A Ca\textsuperscript{2+}-dependent Tryptic Cleavage Site and a Protein Kinase A Phosphorylation Site Are Present in the Ca\textsuperscript{2+} Regulatory Domain of Scallop Muscle Na\textsuperscript{+}-Ca\textsuperscript{2+} Exchanger*

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Minggui Chen‡, Zhimin Zhang, Mary-Anne Tawiah-Boateng, and Peter M. D. Hardwicke§

From the Department of Biochemistry and Molecular Biology, Southern Illinois University, Carbondale, Illinois 62901-4413

Digestion of scallop muscle membrane fractions with trypsin led to release of soluble polypeptides derived from the large cytoplasmic domain of a Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger. In the presence of 1 mM Ca\textsuperscript{2+}, the major product was a peptide of \(-37 kDa\), with an N terminus corresponding to residue 401 of the NCX1 exchanger. In the presence of 10 mM EGTA, \(-16\) and \(-19-kDa\) peptides were the major products. Polyclonal rabbit IgG raised against the 37-kDa peptide also bound to the 16- and 19-kDa soluble trypptic peptides and to a 105–110-kDa polypeptide in the undigested membrane preparation. The 16-kDa fragment corresponded to the N-terminal part of the 37-kDa peptide. The conformation of the precursor polypeptide chain in the region of the C terminus of the 16-kDa tryptic peptide was thus altered by the binding of Ca\textsuperscript{2+}. Phosphorylation of the parent membranes with the catalytic subunit of protein kinase A and \([\gamma-32\text{P}]/\text{ATP}\) led to incorporation of \(^{32}\text{P}\) into the 16- and 37-kDa soluble fragments. A site may exist within the Ca\textsuperscript{2+} regulatory domain of a scallop muscle Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger that mediates direct modulation of secondary Ca\textsuperscript{2+} regulation by cAMP.

The Na\textsuperscript{+}-Ca\textsuperscript{2+} exchangers of the plasma membrane catalyze a secondary active transport process dependent on the Na\textsuperscript{+} electrochemical gradient generated by the Na\textsuperscript{+,K\textsuperscript{-}}-ATPase and play a major role in cellular Ca\textsuperscript{2+} homeostasis in many tissues (1). Three Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger proteins (NCX1, NCX2, and NCX3) have been described in vertebrates (2–5). The molecular biology of the Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger (Culx) from *Drosophila* has been described (6, 7), and an exchanger from squid has been reported (8). An electroneutral Na\textsuperscript{+}-Ca\textsuperscript{2+} antiporter has also been found in mitochondria, where it may be involved in modulating matrix Ca\textsuperscript{2+} in response to changes in cytoplasmic Ca\textsuperscript{2+} concentration (9, 10).

The protein moieties of the plasma membrane Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger proteins are close in overall size (\(-103–106 kDa\)) (4, 11) to the SERCA\textsubscript{1}-type Ca\textsuperscript{2+}-ATPase pumps, which have a molecular mass of \(-110 kDa\) (12). In the case of NCX1, a signal sequence of 32 amino acid residues at the N terminus of the protein is removed post-translationally (13–15). From the N terminus, structure prediction algorithms suggest that five transmembrane segments lead to a large cytoplasmic, followed by four C-terminal transmembrane helices (14–16). Two internal stretches of the f loop, Phe\textsuperscript{407}(375)–Asp\textsuperscript{478}(443) and Thr\textsuperscript{536}(506)–Tyr\textsuperscript{543}(531), show similarity to one another, and are termed the \(\beta\)-1 and \(\beta\)-2 repeats (6). A high affinity Ca\textsuperscript{2+} binding region (\(K_g\), 0.1–3 \(\mu\text{M}\) is present in the large cytoplasmic domain (17–20). This binds Ca\textsuperscript{2+} cooperatively and provides regulation of the exchanger through the I2 mechanism, in which increased levels of cytoplasmic Ca\textsuperscript{2+} activate the enzyme (19). The regulatory Ca\textsuperscript{2+} binding region involves the \(\beta\)-1 repeat and extends through the variable region between the two \(\beta\) repeats to the beginning of \(\beta\)-2 (18). Two acidic triads, one at the C terminus of \(\beta\)-1 (Asp\textsuperscript{726}(694)–Asp\textsuperscript{728}(696)–Asp\textsuperscript{730}(698)), just N-terminal to \(\beta\)-2, are important components of this high affinity Ca\textsuperscript{2+} binding site (18).

The isolated cardiac exchanger shows three bands on silver-stained SDS gels: a glycosylated 120-kDa species corresponding to the native exchanger, a glycosylated 160-kDa polypeptide representing oxidized exchanger, and an unglycosylated polypeptide of 70 kDa, which arises by proteolysis of the 120-kDa protein at the Asp\textsuperscript{528}(506)–Gly or Asp\textsuperscript{532}(508)–Gly bonds in the large cytoplasmic domain (11, 21–23). Digestion of the exchanger with chymotrypsin leads to a loss of regulatory function, probably through proteolysis localized in the cytoplasmic f loop (4).

The study of the protein chemistry of the Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger has been hampered by its low abundance; even in cardiac muscle, the NCX1 exchanger represents only 0.1–0.2% (w/w) of the sarcolemmal membrane protein (24). The mitochondrial exchanger is present at 0.4 \(\mu\text{g}/\text{mg}\) total protein (10). Thus, silver staining has often been necessary to detect the protein on SDS gels (21).

In the past, a number of studies of the Ca-ATPase from the cross-striated adductor muscle of the deep sea scallop have used a deoxycholate-extracted membrane fraction enriched in fragmented SR (25–28). In the course of examining the effect of trypsin on this preparation, it was observed that soluble polypeptides were released by the action of the protease in sufficient amounts for them to be detected by conventional col dodecyl ether; DOC, deoxycholate; PKA, protein kinase A (cAMP-dependent kinase); SL, sarcolemma; MOPS, 3-(N-morpholino)propanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; CAPS, 3-cyclohexylamino)propanesulfonic acid; Tricine, N\textsubscript{2}-hydroxy-1,1-bis (hydroxymethyl)ethyglycine; PBS, phosphate-buffered saline.

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‡ Present address: Open System Div., Information Builders Inc., Two Penn Plaza, 28th Floor, New York, NY 10121.

§ To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, Mail Code 4413, Southern Illinois University, Carbondale, IL 62901-4413. Tel.: 618-453-6449; Fax: 618-453-6440; E-mail: phardwicke@siumed.edu.

The abbreviations used are: SERCA, Sarco(endo)plasmic reticulum calcium ATPase; SR, sarcolemmal reticulum; C\textsubscript{11}E\textsubscript{p}, nonaethyleneglycol dodecyl ether; DOC, deoxycholate; PKA, protein kinase A (cAMP-dependent kinase); SL, sarcolemma; MOPS, 3-(N-morpholino)propanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; CAPS, 3-cyclohexylamino)propanesulfonic acid; Tricine, N\textsubscript{2}-hydroxy-1,1-bis (hydroxymethyl)ethylglycine; PBS, phosphate-buffered saline.
staining of SDS gels with Coomassie Blue. Sequencing of these tryptic fragments showed that they were not derived from the Ca-ATPase but instead possessed N termini identifying them as overlapping stretches of polypeptide originating in the large cytoplasmic domain (I loop) of a Na\textsuperscript{+}\textsuperscript{-}Ca\textsuperscript{2+} exchanger. Because this region of the Na\textsuperscript{+}\textsuperscript{-}Ca\textsuperscript{2+} exchanger is crucial for its regulation through physiological mechanisms and because it may represent a possible target for pharmacological intervention, the polypeptides were further investigated.

**EXPERIMENTAL PROCEDURES**

Deep sea scallops (*Placopecten magellanicus*) were obtained from the Marine Biology Laboratory (Woods Hole, MA).

**Preparation of Native Membranes Enriched in Fragmented Sarco- plasmic Reticulum**—This was carried out essentially as described previously (25–30). Total scallop muscle membranes were separated into fractions enriched in SL (B1 fraction) and SR (B2 fraction) by layering the crude total membranes, suspended in 0.32 M sucrose, 0.1 M KCl, 1 mM CaCl\textsubscript{2}, 20 mM MOPS-Na, pH 7.0, onto a discontinuous gradient comprised of a layer of 0.8 M sucrose on 1.3 M sucrose, both in 0.1 M KCl, 1 mM CaCl\textsubscript{2} 20 mM MOPS-Na, pH 7.0 (29, 30). The SL-enriched fraction (B1) banded at the 0.32–0.8 M sucrose interface, and the SR-enriched fraction (B2) banded at the 0.8–1.3 M interface (25, 29, 30). The B2 fraction was selected.

**Preparation of Membranes Enriched in Sarcolemma**—The B1 fraction was prepared as described above (25, 29, 30).

**Preparation of Deoxycholate-extracted Scallop Membrane Fractions**—This was carried out when necessary with both the SL-rich (B1) and SR-rich (B2) fractions, as described previously (25).

**Preparation of Soluble Fragments from Tryptic Digests**—Membranes were typically suspended at 5–10 mg ml\textsuperscript{-1} in standard media of 20% (v/v) ethylene glycol (Pierce), 0.15 M KCl, 1 mM CaCl\textsubscript{2} or 10 mM EGTA-Na, 50 mM MOPS-Na, pH 7.0. Digestions were at room temperature for 10–15 min. With N\textsubscript{3}-(tosyl-l-phenylalanyl chloromethyl ketone-treated trypsin (Sigma, dissolved in 1 mM KCl at 12,000 units/ml) added in a 1:30 (v/v) volume to membrane protein (giving 400 units of activity/mg total membrane protein). Digestions were terminated by addition of 4:2-aminoethylbenzenesulfonyl fluoride (Calbiochem) to a final concentration of 20 mM, followed by transfer of the sample to ice. The samples were centrifuged at 16,000 \( \times g \) for \( \frac{1}{2} \) h at 4 °C in an Eppendorf microcentrifuge. The supernatant was collected and recentrifuged for 1 h at \( 10^7 \times g \) in a Beckman TL100 bench top ultracentrifuge at 4 °C to remove traces of contaminating membranes.

**Concentration of Soluble Peptides for Use in SDS-PAGE**—Sodium deoxycholate was added to the high speed supernatant from the tryptic digest to a final concentration of 0.025% (w/v), followed by trichloroacetic acid to a final concentration of 6% (w/v). The trichloroacetic acid also deoxycholate was added to the high speed supernatant from the tryptic fraction, followed by DEAE-Sepharose anion exchange chromatography to separate IgG and IgM (33).

**Western Blotting**—SDS gels were first blotted onto polyvinylidene difluoride (Immobilon PSQ), as in the procedure for N-terminal sequencing (see above). Nonspecific binding was blocked with 5% w/v nonfat milk in PBS-T (0.1 M NaCl, 0.1% v/v Tween-20, 0.1 M Na\textsubscript{2}P, pH 7.4) for 1 h at room temperature. The blots were washed twice quickly in PBS-T, followed by one wash for 15 min and two washes for 5 min in PBS-T. The blots were incubated in rabbit anti-37-kDa antibody diluted 1:1000 with 5% (w/v) nonfat milk in PBS-T (0.1 M NaCl, 0.1% v/v Tween-20, 0.1 M Na\textsubscript{2}P, pH 7.4) for 1 h at room temperature. The blots were then washed as before with PBS-T. The blots were then incubated for 1 h in goat anti-rabbit IgG secondary antibody conjugated with horseradish peroxidase (Amersham Pharmacia Biotech) that had been diluted 1:1,500 in 5% (w/v) dried milk in PBS-T. After washing again with PBS-T, the blots were incubated with the ECL luminol system (Amersham Pharmacia Biotech). Protein Concentration—This was by the bicholoronic acid method (34).

**RESULTS**

The starting materials for the work reported here were membrane fractions prepared from the cross-striated part of the adductor muscle of the deep sea scallop. One of these (the B2 fraction; see “Experimental Procedures”) is enriched in fragmented SR but still contaminated by membranes derived from the SL (25, 29). This fraction can be extracted with low (non-solubilizing) concentrations of DOC to remove peripheral membrane proteins and other contaminants (25). On SDS gels of the DOC-extracted B2 fraction, ~90% of the Coomassie Blue-staining material has a mobility corresponding to a molecular mass of 105–115 kDa (Fig. 1 and Ref. 25), consistent with the size of the Ca-ATPase (25, 35, 36). The second scallop muscle fraction (B1), which was used later in the studies, is enriched in SL (29).

Tryptic digests of the DOC-extracted scallop muscle B2 membrane fraction were examined for soluble peptide fragments released by the action of the protease. It was found that a polypeptide of ~37 kDa was the major soluble species formed in the presence of 1 mM Ca\textsuperscript{2+} (Fig. 2), although smaller amounts of soluble ~16- and ~19-kDa peptides were sometimes also present. In the presence of 10 mM EGTA, the pattern of the products was reversed, so that the ~37-kDa fragment was either absent or present in much smaller amounts, and the two smaller polypeptides were the main soluble products. The relative proportions of ~16- and ~19-kDa peptides varied; sometimes the two species were present in approximately the same amounts, whereas in other digests one form predominated. This may mean that the 16- and 19-kDa fragments are susceptible to further proteolysis; in contrast, the ~37-kDa peptide was relatively resistant to the further action of trypsin. All three soluble peptides could be clearly visualized on the gels with standard Coomassie Blue stain. Occasionally, larger soluble polypeptides of ~60 and 40 kDa were observed.

The precursor polypeptide for the soluble peptides had to be very close in size to the scallop SERCA (molecular mass, 110 kDa), because only material of 105–115 kDa was present in sufficient amounts in the starting preparation to account for the amounts of soluble peptides formed (Fig. 1 and Ref. 25).
Conformational Changes in a Na\textsuperscript{+}-Ca\textsuperscript{2+} Exchanger

Fig. 1. The scallop muscle B\textsubscript{2} fraction after extraction with deoxycholate. The scallop muscle B\textsubscript{2} fraction was extracted with deoxycholate as described under “Experimental Procedures” and electrophoresed in the Laemmli Tris-glycine system on a 12.5% gel. Approximately 90\% of the Coomassie Blue-stained material on the gel has a size in the 105–115-kDa range. A small amount of a polypeptide of ~28 kDa is present, probably representing contamination by a component of the SL that resists DOC extraction (29).

Fig. 2. Effect of Ca\textsuperscript{2+} concentration on soluble tryptic fragments produced from a deoxycholate-treated scallop muscle membrane fraction. Digestion of deoxycholate-extracted scallop muscle membrane B\textsubscript{2} was carried out in the presence of EGTA and Ca\textsuperscript{2+} as described under “Experimental Procedures.” The soluble peptides formed in the presence of CaCl\textsubscript{2} and EGTA were concentrated by the DOC-trichloroacetic acid method and electrophoresed in the Tricine SDS-PAGE system. The bands labeled t in lanes 1 and 2 are trypsin (molecular mass, 23.8 kDa) and of its autolysis products. Lane 1, soluble peptides formed from 190 \mu g of total membrane protein in the presence of EGTA. Lane 2, soluble peptides formed from 190 \mu g of total membrane protein in the presence of Ca\textsuperscript{2+}. Lane 3, markers.

There are usually only small and variable traces of other integral protein membrane components on Coomassie Blue-stained SDS gels of the B\textsubscript{2} fraction after DOC extraction, the most significant of these being a 28-kDa protein associated with the SL (25, 29). The latter peptide is too small to be the precursor of the 37-kDa soluble tryptic fragment and in any case is present only at low levels.

The results of N-terminal sequencing of the soluble fragments are shown in Table I, where the data have been aligned to show the apparent relationships between the peptides and some sodium-calcium exchangers. The ~37-kDa peptide produced in the presence of Ca\textsuperscript{2+} and the ~16-kDa polypeptide formed by digestion in the absence of Ca\textsuperscript{2+} had the same N-terminal sequence. Comparison of this N-terminal sequence with known sequences using the EMBL data base (Blitz) indicated that its start corresponded to residue 401(369) of the NCX1 Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger, at the N-terminal end of the \beta-1 repeat in the large cytoplasmic domain (loop f) (2, 4). A lysyl or arginyl residue must precede this sequence for the 37- and 16-kDa fragments to be produced by the action of trypsin. There was no significant similarity to any part of the scallop Ca-ATPase polypeptide chain (36). Assuming an average residue mass of 110 Da, the ~16-kDa fragment was approximately 145 residues long and was likely to correspond closely to the segment of 16-kDa sites that ends within the two acidic triads, Asp\textsuperscript{578}-Asp\textsuperscript{446} and Asp\textsuperscript{330}-Asp\textsuperscript{498} preceding the \beta-2 repeat. The Ca\textsuperscript{2+}-insensitive cleavage site on the scallop precursor represented by the N terminus of the ~16- and ~37-kDa soluble fragments will be designated the T\textsubscript{1} site. The C terminus of the ~16-kDa fragment must be located in a stretch of polypeptide chain inaccessible to trypsin when Ca\textsuperscript{2+} is bound to the precursor protein but more susceptible to proteolysis when Ca\textsuperscript{2+} is unbound. The Ca\textsuperscript{2+}-sensitive proteolytic cleavage site represented by the C terminus of the ~16-kDa fragment will be designated the T\textsubscript{2} site. Satisfactory N-terminal sequencing of the ~19-kDa band peptide was not possible, because of microheterogeneity within that band on SDS gels, but the size of the fragment is consistent with it representing that part of the 37-kDa fragment which is C-terminal to the T\textsubscript{2} cleavage site. There is other evidence supporting this conclusion from the immunological and phosphorylation studies reported below. The tryptic cleavage site corresponding to the C terminus of the 37-kDa fragment, and possibly the C terminus of the 19-kDa fragment, will be designated the T\textsubscript{3} site.

Polyclonal Antibody against the 37-kDa Soluble Fragment

Western blotting of the scallop muscle membrane B\textsubscript{2} fraction against the 37-kDa soluble fragment showed heavy labeling of the 105–110-kDa polypeptide (Fig. 4), which was of the approximate size anticipated for a Na\textsuperscript{+}–Ca\textsuperscript{2+} exchanger. Although only material in the 105–110-kDa region was detected on the blots with Coomassie Blue, Western blots showed in addition traces of a ~60-kDa peptide that bound the antibody. This ~60-kDa membrane-bound fragment may have represented the segment of the scallop muscle Na\textsuperscript{+}–Ca\textsuperscript{2+} exchanger C-terminal to cleavage site T\textsubscript{1}, formed by

Table I

| Scallop soluble 37 kDa | Scallop soluble 16 kDa | Squid NCX-SQ1 (12) | DogNCX1 (6) | RatNCX1 (9) | RatNCX2 (7) | RatNCX3 (8) |
|------------------------|-----------------------|-------------------|------------|------------|------------|------------|
| R/DQFTKTFDVDPHLYYMEN   | R/DQFTKTFDVDPHLYYMEN  | S1a                | S1a        | S1a        | S1a        | S1a        |
| R/DQFTKTFDVDPHLYYMEN   | PDEI-TRVSFDGHYMVEN    | S1a                | S1a        | S1a        | S1a        | S1a        |
| ADD-PTRMYEPFVGYTHVMEN  | ADD-PTRMYEPFVGYTHVMEN | S1a                | S1a        | S1a        | S1a        | S1a        |
| NDVP-SKFFEGTYQCVLEN    | NDVP-SKFFEGTYQCVLEN   | S1a                | S1a        | S1a        | S1a        | S1a        |
| NDVP-SKFFEGTYQCVLEN    | DDDGASRFFPSLYHCVLEN  | S1a                | S1a        | S1a        | S1a        | S1a        |
| PFDASKVFFDFCSIQCVLEN   | PFDASKVFFDFCSIQCVLEN | S1a                | S1a        | S1a        | S1a        | S1a        |
proteolytic damage during the preparation and DOC extraction of the membranes. This polypeptide would have contained the stretch of peptide chain containing the epitopes of the 37-kDa tryptic fragment. (The predicted size for such a fragment, assuming a mean residue mass of 110 Da and a length of ~1,000 residues for the scallop exchanger, would be 62–63 kDa.) Thus, sequence comparisons indicated that the soluble tryptic fragments were not derived from the Ca-ATPase but from a Na⁺-Ca²⁺ exchanger, and Western blots suggested that the precursor of the soluble peptides was in the expected size range for a Na⁺-Ca²⁺ exchanger. The sarcoplasmic reticulum is an internal cytoplasmic cellular membrane and therefore cannot be associated with any secondary active transport process dependent on a Na⁺ electrochemical gradient. Although the Ca-ATPase must account for most of the material in the 105–115-kDa band on SDS gels of the scallop B₂ membrane fraction (20–30), a second, non-Ca-ATPase protein, a Na⁺-Ca²⁺ exchanger, is also present. This enzyme not only has a similar size to the Ca-ATPase but also in addition coincidentally happens to resist extraction by low concentrations of DOC, like the Ca-ATPase. In fact, there is evidence that extraction of the membrane with low concentrations of DOC does not remove all of the non-SR material from the preparation. Thus, traces of a ~28-kDa protein associated with the SL remain in the DOC-extracted B₂ membrane fraction (Fig. 5 and Refs. 25 and 29). Again, the near UV absorption spectrum (not shown) of the DOC-extracted B₂ preparation dissolved in SDS shows that the material contains a trienoic chromophore known to be located in the SL (29). Therefore, some residual structures derived from elements of the SL that contaminate the fragmented SR-enriched B₂ fraction (29) may survive the DOC extraction step and provide a plausible location for the Na⁺-Ca²⁺ exchanger found in this work.

The Precursor of the Soluble Tryptic Fragments Is Also Present in a Membrane Fraction Enriched in Sarcolemma—Because there was a possibility that the precursor of soluble tryptic fragments was located in the SL, the B₁ scallop muscle membrane fraction enriched in SL was prepared as described under “Experimental Procedures.” Digestion of the native (non-DOC extracted) B₂ membranes with trypsin yielded only traces of the soluble peptides (Fig. 5, lanes 1 and 2). However, when the B₁ fraction was first extracted with low concentrations of DOC, according to the protocol used with the B₂ fraction, and then treated with trypsin, the soluble fragments were formed in substantially larger amounts (Fig. 5, lanes 4 and 5). As with digestion of the DOC-extracted B₂ fraction, the 37-kDa fragment was the major soluble product in the presence of Ca²⁺, whereas the 16- and 19-kDa peptides were the predominant species released by digestion in the presence of EGTA.

The increased yield of the soluble tryptic fragments after treatment of the B₁ membrane fraction with DOC was consistent with those peptides originating in the large cytoplasmic domain (f loop) of a Na⁺-Ca²⁺ exchanger, if most of the SL vesicles in the initial preparation had a right-side-out orienta-

**FIG. 3.** Western blot of the soluble tryptic peptides formed in the presence of Ca²⁺ using rabbit anti-scallop 37-kDa fragment IgG. The deoxycholate-extracted B₂ scallop muscle membrane fraction was digested with trypsin, the soluble fraction was concentrated by the DOC-trichloroacetic acid method, and the sample was electrophoresed in the Tricine SDS system. Western blotting using rabbit anti-37-kDa fragment IgG as probe was carried out as described under “Experimental Procedures.” Both the 16- and 19-kDa fragments shared epitopes with the 37-kDa peptide.

**FIG. 4.** Western blot of deoxycholate-extracted scallop muscle B₂ membrane fraction probed with rabbit anti-scallop 37-kDa fragment antibody. Undigested deoxycholate-extracted B₂ membrane fraction was electrophoresed on a Tricine SDS gel. After electrophoretic transfer onto polyvinylidene difluoride, the preparation was probed with rabbit anti-37-kDa peptide antibody as described under “Experimental Procedures.” Lane A, Western blot developed by the luminol method. A strongly labeled zone was present in the 105–110-kDa region. The antibody also detected traces of membrane-bound breakdown products, particularly one species of ~60 kDa, possibly representing a C-terminal fragment produced by proteolysis during preparation. Lane B, the blot stained with Coomassie Blue. The dye detected polypeptide only in the ~110-kDa region.

**FIG. 5.** The soluble fragments are produced by digestion of DOC-extracted scallop sarcolemma. A membrane fraction enriched in SL (B₁ fraction) was prepared as described under “Experimental Procedures.” Part of the preparation was digested with trypsin in the presence of Ca²⁺ or EGTA, according to the usual protocol. The remainder was treated with DOC, exactly as for the B₂ fraction, before digestion with trypsin in the presence of Ca²⁺ or EGTA. Bands labeled t are trypsin (23.8 kDa) and its autolysis products. Lane 1, markers. Lane 2, soluble products from digestion of 300 μg of native SL protein (B₁ fraction) with trypsin in the presence of 1 mM Ca²⁺. The band labeled A is actin (42 kDa) contamination. Lane 3, soluble products from digestion of 300 μg of native SL protein (B₁ fraction) with trypsin in the presence of 1 mM Ca²⁺ (actin has been removed by the deoxycholate extraction). Lane 4, soluble products from digestion of 200 μg of DOC-extracted SL protein (DOC-extracted B₁ fraction) in the presence of 1 mM Ca²⁺ (actin has been removed by the deoxycholate extraction). Lane 5, soluble products from digestion of 200 μg of DOC-extracted SL protein (DOC-extracted B₁ fraction) in the presence of 10 mM EGTA (actin has been removed by the deoxycholate extraction).
**Fig. 6.** Tricine SDS gel of the soluble tryptic cleavage products from the C12E9-insoluble residue of scallop muscle membranes. After treatment of the DOC-extracted B2 membrane fraction with 4% (w/v) C12E9 on ice, as described previously (25), the preparation was centrifuged at 105,000 x g for 1/2 h at 4 °C, the supernatant was removed, and the pellet was sonicated and washed twice in 20% (v/v) ethylene glycol, 0.4 M KCl, 1 mM CaCl2, 50 mM MOPS-Na, pH 7.0. The insoluble residue was resuspended in the same medium and digested with trypsin as described under “Experimental Procedures.” After concentration of the soluble tryptic products by the DOC-trichloroacetic acid method, these were electrophoresed on a Tricine SDS gel. Lane 1, soluble products from digestion of C12E9-insoluble pellet (240 μg of total protein) in the presence of 1 mM CaCl2. Lane 2, soluble products from digestion of C12E9-insoluble pellet (240 μg of total protein) in the presence of 10 mM EGTA. Lane 3, markers.

**Fig. 7.** A ~110-kDa polypeptide in undigested scallop muscle membranes is phosphorylated by the catalytic subunit of PKA. Autoradiogram of a Tricine SDS gel of undigested scallop DOC-extracted B2 membrane fraction treated with PKA and [γ-32P]ATP as described under “Experimental Procedures.” The 105–115-kDa region was strongly labeled with 32P. A peptide of ~60 kDa, probably corresponding to the membrane-bound breakdown product detected by Western blotting, was also labeled with 32P.

**DISCUSSION**

Precise regulation of intracellular Ca2+ levels is critical for the appropriate activation/inactivation of some key cell functions, such as motility and exocytosis. Recently, intracellular Ca2+ has been revealed as a triggering factor for apoptosis (39). Thus, regulation of the channels and transporters determining cell Ca2+ levels is of physiological and pharmacological interest. Sodium-calcium exchange represents one of the most important and widespread mechanisms available to the cell for controlling intracellular Ca2+ concentration. The large cytoplasmic loop of the exchanger is accessible to internal signals reflecting cell status and so has evolved as a regulatory domain mediating the effect of changes in cell Ca2+ concentration on the direction of net Ca2+ movement across the plasma membrane. It is also apparent that external signals may affect the activity of the Na+-Ca2+ exchanger (40). The work reported here suggests that both types of signal may be transduced through the same region of the f loop.

Only material of 105–110 kDa was present in sufficient amounts in the initial DOC-extracted scallop muscle mem-
brane preparation to act as a precursor for the soluble tryptic fragments. The origin of the soluble fragments in a ∼110-kDa species was confirmed by Western blots using rabbit anti-37-kDa peptide IgG. Coincidentally, the precursor is not only close in size to the scallop SR Ca-ATPase, but in addition, like the Ca-ATPase, it resists extraction by low concentrations of DOC; however, unlike the Ca-ATPase it is not solubilized by cold nonionic detergent. The N-terminal sequence of the 37- and 16-kDa soluble fragments shows substantial similarity to a segment of the putative f loop of the well described NCX1 Na\(^{+}\)-Ca\(^{2+}\) exchanger (16). This region in the predicted structure for NCX1 has a location compatible with the presence of a site susceptible to proteolytic enzymes and the production of water-soluble fragments. Because the precursor is also an integral membrane protein with an appropriate molecular mass for a Na\(^{+}\)-Ca\(^{2+}\) exchanger, it is likely to represent an enzyme of that class located in scallop muscle membranes. Such transporters are ubiquitous in animal cells (41), and both plasma membrane and mitochondrial Na\(^{+}\)-Ca\(^{2+}\) exchangers must be present in scallop muscle. The possible overall relationships between the tryptic fragments and the putative parent molluscan Na\(^{+}\)-Ca\(^{2+}\) exchanger are shown in Fig. 9. Gel shift experiments with fusion proteins containing the regulatory Ca\(^{2+}\)-binding site of NCX1 have indicated that a large conformational change occurs in the β1-β2 region when Ca\(^{2+}\) becomes bound (18, 19). The difference in accessibility to trypsin of the region represented by the T2 cleavage site on the scallop exchanger between the Ca\(^{2+}\)-bound and Ca\(^{2+}\)-free states may be a reflection of such a refolding of the large cytoplasmic domain of the exchanger on binding and release of the ligand.

The scallop muscle Na\(^{+}\)-Ca\(^{2+}\) exchanger reported here is 105–110 kDa in size, close to that of the mitochondrial exchanger (10), but smaller than the −120 kDa of the glycolylated NCX1 plasma membrane exchanger. This observation might suggest that the precursor of the soluble tryptic fragments reported here is the scallop mitochondrial exchanger; however, oligomycin-sensitive Ca-ATPase and succinic dehydrogenase activities present in the native scallop membrane preparations (42) are removed by DOC extraction, and there is no indication from SDS gels of significant contamination of the DOC-extracted scallop membrane preparation by the F\(_{0}\)F\(_{1}\)-ATPase (Fig. 1 and Ref. 25). Even in preparations of purified beef heart mitochondria, the Na\(^{+}\)-Ca\(^{2+}\) exchanger is present in only small amounts (0.4 μg/mg total protein (24)).

There was direct evidence consistent with a sarcolemmal location for the precursor of the soluble tryptic fragments. Digestion of the DOC-extracted B1 membrane fraction (SL-enriched) with trypsin yielded soluble peptides, with more 37-kDa fragment being released in the presence of Ca\(^{2+}\) and more 19- and 16-kDa material in the presence of EGTA. The fact that treatment of the membranes with deoxycholate before digestion with trypsin greatly increased the amount of soluble fragments released supports an origin for the soluble tryptic fragments in the Ca\(^{2+}\)-regulatory binding site region of the large cytoplasmic domain (f loop). Presumably, treatment with low concentrations of DOC renders the SL permeable to trypsin and allows the protease access to the cytoplasmic loop of the exchanger on the interior aspect of the membrane. If most of the native vesicles derived from the SL in the preparation were right-side-out, a large increase in the extent of proteolysis of the cytoplasmic domain of the enzyme would be expected after DOC treatment. In fact, ∼70% of the ouabain-inhibitable Na\(^{+}\),K\(^{-}\)-ATPase activity of the scallop muscle B1 membrane fraction has to be unmasked by treatment with 0.2% (w/v) saponin. Because the large cytoplasmic domain of the Na\(^{+}\),K\(^{-}\)-ATPase, which binds the substrate ATP, is located on the inner aspect of the plasma membrane, this finding is consistent with the majority of the native membrane vesicles derived from the scallop SL being right-side-out. The fact that the insoluble residue left after extraction of the membranes with cold nonionic detergent is a good source of the tryptic fragments fits a location for this scallop Na\(^{+}\)-Ca\(^{2+}\) exchanger in some perhaps specialized domain of a cholesterol-rich membrane, such as the SL.

The presence of the precursor of the soluble tryptic fragments in both the B1 (SL-enriched) and B2 (SR-enriched) membrane fractions may be due to the difficulty in completely resolving the scallop SL and SR membrane fractions (29). This problem arises, at least in part, because in the scallop cross-striated adductor muscle, which has no transverse tubules, SR cisternae analogous to the terminal cisternae of vertebrate

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**Fig. 8.** Phosphorylation of deoxycholate-extracted scallop muscle membranes by protein kinase A leads to labeling of the 16- and 37-kDa soluble tryptic peptides but not the 19-kDa species. The deoxycholate-extracted B2 fraction was treated with PKA and [γ-32P]ATP and then digested with trypsin in the presence of Ca\(^{2+}\) or EGTA, as described under “Experimental Procedures.” The soluble peptides were concentrated by the DOC-trichloroacetic acid method and electrophoresed in the Tricine SDS gel system, and the autoradiograms were obtained from the dried gels. Lane 1, phosphorylated soluble peptides after digestion in the presence of EGTA. Lane 2, phosphorylated soluble peptides after digestion in the presence of Ca\(^{2+}\).

**Fig. 9.** Location of cleavage sites generating the tryptic fragments. The relative positions of the scallop tryptic peptides are superimposed on the putative structure of the vertebrate NCX1 Na\(^{+}\)-Ca\(^{2+}\) exchanger (16).
skeletal muscle lie directly beneath the SL (43). The two types of membrane are connected morphologically by foot-type structures resembling those associated with the ryanodine receptor complex seen in the triads of vertebrate muscle. Ryanodine-type Ca$^{2+}$ release channels have been observed in preparations of scallop muscle membranes (44). Possibly, the patches of SL overlying the junctions fulfill some of the functions of the transverse tubules of vertebrate cross-striated muscle. Disruption of the scallop muscle on homogenization may leave pieces of scallop muscle membranes (44). Possibly, the patches of SL structural linkages among the Na$^{+}$-Ca$^{2+}$ exchanger, Na$^{+}$-K$^{+}$ pump, and the Ca$^{2+}$ pump of the SR and showed that the exchanger was concentrated in caveolae of smooth muscle sarcolemma that were in close proximity to the SR. Caveolae are rich in cholesterol and insoluble in cold nonionic detergent (46).

ATP increases the affinity of the transport sites for Ca$^{2+}$ and Na$^{+}$ on the cardiac NCX1 exchanger and the squid giant axon (47). There is evidence that phosphoryl transfer is important for the action of ATP in the squid axon and that a Ca$^{2+}$-dependent protein kinase may be involved in the effect (48). However, in the case of cardiac muscle, protein kinases do not appear to be implicated (49), and a mechanism involving PIP$_2$ interaction with the XIP region may be present (50, 51). Nevertheless, a potential phosphorylation site for cAMP-dependent PKA and calcium calmodulin-stimulated protein kinase is present in the f loop of NCX1, at Ser$^{398(357)}$ in the R$^{385(352)}$KAVS sequence, just N-terminal to the Ca$^{2+}$ regulatory region (2). A possible substrate site for these kinases is present at Thr$^{113}$ in NCX3, on the putative intracellular loop between transmembrane helices 1 and 2 (4). Possible sites for phosphorylation by PKA and protein kinase C exist at Thr$^{262}$ and Thr$^{267}$ in the XIP domain of NCX2 (3, 5). All three vertebrate exchange systems contain a PKA and detected on the 16- and 37-kDa tryptic peptides. Thus, the complete absence of any phosphorylation of the exchange was concentrated in caveolae of smooth muscle sarcolemma that were in close proximity to the SR. Caveolae are rich in cholesterol and insoluble in cold nonionic detergent (46).

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