Interaction of the γ-Subunit of Retinal Rod Outer Segment Phosphodiesterase with Transducin

USE OF SYNTHETIC PEPTIDES AS FUNCTIONAL PROBES

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There is considerable evidence which suggests that the γ-subunit of cGMP phosphodiesterase (PDEγ) is a multifunctional protein which may interact directly with both the catalytic subunits of PDE (PDEαβ) and the α-subunit of transducin (Ta) (Whalen, M., and Bittensky, M. (1989) Biochem. J. 259, 13–19; Griswold-Prenner, I., Young, J. H., Yamane, H. K., and Fung, B. K.-K. (1988) Invest. Ophthalmol. & Visual Sci. 29, (Suppl.) 218). To determine the region of interaction between the multifunctional PDEγ and Ta, and to determine the significance of this interaction, peptides corresponding to various regions of PDEγ were synthesized and tested for their ability to inhibit the GTPase activity of Ta. One of these peptides, PDEγ-3 (bovine amino acid residues 31–45), inhibited the GTPase activity of Ta with an I50 of 450 μM. The peptide (PDEγ-3) was found to inhibit the GTPase activity of Ta by inducing the binding of transducin to the rod outer segment membrane and by altering the GTP/GDP exchange. Analogs of PDEγ-3 were synthesized to determine the required structure of the PDEγ-3 region needed for the interaction of PDEγ with Ta. The results of these studies indicated that the removal of the positively charged amino acids or any of the potential hydrogen-bonding amino acids increased the I50 for the inhibition of the GTPase activity of Ta. Substitution of the hydrophobic amino acids had no effect. These results indicate the hydrophilic interactions may be essential for the binding of PDEγ to Ta and for the inhibition of the GTPase activity of Ta by PDEγ. The observed effects of PDEγ-3 on Ta and on PDE suggest that PDEγ is a multifunctional protein which may play more than one role in the deactivation of the retinal transduction cascade.

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Retinal rod outer segments contain a light-activated guanosine 3′,5′-cyclic monophosphate phosphodiesterase

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The abbreviations used are: PDE, retinal rod outer segment guanosine-3′,5′-cyclic monophosphate phosphodiesterase; PDEα, PDEβ, PDEγ, the three subunits of the PDE; GMPP(NH)P, guanylyl-5′-yl imidodiphosphate; GTPγS, guanosine 5′-O-(thiotriphosphate); β-ME, 2-mercaptoethanol; PMSP, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; RIA, radioimmunoassay; IRBP, interstitial retinol binding protein; BSA, bovine serum albumin; MOPS, 3-(N-morpholino)propanesulfonic acid; ROS, rod outer segment; HPLC, high-performance liquid chromatography.
been determined by protein and cDNA sequencing (9). We have previously reported using this sequence to synthesize peptides corresponding to various regions of the entire PDEy sequence (10). These peptides were tested for their ability to inhibit both PDEy and the GTPase activity of Ta. The results of these experiments indicated the peptide PDEy-3 (bovine amino acid residues 31-45) was the most effective peptide for inhibition of the GTPase activity of Ta. In this report, further data were presented which detail a possible mechanism by which the PDEy subunit inhibits the GTPase activity of Ta.

EXPERIMENTAL PROCEDURES

Materials—Fresh bovine eyes were obtained from a local slaughterhouse (Iowa Beef Packers, Emporia, KS). Buffers, guanosine 5'-triphosphate (GTP), 5'-guanylylimidodiphosphate (GMPP[NH]P), guanosine 5'-diphosphate (GDP), and other reagents were from Sigma. Guanosine 5'-O-(thiotriphosphate) (GTP[S]) was from Boehringer-Mannheim. [γ-32P]GTP (50 Ci/mmol), [35S]GTPyS (120 Ci/ mmol), and [3H]GDP (9 Ci/mmol) were from Du Pont-New England Nuclear. [8-3H]GMPP[NH]P (5 Ci/ml) and [8-3H]GDP (19 Ci/ml) were from ICN Radiochemicals. Carrier-free [35S] was from Amersham Corp. The iodoacetamidocarbonyl amino acids and their resinases were from VEGA Biochemicals, United States Biochemicals Corp., Cleveland, OH, or Sigma. Aquadisc III was from Behring Diagnostics. All other reagents for peptide synthesis were HPLC grade from Pierce, Fisher or Sigma, or Sequenal grade-from Pierce. All materials used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were from Bio-Rad or Sigma.

Preparation of Undepleted Rod Outer Segments—Rod outer segments were prepared by the method of Papermaster and Dreyer (21). Fresh bovine eyes were obtained from a local slaughterhouse within 60 min of slaughter. The eyes were transported in the dark and on ice. Retinas were removed under dim red light and stored without buffer at 70°C.

Typically, a preparation consisted of 50-100 dark-adapted retinas thawed over several hours in the dark. All purification steps were done in dim red light and on ice unless otherwise indicated. The retinas were suspended in ROS-No. 1 (5 mM Tris (pH 7.4), 2 mM MgCl₂, 65 mM NaCl, 1 mM 2-mercaptoethanol (β-ME), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and 34% sucrose (w/w (d = 1.15)) using 1 ml of buffer per retina. The retinas were shaken vigorously for 1 min, shearing the rod outer segments from the rod inner segments (21). The rod outer segments were isolated from the total homogenate at 4000 rpm for 4 min in a SS-34 rotor (Sorvall). The resulting pellet was resuspended in ROS-No. 1 (15 ml/tube) and recentrifuged as above.

The suspended rod outer segments were combined and diluted to three times their volume in ROS-No. 2 (10 mM Tris (pH 7.4), 1 mM MgCl₂, 1 mM β-ME, and 0.5 mM PMSF). The rod outer segments were pelleted at 16,000 rpm for 10 min in a SS-34 rotor, then resuspended in 10 ml of ROS-No. 3 (10 mM Tris (pH 7.4), 1 mM MgCl₂, 1 mM β-ME, 0.5 mM PMSF, and 26.3% sucrose (w/w)).

The suspended rod outer segments were layered (3 ml) on a discontinuous sucrose density gradient of 1 ml each of 1.11, 1.13, and 1.15 g/ml of sucrose in ROS-No. 2. The preparation was centrifuged at 24,000 rpm for 45 min in a SW-27.1 rotor (Beckman).

Purified rod outer segments were removed from the gradient at the 1.11-1.13 g/ml interface and washed with ROS-No. 7 (10 mM Tris (pH 7.4), 100 mM NaCl, 5 mM MgCl₂, 0.1 mM β-ME, and 1 mM PMSF). The rod outer segments were pelleted at 14,000 rpm for 15 min in a SS-34 rotor. This procedure was repeated 2 more times. The washed pellet was resuspended in GTPase buffer (10 mM 3-(4-morpholinyl)propane sulfonic acid (MOPS) (pH 7.4), 60 mM KCl, 2 mM MgCl₂, 1 mM β-ME, and 0.1 mM PMSF) for subsequent assays. The rod outer segment preparation was stored at -70°C and is referred to, herein, as an undepleted rod outer segment preparation.

To make PDEy-depleted rod outer segment membranes, the washed pellet obtained from the above procedure was resuspended in 50 ml of PDE extraction buffer (5 mM Tris (pH 7.4), 0.5 mM MgCl₂, 1 mM β-ME, and 0.1 mM PMSF) (21). The rod outer segment membranes were ruptured by passing the suspension through a 22-gauge needle three times. The suspended rod outer segment membranes were exposed to room light, on ice for 30 min, then pelleted at 16,000 rpm for 30 min in a SS-34 rotor. This procedure was repeated twice.

GTPase Assay—The GTPase assay was performed as previously described (22, 23). Typically, 4 µg of undepleted rod outer segments were used per 100 µl of GTPase buffer. Reactions were initiated by the addition of GTP to a final concentration of 20 µM GTP (20 µM GTP + 0.1 µCi of [γ-32P]GTP per assay tube). The reaction mixture was incubated under room light at 37°C for 20 min, then terminated by the addition of 1 ml of a solution containing 100 µM perchloric acid, 0.1 M KCl, H₃PO₄, and 1 ml of a solution containing 10 mM ammonium molybdate, 20 mM triethylenamine-HCl. The resulting precipitate was collected on a Whatman GF/A glass microfiber filter using a suction filtration apparatus. Each filter was washed with 50 µl of a solution containing 10 mM triethylenamine-HCl, 200 mM perchloric acid, 2.5 mM ammonium molybdate. The quantity of P can be determined by liquid scintillation counting. In the kinetic experiments, the GTP concentration was varied from 10 to 100 µM (final concentration). In some experiments, peptides were preincubated with the undepleted rod outer segment membranes for 5 min at 37°C prior to the addition of substrate.

PDE Activity—PDE activity was determined by the method of Thompson and Appleman (50). The final concentration of the reaction was 50 mM Tris (pH 7.4), 0.1 mM diethylenetriol, 0.5 mM MgCl₂, 1 µm leupeptin, 0.2 mM PMSF, 1 µm pepstatin. The eluate was concentrated with Aquadisc III and the PDEy was pre pared as described (51).

Prelabeling Ta with [35S]GTPyS—The α-subunit of transducin can be eluted from rod outer segment membranes and labeled with nonhydrolyzable analogs of GTP (24). Rather than using [γ-32P]GMP[NH]P (24), another nonhydrolyzable analog of GTP [γ-32P]GTPyS, was used. This analog was used so that subsequent dual labeling will be possible. PDEy-depleted rod outer segment membranes (25 µg) were diluted 1:1 (final volume = 2 ml) with GTPase buffer which contained 600 mM NaCl and 300 µM GTPyS (300 µM GTPyS + 1250 µCi of [γ-32P]GTPyS) and incubated on ice under room light for 12 h. The ROS membranes were then pelleted by centrifuging at 16,000 rpm for 30 min in an SS-34 rotor. The supernatant, containing [35S]GTPyS-labeled Ta (Ta-[35S]GTPyS), was analyzed by SDS-PAGE to check for purity. Transducin (10 µg/ml) was found to be greater than 85% pure when stained with Coomassie Blue. The partially purified Ta-[35S]GTPyS was then used in the nucleotide release assay described below.

Nucleotide Release Assay—Ten µl (10 µg) of the partially purified Ta-[35S]GTPyS was incubated in the presence of various quantities of the PDEy-3, in 100 µl of GTPase buffer for 10 min, at 37°C. Samples were filtered through a Millipore HAWP filter prewashed with GTPase buffer. The filter was then washed three times with 1 ml of GTPase buffer, and bound nucleotide was quantitated by liquid scintillation counting.

[8-3H]GMPP[NH]P Binding Assay—[8-3H]GMPP[NH]P binding was assayed by the method of Stryer et al. (24). An assay consisted of 50 µg of undepleted rod outer segment membranes in 100 µl of GMPP[NH]P-binding buffer (10 mM MOPS (pH 7.4), 60 mM KCl, 30 mM NaCl, 2 mM MgCl₂, 1 mM β-ME, 0.1 M PMSF, and 1 mg/ml BSA). The reaction was initiated by the addition of GMPP[NH]P (20 µM GMPP[NH]P) to 1 ml of a solution containing 10 µM of [8-3H]GMPP[NH]P (5Ci/mmol) per assay tube). The reaction mixture was incubated at room temperature under room light for 30 min. After 30 min, the reaction mixture was filtered through a 0.45-µm Millipore HAWP filter, using a suction filtration apparatus, and washed three times with 1 ml of GMPP[NH]P-binding buffer. The quantity of [8-3H]GMPP[NH]P bound to the filter was determined by liquid scintillation counting. In some experiments, undepleted rod outer segment membranes were preincubated with peptides for 5 min at room temperature prior to the addition of substrate.

[γ-32P]GTPyS/[3H]GDP Binding Assay—A typical assay consisted of 60 µg of dark adapted undepleted rod outer segment membranes (100 µl of GMPP[NH]P-binding buffer (10 mM MOPS (pH 7.4), 60 mM KCl, 30 mM NaCl, 2 mM MgCl₂, 1 mM β-ME, and 0.1 mM PMSF) and 1 ml of a solution containing 10 µM of [γ-32P]GTPyS or both nucleotides (1 µM final concentration each + 1 µCi each labeled nucleotide per assay tube) were added and the reaction initiated by illumination of the undepleted rod outer segment membranes under room light. The reaction mixture was incubated at 0°C for 5 min under room light.
The reaction was terminated by applying 85 μl of the reaction mixture to a 0.45-μm Millipore HAWP filter using a suction filtration apparatus. The filter was washed three times with GMPP(NH)P-binding buffer. The quantity of each labeled nucleotide bound to the filter was determined by liquid scintillation counting using a dual label program. In some experiments, undepleted rod outer segment membranes were preincubated with 50 nmol (2 mM) of the peptide, PDEγ-3 at 37 °C in the dark for 5 min prior to the addition of substrate.

Membrane Reconstitution Assay—Transducin was eluted from PDE-depleted rod outer segment membranes (22, 26) with 5 ml of ROS-NaOH (8.0 mM Tris pH 7.5, 0.1 mM β-ME, 100 mM PMSP) containing 40 μM GTP. The rod outer segment membranes were resuspended by passing them through a 22-gauge needle three times. The rod outer segment membranes were incubated on ice for 20 min and then pelleted by centrifugation for 30 min at 16,000 rpm in a SS-34 rotor. This procedure was repeated three more times, and the supernatants were combined to make a crude transducin preparation (about 85% pure). The rod outer segment membranes were washed six more times in the same manner as described above and the supernatants discarded. Both the crude transducin preparation and this rod outer segment membrane preparation were tested for binding by antipeptide antisera using the solid-phase radioimmunoassay (RIA) described below.

In the reconstitution assay, 17.5 μg of the depleted rod outer segment membrane preparation and 8 μg of the crude transducin preparation were incubated with and without 51 nmol (510 μM) of the peptide, PDEγ-3, and 40 μM GTP on a shaker at room temperature (150 μl total volume) for 100 μl. The rod outer segment membranes were pelleted by centrifugation in a microfuge (Fisher) for 2 min. The supernatants were removed and assayed using antipeptide antisera in a solid-phase RIA. Experiments included incubating 17.5 μg of the depleted rod outer segment preparation alone, and with and without 51 nmol of PDEγ-3, and incubating 8 μg of the crude transducin preparation alone, and with and without 51 nmol of PDEγ-3. Equivalent quantities of human lens membranes were used in place of the depleted rod outer segment membranes as negative controls for background subtraction in the solid-phase RIA. Lens membranes were prepared as described using the NaOH extraction method (27).

Solid-phase Radioimmunoassay—The solid-phase RIA was a modification of the method of Suter (28). Polyethylene tubes (12 × 75 mm) were filled with 0.5 ml of 0.2% glutaraldehyde in 0.1 mM sodium phosphate, pH 5.0, and shaken for 3 h at room temperature. The tubes were then washed three times with 0.1 mM sodium phosphate (pH 6.0). The supernatants from the above described experiments were diluted to 200 μl with 0.1 mM sodium phosphate (pH 8.0), added to each tube, and incubated at 37 °C for 2 h. The tubes were then washed three times with 0.15 M NaCl, 0.05% Tween 20, to block nonspecific binding, and one time with water. Five hundred microliters of 0.1 mM sodium phosphate, 0.9% NaCl, 0.05% Tween 20, 0.02% NaN3 (pH 7.2), was added and the tubes were shaken for 12 h at room temperature. The tubes were then washed three times with 10 mM Tris, 0.05% Tween 20 (pH 8.0), and two times with water. Five hundred microliters of 0.1 mM sodium phosphate, 0.9% NaCl, 0.05% Tween 20, 0.02% NaN3 (pH 7.2), containing 1 μl of 125I-labeled protein A/ml of buffer (1 × 10^6 cpm/tube), was added and the tubes were shaken for 1 h at room temperature. The tubes were washed twice with 10 mM Tris, 0.05% Tween 20 (pH 8.0), twice with water, and then counted in a γ counter (Beckman).

Peptide Synthesis and Purification—Peptides corresponding to 15-amino acid-long segments of bovine PDEγ were synthesized manually by the method of Hodges and Merrifield (14) as modified by Gorman (15) except that cleavage of the peptide from the resin and protecting groups was accomplished using HF in anhydrous trifluoroacetic acid (16) instead of acid. HF cleavage and purification of peptides was accomplished using high-performance liquid chromatography on a Vydac C-18 column using a 0-100% 0.1% trifluoroacetic acid/acetonitrile gradient in 0.1% trifluoroacetic acid/acetonitrile as described elsewhere (17). The peptide was lyophilized off, and the amino acids were dissolved in 100 μl of water. Aliquots of 10 μl were analyzed for amino acid content and were quantitated by comparison with known amino acid standards using a Shimadzu peak integrator.

Circular Dichroism Measurement of PDEγ-3 and PDEγ-3 Analogs—Purified peptides were dissolved in 0.01 M sodium phosphate, 2 mM MgCl2, pH 7.0, to a concentration of 0.5 mg/ml. Samples were scanned from 240 to 190 nm at a rate of 10 nm/min with a time constant of 4, at room temperature on a Jasco J-500A spectropolarimeter using a 0.5-mm quartz cell. The circular dichroism spectrum of PDEγ-3 was compared to standard spectra recorded by Greenfield and Pasman (39).

Miscellaneous Methods—Protein concentrations were determined by the method of Bradford (29) using BSA as a standard. SDS-PAGE was performing as described elsewhere (22). Significant peptides were determined using Student’s t test (49). The concentrations of individual proteins in crude rod outer segment preparations was determined by scanning of autoradiograms of preparations from Western blots. Antiserum directed against each individual protein was reacted with the blot and visualized with 125I-protein A. Quantitation was accomplished by comparing TLC plates with known concentrations of purified proteins (such as purified Ta) using a Gilford multimedia densitometer and Shimadzu integrator.

RESULTS

Inhibition of the GTPase Activity of Ta by PDEγ Protein—PDEγ has been reported to inhibit PDE activity when added back to trypsin-treated PDE (8).

Fig. 1A depicts the inhibition of trypsin-activated PDE by exogenous PDEγ. The I_{50} for PDEγ was around 60 ng of...
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Fig. 1. Effect of PDEγ on PDE activity and GTPase activity. A, trypsin-activated PDE (8) was assayed for PDE activity with varying amounts of PDEγ (50, 51). The assay was for 10 min at 30 °C using 42 ng of PDE preparation per assay and 0–560 ng of Pγ preparation. Based on densitometric scans (see "Experimental Procedures") and estimated molecular weights, the PDEα was 0.002 nmol. At the I50 point (≈60 ng) the Pγ was estimated to be 0.006 nmol. Results are means of triplicate samples. Percent inhibition indicates the activity compared to control PDE activity without added PDEγ. B, GTPase activity was measured as described under "Experimental Procedures." The assay contained 0.785 µg of Ta and 0–11.2 µg of PDEγ per assay. The Ta constituted 6.8% of the total rod outer segment preparation which was added at 12.5 µg/tube. Based on densitometric scans and estimated molecular weights, this is calculated to be 0.021 nmol of Ta and 0.64 nmol of PDEγ. Assays were for 20 min at 37 °C using 20 µM GTP and 1 µCi of [γ-32P]GTP/tube in a final volume of 100 µl. Results are the mean of triplicate samples. Percent inhibition indicates the percent inhibition of GTPase activity of Ta compared to a control without added PDEγ. Histone is type II from calf thymus (Sigma). Standard deviations (S.D.) were ±10%.

PDEγ. Based on densitometric scans of autoradiograms from Western blots (see above) 50% inhibition of PDE activity occurred at a PDEγ:PDEα/β ratio of 3:1. This is close to the predicted ratio of 1:1 and may be higher due to a 30% contamination of the PDE preparation by Ta.

Fig. 1B depicts the inhibition of Ta GTPase activity by the same PDEγ preparation. In this undepleted rod outer segment preparation the I50 for PDEγ inhibition of Ta GTPase activity was around 6 µg at a Ta:PDEγ ratio of 1:30.

The actual I50 may be much lower since this preparation contained 6.8% Ta and considerable PDEα/β. The inhibition of GTPase activity by PDEγ was not the result of nonspecific polycationic effects as histone did not inhibit GTPase activity at up to 200 mg (Fig. 1B).

Inhibition of the GTPase Activity of Ta by PDEγ Peptides—We have previously described the synthesis of peptides of bovine PDEγ (18). These peptides were designated PDEγ-1 through PDEγ-5. Each of these peptides was tested for its ability to inhibit PDE catalytic activity and Ta GTPase activity. The peptide, PDEγ-3, which corresponds to bovine amino acid residues 31–45, was found to be the most effective peptide in inhibiting the GTPase activity of Ta (18). Fig. 2 depicts the dose-response curve for PDEγ-3. The I50, the quantity of peptide needed for 50% inhibition of GTPase activity, was 450 µM. This is about 100-fold higher than that for the native PDEγ protein (Fig. 1). The inhibition of the GTPase activity of Ta by PDEγ-3 was noncompetitive, causing a decrease in the Vmax from 9 to 5 µM/min in the presence of 75 nmol of peptide, leaving the Kα unchanged (18). This inhibition was not due to polycationic effects as polylysine had no inhibitory activity (Fig. 2).

Effect of PDEγ-3 on the Release of GTPγS Bound to Ta in the Absence of Rod Outer Segment Membranes—To further confirm the previously reported kinetic data (18), the effect of PDEγ-3 on the release of GTPγS prebound to Ta in the absence or presence of GDP and in the absence of rod outer
The amount of [35S]GTPγS prebound to Ta in the absence of mixture at concentrations ranging from a similar experiment, [3H]GDP was added to the reaction assay. These results indicated that PDEγ-3 did not decrease GTPγS in the absence of rod outer segment membranes. In rod outer segment membranes, nor did it compete with [35S]GTPγS prebound to Ta compared to a control without added peptide.

The partially purified TCX-[35S]GTPγS was incubated in the presence of various quantities of the peptide, PDEγ-3, and the amount of [35S]GTPγS remaining bound to Ta was determined using a filter binding assay. These results indicated that PDEγ-3 did not decrease the amount of [35S]GTPγS prebound to Ta in the absence of rod outer segment membranes, nor did it compete with [35S]GTPγS in the absence of rod outer segment membranes. In a similar experiment, [3H]GDP was added to the reaction mixture at concentrations ranging from 2 to 100 μM in the absence or presence of 100 nmol of PDEγ-3 and incubated with the partially purified Ta-[35S]GTPγS. These results indicated that there was no exchange of [3H]GDP for [35S]GTPγS prebound to Ta in the presence of PDEγ-3 and in the absence of rod outer segment membranes (data not shown).

Effect of PDEγ-3 on Nucleotide Binding by Ta—Because the binding of GTPγS to Ta was not altered in the absence of rod outer segment membranes, the inhibition of the GTPase activity of Ta may have resulted from an inhibition of the initial GTP/GDP exchange on the rod outer segment membrane. To determine if this was the case, the extent of labeling of Ta with a nonhydrolyzable analog of GTP, GMPP(NH)P, was determined (Fig. 3). The addition of increasing quantities of PDEγ-3 to undepleted rod outer segment membranes resulted in a dose-response curve that was similar to the dose-response curve obtained for the inhibition of the GTPase activity of Ta by PDEγ-3. The IC50 (the quantity of peptide needed for 50% inhibition of GMPP(NH)P binding by Ta) was about 550 μM, compared to the IC50 of 450 μM for the inhibition of the GTPase activity of Ta. These results suggested that the peptide, PDEγ-3, and possibly PDEγ, inhibited the GTPase activity of Ta by altering the GTP/GDP exchange on the rod outer segment membranes.

In a similar experiment, the effect of PDEγ-3 on the absorption and release of [35S]GTPγS from Ta in the absence of rod outer segment membranes was determined (Table I). Ta was prelabeled with [35S]GTPγS as described (24). The resulting supernatant, which contains partially purified Ta-[35S]GTPγS, was incubated with various quantities of PDEγ-3 for 10 min at 37 °C in a total volume of 100 μl of GTPase buffer. Bound nucleotide was determined as described under "Experimental Procedures." The results are from triplicate samples ± S.D. p values were calculated using Student's t test using 0 nmol of peptide as a reference. In this assay, 14 nmol = 140 μM.

<table>
<thead>
<tr>
<th>PDEγ-3 (nmol)</th>
<th>[35S]GTPγS bound (nmol)</th>
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<tbody>
<tr>
<td>0</td>
<td>1.84 ± 0.15</td>
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<tr>
<td>7</td>
<td>1.80 ± 0.05*</td>
</tr>
<tr>
<td>14</td>
<td>1.94 ± 0.07*</td>
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<tr>
<td>36</td>
<td>2.11 ± 0.05</td>
</tr>
<tr>
<td>72</td>
<td>2.20 ± 0.13*</td>
</tr>
<tr>
<td>110</td>
<td>2.02 ± 0.02*</td>
</tr>
<tr>
<td>140</td>
<td>2.09 ± 0.09*</td>
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*0.1 > p > 0.05,
*0.05 > p > 0.02.

FIG. 2. Effect of PDEγ-3 peptides on the GTPase activity of Ta. Undepleted rod outer segment membranes (4 μg) were preincubated 5 min with and without various quantities of the PDEγ-3 peptide or polylysine. The reaction was initiated by the addition of GTP (GTP + 0.1 μCi of γ-[35P]GTP) to a final concentration of 20 μM in a volume of 100 μl of GTPase buffer. After incubation at 37 °C for 20 min, released [35P] was precipitated and quantitated as described under "Experimental Procedures." Results are the means from triplicate samples. Standard deviations (S.D.) were ±10%.

FIG. 3. Effect of PDEγ-3 on GMPP(NH)P Binding by Ta. Undepleted rod outer segment membranes (50 μg) were preincubated 5 min with and without PDEγ-3. The reaction was initiated by the addition of GMPP(NH)P substrate (GMPP(NH)P + 0.1 μCi of [3H]GMPP(NH)P) to a final concentration of 20 μM in a total volume of 100 μl of GMPP(NH)P-binding buffer. After incubation at 25 °C for 30 min, bound nucleotide was quantitated as described under "Experimental Procedures." Results are the means from duplicate samples. Percent inhibition indicates the percent inhibition of GMPP(NH)P binding by Ta compared to a control without added PDEγ-3. The IC50 was about 55 nmol of PDEγ3 or 550 μM.
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The binding of [3H]GDP and [35S]GTPγS to Ta, both separately and together, was determined (Table II). When [3H]GDP (1 μM final concentration) was incubated in the presence of undepleted rod outer segment membranes (previously dark-adapted, then exposed to room light for 5 min) and 200 nmoI of PDEγ-3, there was an increase in the amount of [3H]GDP bound to Ta from 0.54 to 0.72 pmol (33% increase). When [35S]GTPγS was substituted for [3H]GDP (1 μM final concentration), the amount of [35S]GTPγS bound to Ta was about 10 times higher than the amount of [3H]GDP bound to Ta, because the affinity of GTPγS is much higher for Ta in the light than the affinity of GDP for Ta (35). In the presence of 200 nmol (2 mM) PDEγ-3, the amount of [35S]GTPγS bound to Ta was decreased from 8.6 to 6.6 pmol (22% decrease). In the presence of both nucleotides (1 μM final concentration each), no [3H]GDP binding to Ta was detected. In the presence of 200 nmol of PDEγ-3, the amount of [35S]GTPγS bound to Ta was decreased from 11 to 8.4 pmol (25% decrease).

Effect of PDEγ-3 on the Binding of Transducin to Rod Outer Segment Membranes—The inhibition of the exchange of GTP for GDP bound to Ta in the presence of the peptide, PDEγ-3, may be due to an alteration of the binding of Ta to rhodopsin (photoisomerized or nonphotoisomerized), Tβγ, or both rhodopsin and Tγγ on the surface of the rod outer segment membrane. To determine whether this was the case, the effect of PDEγ-3 on membrane binding of transducin was determined.

We have used synthetic peptides to raised antisera to a wide variety of retinal rod outer segment proteins. These proteins include rhodopsin, Ta, Tβ, and Tγ (β- and γ-subunits of transducin), S-antigen (48-kDa protein), interstitial retinol binding protein (IRBP), PDEα, and PDEγ. These antisera were used to determine the purity and quantity of specific proteins in our crude transducin preparation and depleted rod outer segment membrane preparation using a solid-phase RIA.

To determine the effect of PDEγ-3 on the association of Taγγ with rod outer segment membranes (interaction with rhodopsin), we have utilized a crude transducin preparation reconstituted with a depleted rod outer segment membrane preparation. The depleted membrane preparation was tested for the presence of specific rod outer segment proteins using antipeptide antisera and a solid-phase RIA. The depleted rod outer segment membranes tested positive for rhodopsin and negative for S-antigen, IRBP, PDEα, and PDEγ. In this Table II

<table>
<thead>
<tr>
<th>Sample</th>
<th>PDEγ-3</th>
<th>[3H]GDP</th>
<th>[35S]GTPγS</th>
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<tbody>
<tr>
<td></td>
<td>pmol bound</td>
<td>pmol bound</td>
<td>pmol bound</td>
</tr>
<tr>
<td>ROS + [3H]GDP</td>
<td>0.54 ± 0.07</td>
<td>+</td>
<td>0.72 ± 0.03</td>
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<tr>
<td>ROS + [35S]GTPγS</td>
<td>8.6 ± 0.6 (22)</td>
<td>+</td>
<td>6.7 ± 0.2</td>
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<tr>
<td>ROS + both nucleotides</td>
<td>ND</td>
<td>11 ± 1</td>
<td>8.4 ± 1.3 (25)</td>
</tr>
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</table>

Fig. 4. Circular dichroism spectrum for PDEγ-3. Purified peptide was dissolved in 0.01 M sodium phosphate, 2 mM MgCl₂, pH 7.0, to a final concentration of 0.5 mg/ml. The peptide was scanned from 240 to 185 nm at a rate of 10 nm/min, with a time constant of 4, in a 0.5-mm quartz cell at room temperature. The resulting circular dichroism spectrum was compared to standards determined by Greenfield and Fasman (39).
in the absence of membranes (T), nor does the depleted rod outer segment membrane preparation contribute any transducin to the supernatant after centrifugation when incubated in the absence of the crude transducin preparation (ROS). When the crude transducin preparation and the depleted rod outer segment preparation are incubated together, some of the Ta remains bound to the depleted rod outer segments and is, therefore, removed from the supernatant when the membranes are centrifuged (T+ROS). When PDEy-3 is added to the crude transducin preparation and the depleted rod outer segment membranes, essentially all of the Tα is removed from the supernatant after centrifugation of the membranes (T+ROS+Py3). The presence of PDEy-3 appears to enhance the binding of Tα to the rod outer segment membrane. This is not due to PDEy-3 causing the sedimentation of Tα in the absence of rod outer segment membranes or interference of the interaction of Tα-C with Tα because the quantity of Tα in the supernatant is unchanged in the presence of PDEy-3 after centrifugation (T+Py3). Also, the amount of Tα is not increased when depleted membranes are incubated in the presence of PDEy-3 (ROS+Py3).

In a similar experiment, the quantity of Tβ was determined by using antisera to a peptide called Tβ-C. This peptide corresponds to the last 15 amino acids of the C terminus of Tβ. These results were similar to those for Tα, except that more Tβ was bound to the depleted rod outer segment membranes in the absence of PDEy-3, leading to a smaller decrease in the amount of Tβ in the supernatant in the presence of PDEy-3. Also, an analog of PDEy-3, found not to inhibit the GTPase activity of transducin (Analog 1: see Table III and Fig. 8A), was ineffective in inducing the binding of Tα to the rod outer segment membrane (data not shown). Polylysine, when added at 100 mM, likewise, had no effect.

**Table III**

<table>
<thead>
<tr>
<th>PDEγ-3 analogs</th>
<th>Tα-K-Q-R-Q-T-R-Q-F-K-S-K-P-P-K-K-COOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDEγ-3</td>
<td>H3N-K-Q-R-P-T-R-Q-F-K-S-K-P-P-K-K-COOH</td>
</tr>
<tr>
<td>Analog 1</td>
<td>H3N-K-Q-R-P-T-R-Q-F-K-S-K-P-P-K-K-COOH</td>
</tr>
<tr>
<td>Analog 4</td>
<td>H3N-K-Q-R-Q-V-R-Q-F-K-S-K-P-P-K-K-COOH</td>
</tr>
<tr>
<td>Analog 8</td>
<td>H3N-K-Q-R-Q-T-R-Q-F-K-V-K-P-P-K-K-COOH</td>
</tr>
</tbody>
</table>

**Fig. 5. Effect of PDEγ-3 on the interaction of Tα with rod outer segment membranes.** Transducin (T), depleted rod outer segments (ROS), or both transducin and depleted rod outer segments were incubated in the absence or presence of 51 nmol (510 μM) of PDEγ-3 (Py3) at room temperature for 10 min in a total volume of 100 μL of GTPase buffer. The samples were centrifuged, and the resulting supernatants were tested for the quantity of Tα using a solid-phase RIA and antipeptide antisera for Ga-C, a peptide corresponding to the last 15 amino acids of the C terminus of bovine Tα. Results are from six samples (mean ± S.D.). The binding of Tα to equal micrograms of lens membranes was used as a negative control. This value has been subtracted.

**Effect of PDEγ Analogs on the GTPase Activity of Tα.** In our previously published report (18), we analyzed the amino acid sequence of PDEγ for predicted secondary structures based on the statistical method of Chou and Fasman (19) and determined the hydrophobicity plot based on the method of Kyte and Doolittle (20). These analyses indicated a region extending from amino acid residues 29 to 41 predicted to be α-helical by the methods of Chou and Fasman (19). In a similar experiment, the quantity of Tβ was determined by using antisera to a peptide called Tβ-C. This peptide corresponds to bovine PDEγ amino acid residues 31-45. Starred amino acids are substitutions from the original PDEγ sequence used to make the indicated analog. PDEγ-α-helix corresponds to the region extending from amino acid residues 29-41 predicted to be α-helical by the methods of Chou and Fasman (19).
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Fig. 6. $^{125}\text{I}-\text{PDEγ-3}$ binding to membrane homogenates. Peptides were radioiodinated as described under "Experimental Procedures." The radioiodinated peptide, $^{125}\text{I}-\text{PDEγ-3+Tyr}$ (1.2 nmol; 12 μM) was incubated with various quantities of undepleted rod outer segment membranes (●), lens membranes (○), or BSA (×) in 100 μl of GTPase buffer + 1 mg/ml BSA. After incubation at 37 °C for 10 min, bound peptide was determined as described under "Experimental Procedures." Results are the means from duplicate samples.

Fig. 7. Competition of $^{125}\text{I}-\text{PDEγ-3}$ binding by unlabeled peptides. Undepleted rod outer segments were incubated with various quantities of unlabeled PDEγ-3 (○), PDEγ-3+Tyr (×), and IRBP-1 (●). The radioiodinated peptide, $^{125}\text{I}-\text{PDEγ-3+Tyr}$ (1.2 nmol, 12 μM) was added to the rod outer segment membranes to a total volume of 100 μl in GTPase buffer + 1 mg/ml BSA. After incubation at 37 °C for 10 min, bound peptide was quantitated as described under "Experimental Procedures." Results are the means from duplicate samples.

located on the other surface. Most of this predicted α-helical region was located in the peptide, PDEγ-3, which extends from amino acid residues 31 to 45. We used the predicted structure to determine which amino acids could be substituted in PDEγ-3 for the synthesis of PDEγ-3 analogs. These analogs were used to determine functionally important amino acids needed for PDEγ-3 binding to Tα and for the inhibition of the GTPase activity to Tα.

Table III lists the 13 PDEγ-3 analogs which were synthesized, and a peptide corresponding to the predicted α-helical region called PDEγ-α-helix (bovine amino acid residues 29–41). In these analogs, amino acids were substituted to affect the structure of the peptide, to alter the positive charges on the hydrophilic surface or to replace hydrophobic amino acids with hydrophilic or charged amino acids. Amino acids on the hydrophobic surface of the predicted α-helix which were substituted included glutamine 34 and phenylalanine 36. Positively charged amino acids on the hydrophilic surface which were substituted included arginine 33 and arginine 36. Other amino acids substituted, which may be important in hydrogen bonding but contain no positive charge, included glutamine 37, threonine 35, and serine 40. Substitutions were made so as to affect the secondary prediction plot of the intact protein as little as possible (except for Analogs 1, 12, and 13), and, in some cases, to affect the hydrophobicity plot as little as possible (Analogs 1, 3, 6, 7, 9, 12, and 13). The peptides were then tested for their ability to inhibit the GTPase activity of Tα (Fig. 8, A and B).

Fig. 8. Effect of PDEγ-3 analogs on the GTPase activity of transducin. Assays were performed as described in Fig. 2. Results are the means from triplicate samples. Standard deviation values were ±10%. Peptides tested were: A, ●, Analog 1; ○, Analog 2; ×, Analog 3; , Analog 4; □, Analog 5; A, Analog 6; and Δ, Analog 7. ●, Analog 8; □, Analog 9; ×, Analog 10; , Analog 11; □, Analog 12; A, Analog 13; Δ, PDEγ-α-helix. PDEγ-3 (▼) was included in each graph for reference.
Analogs 1, 12, and 13 were made to attempt to disrupt the structure of the peptide, PDEγ-3. In analogs 1 and 13, two different glutamines were substituted with prolines, one on the hydrophobic surface and one on the hydrophilic side, to disrupt any α-helical structure the peptide may obtain upon binding to transducin. These substitutions were made such that the hydrophobicity was not substantially altered. The third peptide, Analog 12, had a proline replaced by a glutamine at the site where there are normally two flanking prolines. This would determine the importance of the flanking prolines in the maintenance of the structure of PDEγ-3, when it is bound to transducin. Circular dichroism measurements of these analogs suggested that they were all in a random coil conformation in solution (data not shown). The circular dichroism spectra for all analogs were identical to the parent peptide PDEγ-3 (see Fig. 4).

When glutamines were replaced with prolines in the region predicted to be an α-helical (Analogs 1 and 13), the inhibition of the GTPase activity of Ta by the peptide analogs was reduced. This suggested that the structure of the peptide may be disrupted by the substitution of the glutamines with prolines. If the structure of this region is indeed α-helical in the intact PDEγ peptide or when the peptide, PDEγ-3 is bound to Ta, then the addition of the prolines could make it more difficult for the peptide to obtain this structure upon binding to transducin. Addition of a glutamine in place of one of the prolines flanking the predicted α-helical region did not decrease the inhibitory effectiveness of the peptide, PDEγ-3, as much as the substitution of amino acids in the predicted α-helical region. This suggests that the structure of this region in PDEγ may depend, in part, on the amino acids in the flanking regions.

Since one side of the predicted α-helix contains a large number of positively charged amino acids (arginines and lysines), substitution of these amino acids would determine the functional importance of these amino acids in the inhibition of the GTPase activity of Ta by the peptide, PDEγ-3. When either of the arginines in the predicted α-helical region were substituted with glutamine and glutamic acid (Analogs 3 and 5, respectively), the peptide's ability to inhibit the GTPase activity of Ta was greatly reduced (I50 > 2 and 2.5 mM, respectively) when compared to the peptide, PDEγ-3 (I50 = 450 μM). When a glutamine adjacent to one of these arginines was replaced by a negatively charged glutamic acid (Analog 11) to neutralize the charge on the adjacent arginine, the decrease in the inhibitory effectiveness of the peptide was not as great as when the arginine was totally replaced (Analogs 3 and 5). When one of these arginines was replaced by another positively charged amino acid (Analog 7), lysine, the ability of the peptide to inhibit the GTPase activity of Ta was not decreased. These results indicate that the positively charged amino acids may be necessary for the binding of PDEγ-3 to transducin and for the inhibition of the GTPase activity to Ta. In contrast, peptides with substitutions made on the hydrophobic surface of the predicted α-helical region (Analogs 2, 6, 9, and 10) were still able to inhibit the GTPase activity of Ta as well as PDEγ-3. When a serine or a threonine was substituted with a valine (Analogs 4 and 8, respectively), the inhibition of the GTPase activity of transducin was reduced, suggesting that there may be other hydrophilic interactions in the binding of PDEγ-3 to transducin and for the inhibition of the GTPase activity of Ta.

An analog corresponding to the predicted α-helical region was also tested for its ability to inhibit the GTPase activity of Ta (Fig. 8B). This peptide inhibited the GTPase activity of Ta as effectively as PDEγ-3. This suggests that the predicted α-helical region alone is able to interact with transducin without the flanking prolines. Although the flanking prolines may not be necessary for the interaction of the predicted α-helical region with transducin, the presence of these prolines may play an important role in maintaining the secondary structure of this region in the intact PDEγ subunit.

**Discussion**

The results of the above experiments (Fig. 1) and those previously reported (18) suggest that PDEγ may regulate the GTPase activity of Ta and, ultimately, the shut-off mechanism of the visual transduction system. Others have suggested that PDEγ may interact directly with Ta (12, 13, 30, 31).

The peptide, PDEγ-3, which corresponds to bovine PDEγ amino acid residues 31-45, contains part of a region of PDEγ which effectively inhibits the GTPase activity of Ta (Fig. 2). Lipkin et al. (40) have reconfirmed our earlier results (18) that the region for the interaction of PDEγ with Ta resides in amino acid residues 25-47. We have shown that this inhibitory region may actually extend between amino acid residues 29-41, as shown by the more specific results using the synthetic peptide PDEγα-helix (Table III) in the GTPase assay (Fig. 8B). This region was predicted to have an α-helical structure containing a large number of positively charged amino acids and amino acids which may participate in hydrogen bonding on the hydrophilic surface of the predicted α-helix. These amino acids may be important for the interaction of PDEγ-3 with transducin as shown by the analog studies described in Fig. 8, A and B. The inhibition of the GTPase activity of transducin is noncompetitive (18) and may result from an alteration of the interaction between transducin α and the rod outer segment membrane, interfering with the GTP/GDP exchange on Ta. This is suggested by the results of the previously reported kinetic experiments (18), by the

![Fig. 9. GTP hydrolytic cycle.](source:https://example.com/fig9.png)
inhibition of guanine nucleotide binding by Ta in the presence of membranes (Fig. 3 and Table II), and by the enhanced binding of transducin to the rod outer segment membrane as shown by the reconstitution experiments (Fig. 5).

The steps leading to the exchange of GTP for GDP prior to their use in the GTPase assays. Preparations have been incorporated into the model depicted in Fig. 9. In this exchange, Ta-GTP is released from Ta and can account when developing a possible mechanism for the inhibition of the GTPase activity of Ta by PDEy.

The GTPase assays, most of the transducin is in the R*-To conformation, TpyTa-GDP (T-GDP) with R* by enhancing the binding of GDP or R*-To state because the preparations were exposed to light prior to their use in the GTPase assays, most of the transducin is in the R*-To-GDP exchange, Ta-GTP is released from Ta and can activate PDE. The forward reactions of these equilibria are driven by the hydrolysis of GTP by Ta (41).

In the undepleted rod outer segment preparations used in the GTPase assays, most of the transducin is in the R* - Tpy-GDP or R* - Tpy state because the preparations were exposed to light prior to their use in the GTPase assays. Preparations of rod outer segment membranes will hydrolyze most of the GTP to GDP and will remain bound to R* as long as exogenous GTP is not present (26, 34). This factor was taken into account when developing a possible mechanism for the inhibition of the GTPase activity of Ta by PDEy.

There are four possible mechanisms by which the peptide, PDEγ, may inhibit the GTPase activity of Ta. These are: 1) decreasing the intrinsic catalytic rate of GTP hydrolysis by Ta through a mechanism not affecting the GTP/GDP exchange, 2) preventing the association of Ta-GTP with GDP bound to Ta, 3) preventing the association of Ta-GTP with GDP by the enhanced binding of Ta-GTP to light and GTP insensitive sites on the rod outer segment membrane, and 4) increased binding of Ta-GTP to R* accompanied by an interference of GTP/GDP exchange (37, 38).

Based on the results reported herein, the inhibition most likely occurs by the increased affinity of Ta for Ta by G3y from the adenylate cyclase system may be indirectly involved in the inhibition of adenylate cyclase activation by Go by increasing the binding of Goα to β-adrenergic receptor and preventing it from associating with and activating adenylate cyclase (47). The same mechanism could regulate the activation of PDE by Ta-GTP. In the molecular model of Hingorani and Ho (32) the binding of GTP to the guanine nucleotide binding domain of Ta moves two α-helices into the receptor binding domain which causes the simultaneous release of Ta-GTP from R* and Ta-GTP. The role of PDEγ may be to increase the affinity of Ta-GDP for Ta-GTP which could lock the receptor binding domain of Ta into the R* - Tpy bound conformation preventing the movement of the two α-helices from the guanine nucleotide binding domain into the receptor binding domain. The overall effect of PDEγ on Ta may be a slight modulation of the cascade, depending on the localized concentrations of each protein within the outer segment.

REFERENCES

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