Regulation of photoreceptor phosphodiesterase catalysis by its non-catalytic cGMP-binding sites

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The photoreceptor 3′:5′-cyclic nucleotide phosphodiesterase (PDE) is the central enzyme of visual excitation in rod photoreceptors. The hydrolytic activity of PDE is precisely regulated by its inhibitory γ subunit (Pγ), which binds directly to the catalytic site. We examined the inhibition of frog rod outer segment PDE by endogenous Pγ, as well as by synthetic peptides corresponding to its central and C-terminal domains, to determine whether the non-catalytic cGMP-binding sites on the catalytic $\alpha\beta$ dimer of PDE allosterically regulate PDE activity. We found that the apparent binding affinity of Pγ for PDE was 28 pM when cGMP occupied the non-catalytic sites, whereas Pγ had an apparent affinity only 1/16 of this when the sites were empty. The elevated basal activity of PDE with empty non-catalytic sites can be decreased by the addition of nanomolar levels of cGMP, demonstrating that the high-affinity non-catalytic sites on the PDE catalytic dimer mediate this effect. No evidence for a direct allosteric effect of the non-catalytic sites on catalysis could be detected for the activated enzyme lacking bound Pγ. The intrinsic affinity of a synthetic C-terminal (residues 63–87) Pγ peptide to bind and to inhibit the hydrolytic activity of activated PDE was enhanced 300-fold in the presence of cGMP compared with cAMP. We conclude that the binding of cGMP to the non-catalytic sites of PDE induces an allosteric change in the structure of the catalytic domain that greatly enhances the interaction of the C-terminus of Pγ with the catalytic domain.

Key words: allosterism, inhibitory subunit, retina, visual transduction.

INTRODUCTION

The photoreceptor 3′:5′-cyclic nucleotide phosphodiesterase (PDE6) is the central effector enzyme in the visual transduction pathway. In rod photoreceptors, visual transduction is initiated by the absorption of light by rhodopsin, an integral membrane protein. Light-activated rhodopsin interacts with transducin, a heterotrimeric G-protein, to catalyse GDP–GTP exchange on its α_t subunit. The activated α_t*–GTP subunit then activates PDE by removing the inhibitory constraint imposed by the inhibitory γ subunits (Pγ) of PDE. Rather than completely dissociating from PDE, the complex of α_t*–GTP–Pγ is believed to remain initially in a complex with the catalytic $\alpha\beta$ heterodimer of rod PDE (P$\alpha\beta$). On activation, PDE hydrolyses cGMP; the subsequent decrease in cytoplasmic cGMP concentration causes dissociation from the cGMP-gated channels. As a result, the channels close, the rod cell becomes hyperpolarized, and the receptor potential is generated (reviewed in [1–3]).

The membrane-associated rod PDE holoenzyme is a heterotetramer ($\alpha\beta\gamma\gamma$) composed of two similar catalytic subunits, α and β, and two identical inhibitory subunits, Pγ. The P$\alpha\beta$ catalytic dimer contains two catalytic sites [4,5] and two high-affinity non-catalytic cGMP-binding sites [6–9].

The PDE holoenzyme is tightly regulated by the Pγ inhibitory subunits. Much work has been done with bovine PDE to elucidate the important sites of interaction of Pγ with P$\alpha\beta$ [10–21]. It has been found that Pγ contains two distinct domains that interact with P$\alpha\beta$: a central cationic region (residues approx. 20–45) and a hydrophobic C-terminal region (residues approx. 63–87). A synthetic peptide corresponding to the central region (24–45-Pγ) was found to bind to bovine PDE with high affinity [16], and it was demonstrated recently that this region could enhance cGMP binding at the non-catalytic sites of activated bovine cone PDE [21]. A second synthetic peptide corresponding to the C-terminal region of Pγ (63–87-Pγ) could completely inhibit PDE activity [16,17] and was found to act by binding directly to the catalytic site and competing with cGMP occupancy of the catalytic site of PDE [19,20].

In addition to PDE6, two other classes of PDE have regulatory cGMP-binding sites that undergo allosteric transitions. For cGMP-stimulated PDE (PDE2), cGMP occupancy of the non-catalytic sites stimulates cyclic-nucleotide hydrolysis at the catalytic site [22]. For the cGMP-binding PDE (PDE5), cGMP binding induces a conformational change required for the catalytic subunit to be phosphorylated by cyclic-nucleotide-dependent protein kinases; whether phosphorylation results in altered PDE5 catalytic activity is currently unclear (see [23] and references therein). For the photoreceptor PDE6 family, there is evidence that cGMP binding and Pγ binding to P$\alpha\beta$ undergo positive co-operativity: when cGMP occupies the non-catalytic sites of frog PDE, Pγ is less likely to be released from P$\alpha\beta$ during the activation of PDE by transducin [24]; when Pγ binds to P$\alpha\beta$, the binding affinity of cGMP for the non-catalytic sites is greatly enhanced [9,25,26]. Whether this positive co-operativity is indirectly mediated through changes in Pγ structure or by means of direct conformational changes in the P$\alpha\beta$ subunits is not known.

In this study we directly examined the ability of endogenous

Abbreviations used: P$\alpha\beta$, catalytic heterodimer of PDE6; Pγ, inhibitory γ subunit of PDE6; PDE, 3′:5′-cyclic nucleotide phosphodiesterase; PDE2, cGMP-stimulated PDE; PDE5, cGMP-binding PDE; PDE6, photoreceptor PDE; ROS, rod outer segments.

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frog Pγ to inhibit frog P$\alpha\beta$ in conditions under which the non-catalytic sites of PDE are occupied (with cGMP as the substrate for hydrolysis) or where the non-catalytic sites are unoccupied (with cAMP as the substrate). This approach takes advantage of the fact that cAMP fails to bind to the non-catalytic sites of frog rod PDE, even at very high nucleotide concentrations [27]. In addition, we investigated the ability of synthetic Pγ peptides corresponding the central (residues 21–46) and C-terminal (residues 63–87) regions of Pγ to affect the hydrolytic activity of frog P$\alpha\beta$, and examined how the interaction between those regions of Pγ and P$\alpha\beta$ are affected by occupancy of the non-catalytic sites. We found that the ability of endogenous Pγ (more specifically, its C-terminal region) to interact with P$\alpha\beta$ is enhanced by occupancy of the non-catalytic sites with cGMP. We conclude that cGMP occupancy of the non-catalytic sites induces an allosteric change in the catalytic domain such that Pγ binds with greater affinity to this region of PDE, thereby increasing the inhibitory potency of Pγ.

MATERIALS AND METHODS

Materials and solutions

[8-^{3}H]cGMP and [8-^{3}H]cAMP were purchased from Dupont–NEN; Percoll was from Pharmacia LKB. All other chemicals were purchased from Sigma. The Pγ central peptide (21–46-Pγ) consists of residues 21–46 of the frog (*Rana pipiens*) sequence [28] and is identical with the bovine rod sequence except for a substitution of alanine for valine at position 21; the C-terminal peptide (63–87-Pγ) is completely conserved in all rod Pγ subunits sequenced so far. Both were synthesized at the Protein Facility in the Department of Pharmacology (University of Washington, Seattle, WA, U.S.A.) and purified by HPLC; the mass was verified by MS. Recombinant bovine Pγ was prepared as described in Artemyev et al. [29]. The Ringer's solution used to isolate the rod photoreceptors contained 105 mM NaCl, 2 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$ and 10 mM Hepes, pH 7.5. The PDE assay medium contained 100 mM Tris/HCl, pH 7.5, 10 mM $MgCl_2$, 0.5 mM EDTA, 2 mM dithiothreitol, 0.5 mg/ml BSA, 5 μM leupeptin and 50 k-i.u./ml aprotinin.

Preparation of PDE

Purified frog (*Rana catesbeiana*) rod outer segments (ROS) were isolated in the dark (under IR illumination) by using modifications of a method described previously [8]. In brief, frog retinas were removed from enucleated eyes; ROS were detached from the retinas by gentle shaking into Ringer's solution containing 5% (v/v) Percoll. The ROS were then typically purified by centrifugation in a discontinuous Percoll gradient consisting of 5%, 30% and 60% Percoll. After dilution of the Percoll with Ringer's solution and a brief centrifugation of the ROS (1 min at 3000 ***g***), the ROS were resuspended in the appropriate assay medium and homogenized at 4 °C with a nylon pestle and glass mortar. No structure was detectable by phase-contrast microscopy after homogenization. Endogenous nucleotides were depleted by incubating the homogenized ROS at room temperature for at least 30 min; this treatment has been shown to remove more than 95% of the endogenous, bound cGMP [8].

The PDE concentration was determined by measuring the rhodopsin concentration spectrophotometrically [30], along with a knowledge of the molar ratio of rhodopsin to PDE (330:1) in frog ROS [8]. This calculation was routinely verified by two other estimates of the PDE concentration in our samples. For one estimate, the maximum extent of cGMP binding to PDE holoenzyme was measured by using 1 μM [^{3}H]cGMP, an amount sufficient to saturate the high-affinity cGMP-binding sites on PDE. The PDE concentration was then calculated on the basis of a stoichiometry of two high-affinity non-catalytic sites per holoenzyme [7,9]. The other method involved measuring the rate of cGMP hydrolysis by transducin-activated PDE at saturating concentrations of cGMP (10 mM) to obtain the V_{max} for our sample. From the relationship $[P\alpha\beta] = V_{max}/k_{cat}$ (where k_{cat} = 4400 cGMP hydrolysed/s per P$\alpha\beta$ for transducin-activated PDE [5]), we calculated the PDE concentration. The three estimates of PDE concentration were internally consistent to within 20%; much of this variability was probably due to differences between animals in the molar ratio of rhodopsin to PDE in different ROS preparations.

To prepare activated PDE lacking bound Pγ subunits (P$\alpha\beta$), we performed limited proteolysis to degrade the inhibitory Pγ subunits [4,31]. PDE (120 nM) (in PDE assay medium lacking protease inhibitors) was incubated with 200 μg/ml tosylphenylalanylchloromethane ('TPCK')-treated trypsin for 10 min at 4 °C, then mixed with a 6-fold excess of soybean trypsin inhibitor. The trypsinization conditions were optimized to minimize exposure to trypsin while achieving maximal PDE activity. The proteolytic activation of PDE can be reversed by the addition of recombinant bovine Pγ: more than 90% of the enzyme activity can be inhibited by addition of 2 mol of Pγ per mol of P$\alpha\beta$ [32]. This indicates that the destruction of Pγ is the primary site of action of trypsin under our experimental conditions.

PDE activity assay

Nucleotide-depleted, homogenized ROS were diluted to 45 μl with PDE assay medium in 0.6 ml microcentrifuge tubes. For experiments in which the synthetic Pγ peptides were used, the ROS were preincubated with the peptides for at least 1.5 h (at 4 °C) before the PDE rate determinations. All samples were brought to room temperature (21 °C) before initiating the activity assays with the addition of 5 μl of substrate. The reaction was stopped by quenching 10 μl samples in 50 μl of 100 mM HCl at various times. To quantify the cGMP that was hydrolysed, samples were analysed with either a radiotracer assay using DEAE anion-exchange chromatography [33] or a colorimetric assay [32]. Total substrate hydrolysis was less than 30% in all experiments. Each PDE rate determination was based on three to six individual time points, during which the time course remained linear. As a control, we typically measured the PDE activity of light-exposed samples that were transducin-activated by the addition of a 10-fold molar excess of guanosine 5′-[γ-thio]triphosphate (over the transducin concentration) 1 min before the addition of substrate. Determinations of the kinetic parameters for cGMP and cAMP as substrates for transducin-activated PDE agreed, within experimental error, with those published previously [5].

Filter binding assay of cGMP binding

The cGMP binding assay in Figure 3 was performed essentially as described in [32], except that PDE inhibitors had to be omitted to measure cyclic-nucleotide hydrolysis and binding simultaneously. Nucleotide-depleted homogenized ROS were diluted with assay medium lacking PDE inhibitors, and the cGMP-binding reaction was initiated by mixing 3 nM PDE (final concentration) with the indicated concentration of [^{3}H]cGMP. After a 3 min incubation at room temperature, two 50 μl samples were filtered and washed on pre-wetted nitrocellulose filters (Millipore HAWP 025). Identical portions were also quenched in

acid at the onset of the incubation and also immediately after the filter binding, and assayed for the extent of [^{3}H]cGMP hydrolysis by using the above-mentioned radiotracer assay.

Data analysis

All experiments were performed three times unless noted otherwise and the results presented are means $\pm$ S.E.M. for n determinations. Fitting of the experimental data was performed by non-linear least-squares analysis with SIGMAPLOT software. Binding curves were generated on the assumption of a single class of non-interacting sites.

RESULTS AND DISCUSSION

cGMP binding to the non-catalytic sites lowers basal activity of PDE holoenzyme

So far, the kinetic properties of frog PDE have been studied primarily with the activated form of the enzyme (i.e. transducin-activated or trypsin-activated), typically with cGMP as the substrate (see Table V in [1] for summary). However, there has been only one report in which the effects of the non-catalytic sites on catalytic activity of PDE holoenzyme have been directly examined. Arshavsky et al. [24] reported that frog PDE holoenzyme displayed no kinetic evidence for direct co-operativity between the non-catalytic and catalytic sites on the enzyme, although they did show that occupancy of the non-catalytic sites with cGMP decreased the catalytic activity of the enzyme and enhanced the overall affinity of Pγ for the P$\alpha\beta$ catalytic dimer.

To explore further how the non-catalytic sites on PDE holoenzyme might regulate the rate of cyclic-nucleotide hydrolysis at the active sites, we compared the catalytic activity of PDE holoenzyme when the non-catalytic sites were either empty or occupied with cGMP. To accomplish this, we relied on the fact that cGMP binds rapidly (less than 1 s under these conditions) and with high affinity (K_d 60 nM [8,9]) to the non-catalytic cGMP-binding sites of the holoenzyme, whereas cAMP is unable to occupy these same binding sites (at most 10% displacement of [^{3}H]cGMP at 20 mM cAMP [27]). We performed this comparison of cGMP and cAMP hydrolytic rates over a wide range of PDE holoenzyme concentrations to alter the equilibrium concentrations of Pγ bound to P$\alpha\beta$:

$$P\alpha\beta + 2P\gamma \rightleftharpoons P\alpha\beta\gamma\gamma \quad (1)$$

The activity assays were performed by incubating PDE with either 1 mM cGMP (full occupancy of non-catalytic sites with cGMP) or 20 mM cAMP (empty non-catalytic sites). These concentrations of cGMP and cAMP were chosen so that each cyclic nucleotide was present at a concentration corresponding to $0.91V_{max}$ for the transducin-activated enzyme (for cGMP, $K_m = 95\ \mu$M and $k_{cat} = 4400$ cGMP/s per P$\alpha\beta$; for cAMP, $K_m =$ 2.0 mM and $k_{cat} = 2500$ cAMP/s per P$\alpha\beta$ [5]; we have made independent measurements in our laboratory of the kinetic parameters for transducin-activated PDE with both cGMP and cAMP as substrates, and our values are in excellent agreement with these). Therefore if the binding of cGMP to the non-catalytic sites does not affect the hydrolytic activity of the PDE holoenzyme, the normalized rate of hydrolysis (v/V_{max}) at 1 mM cGMP or 20 mM cAMP should be equal. (We chose to normalize the rate data to account for possible differences in the catalytic mechanism for cGMP and cAMP, as judged by the 1.8-fold differences in their respective k_{cat} values.) We found that at each PDE concentration we tested, the normalized activity of PDE holoenzyme with cGMP as the substrate was substantially lower than the activity of PDE with cAMP as the substrate (Figure 1). This is consistent with the idea that the binding of cGMP to the non-catalytic sites results in lower rates of hydrolysis at the active sites. From this observation we infer that the binding affinity of Pγ for P$\alpha\beta$ is enhanced by the binding of cGMP.

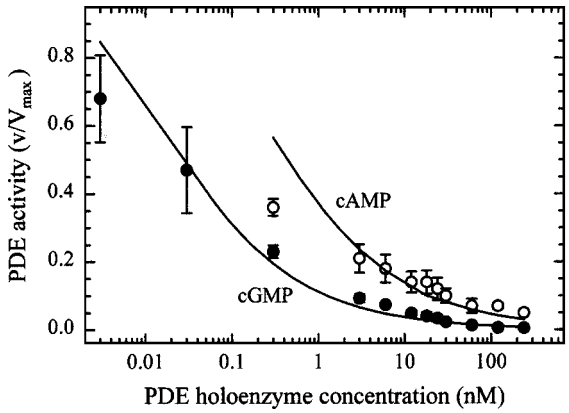

Figure 1 Apparent affinity of Pγ for P$\alpha\beta$ is greater in the presence of cGMP than cAMP

The activity of PDE holoenzyme was determined at various concentrations of homogenized ROS with 1 mM cGMP (●) or 20 mM cAMP (○) as substrate. PDE activity is expressed as a percentage of the maximum rate for transducin-activated PDE under these experimental conditions: 1 mM cGMP, $V_{max} = 4000$ cGMP/s per PDE; 20 mM cAMP, $V_{max} = 2280$ cAMP/s per PDE. The curves represent the best fit to a single-site equilibrium binding of Pγ to a catalytic subunit in the presence of cGMP [K_d (app) $= 28 \pm 7$ pM] or cAMP [K_d (app) $= 440 \pm 59$ pM].

To quantify this effect we assumed a simple equilibrium binding model of a single class of binding sites for Pγ to P$\alpha\beta$, along with measurements of the total P$\alpha\beta$ concentration and a knowledge of the Pγ concentration in ROS [32]. We also initially assumed that nucleotide binding at the active site did not compete with Pγ binding to its binding sites on the enzyme. We found that in the presence of 1 mM cGMP, the K_d (app) for Pγ binding to P$\alpha\beta$ was 28 ± 7 pM. This value was 16-fold lower than when 20 mM cAMP was used and the non-catalytic sites were empty [K_d (app) $= 440 \pm 59$ pM]. The K_d (app) for Pγ binding to frog PDE holoenzyme was significantly weaker than the values reported for bovine rod PDE holoenzyme in the presence of cGMP (at most 10 pM [34]). The model that we used for calculating K_d (app) is a simplification of the actual situation and should not be considered the intrinsic binding affinity of Pγ for P$\alpha\beta$. This is because the affinity of Pγ for P$\alpha\beta$ is composed of two distinct sites of interaction: one with the central region and the other with the C-terminal region of Pγ (see the Introduction). Furthermore, the C-terminal region of Pγ can bind in competition with cyclic nucleotides at the active site [20], complicating the analysis of the intrinsic affinity of full-length Pγ. We shall turn below to the use of synthetic peptides of Pγ to avoid these complicating factors, and emphasize here that the estimates of the ability of full-length Pγ to interact with P$\alpha\beta$ in Figure 1 represent apparent binding affinities that pertain specifically to the conditions of this experiment.

We found that at PDE concentrations lower than those shown in Figure 1 (less than 3 pM for cGMP, less than 300 pM for cAMP) the activity of PDE began to decline, even though not all Pγ had dissociated from P$\alpha\beta$. This effect might have been occurring even at the lowest PDE concentration plotted for each substrate shown in Figure 1, because these two data points fell well below the predicted curve fit. One likely possibility is that

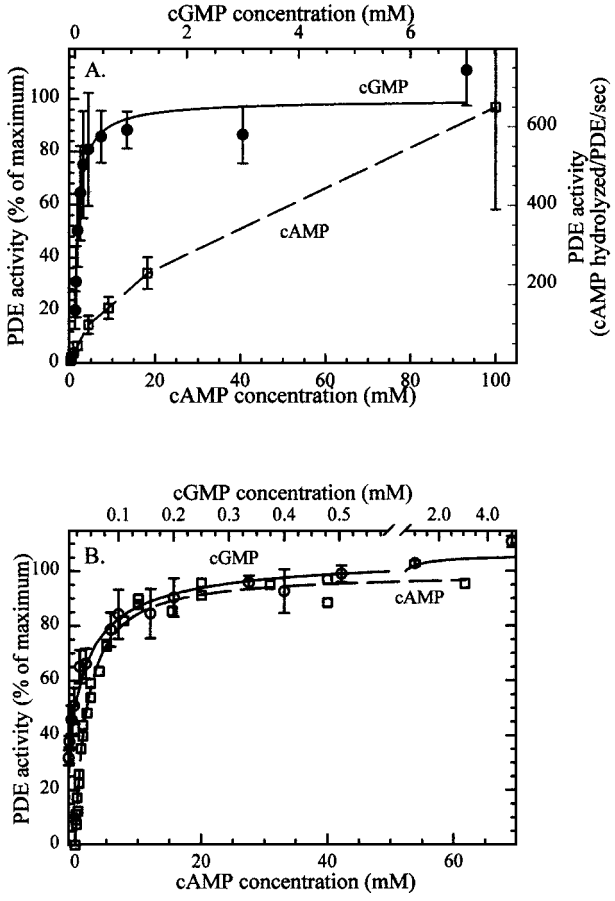

Figure 2 Dependence of hydrolytic activity on substrate concentration for PDE holoenzyme (A) and trypsin-activated Pαβ (B) with cGMP or cAMP as substrate

The dependence of the hydrolytic activity of PDE holoenzyme or trypsin-activated P$\alpha\beta$ was determined with both cGMP (●,○) or cAMP (■,□) as substrates, as described in the Materials and methods section. (**A**) PDE holoenzyme (12 nM) was mixed with the indicated concentrations of cyclic nucleotide and the rate of hydrolysis was determined. The cGMP data points are means ± S.D. for six separate experiments that have been normalized to the V_{max} determined for each experiment. The cAMP data points are means ± S.D. for three separate experiments. Because no V_{max} could be determined under this condition, the right-hand axis reports the absolute value for the cAMP hydrolytic rate. The cGMP data were analysed as a simple hyperbolic function ($K_m = 62 \pm 11$ μM cGMP; $V_{max} = 75 \pm 27$ cGMP hydrolysed/s per PDE; $r = 0.98$), as well as for co-operative behaviour ($h = 0.9 \pm 0.2$). (**B**) The hydrolytic rate of P$\alpha\beta$ was determined as a function of the cGMP (20 pM P$\alpha\beta$) or cAMP (1 nM P$\alpha\beta$) concentration and normalized to the maximum rate determined for each experiment. The circles are the cGMP data compiled from eight experiments; the error bars are the S.D. for three or more determinations, except for cGMP concentrations over 100 μM, where they are the range of two measurements. The solid line is the fit of the data to a hyperbolic function ($K_m = 22 \pm 2$ μM cGMP; $V_{max} = 7870 \pm 150$ cGMP hydrolysed/s per PDE; $r = 0.996$); no co-operativity was detected ($h = 0.9 \pm 0.1$). The cAMP data (□) were compiled from individual measurements from three experiments; the broken line represents a K_m of 1.9 ± 0.1 mM cAMP and a V_{max} of 4580 ± 70 cAMP hydrolysed/s per PDE ($r = 0.995$); no co-operativity was apparent ($h = 1.0 \pm 0.1$).

the frog enzyme is unstable at very low concentrations and begins to denature. The ability to dilute the enzyme further when cGMP was present suggests that the binding of cGMP to the non-catalytic sites of PDE might stabilize the native conformation of the enzyme and protect it from denaturation.

The dependence on PDE concentration of the hydrolytic rate shown in Figure 1 can be extrapolated to the PDE concentration *in vivo* to estimate the basal PDE activity of the dark-adapted rod photoreceptor. From a knowledge of the apparent dissociation constant for Pγ binding [K_d (app) = 28 pM in the presence of cGMP] and the total P$\alpha\beta$ (18 μM P$\alpha\beta$ or 36 μM subunit concentration [8]) and Pγ (equimolar with the catalytic subunit concentration [32]) concentrations in the photoreceptor, we calculated the PDE subunit concentration lacking bound Pγ to be 32 nM. This value represents one fully active PDE catalytic dimer for every 2200 total PDE holoenzyme molecules, and corresponds to 2.2 μM cGMP hydrolysed/s in the rod (assuming a free cytoplasmic cGMP concentration in the dark of 3 μM [1,8]). These values are in good agreement with electrophysiological estimates of dark PDE activity in the amphibian rod [1]. For example, Rieke and Baylor [35] used a noise analysis of the dark current to estimate that 1 in 5000 PDE molecules are fully active in a dark-adapted toad rod. A second estimate comes from Hodgkin and Nunn [36], who calculated a PDE rate of 1 μM cGMP/s turnover in dark-adapted rods.

Another physiological implication of this cGMP-dependent enhancement of Pγ affinity for PDE (Figure 1) relates to photoreceptor light adaptation. Under conditions of prolonged illumination, a sustained light-induced decrease in cytoplasmic cGMP concentration would lead to the dissociation of cGMP from non-catalytic binding sites. This would lower the Pγ affinity for those PDE molecules lacking bound cGMP. One consequence would be an elevation of the basal hydrolytic activity for those PDE molecules that are not light-activated, consistent with measurements *in vivo* of elevated cGMP metabolic flux during continuous illumination of the retina [37].

Evidence for competition between cyclic nucleotides and Pγ at the catalytic site

To test the assumption made above that 1 mM cGMP or 20 mM cAMP were not acting in direct competition with Pγ for binding at the catalytic sites, we next examined whether increasing the cyclic nucleotide concentration at a single enzyme concentration provided evidence for the progressive displacement of bound Pγ from the active site. When cGMP was the substrate, simple Michaelis–Menten kinetics were observed over the concentration range 10 μM to 10 mM; in contrast, saturation behaviour was not observed when cAMP was the substrate (Figure 2A). The K_m for PDE holoenzyme with cGMP as a substrate ($K_m = 62 \pm 11$ μM; $n = 6$) was found to be intermediate between the transducin-activated value ($K_m = 95$ μM [5]) and the trypsin-activated value ($K_m = 22$ μM) (Figure 2B). The k_{cat} obtained for this concentration of PDE holoenzyme (75 ± 27 cGMP hydrolysed/s per PDE; $n = 6$) represents 1.7 % of the maximum transducin-activated rate. No evidence for co-operativity was observed over the entire range of cGMP concentrations [Hill coefficient (h) ≈ 1.0], but this was expected because the non-catalytic cGMP-binding sites on the PDE holoenzyme would be fully occupied even at the lowest cGMP concentration used for activity measurements. We conclude that the PDE holoenzyme with occupied non-catalytic sites exhibits the simple kinetic behaviour expected if 1.7 % of the total holoenzyme population lacked bound Pγ. Furthermore, increasing the cGMP concentration up to 100-fold the K_m did not seem to make a significant change in the concentration of active enzyme by competition of cGMP and Pγ for occupancy of the catalytic site.

When cAMP was the substrate, PDE holoenzyme activity continued to increase as the substrate concentration increased, even up to 100 mM (Figure 2A). Because transducin-activated PDE [5] and trypsin-activated P$\alpha\beta$ (Figure 2B) both have K_m values for cAMP of 2 mM and show saturation behaviour, the

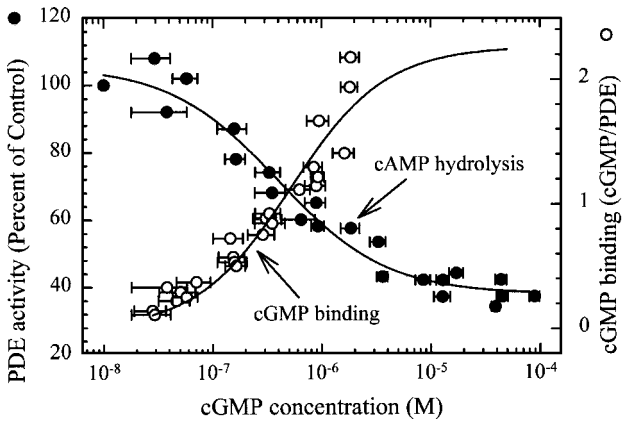

Figure 3 Sub-micromolar levels of cGMP increase the affinity of Pγ for Pαβ

The activity of PDE holoenzyme (3 nM) was determined in the presence of 20 mM cAMP as substrate and increasing concentrations of [^{3}H]cGMP to titrate the non-catalytic sites. Conditions were chosen such that even 10 μM cGMP (0.1 K_m^{cGMP}) did not competitively inhibit 20 mM cAMP (10 K_m^{cAMP}) hydrolysis to a significant extent. The rate of cAMP hydrolysis (●) was measured over a period of 3 min ($IC_{50} = 400 \pm 90$ nM cGMP; mean ± S.D., $r = 0.983$), at the end of which the amount of cGMP bound (○) was measured for cGMP concentrations up to 2 μM by using the filter binding assay ($IC_{50} = 520 \pm 110$ nM, $B_{max} = 2.2 \pm 0.3$ cGMP per PDE; mean ± S.D., $r = 0.956$). Data points represent individual measurements compiled from three separate experiments. Horizontal error bars represent the range of the beginning and ending cGMP concentrations as determined by measurements of [^{3}H]cGMP hydrolysis. In the absence of cGMP the PDE activity was 380 ± 50 cAMP hydrolysed/s per PDE. The IC_{50} for titration of the non-catalytic sites is not equivalent to the intrinsic K_d for cGMP binding, because binding equilibrium was not attained during the brief incubation period required to prevent substantial cGMP hydrolysis.

results for the PDE holoenzyme with cAMP most probably represent direct competition of Pγ and cAMP for binding at the catalytic site. The marked difference between the kinetics of PDE holoenzyme with cGMP and with cAMP as substrates further supports the idea that cGMP enhances the affinity of Pγ for the Pαβ catalytic dimer of PDE by binding to the non-catalytic sites. In the absence of cGMP occupancy at the non-catalytic sites, the Pγ binding affinity is decreased sufficiently to permit high cAMP concentrations to effectively compete with and displace the C-terminal region of Pγ [20], thereby progressively elevating the cAMP hydrolytic rate.

The high-affinity non-catalytic sites are the locus of the cGMP effect on Pγ affinity

Because the comparison in Figure 1 was performed with millimolar levels of cyclic nucleotides, we could not determine whether the high-affinity non-catalytic sites on PDE ($K_d = 60$ nM [8,9]) or another class of low-to-moderate-affinity sites [6,8] was responsible for the differences in hydrolytic activity with cGMP compared with cAMP. Furthermore, because Pγ can compete with high concentrations of cAMP for binding at the catalytic site (Figure 2A), we wanted to examine whether the differences between cGMP and cAMP noted in Figure 1 resulted from cGMP occupancy at the non-catalytic sites. We therefore examined the effect of titrating the high-affinity non-catalytic sites on the ability of PDE to hydrolyse cAMP. We found that sub-micromolar levels of cGMP suppressed the rate of cAMP hydrolysis by 60% (Figure 3). Note that the cGMP concentrations used to titrate the high-affinity binding sites were such that cAMP hydrolysis was not significantly decreased by competitive inhibition. The good correlation between the titration curves in Figure 3 for the non-catalytic sites ($IC_{50} = 520 \pm 110$ nM) and for the suppression of cAMP hydrolysis ($IC_{50} = 400 \pm 90$ nM) leads us to conclude that the high-affinity non-catalytic sites on PDE (the only high-affinity sites with a K_d of less than 1 μM in frog ROS) alter the catalytic properties of PDE by increasing the affinity of Pγ for Pαβ.

Examining Pγ interactions with trypsin-activated frog Pαβ

Treating bovine rod PDE with trypsin to form Pαβ lacking bound Pγ has been shown to be an effective means of removing the inhibitory constraint of Pγ and activating PDE *in vitro* [4,34,38], especially because other methods of extracting Pγ from Pαβ are much less effective in quantitatively removing Pγ from bovine [34] or frog [39] PDE. However, there is substantial variability in previous studies concerning the kinetic parameters for trypsin-activated and transducin-activated PDE (see Table V in [1]). Therefore, before proceeding with experiments measuring the interactions of Pγ with trypsin-activated Pαβ, we first determined its kinetic parameters.

Figure 2(B) shows that the K_m of Pαβ for cGMP was 22 ± 2 μM ($n = 8$), approximately one-fifth of that for cGMP with transducin-activated frog PDE (95 μM [5]). The K_m for cAMP with Pαβ was 1.9 ± 0.1 mM ($n = 3$), which was nearly identical with that for transducin-activated frog PDE (2.0 mM [5]). For both cGMP and cAMP as substrates, the k_{cat} for Pαβ was elevated 1.8-fold ($k_{cat}^{cGMP} = 7870 \pm 150$ cGMP/s per PDE, $n = 8$; $k_{cat}^{cGAP} = 4580 \pm 70$ cAMP hydrolysed/s per PDE, $n = 3$) compared with the respective transducin-activated PDE rate [5]. This difference in maximal activity resulting from transducin activation or trypsin treatment has been noted previously for frog [40] and bovine [41] PDE. Finally, we could see no evidence for co-operativity in the kinetic behaviour of Pαβ as the cGMP concentration was raised from 10 μM to 10 mM. The extent of occupancy of the non-catalytic sites on Pαβ was likely to be increasing at cGMP concentrations of 10 μM or more because the binding affinity of these sites was greatly reduced by the removal of Pγ ($K_d > 10$ μM) (M. R. D'Amours and R. H. Cote, unpublished work). We therefore consider it unlikely that a direct allosteric interaction between the non-catalytic and catalytic sites has a significant role in the photoreceptor Pαβ catalytic dimer, as has been demonstrated for PDE2 [22].

Effects of the synthetic peptide 21–46-Pγ on Pαβ and on PDE holoenzyme

Previous studies have shown that the central (residues approx. 20–45) and the C-terminal (residues 63–87) regions of the Pγ molecule (87 residues) are important for its ability to bind to bovine Pαβ. However, those studies did not examine the effect of non-catalytic site occupancy on the ability of those regions to bind to Pαβ. To examine this, we tested the ability of central and C-terminal synthetic Pγ peptides to affect the hydrolytic activity of frog PDE with either cGMP or cAMP as the substrate.

Pαβ was preincubated with various concentrations of the peptide 21–46-Pγ before the addition of either 10 mM cGMP or 20 mM cAMP to determine whether the peptide altered the rate of cyclic-nucleotide hydrolysis. We found that peptide concentrations up to 2 mM did not significantly inhibit the hydrolysis of either cGMP or cAMP by frog Pαβ (results not shown), which is consistent with results obtained with a similar synthetic peptide and bovine cone PDE [21]. We conclude that there is no allosteric effect of 21–46-Pγ binding to Pαβ that influences catalytic activity at the active site. In addition, incubating 21–46-Pγ with frog Pαβ stimulated cGMP binding slightly but failed to restore fully the high-affinity cGMP binding characteristic of PDE holoenzyme

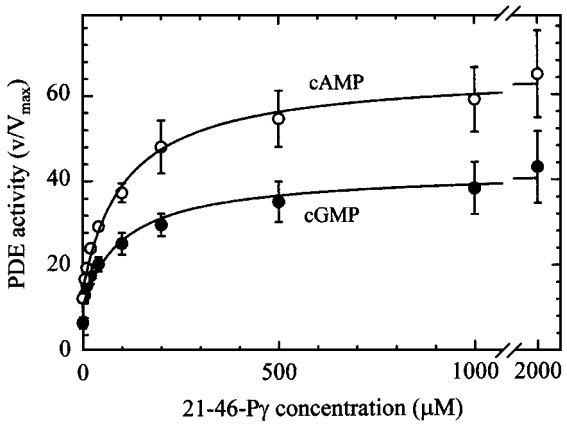

Figure 4 21–46-Pγ can displace endogenous full-length Pγ from PDE holoenzyme

The activity of PDE holoenzyme (3 nM) was determined in the presence of increasing amounts of 21–46-Pγ with 10 mM cGMP (●) or 20 mM cAMP (○) as substrate. The peptide was added before the substrate; assays were performed as described in the Materials and methods section. PDE activity is expressed as a percentage of the maximum transducin-activated rate in the absence of peptide (4360 cGMP hydrolysed/s per PDE or 2270 cAMP hydrolysed/s per PDE). The curves represent the fit of the data to a single-binding-site model for the peptide with IC_{50} values of 104 ± 32 μM (cGMP; $r = 0.984$) and 108 ± 15 μM (cAMP; $r = 0.996$).

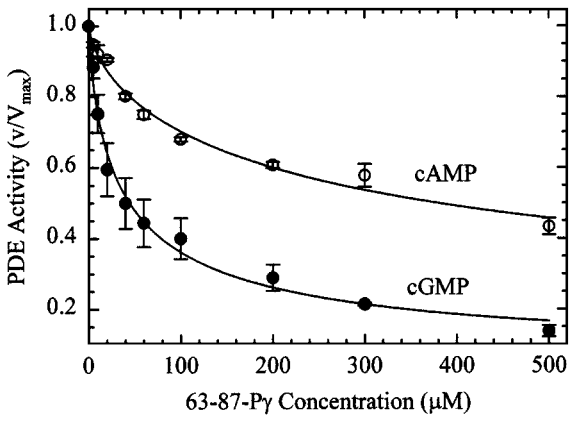

Figure 5 63–87-Pγ is a more potent inhibitor in the presence of cGMP

The activity of Pαβ (3 nM) was determined in the presence of increasing amounts of 63–87-Pγ with 10 mM cGMP (●) or 20 mM cAMP (○) as substrate. PDE activity is expressed as a percentage of the rate in the absence of peptide (7800 cGMP hydrolysed/s per PDE or 4220 cAMP hydrolysed/s per PDE). The following parameters were obtained from the inhibition curves: cGMP, $IC_{50} = 39 \pm 12$ μM, maximum extent of inhibition 99% ($r = 0.995$); cAMP, $IC_{50} = 300 \pm 342$ μM, maximum extent of inhibition 94% ($r = 0.993$).

(results not shown). This is in contrast with evidence that the central region of Pγ can promote cGMP binding to cone catalytic dimers [21] and to bovine rod Pαβ (H. Mou, D. C. Gapp and R. H. Cote, unpublished work). It is likely that the binding affinity of cGMP for frog Pαβ non-catalytic sites is decreased to below the detection limit of our binding assay.

We questioned whether 21–46-Pγ could actually bind to frog PDE, so we tested the ability of 21–46-Pγ to compete with and displace endogenous Pγ from PDE holoenzyme. Because the peptide by itself does not inhibit Pαβ, any increase in the activity of the PDE holoenzyme would indicate competition between 21–46-Pγ and endogenous Pγ for a binding site on Pαβ. Figure 4 demonstrates that increasing concentrations of 21–46-Pγ stimulated the hydrolytic activity of the PDE holoenzyme with either cGMP or cAMP as the substrate. The apparent affinity of the 21–46-Pγ peptide ($IC_{50} \approx 100$ μM) did not depend on which substrate was used, although the maximal extent of activation was somewhat higher when cAMP was present. Because the apparent affinity of full-length Pγ for Pαβ was enhanced with cGMP compared with cAMP (Figure 1), this suggests that occupancy of the non-catalytic sites exerts an effect on a region of Pγ other than the central region (see the next section). It is also noteworthy that the peptide can activate the PDE holoenzyme only to about one-half of the transducin-activated rate. We interpret this result to mean that the peptide to the central region of Pγ can compete with (and displace) only one of the two Pγ molecules bound to the PDE holoenzyme.

Effect of 63–87-Pγ on Pαβ

Having ruled out the central domain of Pγ as being responsible for the effects reported in Figures 1 and 3, we next examined the possibility that cGMP binding to the non-catalytic sites affects the ability of a C-terminal peptide (63–87-Pγ) to bind to and inhibit frog Pαβ.

We found that incubation of increasing amounts of 63–87-Pγ to Pαβ progressively inhibited the cyclic-nucleotide hydrolysis of both cGMP and cAMP, but the inhibitory potency of the peptide was significantly enhanced in the presence of cGMP (Figure 5). With cGMP as a substrate, 63–87-Pγ inhibited Pαβ activity in a dose-dependent manner, with an IC_{50} of 39 μM peptide. With cAMP, an 8-fold greater peptide concentration was needed to suppress 50% of the hydrolytic activity. The magnitude of the cGMP effect on 63–87-Pγ binding agrees well with the 16-fold change reported in Figure 1 with full-length endogenous Pγ.

Assuming that 63–87-Pγ is a simple competitive inhibitor of cyclic-nucleotide hydrolysis at the active site [20], we can calculate the inhibition constant (K_i) of the peptide from the following relationship: $K_i = IC_{50}/(1 + [S]/K_m)$, where [S] is the substrate concentration and the K_m is the Michaelis constant of Pαβ for the appropriate cyclic nucleotide [42]. The K_i of 63–87-Pγ in the presence of cGMP was calculated to be 78 nM, whereas the peptide K_i was 26 μM in the presence of cAMP. This 300-fold enhancement of the intrinsic affinity of the C-terminal peptide for Pγ in the presence of cGMP demonstrates that occupancy of the non-catalytic cGMP-binding sites induces a conformational change in the catalytic Pαβ dimer that promotes higher-affinity binding of Pγ to PDE. Note that a similar analysis of the K_i cannot be applied to the results in Figure 1, because full-length Pγ has two sites of interaction with Pαβ. However, because the experimental conditions were not too different for Figures 1 and 5, it is likely that the intrinsic binding affinity of endogenous Pγ is affected to a similar extent by occupancy of the non-catalytic sites.

CONCLUSIONS

Our results are the first demonstration of allosteric communication between the non-catalytic cGMP-binding sites and the catalytic domain on the PDE6 catalytic dimer. In contrast with PDE2, for which a direct allosteric activation of catalysis has been observed [22], PDE6 catalytic subunits undergo a conformational change on cGMP binding that alters the Pγ-binding site in the catalytic domain so that the affinity of the C-terminal region of Pγ is enhanced approx. 300-fold. Furthermore, we were unable to detect any significant change in catalytic activity

resulting from cGMP binding to the non-catalytic sites in the absence of Pγ. Therefore it is likely that the conformational change induced by cGMP occupancy does not alter the catalytic mechanism itself but rather the interaction of the catalytic domain with its endogenous protein inhibitor, Pγ. In this regard, the non-catalytic sites on PDE6 are functionally more closely related to PDE5 than to PDE2. For PDE5, cGMP binding to the non-catalytic sites induces a conformational change in the catalytic subunits that exposes a site for phosphorylation [23] rather than directly altering catalysis at the active site.

In summary, PDE6 shares with PDE2 and PDE5 the ability of the non-catalytic sites to confer an allosteric change on the catalytic subunits that leads to a unique regulatory mechanism: stimulation of catalysis for PDE2, exposure of a phosphorylation site on PDE5, or inhibition of the active site (via enhanced Pγ binding) for PDE6. It remains to be determined whether the state of cGMP occupancy of the non-catalytic sites of these three classes of PDE serves a physiological function during the excitation, recovery and adaptation phases of the signal transduction pathways in which these enzymes participate.

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