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Purification of the cardiac Na^+–Ca^{2+} exchange protein

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We have used fractionation procedures to enrich solubilized cardiac sarcolemma in the Na^+–Ca^{2+} exchange protein. Sarcolemma is extracted with an alkaline medium to remove peripheral proteins and is then solubilized with decylmaltoside. Next, the exchanger is applied to DEAE-Sepharose and eluted with high salt. The DEAE fraction is applied to WGA-agarose, and a small fraction of protein, enriched in the exchanger, can be eluted by changing the detergent to Triton X-100. This fraction is reconstituted into asolectin proteoliposomes for measurement of Na^+–Ca^{2+} exchange activity and gel electrophoresis. The purified fraction has a Na^+–Ca^{2+} exchange activity of 600 nmol Ca^{2+}/mg of protein per s at 10 μM Ca^{2+} and a purification factor of about 30 as compared with control reconstituted sarcolemmal vesicles. Ca^{2+}–Ca^{2+} exchange and Na^+–Ca^{2+} exchange activities were both present in the same final reconstituted vesicles indicating that the same protein is responsible for both transport activities. SDS-PAGE reveals two prominent protein bands at 70 and 120 kDa. After mild chymotrypsin treatment (1 μg/ml), there is no loss of exchange activity, but the 120 kDa band disappears and the 70 kDa band becomes more dense. This suggests that the 70 kDa band is due to an active proteolytic fragment of the 120 kDa protein. Under non-reducing gel conditions, only a single protein band is seen with an apparent molecular weight of 160 kDa. Antibodies to the purified exchanger preparation are able to immunoprecipitate exchange activity and confirm that the 70 kDa protein derives from the 120 kDa protein. We propose that both the 70 and 120 kDa proteins are associated with the Na^+–Ca^{2+} exchanger.

Introduction

The Na^+–Ca^{2+} exchange transport pathway of cardiac sarcolemma is a highly active and important mechanism for regulating myocardial Ca^{2+}. Many aspects of Na^+–Ca^{2+} exchange have been well characterized in studies using isolated sarcolemmal vesicles (for reviews, see Refs. 1 and 2). More recently, several groups have successfully solubilized and reconstituted transport activity (see, for example, Refs. 3–9). Procedures for the isolation of the Na^+–Ca^{2+} exchange protein would permit many molecular investigations, and there has been a series of reports towards this goal [5–7,10]. Hale et al. [5] described the use of pronase to remove all proteins except the Na^+–Ca^{2+} exchanger from solubilized sarcolemma. They identified a major protein band with a molecular weight of 82 kDa as the exchanger. Further purifi-

Abbreviations: Mops, 4-morpholinepropanesulfonic acid; Caps, 3-(cyclohexylamino)propanesulfonic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; WGA, wheat germ agglutinin; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; DTT, dithiothreitol; NEM, *N*-ethylmaleimide; PMSF, phenylmethylsulfonyl fluoride.

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cation studies, however, have shown that the 82 kDa protein can be separated from exchange activity (Reeves, J.P., personal communication; Carafoli, E., personal communication). Soldati et al. [7] have correlated a 33 kDa protein with exchange activity in the fractions obtained from rate zonal centrifugation of solubilized sarcolemma. However, even their most enriched fraction contains a large number of protein bands. Antibodies produced from proteins in the 30 kDa region of an SDS-polyacrylamide gel partially inhibit exchange activity [11]. In studies using synaptic plasma membranes, Barzilai et al. [6,10] have suggested that the Na^+–Ca^{2+} exchanger is a 70 kDa protein.

There are problems in attempting to isolate the Na^+–Ca^{2+} exchange protein. The exchanger is apparently a high-turnover, low-abundance protein [12]. In addition, there is no specific label which can be followed during fractionation procedures. After solubilization and fractionation, all samples must be reconstituted into liposomes for assay of transport activity.

We report procedures for the purification of the Na^+–Ca^{2+} exchange protein from cardiac sarcolemma. The most prominent proteins in our final preparation have an apparent molecular weight of 70 and 120 kDa. The 70 kDa protein may be an active proteolytic fragment of the 120 kDa protein. Antibodies to the enriched Na^+–Ca^{2+} exchange preparation immunoprecipitate activity and confirm that the 70 and 120 kDa proteins are immunologically related.

Materials and Methods

Solutions. Unless otherwise stated, all solutions were buffered with 10 mM 4-morpholinepropanesulfonic acid (Mops)-NaOH at pH 7.4.

Preparation of sarcolemmal vesicles. Highly purified sarcolemma was isolated from dog ventricles by our previously published methods [13,14]. The sarcolemmal vesicles were suspended in 140 mM NaCl after isolation.

Purification of the Na^+–Ca^{2+} exchanger. Sarcolemmal vesicles (4 mg) were first subjected to alkaline extraction [8]. The vesicles were diluted about 10-fold with cold 10 mM 3-(cyclohexylamino)propanesulfonic acid (Caps), pH 12. Following a 10 min incubation, the extracted vesicles were collected by centrifugation (25 min at 140000 × *g*), and solubilized with 2.4 ml 140 mM NaCl, 10 mM decylmaltoside at 4°C. Following centrifugation to remove nonsolubilized material (20 min at 140000 × *g*), the supernatant was diluted with 1 volume of 140 mM NaCl (20°C). The sample was next applied to the DEAE-Sepharose column (0.6 ml bed volume in a 1.5 cm diameter column pre-equilibrated with 140 mM NaCl, 0.5 mM decylmaltoside). The column was eluted with 1.4 ml of 200 mM NaCl, 0.5 mM decylmaltoside, and then the fraction enriched in Na^+–Ca^{2+} exchange activity was eluted with 2.4 ml of 500 mM NaCl, 3 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS). This fraction was applied twice to the wheat germ agglutinin (WGA)-agarose column (0.4 ml bed volume in a 0.8 cm diameter column pre-equilibrated with 140 mM NaCl, 1 mM CHAPS). The WGA column was washed with 0.8 ml of 500 mM NaCl, 1 mM CHAPS, and Na^+–Ca^{2+} exchange activity was then eluted with 1.0 ml of 10 mg/ml asolectin, 1.5% Triton X-100, 500 mM NaCl, 20 mM Mops-Tris (pH 7.4). Further protein, but little exchange activity, was eluted with 1.0 ml of the same solution containing 0.1 M *N*-acetyl-D-glucosamine. Both columns were run at room temperature.

Reconstitution of solubilized fractions. Samples were reconstituted into proteoliposomes using asolectin. Some fractions in our purification scheme already contained a mixture of asolectin and Triton X-100. Added to those fractions which did not, was one quarter volume of 50 mg/ml asolectin, 7.5% Triton X-100, 0.5 M NaCl, 20 mM Mops-Tris (pH 7.4). The Triton X-100 was then removed to produce proteoliposomes using Bio-Beads SM-2. The mixture was agitated for 20 min at room temperature with the Bio-Beads SM-2. The volume of Bio-Beads was approximately 20% of the reconstitution mixture. To ensure complete removal of the detergent this step was repeated with fresh Bio-Beads. The supernatant was diluted with 140 mM NaCl and spun at 140000 × *g* for 90 min. The resulting pellets were resuspended in 40 μl of 140 mM NaCl for sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Na^+–Ca^{2+} exchange measurements.

Gel electrophoresis. An 8.5% polyacrylamide Laemmli system [15] was used. Samples were first precipitated with cold acetone and extracted with diethyl ether to remove excess lipid. Sample buffer contained either 3.3 mM dithiothreitol (DTT) or 10 mM *N*-ethylmaleimide (NEM). Protein bands were visualized by silver staining [16].

Na^+–Ca^{2+} exchange measurements. Na^+–Ca^{2+} exchange was measured as Na_i^+-dependent Ca^{2+} uptake as described previously [8,9]. Briefly, Na^+-loaded reconstituted vesicles (0.003–0.005 ml) were rapidly diluted into a Ca^{2+} uptake medium (0.25 ml) containing 140 mM KCl, 0.01 mM $CaCl_2$, 0.3 μCi $^{45}CaCl_2$, 0.36 μM valinomycin to initiate Na_i^+-dependent Ca^{2+} uptake. The uptake reaction was quenched after 1.5 s by the addition of 0.03 ml of 140 mM KCl, 10 mM EGTA followed by the further addition of 1.0 ml of cold 140 mM KCl, 1 mM EGTA. The sample was filtered (Sartorius, 0.22 μm), and the filter washed with 2 × 3 ml cold 140 mM KCl, 1 mM EGTA. Blanks were obtained in an identical manner except the Ca^{2+} uptake medium contained NaCl instead of KCl. Blanks corrected for any Ca^{2+} uptake or binding which was not Na^+-gradient dependent.

Antibody production. The Na^+–Ca^{2+} exchanger was purified as described above, emulsified with Freund's complete adjuvant, and injected subcutaneously at multiple sites on the back of a rabbit. Two and four weeks after the initial immunization, this procedure was repeated except with the use of Freund's incomplete adjuvant. Antiserum was collected one week after the final immunization and at one month intervals thereafter.

Immunoblots. Proteins from SDS-PAGE were transferred onto nitrocellulose for 30 min at 100 V in a Bio-Rad Mini Trans-Blot apparatus. Immunoreactions were detected using goat anti-rabbit IgG conjugated to horseradish peroxidase with 4-chloronaphthol as substrate.

Immunoprecipitation. Protein A-Sepharose (0.05 ml) was incubated with 0.5 ml of antiserum or pre-immune serum for 30 min at room temperature with gentle agitation and then washed well with 140 mM NaCl, 10 mM Mops (pH 7.4). Sarcolemma (75 μg) was solubilized with 140 mM NaCl, 10 mM decylmaltoside, spun to remove nonsolubilized material, and incubated with the prepared beads at room temperature with gentle agitation. After 2 h, the beads were removed, the solubilized mixtures were reconstituted as described above, and Na^+–Ca^{2+} exchange activity was measured.

Miscellaneous. Protein was measured by the method of Wang and Smith [17] or by the method of Schaffner and Weissman [18].

Materials. WGA-agarose was from E-Y Laboratories. Protein A-Sepharose, DEAE-Sepharose CL-6B, Triton X-100, CHAPS, and chymotrypsin were from Sigma. Decylmaltoside was from Calbiochem. Bio-Beads SM-2 and nitrocellulose were from Bio-Rad. Asolectin was from Associated Concentrates.

Results

Alkaline extraction, DEAE chromatography, and WGA-affinity chromatography are three fractionation techniques which, when applied in series, are useful for the purification of the Na^+–Ca^{2+} exchange protein. We will briefly describe each of these steps, detail their use in series, and then describe immunological studies.

Alkaline extraction

The use of pH 12 media to enrich sarcolemma in Na^+–Ca^{2+} activity has been previously described [8]. This step doubles specific activity with no loss of total activity (Table I).

DEAE chromatography

The Na^+–Ca^{2+} exchanger readily binds to DEAE-Sepharose and can be eluted by raising the NaCl concentration. The protein elution pattern, however, is very detergent dependent. In initial experiments, sarcolemmal membranes were solubilized with CHAPS and applied to the DEAE. Activity could be eluted when the NaCl was raised above about 250 mM. However, no selective elution occurred. All protein eluted in parallel with the exchanger. Apparently, the ionic interactions are dominated by the zwitterionic CHAPS. Selective elution was initially obtained when the experiment was performed using either octylglucoside or octylthioglucoside as the detergent. In this case, exchange activity was eluted at intermediate NaCl levels with substantial amounts of protein eluting at both lower and higher NaCl. A third elution

pattern was obtained using sarcolemma solubilized with either nonylglucoside or decylmaltoside. Most protein could then be eluted at low NaCl and a fraction enriched in Na^+–Ca^{2+} exchange eluted at higher NaCl levels. Best results were obtained with decylmaltoside, and further experiments used this detergent. Activity was better conserved by running the columns at room temperature rather than at 4°C. Results of a typical experiment are shown in Fig. 1. After reconstitution 77% of the total recovered exchange activity was in fraction D. Typically, the fraction contained 60–80% of total activity and 20% of the eluted protein.

WGA-affinity chromatography

The use of WGA immobilized on agarose is somewhat nonconventional in this study. If sarcolemma is solubilized in either CHAPS or decylmaltoside and then applied to the column most activity binds to the column whereas most protein passes through (Fig. 2). Unexpectedly, most Na^+–Ca^{2+} exchange activity can be eluted by simply changing the detergent to Triton X-100 without the use of specific sugars. The phenomenon has also been observed in another laboratory (Reeves, J.P., personal communication). The remaining protein and a small amount of activity could then be eluted by adding *N*-acetyl-D-glucosamine to the column. An example is shown in Fig. 2. Typically, 50–60% of the total applied exchange activity could be recovered in fraction B after reconstitution.

Purification of the Na^+–Ca^{2+} exchange protein

We have used the alkaline extraction, DEAE, and WGA chromatography in series, and results are shown in Fig. 3 and Table I. Details of the preparation are given in Materials and Methods. As seen in Fig. 3, only two prominent protein bands are left in the final sample (lane D). These proteins have apparent molecular weights of 70 and 120 kDa. An example of this result is also shown in Fig. 4 (lane B). The serially purified protein fraction was used for the experiments shown in Fig. 4. A similar staining pattern is seen with Coomassie blue rather than silver stain (not shown). Under nonreducing conditions (Fig. 4, lane A), only one prominent gel band at 160 kDa is apparent. Use of lower percentage gels (5.5%) did not reveal further protein bands (not shown). The faint band at 160 kDa in Fig. 3, lane D may represent a small amount of non-reduced material and was present in all preparations.

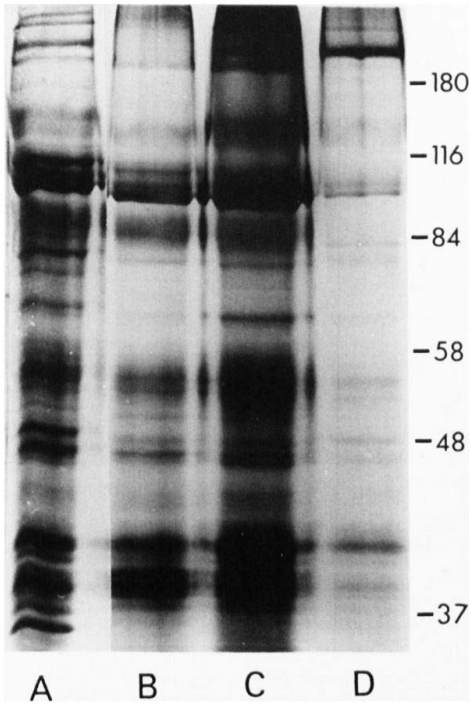

Fig. 1. Fractionation of sarcolemmal proteins on DEAE-Sepharose. 0.4 mg of sarcolemmal vesicles at 3 mg of protein/ml were solubilized with an equal volume of cold 140 mM NaCl, 30 mM decylmaltoside and spun for 12 min in a Beckman Airfuge to remove nonsolubilized material. The supernatant was diluted with one volume of 140 mM NaCl at room temperature and applied to the DEAE column (0.2 ml bed volume, 0.8 cm diameter). Native sarcolemma (5 μg) was applied to lane A. The flow through was fraction B. Fraction C was eluted with 0.5 ml of 200 mM NaCl, 0.5 mM decylmaltoside and fraction D was eluted with 0.5 ml of 500 mM NaCl, 0.5 mM CHAPS. To each fraction was added one quarter volume of 50 mg/ml asolectin, 7.5% Triton X-100, 500 mM NaCl, 20 mM Mops-Tris (pH 7.4). The fractions were then reconstituted by the Bio-Bead method and harvested by centrifugation. After resuspension, about one half of each sample was used for SDS-PAGE as shown. The percentage of the total Na^+–Ca^{2+} exchange activity in fraction B, C, and D was 0, 23, and 77%, respectively.

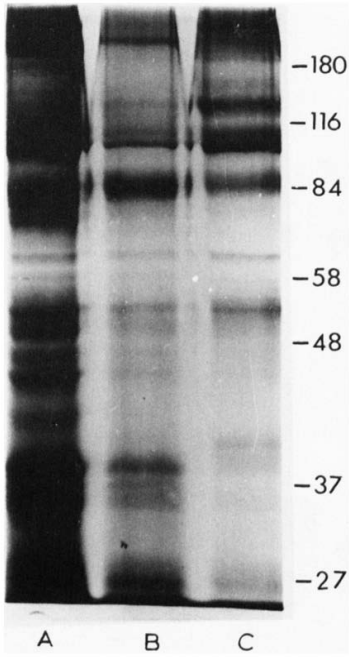

Fig. 2. Fractionation of sarcolemmal proteins on WGA-agarose. Sarcolemma (0.4 mg; 3 mg of protein/ml) was solubilized with one volume of cold 140 mM NaCl, 30 mM decylmaltoside. Nonsolubilized material was removed (12 min spin in a Beckman Airfuge), and the supernatant was diluted with one volume of 140 mM NaCl (room temperature). The sample was applied twice to a WGA-agarose column (0.25 ml bed volume, 0.8 cm diameter), and the flow through was collected (fraction A). The column was then washed with 0.4 ml 140 mM NaCl, 0.5 mM decylmaltoside. Fraction B was eluted with 0.6 ml of 10 mg/ml asolectin, 1.5% Triton X-100, 500 mM NaCl, 20 mM Mops-Tris (pH 7.4). Fraction C was eluted with 0.6 ml of the same solution containing 0.1 M *N*-acetyl-D-glucosamine. Fractions were reconstituted, and the reconstituted vesicles were collected by centrifugation. Samples were used for SDS-PAGE as shown and for Na^+-Ca^{2+} exchange measurements. In this example, the percentage of the total Na^+-Ca^{2+} exchange activity in fractions A, B, and C was 19, 49, and 31%, respectively.

The yield of Na^+-Ca^{2+} exchange activity was 15% of that activity in control reconstituted vesicles and the purification factor was 27-fold. This level of purification was obtained despite an inactivation of 75% of overall activity during the isolation scheme (primarily during the DEAE step). Thus, the true purification achieved is likely to be significantly higher. Activities in the final fraction are compared with control reconstituted vesicles rather than with native sarcolemmal vesicles since exchange activity becomes stimulated upon reconstitution with asolectin [5,7,9]. The exchange activity of the native sarcolemmal vesicles under identical reaction conditions would be about 5 nmol/mg protein per s. Faint protein bands are also present in our final preparation though the pattern of these components was quite variable. Those faint bands which were most consistently present include ones at about 220 and, as mentioned above, 160 kDa. Some proteins do not silver stain well. Thus, we also did autoradiography after radioiodination by the chloramine T method [19], but could detect no other protein bands (not shown).

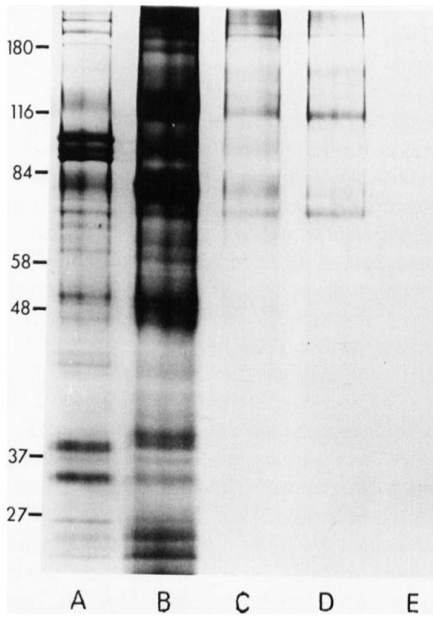

Fig. 3. Purification of the Na^+-Ca^{2+} exchange proteins. See Materials and Methods for details. Lane A, native sarcolemmal vesicles (3 μg). Lane B, sample after alkaline extraction. Lane C, sample after alkaline extraction and DEAE chromatography. Lane D, sample after alkaline extraction, DEAE, and WGA chromatography. The samples in lanes B–D were all first reconstituted with asolectin for Na^+-Ca^{2+} exchange measurements (Table I) before SDS-PAGE. Lane E, control asolectin vesicles (i.e., no protein added).

TABLE I

PURIFICATION OF THE Na^+-Ca^{2+} EXCHANGER

Typical data for serial purification of the Na^+-Ca^{2+} exchanger. The starting material was 4 mg of sarcolemmal vesicles. For details see Materials and Methods. Activities and protein for each fraction were measured after reconstitution. SDS-PAGE of fractions is shown in Fig. 3.

	Protein (μg)	Exchange activity recovered (%)	Exchange specific activity (nmol/mg protein per s)	Purification factor
Control reconstituted vesicles	1640	100	24	1
Alkaline extraction	706	91	53	2
DEAE chromatography	35	25	292	12
WGA chromatography	9	15	654	27

Chymotrypsin treatment

Experiments were done in which the serially purified fraction after the WGA column was exposed to chymotrypsin before reconstitution. Mild conditions which resulted in no loss of activity were chosen. Fig. 4 shows an experiment (lanes B–D) in which increasing amounts of chymotrypsin were used. Low chymotrypsin caused the 120 kDa band to disappear and increased the density of the 70 kDa band (Fig. 4, lane C). In some experiments the 70 kDa band after chymotrypsin treatment appeared as a doublet. After increased chymotrypsin treatment, the 70 kDa band also disappears and a protein band at 36 kDa appears. The appearance of the 36 kDa band did not occur in all experiments, however, and the gel pattern at higher chymotrypsin levels was variable. The band at 24 kDa in lane D is due to chymotrypsin. The low molecular weight protein in lanes A–D was absent in other experiments (see, for example, Fig. 3, lane D) and is assumed to be unimportant. That is, preparations could be obtained which did not contain this protein but did possess high exchange activity.

Since the 70 kDa protein may be a proteolytic fragment of the 120 kDa protein, we performed purification experiments with phenylmethylsulfonyl fluoride (PMSF), a proteinase inhibitor, present throughout. The PMSF had no effect on our final gel pattern. We have not yet tried other proteinase inhibitors.

Ca^{2+}-Ca^{2+} exchange

Cardiac sarcolemmal vesicles exhibit high rates of both Na^+-Ca^{2+} and Ca^{2+}-Ca^{2+} exchange activities [20,21]. It is generally assumed that these activities are alternative modes of the same transport system. To test this assumption, we assayed for Ca^{2+}-Ca^{2+} exchange activity in our final fraction which was highly enriched in Na^+-Ca^{2+} exchange activity. The reconstituted vesicles were first loaded with Ca^{2+} via the Na_i^+-dependent Ca^{2+} uptake reaction. the Ca^{2+}-loaded vesicles were then diluted into efflux media containing either 100 μM EGTA or 40 μM $CaCl_2$. During the 1 min efflux period, 5% of the Ca^{2+} was lost from the vesicles in the presence of EGTA, while 86% of the Ca^{2+} was released in the presence of extravesicular Ca^{2+}. Under our reconstitution conditions it is very unlikely that any one proteoliposome will have more than one protein. The same reconstituted vesicles which were loaded with $^{45}Ca^{2+}$ by Na^+-Ca^{2+} exchange could be emptied of $^{45}Ca^{2+}$ by Ca^{2+}-Ca^{2+} exchange. This is strong evidence that Ca^{2+}-induced Ca^{2+} efflux (Ca^{2+}-Ca^{2+} exchange) and Na^+-Ca^{2+} exchange activity are due to the same protein(s).

Immunological studies

The reconstituted, purified Na^+-Ca^{2+} exchange preparation was used as an antigen to produce polyclonal antibodies in a rabbit. Fig. 5A shows the reaction of these antibodies with the purified exchanger preparation transferred onto nitrocellulose. A strong reaction is seen at 70 kDa and a weaker reaction is at 120 kDa. The reaction at 120 kDa was variable, but always visible. The intensity of the reaction at 70 kDa was always greater than that at 120 kDa. In most cases, the reaction at 120 kDa was greater than for the case

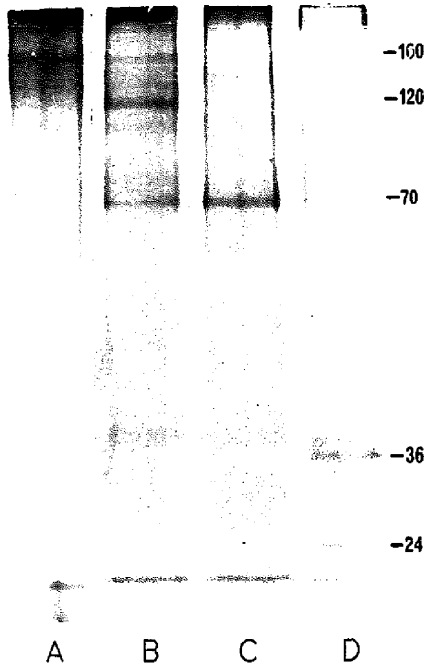

Fig. 4. Effects of chymotrypsin treatment and non-reducing conditions on the Na^+-Ca^{2+} exchanger. Purification procedures as described in Materials and Methods were performed and the enriched exchange fraction was subjected to various treatments. Lanes A and B show the purified exchanger under non-reducing (NEM present) or reducing (DTT present) conditions, respectively. Aliquots of the fraction were treated with either 1 (lane C) or 30 μg/ml (lane D) of chymotrypsin for 10 min at room temperature before reconstitution. The proteolytic reaction was terminated by the addition of PMSF (200 μM). The band at 24 kDa in lane D is due to chymotrypsin. Chymotrypsin treatment, under these conditions, did not affect Na^+-Ca^{2+} exchange activity. The chymotrypsin activity was 60 IU/mg enzyme.

shown in Fig. 5A. Upon mild proteolysis with chymotrypsin (Fig. 5B), the reaction at 120 kDa disappears and the reaction at 70 kDa becomes more intense. Further chymotrypsin treatment (Fig. 5C) causes the 70 kDa band to largely disappear. Thus, the immunoblot largely mirrors the pattern seen on SDS-PAGE (see Figs. 4 and 5) and is consistent with the hypothesis that the 70 kDa protein is a proteolytic fragment of the 120 kDa protein. Immunoblots using native sarcolemmal vesicles rather than the purified exchanger preparation showed the same reaction pattern as seen in Fig. 5A. If the SDS-PAGE was done under non-reducing conditions, only a reaction at 160 kDa was observed on the immunoblot. Incubation of sarcolemmal vesicles with the antibodies does not inhibit Na^+-Ca^{2+} exchange activity. This is different than the results obtained with the antibodies produced by Longoni and Carafoli [11].

To confirm that the 70 and 120 kDa proteins are immunologically related, we did experiments with affinity-purified antibodies. Sarcolemmal proteins were first transferred onto nitrocellulose, incubated overnight with antiserum (1:100 dilution), and then washed extensively with 140 mM NaCl, 10 mM Mops, 0.05% Tween to remove nonspecific binding. The regions corresponding to the 50, 70 and 120 kDa bands were cut out of the nitrocellulose and bound antibodies were eluted with 0.1 M glycine (pH 2.5) and then neutralized with 0.5 M Mops (pH 7.4). These affinity-purified antibodies were then used for immunoreactions against blotted sarcolemmal proteins. The antibodies to the 70 kDa protein reacted with both the 70 and 120 kDa proteins and likewise the antibodies to the 120 kDa protein reacted with both

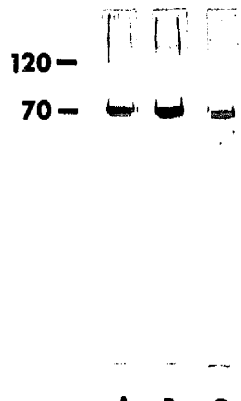

Fig. 5. Immunoreactions with the purified Na^+-Ca^{2+} exchange preparation. Proteins were blotted onto nitrocellulose and incubated with antiserum (1:300 dilution; see Materials and Methods for details). Shown are reactions with the purified exchanger after treatment with 0 (lane A), 1 (lane B), or 30 (lane C) μg/ml chymotrypsin for 10 min at room temperature before reconstitution.

proteins. The antibodies eluted from the 50 kDa region did not react anywhere on the blot and were included as a negative control for non-specific antibody binding.

Immunoprecipitation of exchange activity

Either antiserum to the purified exchanger or pre-immune serum was bound to protein A-Sepharose and incubated with solubilized sarcolemma. After 2 h, the solubilized sarcolemma was reconstituted. We found that $96.8 \pm 1.6\%$ ($n = 3$) of the Na^+–Ca^{2+} exchange activity had been immunoprecipitated by the antiserum as compared with the high activity found after incubation with the pre-immune serum. The use of less antiserum resulted in a corresponding decrease in the amount of Na^+–Ca^{2+} exchange activity which was immunoprecipitated.

Discussion

We have developed fractionation procedures for enriching solubilized sarcolemma in the Na^+–Ca^{2+} exchange protein(s). Under reducing conditions, the two most prominent proteins in our final reconstituted vesicles are at 70 and 120 kDa and we suggest that one or both of these proteins is the exchanger. The 70 kDa protein may be an active proteolytic fragment of the 120 kDa protein, since the density of the 70 kDa band increases as the 120 kDa band decreases with mild chymotrypsin treatment. The results are consistent with the work of Barzilai et al. [6,10] who identified a 70 kDa protein in synaptic plasma membranes as the exchanger.

Antibodies raised against our purified exchanger fraction react strongly with the 70 kDa protein and weakly with the 120 kDa protein. Both cross-reactivity and chymotrypsin experiments suggest that the two proteins are immunologically related (see Results). The antibodies immunoprecipitate Na^+–Ca^{2+} exchange activity.

Under non-reducing conditions, we find only one protein band, at 160 kDa, in our gel patterns. Presumably, the 160 kDa protein is composed of proteins which appear in the gel under reducing conditions. However, the specific origin of this band is unclear. The band always tended to be somewhat broadened (see Fig. 4A) and may represent dimers composed of the 70 and 120 kDa proteins in different combinations. This may account for the observed broadening. The non-additive apparent molecular weight may reflect the anomalous behavior of many proteins on SDS-PAGE [22].

Important steps in our purification scheme are alkaline extraction and the use of anion-exchange chromatography in the absence of exogenous phospholipids and in the presence of a non-ionic detergent. In addition, we selectively eluted exchange activity from a WGA-affinity column by changing detergents rather than by adding *N*-acetyl-D-glucosamine. This is an empirically useful step for which there is no obvious rationale. The dihydropyridine-binding subunit of the skeletal muscle dihydropyridine receptor can also be eluted from WGA columns by changing detergent without the use of a specific sugar [23].

Mild treatment of native sarcolemmal vesicles with chymotrypsin stimulates Na^+–Ca^{2+} exchange activity [24]. This suggests that the exchanger is susceptible to proteolytic cleavage consistent with the present results. We find that the exchange activity of the reconstituted vesicles was unaffected by the proteinase treatment, however, rather than stimulated. This is because reconstitution of the exchanger into asolectin vesicles by itself results in stimulated activity [5,7,9]. Under these conditions, further stimulation by controlled proteolysis cannot be obtained [9].

The specific activity of the Na^+–Ca^{2+} exchanger in our most enriched fraction is about 600 nmol/mg per s at 10 μM Ca^{2+}. The $K_m(Ca^{2+})$ for the exchanger in asolectin vesicles is about 10 μM [9], and we would expect a V_{max} of about 1.2 μmol/mg per s. If the molecular weight of the exchanger is 120 kDa, this would imply a turnover number of about 150 s^{-1} at V_{max}. However, the exchanger has not been purified to homogeneity, and significant inactivation occurs during the isolation scheme. Thus, the true maximal turnover rate is likely to be several times 150 s^{-1}. An estimate of 1000 s^{-1} is consistent with the recent indirect estimate of Cheon and Reeves [12].

Taken together, the purification and immunological data indicate that both the 70 and 120 kDa proteins are associated with sarcolemmal Na^+–Ca^{2+} exchange activity. However, minor

components are present in both the SDS-PAGE and immunoreaction patterns. We cannot eliminate the possibility that a very minor sarcolemmal protein (e.g., either the 160 or 220 kDa protein) is the exchanger. However, the 70 kDa protein is of the same size as the protein identified by Barzilai et al. [6] as the exchanger in brain plasma membranes. It is unlikely that this obvious similarity is coincidence. Perhaps the protein is processed differently in brain tissue so that only the 70 kDa fragment is expressed. Alternatively, there may be different amounts of proteolysis occurring during the different membrane preparations.

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