

## Direct Allosteric Regulation between the GAF Domain and Catalytic Domain of Photoreceptor Phosphodiesterase PDE6\*

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Photoreceptor cGMP phosphodiesterase (PDE6) is the central enzyme in the visual transduction cascade. The PDE6 catalytic subunit contains a catalytic domain and regulatory GAF domains. Unlike most GAF domain-containing PDEs, little is known about direct allosteric communication of PDE6. In this paper, we demonstrate for the first time direct, inter-domain allosteric communication between the GAF and catalytic domains in PDE6. The binding affinity of PDE6 for pharmacological inhibitors or for the C-terminal region of the inhibitory  $\gamma$ -subunit ( $P\gamma$ ), known to directly inhibit PDE6 catalysis, was increased ~2-fold by ligands binding to the GAF domain. Binding of the N-terminal half of  $P\gamma$  to the GAF domains suffices to induce this allosteric effect. Allosteric communication between GAF and catalytic domains is reciprocal, in that drug binding to the catalytic domain slowed cGMP dissociation from the GAF domain. Whereas cGMP hydrolysis was not affected by binding of  $P\gamma$ 1-60,  $P\gamma$  lacking its last seven amino acids decreased the Michaelis constant of PDE6 by 2.5-fold.  $P\gamma$ 1-60 binding to the GAF domain increased vardenafil but not cGMP affinity, indicating that substrate and inhibitor binding sites do not totally overlap. In addition, prolonged incubation of PDE6 with vardenafil or sildenafil (but not IBMX and zaprinast) induced a distinct conformational change in the catalytic domain without affecting the binding properties of the GAF domains. We conclude that while  $P\gamma$ -mediated regulation plays the dominant role in visual excitation,

**the direct, inter-domain allosteric regulation described in this paper may play a feedback role in light adaptational processes during phototransduction.**

The photoreceptor cyclic nucleotide phosphodiesterase (PDE6) is the central enzyme in the vertebrate visual signaling pathway in rods and cones. Phototransduction is initiated when light induces the isomerization of the 11-cis retinal chromophore of rhodopsin, which leads to activation of the photoreceptor-specific G-protein, transducin. Activated transducin then causes activation of PDE6 which results in rapid lowering of cGMP levels, closure of cGMP-gated ion channels, and hyperpolarization of the cell membrane (1-3). Hydrolysis of cGMP by PDE6 must be precisely regulated to control the amplitude and kinetics of the photoresponse. Furthermore, each of these parameters undergoes additional modulation in response to ever-changing conditions of ambient illumination. The PDE6 holoenzyme consists of a catalytic dimer of  $\alpha$ - and  $\beta$ -subunits ( $P\alpha\beta$ ) and two inhibitory  $\gamma$ -subunits ( $P\gamma$ ) that are tightly bound to  $P\alpha\beta$ . Transducin activation of PDE6 results from displacement of the inhibitory constraint of  $P\gamma$  upon activated transducin binding to PDE6. The affinity of  $P\gamma$  for the  $P\alpha\beta$  catalytic dimer is also modulated in a reciprocal manner by noncatalytic cGMP binding to PDE6 at sites distinct from the catalytic site [(4); reviewed in ref. (5)].

Photoreceptor PDE6 is one of five members of the class I phosphodiesterase superfamily that contain tandem regulatory GAF domains [i.e., GAFa and GAFb] (6). The GAF

domains were originally named for their presence in cGMP-regulated PDEs, certain adenylyl cyclases, and the transcription factor Fh1A of bacteria (7). The GAF domains of the vertebrate PDE members contain a functional cyclic nucleotide binding pocket. cGMP is the ligand for PDE2, PDE5, PDE6 and PDE11 (8-13), while cAMP is the ligand for PDE10 (13). For PDE6, the noncatalytic cGMP binding site has been localized to the N-terminal GAF $\alpha$  domain [(14,15); Fig. 1A].

Direct allosteric regulation of catalytic activity induced by binding of cyclic nucleotides to the GAF domains has been well documented for PDE2 and PDE5. For both PDE families, cGMP binding to the GAF domains induces a conformational change that relieves inhibition of catalysis in the active sites, causing stimulation of the enzyme (10,16,17). Furthermore, the binding affinity of inhibitors to the catalytic domains of PDE5 is increased by cGMP addition (18,19). As predicted, this allosteric regulation between the GAF and catalytic domains is reciprocal. For example, in PDE5, some inhibitors enhanced cGMP binding to the GAF domains (11,20). In addition to this direct allosteric communication between GAF and catalytic domains, it has been reported that PDE5 inhibitors can induce a conformational change in the catalytic domain that enhances inhibitor binding affinity in a time-dependent manner (21).

Based on the many similarities between PDE5 and PDE6 (22), direct, inter-domain allosteric communication between the GAF and catalytic domains is predicted for PDE6. However, previous work evaluating whether cGMP binding could influence the catalytic properties of PDE6 has not revealed a direct parallel to the allosteric control exerted on PDE5. For example, cGMP binding to the GAF domains fails to alter either the  $K_M$  or  $k_{cat}$  of the enzyme (4,23,24). Instead, attention has focused on the allosteric control mediated by  $P\gamma$  on both GAF and catalytic domains. The N-terminal region of  $P\gamma$  (Fig. 1B) is known to interact with the GAF domains of the  $P\alpha\beta$  catalytic dimer with a 50-fold higher affinity than the affinity of the C-terminal region of  $P\gamma$  for the catalytic domain of  $P\alpha\beta$ . (24-28). The C-terminal region

of  $P\gamma$  (Fig. 1B) is responsible for blocking the catalytic activity by binding to the catalytic domains of  $P\alpha\beta$  (27-31). The ability of  $P\gamma$  to interact with both GAF and catalytic domains of  $P\alpha\beta$  serves to allosterically link the regulatory and catalytic domains of PDE6 in two ways: (1)  $P\gamma$  binding to the catalytic dimer enhances the binding affinity of cGMP to the GAF domain (32,33); (2) cGMP occupancy of the GAF domain enhances  $P\gamma$  affinity to  $P\alpha\beta$  (4,23,24).

In this paper, we first document that direct allosteric communication between the GAF domains and catalytic domains of PDE6 does indeed occur. Ligand binding to the GAF domains enhances the affinity of inhibitors and  $P\gamma$ 63-87 (i.e., a.a. 63-87 of the  $P\gamma$  sequence) binding to the catalytic dimer. This inter-domain allosteric mechanism is reciprocal, in that inhibitor binding to catalytic domains increases the binding affinity of cGMP to the GAF domains. The magnitude of this direct allosteric regulation in PDE6 is comparable to that seen in PDE5, and may play a role in modulating PDE6 activity during persistent activation of rod photoreceptors which occurs during normal daytime illumination conditions.

## EXPERIMENTAL PROCEDURES

*Materials*—Bovine retinas were purchased from W. L. Lawson, Inc. Synthetic peptide  $P\gamma$ 63-87 was purchased from New England Peptide. Vardenafil and sildenafil were provided by Bayer Healthcare AG. Ultima Gold scintillation fluid was from PerkinElmer Life & Analytical Sciences. Filtration membranes were from Millipore, Bicinchoninic acid (BCA) protein assay reagents were from Pierce, and all other chemicals were from Sigma-Aldrich (St. Louis, MO). Stock solutions of PDE inhibitors were prepared in DMSO, and diluted to less than 1% final concentration before use in assays.

*PDE6 and  $P\alpha\beta$  purification and functional assays*—Bovine rod PDE6 was purified from bovine retinas as described (34).  $P\alpha\beta$  catalytic dimers lacking  $P\gamma$  were prepared by limited trypsin proteolysis and re-purified by gel filtration chromatography prior to use (34). PDE6 catalytic activity was measured in 20 mM

Tris, 10 mM MgCl<sub>2</sub>, 0.5 mg/ml bovine serum albumin either with a phosphate release microplate assay or with a radiotracer assay (35). The PDE6 concentration was estimated based on the rate of cGMP hydrolysis of trypsin-activated PDE6 and a knowledge of the  $k_{\text{cat}}$  of the enzyme [5600 mol cGMP hydrolyzed per P $\alpha\beta$  per s (36)]; this estimate was validated by determining stoichiometric binding of [<sup>3</sup>H]cGMP to PDE6 with a filter binding assay (37). The inhibition potency (IC<sub>50</sub>) of PDE5/6 inhibitors or P $\gamma$ 63-87 was determined in the presence or absence of P $\gamma$  mutants using either 2 mM cGMP or 0.1 mM cAMP as substrates.

*Purification of P $\gamma$  and P $\gamma$  mutants*—Several P $\gamma$  truncation mutants (Fig. 1C) were generated from the full-length coding sequence using standard methods, introduced into the pET11a (Novagen) expression vector, and nucleotide sequences verified. Recombinant P $\gamma$  and mutants (P $\gamma$ 1-45, P $\gamma$ 1-60, and P $\gamma$ 1-80) were expressed in *E. coli* BL21(DE3). The bacterial extract was partially purified by cation exchange chromatography using SP Sepharose, followed by C4 reverse-phase high pressure liquid chromatography (38). The purity (> 95 %) and size of these proteins were evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The inhibitory activity of P $\gamma$  was assessed by its ability to stoichiometrically inhibit P $\alpha\beta$  catalytic dimers (2 P $\gamma$  per P $\alpha\beta$ ) (24). Protein concentrations were determined by the bicinchoninic acid protein assay (39) using bovine gamma globulin as a standard.

*Loading cGMP on the GAF domains of activated PDE6*—Purified P $\alpha\beta$  was pre-incubated with 10 mM EDTA in binding buffer (100 mM Tris, 2 mM MgCl<sub>2</sub> and 0.5 mg/ml bovine serum albumin) for 2 hours at 22°C to inhibit cGMP breakdown before addition of 1  $\mu$ M [<sup>3</sup>H]cGMP and N-terminal region P $\gamma$  peptides (24). The amount of cGMP bound to PDE6 under these conditions was verified to be 1.7-2.0 cGMP per P $\alpha\beta$ . The hydrolytic activity of PDE6 was restored by adding 10 mM MgCl<sub>2</sub> immediately prior to assaying cyclic nucleotide hydrolysis. For experiments in which [<sup>3</sup>H]cGMP dissociation kinetics from P $\alpha\beta$  were measured in

the absence of P $\gamma$ , purified P $\alpha\beta$  was pre-incubated for 2 h with 10 mM EDTA plus 20 mM dipicolinic acid to abolish residual catalytic activity (40). [EDTA alone was unable to protect [<sup>3</sup>H]cGMP from breakdown by P $\alpha\beta$  (lacking P $\gamma$  peptides) during the binding assay.] Release of [<sup>3</sup>H]cGMP from P $\alpha\beta$  was monitored following addition of 1 mM unlabeled cGMP (containing 10 mM MgCl<sub>2</sub> and 5 mM ZnSO<sub>4</sub>), and the time course of [<sup>3</sup>H]cGMP dissociation was monitored in the presence or absence of 100  $\mu$ M vardenafil.

*Data analysis*—Dose-response experiments were analyzed using non-linear regression analysis (Sigmaplot) to fit experimental data to a 3-parameter logistic dose-response function:  $y = a/[1 + (x/x_0)^b]$  where  $a$  is the amplitude,  $b$  is the slope factor, and  $x_0$  is the IC<sub>50</sub> (41). For other experiments, curve-fitting models are described in the figure legends. Except where noted, all experiments were repeated at least three times. Tests of statistical significance for the curve fitting results used the Student's t-test to calculate probability values, as indicated in the figure legends.

## RESULTS

*Binding of ligands to the regulatory GAF domain enhances the ability of vardenafil to bind to the catalytic domain*—Motivated by the analogy of PDE6 with other GAF-containing PDEs, we first explored whether ligand binding to the GAF domains could allosterically alter the properties of the active site of PDE6.

Since both cGMP and the N-terminal region of P $\gamma$  are known to bind to the GAF domains of the PDE6 catalytic dimer (P $\alpha\beta$ ; see Introduction and Fig. 1), we first examined whether the catalytic properties of PDE6 were altered upon binding of these ligands to the GAF domains. The first sixty amino acids of P $\gamma$  (P $\gamma$ 1-60) lack the ability to inhibit catalysis (*data not shown*), and the primary sites of interaction are confined to the GAF domains (28,42). We therefore used the truncated mutant P $\gamma$ 1-60 to determine whether the binding affinity of vardenafil to the catalytic domain of P $\alpha\beta$  was altered when P $\gamma$ 1-60 or P $\gamma$ 1-60 plus cGMP were bound. In order to evaluate catalytic activity

while simultaneously testing the effect of occupancy of the GAF domains, cAMP was used as a substrate for this experiment since it binds very poorly to the GAF domain even at high concentrations (12). Fig. 2 demonstrates that the inhibitory potency of vardenafil was increased approximately 2-fold ( $IC_{50}$  shifted from  $4.3 \pm 0.3$  nM to  $2.2 \pm 0.2$  nM) by P $\gamma$ 1-60 binding to the GAF domain. Experiments using a shorter N-terminal fragment of P $\gamma$  (P $\gamma$ 1-45) showed the same two-fold enhancement of vardenafil binding as were seen with P $\gamma$ 1-60 (*data not shown*), confirming that the GAF-interacting region of P $\gamma$  [localized to a.a. residues 18-45; (24)] was responsible for this inter-domain allostery observed in Fig. 2.

Inclusion of cGMP with P $\gamma$ 1-60 so as to occupy the GAFa cGMP binding pocket failed to further enhance the allosteric effect on vardenafil binding to the P $\alpha\beta$  active site (Fig. 2). [Unfortunately, we were unable to directly measure the allosteric effects of cGMP binding in the absence of the P $\gamma$  N-terminal region, because the high catalytic rate of P $\alpha\beta$  led to hydrolysis of cGMP unless GAF-interacting P $\gamma$  peptides were present to stabilize cGMP binding to the GAFa domain.] It is possible that either P $\gamma$  binding to the GAF domain or cGMP occupancy of the GAFa binding pocket induce the same conformational change in the GAF domains that is transmitted to the catalytic domain.

*Binding of the N-terminal region of P $\gamma$  to the GAF domains enhances the binding affinity of P $\gamma$ 63-87 to the catalytic domain*—The C-terminal region of P $\gamma$  inhibits PDE6 catalysis by interacting with amino acid residues lining the entrance to the catalytic pocket (43); these residues are predicted to be distant from those stabilizing vardenafil binding to the active site (44). Therefore, we questioned whether binding of the N-terminal region of P $\gamma$  to the GAF domains could alter the binding affinity of the C-terminal region of P $\gamma$  to the catalytic domains of P $\alpha\beta$ . Fig. 3 shows that the dose-response curve for P $\gamma$ 63-87 inhibition of cGMP hydrolysis was shifted about two-fold when P $\gamma$ 1-45 or P $\gamma$ 1-60 were bound to the GAF domains. The finding that both vardenafil and P $\gamma$ 63-87 affinity was increased ~2-fold when the N-

terminal half of P $\gamma$  bound to the PDE6 GAF domains indicates that this inter-domain allosteric change is likely to affect the global conformation of the PDE6 catalytic domain.

*Conformational changes in substrate affinity to the active site require P $\gamma$  interactions with the catalytic domain, not the GAF domains*—These novel allosteric effects of P $\gamma$  on vardenafil and P $\gamma$ 63-87 binding to PDE6 differ from earlier work in which cGMP and/or P $\gamma$ 1-45 binding to the GAF domains failed to allosterically alter the kinetic parameters for substrate hydrolysis of the P $\alpha\beta$  active site (4,24). This is unexpected, because cGMP and vardenafil are likely to share some interaction sites within the catalytic pocket, as judged by comparison of crystal structures of PDE5 complexed with 5'-GMP and vardenafil (44,45). To examine this more closely, we measured the Michaelis constant ( $K_M$ ) for cGMP in the presence of two P $\gamma$  truncation mutants, P $\gamma$ 1-60 and P $\gamma$ 1-80. P $\gamma$ 1-60 was chosen because it cannot inhibit catalysis and its primary sites of interaction with P $\alpha\beta$  are within the GAF domain, whereas P $\gamma$ 1-80 partially inhibits catalysis [ $\sim 60\%$  reduction in  $V_{max}$ ; see ref. (30)] and has been shown to interact with sites within the catalytic domain [(28,42); see Fig. 1]. Whereas P $\gamma$ 1-60 failed to affect the  $K_M$  (or  $V_{max}$ ) for cGMP hydrolysis (Fig. 4) [consistent with previous work with P $\gamma$ 1-45 (24)], binding of P $\gamma$ 1-80 to P $\alpha\beta$  increased the  $K_M$  for cGMP from  $9 \pm 0.9$  to  $23 \pm 1.5$   $\mu$ M (Fig. 4). The differences in how cGMP (Fig. 4) and vardenafil (Fig. 2) are affected by P $\gamma$  binding to P $\alpha\beta$  may reflect a local conformational change within the catalytic domain upon binding amino acids 61-80 of P $\gamma$  that is distinct from the inter-domain communication between the GAF and catalytic domains that is induced by the GAF-interacting region of P $\gamma$ .

*Binding of vardenafil to the catalytic domains enhanced cGMP binding to the GAF domains*—Having established in the previous sections the direct allosteric regulation by the GAF domains on the catalytic domain of PDE6, the principle of allosteric linkage (46) requires that this allosteric communication be reciprocal:

occupancy of the active site should induce conformational changes in the GAF domains. In the earliest study of cGMP binding to the GAF domains of PDE6, the non-specific PDE inhibitor 3-isobutyl-1-methylxanthine (IBMX) was reported to allosterically enhance cGMP binding to partially purified, nonactivated frog PDE6 (9). To examine this behavior with our highly purified, activated bovine  $P\alpha\beta$  catalytic dimer, we took special precautions to prevent [ $^3\text{H}$ ]cGMP breakdown by treating  $P\alpha\beta$  with metal ion chelators that completely block cGMP hydrolysis (see *Experimental Procedures*) prior to evaluating the kinetics of [ $^3\text{H}$ ]cGMP dissociation from  $P\alpha\beta$ . Fig. 5 shows that occupancy of the catalytic site with vardenafil slows ~2-fold the release of [ $^3\text{H}$ ]cGMP from the GAF domains when a large excess of unlabeled cGMP was added. Similar results were obtained when the release of [ $^3\text{H}$ ]cGMP from its bound state was induced by a rapid lowering of the free [ $^3\text{H}$ ]cGMP concentration using the “concentration jump” method (47), confirming that the unlabeled cGMP added in Fig. 5 was itself not influencing the outcome of the dissociation kinetics. The ability of vardenafil to allosterically regulate cGMP dissociation kinetics was also observed in experiments where  $P\gamma 1-45$  had been pre-incubated with  $P\alpha\beta$  and [ $^3\text{H}$ ]cGMP (*data not shown*). These results are consistent with the idea that binding of PDE inhibitors to the catalytic domain of the PDE6 catalytic dimer can induce an allosteric change in the cGMP binding pocket located within the GAF $\alpha$  domain that enhances cGMP binding affinity.

*A second conformational change occurs to the catalytic domains upon vardenafil binding—* Because PDE5 undergoes a slow, time-dependent conformational change in its catalytic domain upon drug binding to the active site (21), we sought evidence for a similar allosteric effect on PDE6. Fig. 6 shows that both vardenafil and sildenafil—but not zaprinast or IBMX—increase their own affinities for the active site of  $P\alpha\beta$  approximately two-fold when incubated for 16 hours compared to a 20 min drug incubation. The ability of sildenafil (Fig. 6A), but not zaprinast (Fig. 6B), to induce this shift in

inhibitory potency cannot be ascribed to the overall affinity of drug for the active site, since both drugs have similar inhibition constants for rod PDE6 (48), but rather must reflect differences in how the inhibitors contact residues within the active site.

To test whether this slow conformational change in the catalytic domain is transmitted allosterically to the GAF domain, we examined cGMP dissociation after pre-incubating PDE6 with vardenafil overnight. We found that cGMP dissociation rates were the same regardless of the length of time PDE6 was incubated with vardenafil (*data not shown*). We conclude that this slowly achieved conformational change in the catalytic domain is not communicated to the cGMP binding site on the GAF $\alpha$  domain of PDE6.

## DISCUSSION

This study represents the first demonstration that photoreceptor PDE6 has the ability to undergo allosteric regulation within its catalytic subunits. For over two decades it was thought that unlike other GAF domain-containing PDEs, the catalytic properties of PDE6 are not affected by direct allosteric regulation by the cGMP-binding GAF domains. However, our experiments reveal that there is a mutual allosteric reciprocity between the GAF and catalytic domains. The binding of the N-terminal portion of  $P\gamma$  to the GAF domains alters the catalytic domain conformation, resulting in enhanced affinity of drugs which occupy the active site as well as enhancing the affinity of the C-terminal portion of  $P\gamma$  that binds to amino acid residues in the vicinity of the active site. Conversely, binding of drugs to the active site induces a conformational change in the GAF domains that enhances their binding affinity for cGMP. This direct allosteric regulation occurs in addition to  $P\gamma$ -mediated regulation of PDE6 activation, and may represent a secondary mechanism for its catalytic control.

*Analogies between PDE5 and PDE6 allostereism—*Direct allosteric control of catalytic activity is well documented for PDE5 (see *Introduction*). Binding of cGMP to the GAF domain of PDE5 increases cGMP hydrolysis or

inhibitor binding affinity by 2-5-fold (17-19). Conversely, cGMP binding affinity to the GAF domains of PDE5 is enhanced 2-fold by inhibitors binding to the catalytic domain (11,49). The magnitude of inter-domain allosteric regulation reported for PDE5 is thus comparable to what we describe in this paper for PDE6.

However, there are two major differences between the allosteric relations within PDE5 and PDE6. First, while the cross-regulation of the catalytic and noncatalytic domains in PDE5 extends to both its natural substrates (cyclic nucleotides) and inhibitors, this regulation in PDE6 is restricted only to inhibitors (both its regulatory  $P\gamma$  subunit and chemical compounds such as sildenafil and vardenafil). It appears that in the case of PDE6, direct allosteric regulation by the GAF domain induces a conformational change in the catalytic domain that is sensed by inhibitors, but not by substrates. The ability of  $P\gamma$ 1-80—but not  $P\gamma$ 1-60—to shift the Michaelis constant for cGMP hydrolysis (Fig. 4) suggests that local conformational changes can be induced by the C-terminal domain of  $P\gamma$  that *do* alter the substrate binding pocket. We will discuss the potential structural basis for this below.

A second major difference between PDE5 and PDE6 is that PDE5 activity is also regulated by phosphorylation of its N-terminal region. Phosphorylation of serine-102 in human PDE5 induces a conformational change in the neighboring GAF $\alpha$  domain, resulting in a 4-10-fold increase in binding affinity of cGMP (50,51). This conformational change is further communicated to the catalytic domain, as judged by 2-fold elevated cGMP hydrolytic rate (51-53). This mode of regulation has not been documented for PDE6. Instead, PDE6 has evolved a quite distinct mechanism for regulating its catalytic activity. PDE6 relies on a separate regulatory protein,  $P\gamma$ , whose binding is regulated by activated transducin as well as by occupancy of the PDE6 GAF $\alpha$  domains by cGMP [reviewed in (5,54)].

*Structural basis for the conformational changes in the catalytic domain of PDE6*—Three distinct effects on the catalytic domain of PDE6 have been observed in this study: (1) a reciprocal,

ligand-mediated allosteric communication between the cGMP-binding GAF domains and the catalytic domain; (2) an elevation of the  $K_M$  for cGMP hydrolysis induced only by the  $P\gamma$ 1-80 truncation mutant, and; (3) a slowly occurring intra-domain increase in vardenafil or sildenafil affinity that is not observed for zaprinast or IBMX. Lacking structural data for the PDE6 catalytic domain, we wondered whether the known PDE5 catalytic domain structure (44,45,55) might provide insights on the structural basis for these conformational changes. To examine this, we performed structural homology modeling by threading the PDE6 catalytic domain sequence onto the PDE5 catalytic domain crystal structures (56) containing either no ligand (57), bound vardenafil (58), bound IBMX (55), or bound 5'-GMP (45).

When comparing the un-liganded model of the catalytic domain to that of the vardenafil-containing structure, these two structures are observed to have a very similar  $\alpha$ -helical domain structure overall. Variability in the conformation of the so-called M-loop and H-loop [defined in (55)] suggests that these less structured loop regions are conformationally sensitive (57,58).  $P\gamma$  interacting sites are also located in the vicinity of the M-loop (a.a. 750-760) (43,59). While both the M- and H-loops are believed to provide stabilizing contacts for drug binding, most of the residues interacting with the cGMP substrate are found in the H-loop. This interpretation is further supported by site-directed mutagenesis studies of PDE5 which suggest that residues in the H-loop are more important for stabilizing substrates than inhibitors (57). This might explain why cGMP affinity to the catalytic domains is not affected by  $P\gamma$ 1-60 (Fig. 4) whereas vardenafil or  $P\gamma$ 63-87 inhibition potency *is* increased by  $P\gamma$ 1-60 (Figs. 2 and 3). We speculate that interactions of  $P\gamma$  with the PDE6 GAF domains may induce conformational changes that are transmitted to the H- and/or M-loops in the catalytic domain. In a reciprocal manner, inhibitor binding to the active site of PDE6 may induce conformational changes in the H- or M-loops that are communicated to the GAF domains to alter the conformation of the cGMP binding pocket in the

GAFa domain (Fig. 5). Further work is clearly needed to determine the structural basis for the inter-domain allostereism we report for PDE6 in this paper.

We also showed that certain inhibitors (i.e., vardenafil and sildenafil, but not zaprinast or IBMX) induce a slowly developing increase in drug binding affinity (Fig 6) that is not communicated to the GAF domains of PDE6. This slow shift in drug affinity was observed for PDE5 as well (21). The structure of IBMX-bound PDE5 shows that IBMX interacts primarily with residues in the vicinity of the M-loop but lacks significant contacts with the H-loop (55). In contrast, vardenafil binding to the catalytic domain of PDE5 causes movement of a portion of the H-loop toward the center of the drug binding site, with no major change in M-loop structure (58). We speculate that the conformation of the PDE6 H-loop is affected by vardenafil—but not IBMX—binding, causing an *intra*-domain enhancement of drug binding affinity for vardenafil.

*Physiological implications of direct allosteric regulation of PDE6*—It is well established that transducin activation of PDE6 is the primary regulatory mechanism for the pathway of visual excitation that leads to hyperpolarization of the photoreceptor cell. Specifically, the PDE6 holoenzyme ( $\alpha\beta\gamma$ ) becomes activated when activated transducin  $\alpha$ -subunit displaces the C-terminal region of  $P\gamma$ . A likely scenario is that the N-terminal region of  $P\gamma$  remains associated with PDE6 catalytic dimer upon transient activation of PDE6 [reviewed in (5)]. However, upon persistent illumination, PDE6 may remain activated by transducin for sufficient time for the allosteric communication between the catalytic and GAF domains to trigger the dissociation the N-terminal half of  $P\gamma$  (and bound cGMP) from the GAF domains of an appreciable fraction of PDE6 molecules. Release of  $P\gamma$  and dissociation of bound cGMP from the PDE catalytic subunits have been previously demonstrated to speed up the rate at which transducin-activated PDE6 hydrolyzes its bound GTP, which results in a faster rate of transducin inactivation [and hence PDE6 re-inhibition; (54)]. In this way, allosteric

communication between domains of the PDE6 catalytic dimer could influence the lifetime of activated PDE6, and provide a distinct mechanism of photoreceptor light adaptation characterized by shortened, less sensitive responses to light.

Mutations of PDE6 catalytic subunits are known to cause congenital stationary night blindness or retinal degeneration [reviewed in (5)]. Of particular interest to the present work are those instances where mutations occurring outside the catalytic domain disrupt PDE6 function and lead to visual disturbances or retinal degeneration. One example is the mutation in GAFa (H258N) found in the Rambusch form of autosomal dominant congenital stationary night blindness (60) that alters the affinity of  $P\gamma$  for the catalytic subunits of rod PDE6 (61,62). We speculate that mutations within the GAF domains of PDE6 could also impair inter-domain allosteric communication between the GAF and catalytic domains of PDE6, possibly leading to disruptions in the visual signaling pathway and, ultimately, photoreceptor cell death.

*Conclusion*—This work is the first demonstration of direct allosteric communication within the PDE6 catalytic dimer, and is consistent with allosteric regulation occurring with other PDE families that contain regulatory GAF domains. This inter-domain allosteric communication between the GAF domains and the catalytic domains is reciprocal, in that ligand binding to one domain alters the properties of the other domain. Unlike other PDE families, direct allosteric control of PDE6 is much less important than the G-protein mediated regulation of PDE6 resulting from binding of the  $P\gamma$  subunit to the PDE6 catalytic dimer. Although dis-inhibition of  $P\gamma$  by transducin dominates PDE6 regulation during visual excitation, this newly discovered inter-domain communication between the GAF and catalytic domains may be relevant for fine-tuning the extent and lifetime of PDE6 activation, particularly during conditions of prolonged light adaptation.

## FOOTNOTES

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The abbreviations used are: PDE, cyclic nucleotide phosphodiesterase; PDE6, photoreceptor PDE; P $\alpha\beta$ , catalytic dimer of PDE6  $\alpha$ - and  $\beta$ -subunits; P $\gamma$ , inhibitory  $\gamma$  subunit of PDE6; GAF, regulatory domain of PDE6 named for their presence in cGMP regulated PDEs, certain adenylyl cyclase and the transcription factor Fh1A of bacteria; IBMX, 3-isobutyl-1-methylxanthine.

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### FIGURE LEGENDS

Figure 1. Interaction sites between catalytic and inhibitory subunits of PDE6 and domain organization of  $P\gamma$  mutants. **A.** The catalytic  $\alpha$ - or  $\beta$ -subunit of PDE6 consists of regulatory domain and catalytic domain. The regulatory domain includes two tandem GAF domains. The inter-domain allosteric communication between regulatory and catalytic domain is the subject of this paper. The *asterisk* represents the prenylated, membrane-anchored C-terminus of PDE6. **B.** The 10 kDa  $P\gamma$  inhibitory subunit has two major functional domains: the proline-rich and polycationic region (*a.a. 18-45*, *light gray box*) serves as primary interaction sites with the GAF domains of the PDE6 catalytic dimer; the C-terminal domain of  $P\gamma$  (*a.a. ~70-87*, *dark gray*) directly interacts with the catalytic domain to block cGMP hydrolysis. **C.** Schematic diagram of the  $P\gamma$  truncation mutants and the  $P\gamma$ 63-87 synthetic peptide used in this paper.

Figure 2.  $P\gamma$ 1-60 binding to the PDE6 GAF domains enhanced vardenafil binding affinity to the catalytic sites. Purified  $P\alpha\beta$  (2 nM) was pre-incubated with 10 mM EDTA for 2 h to inhibit PDE activity, followed by incubation with 1  $\mu$ M cGMP and 2  $\mu$ M  $P\gamma$ 1-60 ( $\bullet$ ), 2  $\mu$ M  $P\gamma$ 1-60 only ( $\square$ ), or no addition ( $\blacktriangle$ ). 10 mM  $Mg^{2+}$  was then added to restore catalytic activity, and the inhibitory potency of vardenafil was measured using 0.1 mM cAMP as substrate with the radiotracer assay (*see Experimental Procedures*). cGMP binding assays confirmed retention of bound cGMP during the experiment. The data are the mean ( $\pm$  S.E.M.) of five experiments. The *solid lines* represent the fit to a 3-parameter logistic dose-response equation with  $IC_{50}$  values of  $4.3 \pm 0.3$  nM (no addition),  $2.2 \pm 0.2$  nM ( $P\gamma$ 1-60), and  $2.3 \pm 0.1$  nM ( $P\gamma$ 1-60 plus cGMP). The *asterisks* indicate that the  $IC_{50}$  value was statistically significant ( $p < 0.05$ ) from the control value.

Figure 3. Binding of  $P\gamma$ 1-45 or  $P\gamma$ 1-60 to the GAF domains equally enhanced the binding affinity of  $P\gamma$ 63-87 to the catalytic domain. Purified  $P\alpha\beta$  (0.2 nM) was pre-incubated with 2  $\mu$ M  $P\gamma$ 1-45 ( $\square$ ), 2  $\mu$ M  $P\gamma$ 1-60 ( $\bullet$ ) or no peptide ( $\blacktriangle$ ) for 10 min before adding increasing amounts of  $P\gamma$ 63-87. The PDE activity was measured using 2 mM cGMP as substrate. The data represent the average of four experiments, and curve fitting (*solid line*) yielded the following  $IC_{50}$  values:  $2.2 \pm 0.1$   $\mu$ M (no added peptide);  $1.2 \pm 0.1$   $\mu$ M ( $P\gamma$ 1-45);  $1.5 \pm 0.2$   $\mu$ M ( $P\gamma$ 1-60). The *asterisks* indicate that the  $IC_{50}$  value was statistically significant ( $p \leq 0.05$ ) from the control value.

Figure 4.  $P\gamma$ 1-80 increased the Michaelis-Menten constant of PDE6. Purified  $P\alpha\beta$  (20 pM) was pre-incubated with 200 nM  $P\gamma$ 1-60 ( $\diamond$ ), 200 nM  $P\gamma$ 1-80 ( $\blacktriangle$ ) or no peptide ( $\bullet$ ) for 20 min before addition of increasing amounts of cGMP. The data represent the average of three experiments (except  $P\gamma$ 1-60 where  $n = 2$ ), and were fit to a two-parameter hyperbolic function with the following parameters: no peptides:  $K_M = 9.1 \pm 0.6$   $\mu$ M and  $V_{max} = 6.2 \pm 0.1$  pmol cGMP/s;  $P\gamma$ 1-60:  $K_M = 9.5 \pm 0.9$   $\mu$ M,  $V_{max} = 6.1 \pm 0.1$  pmol

cGMP/s, and; P $\gamma$ 1-80:  $K_M = 22.9 \pm 1.5 \mu\text{M}$ ,  $V_{\text{max}} = 2.4 \pm 0.05 \text{ pmol cGMP/s}$ . The *asterisk* indicates that the  $K_M$  value for P $\gamma$ 1-80 was statistically significant ( $p < 0.05$ ) from the control and P $\gamma$ 1-60 values.

Figure 5. cGMP dissociation kinetics were slowed when vardenafil was bound to PDE6. Purified P $\alpha\beta$  (5 nM) was pre-incubated with 10 mM EDTA, 20 mM dipicolinic acid, and 100  $\mu\text{M}$  vardenafil (■) or 10 mM Tris (●) for 2 hours at 22°C. [ $^3\text{H}$ ]cGMP was then added for 5 min ( $B_{\text{max}} = 2.1 \pm 0.4$  or  $2.2 \pm 0.3$  cGMP per P $\alpha\beta$  in the presence or absence of vardenafil, respectively) before inducing dissociation with unlabeled cGMP (1 mM). The data points represent the mean ( $\pm$  S.E.M.;  $n = 4$ ), while the curves represent the fit of the data to a single-exponential decay. Analysis of the data as a two-component (double-exponential decay) process did not improve the fit to a statistically significant extent. The  $t_{1/2}$  values were  $3.5 \pm 0.3$  min for 100  $\mu\text{M}$  vardenafil and  $2.0 \pm 0.1$  min for the control condition, respectively. The *asterisk* indicates that the  $t_{1/2}$  value was statistically significant ( $p < 0.05$ ) from the control value.

Figure 6. Certain PDE inhibitors induced a slow conformational change in the catalytic domain. 0.2 nM P $\alpha\beta$  was incubated with the indicated PDE inhibitors for 20 min (closed symbol) or overnight (open symbol) before assaying catalytic activity with 2 mM cGMP. The data (mean  $\pm$  S.E.M.,  $n = 3$ ) was fit to the logistic equation with the following  $\text{IC}_{50}$  values: A. vardenafil:  $0.3 \pm 0.02 \mu\text{M}$  (20 min incubation) and  $0.2 \pm 0.02 \mu\text{M}$  (overnight incubation); sildenafil:  $3.0 \pm 0.2 \mu\text{M}$  (20 min) and  $2.0 \pm 0.1 \mu\text{M}$  (overnight); B. zaprinast:  $5.8 \pm 0.2 \mu\text{M}$  (20 min) and  $5.6 \pm 0.2 \mu\text{M}$  (overnight); IBMX:  $1.2 \pm 0.08 \text{ mM}$  (20 min) and  $1.2 \pm 0.01 \text{ mM}$  (overnight). The *asterisk* indicates that the  $\text{IC}_{50}$  value for overnight incubation of drug was statistically significant ( $p < 0.05$ ) from the corresponding 20 min incubation.

Figure 1, Zhang and Cote

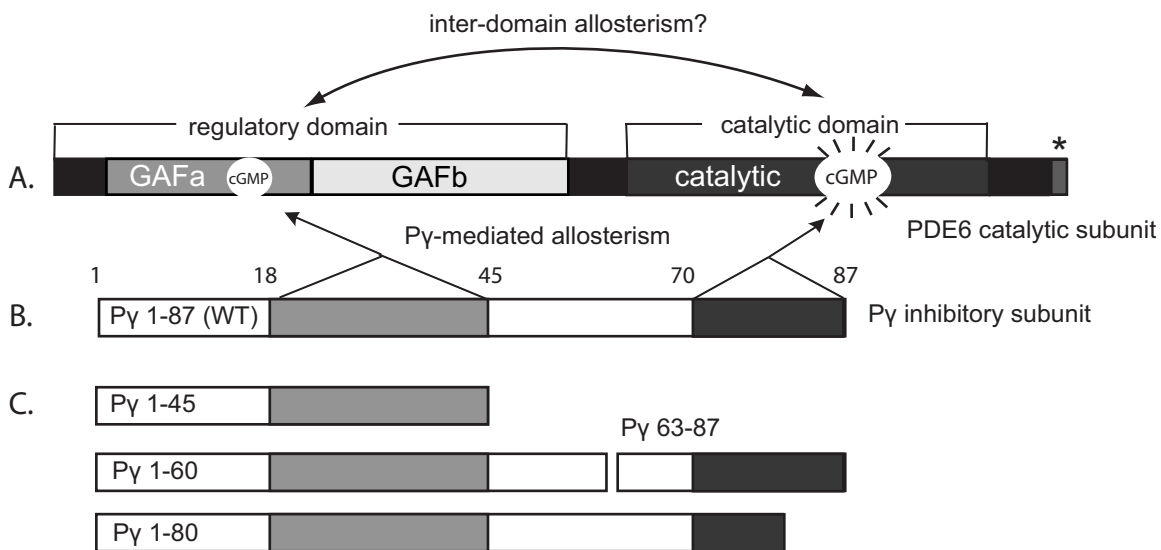


Figure 2, Zhang and Cote

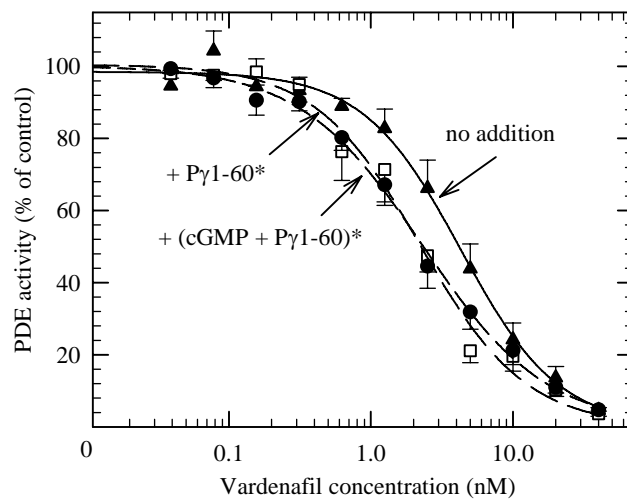


Figure 3, Zhang and Cote

