Enhancement of Phototransduction G Protein-Effector Interactions by Phosphoinositides*

Received for publication, October 20, 2003, and in revised form, December 15, 2003
Published, JBC Papers in Press, December 29, 2003, DOI 10.1074/jbc.M311488200

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Light responses in photoreceptor cells are mediated by the action of the G protein transducin (Gt) 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 Gt on the surface of large unilamellar vesicles containing d-myophosphatidylinositol-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-myophosphatidylinositol-3,4-bisphosphate and d-myophosphatidylinositol-4-phosphate (PI(4)P), but much less by d-myophosphatidylinositol-5-phosphate (PI(5)P) or d-myophosphatidylinositol-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 Gt-GTPγS 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, Gαt, 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 phosphoinosside signaling in rod outer segments and other reports on the modulation of these components by light (1–9). We reported previously (10) that d-myophosphatidylinositol-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 Gαt (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 phosphatidylinerine and phosphatidylethanolamine and PDE6 and between phosphatidylinerine and Gαt (12). Gαt-GTPγS, 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. Gαt is heterogeneous acylated at its N-terminal glycerol residue, and PDE6 has farneyl and geranylgeranyl groups attached to 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 Gαt, PDE6 binds more tightly to lipid bilayers than does Gαt, and, under dilute conditions, we have observed that PDE6 enhances binding of Gαt to vesicles. Moreover, lipid surfaces that favor Gαt activation of PDE6 also enhance its binding to vesicles containing bound PDE6. Both PDE6 and Gαt 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-myophosphatidylinositol-3,4-bisphosphate; PI(3,4)P2; d-myophosphatidylinositol-3,5-bisphosphate; GTPγS, guanosine 5’-O-(3-thiotriphosphate); ROS, rod outer segment; MOPS, 4-morpholinopropansulfonic acid; TBST, Tris-buffered saline with Tween 20.
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% Tween 20) was also used.

**Protein Electrophoresis and Immunoblotting**—SDS-PAGE and immunoblotting were carried out using standard protocols (13). Antibodies used were PDE6α-specific rabbit antiserum from Affinity Bioreagents or PDE6 catalytic subunit-specific antiserum, a gift from Dr. Rick Cote (14), and the Gαs 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 antirabbit 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 (PDE6α), holotransducin (Gαs-GDP), and Gαs-GTPγS 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-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-5-phosphate (PI(5)P), D-myo-phosphatidylinositol-3,5-biphosphate (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 Gαs-GTPγS) were mixed with vesicles (150 μM 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 Gαs or PDE-specific antibodies.
RESULTS

Effect of Phosphoinositides on PDE6 Activation by G_{ot}-GTP\gamma 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_{ot}-GTP\gamma S. As reported previously, reconstitution of PDE6 and G_{ot}-GTP\gamma 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)P₂ was consistently the most potent, followed by PI(3,4)P₂ 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)P₂ 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)P₂. 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_{ot} to Phospholipid Vesicles—In previous experiments with reconstituted vesicles, we observed a strong correlation between PDE6-dependent G_{ot} 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

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 mM G_{ot}-GTP\gamma S (when present); 2 mM cGMP, and 150 mM total phospholipid in the form of synthetic vesicles (when present). To assess maximal PDE activity, samples were treated with 0.2 mg/ml trypsin. 

Ca²⁺ Assays—To determine the influence of [Ca²⁺] on phosphoinositide effects, pH assay samples were buffered with 0.6 or 0.8 mM EGTA and varying amounts of CaCl₂ (0–2.4 mM) added. Free [Ca²⁺] was determined in parallel samples containing 1 µM Fura-2 (Molecular Probes) by measuring fluorescence excitation spectra and calculating [Ca²⁺] as described (17) using a K_d value under our conditions of 224 nM for the binding of Fura-2 to Ca²⁺.

Phospholipase C from Bacillus cereus was obtained from Sigma. ROS membranes were incubated at a concentration of 50 µM rhodopsin with 5 units/ml of enzyme (or no enzyme for the control samples) in pH assay buffer in a volume of 80 µl at 37 °C. After the reaction had proceeded for the indicated times, the sample was diluted to 500 µl 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 µl 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_{ot}-GTP\gamma S diluted in binding 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.

FIG. 2. Enhancement of G_{ot}-GTP\gamma 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_{ot}-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_{ot} antibody. (Note: the supernatant fraction probed with G_{ot} antibody is not shown. Because of the large molar excess of G_{ot}-GTP\gamma S, which does not bind membranes significantly under these conditions without PDE6 (11), the decrease in G_{ot} in the membrane due to vesicle binding is not detectable).
good agreement with the results obtained by activity assays. The strongest binding is to vesicles containing PI(4,5)P2, 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-Gt-GTP/S complex.

**Effects of Divalent Cations on Phosphoinositide Stimulation of PDE Activation**—Because PDE6 and Gt 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. Ca2+ 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 Ca2+ affects the ability of phosphoinositides to stimulate activation of PDE and stimulate binding of PDE or the PDE-Gt-GTP/S complex to membranes. The results, shown in Fig. 3, show at most a modest ability of Ca2+ to stimulate the phosphoinositide effect. It seems unlikely that such small changes play an important physiological role. However, assays at the physiologically relevant [Ca2+]o of 81 nM do demonstrate the greatest enhancement, 3.8-fold, of PDE activation induced by PI(4,5)P2 (Fig. 3A). Mg2+ 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 MgCl2. We detected no significant difference in PDE6 or Gt-GTP/S bound to vesicles in samples with or without millimolar free Mg2+ (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 Gt-GTP/S-PDE6 interactions in membranes from rod outer segments. When ROS membranes were incubated with bacterial phos-
phosphatidylinositol-specific phospholipase C before assaying \( G_{\alpha}\)-GTP\( \gamma \)S-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 PDE\( \gamma \) 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 \( G_{\alpha} \) 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 activity, \( PI(4)P_2 \), \( PI(4,5)P_2 \), and \( PIP(3,4)P_2 \) were much more potent in stimulating \( G \) activation (27, 28), a finding supported by reports of rapid light-mediated changes in the dark, which was rapidly abolished upon illumination. Recently, our standard preparation of ROS membranes was assayed using a novel chromatographic method that can detect PIP and PIP\( _2 \) without radiolabeling (29). PIP was detected at a level of 1% of the level of phosphatidylinerine or ~10 moles per mole of PDE6, and this endogenous PIP likely accounts for the PLC effect we observe. PIP\( _2 \) 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 PIP\( _2 \), was found at 40% of the level of phosphatidylinerine. It will be important to quantify PIP and PIP\( _2 \) 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 phototransduction system might serve as a useful model for understanding the role of phosphoinositide modulation of G protein-effector interactions in desensitization and sensitization.

**Acknowledgments**—We thank Dr. Rick Cote of the University of New Brunswick for providing the PDE6 antibody and Dr. Alecia Gross for helpful comments on the manuscript.

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