Structure-Function Relationships and Localization of the Na/Ca-K Exchanger in Rod Photoreceptors*

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The structural and functional properties of the bovine rod photoreceptor Na/Ca-K exchanger and its distribution in vertebrate photoreceptor cells were studied using a panel of monoclonal antibodies. Antibodies that bind to distinct epitopes along the large hydrophilic N-terminal segment of the exchanger labeled the extracellular surface of the rod outer segment plasma membrane, whereas antibodies against a large hydrophilic loop between the two membrane domains labeled the intracellular side. Enzymatic deglycosylation studies indicated that the exchanger primarily contains O-linked sialo-oligosaccharides located within the N-terminal domain. Removal of the extracellular domain with trypsin or the large intracellular domain with kallikrein did not alter the Na⁺- or K⁺-dependent Ca²⁺ efflux activity of the exchanger when reconstituted into lipid vesicles. Anti-exchanger antibodies were also used to visualize the distribution of the exchanger in the retina by light and electron microscopy. The exchanger was localized to the plasma membrane of rod outer segments. No labeling was observed in the disk membranes, cone photoreceptor cells, or other retinal neurons, and only faint staining was seen in the rod inner segment. These results indicate that the O-linked glycosylated rod Na/Ca-K exchanger is specifically targeted to the plasma membrane of rod photoreceptors and has a topological organization similar to that reported for the cardiac Na/Ca exchanger. The large intracellular and extracellular domains do not directly function in the transport of ions across the rod outer segment plasma membrane, but instead may play a role in protein-protein interactions that maintain the spatial organization of the exchanger in the plasma membrane or possibly regulate transport activity of the exchanger.

The Na/Ca-K exchanger, together with the cGMP-gated channel, plays a crucial role in vertebrate phototransduction and light adaptation by controlling the dynamic level of cytoplasmic Ca²⁺ in the outer segments of rod and cone photoreceptor cells. In the dark, Ca²⁺ entering the outer segment through cGMP-gated cation channels is removed by the Na/Ca-K exchanger, thereby maintaining a steady state cytoplasmic Ca²⁺ level in the range of 220–550 nM (1, 2). Photocexcitation of rhodopsin and activation of the visual cascade system results in the closure of the cGMP-gated channels in the ROS plasma membrane and a hyperpolarization of the photoreceptor cell. Continued extrusion of Ca²⁺ from the ROS by the Na/Ca-K exchanger leads to a significant decrease in intracellular Ca²⁺. This reduction in Ca²⁺ results in the activation of guanylate cyclase by a guanylate cyclase-activating protein called GCAP (3), regulation of channel sensitivity to cGMP by calmodulin (4), and modulation of the phototransduction process by recoverin (5), thereby mediating photorecovery and light adaptation (6).

The rod Na/Ca-K exchanger has been purified from bovine ROS (7, 8), and its primary structure has been determined from its cDNA sequence (9). It has a predicted molecular mass of 130 kDa, but migrates anomalously on SDS-polyacrylamide gels with an apparent mass of ~230 kDa due, in part, to posttranslational glycosylation (10). The rod exchanger is localized in the plasma membrane of ROS (10), where it has recently been shown to exist as a dimer (11).

The rod exchanger exhibits some similarity in structure and function to the cardiac Na/Ca exchanger (NCX1) and its isoforms (NCX2 and NCX3) found in the other tissues (12). Hydropathy profiles suggest that both types of transporters have two membrane domains, each consisting of six transmembrane segments and a large hydrophilic, negatively charged loop connecting the two membrane domains (9, 12). The first membrane spanning segment serves as a cleavable signal sequence and is absent in the mature proteins (9, 13). Both the rod and cardiac exchangers are electrogenic transporters (14, 15), coupling the influx of Na⁺ with the efflux of Ca²⁺ across the cell membrane.

There are, however, notable differences in the structure and function of these two types of exchangers. The rod Na/Ca-K exchanger and the cardiac Na/Ca exchanger show no significant similarity in their primary structure (9, 12). In addition, the rod exchanger contains a large hydrophilic segment at its N terminus that is absent in the other Na/Ca exchangers. At a functional level, the rod exchanger utilizes the energy derived from the influx of 4 Na⁺ and efflux of 1 K⁺ to transport 1 Ca²⁺ against its electrochemical gradient (16, 17). In contrast, the cardiac exchanger and related isoforms are potassium-independent, coupling the influx of 3 Na⁺ to the efflux of 1 Ca²⁺ from the cell (18, 19).

To date there is little direct experimental evidence for the topological organization of the rod exchanger in the ROS plasma membrane, and information concerning the role of specific domains in transport activity of this exchanger is lacking. This is due largely to the limited success in expressing the rod exchanger in heterologous cells for structure-function analyses.

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1 The abbreviations used are: ROS, rod outer segment; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; mAb, monoclonal antibody; DTT, dithiothreitol; GST, glutathione S-transferase; bp, base pair(s).
In this study, we have generated a panel of monoclonal antibodies to specific regions of the bovine rod Na/Ca-K exchanger. These antibodies have been used as probes: 1) to study the topological organization and glycosylation of the exchanger in the ROS plasma membrane, 2) to examine the role of the hydrophilic N-terminal and a large hydrophilic internal domain on sodium- and potassium-dependent Ca^{2+} transport activity of the rod exchanger, and 3) to map the cellular and subcellular distribution in retinal tissue.

**EXPERIMENTAL PROCEDURES**

**Preparation of ROS Membranes**—Bovine ROS were isolated under dim red light from freshly dissected or previously frozen retinas by sucrose density gradient centrifugation and used immediately or stored at −70 °C in light-tight vials (20). ROS membranes were obtained by centrifugation of ROS hypotonically lysed in 10 volumes of 10 mM Hepes-KOH buffer, pH 7.4, containing 1 mM EDTA and 1 mM DTT. The membranes were washed twice in 10 volumes of the same buffer without EDTA by centrifugation at 80,000 × g for 10 min.

**Purification of the Na/Ca-K Exchanger**—The Na/Ca-K exchanger was purified from CHAPS-solubilized ROS membranes by DEAE-Fractogel TSK ion exchange chromatography followed by Af Red-Fractogel TSK chromatography according to the method of Cook and Kaupp (7). For these studies, ROS membranes were solubilized at a concentration of 1 mg/ml in 18 mM CHAPS, 10 mM Hepes-KOH buffer, pH 7.4, 2 mM CaCl₂, 150 mM KCl, and 1 mM DTT prior to exposure to light.

**Generation of the Monoclonal Antibodies**—Monoclonal antibodies were generated from BALB/c mice immunized with either ROS plasma membranes or the purified exchanger as described (21). The PMe 1B3 anti-exchanger antibody has been reported previously (10).

**Constitution and Expression of GST Fusion Proteins**—Bovine retinal Na/Ca-K exchanger cDNA (a generous gift of Dr. Helmut Reiländer) was digested with the indicated restriction enzymes and subcloned in frame into the appropriate pGEX expression vector (Pharmacia) for the production of GST fusion proteins (22). The plasmids were constructed as follows: pbEX1, pbEX2, pbEX3, pbEX4, HindIII to XhoI fragment (bp 524–872) was subcloned into the XhoI fragment (bp2168–2184) was subcloned into the BamHI/SmaI sites of pGEX 3X; pbEX2, AluI fragment (bp 248–524) was subcloned into the SmaI site of pGEX 2T; pbEX3, AluI fragment (bp524–872) was subcloned into the SmaI site of pGEX 2T; pbEX4, HindIII to ScaI fragment (bp 1010–1408) was blunt-ended with Klenow and subcloned into the SmaI site of pGEX 2T; pbEX5, Sau3AI to SmaI fragment (bp 95–445) was subcloned into the BamHI/SmaI sites of pGEX 3X; pbEX6, AluI fragment (bp 2266–2569) fragment was subcloned into the SmaI site of pGEX 1; and pbEX7, AluI fragment (bp 2620–2716) fragment was subcloned into the SmaI site of pGEX 1. The inserts were confirmed by DNA sequencing using Sequenase version 2.0 (U. S. Biochemical Corp.).

**Functional Reconstitution of the Exchanger**—CHAPS-solubilized ROS membranes were reconstituted into liposomes for analysis of Na⁺/Ca²⁺ exchange as described by Cook and Kaupp (7). Briefly, the solubilized membranes (1 mg/ml protein) were added to an equal volume of soybean 1,α-phosphatidylincholine (Sigma type IV) to give a final concentration of 10 mg/ml lipid, 10 mM CHAPS, 10 mM Hepes-KOH buffer, pH 7.4, 100 mM KCl, 2 mM CaCl₂, and 1 mM DTT. The mixture was dialyzed for 18 h against three changes (1 liter each) of reconstitution buffer consisting of 5 mM Hepes-KOH buffer, pH 7.4, 100 mM KCl, and 2 mM CaCl₂. The transmembrane Ca²⁺ gradient was established by first dialyzing the liposomes for 2 h against 1 liter of reconstitution buffer without CaCl₂ and then passing the vesicles through a BioRad (Bio-Rad) ion exchange column equilibrated in the same buffer.

**Calcium efflux assays** were carried out by adding 0.3 ml of liposomes to 1.7 ml of Ca²⁺-free reconstitution buffer containing 50 μM arsenazo III, 2 μM carbonyl cyanide p-trifluoromethoxyphenylhydrazone, and 2 μM valinomycin. Calcium release was initiated by the addition of a known concentration of NaCl and monitored spectrophotometrically using a SLM Aminco DW2000 spectrophotometer in the dual wavelength mode (650–730 nm).

**SDS-Polyacrylamide Gels, Western Blotting, and Protein Concentrations**—Samples were routinely separated on a 8% SDS-polyacrylamide gel and either stained with Coomassie Blue or electrophoretically transferred onto Immobilon-P (Millipore, Bedford, MA) in 25 mM Tris buffer, pH 7.4, containing 190 mM glycine and 50 mM magnesium sulfate. After blotting, the membranes were blocked in phosphate-buffered saline (0.1 M phosphate buffer, pH 7.5, 136 mM NaCl, and 2 mM KCl) containing 0.5% milk and then incubated for 1 h at 25 °C with the mAb hybridoma culture fluids diluted 20-fold with phosphate-buffered saline containing 0.05% milk. After washing the membranes in phosphate-buffered saline, the blots were incubated with sheep anti-mouse immunoglobulin (Ig) conjugated to horseradish peroxidase at 1/5000 dilution. Antibody labeling was detected by enhanced chemiluminescence (Amersham). Protein concentrations were determined by the bichinchoninic acid assay (Pierce) for ROS preparations and by the modified Bradford assay (23) for solubilized ROS membranes. Bovine serum albumin was used as standards in both assays.

**Peptide Synthesis**—The epitopes for some of the mAbs were more precisely mapped using the Epitope Scanning kit (Cambridge Research Biochemicals, Northwick, UK). For these studies, nine amino acid peptides with seven-amino acid overlap were synthesized for analysis by enzyme-linked immunosorbent assays.

**Limited Proteolysis and Deglycosylation**—Intact ROS (1 mg/ml protein) in 2 ml of 10 mM Hepes-KOH buffer, pH 7.4, 20% sucrose, 2 mM KCl and 2 mM CaCl₂ were digested with 2 μg tosylphenylalanylamidomethyl ketone-treated trypsin (Sigma) for 30 min at 25 °C. The ROS were washed three times by centrifugation at 4000 × g for 5 min in the same buffer containing 10 μg/ml soybean trypsin inhibitor. ROS membranes (1 mg/ml protein) in 3 ml of 10 mM Hepes-KOH buffer, pH 7.4, containing 2 mM CaCl₂ and 1 mM DTT were digested with 2 ml of 5 units/ml porcine pancreatic kallikrein (Sigma) for 20 min at 25 °C. The membranes were then washed five times with 0.5 ml of ice-cold 10 mM Hepes-KOH buffer, pH 7.4, containing 1 mM DTT and 100 μg/ml phenylmethylsulfonyl fluoride.

**Deglycosylation studies**, 2.1 mg of ROS in 50 mM sodium acetate, pH 5.2, containing 20% sucrose were first treated with 10 millions of Arthrobacter ureafaciens neuraminidase (Boehringer Mannheim) for 1 h on ice. The ROS membranes were then solubilized in buffer containing 18 mM CHAPS, 10 mM Hepes-KOH buffer, pH 7.4, 150 mM KCl, and 2 mM CaCl₂ and incubated with 100 μl of PMe 2A11-Sepharose 2B (1.5 mg antibody/ml beads) for 30 min at 4 °C. After the matrix was washed six times with CHAPS buffer without DTT, the bound exchanger was treated with 0.5 milliunits of Diplococcus meningosepticum neuraminidase (Boehringer Mannheim) in 20 mM sodium ccdycate-maleate buffer, pH 6.0, for 1 h at 28 °C and eluted with 3% SDS. In some studies, the neuraminidase- and O-glycosidase-treated exchanger, neuraminidase-treated ROS, or untreated ROS were solubilized in 50 mM sodium phosphate buffer, pH 7.5, containing 1% SDS, 1% Nonidet P-40, and 0.5% β-mercaptoethanol and incubated with 1000 units of Flavobacterium meningosepticum endo-N-glycosidase F (New England Biolab) for 1 h at 28 °C.

**Immunolabeling for Fluorescent and Electron Microscopy**—For immunofluorescence microscopy, 10-μm cryosections of bovine retina fixed in 100 mM sodium cacodylate-maleate buffer, pH 7.4, for 1 h on ice. The Rod Na/Ca-K Exchanger.
cocadylate-HCl, pH 7.4 buffer containing 2% gluteraldehyde and 0.2% sucrose for 1 h, post-fixed with 1% osmium tetroxide in the same buffer for 1 h, and embedded in Epon-Araldite resin (Polysciences, Inc., Warrington, PA). In some studies, intact bovine retina tissue was fixed with 1% glutaraldehyde in 100 mM cacodylate-HCl buffer, pH 7.2, containing 100 mM sucrose for 1 h at 4 °C. After rinsing the tissue in the same buffer in the absence of glutaraldehyde, the sample was blocked with 1% glycine and 2% bovine serum albumin in PBS and then labeled with the mAb PMe 2D9 conjugated to gold-dextran particles 10 nm for 4 h (25). The samples were then prepared as described above and viewed under a JEOL 1200EX electron microscope.

RESULTS

Characterization of Monoclonal Antibodies to the Rod Na/Ca-K Exchanger—Eight mAbs to the bovine Na/Ca-K exchanger were generated and initially characterized by Western blotting. As shown in Fig. 1 for antibodies PMe 2D9 and PMe 2A11, the mAbs all specifically labeled the 230-kDa exchanger in both ROS and a highly purified preparation of the exchanger. In addition, the antibodies labeled several smaller proteins that were most evident in preparations derived from frozen retinas. Five mAbs (PMe 2D9, PMe 4G1, PMe 3D12, PMe 7A5, and PMe 6E2) labeled a 190-kDa protein, whereas three mAbs (PMe 2A11, PMe 1B3, and PMe 4G7) labeled 150-kDa and 75-kDa proteins (Fig. 1). These lower molecular mass proteins are generally believed to be proteolytic fragments of the exchanger (10).

Binding Sites for Na/Ca-K Exchanger Monoclonal Antibodies—In order to use the mAbs as probes for structure-function analysis of the exchanger, it was necessary to first map the regions on the exchanger where the antibodies bind. This was accomplished by expressing a series of GST fusion proteins containing selected segments of the exchanger in E. coli (Fig. 2A). As shown in Fig. 2B, the PMe 2D9 antibody specifically labeled overlapping fusion proteins EX1 and EX2, indicating that this antibody binds to an epitope between residues 83 and 148 of the exchanger. The PMe 2A11 antibody labeled EX6 and EX7, but not the other fusion proteins. The EX6 and EX7 fusion proteins do not overlap, but they both contain repeat sequences previously shown to reside in a large segment between putative transmembrane segments 5 and 6 (9). The PMe 2A11 epitope was more precisely identified as the DEDEGEIQA sequence within the repeat region of the exchanger by analyzing PMe 2A11 reactivity to a series of overlapping nine amino acid synthetic peptides.

Similar reactivity studies were carried out for the other mAbs. The fusion proteins and the regions on the exchanger where the various mAbs bind are listed in Table I.

Identification of Intracellular and Extracellular Regions of the Exchanger—mAbs were used with pre-embedding immunogold labeling methods to identify regions of the exchanger that are exposed on the intracellular and extracellular side of the ROS plasma membrane. When hypotonically lysed ROS were labeled with the PMe 2A11 antibody, immunogold particles were localized on the intracellular side of the plasma membrane, i.e. the same side of the membrane to which the disks are attached (Fig. 3A). A similar pattern of labeling had been reported previously for mAb PMe 1B3 (10). In contrast, the PMe 2D9, PMe 3D12, and PMe 6E2 antibodies specifically
labeled the extracellular surface of the plasma membrane when either intact or lysed ROS preparations were used (Fig. 3, B–D). Disk membranes were not labeled with any of the exchanger antibodies. No labeling was observed with the PMe 4G7 antibody, most likely due to the inaccessibility of this epitope in the membrane-bound form of the exchanger.

These results indicate that the N-terminal region of the exchanger containing the binding sites for the PMe 2D9, PMe 3D12, and PMe 6E2 antibodies is localized on the extracellular surface of the ROS plasma membrane, whereas the large internal segment of the exchanger containing the PMe 2A11, PMe 1B3, and PMe 4G7 epitopes is exposed on the intracellular side. These results also confirm earlier studies, indicating that the exchanger is not present in detectable quantities in disk membranes (10).

**Distribution of the Exchanger in Rod Photoreceptor Cells—**
The distribution of the exchanger in bovine retina was visualized by immunofluorescence microscopy. As shown in Fig. 4A, the PMe 2A11 mAb specifically labeled the outer segment layer of the retina. Very dim labeling could be observed in the inner segment upon prolonged exposure (not shown), but other layers of the retina were not labeled. The labeling was specific since no fluorescence was observed when the retina was labeled with the PMe 2A11 antibody in the absence (A) or in the presence (C) of competing peptide. The rod outer segment (ROS), rod inner segment (RIS), and nuclear layers (N) are shown in the differential interference contrast micrograph (B).

The distribution of the exchanger along the plasma membrane of bovine retinal photoreceptor cells was examined by pre-embedding immunogold labeling for electron microscopy. When fixed bovine retina was labeled with the PMe 2D9 antibody, gold particles were found to be distributed along the extracellular surface of the rod photoreceptor outer segment (Fig. 5A). The basal region of the outer segments where new disks form was also labeled, although less intensely (Fig. 5B). Only an occasional gold particle was seen along the plasma membrane of the rod inner segment (RIS). Mitochondria within the inner segment of rods and cones are indicated (m). Bar, 500 nm.

**Enzymatic Deglycosylation—** Previous biochemical studies have shown that the rod Na/Ca-K exchanger is a sialoglycoprotein (10). In order to determine if the rod exchanger contains O-linked or N-linked sialo-oligosaccharides, the exchanger was first treated with neuraminidase and subsequently incubated with either endo-0-glycosidase or endo-N-glycosidase. As shown in Fig. 6, neuraminidase treatment resulted in a decrease in the apparent molecular mass of the

| mAb          | Fusion protein(s) labeled | Deduced epitope residues/sequence | Electron microscopy localization |
|--------------|---------------------------|----------------------------------|---------------------------------|
| PMe 4G1      | EX1                       | 66–74                            | Extracellular                   |
| PMe 2D9      | EX1 and EX2               | 83–148                           | Extracellular                   |
| PMe 7A5      | EX1 and EX2               | 83–148                           | Extracellular                   |
| PMe 3D12     | EX3                       | 175–289                          | Extracellular                   |
| PMe 6E2      | EX4                       | 342–350                          | Extracellular                   |
| PMe 4G7      | EX5                       | 600–728                          | NA*                             |
| PMe 1B3      | EX6                       | 756–856                          | Cytoplasmic                     |
| PMe 2A11     | EX6 and EX7               | Repeat                           | Cytoplasmic                     |

* NA, not available.
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In this study, a panel of monoclonal antibodies was generated against two immunodominant regions of the bovine rod exchanger from 230 to 205 kDa as previously reported (10). Subsequent treatment with O-glycosidase resulted in a further reduction in the apparent molecular mass to 180 kDa (Fig. 5, lane c). Endo-N-glycosidase F had no observable effect on the migration behavior of the untreated, neuraminidase-treated, or O-linked deglycosidase-treated exchanger (data not shown). These results indicate that the rod exchanger contains primarily O-linked sialo-carbohydrate chains, and this posttranslational modification contributes significantly to its migration on SDS-polyacrylamide gels.

Effect of Limited Proteolysis on the Exchanger—The panel of anti-exchanger mAbs was used to identify regions of the exchanger in ROS that are accessible to cleavage by specific proteases. When intact ROS were treated with trypsin under relatively mild conditions and the membrane-bound fragments were analyzed for antibody reactivity by Western blotting, the extracellular epitopes for the PMe 4G1, PMe 2D9, PMe 3D12, and PMe 6E2 antibodies were removed (shown in Fig. 7A for the PMe 2D9 antibody). In contrast, the intracellular epitopes for antibodies PMe 2A11, PMe 1B3, and PMe 4G7 were preserved. These mAbs intensely labeled membrane-bound fragments of 150 kDa, 135 kDa, and 75 kDa (shown in Fig. 7B for PMe 2A11). Extended proteolysis decreased the amount of the 150-kDa fragment and increased the amount of the 75-kDa fragment, indicating that the latter fragment was most likely derived from proteolysis of the 150-kDa fragment.

The effect of limited proteolysis on antibody reactivity was also studied for hypotonically lysed ROS in which the cytoplasmic side of the plasma membrane is accessible. Porcine kallikrein was found to remove the epitopes for the PMe 2A11, PMe 1B3, and PMe 4G7 antibodies (shown in Fig. 7B for PMe 2A11). The mAbs PMe 4G1, PMe 2D9, PMe 3D12, and PMe 6E2 against extracellular epitopes, however, all labeled a 180-kDa membrane-bound fragment.

These results indicate that a large part of the N-terminal extracellular domain of the exchanger, extending up to and possibly beyond amino acid residue 350 (the epitope for PMe 6E2), is removed when intact ROS are digested with trypsin and a significant portion of the large intracellular loop encompassing epitopes for antibodies PMe 4G7 through PMe 2A11 (see Fig. 10) is selectively removed when lysed ROS are treated with kallikrein.

Effect of Limited Proteolysis on Na/Ca-K Exchange Activity—The effect of trypsin and kallikrein treatment on the transport activity of the exchanger was determined by reconstituting the respective membrane fragments into Ca²⁺-containing lipid vesicles and monitoring Na⁺-dependent Ca²⁺ efflux, spectrophotometrically. As shown in Fig. 8(A and B), removal of the extracellular domain with trypsin or the intracellular domain with kallikrein did not significantly alter the Na⁺-dependent Ca²⁺ transport activity of the exchanger. The apparent $K_m$ values for the untreated, trypsin-treated, and kallikrein-treated exchanger were 46.4 ± 4.4 mM (six experiments), 54.4 ± 3.8 mM (three experiments), and 42.3 ± 7.7 mM (three experiments), respectively. The cooperativity of the exchanger for Na⁺, as depicted by the sigmoidal curve, was also similar. The untreated exchanger had a Hill coefficient ($n$) of 3.0 ± 0.4 (6 experiments), whereas the trypsin- and kallikrein-treated exchangers had $n$ values of 2.9 ± 0.2 (three experiments) and 3.1 ± 0.2 (three experiments), respectively.

The $V_{max}$ of both the trypsin- and kallikrein-treated exchangers increased by 2-fold relative to the untreated exchanger. This increase in activity was not due to more efficient detergent solubilization and reconstitution of the exchanger since a similar increase in activity of the exchanger was observed if the exchanger was first reconstituted into lipid vesicles and then treated with the protease (data not shown). A decrease in the activity, however, was observed when the exchanger was subjected to more extensive proteolysis, i.e. higher protease concentrations or prolonging the digestion times.

The potassium dependence of the proteolyzed exchanger was also examined. Removal of the intracellular loop with kallikrein had no effect on the potassium dependence of rod exchanger in the presence or absence of a potassium gradient (Fig. 9, A–C). However, a potassium gradient increased the activity of the both the untreated (data not shown) and kallikrein-treated exchanger. These studies indicate that removal of the intracellular loop does not affect either the K⁺-dependent or -independent Na/Ca transport activity or K⁺ transport through the exchanger.

DISCUSSION

In this study, a panel of monoclonal antibodies was generated against two immunodominant regions of the bovine rod...
treated exchanger (icles in100 m M choline chloride for activity assays in the absence or presence of 2.5 mM symmetrical KCl. In each case Ca\(^{2+}\) exchange activity was measured in the indicated extravesicular KCl concentration. In all cases, Ca\(^{2+}\) release was initiated (arrow) with 50 mM NaCl.

Na/Ca-K exchanger for use as probes to examine the structural and functional properties of this transporter and its distribution in photoreceptor cells. The antibodies that bind to distinct epitopes within the large, hydrophilic N-terminal domain of the exchanger label the extracellular side of rod outer segments, as visualized by immunogold labeling techniques, whereas the antibodies directed against specific sites within the large hydrophilic internal domain of the exchanger label the cytoplasmic surface of the plasma membrane. These studies provide direct experimental evidence in support of the membrane topology of the exchanger that was first proposed by Reilander et al. (9) on the basis of protein sequence and hydropathy profiles. In this model, a long extracellular segment precedes the first hydrophobic domain consisting of five transmembrane segments (H1–H5). A large, negatively charged hydrophilic segment localized on the cytoplasmic side of the plasma membrane is present between this first membrane domain and a second membrane domain consisting of six transmembrane segments (H6–H11). A similar membrane topology has been proposed for the cardiac Na/Ca exchanger, but for this transporter, the large N-terminal extracellular domain is absent (26). The location of the epitopes for the various antibodies in context to the topological model of the rod Na/Ca-K exchanger is shown in Fig. 10.

The role of the large extracellular and intracellular domains of the exchanger in ion transport was examined by limited proteolysis and functional reconstitution. Removal of at least the first 350 amino acids of the extracellular segment did not affect the Na\(^{+}\)-dependent Ca\(^{2+}\) transport activity of the exchanger. Likewise, removal of a substantial portion of the intracellular segment with kallikrein had no significant effect on K\(^{-}\)-dependent or -independent Na\(^{+}\)/Ca\(^{2+}\) exchange. On this basis, we conclude that most, if not all, of the large extracellular and intracellular segments do not play a crucial role in the ion binding or transport by the exchanger. Instead, ion transport across the membrane appears to be mediated by the membrane domains of the exchanger and possibly the proximal hydrophilic segments. The importance of the membrane domains for the function of the exchanger is supported by recent findings indicating that the primary structure of the membrane domains of the bovine and human rod Na/Ca-K exchangers are 94% identical in amino acid sequence, whereas the large extracellular and intracellular domains are only 59% and 45% conserved, respectively (27). Similarly, a recently cloned potas-

FIG. 8. Effect of proteolysis on the Na\(^{+}\)-dependent Ca\(^{2+}\) efflux activity of the Na/Ca-K exchanger. Untreated and trypsin-digested ROS (A) and untreated and kallikrein-digested ROS membranes (B) were solubilized in CHAPS and reconstituted into Ca\(^{2+}\)-containing lipid vesicles for Na\(^{+}\)-dependent Ca\(^{2+}\) efflux assays. The initial velocities (Vi) were normalized to the velocity (V\(_{\text{max}}\)) at 100 mM NaCl, and the data representing the mean from three experiments were fitted to the Hill equation V/V\(_{\text{max}}\) = [Na\(^{+}\)]\(^{f}\)/([Na\(^{+}\)]\(^{f}\) + K\(_{\text{in}}\)) where K\(_{\text{in}}\) and n are the Michaelis constant and the Hill coefficient, respectively. A, the untreated exchanger (○) had a K\(_{\text{in}}\) = 66 mM and n = 3.1 and the trypsin-treated exchanger (●) had a K\(_{\text{in}}\) = 54 mM and n = 2.9. B, the untreated exchanger (△) had a K\(_{\text{in}}\) = 46 mM and n = 2.9 and the kallikrein-treated exchanger (□) had a K\(_{\text{in}}\) = 40 mM and n = 2.5.

FIG. 9. Effect of proteolysis on the K\(^{-}\)-dependent Na/Ca exchange activity. Untreated (A) and kallikrein-digested (B) ROS were solubilized in CHAPS and reconstituted into Ca\(^{2+}\)-containing lipid vesicles in 100 mM choline chloride for activity assays in the absence or presence of 2.5 mM symmetrical KCl. In C, kallikrein-tREATED exchanger was reconstituted into lipid vesicles with 50 mM symmetrical KCl and the exchange activity was measured in the indicated extravesicular KCl concentration. In each case Ca\(^{2+}\) release was initiated (arrow) with 50 mM NaCl.

FIG. 10. Topological model for the rod Na/Ca-K exchanger showing the location of the epitopes for the various monoclonal antibodies used in this study. The model is based on hydrophobicity profiles and localization of the epitopes for antibodies PMe 4G1, PMe 2D9, PMe 3D12, and PMe 6E2 to the extracellular side and the epitopes for antibodies PMe 1B3 and PMe 2A11 to the intracellular side of the ROS plasma membrane. Several possible sites for O-linked glycosylation and conserved consensus sites for N-linked glycosylation are shown. The repeat regions that contain the epitope for PMe 2A11 are shown with shaded bars. The numbers indicate the amino acid positions in the primary structure of the bovine exchanger (9).
sium-dependent Na/Ca exchanger from rat brain that shares an overall amino acid identity of 55% with the bovine rod exchanger, is 80% identical in the membrane spanning segments (28). The lower degree of conservation of the extracellular and intracellular domains is also evident by the general lack of cross-reactivity of the bovine anti-exchanger monoclonal antibodies employed in this study with the rod exchanger of other species. Interestingly, the $V_{\text{max}}$ for Na$^+$-dependent exchange was observed to increase by 2-fold after protease treatment. A similar increase in activity of cardiac Na/Ca exchanger has been reported after proteolysis (29). Cleavage of the large extracellular and intracellular domains may facilitate conformational changes, which the exchanger most likely undergoes as part of the ion transport mechanism as suggested for other transporters (30, 31).

The biochemical properties of the large extracellular domain of rod Na/Ca-K exchanger have not been extensively studied. Earlier studies, however, have indicated that this segment of the exchanger is heavily glycosylated and contains significant amounts of sialic acid residues (10). Studies carried out here using neuraminidase in combination with O- and N-specific endoglycosidases indicate that the exchanger is primarily O-glycosylated. This modification is responsible for a large part of the anomalously slow migration (high apparent molecular mass) of the exchanger by SDS-polyacrylamide gel electrophoresis. The negatively charged intracellular loop also appears to contribute to the retarded migration on SDS gels (9).

[The number of O-linked sialo-glycosylation sites is not currently known; however, Reilander et al. (9) have reported that residues Ser-84, Thr-234, Thr-244, and Thr-245 could not be endoglycosidases indicate that the exchanger is primarily O-glycosylated and that the exchanger, like the cGMP-gated channel is specifically targeted to the plasma membranes. The mechanism for specific targeting of rod photoreceptor membrane proteins to either the plasma membrane or disk membranes remains to be elucidated.

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