Addendum

How Does Regulatory Ca\textsuperscript{2+} Regulate the Na\textsuperscript{+}-Ca\textsuperscript{2+} Exchanger?

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KEY WORDS

Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger, regulatory Ca\textsuperscript{2+}, Ca\textsuperscript{2+} binding domain, Na\textsuperscript{+}-dependent inactivation

ABBREVIATIONS

CBD1 Ca\textsuperscript{2+} binding domain 1

CBD2 Ca\textsuperscript{2+} binding domain 2

NCX1.1 mammalian cardiac Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger

Addendum to:

The Second Ca\textsuperscript{2+}-Binding Domain of the Na\textsuperscript{+}-Ca\textsuperscript{2+} Exchanger Is Essential for Regulation: Crystal Structures and Mutational Analysis.

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ABSTRACT

Spatial and temporal regulation of intracellular Ca\textsuperscript{2+} concentrations is a fundamental requirement for life. The mammalian cardiac Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger serves as the main mechanism for Ca\textsuperscript{2+} efflux after heart contraction. Exchange activity is highly regulated by intracellular Ca\textsuperscript{2+}, which binds two regulatory domains (CBD1 and CBD2) and triggers the full activity of the exchanger. We solved the X-ray crystallographic structure of CBD2 in the presence and absence of Ca\textsuperscript{2+}. Together with mutational analysis of the Ca\textsuperscript{2+} binding sites, this study reveals the crucial role of one of the two bound Ca\textsuperscript{2+} ions and helps propose hypotheses on the mechanism of regulation of the exchanger.

Ca\textsuperscript{2+} is an essential ion involved in a multitude of fundamental life processes that include signal transduction, secretion, metabolic control, synaptic plasticity, expression of genes and muscle contraction.\textsuperscript{1} For Ca\textsuperscript{2+} to be efficiently used, compartmental concentrations have to be regulated in a precise and timely fashion. The mammalian cardiac Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger (NCX1.1) plays an important role in excitation-contraction coupling, serving as the primary mechanism for Ca\textsuperscript{2+} efflux.\textsuperscript{2} NCX1.1 uses the energy stored in the Na\textsuperscript{+} gradient to exchange 3Na\textsuperscript{+} for 1Ca\textsuperscript{2+}.\textsuperscript{3,4} The intracellular levels of Ca\textsuperscript{2+} and Na\textsuperscript{+} are known to also regulate the activity of the exchanger. Rising Na\textsuperscript{+} concentrations lead to Na\textsuperscript{+}-dependent inactivation, while intracellular regulatory Ca\textsuperscript{2+} activates the exchanger by decreasing the extent of Na\textsuperscript{+}-dependent inactivation and by directly activating the exchanger.\textsuperscript{5} Structurally, NCX1.1 has nine predicted transmembrane \(\alpha\)-helices and a large cytoplasmic loop of approximately 500 residues. Hilge and colleagues determined by NMR the structure of two Ca\textsuperscript{2+} binding domains (CBDs) in this loop designated as CBD1 and CBD2. Each CBD displays an immunoglobulin fold that binds Ca\textsuperscript{2+} on one side of the domain.\textsuperscript{6} Although this study greatly increased our knowledge of the structure of the loop, NMR cannot directly observe the position and the number of bound Ca\textsuperscript{2+}. In a subsequent study, we solved the X-ray crystallographic structure of CBD1 and improved our view of the Ca\textsuperscript{2+} binding site by revealing four bound Ca\textsuperscript{2+} ions, instead of the two Ca\textsuperscript{2+} ions estimated by NMR.\textsuperscript{7} In a recent article published in PNAS, we have now solved the X-ray crystallographic structure of CBD2 and accurately described its Ca\textsuperscript{2+} coordination pattern. Combined with mutational and electrophysiological analysis, this work sheds new light on the crucial role of CBD2 in the regulation of Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange activity.\textsuperscript{8}

The gene coding for NCX1 includes a cluster of exons coding a variable region in an unstructured loop of CBD2. Alternative splicing of these exons generates multiple tissue specific variants of NCX1.\textsuperscript{9} In crystallization trials, we used CBD2 of the NCX1.4 splice variant. We solved the structure in the presence and absence of Ca\textsuperscript{2+}. CBD2 binds two Ca\textsuperscript{2+} ions in sites that we have designated as primary and secondary, based on accessibility in the Ca\textsuperscript{2+}-free structure, the overall coordination pattern, and mutational data. The side chains of E516, D578 and E648, the main chain of E580 and two water molecules coordinates the primary Ca\textsuperscript{2+}, while the secondary Ca\textsuperscript{2+} is coordinated by the side chains of D552 and D578, and five water molecules (Fig. 1). In the absence of Ca\textsuperscript{2+}, the side chain of K585 shifts to a similar position as the secondary Ca\textsuperscript{2+} site, forming salt bridges with D552 and E648, and hydrogen bonds the E580 carbonyl oxygen. CBD1 has a glutamate at the position of K585 and it has been shown that its Ca\textsuperscript{2+} binding site unfolds in the absence of Ca\textsuperscript{2+}.\textsuperscript{6} Mutation of K585 to glutamate in CBD2 also results in a loss of tertiary structure of the Ca\textsuperscript{2+} binding site,\textsuperscript{9} and we have shown that it results in a modest decrease in the apparent affinity for regulatory Ca\textsuperscript{2+} (K\textsubscript{a2} values are 1.45 ± 0.18 \(\mu\)M for K585E and 0.92 ± 0.13 \(\mu\)M for WT). The coordination scheme made by K585 in the absence
of Ca\textsuperscript{2+} thereby enables CBD2 to retain its overall tertiary structure via a charge compensation mechanism, while positioning the primary site to facilitate rapid Ca\textsuperscript{2+} binding.

We determined the significance of each Ca\textsuperscript{2+} ion by examining the regulatory properties of full-length NCX1.1 proteins containing mutations at residues that coordinate regulatory Ca\textsuperscript{2+} ions. There are not expected to be any structural differences between NCX1.1 and NCX1.4 in the Ca\textsuperscript{2+} binding region of CBD2 as these splice variants have almost identical Ca\textsuperscript{2+} regulations.\textsuperscript{10} The primary Ca\textsuperscript{2+} binding site mutants, E516L, D578V and E648V (NCX1.4 numbering), lose all Ca\textsuperscript{2+} regulation; that is, they display full exchange activity even without regulatory Ca\textsuperscript{2+}. On the other hand, the D552V mutation of the second Ca\textsuperscript{2+} binding site behaves like the wild type exchanger. This shows the importance of the residues coordinating the primary Ca\textsuperscript{2+} in the regulation of the activity of the exchanger. Considering the fact that binding of Ca\textsuperscript{2+} is the sensor that triggers full activity of the exchanger, our study clearly shows that the primary Ca\textsuperscript{2+} binding site is the key component in exchanger activation by CBD2.

But how is the information of Ca\textsuperscript{2+} binding transmitted to the exchanger? Obviously, the small conformational change that we observe in CBD2 between the presence and absence of Ca\textsuperscript{2+} cannot explain by itself how the regulation of exchanger activity occurs. The information has to somehow be transduced to the transmembrane domains, to perhaps release a steric hindrance that prevents the exchanger from achieving maximal exchange rate. One clue to help understand this regulatory mechanism comes from the regulation exerted by C2 domains. C2 domains are close structural homologs of CBD2 and bind Ca\textsuperscript{2+} in a similar manner; C2 domains are present in many proteins and their role in the regulation of synaptotagmin has been extensively examined due to importance in synaptic membrane fusion.\textsuperscript{7,11} The binding of Ca\textsuperscript{2+} recruits the C2 domains to the plasma membrane using phospholipid headgroups to coordinate the Ca\textsuperscript{2+} and therefore bring the two membranes in close vicinity. Figure 2 depicts this phenomenon by showing the structure of the complex between the C2 domain of protein kinase C\textalpha (PKC\textalpha) and phosphatidylycerine.\textsuperscript{12} Following this lead, we speculate that CBD2 might also be recruited to the membrane or to another part of the exchanger in a Ca\textsuperscript{2+}-dependent manner. In addition, several membrane trafficking proteins have multiple C2 domains.\textsuperscript{13} This is much like the tandem arrangement of CBD1 and CBD2 in the intracellular loop of the exchanger and accentuates the similarity with the C2 domains and a possible mechanism of Ca\textsuperscript{2+} regulation.

Further evidence supporting this hypothesis and reinforcing the role of regulatory Ca\textsuperscript{2+} in exchanger physiology lies in the differences existing between splice variants of NCX that contain either exon A or B. These two exons are mutually exclusive and correspond to residues 570–603 of NCX1.1.\textsuperscript{9} The amino acid differences include the residues involved in Ca\textsuperscript{2+} binding; specifically the key residue D578 in exon A is replaced by an arginine in exon B. The presence of either exon A or B affects exchanger function.\textsuperscript{10} Exon B eliminates the effect of Ca\textsuperscript{2+} on Na\textsuperscript{+}-dependent inactivation. We find that the mutation D578V eliminates all Ca\textsuperscript{2+} regulation. It is probable that the replacement of the aspartate to either arginine or valine eliminates the primary Ca\textsuperscript{2+} binding site of CBD2. However, the presence of arginine instead of valine probably allows different interactions of CBD2 to another part of the exchanger to produce a different phenotype.

Finally, it is notable that DiPolo and Beaugé have recently published that regulatory Ca\textsuperscript{2+} influences the affinities of the intra- and extracellular transport sites for Na\textsuperscript{+} and Ca\textsuperscript{2+} in the NCX of squid axons.\textsuperscript{14} They showed that increasing the degree of saturation of regulatory Ca\textsuperscript{2+} increases the affinity of the external Na\textsuperscript{+} and Ca\textsuperscript{2+} transport sites, while any condition that impairs Ca\textsuperscript{2+} binding to the
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The regulatory site results in a decrease in the affinity of the exchanger for both external ions. Considering that NCX1.1 and the squid axon exchanger share 58% sequence identity and that the key residues of CBD2 involved in NCX1.1 Ca\(^{2+}\) regulation are conserved in the sequence of the squid axon exchanger (E516, D578 and E648), it is likely that the Ca\(^{2+}\) regulation of these two exchangers have a common mechanism. The binding of regulatory Ca\(^{2+}\) to the primary site of CBD2 transduces its information to the exchanger translocation pathway and results in a conformation change that may modify affinity for the transported ions.

Our study of CBD2 sheds new light on the regulation of the Na\(^{+}-\)Ca\(^{2+}\) exchanger. To enhance our comprehension of this highly complex and regulated machinery, much needs to be done. Understanding the functions of the large intracellular loop is important: how do the different domains of the loop interact with each other and with the transmembrane segments? How many of these interactions are Ca\(^{2+}\) dependent? We also do not yet understand how CBD1 and CBD2 interact with one another and how the information of Ca\(^{2+}\) binding to these two domains is integrated. To fully understand the Na\(^{+}-\)Ca\(^{2+}\) exchanger, we will require structures of the full-length protein in different conformational states.

References

1. Carafoli E. Calcium-mediated cellular signals: a story of failures. TIBS 2004; 29:371-9.
2. Bers DM. Excitation-contraction coupling and cardiac contractile force. Boston: Kluwer, 2001.
3. Fujioka Y, Komeda M, Matsuoka S. Stoichiometry of Na\(^{+}-\)Ca\(^{2+}\) exchange in inside-out patches excised from guinea-pig ventricular myocytes. J Physiol 2000; 523: 339-51.
4. Bers DM, Ginsburg KS. Na-Ca stoichiometry and cytosolic Ca-dependent activation of NCX in intact cardiomyocytes. Ann NY Acad Sci 2007; 1099:326-38.
5. Hilgemann DW, Matsuoka S, Nagel GA, Collins A. Steady-state and dynamic properties of cardiac sodium-calcium exchange. Sodium dependent inactivation. J Gen Physiol 1992; 100: 6:950-32.
6. Hilge M, Aelen J, Vuister GW. Ca\(^{2+}\) regulation in the Na\(^{+}/\)Ca\(^{2+}\) exchanger involves two markedly different Ca\(^{2+}\) sensors. Mol Cell 2006; 22:15-25.
7. Nicoll DA, Sawaya MR, Kwon S, Cascio D, Philipson KD, Abramson J. The crystal structure of the primary Ca\(^{2+}\) sensor of the Na\(^{+}/\)Ca\(^{2+}\) exchanger reveals a novel Ca\(^{2+}\) binding motif. J Bio Chem 2006; 281:21577-81.
8. Mercado Besserer G, Oxtoria M, Nicoll DA, Chaptal V, Cascio D, Philipson KD, Abramson J. The second Ca\(^{2+}\)-binding domain of the Na\(^{+}\) Ca\(^{2+}\) exchanger is essential for regulation: Crystal structures and mutational analysis. Proc Nat Acad Sci 2007; 104:18467-72.
9. Quednau BD, Nicoll DA, Philipson KD. Tissue specific and alternative splicing of the Na\(^{+}/\)Ca\(^{2+}\) exchanger isoforms NCX1, NCX2, and NCX3 in rat. Am J Physiol 1997; 272: C1250-61.
10. Dyck C, Ondelchenko A, Elias CL, Quednau B, Philipson KD, Hnatowich M, Hryshko L. Ionic regulation properties of brain and kidney splice variants of the NCX1 Na\(^{+}/\)Ca\(^{2+}\) exchanger. J Gen Physiol 1999; 114:701-11.
11. Martens S, Koelov MM, McMahon HT. How synaptotagmin promotes membrane fusion. Science 2007; 316:1205-8.
12. Verdaguer N, Corbalan-Garcia S, Ochoa W, Fita I, Gomez-Fernandez JC. Ca\(^{2+}\) bridges the C2 membrane binding domain of protein kinase C\(\delta\) directly to phosphatidylinositol. EMBO J 1999; 18; 22:6329-38.
13. Cho W, Stahlisin RV. Membrane binding and subcellular targeting of C2 domains. Biochim Biophys Acta 2006; 1761:838-49.
14. DiPolo R, Reauig L. In the squid axon Na\(^{+}/\)Ca\(^{2+}\) exchanger the state of the Cai-regulatory site influences the affinities of the intra- and extracellular transport sites for Na\(^{+}\) and Ca\(^{2+}\). Eur J Physiol 2008. DOI 10.1007/s00424-007-0430-0.