Phosphorylation by Cyclin-dependent Protein Kinase 5 of the Regulatory Subunit of Retinal cGMP Phosphodiesterase

II. ITS ROLE IN THE TURNOFF OF PHOSPHODIESTERASE IN VIVO*

Retinal cGMP phosphodiesterase (PDE) is regulated by Pγ, the regulatory subunit of cGMP phosphodiesterase, and GTP/Tα, the GTP-bound α subunit of transducin. In the accompanying paper (Matsuura, I., Bondarenko, V. A., Maeda, T., Kachi, S., Yamazaki, M., Usukura, J., Hayashi, F., and Yamazaki, A. (2000) J. Biol. Chem. 275, 32950–32957), we have shown that all known Pγs contain a specific phosphorylation motif for cyclin-dependent protein kinase 5 (Cdk5) and that the unknown kinase is Cdk5 complexed with its activator. Here, using frog rod photoreceptor outer segments (ROS) isolated by a new method, we show that Cdk5 is involved in light-dependent Pγ phosphorylation in vivo. Under dark conditions only negligible amounts of Pγ were phosphorylated. However, under illumination that bleached less than 0.3% of the rhodopsin, ~4% of the total Pγ was phosphorylated in less than 1 s. Pγ dephosphorylation occurred in less than 1 s after the light was turned off. Analysis of the phosphorylated amino acid, inhibition of Pγ phosphorylation by Cdk inhibitors in vivo and in vitro, and two-dimensional peptide map analysis of Pγ phosphorylated in vivo and in vitro indicate that Cdk5 phosphorylates a Pγ threonine in the same manner in vivo and in vitro. These observations, together with immunological data showing the presence of Cdk5 in ROS, suggest that Cdk5 is involved in light-dependent Pγ phosphorylation in ROS and that the phosphorylation is significant and reversible. In an homogenate of frog ROS, PDE activated by light/guanosine 5’-O-(3-thiotriphosphate) (GTPγS) was inhibited by Pγ alone, but not by Pγ complexed with GDP/Tα or GTPγS/Tα. Under these conditions, Pγ phosphorylated by Cdk5 inhibits the light/GTPγS-activated PDE even in the presence of GTPγS/Tα. These observations suggest that phosphorylated Pγ interacts with and inhibits light/GTPγS-activated PDE, but does not interact with GTPγS/Tα in the homogenate. Together, our results strongly suggest that after activation of PDE by light/GTP, Pγ is phosphorylated by Cdk5 and the phosphorylated Pγ inhibits GTPγS/Tα-activated PDE, even in the presence of GTPγS/Tα in ROS.

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The hydrolysis of cGMP by PDE1 in vertebrate ROS is directly involved in visual signal transduction (1, 2). The inactive PDE is composed of Pαβ, catalytic subunits, and two Pγs, regulatory subunits (3–6). In amphibian ROS, PDE is regulated similarly to that in mammalian ROS (7, 8): PDE catalytic activity is controlled by Pγ and GTP/Tα. In frog ROS membranes, bleached rhodopsin stimulates GTP/GDP exchange on Tα (9), and the GTP/Tα formed is released from membrane-bound Tβγ (9, 10). The free GTP/Tα interacts with Pαβγ, and Pγ complexed with GTP/Tα is released from Pαβγ-membranes (10–12). PDE is thereby activated. The release of the Pγ complex is detected even in an isotonic buffer containing Mg2+, and Pγ complexed with GTPγS/Tα can be isolated using sequential column chromatography (10). During PDE activation, Pγ-less Pαβ binds tightly to membranes.2 In the recovery processes of frog ROS, after GTP hydrolysis by Tα, Pγ remains in the complex with GDP/Tα (10). When the GDP/Tα-Pγ complex interacts with membrane-bound Tβγ, Pγ is released from the complex and reassociates with Pαβ, resulting in the turnoff of PDE (10). The Pγ complex with GDP/Tα is very tight, and the GDP/Tα/Pγ complex can be isolated by sequential column chromatography (10).

It has been suggested that Pγ phosphorylation is involved in the PDE regulatory mechanism. Pγ is phosphorylated by PI-stimulated kinase (13), PKC (14), PKA (15), and Pγ kinase (16, 17). In the PI-dependent Pγ phosphorylation (13), threonine 35 or serine 40 in Pγ may be phosphorylated. PKC (14) and PKA (15) phosphorylates threonine 35 in Pγ. The phosphorylated Pγ has a higher inhibitory activity against GTPγS/Tα-activated PDE than that of nonphosphorylated Pγ. The important point in the Pγ phosphorylations by these protein kinases is that the Pγ phosphorylations appear not to occur when Pγ binds to GTPγS/Tα. We have shown that Pγ phosphorylations by PI-stimulated kinase (13) and PKA (15) were inhibited by GTPγS/Tα. In the case of Pγ phosphorylation by PKA (15), the inhibition is due to the unavailability of the phosphorylation site in Pγ, because a Pγ region, including threonine 35, is involved in its interaction

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1 The abbreviations used are: PDE, cGMP phosphodiesterase; Pαβ, the α and β subunits of PDE; Pγ, the γ subunit of PDE; ROS, rod outer segments; Tα and Tβγ, the α and γ subunits of transducin; GTPγTα, the GTP-bound form of Tα; GTPγS, guanosine 5’-O(3-thiotriphosphate); PI, phosphatidylinositol; PKC, protein kinase C; PKA, protein kinase A; Cdk, cyclin-dependent protein kinase; p35, a neuronal activator of Cdk5; p25, a proteolytic digested form of p35; PAGE, polyacrylamide gel electrophoresis; FMRFamide, phenethylsulfonfluoride; DTT, dithiothreitol; MAP kinase, mitogen-activated protein kinase; BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N,N’-N'-tetraacetic acid.

2 V. A. Bondarenko, M. Yamazaki, and A. Yamazaki, unpublished observations.
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with GTP/\(\text{T}a\), and the region is masked when \(P_g\) is complexed with GTP/\(\text{T}a\). The same kind of inhibition was also observed in the ADP-ribosylation of \(P_g\), because the \(P_g\) ADP-ribosylation site (arginine 33 or 36) is masked when \(P_g\) is complexed with GTP/\(\text{T}a\) (18, 19). It is very likely that the phosphorylation of threonine 35 in \(P_g\) occurs when \(P_g\) is complexed with PaB. We have shown that arginine 33 or 36 in \(P_g\) is ADP-ribosylated when \(P_g\) is complexed with PaB (18).

In contrast to these \(P_g\) phosphorylations, \(P_g\) phosphorylation by \(P_g\) kinase appears to be light-dependent and thus can be brought into agreement with in the current model of phototransduction (16, 17). In the phosphorylation, \(P_g\) complexed with GTP/\(\text{T}a\) is the best substrate for \(P_g\) kinase, and the \(P_g\) phosphorylation is dependent upon GTP in ROS membranes. These results indicate that the \(P_g\) phosphorylation occurs after PDE activation. Threonine 22 in \(P_g\) is phosphorylated. The phosphorylated \(P_g\) loses its affinity to GTP/\(\text{T}a\), but gains a 10−15 times higher ability to inhibit PDE activity than that of nonphosphorylated \(P_g\). Thus, the phosphorylated \(P_g\) more effectively inhibits GTP/\(\text{T}a\)-activated PDE than nonphosphorylated \(P_g\), and the inhibition occurs even in the presence of GTP/\(\text{T}a\). These observations imply that 1) the \(P_g\) phosphorylation is probably involved in the recovery phase of phototransduction to the dark state, 2) after activation of PDE, GTP/\(\text{T}a\) may interact with another effector and the interaction may be associated with mechanisms for the recovery of phototransduction, and 3) the lifetime of GTP/\(\text{T}a\)-activated PDE can be regulated by the \(P_g\) phosphorylation when the \(P_g\) phosphorylation functions.

In this series of experiments, we showed that Cdk5 phosphorylates \(P_g\) complexed with GTP/\(\text{T}a\) in vitro (in the accompanying paper (20)) and in vivo (in this paper). In the accompanying paper (20), we have shown that \(P_g\) preserves an amino acid sequence required for the phosphorylation by Cdk5 and that the \(P_g\) kinase is Cdk5 complexed with p35, a Cdk5 activator. We have also demonstrated that recombinant Cdk5/p35 phosphorylates \(P_g\) in a GTP/\(\text{T}a\)-dependent manner in ROS membranes, suggesting that Cdk5 is involved in the phosphorylation of \(P_g\) complexed with GTP/\(\text{T}a\). In the present study, we link these observations with light-dependent \(P_g\) phosphorylation in vivo (21). Using frog photoreceptor outer segments isolated by a new method, we show that Cdk5 is involved in the light-dependent \(P_g\) phosphorylation in vivo, that the \(P_g\) phosphorylation is significant and reversible, and that the \(P_g\) phosphorylation and dephosphorylation are rapid enough to be involved in the recovery phase of phototransduction. Moreover, in an homogenate of photoreceptor outer segments, the phosphorylated \(P_g\) inhibits light/GTP/\(\text{T}a\)-activated PDE, even in the presence of GTP/\(\text{T}a\). These observations suggest that the \(P_g\) phosphorylation verified in the in vitro system (16, 17, 20) functions similarly in functional, isolated photoreceptor outer segments, the phospho-
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Determination of Cdk5 Localization in Frozen Frog Retinas—Fresh frog retina was immediately fixed with 4% paraformaldehyde in 0.13 M phosphate buffer (pH 7.4) for 2 h at 4 °C. After washing with the same buffer (×3), the retina was equilibrated with 30% sucrose solution. Cryo-thick (14 μm thick) slices that bleached (20 CM2050 Bensheim, Germany), and mounted on glass slides. These specimens were blocked with the phosphate-buffered saline buffer containing 1% (w/v) bovine serum albumin and 0.5% Triton X-100 for 30 min at room temperature. Sections were incubated with a Cdk5 antibody at the IgG concentration of 1 ng/ml for 2 h. For controls, the same concentration of the antibody was mixed with the peptide antigen (20 ng/ml) prior to application. Specimens were incubated with a secondary antibody, alkaline phosphatase-conjugated goat anti-rabbit IgG (15 mg/ml), for 1 h. These specimens were washed with the phosphate buffer (×3). Finally, antibody binding sites were visualized with 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt and nitro blue tetrazolium chloride.

Immunological Detection of Phosphorylated Pγ—Before illumination of samples, all manipulations were done under infrared light. Retinas were isolated from frog eye-cups as described above. These retinas were incubated in Ringer’s solution (20 min). After exposure to white light (2% rhodopsin bleached/min) for indicated times, these retinas were quickly frozen as described above. As a control, all procedures were carried out on the reconstituted photoreceptor outer segment. The photoreceptor outer segment layer was isolated from dried retina as described above and then solubilized with 50 μl of 1% SDS containing 100 mM DT. Solubilized outer segment sample was diluted by 10-fold with buffer B (2% Triton X-100, 1% Pharamyly (pH 8–10.5), 0.5% Bio-Lyte (pH 5–7), 0.5% Bio-Lyte (pH 6–8), 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 100 mM odiadic acid, and 9.2% urea in final concentra-
tions). Pγ and phosphorylated Pγ in the solubilized outer segments (50 μg protein) were isolated by two-dimensional gel electrophoresis using Immobiline DryStrip gels, consisting of isoelectric focusing (pH 6–11) in the first dimension and SDS-gradient gel (10–20%) electrophoresis in the second dimension. Pγ and phosphorylated Pγ were blotted on nitrocellulose membranes, and membranes were blocked by 5% milk in Tris-buffered saline containing 0.1% Tween 20. A Pγ-specific antibody diluted in the same blocking buffer was used to identify Pγ, and the bound antibody was detected by a chemiluminescence detection kit. After development of x-ray films, Pγ spots were scanned by Paragon 1200A3 ProScanner and relative density (mm2 × OD) was calculated by Molecular Analyst software (Bio-Rad). The location of phosphorylated Pγ in gels was confirmed using 13P-phosphorylated Pγ.

Detection of Effects of Cdk Inhibitors on Pγ Phosphorylation in Vivo and in Vitro—In the in vivo system, Cdk5 inhibitor, olomoucine (100 μM), or roscovitine (50 μM) was added to retinas incubated in Ringer’s solution containing [32P]Phosphorus. Other experimental conditions were the same as those described above. An inactive analogue of olomoucine, iso-olomoucine (100 μM), was used as a control. Isolation of Pγ and phosphosinomine acid analysis of phosphorylated Pγ were carried out as described above. Effects of these inhibitors on Pγ kinase and recombinant Cdk5/535 were also measured using Pγ phosphorylation in vitro (16, 20).

Two-dimensional Peptide Map Analysis of Pγ Phosphorylated in Vitro and in Vivo—Phosphopeptide maps of Pγ phosphorylated in vitro and in vivo were compared. As Pγ phosphorylated in vitro, frog Pγ (10 μg) was phosphorylated by using ~2–10 μCi of γ-32P-ATP and Pγ kinase, as described previously (16, 20). As Pγ phosphorylated in vivo, after incubation of retinas in Ringer’s solution containing [32P]Phosphorus, Pγ was phosphorylated by 10-min light exposure (20% rhodopsin was bleached), as described above. These phosphorylated Pγs were isolated by immunoprecipitation using a Pγ-specific antibody and SDS-PAGE. Pγ radioactive spots were identified by autoradiography and cut out. Extracted Pγs were digested with trypsin, and the resulting peptides were analyzed using two-dimensional peptide map analysis as described previously (26). Radioactive spots were detected by an image analyzer (BAS2000, Fuji Film).

Measurement of Effects of Nonphosphorylated and Phosphorylated Pγs on Light/32P-activated PDE—Dark-adapted intact frog ROS was prepared from frogs by Percoll density gradient centrifugation (13). The ROS was suspended in buffer C (65 mM KCl, 10 mM NaCl, 10 mM HEPES (pH 7.8), 2 mM MgCl2, 0.2 mM PMSF, 5 mM α-methyl aprotinin), and its aliquot (10 μl) was divided into siliconized plastic tubes. Each tube was tightly sealed with an aluminum foil and stored at ~90 °C. Each frozen ROS was thawed just before use and sheared by passing through a siliconized fine pipette tip. The ROS homogenate (final concentration of rhodopsin, 2.5 μM) was added to 180 ml of buffer C containing 0.45 mM GTPγS and 1 mM BAPTA in a lucen glass chamber with magnetic stirrer. PDE activity was assayed by a pH electrode (Beckman S506A) in the presence or absence of 300 μM Pγ, GDPγS/Pγ, GTPγS/Pγ, and GTPγS/TαS and phosphorylated Pγ. Reaction was started by adding 4 μM cGMP. To stimulate PDE activity, a light flash (100% rhodopsin was given at time 0). After each measurement, continuous white light was applied to see a maximum reaction rate.

Analytical Methods—Pγ phosphorylation by Pγ kinase (16) and recombinant Cdk5/535 (20) was performed as described. Protein concent-
tration was assayed with bovine serum albumin as a standard (27). Concentration of Pγ (10, 20) and recombinant Pγ (20) was meas-
ured as described. It should be emphasized that all experiments were carried out more than two times, and the results were similar. Data shown are representative of these experiments.

RESULTS

Localization of Cdk5 in Frog Retinas—In previous studies (16, 17, 20), we showed that Cdk5 is in a soluble fraction of frog ROS, that Pγ complexed with GTPγS/Tα is the best substrate for Cdk5, and that Pγ phosphorylation by Cdk5 is dependent upon GTPγS in frog ROS membranes. We also showed that all known Pγs have a special phosphorylation motif for proline-
directed kinase, including Cdk5 (20). These biochemical obser-
vations strongly suggest that Cdk5 is in photoreceptor outer segments. In this study, we carried out two experiments to confirm the localization of Cdk5 in photoreceptor outer segments: immunodetection of Cdk5 in outer segments isolated by a new method and an immunohistochemical search for Cdk5 in retina. In the first experiment, photoreceptor outer segments were separated from other neural retinal layers, including photore-
toceptor inner segments (Fig. 1). Light microscopy (~500) showed that outer segment layer contains yellow rod-shape cells (Fig. 1A). The yellow color detected appears to indicate the presence of bleached rhodopsin. The neural retinal layer also contained similar rod-shape cells; however, the color of these cells was white (data not shown). We note that pigment epithelium cells were not contaminated in this preparation, because no black cells were detected in the preparation. To check the purity of the outer segment layer isolated, first we compared the protein profile in the outer segments with that in neural retinal layers. We found that protein profiles are differ-
ent on SDS gels (Fig. 1B, lanes 1). In addition, the purity of the outer segment layer was also examined by comparing of the contents of MAP kinase in the outer segment layer with that in the other neural retinal layers, because, using an immunohis-
tochemical method, we have already suggested that MAP ki-
rase is present in the neural retinal layers, but not in the outer segment layer (20). We found that the immunological signal of MAP kinase was clearly observed in the neural retinal layers, but not in the outer segment layer (Fig. 1B, lanes 3), indicating that MAP kinase contents in the outer segment preparation are not enough to be detected by Western blotting. These observations suggest that the outer segment layer is reasonably sepa-
rated from the other neural retinal layers. Under these condi-
tions, Cdk5 was clearly observed in both outer segment and other neural retinal layers (Fig. 1B, lanes 2). This observation strongly suggests that Cdk5 is present in both the photorecep-
toceptor outer segment layer and the other neural retinal layers in frog retina. This observation also shows that Cdk5 contents in the outer segment layer appears to be less than that in the neural retinal layers. We note that the specificity and sensitiv-
ity of the Cdk5 antibody have already been shown in the accompanying paper (20).

In the immunohistochemical search for Cdk5 in frog retina, the strongest immunological signal was observed in the inner plexiform layer (Fig. 2B). Other strong signals were in the outer plexiform layer and at the interface between the inner segments and the outer nuclear layer. These observations are...
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In previous studies (16, 17, 20), we have shown that Pγ complexed with GTPγS/Ta is the best substrate for Pγ kinase (Cdk5) and that GTPγS is required for Pγ phosphorylation by Cdk5 in photoreceptor outer segment membranes. These observations imply that Pγ phosphorylation is light-dependent in photoreceptors, because binding of GTPγS to Ta is completely dependent upon illuminated rhodopsin (1, 7). Indeed, the light-dependent Pγ phosphorylation was detected in vivo (21). We combined and extended these observations. After incubation with [32P]phosphorus, retinas were illuminated under various light conditions, quickly frozen, and dehydrated. The photoreceptor outer segment layer was then isolated from these retinas as described above. Pγ was isolated from these photoreceptor outer segments by a Pγ-specific antibody and SDS-PAGE, and subsequently phosphorylated amino acids in the Pγ were identified and their radioactivities were measured. Under dark conditions the phosphorylated amino acid was barely detected (Fig. 3A), and only less than 0.5% of the total Pγ was found to be phosphorylated (Fig. 3C). However, a threonine residue in Pγ was clearly phosphorylated in illuminated photoreceptors (Fig. 3A). Two phases of phosphorylation were observed. After an initial rapid phosphorylation, the phosphorylation increased gradually during illumination (Fig. 3B). In the rapid phosphorylation, the Pγ phosphorylation was detected when 0.03% of rhodopsin was bleached (1 s). During the slow phosphorylation, 10 ± 4% of the total Pγ (n = 5) was phosphorylated after bleaching of 10% of rhodopsin (Fig. 3C), and 16 ± 6% of the total Pγ (n = 5) was phosphorylated after bleaching of 20% of rhodopsin (data not shown). Thus, as the rapid phosphorylation, ~4% of the total Pγ was estimated to be phosphorylated after less than 0.3% of the rhodopsin was bleached in less than 10 s (Fig. 3A). These observations indicate that Pγ phosphorylation in photoreceptor outer segments is light-dependent and significant and that the Pγ phosphorylation is fast enough to be involved in the recovery phase of phototransduction. When the light was turned off, the Pγ was dephosphorylated rapidly (Fig. 3, A and B). The Pγ dephosphorylation could be observed in less than 1 s. Together, these results indicate that the Pγ phosphorylation is reversible and that the dephosphorylation is also fast.

We note that incorporation of the radioactivity into a Pγ serine residue was sometimes detected in the in vivo system, especially when the level of the Pγ threonine phosphorylation was increased by high bleaching (Fig. 3A). The incorporation was weak because only less than 10% of the radioactivity incorporated into the threonine residue was observed in the serine residue. As described below, we will show that Cdk5 is involved in the light-dependent phosphorylation of the Pγ threonine. However, the serine phosphorylation seems to be due to Cdk5, because only threonine 22 in Pγ (frog and bovine) is phosphorylated by Cdk5 in vitro (16, 20). Since we do not know the full amino acid sequence of frog Pγ, it is also possible that the Pγ contains another Cdk5 phosphorylation site, including a serine residue, and the phosphorylation of the serine was detected in vivo, but not in vitro, because of structural hindrance. However, this possibility is very slim, because Pγ appears not to have a rigid conformation, and such structural hindrance seems not to be present in Pγ in vitro (20). We also note that the
involvement of PKC and PKA in the serine phosphorylation is unlikely, because these protein kinases have been shown to phosphorylate only threonine 35 in Pγ in vitro (14, 15). It is also possible that the frog Pγ contains a serine phosphorylation site for these protein kinases, and the site could not be phosphorylated by structural hindrance under the in vitro conditions. However, the possibility was small, because Pγ seems not to have a rigid conformation, as described above. In the case of PI-stimulated kinase (13), we suggested that threonine 35 or serine 40 in frog Pγ was phosphorylated in vitro. However, the possibility of serine 40 phosphorylation is also small, because under bleached conditions the site appears to be masked by GTP/Ta in vivo, as described in the Introduction. At present, the mechanism and function of the serine phosphorylation are unknown.

Effects of Cdk Inhibitors on Pγ Phosphorylation in Vitro and in Vivo—We used Cdk inhibitors, olomoucine, and roscovitine to determine whether Pγ phosphorylation in vivo is due to a Cdk. These Cdk inhibitors have been used in vitro (0.2–10 μM) and in vivo (10–100 μM) (29–31). We found that under the in vitro conditions both olomoucine and roscovitine inhibited Pγ phosphorylation by frog Pγ kinase or by recombinant bovine Cdk5/p35 with 50% inhibition ~2 and ~7 μM, respectively (Fig. 4A). Iso-olomoucine, an inactive analog of olomoucine, did not inhibit the Pγ phosphorylation. We also found that 100 μM olomoucine completely inhibited the light-dependent phosphorylation of Pγ threonine in vivo, but iso-olomoucine did not (Fig. 4B). Moreover, 50 μM roscovitine drastically reduced the Pγ threonine phosphorylation. These observations strongly suggest that a Cdk is involved in the light-dependent Pγ phosphorylation.

Pf-stimulated kinase, PKC or PKA, have been shown to phosphorylate Pγ in vitro (13–15). However, it should be emphasized that involvement of these protein kinases in the light-dependent phosphorylation of Pγ threonine (Figs. 3 and 4) is very unlikely for the following reasons. 1) As summarized in the Introduction, these protein kinases appear not to function in the light-dependent Pγ phosphorylation. 2) It is possible that frog Pγ could have another threonine, other than threonine 35, as a phosphorylation site for these protein kinases, and the site would be available in vivo, but not in vitro, because of structural hindrance. However, this possibility is small, because Pγ appears not to have a rigid conformation, as suggested (20). 3) The Cdk inhibitors used are very specific to Cdk5 (31). Very high concentration of these inhibitors is required to inhibit PKC and PKA in vitro; IC50 values for various isozymes of PKC are >100 μM roscovitine and >800 μM olomoucine, and IC50 values for PKA (bovine heart) are >1,000 μM roscovitine and >2,000 μM olomoucine (31). In addition, similar high concentration of these inhibitors is also required for the inhibition of other protein kinases such as cGMP-dependent protein kinase (bovine tracheal smooth muscle, IC50 >1,000) and casein kinase 2 (rat liver, IC50 >2,000) (31).
peptides from \( P_g \) was phosphorylated by \( P_g \) kinase with \( \gamma^{32P}ATP \). As \( P_g \) phosphorylated \( in vitro \), \( P_g \) peptides were incubated in Ringer’s solution containing \( ^{32P} \) phosphate, and \( P_g \) in these retinas was phosphorylated by illumination (10 min). These phosphorylated \( P_g \)s were isolated by a \( P_g \)-specific antibody and SDS-PAGE. These phosphorylated \( P_g \)s were digested with trypsin, and resulting peptides were analyzed using two-dimensional peptide map analysis. a, peptides from \( P_g \) phosphorylated \( in vivo \); b, peptides from \( P_g \) phosphorylated \( in vitro \); c, a mixture of peptides from \( P_g \) phosphorylated \( in vivo \) and \( P_g \) phosphorylated \( in vitro \). * indicates the starting point of the two-dimensional peptide map.

though the effect of these inhibitors on frog kinases is unknown, it is unlikely that frog PI-stimulated kinase, PKC, PKA, or unknown protein kinases, other than Cdk\( s \), could be inhibited by less than 100 \( \mu \)M amounts of these Cdk inhibitors \( in vivo \). Especially, it is very unlikely that any frog protein kinase was completely inhibited by 100 \( \mu \)M olomoucine \( in vivo \) (Fig. 4B).

Evidence for the Involvement of Cdk5 in \( P_g \) Phosphorylation \( in vivo \)—As described above, a Cdk is involved in the \( P_g \) phosphorylation \( in vivo \). To identify the Cdk as Cdk5, two-dimensional phosphopeptide maps of both \( P_g \) phosphorylated by \( P_g \) kinase (Cdk5) \( in vitro \) and \( P_g \) phosphorylated in illuminated retina (10-min illumination) were compared (Fig. 5). We found that only one kind of the radioactive peptide was produced from these \( P_g \)s and that the peptide derived from \( P_g \) phosphorylated \( in vivo \) was found in the same location as that of \( P_g \) phosphorylated \( in vitro \). Together with data showing that only threonine 22 is phosphorylated by Cdk5 \( in vitro \) (16, 20), these observations indicate that the same threonine residue in \( P_g \) is phosphorylated \( in vivo \) and \( in vitro \). These observations imply that the Cdk involved in the light-dependent \( P_g \) phosphorylation \( in vivo \) is Cdk5. Since under our conditions, photoreceptors were illuminated for 10 min (~20% rhodopsin illumination), these results also indicate that the same threonine residue is phosphorylated in these two phases of \( P_g \) phosphorylation. These observations also strongly support our previous conclusion that protein kinases known to phosphorylate \( P_g \) threonine 35 are not involved in the light-dependent phosphorylation of \( P_g \).

Effects of Phosphorylated \( P_g \) on Light/\( GTP_\gamma S \)-activated PDE—In previous studies (16, 17), using systems reconstituted with isolated proteins, we showed that \( P_g \) phosphorylated by \( P_g \) kinase (Cdk5) more effectively inhibits \( GTP_\gamma S/T_\alpha \)-activated PDE than nonphosphorylated \( P_g \). This is because the phosphorylated \( P_g \) loses its affinity to \( GTP_\gamma S/T_\alpha \), but gains a 10–15 times higher ability to inhibit \( GTP_\gamma S/T_\alpha \)-activated PDE than that of nonphosphorylated \( P_g \). In this study, we investigated whether these phenomena were also observed in an homogenate of frog photoreceptor outer segments, a system containing all proteins of outer segments. We checked the effects of non-phosphorylated and phosphorylated \( P_g \)s, alone or complexed with \( T_\alpha \), on the time course of light/\( GTP_\gamma S/T_\alpha \)-dependent activation of PDE (Fig. 6). We found that \( P_g \) complexed with GDP/\( T_\alpha \) could not inhibit the PDE activity; however, the same concentration of \( P_g \) alone inhibited the PDE activity (Fig. 6A). This implies that GDP hydrolysis is not sufficient for the deactivation of light/\( GTP \)-activated PDE in a frog ROS homogenate and that \( P_g \) must be released from GDP/\( T_\alpha \) for the PDE deactivation. This is consistent with previous results obtained in systems reconstituted by isolated proteins (10, 32). We also found that \( P_g \) complexed with \( GTP_\gamma S/T_\alpha \) did not inhibit the light/\( GTP_\gamma S \)-activated PDE; however, \( P_g \) phosphorylated by \( P_g \) kinase (Cdk5) inhibited the light/\( GTP_\gamma S/T_\alpha \)-activated PDE, even in the presence of \( GTP_\gamma S/T_\alpha \) in the homogenate (Fig. 6B). Moreover, we found that in the homogenate, lesser amounts of phosphorylated \( P_g \) were required to inhibit light/\( GTP_\gamma S \)-activated PDE than that of nonphosphorylated \( P_g \) (data not shown). These results indicate that the nonphosphorylated \( P_g \) retains its complex with \( GTP_\gamma S/T_\alpha \) in the homogenate; however, after the \( P_g \) is phosphorylated by Cdk5, the phosphorylated \( P_g \) cannot keep its complex with \( GTP_\gamma S/T_\alpha \) and inhibits effectively \( GTP_\gamma S/T_\alpha \)-activated PDE. We note that the effect of \( P_g \) phosphorylation on the time course of light/\( GTP_\gamma S \)-dependent PDE activation could not be measured by adding ATP to the homogenate, because rhodopsin in the homogenate is also phosphorylated, and the phosphorylated rhodopsin may affect the light/\( GTP_\gamma S \)-activated PDE activity (33, 34). We also note that 300 nM amounts of these \( P_g \)s, free or complexed with GDP/\( T_\alpha \) or \( GTP_\gamma S/T_\alpha \), were used in these experiments, because by using the high concentration of \( P_g \), we tried to clearly show that even a small portion of nonphosphorylated \( P_g \) was not released from its complexes with GDP/\( T_\alpha \) or \( GTP_\gamma S/T_\alpha \). Under similar conditions, previous studies used 35–200 nM \( P_g \) to inhibit light/\( GTP_\gamma S \)-activated PDE activity measured with a pH electrode (35, 36).

**DISCUSSION**

In previous studies (16, 17), we showed that \( P_g \) is phosphorylated by \( P_g \) kinase in a GTP-dependent manner and that the phosphorylated \( P_g \) loses its affinity to GDP/\( T_\alpha \), but gains high affinity to GDP to inhibit GMP hydrolysis by \( P_\alpha \). In the study described in the accompanying paper (20), we have demonstrated that \( P_g \) has a special phosphorylation motif for Cdk5 and that \( P_g \) kinase is Cdk5/p35. In the present study, using an \( in vivo \) system, we have shown the following results: 1) \( P_g \) is phosphorylated in a light-dependent manner, 2) Cdk5 is involved in the \( P_g \) phosphorylation, 3) the \( P_g \) phosphorylation is

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**FIG. 5.** Two-dimensional peptide map analysis of \( P_g \) phosphorylated \( in vitro \) and \( in vivo \). As \( P_g \) phosphorylated \( in vitro \), frog \( P_g \) was phosphorylated by \( P_g \) kinase with \( \gamma^{32P}ATP \). As \( P_g \) phosphorylated \( in vitro \), frog retinas were incubated in Ringer’s solution containing \( ^{32P} \) phosphate, and \( P_g \) in these retinas was phosphorylated by illumination (10 min). These phosphorylated \( P_g \)s were isolated by a \( P_g \)-specific antibody and SDS-PAGE. These phosphorylated \( P_g \)s were digested with trypsin, and resulting peptides were analyzed using two-dimensional peptide map analysis. a, peptides from \( P_g \) phosphorylated \( in vivo \); b, peptides from \( P_g \) phosphorylated \( in vitro \); c, a mixture of peptides from \( P_g \) phosphorylated \( in vivo \) and \( P_g \) phosphorylated \( in vitro \). * indicates the starting point of the two-dimensional peptide map.

**FIG. 6.** Effects of \( P_g \) and phosphorylated \( P_g \) \( P_g \) on light/\( GTP_\gamma S \)-activated PDE. Using an homogenate of intact frog photoreceptor outer segments (2.5 \( \mu \)M rhodopsin), PDE activity was assayed by a pH electrode in 180 \( \mu \)l of buffer C containing 1 mM BAPTA, 50 mM okadaceous acid, 0.45 mM \( GTP_\gamma S \), and 4 mM GMP in the presence or absence of 300 nM of \( P_g \), GDP/\( T_\alpha \)/\( P_g \), \( GTP_\gamma S/T_\alpha /P_g \), or \( GTP_\gamma S/T_\alpha \) and phosphorylated \( P_g \). As a phospho stimulus, a light flash that bleached 0.003% rhodopsin was given at time 0. Under light conditions that illuminated 0.3% of rhodopsin, the PDE activity was 30 \( \mu \)M/s. A, effects of \( P_g \) and GDP/\( T_\alpha /P_g \) on the time course of light/\( GTP_\gamma S \)-dependent PDE activation. a, buffer only; b, GDP/\( T_\alpha /P_g \); c, \( P_g \). B, effects of \( GTP_\gamma S/T_\alpha /P_g \) and \( GTP_\gamma S/T_\alpha \) and phosphorylated \( P_g \) on the time course. a, buffer only; d, \( GTP_\gamma S/T_\alpha /P_g \); e, \( GTP_\gamma S/T_\alpha \) and phosphorylated \( P_g \).
significant and rapid enough to be involved in the recovery phase of phototransduction, 4) dephosphorylation of Py is also rapid, indicating that the Py phosphorylation is reversible. In addition, using immunodetection in highly purified photoreceptor outer segments and an immunohistochemical search of the retina, we have detected significant signals of Cdk5 in photoreceptor outer segments. Moreover, using an homogenate of photoreceptor outer segments, we have demonstrated that the phosphorylated Py inhibits light/GTPγS-activated PDE, even in the presence of GTPγS/Ta. Although factors that regulate the Py phosphorylation are not identified yet, these observations clearly indicate that the Py phosphorylation by Cdk5 can play important roles in PDE regulation.

In this study, we used frog outer segments isolated by a new method. We have shown that the purity of the outer segments isolated by the method is high enough to show the localization of Cdk5 in the outer segments. Nishizawa et al. (37) also recently reported a novel method to isolate bovine photoreceptor cells containing outer segments and the majority of the inner segments. The concept of their method is similar to that of ours, because they used nitrocellulose membranes to attach the photoreceptor cell monolayer. Then, the photoreceptor cell layer was separated from other retinal layers attached to a filter paper. It is unknown why photoreceptor cells isolated by their method contain both outer and inner segments. We, however, speculate that there are several reasons for the difference as follows. 1) The species used to obtain photoreceptors may be critical because the form and size of photoreceptors, especially the structural strength of the ciliary connection, may be different in different species. We used frog retina and they used bovine retina. We have never tried to isolate bovine outer segments by our method. Thus, we do not know whether our method is fitted to isolate inner segment-free outer segments from bovine retina. 2) Dehydration of retina may be important to weaken the structure of ciliary connection. We dehydrate retinas before separation of outer segments from inner segments, but they did not. In any case, both methods will be useful to isolate photoreceptors and their segments and can be improved by attention to the differences in methods and results.

It should be emphasized that this series of studies does not exclude the turnoff mechanism of GTP/Ta-activated PDE by hydrolysis of GTP. We anticipate that both Py phosphorylation and GTP hydrolysis are involved in the deactivation of GTP/Ta-activated PDE in phototransduction and that the Py phosphorylation functions under some special conditions, although the relationship between PDE turnoff by Py phosphorylation and by GTP hydrolysis is unknown now. However, it is clear that GTP hydrolysis is not enough to turnoff GTP/Ta-activated PDE in frog ROS membranes. After hydrolysis of GTP by Ta, Py is still complexed with GDP/Ta, and the Py complex cannot inhibit GTP/Ta-activated PDE (Ref. 10; Fig. 6A). The GDP/Ta/Py complex is easily extracted and isolated from frog ROS membranes (10), indicating that the interaction between GDP/Ta and Py is very tight and specific. Indeed, the GDP/Ta/Py complex is easily prepared by mixing GDP/Ta with Py (32). We have shown that Tγβγ is required to release Py from the GDP/Ta complex in a frog system (10). However, large amounts of Tγβγ may be required for the Py release because of the tight interaction of Py with GDP/Ta. Thus, in addition of the stimulation of GTP hydrolysis by RGS9 (38), the PDE turnoff by GTP hydrolysis may require an unknown mechanism to stimulate the Py release from its complex with GDP/Ta. We do not exclude the possibility that phosphorylation of Py complexed with GDP/Ta is involved in the unknown mechanism, because Py complexed with GDP/Ta is also phosphorylated by pyk-nase (Cdk5), and the phosphorylated Py appears to be released from GDP/Ta (16).

Previous studies have suggested that GTP hydrolysis is required for the deactivation of GTP/Ta-activated PDE in in vivo conditions (39, 40). However, it should be emphasized that these studies do not exclude the PDE deactivation by Py phosphorylation, because ATP was omitted from their experimental conditions (39), or under their conditions the Py phosphorylation might have been suppressed because of the use of a Py mutant that can not interact with GTP/Ta (40). We also note that using hydrolysis-resistant GTP analogs, electrophysiological studies have already suggested a GTP hydrolysis-independent PDE turnoff (41, 42). Under their conditions, Py phosphorylation by Cdk5 could be a mechanism for the GTP hydrolysis-independent PDE turnoff. We also note that the Py phosphorylation mechanism might be involved in previous studies reporting that PDE activity is suppressed by rhodopsin phosphorylation in an homogenate of photoreceptor outer segments. There are many unknown questions in the Py phosphorylation by Cdk5. We have shown that Py phosphorylation by Cdk5 has two phases, rapid and slow. At present the meaning of these two phases of Py phosphorylation is unknown. We also need to explore the regulatory mechanism of the Py phosphorylation by Cdk5. Moreover, electrophysiological study is required to show that Cdk5 is involved in the GTP hydrolysis-independent PDE turnoff described previously (41, 42). Cdk inhibitors we used in this study will be useful for the electrophysiological study. In the Py phosphorylation mechanism, a phosphatase(s) is also crucial for the rapid dephosphorylation of Py, as shown here. In a previous study (17), we suggested that phosphatase 2A might be involved in the dephosphorylation. However, solid data were not obtained for the identification of phosphatase. Moreover, regulation of the phosphatase should also be investigated to understand the total mechanism of the Py phosphorylation. Thus, many experiments are needed to explore the function of the Py phosphorylation in phototransduction. We have shown clues for answers to these questions in this series of studies. Now it is clear that Py is the second protein found to be phosphorylated in a light-dependent manner in phototransduction (rhodopsin is the first protein). The light dependence appears to be due to the requirement of GTP/Ta for the phosphorylation. Thus, the mechanism of the light dependence is different from that of rhodopsin. Studies about rhodopsin phosphorylation clearly show that light-dependent protein phosphorylation is crucial in the regulation of phototransduction such as shut off and adaptation. We anticipate the Py phosphorylation is also important for shut off and adaptation in phototransduction.

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