Structure-Function Analysis of CALX1.1, a Na\(^+\)-Ca\(^{2+}\) Exchanger from *Drosophila*

MUTAGENESIS OF IONIC REGULATORY SITES

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Cytoplasmic Na\(^+\) and Ca\(^{2+}\) regulate the activity of Na\(^+\)-Ca\(^{2+}\) exchange proteins, in addition to serving as the transported ions, and protein regions involved in these processes have been identified for the canine cardiac Na\(^+\)-Ca\(^{2+}\) exchanger, NCX1.1. Although protein regions associated with Na\(^+\) and Ca\(^{2+}\)-dependent regulation are highly conserved among cloned Na\(^+\)-Ca\(^{2+}\) exchangers, it is unknown whether or not the structure-function relationships characteristic of NCX1.1 apply to other exchangers. Therefore, we studied structure-function relationships in a Na\(^+\)-Ca\(^{2+}\) exchanger from *Drosophila*, CALX1.1, which is unique among characterized members of this family of proteins in that µM levels of Ca\(^{2+}\) inhibit exchange current. Wild-type and mutant CALX1.1 exchangers were expressed in *Xenopus* oocytes and characterized electrophysiologically using the giant excised patch technique. Mutations within the putative regulatory Ca\(^{2+}\) binding site of CALX1.1, like corresponding alterations in NCX1.1, led to reduced ability (i.e. D516V and D550I) or inability (i.e. G555P) of Ca\(^{2+}\) to inhibit Na\(^+\)-Ca\(^{2+}\) exchange activity. Similarly, mutations within the putative XIP region of CALX1.1, like XIP in NCX1.1, led to two distinct phenotypes: acceleration (i.e. K306Q) and elimination (i.e. Δ310–313) of Na\(^+\)-dependent inactivation. These results indicate that the respective regulatory roles of the Ca\(^{2+}\) binding site and XIP region are conserved between CALX1.1 and NCX1.1, despite opposite responses to Ca\(^{2+}\). We extended these findings using chimeric constructs of CALX1.1 and NCX1.1 to determine whether or not functional interconversion of Ca\(^{2+}\) regulatory phenotypes was feasible. With one chimera (i.e. CALX-NCX-CALX), substitution of a 193-amino acid segment, from the large intracellular loop of NCX1.1, for the corresponding 177-amino acid segment of CALX1.1 led to an exchanger that was stimulated by Ca\(^{2+}\). This result indicates that the regulatory Ca\(^{2+}\) binding site of NCX1.1 retains function in a CALX1.1 parent transporter and that the substituted segment contains some of the amino acid sequence(s) required for transduction of the Ca\(^{2+}\) binding signal.

The identification of novel Na\(^+\)-Ca\(^{2+}\) exchange proteins has proceeded rapidly in the past 8 years. The family of Na\(^+\)-Ca\(^{2+}\) exchangers includes transporters encoded by unique gene products (1–3), as well as by a variety of alternatively spliced variants (4–7). Promoter elements underlying the tissue-specific expression of alternatively spliced exchangers have been described (8, 9), and developmental changes in splice variant expression have been reported (7). Moreover, exchangers have been cloned from several species and tissue types, and the opportunity now exists for detailed comparative structure-function studies of this family of transport molecules (10).

Regulation of Na\(^+\)-Ca\(^{2+}\) exchange activity by several factors has been described, including Na\(^+\), and Ca\(^{2+}\), with the latter being the most thoroughly studied at the molecular level. First identified in the squid giant axon (11, 12), Ca\(^{2+}\)-dependent regulation is apparent as a stimulation of Na\(^+\)-Ca\(^{2+}\) exchange current in response to µM levels of Ca\(^{2+}\) (13). The basis for this behavior, originally termed I\(_2\) inactivation, is thought to involve entry of the exchanger protein into an I\(_2\) inactive state upon removal of Ca\(^{2+}\) (14). A high affinity Ca\(^{2+}\) binding site has been identified for the canine cardiac exchanger, NCX1.1, which appears to be closely associated with the Ca\(^{2+}\)-dependent regulatory process. This site comprises a 138-amino acid segment of the large intracellular loop of NCX1.1; mutation of specific residues within this region can lead to reductions in both 45Ca\(^{2+}\) binding affinity to fusion proteins (15) and the affinity for functional Ca\(^{2+}\), regulation as assessed electrophysiologically (16).

The cardiac Na\(^+\)-Ca\(^{2+}\) exchanger also undergoes an inactivation process in response to the application of Na\(^+\) (13, 17). This mechanism, termed Na\(^+\)-dependent or I\(_1\) inactivation, is analogous to ion channel gating and involves the exchanger inhibitory peptide (XIP) region at the N terminus of the large cytoplasmic loop of NCX1.1 (18). This amino acid sequence, comprising residues 219–238, was originally identified based upon primary structural similarity with calmodulin binding sites (1). Exogenous application of a peptide corresponding to this amino acid sequence (i.e. XIP) to the intracellular surface of excised membrane patches produces marked inhibition of Na\(^+\)-Ca\(^{2+}\) exchange currents (19, 20). More recent studies have shown that mutations within the XIP region of NCX1.1 are associated with substantial alterations in the rate and extent of I\(_1\) inactivation (18), lending support to the notion that the XIP region of NCX1.1 is intimately involved in the mechanism of Na\(^+\)-dependent inactivation and that exogenous application of XIP may mimic this process. Both of the ionic regulatory mechanisms (i.e. I\(_1\) and I\(_2\)) can be eliminated by limited proteolysis.

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† The abbreviations used are: XIP, exchanger inhibitory peptide; MOPS, 4-morpholinepropanesulfonic acid.
of membrane patches with α-chymotrypsin, apparently converting NCX1.1 into a fully active, deregulated exchanger (13).

Although the role of I1 and I2 regulation in physiological Na\(^+\)-Ca\(^{2+}\) exchange function remains poorly understood, both processes can be readily demonstrated in intact cellular preparations (21, 22). Furthermore, substantial interaction between I1 and I2 regulation has been demonstrated in both structure-function studies and electrophysiological analyses (14, 16, 18). We have recently observed substantial differences in I1 and I2 regulation for alternatively spliced isoforms of CALX1 (23). The fact that alternative splicing has targeted these regulatory mechanisms at least suggests that they may play relevant physiological roles. However, it is unclear how I1 and I2 inactivation occur at the molecular level.

To date, structure-function studies of the Na\(^+\)-Ca\(^{2+}\) exchanger have been restricted to NCX1.1 (16, 18, 20, 24–26), and it is unknown whether or not these findings can be extended to other members of this family of transport proteins. Thus, we combined mutagenesis, chimeric exchanger construction, and electrophysiological measurements to investigate amino acid sequences involved in ionic regulatory mechanisms of CALX1.1, a Na\(^+\)-Ca\(^{2+}\) exchanger from Drosophila melanogaster (27, 28). The Drosophila protein was selected for study because it is unique among characterized exchangers in terms of its negative regulatory response to Ca\(^{2+}\) (29). Targets for mutagenesis of CALX1.1 were selected at or near regions analogous to the regulatory Ca\(^{2+}\)-binding site and XIP region of NCX1.1, and we found striking parallels in terms of altered ionic regulatory properties between the two exchangers. Our results indicate that CALX1.1 and NCX1.1 employ an equivalent site for binding of regulatory Ca\(^{2+}\). Other regions of the CALX1.1 cytoplasmic loop were identified that completely alleviate Ca\(^{2+}\) regulation. Mutations within the XIP region alter the pattern of Na\(^+\)-dependent inactivation, similar to that observed for NCX1.1. Our results indicate that amino acid sequences subserving Na\(^+\)- and Ca\(^{2+}\)-dependent regulatory processes are conserved between CALX1.1 and NCX1.1, irrespective of the fact that these exchangers are regulated by Ca\(^{2+}\) in opposing fashions.

**EXPERIMENTAL PROCEDURES**

**Mutant/Chimera Construction and cRNA Synthesis**—Amino acid substitution and deletion mutations were introduced, essentially as described (30), into CALX1.1 and NCX1.1 cDNA in pBluescript II KS(+) (Stratagene). Six chimeric CALX1.1:NCX1.1 Na\(^+\)-Ca\(^{2+}\) exchangers were constructed by introduction of two unique (silent) restriction sites at corresponding amino acid loci (see Fig. 7) in parent NCX1.1 and CALX1.1 cDNAs (i.e. Bst 1107I and Bss SI sites introduced into CALX1.1 cDNA at nucleotide positions 1193–1198 and 1748–1753 relative to the initiator Met, respectively, and into NCX1.1 cDNA at positions 1085–1090 and 1688–1693 relative to the initiator Met, respectively), followed by standard subcloning procedures. Mutant/chimera cassettes were repaired into unadulterated plasmid CALX1.1 or NCX1.1 cDNA and the subcloned fragments subsequently sequenced (Sequenase 2.0; Amersham Pharmacia Biotech) to verify the authenticity of altered and flanking sequences. Templates for CALX1.1-based mutant/chimera constructs were linearized by digestion with NotI and cRNA subsequently synthesized using T7 Message Machine in vitro transcription kits (Ambion). NCX1.1-based constructs were linearized with HindIII and cRNA synthesized using T3 kits (Ambion).

**Electrophysiology**—Complimentary RNAs (–5 ng) encoding wild type and mutant/chimic constructs were injected into oocytes obtained from Xenopus laevis (29, 31), and outward Na\(^+\)-Ca\(^{2+}\) exchange currents were measured 3–7 days later. Na\(^+\)-Ca\(^{2+}\) exchange activity was assessed using a giant excised patch technique (10) as described (29, 31). Outward (i.e. reverse) exchange currents were examined exclusively because this configuration separates regulatory and transported Ca\(^{2+}\) to opposite surfaces of the cell membrane. Borosilicate glass pipettes (N-51A, Drummond Scientific) were pulled and polished to a final diameter of ~20–35 μm and coated with a Parafilm®-mineral oil mixture to reduce electrical noise and enhance patch stability. Oocytes were placed in 100 mM KOH, 100 mM MES, 20 mM HEPES, 5 mM EGTA, 5 mM MgCl\(_2\), pH 7.0 (30 °C), with MES, and 1–5 GH seals formed via gentle suction after touching the oocyte membrane. Inside-out membrane patches were excised by retraction of the pipette. Pipette solutions contained 100 mM NMG-MES, 30 mM HEPES, 30 mM tetraethylammonium- OH, 16 mM NH\(_4\)SO\(_4\), 8 mM CaCl\(_2\), 6 mM KOH, 0.25 mM ouabain, 0.1 mM niflumic acid, 0.1 mM flufenamic acid, pH 7.0 (30 °C), with MES. Pipettes were connected to the headstage of an Axon Instruments patch clamp amplifier and all command potentials and analyses were conducted using pClamp software (Axon Instruments). Rapid (i.e. ~0.2 s) bath solution changes were accomplished using a custom-built, 20-channel solution switcher. Outward Na\(^+\)-Ca\(^{2+}\) exchange currents were activated by application of Na\(^+\)-containing solutions to the cytoplasmic surface of the patch. Solutions contained 100 mM (Na + Li)-aspartate, 20 mM tetraethylammonium- OH, 20 mM MOPS, 20 mM CsOH, 10 mM EGTA, 1.15 mM Mg(OH)\(_2\), 0–2.3 mM CaCl\(_2\), pH 7.0 (30 °C), with MES or LiOH. Magnesium and Ca\(^{2+}\) were adjusted to yield free concentrations of 1.00 mM and 0–30 μM, respectively, using MAXC software (32). All experiments were performed at 30 °C and all data reported are mean ± S.E.

**RESULTS**

**Regulation and Deregulation of CALX1.1 Na\(^+\)-Ca\(^{2+}\) Exchange Currents**—In the left panel of Fig. 1, fully regulated outward Na\(^+\)-Ca\(^{2+}\) exchange currents are shown from an oocyte membrane patch expressing CALX1.1. The pipette contained 8 mM Ca\(^{2+}\) (i.e. transported Ca\(^{2+}\), ) and the outward current was activated by applying 100 mM Na\(^+\), to the cytoplasmic surface of the patch. In the absence of Ca\(^{2+}\), a large outward current was observed that decayed over seconds to a lower steady-state value, reflecting the I1 inactivation process. Following application of 1 μM Ca\(^{2+}\), a further decrease of current was observed (negative Ca\(^{2+}\), regulation), followed by a return to steady-state values upon Ca\(^{2+}\) removal. Results shown on the right are from a different patch that was treated with 2 mg/ml α-chymotrypsin for ~1 min in order to deregulate CALX1.1. In this case, I1 inactivation was nearly abolished, and outward currents were similar in the presence or absence of 1 μM Ca\(^{2+}\). The results shown are representative of those obtained from 16 regulated patches and 5 deregulated patches.

**Fig. 1. Regulation and deregulation of Na\(^+\)-Ca\(^{2+}\) exchange current for CALX1.1.** In the left panel, regulated outward Na\(^+\)-Ca\(^{2+}\) exchange current is shown from a membrane patch expressing CALX1.1. Current was activated by the application of 100 mM Na\(^+\), in the absence of regulatory Ca\(^{2+}\). A large current was observed that decays to a lower, steady-state value, reflecting the I1 inactivation process. Following application of 1 μM Ca\(^{2+}\), a further decrease of current was observed (negative Ca\(^{2+}\), regulation), followed by a return to steady-state values upon Ca\(^{2+}\) removal. Results shown on the right are from a different patch that was treated with 2 mg/ml α-chymotrypsin for ~1 min in order to deregulate CALX1.1. In this case, I1 inactivation was nearly abolished, and outward currents were similar in the presence or absence of 1 μM Ca\(^{2+}\). The results shown are representative of those obtained from 16 regulated patches and 5 deregulated patches.
activity by α-chymotrypsin was observed in five patches and is qualitatively identical to that observed with NCX1.1 or NCX2 following similar treatment (2, 16).

The Putative Regulatory Ca$^{2+}$ Binding Site—The regulatory Ca$^{2+}$, binding site of NCX1.1 has been localized to amino acids 371–508 in the cardiac Na$^{+}$–Ca$^{2+}$ exchanger, NCX1.1. Two clusters of acidic amino acid residues are prominent within this region, and specific mutations of acidic residues lead to alterations in the affinity of Ca$^{2+}$, binding and functional regulation. In this alignment, we show the analogous acidic clusters for CALX1.1 and X. laevis is not shown because complete amino acid sequences were not available at the time of writing.

Mutagenesis of CALX1.1 Regulatory Sites—Mutations in the regulatory Ca$^{2+}$ binding site of CALX1.1 and a variety of other Na$^{+}$−Ca$^{2+}$ exchangers. Amino acid numbering for Caenorhabditis elegans and X. laevis is not shown because complete amino acid sequences were not available at the time of writing.

Mutations within the regulatory Ca$^{2+}$ binding site of CALX1.1. Outward Na$^{+}$−Ca$^{2+}$ exchange currents are shown for CALX1.1 and three exchangers with mutations in the regulatory Ca$^{2+}$, binding site. All currents were activated by applying 100 mM Na$^{+}$. When present, 1, 3, or 10 μM Ca$^{2+}$, was present prior to and during current activation. Compared with CALX1.1, currents from G555P were insensitive to Ca$^{2+}$, whereas those from D516V and D550I exhibited a lower affinity for negative Ca$^{2+}$, regulation. Current traces for G555P and D550I were filtered at 200 Hz.
Mutagenesis of CALX1.1 Regulatory Sites

Fig. 4. The putative XIP region. The XIP region of NCX1.1 is located between amino acids 219–238, near the N terminus of its large cytoplasmic loop. In this alignment, we show the equivalent region for CALX1.1 and a variety of other Na\(^{+}\)-Ca\(^{2+}\) exchangers. Amino acid numbering for C. elegans and X. laevis is not shown because complete amino acid sequences were not available at the time of writing.

mutations were predicted to mirror the two phenotypic classes of XIP mutants, namely acceleration (i.e. type I) and elimination (i.e. type II) of Na\(^{+}\)-dependent inactivation (18).

Mutations in the XIP Region—Fig. 5 shows typical outward Na\(^{+}\)-Ca\(^{2+}\) currents activated in response to a range of Na\(^{+}\) concentrations to illustrate the I\(_1\) inactivation process in more detail. All records were obtained in the absence of regulatory Ca\(^{2+}\). For CALX1.1, both current magnitude and the extent of current inactivation rose with increasing [Na\(^{+}\)]. For K306Q (analogous to K225Q in NCX1.1), a similar response was observed, although the rate of I\(_1\) inactivation is faster. For example, in response to a pulse of 100 mM Na\(^{+}\), the rate of inactivation for K306Q was 1.27 ± 0.06 s\(^{-1}\) (n = 10) as compared with 0.56 ± 0.04 s\(^{-1}\) (n = 25) for CALX1.1. Also consistent with an accelerated entry of K306Q into the I\(_1\) inactive state as compared with CALX1.1 is the observation that the fraction of steady-state to peak outward current (F\(_{ss}\)) for K306Q is slightly lower than for CALX1.1 (0.21 ± 0.01 versus 0.26 ± 0.01 s\(^{-1}\) for K306Q and CALX1.1, respectively), indicative of a greater proportion of the K306Q exchanger population being in the I\(_1\) inactive state. In contrast, for the deletion mutant Δ310–313 (analogous to Δ229–232 in NCX1.1), I\(_1\) inactivation was apparently eliminated. These results with K306Q and Δ310–313 are directly analogous to those observed for NCX1.1 type I and II mutant exchangers, respectively. Apparently, the XIP-like region of CALX1.1 and the XIP region of NCX1.1 play similar functional roles in the process of Na\(^{+}\)-dependent regulation of exchange activity.

To examine more closely the role of the XIP-like region of CALX1.1 in the process of Na\(^{+}\)-dependent regulation, we performed paired-pulse experiments in order to directly determine the rate constant controlling recovery from the I\(_1\) inactivation process. In all cases, outward currents were activated by 100 mM Na\(^{+}\), the rate constant controlling recovery from the I\(_1\) inactivation mechanism has been eliminated in this mutant. This virtually instantaneous recovery of Δ310–313 was observed in four patches and is qualitatively similar to that of CALX1.1, in which, following deregulation of the exchanger by α-chymotrypsin-treatment, I\(_1\) inactivation was also undetectable (Fig. 6, bottom left panel). Because Δ310–313 does not appear to enter the I\(_1\) inactive state, these data are not plotted as recoveries.

Ca\(^{2+}\)-dependent Regulation of Chimeric Exchangers—The above results indicate that the function of the Ca\(^{2+}\) binding site and XIP region is conserved between CALX1.1 and NCX1.1 in that analogous mutations in either region lead to analogous changes in Ca\(^{2+}\) and Na\(^{+}\)-dependent regulation, respectively. Yet these exchangers exhibit opposite responses to regulatory Ca\(^{2+}\). In an effort to determine the basis for this apparent anomaly and identify protein domains that are responsible for imparting a particular regulatory phenotype, we constructed six chimeric CALX1.1-NCX1.1 exchangers (Fig. 7, bottom panel) and examined their responses to Ca\(^{2+}\). Fig. 7 illustrates this approach. Two silent restriction sites were engineered into

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**Table 1:**

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<thead>
<tr>
<th>Species</th>
<th>Amino Acid Sequence</th>
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<tr>
<td>CALX1.1</td>
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<tr>
<td>Dog NCX1</td>
<td>RLLLFYKRYVRKRAQKQ</td>
</tr>
<tr>
<td>Human NCX1</td>
<td>RLLLFYKRYVRKRAQKQ</td>
</tr>
<tr>
<td>Bow NCX1</td>
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</tr>
<tr>
<td>C. elegans</td>
<td>RLYNQVPLHRYRRSOGM</td>
</tr>
</tbody>
</table>

**Fig. 5.** Mutations within the XIP region of CALX1.1. Outward Na\(^{+}\)-Ca\(^{2+}\) exchange currents are shown for CALX1.1 and two exchangers with mutations in the putative XIP region. Currents were activated by applying the indicated concentrations of Na\(^{+}\), in the absence of Ca\(^{2+}\). CALX1.1 exhibited the typical increase in current, and the extent of Na\(^{+}\)-dependent (i.e. I\(_1\)) inactivation as Na\(^{+}\) was increased. A similar response was observed for K306Q, although the rate and extent of current decay was greater. In contrast, the deletion mutant Δ310–313 did not undergo Na\(^{+}\)-dependent inactivation.
both NCX1.1 and CALX1.1 within regions of perfect sequence conservation among all cloned Na\(^{+}\)-Ca\(^{2+}\) exchangers to optimize our chances of obtaining functional constructs (Fig. 7, middle panel). This allowed construction of exchanger casettes containing 1) the first five transmembrane-spanning segments and XIP region, 2) the regulatory Ca\(^{2+}\) binding domain (currently defined as amino acids 371–508 in NCX1.1) (10, 15, 16) with adjacent sequences, and 3) the alternative splicing region and transmembrane-spanning segments 6–11. We hypothesized that if the regulatory Ca\(^{2+}\) binding site and XIP region were indeed as similar in both exchangers, as our data indicate, then 1) normal Ca\(^{2+}\) binding and I\(_{1}\) inactivation might be preserved, and 2) the approximate location of other domains more directly involved in transduction of the Ca\(^{2+}\) binding signal might be revealed.

Fig. 5 summarizes results from a comparison of the regulatory effects of 1 \(\mu\)M Ca\(^{2+}\), for NCX1.1, CALX1.1, and a chimeric exchanger designated CALX:NCX:CALX. All currents were activated by 100 mM Na\(^{+}\), with Ca\(^{2+}\) present or absent, as indicated. The representative traces shown illustrate the typical stimulatory effect of Ca\(^{2+}\) on NCX1.1 outward current and the inhibition of CALX1.1 exchange activity. For the chimera, however, the majority of which comprises CALX1.1 sequence (see Fig. 7), Na\(^{+}\)-Ca\(^{2+}\) exchange current was also stimulated by regulatory Ca\(^{2+}\), with peak current being more prominently affected than that of steady-state. The graph in Fig. 5, bottom left panel, presents peak current data from 10 NCX1.1 patches and 9 CALX:NCX:CALX patches, covering a range of Ca\(^{2+}\) concentrations. At every concentration examined, NCX1.1 peak current was inhibited by Ca\(^{2+}\), whereas that of the chimera was stimulated. Although it is clear that CALX:NCX:CALX does not behave identically to NCX1.1, it is evident that we have obtained a partial interconversion of regulatory phenotypes. Of the remaining five chimeras in this series, four did not produce measurable levels of Na\(^{+}\)-Ca\(^{2+}\) exchange current. The common feature among this inactive group was that each had dissimilar N- and C-terminal thirds (i.e. CALX:CALX:NCX, CALX:NCX:NCX, NCX:CALX:NCX, NCX:NCX:CALX, and NCX:CALX:CALX). In contrast, the chimera NCX:CALX:NCX yielded measurable outward exchange current that retained Na\(^{+}\)-dependent inactivation but was insensitive to Ca\(^{2+}\), up to 30 \(\mu\)M (data not shown), like G555P (see Fig. 3).

**DISCUSSION**

In this study, we have examined ionic regulation of the *Drosophila Na\(^{+}\)-Ca\(^{2+}\) exchanger CALX1.1 using mutagenesis
been no studies to determine whether or not this site performs a similar function in other exchangers. Thus, we selected for study an exchanger, CALX1.1, with a Ca\(^{2+}\) regulatory phenotype opposite to that of NCX1.1 for two main reasons. First, if the Ca\(^{2+}\) binding site serves a similar functional role in both NCX1.1 and CALX1.1, we anticipated that its affinity could be predictably altered by mutagenesis. Second, we speculated that if the 138-amino acid Ca\(^{2+}\) binding domain of NCX1.1 contained some (or all) of the amino acid determinants directly responsible for transduction of the Ca\(^{2+}\) binding signal, then interchanging this region between CALX1.1 and NCX1.1 presented the greatest likelihood of observing phenotypic differences in the recipient exchanger.

Our results show that NCX1.1-analogous mutations within the regulatory Ca\(^{2+}\) binding site of CALX1.1 lead to parallel changes in the ability of Ca\(^{2+}\) to regulate exchange activity. That is, mutation of corresponding amino acid residues (see Fig. 2) reduces the affinity for Ca\(^{2+}\) regulation, irrespective of whether the regulatory effect is stimulatory (i.e. NCX1.1) or inhibitory (i.e. CALX1.1). This result strongly suggests that the function of the Ca\(^{2+}\) binding site is similar in both exchangers. Considering the striking degree of amino acid sequence similarity between the acidic clusters within the putative Ca\(^{2+}\) binding sites of all exchanger subtypes and species variants shown in Fig. 2, and our present results showing a conserved functional role for exchangers sharing only ~50% overall sequence identity and opposite Ca\(^{2+}\)-dependent regulatory phenotypes, it seems plausible that the regulatory Ca\(^{2+}\) binding site may serve the same function in other, if not all, Na\(^{+}\)-Ca\(^{2+}\) exchangers.

**Na\(^{+}\)-dependent Regulation of CALX1.1**—We have shown that the XIP region serves a similar role in both CALX1.1 and NCX1.1 because analogous mutations produce similar phenotypes in both exchangers. With K306Q, corresponding to K225Q in NCX1.1 (see Fig. 4) (18), current decay into (Fig. 5) and recovery from (Fig. 6) the \(I_1\) inactive state was increased ~2-fold, mirrored by a slight increase in the extent of inactivation as indicated by a lower value of \(F_{ss}\), the fraction of steady-state to peak exchange current. According to the two-state model for \(I_1\) inactivation proposed by Hilgemann et al. (17), the larger \(I_1\) recovery rate constant of K306Q compared with CALX1.1 provides a satisfactory account for the accelerated current decay rate observed with K306Q versus CALX1.1. The observation that \(F_{ss}\) is reduced for K306Q also indicates that entry into the \(I_1\) inactive state has been accelerated. In contrast, the \(I_1\) inactivation process was apparently eliminated altogether in Δ310–313, a result identical to that obtained with the corresponding deletion mutant in NCX1.1, Δ229–232 (18), and with α-chymotrypsin-digested (i.e. deregulated) CALX1.1 (Fig. 6). Although the degree of primary structural similarity within the XIP-like regions of the various exchangers assembled in Fig. 4 is clearly less than for the acidic clusters of the Ca\(^{2+}\) binding site (Fig. 2), it is sufficiently prominent to tentatively conclude that this region may also share a common function in the control of Na\(^{+}\)-dependent inactivation of Na\(^{+}\)-Ca\(^{2+}\) exchangers in general. This possibility is supported by the observation that both CALX1.1 (29) and NCX2 (2), like NCX1.1, are inhibited by XIP, which appears to mimic the \(I_1\) inactivation process.

**Ca\(^{2+}\)-insensitive and Chimeric Exchangers**—Although the function of the XIP region and Ca\(^{2+}\) binding site appears to be conserved between CALX1.1 and NCX1.1, we have no definitive explanation for the observed differences in Ca\(^{2+}\)-dependent regulatory phenotypes. However, we observed that analogous mutations can render both exchangers insensitive to Ca\(^{2+}\) (e.g. G555P in CALX1.1 and G503P in NCX1.1) (see Fig.
3) (18, 33). One possibility is that Ca\(^{2+}\) binds to these exchangers but the transduction process has been disabled. Supporting this notion is a report by Levitsky et al. (15) showing that a fusion protein containing the Ca\(^{2+}\) binding site of NCX1.1, but bearing a G503P equivalent mutation, binds 45Ca\(^{2+}\) indistinguishably from the wild-type sequence. Also consistent with this hypothesis is our result with the chimeric exchanger CALX-NCX-CALX. Here, interchange of the entire Ca\(^{2+}\) binding site and flanking sequences of NCX1.1 with the corresponding region of CALX1.1 led to a transporter that was suppressed by regulatory Ca\(^{2+}\) (see Fig. 8). Thus, the Ca\(^{2+}\) binding function appears to have been retained, and a partial interconversion of phenotypes occurred. Although extrapolation of results from fusion and chimeric proteins to the wild-type exchanger must be made with caution, the above findings suggest that the primary or distinct from this region. Our results with NCX-NCX:CALX:NCX neither support nor refute this notion, irrespective of the fact that the chimeric protein did acquire a novel phenotype (i.e. Ca\(^{2+}\)-insensitive). Although there is little evidence to indicate that Ca\(^{2+}\) binding and transduction domains are modular or discrete, our results are at least suggestive of this possibility. Furthermore, we can conclude that the primary structural alterations associated with these two chimeras are relatively benign with respect to the transport function and Na\(^{+}\)-dependent regulation of the parent molecule. Complete and “symmetric” reversal of Ca\(^{2+}\)-dependent regulatory phenotypes between CALX1.1 and NCX1.1 may be possible through additional studies of this type.

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Structure-Function Analysis of CALX1.1, a Na⁺-Ca²⁺ Exchanger from *Drosophila*:
MUTAGENESIS OF IONIC REGULATORY SITES

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