Recoverin and Rhodopsin Kinase Activity in Detergent-resistant Membrane Rafts from Rod Outer Segments*

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Cholesterol-rich membranes or detergent-resistant membranes (DRMs) have recently been isolated from bovine rod outer segments and were shown to contain several signaling proteins such as, for example, transducin and its effector, cGMP-phosphodiesterase PDE6. Here we report the presence of rhodopsin kinase and recoverin in DRMs that were isolated in either light or dark conditions at high and low Ca\(^{2+}\) concentrations. Inhibition of rhodopsin kinase activity by recoverin was more effective in DRMs than in the initial rod outer segment membranes. Furthermore, the Ca\(^{2+}\) sensitivity of rhodopsin kinase inhibition in DRMs was shifted to lower free Ca\(^{2+}\) concentration in comparison with the initial rod outer segment membranes (IC\(_{50}\) = 0.76 \(\mu\)M in DRMs and 1.91 \(\mu\)M in rod outer segments). We relate this effect to the high cholesterol content of DRMs because manipulating the cholesterol content of rod outer segment membranes by methyl-\(\beta\)-cyclodextrin yielded a similar shift of the Ca\(^{2+}\)-dependent dose-response curve of rhodopsin kinase inhibition. Furthermore, a high cholesterol content in the membranes also increased the ratio of the membrane-bound form of recoverin to its cytoplasmic free form. These data suggest that the Ca\(^{2+}\)-dependent feedback loop that involves recoverin is spatially heterogeneous in the rod cell.

Phototransduction in retinal rod and cone cells is started with the absorption of light by the photopigment rhodopsin, a seven-transmembrane helix receptor. Activated rhodopsin (metarhodopsin II) couples to a heterotrimeric G-protein, transducin, and thereby activates the visual enzymatic cascade that leads to the amplified hydrolysis of cGMP by cGMP-phosphodiesterase (1, 2). Cyclic nucleotide-gated cation channels in the photoreceptor plasma membrane are opened by cGMP in the dark and closed after hydrolysis of cGMP in light. Closure of channels causes hyperpolarization of the cell plasma membrane, which ultimately leads to a reduction of transmitter release at the synapse (3). A decrease in cytoplasmic cGMP release is accompanied by a decrease in free cytoplasmic Ca\(^{2+}\), which is sensed by Ca\(^{2+}\)-sensor proteins that regulate their target proteins in a Ca\(^{2+}\)-dependent fashion. For example, calmodulin influences the ligand sensitivity of the cation channels (4), and guanylate cyclase-activating proteins activate two membrane-bound guanylate cyclases at low Ca\(^{2+}\) concentrations (5–9). Phosphorylation and deactivation of rhodopsin are catalyzed by rhodopsin kinase, which is under Ca\(^{2+}\)-dependent control by the Ca\(^{2+}\)-binding protein recoverin (10–13). These Ca\(^{2+}\)-dependent feedback loops are necessary to restore the dark state of the cell and to adjust the light sensitivity of the photoreceptor cell to different intensities of ambient illumination (1–3, 14).

A photoreceptor cell consists of distinct cellular compartments (outer segment, inner segment, and synaptic terminal), and these compartments differ in their Ca\(^{2+}\) homeostasis and protein content (1, 16). Protein translocation between compartments along the longitudinal axis was observed for some key signaling proteins such as transducin, arrestin, and protein phosphatase 2A (17–20). In addition, a spatial heterogeneity of the cholesterol content in the stacked disk membranes of rod outer segments (ROSs) was observed along the axis of the outer segment. Newly formed disks at the basal part of the outer segment contain a high amount of cholesterol of ~30% of the total lipid content. The percentage of cholesterol decreases during aging of the disk membranes and reaches a mere 5% at the tip end of ROSs (21–23). Cholesterol can inhibit cGMP-phosphodiesterase activity (23) and interferes with formation of photoexcited rhodopsin by influencing membrane acyl chain packing (24). Taken together, these results point to a spatial heterogeneity of visual transduction in ROSs. In fact, single photon responses recorded from the tip of a toad ROS are smaller in amplitude and slower than responses recorded from the base. Background light reduces flash sensitivity at the tip more than at the base (25). Although this spatial heterogeneity of the light response has been known for more than 20 years, it has not been understood at the cellular and molecular levels.

Recent reports have stimulated discussion about the spatial heterogeneity of the rod light response. Detergent-resistant membranes (DRMs) or lipid rafts that contain a high cholesterol/phospholipid ratio have recently been isolated from bovine ROSs (26–30). A light-dependent translocation into DRMs has been demonstrated for transducin; its effector, cGMP-phos-
phodiesterase; the shorter splice variant of arrestin p44; and the RGS9-Gß5L complex (26, 27, 29, 30). ROM-1, a disk membrane protein, which probably functions as an adaptor protein, was copurified with DRM fractions but only showed a modest light-dependent distribution between the DRMs and the detergent-soluble fractions (28). Caveolin and membrane guanylate cyclase (probably retina-specific ROS-GC1) reside in DRMs but do not show any light-dependent translocation (26). Rod function is under dynamic control of Ca2+-mediated feedback loops, and Ca2+ regulates the longitudinal transport of transducin (17), but it is not known whether any signaling proteins different from those mentioned above associate with DRMs or whether Ca2+ is involved in this association. In the present study, we investigated this issue as applied to the Ca2+-sensor recoverin and its target, rhodopsin kinase, to answer questions regarding whether these proteins associate with DRMs and which functional consequences follow from such an interaction.

EXPERIMENTAL PROCEDURES

Isolation of DRMs from Bovine Rod Outer Segments—ROSs from bovine retinae were purified according to a previously published procedure (15). DRMs or lipid rafts were isolated from bovine ROSs by the following procedure of Nair et al. (26), with some modifications. Briefly, ROSs were homogenized in 10% n-glycerol in buffer A (2 mM MgCl2, 1 mM ethyleneglycoltetraacetic acid, and 10 mM Hepes, pH 7.4). For one gradient, 500 µl of ROSs in buffer A were mixed with 2 ml of buffer B (12.5 mM Tris, pH 7.4, 12.5 mM CaCl2, 12.5 mM MgCl2, and 0.63% (w/v) Triton X-100) and incubated for 5 min on ice or at 4 °C. This solution was mixed with 2.5 ml of 80% (w/v) sucrose in an ultracentrifuge tube. The mixture was then carefully overlaid with 4.6 ml of 30% sucrose and 2.3 ml of 50% sucrose. Samples were centrifuged at 54,000 rpm in SW-41 rotor (Beckman) at 4 °C overnight. The whole procedure was performed in either dim red light (dark) or under daylight conditions. In some preparations, CaCl2 was replaced by 1 mM EGTA. When gradients were run for comparison, exactly the same amount of ROS was loaded on the gradient. In addition, we increased the Triton X-100 concentration that was used for solubilization to 1% and 2% (w/v). Alternatively, we isolated DRMs according to the procedure of Boezaart-Battaglia et al. (28). After centrifugation, we collected ~24 fractions (500 µl) of the gradient from bottom to the top using a glass capillary tube and a peristaltic pump.

Determination of Rhodopsin and Cholesterol—Rhodopsin concentration in purified ROSs or in fractions obtained after DRM isolation and fractionation was determined spectrophotometrically at 498 nm using a molar extinction coefficient of 40,000 M−1 cm−1. The amount of rhodopsin was determined spectrophotometrically at 540 nm with 0.5% or 1% (v/v) Triton X-100, the amount of rhodopsin was the rhodopsin/Triton X-100 ratio. For example, when we treated ROSs containing 4.5–4.6 mg/ml rhodopsin with 0.5% or 1% Triton X-100, the amount of rhodopsin was the rhodopsin/Triton X-100 ratio. When gradients were run for comparison, exactly the same amount of ROS was loaded on the gradient. The rhodopsin distribution along the sucrose gradient was monitored by direct examination of the fractions.

RESULTS

We isolated a Triton X-100-insoluble membrane fraction from bovine ROS using a sucrose density gradient centrifugation. Samples were fractionated after the centrifugation step, and the cholesterol content along the gradient was determined by a colorimetric assay. Almost all cholesterol was found in a peak that centered around fraction 6 (Fig. 1A). This distribution pattern of cholesterol was not changed significantly when we performed the whole isolation procedure either in the presence of Ca2+ or EGTA, after illumination, or in the dark (Fig. 1A). The main cholesterol peak coincided with the position of a nearly transparent yellow band at the boundary between 5% and 30% sucrose. This band (boundary fraction) has previously been described (8, 35). Antibodies for Western blotting were from Santa Cruz Biotechnology (Santa Cruz, CA).

The rhodopsin distribution along the sucrose gradient was analyzed by absorption measurements. The main portion of rhodopsin was solubilized by Triton X-100 and found in fractions 12–23 (89%); a smaller portion of ~10% was found at the 5% and 30% sucrose boundary (fractions 5–9) and comigrated with the cholesterol peak (Fig. 1B). Up to 23% rhodopsin was found in DRMs. Fractions 1 and 2 contained only 1% rhodopsin. The presence of rhodopsin in DRMs is consistent with previous observations (27). A critical parameter for solubilization of rhodopsin was the rhodopsin/Triton X-100 ratio. For example, when we treated ROSs containing 4.5–4.6 mg/ml rhodopsin with 0.5% or 1% Triton X-100, the amount of rhodopsin and other proteins in DRMs was similar at both detergent concentrations. However, decreasing the start amount of rhodopsin to 1.7 mg/ml in either 1% or 2% Triton X-100 led to almost complete solubilization of rhodopsin (<1% rhodopsin in DRMs). The cholesterol peak at the boundary between 5% and 30% sucrose also decreased by increasing Triton X-100: whereas at 0.5% Triton X-100, nearly 100% of total cholesterol comigrated with the boundary fraction, it was 37% and 12% of...
total cholesterol at 1% Triton X-100 and 2% Triton X-100, respectively. These results showed that rhodopsin was completely solubilized under conditions that left a significant amount of cholesterol associated with the boundary fraction.

We further tested by Western blotting whether other ROS membrane proteins known to be associated with DRMs (26–30) are present in our DRM preparation. Guanylate cyclase ROS-GC1 and cGMP-phosphodiesterase were present in DRM and non-DRM fractions; transducin showed a clear light-dependent translocation into the DRM fraction (data not shown). Caveolin, a marker protein for lipid rafts, was found almost exclusively in the DRM fraction (Fig. 2A). Interestingly, less caveolin was detected in DRMs after illumination (Fig. 2A). Although the intensity of caveolin staining was variable, we observed this light-dependent distribution of caveolin in two independent fractionation studies. It is known that caveolin associates in a cholesterol-dependent manner with transducin (29) and that transducin undergoes a light-dependent translocation from the outer segment to the inner segment (17, 18). A combination of these two effects could explain our observation.

In summary, we conclude from these results that our DRM preparation from bovine ROS contains the same signaling proteins as reported by other investigators. It is therefore suitable for our further investigations.

When we probed all fractions of the gradient by antibodies against recoverin and rhodopsin kinase, both proteins were detected in the DRM and non-DRM fraction. A comparison of the gradients run in the presence of Ca$^{2+}$ or EGTA under dark or light conditions showed in all cases the presence of recoverin and rhodopsin kinase in DRM of different gradient runs under the indicated conditions. The starting concentration of rhodopsin was 1.84 mg/ml instead of 1.66 mg/ml, and therefore the rhodopsin:Triton X-100 ratio was slightly higher than that in A.
Recoverin and Rhodopsin Kinase in Lipid Rafts

Recoverin in the DRM fraction decreased after illumination in the presence of EGTA (Fig. 2A). Distribution of rhodopsin kinase resembled that of recoverin, but the presence of rhodopsin kinase became most prominent after illumination in the presence of Ca^{2+}. The relative amount of recoverin and rhodopsin kinase in DRMs varied among different preparations, as can be seen best in a comparison of Fig. 2A with Fig. 2B. The effect of switching from Ca^{2+} to EGTA during DRM isolation is more pronounced in Fig. 2B because the presence of EGTA reduced the amount of recoverin and rhodopsin kinase in the DRM fraction.

We next asked whether the inhibition of rhodopsin kinase activity by recoverin in DRMs differs from the inhibition in ROS membranes. In titration experiments, we varied either the recoverin concentration at saturating [Ca^{2+}] or [Ca^{2+}]_{i,eq} at a constant recoverin concentration. At saturating [Ca^{2+}], inhibition of rhodopsin kinase occurred at slightly lower recoverin concentrations (Fig. 3A). However, when we compared the Ca^{2+}-dependent phosphorylation of rhodopsin in ROS membranes with that in isolated DRMs, we observed a significant shift of the IC_{50} to lower free Ca^{2+} concentrations (from 1.91 μM in ROS membranes to 0.76 μM in DRMs) (Fig. 3B). Thus, recoverin was more effective as an inhibitor of rhodopsin kinase in DRMs than it was in ROS membranes. Overall activity of rhodopsin kinase without interference by recoverin was identical in ROS membranes and DRMs for nearly 20 min of incubation. Longer incubation times showed −20% lower kinase activity in ROS membranes.

Because cholesterol is a main constituent of DRMs, we tested how cholesterol influenced the membrane association and inhibitory properties of recoverin. The cholesterol content of native bovine ROS membranes was manipulated by treatment with methyl-β-cyclodextrin, and the binding of recoverin was measured by a centrifugation equilibrium assay. Native ROS membranes contained, on average, 14% cholesterol. Decreasing the cholesterol content to 4.1% also decreased the amount of bound recoverin, whereas an increase of cholesterol to 29.6% increased the amount of bound recoverin at least 2-fold (Fig. 4). These results showed that binding of recoverin to membranes strongly depended on the cholesterol content of the membranes. Control incubations with nonmyristoylated recoverin and arrestin showed no dependence on either Ca^{2+}/EGTA or the percentage of cholesterol (data not shown).

We also tested the recoverin-dependent inhibition of rhodopsin kinase activity when cholesterol in ROS membranes was varied (Fig. 5A). The kinase activity was determined by measuring phosphorylation of rhodopsin. Inhibition of rhodopsin kinase was half-maximal at 6.6 μM recoverin in untreated ROS (14% cholesterol) and shifted to a higher value when cholesterol was lowered (IC_{50} = 10.4 μM at 4.1% cholesterol) or to a lower value when cholesterol was higher (IC_{50} = 4.5 μM at 29.6% cholesterol).
activity was measured as a function of \([\text{Ca}^{2+}]\) free, 29.6%, 14%, and 4.1% cholesterol, respectively. A decrease of \([\text{Ca}^{2+}]\) free in the presence of 20 \(\mu\text{M} \text{CaCl}_2\) decreases the amount of recoverin at which kinase activity is decreased and enforces inhibition of rhodopsin kinase by two means: it decreases the amount of recoverin at which kinase activity is half-maximal, and it shifts the dose-response curve to lower \([\text{Ca}^{2+}]\) free in membranes with 20 \(\mu\text{M} \text{CaCl}_2\). After centrifugation, the amount of bound recoverin was determined.

In our previous work, we used immobilized lipids on sensor chips to explore membrane association of recoverin by surface plasmon resonance spectroscopy (35–37). Here we applied this method to test the influence of cholesterol upon recoverin binding to immobilized lipids. A phospholipid mixture of PE and PC (50:50) was immobilized on a hydrophobic sensor chip, and association of myristoylated recoverin was recorded in the presence of saturating \([\text{Ca}^{2+}]\). The resonance signal exhibited a rapid association phase and a biphasic dissociation phase that is typical for wild-type myristoylated recoverin (Fig. 5A; compare with Fig. 5 in Ref. 35). When the immobilized lipid mixture contained cholesterol (Fig. 7A, top trace), the maximal amplitude of the binding signal increased about 2-fold. Interestingly, the slower phase of the biphasic dissociation signal was more prominent in the presence of cholesterol. Variation of the recoverin concentration revealed the same result: in the presence of cholesterol, the maximal amplitude of the binding signal was at least twice as high as that in the absence of cholesterol (Fig. 7B). Control recordings with protein G in the presence and absence of cholesterol showed no significant difference of sensogram amplitudes, which were similar to the amplitude we reported previously for protein G binding to an immobilized lipid mix (see Fig. 3B in Ref. 35).

In summary, our results suggest that the high cholesterol content of DRMs facilitates binding of recoverin to membranes and enforces inhibition of rhodopsin kinase by two means: it decreases the amount of recoverin at which kinase activity is half-maximal, and it shifts the dose-response curve to lower \([\text{Ca}^{2+}]\) free.

**DISCUSSION**

The light-driven transport of signaling proteins between photoreceptor cell compartments has received growing attention in recent years (17–20). In addition to these longitudinal transport processes, lateral translocation of proteins within disk membranes and into DRMs or lipid rafts has come into focus (26–30). Here we show for the first time that the \([\text{Ca}^{2+}]\) sensor recoverin and its target, rhodopsin kinase, are present in DRMs of bovine rod cells and undergo a \([\text{Ca}^{2+}]\)-dependent translocation within ROS membranes. A decrease of \([\text{Ca}^{2+}]\), by
Two different lipid layers were compared as described in grams were plotted as a function of the injected recoverin concentration. According to our present data, cholesterol has a profound effect on the efficiency with which recoverin controls rhodopsin kinase activity. In cholesterol-containing DRMs, inhibition of rhodopsin kinase at the base of the ROS, which would be consistent with a stronger inhibition of rhodopsin kinase at the base than at the tip, the photoresponse of a dark-adapted cell would become larger and last longer. However, this is opposite to what was observed after single photon absorption (25). However, flash sensitivity in the presence of background light is higher at the base of ROS. If inhibition of rhodopsin kinase is stronger at the base than at the tip, the ratio of membrane-bound recoverin to cytoplasmic free recoverin is increased at high cholesterol content (Fig. 4), which causes more effective inhibitory action of recoverin.

In accordance with the well-described cholesterol gradient in rods (5% at the tip and 30% at the base; see “Introduction”), one could conclude that control of rhodopsin kinase activity by recoverin is spatially heterogenous and thus would contribute to the shape of the phototrcission at the base of a ROS differently than at the tip. It is known that phototransduction involves a complex process that includes both light-dependent and light-independent steps. The above prediction is rather simplified and made under the assumption that other proteins have similar properties in DRMs and outside DRMs. However, it is suggested that recovery is spatially heterogenous and thus would contribute to the shape of the phototransduction at the base of a ROS differently than at the tip. It is known that phototransduction involves a complex process that includes both light-dependent and light-independent steps.

A significant amount of rhodopsin (10–23% of total) was also found in DRMs, but increasing the Triton X-100 concentration led to the complete solubilization of rhodopsin, whereas a significant amount of cholesterol still comigrated with the 5%/30% boundary. These results could indicate that rhodopsin is not associated with DRMs or simply that Triton X-100 has a higher affinity for DRMs than for other membrane proteins. According to our present data, cholesterol has a profound effect on the efficiency with which recoverin controls rhodopsin kinase activity. In cholesterol-containing DRMs, inhibition of rhodopsin kinase became more efficient at lower [Ca\(^{2+}\)] free than in native DRMs and 0.82 \(\mu\)M in ROS membranes with high cholesterol content (29.6%). These values are in the physiological range of free Ca\(^{2+}\) in rod cells. Our data also show that the ratio of membrane-bound recoverin to cytoplasmic free recoverin is increased at high cholesterol content (Fig. 4), which causes more effective inhibitory action of recoverin.

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signal. This reduction in signaling molecules is reminiscent of transgenic mice that harbor a hemizygous knockout of rhodopsin resulting in a reduction of rhodopsin by 50% (46). Photoresponses from these transgenic mice have accelerated rising and recovery phases due to less protein crowding and facilitated diffusion. In fact, they qualitatively resemble the single photon responses from ROS base with faster rising and recovery phases.

In summary, inhibition of rhodopsin kinase by recoverin (i.e., less efficient phosphorylation of rhodopsin) seems to be more pronounced at the base than at the tip of ROS, if we consider the effects of different cholesterol contents. Interpretation of photoresponses published in the literature leads us to suggest that these signaling events are more important under constant photoresponses published in the literature leads us to suggest that these signaling events are more important under constant background light and not under the single photon regime of dark-adapted rods.

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