In rod outer segments the light activation of cGMP phosphodiesterase (PDEαβγ) is accomplished by removal of the γ inhibitory subunit (PDEγ) from the PDEαβ catalytic subunits. A light activation of theinositol signaling pathway also occurs, but there is little information linking these two signal transduction pathways. Here we report that protein kinase C (PKC) purified from bovine rod outer segment phosphorylates the bovine PDEγ with incorporation of 0.9 ± 0.1 mol of phosphate/mol of PDEγ. Phosphorylation of PDEγ increases its ability to inhibit PDEαβ catalytic subunits (trypsin-activated PDE, tPDE) with an IC₅₀ for phosphorylated PDEγ of 26 ± 4 pm and an IC₅₀ of 60 ± 5 pm for unphosphorylated PDEγ. Inhibition of tPDE by PDEγ is characterized by two values of Kᵢ, Kᵢ = 34 pm and Kᵢ = 760 pm. Phosphorylation of PDEγ by PKC eliminates the functional heterogeneity of the PDEγ population resulting in a single value of Kᵢ = 23 pm. Free PDEγ (without PDEαβ catalytic subunits) is a better substrate for PKC than PDEγ in a complex with PDEαβ. Phosphorylation of free PDEγ by PKC is characterized by a value of Vₘₐₓ = 1,550 ± 148 units/mg of PDEγ (Kᵢ = 21.0 ± 1.3 μM). In contrast, phosphorylation of PDEγ in PDEαβγ complex has two values of Vₘₐₓ, Vₘₐₓ = 0.3 ± 0.1 units/mg of PDEγ (Kᵢ = 0.4 ± 0.2 μM) and Vₘₐₓ = 0.7 ± 0.2 units/mg of PDEγ (Kᵢ = 4.6 ± 0.9 μM).

ROS PKC phosphorylates Thr²⁶ in PDEγ. We have previously reported (Morrisson, D. F., Rider, M. A., and Take moto, D. J. (1987) FEBS Lett. 222, 266–270; Lipkin, V. M., Udovichenko, I. P., and Kuznetsov, V. A., Yurovskaya, A. A., Telnykh, E. V., and Skiba, N. P. (1989) Biomed. Sci. (Lond.) 1, 305–308) that the central fragment of PDEγ (24–45) is responsible for binding to PDE catalytic subunits. The new data suggests that this region of PDEγ also includes the site for phosphorylation by PKC and that phosphorylation increases the ability of PDEγ to inhibit PDE catalytic activity. This altered regulation of visual transduction may play a role in desensitization or light adaptation.

Photoreceptor cyclic GMP phosphodiesterase (PDE)¹ is the effecter enzyme in the visual signaling cascade of proteins in the vertebrate photoreceptor cell. The PDE is composed of catalytic PDEα (88 kDa) and PDEβ (85 kDa) subunits and two identical inhibitory subunits (PDEγ, 11 kDa), which inhibit enzyme activity in the dark (reviewed by Stryer (1991)). The primary structures of PDEγ from bovine, mouse, and human have been reported (Ovchinikov et al., 1986; Tuteja and Farber, 1988; Tuteja et al., 1990) and show high homology (96.6%). A central fragment of PDEγ (residues 24–47) is essential for PDEγ interaction with PDE catalytic subunits (Morrisson et al., 1987; Lipkin et al., 1990; Artemyev and Hamm, 1992).

The sequence of events that occurs in the process of vertebrate visual excitation has not yet been completely defined. Several lines of evidence over the past few years have suggested the existence of the phosphoinositol signaling pathway in photoreceptor cells. The light-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate (PtdInsP2) has been demonstrated by biochemical and immunocytochemical studies (Brown et al., 1984; Ghalyami and Anderson, 1984; Hayashi and Amakawa, 1985; Szuts et al., 1986; Das et al., 1987). A phospholipase C that hydrolyzes PtdInsP2 to inositol 1,4,5-trisphosphate has been identified in ROS (Wilde et al., 1986; Gehm and McConnell, 1990), and PKC, a key enzyme in the phosphoinositide pathway, was also identified in ROS (Kelleher and Johnson, 1985; Wolbrink and Cook, 1991). PKC phosphorylates rhodopsin (Kelleher and Johnson, 1986; Newton and Williams, 1991; Newton and Williams, 1993) and arrestin (Weyand and Kuhn, 1990). PtdInsP3 stimulates phosphorylation of PDEγ in ROS, and PKC phosphorylates PDEγ (Hayashi et al., 1991; Udovichenko et al., 1993a, 1993b). A PKC activator, oleoylactylglucerol, decreases the amplitude of the photoreceptor phosphoresponse and dark levels of cGMP up to 40% and depresses the light-stimulated decrease in cGMP levels (Binder et al., 1989). However, the mechanisms by which a PKC participates in the regulation of the visual transduction cascade are not clear. Only in the case of phosphorylation of rhodopsin by PKC has progress been made (reviewed by Newton and Williams (1993)).

The functional consequence of this phosphorylation is a reduced ability to stimulate the light-dependent rhodopsin activation of nucleotide binding subunits (tPDE, trypsin-activated PDE) present in ROS (Kelleher and Johnson, 1986). Information is not complete in the case of other visual transduction cascade proteins. If the regulation of these proteins by PKC does occur, does this alter the cycle by activation of amplification in vision or by desensitization and light adaptation? Here we report that a mechanism regulating PDE func-

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² A scholar of the Kansas Health Foundation of Wichita and a post-doctoral fellow supported by Training Grant CA-09418 from the National Institutes of Health.

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The abbreviations used are: PDE, rod outer segment cyclic GMP phosphodiesterase; ROS, rod outer segments; PDEα and PDEβ, catalytic α and β subunits of PDE; PDEγ, inhibitory subunit of PDE; tPDE, trypsin-activated PDE; PDEαβγ, recombinant PDEα; PKC, protein kinase C; PtdInsP2, phosphatidylinositol 4,5-bisphosphate; GTP-γ-S, guanosine 5′-O-(3-thiotriphosphate); PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; TPCK, 1-tosylamido-2-phenylethyl chloromethyl ketone; tPDE, trypsin-activated PDE, MOPS, 4-morpholinolinesulfonic acid.
tion may involve PDEy phosphorylation catalyzed by the ROS PKC. This kinase phosphorylates Thr\(^{28}\) in PDEy, and phosphorylated PDEy (PDEy-P) demonstrates increased ability to inhibit PDEc\(\beta\) catalytic activity.

**EXPERIMENTAL PROCEDURES**

**Materials**—Fresh bovine eyes were obtained from a local slaughterhouse (Iowa Beef Packers, Emporia, KS). Trypsin (bovine pancreas, type XIII, TPCK-treated; EC 3.4.21.24; 12,000 units/mg), trypsin inhibitor (bovine, type 1-S), calcium-intestinal alkaline phosphatase, and GTPyS were from Sigma. Cyclic [\(^{3}H\)]GMP (15 Ci/mmol), which was purified further by anion-exchange chromatography, and [\(^{32}P\)]ATP (3,000 Ci/mmol) were from DuPont NEN. The t-butoxycarbonyl amino acids and their resins were from Vега Biochemicals, U. S. Biochemical Corp. (Cleveland, OH), or Sigma. Vиде СКС СЛС and ТКС СКС СЛС columns were from P. J. Cоберр (Sейсс, Л. MO), DЕАЕ-СКС СЛС and from DуПоупт.

**Pептид Синтеза и Пурификация**—Pептиды соответствующие к лейнам bovine PDE were synthesized by the method of Merreriff (1963) as modified by Gorman (1984). Pептиды were purified (Morrison et al., 1989) by reverse-phase chromatography on a HPLC Vиде СКС СЛС-18 column. Pептидные концентрации were дetermined by amino acid analyses.

**PDE Purification and Preparation of Trypsin-activated PDE—ROS** were prepared by the method of Pапермастэр and Дрейер (1974). Bovine bovine eyes were obtained from a local slaughterhouse within 1 h of death. Retinas were removed under dim red light and stored without buffer at \(-70^\circ\text{C}\) in the dark. The ROS were purified by centrifugation in sucrose density gradients and washed with isotonic buffer (Pапермастэр and Дрейер, 1974). PDE was eluted by resuspending the pellet in hypotonic buffer (10 mM Tris-C\(_1\), pH 7.4; 1 mM dithiothreitol; 0.1 mM phenylmethylsulfonyl fluoride; 1 mg/ml leupeptin; 1 mg/ml pepstatin). Soluble PDE was concentrated by ion-exchange chromatography on a DEАЕ-СКС СЛС-Sephacel column and was purified by HPLC on a TSK G3000SW column (7.5 x 75 mm) using the buffer of 150 mM MOPS, pH 7.4; 5 mM MgCl\(_2\); 0.25 mM EDTA; 1 mM EGTA; and 1 mM P-mercaptoethanol. PDEy samples were diluted with glycerol to 50% and stored at \(-20^\circ\text{C}\). For preparation of trypsin-activated PDE (tPDE), bovine ROS PDE (500 \(\mu\)l, 100 \(\mu\)M before HPLC) was exposed to TPCK-treated trypsin (5 \(\mu\)l, 1 mg/ml; 12,000 units/mg) for 5 min on ice. The reaction was stopped by the addition of a 5-fold excess of soybean trypsin inhibitor (1 \(\mu\)g inhibits 1.7 \(\mu\)g of trypsin), and tPDE was purified by HPLC as described above. Soybean trypsin inhibitor was purified in advance by gel filtration on a HPLC TSK G3000SW column. Nonpurified soybean trypsin inhibitor contains minor components, which are phosphorylated by kinases, and this makes the identification of PDE phosphorylation difficult to interpret.

**Purification of Wild-type PDEy and Recombinant PDEy**—Wild-type PDEy was separated from PDEc catalytic subunits by reverse-phase HPLC of pure PDE on a Vydac C-4 column. The plasmid for expressing PDEy (clone FXSG). The coding portion of the gene for PDEy has not been published by John Sondek, Yale University. Purification of the recombinant PDEy residing in the plasmid described by Brown and Stryer (1989) may involve PDEy phosphorylation catalyzed by the ROS PKC. Phosphorylated PDEy (PDEy-P) demonstrates increased ability to inhibit UV detection, and protein concentration was further determined by the method of Bradford (1976). The active fractions were pooled and applied to a TSK G3000SW column (7.5 x 75 mm) (0.5-ml injection) equilibrated with 50 mM MOPS, pH 7.4; 1 mM EDTA; 1 mM EGTA; 1 mM \(\beta\)-mercaptoethanol at a flow rate of 0.8 ml/min. Fractions (0.4 ml) were collected and tested for enzyme activity as described below.

**PDE Activity Assay**—Prior to use in assays, [\(^{3}H\)]cGMP was purified as described by Kincaid and Manganelli (1988). PDE activity was determined as described by Hansen et al. (1988). The final concentration of the reaction was 40 mM Tris-Cl, pH 7.4; 5 mM MgCl\(_2\); 100 \(\mu\)M [\(^{3}H\)]cGMP (100,000 cpm/assay) in 100 \(\mu\)l of reaction buffer. Reactions were allowed to proceed for 10 min at 30 \(\circ\text{C}\) and were terminated by placing the tubes in a boiling water bath for 2 min. Snake venom (100 \(\mu\)l, 1 mg/ml) was added to the cooled reaction tubes and incubated for 30 min at 30 \(\circ\text{C}\). The samples were applied to columns of DEАЕ-СКС СЛС-5 (0.5-ml bed volume) and eluted with 1.8 ml of water.

**Pептид Синтеза Activity Assay**—Phosphorylation of proteins and peptides by PKC was accomplished by the method optimized by Huang et al. (1986). The reaction mixture contained 30 mM Tris-Cl, pH 7.5; 6 mM MgCl\(_2\); 0.25 mM EGTA; 0.4 mM CaCl\(_2\); 0.04% Nonidet P-40; 10 \(\mu\)M [\(^{32}P\)]ATP; 100 \(\mu\)g/ml P5; 20 \(\mu\)g/ml 1,2-di-octanoyl-sn-glycerol, and PKC in a final volume of 20 \(\mu\)l. The reaction was initiated by the addition of the kinase, and incubation was for 3 min (for determination of kinetic parameters) or 20 min (for complete incorporation of phosphate into PDEy) at 30 \(\circ\text{C}\). For determination of [\(^{32}P\)] incorporation into substrates, two methods were used. 1) Phosphorylated products were separated by SDS-PAGE followed by autoradiography. Radioactive bands were excised and radioactivity was measured by liquid scintillation spectrometry. This method was used for studying the phosphorylation of PDEy in complex with PDEc catalytic subunits and for the phosphorylation of PDEy peptides. 2) After the phosphorylation reaction, a 10-\(\mu\)l aliquot of each sample was pipetted onto phosphocellulose paper (P81, Whatman) (2 cm x 2 cm) and washed with 75 mM NaCl, 0.10 M Tris, and 25 mM EDTA at 4 \(\circ\text{C}\). The radioactive product was visualized with ninhydrin, and radioactive products were identified by autoradiography.

**Purification of Wild-type PDEy**—Wild-type PDEy was purified as described above. For removal of [\(^{32}P\)]ATP, the phosphorylated products were immobilized on polyclinvidien-dilferuoro membranes (1 x 1 cm, Bio-Rad). The polyclinvidien-dilferuoro membranes were incubated with the phosphorylation reaction mixture for 30 min with 50 mM Tris-Cl, pH 7.5; 50 mM NaCl; 1 mM EDTA; followed by washing with PBS buffer (3 times for 10 min) and Lys-C buffer (50 mM tricine, pH 8.0; 10 mM EDTA; 2 times for 5 min). The polyclinvidien-dilferuoro membranes were transferred into Eppendorf tubes and soaked with 200 \(\mu\)l of Lys-C buffer, and the phosphorylated PDEy was digested with Lys-C (0.25 \(\mu\)g). The radioactive digest products in the solution were monitored by liquid scintillation counting of aliquots, and the
digest was stopped when the reaction reached a plateau (16 h at 37 °C; about 80% of radioactivity in solution). The PDEγ peptides were separated by the reverse-phase chromatography on a HPLC Vydac C-18 column (5 min, buffer B, 0%; from 5 to 25 min the gradient of buffer B from 0% to 70%; buffer A, 0.1% trifluoroacetic acid, 5% acetonitrile in water; buffer B, 0.1% trifluoroacetic acid, 95% acetonitrile in water; detection at 230 nm; flow rate = 1 ml/min). Fractions (0.1 min, 0.1 ml) were collected, and the radioactivity was determined by liquid scintillation counting. A peptide, Q9QRQFK, corresponding to residues 32–39 of bovine PDEγ was used as a standard. This peptide was synthesized by the solid-phase Memfidd (1963) method on an Applied Biosystems automated peptide synthesizer in the Biotechnology Laboratory at Kansas State University, Manhattan, KS. The Q9QRQFK peptide was phosphorylated by ROS PKC with [32P]ATP as described above. For removal of free [32P]ATP, the [32P]peptide was immobilized onto a phosphocellulose paper (P81, Whatman) strip (1 × 2 cm), washed with 75 ml H2O (4 times for 2 min), and eluted with 2 ml ammonium bicarbonate. Ammonium bicarbonate was removed by lyophilization, and the [32P]peptide was dissolved in Lys-C buffer prior to use as a standard for the chromatogram.

**Miscellaneous Methods**—Protein concentrations were determined by the method of Bradford (1976) using bovine serum albumin as standard. Concentration of PDEγ was determined by absorbance of PDEγ in water at 280 nm (extinction coefficient 7,090 M⁻¹ cm⁻¹), as described by Gill and von Hippel (1989). SDS-PAGE was performed as described by Laemmli (1970), using a separating gel of 20% acrylamide, 0.1% bis-acrylamide (for detection of PDEγ) and the sample, which was used for PDEγ phosphorylation, did not change in the presence of cAMP and cGMP and was ineffective without phosphatidylserine, dioctanoylglycerol, and Ca²⁺.

**RESULTS**

**Phosphorylation of PDEγ**—For phosphorylation, pure samples of ROS PKC and PDEγ were used. Pure ROS PKC demonstrates a single band after SDS-PAGE and silver staining, which was used for PDEγ phosphorylation, did not include any detectable minor components after gel silver staining (not shown). The kinetic characteristics of pure PKC (Vmax and Km for histone III-S, Table I) are close to the reported values for PKCs from other sources. Phosphorylation of substrates (histone III-S and PDEγ) by pure ROS PKC does not change in the presence of cAMP and cGMP and was ineffective without phosphatidylserine, dioctanoylglycerol, and Ca²⁺. Taken together these data suggest that the pure ROS PKC does not contain other kinases.

We have found that, in optimal conditions, 1.0 μg of PDEγ is phosphorylated by 55 milliunits of PKC for 20 min (30 °C, 20 μl of assay) with a phosphorylation extent of 0.9 ± 0.1 mol of phosphate/mol of PDEγ (Figs. 1 and 2A). Native PDEγ (purified from ROS) and recombinant PDEγ (rPDEγ) have shown a similar level of phosphorylation (Table I). Phosphorylation of free native PDEγ (without PDEαβ catalytic subunits) by PKC is characterized with a value of Vmax = 1,550 ± 148 units/mg (Km = 21.0 ± 1.9 μM) (Table I). Gel silver staining (Fig. 2B) after separation of phosphorylated products by SDS-PAGE did not identify any degradation products of PDEγ during phosphorylation (Fig. 2). Free PDEγ is the better substrate for PKC than PDEγ in complex with PDEαβ (Table I). This data suggests that the site for phosphorylation is blocked by PDEαβ catalytic subunits. Phosphorylation of PDEγ in PDEαβγ complex was characterized by two values of Vmax: Vmax = 0.3 ± 0.1 units/mg (Km = 0.4 ± 0.2 μM) and Vmax = 0.7 ± 0.2 units/mg (Km = 4.6 ± 0.9 μM) (Fig. 3, Table I). This suggests that the conformational change in the presence of CAMP and cGMP and was ineffective without phosphatidylserine, dioctanoylglycerol, and Ca²⁺.

**TABLE I**

**Kinetic parameters of ROS PKC with different PDEγ substrates**

PKC activity was measured under standard assay conditions containing 30 mM Tris-Cl, pH 7.5; 100 μg/ml phosphatidylinerine, 20 μg/ml 1,2-dioctanoyl-sn-glycerol, 0.04% Nonidet P-40, 10 μM [γ-32P]ATP, 6 mM MgCl₂, 0.4 mM CaCl₂, 0.1–200 μM PDEγ substrates, and 20 ng of pure ROS PKC. The kinetic parameters were expressed as mean ± S.E. of three determinations.

<table>
<thead>
<tr>
<th>PDEγ substrate</th>
<th>Vmax</th>
<th>Km</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDEγ (nmol/mg)</td>
<td>1,550 ± 148</td>
<td>21.0 ± 1.9</td>
</tr>
<tr>
<td>PDEγ-R (0.5 μg)</td>
<td>1,490 ± 77</td>
<td>25.1 ± 1.6</td>
</tr>
<tr>
<td>PDEγ (7–18)</td>
<td>20.1 ± 8.2</td>
<td>102 ± 8</td>
</tr>
<tr>
<td>PDEγ (0.1 μg)</td>
<td>10.0 ± 1.1</td>
<td>205 ± 18</td>
</tr>
<tr>
<td>PDEγ (31–45)</td>
<td>808 ± 44</td>
<td>44.4 ± 4.7</td>
</tr>
<tr>
<td>PDEγ (29–45, T53A)</td>
<td>99.5 ± 7.4</td>
<td>13.8 ± 1.5</td>
</tr>
<tr>
<td>PDEγ (29–45, S40A)</td>
<td>1,309 ± 120</td>
<td>74.0 ± 6.0</td>
</tr>
<tr>
<td>PDEγ (61–72)</td>
<td>5 ± 0.3</td>
<td>180 ± 9</td>
</tr>
<tr>
<td>Histone III-S</td>
<td>1,050 ± 84</td>
<td>142 ± 12</td>
</tr>
</tbody>
</table>

*μg/ml.

**FIG. 1.** Time dependence of recombinant PDEγ (rPDEγ) phosphorylation. Pure rPDEγ (1.0 μg) was incubated with 55 milliunits of ROS PKC at 30 °C with [32P]ATP in a standard assay (20 μl) (see "Experimental Procedures"). After indicated intervals, 2-μl aliquots were used for determination of phosphate incorporation into PDEγ by the phosphocellulose paper method (see "Experimental Procedures"). Specific 32P incorporation into PDEγ was calculated as the difference between total incorporation (PKC + PDEγ) and incorporation of 32P into PKC (without PDEγ).
Retinal Phosphodiesterase Inhibitory Subunit Phosphorylation

Fig. 3. Plot of V/S versus V for phosphorylation of PDEγ in a PDEαβγ complex by ROS PKC. V is the observed rate (units/mg), and S is the concentration of free substrate (μM). Nonphosphorylated PDEγ was phosphorylated for 20 min at 30 °C with [β-32P]ATP, and phosphorylated products were separated by SDS-PAGE. Radioactive bands of PDEγ were excised, and radioactivity was determined by liquid scintillation counting.

Fig. 4A, inhibition of tPDE activity by phosphorylated rPDEγ. PDE activity was determined under standard assay conditions using [3H]cGMP and 0.10 ng of tPDE (10 min, 30 °C). B, Scatchard analysis of rPDEγ inhibition data obtained from the same experiment. The slopes (−Kd) were determined by linear regression analysis. C, Hill plots of rPDEγ inhibition data obtained from the same experiment. The slopes (nH) were determined by linear regression analysis.

Affinity of the PDEγ for Trypsin-activated Phosphodiesterase—The inhibition constant, IC50, for PDEγ and the dissociation constant, Kd, of the complex of the PDEγ with tPDE were determined by measuring the catalytic activity of mixtures of these species. Recombinant PDEγ inhibited tPDE activity with the same affinities as PDEγ isolated from bovine eyes. A treatment of pure rPDEγ and pure native PDEγ with alkaline phosphatase (1 unit/mg of PDEγ, 37 °C for 1 h) did not alter its inhibitory activity (data not shown). Recombinant PDEγ after phosphorylation by ROS PKC inhibits tPDE catalytic activity (IC50 = 60 ± 5 pm) better than nonphosphorylated rPDEγ (IC50 = 60 ± 5 pm) (Fig. 4A; similar results were obtained for native PDEγ).

For the equilibrium PDEγPDEc = PDEγ + PDEc (PDEc is the catalytic subunit of PDE, PDEα, or PDEβ), the concentrations of the species are related by [E]/[I] = ([Eγ] − [Eγ]/Kd, which is the form of the equation derived by Scatchard (1949). [E] is the concentration of the PDEγ–PDEc complex (catalytically inactive); [Eγ] is the total concentration of PDEc, and [I] is the concentration of free PDEγ. The total concentration of tPDE was kept constant, whereas the concentration of PDEγ was varied so that the catalytic activity of tPDE ranged from 2 to 88% of the uninhibited value. Linear (curvilinear) transformation of the inhibition curve was made based on the assumption that for the PDEαβ complex (tPDE) the concentration of tPDE in the assay (5.0 pm) is equivalent to the concentration of PDEα (5.0 pm) and PDEβ (5.0 pm) and that the binding of PDEγ to PDEα or PDEβ totally inhibits the activity of the corresponding catalytic subunit. [E] and [I] were calculated from [E] = (Percent inhibition/100)/[Eγ] and [I] equals ([Eγ] − [E]), where [Eγ] is the total concentration of PDEγ.

Detailed Scatchard analysis for PDEγ inhibition data (Fig. 4B) yields a plot that is curvilinear with downward concavity. In agreement with the finding of Deterre et al. (1988) that two PDEγ are associated with each PDEαβ complex, the Hill plots of inhibition data consistently display slopes (nH) of more than 1.0 (1.21–1.29) in the region passing through the log([E]/[Eγ] − [E])) axis (Fig. 4C). When certain experimental criteria are met (Williams and...
Lefkowitz, 1978), both of these graphic representations suggest either the existence of heterogeneous binding sites with discrete affinities, the existence of positively cooperative site-site interactions among the binding sites, or both. In contrast to the Scatchard plot for PDEγ-p is linear with value of $K_v = 23$ pm. Hill plots of inhibition data for PDEγ-p demonstrate values of $n_H$ close to 1.0 (0.98). These data suggest that phosphorylation of PDEγ not only increases its ability to inhibit PDEαβ catalytic activity but also eliminates the heterogeneous nature of inhibition of PDEαβ by PDEγ.

Identification of the Phosphorylation Site in PDEγ—We have used three approaches for determination of the phosphorylation site(s) in PDEγ for ROS PKC, 1) phosphoamino acid analysis of hydrolysates of $^{32}$P-labeled PDEγ, 2) phosphorylation by ROS PKC of synthetic peptides with native and mutant PDEγ partial sequences, and 3) phosphopeptide mapping after digestion of $^{32}$P-labeled PDEγ by Lys-C proteinase.

1) Phosphoamino acid analysis of hydrolysates of $^{32}$P-labeled PDEγ (phosphorylated in vitro by ROS PKC with incorporation of 0.9 ± 0.1 mol of phosphate/mol of PDEγ) by use of thin-layer electrophoresis identified Thr(P), but not Ser(P) and Tyr(P) (Fig. 5). For this analysis, samples of $^{32}$P-labeled PDEγ were acid hydrolyzed for 1, 2, or 4 h. Longer times of hydrolysis increased the relative yield of Thr(P). Ser(P) and Tyr(P) residues were not identified in any samples of $[^{32}P]PDEγ$.

2) In order to determine which of the 5 possible Thr residues in PDEγ are phosphorylated by ROS PKC, we used synthetic peptides with native and mutant PDEγ sequences as substrates for PKC (Fig. 6). This approach is acceptable for the study of PKC, which recognizes a specific motif (consensus phosphorylation site) in primary structures of target proteins. The principal substrate specificity determinants for PKC are located in short segments of the primary sequence around phosphorylation sites (Pearson and Kemp, 1991). We have found that two peptides that contain Thr$^{35}$: (peptide PDEγ29-45 with a native PDEγ sequence and peptide PDEγ31-45, Ser$^{40}$→Ala) are phosphorylated by ROS PKC: T35-Thr$^{35}$. The role of Thr$^{35}$ in phosphorylation was also demonstrated by the fact that peptide PDEγ(31-45, Thr$^{35}$→Ala) with a mutant PDEγ sequence (substitution Thr$^{35}$→Ala) was a poor substrate for phosphorylation by ROS PKC with a value of $V_{\text{max}}$ about 10 times less than for nonmutant peptide PDEγ(31-45) (Table I).

3) For phosphopeptide mapping, recombinant PDEγ after phosphorylation by ROS PKC with $[^{32}P]ATP$ was subjected to digestion by the endoproteinase Lys-C. This proteinase produces eight peptides from PDEγ including a QRQTRQFK peptide corresponding to residues 32–39 of PDEγ (with Thr$^{35}$). The PDEγ peptides were separated by the reverse-phase chromatography on a HPLC C-18 column (Fig. 7). Liquid scintillation counting of the collected fractions identified a single peak (Fig. 7B). This peak migrated identically with a standard of the synthetic $^{[32}P]QRQTRQFK$ peptide (Fig. 7C) under variable chromatographic conditions (different forms of acetonitrile gradients, up to 100% acetonitrile). Taken together these data suggest that ROS PKC phosphorylates Thr$^{35}$ in PDEγ.

**DISCUSSION**

A long range goal of this project is to provide a molecular explanation for the relationship between the phosphoinositol signal pathway and photoreceptor transduction. Visual trans-

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**Fig. 5. Identification of the amino acids phosphorylated in the PDEγ by ROS PKC.** $^{32}$P-Labeled PDEγ (0.9 mol of phosphate/mol of PDEγ) was subjected to acid hydrolysis (2 h). Phosphoamino acids were separated by electrophoresis on thin-layer cellulose plates, pH 3.5, and then autoradiographed. Authentic Ser(P), Thr(P), and Tyr(P) were run in the same lane and revealed by ninhydrin staining (shown by dot circles).

**Fig. 6. Bovine rod PDEγ structure.** Shown are sequences of peptides that were used for phosphorylation by protein kinase C. Localization of the site for binding to PDEαβ catalytic subunits is from Morrison et al. (1987), Lipkin et al. (1990), and Artemyev and Hamm (1992). Consensus phosphorylation site for PKC is from Pearson and Kemp (1991). Ser and Thr residues are boxed.

**Fig. 7. Peptide mapping of proteolyzed PDEγ.** Recombinant PDEγ (10 mg) was phosphorylated by ROS PKC with $[^{32}P]ATP$ and digested by the endoproteinase Lys-C (see “Experimental Procedures”). The PDEγ peptides were separated on a HPLC C-18 column by using a linear gradient of acetonitrile (5–70% from 5 to 25 min), and a detection at 230 nm (A). Fractions (0.1 min, 0.1 ml) were collected and analyzed by liquid scintillation counting (B). A QRQTRQFK peptide (residues 32–39 in PDEγ) was phosphorylated by ROS PKC with $[^{32}P]ATP$ and used as a standard. C, shown is the result of counting of the 0.1-min fractions after injection of the $[^{32}P]QRQTRQFK$ peptide.
event involving the events from the absorption of a photon by a rhodopsin pigment molecule to the electrical response of the cell (reviewed by Stryer (1991)). The α subunit of transducin (Tu-GTP) stimulates the activity of the phosphodiesterase (PDEαβγ) by removing the inhibitory subunit (PDEγ) from catalytic subunits (PDEαβ) (Fig. 8). The decrease in intracellular concentration of cGMP then leads to the closure of cGMP-regulated cation channels in the plasma membrane, resulting in a lowering of the cytosolic Na⁺ level, hyperpolarization of the cell (reviewed by Stryer 1987).

It is likely that other amino acids, outside of the RQT35 region, which is responsible for binding to PDEγ, have an adjacent hydrophobic residue on the COOH-terminal side of the phosphorylated residue (Pearson and Kemp, 1991). Twenty-three of the reported structures of PKC phosphorylation sites have this composition. Twenty-three of the reported structures of PKC phosphorylation sites have this composition.

ACKNOWLEDGMENTS—We thank John Sondek (Yale University, New Haven, CT) for providing the recombinant PDEγ clone and the protocol for purification of the recombinant PDEγ. We also thank Elin Ulug (Department of Biology, Kansas State University, Manhattan, KS) for help in phosphoamino acid analysis.

REFERENCES

The PDEγ phosphorylation site (Thr35) is located in the PDEγ region, which is responsible for binding to PDEβ3 (Morrison et al., 1987; Lipkin et al., 1990; Artemyev and Hamm, 1992). The location of Thr35 can explain two facts: 1) that phosphorylation of PDEγ changes the function of PDEγ in its ability to inhibit PDEβ3 catalytic activity, and 2) that free PDEγ is a better substrate for PKC than PDEγβ2 complex (in which Thr35 is blocked by catalytic subunits and is not accessible for phosphorylation). The phosphorylation site, Thr35, for ROS PKC, is located in PDEγ region RQT. The structure of this region is in complete correlation with one of the consensus phosphorylation sites (specificity motifs) for PKC, (K/R)XS(T/Y) (Pearson and Kemp, 1991). It is likely that other amino acids, outside of the RQT region but close to Thr35, are also important for specificity of ROS PKC. However, it is clear that ROS PKC, in its ability to recognize a specific motif in PDEγ belongs to a known series of reported PKCs (Pearson and Kemp, 1991).
Retinal Phosphodiesterase Inhibitory Subunit Phosphorylation