The Regulation of the cGMP-binding cGMP Phosphodiesterase by Proteins That Are Immunologically Related to γ Subunit of the Photoreceptor cGMP Phosphodiesterase*

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The cGMP phosphodiesterase from retinal rods (PDE-6) is an αβγ heterotetramer. The α and β subunits contain catalytic sites for cGMP hydrolysis, whereas the γ subunits serve as a protein inhibitor of the enzyme. Visual excitation of photoreceptors enables the activated GTP-bound form of the G-protein transducin to remove the inhibitory action of the γ subunit, thereby triggering PDE-6 activation. The type 5 phosphodiesterase (PDE-5) isoform shares a number of similar characteristics with PDE-6, including binding of cGMP to noncatalytic subunits, the cyclic nucleotide specificity, and inhibitor sensitivities. Although the functional role of PDE-5 remains unclear, it has been shown to be activated by protein kinase A (PKA) (Burns, F., Rodger, I. W. & Pyne, N. J. (1992) Biochem. J. 283, 487–491). Here we report that both the recombinant γ subunit and a peptide corresponding to amino acids 24–46 in this protein inhibited the activation of PDE-5 by PKA. Furthermore, immunoblotting airway smooth muscle membranes with a specific antibody against amino acids 24–46 of the PDE-6 γ subunit identified two major immunoreactive small molecular mass proteins of 14 and 18 kDa (p14 and p18). These appear to form a complex with PDE-5, because PDE activity was immunoprecipitated using antibody against the PDE-6 γ subunit. p14 and p18 were also substrates for phosphorylation by a unidentified kinase that was stimulated by a pertussis toxin-sensitive G-protein. Phosphorylation of p14/p18 in membranes treated with guanine nucleotides correlated with a concurrent reduction in the activation of PDE-5 by PKA. We suggest that p14 and p18 share an epitope common to PDE-6 γ and that this region may interact with PDE-5 to prevent its activation by PKA.

PDEs* are expressed as a family of distinct isoforms (type 1–7) with each subgroup of isoforms containing multiple spliced variants (1). The different members of the PDE family are also differentially regulated by kinases (2–5), calcium/calmodulin (6), cGMP (7, 8), and G-proteins (9). The integration of these diverse cell signals enables the precise co-ordinated regulation of intracellular cyclic nucleotide levels in response to receptor stimulation.

PDE-5 from smooth muscle and PDE-6 from photoreceptors share a number of common properties. They have two tightly bound subunits containing both catalytic and noncatalytic cGMP binding sites, hydrolyze cGMP better than cAMP, and are both inhibited by zaprinast. The noncatalytic cGMP-binding site in PDE-5 is formed from an N-terminal tandem repeat that is indicative of gene duplication, whereas the catalytic site is located in the C-terminal region of the protein and is conserved in a number of PDE isoforms (1, 10).

PDE-6 is expressed in photoreceptor rod cells, where it serves as an effector in the visual transduction signal cascade (11–13). This involves the photoexcitation of rhodopsin, the GDP-GTP cyclical activation of the G-protein, transducin, and the subsequent stimulation of PDE-6 activity by the GTP-bound transducin. PDE-6 is a heterotetrameric protein composed of catalytic α and β subunits and two inhibitory γ subunits. The GTP-bound transducin binds to the γ subunits and displaces them, thereby activating PDE-6. PDE-6γ has two additional functions: first, it increases the affinity for cGMP in the PDE-6 noncatalytic site (14, 15), and second, it participates in the activation of transducin GTPase, thereby terminating the activation of PDE activity by the GTP-bound α subunit of transducin (16). Desensitization of PDE-6 activity might also occur via a recently proposed kinase-directed phosphorylation mechanism (17, 18). When PDE-6γ is removed from PDE-6β in a complex with transducin, it is apparently phosphorylated by an unidentified kinase. The phosphorylated γ subunits appear to render PDE-6 refractory to stimulation by transducin.

PDE-5 is the major cGMP-binding protein in lung (19). It is a homodimer comprised of two identical catalytic subunits, each with the molecular mass of 93 kDa. PDE-5 contains a site (Ser92) that is phosphorylated by protein kinase A (PKA) and protein kinase G (5). The kinase-catalyzed phosphorylation of the PDE-5 is kinetically enhanced by the binding of cGMP to the noncatalytic cGMP-binding site. However, no change in enzyme activity has been observed as a conseuence of the phosphorylation of purified bovine PDE-5 (5). To the contrary, we have demonstrated that the catalytic subunit of PKA can catalyze up to a 10-fold increase in the Vmax of a partially purified preparation of guinea pig lung PDE-5 (20, 21). Activation of PDE-5 by phosphorylation may therefore enhance the rate of cGMP signal termination in intact cells, and this proposal is supported by the observation that cGMP elevating agents elicit rapid transient phosphorylation and activation of PDE-5 in vascular smooth muscle cells (22).

Multiple similarities in the structure and function of PDE-5 and PDE-6 point to the possibility that PDE-5 might contain its
own y-subunits that couple this enzyme to unidentified G-protein-dependent pathway. Here we have characterized the interaction of PDE-5 with recombinant PDE-6y and have identified two small molecular mass proteins in airway smooth muscle cell membranes that appear to share an epitope common to PDE-6y. This epitope region interacts with PDE-5 to prevent its activation by PKA.

EXPERIMENTAL PROCEDURES

Materials

All biochemicals were from Boehringer Mannheim, whereas general chemicals were from Sigma. \(\gamma^3\PPI PATP, \[^{3H}\text{GMP},\) and excitation cation exchange and reverse-phase chromatography (24) from Escherichia coli strain BL21 DE3 transformed with an expression plasmid containing PDEy synthetic gene. The coding sequence for PDEy from the fusion protein (24) was subcloned into an expression vector pET-11a (Novagen) by Dr. J. Sondek (Yale University). The purity of PDEy was estimated to be >95\%, and the PDEy concentration was determined spectrophotometrically at 280 nm using a molar extinction coefficient of 7100.

PDE Assay

The assay of PDE activity was by the two-step radiotracer method (25). Assays were performed at 0.5 \(\mu\text{M}[^{3H}]\text{GMP.}\) All PDE activity measurements were done under conditions of linear rate product formation and where less than 10\% of the substrate was utilized during the assay.

Protein Phosphorylation

Partially purified guinea pig PDE-5 and membranes were separately incubated with an "activation mixture" containing final concentrations of 25 \(\mu\text{M} \text{ATP, 25 mM Heps, pH 7.4, 5 mM MgCl}_2,\) and the catalytic subunit of PKA (10 units). Incubations were performed at 37 °C for 20 min. Samples were then withdrawn for PDE assay. In experiments where the effect of either PDE-6y or a peptide corresponding to amino acids 24–46 of PDE-6y was investigated, we combined each with the PDE prior to the addition of the activation mixture and kinase.

In studies investigating the phosphorylation of PDE-6y peptide (amino acids 24–46), we combined the peptide with the activation mixture and the catalytic subunit of PKA (10 units). In studies using purified protein kinase C (PKC), we added 0.75 \(\text{mM CaCl}_2, 50 \mu\text{g/ml phosphatidyliner,}\) and 0.05 units of PKC to the activation mixture. Each reaction was initiated by adding 1 \(\mu\text{g}\) of \(\gamma^3\PPI PATP.\) Incubations were performed at 37 °C for 60 min and terminated by the addition of 75 \(\mu\text{M orthophosphoric acid.}\) Samples were spotted onto P81 discs and washed in \(3 \times 250 \mu\text{l}\) acetic acid (1\%, v/v) washes. The amount of \(^{32}\PPI P\)-labeled peptide was quantified by liquid scintillation counting.

In experiments where ASM membranes were utilized to assess p14 and p18 phosphorylation, the catalytic subunit of PKA was omitted, and the activation mixture was supplemented with \(\gamma^3\PPI ATP (10 \mu\text{Ci/assay).}\) Incubations were performed at 37 °C for 20 min. At the termination of the incubation, membranes were then harvested by centrifugation at 14,000 \(\times\) g for 10 min at 4 °C and taken for immunoprecipitation with anti-PDE-6y antibody.

In some experiments, ASM membranes were incubated with varying concentrations of \(\text{GppNHp (1–100 \mu\text{M}) and ATP (0.005–50 \mu\text{M}) in 10 mM}\) Tris/HCl, pH 7.4, and 5 \(\mu\text{M MgCl}_2.\) This was performed at 37 °C for 10 min. GppNHp was prepared in 60 \(\mu\text{M NaCl, 5 mM Hepes, pH 7.4, and 5 mM MgCl}_2.\) At the termination of the incubation, membranes were then harvested by centrifugation at 14,000 \(\times\) g for 10 min at 4 °C and taken either for immunoblotting with anti-PDE-6y or for activation with PKA.

Immunoprecipitation

ASM membranes were solubilized in 10 mM \(\text{K}_2\text{HPO}_4/\text{K}_2\text{HPO}_4\) and 0.15 \(\%\) NaCl, pH 6.8 (PBS) containing 1\% (v/v) deoxycholate and 0.1% (v/v) SDS for 60 min at 4 °C. The material was harvested and centrifuged at 14,000 \(\times\) g for 10 min at 4 °C, and 250 \(\mu\text{l}\) of supernatant was taken for the partial purification of the PDE-5. This was achieved using three chromatographic columns; namely (i) DEAE-Sepharose 6B (PDE was eluted with 0.25 M NaCl in phosphate buffer, pH 6.8); (ii) Aff-gel blue agarose (PDE was eluted with 0.35 M potassium thiocyanate in phosphate buffer, pH 6.8); and (iii) zinc chelate adsorbent matrix (PDE activity passes straight through the column). This yields a preparation of enzyme that is approximately 10–20\% pure, based upon maximal isobutylmethylxanthine-stimulated \[^{32}\text{H}]\text{GMP binding to the noncatalytic site of PDE-5.}\) We have previously demonstrated that this enzyme preparation contained a single class of GMP hydrolytic sites, was potently inhibited by zaprinast (IC\textsubscript{50} = 0.68 \(\mu\text{M}\) using 0.5 \(\mu\text{M} \text{GMP),}\) and could be maximally activated up to 10-fold by the catalytic subunit of PKA (20).

Recombinant wild type PDE-6y was purified by a combination of cation exchange and reverse-phase chromatography (24) from E. coli strain BL21 DE3 transformed with an expression plasmid containing PDEy synthetic gene. The coding sequence for PDEy from the fusion protein (24) was subcloned into an expression vector pET-11a (Novagen) by Dr. J. Sondek (Yale University). The purity of PDEy was estimated to be >95\%, and the PDEy concentration was determined spectrophotometrically at 280 nm using a molar extinction coefficient of 7100.

Cell Culture

Guinea Pig ASM Cells—Guinea pig ASM cells was achieved as described previously (23). The cells were maintained in Dulbecco’s modified Eagle’s medium containing 10\% (v/v) fetal calf serum and 10\% (v/v) donor horse serum and was passed twice using trypsin prior to experimentation. Cells were grown to confluence and routinely used at 15–21 days after the initial preparation. Their identity was confirmed to be smooth muscle by the presence of \(\alpha\)-actin, using a smooth muscle-specific mouse anti-\(\alpha\)-actin monoclonal antibody (23).

Membrane Preparation

Guinea Pig Lung Membranes—Lung tissue was homogenized in buffer A, using a Turrex homogenizer, and the subsequently obtained homogenate was filtered through cheese cloth and centrifuged at 10,000 \(\times\) g in a Beckman prep 65 centrifuge for 10 min. The supernatant was removed and reconstituted at 48,000 \(\times\) g for 20 min at 4 °C. The subsequently obtained membrane pellet was resuspended in buffer A.

Protein Purification

Partial purification of guinea pig lung PDE-5 from a "high speed" supernatant fraction was achieved as described previously by us (20). Briefly this involved homogenizing guinea pig lung in phosphate buffer containing 20 mM K\textsubscript{2}HPO/\textsubscript{4}K\textsubscript{2}HPO\textsubscript{4}, pH 6.8, at 4 °C. The homogenate was passed through cheese cloth and centrifuged identically to that described for the preparation of membranes. The high speed (48,000 \(\times\) g) supernatant was taken for the partial purification of the PDE-5. This was achieved using three chromatographic columns; namely (i) DEAE-Sepharose 6B (PDE was eluted with 0.25 M NaCl in phosphate buffer, pH 6.8); (ii) Aff-gel blue agarose (PDE was eluted with 0.35 M potassium thiocyanate in phosphate buffer, pH 6.8); and (iii) zinc chelate adsorbent matrix (PDE activity passes straight through the column). This yields a preparation of enzyme that is approximately 10–20\% pure, based upon maximal isobutylmethylxanthine-stimulated \[^{32}\text{H}]\text{GMP binding to the noncatalytic site of PDE-5.}\) We have previously demonstrated that this enzyme preparation contained a single class of GMP hydrolytic sites, was potently inhibited by zaprinast (IC\textsubscript{50} = 0.68 \(\mu\text{M}\) using 0.5 \(\mu\text{M} \text{GMP),}\) and could be maximally activated up to 10-fold by the catalytic subunit of PKA (20).

Results

PDE-6y Inhibits PKA-activated PDE-5 Activity—To explore the hypothesis that PDE-5 can be regulated by small proteins homologous to PDE-6y, we have tested the ability of recombinant PDE-6y to modulate the activity of the partially purified PDE-5. As shown in Fig. 1A, the incubation of PDE-6y with PDE-5 at 4 °C for 30 min prevented the subsequent activation of PDE-5 by PKA. A doubling in kinase activity does not reduce
the inhibitory effect of PDE-6γ, suggesting that the kinase does not compete with PDE-6γ for PDE-5. It is well documented that PDE-6γ interacts with PDE-6 via a central polycationic region whose role is to increase the affinity between PDE-6γ and PDE-6 catalytic subunits (26–28). To establish whether this region of PDE-6γ is responsible for preventing PDE-5 activation by PKA, we studied the effect of a peptide, whose sequence corresponded to amino acids 24–46 in PDE-6γ on the activation of PDE-5 by PKA. As shown in Fig. 1A, the incubation of PDE-5 with micromolar quantities of the polycationic peptide at 4 °C for 30 min prevented the subsequent activation of PDE-5 by PKA. The effect of PDE-6γ was also concentration-dependent (Fig. 1B), with an approximately 80% inhibition of the PKA-dependent activation of PDE-5 being evident at 0.3 μM PDE-6γ. The addition of PDE-6γ to the activated PDE-5 was without effect (Fig. 1C), suggesting that PDE-6γ elicits its effect by preventing the activation process.

The effect of PDE-6γ was also concentration-dependent (Fig. 1B), with an approximately 80% inhibition of the PKA-dependent activation of PDE-5 being evident at 0.3 μM PDE-6γ. The addition of PDE-6γ to the activated PDE-5 was without effect (Fig. 1C), suggesting that PDE-6γ elicits its effect by preventing the activation process.
It was also necessary to show whether PDE-6<sub>g</sub> is required to be phosphorylated to inhibit PDE-5. This concern was raised after we found that PDE-6<sub>g</sub> can in fact serve as a substrate for PKA (see Fig. 8). A definitive answer came from experiments with the peptide. The peptide does not contain putative sites for the phosphorylation by PKA and indeed was not phosphorylated by this kinase (Fig. 2). The peptide does contain a phosphorylation site for PKC at Thr35 (29) and is vigorously phosphorylated by this kinase (Fig. 2). We conclude from these studies that the phosphorylation of PDE-6<sub>g</sub> subunit by PKA has no influence upon its interaction with PDE-5.

Finally we needed to show that the effect of PDE-6<sub>g</sub> and the polycationic peptide on the regulation of PDE-5 by PKA was not due to inhibition of the kinase. This was established by demonstrating that neither PDE-6<sub>g</sub> nor the polycationic peptide inhibited the PKA-catalyzed phosphorylation of an exogenous substrate, myelin basic protein (Fig. 3).

**Airway Smooth Muscle Membranes Contain Two Proteins Immunoreactive with Anti-PDE-6<sub>g</sub> Antibodies**—We next attempted to establish whether ASM cell membranes express proteins that contain a similar peptide region to that found in PDE-6<sub>g</sub> (amino acids 24–46), because these may serve as inhibitors of PDE-5. Immunoblotting cultured airway smooth muscle membranes with rabbit antibodies raised against amino acids 24–46 of bovine PDE-6<sub>g</sub> identified two immunoreactive staining polypeptides in the low molecular mass range (Fig. 4A). These polypeptides each have an Mr of 14 and 18 kDa (termed here p14 and p18) and migrated close to recombinant PDE-6<sub>g</sub> (see Figs. 8 and 10).

We also show that the detection of p14 and p18 was reduced when the immunizing peptide was combined with antibody (Fig. 4B). p14 and p18 are denoted with arrows. C, a histogram showing the immunoprecipitation of PDE activity with anti-PDE-6<sub>g</sub> antibody. These are representative results from three different membrane preparations.
suggested that these proteins may function like PDE-6γ. It also indicates that a G-protein-dependent mechanism similar to that regulating PDE-6γ may be present in ASM cell membranes. To test this hypothesis we needed to show that PDE-5 could be activated by PKA in isolated membranes and that this could be modified by p14 and p18 in a G-protein-dependent manner. In this regard, Fig. 5 shows that PDE-5 in guinea pig lung and ASM membranes was markedly activated by PKA in the presence of ATP. To assess the role of G-proteins, we have tested the effect of GppNHp (100 μM) to membranes prevented activation in a concentration-dependent manner (Fig. 6). The inhibition was

not due to direct activation of PKA or binding of this guanine nucleotide to the PDE, because GppNHp was without effect on the activation of partially purified PDE-5 by PKA (data not shown). Further, GppNHp did not modulate membrane PDE-5 activity in the absence of PKA (data not shown). p14 and p18 and PKA-dependent Activation of PDE in ASM Membranes Can Be Modulated by GppNHp—We show in Fig. 7A that pertussis toxin treatment of ASM cells, which was used to inactivate G i and G o, reversed the inhibition induced by GppNHp on the PKA-dependent activation of PDE-5. When samples are prepared from membranes treated with GppNHp,
a reduction in the immunoreactivity of p14 and p18 was also detected on immunoblots (Fig. 7B). This reduction was prevented in cells pretreated with pertussis toxin (Fig. 7B) and therefore correlated with the reversal of the inhibitory effect of GppNHp on the PKA-dependent activation of PDE-5.

The corresponding appearance of p14 and p18 in the supernatant of GppNHp-treated membranes was not detected, indicating that the reduced immunoreactivity of p14 and p18 was not due to their displacement from the membrane but rather a consequence of a post-translational modification. This may involve phosphorylation of the antibody recognition epitope supported by endogenous ATP. Alternatively, GppNHp may activate a protease that cleaves p14/p18 to remove the epitope. However, phosphorylation in this region is consistent with the following observations. In Fig. 8 we show that p14 and p18 were phosphorylated by an endogenous kinase when radiolabeled ATP is added, and this could be increased by the addition of GppNHp to membranes. In Fig. 9 we show that the addition of ATP (0.5–50 μM) to membranes also induced a reduction in the immunoreactivity of p14 and p18. However, when membranes were washed with buffer A to remove endogenous nucleotides, GppNHp and ATP were only effective when added together (Fig. 10).

In Fig. 11, we show that the addition of PDE inhibitors (isobutylmethylxanthine and theophylline, 100 μM) at a concentration that is known to completely inhibit PDE-5 activity negated the effect of GppNHp on the phosphorylation and immunoreactivity of p14 and p18. These observations indicate that PDE-5, p14 and p18, unidentified G-protein, and kinase may be linked in a signal transduction cascade, because their ligand bindings appear to modulate their interactions.

**DISCUSSION**

The major observations of this study are: (i) recombinant PDE-6γ and a peptide corresponding to amino acids 24–46 of PDE-6γ prevent the PKA-dependent activation of PDE-5 and (ii) airway smooth muscle cells express two small molecular mass proteins (p14 and p18) that are immunologically related to the inhibitory γ subunit of the photoreceptor cGMP phosphodiesterase. p14 and p18 appear to interact with PDE-5, a major cGMP hydrolyzing enzyme that is present in airway smooth muscle. Phosphorylation of membrane-bound p14/p18 is stimulated by guanine nucleotides, and this correlates with a concurrent reduction in the ability of PKA to activate PDE-5. Phosphorylation of p14/p18 appears to be regulated by a pertussis toxin-sensitive G-protein-dependent kinase. At present we do not know if an additional G-protein is responsible for regulating PDE-5 independently of p14/p18, although our correlative data tend to favor the idea that p14/p18 is responsible for linking a single G-protein to PDE-5. Further evidence for an interaction between PDE-5 and p14/p18 is the formation of a stable complex between these proteins in cell membranes. Experiments with isobutylmethylxanthine and theophylline show that ligand binding to the catalytic site of PDE-5 appears to affect the phosphorylation of p14/p18 by the G-protein-dependent kinase. Ligand binding to catalytic sites is known to increase binding of cGMP to noncatalytic sites in PDE-5, suggesting that interaction of p14/p18 with PDE-5 may be governed by this occupancy. It follows that the association of p14/p18 with PDE-5 may affect the ability of the former to be phosphorylated by the G-protein-dependent kinase.

The identity of the G-protein can be considered on the basis of pertussis toxin sensitivity and may be ascribed to G_{i,α}, because G_{i,α} is not expressed in these cells (30). The G-protein can either bind to p14 and p18 to increase their susceptibility to phosphorylation by an unidentified kinase or increase the activity of this kinase directly. The role of a G-protein in regulating PDE-5 indicates the possibility that this signal transduction pathway may originate from the occupancy of a receptor with extracellular ligand. The proposed model for the regulation of PDE-5 by G-protein is distinct from that explaining the regulation of PDE-6 by its own γ and by transducin. In the latter case, γ inhibits PDE-6 activity whereas G-protein reverses this inhibition. The model proposed for PDE-5 bears some limited analogy with another regulatory mechanism that was recently suggested to modulate activation of PDE-6 in amphibian rod outer segments (17, 18). In this case, when PDE-6γ is complexed with the GTP-bound transducin it is phosphorylated by an unidentified kinase. The phosphorylated γ prevents subsequent activation of PDE-6 by GTP-bound transducin.

Two functional regions in the γ subunit of PDE-6, a polycationic (amino acids 24–46) and a C-terminal domain, interact with both transducin and PDE-6 catalytic subunits (31–34). The C-terminal domain is essential for both the inhibitory action against PDE-6 (26, 31, 32, 35) and for stimulating transducin GTase (36). The role of the polycationic domain is to provide a second site for interaction, which serves to increase the affinity of PDE-6γ for PDE-6β and transducin. However, the peptide corresponding to this region was shown to inhibit the activation of PDE-5 by PKA. The PDE-6γ antibody was raised to this polycationic region, suggesting that p14 and p18 may also contain a similar domain. On the other hand, PDE-5 contains a region that has some homology with the sites in the PDE-6 catalytic subunits that interact with the polycationic region of PDE-6γ (28). In contrast, PDE-5 does not have a region homologous to the site on PDE-6 that was shown to interact with the C terminus of PDE-6γ. Because the polycationic region in PDE-6γ has not been shown to have any catalytic activity on PDE-6, this suggests either that a similar region in p14 and p18 subserves a different function that may be to modulate the activation of PDE-5 by PKA or that this proposed function is conferred to a different domain(s) in p14 and p18. Our finding that the polycationic peptide prevents the PKA-dependent activation of partially purified PDE-5 favors the former proposal.

In conclusion, p14 and p18 are PDE-5-associated proteins that appear to be affected by G-protein and kinase-directed regulation. Along with PDE-6γ they may represent a novel class of proteins that differentially modulate the function of various cyclic nucleotide phosphodiesterases.

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Regulation of PDE-5 by Small Molecular Mass Proteins

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