Ca\textsuperscript{2+} Regulation of Ion Transport in the Na\textsuperscript{+}/Ca\textsuperscript{2+} Exchanger*\textsuperscript{S}

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The binding of Ca\textsuperscript{2+} to two adjacent Ca\textsuperscript{2+}-binding domains, CBD1 and CBD2, regulates ion transport in the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger. As sensors for intracellular Ca\textsuperscript{2+}, the CBDs form electrostatic switches that induce the conformational changes required to initiate and sustain Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange. Depending on the presence of a few key residues in the Ca\textsuperscript{2+}-binding sites, zero to four Ca\textsuperscript{2+} ions can bind with affinities between 0.1 to 20 \textmu M. Importantly, variability in CBD2 as a consequence of alternative splicing modulates not only the number and affinities of the Ca\textsuperscript{2+}-binding sites in CBD2 but also the Ca\textsuperscript{2+} affinities in CBD1.

In the heart, the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger (NCX)\textsuperscript{2} works in concert with the plasma membrane and sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPases to remove Ca\textsuperscript{2+} released into the cytosol after the muscle contraction phase (Refs. 1–5; for general reviews, see Refs. 6 – 8). In this process, NCX predominantly exports one Ca\textsuperscript{2+} ion for the uptake of three Na\textsuperscript{+} ions (9) at an estimated turnover rate of 2500–5000 s\textsuperscript{-1} (10–12).

NCX exists in three isoforms, NCX1 (13), NCX2 (14), and NCX3 (15). Numerous splice variants have been described for NCX1 (16, 17) and NCX3 (18). In NCX1, these are encoded by one of the mutually exclusive exons, A or B, in combination with small cassette exons C–F. In this pool of splice forms, the AD variant (detected in the brain) constitutes the shortest form, and the ACDEF form (found in the heart) constitutes the longest splice variant. Based on the presence of two conserved regions in the transmembrane domain, generally referred to as \alpha-repeats (19), NCX has been assigned to the cation/Ca\textsuperscript{2+} exchanger superfamily (20). Probably originating from an ancient gene duplication event (19, 21, 22), these \alpha-repeat regions were until recently believed to form oppositely oriented re-entrant loops that were suggested to be involved in ion binding and translocation (19, 23).

Over two decades ago (24, 25) it was demonstrated that NCX not only transports Ca\textsuperscript{2+} and Na\textsuperscript{+} but is also regulated by these ions. In pioneering work, Hilgemann et al. (26, 27) dissected the regulatory properties into Ca\textsuperscript{2+}-dependent activation and Na\textsuperscript{+}-dependent inactivation. While initial activation occurs at submicromolar Ca\textsuperscript{2+} concentrations, counteracting Na\textsuperscript{+}-dependent exchanger inactivation may establish when Na\textsuperscript{+} concentrations rise above 15 mM. Strikingly, NCX1 splice variants encoded by exon A can alleviate Na\textsuperscript{+}-dependent inactivation at elevated intracellular Ca\textsuperscript{2+} concentrations, whereas those variants encoded by exon B (28–30) cannot. Furthermore, it appears that exon A-encoded exchanger variants are expressed predominantly in excitable cells, where high Ca\textsuperscript{2+} fluxes are needed (31), whereas exon B-encoded variants are generally restricted to non-excitable cells (18).

Structural Basis for Ca\textsuperscript{2+} Regulation in NCX

Structurally, NCX consists of an \alpha-helical transmembrane domain and a large ~500-residue-long cytosolic loop (Fig. 1A) (32, 33). Residues 371–509 within this loop have been shown to bind Ca\textsuperscript{2+} with high affinity ($K_d \sim 140 – 400 \text{ mM}$) (34, 35). The solution structure of residues 371–509 of canine NCX1 (36) redefined the boundaries (Fig. 1B) of a module earlier described as the Calx-\beta domain (37). A single copy of the Calx-\beta domain is found in integrin \beta4 (37, 38), and multiple copies are present in very large G protein-coupled receptors (39, 40) and extracellular matrix proteins ranging from sponges to humans (41, 42). A sequence identity of 27% for residues 501–650 with residues 371–500 suggested the presence of an unexpected, second Ca\textsuperscript{2+}-binding domain (CBD) in the cytosolic exchanger loop (36). As for the first, the NMR structure of the second CBD was determined in the Ca\textsuperscript{2+}-bound form. Both domains (CBD1 and CBD2) have the architecture of a \beta-sandwich, formed by two antiparallel \beta-sheets, with one \beta-sheet containing strands A, B, and E and the other containing strands C, D, F, and G (Fig. 1B).

Each domain features a \beta-bulge in strand A, but only CBD2 displays a second \beta-bulge in strand G. In contrast, CBD1 possesses a cis-proline at a structurally similar position. The long FG loop in each of the individual domains (residues 468–482 in CBD1 and residues 599–627 in CBD2) is largely unstructured. A variable region comprising 39–75 residues, depending on the splice form, precedes the FG loop in CBD2. Superposition of the ensemble of NMR structures of CBD1 and CBD2 (supplemental Fig. S1) shows the largest differences to be in the BC, CD, and FG loops as well as in parts of the first acidic segment (residues 446–454 and 577–582, respectively). Ile-374/Phe-505, Phe-376/Phe-507, Tyr-422/Phe-553, Phe-431/Phe-562, Phe-456/Phe-587, and Ile-460/Ile-591 form the core of the two domains and are probably important for stability. Intriguingly, at the N-terminal side of the domains, the highly conserved Arg-396/Arg-527 (Fig. 1B) engages in a stacking interaction with the conserved Phe-431/Phe-562. Whereas Arg-527 in CBD2 clearly stabilizes the strictly conserved BC loop, the function of Arg-396 in CBD1 remains unclear.

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2 The abbreviations used are: NCX, Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger; CBD, Ca\textsuperscript{2+}-binding domain; PDB, Protein Data Bank; CLD, catenin-like domain; SAXS, small-angle x-ray scattering; mGlur, metabotropic glutamate receptor; PIP\textsubscript{2}, phosphatidylinositol 4,5-bisphosphate.

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Subsequent x-ray structures of Ca\textsuperscript{2+}/H11001-bound CBDs (CBD1, Protein Data Bank (PDB) code 2DPK (43) and codes 3E9T and 3EAD (44); and CBD2, code 2QVM (45)) have provided a more detailed view of the Ca\textsuperscript{2+}-binding sites. The structures show that the largest contribution to the Ca\textsuperscript{2+}-binding sites of the CBDs originates from two acidic segments, located in the EF loop and at the C terminus of the domains (Fig. 1, B–D), respectively. Interestingly, in CBD2, the EF loop is encoded by one of the mutually exclusive exons, A or B, which determines whether NCX1 can overcome Na\textsuperscript{+}-dependent inactivation. In both CBDs, Ca\textsuperscript{2+}-binding sites are complemented by conserved glutamate (Glu-385/Glu-516) and aspartate (Asp-421/Asp-552) residues in the AB and CD loops, respectively. In CBD1, four Ca\textsuperscript{2+} ions are bound in an arrangement reminiscent of C2 domains (Fig. 1C) (43). In contrast, due to Lys-585 and the absence of acidic counterparts of Glu-454, Asp-499, and Asp-500 of CBD1, the Ca\textsuperscript{2+}-binding sites of CBD2-AD (Fig. 1D), with AD denoting the presence of residues encoded...
forms a salt bridge with Asp-552 and thereby stabilizes the CBD1 are Lys-585 crucially points into Ca\(^{2+}\) and Asp-498–Asp-500. In the case of CBD2-AD, the NMR density was visible for residues corresponding to Asp-447–Glu-451 (PDB code 3E9T, chains C and D) (44), where no electron density was visible for this residue (49).

In the CBD12 interface (46, 47) is not in line with functional studies of CBD1 from Methanococcus jannaschii (NCX_Mj) in the outward conformation (50). The 1.9 Å crystal structure revealed a largely symmetrical transmembrane domain, arranged in the form of two structurally similar halves of five α-helices with opposite topology (Fig. 2A). Helices 2–5 and 7–10 are tightly packed, whereas helices 1 and 6 are somewhat separated from the bundle and tilted at an angle of ~45° with respect to the vertical axis of the membrane. In line with the proposed transport stoichiometry of one Ca\(^{2+}\) ion in exchange for three Na\(^{+}\) ions, four cation-binding sites, one specific for Ca\(^{2+}\) and three that probably bind Na\(^{+}\), were identified in the protein core at the center of the membrane. Strikingly, all residues participating in ion binding originate from the α-repeat regions and are highly conserved in the NCX and Na\(^{+}/Ca^{2+}-K^{+}\) exchanger branches of the cation/Ca\(^{2+}\)-exchanger superfamily (20). In particular, in the center, Glu-54 (Glu-113 in canine NCX1) and Glu-213 (Asp-814) coordinate the Ca\(^{2+}\) ion via their carboxylate groups, whereas the backbone carboxyl oxygen atoms of Thr-50 and Thr-209 complement the binding site. In addition, the Oe2 atoms of Glu-54 and Glu-213 contribute to the middle Na\(^{+}\)-binding site as well as to the Na\(^{+}\)-binding sites toward the extra- and intracellular sides, respectively. The latter two Na\(^{+}\)-binding sites share identical ligand chemistry and geometry, where Ser-77, Ala-206, Thr-209, and Ser-210 form the Na\(^{+}\)-binding site oriented toward the extracellular side, and Ala-47, Thr-50, Ser-51, and Ser-236 constitute the Na\(^{+}\)-binding site closer to the intracellular side. Finally, Asn-81 and Asp-240 complete the coordination scheme of the middle Na\(^{+}\)-binding site.

Two hydrophilic pathways permit independent access to the Na\(^{+}\) - and Ca\(^{2+}\)-binding sites from the extracellular side. Making use of the symmetry of NCX_Mj by applying a molecular dyad defined by the middle Na\(^{+}\) ion and the Ca\(^{2+}\) ion also enabled the construction of a feasible model for the inward-facing conformation that reflects the access pathways from the cytosolic side. The crystal structure of the outward-facing conformation of NCX_Mj and the inward-facing model led to the proposal of a simple and rapid Na\(^{+}/Ca^{2+}\) exchange mechanism that is in agreement with the high turnover rates found for NCX. Together, this work has provided another substantial piece of information to help solve the puzzle of the intact structure of mammalian NCX (Fig. 2B). Presently, residues 1–42, 218–258, and 651–726 (numbering according to the AD splice variant of NCX1), which determine the relative positions of the domains with respect to each other, are the remaining missing pieces.

**Ca\(^{2+}\) Binding Determinants of CBD1 and CBD2**

The thermodynamic properties of the Ca\(^{2+}\)-binding sites of CBD1 and CBD2 have been extensively investigated by isothermal titration calorimetry (46). Initial sequence comparison of all available Na\(^{+}/Ca^{2+}\) exchanger sequences revealed strict conservation of the Ca\(^{2+}\)-coordinating residues in CBD1 (Fig. 1C). The expected similarity of the Ca\(^{2+}\) binding properties of the three known exchanger isoforms was confirmed by the titration of CBD1 of canine NCX1 and mouse NCX2 and NCX3 (46). However, these experiments did not allow their individual macroscopic binding constants to be confidently determined, but only the approximate range, 100–600 nM. These affinities make CBD1 the primary Ca\(^{2+}\) sensor and thus responsible for Ca\(^{2+}\) activation in NCX (26, 34, 36).
In strong contrast to CBD1, sequence analysis of CBD2 has revealed that residue types at positions 552, 578, and 585, located around Ca$^{2+}$/H11001-binding site II (Fig. 1D), can vary depending on the isoform and splice variant. As a consequence, the different isoforms and splice variants bind zero to three Ca$^{2+}$/H11001 ions with affinities 5–50-times lower than those of CBD1 (for details, see Ref. 46).

**Function of Residues Encoded by the Cassette Exons**

An increasing amount of evidence indicates that besides the Ca$^{2+}$/H11001-binding sites of CBD1 and CBD2, residues encoded by the cassette exons may contribute to ion transport regulation in NCX. The first sign that cassette exons can influence Ca$^{2+}$/H11001 affinities of the CBD1 Ca$^{2+}$/H11001-binding sites came from isothermal titration calorimetry experiments (46) with CBD12-AD and CBD12-ACDEF, two-domain constructs representing the brain and heart splice variants. Although the individual CBD structures suggest that both constructs should bind six Ca$^{2+}$ ions, strikingly, the longer ACDEF variant appears to bind only two Ca$^{2+}$ ions with medium affinity, whereas CBD12-AD binds six Ca$^{2+}$ ions, as expected. Completely lacking the high affinity component of CBD1, particle-induced x-ray emission experiments (51) confirmed the coordination of four Ca$^{2+}$ ions in CBD12-ACDEF prior to the start of titration. Thus, residues encoded by the additional cassette exons C, E, and F, located at the opposite end of the CBD2 Ca$^{2+}$/H11001-binding sites, apparently increase the CBD1 Ca$^{2+}$/H11001 affinities substantially. This is in line with the fact that the ACDEF splice variant is found predominantly in the heart, where the exchanger either is required to enter the activated state at considerably lower intracellular Ca$^{2+}$/H11001 concentrations or is even permanently activated (52) to guarantee high Ca$^{2+}$/H11001 fluxes.

**Electrostatic Switches in CBD1 and CBD2**

In analogy to C$_2$ domains (53), analysis of the electrostatic potentials of CBD1 and CBD2 in the absence and presence of Ca$^{2+}$ revealed dramatic differences and suggested that the CBD1 and CBD2 Ca$^{2+}$/H11001-binding sites form electrostatic switches. In particular, in the absence of Ca$^{2+}$, the CBD1 Ca$^{2+}$/H11001-binding sites display a strongly negative electrostatic potential due to the extensive cluster of aspartic and glutamic acid residues (Asp–421, Asp–446, Asp–447, Asp–448, Asp–498, Asp–499, and Asp–500). In strong contrast to CBD1, sequence analysis of CBD2 has revealed that residue types at positions 552, 578, and 585, located around Ca$^{2+}$/H11001-binding site II (Fig. 1D), can vary depending on the isoform and splice variant. As a consequence, the different isoforms and splice variants bind zero to three Ca$^{2+}$/H11001 ions with affinities 5–50-times lower than those of CBD1 (for details, see Ref. 46).

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Asp-500, Glu-385, Glu-451, and Glu-454) and the lack of any basic residues (Fig. 1C). This high density of negative charges not only is responsible for the loss of the structural integrity of the CBD1 Ca\(^{2+}\)-binding sites in the absence of Ca\(^{2+}\) but may also cause repulsion with a highly acidic conserved region consisting of four consecutive glutamate residues (Glu-622–Glu-625) at the beginning of helix 2 in CBD2. Upon binding of four Ca\(^{2+}\) ions, both the negative potential and the repulsion are likely to be strongly reduced.

In comparison, the CBD2 electrostatic potential in the absence of Ca\(^{2+}\) is considerably less negative to the lower number of acidic residues and the presence of basic residues (Arg-547, Lys-583, and Lys-585) around Ca\(^{2+}\)-binding site II. In addition, the different numbers and affinities of the Ca\(^{2+}\)-binding sites in the various isoforms and splice variants determine the strength of the electrostatic switch in CBD2.

**Ca\(^{2+}\) Induces a Twist in the Hinge between CBD1 and CBD2**

SAXS analysis of CBD12-AD and CBD12-ACDEF constructs in the absence and presence of Ca\(^{2+}\) revealed a substantial conformational change upon Ca\(^{2+}\) binding that leads to a compaction of the CBDs (46). Although in the intact exchanger this conformational change is probably constrained by the N- and C-terminal linker regions to the CLD, the SAXS analysis provided the first clue of the effects of Ca\(^{2+}\) binding to the CBD1 Ca\(^{2+}\)-binding sites and indicated that the conformational change induced may not be identical in CBD12-AD and CBD12-ACDEF. Indeed, superposition of the crystal structures of CBD12 from the *Drosophila* Na\(^{+}/Ca\(^{2+}\)* exchanger isoforms CalX1.1 and CalX1.2 shows a rotation by ~9° between them. Comparison of the interfaces of CalX1.1 and CalX1.2 reveals that the number of interactions between CBD1 and CBD2 is reduced in CalX1.2 with respect to CalX1.1. Most prominently, CalX1.2 apparently lacks the salt bridge between Glu-521 and Arg-673 (Fig. 1F), resulting in increased interdomain flexibility.

**The Drosophila Exchanger Remains Enigmatic**

CalX is unique among the characterized NCXs in that micromolar levels of Ca\(^{2+}\) inhibit rather than activate Na\(^{+}/Ca\(^{2+}\) exchange (54). With respect to the CBDs, there are at least three major differences between CalX and the other NCXs. First, CBD1 of CalX lacks most of the FG loop that is disordered in the isolated CBD1 structure of canine NCX1 (36, 43). Similar to the FG loop of CBD2 at the CBD12 interface, the FG loop of CBD1 may rigidify in intact NCX and potentially interact with the CLD. Indeed, Hryshko and co-workers (55) could reconstitute normal Ca\(^{2+}\) activation in a CalX chimera that had Lys-404–Tyr-580 substituted with the corresponding residues of NCX1, indicating that the trimmed FG loop in CBD1 might play a role in Ca\(^{2+}\) inhibition of CalX. Second, CalX lacks three of the four conserved consecutive glutamate residues that might cause repulsion with the CBD1 Ca\(^{2+}\)-binding sites in the absence of Ca\(^{2+}\). Third, CalX does not show Ca\(^{2+}\) binding to CBD2 (PDB code 3E9U (56)) and is therefore incapable of forming an electrostatic switch in the second CBD. However, unlike in the exon B-encoded variants of NCX1, the region around the EF loop of the individual CBD2 of CalX is structured, although the same region is intriguingly disordered in the Ca\(^{2+}\)-bound CBD12 structures (47).

**Potential Mechanism**

The combination of available structural and biophysical data with results from functional studies has resulted in the proposal of a dual electrostatic switch mechanism for Ca\(^{2+}\) regulation in NCX (Fig. 3, A–C (46). As intracellular Ca\(^{2+}\) concentrations rise, Ca\(^{2+}\) ions initially bind to the CBD1 Ca\(^{2+}\)-binding sites. Increased rigidity and a substantial increase in the electrostatic potential of the Ca\(^{2+}\)-binding sites thereby induce a conformational change that leads to a compaction of the CBDs as visualized by SAXS analysis and supported by FRET studies using full-length NCX and CBD12 constructs (57). As a consequence, the tension on the N- and/or C-terminal linker region to the CLD that relays Ca\(^{2+}\) binding and release events to the transmembrane domain is most likely reduced. This initial activation step (Fig. 3B) is referred to as Ca\(^{2+}\)-dependent activation and initiates Na\(^{+}/Ca\(^{2+}\) exchange. Demonstrated by particle-induced x-ray emission analysis (46) and suggested by FRET measurements (57), the Ca\(^{2+}\) concentration at which NCX becomes activated probably depends on the residues encoded by the cassette exons that modulate the affinities of the CBD1 Ca\(^{2+}\)-binding sites. This modulation would, for instance, allow the appropriate response to single Ca\(^{2+}\) transients produced by type 1 metabotropic glutamate receptors (mGluR1) at Purkinje cell synapses, whereas in hippocampal neurons, mGluR5 generates a radically different Ca\(^{2+}\) signal in the form of an oscillatory pattern (8). Although there are no cell-specific expression data for exchanger splice variants in the brain, coexpression of NCX1.4 (encoded by exons A and D) with mGluR1 and of NCX1.5 (encoded by exons A, D, and F) with mGluR5 would make sense, as the latter probably features higher Ca\(^{2+}\) affinities at the CBD1 Ca\(^{2+}\)-binding sites and thereby could remain activated during the series of action potentials.

Recently, several reports have suggested that Ca\(^{2+}\) ions in sited I and II of CBD1 are not essential for Ca\(^{2+}\) regulation (58, 59). Although the impact of the Ca\(^{2+}\) ions in sites III and IV (Fig. 1F) is without doubt most dominant due to their proximity to CBD2, the lack of Ca\(^{2+}\)-binding sites I and II would substantially change the electrostatic potential at the CBD1 Ca\(^{2+}\)-binding sites. The consequence would be a reduction in the repulsion and attraction between the CBD1 Ca\(^{2+}\)-binding sites and CBD2 in the absence and presence of Ca\(^{2+}\), respectively, yet these predicted effects are not reflected in functional studies with NCX1 mutants D421A and E454K (58, 59). Nevertheless, it remains intriguing why sites I and II should not be essential even though all Ca\(^{2+}\)-coordinating residues in CBD1 are strictly conserved.

To sustain Na\(^{+}/Ca\(^{2+}\) exchange, Ca\(^{2+}\) ions probably have to also bind to CBD2 (Fig. 3C), a process that is isoform- and splice variant-specific. Functional (29, 45, 59) and thermodynamic (46) data for exchangers that bind zero to three Ca\(^{2+}\) ions at the CBD2 Ca\(^{2+}\)-binding sites, as well as distinct macroscopic Ca\(^{2+}\) binding constants ranging from 250 nM to 20 μM, strongly indicate that the number and affinities of Ca\(^{2+}\)-binding sites in CBD2 determine the form of the steady-state exchange current.
For instance, exon B-encoded NCX1 splice variants become inactivated in a matter of seconds (29), whereas the NCX1 mutant K585E, which is comparable with the B variant of NCX3 and likely binds two Ca\(^{2+}\)/H\(^{+}\) ions between the CD and EF loops, shows steady-state outward currents above their corresponding peak currents (45, 59). At the same time, this contradicts suggestions that Ca\(^{2+}\)/H\(^{+}\) ions between the CD and EF loops of CBD2 are not essential either (45), thus implying isoform- and splice variant-specific determinants to be irrelevant. In contrast, different properties of the CBD2 Ca\(^{2+}\)/H\(^{+}\)-binding sites may enable optimal adaptation of Na\(^{+}\)/H\(^{+}\)/Ca\(^{2+}\)/H\(^{+}\) exchange to meet special needs within the cell or a specific tissue.

**NCX Inactivation**

As cytosolic Na\(^{+}\) concentrations are low in normal functioning cells, it is unlikely that Na\(^ {+}\)-dependent inactivation plays a substantial role under normal physiological conditions. It could, however, be beneficial by limiting NCX-mediated Ca\(^{2+}\) influx under conditions such as ischemia, in which cytosolic Na\(^{+}\) levels are elevated and phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)) levels are low (52).

As early as 1968, Baker and Blaustein (60) reported that a drop in cytosolic pH could inhibit Na\(^{+}\)/Ca\(^{2+}\) exchange. Extensive studies on the effect of pH were later performed on sarcolemmal vesicles of canine heart (61) and guinea pig ventricular cells (27, 62, 63). Prompted by the proton insensitivity of the transmembrane domain (62), experiments with the CBDs revealed that a mild reduction in cytosolic pH from 7.2 to 6.9 caused an up to 3-fold decrease in the Ca\(^{2+}\) affinities of CBD1 (64). This implies that pH is an additional regulator of NCX activity, as it efficiently modulates Ca\(^{2+}\) affinities of the CBD1 Ca\(^{2+}\)-binding sites.

In addition to its primary function as a Ca\(^{2+}\) efflux mechanism, NCX may also mediate Ca\(^{2+}\) influx, where high cytosolic Na\(^{+}\) concentrations induce a mode of exchange activity that does not require binding of Ca\(^{2+}\) to the CBDs (52, 65). Under these conditions, counteracting Na\(^ {+}\)-dependent inactivation is overcome by high levels of PIP\(_2\), which keep NCX activated (Fig. 3D). Furthermore, it has been shown that PIP\(_2\) strongly interacts with residues 218–237 of NCX and that peptides encoding these residues can inhibit the exchanger (66). As a
result, this region, which also shows some similarity to the calmodulin-binding motif, is referred to as the exchanger inhibitory peptide region. Mutational studies of the exchanger inhibitory peptide region in intact NCX revealed a profound effect of changes in sequence with respect to the interaction with PIP2 as well as Na\(^+\)-dependent inactivation (67).

**NCX in Disease**

NCX is of paramount importance in the heart, as, together with the sarcoplasmic reticulum Ca\(^{2+}\)-ATPase, the exchanger removes \(~99\%\) (\(~30\%\) via NCX and \(~70\%\) via sarcoplasmic reticulum Ca\(^{2+}\)-ATPase) of the Ca\(^{2+}\) released to the cytosol (70). Dysregulation of this Ca\(^{2+}\) balance can lead to cardiac arrhythmias, a leading cause of death in cardiovascular disease. In the failing heart and in patients with ischemia-reperfusion injury (68, 69), in cases of salt-sensitive hypertension (70, 71) or multiple sclerosis (72), NCX appears to operate predominantly in reverse mode, importing rather than exporting Ca\(^{2+}\). The resulting excessive Ca\(^{2+}\) entry may cause the activation of Ca\(^{2+}\)-dependent proteases such as calpain and caspasases (73), subsequently leading to cell apoptosis. As the damaging role of NCX is related mostly to its reverse mode, benzylxophenyl derivatives (74) such as KB-R7943 (75, 76), SEA0400 (77), and SN-6 (78), which specifically inhibit the reverse mode operation of NCX, have attracted a great deal of attention (for review, see Ref. 3). Although experiences with these inhibitors are overall rather positive, thorough testing in relevant preclinical models remains to be performed (79).

**Concluding Remarks**

Over the last 5 years, high resolution NMR and x-ray structures of a large part of the regulatory loop of NCX and the transmembrane domain of a prokaryotic NCX homolog have added a new dimension to our understanding of the regulation and function of this essential transporter. In addition, the role of different splice variants has become increasingly clear. The variability in CBD2 as a consequence of alternative splicing is now known to modulate not only the number and affinities of the Ca\(^{2+}\)-binding sites in CBD2 but also the Ca\(^{2+}\) affinities in CBD1 (46). Furthermore, the CBD12 SAXS solution structures have suggested a conformational change in the hinge between CBD1 and CBD2 upon Ca\(^{2+}\) binding (46), whereas CBD12 x-ray structures have revealed the link between the CBD1 Ca\(^{2+}\)-binding sites and CBD2 (47). This description of the interface includes hydrogen bonds and salt bridges with residues located in the FG loop of CBD2. Future efforts will aim to connect the regulatory effects of the CBDs to the ion-conducting transmembrane domain of NCX.

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**REFERENCES**

1. Blaustein, M. P., and Lederer, W. J. (1999) Sodium/calcium exchange: its physiological implications. *Physiol. Rev.* **79,** 763–854
2. Philipson, K. D., and Nicoll, D. A. (2000) Sodium/calcium exchange: a molecular perspective. *Annu. Rev. Physiol.* **62,** 111–133
3. Annunziato, L., Pignataro, G., and Di Renzo, G. F. (2004) Pharmacology of brain Na\(^+\)/Ca\(^{2+}\) exchanger: from molecular biology to therapeutic perspectives. *Pharmacol. Rev.* **56,** 633–654
4. Lytton, J. (2007) Na\(^+\)/Ca\(^{2+}\) exchangers: three mammalian gene families control Ca\(^{2+}\) transport. *Biochem. J.* **406,** 365–382
5. Brini, M., and Carafoli, E. (2011) The plasma membrane Ca\(^{2+}\)-ATPase and the plasma membrane sodium/calcium exchanger cooperate in the regulation of cell calcium. *Cold Spring Harb. Perspect. Biol.* **3,** a001468
6. Carafoli, E. (2002) Calcium signaling: a tale for all seasons. *Proc. Natl. Acad. Sci. U.S.A.* **99,** 1115–1122
7. Bers, D. M. (2002) Cardiac excitation-contraction coupling. *Nature* **415,** 198–205
8. Berridge, M. J., Bootman, M. D., and Roderick, H. L. (2003) Calcium signaling: dynamics, homeostasis, and remodeling. *Nat. Rev. Mol. Cell Biol.* **4,** 517–529
9. Kang, T. M., and Hilgemann, D. W. (2004) Multiple transport modes of the cardiac Na\(^+\)/Ca\(^{2+}\) exchanger. *Nature* **427,** 544–548
10. Hilgemann, D. W., Nicoll, D. A., and Philipson, K. D. (1991) Charge movement during Na\(^+\) translocation by native and cloned cardiac Na\(^+\)/Ca\(^{2+}\) exchanger. *Nature* **352,** 715–718
11. Niggl, E., and Lederer, W. J. (1991) Molecular operations of the sodium/calcium exchanger revealed by conformation currents. *Nature* **349,** 621–624
12. Egger, M., and Niggl, E. (1999) Regulatory function of sodium/calcium exchange in the heart: milestones and outlook. *J. Membr. Biol.* **168,** 107–130
13. Nicoll, D. A., Longoni, S., and Philipson, K. D. (1990) Molecular cloning and functional expression of the cardiac sarcoslemmal Na\(^+\)/Ca\(^{2+}\) exchanger. *Science* **250,** 562–565
14. Li, Z., Matsuoka, S., Hryshko, L. V., Nicoll, D. A., Bersohn, M. M., Burke, E. P., Litton, R. P., and Philipson, K. D. (1994) Cloning of the NCX2 isoform of the plasma membrane Na\(^+\)/Ca\(^{2+}\) exchanger. *J. Biol. Chem.* **269,** 17434–17439
15. Nicoll, D. A., Quednau, B. D., Qui, Z., Xia, Y. R., Lusis, A. J., and Philipson, K. D. (1996) Cloning of a third mammalian Na\(^+\)/Ca\(^{2+}\) exchanger, NCX3. *J. Biol. Chem.* **271,** 24914–24921
16. Kofuji, P., Lederer, W. J., and Schulze, D. H. (1994) Mutually exclusive and cassette exons underlie alternatively spliced isoforms of the sodium/calcium exchanger. *J. Biol. Chem.* **269,** 5145–5149
17. Lee, S. L., Yu, A. S., and Lytton, J. (1994) Tissue-specific expression of Na\(^+\)/Ca\(^{2+}\) exchanger isoforms. *J. Biol. Chem.* **269,** 14849–14852
18. Quednau, B. D., Nicoll, D. A., and Philipson, K. D. (1997) Tissue specificity and alternative splicing of the Na\(^+\)/Ca\(^{2+}\) exchanger isoforms NCX1, NCX2, and NCX3 in rat. *Am. J. Physiol.* **272,** C1250–C1261
19. Nicoll, D. A., Hryshko, L. V., Matsuoka, S., Frank, J. S., and Philipson, K. D. (1996) Mutation of amino acid residues in the putative transmembrane segments of the cardiac sarcoslemmal Na\(^+\)/Ca\(^{2+}\) exchanger. *J. Biol. Chem.* **271,** 13385–13391
20. Cai, X., and Lytton, J. (2004) The cation/Ca\(^{2+}\) exchanger superfamily: phylogenetic analysis and structural implications. *Mol. Biol. Evol.* **21,** 1692–1703
21. Rapp, M., Granseth, E., Seppälä, S., and von Heijne, G. (2006) Identification and evolution of dual-topology membrane proteins. *Nat. Struct. Mol. Biol.* **13,** 112–116
22. Hilge, M., Aelen, J., Perrakis, A., and Vuister, G. W. (2007) Structural basis for Ca\(^{2+}\) regulation in the Na\(^+\)/Ca\(^{2+}\) exchanger. *Ann. N.Y. Acad. Sci.* **1099,** 7–15
23. Iwamoto, T., Uehara, A., Imanaga, I., and Shigekawa, M. (2000) The Na\(^+\)/Ca\(^{2+}\) exchanger NCX1 has oppositely oriented reentrant loop domains that contain conserved aspartic acids whose mutation alters its apparent Ca\(^{2+}\) affinity. *J. Biol. Chem.* **275,** 38571–38580
24. DiPolo, R. (1979) Calcium influx in internally dialyzed squid giant axons. *J. Gen. Physiol.* **73,** 91–113
25. Hilgemann, D. W. (1990) Regulation and deregulation of cardiac Na\(^+\)/Ca\(^{2+}\) exchange in giant excised sarcolemmal membrane patches. *Nature* **344,** 242–245
26. Hilgemann, D. W., Collins, A., and Matsuoka, S. (1992) Steady-state and dynamic properties of cardiac sodium/calcium exchange. Secondary
modulation by cytoplasmic calcium and ATP. J. Gen. Physiol. 100, 933–961.

27. Hilgemann, D. W., Matsuoka, S., Nagel, G. A., and Collins, A. (1992) Steady-state and dynamic properties of cardiac sodium/calcium exchange. Sodium-dependent inactivation. J. Gen. Physiol. 100, 905–932.

28. Dyck, C., Omelchenko, A., Elias, C. L., Qudnun, B. D., Philipson, K. D., Hnatowich, M., and Hryshko, L. V. (1999) Ionic regulatory properties of brain and kidney splice variants of the NCX1 Na+/Ca2+ exchanger. J. Gen. Physiol. 114, 701–711.

29. Dunn, J., Elias, C. L., Le, H. D., Omelchenko, A., Hryshko, L. V., and Lytton, J. (2002) The molecular determinants of ionic regulatory differences between brain and kidney Na+/Ca2+ exchanger (NCX1) isoforms. J. Biol. Chem. 277, 33957–33962.

30. Hurtado, C., Prociuk, M., Maddaford, T. G., Dibrov, E., Mesaeli, N., Philipson, K. D., and Abramson, D. (2007) The second Ca2+-binding domain of the Na+/Ca2+ exchanger is essential for regulation: crystal structures and mutational analysis. Proc. Natl. Acad. Sci. U.S.A. 104, 18467–18472.

31. Hilge, M., Aelen, J., Foarse, A., Perrakis, A., and Vuister, G. W. (2009) Ca2+ regulation in the Na+/Ca2+ exchanger features a dual electrostatic switch mechanism. Proc. Natl. Acad. Sci. U.S.A. 106, 14333–14338.

32. Wu, M., Tong, S., Gonzalez, J., Jayaraman, V., Spudich, J. L., and Zheng, L. (2011) Structural basis of the Ca2+ inhibitory mechanism of Drosophila Na+/Ca2+ exchanger CALX and its modification by alternative splicing. Structure 19, 1509–1517.

33. Matsuoka, S., Nicoll, D. A., Hryshko, L. V., Levitsky, D. O., Weiss, J. N., and Philipson, K. D. (1995) Regulation of the cardiac Na+/Ca2+ exchanger by Ca2+. Mutational analysis of the Ca2+-binding domain. J. Gen. Physiol. 105, 403–420.

34. Fernàndez-Busquets, X., Kammerer, R. A., and Burger, M. M. (1996) A crystallographic model of the cardiac sarcolemmal Na+/Ca2+ exchanger. J. Biol. Chem. 271, 21577–21581.

35. Wu, M., Le, H. D., Wang, M., Yurkov, V., Omelchenko, A., Hnatowich, M., Nix, I., Hryshko, L. V., and Zheng, L. (2010) Crystal structures of progressive Ca2+-binding states of the Na+/Ca2+ sensor Ca2+-binding domain 1 (CBD1) from the CALX Na+/Ca2+ exchanger reveal incremental conformational transitions. J. Biol. Chem. 285, 2554–2561.

36. Besserer, G. M., Ottolia, M., Nicoll, D. A., Chaptal, V., Cascio, D., Philipson, K. D., and Abramson, J. (2007) The second Ca2+-binding domain of the Na+/Ca2+ exchanger is essential for regulation: crystal structures and mutational analysis. Proc. Natl. Acad. Sci. U.S.A. 104, 18467–18472.
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chamber. J. Biol. Chem. 266, 1014–1020
67. He, Z., Feng, S., Tong, Q., Hilgemann, D. W., and Philipson, K. D. (2000) Interaction of PIP\(_2\) with the XIP region of the cardiac sodium/calcium exchanger. Am. J. Physiol. Cell Physiol. 278, C661–C666
68. Pignataro, G., Gala, R., Cuomo, O., Tortiglione, A., Giaccio, L., Castaldo, P., Sirabella, R., Matrone, C., Canitano, A., Amoroso, S., Di Renzo, G., and Annunziato, L. (2004) Two sodium/calcium exchanger gene products, NCX1 and NCX3, play a major role in the development of permanent focal cerebral ischemia. Stroke 35, 2566–2570
69. Lee, C., Dhall, N. S., and Hryshko, L. V. (2005) Therapeutic potential of novel Na\(^+/\)Ca\(^{2+}\) exchange inhibitors in attenuating ischemia-reperfusion injury. Can. J. Cardiol. 21, 509–516
70. Iwamoto, T., Kita, S., Zhang, J., Blaustein, M. P., Arai, Y., Yoshida, S., Wakimoto, K., Komuro, I., and Katsuragi, T. (2004) Salt-sensitive hypertension is triggered by Ca\(^{2+}\) entry via Na\(^+/\)Ca\(^{2+}\) exchanger type 1 in vascular smooth muscle. Nat. Med. 10, 1193–1199
71. Blaustein, M. P., Zhang, J., Chen, L., and Hamilton, B. P. (2006) How does salt retention raise blood pressure? Am. J. Physiol. Regul. Integr. Comp. Physiol. 290, R514–R523
72. Craner, M. J., Newcombe, J., Black, J. A., Hartle, C., Cuzner, M. L., and Waxman, S. G. (2004) Molecular changes in neurons in multiple sclerosis: altered axonal expression of Na\(_{1.2}\) and Na\(_{1.6}\) sodium channels and Na\(^+/\)Ca\(^{2+}\) exchanger. Proc. Natl. Acad. Sci. U.S.A. 101, 8168–8173
73. Bano, D., Young, K. W., Guerin, C. J., Lefevre, R., Rothwell, N. J., Naldini, L., Rizzuto, R., Carafoli, E., and Nicotera, P. (2005) Cleavage of the plasma membrane Na\(^+/\)Ca\(^{2+}\) exchanger in excitotoxicity. Cell 120, 275–285
74. Iwamoto, T. (2004) Forefront of Na\(^+/\)/Ca\(^{2+}\) exchanger studies: molecular pharmacology of Na\(^+/\)/Ca\(^{2+}\) exchange inhibitors. J. Pharmacol. Sci. 96, 27–32
75. Iwamoto, T., Watano, T., and Shigekawa, M. (1996) A novel isothiourea derivative selectively inhibits the reverse mode of Na\(^+/\)/Ca\(^{2+}\) exchange in cells expressing NCX1. J. Biol. Chem. 271, 22391–22397
76. Iwamoto, T., and Kita, S. (2004) Development and application of Na\(^+/\)/Ca\(^{2+}\) exchange inhibitors. Mol. Cell Biochem. 259, 157–161
77. Iwamoto, T., Kita, S., Uehara, A., Imanaga, I., Matsuda, T., Baba, A., and Katsuragi, T. (2004) Molecular determinants of Na\(^+/\)/Ca\(^{2+}\) exchange (NCX1) inhibition by SEA0400. J. Biol. Chem. 279, 7544–7553
78. Iwamoto, T., Inoue, Y., Ito, K., Sakaue, T., Kita, S., and Katsuragi, T. (2004) The exchanger inhibitory peptide region-dependent inhibition of Na\(^+/\)/Ca\(^{2+}\) exchange by SN-6 (2-[4-(4-nitrobenzyloxy)benzyl]thiazolidine-4-carboxylic acid ethyl ester), a novel benzoxoxyphenyl derivative. Mol. Pharmacol. 66, 45–55
79. Antoons, G., Willems, R., and Sipido, K. R. (2012) Alternative strategies in arrhythmia therapy: evaluation of sodium/calcium exchange as an antiarrhythmic target. Pharmacol. Ther. 134, 26–42