Characterization of the 70 kDa polypeptide of the Na/Ca exchanger

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The Na/Ca exchanger is associated with 160, 120 and 70 kDa polypeptides whose nature is poorly understood. We have purified and characterized the Na/Ca exchanger from bovine cardiac sarcolemmal vesicles (SLVs) by using ion-exchange and affinity chromatographies. The Na/Ca exchanger-enriched fraction was reconstituted into asolectin liposomes [lipid to protein ratio 10:1 (w/w)] that showed Na/Ca exchange activity. Under non-reducing conditions, SDS/PAGE showed a single 70 kDa polypeptide, which was further characterized by immunoblots with different antibodies: SWant, raised against the purified exchanger protein; NH₂-terminus, residues 1–21; NCX1, residues 393–406; and Exon F, residues 622–644. Immunoblots under reducing conditions with SWant, NH₂-terminus and NCX1

showed three bands migrating at 160, 120 and 70 kDa for SLV preparations, whereas Exon F reacted only with the 160 and 120 kDa bands. Under non-reducing conditions, immunoblots with purified reconstituted Na/Ca exchanger showed a single band at 70 kDa reacting with SWant, NH₂-terminus and NCX1 but not with Exon F. We conclude that the 70 kDa protein is associated with Na/Ca exchange activity, has the same N-terminal sequence as the cloned bovine cardiac exchanger, and has its length decreased by at least 35% from its C-terminal portion as compared with that of the wild-type exchanger.

Key words: Na/Ca exchange, heart sarcolemmal vesicles, protein purification.

INTRODUCTION

Na/Ca exchange is of major importance for Ca²⁺ homeostasis in myocardial cells [1–3]. Many molecular and kinetic aspects of the exchanger have been characterized [4,5], and successful solubilization and reconstitution of the exchanger have been performed from different tissues [6–14]. Barzilai et al. [7,14] reported the isolation of two immunologically related polypeptides, one 70 kDa and one 33 kDa, as the Na/Ca exchanger in synaptic plasma membrane.

The canine cardiac Na/Ca exchange protein was purified by using a combination of ion-exchange and affinity chromatographies [11] as 160, 120 and 70 kDa polypeptides whose intensities could be altered by changing the gel conditions. Affinity-purified antibodies specific for the 120 and 70 kDa polypeptides indicate that these two proteins are immunologically related [11]. Durkin et al. [13] purified the bovine heart Na/Ca exchanger by using anion-exchange chromatography. They obtained two major bands migrating at 160 and 120 kDa whose relative intensities could also be changed depending on the electrophoresis conditions used.

The Na/Ca exchanger has been cloned and sequenced [15,16] and a theoretical membrane topology was proposed with 11 α -helical membrane-spanning segments, five upstream and six downstream of a large hydrophilic loop comprising more than 50% of the whole protein. The N-terminus of the canine and bovine 160 and 120 kDa proteins was sequenced and found to be the same N-terminus as the cloned exchanger, starting just after the cleaved signal peptide [11,13].

Gabellini et al. [17] identified a short isoform of the Na/Ca exchanger with an unmodified Na/Ca exchange activity compared with the complete exchanger, which might correspond to the 70 kDa polypeptide observed in sarcolemmal vesicle (SLV) preparations. In another paper [18], the same authors expressed an active heart Na/Ca exchanger isoform lacking the six trans-

membrane segments of the C-terminal portion of the exchanger and a small part of the C-terminus of the cytoplasmic loop. The construct had an apparent molecular mass of 67 kDa, comparable to the 70 kDa protein observed in heart SLVs.

Iwata et al. [19] expressed the cardiac Na/Ca exchanger as a fusion protein with a polyhistidine tag at its C-terminus. Western blotting results, with an antiserum raised against residues 651–688, showed three bands at approx. 160, 120 and 70 kDa, meaning that they had identical C-termini. N-terminal sequencing of the observed 70 kDa fragment showed a cleavage at either position 258 or 270 of the wild-type exchanger amino acid sequence [19].

The nature of the 70 kDa protein therefore remains unclear. Here we report the isolation of an active Na/Ca exchange protein, from bovine heart SLV preparations, as a 70 kDa polypeptide. Immunoblots were performed with antibodies directed against different epitopes, among which was an antibody recognizing the N-terminus (ETEMEG...) sequence of the bovine heart exchanger and another antibody, termed 'Exon F', directed against residues 622–644 in the cytoplasmic hydrophilic loop. Our immunodetection results show that the 70 kDa polypeptide had an N-terminus similar to that of the 120 and 160 kDa proteins described above. Moreover no reaction occurred with the Exon F antibody, suggesting that the 70 kDa fragment lacked part of the C-terminus of the cytoplasmic loop and the six transmembrane segments of the C-terminal of the exchanger.

EXPERIMENTAL

Preparation of bovine heart SLVs

Highly purified SLVs were isolated from bovine heart left ventricle as described previously [10] except that minced ventricles were washed and homogenized immediately after killing, in a solution containing a cocktail of protease inhibitors (0.2 mM

Abbreviations used: DTT, dithiothreitol; NEM, *N*-ethylmaleimide; SLV, sarcolemmal vesicle; WGA, wheatgerm agglutinin.

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PMSF, 2 mg/ml aprotinin, 2 mg/ml pepstatin and 2 mg/ml leupeptin).

Purification of the Na/Ca exchanger protein

The Na/Ca exchanger was purified by using a combination of alkaline extraction, solubilization with decylmaltoside, DEAE-Sepharose ion-exchange chromatography, wheatgerm agglutinin (WGA) affinity chromatography as described by Philipson et al. [11], with the following modifications: first, the alkaline extraction pH was increased from 12 to 12.5, as suggested by Durkin et al. [13]; secondly, the concentration of decylmaltoside was doubled to 20 mM; thirdly, the final concentration of asolectin (Sigma) was decreased to 1/10, to 1 mg/ml, and the Triton X-100 concentration was doubled to 3% (v/v) in the Na/Ca exchange activity elution solution in the WGA chromatography purification step.

Concentration of purified exchanger solution and detergent removal

To concentrate the solubilized purified exchanger fractions without reconstitution, purified Na/Ca exchanger solution was applied to Sephadex G-50 columns (ref. 17-0043-01; Pharmacia Biotech, Roosendaal, The Netherlands) previously equilibrated with a 0.5 mM Mops/Tris solution (pH 7.4 at 37 °C). This desalting step permitted the removal of excess salt and detergent. The Sephadex columns were then centrifuged at 1000 *g* for 1 min and the collected fractions were reconcentrated to the required volume. Those desalting and reconcentration steps were repeated several times until an adequate concentration was reached.

Reconstitution of the purified exchanger

The reconstitution of the purified Na/Ca exchanger protein into asolectin liposomes was as described by Cheon and Reeves [20]: Bio-beads SM-2 were previously soaked and washed with 140 mM NaCl/10 mM Mops (pH 7.4 at 37 °C); the collected proteoliposomes were diluted, centrifuged at 140 000 *g* for 90 min. at 4 °C and then resuspended in 50 μ l of the same solution.

Gel electrophoresis

A 12% (w/v) polyacrylamide Laemmli system was used [21]. The 2 $\times$ sample buffer contained either 0.5% (v/v) mercaptoethanol or 10 mM *N*-ethylmaleimide (NEM). Protein bands were revealed by Gelcode[®] Blue stain reagent (Pierce), an enhanced Coomassie[®] G-250 coloration solution. In brief, after electrophoresis, gel was transferred to a clean tray and rinsed three times for 5 min with 200 ml of deionized water to remove SDS. Gelcode[®] Blue stain (20 ml) was added and the gel was incubated for 1 h, with agitation. Finally, the staining reagent was discarded and gel was agitated in deionized water for 2 h with several changes of water. Kaleidoscope Prestained (Bio-Rad) standards were used for the molecular-mass determination of protein bands.

Proteolysis

Intact reconstituted proteoliposomes were digested with Proteinase K (100 μ g/ml); the digestion reaction was stopped after 1 h by the addition of PMSF to a final concentration of 10 mM. The sample was then diluted in 1 M NaCl/0.5 mM Mops (pH 7.4 at 22 °C) and vortex-mixed for 2–3 min to eliminate the protease, digested peptides and non-membrane-associated digestion products [22,23]. The increase in the ionic strength of the solution (to

1 M NaCl) also decreased possible peptide–lipid interactions. Digested samples were centrifuged at 140 000 *g* for 45 min at 4 °C. The supernatant was discarded and the pellet was resuspended in 0.5 mM Mops (pH 7.4 at 22 °C) and centrifuged under the same conditions to eliminate the remaining proteases, peptides and NaCl. The pellet obtained was then resuspended in the same solution.

Na/Ca exchange activity measurements

The Na/Ca exchange activity in reconstituted proteoliposomes was measured as Na⁺-dependent Ca_o²⁺ uptake by using two previously described protocols [11,20]. In brief, in the first protocol, reconstituted vesicles (50 μ l) were loaded with Na⁺ and diluted in Ca²⁺ uptake medium (140 mM KCl/0.01 mM CaCl₂/0.3 μ Ci of ⁴⁵CaCl₂/0.36 μ M valinomycin) to initiate Na⁺-dependent Ca_o²⁺ uptake. The uptake reaction was quenched after 3 s by the addition of 30 μ l of 140 mM KCl/10 mM EGTA followed by the addition of 1 ml of ice-cold 140 mM KCl/1 mM EGTA. Samples were filtered with 0.22 μ m pore-size nitrocellulose filters (Sartorius), which were then washed twice with 3 ml of cold 140 mM KCl/1 mM EGTA [11]. In the second protocol, a similar procedure was used except that the Ca²⁺ uptake reaction was stopped after 3 s by the addition of 5 ml of ice-cold 140 mM KCl/0.1 mM EGTA and vesicles were harvested by filtration on Whatmann GF/A filters that had previously been soaked in 0.3% polyethyleneimine in water. The filters were then washed twice with 5 ml of ice-cold 140 mM KCl/0.1 mM EGTA, avoiding drying of the filter between rinses. In both protocols, blanks were obtained by replacing KCl with NaCl in the Ca²⁺-uptake medium. In control experiments, activity measurements were performed with asolectin liposomes, reconstituted in the same conditions as for the purified exchanger except that no proteins were added, to evaluate ⁴⁵Ca binding to lipids. All solutions were buffered with 10 mM Mops/Tris, pH 7.4.

Antibody production

Four different antibodies were used to characterize the Na/Ca exchanger in bovine heart SLVs and reconstituted proteoliposomes (purified protein): (1) dog cardiac purified Na/Ca exchanger polyclonal antibody (Swant, Bellinzona, Switzerland); (2) 'NH₂-terminus' antibody, raised against the bovine heart exchanger N-terminal sequence between Glu¹ and Lys²¹; (3) 'NCX1' antibody, raised against a sequence (Thr³⁹³–Phe⁴⁰⁶ upstream of the alternative splicing zone) in the Na/Ca exchanger cytoplasmic loop; and (4) 'Exon F' antibody, raised against the peptide sequence Gly⁶²²–Ala⁶⁴⁴ found in the NCX1.1 bovine heart isoform and corresponding to exon F of the alternative splicing region of the cloned exchanger. The last three antibodies were polyclonal and were obtained from immunized rabbit sera by conventional methods (Eurogentec, Seraing, Belgium). Peptides used in the immunization of rabbits were synthesized by using the fluoren-9-ylmethoxycarbonyl strategy, as described previously [24].

Purification of antibodies

The antibodies NH₂-terminus, NCX1 and Exon F were purified from crude sera by using MPG[®] Glyceryl magnetic beads. In brief, the antigen (NH₂-terminus, NCX1 or Exon F) was first coupled to MPG Glyceryl beads by a covalent bond after treatment of the former with a reducing agent (5 mg/ml 2-mercaptoethanol) to prevent dimerization. Coupled MPG Glyceryl-peptide was then incubated with adequate serum overnight

at 4 °C. The supernatant was removed, excess serum was washed away and bound antibody was eluted with 100 mM glycine, pH 2.5, and neutralized with 0.5 M Mops, pH 7.4.

Immunoblots

Proteins from SDS/PAGE were transferred to nitrocellulose at 220 mA (12 V) for 90 min in a semi-dry blotting apparatus (W.E.P. Company). Immunoreactions were detected with sheep anti-(rabbit IgG) conjugated to an alkaline phosphatase with 5-bromo-4-chloroindol-3-yl phosphate (Sigma) as a substrate.

Miscellaneous

Protein concentration was measured by the method of Schaffner and Weissmann [25] as modified by Newman et al. [26]. The lipid content of samples was determined by a measurement of choline content with a phospholipid enzymic colorimetric test (Boehringer Mannheim).

RESULTS

Purification and reconstitution of the bovine heart Na/Ca exchanger

Na/Ca exchange protein was purified from a preparation of bovine heart SLVs by using a previously published protocol [11]. The detergent (decylmaltoside) concentration was increased from 10 to 20 mM in the solubilization step after alkaline extraction. This resulted in a better solubilization of sarcolemmal proteins, as assayed by SDS/PAGE (results not shown) and an increase in the final yield of purified protein with no drawbacks on the specific activity of Na/Ca exchange throughout the purification process (typical activity values throughout the purification procedure are shown in Table 1). The decrease in asolectin concentration for reconstitution was useful in obtaining a lipid-to-protein ratio at which a single population of vesicles existed

Figure 1 SDS/PAGE [12% (w/v) gel] of purification steps of the Na/Ca exchanger

The starting material was 4 mg of SLV preparation (see the Experimental section for details). Lane 2, control reconstituted native SLV (10 μ g); lane 3, sample after alkaline extraction (10 μ g); lane 4, sample after alkaline extraction and DEAE chromatography (5 μ g); lane 5, sample after alkaline extraction, DEAE and WGA chromatography (5 μ g); lane 6, control asolectin vesicles with no protein added. Samples in lanes 2–5 were all first reconstituted for Na/Ca exchange and protein measurements, as illustrated in Table 1. Protein samples were dissolved in sample buffer containing 10 mM NEM before application to gel lanes. Lane 1 contained standard molecular mass proteins (10 μ l).

[27,28], i.e. reconstituted proteins had the same orientation in all vesicles (see proteolysis results below). Moreover the decrease in asolectin concentration allowed us to perform SDS/PAGE experiments without prior delipidation of reconstituted protein samples, thus avoiding material loss. Indeed, as in comparison

Table 1 Summary of the purification steps of the Na/Ca exchanger

Typical results of Na/Ca exchanger purification throughout all the steps performed (see the Experimental section for purification details). SLV (4 mg) was used as starting material. At each purification step, fractions were reconstituted into asolectin, and activity and protein quantity were measured for each fraction after reconstitution. Control reconstituted vesicles were obtained by solubilizing 4 mg of SLV proteins with 20 mM decylmaltoside followed by reconstitution into asolectin vesicles.

Preparation	Protein (μ g)	Recovered exchange activity (%)	Exchange specific activity (nmol of Ca/s per mg of protein)	Purification (fold)
Control reconstituted vesicles	1849	100	18	1
Alkaline extraction	824	92	37	2
DEAE chromatography	82	68	278	15
WGA chromatography	15	52	1155	64

Table 2 Typical values of Na/Ca activity measurements

Na/Ca exchange activities are shown for purified and reconstituted exchanger (15 μ g) and control protein-free asolectin liposomes. Na_i-dependent Ca_o²⁺ uptake was initiated by incubating 5 μ l of Na⁺-loaded vesicles in Ca²⁺-uptake medium containing 0 mM Na⁺. Blanks were obtained by incubating the vesicles in Ca²⁺-uptake medium containing 140 mM Na⁺. Background values were measured for 0 mM and 140 mM Na⁺ solutions in the absence of vesicles and were subtracted to yield the results shown. Values are the means $\pm$ S.E.M. Abbreviation: n.s., not significant.

Preparation	Line no.	[Na ⁺] (mM)	⁴⁵ Ca uptake (c.p.m.)	P	Stimulation factor	Difference (c.p.m.)	Exchange specific activity (nmol of Ca/s per mg of protein)
Exchanger free liposomes	1	140	62 $\pm$ 24 (8)				
	2	0	66 $\pm$ 13 (8)	Line 2 compared with line 1: n.s.	1	4 $\pm$ 28	—
Purified and reconstituted exchanger	3	140	63 $\pm$ 56 (7)	Line 3 compared with line 4: < 0.001			
	4	0	485 $\pm$ 59 (7)	Line 4 compared with line 2: < 0.001	8	422 $\pm$ 81	1155 $\pm$ 81

Figure 2 Immunoreactions at different stages of the Na/Ca exchanger purification procedure

Proteins were blotted to nitrocellulose, which was then cut and incubated with NCX1 antibody solution (1:1000 dilution; see the Experimental section for details). Lane 2, control reconstituted native SLV (10 µg); lane 3, sample after alkaline extraction (10 µg); lane 4, sample after alkaline extraction and DEAE chromatography (5 µg); lane 5, sample after alkaline extraction, DEAE and WGA chromatography (5 µg); lane 6, control asolectin vesicles with no protein added. Samples in lanes 2–5 were all first reconstituted for Na/Ca exchange and protein measurements, as illustrated in Table 1. Protein samples were dissolved in sample buffer containing 10 mM NEM before application to gel lanes. Lane 1 contained standard molecular mass proteins (10 µl).

with the purification results of Philipson et al. [11], the yield of recovered Na/Ca exchange activity was 52% instead of 15% of the control reconstituted SLVs, and a purification factor of 64-fold instead of 27-fold was obtained, leading to a higher specific activity of Na/Ca exchange (Table 1). Thus in our preparation, specific Na_i-dependent Ca_o²⁺ uptake of the purified and reconstituted exchanger was 1155 ± 81 nmol of Ca²⁺/s per mg of protein (mean $\pm$ S.E.M., $n = 8$). Control experiments performed with reconstituted asolectin vesicles in the absence of proteins showed no uptake, demonstrating that ⁴⁵Ca²⁺ did not bind protein-free liposomes. Table 2 summarizes typical values of Na/Ca exchange activity measurements for purified and reconstituted Na/Ca exchanger and control protein-free asolectin liposomes.

Under non-reducing conditions, SDS/PAGE of the purified bovine heart Na/Ca exchanger showed a single band migrating at 70 kDa for the purified and reconstituted protein fractions. Figures 1 and 2 respectively show SDS/PAGE results and immunoblots, under non-reducing gel conditions, with the use of purified NCX1 antibody at different purification steps: control reconstituted SLV proteins (lane 2), alkaline extraction (lane 3), final elution of DEAE-Sepharose chromatography (lane 4) and Na/Ca exchange activity elution step of WGA-agarose chromatography (lane 5). Because the 70 kDa protein appeared at the last purification step, we performed an immunoblot at different stages of the WGA-agarose purification step: in Figure 3 it can be seen that the 70 kDa polypeptide was selectively eluted at the final purification step (lane 5), after the addition of 3% (v/v) Triton X-100/1 mg/ml asolectin/500 mM NaCl/20 mM Mops/Tris (pH 7.4). The addition of PMSF (0.2 mM) to purification solutions did not alter the SDS/PAGE pattern (results not shown).

Immunoblots

The immunoblot results show that the 70 kDa band was detected in the last purification step only, the 160 and 120 kDa proteins

Figure 3 Immunoreactions at different stages of the WGA chromatography purification step

Sarcolemmal proteins were fractionated on a WGA-agarose column. The starting material was 2 mg of SLV, which was incubated for 10 min in 10 vol. of alkaline extraction solution, centrifuged at 140 000 *g* and solubilized with 1 vol. of cold 140 mM NaCl/20 mM decylmaltoside. Unsolubilized material was removed by centrifugation at 140 000 *g* for 15 min; the supernatant was diluted with 1 vol. of 140 mM NaCl at room temperature. After DEAE chromatography, the sample was applied twice to a WGA-agarose column (0.3 ml bed volume, 0.8 cm diameter) and the flow-through was collected (lane 3; 5 µg of protein). The column was then washed with 0.6 ml of 140 mM NaCl/0.5 mM decylmaltoside (lane 4; 5 µg). Na/Ca exchange protein was eluted with 3% (v/v) Triton X-100/1 mg/ml asolectin/500 mM NaCl/20 mM Mops/Tris (pH 7.4) (lane 5; 5 µg). All fractions were first reconstituted for SDS/PAGE and measurements of Na/Ca exchange activity. The percentages of recovered exchange activity for lanes 3, 4 and 5 were 16%, 0% and 71% respectively (percentage values were normalized to the percentage of recovered Na/Ca exchange activity after DEAE chromatography). Lane 2 contained control reconstituted SLV proteins (10 µg). Lane 1 contained molecular mass standards (10 µl).

being obtained in all previous purification stages. To characterize the purified 70 kDa polypeptide further, a series of immunoblots was performed with four different purified antibodies: SWant, raised against the purified canine Na/Ca exchanger; NH₂-terminus, raised against residues Glu¹-Lys²¹ of the mature bovine heart Na/Ca exchanger; NCX1, raised against residues Thr³⁹³-Phe⁴⁰⁶; and Exon F, raised against residues Gly⁶²²-Ala⁶⁴² (for details on antibody preparation and purification see the Experimental section). Figure 4 shows the localization of the different epitopes of the antibodies used in the Na/Ca exchanger structure. Immunoblots with the four antibodies were run in parallel with either bovine heart SLV preparations or purified Na/Ca exchanger reconstituted into asolectin liposomes. SDS/PAGE of bovine heart SLVs was performed under reducing conditions [10 mM dithiothreitol (DTT) was added to the sample buffer used to dissolve protein samples before loading on the gel], whereas SDS/PAGE of purified and reconstituted exchanger was performed under non-reducing conditions (10 mM NEM). Immunoblots of SLV proteins showed three major bands migrating at 160, 120 and 70 kDa when Swant, NH₂-terminus or NCX1 antibody were used (Figure 5, lanes 4, 3 and 2 respectively). However, immunoblots performed with Exon F antibody showed only two prominent bands at 160 and 120 kDa under the same reducing SDS/PAGE conditions (Figure 5, lane 1).

Immunoblots of purified reconstituted Na/Ca exchanger with Swant, NH₂-terminus or NCX1 antibody (Figure 6, lanes 5, 4 and 2 respectively) showed only one band at 70 kDa, confirming results with SDS/PAGE Gelcode® Coomassie® Blue staining (Figure 1, lane 5). Again, immunolabelling with Exon F antibody failed to detect the 70 kDa polypeptide (Figure 6, lane 3).

Figure 4 Diagram of the membrane topology of the Na/Ca exchanger based on immunoreactions with different antibodies and hydropathy plot analysis [16]

The 70 kDa subunit represents the N-terminus of the exchanger, including the first five transmembrane segments and part of the hydrophilic loop (continuous line, up to Tyr-621). The remaining portion is the C-terminal part, including a portion of the hydrophilic loop and the last six transmembrane segments (dotted line). Epitopes of the antibodies NH₂-terminus, NCX1 and Exon F are shown at their approximate positions with respect to the protein membrane topology. Different regulatory sites are also shown [exchange inhibitory peptide (XIP)- and Ca-binding regions]. Abbreviation: aa, residues.

Figure 5 Immunoreactions with SLV preparations with different antibodies

Proteins were blotted to nitrocellulose, which was then cut and incubated with the corresponding antiserum (1:1000 dilution; see the Experimental section for details). Lane 4, reaction with SWant antibody; lane 3, reaction with NH₂-terminus antibody; lane 2, reaction with NCX1 antibody; lane 1, reaction of Exon F antibody with SLV proteins. The amount of SLV proteins applied to each lane was 10 µg. Lane 5 contained standard molecular mass proteins (10 µl). The sample buffer used to dissolve protein samples before application to gel contained 10 mM DTT.

Figure 6 Immunoreactions with purified and reconstituted Na/Ca exchanger samples with different antibodies

Proteins were blotted to nitrocellulose, which was then cut and incubated with the corresponding antiserum (1:1000 dilution; see the Experimental section for details). Lane 5, reaction with SWant antibody; lane 6, reaction of Swant antibody with control SLV proteins preparation (10 µg); lane 4, reaction of NH₂-terminus antibody with purified and reconstituted exchanger (5 µg); lane 3, reaction of Exon F antibody with purified and reconstituted exchanger (5 µg); lane 2, reaction of NCX1 antibody with intact purified and reconstituted exchanger (5 µg); lane 1, reaction of NCX1 antibody with digested purified and reconstituted exchanger vesicles using Proteinase K (5 µg); lane 7 contained molecular mass standards. The sample buffer used to solubilize purified and reconstituted protein samples contained 10 mM NEM (except the SLV sample in lane 6, to which 10 mM DTT was added) before application to gel lanes.

Figure 7 Immunoreactions with different antibodies, previously incubated or not with the corresponding antigen, with SLV protein preparations

Proteins were blotted to nitrocellulose, which was then cut and incubated with the corresponding antiserum (1:1000 dilution; see the Experimental section for details). Lanes 2 and 3, reaction of NH_2 -terminus antibody before and after the addition of antigen respectively; lanes 4 and 5, reaction of NCX1 antibody before and after the addition of antigen respectively; lanes 6 and 7, reaction of Exon F antibody before and after the addition of antigen respectively. For details of the different antigens used to raise the antibodies, see the Experimental section. Lane 1 contained molecular mass standards (10 μl). Each lane contained 10 μg of protein. The sample buffer used to dissolve protein samples before application to gel contained 10 mM DTT. In lanes 3, 5 and 7, excess antigen (10 $\mu\text{g}/\text{ml}$) was preincubated with the corresponding antibody solution for 24 h before incubation overnight with nitrocellulose sheets.

Figure 8 Immunoreactions with different antibodies, previously incubated or not with the corresponding antigen, with purified and reconstituted Na/Ca exchanger samples

Proteins were blotted to nitrocellulose, which was then cut and incubated with corresponding antiserum (1:1000 dilution; see the Experimental section for details). Lanes 2 and 3, reaction of NH_2 -terminus antibody before and after the addition of antigen respectively; lanes 4 and 5, reaction of NCX1 antibody before and after the addition of antigen respectively; lanes 6 and 7, reaction of Exon F antibody before and after the addition of antigen respectively. Lane 1 contained molecular mass standards (10 μl). Each lane contained 5 μg of protein. The sample buffer used to dissolve protein samples before application to gel contained 10 mM DTT. In lanes 3, 5 and 7, excess antigen (10 $\mu\text{g}/\text{ml}$) was preincubated with the corresponding antibody solution for 24 h before incubation overnight with nitrocellulose sheets.

To verify the specificities of the antibodies used, control immunoblots were performed with antibodies previously incubated with excess antigen peptide (10 $\mu\text{g}/\text{ml}$). Figure 7 shows results obtained with SLV protein preparations; Figure 8 shows

those obtained with purified protein preparations. In all cases the positive reaction was abolished by preincubation of antibody solution with the corresponding antigen before incubation with nitrocellulose, confirming the specificity of the antibodies used.

Proteolysis of intact reconstituted purified Na/Ca exchanger

To determine the orientation of the purified protein in reconstituted asolectin vesicles, intact sealed vesicles were digested with Proteinase K and immunoblots were performed with NCX1 antibody. NCX1 recognizes an epitope (residues 393–406) in the large hydrophilic domain between segments 5 and 6 of the Na/Ca exchanger (see Figure 4 for NCX1 epitope localization), a domain supposed to be intracytoplasmic according to the theoretical membrane topology based on hydropathy plot analysis [15], and as confirmed by monoclonal antibody mapping of the canine cardiac Na/Ca exchanger [29]. Accordingly, after treatment of intact reconstituted purified exchanger with Proteinase K, immunodetection of a polypeptide with a molecular mass ranging from approx. 30 to 60 kDa containing the NCX1 epitope would be expected if the protein were inserted in the normal mode (inside-in) or no such fragment if the insertion mode were inside-out. Immunoblots with NCX1 showed no positive reaction in the expected molecular mass range, indicating that we had a homogeneous population of inside-out reconstituted vesicles (Figure 6, lane 1).

DISCUSSION

The present study resulted in the purification and reconstitution of the bovine cardiac Na/Ca exchanger into asolectin liposomes. The population of liposomes obtained was homogeneous, the exchanger being inserted inside-out, namely with the hydrophilic loop facing the extracellular milieu. The purified protein yield, the recovery of Na/Ca exchange activity and the purification factor were higher than those obtained by Philipson et al. [11]. Moreover, it is most likely that we had more than one protein per liposome under our reconstitution conditions owing to the low lipid-to-protein ratio used.

The present study resulted in a unique 70 kDa band as revealed both by Coomassie Blue staining and immunostaining. That 70 kDa polypeptide resulted from the cleavage of the 160 and 120 kDa proteins at the final elution step in the WGA-agarose purification procedure.

The 70 kDa protein has been proposed to be a proteolytic fragment of the 120 kDa protein [11,13]. It is nevertheless striking that the addition of protease inhibitors to the purification solution did not alter the gel electrophoresis pattern, which was also reported in synaptic plasma membrane preparations [14]. However, the 70 kDa band has been observed in several studies, including the present one, before treatment with chymotrypsin [11,13,14]. One possibility is therefore that the 70 kDa protein is a subunit of the 120 kDa protein.

The 70 kDa protein has been proposed to correspond either to the N-terminus [17,18] or the C-terminus of the exchanger [19]. One major contribution of the present study is the demonstration that the 70 kDa polypeptide seen in both native SLV preparations and the purified protein preparations corresponds to the N-terminal portion of the exchanger. Indeed, immunoblot results with the ' NH_2 -terminus' antibody showed that the 160, 120 and 70 kDa polypeptides in SLV preparations have a common N-terminal portion corresponding to the deduced N-terminus (ETEMEG ...) of the cloned bovine heart exchanger [16]. This had already been demonstrated for the 160 and 120 kDa proteins by sequencing the N-terminus of the canine and bovine ex-

changers [13]. Immunoblots performed on purified protein preparation showed that the 'NH₂-terminus' antibody also labelled the unique 70 kDa band, confirming that the 70 kDa polypeptide has the same N-terminal sequence as the 160 and 120 kDa polypeptides.

That view is also confirmed by the results obtained with the Exon F antibody. This antibody, raised against the last exon of the alternative splicing zone, was located at the C-terminal end of the large hydrophilic loop (residues 622–642), although labelling the 160 and 120 kDa bands in SLV did not detect the 70 kDa band in either SLV or the purified protein preparation. Because the NCX1 antibody, raised against a zone located in the central part of the hydrophilic loop (residues 393–406), reacted with the three bands in SLV and the 70 kDa band in the purified protein preparation, it can be deduced that the 70 kDa polypeptide starts at the N-terminus of the exchanger and ends between residues 407 and 621. This is in agreement with rough protein mass calculation with an average molecular mass of 110 Da per amino acid. In a previous study, an antibody raised against the residues 648–662 of the cloned canine heart exchanger did not bind to the native exchanger but reacted with denatured canine cardiac sarcolemmal protein, including the 70 kDa band [15]. Similarly, in another study, an antibody was raised against the residues 561–688 and was found to detect the three canonical bands in membrane preparations of COS-7 cells transfected with the cardiac Na/Ca exchanger as a fusion protein with a poly-histidine tag at its C-terminus [19]. It is conceivable that the cleavage of the 120 kDa leading to the 70 kDa protein might occur at different but closely located sites (residues 407–663 or 689), depending on the origin and the processing of the protein preparation. Our results are in agreement with the view of Gabellini and co-workers [17,18] that the short isoform (containing the five N-terminal transmembrane domains and a part of the cytoplasmic loop, with a molecular mass of 67 kDa) that they have identified and then expressed could correspond to the 70 kDa polypeptide seen in SLV preparations.

However, our results are in contradiction with those of Iwata et al. [19], who showed that the 70 kDa band contained two polypeptides corresponding to the C-terminus of the exchanger and whose N-termini started at Gly-258 and Gly-270. The present study provides no further insight into the reason for this discrepancy. Nevertheless it is important to note that, in the study by Iwata et al. [19], the Na/Ca exchange activity of reconstituted proteins was not examined; the results are in contradiction with results published later by the same group [17,18].

Taken together, the above results confirm that the 70 kDa polypeptide observed in SLV and purified preparations corre-

sponds to the N-terminal portion of the 120 kDa Na/Ca exchanger and is associated with Na/Ca exchange activity.

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