Phosphorylation of the γ Subunit of the Retinal Photoreceptor cGMP Phosphodiesterase by the cAMP-Dependent Protein Kinase and Its Effect on the γ Subunit Interaction with Other Proteins†

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ABSTRACT: Cyclic GMP phosphodiesterase, a key enzyme in phototransduction, is composed of P$\alpha\beta$ and two Pγ subunits. Interaction of Pγ with P$\alpha\beta$ or with the α subunit (Tα) of transducin is crucial for the regulation of cGMP phosphodiesterase in retinal photoreceptors. Here we have investigated phosphorylation of Pγ by cAMP-dependent protein kinase and its functional effect on the Pγ interaction with P$\alpha\beta$ or Tα in vitro. Pγ, but not Pγ complexed with Tα (both GTP and GDP forms), is phosphorylated. Measurement of ^{32}P radioactivity in phosphorylated Pγ, analysis of phosphorylated Pγ by laser mass spectrometry, identification of phosphoamino acid, and phosphorylation of mutant forms of Pγ indicate that only threonine 35 in Pγ is phosphorylated. Phosphorylation of Pγ mutants also reveals that the C and N terminals of Pγ which are required for the regulation of P$\alpha\beta$ functions are not involved in the Pγ phosphorylation but that arginine 33, which is ADP-ribosylated by an endogenous ADP-ribosyltransferase, is required for the phosphorylation. Phosphorylated Pγ has a higher inhibitory activity for trypsin-activated cGMP phosphodiesterase than nonphosphorylated Pγ, indicating that the Pγ-P$\alpha\beta$ interaction is affected by Pγ phosphorylation. Nonphosphorylated Pγ inhibits both the GTPase activity of Tα and the binding of a hydrolysis-resistant GTP analogue to Tα, while Pγ phosphorylation reduces these inhibitory activities. These observations suggest that a Pγ domain containing threonine 35 is involved in the Pγ-Tα interaction, and Pγ phosphorylation regulates the Pγ-Tα interaction. Our observation suggests that Pγ phosphorylation by cAMP-dependent protein kinase may function for the regulation of phototransduction in vertebrate rod photoreceptors.

Mechanisms to regulate the cGMP level in ROS[1] are fundamental to vertebrate phototransduction (*1*−*3*). The PDE regulation is one of these mechanisms. Illuminated rhodopsin stimulates GTP/GDP exchange on Tα followed by dissociation of GTP·Tα from T$\beta\gamma$. GTP·Tα interacts with PDE. PDE is composed of P$\alpha\beta$ and two Pγ subunits. P$\alpha\beta$ hydrolyzes cGMP (*4*, *5*) and binds cGMP to its cGMP-specific, high-affinity, noncatalytic sites (*6*−*8*). In amphibian ROS, Pγ regulates these P$\alpha\beta$ functions as an inhibitor of cGMP hydrolysis (*9*) and as a stimulator of cGMP binding to the noncatalytic sites (*10*, *11*). The Pγ-GTP·Tα interaction interrupts these Pγ functions by releasing Pγ from P$\alpha\beta$ (*9*−*11*). We have recently indicated that functionally different Pγ is released in different steps of phototransduction (*12*). When cGMP concentration is high (∼5 μM), a Pγ responsible for the inhibition of cGMP hydrolysis is preferentially released, and cGMP is hydrolyzed by P$\alpha\beta$. Thus cGMP concentration in ROS is reduced. When cGMP concentration is low (less than ∼0.5 μM), a Pγ responsible for the stimulation of cGMP binding to the high-affinity sites is released, followed by the rapid release of cGMP from one of these high-affinity sites. Because these noncatalytic sites are the major cGMP binding sites in amphibian ROS (*6*), binding more than 90% of the cellular cGMP (*8*), and cGMP bound to these sites is more than enough to increase cGMP concentration to the dark level, cGMP released from the noncatalytic sites is expected to stimulate the recovery of cytoplasmic cGMP concentration to the dark level.

Post-translational modification of proteins is a general and fundamental mechanism for the regulation of cellular processes. Previous studies have described phosphorylation and

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[1] Abbreviations: ROS, rod outer segments; PDE, cGMP phosphodiesterase; P$\alpha\beta$ and Pγ, $\alpha\beta$ and γ subunits of PDE; Tα and T$\beta\gamma$, α and $\beta\gamma$ subunits of transducin; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; BHT, butylated hydroxytoluene; PI, phosphatidylinositol; PCR, polymerase chain reaction; DTT, dithiothreitol; PMSF, phenylmethylsulfoxyl fluoride; MALDI-TOF mass spectrometry, matrix-assisted laser desoption ionization time-flight mass spectrometry.

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ADP-ribosylation of ROS proteins. Rhodopsin phosphorylation has been studied as a mechanism to terminate the initial step of the light-activated biochemical processes (*2*, *13*, *14*). A 33-kDa protein, phosducin, is phosphorylated by PKA (*15*, *16*), and phosphorylation of phosducin regulates phototransduction through regulation of T$\beta\gamma$ functions (*17*). Pγ is also phosphorylated by several kinases, including PI-stimulated kinase (*18*), PKC (*19*), and Pγ kinase (*20*, *21*). The physiological functions of Pγ phosphorylation by PI-stimulated kinase and PKC are not clear in the current scheme of phototransduction. However, Pγ phosphorylation by Pγ kinase may imply an important mechanism for phototransduction. Pγ complexed with GTP·Tα is phosphorylated by Pγ kinase, and GTP·Tα concomitantly loses its interaction with phosphorylated Pγ. Phosphorylated Pγ, which has higher inhibitory activity for cGMP hydrolysis by P$\alpha\beta$ than nonphosphorylated Pγ does, returns to active (Pγ-less or Pγ-free) PDE and then PDE is inactivated. Therefore, phosphorylation of Pγ by Pγ kinase functions to turn off GTP·Tα-activated PDE without GTP hydrolysis. In addition, guanylyl cyclase (*22*, *23*) and proteins named components I and II (*24*−*26*) have been reported to be phosphorylated. However, the physiological functions of these protein phosphorylations are not clear. In addition, Tα (*27*, *28*) and Pγ (*29*) have been shown to be ADP-ribosylated by an endogenous ADP-ribosyltransferase(s). Characterization of the Tα ADP-ribosylation has not been profoundly studied, while the Pγ ADP-ribosylation was reported in detail (*29*). Arginines 33 and 36 in Pγ are ADP-ribosylated, but only one arginine is modified at a time. Pγ complexed with P$\alpha\beta$ is ADP-ribosylated, but Pγ complexed with Tα (both GTP- and GDP-bound forms) is not ADP-ribosylated, suggesting that a Pγ domain containing these arginines is required for its interaction with Tα, but not with P$\alpha\beta$.

Pγ interactions with other proteins in ROS are pivotal in the phototransduction mechanism. However, studies of the regulatory mechanism of these interactions began only very recently. Pγ phosphorylation by Pγ kinase (*20*) and PKC (*19*) increased its inhibitory activity for cGMP hydrolysis by P$\alpha\beta$, indicating that these Pγ phosphorylations regulate the Pγ interaction with P$\alpha\beta$. Phosphorylation of Pγ by Pγ kinase also abolishes the Pγ-Tα interaction (*21*), and ADP-ribosylation of Pγ may also regulate Pγ interaction with Tα (*29*). In this study, we have extended these studies using the phosphorylation of Pγ by PKA in vitro. This study has shown that this phosphorylation regulates Pγ interactions with P$\alpha\beta$ and Tα. We have also confirmed our previous observation that a Pγ domain containing arginines 33 and 36 is a target for the regulation of the Pγ-Tα interaction.

EXPERIMENTAL PROCEDURES

Materials. Chemical reagents were purchased from the following sources: [γ-^{32}P]GTP, [γ-^{32}P]ATP, [^{35}S]GTPγS, and [^{3}H]cGMP were from DuPont-New England Nuclear; ATP, cAMP, cGMP, GTPγS, GTP, pepstatin, and leupeptin were from Boehringer Manhaim; phosphoserine, phosphothreonine, and phosphotyrosine are from Sigma. Antibodies against peptides derived from the regulatory (amino acid 1−16) and catalytic (amino acid 335−350) subunits of bovine heart PKA were generous gifts from Dr. Yasuo Fukami (Kobe University).

Table 1. Oligonucleotides Used in Site-directed Mutagenesis

mutants	oligonucleotides[a]
T22V	5′-CCCTTTCCTGGGGACGACGGGTCCCC-3′
T35V	5′-GAACTGCCTCACTTGCCGCTGC-3′
N22Del	5′-GACATATACATATGCGTAAGGGCCCGCCG-3′
C10Del	5′-GGGCCAGCGGATCCTACTCCAGGTGGTTG-3′

[a] Underlined letters indicate mutation sites.

Immunological Detection of the Regulatory and Catalytic Subunits of Bovine ROS PKA. Intact ROS were prepared as described (*30*). The ROS preparations (200 μg of protein) were suspended in 100 μL of a buffer (100 mM Tris·HCl (pH 7.5), 5 mM $MgCl_2$, 5 mM DTT, 1 mM EGTA, 0.1 mM PMSF, 0.0025% BHT) and homogenized by passing through #21 gauge needle (×7). The soluble and membrane fractions were separated by centrifugation (40000*g*, 10 min, 4 °C). We refer to the soluble fraction as isotonic extract. The membrane fraction was washed with the same buffer (×3). Then, membrane-bound proteins were extracted by washing with 100 μL of 10 mM Hepes (pH 7.5) containing 5 M urea (×4). We refer to the extract as urea extract. Isotonic and urea extracts were dialyzed with a dialysis tube (molecular weight cut 1000) against water (4 °C) for 1 h and then against 0.1% SDS at room temperature overnight. Then, the samples were lyophilized, dissolved in SDS sample buffer, and analyzed by SDS−polyacrylamide gradient gel electrophoresis (8−16%). The catalytic or the regulatory subunits (150 ng) of bovine heart PKA were also applied to the gel to compare their molecular weights with that of bovine ROS PKA. After electrophoresis, proteins were blotted to nitrocellulose membranes and detected using antibodies. Polyclonal antibodies against peptides derived from the catalytic or the regulatory subunits of bovine heart PKA were used for the first antibody, and anti-rabbit IgG conjugated with alkaline phosphatase was used for the second antibody. Blocking was done by the use of 3% gelatin in a buffer (25 mM Tris·HCl (pH 8.0), 137 mM NaCl, and 2.5 mM KCl). Antibodies were diluted (×1000) with the blocking buffer containing 1% gelatin and 0.05% Tween 20.

Site-Directed Mutagenesis of Pγ. Mutagenesis, expression, and purification of Pγ mutant proteins (R33K, R36K, R33,36L, R33,36K, C18Sub, and N16Sub) were described previously (*12*, *29*). In addition, we prepared new Pγ mutants: T22V and T35V as substituted mutants and C10Del and N22Del as deleted mutants. In T22V and T35V, threonine 22 and threonine 35 were substituted by valine, respectively. In the C10Del mutant, 10 amino acids (amino acid 78−87) are deleted from the C terminal of Pγ. In the N22Del mutant, 22 amino acids (amino acid 2−23) are deleted from the N terminal of Pγ. The amino acid numbers correspond to the mature protein (*31*). A full-length bovine Pγ gene, ligated into the NdeI−BamHI-digested plasmid pET-11A (Novogene), was used in the mutagenesis step. To prepare substituted mutants, two-step PCR was applied for the amplification of bovine Pγ gene (*31*). In the first PCR two primers were used: primer A (5′-GCCAACCTG-CATATGAACCTGGAGCC-3′) and antisense primer (Table 1) for T22V and primer B (5′-GGGGTCGGATCCTAGAT-GATGCCGTACTG-3′) and sense primer (Table 1) for T35V. To amplify the mutant Pγ gene, the second PCR was carried out with the purified product from the first PCR reaction as a primer and primer B (for T22V) or primer A

(for T35V). To generate N- or C-terminal deleted mutants, one-step PCR was applied for the amplification of bovine Pγ gene (*32*). For the N22Del mutant, a primer (Table 1) was used in combination with 5′-GCAGCCGGATCCTA-GATGATACCG-3′. For the C10Del mutant, a primer (Table 1) was used in combination with 5′-TATACATAT-GAACCTGGAACCGCCGAAAG-3′. These amplified Pγ gene products were digested with NdeI and BamHI and cloned into NdeI−BamHI-digested pET-11A. The vector was transferred to *Escherichia coli* BL21 (DE3) (Novogene) for the expression of Pγ. The mutations were confirmed by double-stranded DNA sequencing using the fmol DNA sequencing system (Promega). Protein expression was induced by the addition of 1 mM isopropyl β-D-thiogalactopyranoside. Purification of these Pγ mutant proteins was carried out as described (*29*). We found during purification that C18Sub was sensitive to incubation (80 °C). Therefore, we omitted the heating step from the purification of the mutant. The purity of these mutant proteins was greater than 90%. The running profiles of some Pγ mutant proteins in SDS gels were slightly different from that of wild-type Pγ (Figure 8). These Pγ mutants were identified by the measurement of their inhibitory activities of cGMP hydrolysis and by using Pγ-specific antibodies.

Preparation of Various Pγs. Recombinant bovine Pγ was used for all experiments in this study. Expression and purification of the recombinant Pγ were carried out as described (*29*). It should be emphasized that recombinant Pγ and Pγ purified from bovine ROS (17) were phosphorylated by PKA in a similar manner and that siliconized tubes and pipet tips were used in all experiments except SDS gel electrophoresis. To prepare phosphorylated Pγ, Pγ (50 μg) was incubated in 250 μL of a buffer (20 mM Tris·HCl (pH 7.5), 1 mM DTT, 5 mM $MgCl_2$) with 1 mM ATP and the catalytic subunit of bovine heart PKA (1 μg) (30 °C, 12 h). The mixture was heated (80 °C, 5 min) and centrifuged (345000*g*, 4 °C, 30 min). The supernatant was used as phosphorylated Pγ. The purity of the phosphorylated Pγ was greater than 90%. We note that column chromatography of Pγ (*9*, *20*, *29*) did not increase the purity of Pγ. We also note that phosphorylated Pγ is not sensitive to the heat treatment because phosphorylated Pγ, which was isolated by size exclusion column chromatography without the heat treatment, showed a similar inhibitory activity for cGMP hydrolysis to that of phosphorylated Pγ isolated after heating. The Pγ inhibitory activity has been used to measure the heat sensitivity of Pγ and Pγ mutants. As described above, by the measurement of the inhibitory activity, we found that C18Sub was sensitive to the heat treatment.

Purification of Proteins. Bovine PDE was purified as described (*5*, *33*). Trypsin-activated PDE was prepared from the purified PDE by treatment with trypsin as described (*19*). The activated PDE was further purified by Superose 12 (Pharmacia) that had been equilibrated with a buffer (100 mM Tris·HCl (pH 7.5), 1 mM DTT, 5 mM $MgCl_2$, 1 mM EGTA, 0.1 mM PMSF, and 0.0025% BHT). The purity of the activated PDE was greater than 95%. Under our conditions, PDE specific activity (μmol of cGMP hydrolyzed/mg/min) was increased approximately 5-fold (from 20.3 to 106) by the trypsin treatment. Frog Tα (GTPγS- and GDP-bound forms) and T$\beta\gamma$ were isolated as described (*34*). Urea-treated ROS membranes (frog) were purified as described (*10*).

Measurement of Molecular Ion Mass of Pγ. The molecular masses of Pγ and its phosphorylated species were measured by MALDI-TOF mass spectrometry using a Voyager Elite Biospectrometry Research Workstation (PerSeptive Biosystems, Inc., Framingham, MA) equipped with an N_2 laser ($\lambda = 337$ nm) and operated in linear and positive ion mode with delayed extraction (delay time = 150 ms). Other operating parameters of the Elite were: accelerating voltage, 25 000 V; grid voltage, 92%; guide wire voltage, 0.3%; low mass gate on at $m/z = 4000$. For the preparation of the sample, 0.5 μL of Pγ solution was mixed with 0.5 μL of saturated sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid) in 30% acetonitrile/0.1% trifluoroacetic acid/water. The mixture was spotted on the sample stage and air dried. Signals from 128 scans were averaged and analyzed by GRAMS/386 software (Galactic Industries Corp., Salem, NH). The mass calibration was made at two points: horse heart myoglobin, $[M + H]^+ = 16\,951.55$ (average mass); oxidized insulin B chain, $[M + H]^+ = 3495.95$ (average mass).

Phosphorylation of Pγ and Peptides Derived from Pγ by PKA. Pγ was phosphorylated by the catalytic subunit of bovine heart PKA. The PKA subunit was generously provided by Dr. Yasuo Fukami (Kobe University) or purchased from Upstate Biotechnology Inc. Both PKA subunits showed similar kinase activities for Pγ phosphorylation. The amount of each component was slightly different in each experiment (see each figure legend); however, Pγ phosphorylation was performed in a similar way. The reaction mixture contained Pγ (0.1−0.2 μg), [γ-^{32}P]ATP (10−100 μM, 1.5−3.0 μCi), and the catalytic subunit of PKA (0.04−0.1 μg) in 50 μL of the phosphorylation buffer (10 mM Tris·HCl (pH 7.5) and 5 mM $MgCl_2$). Pγ phosphorylation was initiated by the addition of Pγ and terminated by heating with SDS sample buffer (5 min, 80 °C). These samples were analyzed by SDS−polyacrylamide gradient gel electrophoresis (8−16%) and autoradiography. The band corresponding to Pγ (its apparent molecular weight is 13 000 in gels) was also excised from the gels, and its radioactivity was measured. The radioactivity was proportional to the value obtained by densitometric scanning. Under regular conditions, about 10−20% of the Pγ was phosphorylated. We note that 100% of Pγ could be phosphorylated by PKA when Pγ phosphorylation was carried out under these conditions as shown in Figure 4. Two peptides (MGGPVTPRKGPC and FKQRQTRQFKSC) were also phosphorylated by PKA. Peptides (2 μM or 10 μM) were incubated with 10 μM [γ-^{32}P]ATP (∼1.0 μCi) and the PKA catalytic subunit (0.1 μg) in 50 μL of the phosphorylation buffer. After incubation (30 °C), 50 μL of 300 mM phosphoric acid was added to the reaction mixture to terminate the reaction. The mixture (40 μL) was spotted onto 1.5 × 1.5 cm pieces of Whatman P-81 phosphocellulose (*35*). Then, all of these pieces were washed with 200 μL of 75 mM phosphoric acid (×4) and the radioactivity of each paper was measured.

Phosphoamino Acid Analysis. Phosphorylation of Pγ (0.1 μg) was carried out with the PKA catalytic subunit (0.1 μg) and 10 μM [γ-^{32}P]ATP (∼1.0 μCi) in 50 μL of the phosphorylation buffer (30 °C, 3 h). The mixture was heated

with SDS sample buffer (80 °C, 5 min) and analyzed by SDS−polyacrylamide gradient gel electrophoresis (8−16%). The band corresponding to Pγ was excised and rinsed with 50 mM NH_4HCO_3 (pH 8.9). After the gel piece was minced, the protein was extracted in 1 mL of a solution (50 mM NH_4HCO_3 (pH 8.9), 0.1% SDS, and 2% β-mercaptoethanol) by gentle shaking (room temperature, 24 h). After the addition of 10 μL of bovine serum albumin (1.0 mg/μL), the extract was lyophilized. Dried material was dissolved into 400 μL of water. To the sample was added 800 μL of acetone. Following the incubation (−30 °C, 2 h), precipitate was spun down by centrifugation (13000*g*, 15 min, 0 °C). The pellet was resuspended with cold acetone/ethanol (1:1), and the suspension was incubated (−30 °C, 1 h). Precipitate was spun down and lyophilized and then hydrolyzed with 6 N HCl at 105 °C for 3 h. Water (1 mL) was added to the acid hydrolysate. The sample was lyophilized and mixed with 5 μL of a mixture of three authentic phosphoamino acids (3.3 mg of each phosphoamino acid/mL) as an internal standard. These authentic phosphoamino acids were phosphoserine, phosphothreonine, and phosphotyrosine. Phosphoamino acids were separated on one-dimensional thin-layer electrophoresis (*36*). The thin-layer plate was treated with ninhydrin to detect authentic phosphoamino acids. The ^{32}P-labeled phosphoamino acid was visualized by autoradiography. Radioactive spots were also scraped from the plate, and their radioactivities were measured to estimate the recovery.

Analytical Methods. The activities of PDE and Pγ were assayed as described (*9*). The GTPase activity of Tα and GTPγS binding to Tα was measured as described (*34*). SDS−polyacrylamide gel electrophoresis for Pγ separation was performed as described (*34*). Protein concentrations were assayed with bovine serum albumin as the standard (*37*). The amount of Pγ was assayed by densitometric scanning (*9*). To calculate the Pγ concentration, 9665 was used as the molecular weight of recombinant bovine Pγ although Pγ was detected as a 13 000 band in SDS gels. It should be emphasized that all experiments were carried out more than two times and that the results were similar. Data shown are representative of these experiments.

RESULTS

(1) Phosphorylation of Pγ by PKA. PKA has been reported to be present in vertebrate ROS. For example, our initial study (*6*) has shown that two cAMP binding proteins were present in frog ROS and suggested that these proteins might be the regulatory subunits of PKA. In frog ROS, components I and II were also reported to be phosphorylated by PKA (*24*−*26*), suggesting that PKA is present in frog ROS. In bovine ROS, Lee et al. (*38*) have shown protein kinases which are stimulated by cAMP and suggested that these kinases are similar to PKA that has been characterized in other tissues. They have also shown that phosducin is phosphorylated by PKA in bovine ROS (*15*, *16*, *38*). A search for a phosphorylation motif of PKA in Pγ suggests that some serines and threonines are potential phosphorylated sites by PKA. This information encouraged us to investigate the possibility that bovine Pγ might be phosphorylated by PKA. First, we have confirmed that PKA is present in bovine ROS using antibodies against the catalytic or regulatory subunits of bovine heart PKA. As shown in Figure 1, the antibody against the catalytic subunit recognized a protein

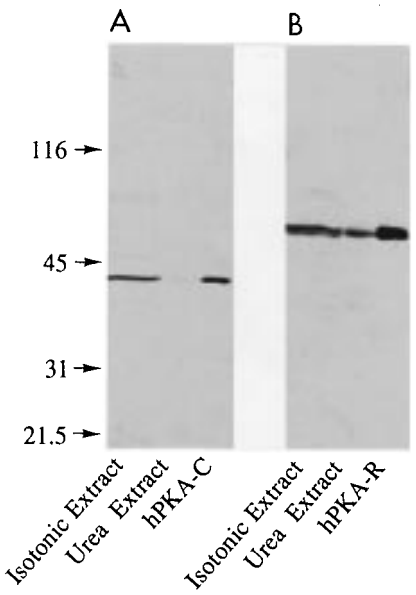

FIGURE 1: Identification of PKA subunits in bovine ROS. After isolation of intact bovine ROS (200 μg protein), isotonic extract and urea extract were prepared as described. Proteins in these preparations were isolated by SDS gel electrophoresis and blotted to nitrocellulose membranes. As controls, the catalytic (hPKA-C) or the regulatory (hPKA-R) subunits of bovine heart were also applied. The catalytic subunit of ROS PKA was identified by using an antibody against the catalytic subunit of bovine heart PKA (A). The regulatory subunit of ROS PKA was also detected by using an antibody against the regulatory subunits of bovine heart PKA (B).

in the isotonic extract, and the molecular weight of the protein was exactly the same as that of the catalytic subunit of bovine heart PKA. The protein was not detected in the urea extract. The antibody against the regulatory subunit also detected a protein in the isotonic extract. Less of the protein was also recognized in the urea extract. The molecular weight of the protein was exactly the same as that of the regulatory subunit of bovine heart PKA. Together with previous studies described above, these observations suggest that PKA is present in bovine ROS and that the ROS PKA is similar to that in bovine heart. Because we could not obtain purified PKA from bovine ROS, we used the catalytic subunit of bovine heart PKA for the phosphorylation of Pγ in this study. As shown in Figure 2, we found that bovine Pγ was phosphorylated by the catalytic subunit. Under our conditions, 13% of Pγ was phosphorylated, assuming one phosphate was incorporated into one Pγ. As shown in Figure 7, the phosphorylation was also time-dependent.

Pγ phosphorylation was also carried out to obtain the highest level of phosphate incorporation into Pγ. After phosphorylation of Pγ with 100 μM [γ-^{32}P]ATP and 1 μg of the catalytic subunit of PKA (33 °C, 12 h), the phosphorylated Pγ was purified using a reverse-phase column as described previously (*9*, *20*, *29*). Measurement of [^{32}P] radioactivity of the phosphorylated Pγ indicates that phosphorylated Pγ contained as much as 1.0 mol of phosphate per Pγ (data not shown). Because the level of phosphorylation appeared to be the highest under these conditions, this observation suggests that the highest value of phosphate incorporated in Pγ by PKA is 1 mol of phosphate/mol of Pγ.

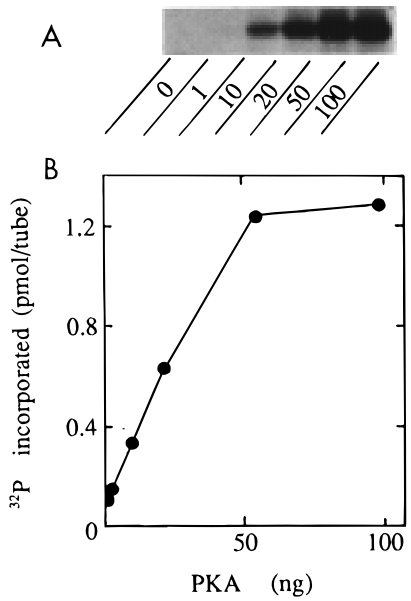

FIGURE 2: Phosphorylation of Pγ by PKA. Pγ (0.2 μg) was phosphorylated with [γ-^{32}P]ATP (100 μM, ~2.5 μCi) and various amounts of the catalytic subunits of PKA (1 h, 33 °C). After phosphorylation, samples were analyzed by SDS−polyacrylamide gel electrophoresis and autoradiography (A). The Pγ band was also excised from gel, and its radioactivity was measured (B).

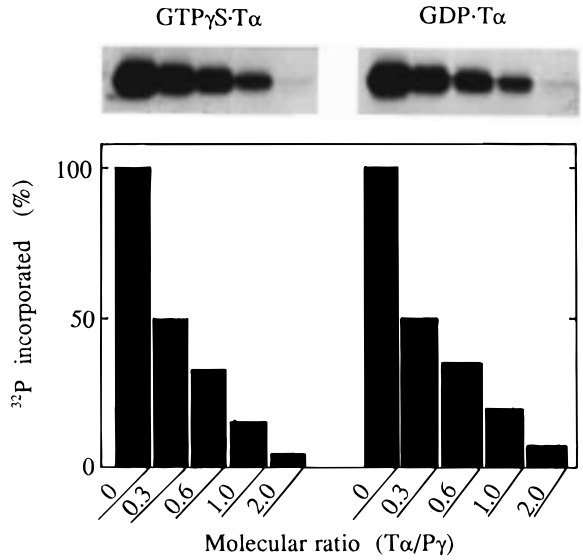

FIGURE 3: Phosphorylation of Pγ complexed with Tα. Pγ (0.2 μg) was phosphorylated by PKA (0.04 μg) in the presence of various amounts of GTPγS·Tα and GDP·Tα. Phosphorylation of Pγ was carried out with [γ-^{32}P]ATP (100 μM, ~2.5 μCi) for 30 min at 33 °C. After phosphorylation, samples were analyzed by SDS−polyacrylamide gel electrophoresis and autoradiography. The Pγ band was also excised and its radioactivity was measured.

Pγ forms a complex with both GTPγS·Tα and GDP·Tα, but not with T$\beta\gamma$ (*9*). Pγ phosphorylation by PKA was inhibited when Pγ was preincubated with the GTPγS- or GDP-bound form of Tα (Figure 3). However, when Kemptide was used as a substrate for PKA, Tα did not inhibit phosphorylation of Kemptide (data not shown), indicating that Tα does not directly inhibit PKA. Under the same conditions, the Pγ phosphorylation was little affected by T$\beta\gamma$ (data not shown). These observations suggest that Pγ complexed with Tα is no longer a substrate for PKA. This also implies that the phosphorylation site(s) of Pγ is masked by Tα when Pγ is complexed with Tα.

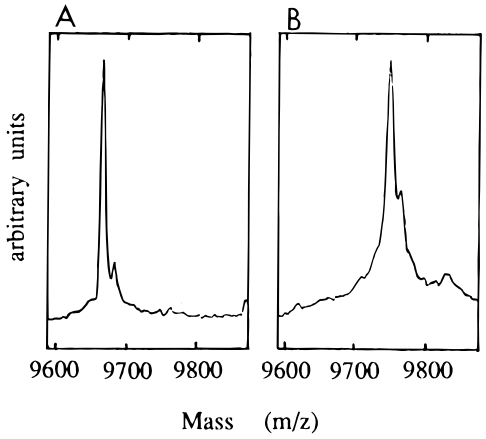

FIGURE 4: Mass spectrum of nonphosphorylated and phosphorylated Pγ. Phosphorylated Pγ was prepared with nonradioactive ATP as described. Molecular ion masses of nonphosphorylated (A) and phosphorylated (B) Pγ were measured by MALDI-TOF mass spectrometry with horse heart myoglobin and oxidized insulin B chain as standards.

(2) Characterization of Pγ Phosphorylation by PKA. (a) Identification of the Number of Phosphorylated Amino Acids. Phosphorylated Pγ was analyzed by MALDI-TOF mass spectrometry. As shown in Figure 4, nonphosphorylated Pγ showed a single peak with a molecular ion mass of 9665.33 Da. With phosphorylated Pγ, a single peak with a molecular ion mass of 9748.92 Da emerged. The difference in the observed masses between nonphosphorylated and phosphorylated Pγ is 83.6 Da. Taking into account the error in the measurement of molecular ion mass of a protein with this molecular weight, these data indicate that a single phosphate group (80.0 Da) is incorporated into a single amino acid in Pγ. This also indicates that 100% of Pγ was phosphorylated under these conditions.

(b) Identification of a Phosphorylated Amino Acid. Following hydrolysis of [^{32}P] phosphorylated Pγ with HCl, phosphoamino acids were identified by thin-layer electrophoresis. As shown in Figure 5, about 90% of the applied ^{32}P radioactivity was recovered with phosphothreonine; however, only negligible radioactivity was detected with phosphoserine and phosphotyrosine. These data indicate that a threonine in Pγ is phosphorylated by PKA.

(c) Identification of the Threonine Phosphorylated in Pγ. Previous studies have shown that threonines 22 (*20*) and 35 (*19*) in Pγ are phosphorylated. First, we investigated, using synthetic peptides, the possibility that one of these threonines is phosphorylated by PKA. As shown in Figure 6, a peptide (FKQRQTRQFKSC) was phosphorylated, but another peptide (MGGPVTPRKGPC) was not. The former peptide corresponds to the Pγ amino acid sequence Phe30 -Ser40, and a cysteine was added to its C terminal. The latter corresponds to the Pγ amino acid sequence Met17-Pro27, and a cysteine was added to its C terminal. These observations suggest that threonine 35, but not threonine 22, is phosphorylated by PKA.

To confirm these observations, and to exclude the possibility that other threonines are phosphorylated by PKA, mutant forms of Pγ were also used as substrates of PKA. As shown in Figure 7A, a substituted mutant, T22V, was phosphorylated by PKA in a manner similar to that of wild-

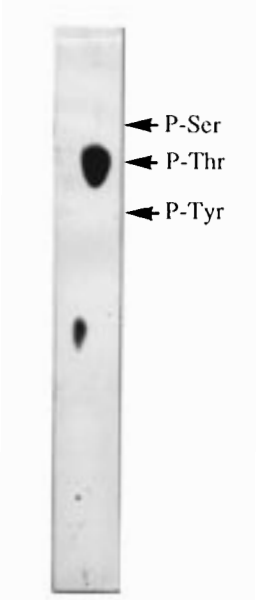

FIGURE 5: Identification of a phosphorylated amino acid in Pγ. After hydrolysis of [^{32}P]-phosphorylated Pγ, the hydrolysate was analyzed using a thin-layer plate as described. Then autoradiography was carried out. Authentic amino acids (P-Ser, phosphoserine; P-Thr, phosphothreonine; and P-Tyr, phosphotyrosine) were visualized with ninhydrin. The profile of authentic amino acids on the thin-layer plate is shown with arrows.

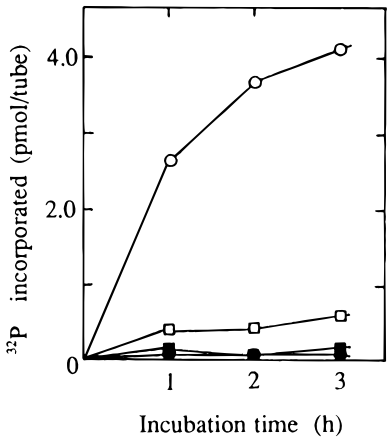

FIGURE 6: Phosphorylation of peptides derived from Pγ. Phosphorylation of peptides (2 μM and 10 μM) was carried out in the presence of [γ-^{32}P]ATP (10 μM, ~1.0 μCi) and the catalytic subunits of PKA (0.1 μg) for various periods at 30 °C. After phosphorylation, samples were spotted on Whatman P-81 phosphocellulose. Their radioactivity was measured: (■, □) 2 μM; (●, ○) 10 μM; (□, ○) FKQRQTRQFKSC; (■, ●) MGGPVTPRKGPC.

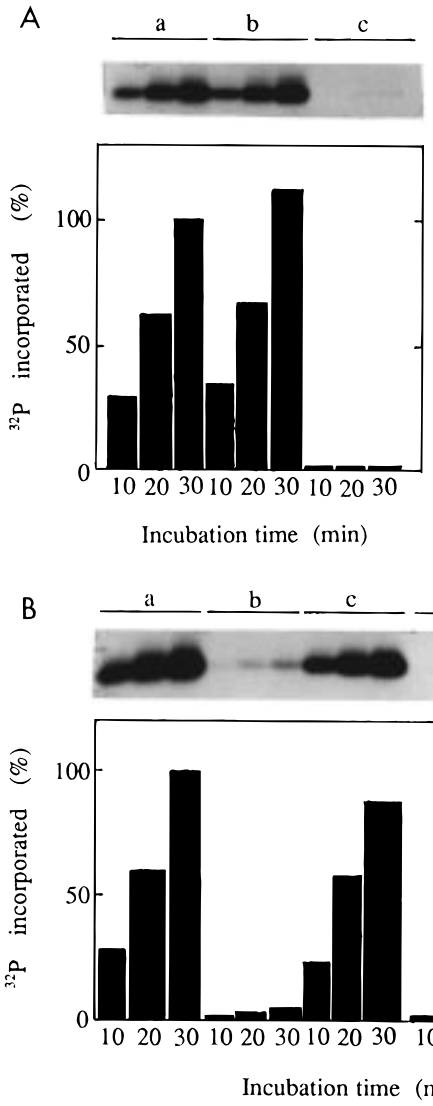

FIGURE 7: Identification of the amino acids involved in Pγ phosphorylation by PKA. Phosphorylation of Pγ mutants (0.2 μg) was carried with the catalytic subunit (0.2 μg) of PKA and of [γ-^{32}P]-ATP (100 μM, ~2.5 μCi) in 50 μL of phosphorylation buffer. Following incubation for various periods at 33 °C, the reaction mixture was mixed with 25 μL of SDS sample buffer and heated at 80 °C for 5 min. After SDS gel electrophoresis and autoradiography, the Pγ band was excised from gel, and its radioactivity was measured. On the basis of the radioactivity of wild-type Pγ after 30 min of incubation, the radioactivity of each mutant was calculated: (A) a, wild-type Pγ; b, T22V; c, T35V; (B) a, wild-type Pγ; b, R33K; c, R36K; d, R33,36K; e, R33,36L.

type Pγ. However, the Pγ mutant T35V was not phosphorylated. Together with the data shown in Figure 4, these observations indicate that only threonine 35 is phosphorylated by PKA. Together with the data shown in Figure 3, these observations also imply that a domain containing this threonine is involved in the Pγ-Tα interaction. We note that this conclusion is consistent with our previous conclusion obtained in the study of Pγ ADP-ribosylation (*29*). We have shown that arginines 33 and 36 in Pγ are ADP-ribosylated by endogenous ADP-ribosyltransferase from frog ROS and that a domain containing these arginines is involved in the Pγ-Tα interaction.

(d) Involvement of other Amino Acids in Pγ Phosphorylation. We investigated a possible role of arginines, which can be ADP-ribosylated by endogenous ADP-ribosyltransferase, in the phosphorylation of threonine 35. We found that the replacement of arginine 33 with lysine, but not arginine 36, abolished Pγ phosphorylation (Figure 7B). In addition, the Pγ phosphorylation was not detected if both arginines were replaced by lysine or leucine. This indicates that arginine 33 is required for the phosphorylation of threonine 35. Arginine 33 appears to form a phosphorylation motif of PKA with thereonine 35.

The C and N terminals in Pγ are crucial for Pγ function. Many groups have shown that the C terminal is involved in Pγ inhibitory activity for cGMP hydrolysis by P$\alpha\beta$ (*12*). The N terminal is required for Pγ stimulation of cGMP binding to noncatalytic sites on frog P$\alpha\beta$ (*12*). We used two different kinds of mutants, substitution and deletion, in each terminal of the Pγ amino acid sequence to investigate involvement

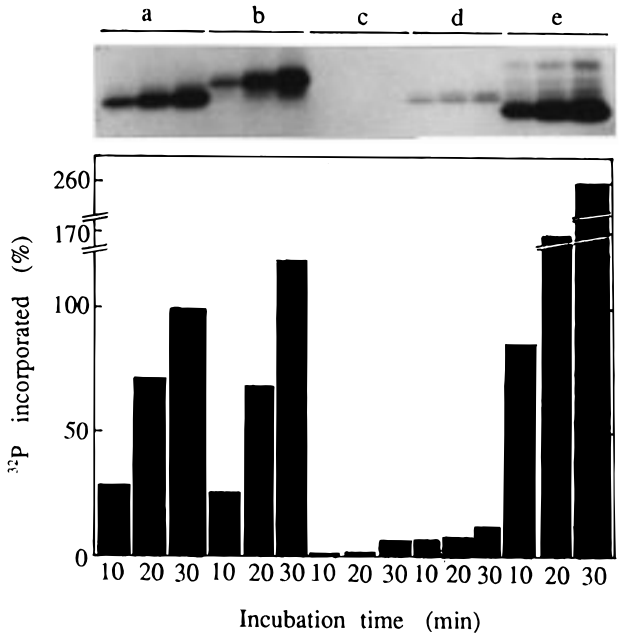

FIGURE 8: No requirement of Pγ functional domains in Pγ phosphorylation by PKA. Phosphorylation of Pγ mutants was carried out as described in the figure legend for Figure 7: a, wild-type Pγ; b, C10Del; c, C18Sub; d, N16Sub; e, N22Del.

of these regions in the phosphorylation of Pγ by PKA. We have already shown that the inhibitory activity for cGMP hydrolysis is completely abolished in Pγ mutants, C10Del (data not shown), or C18Sub (*12*). We have also shown that N16Sub (12) or N22Del[2] has less stimulatory activity for cGMP binding to noncatalytic sites on frog P$\alpha\beta$. As shown in Figure 8, C10Del was phosphorylated like wild-type Pγ; however, phosphorylation of C18Sub was completely abolished. N16Sub was only slightly phosphorylated, while the level of phosphorylation of N22Del was higher than that of wild-type Pγ. These observations indicate the following points: (i) Pγ mutants were phosphorylated when the C- or N-terminal amino acid sequences were deleted. This observation is consistent with data showing that a Pγ peptide corresponding to the Pγ amino acid sequence Phe30-Ser40 was phosphorylated by PKA (Figure 6). This suggests that the C and N terminals in Pγ are not involved in the Pγ phosphorylation by PKA, (ii) C18Sub and N16Sub mutants were not phosphorylated. We speculate that a new amino acid sequence(s) incorporated in the C or N terminal by these substitutions may change Pγ conformation and the conformational change prevents phosphorylation of threonine 35 by PKA, (iii) The level of phosphorylation of N22Del was 2−3 times higher than that of wild-type Pγ. This suggests that the N terminal contains a sequence(s) to inhibit Pγ phosphorylation by PKA. We speculate that relatively slow phosphorylation of Pγ by PKA may be due to the inhibitory effect of the N terminal.

(3) Effect of Phosphorylation on Pγ Interactions with Other Proteins. To understand the physiological functions of Pγ phosphorylation by PKA, the effect of phosphorylation on the interactions between Pγ and other proteins should be clarified. In the current scheme of phototransduction, Pγ interacts with P$\alpha\beta$ or Tα. As shown in Figure 9, phosphorylation of Pγ increased its inhibitory ability by 3−5 times when trypsin-activated PDE was inhibited. This observation suggests that the phosphorylation of Pγ by PKA is functionally involved in the interaction between P$\alpha\beta$ and Pγ.

[2] Unpublished observation by A.Y.

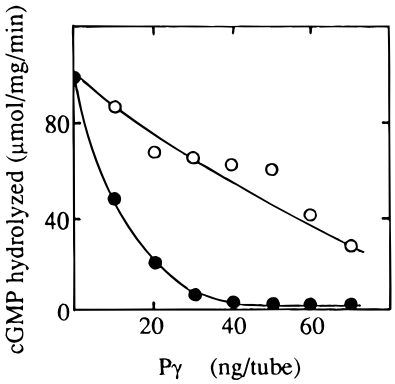

FIGURE 9: Stimulation of the PDE inhibitory activity of Pγ by phosphorylation. Various amounts of phosphorylated Pγ (●) and nonphosphorylated Pγ (○) were incubated with trypsin-activated PDE (180 ng/tube) in 100 μL of 50 mM Tris·HCl (pH 7.5) containing 5 mM $MgCl_2$. Using 4 mM [^{3}H]cGMP (~0.1 μCi), PDE activity was measured at 30 °C for 10 min.

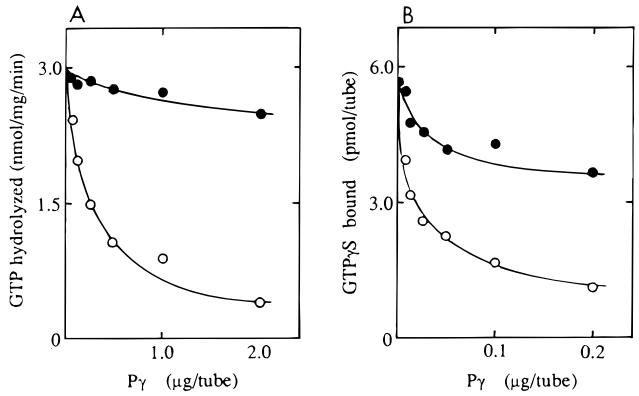

FIGURE 10: Effect of phosphorylated Pγ on the GTPase activity of Tα and GTPγS binding to Tα: phosphorylated Pγ (●) and nonphosphorylated Pγ (○). (A) GTPase activity. GTPase activity was measured in 100 μL of a buffer (10 mM Tris·HCl (pH 7.5), 2 mM DTT, 5 mM $MgCl_2$, and 1 mM EGTA) containing frog Tα (1.8 μg), frog T$\beta\gamma$ (0.5 μg), frog urea-treated ROS membranes (2.0 μg), and various amounts of Pγ. GTP hydrolysis was initiated by the addition of [γ-^{32}P]GTP (2 μM; ~0.1 μCi). Following incubation (30 min, 33 °C), the reaction was terminated by the addition of 500 μL of stop solution (6% charcoal, 10% trichloroacetic acid, and 5 mM NaH_2PO_4). After spinning (1000*g*, 10 min), the radioactivity of the supernatant (100 μL) was measured. (B) GTPγS binding. GTPγS binding to Tα was measured in 100 μL of the buffer, which is used in (A), containing Tα (0.36 mg), T$\beta\gamma$ (0.375 μg), urea-treated ROS membranes (2.0 μg), and various amounts of Pγ. Binding was initiated by the addition of [^{35}S]GTPγS (1 μM; ~0.06 μCi). Following incubation (30 min, 0 °C), 80 μL of the reaction mixture was applied to a membrane filter (Millipore, HA, pore size 0.45 μm) and washed with 4 μL of the same buffer (four times). Then, the radioactivity of the filter was measured.

We have shown that Pγ inhibits both the GTPase activity of Tα and GTPγS binding to Tα under our conditions (*39*). These observations have been used as evidence for the interaction between Pγ and Tα (*21*, *29*). As shown in Figure 10, nonphosphorylated Pγ inhibited both GTPase activity of Tα (86%) and GTPγS binding to Tα (81%); however, phosphorylated Pγ inhibited only 15% of the GTPase activity and 40% of the GTPγS binding. We suggest that, under our conditions, hydrolysis of GTP bound to Tα was so slow

that GTPase activity might not be affected by the inhibition of GTPγS binding to Tα. We also suggest that nonphosphorylated Pγ contaminated in the preparation of phosphorylated Pγ may be responsible for the small inhibition of these Tα functions. In any case, these observations indicate that the phosphorylation of Pγ by PKA regulates its interaction with Tα.

DISCUSSION

PDE, a key enzyme in the regulation of the level of cGMP in retinal photoreceptors, is composed of Pαβ and two Pγ subunits. Pαβ hydrolyzes cGMP (*4*, *5*) and binds cGMP to its high-affinity, cGMP-specific, noncatalytic sites (*6*−*8*). It is clear now that in amphibian ROS Pγ regulates these Pαβ functions as an inhibitor of cGMP hydrolysis (*9*) and as a stimulator of cGMP binding to the noncatalytic sites (*10*, *11*), although the effect of Pγ on the cGMP binding has never been reported in bovine ROS. These functions of Pγ are terminated by Pγ release with GTP·Tα from Pαβ (*9*−*11*). Therefore, the interaction of Pγ with Pαβ or Tα is one of the most crucial reactions in phototransduction. Recent studies have indicated that these Pγ interactions are regulated by Pγ modification. Phosphorylation of Pγ by PKC (*19*) and Pγ kinase (*20*) stimulates Pγ interaction with Pαβ for the inhibition of cGMP hydrolysis by Pαβ. In addition, Pγ phosphorylation by Pγ kinase abolishes Pγ interaction with GTP·Tα (*21*). Moreover, Pγ ADP-ribosylation seems to inhibit Pγ-Tα interaction (*29*). In this study, we have shown that threonine 35 in Pγ is phosphorylated by PKA and that this phosphorylation not only stimulates the Pγ-Pαβ interaction but also inhibits the Pγ-Tα interaction. Therefore, Pγ phosphorylation by PKA may play a role as a molecular switch in phototransduction. We did not measure the effect of phosphorylated Pγ on the cGMP binding to noncatalytic sites on Pαβ because a method to release Pγ from bovine Pαβ has never been established.

This study reinforces the previous conclusion (*29*) that a Pγ domain containing arginine 33 and 36 is involved in the Pγ-Tα interaction. Interestingly, this domain is modified by three different enzymes in vitro: PKC (*19*), ADP-ribosyltransferase (*29*), and PKA (in this study). This suggests that the Pγ domain is crucial for phototransduction. We anticipate that these Pγ modifications occur at the different stages of phototransduction and that the physiological roles of these modifications may be different at each stage of phototransduction. For example, Pγ is ADP-ribosylated when Pγ is complexed with Pαβ and PDE is no longer activated by GTP·Tα (*29*). Therefore, we have speculated that Pγ ADP-ribosylation occurs under the dark conditions. The Pγ ADP-ribosylation may prevent PDE activation from occurring by spontaneous GTP/GDP exchange on Tα. Thus, the ADP-ribosylation may function to reduce noise under the dark conditions and to increase the sensitivity of ROS to photons. However, we do not have any information about a stage for Pγ phosphorylation by PKC and PKA. In the specific stage of Pγ phosphorylation by PKA, the inhibitory effect of the N terminal of Pγ on the phosphorylation of threonine 35 may be released by a new protein−protein interaction. Further study is needed to characterize these Pγ phosphorylations. Moreover, mechanisms for the reversal of these Pγ modifications and the relationship between these modifications should be revealed to understand the real roles of these modifications in phototransduction. In addition, these Pγ modifications should be confirmed under the in vivo conditions. Although definitive data about these Pγ modifications in vivo are lacking, recent studies have suggested that these Pγ modifications occur in vivo. Hayashi (*40*), one of authors in this report, has shown that Pγ is phosphorylated in a light-dependent manner. Moreover, we found that about 50% of Pγ in frog ROS was not released from Pαβ even if excess amounts of GTPγS·Tα were added (*9*), suggesting that the Pγ is insensitive to GTP·Tα. We anticipate that the GTP·Tα-insensitive Pγ is modified by protein kinase and/or by ADP-ribosyltransferase (*29*). Our ongoing study to detect Pγ modifications in vivo and to isolate and characterize the GTP·Tα-insensitive Pγ may reveal the roles of these Pγ modifications in vivo.

PKA is present in vertebrate ROS (*6*, *38*). In this study, we have shown that two different proteins are cross-reacted with antibodies against the regulatory or catalytic subunits of bovine heart PKA, suggesting that PKA similar to heart PKA is present in bovine ROS (Figure 1). Thus, we used bovine heart PKA. It is unfortunate for us that ROS PKA has not been characterized in detail although several proteins in ROS are phosphorylated by PKA (*15*, *16*, *24*−*26*). Moreover, definitive information about the regulatory mechanism of cAMP level in ROS, including retinal adenylyl cyclase and phosphodiesterase, is also missing. We may have to wait for this information to reveal the real function of Pγ phosphorylation by PKA. However, this study clearly indicates that the Pγ domain containing threonine 35 is crucial for the Pγ interaction with Tα and that phosphorylation of the domain regulates Pγ interaction with other proteins. The rod PDE system is a typical G protein dependent signal transduction mechanism. We anticipate that an effector domain required for the interaction with G protein may be modified by a variety of enzymes and that each modification of the domain has a different role for the expression of signals in cells.

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