

Probing the catalytic sites and activation mechanism of photoreceptor phosphodiesterase (PDE6) using radiolabeled PDE inhibitors*

Yu-Ting Liu[†], Suzanne L. Matte[†], Jackie D. Corbin[‡], Sharron H. Francis[‡] and Rick H. Cote^{†1}

From the [†]Department of Molecular, Cellular and Biomedical Sciences, University of New Hampshire, Durham, New Hampshire 03824 and the [‡]Department of Molecular Physiology and Biophysics, Vanderbilt University School of Medicine, Nashville, TN 37232-0615

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Address correspondence to: Rick H. Cote, Department of Molecular, Cellular and Biomedical Sciences, 46 College Rd., University of New Hampshire, Durham, New Hampshire 03824. Tel.: 603-862-2458; Fax: 603-862-4013; E-mail: rick.cote@unh.edu.

Retinal photoreceptor phosphodiesterase (PDE6) is unique among the phosphodiesterase enzyme family 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 is generally assumed that during visual excitation both catalytic sites are relieved of $P\gamma$ inhibition upon binding of two activated transducin molecules. Since PDE6 shares structural and pharmacological similarities with PDE5, we utilized radiolabeled PDE5 inhibitors to probe the catalytic sites of PDE6. The membrane filtration assay we used to quantify [³H]vardenafil binding to PDE6 required histone II-AS to stabilize drug binding to the active site. Under these conditions, [³H]vardenafil binds stoichiometrically to both the α - and β -subunits of the activated PDE6 heterodimer. [³H]vardenafil fails to bind to either the PDE6 holoenzyme or the PDE6 catalytic dimer reconstituted with $P\gamma$, consistent with $P\gamma$ blocking access to the drug binding sites. Following transducin activation of membrane-associated PDE6 holoenzyme, [³H]vardenafil binding increases in proportion to the extent of PDE6 activation. Both [³H]vardenafil binding and hydrolytic activity of transducin-activated PDE6 fail to exceed 50% of the value for the PDE6 catalytic dimer. However, adding a 1000-fold excess of activated transducin can stimulate the hydrolytic activity of PDE6 to its maximum extent. These results demonstrate that both subunits of the PDE6 heterodimer are able to bind ligands to the enzyme active site. Furthermore, transducin relieves $P\gamma$ inhibition of PDE6 in a biphasic manner, with only one-half of the maximum

PDE6 activity efficiently attained during visual excitation.

The superfamily of phosphodiesterase (PDE²) enzymes plays a critical role in maintaining the cellular levels of cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) (1). 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 sub-second changes in cGMP levels (2). During the first steps in vision, photoisomerized rhodopsin activates transducin, which binds GTP and releases its activated α -subunit ($T\alpha$ -GTP) to activate membrane-associated rod PDE holoenzyme by displacing the inhibitory γ subunit ($P\gamma$) from the active sites of the PDE6 catalytic dimer ($P\alpha\beta$). 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 (3,4).

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 ($P\alpha\beta$), 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, (5)], raising the possibility that the catalytic site on the α -subunit in other species is not functional.

Moreover, 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 (6,7), 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 $T\alpha$ -GTP bind to both catalytic subunits of $P\alpha\beta$ releasing the $P\gamma$ inhibition at both active sites (6,8). Other investigators have reported that a single $T\alpha$ -GTP was able to maximally activate the PDE6 catalytic dimer under defined conditions (9-11). The latter work suggests that either the PDE6 catalytic dimer has only one functional active site, or that a single activated $T\alpha$ -GTP can relieve $P\gamma$ inhibition at both $P\alpha\beta$ 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 (12,13) and bovine (10,14) 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, 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 distinct inhibitory $P\gamma$ subunits tightly associated with the catalytic dimer to form an inactive tetrameric holoenzyme (15). PDE6 is also the only family of PDEs in which the catalytic activity is directly regulated by a heterotrimeric G-protein, transducin (2).

PDE6 is most closely related to PDE5 (abundant in vascular smooth muscle) in its biochemical, structural and pharmacological properties (16). Both PDE5 and PDE6 have highly conserved amino acid sequences and 3-dimensional structures (17-20). PDE5 and PDE6 share strong substrate specificity for cGMP compared to cAMP (21). Both can bind cGMP with high affinity at one of their regulatory GAF domains (GAFa) within each catalytic subunit (2,22). Most PDE5-selective inhibitors, including the well-known erectile dysfunction drugs Viagra

(sildenafil) and Levitra (vardenafil), can also potently inhibit PDE6 catalysis as well (23-25).

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 [3 H]vardenafil, we tested the hypothesis that both catalytic domains of the $P\alpha\beta$ 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 [3 H]cGMP was from PerkinElmer Life Sciences and [3 H]vardenafil (26) was a kind gift of Drs. P. Sandner, E. Bischoff, & 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 (27). Rod PDE6 holoenzyme ($P\alpha\beta\gamma\gamma$) 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 (27).

The PDE6 catalytic dimer ($P\alpha\beta$) was prepared from the PDE6 holoenzyme by removing the inhibitory $P\gamma$ subunits through limited trypsin proteolysis (28). A time course of proteolytic activation of PDE6 was determined to ensure that >90% of the $P\gamma$ subunit was destroyed without altering the apparent molecular weight of the catalytic subunits [as judged by SDS-PAGE; (27)].

The concentration of PDE6 was determined by both measurements of catalytic activity under conditions where the k_{cat} was known [5600 s^{-1} ; (29)] and by measurements of [3 H]cGMP binding

under nucleotide-depleted conditions [described in ref. (30)] where the cGMP binding sites in the GAF domains were unoccupied and stoichiometric binding (2.0 cGMP per PDE6 dimer) occurred (31).

Purification of persistently activated transducin α -subunit ($T\alpha$ -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 $T\alpha$ -GTP γ S by centrifugation. The extracted $T\alpha$ -GTP γ S was purified on a Blue Sepharose column (32,33). The concentration of $T\alpha$ -GTP γ S was determined by a colorimetric protein assay. Purified $T\alpha$ -GTP γ S was stored with 50 μ M GTP γ S and 50% glycerol at -20°C .

Preparation and purification of $P\gamma$ and a C-terminal synthetic peptide—Wild-type bovine rod $P\gamma$ (87 amino acids) was expressed in E. coli BL21(DE3) cells and purified to $>97\%$ purity using SP-Sepharose followed by reversed-phase high pressure liquid chromatography (34). The wild-type $P\gamma$ concentration was determined spectrophotometrically using an experimentally determined extinction coefficient of $7550\text{ cm}^{-1}\text{ M}^{-1}$ (31). The inhibitory activity of purified $P\gamma$ was assayed by its ability to stoichiometrically inhibit trypsin-activated bovine rod PDE (29). The spectrophotometric and activity estimates of $P\gamma$ concentration agree to within 10% for all wild-type $P\gamma$ preparations used in this study. The concentration of the synthetic peptide $P\gamma$ 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 MgCl_2 , 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 incubation with an excess of GTP γ S relative to the transducin concentration; the rate of cGMP hydrolysis was then assayed.

Binding of [^3H]varfenafil to catalytic sites on PDE—The membrane filtration assay to quantitate

[^3H]varfenafil binding to PDE6 was adapted from a similar assay for PDE5 (35). 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 (31). Activity measurements were made in 100 mM Tris (pH 7.5) buffer containing 10 mM MgCl_2 , 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 (36) with 10 mM EDTA and 50 μ M varfenafil added to the binding assay solution to prevent cGMP hydrolysis. [Note that varfenafil did not alter cGMP binding, consistent with previous work demonstrating the very low affinity of PDE inhibitors or most cGMP analogs to occupy the cGMP binding sites of the PDE6 GAF domains (24,37).] The rhodopsin concentration was spectrophotometrically determined, using an extinction coefficient of $42000\text{ M}^{-1}\text{ cm}^{-1}$ (38). Protein concentrations were determined by the bicinchoninic acid protein assay (39) using bovine γ -globulin as a standard. Curve fitting and statistical analyses were carried out with Sigmaplot. Unless otherwise noted, all experiments were performed three times.

RESULTS AND DISCUSSION

Histone II-AS stabilizes [^3H]varfenafil 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 varfenafil) also inhibit the catalytic activity of the closely related photoreceptor PDE6 (23-25). To date, varfenafil is the most potent of this class of catalytic site inhibitor, with an inhibition constant for PDE6 of $\sim 1\text{ nM}$ (25). 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 [³H]vardenafil to Pαβ alone. Consistent with previous studies of PDE5 binding to radiolabeled inhibitors (35), we observed that histone II-AS stabilized [³H]vardenafil binding to PDE6 catalytic dimer (Pαβ) in a concentration-dependent manner (Fig. 1). Interestingly, histone II-AS had little effect on cGMP hydrolytic activity, Pγ binding affinity, 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.

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 (40,41), Pγ, and Pγ peptides. Histone VIII-S, which historically has been used to displace Pγ and activate the PDE6 holoenzyme, can stabilize [³H]vardenafil binding to the Pαβ dimer to a certain extent (Fig. 2), but did not reach the maximum binding observed with histone II-AS. Little [³H]vardenafil binding was detected with Pγ or the C-terminal peptide Pγ63-87, as might be expected since the C-terminal region of the inhibitory Pγ subunits competes with drug binding at the active sites (25,42).

[³H]vardenafil binds to PDE6 catalytic dimer, but Pγ 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 Pαβ 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 Pαβ catalytic dimer preparation.

Fig. 3 shows that the binding curve for [³H]vardenafil to purified Pαβ 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, (25)]. This discrepancy may be due to the

requirement for a nanomolar level of PDE6 to reproducibly quantify [³H]vardenafil binding, resulting in titration of the binding 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 [³H]vardenafil binding to purified Pαβ was calculated to be 2.1 ± 0.1 (S.D.; $n = 4$) vardenafil molecules per Pαβ. This result shows that both the α and β catalytic subunits of PDE6 bind vardenafil. In contrast, the same Pαβ reconstituted with Pγ cannot bind [³H]vardenafil to a significant extent under identical experimental conditions (Fig. 3), demonstrating that vardenafil binding is prevented when Pγ inhibits the catalytic site of PDE6. Preliminary experiments with [³H]sildenafil (35) confirmed the ability of both catalytic subunits to stoichiometrically bind drug, but only in the absence of bound Pγ (*data not shown*).

Endogenous activated transducin relieves Pγ 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 thereby 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 (43,44). 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 GTPγS), 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 (Fig. 4). In addition, limited trypsin proteolysis of ROS homogenates (to fully degrade the Pγ subunits and thereby 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 (Fig. 4). These observations agree with the results obtained in Fig. 3 with highly purified PDE6 holoenzyme

and catalytic dimer. Surprisingly, transducin activation of PDE6 failed to activate more than $38 \pm 5\%$ of the full catalytic potential of the enzyme. This level of PDE6 catalytic stimulation by transducin correlated with the observation that only 0.9 ± 0.1 mol [^3H]vardenafil per mol PDE6—45% of the maximum stoichiometry of 2 mol per mol—was able to access the catalytic sites of transducin-activated PDE6 (Fig. 4). 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 $\text{GTP}\gamma\text{S}$ to light-exposed ROS homogenates to progressively activate transducin (and hence PDE6). We observed a strong correlation between stimulation of catalysis and an increase in [^3H]vardenafil binding as the fraction of activated transducin was increased (*data not shown*). These results demonstrate by two independent measures (i.e., stimulation of cGMP hydrolysis and accessibility of vardenafil to the active site) that only one-half of the maximum catalytic activity of PDE6 can be dis-inhibited upon transducin activation.

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 $\text{GTP}\gamma\text{S}$ ($\text{T}\alpha\text{-GTP}\gamma\text{S}$) and added increasing concentrations to either ROS homogenates or to purified, soluble PDE6 holoenzyme. As seen in Fig. 5, increasing the concentration of purified $\text{T}\alpha\text{-GTP}\gamma\text{S}$ added to ROS membrane-attached PDE6 (*open circles*) elevated PDE6 catalytic activity from $35 \pm 4\%$ to $101 \pm 1\%$ of the activity of catalytic dimers in which $\text{P}\gamma$ had been proteolytically removed. This demonstrates that activated $\text{T}\alpha$ —if present in sufficiently high concentrations (~1000-fold excess over endogenous levels)—can fully relieve inhibition by displacing all $\text{P}\gamma$ from its binding sites on the catalytic subunits. Similar results were seen for purified, soluble PDE6 in the absence of ROS disk membranes (Fig. 5, *filled circles*), except that purified PDE6 holoenzyme is much less efficiently activated by purified $\text{T}\alpha\text{-GTP}\gamma\text{S}$ [consistent with previous work; ref. (43)], as judged by the lower extent of activation of soluble PDE6 compared to

membrane-associated PDE6 for any given concentration of $\text{T}\alpha\text{-GTP}\gamma\text{S}$.

Conclusions—Two major conclusions emerge from this work. The first is that each catalytic subunit of the PDE6 heterodimer is able to bind ligands to its active site, and thus is likely to be catalytically active as well (Fig. 6). This conclusion is based on the innovative approach of utilizing radiolabeled vardenafil to quantify the number of drug binding sites relative to the cGMP binding stoichiometry for the PDE6 dimer. The conclusion that both the α - and β -subunits are functionally active is further supported by previous work documenting a stoichiometric relationship between $\text{P}\gamma$ binding to $\text{P}\alpha\beta$ and inhibition of catalysis [i.e., 2 $\text{P}\gamma$ bound per $\text{P}\alpha\beta$ (30,45)], as well as cross-linking studies showing $\text{P}\gamma$ interacting with both catalytic subunits (46-48). Furthermore, the binding of $\text{P}\gamma$ and drug to the active site of the enzyme are mutually exclusive, since high-affinity binding of $\text{P}\gamma$ prevents radiolabeled vardenafil from binding to the active sites of the PDE6 holoenzyme (Figs. 3 & 4). Although not directly demonstrated in this paper, the above-mentioned data support the inference that both subunits are catalytically active as well. This latter point is further substantiated by the observation that recombinant expression of a monomeric catalytic domain of the structurally homologous PDE5 enzyme (16) is catalytically active (49). Further work is needed to unequivocally demonstrate to what extent each catalytic subunit is catalytically active.

The second major conclusion is that activated transducin efficiently activates only one-half of the potential catalytic activity of the $\text{P}\alpha\beta$ catalytic sites, and that the remaining activation of catalysis can only be observed when a large excess of activated transducin is present (Figs. 4 & 5). While our results cannot discriminate the exact mechanism by which transducin activates the PDE6 catalytic heterodimer in this biphasic manner, we present two models that are consistent with our experimental results. The first model (Fig. 6, “B”) assumes that activated transducin binds to two independent, non-identical binding sites on the PDE6 holoenzyme, one of which is efficiently activated by $\text{T}\alpha^*$ while the second, low-affinity site relieves $\text{P}\gamma$ inhibition poorly. This

model is consistent with the observation that $P\gamma$ binds to two non-identical sites on the $P\alpha\beta$ catalytic dimer with a 10-fold difference in binding affinity (29). The catalytic subunit which binds $P\gamma$ more weakly might correspond to the site where $T\alpha^*$ can more readily displace $P\gamma$ and relieve inhibition at this active site. The higher affinity site of interaction of $P\gamma$ with the catalytic dimer might require a much higher concentration of $T\alpha^*$ to effectively displace $P\gamma$ in order to permit catalysis to occur. An alternative model for the biphasic activation of PDE6 by activated transducin is that the binding of $T\alpha^*$ to one catalytic subunit to which $P\gamma$ is bound might allosterically (e.g., negative cooperativity) or sterically reduce the effectiveness with which a second $T\alpha^*$ could bind to and displace $P\gamma$ from the second catalytic subunit (Fig. 6, "C").

The rod photoreceptor outer segment provides a unique cellular milieu for visual transduction and specifically for regulation of PDE6 activation by transducin. The high concentration of transducin [about 500 μM ; (8)] relative to PDE6 (20 μM) ensures the efficiency of propagation of the excitation pathway (3). However, the extremely high affinity of the $P\gamma$ subunits for $P\alpha\beta$ mentioned above [$K_{D1} < 1$ pM and $K_{D2} = 3$ pM; (29)] not only prevents spontaneous activation of PDE6 under dark-adapted conditions, it also poses a challenge for transducin to displace $P\gamma$ from its inhibitory site on the PDE6 catalytic domains during light-induced activation of PDE6 catalysis. Our observation that transducin relieves $P\gamma$ inhibition of PDE6 in a biphasic manner (Fig. 5) may underlie physiological differences in the extent of PDE6 activation during transient, dim light stimuli compared with bright, prolonged light stimulation

(i.e., light adaptation). For example, at light intensities where only a small percentage of the transducin becomes activated, the ability to efficiently activate PDE6 may be limited to about one-half of the full catalytic potential of the enzyme (Fig. 4). This level of activation would still be sufficient to generate a rapid decline in cGMP level, consistent with biochemical and electrophysiological models of the visual excitation and recovery pathways (8). However, prolonged, bright illumination might sufficiently elevate the $T\alpha^*$ concentration high enough to permit the relief of inhibition by $P\gamma$ at its second site of interaction with $P\alpha\beta$. This idea that biphasic activation of PDE6 by transducin may be correlated with the state of dark- versus light-adaptation offers another potential biochemical mechanism for the ability of rod photoreceptors to modulate their light sensitivity over several orders of magnitude (50). Although not experimentally addressed in the current study, it is important that future work also examines the possibility that other PDE6 interacting proteins [e.g., glutamic acid-rich protein-2 (GARP2), prenyl binding protein- δ (PDE δ), etc.; (2)] may modulate transducin-PDE6 interactions as well.

Finally, because mutations in $P\gamma$ have been reported which not only affect its ability to be disinhibited by activated transducin *in vitro* (51,52) but also reduce rod photoreceptor flash sensitivity and photoresponse kinetics *in vivo* (53), the observation of two distinct sites of interaction of transducin with PDE6 holoenzyme has relevance to understanding the molecular basis of loss of visual function and/or retinal degenerative diseases that can result from structural defects in these phototransduction proteins.

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FOOTNOTES

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1 To whom correspondence should be addressed. Tel.: 603-862-2458; Fax: 603-862-4013; E-mail: rick.cote@unh.edu.

2 The abbreviations used are: PDE, phosphodiesterase; PDE6, photoreceptor phosphodiesterase; $P\gamma$, inhibitory subunit of PDE6; $T\alpha$, alpha-subunit of the heterotrimeric G-protein, transducin; PDE5, cGMP-binding, cGMP-specific phosphodiesterase prevalent in vertebrate smooth muscle; $P\alpha\beta$, catalytic heterodimer of rod PDE6; $P\alpha\beta\gamma\gamma$, non-activated PDE6 holoenzyme; $T\alpha$ -GTP γ S, persistently activated alpha-subunit of transducin.

FIGURE LEGENDS

Fig. 1 Histone II-AS stimulates binding of [³H]vardenafil to the active sites of PDE6 catalytic dimer. Purified Pαβ catalytic dimer (6 nM) lacking Pγ 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.

Fig. 2 Comparison of [³H]vardenafil binding to Pαβ stimulated by histones or Pγ. Purified Pαβ (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 μM Pγ or 10 μM Pγ63-87. Vardenafil binding was quantified by the membrane filtration assay. Data represent the mean ± range for duplicate measurements from one representative experiment; similar results were obtained in at least two other experiments (except the Pγ63-87 condition which was tested one other time).

Fig. 3 [³H]vardenafil binds stoichiometrically to each Pαβ catalytic subunit, but only in the absence of Pγ. Purified Pαβ (2.5 nM; filled circles) or Pαβ reconstituted with 10 μM Pγ (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 Pαβ concentration, as estimated by both [³H]cGMP binding assay, as well as hydrolytic activity measurements (which agreed to within 10%). The solid line represents the fit of the data for [³H]vardenafil binding to Pαβ assuming a single class of binding sites (apparent K_D = 7.8 nM and B_{max} = 2.0). The data are representative of 3 similar experiments.

Fig. 4 Transducin-activated PDE6 achieves only one-half of the extent of [³H]vardenafil binding or hydrolytic activity compared to Pαβ. Light-exposed ROS homogenates (containing 4 nM PDE6) 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 were incubated either with 74 nM [³H]vardenafil for 40 min before membrane filtration (black bars) or with cGMP for hydrolytic activity assays (gray bars). Data are the mean ± S.D. of three experiments.

Fig. 5 A large excess of activated transducin α-subunit fully relieves Pγ inhibition of the active sites of the PDE6 catalytic dimer. The indicated concentrations of purified transducin α-subunits with GTPγS bound (Tα-GTPγS) were added to either 1 nM purified PDE6 holoenzyme (filled circles) or 2 nM ROS-PDE6 previously incubated with 50 μM GTPγS 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 ± S.D. of two experiments.

Fig. 6 Model for PDE6 activation by transducin. The nonactivated PDE6 holoenzyme is a catalytic heterodimer (gray) in which each catalytic subunit binds an inhibitory Pγ subunit (black s-shaped rod) at multiple sites within the GAF domains and the catalytic domain [reviewed in (2,15)]. The depiction of the catalytic dimer [based on the structure reported in ref. (18)] shows an active site (ellipse) within each catalytic domain and a cGMP binding site (circle) within the GAF domain. Proteolytic removal of the Pγ subunits (“A”) relieves inhibition of catalysis and permits vardenafil binding to each catalytic site. Two alternative models (B and C) are presented to explain how activation of ROS membrane-associated PDE6 holoenzyme by activated transducin α-subunit (Tα*; dark gray oval) might occur in a biphasic manner. In model “B,” Tα* has one high-affinity binding site on PDE6 holoenzyme that efficiently binds Pγ and thereby relieves inhibition at one active site, with a second, low-affinity binding site that requires high concentrations of Tα* to relieve Pγ inhibition at the second catalytic site. In model “C,” binding of one Tα* to PDE6 holoenzyme (thereby activating PDE6 to one-half of its full catalytic potential) reduces the

effectiveness of a second $T\alpha^*$ to displace the second $P\gamma$ from its inhibitory sites of interaction within the PDE6 catalytic domain. In both models, subsequent dissociation of the $T\alpha^*$ - $P\gamma$ complex from PDE6 catalytic dimer is not depicted but does occur in certain circumstances (54-56).

Figure 1

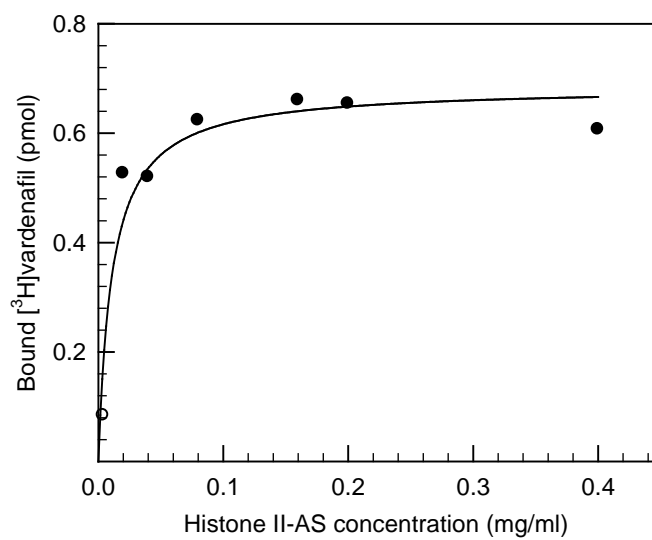


Figure 2

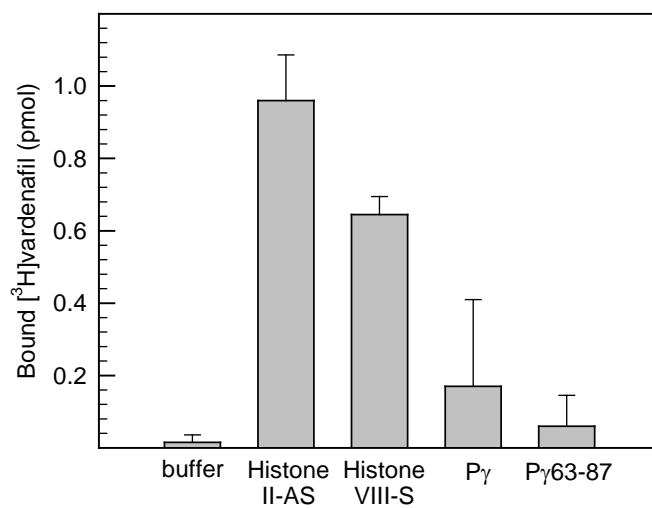


Figure 3

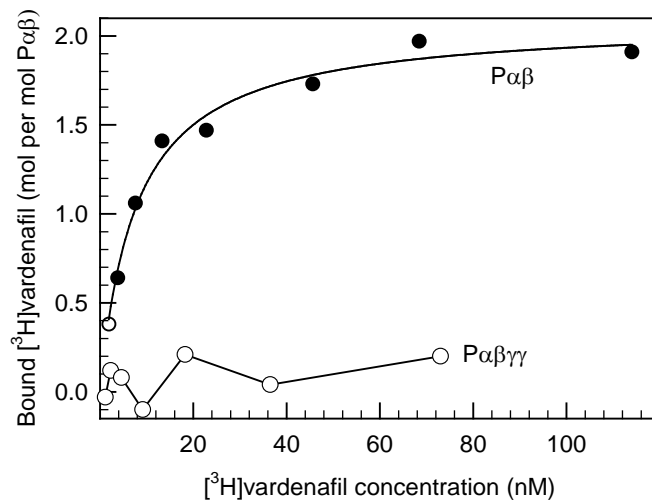


Figure 4

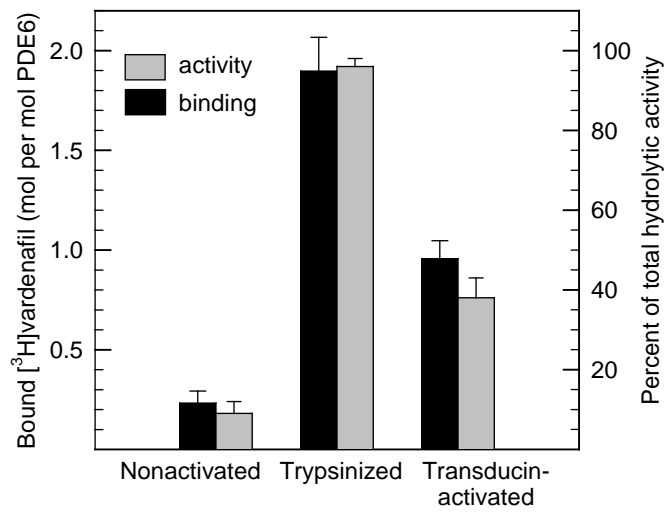


Figure 5

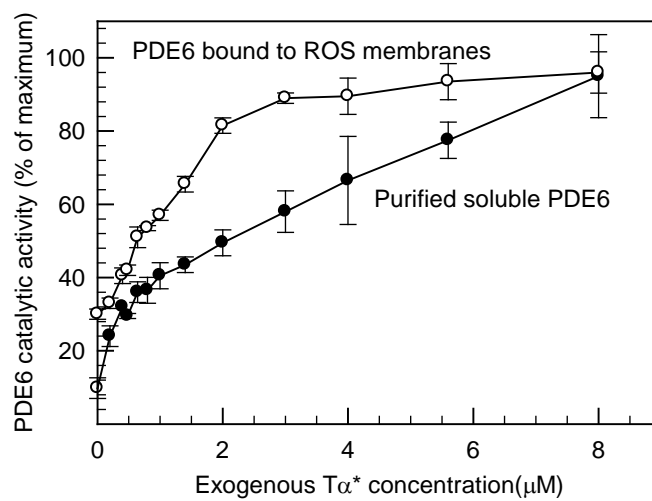


Figure 6

