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Rod Outer Segment Phosphodiesterase Binding and Activation in Reconstituted Membranes†

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ABSTRACT: Light exposure of rhodopsin in rod outer segment (ROS) membranes activates several cyclic GMP phosphodiesterase (PDE) molecules via a GTP-binding protein (G protein). Both PDE and G protein are surface-associated (peripheral) enzymes, which may be extracted from ROS by hypotonic media, individually purified, and recombined in isotonic media with purified rhodopsin–phospholipid vesicles to yield membranes of low dark and high light phosphodiesterase activity. In isotonic media, the PDE strongly associates with phospholipid membranes as well as with ROS and rhodopsin–phospholipid membranes. Because only membrane-associated PDE is readily light activated, the PDE activity saturates when the available binding sites are occupied. At a constant G-protein concentration, the PDE activity observed at saturation is 4 times greater for unilamellar rhodopsin–phospholipid vesicles with a lipid to rhodopsin ratio of 460 than for those with a ratio of 120. Thus, PDE association with membrane in isotonic media is dependent on the phospholipid content rather than the rhodopsin content. Several G proteins per PDE are necessary to maximize the PDE activity of reconstituted membranes; therefore, a weak association between activated G protein and PDE is indicated. Both peripheral enzymes readily transfer between membrane surfaces. Rhodopsin–phospholipid vesicles devoid of enzyme activity were exposed to a light flash and then mixed in the dark in isotonic media with unilluminated ROS membranes which contained PDE and G protein. PDE activity was observed within 2 s after mixing. Subsequent separation and evaluation of the denser ROS membranes and the less dense vesicles demonstrated that both PDE and G protein were associated with the vesicles as well as the ROS membranes. This peripheral protein transfer is at least 10^3 times faster than previously identified lipid exchange between vesicles or the transfer of integral protein between membranes and vesicles. The transfer may be fast enough to be physiologically relevant in this membrane system, and similar transfer processes are likely in experiments with other peripheral proteins.

Light absorption by the 11-*cis*-retinal of the transmembrane rhodopsin (Rh)[1] produces spectral and structural changes in the protein which result in the activation of several photoreceptor outer segment enzymes (O'Brien, 1982; Pober & Bitensky, 1979). In one activation sequence, a photoproduced intermediate of rhodopsin (Rh*) interacts with the membrane surface associated G protein to facilitate the exchange of GTP for GDP (Fung & Stryer, 1980). This G-protein complex (G_{GTP}) in turn activates a second membrane surface associated enzyme, a cGMP phosphodiesterase (PDE) (Fung et al., 1981), which catalyzes the hydrolysis of cGMP with a turnover of 10^3 s^{-1} (Yee & Liebman, 1978). The two-step amplification (a single Rh* may catalyze the binding of GTP to $\sim 10^2$ G proteins, each of which may activate a PDE) results in the hydrolysis (in vitro) of 10^5 cGMP per Rh* per s (Liebman & Pugh, 1979; Woodruff & Bownds, 1979).

A current hypothesis for the activation sequence is given by the following equations, where G_{GDP} represent G protein with bound GDP and G_{GTP}* symbolizes G protein with bound GTP, with the asterisk indicating an activated species. More information is necessary about this sequence of reactions and the reactants before a kinetic theory can be formulated for the activation and inactivation of Rh, G, and PDE. It is known that rhodopsin is a transmembrane protein (Fung & Hubbell,

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[1] Abbreviations: ROS, rod outer segment(s); Rh, rhodopsin; PDE, phosphodiesterase; GTP, guanosine 5′-triphosphate; GDP, guanosine 5′-diphosphate; cGMP, guanosine cyclic 3′,5′-phosphate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; PMSF, phenylmethanesulfonyl fluoride; DTT, dithiothreitol; PC, egg phosphatidylcholine; PE, egg phosphatidylethanolamine; EDTA, ethylenediaminetetraacetic acid; G protein, GTP-binding protein; SDS, sodium dodecyl sulfate; GTPase, guanosinetriphosphatase; PAGE, polyacrylamide gel electrophoresis; TLC, thin-layer chromatography.

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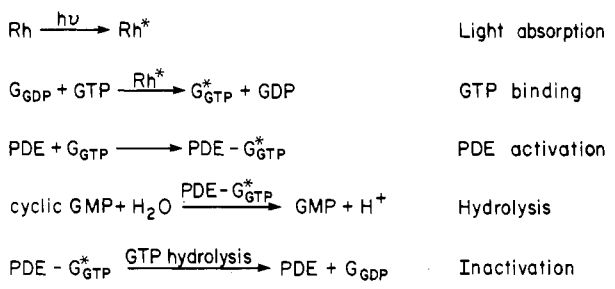

1978) and that the G protein and PDE are associated with the membrane surface under isotonic conditions (Baehr et al., 1979, 1982). It remains to be established where the G protein and PDE reside on the rhodpsin–lipid membrane surface, and whether the reactants find one another by membrane surface diffusion or by diffusion through the aqueous phase, or by a combination of both.

During our studies of this problem, we have observed that an Rh* on one membrane vesicle can activate peripheral enzymes that originate on another membrane vesicle (O'Brien & Tyminski, 1981). This report summarizes our findings. In addition, the reconstitution of the G protein–phosphodiesterase system is described, and the binding of PDE to phospholipid membranes surfaces is demonstrated.

Experimental Procedures

Materials. Frozen bovine retinas were obtained from American Stores Packing. Buffers included tris(hydroxymethyl)aminomethane (Tris) from Sigma and *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (HEPES) from Calbiochem. Nucleotides and phenylmethanesulfonyl fluoride (PMSF) were obtained from Sigma. Dithiothreitol (DTT) was purchased from Calbiochem. Protein content was determined by the Bradford (1976) technique (Bio-Rad Laboratories). The assays for G protein were standardized with purified G protein. Guanosine [γ-^{32}P]triphosphate was obtained from New England Nuclear. Lipids for preparation of phospholipid membrane vesicles were obtained from Lipid Products or Avanti Biochemicals.

Isolation of Rod Outer Segments (ROS). All procedures concerning rhodopsin were carried out at 4 °C under dim red light (Kodak safelight filter 2) or in complete darkness.

Rod outer segments (ROS) were isolated according to the procedure of Yee & Liebman (1978) with several modifications. Frozen bovine retinas were thawed at room temperature for 1 h and suspended in an argon-saturated isotonic medium of 45% sucrose (density 1.20 g/mL). The isotonic buffer (B_I) consisted of 120 mM NaCl, 2 mM $MgSO_4$, 2 mM DTT, 0.05 mM PMSF, and 10 mM Tris, pH 8.0. After the retinas were completely thawed, the rod outer segments were removed from the retinas with mild shaking. The suspensions were overlaid with B_I and centrifuged at 27000*g* for 20 min. The suspended ROS contained in the supernatant were decanted and washed with 1 volume of B_I. The resulting pellets were resuspended with mild shaking in 38% sucrose (density 1.17 g/mL) in B_I, overlaid with B_I, and centrifuged at 27000*g* for 20 min. The ROS were removed from the B_I–sucrose interface with a disposable pipet and washed twice with B_I. Absorbance ratios of 280 nm:500 nm ranged from 2.6 to 3.2 with yields of 15–17 nmol of rhodopsin/retina.

Rhodopsin in Detergent. Purified rhodopsin in tridecyltrimethylammonium bromide was prepared as described by Hong & Hubbell (1973).

Rhodopsin–Phospholipid Membrane Vesicles. Rhodopsin–phospholipid membrane vesicles were prepared as described by O'Brien et al. (1977, 1979). Rh–PC/PE (1:50/50) represents a rhodopsin–egg phosphatidylcholine/egg phosphatidylethanolamine membrane where the desired molar ratio of the components is 1:50/50. The membranes were homogeneous upon sucrose density centrifugation, and the actual lipid content was determined by phosphorus analysis.

Purification of ROS. ROS were purified, when required, by discontinuous sucrose density gradient centrifugation (Papermaster & Dreyer, 1974). Stepwise gradients of sucrose in B_I were prepared in 9.5-mL density increments of 1.15, 1.13, 1.11, and 1.10 g/mL. The gradients were fractionated into 1-mL volumes and assayed spectrophotometrically. After the combined, purified ROS were washed once with B_I, the absorbance ratio of 280 nm:500 nm was typically 2.3–2.5.

Sodium Dodecyl Sulfate (SDS)–Polyacrylamide Gel Electrophoresis. SDS–polyacrylamide gel electrophoresis was performed in a slab gel apparatus (Bio-Rad Model 200) by the discontinuous buffer system of Laemmili (1970) combined with the acrylamide concentrations of Baehr et al. (1979). Gel thickness was 0.75 mm. Acrylamide and *N,N'*-methylenebis(acrylamide) concentrations were 15% and 0.08%, respectively. A 20–25-μg portion of protein was applied to each slot. Protein bands were visualized with Coomassie brilliant blue R-250.

Isolation of Soluble and Peripheral Proteins. Isolated ROS were freed of cytoplasmic and membrane surface associated proteins by repetitive washings with isotonic and hypotonic buffers, respectively. The isotonic buffer (B_I) is described above. The hypotonic buffer contained 10 mM Tris, pH 8.0, 2 mM DTT, and 1 mM EDTA. The ROS were increasingly more difficult to pellet after the first hypotonic wash.

Purification of PDE and G Protein. PDE and G protein were purified by the procedure of Kuhn (1980, 1982). This method takes advantage of the light-induced binding of the G protein to the membrane surface to separate it from the PDE under hypotonic conditions.

Purified ROS were diluted to twice their volume with B_I, pH 7.5. Each sample tube was flushed with argon, capped, and exposed to room light at 20 °C for 1 min. Bleached suspensions were cooled on ice for 1 min and then pelleted. The supernatant was decanted, and the pellets were resuspended in hypotonic buffer of 1 mM DTT, 0.05 mM PMSF, and 5 mM Tris, pH 7.5. The suspension was incubated at 20 °C and then cooled on ice. In the dark, the suspensions were diluted with an equal volume of hypertonic buffer to return to isotonic conditions. After incubation at 20 °C, the suspension was pelleted, and the supernatant was decanted. Under room light, the pellets were resuspended in hypotonic buffer to remove the PDE, incubated at 20 °C, cooled to 4 °C, and centrifuged. The hypotonic wash was repeated 3 times. The PDE washes were combined and concentrated, from which 1.8 mg of PDE was isolated per 1000 nmol of rhodopsin.

After completion of the PDE washes, the pellet was resuspended in a buffer of 0.1 mM GTP, 0.1 mM EDTA, 1 mM DTT, and 10 mM Tris, with a disposable pipet. Incubation at 20 °C for 4 min facilitated removal of G protein from the membranes. A second washing of the pellet produced relatively small amounts of protein released into the supernatant. Yields of 4.3–4.8 mg of G protein per 1000 nmol of rhodopsin were obtained.

Purification of PDE. The concentrated PDE wash was purified on a Sephacryl S-200 column that was equilibrated with a buffer of 10 mM HEPES, pH 7.8, 100 mM NaCl, 2 mM DTT, and 0.1 mM EDTA. Appropriate column fractions were combined and concentrated to produce a solution of PDE

in 50% glycerol, 10 mM HEPES, pH 7.8, 120 mM NaCl, 2 mM DTT, 2 mM $MgSO_4$, and 0.1 mM EDTA. The PDE was stored at −20 °C.

Purification of G Protein. Excess GTP was removed by passing the concentrated GTP wash through a Sephadex G-50 column equilibrated with the same buffer used in the PDE chromatography. Appropriate fractions were combined and concentrated by dialysis against 50% glycerol, 10 mM HEPES, pH 7.8, 120 mM NaCl, 2 mM DTT, 2 mM $MgSO_4$, and 0.1 mM EDTA. The purified G protein was stored at −20 °C.

GTPase Assays. GTPase activity was assayed according to the procedure of Neufield & Levy (1969) with several modifications. The inorganic phosphate was rendered soluble in isobutyl alcohol by complexation with acidic ammonium molybdate (~8 g/500 mL + 35 mL of concentrated H_2SO_4) followed by reduction with $FeSO_4$ (5% in 1% H_2SO_4) (Sumner, 1944).

PDE Assay. The assay is based on the technique of Cheung (1969), which was first applied to ROS PDE by Yee & Liebman (1978).

All operations during the assay were shielded from the room safelights, and an FJW Industries image converter with infrared illuminator was used to align the sample properly in the thermostated bath. Membranes stored in the dark under argon at 4 °C were diluted in a moderate ionic strength buffer (usually B_I) with 10 mM Tris or HEPES, pH 7.8–8.0. The sample was allowed to thermally equilibrate at 25 °C, unless otherwise specified, in the thermostated bath. Aliquots of GTP or GTP–ATP and cGMP were added to give a sample volume of 2.0 mL; in later experiments, a volume of 0.5 mL was used.

The sample pH was monitored with a combination pH electrode (Corning 476050, A. H. Thomas 4094-L15, or Microelectrodes MI-410) and amplified with a Keithley electrometer (Model 610B). After addition of saturating levels of the substrate (~1 mM cGMP), the dark activity was determined, and the sample was exposed to a 1-ms flash (Vivitar Model 283 with a Corning GS5-60 filter) 15 cm from the sample. Rhodopsin bleaching was calibrated by spectrophotometry of suspensions before and after flashes. Flash output was attenuated by calibrated neutral-density filters (Optical Industries, Inc.).

Mixing Protocol. In these experiments, the PDE activity was assayed as described above. A sample of enzyme-depleted ROS or rhodopsin–lipid membrane vesicles was combined with isotonic buffer, GTP, and cGMP. After temperature equilibration, the sample was exposed to a calibrated flash to bleach some of the rhodopsin. After a selected time interval, the sample suspension was treated in the dark with an unexposed sample of ROS membranes, which contained the peripheral enzymes. The final sample volume was 2.0 mL. After the new PDE activity was determined, the sample was usually exposed to a second light flash to fully activate all the enzymes in the sample.

Results

ROS PDE Activity. The V_m was 3 μM H^+ s^{-1} (μM rhodopsin)$^{-1}$ for PDE-mediated hydrolysis of cGMP of light-exposed fragmented bovine ROS (5 μM rhodopsin) derived from frozen retinas. The ratio of light to dark activity ranged from 20 to 50 in various samples. We found, as have others (Liebman & Pugh, 1979), that the V_m for ROS membranes from fresh bovine retinas is usually 3–4 times greater than for ROS from frozen retinas. The difference is probably due to denaturation and loss of both G protein and PDE.

The activity observed is dependent on the presence of light-exposed rhodopsin (Rh*), G protein, and PDE in the sample. The activity saturates at high bleach levels of rhodopsin (>10^{-3} fraction bleached). The preferred substrate is cGMP, which is estimated to have a cytoplasmic concentration of 30–60 μM (Woodruff et al., 1977). We found an enzyme Michaelis constant of 85 μM cGMP, which compares to the reported value of 70 ± 20 μM cGMP (Yee & Liebman, 1978). The enzyme activity requires Mg^{2+} or Mn^{2+} and a nucleoside triphosphate cofactor, GTP. Nonhydrolyzable analogues can be substituted for GTP.

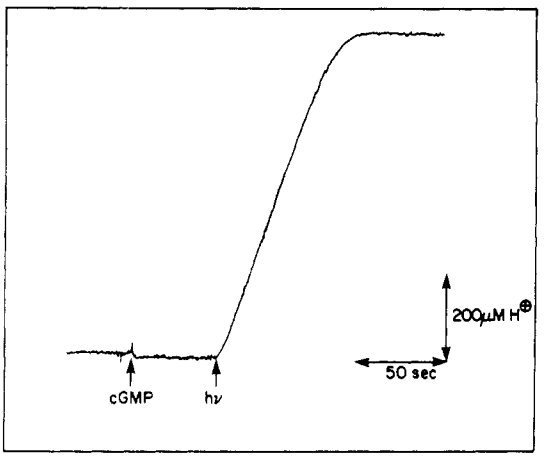

FIGURE 1: Time course of pH for a reconstituted rhodopsin membrane of Rh–PC (1:100) (2.6 μM Rh), PDE (0.05 μM), and G protein (0.5 μM) in isotonic buffer (10 mM HEPES, pH 7.9, and 250 μM GTP at 25 °C). An aliquot of substrate was added in the dark to give 1 mM cGMP. The sample was then exposed to a 1-ms light flash, which bleached 0.04 of the Rh. The sample pH changed until the cGMP was exhausted.

The high level of light-induced PDE activity in ROS membranes is dependent on buffers of moderate ionic strength, e.g., B_I. Hypotonic washes of the ROS membranes reduce the activity to <4% of this normal level. If the hypotonic supernatant is restored to a well-washed ROS membrane and the buffer is brought to moderate ionic strength, the light-induced enzyme activity is again observed. The following observations suggest that the enzyme activity is membrane associated.

After sedimentation of light-exposed ROS membranes in B_I and separation of the sample into supernatant and pellet, >96% of the activity is with the pellet. Any PDE removed from the membrane by sedimentation in buffer of moderate ionic strength does not yield significant activity. Furthermore, if the ROS membranes in B_I are exposed to light, pelleted, and then redispersed in hypotonic buffer with 1 mM GTP and 2 mM Mg^{2+}, which removes at least 90% of the PDE and G protein from the membrane, there is little enzyme activity in the supernatant. Thus, removal of activated PDE from the membrane surface significantly reduces its activity.

Reconstituted PDE Activity. Light-induced phosphodiesterase activity can also be restored to a well-washed ROS membrane or reconstituted with an Rh–lipid membrane by the addition of purified PDE and purified G protein. Figure 1 shows the time course of pH change for a sample reconstituted from Rh–PC (1:100) (2.6 μM Rh) with added 0.05 μM PDE and 0.5 μM G protein in isotonic 10 mM Hepes buffer, pH 7.9. This sample has a V_m of 3.8 μM H^+ s^{-1} (μM rhodopsin)$^{-1}$ in the light and shows exceptionally low dark PDE activity. Usually the ratio of light to dark activity in these reconstituted systems is >20 if care is taken not to expose the rhodopsin membranes to red light during the reconstitution steps prior to the assay and PDE proteolysis is minimized by inclusion of an inhibitor, e.g., PMSF, in the ROS isolation.

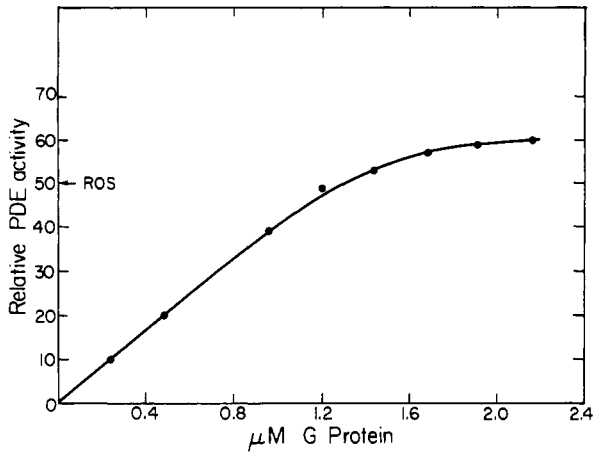

FIGURE 2: Effect of G-protein concentration on light-induced PDE activity. The PDE activity relative to a ROS sample (5 μM Rh) (indicated by the arrow) is shown for samples of Rh–PC/PE (1:50/50) membranes (5 μM Rh) with purified PDE (0.044 μM) at various concentrations of purified G protein.

The saturated or maximum light-induced PDE activity in a reconstituted system is dependent on the concentration of both G protein and PDE and the membrane surface area. The observed maximum light-induced activity of Rh–PC (1:100) membranes (2.6 μM rhodopsin) at a constant PDE concentration increased with the amount of added purified G protein. Saturation of the light-induced activity for this membrane occurred at about 0.5 μM G protein, which is estimated to be ~10 G proteins for each PDE.

A similar experiment is shown in Figure 2 for sonicated unilamellar Rh–PC/PE (1:50/50) membranes (5 μM rhodopsin) with an estimated 0.044 μM PDE. The maximum light-induced PDE activity increases with added G protein and gradually aproaches saturation at about 1.5 μM G protein (Rh:G ~ 3.3). This limit may be imposed by the G-protein binding saturation of the rhodopsin membrane surface, which has been reported for ROS disks to be 4 Rh/G (Baehr et al., 1982).

Clearly, multiple G proteins per PDE are necessary to fully activate the PDE in these reconstituted samples. PDE activation is currently considered to be due to G_{GTP} binding with PDE, with the resultant change in interaction of the PDE inhibitor (γ subunit) (Hurley, 1982; Hurley & Stryer, 1982). Inefficiencies in this process may be a consequence of weak binding of G_{GTP} to PDE, as suggested recently by Liebman & Pugh (1982). Weak binding can be interpreted in terms of the rates of formation of the PDE–G_{GTP} complex and the competitive rate of deactivation by hydrolysis of the bound GTP. There could also be an unspecified competitive loss process for G_{GTP}.

At a given G-protein concentration, the maximum light-induced PDE activity increases with added PDE until a plateau is approached. Since the PDE is readily light activated only when it is membrane associated, the activity saturates at the PDE concentration where the available PDE binding sites are occupied. Figure 3 shows the PDE activity of sonicated Rh–PC/PE (1:250/250) (lipid:rhodopsin ratio was 460 as determined by phosphorus analysis) membranes as a function of added PDE for two different G-protein concentrations. The relative PDE activity saturates at an estimated 0.11 or 0.12 μM PDE for G-protein concentrations of 0.40 and 0.67 μM, respectively. This corresponds to an apparent stoichiometry of one PDE molecule for every 4.9 ± 0.2 rhodopsins and 2200 ± 100 lipid molecules. Because these are sonicated vesicles with an average diameter of 340 Å (Tyminski et al., 1982),

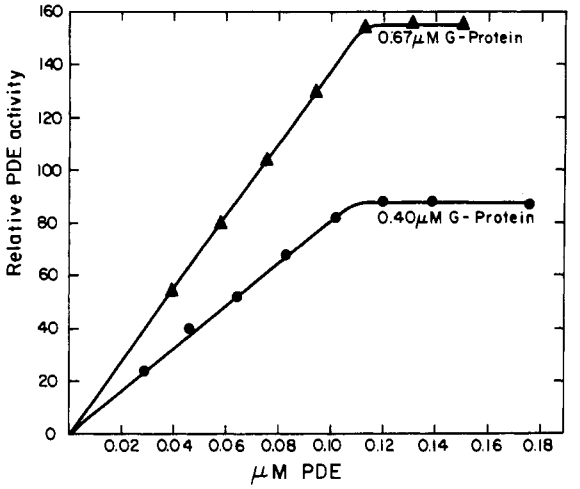

FIGURE 3: Effect of PDE concentration on light-induced PDE activity. The relative PDE activity is the same as in Figure 2 for samples of Rh–PC/PE (1:250/250) (lipid:rhodopsin = 460) (0.55 μM Rh) at various concentrations of purified PDE, and at two G-protein levels: 0.40 μM (●); 0.67 μM (▲).

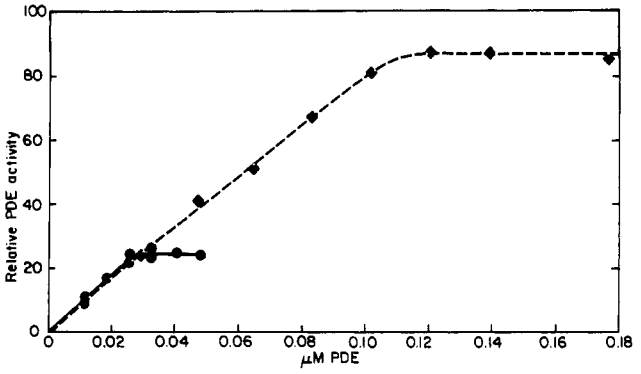

FIGURE 4: Comparison of light-induced PDE activity for Rh–PC/PE (1:250/250) (lipid:rhodopsin = 460) (0.55 μM Rh) and Rh–PC/PE (1:50/50) (lipid:rhodopsin = 120) (0.53 μM Rh) membranes at constant G-protein concentration (0.40 μM) and variable PDE concentrations. The relative PDE activity is the same as in Figure 2. The activity is shown for Rh–PC/PE (1:250/250) (♦) and Rh–PC/PE (1:50/50) (●).

0.66 of the lipids (about 1500 lipids/PDE) are on the outside half of the vesicle bilayer, the region available for interaction with the PDE. Again, note that the relative PDE activity found in these experiments is proportional to the G-protein concentration.

The effect of PDE concentration on the light-induced PDE activity was also determined for sonicated Rh–PC/PE (1:50/50) (lipid:rhodopsin ratio was 120) membranes. The activity saturated at a significantly lower level than observed for Rh–PC/PE (1:250/250) membranes (Figure 4). The PDE activity at saturation is 3.8 times as great in the reconstitution of Rh–PC/PE (1:250/250) as with Rh–PC/PE (1:50/50). The rhodopsin ratio is 1, and the lipid ratio is 4. The maximum light-induced PDE activity parallels the lipid rather than the rhodopsin content of the membrane.

The protein content in these experiments was determined by the Bradford (1976) assay, which was standardized with G protein. The same standardization was assumed for PDE; thus, the values for PDE may be subject to a systematic error as large as a factor of 2. The estimated amount of PDE at surface saturation of these membranes may be affected by the presence of glycerol in the reconstitution and binding experiments. Both G protein and PDE were stored in 50% glycerol isotonic buffer, and after addition and dilution with the

Table I: Mole Ratios of PDE to Rhodopsin and Lipid at Saturation of the Membrane Surface

membrane	estimated mole ratios	
	PDE:Rh	PDE:lipid[a]
Rh–PC (1:100)	0.05 ± 0.006	$(4.2 \pm 0.4) \times 10^{-4}$
Rh–PC (1:500)	0.26 ± 0.03	$(4.8 \pm 0.6) \times 10^{-4}$
Rh–PC/PE (1:250/250)	0.23 ± 0.04	$(5.0 \pm 0.7) \times 10^{-4}$

[a] Ratios calculated from the lipid:rhodopsin ratio determined by phosphorus analysis for each membrane were the following: Rh–PC (1:100), 119; Rh–PC (1:500), 540; Rh–PC/PE (1:250/250), 460.

membranes and nucleotides, the final isotonic buffer was usually 10% glycerol. Furthermore, the estimated amount of PDE required to saturate a membrane surface will deviate from the values above if some of the PDE is denatured, because denatured PDE detectable by the protein assay will not contribute to the light-induced activity. The major conclusion of these experiments, that the amount of PDE bound to the membrane surface is dependent on the lipid content rather than the rhodopsin content, is not affected by these reservations.

PDE Binding to Membranes. Further support for this conclusion comes from assays of the binding of PDE to different Rh–lipid membranes. Purified PDE was allowed to equilibrate with the membrane, and the membranes were pelleted by a 30-s spin in an airfuge. The protein contents of the supernatant and the pellet were determined, as well as the rhodopsin content of the pellet. The protein assays were supplemented by SDS gel electrophoresis of the pelleted membranes, which showed staining patterns due to PDE, rhodopsin monomer, and some dimer. Table I shows the mole ratios of PDE to rhodopsin and to lipid content at saturation for three membranes. The estimated mole ratio of Rh to PDE varied from 20 to about 4 as the lipid content was increased from 100 lipids/Rh to 500 lipids/Rh, whereas the calculated mole ratio of lipid to PDE remained resonably constant at about 2000. Both techniques, one based on protein analysis and the other on enzyme activity, yield the same conclusion that the amount of PDE associated with the membrane is a function of the lipid content rather than the rhodopsin content.

PDE Transfer between Membranes. The residence time of the PDE at a particular site remains to be established. If the peripheral enzymes, are tightly associated with the ROS disk membrane surface, then an Rh* will be able to activate only enzymes in its immediate domain. The size of the domain will be a function of the lifetime of Rh* and the lateral motion of the transmembrane protein Rh* and the surface-associated enzymes. The maximum domain will be limited by the size of the membrane particles or vesicles, because strong association of the enzymes with the membrane surface will preclude intervesicle activation of enzymes of Rh*. However, the domain can exceed the size of the membranes or vesicles if the enzymes can move from surface to surface. The following experiment demonstrates that intervesicle activation does occur in aqueous suspensions of membrane vesicles (Figure 5).

A sample of Rh–PC (1:100) (10 nmol of Rh) membrane vesicles in moderate ionic strength buffer with GTP and cGMP at 25 °C was flashed with sufficient light to bleach 4% of the rhodopsin, and the rate of pH change was not affected. This lack of enzyme activity is expected, because the Rh–PC membranes are free of G protein and PDE. One to two minutes after the light flash, the stirred vesicle suspension was treated in the dark with an unexposed sample of ROS membranes similar in activity to that described above. The ROS membranes were previously washed and vortexed with isotonic buffer to remove loosely associated proteins, yet have surface-associated PDE and G-protein enzymes. The pH record

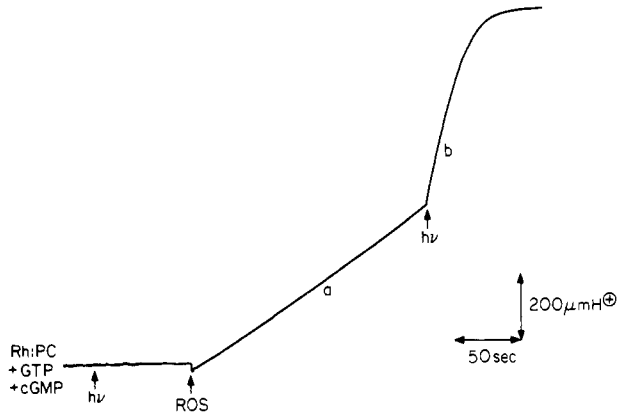

FIGURE 5: pH profile for a mixing experiment. Initially a sample of Rh–PC (1:100) (10 nmol of Rh), GTP (0.5 μM), and cGMP (2 μM) in 1.9 mL of isotonic buffer and 10 mM Tris, pH 7.9, at 25 °C was mixed and then exposed to a light flash (0.04 bleach). A 0.1-mL aliquot of dark bovine ROS (100 μM Rh) in isotonic buffer was added as indicated by the arrow. After a period of moderate pH change (line segment a), the sample was exposed to a second light flash to fully activate the enzyme system.

(Figure 5) shows an artifact due to mixing (1–2 s) followed immediately by a change in the pH. This pH change has the characteristics identified with cGMP hydrolysis: dependence on GTP, cGMP, Mn^{2+} or Mg^{2+}, the peripheral enzymes, and bleached rhodopsin. The production of protons is due to light activation of PDE. This raises the question of how the bleached rhodopsin (Rh*) on the Rh–PC vesicles activated G protein and PDE that originated on another membrane (ROS) surface.

The pH profile shown in Figure 5 consists of three phases: the initial stable pH before mixing; the moderate increase in pH (line segment a), which is $(dH^+/dt)_a$; the the steeper increase in pH due to complete enzyme activation (line segment b), which is $(dH^+/dt)_b$. If we represent the dark activity of the ROS membranes as $(dH^+/dt)_d$ which is usually 0.1 μM H^+ s^{-1} (μM rhodopsin)$^{-1}$ or $^1/_{30}$th of $(dH^+/dt)_b$, then the expression

$$\frac{(dH^+/dt)_a - (dH^+/dt)_d}{(dH^+/dt)_b}$$

gives the fraction of PDE activity initiated by the Rh* on the Rh–lipid membrane vesicles compared to the total PDE activity in the sample. The activity initiated by Rh* is observed in Figure 5 as soon as the 1–2-s mixing artifact is complete and remains reasonably constant until the mixed sample is exposed to a light flash. Thus, the processes that result in this activation must be substantially complete in less than 1–2 s.

If we repeat the experiment in Figure 5, with sonicated Rh–PC (1:500) vesicles rather than Rh–PC (1:100) membranes, we can utilize the buoyancy of the sonicated vesicles to separate them from the ROS membranes. ROS membranes can be completely pelleted at 25000*g* for 1 min, whereas sonicated Rh–PC (1:500) membrane vesicles do not pellet under these conditions. When sonicated Rh–PC (1:500) (2 nmol of Rh) vesicles were exposed to a 4% bleaching flash and then mixed with unexposed ROS (10 nmol of Rh) membranes, the pH changed in the same manner as shown in Figure 5. The reaction mixture was centrifuged in the dark, and the ROS and Rh–PC (1:500) vesicles were separated. Each was analyzed for protein content by SDS–PAGE. Both fractions displayed the polypeptide bands associated with PDE and G protein, as well as rhodopsin. Therefore, some of both enzymes, PDE and G protein, necessary for light-induced PDE

Table II: Effect of Acceptor Membrane Surface on the Fraction of Enzyme Activity Transferred

Rh:PC ratio	acceptor membrane Rh (nmol)	acceptor membrane PC (μmol)	ROS Rh (nmol)	ROS lipid (μmol)	fraction of activity transferred
1:100	10	1	10	0.7	0.16
1:500	10	5	10	0.7	0.36
1:500	5	2.5	10	0.7	0.35
1:500	2	1	10	0.7	0.21

activity, transferred to the rhodopsin–lipid vesicles.

When the light-exposed sonicated Rh–PC (1:500) vesicles and dark ROS membranes are mixed and then separated in the dark and each analyzed separately for PDE activity, the activity was found with the Rh–PC (1:500) vesicles (supernatant) and not with the ROS membranes (pellet). These observations indicate that $(dH^+/dt)_a$ is due to G protein and PDE that have transferred to the light-exposed Rh–lipid vesicles and are activated on those vesicles by Rh*.

Generally the mixing experiments were conducted in the presence of 250 μM GTP; however, the fraction of activity transferred was independent of the GTP concentration from 1 to 500 μM GTP. Indeed, if a mixed sample of sonicated light-exposed Rh–PC (1:500) vesicles and dark ROS membranes, that do not contain GTP, is separated by centrifugation and then treated with 250 μM GTP, the supernatant (Rh–PC vesicles) displays PDE activity. Therefore, both PDE and G_{GDP} transferred to the Rh–PC (1:500) vesicles. The GTP is crucial to the amplified interaction of G protein with Rh* but is not required for the transfer of G protein to the Rh*–lipid vesicles.

A potentially important variable in the mixing experiment is the time delay between exposure of the Rh–lipid membranes and addition of the dark ROS membranes. The fraction of enzyme activity transferred was insensitive to a time delay of 15–100 s. The rest of the experiments described below had delay times of 50–70 s. Times <15 s could not be conveniently achieved by the present protocol. At extended delay times >300 s, a significant decrease in the fraction of enzyme activity was found. This is presumably due to relaxation of the light-excited rhodopsin intermediate. Other groups have demonstrated this intermediate to be metarhodopsin II (meta II) (Parkes et al., 1979). Schnetkamp et al. (1979) report a $t_{1/2}$ at 25 °C of 250 s for bovine meta II relaxation in broken ROS membranes.

The fraction of enzyme activity transferred is sensitive to the relative surface area of the donor membrane (dark ROS) and acceptor membrane (Rh*–lipid) and increases with increased amounts of acceptor membrane. The data in Table II show that a change from 1:100 Rh:PC to 1:500 Rh:PC at constant rhodopsin and bleached rhodopsin concentrations increases the fraction of enzyme activity transferred from 0.16 to 0.36. Also, an increase in the amount of 1:500 Rh:PC from 1 to 5 μmol of PC, compared to a constant ~0.7 μmol of ROS lipid, results in an increase in the fraction of enzyme activity transferred from 0.21 to 0.36.

Since the fraction of enzyme activity transferred is dependent on the lipid content of the light-exposed acceptor membranes, the addition of dark Rh–PC or PC should provide competitive sites for some of the enzyme. This portion of the enzyme will not be available for activation by the Rh* in the light-exposed Rh–PC. This prediction is confirmed by the observation that the fraction of enzyme activity transferred from dark ROS (5 μM Rh) to light-exposed Rh–PC (1:100) (5 μM Rh) membranes is decreased from 0.17 to 0.09 if an additional 10 nmol of dark Rh–PC (1:100) membranes is included in the 2.0-mL sample mixture. An equivalent effect is found if PC membranes are used in place of dark Rh–PC (1:100) membranes. These observations suggest that the maximum light-induced PDE activity in ROS membranes could be decreased by the addition of lipid vesicles to the ROS membrane suspension before light exposure. Indeed, that is the case; the normal V_m of about 3 μM H^+ s^{-1} (μM rhodopsin)$^{-1}$ is decreased to about half by the addition of a comparable quantity of egg PC vesicles.

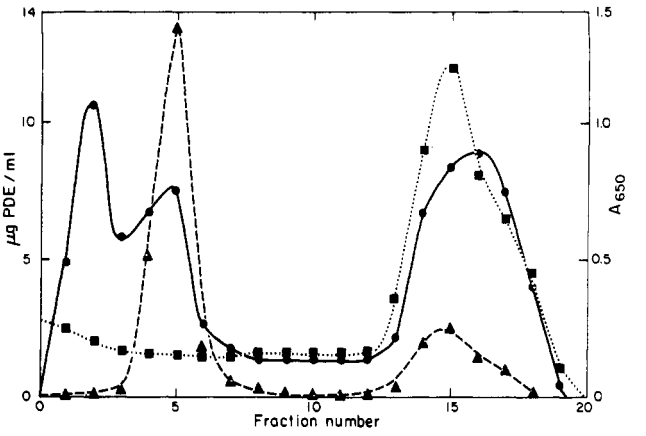

FIGURE 6: Profile of PDE distribution in a fractionated sucrose gradient. Rh–PC/PE (1:250/250) membranes with bound PDE were mixed with PC bilayers and vesicles, layered onto a 1–15% sucrose gradient, and spun at 100000*g* to equilibrium. The fractions collected are in order from low to high sucrose content. The fractions were assayed for PDE content by trypsin activation (●) and for membrane turbidity by light scattering at 650 nm (▲). The PDE content of fractions from a control of Rh–PC/PE (1:250/250) membranes with bound PDE and without PC bilayers is also shown (■).

The ease of PDE movement from a rhodopsin membrane surface to another lipid surface is shown by the following experiment. Rh–PC/PE (1:250/250) membranes were combined with PDE under isotonic conditions. The mixture was allowed to equilibrate and then centrifuged to pellet the membranes with bound PDE, and the supernatant and unbound PDE were removed. The membranes with bound PDE were dispersed with a comparable amount of PC bilayers. The mixture was immediately layered onto a 1–15% sucrose density gradient and centrifuged at 100000*g* to separate the membrane and PC bilayers. The data in Figure 6 result from analysis of the gradient fractions for PDE content (trypsin activation), for membrane or bilayer content (turbidity), and for protein content of the rhodopsin-containing fractions. Also shown is the PDE content for a control gradient of Rh–PC/PE (1:250/250) membranes with bound PDE. In both the control and the mixed-membrane experiments, PDE was found in the rhodopsin-containing fractions 14–18. However, in the presence of PC bilayers, a large proportion of the PDE was also found in the less dense fractions 1–6. The distribution profile shows a double maximum for PDE at fractions 2 and 5. Fraction 5 corresponds to the peak of turbidity due to the PC bilayers. Analysis of fractions 1–6 by TLC showed the presence of PC in each fraction even though the turbidity was greatest for fraction 5. Fractions 1–3 were composed of small PC vesicles, whereas the extended bilayers were found in fractions 4–6. The TLC data for fractions 1–6 also showed that <5% of the lipid was PE; therefore, little if any of the lipid in these fractions originated from the Rh–PC/PE membranes. The PDE in these fractions resulted from peripheral protein transfer from the Rh–PC/PE membranes to the PC bilayers and vesicles, which did not appear to be accompanied

by lipid transfer. The transfer occurred under isotonic conditions during the time of mixing and centrifugation start up. About 40% of the PDE was found in the lipid bilayer containing fractions, and 45% of the PDE remained with the Rh–PC/PE membranes. The balance was in the intermediate nonmembrane fractions.

Discussion

The experimental observations reported above demonstrate (1) that light-induced PDE activity may be reconstituted from rhodopsin-containing membranes, purified G protein, and purified PDE, (2) that the association of ROS phosphodiesterase with membranes is dependent on the lipid content rather than the rhodopsin content, and (3) that both peripheral enzymes are readily transferred between membrane surfaces under isotonic conditions.

Phospholipid association of the ROS PDE is central to each of the following observations reported here: the maximum PDE activity in reconstituted systems is dependent on the lipid surface area (Figure 4); the bound PDE is dependent on the membrane lipid content (Table I); the addition of PC vesicles to Rh–PC/PE membranes with bound PDE results in transfer of some of the PDE to the PC vesicles (Figure 6); the fraction of enzymatic activity transferred from dark ROS to light-exposed Rh–PC vesicles is dependent on the relative surface area of the membranes (Table II); and the V_m of ROS membranes is diminished by the addition of egg PC vesicles.

The transfer of the peripheral enzymes, G protein and PDE, from the dark ROS membranes to the light-exposed Rh–lipid membranes must occur in less than 1–2 s (Figure 5). The speed of the transfer makes it unlikely that this phenomenon is related to processes such as lipid diffusion or lipid transfer between vesicles or intrinsic membrane protein transfer between vesicles. McLean & Phillips (1981) found a half-time of hours for the transfer of cholesterol or phosphatidylcholine between lipid vesicles at 37 °C. Nichols & Pagano (1981) measured the transfer of a fluorescent lipid analogue between vesicles and found the half-time to be tens of minutes at 20 °C. Cook et al. (1980) observed the transfer of erythrocyte acetylcholinesterase to dimyristoyl-PC vesicles with a half-time of about 1 h at 28 °C. Their experiments used membrane lipid concentrations of about 10^{-2} M, whereas the lipid concentrations in our study were usually $<10^{-3}$ M. The transfer of peripheral enzymes is at least 10^3 times faster than the phenomena reported previously and approaches a physiologically interesting time frame. Resolution of the kinetics of peripheral enzyme transfer will require faster response techniques than those used here. In this regard, a light-scattering phenomenon of $\sim 10^{-1}$ s was associated with the light-induced binding of G protein to ROS membranes (Kuhn et al., 1981).

The model of the ROS disk membranes and peripheral enzymes that emerges from these results is one of a transmembrane protein rhodopsin in a lipid bilayer with the PDE molecules associated with the lipid surface. The dynamic nature of the disk membrane is well established from the rapid rotational (Cone, 1972) and lateral (Poo & Cone, 1973; Liebman & Entine, 1974) diffusion of rhodopsin in these membranes. We now have evidence that the G protein and PDE are also in rapid motion on the disk membrane surface. The location of the G protein on the ROS disk membrane surface in the dark is still uncertain. In the light in the absence of GTP it binds to rhodopsin (Kuhn, 1980), and in the presence of GTP the G protein or the α subunit (Fung, 1983) prefers to associate with PDE in an undefined manner.

In the mixing experiments, the observed transfer of G protein may occur in association with some of the PDE as it transfers and binds to the newly available lipid surface (O'Brien, 1982). Alternatively, the G protein may transfer directly to light-exposed or dark rhodopsins on the Rh–lipid membranes. If G_{GDP} transfer between membranes is light dependent, then enzyme hopping of G to Rh* must be considered as an alternative to lateral diffusion of Rh* (Liebman & Pugh, 1979) as an activation mechanism. The nature of the G-protein transfer and its possible physiological significance must await determination of the binding constants for G_{GDP} and G_{GTP} with the membrane surface, and whether the observed transfer of G_{GDP} is light dependent.

The transfer of PDE and G protein reported here suggests two possible explanations for the mixing experiments described by Bitensky and co-workers [see Pober & Bitensky (1979)]. The sensitivity of PDE activity to the degree of light exposure of frog and bovine ROS membranes was ascertained either by uniform membrane illumination to give different bleach levels or by an admixture procedure where a proportion of the membranes was given a full bleach followed by mixing with dark ROS membranes (Keirns et al., 1975). In bovine ROS, half-maximal activation of the PDE was observed with a mixture of 10% bleached membranes and 90% unilluminated membranes. Keirns et al. (1975) assayed the activity, starting 5 min after mixing. Our mixing experiments suggest that the activation was probably complete in a second or so, and indeed, we repeated the admixture experiment and observed the change in activity within a few seconds of mixing the light and dark ROS membranes. In these experiments, both illuminated and unilluminated ROS membranes contained peripheral enzymes; thus, the activation does not necessarily involve movement of PDE from one membrane to another, yet sufficient G_{GTP} must be formed by 10% of the rhodopsin membranes to activate 50% of the PDE. The relative quantities of Rh, G protein, and PDE in the experiments of Keirns et al. (1975) are unknown; thus, it is not possible to distinguish whether only G_{GTP} or both G_{GDP} and G_{GTP} diffused between the bleached and the dark ROS membranes. In the first case, the bleached rhodopsin interacts only with the G_{GDP} present on the light-exposed membranes. The G_{GTP} formed then diffuses between membranes and activates the PDE on both light and dark ROS. This requires at least 5 times as much G protein as PDE in the bovine ROS used, a reasonable expectation. In the second case, the bleached rhodopsin interacts with G_{GDP} from both dark- and light-exposed membranes, and this can occur only if G_{GDP} can diffuse between membranes. Both possibilities require G_{GTP} be able to diffuse between membranes to activate 50% of the PDE. Keirns et al. (1975) also found that for frog ROS, half-maximal activation of the PDE resulted from an admixture of 1% bleached membranes and 99% dark membranes. If only the G_{GDP} on the bleached membrane is converted to G_{GTP} (case 1), then in the frog ROS used there must have been at least 50 times as much G protein as PDE. Whether the ratio of G protein to PDE was greater on the frog than the bovine ROS membranes is unknown, and thus we cannot exclude the possibility of G_{GDP} diffusion from a dark membrane to a light-exposed membrane, where it is converted to G_{GTP} and is then able to activate PDE.

Wheeler et al. (1977) used a similar mixing protocol in studying the GTPase activity of frog ROS membranes. They found that an admixture of 1% fully bleached washed ROS membranes with 99% unbleached unwashed ROS membranes gave full GTPase activity. The membranes to be bleached were washed to remove GTPase activity before the mixing experiment. This suggests that the G_{GDP} activated by bleached rhodopsin originated on the unexposed ROS membranes and

transferred to the illuminated ROS membranes. It is uncertain whether this transfer was promoted by the presence of the bleached rhodopsin or occurred simply because of the additional membrane and dark rhodopsin content in the mixture followed by activation by Rh*. In our experiments and in those of Wheeler et al. (1977), both possibilities must be considered, and further experiments are required to establish their physiological relevance.

Registry No. PDE, 9068-52-4.

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