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cGMP binding sites on photoreceptor phosphodiesterase: Role in feedback regulation of visual transduction

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ABSTRACT A central step in vertebrate visual transduction is the rapid drop in cGMP levels that causes cGMP-gated ion channels in the photoreceptor cell membrane to close. It has long been a puzzle that the cGMP phosphodiesterase (PDE) whose activation causes this decrease contains not only catalytic sites for cGMP hydrolysis but also noncatalytic cGMP binding sites. Recent work has shown that occupancy of these noncatalytic sites slows the rate of PDE inactivation. We report here that PDE activation induced by activated transducin lowers the cGMP binding affinity for noncatalytic sites on PDE and accelerates the dissociation of cGMP from these sites. These sites can exist in three states: high affinity (Kd = 60 nM) for the nonactivated PDE, intermediate affinity (Kd ~ 180 nM) when the enzyme is activated in a complex with transducin, and low affinity (Kd > 1 μM) when transducin physically removes the inhibitory subunits of PDE from the PDE catalytic subunits. Activation of PDE by transducin causes a 10-fold increase in the rate of cGMP dissociation from one of the two noncatalytic sites; physical removal of the inhibitory subunits from the PDE catalytic subunits further accelerates the cGMP dissociation rate from both sites >50-fold. Because PDE molecules lacking bound cGMP inactivate more rapidly, this suggests that a prolonged cGMP decrease may act as a negative feedback regulator to generate the faster, smaller photoresponses characteristic of light-adapted photoreceptors.

The chain of events responsible for visual excitation in retinal rod photoreceptors is well documented (reviewed in refs. 1–9). Photoc excitation of rhodopsin results in activation of transducin, the heterotrimeric guanine nucleotide-binding protein (G protein) of photoreceptor cells, which then binds and activates its effector enzyme, cGMP phosphodiesterase (PDE). The acceleration of PDE activity results in a drop in the cytoplasmic concentration of cGMP, which causes the closure of cGMP-gated plasma membrane cationic channels and cell hyperpolarization. The visual excitation cascade is inactivated by several processes that serve to restore the dark-adapted condition. These events include phosphorylation of rhodopsin by rhodopsin kinase and binding of arrestin to phosphorylated rhodopsin, hydrolysis of GTP on transducin by its intrinsic GTPase activity, restoration of PDE inhibition by its γ subunits, and resynthesis of cGMP by guanylate cyclase.

Nonactivated rod PDE is a heterotrimer consisting of two similar α and β catalytic subunits and two identical γ subunits which serve as a protein inhibitor of the enzyme (reviewed in refs. 2, 5, and 6). Each of the α and β subunits contains one catalytic site for cGMP hydrolysis as well as one or, probably, two noncatalytic cGMP binding sites (Fig. 1A; refs. 7–10). The inhibitory constraint of the PDE γ subunits is released when activated transducin α subunit (αGTP) binds to the PDE heterotrimer and either displaces PDE γ subunits or causes their physical removal from the PDE catalytic subunits (11–17). PDE inactivation and reassociation of the holoenzyme (αGTPγ) results when transducin becomes inactivated following GTP hydrolysis.

PDE (18, 19), and specifically its inhibitory γ subunit (18, 20), can act as a GTPase-activating protein to accelerate the hydrolysis of GTP bound to activated transducin. Hence, PDE appears capable of regulating its activated lifetime. Furthermore, studies with amphibian rod outer segments (ROS) showed that the mechanism of PDE activation by transducin, as well as the extent of GTPase stimulation, depends on the state of occupancy of cGMP on noncatalytic PDE binding sites (12, 18). When the sites are occupied by cGMP, transducin activation results in formation of a complex of αGTP and activated PDE αβP2 holoenzyme (Fig. 1B); in this case, the inhibitory γ subunits are displaced from their site of inhibition but do not dissociate from the catalytic αβ subunits. Transducin GTPase activity in this case is relatively slow, and PDE stays active longer. When the cGMP binding sites are empty, activated transducin physically removes PDE γ subunits from the catalytic subunits (Fig. 1C), and the γ subunits now maximally accelerate GTPase activity. In this model, binding of cGMP to the noncatalytic cGMP binding sites serves to regulate the lifetime of activated transducin, and hence the lifetime of activated PDE. This negative feedback scheme could represent an element of the biochemical mechanism of photoreceptor light adaptation wherein sustained photoactivation of the transduction cascade results in smaller and faster photoresponses to flashes of light.

To examine this hypothesis, we have studied the dynamics of cGMP binding and dissociation at the PDE noncatalytic sites to determine (i) whether this binding occurs in the physiological range of cGMP concentration changes, (ii) whether the cGMP dissociation occurs on the time scale of photoreceptor light adaptation, and (iii) whether PDE γ subunits can directly influence cGMP binding to PDE noncatalytic sites. We find that PDE activation lowers the binding affinity of cGMP and accelerates the dissociation kinetics of these sites via changes in the association of the PDE γ subunit to the catalytic subunits. These data support a model in which a sustained decrease in cGMP concentration that occurs during continuous illumination will promote the release of bound cGMP from noncatalytic sites on activated PDE. The time scale of cGMP dissociation from PDE, tens of seconds, correlates with the onset of some aspects of rod light adaptation observed in physiological studies.

Abbreviations: PDE, cGMP phosphodiesterase; ROS, rod outer segment(s); GTP[γS], guanosine 5'-[γ-thio]triphosphate; αGTP, activated α subunit of transducin.

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FIG. 1. Model of the reciprocal regulation of noncatalytic cGMP binding sites, PDE γ subunits, and catalytic activity of rod photoreceptor PDE. (A) In the dark, nonactivated PDE consists of two inhibitory γ subunits which inhibit catalytic activity at the active site (V-shaped notch) on the α and β subunits; the regulatory cGMP (G) binding sites are represented in their highest-affinity conformation. (B) Activated transducin α subunit (αγ-GTP) partially displaces the PDE γ subunits, completely relieving inhibition of catalysis and lowering somewhat the cGMP binding affinity at noncatalytic sites. (C) The activated PDE αβ dimer has the same catalytic activity as in B, but the complete dissociation of γ subunits results in a large reduction in cGMP binding affinity at regulatory sites (see text).

MATERIALS AND METHODS

Materials and Solutions. [8-3H]cGMP was purchased from DuPont/NEN and Percoll from Pharmacia LKB. Zaprinast was a gift of May and Baker (Dagenham, U.K.). Other chemicals were obtained from Sigma. The Ringer’s solution used to isolate rod photoreceptors contained 105 mM NaCl, 2 mM KCl, 2 mM MgCl2, 1 mM CaCl2, and 10 mM Hepes (pH 7.5). The “intracellular” medium used in all experiments contained 77 mM KCl, 35 mM NaCl, 2.0 mM MgCl2, 1.0 mM dithiothreitol, 1.0 mM CaCl2, 1.16 mM EGTA, 10 mM Hepes (pH 7.5, pCa 6.7).

Preparation of ROS Membranes. Live bullfrogs (Rana catesbeiana or Rana grylio) were purchased from commercial sources and maintained with feeding on a 12-h light, 12-h dark cycle at least 2 weeks before use. Isolation of ROS was done in the dark under infrared illumination, as described (21). In brief, animals were decapitated, and retinas were removed, placed into Ringer’s solution containing 5% Percoll, and shaken gently to detach ROS. The isolated ROS were then purifed on a Percoll gradient. Intact ROS were then centrifuged free of Percoll in Ringer’s solution and then disrupted in a motorized Potter–Elvehjem homogenizer (creating a membrane suspension with no structure detectable by light microscopy). The ROS membrane preparation was incubated for 30 min at room temperature to deplete endogenous nucleotides, including bound cGMP (22). The rhodopsin concentration was determined spectrophotometrically (23).

Preparation of Nonactivated PDE, αγ-Guanosine 5’(γ-thio)Triphosphate (GTPγS)-Activated PDE, and Activated PDEαβ Dimers. The finding that the PDE is the only protein in amphibian ROS which binds cGMP with high affinity (i.e., in the submicromolar range; refs. 7 and 22) permitted us to study the dynamics of PDE noncatalytic sites under relatively physiological conditions in unfraccionated ROS membrane preparations. We verified this premise by purifying amphibian PDE to homogeneity (12) and determining that the high-affinity cGMP binding could be solely ascribed to PDE (apparent equilibrium dissociation constant (Kd) = 60 nM, maximum site density (Bmax) = 2 cGMP per PDE holoenzyme; V.Y.A. and R.H.C., unpublished observations). Lower-affinity cGMP binding sites present in ROS (22) will not bind [3H]cGMP to any significant extent under the experimental conditions (<1 μM cGMP) used in this study, nor would they be easily detected by the filter binding assay used for our cGMP binding determinations.

The PDE content of ROS membrane preparations was calculated by assuming a ratio for PDE/rhodopsin of 1:270 (24). To ensure that ROS membrane preparations contained sufficient PDE γ subunits to maintain nonactivated PDE in its heterotetrameric form (αβγγ), =1 additional PDE γ subunit per PDE molecule was routinely added to ROS membrane preparations before cGMP addition. To prepare PDE activated by transducin, a poorly hydrolyzable analog of GTP, GTPγS, was added to illuminated ROS membranes in sufficient amounts to activate all transducin (~2-fold molar excess).

Membranes containing PDE depleted of most of the γ subunits were prepared as described (12, 15). In brief, illuminated ROS suspensions containing 20 μM rhodopsin were incubated for 1 min with 10 μM GTP in intracellular medium and then diluted 10-fold with the same medium and immediately centrifuged at 40,000 × g. After this procedure most of the PDE γ subunit was found in the supernatant in a soluble complex with transducin. The membrane pellet was washed once to remove the residual GTP and finally resuspended in a Potter–Elvehjem homogenizer. A combination of PDE γ quantitation by the Western blot analysis along with direct determinations of PDE hydrolytic activity (described in detail in ref. 12) showed that 70–80% of the PDE in this preparation was present as the αβ dimer, while the rest retained one or two γ subunits.

Preparation of Recombinant PDE γ Subunit. Recombinant PDE γ subunit was purified by a combination of cation-exchange and reverse-phase chromatography (25) from Escherichia coli BL21(DE3) transformed with an expression plasmid containing a synthetic PDE γ subunit gene. The gene was originally prepared as a fusion protein (25). The coding sequence for PDE γ subunit was subcloned into the expression vector PET-11a (Novagen) by J. Sondek (Yale University). The concentration of PDE γ subunit was determined spectrophotometrically at 280 nm by using a molar extinction coefficient of 7100.

Filter Binding Assay of cGMP Binding. Nucleotide-depleted ROS membrane preparations described above were mixed with an equal volume of intracellular medium containing [3H]cGMP and a type V-specific inhibitor of PDE, zaprinast (26). Zaprinast was prepared as a 400 mM stock solution in 1-methyl-2-pyrrolidinone and diluted to the final concentration of 1 mM in the intracellular medium. Simultaneous measurements of PDE activity during the filter binding assay (12, 22) showed that <10% of the [3H]cGMP was hydrolyzed under all experimental conditions. Zaprinast (1 mM) was found to have no discernible effect on cGMP binding to PDE noncatalytic sites (K. R. McCarthy and R.H.C., unpublished observations). For experiments in which GTPγS was used, we ascertained that undetectable amounts of cGMP were being synthesized from GTPγS via endogenous guanylate cyclase activity in ROS.

For equilibrium binding measurements, incubations proceeded for 2 min at room temperature, and then 25-μ
portions were added to prewet nitrocellulose filters (Millipore, HAWP 025) on a vacuum manifold system. The filters were then rinsed with three 1-ml portions of ice-cold intracellular medium. Sample application and filter washing were completed within 4 sec. Nonspecific binding (<1% of the total dpm) was determined as described (22). Filter-bound radioactivity was measured in a scintillation counter and the data were analyzed with the nonlinear, weighted least-squares curve-fitting programs EBDA, LIGAND, and KINETIC (27) and SIGMAPLOT (Jandel, Corte Madera, CA).

RESULTS

To characterize the noncatalytic sites in both inactive and activated PDE, we used three different ROS preparations (see Materials and Methods) which are shown schematically in Fig. 1. The first is a suspension of homogenized ROS containing nonactivated PDE holoenzyme (Fig. 1A). The second preparation is the same ROS preparation to which GTP[γS] has been added to persistently activate transducin. In the presence of cGMP the activated PDE heterotetramer exists in a complex with two molecules of α2–GTP[γS] (Fig. 1B). The third preparation consists of homogenized ROS suspensions depleted of most of the PDE γ subunits; hence, PDE exists primarily as the activated αβ dimer (Fig. 1C).

PDE Activation Results in a Decrease in the Affinity of Noncatalytic cGMP Binding Sites. Equilibrium binding of cGMP to nonactivated PDE exhibited a KD of 57 ± 7 nM (mean ± SD) and a Bmax of 0.006 ± 0.001 mol of cGMP per mol of rhodopsin (Fig. 2, top curve), in agreement with a previous estimate (22). The maximum amount of cGMP binding is equivalent to 1.6 ± 0.3 mol of cGMP bound per mol of PDE holoenzyme, consistent with the binding stoichiometry of 2 mol of cGMP per mol of holoenzyme observed in bovine rod PDE-β),

Activation of PDE by α2–GTP[γS] reduced the apparent binding affinity by a factor of 3 (Fig. 2, middle curve), with only a minor reduction in Bmax. This particular experiment does not represent a true equilibrium binding situation, because addition of increasing amounts of cGMP to a ROS preparation containing GTP[γS] should enhance binding of PDE γ subunits to PDE αβ dimers, which in turn should enhance cGMP binding affinity at noncatalytic sites on PDE. PDE αβ dimers were prepared by physical removal of 70–80% of the inhibitory γ subunit, an 80% reduction in the maximum extent of [3H]cGMP binding was observed (Fig. 2, bottom curve). The high-affinity CGMP binding sites had not been destroyed, since the addition of exogenous PDE γ subunit restored high-affinity cGMP binding in a dose-dependent manner (see below). Rather, we conclude that the binding affinity of these sites has been reduced beyond the range of detection of the filter binding assay (KD > 1 μM).

[Low-affinity CGMP binding in this preparation could be detected by a centrifugal separation technique (22, 28), but the KD in this case was too high (>7 μM) for reliable determinations.] These results are consistent with an earlier observation of a reduction of CGMP binding upon activation of frog PDE (8). The small amount of high-affinity CGMP binding observed in the bottom curve of Fig. 2 is consistent with residual PDE holoenzyme present in this PDE αβ dimer preparation (see Materials and Methods).

PDE γ Subunits Regulate the Binding Affinity of CGMP to Noncatalytic Sites. The above results suggest a reciprocal relationship between binding of PDE γ subunit to PDE αβ dimers and the binding affinity of CGMP to the noncatalytic binding sites of PDE. To test this possibility, we first prepared ROS depleted of PDE γ subunits to obtain a preparation composed primarily of PDE αβ dimers. Addition of increasing amounts of PDE γ subunits to this preparation resulted in a progressive restoration of high-affinity CGMP binding (Fig. 3). Full restoration of high-affinity CGMP binding was observed when two to three PDE γ subunits per PDE αβ dimer were added to this activated PDE preparation. Because each catalytic subunit has one high-affinity noncatalytic CGMP binding site, this indicates that binding of PDE γ subunit to both catalytic subunits is necessary to completely convert both noncatalytic sites from low to high affinity.

PDE Activation Accelerates CGMP Dissociation from the Noncatalytic Sites. Since the cytoplasmic CGMP concentration in rod photoreceptors is estimated to be 1–5 μM and to decrease upon illumination to levels similar to those studied in Fig. 2 (1), one physiological consequence of photoreceptor illumination may be a net dissociation of CGMP from noncatalytic binding sites on PDE. However, the extent of CGMP dissociation will most likely depend on the (i) duration of the light stimulus, (ii) the state of activation of PDE, and (iii) the dissociation rate constant for CGMP release from PDE noncatalytic sites. To determine whether CGMP dissociation occurred on a physiological time scale, we first equilibrated the PDE preparations depicted in Fig. 1 with micromolar levels of [3H]cGMP to occupy noncatalytic binding sites and
then monitored the rate of its displacement after adding an excess of unlabeled cGMP. PDE activation increased the rate of [3H]cGMP dissociation from minutes to seconds (Fig. 4). Nonactivated PDE holoenzyme released [3H]cGMP in a slow, monotonic fashion (t1/2 = 5.8 min; Fig. 4, top curve). The PDE holoenzyme in a complex with activated transducin showed accelerated, biphasic dissociation kinetics (Fig. 4, middle curve), consistent with two classes of binding sites present in approximately equal concentration but differing 10-fold in dissociation rates (t1/2 = 25 sec and 4.4 min). This result suggests that the high-affinity noncatalytic cGMP binding sites on PDE α and β subunits are not equivalent, and may explain why the decrease in total cGMP concentration in intact photoreceptors is always <50% during the first few tens of seconds of continuous illumination (29–32). The dissociation of bound cGMP from the PDE αβ dimer (Fig. 4, bottom curve) was accelerated >50-fold compared with nonactivated PDE holoenzyme. Approximately 80% of the total cGMP dissociated more rapidly than the time resolution of our method (∼4 sec); the 20% of the cGMP dissociating with a slow rate may be attributed to the fraction of inactive PDE still retaining γ subunits.

**DISCUSSION**

Our observations can be summarized by the model depicted in Fig. 1. In the absence of illumination, PDE is not activated and the noncatalytic cGMP binding sites are present in their highest-affinity conformation (Fig. 1A). Tight binding of cGMP ensures tight binding of the inhibitory γ subunits and the low basal level of PDE catalytic activity characteristic of dark-adapted photoreceptors (12). The binding affinity for cGMP at the noncatalytic sites is lowered severalfold when activated transducin partially displaces PDE γ subunits from the PDE catalytic subunits (Fig. 1B); under this condition, we predict the relatively rapid dissociation of half of the bound cGMP on PDE. When PDE γ completely dissociates from PDE αβ (Fig. 1C), the binding affinity of cGMP is greatly reduced, and any bound cGMP is expected to dissociate very rapidly from both noncatalytic binding sites on the αβ dimer.

This reciprocal regulation of PDE by its inhibitory γ subunit and by cGMP may play a role in light adaptation of amphibian rod photoreceptors. Consider first the response of a dark-adapted rod photoreceptor to a flash of light. Transient activation of PDE (from A to B in Fig. 1) will cause a temporary drop in cytoplasmic cGMP concentration but little dissociation of bound cGMP on the time scale of the flash response. Because cGMP remains bound to the noncatalytic sites, PDE γ subunits remain bound to PDE αβ (12), and hence transducin activation is relatively long-lived (18). This accounts for the larger, slower photoresponses characteristic of dark-adapted rod photoreceptors. Continuous illumination sufficient to activate most of the PDE will cause a sustained drop in cytoplasmic cGMP levels. In response to this cGMP decrease, we predict a gradual transition of PDE from B to C (Fig. 1) as dissociation of bound cGMP occurs over tens of seconds. The form in Fig. 1C inactivates more rapidly than that in Fig. 1B because the ability of dissociated PDE γ subunit to activate transducin GTPase is more pronounced than that of PDE holoenzyme (18). As a result the number of activated PDE molecules is expected to decrease with sustained continuous illumination, and partial restoration of free cytoplasmic cGMP levels and of the cGMP-gated ionic conductance is predicted. If this light-adapted photoreceptor is now exposed to a flash of light, a smaller and faster photoresponse is predicted; the amplitude and kinetics of the flash response might be determined by the relative abundance in...
this photoreceptor of the three forms of PDE depicted in Fig. 1. Thus, we propose that the noncatalytic cGMP-binding sites on PDE serve as a sensor of the level of background illumination in order to regulate desensitization of the cGMP transduction pathway. This hypothesis about the role of cGMP in photoreceptor light adaptation is consistent with electrophysiological evidence which demonstrates a gradual acceleration of the recovery of the photoresponse when amphibian rod photoreceptors are exposed to continuous illumination on the time scale of tens of seconds (33, 34). Our proposed mechanism is distinct from, but complementary to, those aspects of light adaptation that are believed to be regulated by calcium (reviewed in refs. 1–4).

It is important to note that our model is based on two assumptions. The first is that the GTPase activity of transducin, rather than some other mechanism (see ref. 35), determines the rate of PDE inactivation during the recovery phase of the photoresponse. The second assumption is that the reassociation of PDE γ with PDE αβ occurs more rapidly than the transducin GTPase reaction. Recent experiments supporting these assumptions have demonstrated that the rates of PDE turnover and transducin GTPase in disrupted frog ROS correlate well both in the presence and in the absence of cGMP binding in the PDE noncatalytic sites (V.Y.A., C. L. Dumke and M.D.B., unpublished work).

One interesting point which is not addressed by the experiments in this paper is the exact sequence of events which occur during the transition from B to C in Fig. 1. Our data do not allow us to distinguish whether cGMP dissociation from noncatalytic sites precedes or follows the dissociation of the α–GTP–PDE γ complex from PDE αβ.

The results shown in Fig. 4 for α–GTP[y]S-activated PDE indicate that the two noncatalytic cGMP binding sites on PDE do not dissociate equally rapidly under certain conditions. We cannot distinguish at present whether the cGMP binding sites on the α and β subunits are intrinsically different or whether dissociation of one cGMP molecule from the holoenzyme allosterically induces a reduced cGMP dissociation from the other noncatalytic site. The observation that the total cellular cGMP concentration seldom drops below 50% of its dark-adapted value even after prolonged illumination (29–32), along with the fact that the high-affinity cGMP site density in ROS is practically equal to the total cGMP concentration, suggests that one noncatalytic cGMP binding site on PDE may tightly bind half of the total cellular cGMP even after sustained photoreceptor stimulation. This suggests that dissociation of only one cGMP molecule from transducin-activated PDE may be sufficient to induce the GTPase-activating activity of PDE.

Our results demonstrate that the dynamics of cGMP binding to noncatalytic binding sites on PDE occur over a range of cGMP concentrations similar to the predicted cytoplasmic free cGMP levels in living rod photoreceptors. Furthermore, while changes in the site occupancy upon PDE activation are too slow to be involved in visual excitation, the dissociation kinetics do correlate with some aspects of light adaptation (as judged from electrophysiological studies; refs. 33 and 34). Our results, combined with previous observations (12, 18, 36), suggest that the light-induced decrease in cGMP levels in amphibian photoreceptors feeds back on the transduction cascade to decrease the lifetime of activated PDE and thus causes desensitization of visual excitation during prolonged stimulation. The control of cGMP concentration in retinal photoreceptors by means of allosteric interaction with its effector enzyme may represent a desensitization mechanism that may be of general importance in other signal transduction systems employing second messengers.

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