Protein kinase C in rod outer segments: effects of phosphorylation of the phosphodiesterase inhibitory subunit

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The inhibitory subunit (PDEγ) of the cGMP phosphodiesterase (PDE$\alpha\beta\gamma_2$) in rod outer segments (ROS) realizes its regulatory role in phototransduction by inhibition of PDE$\alpha\beta$ catalytic activity. The photoreceptor G-protein, transducin, serves as a transducer from the receptor (rhodopsin) to the effector (PDE) and eliminates the inhibitory effect of PDEγ by direct interaction with PDEγ. Our previous study [Udovichenko, Cunnick, Gonzalez and Takemoto (1994) J. Biol. Chem. **269**, 9850–9856] has shown that PDEγ is a substrate for protein kinase C (PKC) from ROS and that phosphorylation by PKC increases the ability of PDEγ to inhibit PDE$\alpha\beta$ catalytic activity. Here we report that transducin is less effective in activation of PDE$\alpha\beta(\gamma_p)_2$ (a complex of PDE$\alpha\beta$ with phosphorylated PDEγ, PDEγ_p) than PDE$\alpha\beta\gamma_2$. PDEγ_p also increases the rate constant of GTP hydrolysis of transducin (from 0.16 s^{-1} for non-phosphorylated PDEγ to 0.21 s^{-1} for PDEγ_p). These data suggest that phosphorylation of the inhibitory subunit of PDE by PKC may regulate the visual transduction cascade by decreasing the photoresponse.

INTRODUCTION

Visual excitation in vertebrates is mediated by a light-activated enzymic cascade [rhodopsin; transducin; phosphodiesterase (PDE)] that results in the hydrolysis of cGMP by PDE. The light-induced decrease in the level of cGMP closes cation-specific channels, which leads to the hyperpolarization of the plasma membrane and generation of the neural signal [1–9]. The bovine rod holoPDE 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 [10,11]. During phototransduction the interaction between the GTP-bound form of transducin (α_t-GTP) (α is the α-subunit of transducin) and PDEγ is critical for PDE activation. It has been reported that PtdIns(4,5)P_2 stimulates phosphorylation of PDEγ [12] and the kinase that was responsible for this phosphorylation was identified as rod outer segment (ROS) protein kinase C (PKC) [13,14].

For two proteins (the receptor, rhodopsin, and the effector, PDE) the functional effects of ROS PKC phosphorylation have been shown. PKC phosphorylates rhodopsin both *in situ* and *in vitro* and the functional consequence of this phosphorylation is the reduced ability of rhodopsin to activate transducin [15]. Phosphorylation of PDEγ by PKC increases its ability to inhibit PDE$\alpha\beta$ catalytic activity. Here we report that another result of this phosphorylation is the decreasing ability of α_t-guanosine 5′-[γ-thio]triphosphate (α_t-GTP[S]) to activate PDE$\alpha\beta(\gamma_p)_2$. Consequently, ROS PKC is implicated in the desensitization of visual transduction at at least two points, at receptor and effector levels.

EXPERIMENTAL PROCEDURE

Materials

Fresh bovine eyes were obtained from a local slaughterhouse (IBP, Emporia, KS, U.S.A.). GTP[S] was from Sigma (St. Louis, MO, U.S.A.), cyclic [8-^{3}H]GMP (15 Ci/mmol), which was purified further by anion-exchange chromatography, and [γ-^{32}P]ATP (3000 Ci/mmol) were from Du Pont–New England Nuclear. The t-butoxycarbonyl amino acids and their resins were from Vega Biochemicals, United States Biomedical Corp. (Cleveland, OH, U.S.A.) or Sigma. Vydac HPLC columns and TSK HPLC columns were from P. J. Cobert Associates (St. Louis, MO, U.S.A.), and DEAE-Sephacel, S-Sepharose, the FPLC Superose 12 HR 10/30 column and the FPLC Mono-S HR 5/5 column were from Pharmacia LKB Biotechnology.

PDE purification

ROS were prepared by the method described in [16]. Fresh bovine eyes were obtained from a local slaughterhouse within 1 h of slaughter. Retinas were removed under dim red light and stored without buffer at −70 °C in the dark. The ROS were purified by centrifugation in sucrose density gradients and washed with isotonic buffer [16]. PDE was eluted by resuspending the pellet in hypotonic buffer [10 mM Tris/HCl, pH 7.4/5 mM $MgCl_2$/1 mM dithiothreitol (DTT)/0.1 mM PMSF/1 μg/ml leupeptin/1 μg/ml pepstatin]. Soluble PDE was concentrated by ion-exchange chromatography on a DEAE-Sephacel column and was purified by HPLC on a TSK G3000SW column (7.5 mm × 75 mm) using a buffer of 150 mM Mops, pH 7.4/5 mM $MgCl_2$/1 mM 2-mercaptoethanol. Pure PDE samples were diluted with glycerol to 50 % (v/v) and stored at −20 °C. For preparation of trypsin-activated PDE (tPDE), bovine ROS PDE (500 μl, 100 μg/ml; before HPLC) was exposed to tosylphenylalanyl chloromethyl ketone (TPCK)-treated trypsin (5 μl, 1 mg/ml; 12000 units/mg) for 5 min on ice. The reaction was stopped by the addition of a 5-fold excess of soybean trypsin inhibitor (1 mg inhibits 1.7 mg of trypsin) and tPDE was purified by HPLC as described above. Soybean trypsin inhibitor was

Abbreviations used: ROS, rod outer segments; PDE, phosphodiesterase; PDEα and PDEβ, catalytic α- and β-subunits of PDE; PDEγ, inhibitory subunit of PDE; PDEγ_p, phosphorylated PDEγ; rPDEγ, recombinant PDEγ; tPDE, trypsin-activated PDE; α, α-subunit of transducin; α_t-GTP, GTP-bound form of transducin; PKC, protein kinase C; PS, phosphatidylserine; DG, 1,2-dioctanoyl-*sn*-glycerol; DTT, dithiothreitol; GTP[S], guanosine 5′-[γ-thio]triphosphate; CAPS, 3-cyclohexylamino-1-propanesulphonic acid; TPCK, tosylphenylalanyl chloromethyl ketone.

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purified in advance by gel-filtration on a HPLC TSK G3000SW column.

Transducin purification

Transducin was eluted by resuspending the pellet (after extraction of PDE) in hypotonic buffer with 10 μM GTP[S]. Soluble transducin was concentrated by ion-exchange chromatography on a DEAE-Sephacel column. Transducin α- and $\beta\gamma$-subunits were separated by FPLC on a Superose 12 column using a buffer consisting of 120 mM KCl, 100 μM $MgCl_2$, 20 mM Tris/HCl (pH 7.5) and 1 mM 2-mercaptoethanol.

Purification of wild-type PDEγ and recombinant PDEγ

Wild-type PDEγ was separated from PDE catalytic subunits by reverse-phase HPLC of pure PDE on a Vydac C-4 column. The plasmid for expressing full-length PDEγ was a gift from J. Sondek (Yale University, New Haven, CT, U.S.A.), and was made using standard PCR to introduced *Nde*I and *Bam*HI sites flanking the gene for PDEγ. The amplified gene was then subcloned into pET11a (Novagen, Madison, WI, U.S.A.) between *Nde*I and *Bam*HI sites. The template for the PCR was the synthetic gene for PDEγ residing in the plasmid [17] (clone FXSG). The coding portion of the gene for PDEγ has not been altered. Except for the coding portion of PDEγ, no other portion of the FXSG clone was present in the pET11a construct. Purification of the recombinant PDEγ (rPDEγ) was done as described [18].

PDE activity assay

Before being used in assays, [^{3}H]cGMP was purified [19]. PDE activity was determined as described previously [20]. The final concentrations in the reaction mixture were 40 mM Tris/HCl (pH 7.4), 5 mM $MgCl_2$, 100 μM [^{3}H]cGMP (100000 c.p.m./assay) in a final volume of 100 μl. Reactions were allowed to proceed for 10 min at 30 °C and were terminated by placing the tubes in a water bath at 70 °C for 2 min. The incubation at 70 °C in contrast to the incubation at 100 °C leads to a decrease in the non-enzymic hydrolysis of cGMP. Snake venom (100 μl, 1 mg/ml) was added to the cooled reaction tubes and the samples were incubated for 30 min at 30 °C. The samples were applied to columns of DEAE-Sephacel (0.5 ml bed volume) and eluted with 1.8 ml of water. Radioactivity was measured by liquid scintillation spectrometry. Specific hydrolysis of cGMP was determined as the difference between total hydrolysis of cGMP and non-enzymic hydrolysis of cGMP (without PDE).

Purification of PKC

For purification of PKC, ROS membranes after extraction of PDE (see the section on PDE purification) and transducin (see the section on transducin purification) were used. PKC was extracted by suspending the depleted ROS membranes in buffer with 10 mM Tris/HCl (pH 7.5), 2 mM EGTA, 2 mM EDTA, 1 mM DTT, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin and 0.5 mM PMSF (buffer D), followed by centrifugation (100000 ***g*** for 1 h). PKC extract was applied to a DEAE-Sephacel column (1 × 5 cm) equilibrated with buffer D at a flow rate of 1 ml/min. The column was then washed with five column volumes of buffer D and eluted with a linear gradient (16 ml) of 0 to 0.4 M NaCl/buffer D. Fractions (0.5 ml) were collected and tested for PKC activity and the protein concentrations were measured. GTP[S] present in the fractions made it difficult to obtain correct values for proteins by UV detection, therefore protein concentration was also determined using the method described in [21]. The active fractions were pooled and applied to a TSK G3000SW column (7.5 × 75 mm) (0.5 ml injection) equilibrated with 20 mM Tris/HCl (pH 7.5)/100 mM NaCl/0.5 mM EDTA/0.5 mM EGTA/5 mM 2-mercaptoethanol, at a flow rate of 0.8 ml/min. Fractions (0.4 ml) were collected and tested for enzyme activity as described below. Pure PKC samples were diluted with glycerol to 50 % and stored at −20 °C.

PKC activity assay

Phosphorylation of histone IIIS and PDEγ by PKC was accomplished as described [22]. The reaction mixture contained 20 mM Tris/HCl (pH 7.5), 15 mM $MgCl_2$, 0.1 mM EGTA, 62.5 μM EDTA, 0.1 % (v/v) Triton X-100, 10 μM [γ-^{32}P]ATP (100000–500000 c.p.m.), 200 μg/ml phosphatidylserine, 15 μg/ml 1,2-dioctanoyl-*sn*-glycerol/10 % (v/v) glycerol and 1 ng/μl PKC, in a final volume of 20 μl. The concentration of $CaCl_2$ was taken to 70 μM in order to create a 100 nM concentration of [Ca^{2+}] free in this assay. For preparative phosphorylation of PDE, 0.5–5 ml assays were used. The reactions were initiated by addition of the kinase and incubation was for 3 min (for determination of kinetic parameters) or 20 min (for complete incorporation of phosphate into PDEγ) at 30 °C. After the phosphorylation reaction a 10 μl aliquot of each sample was pipetted onto phosphocellulose paper (P81; Whatman) strips (1 × 2 cm), washed with 75 mM H_3PO_4 (4 × 2 min), and radioactivity was measured by liquid scintillation spectrometry. Specific incorporation of ^{32}P into substrate was determined as the difference between total incorporation of ^{32}P (PKC+ substrate) and incorporation of ^{32}P into PKC without substrate (autophosphorylation of PKC). One unit (U) of protein kinase activity is defined as the amount of enzyme catalysing the incorporation of 1 nmol of phosphate from ATP into histone IIIS per min at 30 °C under the assay conditions.

Separation of phosphorylated PDEγ (PDEγ_p) from non-phosphorylated PDEγ

Recombinant PDEγ was purified as described in the Experimental section. After PDEγ phosphorylation by ROS PKC, the products of the reaction (0.5–5 ml) were loaded onto S-Sepharose (0.5 ml, equilibrated with 50 mM Tris/HCl, pH 7.5/20 mM NaCl). The column was washed with 2 ml of the same buffer and the inhibitory subunit was eluted by 1 M NaCl/50 mM Tris/HCl, pH 7.5. Fractions containing PDEγ were pooled, lyophilized, dissolved in water and applied to a Sephadex G-10 column. Gel-filtration was performed in buffer with 20 mM Na-CAPS (CAPS is 3-cyclohexylamino-1-propanesulphonic acid), pH 10.0/20 mM NaCl. PDEγ_p and PDEγ were separated by ion-exchange chromatography on an FPLC Mono-S HR 5/5 column (2 % buffer B, 5 min; 80 % buffer B, 5–20 min) at a flow rate of 1 ml/min with detection at 280 nm (buffer A: 20 mM Na-CAPS, pH 10.0; buffer B: 20 mM Na-CAPS, pH 10.0/1 M NaCl). Fractions (0.1 min; 0.1 ml) were collected and PDEγ_p was detected by SDS/PAGE and/or by liquid scintillation counting of radioactivity when [^{32}P]PDEγ_p was used as a standard.

Reconstitution of holo-PDE with recombinant PDEγ and PDEγ_p

Reconstitution was performed as described previously [18]. Illuminated ROS membranes after sucrose-density-gradient centrifugation were washed twice with buffer C (120 mM KCl/100 μM $MgCl_2$/20 mM Tris/HCl, pH 7.5) and incubated with 2 mM recombinant PDEγ or PDEγ_p (with 25 μM rhodopsin) for

20 min at room temperature in buffer C with 200 μM GTP. Membranes were washed twice with buffer C to remove the excess recombinant PDEγ, PDEγ_p and the displaced native PDEγ. Concentration of PDE in membranes was determined by using a value of specific activity (V_{max}) for pure tPDE (hydrolysis of 123050 nM cGMP/min per mg of tPDE at 30 °C under the assay conditions). For determination of PDE concentration in the reconstituted systems, the aliquots of membranes (20 μl, 1 mg/ml protein) were exposed to TPCK-treated trypsin (2 μl, 1 mg/ml; 12000 U/mg) for 5 min on ice. The reaction was stopped by the addition of a 5-fold excess of soybean trypsin inhibitor (1 mg inhibits 1.7 mg of trypsin), and tPDE activity was determined as described above. For activation of PDE in the reconstitution systems by pure α_t-GTP[S], the membranes (10 μl, 1 ng PDE; buffer C) were mixed with α_t-GTP[S] (0–800 μM in buffer C) and incubated for 5 min at 30 °C. After that, 10 μl of cGMP solution (120 mM Tris/HCl, pH 7.4/15 mM $MgCl_2$/300 μM [^{3}H]cGMP, 100000 c.p.m.) was added and reactions were allowed to proceed for 10 min at 30 °C.

GTPase measurements

GTPase activity of transducin was performed as described [23]. After sucrose-density-gradient centrifugation, ROS were bleached and washed with an isotonic buffer (100 mM KCl/2 mM $MgCl_2$/1 mM DTT/10 mM Tris/HCl, pH 7.5) and three times by a hypotonic buffer (5 mM Tris/HCl, pH 8.0/0.5 mM EDTA/1 mM DTT). Before use the membranes were incubated for 5 h at room temperature. The reaction mixture contained 20 mM Tris/HCl, pH 7.5, 5 mM $MgCl_2$, 0.2 μM [γ-^{32}P]GTP (100000 d.p.m./assay) and 20 μM rhodopsin, in a final volume of 20 μl. The reaction was stopped by the addition of 100 μmol of 6 % (v/v) perchloric acid, and ^{32}P formation was measured as described in [24,25].

Western blot analysis

SDS/PAGE was performed on Laemmli-type mini-slab gels [26]. After separation by SDS/PAGE, proteins were transferred to nitrocellulose using a Transphor apparatus (Hoefer Scientific Instruments) in a buffer of 3 mM Na_2CO_3/10 mM $NaHCO_3$/10 % (v/v) methanol (2.5 V/cm; 1 h at room temperature). Blots were blocked for 30 min with 2 % (v/v) BSA. Antisera or purified antibodies were added at 1:100 with 2 % (v/v) BSA and 0.5 mM PMSF and incubated at room temperature for 2 h. Blots were washed three times with 30 mM sodium phosphate, pH 7.5/150 mM NaCl (buffer E) and incubated with ^{125}I-Protein A (100–200 Ci/mmol; 2×10^6 c.p.m./ml) in buffer E with 2 % (v/v) BSA for 1 h, followed by washing three times with buffer E. Exposure of the radioactive blots (usually overnight) to Cronex X-ray film and subsequent development revealed the proteins. Protein A was iodinated in a mixture of 5 μl of Na^{125}I (0.5 mCi; 2200 Ci/mmol), 2 μl of Protein A (10 μg/ml), 2 μl of 0.5 M potassium phosphate, pH 7.5, and 1 μl of chloramine T (2 mg/ml) for 1 min with mixing. The specific activity of ^{125}I-Protein A was approx. 100–200 Ci/mmol.

Miscellaneous methods

Protein concentrations were determined by the method described in [21] using BSA or PDEγ as standards. The concentration of PDEγ was determined by absorbance of PDEγ in water at 280 nm (ϵ 7090 $M^{-1} \cdot cm^{-1}$), as described [27]. SDS/PAGE was performed as described [26], using a separating gel of 16 % acrylamide/0.08 % bisacrylamide for detection of PKC, or 22 % acrylamide/0.11 % bisacrylamide for detection of PDEγ.

RESULTS

ROS PKC purification

Table 1 and Figure 1 give details of the ROS PKC purification results. The procedures include preparation of ROS membranes by centrifugation in a sucrose density gradient, followed by washing with isotonic and hypotonic buffers containing Mg^{2+}. Under these conditions, PKC bound tightly to membranes and could be extracted with hypotonic buffer containing EGTA and EDTA. ROS PKC was purified to homogeneity by successive chromatography on DEAE-Sephacel and HPLC TSK G3000SW. PKC exhibits an M_r of approx. 83000, as determined by SDS/PAGE. By HPLC gel-filtration on TSK G3000SW, an M_r of 85000 was calculated, indicating that the enzyme, in solution, is monomeric. ROS PKC undergoes autophosphorylation. The purified PKC has a specific activity (V_{max}) for histone IIIS of 1050 ± 84 units/mg and $K_m = 142 \pm 12$ μg/ml. The PKC purification procedure described yields about 21 μg of pure PKC from 400 retinas with a recovery of 40 %. The V_{max} and K_m values for phosphorylation of PDEγ by PKC have been reported previously [28,29].

The advantage of this described procedure for ROS PKC purification is the ability to use the same ROS membranes for

Table 1 Purification of PKC from bovine ROS

Fraction	Protein (μg)	Total activity (units)	Specific activity (units · mg^{-1})	Fold purification	Yield (%)
Soluble protein extract*	1260†	54.0‡	43	1	100
DEAE-Sephacel eluate	203	34.4	169	4.0	63
TSK-3000 eluate	21	22.1	1050	24.8§	40

* ROS membranes were purified by centrifugation in a sucrose density gradient, followed by washing with isotonic and hypotonic buffers, and PKC was solubilized in buffer with 2 mM EGTA and 2 mM EDTA (see Experimental section).

† Values shown are based on 400 bovine retinas (210 g wet weight).

‡ Activity was determined by using histone IIIS as a substrate.

§ Fold purification was determined using depleted ROS as a starting point.

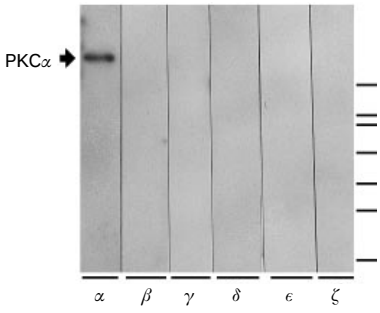

Figure 1 Western blot of pure ROS PKC

Samples of pure ROS PKC (100 ng of protein per lane) were separated by SDS/PAGE, followed by electrophoretic transfer to nitrocellulose membranes. Each membrane was incubated with type α-, β-, γ-, δ-, ϵ- or ζ-PKC-specific antibody as indicated. All antibodies were used at a 1:100 dilution and were purified before use by affinity chromatography on peptide–agarose columns. The immunoreactive bands were detected by autoradiography after incubation with ^{125}I-Protein A. An autoradiograph (12 h exposure at −70 °C) is shown. Molecular mass standards (M_r: 97400, 66000, 45000, 36000, 29000, 24000, 20100 and 14000) are indicated by marks to the right.

consistent extraction of ROS proteins (PDE by hypotonic buffer with Mg^{2+}, transducin by hypotonic buffer with Mg^{2+}, and GTP and PKC by hypotonic buffer with EDTA/EGTA). The abundance of PKC in ROS is about 0.4–0.8 $U \cdot mg^{-1}$ and a 1000–2000-fold purification is required for preparation of pure PKC. Our procedure of washing the membranes with isotonic and hypotonic buffers (before PKC extraction) allows a considerable enrichment of ROS PKC-containing membranes. After extraction of PKC by EDTA/EGTA the specific activity of ROS PKC was 43 $U \cdot mg^{-1}$ and approx. 25-fold purification was required for preparation of pure PKC.

Identification of the ROS PKC isoenzyme

The synthetic peptides used for antisera production contained sequences unique to PKCα, PKCβ, PKCγ, PKCδ, PKCϵ and PKCζ [30–32] are described in the Experimental section. Peptides corresponded to sequences from the V3 region of PKCα, PKCβ and PKCγ, or sequences from the C-terminus (V5 region) of PKCδ, PKCϵ and PKCζ. The structure of the PKCβ synthetic peptide is common to PKCβI and PKCβII (these subspecies differ from each other only in a short stretch at their C-terminal end region, V5). The sequences corresponding to these peptides are highly conserved in rabbit, rat, bovine and human genomes [30,31]. A computer search indicated that these peptides are not homologous to known sequences of other proteins. The specificity of antibodies raised against the peptide immunogens have been reported [13]. Previously we have reported [13] that in bovine ROS membranes a single major immunoreactive species of M_r 83000 was detected with anti-PKCα antibodies. In order to identify the subtype of pure ROS PKC we used antibodies specific for PKCα, PKCβ, PKCγ, PKCδ, PKCϵ and PKCζ and have shown that purified ROS PKC is an α type PKC (Figure 1). For correct interpretation of these results, all antibodies were checked for their ability to identify different types of PKC in brain.

Separation of PDEγ and PDEγ_p

The level of PDEγ phosphorylation by ROS PKC varied and was usually 60–90 %. In order to study the biochemical properties of PDEγ_p we separated PDEγ_p from non-phosphorylated PDEγ by FPLC ion-exchange chromatography on a Mono-S column. Conditions for PDEγ_p separation were optimized by using Na-CAPS buffer with a pH of 10.0, which is close to the isoelectric points of non-phosphorylated PDEγ (10.17) and PDEγ_p (9.44). The isoelectric points were determined by computer analysis of PDEγ structure. Recovery of PDEγ_p after isocratic elution by 0.8 M NaCl was usually 30–35 %, with satisfactory separation of PDEγ and PDEγ_p (Δ 0.5 min). If a gradient of NaCl concentration was used, this resulted in a more complete separation of PDEγ and PDEγ_p (Δ 3 min), however, this dramatically decreased the recovery of PDEγ_p to 3–5 %. For all experiments with PDEγ_p we used pure samples of PDEγ_p.

Effect of PDEγ_p on GTPase activity of transducin

It has previously been reported [23,33–35] that PDEγ in the presence of ROS membranes stimulates GTPase activity of α_t-GTP. For these experiments, PDE was removed from ROS membranes by washing with hypotonic buffer, and depleted membranes were used for determination of GTPase activity. The 'single turnover' technique used for the study of GTPase activity of transducin was as described previously [23]. The rate of GTP hydrolysis for membranes (1 mg/ml membrane protein) without

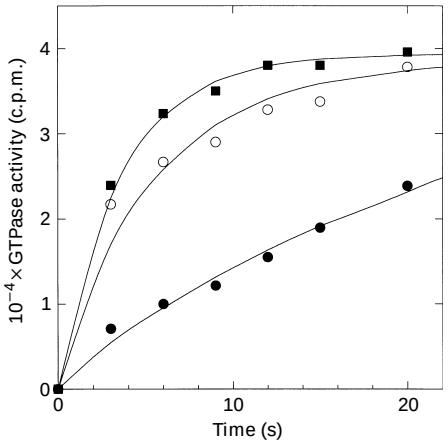

Figure 2 Effect of PDEγ_p on GTP hydrolysis by transducin

GTPase activity of transducin was determined in depleted ROS membranes (1 mg/ml membrane protein) that did not contain PDE (●), and in the presence of 0.5 μM recombinant PDEγ (○) or 0.5 μM PDEγ_p (□). At indicated times the reaction mixture was quenched with 6% perchloric acid. The rate constants of GTP hydrolysis were determined by single exponential fits of the data.

PDEγ was 0.05 s^{-1} (Figure 2), which was almost identical with that previously reported (0.058 s^{-1}, [24]). As shown in Figure 2, PDEγ_p (0.5 μM) accelerates GTPase activity of α_t-GTP somewhat more effectively than non-phosphorylated PDEγ (0.5 μM) from 0.16 s^{-1} for non-phosphorylated PDEγ to 0.21 s^{-1} ($P < 0.006$).

Activation by transducin of the holo-PDE reconstituted with PDEγ_p

Trypsin digests not only PDEγ but also PDE$\alpha\beta$ catalytic subunits, and preparation of tPDE has its own limitation in studies of PDE activation by transducin. Native PDEγ has been replaced by exogenous PDEγ (PDEγ_p) after incubation of ROS in the presence of GTP with an excess of wild-type PDEγ or PDEγ_p. After washing the membranes in buffer C to remove the excess free PDEγ, reconstituted holo-PDE was used for activation by α_t-GTP[S]. Figure 3 shows the activation by increasing amounts of α_t-GTP[S] of holo-PDE reconstituted with either wild-type PDEγ or PDEγ_p. The activation by purified α_t-GTP[S] was less effective when PDEγ_p was used for reconstitution.

These data suggest that phosphorylation of the inhibitory subunit of PDE by ROS PKC may regulate visual transduction by decreasing the photoresponse during light adaptation.

DISCUSSION

The rod visual-transduction cascade includes the events from the absorption of a photon by a rhodopsin molecule to the electrical response of the rod cell. The photoexcited rhodopsin then activates holo-transducin ($\alpha\beta\gamma$) by promoting the exchange of GTP for bound GDP on α_t. During phototransduction, the interaction between the GTP-bound form of transducin (α_t-GTP) and PDEγ is critical for PDE activation. The resulting decrease in cGMP concentration then leads to the closure of the cGMP-gated cation channels in the plasma membrane, resulting in a lowering of Na^+, hyperpolarization of the membrane and generation of the neural signal. The activated α_t-GTP is returned to the resting α_t-GDP state by its intrinsic GTPase activity. This

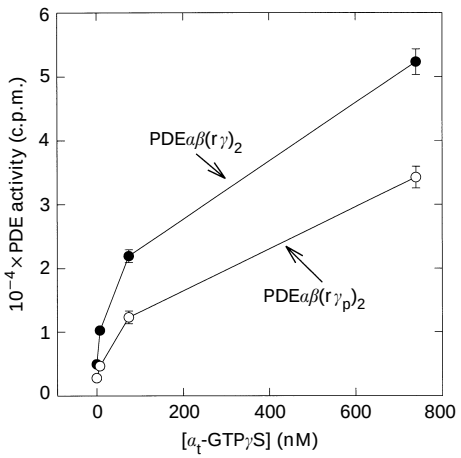

Figure 3 Activation, by α_t-GTP[S], of holo-PDE in the reconstitution system

Native PDEγ in non-depleted ROS membranes was substituted by rPDEγ or PDEγ_p after incubation of membranes (25 μM rhodopsin) with 1.5 μM rPDEγ or PDEγ_p in the presence of 200 μM GTP. Exogenous α_t-GTP[S] was used for activation of PDE in both PDE$\alpha\beta$(rγ)$_2$ (●) and PDE$\alpha\beta$(γ_p)$_2$ (○) reconstitution systems. PDE activity was determined under standard assay conditions using [^{3}H]cGMP.

results in the rebinding of α_t-GDP to the transducin $\beta\gamma$ and in the release of the inhibitory PDEγ subunit, which then rebinds to and inhibits the PDE$\alpha\beta$ catalytic subunits [1–9].

Although the nature of the receptor and G-protein for the phosphoinositide system in ROS is still unclear, for two proteins (receptor, rhodopsin and effector, PDE) the functional effects of ROS PKC phosphorylation have been shown [14,15,28,36,37]. As our data suggest (reported in this paper and previously [28]), phosphorylation of PDEγ by ROS PKC has multiple effects on the process of inhibition-activation of PDE. (1) Phosphorylation of PDEγ increases its ability to inhibit PDE$\alpha\beta$ catalytic activity (tPDE). (2) The other functional result of PDEγ phosphorylation is the elimination of the functional heterogeneity of the PDEγ population. A possible physiological role for the two classes of the binding sites (for non-phosphorylated PDEγ) could be in the graded response of PDE to bleaches of varying intensity [38]. (3) For a reconstituted system with PDE$\alpha\beta$(γ_p)$_2$ (a complex of PDE$\alpha\beta$ with phosphorylated PDEγ), the transducin is less effective in activation of PDE. (4) PDEγ_p also increases the rate constant of GTP hydrolysis of transducin. Therefore, the phosphorylation of PDEγ principally changes the inhibition properties of PDEγ, and a physiological role for these changes could be to provide a more effective turn-off of light-activated PDE. This altered regulation of visual transduction may play a role in light adaptation.

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