J. Membrane Biol. 106, 211–218 (1988)

The Journal of
Membrane Biology

Evidence for High Molecular Weight Na-Ca Exchange in Cardiac Sarcolemmal Vesicles

Calvin C. Hale†‡, Steven B. Kleiboeker†‡, Carol G. Carlton‡, Michael J. Rovetto‡§, Chan Jung‖, and H.D. Kim★

Departments of †Veterinary Biomedical Sciences, ‡The John M. Dalton Research Center, §Physiology, and ★Pharmacology, University of Missouri-Columbia, Columbia, Missouri 65211 and ‖Veterans Administration Hospital, Buffalo, New York 14215

Summary. Cardiac sarcolemma (SL) vesicles were subjected to irradiation inactivation-target sizing analyses and gel permeation high performance liquid chromatography (HPLC) to ascertain the weight range of native Na-Ca exchange. Frozen SL vesicle preparations were irradiated by electron bombardment and assayed for Na-Ca exchange activity. When applied to classical target sizing theory, the results yielded a minimum molecular weight (M_r) of approximately 226,000 ± 20,000 SD (n = 6). SL vesicle proteins were solubilized in 6% sodium cholate in the presence of exogenous phospholipid and fractionated by size on a TSK 30XL HPLC column. Eluted proteins were mixed 1:1 with mobile phase buffer containing 50 mg/ml soybean phospholipid and reconstituted by detergent dilution. The resulting proteoliposomes were assayed for Na-Ca exchange activity. Na-Ca exchange activity eluted in early fractions containing larger proteins as revealed by SDS-PAGE. Recovery of total protein and Na-Ca exchange activity were 91 ± 7 and 68 ± 11%, respectively. In the peak fraction, Na-Ca exchange specific activity increased two- to threefold compared to reconstituted controls. Compared to the elution profile of protein standards under identical column conditions, sodium cholate solubilized exchange activity had a minimum M_r of 224,000 Da. Specific $^{45}Ca^{2+}$-binding SL proteins with M_r of 234,000, 112,000, and 90,000 Da were detected by autoradiography of proteins transferred electrophoretically to nitrocellulose.

These data suggest that native cardiac Na-Ca exchange is approximately 225,000 Da or larger. The exact identification and purification of cardiac Na-Ca exchange protein(s) remains incomplete.

Key Words Na-Ca exchange · sarcolemma · reconstitution · HPLC · target sizing · cardiac

Introduction

Many "excitable" cells contain a Na-Ca exchange mechanism on their plasma membrane, which may play a role in either excitation-secretion or excitation-contraction coupling. In cardiac myocytes, Na-Ca exchange is likely to affect Ca^{2+} homeostasis by lowering cytosolic Ca^{2+} levels during diastole [*cf.* 25, 28]. In 1979, Reeves and Sutko demonstrated Na-Ca exchange in subcellular vesicle preparations [26]. Highly purified sarcolemma (SL) vesicle preparations have enabled description of important parameters of the exchange process such as electrogenicity, kinetics, and stoichiometry as well as advances toward purification [*cf.* 25]. Three laboratories have reportedly identified protein(s) responsible for catalyzing the exchange of Na^+ for Ca^{2+} in cardiac SL vesicles. In the first report, Hale et al. detailed a protease digestion procedure in which detergent-solubilized cardiac SL vesicle proteins were rapidly degraded prior to reconstitution [6]. The resulting proteoliposomes contained significant Na-Ca exchange activity that escaped degradation. Analysis of these preparations by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) revealed the presence of a diffuse 82,000-Da protein. The second report was that of Michalak and coworkers who reported that monoclonal antibody 44D7, originally raised against non-T non-B acute lymphoblastic leukemia cells, inhibited Na-Ca exchange in cardiac and skeletal muscle SL vesicles [15,20]. Under nonreducing conditions, immunoprecipitates of SL protein by 44D7 contained a major polypeptide with an apparent M_r of 125,000 Da by SDS-PAGE. The intensity of this band was decreased by the reducing agent dithiothreitol (DTT) and two bands of apparent M_r of 95,000 and 38,000 Da were observed. The third report was from Carafoli and coworkers who used rate zonal centrifugation to facilitate density separation of cardiac SL vesicles and reconstituted proteoliposomes [31]. They determined that a SL protein with an apparent M_r of 33,000 Da best correlated with Na-Ca exchange activity in these preparations. Additionally, "specific" polyclonal antibodies prepared against this protein not only inhibited Na-Ca exchange but, under nonreducing conditions, recognized proteins of 33,000, 70,000, and 140,000 Da M_r following

SDS-PAGE and electrophoretic transfer to nitrocellulose [16]. Under reducing conditions, the 140,000-M_r protein was not recognized. They proposed that the basic unit of cardiac Na-Ca exchange was a monomer of 33,000 with the 140,000-M_r tetramer representing the functional form. The 70,000-M_r protein in this scheme represented an intermediate dimeric state in the monomer-tetramer transition.

In addition to proteins identified in the heart, Barzilai et al. described a 70,000-Da protein in brain synaptosomes that purified with Na-Ca exchange activity upon density gradient centrifugation [4]. Relationship, if any, between this protein and the 70,000-Da intermediate dimeric protein described above is unclear. In any event, an antibody prepared against the protein in brain has been reported capable of immunoprecipitation of the functional protein [3].

Thus the protein(s) responsible for catalyzing cardiac Na-Ca exchange has not been conclusively identified. Antibodies apparently capable of inhibiting the exchange process have no cross-recognition with other reported proteins. In this report, we describe the results of radiation target analysis and high pressure liquid chromatography (HPLC) fractionation of Na-Ca exchange activity and the identification of specific Ca^{2+}-binding SL proteins. Based on the data, we conclude that the native cardiac Na-Ca exchanger is approximately 225,000 M_r or larger.

Materials and Methods

Preparation and Handling of SL Vesicles

Cardiac SL vesicles were prepared from trimmed bovine ventricular tissue obtained from a local abattoir by the method of Kuwayama and Kanazawa [12] as modified by Slaughter, Sutko and Reeves [30]. In experiments where vesicle protein was subjected to HPLC, the final washes for the vesicle preparations contained 160 mM sodium phosphate, 20 mM MOPS/Tris buffer, at pH 7.4. SL vesicle preparations were rapidly frozen in cryovials with liquid N_2 and stored at −70°C until used for study. For target sizing protocols, 0.5 ml aliquots were rapidly frozen in planchets and stored at −70°C. Na-Ca exchange activity in freshly prepared SL vesicle preparations was unaltered by one freeze-thaw cycle.

Solubilization and HPLC Fractionation of SL Vesicle Protein

Vesicles were thawed at 37°C, pelleted by ultracentrifugation (180,000 × g; 0.5 hr). The pellet was resuspended and solubilized in 6% sodium cholate buffered with 100 mM sodium phosphate, 2.5 mg/ml soybean phospholipids (Asolectin; Associated Concentrates), pH 7.3 at a final protein concentration of 10 mg/ml. This mixture remained at room temperature for 30 min with occasional agitation on a vortex mixer. The extracted, solubilized proteins were recovered from the supernatant fluid of this mixture following 0.5 hr centrifugation at 100,000 × g. The unsolubilized, pelleted fraction was discarded. Extracted proteins (0.2 ml) were injected onto a TSK 30XL column (7.7 × 300 mm, 5.0 ml void; BioRad) connected to a Perkin-Elmer Series 4 HPLC. The column resin had been previously equilibrated with a mobile phase of the above phosphate-lipid buffer containing 2% sodium cholate. The column flow rate was 0.3 ml/min and column effluent fraction was collected at 1-min intervals.

Reconstitution of SL Vesicle Protein

Reconstitution of sodium cholate solubilized SL protein into proteoliposomes was accomplished by the detergent-dilution method described by Miyamoto and Racker [21] and modified by Hale et al. [6]. HPLC fractions and control extracts were mixed 1 : 1 with column equilibration buffer containing 50 mg/ml soybean phospholipid followed by a five-fold dilution into ice-cold buffer of 160 mM NaCl, 20 mM MOPS/Tris, pH 7.4. Reconstituted proteoliposomes were recovered by two successive ultracentrifugation washes (180,000 × g; 2.0 hr) in the dilution buffer.

Electrophoretic Techniques

The Tris-glycine system of Laemmli was used for electrophoresis in 0.1% sodium dodecyl sulfate (SDS) with the percent acrylamide in the resolving gel indicated in the figure legends [13]. Samples were prepared and visualized as previously described [6]. Electrophoretic transfer onto nitrocellulose (Western blot) was performed by the method of Towbin, Staehelin and Gordon [33]. Calcium-binding proteins, which had been electrophoretically transferred onto nitrocellulose, were visualized using autoradiography as described by Ebashi and coworkers [18]. Briefly, 150 μg of bovine SL vesicle proteins were fractionated on a 5% SDS polyacrylamide gel followed by electrophoretic transfer onto a nitrocellulose membrane at room temperature at 100 mA for 12 hr. The extended time insured a more complete transfer of proteins with M_r greater than 100,000. The blot was then washed at room temperature for 1 hr (two changes) with 60 mM KCl, 5 mM $MgCl_2$, 10 mM MOPS/Tris, pH 6.8, followed by a 10-min incubation in the same buffer containing 1 mCi $^{45}Ca^{2+}$/liter. Finally, the blot was rinsed 5 min with dH_2O, dried at 37°C and subjected to autoradiography. Radioactive peaks were quantified using a scanning densitometer (Hoefer Scientific Instruments, Model 6S300).

Radiation Target Sizing of Cardiac Na-Ca Exchange

Five hundred μl of freshly prepared SL vesicles (~5–10 mg protein) were placed in aluminum planchets and rapidly frozen in liquid N_2. Samples (including controls that were not irradiated) were packed in dry ice and shipped from Columbia, MO, to Buffalo, NY, for electron bombardment. Samples were then shipped back to Columbia, MO, for assay. At no time during transport or handling during irradiation treatment were samples allowed to thaw. The irradiation inactivation was performed as previously described by Jung et al. with some modification [8]. Briefly, planchets containing frozen samples were passed under an electron beam of uniform geometry and frequency receiving 0.675

MeV at a dose rate of 0.5 MeV/min/pass. Radiation target analyses of Na-Ca exchange activity were performed as described by Kepner and Macey [11].

Miscellaneous Assays

Na-Ca exchange activity was measured in native SL vesicles and reconstituted proteoliposomes by the method of Reeves and Sutko [27]. Na-Ca exchange was quantified as the difference in $^{45}Ca^{2+}$ accumulation into vesicles in the presence or absence of an outwardly directed Na^+ gradient. Protein assays for native SL vesicles were performed by the method of Lowry [17]. To avoid interference from the high concentrations of exogenous lipid and low protein content, reconstituted proteoliposome protein assays were performed by the method of Schaffner and Weissmann [29] as described by Kaback and coworkers [22].

Based on the procedure of Ahmed and Reis [1], the hydrolysis rate of 2, 8, ^{3}H-5′-adenosine monophosphate (AMP) to ^{3}H-adenosine was used to estimate 5′nucleotidase activity. The reaction was initiated by rapid mixing of 40 to 80 μg bovine SL vesicle protein with 10 μM AMP, 10 μCi ^{3}H-AMP/ml in 160 mM NaCl, 20 mM MOPS/Tris, 2 mM $MgSO_4$, pH 7.4 at 37°C. Samples were taken from the reaction vessels at timed intervals up to 5 min. These were immediately mixed with ice-cold perchloric acid to give a final acid concentration of 4.6% (wt/vol). The protein was pelleted by centrifugation (13,000 × g, 5 min) and the supernatant fluid was removed, neutralized with 2 M K_2CO_3, 0.5 M MOPS. Aliquots were loaded on 7 × 40 mm columns of PEI cellulose (Sigma) equilibrated with dH_2O. Nucleosides, including ^{3}H-adenosine, were eluted with dH_2O. AMP was eluted with 3, 1 ml volumes of 50 mM KH_2PO_4. The column eluates were collected and the radioactivities were determined by liquid scintillation spectroscopy. In all cases the time courses for AMP hydrolysis were determined and were found to be linear over several minutes. The rates were calculated from linear time course curves over which no more than 20% of the AMP was hydrolyzed.

Results

Radiation Target Analysis of Na-Ca Exchange in Cardiac SL Vesicles

Initial attempts to assess Na-Ca exchange by radiation target analysis used SL vesicle preparations subjected to radiation doses up to 15 Mrad. These preliminary experiments clearly indicated that Na-Ca exchange activity was substantially decreased by irradiation doses less than 5 Mrad and was essentially inactivated by higher doses. Subsequent experiments were confined to radiation levels between 1 and 4 Mrad. The sensitivity of Na-Ca exchange to electron bombardment inactivation is shown in Fig. 1. Na-Ca exchange activity was inactivated by relatively low-dose radiation. In addition to Na-Ca exchange, the same SL vesicle preparations were assayed for 5′-nucleotidase, cytochalasin B binding, and glucose transport [5]. Inactivation of D-glucose (*vs.* L-glucose) transport and cytochalasin B binding to the glucose transporter did not parallel Na-Ca exchange inactivation, as much larger doses were required for inactivation. This strongly suggests that irradiation, especially at lower doses which inactivated Na-Ca exchange activity, did not produce significant nonspecific membrane disruption. As an internal control, 5′-nucleotidase inactivation yielded a target size of 81,500 ± 5,000 Da, which is consistent with previously reported values of about 73,000 Da [2, 32]. The results of target size analyses using six different SL vesicle preparations are shown in the Table. As indicated in the Table, the molecular weight for cardiac Na-Ca exchange predicted by radiation target sizing is 226,000 ± 20,000 (SD). For reasons indicated in the Discussion, this value may be a minimum molecular weight.

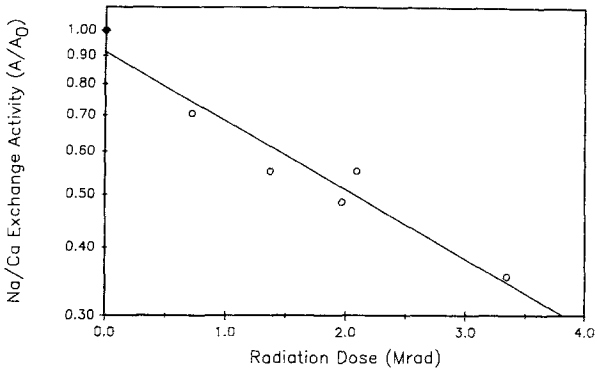

Fig. 1. Loss of Na-Ca exchange activity in cardiac SL vesicles following irradiation by high energy electrons. Native cardiac SL vesicles were irradiated in the frozen state as described in Materials and Methods and [8]. Data shown were from a single experiment. Each datum point represents the average of triplicate determinations. In this experiment, target size analysis M_r was 225,000 Da

Table. Radiation target analyses of Na-Ca exchange in cardiac sarcolemmal vesicles[a]

Experiment	Mol wt (Da)
1	223,000
2	258,000
3	216,000
4	225,000
5	195,000
6	241,000
Mean	226,000 ± 20,000 (SD)

[a] Summary of Na-Ca exchange irradiation inactivation sizing experiments. Six separate bovine SL vesicle preparations were subjected to irradiation inactivation and target sizing analyses. Each experiment was conducted individually over the course of one year.

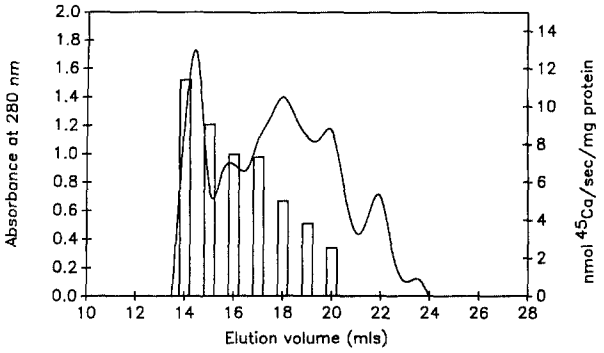

Fig. 2. Elution pattern of sodium cholate solubilized cardiac SL vesicle proteins and Na-Ca exchange from gel permeation HPLC. Native cardiac SL vesicle proteins were detergent solubilized and fractionated by gel permeation HPLC. The column effluent was monitored at 280 nm. Individual column fractions were reconstituted and assayed for Na-Ca exchange activity

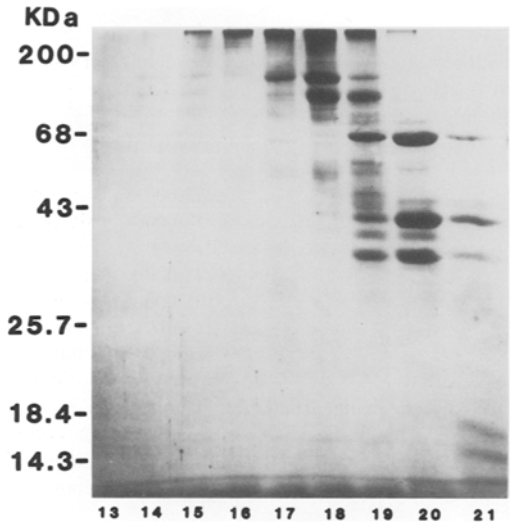

Fig. 3. SDS-PAGE of cardiac SL vesicle proteins fractionated by gel permeation HPLC. HPLC column fractions (150 μl) were subjected to SDS-PAGE (10% acrylamide) as described in Materials and Methods. Proteins were visualized with Coomassie blue R-250

Fractionation of Cardiac SL Vesicle Proteins by HPLC Gel Permeation

Cardiac SL vesicle proteins solubilized in the detergent sodium cholate were fractionated on the basis of size using a TSK 30XL column. The absorbance of the column effluent was continuously monitored at 280 nm during elution of the solubilized SL protein. Column fractions were collected and reconstituted as described in Materials and Methods. Na-Ca exchange activity eluted as shown in Fig. 2. The elution pattern of exchange activity is normalized to the protein content of each reconstituted fraction.

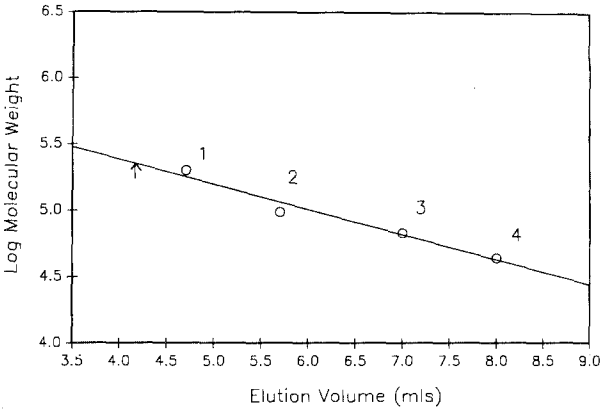

Fig. 4. M_r estimate of sodium cholate solubilized cardiac Na-Ca exchange by gel permeation HPLC. Gel permeation HPLC of Na-Ca exchange was performed as described in Materials and Methods. Protein standards were fractionated under identical column conditions. The elution of protein standards was monitored by absorbance at 280 nm and SDS-PAGE (*not shown*). Profile shown represents the leading edge of eluted standards. (*1*) myosin, 200,000 Da; (*2*) phosphorylase *b*, 97,400 Da (*3*) bovine serum albumin, 68,000 Da; (*4*) ovalbumin, 43,000 Da. The arrow indicates the elution position of the leading edge of peak Na-Ca exchange specific activity

Highest specific activity, 11.4 nmol Ca^{2+}/mg protein/sec, was recovered in fraction 14. HPLC routinely produced a two- to threefold increase in specific activity when compared to unfractionated, reconstituted controls. For example, in the experiment shown in Fig. 2, the activity of the control activity was 5.0 nmol Ca^{2+}/mg protein/sec. Total recovery of protein was 91 ± 7% and recovery of Na-Ca exchange activity was 68 ± 11% (n = 4). HPLC gel permeation resulted in nearly a 10-fold increase in recovery of Na-Ca exchange activity over previously reported methods [6, 34] and was presumably due to the shorter time of fractionation or contact with the column matrix.

Figure 3 illustrates the protein composition of TSK 30XL column fractions following SDS-PAGE. Fractionation of SL protein was according to size by the column with larger proteins eluting in earlier fractions. Both earliest and highest specific activity Na-Ca exchange eluted in fractions containing high M_r proteins (Fig. 2); whereas fractions with little exchange activity eluted later and contained smaller proteins including those in the 33,000 Da range. The presence of significant Na-Ca exchange activity in fraction 14 in conjunction with the paucity of protein would account for the high specific activity in that fraction and suggests that Na-Ca exchange may be a minor component in terms of total SL protein. Selected protein M_r standards were used to calibrate the size elution pattern of the column and are shown in Fig. 4. Peak Na-Ca exchange activity

eluted one fraction before 200,000 Da myosin. The apparent M_r of cardiac Na-Ca exchange by gel permeation HPLC was determined to be approximately 224,000 Da or larger from data obtained in experiments like those shown in Fig. 4. The 224,000 Da estimation by HPLC gel permeation is likely to represent a minimum since the elution of activity appeared near or at the column void volume, yet ahead of the 200,000 Da myosin. These data suggest that solubilized exchange protein(s) forms a large complex (>200,000 Da); however, under conditions reported here, an exact M_r cannot be estimated.

Detection of High Molecular Weight Ca^{2+}-Binding SL Vesicle Protein

Three major high molecular weight Ca^{2+}-binding proteins were detected as radioactive bands by autoradiography. The apparent M_r of these proteins as shown in Fig. 5 were 234,000, 112,000, and 90,000 Da. Radiolabeled Ca^{2+} bound to these electrophoretically transferred SL proteins even though the membrane had been incubated in 160 mM KCl and 5 mM $MgCl_2$ prior to and during incubation with $^{45}Ca^{2+}$. This suggested that the $^{45}Ca^{2+}$ binding observed was not due to radiolabel association with nonspecific negative charges on blotted proteins since the monovalent and divalent cations did not block $^{45}Ca^{2+}$ binding. Additionally, when the blotted membrane proteins were incubated with 160 mM KCl and 10 mM $LaCl_3$ prior to addition of $^{45}Ca^{2+}$, radioactive bands were not observed. We also did not observe any effects of 160 mM NaCl or 5 mM $MgCl_2$ on $^{45}Ca^{2+}$ binding to SL protein, although omission of $MgCl_2$ from either KCl or NaCl buffers increased the background on autoradiograms as previously reported [18]. The same three proteins bound $^{45}Ca^{2+}$ whether subjected to reducing or nonreducing SDS-PAGE.

It was our experience that the 234,000 M_r protein did not transfer efficiently (or at all) from 7.5% or higher concentration polyacrylamide gels if the time of transfer at 100 mA was less than 3 hr. Minimum transfer time at 100 mA for the 234,000 M_r $^{45}Ca^{2+}$-binding protein in 5 or 7.5% polyacrylamide gels was 12 hr. This was determined by staining the polyacrylamide gel with Coomassie blue R-250 following electrophoretic transfer to assess blotting efficiency.

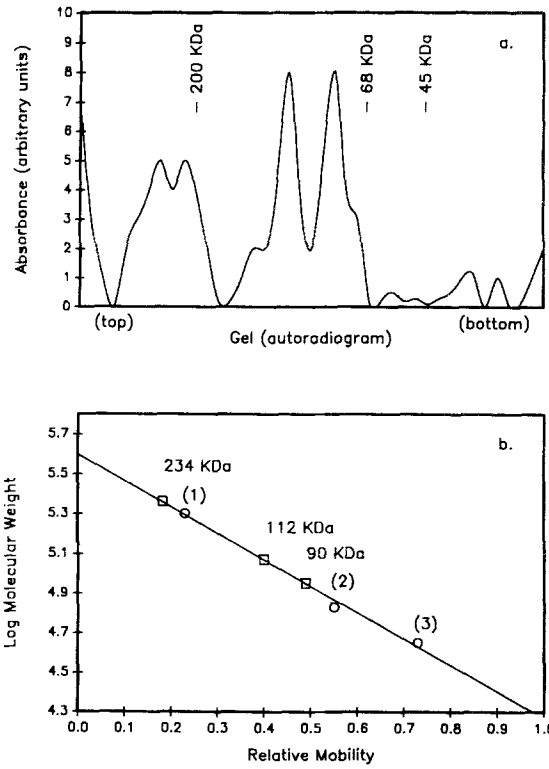

Fig. 5. Detection of cardiac SL $^{45}Ca^{2+}$-binding proteins. Cardiac SL proteins (100 μg/lane) were electrophoretically transferred to a nitrocellulose membrane following SDS-PAGE (% acrylamide). Blotted proteins were subjected to incubation in the presence of $^{45}Ca^{2+}$, label-free washes, drying, and autoradiography. (*a*) densitometric tracing of exposed film. Indicated are positions of standard protein M_r markers; (*b*) relative mobility plot indicating M_r determinations for the three major peaks identified in panel (*a*). Circles indicate M_r standards (*see* Fig. 4). Squares indicate relative mobility of absorbance peaks seen in (*a*). The M_r of each peak is shown

Discussion

There is currently no agreement on the identification or M_r of cardiac Na-Ca exchange. Thus far, antibodies reported to inhibit exchange activity have not been shown to recognize the same proteins, which raises the possibility of inhibition artifacts. Clearly, more definitive experiments with additional antibodies would be useful. For example, no laboratory has yet reported immunoprecipitation of cardiac Na-Ca exchange activity prior to reconstitution of detergent-solubilized SL proteins, although as mentioned earlier, this has been reported in brain [3]. It would appear that additional immunological information beyond simple inhibition of activity will be required to ascertain whether or not an antibody is specifically directed against Na-Ca exchange protein.

We report in the present study three independent determinations that, taken together, provide evidence that the native M_r of cardiac Na-Ca exchange transporting protein in SL vesicles is 225,000 Da or larger. Radiation target analysis and sizing by gel permeation HPLC provide direct evi-

dence that the native carrier may be larger than previous reports indicate. The identification of specific Ca^{2+}-binding proteins, especially in the 200,000 Da range, is indirect and circumstantial.

When considering the validity of the apparent 226,000 Da M_r determined by radiation target analysis, two questions must be addressed. First, what is the range of accuracy of this technique; and second, what effect did the inactivation process have, if any, on the carbohydrate moiety of Na-Ca exchange, a reputed glycoprotein [23]? Kempner and Schlegel have reported that the accuracy of the radiation-inactivation technique is approximately 14% [10]. Given this error, the low estimate for the M_r would be approximately 194,000 Da, which is still considerably higher than previously reported exchange proteins. In a review of over 30 enzymes subjected to irradiation inactivation, Kempner and Schlegel reported good agreement between known M_r and target size analyses of over half the enzymes studied [10]. A substantial number of enzymes, however, yielded apparent M_r by target sizing that were lower than previously described values. This discrepancy was resolved by the observation that *all* low values corresponded to the subunit sizes of these enzymes.

A recent report applied target inactivation analysis to the ryanodine receptor, a membrane protein located on the sarcoplasmic reticulum [19]. These data indicated that the target M_r was smaller than the polypeptide that binds ryanodine. They suggested that target sizing in this case yielded a minimum M_r because the ryanodine binding site represented a portion of the functional structure and only a domain of the active polypeptide. It cannot be determined from the present study whether the target size of 226,000 Da for cardiac Na-Ca exchange is a close approximation or a minimum.

Many plasma membrane proteins are glycoproteins. Cardiac Na-Ca exchange activity in SL vesicles has been reported to be stimulated by endoglycosidases, which suggests that this transporter contains carbohydrate [23]. It also has been proposed that a carbohydrate moiety may contribute to the resistance of this carrier to proteolytic degradation [6, 24, 34]. Lectin affinity column chromatography has been used to separate Na-Ca exchange activity from the 82,000-Da protein, strongly suggesting this protein does not catalyze the exchange process (C.C. Hale & J.P. Reeves, *unpublished data*). In addressing the question of the influence of carbohydrate on target size analysis, Kempner and coworkers found that, without exception, only the protein portion of an enzyme was sensitive to the loss of activity observed during radiation inactivation [9]. This was true for artificially created glycoproteins as well as native receptors and enzymes, even when the contribution of carbohydrate to the overall M_r was as high as 50%. Given this observation, it is likely that the carbohydrate portion of Na-Ca exchange had little or no influence on the M_r determined by radiation inactivation.

Gel permeation HPLC data clearly indicated that sodium cholate solubilized SL vesicle proteins were fractionated on the basis of size and that Na-Ca exchange eluted with larger proteins (Figs. 2–4). Fractionation by gel permeation of sodium cholate solubilized membrane proteins occurs because of the small micelle size of this detergent [7]. Since authentic membrane protein standards were not available, we utilized soluble protein standards fractionated under identical column conditions. Although soluble globular proteins have been found to be generally suitable as HPLC M_r standards, caution must be exercised as the error in either direction has been reported to be as large as 10–20% [14]. The standard proteins were fractionated by the column on the basis of M_r and, as shown in Fig. 3, sodium cholate solubilized SL proteins also were fractionated by size as judged by SDS-PAGE. Thus, although there is inherent error in this method with regards to precise estimations of M_r, it can provide an approximation of size and the data strongly suggest that native Na-Ca activity is larger than previously reported. Although fraction 14 contained predominately high M_r proteins as well as peak specific activity, SDS-PAGE analysis indicated that some smaller SL proteins were present in low concentrations in this fraction (Fig. 3). One possibility for the presence of these proteins is that they represent subunits of a multimeric Na-Ca exchange protein complex, which solubilized and fractionated intact on the column. The existence of a large complex of smaller protein subunits capable of catalyzing Na-Ca exchange, is not consistent with the M_r obtained in target sizing experiments (*see* the Table) [10]. Regardless of these considerations, a 33,000 Da protein eluted in fractions following peak exchange activity (Fig. 3). All electrophoretograms presented in the current study utilized *b*-mercaptoethanol as a reducing agent. If a tetramer complex (or double tetramer of 280,000 Da) of 33,000 Da protein eluted with larger proteins (under the nonreducing conditions of the HPLC experiments), individual monomers would have been present and visualized by SDS-PAGE in peak activity fractions. This was not the case. Another possibility is that Na-Ca exchange may exist as a dimer of two 125,000 Da proteins [15, 20]. This would place the M_r for Na-Ca exchange at nearly 250,000 Da. As described above, a subunit M_r of 125,000 Da is not consistent with the 226,000 Da M_r determined by irradiation inactiva-

tion since the latter is likely to represent a minimum M_r.

Ebashi and coworkers described a convenient method for identification of Ca^{2+}-binding proteins [18]. Fortuitously, many Ca^{2+}-binding proteins retain the ability to specifically bind $^{45}Ca^{2+}$ when transferred to a nitrocellulose membrane following SDS-PAGE. Specificity of Ca^{2+}-binding proteins for $^{45}Ca^{2+}$ was dependent upon factors such as pH and $MgCl_2$ as had been originally reported [18]. We detected three major Ca^{2+}-binding proteins in cardiac SL vesicles with apparent M_r of 234,000, 112,000, and 90,000 Da (Fig. 6). While this detection method did not relate Ca^{2+} binding to specific transport or enzymatic activity (e.g., differential binding in the presence of Na^+), it did allow the detection of specific Ca^{2+}-binding proteins. By inference, the data suggest that the detected proteins are somehow involved with Ca^{2+} either in terms of regulation of their activity or Ca^{2+} transport. The presence of a Ca^{2+}-binding protein of 234,000 Da M_r is consistent and supportive of the data described above indicating a native M_r of cardiac Na-Ca exchange of approximately 225,000 Da. The exact nature of the interaction of Ca^{2+} with these three proteins is unknown.

In summary, target sizing analyses and gel permeation chromatography data tentatively place the M_r of native Na-Ca exchange in cardiac SL vesicles at approximately 225,000 Da or larger. Three Ca^{2+}-binding proteins have been detected in these preparations. Identification of the larger of these proteins as the cardiac Na-Ca exchanger is strictly circumstantial. In this report, we have presented data that suggest the size of native cardiac Na-Ca exchange is larger than heretofore reported. Although, conclusive identification of protein(s) constituting cardiac Na-Ca exchange remains incomplete, the present work may provide future direction in terms of purification and identification techniques amenable to larger proteins.

The authors would like to thank L. Novela, A. Long, and J. Carlton for expert technical assistance. This work was supported by the National Science Foundation, Grant No. DCB 8602234, the American Heart Association—Missouri Affiliate, and the Edward Mallinckrodt Foundation. SBK was the recipient of a Dalton Research Fellowship.

References

1. Ahmed, Z., Reis, J.L. 1958. The activation and inhibition of 5′-nucleotidase. *Biochem. J.* **69:**386–387
2. Baron, M.D., Luzio, J.P. 1987. The synthesis and turnover of 5′-nucleotidase in primary cultured hepatocytes. *Biochim. Biophys. Acta* **927:**81–85
3. Barzilai, R., Rahamimoff, H. 1987. Immunological Identification of the synaptic plasma membrane Na^+-Ca^{2+} exchanger. *J. Biol. Chem.* **262:**10315–10320
4. Barzilai, A., Spanier, R., Rahamimoff, H. 1984. Isolation, purification, and reconstitution of the Na^+ gradient-dependent Ca^{2+} transporter (Na^+-Ca^{2+} exchanger) from brain synaptic membranes. *Proc. Natl. Acad. Sci. USA* **81:**6521–6525
5. Dale, W.E., Tsai, Y.-S., Yung, C.Y., Hale, C.C., Rovetto, M.J., Kim, H.D. 1988. Kinetic characterization and radiation-target sizing of the glucose transporter in cardia sarcolemmal vesicles. *Biochim. Biophys. Acta (in press)*
6. Hale, C.C., Slaughter, R.S., Ahrens, D.C., Reeves, J.P. 1984. Identification and partial purification of the cardiac sodium-calcium exchange protein. *Proc. Natl. Acad. Sci. USA* **81:**6569–6573
7. Helenius, A., McCaslin, D.R., Fries, E., Tanford, C. 1979. Properties of detergents. *Meth. Enzymol.* **56:**734–758
8. Jung, C.Y., Hsu, T.L., Hah, J.S., Cha, C., Haas, M.N. 1980. Glucose transport carrier of human erythrocytes. *J. Biol. Chem.* **255:**361–364
9. Kempner, E.S., Miller, J.H., McCreery, M.J. 1986. Radiation target analysis of glycoproteins. *Anal. Biochem.* **156:**140–146
10. Kempner, E.S., Schlegel, W. 1979. Size determination of enzymes by radiation inactivation. *Anal. Biochem.* **92:**2–10
11. Kepner, G.R., Macey, R.I. 1968. Membrane enzyme systems. Molecular size determinations by radiation inactivation. *Biochim. Biophys. Acta* **163:**188–203
12. Kuwayama, H., Kanazawa, T. 1982. Purification of cardiac sarcolemmal vesicles: High sodium pump content and ATP-dependent calmodulin-activated calcium uptake. *J. Biochem.* **91:**1419–1426
13. Laemmli, V.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature (London)* **227:**680–685
14. LeMaire, M., Aggerbeck, L.P., Monteilhet, C., Andersen, J.P., Moller, J.V. 1986. The use of high-performance liquid chromatography for the determination of size and molecular weight of proteins: A caution and a list of membrane proteins suitable as standards. *Anal. Biochem.* **154:**525–535
15. Letarte, M., Quakenbush, E.J., Baumal, R., Michalak, M., 1986. Correlations between the 44D7 antigenic complex and the plasma membrane Na^+-Ca^{2+} exchanger. *Biochem. Cell Biol.* **64:**1160–1169
16. Longoni, S., Carafoli, E. 1987. Identification of the Na^+/Ca^{2+} exchanger of calf heart sarcolemma with the help of specific antibodies. *Biochem. Biophys. Res. Commun.* **145:**1059–1063
17. Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:**265–275
18. Maruyama, K., Mikawa, T., Ebashi, S. 1984. Detection of calcium binding proteins by ^{45}Ca autoradiography on nitrocellulose membrane after sodium dodecyl sulfate gel electrophoresis. *J. Biochem.* **95:**511–519
19. McGrew, S.G., Boucek, R.J., McIntyre, J.O., Jung, C.Y., Fleischer, S. 1987. Target size of the ryanodine receptor from junctional terminal cisternae of sarcoplasmic reticulum. *Biochemistry* **26:**3183–3187
20. Michalak, M., Quakenbush, E.J., Letarte, M. 1986. Inhibition of Na^+/Ca^{2+} exchanger activity in cardiac and skeletal muscle sarcolemmal vesicles by monoclonal antibody 44D7. *J. Biol. Chem.* **261:**92–95
21. Miyamoto, H., Racker, E.F. 1980. Solubilization and partial

purification of the Ca^{2+}/Na^+ antiporter from the plasma membrane of bovine heart. *J. Biol. Chem.* **255:**2656–2658
22. Newman, M.J., Foster, D.L., Wilson, T.H., Kaback, H.R. 1981. Purification and reconstitution of functional lactose carrier from *Escherichia coli*. *J. Biol. Chem.* **256:**11804–11808
23. Peña, P. de la, Hale, C.C., Reeves, J.P. 1985. The cardiac sarcolemmal Na-Ca exchanger is a glycoprotein. *Biophys. J.* **47:**271a
24. Philipson, K.D., Nishimoto, A.Y. 1982. Stimulation of Na^+-Ca^{2+} exchanger in cardiac sarcolemmal vesicles by proteinase pretreatment. *Am. J. Physiol.* **243:**C191–C195
25. Reeves, J.P. 1985. The sarcolemmal sodium-calcium exchange system. *In:* Regulation of Calcium Transport Across Muscle Membranes. A.E. Shamoo, editor. pp. 77–127. Academic, New York
26. Reeves, J.P., Sutko, J.L. 1979. Sodium-calcium ion exchange in cardiac membrane vesicles. *Proc. Natl. Acad. Sci. USA* **76:**590–594
27. Reeves, J.P., Sutko, J.L. 1983. Competitive interactions of sodium and calcium with the sodium-calcium exchange system of cardiac sarcolemmal vesicles. *J. Biol. Chem.* **258:**3178–3182
28. Reuter, H. 1982. Na-Ca countertransport in cardiac muscle. *In:* Membranes and Transport. A.N. Martanasi, editor. pp. 623–631. Plenum, New York
29. Schaffner, W., Weissmann, C. 1973. A rapid, sensitive, and specific method for the determination of protein in dilute solution. *Anal. Biochem.* **56:**502–514
30. Slaughter, R.S., Sutko, J.L., Reeves, J.P. 1983. Equilibrium calcium-calcium exchange in cardiac sarcolemmal vesicles. *J. Biol. Chem.* **258:**3183–3190
31. Soldati, L, Longoni, S., Carafoli, E. 1985. Solubilization and reconstitution of the Na^+/Ca^{2+} exchanger of cardiac sarcolemma. *J. Biol. Chem.* **260:**13321–13327
32. Thompson, L.F., Ruedi, J.M., Low, M.G. 1987. Purification of 5′-nucleotidase from human placenta after release from plasma membranes by phosphotidylinositol-specific phospholipase C. *Biochem. Biophys. Res. Commun.* **145:**118–125
33. Towbin, H., Staehelin, T., Gordon, J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76:**4350–4354
34. Wakabayashi, S., Goshima, K. 1982. Partial purification of Na^+-Ca^{2+} antiporter from plasma membrane of chick heart. *Biochim. Biophys. Acta* **693:**125–133

Received 5 April 1988; revised 19 August 1988

Note Added in Proof

Additional support for high M_r Na-Ca exchange protein appears in the recent publication of Cook and Kaupp who found the rod outer segment exchanger has an apparent molecular weight of 220,000 Da (Cook, N.J., Kaupp, U.B. 1988. *J. Biol. Chem.* **261:**11382–11388).