Drosophila Neurocalcin, a Fatty Acylated, Ca\textsuperscript{2+}-binding Protein that Associates with Membranes and Inhibits in Vitro Phosphorylation of Bovine Rhodopsin\textsuperscript{*}

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Neurocalcins belong to a family of neuronal specific EF hand Ca\textsuperscript{2+}-binding proteins defined by recoverin. Previously, we reported the cloning and initial characterization of neurocalcin in Drosophila melanogaster (Teng, D. H.-F., Chen, C.-K., and Hurley, J. B. (1994) J. Biol. Chem. 269, 31900–31907). We showed that the Drosophila neurocalcin protein (DrosNCa) is expressed in neurons and that bacteriologically expressed recombinant DrosNCa (rDrosNCa) can be myristoylated. Here, we present two lines of evidence that DrosNCa is fatty acylated in vivo. First, the mobility of affinity-purified native DrosNCa on two-dimensional gel electrophoresis is identical to that of myristoylated rDrosNCa and distinct from that of nonacylated rDrosNCa. Second, the membrane binding properties of native DrosNCa are similar to those of myristoylated rDrosNCa; both of these proteins bind to membranes at 0.2 mM Ca\textsuperscript{2+}, whereas nonacylated rDrosNCa always remains soluble. It has been shown that recoverin inhibits the phosphorylation of rhodopsin when Ca\textsuperscript{2+} is present (Kawamura et al., 1993) and that a dependent recoverin/rhodopsin kinase interaction underlies the inhibitory effect of recoverin (Chen et al., 1995). Given the similarities between recoverin and neurocalcin, we examined the effect of DrosNCa on rhodopsin phosphorylation. We find that rDrosNCa is capable of inhibiting bovine rhodopsin phosphorylation in vitro in a Ca\textsuperscript{2+}-dependent manner. The inhibitory activity of rDrosNCa is enhanced by myristoylation, and the potency of its effect is similar to that of recoverin. Two other related EF hand proteins, guanylate cyclase-activating protein-2 and calmodulin, are only poor inhibitors in these phosphorylation assays. In vitro inhibition of rhodopsin phosphorylation therefore appears to be an assayable property of a subset of recoverin-like proteins.

Neurons are highly specialized cells that have evolved elaborate mechanisms to sense, integrate, and transmit signals. The calcium ion (Ca\textsuperscript{2+}) is a universal second messenger that is a key player in neuronal signaling. Dynamic fluxes in the intracellular concentration of Ca\textsuperscript{2+} during neuronal excitation, recovery, and adaptation or potentiation, are interpreted by Ca\textsuperscript{2+}-binding proteins that act as transducers; the binding or release of Ca\textsuperscript{2+} causes these proteins to switch into an "on" or "off" state in the regulation of effector activity.

Many Ca\textsuperscript{2+}-sensing transducers are members of the EF hand superfamily of Ca\textsuperscript{2+}-binding proteins (Moncrief et al., 1990), one of the best characterized being calmodulin. In the past few years, a new and rapidly expanding EF hand subfamily has been defined by recoverin and its cognates (Dizhoor et al., 1991; Hurley et al., 1993). Electrophysiological analyses on mammalian recoverin and frog S-modulin reveal that these photoreceptor proteins prolong photoexcitation in high free Ca\textsuperscript{2+} concentrations (Gray-Keller et al., 1993; Kawamura, 1993). Accumulated biochemical evidence suggests that recoverin/S-modulin delays photorecovery by preventing rhodopsin kinase from phosphorylating rhodopsin in high Ca\textsuperscript{2+} conditions (Kawamura et al., 1993; Chen et al., 1995; Klencin et al., 1995). Indeed, Chen and co-workers (1995) have recently found that recoverin directly binds to rhodopsin kinase at high Ca\textsuperscript{2+} levels. Recoverin is heterogeneously fatty acylated on its amino terminus (Dizhoor et al., 1992), and this posttranslational modification enhances the association of recoverin with membranes in high Ca\textsuperscript{2+} conditions (Dizhoor et al., 1993). However, the presence of a fatty acyl moiety on recoverin is not necessary for its inhibition of rhodopsin kinase (Chen et al., 1995). Flaherty et al. (1993) have shown that the crystallographic structure of recoverin, with Ca\textsuperscript{2+} bound in its EF3 site, is compact and that the protein is composed of two domains, each having one functional EF hand. The three-dimensional structure of recoverin also reveals the presence of a hydrophobic crevice composed of aromatic and aliphatic amino acid residues distributed in the N-terminal half of the protein that may be involved in interactions with a target factor(s). Interestingly, Polans et al. (1991) have found that recoverin is an antigen in the autoimmune response of people inflicted with cancer-associated retinopathy, but its role in this degenerative disease is unclear.

Numerous vertebrate cognates of recoverin have been identified including visinin (Yamagata et al., 1990), multiple isoforms of neurocalcin (Hidaka and Okazaki, 1993; Terasawa et al., 1992), hippocalcin (Kobayashi et al., 1992), and neuronal visinin-related proteins like Vilip (Lenz et al., 1992) and NVPs (Kajimoto et al., 1993). Protein sequence comparisons indicate that recoverin, S-modulin, and visinin define a distinct subclass that is expressed in photoreceptor cells. Another subclass of these neuronaly expressed Ca\textsuperscript{2+}-binding proteins is comprised of bovine neurocalcin, rat hippocalcin, rat NVPs, and chicken Vilip. The evolutionary diversity of these EF hand recoverin-like proteins is further exemplified by the recent discoveries of yet another subclass defined by GCAP-1 (Palczewski et al., 1994) and GCAP-2 (Dizhoor et al., 1995), two mammalian photoreceptor proteins that activate membrane guanylate cyclase when the free Ca\textsuperscript{2+} levels are low.

Two invertebrate homologues of recoverin have been re-
ported to date: Drosophila frequen and neurecalcin. Pongs et al. (1994) identified frequen from studies on the Shaker-like V7 mutants of D. melanogaster. They proposed that the over-expression of frequen in V7 mutants causes the augmented facilitation of neurotransmitter release at neuromuscular junctions. In addition, Pongs et al. reported that recombinant frequen is capable of activating bovine rod outer segment membrane guanylate cyclase in a Ca2+-dependent manner in vitro, but the in vivo target of frequen has not been established. In a search for homologues of frequen in D. melanogaster, we discovered Drosophila neurecalcin (nca), a gene coding for a protein that is 88% identical to the bovine neurecalcin α isoform on the primary sequence level (Teng et al., 1994). Initial characterization revealed that the Drosophila neurecalcin protein (DrosNca) is expressed in neurons and that bacterially expressed recombinant DrosNca could be myristoylated by N- acyl transferase. Here, we present evidences that native DrosNca is fatty acylated in vivo and that this modification significantly enhances its Ca2+-dependent association with Drosophila membranes. In addition, we have found that at high Ca2+ concentrations in reconstituted assays, both recombinant myristoylated and nonacylated DrosNca are capable of inhibiting the phosphorylation of bovine rhodopsin by rhodopsin kinase, but the recombinant DrosNca proteins neither stimulate nor inhibit mammalian photoreceptor membrane guanylate cyclase in high or low Ca2+ conditions.

EXPERIMENTAL PROCEDURES

Materials

Phenyl-Sepharose CL-4B and CNBr-Sepharose were purchased from Pharmacia Biotech Inc. Protein quantitation reagent, amylphosphate, and two-dimensional gel protein standards were obtained from Bio-Rad. (γ-32P)ATP (3000 Ci/mmol) was purchased from DuPont NEN. ECL detection kit for Western analyses was procured from Amersham Corp. Myristoyltransferase was kindly provided by R. Hughes and A. Dizioor.

Methods

Native gel electrophoresis and Western analyses were performed as described by Teng et al. (1994). Two-dimensional gel electrophoresis was done according to the protocols of Bio-Rad. Anti-DrosNca rabbit antibodies 6094 and 6515 were generated and affinity-purified as described previously (Teng et al., 1994).

Purification of Recombinant Recoverin and Neurecalcin

Myristoylated and nonacylated recombinant bovine recoverin (rBovRv) and recombinant Drosophila neurecalcin (rDrosNca) were expressed in E. coli as described by Ray et al. (1992). rDrosNca and rBovRv were isolated as described previously (Teng et al., 1994) with the following modification: proteins that were bound to phenyl-Sepharose CL-4B matrix in buffer containing 1 mM CaCl2 were eluted with 10 mM Tris (pH 7.5), 2 mM MgCl2, 0.2 mM PMSF, 5 mM β-ME, 5 μg/ml aprotinin, and 5 μg/ml leupeptin containing either 2 mM EGTA or 0.2 mM CaCl2. Each homogenate was placed on ice for 10 min and then spun at 100,000 × g for 30 min, 4°C. The resulting supernatant and pellet fractions were analyzed on SDS-PAGE. The concentration of protein was determined by the Bio-Rad protein assay.

Rhodopsin Phosphorylation—Urea-washed rod outer segment membranes were prepared as described in Chen et al. (1995) with modifications. The RK eluate was dialyzed against Tris-HCl 20 mM (pH 7.5), 5 mM MgCl2. The purified enzyme was examined by SDS-PAGE and estimated to be more than 90% pure. The concentration of the protein was determined by the Bio-Rad protein assay.

RESULTS

Myristoylated and Nonacylated Recombinant Drosophila Neurecalcin—To investigate and compare the biochemical properties of the fatty acylated and unmodified forms of DrosNca, milligram quantities of myr-rDrosNca and non- rDrosNca were expressed in E. coli and subsequently purified. The masses of myr-rDrosNca and non-rDrosNca were 21,975.51 and 21,764.17 Da, respectively, as determined by electrospray mass spectrometry, and are consistent with the calculated masses of acylated and unmodified polypeptide products of the nca gene. The isolated proteins were examined by native and SDS-polyacrylamide gel electrophoreses. Under
native conditions (Fig. 1A), the Ca\(^{2+}\)-free (EGTA) forms of the two proteins migrated faster than their Ca\(^{2+}\)-bound forms, and myr-rDrosNCa (m) migrated further than non-rDrosNCa (n). As shown in Fig. 1B, in denaturing SDS-PAGE conditions with the addition of \(\beta\)-ME, both proteins resolved as single bands that migrated more slowly in the absence of Ca\(^{2+}\) than in its presence, and myr-rDrosNCa was slightly faster than non-rDrosNCa.

When myr-rDrosNCa and non-rDrosNCa were subjected to SDS-PAGE without \(\beta\)-mercaptoethanol, the formation of dimers was detected (Fig. 1C). Under these nonreducing conditions, multiple forms of monomeric (arrows) and dimeric (arrowhead) DrosNCa were observed, and their mobilities differed from those of the \(\beta\)-ME-treated proteins (Fig. 1B). Considering that three cysteine residues are present in DrosNCa, these results suggest that rDrosNCa can form intra- and/or intermolecular disulfide bonds. Curiously, the distribution of the monomeric and dimeric species for myr- and non-rDrosNCa were different, suggesting that lipid modification could influence the formation of the disulfide linkages.

Tryptophan Fluorescence—DrosNCa, like bovine neurocalcin, has two tryptophan residues located at positions 30 and 103 in EF1 and EF3, respectively (Teng et al., 1994); these two tryptophans are conserved throughout the recoverin family. The fluorescence change of myr-rDrosNCa upon binding Ca\(^{2+}\) is very similar to the change observed for myristoylated recombinant bovine neurocalcin \(\delta\) (Ladant, 1995).

Compared with the Ca\(^{2+}\)-free form (HEDTA), the maximum fluorescence intensity of myr-rDrosNCa in 0.1 mM free Ca\(^{2+}\) was approximately 2-fold higher, but the emission maximum of 335 nm was not significantly different (Fig. 2A). This change in the amplitude of the signal was irreversible and appeared to be Ca\(^{2+}\)-specific because MgCl\(_2\) did not induce any variation in the fluorescence spectrum of the protein (data not shown). The fluorescence of myr-rDrosNCa in its Ca\(^{2+}\)-bound form was similar that of non-rDrosNCa. However, unlike myr-rDrosNCa, non-rDrosNCa did not show a substantial increase in fluorescence upon Ca\(^{2+}\) binding (Fig. 2B), even when up to 1 mM free Ca\(^{2+}\) was added (data not shown). In contrast, nonacylated bovine neurocalcin \(\delta\) has been reported to undergo a 1.5-fold increase of its fluorescence when it binds Ca\(^{2+}\) (Ladant, 1995). From these data, we infer that the Ca\(^{2+}\)-induced variation of the fluorescence of myr-rDrosNCa is mainly due to a change in the position of its myristoyl group, which influences the environment of one or more of its tryptophan residues.

Native Drosophila Neurocalcin—We have previously shown that recombinant DrosNCa can be myristoylated by N-myristoyl transferase (Teng et al., 1994). Since lipid modification has yet not been demonstrated to occur on Drosophila proteins, we wondered if native DrosNCa was acylated. To address this issue, we purified the protein from wild type Canton-S Drosophila adult extracts by affinity chromatography using a column of affinity-purified AP6515 anti-rDrosNCa antibodies. Fig. 3A shows a sample of the fly head extract (lane 2) that was loaded onto the antibody column and the proteins recovered in the low pH elution (lane 3). A band was detected in the eluted fraction that migrated with an apparent mass of 22 kDa, the expected size for DrosNCa. To ascertain that the eluted 22-kDa band was native DrosNCa, we performed Western blot analyses using AP6094, a different affinity-purified anti-rDrosNCa antibody. Fig. 3B shows that the 22-kDa protein was recognized by AP6094 (lane 3) but not detected by the mouse anti-rabbit secondary antibodies alone (data not shown) and that it was depleted from the flow-through fraction after passage over the AP6515-Sepharose column (lane 2).

Two-dimensional gel electrophoresis was performed to determine if native DrosNCa behaved like myr-rDrosNCa or non-rDrosNCa. As shown in Fig. 4A, myr-rDrosNCa resolved at a more acidic pH relative to non-rDrosNCa in the IEF dimension, and myr-rDrosNCa migrated slightly faster than non-rDrosNCa in the SDS-PAGE dimension as previously observed in Fig. 1. We found that in IEF gels having different gradients of ampholytes, myr-rDrosNCa consistently resolved at a more acidic pH relative to the 5.2 pI, 45-kDa standard (arrow), whereas non-rDrosNCa settled at a more basic pH. This result...
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is consistent with the fact that a fatty acyl modification on DrosNCa results in the loss of one positive charge in the α-amino group of the N-terminal glycine residue. The apparent pl values of both rDrosNCa proteins are close to the calculated pl of 5.1 for the deduced nca polypeptide product.

Previously, we had reported that AP6094 weakly cross-reacted to rat hippocalcin. This observation raised the possibility that these antibodies would detect homologues or different isoforms of DrosNCa in fly preparations. We therefore performed Western analysis on proteins extracted from Drosophila adult heads that had been separated on a two-dimensional gel. As shown in Fig. 4B, only one spot was recognized by AP6094 in the immunoblot. Under these two-dimensional gel conditions, rat hippocalcin was clearly separated from myr-rDrosNCa and non-rDrosNCa (data not shown). Thus, these data suggest that AP6094 specifically recognizes a single antigen in Drosophila adult heads, that being native DrosNCa.

To ascertain if the gel mobility of purified native DrosNCa was similar to either myr-rDrosNCa or non-rDrosNCa, mixtures of equivalent quantities of native and recombinant DrosNCa were separated on two-dimensional gels, transferred, and probed with AP6094. Whereas non-rDrosNCa and the native protein resolved as two distinct spots (Fig. 4C), the immunoblot signal for myr-rDrosNCa and the native protein comigrated (Fig. 4D). From these results, we infer that DrosNCa is fatty acylated in vivo.

Ca$^{2+}$-dependent Translocation of DrosNCa—Studies on recovery (Dizhoor et al., 1993), hippocalcin (Kobayashi et al., 1993), and bovine neurocalcins (Ladant, 1995) have shown that the fatty acylated forms of these two proteins bind to membranes in high Ca$^{2+}$ concentrations. To determine if native DrosNCa undergoes Ca$^{2+}$-dependent translocation, Canton-S adult heads were homogenized in 10 mM NaCl, Tris buffer with either 1 mM CaCl$_2$ or 2 mM EGTA and partitioned by centrifugation, and the resulting pellet and supernatant fractions were analyzed on immunoblots using AP6094 (Fig. 5A). In the presence of Ca$^{2+}$, almost all of DrosNCa was found in the pellet, whereas 70% of the protein was present in the soluble fraction in the presence of EGTA. When a similar experiment was done using 100 mM NaCl, Tris buffer with EGTA, only 40% of DrosNCa was found in the supernatant (data not shown). The data reveal that native DrosNCa binds to membranes and/or cytoskeleton when the levels of free Ca$^{2+}$ are high and that its affinity for membranes increases with the ionic strength of its solvent environment. The translocation properties of DrosNCa are therefore similar to those of recovery, hippocalcin, and bovine neurocalcins.

**Fig. 3.** Purification of native Drosophila neurocalcin. A, native DrosNCa was purified from a soluble extract of Canton-S adult heads by affinity chromatography using an AP6515 anti-DrosNCa antibody column. Fractions obtained from this purification scheme were subjected to SDS-PAGE on a 15% gel stained with Coomassie Blue. Lane 1, molecular mass standards (sizes indicated are in kDa); lane 2, 40 μg of protein from the Canton-S adult head extract; lane 3, approximately 1 μg of protein recovered in the low pH elution. B, Western blot. The following fractions were separated on a 15% SDS-polyacrylamide gel, transferred onto nitrocellulose, and probed with 0.2 mg of AP6094. Lane 1, 40 μg of protein from the soluble Canton-S head extract; lane 2, 40 μg of protein from the flow-through fraction of the AP6515 column; lane 3, 4 μl of the low pH eluate. Based on densitometric scans and comparison to signals of rDrosNCa standards, it was estimated that there was originally 12.5 μg of DrosNCa in the 38 mg of protein in the Drosophila head extract, and approximately 1.7 μg of DrosNCa was recovered in the low pH elution. This corresponds to a yield of 14% from the antibody column.

**Fig. 4.** Two-dimensional gel electrophoresis. A, a mixture of 1.5 μg of non-rDrosNCa (n), 1.5 μg of myr-rDrosNCa (m), and 8 μg of two-dimensional molecular mass standards was first resolved in an IEF gel and then separated on a 15% SDS-polyacrylamide gel; the proteins were stained with Coomassie Blue. B, two-dimensional Western blot of 40 μg of total protein from a soluble fraction of Canton-S adult heads homogenized in 0.5% SDS and 5 mM EGTA. C, two-dimensional immunoblot of a mixture of 5 ng of non-rDrosNCa, 5 ng of purified native DrosNCa, and 8 μg of two-dimensional molecular mass standards. D, a mixture of 5 ng of myr-rDrosNCa, 5 ng of purified native DrosNCa and 8 μg of two-dimensional standards. The blots shown in B–D were initially stained with Ponceau-S to visualize the proteins, and subsequently probed with AP6094 primary antibody and a goat anti-rabbit HRP-conjugated secondary antibody and detected by using chemiluminescence. All of the two-dimensional gels were resolved in the presence of 2.5 mM EGTA.
proteins have similar Ca\textsuperscript{2+} sequences of DrosNCa and BovRv are 54% identical and the two recombinant protein was reconstituted with washed binding abilities of myr-rDrosNCa and non-rDrosNCa. Briefly, translocation was investigated by comparing the membrane binding in the presence of 0.2 mM CaCl\textsubscript{2}, most of the myr-rDrosNCa was located in the pellet, whereas it was almost completely soluble in the presence of EGTA. In contrast, non-rDrosNCa was always found in the supernatant in the presence of EGTA. These results show that the association of recombinant DrosNCa to the particulate fraction is dependent on its fatty acyl modification. Ca\textsuperscript{2+}-dependent inhibition of Bovine Rhodopsin Phosphorylation—It has been shown that recoverin inhibits the in vitro phosphorylation of rhodopsin by rhodopsin kinase (Kawamura et al., 1993; Chen et al., 1995; Klenchin et al., 1995) and that recoverin directly interacts with rhodopsin kinase in a Ca\textsuperscript{2+}-dependent manner (Chen et al., 1995). Since the primary sequences of DrosNCa and BovRv are 54% identical and the two proteins have similar Ca\textsuperscript{2+}-dependent membrane binding properties, we examined if DrosNCa can affect rhodopsin phosphorylation in vitro. Urea-washed bovine rod outer segment membranes were reconstituted with purified recombinant bovine rhodopsin kinase in the presence or absence of myr- or non-rDrosNCa, and light-dependent rhodopsin phosphorylation was analyzed. When 5 \mu M of non- or myr-rDrosNCa were added in the assays, an inhibition of rhodopsin phosphorylation was observed in 0.1 mM Ca\textsuperscript{2+} but not in the presence of 1 mM EGTA (Fig. 6A); 65 and 85% inhibitions were observed for nonacylated and myristoylated proteins, respectively, at 0.1 mM Ca\textsuperscript{2+}. To analyze the potency of the Ca\textsuperscript{2+}-dependent inhibition of rhodopsin phosphorylation by DrosNCa, we compared the activity of non- and myr-rDrosNCa to those of non- and myr-BovRv. Increasing concentrations of the recombinant myristoylated or nonacylated forms of DrosNCa or BovRv were added to reactions in the presence of 0.1 mM Ca\textsuperscript{2+}, and the percentage of inhibition of rhodopsin phosphorylation was determined. Surprisingly, DrosNCa showed inhibitory effects similar to that of BovRv. The plots of both myristoylated (closed squares) and nonacylated (open squares) DrosNCa were basically superimposed with those of myristoylated (closed circles) and nonacylated (open circles) BovRv, respectively (Fig. 6B). The EC\textsubscript{50} values were approximately 0.8 \mu M for the myristoylated proteins and 4 \mu M for the nonacylated proteins. We wanted to determine if the inhibition of rhodopsin phosphorylation was an activity exhibited by other Ca\textsuperscript{2+}-binding proteins.
DISCUSSION

In this paper, we present two lines of evidence indicating that Drosophila neurocalcin is a Ca$^{2+}$-binding protein that is fatty acylated in vivo. First, on two-dimensional gels, native DrosNCa comigrates with myristoylated rDrosNCa and is distinctly separated from nonacylated rDrosNCa. Second, the membrane binding properties of native DrosNCa are similar to those of myr-rDrosNCa; both proteins bind to membranes in high Ca$^{2+}$ conditions and become more soluble when free Ca$^{2+}$ levels decrease. This study therefore provides the first evidence of the existence of protein acylation in Drosophila. Definitive elucidation of the type of lipid modification(s) on DrosNCa in vivo will have to be obtained by mass spectrometry analyses.

The Ca$^{2+}$-dependent membrane association ability of rDrosNCa is greatly enhanced by fatty acylation. It is likely that Drosophila translocation occurs by the Ca$^{2+}$-myristoyl switch mechanism that has been proposed for recoverin (Zozulya and Stryer, 1992; Dizhoor et al., 1993; Hughes et al., 1995; Tanaka et al., 1995). In this model, the fatty acyl moiety of the Ca$^{2+}$-free form of the protein resides in a hydrophobic pocket composed of aromatic and aliphatic amino acid residues distributed in the N-terminal half of the protein. The binding of Ca$^{2+}$ to recoverin induces the extrusion of its lipid moiety and N-terminal α-helix into solution, thereby unmasking the hydrophobic pocket; this conformation favors interactions with membranes, cytoskeleton, and/or a target factor(s). The fluorescence of rDrosNCa in the presence and absence of Ca$^{2+}$ is consistent with the Ca$^{2+}$-myristoyl switch model. First, the variation of fluorescence of myr-rDrosNCa seems to be mainly due to a movement of its myristoyl group, since no major fluorescence change occurs upon Ca$^{2+}$-binding to non-acylated rDrosNCa. Second, the fluorescence of myr-rDrosNCa in its Ca$^{2+}$-bound form is very similar to that of the protein lacking the myristoyl modification. Taken together, these observations suggest that the observed fluorescence change of myr-rDrosNCa upon Ca$^{2+}$-binding is due to the movement of its fatty acyl moiety from a protein environment into solution.

Function of Recoverin-like Ca$^{2+}$ Sensors—Like calmodulin, recoverin and its homologues appear to have descended from a common ancestor that had four EF hand sites. All of the recoverin-like proteins identified to date have sequence determinants in their N termini necessary for fatty acylation, a modification that appears to be important for their Ca$^{2+}$-dependent translocation properties. The discovery of multiple subclasses of neuronal recoverin-like proteins suggest that these cognates perform specialized cellular functions. Several of these cognates are coexpressed in a given neuron. These EF hand proteins have varying affinities for Ca$^{2+}$ (Cox et al., 1994; Ladant, 1995; Chen et al., 1995; Klenchin et al., 1995; Palczewski et al., 1994; Dizhoor et al., 1995), suggesting that their regulatory activities would be performed in different ranges of Ca$^{2+}$ concentrations. It has been proposed that recoverin and S-modulin delay photorecovery by inhibiting the receptor-quenching activity of rhodopsin kinase at high Ca$^{2+}$ levels (Kawamura et al., 1993; Chen et al., 1995; Klenchin et al., 1995). In contrast, GCAP-1 and GCAP-2 appear to promote photorecovery by stimulating membrane guanylate cyclase at low Ca$^{2+}$ concentrations (Palczewski et al., 1994; Dizhoor et al., 1995). Drosophila frequenin has been reported to stimulate bovine photoreceptor membrane guanylate cyclase in low Ca$^{2+}$ conditions (Pongs et al., 1994); however, DrosNCa does not exhibit this activity in similar in vitro assays.2

In this paper, we report that DrosNCa is capable of inhibiting the phosphorylation of bleached rhodopsin in reconstituted assays in a Ca$^{2+}$-dependent manner. We show that its potency is similar to that of recoverin and that its inhibitory activity, like that of recoverin, is enhanced by myristoylation. Our results on BovRv are in agreement with the findings of Chen et al. (1995) but in contrast to Calvert et al. (1995), who report that myristoylated and nonacylated BovRv have similar inhibitory potencies. It seems plausible that differences between our experimental procedures and those of Calvert et al. may account for the discrepancies in results. Three experimental differences stand out in particular: (i) in Calvert et al., 100% of the rhodopsin was bleached during the experiment, whereas we bleached only 0.05% of the rhodopsin; (ii) we used recombinant rhodopsin kinase that is produced using the baculovirus-Sf9 expression system, whereas Calvert et al. extracted the enzyme from bovine photoreceptors; and (iii), the methods of purifying rhodopsin kinase differed, e.g., the rhodopsin kinase preparations of Calvert et al. included transducin, arrestin, and 0.4% Tween 20, whereas our protein preparations were highly purified and did not contain detergent. We have found that detergent can interfere with the Ca$^{2+}$-dependent association of rhodopsin kinase and recoverin.3

Although our experiments do not explore the mechanism by which DrosNCa inhibits rhodopsin phosphorylation by rhodopsin kinase, the similarity between the inhibitory effects of DrosNCa and recoverin suggests that these two proteins act through the same mechanism(s). It has been shown that recoverin and rhodopsin kinase associate in a Ca$^{2+}$-dependent manner and that this interaction is required for the inhibitory effect of recoverin on rhodopsin phosphorylation (Chen et al., 1995). One might therefore postulate that a direct interaction between DrosNCa and rhodopsin kinase is necessary for the inhibition of rhodopsin phosphorylation by DrosNCa. Further investigations are required to test this hypothesis. Ca$^{2+}$-dependent inhibition of rhodopsin phosphorylation has also been observed using another recoverin cognate, hippocalbin, but other recoverin-related proteins like GCAP-2 or the more distant calmodulin are only poor inhibitors in these phosphorylation assays. As such, the capacity of in vitro inhibition of rhodopsin phosphorylation appears to be a newly discovered property of a subset of recoverin-like proteins including recoverin, neurocalcin, and hippocalbin. This is further supported by recent evidence that neuronal recoverin-like proteins of the neuronal calcium sensor family inhibit in vitro phosphorylation of rhodopsin (De Castro et al., 1995).

Rhodopsin kinase belongs to a subfamily of serine/threonine kinases that desensitize activated seven-transmembrane receptor proteins involved in signal transduction. Cognates of rhodopsin kinase include mammalian βARK1, βARK2, and G protein-coupled receptor kinases 4–6, as well as Drosophila G protein-coupled receptor kinases 1–4 (Cassill et al., 1991; Inglese et al., 1993). Given that recoverin-like proteins colocalize with homologues of rhodopsin kinase in neuronal tissues and that several cognates of recoverin inhibit rhodopsin phosphorylation in a Ca$^{2+}$-sensitive manner, it is tempting to speculate that members of these two protein subfamilies interact to confer Ca$^{2+}$-dependent attenuation to seven-transmembrane receptor quenching events. However, at least two issues raise questions.

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2 A. Dizhoor, personal communication.

3 C.-K. Chen, unpublished observations.
about the ability of recoverin-like proteins to control receptor activity in vivo. First, recoverin and its cognates appear to be exclusively expressed in neuronal tissues, whereas GRKs and seven-transmembrane receptors are present in non-neuronal cells as well. Second, the documented data on the inhibitory effects of recoverin and its cognates are based on in vitro studies using mammalian rhodopsin kinase. If the recoverin and rhodopsin kinase subfamilies interact as proposed, one should be able to demonstrate that recoverin-like proteins also inhibit βARK1, βARK2, and/or G protein-coupled receptor kinases 4–6. So far, Chen et al. (1995) have found that recoverin cannot inhibit βARK1 in reconstituted assays.

Neuronal signaling is attenuated by mechanisms involving the universal second messenger Ca\(^{2+}\). Recoverin and its homologues probably function as Ca\(^{2+}\)-sensing regulators in neuronal processes, but their precise mode(s) of action remains unresolved. It is plausible that each recoverin-like protein differentially controls a receptor kinase. Alternatively, it is conceivable that these EF hand proteins, like calmodulin, may interact with multiple targets. Indeed, multiple proteins have been reported to associate with recoverin and neurocalcin in a Ca\(^{2+}\)-dependent manner (Chen et al., 1995; Okazaki et al., 1995). Further studies are therefore required to elucidate the physiological roles of this diverse class of Ca\(^{2+}\)-binding proteins.

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