Regulation of phosducin phosphorylation in retinal rods by Ca^{2+}/calmodulin-dependent adenylyl cyclase

(visual signal transduction/G protein/cAMP-dependent protein kinase/light adaptation)

Barry M. Willardson, Jon F. Wilkins, Tatsuro Yoshida, and Mark W. Bitensky[†]

Biophysics Group, Physics Division, Los Alamos National Laboratory, University of California, Los Alamos, NM 87545

ABSTRACT The phosphoprotein phosducin (Pd) regulates many guanine nucleotide binding protein (G protein)-linked signaling pathways. In visual signal transduction, unphosphorylated Pd blocks the interaction of light-activated rhodopsin with its G protein (G$_t$) by binding to the $\beta\gamma$ subunits of G$_t$ and preventing their association with the G$_t\alpha$ subunit. When Pd is phosphorylated by cAMP-dependent protein kinase, it no longer inhibits G$_t$ subunit interactions. Thus, factors that determine the phosphorylation state of Pd in rod outer segments are important in controlling the number of G$_t$s available for activation by rhodopsin. The cyclic nucleotide dependencies of the rate of Pd phosphorylation by endogenous cAMP-dependent protein kinase suggest that cAMP, and not cGMP, controls Pd phosphorylation. The synthesis of cAMP by adenylyl cyclase in rod outer segment preparations was found to be dependent on Ca^{2+} and calmodulin. The Ca^{2+} dependence was within the physiological range of Ca^{2+} concentrations in rods ($K_{1/2} = 230 \pm 9$ nM) and was highly cooperative ($n_{app} = 3.6 \pm 0.5$). Through its effect on adenylyl cyclase and cAMP-dependent protein kinase, physiologically high Ca^{2+} (1100 nM) was found to increase the rate of Pd phosphorylation 3-fold compared to the rate of phosphorylation at physiologically low Ca^{2+} (8 nM). No evidence for Pd phosphorylation by other Ca^{2+}-dependent kinases was found. These results suggest that Ca^{2+} can regulate the light response at the level of G$_t$ activation through its effect on the phosphorylation state of Pd.

Phosducin (Pd) regulates the activity of many guanine nucleotide binding protein (G protein) signaling pathways, including those linked to G$_s$, G$_i$, G$_o$, and retinal rod G protein (G$_t$) (1, 2). Unphosphorylated Pd binds to the $\beta\gamma$ subunits of G proteins and blocks their function in the receptor activation of Gα (1–3) and also their direct interaction with effector proteins (4, 5). When Pd is phosphorylated by cAMP-dependent protein kinase (PKA) (6), it does not affect these G$\beta\gamma$ functions (1, 3–5). Because G-protein-mediated signals can control cellular [cAMP] and PKA activity, it has been suggested that such signals may affect their own feedback regulation by determining the phosphorylation state of Pd (1, 3).

In retinal rods, light activation of rhodopsin (Rho) induces a decrease in the free cytosolic [cGMP] as a result of Rho activation by G$_t$ and G$_t\alpha$·GTP activation of cGMP phosphodiesterase. The fall in [cGMP] causes cGMP-gated cation channels in the rod outer segment (ROS) plasma membrane to close, decreasing the influx of Na$^+$ and Ca^{2+}. The resulting hyperpolarization generates the neural signal. The light-induced decrease in influx of Ca^{2+}, coupled with the light-insensitive efflux of Ca^{2+} from the outer segment by the Na$^+$,K$^+$/Ca^{2+} exchanger, causes the internal [Ca^{2+}] to decrease from $\approx$500 to $\approx$50 nM during a light response (7). These changes in [Ca^{2+}] orchestrate a number of biochemical events

believed to be involved in light adaptation. In the light-adapted state, the amplitude and duration of photoresponses are diminished (reviewed in ref. 8). Low intracellular [Ca^{2+}] may contribute to light adaptation by activating guanylyl cyclase and increasing cGMP synthesis (9) and by activating Rho kinase and decreasing the lifetime of light-activated Rho (Rho*) (10). Pd has also been considered as a potential mediator of light adaptation (3, 11). Pd becomes dephosphorylated in response to light (12), and dephosphorylated Pd blocks activation of G$_t$ by Rho* through binding to G$_t\beta\gamma$ and inhibiting their interaction with G$_t\alpha$. Here we report that the adenylyl cyclase activity in ROS preparations is regulated by Ca^{2+} and calmodulin. Through its effect on PKA, this adenylyl cyclase brings Pd phosphorylation under the control of Ca^{2+}.

MATERIALS AND METHODS

Cyclic Nucleotide Dependencies of Pd Phosphorylation. Experiments to measure the cyclic nucleotide dependence of Pd phosphorylation by ROS PKA were carried out in the dark under infrared illumination. Intact ROSs were isolated from fresh dark-adapted bovine retinas (J.A. & W. L. Lawson, Lincoln, NE) as described (13). These ROSs (20 μM Rho), disrupted with a 27-gauge needle and syringe, were incubated with 5 μM Pd, 1 mM [γ-^{32}P]ATP ($\approx$200 mCi/mmol; 1 Ci = 37 GBq), and a known amount of either 8-Br-cAMP or 8-Br-cGMP, at a total vol of 10 μl in phosphorylation buffer (20 mM Hepes, pH 7.5/100 mM KCl/2 mM MgCl$_2$/1 mM dithiothreitol (DTT)/0.2 mM EGTA) at 25°C. Phosphorylation was initiated by the addition of the [γ-^{32}P]ATP and quenched by the addition of 4 μl of a 4-fold concentrate of Laemmli denaturing sample buffer after 10, 15, 25, or 40 sec of incubation. Pd was isolated by SDS/PAGE, and its phosphorylation state was determined by excising the Pd band from the gel and counting the ^{32}P in a scintillation counter. The hydrolysis-resistant 8-bromo analogs of the cyclic nucleotides sustained no measurable hydrolysis over the course of the experiment (data not shown). Initial rates of phosphorylation were determined by fitting the data from the four time points to the equation $p = p^*_{max}[1 - \exp(-t^* v_0/p_{max})]$, where p is the degree of phosphorylation, p_{max} is the maximum achievable degree of phosphorylation, t is the time of incubation, and v_0 is the initial rate of phosphorylation.

Measurement of Adenylyl Cyclase Activity. Experiments were performed in the dark under infrared illumination. Intact ROSs were disrupted with a 27-gauge needle and syringe in cyclase buffer (40 mM Mops, pH 7.1/56 mM KCl/8 mM NaCl/5 mM MgCl$_2$/2 mM isobutylmethylxanthine/6 mM phospho*enol*pyruvate/0.02 mg of pyruvate kinase per ml). The disrupted ROSs (20 μM Rho) were incubated at various free

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Abbreviations: Pd, phosducin; G protein, guanine nucleotide binding protein; G$_t$, retinal rod G protein, transducin; PKA, cAMP-dependent protein kinase; ROS, retinal rod outer segment; Rho, rhodopsin; Rho*, light-activated rhodopsin.
[†]To whom reprint requests should be addressed.

[Ca^{2+}] and 1 mM [α-^{32}P]ATP ($\approx$200 mCi/nmol) for 10 min at 37°C in cyclase buffer. The free [Ca^{2+}] was set by buffering various total [Ca^{2+}] with 2 mM EGTA. The free [Ca^{2+}] in each sample was determined by fluorescence measurements using the Ca^{2+}-sensitive dye Fura-2 with a K_d of 135 nM for the ionic strength and pH conditions of the experiment (14). The reaction was initiated by addition of the [α-^{32}P]ATP and was quenched by addition of 5 μl of 200 mM EDTA to the 25-μl reaction volume. The quenched reaction mixture was immediately heated to 95°C for 2 min and then centrifuged for 5 min at 16,000 × *g* to clear the supernatant. cAMP was isolated by polyethylenimine TLC separation of 5 μl of the supernatant in 200 mM LiCl (9) and quantified in a scintillation counter. Adenylyl cyclase activity was linear within the first 10 min of incubation (data not shown).

Washing of ROSs to Remove Soluble Proteins. ROSs were washed in the dark under infrared illumination to remove soluble proteins as follows. Intact ROSs were disrupted by passing through a 27-gauge needle, and the ROSs were kept on ice throughout the procedure to prevent resealing of the membrane fragments. The ROSs were suspended in isotonic buffer [10 mM Hepes, pH 7.5/100 mM KCl/20 mM NaCl/1 mM EDTA/1 mM DTT/0.2 mM phenylmethylsulfonyl fluoride (PMSF)] and pelleted three times by centrifugation for 12 min at 6000 × *g*. The third pellet was resuspended in a 1:1 mixture of isotonic buffer and isolation buffer (20 mM Hepes, pH 7.4/10 mM D-glucose/5% Ficoll/20% sucrose/1 mM DTT/0.1 mM PMSF) and pelleted by centrifugation for 17 min

at 6000 × *g*. The washed ROSs were then suspended in isolation buffer.

Measurement of Ca^{2+} Dependence of Pd Phosphorylation. All procedures were carried out in the dark under infrared illumination. Pd was purified to >95% purity from bovine retinas as described (3). Intact ROSs, disrupted with a 27-gauge needle and syringe, were incubated at 8 or 1100 nM free [Ca^{2+}] and 1 mM ATP at 37°C in a 13-μl reaction volume of cyclase buffer. Free [Ca^{2+}] was buffered and measured as described above. After 45 sec of incubation, 4 μl of 2.4 mM [γ-^{32}P]ATP ($\approx$200 mCi/mmol) was added to the mixture; 15 sec later, 3 μl of 33 μM Pd was added to begin phosphorylation. After 15 sec of phosphorylation, 4 μl of a 4-fold concentrate of Laemmli denaturing sample buffer was added. Pd was isolated from 9 μl of the final 24-μl vol by SDS/PAGE. The Pd band was excised and the incorporated ^{32}P was quantified with a scintillation counter. Initial rates of phosphorylation were estimated from the phosphorylation occurring in 15 sec.

RESULTS AND DISCUSSION

The cyclic nucleotide dependencies of Pd phosphorylation by the PKA in bovine ROS preparations are shown in Fig. 1. The hydrolysis-resistant 8-bromo analog of cAMP activated the kinase with a $K_{1/2}$ of 0.56 ± 0.09 μM. The 8-Br-cAMP dependence was cooperative (n_{app} = 1.4 ± 0.3), consistent with two cooperatively interacting sites on each regulatory subunit of PKA (15). The response saturated at $\approx$2 μM 8-Br-cAMP at a rate of 150 ± 40 pmol per min per nmol of Rho. 8-Br-cGMP was a 500-fold weaker activator of the endogenous kinase. The $K_{1/2}$ for activation with 8-Br-cGMP was 330 ± 115 μM. Maximal activation was achieved at 1 mM, and the cooperativity of the activation was similar to that of 8-Br-cAMP (n_{app} = 1.3 ± 0.5). The poor activation by 8-Br-cGMP could not be attributed to hydrolysis of the nucleotide because in these dark-adapted ROS preparations no significant hydrolysis of either 8-Br-cGMP or 8-Br-cAMP occurred within the incubation time of the experiment.

These data suggest that it is cAMP, and not cGMP, that is responsible for the activation of PKA in the rod. It is possible that the fluctuations in free [cAMP] that occur in response to light could span the range over which the PKA is activated. The light-mediated decrease in free [cAMP] in ROSs has not been quantified, but total ROS cAMP is $\approx$5 μM (16) and decreases $\approx$50% in the light-adapted state (16, 17). It has been suggested that cGMP could control outer segment PKA activity because
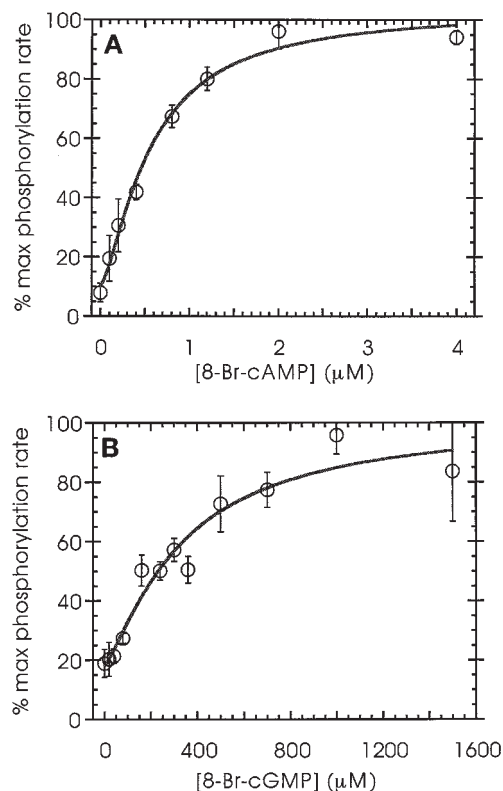

FIG. 1.   Effect of cyclic nucleotide concentration on phosphorylation rate of Pd by ROS PKA. Initial rates of Pd phosphorylation were measured as described at the indicated concentrations of 8-Br-cAMP (*A*) or 8-Br-cGMP (*B*). The initial rate data were fit to the Hill equation. Half-maximal stimulation occurs at 0.56 ± 0.09 μM 8-Br-cAMP with a cooperativity of 1.4 ± 0.3. With 8-Br-cGMP, half-maximal stimulation occurs at 328 ± 115 μM, with a cooperativity of 1.3 ± 0.5. The maximal phosphorylation rate was determined to be 150 ± 40 pmol of ^{32}P incorporated per min per nmol of Rho (*n* = 7). Data points represent average values from three experiments ± SD.
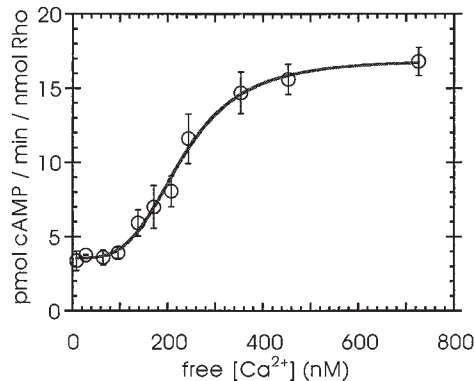
FIG. 2.   Effect of [Ca^{2+}] on ROS adenylyl cyclase activity. ROS adenylyl cyclase activity was measured at the indicated concentrations of free Ca^{2+} as described. Data were fit to the Hill equation. The curve shows a cooperative dependence on [Ca^{2+}] (n_{app} = 3.6 ± 0.5), which falls within the range of physiological [Ca^{2+}] in the rod (K_m = 230 ± 9 nM). Data points represent average values from four to six experiments ± SD. Similar results were obtained from five different ROS preparations.

of its high concentration in ROSs. However, the total [cGMP] in a ROS is ≈ 50 μM and also decreases $\approx 50\%$ upon light activation (18–20). From electrophysiological measurements of the cGMP-gated channel, light activation has been estimated to decrease the free [cGMP] from ≈ 5 to <1 μM (21). Thus, the range of variation for free [cGMP] lies ≈ 100-fold lower than the $K_{1/2}$ for stimulation of the rod PKA. From these results, it appears that cGMP does not regulate PKA activity in ROSs. Similar cyclic nucleotide dependencies of PKA activity were found in amphibian rods for phosphorylation of components I and II, which may be Pd analogs in amphibian photoreceptors (22).

The probability that rod PKA is regulated solely by cAMP stimulates renewed interest in factors that control rod adenylyl cyclase. Since Ca^{2+} levels in ROSs reflect recent light signaling, and neuronal adenylyl cyclase is commonly regulated by Ca^{2+}/calmodulin, the possibility that ROS adenylyl cyclase might be regulated by Ca^{2+} was tested. In bovine ROS preparations in which soluble components are retained, adenylyl cyclase activity exhibited a striking dependence on free $[Ca^{2+}]$ over its physiological range (7, 23–25) in ROS cytosol (Fig. 2). The activity increased >4-fold when free $[Ca^{2+}]$ was raised from 100 to 400 nM. Half-maximal activation occurred at 230 $\pm$ 9 mM Ca^{2+}. The Ca^{2+} dependence was strongly cooperative, with an n_{app} of 3.6 $\pm$ 0.5. Thus, ROS adenylyl cyclase can respond with great sensitivity to those changes in ROS cytosolic $[Ca^{2+}]$ that occur in response to light.

Calmodulin appears to be the Ca^{2+}-binding protein responsible for regulation of ROS adenylyl cyclase. A peptide inhibitor of calmodulin derived from Ca^{2+}/calmodulin-dependent protein kinase II (26) was found to inhibit Ca^{2+} activation of the cyclase (Fig. 3). At saturating inhibitor concentrations adenylyl cyclase activity was reduced to ≈ 2.5 pmol of cAMP synthesized per min per nmol of Rho, the level found at low $[Ca^{2+}]$ in the absence of the inhibitor. Half-maximal inhibition occurred at ≈ 20 μM inhibitor.

A second experimental format confirmed that calmodulin regulates ROS adenylyl cyclase. Intact ROSs were disrupted and washed with isotonic buffer to deplete cytosolic proteins. Adenylyl cyclase activity in these ROSs was 2.7 pmol of cAMP synthesized per min per nmol of Rho and was not affected by the addition of Ca^{2+} (Fig. 4*A*). When bovine brain calmodulin was added, Ca^{2+} activation of adenylyl cyclase was restored to

approximately the same level found in unwashed preparations. Only a minimal amount of calmodulin-induced activation of the cyclase was observed at low $[Ca^{2+}]$. The difference between the high and low $[Ca^{2+}]$ curves gives the true Ca^{2+}/calmodulin dependence (Fig. 4*B*). This curve was best fit by a hyperbolic function, with half-maximal activation occurring at 0.4 $\pm$ 0.05 μM calmodulin. This indicates that the interaction between brain calmodulin and adenylyl cyclase was not cooperative. The cooperative activation of adenylyl cyclase by Ca^{2+} was thus probably a result of cooperative binding of Ca^{2+} to calmodulin rather than cooperative binding of Ca^{2+}/calmodulin to adenylyl cyclase. This is consistent with evidence that calmodulin has four Ca^{2+}-binding sites, which interact cooperatively in the presence of a ligand (27, 28). However, the possibility that endogenous ROS calmodulin binds adenylyl cyclase differently than brain calmodulin was not excluded. These data demonstrate that rod adenylyl cyclase belongs to the Ca^{2+}/calmodulin-dependent family of cyclases, which includes types I, III, and VIII (29–31). Adenylyl cyclase type I is specific to neurons, and its mRNA has been found in high concentration in the cytoplasm of bovine photoreceptor cells (32). Thus, this appears to be the most likely candidate for the ROS adenylyl cyclase.

The Ca^{2+}/calmodulin regulation of adenylyl cyclase suggests that Ca^{2+} could regulate Pd phosphorylation through its effect on PKA activity. This possibility was tested by preincubating ROS for 1 min in the presence of ATP to allow cAMP synthesis prior to measuring Pd phosphorylation. During the preincubation, $[Ca^{2+}]$ was buffered to levels at which adenylyl cyclase would be maximally active (1100 nM free Ca^{2+}) or
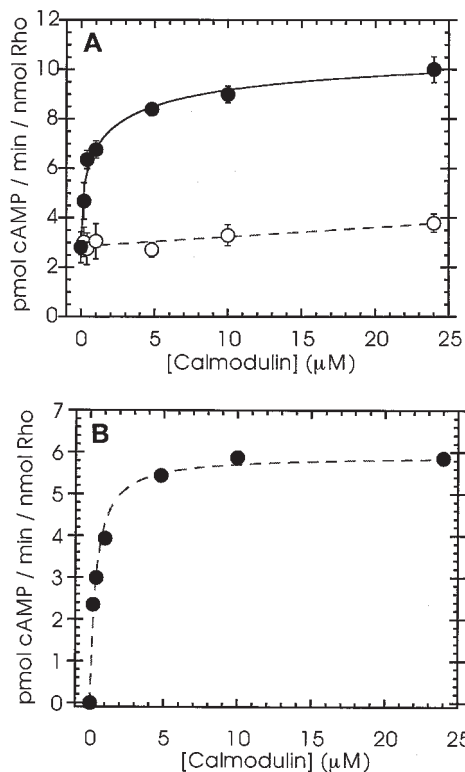
FIG. 4. Effect of exogenous calmodulin on ROS adenylyl cyclase activity. (*A*) Adenylyl cyclase activity in ROSs washed to remove soluble proteins was measured as described at 8 nM ($\bigcirc$) and 726 nM ($\bullet$) free Ca^{2+} in the presence of the indicated amounts of bovine brain calmodulin (Calbiochem). Data points represent average values from four to six experiments $\pm$ SD. Curves represent a fit of the data to the Hill equation. (*B*) Net activation of adenylyl cyclase by Ca^{2+}/calmodulin was determined from the difference of the high and low Ca^{2+} curves from *A*. A simple hyperbolic function fit the curve with half-maximal activation occurring at 0.4 $\pm$ 0.05 μM calmodulin.
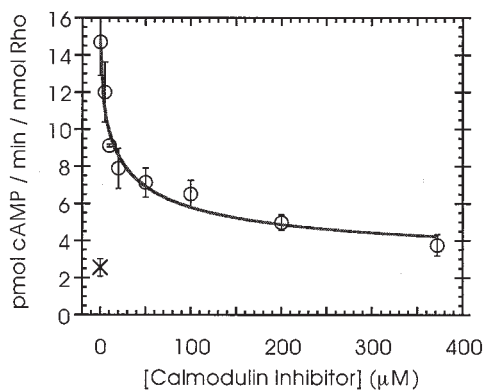

FIG. 3. Inhibition of ROS adenylyl cyclase activity with a calmodulin-binding peptide. ROS adenylyl cyclase activity was measured as described at saturating (726 nM) free $[Ca^{2+}]$ ($\bigcirc$) and low (8 nM) $[Ca^{2+}]$ ($\times$) in the presence of the indicated amounts of a synthetic peptide from the calmodulin-binding domain of Ca^{2+}/calmodulin-dependent protein kinase II (Calbiochem) (26). Data fit the equation $v = \{(v_{max} - v_{min})/(1 + ([peptide]/C)^n\} + v_{min}$, where v is the rate of adenylyl cyclase activity, C is the IC_{50} for the inhibitor, and n is a slope factor. The curve fit showed complete inhibition at saturating concentrations of the peptide. Data points represent average values from four to six experiments $\pm$ SD. The peptide was without effect at 8 nM Ca^{2+} (data not shown).
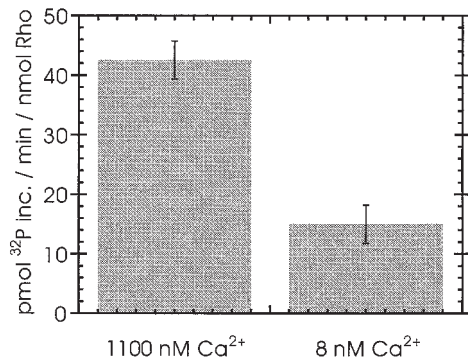
FIG. 5.   Effect of preincubation at high or low $[Ca^{2+}]$ on rates of Pd phosphorylation by ROS PKA. Rates of phosphorylation of Pd by ROS PKA were measured after preincubation of the ROS at high (1100 nM) or low (8 nM) $[Ca^{2+}]$ as described. Phosphorylation rates demonstrated a 2.8-fold increase when preincubation was carried out at a $[Ca^{2+}]$ that was saturating with respect to adenylyl cyclase activity. From the data in Fig. 2, the [cAMP] in the high and low Ca^{2+} samples are estimated to be 220 and 46 nM, respectively. Bars represent averages of four experiments $\pm$ SD.

minimally active (8 nM free Ca^{2+}). After preincubation, the rate of Pd phosphorylation was measured. At high $[Ca^{2+}]$, the rate of Pd phosphorylation was 2.8-fold greater than at low $[Ca^{2+}]$ (Fig. 5). From the Ca^{2+}-dependent activity of the adenylyl cyclase, the [cAMP] in the high and low Ca^{2+} samples were estimated to be 220 and 46 nM, respectively. In the

absence of a preincubation period, no dependence of Pd phosphorylation on $[Ca^{2+}]$ was observed (data not shown), suggesting that cAMP accumulation was necessary for the Ca^{2+}-dependent increase in phosphorylation and that direct Ca^{2+}-induced activation of a Pd kinase was not occurring.

This finding has implications for regulation of rod sensitivity by Ca^{2+}, since the light-mediated fall in $[Ca^{2+}]$ could lead to a decrease in the rate of Pd phosphorylation. A potential mechanism for this regulation is depicted in Fig. 6. After a light stimulus (Fig. 6A), the decreased rate of cAMP synthesis at low Ca^{2+}, coupled with the increased rate of cAMP hydrolysis by activated phosphodiesterase, would cause free [cAMP] to fall below the concentration required to activate PKA. Rod phosphatases would then dephosphorylate Pd unopposed by the activity of PKA. (It is not known whether Pd phosphatases are also regulated by cAMP or Ca^{2+}, but a paradigm in which Pd phosphorylation and dephosphorylation are reciprocally regulated has advantageous regulatory features that warrant investigation.) This mechanism could explain the observed decrease in Pd phosphorylation that occurs upon light activation (12). Unphosphorylated Pd would then compete with $G_t\alpha$ for binding to $G_t\beta\gamma$. Since the amount of Pd in ROSs is approximately equal to the amount of G_t (ref. 35, J.F.W., M.W.B., and B.M.W., unpublished data), a significant amount of $G_t\beta\gamma$ could be sequestered and thus the number of $G_t\alpha$ activated by a given Rho* would decrease. This would contribute to the reduced sensitivity found in light-adapted rods.

Possible steps involved in the recovery from Pd inhibition of G_t activity are depicted in Fig. 6B. Upon termination of the
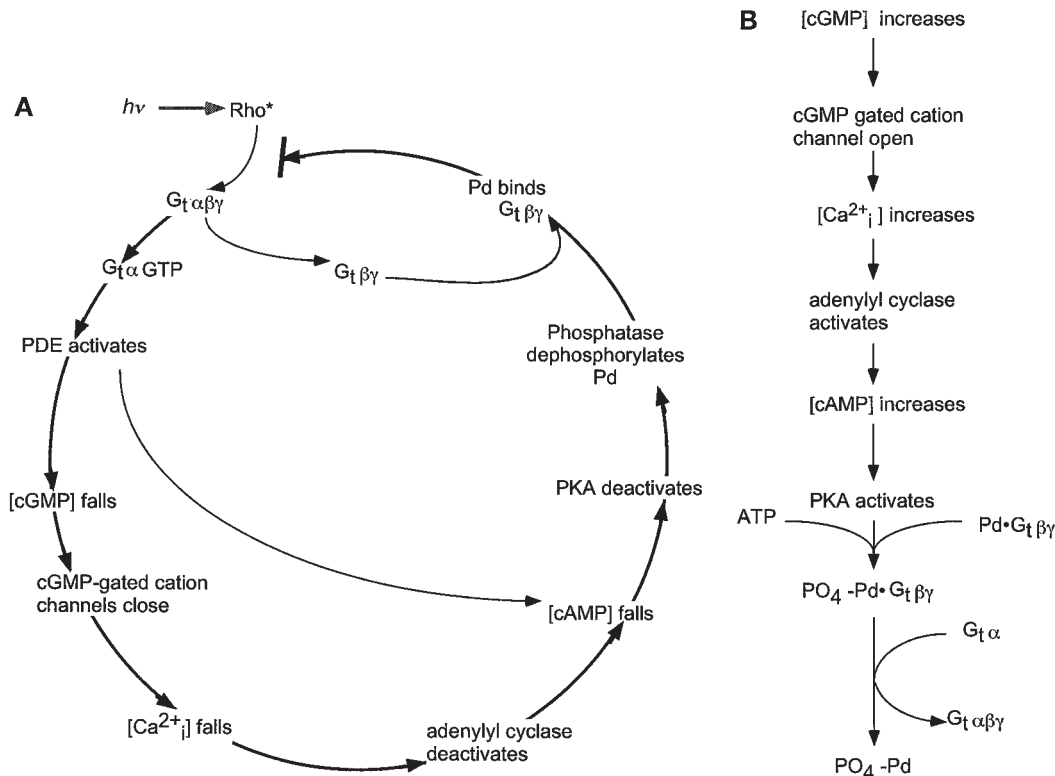

FIG. 6.   Schematic representation of Ca^{2+}-dependent regulation of G_t activity by Pd. (A) Proposed down-regulation of the light activation cascade by Pd. Absorption of light by Rho initiates G_t-mediated activation of phosphodiesterase (PDE) that results in cGMP-gated cation channel closure and a decrease in internal Ca^{2+} in the ROSs. The decrease in Ca^{2+} results in a decrease in the activity of the Ca^{2+}/calmodulin-dependent adenylyl cyclase and, combined with hydrolysis of cAMP by PDE, results in a decrease in [cAMP]. PKA is thereby inactivated and phosphatase dephosphorylates Pd. Pd then binds $G_t\beta\gamma$ and excludes it from interacting with $G_t\alpha$. Rho* can no longer activate $G_t\alpha$ in the absence of $G_t\beta\gamma$. Thus, further activation of G_t is inhibited. (B) Recovery of sensitivity with respect to G_t activation as the rod dark adapts. The events that lead to an increase in [cGMP] (36, 37) are not depicted. The binding of cGMP to the cation channels results in opening of the channels and an influx of Ca^{2+} into the ROS. Ca^{2+}/calmodulin-dependent adenylyl cyclase becomes activated and synthesis of cAMP is increased. Increased [cAMP] activates PKA, and PKA phosphorylates Pd in its complex with $G_t\beta\gamma$. The phosphorylated Pd dissociates from $G_t\beta\gamma$ as $G_t\alpha$ binds $G_t\beta\gamma$. The $G_t\alpha\beta\gamma$ heterotrimer is thus reformed and is able to be activated by Rho* in the next light response.

light signal, Ca^{2+} levels in the ROS cytosol would recover after reopening of the cGMP-gated ion channels. Once the $[Ca^{2+}]$ achieved dark-adapted levels, ROS adenylyl cyclase would be activated and [cAMP] would increase. This would result in activation of ROS PKA and phosphorylation of Pd in the Pd–$G_t\beta\gamma$ complex. $G_t\alpha$ could then readily compete with the phosphorylated Pd for binding to $G_t\beta\gamma$. In this manner, the full complement of G_t would become available for activation. Thus, the striking sensitivity to light that is found in dark-adapted rods would be fully restored. In this manner, $[Ca^{2+}]$ may modulate the phosphorylation state of Pd and thereby regulate the fraction of the total G_t pool that is available for activation by Rho.

The phosphorylation rate of Pd is significantly inhibited when it is complexed with $G_t\beta\gamma$. At the levels of PKA activity found in intact ROS preparations, the $t_{1/2}$ for the phosphorylation of Pd in the Pd–$G_t\beta\gamma$ complex would be ≈ 3.5 min (J.F.W., M.W.B., and B.M.W., unpublished data). This slow rephosphorylation of Pd in the complex provides a time interval after the light response during which the rod exhibits reduced sensitivity. Thus, the Pd–$G_t\beta\gamma$ complex could comprise a molecular memory that records recent light-signaling activity.

An important question yet to be addressed concerns the spatial localization of the components of this regulatory cascade. This will ultimately determine whether these proteins have an opportunity to interact *in vivo*. G_t is associated with the cytoplasmic face of the ROS disc membrane. Pd has been localized to the cytosol of both the outer and inner segments. The data presented in Fig. 4 show that calmodulin is cytosolic and, despite its fatty acid acylation, PKA is a cytosolic protein kinase (15). Thus, all of these proteins would be able to interact in close proximity. The Ca^{2+}/calmodulin-dependent adenylyl cyclase is an integral membrane protein (36). Its distribution in the rod is not known, but large amounts of Ca^{2+}/calmodulin-dependent type I adenylyl cyclase mRNA are found in the rod inner segment (32). Whether the expressed cyclase localizes to the outer segment plasma membrane or disc membranes or to inner segment membranes is not known. Our ROS preparations are not completely free of inner segments; thus, we cannot conclude definitively that the cyclase localizes only to the outer segment. However, the fact that the cyclase mRNA is abundant in rods strongly suggests that the cyclase activity that we observe is associated with the rods and is not a contaminant from other retinal cell types.

A more complete understanding of the molecular processes involved in light adaptation is emerging. $[Ca^{2+}]$ plays an important role in orchestrating these events through several different Ca^{2+}-binding proteins, including recoverin/S-modulin (10, 37), guanylate cyclase-activating protein (33), and calmodulin. Now, in addition to regulating guanylyl cyclase (9) and Rho kinase activities (10), Ca^{2+} may also control the magnitude of G_t activation and signal amplification by its effect on Pd phosphorylation through regulation of adenylyl cyclase.

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