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One of the Ca^{2+} binding sites of recoverin exclusively controls interaction with rhodopsin kinase

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Abstract

Recoverin is a neuronal calcium sensor protein that controls the activity of rhodopsin kinase in a Ca^{2+}-dependent manner. Mutations in the EF-hand Ca^{2+} binding sites are valuable tools for investigating the functional properties of recoverin. In the recoverin mutant E121Q (Rec^{E121Q}) the high-affinity Ca^{2+} binding site is disabled. The non-myristoylated form of Rec^{E121Q} binds one Ca^{2+} via its second Ca^{2+}-binding site (EF-hand 2), whereas the myristoylated variant does not bind Ca^{2+} at all. Binding of Ca^{2+} to non-myristoylated Rec^{E121Q} apparently triggers exposure of apolar side chains, allowing for association with hydrophobic matrices. Likewise, an interaction surface for the recoverin target rhodopsin kinase is constituted upon Ca^{2+} binding to the non-acylated mutant. Structural changes resulting from Ca^{2+}-occupation of EF-hand 2 in myristoylated and non-myristoylated recoverin variants are discussed in terms of critical conditions required for biological activity.

Keywords: calcium signalling; neuronal calcium sensor; phototransduction.

Introduction

Recoverin belongs to a family of so-called neuronal calcium sensor (NCS) proteins, which can be further divided into five subfamilies entitled the frequenins, visinin-like proteins (VILIPs), recoverins, guanylate cyclase-activating proteins (GCAPs) and Kv-channel interacting proteins (KChIPs) (Braunewell and Gundelfinger, 1999; Burgoyne et al., 2004). These proteins are mainly expressed in the nervous system and sense changes in intracellular Ca^{2+} concentrations. They typically undergo Ca^{2+}-induced conformational changes and control their target proteins in a Ca^{2+}-dependent fashion. Recoverin is among the best-studied NCS proteins and some aspects of its three-dimensional structure and its functional properties are representative of other members of the NCS family (Ames et al., 1996).

Mammalian recoverin and its amphibian orthologue S-modulin are mainly expressed in the photoreceptor cells of the retina (Dizhoor et al., 1991; Lambrecht and Koch, 1991; Kawamura and Murakami, 1991). Recent physiological recordings in transgenic mice lacking recoverin (Makino et al., 2004) confirmed earlier biochemical results, that recoverin inhibits rhodopsin kinase in dark-adapted photoreceptor cells when the cytoplasmic Ca^{2+} concentration is high (Kawamura et al., 1993; Gorodovikova et al., 1994). Relief of inhibition is triggered by the light-induced decrease in cytoplasmic Ca^{2+} concentration and, as a result, the inactivation of photoexcited rhodopsin by phosphorylation is facilitated (Kawamura et al., 1993; Gorodovikova et al., 1994; Chen et al., 1995; Klenchin et al., 1995).

A central aspect of the functional properties of recoverin is the Ca^{2+}-myristoyl switch (Zozulya and Stryer, 1992). Like most other members of the NCS protein family, recoverin is posttranslationally acylated (mainly myristoylated) at its amino-terminal Gly. In the Ca^{2+}-free state, the myristoyl group is buried within a hydrophobic pocket (Tanaka et al., 1995). Sequential binding of Ca^{2+} to its third and second EF-hands (the first and fourth EF-hands are not functional) triggers a conformational change that leads to exposure of the myristoyl group (Permyakov et al., 2000; Senin et al., 2002). Recoverin with an exposed myristoyl group strongly binds to biological and artificial membranes (Zozulya and Stryer, 1992; Dizhoor et al., 1993; Lange and Koch, 1997; Senin et al., 2002). Mutation studies showed that the second EF-hand controls the mean residence time of recoverin at the membranes and thereby has a strong influence on the inhibition of rhodopsin kinase (Senin et al., 2002). Furthermore, the myristoylated form of the mutant E85Q (Rec^{E85Q}), which does not bind Ca^{2+} to EF-hand 2, represents an intermediate state of recoverin during its Ca^{2+}-induced conformational change (Ames et al., 2002; Senin et al., 2002). On the other hand, the myristoylated form of the mutant Rec^{E121Q} (impaired in Ca^{2+} binding to EF-hand 3) is probably locked in the Ca^{2+}-free state, with the myristoyl group completely buried (Senin et al., 2002). According to these observations, an unresolved question is whether binding of Ca^{2+} to EF-hand 2 without prior binding to EF-hand 3 may lead to a functional variant of recoverin and how this behaviour will be modulated by the myristoylation state. We tried to address this question by investigating properties of the myristoylated and non-

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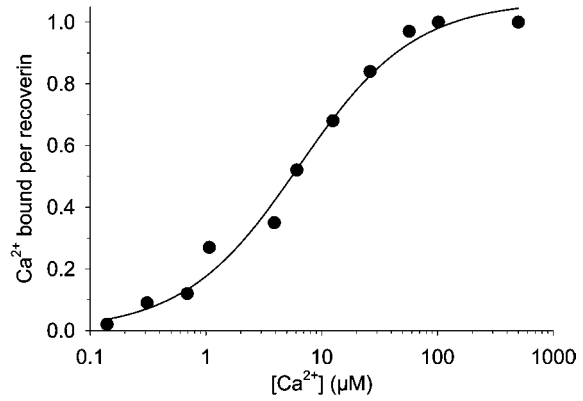

Figure 1 $[^{45}Ca]^{2+}$-binding to the non-myristoylated recoverin mutant Rec^{E121Q}.
Non-myristoylated Rec^{E121Q} was incubated with increasing free Ca^{2+} concentration. Half-maximal binding was achieved at 6.4 μM Ca^{2+}.

myristoylated forms of the mutant Rec^{E121Q}, which has a functional EF-hand 2 and a non-functional EF-hand 3.

Results and discussion

Ca^{2+} binding

Ca^{2+} binding in myristoylated recoverin is believed to occur in a sequential fashion, with the high-affinity EF-hand 3 being occupied prior to the lower-affinity EF-hand 2 (see above). Consistent with this model, it was previously shown that the myristoylated recoverin mutant Rec^{E121Q} does not bind Ca^{2+} up to millimolar concentrations of free Ca^{2+} (Senin et al., 2002). Thus, prevention of Ca^{2+} binding to the third EF-hand keeps the protein in a closed conformation when the myristoyl group is present. Previous work also showed that myristoylated and non-myristoylated recoverin mutant Rec^{E85Q} (second EF-hand disabled) each bind one Ca^{2+} to their intact third EF-hand (Ames et al., 2002; Senin et al., 2002; Weiergräber et al., 2003). According to these results, it has remained unclear whether the non-myristoylated EF-3 mutant Rec^{E121Q} will still bind Ca^{2+} to the low-affinity site. We addressed this question by a direct $[^{45}Ca]^{2+}$ binding assay (Figure 1) and determined an apparent K_d of 6.4 μM for binding of one Ca^{2+} to Rec^{E121Q}, which is consistent with the low-affinity Ca^{2+} binding site with K_d=6–7 μM in wild-type recoverin (Ames et al., 1995).

Interaction with phenyl agarose

In order to gain more insight into the functional interdependence of EF-hands 2 and 3, we investigated some key properties of recoverin, including exposure of critical hydrophobic residues and inhibition of rhodopsin kinase.

Binding of recoverin to phenyl agarose is thought to depend on the Ca^{2+}-induced exposure of hydrophobic residues and not on the presence of the myristoyl group (Zozulya and Stryer, 1992). We applied this assay to test whether the myristoylated and non-myristoylated recoverin variants could bind to phenyl agarose in a fashion

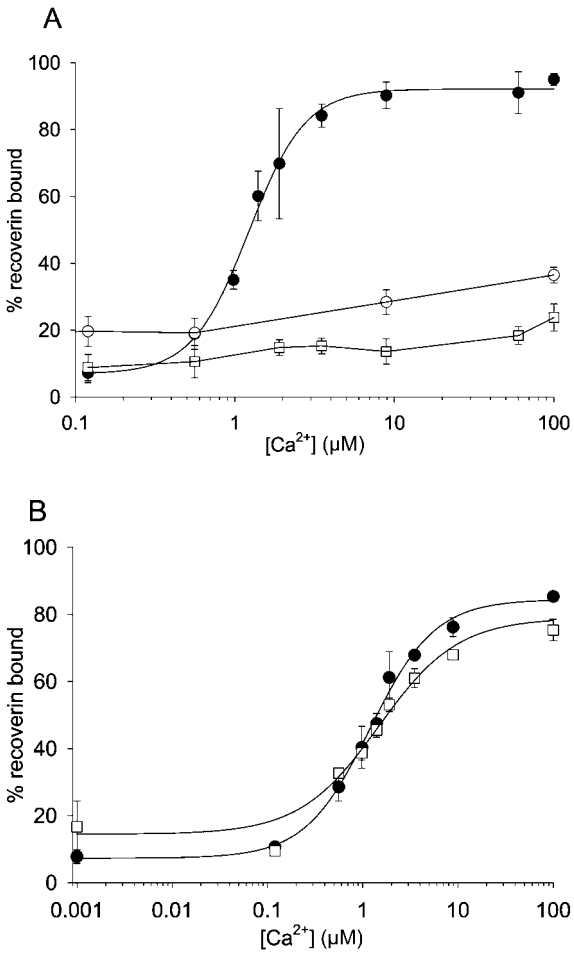

Figure 2 Binding of recoverin and its mutants to phenyl agarose.
(A) Myristoylated wild-type recoverin (●) and myristoylated mutants Rec^{E85Q} (□) and Rec^{E121Q} (○) were incubated with phenyl agarose at different free Ca^{2+} concentrations and the amount of bound protein was determined as described under materials and methods. Half-maximal binding of wild-type recoverin occurred at 1.5 μM Ca^{2+}. Myristoylated recoverin mutants only exhibited some non-specific adsorption to the hydrophobic matrix. (B) Non-myristoylated wild-type recoverin (●) and mutant Rec^{E121Q} (□) bound to phenyl agarose in a similar fashion. In contrast, non-myristoylated Rec^{E85Q} did not bind to phenyl agarose (see Figure 2 in Weiergräber et al., 2003).

similar to the wild type (Figure 2). While binding of myristoylated wild-type recoverin to phenyl agarose was half-maximal at 1.5 μM, similar to the value reported previously (Zozulya and Stryer, 1992), we observed no Ca^{2+}-dependent binding to phenyl agarose for myristoylated Rec^{E85Q} and Rec^{E121Q} (Figure 2A). This result indicates that both mutants do not expose hydrophobic amino-acid side chains necessary for interaction with hydrophobic matrices. However, using the non-myristoylated recoverin forms, a different picture emerged. Non-myristoylated Rec^{E121Q} bound to phenyl agarose, similar to non-myristoylated wild-type recoverin (Figure 2B), which is in contrast to the behaviour of non-myristoylated Rec^{E85Q} that does not bind to phenyl agarose (Weiergräber et al., 2003). Thus, the Ca^{2+}-dependent exposure of critical hydrophobic amino-acid side chains is a function of both the integrity of EF-hands and the myristoylation state.

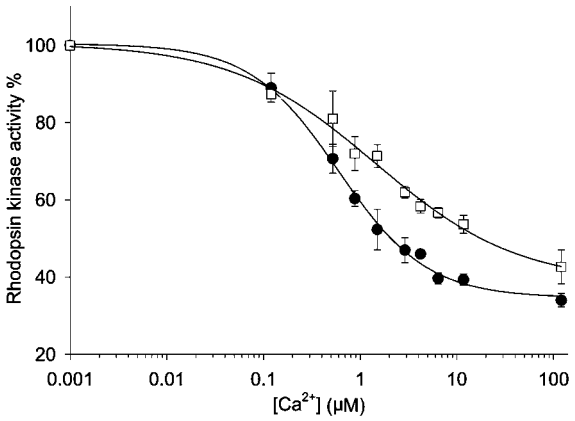

Figure 3 Inhibition of rhodopsin kinase by non-myristoylated wild-type recoverin (●) and mutant Rec^{E121Q} (□) as a function of the free Ca^{2+} concentration.
Inhibition was half-maximal at 0.8 μM Ca^{2+} for wild-type recoverin and at 3 μM Ca^{2+} for Rec^{E121Q}. Myristoylated Rec^{E121Q} did not inhibit rhodopsin kinase (data not shown and Figure 3 in Alekseev et al., 1998).

Control of rhodopsin kinase activity

Myristoylated recoverin mutants Rec^{E85Q} and Rec^{E121Q} and non-myristoylated Rec^{E85Q} are unable to inhibit rhodopsin kinase (Alekseev et al., 1998; Weiergräber et al., 2003). However, non-myristoylated Rec^{E121Q} showed significant inhibition of rhodopsin kinase, with maximum inhibition at high Ca^{2+} concentrations, similar to the wild-type control (Figure 3). The IC_{50} for Ca^{2+} (Ca^{2+} concentration at which inhibition is half-maximal) was shifted from approximately 1 to 3 μM.

Structural implications

The solution structure of myristoylated wild-type recoverin with two bound Ca^{2+} revealed the presence of a large hydrophobic region on the solvent-accessible surface, including aromatic (Phe ^{23}, Trp^{31}, Phe^{35}, Phe^{56}, Phe^{57}, Phe^{83}) and aliphatic (Leu^{28}, Ile^{44}, Leu^{81}, Leu^{90}) side chains (Ames et al., 1997). Mutational studies using the amphibian orthologue S-modulin have led to the identification of a putative interaction surface for rhodopsin kinase, which roughly coincides with the phenyl agarose binding site mentioned above. Based on phenotypic criteria, the seven amino acid residues involved were assigned higher (Phe^{23}, Glu^{27}, Phe^{56}, Thr^{93}) or lower significance (Thr^{21}, Phe^{57}, Lys^{101}) (Tachibanaki et al., 2000).

In myristoylated recoverin, partial unclamping of the acyl chain and reorganisation at the domain interface induced by Ca^{2+} binding to EF-3 is a prerequisite for occupation of EF-2, which finally leads to a large conformational change in the N-terminal domain and complete release of the myristoyl group (Ames et al., 1997). In agreement with this model, we found that myristoylated Rec^{E121Q} is unable to bind any Ca^{2+}. In the absence of the myristoyl chain, however, EF-2 is capable of binding Ca^{2+} even if EF-3 is inactivated. Our results indicate that non-myristoylated Rec^{E121Q} with Ca^{2+} bound to EF-2 only exposes surface features allowing for binding to phenyl agarose, as well as for interaction with rhodopsin kinase (see above). We infer from these findings that the configuration of critical residues under these conditions is likely to be similar to the wild type with two Ca^{2+}. The shift in the IC_{50} of non-myristoylated Rec^{E121Q} with respect to the wild type indicates that Ca^{2+} binding to EF-2 may be slightly facilitated by previous occupation of EF-3, even in the non-myristoylated protein.

Since the surface regions probably mediating phenyl agarose binding and interaction with rhodopsin kinase are located on the N-terminal domain, their exposure is likely to be controlled by Ca^{2+} binding to EF-2. Indeed, the recoverin mutant Rec^{E85Q}, which is unable to bind Ca^{2+} to EF-2, does not display any inhibitory activity with respect to rhodopsin kinase or phenyl agarose binding (Weiergräber et al., 2003). This is true irrespective of the myristoylation state, although we have shown recently that the conformations of the myristoylated and non-myristoylated E85Q mutants differ dramatically (Weiergräber et al., 2003). Figure 4 illustrates the distribution of hydrophobic side chains (upper row) and residues believed to interact with rhodopsin kinase (bottom row) for the myristoylated and non-myristoylated E85Q mutants (Ca^{2+} bound to EF-3 only) and the myristoylated wild type (Ca^{2+} bound to EF-3 and EF-2). The latter is considered representative of the Ca^{2+}-saturated non-myristoylated wild type as well, since the extruded acyl chain does not significantly interact with the protein moiety. Thus, the differences between each of the mutants and the wild type reflect the structural changes induced by occupation of EF-2.

In the case of myristoylated Rec^{E85Q} (left), the distribution of critical residues is clearly very different from the myristoylated wild type with two Ca^{2+} (right). In contrast, the overall structure of the non-myristoylated mutant (middle) is more similar to the wild type, but still displays significant differences, including exposure of the hydroxyl oxygen of Tyr^{86} (indicated by an asterisk) within the hydrophobic patch and steric arrangement of the side chains involved in interaction with rhodopsin kinase.

Our findings support a model in which Ca^{2+} binding to EF-hand 2 in recoverin serves as a master switch exerting immediate control on the structural features governing biological activity. In the myristoylated wild type, occupation of EF-3 enables subsequent binding of Ca^{2+} to EF-2 via conformational changes at the domain interface, whereas in the non-acylated protein this is not strictly required.

Materials and methods

Preparation of ROS and urea-washed ROS

Bovine (rod outer segment) ROS was prepared from fresh bovine retinae and stored at -80°C as previously described (Koch et al., 1994). Urea-washed ROS membranes were prepared by homogenisation of ROS in 5 M urea in 20 mM Tris-HCl (pH 7.5) in the dark (Shichi and Somers, 1978). Then ROS membranes were incubated on ice for 5 min and centrifuged at 100 000 *g* for 40 min at 4°C. The pellet was resuspended and washed three times with buffer A (20 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) and stored at -80°C.

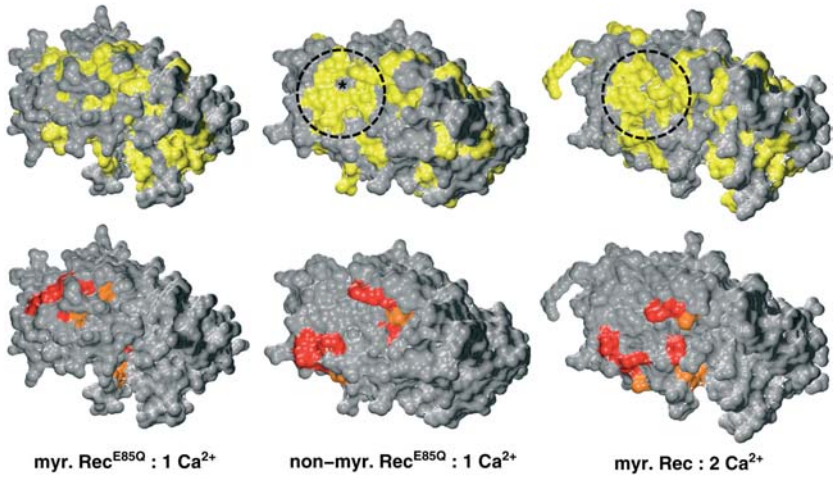

Figure 4 Solvent-accessible surfaces of different recoverin variants revealing structural changes related to Ca^{2+} binding in EF-2. Left to right: myristoylated Rec^{E85Q} with Ca^{2+} in EF-3 (PDB-ID 1LA3; Ames et al., 2002); non-myristoylated Rec^{E85Q} with Ca^{2+} in EF-3 (PDB-ID 1OMV; Weiergräber et al., 2003); and myristoylated wild-type recoverin with Ca^{2+} in EF-3 and EF-2 (PDB-ID 1JSA; Ames et al., 1997). Upper row, distribution of apolar side chains (yellow). The circle marks a hydrophobic patch present in a subset of recoverin structures; the hydroxyl oxygen of Tyr^{86} exposed in non-myristoylated Rec^{E85Q} is indicated by an asterisk. Bottom row, residues critical for interaction with rhodopsin kinase (Tachibanaki et al., 2000) highlighted in red (higher significance) and orange (lower significance). Arrangement of these side chains in the myristoylated and non-myristoylated Rec^{E85Q} mutants with Ca^{2+} bound to EF-3 differ significantly from each other and from the myristoylated wild-type with two Ca^{2+} ions.

Preparation of recoverin and recoverin mutants

Wild-type myristoylated and non-myristoylated recoverin were heterologously expressed in *E. coli* and purified as previously described (Senin et al., 2002). Mutagenesis of recoverin mutants (Rec^{E85Q}, Rec^{E121Q}) was carried out as described by Alekseev et al. (1998). Recombinant myristoylated and non-myristoylated recoverin mutants were heterologously expressed in *E. coli* strain BL21 and purified as before. In order to attach a myristoyl group at the N-terminus of recoverin and recoverin mutants, the plasmid pBB-131 containing the N-terminal myristoyl transferase 1 (NMT1) from *Saccharomyces cerevisiae* (kindly provided by Dr. J.I. Gordon, Washington University School of Medicine, St. Louis, USA) was coexpressed with the corresponding recoverin plasmid (pET11d). The degree of myristoylation was determined by reversed-phase HPLC analysis as described by Hwang and Koch (2002). The analysis was based on a previously published HPLC analysis of recoverin (Sanada et al., 1995). The degree of myristoylation was 95% in the case of the mutant forms.

$[^{45}Ca]^{2+}$ binding assay

All buffers and protein solutions used in Ca^{2+} titration experiments were passed over a Chelex column (Bio-Rad, Munich, Germany) to remove residual amounts of Ca^{2+}. Chelex resin was prepared and equilibrated according to the manufacturer's instructions. Binding of $[^{45}Ca]^{2+}$ to protein samples, which contained 50, 100 or 440 μM protein dissolved in 20 mM HEPES, pH 7.5 (volume 0.5 ml), was determined as previously described (Ames et al., 1995; Senin et al., 2002).

Phenyl agarose binding assay

The phenyl agarose binding assay was carried out according to a published procedure (Zozulya and Stryer, 1992). Briefly, 2 μM wild-type recoverin or its mutants E85Q or E121Q were mixed with 100 μl of phenyl agarose (Sigma, Munich, Germany) and incubated at 37°C (Eppendorf thermomixer 5436, 1000 rpm) for 15 min in 20 mM HEPES (pH 7.5), 150 mM NaCl, 20 mM $MgCl_2$, 1 mM dithiothreitol, 3 mM EGTA and 0–50 mM $CaCl_2$ (total volume 1 ml). The free Ca^{2+} concentration was calculated using the program Webmax 2.0 (Stanford University, CA, USA). The mixture was centrifuged for 15 min (14 000 *g*, table-top centrifuge Eppendorf model 5415), and protein concentration in the supernatant was determined using a Bradford protein assay (Bio-Rad); the amount of bound protein was calculated accordingly.

Rhodopsin phosphorylation

Phosphorylation of rhodopsin was assayed as previously described (Senin et al., 1995) at 25°C in a reaction mixture (150 μl) containing 20 mM Tris-HCl, (pH 7.5), 150 mM NaCl, 3 mM $MgCl_2$, 200 μM [γ-^{32}P]ATP (6–9×10^5 cpm/nmol), approximately 0.7 U of rhodopsin kinase, urea-washed ROS membranes (10 μM rhodopsin) and 3 μM recoverin or its mutants. Immediately after illumination of the mixture (0.2% bleaching of rhodopsin) ATP was added to start the reaction, which was stopped 20 min later by the addition of the 4× 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. The free Ca^{2+} concentration was calculated and adjusted as described above.

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