The Journal of Neuroscience, May 1990, 10(5): 1486–1494

Immunological Characterization and Localization of the Na^+/Ca^{2+}-Exchanger in Bovine Retina

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The sodium/calcium exchanger was purified from bovine retinal rod outer segment membranes and used for the immunization of New Zealand White rabbits. A polyclonal antibody was produced which was found to bind specifically to the 230 kDa Na^+/Ca^{2+}-exchanger protein as assessed by Western blotting. The antibody did not bind to the high-molecular-weight "rim protein," thereby demonstrating that this protein is distinct from the rod outer segment Na^+/Ca^{2+}-exchanger. We used the polyclonal antibody for immunohistochemically localizing the exchange protein in bovine retina. Fluorescent light microscopy revealed intensive immunolabeling of the photoreceptor outer segments, whereas other retinal cell layers exhibited minimal binding. Using the electron microscopic immunogold method, we found specific antibody binding to the extracellular side of rod outer segment plasma membrane. Rod disk membranes, rod inner segments, and cone photoreceptors displayed no significant labeling. We therefore conclude that the Na^+/Ca^{2+}-exchanger is localized primarily in the rod outer segment plasma membrane, the most appropriate localization considering its proposed role in the process of vertebrate phototransduction.

Light hyperpolarizes vertebrate photoreceptors (Baylor et al., 1979; Stryer, 1986) by inducing the closure of "light-dependent cation channels" localized primarily in the plasma membrane of the outer segment (Cook et al., 1989). These channels are now known to be directly and cooperatively gated by cyclic GMP (Fesenko et al., 1985; Haynes et al., 1986). Photoactivation of rhodopsin initiates channel closure through activation of an enzymatic cascade consisting of the GTP-binding protein transducin and the cGMP-hydrolyzing enzyme phosphodiesterase (Yee and Liebman, 1978; Fung et al., 1981). Although the "dark current" carried by these channels is mainly constituted by sodium ions flowing into the cell, the light-dependent channels are also permeable to divalent cations (Capovilla et al., 1983; Yau and Nakatani, 1984a; Hodgkin et al., 1985). This results in a steady influx of calcium ions (constituting 10–15% of the dark current) into the photoreceptor outer segment under dark conditions.

Calcium ions entering the photoreceptor cell are rapidly expelled by a highly active potassium-dependent electrogenic sodium/calcium exchanger (Yau and Nakatani, 1984b; Schnetkamp, 1986; Lagnado et al., 1988; Cervetto et al., 1989; Schnetkamp et al., 1989). The Na^+/Ca^{2+}-exchanger has been shown to continue operating after light exposure (Yau and Nakatani, 1984b). Thus, in addition to hyperpolarization (i.e., abolition of Na^+ and Ca^{2+} influx through the cGMP-gated channel), light also induces calcium release from rod photoreceptors (Gold and Korenbrot, 1980; George and Hagins, 1983; Schröder and Fain, 1984) and a corresponding reduction of the cytosolic calcium concentration (McNaughton et al., 1986).

In a previous report (Cook and Kaupp, 1988), we described the chromatographic purification of the bovine ROS Na^+/Ca^{2+}-exchanger. After functional reconstitution by a dialysis procedure (Cook et al., 1986), the purified protein was found to exhibit properties similar to the exchange protein *in situ.* The availability of the rod photoreceptor Na^+/Ca^{2+}-exchanger in its purified form allows the generation of specific antibodies which should permit the immunological characterization of the exchange protein, and which may be useful in the search for immunologically related Na^+/Ca^{2+}-exchange proteins in other tissues. In this study we report the generation and characterization of a polyclonal antibody to the bovine rod outer segment Na^+/Ca^{2+}-exchanger. We have used the antibody to further characterize the exchange protein and to determine its localization in bovine retinal tissue.

Materials and Methods

Materials. Goat anti-rabbit IgG conjugates (HRP, alkaline phosphatase, FITC) were supplied by Sigma. Gold-coupled goat anti-rabbit IgG was from Janssen. BSA, Tween 20, alkaline phosphatase substrates, and HRP substrates were obtained from Sigma. All other reagents and materials were of analytical grade or better and were from the same sources as described elsewhere (Cook and Kaupp, 1988).

Purification of the rod photoreceptor Na^+/Ca^{2+}-exchanger and generation of polyclonal antibody. The Na^+/Ca^{2+}-exchange protein was purified by a combination of anion-exchange chromatography on DEAE Fractogel TSK, affinity chromatography on AF-Red Fractogel TSK and lectin-affinity chromatography on Con A–Sepharose 4B as previously described (Cook and Kaupp, 1988). The purified Na^+/Ca^{2+}-exchanger used for the generation of antibodies and for ELISA competition assays exhibited apparent homogeneity as assessed by SDS–polyacrylamide electrophoresis. Antiserum was generated by subcutaneous injection (50 μg Na^+/Ca^{2+}-exchange protein per injection) with Freund's complete adjuvant at multiple sites along the spines of New Zealand White rabbits. This was followed by subsequent injections with incomplete adjuvant 1 month later and then at 2 week intervals.

Western blotting and ELISA. SDS electrophoresis was carried out on 5–15% acrylamide gradient gels (with a 3% acrylamide stacking gel) with the buffer system of Laemmli (1970) using a "Mighty Small" apparatus

Received May 30, 1989; revised Nov. 8, 1989; accepted Nov. 24, 1989.

This work was supported by the Deutsche Forschungsgemeinschaft (SFB 169), the Max-Planck Gesellschaft, and a Leibniz-Programm grant to Prof. Hartmut Michel.

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from Hoefer. Gels were stained with Coomassie blue R250 or electrotransferred overnight at 300 mA onto Immobilon membrane (Millipore) in 25 mM Tris-acetate buffer (pH 8.3) containing 10% (vol/vol) methanol and 0.05% (wt/vol) SDS using a Hoefer TE42 transfer unit. After blocking with BSA, blots were incubated with rabbit antiserum and antibody binding was detected using goat anti-rabbit IgG alkaline phosphatase conjugate and the substrates 5-bromo-4-chloro-3-indolylphosphate and nitro blue tetrazolium.

ELISA was carried out using a Titertek Multiskan Plus MK II apparatus (Flow). ROS membranes [100 μg protein in 0.05 M sodium carbonate buffer, pH 9.6 containing 0.1% (wt/vol) Tween 20 per well] were applied to E.I.A. II Plus microtitration plates (Flow) overnight at 4°C, then rinsed 4 times with PBS 0.1% (wt/vol) Tween 20 and blocked (1 hr, 30°C) with 1% (wt/vol) BSA in PBS. The plates were then treated (4 hr at room temperature) with antiserum or preimmune serum diluted in PBS containing 1% (wt/vol) BSA and 0.1% (wt/vol) Tween 20. After 4 rinses, antibody binding was detected using goat anti-rabbit IgG HRP conjugate and tetramethylbenzidine/H_2O_2 substrate. After incubation (20 min for Fig. 1; 10 min for Fig. 2*a*) at room temperature, the reaction was stopped by addition of 0.4 M (final concentration) H_2SO_4, and plates were read at 450 nm. All ELISA experiments were carried out on duplicate samples.

Immunofluorescence microscopy. Bovine eyes were obtained shortly after animal death from a local slaughterhouse and immediately prepared for microscopy. Strips of the eye capsule together with the adhering retina were frozen in liquid nitrogen-cooled propane. The retina, together with the pigment epithelium layer, was then broken off and placed, still frozen, on liver tissue blocks which were mounted onto metal holders fitted to a cryomicrotome (Slee, Mainz, FRG). Cryosections, 5 μm thick, were cut and thaw-mounted onto Alcian blue (0.005%) coated glass slides, dried, and stored in the cold until immunostaining. Sections on slides were fixed briefly with acetone, then treated with 10 mM PBS containing 1% BSA, 0.1% Triton X-100, and 0.2% Tween 20 and then incubated for 1 hr at room temperature with the specific polyclonal antiserum or with the corresponding preimmune serum (both at a dilution of 1:6). Antibody binding was visualized with FITC goat anti-rabbit IgG. Sections were viewed in a light microscope (Zeiss) equipped with an epifluorescence device.

Electron microscopy and immunogold labeling. For a typical experiment, the front half of a bovine eye was excised and the vitreous humor and lens removed. The eye cup was then filled with fixative consisting of 4% paraformaldehyde and 0.1% glutaraldehyde or 1% glutaraldehyde, both in 10 mM PBS. After fixation (1.5 hr), the retina was removed from the eye capsule, cut into small pieces, washed overnight with 50 mM NH_4Cl, dehydrated, embedded in LR White resin (Craig and Miller, 1984), and polymerized at 58°C. Ultrathin sections were placed on Formvar-coated nickel grids and used the next day for immunogold labeling.

Isolated outer segments were fixed with 4% paraformaldehyde/0.2% glutaraldehyde in 10 mM PBS, pH 7.4, containing 15% sucrose for about an hour. For immunostaining, the fixed material was adsorbed onto Alcian blue-coated glass slides (Molday et al., 1987). After immunolabeling, outer segments were refixed with 1% glutaraldehyde in 10 mM PBS and in 1% OsO_4 in 0.1 M sodium cacodylate buffer, dehydrated, and embedded in Spurr's resin (Spurr, 1969).

For electron microscopic immunostaining of sections, the postembedding immunogold method was applied. Thin sections were first treated with saturated sodium metaperiodate, then with 50 mM NH_4Cl, and finally with 10 mM PBS, pH 7.3, containing 1% BSA, 0.1% Triton X-100, and 0.2% Tween 20. Incubation with the antiserum (1:100–1:400 dilution) was performed overnight, and antibody binding was detected using second antibody (goat anti-rabbit IgG) coupled to gold particles (15 nm diameter). In controls, instead of the specific antibody, the preimmune serum at the corresponding dilution was applied to the sections. Thin sections were analyzed with a Philips EM 300 electron microscope.

In the case of isolated ROS, preembedding immunostaining was performed. Fixed outer segments adsorbed onto glass slides were incubated with antiserum or preimmune serum (1:50 dilution) and then treated with goat anti-rabbit IgG coupled to gold particles. All sections were double contrasted with uranyl acetate and lead citrate before electron microscopy.

Estimation of particle density of immunogold-labeled membranes. To quantify the immunogold labeling intensity on plasma membranes of outer and inner segments of rods and cones and of rod disk membranes gold particles were counted along the membranes. Enlarged prints (40,000 or 60,000×) were used for measuring membrane length and number of particles along the membranes on a digitizing tablet connected to a Morphomat 10 from Zeiss. Data were collected from 2 immunostainings of 3 bovine eyes. In these preparations, the preimmune serum and the specific antibody serum were applied at a dilution of 1:100. The labeling intensity is expressed as particles per micron of membrane length, and the data from 3 animals (3 eyes) were taken together (n = 3). The means were used for t test calculations (see Table 1).

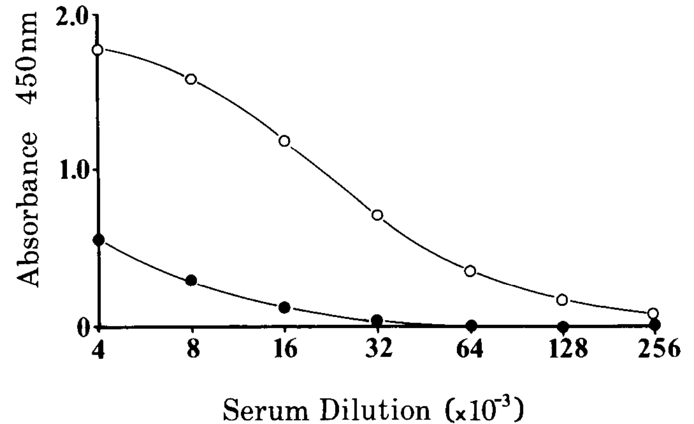


Figure 1. ELISA analysis of antiserum and preimmune serum binding to ROS membranes. Symbols: ○, Na^+/Ca^{2+}-exchanger antiserum; ●, preimmune serum.

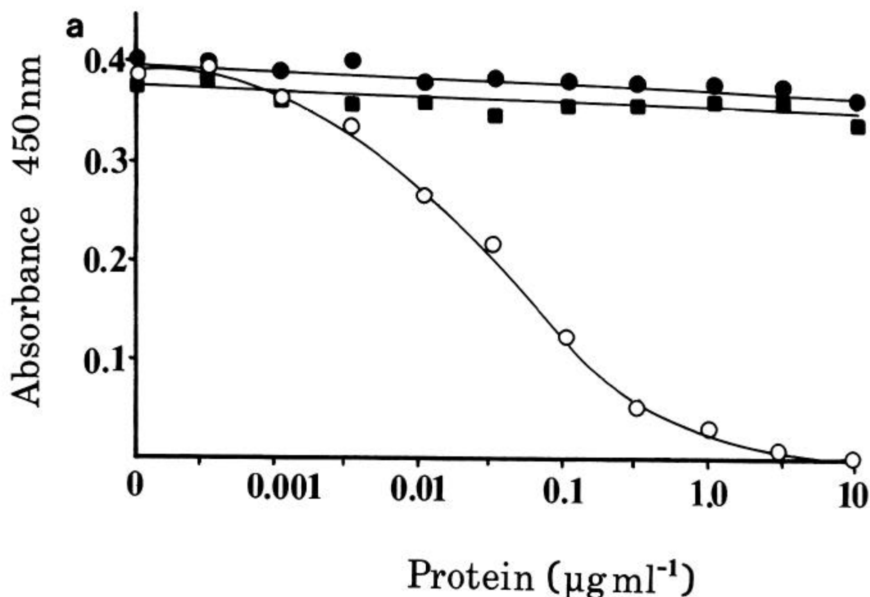
Results

Characterization of the anti-Na^+/Ca^{2+}-exchanger antiserum

We investigated the binding of Na^+/Ca^{2+}-exchanger antibody to rod photoreceptor membranes using ELISA. As can be seen in Figure 1, the antiserum exhibited a much greater immunoreactivity to these membranes than the preimmune serum did. The specificity of this immunoreactivity was investigated by ELISA competitive inhibition studies using purified Na^+/Ca^{2+}-exchange protein and Western blotting. As can be seen in Figure 2*a*, the presence of added purified Na^+/Ca^{2+}-exchanger was found to block the binding of antiserum to ROS membranes and reduce the immunoreactivity to the level displayed by the preimmune serum. Western blot analysis (Fig. 2*b*) also showed that the antiserum specifically reacts with the 230 kDa Na^+/Ca^{2+}-exchanger. We can therefore conclude that the difference in antibody binding of the antiserum relative to that of the preimmune serum, as seen in the immunohistochemical studies described below, is due exclusively to immunoreactivity against the Na^+/Ca^{2+}-exchanger.

ROS Na^+/Ca^{2+}-exchanger is immunologically distinct from the "rim protein"

We have previously reported (Cook and Kaupp, 1988) that the rod photoreceptor Na^+/Ca^{2+}-exchanger has a similar molecular weight to another well-characterized ROS protein, the so-called "rim protein." We noted, however, that these 2 proteins elute at different salt concentrations from a DEAE anion-exchange column and therefore suggested that they are distinct from one another. In this study we have been able to confirm this conclusion by electrophoresis and Western blotting. In Figure 2*b*, *A* shows 5–15% acrylamide gradient electrophoresis of purified Na^+/Ca^{2+}-exchange protein and ROS membranes (lanes a and b, respectively). Under this electrophoresis system, the Na^+/Ca^{2+}-exchanger migrates as a protein of molecular weight 230



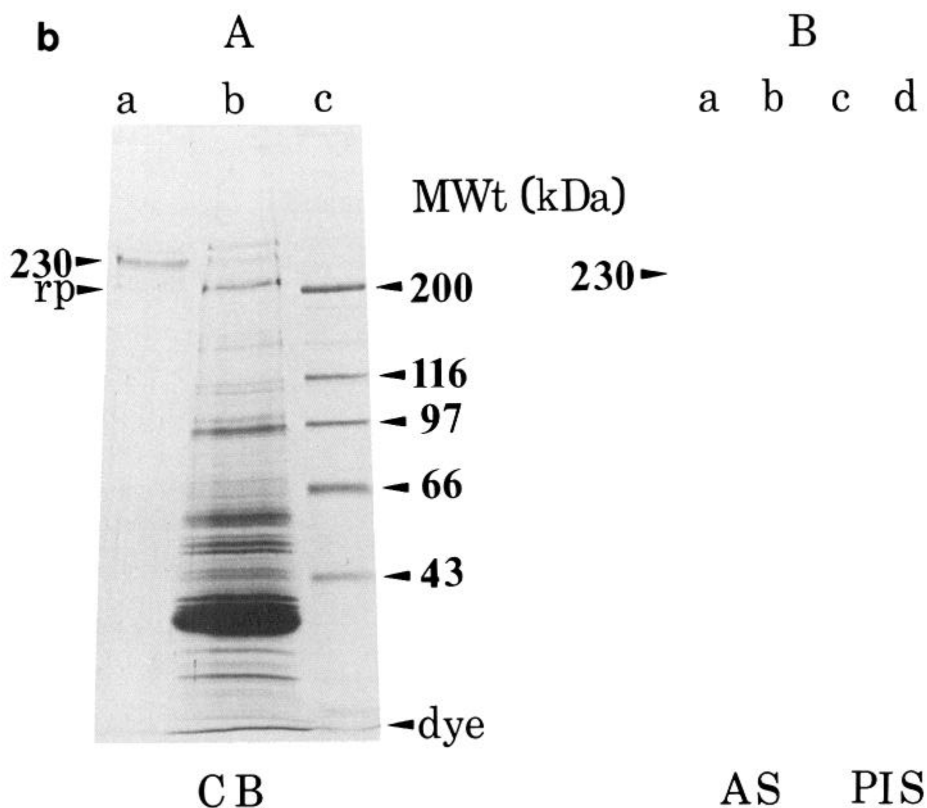


Figure 2. Specificity of the antiserum for the ROS Na^+/Ca^{2+}-exchanger. *a,* ELISA-competition analysis. Immobilized ROS membranes were incubated with antiserum (diluted 2×10^4-fold) in the presence of the increasing concentrations of the following proteins: BSA (●), rhodopsin (■), and purified Na^+/Ca^{2+}-exchange protein (○). In this experiment, the preimmune serum immunoreaction (absorbance = 0.049 at 2×10^4-fold dilution) has been subtracted from the values for antiserum binding (i.e., an absorbance of zero means that the sample has the same immunoreactivity as preimmune serum). *b,* Western blot analysis. *A,* Coomassie blue (*CB*)-stained gel of (*a*) purified Na^+/Ca^{2+}-exchange protein (0.5 μg), (*b*) ROS membranes (20 μg protein), and (*c*) molecular-weight markers. *B,* Corresponding Western blot of lanes a and b from *A*: *a,* purified Na^+/Ca^{2+}-exchanger (0.1 μg) incubated with antiserum (*AS*); *b,* ROS membranes (20 μg protein) incubated with antiserum; *c,* purified Na^+/Ca^{2+}-exchanger incubated with preimmune serum (*PIS*); and *d,* ROS membranes incubated with preimmune serum. In all cases, the antiserum or preimmune serum dilution during Western blotting was 1:5000. The electrophoretic mobility of the "rim protein" (see Results) is denoted by *rp.*

kDa, i.e., clearly larger than the rim protein which migrates at about 210 kDa. Gradient electrophoresis also reveals that the Na^+/Ca^{2+}-exchanger is a relatively minor component of ROS membranes, representing about 0.1–0.3% of total membrane protein (as determined by gel scanning).

Western blotting also demonstrated that these 2 proteins are immunologically different: Figure 2*b*, *B*, lane a shows antiserum labeling of purified Na^+/Ca^{2+}-exchanger, and lane b shows labeling of ROS membranes after electrophoresis and electrotransfer onto Immobilon membrane. In ROS membranes, the antiserum specifically labels a protein of identical molecular weight to the purified Na^+/Ca^{2+}-exchanger, and not the more prominent rim protein. The subcellular localization of Na^+/Ca^{2+}-exchanger immunoreactivity (see below) also confirms that it is not the rim protein.

Immunolocalization of the Na^+/Ca^{2+}-exchanger in bovine retina

Incubation of retinal cryosections with the specific polyclonal antibody results in intensive labeling of the layer where the outer

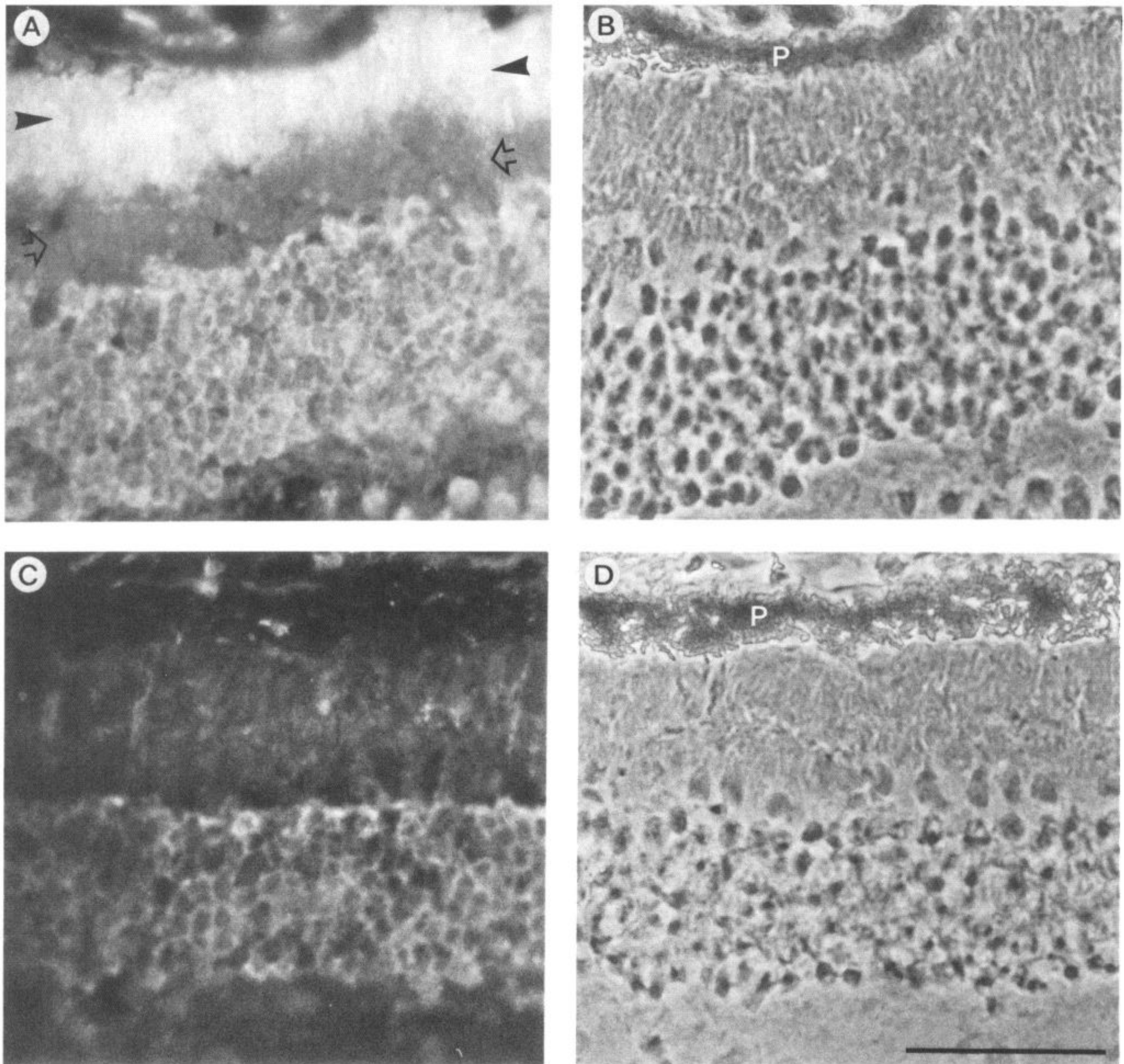


Figure 3. Immunofluorescent labeling of cryosections of bovine retina. *A*, Labeling with Na^+/Ca^{2+}-exchanger antiserum. Antibodies against the Na^+/Ca^{2+}-exchanger protein bind to the outer segment layer of photoreceptor cells (*solid arrowheads*). The inner segments (*open arrows*) are not labeled. *B*, Phase-contrast microscopy of the cryosection in *A*. *P*, pigment epithelium layer. *C*, Labeling with preimmune serum. Some nonspecific labeling of bipolar cells is seen. *D*, Phase-contrast microscopy of the cryosection in *D*. Scale bar, 50 μm.

segments of bovine photoreceptor cells are located (Fig. 3*A*). In the respective control (Fig. 3*C*) using the preimmune serum instead of the specific antiserum, the outer segment layer is not labeled, whereas other elements of the retina (e.g., the bipolar cells) show a similar degree of immunofluorescence to that seen with the specific antiserum. This residual labeling of nonphotoreceptor structures was concluded to be nonspecific.

In order to determine whether the outer segments of cone cells (not identifiable in cryosections under light microscopy) were also labeled, we performed immunostaining at the electron microscopic level. Figure 4 shows electron micrographs of immunolabeled rod (Fig. 4*A*) and cone (Fig. 4*C*) photoreceptors with the specific antiserum. Under the electron microscope, cone cells could be identified, e.g., by the high density of mitochondria in their inner segment. In contrast to rods, the outer segment of cone photoreceptors did not show significant Na^+/Ca^{2+}-exchanger-antibody binding. The density and distribution of particles over cone cells immunostained with the specific antiserum (Fig. 4*C*) is similar to controls where the preimmune serum was applied (Fig. 4*D*). Neither rod nor cone inner segments showed specific antibody binding.

The above observations were confirmed by quantitative analysis of immunogold-labeled sections (Table 1). The particle density on the plasma membrane of cone outer (COS plasma membrane) and inner (CIS plasma membrane) segments was about 1 per micron of membrane in sections stained with either preim-

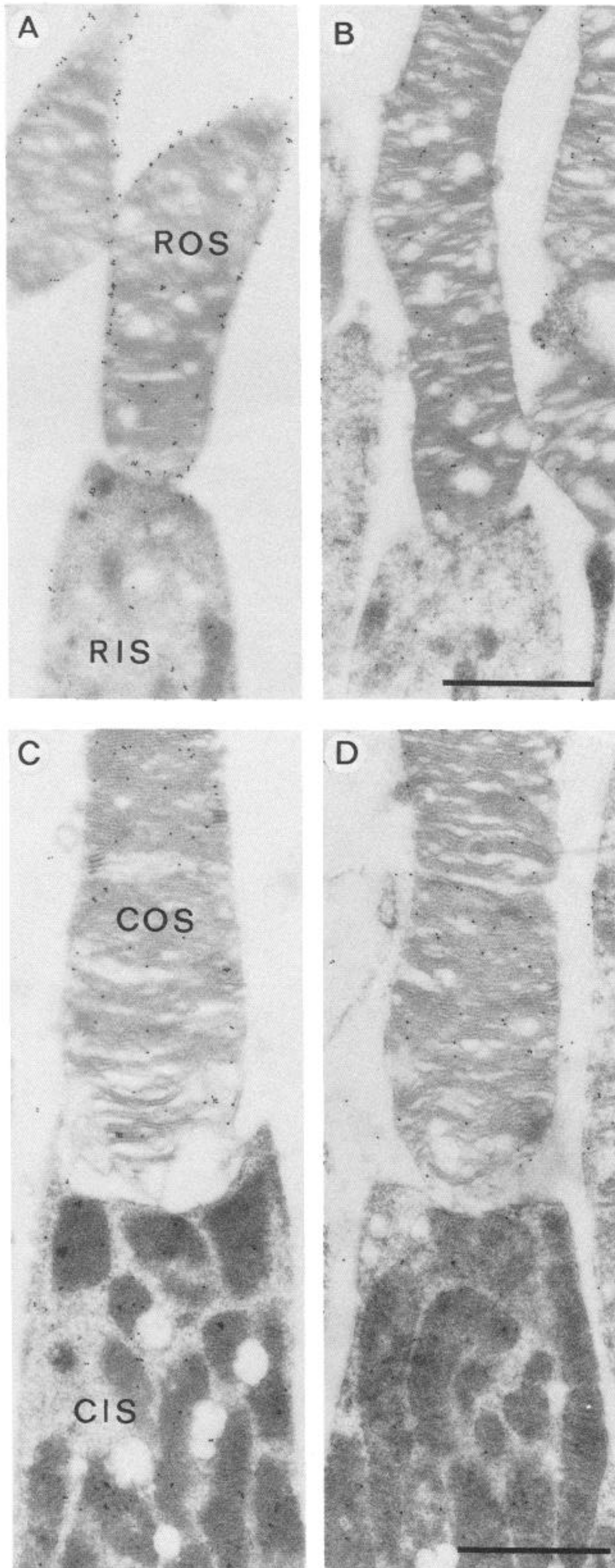


Figure 4. Electron microscopy of immunogold-labeled rod and cone photoreceptors. *A,* After incubation with Na^+/Ca^{2+}-exchanger antiserum (1:400), intense labeling occurs over the plasma membrane of rod outer segments (*ROS*) and is absent over the plasma membrane of rod inner segments (*RIS*). It is also absent over cone outer (*COS*) and inner (*CIS*) segments (*C*). Background labeling is documented by applying the preimmune serum (1:400) to rod (*B*) and cone (*D*) photoreceptors. Under our conditions, only background labeling can be detected over cone cells. The labeling of disk membranes of rods or cones with the preimmune serum is about the same as in (*A*) or (*C*), where the specific antiserum was used. Scale bars, 1 μm.

mune serum or with specific antiserum. Similar values were also obtained for rod inner (RIS plasma membrane) segment plasma membrane immunolabeled with either preimmune serum or with the antiserum. The plasma membrane of the rod outer segment was found to be the only photoreceptor membrane to show enhanced immunolabeling by the antiserum, the particle density being about 4 times greater than that after immunolabeling with preimmune serum.

Subcellular localization of anti-Na^+/Ca^{2+}-exchanger immunoreactivity in rod photoreceptors

Antibodies were found to bind intensely to the plasma membrane of ROS, the immunogold particle density there being about 7 times greater than that of the ROS disk membrane (Table 1). Particle density analysis also revealed a 5-fold higher immunogold density of the ROS plasma membrane relative to that of RIS plasma membrane. Immunolabeling with preimmune serum gave similar particle densities for all 3 membranes, thereby demonstrating that the immunoreactivity of the specific antiserum to rod photoreceptors is localized primarily to the ROS plasma membrane.

From immunolabeling experiments on cross-sectioned photoreceptors the same label distribution was obtained. After treatment with antiserum the gold label is concentrated over the periphery of the ROS (Fig. 5*A*), whereas in controls with the preimmune serum (Fig. 5*B*), no prominent labeling of the ROS plasma membrane is detected. With both antiserum and preimmune serum a low amount of nonspecific gold labeling of disk membranes can be seen. In Figure 5*A,* cross sections of both the outer and inner segments can be seen. The specific label is located over the plasma membrane of rod outer segments, but not over the plasma membrane of the inner segments. Some gold label can also be seen over the cytoplasm of inner segments; however, this can be considered to be nonspecific since treatment with preimmune serum gave similar results.

An interesting question is whether the Na^+/Ca^{2+}-exchanger protein is located in the outer rim of disk membranes or in the plasma membrane itself. Micrographs like that of Figure 6, where the plasma membrane and disk membranes have dissociated from each other clearly demonstrate that the gold marker is located along the plasma membrane of rod outer segments (Fig. 6, *A–C*). On the other hand, at places where the plasma membrane has been stripped off from the outer segment (Fig. 6*D*), no labeling of disk rims is found. These observations suggest that the antigen resides essentially in the plasma membrane of rod outer segments and not in the disk membranes.

In order to determine whether antibody binding occurs on the cytoplasmic or extracellular side of the plasma membrane, we performed preembedding immunolabeling of purified ROS. Figure 7 shows that the immunogold particles are located on the outside of the outer segments [this is easily visible at higher magnification (Fig. 7, inset)], thereby demonstrating that antigenicity exists at the extracellular side of the ROS plasma membrane.

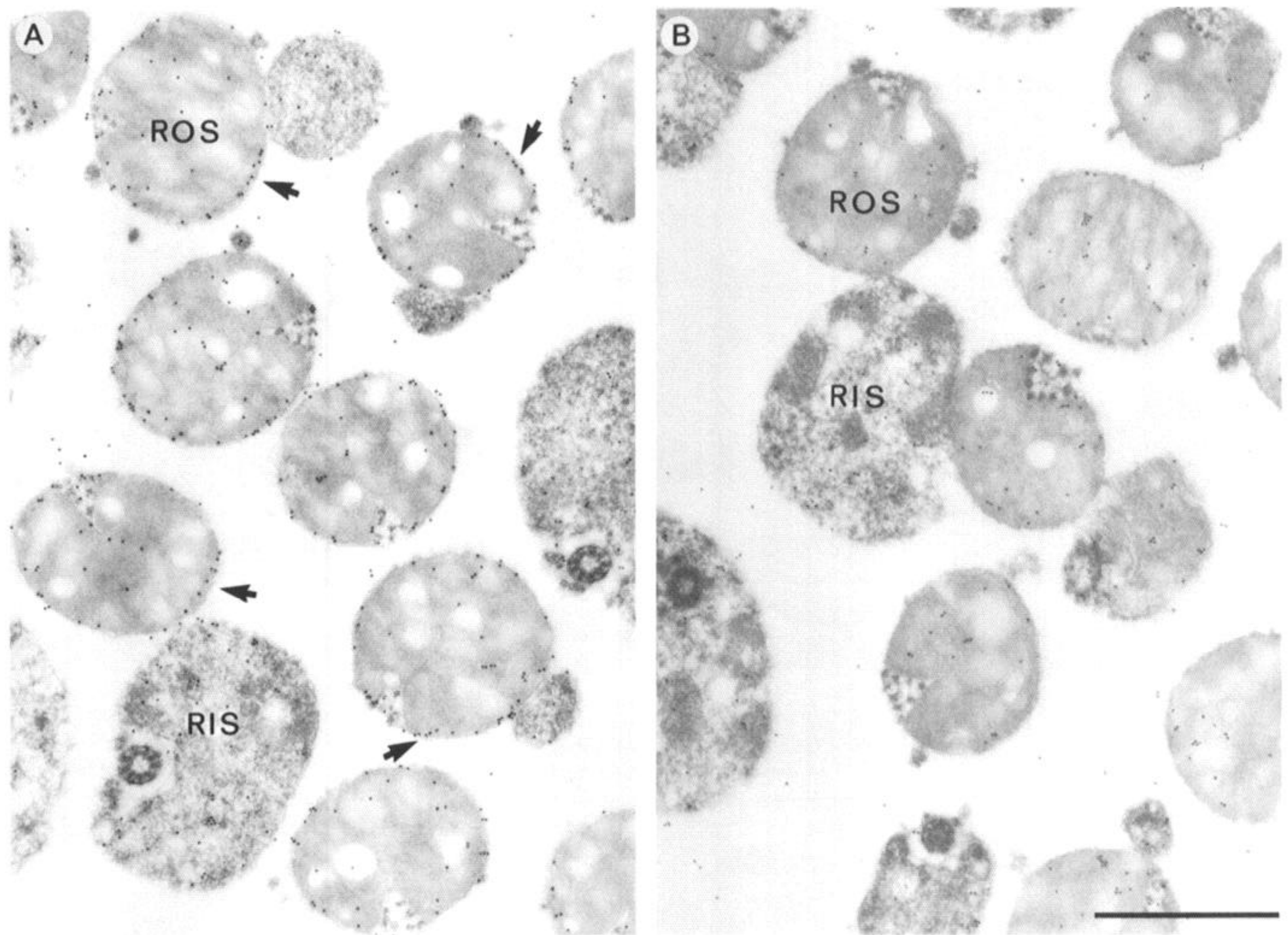


Figure 5. Electron microscopy of immunogold-labeled cross-sectioned rod outer (*ROS*) and rod inner (*RIS*) segments. *A,* With antiserum the labeling is concentrated over the plasma membrane of ROS (*arrows*). RIS display no specific labeling of the plasma membranes. *B,* With control preimmune serum, no intense labeling of plasma membranes is found. Scale bar, 1 μm.

Discussion

In this report, we describe the production and characterization of a polyclonal antibody to the 230 kDa Na^+/Ca^{2+}-exchanger purified from bovine rod outer segments. We were able to use this antibody to demonstrate that the Na^+/Ca^{2+}-exchange protein is distinct from the "rim protein," a relatively abundant high-molecular-weight protein in ROS which has been extensively characterized (Papermaster et al., 1978; Molday and Molday, 1979; MacKenzie and Molday, 1982; Szuts, 1985). We also found electrophoretic conditions where the Na^+/Ca^{2+}-exchanger migrated as a protein with an apparent molecular weight of 230 kDa and the rim protein with an apparent molecular weight of 210 kDa. The subcellular localization of Na^+/Ca^{2+}-exchanger immunoreactivity, i.e., the lack of immunoreactivity at the rims of ROS disk membranes, further confirms that these 2 proteins are distinct from each other.

In addition to the rim protein, the existence of other high-molecular-weight ROS glycoproteins has been recently described (Molday and Molday, 1987a, b; Polans and Burton, 1988). These glycoproteins were all demonstrated to be specifically localized in the plasma membrane of the photoreceptor outer segment. Given the immunocytochemical results reported above, it would be interesting to deterrmine whether any of the proteins described by these authors are identical to the Na^+/Ca^{2+}-exchanger.

Using the polyclonal antibody, we were also able to show that the exchanger is located in the outer segment of rod (but not cone) photoreceptors of the bovine retina. We take the lack of immunoreactivity with cone photoreceptors as an indication that the cone and rod Na^+/Ca^{2+}-exchange proteins are immunologically different, and not as an indication that cone photoreceptors do not possess a Na^+/Ca^{2+}-exchange system [for which there is much evidence to the contrary (Nakatani and Yau, 1989)]. It is also conceivable that the immunoreactivity of the cone Na^+/Ca^{2+}-exchanger is less stable under the tissue preparation conditions used than that of rod photoreceptors or that the amount of exchanger in cones is too small to be detected under these conditions.

We were also able to show that within the rod photoreceptor, the Na^+/Ca^{2+}-exchanger is located primarily in the plasma membrane of the outer segment. Na^+/Ca^{2+}-exchanger antibody binding was found to be localized to the extracellular side of this membrane. We note, however, that there exist several reports in the literature describing the existence of Na^+/Ca^{2+}-exchange fluxes across the ROS disk membrane (Schnetkamp et al., 1977;

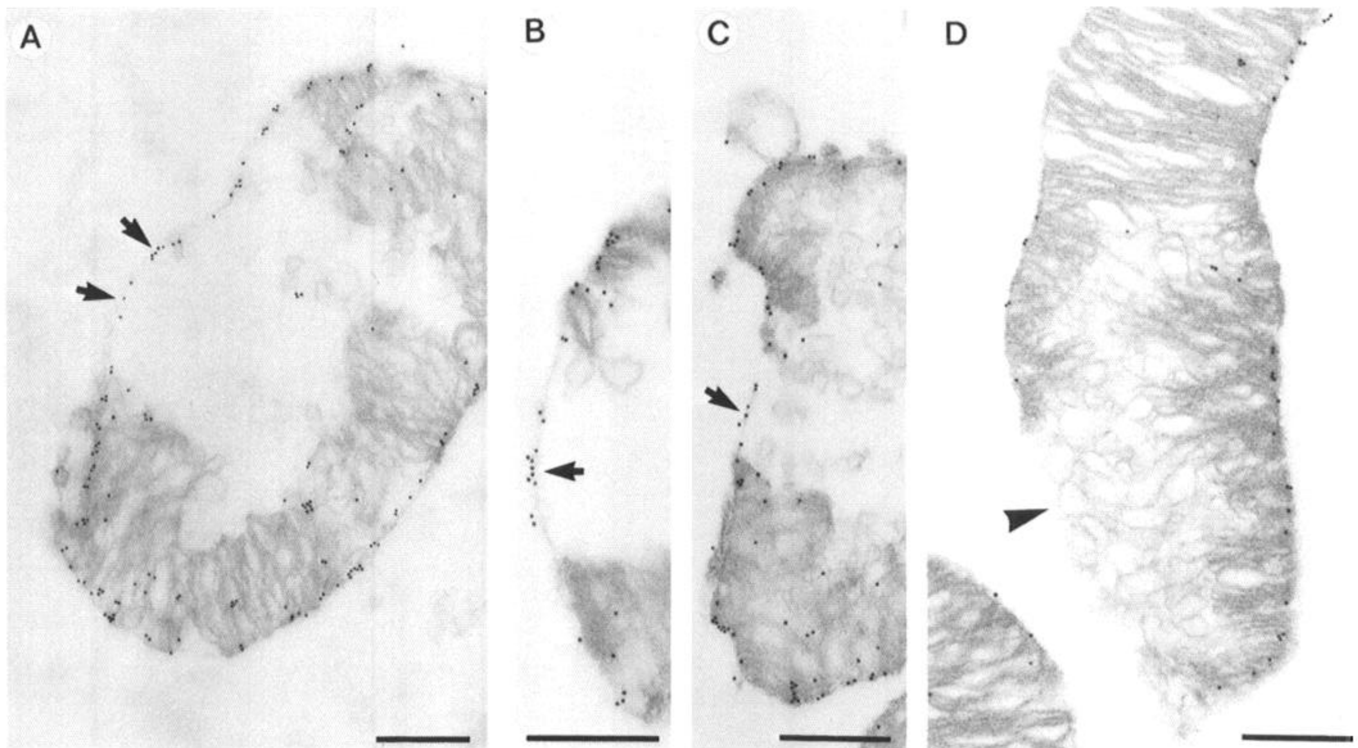


Figure 6. Electron microscopy of immunogold-labeled rod outer segments with detached plasma membrane. *A–C,* Heavily labeled sections of plasma membranes (*arrows*) after dissociation from the disk membrane. *D,* No labeling is detected at the rims of disk membranes (*arrowhead*) at a site devoid of plasma membrane. Scale bars, 0.5 μm.

Schnetkamp, 1986; Schnetkamp and Bownds, 1987). We believe that our results can be reconciled with these reports as follows: (1) our immunolocalization studies do not eliminate the possibility that residual Na^+/Ca^{2+}-exchange activity may exist in the ROS disk membrane (however, if this is so, it is clearly far lower than the Na^+/Ca^{2+}-exchange activity of the ROS plasma membrane); or (2) the Na^+/Ca^{2+}-exchange activity observed in disk membrane preparations may be an artifact arising from the

Table 1. Determination of particle density on plasma and disk membranes of rod outer (ROS) and rod inner (RIS) segments and of cone outer (COS) and cone inner (CIS) segments[a]

Membrane	Parameter	Preimmune serum	Specific antiserum	p^b
ROS plasma membrane	Particles	2421	9117	
	Membrane length (μm)	1566.46	1569.21	
	Particle density/μm	1.54 ± 0.05	5.82 ± 0.67	<0.001
RIS plasma membrane	Particles	797	1494	
	Membrane length (μm)	846.68	1370.40	
	Particle density/μm	0.9 ± 0.10	1.13 ± 0.21	NS
ROS disk membrane	Particles	1136	1113	
	Membrane length (μm)	1207.81	1421.80	
	Particle density/μm	0.95 ± 0.15	0.78 ± 0.02	NS
COS plasma membrane	Particles	280	148	
	Membrane length (μm)	236.61	132.84	
	Particle density/μm	1.24 ± 0.24	1.1 ± 0.24	NS
CIS plasma membrane	Particles	320	145	
	Membrane length (μm)	316.03	166.44	
	Particle density/μm	0.99 ± 0.06	0.83 ± 0.05	n.d.

We also determined p by comparing the means of ROS and RIS plasma membranes stained with the specific antiserum ($p < 0.001$) and comparing the means of specific antiserum stained ROS plasma membranes with ROS disk membranes ($p < 0.001$).

[a] Data were collected from 2 immunostainings (dilution 1:100) of 3 animals (1 eye each). Means ± SD are given.

[b] p was determined using Student's t test for unpaired data comparing the means of both preimmune serum and specific antiserum.

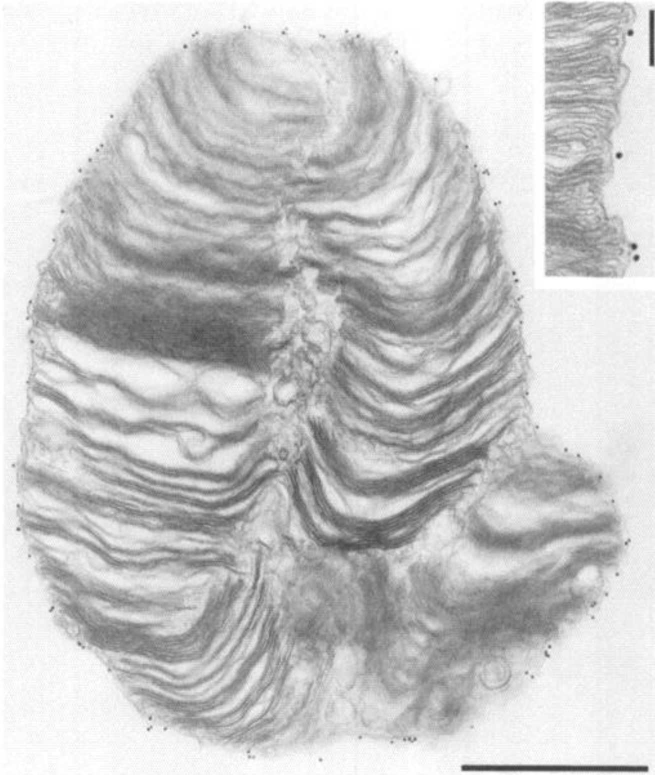


Figure 7. Preembedding immunogold labeling of isolated rod outer segments. The antibody can clearly be seen to bind to the extracellular side of the plasma membrane; this is more clearly seen at higher magnification (*inset*). Scale bar, 1 μm (inset, 0.1 μm).

fusion of plasma membrane components after permeabilization of the outer segment. We have previously presented evidence for such a fusion phenomenon when investigating the subcellular localization of the ROS cGMP-gated cation channel (Cook et al., 1989).

From biochemical and physiological studies it has been shown that calcium is an important ion participating in the light adaptation and restoration of vertebrate photoreceptors (Matthews et al., 1988; Pugh and Altman, 1988; Nakatani and Yau, 1988). It is generally accepted that in the dark calcium enters the photoreceptor outer segment via the cGMP-gated cation channels and is extruded by the Na^+/Ca^{2+}-exchanger, thus maintaining cytosolic calcium homeostasis. After illumination, the cytosolic calcium concentration decreases as a consequence of the closing of the cation channels and of the continuing activity of the Na^+/Ca^{2+}-exchanger. The resulting low cytosolic calcium is believed to directly or indirectly activate guanylate cyclase (Pepe et al., 1986; Koch and Stryer, 1988), thereby replenishing the cytosolic cGMP concentration and returning the photoreceptor cell to its preillumination state. Our results indicate that the Na^+/Ca^{2+}-exchanger is ideally localized to carry out its role in such a transduction process: in the outer segment plasma membrane the exchanger can directly remove calcium from the cytosol and expel it into the extracellular space.

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