Evidence for cardiac sodium–calcium exchanger association with caveolin-3

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Abstract The interaction of cardiac Na^+–Ca^{2+} exchange (NCX1) with caveolin proteins was investigated in sarcolemmal vesicles. Western blots of sarcolemmal vesicles revealed the presence of caveolin-1, -2, and -3. NCX1 co-fractionated more closely with caveolin-3 than caveolin-1 on sucrose density gradients. NCX1 has five possible caveolin-binding motifs and NCX1 co-precipitated specifically with caveolin-3. Molecular sieve column chromatography indicated that this co-precipitation was not due to incomplete solubilization of lipid raft microdomains. Cholesterol chelation in vesicles decreased NCX1 transport activity and caveolin-3 co-precipitation. NCX1 may play a role in caveolar transmembrane signaling in addition to its role in excitation–contraction coupling. **© 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Caveola; Sarcolemma; Cardiac Na^+–Ca^{2+} exchange

1. Introduction

The cardiac Na^+–Ca^{2+} exchanger (NCX1) moves Ca^{2+} across the myocyte sarcolemma and helps regulate myocardial contractility. NCX1 transports Ca^{2+} into the cell during systole (backward mode) and Ca^{2+} out of the cell during diastole (forward mode). Because it plays a role in myocardial Ca^{2+} homeostasis, NCX1 could be an important regulator for signaling factors involved in raising or lowering inotropic activity of the heart. For many types of cells, the signaling factors are now known to be concentrated in microdomains called caveolae [1,2]. Caveolae composition is cell specific and characteristically they contain isoforms of the known caveolin proteins (caveolin-1, -2, and -3). Caveolins bind to and regulate the activity of many components of receptor signaling pathways through their 'scaffolding' domain [1,2].

Recent animal and human clinical studies have reported an increase or upregulation of NCX1 in heart failure (HF) [3–5], although reports linking NCX1 function to HF are still rare [6]. The muscle specific isoform, caveolin-3, has also been shown to increase in HF [7]. The increase in caveolin-3 and sarcolemmal caveolae in HF is thought to enhance nitric oxide signaling [7]. In endothelial cells, nitric oxide synthase copurified with caveolin-1 and NCX1 in sucrose density gradients [8]. However, the NCX1 protein was observed in all fractions and therefore a clear association of NCX1 with caveolin protein in endothelial cells has not been established. There are no previous reports of NCX1 association with caveolins in cardiac myocytes.

In the present study, we examined the interaction of NCX1 protein with caveolin proteins in cardiac sarcolemmal vesicles. Sarcolemmal vesicles, which are prepared from myocardial tissue composed of myocytes as well as endothelial cells and smooth muscle cells, contained caveolin-1, -2, and -3. Our data demonstrate that NCX1 co-precipitates with the muscle specific isoform, caveolin-3. It is possible that some sarcolemmal NCX1 transporters may play a role in caveolar transmembrane signaling, in addition to its better-understood role in excitation–contraction coupling.

2. Materials and methods

2.1. Preparation of bovine cardiac sarcolemmal (BSl) vesicles

BSl vesicles were prepared as described [9]. The final vesicle product was suspended in 160 mM NaCl, 20 mM 3-[*N*-morpholino]propanesulfonic acid (MOPS)/Tris, pH 7.4. Vesicles were maintained at −70°C prior to use.

2.2. Preparation of Sepharose bead immobilized antibodies and immunoprecipitation

NCX1 antibody (Affinity Bioreagents, Golden, CO, USA), caveolin-3 antibody (Transduction Laboratories, Lexington, KY, USA) and non-immunogenic IgG were coupled to CNBr 4B Sepharose beads (Sigma, St. Louis, MO, USA) per the manufacturer's instructions. Beads were extensively washed after coupling and equilibrated in extraction buffer consisting of 60 mM octylglucoside detergent, 160 mM NaCl, and 20 mM MOPS adjusted to a final pH of 7.4 with Tris.

BSl vesicles (4 mg) were thawed, pelleted by centrifugation, and resuspended in extraction buffer (5 ml). The resuspended mixture was maintained on ice for 30 min with periodic vortex mixing. The detergent extract was subjected to centrifugation (160 000 × *g* for 15 min). Detergent-solubilized proteins were recovered from the supernatant fraction and mixed with bead-immobilized NCX1 antibody, caveolin-3 antibody or non-immunogenic IgG (40 μl beads/ml extract) and allowed to gently mix in a rotating mixer at 4°C for 18 h. Beads were extensively washed with several changes of extraction buffer. The final bead pellets were resuspended in sodium dodecyl sulfate–polyacrylamide gel electrophoresis reducing sample buffer and boiled for 6 min prior to electrophoretic procedures.

2.3. Sucrose density gradient membrane fractionation

Ventricular tissue membranes [9] were applied to a 5–35% discontinuous sucrose gradient as previously described [10]. 2 ml gradient

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Abbreviations: HF, heart failure; XIP, exchange inhibitory peptide; BSl, bovine cardiac sarcolemmal; MOPS, 3-[*N*-morpholino]propanesulfonic acid

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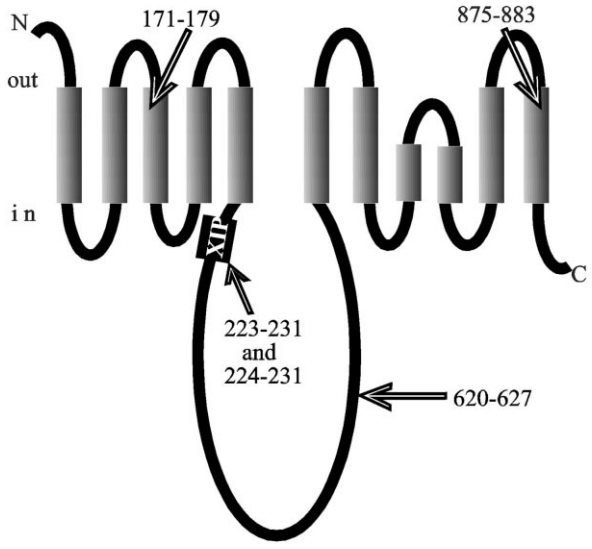

Fig. 1. Predicted location of NCX1 caveolin-binding motifs. The hydropathy plot model of NCX1 is based upon topological analysis [17]. Transmembrane regions are represented by shaded cylinders and extramembranal loops are black lines. Endogenous exchange inhibitory peptide (XIP) is shown as a black box. Arrows designate the approximate location of the NCX1 caveolin-binding motifs. Two (amino acids 171–179 and 875–883) are modeled to be in transmembrane helices near the external face of the sarcolemma membrane. Amino acids 223–231, 224–231, and 620–627 are all modeled to exist on the protein's large cytoplasmic loop. The first two segments actually overlap within the endogenous XIP domain and the third segment (620–627) correlates with a cardiac specific splice variant.

fractions were collected and concentrated by 10% trichloroacetic acid precipitation prior to electrophoretic analysis.

2.4. Molecular sieve column chromatography

BSl vesicle protein was solubilized as in immunoprecipitation studies and subjected to molecular sieve column chromatography by Sephadex G150-120 as previously described [11]. Column performance (fractionation by size) was determined using a commercially available kit (Gel Filtration Calibration Kit, Pharmacia, Piscataway, NJ, USA).

2.5. NCX1 transport assay

NCX1 activity was determined as previously described [9]. Three second transport time points were performed on at least three different vesicle preparations. All points are the result of triplicate determinations.

2.6. Chemicals and reagents

Unless otherwise noted, all reagents were obtained from Sigma, St. Louis, MO, USA.

3. Results and discussion

3.1. Caveolin-binding motifs in NCX1

The scaffolding domain of caveolin is the region of the protein that associates with or binds to other proteins sequestered in caveolae [1]. Two related caveolin-binding motifs, i.e. motifs present on caveolin-associated proteins that bind to the scaffolding region, have been described. The two motifs are: ϕXϕXXXXϕ and ϕXXXXϕXXϕ, where ϕ is aromatic amino acids Trp (W), Phe (F) or Tyr (Y), and X is any amino acid [1]. To associate directly with caveolins, a protein must contain this motif. A scan of NCX1 primary amino acid sequence indicated the presence of five potential caveolin-binding motifs located at amino acids 171–179, 223–231, 224–231, 620–627, and 875–883 (Fig. 1). Interestingly, three of these motifs are located at regions of the protein which have special significance. Two are nestled within the transporter's endogenous exchange inhibitory peptide domain, a region of NCX1 involved in regulation of transport [9,12]. A third at amino acids 620–627 is an alternative splicing exon found in NCX1 but not other exchanger isoforms [13]. Because the caveolin scaffolding domain, which associates with the binding motif, is located at or near the cytoplasmic face of the plasma membrane, it is unlikely that the motifs at amino acids 171–178 or 875–883 on the extracellular side of the membrane are actual caveolin-binding sites.

3.2. The presence of caveolin protein in cardiac sarcolemmal vesicle preparations

Western blots of BSl vesicle proteins (15 μg) were probed with antibodies against NCX1, caveolin-1, -2, and -3 (Fig. 2a).

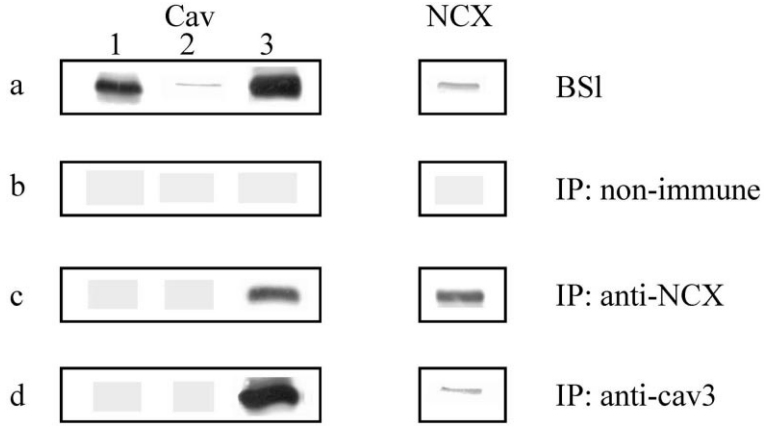

Fig. 2. Western blot analysis of caveolin and NCX1 proteins. Western blot (immunoblot) analysis of sarcolemma vesicle proteins and immunoprecipitated proteins are detailed in Section 2. a: Blots of sarcolemmal vesicle proteins separated in 10% polyacrylamide gels were probed with antibodies against caveolin-1, caveolin-2, and caveolin-3 or NXC1 as indicated. All three caveolin isoforms were present in the vesicle preparations; however, the observed level of caveolin-2 was low. b–d: Blots of immunoprecipitated proteins. Detergent-solubilized BSl proteins were incubated with Sepharose bead coupled non-immune antibody (b), anti-NCX1 antibody (c) or anti-caveolin-3 antibody (d). Blots were then probed with anti-caveolin-1, anti-caveolin-2, and anti-caveolin-3 antibodies or anti-NXC1 antibody as indicated. For (c), six-fold more sample was loaded on gels to visualize NCX1 compared to the sample volume used to visualize caveolin-3.

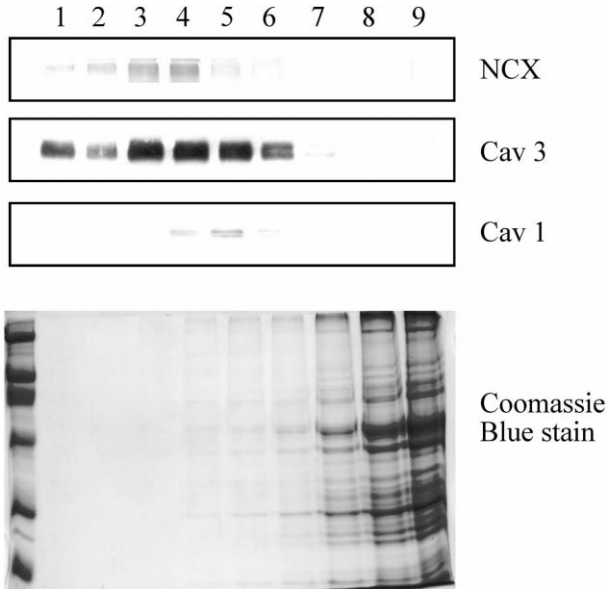

Fig. 3. Sucrose gradient analysis of bovine myocardial membranes. A crude membrane homogenate of bovine myocardial membranes was prepared as described in Section 2. Upper: Sucrose density gradient fractions 1–9 as labeled were subjected to Western blot analysis. Lower: Coomassie blue-stained protein pattern of the same gradient fractions.

All three caveolin isoforms were present in BSl vesicles although caveolin-2 was barely detectable. These were also observed in sarcolemmal membrane vesicles from canine and porcine myocardium (not shown). No labeling was observed when blots were probed with non-immune antibodies (not shown). The myocardial tissue used to prepare the membranes is comprised of myocyte, smooth muscle, and endothelial cells. Much or all of caveolin-1 and -2 may be from endothelial cells, as it has been reported that differentiated muscle cells mainly express caveolin-3 [14].

3.3. Gradient fractionated myocardial membranes

The 'standard' sarcolemmal vesicle preparation [9] uses a single sucrose step gradient to separate the lighter plasma membrane fractions from other, higher density cellular membranes. We further fractionated the light membrane fraction using a multi-step gradient previously reported to fractionate caveolar proteins [10] (Fig. 3). The peak of NCX1 and caveolin-3 protein tended to co-localize more closely in fractions 3 and 4, while caveolin-1 protein peaked in fractions 4 and 5. This suggests that NCX1 and caveolin-3 may reside in the same light membrane fraction while caveolin-1 appears to be in slightly higher density membranes. The same result, i.e. the co-fractionation of NCX1 and caveolin-3, was observed on gradients containing total cellular (soluble and membrane) protein (not shown). It is likely that caveolae with either caveolin-3 or -1 arise from different cell types (myocytes and endothelial cells, respectively).

3.4. Co-precipitation of caveolin-3 and NCX1

To address the question as to which, if any, of the caveolin protein isoforms associate with NCX1, co-immunoprecipitation experiments were performed. Sepharose beads coupled to monoclonal antibodies against NCX1, caveolin-3 or non-immunogenic IgG were incubated with detergent-extracted sarcolemmal proteins. The beads were extensively washed with the extraction buffer to remove non-specifically associated protein. The immunoprecipitate was subjected to Western blot analysis. The caveolin isoforms and NCX1 did not non-specifically precipitate with control IgG (Fig. 2b). In Fig. 2c the presence of NCX1 and caveolin-3 was detected following precipitation with NCX1 antibody. Caveolin-1 and -2, while present in the sarcolemmal vesicle membrane preparations (Fig. 2a), did not co-immunoprecipitate with NCX1. NCX1 and caveolin-3 were detected following precipitation with immobilized anti-caveolin-3 (Fig. 2d). The results shown in Fig. 2b–d suggest that cardiac NCX1 specifically associates with caveolin-3, which is the muscle specific caveolin isoform.

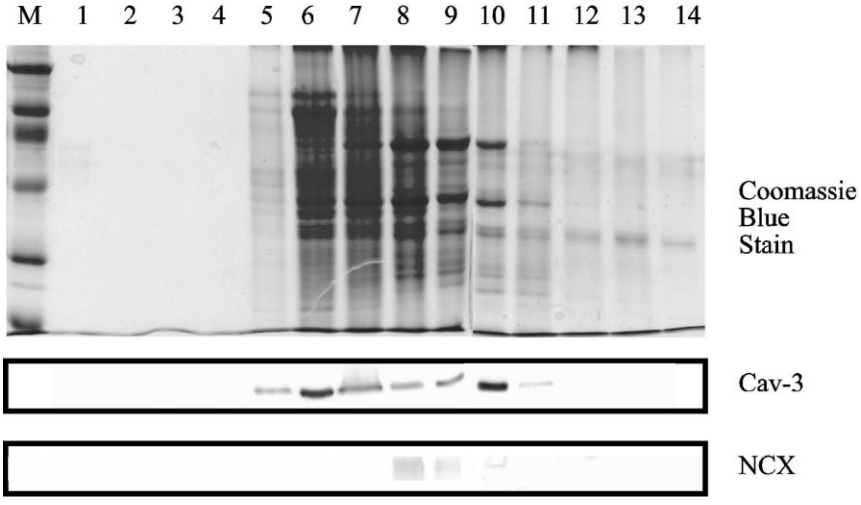

Fig. 4. Molecular sieve column chromatography fractionation of detergent-solubilized BSl vesicles. Detergent-solubilized BSl proteins were fractionated by molecular sieve column chromatography (Section 2 and as previously described [11]). Shown is the protein elution profile as visualized by Coomassie blue staining (top) and Western blot analysis of the fractions by anti-NCX1 and anti-caveolin-3 antibodies. Column fractions are numbered on the top. Calibration of the column indicated the void volume as fraction 4. NCX1 was detected in fractions 8–10, which were also the peak fractions for 120 kDa aldolase (from the commercial calibration kit). The results shown are representative of data obtained from two separate molecular sieve columns.

3.5. Molecular sieve column chromatography fractionation of BSl vesicle proteins

To exclude the possibility that the co-precipitations (Fig. 2) are due to the presence of the remnants of lipid raft microdomains, we fractionated detergent-solubilized protein by molecular sieve column chromatography. The column matrix utilized in these experiments excluded proteins or complexes (like large lipid raft microdomains) greater than 300 kDa. NCX1 was not observed in the void volume fractions but rather eluted with proteins in the size range included by the column matrix (Fig. 4). This suggests that NCX1 was completely solubilized by the detergent treatment and not part of a lipid raft microdomain. Caveolin-3 was observed in many fractions as expected because it binds to signaling proteins of different sizes. It has also been reported that in the presence of octylglucoside, caveolin monomers can form oligomers as large as 350 kDa [15].

3.6. Cholesterol depletion of caveolae and co-precipitation

Functional and structural integrity of caveolae is dependent upon a critical level of cholesterol in the plasma membrane and the caveolae itself [2,16]. β-Cyclodextrin, by binding cholesterol, has been shown to disrupt function and structure of caveolae [16]. The disappearance of caveolae occurs because caveolin protein requires cholesterol to remain in the membrane [15,16]. Without caveolin protein in the membrane microdomain, caveolae cannot retain structural integrity. BSl vesicles were treated with 0.2 and 2.0% (w/v) cyclodextrin for 1 h on ice prior to solubilization and immunoprecipitation with immobilized NCX1 antibody. In the same vesicles, β-cyclodextrin diminished the level of caveolin-3 that co-precipitated with NCX1, while the level of NCX1 was not affected (Fig. 5a). Densitometric analysis indicated that caveolin-3 co-precipitation was reduced by 42 and 54% for 0.2 and 2.0% β-cyclodextrin, respectively. These data suggest that NCX1 and caveolin-3 are likely inserted in a lipid bilayer with cholesterol and that cholesterol may be required for the NCX1/caveolin-3 interaction. In other experiments, NCX1 transport activity was evaluated in BSl vesicles preincubated with β-cyclodextrin (Fig. 5b). A dose-dependent decrease of NCX1 transport activity was observed which paralleled the decrease in co-precipitated caveolin-3 shown in Fig. 5a. In both experiments, even at the highest concentration of β-cyclodextrin (2%), co-precipitation of caveolin-3 and NCX1 activity were diminished but not abolished. Passive $^{45}Ca^{2+}$ equilibration in vesicles treated with β-cyclodextrin was not different from control (untreated) vesicles, indicating that this treatment did not cause these vesicles to become leaky.

In this report, we describe the association of NCX1 with the muscle specific caveolin isoform, caveolin-3. While all three known isoforms of caveolin protein are present in cardiac sarcolemmal vesicle membrane preparations, only caveolin-3 co-precipitates with NCX1. NCX1 can also be co-precipitated with immobilized caveolin-3 antibody. The low level of NCX1 observed in anti-caveolin-3 precipitations likely reflects the ability of caveolin-3 to associate with a wide variety of proteins besides NCX1. In other words, NCX1 may exclusively associate with caveolin-3, while caveolin-3 can potentially bind to many different proteins within caveolae [1,2]. These observations strongly implicate the presence and partitioning of some NCX1 protein in myocyte caveolae. In light of recent reports indicating increased caveolin-3 and NCX1 protein in HF, it is possible that the increased expression of these two proteins may not be casually unrelated events [3–7]. Concentration of NCX1 in caveolae has potential ramifications in terms of exchanger function beyond Ca^{2+} homeostasis in myocardial contractility. In cardiac myocytes, it remains to be shown if NCX1 has additional functions beyond cytosolic Ca^{2+} homeostasis related to contractility, but the possibility is intriguing.

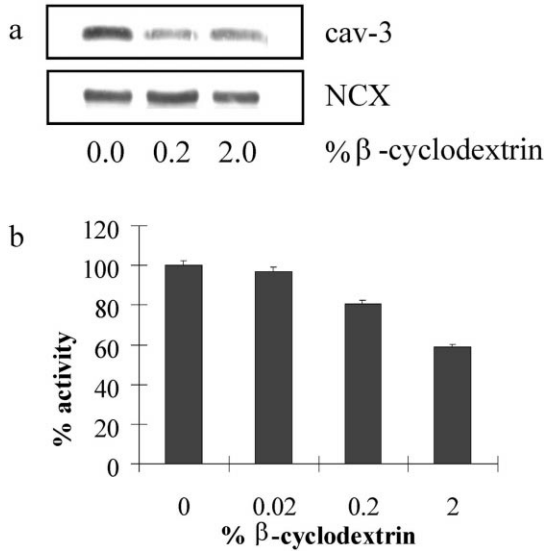

Fig. 5. The effect of β-cyclodextrin on NCX1 activity and caveolin-3 co-precipitation. Vesicles were pretreated with β-cyclodextrin for 1 h on ice. The cholesterol-depleted membranes were assayed for NCX1 transport activity or detergent-solubilized and immunoprecipitated with immobilized anti-NCX1 antibody. a: Upper: blots probed with anti-caveolin-3 antibody; lower: blots probed with anti-NCX1 antibody. b: The effect of β-cyclodextrin on NCX1 transport activity ($n = 5$). Passive $^{45}Ca^{2+}$ equilibration in β-cyclodextrin-treated vesicles was not different from control vesicles, indicating treatment did not make the vesicles leaky (not shown). The amount of the NCX1 precipitated and pellet size were not affected by β-cyclodextrin treatment.

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