Identification of the Sodium–Calcium Exchanger as the Major Ricin-Binding Glycoprotein of Bovine Rod Outer Segments and Its Localization to the Plasma Membrane†

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ABSTRACT: After neuraminidase treatment the Na^+/Ca^{2+} exchanger of bovine rod outer segments was found to specifically bind *Ricinus communis* agglutinin. SDS gel electrophoresis and Western blotting of ricin-binding proteins purified from rod outer segment membranes by lectin affinity chromatography revealed the existence of two major polypeptides of M_r 215K and 103K, the former of which was found to specifically react with PMe 1B3, a monoclonal antibody specific for the 230-kDa non-neuraminidase-treated Na^+/Ca^{2+} exchanger. Reconstitution of the ricin affinity-purified exchanger into calcium-containing liposomes revealed that neuraminidase treatment had no significant effect on the kinetics of Na^+/Ca^{2+} exchange activation by sodium. We further investigated the density of the Na^+/Ca^{2+} exchanger in disk and plasma membrane preparations using Western blotting, radioimmunoassays, immunoelectron microscopy, and reconstitution procedures. The results indicate that the Na^+/Ca^{2+} exchanger is localized in the rod photoreceptor plasma membrane and is absent or present in extremely low concentrations in disk membranes, as we have previously shown to be the case for the cGMP-gated cation channel. Previous reports describing the existence of Na^+/Ca^{2+} exchange activity in rod outer segment disk membrane preparations may be due to the fusion of plasma membrane components and/or the presence of contaminating plasma membrane vesicles.

Vertebrate rod photoreceptors possess a guanosine 3′,5′-cyclic phosphate (cGMP)[1]-gated cation channel in the plasma membrane of their outer segments (Fesenko et al., 1985; Yau & Nakatani, 1985). Under dark conditions, a significant number of these channels exist in the open state and mediate a steady influx of positive charges (the so-called "dark current") into the cell. Sodium ions entering the outer segment diffuse through to the photoreceptor inner segment, and are actively expelled by a Na^+/K^+-ATPase (Sillmann et al., 1969; Hagins et al., 1970). Calcium ions, which carry about 10–15% of the dark current (Hodgkin et al., 1985), are removed from the cytosol via a highly active Na^+/Ca^{2+} exchanger in the photoreceptor outer segment (Yau & Nakatani, 1984; Schröder & Fain, 1984). This Na^+/Ca^{2+} exchange process has been shown to be potassium dependent (Schnetkamp, 1986; Cervetto et al., 1989) and electrogenic (Yau & Nakatani, 1984). Although investigations of the Na^+/Ca^{2+} exchange process have mainly been directed at plasma membrane transport (Yau & Nakatani, 1984; Lagnado et al., 1988; Nakatani & Yau, 1989; Cervetto et al., 1989), Na^+/Ca^{2+} exchange across the disk membrane of permeabilized ROS has also been reported (Schnetkamp et al., 1977; Schnetkamp, 1986; Schnetkamp & Bownds, 1987).

Recently, the Na^+/Ca^{2+} exchanger of bovine ROS has been purified and identified as a 220-kDa glycoprotein (Cook & Kaupp, 1988). Reconstitution studies showed that the purified protein exhibited properties similar to the Na^+/Ca^{2+} exchanger in situ. Identification of the ROS Na^+/Ca^{2+} exchange protein opens the door to studies directed at the characterization and localization of this important transport protein in photoreceptors. In this paper, we describe the identification of the ROS Na^+/Ca^{2+} exchanger as a ricin-binding protein previously shown to be specific for the ROS plasma membrane (Molday & Molday, 1987a). Using immunological and reconstitution techniques, we have determined the relative densities of the Na^+/Ca^{2+} exchanger in highly purified preparations of bovine ROS disk and plasma membranes (Molday & Molday, 1987b).

Experimental Procedures

Materials. Ricinus communis II–agarose was purchased from Medac (Hamburg, FRG). *Arthrobacter ureafaciens* neuraminidase was purchased from Boehringer Mannheim (Indianapolis, IN). Goat anti-mouse IgG–alkaline phosphatase conjugate, bovine pancreas trypsin, alkaline phosphatase substrates, FCCP, valinomycin, and D-galactose were obtained from Sigma (Munchen, FRG), and Immobilon membrane was from Millipore (Eschborn, FRG). All other chemicals and reagents were of the highest grade available and obtained from

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[1] Abbreviations: ROS, rod outer segment(s); cGMP, guanosine 3′,5′-cyclicphosphate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate; arsenazo III, 3,6-bis[(2-arsonophenyl)azo]-4,5-dihydroxy-2,7-naphthalenedisulfonic acid; SDS, sodium dodecyl sulfate; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)-phenylhydrazone; Con A, concanavalin A; ricin, *Ricin communis* agglutinin 120; ELISA, enzyme-linked immunosorbent assay; RIA, radioimmune assay; M_r, molecular weight; kDa, kilodalton(s); Ig, immunoglobulin.

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the sources mentioned in previous publications (Cook et al., 1986; Molday & Molday, 1987b; Cook & Kaupp, 1988).

Preparation of ROS Membranes and Generation of Monoclonal Antibody PMe 1B3. ROS were routinely prepared under dim red light from dark-adapted bovine retinas using continuous sucrose density gradient procedures (Molday & Molday, 1987b). For the preparation of ROS disk and plasma membrane fractions, ROS (50–80 mg of protein) in 4 mL were treated with 0.1 unit of neuraminidase for 2 h, labeled with ricin–gold–dextran particles (approximate diameter 15 nm), hypotonically lysed in 0.02 M Tris–acetate buffer, pH 7.2, and either treated with 0.2 μg/mL trypsin for 20 min at 4 °C or incubated in 2 mM Tris buffer, pH 7.2, overnight at 4 °C to dissociate the disks from the plasma membrane as previously described (Molday & Molday, 1987b). After the reaction was stopped with excess soybean trypsin inhibitor (4 μg/mL), the disk membranes were separated from the ricin–gold–dextran-labeled plasma membrane by sucrose density gradient centrifugation (Molday & Molday, 1987b). The disk and plasma membrane fractions were either used immediately or stored in liquid nitrogen until needed.

Monoclonal antibody PMe 1B3 was obtained from hybridoma cells generated by the fusion of NS-1 cells with spleen cells from mice immunized 3 times with 20 μg of ROS plasma membrane protein in complete Freunds adjuvant as described elsewhere (MacKenzie & Molday, 1982).

Purification and Reconstitution of the ROS Na^+/Ca^{2+} Exchanger and ROS Ricin-Binding Proteins. Purification of the Na^+/Ca^{2+} exchange protein was achieved by a three-step chromatographic procedure as previously described (Cook & Kaupp, 1988). In some experiments, the purified Na^+/Ca^{2+} exchanger was subsequently treated with neuraminidase (0.025 unit/mL). During the course of this study, we also developed a procedure for the purification of the Na^+/Ca^{2+}, exchanger by ricin affinity chromatography. Briefly, ROS membranes (20 mg of rhodopsin) treated with neuraminidase as described above and stripped of peripheral proteins were solubilized in 20 mL of buffer A [10 mM Hepes–KOH, pH 7.4, 0.15 M KCl, 10 mM $CaCl_2$, 1 mM dithiothreitol, 1.1% (w/v) CHAPS, and 0.22% (w/v) asolectin containing the following protease inhibitors: 0.1 mM diisopropyl fluorophosphate, 2 μg mL^{-1} aprotonin, and 2 μg mL^{-1} leupeptin] and applied to a ricin–agarose column (bed volume 5 mL) equilibrated with buffer B [identical with buffer A, except with 0.8% (w/v) CHAPS and 0.17% (w/v) asolectin] at a flow rate of 0.3 mL min^{-1}. After washing with buffer B, the column was eluted with buffer B containing 0.2 M galactose. Column fractions were detected by monitoring the column effluent's absorbance at 280 nm.

Reconstitution of the Na^+/Ca^{2+} exchanger into asolectin liposomes was achieved by using a previously described dialysis procedure (Cook et al., 1986). Briefly, a concentrated asolectin suspension was added to solubilized exchanger to give a final asolectin concentration of 10 mg mL^{-1} and a CHAPS concentration of 0.6% (w/v) and then dialyzed (48 h, three changes) against 10 mM Hepes–KOH, pH 7.4, 0.1 M KCl, 0.1 mM dithiothreitol, and 2 mM $CaCl_2$. The liposomes were then dialyzed against the same buffer without $CaCl_2$ in order to establish a transmembrane calcium gradient.

Spectrophotometric Determination of Na^+-Activated Ca^{2+} Efflux. Sodium-induced calcium effluxes from liposomes containing the Na^+/Ca^{2+} exchange protein were determined with the metallochromic dye arsenazo III (50 μM) using an Aminco DW-2000 spectrophotometer with stirring attachment operating in the dual wavelength mode (650–730 nm). Reagents (e.g., NaCl) were injected into the cuvette (sample volume 2 mL) through an orifice in the lid of the cuvette holder, and the resulting efflux signals were calibrated by adding known amounts of $CaCl_2$ to the sample.

Immunoprecipitation Experiments. Immunoprecipitation of the Na^+/Ca^{2+} exchanger from solubilized extracts was performed by using PMe 1B3 coupled to protein A–Sepharose 4B via rabbit-anti mouse IgG as described elsewhere (Cook et al., 1989). Immunoprecipitated extracts were tested for Na^+/Ca^{2+} exchange activity by reconstitution into calcium-containing liposomes as described above.

Solid-Phase Competitive Inhibition Immunoassays. The binding of monoclonal antibody PMe 1B3 to ROS membranes was determined by ELISA or RIA. For ELISA experiments, ROS membranes (100 μg of protein) in 50 mM sodium carbonate, pH 9.6, and 0.1% (w/v) Tween 20 were applied overnight to EIA II Plus microtitration plates (Flow) and treated with PMe 1B3. Antibody binding was detected by using goat anti-mouse IgG–alkaline phosphatase conjugate and *p*-nitrophenyl phosphate as the substrate. Plates were read by using a Titertek Multiskan Plus MK II spectrophotometer operating in the dual wavelength mode (405–450 nm). Indirect solid-phase RIA was performed by using Flex vinyl microtiter wells as previously described (MacKenzie & Molday, 1982).

Electrophoresis and Western Blotting. Electrophoresis was carried out on 6% continuous minigels (Figure 3) or on 6–16% acrylamide gradient gels (Figures 1 and 8). Gels were then either stained with Coomassie blue R-250 or subjected to electrotransfer onto Immobilon or nitrocellulose membranes for Western blotting. Membranes were blocked with 0.5% Tween 20 or bovine serum albumin, incubated with either ^{125}I-ricin (Molday & Molday, 1987b) or monoclonal antibody PMe 1B3 hybridoma culture fluid (diluted 1:10), and then developed by using either ^{125}I-labeled goat anti-mouse Ig (Molday et al., 1987) or goat anti-mouse IgG–alkaline phosphatase conjugate with the substrates 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium.

Immunogold–Dextran Labeling for Electron Microscopy. Bovine ROS lysed in 20 mM Tris–acetate, pH 7.4, were incubated with 0.2 mL of undiluted PMe 1B3 hybridoma cell culture supernatant for 60 min, washed in 20 mM Tris buffer by repeated centrifugation, and incubated for 60 min with 0.2 mL of goat anti-mouse Ig gold–dextran conjugate (9-nm gold particles) having an A_{520} of 0.5 (Hicks & Molday, 1986). After being labeled with the gold–dextran reagents, the ROS sample was washed in phosphate-buffered saline, fixed in 1% glutaraldehyde in phosphate-buffered saline for 30 min, and adsorbed to Thermanox coverslips (Nunc Inc., Naperville, IL) for 4 h. The coverslips were then rinsed in 0.1 M cacodylate buffer, pH 7.2, containing 0.1 M sucrose, postfixed in 1% osmium tetroxide in the same buffer, dehydrated in ethanol, and embedded in Epon–Araldite resin for thin sectioning. Sections stained with uranyl acetate and lead citrate were viewed under a JEOL 1200 EX electron microscope.

Results

Identification of the ROS Na^+/Ca^{2+} Exchanger as a Ricin-Binding Protein. ROS membranes have previously been shown to contain several high molecular weight proteins, in the range 200K–240K (Wong & Molday, 1986). One of these proteins of apparent M_r 230K in gradient polyacrylamide gels was shown to bind *Ricinus communis* agglutinin after treatment of ROS membranes with neuraminidase and to be specifically located in the ROS plasma membrane (Molday & Molday, 1987a). As shown in Western blots of ROS membranes labeled with ricin, the apparent molecular weight

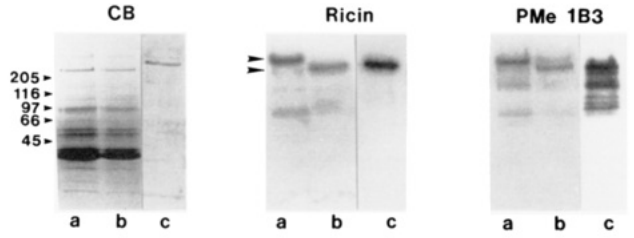

FIGURE 1: Binding of ricin and monoclonal antibody PMe 1B3 to ROS membranes and the Na^+/Ca^{2+} exchange protein treated with neuraminidase. Untreated ROS membranes (lane a), neuraminidase-treated ROS membranes (lane b), or purified, neuraminidase-treated Na^+/Ca^{2+} exchange protein (lane c) were subjected to SDS gel electrophoresis on 6–16% gradient gels and either stained with Coomassie blue (CB) or transferred to Immobilon membranes and treated either with neuraminidase followed by ^{125}I-labeled ricin or with PMe 1B3 antibody followed by ^{125}I-labeled goat anti-mouse Ig. Arrows on the ricin Western blot indicate the decrease in molecular weight due to pretreatment of ROS with neuraminidase. The purified exchange protein was run on a separate gel.

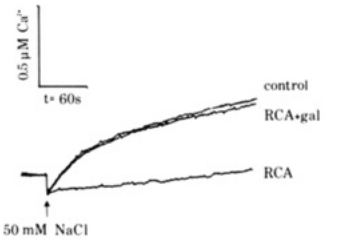

FIGURE 2: Ricin lectin affinity precipitation of the rod outer segment Na^+/Ca^{2+} exchanger. Solubilized, neuraminidase-treated ROS membranes (1.0 mg of rhodopsin mL^{-1} in buffer A) were incubated at 4 °C for 30 min with ricin–agarose (RCA), ricin–agarose and 0.2 M galactose (RCA + gal), or agarose alone (control). After centrifugation in a bench-top centrifuge, unsedimented proteins were reconstituted into calcium-containing liposomes (0.5 mg of rhodopsin mL^{-1}, 10 mg of phospholipid mL^{-1}). Liposomes (0.4 mL) were tested for Na^+-activated Ca^{2+} efflux by using the metallochromic dye arsenazo III as described under Experimental Procedures.

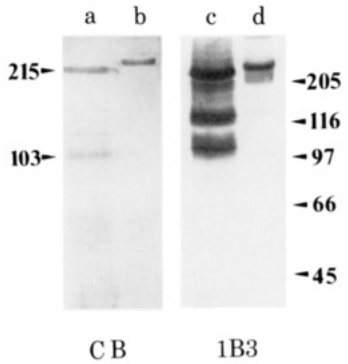

FIGURE 3: Purification of the Na^+/Ca^{2+} exchanger by ricin affinity chromatography. Bovine ROS were treated with 0.025 unit/mL neuraminidase, subjected to hypotonic lysis to remove peripheral proteins, solubilized in CHAPS, and applied to a ricin–agarose column. The fraction eluted with 0.2 M galactose was compared with the Na^+/Ca^{2+} exchanger purified by the method of Cook and Kaupp (1988). SDS gel electrophoresis was carried out on 6% polyacrylamide gels, and proteins were electrotransferred to a Immobilon membrane before immunoblotting with monoclonal antibody PMe 1B3 followed by development with goat anti-mouse IgG–alkaline phosphatase. (a) Neuraminidase-treated ROS ricin-binding proteins (Coomassie blue); (b) purified Na^+/Ca^{2+} exchanger (without neuraminidase treatment); (c) immunoblot of lane a; (d) immunoblot of lane b.

of this protein decreases slightly (approximately 15K) when ROS are treated with neuraminidase prior to electrophoresis (Figure 1). Monoclonal antibody PMe 1B3 generated from a mouse immunized with ROS plasma membranes was also found to bind to a protein of the same molecular weight in ROS membranes (Figure 1). The apparent molecular weight of this protein decreased after neuraminidase treatment in a similar manner to that observed for ricin-labeled Western blots.

Given that the ROS Na^+/Ca^{2+} exchanger is also a glycoprotein of similar molecular weight (Cook & Kaupp, 1988), we were interested to determine whether or not the exchange protein binds ricin and the PMe 1B3 monoclonal antibody. To this end, the Na^+/Ca^{2+} exchange protein was purified from solubilized ROS by column chromatography as previously described (Cook & Kaupp, 1988) and treated with neuraminidase. As shown in Figure 1, both ricin and the PMe 1B3 antibody labeled the high molecular weight Na^+/Ca^{2+} exchange protein as determined by Western blotting. The PMe 1B3 antibody also labeled protein bands of lower apparent molecular weight. The number and intensity of these bands varied with preparations and storage conditions (see Figure 3 for comparison) and appear to be proteolytic fragments of the Na^+/Ca^{2+} exchanger.

Purification of the Na^+/Ca^{2+} Exchanger by Ricin Affinity Chromatography. The interaction of the Na^+/Ca^{2+} exchanger with ricin was further investigated by using ricin–agarose chromatography. Neuraminidase-treated, solubilized ROS membranes were incubated with ricin–agarose, and unbound proteins were tested for Na^+/Ca^{2+} exchange activity by reconstitution into Ca^{2+}-containing liposomes. As can be seen in Figure 2, Na^+/Ca^{2+} exchange activity could be completely and specifically removed from solubilized ROS membranes by this procedure. In the presence of galactose, an inhibitor of ricin binding, the exchange activity was not precipitated with ricin–agarose.

To further confirm that the Na^+/Ca^{2+} exchanger is the high molecular weight plasma membrane specific glycoprotein, we undertook Western blot analysis of ROS ricin-binding proteins purified by lectin affinity chromatography (Figure 3). SDS electrophoresis of affinity-purified ROS ricin-binding proteins on 6% polyacrylamide gels revealed two major polypeptides of M_r 215K and 103K. These components presumably correspond to the previously described plasma membrane specific ricin-binding proteins which exhibit apparent molecular weights of 230K and 110K when electrophoresed on 6.5–15% polyacrylamide gels (Molday & Molday, 1987a,b). The ROS Na^+/Ca^{2+} exchanger, purified as previously described (Cook & Kaupp, 1988), exhibited an apparent molecular weight of 230K by electrophoresis on 6% polyacrylamide minigels.

In order to verify that the Na^+/Ca^{2+} exchanger is present in the ricin-binding protein fraction, we performed immunoblotting analysis with PMe 1B3, a monoclonal antibody which specifically binds to the Na^+/Ca^{2+} exchanger (see Figure 3, lane d). The 215-kDa component was found to strongly react with this antibody, thereby confirming its identity as the ROS Na^+/Ca^{2+} exchanger. Immunoblotting also revealed the presence of several other lower molecular weight immunoreactive bands most of which were barely visible on the Coomassie blue stained gel. These bands presumably represent minor proteolysis products of the Na^+/Ca^{2+} exchanger which transfer more efficiently than undegraded exchange protein. The lower molecular weight (103K–110K) ricin-binding protein appears to be immunologically distinct from the Na^+/Ca^{2+} exchanger.

Effect of Neuraminidase Treatment on the Na^+/Ca^{2+} Exchange Activity. The 15K decrease in molecular weight of the Na^+/Ca^{2+} exchanger after ricin affinity purification is presumably due to the loss of sialic acid residues during neuraminidase treatment. The removal of such a large amount

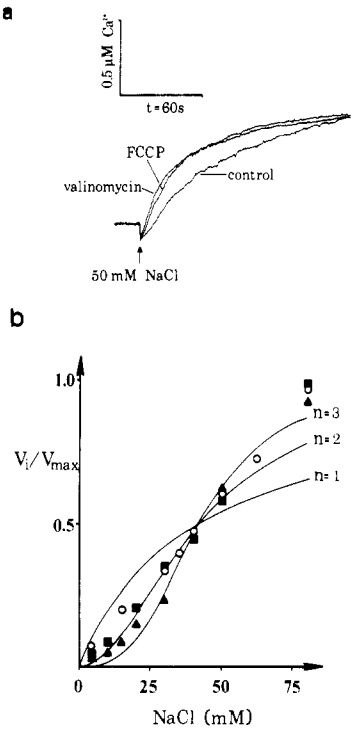

FIGURE 4: Characterization of the Na^+/Ca^{2+} exchanger purified by ricin affinity chromatography. Ricin-binding proteins purified from neuraminidase-treated, solubilized ROS membranes (10 mg of rhodopsin) by ricin–agarose chromatography were incorporated into 8.0 mL of liposomes (10 mg of phospholipid mL^{-1}). Na^+/Ca^{2+} exchange activity was determined on 0.4-mL liposome aliquots using the metallochromic dye arsenazo III. (a) Stimulation of Na^+/Ca^{2+} exchange by FCCP and valinomycin. The cuvette contained a 2 μM sample of the indicated ionophore. (b) Normalized initial efflux rates as a function of sodium concentration. Results from three different experiments with (open symbols) or without (closed symbols) 2 μM valinomycin are shown. The solid curves are plotted for n (the number of Na^+ ions which activate the efflux of one Ca^{2+} ion) = 1, 2, or 3 using a K_m of 42 mM according to the formula previously described (Cook & Kaupp, 1988).

of these negatively charged sugars would also change the surface charge of the Na^+/Ca^{2+} exchanger and would possibly affect its activity. As can be seen in Figure 4, this does not appear to be the case. Figure 4a shows that the Na^+/Ca^{2+} exchange activity can be stimulated by addition of FCCP or valinomycin; i.e., the Na^+/Ca^{2+} exchanger remains electrogenic. These ionophores will act to dissipate the buildup of an unfavorable membrane potential, a consequence of the electrogenicity of Na^+/Ca^{2+} exchange. Valinomycin was found to stimulate Na^+/Ca^{2+} exchange more than 3-fold, giving a turnover number of ~100 Ca^{2+} ions s^{-1} $exchanger^{-1}$ at 50 mM Na^+ [i.e., 3-fold higher than our previously reported value (Cook & Kaupp, 1988), and in better agreement with the results of Cheon and Reeves (1988), who demonstrated that the Na^+/Ca^{2+} exchanger of cardiac sarcolemma is a low-density, high-turnover system]. A turnover number of ~100 Ca^{2+} s^{-1} $exchanger^{-1}$ is probably still low for the potential maximal turnover rate for the ROS Na^+/Ca^{2+} exchanger for the reasons (partial denaturation, absence of acidic lipids, etc. during reconstitution) previously described (Cook & Kaupp, 1988).

Figure 4b shows the Na^+ concentration dependence of the Na^+/Ca^{2+} exchanger after purification by ricin affinity chromatography. Sodium activated the half-maximal Ca^{2+} efflux rate at a concentration (EC_{50}) of 42 mM, i.e., similar to the value (35 mM) reported for the non-neuraminidase-treated Na^+/Ca^{2+} exchanger after reconstitution (Cook & Kaupp, 1988). The normalized efflux rates also showed a distinct sigmoidicity, indicative of the stoichiometry of the exchange process (Yau & Nakatani, 1984; Cervetto et al., 1989).

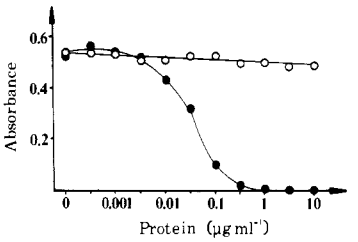

FIGURE 5: ELISA competition analysis of PMe 1B3 binding to ROS membranes. Microtiter plates were treated with ROS membranes (100 μg of protein) and incubated with monoclonal antibody PMe 1B3 (at a 20-fold dilution of hybridoma culture supernatant) in the presence of different concentrations of (●) purified Na^+/Ca^{2+} exchange protein) or (○) rhodopsin. Antibody binding was determined by using goat anti-mouse IgG–alkaline phosphatase.

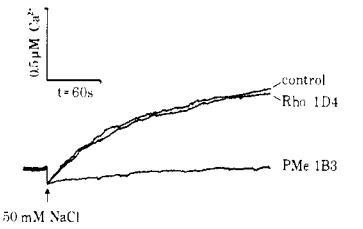

FIGURE 6: Immunoprecipitation of the Na^+/Ca^{2+} exchanger with monoclonal antibody PMe 1B3. Solubilized Na^+/Ca^{2+} exchanger extracts were incubated with protein A–Sepharose 4B treated with antibodies as described under Experimental Procedures. As controls, rabbit anti-mouse IgG–protein A–Sepharose 4B was incubated with exchanger extract without antibody, or after treatment with a monoclonal antibody against rhodopsin (Rho 1D4). After centrifugation in a bench-top centrifuge, unbound proteins were tested for Na^+/Ca^{2+} exchange activity by reconstitution into calcium-containing liposomes (0.5 mL of extract mL^{-1} liposomes, 10 mg of phospholipid mL^{-1}). Liposomes (0.4-mL aliquots) were spectrophotometrically tested for Na^+-activated Ca^{2+} efflux with arsenazo III.

PMe 1B3 Monoclonal Antibody Binding to the ROS Na^+/Ca^{2+} Exchanger. We further investigated the specificity of PMe 1B3 for the ROS Na^+/Ca^{2+} exchanger using competitive inhibition ELISA. As can be seen in Figure 5, the addition of purified Na^+/Ca^{2+} exchange protein was found to totally and specifically block the binding of this monoclonal antibody to ROS membranes, thereby demonstrating the PMe 1B3 binding to ROS membranes is due to binding to the Na^+/Ca^{2+} exchanger alone.

Monoclonal antibody PMe 1B3 could also be used to confirm that the 230-kDa protein is indeed the ROS Na^+/Ca^{2+} exchanger by immunoprecipitation analysis (Figure 6). In this experiment, solubilized extracts were treated with protein A–Sepharose 4B to which selected antibodies had been coupled, followed by functional reconstitution of the unbound proteins to test for Na^+/Ca^{2+} exchange activity. Monoclonal antibody PMe 1B3 completely immunoprecipitated the Na^+/Ca^{2+} exchange activity, whereas control antibodies (e.g., Rho 1D4 against rhodopsin) were without effect.

Relative Abundance of the Na^+/Ca^{2+} Exchanger in ROS Plasma and Disk Membranes. The existence of a specific monoclonal antibody to the ROS Na^+/Ca^{2+} exchanger permitted the determination of the relative abundance of the Na^+/Ca^{2+} exchange protein in the bovine rod photoreceptor disk and plasma membrane. Immunocytochemical studies using the PMe 1B3 antibody were carried out on hypotonically lysed ROS. These preparations consist of inverted plasma

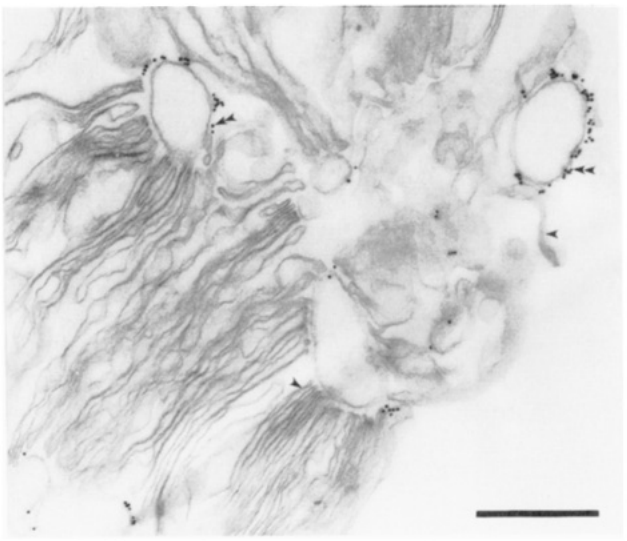

FIGURE 7: Electron microscopic localization of the PMe 1B3 binding site to the cytoplasmic side of the ROS plasma membrane. Rod outer segments lysed in 20 mM Tris buffer, pH 7.4, were indirectly labeled with PMe 1B3 followed by goat anti-mouse IgG–gold–dextran particles (average diameter 9 nm). Gold particles (double arrows) can be seen labeling the accessible cytoplasmic surface of inverted plasma membrane vesicles containing attached unlabeled disks (arrows). The inserted bar represents 0.2 μm.

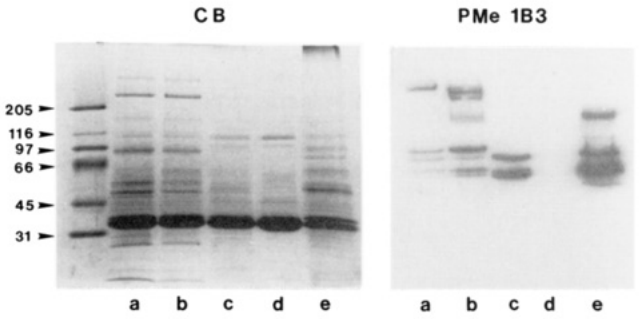

FIGURE 8: Subcellular localization of the Na^+/Ca^{2+} exchanger: Western blot analysis. Coomassie blue stained SDS–6–16% polyacrylamide gradient gels (left panel) and PMe 1B3 labeled Western blots (right panel) of (a) ROS membranes, (b) neuraminidase-treated ROS membranes, (c) neuraminidase-treated ROS membranes after mild trypsinization, (d) purified ROS disk membranes, and (e) purified ROS plasma membranes.

membrane vesicles with disks still attached to their cytoplasmic surface (Molday & Molday, 1987a,b). As shown in Figure 7, the cytoplasmic surfaces of inverted plasma membrane vesicles which are accessible to the immunochemical reagents are intensely labeled with gold particles whereas the disks are not labeled. These results indicate that the Na^+/Ca^{2+} exchanger is localized in the plasma membrane of ROS and the the antigenic site of the PMe 1B3 antibody is exposed on the cytoplasmic surface of the plasma membrane.

To obtain more information about the subcellular localization of the Na^+/Ca^{2+} exchanger, various ROS membrane fractions were exposed to gradient electrophoresis followed by immunoblotting with PMe 1B3. Figure 8 (left panel) shows a Coomassie blue stained SDS gel of various ROS membrane fractions and (right panel) the corresponding Western blot labeled with PMe 1B3. In untreated membranes (lane a), the antibody reacted strongly with a 230-kDa polypeptide and with some lower molecular weight bands, probably proteolytic fragments of the Na^+/Ca^{2+} exchanger. After neuraminidase treatment (lane b), the molecular weight of the 230-kDa polypeptide was slightly reduced, and the immunostaining of the

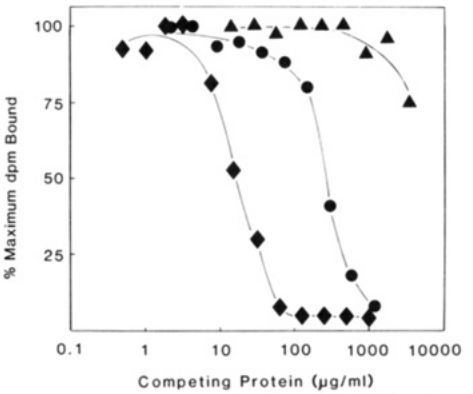

FIGURE 9: Subcellular localization of the Na^+/Ca^{2+} exchanger: RIA competition analysis. Microtiter plates treated with ROS membranes were incubated with monoclonal antibody PMe 1B3 hybridoma cell culture fluid (dilution 1:30) in the presence of different amounts of solubilized ROS membrane extracts: (●) ROS total membranes; (♦) purified ROS plasma membranes; (▲) purified ROS disk membranes. Binding of the monoclonal antibody to the ROS membranes was determined by using ^{125}I-labeled goat anti-mouse Ig as a secondary antibody.

proteolytic fragments was increased, suggesting that further proteolysis had occurred. Mild trypsinization (lane c), in order to digest the cytoskeletal system, eliminated immunoreactivity in the high molecular weight region and increased the abundance of immunoreactive bands in the region 50–90 kDa. After ricin–gold treatment and density centrifugation, the trypsinized membranes were separated into purified disk and plasma membrane fractions. The disk membrane fraction (lane d) was found to be devoid of immunoreactivity whereas the plasma membrane fraction (lane e) exhibited intense labeling.

A more quantitative estimate of the relative densities of the Na^+/Ca^{2+} exchanger in ROS plasma and disk membranes could be obtained by solid-phase RIA competitive inhibition assays. In this experiment, ROS membranes were adsorbed onto microtiter plates and incubated with PMe 1B3 in the presence of purified membrane fractions. As shown in Figure 9, plasma membrane extracts blocked immunoreactivity at protein concentrations about 200-fold lower than the disk membrane protein concentration required to give the same degree of inhibition.

To provide further evidence that the functional Na^+/Ca^{2+} exchanger is located exclusively in the ROS plasma membrane, we solubilized purified plasma and disk membrane fractions and tested them for Na^+/Ca^{2+} exchange activity by functional reconstitution. As can be seen from Figure 10A, the reconstitution of plasma membrane proteins purified from trypsinized ROS membranes into calcium-containing liposomes resulted in a large efflux signal upon addition of Na^+, whereas the disk membrane fraction was devoid of activity. When trypsinization was not employed during the membrane purification procedure (Figure 10B), some residual Na^+/Ca^{2+} exchange activity was observed in the disk membrane fraction. This may indicate that, in the absence of trypsinization, some plasma membrane material may remain attached to the disks, as we have suggested elsewhere (Cook et al., 1989). This may explain other reports in the literature (Schnetkamp et al., 1977; Schnetkamp, 1986; Schnetkamp & Bownds, 1987) describing Na^+/Ca^{2+} exchange activity in disk membrane extracts or in ROS preparations where the plasma membrane has been permeabilized. Our results also indicate that, even though trypsinization results in extensive cleavage of the ROS Na^+/Ca^{2+} exchanger, it still retains normal activity.

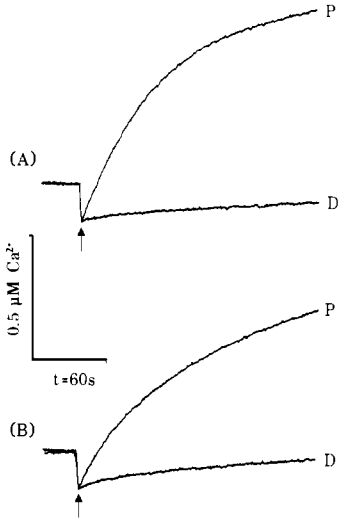

FIGURE 10: Subcellular localization of the Na^+/Ca^{2+} exchanger determined by solubilization and reconstitution. Purified membrane fractions were solubilized and reconstituted into calcium-containing liposomes (10 mg of phospholipid mL^{-1}) to test for Na^+-activated Ca^{2+} efflux. At the time point indicated by the arrows, 50 mM NaCl was injected into the cuvette. (A) Reconstituted disk (D) and plasma (P) membrane extracts prepared from neuraminidase-treated ROS membranes after mild trypsinization. In both cases, liposomes containing 15 μg of protein were present in the cuvette. (B) Reconstituted disk (D) and plasma (P) membrane extracts prepared from ROS membranes without trypsinization. In both cases, liposomes containing 24 μg of protein were present in the cuvette.

DISCUSSION

Glycoprotein Properties of the ROS Na^+/Ca^{2+} Exchanger. The results presented in this paper indicate that the ROS Na^+/Ca^{2+} exchanger is heavily glycosylated. The exchange protein has been shown to bind to concanavalin A (Cook & Kaupp, 1988), wheat germ agglutinin (Nicoll & Applebury, 1988), and, after neuraminidase treatment, *Ricinis communis* agglutinin (this study). Neuraminidase treatment resulted in a decrease in molecular weight of about 15K, suggesting that the ROS Na^+/Ca^{2+} exchanger has a high sialic acid content, possibly in the form of polysialic acid. In addition, the fact that the exchange protein binds to Con A suggests that terminal α-mannose residues are also present, and ricin binding after neuraminidase treatment indicates the presence of internal β-galactose or *N*-acetyl-β-galactose.

The function of the Na^+/Ca^{2+} exchanger carbohydrate is unknown. Previous microscopy studies (Uehara et al., 1983; Hicks & Molday, 1985) have shown that intense ricin binding is observed at the basal part of the rod outer segment where disks are continuous with the plasma membrane. This could mean that the Na^+/Ca^{2+} exchanger is inserted into the plasma membrane as a glycoprotein with terminal galactose and is then modified by sialylation as the disk and plasma membranes separate from each other, further along the outer segment. Photoreceptor glycoproteins have also been speculated to play a role in the recognition and subsequent phagocytosis of the distal part of the outer segment by the pigment epithelium (O'Brien, 1976). Since we have shown here that the Na^+/Ca^{2+} exchanger is located exclusively in the ROS plasma membrane, its extensive carbohydrate content (which presumably extends into the extracellular matrix) may indeed be one of the first points of contact with the pigment epithelium. Alternatively, it may be involved in weak interactions with one or more components of the interphotoreceptor matrix.

The high content of the negatively charged sugar sialic acid may induce extracellular surface charge effects on the Na^+/Ca^{2+} exchange protein and thereby influence the Na^+ dependence of the exchange process. From the results presented here, this does not appear to be the case: the desiaylated Na^+/Ca^{2+} exchanger retains its electrogenicity and exhibits a similar Na^+ dependence to that of the untreated protein.

Na^+/Ca^{2+} Exchanger Is Localized in the ROS Plasma Membrane. Immunochemical studies indicate that the density of the Na^+/Ca^{2+} exchanger in the plasma membrane is at least 200-fold greater than that of the disk membrane. The small amount of immunoreactivity found in the disk membrane fraction may be due either to a small contamination of plasma membrane in these preparations or to the presence of residual exchanger in disks. Solubilization and reconstitution studies indicated the presence of residual Na^+/Ca^{2+} exchange activity in disk membrane preparations if the cytoskeletal system was not digested by mild trypsinization. This observation may explain other reports (Schnetkamp & Bownds, 1987) describing the existence of Na^+/Ca^{2+} exchange activity across the disk membrane: permeabilization of the ROS (in order to make the disk membrane accessible) may have resulted in fusion of plasma membrane fragments connected to the disk membrane by the cytoskeleton. More recently, Bauer (1988) has presented results indicating that both the cGMP-dependent channel and the Na^+/Ca^{2+} exchanger are present in a distinct population of ROS membrane vesicles. This vesicle population is suggested to be derived from the plasma membrane of ROS.

The density of the Na^+/Ca^{2+} exchanger in the ROS plasma membrane is difficult to determine, since this protein appears to be relatively susceptible to proteolysis (thereby complicating electrophoretic densitometry) during membrane preparation. However, we have previously shown (Cook & Kaupp, 1988) that when total ROS membrane proteins are reconstituted into calcium-containing liposomes, Na^+ can induce a calcium efflux about 3-fold greater than cGMP can through activation of the cGMP-gated channel. Since the Na^+/Ca^{2+} exchanger (but not the channel protein) will mediate Ca^{2+} efflux regardless of its orientation in the liposome membrane, we concluded that the exchange protein is about 1.5-fold more abundant than the cGMP-gated channel which is also localized exclusively in the plasma membrane. This would correspond to a plasma membrane density of $\sim$450 Na^+/Ca^{2+} exchangers μm^{-2}. It is also possible to obtain a rough estimate of the density of the Na^+/Ca^{2+} exchanger in the ROS plasma membrane by liposome density analysis of the magnitude of efflux signals such as those shown in Figure 10. Using light-scattering analysis, we established that the reconstitution conditions used yield $\sim 4.5 \times 10^{13}$ liposomes mL^{-1} (Cook et al., 1986). We found that when 75 μg of plasma membrane protein purified after mild trypsinization was incorporated into 1 mL of liposomes (10 mg of asolectin mL^{-1}), 22% of the Ca^{2+} content could be released by Na^+ (i.e., $\sim 10^{12}$ liposomes have at least one exchange protein). When an apparent molecular weight of 220K was used, this would correspond to 3.7 μg of Na^+/Ca^{2+} exchanger protein per 75 μg of plasma membrane protein (i.e., about 4% of total plasma membrane protein). Given that ROS membranes have about 27 000 rhodopsin molecules μm^{-2}, this yields a density of $\sim$200 exchangers μm^{-2} (assuming one exchanger protein occupies 5.5-fold more space than the 40-kDa rhodopsin) in the plasma membrane. This value presumably underestimates the exchanger density because (i) it is possible that some of the liposomes contain more than one exchanger protein and (ii) some denaturation of the Na^+/Ca^{2+} exchanger may have occurred during solubilization and reconstitution. At any rate, given this approximate density and the high turnover rate of the Na^+/Ca^{2+} exchange process (Cervetto et al., 1989), the ROS Na^+/Ca^{2+} exchanger would

be able to comfortably maintain the cytosolic Ca^{2+} in ROS at submillimolar concentrations.

Registry No. Na, 7440-23-5; Ca, 7440-70-2.

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Binding of Proteins to Specific Target Sites in Membranes Measured by Total Internal Reflection Fluorescence Microscopy†

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ABSTRACT: A new quantitative technique for measuring the binding of proteins to membranes is described. The method is based on a combination of total internal reflection fluorescence microscopy and the preparation of supported planar bilayers. Specific and reversible binding of a fluorescence-labeled monoclonal antibody to lipid haptens that were embedded in supported bilayers has been measured by this technique and compared to binding experiments that were conducted on membrane vesicles in solution. Equilibrium binding constants and kinetic parameters have been determined and used to expand the picture of the antibody–lipid hapten reaction. Estimates demonstrate that this technique is capable of measuring a broad range of binding constants (down to about 10^4 M^{-1}) using only small amounts of ligand and receptor.

An increasingly large number of cellular receptors have been identified for which quantitative thermodynamic and kinetic data on their interactions with ligands are highly desirable. Binding studies on whole cells by radioligand assays and related methods only yield semiquantitative data because of the usually high unspecific background binding and other complications (Klotz, 1982). Unspecific binding to the support and the undefined state of the receptor molecules affect solid-phase assays and other methods with solubilized receptors (Engel, 1984). In many cases, the functional reconstitution of receptors in synthetic lipid vesicles and other model membranes was successful (Klausner et al., 1984; Montal et al., 1981). However, the currently available methods for monitoring binding to membrane-bound receptors still suffer from a number of intrinsic and technical problems. Because of scattering problems, spectroscopic techniques in solution can usually be performed only with small sonicated vesicles at moderate concentrations. Some physical properties of such vesicles are quite distinct from those found in less curved membranes. Especially when large protein ligands with moderate binding constants are considered, it is often difficult to determine the amount of bound ligand in the presence of excess free ligand in solution. It is known that binding is often accompanied by an association of receptor molecules (Metzger et al., 1986; Schlessinger, 1988) and critically depends on the mobility of the receptors in the membrane (Axelrod, 1983). Multivalency of large ligands may also induce changes of the state of association and the receptor mobility. The latter parameters are determinable by microscopic techniques such

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