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Regulation of mammalian photoreceptor phosphodiesterase (PDE) by its noncatalytic GAF domains and its two small subunits, gamma and delta

Hongmei Mou
University of New Hampshire, Durham

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REGULATION OF MAMMALIAN PHOTORECEPTOR PHOSPHODIESTERASE (PDE) BY ITS NONCATALYTIC GAF DOMAINS AND ITS TWO SMALL SUBUNITS, GAMMA AND DELTA

BY

HONGMEI MOU
B.S. Biochemistry, Beijing Normal University, China, 1993
M.S. Molecular Biology, Chinese Academy of Science, 1996

DISSERTATION

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This dissertation has been examined and approved.

Dissertation Director, Dr. Richard H. Cote
Associate Professor of Biochemistry

Dr. Clyde L. Denis
Professor of Biochemistry

Dr. Thomas M. Laue
Associate Professor of Biochemistry

Dr. Winsor H. Watson, III
Associate Professor of Zoology

Dr. Gale B. Carey
Associate Professor of Animal and Nutritional Sciences

December 14, 2001
Date

DEDICATION

I dedicate this dissertation to my parents, Xiulin Ai and Jianzhong Mou

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ABSTRACT

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cGMP phosphodiesterase (PDE) is the central effector of visual transduction in retinal rod photoreceptors. Upon activation by transducin (a G-protein), PDE hydrolyzes cytoplasmic cGMP to induce the hyperpolarization of the plasma membrane in the outer segment of the rod cell. The extent and lifetime of activation of PDE must be precisely regulated in order to control the exquisite sensitivity, speed, and adaptational properties of the visual transduction pathway. The overall goal of this work was to investigate the possible regulatory mechanisms of PDE in mammalian rod photoreceptors by its noncatalytic cGMP binding sites on the PDE catalytic dimer (Pαβ), and by the interactions between the inhibitory Pγ subunit and Pαβ in their phosphorylated and non-phosphorylated states.

First, the binding of cGMP to the noncatalytic sites on membrane-associated PDE (PDE-M) and soluble PDE (PDE-S) was characterized. We found that PDE-M holoenzyme contains 2 non-identical high-affinity cGMP binding sites; one of the cGMP binding sites is functionally nonexchangeable. In contrast, PDE-S can release and bind

cGMP at both noncatalytic sites. Activation of PDE reduces more than 100-fold its cGMP binding affinity at one site, while the second cGMP binding site exhibits a 3-fold reduction in binding affinity. We postulate that cGMP dissociation from these two non-identical binding sites might contribute differently to controlling the activation and inactivation kinetics of PDE6 during visual transduction in rod photoreceptors.

We also studied interactions between Pγ and the mammalian Pαβ catalytic dimer. It was found that Pγ binds to two distinct sites on the catalytic Pαβ dimer. cGMP occupancy at the noncatalytic binding sites is responsible for this binding heterogeneity. Although cGMP binding positively enhances Pγ binding affinity to Pαβ, it has no effect on PDE catalytic activity. Two major domains on Pγ interact with Pαβ independently. The N-terminal half of Pγ functions to restore and stabilize cGMP binding at the GAF domain with a binding affinity 50-fold greater than its C-terminal, inhibitory region.

Phosphorylation is an important regulatory mechanism in the visual transduction pathway. Our results showed that both Pγ and Pαβ could be stoichiometrically phosphorylated in vitro. Pγ phosphorylation has modest effects on its binding affinity to Pαβ and its overall inhibitory potency. However, phosphorylation at Thr^{22} mildly decreases the ability of the central region of Pγ to bind to Pαβ and to stabilize cGMP binding at the GAF domains. Similar to PDE5, phosphorylation of photoreceptor Pαβ regulates cGMP binding at the GAF domains. These results suggest a potential role of Pαβ phosphorylation in regulating PDE by regulating cGMP levels in photoreceptor cells.

INTRODUCTION

A. Vision and Photoreceptor Cells

Cells process a diverse range of stimuli, from hormones and neurotransmitters to sensory signals, such as odorants and light. Light is perceived within the retina of the eye. The retina consists of five distinct layers: outer nuclear layer (ONL, containing photoreceptor cells), outer plexiform layer (OPL, consisting of synaptic processes of photoreceptor, bipolar and horizontal cells), inner nuclear layer (INL, containing bipolar, horizontal and amacrine cells), inner plexiform layer (IPL, consisting of synaptic contacts between processes of bipolar, amacrine and ganglion cells), and ganglion cell layer (containing ganglion cells). Vertebrate photoreceptors consist of two types of cells: rods and cones. Cone photoreceptor cells, which have higher temporal resolution than rods, function in bright light and are responsible for color vision. In humans, there are three different classes of cones, long-wavelength cones (red cones, 560-565 nm maximal sensitivity), medium-wavelength cones (green cones, 535-540 nm maximal sensitivity) and short-wavelength cones (blue cones, 440 maximal sensitivity). Rods are the receptors for dim light conditions and night vision. Compared to cones, rods are achromatic and have lower temporal resolution. Many more rods than cones (20:1) exist in mammalian retinas. For example, a human retina has about $1.2x10^8$ rods but only $3x10^6$ cones. Both types of photoreceptors function to transduce a photon of light into atomic motion and then into a discrete nerve impulse. The information provided by the photoreceptors is further processed by an intricate array of nerve cells within the retina,

then sent to the brain by the fibers of the optic nerve, and ultimately is processed by the visual cortex.

As shown in Figure I.1, photoreceptors are highly compartmentalized and consist of two major segments, an inner segment (IS) and an outer segment (OS), which are joined by a slender immotile connecting cilium. The outer segment of a rod is specialized for photoreception, containing a stack of about 1000 discs. The visual receptor molecules are densely packed in these membranous structures. The composition of the OS is better characterized than any other membrane involved in signal transduction: the mass is half protein and half lipid (Fliesler and Anderson, 1983). Rhodopsin and transducin are the two most abundant proteins in the OS and comprise 70% and 17% of the total protein, respectively. The remaining proteins are involved in regulating cyclic nucleotide metabolism (such as phosphodiesterase and guanylate cyclase) and protein phosphorylations. The inner segment is rich in mitochondria and ribosomes and functions to generate ATP at a very rapid rate and to synthesize proteins actively (Hurley, 1992; Fein and Szuts, 1982).

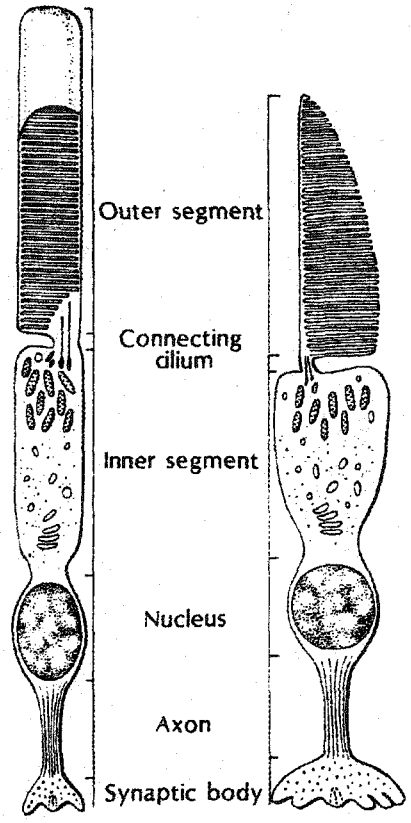

Figure I.1 Rod and Cone photoreceptors

B. Visual Transduction Pathway

1. Visual Excitation

The photoresponse in vertebrate photoreceptors is now understood at a molecular level. It begins when a photon of light activates an enzymatic cascade involving rhodopsin (R), transducin (G_t) and phosphodiesterase (PDE) (Figure I.2).

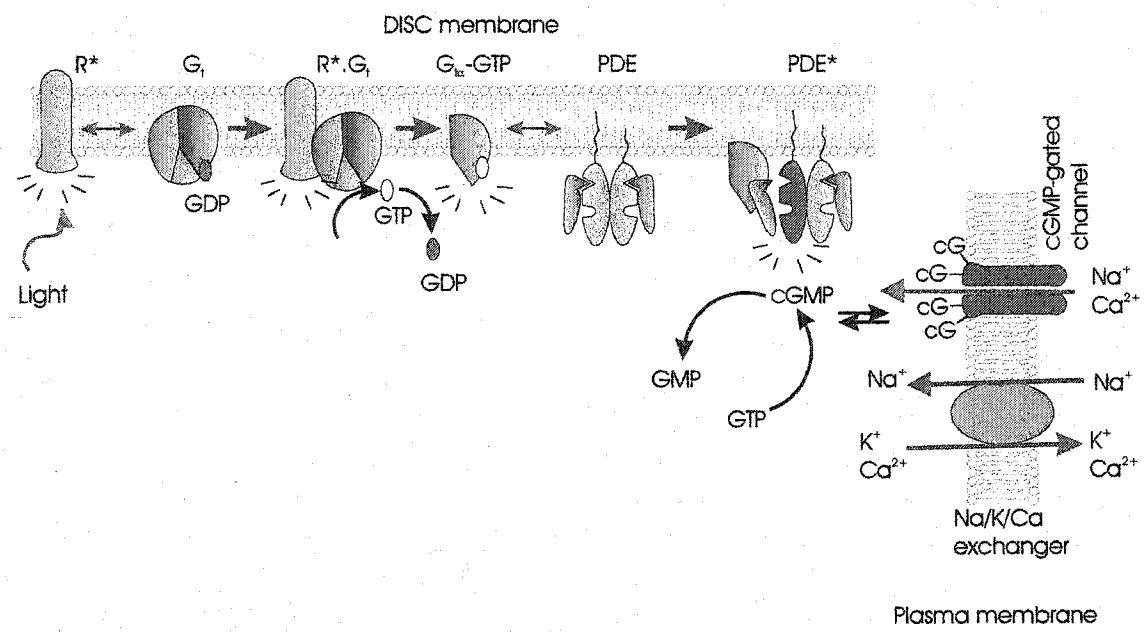

Figure I.2. **Schematic of the visual excitation cascade**

Rhodopsin is a 40-kDa integral membrane protein containing seven trans-membrane helices. The 11-cis-retinal chromophore lies in a hydrophilic pocket of the protein, near the center of the membrane. Transducin is a member of the heterotrimeric G-protein family and functions as a signal-coupling protein in visual excitation. This peripheral membrane protein consists of α (39 kDa), β (36 kDa), and γ (8 kDa) subunits. The α subunit ($G_t\alpha$) has a guanyl-nucleotide-binding site, and it interconverts between an inactive GDP-bound state and an active GTP-bound state. Photoexcited rhodopsin (R*) activates transducin by forming a complex with it and catalyzing the exchange of GTP

for bound GDP. The binding of GTP to transducin leads to the release of R* for another round of transducin activation. Indeed, ~100 transducin can be activated during the lifetime of a single R*. The conformational change induced by the binding of GTP also induces the dissociation of $G_t\alpha$-GTP from $G_t\beta\gamma$. $G_t\alpha$-GTP, the active form of transducin, will in turn activate phosphodiesterase (PDE), the central effector enzyme in the photoresponse. Rod PDE is a tetramer consisting of a catalytic heterodimer ($P\alpha\beta$) to which two inhibitory γ subunits ($P\gamma$) bind. Transducin activation causes $P\gamma$ to be displaced from its inhibitory sites on $P\alpha\beta$. Hydrolysis of cGMP by activated PDE decreases the cGMP level in the photoreceptor cells. cGMP is a diffusible intracellular second messenger used in many signal transduction systems. In rod photoreceptors, R, G_t and PDE are localized to the disk membrane. cGMP is thus responsible for transmitting the visual excitation signal from the disk membrane to the plasma membrane (Lagnado, 2000).

The plasma membrane of the rod photoreceptor contains cGMP-gated cationic channels. In the dark when cGMP levels are high, the cGMP is bound to the channels and the channels are kept open. Sodium ions flow into the outer segment because the electrochemical gradient for Na^+ is large. This gradient is maintained by Na^+-K^+ ATPase pumps located in the inner segment. When cGMP levels decrease following light activation, cGMP dissociates from the channels, causing the channel to close. Consequently, the influx of Na^+ decreases, and the plasma membrane becomes hyperpolarized (more negative on the inside). This light-induced hyperpolarization is then passively transmitted by the plasma membrane from the outer segment to the

synaptic body (for review, see (Pugh, Jr. and Lamb, 2000; Lagnado, 2000; Palczewski and Saari, 1997).

2. Termination and adaptation of the photoresponse

After the light is extinguished, the rod photoreceptor cell quickly recovers from light excitation so that it can respond to the next light stimulus. In addition to response termination, photoreceptor cells are also able to adjust their light sensitivity over a broad range of background levels of illumination. This process, called light adaptation (or background adaptation), maintains the working range of the transduction cascade within a physically useful region of light intensities. The photoreceptor's sensitivity is also reduced by previous exposure to light bright enough to bleach a substantial fraction of the photopigment (greater than 5%). This desensitization and subsequent recovery of sensitivity occurring in darkness is called bleaching adaptation. The recovery from bleaching adaptation is also known as dark adaptation. There is a strong similarity between bleaching adaptation and background adaptation. During bleaching adaptation, the retina behaves as if it is being illuminated by a steady light of gradually decreasing intensity.

Although photoreceptor recovery and adaptation is not the simple reverse of the photo-excitation, response recovery and adaptation mechanisms feed back onto many steps of the excitation pathway (for reviews, see (Fain et al., 2001; Pugh, Jr. and Lamb, 2000; Pugh, Jr. et al., 1999; Detwiler and Gray-Keller, 1996; Fain et al., 1996)).

a. Termination and desensitization of R*

First, activated rhodopsin needs to be deactivated so that it does not continue to catalyze the activation of transducin. Deactivation of rhodopsin is initiated by rhodopsin phosphorylation at multiple sites in its carboxyl-terminal region by rhodopsin kinase (RK). The C-terminus of rhodopsin contains seven serines and threonines that are capable of being phosphorylated *in vitro*. Nevertheless, it is likely that *in vivo* only one or a few amino acids get phosphorylated and contribute to rhodopsin turnoff (Ohguro and Palczewski, 1995; Kennedy et al., 2001). After phosphorylation, rhodopsin itself has decreased ability to bind with transducin and to stimulate the GTP/GDP exchange on $G_t\alpha$ (Langlois et al., 1996; Xu et al., 1997). A complete inactivation of R* requires the binding of arrestin (Gurevich and Benovic, 1993; Wilden, 1995; Krupnick and Benovic, 1998; Krupnick et al., 1997; Krupnick and Benovic, 1998). Arrestin binds specifically the phosphorylated rhodopsin at its carboxyl-terminal sequence (Krupnick et al., 1994). Once bound, arrestin completely blocks the interaction of transducin with rhodopsin.

b. Termination of Gtα*-PDE*

To return to the dark state, activated PDE also needs to be turned off so that it stops hydrolyzing cGMP. The turnoff of activated PDE is a key step and also a rate-limiting step of response termination. $G_t\alpha$* is deactivated by the hydrolysis of GTP bound to $G_t\alpha$* and PDE activity is inhibited upon re-association of the catalytic subunits of PDE with Pγ. $G_t\alpha$ has an intrinsic GTPase activity (Fung et al., 1981). However, turnover numbers of intrinsic GTPase are reported to be 1-2 min^{-1}, nearly 100-fold too slow (Sitaramayya and Liebman, 1983) to account for turnoff of the electrical response. Therefore, there should exist GTPase-activating proteins (GAPs) in the rod outer segment.

The first GAP identified was Pγ itself (Arshavsky and Bownds, 1992). Later, it was reported that Pγ alone is insufficient for activating transducin GTPase and that one or more factors in addition to Pγ must also be present in the outer segment (Angleson and Wensel, 1993; Angleson and Wensel, 1994; Antonny et al., 1993; Arshavsky et al., 1994). This supplementary factor was found to be RGS9-1(He et al., 1998). RGS9-1 is a member of the RGS (the regulators of G protein signaling) family of GTPase accelerating proteins. In the photoreceptor cell, RGS9-1 forms a tight complex with a G protein β subunit 5 type long splice variant (Gβ5L) and functions to stabilize the $G_t\alpha$- GTP in a conformation most favorable for hydrolyzing bound GTP (Slep et al., 2001) (Bourne, 1997). Actually, Pγ itself does not activate transducin GTPase but it enhances the catalytic action of RGS9-1 by recruiting RGS9-1 for hydrolytic transition-state stabilization (Slep et al., 2001). Deletion of the RGS9-1 gene in mice results in profound slowing of photoresponse recovery and GTP hydrolysis by transducin in both rods (Chen et al., 2000) and cones (Lyubarsky et al., 2001). Immunolocalization of RGS9-1 reveals that RGS9-1 is present in cones at significantly higher levels than in rods (Cowan et al., 1998). This might account in part for the faster response kinetics and lower sensitivity of cones compared to rods (Lyubarsky et al., 2001; Cowan et al., 1998).

c. Restoration of cGMP level by cGMP synthesis

Activation of the PDE hydrolyzes free cGMP, and the free concentration of cGMP declines rapidly. Therefore, the restoration of the dark state also requires the re-synthesis of cyclic GMP so that the free cGMP concentration can reach a steady-state concentration to produce a steady-state probability of opening of the cGMP-gated channels. The synthesis of cGMP is largely produced by specialized photoreceptor

guanylyl cyclases (retGC-1 and retGC2) (Lowe et al., 1995; Shyjan et al., 1992; Yang et al., 1995). Both retGC-1 and retGC2 are members of a family of membrane-bound cyclases and present in both rod and cone outer segments. However, retGC-1 is apparently more abundant in cones.

3. Role of Ca^{2+} in regulation of visual termination and adaptation

The concentration of Ca^{2+} is regulated by both the cGMP-gated channel and the Na^{+}/Ca^{2+}-K^{+} exchanger. The cGMP-gated channels of both rods and cones are permeable to a variety of monovalent cations (including Na^{+}, K^{+}, and Li^{+}) as well as divalent cations (including Ca^{2+}). In darkness, the cGMP-gated channels are partially open and there is a steady influx of Ca^{2+} into the photoreceptor. Ca^{2+} is transported out of the outer segments by a Na^{+}/Ca^{2+}-K^{+} exchanger which exchanges four Na^{+} (inward) for one Ca^{2+} and one K^{+} (outward). In the light, the influx of Ca^{2+} into the outer segment decreases when the cGMP-gated channels close. However, Na^{+}/Ca^{2+}-K^{+} exchange is not affected directly by illumination so that Ca^{2+} continues to be transported out of the photoreceptor cells. This results in a decrease in the intracellular Ca^{2+} concentration. The concentration of Ca^{2+} is ~500-700 nM in darkness and is ~30-50 nM in saturating light (~10-20 fold change in the free Ca^{2+} concentration) (Matthews and Fain, 2001; Sampath et al., 1999).

Ca^{2+}-dependent modulation of visual transduction is the most important mechanism for light adaptation in both rods and cones. Ca^{2+} regulates most of the turnoff and restoration processes accompanying the photoresponse, including (a) alteration of the lifetime of R* by modulation the activity of rhodopsin kinase (b) regulation of GC activity; (c) regulation of the affinity of the outer segment channels for cGMP. Recently,

Burns and Balyor have suggested that Ca^{2+} is also involved in the alteration of the gain of the transduction cascade (Burns and Baylor, 2001).

a. Modulation of R* shutoff

During the response recovery and light adaptation, Ca^{2+} and recoverin (Rec) alter the lifetime of R* by modulating the activity of RK. In the dark when the Ca^{2+} concentration is high, most of the recoverin is in the Ca^{2+}-bound state. Rec-Ca^{2+} forms a complex with RK at the membrane, blocking its activity. When Ca^{2+} drops during the light response, Rec releases its Ca^{2+} and dissociates from RK. The elevated concentration of free RK permits the rapid phosphorylation of R*. Arrestin then binds, substantially quenching the R* activity. Recoverin has been shown to be important in mediating Ca^{2+}-dependent modulation of RK activity *in vitro* (Chen et al., 1995) and *in vivo* (Xu et al., 1997).

b. Regulation of cGMP synthesis

The activity of the photoreceptor specific guanylate cyclase (ret-GC) is Ca^{2+}-sensitive (Lolley and Racz, 1982) and its cyclase activity increases as the Ca^{2+} concentration declines (Dizhoor et al., 1991; Gorczyca et al., 1994; Frins et al., 1996; Koutalos et al., 1995). At least two small-molecular-weight Ca^{2+}-binding proteins, named as GC activating proteins (GCAPs), confer such a Ca^{2+} sensitivity on retGC (Cuenca et al., 1998; Kachi et al., 1999). GCAP has three Ca^{2+}-binding EF-hand regions (Palczewski et al., 1994) and has high affinity for Ca^{2+}. In a low Ca^{2+} situation, unoccupied GCAPs stimulate the activity of retGC through dimerization of cyclase subunits (Yu et al., 1999). When the Ca^{2+} concentration is elevated, GCAP in its Ca^{2+}-loaded form inhibits the catalysis of retGCs. In addition to GCAPs, another GC regulatory protein, called GC

inhibitory protein (GCIP) was recently discovered in amphibian photoreceptors (Li et al., 1998). GCIP is also a small molecular-weight protein and shares some conserved sequences with GCAP. GCIP does not stimulate ret-GC in low Ca^{2+} concentration, but it inhibits the ret-GC activity when Ca^{2+} concentration rises. The role of GCIP in cyclase regulation is presently unclear.

c: Regulation of cGMP-gated channel

The cGMP-gated channel from bovine rods also can be modulated by Ca^{2+} (Hsu and Molday, 1993; Gordon et al., 1995; Bauer, 1996; Haynes and Stotz, 1997). In the β subunit of the channel, there exists a calmodulin (CaM) binding site which binds CaM at high Ca^{2+} concentration (Chen et al., 1994; Weitz et al., 1998). Lowering Ca^{2+} concentration over the physiological range during illumination releases the CaM from the channel, increasing the affinity of the channel for cGMP and leading to opening more channels. Ca^{2+} also modulates the sensitivity of channel for cGMP by stimulating the phosphorylation of the channel. One specific tyrosine (Tyr^{498}) in the α-subunits appears to be site of the phosphorylation by a membrane-associated protein kinase. After phosphorylation, the sensitivity of cGMP-gated channel increases so that less cGMP is required to keep it open (Gordon et al., 1992).

C. Overview of PDE Superfamily

At present, 11 classes of PDE (>20 different genes products) have been reported (Gopal et al., 2001). Each family is distinguished by its unique combination of enzymatic characteristics, pharmacological inhibitory profiles and their distinct regulatory mechanism, as well as their specific tissue, cellular and sometimes also subcellular distributions (Table I.1).

1. Catalytic Domains

All PDEs have an approximately 270 amino acid homology region in the carboxyl half which forms the catalytic domain (Charbonneau, 1990; Conti, 2000). Different PDEs have different substrate specificities. PDE1, PDE10 and PDE11 hydrolyze both cAMP and cGMP well. PDE2, PDE3, PDE4, PDE7, and PDE8 prefer cAMP as substrate while PDE5, PDE6 and PDE9 prefer cGMP as substrate. PDE7 and PDE8 have the highest affinity for cAMP and PDE9 has the highest affinity for cGMP. Of all PDE types, PDE6 has the highest k_{cat}/K_m value (Mou et al., 1999a; Norton et al., 2000; Leskov et al., 2000), which approaches the substrate diffusion limit.

Table I.1: Phosphodiesterase superfamily

DPE class (Type)		Common name	Major location	Type of regulatory domain	Preferred Substrate	Selective Inhibitor	Reference
PDE1	PDE1A PDE1B PDE1C	Ca^{2+} calmodulin dependent PDE	Brain, heart, nose	Ca^{2+}/CaM, phosphorylation.	cAMP cGMP	SCH 51866	(Kakkar et al., 1999; Zhao et al., 1997)
PDE2	PDE2A	cGMP-stimulated PDE	Brain, heart, liver	GAF	cAMP	EHNA	(Haslam et al., 1999; Juilfs et al., 1999)
PDE3	PDE3A PDE3B	cGMP-inhibited PDE	Heart, adipose tissue	Phosphorylation	cAMP		(Shakur et al., 2000)
PDE4	PDE4A PDE4B PDE4C PDE4D	cAMP-specific PDE	Lung, yeast	Phosphorylation	cAMP	Rolipram Ro 201724	(Conti, 2000; Torphy, 1998)
PDE5	PDE5A	cGMP binding PDE	Lung, smooth muscle	GAF, phosphorylation	cGMP	Sildenafil Zaprinast	(Francis et al., 2000; Corbin and Francis, 1999)
PDE6	PDE6A PDE6B PDE6C	Photoreceptor PDE	Photoreceptor cells	GAF, γ	cGMP	Zaprinast E4021	(Francis et al., 2000)
PDE7	PDE7A	High-affinity cAMP-specific PDE, rolipram-insensitive	Olfactory cells, brain, skeletal muscle	?	cAMP	Not reported	(Loughney and Ferguson, 1996; Conti et al., 1995)
PDE8	PDE8A PDE8B	High-affinity cAMP-specific PDE, IBMX-insensitive	Thyroid gland	PAS	cAMP	Not reported	(Soderling and Beavo, 2000)
PDE9	PDE9A	High-affinity cGMP-specific PDE	Spleen, small intestine, brain	?	cGMP	Not reported	(Soderling and Beavo, 2000; Juilfs et al., 1999)
PDE10	PDE10A	cAMP PDE and cAMP-inhibited cGMP PDE	Brain, skeleton muscle	GAF	cAMP and cGMP	Not reported	(Soderling and Beavo, 2000)
PDE11	PDE11A	cAMP and cGMP hydrolyzing PDE	Prostate	GAF	cGMP and cAMP	Not reported	(Yuasa et al., 2001; Yuasa et al., 2000; Fawcett et al., 2000)

2. Regulatory Domains

PDEs have regulatory domains in the N-terminal half of the primary sequence. For example, PDE1 has a CaM binding site immediately adjacent to the N-terminus, which can bind Ca^{2+}-CaM with high affinity. PDE4 has a PAS domain (named for their occurrence in Drosophila **P**eriod protein, vertebrate **a**ryl hydrocarbon nuclear translocator, and Drosophila **S**ingle-minded protein) in the N-terminal region but its function is presently unclear. PDE2, PDE5, PDE6, PDE10 and PDE11 have two cGMP binding repeats, called GAF domains (named for their occurrence in cyclic **G**MP –regulated PDEs, some **a**denylyl cyclase and the bacterial transcription factor **F**h1A) (Aravind and Ponting, 1997) (Fig. I.3). The GAF domains of all PDEs are set apart from most others GAF domain-containing proteins by the presence of a $(R/K)X_mNK_nD$ motif (Turko et al., 1996; Granovsky et al., 1998b; Ho et al., 2000). PDE6-GAF has the highest affinity for cGMP (Gillespie and Beavo, 1989; Mou et al., 1999a; Norton et al., 2000). Homologous GAF domains in PDE10A and PDE11A seem to lack a high-affinity allosteric cGMP binding ability (Soderling et al., 1999; Fawcett et al., 2000). cGMP occupancy of the GAF domains regulates, directly or indirectly, the catalytic rate of these PDE enzymes. cGMP binding to the GAF domains of PDE2 allosterically increases the affinity of the catalytic site for substrate by direct allosteric interactions. cGMP binding to the GAF domains of PDE5 makes it a very good substrate for PKG (cGMP-dependent protein kinase) or PKA (cAMP-dependent protein kinase). Once phosphorylated, catalysis is stimulated at the active sites.

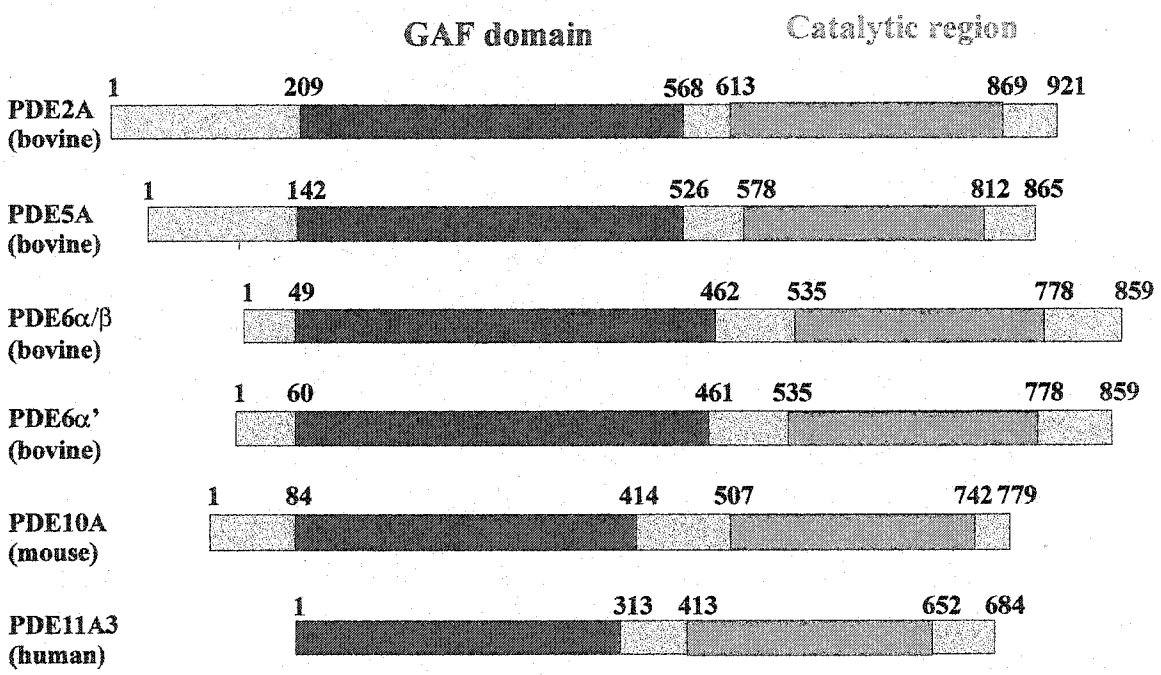

Figure I.3. **The domain structure of GAF-containing phosphodiesterases.**

3. Regulation of PDE by phosphorylation

PDE1, PDE3, PDE4, and PDE5 are reported to be regulated and activated by serine/threonine phosphorylation mechanisms. The kinases responsible for phosphorylation and the possible functions of phosphorylation are listed in Table I.2. Phosphorylation of PDE1 by PKA or CaM kinase II has not been shown to alter the catalytic properties of this enzyme directly, but it does decrease the affinity of the enzyme for Ca^{2+}/CaM (Hashimoto et al., 1989; Sharma and Wang, 1985; Sharma and Wang, 1986; Florio et al., 1994). In the cell, this is equivalent to decreasing the affinity for Ca^{2+}. The phosphorylation of PDE3 by PKA or insulin-dependent kinase stimulates its activity directly and is used as a mechanism for attenuating the amplitude of the cAMP signal in the cell (Macphee et al., 1988; Rascon et al., 1994; Smith et al., 1991;

Ahmad et al., 2000). Phosphorylation also is an important mechanism of regulation of the activity of PDE4 to rapidly decrease cAMP levels (Sette et al., 1994a). As mentioned above, the major effect of phosphorylation of PDE5 is to cause a modest decrease in the K_m for cGMP and to allosterically enhance the cGMP-binding abilities (Corbin et al., 2000).

Table I.2. Phosphorylation of PDE superfamily

PDE	Type	Protein kinase*	Function	References
PDE1	PDE1A PDE1B PDE1C	PKA CaMII ?	decrease the affinity of enzyme for CaM	(Hashimoto et al., 1989; Sharma and Wang, 1985; Sharma and Wang, 1986; Florio et al., 1994)
PDE3	PDE3A PDE3B	PKA insulin-dependent kinase	Increase the enzyme activity for cAMP	(Macphee et al., 1988; Rascon et al., 1994; Smith et al., 1991)
PDE4	PDE4A PDE4B PDE4C PDE4D	? ? ? PKA	Increase the enzyme activity for cAMP	(Sette et al., 1994b)
PDE5	PDE5A	PKA PKG	Increase the affinity of enzyme for cGMP at the active sites and the noncatalytic cGMP binding sites	(Corbin et al., 2000; Turko et al., 1998)

*: PKA: cAMP-dependent kinase; PKG: cGMP-dependent kinase; PKC: protein kinase cdk5: cyclin-dependent kinase type 5; MAP kinase: myosin-dependent kinase; CaMII: CaM-dependent kinase II

D. Structure and Regulation of PDE6

1. PDE Structure and Function

Photoreceptor PDE (PDE6) is the key effector enzyme in the vertebrate visual transduction cascade. There are several isoforms of PDEs in photoreceptors: a membrane-associated rod PDE (PDE-M), a soluble rod PDE (PDE-S) and a cone PDE (PDE-C).

a. PDE-M

PDE-M is the most abundant photoreceptor PDE and is a tetramer containing two similar but nonidentical catalytic subunits, α (99.2 kDa) and β (98.3 kDa), as well as two identical γ subunits (9.7 kDa) (Baehr et al., 1979; Ovchinnikov et al., 1986; Ovchinnikov et al., 1987; Lipkin et al., 1988; Deterre et al., 1988). On each Pα and Pβ, there is a catalytic site and a noncatalytic cGMP binding site. Pγ functions to inhibit the cGMP hydrolysis at the active sites (Hurley and Stryer, 1982) as well as to stimulate and stabilize cGMP binding at noncatalytic cGMP binding sites (Yamazaki et al., 1982; Cote et al., 1994; Mou et al., 1999b; Cote et al., 1994). Pγ also has recognition sites for binding transducin (Yamazaki et al., 1990; Granovsky et al., 1998a; Ford et al., 1998; Skiba et al., 1995; Skiba et al., 1995; Morrison et al., 1989; Cunnick et al., 1990).

b. PDE-S

PDE-S has the same Pα, Pβ and Pγ subunits to those of PDE-M, except that PDE-S has a unique subunit, Pδ. Pδ was first identified as a 17 kDa protein that immunoprecipitated with bovine retinal PDE (Hurwitz et al., 1985), and is supposed to keep PDE-S soluble by interacting with the methylated, prenylated C-terminus of the PDE catalytic subunits (Cook et al., 2000).

c. PDE-C

The subunit stoichiometry of cone PDE is not as well defined as other isoforms of PDE6 but is thought to consist of two identical α'-subunits (Pα'), one or two 9.3 kDa inhibitory γ subunits (Pγ'), and one or two 17 kDa δ subunits (Pδ). However, the immunocytochemistry experiment on bovine retinal sections indicates that Pδ exists only rod outer segments (Florio et al., 1996), suggesting that Pδ bound to PDE-C may be a artifact.

2. Regulation of PDE6 function by interaction of Pγ with PDE

Interactions between Pγ and the Pαβ are essential for the regulation of PDE function in vertebrate rod photoreceptors during visual transduction (Fig. I.4). Although extensive studies have shown the significance of the interaction between Pαβ and Pγ, actual binding affinity of Pγ for Pαβ has never been conclusively determined. By measuring the effects of dilution on the catalytic activities of membrane-bound native PDE and purified PDE in solution, the dissociation constant of the bovine rod PDE has been estimated indirectly to be less than 10 pM (Wensel and Stryer, 1986) and of the frog rod PDE to be about 28 pM (D'Amours and Cote, 1999). High affinity binding of Pγ with Pαβ results in very low basal activity in the dark-adapted state.

Previous peptide studies have indicated that two major regions on Pγ provide the major interactions with Pαβ: (a) the carboxyl terminus amino acids (Lipkin et al., 1988; Artemyev and Hamm, 1992; Takemoto et al., 1992; Brown, 1992; Skiba et al., 1995; Granovsky et al., 1997)and (b) the central polycationic region (Granovsky et al., 1998a;

Artemyev et al., 1996). The C-terminal region of Pγ, especially the last 7-10 amino acids, interacts directly with the active sites (Artemyev and Hamm, 1992; Granovsky et al., 1997). The binding of the C-terminal region to the active sites is regulated in a reciprocal manner by noncatalytic cGMP occupancy at the GAF domain of frog PDE (D'Amours et al., 1999). The central lysine-rich region of Pγ also interacts with Pαβ with high affinity, but the function of this region of Pγ is not quite clear. Cross-linking experiments indicated that the site on PαB that interacts with Pγ is between the second GAF domains and catalytic sites (Natochin and Artemyev, 1996), raising the possibility that the central region of Pγ might be responsible for the regulation of noncatalytic cGMP binding. Actually, it has been reported that a Pγ central peptide (Pγ24-45) was able to restore high affinity cGMP binding to bovine cone PDE (Granovsky et al., 1998b).

3. Regulation of PDE function by interaction of Pγ with transducin

a. Models for transducin activation of PDE

When photoreceptor cells are exposed to light, $G_t\alpha^*$ -GTP binds to Pγ and displaces it from its inhibitory site on Pαβ, causing activation of PDE. The activation of PDE by transducin is the key step in the visual transduction and has been investigated extensively. A number of models have been proposed for the mechanism of PDE activation and for the subunit composition of the activated complexes. In addition, the activation process has been reconstituted on membranes with purified PDE and purified transducin (Fung and Nash, 1983; Tyminski and O'Brien, 1984; Wensel and Stryer, 1986; Bennett and Clerc, 1989; Malinski and Wensel, 1992; Otto-Bruc et al., 1993; Melia et al., 2000).

The first model is that two transducins bind a single PDE with equal affinity and each transducin contributes equally to PDE activation (Wensel and Stryer, 1990). To support this, co-immunoprecipitation experiments have revealed a GTP-specific interaction between $G_t\alpha$ and $P\gamma$ in a one-to-one stoichiometry (Fung and Griswold-Prenner, 1989). A direct measurement also suggested that $G_t\alpha$ bound per PDE ratio at saturation is close to 2 (Clerc and Bennett, 1992).

However, there is evidence that the binding of the first high affinity $G_t\alpha^*$ to PDE elicit 80-100% of the maximal activity of PDE, whereas the binding of the second low affinity $G_t\alpha^*$ to PDE elicits little or no additional activation of PDE (Bruckert et al., 1994). In this case, PDE would be nearly maximally activated through its interaction with only one $G_t\alpha^*$ in the physiological situation. The interaction of the second transducin to PDE might function to ensure a rapid deactivation of $G_t\alpha^*$ through the enhancement of the rate of GTP hydrolysis (Bruckert et al., 1994). A recent reconstitution experiment with purified PDE and transducin on cationic membranes was consistent with this model that PDE is maximally activated when one $G_t\alpha$-GTPγS bound to the vesicles per PDE (Melia et al., 2000). If this is true, then either there is only one catalytic site on PDE that can be relieved of its inhibitory constraint by $P\gamma$ (Berger et al., 1999), or the two catalytic sites on PDE are simultaneously activated by a single $G_t\alpha$-GTPγS.

The third model is that a higher affinity transducin contributing 30% or less of the catalytic activity, with occupation of a second lower affinity transducin needed for full activation (Bennett and Clerc, 1989; Clerc et al., 1992). This model has suggested that transducin activation could stimulate the maximum catalytic potential of the $P\alpha\beta$ catalytic dimer. However, Norton et al. showed that the rate of cGMP hydrolysis for

taPDE is only half of the rate of trypsinized PDE (Norton et al., 2000). Therefore, transducin activation fails to relieve completely Pγ inhibition at both active sites. Activation of PDE catalysis by one, not two, activated transducin molecules in frog ROS homogenates further confirms that the third model is unlikely.

b. Sites of interaction of $G_t\alpha$ with Pγ

There are multiple distinct sites of interaction between activated $G_t\alpha$ and Pγ (Fig. I.4). The interaction between $G_t\alpha$-293-314 and Pγ46-87 is important for PDE activation, especially the residue Ile^{87}, which is involved in relieving the inhibitory constraint of Pγ to allow the entry of substrate into the active pocket (Slep et al., 2001; Sondek et al., 1994). Trp^{70} is important for Pγ GAP activity (Slepak et al., 1995) A second site of interaction involves the polycationic region Pγ24-45 and residues 250-275 on the $G_t\alpha$ subunit (Cunnick et al., 1994; Artemyev et al., 1993). This interaction is supposed to function as an anchor for transducin to form a complex with PDE6 during activation. A $G_t\alpha$-induced conformational change in the Pγ24-46 region may result in a decreased affinity of Pγ for Pαβ and an increased affinity for $G_t\alpha$ (Artemyev et al., 1993).

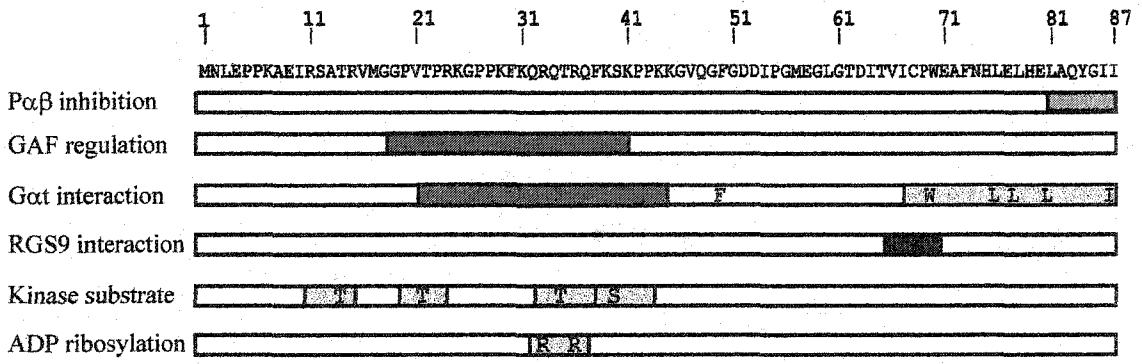

Figure I.4. **Model of multiple sites of interaction of Pγ with Pαβ, Gtα, RGS9 and other proteins.**

4. Regulation of PDE6 function by its GAF domains

The physiological function of the noncatalytic sites on PDE6 differs from what is known about the regulatory properties of the PDE2 or PDE5 GAF domains. Two possible regulatory mechanisms have been proposed for noncatalytic cGMP binding sites: (1) regulating the free cGMP concentration by binding and buffering the cytoplasmic cGMP concentration (Yamazaki et al., 1996) and (2) regulating the duration of PDE6 activation by altering Pγ ability to act as a GAP for transducin inactivation (Cote et al., 1994).

a. Regulating the free cGMP concentration

In dark-adapted rod outer segments, the cytoplasmic cGMP concentration is about 3.5 μM, consistent with predictions of the cytoplasmic cGMP concentration based on activation of the cGMP-gated ion channel of rod photoreceptors. However, the total cGMP concentration is 60 μM (Cote and Bownds, 1984). About 95% of the total cellular cGMP is bound to two classes of cGMP binding sites on PDE (Cote and Brunnock, 1993). During visual excitation when cytoplasmic cGMP concentration is decreased, the bound

cGMP is dissociated from the lower affinity cGMP binding sites. However, whether this mechanism can help to restore the cGMP concentration in the cytoplasmic is questionable. First, kinetics of cGMP dissociation from the high affinity binding sites is too slow to participate in restoring cGMP concentration during the time of visual excitation and recovery (Cote and Brunnock, 1993; Cote et al., 1994; Yamazaki et al., 1996; Norton et al., 2000; Mou et al., 1999a). Second, only 50% or less of bound cGMP can be released to buffer the cytosolic cGMP concentration during the visual excitation (Cote et al 1984; Cote et al., 1986; Cote et al., 1989). Finally, the dissociated cGMP would be hydrolyzed in milliseconds, if PDE remained activated (Calvert et al., 1998; Arshavsky et al., 1992).

b. Regulating the lifetime of $G_t\alpha$* by altering Pγ affinity for acting as a GAP

It has been reported that cGMP binding to the PDE noncatalytic sites modulates, in a reciprocal way, the binding affinity of Pγ to both amphibian Pαβ (D'Amours and Cote, 1999) and mammalian Pαβ (Mou et al., 1999a; Mou and Cote, 2001a). In amphibian PDE, cGMP binding to the non-catalytic sites lowers basal activity of PDE holoenzyme. By gradually diluting frog ROS homogenates to the picomolar concentration of PDE, D'Amours and Cote have estimated that the apparent binding affinity of Pγ for PDE was 28 pM when cGMP occupied the non-catalytic sites, whereas the binding affinity of Pγ was 16-fold weaker when the sites were empty (D'Amours and Cote, 1999). In mammalian PDE, cGMP binding to the non-catalytic sites increase the binding affinity of Pγ at least 10-fold at one of the binding site (Mou and Cote, 2001b).

Pγ, in combination with RGS9 and Gβ5L, serves as a GTPase-activating protein for transducin, accelerating transducin inactivation (Arshavsky et al., 1992; Arshavsky and Bownds, 1992; Makino et al., 1999; Skiba et al., 2001; Skiba et al., 2000; Kovoor et

al., 2000; Chen et al., 2000; Cowan et al., 1998; He et al., 1998). Pγ in its dissociated form has a higher GAP activity than Pγ bound to the PDE-transducin complex (Tsang et al., 1998; Cowan et al., 2000). Therefore, noncatalytic cGMP binding to the GAF domains of PDE regulates some aspects of photoreceptor light adaptation by altering the ability Pγ to act as a GAP.

Fig I.5 describes a model of how the GAF domain regulates the lifetime of PDE during visual excitation. When the sites are occupied by cGMP, Pγ has a higher affinity for PDE and PDE forms a complex with activated transducin. In this state, the rate of GTP hydrolysis is relatively slower, and PDE stays active for a relatively longer time. If photoreceptors are exposed to continuous light, the cytoplasmic cGMP will be lowered, inducing cGMP dissociation from the GAF domains. Emptying cGMP binding sites induces a conformational change on Pαβ so that Pγ has relatively lower affinity for PDE. Activated transducin then removes Pγ from the PDE catalytic subunits. As a consequence, the rate of GTP hydrolysis is accelerated by the Pγ-RGS9-1-Gβ5L complex and the duration of PDE activation is reduced.

Whether or not the two PDE noncatalytic cGMP binding sites play an equal role in the regulation of GTPase activity is not clear. In the nonactivated state, cGMP dissociation from each of the PDE noncatalytic sites is independent of the occupation state of the other site and there is a lack of cooperativity in cGMP binding to the noncatalytic sites (Cote and Brunnock, 1993; Mou et al., 1999a; Norton et al., 2000). However, once activated by transducin, cGMP dissociation from two noncatalytic binding sites becomes heterogeneous. Removing the Pγ subunit results in rapid release of cGMP from only one of the noncatalytic sites on frog PDE, and cGMP dissociation from

the second binding site remains similar to that of the nonactivated PDE (Norton et al., 2000). It is possible that cGMP dissociation from two different noncatalytic sites act to regulate transducin GTPase based on the duration and/or intensity of light stimulation. The former one is important to regulate the lifetime of PDE* in a short light response, and the latter one is essential to regulate the lifetime of PDE* during adaptation to continuous illumination.

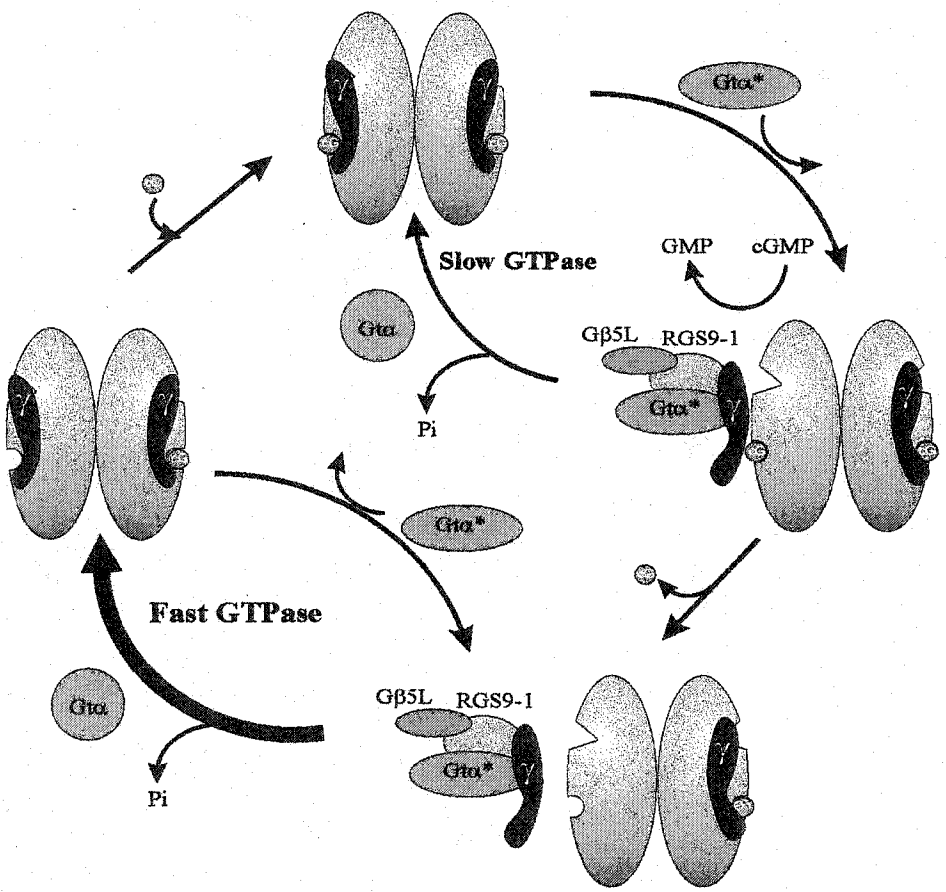

Figure I.5. **Model for how cGMP binding to the GAF domains on PDE determines the rate at which PDE is inactivated by transducin GTPase.**

5. Regulation of PDE function by GARP

GARPs are a class of glutamic acid-rich proteins. In rod photoreceptor cells, three GARPs have been found: GARP1 (130 kDa) and GARP2 (62 kDa) are both soluble, while the third one is the β-subunit of the cyclic nucleotide-gated channel (240 kDa) (Sugimoto et al., 1991; Körschen et al., 1995; Ardell et al., 1995; Finn et al., 1996).

In mammalian rod outer segments, GARPs are restricted to the rim region and incisures of discs and function as multivalent proteins that interact with the some key players of cGMP signaling, such as PDE and guanylate cyclase, and retina-specific ATP-binding cassette transporter (ABCR) (Körschen et al., 1999). The assembly of these signaling molecules into macromolecular complexes may provide intracellular signaling pathway with higher specificity, sensitivity and speed in rod photoreceptors. Recently, work done in our lab suggested that GARP2 also exists in frog photoreceptor cells (Maftei et al., 2000). Immunoaffinity chromatography and co-immunoprecipitation analyses suggested that frog GARP2 forms a tight complexes with PDE, transducin, and RGS9 (Maftei et al., 2000). No similar GARPs have been found in cones (Körschen et al., 1999). GARP2 potently inhibits PDE activity and associates more strongly with light-activated than nonactivated PDE (Körschen et al., 1999). This sequestration of PDE independent of inactivation of transducin might be a an important inactivation mechanism at high light intensities, when a significant fraction of active PDE is reaching the disc margin. In this way, GARPs may function to down-regulate cGMP turnover in the daylight when rod function is saturated.

6. Regulation of PDE6 function by post-translational modifications

a: Isoprenylation/methylation of PDE6

Many proteins in the visual excitation pathway (such as rhodopsin, transducin and PDE) as well as other regulators of transduction [rhodopsin kinase (RK), arrestin and its homologues, RGS9-Gβ5L, etc.] are either membrane-associated or integral membrane protein. About 70-80% of the phosphodiesterase activity found in bovine rod outer segments is membrane-bound (Cook et al., 2001). In frog rod outer segments, > 90% rod PDE is membrane-associated (Norton et al., 2000). The association of multiple cascade components to the membrane facilitates the interaction of these proteins during signaling so that vertebrate photoreceptors can respond to light as a 2-dimentional collision process, ensuring both a high degree of signal amplification and fast signal termination on the physiological subsecond time scale.

Rod PDE is anchored to the disc membrane through its C-terminal isoprenylated and carboxymethylated modifications (Cook et al., 2001; Qin et al., 1992). Limited proteolysis of the C-terminus of the PDE catalytic subunits correlates with loss of membrane binding (Catty and Deterre, 1991; Wensel and Stryer, 1986; Ong et al., 1989), suggesting that C-terminal modifications are the major contributor to PDE interaction with disc membranes. The Pα subunit is modified by a farnesyl (C-15) group on the cysteine of the C-terminal motif CCVQ, whereas the Pβ subunit is modified by a geranylgeranyl (C-20) group on the cysteine of CRIL (Qin et al., 1992; Anant et al., 1992). In addition, both C-termini are methylated (Ong et al., 1989).

The 17 kDa Pδ subunit has previously been shown to co-purify with the soluble form of PDE6, and it functions to solubilize the membrane-bound form by interaction

with the methylated, isoprenylated C-terminus of the PDE catalytic subunits (Cook et al., 2000). After released from the disc membrane, PDE cannot be activated by transducin. Therefore, solubilization of PDE by Pδ might be a possible desensitization mechanism during light adaptation. The binding of Pδ to membrane-associated PDE is regulated by the C-terminal prenylation and methylation of the catalytic subunits. Prenylation and methylation are both potentially reversible and regulated by prenyl (methyl) transferase and prenylester (methylester) hydrolase (Rando, 1996).

b: ADP-ribosylation of Pγ

ADP-ribosylation of Pγ has also been demonstrated as a potential mechanism for the regulation of the interaction between Pγ and $G_t\alpha$. Rod outer segments contain two endogenous glycosylphosphatidylinositol-anchored ADP-ribosyltransferase isozymes, and these two isozymes have similar properties in ADP-ribosylating Pγ. Two arginines (Arg^{33} and Arg^{36}) in the central region of Pγ are ADP-ribosylated when Pγ is free or complexed with the catalytic subunits of PDE (Bondarenko et al., 1999; Bondarenko et al., 1997). After ADP-ribosylation, Pγ loses its interaction with $G_t\alpha$-GTP (Bondarenko et al., 1999). ADP-ribosylation of Pγ is reversible. Bondarenko et al. have shown that a soluble fraction of ROS contains an enzyme(s) to release the radioactivity of [^{32}P]ADP-ribosylated Pγ in a concentration- and time-dependent manners (Bondarenko et al., 1999). This suggests that reversible Pγ ADP-ribosylation may function as a negative regulator of transducin-dependent activation of PDE in phototransduction.

c. Phosphorylation of Pγ and Pαβ

There are 8 potential phosphorylation sites in the Pγ sequence. The central region of Pγ contains most of the predicted phosphorylation sites. To date, several protein

kinases have been found to exist in ROS and some of them have predicted phosphorylation sites on Pγ (Table I.3).

Pγ phosphorylation has been studied both *in vivo* (Hayashi, 1994; Matsuura et al., 2000) and *in vitro* by several protein kinases, including phosphatidylinositol (PI)-stimulated kinase (Hayashi et al., 1991), protein kinase C (PKC) (Udovichenko et al., 1994), PKA (Xu et al., 1998), and cdk5 (originally termed "Pγ kinase") (Tsuboi et al., 1994b; Tsuboi et al., 1994a; Hayashi et al., 2000; Matsuura et al., 2000).

Table I.3: Protein kinases found in the rod outer segment of photoreceptors

Protein kinase found in ROS*	Reference	Consensus Sequence	Potential sites on Pγ
RK	(Lee et al., 1981)	-	Thr^{62}
PKA	(Walter, 1984)	RX_{1-2}**S/T**X	Thr^{35}
PKC	(Kelleher and Johnson, 1985; Udovichenko et al., 1997)	X**S/T**XR/K	Thr^{35}
PKG	(Taylor and Uhler, 2000)	$(R/K)_{2-3}$X**S/T**X	Thr^{35}
MAP kinase	(Ko et al., 2001)	P**X S/T P**	Thr^{22}
Cdk5 (Pγ kinase)	(Hayashi et al., 2000)	K**S/T**PXK	Thr^{22}
CaMII	(Liu et al., 2000)	RXX**S/T**X	Thr^{14}
CKII	(Hollander et al., 1999)	X**S/T**XXD/E	Not Found
Protein-tyrosine kinase (such as Src)	(Bell et al., 2000)	-	Tyr^{84}

*: RK: Rhodopsin kinase; PKA: cAMP-dependent kinase; PKG: cGMP-dependent kinase; PKC: protein kinase cdk5: cyclin-dependent kinase type 5; MAP kinase: mitogen-activated protein kinase; CaMII: CaM-dependent kinase II

The physiological function of Pγ phosphorylation is not clear in the current scheme of phototransduction. However, Pγ phosphorylation by the PKA, PKC or cdk5 has been reported to enhance the inhibitory effect of Pγ on Pαβ catalysis (Xu et al., 1998; Udovichenko et al., 1994). In addition, phosphorylation of Pγ by PKC is reported to

eliminate the binding heterogeneity of Pγ to Pαβ, resulting in a single class of Pγ binding sites (Udovichenko et al., 1994). These earlier studies suffered from several deficiencies that limited their usefulness. For example, the concentration of PDE used in their assays was in the nanomolar range, whereas the Pγ affinity occurs in the pM range; thus, Pγ should inhibit the catalysis of PDE in a concentration-dependent manner and the effect of Pγ phosphorylation would be obscured. In addition, PDE preparations used in the earlier studies also contained transducin which can also bind Pγ, further complicating analysis of Pγ phosphorylation.

Yamazaki and his colleagues have also suggested that phosphorylated Pγ loses or decreases its ability to interact with $G_t\alpha^*$. Phosphorylated Pγ has less ability to inhibit GTPγS binding to $G_t\alpha$ or to inhibit GTPase activity of $G_t\alpha$, but it gains a 10-15 times higher ability to inhibit $G_t\alpha$-activated PDE than that of non-phosphorylated Pγ (Xu et al., 1998; Tsuboi et al., 1994b; Hayashi et al., 2000). They proposed that the Pγ phosphorylation is probably involved in the PDE inactivation and light recovery phase of phototransduction and that it operates without shutoff of $G_t\alpha^*$. They also reported that Pγ in frog ROS homogenates can be phosphorylated *in vivo* in a light-stimulated manner (Tsuboi et al., 1994b; Hayashi et al., 2000). However, the total phosphorylation level of Pγ is rather low (<10%) so that the real physiological function of Pγ phosphorylation is remained uncertain.

To date, there is only one report demonstrating phosphorylation of PDE catalytic subunits *in vitro* by recombinant PKA or PKC (Udovichenko et al., 1993). PKC phosphorylates only Pα subunit of PDE holoenzyme or trypsinized PDE. In contrast, PKA phosphorylates both Pα and Pβ subunits. In addition, PKA does not phosphorylate

trypsin-activated PDE. Since trypsinization removes the part of C-terminal regions from Pαβ, it is possible that PKA phosphorylates PDE catalytic subunits at its C-terminal region while PKC phosphorylates PDE catalytic subunits at other region. Udovichenko et al. reported that a synthetic peptide AKVISNLLGPREAAV (Pα30-44) inhibited phosphorylation of PDE catalytic subunits by PKC from ROS, suggesting that Ser^{34} of Pα subunit is a likely phosphorylation sites for PKC. Currently, the function of PDE catalytic subunit phosphorylation on PDE regulation is not clear.

E: Goal and Hypotheses to be Tested

The overall goal of my dissertation research to define the regulatory mechanisms that determine how long, and to what extent, this central enzyme in vision remains activated following exposure of mammalian rod photoreceptors to light. My whole work is divided into three sections:

(1) Study how cGMP catalysis and binding to noncatalytic sites on mammalian rod PDE is regulated by its small molecular weight subunits (γ and δ)

Photoreceptor PDE (PDE6) associated with membranes in rods is an oligomer consisting of similar α and β catalytic subunits and two identical inhibitory γ subunits (αβγγ) (Deterre et al., 1988). On each α and β subunit there is one catalytic site preferring cGMP to cAMP as a substrate, and one noncatalytic site which binds cGMP with high affinity. cGMP is the primary intracellular messenger for controlling the physiological response of both rod and cone photoreceptor cells to light (Pugh, Jr. and Lamb, 1993; Yau, 1994; Pugh, Jr. et al., 1999). Characterization of the binding of cGMP to the noncatalytic sites in PDE will help to evaluate their role in regulating PDE during

phototransduction. For the photoreceptor PDE, most of our knowledge about the role of the noncatalytic sites in PDE regulation has been obtained with amphibian rod PDE. It has been found that cGMP occupancy at the noncatalytic binding sites of frog photoreceptor PDE enhances the association of Pγ with Pαβ in a reciprocal manner, and it has been postulated that cGMP binding at the noncatalytic binding sites may determine whether the Pγ subunit can participate as an accessory GTPase-activating protein for accelerating transducin inactivation (Arshavsky et al., 1992; Cote et al., 1994). In addition, the noncatalytic sites on frog rod PDE have been postulated to serve as a cytoplasmic buffer for cGMP, possibly serving to help restore cGMP levels during recovery from the light stimulation (Cote and Brunnock, 1993; Yamazaki et al., 1996). Compared to amphibian PDE, much less is known about the cGMP binding properties to noncatalytic cGMP-binding sites on photoreceptor PDE from mammalian rod photoreceptors. In this part of my work, my goal is to characterize the properties of cGMP binding to noncatalytic sites on mammalian rod photoreceptor PDE, and also investigate how they are regulated by Pγ and Pδ.

(2) Functionally map the domains on Pγ that interact with and regulate PDE function

Interactions between Pγ and the Pαβ catalytic dimer are essential for the regulation of cGMP levels in vertebrate rod photoreceptors during visual transduction. Pγ has multiple functions: it functions to inhibit the cGMP hydrolysis at the active sites (Hurley and Stryer, 1982) as well as to stimulate and stabilize cGMP binding at noncatalytic cGMP binding sites (Yamazaki et al., 1982; Cote et al., 1994); Pγ also has recognition sites for binding transducin (Skiba et al., 1995; Morrison et al., 1989;

Cunnick et al., 1990); In addition, Pγ serves as a GTPase-activating protein (GAP) together with RGS9 (Arshavsky & Bownds, 1992; He et al., 1997). By using synthetic peptides of Pγ or site-directed mutants of recombinant Pγ, it has been found that Pγ contains at least two regions that interact with Pαβ: a central cationic region and a hydrophobic C-terminal region (Artemyev & Hamm, 1992; Takemoto et al., 1992). Cross-linking studies have shown that the carboxyl-terminus of Pγ interacts with the catalytic sites of Pαβ both physically and functionally (Artemyev et al., 1996). The central region is also important in the interaction of Pγ with Pαβ but does not directly contribute to PDE inhibition (Artemyev et al., 1996). Cross-linking experiments have also localized a region just past the end of the second noncatalytic cGMP-binding domain that binds to the central polycationic domain of Pγ (Artemyev et al., 1996). This suggests that the central region of Pγ might be responsible for the regulation of noncatalytic cGMP binding. It has been reported that a Pγ central peptide (Pγ24-45) was able to restore high affinity cGMP binding to bovine cone PDE (Granovsky et al., 1998), but a similar peptide has no major effect on frog rod PDE (D'Amours & Cote, 1999). Thus, how Pγ stimulates and stabilizes high affinity cGMP binding is still elusive. In this part, I will first measure carefully the binding affinity of the full-length Pγ to PDE catalytic Pαβ subunits. Then I will functionally map the distinct domains on the inhibitory Pγ subunit which regulate cGMP binding at the noncatalytic binding sites as well as cGMP catalysis at the active sites of mammalian rod PDE6. I will also identify the region that contributes most to the high binding affinity of Pγ to Pαβ. This work will help us to understand how cGMP binding at noncatalytic binding sites and cGMP catalysis at the active sites of mammalian rod PDE6 are regulated by distinct domains of the inhibitory Pγ subunit.

(3) Investigate the role of Pγ and Pαβ phosphorylation in regulating PDE during phototransduction.

A role for Pγ phosphorylation in modulating the interaction between Pγ and Pαβ as well as with the transducin α subunit has been suggested in several studies (Xu et al., 1998; Tsuboi et al., 1994a and 1994b). In addition, several protein kinases known to exist in rod photoreceptors have potential phosphorylation sites on Pγ. However, the mechanism for PDE to be regulated by Pγ phosphorylation during the photoresponse is still not known. Furthermore, we do not know if Pαβ phosphorylation also participates in regulating photoreceptor PDE. In this work, I will examine how Pγ or Pαβ phosphorylation regulates PDE by modifying the interaction between Pγ and Pαβ, as well as the catalytic properties of PDE at its active site and the noncatalytic cGMP binding affinity to its GAF domains. I hypothesize that Pγ or Pαβ phosphorylation does not regulate directly Pγ binding affinity or PDE catalysis, but rather serves to regulate its allosteric cGMP binding properties.

Chapter I

cGMP binding to noncatalytic sites on mammalian rod photoreceptor phosphodiesterase is regulated by binding of its γ and δ subunits*

Abstract

The binding of cGMP to the noncatalytic sites on two isoforms of the phosphodiesterase (PDE) from mammalian rod outer segments has been characterized to evaluate their role in regulating PDE during phototransduction. Nonactivated, membrane-associated PDE (PDE-M; $\alpha\beta\gamma_2$) has one exchangeable site for cGMP binding; endogenous cGMP remains nonexchangeable at the second site. Non-activated, soluble PDE (PDE-S; $\alpha\beta\gamma_2\delta$) can release and bind cGMP at both noncatalytic sites; the δ subunit is likely responsible for this difference in cGMP exchange rates. Removal of the δ and/or γ subunits yields a catalytic αβ dimer with identical catalytic and binding properties for both PDE-M and PDE-S: high affinity cGMP binding is abolished at one site ($K_D > 1$ μM); cGMP binding affinity at the second site ($K_D \sim 60$ nM) is reduced 3-4 fold compared to he nonactivated enzyme; the kinetics of cGMP exchange to activated PDE-M and PDE-S are accelerated to similar extents. The properties of nonactivated PDE can be restored upon addition of γ subunit. Occupancy of the noncatalytic sites by cGMP may modulate the interaction of the γ subunit with the αβ dimer and thereby regulate

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cytoplasmic cGMP concentration and the lifetime of activated PDE during visual transduction in photoreceptor cells.

Introduction

The cGMP phosphodiesterase (PDE)[1] present in rod and cone retinal photoreceptors is the central effector enzyme for vertebrate visual excitation. Photo-activation of the visual pigment rhodopsin leads to G-protein (transducin) activation, which then proceeds to the activation of PDE. The activated α-subunit of transducin (α_t-GTP) is believed to activate the membrane-associated rod PDE holoenzyme (subunit stoichiometry: $\alpha\beta\gamma_2$) by displacing the inhibitory γ subunits (Pγ) from the active sites of the catalytic heterodimer (Pαβ). The enhanced hydrolytic activity of the activated PDE rapidly reduces cytoplasmic cGMP levels, and then leads to dissociation of bound cGMP from the cGMP-gated ion channel, closure of the ion channel, and finally a hyperpolarization of the membrane. These steps constitute the excitation pathway for vertebrate visual transduction (for reviews, see (Pugh, Jr. and Lamb, 1993; Helmreich and Hofmann, 1996; Palczewski and Saari, 1997; Helmreich and Hofmann, 1996; Palczewski and Saari, 1997)).

The photoreceptor PDE is a member of a family of phosphodiesterases that all share the ability to hydrolyze cyclic nucleotides. The PDE in rods and cones (classified as PDE6; (Beavo, 1995)) is most closely related to the cGMP-specific PDE (PDE5) based on several criteria, including overall amino acid sequence similarity, substrate preference for cGMP over cAMP, inhibition of catalysis by isozyme-selective drugs, and the presence of a consensus sequence in the N-terminal half of the protein that represents noncatalytic cGMP binding domains. In the case of PDE5, binding of cGMP to the noncatalytic cGMP binding sites may regulate activity indirectly via protein phosphorylation of the enzyme (reviewed in ref. (Burns et al., 1996)). The cGMP-

stimulated PDE (PDE2) also contains noncatalytic cGMP binding sites that allosterically regulate catalysis at the active site (Martins et al., 1982).

For the photoreceptor PDE, most of our current knowledge about the role of the noncatalytic sites in PDE regulation has been obtained with amphibian rod PDE. It has been found that cGMP occupancy at the noncatalytic binding sites of frog photoreceptor PDE enhances the association of Pγ with Pαβ in a reciprocal manner, and it has been postulated that cGMP binding at the noncatalytic binding sites may determine whether the Pγ subunit can participate as an accessory GTPase-activating protein for accelerating transducin inactivation (Yamazaki et al., 1980; Yamazaki et al., 1982; Arshavsky et al., 1992; Cote et al., 1994; Yamazaki et al., 1982; Arshavsky et al., 1992; Cote et al., 1994). In addition, the noncatalytic sites on frog rod PDE have been postulated to serve as a cytoplasmic buffer for cGMP, possibly serving to help restore cGMP levels during recovery from the light stimulation (Cote and Brunnock, 1993; Yamazaki et al., 1996; Yamazaki et al., 1996).

Much less is known about the function of noncatalytic cGMP binding sites on photoreceptor PDE during visual transduction in mammalian rod photoreceptors. Two distinct isoforms of rod PDE have been described in bovine rod photoreceptors, a membrane-associated PDE (PDE-M) and a soluble PDE (PDE-S) (Baehr et al., 1979; Gillespie et al., 1989; Gillespie et al., 1989). The only known difference between the two isoforms is the presence of a 17 kDa δ subunit (Pδ) associated with PDE-S but not PDE-

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1. The abbreviations used are: PDE, photoreceptor phosphodiesterase; PDE-M, membrane-associated bovine rod PDE; PDE-S, soluble bovine rod PDE; nPDE, non-activated PDE; tPDE, trypsinized PDE; Pαβ, catalytic dimer of PDE; Pγ, inhibitory 10 kDa γ subunit of PDE; Pδ, 17 kDa δ subunit associated with PDE-S; K_D, dissociation constant.

M (Gillespie et al., 1989). The Pδ subunit can solubilize membrane-associated PDE *in vitro* (Florio et al., 1996), but its role in visual transduction has not been determined. The noncatalytic cGMP binding sites on bovine PDE-M have been shown to bind and release cGMP very slowly (Gillespie and Beavo, 1989a), and little is known about the cGMP binding properties of PDE-S. Thus, it remains unclear whether the noncatalytic sites on mammalian rod PDE play a similar physiological role to what has been proposed for the amphibian system.

In this chapter, we first show that PDE-M and PDE-S in their nonactivated states have different cGMP binding properties. Both high affinity cGMP sites on PDE-S are slowly exchangeable, whereas PDE-M contains one exchangeable and one nonexchangeable noncatalytic site. The presence of the δ subunit in PDE-S suggests a role for this subunit in enhancing cGMP exchange at one noncatalytic site that is nonexchangeable in PDE-M. Second, activation of the two isoforms leads to similar behavior of PDE-S and PDE-M in terms of: (1) a large decrease in cGMP binding affinity at one noncatalytic site; (2) a ~10-fold acceleration of cGMP exchange at the remaining high affinity site; and (3) similar hydrolytic rates at the active site. While it is unlikely that cGMP binding and release at the noncatalytic sites regulate PDE activity during the early events of visual excitation, changes in cGMP occupancy at the noncatalytic sites may help regulate the lifetime of activated PDE during later stages of the visual transduction pathway.

EXPERIMENTAL PROCEDURES

Materials--Bovine retinas were purchased from W.L. Lawson, Inc. Radiochemicals were obtained from NEN Life Sciences Products, and scintillation fluid (Ultima Gold) was from Packard Instrument Co. Zaprinast was a gift of Rhone-Poulenc Rorer (Dagenham, U.K.). Sulfolink coupling gel was obtained from Pierce. Pefabloc was obtained from Boehringer-Mannheim. cGMP antisera for radioimmunoassays were purchased from Chemicon. Filtration and ultrafiltration products were from Millipore. Superdex 200 and Mono Q columns, as well as pGEX-2T vector and glutathione-Sepharose 4B were from Pharmacia. The thrombin cleavage capture kit was from Novagen. The reverse-phase columns for Pγ purification were from Vydac. Electrophoretic reagents and immunochemicals were from Bio-Rad. All other chemicals were obtained from Sigma.

Preparation of nonactivated PDE-S (nPDE-S) and PDE-M (nPDE-M)—Both PDE-S and PDE-M were first partially purified from frozen bovine retinas by modifications of the ion exchange and immunoaffinity chromatography procedures described previously (Gillespie et al., 1989; Gillespie and Beavo, 1989a; Gillespie and Beavo, 1988; Gillespie and Beavo, 1989a; Gillespie et al., 1989). Under infrared illumination, thawed retinas were stirred in a beaker containing 1.5 ml per retina of ROS Buffer (60 mM KCl, 30 mM NaCl, 2 mM $MgCl_2$, 20 mM MOPS, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, pH 7.2) containing 47.5% (w:v) sucrose. The retinal homogenate was centrifuged for 1 h at 8,000 x g (4°C), and the supernatant (containing both membrane-associated and soluble PDE isozymes) was removed from the pellet. The pellet was homogenized in 0.4 ml ROS Buffer per retina to extract additional soluble PDE

isozymes, and then centrifuged at 96,000 x g for 45 min at 4°C. The supernatant resulting from this spin was pooled with the soluble PDE isozymes obtained below.

The sucrose-containing supernatant from the first centrifugation was diluted 2-fold with ROS Buffer, and then centrifuged (23,000 x g for 45 min at 4°C). The pellet contained crude ROS which were used to extract PDE-M. The supernatant of this spin (containing soluble PDE isozymes) was combined with the other soluble PDE-containing supernatant. DE-52 anion exchange chromatography was employed to separate cone PDE from PDE-S, essentially as described by Gillespie and Beavo (Gillespie and Beavo, 1988). PDE-S was further purified by immunoaffinity chromatography using the monoclonal antibody ROS1 (Hurwitz et al., 1984) immobilized on a Sulfolink agarose gel.

The crude ROS pellet (containing PDE-M) was resuspended in ROS buffer, homogenized, and centrifuged at 27,000 x g for 45 min at 4°C to remove soluble proteins. After repeating this isotonic washing and centrifugation of the ROS membranes twice, the ROS membranes were exposed to light at 4°C. PDE-M was extracted four times from the ROS membranes by the following sequence: (1) resuspension and homogenization of the ROS membrane pellet in 0.5 ml/retina of 10 mM Tris, 1 mM $MgCl_2$, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride (pH 7.5); (2) centrifugation at 96,000 x g for 45 min at 4°C; (3) collection of the PDE-M-containing supernatant. Immunoaffinity purification of the hypotonically extracted PDE-M was then performed.

Immunopurified PDE (PDE purity, ~80%) was concentrated in a Centricon concentrator, and placed in storage buffer (100 mM NaCl, 2 mM $MgCl_2$, 10 mM Tris, 2 mM dithiothreitol, 0.4 mM Pefabloc, pH 7.5) containing 50% glycerol at –20°C. Both

PDE-S and PDE-M were further purified by gel filtration chromatography on a Superdex 200 HR 10/30 column equilibrated with Buffer A (120 mM NaCl, 5 mM $MgCl_2$, 25 mM HEPES, pH 7.5, 2 mM dithiothreitol, 0.2 mM Pefabloc). After elution, the purified PDE was concentrated and stored as described above. SDS polyacrylamide gel electrophoresis of the purified, non-activated PDE-S (nPDE-S) and non-activated PDE-M (nPDE-M; Fig. 1A, lanes 5 and 6, respectively) shows that >90% of the Coomassie-stained bands represent the catalytic and low molecular weight subunits of PDE. For five different purified PDE preparations, we recovered 2.6 ± 1.1 nmol of purified nPDE-M and 1.0 ± 0.4 nmol nPDE-S per 100 bovine retinas using these procedures.

Preparation of trypsinized PDE-M (tPDE-M) and PDE-S (tPDE-S)—To prepare Pαβ dimers from nPDE-S and from nPDE-M, limited proteolysis with L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin was performed (Baehr et al., 1979; Miki et al., 1975; Hurley and Stryer, 1982; Baehr et al., 1979; Hurley and Stryer, 1982). Purified nPDE (0.8 – 1.0 mg/ml) was prepared in 25 mM MOPS (pH 7.5) containing 25% glycerol, and incubated at 4°C with 50 μg/ml of trypsin for 35-40 min. These conditions were optimized to obtain complete enzyme activation (using a PDE activity assay) without causing the appearance of a 70 kDa proteolytic fragment of the catalytic subunits (as judged by Coomassie-stained SDS polyacrylamide gels of the trypsinized enzymes [Fig. 1.1A, lanes 3 and 4; see also (Catty and Deterre, 1991; Artemyev et al., 1996; Artemyev et al., 1996)]. Proteolysis was halted with a 10-fold weight excess of soybean trypsin inhibitor. Analysis of the trypsinized PDE-S (tPDE-S) preparation at this stage showed that the Pδ subunit was not degraded to a significant extent (Fig. 1.1B, lane 4).

The trypsinized PDE (tPDE) was then diluted >500-fold into Buffer A to enhance dissociation of the low molecular weight subunits and their proteolytic fragments. Ultrafiltration (BioMax, 30,000 molecular weight cut-off) of the tPDE was performed to concentrate the enzyme. Substantial amounts of a ~4,500 kDa band that immunoreacted with the C-terminal Pγ polyclonal antibody (UNH9710-4P) remained in the tPDE samples at this stage (Fig. 1.1B, lanes 3 and 4).

To prepare pure Pαβ preparations from the trypsinized enzyme preparations, the tPDE was further purified by Superdex-200 chromatography using Buffer A as the mobile phase. This was followed by anion exchange chromatography on a Mono Q HR 5/5 column equilibrated in 250 mM NaCl, 1 mM $MgCl_2$, 25 mM Tris (pH 7.5), 1 mM DTT, and 0.2 mM phenylmethylsulfonyl fluoride, using a linear gradient from 250 to 500 mM NaCl to elute Pαβ dimers. The purity of the Pαβ-S or Pαβ-M prepared from tPDE-S or tPDE-M, respectively, was found to be >90% by Coomassie stained gels (Fig. 1.1A, lanes 3 and 4), with undetectable levels of Pγ or Pδ immunoreactivity (Fig. 1.1B, lanes 5 and 6).

Determination of the catalytic constant (k_{cat}) for trypsin-activated PDE—We routinely determined the concentration of native PDE by measuring the maximum hydrolytic activity (V_{max}) of a PDE-containing sample following limited proteolysis to fully activate the enzyme (described above); using activity measurements instead of total protein determinations to estimate the PDE concentration distinguishes native (i.e., non-denatured) PDE from denatured enzyme and minor impurities present in our PDE preparations.

To calculate the concentration of enzyme from the equation, $[P\alpha\beta] = V_{max}/k_{cat}$, we needed to accurately determine the catalytic constant (k_{cat}) for our PDE-M and PDE-S preparations. The determination of the k_{cat} required independent knowledge of the PDE concentration, which was obtained by total protein determinations (Bradford method) and by measuring the maximum cGMP binding site density (B_{max}). The latter approach relies on observations that bovine nPDE-M can bind 2.0 mol of cGMP per mol catalytic dimer (Gillespie and Beavo, 1989a) and that tPDE is capable of binding only 1 mol of [^{3}H]cGMP per mol of catalytic dimer under our conditions (see Results). For the case of PDE-M, the trypsin-activated enzyme has a $k_{cat} = 5550 \pm 100$ mol cGMP hydrolyzed/s/mol [^{3}H]cGMP bound (n = 24) at 22°C. Trypsin-activated PDE-S is able to hydrolyze 5620 ± 110 mol cGMP/s/mol [^{3}H]cGMP bound (n = 16). We have used the average value, k_{cat} = 5600 cGMP hydrolyzed/P$\alpha\beta$/s, in our calculations of PDE concentration. This value is $22 \pm 3\%$ (n = 4) higher than that obtained in identical PDE-S and PDE-M samples in which the Bradford assay was used to quantitate PDE concentration (k_{cat} = 4930 cGMP hydrolyzed/PDE/s).

Purification of Pγ and Pδ—The recombinant bovine rod Pγ subunit was expressed in bacteria as described in (Artemyev et al., 1998) with the following modifications. Following cation exchange chromatography, Pγ was chromatographed on a preparative C18 reverse-phase column (300 Å, 22 x 250 mm) using a linear gradient of 30-50% acetonitrile in 0.1% trifluoroacetic acid. The purity of the Pγ peak was verified on an analytical Vydac C4 reverse-phase column (300 Å, 4.6 x 25 mm) and by SDS polyacrylamide gel electrophoresis (Fig. 1.1A, lane 1). The concentration of Pγ was routinely measured spectrophotometrically after having empirically determined the

extinction coefficient (ε_{277} = 7550 cm-M^{-1} in 45% acetonitrile, 0.1% trifluoroacetic acid) in conjunction with amino acid analysis. The inhibitory activity of Pγ preparations was assessed as described previously (Hamilton et al., 1993), and was typically >95% active.

The recombinant Pδ subunit was expressed and purified by two different methods (this work was done by Terry A. Cook, University of Washington, Seattle, WA). In the first method, the Pδ subunit was overexpressed in Sf9 cells using a baculovirus expression system; the protein was then purified by anion exchange chromatography and ultrafiltration, as described in detail in Florio et al. (Florio et al., 1996). The second method was to subclone the 17K-11 Pδ coding sequence (Florio et al., 1996) into the pGEX2T expression vector and to express the Pδ-GST fusion protein following standard procedures. Following purification on a glutathione-Sepharose affinity column, the fusion partner was cleaved and the protease removed with the use of a thrombin cleavage capture kit according the manufacturer's instructions. The Pδ was concentrated by ultrafiltration and stored in 50% glycerol at –20°C. [Upon sequencing the Pδ-GST construct, a PCR artifact was discovered that caused an amino acid substitution at position 85 from glutamine to arginine. No functional differences could be detected when the cleaved fusion protein containing the substitution was compared with the Pδ expressed from Sf9 cells.]

The concentration of Pδ was determined using the Bradford assay. The purity of different Pδ preparations was found to exceed 80% for both methods (e.g., Fig. 1.1A, lane 2); immunoblot analysis with the 17K-II Pδ antibody revealed a single immunoreactive band at ~17 kDa.

Measurements of cGMP binding to noncatalytic sites on PDE—Two different methods were used to quantitate cGMP binding to the noncatalytic sites on PDE, cGMP radioimmunoassay and a membrane filtration assay. Purified, concentrated PDE was first diluted in Buffer B (77 mM KCl, 35 mM NaCl, 2.0 mM $MgCl_2$, 1.0 mM $CaCl_2$, 1.18 mM EGTA ($[Ca^{2+}_{free}]$ = 240 nM), 10 mM HEPES, pH 7.5) supplemented with final concentrations of: 1.0 mM dithiothreitol, 0.5 μg/ml leupeptin, 0.2 mM Pefabloc, and 0.7 μg/ml pepstatin. In experiments where depletion of endogenous nucleotides was attempted by incubating the PDE at 37°C (24 h for nPDE, 2 h for tPDE), Buffer B was supplemented with 5-fold greater concentrations of protease inhibitors and dithiothreitol, as well as 0.1 mg/ml bovine serum albumin and 30% glycerol [to minimize loss of PDE activity (<20% over the 24 h period)]; before use, the nucleotide-depleted PDE was diluted >10-fold in Buffer B lacking the supplements.

To measure the amount of endogenous cGMP remaining bound to PDE following purification, a cGMP radioimmunoassay was used. Briefly, PDE-containing samples were quenched with 50% HCl in ethanol, then centrifuged to remove precipitated material. The acidic supernatant was dried down under vacuum, and the neutralized sample was resuspended in 0.1 M citrate, pH 6.2. The radioimmunoassay was performed as described in Cote et al. (Cote, 2000), with an operating range of 0.15 to 3 pmol of cGMP per sample, as judged by comparison with a standard curve using known amounts of cGMP treated identically to the unknown samples.

To determine the equilibrium and kinetic properties of [^{3}H]cGMP binding to the various PDE preparations, a membrane filtration assay was used (Cote, 2000). PDE-containing samples in Buffer B were incubated with the indicated concentrations of

[^{3}H]cGMP in the presence of 0.1 or 0.8 mM zaprinast, a photoreceptor PDE catalytic site inhibitor (Gillespie and Beavo, 1989b). The higher zaprinast concentration was used with activated PDE samples to insure that the extent of [^{3}H]cGMP hydrolysis during the incubation period was <10% under all conditions tested. In separate experiments, we verified that 2 – 1000 μM zaprinast was unable to compete with [^{3}H]cGMP bound to the noncatalytic sites of nPDE-M to which excess Pγ had been added to prevent ligand hydrolysis.

Following incubation of PDE with the radiolabeled cGMP, portions were directly filtered on prewet nitrocellulose membranes (Millipore HA membrane, 0.45 μm), and quickly (< 4 s) rinsed with three 1-ml portions of ice-cold Buffer B Nonspecific binding was determined as described previously (Cote and Brunnock, 1993). The membrane filtration technique is useful for measuring relatively high affinity ligand binding (K_D < 1 μM); lower affinity cGMP binding sites are likely to release bound cGMP during the washing procedure and may go undetected.

Analytical procedures—A colorimetric, coupled-enzyme assay was employed for measurements of cGMP hydrolytic rates, as described in detail in (Cote, 2000). SDS polyacrylamide gel electrophoresis followed the procedure of Laemmli (Laemmli, 1970). Immunoblot analyses of the Pγ and Pδ subunits were performed with polyclonal antiserum UNH9710-4P to the C-terminal region (a.a. 63-87) of Pγ or antiserum 17K-II to residues 23-41 of the Pδ protein (Florio et al., 1996) along with a goat anti-rabbit secondary antibody coupled to horse-radish peroxidase, using standard protocols (Gallagher, 1998). Protein concentrations were estimated with the Bradford assay (Bradford, 1976) using bovine serum albumin as a standard; a correction factor of 1.2

was applied for estimates of purified PDE catalytic subunit concentrations (Gillespie and Beavo, 1989a). Data analysis was performed using Sigmaplot (SPSS, Inc.) and KELL (Biosoft). All experiments were repeated at least three times, and error estimates represent the S.E.M.

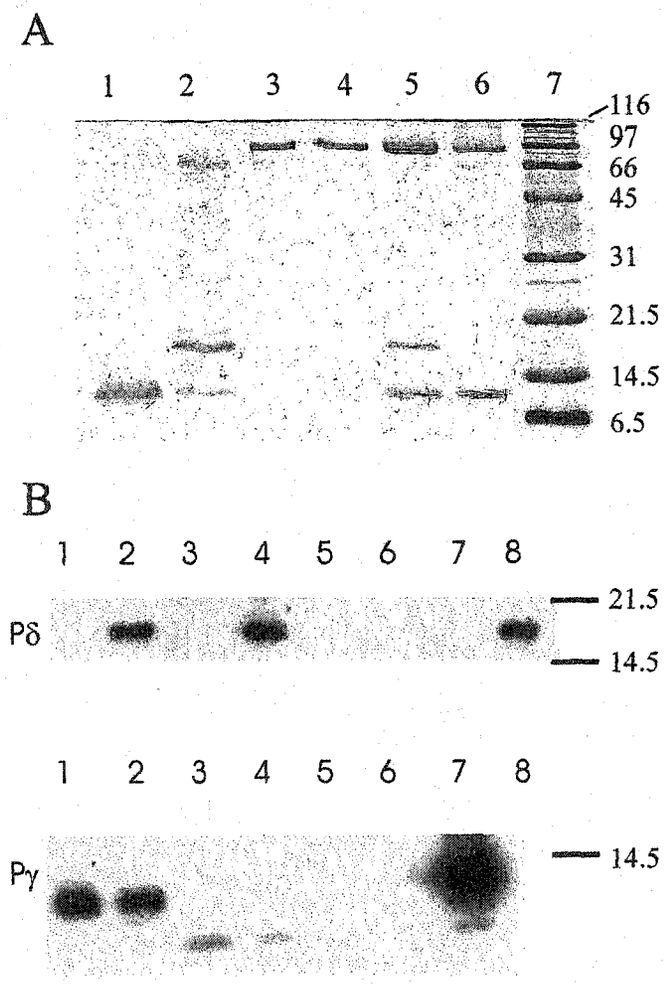

Figure 1.1. **Purity and subunit composition of nonactivated and trypsin-activated PDE-M and PDE-S enzymes used in this study.** A. 15% SDS polyacrylamide gels stained with Coomassie Blue. Purity of proteins analyzed was ≥90% except where noted. Lane 1, recombinant bovine Pγ (0.42 nmol); Lane 2, recombinant bovine Pδ (2.5 μg; ~80% purity). Lane 3, purified Pαβ-S (80 pmol); Lane 4, purified Pαβ-M (80 pmol); Lane 5, nPDE-S (120 pmol); Lane 6, nPDE-M (80 pmol; Lane 7, molecular weight markers (in kDa). B. Immunoblot analysis using Pδ antibody 17K-II (upper panel) or Pγ antibody UNH9710-4P (lower panel). In lanes 1 – 6, 1 pmol of the indicated enzyme was loaded. Lane 1, nPDE-M; Lane 2, nPDE-S; Lane 3, tPDE-M after concentration but prior to gel filtration, showing an immunoreactive 4.5 kDa Pγ proteolytic product; Lane 4, tPDE-S before concentration or purification, showing undegraded Pδ immunoreactivity and a 4.5 kDa Pγ peptide; Lane 5, purified Pαβ-M; Lane 6, purified Pαβ-S; Lane 7, Pγ (6 pmol); Lane 8, Pδ (20 ng); Bars, molecular weight markers.

RESULTS

The soluble and membrane-associated isoforms of bovine rod PDE have different cGMP binding properties in their nonactivated states—As reported previously, the nonactivated, membrane-associated PDE (nPDE-M; $\alpha\beta\gamma_2$) of bovine rod photoreceptors binds 2 mol of cGMP per mol PDE very tightly and exchanges its bound cGMP very slowly (Gillespie and Beavo, 1989a). To directly compare the cGMP binding properties of the soluble PDE (nPDE-S; $\alpha\beta\gamma_2\delta$) with nPDE-M, we measured both the amount of endogenous cGMP remaining bound to purified, nonactivated PDE (nPDE) by cGMP radioimmunoassay, and the amount of exchangeable [^{3}H]cGMP binding sites by a membrane filtration assay (see "Experimental Procedures"). For the case of the membrane-associated isoform, we found that purified nPDE-M retained 1.9 ± 0.04 mol cGMP per PDE holoenzyme ($\alpha\beta\gamma_2$) as judged by a cGMP radioimmunoassay (Fig. 1.2). Approximately 0.1 ± 0.04 mol [^{3}H]cGMP was able to bind to nPDE-M in this condition. These results are identical to previous measurements where tightly bound, endogenous cGMP (1.8 ± 0.3 mol cGMP/PDE) as well as [^{3}H]cGMP binding (0.1 mol cGMP/PDE) were analyzed by HPLC (Gillespie and Beavo, 1989a). Incubation of nPDE-M for 24 h at 37°C resulted in the loss of 1 mol of endogenous cGMP per PDE with the concomitant ability to bind 1 mol of [^{3}H]cGMP per PDE (Fig. 1.2). We conclude that nPDE-M consists of 2 high affinity, noncatalytic cGMP binding sites, of which only one is able to release its bound cGMP under these conditions.

The nonactivated, soluble isoform of rod PDE (nPDE-S) also retains bound endogenous cGMP following its extensive purification (1.7 ± 0.04 mol cGMP/mol PDE; Fig. 2). Upon addition of exogenous [^{3}H]cGMP, the total binding stoichiometry is 2.0 ±

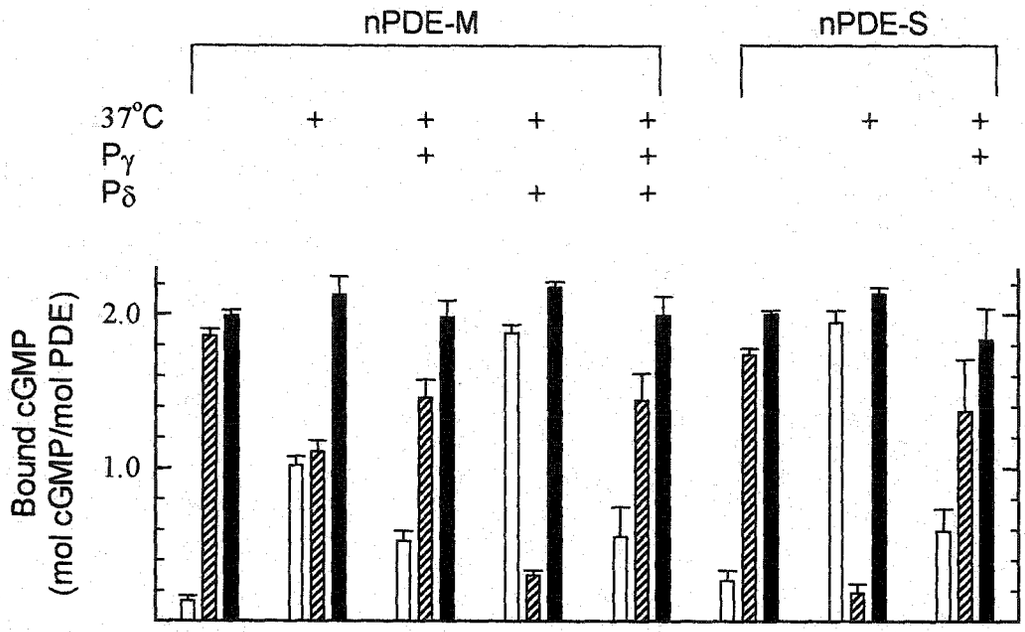

Figure 1.2. **Effects of temperature and added Pγ and Pδ subunits on the noncatalytic cGMP binding sites of nPDE-M and nPDE-S.** Purified nPDE-M and nPDE-S were diluted to 50 nM concentration in Buffer B (containing the higher levels of supplements, including 30% glycerol and 0.1 mg/ml bovine serum albumin as stabilizers), as described in the Experimental Procedures. The PDE samples were then incubated under the following conditions, as indicated in the heading: –37°C, 24 h at 4°C; +37°C, 24 h at 37°C; +Pγ, addition of 10 mol Pγ/mol Pαβ to PDE at onset of 37°C incubation; +Pδ, addition of 5 mol Pδ/mol Pαβ at onset of 37°C incubation. After the incubation period, portions were removed for analysis of endogenous cGMP remaining bound by cGMP radioimmunoassay (hatched bars). Other portions were incubated with 1 μM [^{3}H]cGMP for 30 min at 4°C, and then membrane filtration performed (open bars). The y-axis is expressed as mol cGMP bound per mol PDE holoenzyme for each assay, as well as the sum of the endogenous and [^{3}H]cGMP binding (filled bars).

0.1 mol cGMP/mol PDE, identical to nPDE-M. One major difference between nPDE-M and nPDE-S is the ability of the soluble isoform to release ~90% of its endogenous cGMP upon 24 h incubation at 37°C, as well as being able to bind 1.9 ± 0.08 mol [^{3}H]cGMP/PDE. We conclude that both high affinity noncatalytic binding sites on nPDE-S are able to undergo nucleotide exchange, whereas only one site is exchangeable in nPDE-M.

One obvious difference between nPDE-M and nPDE-S is the presence of the Pδ subunit associated with nPDE-S. To test whether the Pδ subunit could account for the different cGMP binding properties noted above, we incubated nPDE-M with an excess of recombinant Pδ subunit. As shown in Fig. 1.2, incubation with Pδ can cause the release of endogenous cGMP from the formerly "nonexchangeable" binding site on nPDE-M; only 0.3 ± 0.03 mol cGMP/mol PDE remain bound after a 24 h incubation at 37°C, while addition of [^{3}H]cGMP to the nucleotide-depleted enzyme permits 1.9 ± 0.05 mol cGMP/mol PDE to bind. This result indicates that Pδ can bind to nPDE-M (in accord with a previous reconstitution study (Florio et al., 1996)) and enhance the rate of cGMP exchange at one of the noncatalytic cGMP binding sites that is essentially nonexchangeable when Pδ is absent.

We also examined the effects of adding a 10-fold molar excess of Pγ on the cGMP binding properties of nPDE-M and nPDE-S. In all cases where Pγ was added, the amount of endogenous cGMP that was retained following the 24 h incubation was increased to ~ 1.4 mol cGMP/mol PDE, regardless of whether the Pδ subunit was also added in molar excess (Fig. 1.2). The ability of [^{3}H]cGMP to bind was reduced in a corresponding manner (~0.5 – 0.6 mol [^{3}H]cGMP/mol PDE) for both nPDE-M and

nPDE-S. We conclude that the Pγ subunit acts to reduce the rate of cGMP dissociation from exchangeable noncatalytic sites on both nPDE-M and nPDE-S.

Activation of PDE by limited proteolysis of the Pγ subunit alters the cGMP binding properties of PDE—Since addition of Pγ reduces the amount of endogenous cGMP released as well as the amount of [^{3}H]cGMP that could associate with the noncatalytic sites of nPDE-M or nPDE-S (Fig. 1.2), we reasoned that removal of the Pγ subunits from the holoenzyme would enhance the ability of cGMP to exchange between the bound and free state. To test this, we exposed a preparation of purified nPDE-S to mild trypsin proteolysis (Hurley and Stryer, 1982) for various times at 4°C. As described in the "Experimental Procedures," this treatment degrades the Pγ subunit and relieves inhibition of catalysis at the active site, with no detectable effect on the apparent molecular weights of Pδ or Pαβ on SDS polyacrylamide gels (Fig. 1.1). We then measured the ability of 1 μM [^{3}H]cGMP to occupy the noncatalytic sites, as well as the maximum hydrolytic rate of tPDE-S. Fig. 1.3 shows that the time course of activation by trypsin correlated well with the ability to bind up to 1 mol [^{3}H]cGMP per catalytic dimer. [Identical trypsin time course experiments performed on nPDE-M gave essentially identical results to those shown for nPDE-S in Fig. 1.3 (*data not shown*).] Separate measurements by radioimmunoassay of the endogenous cGMP remaining bound to fully activated tPDE-S at the time when the [^{3}H]cGMP was added (< 0.3 cGMP/PDE) indicated that >85% of the total bound cGMP had dissociated and been degraded by the activated enzyme. Therefore, the inability to bind more than 1 mol cGMP per mol PDE could not be due to endogenous bound cGMP preventing [^{3}H]cGMP association. One possible conclusion from this result is that the proteolytic destruction of Pγ had lowered

(below the level of detection by membrane filtration) the binding affinity for cGMP at only one of the two noncatalytic sites. Another possibility we considered was that trypsin had damaged the catalytic subunits so that high affinity cGMP binding at one of the noncatalytic sites was permanently lost.

To distinguish these possibilities, we prepared purified catalytic dimers of PDE-S (Pαβ-S) from which all traces of the low molecular weight subunits had been removed (see "Experimental Procedures" and Fig. 1.1); this Pαβ-S preparation also lacked detectable amounts of endogenous cGMP at the noncatalytic sites. Purified Pαβ-S was able to bind 1 mol cGMP per mol PDE in the absence of Pγ, in agreement with the results with tPDE-S in Fig. 1.3. Following addition of increasing amounts of Pγ to Pαβ-S, we observed a progressive increase in [^{3}H]cGMP binding that correlated with the inhibition of hydrolysis at the active site (Fig. 1.4). Addition of 2 mol Pγ per mol Pαβ-S permitted an additional 0.9 mol [^{3}H]cGMP per mol Pαβ-S to bind, and inhibited 90% of the hydrolytic activity of the enzyme. [Identical results were obtained upon titration of Pαβ-M with Pγ (*data not shown*).] In none of our experiments did we detect an effect of adding Pδ on cGMP binding to high affinity sites on Pαβ-S or Pαβ-M.

Several interesting conclusions can be drawn from the above results. First, purified Pαβ dimers derived from PDE-S and PDE-M are able to bind 1 mol cGMP per Pαβ in the complete absence of Pγ, Pδ or their proteolytic fragments. Second, it follows that removal of Pγ from PDE reduces the binding affinity of cGMP for one of the two noncatalytic sites below the level of detection of binding assay (K_D > 1 μM). Third, the proteolytic treatment of Pαβ is reversible upon addition of stoichiometric amounts of Pγ.

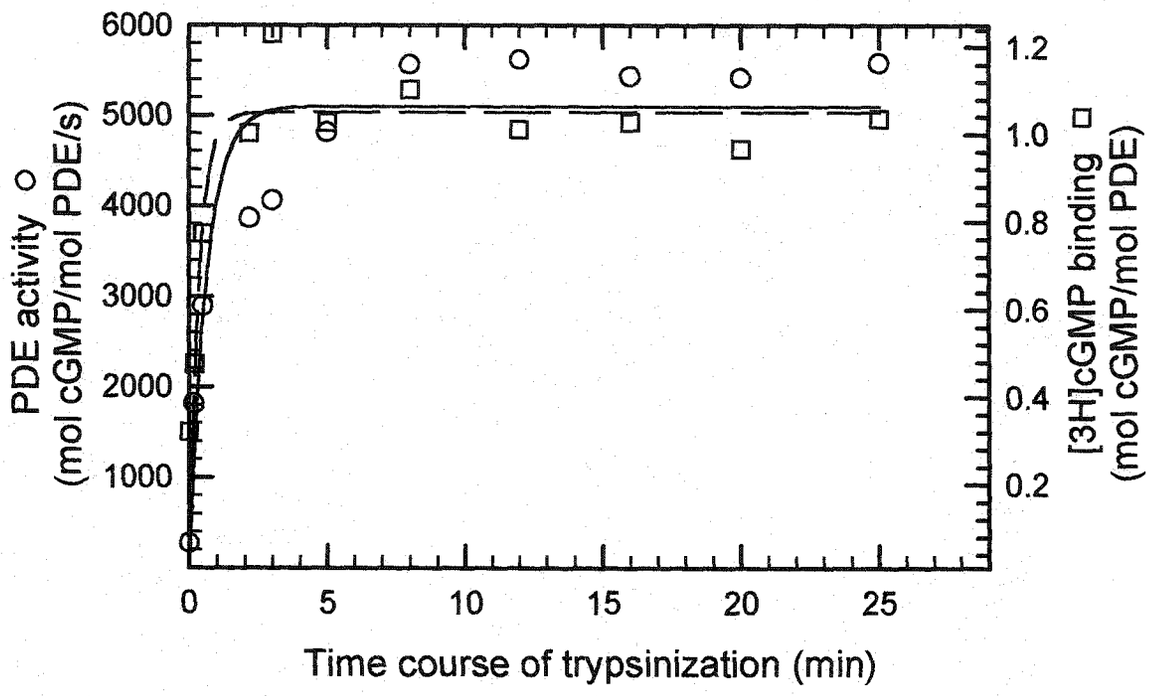

Figure 1.3. **Activation of nPDE-S by limited proteolysis follows the same time course as ability to bind [^{3}H]cGMP to one noncatalytic site on PDE.** Purified nPDE-S was incubated with trypsin (45 μg/ml) at 4°C as described in "Experimental Procedures." At the indicated times, portions were treated with soybean trypsin inhibitor to stop proteolysis, and the tPDE-S (590 nM) was then incubated at 37°C for 30 min to permit release and degradation of any rapidly exchangeable cGMP. The enzymatic activity (using 2 mM cGMP as substrate; circles) or ability to bind 1 μM [^{3}H]cGMP (5 min at 37°C, to minimize binding to non-activated sites; squares] were determined as described in the "Experimental Procedures." These data points are from one of three representative experiments, and the curves represent a fit to a single exponential model.

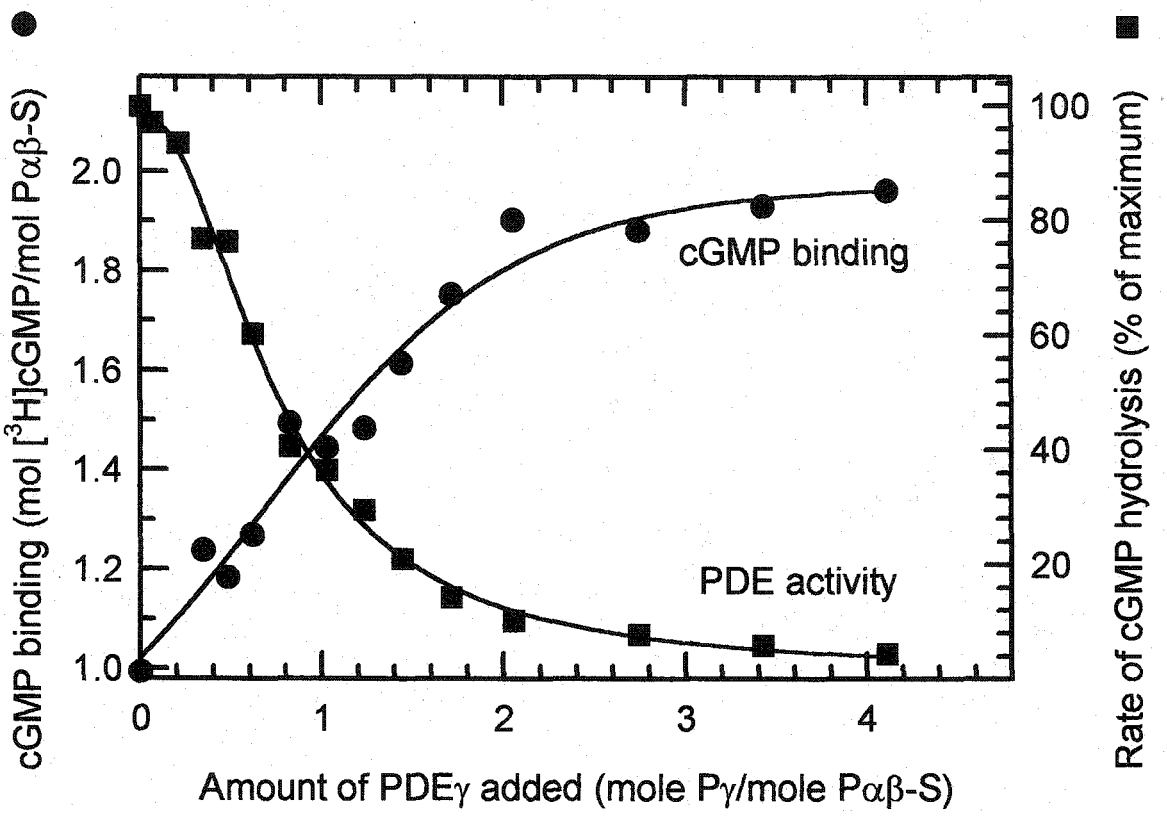

Figure 1.4. **Addition of stoichiometric amounts of Pγ is sufficient to restore cGMP binding to the second high affinity noncatalytic binding site of Pαβ-S.** Purified Pαβ-S was first depleted of endogenous cGMP by incubation at 37°C (<0.1 mol cGMP bound/mol Pαβ-S), as described in "Experimental Procedures." The indicated amounts of purified Pγ were incubated with Pαβ-S for 30 min at 4°C prior to commencing activity (1.4 nM PDE) and binding assays (5 nM PDE). The hydrolytic activity was measured with 10 mM cGMP as substrate and normalized to the rate in the absence of added Pγ (5240 mol cGMP hydrolyzed/mol PDE/s). 1 μM [^{3}H]cGMP was incubated for 30 min at 37°C prior to filter binding. The symbols represent individual data points from one of three similar experiments.

This shows that the Pγ subunit is the major target of action of trypsin, and that the limited proteolysis does not destroy the ability of Pαβ to bind cGMP at the "low affinity" site if Pγ is added back. Finally, in contrast to the two non-identical cGMP binding sites on Pαβ, no difference in binding affinity of Pγ to the two Pγ binding sites on Pαβ could be discerned for either PDE-S or PDE-M. We infer this from the observation that addition of 1 Pγ per Pαβ restores approximately one-half of the total cGMP binding that requires Pγ binding to Pαβ.

Kinetic and equilibrium analysis of cGMP binding to noncatalytic sites on PDE-S and PDE-M—To better understand the regulation of cGMP binding at the two non-identical binding sites on the soluble and membrane-associated rod PDE isoforms, we analyzed the kinetics and equilibrium binding of cGMP to both nPDE and Pαβ preparations for both PDE-S and PDE-M. Equilibrium binding measurements were performed at 37°C over the range of 2 to 400 nM cGMP for each PDE preparation, and the K_D and B_{max} determined (Fig. 1.5; see Table 1.1 for average values from several experiments). In all cases, the equilibrium binding data fit a model for a single class of non-interacting binding sites, even in the case of nPDE-S where the stoichiometry of binding indicated 2 binding sites per holoenzyme (Table 1.1). For the case of PDE-S, limited trypsin proteolysis of the enzyme resulted in the loss of detectable cGMP binding at one of the noncatalytic sites, and a 4-fold reduction in cGMP binding affinity at the remaining site. For the case of nPDE-M where only one site is available to exchange cGMP (the other retaining bound endogenous cGMP; Fig. 1.2), trypsinization reduces the binding affinity 3-fold; note, however, that we cannot distinguish whether the

exchangeable or nonexchangeable site is responsible for the cGMP binding we detect in activated Pαβ-M.

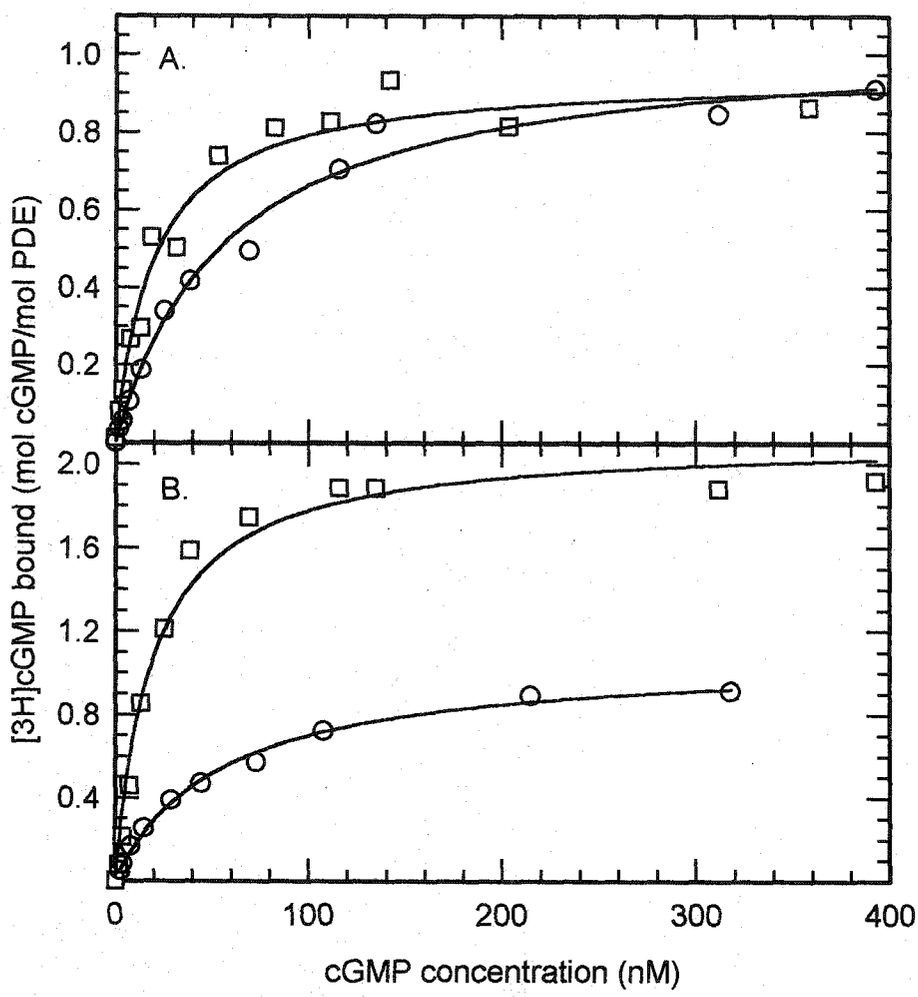

Figure 1.5. **Equilibrium binding of cGMP to nonactivated and activated rod PDE isozymes.** Purified PDE preparations were first incubated at 37°C to remove exchangeable, bound cGMP, as described in "Experimental Procedures." Except for nPDE-M (~1 mol endogenous cGMP bound/mol PDE), each PDE preparation retained < 0.1 mol endogenous cGMP/mol PDE following nucleotide depletion. Each preparation was diluted to 5 nM (final concentration) in Buffer B, and incubated with the indicated concentrations of [^{3}H]cGMP at 37°C for 30 min prior to membrane filtration. Curves represent the fit to a single-site binding model. A. Binding of nPDE-M (squares), K_D = 20 nM and B_{max} = 0.95 mol cGMP/mol PDE; binding to Pαβ-M (circles), K_D = 58 nM and B_{max} = 1.04 mol cGMP/mol PDE. B. Binding of nPDE-S (squares), K_D = 19 nM, B_{max} = 2.12 mol cGMP/mol PDE; binding to Pαβ-S (circles), K_D = 54 nM and B_{max} = 1.08 mol cGMP/mol PDE. The symbols represent individual data points from a single experiment; see Table I for average values for n determinations.

It is evident from comparing the K_D values of the two isoforms that the single exchangeable noncatalytic binding site we detect in nPDE-M has the same binding affinity for cGMP as the 2 sites that bind [^{3}H]cGMP in nPDE-S. Likewise, trypsin activation of each isoform results in a loss of detectable binding to one of the two cGMP binding sites per Pαβ, and a uniform decrease in binding affinity (K_D ~ 60 nM) of cGMP at the other site. These results strongly suggest that PDE-M and PDE-S have very similar or identical cGMP binding sites on the Pαβ dimer. Further, association of Pγ and Pδ with Pαβ each have distinct effects in modulating cGMP binding to the two noncatalytic sites of PDE.

Kinetic analysis of the association and dissociation kinetics of cGMP binding to PDE provides an additional approach for understanding how cGMP binding to the noncatalytic sites is regulated. Fig. 6 shows that when the Pγ and Pδ subunits are removed from the catalytic dimer to form Pαβ-S, both the association and dissociation rates of cGMP with the one remaining high affinity noncatalytic site are accelerated . The association rate constant (k_{+1}) is increased 6-fold upon removal of Pγ and Pδ from Pαβ-S, while the dissociation rate constant (k_{-1}) increases 16-fold (Table 1.1). Kinetic analysis of the noncatalytic sites on PDE-M (Table 1.1) is in general agreement with the results obtained with PDE-S. Upon removal of Pγ, the k_{+1} of Pαβ-M for the one high affinity binding site is increased 4-fold compared to nPDE-M, while the k_{-1} is increased 17-fold by Pγ removal. The similar kinetic behavior of PDE-M and PDE-S indicates that the binding of Pγ--rather than Pδ--is the major factor affecting the ability of cGMP to bind to and dissociate from the noncatalytic sites on both PDE-S and PDE-M. Furthermore, the interaction of Pγ with Pαβ has its greatest effect on the cGMP dissociation rate constant,

rather than the k_{+1} or the K_D. This is intriguing since it is the rate of cGMP dissociation from noncatalytic sites on PDE that is most relevant to proposed physiological roles of these sites in regulating PDE during recovery or light adaptation (see Discussion).

While we cannot measure detectable [^{3}H]cGMP binding to the second noncatalytic site on Pαβ with our filter binding assay, some minimum values of the binding parameters can be inferred. The time required for filtration (~4 sec) sets a limit to highest dissociation rate constant that can be measured (Cote, 2000). Since the half-time for cGMP dissociation from the higher affinity site on Pαβ-S or Pαβ-M is ~100 sec (Table 1.1), the lower affinity site must have a dissociation rate at least 25-fold larger in order for any bound cGMP to be released (and therefore not detected) during the filtration process. This implies that the K_D for the lower affinity site is likely to be >25-fold larger (>1.5 μM) than the value measured for Pαβ-S and Pαβ-M. We conclude that activation of PDE reduces the binding affinity at one site only 3-4-fold, while the other binding site undergoes a >25-fold reduction in affinity with a concomitant acceleration of the cGMP dissociation rate.

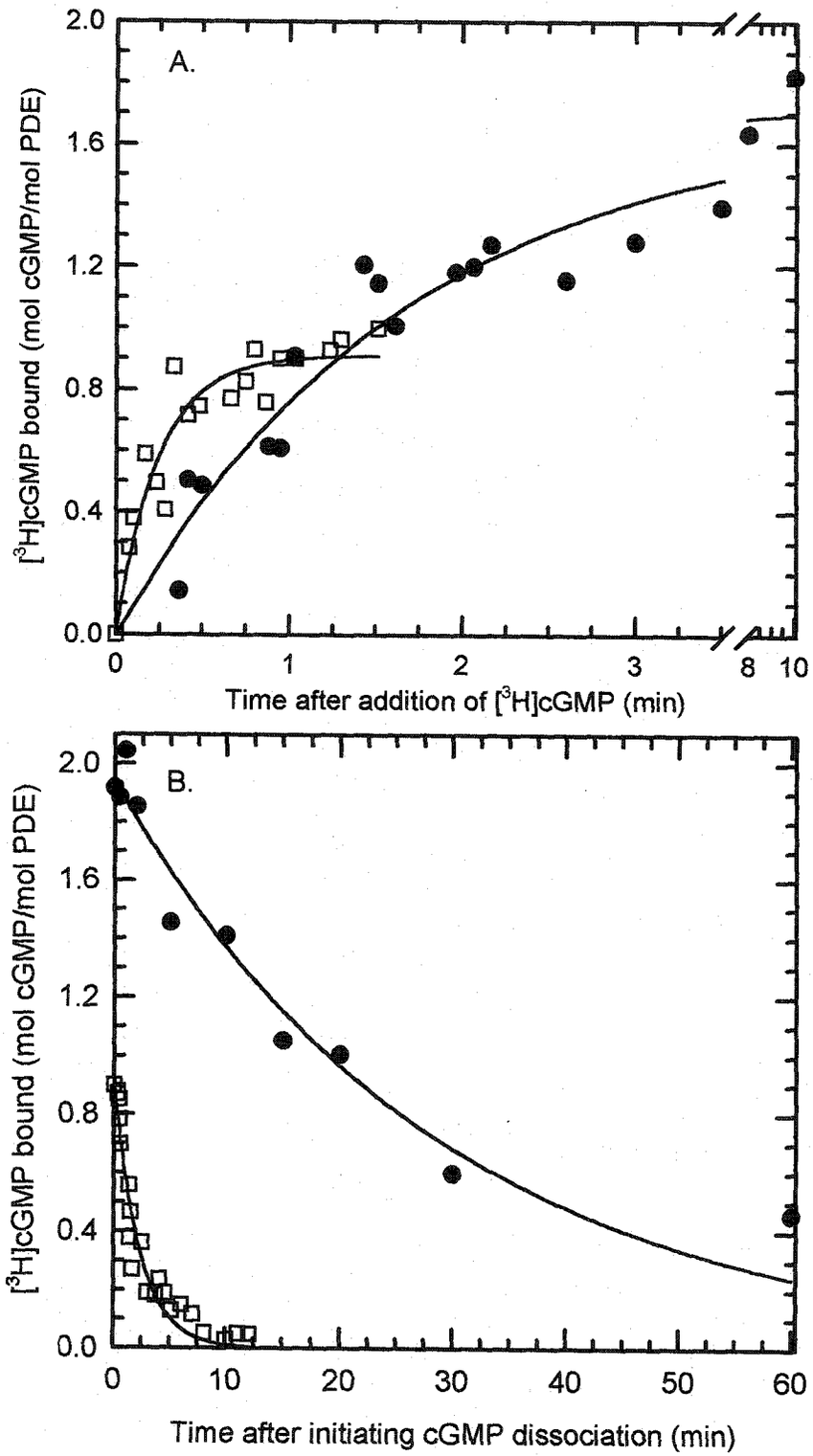

Figure. 1.6. **The kinetics of cGMP association and dissociation to PDE-S are accelerated upon enzyme activation.** Purified nPDE-S and Pαβ-S were depleted of their endogenous nucleotides by incubation at 37°C for 16 and 2 h, respectively. A. Association kinetics were obtained at 37°C by adding ~500 nM [^{3}H]cGMP to 8 nM nPDE-S (circles) or Pαβ-S (squares) at time zero, and filtering samples at the indicated times. The data were analyzed as a pseudo-first order reaction with the association rate constant $k_{+1} = (k_{obs} - k_{-1})/[cGMP]_{tot.}$, where k_{obs} is the first-order rate constant for the single exponential, k_{-1} is the dissociation rate constant (determined in part B.), and $[cGMP]_{tot.}$ is the total [^{3}H]cGMP concentration. The data fit a single exponential function for both nPDE-S ($k_{obs} = 9.8 \pm 1.0 \times 10^{-3}$ sec^{-1}; $B_{max} = 1.7 \pm 0.1$ [^{3}H]cGMP bound/PDE) and Pαβ-S ($k_{obs} = 0.068 \pm 0.011$ sec^{-1}; $B_{max} = 0.9 \pm 0.1$ [^{3}H]cGMP/PDE). B. Dissociation kinetics were determined at 37°C by first adding 500 nM [^{3}H]cGMP to ~6 nM nPDE-S (circles) or Pαβ-S (squares) and incubating for 30 or 2 min, respectively. Dissociation was initiated by addition of 1 mM (final concentration) unlabeled cGMP, and samples were filtered at the indicated times thereafter. The curves represent the fit of the data to a single exponential decay function for both nPDE-S ($k_{-1} = 5.8 \pm 0.6 \times 10^{-4}$ s^{-1}; $B_{max} = 1.9 \pm 0.1$ cGMP/PDE) and Pαβ-S ($k_{-1} = 7.3 \pm 0.7 \times 10^{-3}$ s^{-1}; $B_{max} = 0.9 \pm 0.1$ cGMP/PDE). The symbols represent individual determinations from one experiment; see Table I for average values for n separate determinations of the rate constants.

TABLE 1.1

Comparison of the [^{3}H]cGMP binding properties of bovine rod PDE isoforms

The experimental conditions for the equilibrium binding measurements and the kinetics measurements are provided in the legends to Figs. 1.5 and 1.6, respectively. The data represents the mean (± S.E.M.); the value in parentheses is the number of independent determinations.

PDE[a]	Max. [^{3}H]cGMP binding (cGMP/PDE)	Measured K_D (nM)	Dissociation kinetics, k_{-1} (sec^{-1})	Association kinetics k_{+1} ($M^{-1}sec^{-1}$)	Kinetic $K_D = k_{-1}/k_{+1}$ (nM)[c]
nPDE-M[b]	1.00 ± 0.04 (3)	18 ± 2 (3)	3.9 ± 0.1 x 10^{-4} (3)	2.2 ± 0.6 x 10^4 (4)	18
Pαβ-M	1.02 ± 0.04 (4)	56 ± 4 (4)	6.8 ± 1.0 x 10^{-3} (5)	9.8 ± 1.9 x 10^4 (8)	71
nPDE-S	1.85 ± 0.09 (4)	14 ± 2 (4)	4.5 ± 0.9 x 10^{-4} (5)	2.3 ± 0.4 x 10^4 (4)	20
Pαβ-S	0.93 ± 0.03 (3)	57 ± 13 (3)	7.2 ± 0.4 x 10^{-3} (5)	1.3 ± 0.2 x 10^5 (4)	55

[a]nPDE refers to PDE holoenzyme containing the low molecular subunits; Pαβ refers to activated PDE catalytic dimer lacking Pγ and/or Pδ. The -M and -S suffix refer to membrane-associated and soluble forms of PDE, respectively.

[b]nPDE- M has one nonexchangeable site (Fig. 1.2) which cannot be detected in these experiments.

[c]The kinetic K_D is in general agreement with the measured K_D, indicating the internal consistency of the equilibrium binding and kinetic data.

DISCUSSION

We have shown that mammalian rod photoreceptor PDE isoforms can undergo substantial changes in cGMP binding to two non-identical, high affinity noncatalytic sites upon activation of the enzyme by removal of the Pγ subunits. We also report that the soluble and membrane-associated isoforms of bovine rod PDE have different binding properties in their nonactivated states. Finally, our results demonstrate that the Pγ subunit is primarily responsible for altering cGMP exchange upon activation, and that the Pδ subunit may interact with Pγ binding to the enzyme to permit cGMP exchange at a site on non-activated PDE that is nonexchangeable in PDE-M but exchangeable in PDE-S.

The two high affinity noncatalytic binding sites on bovine rod PDE are not identical—Previous work established that the PDE-M holoenzyme contains 2 high affinity cGMP binding sites that retain bound cGMP even following extensive purification (Gillespie and Beavo, 1989a). We have extended that work to demonstrate that the soluble isoform of rod PDE also contains 2 high affinity cGMP binding sites. Of greater significance is the demonstration that the two noncatalytic sites are not identical in their cGMP binding properties. The presence of one functionally nonexchangeable cGMP binding site on nonactivated PDE-M is in marked contrast to the two exchangeable sites found in bovine rod PDE-S (Fig. 1.2), cone PDE (Gillespie and Beavo, 1988), or the amphibian membrane-associated rod PDE (Cote et al., 1994).

Activation of bovine rod PDE reveals a fundamental difference between the two non-identical noncatalytic sites on both bovine rod isoforms. Removal of the Pγ subunits from PDE-M or PDE-S eliminates detectable binding ($K_D > 1$ μM) of cGMP to one of the two classes of noncatalytic sites on the Pαβ dimer. This effect represents a shift of ≥

100-fold in overall cGMP binding affinity at this site, and a rate of dissociation that is too fast to detect by filter binding ($t_{1/2} < 4$ s). The remaining cGMP binding sites exhibit a 3-fold reduction in overall binding affinity upon removal of Pγ compared to the [^{3}H]cGMP binding sites on nPDE-M or nPDE-S. We conclude that the Pγ subunit not only acts in its well-known role as an inhibitor of cyclic nucleotide catalysis at the active sites on Pαβ, but also serves to regulate the affinity of interaction of cGMP with the two distinct noncatalytic sites on the Pαβ dimer. The amphibian PDE, in contrast, shows no detectable cGMP binding to Pαβ when the Pγ subunits are removed by limited proteolysis (Yamazaki et al., 1980; Yamazaki et al., 1982; Yamazaki et al., 1982) or by extraction with activated transducin (Cote et al., 1994).

No intrinsic differences in the catalytic Pαβ dimers derived from PDE-S and PDE-M—Based on the results of this study and previous work (Gillespie et al., 1989; Florio et al., 1996; Florio et al., 1996), it appears very likely that the catalytic subunits are identical in PDE-S and PDE-M. The molecular weights of α and β are indistinguishable on SDS polyacrylamide gels (Gillespie et al., 1989), the catalytic constant of tPDE-S is identical to that of tPDE-M [see "Experimental Procedures" and ref. (Gillespie et al., 1989)], and the noncatalytic cGMP binding sites show very similar kinetic and equilibrium binding behavior for the purified Pαβ dimers derived from PDE-S and PDE-M (Figs. 5 – 6 and Table I).While a definitive statement awaits direct amino acid sequence comparisons of the α and β subunits of PDE-S and PDE-M, all available evidence indicates that binding of the Pδ subunit to the PDE holoenzyme confers the differences in behavior seen with nPDE-S and nPDE-M.

Two distinct actions of Pδ on PDE—We report a new function that can be ascribed to the binding of the Pδ subunit to rod PDE: the ability of Pδ to permit cGMP release from a second noncatalytic binding site on nPDE-S that remains functionally nonexchangeable in PDE-M. How this effect of Pδ on PDE-S relates to the other known function of Pδ—namely to solubilize PDE that would otherwise remain attached to rod outer segment disk membranes (Florio et al., 1996)—is not clear at present. However, membrane association of PDE is not required for Pδ binding, since purified nPDE-M to which recombinant Pδ has been added is able to exchange endogenous cGMP at both noncatalytic sites in the absence of membrane attachment.

Physiological significance of noncatalytic cGMP binding sites on mammalian rod PDE isoforms—In the dark-adapted photoreceptor, cytoplasmic cGMP levels are in the low micromolar range, and both noncatalytic sites on nPDE-S and nPDE-M will be fully occupied. The mechanism by which transducin activates PDE to rapidly lower cytoplasmic cGMP levels during visual excitation is well understood, and quantitatively accounts for the rising phase of the photoresponse (for review, see (Olson and Pugh, Jr., 1993)). Thus, there is no need to invoke the noncatalytic cGMP binding sites on bovine rod PDE to describe the regulation of PDE during the initial events of photo-excitation. We consider instead possible ways the noncatalytic cGMP binding sites may be involved in later stages of the visual transduction pathway, namely recovery from light stimulation and/or light adaptation.

A role for the noncatalytic sites to buffer free cGMP levels during the recovery phase of the flash photoresponse has been proposed for amphibian rod photoreceptors (Yamazaki et al., 1996). In this model, the noncatalytic sites serve to buffer cytoplasmic

cGMP levels in the dark, and upon PDE activation release bound cGMP to accelerate the return to the resting state. Our data show that activation of mammalian rod PDE by removing the Pγ subunit results in the immediate loss of detectable binding to one of the noncatalytic sites on both PDE-M and PDE-S. This rapidly dissociating class of sites has the potential to release its bound cGMP on the same time scale as recovery phase of the photoresponse (<1 sec in mammalian photoreceptors (Baylor et al., 1984; Lyubarsky and Pugh, Jr., 1996; Lyubarsky and Pugh, Jr., 1996)). However, upon cGMP release from the bound state, it is likely that the free cGMP would be destroyed by the still-active catalytic sites, and therefore not contributes to elevating the cytoplasmic cGMP levels during photoresponse recovery. This conclusion is supported by recent measurements of cGMP dissociation and hydrolysis in amphibian rod outer segment suspensions (Calvert et al., 1998).

An alternative hypothesis is that the noncatalytic sites on PDE may indirectly regulate the lifetime of transducin-activated PDE via modulation of Pγ binding affinity to Pαβ (Cote et al., 1994). This idea is based on the following observations in amphibian and mammalian photoreceptors: removal of bound cGMP from the noncatalytic sites in frog ROS reduces Pγ binding affinity to Pαβ (Arshavsky et al., 1992) and correlates with an accelerated transducin GTPase activity (Arshavsky and Bownds, 1992); in bovine ROS, Pγ serves as a GTPase accelerating factor (Angleson and Wensel, 1994; Arshavsky et al., 1994; Arshavsky et al., 1994) acting in concert with the RGS9 protein (He et al., 1998; Tsang et al., 1998; Tsang et al., 1998). Accordingly, cGMP dissociation from the rapidly dissociating class of sites on activated PDE might signal Pγ to interact with RGS9

to stimulate GTPase activity of transducin to inactivate PDE during the recovery phase of the photoresponse.

cGMP dissociation from the second, high affinity class of binding sites on bovine rod PDE is too slow ($t_{1/2}$ ~ 1.6 min; Table 1) to be implicated in the recovery process of the dark-adapted photoresponse. It is possible that cGMP dissociation from these noncatalytic sites may act to gradually reduce the lifetime of activated PDE during adaptation to continuous illumination, and thereby lead to the smaller, faster responses characteristic of the light-adapted state. Coles and Yamane (Coles and Yamane, 1975) and Cervetto et al. (Cervetto et al., 1984) have shown a gradual acceleration of recovery of the flash photoresponse when amphibian rod photoreceptors are exposed to continuous illumination; this response acceleration slowly develops over the same general time frame as cGMP dissociation from the higher affinity noncatalytic sites on PDE. Future work is needed to establish whether a strict temporal correlation exists between occupancy of cGMP at the noncatalytic sites and the kinetics of the recovery phase of the light-adapted photoresponse, and whether these studies performed with lower vertebrates are relevant to the regulation of mammalian rod phototransduction.

In conclusion, cGMP binding and dissociation at the noncatalytic sites on the photoreceptor PDE may indirectly regulate the enzyme by affecting the strength of the interaction of the Pγ subunit with the catalytic subunits. This in turn may direct whether Pγ interacts with the Pαβ dimer to regulate catalytic activity, or with transducin and its GTPase accelerating protein, RGS9, to regulate the kinetics of transducin inactivation. In addition, there is conflicting evidence at present (Arshavsky et al., 1992; Granovsky et al., 1998; Granovsky et al., 1998) as to whether the noncatalytic sites can allosterically

regulate hydrolytic activity at the active sites, as has been demonstrated for PDE2 (Martins et al., 1982). The results in this paper provide a strong foundation for unraveling the complex interplay between the noncatalytic and catalytic sites on photoreceptor PDE and the low molecular weight Pγ and Pδ subunits that interact with and regulate the action of this central effector enzyme in visual transduction.

Chapter II

The catalytic and GAF domains of the rod cGMP phosphodiesterase (PDE6) heterodimer are regulated by distinct regions of its inhibitory γ subunit*

ABSTRACT

The central effector of visual transduction in retinal rod photoreceptors, cGMP phosphodiesterase (PDE6), is a catalytic heterodimer (αβ) to which low molecular weight inhibitory γ subunits bind to form the nonactivated PDE holoenzyme ($\alpha\beta\gamma_2$). While it is known that γ binds tightly to αβ, the binding affinity for each γ subunit to αβ, the domains on γ that interact with αβ, and the allosteric interactions between γ and the regulatory and catalytic regions on αβ, are not well understood. We show here that the γ subunit binds to two distinct sites on the catalytic αβ dimer ($K_{D1} < 1$ pM, $K_{D2} = 3$ pM) when the regulatory GAF domains of bovine rod PDE6 are occupied by cGMP. Binding heterogeneity of γ to αβ is absent when cAMP occupies the noncatalytic sites. Two major domains on γ can interact independently with αβ, with the N-terminal half of γ binding with 50-fold greater affinity than its C-terminal, inhibitory region. The N-terminal half of γ is responsible for the positive cooperativity between γ and cGMP binding sites on αβ, but has no effect on catalytic activity. Using synthetic peptides, we identified regions of the amino acid sequence of γ that bind to αβ, restore high-affinity cGMP binding to low-

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affinity noncatalytic sites, and retard cGMP exchange with both noncatalytic sites. Subunit heterogeneity, multiple sites of γ interaction with $\alpha\beta$, and positive cooperativity of γ with the GAF domains are all likely to contribute to precisely controlling the activation and inactivation kinetics of PDE6 during visual transduction in rod photoreceptors.

Station. This work was supported by National Institutes of Health Grant EY-05798 (to R.H.C.).

INTRODUCTION

The extent and lifetime of activation of the photoreceptor cGMP phosphodiesterase (PDE6[1]; E.C. 3.1.4.35) must be precisely regulated in rod and cone cells in order to control the exquisite sensitivity, speed, and adaptational properties of the visual transduction pathway in vertebrate photoreceptors. The membrane-associated rod photoreceptor PDE6 consists of a dimer of two homologous catalytic subunits (Pαβ) to which two low molecular weight inhibitory subunits (Pγ) bind (holoenzyme stoichiometry: $\alpha\beta\gamma_2$). The catalytic subunits contain GAF domains which are responsible for high-affinity, noncatalytic binding of two cGMP molecules per holoenzyme. It is well established that relief of the inhibitory constraint on PDE6 arises from the binding of activated heterotrimeric G-protein (transducin) to Pγ following photoactivation of the visual pigment, rhodopsin (reviewed in refs. (Pfister et al., 1993; Pugh, Jr. and Lamb, 1993; Bownds and Arshavsky, 1995; Artemyev et al., 1998; Pugh, Jr. and Lamb, 1993; Bownds and Arshavsky, 1995; Artemyev et al., 1998)). However, the strength of the interaction between Pγ and Pαβ has been difficult to quantitate and K_D values vary widely [from picomolar (Wensel and Stryer, 1986; Hamilton et al., 1993; Skiba et al., 1995; Hamilton et al., 1993; Skiba et al., 1995) up to nanomolar values (Otto-Bruc et al., 1993; Yamazaki et al., 1996b; Yamazaki et al., 1996b)]. In addition, it has not been conclusively demonstrated whether both Pγ molecules bind with equal affinity to Pαβ to

[1] The abbreviations used are: PDE, cyclic nucleotide phosphodiesterase; PDE6, PDE from photoreceptor cells; Pαβ, catalytic heterodimer of rod PDE6; Pγ, inhibitory 10-kDa γ subunit of rod PDE6; α_t, α-subunit of rod photoreceptor G-protein, transducin; K_D, dissociation constant; k_{cat}, turnover number for activated PDE6, based on the Pαβ concentration; K_I, inhibition constant; RGS-9, regulator of G-protein signaling-9; LY, Lucifer Yellow.

form the nonactivated holoenzyme [although two different binding sites on Pαβ have been inferred using mutant Pγ; ref. (Berger et al., 1999)]. Finally, recent evidence suggests that binding of activated transducin to PDE6 relieves inhibition at only one of the two active sites, further supporting the idea of catalytic subunit heterogeneity with respect to Pγ binding (Melia et al., 2000; Norton et al., 2000; Norton et al., 2000).

Use of synthetic peptides to defined regions of Pγ, as well as mutagenesis of the Pγ subunit, have revealed that Pγ has multiple sites of interaction with rod Pαβ, with the α-subunit of activated transducin (α_t*), and with the Regulator of G-protein Signaling-9 (RGS-9). The C-terminal residues of Pγ (amino acids 77 – 87) have been shown to interact directly with the catalytic sites of Pαβ to inhibit catalysis (Skiba et al., 1995; Lipkin et al., 1988; Brown, 1992; Granovsky et al., 1997; Brown, 1992; Skiba et al., 1995; Granovsky et al., 1997). A second major site of interaction between Pγ and Pαβ has been identified in the lysine-rich central portion (amino acids 24-45) of the Pγ sequence (Lipkin et al., 1988; Artemyev and Hamm, 1992; Takemoto et al., 1992; Lipkin et al., 1993; Natochin and Artemyev, 1996; Artemyev and Hamm, 1992; Takemoto et al., 1992; Lipkin et al., 1993; Natochin and Artemyev, 1996), but the function of this interaction is unclear. α_t* has been shown to interact with Pγ at two distinct regions: one in its C-terminal domain (amino acid residues 65 – 87), and the second in the central, lysine-rich region of Pγ (Lipkin et al., 1988; Brown, 1992; Artemyev and Hamm, 1992; Artemyev et al., 1992; Slepak et al., 1995; Slep et al., 2001; Artemyev et al., 1992; Artemyev and Hamm, 1992; Brown, 1992; Slepak et al., 1995; Slep et al., 2001). Finally, Pγ also serves to potentiate the GTPase-activating protein function of RGS-9 by

interacting with the protein in the neighborhood of W70 (Slepak et al., 1995; Slep et al., 2001; Tsang et al., 1998; Tsang et al., 1998; Slep et al., 2001).

In this paper, we show that the K_D of Pγ binding to rod PDE6 is in the picomolar to sub-picomolar range. Furthermore, Pγ does not bind with equal affinity to the two sites on Pαβ when cGMP occupies the noncatalytic regulatory sites located on the Pαβ dimer. We also demonstrate that the central region of Pγ stabilizes high-affinity cGMP binding to the noncatalytic sites, and the affinity of the central region of Pγ exceeds by 50-fold the affinity of its C-terminal region. Finally, using a series of synthetic peptides, we identify important residues in the central region that contribute to the stabilization of Pγ binding to Pαβ.

EXPERIMENTAL PROCEDURES

Materials—Bovine retinas were purchased from W.L. Lawson, Inc. The Pγ mutant, Pγ1-45C [consisting of the first 45 amino acids of bovine rod Pγ plus a C-terminal cysteine residue; ref. (Skiba et al., 1996)] was a kind gift of Dr. N.P. Skiba, while zaprinast was generously supplied by Rhone-Poulenc Rorer (Dagenham, UK). Crude synthetic peptides were prepared at New England Peptide or at the protein facility at the University of New Hampshire. Radiochemicals were from PerkinElmer Life Sciences. Gel electrophoresis and immunoblotting supplies were from Bio-Rad Laboratories. Ultima Gold scintillation fluid was from Packard Instrument Co., filtration and ultrafiltration products were from Millipore, protein assay reagents were from Pierce Chemical Co., and all other chemicals were from Sigma.

Preparation of PDE6—Membrane-associated bovine rod PDE6 was purified to >90% homogeneity from frozen bovine retinas as described previously (Mou et al., 1999). The resulting nonactivated PDE holoenzyme (subunit stoichiometry: $\alpha\beta\gamma_2$) was stored in 50% glycerol at –20°. The PDE6 catalytic heterodimer (Pαβ) was prepared by limited proteolysis of the Pγ subunits, followed by Mono Q ion-exchange chromatography to remove proteolytic fragments of Pγ (Mou et al., 1999). The Pαβ preparation was >95% pure as judged from Coomassie-stained SDS-PAGE. No shift in the apparent molecular weight of the α or β subunit was observed following this treatment. [Attempts to prepare Pαβ without resorting to proteolytic digestion of Pγ were unsuccessful.] Immunoblot analysis with an anti-peptide Pγ antibody (UNH9710) directed to the C-terminal region (amino acids 63-87) of Pγ revealed no detectable full-length Pγ; a 5 kDa band representing the major proteolytic product of Pγ exhibited <5% of the original Pγ

immunoreactivity (Mou et al., 1999; Cote, 2000; Mou et al., 1999). To remove endogenous cGMP bound to the noncatalytic regulatory sites on Pαβ, the Pαβ was incubated at 37° for 30 min prior to use.

Preparation and purification of Pγ and Pγ1-45C—Full-length bovine rod Pγ or the N-terminal fragment, Pγ1-45C, was expressed in E. coli using the pET11a expression vector (Slepak et al., 1995) and purified as described previously (Artemyev et al., 1998; Mou et al., 1999; Mou et al., 1999). The total Pγ concentration was initially determined spectrophotometrically (ε_{277} = 7550 cm M^{-1}), and verified by assaying its inhibitory activity (Cote, 2000); these two estimates varied by <10%. The concentration of Pγ1-45 was measured with a colorimetric protein assay (Smith et al., 1985). To fluorescently label Pγ1-45C with Lucifer Yellow (Pγ1-45C-LY), the procedure of Artemyev et al. (Artemyev et al., 1992) was used, followed by purification of Pγ1-45C-LY by reversed-phase high-pressure liquid chromatography.

Peptide purification—Following automated peptide synthesis, peptides were cleaved from the resin with anhydrous HF and lyophilized. Crude, acidic peptides (Pγ55-75 and Pγ63-87) were first purified by anion-exchange chromatography using DEAE Sephadex A-25 and a linear NaCl gradient at pH 7.5. Other peptides were initially purified by cation exchange chromatography on CM Sephadex C25 using a linear NH_4HCO_3 gradient at pH 8.0. All peptides were further purified by reversed-phase high-pressure liquid chromatography on a 22 x 250 mm, 300 angstrom C_{18} column (Vydac), using a linear gradient of 30 – 70% acetonitrile in 0.1% trifluoroacetic acid. After lyophilization, each peptide was resuspended in 10 mM Tris, pH 7.5, and its concentration determined

with the bicinchoninic acid protein assay (Smith et al., 1985) using bovine serum albumin as a standard.

Analytical procedures and data analysis—The concentration of PDE and its catalytic activity were determined as described in detail elsewhere (Mou et al., 1999; Cote, 2000; Cote, 2000). Bovine serum albumin (250 μg/ml) was added when assaying enzyme concentrations in the sub-nanomolar range to prevent loss of activity. A membrane filtration assay was used to determine the equilibrium and kinetic properties of cGMP binding to high-affinity noncatalytic sites on PDE (Mou et al., 1999; Cote, 2000; Cote, 2000). The PDE inhibitor, zaprinast (0.1 – 1.0 mM), was included in the assay solution to insure that <10% hydrolysis of [^{3}H]cGMP occurred. Nonspecific binding was determined as described previously (Cote and Brunnock, 1993). The fluorescence assay of Pγ1-45C-LY binding to Pαβ was conducted in an Aminco-Bowman Series 2 spectrofluorimeter using an excitation wavelength of 430 nm and an emission wavelength of 520 nm (Artemyev et al., 1992).

Except where noted, experiments were performed three times, and the results presented as the mean ± standard error of the mean (SEM). Nonlinear regression analysis of the data was performed using Sigmaplot 2000 (SPSS, Inc.).

For the results in Figs. 1 and 2, two different curve-fitting equations were used to fit the Pγ binding data. The first equation represents the standard equilibrium binding equation for a single class of noninteracting sites:

$$B = \frac{B_{max} \cdot [P\gamma]}{K_D + [P\gamma]} \qquad (Eq.1)$$

where B is the amount of Pγ bound, B_{max} is the maximum extent of binding, K_D is the dissociation constant, and the free Pγ concentration, [Pγ], is assumed to be approximated by the total added Pγ concentration (i.e., $[P\gamma] \cong [P\gamma]_T$).

Because the free Pγ concentration was not experimentally measured, Eq. 1 can only be used to estimate the K_D when ligand depletion from solution is not significant. For high-affinity binding reactions, this condition is met when the total number of binding sites (P_T) is near the K_D. However, if we substitute the conservation of mass equation ($[P\gamma]_T = B + [P\gamma]$) into Eq. 1, we can estimate the K_D for any P_T without having to assume that $[P\gamma] \cong [P\gamma]_T$:

$$B = \frac{B_{max}}{P_T}\left(-x - \frac{\sqrt{x^2 - 4P_T \cdot [P\gamma]_T}}{2}\right) \qquad (Eq.2)$$

where, $x \equiv -K_D - P_T - [P\gamma]_T$. This analytical approach was previously used to study high-affinity binding of GTPγS to transducin (Malinski et al., 1996).

RESULTS

Quantitative analysis of Pγ binding to Pαβ—Given the >100-fold range of reported K_D values for Pγ binding to PDE6 in the literature (see Introduction), we suspected that stoichiometric binding of Pγ to Pαβ was occurring in those instances where the Pαβ concentration exceeded the intrinsic K_D value for the binding reaction. To directly test this, we examined the ability of Pγ to inhibit catalysis as a function of the Pαβ concentration (Fig. 2.1A). At Pαβ concentrations ≥ 50 pM, there was a linear increase in the extent of inhibition up to the plateau as the Pγ concentration was increased; the concentration of Pγ needed to attain >90% inhibition was approximately twice the concentration of Pαβ. This behavior represents a titration phenomenon in which added Pγ stoichiometrically binds to Pαβ with very high affinity until essentially all of the binding sites are occupied (2 mol Pγ per mol Pαβ). At Pαβ concentrations ≤ 10 pM, the inhibition curve departed from stoichiometric behavior, and an excess of Pγ was needed to fully inhibit the enzyme (Fig. 2.1A).

The Pγ binding data in Fig. 2.1A was analyzed using two equations for equilibrium binding of Pγ to Pαβ. The first approach (Eq. 1) assumed that the concentration of free Pγ, [Pγ], was approximately equal to the total added Pγ concentration, $[P\gamma]_T$ (i.e., no ligand depletion). When Eq. 1 was used to estimate the binding affinity of Pγ at each concentration of Pαβ tested, we found that the value of the apparent K_D increased in direct proportion to the concentration of Pαβ. (Fig. 2.1B, circles). For example, at 1 pM Pαβ, the calculated K_D for Pγ binding was 1.6 pM, while

at a concentration of 500 pM Pαβ, the curve-fitting to Eq. 1 gave an apparent K_D = 300 pM.

We re-examined the Pγ binding data with Eq. 2 (which is not constrained by the assumption that $[P\gamma] = [P\gamma]_T$) to estimate the apparent K_D for Pγ binding. Over the concentration range of 1 to 500 pM Pαβ, the calculated K_D showed little dependence on the Pαβ concentration. The convergence of the two estimates for the apparent K_D as the Pαβ concentration was lowered to 1 pM indicated that both Eq. 1 and Eq. 2 returned similar estimates when $P_T \leq K_D$ and when the free Pγ concentration could be approximated as the total Pγ concentration. We conclude that the most reliable condition for determining the binding affinity of Pγ is when the Pαβ concentration is in the low pM range.

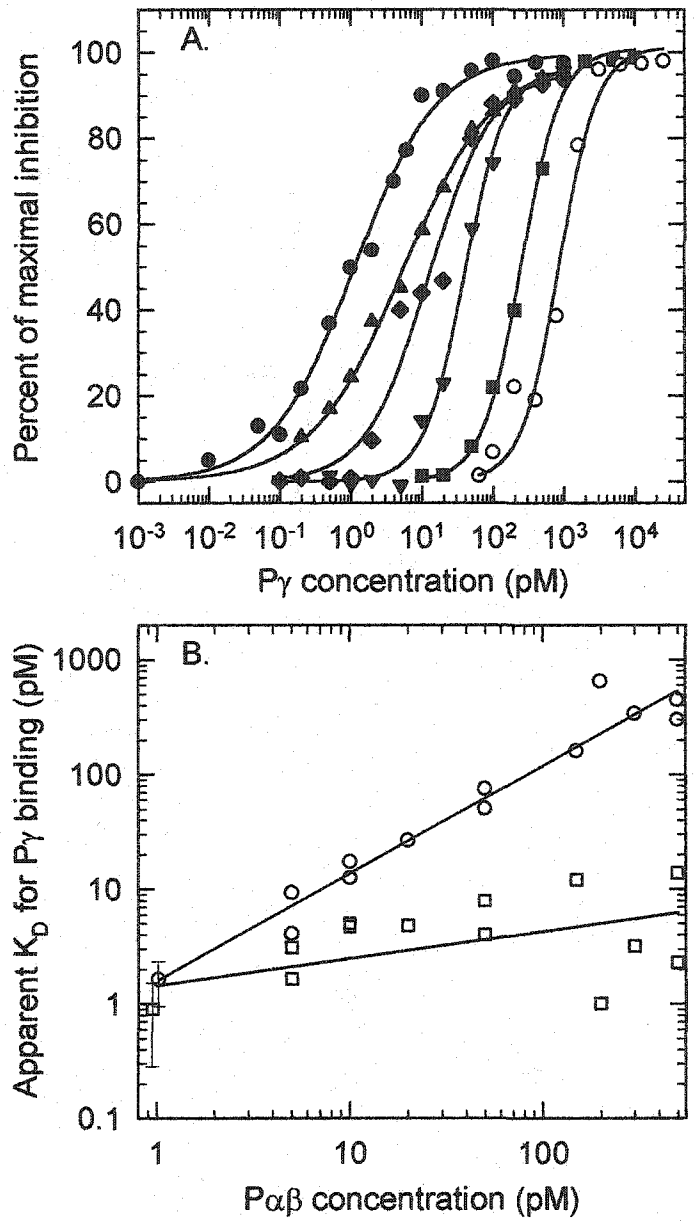


Figure 2.1. **Inhibition of catalytic activity by Pγ at various concentrations of Pαβ.** Pαβ and Pγ were prepared as described in *Experimental Procedures*. *A*. The ability of increasing amounts of Pγ to bind to and inhibit hydrolysis of cGMP was determined at the following Pαβ concentrations (in pM): 1.0 (●), 5.0 (▲), 10 (♦), 50 (▼), 200 (■), and 500 (○). The substrate concentration was 100 μM cGMP at ≤ 50 pM Pαβ or 2.0 mM at 200 and 500 pM Pαβ. The curves represent the fit of the data to Eq. 2. *B*. The affinity of Pγ binding to Pαβ (apparent K_D) was estimated using either Eq. 1 (○) or Eq. 2 (□) at each indicated Pαβ concentration. The points represent individual determinations except for 1 pM (mean ± SEM, n = 6).

Heterogeneity in Pγ binding to Pαβ—Closer examination of the results at the lowest Pαβ concentration we could easily test (1 pM) indicated that the two Pγ binding sites on Pαβ were not identical when we assayed Pγ inhibition of cGMP hydrolysis (Fig. 2.2, *circles*). When 1 pM Pαβ was incubated with up to 1 mol Pγ per mol Pαβ, stoichiometric binding of Pγ to Pαβ was observed. This is evident from the fact that the data fit a straight line for the first 50% of the binding curve (Fig. 2.2, *dotted line*). After binding 1.0 Pγ per Pαβ, the remaining Pγ binding sites required an excess of free Pγ in order fully inhibit Pαβ. Attempts to fit the entire binding curve to Eq. 1 (*upper dashed line*, Fig. 2.2) or Eq. 2 failed to generate statistically valid regression coefficients, so we fit the data to a modified form of Eq. 1 that included two independent classes of binding sites. This approach gave the best fit to the experimental data (Fig. 2.2, *solid line*), and resolved two classes of Pγ binding sites with K_D values of 0.3 and 7 pM. [The 0.3 pM value is an upper limit for the higher affinity K_D. Because this site exhibits titration behavior, the true binding affinity may be greater than the curve-fitting estimate.] Note that the data cannot distinguish between two independent classes of Pγ binding sites and a model in which the first Pγ to bind reduces the binding affinity of Pαβ for the second Pγ (negative cooperativity).

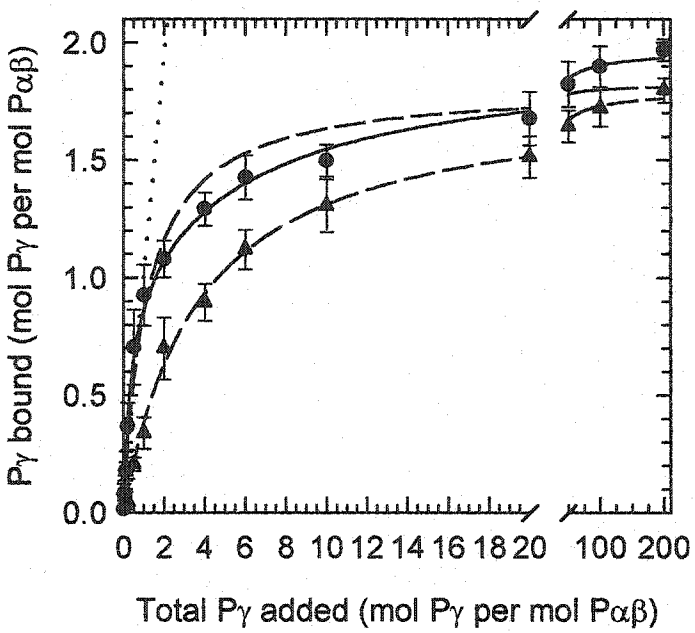


Fig. 2.2. **Pγ binding affinity at 1 pM Pαβ reveals two classes of binding sites in the presence of cGMP.** Pαβ (1.0 pM) was incubated with various concentrations of Pγ at 4° for 10 min, and then the extent of inhibition of cGMP (●, 100 μM concentration; n = 7) or cAMP (▲, 500 μM concentration; n = 6) hydrolysis was determined at 22°. The cAMP data was well fit as a single class of Pγ binding sites using either Eq. 1 (*dashed line*: $K_D = 3.7 \pm 0.3$ pM, $B_{max} = 1.80 \pm 0.03$ mol Pγ bound per mol Pαβ) or Eq. 2 ($K_D = 2.6 \pm 0.3$ pM, $B_{max} = 1.76 \pm 0.04$ Pγ per Pαβ; not shown). The cGMP data did not fit a single site model using either Eq. 1 (*dashed line*) or Eq. 2 (not shown), as judged by statistical analysis of the regression. For values of total added Pγ ≤ 1.0, the binding curve fit a linear model (*dotted line*), consistent with stoichiometric binding ($K_D < 1$ pM) of 1 mol Pγ per mol Pαβ in the presence of cGMP. A two-site model was also used (by extension of Eq. 1) to fit the entire data set (*solid line*), and resolved the binding curve into two classes of sites with $K_{D1} = 0.3 \pm 0.1$ pM and $K_{D2} = 6.6 \pm 2.3$ pM.

cGMP binding to noncatalytic sites on Pαβ is responsible for Pγ binding heterogeneity— We have previously demonstrated that bovine rod Pαβ (in the absence of Pγ) contains high- (K_D = 60 nM) and low-affinity (K_D > 1 μM) cGMP binding sites, and that the low-affinity site is restored to its high-affinity state upon Pγ addition (Mou et al., 1999). We hypothesized that the Pγ binding heterogeneity in Fig. 2.2 might result from heterogeneity in cGMP binding to the noncatalytic sites. To test this, we needed to measure Pγ binding when the noncatalytic sites are not occupied with cGMP. cAMP is an alternate substrate for PDE6 catalysis and binds with negligible affinity to amphibian PDE6 (K_D > 40 mM) (Hebert et al., 1998). Fig. 2.3 (*triangles*) demonstrates that cAMP is able to weakly bind to bovine Pαβ, as judged by its ability to compete with cGMP binding to the one high-affinity site present on Pαβ (Mou et al., 1999). Assuming simple competition between cGMP and cAMP binding, cAMP binds to the high-affinity noncatalytic site with a K_D ~ 200 μM.

When we re-examined the binding affinity of Pγ to Pαβ in the presence of 500 μM cAMP, we found that Pγ binds to Pαβ with a single K_D value of 3 pM (Fig. 2.2, *triangles*). No evidence of binding site heterogeneity or cooperativity was evident when Pγ interaction with Pαβ was assayed based on its inhibition of cAMP hydrolysis. This implies that cAMP binding to noncatalytic sites does not affect Pγ binding to Pαβ. In contrast, binding of cGMP to noncatalytic sites induces an allosteric transition that increases Pγ binding affinity ≥ 10-fold to one site on Pαβ. The other Pγ binding site maintains an affinity for Pαβ similar to the K_D observed when cAMP is present.

Lack of direct allosteric communication between the catalytic and noncatalytic sites on bovine Pαβ—Because cGMP binding induces a conformational change in Pαβ that enhances Pγ affinity, we hypothesized that the GAF domain might also allosterically regulate hydrolytic activity in the catalytic domain [in analogy to the cGMP-stimulated PDE, PDE2; ref. (Martins et al., 1982; Yamamoto et al., 1983; Yamamoto et al., 1983)]. To test this, we compared the kinetic parameters of Pαβ using cGMP or cAMP as the substrate. Table 2.1 shows that when cGMP is the substrate, Pαβ achieves a catalytic efficiency ($k_{cat}/K_M = 4x10^8\ M^{-1}s^{-1}$) approaching the diffusion-controlled limit for a bimolecular collision. PDE6 has a 100-fold greater specificity for cGMP compared to cAMP, as judged by the decrease in the k_{cat}/K_M value when cAMP is the substrate. Most of the reduction in substrate specificity for cAMP can be ascribed to the 65-fold increase in its K_M value (Table 2.1). For both cGMP and cAMP, no cooperativity could be detected (Table 2.1), but this is not unexpected since the high-affinity noncatalytic site on Pαβ is occupied over most of the concentration range we were able to test.

Table 2.1

Kinetic parameters for cyclic nucleotide hydrolysis by bovine rod Pαβ

Pαβ was depleted of endogenous, bound cGMP by incubation at 37° for 30 min. 5 pM Pαβ (for cGMP as substrate) or 50 pM Pαβ (for cAMP measurements) was added to 0.1 – 2000 μM cGMP or 15 μM to 60 mM cAMP and the initial rate of hydrolysis determined.

Condition	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M ($M^{-1}s^{-1}$)	Hill coefficient
cGMP substrate	14 ± 1.0	5440 ± 80	$3.9x10^8$	0.9 ± 0.1
cAMP substrate	910 ± 100	3060 ± 100	$3.4x10^6$	0.9 ± 0.1
cAMP substrate + cGMP occupying noncatalytic sites[a]	830 ± 60	3160 ± 70	$3.8x10^6$	1.0 ± 0.1

[a]To load cGMP on the noncatalytic sites of Pαβ, 200 nM Pαβ was first incubated with 5 μM cGMP, 100 μM zaprinast, and 10 μM Pγ1-45. To remove unbound low molecular weight compounds, the Pαβ was concentrated by ultrafiltration. The Pαβ was washed three times by resuspending in buffer (containing Pγ1-45) and re-concentrating. The final Pαβ preparation contained 1.1 – 1.6 mol cGMP bound per mol Pαβ

To directly test whether cGMP occupancy of the noncatalytic sites affected catalysis, we first pre-incubated Pαβ with cGMP and the N-terminal half of Pγ (Pγ1-45) to load cGMP onto both noncatalytic sites. [Pγ1-45 has no effect on catalysis of Pαβ (*data not shown*), but stabilizes high-affinity cGMP binding at both noncatalytic sites on Pαβ; see below.] The complex of Pαβ with bound Pγ1-45 and cGMP was then incubated with increasing concentrations of cAMP to determine its kinetic parameters. No significant change in K_M, k_{cat} or Hill coefficient could be discerned when compared with Pαβ incubated with cAMP alone (Table 2.1). These results confirm and extend previous observations with amphibian PDE6 (Arshavsky et al., 1992; D'Amours and Cote, 1999; D'Amours and Cote, 1999) that no direct allosteric mechanism regulates catalysis via the state of occupancy of the GAF domains on PDE6, as is the case for PDE2 (Martins et al., 1982; Yamamoto et al., 1983; Yamamoto et al., 1983). Thus, the differences in the K_M and k_{cat} values for cAMP and cGMP are due to differences in substrate specificity at the active site, not on the state of occupancy of the GAF domain.

The N-terminal half of Pγ binds to Pαβ with much greater affinity than the C-terminal region—Previous work has documented that two major domains of interaction with Pαβ exist on Pγ: the C-terminal residues and the polycationic central region (see Introduction). To understand how distinct binding domains on Pγ contribute to the very high-affinity binding of Pγ to Pαβ, we assayed the ability of these individual domains of Pγ to bind to Pαβ. We first assayed the ability of the C-terminal peptide, Pγ63-87, to bind to, and inhibit, cyclic nucleotide hydrolysis at the active site. Based on previous work (Granovsky et al., 1997), we expected that Pγ63-87 would act as a simple competitive inhibitor with respect to cyclic nucleotides. When cAMP is used as substrate, Pγ63-87

acts as a simple competitive inhibitor of catalysis with an inhibition constant, K_I, equal to 3.5 μM (Fig. 2.4). The affinity of this inhibitory domain is 6 orders of magnitude weaker than the entire Pγ molecule (K_D = 3 pM), indicating that the N-terminal 62 amino acids most likely contain the major site(s) of high-affinity interaction.

To directly measure the binding affinity of the N-terminal half of Pγ to Pαβ, we assayed the interaction of Pγ1-45 covalently labeled with Lucifer Yellow (Pγ1-45-LY) with Pαβ. Fig. 2.5A demonstrates that either in the absence of cyclic nucleotides or in the presence of cAMP, Pαβ binds to Pγ1-45-LY as a single class of binding sites with a K_D = 68 ± 15 or 62 ± 14 nM, respectively. In both cases, the maximum extent of binding was the same.

To test how the fluorescent probe altered the binding affinity of Pγ1-45, we also tested the effectiveness of Pγ1-45 in restoring high-affinity cGMP binding to the low-affinity site on Pαβ (Mou et al., 1999). In the absence of Pγ or Pγ1-45, Pαβ is able to bind 1 mol cGMP per mol Pαβ at micromolar levels of [^{3}H]cGMP (see Fig. 2.3, *triangles*). Addition of increasing amounts of Pγ1-45 in the presence of 600 nM [^{3}H]cGMP stimulates cGMP binding to a second site on Pαβ (Fig. 2.5B) with a K_D = 80 nM, a value in good agreement with the K_D for binding of Pγ1-45-LY to Pαβ.

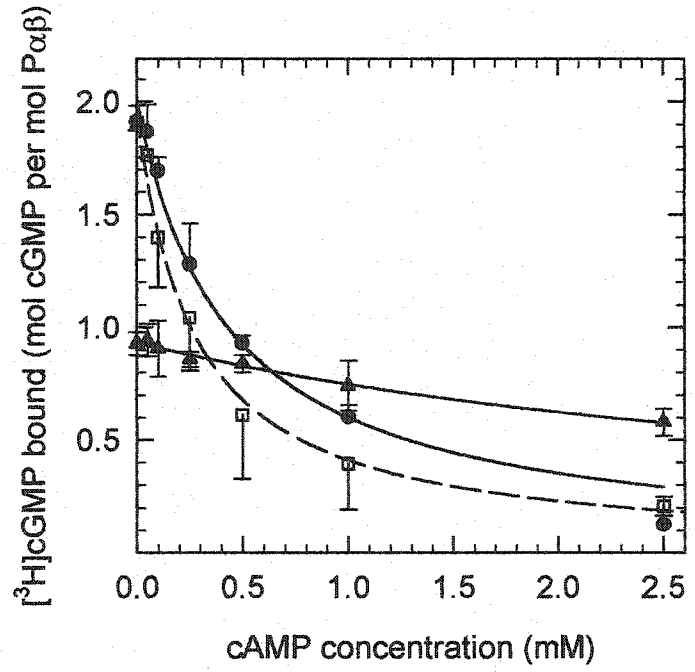


Figure 2.3. **cAMP binding affinity for the noncatalytic sites of bovine rod Pαβ in the absence of Pγ or upon addition of Pγ or Pγ1-45.** 1 μM [^{3}H]cGMP and the indicated concentrations of cAMP were added to 10 nM Pαβ in the absence of Pγ (▲), or following pre-incubation with 20 nM Pγ (●) or 10 μM Pγ1-45 (□, mean ± range for n = 2). The ability of cAMP to compete with [^{3}H]cGMP binding to noncatalytic sites was determined by filter binding, and the data was fit to a single class of non-interacting sites to obtain the following $K_{1/2}$ and B_{max} values, respectively: Pαβ, 4.0 mM, 0.9 cGMP/Pαβ; Pαβ+Pγ, 0.4 mM, 2.0 cGMP/Pαβ; Pαβ+Pγ1-45, 0.3 mM, 2.0 cGMP/Pαβ. The K_D for cAMP binding was calculated from the $K_{1/2}$ value (Cheng and Prusoff, 1973) using K_D = 60 nM for cGMP binding.

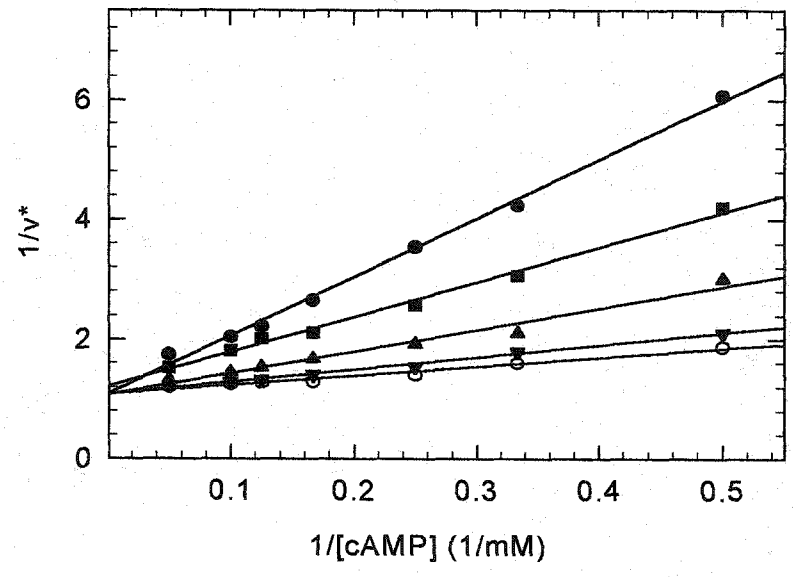


Figure 2.4. **Competitive inhibition of Pγ63-87 with cAMP at the active site of Pαβ.** Pαβ (2 nM) was incubated with 1.0 (open circles), 2.0 (upside-down triangles), 5.0 (triangles), 10 (squares) or 20 (filled circles) μM Pγ63-87 for 10 min at room temperature before adding the indicated amounts of cAMP and determining the initial rate. The data were plotted as the reciprocal of fraction of the maximum rate ($v^* = v/V_{max}$) versus the reciprocal of the substrate concentration. The inhibition constant, K_I, for Pγ63-87 was calculated to be 2.9 ± 0.3 μM from a replot of the slopes versus the corresponding inhibitor concentrations. The data is representative of three experiments with an average $K_I = 3.5 \pm 0.5$ μM.

We conclude that the N-terminal half of Pγ (Pγ1-45) binds to Pαβ about 50-fold more tightly than the C-terminal region (Pγ63-87). Furthermore, if each binding domain were to interact with Pαβ independently, the sum of the interaction energies of Pγ1-45 and Pγ63-87 ($K_D \sim 10^{-13}$ M) would account reasonably well with the measured K_D for full-length Pγ.

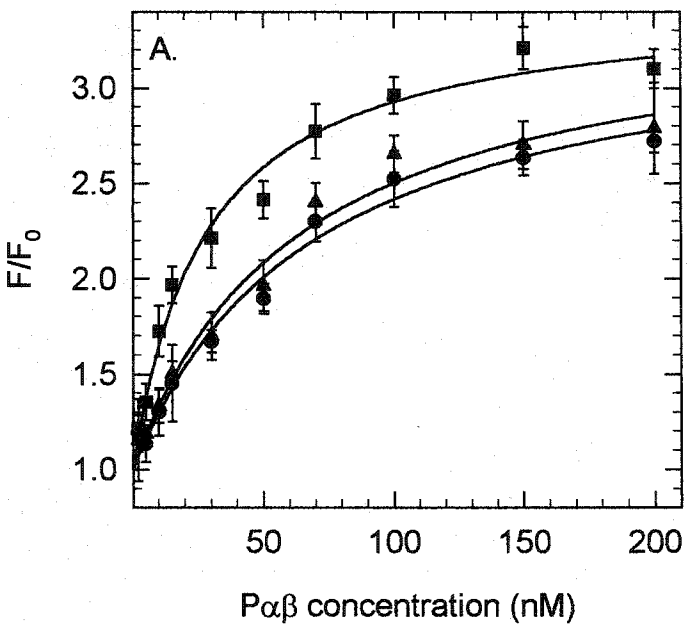
Relationship between occupancy of the GAF domain and Pγ binding affinity—In Fig. 2.2, we showed that heterogeneity in Pγ binding to Pαβ (assayed by inhibition of cyclic nucleotide hydrolysis) was dependent on the state of occupancy of the noncatalytic sites. The cGMP-dependent enhancement of full-length Pγ binding affinity is also observed with the fluorescently labeled N-terminal half of Pγ. Fig. 2.5A shows that Pγ1-45-LY undergoes a 2.3-fold increase in binding affinity, with no significant change in maximum binding, when the GAF domains are occupied by cGMP compared to empty or cAMP-filled sites. Thus, occupancy of the noncatalytic site by cGMP is required to induce the conformational change in Pαβ that enhances Pγ binding affinity.

These results and our previous study with bovine rod Pαβ (Mou et al., 1999) are in accord with a simple reciprocal relationship: cGMP occupancy of the noncatalytic sites enhances Pγ affinity to one of its binding sites and, conversely, Pγ binding to Pαβ enhances cGMP binding affinity to its low-affinity noncatalytic site. Unexpectedly, this correlation does not apply when cAMP occupies the noncatalytic sites. Fig. 2.3 shows that addition of Pγ or Pγ1-45 to Pαβ enhances 10-fold the ability of cAMP to compete with cGMP for binding to both noncatalytic sites. This suggests that Pγ binding induces a conformational change in Pαβ that enhances the affinity of both cGMP and cAMP for

Pαβ. However, Fig. 2.5A reveals that cAMP occupancy of the noncatalytic sites is not sufficient to induce an increase in Pγ1-45-LY binding to Pαβ. It appears that cGMP, but not cAMP, is needed to induce a conformational change in Pαβ that enhances Pγ binding affinity. This conclusion is supported by experiments with cGMP analogs that show intermediate effects on the relationship between cyclic nucleotide occupancy and enhanced Pγ binding affinity (H.M. and R.H.C., unpublished observations).

Defining the amino acid residues responsible for high-affinity interactions of Pγ with Pαβ—We prepared a set of synthetic peptides corresponding to various regions of the Pγ primary sequence to identify regions of Pγ that stabilized high-affinity binding of Pγ. We first measured the ability of these peptides to compete with full-length, endogenous Pγ bound to nonactivated PDE6, as judged by the increase in catalytic activity at the active site. Fig. 2.6A shows that two peptides, Pγ1-45 and Pγ18-41, were able to compete with full-length Pγ to activate PDE6 to 70-80% of its maximal rate and with $K_{1/2}$ values of 1 and 11 μM, respectively. The Pγ21-46 peptide showed a significant drop in its ability to compete with Pγ when compared with Pγ18-41, suggesting that amino acid residues 18-20 may stabilize a peptide conformation of Pγ18-41 that enhances its interaction with Pαβ. Several other peptides were able to stimulate cGMP hydrolysis to a limited extent (20-40% activation) and with low affinity ($K_{1/2}$ ~ 400 – 500 μM), including Pγ1-18, Pγ10-30, and Pγ35-56. The peptide Pγ55-75 was completely ineffective in competing with Pγ. These results indicate that the interaction of Pγ with Pαβ may consist of multiple low-affinity sites of interaction along the N-terminal half of the Pγ

molecule, with high-affinity interactions occurring in the region of amino acid residues 18 through 41.



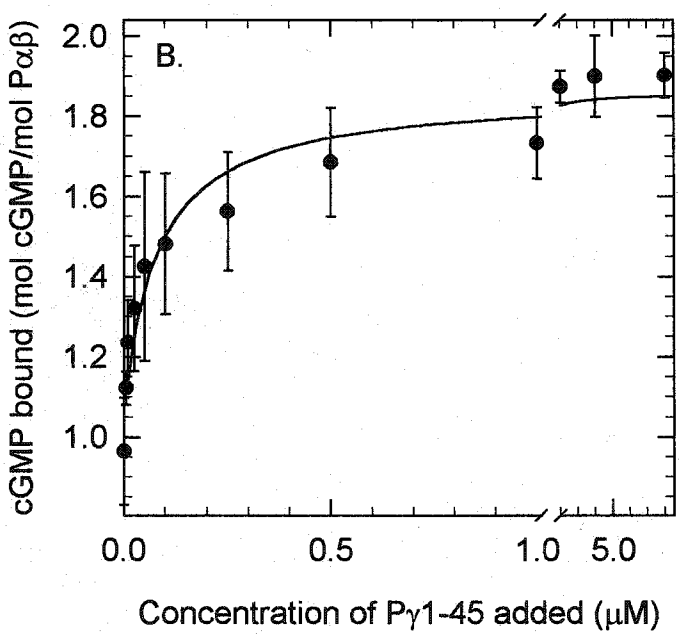


Figure 2.5. **High-affinity binding of Pγ1-45 to Pαβ.** *A.* The relative increase in fluorescence (F/F_0) of Pγ1-45 labeled with Lucifer Yellow (Pγ1-45-LY) was measured after addition of the indicated amounts of Pαβ. The buffer used for these assays contained 10 mM HEPES, pH 7.8, 100 mM NaCl, 1 mM $MgCl_2$, 200 μM zaprinast, and either no nucleotide (●), 10 mM cAMP (▲) or 10 μM cGMP (■). The curves represent the fit of each data set to a hyperbolic function: no nucleotide, $K_D = 68 \pm 15$ nM, $(F/F_0)_{max} = 3.3$; cAMP, $K_D = 62 \pm 14$ nM, $(F/F_0)_{max} = 3.4$; cGMP, $K_D = 28 \pm 5$ nM, $(F/F_0)_{max} = 3.5$. No evidence for cooperativity was detected using the Hill equation to fit the data. *B.* The ability of Pγ1-45 to restore high-affinity cGMP binding to a low affinity class of noncatalytic sites on Pαβ (10 nM) was measured following incubation of Pαβ with the indicated concentration of Pγ1-45 and 600 nM [^{3}H]cGMP. The data represents the average of 4 different experiments, and the curve is the fit of the data to a hyperbolic function ($K_D = 83$ nM; $B_{max} = 1.9$ mol cGMP bound per mol Pαβ).

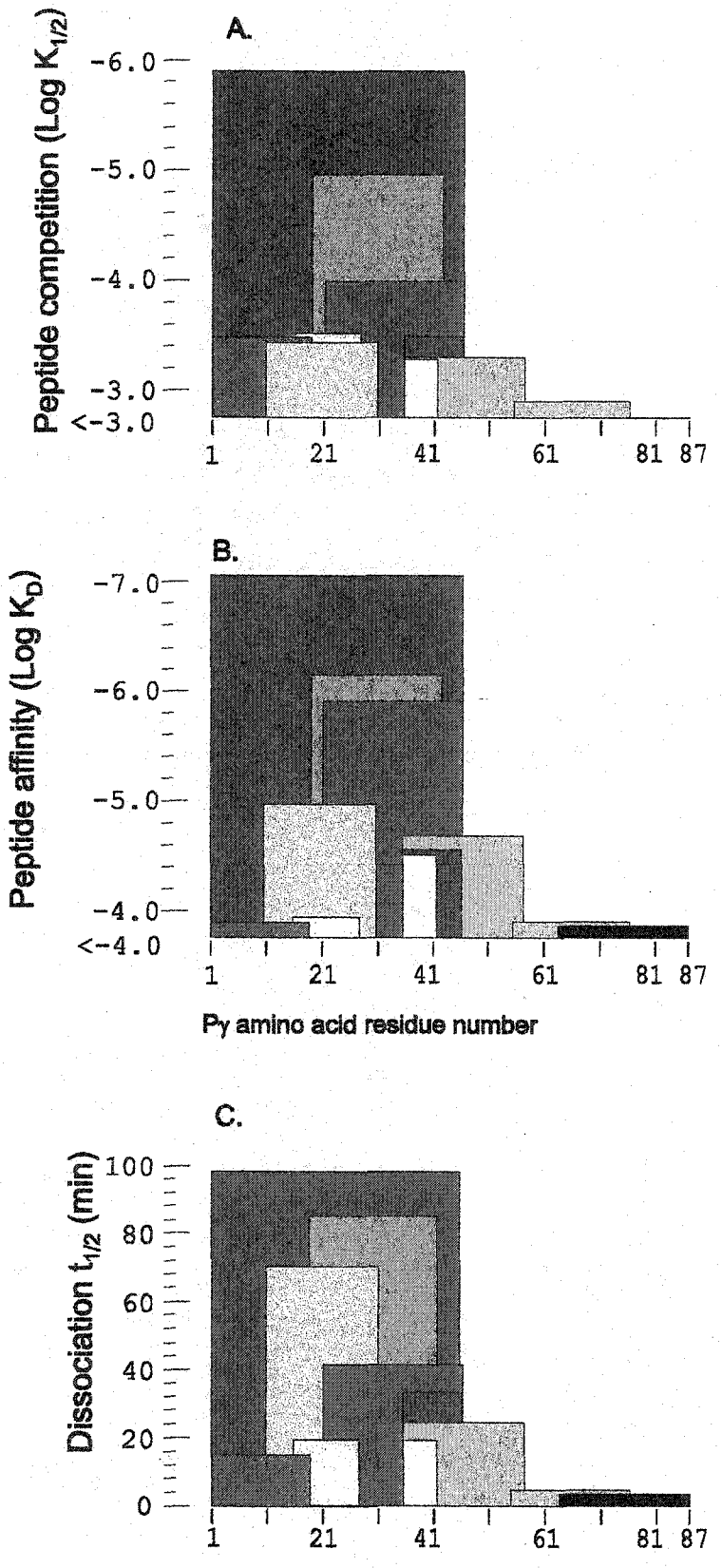


Figure 2.6. **Ability of Pγ peptides to interact with Pαβ.** *A.* The catalytic activity of nonactivated PDE6 holoenzyme (5 nM) was tested following incubation of PDE6 with increasing concentrations of Pγ peptides. The maximum extent of activation and the $K_{1/2}$ for the Pγ peptides were determined by fitting the concentration dependence to a hyperbolic function. The ordinate represents the log($K_{1/2}$) for each peptide and the abscissa visually depicts each Pγ peptide tested. The maximum percent activation of cGMP hydrolysis induced by each peptide was: Pγ1-45, 81%; Pγ18-41, 69%; Pγ10-30, 42%; Pγ35-56, 32%; Pγ21-46, 25%; Pγ1-18, 18% ; Pγ15-26, 14%; Pγ35-46, 11%; Pγ35-41, 9%; Pγ55-75, <5%. *B.* The ability of Pγ peptides to restore high-affinity cGMP binding to low affinity sites on Pαβ was determined as described in the legend to Fig. 5B, and the K_D values plotted on the ordinate on a log scale. The B_{max} for restoring high-affinity binding (% of restoration observed with Pγ = 100%) was: 100% (Pγ, Pγ1-45, Pγ10-30, Pγ18-41), 90% (Pγ21-46), 70% (Pγ35-56, Pγ35-46), 40% (Pγ35-41), and 0% (Pγ1-18, Pγ15-26, Pγ55-75, Pγ63-87). *C.* The [^{3}H]cGMP dissociation kinetics were determined at 22° by pre-incubating 5 nM Pαβ with 0.6 μM [^{3}H]cGMP and 200 μM of the indicated Pγ peptide until binding equilibrium was attained. Dissociation of bound [^{3}H]cGMP was initiated by addition of 2 mM unlabeled cGMP, and the data fit to a single exponential decay function to determine the $t_{1/2}$ from the equation: $t_{1/2} = 0.693/k_{-1}$, where k_{-1} is the dissociation rate constant.

We showed above that the N-terminal half of Pγ, Pγ1-45, could restore high-affinity cGMP binding to a low affinity class of noncatalytic sites on Pαβ (Figs. 2.3 & 2.5B). To better define this region on Pγ, we tested the ability of peptides to bind to Pαβ and restore high-affinity cGMP binding to this class of sites. Fig. 2.6B shows that Pγ18-41 and Pγ21-46 both fully restored cGMP binding to Pαβ with a binding affinity reduced approximately 10-fold compared to Pγ1-45. Pγ10-30 was also able to fully restore cGMP binding, but its interaction with Pαβ was 10-fold further reduced. Even though Pγ15-26 failed to interact with Pαβ to restore cGMP binding, the overall results with several other peptides suggest that residues 18 to 30 of Pγ are important in stabilizing Pγ binding to Pαβ.

We also tested peptides covering amino acids 35 – 56 of the Pγ sequence (Fig. 2.6B). While full restoration of cGMP binding could not be observed with Pγ35-56 or smaller peptides, all three peptides showed K_D values in the 20 – 30 μM range. The effectiveness of the shortest peptide, Pγ35-41, to stimulate cGMP binding to Pαβ indicates that these residues are important in direct binding to Pαβ and in stabilizing a high-affinity conformation of the second noncatalytic site on PDE6.

Although Pγ1-45 has 10-fold higher affinity for Pαβ than Pγ18-41, we could not detect any interaction of the peptide Pγ1-18 with Pαβ in terms of restoring high-affinity cGMP binding (Fig. 2.6B). It is possible that some of the N-terminal residues may stabilize a conformation of Pγ1-45 that favors its binding to Pαβ compared to Pγ18-41. Two other peptides, Pγ55-75 and Pγ63-87, completely lacked the ability to restore cGMP binding even at 300 μM peptide concentrations.

The binding affinity of cGMP for the noncatalytic sites was determined in the presence of 200 μM of each peptide to examine whether the low- and high-affinity noncatalytic sites could be distinguished (*data not shown*). All Pγ peptides that were competent to restore cGMP binding to the low-affinity sites failed to alter the K_D for cGMP binding (K_D range: 51 – 63 nM). No evidence for binding site heterogeneity could be detected. We conclude that binding of Pγ or central region Pγ peptides induces a conformational change in the low-affinity noncatalytic site that enhances its affinity for cGMP to a value indistinguishable from the high-affinity noncatalytic site.

The restoration of cGMP binding shown in Fig. 2.6B describes the ability of Pγ peptides to act on the low-affinity noncatalytic sites on Pαβ. To examine both high- and low-affinity noncatalytic sites, we examined whether Pγ peptides could alter the rate of [^{3}H]cGMP dissociation from the noncatalytic sites of Pαβ. Fig. 2.6C shows that most of the peptides were able to retard the rate of [^{3}H]cGMP dissociation from the noncatalytic sites. In all instances, the dissociation kinetics was fit to a single exponential loss of [^{3}H]cGMP binding; no evidence for two distinct classes of sites was evident (*not shown*). As expected, Pγ1-45 retarded most effectively cGMP exchange at both noncatalytic sites on Pαβ, causing a 16-fold increase in the half-time for [^{3}H]cGMP release from noncatalytic sites. The Pγ peptides Pγ18-41 and Pγ10-30 also retarded cGMP exchange by greater than 10-fold. Comparison of Pγ18-41 ($t_{1/2}$ = 84 min) with Pγ21-46 ($t_{1/2}$ = 41 min) supports the importance of amino acid residues 18-20 in stabilizing binding of Pγ peptides to Pαβ. The Pγ peptide, Pγ35-46, is nearly as effective as the larger Pγ21-46 in

slowing the rate of cGMP dissociation from Pαβ, while the seven amino-acid Pγ35-41 also acted to increase the half-time for cGMP dissociation 3-fold.

Two peptides, Pγ55-75 and Pγ63-83, were unable to affect cGMP dissociation compared to Pαβ lacking Pγ peptides ($t_{1/2}$ = 6 min), in agreement with their inability to restore cGMP binding to low affinity sites (Fig. 2.6B). The fact that the Pγ1-18 and Pγ15-26 peptides slowed cGMP dissociation ~ 3-fold from the high-affinity noncatalytic sites of Pαβ (Fig. 2.6C) without being able to restore cGMP binding to the low-affinity site (Fig. 2.6B) suggests that this region of Pγ may affect only the high-affinity GAF domain of PDE6.

DISCUSSION

This paper shows that Pγ binds to two distinct high-affinity binding sites on Pαβ when cGMP occupies the noncatalytic sites. Our work also describes the reciprocal allosteric regulation of PDE6 resulting from Pγ binding to Pαβ and cGMP binding to high-affinity sites in the GAF domains of Pαβ. Since no direct allosteric communication between the GAF domains and the active sites is detected, we conclude that the Pγ subunit is required to facilitate allosteric communication between the regulatory and catalytic domains of PDE6. Finally, we have mapped regions within the N-terminal half of the molecule that interact with Pαβ, some of which induce conformational changes at the GAF domain.

Implications of high-affinity, two-site Pγ binding to Pαβ for PDE function. Our quantitative analysis of Pγ binding to Pαβ helps to make sense of the wide range of apparent binding affinities reported in the literature. The majority of studies of the Pγ affinity for Pαβ have been carried out with concentrations of enzyme much greater than the K_D value for Pγ binding. Under these conditions, Pγ will bind in a stoichiometric manner (Fig. 2.1). Even when the appropriate model is applied to the binding data (i.e., Eq. 2), it is difficult to extract meaningful values for the K_D values. For example, we were unable to satisfactorily fit data obtained at > 800 pM Pαβ concentrations to Eq. 2, primarily because the estimate of the free Pγ concentration is very uncertain (H.M and R.H.C., unpublished). In previous reports where the PDE concentration was lowered into the picomolar range [e.g., refs. (Wensel and Stryer, 1986; Hamilton et al., 1993; D'Amours and Cote, 1999; Hamilton et al., 1993; D'Amours and Cote, 1999)], the

binding affinities of Pγ for Pαβ were much closer to the values we report. Previous evidence supporting two distinct Pγ binding sites on Pαβ has mostly been obtained indirectly, based on heterogeneity in transducin activation of PDE6 (Melia et al., 2000; Norton et al., 2000; Bennett and Clerc, 1989; Bruckert et al., 1994; Bruckert et al., 1994; Melia et al., 2000; Norton et al., 2000). However, Berger et al. (Berger et al., 1999) have reported two classes of Pγ binding sites on Pαβ when probed with a fluorescently labeled mutant of Pγ.

The functional heterogeneity in Pγ binding to Pαβ is a consequence of the state of occupancy of the noncatalytic sites with cGMP (Fig. 2.2)—but not cAMP. Since the membrane-associated bovine rod PDE6 holoenzyme has one exchangeable and one nonexchangeable cGMP binding site (Mou et al., 1999), we hypothesize that the very high affinity (K_D < 1 pM) Pγ binding site may correlate with this nonexchangeable cGMP site. This Pγ binding site associated with the nonexchangeable cGMP site is unlikely to function during visual transduction. Instead, it may serve a structural role in stabilizing the native conformation of the PDE6 holoenzyme. This idea is supported by studies showing that mutations in the GAF domains of PDE6 [(Gal et al., 1994); reviewed in ref. (Dryja et al., 1999)] or a disrupted or mutated Pγ gene (Tsang et al., 1996) can affect the levels of expression and/or activation of PDE6. It might also explain the difficulty in expressing functional PDE6 in various expression systems (Piriev et al., 1993; Qin and Baehr, 1994; Granovsky et al., 1998; Qin and Baehr, 1994; Granovsky et al., 1998) if co-expression of catalytic and Pγ subunits must occur to properly fold the nascent polypeptide chains of PDE6.

Allosteric regulation of PDE6 requires Pγ to communicate between the GAF domain and the catalytic domain. In addition to a catalytic domain near the C-terminus that is conserved in all members of the vertebrate PDE superfamily, most PDE families contain N-terminal domains that serve regulatory functions. It has been proposed (Conti, 2000) that a common regulatory feature of the N-terminal domain is to alter the inhibitory constraint on catalysis via conformational changes in the catalytic dimer. Almost half of the known PDE families (PDE2, PDE5, PDE6, PDE10, and PDE11) contain two tandem GAF domains (Aravind and Ponting, 1997) which in most cases code for functional, noncatalytic cGMP binding sites. For PDE2 and PDE5, binding of cGMP to the GAF domain induces a conformational change in the catalytic dimer that either directly or indirectly stimulates catalysis (Martins et al., 1982; Yamamoto et al., 1983; Burns et al., 1992; Corbin et al., 2000; Yamamoto et al., 1983; Burns et al., 1992; Corbin et al., 2000). For PDE6, we show that loading the GAF domain with cGMP has no direct influence on PDE6 kinetic parameters (Table 2.1).

Nonetheless, cGMP binding to the GAF domain of PDE6 does induce a conformational change in the catalytic dimer. Occupancy of the noncatalytic sites enhances the interaction of Pγ (Fig. 2.2) or Pγ1-45 (Fig. 2.5A) to the catalytic dimer. This effect is specific for cGMP (Fig. 2.5A). Conversely, addition of Pγ to bovine rod Pαβ enhances cyclic nucleotide binding affinity to the noncatalytic sites (Fig. 2.3) in a reciprocal manner. For cGMP, Pγ acts to restore high-affinity binding to a low affinity class of sites on bovine Pαβ; the other, high-affinity class of cGMP sites undergoes no change in affinity (Mou et al., 1999). For cAMP, addition of Pγ or Pγ1-45 to Pαβ increases the binding affinity 10-fold to both noncatalytic sites on Pαβ (Fig. 2.3).

We conclude that unlike PDE2 or PDE5, the GAF domain in the PDE6 catalytic subunit is allosterically uncoupled from its catalytic domain unless Pγ is bound to bridge the two domains. Furthermore, the heterogeneity in Pγ binding to Pαβ suggests that only one Pγ binding site on Pαβ is sensitive to cGMP occupancy of the noncatalytic sites. Future efforts will identify which catalytic subunit contains the cGMP-sensitive Pγ binding site and the high-affinity noncatalytic site on Pαβ.

Pγ is a multi-functional subunit with several sites of interaction with the PDE6 transduction complex. The Pγ subunit of PDE6 contains within its 87 amino acid sequence numerous sites of interactions with Pαβ, transducin, RGS-9 and perhaps other proteins (see Introduction). In this study, we have focused on Pγ-Pαβ interactions, and find that the N-terminal half of Pγ binds 50-fold more tightly to the catalytic dimer than the C-terminal region (Pγ63-87). This high-affinity binding domain in the region of residues 18 – 41 of Pγ explains why α_t* activation of the PDE6 holoenzyme sometimes displaces the inhibitory constraint of Pγ without causing complete dissociation of Pγ from Pαβ (Wensel and Stryer, 1986; Clerc and Bennett, 1992; Clerc and Bennett, 1992). The correlation of Pγ dissociation from transducin-activated PDE with loss of cGMP from the GAF domain (Norton et al., 2000) also makes sense based on the reduced binding affinity of Pγ or Pγ1-45 in the absence of cGMP (Figs. 2.2 & 2.5).

Within the central region of the Pγ subunit, amino acids 18 – 20 and 35 – 41 both contribute to stabilizing Pγ binding to Pαβ (Fig. 2.6). Other Pγ interaction sites probably exist within this polycationic region that went undetected with our selected peptides, including potential sites of regulation via phosphorylation at T22 and/or T35 (Tsuboi et

al., 1994; Xu et al., 1998; Matsuura et al., 2000; Xu et al., 1998; Matsuura et al., 2000) or via ADP-ribosylation at R33 or R36 (Bondarenko et al., 1997).

Our results demonstrate that the N-terminal 18 amino acids of Pγ play no direct role in inhibiting catalysis or stimulating high-affinity cGMP binding to Pαβ (Fig. 2.6), contrary to a previous study (Yamazaki et al., 1996a). However, the N-terminus of Pγ probably contains weak sites of interaction with Pαβ and may help stabilize a high-affinity conformation of the 18-41 region of Pγ.

Significantly, the one peptide that shows no detectible interaction with Pαβ, namely Pγ55-75, contains a major site of interaction with activated transducin, α_t* (see Introduction). A recent structural determination of the interaction of the C-terminal half of Pγ with α_t* has shown that α_t^* binds to several residues in the vicinity of W70 of Pγ to cause the displacement of the C-terminal residues of Pγ known to block the active site (Slep et al., 2001).

Summary. Our results extend previous models postulating two major sites of interaction between PDE6 and activated transducin—both of which are mediated through the inhibitory Pγ subunit. The extreme C-terminus of Pγ functions to block catalysis at the active site, but has relatively low affinity for Pαβ. It is the high-affinity interactions of the central, polycationic region of Pγ (particularly residues 18-41) that stabilize binding to Pαβ, thereby insuring that a very small fraction of PDE6 holoenzyme is catalytically active prior to light activation. High levels of cGMP in dark-adapted photoreceptor cells result in saturation of the noncatalytic sites on PDE6, further enhancing Pγ affinity to one binding site on the PDE6 holoenzyme. Upon activation of the phototransduction cascade, binding of activated transducin to the PDE6

holoenzyme—specifically in the vicinity of W70 of Pγ—is sufficient to displace the Pγ C-terminus and cause activation at one active site on Pαβ. [The very high-affinity Pγ binding site (K_D < pM) when cGMP is present likely prevents α_t* displacement of Pγ at the second active site (Melia et al., 2000; Norton et al., 2000; Norton et al., 2000)]. At early times following light stimulation, α_t* remains associated with the PDE6 holoenzyme, and does not physically dissociate as a α_t*-Pγ complex. Following PDE6 activation, cGMP levels in the outer segment drop and remain low until the inactivation process re-inhibits PDE6. The positive cooperativity between the GAF domains and the central region of Pγ suggests that a sustained lowering of cGMP levels (e.g., during light adaptation) will lead to cGMP dissociation and a lowered Pγ affinity for Pαβ. This cGMP-dependent allosteric transition could cause the multi-functional Pγ subunit to become available to interact with RGS-9 to facilitate an enhanced GTPase rate on α_t*, consistent with biochemical and structural studies (Slep et al., 2001; Arshavsky and Bownds, 1992; Calvert et al., 1998; He et al., 1998; Calvert et al., 1998; He et al., 1998; Slep et al., 2001).

While homology modeling of the GAF domain (Ho et al., 2000) and the catalytic domain (Xu et al., 2000; Granovsky and Artemyev, 2000; Granovsky and Artemyev, 2000) of PDE complement recent structural information on Pγ binding to α_t* (Slep et al., 2001), we still lack crystal structures revealing the interactions of each Pγ molecule with the α and β subunits of the rod PDE6 heterodimer. The present study provides the biochemical basis for understanding how the central and C-terminal domains of Pγ bridge the regulatory and catalytic domains of the rod PDE6 heterodimer to control the

magnitude and duration of PDE activation during visual excitation, recovery, and light adaptation.

Chapter III

Regulation of PDE6 by phosphorylation of the inhibitory γ subunits and the catalytic αβ subunits

Abstract

Many proteins in the visual transduction pathway become phosphorylated upon light activation. Since PDE6 is the key effector enzyme in phototransduction, it is quite possible that PDE function is regulated by reversible phosphorylation of its subunits. In this study, we first examined the effect of phosphorylation of the inhibitory rod PDE γ subunit (Pγ) on its interaction with the PDE6 catalytic subunits (Pαβ) after phosphorylation at Thr^{22} by mitogen-stimulated protein kinase (MAPK) and at Thr^{35} by cAMP-dependent protein kinase A (PKA). Our results showed that phosphorylation of Pγ neither abolishes the heterogeneity in Pγ binding to Pαβ nor greatly affects the potency of Pγ to inhibit both trypsinized PDE and transducin-activated PDE. However, phosphorylation at Thr^{22} mildly decreases the ability of the central region of Pγ to bind to Pαβ and to stabilize the cGMP binding at the GAF domains. Pγ bound to Pαβ is a poor substrate for phosphorylation, but transducin activation enhances its ability to be phosphorylated in bovine rod outer segments (ROS). Pαβ can also be phosphorylated by PKA and MAPK under *in vitro* conditions. PKA prefers Pβ as a substrate while MAPK can phosphorylate both Pα and Pβ. An unknown protein kinase from ROS membrane associates with PDE with a moderate affinity and phosphorylates PDE at its α subunit. No alteration in k_{cat} and K_m has been detected after the phosphorylation of Pαβ. However,

cGMP occupancy at the noncatalytic sites reciprocally regulates the phosphorylation of Pαβ. These results suggest a potential role of Pγ and Pαβ phosphorylation in regulating PDE6 by regulating cGMP levels in the photoreceptor cells. Other possible physiological roles of Pγ and Pαβ phosphorylation in regulating PDE during phototransduction are discussed.

Introduction

Photoactivation of the visual pathway starts when a photon of light stimulates a molecule of rhodopsin, initiating a transduction cascade involving the sequential activation of rhodopsin (R), transducin (G_t) and cGMP phosphodiesterase (PDE). PDE is the key effector enzyme in visual transduction and functions to hydrolyze the second messenger cGMP upon activation [for reviews, see (Pugh, Jr. et al., 1999)]. The decrease in cGMP concentration causes dissociation of cGMP from the cGMP-gated ion channels. This causes the channels to close, resulting in a hyperpolarization of the membrane.

Protein phosphorylation plays a central role in regulating the function of several phototransduction proteins. Many protein kinases have been found to exist in vertebrate rod outer segments: rhodopsin kinase (RK) (Lee et al., 1981); cyclin-dependent protein kinase 5 (cdk5) (Hayashi et al., 2000); protein kinase C (PKC) (Kelleher and Johnson, 1985; Udovichenko et al., 1997; Udovichenko et al., 1997); cAMP-dependent kinase (PKA) (Walter, 1984), casein kinase II (CKII) (Hollander et al., 1999); Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) (Liu et al., 2000); cGMP-dependent kinase (PKG) (Taylor and Uhler, 2000), mitogen-stimulated protein kinase (MAPK) (Ko et al., 2001) and protein-tyrosine kinase (Bell et al., 2000). Rhodopsin phosphorylation by RK has been studied as a mechanism to terminate the initial step of the light-activated biochemical process (Wilden, 1995; McDowell et al., 2001; Hurley et al., 1998; Krupnick and Benovic, 1998; Wilden, 1995; Hurley et al., 1998; Krupnick and Benovic, 1998; Kennedy et al., 2001; McDowell et al., 2001). Phosphorylation of phosducin by CaMKII regulates transducin through regulation of $G_t\beta\gamma$ function (Bauer et al., 1992; Lee et al., 1992; Gaudet et al., 1999; Thulin et al., 2001; Gaudet et al., 1999; Bauer et al., 1992; Lee

et al., 1992). Phosphorylation of the cGMP-gated channel by a tyrosine kinase is another mechanism to regulate cGMP sensitivity in addition to calmodulin regulation (Muller et al., 2001). RGS9-1 (regulator of G protein signaling 9) is also found to be phosphorylated by an endogenous protein kinase in rod outer segments (Hu et al., 2001).

Most work on PDE6 phosphorylation has focused on Pγ phosphorylation. Currently, Pγ phosphorylation has been studied *in vivo* (Hayashi, 1994; Hayashi et al., 2000; Hayashi et al., 2000) and *in vitro* using several protein kinases including phosphatidylinositiol (PI)-stimulated kinase (Hayashi et al., 1991), PKC (Udovichenko et al., 1994; Udovichenko et al., 1996; Udovichenko et al., 1994), PKA (Xu et al., 1998), cdk5 (Tsuboi et al., 1994b; Tsuboi et al., 1994a; Sharma et al., 1999; Hayashi et al., 2000; Matsuura et al., 2000; Hayashi et al., 2000; Sharma et al., 1999; Tsuboi et al., 1994b; Tsuboi et al., 1994a). The physiological function of Pγ phosphorylation in the current scheme of phototransduction is not well understood. The central region of Pγ contains most of the predicted phosphorylation sites and the central domain of Pγ is also a major site at which Pγ interacts with Pαβ (Artemyev and Hamm, 1992; Mou and Cote, 2001; Mou and Cote, 2001; Mou and Cote, 2001). Yamazaki and colleagues have proposed that Pγ phosphorylation at either Thr^{35} (by PKA) or at Thr^{22} by (cdk5) enhances almost 10-fold the interaction of Pγ with Pαβ (Tsuboi et al., 1994b; Xu et al., 1998; Hayashi et al., 2000; Tsuboi et al., 1994b; Tsuboi et al., 1994a; Matsuura et al., 2000; Hayashi et al., 2000). However, their conclusions are open to alternative interpretations. For example, they have not carefully evaluated the K_D of Pγ binding to Pαβ with or without phosphorylation. Actually, the experimental conditions chosen (nanomolar concentration of PDE) in their studies would prevent the Pγ binding affinity for bovine or frog Pαβ

from being discerned because Pγ interacts with Pαβ with such high affinity (K_D < 10 pM) (Wensel and Stryer, 1986; Mou and Cote, 2001; Mou and Cote, 2001) that both non-phosphorylated Pγ and phosphorylated Pγ should inhibit the catalysis of Pαβ in a titration manner. In addition, they have not studied if Pγ phosphorylation would affect cGMP binding to GAF domains on PDE6. The effect of Pγ phosphorylation on the ability of Pγ to serve as a GTPase-activating protein (GAP) for transducin has also been reported. Xu et al. have shown that, once phosphorylated, Pγ had less ability to deactivate transducin and displace bound GTPγS from its binding site on $G_t\alpha$ (Xu et al., 1998). However, direct measurements of Pγ-transducin interactions were not performed in their study.

Several members of the PDE family are regulated and activated by serine/threonine phosphorylation of the catalytic subunits, including PDE1(Florio et al., 1994; Florio et al., 1994), PDE3 (Macphee et al., 1988), PDE4 (Lim et al., 1999) and PDE5 (Thomas et al., 1990; Beltman et al., 1993; Corbin et al., 2000; Beltman et al., 1993; Corbin et al., 2000). Phosphorylation of PDE5 may be most relevant to PDE6, since the structure of PDE5 is the most closely related to PDE6 (McAllister-Lucas et al., 1993; Tcheudji et al., 2001; Tcheudji et al., 2001). Phosphorylation of PDE5 is allosterically enhanced by cGMP occupancy of cGMP binding sites (Turko et al., 1998). No major effect of phosphorylation on the maximum rate of catalysis and the potency of PDE5 inhibitors has been described. However, a modest decrease in the K_m for cGMP hydrolysis and K_D for cGMP binding has been observed (Corbin et al., 2000). To date, only one report has suggested that PDE6 serves a substrate for PKA and ROS PKC *in vitro* (Udovichenko et al., 1993). They showed that PKA phosphorylated PDE at both subunits, but removal of Pγ by trypsinization abolished the phosphorylation by PKA.

PKC only phosphorylated the Pα subunit and this phosphorylation was independent of trypsinization. A synthetic peptide AKVISNLLGPREAAV (Pα30-44) inhibited phosphorylation of PDE catalytic subunits by PKC from ROS, suggesting that Ser^{34} of Pα subunit is a potential phosphorylation site for PKC. The effect of phosphorylation on PDE6 catalysis and cGMP binding properties has not been described. In addition, no *in vivo* phosphorylation of PDE6 has been reported.

In this study, we have repeated the stoichiometric phosphorylation of Pγ at Thr^{35} by PKA and at Thr^{22} by MAP kinase *in vitro*. We have measured the K_D of nonphosphorylated and phosphorylated Pγ binding to Pαβ and found no apparent effect of phosphorylation on the ability of Pγ to inhibit both trypsinized and transducin-activated PDE. However, phosphorylation of Pγ at Thr^{22} mildly decreases the ability of the central region of Pγ to stabilize cGMP binding to the GAF domains and to displace the endogenous full-length Pγ from its binding sites in the catalytic domain of PDE. We have also observed an increase in Pγ phosphorylation upon light activation of bovine ROS, but the stoichiometry is low. Based on these observations, we conclude that the phosphorylation of Pγ is unlikely to regulate PDE catalysis directly, but may be involved in regulating its noncatalytic cGMP binding. It remains possible that other signaling pathways intersect with visual excitation by recognizing Pγ in its phosphorylated state.

Our results show that PDE catalytic subunits are also substrates for phosphorylation by PKA, MAP kinase, and by an unknown ROS membrane-associated "PDE6 kinase". When the GAF domains lack bound cGMP, Pαβ phosphorylation is enhanced, opposite to the case for PDE5. We have not found the apparent effect of PDE6 phosphorylation on its catalysis. However, phosphorylation of Pαβ restores high affinity

cGMP binding to low-affinity noncatalytic sites. The possible physiological role of Pαβ phosphorylation in regulating PDE during phototransduction will be discussed.

Material and Experimental Procedures

Materials—Bovine retinas were purchased from W.L. Lawson, Inc. [^{3}H]cGMP was from NEN Life Sciences Products and [γ-^{32}P]ATP was from ICN Biochemicals. Filtration and ultrafiltration products were from Millipore. Scintillation fluid (Ultima Gold) was from Packard Instrument Corporation. Zaprinast was a gift of Rhone-Poulenc Rorer (Dagenham, UK). BCA protein assay reagent was from Pierce. Superdex 200 and Mono Q columns were from Pharmacia, and the C18 reverse-phase HPLC column (300A, 22 x 250 mm) was from Vydac. cAMP-dependent protein kinase (PKA) catalytic subunit and MAP kinase 2 (Co-expressed with Erk2) were from Upstate Biotechnology. Bacterial strains containing Pγ22C and Pγ35C are kind gifts of Dr. N.O. The mutants Pγ22C and Pγ35C both had a substitution of Ser at Cys^{68} of the bovine rod Pγ sequence, in addition to a Cys substitution for the naturally occurring Thr at positions 22 or 35, respectively (Granovsky et al., 1998). Artemyev and bacterial strains containing Pγ1-45C (Pγ amino acid residues 1-45 plus a cysteine tagged at the C-terminal end) was provided by Dr. N.P.Skiba (Skiba et al., 1996). All other chemicals were obtained from Sigma.

Preparation of bovine rod outer segments (ROS) -- Purified bovine ROS was prepared from frozen retinas in the dark under IR illumination following the method of McDowell (McDowell, 1993). 50 retinas were suspended with 45 ml of solution A (0.1 M potassium phosphate monobasic, pH 7.0, 1.0 mM $MgCl_2$, 0.1 mM Na_2-EDTA, 1 mM DTT, 0.2 mM PMSF) in a flask. The ROS were detached from the retina by stirring on a magnetic plate for 10 min while keeping the flask on ice. After spinning at 5000 rpm for 30 minutes, the crude ROS was collected through 4 layers of gauze. The sucrose was diluted 1.5 fold with solution A, followed by spinning for 1 hour at 7500 rpm. The ROS-containing pellet

was resuspended in solution A supplemented with 25.5% of sucrose (d = 1.105 g/ml), then loaded on a discontinuous sucrose gradient containing 5 ml of 27.1% sucrose (d = 1.135 g/ml) and 5 ml 32.3% of sucrose (d = 1.115 g/ml). The sucrose gradient was centrifuged at 27,000 rpm in SW27.1 rotor at 4°C for 1 hour. The ROS layer found in the lower interface (1.115 g/ml and 1.135 g/ml) was removed with 15 g needle. The ROS was then diluted with solution A and centrifuged (17K in SS-34 rotor for 60 minutes). The purified ROS pellet was stored at –80°C until use.

Preparation of non-activated membrane-associated PDE (PDE-M) and Pαβ dimer -- Bovine rod membrane-associated PDE was prepared from frozen bovine retinas and purified by using ion-exchange, immunoaffinity and gel-filtration chromatography using procedures described previously (Mou et al., 1999). The purified PDE was concentrated with Biomax-50K and stored in PDE storage buffer (100 mM NaCl, 2 mM $MgCl_2$, 10 mM Tris, 7.5, 2 mM dithiothreitol, 0.4 mM Pefabloc) containing 50% glycerol at –20°C. PDE catalytic subunits (Pαβ) were prepared by limited proteolysis of purified PDE by L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin and Pγ fragments were removed by Mono-Q chromatography, as described in detail previously (Mou et al., 1999). The purity of the Pαβ was found to be >95%. The concentration of PDE was determined as described previously (Mou et al., 1999; Cote, 2000; Cote, 2000).

Purification and quantitation of Pγ and Pγ mutants (Pγ22C, Pγ 35C and truncated mutant Pγ1-45C) -- The recombinant bovine rod full-length Pγ and Pγ mutants were expressed using the pET11a-Pγ, pET11a-Pγ22C, pET11a-Pγ35C and pET11a-Pγ1-45C vectors. The expression and purification procedures were the same as described in Mou et al. (Mou et al., 1999). The concentration of purified Pγ, Pγ22C and Pγ35C were measured

spectrophotometrically at 277 nm with an extinction coefficient of 7550 $cm \cdot M^{-1}$ (Cote, 2000). The concentration of purified Pγ1-45C was determined by the bicinchoninic acid (BCA) protein assay with bovine serum albumin (BSA) as the protein standard.

Pγ peptide synthesis and purification--Pγ18-41 and two singly phosphorylated peptides [Pγ18-41(22P) and Pγ18-41(35P)] were synthesized at the Protein Facility in the Department of Biochemistry and Molecular Biology at the University of New Hampshire. Cleavage of peptides from the resin and protecting groups were accomplished with anhydrous hydrofluoric acid (HF). The peptides were first purified with cation exchange chromatography on CM Sephadex C25 equilibrated with 1 mM NH_4HCO_3, pH 8.0 and eluted with a linear 0-1.0 M NH_4HCO_3 gradient. After ion-exchange chromatography, the peptides were further purified with a preparative C18 reverse-phase column (300A, 22 x 250 mm) using a linear gradient of 30-70% acetonitrile in 0.1% trifluoroacetic acid. The dried peptides were resuspended in 10 mM Tris, pH 7.5. The concentration of Pγ peptides was routinely measured by BCA protein assay.

Phosphorylation of purified recombinant Pγ, Pγ mutants, PDE-M by PKA or/and MAP kinase -- The phosphorylation assay was performed in final volume of 30-100 μl assay dilution buffer [20 mM MOPS, pH 7.2, 25 mM β-glycerol phosphate, 5 mM EGTA, 1mM sodium orthovanadate, 1mM dithiothreitol, 15 mM $MgCl_2$], plus 100 μM ATP (with or without 0.02-0.05 μCi/μl [γ-^{32}P]ATP), 1:50 phosphatase inhibitor cocktail-1 (Sigma, cat. # P2850; a mixture of inhibitors that will inhibit the L-isozymes of alkaline phosphatase as well as serine/threonine protein phosphatases such as PP1 and PP2A) and the desired concentration of Pγ, Pγ mutants or PDE-M. The phosphorylation reaction was initiated by adding PKA (~ 20 ng/μg protein) or MAP kinase (10-20 ng/μg protein). Two

methods were used to detect the incorporation of [^{32}P]. The first one was to apply the phosphorylation mixture to P81 phosphocellulose paper squares (Roskoski, Jr., 1983), then rinsed three times in 0.75% phosphoric acid to remove the free [^{32}P]ATP. The radioactivity was measured in a scintillation counter. The second method was to first separate the proteins by SDS-PAGE and then detect incorporation of [^{32}P] by autoradiography.

In situ phosphorylation of Pγ and PDE catalytic subunits in bovine ROS -- Bovine ROS homogenates ([rhodopsin] = 50-100 μM) were incubated with 200-500 μM [γ-^{32}P]ATP under nonactivated (in the darkness and without GTPγS) or activated (exposed to light and in the presence of 100 μM GTPγS) conditions. The free [^{32}P]ATP and soluble proteins were removed by centrifugation at 13000 rpm for 10 minutes (loss of Pγ even in the present of GTPγS was less than 5%). For the detection of Pγ phosphorylation, Pγ was extracted by adding a final concentration of 20-40% of acetonitrile and heating at 80°C for 10-20 min. For the detection of PDE catalytic subunit phosphorylation, PDE-M was first extracted from ROS by washing ROS membranes at least three times with hypotonic buffer (5 mM Tris, pH 7.5, 1 mM $MgCl_2$, 1 mM DTT, 0.2 mM Pefabloc) or 10 mM CHAPS in ROS buffer. After extraction, Pγ was partially purified on a Sep-Pak C18 cartridge using 50% of acetonitrile/0.1 % trifluoroacetic acid to elute Pγ; PDE-M was concentrated with 10K Centricon ultrafiltration device. The stoichiometry of phosphorylation was determined by comparison with [^{32}P] incorporation into recombinant Pγ or purified PDE-M by PKA *in vitro*.

Assay for PDE hydrolytic activity and measurements of cGMP binding to noncatalytic sites on Pαβ -- Two different methods (a radiotracer assay and a colorimetric assay) were

used to quantify the PDE hydrolytic activity (Cote, 2000). The equilibrium binding and kinetics of [^{3}H]cGMP binding to the noncatalytic sites were measured with a filter binding assay as described (Cote, 2000). For experiments in which the Pγ or Pγ peptides were used, Pγ or Pγ peptides were added to the mixtures 10 min before cGMP or cAMP.

Data analysis – All experiments were performed at least three times and the results presented are the mean ± S.E.M. unless noted otherwise. Fitting of the data was performed using Sigmaplot (SPSS, Inc.), as described in the legends. Quantitation of protein phosphorylation after autoradiography was performed using Quantiscan (Biosoft).

Results

Part I: Regulation of PDE6 by Pγ phosphorylation

Pγ is phosphorylated by PKA at Thr^{35} and by MAP kinase at Thr^{22}-- Previous phosphorylation studies have shown that Thr^{35} and Thr^{22} on rod Pγ can be phosphorylated by PKA and cdk5 *in vitro* (Xu et al., 1998; Matsuura et al., 2000; Matsuura et al., 2000). Here, we confirmed that recombinant Pγ could achieve stoichiometric levels of phosphate incorporation when incubated with PKA or MAP kinase. As shown in Fig. 3.1.A, the phosphorylation level of wild-type Pγ by PKA was close to 1 mole of phosphate incorporated per mole of Pγ. The site of phosphorylation on Pγ by PKA was confirmed by ^{32}P labeling of T35C and T22C Pγ mutants. Incubation of the T22C mutant with PKA resulted in stoichiometric phosphate incorporation, whereas the T35C mutant lacked a significant level of phosphorylation. Phosphorylation of Pγ by MAP kinase has not been previously described, but based on the substrate specificity of MAP kinase [Pro-X-(Thr/Ser)-Pro; (Stokoe et al., 1992)], we predicted that the proline-rich region of Pγ would support phosphorylation at Thr^{22}. As shown in Fig. 3.1.B, about 1 mol phosphate was incorporated into Pγ. The Pγ mutant in which Thr^{22} was replaced with cysteine failed to incorporate significant amounts of [^{32}P] compared to wild-type Pγ or Pγ substituted at Thr^{35} with a cysteine residue, suggesting that Thr^{22} was the site of phosphorylation. Additional support for the PKA and MAP kinase substrate specificity was observed when the Pγ peptides (Mou and Cote, 2001) were used for phosphorylation. The peptides containing Thr^{35} (but lacking Thr^{22}) could be phosphorylated only by PKA, while the peptides containing Thr^{22} (but lacking Thr^{35}) could be phosphorylated by only MAP kinase (data not shown).

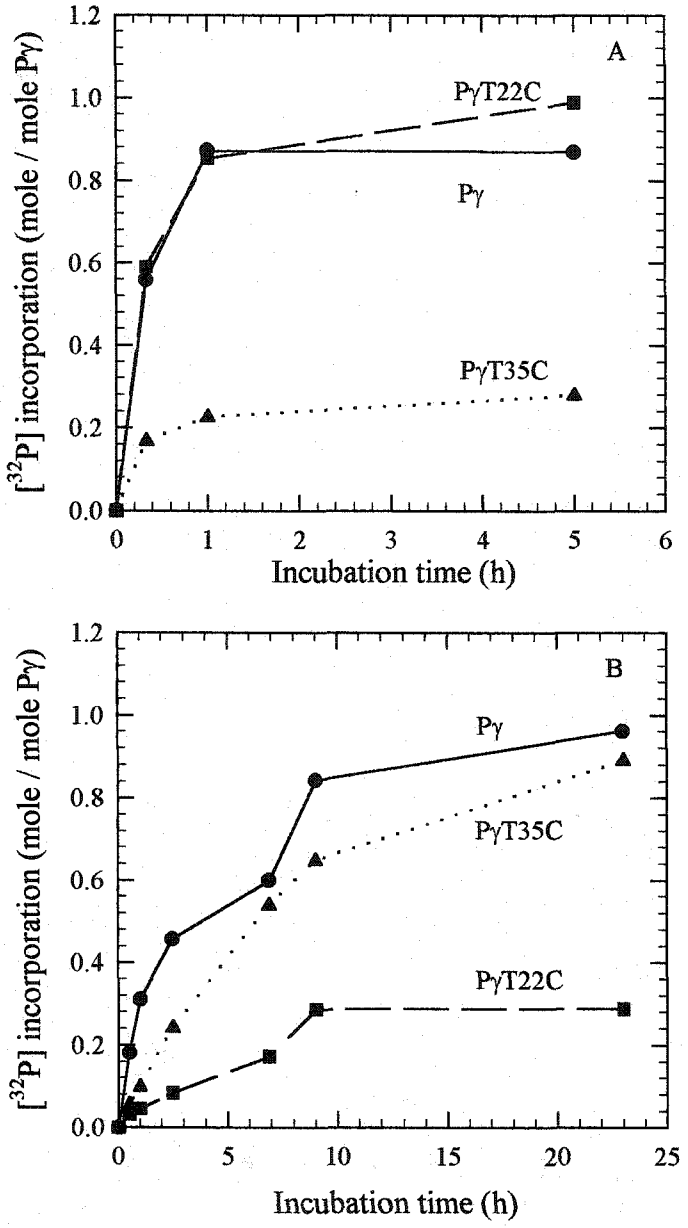


Figure 3.1. **Phosphorylation of Pγ and Pγ mutants (Pγ35C and Pγ22C) by PKA or MAP kinase.** The ability of PKA and MAP kinase to phosphorylate bovine rod Pγ at Thr^{35} and Thr^{22} was determined by ^{32}P labeling of wild type Pγ and two Pγ mutants: PγT35C and PγT22C. The phosphorylation assay was performed in a final volume of 50 µl assay dilution buffer containing 1.0 µM final concentration of Pγ or Pγ mutants, 100 µM [γ-^{32}P]ATP and either 50 ng PKA per tube (A) or 50 ng MAP kinase per tube (B). After incubating at 30°C for various times, aliquots were applied to a P81 phosphocelluse paper squares (see Experimental Procedures)

Pγ phosphorylation at either Thr^{22} or Thr^{35} only slightly modifies its inhibitory activity to Pαβ--The inhibition of the catalysis of PDE by Pγ is the primary mechanism to regulate PDE function in visual transduction. Thus, it is important to investigate if Pγ phosphorylation will affect its inhibitory function. Previous studies have proposed that Pγ phosphorylation either at Thr^{35} or Thr^{22} greatly enhanced the inhibitory potency of Pγ (Tsuboi et al., 1994b; Udovichenko et al., 1994; Xu et al., 1998; Matsuura et al., 2000; Tsuboi et al., 1994a; Tsuboi et al., 1994b; Udovichenko et al., 1994; Matsuura et al., 2000). However, in most cases, nanomolar concentrations of PDE were used and a predicted stoichiometric inhibition of catalysis of Pγ or phosphorylated Pγ should be observed (Wensel and Stryer, 1986; Mou and Cote, 2001; Mou and Cote, 2001). It is not clear how an enhancement of Pγ inhibition could be detected under these conditions. Therefore, we decided to re-examine whether phosphorylation of Pγ can directly alter its potency to inhibit PDE catalysis. We used a very low concentration of PDE (1 pM) to ensure that the equilibrium binding of Pγ and Pαβ could be observed (Fig. 3.2). A 1-site model did not fit well to the binding data, so a two-site model (Mou and Cote, 2001) was chosen. The results shown in Fig. 3.2 indicated that Pγ binding heterogeneity was not abolished upon phosphorylation at either or both threonine positions. In all four cases, the very high affinity binding site had a predicted K_D value of < 0.5 pM and displayed titration behavior. Such an extremely high affinity makes this Pγ binding site highly unlikely to be regulated by phosphorylation. Indeed, this Pγ might never dissociate from the PDE catalytic dimer during phototransduction, and perhaps functions as a scaffolding protein to stabilize PDE in its native conformation. For the second, lower affinity Pγ binding site, phosphorylation at Thr^{22} or Thr^{35} decreased the interaction of Pγ with Pαβ 2

to 2.5-fold, which was in sharp contrast to the 10-fold enhancing effects of Pγ phosphorylation reported previously (Tsuboi et al., 1994b; Udovichenko et al., 1994; Xu et al., 1998; Matsuura et al., 2000). Based on the above results, we concluded that phosphorylation had only a mild effect to decrease the binding affinity of Pγ to Pαβ.

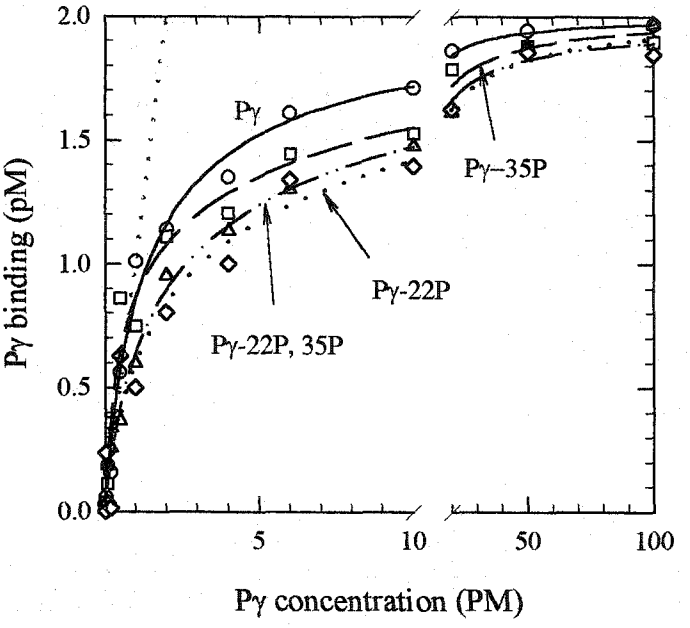


Figure 3.2. **Affinity of Pγ for Pαβ was not greatly affected by phosphorylation at Thr^{22}, Thr^{35} or both sites.** Bovine Pαβ was prepared by limited trypsin proteolysis and purified before use (see *Experimental Procedures*). Pγ phosphorylated at position Thr^{22}, Thr^{35} or doubly phosphorylated at both sites ($Thr^{22,35}$) was prepared by incubating Pγ with MAP kinase, PKA, or both kinases. Non-phosphorylated Pγ was treated identically, except that the protein kinase was omitted. To measure the binding affinity of Pγ or phosphorylated Pγ for Pαβ, Pαβ was diluted to 1 pM and then incubated for 10 min with the indicated concentration of Pγ or phosphorylated Pγ. Inhibition of cGMP (100 μM) hydrolysis was determined with the radiotracer assay. The data (averaged from three experiments) were fit to a double hyperbolic function, assuming two equal classes of binding sites. The higher affinity class of sites had predicted K_D values of < 0.5 pM in all four cases and displayed titration behavior (dotted line). The lower affinity class of sites had K_D values of 2.7 (Pγ, *circles*), 4.9 pM (Pγ35P, *squares*). 6.3 pM (Pγ22P, *triangles*) and 6.9 pM (Pγ22P35P, *diamonds)*

Pγ phosphorylation does not modify its interaction with transducin-activated PDE --- Several studies have suggested that Pγ phosphorylation reduces or abolishes the affinity of Pγ for activated transducin α-subunit ($G_t\alpha^*$) so that Pγ will dissociate from $G_t\alpha^*$ upon phosphorylation and re-inhibit the catalysis of PDE (Xu et al., 1998; Hayashi et al., 2000; Hayashi et al., 2000). If this is true, the phosphorylation of Pγ would serve to deactivate PDE without the turnoff the $G_t\alpha^*$. In this study, we re-examined if Pγ phosphorylation would modify its ability to inhibit transducin-activated PDE. It had previously been shown that inhibition of transducin-activated PDE (taPDE) in frog ROS homogenates requires excess Pγ since Pγ binds to $G_t\alpha$ - GTPγS as well as to the active site of PDE (Norton et al., 2000). As shown in Fig.3.3, the IC_{50} values for Pγ to inhibit bovine taPDE was 3.9 Pγ/PDE, which was somewhat lower than in frog ROS homogenates (Norton et al., 2000). Pγ phosphorylated at Thr^{22} (IC_{50} = 3.5 Pγ/PDE) or at Thr^{35} (IC_{50} = 4.3 Pγ/PDE) did not differ significantly from the non-phosphorylated control. Doubly phosphorylated Pγ (Pγ22P35P) was also tested, and the inhibitory curve was also similar to that of the Pγ itself (data not shown). Therefore, the reported dramatic effect of Pγ phosphorylation on inhibition of taPDE was not observed in our study. This indicated that Pγ has the similar affinity for transducin-activated PDE regardless of its state of phosphorylation.

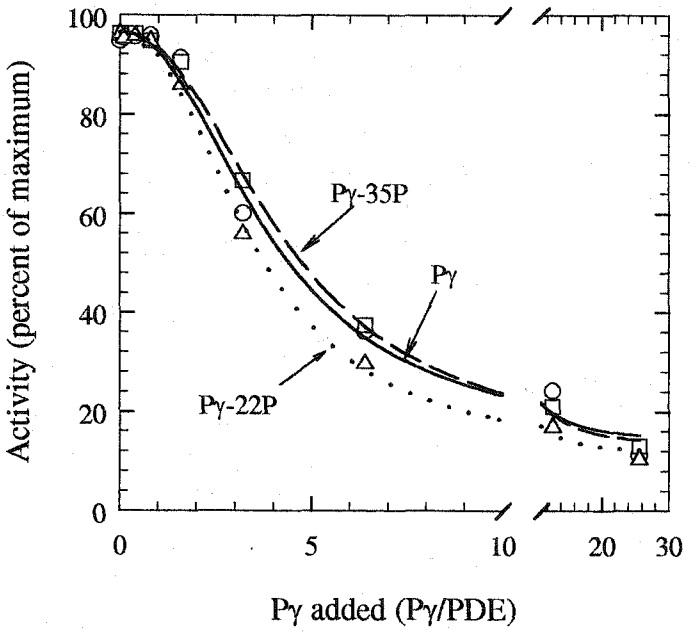


Figure 3.3. **Affinity of Pγ for activated transducin was not greatly affected by phosphorylation at Thr^{22} or Thr^{35}.** Purified bovine ROS (20-30 nM PDE) was incubated with increasing amounts of Pγ or phosphorylated Pγ for 10 minutes. 100 μM GTPγS was added 1 minute prior to 10 mM cGMP addition and PDE activity was measured. Maximal transducin-activated PDE activity (1100-1200 s^{-1}) was defined as the activity in the absence of added Pγ. The IC_{50} value for each Pγ was determined by fitting the data to a 4 parameter logistic function: non-phosphorylated Pγ (*circles*) IC_{50} = 3.9 Pγ/PDE; Pγ35P (*squares*), IC_{50} = 4.3 Pγ/PDE; Pγ22P (*triangles*), IC_{50} = 3.5 Pγ/PDE. This experiment is representative of three others.

Pγ phosphorylation has a moderate effect on the affinity of the Pγ central peptides for Pαβ -- It is possible that the effect of phosphorylation is confined to the central region of Pγ where the phosphorylation sites are located. The effect of phosphorylation on the overall binding affinity of Pγ to Pαβ might be offset in large part by other regions of Pγ in which its interaction with Pαβ is not modulated by phosphorylation. Therefore, we tested whether phosphorylation has any effect on the interaction of the Pγ central region for Pαβ. The central region of bovine rod Pγ is able to restore high-affinity cGMP binding to a low-affinity noncatalytic binding site on bovine PDE and to stabilize cGMP binding to both binding sites (Mou and Cote, 2001). In this study, we have synthetically prepared peptides corresponding to amino acids 18-41 with and without phosphothreonine residues at Thr^{22} or Thr^{35}. As shown in Fig.3.4, phosphorylation at either Thr^{22} or Thr^{35} did not affect the ability of Pγ18-41 to restore cGMP binding to a second low-affinity class of binding sites. In all cases, the highest cGMP binding levels approached the expected ~2 moles of cGMP bound per mole of PDE. After initiating cGMP dissociation from the noncatalytic sites, however, a reduced affinity of Pγ18-41(22P) can be inferred based on the 2-fold faster rate of cGMP dissociation. In contrast, the phosphorylation at position 35 enhanced 1.7-fold the ability of the peptide to stabilize cGMP binding to Pαβ. We conclude that phosphorylation at Thr^{22} weakens the intrinsic affinity of the central region of Pγ to interact with PDE6 while phosphorylation at Thr^{35} may have a slight stabilizing effect on binding.

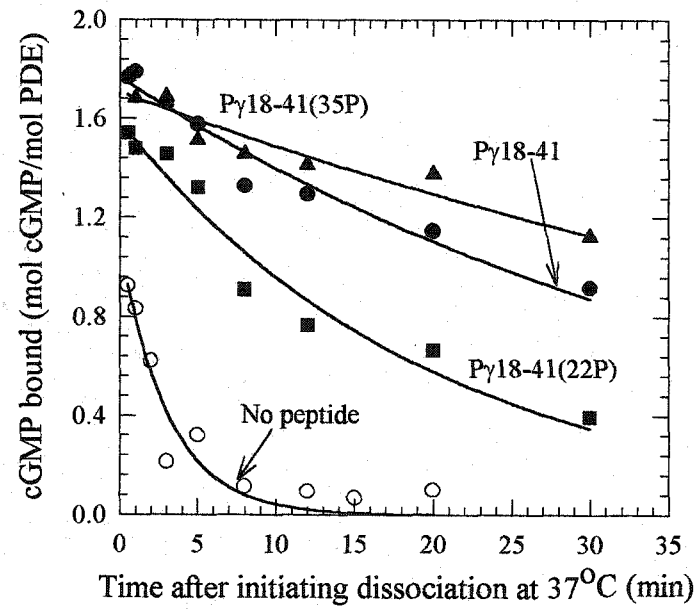


Figure 3.4. **Phosphorylation of the Pγ18-41 peptide at Thr[22] reduced its affinity for Pαβ as indicated by its potency to stabilize cGMP binding to Pαβ:** 5 nM bovine Pαβ was incubated with 0.6 μM [^{3}H]cGMP with or without 20 μM of the indicated Pγ18-41 synthetic peptides. Dissociation of bound [^{3}H]cGMP was initiated at time zero with 2 mM cold cGMP, and portions filtered at the indicated times. The curves represent a single exponential decay function: Pγ18-41 (filled circles), $t_{1/2}$ = 29.5 min; Pγ18-41(22P) (squares): $t_{1/2}$ = 13.8 min; Pγ18-41(35P) (triangles), $t_{1/2}$ = 50.2 min; no peptide (open circles), $t_{1/2}$ = 2.15 min. This experiment is representative of three others.

Next, we examined whether phosphorylation of Pγ peptides altered their ability to displace endogenous Pγ from its binding sites on the PDE holoenzyme (Pαβγγ). This competition experiment took advantage of the fact that the central region of Pγ binds with high affinity to Pαβ but is unable to inhibit catalysis (Mou and Cote, 2001). Both the central peptide (Pγ18-41) and the N-terminal half of Pγ (consisting of amino acid residues 1-45) were tested. Pγ1-45 with a phosphorylated residue at position 22 or 35 was prepared by incubation with MAP kinase or PKA, respectively. The data in Fig.3.5 illustrated that Pγ1-45 could compete with full-length Pγ to induce 70-100% of the maximal catalytic activity, while Pγ18-41 could stimulate up to 60% of the maximal activity. Phosphorylation at position 22 reduced 4-fold the ability of the peptide to stimulate catalytic activity. This result is consistent with our direct binding measurements of Pγ with Pαβ in Fig. 4 that Pγ phosphorylation at Thr^{22} decreases its interaction with PDE6. We also noticed that although Pγ1-45 also showed a significant drop in the stimulated catalysis when Thr^{22} was phosphorylated, this effect was more apparent in shorter peptide (Pγ18-41), where the maximal activation dropped from 58% to 16% when Thr^{22} was phosphorylated. This result supported the idea that the effect of phosphorylation is confined to a small region of Pγ where the phosphorylation sites are located.

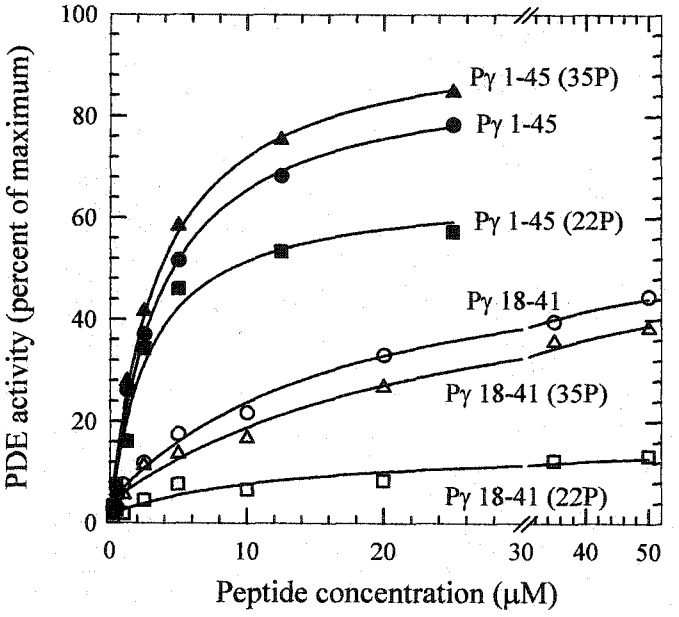


Figure 3.5. **Phosphorylation at Thr^{22} reduced the ability of Pγ peptides to displace bound Pγ.** Bovine PDE holoenzyme (5 nM) was incubated with increasing concentrations Pγ peptides to exchange with the endogenous Pγ bound to Pαβ. In the absence of peptide, the basal activity was 200 cGMP/PDE/sec, about 4 % of the fully activated rate. The extent of activation and the $K_{1/2}$ values for each peptides were determined by fitting the concentration dependence to a hyperbolic function: Pγ1-45, V_{max} = 89.3 %, $K_{1/2}$ = 3.6 μM; Pγ1-45 (35P), V_{max} = 97.0 %, $K_{1/2}$ = 3.5 μM. Pγ1-45 (22P), V_{max} = 65.7 %, $K_{1/2}$ = 2.8 μM; Pγ18-41, V_{max} = 57.8 %, $K_{1/2}$ = 17.2 μM; Pγ18-41 (35P), V_{max} = 56.2 %, $K_{1/2}$ = 25.2 μM; Pγ18-41 (22P), V_{max} = 15.6 %, $K_{1/2}$ = 13.0 μM; This experiment is representative of four others.

Pγ complexed with PDE is a poor substrate for phosphorylation ---- To test the conditions under which Pγ is readily phosphorylated, we first checked if Pγ complexed with PDE catalytic subunits was a good substrate for phosphorylation. Equal amounts of Pγ (either bound to bovine rod Pαβ or free in solution) were incubated with protein kinases and the extent of phosphorylation was measured at various times (Fig.3.6). We found that when Pγ was bound to Pαβ, very low levels of phosphorylation were detected. In contrast, free Pγ was readily phosphorylated with both PKA and MAP kinase, consistent with the results obtained with PKC (Udovichenko et al., 1994) and cdk5 (Matsuura et al., 2000). We confirmed this result by phosphorylating an increasing amount of recombinant Pγ in the absence or in the presence of fixed amount of Pαβ (Fig.3.7). When the added Pγ concentration was less than the concentration of binding sites on Pαβ, Pγ formed a complex with Pαβ and was poorly phosphorylated. Once all binding sites on Pαβ were occupied, PKA was able to incorporate phosphate into Pγ as easily as into free Pγ lacking Pαβ. We conclude that free Pγ is a better substrate for both kinases than Pγ complexed as Pαβγγ, suggesting that Thr^{35} and Thr^{22} are involved in the interaction of Pγ with Pαβ.

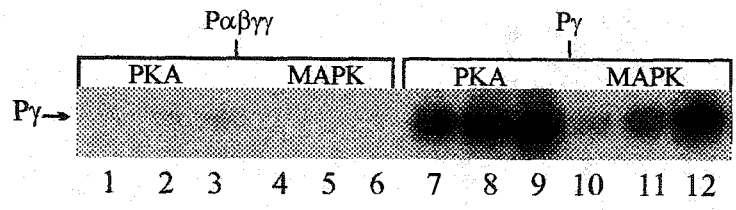


Figure 3.6. **Pγ bound to Pαβ was a poor substrate for phosphorylation.** Purified bovine PDE6 holoenzyme (~100 nM, lane 1-6) or free Pγ (~200 nM, same total Pγ concentration, lane 7-12) were phosphorylated in a final volume of 30 µl assay dilution buffer containing 50 µM [γ-^{32}P]ATP and either 50 ng PKA (lanes 1-3 and 7-9) or MAP kinase (4-6 and 10-12) per tube. The reactions were stopped with 10 µl of 5x gel loading buffer after 10 min (lane 1, 4, 7, 10), 30 min (2, 5, 8, 11) or 2 h (3, 6, 9, 12). The proteins were separated by SDS-PAGE and the incorporation of [^{32}P] into Pγ was detected by autoradiography.

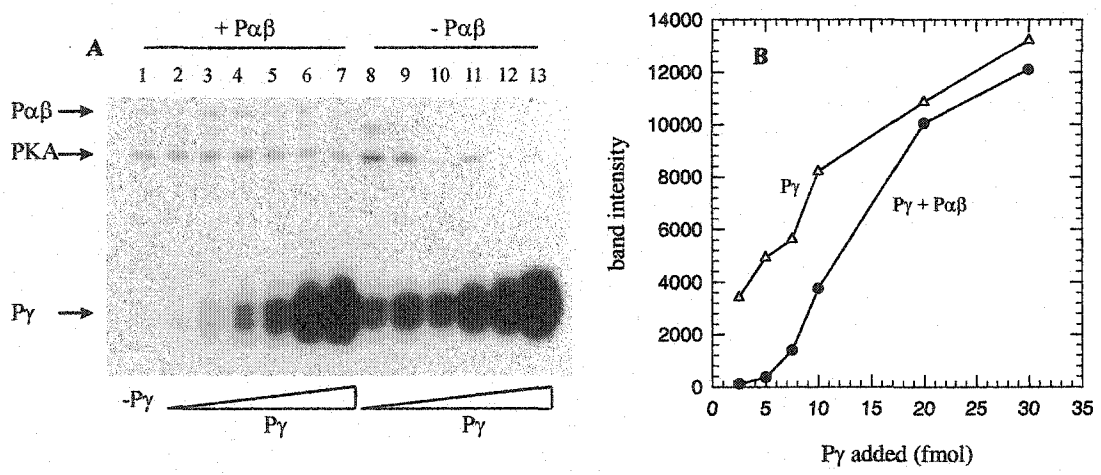


Figure 3.7. **Phosphorylation of Pγ by PKA in the presence of Pαβ.** The phosphorylation Pγ by PKA in the absence or in the presence of Pαβ was performed in a final volume of 30 µl assay dilution buffer containing 50 µM [γ-^{32}P]ATP and 3.4 fmol purified Pαβ (lane 1), increasing amounts of recombinant Pγ (from 2.5 – 30 fmol) in the presence of ~3.4 fmol Pαβ (lane 2-7), or in the absence of Pαβ (lane 8-13). 50 ng PKA/ tube was added to initiate the phosphorylation. After incubating at ~21°C for the 3 hours, proteins were quenched and separated on 15% SDS-acrylamide gel and the incorporation of ^{32}P into Pγ was detected by autoradiography (A). The Pγ binds bands were also scanned and the band intensity (in arbitrary units) was plotted versus the amount of Pγ added (B).

We considered the possibility that transducin activation of PDE6 might induce a conformational change in the central region of Pγ, allowing Pγ phosphorylation sites to be exposed. To test this, we incubated bovine ROS homogenates with [γ-^{32}P]ATP and phosphatase inhibitors under dark-adapted and light-activating conditions (i.e., light plus 100 μM GTPγS to activate transducin; see *Experimental Procedures*). We found that under dark-adapted conditions (nonactivated), almost no Pγ in ROS could be phosphorylated (Fig.3.8, lane 1, 4). For transducin-activated samples, some [^{32}P] was incorporated into Pγ (lane 2), but the total extent of Pγ phosphorylation was less than 3% of the level for free Pγ. Incubation of bovine ROS with the recombinant PDE δ subunit (Pδ) (lane 3) before adding [γ-^{32}P]ATP only slightly changed the degree of Pγ phosphorylation, suggesting that membrane-association of PDE is not important for Pγ phosphorylation. Furthermore, addition of a PKA inhibitor to ROS did not abolish Pγ phosphorylation (lane 5), suggesting that PKA is not the only protein kinase responsible for Pγ phosphorylation in bovine ROS homogenates. Based on the above results, we conclude that Pγ could be phosphorylated in an activation-stimulated manner, although the extent of phosphorylation does not approach stoichiometric levels. However, the sub-stoichiometric phosphorylation of Pγ in ROS constrains its potential role in regulating PDE in visual transduction pathway.

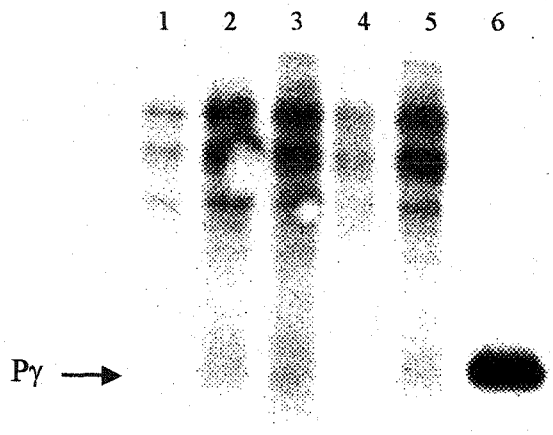


Figure 3.8. **Phosphorylation of Pγ in dark-adapted or light-activated bovine ROS.** Purified bovine ROS homogenates (60 μM rhodopsin) containing phosphatase inhibitors were incubated with 100 μM [γ-^{32}P]ATP for 5 h with in the dark (-GTPγS, lane 1, 4) or in the light (+100 μM GTPγS, lane 2, 3, 5). To investigate the effect of Pδ on Pγ phosphorylation, 2 μM of the recombinant Pδ (lane 3) was incubated with bovine ROS at 4°C overnight before phosphorylation. For lane 5, 20 μl of the PKA inhibitor was added to test whether the endogenous PKA activity is responsible for Pγ phosphorylation in ROS. After quenching the phosphorylation reaction, Pγ was released from ROS membranes with 20% acetonitrile, and partially purified on a Sep-Pak C-18 column. The same amount of recombinant Pγ was phosphorylated by PKA in vitro (lane 6) to estimate the stoichiometry of Pγ phosphorylation in bovine ROS.

Part II: Regulation of PDE6 by Pαβ phosphorylation

Phosphorylation of PDE catalytic subunits by PKA and MAP kinase – In part I, we have shown that both PKA and MAP kinases could phosphorylate Pγ stoichiometrically. In this part, we investigated whether these two kinases could phosphorylate the PDE α and β catalytic subunits *in vitro*. As shown in Fig.3.9, ^{32}P was incorporated into PDE catalytic subunits with either PKA or MAP kinase. Quantitation of the amount of ^{32}P incorporated into phosphorylated PDE (Fig.3.10) indicated that phosphorylated PDE contained a maximum of ~1.0 mol of phosphate per PDE when PKA was used as the kinase. Longer incubations could not enhance further phosphate incorporation into PDE, suggesting that PKA only phosphorylates one site on either Pα or Pβ. This result was inconsistent with a previous report that both PDE catalytic subunits can be phosphorylated by PKA (Udovichenko et al., 1993). However, in agreement with Udovichenko et al. (Udovichenko et al., 1993), we found that Pαβ, prepared by limited-trypsinization, could not be phosphorylated by PKA (data not shown). In addition, trypsinization also gradually removed the radioactivity from [^{32}P]-labeled PDE by PKA (data not shown). It is possible that the potential phosphorylation site for PKA is localized in the C-terminal region of Pαβ that is sensitive to trypsin proteolysis (Catty and Deterre, 1991). When MAP kinase was used to phosphorylate PDE, more than 1.0 mol of phosphate per PDE was incorporated. Longer time of incubation resulted in about 1.4 mol of phosphate per PDE (Fig.3.10, triangles). Therefore, MAP kinase phosphorylates Pαβ at two or more different sites. Similar to the case of PKA, the potential sites for MAP kinase are sensitive to proteolysis by trypsin (data not shown).

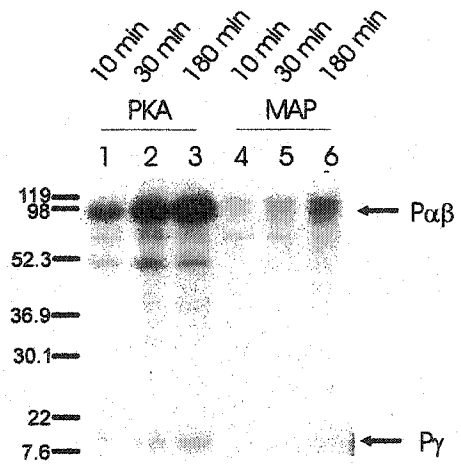


Figure 3.9. **Phosphorylation of the catalytic subunits of the PDE holoenzyme by PKA and MAP kinase.** Purified PDE holoenzyme was diluted to a final concentration of 200 nM with assay dilution buffer and 100 μM [γ^{32}P] ATP. Phosphorylation was initiated at room temperature by adding PKA (5 ng kinase/μg protein) or MAP kinase (5 ng kinase/μg protein) to the reaction mixture. After incubating at room temperature for various times, 20 μl aliquots were quenched and phosphorylation of PDE was detected by 15% SDS-PAGE followed by autoradiography.

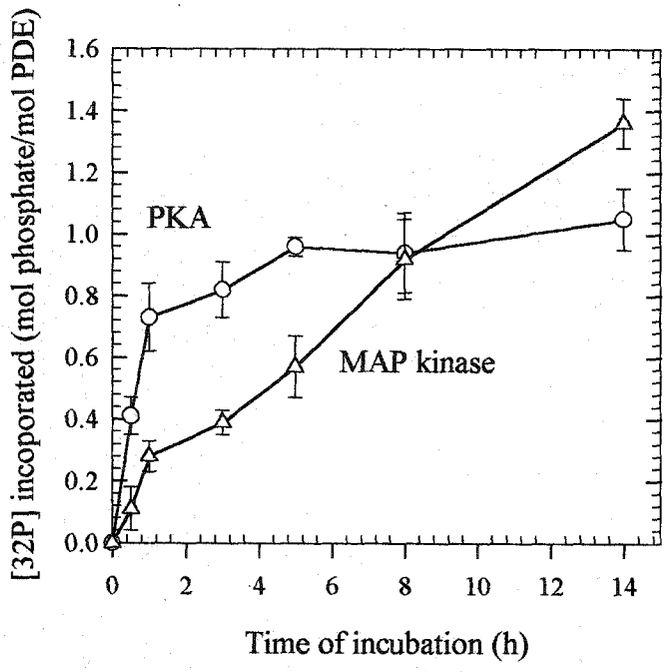


Figure 3.10. **Time course of phosphorylation of bovine PDE by PKA and MAPK.** Purified bovine PDE holoenzyme (15 pmol) was phosphorylated with 100 μM [γ-^{32}P]ATP (0.5 Ci/ μmol PDE) and 200 ng of PKA (circles) or MAP kinase (triangles) at 22°C in assay dilution buffer. At various times, aliquots were withdrawn, spotted onto phosphocellulose P81 paper and immersed in 75 mM phosphoric acid to terminate the reaction. The radioactivity was measured by liquid scintillation counting.

Phosphorylation of PDE by an unknown endogenous PDE6 kinase -- After establishing that PDE holoenzyme could be phosphorylated by PKA and MAP kinase *in vitro*, we next wanted to determine if PDE served as a substrate for phosphorylation *in situ*. After incubating bovine ROS homogenates with exogenous [γ-^{32}P]ATP, we found that ^{32}P could be incorporated into PDE catalytic subunits in a light-stimulated manner. However, the total level of phosphorylation was low (about 0.1-0.3 mol phosphate per mol PDE), compared to the control containing the same amount of purified PDE with exogenous PKA added (data not shown). In bovine ROS homogenates, PDE interacts with many proteins to form a supramolecular complex (Körschen et al., 1999). It is possible that these interactions might block the PDE phosphorylation sites so to result in a low stoichiometry of phosphorylation.

When we separated ROS membranes from soluble proteins followed by washing ROS membrane extensively with isotonic buffer, we found that ROS membranes retained their ability to incorporate phosphate into PDE catalytic subunits upon addition of the exogenous [γ-^{32}P]ATP (data not shown). This suggested that a membrane-associated kinase was responsible for the phosphorylation of PDE6. This membrane-associated protein kinase could be extracted by hypotonic buffer together with PDE-M (Fig.3.11, lane 1). It bound PDE-M tightly and was resistant to several purification steps (lane 2-4). PDE-M had two activity peaks on Mono-Q chromatography. PDE-M in peak I which contained no kinase activity was loaded on the gel-filtration to get purified PDE-M sample free of the endogenous kinase (lane 5). PDE-M in peak II was also further purified on gel-filtration without loss of kinase activity (data not shown), suggesting that

this endogenous kinase complexed with PDE with a high affinity. Soluble PDE fractions were also checked. After separating cone PDE (PDE-C) and soluble rod PDE (PDE-S) on a Q-Sepharose column, PDE-C could be phosphorylated by an unknown protein (lane 6), while no kinase activity was observed in the PDE-S peak (lane 7).

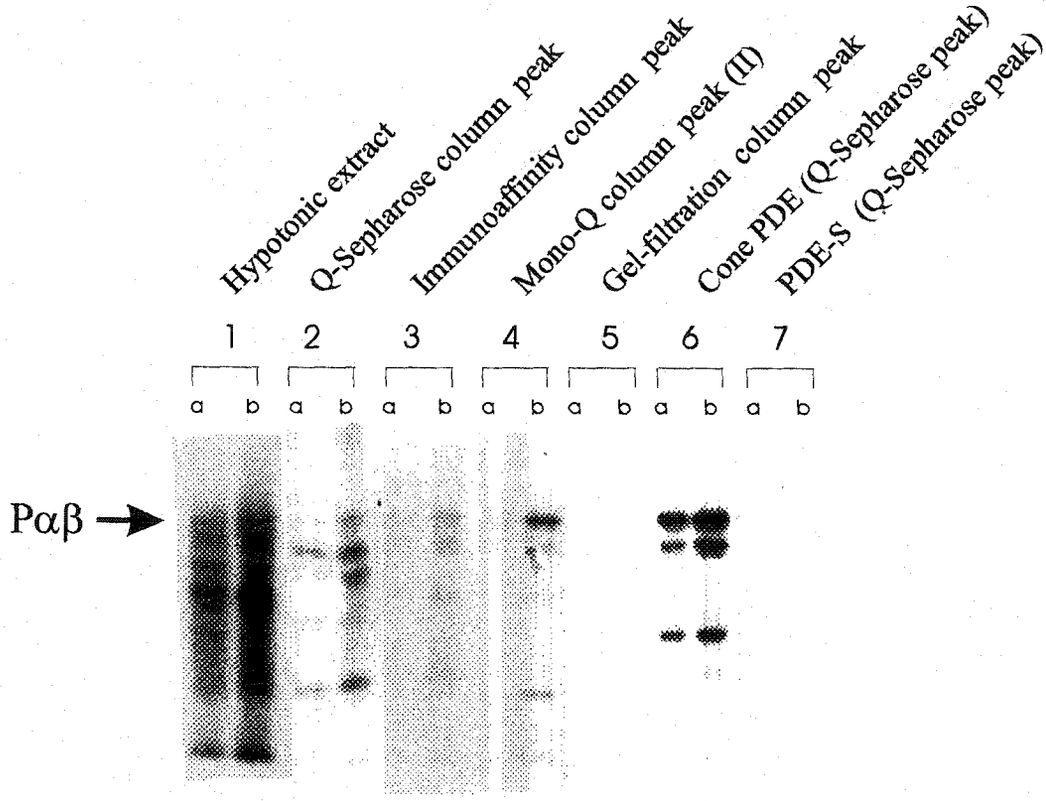


Figure 3.11. **Phosphorylation of PDE at different purification steps.** Bovine rod PDE was extracted from ROS membrane with hypotonic buffer (lane 1) and purified sequentially on a Q-Sepharose ion-exchange column (lane 2), ROS-1 immunoaffinity column (lane 3) and Mono-Q ion exchange column (lane 4). Two PDE activity peaks were detected after Mono-Q chromatography. The PDE peak II containing kinase activity was loaded in lane 4. The PDE peak I free of kinase activity was further purified on a Superdex 200 gel-filtration column (lane 5). Soluble proteins from bovine retinal extract were loaded on a Q-Sepharose ion-exchange column to separate cone PDE (lane 6) and PDE-S (lane 7). The same amount of PDE from each purification step (based on an activity assay) was incubated with 100 μM [γ-^{32}P] in the assay dilution buffer. No exogenous protein kinase was added. After incubating the phosphorylation mixtures at room temperature for 0.5 (a) or 2 h (b), all samples were quenched and separated on 15 % SDS-PAGE followed by autoradiography.

In order to identify the catalytic subunit of PDE being phosphorylated by protein kinases, we used a low percentage bisacrylamide gel (15% acrylamide and 0.04% bisacrylamide) to separate Pα and Pβ (Catty and Deterre, 1991). We found that the endogenous kinase phosphorylated only the Pα subunit, and PKA preferably phosphorylated the Pβ subunit. MAP kinase could phosphorylate PDE Pα and Pβ equally well (Fig.3.12). Thus, it appeared that the endogenous kinase is neither PKA nor MAP kinase. To date, we have not identified the protein kinase responsible for phosphorylating PDE *in vivo*. However, PKC is one candidate for the PDE kinase, since it is present in rod outer segment (Wolbring and Cook, 1991), and has been reported to phosphorylate only the Pα subunit *in vitro* (Udovichenko et al., 1993).

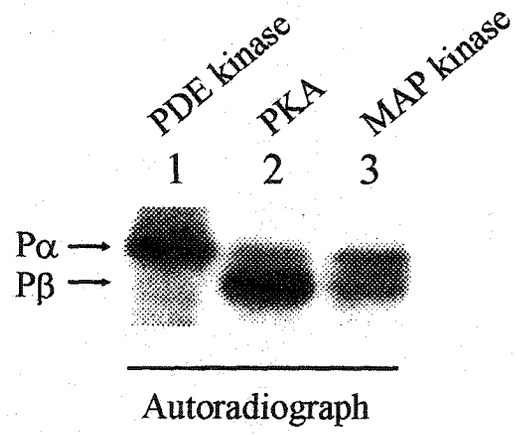


Figure 3.12. **Subunit specificity for phosphorylation by an endogenous kinase, PKA or MAP kinase.** For lane 1, PDE purified by ROS-1 affinity chromotography was diluted to ~50 nM with assay dilution buffer and a final concentration of 100 μM [γ-^{32}P]ATP. No exogenous kinase was added. For lane 2 and 3, PDE was purified by ROS-1 affinity chromatography, Mono-Q chromatography and gel-filtration chromatography (see Fig. 11); only trace amount of endogenous kinase have been detected to exist in this purified PDE sample. PDE was phosphorylated in the similar way except that PKA (5 ng kinase/μg protein) or MAP kinase (5 ng kinase/μg protein) was used. All reactions were incubated at 22°C for 5 hours. Pα and Pβ were separated on a 15% acrylamide, 0.04% bisacrylamide gel (18 h at 100 V), followed by autoradiography.

Depletion of noncatalytic cGMP binding enhances PDE catalytic subunit phosphorylation -- Work done with PDE5 has suggested that binding of cGMP to allosteric sites of PDE5 is required to expose the site of phosphorylation (Ser^{92}) by the cGMP-dependent protein kinase (Thomas et al., 1990; Turko et al., 1998; Francis et al., 1990; Turko et al., 1998). Since PDE6 is the closest to PDE5 both structurally and functionally, it is plausible that the GAF domains also allosterically regulate the phosphorylation of PDE6. In order to investigate this hypothesis, PDE holoenzyme with different states of cGMP occupancy were tested. PDE was depleted of its endogenous cGMP by incubating at 37°C overnight. Pδ was added to one PDE preparation to facilitate the dissociation of cGMP from the non-exchangeable binding sites (Mou et al., 1999). The results in Fig.3.13 show that depletion of cGMP from its binding sites enhanced the phosphorylation by increasing the rate of [^{32}P] incorporation. This enhancement was not due to the interaction of Pδ with PDE, since Pδ itself could not change the rate and degree of PDE phosphorylation when both noncatalytic cGMP binding sites were occupied (Fig.3.14).

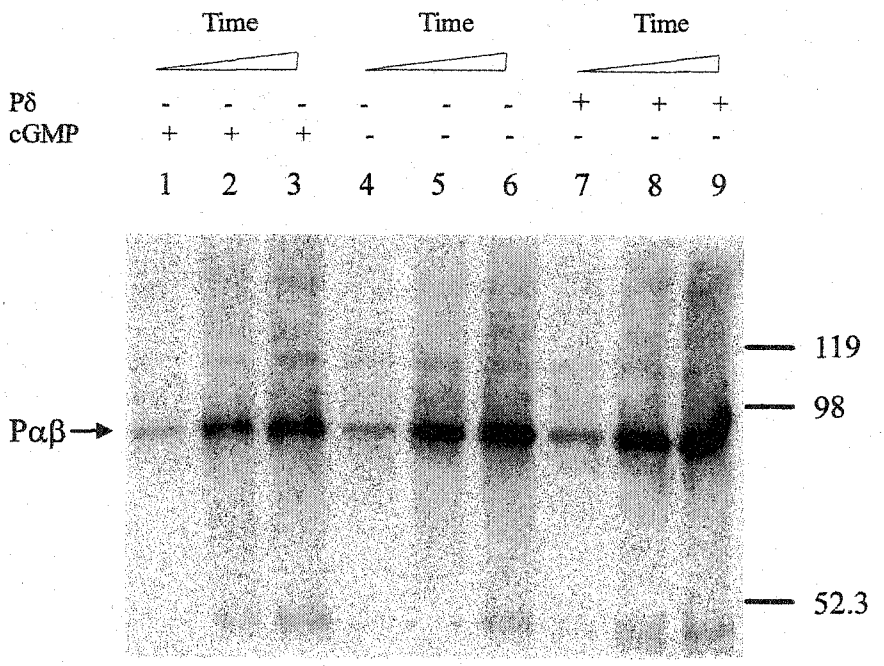


Figure 3.13. **Depletion of cGMP from the noncatalytic cGMP binding sites enhanced the phosphorylation of PDE holoenzyme.** ROS1-affinity chromotography purified bovine rod PDE-M was incubated at 37°C overnight to deplete the endogenous cGMP from its GAF domains. for one samples (lanes 7-9), Pδ was added to allow cGMP exchange at an otherwsie nonexchangeable site (Mou et al., 1999). After overnight incubation, PDE lost less than 20% its total PDE activity. For lane 1-3, PDE-M was replenished with 20 μM cGMP and 200 μM zaprinast immediately before phosphorylation. PDE was diluted to ~50 nM with assay dilution buffer and incubated with a final concentration of 100 μM [γ-^{32}P]ATP. No exogenous kinase was added. The reactions were stopped at 0.5, 3 and 6 hours by addition of 10 μl of gel sample buffer. PDE was separated on a 15% acrylamide, 0.04% bisacrylamide SDS-PAGE (18 h at 100 V), followed by autoradiography.

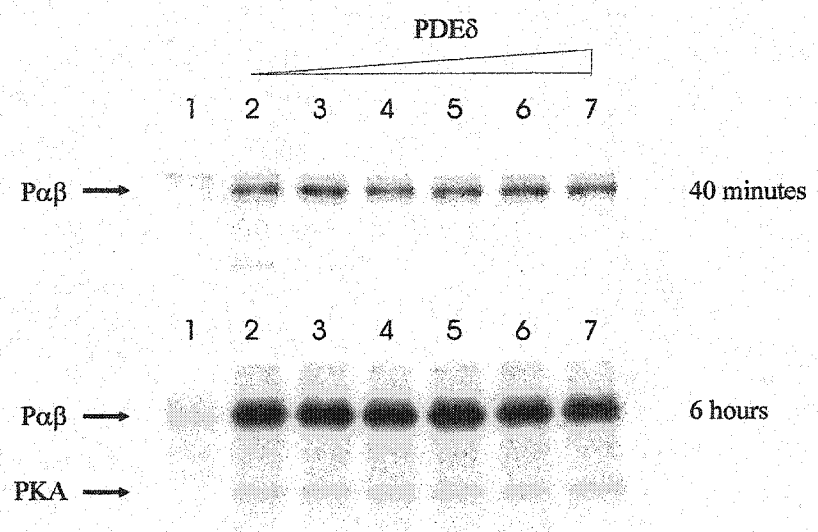


Figure 3.14. **Interaction of Pδ with PDE has no effect on the rate or extent of PDE phosphorylation when the noncatalytic cGMP binding sites are occupied.** 50 nM PDE holoenzyme (gel-filtration purified) was phosphorylated by 50 ng PKA in assay dilution buffer containing 200 μM zaprinast, 20 μM cGMP, 100 μM [γ-^{32}P] ATP and increasing concentrations of Pδ (lane 1 and lane 2: 0 nM; lane 3: 30 nM; lane 4: 60 nM; lane 5: 120 nM; lane 6: 300 nM; lane 7: 600 nM). PKA was omitted in lane 1. After incubating at 22°C for 40 minutes or 6 hours, the reactions were stopped by addition of 10 μl of electrophoresis sample buffer. PDE was separated by 10% SDS-PAGE, followed by autoradiography.

Effect of phosphorylation on the regulation of PDE function---- The possible function of PDE6 catalytic subunit phosphorylation has been studied. After phosphorylation, both PDE3 and PDE5 gain a higher k_{cat} and lower K_m for substrate at the active sites (Macphee et al., 1988; Beltman et al., 1993; Turko et al., 1998; Corbin et al., 2000; Macphee et al., 1988; Corbin et al., 2000; Turko et al., 1998). For PDE6, no alteration in k_{cat} or K_m has been observed upon phosphorylation of the catalytic subunits (data not shown). However, we discovered that the cGMP binding stoichiometry of bovine rod PDE at the noncatalytic sites was increased from 2 to 3-4 cGMP bound per Pαβ if ROS homogenates were incubated beforehand with ATP. The total amount of increase varied in different ROS preparations from no change to 2 cGMP per PDE (Fig.3.15). The reason for this variation is not known. In addition, this effect of phosphorylation on cGMP binding is much less apparent with purified PDE6. It is also not clear whether there is a direct relationship between the stoichiometry of phosphorylation and the extent of cGMP binding to Pαβ. The increase in cGMP binding stoichiometry upon ATP addition was also seen in frog ROS homogenates when exogenous ATP was added (Grazio, 1997). It is possible that phosphorylation of PDE6 induces a conformational change in the GAF domains on Pαβ so to convert low affinity cGMP binding sites to a state of higher binding affinity for cGMP.

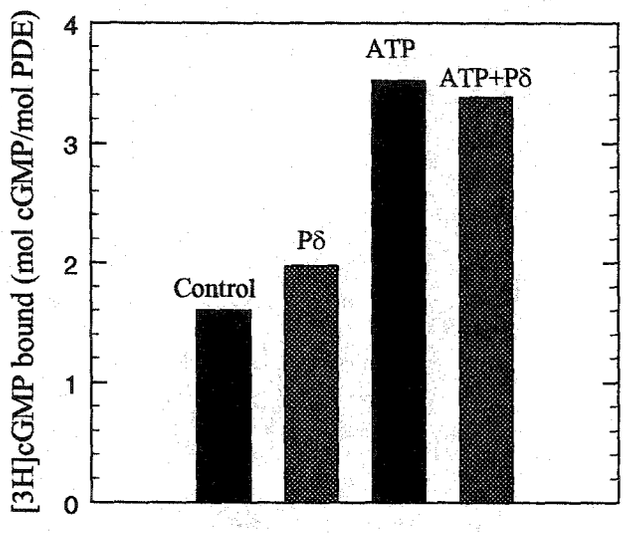


Figure 3.15. **Effect of PDE phosphorylation on cGMP binding to the GAF domains.** Purified bovine ROS was first diluted to 6.6 μM rhodopsin (~20 nM PDE) in 77 mM KCl, 35 mM NaCl, 2.0 mM $MgCl_2$, 1.0 mM $CaCl_2$, 1.18 mM EGTA ($[Ca^{2+}_{free}]$ = 240 nM), 10 mM HEPES, pH 7.5, 5.0 mM dithiothreitol, 2.5 μg/ml leupeptin, 1 mM Pefabloc, 3.5 μg/ml pepstatin, and 200 μM zaprinast. 1 μM of [^{3}H]cGMP was added to the binding mixture followed by incubating at 30°C for 24 hours to exchange the endogenous nucleotides for radioactive-labeled cGMP (column 1). The function assay determined after incubation indicated that loss of PDE activity was less than 20% over the 24 h period. To investigate the effect of Pδ and phosphorylation of Pαβ on the noncatalytic cGMP binding, 2 μM purified Pδ (column 2) or 200 μM of ATP (column 3) or 2 μM Pδ plus 200 μM ATP (column 4) was also added to the binding mixture. The amount of [3H]cGMP bound to PDE was determined by filter binding assay: cGMP binding to bovine ROS itself: V_{max} = 1.60 mol cGMP/mol PDE; bovine ROS + Pδ: V_{max} = 1.97 mol cGMP/mol PDE; bovine ROS + ATP: V_{max} = 3.52 mol cGMP/ mol PDE; bovine ROS + Pδ + ATP: V_{max} = 3.38 mol cGMP/mol PDE. This figure represents one of three similar experiments.

Discussion

Functional effects and potential physiological role of phosphorylation of Pγ at Thr^{22} and Thr^{35}. Several G-protein independent pathways for modulating PDE6 activity and lifetime have been suggested, such as GARP2-dependent inactivation of PDE6 (Körschen et al., 1999) and solubilization of PDE6 by Pδ (Cook et al., 2001). In this work, the role of Pγ phosphorylation in modulating the interaction between Pγ and Pαβ was studied. Our results failed to support the previous idea that Pγ phosphorylation is involved in a G-protein-independent pathway by directly enhancing the inhibitory ability of Pγ (Tsuboi et al., 1994a; Tsuboi et al., 1994b; Xu et al., 1998; Matsuura et al., 2000). We did not detect an increase in Pγ binding affinity or its inhibitory potency for PDE6 upon phosphorylation of Pγ at either Thr^{35} or Thr^{22}. On the contrary, we observed that Pγ phosphorylation decreases the ability of the central region of Pγ to bind to PDE, as well as modestly reducing its inhibitory potency. The reason for the discrepancy between our results and previously published work is probably due to the different experimental conditions used, as well as different interpretation of the observed results. In most of the published papers, nanomolar concentrations of PDE were used and it was difficult to extract meaningful binding affinity values. In this study, we used conditions where the K_D for Pγ could be accurately assessed as a result of phosphorylation.

Although no direct modulation of Pγ phosphorylation in the interaction of Pγ with PDE has been found, we did observe that transducin activation stimulates Pγ phosphorylation in bovine ROS, in agreement with previous reports (Tsuboi et al., 1994b; Hayashi et al., 2000). Together with the finding that Pγ complexed with PDE is a poor

substrate for phosphorylation, we suggested that the condition under which Pγ is likely to be phosphorylated is when Pγ is displaced from Pαβ binding sites by activated transducin. However, less than 5% of total pool of Pγ could be phosphorylated in bovine ROS, which makes the importance of this post-translation modification of Pγ in visual transduction questionable. It is unlikely that such a small effect could act directly onto PDE. We speculate that the light-induced increase in Pγ phosphorylation may have some alternative function in connecting the visual transduction pathway with other signaling pathways in the rod outer segment. The central region of Pγ contains a potential Type II SH3 domain ligand binding site, which is known to be involved in protein-protein interactions. The fact that Pγ is expressed in non-retinal cells (Tate et al., 1998) and can serve as a novel intermediate regulating p42/p44 MAP kinase signaling (Wan et al., 2001) suggests that Pγ may indeed act on other signaling pathways. Whether Pγ phosphorylation regulates its function in these novel pathways in photoreceptors is a subject for further study.

At this time, we are not clear which residues on Pγ are phosphorylated *in vivo*. To clarify this question, we will need to extract Pγ from the intact retinas or intact ROS under different dark and light conditions and to perform the mass spectrometric analysis of Pγ to determine the occurrence and site(s) of Pγ phosphorylation.

Functional effects and potential physiological role of phosphorylation of the catalytic subunits of PDE6: The occurrence and significance of phosphorylation of the PDE6 catalytic dimer for PDE6 regulation was studied in this work since many of known PDE families (PDE1, PDE3, PDE4, PDE5) have been reported to be regulated in a phosphorylation-dependent manner. In addition, phosphorylation of PDE6 catalytic dimer might represent an additional G-protein-independent pathway during light adaptation.

Our results showed that the PDE catalytic dimer is a substrate for several protein kinases, including PKA and MAP kinase *in vitro*, as well as an unknown endogenous, membrane-associated "PDE6 kinase" from bovine ROS. Protein kinase activity co-purifies with bovine PDE through several stages of purification, and represents one candidate for a PDE-binding protein.

No direct regulation of catalysis by the phosphorylation of PDE6 catalytic dimer has been found in our work. However, our results showed for the first time that the phosphorylation of PDE6 is allosterically regulated by its GAF domains. Depletion of cGMP from the GAF domains enhances phosphorylation of the PDE6 catalytic dimer, suggesting that occupancy of the GAF domains of PDE6 elicits a conformation change that masks the phosphorylation sites on Pα and/or Pβ. After Pαβ phosphorylation, low affinity cGMP binding sites are converted to their high affinity state, similar to the case for PDE5 (Corbin et al., 2000). Therefore, it is possible that Pαβ phosphorylation represents a negative feedback mechanism to regulate cGMP levels and to regulate the lifetime of active PDE during light adaptation. In this scenario, when photoreceptors are exposed to bright light or continuous light, the cytoplasmic cGMP will be decreased to a certain level to induce cGMP dissociation from the GAF domains. The unoccupied GAF domains in turn induce a conformational changes in Pαβ to decrease Pγ affinity for better GAP activity, consequently shortening the lifetime of active PDE. However, the empty GAF domains also will enhance the phosphorylation of Pαβ. Upon Pαβ phosphorylation, high affinity cGMP binding is restored to the low affinity binding sites leading to re-binding of cGMP which also promote Pγ association with Pαβ.

SUMMARY

The major objective of this study is to explore what is the functional significance of cGMP binding, as well as Pγ and Pαβ phosphorylation, for the mechanism of PDE regulation.

First, we characterized the cGMP binding properties to bovine rod PDE. We showed that two mammalian rod photoreceptor PDE isoforms (soluble and membrane-associated PDE) have two non-identical, high-affinity noncatalytic sites. Interaction of Pδ with PDE-S permits cGMP release from a second noncatalytic binding site on nPDE-S that remains functionally nonexchangeable in PDE-M. The Pγ subunit not only acts as an inhibitor of cyclic nucleotide catalysis at the active sites on Pαβ, but also serves to regulate the binding affinity of cGMP on the Pαβ dimer. Upon activation of PDE by removal of the Pγ subunits, one of the two classes of noncatalytic sites on the Pαβ dimer eliminates its detectable binding (K_D > 1 μM) of cGMP. The remaining cGMP binding sites exhibit a 3-fold reduction in overall binding affinity. In addition, we also demonstrated that Pγ subunit is primarily responsible for altering cGMP exchange upon activation.

Interactions between Pγ and the Pαβ are essential for the regulation of PDE function in vertebrate rod photoreceptors during visual transduction. In this study, we for the first time measured K_D of Pγ binding to mammalian rod PDE and found that the value is in the range of picomolar to sub-picomolar. Pγ binds to two distinct high-affinity binding sites on Pαβ when cGMP occupies the noncatalytic sites. Our work also

describes the reciprocal allosteric regulation of PDE6 resulting from Pγ binding to Pαβ and cGMP binding to high-affinity sites in the GAF domains of Pαβ. By using synthetic peptides, we revealed that Pγ has multiple sites of interaction with rod Pαβ: the C-terminus functions to inhibit the catalysis, while the central region functions to restore and stabilize cGMP binding to the GAF domain. We also mapped regions within the N-terminal half of the molecule that interact with Pαβ, some of which induce conformational changes at the GAF domain.

The role of Pγ phosphorylation in modulating the interaction between Pγ and Pαβ was also studied. Our results showed that Pγ binding affinity or its inhibitory potency for PDE6 was not increased upon phosphorylation of Pγ at either Thr^{35} or Thr^{22}. In contrast, Pγ phosphorylation decreases the ability of the central region of Pγ to bind to PDE, as well as modestly reduces its inhibitory potency. We speculate that the light-induced increase in Pγ phosphorylation may have some alternative function in connecting the visual transduction pathway with other signaling pathways in the rod outer segment, rather to be involved in a G-protein-independent pathway by directly enhancing the inhibitory ability of Pγ .

Finally phosphorylation of the PDE6 catalytic dimer was studied to evaluate if it represents a G-protein-independent pathway to regulate PDE during light adaptation. Our results showed that the PDE catalytic dimer is a substrate for several protein kinases, including PKA and MAP kinase *in vitro*, as well as an unknown endogenous, membrane-associated "PDE6 kinase" from bovine ROS. We also for the first time showed that the phosphorylation of PDE6 is allosterically regulated by its GAF domains. Depletion of cGMP from the GAF domains enhances phosphorylation of the PDE6 catalytic dimer.

After Pαβ is phosphorylated, low affinity cGMP binding sites are converted to their high affinity state, similar to the case for PDE5. Therefore, it is possible that Pαβ phosphorylation represents a negative feedback mechanism to regulate cGMP levels and to regulate the lifetime of active PDE during light adaptation.

The above work helps us understand how PDE is regulated during the visual transduction and light adaptation. In dark-adapted rod outer segments, the cytoplasmic cGMP concentration is about 4 μM. In this condition, both noncatalytic sites on nPDE-S and nPDE-M will be fully occupied. Pγ binds to Pαβ tightly and inhibits its catalysis. When photoreceptor cells are exposed to light, $G_t\alpha^*$ -GTP binds to Pγ and displaces it from its inhibitory site on Pαβ, causing activation of PDE. During the recovery phase of the flash photoresponse, activation of PDE results in the immediate loss of cGMP binding to one of the GAF domains on both PDE-M and PDE-S. As a result, its bound cGMP is released rapidly to the cytoplasmic space on the same time scale as recovery phase of the photoresponse to buffer free cGMP levels. However, upon cGMP release from the bound state, it is likely that the free cGMP would be destroyed by the still-active catalytic sites, and therefore not contributes to elevating the cytoplasmic cGMP levels during photoresponse recovery. Activation of PDE also reduces cGMP binding affinity 3-4 fold at the second cGMP binding site on PDE.

The noncatalytic sites might be indirectly involved in regulating the lifetime of transducin-activated PDE via modulation of Pγ binding affinity to Pαβ. In chapter II, we showed that there exists a reciprocal relationship between cGMP binding to the GAF domains and the binding affinity of Pγ to Pαβ. In bovine ROS, Pγ serves as a GTPase accelerating factor, acting in concert with the RGS9 protein. Therefore, dissociation of

bound cGMP from the noncatalytic sites reduces Pγ binding affinity to Pαβ and Pγ is partitioned from Pαβ to transducin. Consequently, Pγ interacts with RGS9 to stimulate GTPase activity of transducin to inactivate PDE during the recovery phase of the photoresponse.

Dissociation of Pγ from Pαβ together with $G_t\alpha$ also makes Pγ a good substrate for phosphorylation. Upon phosphorylation, Pγ may act on other proteins in a phosphorylation-dependent manner so that the several signaling pathways are connected. In addition to Pγ, PDE catalytic subunits are phosphorylated when the GAF domains becomes empty. Phosphorylation of Pαβ will elicit a conformation change on Pαβ so that low affinity cGMP binding sites are converted to their high affinity state, which also promotes Pγ re-association with Pαβ. In this scenario, Pαβ phosphorylation serves as a negative feedback mechanism to regulate cGMP levels and to regulate the lifetime of active PDE during light adaptation.

Although our work has made a significant advance in our understanding of the regulation of PDE during the visual excitation and adaptation, there are still many questions needing to be answered. For example, which cGMP binding site on PDE-M is the non-exchangeable site? Is the heterogeneity in Pγ binding correlated with the heterogeneity in two GAF domains as well as the different subunit composition of Pαβ? How does Pγ phosphorylation communicate with other signal pathways? Which endogenous protein kinase is responsible for phosphorylating Pαβ? Under what condition is Pαβ phosphorylated during visual transduction, and how does its phosphorylation contribute to a novel negative feedback mechanism to regulate cGMP levels and PDE function. All of these questions will be subjects for further study.

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