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

Received for publication, March 5, 2004, and in revised form, August 30, 2004
Published, JBC Papers in Press, September 7, 2004, DOI 10.1074/jbc.M402516200

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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 μM in DRMs and 1.91 μM 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-β-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 cytosplasmic 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 involving 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 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 (ROSSs)† 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.

† The abbreviations used are: ROS, rod outer segment; DRM, detergent-resistant membrane; PE, phosphatidylethanolamine; PC, phosphatidylincholine; HPLC, high pressure liquid chromatography; [Ca\(^{2+}\)\_free\] = free Ca\(^{2+}\) concentration.

* This work was supported by grants from the Deutsche Forschungsgemeinschaft (to K.-W. K.); a grant from the Forschungszentrum Jülich (to I. I. S., P. P. P., and K.-W. K.); the Russian Fund for Basic Research (to P. P. P.); and INTAS Projects (to I. I. S., K. W. K.). The costs of publication of this article were defrayed by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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This paper is available on line at http://www.jbc.org
phodiesterase; the shorter splice variant of arrestin p44; and the RGS9-Gβ5L 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 Ca\(^{2+}\)-mediated feedback loops, and Ca\(^{2+}\) 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 Ca\(^{2+}\) is involved in this association. In the present study, we investigated this issue as applied to the Ca\(^{2+}\)-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 (10 mg of rhodopsin) in buffer A (420 mM NaCl, 10 mM HEPES, pH 7.4, 1.25 mM CaCl\(_2\), 1.25 mM MgCl\(_2\), and 0.63% (v/v) Triton X-100) were homogenized with glass beads in a Potter-Elvehjem homogenizer, and the homogenate was centrifuged at 250,000 \(\times\) g for 30 min at 4 °C. The supernatant was carefully layered on top of 31% sucrose in 10 mM Tris-HCl (pH 7.5) and 1 mM cholesterol. Mixtures were incubated in the dark at 25 °C for 2 h.

**Purification of Recoverin and Phosphorylation of Rhodopsin—** Myristoylated recoverin was heterologously expressed in Escherichia coli and purified from a cell extract as described previously (35, 36). Phosphorylation of rhodopsin was assayed as described in Ref. 35 at 25 °C in the reaction mixture (50 \(\mu\)l) containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 3 mM MgCl\(_2\), 200 \(\mu\)M [gamma-\(^{32}\)P]ATP (1–3 \times 10\(^{5}\) cpm/nmol), about 1 unit of rhodopsin kinase, ROS membranes or DRMs (see figure legends), and myristoylated recoverin (see figure legends). Free calcium concentration ([Ca\(^{2+}\)]\(_{free}\)) was adjusted as described previously (35) and varied as indicated in the corresponding figures. Immediately after illumination of the mixture (100% bleaching of rhodopsin), ATP was added to start the reaction, which was stopped 20 min later or by the addition of the 2 \(\times\) SDS-PAGE sample buffer. After SDS-PAGE of the samples, zones of rhodopsin were cut out, and \(^{32}\)P incorporation was estimated by Cherenkov counting.

**Surface Plasmon Resonance Spectroscopy—** Hydrophobic L1 sensor chips (Biacore, Stockholm, Sweden) were used to immobilize lipid mixtures of phosphatidylcholine, phosphatidylethanolamine, and cholesterol. Recoverin was applied in the mobile phase in running buffer (10 mM Hepes, pH 7.5, 150 mM KCl, 20 mM MgCl\(_2\), and 0.2 mM CaCl\(_2\)) at a flow rate of 5 \(\mu\)l/min. Details of surface plasmon resonance experiments and analysis have been described elsewhere (35–37).

**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 Ca\(^{2+}\) 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 identified as DRMs or lipid rafts from ROS (26–28).

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 25% rhodopsin was found in DRMs. Fractions 1 and 2 contained only 5% 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% (v/v) 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 DRMs 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+}]_{med} 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).
those described in cholesterol, the IC50 was 2.43

**FIG. 5.** *Rhodopsin kinase activity at different cholesterol levels*. A, Rhodopsin kinase activity was determined as a function of recoverin concentration. Different cholesterol levels in membranes were adjusted by treatment with methyl-β-cyclodextrin (●, 29.6%; ○, 14%; ▼, 4.1%). Half-maximal inhibition was at 4.5, 6.6, and 10.4 μM recoverin at 29.6%, 14%, and 4.1% cholesterol, respectively. B, Rhodopsin kinase activity was measured as a function of [Ca2+]free in the presence of 20 μM recoverin. Cholesterol adjustment and symbols are the same as those described in A. IC50 values of [Ca2+]free were 0.82 (29.6% cholesterol), 2.43 (14% cholesterol), and 4.31 μM (4.1% cholesterol).

Inhibition of rhodopsin kinase by recoverin depends also on [Ca2+]free. (11–13). We tested whether a change in the cholesterol content of native ROS membranes has any influence on the Ca2+-dependent activity of rhodopsin kinase. Increasing the cholesterol content from 4.1% to 29.4% shifted the dose-response curve to lower [Ca2+]free, and the IC50 changed from 4.31 to 0.92 μM [Ca2+]free (Fig. 5B). At intermediate levels of cholesterol, the IC50 was 2.43 μM. These results were consistent with our observation that inhibition of rhodopsin kinase by recoverin is more efficient and occurs at lower [Ca2+]free in DRMs than in ROS membranes. The results also implicate that the shift in the dose-response curve shown in Fig. 3 cannot be attributed to detergent treatment because no detergents were needed to adjust different cholesterol levels. Furthermore, the binding experiments shown in Fig. 4 indicate that recoverin is bound more strongly to membranes when higher cholesterol levels are adjusted.

We also tested the influence of cholesterol on the membrane association of recoverin at saturating [Ca2+] using phospholipid vesicles containing phosphatidylethanolamine (PE) and phosphatidylcholine (PC) at a ratio of 50:50 without cholesterol or with increasing amounts of cholesterol (5–50%) by keeping the PC:PE ratio constant. Binding of recoverin to the vesicles was tested by an equilibrium centrifugation assay. Vesicles without cholesterol showed less than half of recoverin binding compared with vesicles containing increasing amounts of cholesterol in addition to PE and PC (Fig. 6).

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 [Ca2+]. The resonance signal exhibited a rapid association phase and a biphasic dissociation phase that is typical for wild-type myristoylated recoverin (Fig. 7A; 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 [Ca2+]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 Ca2+-sensor recoverin and its target, rhodopsin kinase, are present in DRMs of bovine rod cells and undergo a Ca2+-dependent translocation within ROS membranes. A decrease of Ca2+, by
against such a role under in vivo conditions is the experimentally observed high IC$_{50}$ for [Ca$^{2+}$]$_{free}$, which is about 1 order of magnitude higher than the cytoplasmic [Ca$^{2+}$]$_{free}$ in a dark-adapted rod cell (41). However, it has been argued that the IC$_{50}$ value can be shifted into the physiologically relevant range when the data on rhodopsin kinase inhibition are extrapolated to the membrane-rich in vivo conditions of the rod cell (13, 42). Our results may provide an experimentally based solution to this problem because we show that high cholesterol content in membranes can shift the IC$_{50}$ to lower [Ca$^{2+}$]$_{free}$: 0.76 μM in native DRMs and 0.82 μM 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.

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 heterogeneous and thus would contribute to the shape of the photoresponse at the base of a ROS differently than at the tip. It is known that photoreponses from rods depend on the longitudinal position of photon absorption (25). Responses from the base of a ROS are faster and have a larger peak amplitude; in the presence of background light absorption, sensitivity is lower at the tip than at the base of ROS. If inhibition of rhodopsin kinase is stronger at the base than at the tip, the photoreponse 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 the ROS, which would be consistent with a stronger inhibition of rhodopsin kinase at the base.

The above prediction is rather simplified and made under the assumption that other proteins have similar properties in DRMs and outside DRMs. However, To is suggested to have a reduced coupling to rhodopsin in DRMs (26). Furthermore, cholesterol inhibits cGMP-phosphodiesterase activity and metarhodopsin II formation (23, 24); cholesterol inhibits the latter by influencing the acyl chain packing of surrounding lipids. Finally, the splice variant of arrestin p44 that is found in DRMs after illumination (26) can be bound to nonphosphorylated metarhodopsin II with a rather low off-rate (0.07 s$^{-1}$) and would thereby prevent transducin activation (45). Together, these findings suggest that phototransduction in DRMs is less efficient.

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 potency to solubilize rhodopsin than to solubilize cholesterol. We cannot distinguish between these possibilities. Furthermore, our data do not allow us to draw any conclusions about the preexistence of rafts before treatment with detergent. However, we emphasize that the existence or nonexistence of rafts is irrelevant to our observation that cholesterol has a significant impact on membrane association of recoverin and on inhibition of rhodopsin kinase. Thus, taking the cholesterol gradient in ROSs into account, we assume that rhodopsin in a cholesterol rich-environment is more restricted in diffusion and that phototransduction in DRMs works less efficiently (see above for a discussion of the literature). As a consequence, the base of the ROS would contain a significant amount of signaling proteins in a “caged-like state” unable to transmit the light
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 background light and not under the single photon regime of dark-adapted rods.

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doi: 10.1074/jbc.M402516200 originally published online September 7, 2004

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