Rhodopsin Kinase Inhibition by Recoverin

FUNCTION OF RECOVERIN MYRISTOYLATION*

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Recoverin is a Ca\(^{2+}\)-binding protein that may play a role in vertebrate photoreceptor light adaptation by imparting Ca\(^{2+}\) sensitivity to rhodopsin kinase. It is heterogeneously acylated (mostly myristoylated) at its aminoterminal glycine. Recent studies have shown that recoverin myristoylation is necessary for its Ca\(^{2+}\)-dependent membrane association and cooperative Ca\(^{2+}\) binding. We have addressed several issues concerning the role of recoverin myristoylation with respect to inhibition of rhodopsin kinase. We find that 1) myristoylation of recoverin is not necessary for inhibition of rhodopsin kinase, 2) myristoylation of recoverin induces a cooperative Ca\(^{2+}\)-dependence for rhodopsin kinase inhibition, and 3) each Ca\(^{2+}\)-binding site on the nonmyristoylated recoverin partially inhibits rhodopsin kinase. The available data suggest that the functions of recoverin myristoylation in the living rod are to induce a sharp Ca\(^{2+}\) dependence of rhodopsin kinase inhibition and to bring this dependence into the rod's physiological Ca\(^{2+}\) concentration range.

In the vertebrate photoreceptor recoverin may provide a Ca\(^{2+}\)-dependent feedback system involved in light adaptation by binding Ca\(^{2+}\) in the dark, when Ca\(^{2+}\) levels are high, and releasing it when Ca\(^{2+}\) levels drop upon illumination (1–5). Ca\(^{2+}\)-recoverin inhibits RK,\(^{1}\) and release of this inhibition would accelerate the inactivation of Rho* when Ca\(^{2+}\) levels drop. In a previous study (4) we have demonstrated that Ca\(^{2+}\) bound recoverin acts through a direct interaction with RK to decrease its catalytic activity (see also Refs. 2 and 5).

Recoverin belongs to a family of proteins post-translationally modified by the covalent addition of myristate to their aminoterminal glycine residues (3). Myristoylation of recoverin is necessary for its Ca\(^{2+}\)-dependent association with photoreceptor membranes (6–8). This membrane binding serves to bring inhibition of RK by recoverin into the physiological Ca\(^{2+}\) range under in vivo conditions (4, 6). Recently it was demonstrated that the myristoylation of recoverin induces cooperative Ca\(^{2+}\) binding to recoverin (10). A previous study has asserted that only one Ca\(^{2+}\)-binding site on recoverin is involved in the inhibition of Rho* phosphorylation, and that the other is involved in membrane binding (8). We have studied recoverin myristoylation with regard to inhibition of RK in order to further define its function and have addressed the following issues. 1) Is myristoylation of recoverin necessary for RK inhibition? 2) Is myristoylation of recoverin necessary for the inhibition of RK to be cooperative with respect to Ca\(^{2+}\) concentration? 3) Do both Ca\(^{2+}\)-binding sites on recoverin act to promote the inhibition of RK?

EXPERIMENTAL PROCEDURES

Materials and Solutions—[\(^{32}\)P]ATP was purchased from DuPont NEN, 1× CaCl\(_2\) solution from BDH, dibromo-BAPTA PM from Molecular Probes, and bis-Tris propane from Calbiochem. Other chemicals were obtained from Sigma. The standard buffer used in all experiments contained 110 mM potassium gluconate, 20 mM HEPES, pH 7.5, and 2 mM MgCl\(_2\).

ROS Preparation and RK Extraction—Bovine ROS were purified under infrared illumination as described elsewhere (11). The same method of bovine ROS isolation was used for RK extracts, but all sucrose solutions were prepared in 10 mM Tris, 5 mM MgCl\(_2\), pH 7.5, and the procedure was performed in room light. RK was extracted as previously described (12).

Expression and Purification of Recombinant Bovine Recoverin—Recombinant bovine myristoylated and nonmyristoylated recoverins were produced using a bacterial expression system (7) that was a generous gift from Dr. J. Hurley. For the myristoylated form of recoverin, the extent of myristoylation was determined by electrospray ionization mass spectrometry and found to be greater than 95%. Both forms of recoverin were purified by a published procedure (13) and stored at –70 °C. The concentrations of each form of recoverin were determined by absorbance at 280 nm using a molar extinction coefficient of 36,400 (4).

Calcium Buffering—A set of 4× stock solutions with different CaCl\(_2\) concentrations and a fixed dibromo-BAPTA concentration in standard buffer were prepared as described in Klenchin et al. (4) (1 × 3 mM dibromo-BAPTA).

RK Activity Assay—RK activity was measured as described previously (4). Briefly, urea-treated ROS membranes were used as substrate (10 μg rhodopsin, final concentration). Urea-treated ROS membranes, a ROS extract containing RK, a CaCl\(_2\)/dibromo-BAPTA stock solution, and recoverin if necessary were mixed in 15 μl final volume, in the dark, the suspension was illuminated bleaching all Rho, and 5 μl of \([\^{32}\)P]ATP (0.2–0.4 mM) were added. After 1–2 min the reaction was stopped by addition of 100 mM EDTA, 100 mM KF, pH 7.5. 32P incorporation was measured by filtration of samples through nitrocellulose filters.

RESULTS AND DISCUSSION

Only the Ca\(^{2+}\)-bound form of recoverin is able to inhibit RK. The potency of inhibition of RK by myristoylated and nonmyristoylated recoverin can thus be compared when all Ca\(^{2+}\)-binding sites are occupied. The dependence of the inhibition of RK on the concentration of myristoylated and nonmyristoylated recoverin at saturating Ca\(^{2+}\) levels is shown in Fig. 1. Myristoylated and nonmyristoylated recoverin (filled and open symbols, respectively) are practically the same with respect to their inhibition of RK activity. Both recombinant forms of re-
coverin show a $K_{1/2}$ of inhibition of about 3.5 $\mu$M, the same as the native bovine protein (4), and at sufficiently high concentrations are able to fully suppress RK activity ($I_{\max} = 96\%$, Fig. 1). Thus, myristoylation of recoverin does not affect the ability of recoverin to bind and inhibit RK. Several laboratories have reported that myristoylation of recoverin is necessary for its Ca$^{2+}$-dependent association with membranes (6–8), suggesting that it might be involved in recoverin inhibition of RK. Our data provide direct evidence against this idea and are consistent with previous conclusions (4, 8) that the association of recoverin with membranes is not necessary for its inhibitory activity toward RK.

Myristoylation of recoverin has been shown to radically change the way recoverin binds Ca$^{2+}$. From direct Ca$^{2+}$-binding measurements Ames et al. (10) conclude that nonmyristoylated recoverin has two independent Ca$^{2+}$-binding sites with affinities of 110 $\mu$M and 6.9 $\mu$M, whereas the presence of the myristoyl group results in cooperative Ca$^{2+}$ binding with an apparent $K_{1/2} = 17 \mu$M. If this is the case and if the hypothesis proposed earlier by us is correct (4) (namely, that binding of recoverin to RK is necessary and sufficient for the RK inhibition and is a strict function of Ca$^{2+}$), then the difference in Ca$^{2+}$ binding by recoverin should be observed in the Ca$^{2+}$ dependence of RK inhibition for the respective forms of recoverin. Indeed, the Ca$^{2+}$ dependence of RK inhibition is strikingly different for myristoylated and nonmyristoylated recoverin (Fig. 2). Fitting the data with the Hill equation consistently shows a Hill coefficient (n) less than 1 and $K_{1/2}$ of $\sim 0.35 \mu$M for the nonmyristoylated recoverin, while the best fit for the myristoylated form gives n = 1.9 and a much higher $K_{1/2}$ (5.3 $\mu$M; Fig. 2). Thus, myristoylated recombinant recoverin behaves much as the native form with respect to positive cooperativity with n = 2 and a $K_{1/2}$ in the micromolar range (4).

Nonmyristoylated recoverin inhibits RK activity with apparent negative cooperativity (n = 0.8). This data, considered with the direct Ca$^{2+}$-binding study (10), suggests an independent and additive action of the two Ca$^{2+}$-binding sites on nonmyristoylated recoverin with respect to RK inhibition rather than true negative cooperativity. The data for nonmyristoylated recoverin can be fit with the following equation

$$P = P_{\max}\left(1 - \left[a \frac{C_{\text{Ca}^{2+}}}{C_{\text{Ca}^{2+}} + K_a} + b \frac{C_{\text{Ca}^{2+}}}{C_{\text{Ca}^{2+}} + K_b}\right]\right)$$  (Eq. 1)

where P is the proportion of maximal phosphate incorporation by RK activity, $P_{\max}$ at the indicated Ca$^{2+}$ concentration, a and b are amplitude factors that reflect the contribution of the two Ca$^{2+}$-binding sites, and $K_a$ and $K_b$ are constants that reflect the Ca$^{2+}$ range of RK inhibition upon the occupancy of these sites (see legend to Fig. 2). If the occupancy of only the first site is sufficient for RK binding and inhibition, then in experiments of the sort shown in Fig. 2 one would expect to find an apparent $K_{1/2}$ slightly less than the $K_b$ for the high affinity Ca$^{2+}$ binding site (110 $\mu$M) (10). Similarly, if both sites have to be occupied by Ca$^{2+}$ to observe full inhibition, the observed $K_{1/2}$ would be close to the $K_a$ of the lower affinity site, in the micromolar range (10). In both cases, the Hill coefficient should equal 1. None of these predictions is seen experimentally, indicating that occupation of each Ca$^{2+}$-binding site on recoverin leads to a partial inhibition of RK activity.

Our data and conclusions differ, in part, from those of Kawamura et al. (8). Based on Ca$^{2+}$ titration experiments analogous to the ones shown in Fig. 2, they also conclude that there is essentially no difference in the inhibition of RK by myristoylated and nonmyristoylated recoverin. Such experiments are not sufficient to prove this point. What is required are recoverin titration experiments of the sort shown in Fig. 1. Our data on the Ca$^{2+}$ dependence of RK inhibition do unequivocally show a difference between myristoylated and nonmyristoylated recoverin. Closer examination of the data of Kawamura et al. reveals a qualitatively similar difference for the two forms of recoverin (Ref. 8, Fig. 2). Possible errors in Ca$^{2+}$ buffering (see Ref. 4 for discussion) and large error bars may have led the authors to ignore this small difference. Kawamura et al. take their data, that there is no difference in Ca$^{2+}$-dependent inhibition of RK by myristoylated and nonmyristoylated recoverin and that nonmyristoylated recoverin does not bind to membranes, to suggest that there are two distinct Ca$^{2+}$-binding sites on recoverin. One of these sites is responsible for the inhibition of RK, and the other is responsi-
ble for membrane association. Our data (Fig. 2) rule out this model by showing that occupation of each Ca\textsuperscript{2+}-binding site on recoverin leads to a partial inhibition of RK activity.

The influence of recoverin myristoylation on its Ca\textsuperscript{2+}-dependent inhibition of RK has also been recently addressed by Chen et al. (5) who report that Ca\textsuperscript{2+} titration curves of recoverin inhibition by nonmyristoylated and myristoylated recoverins have the same apparent K\textsubscript{1/2} of 3 \(\mu\text{M}\) free Ca\textsuperscript{2+} and also that myristoylated recoverin is a much more potent inhibitor of RK than the nonacylated form (apparent K\textsubscript{1/2} is 0.8 \(\mu\text{M}\) versus 8 \(\mu\text{M}\) recoverin at saturated Ca\textsuperscript{2+}). These results are entirely at variance with our own. We do not attempt to explain this, but note that our findings are consistent with all previous studies of Ca\textsuperscript{2+}/recoverin/RK interaction (1, 2, 4, 10). It has been shown that nonmyristoylated recoverin binds Ca\textsuperscript{2+} better (10) and that Ca\textsuperscript{2+} binding to recoverin is sufficient for binding to and inhibition of RK (1, 2, 4, 5). It follows then that the Ca\textsuperscript{2+} dependence of nonmyristoylated recoverin inhibition of RK should have a K\textsubscript{1/2} lower than that for myristoylated recoverin. This is exactly what we observe (Fig. 2). Also, similar results have been obtained by Kawamura et al. (8) (see above).

Most of the recoverin found in the retina is acylated by myristate or closely related fatty acids (3). Thus, recombinant myristoylated recoverin should be similar to the native protein. We find this to be the case both with respect to Ca\textsuperscript{2+} titration of RK inhibition and recoverin titration at saturated Ca\textsuperscript{2+} (4; present report). The high half-saturating recoverin concentration that we find for the recombinant protein (–3.5 \(\mu\text{M}\)) is consistent with what we and others observe for native bovine recoverin (5–7 \(\mu\text{M}\) (2) and 3.4 \(\mu\text{M}\) (4)) and for the frog protein (–7.5 \(\mu\text{M}\) (1)); this contrasts with the K\textsubscript{1/2} of 0.8 \(\mu\text{M}\) found by Chen et al. (5). Our finding that the myristoyl group has little or no effect on recoverin interaction with RK (Fig. 1) is consistent with our observation that RK inhibition by Ca\textsuperscript{2+}-recoverin is not sensitive to relatively high detergent concentrations (as high as 0.4% Tween 80), but Ca\textsuperscript{2+}/myristoyl-dependent binding of recoverin to membranes is diminished by Tween 80.\textsuperscript{2} This suggests that hydrophobic interaction is not of crucial importance for recoverin binding to RK.

It is interesting to note that myristoylation of recoverin has opposing consequences on recoverin function. On one hand, it induces cooperative Ca\textsuperscript{2+} binding, a feature that allows efficient detection of changes in Ca\textsuperscript{2+} concentrations in photoreceptors. On the other hand, it significantly increases the Ca\textsuperscript{2+} range over which inhibition of RK by recoverin occurs in vitro (Fig. 2), bringing it far from the reported in vivo range of free Ca\textsuperscript{2+} concentrations (200–600 nM in the darkness and much lower in bright light) (14–17). In a previous report we point out that a K\textsubscript{1/2} for recoverin inhibition of RK in the micromolar free Ca\textsuperscript{2+} range under dilute, in vitro conditions is expected, because Ca\textsuperscript{2+}-dependent membrane association of recoverin effectively reduces this K\textsubscript{1/2} to about 270 nM Ca\textsuperscript{2+} under more concentrated in vivo conditions (4). This effect arises because Ca\textsuperscript{2+}-bound myristoylated recoverin binds to membranes and no longer participates in free solution equilibrium. Thus, a higher total concentration of Ca\textsuperscript{2+}-recoverin results.

From the available data to date we propose two roles for recoverin myristoylation relevant to photoreceptor physiology. First, it imparts a sharp calcium sensitivity range, allowing it to act as more of a "switch" in sensing Ca\textsuperscript{2+}; second, it induces Ca\textsuperscript{2+}-dependent association of recoverin with photoreceptor membranes, allowing recoverin to act in the physiological Ca\textsuperscript{2+} range of the photoreceptor.

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\textsuperscript{2} P. D. Calvert and M. D. Bownds, unpublished observation.