Functional Analysis of a Disulfide Bond in the Cardiac Na⁺-Ca²⁺ Exchanger*

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The electrophoretic mobility of the cardiac Na⁺-Ca²⁺ exchange protein is different under reducing and non-reducing conditions. This mobility shift is eliminated in a cysteine-less exchanger, suggesting that the presence or absence of an intramolecular disulfide bond alters the conformation and mobility of the exchanger. Using cysteine mutagenesis and biochemical analysis, we have identified the cysteine residues involved in the disulfide bond. Cysteine 792 in loop h of the exchanger forms a disulfide bond with either cysteine 14 or 20 near the NH₂ terminus. Because the NH₂ terminus is extracellular, the data establish that loop h must also be extracellular. A rearrangement of disulfide bonds has previously been implicated in the stimulation of exchange activity by combinations of reducing and oxidizing agents. We have investigated the role of cysteines in the stimulation of the exchanger by the combination of FeSO₄ and dithiothreitol (Fe-DTT). Using the giant excised patch technique, we find that stimulation of the wild type exchanger by Fe-DTT is primarily due to the removal of a Na⁺-dependent inactivation process. Analysis of mutated exchangers, however, indicates that cysteines are not responsible for stimulation of the exchange activity by Fe-DTT. Ca²⁺ blocks modification of the exchanger by Fe-DTT. Disulfide bonds are not involved in redox stimulation of the exchanger, and the modification reaction is unknown. Modulation of Na⁺-dependent inactivation may be a general mechanism for regulation of Na⁺-Ca²⁺ exchange activity and may have physiological significance.

The cardiac Na⁺-Ca²⁺ exchanger (NCX1.1; Ref. 1) is a membrane protein that plays a key role in Ca²⁺ homeostasis in mammalian myocardium. It is the main mechanism for Ca²⁺ extrusion during diastole in cardiomyocytes (2). The exchanger uses the Na⁺ gradient generated by the Na⁺,K⁺-ATPase to couple the extrusion of one Ca²⁺ ion in exchange for three Na⁺ ions. Topological models based on hydrophathy analysis and experimental data propose nine TMSs arranged in two clusters separated by a large intracellular loop (3, 4). The NH₂ terminus is extracellular (5), whereas the COOH terminus is intracellular (3, 4, 6). The NH₂-terminal cluster contains TMSs I–5, with a possible reentrant loop between TMSs 2 and 3 (4), whereas the COOH-terminal cluster is proposed to have four TMSs with a possible reentrant loop between TMSs 7 and 8 (3). The large intracellular loop (loop f) together with the loop connecting the first two transmembrane segments (loop b) are important for exchanger regulation (7–11). TMSs 2–3 and 8–9 display intramolecular homology and are referred to as the a₁ and a₂ repeats, respectively (12). The a repeats are proposed to face opposite sides of the membrane and may be part of the ion translocation pathway (3, 4, 13).

Na⁺-Ca²⁺ exchanger activity is sensitive to treatments with combinations of redox agents (14). The activity of the protein is enhanced, for example, after exposing preparations of cardiac sarcolemmal vesicles to DTT and FeSO₄. Similar experiments using the sulphydryl-specific reagents glutathione and glutathione disulfide also up-regulated exchanger activity. It was proposed that the molecular mechanism underlying this up-regulation involved a thiol-disulfide interchange in the exchanger protein.

The apparent molecular mass of purified exchanger protein from canine cardiac sarcolemma varies on SDSPAGE depending on conditions (15). Exchanger protein treated with the reducing agent DTT appears as two bands of molecular masses 70 and 120 kDa (and a faint 140-kDa band). The 120-kDa band corresponds to the mature protein, whereas the 70-kDa band is an active proteolytic fragment (15). Under nonreducing conditions, the apparent molecular mass of the protein shifts from 120 to 160 kDa. The origin of the 160-kDa band is not clearly understood. However, such differences in migration properties are consistent with the presence of an intramolecular disulfide bond.

Here we report the characterization of an intramolecular disulfide bond in the cardiac Na⁺-Ca²⁺ exchanger protein (NCX1.1). We have identified the cysteine residues involved in this disulfide bond and have analyzed the role of disulfide bonds in exchanger function and regulation.

MATERIALS AND METHODS

Generation of Mutant Exchangers—Mutants with cysteines reintroduced into the Cys-less background are denoted with the introduced cysteine only (e.g. C14 or C14/C20). Mutants in the wild type background are denoted with the number of the residue and the amino acid substituted (e.g. C14A or C14A/C20A). All mutations were generated as described previously (3). C14/C20 was generated by replacing the BamHI-NcoI fragment of the Cys-less exchanger with the BamHI-NcoI fragment from the wild type NCX1.1 cDNA. Constructs C14 or C20 were generated by replacing the BamHI-NcoI fragment with the dithiothreitol; NEM, N-ethylmaleimide; IAA, iodoacetimide; PAGE, polyacrylamide gel electrophoresis; MES, 4-morpholineethanesulfonic acid.

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BanHI-NcoI fragment isolated from mutants C20A or C14A, respectively. The C792 construct was created by subcloning the Nsi-KpnI fragment from mutant C786A into the Cys-less background.

Expression of Exchanger Proteins—cRNA was prepared using the mMessage mMachine in vitro RNA synthesis kit (Ambion) and injected into Xenopus laevis oocytes. All constructs were assayed for functional expression by measuring Na\textsuperscript{+}-dependent Ca\textsuperscript{2+} uptake as described by Doering et al. (10).

Electrophoresis of Exchanger Proteins—48–72 h after cRNA injection, 10 oocytes were incubated in 1 ml of Barth’s solution supplemented with 10 mM IAA or NEM for 20 min at room temperature. Oocytes were transferred to Eppendorf tubes and suspended in 100 μl of homogenization buffer (phosphate-buffered saline supplemented with 1% Triton X-100 and 25 mM IAA or NEM). The samples were briefly homogenized using a bath sonicator, and the homogenate was centrifuged in an Eppendorf microfuge for 10 min. The supernatant was transferred to a Spin X-filter (Costar) and centrifuged again for 5 min at top speed. 15 μl of sample buffer (6% SDS (w/v), 125 mM Tris-HCl, pH 6.8, 20% v/v glycerol) containing 10% (v/v) 2-mercaptoethanol or 25 mM NEM were added to 30 μl of the filtrate. The 2-mercaptoethanol or NEM were added from stock solutions to the sample buffer immediately before use. 30 μl of sample were subjected to electrophoresis on discontinuous 7.5% SDS-polyacrylamide gels (15).

Western Blots—Proteins in SDS-PAGE gels were transferred to nitrocellulose membranes (Bio-Rad). Blots were probed with exchanger-specific monoclonal antibodies (C2C12) as described previously (16). Western blots signals were detected by chemiluminescence using an ECL kit (Amersham Pharmacia Biotech; see Figs. 2A and 3B) or by the color development of horseradish peroxidase/diaminobenzidine (Ref. 5; see Figs. 1 and 2B).

Electrophysiology—Outward Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange currents were recorded using the inside-out giant patch technique (17). Borosilicate glass pipettes were pulled and fire-polished to a final inner diameter of about 20–30 μm and coated with a mixture of paraffin and mineral oil. To excise a membrane patch, the vitelline membrane of the oocyte was manually removed in an isotonic solution also present during seal formation (110 mM KCl, 10 mM HEPES, 2 mM MgCl\textsubscript{2}, pH 7). After membrane excision, solutions were rapidly changed using an automated 20-channel solution switcher. Recordings of exchange current were obtained using the following solutions: pipette solution, 100 mM N-methylglucamine, 20 mM HEPES, 20 mM tetraethylammonium hydroxide, 0.1 mM niflumic acid, 0.05 mM ouabain, 8 mM Ca(OH)\textsubscript{2}, 20 mM CsOH, pH 7 (using MES); or bath solution, 100 mM CsOH or NaOH, 20 mM tetraethylammonium hydroxide, 20 mM HEPES, 10 mM EGTA, 20 mM CsOH, 0 or 5.75 mM Ca(OH)\textsubscript{2} (0 or 1 μm free Ca\textsuperscript{2+}), pH 7 (using MES). A high Ca\textsuperscript{2+} concentration was prepared by adding 10 μM Ca(OH)\textsubscript{2} in the absence of EGTA. Ca\textsuperscript{2+} concentrations were calculated according to the Chelator program (18).

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

RESULTS

The apparent molecular mass of the protein remains at 120 kDa.

The shift in the electrophoretic mobility for the wild type exchanger under reducing and nonreducing conditions can thus be explained by the presence of an intramolecular disulfide bond. When such a bond is present, the protein may not denature fully, and its mobility is altered compared with the reduced and more denatured protein. To identify the residues involved in the mobility shift, we analyzed mutant exchanger proteins with each of the 15 cysteine residues replaced.

Functional Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange activity is still present after replacement of any one or more of the 15 native cysteine residues (3). We examined the electrophoretic mobility characteristics of cysteine mutants expressed in Xenopus oocytes. The effects of removal of each of the cysteines was tested. The results can be summarized as follows (Fig. 2). The mobility shift to 160 kDa under nonreducing conditions, indicative of the formation of a disulfide bond is eliminated by mutation of either cysteine 792 or by mutation of both cysteines 14 and 20. Thus, mutants C792A, C14A/C20A, and C14A/C20A/C792A all give rise to exchanger proteins that have electrophoretic mobilities identical to those of the Cys-less exchange protein (Fig. 2A). The apparent molecular mass for these exchangers under both reducing and nonreducing conditions is 120 kDa. Mutation of either cysteine 14 or cysteine 20 alone gives rise to proteins that behave like the wild type exchanger (Fig. 2A).

Thus, the data indicate that the mobility shift is due to the formation of a disulfide bond between cysteine 792 and either cysteine 14 or 20. In the mutant C14A, a disulfide bond between cysteines 20 and 792 is present, whereas with C20A, the disulfide is between cysteines 14 and 792. The presence of either disulfide bond induces the mobility shift to 160 kDa.

The disulfide-sensitive mobility shift was not affected by replacement of any other cysteine residues. Representative results are shown in Fig. 2B. Mutants C730A and C383A/C387A both give rise to proteins with electrophoretic mobilities identical to those of the wild type exchanger. There is a shift from the 120-kDa band under reducing conditions to a 160-kDa band under nonreducing conditions. Similar results were obtained using mutants C122A, C151A, C210A, C485A, C557A, C738A, C768A, C914A, and C933A (not shown). Functional properties of these mutants have been described previously (3).

These results indicate that both cysteines 14 and 20 are capable of interacting with cysteine 792 to form a disulfide bond in the Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger protein. This bond is not artifically generated during or after homogenization of the oocytes. The intact oocytes were incubated with 10 mM IAA or NEM, and the homogenization was performed in the presence of 10 mM IAA or NEM. These conditions would prevent formation of any aberrant disulfide bonds. The increase in the mo-
lecular mass is not due to alkylation because the mobility shift is specific to exchanger proteins in which the combination of cysteine 792 with cysteines 14 and/or 20 is preserved. Also, identical results were obtained if IAA and NEM treatments were omitted.

To confirm and further assess our conclusions, exchanger constructs were generated in which C14, C20, or C14/C20 were omitted. All constructs were expressed in Xenopus oocytes, and electrophysiological properties were characterized using the giant patch technique in the inside-out configuration. This technique has been used extensively to characterize the Na\(^{+}\)-Ca\(^{2+}\) exchanger; the Na\(^{+}\)-induced currents are unequivocally due to exchange activity (3, 5, 7–11, 13, 17). Exchange currents, which are absent in control, H\(_2\)O-injected oocytes, display properties characteristic of Na\(^{+}\)-Ca\(^{2+}\) exchange.

Fig. 3 shows representative outward Na\(^{+}\)-Ca\(^{2+}\) exchanger currents and their activation by 100 \(\mu\)M FeSO\(_4\) and 3 mM DTT (Fe-DTT). Upon application of internal Na\(^{+}\) (100 mM), an outward exchange current was elicited when Ca\(^{2+}\) (8 mM) was present in the pipette. The exchange current decayed exponentially with a time constant of several seconds reaching a steady state that was 28 \(\pm\) 2% \((n = 31)\) of peak current (100 mM Na\(^{+}\), 1 \(\mu\)M Ca\(^{2+}\)). This decay process is known as Na\(^{+}\)-dependent inactivation (19). As shown in Figs. 4 and 5, cytoplasmic application of Fe-DTT in the presence of 100 mM Na\(^{+}\) and contaminant Ca\(^{2+}\) (i.e., in the absence of any Ca\(^{2+}\) buffer; Fe treatment could not be done in the presence of EGTA) induced a significant activation of the exchange current \((n = 30\) of 37). After activation, steady state current increased to 76 \(\pm\) 3% of peak current. (Current during the Fe-DTT treatment is not readily interpretable because of the unknown concentration of Ca\(^{2+}\), which has a large effect on the Na\(^{+}\)-dependent inactivation process (19).)

Stimulation of the wild type exchanger activity by Fe-DTT was characterized by either a decrease or a complete removal of the Na\(^{+}\)-dependent inactivation leading to an augmentation of the steady state current (as indicated by the double arrow in Fig. 4). After exposure to Fe-DTT, steady state current increased by 7.8 \(\pm\) 2.2fold \((n = 30, 100\) mM Na\(^{+}\), 1 \(\mu\)M Ca\(^{2+}\)) whereas activation of the peak current was less pronounced. On average, peak current increased by 2.2 \(\pm\) 0.6 fold \((n = 30)\).
Contaminant Ca$^{2+}$ and is obtained by adding 10 mM DTT perfused in the presence of 100 mM Na$^+$ and contaminant [Ca$^{2+}$], in the absence of any calcium buffer. Fe-DTT was applied in the bath (cytoplasmic side) for about 1 min. Exposure to Fe-DTT induced an increase of the steady state current as indicated by the double arrow. Dashed lines indicate steady state current level in control condition and after Fe-DTT.

Control  

Na$^+$  

1 µM Ca$^{2+}$  

100 pA  

10 s  

Fe-DTT  

Na$^+$  

1 µM Ca$^{2+}$  

100 pA  

10 s  

Fig. 4. Stimulation by Fe-DTT of outward Na$^+$$-$$\text{Ca}^{2+}$ exchange current. A giant patch was excised from an oocyte expressing the wild type exchanger, NCX1.1. The current was evoked by rapid replacement of 100 mM Ca$^+$ with 100 mM Na$^+$ at the cytoplasmic surface. Ca$^{2+}$ (8 mM) was always present in the pipette. [Ca$^{2+}$], concentrations are indicated. Currents were recorded at a holding potential of 0 mV. Exchange activity was stimulated by application of 100 µM FeSO$_4$ and 3 mM DTT perfused in the presence of 100 mM Na$^+$ and contaminant [Ca$^{2+}$], and in the absence of any calcium buffer. Fe-DTT was applied in the bath (cytoplasmic side) for about 1 min. Exposure to Fe-DTT induced an increase of the steady state current as indicated by the double arrow. Dashed lines indicate steady state current level in control condition and after Fe-DTT.

Fig. 5. Effects of redox modification on regulation of Na$^+$$-$$\text{Ca}^{2+}$ exchange current. Traces illustrate outward currents of the wild type exchanger under control conditions (left panels) and after Fe-DTT treatment for 1–3 min (right panels). Data are shown at three different levels of regulatory Ca$^{2+}$: 1 µM (top panels), 15 µM (middle panels), and 0 mM (10 mM EGTA; bottom panels). The level of 15 µM Ca$^{2+}$ is approximate and is obtained by adding 10 µM Ca$^{2+}$ to a bath solution containing contaminant Ca$^{2+}$ and no EGTA.

However, in 10 experiments of 30, a 26 ± 5% reduction in peak current was observed after exposure to Fe-DTT even though an increase (127 ± 18%) in the steady state current was still observed. Stimulation of exchanger activity was not reversed upon removal of Fe-DTT or by subsequent addition of DTT (n = 3) after Fe-DTT activation.

As observed previously (20), cytoplasmic Ca$^{2+}$ also regulates exchange activity (Fig. 5). When [Ca$^{2+}$], is raised from 1 to 15 µM, the peak current increases and the Na$^+$-dependent inactivation is eliminated. In addition, when [Ca$^{2+}$], is removed, exchanger current declines to a low Ca$^{2+}$-insensitive steady state level. Exposure of the patch to internal Fe-DTT induced an increase of the Ca$^{2+}$-insensitive steady state current by 6.3 ± 1.2 fold (n = 21; Fig. 5, middle panels). The outward current elicited by application of cytoplasmic Na$^+$ in the absence of regulatory Ca$^{2+}$ retains Na$^+$-dependent inactivation, and this inactivation is reduced by application of Fe-DTT (Fig. 5, bottom panels). In these experiments, steady state current increased from 18 ± 1% to 57 ± 11% of peak current (n = 4). Thus, redox stimulation removes Na$^+$-dependent inactivation and appears to reduce Ca$^{2+}$ regulation.

Exchange activity was stimulated only by the combination of FeSO$_4$ and DTT. When either DTT (3 mM, n = 5) or FeSO$_4$ (100 µM, n = 4) was tested alone, no stimulation of the transport activity was observed (although in one additional experiment FeSO$_4$ was able to mimic the effect of Fe-DTT). Furthermore, in contrast to the results of Reeves et al. (14) other redox reagents such as GSH-GSSH (n = 3), xanthine-xanthine oxidase (n = 3), H$_2$O$_2$ (n = 3), and FeSO$_4$ + H$_2$O$_2$ (n = 1) were unable to evoke exchanger activation. H$_2$O$_2$ caused a decrease in exchange current.

To determine whether the disulfide bond between C792 and either C14 or C20 were involved in the redox stimulation of the exchanger, we studied the effects of Fe-DTT on various mutants (Fig. 6). We examined the Cys-less exchanger with cysteines 14, 20, and 792 reconstituted (top) and the wild type exchanger with cysteine 14, 20, or 792 mutated to alanine. All four mutants displayed Na$^+$-dependent inactivation and Ca$^{2+}$-dependent inactivation and promoted an increase in steady state current. The data indicate that cysteines C14, C20, and C792 are not involved in the mechanism of action of Fe-DTT. Because the mutant Cys-less plus C14/C20/C792 was activated by Fe-DTT (Fig. 6; steady state current increased from 12 ± 6 to 55 ± 4% of peak current (n = 3)), these cysteines are the only possible candidates to be responsible for the redox modulation. Consequently, at least one of the mutants (in particular C792A) should be resistant to stimulation by Fe-DTT if there is a link between disulfide bonds and redox stimulation. This is not the case, because mutants C14A (n = 3), C20A (n = 4), and C792A (stimulation in four of five experiments) were all activated by Fe-DTT. In these three cases, the steady state exchange current was 12 ± 9, 21 ± 6, and 31 ± 11% of peak current before
The mechanism by which Fe-DTT activates the Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger is not clear. However, redox reagents did not stimulate the exchanger if high cytoplasmic Ca\textsuperscript{2+} was present during the treatment with Fe-DTT (n = 4). Fig. 7A shows representative traces of exchanger current recorded from the same patch under control conditions and after application of Fe-DTT in the presence of high and low [Ca\textsuperscript{2+}]. Outward current was not significantly stimulated (1.3 ± 0.3-fold increase at steady state) by perfusion with Fe-DTT in the presence of 1 mM [Ca\textsuperscript{2+}]. Stimulation was observed after a subsequent treatment with Fe-DTT in the presence of low [Ca\textsuperscript{2+}]. In addition, a mutant that lacks [Ca\textsuperscript{2+}], regulation (Δ680–685) was not stimulated by Fe-DTT (Fig. 7B; n = 5).

**DISCUSSION**

Early experimental observations (15, 21) indicated that the purified Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger appeared on SDS-PAGE as three main bands with molecular masses of 70, 120, and 160 kDa. The 70-kDa band is an active proteolytic fragment that may represent either a COOH-terminal (22) or NH\textsubscript{2}-terminal (23) fragment of the exchanger. The 120-kDa band corresponds to the mature protein. The origin of the 160-kDa band has remained unclear though the intensity of this band is dependent on gel conditions. Under reducing conditions, the 160-kDa band is weak, and the 120-kDa band is more intense. Under nonreducing conditions, the 120-kDa band disappears, and the 160-kDa band becomes prominent (15). We demonstrate here, using mutagenesis and biochemical analysis, that this mobility shift is due to an intramolecular disulfide bond between cysteine 792 and either cysteine 14 or 20. Disulfide bond formation is unlikely to represent a dimerization reaction between two exchanger molecules. The mobility shift is too modest for intermolecular, rather than intramolecular, bond formation. Also, there is unlikely to be an intermolecular bond formed between the exchanger and some other protein because the mobility shift occurs even with purified exchanger protein.

The Cys-less exchanger shows no difference in electrophoretic mobility under reducing and nonreducing conditions (Fig. 1), demonstrating the importance of cysteine residues in a conformation-dependent mobility shift. The involvement of specific cysteine residues was determined in two sets of experiments. First, we removed individual cysteines from the wild type exchanger (Fig. 2). Second, we reintroduced specific cysteines into the Cys-less exchanger (Fig. 3). Identical results were obtained in both cases; the mobility shift required the presence of cysteine 792 in combination with either cysteine 14 or 20. The disulfide bond appears to have some functional significance. In an earlier study (3), we noted that mutation of either cysteine 20 or 792 to alanine decreased expression of activity by more than 50%. Possibly, other cysteines in the exchanger can also form intramolecular disulfide bonds. If this occurs, however, these disulfide bonds do not induce any mobility shift. Also, our previous study, in which the Cys-less exchanger was initially constructed, would suggest that such an undetected disulfide bond has no important functional role (3).

Cysteines 14 and 20 are near the NH\textsubscript{2} terminus of the protein. This segment of the protein is localized on the extracellular side of the membrane (4–6). In initial topological models (24), cysteine 792 was assigned to the intracellular loop connecting TMSs 7 and 8 (loop h). More recently, the exchanger has been subjected to detailed topological analysis based on cysteine substitution analysis (3, 4), and loop h has been reasigned to be extracellular. Because cysteines 14 and 20 are extracellular, cysteine 792 must also be extracellular for disulfide bond formation to occur. Thus, our results confirm the extracellular location of loop h.

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[2] D. A. Nicoll, M. Ottolia, and K. D. Phillipson, unpublished observation.
Cysteines 14, 20, and 792 are distant in the linear sequence but must be in close proximity in the native protein. This would also suggest that TMS 1 and TMSs 6 and 7 (which adjoin loop h in our current model) are near each other in the folded protein structure. However, TMS 1 does not begin until about residue 40 and cysteine 20 may extend some distance from TMS1; thus the actual proximity of TMSs 1, 6, and 7 is not clear.

Reeves et al. (14) had previously observed that treatment of cardiac sarcolemmal vesicles with Fe-DTT activated the Na\(^+\)-Ca\(^{2+}\) exchanger as measured using \(^{44}\)Ca\(^{2+}\) fluxes. We are able to reproduce this observation by measuring currents across giant excised patches of oocytes expressing the cloned cardiac exchanger NCX1.1. Mechanistically, the major consequence of redox treatment is the removal of Na\(^+\)-dependent inactivation (Fig. 4). Removal of Na\(^+\)-dependent inactivation has also been implicated in the stimulation of the exchanger by the second messenger PIP\(_2\) (25). Perhaps modulation of Na\(^+\)-dependent inactivation is a general mechanism for regulation of exchange activity.

Reeves et al. (14) had hypothesized that the redox stimulation was due to the rearrangement of a disulfide bond. That is, the reduction of one disulfide bond and the formation of a second different disulfide bond might occur. This model seems to be consistent with our finding that cysteine 792 could form a covalent linkage with either of two different cysteines. Perhaps, a cysteine 20/792 disulfide could shift to a cysteine 14/792 disulfide under the influence of Fe-DTT.

Our results, however, eliminate the possibility of cysteine involvement in redox stimulation of Na\(^+\)-Ca\(^{2+}\) exchange activity. First, we have previously noted that the Cys-less exchanger demonstrates both Na\(^+\)-dependent inactivation and Ca\(^{2+}\) regulation (3). This would suggest that none of the 15 endogenous cysteines are involved in exchanger regulation. Second, our results here demonstrate that a disulfide rearrangement could not be involved in redox stimulation. The Cys-less exchanger in which only cysteines 14, 20, and 792 have been reintroduced displays redox stimulation (Fig. 6). Therefore, if a disulfide bond is involved in the effect, it must involve these residues. However, the exchanger can still be stimulated by Fe-DTT after elimination of any one of these residues. This result is highly unlikely if a disulfide rearrangement is involved in redox stimulation. The mechanism of stimulation of the exchanger by Fe-DTT is thus unknown but may involve some other modification of the protein or the lipid environment. We have found that redox stimulation of the exchanger is blocked if high Ca\(^{2+}\) is present during the Fe-DTT treatment (Fig. 7A). Likewise, a mutant exchanger that displays no Ca\(^{2+}\) regulation cannot be stimulated by Fe-DTT (Fig. 7B). Perhaps a specific Ca\(^{2+}\)-dependent conformation is required for Fe-DTT action.

Reeves et al. (14) also found that the effects of Fe-DTT on exchange activity were blocked by the presence of Ca\(^{2+}\) in the incubation medium.

Stimulation of cardiac Na\(^+\)-Ca\(^{2+}\) exchange activity by redox modification could have physiological implications. For example, the generation of oxygen-derived free radicals during ischemia/reperfusion could induce redox modification of the exchanger. In this regard, Goldhaber (26) found that free radicals stimulated the exchange current of ventricular myocytes. Perhaps, a similar mechanism to that observed here is involved.

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