Involvement of cGMP-dependent protein kinase in the relaxation of ovine pulmonary arteries to cGMP and cAMP

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Dhanakoti, Srinivas N., Yuansheng Gao, Minh Q. Nguyen, and J. Usha Raj. Involvement of cGMP-dependent protein kinase in the relaxation of ovine pulmonary arteries to cGMP and cAMP. J. Appl. Physiol. 88: 1637–1642, 2000.—Agnost-induced smooth muscle relaxation occurs following an increase in intracellular concentrations of cGMP or cAMP. However, the role of protein kinase G (PKG) and/or protein kinase A (PKA) in cGMP- or cAMP-mediated pulmonary vasodilation is not clearly elucidated. In this study, we examined the relaxation responses of isolated pulmonary arteries of lambs (age = 10 ± 1 days), preconstricted with endothelin-1, to increasing concentrations of 8-bromo-cGMP (8-Br-cGMP) or 8-Br-cAMP (cell-permeable analogs), in the presence or absence of Rp-8-β-phenyl-1, N2-etheno-bromoguanosine cyclic monophosphodiesterate (Rp-8-PET-Br-cGMPS) or KT-5720, selective inhibitors of PKG and PKA, respectively. When examined for specificity, Rp-8-Br-PET-cGMPS abolished PKG, but not PKA, activity in pulmonary arterial extracts, whereas KT-5720 inhibited PKA activity only. 8-Br-cGMP-induced relaxation was inhibited by the PKG inhibitor only, whereas 8-Br-cAMP-induced relaxation was inhibited by both inhibitors. A nearly fourth higher concentration of cAMP than cGMP was required to relax arteries by 50% and to activate PKG by 50%. Our results demonstrate that relaxation of pulmonary arteries is more sensitive to cGMP than cAMP and that PKG plays an important role in both cGMP- and cAMP-mediated relaxation.

protein kinase A; protein kinase G; lung; smooth muscle; lambs

ELEVATION OF INTRACELLULAR concentrations of second messengers such as cGMP and cAMP is involved in mediating the vasorelaxant effect of a variety of vasoactive agonists, such as nitric oxide, prostaglandins, and β-adrenergic agents (2, 21). In the transition from fetal to neonatal life at birth, there is a dramatic fall in pulmonary vascular resistance that is brought about by a host of events, including the production of increased amounts of intracellular cGMP and cAMP, secondary to the increased synthesis of various agonists, such as endothelial-derived nitric oxide, prostacyclin, prostaglandin E2 (PGE2), and bradykinin (11). The relative importance of these two cyclic nucleotides in bringing about pulmonary vasodilation at birth is not known. The mechanisms by which cGMP plays an important role in smooth muscle relaxation (13, 26, 28) include 1) activation of cGMP-dependent protein kinase (PKG), 2) cGMP-gated channel proteins, and 3) cGMP-regulated phosphodiesterases (PDE). Recently (12), it was reported that PKG may not be involved in regulating basal tone in rat pulmonary vasculature.

We have shown (16–20) that there is heterogeneity in relaxation responses of pulmonary arteries and veins of near-term fetal and newborn lambs to cGMP- and cAMP-elevating agents. Based on these data, we hypothesized that cGMP is more potent than cAMP in mediating pulmonary vasodilation. We also wished to determine whether cGMP-induced relaxation is primarily mediated by PKG and if PKG is involved in cAMP-mediated relaxation. Therefore, in this study we investigated the role of PKG in both cGMP- and cAMP-mediated pulmonary vasodilation by 1) examining the relaxation responses of ovine pulmonary arteries to 8-bromo-cGMP (8-Br-cGMP) and 8-Br-cAMP, in the absence or presence of selective PKG inhibitor Rp-8-β-phenyl-1, N2-etheno-bromoguanosine cyclic monophosphodiesterate (Rp-8-PET-cGMPS, inhibition constant (Ki) = 0.03 µM), or the selective PKA inhibitor KT-5720 (Ki = 0.056 µM) and 2) determining the activation of PKG by cGMP and cAMP in pulmonary arterial extracts. Our results indicate that ovine pulmonary arteries are more sensitive to relaxation induced by cGMP than cAMP and that activation of PKG plays a major role in both cGMP- and cAMP-induced relaxation.

MATERIALS AND METHODS

Materials. Rp-8-Br-PET-cGMPS was obtained from Biology Life Sciences Institute. KT-5720 was provided by Dr. Douglas Palmer (Kamiya Biomedical). IBMX, indomethacin, and nitroarginine were obtained from Aldrich Chemical. Cyclic nucleotide analogs of cGMP and cAMP and endothelin-1 were purchased from Peptide International. BPEtide was purchased from Biomol, and Kemptide and PKA (5–24) inhibitor were from Peninsula Laboratories. cGMP, cAMP, BSA (fraction V), ATP, and all other chemicals used in this study were purchased from Sigma Chemical. Phosphocellulose papers (P-81) were purchased from Whatman and [γ-32P]ATP from Dupont-New England Nuclear.

Lungs from 14 lambs (Nebeker Ranch, Lancaster, CA) of either sex, age = 10 ± 1 days, were used. Lambs were anesthetized with ketamine-HCl (30 mg/kg im) and then killed with an overdose of pentobarbital sodium. The lungs were immediately removed, and fourth-generation pulmonary arteries (outside diameter = 2.0–2.5 mm) were dissected free of parenchyma and cut into rings (3-mm lengths).
Portions of pulmonary arteries were quickly frozen in liquid nitrogen and stored at −80°C.

Vessel tension experiments in organ chambers. Vessel rings were suspended in organ chambers filled with 10 ml of modified Krebs-Ringer bicarbonate solution [composition (in mM): 118.3 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.2 MgSO₄, 1.2 KH₂PO₄, 25.0 NaHCO₃, 11.1 glucose] maintained at 37 ± 0.5°C and aerated with 95% O₂-5% CO₂ (pH 7.4). Each ring was suspended by two stirrups passed through the lumen. One stirrup was anchored to the bottom of the organ chamber; the other one was connected to a strain gauge (model FT03C, Grass Instrument, Quincy, MA) for the measurement of isometric force (16–20).

At the beginning of the experiment, each vessel ring was stretched to its optimal resting tension. This was achieved by gradually stretching the vessel with weights in 0.2-g increments until the active contraction of the vessel ring to 100 mM KCl reached a plateau. The optimal resting tension for the pulmonary arteries was 0.28 ± 0.03 gm dry wt of tissue (n = 30). After the vessels were brought to their optimal resting tension, 1 h of equilibration was allowed. Indomethacin (0.01 mM; an inhibitor of cyclooxygenase) and nitro-L-arginine (0.1 mM; an inhibitor of nitric oxide synthase) were added to eliminate any involvement of endogenous prostanooids and endothelium-derived nitric oxide (16–20). Indomethacin and nitro-L-arginine did not affect the resting tension of the arteries.

Measurement of cGMP- and cAMP-induced relaxation. Thirty minutes after administration of indomethacin and nitro-L-arginine, vessels were constricted with 3 mM endothelin-1. After the contractions became stable, the effects of varying concentrations of 8-Br-cGMP and 8-Br-cAMP were determined.

Role of PKG and PKA in cGMP-induced relaxation. Thirty minutes after administration of indomethacin and nitro-L-arginine, Rp-8-Br-PET-cGMP (0.03 mM), or KT-5720 (0.05 mM), or solvent was added to the organ chamber. These inhibitors did not affect resting tension of the arteries. Control vessels and vessels treated with Rp-8-Br-PET-cGMPs and KT-5720 were constricted with endothelin-1 after a 30-min equilibration time. After contractions became stable, the effect of 8-Br-cGMP (0.1–300 µM) was determined. All experiments were carried out in parallel. For each vessel ring, only one inhibitor was tested.

Role of PKG and PKA in cAMP-induced relaxation. The experimental protocol was similar to that described above except that 8-Br-cAMP (0.1–300 µM) was used in place of 8-Br-cGMP.

Biochemical studies. Freshly frozen pulmonary arteries were employed for biochemical assays. Arteries were homogenized in a buffer containing 50 mM Tris-HCl (pH 7.4) at 22°C, 10 mM EDTA, 2 mM dithiothreitol, 1 mM IBMX (a general inhibitor of phosphodiesterases), 0.1 mM nitro-L-arginine, and 0.01 mM indomethacin. Homogenization was carried out on ice, using a Potter-Elvejhem homogenizer, followed by sonication on ice and centrifugation at 13,000 g for 10 min at 4°C. Supernatant from the arterial extract was immediately used for determination of PKG and PKA activity.

PKG activity in extracts of pulmonary arteries. PKG assay is based on the incorporation of 32P from [γ-32P]ATP into a specific PKG peptide substrate, BPDEtide. The peptide sequence is derived from the phosphorylation sequence of cGMP-binding cGMP-specific phosphodiesterase (PDE5) by PKG (6). Assays were carried out as described by Corbin's group (22), with slight modifications. Briefly, reactions were started by the addition of aliquots (10 µl) of arterial extract to an assay mixture in a total volume of 40 µl, containing 50 mM Tris-HCl (pH 7.4) at 22°C, 20 mM MgCl₂, 0.1 mM IBMX, 150 µM BPDEtide, 1 µM PKA-(5–24) inhibitor, a synthetic peptide inhibitor of PKA, (Kᵢ = 0.9 nM) and 0.2 mM [γ-32P]ATP (400 counts·min⁻¹·pmol⁻¹). The reaction mixture was incubated at 30°C for 10 min in the absence or presence of 5 µM cGMP, and the reaction was then terminated by spotting 40 µl onto P-81 phosphocellulose (2 cm × 2 cm) strips and immediately placing them in 75 mM ice-cold phosphoric acid. The strips were then washed and processed as described by Roskowski (31). The radioactivity in the strips was counted by using a Beckman scintillation counter. Counts obtained in the absence or presence of cGMP were corrected for nonspecific binding of [32P]ATP and peptide to the P-81 strips and for counts obtained in the absence of peptide substrate. Protein content in extracts of pulmonary arteries was determined by Bradford's procedure (3) using BSA (fraction V) as the standard.

PKG activity in extracts of pulmonary arteries. PKA assay (22) was carried out in similar fashion to that of PKG, except that BPDEtide was replaced with a specific PKA peptide substrate, kemptide (130 µM), and PKA inhibitor was omitted. Assay was carried out in the absence or presence of 2 µM cAMP. All assays were performed in triplicate.

Our preliminary data showed that PKG activity (pmol·min⁻¹·mg protein⁻¹) 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, respectively) in extracts of freshly isolated or frozen and stored (−80°C) pulmonary arteries. Also, in preliminary experiments, we found that PKG activity was linear up to 15 µg of extract protein, and up to 15 min of incubation, and the activity was saturated with 5 µM exogenous cGMP, whereas, for PKA, the exception was that activity was saturated with 2 µM cAMP. Inclusion of a variety of protease (leupeptin, pepstatin, antipain, aprotinin, phenylmethylsulfonyl fluoride) and phosphatase (calyculin A, cyperrhetin, sodium vanadate) inhibitors in the homogenization buffer 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.

Effect of inhibitors on PKG and PKA activity. Initially, the effects of increasing concentrations of Rp-8-Br-PET-cGMPs (≥30 µM) and KT-5720 (≥100 µM) on the activities of PKG and PKA in arterial extracts in the presence or absence of 5 µM cGMP and 2 µM cAMP, respectively, were determined. Subsequently, their effect was determined at 30 and 50 µM for Rp-8-Br-PET-cGMPs and KT-5720, respectively.

Activation of PKG by cGMP and cAMP. Pulmonary arterial extracts were incubated at 0°C for 30 min with increasing concentrations of cGMP or cAMP (0–5 µM), followed by determination of PKG activity as described in PKG activity in extracts of pulmonary arteries.

Data analysis. Pulmonary arterial relaxation is expressed as change in tension (%). Activity of PKG and PKA is expressed as picomoles·min⁻¹·mg protein⁻¹ incorporated into the peptide per minute per milligram protein. Data are expressed as means ± SE. When mean values of two groups were compared, Student’s t-test for unpaired observations was employed. When the mean values of the same group before and after stimulation were compared, Student’s t-test for paired observations was used. Comparison of mean values of more than two groups was analyzed with one-way ANOVA, with Student-Newman-Keuls test for post hoc testing of multiple comparison. All these analyses were performed using a commercially available statistics package (SigmaStat, Jandel Scientific, San Rafael, CA). Values were considered significant at P <
Comparison of cGMP and cAMP-induced relaxation of pulmonary arteries. A representative trace, as shown in Fig. 1, depicts the change in tension (g) of pulmonary arteries, previously raised by 3 nM of endothelin-1, in response to increasing concentrations (10 nM to 300 µM) of 8-Br-cGMP or 8-Br-cAMP, observed over a 4-h period. The control tensions raised by endothelin-1 were 1.12 ± 0.13 and 0.98 ± 0.10 g/mg tissue for 8-Br-cGMP- and 8-Br-cAMP-treated vessels (n = 5 animals/group), respectively. Maximal relaxation responses were 92.1 ± 4.9 and 59.1 ± 6.8% for vessels treated with 300 µM 8-Br-cGMP or 8-Br-cAMP, respectively. The calculated EC50 values were 35.5 ± 5.2 and 120.2 ± 15.8 µM for 8-Br-cGMP and 8-Br-cAMP, respectively. The data suggest that newborn pulmonary arteries are more sensitive to relaxation by cGMP than by cAMP.

Involvement of PKG or PKA in cGMP-induced relaxation of arteries. Experiments were carried out in the presence of cell-permeable inhibitors of PKG and PKA. The inhibitors, Rp-8-Br-PET-cGMPS (a potent inhibitor of PKG, Ki = 0.035 µM) and KT-5720 (a potent inhibitor of PKA, Ki = 0.056 µM), did not significantly affect resting tension in the arteries. Pretreatment with Rp-8-Br-PET-cGMPS significantly inhibited 8-Br-cGMP-induced relaxation of arteries; only ~40% relaxation was observed with 300 µM 8-Br-cGMP in the presence of 8-Br-PET-cGMPS (Fig. 2). KT-5720 did not have any effect.

Involvement of PKA or PKG in cAMP-induced relaxation of arteries. As shown in Fig. 3, the maximal relaxation response of arteries was ~59% with 300 µM 8-Br-cAMP, and this relaxation was inhibited by both KT-5720 (50 µM) and Rp-8-Br-PET-cGMPS (30 µM). There was no significant difference (P > 0.05) between the two inhibitors in their inhibitory effect on relaxation induced by 8-Br-cAMP.

Effect of inhibitors on PKG and PKA activity in extracts of pulmonary arteries. The effect of the two inhibitors employed in our vessel tension studies on PKG and PKA activity in extracts of arteries was determined. Dose-dependent inhibition of exogenous, cGMP-stimulated PKG activity with Rp-8-Br-PET-cGMPS (≥30 µM) and that of cAMP-stimulated PKA activity with KT-5720 (≤100 µM) was observed (Fig. 4). There was no difference in PKG activity observed with 50–100 µM KT-5720, and, therefore, the effect of KT-5720 at 50 µM was examined in subsequent experiments. As shown in Fig. 5A exogenous cGMP (5 µM) stimulated PKG activity by more than 3.6-fold (38 ± 4.5 vs. 137 ± 18 pmol·min⁻¹·mg protein⁻¹), and this stimulated activity was abolished in the presence of the PKG inhibitor but was unaffected in the presence of the PKA inhibitor. Both of the inhibitors did not affect PKG activity determined in the absence of cGMP. Exogenous cAMP (2 µM) stimulated PKA activity by threefold (420 ± 21 vs. 1,263 ± 75 pmol·min⁻¹·mg protein⁻¹). This stimulation was abolished by the PKA inhibitor but was unaffected by the PKG inhibitor (Fig. 5B). PKA activity without added cAMP was not different in the presence of the two inhibitors. PKA activity in pulmonary arterial extracts is nearly 10-fold higher than that of PKG activity (Fig. 5).

Activation of PKG by cGMP and cAMP. Assay of PKG activity following preincubation of arterial extracts with increasing concentrations of cGMP or cAMP revealed that cAMP can cross-activate PKG, albeit, at a 10-fold higher concentration than cGMP (Fig. 6).

DISCUSSION

Vasoactive agonists cause smooth muscle relaxation following increases in intracellular concentrations of cGMP or cAMP. In this study, we examined whether
cGMP or cAMP was more potent in causing relaxation of pulmonary arteries of newborn lambs. Because the involvement of PKG in pulmonary vasodilation was questioned in a recent publication (12), and its involvement in cAMP-mediated relaxation in this vascular bed is not known, we also examined the role of PKG in pulmonary arterial relaxation. Our data show that pulmonary arterial smooth muscle relaxation is more sensitive to cGMP than to cAMP and that PKG is involved in relaxation mediated by cGMP or cAMP. By employing phosphodiesterases-resistant, cell-permeable analogs of cGMP and cAMP, we found that relaxation of isolated pulmonary arteries is more sensitive to cGMP than to cAMP. The EC50 of 8-BrcGMP (35.5 ± 5.2 µM) in causing relaxation of pulmonary arteries of newborn lambs is in agreement with our previous results (16). Previously, it has been shown that cGMP analogs are more effective than cAMP analogs in relaxing both pig coronary arteries and guinea pig tracheal smooth muscle (14).

Because cross-activation of PKG by cAMP (22) and PKA by cGMP (8) can occur in cells of systemic vessels and basal intracellular cAMP content is higher than cGMP (17, 16), we employed selective PKG and PKA inhibitors to ascertain the contributions of PKG or PKA in cGMP- or cAMP-induced relaxation of pulmonary arteries. Although the selectivity of these inhibitors has been examined with purified enzymes (4, 15, 23), we chose the concentrations of the inhibitors only after determining their effects in abolishing PKG or PKA activity in arterial extracts. PKG and PKA activity was abolished only by their respective inhibitors. Similar to activity inhibition, a significant inhibition of cGMP-induced relaxation of pulmonary arteries was observed only with the PKG inhibitor, suggesting that PKG is involved in relaxant effects of cGMP. In contrast, cAMP-induced relaxation was inhibited to the same degree by both PKG and PKA inhibitors, suggesting that PKG is also involved in mediating cAMP-induced relaxation. We did not use both inhibitors in our studies because we were primarily interested in determining the involvement of PKG or PKA in cyclic nucleotide-mediated pulmonary arterial relaxation.

Although we determined PKG or PKA activity in pulmonary arteries (outside diameter, 0.4–2.5 mm), we do not know whether the activities will be different in arteries of varying sizes. Previously, we showed (16) that endothelium-dependent relaxation of small pulmonary arteries (0.4–0.7 mm) is greater than that of large arteries (1.5–2.5 mm) in response to nitric oxide, ACh, and bradykinin. This differential response between large and small arteries may be due to the difference in the activity of soluble guanylyl cyclase, as higher cGMP content was observed in the small arteries in response to the employed agonists (16). Thus it is possible that, compared with large arteries, PKG may play a more...
important role in cGMP-mediated relaxation of small arteries.

Although there are no reports on the activities of PKG and PKA in isolated pulmonary vessels, there are suggestions that most of the PKG activity in the whole lung is attributable to its activity in smooth muscle cells (13a). As suggested by Corbin et al. (13, 14), great caution must be exercised in interpreting results obtained with PKG and PKA inhibitors, and, moreover, use of inhibitors that inhibit phosphodiesterase, nitric oxide synthase, and cyclooxygenase, as employed in this study, definitely needs careful comparison. Because our findings are with pulmonary arteries, we have interpreted our data by comparing the results of physiological vessel tension experiments and biochemical assays in the presence and absence of increasing concentrations of the inhibitors.

Our results support the hypothesis that PKG is primarily involved in cGMP-induced smooth muscle relaxation and is in agreement with the findings of other investigators (1, 7, 27). It has been shown that pulmonary blood flow in fetal lambs is greatly reduced when infused with another PKG inhibitor, KT-5823 (7). In rat pulmonary arterial smooth cells (1), 8-Br-cGMP induced an increase in whole cell K⁺ currents, which is used as an index of relaxation, and this increase was completely blocked by H-8, an agent that inhibits both PKG and PKA. Activation of PKG by intracellular cGMP is thought to be involved in the lowering of intracellular Ca²⁺ concentrations ([Ca²⁺]) leading to vascular smooth muscle relaxation (5, 21, 26, 32). Felbel et al. (10) were the first to demonstrate that when PKG was introduced to isolated tracheal smooth muscle cells, [Ca²⁺] levels decreased. Later, Lincoln’s group (9, 27, 30) showed that introduction of PKG, but not the catalytic subunit of PKA, into passaged rat aortic smooth muscle cells that express very little PKG produced significant decreases in [Ca²⁺] levels. Interestingly, they also demonstrated that PKG brought about decreases in [Ca²⁺], following increases in cAMP, suggesting that PKG can be cross-activated by cAMP and mediates the actions of cAMP in smooth muscle relaxation. Recently, in a PKG-deficient mouse model, PKG was shown to be essential in nitric oxide-cGMP-mediated relaxation of aortic smooth muscle (29).

Our studies on the role of PKG in cGMP-induced pulmonary vasodilation differ in a number of ways from those of a recent report (12). It was reported that hypoxic pulmonary vasoconstriction (HPV) was not augmented when two PKG inhibitors, Rp-8-pCPT-cGMPS and H-8, were separately infused in isolated lungs of normotensive and hypertensive rats, but HPV was augmented when lungs were infused with ODQ, a soluble guanylate cyclase inhibitor. Rp-8-pCPT-cGMPS is less membrane permeable and less potent (by 17-fold) than Rp-8-Br-PET-cGMPS (4, 15) in inhibiting PKG, whereas H-8 inhibits both PKG and PKA. In our studies, we used Rp-8-Br-PET-cGMPS (4, 15), which is more cell permeable and more potent in inhibiting PKG than Rp-8-pCPT-cGMPS (4, 15), whereas KT-5720 (Ki = 0.33 µM) (23) specifically inhibits PKA activity. Moreover, in this report, a change in back phosphorylation of inositol triphosphate receptors, a very indirect measure of PKG activity, was used.

![Fig. 5. Effects of inhibitors on PKG (A) and PKA (B) activity in extracts of pulmonary arteries of newborn lambs. Activity was determined as described in MATERIALS AND METHODS. Values are means ± SE, n = 5. *Significant difference (P < 0.05) from control.](http://jap.physiology.org/)
as an indicator of such activity (24). With this method, only a 25% inhibition of PKG activity was observed with Rp-8-pCPT-cGMPS. Also, we utilized isolated vessels that were incubated with the inhibitor, a procedure that may have allowed the inhibitor to more effectively permeate the smooth muscle cells than in the situation of isolated, perfused lungs infused with the inhibitor.

In conclusion, our data demonstrate that relaxation of pulmonary arteries of newborn lambs is more sensitive to cGMP than to cAMP and that PKG contributes significantly to cGMP- or cAMP-mediated pulmonary smooth muscle relaxation.

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