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INHIBITION OF RHODOPSIN PHOSPHORYLATION BY NON-MYRISTOYLATED RECOMBINANT RECOVERIN

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SUMMARY: Bovine recoverin regulates rhodopsin phosphorylation and controls photoreceptor light sensitivity in a Ca^{2+}-dependent manner. Recoverin is post-translationally modified with lipids (myristic acid or related lipids) at its N-terminus. Since with this lipid modification (N-myristoylation), recoverin associates with rod outer segment membranes in a Ca^{2+}-dependent manner, N-myristoylation has been suggested to be important for the function of this protein. To study the role of this modification, we obtained recombinant non-myristoylated recoverin in E. coli and studied its functional properties. Here, we report that recombinant non-myristoylated recoverin inhibits rhodopsin phosphorylation at Ca^{2+} concentrations of 30 nM - 10 μM in a similar way as native N-myristoylated recoverin does. Thus, our result showed that N-myristoylation is not essential for the Ca^{2+}-dependent inhibition of rhodopsin phosphorylation by recoverin. © 1994 Academic Press, Inc.

In vertebrate rod photoreceptors, light activates the visual pigment, rhodopsin, which ultimately leads to hydrolysis of cGMP and closure of the cGMP-gated channel to induce membrane hyperpolarization (1,2). Photoreceptors are desensitized during light-adaptation. A light-induced decrease in intracellular Ca^{2+} concentration has been shown to be the underlying mechanism of light-adaptation (3,4). S-modulin present in frog photoreceptors (5) or its bovine counterpart recoverin (6,7) detects this decrease in Ca^{2+} concentration and reduces the efficiency of phototransduction to desensitize the cell. This regulation, or the S-modulin effect, is attained by dis-inhibition of rhodopsin phosphorylation (8,9), the inactivation mechanism of light-activated rhodopsin.

The N-terminal glycine of recoverin is post-translationally modified with lipids related to myristic acid (10), which is known as N-myristoylation (see ref. 11 for review). This modification has been shown to be essential for the association of recoverin with rod outer segment (ROS) membranes at high Ca^{2+} concentrations (12,13). Since Ca^{2+}-dependent association of S-modulin with ROS membranes has been observed in intact photoreceptors (5), it has been suggested that N-myristoylation is essential for the function of recoverin (12,13). In the present study, the role of the N-myristoylation in recoverin was examined by comparing the functional properties of native (myristoylated) and recombinant (non-myristoylated) recoverins.

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MATERIALS AND METHODS

Expression and purification of recombinant recoverin Bovine recoverin cDNA clone (pTrec 2) (14) was obtained from the American Type Culture Collection (number 77328). Recombinant recoverin was overproduced in E. coli as described by Ray et al. (14). After induction, bacteria were collected by centrifugation, resuspended in a lysis buffer (50 mM Tris-HCl, 2 mM EDTA, 0.1 % (v/v) Triton X-100, pH 8.0), frozen in liquid nitrogen, and sonicated for 3-5 min. After centrifugation, the supernatant was made 40 % ammonium sulfate and centrifuged. The supernatant was subjected to isoelectric precipitation at pH 4.3 to obtain recoverin in the precipitate.

Purification of recombinant recoverin was carried out based on the method of Polans et al. (15) and Kawamura et al. (16). The precipitate was resuspended in 50 mM HEPES, 2 mM $CaCl_2$, 0.1 M NaCl (pH 7.5) and applied to a Phenyl-Sepharose column. Recoverin was eluted with the same buffer containing 10 mM EDTA instead of $CaCl_2$ and was further purified with a DEAE column.

Native recoverin was obtained in a similar way using fresh retina as described (9).

Preparation of frog rod outer segment membranes Frog ROS containing rhodopsin was obtained under complete darkness in a potassium-gluconate buffer (K-gluconate buffer) containing 115 mM potassium gluconate, 2.5 mM KCl, 2 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM $CaCl_2$, 0.2 mM EGTA and 10 mM HEPES (pH 7.5). ROS was fragmented by vigorously sucking into and ejecting from a thin needle (# 28 gauge) 4 times. The resultant ROS fragments, or ROS membranes were washed with the K-gluconate buffer twice to eliminate endogenous S-modulin. The membranes were stored overnight at 4 °C in the presence of 0.1 mM ATP and 0.1 mM GTP in the K-gluconate buffer. Just before the experiment, the membranes were washed once more to eliminate the residual, if any, S-modulin.

Membrane-association of recoverins and other proteins ROS membranes containing 6 nmoles of rhodopsin were first mixed with 0.5 ml of the K-gluconate buffer containing 0.25 mg bovine serum albumin to block the non-specific association sites of the membranes. After washing the membranes two times by centrifugation (100,000 x g, 15 min) with 2 ml of the K-gluconate buffer supplemented with 1 mM Ca^{2+} (1 mM Ca^{2+}/K-gluconate buffer), 20 pmoles of recoverin or other proteins in 10 μl of a 1 mM Ca^{2+}/K-gluconate buffer were added to the membranes. The mixture was incubated at room temperature for 30 min and then centrifuged to extract the protein that did not associate with the membranes at this high (1 mM) Ca^{2+} concentration. Extraction was repeated one more time. The two extracts obtained at this high Ca^{2+} concentration were combined and applied to SDS-PAGE to quantitate the protein.

The precipitated membranes were then mixed with 10 μl of a 10 mM EGTA/K-gluconate buffer and centrifuged to obtain the extract containing the protein that was liberated from the membranes by lowering the Ca^{2+} concentration. The extraction was repeated and the two extracts obtained at this low (<1 nM) Ca^{2+} concentration were combined and applied to SDS-PAGE to quantitate the protein that associated with the membranes at the high Ca^{2+} concentration. Calmodulin used in this study was obtained from Sigma (P-2277).

Rhodopsin phosphorylation Rhodopsin phosphorylation was carried out as described previously (8). In brief, ROS membranes (15 μl) containing 10-15 μM rhodopsin plus exogenous protein were mixed in the dark with a reaction mixture (10 μl) consisting of 0.1 mM ATP, Γ-[^{32}P]ATP (24 MBq/μmol of ATP), 0.5 mM GTP and 4 mM cGMP. Rhodopsin phosphorylation was initiated by giving a weak light flash bleaching 3.5 x 10^6 rhodopsin molecules per ROS. We used a glass test tube to minimize Cerenkov radiation. The phosphorylation reaction was terminated by addition of 180 μl of ice-chilled 10 % (w/w) trichloroacetic acid. Our HPLC analysis showed that more than 95 % of added ATP still remained at the end of the reaction, which excludes the possibility that the inhibition of rhodopsin phosphorylation observed in the present and previous studies is not due to the reduction in the ATP concentration.

After centrifugation (7,000 x g, 15 min), the precipitate was washed once with the K-gluconate buffer and dissolved into a sample buffer for SDS-PAGE. The incorporation of ^{32}P into rhodopsin was detected either by autoradiography or counting of the ^{32}P activity in the dissected rhodopsin bands of the SDS-PAGE gel. Ca^{2+} concentration was buffered using a Ca^{2+}/EGTA buffering system (17).

RESULTS

Membrane-association of native and recombinant recoverins Previous reports showed that native S-modulin and native recoverin associate with ROS membranes at high Ca^{2+} concentrations (5,12,13,18), but that recombinant recoverin expressed in E. coli does not (12,13). The inability of the membrane-

association is due to the lack of N-myristoylation in recombinant recoverin (12,13). Indeed, E. coli does not have N-myristoylation activity (11).

To confirm that our recombinant recoverin expressed in E. coli is not N-myristoylated, membrane-association of the recombinant protein was examined (Fig. 1). Native recoverin or recombinant recoverin was mixed with ROS membranes at 1 mM Ca^{2+} and then centrifuged. Each supernatant was applied to SDS-PAGE to quantitate the protein that did not associate with the membranes (Fig. 1A, left lane for each protein indicated). Then a 10 mM EGTA/K-gluconate buffer was added to the precipitate and centrifuged to quantitate the protein that was liberated from the membranes at low Ca^{2+} concentrations (<1 nM). The supernatant was analyzed by SDS-PAGE (Fig. 1A, right lane). The protein amount in each extract was calculated as a percentage of the total of the protein extracted (Fig. 1B). Native recoverin was found mainly in the second low Ca^{2+} extract while recombinant recoverin was found mainly in the first high Ca^{2+} extract. The result indicated that our recombinant recoverin does not associate with ROS membranes but that native recoverin does at a high (1 mM) Ca^{2+} concentration. Thus, the result showed that our recombinant recoverin is non-myristoylated.

Further support about the lack of N-myristoylation came from additional two studies. First, with a protein sequencer, we confirmed that the sequence of the N-terminal 7 amino acids of the recombinant is identical to that of native recoverin. This result also showed that the N-terminus is not modified in our recombinant recoverin, since N-myristoylated proteins cannot be cleaved by Edman degradation. Second, our reversed phase HPLC analysis showed that the recombinant recoverin is eluted at less hydrophobic conditions than native recoverin probably because of the lack of N-myristoylation (not shown).

In Fig. 1, membrane-association of chick NCS-1 (19) and calmodulin was also examined. NCS-1 has high homology to *Drosophila* frequenin (20), is another member of the protein family that includes S-modulin and recoverin, and possesses a consensus amino acid sequence for N-myristoylation. NCS-1 used in this study was produced in E. coli and was shown to be non-myristoylated by mass spectrometry. Both NCS-1 and calmodulin, which is also non-myristoylated, bound little to the membranes at high Ca^{2+} concentrations (Fig. 1). These results support the idea that N-myristoylation is essential for the membrane-association (12,13) and that our recombinant recoverin is non-myristoylated.

Inhibition of rhodopsin phosphorylation by non-myristoylated recoverin at high Ca^{2+} concentrations The effect of non-myristoylated recoverin on rhodopsin phosphorylation was investigated as a function of Ca^{2+} concentration (Fig. 2). Previous electrophysiological work suggested that S-modulin associates with disk membranes at a high (1 μM) Ca^{2+} concentration and that it regulates the photoreceptor sensitivity in the membrane-associated form (5). Since N-myristoylation is essential for the association with the membranes (Fig. 1 and refs. 12 and 13), we anticipated that non-myristoylated recoverin would not influence rhodopsin phosphorylation. To our surprise, however, non-myristoylated recoverin inhibited rhodopsin phosphorylation at high, but physiological Ca^{2+} concentrations (right-half panel in Fig. 2A) in a similar way as native recoverin did (left-half panel).

To quantitate the result, rhodopsin bands were dissected from the SDS-PAGE gel and incorporation of ^{32}P into rhodopsin was counted (Fig. 2B and C). The inhibition of rhodopsin phosphorylation took place on both native and recombinant recoverins at a Ca^{2+} concentration range between 30 nM and 10 μM, and the extent of the inhibition was higher with the recombinant recoverin

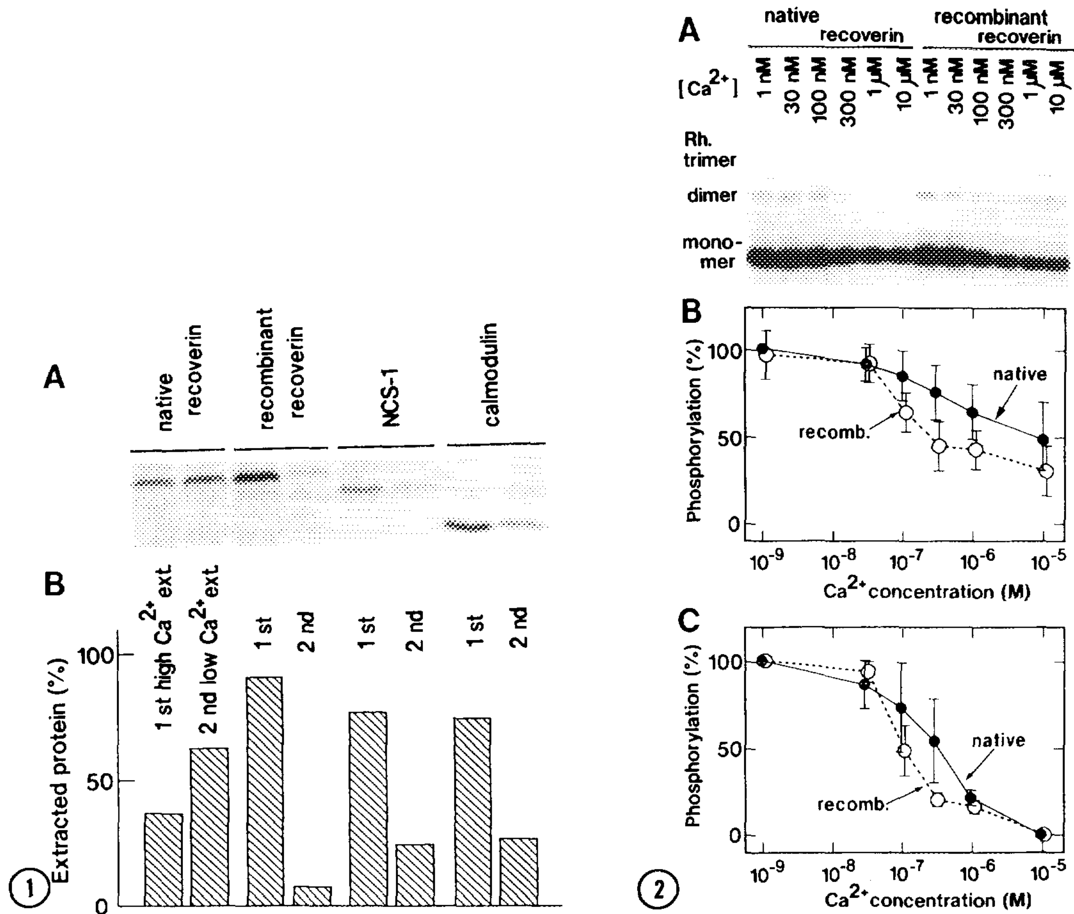

Figure 1. Binding of native recoverin, recombinant recoverin, NCS-1 and calmodulin to ROS membranes.

A. Extraction of proteins at a high and a subsequent low Ca^{2+} concentration (see MATERIALS AND METHODS). Proteins indicated were mixed with ROS membranes first at a high (1 mM) Ca^{2+} concentration. Unbound proteins were removed by centrifugation and supernatants were applied to SDS-PAGE for staining with Coomassie Brilliant Blue (left lane for each protein). Proteins that associated with the membranes at 1 mM Ca^{2+} were obtained by subsequent extractions with an EGTA solution (right lane).

B. Amount of protein present in extracts shown in *A*. The density of the protein band in *A* was quantitated. For each lane, the protein amount is expressed as the fraction of the total protein obtained in the first plus the second extracts.

Figure 2. Effect of native and recombinant recoverins on rhodopsin phosphorylation.

A. Autoradiograph of rhodopsin phosphorylation. Rhodopsin phosphorylation was carried out in the presence of native and recombinant recoverins (final concentrations; 5 μM each) at the various Ca^{2+} concentrations indicated. Each sample was applied to SDS-PAGE and autoradiographed. In our gel system, rhodopsin is present mainly as a monomer but a small portion is a dimer or a trimer.

B. Pooled data (n=5) obtained by counting ^{32}P in the dissected rhodopsin bands of the SDS gel. In an experiment as in *A*, each data point was normalized to the count obtained in the presence of native recoverin at 1 nM Ca^{2+}. Closed and open circles show the results in the presence of native and recombinant recoverins, respectively.

C. Pooled data (n=5) normalized at both 1 nM and 10 μM Ca^{2+}. The data points at 1 nM and 10 μM Ca^{2+} are set at 100 % and 0 %, respectively for both native (closed circle) and recombinant recoverins (open circles). Other data points are expressed as relative values to this scale.

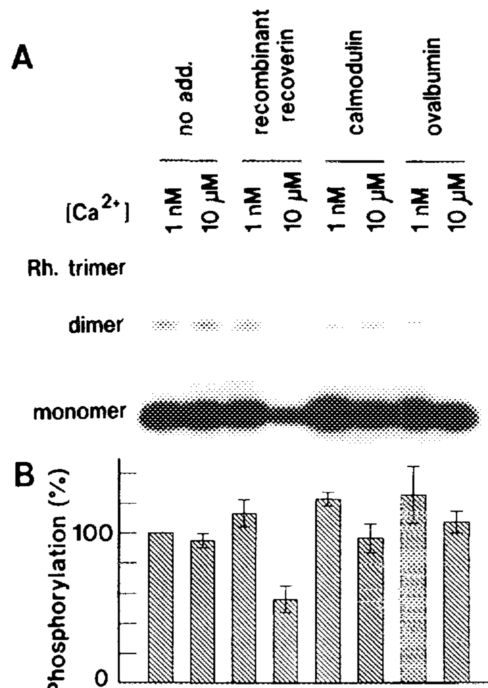

Figure 3. Specific effect of recoverin on rhodopsin phosphorylation.
A. Autoradiograph of rhodopsin phosphorylation at Ca^{2+} concentrations indicated; in the absence of exogenous proteins (no add.); in the presence of recombinant recoverin (4.5 μM), calmodulin (7.4 μM) and ovalbumin (4.5 μM).
B. Pooled data of incorporation of ^{32}P into rhodopsin bands. Incorporation of ^{32}P at 1 nM Ca^{2+} with no addition was set at 100 %. Other data are expressed as ratios to this value. Each value is expressed as the mean ± SD (n=4) except in the experiment of ovalbumin where the value is expressed as the mean ± the range of variations (n=2).

(Fig. 2B). It might be the case that some of our native recoverin had lost its activity during the course of purification and thus the inhibition with native recoverin was lower than that with recombinant recoverin. To overcome this possibility, we compared the Ca^{2+}-dependency of the inhibition caused by recombinant recoverin with that caused by native recoverin. In this comparison, the levels of phosphorylation at 1 nM Ca^{2+} and 10 μM Ca^{2+} were set at 100 % and 0 %, respectively. The result shown in Fig. 2C indicated that the inhibition profile of recombinant recoverin was not different from that of native recoverin: Ca^{2+} concentrations required for half-inhibition were ≈100 nM and ≈300 nM for non-myristoylated and N-myristoylated recoverins, respectively, but these values are close to each other. Therefore, we concluded that the Ca^{2+}-dependent inhibition of rhodopsin phosphorylation is similar in N-myristoylated and non-myristoylated recoverins and that N-myristoylation is not essential for this inhibition.

<u>Specific inhibition of rhodopsin phosphorylation by the S-modulin protein family</u> REcoverin and possibly S-modulin contain 4 EF hand structures (21), and are members of the calcium binding protein super family which includes calmodulin, troponin C and parvalbumin. To examine the specificity of the S-modulin effect, we examined the effect of calmodulin and more generally that of ovalbumin on the inhibition of the rhodopsin phosphorylation (Fig. 3). The result showed that the inhibition is observed specifically in the presence of recoverin. Calmodulin slightly reduced the phosphorylation at 10 μM

Ca^{2+}, but the effect was small. The slight effect could be due to contaminated CNS-1 in the commercially available calmodulin. Indeed, on the SDS-PAGE gel, we detected a faint protein band at the position close to CNS-1 in the calmodulin sample used. Our result, therefore, strongly suggests that the S-modulin effect is specific.

DISCUSSION

The present work showed that non-myristoylated recoverin does not associate with ROS membranes (Fig. 1) but yet it does inhibit rhodopsin phosphorylation at physiological Ca^{2+} concentrations (Fig. 2). Our result, therefore, indicates that N-myristoylation and thus membrane-association of recoverin is not required for the inhibition of rhodopsin phosphorylation.

The above finding suggests the presence of two distinct Ca^{2+}-binding sites in recoverin. According to previous studies, half-effective Ca^{2+} concentrations are different in the inhibition of rhodopsin phosphorylation (100–300 nM, Fig. 2 and also in refs. 8 and 9) and in the membrane-association of S-modulin or recoverin (2–10 μM; refs. 12 and 16). This difference, however, has not been well recognized, because the two Ca^{2+} effects are observed simultaneously in native recoverin or S-modulin and therefore it has not been clear whether the underlying mechanisms are different or not. Present work unequivocally showed that the mechanisms are different: in non-myristoylated recoverin, one Ca^{2+} effect (inhibition of rhodopsin phosphorylation) was observed but the other effect (membrane-association) was not. Thus, there must be at least two functionally distinct Ca^{2+}-binding sites in recoverin: one is a high affinity site(s) with a K_D value of 100 – 300 nM and is responsible for the inhibition of rhodopsin phosphorylation, and the other is a low affinity site(s) with a K_D value of 2–10 μM and is responsible for the membrane-association. Since the Ca^{2+} concentration in intact rods is below μM level (22,23), Ca^{2+} binding to the high affinity site(s) would be a physiological reaction. According to a 3D-structural analysis, recoverin has 4 EF hands, and EF2 and EF3 are the Ca^{2+}-binding sites (21). It will be interesting to see which site(s) is responsible for the inhibition of rhodopsin phosphorylation and which for the membrane-association.

Even though the membrane-association does not seem to be essential for the inhibition of rhodopsin phosphorylation, S-modulin or recoverin must interact with a physiological target (probably protein) molecule in the membrane at physiological Ca^{2+} concentrations. Perhaps the membrane-association so far observed at Ca^{2+} concentrations above μM level (Fig. 1 and also in refs. 12, 13 and 16) does not reflect the interaction between recoverin and the physiological target protein that is involved in the inhibition of rhodopsin phosphorylation. The potential candidates of the physiological target protein of S-modulin or recoverin are rhodopsin kinase and rhodopsin (5). Indeed, Ca^{2+}-dependent direct binding of recoverin to rhodopsin kinase was observed (24). However, the Ca^{2+} concentration used in this study was very high and 0.1 mM which is the range where the membrane-association of recoverin occurs (12,13,16). It is thus possible that the binding of recoverin to rhodopsin kinase observed is due to the same hydrophobic interaction that mediates the membrane-association of recoverin. Even though rhodopsin kinase is a highly probable candidate, the binding of recoverin to this protein should be examined carefully at physiological Ca^{2+} concentrations where only the inhibition of rhodopsin phosphorylation is observed.

N-myristoylation occurs in many proteins (11). In some of these proteins, this modification is essential for their functions. However, in the case of recoverin and probably S-modulin also, this modification does not play a central role in the inhibition of rhodopsin phosphorylation. It might be the case that N-myristoylation functions as a membrane-anchor to establish a tight interaction between recoverin and the physiological target at extremely high Ca^{2+} concentrations. Alternatively, this modification may have some additional physiological roles. Obviously, further studies are required to elucidate the potential role of N-myristoylation in this protein family.

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