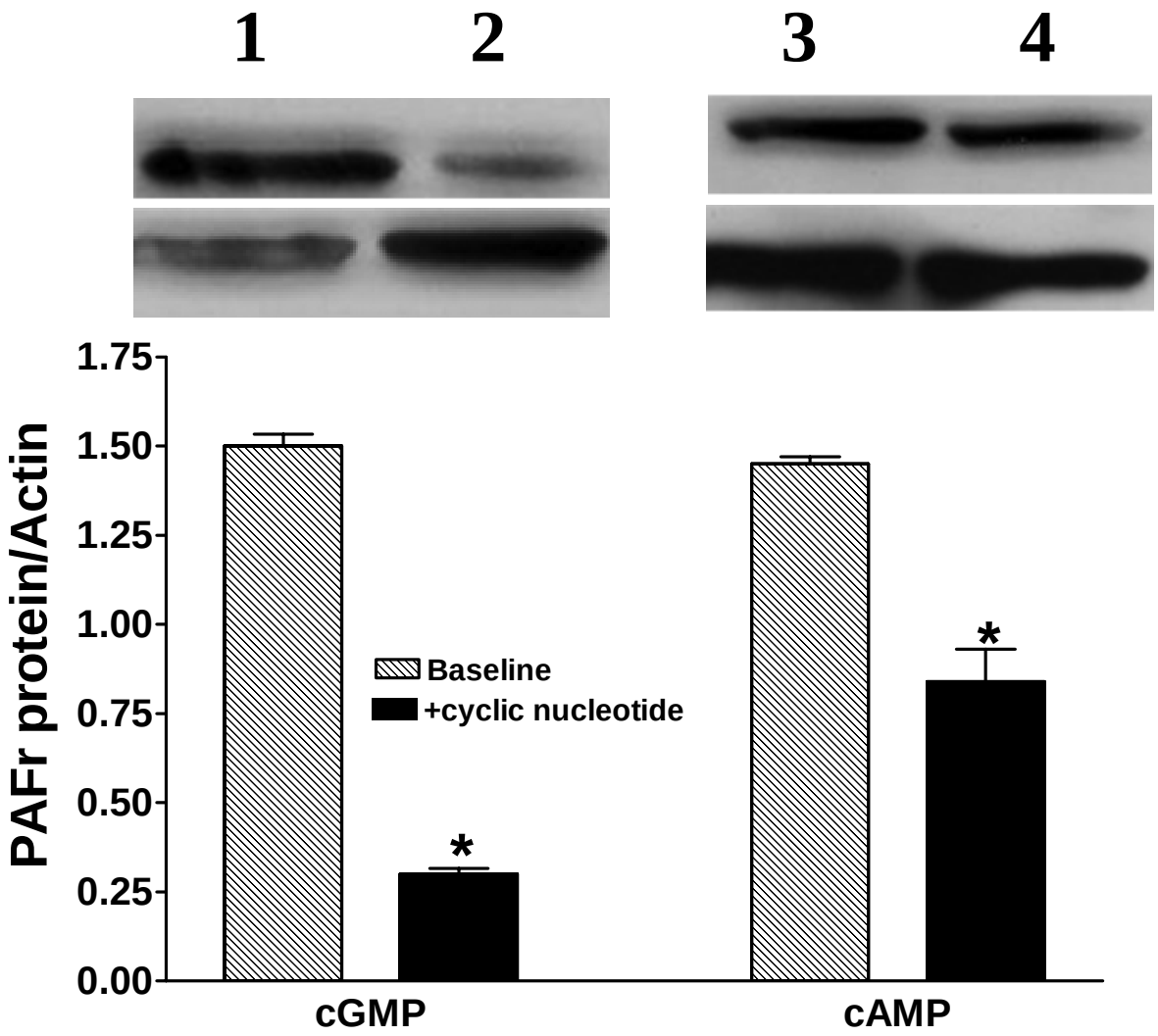


Figure 8a.

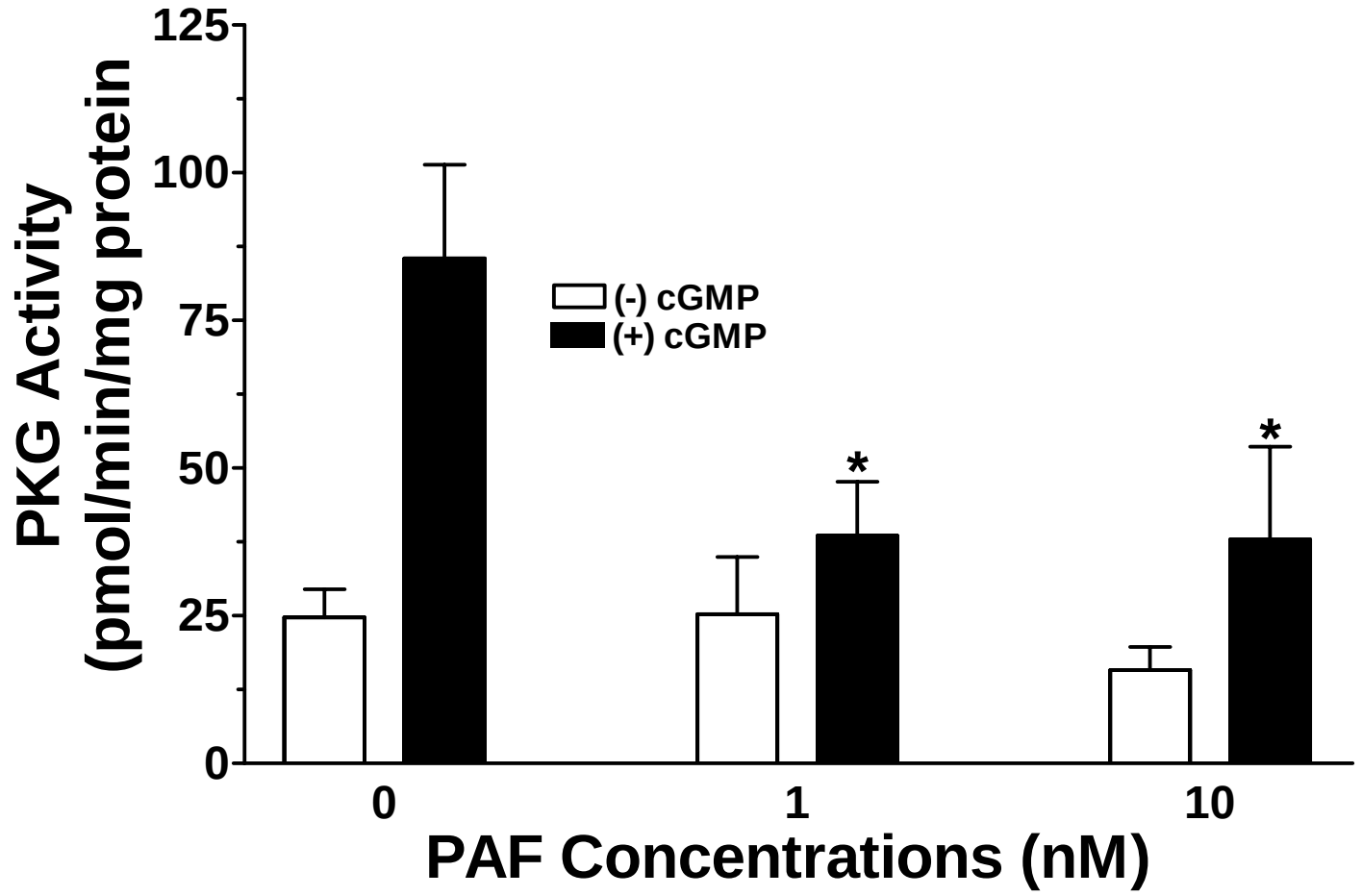


Figure 8b.