A New Topological Model of the Cardiac Sarcolemmal Na\(^+\)-Ca\(^{2+}\) Exchanger*

(Received for publication, August 26, 1998, and in revised form, October 19, 1998)

Debora A. Nicoll‡, Michela Ottolia, Liyan Lu, Yujuan Lu, and Kenneth D. Philipson

From the Departments of Physiology and Medicine and the Cardiovascular Research Laboratories, UCLA School of Medicine, Los Angeles, California 90095-1760

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Printed in U.S.A.

The current topological model of the Na\(^+\)-Ca\(^{2+}\) exchanger consists of 11 transmembrane segments with extracellular loops a, c, e, g, i, and k and cytoplasmic loops b, d, f, h, and j. Cytoplasmic loop f, which plays a role in regulating the exchanger, is large and separates the first five from the last six transmembrane segments. We have tested this topological model by mutating residues near putative transmembrane segments to cysteine and then examining the effects of intracellular and extracellular applications of sulfhydryl-modifying reagents on exchanger activity. To aid in our topological studies, we also constructed a cysteineless Na\(^+\)-Ca\(^{2+}\) exchanger. This mutant is fully functional in Na\(^+\) gradient-dependent \(^{40}\text{Ca}^{2+}\) uptake measurements and displays wild-type regulatory properties. It is concluded that the 15 endogenous cysteine residues are not essential for either activity or regulation of the exchanger. Our data support the current model by placing loops c and e at the extracellular surface and loops d, j, and l at the intracellular surface. However, the data also support placing Ser-788 of loop h at the extracellular surface and Gly-837 of loop i at the intracellular surface. To account for this data, we propose a revision of the model that places transmembrane segment 6 in cytoplasmic loop f. Additionally, we propose that putative transmembrane segment 9 does not span the membrane, but may form a “P-loop”-like structure.

The Na\(^+\)-Ca\(^{2+}\) exchanger NCX1 is a plasma membrane protein that exchanges three Na\(^+\) ions for one Ca\(^{2+}\) ion. The highest levels of exchange activity have been observed in cardiac myocytes where the NCX1.1 splice variant is expressed (1–3). NCX1.1 and other splice variants are present in a wide array of other cell types (1, 2, 4–7). In the myocyte, NCX1.1 is prominent in Ca\(^{2+}\) extrusion during cardiac relaxation (8) and may also be involved in Ca\(^{2+}\) influx during contraction (9–11). NCX1.1 has been cloned, sequenced, and expressed in Xenopus oocytes (3) and insect (12) and mammalian (13) cell lines.

The mature NCX1 proteins, formed after cleavage of a signal peptide, have been modeled to have 11 α-helical transmembrane segments. An amino-terminal hydrophobic domain (TMS1–5)\(^1\) is separated from a carboxyl-terminal domain (TMS6–11) by a long cytoplasmic loop, loop f (Fig. 1A). Segment a, at the amino terminus, has been shown to be extracellular (14, 15), and loops b (14, 16) and f (14, 17–19) have been shown to be intracellular, but the rest of the topology of NCX1 has not yet been experimentally determined.

Portions of loops b (16) and f (18) are involved in regulatory properties of the exchanger, whereas the two hydrophobic domains are important in ion binding and transport (20). The two hydrophobic domains share a repeated motif that has been designated α (21). The α-1 repeat encompasses portions of TMS2 and TMS3 in the amino-terminal domain, and the α-2 repeat encompasses portions of TMS8 and TMS9 in the carboxyl-terminal domain (Fig. 1A). The α-1 and α-2 repeats have been proposed to have similar roles in the exchange mechanism since they have similar sequences and are both modeled to be near the extracellular surface, and parallel mutations have similar effects on exchange activity (20).

The importance of cysteine residues in the exchanger has been examined previously. The activity of wild-type NCX1 expressed in Xenopus oocytes is not inhibited by application of several sulfhydryl-modifying reagents (16). Thus, accessible cysteines of NCX1 in the oocyte membrane are not critical for ion translocation. However, Pierce et al. (22) found that the exchanger, in sarcolemmal membrane vesicles, was sensitive to sulfhydryl reagents. Also, disulfide bonds are present in the exchanger as determined by gel mobility differences under reducing and nonreducing conditions in mutant (23) and wild-type (24) exchanger preparations. Finally, Reeves et al. (25) found that redox modification of the exchanger stimulated activity and suggested that the stimulation was due to a rearrangement of disulfide bonds.

We have further examined the topology of NCX1.1. Mutants with introduced cysteines were constructed and examined for sensitivity to membrane-impermeable sulfhydryl reagents. We have also examined the role of cysteine residues in the exchanger by replacing all of the cysteines and characterizing the cysteineless exchanger.

MATERIALS AND METHODS

The modified canine NCX1.1 cDNA (20) was used for all studies. Mutations were generated in 300–500-base pair cassettes using the Sculptor in vitro mutagenesis kit (Amersham Pharmacia Biotech) or the QuickChange site-directed mutagenesis kit (Stratagene). All mutations were verified by sequencing. In S875C, an additional mutation (A873T) was inadvertently generated. All other mutants contained only the intended mutation. Full-length exchangers were constructed by subcloning the mutagenized cassettes into the wild-type exchanger. The cysteineless exchanger (clone H) was constructed by a series of subclon-

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* This work was supported by National Institutes of Health Research Grant HL49101 (to K. D. P.); by a grant from the American Heart Association, Greater Los Angeles Affiliate (to M. O.); and by the Laubisch Fund. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Cardiovascular Research Lab., MRL 3645, UCLA School of Medicine, Los Angeles, CA 90095-1760. Tel.: 310-825-5137; Fax: 310-206-5777; E-mail: dnicoll@mednet.ucla.edu.

1 The abbreviations used are: TMS, transmembrane segment; MTS, methanethiosulfonate; MTSET, [2-(trimethylammonium)ethyl] methanethiosulfonate bromide; MTSES, sodium (2-sulfonatoethyl) methanethiosulfonate; MTSEA, 2-(aminoethy)l methanethiosulfonate hydrobromide.
ing and mutagenic steps. The entire clone H was verified by sequencing. In some instances, cysteines were introduced into the cysteineless background. The names of these mutants are preceded by H: (e.g. H:A792C). All mutants without the H: indication are in the wild-type background (e.g. V168C).

cRNA was synthesized using mMessage mMachine (Ambion Inc.) and injected into Xenopus oocytes. Na\(^{+}\)-Ca\(^{2+}\) exchange activity was measured as Na\(^{+}\) gradient-dependent uptake of 45Ca\(^{2+}\) into oocytes as described (16). To determine the effects of MTS reagents (Toronto Research Chemicals) on exchange activity, the oocytes were preincubated for 10 min with the reagent in the bathing medium or injected with MTS reagents at a final concentration of \(10 \text{ mM}\) as described previously (16). All data are presented as means \(\pm\) S.E.

Na\(^{+}\)-Ca\(^{2+}\) exchange activity was also examined as outward exchange current using the inside-out giant patch technique (26). Borosilicate glass pipettes were pulled, fire-polished to a final inner diameter of \(20–30 \mu\text{m}\), and coated with a mixture of Parafilm and mineral oil. To excise a membrane patch, the oocyte vitelline membrane was first removed manually in an isotonic solution identical to the solution for seal formation (110 mM KCl, 10 mM HEPES, 2 mM CaCl\(_2\), and 2 mM MgCl\(_2\), pH 7). After membrane excision, solutions were rapidly changed using a computer-controlled 20-channel solution switcher. Recordings of the exchange current were obtained using the following solutions: pipette solution (100 mM N-methylglucamine, 20 mM HEPES, 20 mM tetaethylammonium hydroxide, 8 mM Ca(OH)\(_2\), 0.1 mM niflumic acid, and 0.15 mM ouabain, pH 7 (using methanesulfonic acid)) and bath solution (100 mM CsOH or NaOH, 20 mM tetaethylammonium hydroxide, 20 mM HEPES, 10 mM EGTA, and 0 or 5.75 mM Ca(OH)\(_2\) (0 or 1 \(\mu\)M free Ca\(^{2+}\)), pH 7 (using methanesulfonic acid)). Solutions with higher free Ca\(^{2+}\) concentration were prepared by adding 10 \(\mu\)M Ca(OH)\(_2\) in the absence of EGTA. Calcium concentrations were calculated according to the Chelator program (27).

PClamp software was used for data acquisition and analysis. Data were acquired on line at 4 ms/point and filtered at 50 Hz using an 8-pole Bessel filter. Experiments were performed at 35 °C and a holding potential of 0 mV.

RESULTS

Stimulation of the Wild-type Exchanger by Intracellular MTS-SET—The strategy for examining the topology of the exchanger consisted of introducing cysteine residues at sites modeled to be near the membrane surface. These residues might be accessible to sulfhydryl reagents, and modification of the residues might have functional consequences. Mutant exchangers were expressed in Xenopus oocytes, and the effects of MTS sulfhydryl-modifying reagents on activity were examined. The reagents MTSET (positively charged), MTSES (negatively charged), and MTSEA (neutral and positively charged species in equilibrium) were used. Oocytes were either incubated in or injected with the MTS reagent to determine if the introduced cysteine was reactive with extracellular or intracellular reagent, respectively. This strategy was previously used to map loop b of the exchanger to the intracellular surface (16). Although the wild-type exchanger contains 15 native cysteines, this approach was possible because the wild-type exchanger, expressed in Xenopus oocytes, is not inhibited by application of any of the sulfhydryl reagents used in this study (16). This is demonstrated in Fig. 2A for MTSET, MTSES, and MTSEA.

However, as noted previously (16), the wild-type exchanger expressed in oocytes is sometimes stimulated by intracellular
application of MTSET (Fig. 2A). The extent of stimulation is a function of the level of exchange activity expressed in the oocyte (Fig. 2B). When the activity is low (<20 pmol/oocyte/10 min), stimulation is inversely related to the level of activity. At high levels of activity, stimulation is low or nonexistent. Stimulation is at least partially reversible by treating cells with dithiothreitol (Fig. 2C) and requires expression of a functional exchanger since oocytes injected with nonfunctional mutant exchangers do not display exchange activity following injection of MTSET (data not shown).

Stimulation is not restricted to intracellular MTSET. Both MTSES and MTSEA also sometimes stimulate the exchanger. Stimulation by these reagents has most often been observed with mutants when low levels of activity were expressed. For example, mutant Y184C is stimulated by intracellular MTSEA (Fig. 2D). The extent of stimulation by MTSEA appears to follow the same relationship as seen for the extent of stimulation of the wild-type exchanger by MTSET (Fig. 2B, closed circles).

The Cysteineless Exchanger—Although the wild-type exchanger is not inhibited by sulphydryl-modifying reagents when expressed in oocytes, it is still possible that mutants with introduced cysteine residues have altered conformations that result in increased accessibility of native cysteines. Thus, further aid in topological analysis and also to determine the importance of native cysteine residues, a cysteineless exchanger was constructed. Initially, each individual cysteine residue (except Cys-383 and Cys-387, which were mutated together) was mutated to an alanine to determine if any single cysteine is crucial to exchange activity. In all cases, except for Cys-730, the cysteine-to-alanine mutation resulted in a functional exchanger (Fig. 3). Although mutant C730A was inactive, the exchanger did tolerate a serine residue at this position. Two of the cysteine mutants (C20A and C792A) expressed ~50% of wild-type activity. These two residues have been shown to form a disulfide bond (23). Two other mutants (C383A,C387A and C933A) expressed ~150% of wild-type activity. Different levels of activity may reflect the levels of expression at the cell surface or altered turnover rates.

Thus, the cysteineless exchanger contained a serine at position 730 and an alanine at all other cysteine positions. This construct is designated HH, and the names of any mutants in the cysteineless background are preceded by H (e.g. H:G837C). The cysteineless exchanger exhibits near wild-type levels of exchange activity (Fig. 4) as measured by 45Ca2+ uptake. Like the wild-type exchanger, it was sometimes stimulated by intracellular MTSET application (Fig. 2D). The amount of stimulation of the cysteineless exchanger by intracellular MTSET was also related to the level of exchanger activity (data not shown).

To further compare the cysteineless and wild-type exchangers, electrophysiological properties were studied using the giant patch technique (26). The characteristics of the cysteineless Na+-Ca2+ exchange current were similar to those of the wild-type current measured under the same conditions (Fig. 5). Application of cytoplasmic Na+, when Ca2+ (8 mM) was present in the pipette, elicited an outward current as the applied Na+ exchanged with pipette Ca2+. The exchange current peaked and then decayed with time (left traces). The decay process is known as Na+-dependent inactivation (28).

Fig. 5 (right traces) demonstrates that the wild-type and cysteineless exchangers display normal regulation by nontransported cytoplasmic Ca2+ (29). For both exchangers, the peak currents increased when the cytoplasmic Ca2+ concentration was raised to 15 μM; the currents declined to zero when the regulatory Ca2+ was removed from the bath solution; and Na+-dependent inactivation was attenuated by the high internal Ca2+ concentrations. When the regulatory Ca2+ concentration was increased from 1 to 15 μM, inactivation was virtually eliminated. The small level of inactivation seen in the trace for the cysteineless exchanger was seen occasionally with both the wild-type and cysteineless exchangers. These effects of regulatory Ca2+ on the exchange activity and inactivation process are characteristic of NCX1 (29). These findings have been verified.
in another laboratory. Thus, the primary biophysical characteristics of the exchanger were maintained in the cysteineless mutant.

The Amino-terminal Hydrophobic Domain of the Exchanger: Evidence in Support of the Model—Cysteines that were introduced at five different sites were useful in determining the topology of the amino-terminal portion of the exchanger. These were cysteines introduced at positions 135, 138, 168, 184, and 188 (H:T135C, G138C, V168C, Y184C, and S188C, respectively). Position 135 is modeled to be in extracellular loop c between TMS2 and TMS3, and position 138 is modeled to be near the interface of loop c and TMS3. Position 168 is modeled to be at the intracellular interface of loop d and TMS4, and positions 184 and 188 are modeled to be near the extracellular interface of TMS4 and loop e (Fig. 1A).

Exchange activity was present in mutants with cysteine introduced in loop c, d, or e, although mutations in loop c significantly reduced activity. As determined by Na$^+$ gradient-dependent uptake of $^{45}$Ca$^{2+}$ into oocytes expressing each of the mutants was determined (see “Materials and Methods”), and the activity was normalized to wild-type activity. The number of experiments performed is indicated by the number at the bottom of each column.

To examine the topological distribution of the introduced cysteine residues, the effects of membrane-impermeant sulfhydryl reagents on the activity of mutants H:T135C, G138C, V168C, Y184C, and S188C were examined. Mutants H:T135C, G138C, and S188C were inhibited by extracellular applications of the sulfhydryl reagent MTSET (Fig. 6). A modest inhibition of the activity of mutants H:T135C and G138C by intracellular applications of the sulfhydryl reagent MTSET was observed. When cysteine was added to the bathing medium during injection to scavenge leaked MTSET, the inhibition was relieved (data not shown; see “Discussion”). Y184C was inhibited only by extracellular MTSEA. In contrast, mutant V168C was inhibited by intracellular (but not extracellular) application of MTSET or MTSES. Hence, loop c appears to be extracellular and loop d intracellular, as previously modeled. Y184C and S188C were stimulated by intracellular MTS reagents in addition to being inhibited by extracellular reagents. Nevertheless, we conclude that loop e is extracellular, as previously modeled (see “Discussion”).

The Carboxyl-terminal Hydrophobic Domain: Evidence for a New Model—Ser-875 is modeled to be in TMS10 near intracellular loop j, and Gly-870 is modeled to be in loop j (Fig. 1). Mutants containing cysteines at these positions displayed near wild-type levels of exchange activity (Fig. 4). S875C was inhibited by intracellular (but not extracellular) application of MTSEA. In contrast, mutation of another residue in loop e (D157C) resulted in wild-type levels of activity.

To examine the topological distribution of the introduced cysteine residues, the effects of membrane-impermeant sulfhydryl reagents on the activity of mutants H:T135C, G138C, V168C, Y184C, and S188C were examined. Mutants H:T135C, G138C, and S188C were inhibited by extracellular applications of the sulfhydryl reagent MTSET (Fig. 6). A modest inhibition of the activity of mutants H:T135C and G138C by intracellular applications of the sulfhydryl reagent MTSET was observed. When cysteine was added to the bathing medium during injection to scavenge leaked MTSET, the inhibition was relieved (data not shown; see “Discussion”). Y184C was inhibited only by extracellular MTSEA. In contrast, mutant V168C was inhibited by intracellular (but not extracellular) application of MTSET or MTSES. Hence, loop c appears to be extracellular and loop d intracellular, as previously modeled. Y184C and S188C were stimulated by intracellular MTS reagents in addition to being inhibited by extracellular reagents. Nevertheless, we conclude that loop e is extracellular, as previously modeled (see “Discussion”).

* D. W. Hilgemann, personal communication.
one of the exchanger that places loop j at the intracellular surface. In support of an intracellular disposition of the carboxyl terminus, mutation of the carboxyl-terminal amino acid Phe-938 to serine is modeled to be in loop h near the intracellular side of TMS7. However, when a cysteine residue was introduced at position 788 in either the cysteineless (H:S788C) or wild-type (S788C) background, the mutant exchanger was inhibited by intracellular (and not extracellular) application of MTSET (Fig. 6). This suggests that if MTSEA permeates the oocyte membrane, it does so at a low rate. Mutants S875C and H:F938C were sensitive to intracellular applications of MTS reagents, but extracellular applications had very little effect. Each of the exchangers with a mutation in an extracellular loop was sensitive to intracellular applications of MTS reagents, but extracellular applications had very little effect. Each of the exchangers with a mutation in an extracellular loop was sensitive to extracellular applications of MTS reagents. Mutants in extraacellular loop c were also mildly inhibited by intracellular application of MTS reagents (Fig. 6). The inhibition by extracellular MTS reagents appears to be due to leakage of the reagent during injection since including extracellular cysteine during injection removed the sensitivity (data not shown).

Three extracellular loops (c, e, and h) and four intracellular loops (d, i, j, and l) were localized in this study. Each of the exchangers with a mutation in an extracellular loop was sensitive to intracellular applications of MTS reagents, but extracellular applications had very little effect. Each of the exchangers with a mutation in an extracellular loop was sensitive to extracellular applications of MTS reagents. Mutants in extracellular loop c were also mildly inhibited by intracellular application of MTS reagents (Fig. 6). The inhibition by intracellular MTS reagents appears to be due to leakage of the reagent during injection since including extracellular cysteine during injection removed the sensitivity (data not shown).

A way to test if a hydrophobic region is likely to be a transmembrane segment is by alanine insertion mutagenesis. It has been observed that inserting single amino acids near the central core of transmembrane helices in lactose permease disrupts protein function (30). Three mutants were constructed with an alanine inserted in different hydrophobic sections between loop f and Ser-788. Mutant 701iA had an alanine inserted after residue 701 in a short hydrophobic region of loop f. Mutants 749iA and 774iA had alanines inserted into the middle of putative TMS6 and TMS7, respectively. Both mutants 701iA and 749iA expressed Na\(^{+}\)-Ca\(^{2+}\) exchange activity (n = 2), whereas no activity was observed in oocytes injected with RNA for mutant 774iA (n = 6; data not shown). This suggests that TMS7, but not putative TMS6, is a transmembrane segment.

Gly-837 is modeled to be at the interface of extracellular loop i and TMS9 of the α2 repeat (Fig. 1). To examine the topology of loop i, mutant G837C was constructed and analyzed by sulphydryl modification. This mutant displayed a decreased level of expression relative to the wild-type exchanger (Fig. 4). Mutant G837C was inhibited by intracellular application of either MTSET or MTSES (Fig. 6). Thus, it appears that Gly-837 is located at the intracellular rather than the extracellular surface, as modeled.

To verify that sulphydryl modification occurred at the cysteine introduced at position 837, and not at a wild-type cysteine that may have become accessible following mutagenesis, the G837C mutation was also introduced into the cysteineless background (H:G837C). Mutant H:G837C expressed low exchange activity (~10–20% of wild-type activity), but was still inhibited by intracellular (and not extracellular) application of MTSES (n = 2).

**DISCUSSION**

The Technique—In this study, 66 amino acid residues were mutated to cysteine. Most mutants, 47, were uninformative in preliminary experiments with MTS reagents, and 9 of the mutants did not express exchange activity. These mutants were not studied further. Only 10 mutations proved useful for determining the topology of the exchanger. The probability of successfully choosing a residue to mutate to cysteine may correlate with how well the exchanger was initially modeled. In the N-terminal half, where the data support the initial model, 5 of 20 mutants were useful. In the C-terminal half, only 5 of 49 mutants were useful.

This technique relies on the permeability characteristics of the sulphydryl reagents. We assume that MTSET, MTSES, and MTSEA, which are predominantly present as charged forms at physiologic pH, are impermeable. However, it has been reported that the reagent MTSEA is membrane-permeable (31). Our results suggest that if MTSEA permeates the oocyte membrane, it does so at a low rate. Mutants S875C and H:F938C were sensitive to MTSEA only when injected into the oocyte. Incubating the oocyte in a solution containing MTSEA had no effect (Fig. 6). If the oocyte membrane was highly permeable to MTSEA, then preincubation would also inhibit exchange activity.

Three extracellular loops (c, e, and h) and four intracellular loops (d, i, j, and l) were localized in this study. Each of the exchangers with a mutation in an extracellular loop was sensitive to intracellular applications of MTS reagents, but extracellular applications had very little effect. Each of the exchangers with a mutation in an extracellular loop was sensitive to extracellular applications of MTS reagents. Mutants in extracellular loop c were also mildly inhibited by intracellular application of MTS reagents (Fig. 6). The inhibition by intracellular MTS reagents appears to be due to leakage of the reagent during injection since including extracellular cysteine during injection removed the sensitivity (data not shown). Loop e mutants Y184C and S188C were stimulated by intracellular application of MTS reagents. The stimulation appears to be

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*Additional residues mutated to cysteine (or reintroduced into the cysteineless background) were as follows: Ala-43, Ser-58, Asp-62, Ala-106, Ser-117, Glu-120, Cys-122, Asp-157, Thr-172, Ser-190, Glu-196, Asp-218, Asp-740, Tyr-741, His-744, Lys-751, Pro-758, Tyr-762, Trp-763, Asn-784, Gly-785, Trp-786, Asp-785, Lys-786, His-789, Phe-790, Gly-791, Cys-792, Thr-793, Ile-794, Gly-809, Val-804, Gly-809, Thr-815, Lys-819, Ala-821, Ala-822, Thr-823, Asp-825, Tyr-827, Asp-829, Ser-831, Val-835, Thr-836, Asp-839, Ala-845, Asp-855, Thr-857, His-858, Thr-871, Tyr-892, Arg-893, Lys-909, Tyr-923, Phe-926, and Ser-927 (boldface indicates mutants with no activity).
similar to the stimulation of the wild-type exchanger (see below) since the extent of stimulation correlates with the level of expressed activity (Fig. 2B, closed circles). However, we cannot rule out other possible explanations for this stimulation such as accessibility of the residues from both sides of the membrane.

**Effect of Intracellular MTSET Applications on the Wild-type Exchanger**—Injections of MTSET into oocytes expressing the wild-type exchanger sometimes stimulated exchanger activity by as much as 5-fold (Fig. 2, A and B). Some of the other mutants examined were also stimulated by MTSET and other MTS reagents. For example, mutants Y184C and S188C were stimulated by intracellular MTSEA and MTSET, respectively (Fig. 6). A similar effect was previously seen with the MTS reagent methyl methanethiosulfonate on exchanger activity in sarcosomal membranes (22). The extent of stimulation by intracellular MTS reagents correlates with the level of exchange activity expressed in the oocytes (Fig. 2B). At low exchange activity, the stimulation was greater than at high levels of activity. The stimulation of activity appears to take place via chemical modification of a free sulfhydryl since it can be at least partially reversed by reduction with dithiothreitol (Fig. 2C). However, the stimulation does not occur through direct modification of an exchanger sulfhydryl residue since it is also observed with the cysteineless exchanger (Fig. 2D).

Perhaps there is an exchanger inhibitory factor that is present at low concentrations and that contains a free sulfhydryl to react with MTS reagents and to relieve inhibition of the exchanger. Thus, when expression of the exchanger is low, all exchangers would be inhibited by interaction with the factor. MTSET would relieve inhibition and cause stimulation of exchange activity. At higher levels of exchanger expression, the concentration of the exchanger would exceed that of the factor. Addition of MTSET would relieve inhibition of only a small fraction of the exchangers and therefore would not significantly increase activity.

**The Cysteineless Exchanger**—The cysteineless exchanger, despite having 15 mutations, has characteristics similar to the wild-type exchanger. Similar levels of $[^{45}\text{Ca}^2+]_{\text{uptake}}$ were observed for both the wild-type and cysteineless exchangers (Fig. 4). However, the level of current recorded from oocytes expressing the cysteineless mutant was usually less than that of the wild-type current (data not shown). It is difficult to compare the activity measured with the two different methods, and the explanation for this discrepancy is unclear. The electrophysiological studies indicate that the cysteineless exchanger shares the biophysical properties of the wild-type exchanger (Fig. 5). The cysteineless exchanger current is activated by Na$^+$ and shows Na$^+$-dependent inactivation and Ca$^{2+}$-regulation. Thus, there appears to be no absolute requirement for any of the endogenous cysteines in ion transport or regulation, although more detailed characterization may indicate more subtle effects. In some cases where cysteines have been introduced into wild-type and cysteineless backgrounds, slight differences are seen. For example, mutant H:G837C expresses lower levels of exchange activity than mutant G837C. Also, mutant S788C appears to be less sensitive to extracellular MTSET than mutant H:S788C (Fig. 6).

For the exchangers with individual cysteines replaced by alanine, only mutants C20A, C792A, and, to a lesser extent, C485A had reduced activity, whereas only mutant C933A and double mutant C833A,C837A had stimulated activity. Reeves et al. (25) previously found that the exchanger is stimulated by the simultaneous presence of both a reductant and an oxidant. They proposed a mechanism for redox modulation that involves the rearrangement of disulfide bonds. By gel shift analysis, it has been shown that Cys-792 forms a disulfide bond with either Cys-20 or Cys-14 (23). These residues (Cys-14, Cys-20, and Cys-792) may be involved in redox stimulation, although other residues such as Cys-383, Cys-387, and/or Cys-933 may also have a role.

The only position where cysteine could not be replaced by an alanine was at 730, where there was no exchange activity following a cysteine-to-alanine mutation. Wild-type levels of activity were observed, however, when the cysteine was mutated to the more chemically conserved serine. This cysteine is in the middle of an extremely hydrophilic region consisting of one basic and nine acidic residues in a stretch of 12 residues, and it would appear that the somewhat hydrophilic nature of the cysteine must be maintained at this location.

**A New Model for NCX1**—The initial model for the Na$^+$-Ca$^{2+}$ exchanger (Fig. 1, A and B) was constructed on the assumption that stretches of $−20$ consecutive hydrophobic amino acids in the primary sequence form transmembrane $α$-helices. Also, since the regions designated $α-1$ and $α-2$ share significant sequence similarity, it seemed reasonable that they would share similar structure and transmembrane disposition. With this model in mind, we introduced cysteine residues into locations modeled to be near the membrane surface and examined the effects of membrane-impermeable sulfhydryl-modifying reagents on exchange activity. The results support the dispositions of loops c–e in the N-terminal half and loops j and l in the C-terminal half.

Based on the results presented here as well as previous results from several laboratories, we propose the modified exchanger model presented in Fig. 1B. The most prominent feature of the modified model is that the C-terminal half is significantly altered, whereas the N-terminal half remains unchanged.

The N terminus, comprising segment a, is clearly extracellular. The exchanger mRNA encodes for a signal peptide that is cleaved (15, 32), leaving the N terminus outside. Also, Asn-9 is glycosylated (15), further supporting an extracellular disposition. Finally, following introduction of a FLAG epitope after Gly-8 of rat brain NCX1.5, the exchanger can be detected by extracellular application of anti-FLAG antibody (14).

There are three pieces of evidence that support the intracellular location of loop b. First, mutants S100C and L102C are...
both sensitive to intracellular application of intracellular MTS reagents (16). Second, mutant N101C displays altered regulatory properties (16). The exchanger regulatory properties are manifested at the intracellular surface, suggesting that Asn-101 is also inside. Third, an antibody raised against a loop β peptide of rat NCX1.5 binds only to permeabilized cells expressing NCX1.5, suggesting that the epitope is intracellular (14).

In this work, we have identified the locations of loops c–e. Loop c is extracellular since mutants H:TI35C and G138C are inhibited by extracellular application of MTS reagents (Fig. 6), and loop d is intracellular since mutant V168C is inhibited by intracellular application of MTS reagents. Loop e is extracellular since mutants Y184C and S188C are inhibited by extracellular MTS reagents. This conclusion for loop e is not unequivocal since mutants Y184C and S188C were also stimulated by intracellular reagents. We suspect that the stimulation is related to the stimulatory phenomenon also sometimes seen with the wild-type and cysteineless exchangers (Fig. 2). Nevertheless, an extracellular location for loop e has also been strongly implied by the work of Sahin-Töth et al. (33), in which glycosylation was observed at a site introduced on an exchanger truncated in loop e.

There are a number of data that support intracellular placement of loop f. The peptide XIP (exchanger inhibitory peptide), corresponding to a portion of loop f, inhibits the exchanger at the intracellular surface (17). Deletion and single site mutants of loop f modify intracellular regulatory properties (18). Also, antibody epitopes on loop f have been mapped to the intracellular surface (14, 19).

Hence, the initial model for the N-terminal half of the exchanger, consisting of five transmembrane segments, is supported by many data. There are, however, no data to indicate the structure of the transmembrane segments (α-helical, β-sheet, or other), nor have the exact beginnings and ends of transmembrane segments been determined.

In the C-terminal half of the exchanger, the results with two mutants, S788C in loop h and G837C in loop i, were not as predicted from the model. S788C was modeled to be at the intracellular surface, but is sensitive to extracellular MTSET, and G837C was modeled to be extracellular, but is sensitive to intracellular MTSET and MTSES. We have also found that Cys-792 in loop h of the wild-type exchanger is involved in disulfide bond formation with a cysteine in extracellular segment a (23), further supporting an extracellular disposition of loop h. Based on these results, TMS8 must actually be in the opposite orientation to that in the initial model (Fig. 1). This conclusion indicates the need for alterations to the model. First, the exchanger protein must traverse the membrane an odd number of times between loop f and residue 788. Assuming only α-helical transmembrane segments, then either TMS6 or TMS7 does not cross the membrane, or there is a third transmembrane segment between loop f and residue 788.

We have tentatively remodeled the exchanger by removing TMS6, but keeping TMS7 in the membrane (Fig. 1B). There are not likely to be any transmembrane segments in loop f between residues 240 and 685 since an exchanger with these residues deleted is functional (18). Between residues 685 and 788, there are only three hydrophobic stretches, two of which are putative TMS6 and TMS7 and the third of which is a short stretch including residue 701. Inserting an alanine into the middle of putative TMS6 or after residue 701 does not affect exchange activity, but inserting an alanine into putative TMS7 disrupts exchange activity. This suggests that TMS7 does traverse the membrane. Alanine insertion mutagenesis has not yet been extensively used to identify transmembrane segments, and our assignment of TMS6 and TMS7 based on this technique is not unequivocal. Additionally, however, TMS6 contains three hydrophilic residues (Asp-740, His-744, and Lys-751) that might be functionally important if in a transmembrane segment, and yet mutation of each of these residues does not disrupt exchange activity (20).

The second modification that must be made to the model is to account for residues 837 and 870 both being on the intracellular side of the exchanger. Thus, the protein either must not traverse the membrane or does so an even number of times between those two residues. Since only 32 amino acids separate residues 837 and 870, this section of the exchanger cannot comprise two α-helical transmembrane segments. However, TMS9 may exist in an alternative conformation that allows it to enter the membrane. We have speculatively modeled TMS9 in this manner (Fig. 1B) since it contains the sequence GIG immediately following the α-2 repeat. This is similar to the signature GYG sequence in the pore-forming “P-loop” region of potassium channels (34).

The results with G870C, S875C, and H:F938C support placing loops j and l on the intracellular side. Cook et al. (14) have also suggested that the C terminus (loop l) is intracellular. Loop k is the only extracellular loop in the modified model for which no topological data are available. The amino acid sequence of loop k actually suggests that it is not extracellular. Five of the 15 residues in loop k are basic (lysine and arginine). Membrane-spanning sections of proteins generally have a net positive charge on the cytoplasmic side and few arginines or lysines on the extracellular side (35). Thus, the existence of TMS10 and TMS11 is not yet verified.

One interesting aspect of the modified model of the exchanger is that the α-repeat regions face opposite sides of the membrane (Fig. 1B). Gly-138 in the α-1 repeat is at a position equivalent to Gly-837 in the α-2 repeat, and yet mutant G138C is accessible to extracellular and G837C to intracellular solutions. The exchanger therefore appears to be more like the aquaporins (36) in that the repeated domains have opposite orientations compared with the ion channels, which contain repeated domains on the same side of the membrane (34). Perhaps the α-repeats form two separate ion interaction domains on opposite sides of the membrane or perhaps act in concert to form a single ion interaction domain. In either case, the protein environment that an ion senses on one side of the membrane might resemble the environment it senses on the other side.

Also of note in the new model of the exchanger is that the apparent structure near the α-repeats is different. In the vicinity of the α-1 repeat, the polypeptide chain apparently traverses the membrane twice to form TMS2 and TMS3. In the vicinity of the α-2 repeat, the polypeptide chain crosses the membrane only once to form TMS8, and then the chain either may loop in and out of the membrane as shown or does not reenter the membrane. The α-repeats may form part of the ion-binding sites, and speculatively, the GIG motif forms a hairpin loop that serves as a pivot for conformational changes, allowing for alternating access to the ion-binding sites.

Acknowledgment—We are grateful to Dr. Scott John for comments on this manuscript.

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Topology of the Na\textsuperscript{+}-Ca\textsuperscript{2+} Exchanger

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