Signal Transduction in the Visual Cascade Involves Specific Lipid-Protein Interactions*

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In retinal rod photoreceptor cells, transducin (Gt) and cyclic GMP phosphodiesterase (PDE) are peripherally anchored to the cytoplasmic surface of the disk sacculae. We have examined the role of specific phospholipids in the interaction of these proteins with native osmotically intact disk vesicles, employing spin-labeled phospholipid analogues (2% of total phospholipids) and bovine serum albumin back-exchange assay. Inactive GDP-bound transducin exclusively reduced the extraction of negatively charged phosphatidylserine. The effect disappeared upon activation of the G-protein with GTP (Gts). PDE affected the extraction of the zwitterionic phosphatidycholine and, to a smaller extent, of phosphatidylethanolamine. When active GtGTPγS interacted with the PDE to form the active effector, the interaction with phosphatidylserine was specifically enhanced. Each copy of the G-protein bound 3 ± 1 molecules of phosphatidylserine, whereas the PDE bound a much larger amount (70 ± 10) of a mixture of phosphatidylcholine and ethanolamine. The results are interpreted as a head group-specific and state-dependent interaction of the signaling proteins with the phospholipids of the photoreceptor membrane.

The transduction of the light signal in the retinal rod photoreceptor is a well studied model system for G-protein-coupled signal transduction. It involves the sequential activation of rhodopsin, transducin (Gt),1 and cGMP phosphodiesterase (PDE) on the cytoplasmic surface of the disk sacculae that fill the rod outer segment (ROS). Absorption of light transforms rhodopsin into the activated metarhodopsin II state, which then interacts with Gt to rapidly catalyze the exchange of GDP to GTP in the nucleotide-binding site of the Gtα-subunit. Gtα-GTP dissociates and couples to the effector PDE (1, 2). Activation of PDE results in a decrease in the cytosolic cGMP concentration which in turn leads to closure of cGMP-regulated channels in the plasma membrane, hyperpolarization, and neuronal signaling (see Ref. 3).

Heterotrimeric Gt is peripherally attached to the disk membrane by weak hydrophobic and ionic interactions. Both N-terminal acylation of the Gtα-subunit and C-terminal farnesylation of the Gtα-subunit are required for membrane association of Gt (4). Electrostatic interactions, especially of Gtβγ, further enhance the membrane binding to negatively charged surfaces or to vesicles containing the acidic lipid phosphatidylserine (5–7). Inactive transducin in its GDP-bound form is attached as a GtGTPγS heterotrimer to the membrane. Gt couples to activated rhodopsin, triggering GDP release as a first step of catalytic nucleotide exchange. The interaction with the receptor occurs directly from the membrane-bound state of the holoprotein. After activation of transducin, Gtα dissociates from Gtβγ and couples stoichiometrically to the effector PDE.

The PDE is a heterotetrameric protein composed of the dissociable αβ complex (8) and two identical γ-subunits (9). Full activation of the enzyme requires the binding of activated (GTP bound) Gt to both of its inhibitory γ-subunits (see Ref. 10). Like Gt, the PDE complex is weakly associated to the surface of the disk membrane by geranylgeranylation of PDE-β and farnesylation of PDE-α (11, 12). The geranylgeranyl moiety was mainly made responsible for membrane attachment, whereas the farnesyl group may play a role in protein-protein interactions. It is known that efficient activation of PDE by active GtαGTP requires the presence of membranes, and protein-membrane interactions co-determine the interaction of Gtα-GTP with the PDE (13). PDE activation studies on vesicles of different lipid composition showed that the activity of PDE depended on the nature of the lipid. The highest PDE activation was found on large unilamellar vesicles composed of the unsaturated phospholipid dioleoylphosphatidylcholine (14).

This study provides direct information on the phospholipid interaction of the signaling proteins in native disk membranes. In particular, we are interested in the dynamics of this protein-lipid interaction during activation of the signal cascade. To characterize this interaction, we incorporate spin-labeled phospholipids into disk membranes, and we probe their accessibility to extraction by bovine serum albumin (BSA) (15, 16). In a previous study, employing this approach, we have shown that the interaction of rhodopsin with phosphatidylserine (PS) is dependent on its state of the activation, in agreement with earlier results (17) obtained by chemical labeling of PS in which rhodopsin was found to protect some of the PS of the membrane. During the lifetime of the active metarhodopsin II conformation, one molecule of PS became released by rhodopsin (18). We have now measured the extractability of BSA of...
spin-labeled phospholipid analogues from the disk membrane in the absence or presence of Gt and PDE, both in the dark and under conditions of light activation. In the present study, we identified privileged interactions of Gt and PDE phospholipids. Gt anchors in a small cluster of PS, whereas PDE binds to a large “cushion” of more than \( \pm 10 \) molecules of phosphatidylcholine (PC) and phosphatidylethanolamine (PE).

**EXPERIMENTAL PROCEDURES**

**Materials**—Short chain spin-labeled phospholipid analogues 1-palmitoyl-2-(4-doxyl-pentanoyl)-sn-glycero-3-phosphocholine (SL-PC), 1-palmitoyl-2-(4-doxyl-pentanoyl)-sn-glycero-3-phosphoserine (SL-PSE), and 1-palmitoyl-2-(4-doxyl-pentanoyl)-sn-glycero-3-phosphoethanolamine (SL-PE) were synthesized as described previously (16, 19).

**Preparation of Rod Outer Segments (ROS) and Disk Vesicles**—ROS were purified from freshly dissected and frozen bovine retinae and prepared in the dark according to Kühn (20). Osmotically intact disk vesicles were prepared by extraction of soluble and membrane-associated proteins as described (18, 21) and resuspended in 1.3-bis[(tris(hydroxymethyl)methylamino)- propane (BTP) buffer (2% (v/v) sucrose solution, 130 mM NaCl, 20 mM BTP, pH 7.4, 1 mM MgCl\(_2\)) or Heps buffer (5% (v/v) sucrose, 130 mM Heps, pH 7.4, osmotic pressure 320 \( \pm 5 \) mosmol) as indicated in the figure legends. To prepare isolated disks, the suspension was pressed through a filter with 2-\( \mu \)m pores (Nuclepore filter) and used immediately. The concentration of rhodopsin was determined spectrophotometrically using \( \varepsilon_{\text{max}} = 40,000 \, \text{M}^{-1} \, \text{cm}^{-1} \).

To minimize lipid peroxidation during the experiment, we always used disk membranes that were freshly prepared and used immediately for spin label incorporation. Intact ROS were prepared with their own complement of cytosolic anti-oxidants (prepared according to Schnetkamp (22)) and resuspended in a Ficoll/sucrose solution that gave the same results as ROS prepared according to Kühn (20), containing the EDTA, diethiothreitol, and taurine). Also, disks in Ficoll/sucrose solution (22) and disks in salt/buffer/sucrose solution (see above) showed the same results.

**Protein Preparation**—Gt and PDE were purified from bovine retina essentially as described (23) and stored in 20 mM Tris buffer, pH 7.0, EDTA, diethiothreitol, and taurine). Also, disks in Ficoll/sucrose solution (22) and disks in salt/buffer/sucrose solution (see above) showed the same results.

**Centrifugation Assay**—The relative amount of soluble and membrane-associated Gt and PDE was determined using the centrifugation assay (24, 25). The applications to the gels were normalized as follows. Aliquots (100 \( \mu \)l) of the samples used for the ESR/spin-label experiments (30 \( \mu \)M rhodopsin reconstituted with 5 \( \mu \)M Gt, and/or 1 \( \mu \)M PDE) were incubated at temperature and pelleted by centrifugation (5 min, 52,000 \( \times \) g, 4 °C). After complete removal of the supernatant, each pellet was resuspended in 100 \( \mu \)l of buffer to yield the initial membrane concentration. To determine the amount of protein either bound to the membrane pellet or present in the supernatant, the same volume of supernatant or resuspended pellet was analyzed by SDS-PAGE.

**Measurement of Transbilayer Distribution of Spin-label Analogues**—The SL-PL were dissolved in chloroform/methanol (1:1, v/v), transferred to a glass tube, dried under nitrogen, and mixed with the desired volume of buffer, leading to a suspension of SL-PL micelles. For labeling in the dark, disk membrane suspensions were incubated with 5 \( \mu \)M rhodopsin, incubated with 5 \( \mu \)M diisopropyl fluorophosphate) were mixed with the label solutions corresponding to time 0 for all kinetic measurements. After 1 min on ice to extract all lipid analogues accessible to BSA (21). After centrifugation supernatant and pellet were frozen for further analysis.

**Influence of Proteins on Analogue Extractability**—To investigate the influence of PDE, washed disk vesicles (30 \( \mu \)M rhodopsin) were either incubated with 1–3 \( \mu \)M PDE at 20 °C for 30 min in the dark before labeling, or PDE (0.5–3 \( \mu \)M) was added to disk membranes after SL-PL had equilibrated between the two membrane leaflets (see below). To remove PDE from the disk membranes after incubation, the pellets of the spin-labeled disk membranes were washed in solution of low salt concentration (8.5% sucrose (w/v), 20 mM Heps, pH 7.4, osmotic pressure 310 \( \pm 10 \) mosmol) for 2 min (52,000 \( \times \) g), and the concentrations of SL-PL in the supernatant and in the membrane pellet were measured by ESR spectroscopy as described below.

To assess the influence of Gt, SL-PL were first allowed to equilibrate across the disk membranes (30 \( \mu \)M rhodopsin) in the dark. Subsequently, transducin (5 \( \mu \)M) was added, and activation of G-protein was started by adding GTTP\( \gamma \)S (30 \( \mu \)M final concentration). ESR signal intensities were corrected for the dilution effect caused by the addition of PDE, transducin, and/or GTTP\( \gamma \)S.

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**RESULTS**

**Membrane Binding of Gt and PDE**—In vitro, membrane binding of both Gt and PDE depends on several factors, including protein and membrane concentration, ionic strength, pH, and temperature. We therefore used a centrifugation assay to quantify membrane binding of both proteins under the conditions of the spin-label experiments (24, 26).

At the high membrane concentration (30 \( \mu \)M rhodopsin), about 70% of the Gt and PDE added bound to the membrane in the dark (Fig. 1, lanes 2 and 3). As expected, activation of Gt by GTTP\( \gamma \)S resulted in a substantial dissociation of Gt from the membranes (Fig. 1, lane 4), whereas Gt activation in the presence of PDE enhanced membrane binding of PDE (Fig. 1, lane 5). In addition, in the latter case a fraction of Gt was retained on the membranes, consistent with enhanced membrane binding of the active PDE-Gt\( \alpha \)GTTP\( \gamma \)S complex formed (27, 28).

**BSA Extraction of Spin-labeled Phospholipid Analogues**—To identify and characterize the interaction of Gt and PDE with spin-labeled phospholipid analogues (SL-PL), we measured the BSA-extractable amounts of those analogues from disk membranes in the absence or presence of proteins. Briefly, the technique (18, 21) consists of the following: (i) incorporation of SL-PL into the outer membrane leaflet, (ii) extraction of SL-PL localized in the outer leaflet with BSA after various incubation times, and (iii) centrifugation of membranes and quantification of SL-PL in the pellet (i.e. lipid analogues which remained in the membrane) and in the supernatant (i.e. BSA-extracted lipid analogues) by ESR spectroscopy. Upon mixture with membranes, analogues incorporated into the outer membrane leaflet within a few seconds (29). Subsequently, the extractable amount of SL-PL decreases within minutes (Fig. 2A), which is explained by a redistribution of the SL-PL between outer and inner leaflet of the membrane. The final level of SL-PL available for back-exchange with BSA reflected the equilibrium distribution of SL-PL. We have discussed previously (21) to what degree the distribution between the outer and inner leaflet of the membranes of the SL-PL is a correct measure of the endogenous phospholipid species. It can be stated that in the
Molecular mass standard (left) show no residual Gt or PDE but a fraction control membranes (lane 1) and pellets (B) were analyzed by SDS-PAGE. The supernatants (used for ESR spin-label experiments were pelleted by centrifugation. investigated in a separate sample in which excess GTP/H9253/H11011 measured for SL-PC or SL-PE (fects the extraction of SL-PS. A much smaller, if any, effect was membrane-associated inactive holoprotein that specifically af-

case of PS, all available techniques yielded consistent results (17, 30,31). No direct assay was available for PC, so that these data cannot be directly compared. For PE, chemical probing of the native lipid yielded higher asymmetries than found with the SL-PL (21, 32).

The three SL-PL analogues reached a stable equilibrium distribution with a mean half-time of less than 5 min. The data indicate (Fig. 2A) an almost symmetrical distribution of spin-labeled phosphatidylcholine (SL-PC) and phosphatidylyethanolamine (SL-PE), and a more asymmetrical distribution of spin-labeled phosphatidylserine (SL-PS), i.e. 75% of the spin label in the outer leaflet. This is the same distribution as was previously reported under slightly different conditions (see “Experimental Procedures”) (18, 21).

Transducin Reduces the BSA Extraction of Spin-labeled PS—To investigate the interaction of Gt with SL-PL, Gt (5 μM) was added to the membranes after complete equilibration of SL-PL between the two leaflets. Subsequently, the extraction of SL-PL by BSA (1 min for each aliquot) was assayed in 5-min intervals and compared with that of samples without transducin. The influence of Gt activation on lipid interaction was investigated in a separate sample in which excess GTPyS was co-injected together with Gt. The sample was left in the dark to keep the amount of activated rhodopsin as low as possible. Even under these conditions Gt is activated within seconds by a small fraction of endogenous opsin/all-trans-retinal complexes present in the membranes (33).

Fig. 2B shows the fraction of SL-PL that remained in the membranes after BSA extraction in the presence of inactive or active Gt. SL-PS becomes significantly less extractable upon addition of Gt. In the presence of 5 μM Gt, additional 5.2 ± 0.6% of SL-PS was not accessible to BSA. This was not observed upon activation of Gt with GTPyS, indicating that it is the membrane-associated inactive holoprotein that specifically affects the extraction of SL-PS. A much smaller, if any, effect was measured for SL-PC or SL-PE (−2% less extractable SL-PE with 5 μM Gt). Although we found consistently a slightly lower level of extraction, the effect was not significant, neither with nor without GTPyS.

The extraction of SL-PS was repeated with different amounts of Gt. The membrane-bound non-extractable amount of SL-PS increased with Gt concentration (Fig. 2C). Similar results were obtained in the absence and presence of 1 mM MgCl2 (see “Experimental Procedures”).
FIG. 3. A–C, influence of PDE binding on extraction of spin-labeled phospholipids from disk vesicles. The kinetics of the non-extractable fraction of SL-PL in the dark at 20 °C is plotted (see Fig. 2A) for the sample with PDE preincubation (3 μM for 30 min; open symbols) and without PDE (filled symbols) in A–C (A, SL-PS; B, SL-PC; C, SL-PE). Insets show the kinetics for a time interval of 50–90 min in an alternative protocol, where PDE was added after SL-PL equilibration in the disk membrane. Spin-labeled analogues (2% of total endogenous phospholipids) were allowed to equilibrate across the disk membranes in Hepes in the dark at 20 °C (see Fig. 2A). At the times indicated, the percentage of non-extractable SL-PL is presented in the dark (filled symbols). After 60 min PDE (1 μM as final concentration) was added to the spin-labeled vesicle suspension in BTP and Hepes and BSA extraction was followed (open symbols) in A–C (see above). Means ± S.D. are given for three independent preparations.

PDE Reduces the BSA Extraction of Spin-labeled PC and PE—To measure the influence of PDE on BSA extraction of SL-PL, disk membranes were preincubated with PDE (1–3 μM PDE, 30 min) in the dark to reach an equilibrium between membrane-bound and -soluble PDE. Subsequently, SL-PL were added, and their BSA-extractable portion was followed as described above (Fig. 3). In an alternative protocol, PDE was added to the disk membranes after SL-PL equilibration between both membrane leaflets (Fig. 3, insets).

Fig. 3 shows the amount of SL-PL that remained in the membranes after BSA extraction, with and without pre-equilibration of the membranes with PDE. As seen in Fig. 3A, PDE had no effect on the extractability of SL-PS and did not affect the asymmetric distribution of PS between leaflets. In contrast to SL-PS, both SL-PC and SL-PE were significantly less extractable in the presence of PDE (Fig. 3, B and C). Under these conditions (3 μM PDE) about 17% less of SL-PC and 12% less of SL-PE were found in the BSA extract. Interestingly, the effect on SL-PL extractability was complete before the first aliquot was taken (i.e. within 1 min, see below). As Fig. 3 shows, both the time course and the magnitude of subsequent transbilayer movement of SL-PC and SL-PE remained unchanged.

When the PDE was added after a stable plateau of SL-PL transbilayer distribution was reached (Fig. 3, insets), SL-PS extraction was not affected (Fig. 3A, insets), but both SL-PC and SL-PE became significantly less extractable. The effect was already recognizable at the first data point, i.e. 1 min after addition of 1 μM PDE (Fig. 3, B and C, insets); 6% of the SL-PC and 4% of the SL-PE resisted the BSA extraction. On a relative scale, this effect is similar to that seen for membranes preincubated with PDE. When the extraction was repeated with different amounts of PDE (preincubation protocol), the membrane-bound non-extractable amount of both SL-PC and SL-PE increased with PDE, whereas no influence of PDE was seen in the case of SL-PS (Fig. 4). The PDE-related non-extractable amount of SL-PC and SL-PE did not saturate within the concentration range investigated. Unfortunately, the onset of membrane aggregation above 3 μM PDE prevented the study of higher PDE concentrations.

The Effect of PDE Is Reversible—To test the reversibility of the PDE effect, disks were preincubated with 2 μM PDE (30 min in the dark); SL-PC was added, and aliquots were taken after various incubation times for the back-exchange assay (PC control in Fig. 5A). In parallel, aliquots were taken, and the disks were pelleted by centrifugation and treated with low ionic strength buffer to release PDE from membranes (see “Experimental Procedures”). Quantitative removal of PDE was confirmed by analysis of the samples with the centrifugation assay (data not shown). As seen in Fig. 5A, the release of PDE from the disk membranes at low ionic strength resulted in a complete and immediate reversal of the PDE effect on SL-PC extractability. To verify that the washing procedure per se did not affect the extraction of analogues, the pellet of spin-labeled, PDE-bound membranes was washed with isotonic buffer (data not shown); PDE remained membrane-associated, and the amount of extracted analogues could not be reversed to the level of the control. The reversibility of the PDE effect was also seen when the membranes containing the labeled lipid were persistently incubated with BSA with or without PDE (Fig. 5B).
Effect of PDE Activation on the Extraction of Spin-labeled Lipids—We asked next whether the simultaneous presence of Gt and PDE and/or their interaction in the active Gt-GTP-PDE complex affected the extraction of SL-PL. By using the protocol of Figs. 2 and 3, Gt, PDE, and GTP/S were successively added to the membrane suspension (Fig. 6). Consistent with the results described above addition of Gt resulted in a reduced extraction of SL-PS but not of SL-PE or SL-PC. The subsequent addition of PDE reduced the extractability of SL-PC and to a lower degree that of SL-PE but did not influence that of SL-PS. Thus, in the presence of both Gt and PDE, the effect of the respective proteins is simply superimposed, demonstrating that, in their inactive form, the two proteins act independently on the membrane lipids.

Although we observed a reversal of the Gt-SL-PS interaction upon activation of Gt (see above), this was not seen when Gt was activated in the presence of PDE. Under this condition, reduced extractability of SL-PS was not reverted (Fig. 6A), although a significant fraction of active Gt dissociated from the membranes (see above and Fig. 1). Activation of Gt and PDE resulted in a significant further decrease of SL-PC extraction (Fig. 6B), consistent with the enhanced membrane binding of PDE (see above and Fig. 1). Interestingly, the effect was not seen with SL-PE (Fig. 6C).

DISCUSSION

We have examined the interaction with membrane phospholipids of two proteins involved in visual signal transduction, namely, the G-protein transducin (Gt) and the effector, a cyclic GMP phosphodiesterase (PDE). In the native, osmotically intact disk vesicles, each rhodopsin molecule is surrounded by about 65 lipids (34). With the membrane composition (35) and the asymmetry of the membrane (Fig. 2A), an average of 7 PS, 16 PC, and 16 PE per rhodopsin is found in the outer leaflet (to which Gt and PDE anchor). A BSA back-exchange technique was applied to assess and quantify lipid-protein interaction in native membrane preparations. To this end, the different amount of extracted spin-labeled phospholipid analogue in the presence or absence of the respective protein was taken as a measure. Disk membranes were reconstituted with Gt and

FIG. 5. Effect of PDE on SL-PC extraction is reversible. A, spin-labeled lipids (2% of total phospholipids) were added to the disk vesicles and resuspended in sucrose/Hepes buffer (see “Experimental Procedures” and protocol in Fig. 2A). Kinetics of the fraction of non-extractable SL-PC in rod disk membranes in the dark in the presence of PDE (2 μM, white circles), without PDE (black circles), and after removal of PDE from the disk vesicles by washing in buffer with reduced salt concentration (gray circles, see “Experimental Procedures”). The enhanced onset after the kinetics in the presence of PDE reflects the interaction between PDE and SL-PC (see “Discussion”). Data points are from two independent measurements. B, SL-PC (2% of total endogenous phospholipids) was allowed to equilibrate across the disk membranes in the dark at 20°C (see Fig. 2A). At t = 60 min, PDE (2 μM final concentration; gray triangles) was added. Control, without PDE (black circles). At t = 75 min both samples were mixed with BSA (final concentration 3% (w/v); white circles and triangles, respectively) and incubated further at 20°C. The amount of non-extractable SL-PC is presented from three independent measurements.

FIG. 6. A–C, influence of Gt and PDE binding on disk membranes on the SL-PL extraction by BSA from the membrane in the dark and after activation. Spin-labeled analogues (2% of total endogenous phospholipids) were allowed to equilibrate across the disk membranes in the dark at 20°C (see Fig. 2A) The percentage of non-extractable SL-PL is plotted versus time, for samples after Gt binding (5 μM) (gray symbols), after PDE incubation (1–1.5 μM) (white) in the dark, and after activation with GTPγS (30 μM) (black-pointed) compared with SL-PL redistribution without protein binding in the dark (black symbols) in A–C (A, SL-PS; B, SL-PC; C, SL-PE; means ± S.D. for at least three independent preparations).
PDE, alone or in combination, and in their active or inactive states. The salient result of the work is that the lipid interactions of the two proteins are lipid head group-specific and depend on the activation state of the protein. We find an interaction of Gt only with the negatively charged SL-PL phosphatidylserine, and of the PDE only with the zwitterionic SL-PL phosphatidylcholine and phosphatidylethanolamine. The two proteins differ not only in the type but also in the amount of lipids they can bind.

The Gt Heterotrimer Is Anchored in a Small Cluster of Phosphatidylserine Molecules—Binding of Gt to rod disk membranes causes a reduced extraction of spin-labeled PS by BSA. This was not seen in the case of SL-PC and SL-PE, indicating a head group-specific lipid interaction of Gt. Under our conditions, 5% of the total SL-PS incorporated into disk membranes could not be extracted by BSA after membrane binding of Gt. This result was obtained in both the absence and presence of 1 mM MgCl₂. Given the fraction and (asymmetric) distribution of PS in the disk membrane, the 5% effect on total lipid translates into 0.7 mol % of PS. Gt heterotrimer is acylated on the α-subunit and prenylated on the γ-subunit, and membrane association of the inactive protein is primarily due to the penetration of both of these lipophilic modifications into the hydrocarbon region of the membrane (4). Because only the outer leaflet can contribute to anchoring, the effect of Gt on SL-PS would account for three molecules of outer leaflet SL-PS bound to Gt.

How do the specific properties of Gt anchoring fit to such a selective lipid interaction? It is known that the highly unsaturated lipid environment of the disks favors transducin binding (14, 36). In bovine disk membranes, phospholipids contain a very high level of polyunsaturated fatty acids; PS has the highest level (37). More importantly, calculations based on the crystal structure of Gβγ have led to the conclusion that the negatively charged Gβγ subunit complex is highly polarized and displays a region of positive electrostatic potential surrounding the site of farnesylation. This may explain why Gβγ binds strongly to vesicles consisting of acidic phosphatidylserine (5, 38) and to negatively charged monolayers, whereas the Gαα-subunit does not show electrostatic attraction (6, 38).

The attached protein should be oriented in such a way that the farnesyl moiety can be fully inserted into the membrane with a negatively charged surface enhancing the membrane partitioning of transducin (7). So the electrostatic interaction of Gβγ with negative charges of the membrane surface may lead to an orientation of Gt heterotrimer and synergistically favor its anchoring. Evidence has been provided (6) that in the Gt holoprotein, myristoyl and farnesyl modifications on Gαα and Gβγ, respectively, are sufficiently close to one another to act as one hydrophobic anchor. We can now propose that 3 ± 1 molecules of PS accommodate this myristoyl/farnesyl anchor, making optimized dual use of electrostatic and hydrophobic interactions.

The permanent activation of transducin by GTPγS leads to Gαα and Gβγ dissociation from the membrane (24), which explains the absence of the effect on PS extraction.

The PDE Tetramer Binds a Cushion of about 70 PC and PE Phospholipids—Studies of the equilibrium between soluble and membrane-bound native PDE found in rod outer segments suggested that PDE is associated to the disk membrane through anchoring to phospholipids (39). Based on the effect of PDE on BSA extraction of SL-PL, and along the same lines as discussed above for the G-protein, the PDE preferentially interacts with the phospholipids PC and PE. With the PDE no significant influence was found on the extraction of the negatively charged phospholipid SL-PS. The resistance to BSA extraction of SL-PC and SL-PE was shown to increase in a linear proportion to the amount of PDE added, with a maximal effect of 17 and 12% for SL-PC and SL-PE, respectively. In consideration of the native concentration of PDE (rhodopsin/PDE = 50:1) and the incomplete membrane binding under the conditions of our experiments (70% of added PDE was membrane-bound, see above), the native density of PDE on the membrane surface was reached after adding ~1 μM PDE. Under these conditions extraction of analogues by BSA was reduced by about 6 and 4% of total spin-labeled PC and PE analogues, respectively, as compared with control membranes. With the known molar ratio to rhodopsin of each of the two lipids and of the PDE, one arrives at the conclusion that about 40 molecules of PC and 30–40 of PE are excluded from BSA extraction per molecule of membrane-bound PDE (Table I).

These results exclude artifacts like membrane aggregation, which could reduce the overall access of BSA to the membranes, thus reducing extraction of SL-PL. Such effects would affect all three lipids. The effects are of a remarkable distinct specificity, i.e. PDE does not affect PS and Gt does not affect PC or PE. Moreover, we can state that, despite the specificity, the effect does not result from tight binding of the label to the protein at least for PDE. If this were the case, removal of PDE would co-extract label (i.e. would act like BSA) which was not seen. This is consistent with the observation that prolonged incubation with BSA allows us to extract SL-PC even in the presence of bound PDE to the same extent as observed for control samples (absence of PDE, see Fig. 5B).

| Phospholipid | Non-extractable SL-PL after addition of Gt or PDE | No. lipid per protein |
|--------------|-----------------------------------------------|----------------------|
|              | % of total SL-PL added | Gt molecule | PDE molecule |
| 5 μM Gt | 3 μM PDE | |
| PC | 0.1 ± 0.3 | 17 ± 2.0 | a | 42 ± 5 |
| PE | 2.4 ± 0.2 | 12 ± 1.8 | a | 33 ± 5 |
| PS | 5.2 ± 0.6 | 0.4 ± 0.01 | 3 ± 1 | a |

* A No calculation due to the not significant experimental data.  
* Significant at p > 0.05 compared with the control level.

Fig. 7. Model for Gt and PDE membrane interaction in the inactive state. Models of rhodopsin (left), Gt (middle), and PDE (right) and their putative interaction with the respective phospholipids (see Table I). Note that the structures in lower part (rhodopsin, Protein Data Bank code 1HZX; Gt, Protein Data Bank code 1GOT) are not to scale.

Anchoring of Signal Proteins to Specific Lipid Head Groups

Gt or PDE addition to washed disk membranes

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Fig. 7. Model for Gt and PDE membrane interaction in the inactive state. Models of rhodopsin (left), Gt (middle), and PDE (right) and their putative interaction with the respective phospholipids (see Table I). Note that the structures in lower part (rhodopsin, Protein Data Bank code 1HZX; Gt, Protein Data Bank code 1GOT) are not to scale.
The onset of the SL-PL redistribution curve and its entire time course in the presence of PDE is shifted to higher values (Fig. 3), indicating that the lipid-PDE interaction is present from the very beginning of the extraction. Several reasons argue that this fast kinetics reflects a recruitment of analogues to PDE, and not a PDE-induced translocation of the analogues to the inner membrane leaflet. The linearity of the dose-response curve (Fig. 4) is also consistent with a direct effect, in which each molecule of PDE bound to the membrane surface exerts the same relative effect. We therefore assume that the lipid analogues (and presumably also the native phospholipids PC and PE) interact directly with the PDE.

**PDE Activation and Formation of Signaling Phospholipid Complexes**—The exchange of GDP for GTP or GTPγS in the nucleotide-binding site of Gα leads to separation of Gα and Gβγ. To fully activate the PDE holoprotein, two copies of the activated Gα-GTPγS must bind to the PDE (40). Native PDE is anchored to the disk membrane by isoprenylation and carboxymethylation of the C termini of its PDEα- and PDEβ-subunits. It is well documented that the ability of Gα to activate the PDE is sensitively dependent on the presence and/or composition of membranes (14, 39), and several studies (28, 39) have shown that PDE is more tightly membrane-bound when activated by Gα. The interaction between Gα-GTP and PDEαβ causes a fast change in the light scattering of the membrane, which precedes the enzymatic activity (23).

The conclusion from these previous studies that the activation of PDE both requires the presence of the disk membrane and affects its properties is supported and expanded by our results. It was found that the formation of the PDE-Gα-GTPγS complex generates a fraction of spin-labeled phospholipids non-accessible to BSA extraction, as compared with the PDE and Gα constituents. Notably, however, the specifics of phospholipid interaction with the complex (Fig. 6) does not simply reflect the properties of the constituents. The complex does not bind any PS (as does the inactive Gα holoprotein) and displays a preference for PC, which may explain why activated PDE binds tighter to the membrane.

**Relationship to Lipid Rafts**—Our finding that protein-lipid interaction depends on the activation state of the proteins involved emphasizes the role of membrane plasticity and the temporary formation of lateral lipid domains in signal transduction. The question arises as to how these domains relate to the preformed micromodules, known as rafts, which are identified as detergent-insoluble fractions. The current data do not allow us to decide whether the PDE binds to preformed rafts or protein binding to the membrane induces a lateral inhomogeneity in the lipid distribution. Recent analyses (41, 42) have suggested that both Gα and PDE change their localization within the disk membrane between rafts and the surrounding bulk fluid membrane in a state-dependent manner. For Gα, such behavior would fit into the more general notion that G-proteins, including those of the Gα family (to which Gα belongs), tend to enrich in rafts (43). Reduced activation was seen under conditions of Gα recruitment to rafts, consistent with a reduction in the diffusion speed, and thus with the diffusional encounter frequency between the proteins, which limits the activation process (44). Therefore, by taking into account our results on Gα-PS interaction, we may anticipate that an optimal activation/deactivation cycle of the G-protein requires the time-ordered binding of the protein to different lipid microdomains. Indeed, it is possible and even experimentally supported in the case of Gα (42) that the proteins are translocated to rafts in the deactivation phase of the rod phototransduction. The future combination of our approach with illumination protocols may provide insight into how the sets of interacting phospholipid identified here are involved in these processes.

**Phospholipid Footprints of Peripherally Membrane-attached Signaling Proteins**—This study brings up a new aspect of visual signal transduction, namely that the two key signaling proteins use very specific sets of phospholipids to interact with the membrane. We have found that the amount of labeled lipid that resisted extraction by BSA did not exceed the number required to cover the likely footprint of the protein on the membrane. The G-protein is anchored by only a few molecules of PS. Based on known structural details, a likely arrangement of the negatively charged PS head groups can be envisaged (Fig. 7). In the case of the PDE effector, membrane association is mediated by a more extended interaction with both PC and PE. Although the structure of PDE holoprotein is unknown, we propose that its footprint on the membrane is much larger, comprising about 70 molecules of PC and PE. These differences between anchor- and cushion-like phospholipid interaction may reflect the specifics of interaction during signal transduction. One may speculate that a small anchor leaves Gα sufficient freedom for the necessary rapid (1 ms at 34 °C) and reliable multiple interaction of many Gα with the activated receptor. On the other hand, the time delay of interaction between Gα and PDE is on the order of 5 ms (23), allowing a longer time for stoichiometric interaction. The detailed mechanisms of these membrane-protein interactions remain to be elucidated, but it can be expected that they will make a significant contribution to the optimized function of the G-protein-coupled transduction pathway in rods.

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