

# Phosphorylation of bovine rod photoreceptor cyclic GMP phosphodiesterase

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The cyclic GMP phosphodiesterase (PDE) of retinal rods plays a key role in phototransduction and consists of two catalytic subunits (PDE $\alpha$  and PDE $\beta$ ) and two identical inhibitory subunits (PDE $\gamma$ ). Here we report that PDE $\alpha$  and PDE $\gamma$  are phosphorylated by protein kinase(s) C (PKC) from brain and rod outer segments (ROS). These same two types of PKC also phosphorylate PDE $\alpha$  in trypsin-activated PDE (without PDE $\gamma$ ). In contrast, cyclic-AMP-dependent protein kinase catalytic subunit phosphorylates both PDE $\alpha$  and PDE $\beta$ , but not PDE $\gamma$ . This kinase does not phosphorylate trypsin-activated PDE. The

synthetic peptides AKVISNLLGPAAV (PDE $\alpha$  30–44) and KQRQTRQFKSKPPKK (PDE $\gamma$  31–45) inhibited phosphorylation of PDE by PKC from ROS. These data suggest that sites (at least one for each subunit) for phosphorylation of PDE by PKC are localized in these corresponding regions of PDE $\alpha$  and PDE $\gamma$ . Isoenzyme-specific PKC antibodies against peptides unique to the  $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ ,  $\epsilon$  and  $\zeta$  isoforms of protein kinase C were used to show that a major form of PKC in ROS is PKC $\alpha$ . However, other minor forms were also present.

## INTRODUCTION

Retinal rod outer segment (ROS) cyclic GMP (cGMP) phosphodiesterase (PDE) is activated by light through a signal transduction from rhodopsin to transducin. The light-induced decrease in the level of cyclic GMP closes cation-specific channels, which leads to the hyperpolarization of the plasma membrane and generation of the neural signal (reviewed by Stryer, 1991). Bovine rod PDE consists of three kinds of polypeptide chains: catalytic subunits  $\alpha$  (88 kDa, PDE $\alpha$ ) and  $\beta$  (84 kDa, PDE $\beta$ ), and two identical inhibitory  $\gamma$  subunits (13 kDa, PDE $\gamma$ ) (Baehr et al., 1979; Deterre et al., 1988). PDE activation during phototransduction results from removal of PDE $\gamma$  by transducin  $\alpha$ -GTP (T $\alpha$ -GTP), the  $\alpha$  subunit of the GTP-binding regulatory protein present in ROS.

Post-translational processing has been found to occur on all PDE subunits. The C-terminus of both the PDE $\alpha$  and the PDE $\gamma$  subunits contains a signal sequence for a complex series of post-translational modifications. Bovine rod PDE $\alpha$  and PDE $\beta$  are methyl-esterified at a C-terminal cysteine residue (Swanson and Applebury, 1983; Ong et al., 1989; Catty and Deterre, 1991; Anant et al., 1992), and are differentially prenylated, by farnesylation and geranylgeranylation respectively (Anant et al., 1992). Prenyl modification of PDE may play a role in membrane attachment and in correctly positioning the PDE molecule for phototransduction (Catty and Deterre, 1991; Anant et al., 1992). PDE $\gamma$  is phosphorylated by cytosolic protein kinase(s) derived from intact frog ROS (Hayashi et al., 1991). The role of this phosphorylation is unclear, and there is no information about phosphorylation of PDE catalytic subunits. Post-translation modification of PDE may be a critical step for normal function of PDE. In this paper we report that PDE $\alpha$  and PDE $\gamma$  are phosphorylated by a protein kinase C (PKC). Cyclic-AMP-dependent protein kinase catalytic subunit (APK) phosphorylates PDE $\alpha$  and PDE $\beta$ .

## EXPERIMENTAL

### Materials

Fresh bovine eyes were obtained from a local slaughterhouse (Iowa Beef Packers, Emporia, KS, U.S.A.). PKC (rat brain) was from Calbiochem (La Jolla, CA, U.S.A.) (lot 151191). APK (bovine heart) (lot 110H9640) and trypsin [bovine pancreas, type XIII, treated with tosylphenylalanylchloromethane ('TPCK'); EC 3.4.21.4; 12000 units/mg] were from Sigma (St. Louis, MO, U.S.A.). Trypsin inhibitor (soybean, type 1-S) was also from Sigma, and was purified further by h.p.l.c. gel-filtration. [8-<sup>3</sup>H]-cGMP (5 Ci/mmol), which was purified further by anion-exchange chromatography, [ $\gamma$ -<sup>32</sup>P]ATP (3000 Ci/mmol) and Na<sup>125</sup>I (2200 Ci/mmol) were from Du Pont–New England Nuclear; L-[4,5-<sup>3</sup>H]leucine (143 Ci/mmol) was from Amersham (Arlington Heights, IL, U.S.A.). The t-butoxycarbonyl (t-Boc) amino acids and their resins were from Vega Biochemicals, United States Biomedical Corp. (Cleveland, OH, U.S.A.), or Sigma. Vydac h.p.l.c. columns and TSK h.p.l.c. columns were from P. J. Cobert Associates (St. Louis, MO, U.S.A.), Affi-Gel 10 was from Bio-Rad, DEAE-Sephacel and Sephadex G-25 were from Pharmacia LKB Biotechnology, nitrocellulose was from S & S, X-ray film was from Du Pont, and developing solutions were from Kodak.

### Peptide synthesis and purification

Peptides were synthesized manually from *N*-t-Boc-L-amino acid derivatives by the method of Merrifield (1963) as modified by Gorman (1984). Cleavage of the peptides from the resins and protecting groups was accomplished with anhydrous HF (Stewart and Young, 1984). Cleaved peptides were purified (Morrison et al., 1989) by reverse-phase chromatography on a h.p.l.c. Vydac C-18 column. Peptide concentrations were determined by amino acid analyses (Lockhart et al., 1982) by using a reverse-phase

Abbreviations used: ROS, rod outer segments; cGMP, cyclic GMP; PDE, retinal ROS cGMP phosphodiesterase; PDE $\alpha$  and PDE $\beta$ , catalytic  $\alpha$  and  $\beta$  subunits of PDE; PDE $\gamma$ , inhibitory  $\gamma$  subunit of PDE; PKC, protein kinase C; PKC $\alpha$ , PKC $\beta$ , PKC $\gamma$ , PKC $\delta$ , PKC $\epsilon$  and PKC $\zeta$ , the  $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ ,  $\epsilon$  and  $\zeta$  isoenzymes of PKC (Ogita et al., 1990); APK, cyclic-AMP-dependent protein kinase catalytic subunit; KLH, keyhole-limpet haemocyanin; PMSF, phenylmethanesulphonyl fluoride; DTT, dithiothreitol; GTP[S], guanosine 5'-[ $\gamma$ -thio]triphosphate.

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h.p.l.c. Vydak C-18 column and *o*-phthalaldehyde as a detecting reagent.

#### Antisera production, purification and characterization

The peptides were coupled to keyhole-limpet haemocyanin (KLH) as previously described (Oppert et al., 1991). Rabbits were injected three or four times, subcutaneously, every 2 weeks with 400  $\mu$ l of a suspension (1:1) of KLH-peptide/Freund's complete adjuvant (first injection) or KLH-peptide/Freund's incomplete adjuvant (subsequent injections). Sera were tested by using a radioimmunoassay and/or Western-blot analysis.

Antibodies were purified by affinity chromatography with peptide-agarose. Each peptide (2 ml, 10 mg/ml) was mixed with 1 g of Affi-Gel 10 (Bio-Rad) and rotated (on a nutator) for 16 h at 4 °C. Unreacted *N*-hydroxysuccinimide ester groups on the agarose were blocked by addition of 1.0 M ethanolamine hydrochloride, pH 8.0, for 1 h at 4 °C. For antibody purification, 5 ml of serum was mixed with 20 ml of buffer A [30 mM sodium phosphate, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% polyvinylpyrrolidone ( $M_r$  40000), 0.1% Tween 20] and precipitated material was removed by centrifugation at 40000 *g* for 30 min (SS-34, Sorvall). The column with affinity support (0.5 ml) was washed with buffer A and diluted serum was applied to the column at room temperature. After unbound proteins had passed through the column, the column was eluted with 0.1 M glycine/HCl, pH 3.5 (2 ml), and monospecific antibodies were collected in tubes with 0.2 M Hepes, pH 8.0. Pure antibodies were stored in 50% glycerol at -20 °C with 0.5 mM phenylmethanesulphonyl fluoride (PMSF) and 1 mg/ml leupeptin.

The solid-phase radioimmunoassay was a modification of the method of Suter (1982) as previously described (Takemoto et al., 1992).

#### Electrophoresis and Western blot analysis

SDS/PAGE was performed on Laemmli-type mini-slab gels (Laemmli, 1970) by using high-resolution conditions optimized by Catty and Deterre (1991) for separation of the PDE $\alpha$  and PDE $\beta$  polypeptides (16% acrylamide and 0.08% bisacrylamide in the separating-gel bed). In some experiments for identification of peptides with low molecular mass (about 2 kDa) the separating gel consisted of two parts: top one-third with 16% acrylamide/0.08% bisacrylamide and bottom two-thirds with 30% acrylamide/0.15% bisacrylamide. After separation by SDS/PAGE, proteins were transferred to nitrocellulose. Blots were blocked for 30 min with 2% BSA in buffer A. Antisera or pure antibodies were added at 1:100 in buffer A with 2% BSA and 0.5 mM PMSF and incubated at room temperature for 2 h. Blots were washed three times with buffer A and incubated with <sup>125</sup>I-Protein A (100–200 Ci/mmol, 2  $\times$  10<sup>6</sup> c.p.m./ml) in buffer A with 2% BSA for 1 h, followed by washing three times with buffer A. Exposure of the radioactive blots (usually overnight) to Cronex X-ray film and subsequent development revealed the proteins.

#### ROS preparation

ROS were prepared by the method of Papermaster and Dreyer (1974). Fresh bovine eyes were obtained from a local slaughterhouse within 1 h 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 in the dark. All procedures were performed under dim red light unless noted otherwise. Each retina was suspended in 1 ml of ROS buffer 1 [10 mM Tris/HCl, pH 7.4, 2 mM MgCl<sub>2</sub>, 65 mM NaCl, 1 mM dithiothreitol (DTT), 0.5 mM PMSF, 34% (w/w) sucrose] and shaken vigorously for 1 min. After centrifugation at 4000 rev./min for 5 min (Sorvall SS-34 rotor), the pellet was resuspended in 15 ml of ROS 1 and re-centrifuged as above. Pooled supernatants were diluted 1:2 in ROS buffer 2 (10 mM Tris/HCl, pH 7.4, 1 mM MgCl<sub>2</sub>, 1 mM DTT, 0.5 mM PMSF) and centrifuged at 7000 rev./min for 10 min (Sorvall SS-34 rotor). Pelleted ROS were resuspended in 12 ml of ROS buffer 3 (10 mM Tris/HCl, pH 7.4, 1 mM MgCl<sub>2</sub>, 1 mM DTT, 0.5 mM PMSF, 26.3% sucrose), suspended by forcing through a 26-gauge needle and layered (2 ml/tube) on to a discontinuous sucrose-density gradient consisting of 1 ml each of 1.11, 1.13 and 1.15 g/ml sucrose in ROS buffer 2. After centrifugation at 24000 rev./min (55000 *g*) for 45 min (Beckman SW50.1 rotor), ROS discs were collected at the 1.11/1.13 g/ml sucrose interface. ROS discs were then washed with ROS buffer 7 (10 mM Tris/HCl, pH 7.4, 100 mM NaCl, 5 mM MgCl<sub>2</sub>, 1 mM DTT, 0.1 mM PMSF) and centrifuged at 18000 rev./min for 30 min (Sorvall SS-34 rotor). After washing twice more as described above, the ROS membranes were resuspended in ROS buffer 7 and stored at -70 °C.

#### PDE purification and preparation of trypsin-activated PDE

ROS membranes were washed three times in ROS buffer 7 and pelleted each time at 18000 rev./min for 30 min (Sorvall SS-34 rotor). Soluble PDE was eluted from the washed membranes by resuspending the pellet in ROS buffer 8 (10 mM Tris/HCl, pH 7.4, 1 mM DTT, 0.1 mM PMSF, 1  $\mu$ g/ml leupeptin, 1  $\mu$ g/ml pepstatin), suspended by forcing through a 26-gauge needle, incubating under bright light for 30 min on ice, and pelleting the membranes at 18000 rev./min for 30 min (Sorvall SS-34 rotor). The elution was repeated twice and the pooled supernatants were re-centrifuged at 100000 *g* for 1 h. Soluble PDE was then concentrated by column ion-exchange chromatography with DEAE-Sephacel. PDE was further purified by h.p.l.c. on a TSK G3000SW column (7.5 mm  $\times$  75 mm) using a buffer of 150 mM Mops, pH 7.4, 5 mM MgCl<sub>2</sub> and 1 mM  $\beta$ -mercaptoethanol. Pure PDE samples were stored in 50% glycerol at -20 °C. Purity of PDE was assessed by SDS/PAGE. For preparation of trypsin-activated PDE, bovine ROS PDE (500  $\mu$ l, 100  $\mu$ g/ml; before h.p.l.c.) was exposed to TPCCK-treated trypsin (5  $\mu$ l, 1 mg/ml; 12000 units/mg) for 5 min on ice. The reaction was stopped by addition of a 5-fold excess of soybean trypsin inhibitor (1 mg inhibits 1.7 mg of trypsin) and trypsin-activated PDE was purified by h.p.l.c. as described above. Soybean trypsin inhibitor was purified in advance by gel filtration on a h.p.l.c. TSK G3000SW column. Non-purified soybean trypsin inhibitor contains minor components which are phosphorylated by kinases, and this makes the identification of PDE phosphorylation difficult to interpret.

#### PDE activity assay

Before use in the assay, [<sup>3</sup>H]cGMP was purified as described by Kincaid and Manganiello (1988). Radiolabelled substrate was applied to a column (0.5 cm  $\times$  2 cm) of DEAE-Sephadex A-25 equilibrated with water. The column was washed with 4 ml of water, followed by exactly 800  $\mu$ l of 50 mM HCl, then eluted with 3 ml of 50 mM HCl. Fractions were neutralized with 1 M Tris/HCl, pH 7.5. PDE activity was determined as described by Hansen et al. (1988). The final concentrations in the reaction

mixture were 40 mM Tris/HCl, pH 7.4, 5 mM MgCl<sub>2</sub> and 100 μM [<sup>3</sup>H]cGMP (100000 c.p.m./assay) in a final volume of 100 μl. The reaction was allowed to proceed for 10 min at 30 °C, and was terminated by placing the tubes in a boiling-water bath for 2 min. Snake venom (100 μl, 1 mg/ml) was added to the cooled reaction tubes and 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.

### PDE phosphorylation and protein kinase activity assays

Phosphorylation of PDE by PKC was accomplished by methods optimized by Huang et al. (1988), by using lipid/detergent mixed micelles. The reaction mixture contained 30 mM Tris/HCl, pH 7.5, 6 mM MgCl<sub>2</sub>, 0.25 mM EGTA, 0.4 mM CaCl<sub>2</sub>, 0.04% Nonidet P-40, 10 μM [<sup>32</sup>P]ATP, 100 μg/ml phosphatidylserine, 20 μg/ml dioctanoylglycerol, 0.1–1 μg of PDE, and PKC in a final volume of 10–25 μl. The reaction mixture of PDE phosphorylation by APK contained 30 mM Tris/HCl, pH 7.4, 2 mM MgCl<sub>2</sub>, 10 μM [<sup>32</sup>P]ATP, 0.1–1 μg of PDE, and APK in a final volume of 10–25 μl. The reactions were initiated by addition of the appropriate kinase, and incubation was for 5 min (for PKC) or 15 min (for APK) at 30 °C. Reactions were stopped by the addition of 20 μl of electrophoresis sample buffer, followed by SDS/PAGE. For determination of incorporation of <sup>32</sup>P into histone, the reaction mixture included 1 mg/ml histone III. After incubation, tubes were placed on ice and reactions were stopped by addition of 200 μl of ice-cold 0.5% BSA, immediately followed by 800 μl of ice-cold 25% (w/v) trichloroacetic acid (Kuo and Greengard, 1970). After 30 min incubation on ice, samples were applied to Whatman GF/F filters and washed three times with ice-cold 10% trichloroacetic acid and twice with acetone before drying and scintillation counting.

### Transcription and translation *in vitro*

Plasmids pGE $\alpha$ P and pGE $\beta$ P were a gift from Nikolai Khrantsov (Shemyakin Institute of Bioorganic Chemistry, Moscow, Russia). These plasmids consist of PDE $\alpha$  cDNA (pGE $\alpha$ P) and PDE $\beta$  cDNA (pGE $\beta$ P), which were inserted into pGEM-2 Vector (Promega Corp., Madison, WI, U.S.A.) under the control of the SP6 promoter (Ishchenko et al., 1989; Lipkin et al., 1990b,c). Plasmids pGE $\alpha$ P and pGE $\beta$ P were linearized with *Pvu*II and *Hind*III respectively, and transcription and translation *in vitro* were performed as described by Zozulya et al. (1990) and Lipkin et al. (1990a) by using a nuclease-treated rabbit reticulocyte lysate (Promega). The usual yield was 6.1 ± 2.5 μg/ml PDE $\alpha$  and 2.7 ± 1.3 μg/ml PDE $\beta$ . These proteins were used as molecular-mass markers.

### Purification of ROS PKC

For this, membranes after extraction of PDE (as described above) and transducin (T $\alpha$ -GTP[S]) were used. T $\alpha$ -GTP[S] was eluted by resuspending the pellet in ROS buffer 8 with 10 μM GTP[S], suspended by forcing through a 26-gauge needle and pelleting the membranes at 18000 rev./min for 30 min (Sorvall SS-34 rotor). The elution was repeated twice. PKC was extracted by suspending the depleted ROS membranes in buffer of composition 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 B), followed by

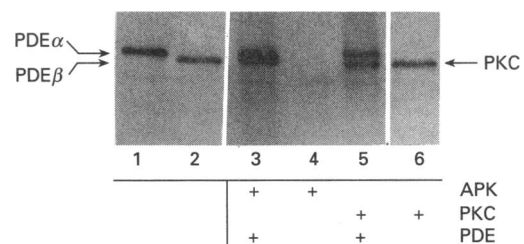
centrifugation (100000 g for 1 h). The PKC extract was applied to a DEAE-Sephacel column (1 cm × 5 cm) equilibrated with buffer B at a flow rate of 1 ml/min. The column was then washed with 5 column vol. of buffer B and PKC was eluted with a linear gradient (16 ml) of 0–0.4 M NaCl in buffer B; 1 ml fractions were collected and tested for PKC activity and protein concentration. GTP[S] present in the fractions made it difficult to obtain a correct protein concentration by u.v. 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 mm × 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 above.

### RESULTS

Studies on the phosphorylation of PDE by PKC were accomplished in two systems. The first utilized a commercial PKC from brain, which consists mostly of the  $\alpha$ -isozyme form. This enzyme and a commercial catalytic subunit of APK were utilized to phosphorylate a purified PDE from ROS. The second system utilized PKC isolated from ROS.

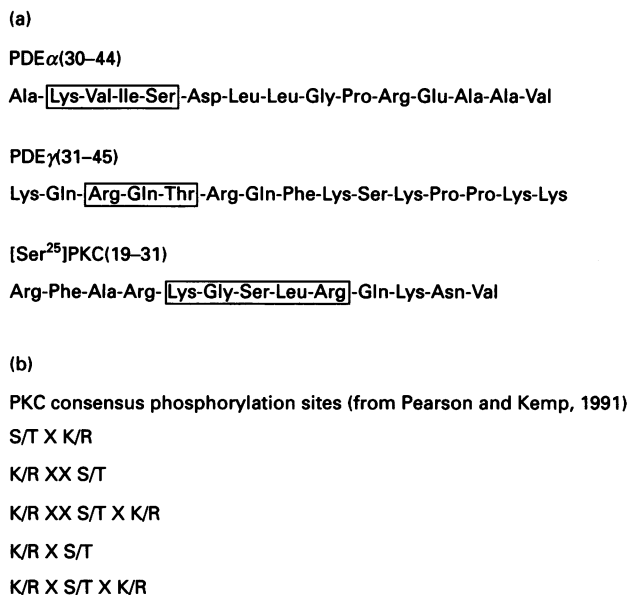
Bovine PDE (PDE $\alpha\beta\gamma_2$ ), purified from dark-adapted ROS membranes, was phosphorylated by PKC and APK (Figure 1). PKC phosphorylates the PDE $\alpha$  (Figure 1) and PDE $\gamma$  (results not shown) subunits of PDE. PDE $\alpha$  from a trypsin-activated PDE is also phosphorylated by PKC to the same level as control PDE (results not shown). Since trypsin activation of PDE removed PDE $\gamma$  (Miki et al., 1975; Hurley and Stryer, 1982), this means that PDE $\gamma$  is not necessary for PDE $\alpha$  phosphorylation by brain PKC. The APK from bovine heart phosphorylates both the PDE $\alpha$  and PDE $\beta$  subunits (Figure 1), but not PDE $\gamma$ . This kinase does not phosphorylate trypsin-activated PDE (results not shown).

In order to identify the sites on PDE $\alpha$  and PDE $\beta$  which are phosphorylated by PKC, we synthesized two peptides, corresponding to PDE $\alpha$  residues 30–44 (AKVISNLLGPAAV) (Lipkin et al., 1990c) and PDE $\gamma$  residues 31–45 (KQRQTRQFKSKPPKK) (Ovchinnikov et al., 1986) (Figure 2). PDE $\alpha$  contains a Ser at residue 34 which is absent from PDE $\beta$



**Figure 1** Phosphorylation of PDE from bovine ROS by APK and PKC

For phosphorylation pure PDE after gel-filtration on a TSK G3000SW column was used. The reaction mixture for PDE phosphorylation by APK contained 30 mM Tris/HCl, pH 7.4, 2 mM MgCl<sub>2</sub>, 10 μM [<sup>32</sup>P]ATP (5 μCi), 0.1 μg of PDE and 0.1 μg of APK (10 μl). The reaction mixture for phosphorylation by PKC from rat brain contained 30 mM Tris/HCl, pH 7.5, 6 mM MgCl<sub>2</sub>, 0.25 mM EGTA, 0.4 mM CaCl<sub>2</sub>, 0.04% Nonidet P-40, 10 μM [<sup>32</sup>P]ATP (5 μCi), 100 μg/ml phosphatidylserine, 20 μg/ml dioctanoylglycerol, 0.1 μg of PDE and 0.1 μg of PKC in a final volume of 10 μl. Reactions were incubated for 30 min. Reaction products were separated by SDS/PAGE, followed by fluorography (lanes 1 and 2) or autoradiography (lanes 3–6). Lanes 1 and 2 show catalytic subunits of PDE:  $\alpha$  (lane 1) and  $\beta$  (lane 2) were synthesized in translation system *in vitro* with [<sup>3</sup>H]leucine and used as molecular-mass standards.



**Figure 2** (a) Amino acid sequences of synthetic peptides, and (b) structures of PKC motifs

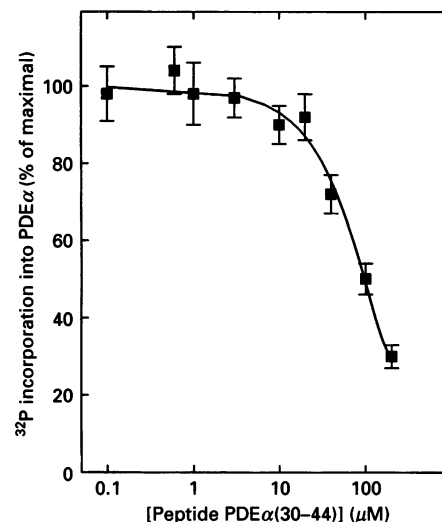
(a) Sequences of PDE $\alpha$ (30–44) (bovine rod PDE; Lipkin et al., 1990b,c) and PDE $\gamma$ (31–45) (bovine rod PDE; Ovchinnikov et al., 1986), which contains consensus phosphorylation sites for PKC. Peptide [Ser<sup>25</sup>]PKC(19–31) was also synthesized and used as a control in some experiments. The pseudosubstrate analogue peptide [Ser<sup>25</sup>]PKC(19–31) is derived from the inhibitor peptide PKC(19–36) by replacing alanine with serine, which can then be phosphorylated by PKC (House and Kemp, 1987). Consensus sites are boxed. (b) Structures of PKC motifs are from Pearson and Kemp (1991). K/R and S/T means Lys or Arg, and Ser or Thr.

(Lipkin et al., 1990c). This sequence contains a consensus phosphorylation site motif for PKC, KXXS (Pearson and Kemp, 1991). A region of PDE $\gamma$  peptide 31–45 also contains a PKC motif RXT (Pearson and Kemp, 1991) and is rich in the basic amino acids preferred for effective phosphorylation by PKC. These peptides were used either as PKC substrates in competition binding experiments (Figure 3) or directly (Figure 4).

PDE $\alpha$  phosphorylation by brain PKC is inhibited by PDE $\alpha$  peptide 30–44 in a dose-dependent manner (Figure 3). The PDE $\gamma$  peptide 31–45 not only inhibits phosphorylation of PDE $\gamma$  (results not shown), but is also phosphorylated by PKC from rat brain (Figure 4). These data suggest that PKC phosphorylates specific sites on PDE $\alpha$  and PDE $\gamma$  which are unique to each subunit.

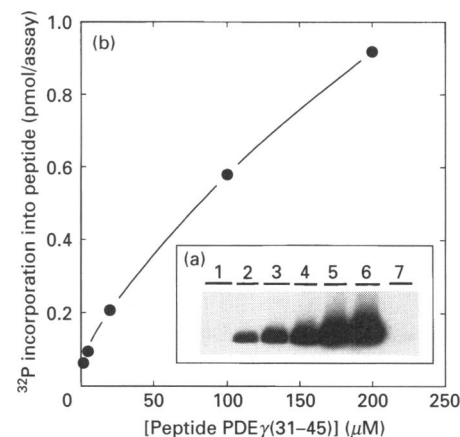
In a PDE assay *in vitro*, purified PDE activity is not altered by phosphorylation with either APK (Figure 5b; 1 and 2) or by brain PKC (Figure 5b; 3 and 4). Likewise, dephosphorylation by alkaline phosphatase had no effect on PDE activity (Figure 5a).

After establishing that ROS PDE $\alpha$  and PDE $\gamma$  serve as substrates *in vitro* for PKC, we wished to determine if PDE served as a substrate for PKC from ROS. PDE interacts with many proteins in ROS, and these interactions could be affected by phosphorylation. To begin to study the effects of ROS PKC on PDE, we initiated purification and characterization of PKC from ROS. PKC isoforms are known to exist in many tissues. However, it should be mentioned that, thus far, most proteins which have been sequenced from rod or cone sources have been found to be unique gene products. This has been reported for PDE, transducin and arrestin (reviewed by Stryer, 1991). It is therefore possible that unique kinases or phosphatases will be



**Figure 3** Phosphorylation of PDE $\alpha$  by PKC in the presence of peptide PDE $\alpha$ (30–44)

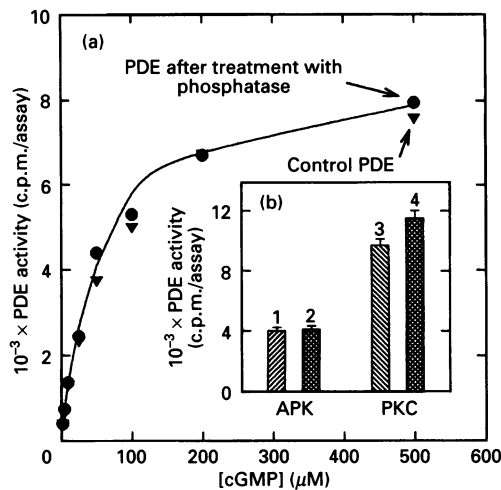
Phosphorylation of PDE by PKC from brain was done in a standard assay (see the Experimental section) with 10  $\mu$ M [ $\gamma$ -<sup>32</sup>P]ATP (5  $\mu$ Ci) and different concentrations of peptide PDE $\alpha$ (30–44) at 30 °C for 30 min. After the phosphorylation reaction and separation of proteins by SDS/PAGE, PDE $\alpha$  bands were excised and radioactivity was determined by scintillation counting. Incorporation of <sup>32</sup>P into PDE $\alpha$  without peptide PDE $\alpha$ (30–44) was taken as 100%. The results of a single experiment are shown; this experiment was repeated twice with the same result.



**Figure 4** Phosphorylation of a peptide PDE $\gamma$ (31–45) by PKC

Phosphorylation of peptide PDE $\gamma$ (31–45) by PKC from brain was done in the standard assay (see the Experimental section) with 10  $\mu$ M [ $\gamma$ -<sup>32</sup>P]ATP (5  $\mu$ Ci) and different concentrations of peptide PDE $\gamma$ (31–45) at 30 °C for 60 min. (a) After the phosphorylation reaction, peptides were separated by SDS/PAGE and phosphorylated peptides were revealed by autoradiography. Lane 1, reaction mixture in the absence of PKC and peptide; lanes 2–6, different concentrations of PDE $\gamma$ (31–45) in a phosphorylation assay (2, 5, 20, 100 and 200  $\mu$ M respectively, lanes 2–6); lane 7, phosphorylation without peptide. (b) Radioactive bands were excised and radioactivity was determined by scintillation counting. Specific <sup>32</sup>P incorporation into peptide was determined as the difference between total radioactivity in the band and radioactivity without peptide. Results are of a single gel. This experiment was repeated twice more with the same result.

present in ROS, such as the rhodopsin kinase (Thompson and Findlay, 1984). In order to aid in preliminary identification of the ROS PKC, isoform-specific peptide antibodies were developed



**Figure 5** Effect of phosphatase and protein kinases on PDE activity

(a) Pure PDE was treated with alkaline phosphatase (1 unit of alkaline phosphatase activity per  $\mu\text{g}$  of PDE) for 30 min at  $30^\circ\text{C}$ , and PDE activity was determined at different cGMP concentrations as described in the Experimental section. (b) PDE was phosphorylated (as described in the Experimental section, but without  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ ) by PKC from brain (bars 3 and 4) or APK from heart (bars 1 and 2), and PDE activity was determined in standard assay conditions with  $100\ \mu\text{M}$  cGMP. For controls (bars 1 and 3) protein kinases were treated for 2 min at  $100^\circ\text{C}$  before the assay to destroy enzyme activity. PDE activity was expressed in c.p.m., as means  $\pm$  S.E.M. of three independent measurements.

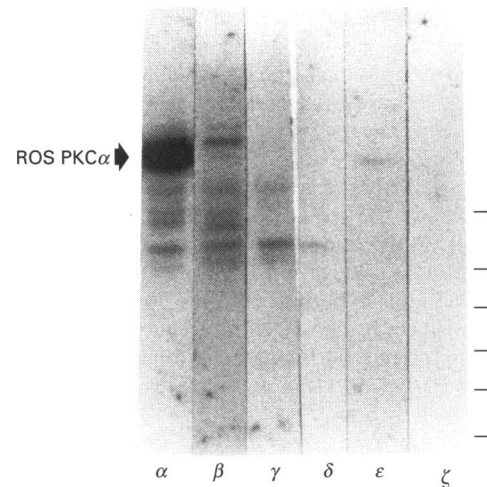
**Table 1** Amino acid sequences of synthetic peptides for preparation of isoenzyme-specific PKC antibodies

Peptides were synthesized by the method of Merrifield (1963) as modified by Gorman (1984). All peptides contained three additional Lys residues on the C-terminus (not shown in the Table) for cross-linking to KLH (see the Experimental section). Peptide-KLH conjugates were used for rabbit injection and antibody production. PKC isoenzyme sequences were obtained from Ono et al. (1988, 1989), Makowske et al. (1988) and Henrich (1991). Peptides were synthesized on the basis of sequences unique to the  $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ ,  $\epsilon$  or  $\zeta$  isoforms of PKC or a sequence conserved in all six forms ('con-pep') (see the Results section).

PKC isoenzyme	Peptide	Amino acid residues	Sequence
PKC $\alpha$	$\alpha$ -pep	313–326	AGNKVISPSDDRQ
PKC $\beta$	$\beta$ -pep	313–329	GPKTPEEKANTISKED
PKC $\gamma$	$\gamma$ -pep	306–318	NYPLELYERVRTG
PKC $\delta$	$\delta$ -pep	662–673	SFVNPKEYEQFLE
PKC $\epsilon$	$\epsilon$ -pep	726–737	KGFSYFGEDLMP
PKC $\zeta$	$\zeta$ -pep	577–592	GFEYINPLLLSAEESV
Consensus	cons-pep	381–394( $\gamma$ )	ILKDKVIVQDDVD

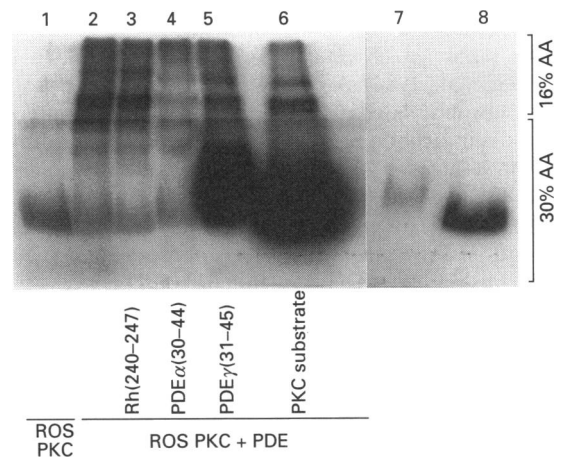
(Table 1). Peptides were synthesized based on sequences unique to the  $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ ,  $\epsilon$  or  $\zeta$  isoforms of PKC or to a sequence conserved in all six forms (Ono et al., 1988, 1989; Makowske et al., 1988; Henrich, 1991). Peptides corresponded to sequences from the V3 region of PKC $\alpha$ , PKC $\beta$  and PKC $\gamma$ , or to sequences from C-terminus (V5 region) of PKC $\delta$ , PKC $\epsilon$  and PKC $\zeta$ . The structure of  $\beta$ -peptide is common for PKC $\beta$ I and PKC $\beta$ II (these subspecies differ from each other only in a short range at their C-terminal end region, V5).

Antisera did not cross-react with other peptides. These antisera were used in Western blots of ROS preparations (Figure 6). It is



**Figure 6** Western blots of PKC isoenzymes from bovine ROS

ROS were prepared as described in the Experimental section and soluble proteins were extracted by suspension of ROD in hypotonic buffer with EDTA and EGTA. Soluble protein extracts ( $25\ \mu\text{g}$  of protein in each lane) were separated by SDS/PAGE, followed by electrophoretic transfer to nitrocellulose membranes. Each membrane was incubated (see the Experimental section) with type  $\alpha$ -,  $\beta$ -,  $\gamma$ -,  $\delta$ -,  $\epsilon$ - or  $\zeta$ -specific antibody as indicated below the lanes ( $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ ,  $\epsilon$  or  $\zeta$ ). All antibodies were used at 1:100 dilution and were purified before use (see the Experimental section). The immunoreactive bands were detected by autoradiography after incubation with  $^{125}\text{I}$ -Protein A. An autoradiograph (12 h exposure at  $-70^\circ\text{C}$ ) is shown. Molecular-mass standards (66, 45, 36, 29, 24 and 20.1 kDa, from top to bottom) are indicated by marks to the right.



**Figure 7** Phosphorylation of PDE by ROS PKC in the presence of different peptides

Purified ROS PKC was used for PDE phosphorylation in a standard assay for 60 min at  $30^\circ\text{C}$ . Reaction products were separated by SDS/PAGE, followed by autoradiography. The separating gel consisted of two parts: top one-third with 16% acrylamide (AA) and bottom two-thirds with 30% acrylamide. Phosphorylation was done in the absence (lane 1) or in the presence (lanes 2–8) of  $0.1\ \mu\text{g}$  of PDE. For competition assays, peptides corresponding to rhodopsin (Rh) residues 240–247 (lane 3), PDE $\alpha$ (30–44) (lane 4), PDE $\gamma$ (31–45) (lanes 5 and 7) and a synthetic substrate for PKC (House and Kemp, 1987),  $[\text{Ser}^{25}]\text{PKC}(19\text{--}31)$  (lanes 6 and 8) were included in the reaction. The concentration of  $[\text{Ser}^{25}]\text{PKC}(19\text{--}31)$  in the phosphorylation assay was  $10\ \mu\text{M}$ ; other peptides were used at  $200\ \mu\text{M}$ . The autoradiograph of lanes 7 and 8 is from the same gel as lanes 5 and 6, but at different exposure times (for 12 h at  $-70^\circ\text{C}$ , lanes 1–6, or 15 min at room temperature, lanes 7 and 8).

apparent that the major form of PKC (85 kDa) is PKC $\alpha$ -reactive, but minor forms of PKC- $\beta$ , - $\gamma$  and - $\epsilon$  were also present

in a soluble protein preparation of ROS. Similar results were obtained by using whole ROS (results not shown). Although these antisera may not identify a ROS-unique PKC, the Western-blot results in Figure 6 suggest that one major PKC is an  $\alpha$  isoform, as previously suggested by use of column chromatography (Wolbring and Cook, 1991).

In order to further characterize the PKC from ROS, we purified PKC and showed that ROS PKC and PKC from brain phosphorylate PDE $\alpha$  and PDE $\gamma$  in a similar manner (results not shown). For PKC purification, ROS membranes after washing with isotonic and hypotonic buffers were used. PKC was solubilized with chelating reagents and purified by ion-exchange chromatography and gel filtration. We have previously shown that peptide PDE $\alpha$ (30–44) is a competitive inhibitor of PDE $\alpha$  phosphorylation by brain PKC (Figure 3). However, this peptide was not a substrate (results not shown). The peptide PDE $\gamma$ (31–45) is phosphorylated by brain PKC (Figure 4). When used in a competition assay with ROS PKC, the peptide PDE $\alpha$ (30–44) inhibited the phosphorylation of PDE $\alpha$  by ROS PKC (Figure 7). The peptide PDE $\gamma$ (31–45) and a synthetic substrate for PKC, [Ser<sup>25</sup>]PKC(19–31), are phosphorylated by ROS PKC (Figure 7). These data suggest that sites (at least one for each subunit) for phosphorylation of ROS PDE by ROS PKC are localized in corresponding regions of PDE $\alpha$  and PDE $\gamma$ .

## DISCUSSION

Phosphorylation of proteins plays one of the most fundamental roles in cell regulation. In vertebrate rod photoreceptors, one of the well-studied mechanisms of termination of phototransduction is achieved by the phosphorylation of multiple serine and threonine residues in the C-terminal region of rhodopsin by rhodopsin kinase, a 68 kDa cytosolic protein (Wilden et al., 1986; Miller et al., 1986). Arrestin, a 48 kDa cytosolic protein, is bound to phosphorylated rhodopsin to prevent it from interacting with transducin. Rhodopsin dephosphorylation is performed by protein phosphatase 2A (Fowles et al., 1989; Palczewski et al., 1989). The possibility that PKC is also involved in phototransduction by phosphorylating rhodopsin was explored *in situ* and *in vitro* (Kelleher and Johnson, 1986; Newton and Williams, 1991). The functional consequence of PKC phosphorylation of rhodopsin was a decreased ability to stimulate the light-dependent rhodopsin activation of GTP[S] binding to transducin (Kelleher and Johnson, 1986). Limited proteolysis of rhodopsin, phosphorylated *in situ*, indicates that PKC modifies rhodopsin on a domain distinct from that recognized by rhodopsin kinase (Newton and Williams, 1991). PKC has the same affinity for unbleached and bleached rhodopsin and may be involved in light adaptation at low light levels, where most of the rhodopsin is unbleached, whereas rhodopsin kinase desensitizes rhodopsin at high light levels (Newton and Williams, 1991).

The outer segment of vertebrate rod photoreceptors has been reported to be a rich source of PKC (Kelleher and Johnson, 1985; Wolbring and Cook, 1991). There is about 1 mol of PKC per 2000 mol of rhodopsin (Kelleher and Johnson, 1985). Immunocytochemical analysis of rat retina showed that PKC is concentrated in photoreceptor outer segments as well as in outer and inner plexiform layers (Wood et al., 1988). PKC was purified from bone ROS (Kelleher and Johnson, 1985; Wolbring and Cook, 1991) and has a molecular mass of approx. 85 kDa. Addition of the PKC activator oleoylacetyl-glycerol to electropermeabilized ROS decreases the amplitude of the photoresponse and dark levels of cGMP up to 40%, and depresses the light-stimulated decrease in cGMP levels (Binder et al., 1989).

The light-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate in photoreceptor cells has been demonstrated by biochemical and immunocytochemical studies by many research groups (Brown et al., 1984; Ghalayini & Anderson, 1984; Hayashi & Amakawa, 1985; Hayashi et al., 1987; Szuts et al., 1986; Das et al., 1987). In this regard, a phospholipase C that hydrolyses phosphatidylinositol 4,5-bisphosphate has been identified in ROS (Gehm and McConnell, 1990). However, the true function of the phosphoinositides in the phototransduction mechanism has not been clarified yet. In order to study the role of phosphorylation in the visual transduction process, we have studied the phosphorylation of PDE, a key enzyme in signal transduction, by PKC and APK.

The PKC from brain or the partially purified PKC from ROS both phosphorylated PDE $\alpha$  and PDE $\gamma$ . PDE $\alpha$  phosphorylation was not altered by trypsin activation, a process which removes the PDE $\gamma$  (Hurley and Stryer, 1982) and a C-terminal portion of PDE $\alpha$  (Catty and Deterre, 1991). Preliminary results using peptide PDE $\alpha$ (30–44) suggest that this is a major phosphorylation site on PDE $\alpha$ , one which would not be removed by trypsin activation. It is noteworthy that the peptide does not itself serve as a substrate, but only competes. This may be because the peptide does not contain all of the optimum site. In contrast, the peptide PDE $\gamma$ (31–45) both competes for PKC phosphorylation of PDE $\gamma$ , and is itself a substrate.

In our Western blots, the major form of PKC was immunoreactive with an antipeptide antiserum directed against a region of brain PKC $\alpha$  isoform. This does not necessarily imply that the ROS PKC is identical in sequence with brain PKC $\alpha$ , since multiple isoforms are known to exist. Indeed, when separation was attempted in DEAE-Sephacel and h.p.l.c. TSK-3000 columns, multiple PKC activity peaks were observed in ROS supernatants (results not shown). Some did not react with anti-PKC $\alpha$  antisera. The results with peptide competition further suggest that multiple PKC isoforms may exist. Both peptides competed, yet varied in sequence. It is therefore possible that other distinct ROS forms of PKC may exist.

The role of PKC phosphorylation of PDE $\alpha$  or PDE $\gamma$  has not been studied in detail. As shown recently by Hayashi et al. (1991), PDE $\gamma$  is phosphorylated by unknown cytosolic protein kinase(s), derived from intact frog ROS. Our study suggests that PKC from brain, as well as PKC from ROS, phosphorylates PDE $\gamma$  (in complex with PDE $\alpha\beta$ ). This kinase is membrane-bound in the presence of bivalent cations and can be solubilized in hypotonic buffer with chelating reagents. For PDE $\gamma$  phosphorylation, Hayashi et al. (1991) used a supernatant (designated as 'crude kinase') after suspension of ROS membranes in isotonic buffer without bivalent cations and chelating reagents. So it is not clear if PKC is present in solution under these conditions. If not, then this implies that there are at least two types of kinases which can phosphorylate PDE $\gamma$ . Binder et al. (1989) found evidence for only three protein kinases in ROS: a PKC-like activity, APK and rhodopsin kinase. On the other hand, the low-molecular-mass proteins, components I and II (12 and 11 kDa), in frog ROS are preferential substrates of cGMP-dependent protein kinase (Polans et al., 1979; Hermolin et al., 1982). We did not see phosphorylation of PDE $\gamma$  by APK from bovine heart. Maybe this kinase is not an effective catalyst when bovine PDE $\gamma$  is used as a substrate.

Although the phosphorylation does not alter PDE activity *in vitro*, we have not determined effects on PDE interaction with other proteins. For these data, it appears that PKC $\alpha$  and possibly other isoforms of PKC exist in ROS and exhibit substrate specificity for differing sequences on two distinct subunits of PDE.

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