Suppression of GTP/Tα-Dependent Activation of cGMP Phosphodiesterase by ADP-Ribosylation by Its γ Subunit in Amphibian Rod Photoreceptor Membranes†

Vladimir A. Bondarenko,‡ Matsuyo Yamazaki,‡ Fumio Hayashi,§ and Akio Yamazaki*,‡,||

Kresge Eye Institute and Departments of Ophthalmology and Pharmacology, Wayne State University, School of Medicine, Detroit, Michigan 48201, and Department of Biology, Faculty of Science, Kobe University, Kobe, 657, Japan

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ABSTRACT: Our previous study has shown that Pγ, the regulatory subunit of cGMP phosphodiesterase (PDE), is ADP-ribosylated by endogenous ADP-ribosyltransferase when Pγ is free or complexed with the catalytic subunits of PDE in amphibian rod photoreceptor membranes. The Pγ domain containing ADP-ribosylated arginines was shown to be involved in its interaction with Tα, a key interaction for PDE activation. In this study, we describe a possible function of the Pγ ADP-ribosylation in the GTP/Tα-dependent PDE activation. When rod membranes were preincubated with or without NAD and washed with a buffer containing GTP, the PDE activity of NAD-preincubated membranes was increased by the GTP-washing only to ∼50% of that of membranes preincubated without NAD. The Pγ release by the GTP-washing from these NAD-preincubated membranes was also suppressed to ∼50% of that preincubated without NAD. Taking into consideration that ∼50% of Pγ is ADP-ribosylated under these conditions, these observations suggest that the ADP-ribosylated Pγ cannot interact with GTP/Tα. We have also shown that a soluble fraction of ROS contains an enzyme(s) to release the radioactivity of [^{32}P]ADP-ribosylated Pγ in concentration- and time-dependent manners, suggesting that the Pγ ADP-ribosylation is reversible. Rod ADP-ribosyltransferase solubilized from membranes by phosphatidylinositol-specific phospholipase C was separated into two fractions by ion-exchange columns. Biochemical characterization of these two fractions, including measurement of the K_m for NAD and Pγ, estimation of their molecular masses, ADP-ribosylation of Pγ arginine mutants, effects of ADP-ribosyltransferase inhibitors on the Pγ ADP-ribosylation, and effects of salts and pH on the Pγ ADP-ribosylation, indicates that rod ADP-ribosyltransferase contains two isozymes, and that these two isozymes have similar properties for the Pγ ADP-ribosylation. Our observations strongly suggest that the negative regulation of PDE through the reversible Pγ ADP-ribosylation may function in the phototransduction mechanism.

The hydrolysis of cGMP by cyclic GMP phosphodiesterase (PDE)[1] is a crucial step for phototransduction in vertebrate photoreceptor ROS (*1*−*4*). Illuminated rhodopsin stimulates GTP/GDP exchange on Tα, followed by dissociation of GTP/Tα from Tβγ. GTP/Tα activates PDE, resulting in a fall in the cytoplasmic cGMP concentration, closure of cGMP-gated channels, and hyperpolarization of plasma membranes. PDE is composed of Pαβ and two Pγ subunits. Pαβ hydrolyzes cGMP (*5*, *6*) and binds cGMP to its high-affinity, noncatalytic sites (*7*−*9*). In amphibian photoreceptors, it has been clear that Pγ regulates these Pαβ functions. Pγ inhibits cGMP hydrolysis by Pαβ (*10*, *11*) but stimulates cGMP binding to noncatalytic sites (*12*, *13*). GTP/Tα regulates these Pγ functions by releasing Pγ from Pαβ in vitro (*10*, *11*). Different interactions between Pαβ and Pγ have been suggested for the expression of these two functions; i.e., functionally different Pγs appear to be released by GTP/Tα in different steps of phototransduction (*14*, *15*). When the cGMP concentration is at the dark level, Pγ responsible for the inhibition of cGMP hydrolysis is released. Consequently, cGMP is hydrolyzed by Pγ-less PDE. When the cGMP concentration becomes low, Pγ responsible for the stimulation of cGMP binding is released, and the affinity of these noncatalytic sites to cGMP is reduced. Consequently, cGMP is released from these noncatalytic sites. In mammalian photoreceptors, Pγ has been shown to inhibit cGMP hydrolysis (*16*). However, the release of Pγ by GTP/Tα is controversial even in the in vitro system (*17*−*20*), and the regulation by Pγ of cGMP binding to noncatalytic sites on Pαβ has never been described.

Recent studies have shown that phosphorylation of Pγ is involved in the regulation of its interaction between Pαβ

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* To whom correspondence should be addressed at the Kresge Eye Institute, Wayne State University, School of Medicine, 4717 St. Antoine Blvd., Detroit, MI 48201. Telephone: 313-577-2009. Fax: 313-577-0238. E-mail: akio_yamazaki@wayne.edu.

‡ Kresge Eye Institute and Department of Ophthalmology, Wayne State University.

§ Department of Biology, Kobe University.

|| Department of Pharmacology, Wayne State University.

[1] Abbreviations: ROS, rod outer segment(s); PDE, cGMP phosphodiesterase; Pαβ, α and β subunits of PDE; Pγ, regulatory subunit of PDE; Tα, α subunit of transducin; Tβγ, βγ subunit of transducin; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); PMSF, phenylmethanesulfonyl fluoride; DTT, dithiothreitol; PI, phosphatidylinositol; GPI, glycosylphosphatidylinositol; SDS−PAGE, SDS−polyacrylamide gel electrophoresis; ADP-RT-1 and ADP-RT-2, two isozymes of rod ADP-ribosyltransferase.

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and Pγ in vitro. Pγ is phosphorylated by several kinases, such as PI-stimulated kinase (*21*), PKC (*22*), PKA (*23*), and Pγ kinase (*24*, *25*). Pγ phosphorylated by PKC and PKA has a higher inhibitory activity against Pγ-less PDE. However, the real function of these Pγ phosphorylations is not clear in the current model of phototransduction because only free Pγ is used as a substrate for these phosphorylations, and their phosphorylation steps in the phototransduction mechanism are unknown. However, the Pγ phosphorylation by Pγ kinase may imply an important mechanism for phototransduction. We recently identified this kinase as cyclin-dependent protein kinase 5 (*26*). In this system, Pγ complexed with GTP/Tα is phosphorylated, and the Pγ loses its ability to interact with GTP/Tα. The phosphorylated Pγ, which has higher affinity to Pγ-less Pαβ than that of nonphosphorylated Pγ, returns to and inhibits the Pαβ. These observations indicate that GTP/Tα-activated PDE can be deactivated without GTP hydrolysis in ROS membranes, and that the lifetime of GTP/Tα-activated PDE can be regulated by the Pγ phosphorylation. It is possible that the mechanism may function in retinal photoreceptors because the light-dependent Pγ phosphorylation has been shown in an in vivo system (*27*).

ADP-ribosylation of Pγ has also been demonstrated as a potential mechanism for the regulation of the interaction between Pγ and Tα. Pγ, free or complexed with Pαβ, has been shown to be ADP-ribosylated by endogenous ADP-ribosyltransferase in frog ROS membranes (*28*). Arginines 33 and 36 in Pγ are similarly ADP-ribosylated, but only one arginine is modified at a time. Interestingly, substitution of these arginines to leucines changes the Pγ effect on Tα functions, GTP binding and GTP hydrolysis. Moreover, the Pγ ADP-ribosylation is inhibited when Pγ is complexed with Tα (both GTP- and GDP-bound forms). We have used these phenomena to show that the Pγ domain that contains these two arginines is required for its interaction with Tα, and that positive charges of these arginines are crucial for the interaction with Tα. These observations also imply that the Pγ ADP-ribosylation may be involved in the regulation of PDE.

In this study, we have focused on the function of the Pγ ADP-ribosylation in PDE regulation in vitro. We have shown that the activation of PDE by GTP/Tα, i.e., the release of Pγ from Pαβ by GTP/Tα, is hampered by preincubation of ROS membranes with NAD under the conditions for the Pγ ADP-ribosylation. Moreover, we have suggested that the Pγ ADP-ribosylation is reversible, because ADP-ribosylarginine hydrolase activity is detected in a soluble fraction of ROS. To strengthen our conclusion, we also solubilize rod ADP-ribosyltransferase by PI-specific phospholipase C. We show that the rod ADP-ribosyltransferase contains two isozymes, and that these isozymes have the same characteristics for the Pγ ADP-ribosylation. This study suggests that the Pγ ADP-ribosylation may function as a negative regulator of GTP/Tα-dependent activation of PDE in phototransduction.

EXPERIMENTAL PROCEDURES

Materials. Mono Q HR 5/5 (5 × 50 mm) and Mono S HR 5/5 (5 × 50 mm) were purchased from Pharmacia Biotech Inc. Bio-Sil TSK 250 (7.5 × 300 mm) was from Bio-Rad. Chemical reagents were obtained from the following sources: [*adenylate*-^{32}P]NAD and [^{3}H]cGMP from New England Nuclear; GTP, NAD, and cGMP from Boehringer Mannheim; benzamide, novobiocin, PMSF, leupeptin, pepstatin A, and benzamidine from Sigma; PI-specific phospholipase C isolated from *Bacillus thuringiensis* from ICN; Immobilon-P from Millipore. A Pγ-specific antibody was prepared using a peptide corresponding to 24R-46G of bovine Pγ. Frog Pγ is also detected by the antibody because frog Pγ contains the same peptide sequence.

Preparation of ROS Membranes and Pγ. Under dim red light, ROS were prepared from dark-adapted bullfrogs (*Rana catesbiana* or *Rana grylio*) by 46% sucrose flotation in buffer A [100 mM Tris/HCl (pH 7.5), 5 mM DTT, 0.1 mM PMSF, 5 μM leupeptin, 5 μM pepstatin A, and 1 mM benzamidine]. Pγ- and transducin-depleted ROS membranes were prepared as described (*28*). As GTP-activated PDE, Pγ-less PDE/membranes were prepared as described (*11*). Frog Pγ was extracted by a GTP-containing buffer and purified as described (*11*). Recombinant bovine Pγ and its arginine mutants were expressed and purified as described (*28*). [^{32}P]ADP-ribosylated Pγ was prepared as described (*28*). We note that ∼20% of Pγ in the preparation was ADP-ribosylated although ∼50% of Pγ is ADP-ribosylated in ROS membranes (*28*). It should be emphasized that such low incorporation of ADP-ribose into Pγ did not affect our conclusions because our conclusions were obtained using linear portions of Pγ ADP-ribosylation and not dependent on the level of Pγ ADP-ribosylation. The reason for the low incorporation of ADP-ribose into Pγ is unknown. Siliconized tubes and pipet tips were used in all experiments using Pγ expect SDS−PAGE.

Preparation of ADP-Ribosyltransferase and Its Isozymes. Pγ- and transducin-depleted ROS membranes (*28*) and a preparation solubilized from Pγ- and transducin-depleted ROS membranes were used as ADP-ribosyltransferase. To prepare solubilized ADP-ribosyltransferase, Pγ- and transducin-depleted ROS membranes (120 mg of protein) were suspended in 10 mL of buffer B [10 mM Tris/HCl (pH 7.5), 1 mM EDTA, 1 mM DTT, and 0.1 mM PMSF] containing 1 unit of PI-specific phospholipase C. Following incubation (37 °C, 30 min), a supernatant was obtained by centrifugation (200000*g*, 30 min, 4 °C). In the supernatant (∼8 mg of protein), ∼90% of the total ADP-ribosyltransferase activity was detected (Figure 3). The supernatant was applied to a Mono Q column which had been equilibrated with buffer C [10 mM Tris/HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM DTT, and 0.1 mM PMSF]. After the column was washed with buffer C, ADP-ribosyltransferase was eluted with a NaCl gradient in buffer C. The column conditions were the following: flow rate, 1.0 mL/min; fraction volume, 0.5 mL. Under these conditions, two isozymes, ADP-RT-1 and ADP-RT-2, were isolated (Figure 4A). These preparations were used to check their biochemical properties. In addition, these isozymes were separated using a Mono S column. Before the supernatant was applied to a Mono S column, the pH of the solubilized enzyme was adjusted to 4.2 by dialyzing against buffer D [10 mM citrate/phosphate (pH 4.2), 1 mM DTT, and 0.1 mM PMSF]. Then, the sample was applied to a Mono S column which had been equilibrated with buffer D. After the column was washed with the same buffer, ADP-ribosyltransferase was eluted with 20 mL of a linear gradient of NaCl (0−1 M) in buffer D. The column conditions were

as follows: flow rate, 1.0 mL/min; fraction volume, 0.5 mL. Under these conditions, ~20% of the total activity was found in a flow-through fraction, and ~50% of the total activity was found to bind to the column and to be eluted as a peak fraction (data not shown). Contents of these isozymes in these fractions will be described in the text.

Measurement of ADP-Ribosyltransferase Activity. The activity of ADP-ribosyltransferase was measured in 50 μL of 10 mM Tris/HCl (pH 7.5) containing Pγ (0.1 μg) and NAD (20 μM, ~0.5 μCi) at 33 °C. The reaction was initiated by addition of NAD and terminated by heating with SDS-sample buffer for 2 min at 80 °C. The reaction products were analyzed by SDS−PAGE and autoradiography as described (*28*). The radioactivity of Pγ bands in gels was also measured as described (*28*). It should be emphasized that all fractions in column chromatography were dialyzed against buffer E [10 mM Tris/HCl (pH 7.5), 1 mM DTT, and 0.1 mM PMSF] before the enzyme activity was measured, because the activity of solubilized ADP-ribosyltransferase is affected by salt concentration in a reaction mixture (Figure 7B). We also note that the enzymatic activity of rod ADP-ribosyltransferase isozymes is shown as ADP-ribose incorporated per incubation period per tube, because the protein concentration of each fraction in the Mono Q chromatography was too small for measurement.

Measurement of Molecular Mass of ADP-Ribosyltransferase Solubilized with PI-Specific Phospholipase C. ADP-ribosyltransferase solubilized with PI-specific phospholipase C (140 μL, 120 μg of protein) was applied to a TSK 250 column which had been equilibrated with buffer F [20 mM Tris/HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM DTT, 0.1 mM PMSF, and 100 mM NaCl]. Conditions for the chromatography were as follows: flow rate, 0.5 mL/min; fraction volume, 250 μL. After dialysis of all fractions against buffer E, the enzyme activity was measured. As molecular mass standards, ovalbumin (45 kDa) and chymotrypsinogen A (25 kDa) were also applied and eluted under the same conditions.

Measurement of Pγ ADP-Ribosylation in Gels. The molecular mass of two isozymes of rod ADP-ribosyltransferase was also measured by in-gel detection of Pγ ADP-ribosylation (Figure 4A) (*29*, *30*). In the system, Pγ (200 μg) was added to 10 mL of a gel mixture, and gels were kept at room temperature (24 h). ADP-ribosyltransferase fractions (50 μL) were suspended in sample buffer [50 mM Tris/HCl (pH 6.8), 1% SDS, 0.0025% bromphenol blue, and 10% glycerol] and incubated (5 min, 34 °C). After SDS−PAGE, these isozymes were renatured by incubation of these gels with buffer E containing 0.1% Triton-X 100 (30 min, 20 °C) (×2). ADP-ribosylation of Pγ was measured by incubating these gels (120−240 min, 33 °C) with 8 mL of buffer E containing NAD (20 μM, ~0.4 mCi). Gels were stained and dried, and ADP-ribosylated Pγ was detected by autoradiography.

Analytical Methods. SDS−PAGE was performed as described (*31*). Activities of PDE and Pγ were assayed as described (*11*). GTPγS binding to various ROS membranes was performed as described (*31*). Protein concentration was measured with bovine serum albumin as standard (*32*). To calculate the Pγ concentration, 9625 and 9669 were used as molecular weights of frog and recombinant bovine Pγ (*24*), respectively, although Pγ was detected as a 13 000 band in SDS-gels. It should be emphasized that all experiments were carried out more than 2 times, and results were similar. Data shown are representative of these experiments.

RESULTS

(1) Effect of Preincubation with NAD on GTP/Tα-Dependent Activation of PDE and Pγ Release. In amphibian ROS membranes, Pγ is released by GTP/Tα for the activation of PDE. This conclusion is based on the following observations: (a) After ROS membranes are incubated with GTP of GTPγS, PDE activity in these membranes is increased to ~10 times the basal activity (*10*, *11*). We note that the PDE activity is measured after these guanine nucleotides are washed out. (b) Pγ is released by GTPγS or GTP from ROS membranes (*10*, *11*). When GTPγS is used, Pγ complexed with GTPγS/Tα can be isolated. If GTP is used, Pγ complexed with GDP/Tα can be isolated, indicating that Pγ has high affinity to Tα even after GTP bound to Tα is hydrolyzed to GDP. Since only GTP/Tα can active PDE, we have speculated that GTP/Tα also interacts with P$\alpha\beta$, and that the interaction makes the interaction between Tα and Pγ possible (*33*). In this study, the effect of the Pγ ADP-ribosylation on the GTP/Tα-dependent activation of PDE was investigated by measuring GTPγS/Tα-activated PDE activity and GTPγS/Tα-dependent Pγ release in ROS membranes incubated with or without NAD. As shown in Figure 1A, lanes c and d, if ROS membranes were preincubated without NAD, the PDE activity was stimulated by GTPγS-washing, and the activity was ~6 times of the basal activity. However, when ROS membranes were preincubated with NAD, the activity was decreased to ~50% of that observed in membranes preincubated without NAD (Figure 1A, lanes b and d). The basal activity of PDE in membranes was not affected by the NAD incubation (Figure 1A, lanes a and c). We note that the PDE activity was measured after free NAD and GTPγS were washed out. When the contents of Pγ in extracts were measured using inhibition of PDE activity in Pγ-less PDE/membranes, the PDE activity with the extract from NAD-treated membranes was higher than that with the extract from control membranes (Figure 1B). This suggests that the GTPγS extract from NAD-treated membranes contains less Pγ than that of control membranes. Dot-blotting of each GTPγS extract to an Immobilon-P membrane and visualization of the blotted Pγ by a Pγ-specific antibody showed that the Pγ content in the extract of NAD-pretreated ROS membranes was less than ~50% of that in the extract from membranes preincubated without NAD (Figure 1C). In our previous study (*28*), we have shown that under similar conditions only Pγ is ADP-ribosylated by endogenous ADP-ribosyltransferase and ~50% Pγ is modified. We have also indicated that a Pγ domain containing ADP-ribosylation sites (arginines 33 and 36) is crucial for its interaction with Tα. Together, these observations suggest that only Pγ ADP-ribosylation was involved in the NAD effect, and that the release of Pγ was suppressed by the ADP-ribosylation because the ADP-ribosylated Pγ lost its ability to interact with GTP/Tα. We note that the level of GTPγS binding to ROS membranes was not changed by the NAD treatment when GTPγS binding to these ROS membranes was compared after incubation of these membranes with or without NAD (data not shown). We also note that ADP-ribosylation of Tα (*34*, *35*) was negligible under our conditions (data not shown). These observations suggest that

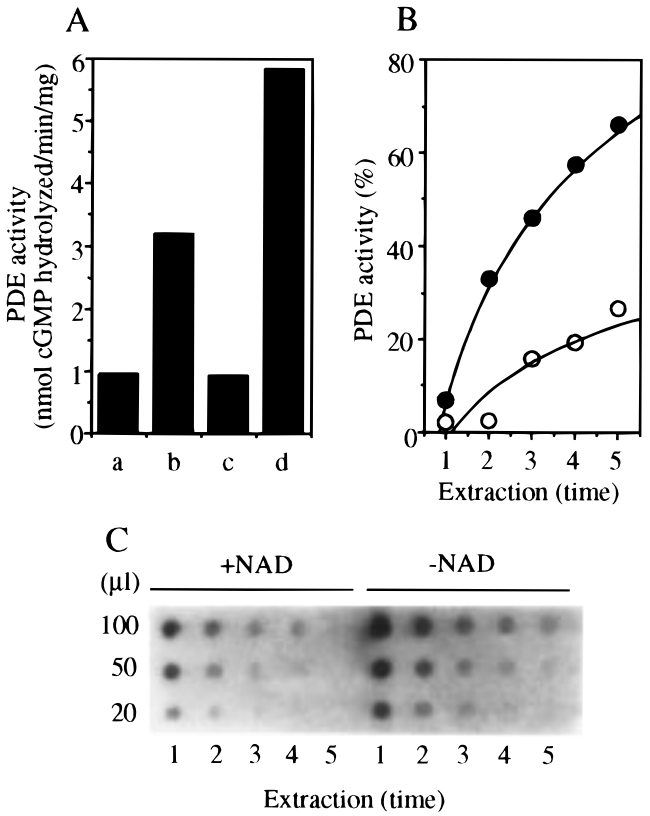

FIGURE 1: Inhibitory effect of preincubation of ROS membranes with NAD on the GTP/Tα-dependent PDE activation and Pγ release. After ROS membranes of 20 frogs were washed with 5 mL of buffer G [10 mM Tris/HCl (pH 7.5), 5 mM DTT, 5 mM $MgCl_2$, 1 mM EGTA, and 0.1 mM PMSF] (×7), the ROS membranes suspended in 6 mL of buffer G were divided into two portions. One portion (27.5 mg of protein) was incubated with NAD (final concentration, 50 μM) for 1 h at 33 °C, and the other portion, as a control, was incubated without NAD under the same conditions. These ROS membranes were washed with 5 mL of buffer G (×2) to remove residual NAD and suspended in 1 mL of buffer G. Each ROS membrane preparation was further divided into two portions. These ROS membranes were incubated with or without 10 μM GTPγS (final concentration) for 10 min at 0 °C, and supernatant and membrane fractions were separated by centrifugation for Pγ extraction (15 min, 2000000*g*, 4 °C). The Pγ extraction was carried out 7 times, and each supernatant was collected separately. After extraction of Pγ, ROS membranes were further washed with 2 mL of buffer G (×3) to remove residual GTPγS and suspended in 2 mL of buffer G. (A) PDE activity in ROS membranes. PDE activity in membranes was measured after appropriate dilution. Lanes a and b, membranes incubated with NAD; lanes c and d, membranes incubated without NAD; lanes a and c, membranes incubated without GTPγS; lanes b and d, membranes incubated with GTPγS. (B) Pγ in supernatants measured by the inhibition of PDE activity. Pγ extracted from ROS membranes, which were treated with or without NAD, was measured with Pγ-less PDE/membranes. (●) PDE activity with supernatants extracted from NAD-treated membranes; (○) PDE activity with supernatants from control membranes. (C) Pγ in supernatants detected by a Pγ-specific antibody. Pγ in supernatants was detected using a Pγ-specific antibody after dot-blotting of supernatants (20, 50, and 100 μL) to an Immobilon-P membrane. +NAD, extracts from NAD-treated membranes; −NAD, extracts from control membranes.

the amounts of GTPγS/Tα in these membranes were not changed by the NAD treatment, and that Tα was not modified by endogenous ADP-ribosyltransferase. Thus, it is concluded that inhibition by the NAD preincubation of the GTP/Tα-dependent PDE activation did not result from the NAD effect on Tα.

(2) ADP-Ribosylarginine Hydrolase Activity in the Soluble Fraction of ROS. If Pγ ADP-ribosylation is involved in the regulation of PDE in vivo, Pγ ADP-ribosylation should be reversible. We found ADP-ribosylarginine hydrolase activity in a soluble fraction of ROS. As shown in Figure $2A_1$, the Pγ ADP-ribosylation was reduced by the soluble fraction in a concentration-dependent manner. Interestingly, ADP-ribosylation levels of Pγ incubated with large amounts of the supernatant (8.7 and 26 μg) were lower than that of Pγ preincubated without the supernatant (*0 μg), suggesting the release of ADP-ribose from ADP-ribosylated Pγ. The effect of the supernatant was also detected in a time-dependent manner (Figure $2A_2$). All ADP-ribosylation levels of Pγ incubated with the soluble fraction were lower than that of Pγ which was not incubated with the supernatant, suggesting the release of ADP-ribose from the ADP-ribosylated Pγ. We note that under these conditions Pγ was constantly ADP-ribosylated during incubation with the supernatant. To confirm the release of ADP-ribose from ADP-ribosylated Pγ, the direct effect of the soluble fraction on [^{32}P]ADP-ribosylated Pγ was also checked. As shown in Figure 2B, radioactivity of the ADP-ribosylated Pγ was reduced in a time-dependent manner when the Pγ was incubated with the soluble fraction. We note that without the soluble fraction no reduction was detected in the entire incubation period (data not shown). These observations suggest that an enzyme catalyzing the release of ADP-ribose from ADP-ribosylated proteins, ADP-ribosylarginine hydrolase, is present in the soluble fraction of ROS. This observation implies that the Pγ ADP-ribosylation is reversible.

(3) Solubilization of Rod ADP-Ribosyltransferase by PI-Specific Phospholipase C. To strengthen our conclusions described above, we tried to isolate ADP-ribosyltransferase from frog ROS. Preliminary experiments indicated that all activity of ADP-ribosyltransferase was found in the membrane fraction, and that the enzyme was sedimented in the rhodopsin fraction in a sucrose gradient. These observations strongly suggest that ADP-ribosyltransferase is in ROS membranes. However, the ADP-ribosyltransferase was not effectively solubilized with nonionic detergents, such as Triton X-100, Nonidet P-40, and *n*-dodecyl-β-D-maltoside (data not shown). Thus, we speculated that the enzyme might be a GPI-anchored protein as other ADP-ribosyltransferase (*36*, *37*). We found that rod ADP-ribosyltransferase was solubilized by PI-specific phospholipase C. As shown in Figure 3, the enzyme activity in a supernatant was increased with the concentration of PI-specific phospholipase C, and the enzyme activity in membranes was decreased almost proportionally. Under our conditions, ∼90% of the total activity was released by the PI-specific phospholipase C. Since PI-specific phospholipase C is able to cleave specifically PI into diglyceride and *myo*-inositol cyclic 1,2-phosphate (*38*), these observations indicate that rod ADP-ribosyltransferase is a GPI-anchored protein. The molecular mass of the solubilized enzyme measured by a TSK-250 size exclusion column is ∼45 kDa (data not shown).

(4) Two Isozymes of Rod ADP-Ribosyltransferase. After solubilization of rod ADP-ribosyltransferase by PI-specific phospholipase C, the enzyme preparation was applied to a Mono Q column. As shown in Figure 4A, rod ADP-ribosyltransferase was eluted as two enzymatic peaks. The first peak of rod ADP-ribosyltransferase was eluted by ∼350

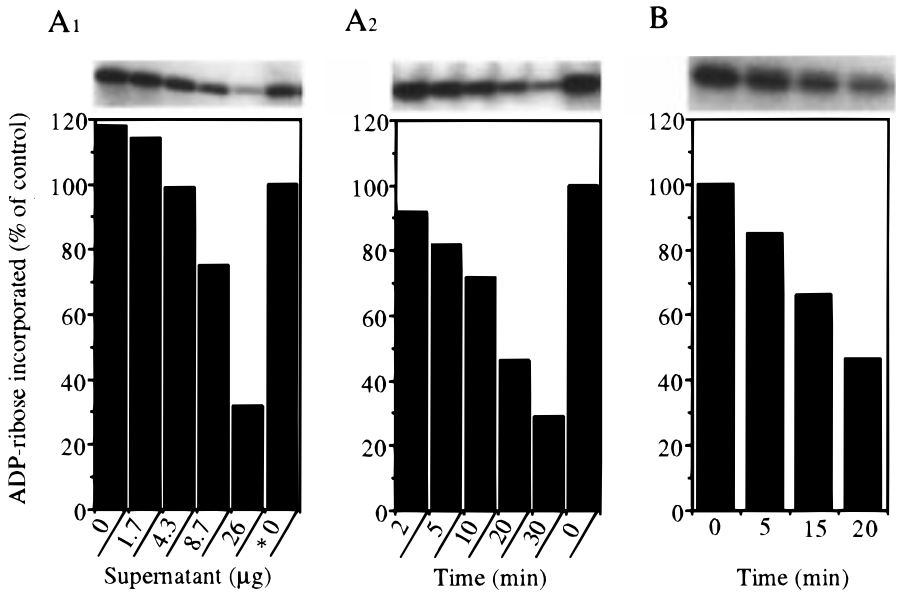

FIGURE 2: Effect of a ROS-soluble fraction on the ADP-ribosylated Pγ. (A) Effect of a ROS supernatant under the conditions for Pγ ADP-ribosylation. (A_1) Amounts of the supernatant. After ROS from two frogs were suspended in 0.5 mL of buffer G, the membrane and soluble fractions were separated by centrifugation (15 min, 200000*g*, 4 °C). The membrane fraction was further washed with 2 mL of buffer G (×6). The supernatant fraction was also further centrifuged (15 min, 200000*g*, 4 °C) to exclude completely the membrane fraction. Pγ (0.2 μg) was ADP-ribosylated in 30 μL of the reaction mixture containing the membrane fraction (42 μg) for 30 min at 33 °C. Then 20 μL of the supernatant containing the indicated amounts of proteins was added to the reaction mixture, and the mixture was further incubated (22 min). The reaction was terminated by incubating (5 min, 80 °C) with SDS-sample buffer. Pγ ADP-ribosylation was monitored by SDS−PAGE and autoradiography, and the radioactivity of Pγ bands was also measured. The asterisk indicates the level of Pγ ADP-ribosylation before incubation with supernatant. (A_2) Time course. Pγ ADP-ribosylation was carried out in 156 μL of the reaction mixture containing Pγ (1.2 μg), membranes (252 μg), and NAD (19.2 μM, ~50 μCi) for 30 min at 33 °C. Then the supernatant (144 μL, 163 μg) was added to the reaction mixture and further incubated at 33 °C. After incubation for the indicated periods, the reaction mixture (50 μL) was taken out, mixed with 30 μL of SDS-sample buffer, and heated at 80 °C for 5 min to terminate the reaction. SDS−PAGE and autoradiography were carried out as described. The radioactivity of Pγ bands was also measured. (B) Effect of a ROS supernatant on the release of radioactivity from [^{32}P]ADP-ribosylated Pγ. [^{32}P]ADP-ribosylated Pγ (0.64 μg) was incubated (33 °C) with a ROS soluble fraction (67 μg) in 160 μL of buffer H [10 mM Tris/HCl (pH 7.5), 5 mM $MgCl_2$, and 1 mM DTT]. After incubation for the indicated periods, an aliquot (50 μL) was taken out, and 30 μL of SDS-sample buffer was added to the aliquot and heated at 80 °C for 5 min. These samples were analyzed as described. In the [^{32}P]ADP-ribosylated Pγ preparation, ~23% of Pγ was ADP-ribosylated, and its radioactivity was 10 440 cpm.

mM NaCl and contained ~10% of the eluted activity. The second peak was eluted by ~500 mM NaCl and contained ~90% of the eluted activity. These observations indicate that the preparation contains two fractions of ADP-ribosyltransferase. The molecular masses of ADP-ribosyltransferase in these fractions were also estimated by in-gel detection of Pγ ADP-ribosylation. These molecular masses are 40 and 45 kDa, respectively (Figure 4A, upper panel). These molecular masses are in the range measured by the size-exclusion column chromatography as described above. These observations indicate that there are two different enzymes for the Pγ ADP-ribosylation in the rod ADP-ribosyltransferase preparation. When the pH of the preparation was adjusted to 4.2 by dialyzing against a citrate/phosphate buffer and the preparation was applied to a Mono S column that had been equilibrated with the same buffer, ~23% of the applied activity was found in a flow-through fraction and ~50% of the applied activity was eluted as a single peak by ~280 mM NaCl (data not shown). When the peak fraction in the Mono S column was further applied to a Mono Q column under the same conditions as those shown in Figure 4A, all enzymatic activity was eluted in the same fraction of a second peak activity in the Mono Q column chromatography (Figure 4B, upper panel). These observations suggest that the enzyme bound to the Mono S column is the same as the enzyme in the second peak in the Mono Q chromatography, and that the enzyme fraction binds to both Mono Q and Mono S columns under our conditions. When the flow-through fraction in the Mono S chromatography was applied to a Mono Q column under the same conditions as those shown in Figure 4A, two enzymatic activities were eluted (Figure 4B, lower panel). The first peak (~50% of the eluted activity) was eluted in the same fractions as that in the first peak in Figure 4A, and the second peak (~50% of the eluted activity) was eluted in the same fractions as that in the second peak in Figure 4A. These observations suggest that the flow-through fraction of the Mono S column chromatography contains the first and second peak fractions in the Mono Q column shown in Figure 4A, and that the first peak enzyme in the Mono Q column (Figure 4A) could not bind to the Mono S column under our conditions. Together, these observations indicate that rod ADP-ribosyltransferase contains at least two enzymes, ADP-RT-1 and ADP-RT-2. Data depicted in the lower panel of Figure 4B also suggest that some fractions of the second peak enzyme in the Mono Q column (Figure 4A) could not bind to the Mono S column. We are not sure whether the second peak in the Mono Q column could be further divided into two different fractions or a part of the second peak enzyme simply could not bind to the Mono S column under the conditions.

(5) Biochemical Characterization of ADP-RT-1 and ADP-RT-2. We conclude that the basic properties of these two isozymes for the Pγ ADP-ribosylation appear to be similar,

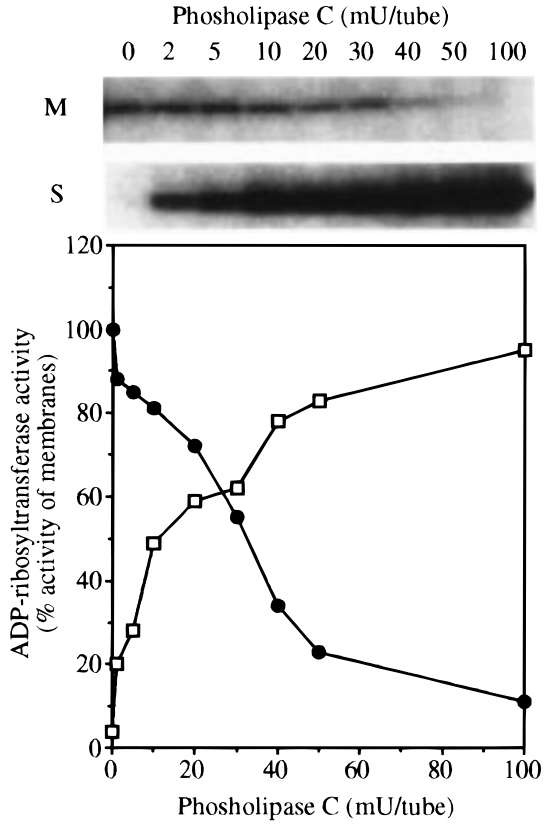

FIGURE 3: Solubilization of rod ADP-ribosyltransferase by PI-specific phospholipase C. Pγ- and transducin-depleted ROS membranes (2.3 mg of protein) were suspended in 800 μL of buffer B. To 80 μL of the sample were added the indicated milliunits (mU) of PI-specific phospholipase C and incubated (37 °C, 30 min). Supernatant and membrane fractions were separated by centrifugation (200000*g*, 30 min, 4 °C). The membrane fraction was suspended in the same volume of buffer B. ADP-ribosyltransferase activities in these supernatant (○) and membrane (●) fractions were measured ad described. Pγ ADP-ribosylation was monitored by SDS−PAGE and autoradiography. We note that the volume of the supernatant fraction added to the reaction mixture was 10 times larger than that of the membrane fraction. Thus, the Pγ ADP-ribosylation by the supernatant fraction was much higher than that by the membrane fraction in autoradiography. The ADP-ribosyltransferase activity of these membranes (100%) was 6.6 pmol of ADP-ribose incorporated min^{-1} (mg of protein)$^{-1}$. Autoradiography: M, membranes; S, supernatant.

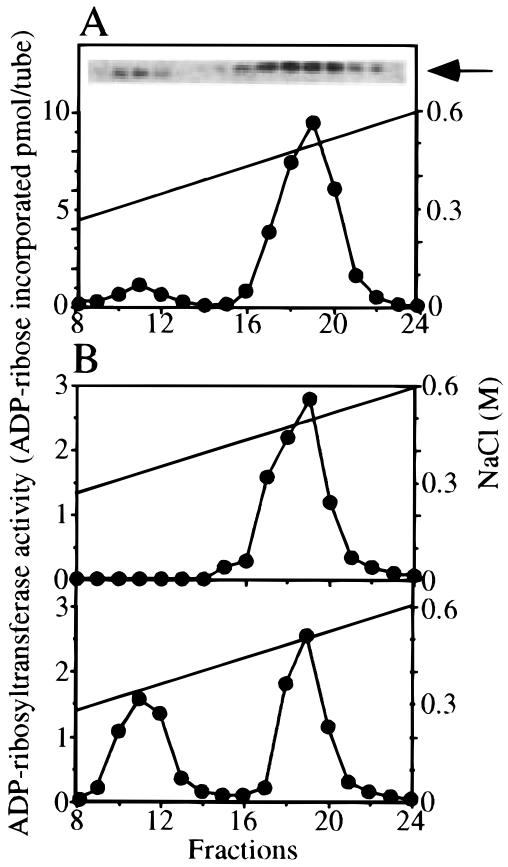

FIGURE 4: Mono Q column chromatography of rod ADP-ribosyltransferase solubilized by PI-specific phospholipase C. (A) Mono Q column chromatography of the solubilized enzyme. ADP-ribosyltransferase solubilized by PI-specific phospholipase C from Pγ- and transducin-depleted membranes (120 mg) was applied to a Mono Q column which had been equilibrated with buffer C. After the column was washed with buffer C, the enzyme was eluted with a linear gradient of NaCl (0−1 M) in buffer C. ADP-ribosyltransferase activity in each fraction was monitored for 30 min. In-gel measurement of the enzymatic activity (120 min, 33 °C) was also carried out using Pγ as substrate in gels to estimate molecular masses of these isozymes, as shown in the top of panel A. An arrow indicates 45 kDa (the second peak). (B) Mono Q chromatography of bound and flow-through fractions in Mono S column chromatography. ADP-ribosyltransferase was solubilized from Pγ- and transducin-depleted ROS membranes as described. After the pH of a ADP-ribosyltransferase preparation (0.7 mg) was adjusted to 4.2 with buffer D, the sample was applied to a Mono S column which had been equilibrated with buffer D, and eluted with 20 mL of a linear gradient of NaCl (0−1 M) in buffer D. The flow-through fraction (0.2 mg of protein) and the bound fraction (30 μg of protein) were dialyzed against buffer E. These samples were separately applied to a Mono Q column and chromatographed under the same conditions as described in (A). ADP-ribosyltransferase activity in each fraction was measured by Pγ ADP-ribosylation. Upper panel, the bound fraction from the Mono Q column; lower panel, the flow-through fraction from the Mono S column.

but the two isozymes have different properties for the following reasons.

(a) The K_m Values of ADP-RT-1 and ADP-RT-2 for NAD and Pγ. The K_m values for Pγ of these isozymes are ∼2.5 μM and ∼2.0 μM, respectively. The K_m values for NAD are ∼15 μM and ∼20 μM, respectively.

(b) ADP-Ribosylation of Pγ Mutants. Bovine Pγ contains five arginine residues: Arg-11, Arg-15, Arg-24, Arg-33, and Arg-36. In a previous study (*28*), we have shown that Arg-33 and Arg-36 in Pγ can be ADP-ribosylated by ADP-ribosyltransferase in Pγ- and transducin-depleted ROS membranes; however, only one ADP-ribose is incorporated at a time. In this study, ADP-ribosylation of Pγ and its arginine mutants was also investigated using ADP-RT-1 and ADP-RT-2 preparations. As shown in Figure 5, ADP-ribosylation by these enzymes was abolished if both Arg-33 and Arg-36 were replaced by lysines (R33,36K and R11,15,24,33,36K). However, the ADP-ribosylation was not affected by mutation of other arginines in Pγ (R11,15K and R15,24K). We note that R33K and R36K were also ADP-ribosylated by these two isozyme preparations (data not shown). Together, we conclude that both enzyme preparations can similarly ADP-ribosylate Arg-33 and Arg-36, but one arginine is ADP-ribosylated at a time.

(c) The Effect of Inhibitors for ADP-Ribosyltransferase. As shown in Figure 6, both isozymes are sensitive to inhibitors of ADP-ribosyltransferase, novobiocin (*28*, *39*) and benzamide (*40*); however, the sensitivity of ADP-RT-2 to these inhibitors is much higher than that of ADP-RT-1.

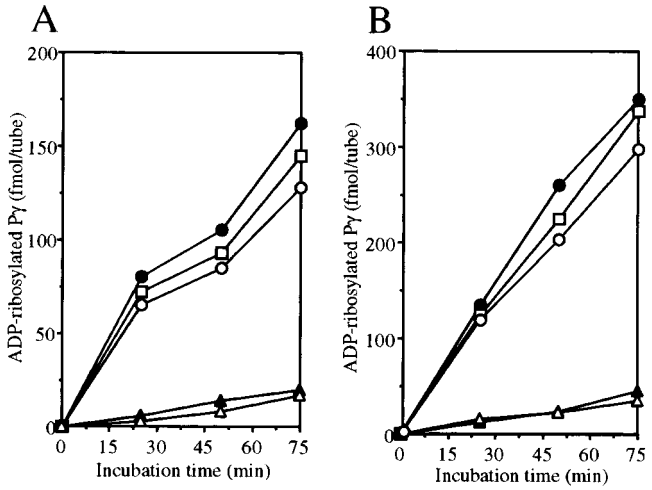

FIGURE 5: ADP-ribosylation of Pγ and its arginine mutants by two ADP-ribosyltransferase isozymes. Pγ (●) and its mutants, R11,15K (□), R15,24K (○), R33,36K (▲), and R11,15,24,33,36K (△), were ADP-ribosylated with two isozymes. These isozymes were isolated by a Mono Q column, as described in Figure 4A. Pγ and its mutants (0.4 μg), NAD (20 μM, ~2 μCi), and each isozyme were incubated in 200 μL of 10 mM Tris/HCl (pH 7.5) at 37 °C. After incubation for the indicated periods, an aliquot (50 μL) was taken out. SDS-sample buffer (20 μL) was added to the aliquot and heated at 80 °C for 5 min to stop the reaction. The ADP-ribosylated Pγ was monitored using SDS−PAGE as described. (A) ADP-RT-1; (B) ADP-RT-2.

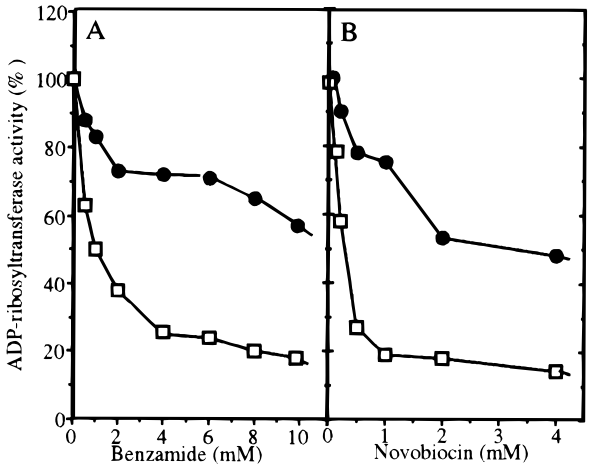

FIGURE 6: Effect of inhibitors of ADP-ribosyltransferase on the Pγ ADP-ribosylation by two ADP-ribosyltransferase isozymes. The Pγ ADP-ribosylation was assayed in the presence of ADP-ribosyltransferase inhibitors: benzamide (A) and novobiocin (B). Pγ (0.1 μg) was incubated in 50 μL of 20 mM Tris/HCl (pH 7.5) containing NAD (20 μM, ~0.5 μCi) and ADP-RT-1 (●) or ADP-RT-2 (□) for 60 min at 33 °C. The ADP-ribosylated Pγ was monitored using SDS−PAGE as described. The enzymatic activities of ADP-RT-1 and ADP-RT-2 without inhibitor (100%) were 1.1 and 1.3 pmol of ADP-ribose incorporated/tube, respectively.

(d) The Optimum pH for the Enzymatic Activity. As shown in Figure 7A, the enzymatic activities of both isozymes were detected in a wide range of pHs in their reaction buffers. However, the optimum pHs for the activity of ADP-RT-1 and ADP-RT-2 were ~7.0 and ~4.5, respectively. We note that Pγ treated with different pHs (pH 2−12) showed the same inhibitory activity on Pγ-less PDE/membranes, indicating that these observations are not due to the sensitivity of Pγ to the pH range. Thus, we conclude that these observations are due to the sensitivity of the isozymes to a different pH range.

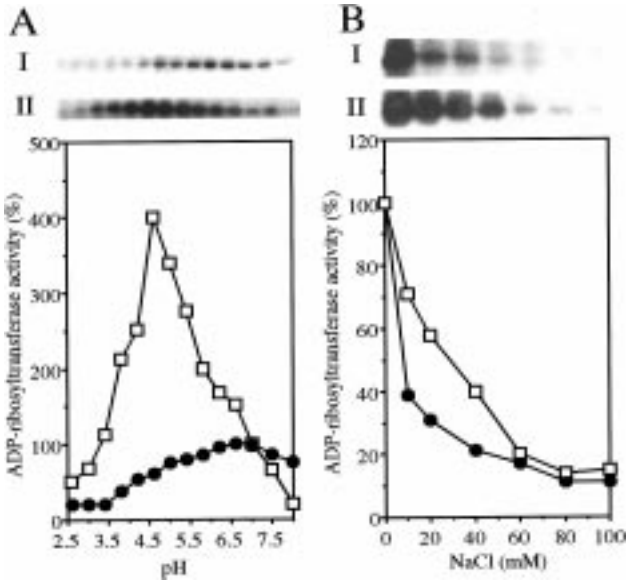

FIGURE 7: Effect of pH and NaCl concentration on the Pγ ADP-ribosylation by two ADP-ribosyltransferase isozymes. Two isozymes were prepared from Pγ- and transducin-depleted ROS membranes as described in Figure 4A. (A) pH. The Pγ ADP-ribosylation was assayed in 50 μL of 20 mM citrate/phosphate (pH 2.6−6.0) or phosphate (pH 6.5−8.0) buffers containing Pγ (0.1 μg), NAD (20 μM, ~0.5 μCi), and ADP-RT-1 (●) or ADP-RT-2 (□) for 60 min at 33 °C. The ADP-ribosylated Pγ was monitored using SDS−PAGE as described. ADP-ribosylated Pγ in gels is also shown: I, ADP-RT-1; II, ADP-RT-2. The enzyme activities of ADP-RT-1 or ADP-RT-2 at pH 7.0 (100%) were 0.7 and 1.3 pmol of ADP-ribose incorporated/tube, respectively. (B) NaCl. The Pγ ADP-ribosylation (60 min at 33 °C) was assayed in 50 μL of 10 mM Tris/HCl (pH 7.5) containing Pγ (0.1 μg), NAD (20 μM, ~0.5 μCi), and ADP-RT-1 (●) or ADP-RT-2 (□) in the presence of various concentrations of NaCl. The ADP-ribosylated Pγ was monitored using SDS−PAGE. ADP-ribosylated Pγ in gels is also shown: I, ADP-RT-1; II, ADP-RT-2. The maximal activities (100%) of ADP-RT-1 and ADP-RT-2 were 1.7 and 2.3 pmol of ADP-ribose incorporated/tube, respectively.

(e) The Sensitivity of These Enzymes to NaCl. Both enzymes are very sensitive to the NaCl concentration in reaction mixtures; however, their sensitivities are different. In the presence of 10 mM NaCl, ~60% of the ADP-RT-1 activity was inhibited; however, only ~30% of the ADP-RT-2 activity was reduced (Figure 7B). We note that high concentration of other salts, such as Tris/HCl (pH 7.5) and KCl, also inhibited the Pγ ADP-ribosylation (data not shown), indicating that salt concentration may be an important factor for the Pγ ADP-ribosylation by these solubilized isozymes. We note that the Pγ ADP-ribosylation was detected even in the presence of 100−200 mM salts if the enzyme was not solubilized with PI-specific phospholipase C (data not shown). We believe that solubilized isozymes are more sensitive to salt concentration than membrane-bound isozymes, and that these isozymes are not sensitive to physiological concentrations of salts in photoreceptors. In this study, we used their salt sensitivities to show that these isozymes are different.

DISCUSSION

In a previous study (*28*), we have shown that Pγ, free or complexed with P$\alpha\beta$, is ADP-ribosylated by an endogenous ADP-ribosyltransferase, and that a Pγ domain containing ADP-ribosylated sites is required for its interaction with Tα.

We used Pγ ADP-ribosylation as a tool to identify the Pγ domain. In this study, we investigated the possible function of Pγ ADP-ribosylation in the GTP/Tα-dependent activation of PDE. We have shown that GTP/Tα-dependent activation of PDE in ROS membranes is inhibited by preincubation of these membranes with NAD. The inhibition is due to the suppression of GTP/Tα-dependent Pγ release. Since only Pγ was ADP-ribosylated under our conditions, we conclude that the inhibition of Pγ release by NAD results from the Pγ ADP-ribosylation. In addition, we have suggested that ADP-ribosylarginine hydrolase, an enzyme that releases ADP-ribose from ADP-ribosylated arginine, is in the soluble fraction of ROS. This implies that the Pγ ADP-ribosylation is reversible. To reinforce our conclusion, we also tried to solubilize and characterize rod ADP-ribosyltransferase. We have shown that rod ADP-ribosyltransferase is solubilized by PI-specific phospholipase C, indicating that the enzyme is a GPI-anchored protein. We believe that rod ADP-ribosyltransferase is the first GPI-anchored protein found in ROS. We have also shown that the solubilized enzyme contains two isozymes, which can be separated by Mono S and Mono Q columns. These two isozymes have similar properties for Pγ ADP-ribosylation. Overall, we conclude that the PDE activation by GTP/Tα is inhibited by Pγ ADP-ribosylation through inhibition of the release of Pγ by GTP/Tα.

We think that this study is only the first step to understand the function of Pγ ADP-ribosylation in the GTP/Tα-dependent PDE activation, and that more information is needed to reveal the real function of Pγ ADP-ribosylation in phototransduction. For example, due to the difficulty in preparation of highly ADP-ribosylated Pγ, we are not sure whether ADP-ribosylation of Pγ changes its affinity to Pαβ. For the same reason, we could not reconstitute Pαβ complexed with ADP-ribosylated Pγ. We also could not confirm that ADP-ribosylated Pγ is not released by GTP/Tα in the reconstituted system. Moreover, we do not know whether ADP-ribosylation of Pγ occurs in vivo. Furthermore, we do not know the relationship between Pγ phosphorylation and Pγ ADP-ribosylation. The Pγ domain containing the ADP-ribosylated site (*28*) also includes threonine 35, which can be phosphorylated by PKC (*22*) and PKA (*23*) in vitro. Thus, the close relationship between Pγ phosphorylation and Pγ ADP-ribosylation is expected. We believe that these studies will confirm the role of Pγ ADP-ribosylation in PDE regulation, and that the Pγ ADP-ribosylation, if confirmed by these studies, opens a new regulatory mechanism of PDE in phototransduction.

This study suggests the possibility that some PDE may not accept GTP/Tα signals because Pγ is ADP-ribosylated and the Pγ ability to interact with Tα is abolished. This possibility is reinforced by observations suggesting that ROS contains all enzymes required for the reversible processes. Has this possibility been predicted in previous studies? We have shown that ~50% of Pγ is not sensitive to GTP/Tα in amphibian ROS membranes (*11*). Pγ cannot be released even when excess GTPγS/Tα is added. We have also shown that all bovine PDE cannot be necessarily activated in the presence of excess amounts of GTPγS/Tα even with overnight incubation (*41*). At present, we do not know the reason some PDE cannot be activated by GTP/Tα. However, we anticipate that Pγ ADP-ribosylation may be involved in the GTP/Tα insensitivity of Pγ. If so, it is possible that the Pγ ADP-ribosylation may be a mechanism to prevent spontaneous activation of Tα. Pγ ADP-ribosylation has been detected when Pγ is free or complexed with Pαβ (*28*). Since it is difficult to imagine that free Pγ is present in ROS, Pγ complexed with Pαβ may be the real substrate for rod ADP-ribosyltransferase in ROS. If so, Pγ ADP-ribosylation may occur in dark conditions, and PDE containing ADP-ribosylated Pγ cannot be activated by GTP/Tα even if GTP/Tα is formed under the dark conditions. Under these conditions, reversible ADP-ribosylation will be important. We have suggested that ADP-ribosylarginine hydrolase is present in ROS, and that the Pγ ADP-ribosylation is reversible in ROS. However, we note that this may not be a simple reversible reaction. We found that the Pγ ADP-ribosylation was also detected even in a ROS homogenate; however, the levels of the ADP-ribosylation were changed (data not shown). These observations may suggest that enzymatic activities of ADP-ribosyltransferase and ADP-ribosylarginine hydrolase may be different under different conditions. We anticipate that both ADP-ribosyltransferase and ADP-ribosylarginine hydrolase systems are co-regulated to regulate the total volume of available PDE for phototransduction.

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