Solubilization, Purification, and Reconstitution of the Sodium-Calcium Exchanger from Bovine Retinal Rod Outer Segments*

Neil J. Cookt and U. Benjamin Kaupp
From the Universität Osnabrück, Fachbereich 5 Biologie/Chemie, Abteilung Biophysik, Postfach 4469, D-4500 Osnabrück, Federal Republic of Germany

We have previously described a method for the solubilization and reconstitution of the cGMP-gated cation channel from the membranes of bovine rod outer segments (Cook, N. J., Zeilinger, C., Koch, K.-W., and Kaupp, U. B. (1986) Biochim. Biophys. Acta 861, 17033-17039). Here we report that not only cGMP but also sodium is capable of releasing entrapped calcium from liposomes reconstituted with total rod outer segment membrane proteins. Other alkali cations tested were unable to induce calcium efflux; therefore, we concluded that the sodium-induced calcium efflux was due to the sodium-calcium exchanger. Sodium was found to activate calcium efflux from these liposomes with an EC₅₀ of ~35 mM, comparable to values reported for the sodium-calcium exchanger in native rod outer segment membranes. We found that reconstitution of the sodium-calcium exchanger is quantitative and used this method to assay the exchanger protein during purification using conventional protein chromatographic techniques. In this way, we were able to purify and identify as the rod outer segment sodium-calcium exchanger a glycoprotein of apparent Mₛ = 220,000 to >90% homogeneity. The specific activity of the purified protein at room temperature was 8.2 µmol of Ca²⁺ exchanged min⁻¹ mg⁻¹ of protein at 50 mM Na⁺, corresponding to a turnover number of ~30 Ca²⁺ (or 90 Na⁺) s⁻¹ exchanger⁻¹. The Mₛ = 220,000 protein reported here appears to be distinct from another protein ("rim protein") with an identical Mₛ known to exist in these membranes.

Light results in the hyperpolarization of vertebrate rod photoreceptors by inducing the closure of cation-specific channels present in the plasma membrane (1, 2). These "light-dependent" channels are directly gated by cGMP via a cooperative mechanism (3–6). Although their primary function is to transport monovalent cations, these channels are also appreciably permeable to divalent cations (7). In the dark, the light-dependent channels mediate an inward current due to light also induces calcium release from rod photoreceptors by inducing the closure of cation-specific channels present in the plasma membrane, light also induces calcium release from rod photoreceptors (10–12) and a corresponding decrease of internal calcium (14), since the Na-Ca exchanger apparently operates independently of light.

The ROS Na-Ca exchanger shares many properties of Na-Ca exchange systems in other cells (see Ref. 15 for a recent review). It operates electrogenically with a stoichiometry of 3 Na⁺ for each Ca²⁺ exchanged (16), is capable of Ca-Ca exchange under steady state conditions (17, 18), is highly specific for sodium compared to other alkali cations (16, 19), and can change the internal Ca²⁺ concentration of ROS by as much as 0.5 mM s⁻¹ (19). Although the ROS cGMP-gated channel has been purified (20) and biochemically (20–22) and electrophysiologically (23) investigated, little work has been directed toward a biochemical characterization of the ROS Na-Ca exchange protein. The purification of the ROS Na-Ca exchange protein has been hampered by the absence of a method for detecting the protein after solubilization from its native membrane. In a previous report (24), we demonstrated that the ROS cGMP-gated channel can be effectively assayed after solubilization by reconstitution into asolectin liposomes, followed by spectroscopic measurement of cGMP-activated calcium fluxes. Here we have employed a similar reconstitution method followed by the spectroscopic determination of sodium-activated calcium fluxes to assay the ROS Na-Ca exchange protein. We were able to purify and subsequently identify a protein of apparent Mₛ = 220,000 as the ROS Na-Ca exchange protein. An initial characterization of the purified and reconstituted protein is presented.

**EXPERIMENTAL PROCEDURES**

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†Recipient of a long term fellowship from the European Molecular Biology Organization. To whom all correspondence and reprint requests should be addressed: Max-Planck Institut für Biophysik, Abteilung Molekülare Membranbiologie, Heierich-Hoffmann-Str. 7, D-6000 Frankfurt 71, Federal Republic of Germany.

**Materials**

CHAPS and Heps were supplied by Serva (Heidelberg, Federal Republic of Germany). Cyclic GMP, arsenazo III (98% pure), calcium ionophore (A23187), DTT, α-methyl-d-mannoside, and protease inhibitors were from Sigma (München, FRG). Soybean phosphatidylcholine (asolectin) Type IV-S from Sigma was further purified as previously described (24). DEAE-Flactogel-TSK and AF Red Fractogel-TSK were supplied by Merck (Darmstadt, FRG) and Sepharose 4B and concanavalin A-Sepharose 4B were from Pharmacia LKB Biotechnology Inc., (Freiburg, FRG). Dialysis tubing (inside diameter 1 and 8/32 inches) was from Medicel International Ltd. (London, United Kingdom). Electrophoresis chemicals (acrylamide (4 × crystallized), N,N′-methylenebisacrylamide (4 × crystallized), TEMED, SDS, and Coomassie Brilliant Blue R-250) were supplied by Serva.

The abbreviations used are: ROS, rod outer segment; Heps, N-2-hydroxycetylperazine-N'-2-ethanesulfonic acid; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; DTT, dithiothreitol; TEMED, N,N′,N″,N‴-tetramethylethylenediamine; SDS, sodium dodecyl sulfate; Con A, concanavalin A; FPLC, fast protein liquid chromatography.

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Electrophoresis molecular weight standards myosin (Mr = 205,000), β-galactosidase (Mr = 116,000), phosphorylase b (Mr = 97,000), bovine serum albumin (Mr = 66,000), ovalbumin (Mr = 45,000), and carbonic anhydrase (Mr = 29,000) were from Sigma. All other chemicals were from Merck and of the highest grade available.

Preparation of Bovine ROS Membranes

ROSs were prepared by a sucrose gradient method exactly as previously described (22) and stored at -20 °C for no longer than 14 days in tubes wrapped in thick aluminum foil. Before use, ROS membranes were thawed and hypotonically extracted three times in 10 mM Hepes-KOH, pH 7.4, 1 mM EDTA, and 1 mM DTT to remove peripheral proteins. Rhodopsin concentration was determined by the method of de Grip et al. (25). All procedures up to and including membrane solubilization were carried out under dim red light or darkness. All steps after solubilization were carried out in a cold room (4 °C) or on ice under normal laboratory lighting.

Reconstitution of the Solubilized Na-Ca Exchanger into Phospholipid Liposomes

Reconstitution of the Na-Ca exchanger from solubilized ROS membranes or from column fractions was carried out as previously described for the ROS cGMP-gated channel (24). Typically, 1 ml of a concentrated phospholipid-detergent extract was added to 1 ml of sample to give the following final concentrations: 10 mM Hepes-KOH, pH 7.4, 1 mM DTT, 10 mg ml⁻¹ soybean phosphatidylcholine, and 10 mM CHAPS (calcium and KCl concentrations were normally present in the dialysis buffer). Dialysis was carried out against three changes of 10 mM Hepes-KOH, pH 7.4, 0.1 mM DTT, 0.1 M KCl, and 2 mM CaCl₂ finally against one change of the same buffer without calcium.

Spectroscopic Flux Measurements and Determination of Na-Ca Exchange Activity

Sodium-, cGMP-, or ionophore-activated calcium efflux from liposomes was monitored with an Aminco DW-2A spectrophotometer operating in the dual wavelength mode (650-730 nm) using the calcium-indicator dye arsenazo III (20, 24). The application of arsenazo III to the measurement of calcium efflux from ROS disks has been described in depth elsewhere (26). Final concentrations in the cuvette (volume, 2 ml) were: arsenazo III, 50 µM; Hepes-KOH, pH 7.4, 10 mM; KCl, 0.1 M. Calcium efflux was activated by injecting sodium, cGMP, or A23187 into the cuvette using a Hamilton syringe coupled to the cuvette cap. Sodium-activated calcium efflux was relatively slow, and initial rates of efflux could be easily determined by tangent fitting. Na-Ca exchange activity is expressed as nanomoles of Ca²⁺ exchanged to the extracellular compartment per minute after the injection of 50 mM NaCl into the cuvette. Calcium release signals were calibrated by injecting known amounts (usually 1 nmol) of Ca²⁺ into the cuvette. For kinetic analysis, recordings were stored in a transient recorder (Tracer TN-1600) and transferred to a PDP 11/24 computer.

Chromatographic Procedures

All chromatographic procedures were carried out in a cold room (4 °C) under normal lighting. All buffers were freshly prepared and contained the following protease inhibitors: aprotinin, 5 µg ml⁻¹; leupeptin, 2 µg ml⁻¹; diisopropyl fluorophosphate, 0.02% (v/v). DEAE-Fractogel-TSK Chromatography—Anion exchange chromatography was carried out exactly as described for the first step in the purification of the cGMP-gated channel from bovine ROS membranes (20). Briefly, ROS membranes (typically, 50-70 mg of rhodopsin) were solubilized in 10 mM Hepes-KOH, pH 7.4, 1 mM DTT, 10 mM CaCl₂ (HDC buffer) containing 1.1% (w/v) CHAPS, 0.22% (w/v) soybean phosphatidylcholine, and 0.15 M KCl at a rhodopsin concentration of ~1 mg ml⁻¹. After centrifugation, the extract was directly applied to a DEAE-Fractogel-TSK column (0.9 × 4 cm) equilibrated with HDC buffer containing 0.8% (w/v) CHAPS, 0.17% (w/v) soybean phosphatidylcholine (HDCChP buffer), and 0.15 M KCl. After application of the sample, the column was washed with HDCChP/0.15 M KCl buffer, then eluted in a single step with HDCChP buffer containing 0.7 M KCl. The protein fraction eluted at high salt contained the Na-Ca exchange activity and was used for the next purification step. The flow rate throughout this chromatographic step was 0.5 ml min⁻¹.

AF Red Fractogel-TSK Chromatography—The Na-Ca fraction which contained the Na-Ca exchanger protein eluted at 0.7 M KCl from the DEAE-Fractogel-TSK column was directly applied to an AF Red Fractogel-TSK column (0.9 × 6.0 cm) equilibrated with HDCChP/0.7 M KCl buffer. The protein fraction which did not bind to this column matrix at 0.7 M KCl contained the Na-Ca exchange activity and was used for further purification steps (Con A-Sepharose 4B). The flow rate throughout this chromatographic step was 0.25 ml min⁻¹.

Con A-Sepharose 4B Chromatography—The protein fraction which did not bind to the AF Red Fractogel-TSK column at 0.7 M KCl was adjusted to contain 1 mM MgCl₂ and 1 mM MnCl₂. After application to a Con A-Sepharose 4B column (0.7 × 8 cm) equilibrated with HDCChP buffer containing 0.1 M KCl, 1 mM MgCl₂, and 1 mM MnCl₂. After sample application, the column was washed with the buffer used for column equilibration and subsequently the purified Na-Ca exchanger was eluted with the same buffer containing 0.1 M α-methyl-D-mannoside. The flow rate throughout this chromatographic step was 0.25 ml min⁻¹.

Sepharose CL-4B Chromatography—As an alternative to the Con A-Sepharose 4B purification step, the Na-Ca exchange protein could also be further purified from the protein fraction which did not bind to the AF Red Fractogel-TSK column at 0.7 M KCl by gel filtration on Sepharose CL-4B. The 0.7 M KCl nonbound protein fraction from the AF Red Fractogel-TSK was concentrated to a volume of 2 ml using an Amicon ultrafiltration cell and then applied to a Sepharose CL-4B column (1.6 × 100 cm) equilibrated with HDCChP buffer containing 0.15 M KCl. The column was eluted with the same buffer as that used for equilibration, and fractions (5.7 ml) were collected for reconstitution and electrophoresis. The flow rate throughout this chromatographic step was 0.6 ml min⁻¹.

PPLC DEAE-chromatography of ROS Membrane Proteins—In a typical experiment, ROS membranes (20 mg of rhodopsin) were solubilized in 20 ml of HDC buffer containing 1.1% CHAPS and 0.22% phosphatidylcholine. After centrifugation, the extract was applied as a series of 2-ml injections followed by 0.5-ml washes to a DEAE-Fractogel-TSK column (0.5 × 5 cm) fitted to a Pharmacia FPLC system and equilibrated with KCl-free HDCChP buffer. After washing with 2 ml of equilibration buffer, proteins were eluted with an isotonic salt gradient (0-0.5 M KCl) applied over 100 ml at a flow rate of 1 ml min⁻¹. Fractions (volume, 4 ml) were collected and tested for Na-Ca exchange activity and analyzed for protein content by SDS-gel electrophoresis.

Electrophoresis, Protein Assay

SDS-gel electrophoresis was carried out according to the method of Laemmli (27). The stacking gel contained 3% acrylamide and the separation gel 7.5%. Samples were denatured by suspension in 3% SDS and 1% β-mercaptoethanol (final concentrations) without boiling before electrophoresis. Gels were stained with Coomassie Blue R-250. Protein content was determined according to the method of Read and Northcote (28) using bovine serum albumin as the standard.

RESULTS

Solubilization and Reconstitution of the Na-Ca Exchanger from ROS Membranes

In Fig. 1, it can be seen that not only cGMP but also NaCl releases entrapped Ca²⁺ from liposomes formed after reconstitution of solubilized ROS total membrane proteins according to our previously described method (24). The amplitude of the Na⁺-induced Ca²⁺ release signal was always significantly (≈3-fold) larger than the cGMP-activated signal. Assuming that both proteins are reconstituted with comparable efficiencies, and given that the Na-Ca exchanger (but not the cGMP-gated channel) will mediate Ca²⁺ release regardless of its orientation in the liposome membrane, this suggests that the density of the Na-Ca exchanger in ROS membranes is at least comparable or even greater than that of the cGMP-gated channel.

In Fig. 2, the alkali cation specificity of the calcium release signal is investigated. Of all the alkali cations tested, only

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FIG. 1. cGMP- and sodium-induced calcium release from liposomes reconstituted with total ROS membrane proteins. Calcium-containing liposomes were prepared from solubilized ROS membranes as previously described (24). In both traces, the cuvette contained 0.3 ml of liposomes (0.375 mg of rhodopsin ml⁻¹, 10 mg of phospholipid ml⁻¹), NaCl, cGMP, and ionophore were injected into the cuvette at the time points indicated by the arrows.

Na⁺ was found to be effective in inducing calcium release (the rapid decrease in absorption immediately after the injection of the cation solution into the cuvette is presumably attributable to both dilution of the dye and an increase in ionic strength which induces a decrease in the affinity of arszenazo III for Ca²⁺). This specificity correlates well with the known specificity of the Na-Ca exchanger in native ROS membranes (16, 19) and of Na-Ca exchange systems in other cells (15). From this experiment, we conclude that the Ca²⁺-indicating absorption change is indeed due to ROS Na-Ca exchanger activity and does not result from a nonspecific release mechanism due to an increase in ionic strength outside the liposome membrane.

In order to further investigate whether the reconstituted Na-Ca exchanger is in a physiologically relevant condition, we determined the Na⁺ concentration dependence of the Na⁺-induced Ca²⁺ release. In Fig. 3A, calcium efflux signals generated by the injection of different Na⁺ concentrations into the cuvette are shown. The initial rate of Ca²⁺ efflux typically saturated at ≈100 mM NaCl. Higher sodium concentrations were often found to generate a submaximal rate of efflux. In Fig. 3B, normalized efflux rates are plotted as a function of the sodium concentration. Sodium activated the half-maximal Ca²⁺ efflux at a concentration (EC₅₀) of ≈35 mM. This value is in good agreement with an EC₅₀ = 30 mM in the presence of KCl reported by Schnetkamp (19) for the Na-Ca exchanger in the native ROS membrane. From Fig. 3B, it can also be seen that the Na⁺ dependence plot of Ca²⁺ efflux exhibits a distinct sigmoidicity suggesting that more than one Na⁺ is exchanged for each Ca²⁺ (as is known to be the case from studies directed at the stoichiometry or electrogenicity of Na-Ca exchange (16, 19, 30, 46)). The solid lines in Fig. 3B were calculated according to the formula (49, 50):

\[ V/V_{\text{max}} = \frac{[\text{Na}^+]^n}{K_n^+ + [\text{Na}^+]^n} \]

for n = 1, 2, and 3 (which denotes the number of Na⁺ ions involved in the exchange process) and assuming a value for Kₙ (EC₅₀) of 35 mM.

Functional Reconstitution as an Assay for the Na-Ca Exchanger Protein

The data shown in Figs. 2 and 3 demonstrate that the reconstituted Na-Ca exchanger exists in a physiologically relevant state, comparable to that of the ROS Na-Ca exchan-
ger in situ. In Fig. 4, Ca\(^{2+}\) effluxes generated by a constant sodium ion concentration (50 mM) from liposomes reconstituted with different amounts of protein (and therefore different amounts of Na-Ca exchanger) are shown. Both the signal amplitude and the initial rate of efflux increase with increasing protein concentrations, demonstrating that the reconstitution procedure is quantitative. The Ca\(^{2+}\) efflux velocity was always sufficiently slow to allow the accurate determination of initial rates of efflux and was therefore used as a measure of Na-Ca exchange activity during chromatographic purification.

**Purification and Identification of the ROS Na-Ca Exchanger Polypeptide**

**DEAE-Fractogel-TSK and AF Red Fractogel-TSK Chromatography**—During early experiments on the ROS Na-Ca exchanger, we simultaneously assayed both the Na-Ca exchanger and the cGMP-gated channel protein in fractions eluting from DEAE-Fractogel-TSK and AF Red Fractogel-TSK columns while purifying the cGMP-gated channel from ROS membranes (20). The Na-Ca exchange activity was found to be carried through with the cGMP-gated channel activity during anion exchange chromatography on DEAE-Fractogel-TSK. The two activities could subsequently be separated by a further chromatographic step using AF Red Fractogel-TSK. The Na-Ca exchange protein could be recovered in the protein fraction which did not bind to the AF Red Fractogel-TSK column at 0.7 M KC\(_1\) with a good yield (>40%) and high purification factor (65-fold) (Table I). Therefore, in most experiments, the 0.7 M KC\(_1\) nonbound fraction from the AF Red Fractogel-TSK column, usually obtained while routinely purifying the cGMP-gated channel protein, was used as the source for Na-Ca exchanger for further purification (Con A-Sepharose 4B or Sepharose CL-4B chromatography, see below). For SDS-polyacrylamide gel analysis of column fractions up to this stage, see Ref. 20.

**Con A-Sepharose 4B Chromatography**—The Na-Ca exchanger-containing extract from the AF Red Fractogel-TSK chromatography step was further purified by lectin affinity chromatography on Con A-Sepharose 4B. The Na-Ca exchanger protein binds to this matrix (thereby demonstrating that the Na-Ca exchanger is a glycoprotein) and could be eluted with buffer containing 0.1 M α-methyl-d-mannoside. This final purification step yielded a 108-fold purified extract (relative to solubilized ROS membranes) with a yield of 21% (Table I).

SDS-polyacrylamide gel electrophoresis analysis of fractions obtained during the ConA-affinity chromatography step is shown in Fig. 5. The protein fraction eluted by α-methyl-d-mannoside and containing the Na-Ca exchanger activity was found to consist of one single polypeptide of apparent \(M_\text{r} = 220,000\). Occasionally, low molecular weight components (possibly rhodopsin) were observed, but their absence or presence could not be correlated with Na-Ca exchange activity.

**Sepharose CL-4B Gel Filtration**—In order to confirm that the \(M_\text{r} = 220,000\) polypeptide is indeed the ROS Na-Ca exchanger, the protein fraction which did not bind to the AF Red Fractogel-TSK column at 0.7 M KC\(_1\) was further purified by gel filtration on Sepharose CL-4B (Fig. 6). Fractions were collected and analyzed for Na-Ca exchange activity by reconstitution and for protein content by electrophoresis. A single peak of activity (eluting just behind the void volume) was detected which corresponded perfectly to the elution profile of the \(M_\text{r} = 220,000\) polypeptide (Fig. 6, inset). The most active fractions (fractions 13 and 14) from this chromatographic separation consisted of essentially pure \(M_\text{r} = 220,000\) polypeptide.

**Is the Na-Ca Exchange Protein the “Rim Protein”?**

The above results demonstrate that the glycoprotein of \(M_\text{r} = 220,000\) constitutes the bovine ROS Na-Ca exchanger. In vertebrate ROS, a membrane protein of this molecular weight has been extensively characterized (31–33). This protein, referred to as the rim protein, has been shown to bind both concanavalin A and wheat germ agglutinin (32, 33), to span the disk membrane (32), to undergo light-regulated phosphorylation in frog ROS (34), and to be localized along the margins and incisures of frog ROS disks (31). Given the extensive biochemical characterization to which this protein has been subjected, it was of interest to determine whether or not the rim protein and the Na-Ca exchanger are one and the same protein.

To this end, we carried out FPLC DEAE-chromatography of total ROS membrane proteins to determine if there exists more than one polypeptide of \(M_\text{r} = 220,000\). As shown in Fig. 7, this is indeed the case. One protein of \(M_\text{r} = 220,000\) eluted from the DEAE-Fractogel-TSK FPLC column at about 0.12 M KC\(_1\) (fractions 9–14) and a second, corresponding to the Na-Ca exchange activity peak, eluted at about 0.25 M KC\(_1\) (fractions 18–21). Since the protein eluting at 0.12 M KC\(_1\) is present in much greater abundance than the protein at 0.25 M KC\(_1\), it probably constitutes the rim protein. We conclude that the \(M_\text{r} = 220,000\) protein that we have purified and identified as the bovine ROS Na-Ca exchanger is probably distinct from the previously reported rim protein. However, we cannot eliminate the possibility that some of the above properties attributed to the rim protein are also properties of the Na-Ca exchanger since the above reports have identified the rim protein purely on the basis of its electrophoretic mobility, a method which would not resolve the two proteins discussed here. It is also conceivable that the \(M_\text{r} = 220,000\)
**Table I**

Purification of the sodium-calcium exchanger from bovine rod outer segment membranes

One "unit" of exchanger activity is defined as that amount needed to exchange 1 nmol of Ca^{2+} min^{-1} at 50 mM NaCl. Activity in the corresponding column fractions (DEAE-Fractogel-TSK nonbound fraction at 0.15 M KCl, AF Red Fractogel-TSK bound fraction at 0.7 M KCl, and Con A-Sepharose 4B nonbound fraction) was negligible or not detected. See Ref. 20 for SDS electrophoresis of the first two chromatography steps, Fig. 5 for SDS electrophoresis of the Con A-Sepharose 4B step.

| Fraction                    | Volume | Units/ml | Total units | Protein | Units/mg protein | Recovery | Purification |
|-----------------------------|--------|----------|-------------|---------|-----------------|----------|--------------|
| Solubilized membranes       | 85     | 51.2     | 4352        | 0.675   | 75.8            | 100      | 1            |
| DEAE-Fractogel-TSK bound    | 4.8    | 824      | 3960        | 0.270   | 3052            | 91       | 40           |
| AF Red Fractogel-TSK non-   | 9.4    | 190      | 1788        | 0.0386  | 4928            | 41.1     | 65           |
| Con A-Sepharose 4B bound    | 5.0    | 118      | 922         | 0.0144  | 8166            | 21       | 108          |

**Fig. 5.** SDS-polyacrylamide gel electrophoresis of the Con A-Sepharose 4B chromatography experiment. The stacking gel contained 3% acrylamide and the separation gel 7.5% and was stained with Coomasie Brilliant Blue. The lanes contained: a, molecular weight markers; b, 120 μl of the protein fraction which did not bind to the AF Red Fractogel-TSK column and which was applied to the Con A-Sepharose 4B column; c, 120 μl of the protein fraction which did not bind to the Con A column (devoid of Na-Ca exchange activity); and d, 120 μl of the protein fraction (purified Na-Ca exchanger) which was eluted from the Con A column with buffer containing 0.1 M α-methyl-D-mannoside.

During the course of the purification protocol, we routinely tested column fractions for Na-Ca exchange activity by functional reconstitution, a procedure which is quantitative and reproducible. We have previously employed a similar strategy to purify the cGMP-gated cation channel from bovine ROS membranes. The long dialysis time inherent to the reconstitution procedure does however mean that the target protein cannot be assayed in fractions eluted from a column before the next chromatographic step is performed. Therefore, step-elution chromatography must be employed except for the very last step in a purification protocol (see Ref. 21 for a description of how the conditions for a step-elution procedure can be optimized).

During all purification procedures, we included protease inhibitors, calcium, and exogenous phospholipid (soybean phosphatidylcholine) in all buffers in order to stabilize the Na-Ca exchanger protein. The presence of calcium and phospholipid has also been found to stabilize the function of several other membrane proteins (see for example Refs. 35-38) during the course of their purification. This information may be important when attempting to chromatographically purify Na-Ca exchanger activity from other sources.

In order to confirm the identity of the Na-Ca exchanger polypeptide, we correlated activity profiles with the elution profiles of the $M_r = 220,000$ protein on gel filtration and FPLC anion exchange columns. Exchange activity was correlated well with the $M_r = 220,000$ polypeptide and not with...
by the thicker continuous diagonal line.

effluent at activity (nanomoles of Ca\(^{2+}\) released by 50 mM NaCl ml\(^{-1}\) column fraction) profile activity correlates with the elution profile of a but present at high specific activity, was responsible for Na-

any other proteins of molecular weights similar to those reported for Na-Ca exchanger proteins from other sources (see below).

**Liposome Density Analysis**—We also considered the possibility that another protein, not detected by electrophoresis but present at high specific activity, was responsible for Na-Ca exchange activity in the final purified extract. We could eliminate this possibility as follows. When the final ConA-Sepharose-purified extract was reconstituted at a protein concentration of 7.2 \(\mu\)g of protein ml\(^{-1}\) and a phospholipid concentration of 10 mg ml\(^{-1}\), 34% of the entrapped calcium could be released by NaCl. Thus, we conclude that, under these conditions, 34% of the liposomes contain at least one copy of the Na-Ca exchanger.

From quasielastic light scattering (24), we determined a mean liposome radius of 72 nm. From this value and using the procedure described previously (24), we calculated that, under the above conditions, there are \(3.2 \times 10^{13}\) liposomes ml\(^{-1}\) in our reconstitution system. When 34% of these liposomes have a Na-Ca exchanger copy, there are \(10^{15}\) exchangers ml\(^{-1}\), i.e. 18 pmol of exchanger ml\(^{-1}\) of liposomes. At 7.4 \(\mu\)g of protein ml\(^{-1}\), this value corresponds to one exchanger for every 1.8 \(M_t = 220,000\) proteins (this ratio would be even lower if one considers the possibility that some of the liposomes might contain more than one Na-Ca exchanger protein). If some other protein, not visible on the gel, were responsible for the exchanger activity, it would have to be present at this molar ratio (or lower) which is clearly not the case.

**Properties of the Purified and Reconstituted Na-Ca Exchanger**—As shown in Table I, we were able to purify the ROS Na-Ca exchanger to a specific activity of 8166 nmol of Ca\(^{2+}\) exchanged at 50 mM Na\(^{+}\) min\(^{-1}\) mg\(^{-1}\) of protein. This corresponds to a turnover number of about 30 Ca\(^{2+}\) (or 90 Na\(^{+}\) s\(^{-1}\) for each \(M_t = 220,000\) polypeptide. The actual maximal turnover number is probably higher given the following factors: (i) denaturation of the exchanger protein may have occurred during the reconstitution procedure; (ii) 50 mM Na\(^{+}\) does not produce the maximal efflux rate; (iii) in our system the membrane potential has not been clamped; and (iv) reconstitution with acidic phospholipids may enhance Na-Ca exchange activity as has been shown to be the case for the cardiac sarcolemmal Na-Ca exchanger (42, 44). We remark that if some other protein, not visible on the gel, is responsible for Na-Ca exchange activity, its turnover number for Ca\(^{2+}\) must be several orders of magnitude greater (i.e. probably unrealistic for an exchange protein) than the value reported above.

From Fig. 1, we inferred that the Na-Ca exchanger is present in ROS membranes at a density at least equal to, but possibly higher than, the cGMP-gated channel. Thus, under dark conditions where only a few percent of the light-regulated channels are open (1), the number of Na-Ca exchangers that will be operating will far exceed (=30-fold) the number of open channels. Therefore, it can be concluded that the Na-Ca exchange activity in ROS is sufficient to dispose of Ca\(^{2+}\) ions entering the cell via the cGMP-gated conductance and that the internal Ca\(^{2+}\) concentration is very low in darkness.

**Density of the Na-Ca Exchanger in ROS Membranes**—From Table I, it can be seen that approximately 72 \(\mu\)g of \(M_t = 240,000\) protein can be purified from solubilized membranes (57.4 mg of protein) with a recovery of 21%. This corresponds to =0.34 mg of exchanger protein, 57.4 mg\(^{-1}\) of ROS membrane protein, i.e. the Na-Ca exchanger represents about 0.6% of total ROS membrane protein. Given that rhodopsin constitutes =90% of total ROS membrane protein, this value yields a Na-Ca exchanger:rhodopsin of about 1:800. This would correspond to an exchange density of about 30 \(\mu\)m\(^{-2}\) if the Na-Ca exchanger is assumed to be distributed uniformly throughout the ROS disk and plasma membranes. On the other hand, if the Na-Ca exchanger is assumed to exist exclusively in the ROS plasma membrane (which represents about 5% of total ROS membrane), the density will be =600 \(\mu\)m\(^{-2}\), i.e. very similar to that of the cGMP-gated channel (as was implicated from the results in Fig. 1).

**Purification of Na-Ca Exchangers from Other Tissues**—Attempts to identify and purify the membrane protein(s) responsible for Na-Ca exchange activity from other sources have suffered from the lack of a suitable assay or probe once the Na-Ca exchanger protein has been solubilized from its native membrane. Until now, purification attempts have been based on selective extraction or selective proteolysis procedures in order to enrich Na-Ca exchange activity (as assessed by reconstitution) (39, 40). From that work, proteins of \(M_t =

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**Fig. 7.** FPLC DEAE-Fractogel-TSK chromatography of total ROS membrane proteins. Top, absorbance of the column effluent at 280 nm (continuous thin line) and the Na-Ca exchanger activity (nanomoles of Ca\(^{2+}\) released by 50 mM NaCl ml\(^{-1}\) column fraction) profile ( ). The isocratic KCl gradient is represented by the thicker continuous diagonal line. Bottom, SDS electrophoresis (7.5% acrylamide) of column fractions reveals that Na-Ca exchange activity correlates with the elution profile of a \(M_t = 220,000\) protein (fractions 18–21) which is not the \(M_t = 220,000\) rim protein (fractions 9–14).
proteins have been shown to immunoprecipitate Na-Ca exchanger from bovine photoreceptor cell membranes and 70,000 protein from bovine photoreceptor cells to reducing agents and chymotrypsin (48). It would be interesting to investigate if the techniques described here can be applied to the purification of Na-Ca exchange proteins from other tissues.

In conclusion, we report for the first time the purification to apparent homogeneity of the Na-Ca exchanger protein from bovine ROS. The availability of the protein in its purified form will greatly aid studies directed at understanding how the Na-Ca exchanger operates and is regulated in the photoreceptor cell in situ. The understanding of the molecular properties of the ROS Na-Ca exchanger may also lead to a greater understanding of how Na-Ca exchange functions in other cells.

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