Communication

The γ Subunit of Rod cGMP-Phosphodiesterase Blocks the Enzyme Catalytic Site*

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Alexey E. Granovsky, Michael Natochin, and Nikolai O. Artemyev

From the Department of Physiology and Biophysics, University of Iowa College of Medicine, Iowa City, Iowa 52242

Cyclic GMP phosphodiesterase (PDE) is the effector enzyme in the visual transduction cascade of vertebrate photoreceptor cells. In the dark, the activity of the enzyme α and β subunits (PDE) is inhibited by two γ subunits (Pγ). Previous results have established that approximately 5–7 C-terminal residues of Pγ comprise the inhibitory domain. To study the interaction between the Pγ C-terminal region and Pβ, the Pγ mutant (Cys68Ser, and the last 4 C-terminal residues replaced with cysteine, Pγ1–83Cys) was labeled with the fluorescent probe 3-(bromoacetyl)-7-diethylaminocoumarin (BC) at the cysteine residue (Pγ1–83BC). Pγ1–83BC was a more potent inhibitor of PDE activity than the unlabeled mutant, suggesting that the fluorescent probe in part substitutes for the Pγ C terminus in PDE inhibition. HolopDE (PDEγ–2) had no effect on the Pγ1–83BC fluorescence, but the addition of Pββ to Pγ1–83BC resulted in an approximately 8-fold maximal fluorescence increase. A Kd for the Pγ1–83BC-Pββ interaction was 4.0 ± 0.5 nm. Zaprinast, a specific competitive inhibitor of PDE, effectively displaced the Pγ1–83BC C terminus from its binding site on Pββ (IC50 = 0.9 mM). cGMP and its analogs, 8-Br-cGMP and 2′-butyryl-cGMP, also competed with the Pγ1–83C terminus for binding to Pββ. Our results provide new insight into the mechanism of PDE inhibition by showing that Pγ blocks the binding of cGMP to the PDE catalytic site.

In the visual transduction cascade of rod photoreceptor cells, the photoexcited visual receptor, rhodopsin, interacts with the rod G-protein, transducin, and stimulates the exchange of GTP for bound GDP. The GTP-bound α subunit of transducin dissociates from rhodopsin and the transducin βγ subunits and activates the effector enzyme, cGMP phosphodiesterase (PDE), by relieving the inhibitory constraint imposed by two identical inhibitory subunits of PDE (Pγ) on the enzyme αβ catalytic subunits (PDE). Insights into the Pγ-Pβ interaction are critical for understanding the mechanisms of PDE inhibition by Pγ and PDE activation by transducin. Approximately 5–7 C-terminal amino acid residues of Pγ are involved in the inhibitory interaction with Pββ (5–9). Recently, using a cross-linking approach we have identified a site on Pγ for binding of the Pγ C terminus as a region Pγ751–763 within the PDE catalytic domain (10). The finding suggests that the Pγ C terminus either occupies the site for binding and catalysis of cGMP or induces local conformational changes of the PDE catalytic site that block cGMP hydrolysis. Here, we study the interaction between the C terminus of Pγ and Pββ using a novel fluorescence assay to elucidate the mechanism of PDE inhibition by Pγ.

EXPERIMENTAL PROCEDURES

Materials—cGMP was obtained from Boehringer Mannheim. 3-(bromoacetyl)-7-diethylaminocoumarin (BC) was purchased from Molecular Probes. Bovine rod outer segment (ROS) membranes were prepared by the method of Papaster and Dreyer (11). PDE was extracted from ROS membranes as described in Ref. 12. PDE and trypsin-activated PDE (tPDE) were prepared and purified as described previously (6). The purified proteins were kept in 40% glycerol at −20 °C. The Pγ subunit was expressed in Escherichia coli and purified as described in Ref. 9. The Pγ mutants PγCys68Ser and Pγ1–83Cys (Cys68Ser and the last 4 C-terminal residues, Tyr-Gly-Ile-Ile, replaced with a single cysteine) were obtained as described in Ref. 10.

Preparation of Trypsin-activated PDE, Pγ and Pγ Mutants—Bovine rod outer segment (ROS) membranes were prepared by the method of Papaster and Dreyer (11). PDE was extracted from ROS membranes as described in Ref. 12. PDE and trypsin-activated PDE (tPDE) were prepared and purified as described previously (6). The purified proteins were kept in 40% glycerol at −20 °C.

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Fluorescence Spectrophotometer (Hitachi) in 1 ml of 80 mM Tris-HCl (pH 7.6) containing 100 mM NaCl and purified by reversed-phase HPLC on a C-4 column Microsorb-MW (Rainin) using a 0–100% gradient of acetonitrile, 0.1% trifluoroacetic acid. The preparations of Pγ1–83BC and PγBC contained no free BC. Using ε445 = 53,000 for BC, the molar ratio of BC incorporation into Pγ and Pγ1–83BC was greater than 0.5 mol/mol. Pγ1–83BC was prepared by labeling of peptide Pγ24–45Cys and purified as described in Ref. 13. A Pγ mutant, PγCys68Ser, and a peptide, Pγ24–45, that contain no cysteine were not derivatized with BC under similar conditions, suggesting the selectivity of cysteine labeling.

Fluorescent Assays—Fluorescent assays were performed on a F-2000 Fluorescence Spectrophotometer (Hitachi) in 1 ml of 80 mM Tris-HCl buffer (pH 7.6) containing 2 mM MgCl2. Fluorescence of Pγ1–83BC, PγBC, or Pγ24–45BC was monitored with excitation at 445 nm and emission at 495 nm. The assays were carried out at equilibrium, which was typically reached less than 3 s after mixing of the components. The concentration of labeled polypeptides was determined using ε445 = 53,000. Where indicated, zaprinast was added from 1 mM stock solution to an assay buffer. The KM values in Figs. 2 and 4 were calculated by fitting the data to Equation 1,

\[ F / F_0 = 1 + (F / F_{max} - 1) \times X / (K_m + X) \]

where \( F_0 \) is a basal fluorescence of Pde, \( F / F_{max} \) is the maximal relative increase of fluorescence, and \( X \) is a concentration of free tPDE.

The IC50 values in Figs. 3 and 5 were calculated by fitting the data to the one site competition equation with variable slope.
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1–83BC and P-ab

terminus and P-ab containing 100 mM NaCl and 1 mM MgCl2. The reaction was initiated by

Ki with an apparent

were 215 and 480 1/g

83BC increased the fluorescence of the probe many fold in a

1g

In agreement with previous data (17), the addition of P-ab

can substitute for the P-ab 1g P-ab C-terminal residues, Gly-Ile-Ile, are replaced with the fluorescent probe BC, and substantially restores the inhibitory potential of the P-ab 1–83Cys mutant. Furthermore, the apparent K_i for tPDE inhibition by P-ab 1–83BC was influenced by the cGMP concentration in the assay. Decrease in cGMP concentration from 3 to 0.3 mM resulted in a reduction of the apparent K_i value from 5.4 to 2.1 nM (Fig. 1A). In control experiments, labeling of wild-type P-C with BC at Cys386 did not notably affect the ability of P-C to inhibit tPDE (not shown). Both P-C and P-C BC fully inhibited tPDE with K_i values less than 0.25 nM (not shown), and the K_i values were not affected by the concentration of cGMP in the assay (not shown). Next we examined effects of P-C 1–83BC and P-C on K_m values of cGMP hydrolysis by tPDE. A K_m of 90 μM was calculated from the Michaelis-Menten plot for tPDE (Fig. 1B). This K_m is consistent with earlier estimates (17). In the presence of 3.5 and 8 nM of P-C 1–83BC, the apparent K_m values were 215 and 480 μM, and V_max values were 95 and 80%, respectively (Fig. 1B). The inhibitory interaction between P-C 1–83BC and P-ab was not purely competitive with cGMP, because the V_max values were also affected by P-C 1–83BC (Fig. 1B). In agreement with previous data (17), the addition of P-C did not significantly change the K_m for cGMP hydrolysis by tPDE (not shown).

Binding of P-C 1–83BC to P-ab—Addition of tPDE to P-C 1–83BC increased the fluorescence of the probe many fold in a dose-dependent manner (Fig. 2). The binding curve shows a single class of binding sites with K_d = 4.0 ± 0.5 nM and a maximal fluorescence enhancement F/F_0 = 8.4 ± 0.2. No change in the fluorescence of P-C 1–83BC was detected upon the addition of holodPDE or tPDE reconstituted with P-C. Furthermore, addition of P-C to the P-C 1–83BC-P-ab complex readily reduced fluorescence to a basal F_0 level, suggesting that the fluorescent increase reflects a specific interaction between P-C and P-ab. The curves (●) or in the presence of 3.5 (●) and 8 nM of total P-C 1–83BC (●) were determined at varying concentrations of cGMP. The curves (●, K_m = 90 ± 10 μM; ■, K_m = 215 ± 23 μM, V_max = 95%; and ○, K_m = 480 ± 76 μM, V_max = 80%) fit the Michaelis-Menten equation with r values of 0.99, 0.99, and 0.98, respectively.

Effect of Zaprinast on the Interaction between P-C 1–83BC and P-ab—Zaprinast is a well characterized competitive inhibitor of photoreceptor PDEs and cGMP-binding, cGMP-specific PDE (19, 20). We have investigated effects of zaprinast on the interaction between P-C 1–83BC and P-ab. Zaprinast had no effect on the basal fluorescence of P-C 1–83BC. Addition of increasing concentrations of zaprinast resulted in a complete reversal of the fluorescent enhancement of P-C 1–83BC bound to P-ab (IC_50 of 0.9 μM) (Fig. 3). Zaprinast was effective in blocking the P-C 1–83BC-P-ab interaction within a pharmacologically relevant range of concentrations. A K_d value of 140 nM for zaprinast binding to rod PDE catalytic sites was calculated based on inhibition of PDE activity by zaprinast (19). Our assay does not allow us to calculate the true K_d value for the zapri-
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The decrease in fluorescence of the Pγ1–83BC–tPDE complex had no effect on the fluorescence increase of Pγ24–45BC caused by its binding to tPDE (not shown). At a higher concentration (10 μM), zaprinast reduced the maximal increase in fluorescence of the Pγ24–45BC–Paβ complex by only ~15% without affecting the Kd of zaprinast (Fig. 4) approached the Kd for the Pγ24–45BC–Paβ complex. This supports the notion that zaprinast competes with the Pγ1–83BC C terminus for binding to Paβα and does not affect the interaction of the polycationic region, Pγ24–45, with Paβα.

Effects of cGMP and Its Analogs on the Interaction between Pγ1–83BC and Paβα—The relative potency of zaprinast in competition with the Pγ1–83BC C terminus for binding to Paβα indicated that cGMP and its analogs might be effective as well. Effects of cGMP on the Pγ1–83BC–Paβα interaction were tested in the presence of Mg2+, Mg2+ participates in binding of cGMP to the PDE catalytic site and is critical for cGMP hydrolysis. In the presence of Mg2+, cGMP reversed the fluorescent enhancement of Pγ1–83BC bound to Paβα with an apparent IC50 of 0.77 nM (Fig. 5A). Presumably, cGMP would be significantly more effective in the absence of cGMP hydrolysis. In the presence of EDTA, cGMP was ~2.5-fold less potent (IC50 of 1.9 mM, Fig. 5A). Because cGMP is not hydrolyzed in the presence of EDTA, the data suggest that Mg2+ enhances affinity of cGMP for the catalytic site by more than 2.5-fold. We also tested two cGMP analogs, 8-Br-cGMP and 2′-butyrylcGMP, because they block ROS-PDE activity with a relatively high affinity (21). Both cGMP analogs competed with the Pγ1–83BC C terminus for binding to Paβα (Fig. 5B). The IC50 values from the competition curves for 8-Br-cGMP and 2′-butyrylcGMP were 0.42 mM and 0.58 mM, respectively. Perhaps, an IC50 for 2′-butyrylcGMP was higher than an IC50 for 8-Br-cGMP because tPDE hydrolyzed 2′-butyrylcGMP with a rate of ~15% of cGMP hydrolysis, whereas 8-Br-cGMP was resistant to the hydrolysis (not shown).

To examine the effects of zaprinast on the apparent Kd of Pγ1–83BC binding to Paβα, the binding curves were obtained in the presence of different concentrations of zaprinast (Fig. 4). Increasing concentrations of zaprinast reduced the fluorescent enhancement of Pγ1–83BC by Paβα and increased the apparent Kd of the Pγ1–83BC binding to Paβα. The apparent Kd calculated from the binding curves in the presence of 2 and 4 μM of zaprinast were 14.7 ± 5.9 and 22.6 ± 1.3 nM, respectively. The decrease in fluorescence of the Pγ1–83BC–Paβα complex and the increase in apparent Kd values suggest that zaprinast competitively displaces the fluorescently labeled Pγ1–83BC C terminus from the binding pocket on Paβα. Dipyridamole, another potent competitive inhibitor of photoreceptor PDEs (19), was unsuitable for studies using our assay. Dipyridamole is highly fluorescent with maximal emission at 480 nm.

Effects of Zaprinast on the Interaction between Pγ24–45BC and Paβα—To investigate if zaprinast can compete for binding between the polycationic region of Pγ, Pγ24–45, and Paβα, we utilized an assay of interaction between a synthetic peptide, Pγ24–45Cys, labeled with BC and tPDE (13). Zaprinast at concentrations that completely reversed the fluorescent enhancement of the Pγ1–83BC–Paβα complex had no effect on the fluorescent increase of Pγ24–45BC caused by its binding to tPDE (not shown). At a higher concentration (10 μM), zaprinast reduced the maximal increase in fluorescence of the Pγ24–45BC–Paβ complex by only ~15% without affecting the Kd of zaprinast. The Kd values for Pγ24–45BC binding to Paβα in the presence of 10 μM of zaprinast or with no zaprinast added were indistinguishable (~26 nM) (not shown). Interestingly, the apparent Kd values for Pγ1–83BC binding to Paβα at higher concentrations of zaprinast (Fig. 4) approached the Kd for the Pγ24–45BC–Paβ complex. This supports the notion that zaprinast competes with the Pγ1–83BC C terminus for binding to Paβα and does not affect the interaction of the polycationic region, Pγ24–45, with Paβα.

FIG. 2. Binding of Pγ1–83BC to tPDE. The relative increase in fluorescence (F/Fo) of Pγ1–83BC (10 nM) was determined after the addition of increasing concentrations of tPDE and is plotted as a function of the free tPDE concentration. The relative increase in fluorescence (F/Fo) enhancement of Pγ1–83BC was determined after the addition of increasing concentrations of tPDE and is plotted as a function of the free tPDE concentration. The binding curve (Kd = 4.0 ± 0.5 nM, maximum F/Fo = 8.4 ± 0.2) fits the data with r = 0.98.

FIG. 3. Competition between Pγ1–83BC and zaprinast for binding to Paβα. tPDE (total concentration, 6 nM) was added to Pγ1–83BC (10 nM), and then the fluorescence was measured before and after the addition of increasing concentrations of zaprinast. The relative fluorescent change (F/Fo) is plotted as a function of zaprinast concentration. The curve (IC50 = 0.90 ± 0.02 μM and Hill slope = 1.1) fits the data with r = 0.98.

FIG. 4. Effect of zaprinast on apparent Kd for Pγ1–83BC binding to Paβα. Zaprinast was added to Pγ1–83BC (10 nM) at indicated concentrations, and then the relative increase in fluorescence (F/Fo) of Pγ1–83BC was determined after the addition of increasing concentrations of tPDE. The calculated apparent Kd values for Pγ1–83BC binding to tPDE in the presence of 0.5, 2 and 4 μM of zaprinast were 5.1 ± 0.7, 14.7 ± 0.9, and 22.6 ± 1.3 nM, respectively. ▲, 5 × 10−6 zaprinast; ▲, 2 × 10−6 zaprinast; ▼, 4 × 10−6 zaprinast.
the dark and for inactivation of the enzyme upon recovery of the photoreceptor cell from light stimulation. The Pγ subunits bind to Pαβ with very high affinity (Kd < 100 pm) (22). The high affinity of the Pγ-Pαβ interaction is provided by two major binding sites on Pγ, the central polycationic region, Pγ-24–45, and the C-terminal 5–7 amino acid residues (5–9). The main role of the Pγ-24–45 region is to enhance the affinity of Pγ interaction with Pαβ. The C terminus of Pγ is critical for PDE inhibition. Truncations of the Pγ-C-terminal residues lead to a loss of the Pγ inhibitory function (5, 8, 9). Peptides corresponding to the C-terminal region of Pγ can fully inhibit PDE activity (6, 7, 9). Recently, we have shown that the C-terminal region of Pγ binds within the catalytic domain of PDE (10). This finding raised the possibility that Pγ may inhibit PDE activity by physically blocking the binding site for cGMP. An alternative mechanism of PDE inhibition by Pγ would be a local conformational change of the PDE catalytic site that prevents cGMP hydrolysis. Standard analysis for competitive (noncompetitive) inhibition of cGMP hydrolysis may not discriminate between the two mechanisms because Pγ binds to Pαβ very tightly (Kd < 100 pm) compared with cGMP binding (Km for cGMP is within 17–80 μM range) (17, 19, 22). The large differences in affinity and very slow off-rates of Pγ from Pαβ (>10 min) (8, 22) may not allow cGMP to compete with Pγ bound to Pαβ. It has been shown previously that the addition of Pγ to PDE caused very little change in the apparent Km value (17). Indeed, in our experiments the Km value of 90 μM was unaffected by the addition of the Pγ.

To study the mechanism of PDE inhibition by Pγ, we developed an assay that reports binding of the Pγ C terminus to Pαβ. The assay utilizes a Pγ mutant with the C-terminal amino acid residues replaced with a fluorescent probe, BC. The fluorescently labeled mutant, Pγ-1–83BC, was a more potent inhibitor of PDE activity than the unlabeled mutant, Pγ-1–83Cys, suggesting that the probe interacts with the inhibitory pocket on Pαβ. Addition of Pγ-1–83BC to Pαβ led to a dose-dependent increase of the apparent Km values for cGMP hydrolysis. Binding of Pγ-1–83BC to Pαβ produced a large 8-fold increase in the probe fluorescence. Zaprinast, a specific competitive inhibitor of photoreceptor PDEs, effectively competed for the interaction between Pγ-1–83BC and Pαβ, but had no effect on binding of the polycationic region, Pγ-24–45, to Pαβ. Perhaps the fact that Pγ-1–83BC binds to Pαβ (Kd of 4 nm) less tightly than Pγ has helped zaprinast to compete for the Pγ-1–83BC-Pαβ interaction. cGMP and its analogs, 8-Br-cGMP and 2′-butyryl-cGMP, were also effective in blocking the interaction between the Pγ-1–83BC C terminus and Pαβ using the fluorescent assay. Effects of cGMP and its analogs on the Pγ-1–83BC binding to Pαβ cannot be attributed to the noncatalytic cGMP-binding sites of PDE, because bovine rod PDE contains two molecules of tightly bound cGMP with an extremely slow off-rate (τ1/2 = ~4 h) (23).

Overall, our data strongly suggest that Pγ inhibits PDE activity by physically blocking access of the substrate, cGMP, to the PDE catalytic site. The region of Pα, Pα-751–763, that interacts with the C terminus of Pγ (10) is adjacent to the NKD motif. In G-proteins, the NKD motif specifies binding of the GTP guanine ring (24, 25). Based on our results, it is likely that the NKD motif is involved in the binding of cGMP by photoreceptor PDEs.

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