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**CHARACTERIZATION OF THE ACTIVATION AND ALLOSTERIC
REGULATION OF PHOTORECEPTOR PHOSPHODIESTERASE (PDE6)**

BY

YU-TING LIU

Bachelor of Medicine, Inner Mongolia Medical College, 2002

THESIS

Submitted to the University of New Hampshire
in Partial Fulfillment of
the Requirements for the Degree of

Masters in Science
in
Biochemistry

September, 2007

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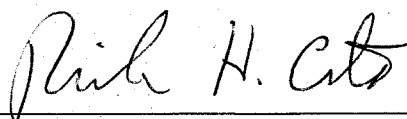
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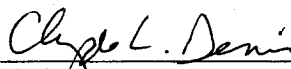
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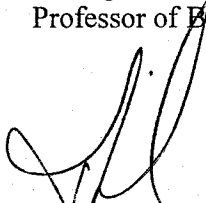
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Date

DEDICATION

I'd like to split this dedication into two parts: one is to my wonderful parents, JiYing Qiu and ZhenZhong Liu, and the other to my beloved husband, Gang Yao.

ACKNOWLEDGEMENT

I would like to thank all my family members, here and overseas, for their constant love, understanding and support throughout the years.

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ABSTRACT

CHARACTERATION OF THE ACTIVATION AND ALLOSTERIC REGULATION OF PHOTORECEPTOR PHOSPHODIESTERASE (PDE6)

by

Yu-Ting Liu

University of New Hampshire, September, 2007

Photoreceptor phosphodiesterase (PDE6) amplifies visual signals in rod and cone photoreceptors. We developed an assay to quantify binding of radiolabeled PDE inhibitors to the catalytic sites of PDE6 to explore the activation and regulation of PDE6. We determined that both catalytic domains on the PDE6 heterodimer are catalytically active and equivalent. Only one catalytic subunit on PDE6 readily binds to and becomes activated by the G-protein, transducin. The other PDE6 catalytic site requires a large excess of activated transducin to fully activate PDE6. We conclude that transducin activates the two catalytic subunits of rod PDE6 differently. We also demonstrated direct allosteric communication between the regulatory GAF domain and catalytic domain of PDE6: binding of a fragment of the inhibitory gamma subunit to the GAF domain stabilized radiolabeled PDE inhibitor binding to the catalytic sites; conversely, occupancy of the catalytic site by PDE inhibitors altered cGMP binding to the GAF domain.

CHAPTER 1

INTRODUCTION

A. Phosphodiesterase Superfamily

Cyclic nucleotide phosphodiesterases (PDEs) are enzymes that regulate the cellular levels of the second messengers, cAMP and cGMP. There are 11 different vertebrate PDE families (Beavo et al., 2006), with each family typically having several different isoforms and splice variants (Table 1.1). These distinct PDE families differ in their amino acid sequences, kinetic properties, modes of regulation, intracellular localization, cellular expression and inhibitor sensitivities.

A1. Rod and Cone Photoreceptor PDE6

Photoreceptor phosphodiesterase (PDE6) is the central effector enzyme in phototransduction in rod and cone photoreceptor cells. PDE6 is unique among PDE families for its regulation by a G-protein, transducin, for its isoprenylated C-terminus which attaches to the disk membrane, and for the regulation of its catalytic activity by inhibitory γ -subunits ($P\gamma$). Few differences between rod and cone PDE6 have been reported. Rod PDE6 is a tetramer, in which two $P\gamma$ tightly bind to non-identical α and β catalytic subunits ($P\alpha\beta$) (Baehr et al., 1979; Deterre et al., 1988). In contrast, cone PDE6 contains two identical α' catalytic subunits which are highly homologous to the rod α and

β subunits. The cone γ' -subunit ($P\gamma'$) is 3 amino acids smaller than rod $P\gamma$ but the amino acid sequences are very highly conserved (Ovchinnikov et al., 1986; Hurley et al., 1998).

Family	Number of genes	Substrate specificity	Tissue distribution	Specific Inhibitor
PDE1	3	dual	heart, brain, muscle, etc.	vinpocetine
PDE2	1	dual	adrenal, heart, lung, etc.	EHNA
PDE3	2	dual	heart, lung, liver, etc.	cilostamide
PDE4	4	cAMP	Sertoli cells, kidney, brain, etc.	rolipram, Ro201724
PDE5	1	cGMP	lung, platelets, smooth muscle	tadalafil (vardeafil, sildenafil)
PDE6	3	cGMP	photoreceptors	zaprinast (vardeafil, sildenafil)
PDE7	2	cAMP	skeletal muscle, heart, kidney, etc.	benzothieno- and benzothiadiazine dioxides
PDE8	2	cAMP	testis, ovary, small intestine, colon	none
PDE9	1	cGMP	spleen, intestine, kidney, heart, brain	BAY 73-6691
PDE10	1	dual	brain, testis	none
PDE11	1	dual	skeletal muscle, prostate	none

Table 1.1 Phosphodiesterase Superfamily. The Phosphodiesterase (PDE) superfamily contains 11 distinct members that degrade cellular second messenger cAMP and cGMP. The table contains the number of genes encoded each PDE, the substrate selectivity toward cAMP or cGMP, the existence in certain tissues, and the specific inhibitors reported for each family member to date. Inhibitors in parentheses are ones that should be considered PDE5/6 selective inhibitors. The information compiled from (Beavo et al., 2006).

PDE6 regulation by $P\gamma$ is essential for operation of the phototransduction cascade. Bovine rod PDE6 catalytic subunits contain two non-identical $P\gamma$ binding sites, both of which bind with very high affinity (Mou and Cote, 2001). Rod PDE6 has a higher affinity for $P\gamma$ than cone PDE6 does for its corresponding $P\gamma'$ (Hamilton et al., 1993).

Because the physiological responses of rods and cones to light differ greatly in their sensitivity, kinetics and adaptation properties, it is reasonable to hypothesize that some of these differences might be due to differences in the biochemical and/or regulatory properties of rod and cone PDE6. Prior studies of the enzymological properties of PDE6 indicated that they share similar kinetic properties [K_M and k_{cat} have been reported in rod and cone PDE6 (Gillespie and Beavo, 1988; Huang et al., 2004; Mou and Cote, 2001; Valeriani, 2004)]. However, most enzymatic studies have been focused on rod PDE6 [bovine $k_{cat} = 5440 \text{ s}^{-1}$; frog $k_{cat} = 7500 \text{ s}^{-1}$; (Norton et al., 2000; Mou and Cote, 2001)]. The results for cone PDE6 vary. For example, cone PDE6 k_{cat} has been reported to range from $3500\text{--}5600 \text{ s}^{-1}$ (Granovsky et al., 1998; Gillespie and Beavo, 1988; Valeriani, 2004).

The $P\gamma$ concentration is equal to PDE6 catalytic subunits in rod outer segments (Norton et al., 2000). In the dark-adapted state, almost all PDE6 exists as the non-activated holoenzyme ($P\alpha\beta\gamma\gamma$) by the binding of $P\gamma$ to $P\alpha\beta$ with very high affinity (Wensel and Stryer, 1986). The basal activity of rod PDE6 is equivalent to only 1 out of 2200 $P\alpha\beta$ being fully active [i.e., lacking $P\gamma$ (Rieke and Baylor, 1996; D'Amours and Cote, 1999)]. Removing $P\gamma$ from the catalytic dimer by limited proteolysis (Hurley and Stryer, 1982), by competition with polycationic proteins (Miki et al., 1975), or by extraction of $P\gamma$ by binding to activated transducin (Yamazaki et al., 1983) relieves $P\gamma$

inhibition and stimulates catalysis. The catalytic mechanism of PDE6 that involves transducin is not well understood at the molecular level. Chapter 2 presents experiments to explore how activated transducin causes PDE6 activation.

Studies on PDE6 inhibition by various classes of PDE6 inhibitors showed that inhibitors studied to date poorly discriminate rod and cone PDE6 (Gillespie and Beavo, 1989b; D'Amours et al., 1999; Zhang et al., 2005). Thus, further work is needed to examine the pharmacological properties of rod and cone PDE6 to determine whether differences in the active site can be discovered. Experiments described in Chapter 3 address this issue, which is of importance in evaluating whether difference in rod and cone PDE6 can account for differences in rod and cone cell light responses.

A2. Catalytic Subunits of PDE6

PDE6 catalytic subunits consist of a heterodimer of α and β subunits (Figure 1.1). Each catalytic subunit has a catalytic domain (similar in all 11 PDE families) attached to an N-terminal regulatory domain comprised of two tandem GAF domains (GAF α and GAF β) (Cote, 2006). In addition, a C-terminal isoprenylation motif allows the enzyme to be tethered to the photoreceptor disk membrane by farnesyl and geranylgeranyl groups (Figure 1.2) (Anant et al., 1992; Qin et al., 1992).

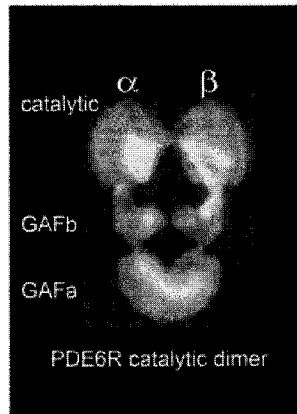


Figure 1.1 The Molecular Organization of PDE6. The organization of catalytic domains and two tandem GAF domains of rod PDE6 catalytic subunits is depicted as a 3-D model analyzed by electron microscopy (Kameni Tcheudji et al., 2001). The $\text{P}\gamma$ subunits could not be visualized at this level of resolution.

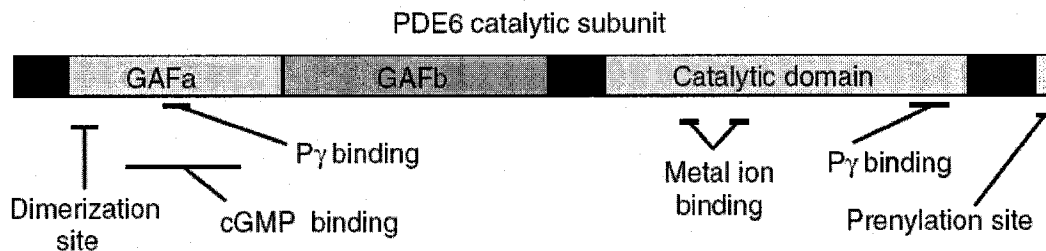


Figure 1.2 Domain Organization of the Catalytic Subunits of PDE6. The catalytic subunit of PDE6 consists of regulatory GAFa and GAFb domains, a catalytic domain, and a prenylated C-terminus. The noncatalytic cGMP binding and the N-terminal $\text{P}\gamma$ binding interaction sites are within GAFa domains and the GAFa domain also serves as the main functional site for dimerization. Metal ion binding within the catalytic site is critical for catalysis. The C-terminal region of $\text{P}\gamma$ binds to the catalytic site to inhibit the catalysis of PDE6.

A2a. Catalytic Domain of PDE6

The catalytic domain of PDE6 is comprised of approximately 280 amino acid residues located in the C-terminal part of the molecule and is highly conserved among all known PDE families (Cote, 2006). Because of the difficulty in expressing functional PDE6 catalytic subunits in heterologous systems (Granovsky et al., 1998; Muradov et al., 2006), no crystal structure or site-directed mutagenesis of PDE6 has been reported. The constructed chimeric protein containing both PDE5 and PDE6 sequences with structural homology modeling was studied for understanding the structure of the PDE6 catalytic domain. Activated rod PDE6 catalytic dimer has a ~1000-fold higher turnover number for cGMP than for the closely related PDE5 family. The basis for this difference is not entirely understood, but is likely due in part to differences of the metal-binding pocket of the active site of the two enzyme families. In one study, the k_{cat} was increased 10-fold when substituting two PDE5 residues for their PDE6 counterparts in the active site near the metal binding site (Granovsky and Artemyev, 2001). The histidine residues which bind zinc and magnesium in the active site of PDE6 are likely to be homologous to similar residues in the active site of PDE5 (Francis et al., 2000). Binding of divalent cations is critically important for both hydrolytic activity and enzyme stability of PDE6 (He et al., 2000). The presence of a “glutamine switch” in PDE6 likely confers substrate specificity for cGMP over cAMP (Zhang et al., 2004).

A2b. GAF Domains

The N-terminal half of the PDE6 catalytic subunits contains two structural motifs called GAF domains (GAFa and GAFb) because of their presence in cGMP-regulated

PDE, adenylate cyclases, and the *E. coli* protein Fh1A (Aravind and Ponting, 1997). The tandem GAF domains of PDE6 are highly conserved in amino acid sequence within the PDE6 family, as well as showing strong sequence similarity to the other four GAF-containing PDE families (PDE2, PDE5, PDE10, and PDE11). For PDE2, PDE5 and PDE6, binding of cGMP to the regulatory GAF domains has been demonstrated experimentally (Gillespie and Beavo, 1989a; Martins et al., 1982; Thomas et al., 1990; Yamazaki et al., 1982). Noncatalytic cGMP binding has been shown to directly stimulate the catalytic activity of PDE2 and PDE5 (Martins et al., 1982; Rybalkin et al., 2003). The functional significance of the GAF domains of PDE6 is not well understood. In PDE6, cGMP binding to regulatory GAF domains enhances the affinity of the inhibitory P γ subunits for the catalytic subunits, but does not appear to influence the catalytic activity independent of P γ binding. Reciprocally, P γ binding enhances the affinity of cGMP binding to the GAF domains (Cote et al., 1994; Mou and Cote, 2001).

Determination of the crystal structure of PDE2A GAFa-GAFb domains with cGMP bound revealed that the cGMP binding site lies within the PDE2 GAFb domain (Martinez et al., 2002). Furthermore, the residues stabilizing cGMP binding represent a novel cGMP binding motif. Sequence comparison of PDE2, PDE5 and PDE6 predict that the GAFa—rather than GAFb—domain is the likely site for cGMP binding in both PDE5 and PDE6 (Martinez et al., 2002). This prediction has been confirmed for PDE5 (Liu et al., 2002; Sopory et al., 2003) and PDE6 (Huang et al., 2004; Muradov et al., 2004) through the identification of several amino acid residues important for high-affinity interactions with cGMP. Specifically, several residues essential for stabilizing high-

affinity cGMP binding to PDE6 were only found within or close to the H4 helix in cGMP-binding PDEs (Huang et al., 2004; Muradov et al., 2004).

The structural model of the PDE6 holoenzyme (Figure 1.1) suggests that the GAFa domains on the catalytic subunits of rod and cone PDE6 also contain the dimerization sites which are critical for forming tightly bound catalytic dimers (Kameni Tcheudji et al., 2001). A mutagenesis study in the N-terminal region of the GAFa domain confirmed this function and also pointed out that the formation of $\alpha\beta$ catalytic heterodimer is strongly selected over $\alpha\alpha$ or $\beta\beta$ formation (Muradov et al., 2003). This work also supported early reports of rod heterodimer formation (Artemyev et al., 1996b).

There are also interaction sites within the GAFa domain which bind to the N-terminal inhibitory $P\gamma$ subunits of PDE6 (see section A3 below). The binding of $P\gamma$ to the two GAFa domains on the PDE6 catalytic dimer is non-identical, as is also true for noncatalytic cGMP binding, and this heterogeneity suggests structural differences of the GAFa domains of the α -subunit and β -subunit (Guo et al., 2005).

Unlike well discovered GAFa domain, the function of GAFb domain remains unclear. The GAFb domain of PDE6 contains a 29-amino acid sequence inserted in its signature residues (NFKDE) that is absent in the GAFa domain sequence; this GAFb insert may impair cGMP binding at this site. A mutagenesis study determined that an asparagine residue in the PDE6 GAFb domain is critical for cGMP binding. This result may be due to indirect stabilization of cGMP binding in GAFa domain by conformational change of the GAFb domain (Granovsky et al., 1998). Although there is no evidence regarding the role of GAFb, it is possible that GAFb helps stabilize the formation of the $P\alpha\beta$ catalytic dimer (Muradov et al., 2003).

A3. Inhibitory Subunits

PDE6 is unique among the PDE families in having its catalytic activity regulated by its inhibitory P γ subunit. Two regions of P γ are primarily involved in its interaction with PDE6 catalytic subunits (Figure 1.3), the central polycationic region (residues 21-45 of rod P γ) and the P γ C-terminus. The polycationic region makes a major contribution to the overall affinity of P γ for PDE6 catalytic subunits (Artemyev and Hamm, 1992; Mou and Cote, 2001). A cross-linking study using a photoexcitable peptide probe corresponding to the polycationic region of P γ revealed incorporation of the probe into the GAFa domain of rod Pa at residues Met¹³⁸Gly¹³⁹ (Muradov et al., 2002). In addition, mutagenesis of PDE6-specific amino acids in the vicinity of this site can abolish binding of P γ to the catalytic dimer (Muradov et al., 2004).

The C-terminus of P γ represents the key inhibitory domain and apparently directly occludes the catalytic cavity (Granovsky et al., 2000). By direct binding of its C-terminal residue to the catalytic pocket, P γ blocks the access of cGMP binding to the catalytic sites of PDE6 (Artemyev et al., 1996a; Granovsky et al., 1997).

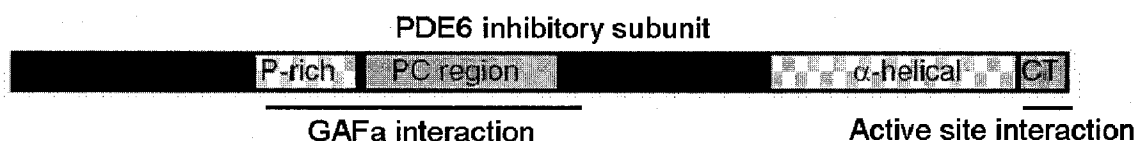


Figure 1.3 Domain Organizations of the Inhibitory Subunits of PDE6. The proline-rich region (P-rich) and polycationic region (PC region) at the N-terminal region of P γ subunits is responsible for the P γ interaction with GAFa domain of PDE6. The α -helical region (α -helical), and the C-terminal (CT) residues are responsible for the P γ binding to the active site of PDE6.

A4. Pharmacology of PDE6

The substantial homology of PDE6 with PDE5 has implications for development of inhibitors targeting PDE5 or PDE6. Of the 11 mammalian PDE families, Rod and cone PDE6 is most closely related to PDE5 (abundant in vascular smooth muscle) in its biochemical, structural and pharmacological properties (Cote, 2004). Both PDE5 and PDE6 have highly conserved amino acid sequences (Figure 1.4) and 3-dimensional structures (Cote, 2004). PDE5 and PDE6 share strong substrate specificity for cGMP compared to cAMP (Beltman et al., 1995). Most PDE5-selective inhibitors, including sildenafil (Viagra; Pfizer) and vardenafil (Levitra; Bayer Pharmaceuticals)—two drugs approved for treatment of male erectile dysfunction—also potently target PDE6.

Indeed, a major challenge in developing PDE5-specific inhibitors for therapeutic purposes is the similarity in the catalytic sites of PDE5 and PDE6 with respect to drug binding. Preclinical and clinical data on the effects of sildenafil have revealed significant but transitory effects on visual function, presumably through inhibition of photoreceptor PDE6 (Laties and Zrenner, 2002). Sildenafil is a highly selective inhibitor of PDE5 ($K_i = 4$ nM) with one exception, namely its potent inhibition of rod and cone PDE6 ($K_i = 30$ nM) (Ballard et al., 1998; Zhang et al., 2005). Tadalafil and vardenafil, two other approved drugs, show lesser effects on visual function (Uckert et al., 2003). Electroretinogram studies have shown that PDE5 inhibitors exert a modest effect on visual function (Luu et al., 2001).

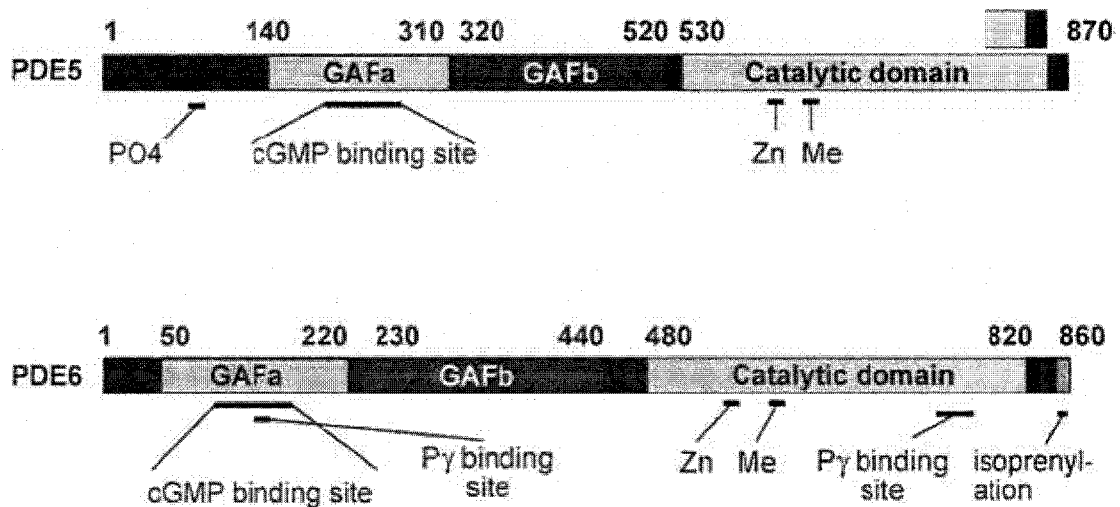


Figure 1.4 Similar Domain Organizations between PDE5 and PDE6. The catalytic subunits of PDE5 and PDE6 share a similar domain organization. Both enzymes contain regulatory GAF domains which are responsible for noncatalytic cGMP binding. In addition, PDE6 GAFa also contains a region that interacts with the inhibitory γ -subunit. The catalytic domains of PDE5 and PDE6 are also highly homologous, with a similar catalytic core containing metal ion binding sites. For PDE6, the Py binding site within the catalytic domain is responsible for the inhibition of hydrolysis upon Py binding. PDE6 contains a farnesyl (α subunit) or geranylgeranyl (β subunit) group attached to its C-terminus while PDE5 has a phosphorylation site at its N-terminus.

B. Visual Transduction

Cyclic nucleotides (cAMP and cGMP) are second messengers in all eukaryotic cells. As the primary intracellular messenger for the light response in rod and cone photoreceptor cells, the cellular level of cGMP is precisely controlled by the balance of its synthesis (via guanylate cyclase) and its degradation (via PDE6). In the dark, cGMP is maintained at a high concentration that permits opening of some of the cGMP-gated ion channels. When exposed to light, the acceleration of PDE6 hydrolysis causes a rapid drop in cGMP levels leading to the closure of cGMP-gated ion channel and membrane hyperpolarization. It is obvious that the lifetime of activated PDE6 must be a highly

regulated process. The activation of guanylate cyclase (regulated by calcium) to synthesize cGMP is coupled with PDE6 inactivation to quickly recover cGMP levels to their dark-adapted state.

B1. Rod and Cone Cells

The first step in seeing occurs in the retina where two classes of photoreceptor cells rods and cones (Figure 1.5), convert light into electrical signals which can be transmitted neurally to the brain. In darkness, the visual transduction pathway in rod photoreceptors has high photosensitivity and high amplification sufficient to detect even a single photon. But, during daylight illumination conditions, the rod photoresponse is saturated and vision depends on cone photoreceptors. Cone photoreceptors are much less sensitive than rods, but possess the important property that their photoresponses do not saturate even at the brightest illumination conditions. In addition, most vertebrates have only one visual pigment for rod and two or more types of cone photoreceptors that differ in the spectral sensitivity of their visual pigments. (Rodieck, 1998; Fain, 2003)

Although rod and cone photoreceptors share the same overall biochemical machinery to carry out phototransduction, they differ in many respects: (1) cellular anatomy; (2) the central phototransduction proteins (including rod and cone PDE6 subunits) in cones are homologous but not identical to those in rods; (3) a few photoreceptor proteins (e.g., GARP2) are only found in one photoreceptor type, or differentially expressed (e.g., RGS-9) in rods and cones. These differences are likely to account for some or all of the differences in rod and cone phototransduction pathways.

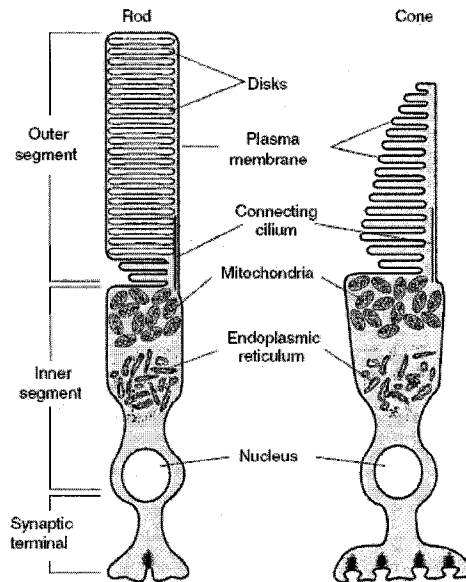


Figure 1.5 Schematic Diagram of Vertebrate Rod and Cone Photoreceptors. The outer segment of a rod photoreceptor (left) consists of separate, flattened disk membranes which are stacked together. For cone cells (right), the outer segments are formed as continuous invaginations of the plasma membrane. Connected to the outer segment is the mitochondria-rich ellipsoid portion of the inner segment which communicates with second-order retinal neurons.

The outer segment of rod and cone photoreceptors is the compartment where phototransduction occurs and where initial membrane hyperpolarization is triggered. The phototransducing outer segment portion of the cell consists of a densely packed membranous system optimized for photon capture and signal transduction. Thousands of separate, flattened disk membranes are stacked together in the rod outer segment, which are distinct from the plasma membrane. For cone cells, the outer segments are formed as continuous invaginations of the plasma membrane. The closely packed disk membranes in the outer segments greatly reduce free diffusion in the cytoplasm, resulting in the restricted spread of the visual excitation pathway. (Rodieck, 1998)

B2. Visual Excitation Pathway

The disk membrane of the photoreceptor outer segment is the location where the protein components of visual excitation transform a photon of light into an amplified signaling pathway that leads to PDE6 activation and drop in cGMP levels. The drop in cellular cGMP causes closure of a plasma membrane channel that triggers a receptor potential. The central components of this disk membrane-localized signaling pathway consist of the integral membrane protein, rhodopsin, the heterotrimeric G-protein, transducin, and the photoreceptor PDE (PDE6). Rhodopsin is a 40 kDa, seven transmembrane segment receptor to which the chromophore 11-cis retinal is bound. The dense packing of rhodopsin molecules in the disk membrane (equivalent solution concentration of 6 mM) ensures a high probability of photon capture. Transducin is a peripheral membrane protein anchored to the disk membrane by both its myristoylated α -subunit and by its farnesylated γ -subunit (which exists in tight complex with its β -subunit). PDE6 is also confined to the disk membrane by isoprenylation of its catalytic subunits. The high concentrations of rhodopsin, transducin (0.6 mM), and PDE6 (20 μ M)—along with their confinement to the disk membrane—serve to optimize the sensitivity, speed, and amplification properties of these initial events in vertebrate phototransduction.

The first step of seeing begins with the photoisomerization of 11-cis retinal to the all-trans isomer that is covalently bound to rhodopsin (Figure 1.6). Activated rhodopsin (R^*) undergoes a conformational change in its cytoplasmic loops that allows this transmembrane receptor to bind and activate transducin. Transducin activation occurs when the $T\alpha$ -GDP subunit (inactive) undergoes nucleotide exchange, and $T\alpha$ -GTP (active) dissociates from the transducin $\beta\gamma$ dimer. The released $T\alpha$ -GTP then binds to the

nonactivated PDE6 holoenzyme and displaces the inhibitory $P\gamma$ subunit to accelerate cGMP hydrolysis at the active site of PDE6. The rapid drop in cytoplasmic cGMP levels leads to dissociation of cGMP from the ligand-gated cation channel, and the channel changes from the open (dark) state to the closed (light) state. Membrane hyperpolarization results in generation of the receptor potential at the photoreceptor synapse.

As the main effector enzyme emphasizing visual signaling in photoreceptors, the activity of PDE6 has to be precisely regulated. A greater than 100-fold activation of PDE6 by transducin is coupled to catalytic rates for cGMP hydrolysis that approach the diffusion-controlled limit. In summary, PDE6 functions with high efficiency and in a highly controlled manner to regulate the cellular level of cGMP in the outer segment on the millisecond time scale required for vision (Pugh, Jr. and Lamb, 1993; Arshavsky et al., 2002).

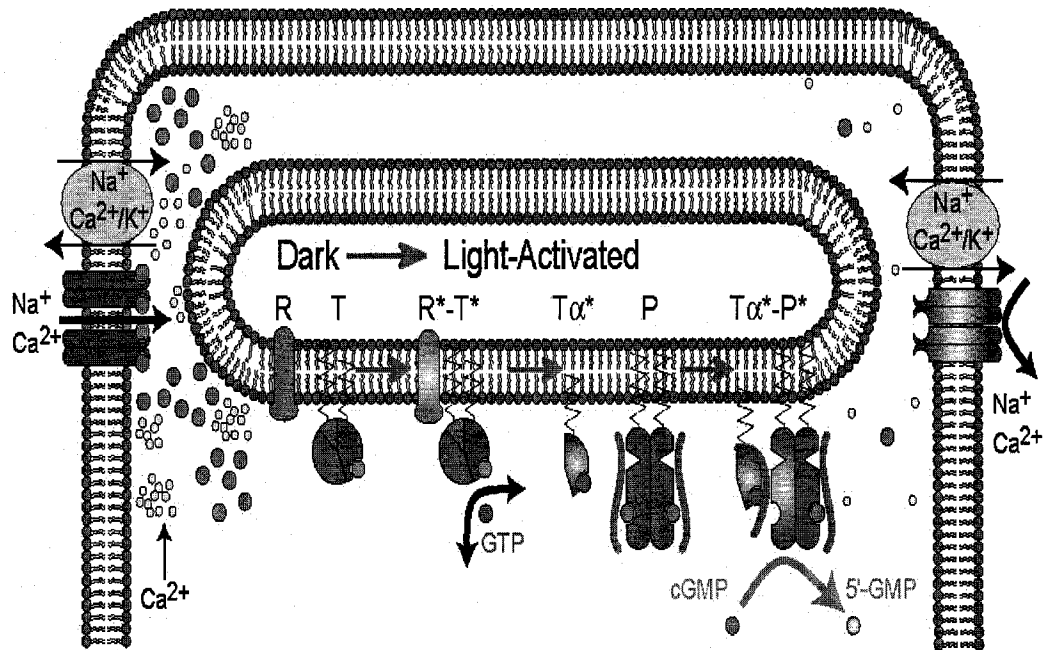


Figure 1.6 The Photo-excitation Pathway in Vertebrate Rod Photoreceptors. The initial events of phototransduction occur on the physically separate disc membranes in the outer segment of rod cells. When light reaches the retina, photo-activation of rhodopsin (R^*) catalyzes the activation of hundreds of heterotrimeric G-proteins, transducin (T). Then, T α -subunit ($T\alpha$) undergoes nucleotide exchange from GDP to GTP leaving transducin $\beta\gamma$ -subunits. The associated $T\alpha^*$ -GTP subunit then interacts with the PDE6 holoenzyme (P), displacing the γ -subunit of PDE6 and relieving inhibition of catalysis at one catalytic subunit. The activation of PDE6 results in rapid hydrolysis of cytoplasmic cGMP. The drop in cGMP levels in the outer segment causes the closure of cGMP-gated ion channels and membrane hyperpolarization.

B3. Transducin Regulates PDE6

Upon transducin activation, the $P\gamma$ subunits of PDE6 are displaced from $P\alpha\beta\gamma\gamma$ by $T\alpha$ -GTP, relieving the hydrolytic activity at the catalytic site of PDE6 (Stavenga et al., 2000; Arshavsky et al., 2002). Whether each activated transducin $T\alpha$ -GTP activates one or both PDE6 catalytic subunits is unclear at present. Some studies suggest that two activated transducins bind the PDE6 catalytic dimer (i.e., 1:1 subunit molar ratio) to

stimulate hydrolytic activity to its full extent (Wensel and Stryer, 1990; Leskov et al., 2000). Other reports suggest that single Ta-GTP was able to maximally activate the PDE6 catalytic dimer (i.e. 1:2 subunit molar ratio) under certain conditions (Bruckert et al., 1994; Melia et al., 2000; Yamazaki et al., 2002). The latter study suggested that either PDE6 catalytic dimer has only one functional active site, or that a single Ta-GTP can relieve $P\gamma$ inhibition at both active sites. Furthermore, it has been reported that activated transducin can activate PDE6 to about one-half of the rate that is seen if the γ -subunits are physically removed from frog (Whalen et al., 1990; Norton et al., 2000) or bovine PDE6 (Melia et al., 2000; Gillespie, 1990). This has led to conflicting models of transducin activation of PDE6 in which transducin is hypothesized to relieve $P\gamma$ inhibition at either one or both catalytic sites of PDE6.

Transducin activation also reduces $P\gamma$ affinity to the regulatory GAF domains of PDE6, as judged by a 10-fold increase in the dissociation rate of cGMP from one GAF domain and a concomitant dissociation of one $P\gamma$ from the holoenzyme. The second GAFa domain of PDE6 retains high affinity for cGMP, and the second $P\gamma$ remains associated with the PDE6 catalytic dimer (Norton et al., 2000; Cote et al., 1994). The reciprocal positive cooperativity of cGMP and $P\gamma$ binding (mentioned in Section A2b) may be relevant to understanding the transducin activation mechanism. In particular, the heterogeneity and positive cooperativity of $P\gamma$ and cGMP binding to the two catalytic subunits of PDE6 suggest that activated Ta-GTP may interact differently with the two catalytic subunits as well. The consequences of PDE6 allostereism on the transducin activation mechanism are poorly understood, but may be critically important to a full

understanding of the molecular mechanisms regulating the physiological response of photoreceptor cells to light.

C. Research Goals

Considering the fact that the visual transduction pathway is the best studied G-protein coupled signaling pathway, it is surprising that many questions about the function and regulation of PDE6 remain unanswered. For example, rod PDE6 exists as a $\text{P}\alpha\beta$ catalytic dimer under all conditions that retain its enzymatic activity, but it is not clear whether one or both catalytic domains are active (Figure 1.7). Importantly, there is no consensus in the literature on the issue of whether transducin can relieve the inhibition of $\text{P}\gamma$ at one or both active sites of PDE6 (Figure 1.7). Another mystery is the role of the regulatory GAF domains of PDE6. For two other GAF-containing PDEs (PDE2 and PDE5), cGMP binding to the GAF domains induces direct conformational changes within the catalytic subunits that relieves inhibition of catalysis at the active site of the enzyme (Martins et al., 1982; Yamamoto et al., 1983; Corbin et al., 2000; Okada and Asakawa, 2002; Rybalkin et al., 2003). No direct allosteric communication between the GAF and catalytic domains of PDE6 has been reported to date (Arshavsky et al., 1992; Mou and Cote, 2001), although the close similarities of PDE5 and PDE6 suggest that PDE6 might also retain this direct allosteric mechanism.

In this thesis, I used the ability of PDE5 inhibitors to bind to the PDE6 active site to probe the properties of the catalytic mechanism of PDE6. Using radiolabeled [^3H]sildenafil and [^3H]vardenafil provides a unique opportunity to test the accessibility of the catalytic sites of PDE6 catalytic dimer by the direct binding of inhibitor to the active

sites. By determining the maximum stoichiometry of inhibitor binding to PDE6, I will address the unanswered question of whether one or both catalytic sites are functional. Once this is determined, radiolabeled inhibitors will also be used to explore the mechanism of transducin activation of PDE6, specifically whether transducin can release $P\gamma$ inhibition at one or both active sites. A third goal of my work is to examine the direct allosteric communication between the regulatory GAF domains and catalytic domains of PDE6 by examining changes in cGMP binding (to GAF domains) or inhibitor binding (to catalytic domains) when ligand occupies the other binding site. Finally, I will examine potential differences in the active sites of rod and cone PDE6 by testing a set of inhibitor compounds for their potency and relative selectivity for the purified rod PDE6 versus cone PDE6.

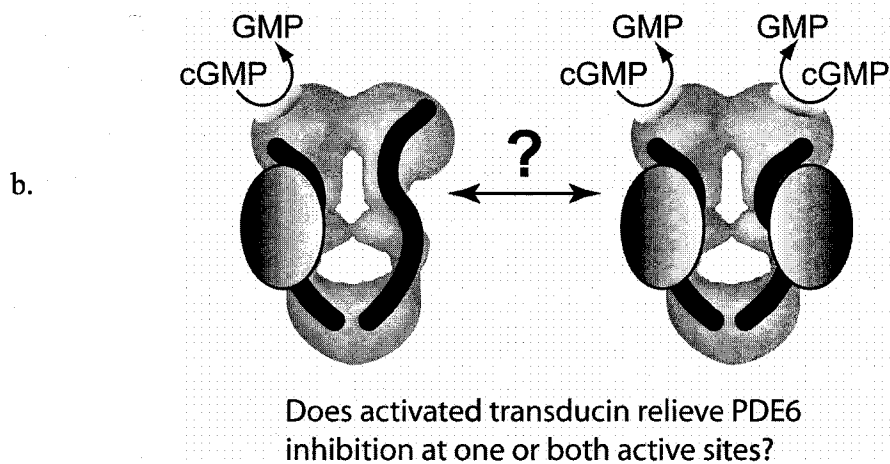
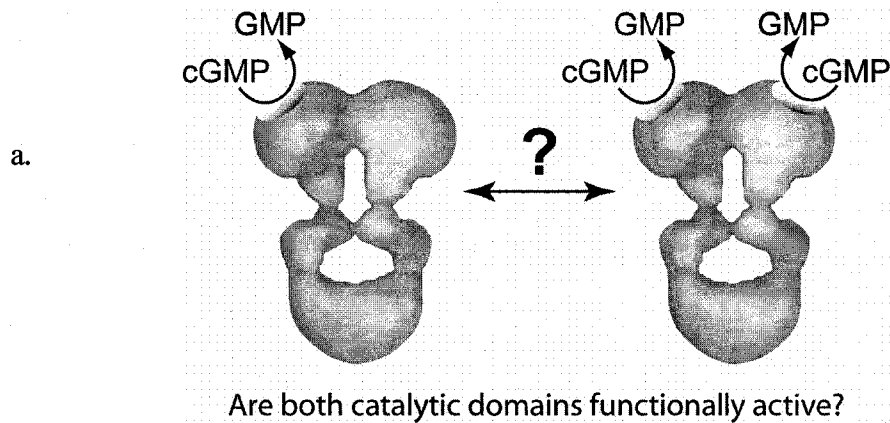


Figure 1.7 Regulation of PDE6 catalytic activity. The prevailing model of rod phototransduction asserts that the PDE6 catalytic dimer contains two active sites, both of which catalyze cGMP hydrolysis (part a, figure to right), and both of which are relieved of $P\gamma$ inhibition by the binding of two transducin α_T^* molecules (part b, figure to right). However, an alternative model has been proposed in which PDE6 has only one functional active site (part a, figure to left). Furthermore, it has been reported that transducin is only capable of activating PDE6 on ROS membranes to about 50% of the full catalytic rate of the $Pa\beta$ catalytic dimer, which suggests that transducin is only capable of activating one catalytic site on PDE6 (part b, figure to left). The work in this thesis seeks to resolve these unanswered questions.

CHAPTER 2

PROBING THE CATALYTIC SITES AND ACTIVATION MECHANISM OF PHOTORECEPTOR PHOSPHODIESTERASE (PDE6) USING RADIOLABELED PDE INHIBITORS

Abstract

Retinal photoreceptor phosphodiesterase (PDE6) is unique among the eleven vertebrate phosphodiesterase families not only for its catalytic heterodimer, but also for its regulatory γ subunits ($P\gamma$) whose inhibitory action is released upon binding to the G-protein, transducin. It has been assumed—but never demonstrated—that during visual excitation both PDE6 catalytic sites are relieved of $P\gamma$ inhibition by the binding of two activated transducin molecules. Since PDE6 shares significant structural and pharmacological similarities with PDE5, we utilized radiolabeled PDE inhibitors lacking selectivity for PDE5 versus PDE6 to probe the catalytic sites of rod PDE6. The membrane filtration assay we used to quantify [^3H]vardenafil binding to PDE6 required histone II-AS to stabilize drug binding to PDE6. Under these conditions, [^3H]vardenafil binds stoichiometrically to both the α and β catalytic subunits of the activated PDE6 heterodimer. [^3H]vardenafil is unable to bind to the PDE6 holoenzyme ($\alpha\beta\gamma\gamma$) consistent with $P\gamma$ blocking access to the drug binding sites. Following transducin activation of PDE6 holoenzyme on rod outer segment membranes, [^3H]vardenafil binding increases

proportionally as PDE6 is progressively activated by transducin. However, both [³H]vardenafil binding and hydrolytic activity of transducin-activated PDE6 approach only one-half of the value for PDE6 catalytic dimer lacking bound P γ subunits. Addition of a 1000-fold excess of purified, activated transducin α -subunits reveals a second, low affinity binding site that can stimulate the hydrolytic activity of PDE6 to its maximum extent. These results demonstrate that each catalytic domain in the rod PDE6 heterodimer stoichiometrically binds radiolabeled inhibitor, and possesses full catalytic activity. Furthermore, activated transducin α -subunit has high and low affinity binding sites such that endogenous activated transducin activates only one-half of the maximum catalytic activity of the PDE6 catalytic dimer during visual excitation.

Introduction

The superfamily of phosphodiesterase (PDE) enzymes plays a critical role in maintaining the cellular levels of cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) (Beavo et al., 2006). Photoreceptor phosphodiesterase (PDE6) is the central effector responsible for lowering cGMP levels in photoreceptor cells following light stimulation. The PDE6 activation mechanism, its catalytic efficiency, and its substrate specificity are all designed to optimize the ability of photoreceptors to rapidly respond to light stimuli with subsecond changes in cGMP levels (Cote, 2006). During the first steps in vision, photoisomerized rhodopsin activates transducin, which binds GTP and releases its α -subunit (α -GTP) to activate membrane-associated rod PDE holoenzyme by displacing P γ from the active sites of P $\alpha\beta$. The drop in cGMP that results from PDE6 activation causes cGMP-gated ion channels to close,

resulting in membrane hyperpolarization that is transmitted to second-order retinal neurons (Stavenga et al., 2000; Arshavsky et al., 2002).

Considering the wealth of quantitative information about the phototransduction pathway, it is surprising that important aspects of PDE6 function and regulation remain unknown. For example, rod PDE6 usually exists as a tightly associated catalytic dimer of α - and β -subunits ($\text{P}\alpha\beta$), but there are still questions about whether one or both of the catalytic domains are active. Underscoring this point is the fact that chicken rod photoreceptor PDE6 apparently contains only one functional catalytic subunit [β -subunit, (Huang et al., 2004)], raising the possibility that the catalytic site on the α -subunit in other species is not functional. More importantly, there is no consensus in the literature on the issue of whether transducin can fully activate PDE6 catalysis. Although it has been assumed that transducin can activate PDE6 in a 1:1 molar ratio (Wensel and Stryer, 1990; Leskov et al., 2000), the question of whether one or both PDE6 catalytic sites become activated by transducin during visual excitation has never been demonstrated. In some instances, it has been reported that two Ta-GTP bind to both catalytic subunits of $\text{P}\alpha\beta$ releasing the $\text{P}\gamma$ inhibition at both active sites (Pugh and Lamb, 2000; Wensel and Stryer, 1990). Other investigators have reported that a single Ta-GTP was able to maximally activate the PDE6 catalytic dimer under defined conditions (Bruckert et al., 1994; Melia et al., 2000; Yamazaki et al., 2002). The latter work suggests that either the PDE6 catalytic dimer has only one functional active site, or that a single activated Ta-GTP can relieve $\text{P}\gamma$ inhibition at both $\text{P}\alpha\beta$ active sites. Furthermore, it is reported that transducin can activate PDE6 to about one-half of the rate that is seen if the γ -subunits are physically removed from PDE6 in frog (Whalen et al., 1990; Norton et al., 2000) and bovine (Melia

et al., 2000; Gillespie, 1990) rod outer segments. This has led to conflicting models of transducin activation of PDE6 in which transducin is hypothesized to relieve $P\gamma$ inhibition at either one or both catalytic sites of PDE6.

PDE6 differs in several fundamental ways from the other ten classes of mammalian phosphodiesterases. Rod PDE6 is the only PDE that exists as a catalytic heterodimer ($\text{Pa}\beta$), whereas cone PDE6 and the other ten PDE families are all believed to be homodimers. Unlike other PDE families, rod and cone PDE6 catalytic activity is primarily regulated by its inhibitory $P\gamma$ subunits tightly associated with $\text{Pa}\beta$ to form an inactive tetrameric holoenzyme ($\alpha\beta\gamma\gamma$). PDE6 is also the only family of PDEs in which the catalytic activity is directly regulated by a heterotrimeric G-protein, transducin (Cote, 2006).

PDE6 is most closely related to PDE5 (abundant in vascular smooth muscle) in its biochemical, structural and pharmacological properties (Cote, 2004). Both PDE5 and PDE6 have highly conserved amino acid sequences and 3-dimensional structures (McAllister-Lucas et al., 1993; Kameni Tchoudji et al., 2001). PDE5 and PDE6 share strong substrate specificity for cGMP compared to cAMP (Beltman et al., 1995). Both can bind cGMP with high affinity at their regulatory GAF domains (Francis et al., 2006; Cote, 2006). Most PDE5-selective inhibitors, including the well-known erectile dysfunction drugs Viagra (sildenafil) and Levitra (vardenafil), can also potently inhibit PDE6 catalysis as well (Gillespie and Beavo, 1989b; D'Amours et al., 1999; Zhang et al., 2005).

We used the ability of PDE5 inhibitors to bind with high affinity to PDE6 to probe the active sites of the enzyme and to better elucidate the activation mechanism by

transducin. Using [³H]vardenafil, we tested the hypothesis that both catalytic domains of the Paβ dimer are catalytically active and functionally equivalent. We then evaluated whether binding of activated transducin to the PDE6 holoenzyme relieves inhibition at one or both of the active sites in the PDE6 dimer.

Experimental Procedures

Materials—Bovine retinas were purchased from W.L. Lawson, Inc.. Superdex 200 and Mono-Q columns were from GE Healthcare, Inc., and the C18 reversed-phase column (300A, 22×250 mm) was from Vydac. Filtration and ultrafiltration products were from Millipore. Scintillation fluid (Ultima Gold-XR) and [³H]cGMP was from PerkinElmer Life Sciences and [³H]vardenafil was a kind gift of Drs. P. Sandner & U. Pleiss (Bayer Healthcare AG). Protein assay reagents were from Pierce and all other chemicals were obtained from Sigma.

Preparation of bovine rod outer segments (ROS), PDE holoenzyme and PDE heterodimer—Bovine rod outer segments (ROS) were prepared from frozen bovine retinas under dark-adapted conditions on a discontinuous sucrose gradient (Pentia et al., 2005). PDE6 holoenzyme (Pαβγγ) was extracted with a hypotonic buffer from illuminated ROS homogenates and purified by Mono-Q anion-exchange chromatography and Superdex 200 gel filtration chromatography. The purified PDE6 (> 95% pure) was then concentrated by ultrafiltration and stored with 50% glycerol at -20°C (Pentia et al., 2005).

The PDE6 catalytic dimer (Paβ) was prepared from the PDE6 holoenzyme by removing the inhibitory Pγ subunits through limited trypsin proteolysis (Hurley and

Stryer, 1982). A time course of proteolytic activation of PDE6 was determined to ensure that >90% of the P γ subunit was destroyed without altering the apparent molecular weight of the catalytic subunits [as judged by SDS-PAGE; (Pentia et al., 2005)].

The concentration of PDE6 was determined by both measurements of catalytic activity under conditions where the k_{cat} was known [5600 s⁻¹; (Mou and Cote, 2001)] and by measurements of [³H]cGMP binding under conditions where maximum binding occupancy was achieved [(B_{max} = 2.0 cGMP per PDE6; (Cote, 2000)].

Purification of persistently activated transducin α -subunit (Ta-GTP γ S)—Transducin α -subunits were extracted from the PDE6-depleted ROS membranes by adding 50 μ M GTP γ S (in low salt buffer) to the ROS membranes and recovering the solubilized Ta-GTP γ S by centrifugation. The extracted Ta-GTP γ S was purified on a Blue Sepharose column (Kleuss et al., 1987; Wensel et al., 2005). The concentration of Ta-GTP γ S was determined by a colorimetric protein assay. Purified Ta-GTP γ S was stored with 50 μ M GTP γ S and 50% glycerol at -20°C.

Preparation and purification of P γ and P γ 63-87—Wild-type bovine rod P γ (87 amino acids) and the N-terminal truncated P γ mutant, P γ 1-45C (Skiba et al., 1996) were expressed in *E. coli* and purified to >97% purity using SP-Sepharose followed by reversed-phase high pressure liquid chromatography (Granovsky et al., 2000). The wild-type P γ concentration was determined spectrophotometrically using an experimentally determined extinction coefficient of 7550 cm⁻¹ M⁻¹ (Cote, 2000). The inhibitory activity of purified P γ was assayed by its ability to stoichiometrically inhibit trypsin-activated bovine rod PDE (Mou et al., 1999). The spectrophotometric and activity estimates of P γ concentration agree to within 10% for all wild-type P γ preparations used in this study.

The concentration of the synthetic peptide P γ 63-87 (New England Peptide, Inc.) was determined by a protein assay.

Transducin activation of ROS PDE—Purified ROS were resuspended in buffer A (20 mM MOPS, 2 mM MgCl₂, 30 mM KCl, 120 mM NaCl, pH 7.4) at a concentration of 30 μ M rhodopsin, and then passed through a 26 gauge insulin needle ten times under dim red light. The concentration of membrane-associated PDE was estimated based on its stoichiometric ratio to rhodopsin (300 rhodopsin per PDE6) and its maximum hydrolytic activity after trypsin proteolysis. After ROS homogenates were fully bleached by light to activate rhodopsin, PDE6 was activated by incubating with an excess of GTP γ S relative to the transducin concentration and the rate of cGMP hydrolysis assayed.

Binding of [³H]vardenafil to catalytic sites on PDE—The membrane filtration assay to quantitate [³H]vardenafil binding to PDE6 was adapted from a similar assay for PDE5 (Blount et al., 2004). The standard binding assay buffer contained histone Type II-AS (0.2 mg/ml). To reduce nonspecific binding, samples were diluted 20-fold with ice-cold wash buffer (10 mM Tris, pH 7.5, 0.1% Triton X-100) immediately before applying the sample onto pre-wet Millipore HAWP 025 membrane filters. Filters were washed 8 times with 1-ml ice-cold wash buffer.

Analytical methods: The rate of cGMP hydrolysis was determined by a phosphate release assay (Cote, 2000). Activity measurements were made in 100 mM Tris (pH 7.5) buffer containing: 10 mM MgCl₂, 0.5 mg/ml BSA, 0.5 mM EDTA, 2 mM dithiothreitol. All rate measurements were obtained from four individual time points at saturating cGMP concentrations (10 mM) and less than 30% substrate was consumed during this time. The [³H]cGMP membrane filtration binding assay was used to determine the

stoichiometry of cGMP binding under various conditions (Cote, 2005) with 10 mM EDTA and 50 μ M vardenafil added to the binding assay solution to prevent cGMP hydrolysis. The rhodopsin concentration was spectrophotometrically determined, using an extinction coefficient of $42000 \text{ M}^{-1}\text{cm}^{-1}$ (Bownds et al., 1971).

Results and Discussion

Histone II-AS stabilizes [³H]vardenafil binding to the catalytic sites of PDE6 catalytic dimer—Previous work has shown that most of the so-called PDE5-selective inhibitors (e.g., zaprinast, E4021, sildenafil, and vardenafil) also inhibit the catalytic activity of the closely related photoreceptor PDE6 (Gillespie and Beavo, 1989b; D'Amours et al., 1999; Zhang et al., 2005). To date, vardenafil is the most potent of this class of catalytic site inhibitor, with an inhibition constant for PDE6 of $\sim 1 \text{ nM}$ (Zhang et al., 2005). As such, it represents a useful tool for probing the active sites of PDE6 in its nonactivated and activated states. Initial experiments measuring [³H]vardenafil binding to purified PDE6 using a membrane filtration assay revealed high nonspecific binding and variability in total binding. There was no detected binding of vardenafil to Pa β alone. Consistent with previous studies of PDE5 binding to radiolabeled inhibitors (Blount et al., 2004), we observed that histone II-AS stabilized [³H]vardenafil binding to PDE6 catalytic dimer (Pa β) in a concentration-dependent manner (Figure 2.1). Interestingly, histone II-AS had minor effects on cGMP hydrolytic activity at the active site or the ability of [³H]cGMP to bind to the regulatory GAF domains (*data not shown*). The mechanism of histone II-AS effects on PDE5 or PDE6 is still not clear, but one likely

possibility is that histone II-AS slows down drug dissociation during the washing step of the filter binding assay.

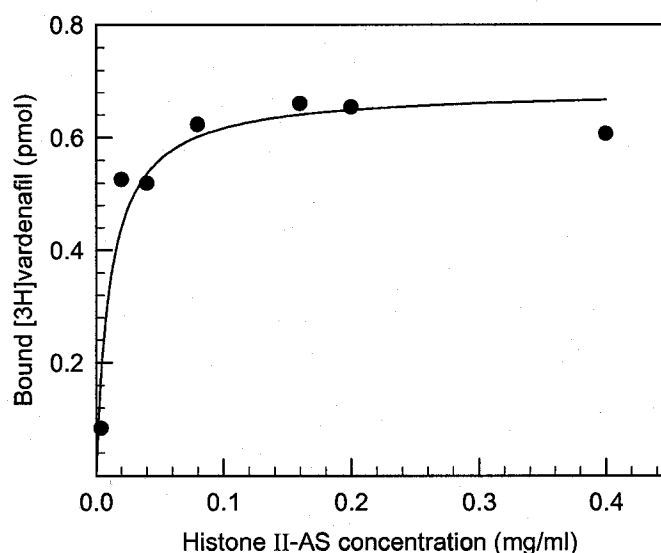


Figure 2.1 Histone II-AS Stimulates Binding of [³H]vardenafil to the Active Sites of PDE6 Catalytic Dimer. Purified P $\alpha\beta$ catalytic dimer (6 nM) lacking P γ was incubated with 70 nM [³H]vardenafil and increasing amounts of histone II-AS at room temperature for 40 min. The amount of radiolabeled drug was determined by a membrane filtration assay (see Experimental Procedures). Data represent one of three similar experiments.

To further characterize the unexpected stabilization of [³H]vardenafil binding by histone II-AS, we tested several other known PDE6-interacting compounds, including histone Type VIII-S (Miki et al., 1975; Hurwitz et al., 1984), P γ , and P γ peptides. Histone VIII-S, which historically has been used to displace P γ and activate the PDE6 holoenzyme, can stabilize vardenafil binding to the P $\alpha\beta$ dimer to a certain extent (Figure 2.2), but did not reach the maximum binding observed with histone II-AS. Little [³H]vardenafil binding was detected with P γ or the C-terminal peptide P γ 63-87, as might

be expected since the C-terminal region of the inhibitory $P\gamma$ subunits competes with drug binding at the active sites (Granovsky and Artemyev, 2000; Zhang et al., 2005).

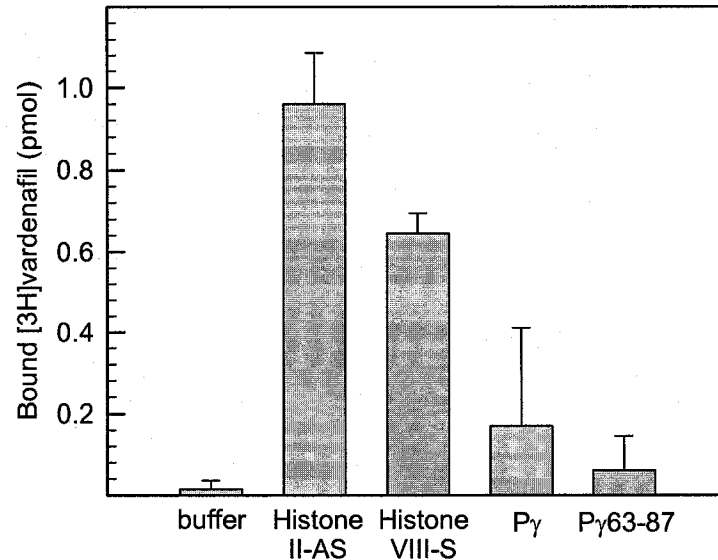


Figure 2.2 Comparison of [³H]vardenafil Binding to Pa β Stimulated by Histones or P γ . Purified Pa β (5 nM) and 70 nM [³H]vardenafil were incubated with the following for 40 min at room temperature: 10 mM Tris (pH 7.5), 0.2 mg/ml histone II-AS, 0.2 mg/ml histone VIII-S, 10 μ M P γ or 10 μ M P γ 63-87. Vardenafil binding was quantified by the membrane filtration assay.

[³H]vardenafil binds to PDE6 catalytic dimer, but P γ blocks inhibitor binding to the holoenzyme—It is well established that rod PDE6 consists of α and β catalytic subunits, and it has been assumed, but never demonstrated, that each catalytic domain of Pa β is active. The binding assay for [³H]vardenafil allowed us to directly test this by comparing catalytic activity, cGMP binding stoichiometry and [³H]vardenafil binding in the same Pa β catalytic dimer preparation.

Figure 2.3 shows that the binding curve for [³H]vardenafil to purified Pa β displays a single class of drug binding sites. The apparent binding affinity for vardenafil is high, but lower than the reported value of the inhibition constant [$K_i = 0.7$ nM, (Zhang

et al., 2005)]. This discrepancy is due to the requirement for nanomolar levels of PDE6 to reproducibly quantify [³H]vardenafil binding, resulting in titration of the binding

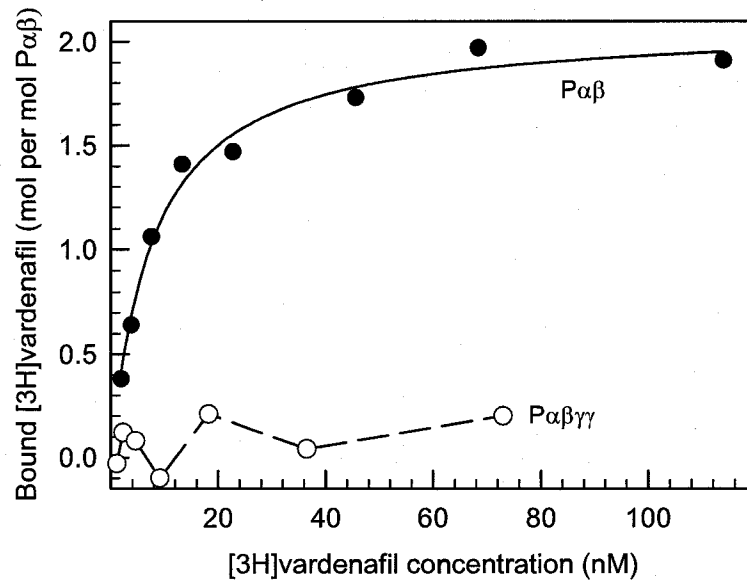


Figure 2.3 [³H]vardenafil Binds Stoichiometrically to Each Pa_β Catalytic Subunit, But Only in the Absence of P_γ. Purified Pa_β (2.5 nM; filled circles) or Pa_β reconstituted with P_γ (10 μM; open circles) was incubated with 0.2 mg/ml histone II-AS and the indicated concentration of [³H]vardenafil. Samples were incubated for 40 min before membrane filtration. Vardenafil binding was normalized to the Pa_β concentration, as estimated by both [³H]cGMP binding assay, as well as hydrolytic activity measurements (which agreed to within 10%). [³H]vardenafil binding to Pa_β was fit assuming a single class of binding sites ($K_D = 7.8$ nM and $B_{max} = 2.0$). The data is representative of 3 similar experiments.

site as the vardenafil concentration is increased [This interpretation is supported by experiments in which the apparent K_D for [³H]vardenafil binding decreased as the PDE6 concentration was lowered (*data not shown*)]. The maximum extent of vardenafil binding to purified Pa_β was calculated to be 2.0 vardenafil molecules per Pa_β. This result shows that both the α and β catalytic subunits of PDE6 bind vardenafil equally well. In contrast, the same Pa_β reconstituted with P_γ cannot bind [³H]vardenafil to a significant extent

under identical experimental conditions (Figure 2.3), demonstrating that vardenafil binding is prevented when $P\gamma$ inhibits the catalytic site of PDE6.

Endogenous activated transducin relieves $P\gamma$ inhibition of only one-half of the full catalytic potential of PDE6 on ROS membranes—To evaluate the extent to which transducin can stimulate PDE6 catalysis and permit binding of [^3H]vardenafil to the PDE6 catalytic sites, we used ROS homogenates in which the key proteins of visual excitation (rhodopsin, transducin, and PDE6) remain associated with the disk membrane. The use of ROS homogenates was necessitated by the well-established fact that transducin poorly activates rod PDE6 when both proteins are not bound to ROS disk membranes (Wensel and Stryer, 1988; Gillespie and Beavo, 1988). The analysis of these experiments was simplified because PDE6 is the only enzyme present in ROS homogenates capable of breaking down cGMP and of binding vardenafil.

We found that when transducin is inactive (i.e., in the absence of $\text{GTP}\gamma\text{S}$), PDE6 hydrolytic activity in these ROS homogenate preparations was low (<10% of the fully activated rate) as was the ability of PDE6 to bind [^3H]vardenafil (Figure 2.4). In addition, limited trypsin proteolysis of ROS homogenates (to fully degrade the $P\gamma$ subunits and activate PDE6 catalysis) resulted in stoichiometric binding of [^3H]vardenafil (1.9 ± 0.2 mol vardenafil per mol PDE6 catalytic dimer) and complete activation of cGMP hydrolysis (Figure 2.4). Surprisingly, transducin activation of PDE6 failed to activate more than $38 \pm 5\%$ of the full catalytic potential of the enzyme. The maximum extent of PDE6 catalytic stimulation was consistent with this observation in that only 0.9 ± 0.1 mol vardenafil per mol PDE6—not 2 mol per mol—was able to access the catalytic sites of transducin-activated PDE6.

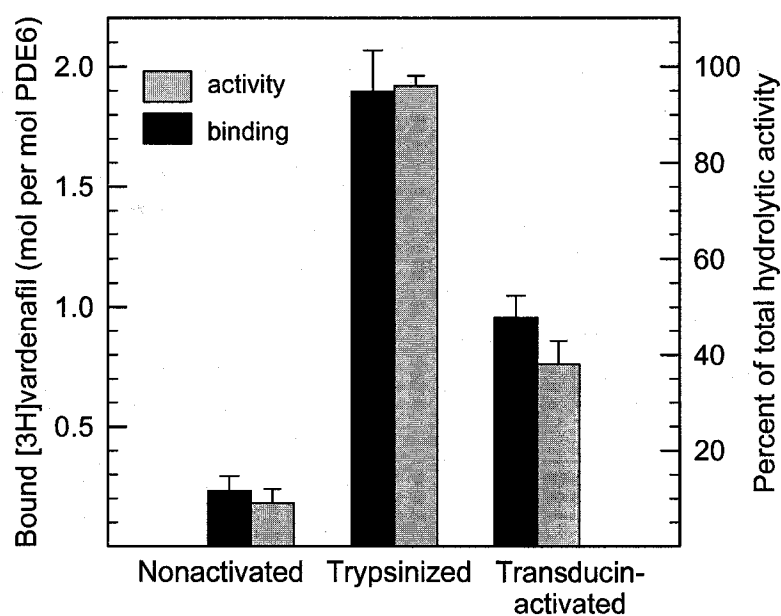


Figure 2.4 Transducin-activated PDE6 Achieves Only One-half of the Extent of [³H]vardenafil Binding or Hydrolytic Activity Compared to Paβ. Light-exposed ROS homogenates were prepared (see Experimental Procedures) and portions were incubated with either buffer (nonactivated) or with 50 μM GTPγS (to activate transducin). A separate portion was exposed to trypsin to maximally activate PDE6 catalysis. The samples (containing 4 nM PDE6) were incubated with 74 nM [³H]vardenafil for 40 min before membrane filtration (black bars) and activity assays (gray bars). Data are the mean ± S.D. of three experiments.

To further explore this relationship between stimulation of PDE6 catalysis by transducin and the availability of the PDE6 active site to bind radiolabeled drug, we added increasing amounts of GTPγS to light-exposed ROS homogenates to progressively activate transducin (and hence PDE6). Figure 2.5 shows an excellent correlation between stimulation of catalysis and [³H]vardenafil binding as the fraction of activated transducin was increased. These results also confirmed that a maximum of approximately one-half of the catalytic sites on PDE6 are dis-inhibited upon transducin activation, as judged by

both accessibility of vardenafil to the PDE6 active site as well as by the extent of stimulation of cGMP hydrolysis.

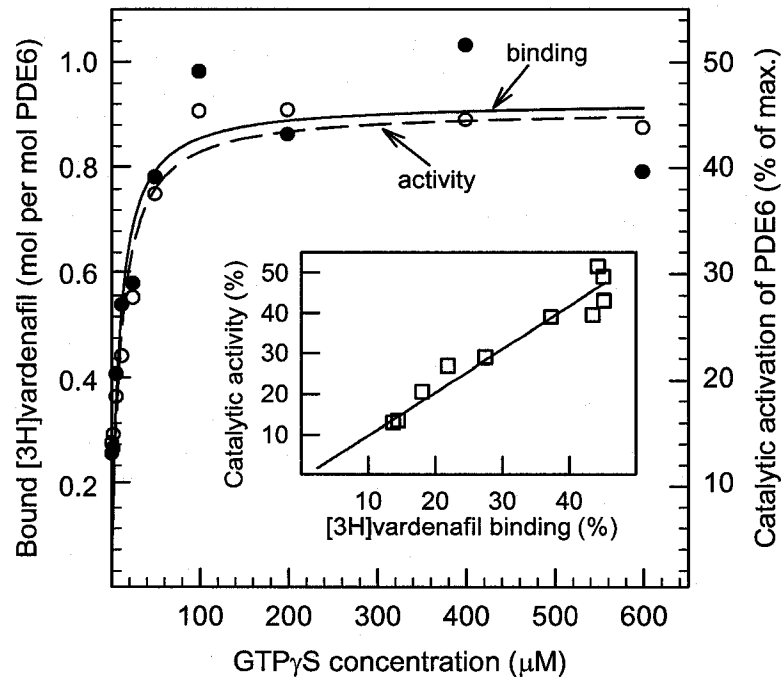


Figure 2.5 [3H]vardenafil Binding Correlates with the Extent of PDE6 Activation by Transducin. Increasing concentrations of GTP γ S were added to ROS homogenates (10 nM PDE6) to progressively activate PDE6. The extent of PDE6 activation was quantified by measuring cGMP hydrolytic rates (filled circles) and normalized to the activity of the same sample activated by limited trypsin proteolysis. In separate samples, the amount of [3H]vardenafil bound was determined (open circles). A correlation plot (inset) shows a linear relationship of activation and vardenafil binding ($r^2=0.96$).

Because this result differs from the commonly held view that transducin can fully activate PDE6 during visual excitation, we next explored whether addition of exogenous, activated transducin (i.e., greater than the endogenous levels present in the ROS homogenates) could further stimulate PDE6 catalysis. We purified the α -subunit of transducin bound to GTP γ S (Ta-GTP γ S) and added increasing concentrations to either ROS homogenates or to purified, soluble PDE6 holoenzyme. As seen in Figure 2.6, addition of increasing concentrations of purified Ta-GTP γ S to ROS homogenates (open

circles) elevated PDE6 catalytic activity from $35 \pm 4\%$ to $101 \pm 1\%$ of the activity of catalytic dimers in which Py had been proteolytically removed. This demonstrates that a second activated Ta can bind to a second Py subunit and displace it from its active site, but does so with a relatively low binding affinity (i.e., requiring a greater than 1000-fold excess of exogenous activated Ta). Similar results were seen for purified, soluble PDE6 in the absence of ROS disk membranes (Figure 2.6), but in this instance an even greater concentration of $\text{Ta-GTP}\gamma\text{S}$ was needed to fully activate the enzyme.

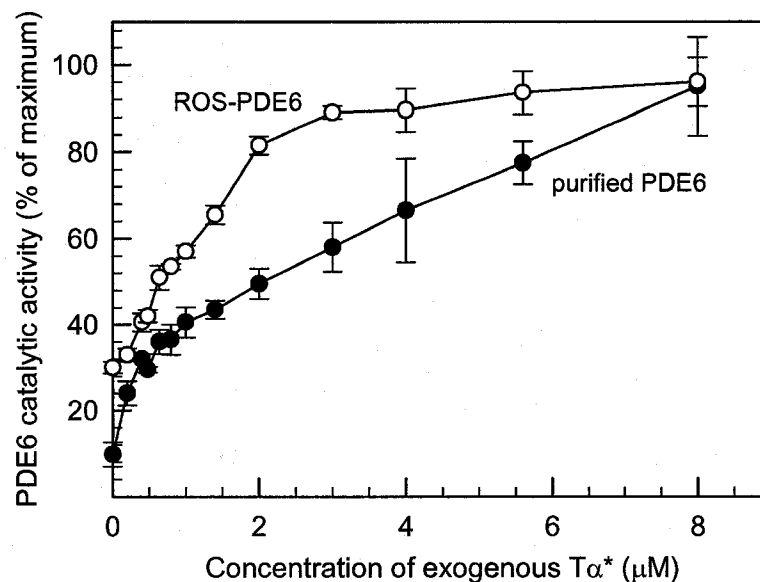


Figure 2.6 A Large Excess of Activated Transducin α -subunit Fully Relieves Py Inhibition of Both Active Sites of the PDE6 Catalytic Dimer. The indicated concentrations of purified transducin α -subunits with $\text{GTP}\gamma\text{S}$ bound ($\text{T}\alpha\text{-GTP}\gamma\text{S}$) was added to either 1 nM purified PDE6 holoenzyme (filled circles) or 2 nM ROS-PDE6 previously incubated with 50 μM $\text{GTP}\gamma\text{S}$ to activate endogenous transducin (open circles). In both cases, hydrolytic rates were normalized by comparison to the catalytic activity of samples that had been treated with trypsin to fully activate PDE6 catalysis. Data are the mean \pm S.D. of two experiments.

Conclusions

Two major conclusions emerge from this work. The first is that each catalytic subunit of the rod heterodimer possesses full catalytic activity (Figure 2.7, left-most diagram). This conclusion was demonstrated by the innovative approach of utilizing radiolabeled vardenafil to quantify the number of binding sites with respect to the catalytic activity and cGMP binding stoichiometry of identical Pa β preparations. The second major conclusion is that activated transducin only activates one-half of the active sites of PDE6 with high efficiency, and that a second low affinity transducin binding site on PDE6 is observed only when a large excess of exogenous transducin is present.

The photoreceptor outer segment provides a unique cellular environment for visual transduction and specifically for regulation of PDE6 activation by transducin. The high concentration of transducin [about 500 μ M; (Pugh and Lamb, 2000)] relative to PDE6 (20 μ M) ensures the efficiency of propagation of the excitation pathway (Arshavsky et al., 2002). However, the high affinity of the P γ subunits for Pa β not only prevent “dark” activation of PDE6 under dark-adapted conditions [the basal activity of rod PDE6 is equivalent to only 1 out of 2200 Pa β being fully active (Rieke and Baylor, 1996; D'Amours and Cote, 1999)], it also poses a challenge for transducin to displace P γ during the activation mechanism. Our observation that transducin only activates PDE6 activity to its half-maximum potential suggests that only one P γ site is dis-inhibited during visual excitation. The second P γ site may only be dis-inhibited upon prolonged illumination, in which a sustained drop in the cGMP concentration within the outer segments may cause dissociation of cGMP from the PDE6 GAF domain. This in turn would decrease the P γ affinity, thereby allowing the second catalytic site of PDE6 to be activated by transducin.

Although we observed the complete activation of PDE6 only when exogenous Ta-GTP γ S was added in vitro, in fact the transducin concentration in the outer segment of a living photoreceptor easily achieved the concentration needed to activate both catalytic sites of PDE6. The idea that two distinct transducin sites on PDE6 are used for dark-adapted and light-adapted conditions provides a biochemical rationale for the ability of photoreceptors to modulate sensitivity over 2-3 orders of magnitude.

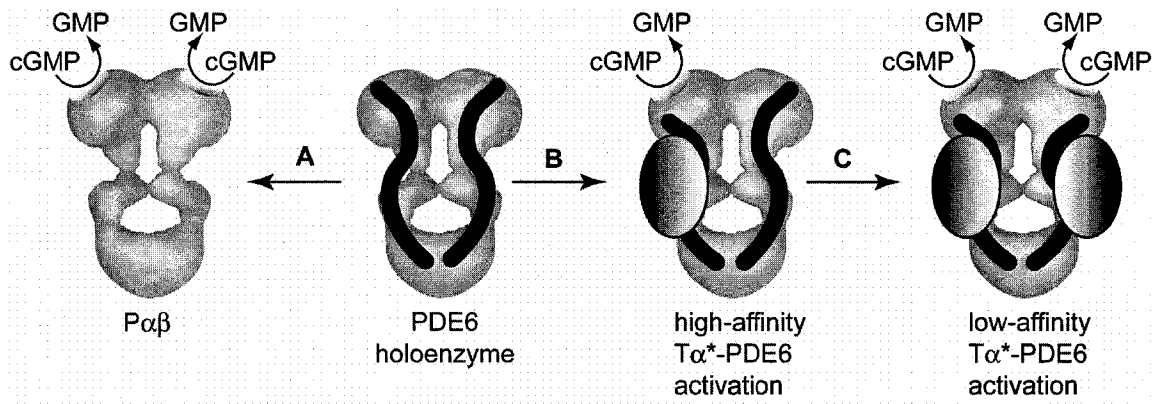


Figure 2.7 Mechanism of PDE6 Activation. In its nonactivated state, the catalytic activity of rod PDE6 holoenzyme is fully inhibited by two Py subunits (black s-shaped rods) binding to the $\text{Pa}\beta$ heterodimer (represented as having one catalytic domain and a tandem GAF regulatory domain per catalytic subunit). Physical removal of both Py subunits by limited proteolysis (Process A) allows full catalytic activity of each of the two active sites. Activation of ROS-membrane associated PDE6 by endogenous transducin results in activation of one-half of the full catalytic potential of PDE6, most likely by binding to and displacing a lower affinity Py subunit from its interaction site on one catalytic subunit (Process B). Addition of a large excess of Ta-GTP γ S reveals a second transducin interacting site that displaces the second Py subunit from its higher affinity interaction with the catalytic site, thereby leading to full catalytic activation (Process C).

CHAPTER 3

BIOCHEMICAL APPROACHES TO STUDY THE CATALYTIC AND ALLOSTERIC PROPERTIES OF PHOTORECEPTOR PHOSPHODIESTERASE (PDE6)

Introduction

PDE6 is the central enzyme in phototransduction in rod and cone photoreceptor cells. Rod PDE6 is a tetramer, consisting of two inhibitory γ subunits tightly bound to α and β subunits (Baehr et al., 1979; Deterre et al., 1988). Cone PDE6 contains two identical α' subunits, to which cone-specific γ' subunits bind with unknown stoichiometry. The cone γ' subunit is slightly smaller than rod γ (Hamilton and Hurley, 1990) and the affinity of cone γ' subunits binding to PDE6 is weaker than rod γ subunits (Hamilton et al., 1993; Valeriani, 2004). Although the physical responses of rod and cone to light differ, rod and cone PDE6 have similar kinetic properties [i.e. similar K_M and k_{cat} values (Gillespie and Beavo, 1988; Huang et al., 2004; Mou and Cote, 2001)].

PDE6 Pharmacology

The rod and cone photoreceptor PDE6 belongs to a superfamily of 11 distinct cyclic nucleotide PDEs (Beavo et al., 2006). Not only are rod and cone PDE6 most

closely related to PDE5 (abundant in vascular smooth muscle) in their biochemical and structural properties (Cote, 2004), most drugs that selectively and potently target PDE5 also inhibit PDE6 as well. Preclinical and clinical data on the effects of sildenafil have revealed significant but transitory effects on visual function, presumably through inhibition of photoreceptor PDE6 (Laties and Zrenner, 2002). Tadalafil and vardenafil, two other approved drugs, show smaller effects on visual function (Uckert et al., 2003). Remarkably little is known about the effects of PDE5-selective drugs on cGMP metabolism in photoreceptors (Zhang et al., 2005). Of the few reports comparing the pharmacology of purified rod and cone PDE6 to date, few compounds have been shown to discriminate these two isoforms. In this study, we surveyed the potency and selectivity of a series of PDE inhibitors originally designed to target PDE5, in the hope of identifying compounds that discriminate purified rod and cone PDE6, and thereby provide insights into differences in the catalytic sites of the two isoforms.

Function of Regulatory GAF Domains of PDE6

Five members of the cyclic nucleotide phosphodiesterase (PDE) superfamily contain N-terminal regulatory domains that consist of a tandem pair of GAF domains. For PDE2 and PDE5, cGMP binding to the N-terminal regulatory GAF domains induce conformational changes that relieve inhibition of catalysis in the C-terminal active sites of the enzyme (Martins et al., 1982; Yamamoto et al., 1983; Corbin et al., 2000; Okada and Asakawa, 2002; Rybalkin et al., 2003). For PDE6, its regulatory GAF domains contain both cGMP binding sites and Py binding sites. Occupancy of the PDE6 GAF domains by cGMP enhances Py affinity for its catalytic subunits (Yamazaki et al., 1990;

Cote et al., 1994; D'Amours and Cote, 1999). No direct allosteric communication between the GAF and catalytic domains of PDE6 has been reported to date (Arshavsky et al., 1992; Mou and Cote, 2001). In this chapter, we report that occupancy of the GAF domains by an N-terminal P γ peptide stabilizes binding of PDE inhibitor to the catalytic sites. Other regions of P γ (or full length P γ) fail to stabilize inhibitor binding. Unlike zaprinast and IBMX, the binding of two PDE5/6-selective inhibitors (sildenafil and vardenafil) to PDE6 catalytic sites lead to the shift of the low affinity cGMP binding site to a higher affinity site when P γ is absent. This provides evidence for direct allosteric communication between regulatory and catalytic domains of the PDE6 catalytic dimer.

Experimental Procedures

Materials—Bovine retinas were purchased from W.L. Lawson, Inc.. Superdex 200 and Mono-Q columns were from GE Healthcare, and the C18 reversed-phase column (300 angstroms, 22×250 mm) was from Vydac. Filtration and ultrafiltration products were from Millipore, except for glass fiber filters (catalog #66085, Gelman, or #32, Schleicher and Schull). Scintillation fluid (Ultima Gold-XR) and [³H]cGMP were purchased from PerkinElmer Life Sciences. [³H]vardenafil was a kind gift of Drs. P. Sandner & U. Pleiss (Bayer Healthcare AG). [³H]sildenafil was a kind gift of Drs. Jackie Corbin and Sharron Francis (Vanderbilt University School of Medicine). Other PDE inhibitor compounds were a kind gift of Dr. Vince Florio (ICOS Corp.). Protein assay reagents were from Pierce and all other chemicals were obtained from Sigma.

Preparation of bovine rod outer segments (ROS), rod PDE6 holoenzyme (PDE6R) or rod PDE6 heterodimer (Pa β)—PDE6R and Pa β were purified from ROS,

which was prepared from bovine retina under dark-adapted conditions; as described in chapter 2 (Pentia et al., 2005).

Preparation of bovine cone PDE6 (PDE6C) and cone catalytic dimer (Pa'₂)—

The soluble PDE6C was extracted from commercial frozen bovine retinas under dark-adapted conditions and purified with Q-Sepharose resin using a Pharmacia XK26 column (Pentia et al., 2005). The partially purified PDE6C was stored in 50% glycerol at -20 °C. Pa'₂ was prepared by limited proteolysis, as described in chapter 2 for rod Paβ. The concentration of PDE6C was determined by measurements of catalytic activity under conditions where the k_{cat} was known [4200 cGMP per Pa'₂ per second; (Valeriani, 2004)].

Assay of inhibition of PDE6 hydrolysis by inhibitor compounds—

PDE inhibitors were prepared in DMSO (100 mM stock concentration) due to their poor solubility in water. Paβ or Pa'₂ (2 pM final concentration) purified from the same initial retinal homogenates (see above) were pre-incubated with inhibitors before addition of 1 μM [³H]cGMP. The hydrolytic reaction was then stopped by addition of HCl at four different time points (1, 3, 5, 7 min). Control samples, either lacking PDE6 (negative control) or permitted to fully degrade the [³H]cGMP for 40 min (positive control), were included in all experiments. Following treatment of each sample with 5'-nucleotidase to degrade 5'-GMP to guanosine, the [³H]guanosine was quantified by chromatography using DEAE-Sephadex A-25 resin (Cote, 2000).

Preparation and purification of Pγ and Pγ1-45—

Full length bovine rod Pγ (87 amino acids) was expressed in E. coli, purified and tested as described (Granovsky et al., 2000). The truncated Pγ mutant, Pγ1-45C (Skiba et al., 1996) was expressed in E. coli

and purified to >97% purity using SP-Sepharose followed by reversed-phase high pressure liquid chromatography (Granovsky et al., 2000). The concentration of the synthetic peptide P γ 63-87 (New England Peptide) was determined by a protein colorimetric assay.

Transducin activation of ROS PDE6—Transducin-activated PDE6 was prepared exactly as described in Chapter 2.

Binding of [³H]vardenafil to catalytic sites on PDE6— The membrane filtration assay to quantitate [³H]vardenafil binding to PDE6 was adapted from a similar assay for PDE5 (Blount et al., 2004). The standard binding assay buffer contained histone II-AS (0.2 mg/ml). To routinely reduce nonspecific binding, samples were diluted 20-fold with ice-cold wash buffer (10 mM Tris, pH 7.5, 0.1% Triton X-100) immediately before applying the sample onto pre-wet membrane filters (Millipore, 0.45 μ M HA). Filters were washed 8 times with 1-ml ice-cold wash buffer, and radioactivity trapped on the filter quantified by scintillation counting.

Binding of [³H]cGMP to GAF domain on PDE6—Purified PDE6 holoenzyme was carefully trypsinized to reach 90% of its maximum hydrolytic activity, followed by a 3 hour incubation at 30°C to degrade endogenous cGMP present in the PDE6 sample. The cGMP-depleted, activated PDE6 was then treated with 10 mM EDTA to chelate divalent cations and prevent hydrolysis of [³H]cGMP during the binding assay. 5 nM EDTA-treated PDE6 and 1 μ M [³H]cGMP were incubated in a 37°C water bath for 5 min before membrane filtration (Cote, 2005). Parallel measurements of the PDE6 concentration were based on hydrolytic activity measurements.

Analytical methods—Except where noted, the rate of cGMP hydrolysis was routinely determined by a phosphate release assay (Cote, 2000). Activity measurements were made in 100 mM Tris (pH 7.5) buffer containing 10 mM MgCl₂, 0.5 mg/ml BSA, 0.5 mM EDTA, and 2 mM dithiothreitol. All rate measurements were obtained from four individual time points at saturating cGMP concentrations (10 mM) and less than 30% substrate was consumed during this time. The rhodopsin concentration was spectrophotometrically determined by difference spectroscopy, using an extinction coefficient of 42,000 M⁻¹cm⁻¹ (Bownds et al., 1971).

The inhibition constant (K_i) was calculated from the sigmoidal concentration dependence curve using the equation: $K_i = IC_{50} / (1 + [S]/K_M)$ (Cheng and Prusoff, 1973), where IC_{50} is the concentration of inhibitor that reduces catalytic activity *in vitro* by 50%, $[S]$ is the substrate concentration, and K_M is the Michaelis constant. According to this equation, if the concentration of cGMP is much lower than K_M , then $K_i \sim IC_{50}$. The following values for K_M were used: 14 μ M for purified bovine rod PDE6 (Mou and Cote, 2001); 7 μ M for purified bovine cone PDE6 (Valeriani, 2004). Unless noted, all experiments were repeated at least three times, and average values are reported. Curve fitting was performed using Sigmaplot.

Results and Discussion

A. Inhibitor Binding to the Catalytic Site of Rod PDE6 versus Cone PDE6

Previous studies of the enzymological properties of PDE6 indicated that rod and cone PDE6 share similar kinetic properties (Gillespie and Beavo, 1988; Huang et al., 2004; Mou and Cote, 2001; Valeriani, 2004). PDE6 inhibitors, which potently inhibit rod PDE6 (PDE6R) can strongly target cone PDE6 (PDE6C) as well. We tested a novel set of PDE5 inhibitors developed by ICOS for their ability to inhibit purified PDE6 in the hope that structural difference in the drug binding sites might be uncovered. Both PDE6R and PDE6C were tested in their fully activated state in which the inhibition by P γ subunits were physically removed by limited proteolysis. Dose-response curves were generated for each inhibitor (a representative example is presented in Figure 3.1), and the drug inhibition constant (K_i) was calculated (see Methods) based on the IC_{50} obtained by curve-fitting analysis. The results of testing ten such inhibitors are summarized in Table 1.1.

The results showed that B₄ and C₁ were the most potent in inhibiting rod and cone PDE6, while A₃ and B₃ were less potent inhibitors of all the compounds tested. The potency of all ten inhibitors tested was much lower than the well-known PDE5/6 inhibitor, vardenafil ($K_i < 1$ nM). When examining pharmacological differences between rod and cone PDE6, none of the compounds tested showed a strong preference for inhibiting rod or cone PDE6 isozymes. Inhibitors A₃, B₃, and B₄ showed about 2-fold preference for inhibiting PDE6C compared to the PDE6R, whereas inhibitor B₁ showed a 3-fold preference for binding to PDE6R compared to PDE6C. These minor differences

may be due to subtle differences in amino acid residues contacting these inhibitors within the catalytic site and/or small differences in the conformation of the active sites of the rod and cone enzymes.

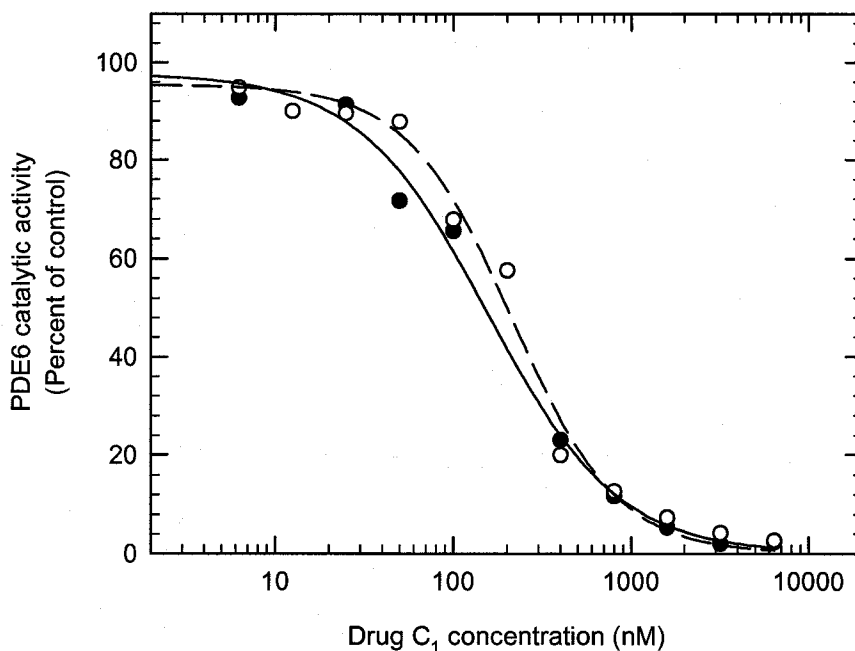


Figure 3.1 Dose-response Curves for Inhibition of PDE6R and PDE6C by Compound C₁. Purified PDE6R and PDE6C were activated by destroying the P γ subunits (see Methods) and then 2 pM concentration of PDE6R (filled circles) or PDE6C (open circles) were incubated with PDE inhibitor “C₁” at the indicated concentration for 15 min. 1 μ M [³H]cGMP was then added to assay catalytic activity using a radiotracer assay (see Methods). The data points for each enzyme were fit to a 3-parameter sigmoidal function using Sigmaplot. The K_i values for this individual experiment (typical of 3 other experiments) were: PDE6R, IC₅₀ = 155 nM, PDE6C, IC₅₀ = 213 nM.

PDE6 varies greatly in its ability to be inhibited by various classes of inhibitor compounds (Zhang et al., 2005). These may reflect the unique catalytic properties of the photoreceptor enzyme. PDE6 operates with very high catalytic efficiency for cGMP.

While the low affinity of substrate (K_M = 14 μ M for cGMP) and the high catalytic

constant (up to 8000 cGMP hydrolyzed per PDE6 per sec) of PDE6 are ideally suited for the millisecond time-scale activation of PDE6 required for visual transduction, there appears to be little distinction in the active sites of the rod and cone isoforms as judged by this set of test compounds.

ICOS compound	Rod IC ₅₀ (nM)	Cone IC ₅₀ (nM)	Selectivity ratio
			(PDE6R/PDE6C)
A ₁	205 ± 70	240 ± 40	0.9
A ₂	320 ± 250	240 ± 150	1.3
A ₃	1350 ± 850	1200 ± 1300	1.8
B ₁	650 ± 350	1900 ± 1900	0.3
B ₂	590 ± 100	660 ± 140	0.9
B ₃	6150 ± 3060	2550 ± 730	2.4
B ₄	59 ± 3	30 ± 4	1.8
C ₁	84 ± 15	70 ± 40	1.6
C ₂	540 ± 320	380 ± 230	1.4
C ₃	450 ± 110	370 ± 30	1.2

Table 3.1 PDE5 Inhibitor K_i Measurements on Rod PDE6 versus Cone PDE6. PDE5 inhibitor compounds were supplied by ICOS with their molecular weight, chemical formula and structure, and were given arbitrary designations. IC₅₀ values were determined as described in Fig. 3.1. The selectivity ratio was calculated as the ratio of the IC₅₀ values for PDE6R divided by PDE6C. Data represent the average of two (or three) experiments ± S.D.

B. Developing a Procedure for [³H]vardenafil Binding to PDE6

Methods to lower nonspecific binding of [³H]vardenafil during the membrane filtration binding assay—The radiolabeled PDE inhibitor [³H]vardenafil allows us to not only directly measure vardenafil binding affinity to PDE6 but also to assay whether the active sites are accessible. When we first attempted to use the filter binding conditions used for [³H]cGMP with PDE6 (Cote, 2005) or [³H]vardenafil with PDE5 (Blount et al.,

2004), we observed very high nonspecific binding (30 times higher than a typical [³H]cGMP binding assay). This level of nonspecific binding obscures actual binding. Whereas the nonspecific binding for a typical [³H]cGMP filter binding assay is lower than 0.5% of the total DPMs under standard assay conditions (see Methods), greater than 10% [³H]vardenafil (or [³H]sildenafil) DPMs were bound in the absence of PDE6.

In order to reduce this high nonspecific binding of [³H]vardenafil (or [³H]sildenafil), we first tested the washing solution used to rinse the membranes after applying the sample. The same amount of [³H]vardenafil as used for the binding assay was applied to pre-wetted filter membranes (Millipore 0.45 μm HA), then washed four times with 1 ml wash buffer (containing different additives). Figure 3.2 shows that increasing concentrations of salt did not lower the nonspecific binding effectively, nor did 1 mM EDTA. Detergent (0.1% Triton X-100) was found to be more useful in lowering nonspecific binding to about 25% of the total DPMs, but still higher than acceptable for accurate measurements of specific binding of [³H]vardenafil (or [³H]sildenafil) to PDE6.

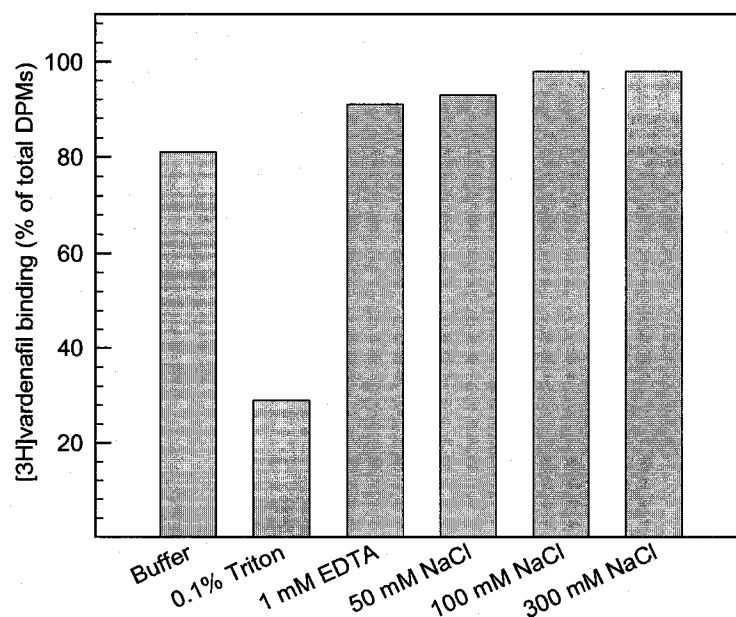


Figure 3.2 Various Wash Buffers Tested to Reduce [³H]vardenafil Nonspecific Binding. [³H]vardenafil (176,000 DPM in a 50 μ l volume) was directly applied onto pre-wet mixed cellulose acetate filter membranes (Millipore 0.45 μ m HA) under vacuum, and then washed 4 times with 1 ml of ice-cold 10 mM Tris buffer or buffer containing 0.1% Triton X-100, 1 mM EDTA, or 50 mM, 100 mM or 300 mM NaCl. The extent of [³H]vardenafil nonspecific binding was then determined by scintillation counting of the filters.

We assumed that the [³H]vardenafil was adhering to the membrane support, so it seemed reasonable to test various types of membrane filters next. The results in Figure 3.3 show that two types of glass fiber filters have the ability to lower the nonspecific binding of [³H]vardenafil, especially glass fiber #66075. Unfortunately, we were unable to use either type of glass fiber filter because of its inability to retain PDE6 on the filter during washing (*data not shown*). Since all other types of filter membranes showed a higher level of nonspecific binding, we continued to use the Millipore 0.45 μ m HA membrane (routinely used for [³H]cGMP binding assay) for all subsequent experiments.

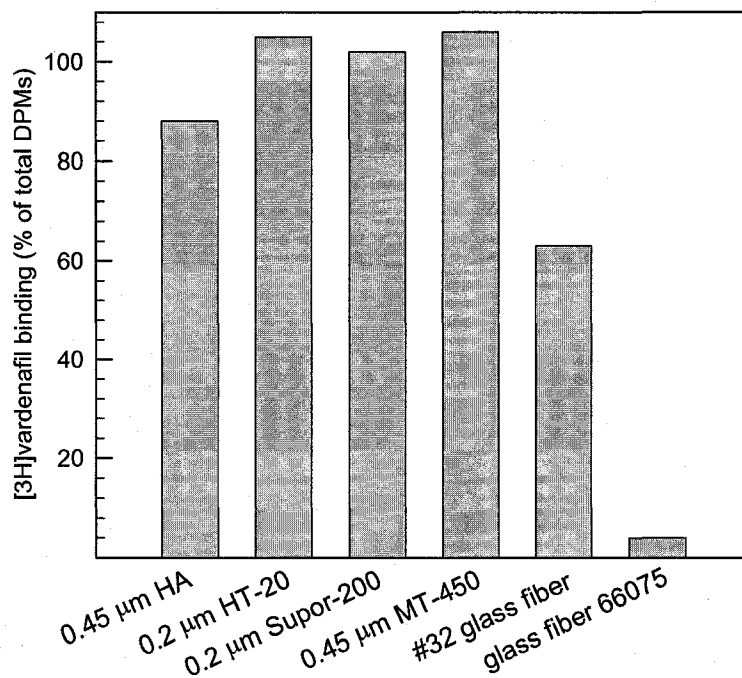


Figure 3.3 Testing Different Types of Filter Membranes to Reduce [³H]vardenafil Nonspecific Binding. The following filter membranes were pre-wet under vacuum: 0.45 μm HA (Millipore), 0.2 μm HT-20 (Gelman), 0.2 μm Supor-200 (Gelman), 0.45 μm T-450, Schleicher & Schuell #32 glass fiber filter 25 mm, or Gelman Sciences glass fiber filter 66075. After applying 50 μl portions of [³H]vardenafil (176,000 DPM) to each type of membrane, the membrane was washed 4 times with ice-cold 10 mM Tris (pH 7.5). The [³H]vardenafil nonspecific binding was then determined by scintillation counting of filters mixed with 4 ml Ultima Gold-XR.

We next tested the effect of pH on nonspecific drug binding. As Figure 3.4 shows, at the three pH values tested, about 80% of [³H]vardenafil was remained on the filter membrane after four washes. Wider ranges of pH were not tested, because of instability of PDE6 below pH 6.5 or above pH 8.5.

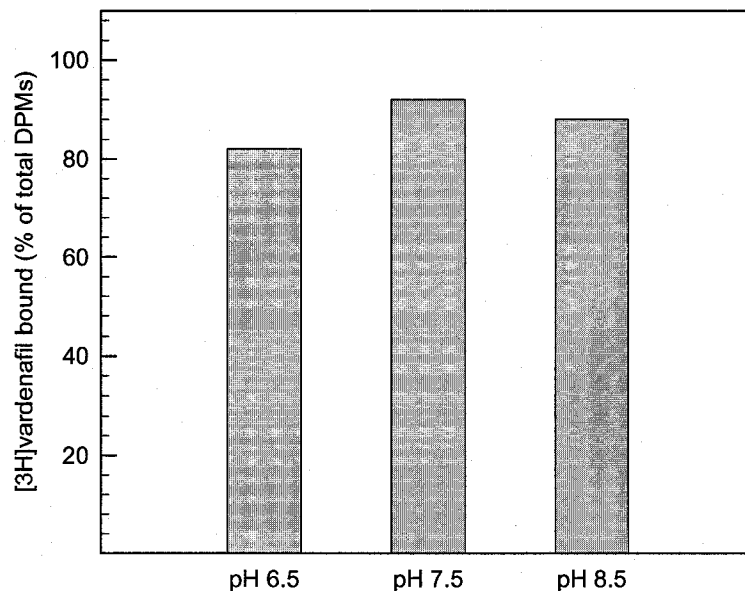


Figure 3.4 Evaluating the effects of pH of the washing buffer on [³H]vardenafil nonspecific binding. [³H]vardenafil (176,000 DPM in a 50 μ l volume) was applied onto pre-wet filter membrane (Millipore 0.45 μ m HA) under vacuum. The filters were washed by 4 times with 1 ml ice-cold 10 mM Tris (pH 7.5) adjusted to the following pH: 6.5, 7.5, or 8.5. The [³H]vardenafil nonspecific binding was then determined by scintillation counting.

We then speculated that varying the volume of the sample applied to the filter or the volume and number of washes could influence [³H]vardenafil nonspecific binding. As showed in Figure 3.5, a larger, 1-ml sample volume is better than a 50 μ l sample volume. In addition, 3 ml per wash is more effective than 1 ml of wash volume under all conditions tested for lowering the nonspecific binding. While at least four washes are needed to substantially reduce nonspecific binding, 6-8 washes are preferable to further reduce the nonspecific binding.

From the previous experiments we conclude that the optimal conditions for lowering nonspecific binding for [³H]vardenafil (or [³H]sildenafil) to membrane filters were: (1) 0.1% Triton X-100 in wash buffer; (2) Increasing the 50 µl reaction volume to 1 ml immediately before applying to the filter; (3) wash with 1 ml wash buffer 8 times. This method reduced the NSB to 3-5% of total DPMs and permitted us to quantify specific binding of radiolabeled inhibitors to PDE6.

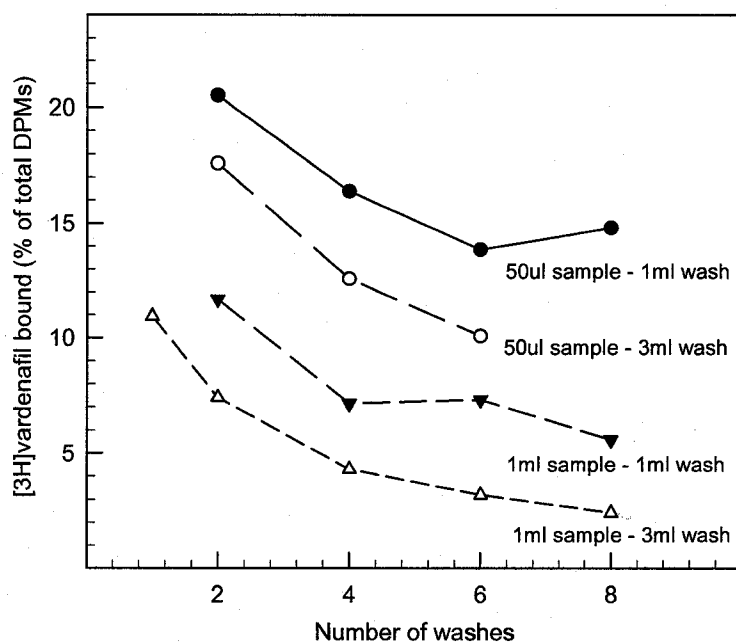


Figure 3.5 Effects of Sample Volume and Wash Number and Volume on [³H]vardenafil Nonspecific Binding. [³H]vardenafil (177,000 DPM per sample) was applied onto pre-wet filter membranes (Millipore 0.45 µM HA) under vacuum in a sample volume of 50 µl (circles) or 1 ml (triangles) and washed with either 1 ml (filled) or 3 ml (open) ice-cold 10 mM Tris (pH 7.5). The nonspecific binding on the filter membrane was qualified by scintillation counting.

[³H]varденафил binding is stabilized by a P γ peptide during the filter binding assay—Although adding Triton X-100, increasing the sample volume, and increasing the filter washing successfully reduced the nonspecific binding to about 3-5% of total DPMs, initially we were unable to reliably detect [³H]varденафил binding to PDE6 (i.e. no signal was detectable above the nonspecific binding). Therefore, histone II-AS was introduced into the binding solution (see Chapter 2) since it had been previously used in [³H]varденафил binding assay of PDE5 (Blount et al., 2004). We also tested several other compounds, including PDE6-interacting compounds: histone VIII-S (Miki et al., 1975; Hurwitz et al., 1984), P γ , and two P γ peptides (P γ 1-45 and P γ 63-87). The effects of histone VIII-S, P γ , and P γ 63-87 on [³H]varденафил binding have already been described in Chapter 2. Surprisingly, P γ 1-45, which is known to stabilize high affinity cGMP binding to the GAF domains (Mou and Cote, 2001), is also able to stabilize [³H]varденафил binding to the catalytic domains (Figure 3.6). There is no detected binding of varденафил to Pa β alone, but the binding was greatly enhanced by addition of histone II-AS or P γ 1-45. The P γ 1-45 stabilization of varденафил binding to the catalytic site suggests an allosteric effect on the active site though GAF domain upon P γ interaction with the GAF domain. The mechanism of action of histone II-AS is not clear.

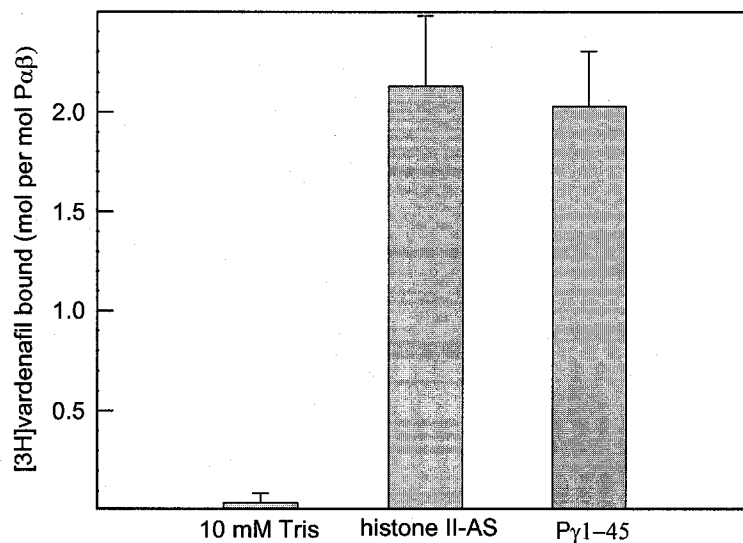


Figure 3.6 Comparison of [³H]vardenafil Binding to Paβ Stimulated by Histone II-AS or Pγ1-45. Purified Paβ (5 nM) and 70 nM [³H]vardenafil were incubated with histone II-AS (0.2 mg/ml) or Pγ1-45 (10 μM) for 40 min at room temperature. Vardenafil binding was quantified by the membrane filtration assay. Data represents the average of three experiments ± S.D.

Histone II-AS has minor effects on the catalysis of PDE6 catalytic dimer, PDE6 holoenzyme, and ROS membrane-associated PDE6—We tested the effect of histone II-AS on PDE6 catalysis, since we wanted to routinely use it to stabilize [³H]vardenafil inhibitor binding. While histone II-AS can dramatically increase the detectable vardenafil binding signal to catalytic sites of Paβ (Figure 3.6), only minor effects were observed on PDE6 hydrolytic activity (when tested at the same concentration as used in the membrane filtration assay; Figure 3.7). Our results demonstrated that histone II-AS increased the basal activity of PDE6 holoenzyme to about 10 percent of its maximum value. Interestingly, histone II-AS can also decrease the catalysis of Paβ by about 10 percent.

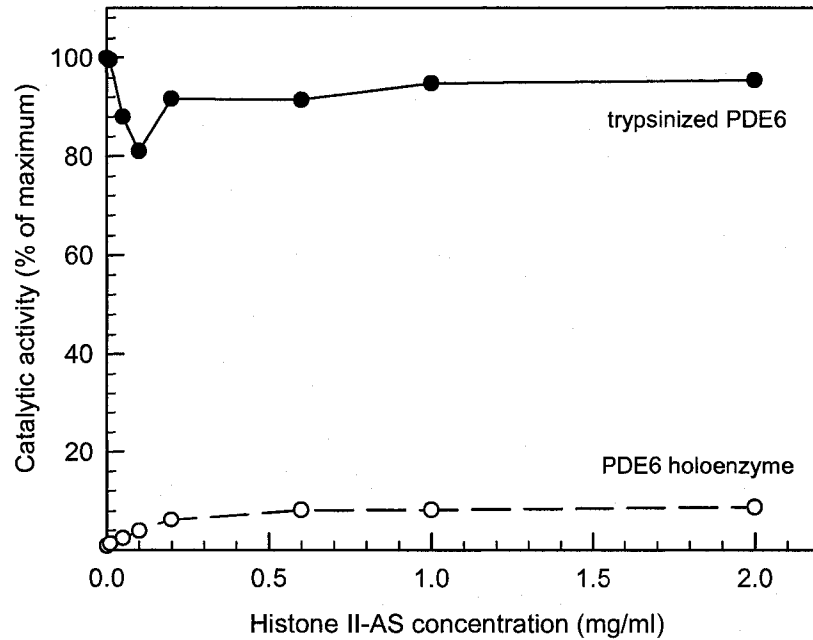


Figure 3.7 Histone II-AS Effects on Trypsinized PDE6 and PDE6 Holoenzyme. Increasing amounts of histone II-AS were incubated with trypsinized PDE6 (5 nM) or PDE6 holoenzyme (5 nM) for 10 min at room temperature. The catalytic activity was then determined by hydrolytic activity measurement using the phosphate release assay. Data represent one of three similar experiments.

We then further tested the histone II-AS effect on ROS-PDE6 in its trypsin- or transducin-activated state, and at the same histone II-AS concentration (0.2 mg/ml) we had used for [³H]vardenafil binding to purified PDE6. We found that 0.2 mg/ml histone II-AS can increase about 30% the catalytic rate of transducin-activated PDE6. Because of this, we lowered the concentration of histone II-AS (0.05mg/ml) to test the effect on the catalysis of transducin-activated PDE6 as the GTP γ S concentration was increased. Figure 3.8 shows that transducin-activation of ROS-PDE6 is unaffected when 0.05 mg/ml histone II-AS is present. Although higher concentrations of histone II-AS can affect the extent of transducin activation, a low concentration of 0.05 mg/ml (suitable for [³H]vardenafil binding assays) does not affect the catalytic properties of ROS-PDE6.

These results suggest that histone II-AS not only may slow down the rapid exchange of radiolabeled inhibitor at the active site (useful for the binding assay), it may also decrease the affinity of $P\gamma$ for PDE6 under conditions where transducin activation is being studied.

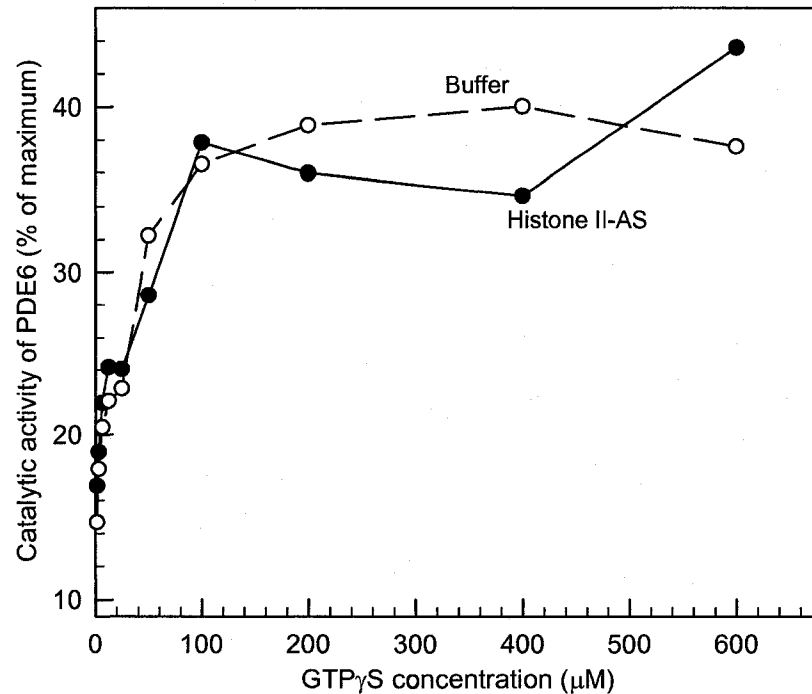


Figure 3.8 Low Concentrations of Histone II-AS Do Not Affect the Catalytic Activity of Transducin-activated PDE6. Increasing concentrations of GTP γ S were added to ROS homogenates (10 nM PDE6) to progressively activate PDE6. Then, 0.05 mg/ml histone II-AS (filled circles) or buffer (open circles) was added, and the extent of PDE6 activation was quantified by measuring cGMP hydrolytic rates and normalized to the activity of the same sample fully activated by limited trypsin proteolysis.

Histone II-AS decreases the [3 H]cGMP binding affinity to $Pa\beta$ complexed with $P\gamma 1-45$ —In order to further explore the effects of histone II-AS on PDE6, we tested whether histone II-AS affected [3 H]cGMP binding to the regulatory GAF domain. PDE6 has two non-identical cGMP binding sites. One site can bind cGMP with high affinity in the absence of $P\gamma$, while the other, low-affinity site requires $P\gamma$ to stabilize cGMP binding to the second site (Mou and Cote, 2001).

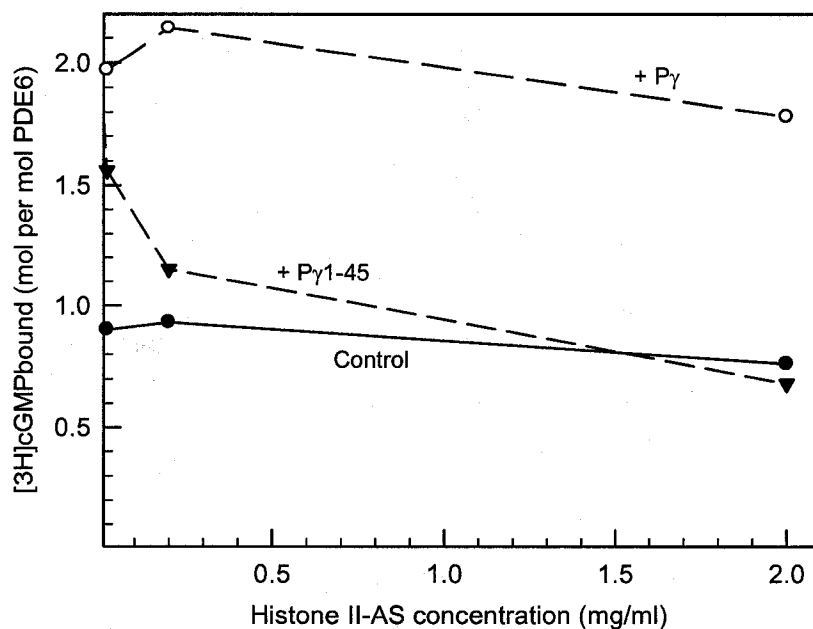


Figure 3.9 Histone II-AS Lowers [^3H]cGMP Binding to the Noncatalytic cGMP Binding Sites of Pa β Reconstituted With P γ 1-45. Nucleotide-depleted Pa β (4 nM) was incubated with the indicated concentration of histone II-AS along with buffer (filled circles), 10 μM P γ (open circles), or 10 μM P γ 1-45 (filled triangles) at 37°C for 5 min. [^3H]cGMP (10 μM) was added for 5 min and binding quantified by the filter binding assay.

As expected, only one [^3H]cGMP bound to Pa β at its high affinity binding site; cGMP binding to Pa β was unaltered by histone II-AS (Figure 3.9, filled circles). When P γ or P γ 1-45 was added 1.6 or 2.0 [^3H]cGMP were bound per PDE6, confirming that the low affinity binding site was converted to a high affinity binding site (Figure 3.9). Histone II-AS did not significantly affect the binding of [^3H]cGMP to PDE6 holoenzyme (Pa $\beta\gamma\gamma$). However Pa β reconstituted with P γ 1-45 showed a decrease in [^3H]cGMP binding with increasing amounts of histone II-AS (Figure 3.9, triangles). These results suggest that histone II-AS may be able to compete with P γ 1-45—but not full-length P γ —

in its binding to Pa β , and that histone II-AS cannot by itself stabilize cGMP binding to the low affinity binding site.

Anomalous effects of histone VIII-S on PDE6 catalytic properties—Histone VIII-S, which historically has been used to activate PDE6 holoenzyme (Miki et al., 1975; Hurwitz et al., 1984), can stabilize vardenafil binding to a certain extent (see Chapter 2). We reexamined the effect of histone VIII-S on the catalysis of nonactivated PDE6 holoenzyme or trypsinized PDE6, and surprisingly found that histone VIII-S was not able to activate PDE6 holoenzyme (Figure 3.10, filled circles). Also to our surprise, histone VIII-S can fully inhibit the catalysis of trypsinized PDE6 (Figure 3.10, open circles). The reasons for this unexpected behavior are not understood, but may reflect batch-to-batch variations in the histone VIII-S used in different labs.

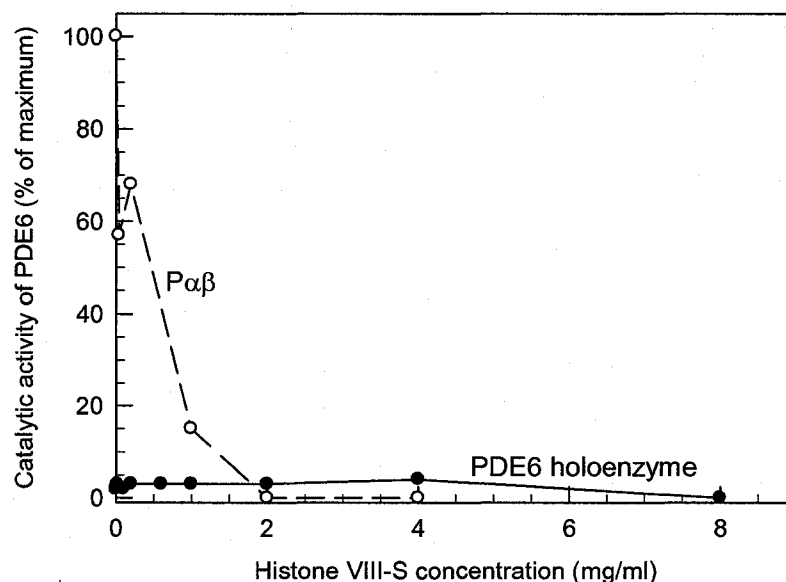


Figure 3.10 Histone VIII-S Inhibits the Catalytic Activity of Trypsinized PDE6. PDE6 holoenzyme (2.2 nM; filled circles) or trypsin-treated PDE6 (1.5 nM; open circles) was incubated with the indicated amounts of histone VIII-S, then PDE6 catalytic activity was measured and normalized to the maximum catalytic activity of Pa β lacking histone VIII-S.

[³H]sildenafil binds to PDE6 catalytic dimer lacking P γ , but fails to bind to the holoenzyme—Radiolabeled inhibitors give us an unique opportunity to evaluate the catalytic sites of PDE6. As reported in Chapter 2, [³H]vardenafil binds to PDE6 catalytic dimer with high affinity ($K_D = 7$ nM) and P γ blocks the access of inhibitor binding to the active site. Consistent with this observation, [³H]sildenafil bound to PDE6 with high affinity ($K_D = 43$ nM), in good agreement with the previously reported $K_i = 11$ nM (Zhang et al., 2005). Furthermore, the binding curve was consistent with only one class of binding sites (Figure 3.11). Two sildenafil stoichiometrically bound to one PDE6 ($B_{max} = 2.2$) demonstrating that the catalytic sites of PDE6 are both available for sildenafil, which is in agreement with our conclusion from [³H]vardenafil binding (Chapter 2). Furthermore, the simple hyperbolic behavior of drug binding suggests the two catalytic sites are identical. In contrast, sildenafil failed to bind to PDE6 when Pa β was reconstituted with P γ , regardless of whether P γ was added before or after [³H]sildenafil addition (Figure 3.11).

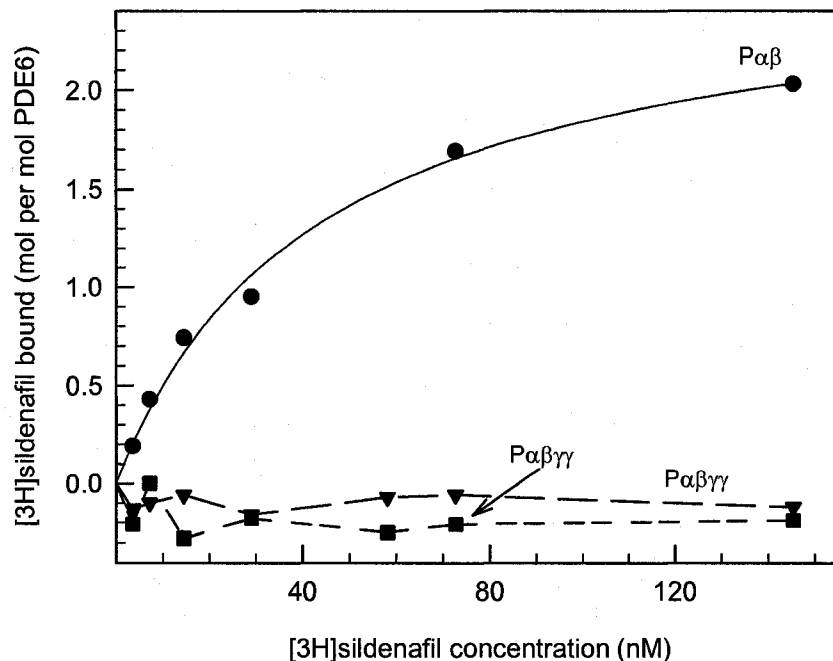


Figure 3.11 [³H]sildenafil Binds Stoichiometrically to Each Pa β Catalytic Subunit, But Only In the Absence of P γ . Purified Pa β (5 nM; filled circles) or Pa β reconstituted with 10 μ M P γ was incubated with 0.2 mg/ml histone II-AS and 70 nM [³H]sildenafil (added after P γ addition, squares; or before P γ addition, triangles). Samples were incubated for 40 min before membrane filtration. Sildenafil binding was normalized to the Pa β concentration, as estimated by both [³H]cGMP binding assay, as well as by hydrolytic activity measurements (which agreed to within 10%). [³H]sildenafil binding to Pa β was fit assuming a single class of binding sites ($K_D = 42.9$ nM and $B_{max} = 2.2$). Data is representative of two experiments.

[³H]vardenafil binds to ROS-PDE6 with lower affinity compared with highly purified PDE6—The experiments with highly purified PDE6 (above and in Chapter 2) concluded that two molecules of inhibitor can bind to each Pa β heterodimer, but that binding is blocked when P γ is associated with the catalytic dimer. We next tested the affinity of [³H]vardenafil for the catalytic sites under more physiological conditions, namely where PDE6 is still attached to the disk membrane in its trypsin- or transducin-activated states.

Figure 3.12 shows that [³H]vardenafil binding to trypsin-activated ROS-PDE6 is consistent with a single class of binding sites with a lower apparent affinity than it binds to purified trypsinized PDE6. Under the conditions where P_γ inhibition was fully relieved on ROS-PDE6, the catalytic subunits bound 1.4 drug molecules per catalytic dimer. [The failure to reach the anticipated 2.0 mol drug per mol PDE6 was not readily explained.] No binding was detected with nonactivated ROS-PDE6 (*data not shown*) demonstrating that the highly specific drug molecules do not bind to other cellular components and that the P_γ subunit prevented inhibitor binding to catalytic sites on ROS-PDE6 as well. [³H]vardenafil was also tested with ROS-PDE6 following transducin activation. The vardenafil binding to transducin-activated ROS-PDE6 (Figure 3.12, open circles) has a nearly 6-fold lower affinity compared with its binding to trypsin-activated ROS-PDE6 (Figure 3.12, filled circles). Since the P_γ subunits were degraded by the trypsinization protocol, this lower apparent affinity of vardenafil for transducin-activated ROS-PDE6 may be due to competition at the catalytic site between drug and P_γ which is displaced (but not completely dissociated) upon transducin activation.

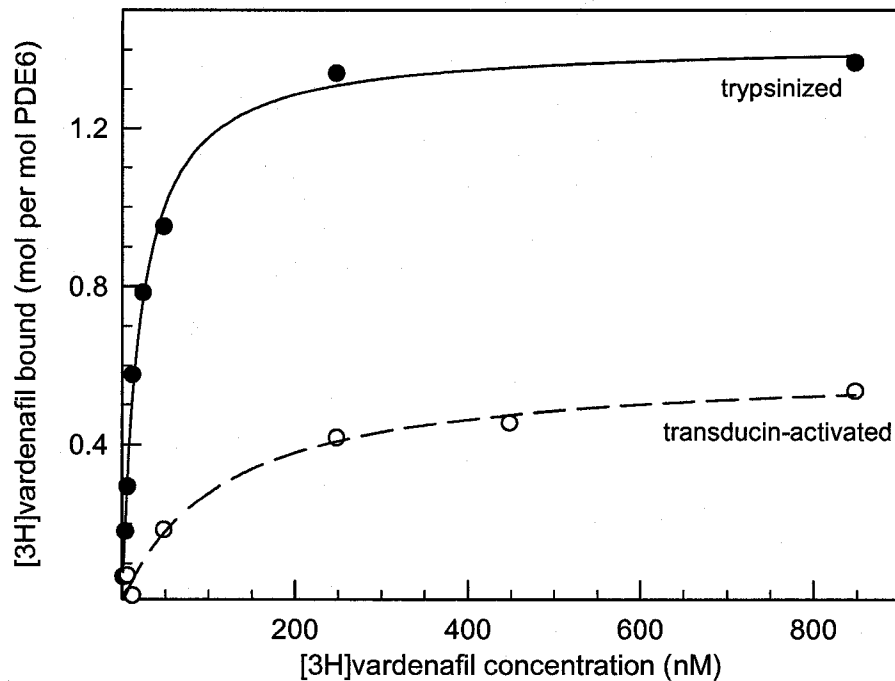


Figure 3.12 [^3H]vardenafil Binds With Higher Affinity to Trypsinized ROS-PDE6 than Transducin Activated ROS-PDE6. Trypsinized ROS homogenates (4 nM; filled circles) and transducin-activated ROS homogenates (10 nM; open circles) were incubated with 0.05 mg/ml histone II-AS and increasing concentrations of [^3H]vardenafil. Samples were incubated for 40 min before membrane filtration. [^3H]vardenafil binding to PDE6 was fit assuming a single class of binding sites (trypsin-activated: $K_D = 20$ nM, $B_{\max} = 1.4$ vardenafil per PDE6; transducin-activated: $K_D = 112$ nM, $B_{\max} = 0.6$ vardenafil per PDE6).

C. Allosteric Communication Between the Regulatory Domain and the Catalytic Site of PDE6

Drug binding at the catalytic site of PDE6 alters the cGMP binding properties to the GAF domain—Under the condition where there is no $\text{P}\gamma$ inhibition of the $\text{Pa}\beta$ active sites, cGMP can bind to one high affinity binding site (within the GAFa domain on $\text{Pa}\beta$), as judged by a [^3H]cGMP filter binding assay (Mou et al., 1999). Upon $\text{P}\gamma$ binding, a low affinity cGMP binding site undergoes a change from low affinity to high affinity, making the second cGMP binding to PDE6 detectable with the filter binding assay. In order to

prevent the hydrolysis of cGMP at the catalytic sites, EDTA and/or PDE inhibitors were used to inhibit the catalytic activity during cGMP binding to the regulatory domain. We wished to examine whether the manner of inhibition of catalysis had an effect on cGMP binding to the GAF domain, since occupancy of the active site with drug might allosterically influence cGMP binding in a different manner than, for example, metal ion chelation at the active site.

Consistent with published work, 0.8 ± 0.1 cGMP per Pa β was detected when only EDTA was used to prevent the hydrolysis of cGMP, while 1.8 ± 0.1 cGMP bound per PDE6 catalytic dimer when P γ was added back (Figure 3.13). The PDE inhibitors tadalafil and zaprinast showed similar results as EDTA, with only 1.0 - 1.4 cGMP per PDE6 in the absence of P γ , and a stimulation of cGMP binding to about 2.0 mol cGMP per mol PDE6 upon P γ addition. Surprisingly, the presence of sildenafil and vardenafil resulted in 1.8 ± 0.2 and 1.5 ± 0.1 [3 H]cGMP bound per Pa β in the absence of P γ , with an insignificant increase in cGMP binding when Pa β was reconstituted with P γ . We further tested the stimulatory effect of sildenafil and vardenafil on cGMP binding to Pa β by studying cGMP binding as a function of drug concentration (Figure 3.14). In general, the dose-response relationship for vardenafil and sildenafil is consistent with drug binding at the active site (and not some other binding site) being responsible for the stimulating action of these drugs on [3 H]cGMP binding to the GAF domain. The enhanced binding of cGMP to the second class of lower affinity binding sites suggested that the second cGMP binding site undergoes a conformational change from low affinity to high affinity upon the occupancy of sildenafil or vardenafil at the active site.

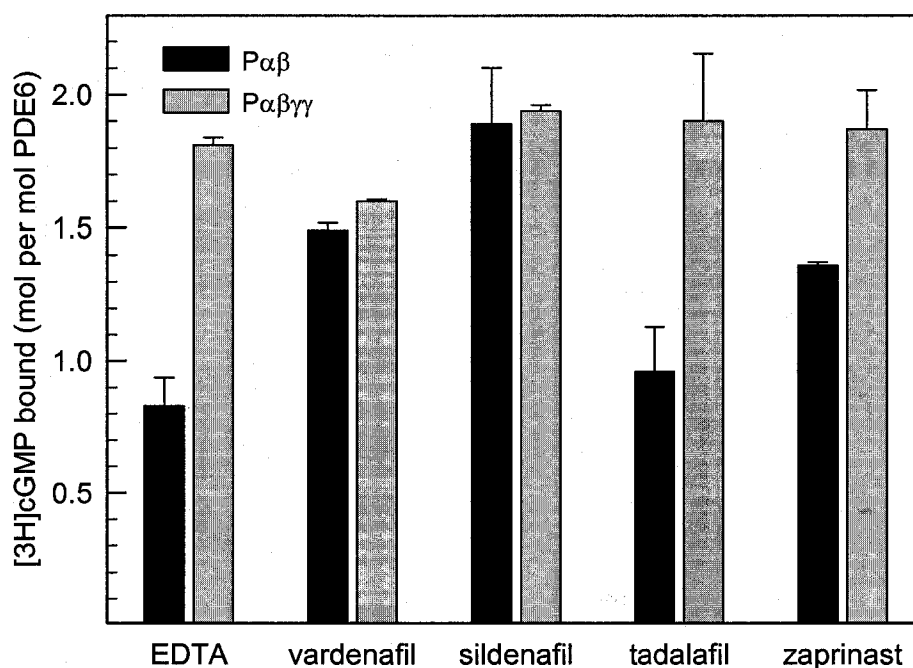


Figure 3.13 Occupancy of the Catalytic Sites by Certain Inhibitors Alters the cGMP Binding to Regulatory GAF Domain. cGMP depleted, trypsinized PDE6 (3.7 nM) was treated with the following to prevent the hydrolysis of [³H]cGMP: 10 mM EDTA, 100 μM vardenafil, 100 μM sildenafil, 100 μM tadalafil, or 100 μM zaprinast. Then, the inhibitor-treated PDE6 was mixed with 10 μM [³H]cGMP with buffer (black) or 10 μM Pγ (gray) and incubated at 37°C for 5 min before membrane filtration. Parallel measurements of PDE6 concentration were tested based on hydrolytic activity. Data are the mean ± S.D. of two experiments.

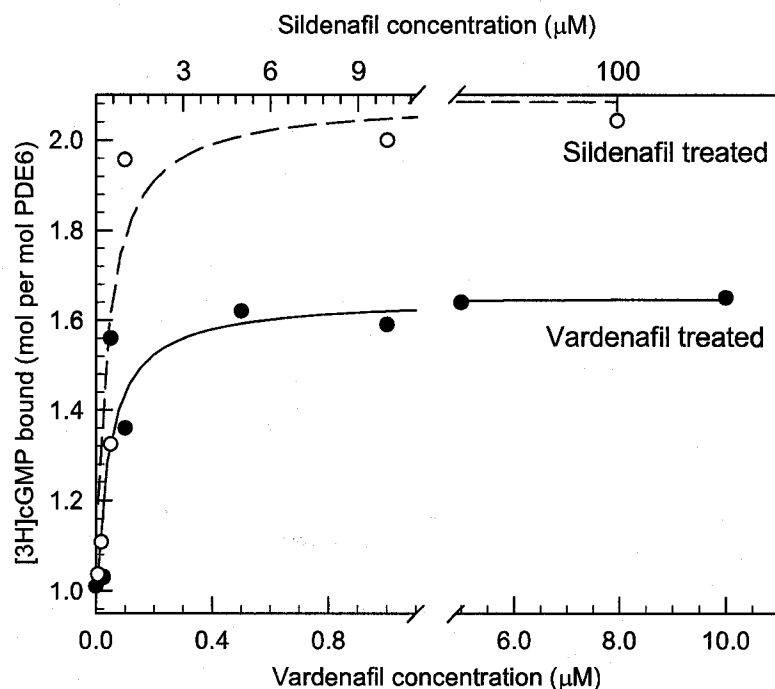


Figure 3.14 cGMP Binding to Regulatory GAF Domain is Enhanced by Vardenafil or Sildenafil In a Concentration Dependent Manner. cGMP-depleted trypsinized PDE6 was treated with the indicated concentration of vardenafil (filled circles) or sildenafil (open circles). Then the PDE6 was mixed with 10 μM [^3H]cGMP and incubated at 37°C for 5 min before membrane filtration. PDE6 concentration was estimated based on its maximum hydrolytic activity (see Methods). [^3H]cGMP binding to PDE6 was fit assuming a single class of binding sites (vardenafil: $K_{1/2} = 0.01$ nM and $B_{\text{max}} = 1.6$ cGMP/PDE6; sildenafil: $K_{1/2} = 0.05$ μM and $B_{\text{max}} = 2.0$ cGMP/PDE6). Data represents one of two experiments.

PDE6 GAF domains may allosterically affect the catalytic site through $P\gamma$ subunits of PDE6—As demonstrated in Section B, $P\gamma 1-45$ was able to stabilize high affinity vardenafil binding when assayed in the absence of histone II-AS. This result suggested a direct allosteric effect between the GAF and catalytic domain. To further explore this phenomenon, we examined the concentration dependence of $P\gamma 1-45$ stimulation. As shown in Figure 3.15, $P\gamma 1-45$ is able to stabilize [^3H]vardenafil binding to the catalytic domains in a concentration-dependent manner. A comparison of the dose-response

curves shows that ~10-fold greater P γ 1-45 is needed to enhance vardenafil binding compared to cGMP binding. This occurs over a range of P γ 1-45 concentrations that have no direct effect on the hydrolytic activity at the catalytic domain (*not shown*). The greater potency of P γ 1-45 to stimulate cGMP binding may be explained as a “local” stabilization of cGMP binding to the GAFa domain by P γ 1-45 interaction with GAFa. In contrast, the lower potency of P γ 1-45 to alter vardenafil binding may be explained as a more “distant” effect that requires conformational change be transmitted from the GAFa to the catalytic domain.

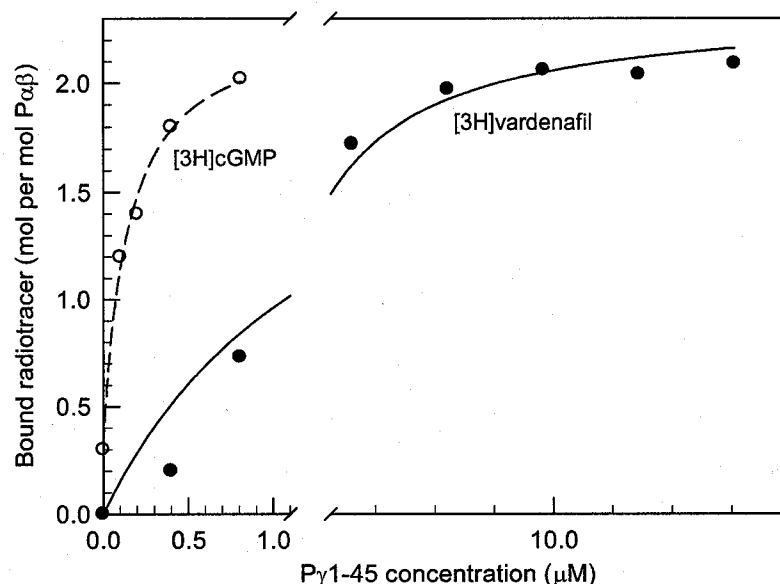


Figure 3.15 P γ 1-45 Stabilizes [3 H]vardenafil and [3 H]cGMP Binding to Pa β Catalytic Subunits Over Different Concentration Ranges. Purified Pa β (1 nM), [3 H]vardenafil (70 nM; filled circles) and the indicated concentrations of P γ 1-45 were incubated for 40 min before membrane filtration. Alternatively, cGMP-depleted, trypsinized PDE6 (6.7 nM final concentration) was first treated with 10 mM EDTA to prevent cGMP hydrolysis and then incubated with 10 μ M [3 H]cGMP and the indicated amount of P γ 1-45 at 37°C for 5 min before membrane filtration (open circles). Vardenafil binding and cGMP binding were normalized to the Pa β concentration, as estimated by hydrolytic activity measurements. [3 H]vardenafil binding to Pa β was fit (2-parameter hyperbola) assuming a single class of binding sites ($K_{1/2} = 1.4 \mu$ M and $B_{max} = 2.3$ cGMP/PDE6). [3 H]cGMP binding data was fit to a 3-parameter hyperbolic function with $K_{1/2} = 97$ nM and $B_{max} = 2.0$ cGMP per PDE6. Note different scales for x-axis.

Conclusion

Through developing the filter binding assay for [³H]varденаfil to PDE6, we discovered that maximal binding required either histone II-AS or the N-terminal fragment of the inhibitory subunit of PDE6. There is no detected binding of vardenafil to Paβ alone, but the binding was greatly enhanced by addition of certain peptides or proteins. While histone II-AS, histone VIII-S, Pγ1-87 and Pγ63-87 can each stimulate somewhat vardenafil binding to Paβ, only histone II-AS and Pγ1-45 stabilize binding sufficiently so that both active sites of Paβ can be labeled. The stimulation of drug binding at the active site by Pγ1-45 demonstrated a direct allosteric communication between the GAF and catalytic domains. Pγ1-45 is the N-terminal part of Pγ subunits that believed to bind only to the regulatory GAF domains of PDE6, and lacks contact sites with the catalytic domain (Guo et al., 2006). Thus, we propose that the enhancement of inhibitor binding within the catalytic domain is caused by the conformational change induced by Pγ1-45 binding to the regulatory GAF domains.

The function of histone II-AS is still unclear, but it might be the histone II-AS, which is polycationic, mimics the polycationic region of Pγ1-45. Further work with chemically defined histones or peptides is needed to better define the mechanism of action of histone II-AS on PDE6 conformation.

FUTURE DIRECTIONS

One major conclusion of this thesis is that endogenous transducin can relieve Py inhibition at only one active site of PDE6, but that a second site can be detected when exogenous transducin is added to ROS membranes (Chapter 2). However, the current work failed to directly demonstrate the stoichiometry of transducin binding to PDE6. To address this question, cross-linking experiments could be performed. If the physical interaction site of PDE6 and transducin can be coupled successfully by the cross-linker, then native gel electrophoresis or size exclusion chromatography could be used to demonstrate whether one or two transducins bind per PDE6. For these experiments, both low and high concentrations of transducin would be needed to compare different interaction sites. A second approach to this question is to use analytical ultracentrifugation to determine the binding stoichiometry of transducin to PDE6. By detecting a shift in the sedimentation properties of labeled PDE6 when adding increasing amount of transducin, the added mass and/or changes in overall shape of the complex can be calculated.

This work also highlights the importance of membrane association of transducin and PDE6 for efficient activation of PDE6 during visual transduction (Chapter 2). Future experiments should be carried out to determine whether membrane binding alone or protein concentration on the membrane is the critical factor for efficient PDE6 activation by transducin. The composition of the membranes used for tethering transducin and

PDE6 should also be examined; for example, are membrane vesicles prepared from purified phospholipids as effective as ROS membranes? If ROS membranes are more effective in stimulating PDE6 activation by transducin, then the identity of the stimulatory factors needs to be determined.

Another area that requires further study is the mechanism of action of histones on PDE6. While our work suggests that histone II-AS competes with P γ (particularly its polycationic region) for a common binding site on PDE6 catalytic dimer, the location of the interaction site, its affinity, and whether endogenous proteins (e.g., GARP2) may act similarly to histone II-AS, are all open questions. The ability of histone II-AS to stimulate drug binding (Chapters 2 and 3) is also not understood, and requires further work to determine whether histones exert a long-range effect via the GAF domains, or instead may bind directly to the catalytic domain to stabilize drug binding. Likewise, the effects of histone VIII-S we observe run counter to the published literature, and require further study to understand how this class of histone interacts with PDE6.

Finally, further work is needed to determine the topology of the catalytic site of PDE6 and its ability to bind drugs in a selective manner. For example, compound B₃ (Chapter 3) shows selectivity of PDE6R compared to PDE5 (but no significant rod-cone selectivity). Analysis of additional inhibitor compounds may further delineate the subtle differences that distinguish PDE5 from PDE6 and rod PDE6 from cone PDE6.

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