

Enhancement of Phototransduction G Protein-Effector Interactions by Phosphoinositides*

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Feng He, Muling Mao‡, and Theodore G. Wensel§

From the Department of Biochemistry and Molecular Biology, Baylor College of Medicine, Houston, Texas 77030

Light responses in photoreceptor cells are mediated by the action of the G protein transducin (G_t) on the effector enzyme cGMP phosphodiesterase (PDE6) at the surface of disk membranes. The enzymatic components needed for phosphoinositide-based signaling are known to be present in rod cells, but it has remained uncertain what role phosphoinositides play in vertebrate phototransduction. Reconstitution of PDE6 and activated $G_{\alpha t}$ on the surface of large unilamellar vesicles containing D-*myo*-phosphatidylinositol-4,5-bisphosphate (PI(4,5)P₂), stimulated PDE activity nearly 4-fold above the level observed with membranes containing no phosphoinositides, whereas G protein-independent activation by trypsin was unaffected by the presence of phosphoinositides. PDE activity was similarly stimulated by D-*myo*-phosphatidylinositol-3,4-bisphosphate and D-*myo*-phosphatidylinositol-4-phosphate (PI(4)P), but much less by D-*myo*-phosphatidylinositol-5-phosphate (PI(5)P) or D-*myo*-phosphatidylinositol-3,5-bisphosphate. Incubation of rod outer segment membranes with phosphoinositide-specific phospholipase C decreased G protein-stimulated activation of endogenous PDE6, but not trypsin-stimulated PDE activity. Binding experiments using phosphoinositide-containing vesicles revealed patterns of PDE6 binding and PDE6-enhanced $G_{\alpha t}$ -GTP γ S binding, consistent with the activation profile PI(4,5)P₂ > PI(4)P > PI(5)P ~ control vesicles. These results suggest that enhancement of effector-G protein interactions represents a possible mechanism for modulation of phototransduction gain by changes in phosphoinositide levels, perhaps occurring in response to long-term changes in illumination or other environmental cues.

The G protein cascade of vertebrate vision is mediated by the action of the α subunit of the heterotrimeric G protein transducin, $G_{\alpha t}$ ¹ on a cGMP-specific phosphodiesterase, PDE6. In

many signaling cascades there is cross-talk between cyclic nucleotide second messenger signaling and signaling by phosphoinositides and their metabolic products. There have been a number of reports on the presence of the components of phosphoinositide signaling in rod outer segments and other reports on the modulation of these components by light (1–9). We reported previously (10) that D-*myo*-phosphatidylinositol-4,5-bisphosphate (PI(4,5)P₂), added in micellar form, stimulates PDE6 activation in rod cells in *Xenopus laevis* outer segment membranes, as measured with electrophysiological methods, and in bovine rod outer segments, as measured using an enzymatic assay. The cGMP-gated cation channel of rod outer segments was also found to be inhibited by PI(4,5)P₂. These results left in question whether lipids delivered in this manner act at a site relevant for physiological regulation of PDE activation in rod cells, where they would be presented at the surface of the disk membrane. They also left in question whether other phosphoinositides, such as PIP, a more abundant lipid in rod cells than PIP₂, may also play a role.

An important role for phospholipids in G protein-effector interactions is well established, particularly in photoreceptors. The presence of phospholipid bilayers dramatically enhances activation of PDE6 by $G_{\alpha t}$ (see Ref. 11 and the references cited therein), and the magnitude of the effect is determined by the character of the head group and the side chains. A recent study demonstrated specific interactions between phosphatidylcholine and phosphatidylethanolamine and PDE6 and between phosphatidylserine and $G_{\alpha t}$ (12). $G_{\alpha t}$ -GTP γ S, in contrast, did not bind L- α -phosphatidylserine (PS) but enhanced interactions of PDE6 with L- α -phosphatidylcholine (PC). PDE6 and transducin are both peripheral membrane proteins that can be readily isolated in soluble form and whose membrane attachment is assisted by covalently attached lipids. $G_{\alpha t}$ is heterogeneously acylated at its N-terminal glyceryl residue, and PDE6 has farnesyl and geranylgeranyl groups attached in thioether linkages to C-terminal methyl-esterified methionyl residues on the α and β subunits. Because it has two attached lipids as compared with one for $G_{\alpha t}$, PDE6 binds more tightly to lipid bilayers than does $G_{\alpha t}$, and, under dilute conditions, we have observed that PDE6 enhances binding of $G_{\alpha t}$ to vesicles. Moreover, lipid surfaces that favor $G_{\alpha t}$ activation of PDE6 also enhance its binding to vesicles containing bound PDE6. Both PDE6 and $G_{\alpha t}$ are acidic proteins with large net negative charges at neutral pH, yet they bind fairly tightly to negatively charged rod outer segment disk membranes and to vesicles containing negatively charged lipids. Thus, in contrast to many peripheral proteins whose attraction to membranes is largely electrostatic and therefore somewhat nonspecific in nature,

D-*myo*-phosphatidylinositol-3,4-bisphosphate; PI(3,5)P₂, D-*myo*-phosphatidylinositol-3,5-bisphosphate; GTP γ S, guanosine 5'-O-(3-thiotriphosphate); ROS, rod outer segment; MOPS, 4-morpholinepropanesulfonic acid; TBST, Tris-buffered saline with Tween 20.

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§ To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, Baylor College of Medicine, 1 Baylor Plaza, Houston, TX 77030. Tel.: 713-798-6996; Fax: 713-798-1625; E-mail: twensel@bcm.tmc.edu.

¹ The abbreviations used are: $G_{\alpha t}$, G protein transducin α subunit; PDE, phosphodiesterase; PDE6, photoreceptor cGMP-specific phosphodiesterase; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; PI, D-*myo*-phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP₂, phosphatidylinositol 4,5-bisphosphate; PI(5)P, D-*myo*-phosphatidylinositol-5-phosphate; PI(4,5)P₂, D-*myo*-phosphatidylinositol-4,5-bisphosphate; PI(3,4)P₂,

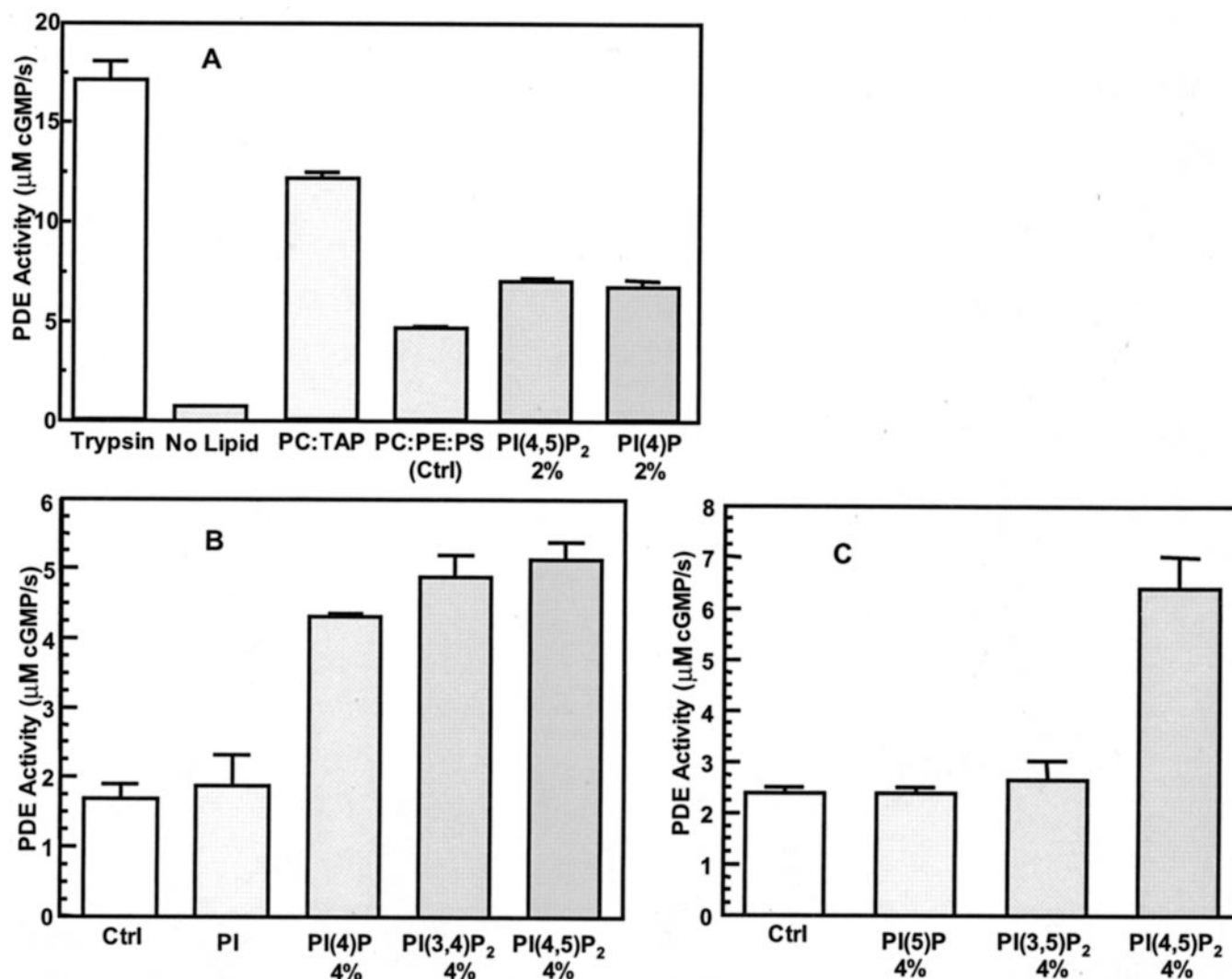


FIG. 1. Effects of phosphoinositides on PDE activation by transducin. PDE activity was monitored continuously by pH recording in the presence of vesicles, and the increase in activity upon the addition of $G_{\alpha t}$ -GTP γ S is plotted. Control vesicles (*ctrl*) contain 50:35:15 (%) of PC/PE/PS (molar ratio). PI, PI(4)P, PI(3,4)P₂, PI(4,5)P₂, PI(5)P₂ and PI(3,5)P₂ vesicles have the same lipid mixture with 2% (A) or 4% (B and C) of the total replaced by the indicated phosphoinositides (*i.e.* 48:34:14:4 (%) of PC/PE/PS/phosphoinositide). PC:TAP vesicles contain 20% dioleoyltrimethylammonium propane and 20% dioleoylphosphatidylcholine (11). Conditions were 200- μ l final volume of pH assay buffer, pH 8, 20 nM PDE, 150 μ M total lipid, 2 mM cGMP, and 150 nM $G_{\alpha t}$ -GTP γ S at room temperature. Experiments within each panel were carried out on the same day using the same batches of PDE6 and $G_{\alpha t}$ -GTP γ S, but these were different between panels.

these phototransduction proteins seem to rely on hydrophobic interactions between the hydrocarbon phase of the membrane and their attached lipid chains and on specific interactions with certain head groups. Here we explore the ability of phosphorylated inositol phospholipids to modulate these interactions as a possible mechanism for modulating the efficiency of phototransduction.

EXPERIMENTAL PROCEDURES

Buffers—Standard buffers include a PDE pH assay buffer (20 mM MOPS, pH 8, 50 mM NaCl, 2 mM MgCl₂, 0.1 mM EDTA and 1 mM DTT), a binding buffer for immobilized phospholipids (300 mM NaCl, 50 mM Tris-HCl, pH 7, 1 mM CaCl₂, 1 mM MgCl₂, and 1 mM MnCl₂), and a washing buffer for immobilized lipids (300 mM NaCl, 25 mM Tris-HCl, pH 8). For all these buffers, 1 mM dithiothreitol and ~20 mg/liter solid phenylmethylsulfonyl fluoride were added before use. Tris-buffered saline with Tween 20 (TBST; 10 mM Tris-HCl, pH 8, 150 mM NaCl, and 0.1% (v/v) Tween 20) was also used.

Protein Electrophoresis and Immunoblotting—SDS-PAGE and immunoblotting were carried out using standard protocols (13). Antibodies used were PDE6 α -specific rabbit antiserum from Affinity Bioreagents or PDE6 catalytic subunit-specific antiserum, a gift from Dr. Rick Cote (14), and the $G_{\alpha t}$ antibody (Santa Cruz Biotechnologies); these were used at dilutions of 1:500 to 1:1000. The secondary antibody

used was a horseradish peroxidase-conjugated (Promega) goat anti-rabbit antibody with detection by chemiluminescence. Films exposed to the blots were scanned, and the optical densities were quantified using Unscan-it® software (Silk Scientific Corporation).

Purification of Proteins and Rod Outer Segment (ROS) Membranes—Bovine ROS membranes were purified as described (15). PDE6 (PDE $_{\alpha\beta\gamma}$), holotransducin ($G_{\alpha\beta\gamma}$ -GDP), and $G_{\alpha t}$ -GTP γ S were purified from ROS as described previously (11, 16).

Vesicle Preparation—Vesicles of 100-nm diameter were prepared by extrusion as described previously (11, 16). Lipids used were as follows: PS (brain, porcine-sodium salt), PC (egg, chicken), and L- α -phosphatidylethanolamine (PE) (egg, chicken) from Avanti Polar Lipids; D-*myo*-phosphatidylinositol-4-phosphate (PI(4)P) (bovine brain) and PI(4,5)P₂ (bovine brain) from Calbiochem; and D-*myo*-phosphatidylinositol (PI), D-*myo*-phosphatidylinositol-5-phosphate (PI(5)P), and D-*myo*-phosphatidylinositol-3,5-bisphosphate (PI(3,5)P₂) from Echelon Biosciences Inc.

Vesicle Binding Assays—Assays were carried out essentially as described previously (11). Briefly, purified proteins (20 nM PDE6 holoenzyme and/or 150 nM $G_{\alpha t}$ -GTP γ S) were mixed with vesicles (150 μ M total phospholipid) and, after 10 min of incubation, spun at 44,000 rpm for 20 min in a TLA-100.3 tabletop ultracentrifuge rotor at 4 °C. The supernatant was removed, and equivalent proportions of the total pellet and supernatant fractions were analyzed by SDS/PAGE and immunoblotting with $G_{\alpha t}$ - or PDE-specific antibodies.

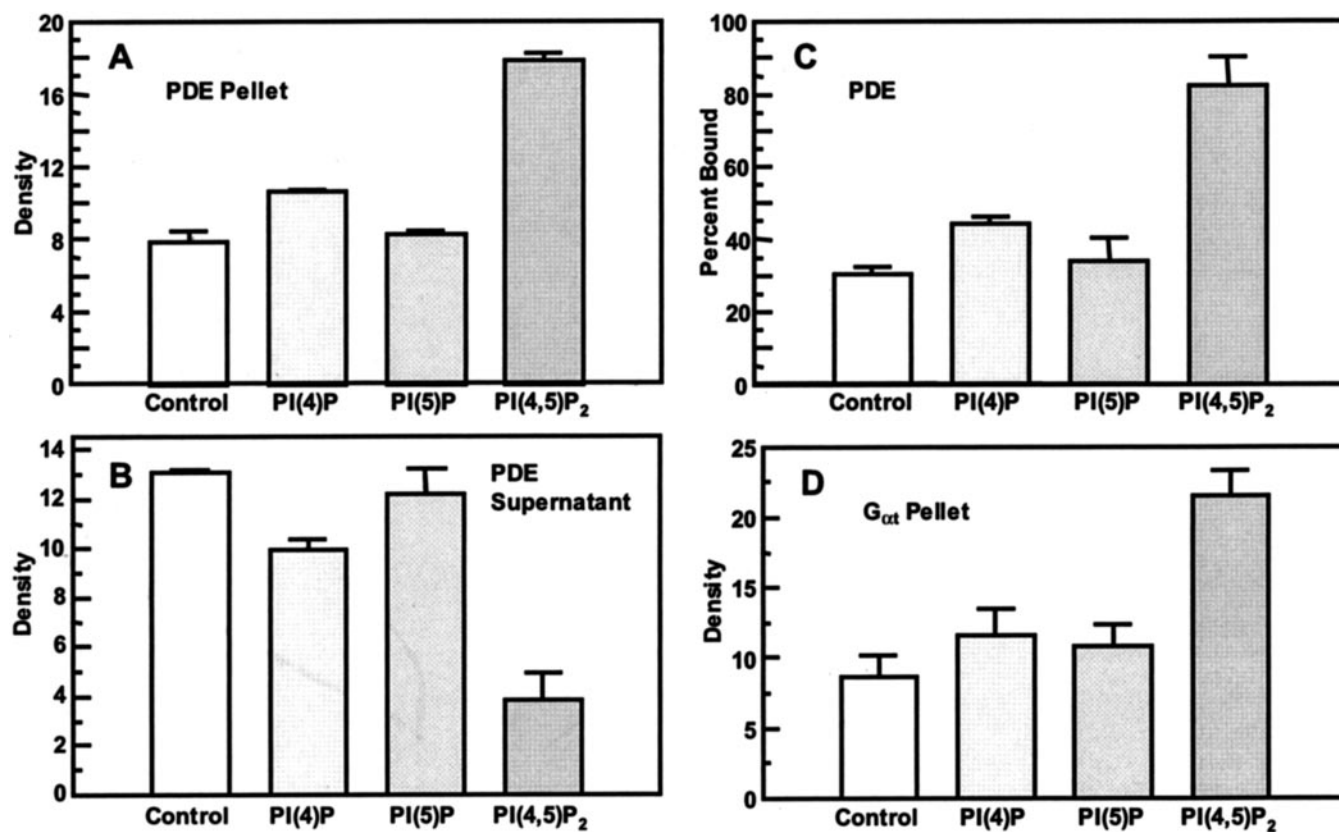


FIG. 2. Enhancement of $G_{\alpha t}$ -GTP γ S-PDE6 complex binding to membranes by phosphoinositides. Sucrose-loaded vesicles and proteins were incubated at the same concentrations in the same buffer as in Fig. 1, and the vesicles were separated by ultracentrifugation. Equal fractions of the total samples from supernatant or pellet were loaded on a gel for SDS-PAGE and immunoblotting and probed with PDE6- or $G_{\alpha t}$ -specific antisera. A–D, results of densitometric measurements of the bands in films exposed by chemiluminescence. A, pellet, probed with PDE antibody. B, supernatant, probed with PDE antibody. C, percentage of PDE binding calculated from the data of panels A and B. D, pellet probed with $G_{\alpha t}$ antibody. (Note: the supernatant fraction probed with $G_{\alpha t}$ antibody is not shown. Because of the large molar excess of $G_{\alpha t}$ -GTP γ S, which does not bind membranes significantly under these conditions without PDE6 (11), the decrease in $G_{\alpha t}$ in the membrane due to vesicle binding is not detectable).

Phosphodiesterase Assays—Assays of PDE-catalyzed cGMP hydrolysis were carried out as described (11) by continuous pH recording with the following standard conditions in pH assay buffer: 20 nM PDE; 150 nM $G_{\alpha t}$ -GTP γ S (when present); 2 mM cGMP; and 150 μ M total phospholipid in the form of synthetic vesicles (when present). To assess maximal PDE activity, samples were treated with 0.2 mg/ml trypsin.

Ca^{2+} Assays—To determine the influence of $[Ca^{2+}]$ on phosphoinositide effects, pH assay samples were buffered with 0.6 or 0.8 mM EGTA and varying amounts of $CaCl_2$ (0–2.4 mM) added. Free $[Ca^{2+}]$ was determined in parallel samples containing 1 μ M Fura-2 (Molecular Probes) by measuring fluorescence excitation spectra and calculating $[Ca^{2+}]$ as described (17) using a K_d value under our conditions of 224 nM for the binding of Fura-2 to Ca^{2+} .

Phospholipase Treatment—Phosphatidylinositol-specific phospholipase C from *Bacillus cereus* was obtained from Sigma. ROS membranes were incubated at a concentration of 50 μ M rhodopsin with 5 units/ml of enzyme (or no enzyme for the control samples) in pH assay buffer in a volume of 80 μ l, at 37 °C. After the reaction had proceeded for the indicated times, the sample was diluted to 500 μ l and then centrifuged at 40,000 rpm for 20 min at 4 °C (TLA-100.3 rotor). The supernatant was removed, and then the ROS membranes were washed again with PDE assay buffer and finally resuspended in 40 μ l of PDE assay buffer for adding to phosphodiesterase assays.

Phospholipid Filter Binding Assay—Filter binding assays were performed using immobilized lipid membranes (Echelon Biosciences, Inc.) according to the manufacturer's instructions. The membrane was blocked in TBST/0.1% ovalbumin for 1 h at room temperature and then incubated with 2.25 nM PDE6 or 5 nM $G_{\alpha t}$ -GTP γ S diluted in blocking solution at 4 °C overnight. The membrane was extensively washed in TBST prior to standard immunoblotting with the appropriate primary antibody (see above). After final washing, ECL detection was used to indicate binding of protein to lipids.

RESULTS

Effect of Phosphoinositides on PDE6 Activation by $G_{\alpha t}$ -GTP γ S—To present phosphoinositides in a more physiologically relevant context than the addition of micelles employed previously (10), we prepared large unilamellar vesicles with fixed proportions of PE, PC, and PS containing various phosphoinositides. These were then reconstituted with purified PDE6 and activated transducin in the form $G_{\alpha t}$ -GTP γ S. As reported previously, reconstitution of PDE6 and $G_{\alpha t}$ -GTP γ S on vesicles greatly enhances the activation of PDE, even in the absence of phosphoinositides (Fig. 1). However, the presence of phosphoinositides phosphorylated at the 4-OH position enhanced the activation even further, by as much as a factor of 3.8 (see below). $PI(4,5)P_2$ was consistently the most potent, followed by $PI(3,4)P_2$ and $PI(4)P$. In contrast, PI and $PI(5)P$ had little effect on PDE activation above that observed with the control vesicles containing only PC, PE, and PS. $PI(3,5)P_2$ had some enhancement activity, which, over a large number of experiments, ranged between <10% to as much as 44% of the activity of $PI(3,4)P_2$. Thus the effect is specific for certain isomers of the phosphoinositides, with a preference for those phosphorylated at the 4 position.

Binding of PDE and $G_{\alpha t}$ to Phospholipid Vesicles—In previous experiments with reconstituted vesicles, we observed a strong correlation between PDE6-dependent $G_{\alpha t}$ binding to vesicles and PDE activation (11, 16). Therefore, we assayed binding of PDE6 and transducin to vesicles with different phosphoinositides incorporated. The results, shown in Fig. 2, are in

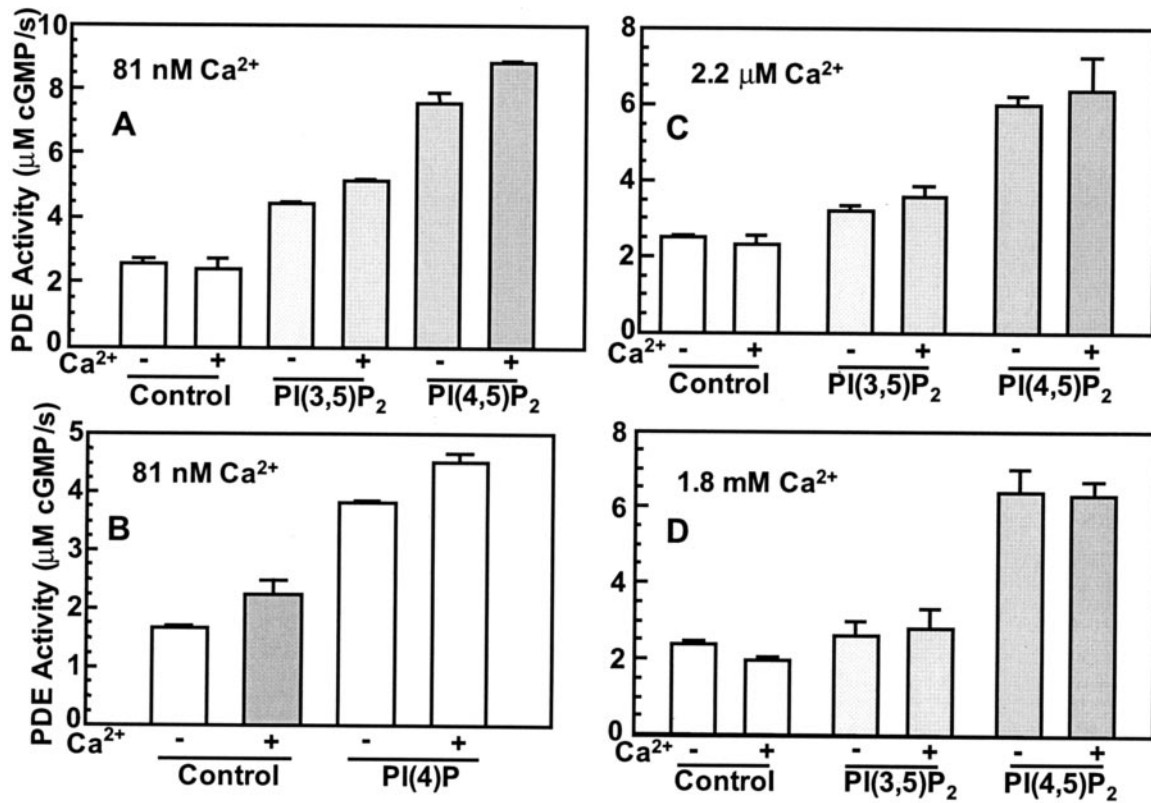


FIG. 3. Effects of divalent cations on PDE activation. Conditions were the same as in Fig. 1, except that samples contained, in addition, 0.6 mM EGTA (Panels A, B) or 0.8 mM EGTA (Panels C, D) and varying amounts of $CaCl_2$ to give the indicated free $[Ca^{2+}]$, as assayed using Fura-2 fluorescence in parallel samples of identical composition except for the addition of 1 μM Fura-2.

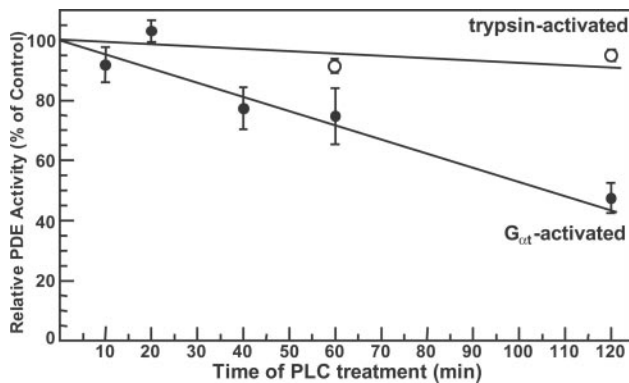


FIG. 4. Decrease in PDE activation efficiency upon treatment with phosphatidylinositol-specific phospholipase C. Bovine ROS membranes were treated with pH assay buffer containing *B. cereus* PI-specific PLC (5 units/ml; Sigma) at 37°C for the indicated times, while control ROS membranes were treated for the same time with pH assay buffer. After treatment, ROS membranes were washed with pH assay buffer, and then the PDE activity was monitored by pH assay in 200-μl final volume containing treated ROS membranes (2 μM photoisomerized rhodopsin), 2 mM cGMP, and 50 μM GTPγS. Total PDE activity remaining after treatment was assayed after trypsin digestion to remove the inhibitory PDE6γ subunits. The activity of the PLC-treated sample at each time point is plotted as a function of the activity of the control sample after the same incubation time without PLC.

good agreement with the results obtained by activity assays. The strongest binding is to vesicles containing $PI(4,5)P_2$, which is the most potent activator, and there is somewhat weaker binding to $PI(4)P$, which also activates less potently. The addition of $PI(5)P$ to vesicles only slightly enhanced binding of the PDE6- $G_{\alpha t}$ -GTPγS complex.

Effects of Divalent Cations on Phosphoinositide Stimulation of PDE Activation—Because PDE6 and $G_{\alpha t}$ are both negatively charged and the most potent lipid mixtures for stimulating

their interactions include synthetic lipids with positive charges (11), it seems reasonable to propose that divalent cations may play a role in the interactions of these negatively charged protein surfaces with lipids possessing multiple negative charges. Ca^{2+} plays a critical role in the recovery phase of phototransduction and changes dramatically in response to light, as it is extruded from the cell upon closure of the major influx route, the cGMP-gated channels. Therefore we tested whether the concentration of Ca^{2+} affects the ability of phosphoinositides to stimulate activation of PDE and stimulate binding of PDE or the PDE- $G_{\alpha t}$ -GTPγS complex to membranes. The results, shown in Fig. 3, show at most a modest ability of Ca^{2+} to stimulate the phosphoinositide effect. It seems unlikely that such small changes play an important physiological role. However, assays at the physiologically relevant $[Ca^{2+}]$ of 81 nM do demonstrate the greatest enhancement, 3.8-fold, of PDE activation induced by $PI(4,5)P_2$ (Fig. 3A). Mg^{2+} is essential for PDE enzymatic activity (18, 19), so we were not able to assay directly its role in the phosphoinositide enhancement of activity. However, we were able to measure its effect on the enhancement of membrane binding by phosphoinositides by carrying out vesicle binding assays such as those shown in Fig. 2 with 2 mM EDTA in the presence or absence of 4 mM $MgCl_2$. We detected no significant difference in PDE6 or $G_{\alpha t}$ -GTPγS bound to vesicles in samples with or without millimolar free Mg^{2+} (data not shown). Thus, divalent cations do not play a major role in the interaction between the negatively charged protein complex and the negatively charged lipids.

Effect of Phosphoinositide Depletion by Phospholipase C—If endogenous phosphoinositides present in disk membranes act to enhance transducin activation of PDE6, then depletion of those lipids would be expected to decrease the $G_{\alpha t}$ -GTPγS-PDE6 interactions in membranes from rod outer segments. When ROS membranes were incubated with bacterial phospho-

phatidylinositol-specific phospholipase C before assaying G_{αt}-GTPγS-stimulated PDE6 activity, the activity was found to be lower in the phospholipase samples (Fig. 4). The relative G protein-stimulated activity decreased over a 2-h PLC incubation, whereas the total PDE activity, triggered by trypsinization of the PDEγ inhibitory subunits, remained nearly constant. These results are consistent with the loss, upon phospholipase treatment, of endogenous phosphoinositides in ROS membranes that enhance PDE6-G protein interactions.

Binding of Immobilized Phosphoinositides by PDE6 and Transducin—In addition to assaying phosphoinositide binding using vesicles, we also tested PDE6 and G_{αt} binding to commercially available filter membranes containing immobilized phospholipids, including various phosphoinositides. When a PDE6 heterotetramer was incubated with filters containing immobilized phospholipids, which were then washed and probed with PDE6-specific antibodies, binding of PDE6 to specific phosphoinositides was easily detectable (data not shown). However, there was little correlation between the specificity of binding of PDE to immobilized phospholipids and the effects of different phosphoinositides on PDE activation. PI(4)P, PI(4,5)P₂, and PI(3,4)P₂ were much more potent in stimulating G_t activation of PDE than were PI(5)P and PI(3,5)P₂, but the preference was reversed in binding assays using immobilized phospholipids, where the strongest binding was seen with PI(5)P and PI(3,5)P₂. We were also unable to detect significant enhancement of G_{αt}-GTPγS binding to the immobilized phosphoinositides in the presence of PDE6. We conclude that the patterns of specificity of these proteins binding to lipids as presented on these membranes are not representative of their binding to either reconstituted lipids or, in all likelihood, native membranes.

DISCUSSION

Nearly two decades of investigation have failed to uncover convincing evidence that phosphoinositides play a major role in the activation phase of dim flash responses in rod cells. Experiments with gecko rods suggest that inositol 1,4,5-trisphosphate (InsP₃) does not play an important role in phototransduction (20). However, it is possible that phosphoinositides in general and their ability to modulate G protein-effector interactions as documented here, in particular, may have a role to play in the changes in sensitivity and kinetics that occur under varying background lighting conditions. Light and dark adaptation, although more subtle than the immediate dramatic responses of dark-adapted rod cells to light, are also more complex processes (21–26). They are known to have multiple components, not all of which have been definitively identified. An interesting possibility is that phospholipase C activation, either directly by G protein subunits or by changes in intracellular Ca²⁺, could modulate PDE6 activation efficiency. Alternatively, synthesis of phosphoinositides by regulation of lipid kinases or the enzymes responsible for PI synthesis could serve as a point of regulation during adaptation.

Immunostaining experiments using antibodies specific for PI(4,5)P₂, revealed strong immunostaining of rod outer segments in the dark, which was rapidly abolished upon illumination (27, 28), a finding supported by reports of rapid light-induced hydrolysis of PIP₂ (8, 9). Biochemical experiments (1, 2) have indicated that rod outer segments contain the enzymes necessary for phosphorylation of PI and PIP as well as phospholipase C, which can degrade PIP and PIP₂. These previous results suggest that there may be fairly high levels of PIP₂ and, possibly, PIP in the dark and that they can be reduced in

response to illumination. Recently, our standard preparation of ROS membranes was assayed using a novel chromatographic method that can detect PIP and PIP₂ without radiolabeling (29). PIP was detected at a level of 1% of the level of phosphatidylserine or ~10 moles per mole of PDE6, and this endogenous PIP likely accounts for the PLC effect we observe. PIP₂ levels were undetectably low, likely as a result of the operation of lipid phosphatases and phospholipases in our ATP-depleted preparation from frozen retinas. Phosphatidylinositol, the precursor for PIP and PIP₂, was found at 40% of the level of phosphatidylserine. It will be important to quantify PIP and PIP₂ levels *in vivo* in light-adapted and dark-adapted conditions.

Sites for sensitivity modulation in G protein signaling pathways are coming under increasing scrutiny as potential sites for therapeutic intervention with drugs that might fine tune rather than completely block certain pathways. The photo-transduction system might serve as a useful model for understanding the role of phosphoinositide modulation of G protein-effector interactions in desensitization and sensitization.

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