Interactions between Ca\textsuperscript{2+} binding domains of the Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger and secondary regulation

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The Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger (NCX) is a plasma membrane protein particularly abundant in cardiomyocytes where it plays a prominent role in Ca\textsuperscript{2+} extrusion. In addition to being transported, cytoplasmic Ca\textsuperscript{2+} and Na\textsuperscript{+} regulate NCX activity by activating and inhibiting ion transport, respectively. There are two Ca\textsuperscript{2+} binding domains within the exchanger, CBD1 and CBD2, which have been crystallized and detailed structural information obtained. We have recently studied the roles of residues coordinating Ca\textsuperscript{2+} in both CBD1 and CBD2. To gain further insight into NCX regulation, we investigate here the presence of possible functional interactions between the two CBDs. This study reveals the important role of CBD organization in Ca\textsuperscript{2+} regulation of the exchanger.

As early as 1979 a need for a ‘finite’ amount of intracellular Ca\textsuperscript{2+} was found to be necessary for Na\textsuperscript{+}-Ca\textsuperscript{2+} Exchanger (NCX) activity in the squid axon.1 Since then, the modulatory properties of cytoplasmic Ca\textsuperscript{2+} have been studied in detail.2-4 Cytoplasmic regulatory Ca\textsuperscript{2+} activates NCX and also slows NCX entry into a Na\textsuperscript{+}-dependent inactivated state.3 The cloning of NCX6 enabled the study of the molecular details of cytoplasmic Ca\textsuperscript{2+} binding. Through a series of deletion and single site mutations, Ca\textsuperscript{2+}-binding was localized to a large intracellular loop between transmembrane segments 5 and 6.3 (see Fig. 1). More specifically, Ca\textsuperscript{2+} was found to bind to an acidic region in what was known as the “β-1 repeat” because it was the first of a tandem pair of domains with significant sequence similarity. Mutations of acidic residues in the β-1 repeat resulted in a significantly lowered affinity for regulatory Ca\textsuperscript{2+} without totally removing Ca\textsuperscript{2+} regulation.9,10

NMR and crystal structures of the β-repeats have greatly advanced our understanding of how regulatory Ca\textsuperscript{2+} binds to NCX.11-18 These studies showed that both β-1 and β-2 domains were capable of binding Ca\textsuperscript{2+}, not just β-1. The regions were renamed CBD1 and CBD2 for Calcium binding domain. As shown in Figure 1, each domain is comprised of a 7-strand antiparallel β-sandwich with Ca\textsuperscript{2+} binding sites at the head and an unstructured loop F-G at the tail of the sandwich. The CBD structures were determined independently of one another so the architecture of the conjunct Ca\textsuperscript{2+} sensors in not yet known. However, since the connection between the two CBDs is very short they must be arranged in some type of head-to-tail conformation. Thus, it is clear that the Ca\textsuperscript{2+} binding sites of the two CBDs are not in proximity.

CBD1 can bind four Ca\textsuperscript{2+} ions (Ca1-Ca4 at sites 1–4), and CBD2 binds two ions. Both CBDs primarily coordinate Ca\textsuperscript{2+} through aspartate and glutamate residues. In recent work, we focused on determining the relative roles of the four CBD1 Ca\textsuperscript{2+}-binding sites in NCX Ca\textsuperscript{2+} regulation. Single-site mutants were generated in the CBD1 region of the full length NCX and their effects on Ca\textsuperscript{2+} affinity were investigated electrophysiologically.19 The most striking observation is that the preclusion of binding of Ca\textsuperscript{2+} to sites 1 and 2 is not critical for regulation. In sharp
However, we found that deletion of amino acids 596–663 in exons ACDEF in the F-G loop of CBD2 had no effect on the biophysical properties of NCX.19 To test for the importance of the B-C strand-connecting loop, we neutralized the only charged residue, arginine 532 to cysteine (R532C) and determined the effects on outward NCX current.

Figure 2A shows outward current recorded from oocytes expressing the exchanger carrying the mutation. Recordings were performed as described previously.19 Similar to the wild type NCX (WT), the current peaked and then decayed over time (Na+-dependent inactivation). Raising intracellular Ca²⁺ concentration relieved the inactivation and increased the current.

The CBD2 F-G strand-connecting loop (aa 596–663) is of particular interest as this is a site of alternative splicing. All NCX1 isoforms contain either exon A or B, exon D and various combinations of exons C, E and F. Thus, it is tempting to speculate that the combination of exons may have an impact on NCX1 regulation. However, we found that deletion of amino acids 596–663 in exons ACDEF in the F-G loop of CBD2 had no effect on the biophysical properties of NCX.19

To test for the importance of the B-C strand-connecting loop, we neutralized the only charged residue, arginine 532 to cysteine (R532C) and determined the effects on outward NCX current. Figure 2A, shows outward current recorded from oocytes expressing the exchanger carrying the mutation. Recordings were performed as described previously.19 Similar to the wild type NCX (WT), the current peaked and then decayed over time (Na+-dependent inactivation). Raising intracellular Ca²⁺ concentration relieved the inactivation and increased the current. Figure 2B shows the dependency of the peak current on intracellular Ca²⁺ concentration. A slight decrease in the affinity for Ca²⁺ was observed in the contrast, mutations of residues involved in coordinating Ca³⁺ and Ca⁴⁺ drastically decrease the sensitivity of the exchanger for Ca²⁺. Of note was a 7-residue mutant, which should remove all Ca²⁺ binding to CBD1, but still yielded an NCX that was capable of being regulated by intracellular Ca²⁺ albeit with much reduced affinity. This implied an underlying role of CBD2 in ionic regulation. Indeed, mutations to the primary Ca²⁺-binding site of CBD2 caused deregulation of NCX. This was surprising because CBD2 has a low affinity for Ca²⁺ (~5 μM)20 and the structural differences in the Ca²⁺-bound and -free states are minimal.11 In contrast, CBD1 has an affinity for Ca²⁺ in the physiologic range (~200 nM)20,21 and appears to undergo large structural changes upon release of Ca²⁺.14,21,22

These observations give hints as to how Ca²⁺ binding to the CBDs might put NCX into a more active state. One proposal is that there is an electrostatic interaction between the two CBDs;15 Ca²⁺ binding changes that interaction and induces a twist in the hinge region between the two CBDs thus contracting their conformation with the end result being an increase in NCX activity. This proposal requires a close proximity of charged residues of the two CBDs. Candidates for the charged regions are the Ca²⁺ binding site at the head of CBD1 and the loops connecting the B-C, D-E and F-G strands at the tail of CBD2.

The CBS structure of the Na⁺⁻Ca²⁺ exchanger. Diagrammatic representation of the proposed topology of NCX. The crystal structures of the individual Ca²⁺-binding domains are shown as CBD1 (left, PDB ID 2DPK) and CBD2 (right, PDB ID 2QVM). Ca²⁺ ions are indicated as spheres. The organization of the conjunct CBDs is unknown, thus the displayed orientation of the CBDs is arbitrary within the constraint that CBD1 and 2 are connected by a short linker and therefore the C-terminus of CBD1 is expected to be in close proximity to the N-terminus of CBD2. This model positions the charged residues R532C (within loop B-C) and D565 and E566 (loop D-E) of CBD2 in the vicinity of CBD1 Ca²⁺ binding site. These residues may interact with CBD1 playing a role in NCX Ca²⁺ regulation. In Figure 2, we demonstrate that replacement of Arg123 has a minimal effect in the exchanger Ca²⁺ regulation. The function of D565 and E566 has yet to be determined. The location at which the seven alanines (7-Ala) were inserted is also indicated.
R532C NCX. These effects, however, are minimal and rule out a prominent role of this residue in NCX regulation. To explore global interactions between the CBDs, we inserted a linker of 7 alanines between H501 of CBD1 and A502 of CBD2 to alter the interactions between the CBDs (Fig. 1). This mutant, NCX-7Ala, shows an apparent decrease in affinity for cytoplasmic Ca\textsuperscript{2+} compared to WT as measured by the dependence of peak currents on Ca\textsuperscript{2+} concentration (Fig. 2A and B). We do not anticipate that the 7 alanine insertion affects the affinity of CBD1 for Ca\textsuperscript{2+} since the apparent affinity of a YFP-CBD1-CFP fusion protein is identical whether the CFP is fused after CBD1 for Ca\textsuperscript{2+} since the apparent affinities of the CBDs decrease with the CBDs (Fig. 2A and B). We do not anticipate that the 7 alanine insertion affects the affinity of CBD1 for Ca\textsuperscript{2+} since the apparent affinities of the CBDs decrease with the CBDs (Fig. 2A and B). We do not anticipate that the 7 alanine insertion affects the affinity of CBD1 for Ca\textsuperscript{2+} since the apparent affinities of the CBDs decrease with the CBDs (Fig. 2A and B). We do not anticipate that the 7 alanine insertion affects the affinity of CBD1 for Ca\textsuperscript{2+} since the apparent affinities of the CBDs decrease with the CBDs (Fig. 2A and B).

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