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20 Years from NCX Purification and Cloning: Milestones

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Abstract

The Na^+/Ca^{2+} exchanger protein was first isolated from cardiac sarcolemma in 1988 and cloned in 1990. This allowed study of Na^+/Ca^{2+} exchange at the molecular level to begin. I will review the story leading to the cloning of NCX and the research that resulted from this event. This will include structure-function studies such as determination of the numbers of transmembrane segments and topological arrangement. Information on ion transport sites has been gathered from site-directed mutagenesis. The regions involved in Ca^{2+} regulation have been identified, analyzed, and crystallized.

We have also generated genetically altered mice to study the role of NCX in the myocardium. Of special interest are mice with atrial- or ventricular-specific KO of NCX that reveal new information on the role of NCX in excitation-contraction coupling and in cardiac pacemaker activity.

Keywords

Na^+/Ca^{2+} exchange; Ca^{2+} transport; excitation-contraction coupling; Ca^{2+} regulation; Na^+ transport; cardiac pacemaking

2. Introduction

This chapter will provide a brief overview of the progress in the development and use of molecular tools to study the Na^+/Ca^{2+} exchanger, NCX1. Focus will be on advances made in the laboratory of the author. This is not to mean that major breakthroughs did not occur in other laboratories. For example, Na^+/Ca^{2+} exchange research was initiated by seminal measurements in cardiac tissue (Reuter and Seitz, 1968) and in the squid axon (Baker et al., 1969). Study of regulation of the exchanger was largely initiated and developed by a large body of work from Reinaldo DiPolo and Luis Beauge (2006). Reeves and Sutko (1979) stimulated the application of biochemical techniques to study Na^+/Ca^{2+} exchange with the development of an assay using cardiac sarcolemmal vesicles. Na^+/Ca^{2+} exchange research received a strong boost by the development of the giant excised patch technique by Don Hilgemann (1990). All of these advances were essential to stimulate the research described below.

The exchanger that has received the most attention at the molecular level is that present in cardiac sarcolemma, now known as NCX1, which will be the focus of this chapter. The application of molecular tools to cardiac physiology has also been a productive area of research and some examples will be presented.

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3. Isolation of the Na^+/Ca^{2+} exchange protein

Isolation of the Na^+/Ca^{2+} exchange protein was a nontrivial task. The exchanger is in low abundance and membrane proteins are almost always difficult with which to work. The protein must first be solubilized with detergent prior to any fractionation. In the case of the exchanger, there was no way to identify the protein except by function. So, every fraction from the separation procedures needed to be reconstituted into liposomes to be assayed for activity using a $^{45}Ca^{2+}$ uptake assay.

The successful isolation procedure (Philipson et al., 1988) identified a 120 kDa protein as the Na^+/Ca^{2+} exchanger. The protein migrated as a 160 kDa band under nonreducing conditions due to disulfide bond formation. Proteolysis reduced the size to 70 kDa though functional exchange activity was retained. However, the quantity of protein that could be isolated was only several micrograms – not enough for most biochemical approaches. The one useful purpose for the isolated exchanger protein was to produce anti-exchanger polyclonal antibodies.

4. Cloning of the Na^+/Ca^{2+} exchanger

We made use of our polyclonal antibody to clone the Na^+/Ca^{2+} exchanger (Nicoll et al., 1990). We screened a λ phage expression library to find a partial clone that expressed a portion of a protein that reacted with the antibodies. After much effort, we found a full-length clone by screening an unamplified homemade library. At this point, we were unsure if the protein encoded by the clone was truly the Na^+/Ca^{2+} exchanger. This was because the identity of the clone was dependent on the quality of our antibodies and the antigen used to produce the antibodies had not been completely pure. cRNA was synthesized from the clone and injected into oocytes. The injected oocytes displayed Na^+ gradient-dependent uptake of $^{45}Ca^{2+}$ confirming that the first exchanger, NCX1, had been cloned (Nicoll et al., 1990). Subsequently, we cloned NCX2 (Li et al., 1994) and NCX3 (Nicoll et al., 1996b). We and others have noticed the presence of several splice variants of the NCX clones.

DNA sequencing revealed an open-reading frame of 970 amino acids with multiple hydrophobic segments. The initial 32 amino acids represent a cleaved leader peptide and are not present in the mature protein. One site (Asn9) is glycosylated. Hydropathy analysis suggested the presence of 12 transmembrane segments (TMSs) though experimental analysis indicates 9 TMSs (Nicoll et al. 1999; Iwamoto et al., 2000). A large intracellular hydrophilic loop was present between the N- and C-terminal groups of TMSs (Fig. 1).

We noted a region at the beginning of the large intracellular loop with characteristics similar to a calmodulin-binding region. Subsequently, we found that a 20 amino acid peptide with this sequence was a potent inhibitor of NCX (Li et al., 1991). The peptide is known as XIP (exchanger inhibitory peptide). It appears that the endogenous XIP region of NCX is involved in autoregulation though the exact role of the XIP region is still not fully defined. Mutations of the XIP region have major effects on a process known as Na^+-dependent inactivation (Matsuoka et al., 1997).

Another feature of the large intracellular loop is a region of extensive alternative splicing. Different combinations of 6 small exons denoted A through F are expressed in a tissue specific manner. Exons A and B are mutually exclusive and one or the other is always present. The presence of a specific splice variant bestows distinctive regulatory properties to an exchanger though the effects are often subtle and of unclear physiological significance.

5. Structure and function

A current model of the exchanger NCX1 with 9 TMSs and a large intracellular loop is shown in Fig. 1. The model is based on cysteine accessibility experiments and epitope mapping (Nicoll et al. 1999; Iwamoto et al., 2000). Interestingly, a bacterial homologue of NCX has been determined to have 10 TMSs (Saaf et al., 2001). It remains to be confirmed whether the mammalian and bacterial homologues truly have different topologies.

5.1 Helix packing

The topology studies give us an idea of the two dimensional arrangement of NCX. We have used a crosslinking approach to begin to obtain information on the three dimensional organization of the TMSs. The approach determines the proximity of an individual TMS to other TMSs. A series of studies provides a model (Ren et al., 2010) in which the N-terminal group of TMSs is segregated from the C-terminal group of TMSs.

5.2 α Repeats and mutational analysis

The α repeats (α-1 and α-2) of NCX are two regions of intramolecular homology (Schwartz and Benzer, 1997). As shown in Fig. 1, α-1 spans portions of TMSs 2 and 3 while α-2 encompasses a portion of TMS7 and a portion of a reentrant loop between TMSs 7 and 8. The intramolecular homology strongly suggests that the exchanger evolved from a gene duplication event. The fact that only the α repeats have retained intramolecular homology through evolution suggests that these regions have key roles in the ion translocation process. Mutational analysis confirms this assertion. Mutation of several residues in the α repeats has strong effects on transport properties (Nicoll et al., 1996a; Ottolia et al., 2005; also see chapter by M. Ottolia and K.D. Philipson in this volume). We have characterized mutations of the α repeats that eliminate exchange activity or alter Na^+ and Ca^{2+} affinities.

It is also striking that homologous α repeats are present in all classes of the cation/Ca^{2+} superfamily (Cai and Lytton, 2004). Thus, the α repeats display both intra- and intermolecular homology. The α repeats are present, for example, in the NCXs, NCKXs, and in prokaryotic exchangers.

6. Regulation

The most prominent regulatory mechanisms of NCX1 are induced by Na^+ and Ca^{2+}. That is, in addition to being the substrates for transport, Na^+ and Ca^{2+} have separate regulatory roles. Although regulation of Na^+/Ca^{2+} exchange activity by Ca^{2+} has been studied for quite some time (DiPolo, 1979), the development of the giant excised patch technique by Hilgemann (1990) greatly facilitated study of regulation at the molecular level. The use of the giant patch to measure exchanger currents allowed Na^+ regulation (Na^+-dependent inactivation) of the exchanger to be described for the first time. Although experimentally Na^+ regulation can be quite prominent, the physiological role of this process is unknown. Na^+ regulation may be involved in the effects of PIP_2 on exchange activity (Hilgemann and Ball, 1996). It was fortuitous that the cloning of NCX and the development of the giant patch were both described in 1990. The giant patch approach was well suited for analysis of mutant exchangers expressed in *Xenopus* oocytes.

Ca^{2+} regulation is also most readily studied in giant excised patches. In a typical experiment, a giant patch of plasma membrane is excised from an oocyte expressing a wild type or mutant Na^+/Ca^{2+} exchanger. With Ca^{2+} in the patch pipette at the extracellular surface, an exchange current can be elicited by rapid application of Na^+ into the bath at the intracellular surface. However, having Na^+ and Ca^{2+} on opposite sides of the membrane is not sufficient by itself to initiate exchange. A low concentration of Ca^{2+} must be added to the Na^+ to bind

to a regulatory site to activate exchange activity (Hilgemann, 1990). With an exchanger clone in hand, we set out to locate the Ca^{2+} regulatory site.

We analyzed an exchanger from which we had deleted a large portion of the intracellular loop (Matsuoka et al., 1993). Loop deletion eliminated Ca^{2+} regulation and we speculated that a Ca^{2+} regulatory site was present on the large intracellular loop. This was the first published study using a mutated Na^+/Ca^{2+} exchanger. Subsequently, we employed the $^{45}Ca^{2+}$ overlay technique to localize a Ca^{2+}-binding region within the large intracellular loop (Levitski et al., 1994). We found a region of about 140 amino acids that bound Ca^{2+} with high affinity. This region has excellent overlap with what is now known as the first Ca^{2+}-binding domain (CBD1). Mutations of specific acidic amino acid residues eliminated Ca^{2+} binding.

Although we had found a Ca^{2+}-binding region, we initially had no evidence that this region was involved in Ca^{2+} regulation. To address this question, we combined site-directed mutagenesis with electrophysiological measurements using the excised patch technique. We found an excellent correlation between mutations that eliminated the binding of $^{45}Ca^{2+}$ and mutations that disrupted Ca^{2+} regulation (Matsuoka et al., 1995). Thus, we were confident that we had identified a region of the Na^+/Ca^{2+} exchanger that was involved in Ca^{2+} regulation.

An advance in the understanding of NCX Ca^{2+} regulation was the discovery by Hilge et al. (2006) that there was a second Ca^{2+} binding region adjoining the region that we had initially identified. The two Ca^{2+}-binding regions are now known as Ca^{2+}-binding domains 1 and 2 (CBD1 and CBD2). These two domains are the same as the β repeats noted by Schwartz and Benzer (1997) from sequence analysis. Hilge et al. (2006) also determined structures of CBD1 and CBD2 by NMR. The overall structures of CBD1 and CBD2 are similar comprised of a β sandwich fold with the Ca^{2+} bound by connecting loops. Shortly thereafter, we determined crystal structures of both CBDs that detailed features of the Ca^{2+}-binding sites (Nicoll et al., 2006; Besserer et al., 2007). Knowing the nature of the Ca^{2+}-binding sites allowed detailed mutational studies to probe the relative roles of CBD1 and CBD2 (Ottolia et al., 2009).

CBD1 is the more dominant player in Ca^{2+} regulation and is primary in determining the apparent Ca^{2+} affinity for regulation. We find that an intact CBD2 is also essential in regulation though the exact role of CBD2 Ca^{2+} binding is unclear. Overall, our current understanding of NCX Ca^{2+} regulation is incomplete. Essentially nothing is known of how the binding of regulatory Ca^{2+} is transduced to the transmembrane segments.

We have been successful in using fluorescent resonance energy transfer (FRET) techniques to study Ca^{2+}-induced conformational changes of the exchanger. We have used two approaches. First, we have coexpressed in *Xenopus* oocytes full-length exchangers in which either CFP or YFP has been inserted into the large intracellular loop (John et al., 2011). We then monitor fluorescence generated at the plasma membrane using a novel preparation of sheets of plasma membrane (Ottolia et al., 2007). We detect FRET indicating that exchangers are in close proximity to one another. Analysis indicates the presence of NCX dimers. A striking finding was that the interaction between exchangers was very sensitive to the level of regulatory Ca^{2+}. That is, conformational changes induced by the binding of Ca^{2+} changed the proximity of at least a portion of the intracellular loops of adjacent exchangers. Further research will resolve the significance of this finding.

Second, we have applied FRET to the study of individual CBDs (John et al., 2011). We expressed CBD1, CBD2, or CBD12 (CBD1 and CBD2 linked as occurs in the intact

exchanger) with CFP and YFP attached to both the N- and C-termini of the protein. Each construct was targeted to the plasma membrane of the oocyte with an appropriate tag. This allowed us to ascertain the Ca^{2+} dependence of Ca^{2+} binding by monitoring conformational changes by FRET. As expected from previous studies, CBD1 bound Ca^{2+} with a higher affinity than CBD2. Unexpectedly, CBD12 bound Ca^{2+} with a substantially higher affinity than either CBD1 or CBD2. The data were most consistent with a model in which the interaction of CBD2 with CBD1 increased the binding affinity of CBD1 for Ca^{2+}. The higher binding affinity of CBD12 reflects the Ca^{2+}-binding properties of the intact Na^+/Ca^{2+} exchanger.

7. Cardiac-specific knockout of the Na^+/Ca^{2+} exchanger

In addition to structure/function studies, we also do physiological studies using mice with genetically altered levels of the Na^+/Ca^{2+} exchanger. Some of our more interesting experiments have used mice with a cardiac-specific knockout of the exchanger. Three studies are of special interest:

A. We created a ventricular-specific knockout of the Na^+/Ca^{2+} exchanger (Henderson et al., 2004). Ca^{2+} enters cardiac myocytes with each excitation through the voltage-dependent Ca^{2+} channel. To maintain Ca^{2+} homeostasis, an equal amount of Ca^{2+} must be extruded from the cell and the exchanger is the primary Ca^{2+} efflux mechanism. Surprisingly, the mouse survives under these conditions but the myocardium undergoes remarkable adaptations to allow survival. One striking adaptation is that Ca^{2+} influx is markedly decreased by 80% by downregulation of the L type Ca^{2+} current and by shortening of the action potential (Pott et al., 2005). With a diminution of Ca^{2+} influx, a powerful Ca^{2+} efflux mechanism is no longer needed. Under these conditions, the PMCA is an adequate Ca^{2+} efflux mechanism. The mechanism of downregulation of the Ca^{2+} channel is of interest: In the absence of the exchanger, Ca^{2+} appears to accumulate in the diadic cleft and directly inactivate Ca^{2+} channels. Thus, we have uncovered an important autoregulatory mechanism. When Ca^{2+} efflux is limited, there is feedback to limit Ca^{2+} influx (Pott et al., 2007).

B. There has been a controversy in the field of cardiac excitation-contraction coupling regarding the role of the Na^+/Ca^{2+}exchanger in Ca^{2+}influx. More specifically, it has been hypothesized that Na^+ influx during the action potential causes a local increase in Na^+ that transiently reverses the exchanger and the resultant Ca^{2+} influx contributes to the Ca^{2+}-induced Ca^{2+} release process (LeBlanc and Hume, 1990). To test this controversial hypothesis, we examined the effects of blockade of the Na^+ channel in myocytes isolated from wild type mice and from mice with a cardiac-specific KO of the Na^+/Ca^{2+} exchanger. Blockade of the Na^+ channel had a substantial effect on the action potential-induced Ca^{2+} transient in wild type myocytes but had no effect on the KO myocytes (Larbig et al., 2010; also see chapter by J. Goldhaber et al. in this volume). The results strongly support a role for Ca^{2+} influx mediated by the Na^+/Ca^{2+} exchanger as an important contributor to cardiac contraction.

C. The KO experiments described above make use of a ventricular-specific KO of the exchanger. We have now also generated a mouse with an atrial-specific KO of the Na^+/Ca^{2+} exchanger including the sino-atrial (SA) node. This is of particular interest as there has been much recent, and controversial, work indicating a key role for the exchanger in generating the pacemaker current of the SA node (Lakatta et al., 2010). Consistent with this hypothesis, we find the SA node of the KO mice to be electrically silent and no P wave (due to atrial depolarization) is evident on

electrocardiograms. These results need to be confirmed but preliminarily our results are consistent with the exchanger being an essential component of cardiac pacemaking.

8. Final comment

The cloning of NCX1 in 1990 opened the door to molecular studies of the Na^+/Ca^{2+} exchanger. It will be exciting to follow futures advances in our understanding of this intriguing molecule.

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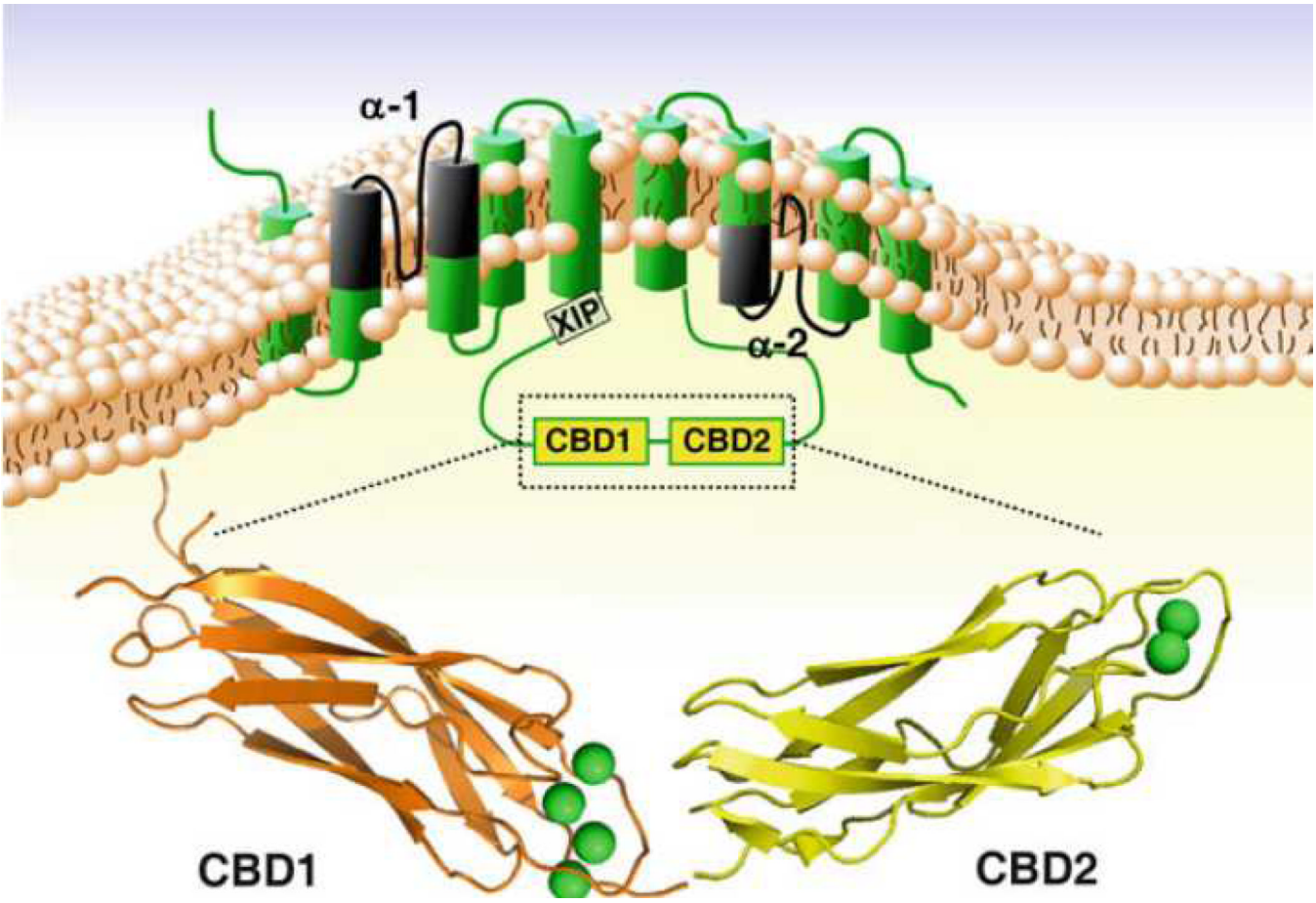

Figure 1.

Two-dimensional model of the Na^+/Ca^{2+} exchanger (NCX1). The extracellular surface is on top and shown are 9 TMSs. The α repeat regions (α-1 and α-2), critical for transport, are in black. The XIP region is involved in autoregulation and binds PIP_2. The two Ca^{2+}-binding domains (CBD1 and CBD2) regulate exchange activity. The structures of CBD1 and CBD2 are known and are shown as ribbon diagrams as determined by X-ray crystallography. Green balls represent bound Ca^{2+}.