Phosducin (Pd) is a widely expressed phosphoprotein that regulates G-protein (G) signaling. Unphosphorylated Pd binds to Gβγ subunits and blocks their interaction with Gα. This binding sequesters Gβγ and inhibits both receptor-mediated activation of Gα and direct interactions between Gβγ and effector enzymes. When phosphorylated by cAMP-dependent protein kinase, Pd does not affect these functions of Gβγ. To further understand the role of Pd in regulating G-protein signaling in retinal rod photoreceptor cells, we have measured the abundance of Pd in rods and examined factors that control the rate of Pd phosphorylation. Pd is expressed at a copy number comparable to that for the rod G-protein, transducin (Gt). The ratio of rhodopsin (Rho) to Pd is 15.5 ± 3.5 to 1. The rate of Pd phosphorylation in rods was dependent on [cAMP]. 

**Experimental Procedures**

Phosducin (Pd) is a regulatory phosphoprotein that is expressed in many cell types (1–4). Pd regulates G-protein (G) signaling pathways by competing with Gα for binding to Gβγ subunits (5). The efficacy of Pd binding to Gβγ is determined by its phosphorylation state (1, 5–7). Unphosphorylated Pd sequesters Gβγ and prevents receptor-mediated Gα activation (1, 5, 8). Binding of unphosphorylated Pd also blocks the direct interaction between Gβγ and effector enzymes (6, 7). When Pd is phosphorylated by cAMP-dependent protein kinase (PKA), it no longer affects these Gβγ functions (1, 5–7). Thus, the phosphorylation state of Pd determines the magnitude of G-protein-mediated signal amplification by regulating the availability of Gβγ. Since many G-protein-linked pathways affect intracellular cAMP levels, and thereby affect PKA activity, Pd can serve as a feedback regulator of G-protein-mediated signal transduction.

In vertebrate rod photoreceptor cells, the photoresponse is mediated by a G-protein-linked pathway (reviewed in Ref. 9). Photon capture by rhodopsin (Rho) causes photosensitization of its prosthetic group, 11-cis-retinal, to the all-trans isomer. This isomerization results in a conformational change that converts Rho to its active form, Meta II rhodopsin (Rho*). Rho* then binds to the retinal G-protein, transducin (Gt), allowing bound GDP to be exchanged for GTP on Gt. The released Gt-GTP then activates rod phosphodiesterase (PDE). Activated PDE lowers cytosolic cGMP concentrations, thereby closing the cGMP-gated cation channels in the rod plasma membrane. Closure of the cation channels results in hyperpolarization of the plasma membrane, thereby generating a neural impulse. Ion channel closure also reduces the flow of Ca2+ into the rod. Ca2+ continues to exit through a Na+,K+ / Ca2+ exchanger, causing the cytosolic Ca2+ concentration to fall in response to light.

Rod cells can readily adjust their sensitivity to ambient light conditions and respond to photon fluxes spanning 3 orders of magnitude. Fully dark adapted rods have single-photon sensitivity. In this regime, a single Rho* can initiate hydrolysis of ~105 molecules of cGMP. With protracted or repeated exposure to light, however, this amplification mechanism is dramatically down regulated. Light adaptation causes a decrease in both amplitude and duration of the response resulting from a given light signal (reviewed in Ref. 10). Light-dependent reactions that appear to contribute to light adaptation include phosphorylation of Rho and arrestin binding (11), activation of guanylyl cyclase (12), and dephosphorylation of phosducin (5). The light-induced fall in Ca2+ concentration appears to trigger these adaptive events (12–14).

There is a correlation between the phosphorylation state of Pd and the degree of light adaptation in the rod. Pd is phosphorylated in the dark and becomes dephosphorylated upon exposure to light (15). In the dark the dephosphorylated Pd can bind to Gβγ and prevent the activation of Gt by Rho* (5, 8). The period during which Pd remains bound to Gβγ defines a light-adapted state that persists until Pd is rephosphorylated. We have examined the rates of phosphorylation of Pd, both free and complexed with Gβγ. The results provide evidence that the Pd-Gβγ complex could encode a molecular memory in retinal rods. The duration of this memory results from the stability of the Pd-Gβγ complex and the relatively slow rate of its phosphorylation. It is also shown that Pd is present in sufficient quantities to significantly reduce the amount of available Gt. Thus, the properties of Pd suggest that it could play a fundamental role in light adaptation.
Kinetics of Phosducin Phosphorylation

isolated from dark-adapted bovine retinas (J. A. Lawson and W. L. Lawson, Lincoln, NE) by a previously described method (5). This Pd was >95% pure, as determined by SDS-PAGE. Ten-μl samples of known concentration of Pd ranging from 0.1 to 0.5 μM were incorporated into Laemmli buffer (20 mM HEPES, pH 7.5, and 500 mM NaCl) and placed on ice before and after disruption with a 27-gauge needle and syringe. The rate of Rho phosphorylation was inhibited 87.3 ± 2.3% (n = 3) in the intact ROS, indicating that most of the ROS intact. Rho concentrations in the ROS preparation were determined by dark/light difference spectroscopy using an extinction coefficient for Rho of 40,000/M at 500 nm. The concentration of Rho in the ROS was also quantified in an analogous manner as a control for the mechanism of regulation of Pd phosphorylation.

Identity of the Endogenous ROS Kinase—

To understand the role of Pd in the regulation of rod phototransduction, it is necessary to determine the amount of Pd expressed in ROS. Since Pd functions by sequestering Gt, the two would have to be expressed at comparable copy number in the rod for Pd to function as a meaningful regulator of Gt. The quantity of Pd present in intact ROS preparations was determined by quantitative Western blots, as described under "Experimental Procedures." A photograph of one of the blots is presented in Fig. 1A, and the corresponding densitometry data are shown in Fig. 1, B and C. Pd was present at one Pd per 15.5 ± 3.5 Rho (n = 7). Gt has been reported as being present at approximately one Gt per 10 Rho (22–24). Pd is thus present in nearly equimolar amounts with Gt.

The amount of arrestin was also quantified as a control to determine whether soluble proteins were retained in our intact ROS prep. Arrestin was found to be present at one arrestin per 5.0 ± 1.5 Rho (n = 6), indicating that few soluble proteins were lost during the isolation of the intact ROS. Previous determinations of arrestin concentration gave values of approximately one arrestin per 10 Rho (25–27). The quantities of Pd present in ROS make it a good candidate for a major regulatory role in visual signal transduction and underscore the importance of the mechanism of regulation of Pd phosphorylation.

2 Cosmasse Blue-stained gels of intact ROS prepared in this manner do not show a band where Pd migrates with an intensity equal to 1/16th of the Rho band (16). This apparent discrepancy between the quantitative Western blots and Cosmasse Blue-stained gels is probably a result of poor staining of Pd with Cosmasse Blue compared to Rho staining in SDS-PAGE. The binding of Cosmasse Blue R or G to protein is proportional to the positively charged residues on the protein and its hydrophobicity (41). Pd is a very hydrophilic protein, whereas Rho is very hydrophobic. SDS is a similar molecule to Cosmasse Blue in that both have negatively charged groups and hydrophobic moieties (41). Appropriately, the binding of SDS to Pd is significantly less than its binding to Rho. In SDS gels, Pd migrates more slowly than predicted by its molecular weight because of high SDS binding. The quantitative Western blot is superior to comparisons of Cosmasse Blue stain intensity for determining Rho/Pd ratios because it avoids the ambiguities of quantitatively significant differences in the binding of the dye to the different proteins.
The data in Fig. 2 show the inhibition of Pd phosphorylation by the IP-20 peptide, which is specific for the α and β isoforms of PKA (30). Half-maximal inhibition occurred at a concentration of 156 ± 37 nM, and the data indicate complete inhibition at saturating concentrations of the peptide. This result confirms that PKA is the endogenous kinase responsible for Pd phosphorylation.

Endogenous Activation of ROS PKA—PKA is activated primarily by cAMP but can also be activated by cGMP at higher concentrations. In the dark-adapted state, ROS contain 50 μM total cGMP (31–33) and 5 μM total cAMP (34). Upon light-stimulated activation of the rod cell, the total concentration of both nucleotides drop by ~50% (31–35). The drop in free cAMP in ROS that results from light activation has not been measured, but free cGMP levels drop from ~5 μM to <1 μM (36). Both nucleotides have been considered as possible activators of ROS PKA. To understand the kinetics of Pd phosphorylation, it is important to determine which of the two activates PKA in vivo.

Pd phosphorylation by ROS PKA was measured at various concentrations of 8-Br-cAMP and 8-Br-cGMP (Fig. 3). The hydrolysis-resistant 8-bromo analogs were used to prevent inaccuracies resulting from PDE activity. No measurable hydrolysis of either nucleotide occurred over the course of the experiment (data not shown). Initial rates of phosphorylation were determined as described under "Experimental Procedures." The dependencies of the initial rates of Pd phosphorylation for cAMP and cGMP are shown in Fig. 3. The curve fits of the data were generated with the Hill equation. The curve for the 8-Br-cAMP data gives a $K_m$ of 0.56 ± 0.09 μM with an $n_{app}$ of 1.4 ± 0.3. The curve for the 8-Br-cGMP data yields a $K_m$ of 330 ± 115 μM and an $n_{app}$ of 1.3 ± 0.5.
Fig. 4. Effects of Pd concentration on the rate of Pd phosphorylation. Rates of Pd phosphorylation by ROS PKA were measured at the indicated concentrations of Pd. Measurements were carried out at 20 μM Rho in the presence of 10 μM 8-Br-cAMP as described under “Experimental Procedures.” Bars, the S.D. of the data from three separate experiments. The data were fit to the Hill equation, yielding a K_m of 9.7 ± 1.2 μM and a high degree of cooperativity (n_app = 2.2 ± 0.5). The maximal rate presented in this figure, based on the phosphorylation occurring in 10 s, is 390 ± 70 pmol PO_4 incorporated/min/nmol Rho (n = 3).

Fig. 5. Inhibition of the phosphorylation rate of Pd by G_βγ. Rates of Pd phosphorylation by ROS PKA were measured in the presence of the indicated concentrations of G_βγ. Measurements were carried out at 20 μM Rho and 1.5 μM Pd in the presence of 10 μM 8-Br-cAMP as described under “Experimental Procedures.” Bars, the S.D. of the data from three separate experiments. The data were fit to the equation:

\[ V = \frac{V_{max} - V_{min}}{1 + (K_{bg})} + V_{min}, \]

where V is the rate of Pd phosphorylation, n is a slope factor, and K_{bg} is the concentration of G_βγ at which half-maximal inhibition occurred. The curve fit yields a K_{bg} of 0.78 ± 0.11 and a slope factor of 0.93 ± 0.34. The values for V_{max} and V_{min} were determined to be 128 and 2.59 pmol PO_4 incorporated/min/nmol Rho, respectively. This corresponds to a 50-fold inhibition at saturating concentrations of G_βγ.

Kinetics of Phosphoducin Phosphorylation

Inhibition of Pd Phosphorylation by G_βγ—When Pd is dephosphorylated, it is found primarily in a complex with G_βγ. Thus, the more significant rate of Pd phosphorylation is that measured in the presence of G_βγ, a rate considerably slower than that for Pd alone. The extent of the inhibition by G_βγ was determined by running the phosphorylation experiments in the presence of increasing amounts of G_βγ. The data presented in Fig. 5 show that half-maximal inhibition occurs at 0.8 ± 0.1 μM G_βγ. The curve fit indicates that there is a 50-fold inhibition in the rate of Pd phosphorylation at saturating concentrations of G_βγ. The rate of phosphorylation of the PdG_βγ complex would, therefore, be 10.3 ± 3.6 pmol PO_4 incorporated/min/nmol Rho. The presence of G_βγ showed no effect on the rate of phosphorylation of histone type III-S by PKA (data not shown), indicating that the inhibition of Pd phosphorylation is due to the interaction of G_βγ with Pd and not due to an interaction with PKA.

Mechanism of Pd Phosphorylation in the Presence of G_βγ—These data raise the question of the nature of the inhibition of Pd phosphorylation by G_βγ. The mechanism of inhibition determines whether the rate-determining step is the dissociation or the phosphorylation of the PdG_βγ complex. In a competitive model, the binding of G_βγ to Pd could completely block phosphorylation. The complex would have to dissociate to allow PKA to phosphorylate Pd. Alternatively, in a noncompetitive model, PKA could phosphorylate Pd while it is bound to G_βγ, but at a slower rate than that for free Pd. This question was addressed in an experimental format with purified Pd and G_βγ and purified bovine heart PKA catalytic subunit (Fluka). G_βγ inhibition of Pd phosphorylation was measured at three different PKA activity levels. If G_βγ were a competitive inhibitor of Pd phosphorylation, one would expect all of the inhibition curves to show complete inhibition at saturating concentrations of G_βγ. However, the curves shown in Fig. 6 each reach different levels of maximal inhibition. Since the addition of more PKA increases the rate of phosphorylation at maximal inhibition, PKA must be able to phosphorylate the PdG_βγ complex. This noncompetitive inhibition means that the K_m for the phosphorylation of the complex should be the same as that for free Pd. However, the maximal rate of Pd phosphorylation (V_{max}) in the complex will be 50-fold lower.
Kinetics of Phosducin Phosphorylation

**Fig. 6.** Inhibition of phosphorylation by G<sub>Bγ</sub> at different concentrations of PKA. Rates of Pd phosphorylation by three different concentrations of PKA were measured in the presence of the indicated concentrations of G<sub>Bγ</sub>. Purified bovine heart PKA (Fluka) was used at activity levels of 0.5 units/μl (C), 0.25 units/μl ( ), and 0.125 units/μl ( ), as indicated by the supplier. Measurements were carried out in the presence of 10 μM 8-Br-cAMP as described under “Experimental Procedures.” Bars, the S.D. of the data from three separate experiments. The data from each curve were fit to the equation \( V = (V_{max} - V_{min})K_{bg}\frac{1}{2} + V_{min} \), where \( V \) is the rate of Pd phosphorylation and \( K_{bg}\frac{1}{2} \) is the concentration of G<sub>Bγ</sub> at which half-maximal inhibition occurred. The values for \( V_{max} \) determined for the three curves were 43 ± 6%, 31 ± 6%, and 23 ± 3% of the maximum phosphorylation rate for the 0.5, 0.25, and 0.125 unit/μl samples, respectively.

**Discussion**

Pd is expressed in many cell types that use G-protein-mediated signal transduction. Its ability to bind the βγ subunits of G-proteins and to inhibit Gβγ functions raises questions regarding the nature of its role in signal regulation. We have shown that ROS PKA is activated in vivo by cAMP and not cGMP. Knowledge of the time and time scale of the changes in cAMP is thus crucial to a complete understanding of the regulation of Pd phosphorylation. The fall in cAMP that occurs upon light activation is the product of two events that result from light stimulation. Activated PDE will hydrolyze cAMP, albeit Pd-mediated light adaptation by determining the timescale for the rephosphorylation of Pd. Phosphorylation of Pd will not occur in the dark-adapted state, which is characterized by large signal amplification.

The data were gathered using ROS preparations in which soluble proteins were retained; therefore, the measured phosphorylation rates should be a reasonable reflection of the in vivo ROS activity. Furthermore, under physiological conditions, the phosphorylation rate is saturated with respect to Pd and G<sub>Bγ</sub>. The rate is dependent on the concentration of free cAMP, and those levels have yet to be measured; but under dark conditions in which PDE is inactivated and Ca<sup>2+</sup> levels are high, the rate may be saturated with respect to cAMP as well. The rates presented here should, therefore, reasonably reflect the in vivo phosphorylation rates. Thus, mammalian rods may have a period of molecular memory, the duration of which is defined by a time of ~3 min, in which light responses are down-regulated as a result of a persistent Pd<sub>Bγ</sub> complex.

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