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Binding and activation of rod outer segment phoshodiesterase and guanosine triphosphate binding protein by disc membranes: influence of reassociation method and divalent cations

James L. Miller [a], Burton J. Litman [a] and Edward A. Dratz [b]

[a] *Department of Biochemistry, University of Virginia, School of Medicine, Charlottesville, VA 22908 and* [b] *Department of Chemistry, Montana State University Bozeman, MT 59717 (U.S.A.)*

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Attempts to optimize the recovery of light-stimulated phosphodiesterase activity following reassociation of the hypotonically extractable proteins derived from retinal rod segments with hypotonically stripped disc membranes lead to the following observations: (1) the best reassociations were obtained by mixing proteins and stripped disc membranes under hypotonic conditions and slowly increasing the salt concentration; (2) the binding of G-protein and phosphodiesterase to stripped disc membrane occurs in less than 5 minutes and (3) the recovery of light-stimulated phoshodiesterase activation in response to subsaturating stimulus levels requires 2–3 h to plateau. Stripped disc membranes and proteins were reassociated in 'isotonic' buffers containing KCl/NaCl, KCl/NaCl plus Mg^{2+}, or KCl/NaCl plus Ca^{2+}. Large fractional rhodopsin bleaches produced nearly identical light-stimulated phosphodiesterase activities in each of these samples and in the control rod outer segment membranes. Rod outer segment membranes and reassociated stripped disc membrane samples containing divalent cations showed similar phosphodiesterase activities in response to low fractional rhodopsin bleaches (e.g. $\leq 0.1\%$), however, samples devoid of divalent cations during reassociation required rhodopsin bleaches up to 10-fold larger to elicit comparable phosphodiesterase activities. These results suggest that not all phosphodiesterase and/or G-protein molecules bound to the disc membrane surface are equivalent with regard to their efficiency of activation by bleached rhodopsin and that divalent cations can modulate the distribution of G-protein and/or phosphodiesterase between these populations.

Introduction

The vertebrate rod outer segment contains a number of enzymes that are activated in the presence of photobleached rhodopsin (rho*). Rho* binds, activates, and releases a GTP-binding protein (G-protein) [1–4] such that a single rho* serially activates numerous G-protein molecules. An activated G-protein is capable of activating a cGMP phosphodiesterase [5,6] apparently by inducing the release of an inhibitory subunit from the cGMP phosphodiesterase [7,8]. The activation of the cGMP phosphodiesterase following illumination has been implicated in both a rapid, steady-state flux of cGMP in whole retinas [9] and a reduction of cGMP levels in the rod outer

Abbreviations: G-protein, GTP-binding protein, transducin; cGMP, cyclic guanosine 3′,5′-monophosphate; GTP, guanosine triphosphate; EDTA, ethylenediaminetetraacetic acid; DTPA, diethylenediaminepentaacetic acid; CTAB, hexadecyl(cetyl)trimethylammonium bromide.

Correspondence: B.J. Litman, Department of Biochemistry, University of Virginia, School of Medicine, Charlottesville, VA 22908, U.S.A.

segment [10]. It now appears that a decrease in cGMP concentration closes the light-sensitive sodium channels in the retinal rod [11–14] and leads to the hyperpolarization of the plasma membrane that excites the cell.

The cGMP phosphodiesterase and G-protein are associated with the disc membrane in moderate ionic strength solutions, in vitro, but dissociate from the disc membrane surface at extremes of ionic strength [1,2]. The reversible dissociation of these peripheral proteins from disc membranes has been exploited by several laboratories to study their activation by rhodopsin in both discs and reconstituted vesicles [15–21]. We have previously reported that the order and method used to recombine the G-protein and cGMP phosphodiesterase with stripped disc membranes influences the resultant recovery of the light-stimulated cGMP phosphodiesterase activity [20]. Additional parameters also influence the interaction of peripheral enzymes with the disc membrane surface and the subsequent recovery of light activatable enzymatic activities. These include divalent cations, the concentration of proteins and membranes used during the reassociation, and the state of membrane aggregation during reassociation. In order to better understand the interaction of cGMP phosphodiesterase and G-protein with the disc membrane surface and their activation by rho*, we have studied the binding and recovery of light-stimulated cGMP phosphodiesterase and G-protein (vide infra) activities following reassociation with hypotonically stripped disc membranes.

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Methods

Isolation of rod outer segments

Frozen bovine retinas were obtained from James and Wanda Lawson Inc, (Lincoln, NE). Fresh bovine retinas were obtained from a local slaughterhouse. The eyes were dissected into isotonic buffer which contained 50% sucrose and 100 Kallikrein inhibitor units (KIU) per ml of the protease inhibitor, Trasylol (Mobay Pharmaceuticals). Rod outer segment preparations were performed in dim red light (Kodak Wratten No. 2) at 4 °C. 'Isotonic buffer' contained 60 mM KCl, 30 mM NaCl, 10 mM Tris (pH 8.0), 2 mM $MgCl_2$, 1 mM dithiothreitol, 50 μM DTPA, and 100 Kallikrein inhibitor units per ml 'Trasylol'. 'Hypotonic buffer' contained 10 mM Tris (pH 8.0), 2 mM EDTA, 2 mM dithiothreitol and 100 Kallikrein inhibitor units Trasylol.

Rod outer segments were prepared as previously described [20] with the following modifications. After the first centrifugation step in 34–35% sucrose and isotonic buffer, the supernatant was collected and diluted with isotonic buffer to an apparent sucrose concentration of 32% using refractive index measurements. The suspension was overlayed with isotonic buffer and centrifuged in a Beckman SW-28 rotor at 20000 rpm for 90 min. Rod outer segments are collected from the carpet at the sucrose-buffer interface, and were centrifuged at 20000 rpm in a Sorvall SS-34 rotor for 20 min. The supernatant was removed and the rod outer segment pellet was overlayed with fresh isotonic buffer such that the rhodopsin concentration, if resuspended, would have been 5 mg/ml. Rhodopsin concentrations were determined by recording the difference in absorbance of 500 nm light by a sample in a buffered, isotonic solution containing 1.5% (w/v) of the detergent CTAB and assuming an absorption coefficient for rhodopsin of 40000 $mol^{-1} \cdot cm^{-1}$ and a molecular weight of 40000 $g \cdot mol^{-1}$. Samples were used immediately or stored as pellets at −80 °C.

Hypotonic stripping of rod disc membranes

Rod outer segments were centrifuged to clarify the supernatant. This isotonic supernatant was stored on ice and later combined with hypotonic supernatants. Rod outer segments were resuspended to a final rhodopsin concentration of 4–8 mg/ml in hypotonic buffer and incubated on ice, in complete darkness, for 45 min with occasional mixing. This mixture was centrifuged for 15 min in a Beckman TY-65 rotor at 50000 rpm. The hypotonic resuspension and centrifugation steps were repeated one additional time. The hypotonic supernatants were combined and, if necessary, concentrated in an Amicon ultrafiltration cell using a PM-10 filter. The hypotonic extract was concentrated until the volume equalled that of the

isotonic supernatant and then both supernatants were pooled. Concentration continued until the volume corresponded to a protein concentration equivalent to the level of enzyme extractable from rod outer segments at a concentration of 4 mg rhodopsin per ml. This mixture is designated 'protein extract'. If the protein extract was to be stored, the protein mixture was concentrated to an equivalent of 8 mg rhodopsin per ml and then combined with ice cold glycerol (Baker, USP Grade) to a final glycerol concentration of 40% (v/v).

Disc membranes devoid of detectable cGMP phosphodiesterase and rhodopsin kinase activity were prepared by additional hypotonic washes and centrifugation in 5% Ficoll (22), 100 μM $MgCl_2$, and 50 μM DTPA. 'Ficoll floated' disc membranes are designated stripped disc membranes.

Reassociation with extrinsic proteins

Method I. Stripped disc membranes in isotonic buffer and the protein extract were added separately to the assay mixture (which contained 1–2 mM cGMP and 50 μM GTP in isotonic buffer) and assayed immediately.

Method II. Stripped disc membranes in isotonic buffer were mixed with protein extract and incubated on ice (usually three hours) prior to the assay.

Method III. Stripped disc membranes in hypotonic buffer were mixed with concentrated protein extract and placed in a dialysis bag (mol. wt. cut-off 4000–8000). The mixture was dialyzed against 100 volumes of isotonic buffer containing 15% (v/v) glycerol for three hours. The concentration of rhodopsin was maintained at ≥ 2 mg/ml (50 μM) during dialysis.

When alternative reassociation protocols were compared directly, aliquots from the same protein extracts were used and the rhodopsin concentration in Methods II and III reassociations were equal. All protein extracts were mixed with stripped disc membranes in proportion to the amount originally present in the rod outer segment preparation.

Measurement of membrane aggregation

Turbidity measurements were performed in a Cary 219 spectrophotometer equipped with a sample stirring attachment. The disc membrane suspension contained 1 mg/ml rhodopsin in 10 mM Tris (pH 8.0) and 2 mM EDTA. The sample was stirred in a 1 cm $\times$ 1 cm fluorescence cuvette equilibrated to room temperature (22°C) and the turbidity was measured as apparent absorbance at 730 nm. Turbidity changes were recorded following addition of either hypotonic buffer that contained 10 mM Tris (pH 8.0), 2 mM EDTA or a hypertonic buffer that contained 100 mM Tris (pH 8.0), 600 mM KCl, 300 mM NaCl, 20 mM $MgCl_2$, and 1 mM EDTA.

Binding of cGMP phosphodiesterase and G-protein to stripped disc membranes

All manipulations were performed in complete darkness or under infrared illumination with an infrared image converter (Electro-optics, Nutley, NJ). Binding experiments utilized the 'protein extract' and stripped disc membranes as described above. Stripped disc membranes were resuspended in isotonic buffer to a concentration of 4 mg rhodopsin per ml. The protein extract was concentrated to a volume equivalent to an extract of 4 mg rhodopsin/ml. Equal volumes of stripped disc membranes and the protein extract were mixed at 4°C. A 100 μl aliquot was immediatley withdrawn and centrifuged (100000 $\times$ g, 30 s, in a Beckman airfuge) and the supernatant carefully collected. Additional aliquots were collected at time intervals up to three hours.

SDS-polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis was performed in a modified Laemmli [23] gel system using a 12% acrylamide gel with a 30 : 1.1 acrylamide/bisacrylamide ratio according to Farrer [24].

Assay of cGMP phosphodiesterase activity

All manipulations of rhodopsin containing membranes for cGMP phosphodiesterase assays were performed in complete darkness or under infrared illumination using the method of Liebman and Evansczuk [25] as previously described [19–21]. The assay mixture contained 5 μM rhodopsin, 1 to 2 mM cGMP, and 50 μM GTP in pH 8 isotonic buffer. The sample was placed in a cuvette (10 mm light path) in a clear plexiglass

chamber thermostated to 30°C. The pH was measured using a Thomas Model T-17 or Microelectrode Model 410 pH electrode in conjunction with an Orion Model 810 Ion Analyzer. The digitized output was recorded by a DEC 11-23 micro-computer and the data stored on disk. The calibration of rhodopsin bleaches was performed according to Miller and Dratz [20].

Results and Discussion

Stability of cGMP phosphodiesterase and G-protein in the protein extract

The addition of glycerol to protein extracts and reassociated stripped disc membrane samples during storage, improved the reproducibility of light-dependent cGMP phosphodiesterase activation. In the absence of glycerol, the recovery of light-initiated cGMP phosphodiesterase activity (measured following flash excitation in the presence of stripped disc membranes) decreased by approx. 30% during the first 24 h when the eluted, concentrated proteins were stored at 0–4°C. The loss of light sensitivity did not appear to be due to any inherent lability of the cGMP phosphodiesterase since incubation with either protamine sulfate or short term trypsin digestion resulted in full recovery of cGMP phosphodiesterase activity [5,26]. We therefore suspected that the loss of light-activatable cGMP phosphodiesterase activity was due to the loss of G-protein function. When 40% glycerol (v/v) was included in the protein extract, 90% of the light-activatable cGMP phosphodiesterase activity could be retained for up to two weeks, when stored at −20°C, with only a small reduction in sensitivity. Sensitivity was measured by the percent rhodopsin bleach required to produce $V_{max}/2$ of the cGMP phosphodiesterase. Control experiments, in which glycerol was added either to a suspension of rod outer segments prior to dilution or to the assay mixture, did not exhibit any effect of the glycerol on either sensitivity or the V_{max} of the cGMP phosphodiesterase.

Reassociation of disc membranes with protein extract

Aliquots of stripped disc membranes were mixed with freshly prepared protein extract to yield a final rhodopsin concentration of 50 μM and treated according to Methods II and III. A third aliquot was mixed in the reaction cuvette according to Method I. Fig. 1 shows the light-stimulated cGMP phosphodiesterase activity in each of these preparations as a function of the percent rhodopsin bleached. The V_{max} of the cGMP phosphodiesterase in isolated rod outer segment and Method III reassociation samples ranged from 3.5–8 moles H^+ per second per mole rhodopsin. All of these preparations displayed half-maximal cGMP phosphodiesterase activity when approx. 0.02% of the rhodopsin was bleached. Reassocia-

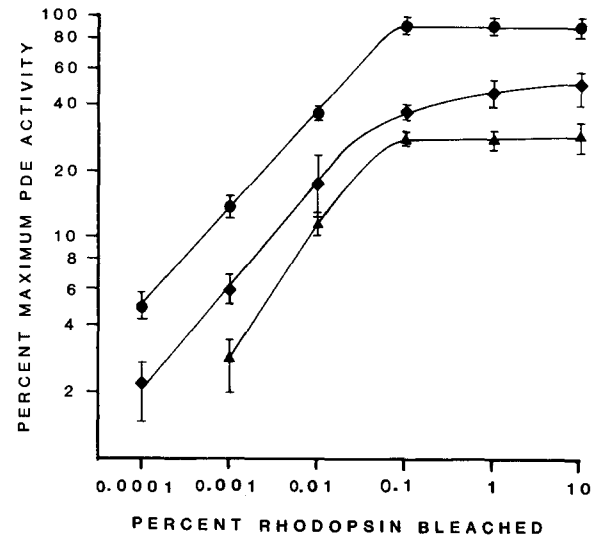

Fig. 1. Light titration of light-initiated cGMP phosphodiesterase (PDE) activity produced by alternative protein-membrane reassociation protocols. Hypotonically stripped, Ficoll floated, disc membranes were reassociated with the same protein extract according to Method I (●), Method II (◆), and Method III (●) as described in the text. 100% cGMP phosphodiesterase activation corresponds to the maximum level of cGMP phosphodiesterase activity produced by a 10% rhodopsin bleach in any of the reassociated preparations. In this experiment, the Method III reassociation recovered > 90% of the original cGMP phosphodiesterase activity that was present in each of the freshly prepared rod outer segment membranes from which the protein extracts were derived and 100% of the phosphodiesterase activity remaining in the rod outer segments at the time the reassociated mixtures were assayed. The data were averaged for three different protein extracts used in separate experiments. Error bars correspond to the range of experimental values obtained. The V_{max} of the cGMP phosphodiesterase (corresponding to 100% on the ordinate axis) in these experiments ranged from 3.5 to 8 moles H^+ released per second per mole rhodopsin. cGMP phosphodiesterase assays were performed as described in the text.

tion by Method III in this experiment yielded ≥ 90% of the cGMP phosphodiesterase activity present in the initial rod outer segment starting material.

The cGMP phosphodiesterase activity recovered by the dialysis reassociation (Method III) generally ranged from 75 to 100% of that in the rod outer segment preparation. The majority of dialysis reassociations recover ≥ 85% of the original cGMP phosphodiesterase activity. Reassociation Methods I and II generally recovered 20 to 60% of the original cGMP phosphodiesterase activity, respectively, with the majority of Method I and II reassociations recovering less than 50% of the light-initiated cGMP phosphodiesterase activity.

Control experiments were performed to determine if the concentration steps used during preparation of the protein extract affected the behavior of the various reassociated preparations. Rod outer segments were resuspended to a concentration of 200 μM (8 mg/ml) for each of the two hypotonic washing steps and the two supernatants were pooled and combined with stripped disc membranes according to the three reassociation protocols. The results from these experiments were very similar to the data shown in Fig. 1.

Ionic strength-dependent disc membrane aggregation

The most efficient reassociation of cGMP phosphodiesterase and G-protein with stripped disc membranes were obtained when the disc membranes and extrinsic proteins were combined in hypotonic buffer and the salt concentrations increased gradually (Method III). Previously, Gaw [27] had demonstrated that salts tend to strongly aggregate disc membrane vesicles. This observation suggested that ionic strength-dependent disc membrane aggregation could potentially affect the level of cGMP phosphodiesterase activation recovered following reassociation and it was of interest to determine whether aggregation occurred under the conditions of the current experiments. Lewis et al. [28] and Carretta and Stein [29–31] have demonstrated that disc membrane aggregation is accompanied by an increase in apparent absorbance near 730 nm. Therefore, we were able to test for ionic strength-dependent aggregation by

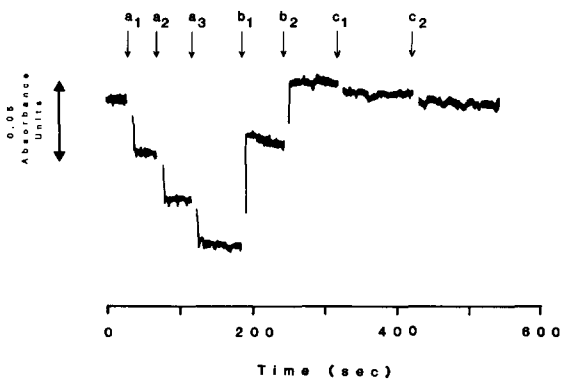

Fig. 2. Ionic strength-dependent turbidity changes in a suspension of stripped disc membranes containing 25 μM rhodopsin. Measurements were performed as described under Methods. Arrows indicate additions of 60 μl of hypotonic buffer (a_1, a_2 and a_3), 30 μl of hypertonic buffer containing 100 mM Tris (pH 8.0), 600 mM KCl, 300 mM NaCl, 20 mM $MgCl_2$, and 1 mM EDTA (b_1 and b_2) or 60 μl of the same hypertonic buffer (c_1 and c_2).

measuring turbidity changes of a hypotonic suspension of stripped disc membranes due to exposure to cGMP phosphodiesterase assay buffer conditions. A suspension containing 25 μM rhodopsin possessed an initial apparent absorbance at 730 nm of approx. 1.2 absorbance units. Addition of small amounts of hypotonic buffer to the cuvette (Fig. 2, additions indicated by 'a_1', 'a_2' and 'a_3') decreased the apparent absorbance as expected, due to the dilution of the scattering species. Subsequent addition of a hypertonic buffer contining 10 times the concentration of the isotonic buffer (Fig. 2, additions indicated by 'b_1' and 'b_2') actually reversed the effect of dilution and caused an increase in the apparent absorbance. Further addition of the hypertonic buffer (Fig. 2, indicated by 'c_1' and 'c_2') demonstrated that 85% of the salt dependent turbidity change followed an increase in the salt concentration of the suspension to 0.7 mM Mg^{2+} and 30 mM monovalent ions. These turbidity measurements indicated that increasing the ionic strength of the stripped disc membranes suspension produced an increase in disc vesicle aggregation. Presumably, these aggregated disc vesicles possess a reduced surface area that is accessible for protein binding relative to the combined surface areas of disaggregated discs. The effect of disc vesicle aggregation on the subsequent activation of the

cGMP phosphodiesterase may be several-fold. The membrane-associated forms of these proteins are favored in the presence of moderate ionic strength environments. If a hypotonic protein mixture is suddenly diluted into a moderate ionic strength environment, the proteins will rapidly bind to any available membrane surface. Under aggregated conditions, the cGMP phosphodiesterase and G-protein would first bind to the surfaces of disc vesicle aggregates and would require time to redistribute uniformly over the total disc surfaces. The time required for this redistribution may account for the increased efficiency of cGMP phosphodiesterase activation of Method II reassociation over Method I reassociation (see Fig. 1).

Time-course of cGMP phosphodiesterase and G-protein binding to disc membranes and the recovery of light-initiated cGMP phosphodiesterase activity

In order to determine if the binding of the cGMP phosphodiesterase and G-protein to the disc membrane surface parallels the recovery of light-initiated cGMP phosphodiesterase activity, the following experiments were performed. The protein extract and stripped disc membranes were mixed under infrared illumination according to Method II and incubated in complete darkness. Aliquots were removed after 0–3 h incubation, and assayed for light initiated cGMP phosphodiesterase activity or centrifuged (100 000 × g, 30 s in a Beckman airfuge) to separate the popula-

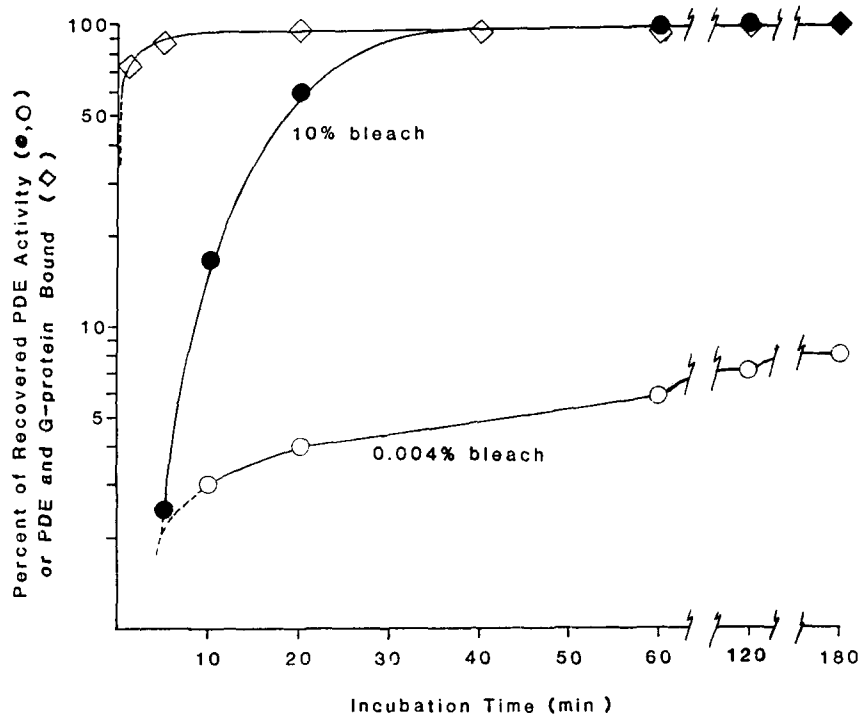

Fig. 3. Time-course of the binding of cGMP phosphodiesterase (PDE) and G-protein to disc membranes and the recovery of light-initiated cGMP phosphodiesterase activity. Stripped disc membranes and protein extracts were combined according to Method II. Aliquots were removed at the indicated times and either added to the cGMP phosphodiesterase assay mixture or centrifuged to separate the membrane bound proteins from the soluble proteins. For the cGMP phosphodiesterase assay, flashes of light bleaching 0.004 (lower curve, ○) and 10% (center curve, ●) of the rhodopsin present were delivered at the indicated times (±2 min). Assay conditions were as described in the text. 100% of the recovered cGMP phosphodiesterase activity corresponded to 6 moles H^+ per second per mole rhodopsin in this particular experiment. In the binding experiment, '100% bound' corresponds to a maximal binding of 80% of the G-protein and 70% of the cGMP phosphodiesterase initially present.

tions of membrane bound and soluble cGMP phosphodiesterase and G-protein. The proteins present in the supernatants were separated by SDS-polyacrylamide gel electrophoresis and the binding of the proteins to the disc membranes was measured as the loss of staining intensities of their respective bands from Coomassie-stained SDS gels.

Fig. 3 compares the time-course of cGMP phosphodiesterase and G-protein binding to stripped disc membranes with the recovery of light-stimulated cGMP phosphodiesterase activity. Under the conditions used, a maximum of 80% of the G-protein and 70% of the cGMP phosphodiesterase bound to the disc membranes. Binding data are expressed as a percentage of these maximal values. Parallel binding experiments were performed following reassociation by Methods I and III. These latter preparations exhibited the same equilibrium distribution of cGMP phosphodiesterase and G-protein between soluble and membrane associated populations as was observed following reassociation by Method II (data not shown). The V_{max} of the cGMP phosphodiesterase in the reassociated preparations shown in Fig. 3 was 6 moles H^+ per mole rhodopsin per second which was 80% of the rod outer segment control activity (the highest cGMP phosphodiesterase activity recovered by Method II).

The binding of cGMP phosphodiesterase and G-protein to stripped disc membranes were significantly faster than the recovery of light-initiated cGMP phosphodiesterase activity. Following reassociation by Method II, the binding of cGMP phosphodiesterase and G-protein had reached 90% of the equilibrium distribution in less than 5 min. The recovery of light-induced cGMP phosphodiesterase activity in response to 10% rhodopsin bleaches required approx. 0.5 h of dark incubation to reach 'completion'. The cGMP phosphodiesterase activity elicited by low fractional rhodopsin bleaches (0.004%), in contrast, required 2–3 h of dark incubation to approach an approximately constant level of cGMP phosphodiesterase activation.

Fig. 3 indicates that the recovery of light stimulated cGMP phosphodiesterase activity proceeds without a detectable change in the fraction of the cGMP phosphodiesterase and G-protein that is bound to the disc membrane surfaces. The combination of a rapid association of the cGMP phosphodiesterase and G-protein with stripped disc membranes and a kinetically slower recovery of light-initiated cGMP phosphodiesterase activation suggests that not all membrane-associated molecules are equivalent in terms of their efficiency of activation by rho*. One may interpret these results to indicate that subsequent to the association of the cGMP phosphodiesterase and G-protein with the disc membrane surface, the binding interactions slowly change in favor of some preferred (lower energy) configuration. Apparently these energetically preferred states exhibit an enhanced efficiency of cGMP phosphodiesterase activation relative to those configurations present early in the binding process. The light titration curves, obtained for the various reassociation methods (Fig. 1) show that the reassociation method employed influences the final efficiency of cGMP phosphodiesterase activation. The distinction between efficiently activated and less efficiently activated cGMP phosphodiesterase and G-protein populations, therefore, is more complex than the distinction between membrane-associated and soluble cGMP phosphodiesterase and G-protein.

Influence of divalent cations on recovery of cGMP phosphodiesterase activity

In addition to the method of mixing salts, protein extracts, and stripped disc membranes, the influence of divalent cations in the buffer used for reassociation were also investigated. Three 'isotonic buffers' were used in these experiments: (1) Iso : Ca^{2+} (identical to 'isotonic buffer', but with 2 mM Ca^{2+} substituted for 2 mM Mg^{2+}), (2) Iso : Mg^{2+} (identical to 'isotonic buffer'), and (3) Iso : ND (containing 10 mM Tris, (pH 8.0), 65 mM KCl, 30 mM NaCl, 1 mM dithiothreitol, and 50 μM DTPA, with no divalent cations added). The extrinsic proteins were reassociated according to Method III and the above buffers were substituted for 'isotonic buffer' at the dialysis step. The Mg^{2+} required for cGMP phosphodiesterase activation was introduced into the assay mixtures less than 20 s before the activating flash if not already present.

Fig. 4 compares the relative cGMP phos-

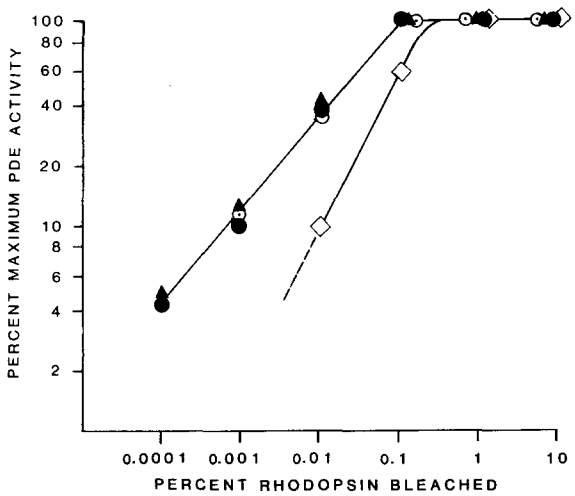

Fig. 4. Effect of divalent cations on the light titration of cGMP phosphodiesterase (PDE) activity using Method III (dialysis) reassocaitions. Stimulus-response curves for rod outer segment (●) and reassociations are shown carried out in three different buffers (see text for details). Symbols correspond to Iso : Mg^{2+} (▲), Iso : Ca^{2+} (⊙), and Iso : ND (◇) reassociations. A special isotonic buffer that contained no added divalent cations was used in the cGMP phosphodiesterase assays and 2 mM $MgCl_2$ was added to the assay mixture of the Iso : Ca and Iso : ND reassociations up to 20 seconds prior to the activating flash.

phodiesterase activities for samples reassociated with different divalent cation compositions as a function of the percent rhodopsin bleach. The stimulus-response behavior of rod outer segment, Iso : Ca^{2+}, and Iso : Mg^{2+} preparations were essentially indistinguishable at all of the bleaching levels tested. In this particular preparation, the V_{max} of the rod outer segment control, the Iso : Ca^{2+} and the Iso : Mg^{2+} reassociations was 3.5 moles H^+ per mole rhodopsin per second and rhodopsin bleaches of approx. 0.01% were required to produce $V_{max}/2$ of the cGMP phosphodiesterase.

Similar cGMP phosphodiesterase activities were observed for reassociations carried out in Ca^{2+}, Mg^{2+}, and 'divalent free', K^+/Na^+ isotonic buffers if assayed with large fractional rhodopsin bleaches. At low fractional bleaches, however, the 'divalent free' reassociations exhibited a lower efficiency of cGMP phosphodiesterase activation (Fig. 3). The enhanced ability of Ca^{2+} and Mg^{2+} containing isotonic buffers relative to 'divalent free', K^+/Na^+ isotonic buffers to reassociate the cGMP phosphodiesterase and G-protein activities with stripped disc membranes assayed by low fractional rhodopsin bleaches (see Fig. 3) suggests that some divalent cation specific interaction modulates the number of cGMP phosphodiesterases that are activated by rho*. When these reassociations were centrifuged to separate the membrane associated from the soluble proteins, no differences between the Iso : Ca^{2+}, Iso : Mg^{2+}, and the Iso : ND reassociations were observed (data not shown). This result indicated that the differences in the recovered cGMP phosphodiesterase activity between these samples did not arise from differences in the amount of protein associated with the disc membrane surface. Knowles and MacKenzie [32] also found little difference in the distribution of cGMP phosphodiesterase activity between supernatant and membrane fractions of rod disc membrane suspensions following centrifugation from buffers containing varying levels of NaCl, $CaCl_2$, or $MgCl_2$. In these latter studies, however, cGMP phosphodiesterase activation was measured only in the presence of saturating levels of rho*.

We can suggest several potential mechanisms to accommodate the effect of divalent cations on the recovery of light-initiated cGMP phosphodiesterase activation. One possibility is that divalent cations are required to mediate the binding of the G-protein (and cGMP phosphodiesterase) to specific disc membrane binding sites (e.g. phosphatidylserine) or may influence the 'intramolecular' interactions between G-protein and cGMP phosphodiesterase subunits. Alternatively, divalent cations might be required during reassociation for the most efficient interaction between the G-protein and rho*. Finally, because cGMP phosphodiesterase and G-protein posess a net negative charge [26,33], divalent cations might more effectively shield the negatively charged phospholipids present on the disc membrane surface than monovalent cations alone and thus stabilizing the interactions of extrinsic proteins with the membrane surface. Although the present experiments indicated that Ca^{2+} and Mg^{2+} ions can influence the efficiency of the cGMP phosphodiesterase activation cascade, we could not determine if Ca^{2+} and Mg^{2+} exert their influence by interaction with identical or different sites. For example, Mg^{2+}

might 'optimize' cGMP phosphodiesterase and G-protein subunit interactions whereas Ca^{2+} might facilitate the interactions of extrinsic proteins with the membrane surface.

Conclusion

Our experiments demonstrate that the light-initiated cGMP phosphodiesterase activation in reassociated preparations can be modulated by the nature of the interaction between extrinsic proteins and disc membrane vesicles. Different lines of evidence support the existence of sub-populations of membrane associated enzymes that differ in their efficiency of activation by rho*. The distribution of cGMP phosphodiesterase and/or G-protein between these populations is influenced by such factors as reassociation protocol, the state of membrane aggregation, and the ionic strength of the disc membrane suspension during reassociation. Divalent cations can further influence the recovery of light initiated cGMP phosphodiesterase activity apparently by facilitating the interaction between extrinsic proteins and rho*.

Acknowledgments

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