# *N*-Myristoylation of recoverin enhances its efficiency as an inhibitor of rhodopsin kinase

Ivan I. Senin[a], Aminullah A. Zargarov[b], Andrey M. Alekseev[b], Elena N. Gorodovikova[a], Valery M. Lipkin[b], Pavel P. Philippov[a,*]

[a]*Department of Enzymology, A.N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, Moscow 119899, Russian Federation*
[b]*Branch of Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Puschino, Moscow Region 142292, Russian Federation*

Received 18 September 1995

**Abstract Recoverin, a recently identified member of the EF-hand superfamily of $Ca^{2+}$-binding proteins, is capable to inhibit rhodopsin phosphorylation by rhodopsin kinase at high but not at low free [$Ca^{2+}$]. The N-terminal glycine residue of retinal recoverin is heterogeneously acylated with myristoyl or related *N*-acyl group. To clarify the role of the N-terminal acylation of recoverin in its inhibitory action upon rhodopsin phosphorylation, we compared the efficiency of myristoylated and non-myristoylated forms of recombinant recoverin as inhibitors of rhodopsin kinase activity. We have found that rhodopsin phosphorylation by purified rhodopsin kinase, which does not depend on free [$Ca^{2+}$] in the absence of recoverin, is regulated by $Ca^{2+}$ in the presence of both forms of the recombinant protein. $EC_{50}$ values for $Ca^{2+}$ are the same (2 $\mu$M) for the myristoylated and non-myristoylated forms; the Hill coefficients of 1.7 and 0.9, respectively, indicate that the effect is cooperative with respect to $Ca^{2+}$ only for myristoylated recoverin. In the presence of $Ca^{2+}$, both forms of recoverin taken at saturated concentrations cause an almost equal inhibition of rhodopsin phosphorylation. However, the inhibitory action of the myristoylated form occurs at much lower its concentrations than that of the non-myristoylated form ($EC_{50}$ are 0.9 and 6.5 $\mu$M, respectively).**

*Key words:* Photoreception; Phosphorylation; Rhodopsin; Rhodopsin kinase; Recoverin; Calcium-binding protein; Bovine retina rod cell

## 1. Introduction

Illumination of the retina rod cell triggers the signal transduction in the cascade Rh – transducin – cGMP-phosphodiesterase that results in hydrolysis of cGMP and consequent closure of cation specific channels in the ROS plasma membrane which accompanies by a drop of free [$Ca^{2+}$] from about 550 to 200 nM [1]. The light-induced decrease of free [$Ca^{2+}$] in turn activates the photoreceptor guanylate cyclase thus stimulating the process of the cell recovery [2]. Originally, Rc was suggested to mediate the $Ca^{2+}$ effect upon guanylate cyclase in bovine ROS [3,4], however, this suggestion was not yet confirmed [5,6]. S-modulin, the Rc homolog in frog ROS, was shown to regulate RK in $Ca^{2+}$-dependent manner [7]. This fact and the ability of Rc to form a $Ca^{2+}$-dependent complex with 67-kDa protein, presumably RK, in bovine ROS [6] had been the first indications at the possible function of Rc which, as was confirmed later [8,9], acts as an inhibitor of the RK activity at high [$Ca^{2+}$]. *N*-Myristoylation of Rc [10] is essential for its association with photoreceptor membranes that might be important for the Rc functional activity [11,12]. To answer the question whether *N*-myristoylation of Rc is essential for manifesting its inhibitory action upon RK we have compared the inhibitory efficiency of m-Rc and n-Rc in the reconstituted system consisting of urea-washed ROS membranes, purified RK and Rc.

## 2. Experimental

In order to express the Rc gene in *E. coli* the corresponding cDNA were inserted into pET11d (Biolabs, USA) under the control of the T7 phage promoter [13] by using the flunking *Nco*I and *Bam*HI sites. This vector was designated as pET11d-rec. The host strain for expression was BL21 (DE3). To produce the m-Rc the overproducing strain pET11d rec/pBB131/BL21(DE3) carrying the yeast myristoyl-CoA: protein *N*-myristoyltransferase expression vector [14] was grown in LB medium with an addition of myristic acid (5 mg/l of growth medium) immediately after the induction with isoporopyl-$\beta$-D-thiogalactoside. The recombinant m-Rc and n-Rc as well as the retinal Rc have been isolated using salting-out procedure (70%–90% saturation of ammonium sulphate) followed by calcium-dependent hydrophobic chromatography on Phenyl-Sepharose [11] and MonoQ columns [3].

ROS prepared from fresh or frozen bovine retina under dim red light [15] were frozen in liquid nitrogen and stored at −70°C. Before using in the RK assay, ROS membranes were washed several times with 6 M urea. Rh concentration was determined by the difference in the optical densities of the samples at 500 nm before and after illumination in the presence of 1% Triton X-100, taking $\varepsilon$ = 42,000 [16]. The amino acid analysis and the Coomassie blue binding method [17] were used to determine other protein concentrations. RK was purified according to [18]. SDS-PAGE was performed according to [19].

$Ca^{2+}$/EGTA buffers were prepared as described in [20]; free [$Ca^{2+}$] in the buffers was determined by using the fluorescent indicator Fura-2 [21] or $Ca^{2+}$-sensitive electrode [22].

RK was assayed as described earlier [9] at 25°C in the reaction mixture (100 $\mu$l) containing 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2 mM $MgCl_2$, 400 $\mu$M [$\gamma$-$^{32}$P]ATP ($6.7 \times 10^5$ cpm/nmol), about 0.5 $\mu$g RK, urea-washed ROS membranes (80 $\mu$g Rh), [Rc] as indicated; $CaCl_2$ and EGTA were added to obtain the free [$Ca^{2+}$] shown in the figures. Immediately after illumination of the mixture (0.6% bleaching of Rh) ATP were added to start the reaction which was stopped 30 min later by the addition of the SDS-PAGE sample buffer. After SDS-PAGE of the samples, zones of Rh were cut out and $^{32}$P incorporation was estimated by Cherenkov counting in plastic tubes. The counting level was 21,000–170,000 and 10,000–12,000 in the bleached and dark samples, correspondingly; nonspecific $^{32}$P binding to the gel strips in the absence of Rh phosphorylation did not exceed 500 cpm/sample.

* Corresponding author. Fax: (7) (095) 939 09 78.
E-mail: ppp@enzlab.genebee.msu.su

*Abbreviations*: ROS, rod outer segment; cGMP, 3′,5′-cyclic guanosine monophosphate; Rh, rhodopsin; RK, rhodopsin kinase; Rc, recoverin; m-Rc and n-Rc, myristoylated and non-myristoylated recombinant recoverin, respectively.


*SSDI* 0014-5793(95)01187-0

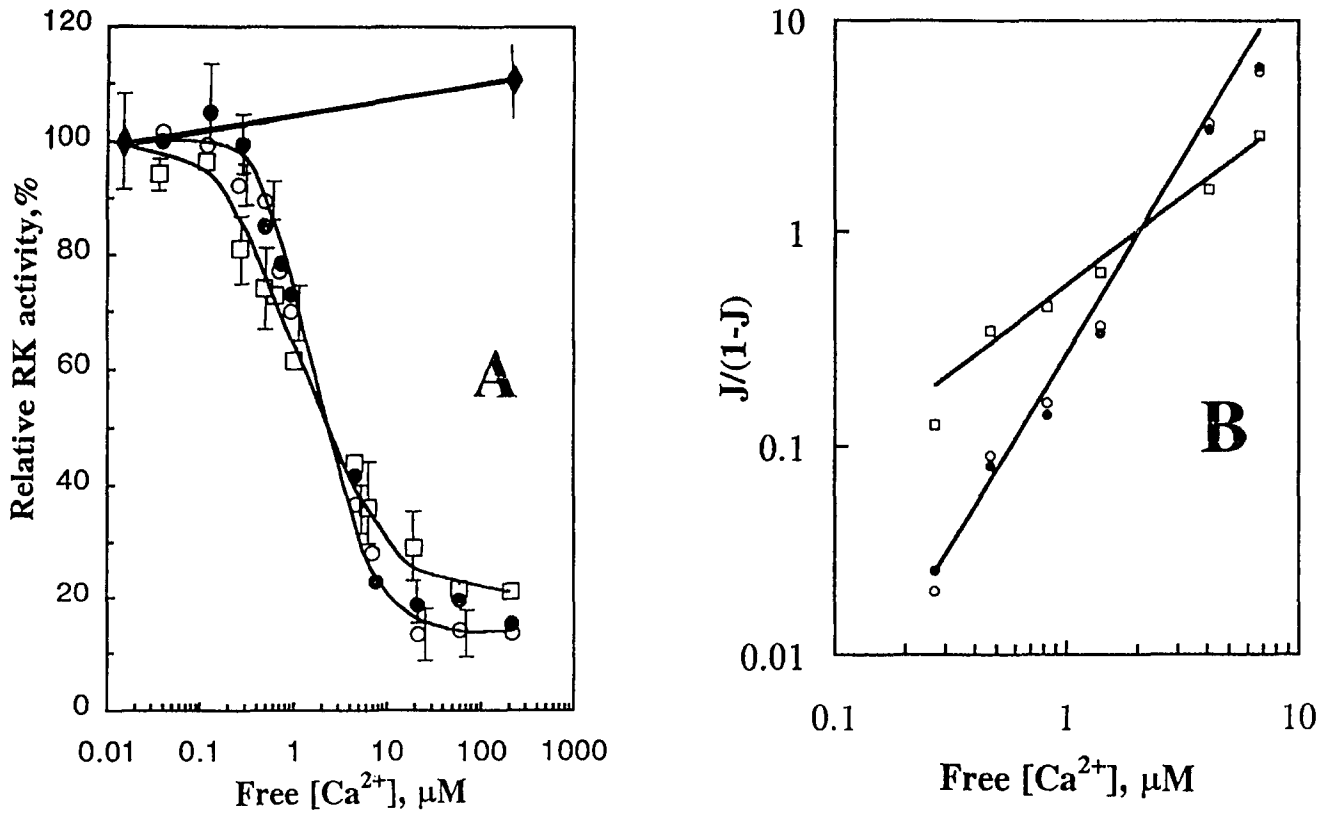


Fig. 1. (A) Dependence of the RK activity on $[Ca^{2+}]$ in the reconstituted sysem consisting of urea-washed ROS membranes (0.6% bleaching of Rh), RK and Rc. The saturated concentrations of Rc were used: 10 $\mu$M retinal (●) and m-Rc (○), 20 $\mu$M n-Rc (□); the level without Rc (♦). Points represent the average values ($n$ = 2) with S.D. bars; the dark level of the activity independent of $[Ca^{2+}]$ was subtracted in the each cases. (B) Corresponding Hill plots of the data.

## 3. Results

Recombinant n-Rc was produced in *E. coli* coinfected with recombinant plasmids encoding Rc. m-Rc was obtained by coexpressing Rc and myristoyl-CoA:protein *N*-myristoyltransferase in *E. coli* in the presence of [9,10($n$)-$^3$H]myristate. Both the proteins purified to >95% homogeneity by chromatography on Phenyl-Sepharose and MonoQ columns (Pharmacia) gave the cross-reaction with antibodies against retinal Rc (data not shown).

One can see in Fig. 1A that phosphorylation of Rh in the content of urea-washed ROS membranes by purified RK does not depend on free $[Ca^{2+}]$ in the absence of Rc. In the presence of Rc, RK becomes $Ca^{2+}$-sensitive: an increase of free $[Ca^{2+}]$ is accompanied with a decrease of the RK activity. $EC_{50}$ for $Ca^{2+}$ is equal to 2 $\mu$M irrespective of the Rc forms used and in all the cases almost full suppression of the $^{32}$P incorporation in Rh is revealed at sufficiently high free $[Ca^{2+}]$. The Hill coefficient of 1.7 in the case of retinal Rc and m-Rc, in comparison with 0.9 for n-Rc, indicates that the effect is cooperative with respect to $Ca^{2+}$ only for the *N*-acylated proteins (Fig. 1B). It is interesting to note that under our experimental conditions the so-called 'high-gain' phosphorylation [23] was revealed if the $^{32}$P incorporation was expressed as a molar ratio to bleached Rh. In accordance with [24] the high-gain effect was fully quenched in the presence of Rc (data not shown).

Investigation of the RK activity dependence on the Rc concentration at saturated free $[Ca^{2+}]$ (Fig. 2) shows that at the saturated Rc concentrations acylated and non-acylated forms of Rc cause an almost equal inhibition of the RK activity. However, the inhibitory action of the acylated Rc manifests at the significantly lower concentration than in the case of its non-acylated form: the $EC_{50}$ values are equal to 1.3 $\mu$M for retinal Rc, 0.9 $\mu$M for m-Rc and 6.5 $\mu$M for n-Rc.

Therefore, one may conclude that although the presence of acyl group on the N-terminus of Rc is not absolutely essential for manifestation of its inhibitory activity in the reaction of Rh phosphorylation, *N*-myristoylation of Rc enhances its efficiency as an inhibitor of the RK activity.

## 4. Discussion

Rc which belongs to a new EF-hand superfamily of $Ca^{2+}$-binding proteins was originally revealed [3,25] due to its ability to bind to immobilized delipidated Rh. Recently crystallographic structure of Rc has been solved [26] and the Rc three-dimensional structure obtained predicts two functional $Ca^{2+}$-binding sites (of the fore EF-hand like structures which are present in the Rc molecule) and a hydrophobic cleft. In the presence of $Ca^{2+}$ the myristoyl residue and the cleft are exposed whereas in the $Ca^{2+}$-free form of Rc they are buried inside the protein molecule [11].

The present data show that in the reconstituted system urea-washed membranes – RK – Rc the increase of free $[Ca^{2+}]$ is followed by the decrease of Rh phosphorylation irrespective of which the form of Rc – retinal, m-Rc or n-Rc – was used.

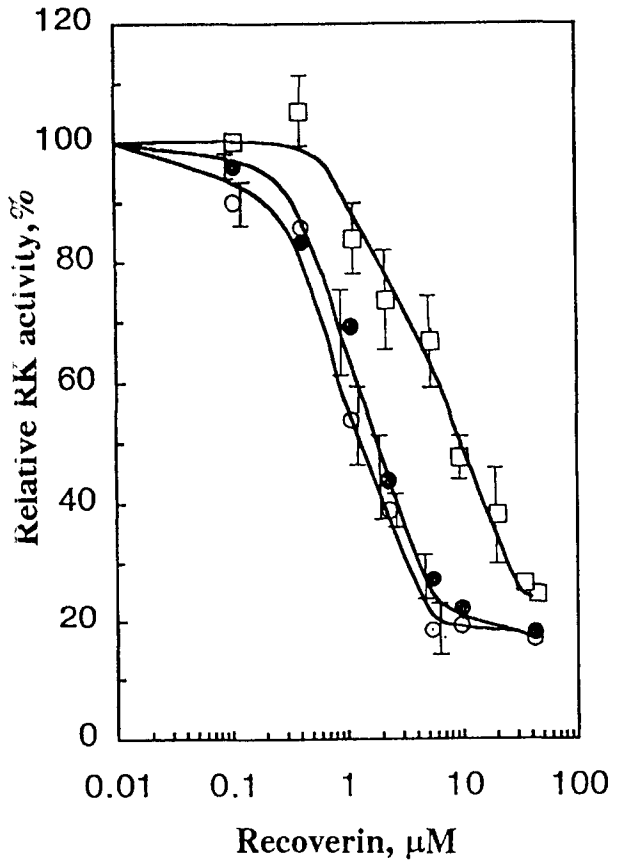


Fig. 2. Dependence of the RK activity on Rc concentration in the reconstituted sysem consisting of urea-washed ROS membranes (0.6% bleaching of Rh), RK and Rc at saturated free [$Ca^{2+}$] (200 $\mu$M). (●), retinal Rc; (○), m-Rc; (□), nRc. Points represent the average values ($n$ = 2) with S.D. bars; the dark level of the activity independent of Rc concentration was subtracted in the each cases.

However, it should be stressed that the Rc potency as an inhibitor of the RK activity is several times higher when N-terminus of Rc is acylated.

One may suggest that a different efficiency of retinal Rc ($EC_{50}$ = 1.3) and m-Rc ($EC_{50}$ = 0.9) as inhibitors of RK is due to the fact that retinal Rc, in contrast to m-Rc, is heterogeneously acylated [10]. Since under our experimental conditions only a small portion of Rc, irrespective of its acylation, was bound to the membranes (our unpublished data) one may suggest that in the reconstituted system a different inhibitory efficiency of retinal Rc and m-Rc, on the one hand, and n-Rc, on the other hand, is due to participation of the acyl residue in the interaction between Rc and RK.

It is interesting to mark that the $N$-acylated Rc manifests cooperativity with respect to $Ca^{2+}$ (Hill coefficient is equal to 1.7) whereas the cooperativity is not revealed in the case of the non-acylated protein (the coefficient = 0.9). A similar effect was demonstrated earlier in the experiments in which $Ca^{2+}$ binding to m-Rc and n-Rc had been compared [27].

Under our experimental conditions the level of Rh phosphorylation decreased as free [$Ca^{2+}$] was elevated. Irrespective of the Rc forms used $EC_{50}$ for $Ca^{2+}$ was equal to 2 $\mu$M that is close to the $EC_{50}$ values which was obtained in the similar reconstituted system [24,28]. However these values are substantially higher than free [$Ca^{2+}$] in the physiological conditions and than the $EC_{50}$ values which were previously communicated for Rc [7–9] -containing systems. It is not clear, however, what is the cause of this discrepancy but it should be marked that (i) the ROS suspension, i.e. more physiological conditions, was used in the most of earlier works, and (ii) the concentrations of free $Ca^{2+}$ shown in these works could be unreliable since the free [$Ca^{2+}$] calculations were used without their direct measurements.

According to [11] the $Ca^{2+}$ affinity of Rc drastically increases as the concentration of the ROS membranes in the reconstituted system approaches the in vivo value. Since under our experimental conditions concentrations of the components, including ROS membranes, are many times lower that in vivo the high value of $EC_{50}$ for $Ca^{2+}$ in the reconstituted system (2 $\mu$M in the present work) does not exclude the possibility of Rc participating in the $Ca^{2+}$-sensitive control of RK in retinal rod cells.

Therefore, under our experimental conditions two functions of $N$-terminal acylation of Rc have been revealed: (i) enhancing the Rc inhibitory efficiency with respect to RK and (ii) conferring cooperativity with respect to $Ca^{2+}$ on the Rc inhibitory effect in the reaction of Rh phosphorylation.

*Acknowledgements*: We are indebted to Dr. B. Shmukler for the isolation of recoverin gene, to Dr. S. Zozulya for gifting the $N$-myristoyltransferase gene, to Dr. V. Zinchenko for helping us in $Ca^{2+}$-measurements, to Dr. A.M. Dizhoor, C.-K. Chen and J.B. Hurley for helpful discussion. This work was supported in part by grants from the Russian Foundation for Basic Research (94-04-11673 and 93-04-7310), the International Science Foundation (MJ9000, MJ9300, MU 5000 and MU 5300), the 'International Projects' of the Ministry of Science of the Russian Federation, the International Soros Science Education Program (I.I.S.).

## References

[1] Gray-Keller, M.P.and Detwiler, P.W. (1994) Neuron 13, 849–861.
[2] Koch, K.-W. and Stryer, L. (1988) Nature 334, 64–66.
[3] Dizhoor, A.M., Ray, S., Kumar, S., Niemi, G., Spencer, M., Brolley, D., Walsh, K.A., Philippov, P.P., Hurley, J.B. and Stryer, L. (1991) Science 251, 915–918.
[4] Lambrecht, H.-G. and Koch, K.-W. (1991) EMBO J. 10, 793–798.
[5] Hurley, J.B., Dizhoor, A.M., Ray, S. and Stryer, L. (1993) Science 260, 740.
[6] Gorodovikova, E.N. and Philippov, P.P. (1993) FEBS Lett. 335, 277–279.
[7] Kawamura, S., Hisatomi, O., Kayada, S., Tokunaga, F. and Kuo, C.-H. (1993) J. Biol. Chem. 268, 14579–14582.
[8] Gorodovikova, E.N., Gimelbrant, A.A., Senin, I.I. and Philippov, P.P. (1994) FEBS Lett. 349, 187–190.
[9] Gorodovikova, E.N., Senin, I.I. and Philippov, P.P. (1994) FEBS Lett. 353, 171–172.
[10] Dizhoor, A.M., Ericsson, L.H., Johnson, R.S., Kumar, S., Olshevskaya, E.V., Zozulya, S., Neubert, T.A., Stryer, L., Hurley, J.B. and Walsh, K.A. (1993) J. Biol. Chem. 267, 16033–16036.
[11] Zozulya, S. and Stryer, L. (1992) Proc. Natl. Acad. Sci. USA 89, 11569–11573.
[12] Dizhoor, A.M., Chen, C.-K., Olshevskaya, E.V., Sinelnikova, V.V., Philippov, P.P. and Hurley,J.B. (1993) Science 259, 829–832.
[13] Hirel, P.H., Schmitter, M.J., Dessen, P., Fayat, G., Blanquet, S. (1989) Proc. Natl. Acad. Sci. USA 86, 8247–8251.
[14] Towler, D.A., Gordon, J.I., Adams, S.P., Glaser, L. (1988) Annu. Rev. Biochem. 57, 69–99.
[15] Schnetkamp, P.P.M., Klompmakers, A.A. and Daemen, F.J.M. (1979) Biochim. Biophys. Acta 552, 379–389.
[16] Aton, B.R., Litman, B.J. and Jackson, M.L. (1984) Biochemistry 23, 1737–1741.

[17] Bradford, M. (1976) Anal. Biochem. 72, 248–254.
[18] Palczewski, K., McDowell, J. and Hargrave, P.A. (1988) J. Biol. Chem. 263, 14067–14073.
[19] Laemmli, U.K. (1970) Nature 227, 680–685.
[20] Sitaramayya, A. and Margulis, A. (1992) Biochemistry 31, 10652–10656.
[21] Tsien, R. and Pozzan, T. (1989) Methods Enzymol. 12, 230–262.
[22] Orchard, C.H., Boyett, M.R., Fry, C.H. and Hunter, M. (1991) pp. 83–113, Oxford University Press, Oxford, New York and Tokyo.
[23] Binder, B.M., Biernbaum, M.C. and Bownds, M.D. (1990) J. Biol. Chem. 265, 15333–15340.
[24] Chen, C.-K., Inglese, J., Lefkowitz, R.J. and Hurley, J.B. (1995) J. Biol. Chem. 270, 18060–18066.
[25] Dizhoor, A.M., Nekrasova, E.R. and Philippov, P.P. (1991) Biokhimiya (in Russ.) 56, 225–228.
[26] Flaherty, K.M., Zozulya, S., Stryer, L. and McKay, D.B. (1993) 75, 709–716.
[27] Ames, J.B., Porumb, T., Tanaka, T., Ikura, M. and Stryer, L. (1995) J. Biol. Chem. 270, 4526–4533.
[28] Klenchin, V.A., Calvel, P.D. and Bownds, M.D. (1995) J. Biol. Chem. 270, 16147–16152.