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Enrichment of Na^+-Ca^{2+} exchange in cardiac sarcolemmal vesicles by alkaline extraction

Kenneth D. Philipson [a], Alicia A. McDonough [b], Joy S. Frank [a] and Robert Ward [a]

[a] *Departments of Medicine and Physiology and the American Heart Association, Greater Los Angeles Affiliate, Cardiovascular Research Laboratories, University of California, Los Angeles, Los Angeles CA 90024 and* [b] *Department of Physiology and Biophysics, University of Southern California School of Medicine, Los Angeles, CA 90033 (U.S.A.)*

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Exposure of canine cardiac sarcolemmal vesicles to alkaline media ($\geq$ pH 12) results in the extraction of 33% of the protein. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis shows that specific proteins are being solubilized. Most of the phospholipid and sialic acid remains with the pellet after centrifugation. Electron microscopy reveals that alkaline treatment does not cause gross morphological damage to the vesicles, although freeze-fracture demonstrates some aggregation of intramembrane particles. The data indicate that high pH probably removes peripheral proteins and leaves the integral proteins in place. We find complete recovery of Na^+-Ca^{2+} exchange activity in alkaline-extracted membranes after solubilization and reconstitution. These vesicles contain only 50% of the protein of vesicles reconstituted from control sarcolemma. Thus, the specific activity of Na^+-Ca^{2+} exchange is doubled. Alkaline extraction is a useful and reproducible procedure for enrichment of the Na^+-Ca^{2+} exchange protein. ($Na^+ + K^+$)-ATPase is completely inactivated by exposure to pH 12 medium though immunodetection shows that the ($Na^+ + K^+$)-ATPase proteins are not extracted. We detect both α and $\alpha+$ forms of ($Na^+ + K^+$)-ATPase and deduce that the Na^+ pump proteins do not comprise a major fraction of sarcolemmal protein.

Introduction

The Na^+-Ca^{2+} exchange system of cardiac sarcolemma catalyzes an electrogenic counter-transport of Na^+ ions for Ca^{2+} with a probable stoichiometry 3 to 1. In isolated sarcolemmal vesicles, Na^+-Ca^{2+} exchange is a highly active process and has been characterized in much detail (for reviews, see Refs. 1, 2). In vivo, Na^+-Ca^{2+} exchange can move Ca^{2+} in either direction across the sarcolemmal membrane. The Na^+ gradient, Ca^{2+} gradient, and membrane potential determine the direction of net Ca^{2+} flux. The physiological significance of Na^+-Ca^{2+} exchange is controversial (for reviews, see Refs. 3–5).

Attempts have begun to isolate the Na^+-Ca^{2+} exchange protein(s). Success would allow ini-

Abbreviations: Caps, 3-(cyclohexylamino)propanesulfonic acid; Mops, 4-morpholinepropanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Correspondence: K.D. Philipson, Cardiovascular Research Laboratory, A3-381 CHS, UCLA School of Medicine, Los Angeles, CA 90024, U.S.A.

tiation of study at a more molecular level. Towards this end, several groups have reported on the solubilization and reconstitution of Na^+-Ca^{2+} exchange activity [6–9]. Initial suggestions have been that the molecular weight of the exchange protein is either 70 [10], 82 [11], or 33 [12] kDa.

We report here a simple method to enrich the Na^+-Ca^{2+} exchange of sarcolemmal membranes by alkaline extraction. This approach should be useful in the purification of sarcolemmal proteins. In addition, we characterize the effects of alkaline treatment on the $(Na^+ + K^+)$-ATPase and on sarcolemmal morphology.

Materials and Methods

Sarcolemmal isolation. Highly purified sarcolemmal vesicles were isolated from canine ventricles as described previously [13] with minor modifications [28].

Alkaline extraction. Sarcolemmal vesicles (3–5 mg/ml) were diluted 10-fold with cold 10 mM Caps, pH 12 (adjusted with NaOH) (McDonough, A.A., unpublished data). Control vesicles were diluted with cold 140 mM NaCl, 10 mM Mops-Tris, pH 7.4.

Solubilization and reconstitution. Sarcolemmal pellets (either control or after alkaline extraction) from about 0.16 mg of initial protein were solubilized in 0.16 ml of cold asolectin (25 mg/ml; Associated Concentrates, Woodside, NY), 2% sodium cholate, NaCl (0.5 M), Mops (20 mM, adjusted to pH 7.4 with Tris). The mixture was spun for 15 min in an Airfuge to remove non-solubilized material. The clear supernatant was reconstituted by a 6-fold dilution with cold sodium phosphate (124 mM, pH 7.4). Reconstituted vesicles were harvested by centrifugation at $140\,000 \times g$ for 90 min and resuspended in the same sodium phosphate solution. The sequence is similar to the cholate-dilution procedure initially described by Miyamoto and Racker [6] for reconstituting Na^+-Ca^{2+} exchange activity.

Na^+-Ca^{2+} exchange. Na^+-loaded reconstituted vesicles (0.005 ml; about 0.4–0.8 mg/ml) were rapidly diluted into a Ca^{2+} uptake medium (0.25 ml) containing KCl (140 mM), $CaCl_2$ (0.01 mM), $^{45}CaCl_2$ (0.3 μCi), valinomycin (0.36 μM), Mops-Tris (10 mM), pH 7.4 to initiate Na_i^+-dependent Ca^{2+} uptake. Initial rates were determined by quenching the transport reaction after 1.5 s by the automated addition of 0.03 ml of KCl (140 mM), EGTA (10 mM). A further addition of 1.0 ml of cold KCl (140 mM), EGTA (1 mM) was immediately made. The mixture was filtered (Sartorius, 0.22 μm), and the filter washed with 2×3 ml cold KCl (140 mM), EGTA (1 mM). 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 which was not Na^+-gradient dependent and were typically less than 5% of values in KCl. These techniques for measuring electrogenic Na^+-Ca^{2+} exchange as Na_i^+-dependent Ca^{2+} uptake have been used extensively in previous studies [1,2].

Electron microscopy. All tissue was examined on a JEOL 100 CX microscope. For thin-section electron microscopy, sarcolemmal vesicles were centrifuged to form a loose pellet, exposed to 2% glutaraldehyde and post-fixed in buffered (0.1 M sodium cacodylate) osmium tetroxide (1%). The tissue was dehydrated in ethanol and embedded in Epm 812. For freeze-fracture electron microscopy, pelleted sarcolemmal vesicles were fixed in 2% glutaraldehyde, rinsed in buffer and then exposed to glycerol (12% for 10 min, 25% for 30 min). The pelleted sarcolemmal preparation was rapidly frozen in Freon 22 at the liquid-solid interface. Frozen tissue was transferred to the cold stage of a Balzers 301 Freeze-Etch unit equipped with an electron beam gun and a quartz-crystal monitor. Fracture was carried out at $-150\,°C$ in a vacuum of $3 \cdot 10^{-7}$ torr. The replicas were generated as previously described [13].

Antibody production and immunodetection of $(Na^+ + K^+)$-ATPase. Preparation of antibodies to purified guinea pig kidney $(Na^+ + K^+)$-ATPase has been described previously [14]. Antibodies were used to detect $(Na^+ + K^+)$-ATPase by a modification of the immunodetection procedure of Renart et al. [15] as described by McDonough and Schmitt [16].

Miscellaneous. Phospholipid content was determined as described previously [17]. Sialic acid was quantitated by the method of Warren [18]. protein was determined by the method of Lowry et al. [19] or by the method of Wang and Smith

[20] for reconstituted vesicles. Data are presented as means ± S.E.

Results

Alkaline extraction of sarcolemma

When sarcolemmal vesicles are exposed to pH 12 solution (see Materials and Methods for details), the vesicle suspension clarifies and about one third of the protein is extracted from the membrane (Table I). SDS-PAGE electrophoresis (Fig. 1) shows that the proteins of the native sarcolemma (lane A) specifically distribute into the supernatant (lane C) or pellet (lane D). The overall gel pattern of the sarcolemma is well conserved (compare lane A with lanes C plus D). The only notable exception is in the 100 kDa region where there is an apparent loss of protein after pH 12 treatment. Lesser amounts of sialic acid and phospholipid are extracted (15.7% and 11.3%, respectively) (Table I).

Na^+-Ca^{2+} exchange after alkaline treatment

After alkaline treatment the remaining membranes demonstrate no Na^+-Ca^{2+} exchange exchange activity. Membrane permeability barriers are possibly destroyed by the high pH, however, so we also assayed for transport activity after solubilization and reconstitution of the membranes. As shown in Table II, the Na^+-Ca^{2+} exchange activity of vesicles reconstituted from alkaline-extracted sarcolemma is approximately double that of control reconstituted vesicles. Total Na^+-Ca^{2+} exchange activity, however, is unchanged. Specific activity is increased due to a partial purification of the exchanger. About one half of all protein has been removed and no apparent activation or inactivation of exchange activity results from base treatment.

TABLE I

ALKALINE EXTRACTION OF SARCOLEMMA

Sarcolemmal vesicles were treated with pH 12 medium and centrifuged as described in Materials and Methods. $n = 3$.

	Native vesicles	Distribution after pH 12 treatment (% of total)	
		supernatant	pellet
Sialic acid	80.7 ± 18.2 nmol/mg	15.7 ± 1.5	84.3 ± 1.5
Phospholipid	2.0 ± 0.1 μmol/mg	11.3 ± 2.9	88.7 ± 2.9
Protein		33.0 ± 1.5	67.0 ± 1.5

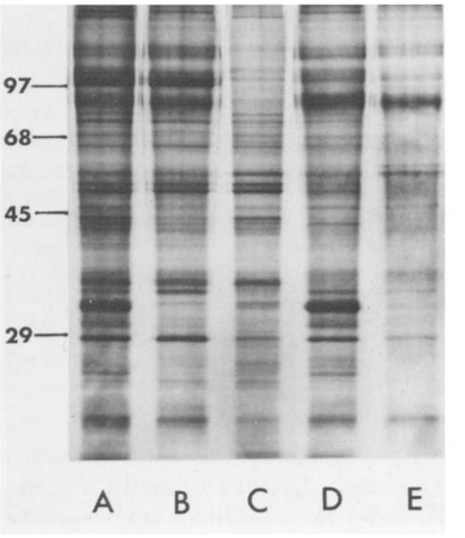

Fig. 1. Distribution of sarcolemmal proteins after alkaline extraction and reconstitution. Photograph of a silver-stained SDS-PAGE pattern is shown. Lane A: native sarcolemmal vesicles (8 μg protein). Lane B: vesicles (6 μg protein) reconstituted from native sarcolemma. Lanes C and D: supernatant and pellet, respectively, after alkaline extraction of sarcolemmal vesicles (8 μg initial protein). Lane E: vesicles (2 μg protein) reconstituted from alkaline-extracted sarcolemmal vesicles. A 10% polyacrylamide Laemmli system was used. Samples were first precipitated with trichloroacetic acid and extracted with diethyl ether to remove excess lipid. Molecular weight markers were carbonic anhydrase, ovalbumin, bovine serum albumin, and phosphorylase B.

Treatment with pH 12 medium only removes 33% of the sarcolemmal protein (Table I) but, in addition, some of the remaining protein is rendered unsuitable for reconstitution. Thus, only 50% of the protein of reconstituted vesicles is found in vesicles reconstituted from alkaline-extracted sarcolemma. Possibly the high pH denatures certain proteins such that they can no longer be solubilized and reconstituted. This finding is reflected in the gel patterns of Fig. 1. Lanes A and B contain sarcolemma and reconstituted sarcolemma, respectively. The patterns are generally similar. Lanes D and E contain alkaline-extracted sarcolemma and vesicles reconstituted from these membranes. Larger differences are apparent than between lanes A and B.

TABLE II

Na^+-Ca^{2+} EXCHANGE OF VESICLES RECONSTITUTED FROM CONTROL AND ALKALINE-EXTRACTED SARCOLEMMA

Sarcolemmal vesicles (0.20 mg) were treated at pH 12 (or pH 7.4 for controls) and centrifuged. The pellets were solubilized and reconstituted and assayed for Na^+-Ca^{2+} exchange activity. See Materials and Methods for details. $n = 5$.

	Protein reconstituted (mg)	Na^+-Ca^{2+} exchange	
		specific activity (nmol/mg per s)	total activity (nmol/s)
Reconstituted control sarcolemma	0.051 ± 0.001	30.6 ± 5.8	1.56 ± 0.26
Reconstituted alkaline-extracted sarcolemma	0.025 ± 0.001	63.5 ± 13.5	1.60 ± 0.25
% of control	49.0	208.8	102.6

The Na^+-Ca^{2+} exchange activity of native sarcolemmal vesicles is about 6.0 nmol Ca^{2+}/mg protein per s under the conditions used here for transport measurements. After solubilization and reconstitution in asolectin, activity is 30.6 nmol Ca^{2+}/mg protein per s (Table II). As pointed out by others [11,12], the reconstitution procedure by itself stimulates exchange activity. Thus, the increased exchange activity of reconstituted vesicles does not indicate that purification has been achieved. Nevertheless, it is still valid to use the activities of vesicles reconstituted from control or alkaline-extracted sarcolemma to indicate a relative purification.

Extraction conditions

In preliminary experiments, many variations of the extraction procedures were tested. Although we did alkaline extractions of cardiac sarcolemma in a hypotonic medium, the presence of salts had little effect on results. For example, pH 12 treatment in a 140 mM NaCl medium did not change the removal of sarcolemmal protein nor subsequent Na^+-Ca^{2+} exchange activity after solubilization and reconstitution. The presence of either 200 μM Ca^{2+} or 200 μM EDTA caused small decreases in the amount of protein solubilized. pH 12 gave better extraction than pH 11.5, but higher solutions did not improve results. At pH values less than 11.5 little protein was extracted. About 50% of total Na^+-Ca^{2+} exchange activity was able to survive incubation in 0.1 M NaOH. Alkaline extraction was similar at both longer incubation periods (up to 60 min) and higher temperatures (up to 37°C).

Other extraction methods

High pH has been used to selectively solubilize proteins from red blood cell membranes [21]. With the red cell membranes several other perturbants also extract the same protein components [21]. For the sarcolemmal membrane, however, none of these reagents could duplicate the action of high pH. Membranes were first incubated with different reagents and then centrifuged. Pellets were solubilized and reconstituted as described for the alkaline extraction experiments. Lithium diiodosalicylate (30 mM) caused complete inactivation of the Na^+-Ca^{2+} exchanger and little protein extraction. Guanidine (4 M) solubilized about 50% of the sarcolemmal protein but also completely inactivated the exchanger. Both of these reagents are reported to act on erythrocyte membranes in a manner analogous to high pH. We also investigated the effects of two chaotropic agents on sarcolemmal membranes. Lithium bromide (1 M) caused about a 20% loss of both protein and exchange activity, and sodium perchlorate (1.2 M) neither solubilized any protein nor affected the Na^+-Ca^{2+} exchanger. Urea (6 M) non-selectively removed 28% of the sarcolemmal protein but also inactivated about 50% of exchanger activity.

Electron microscopy

The suspension of sarcolemmal vesicles clarifies after addition of the pH 12 extraction medium. We examined the state of the vesicles after being subjected to alkaline treatment by both thin section and freeze-fracture electron microscopy. Despite a marked change in visual appearance of the sarcolemmal suspension, vesicular structure is

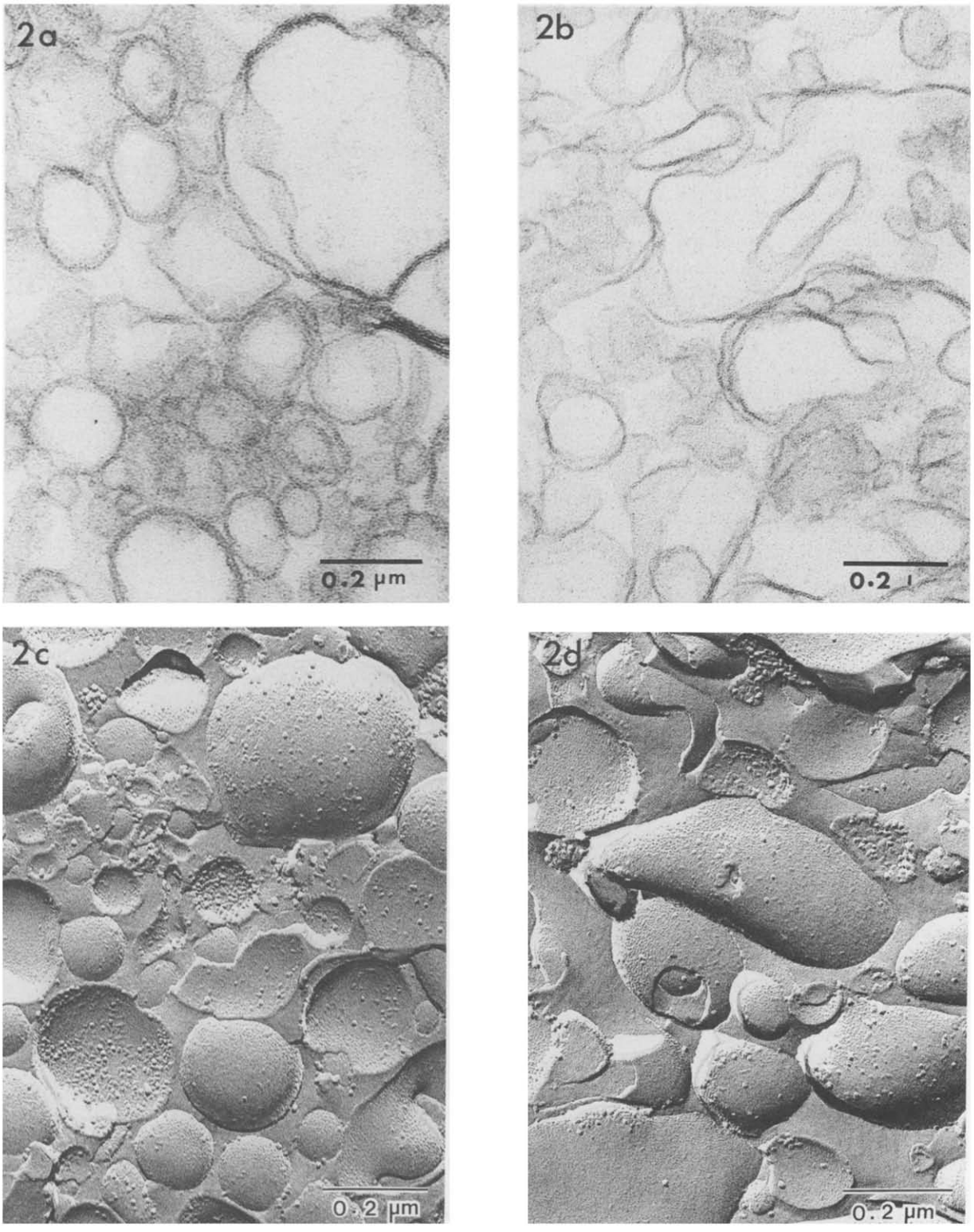

Fig. 2. Electron microscopy of alkaline-treated sarcolemma. Panels a, b: thin-section electron micrographs showing the configuration of vesicles from control (a) and pH 12-treated sarcolemma (b). The heterogeneity in vesicle size is typical of this preparation. After pH 12 treatment, however, the large vesicles appear to be collapsed. Panels c, d: freeze-fracture electron micrographs illustrating the intramembrane particle distribution in these vesicles (c, control). The sheet-like form of the collapsed vesicles is more easily seen in the fractured membranes (d).

maintained (Figs. 2, a and b). The larger diameter vesicles in the sarcolemmal preparation appeared to collapse, however, to a more flattened configuration after pH 12 treatment (Figs. 2, a and b). In the freeze-fractured preparations this gave the appearance of more sheet-like membrane structures (compare Figs. 2, c and d). In many cases, the pH 12-treated sarcolemma also appeared to have more aggregated intramembrane particles than the control preparations (not shown).

($Na^+ + K^+$)-ATPase after alkaline extraction

No ($Na^+ + K^+$)-ATPase activity could be detected in either the extracted protein or the pellet after alkaline treatment. The catalytic, or α subunit, of the ($Na^+ + K^+$)-ATPase has a molecular weight of approx. 100 kDa. As mentioned earlier, the prominent bands of the sarcolemma of this molecular weight are poorly conserved after alkaline treatment (compare lane A with lanes C plus D in Fig. 1). The loss of protein bands in this region of the gel may correlate with the loss of ($Na^+ + K^+$)-ATPase activity. To test this hypothesis, we performed immunoblots using an antibody generated against highly purified guinea pig kidney ($Na^+ + K^+$)-ATPase. This antibody recognizes both α and $\alpha+$ forms of the enzyme [16]. As shown in Fig. 3, substantial amounts of α subunits remain in the pellet (lane C) after alkaline treatment and centrifugation. In some experiments, alkaline treatment produced in the pellet small amounts of higher molecular weight aggregates of the α subunits. Neither α subunit was ever detected in the extracted proteins (lane B). The results indicate that the ($Na^+ + K^+$)-ATPase was being inactivated by the alkaline treatment and that much of the α subunit still migrated with a molecular weight of approx. 100 kDa. The loss of bands in this region of the gel was probably due primarily to loss of proteins other than the α subunit of the ($Na^+ + K^+$)-ATPase.

In addition, we detected both α and $\alpha+$ forms of the enzyme in the canine cardiac sarcolemma. For comparison, immunoblots of both guinea pig brain and kidney ($Na^+ + K^+$)-ATPase are shown. These tissues have previously been determined to have only the α form (kidney) or both the α and $\alpha+$ forms of ($Na^+ + K^+$)-ATPase (brain) [16]. The immunoreactive band at 77 kDa probably

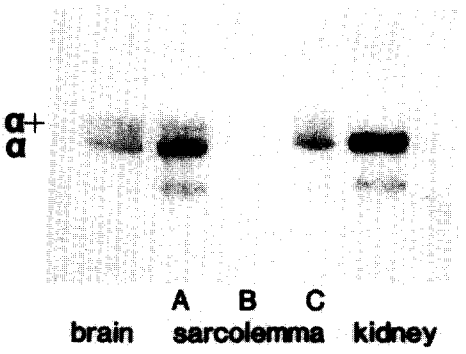

Fig. 3. Immunodetection of the α subunit of ($Na^+ + K^+$)-ATPase. Lanes contain 12 μg guinea pig brain microsomes; 12.5 μg canine cardiac sarcolemma (A); the supernatant (B) and pellet (C) after alkaline extraction and centrifugation of 12.5 μg sarcolemma; 2 μg purified guinea pig kidney ($Na^+ + K^+$)-ATPase. Samples were separated by SDS-PAGE (7.5% acrylamide), as described by Schmitt and McDonough [27] blotted onto diazophenylthioether paper, probed with antibodies to guinea pig ($Na^+ + K^+$)-ATPase, incubated with ^{125}I-labeled protein A, and detected by autoradiography. See Refs. 16 and 27 for further details.

represents a characterized proteolytic degradation product of the α subunit [22]. Significant amounts of the β-subunit of the ($Na^+ + K^+$)-ATPase were also found in the pellet after extraction with none being immunodetectable in the extracted protein (not shown).

Discussion

We find that treatment of canine cardiac sarcolemma at high pH extracts specific proteins from the membrane. The protein(s) responsible for Na^+-Ca^{2+} exchange activity are not extracted and remain fully active. Use of alkaline treatment to extract membrane proteins was first described in detail for red cell membranes [21]. Several other protein perturbants (e.g., guanidine or lithium diiodosalicylate) selectively extract the same proteins from red cell membranes as does alkaline treatment [21]. With the cardiac sarcolemma, however, only high pH has this effect. In the red cell system, it was suggested that alkaline treatment removes peripheral loosely bound proteins. No glycoproteins, sialic acid, or phospholipid were extracted [21]. These data are generally similar to the findings with cardiac sarcolemma, although we

extracted some of the sialic acid and phospholipid (16 and 11%, respectively) at pH 12. Electron microscopy confirms that alkaline treatment probably removes peripheral proteins in that substantial amounts of protein are extracted without gross morphological damage. Apparent clustering of intramembrane particles after alkaline treatment may be due to removal of cytoskeletal elements which normally restrict the mobility of integral proteins.

The resistance of Na^+-Ca^{2+} exchange activity to alkaline treatment is striking. After solubilization and reconstitution, we recover 100% of the exchange activity. Since half of the protein has been removed, exchanger specific activity is doubled compared to control reconstituted vesicles. Substantial exchange activity can be recovered even after exposure to 0.1 M NaOH. The extraction procedure is highly reproducible and should represent a useful first step in schemes for isolation of the Na^+-Ca^{2+} exchanger and other sarcolemmal integral proteins. The glucose transporter of red cell membranes is also pH 12 resistant, and alkaline extraction has recently been useful in precedures for isolation of this transporter [23]. The Na^+-Ca^{2+} exchanger is also highly resistant to inactivation by proteinases [7,11,24]. Possibly, only very limited portions of the protein are exposed at the membrane interface, and the surrounding lipid environment protects the enzyme from both proteinases and alkaline insult.

Although $(Na^+ + K^+)$-ATPase was completely inactivated by exposure to pH 12 medium, this enzyme was not extracted as shown by immunodetection. This finding is consistent with alkaline extraction only removing non-integral proteins. Surprisingly, we detected the presence of both α and $\alpha+$ forms of the $(Na^+ + K^+)$-ATPase. Guinea pig cardiac sarcolemma has previously been shown to have only the α form of the Na^+ pump [16] whereas brain tissue of all species tested has both α and $\alpha+$ forms [25]. More detailed characterization of the α forms of heart $(Na^+ + K^+)$-ATPase will be presented elsewhere [29]. After extraction of peripheral proteins, the gel density of proteins having a molecular weight of approx. 100 kDa is relatively low (Fig. 1, lane D) although the $(Na^+ + K^+)$-ATPase is still present. Thus, the $(Na^+ + K^+)$-ATPase does not constitute a major fraction of sarcolemmal protein. On the basis of freeze-fracture P-face particle density and quantitation of ouabain-binding sites, Kordylewski et al. [26] have suggested that the $(Na^+ + K^+)$-ATPase may account for 16% of all intramembrane particles. Our data suggest that this may be an overestimate.

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