STRUCTURE-FUNCTION STUDIES OF *DROSOPHILA* Na^+-Ca^{2+} EXCHANGER PROTEINS

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By
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NaCl, sodium chloride

KCl, potassium chloride

$MgSO_4$, magnesium sulfate

HEPES, (N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid])

$NaHCO_3$, sodium bicarbonate

$CaCl_2$, calcium chloride

BSA, bovine serum albumin

$CaCO_3$, calcium carbonate

KOH, potassium hydroxide

NMG-MES, N-methyl-D-glucamine-(2-[N-morpholino] ethanesulfonic acid

TEA-OH, tetraethylammonium hydroxide

$Mg(OH)_2$, magnesium hydroxide

CsOH, cesium hydroxide

MOPS, (3-[N-morpholino] propanesulfonic acid)

EGTA, ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetra-acetic acid

K_2HPO_4, potassium phosphate

MES, (2-[N-morpholino] ethanesulfonic acid

Abstract

The cardiac Na^+-Ca^{2+} exchanger, NCX1.1, is the most extensively studied member of this multi-gene family of ion counter-transporters. The Na^+-Ca^{2+} exchanger mediates transsarcolemmal Ca^{2+} transport and thus plays a key role in regulating cardiac contractility. To accomplish the movement of Ca^{2+} against its concentration gradient, the Na^+-Ca^{2+} exchanger utilizes the energy stored within the Na^+ electrochemical gradient. The Na^+-Ca^{2+} exchanger is an electrogenic transporter with a stoichiometry of $3Na^+$:$1Ca^{2+}$. In addition to being transported, cytoplasmic Na^+_i and Ca^{2+}_i regulate exchange activity. Within the large cytoplasmic loop of the exchanger, amino acid sequences have been identified that play critical roles in these ionic regulatory processes. However, these regulatory properties are not unique to NCX1.1. Similar responses to cytoplasmic Na^+_i and Ca^{2+}_i have been observed for most other exchanger subtypes and alternatively spliced isoforms. Protein regions involved in Na^+_i- and Ca^{2+}_i-dependent regulation for NCX1.1 appear to be highly conserved for all exchangers characterized to date. While this high degree of sequence similarity suggests a conserved functional role for these protein domains, it is unknown whether the structure-function relationships of NCX1.1 apply to any other exchanger. To examine this possibility, structure-function studies targeting these regulatory domains were performed with a *Drosophila* Na^+-Ca^{2+} exchanger, CALX1.1. Although NCX1.1 and CALX1.1 are both regulated by Na^+_i and Ca^{2+}_i, an opposite response to regulatory Ca^{2+}_i is observed between these two exchangers. That is, whereas Ca^{2+}_i activates NCX1.1 exchange activity, CALX1.1 is inhibited.

Wild-type and mutant exchangers were expressed in *Xenopus laevis* oocytes and characterized electrophysiologically using the giant excised patch clamp technique. Mutations within the regulatory Ca^{2+}_i binding site of the cardiac exchanger altered the affinity for Ca^{2+}_i regulation. Like NCX1.1, analogous modifications in the *Drosophila* exchanger, CALX1.1, alter Ca^{2+}_i regulation in a predictable fashion. The amino acid sequence mediating Na^+_i-dependent regulation is localized to the large cytoplasmic loop and is termed the eXchange Inhibitory Peptide, or XIP, region. Structure-function studies with NCX1.1 have shown alterations in Na^+_i-induced inactivation when residues within this region were mutated. Corresponding mutations in CALX1.1 produced responses similar to those observed for NCX1.1. Thus, the regulatory Ca^{2+}_i binding site and XIP region appear to serve similar functions in CALX1.1 and NCX1.1. Therefore, the opposite responses to regulatory Ca^{2+}_i must reside in, as yet, undefined portions of the exchanger molecule. To identify a single or multiple regions involved in the transduction of the Ca^{2+}_i binding signal, chimaeric exchangers were constructed. One chimaera, comprised primarily of CALX1.1, with a portion of substituted NCX1.1 cytoplasmic loop including the regulatory Ca^{2+}_i binding site and adjacent sequences, was stimulated by regulatory Ca^{2+}_i. Therefore, this phenotypic conversion suggests that the substituted amino acid sequence contains some or all of the region responsible for the transduction of the Ca^{2+}_i binding signal.

The second major aspect of this thesis involves the study of two alternatively spliced isoforms of the Na^+-Ca^{2+} exchanger identified in *Drosophila melanogaster*.

These isoforms, called CALX1.1 and CALX1.2, differ by five amino acids in a region near the C-terminus of the large cytoplasmic loop. This region is of great interest as the analogous region in mammalian exchangers undergoes alternative splicing to produce tissue-specific isoforms. At present, the consequences of tissue-specific alternative splicing on exchanger function are unknown. To determine whether the sequence difference observed for *Drosophila* isoforms influenced intrinsic regulatory properties, we examined Na^+_i- and Ca^{2+}_i-dependent regulation for both exchangers using the giant excised membrane patch clamp technique. Significant differences between these isoforms were observed for both regulatory processes. Although both exchangers exhibited Na^+_i-dependent inactivation, there was a pronounced difference in the rate of recovery from the Na^+_i-induced inactive state. Negative regulation by Ca^{2+}_i was observed for both exchangers, but both the extent and affinity of this inhibition was markedly different. Furthermore, regulatory Ca^{2+}_i elicited opposite responses with respect to steady-state current levels. Whereas Ca^{2+}_i stabilized I_1 inactivation for CALX1.1, resulting in lower steady-state levels, regulatory Ca^{2+}_i alleviated I_1 inactivation for CALX1.2. These findings suggest alternative splicing may play a role in tailoring regulatory properties of Na^+-Ca^{2+} exchange proteins to accommodate tissue-specific demands.

Introduction

The Na^+-Ca^{2+} exchange process, first identified in the late 1960's (Baker et al. 1969; Reuter and Seitz, 1968), is mediated by a single transmembrane spanning protein located in the plasma membrane of various tissues (Gleason et al. 1994; Furman et al. 1993; Dominguez et al. 1991; Balasubramanyam et al. 1994; Blaustein, 1988). The Na^+-Ca^{2+} exchanger has been shown to play an important role in diverse physiological functions in a wide array of tissues (McDaniel et al. 1993; Kofuji et al. 1992; Komuro et al. 1992). In the heart, for example, the Na^+-Ca^{2+} exchanger is the primary mechanism for Ca^{2+} efflux following a cardiac contraction (Mullins, 1979; Crespo et al. 1990). On a beat-to-beat basis, the Ca^{2+} equivalent that has entered the myocyte *via* voltage-dependent Ca^{2+} channels upon depolarization is ultimately removed by Na^+-Ca^{2+} exchange (Bridge et al. 1990). Thus, the Na^+-Ca^{2+} exchanger plays a critical role in regulating Ca^{2+} homeostasis and cardiac contractility (Mullins, 1979; Crespo et al. 1990). However, with the ability to function in either the Ca^{2+} influx or efflux mode (*i.e.* reversible transporter), it has been suggested that Ca^{2+} influx mediated by the exchanger may also play a role in the Ca^{2+}-induced Ca^{2+}-release process (Levi et al. 1994; Leblanc and Hume, 1990). Furthermore, in the nephron, Na^+-Ca^{2+} exchange mediates Ca^{2+} re-absorption in the nephron (Dominguez et al. 1992; Windhager et al. 1991), whereas in neuronal tissue, the exchanger appears to play an important role in Ca^{2+} efflux, and thus, excitation-secretion coupling (Blaustein et al. 1996).

Since the isolation and cloning of the cardiac Na^+-Ca^{2+} exchanger (Philipson et al. 1988; Nicoll et al. 1990), many unique gene products and alternatively spliced isoforms of the Na^+-Ca^{2+} exchanger have been identified suggesting this group of membrane proteins may represent a superfamily of ion transporters (Lee et al. 1994; Schwarz and Benzer, 1997; Ruknudin et al. 1997; Kofuji et al. 1994; Nicoll et al. 1996; Li et al. 1994). With the cloning of Na^+-Ca^{2+} exchange proteins, molecular biological tools and electrophysiological measurements have allowed a detailed examination of exchanger function. To date, several intrinsic and extrinsic forms of regulation have been identified which influence exchange activity of the cardiac Na^+-Ca^{2+} exchanger (Philipson, 1990; Doering and Lederer, 1993; Hilgemann et al. 1992; Hilgemann, 1990; Bridge et al. 1988; Condrescu et al. 1995; Hilgemann and Collins, 1992). For example, two intrinsic forms of ionic regulation have been observed for NCX1.1 using the giant excised patch technique (Hilgemann et al. 1992a; Hilgemann et al. 1992a; Hilgemann, 1990a). These regulatory processes, mediated by the transported ions themselves (Na^+ and Ca^{2+}), have since been observed for other exchanger subtypes and alternatively-spliced isoforms (unpublished data) (Linck et al. 1998; Hryshko et al. 1996; Li et al. 1994).

As for NCX1.1, ionic regulation has been observed for the *Drosophila* Na^+-Ca^{2+} exchanger, CALX1.1 (Hryshko et al. 1996). Although exhibiting both Na^+_i- and Ca^{2+}_i-dependent regulation, CALX1.1 is unique among all characterized Na^+-Ca^{2+} exchangers in that cytoplasmic Ca^{2+}_i inhibits exchange activity. For all other characterized exchangers, regulatory Ca^{2+}_i stimulates exchange activity.

The Na^+-Ca^{2+} exchanger is modeled to contain 11 transmembrane spanning segments (Nicoll et al. 1990). A large cytoplasmic loop, located between segments five and six, has been shown using deletion mutagenesis to mediate the regulation of exchange activity, whereas the transmembrane segments were sufficient for ion transport (Matsuoka et al. 1993). Within this cytoplasmic loop, structure-function studies have identified amino acid sequences involved in ionic regulation of NCX1.1. Mutations targeting amino acid residues within these regions significantly altered Na^+_i- and Ca^{2+}_i-dependent regulatory properties of NCX1.1 (Hilgemann, 1990; Matsuoka et al. 1995).

To date, all structure-function studies have been restricted to the cardiac Na^+-Ca^{2+} exchanger, NCX1.1. There is no information on structure-function relationships for any other member of this transport superfamily. Thus, to determine the possibility of a conserved functional role for these amino acid sequences mediating ionic regulation in NCX1.1, structure-function studies were performed with the *Drosophila* Na^+-Ca^{2+} exchanger, CALX1.1. Electrophysiological measurements were used to detect changes in ionic regulation as a result of molecular manipulations within putative regulatory regions. Amino acid residues, analogous to those involved in ionic regulation for NCX1.1, were targets for mutagenesis in CALX1.1.

Defects in ion channels or transporters produce a broad spectrum of human disease. Examples include cardiac hypertrophy, failing myocardium and the long QT syndrome. In hypertrophy and heart failure, decreased expression of the SR-

Ca^{2+}- ATPase (mRNA and protein levels) have been reported (Meyer et al. 1995; Mercadier et al. 1990). The reduced levels of SR-Ca^{2+}-ATPase in hypertrophy and heart failure may play an important role in the observed alterations of Ca^{2+} handling associated with these diseases (Schwinger et al. 1992; Beuckelmann et al. 1992). The ion channel defects SCN5A (voltage-gated Na^+ channel) and HERG (rectifying K^+ channel) have been associated with the long QT syndrome (Kass and Davies, 1996). With respect to Na^+-Ca^{2+} exchange, alterations in activity have been observed in cardiac disease. In hypertrophied and failing hearts, the upregulation of Na^+-Ca^{2+} exchange activity is thought to support contractility that would otherwise be impaired due to a decreased Ca^{2+} current (Litwin and Bridge, 1997; Flesch et al. 1996). While functional alterations of exchange activity have been observed under disease conditions, it is unknown whether genetic alterations may predispose individuals to cardiac disease. The study of this possibility has only recently become a realistic goal within basic cardiac research with the cloning of different exchange proteins. While structure-function studies demonstrate how dramatically Na^+-Ca^{2+} exchange properties can be altered, it remains to be established if similar "naturally occurring" mutations occur in humans.

Recently, unique gene products and alternatively spliced isoforms of Na^+-Ca^{2+} exchange proteins have been identified in a wide array of mammalian tissues (Lederer et al. 1996; Lee et al. 1994; Quednau et al. 1997; Kofuji et al. 1994; Barnes et al. 1997). Similarly, splice variants have also been identified for the *Drosophila* Na^+-Ca^{2+} exchanger (Schwarz and Benzer, 1997; Ruknudin et al. 1997). At present,

the physiological significance of alternative splicing is unknown. Although experimental evidence is lacking, this process may be involved in directing tissue-specific isoforms to their target tissues, or in modifying exchanger function to accommodate tissue-specific requirements. To answer the question of whether alternative splicing influences Na^{+}-Ca^{2+} exchange function, we examined the splice variants from *Drosophila melanogaster*, CALX1.1 and CALX1.2 electrophysiologically. Our results demonstrate substantial differences in the functional properties of these isoforms. For the first time, these data provide evidence that alternative splicing plays a role in tailoring the regulatory profiles of unique exchangers.

Literature Review

Biosynthesis and Topological Arrangement of the Na^+-Ca^{2+} Exchanger

The canine cardiac Na^+-Ca^{2+} exchanger, NCX1.1, was first cloned in 1990 (Nicoll et al. 1990). Initially, the open reading frame encoding for this transport protein was thought to be 970 amino acids in length. Based on hydropathy analysis, the Na^+-Ca^{2+} exchanger was modeled to contain 12 transmembrane segments with a large hydrophilic loop situated between transmembrane segments six and seven (Nicoll et al. 1990). The exchanger could be divided into three regions: 1) a hydrophobic segment containing the six N-terminal transmembrane segments, 2) a large hydrophilic loop and 3) a hydrophobic segment containing the six C-terminal transmembrane segments. Although the division of the Na^+-Ca^{2+} exchanger into long hydrophobic and hydrophilic regions was similar to that observed for other ATP-dependent cation pumps (Shull and Greeb, 1988) and ion exchangers (Kudrycki et al. 1990; Sardet et al. 1989), the Na^+-Ca^{2+} exchanger showed almost no sequence similarity to any other protein with the exception of a 23 amino acid sequence that was 48 % identical to the α - subunit of the Na^+-K^+-ATPase transporter (Devereux et al. 1984). This region, at an analogous position between the proteins, was thought to play a similar functional or structural role.

Although the initial hydropathy analysis of the cardiac exchanger suggested 12 transmembrane segments, there was evidence supporting the presence of a signal peptide at the N-terminal region which would be cleaved during processing of the protein (Kozak, 1989). The consensus cleavage site was situated between amino

acids 32 and 33 (von Heijne, 1986). Sequence analysis of the N-terminal region of the cardiac exchanger showed that the protein sequence began at the predicted cleavage site (Hilgemann, 1990), indicating that the first transmembrane segment was cleaved resulting in an integral membrane protein with 11 transmembrane segments. Thus, the mature peptide of the cardiac exchanger is encoded by a 938 amino acid reading frame. A similar topological arrangement has now been proposed for other Na^+-Ca^{2+} exchangers such as the brain (Li et al. 1994) and retinal Na^+-Ca^{2+}-K^+ exchanger (Reilander et al. 1992) which are the products of different genes. While rare for ion transporters, signal peptides are present in other similar proteins. For example, it appears that a cleaveable signal peptide is present for the Na^+-Ca^{2+}-K^+ exchanger (Papermaster, 1982) with the cleavable leader sequence located between residues 65-66 since residue 66 is the first amino acid at the N-terminus of the mature peptide.

Like other membrane transporters, the cardiac Na^+-Ca^{2+} exchanger exhibits glycosylation on the extracellular surface (Hryshko et al. 1993). Although six potential glycosylation sites have been identified based on the primary amino acid sequence, glycosylation has been shown to occur only at residue nine of the Na^+-Ca^{2+} exchanger (Hryshko et al. 1993). Despite the observation that glycosylation does not affect the activity of the cardiac exchanger (Hryshko et al. 1993), glycosylation sites provide important information with respect to topological organization. That is, glycosylation indicates positions of the protein facing the extracellular surface. Since the asparagine located at position nine is glycosylated,

this indicates that the N-terminal region of the exchanger is situated on the extracellular surface (Hryshko et al. 1993). Like the cardiac exchanger, other membrane transporters also exhibit some degree of glycosylation. For example, the Na^+-Ca^{2+}-K^+ photoreceptor exchanger is thought to undergo substantial glycosylation. This in part, explains the discrepancy observed between the actual molecular weight (210-220 kDa) and the predicted molecular weight (130 kDa) based on the amino acid sequence of the exchanger (Papermaster, 1982).

Although a complete topological organization of the exchanger has yet to be determined, portions of the exchanger have been localized to either the intra- or extracellular surface. The large hydrophilic loop situated between the N- and C-terminal transmembrane segments has been experimentally demonstrated to face the cytoplasmic surface (Porzig et al. 1993; Matsuoka et al. 1993). Within this loop, protein regions involved in regulating exchange activity have been identified (Matsuoka et al. 1993). Amino acid sequences between transmembrane segments two and three and eight and nine, termed the α repeats, appear to face the extracellular surface (Nicoll et al. 1990). These sequences are highly conserved among other Na^+-Ca^{2+} exchangers (Philipson et al. 1996) and play an important role in ion binding and translocation (Nicoll et al. 1996a). The amino acid sequence between transmembrane segments four and five is thought to face the extracellular surface (Nicoll et al. 1990). This region shows sequence similarity to the Na^+,K^+ pump and plays a role in controlling exchanger function (Nicoll et al. 1996a). Furthermore, cysteine mutagenesis and sulfhydryl modification have identified the

amino acid sequence between transmembrane segments two and three as being located on the cytoplasmic surface (Doering et al. 1998). This region may, in part, be responsible for the link between ion transport and regulation of exchanger activity (Doering et al. 1998).

Cellular localization of the Na^+-Ca^{2+} exchanger

Although the Na^+-Ca^{2+} exchanger has been identified in cell membranes of various tissues (Gleason et al. 1994; Furman et al. 1993; Dominguez et al. 1991; Balasubramanyam et al. 1994; Blaustein, 1988), the precise location and distribution within these membranes remains largely unexplored. In cardiac muscle, for example, the Na^+-Ca^{2+} exchanger has been identified at the surface membrane (Eisner and Lederer, 1985). Since a nonuniform distribution of the exchanger protein has been observed in nerve and smooth muscle cells, it was suggested that the Na^+-Ca^{2+} exchanger may be localized to specific regions of the plasma membrane of cardiac cells (Moore et al. 1991). Using adult guinea pig and rat heart cells, the exchanger was identified within the sarcolemmal membrane, intercalated disks and T-tubules (Kieval et al. 1992). These results suggested the exchanger was equally distributed within the sarcolemma of the cardiac myocytes (Kieval et al. 1992). Furthermore, these findings demonstrated the absence of the Na^+-Ca^{2+} exchanger within intracellular membranes (*i.e.* nuclear membrane) or organelles (Kieval et al. 1992). In contrast to these findings, a nonuniform distribution of the Na^+-Ca^{2+} exchanger within the sarcolemmal membrane has also been shown (Frank et al. 1992). Here, strong labeling of the Na^+-Ca^{2+} exchanger in T-tubules of guinea pig myocytes was

observed, whereas the sarcolemmal membrane exhibited patchy distribution of the protein and only ≈ 50 % of the intercalated discs showed labeling for the exchanger (Frank et al. 1992). In frog skeletal muscle, the Na^+-Ca^{2+} exchanger is an important calcium transporting protein (Caputo and Bolanos, 1978). Here, the exchanger is found in T-tubules and may play an important role in Ca^{2+} efflux following muscle stimulation.

The presence of the exchanger on all membranes in contact with the extracellular surface allows for substantial Ca^{2+} transport across the sarcolemma, which in turn, may provide a means for spatial regulation of intracellular Ca^{2+} concentration. The distance between the membrane and any region within the cytoplasm is relatively small in cardiac cells. The observation that Na^+-Ca^{2+} exchange can produce relaxation following caffeine induced Ca^{2+} release from the sarcoplasmic reticulum in the presence of abolished SR CaATPase activity supports the notion that the Na^+-Ca^{2+} exchanger is present over the entire sarcolemma and thus has rapid access to the entire cytoplasmic Ca^{2+} pool (Bridge et al. 1990).

Since the distribution of L-type Ca^{2+} channels is similar to that observed for the Na^+-Ca^{2+} exchanger (*i.e.*, within the T-tubules and sarcolemma), this spatial organization may facilitate a functional interaction between these transport proteins in excitation-contraction coupling, where the Ca^{2+} equivalent that enters the cell via Ca^{2+} channels is removed by Na^+-Ca^{2+} exchange (Bridge et al. 1990). However, it has been suggested that the Na^+-Ca^{2+} exchanger may be excluded from the functional

domain of Ca^{2+} surrounding the L-type Ca^{2+} channel/SR-Ca release channel complex (Adachi-Akahane et al. 1996; Sham et al. 1995).

Although the mechanism for localization of the exchanger is unknown, it has been hypothesized that the Na^{+}-Ca^{2+} exchanger interacts with cytoskeletal elements to restrict its movement along the plane of the membrane. Ankyrin proteins have been shown to link membrane cytoskeleton and integral membrane proteins (Bennett, 1992). Since various proteins such as the Na^{+}-K^{+}-ATPase (Nelson and Veshnock, 1987; Morrow et al. 1989) and the voltage-dependent Na^{+} channels of the brain (Srinivasan et al. 1988) have been shown to interact with ankyrin to localize its position, it was suggested that the Na^{+}-Ca^{2+} exchanger also interacted with ankyrin proteins to restrict its localization within the membrane. Ankyrin proteins are localized to T-tubular and surface sarcolemma membrane in myocytes and shown to bind the cardiac exchanger with a high affinity (Li et al. 1993). These results support the notion that ankyrin may play a role in localizing the cardiac exchanger to regions of the sarcolemma.

Transport and Electrogenicity of Na^{+}-Ca^{2+} Exchange Activity

The Na^{+}-Ca^{2+} exchanger is an integral membrane protein involved in the secondary active transport of three Na^{+} in exchange for each Ca^{2+} (Reeves and Hale, 1984; Kimura et al. 1986). Based upon the stoichiometry of the exchange process, the Na^{+}-Ca^{2+} exchanger is an electrogenic transporter with the net movement of one positive charge per exchange cycle (Lagnado and McNaughton, 1990). The transport cycle has been demonstrated to follow a consecutive rather than a

simultaneous mechanism, where Na^+ and Ca^{2+} are translocated across the plasma membrane in separate steps (Khananshvili, 1990; Hilgemann et al. 1991). An eight-state transport cycle has been suggested as the mechanism responsible for the exchange process (Hilgemann et al. 1991). Here, upon binding of three Na^+ ions, the exchanger occludes and translocates Na^+ across the plasma membrane where de-occlusion and release of Na^+ occurs. At this point, the exchanger binding sites are vacant and able to bind Ca^{2+}, followed by occlusion and translocation across the plasma membrane where Ca^{2+} is released, thus completing the transport cycle. Using cardiac myocytes (Hilgemann, 1989; Hilgemann, 1990) and *Xenopus* oocytes expressing the cardiac Na^+-Ca^{2+} exchanger (Nicoll et al. 1990), this net movement of one positive charge has been associated with Na^+ translocation. Furthermore, the electrogenic step appears to occur at the extracellular surface of the Na^+-Ca^{2+} exchanger (Hilgemann et al. 1991). Since the energy stored within the Na^+ electrochemical gradient is used for the uphill transport of Ca^{2+}, the Na^+-Ca^{2+} exchanger may either move Ca^{2+} into or out of the cytoplasm across the cell membrane depending upon the prevailing electrochemical gradients (Barcenas-Ruiz et al. 1987).

Regulation of Na^+-Ca^{2+} exchange activity

1) Ca^{2+}-Dependent Regulation

In addition to transporting Ca^{2+}, the Na^+-Ca^{2+} exchanger is also regulated by cytoplasmic Ca^{2+}, an effect initially observed in squid giant axon (Baker and McNaughton, 1976) and intact myocytes (Kimura et al. 1986). More recently, this

Ca^{2+}-dependent regulatory process has been studied in great detail using giant excised membrane patches from *Xenopus laevis* oocytes expressing Na^{+}-Ca^{2+} exchanger proteins (Hilgemann et al. 1992a; Hilgemann et al. 1992a; Hilgemann, 1990a) Although Ca^{2+}-dependent regulation of exchange activity is most readily observed for outward Na^{+}-Ca^{2+} exchange using the giant excised patch clamp technique (*i.e.* Ca^{2+} influx mode or reverse Na^{+}-Ca^{2+} exchange current), it has been shown that Ca^{2+} regulates both outward and inward exchange currents (Matsuoka et al. 1995). In other words, forward and reverse (*i.e.* Ca^{2+} efflux or Ca^{2+} influx, respectively) Na^{+}-Ca^{2+} exchange currents are regulated by cytoplasmic Ca^{2+}.

Since the cloning of the Na^{+}-Ca^{2+} exchanger (Nicoll et al. 1990), molecular biological techniques could be used to study regulatory properties associated with Na^{+}-Ca^{2+} exchange activity. For example, with NCX1.1, deletion of the large cytoplasmic loop comprising ≈ half the protein mass resulted in a Na^{+}-Ca^{2+} exchanger that was capable of ion transport but no longer exhibited Ca^{2+}-dependent regulation (Matsuoka et al. 1993). This observation illustrated that the transmembrane segments were sufficient for ion transport and suggested that the large cytoplasmic loop contained a Ca^{2+}-binding domain where regulatory Ca^{2+} binds to, and elicits its influence on Na^{+}-Ca^{2+} exchange activity (Matsuoka et al. 1993; Levitsky et al. 1994; Matsuoka et al. 1995). Thus, the amino acid sequence within the cytoplasmic loop where regulatory Ca^{2+} binds (*i.e.* the regulatory Ca^{2+} binding site) is distinct from the Ca^{2+} transport site (Matsuoka et al. 1993; Levitsky et al. 1994; Matsuoka et al. 1995). Fusion protein analysis and $^{45}Ca^{2+}$ overlay

studies have further delineated this regulatory Ca^{2+} binding site within the cytoplasmic loop (Levitsky et al. 1994). This region, spanning 138 amino acids near the center of the loop, exhibits two highly acidic sequences each of which contains three sequential aspartic acid residues and binds Ca^{2+} with a K_d value in the micromolar range (Levitsky et al. 1994). Thus, Ca^{2+}-dependent regulation is mediated by a high affinity regulatory Ca^{2+} binding site. Mutation of acidic residues within the Ca^{2+} binding site reduced the affinity for $^{45}Ca^{2+}$ binding (Levitsky et al. 1994) and Ca^{2+} regulation as assessed using the giant excised patch clamp technique (Matsuoka et al. 1995). These results support the notion that the regulatory Ca^{2+} binding site plays a role in mediating secondary Ca^{2+} regulation of Na^{+}-Ca^{2+} exchange activity for NCX1.1. Although Ca^{2+}-dependent regulation has been observed under experimental conditions, the physiological significance of this regulatory process has yet to be determined. One possible hypothesis is that Ca^{2+}-dependent regulation prevents intracellular Ca^{2+} levels from becoming lowered beyond physiological levels (Hilgemann, 1988). For example, as the intracellular Ca concentration is lowered, Ca^{2+} is removed from the Ca^{2+} binding site, exchange activity is inhibited, and thus, further Ca^{2+} efflux is prevented.

For all mammalian Na^{+}-Ca^{2+} exchangers including distinct gene products and alternatively spliced isoforms, regulatory Ca^{2+} stimulates exchange activity (Hilgemann et al. 1992a; Hilgemann, 1990a; Matsuoka et al. 1993a; Matsuoka et al. 1995a; Li et al. 1994a). Consistent with the observation of nearly abolished Na^{+}-Ca^{2+} exchange current in the absence of regulatory Ca^{2+}, despite infinite ionic

gradients favoring exchange, is the hypothesis that in the absence of Ca^{2+}, Na^{+}-Ca^{2+} exchange proteins enter an inactive state that inhibits the exchanger from participating in current production (Hilgemann et al. 1992a; Hilgemann et al. 1992a; Hilgemann, 1990a). In other words, regulatory Ca^{2+} inhibits this inactive state, which in turn allows a greater number of exchangers to contribute to current production. Furthermore, it has also been shown that at low regulatory Ca^{2+} concentrations, the affinity for transport Na^{+} is decreased (Matsuoka et al. 1995). Thus, in addition to affecting the fraction of exchangers found in the active or inactive state, Ca^{2+}-dependent regulation may also influence transport properties of Na^{+}-Ca^{2+} exchange.

In contrast to the stimulatory effect observed for mammalian exchangers, regulatory Ca^{2+} inhibits Na^{+}-Ca^{2+} exchange current for the *Drosophila* Na^{+}-Ca^{2+} exchanger (Hryshko et al. 1996). Despite showing a greater than 75 % amino acid sequence identity of the regulatory Ca^{2+} binding site with mammalian exchangers (Matsuoka et al. 1995; Hryshko et al. 1996; Li et al. 1994), the *Drosophila* exchanger, termed CALX1.1, is unique in terms of its response to regulatory Ca^{2+}.

2) Na^{+}-Dependent Regulation

In addition to being transported by the Na^{+}-Ca^{2+} exchanger, Na^{+} regulates exchange activity from the cytoplasmic surface of the exchanger (Hilgemann, 1990). Upon activation of outward exchange activity by application of Na^{+}, current rapidly rises to a peak level, followed by a slow, time-dependent inactivation to a lower steady-state current value (Hilgemann, 1990). This inactivation process is mediated

by Na^+ and is referred to as Na^+-induced inactivation. Upon binding three Na^+ ions, the Na^+-Ca^{2+} exchanger may either enter the transport cycle resulting in current production, or, enter the Na^+-induced, or I_i, inactive state (Hilgemann, 1990). Therefore, within the exchanger population, the distribution of the exchangers between the active and inactive states modulates steady-state current levels. With increasing Na^+ concentrations, the fraction of exchangers in the 3 Na^+ loaded state is increased, augmenting both peak current and the extent of Na^+-induced inactivation (Hilgemann et al. 1992a; Hilgemann et al. 1992a; Hilgemann, 1990a).

Within the large cytoplasmic loop of the Na^+-Ca^{2+} exchanger, a putative auto regulatory domain mediating Na^+-dependent regulation was initially identified using deletion mutagenesis (Matsuoka et al. 1993). This 20 amino acid sequence, located at the N-terminus of the cytoplasmic loop, contained hydrophobic residues interspersed with basic residues: an arrangement characteristic of calmodulin binding sites (O'Neil and DeGrado, 1990). Since calmodulin binding sites represent autoinhibitory domains in many proteins (Enyedi et al. 1989), it was thought this amino acid sequence may be functionally important in regulation of Na^+-Ca^{2+} exchange activity. To determine a possible role for this protein domain, a peptide corresponding to this positively charged 20 amino acid sequence was synthesized and applied to membrane patches expressing Na^+-Ca^{2+} exchange proteins. This peptide, termed the eXchange Inhibitory Peptide (XIP), potently and reversibly inhibited exchange current for all Na^+-Ca^{2+} exchangers examined (Li et al. 1991; Kleiboeker et al. 1992). Although the exogenous XIP peptide binds calmodulin with

a moderately high affinity, it appears that calmodulin does not bind to the endogenous XIP region of the exchanger or affect exchange activity. Furthermore, although the exogenous XIP peptide binds directly to the Na^+-Ca^{2+} exchanger within sarcolemmal membranes to affect exchange activity (Matsuoka et al. 1993; Kleiboeker et al. 1992), there is no convincing evidence supporting a direct interaction between the endogenous XIP region and neighbouring protein domains of the exchanger. When bound to the exchanger, exogenous XIP inhibits Na^+-Ca^{2+} exchange activity (Matsuoka et al. 1993; Kleiboeker et al. 1992). It has been suggested that exogenous XIP interacts with the regulatory Ca^{2+} binding site, thus, this regulatory site may then bind Ca^{2+} and exogenous XIP simultaneously (Hale et al. 1997). Since many results have shown anionic sarcolemmal lipids to regulate exchange activity, it has been hypothesized that the endogenous XIP region interacts with membrane lipids to mediate this form of regulation (Ford and Hale, 1996; Hilgemann and Ball, 1996; Shannon et al. 1994). For example, the cardiac Na^+-Ca^{2+} exchanger is influenced by the levels of PIP_2 in membrane adjacent to the exchanger (Hilgemann and Ball, 1996). When conditions are favorable for this protein-membrane interaction, Na^+-Ca^{2+} exchange activity is stimulated (Ford and Hale, 1996; Hilgemann and Ball, 1996; Shannon et al. 1994).

Mutation of residues within the endogenous XIP region resulted in two distinct phenotypes with respect to Na^+-dependent regulation for the cardiac Na^+-Ca^{2+} exchanger (Matsuoka et al. 1997). The Na^+-induced inactivation process was

either accelarated or eliminated following point or deletion mutations within the XIP region (Matsuoka et al. 1997).

3) Effects of MgATP and Phosphorylation on Na^+-Ca^{2+} Exchange Activity

Phosphorylation of Ca^{2+} channels (Levitan, 1985) and Ca^{2+} ATPases (Caroni and Carafoli, 1981) have been implicated in modifying transsarcolemmal Ca^{2+} fluxes. Since the Na^+-Ca^{2+} exchanger plays a role in the dynamics of Ca^{2+} transport, it is possible that ATP may either directly affect or be used as a substrate in the phosphorylation of the exchanger. This process could coordinate exchange activity to maintain Ca^{2+} homeostasis under a variety of conditions. Although putative phosphorylation sites have been identified for each Na^+-Ca^{2+} exchanger, the role of these sites have yet to be determined.

In cardiomyocytes, in addition to being stimulated by cytoplasmic Ca^{2+}, MgATP has been shown to stimulate exchange activity (Hilgemann, 1990), whereas ATP depletion is inhibitory (Haworth et al. 1987; Condrescu et al. 1995). Furthermore, it has been shown that MgATP influences the Na^+- and Ca^{2+}-dependent regulatory processes of membrane patches from cardiomyocytes (Hilgemann et al. 1992a; Collins et al. 1992a) and *Xenopous* oocytes expressing Na^+-Ca^{2+} exchanger proteins (Hilgemann, 1990). For *Xenopus* oocyte membrane patches, ATP was found to decrease the extent of Na^+-induced inactivation (Hilgemann et al. 1992a). This may be the result of a decreased rate of entry into the I_1 inactive state, or the accelerated recovery from Na^+-induced inactivation. More recently, it has been shown that the upregulation of the cardiac Na^+-Ca^{2+} exchange activity by MgATP

may be correlated with phosphorylation of the exchanger via a protein kinase C dependent pathway (Iwamoto et al. 1996). Furthermore, phosphorylation of the squid axon (DiPolo and Beauge, 1987) and smooth muscle Na^+-Ca^{2+} exchanger (Iwamoto et al. 1995) has been shown to result in stimulation of exchange activity. Thus, it appears the phosphorylation of various Na^+-Ca^{2+} exchange proteins may play an important role in modulating exchange activity under physiological conditions.

Na^+-Ca^{2+} Exchange Proteins - Unique Gene Products

Although predominantly found in cardiac tissue (Kofuji et al. 1992; Komuro et al. 1992; Nicoll et al. 1996), NCX1.1 has recently been identified and cloned from other tissues and species (DiPolo and Beauge, 1991; Diebold et al. 1992; Cooper et al. 1984; Condrescu et al. 1995). The NCX1.1 exchanger is expressed at varying levels in all human and rat tissues examined with the greatest expression observed in cardiac tissue (Kofuji et al. 1992). Furthermore, the NCX1 exchanger exhibits a high degree of homology within and between species. The chromosomal location of the NCX1 gene has been shown to vary between species. In humans, the NCX1 gene is found on the short arm of chromosome 2 (Shieh et al. 1992), whereas in the mouse, the transporter gene is located on chromosome 17 (Nicoll et al. 1996b).

1) NCX2

A second mammalian Na^+-Ca^{2+} exchanger has recently been cloned from a rat brain cDNA library (Nicoll et al. 1996b). This Na^+-Ca^{2+} exchanger, NCX2, is the product of a different gene as compared with NCX1 (Nicoll et al. 1996b). The gene

encoding this second Na^+-Ca^{2+} exchanger is predicted to encode for a protein of 921 amino acids (Li et al. 1994) and is located on chromosome 14 (Li et al. 1994) and 7 (Nicoll et al. 1996b) for human and mouse, respectively. NCX2 is 61 and 65 % identical at the nucleotide and amino acid levels respectively compared to NCX1.1 (Li et al. 1994). Furthermore, based on sequence analysis, it is predicted that NCX2 exhibits a similar topological organization with 11 transmembrane segments and a large cytoplasmic loop as compared to NCX1.1 (Li et al. 1994). In contrast to observing alternatively-spliced isoforms for NCX1 (Kofuji et al. 1993; Lee et al. 1994; Kofuji et al. 1994), it appears that NCX2 does not undergo alternative-splicing (Quednau et al. 1997). A 37 amino acid deletion is observed for NCX2 in the cytoplasmic loop where alternative splicing occurs in NCX1 (Quednau et al. 1997).

The NCX2 exchanger exhibited functional properties that were similar to those observed for NCX1.1 (Li et al. 1994). For example, regulatory Ca^{2+} was observed to stimulate Na^+-Ca^{2+} exchange currents for both NCX1.1 and NCX2 (Hilgemann et al. 1992a; Hilgemann, 1990a; Matsuoka et al. 1993a; Matsuoka et al. 1995a; Li et al. 1994a). Furthermore, cellular ATP depletion had a similar inhibitory effect on exchange currents for both mammalian exchangers (Linck et al. 1998). In terms of transport properties, NCX2 exhibits a similar affinity for transport Na^+ as compared to NCX1.1 (Li et al. 1994). Thus, although NCX1.1 and NCX2 are found predominantly in different tissues, it appears they may be regulated in a similar fashion to perform tissue-specific functions.

2) NCX3

A third mammalian Na^+-Ca^{2+} exchanger has recently been cloned from a rat brain cDNA library (Nicoll et al. 1996b). This exchanger, NCX3, is the product of a different gene as compared with NCX1 and NCX2 (Nicoll et al. 1996b). The NCX3 gene encodes for a protein of 927 amino acids and is found on chromosome 12 in the mouse (Nicoll et al. 1996b). Based upon sequence analysis, it is predicted that NCX3 exhibits a similar topological organization as compared with NCX1.1. and NCX2 (Nicoll et al. 1996b). Furthermore, the NCX3 exchanger is 73 and 75 % identical at the amino acid level with NCX1.1 and NCX2, respectively (Nicoll et al. 1996b). Similar to NCX2, NCX3 is predominantly found in brain and skeletal muscle (Li et al. 1991; Nicoll et al. 1996) and exhibits a 37 amino acid deletion in the cytoplasmic loop of the exchanger which undergoes alternative splicing to produce tissue-specific isoforms in NCX1 (Quednau et al. 1997). However, despite the observed deletion in the cytoplasmic loop, developmentally regulated alternatively-spliced isoforms have been identified for NCX3 (Quednau et al. 1997).

The functional properties of NCX3 were studied and compared to the previously cloned mammalian exchangers. As for NCX1.1 and NCX2, regulatory Ca^{2+} stimulated Na^+-Ca^{2+} exchange currents of NCX3 (Linck et al. 1998). However, whereas NCX1.1 and NCX2 exchange currents were inhibited by the depletion of cellular ATP, NCX3 activity appeared insensitive to reduced ATP concentrations (Linck et al. 1998). Furthermore, as observed for NCX1.1, NCX3 exchange activity was slightly stimulated by the activation of protein kinase A or C (Linck et al. 1998).

Thus, some similarities with respect to functional properties are observed between the NCX1.1 and NCX3 Na^+-Ca^{2+} exchangers.

3) The *Drosophila* Na^+-Ca^{2+} Exchanger: CALX1

Sodium-calcium exchange proteins have been identified in a wide array of species including squid (Baker et al. 1969) and barnacle (Niggli and Lederer, 1991). Surprisingly, these Na^+-Ca^{2+} exchange proteins exhibit functional properties similar to those observed for mammalian exchangers (Li et al. 1994). Recently, a Na^+-Ca^{2+} exchanger has been identified and cloned from the fruit fly, *Drosophila melanogaster* (Schwarz and Benzer, 1997; Ruknudin et al. 1997). The *Drosophila* Na^+-Ca^{2+} exchanger, CALX1.1, is found on chromosome 3 (Ruknudin et al. 1997) and is 49 % identical to NCX1.1 at the amino acid level (Hryshko et al. 1996). Based on hydropathy analysis, CALX1.1 exhibits a similar topological arrangement as other characterized exchangers with a large hydrophilic loop located between the N- and C-terminal transmembrane segments (Ruknudin et al. 1997). Within the cytoplasmic loop of CALX1, an alternative-splice site has been identified at a region that corresponds to the alternative-splice site in NCX1.1 (Ruknudin et al. 1997).

Two forms of ionic regulation influence CALX1.1 exchange activity (Hryshko et al. 1996). Similar to NCX1.1, Na^+- and Ca^{2+}-dependent regulatory processes have been observed for the *Drosophila* Na^+-Ca^{2+} exchanger (Hryshko et al. 1996). Previously, a putative regulatory Ca^{2+} binding site and XIP region have been identified within the cytoplasmic loop of CALX1.1. However, despite the sequence similarity of the regulatory Ca^{2+} binding site between the cardiac and *Drosophila*

exchangers, CALX1.1 is inhibited by regulatory Ca^{2+} whereas NCX1.1 is activated (Hryshko et al. 1996). Thus, CALX1.1 is unique among all characterized exchangers in that regulatory Ca^{2+} inhibits exchange activity. Furthermore, the inhibition of CALX1.1 exchange activity occurs over the same concentration range that is observed to stimulate NCX1.1 (Hryshko et al. 1996). Moreover, Na^+-induced inactivation is observed for both NCX1.1 and CALX1.1. That is, as cytoplasmic Na^+ concentrations are increased, a greater extent of Na^+-induced inactivation is observed.

Mammalian Alternatively-Spliced Isoforms of the Na^+-Ca^{2+} Exchanger

In addition to identifying unique gene products of the Na^+-Ca^{2+} exchanger, several alternatively-spliced isoforms have been discovered for NCX1 and NCX3 (Nakasaki et al. 1993; Lee et al. 1994; Quednau et al. 1997; Kofuji et al. 1994). For NCX1, a variable domain spanning 110 amino acids near the C-terminal end of the cytoplasmic loop undergoes alternative-splicing to produce tissue-specific splice-variants (Lee et al. 1994; Kofuji et al. 1994). This region, the alternative splice site, contains two mutually exclusive exons, A and B, as well as four cassette exons, C, D, E and F (Kofuji et al. 1994). Whereas the mutually exclusive exon B is found in all rat tissues except heart, exon A is preferentially expressed in cardiac, brain and skeletal tissue (Quednau et al. 1997). Although this exon arrangement may allow up to 32 Na^+-Ca^{2+} exchanger isoforms (Kofuji et al. 1994), to date, only twelve splice variants for NCX1 have been identified (Kofuji et al. 1993; Lee et al. 1994; Quednau et al. 1997; Kofuji et al. 1994). Furthermore, the regional expression of the

alternatively-spliced isoforms for NCX1 appear to be regulated by three tissue-specific promoters that control the splicing pattern within the cytoplasmic loop (Barnes et al. 1997).

Three alternatively-spliced isoforms of NCX3 have been identified in brain and skeletal muscle of rat (Quednau et al. 1997). For NCX3, a 37 amino acid deletion exists in the region of the cytoplasmic loop which corresponds to the alternative-splice site in NCX1.1 (Quednau et al. 1997). Thus, since the deletion observed for NCX3 spans the amino acid equivalent of exons D, E and F in NCX1, the splice-variants of NCX3 may contain either of the mutually exclusive exons, A or B, whereas exon C may or may not be present (Quednau et al. 1997).

Although NCX1 is encoded by a single gene, it is involved in performing diverse physiological processes in a wide array of tissues (McDaniel et al. 1993; Kofuji et al. 1992; Komuro et al. 1992). For example, in cardiac tissue, NCX1.1 plays a critical role in regulating cardiac contractility, whereas in the kidney, Na^+-Ca^{2+} exchange is the primary mechanism for Ca^{2+} reabsorption and extracellular Ca^{2+} homeostasis (Bindels et al. 1992; Bourdeau et al. 1993; Reilly et al. 1993). Thus, alternative-splicing of the primary transcript encoding NCX1 may be involved in tailoring functional properties of the exchanger to accommodate tissue-specific demands. Support for this notion comes from studies showing that the alternative splice site may play a role in Ca^{2+}-dependent regulation (Matsuoka et al. 1993).

Alternatively-Spliced Isoforms of the *Drosophila* Na^+-Ca^{2+} Exchanger

Two alternatively-spliced isoforms have been identified for the *Drosophila* Na^+-Ca^{2+} exchanger (Schwarz and Benzer, 1997; Ruknudin et al. 1997). In *Drosophila*, the alternative splice site appears to occur at a region within the large cytoplasmic loop corresponding to the splice site of NCX1 (Ruknudin et al. 1997). Within this region, a five amino acid difference is observed between the two *Drosophila* isoforms, CALX1.1 and CALX1.2 (Ruknudin et al. 1997). Although the physiological significance of ionic regulation is unknown, alternative-splicing may play a role in tailoring regulatory properties of the Na^+-Ca^{2+} exchanger to modify its function to meet tissue-specific demands.

Role of Na^+-Ca^{2+} Exchange in Excitation-Contraction Coupling

Although extracellular and intracellular Ca^{2+} are required to elicit a cardiac contraction (Wohlfart and Noble, 1982; Chapman, 1983), the amount contributed from these two sources varies between species examined (Fabiato, 1982; Bers, 1985). In cardiac muscle, excitation-contraction coupling occurs *via* a process termed Ca^{2+}-induced Ca^{2+}-release (Fabiato, 1985). Here, depolarization of the cardiac sarcolemma including T-tubules causes the opening of voltage-dependent Ca^{2+} channels (L-type Ca^{2+} channels) allowing the passage of Ca^{2+} across the plasma membrane. This calcium current (I_{Ca}), termed "trigger" Ca^{2+}, elicits a much larger and graded release of Ca^{2+} from the sarcoplasmic reticulum (Callewaert et al. 1988; Cleemann and Morad, 1991; Beuckelmann and Wier, 1988; Nabauer et al. 1989). The rapid activation of the SR-Ca^{2+} release channels requires a high Ca^{2+}

concentration in the immediate vicinity of these channels (Cannell et al. 1995; Cheng et al. 1993). This direct interaction between the L-type Ca^{2+} channels in the sarcolemma and the SR-Ca^{2+} release channels (ryanodine receptors) in the sarcoplasmic reticulum is facilitated by their close proximity within the diads of cardiac muscle (Santana et al. 1996; Cheng et al. 1996; Cheng et al. 1996). This spatial arrangement appears to be disrupted in cardiac hypertrophy and heart failure leading to a reduced excitation-contraction coupling efficiency under these disease conditions (Gomez et al. 1997). Results showing the activation of several SR-Ca^{2+} release channels in response to Ca^{2+} entry from each L-type Ca channel (Lopez-Lopez et al. 1995; Cannell et al. 1995) suggests that Ca^{2+} entering via the L-type Ca^{2+} channels is the major stimulus responsible for the rise in intracellular Ca^{2+} concentrations during excitation-contraction coupling (Reuter, 1973; Beeler, and Reuter, 1970; Cleemann and Morad, 1991). Furthermore, the release of "activator" Ca^{2+} from the sarcoplasmic reticulum results in a cardiac contraction.

In contrast to Ca^{2+}-induced Ca^{2+}-release observed for cardiac muscle, SR-Ca^{2+} release in skeletal muscle is triggered by an intramembrane charge movement within the sarcolemma following depolarization. That is, whereas Ca^{2+} activates SR-Ca^{2+} release channels in cardiac muscle, charge movement in skeletal muscle is responsible for the opening of these channels, thereby permitting Ca^{2+} release from the sarcoplasmic reticulum into the cytoplasm (Anderson et al. 1989).

Following a cardiac contraction, intracellular Ca^{2+} levels must be restored to resting levels to allow for proper relaxation of the cardiac tissue and adequate filling

of the heart chambers prior to the next contraction. Located within the membrane of the sarcoplasmic reticulum, SR-Ca^{2+} ATPase transporters are involved in the re-uptake of Ca^{2+} that has been released into the cytoplasm upon excitation *via* an ATP dependent mechanism. This process reduces the Ca^{2+} transient produced during excitation-contraction coupling thus contributing to the relaxation of cardiac tissue. In rabbit ventricular myocytes, it has been shown that SR-Ca^{2+} uptake is responsible for ≈ 75 % of relaxation (Hryshko et al. 1989). Similar results were observed for rabbit ventricular muscle (Bers and Bridge, 1989). Furthermore, a Ca^{2+}-ATPase and Na^{+}-Ca^{2+} exchanger within the sarcolemma of the myocytes contribute to the reduction of the intracellular Ca^{2+} concentration. Sodium-calcium exchange has been shown to be the primary mechanism for Ca^{2+} efflux following a cardiac contraction, thus playing a major role in influencing resting Ca^{2+} levels and the Ca^{2+} content of the sarcoplasmic reticulum (Mullins, 1979; Crespo et al. 1990). For Na^{+}-Ca^{2+} exchange, the extent to which this transporter participates in reducing the intracellular Ca^{2+} concentration depends upon the species examined. For example, in rat cardiac myocytes, the Na^{+}-Ca^{2+} exchanger removes 7 % of the intracellular Ca^{2+}, whereas for rabbit myocytes, the fraction removed is 28 % (Bassani et al. 1994). It has also be shown that the Ca^{2+} equivalent that enters the cell upon depolarization through the L-type Ca^{2+} channels is ultimately extruded via the Na^{+}-Ca^{2+} exchanger (Bridge et al. 1990). However, despite transporting a much smaller fraction of Ca^{2+} as compared to the SR-Ca^{2+} ATPase, the Na^{+}-Ca^{2+} exchanger is capable of producing relaxation following contraction in the absence of SR-Ca^{2+} ATPase

activity (Bers and Bridge, 1989). Here, in the absence of SR-Ca^{2+} ATPase activity, the rate of relaxation was reduced by 70 % (Bers and Bridge, 1989). On the other hand, the rate of relaxation was reduced by ≈ 30 % in the absence of Na^{+}-Ca^{2+} exchange activity with intact SR-Ca^{2+} ATPase activity (Bers and Bridge, 1989). The amount of Ca^{2+} removed by the sarcolemmal Ca^{2+}-ATPase appears insignificant as compared with that transported by the Na^{+}-Ca^{2+} exchanger during diastole or under resting conditions (Bers, 1991; Wier, 1990). For example, in rabbit ventricular muscle, the rate of relaxation mediated by sarcolemmal Ca^{2+}-ATPase activity, where Na^{+}-Ca^{2+} exchange and SR-Ca^{2+} ATPase activity is abolished, was approximately 1,200 % slower as compared with muscle preparations where these processes were intact (Bers and Bridge, 1989). Thus, calcium efflux mediated by the sarcolemmal Ca^{2+}-ATPase may be involved only in fine tuning of Ca^{2+} concentrations within myocytes over a long-term period.

The extent to which Ca^{2+} is removed by the Na^{+}-Ca^{2+} exchanger has also been shown to vary during development (Vetter et al. 1987; Nakanishi and Jarmakani, 1981). Prior to the complete development of T-tubular systems and the sarcoplasmic reticulum in immature hearts, the Na^{+}-Ca^{2+} exchanger plays a significant role in Ca^{2+} fluxes across the sarcolemma. For example, adult hearts of rat and rabbit contained an eight and six fold reduction of Na^{+}-Ca^{2+} exchange protein, respectively, as compared with late fetal rabbit and newborn rat hearts (Boerth et al. 1994). Thus, for rat and rabbit myocytes, exchanger transcript levels peaked near birth and declined postnatally (Boerth et al. 1994).

Although Na^+-Ca^{2+} exchange is the primary mechanism for transsarcolemmal Ca^{2+} efflux (O'Neill et al. 1991; Cannell, 1991; Barry and Bridge, 1993), it has recently been speculated that reverse Na^+-Ca^{2+} exchange (*i.e.* Ca influx mode) may play a role in contributing to Ca^{2+}-induced Ca^{2+}-release during excitation-contraction coupling (Nuss and Houser, 1992; Leblanc and Hume, 1990). For example, Ca^{2+} transients and cardiac contractions were observed following stimulation despite inhibition of L-type Ca^{2+} channels by either Ca^{2+} channel blockers or positive potentials, therefore suggesting Ca^{2+} influx is occurring *via* another mechanism (Howlett et al. 1998; Leblanc and Hume, 1990). This secondary Ca^{2+} influx was thought to occur via the Na^+-Ca^{2+} exchanger. Since the Na^+-Ca^{2+} exchanger is an electrogenic transporter (Beuckelmann and Wier, 1989; Kimura et al. 1987), depolarization to potentials more positive than the reversal potential of the exchanger favors Ca^{2+} influx (Shattock and Bers, 1989; Earm and Noble, 1990), which in turn was shown to trigger Ca^{2+}-induced Ca^{2+}-release (Evans and Cannell, 1997; Leblanc and Hume, 1990; Sham et al. 1992). Furthermore, elevated Na^+ concentrations associated with the Na^+ current entering voltage-dependent Na^+ channels upon initial depolarization of the sarcolemma induced reverse Na^+-Ca^{2+} exchange activity (*i.e.* Ca^{2+} influx) and sufficient Ca^{2+} entry to activate SR-Ca^{2+} release channels (Lipp and Niggli, 1994; Leblanc and Hume, 1990). The ability of reverse Na^+-Ca^{2+} exchange to activate contraction required a functional sarcoplasmic reticulum, supporting the notion that Ca^{2+} influx *via* the Na^+-Ca^{2+} exchanger is capable of triggering SR Ca^{2+} release. It has also been speculated that upon depolarization, a voltage-gated release

mechanism activates SR-Ca^{2+} release channels leading to a cardiac contraction (Schneider and Chandler, 1973; Howlett et al. 1998). This channel activation may be similar to that observed for the opening of the SR-Ca^{2+} release channels *via* intramembrane charge movement in skeletal muscle.

In contrast to adult hearts, the sarcoplasmic reticulum of the immature heart has a reduced capacity to regulate Ca^{2+} levels (Arai et al. 1992; Pegg and Michalak, 1987; Kojima et al. 1990). Despite a greater dependence on transsarcolemmal Ca^{2+} fluxes early in development for regulating cardiac contractions, a decrease in the number of functional voltage-sensitive Ca^{2+} channels was observed in the immature heart (Wetzel et al. 1993; Huynh et al. 1992; Wetzel et al. 1991; Renaud et al. 1984). It has been suggested that the Na^{+}-Ca^{2+} exchanger is capable of providing an alternative mechanism for Ca^{2+} influx early in development to trigger cardiac contractions (Wetzel et al. 1993; Huynh et al. 1992; Nakanishi and Jarmakani, 1981). Thus, based upon these results, it appears the Na^{+}-Ca^{2+} exchanger may play a prominent role in both contraction and relaxation of cardiac muscle.

In contrast to these findings, there have been a number of reports disputing the role of reverse Na^{+}-Ca^{2+} exchange (*i.e.* Ca influx) in Ca^{2+}-induced Ca^{2+}-release. For example, several groups have demonstrated that Ca^{2+} entry mediated by reverse Na^{+}-Ca^{2+} exchange activity following elevated Na^{+} concentrations is inadequate to trigger SR-Ca^{2+} release (Cannell et al. 1996; Sipido et al. 1995; Bouchard et al. 1993; Sham et al. 1992). It has also been shown that following the activation of β-adrenergic receptors or protein kinase A in rat heart cells, voltage-dependent Na^{+}

channels allow the passage of Ca^{2+} in addition to Na^{+} ions in a process called slip-mode conductance (Santana et al. 1998). This Ca^{2+} entry *via* the Na^{+} channels acts as a trigger for SR-Ca^{2+} release, and thus, may down play the importance of reverse Na^{+}-Ca^{2+} exchange activity stimulated by elevated Na^{+} concentrations in triggering SR-Ca^{2+} release (Santana et al. 1998). Furthermore, reverse Na^{+}-Ca^{2+} exchange activity caused by membrane potentials positive to the reverse potential of the exchanger have also been shown to have a negligible role in triggering Ca^{2+} release from the sarcoplasmic reticulum through-out the duration of the action potential (Sipido et al. 1997). Moreover, physiological membrane potentials failed to trigger sarcoplasmic reticulum Ca^{2+} release in transgenic mice overexpressing the Na^{+}-Ca^{2+} exchanger protein (Adachi-Akahane et al. 1997). Thus, due to results supporting or disputing a role of reverse Na^{+}-Ca^{2+} exchange in Ca^{2+}-induced Ca^{2+}-release, the precise role of the Na^{+}-Ca^{2+} exchanger in excitation-contraction coupling has yet to be determined.

METHODS

Preparation of *Xenopus laevis* oocytes

Xenopus laevis were anaesthetized in 250 mg/l ethyl *p*-aminobenzoate (Sigma) in deionized ice-water for 30 min, the oocytes removed and washed in Solution A (mM: 88 NaCl, 15 HEPES, 2.4 $NaHCO_3$, 1.0 KCl, 0.82 $MgSO_4$; pH 7.6). The follicles were teased apart, the oocytes (≈ 5 ml) transferred to 5 ml Solution A containing 80 mg collagenase (Type II; Worthington) and incubated for 40 - 45 min at room temperature with gentle agitation. The oocytes were washed free of collagenase in Solution B (Solution A *plus* 0.41 mM $CaCl_2$ *plus* 0.3 mM $Ca(NO_3)_2$ containing 1 mg/ml BSA (Fraction V; Sigma), transferred to 5 ml of 200 mM K_2HPO_4 containing 1 mg/ml BSA and incubated for 11 - 12 min at room temperature with gentle agitation. Following several washes in Solution B *plus* BSA, defolliculated stage V and VI oocytes were selected and incubated at 16 °C overnight in Solution B.

Synthesis of CALX1.1, CALX1.2 and NCX1.1 cRNAs

Complimentary DNAs encoding CALX1.1, CALX1.2 and NCX1.1 contained in pBluescript II SK(+) (Stratagene) were linearized with *Not* I (CALX1.1, CALX1.2) or *Hind* III (NCX1.1). Complimentary RNAs where then synthesized using T7 mMessage mMachine *in vitro* transcription kits (Ambion), according to the manufacturer's instructions. Following injection with ≈ 5 ng of cRNA encoding CALX1.1, CALX1.2 or NCX1.1, the oocytes were maintained at 16 °C for up to 8

days. Electrophysiological measurements were typically obtained from day 3 to 6 post-injection.

Mutant/Chimaera Construction and cRNA Synthesis

Amino acid substitution and deletion mutations were introduced, essentially as described (Kunkel et al. 1991), into CALX1.1 and NCX1.1 cDNA in pBluescript II SK(+) (Stratagene). Six chimaeric CALX1.1:NCX1.1 Na^{+}-Ca^{2+} exchangers were constructed by introduction of two unique (silent) restriction sites at corresponding amino acid locations (see Figure 12) 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/chimaera cassettes were repaired into unadulterated plasmid CALX1.1. or NCX1.1 cDNA and the subcloned fragments subsequently sequenced (Sequenase 2.0; Amersham) to verify the authenticity of altered and flanking sequences. Templates for CALX1.1-based mutant/chimaeric constructs were linearized by digestion with *Not I* and cRNA subsequently synthesized using T7 mMessage mMachine *in vitro* transcription kits (Ambion). NCX1.1-based constructs were linearized with *Hind III* and cRNA synthesized using T3 kits (Ambion).

Measurement of Na^+-Ca^{2+} exchange activity

The giant excised membrane patch technique was used to measure outward Na^+-Ca^{2+} exchange currents (Hilgemann, 1990; Hryshko et al. 1996; Trac et al. 1997). Borosilicate glass pipettes were pulled and polished to a final inner diameter of ≈ 20 - 30 μm and coated with a Parafilm™:mineral oil mixture to enhance patch stability and reduce electrical noise. Following removal of the vitellin layer by dissection, oocytes were placed in (mM) 100 KOH, 100 MES, 20 HEPES, 5 EGTA, 5 $MgCl_2$; pH 7.0 at 30 °C with MES, and GΩ seals were formed *via* gentle suction (Figure 1a). Membrane patches (inside-out configuration) were excised by progressive movements of the pipette tip. Rapid bath solution (*i.e.*, intracellular) changes were accomplished using a custom-built, computer-controlled, 20-channel solution switcher. Axon Instruments hardware (Axopatch 200a) and software (Axotape) were used for data acquisition and analysis. Pipette (*i.e.*, extracellular) solutions contained (mM) 100 NMG-MES, 30 HEPES, 30 TEA-OH, 16 sulfamic acid, 8 $CaCO_3$, 6 KOH, 0.25 ouabain, 0.1 niflumic acid, 0.1 flufenamic acid; pH 7.0 at 30 °C with MES. Outward Na^+-Ca^{2+} exchange currents were elicited by switching from Li^+_i- to Na^+_i-based bath solutions containing (mM) 100 [Na^++ Li^+]-aspartate, 20 MOPS, 20 TEA-OH, 20 CsOH, 10 EGTA, 0 - 2.3 $CaCO_3$, 1.0 - 1.5 $Mg(OH)_2$; pH 7.0 at 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 (Bers et al. 1994). All experiments were conducted at 30 ± 1 °C.

Statistical Analysis

All data reported are means ± SEM, unless otherwise indicated. Statistical comparisons were performed using the two-tailed student's t-test (Microcal Software Inc., Northampton, MA, USA). Significant differences were defined by a probability of less than 0.05.

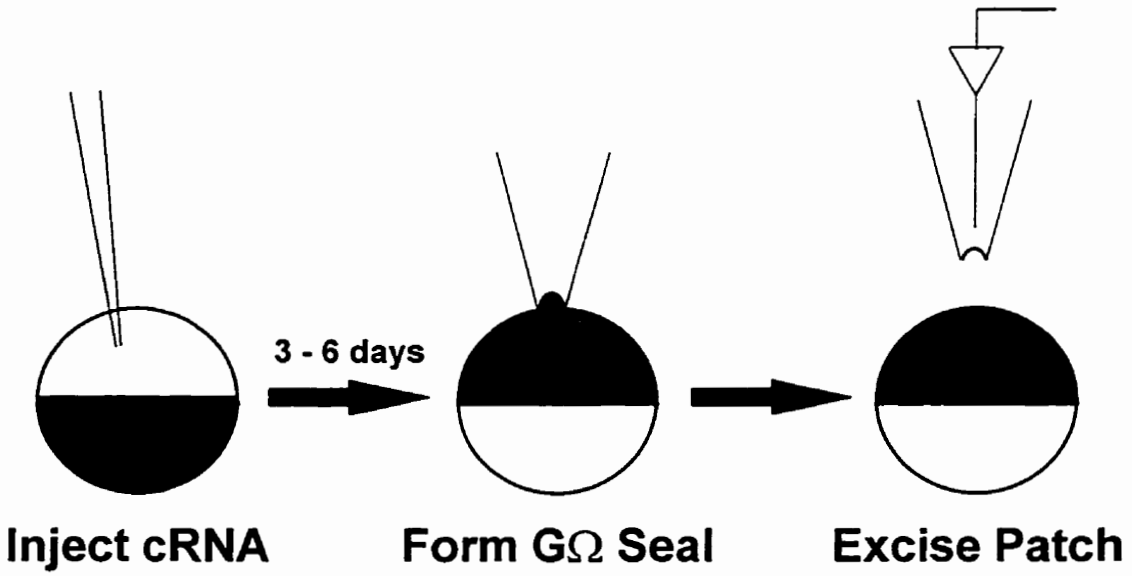

Figure 1a. The Expression and Electrophysiological Characterization of Na^+-Ca^{2+} Exchange Proteins. Complimentary RNA encoding Na^+-Ca^{2+} exchange protein was injected into *Xenopus laevis* oocytes (*left panel*). Following 3-6 days to allow for protein expression, gigaohm seals were formed between oocyte membrane and pipette tip (*middle panel*). The membrane patch was then excised by retraction of the pipette tip from the oocyte (*right panel*)

RESULTS

Part A: Structure-function Studies

1) Typical Outward Na^+-Ca^{2+} Exchange Current

The giant excised membrane patch clamp technique is used to remove patches of membrane from oocytes expressing the Na^+-Ca^{2+} exchangers (Hilgemann, 1989). Once removed (*left panel*, Figure 1b), we now have access to the cytoplasmic surface of the membrane patch. For all experiments shown, the pipette or extracellular solution contained 8 mM Ca, (transport Ca^{2+}_o). By applying Na^+_i to the cytoplasmic or bath side of the membrane patch, we now have conditions favourable for Na^+-Ca^{2+} exchange current. The stoichiometry of this consecutive transport cycle is $3Na^+$:$1Ca^{2+}$ (Khananshvili, 1990; Reeves and Hale, 1984; Kimura et al. 1986; Hilgemann et al. 1991). Thus, the Na^+-Ca^{2+} exchanger is an electrogenic transporter allowing for electrophysiological characterization of exchange activity. In the *right panel* of Figure 1b, we show a typical Na^+-Ca^{2+} exchange current elicited by the application of Na^+_i to the cytoplasmic surface of the membrane patch. Initially, we observe a rapid rise of exchange current to a peak level, followed by a slow, time-dependent inactivation to a lower steady-state current level (Hilgemann, 1990). Note that the exchanger is operating in the Ca^{2+} influx, or reverse mode of transport. Since the net movement of charge is directed into the pipette (extracellular surface), this exchange current is referred to as outward Na^+-Ca^{2+} exchange current. Here, for outward exchange currents, bath Na^+_i exchanges for pipette Ca^{2+}_o. Furthermore,

current values are highly variable from one oocyte to another. At present, the reason for this differences remains unknown.

2) Na^+_i Regulation

In addition to being transported by the Na^+-Ca^{2+} exchanger, Na^+_i can also regulate exchange activity (Hilgemann, 1990). In Figure 2, Na^+-Ca^{2+} exchange currents for NCX1.1, the cardiac Na^+-Ca^{2+} exchanger, were activated by applying various Na^+_i concentrations to the cytoplasmic surface of the membrane patch. With increasing Na^+_i concentrations, the magnitude of peak current and the extent of current inactivation increase. This slow time-dependent inactivation to lower steady-state levels is referred to as Na^+_i-induced, or I_1 inactivation. Therefore, as Na^+_i levels are elevated, Na^+_i regulation can be seen as an increase in the extent of current inactivation. This form of regulation has been observed for all characterized Na^+-Ca^{2+} exchangers including CALX1.1, the *Drosophila* Na^+-Ca^{2+} exchanger (Hilgemann, 1990; Hryshko et al. 1996; Li et al. 1994).

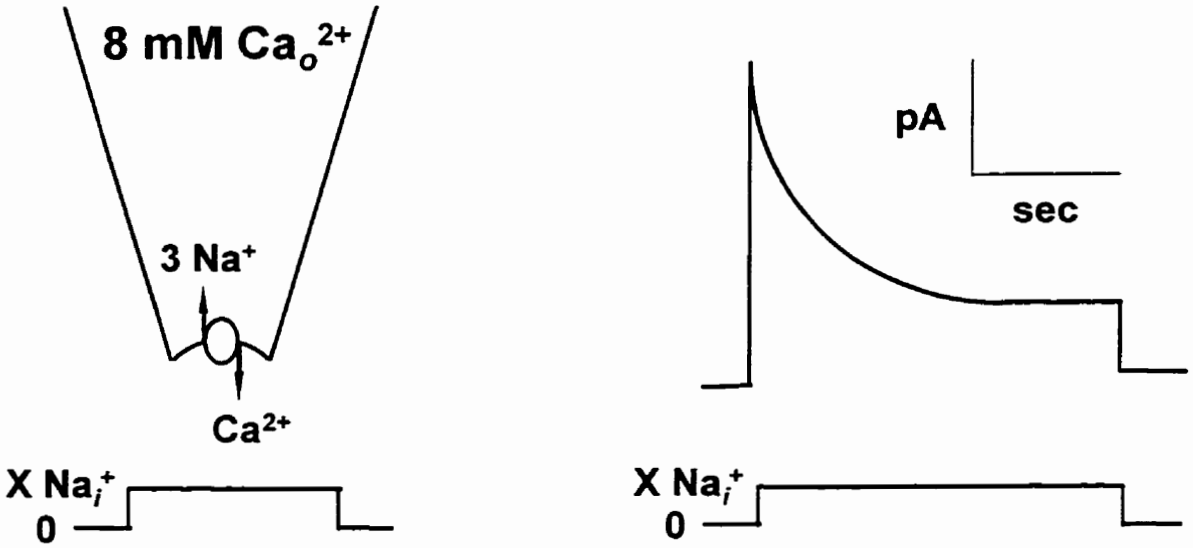

Figure 1b. Typical Na^+-Ca^{2+} Exchange Current. In the *left panel*, an excised membrane patch expressing Na^+-Ca^{2+} exchangers is shown located at the tip of a pipette. In this configuration, the Na^+-Ca^{2+} exchanger transports 3 cytoplasmic Na^+_i ions into the pipette (*i.e.*, extracellular surface) in exchange for 1 Ca^{2+}_o. The net movement of 1 positive charge associated with Na^+_i translocation is in the outward direction (*i.e.*, into the pipette), and is therefore referred to as outward exchange current. The *right panel* illustrates a typical outward Na^+-Ca^{2+} exchange current, where the Na^+-Ca^{2+} exchanger is operating in the Ca^{2+} influx mode of transport.

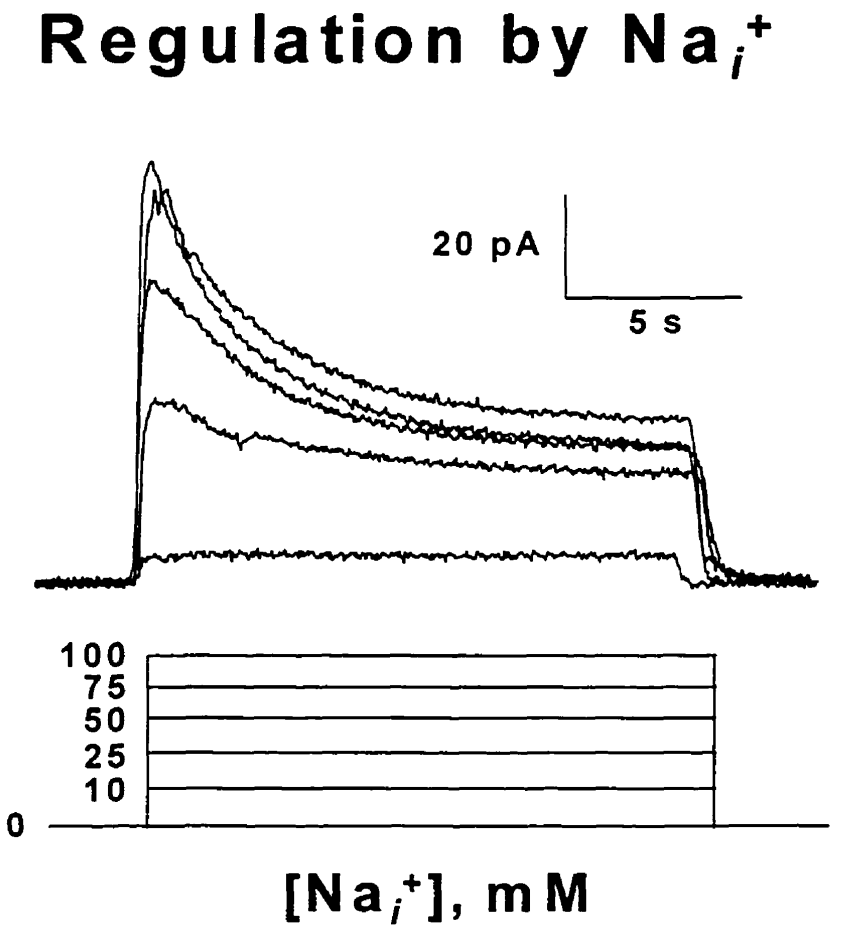

Figure 2. Regulation of Na^+-Ca^{2+} exchange current by Na^+_i. The indicated Na^+_i concentrations were used to activate exchange currents for NCX1.1. With increasing Na^+_i concentrations, the magnitude of peak and extent of current inactivation increases.

3) Ca^{2+}_i Regulation

In addition to Na^+_i-dependent regulation, exchange activity is also regulated by Ca^{2+}_i from the cytoplasmic surface (Hilgemann, 1990). Figure 3 shows the activation of Na^+-Ca^{2+} exchange for NCX1.1 by the application of 100 mM Na^+_i with the indicated Ca^{2+}_i concentrations. For outward exchange currents, regulatory Ca^{2+}_i and bath Na^+_i are applied to the cytoplasmic side of the Na^+-Ca^{2+} exchanger. With an elevation of Ca^{2+}_i concentration, peak current increases and the extent of Na^+_i-induced inactivation, or I_1, becomes less pronounced resulting in larger steady-state current levels (Hilgemann et al. 1992a). The stimulatory effect of regulatory Ca^{2+}_i is observed for all characterized Na^+-Ca^{2+} exchangers with the exception of the *Drosophila* Na^+-Ca^{2+} exchanger, CALX1.1 (Hryshko et al. 1996). Note, the decrease in peak current at higher Ca^{2+}_i concentrations (30,100 μM) can be attributed to competition of Na^+_i and Ca^{2+}_i at the cytoplasmic transport site, rather than an inhibitory effect of regulatory Ca^{2+}_i at these elevated levels.

Regulation by Ca^{2+}_i

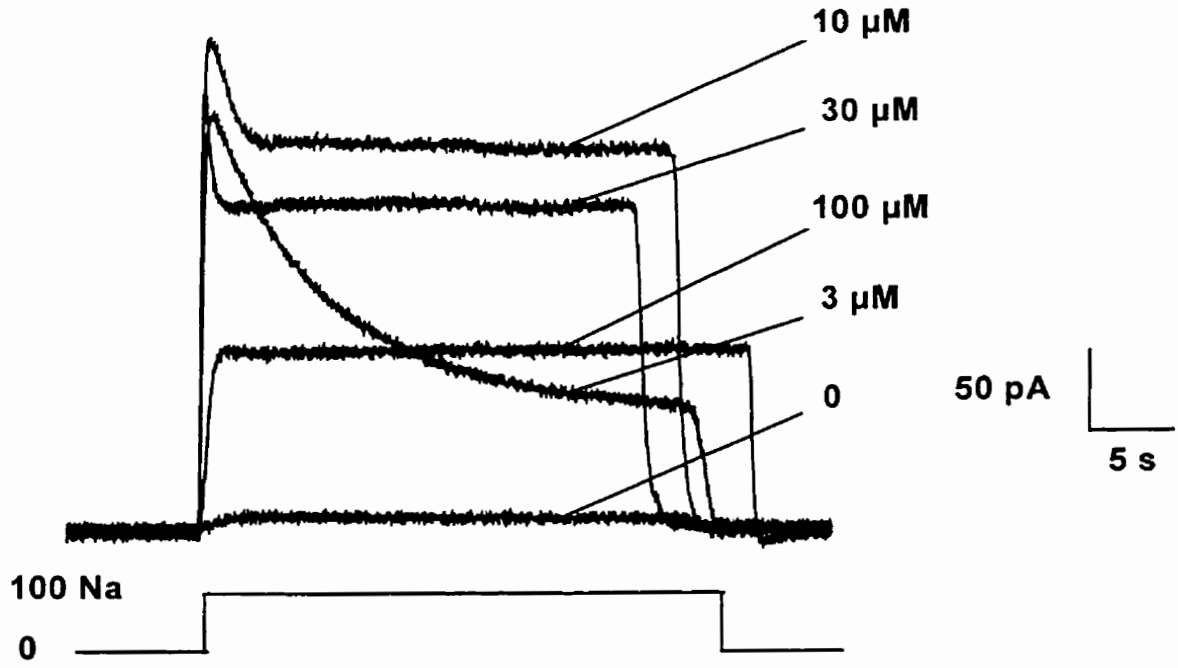

Figure 3. Regulation of Na^+-Ca^{2+} Exchange Current by Ca^{2+}_i. Exchange currents for NCX1.1 were activated by 100 mM Na^+_i in the presence of the indicated Ca^{2+}_i concentration. With increasing Ca^{2+}_i concentrations, peak current is augmented and the extent of Na^+_i-induced inactivation is reduced.

4) Phenotypic Differences of CALX1.1 and NCX1.1 in Response to Regulatory Ca^{2+}_i

Figure 4 (*bottom right*) shows outward Na^+-Ca^{2+} exchange current for CALX1.1. Exchange current was triggered by the application of 100 mM Na^+_i in the absence of regulatory Ca^{2+}_i. At steady-state current levels, application of 1 μM regulatory Ca^{2+}_i causes a further decrease in exchange current, which then recovers to steady-state values upon removal of Ca^{2+}_i. This inactivation of exchange current caused by Ca^{2+}_i application at steady-state current levels illustrates the inhibitory effect of regulatory Ca^{2+}_i for CALX1.1. For NCX1.1, this Ca^{2+}_i-dependent activation of outward Na^+-Ca^{2+} exchange current is opposite to that observed for CALX1.1 (*top left*). Thus, in contrast to CALX1.1, regulatory Ca^{2+}_i stimulates exchange current for NCX1.1. That is, removal of Ca^{2+}_i at steady-state levels results in decreased Na^+-Ca^{2+} exchange current. The remaining figures (*bottom left, top right*) show different permutations of this protocol illustrating the inhibitory and stimulatory effect of regulatory Ca^{2+}_i, for CALX1.1 and NCX1.1 respectively.

5) Deregulation of Na^+-Ca^{2+} Exchange Current

In the *left panel* of Figure 5, negative Ca^{2+}_i regulation is shown for CALX1.1. However, following treatment with 2 mg/ml of α-chymotrypsin for ≈ 60 s (*right panel*), negative Ca^{2+}_i regulation is no longer observed (*i.e.* magnitude of peak and steady-state current levels are similar in the presence or absence of regulatory Ca^{2+}_i). Deregulated Na^+-Ca^{2+} exchange currents were also observed for NCX1.1 and NCX2 following α-chymotrypsin treatment (Matsuoka et al. 1995; Li et al. 1994).

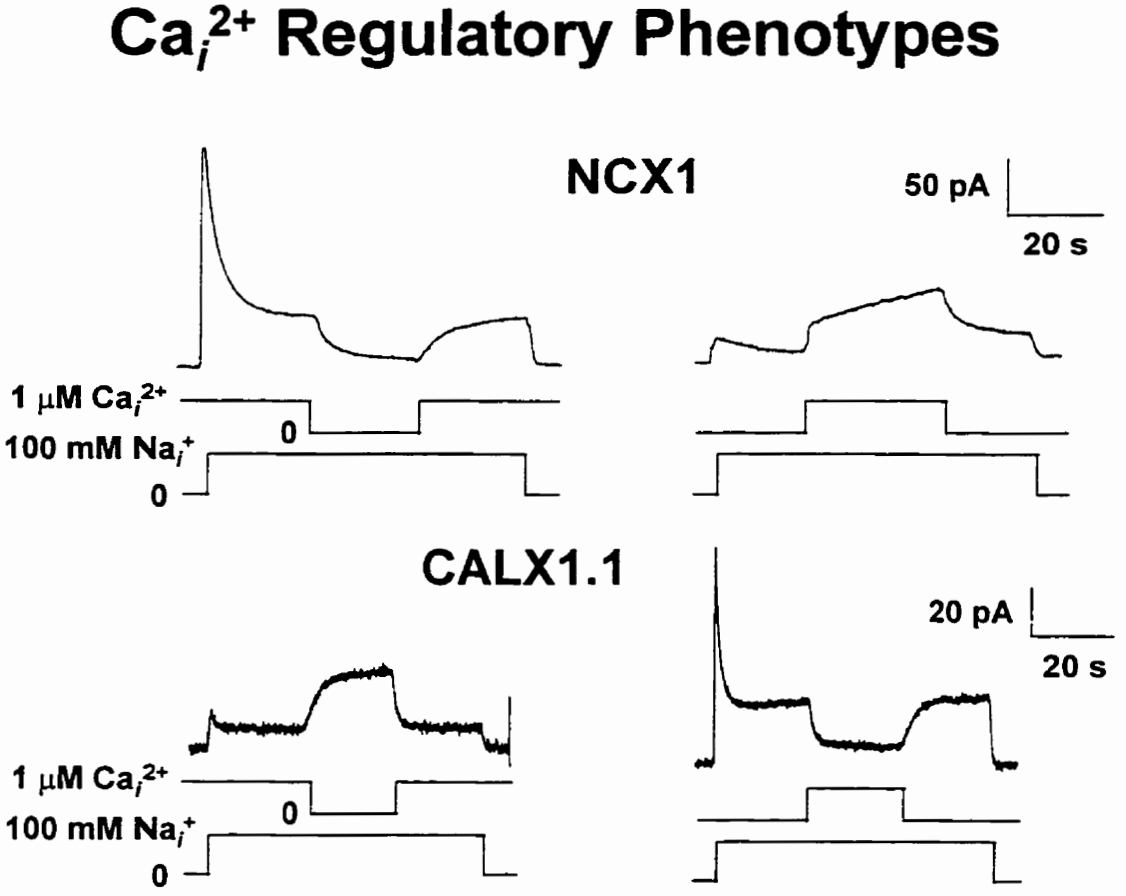

Figure 4. Phenotypic Differences in Response to Regulatory Ca^{2+}_i for NCX1.1 and CALX1.1. The *top left panel* shows exchange current for NCX1.1 in the presence of regulatory Ca^{2+}_i. At steady-state current levels, removal of regulatory Ca^{2+}_i results in a decrease of exchange current which then recovers when regulatory Ca^{2+}_i is restored. In contrast, for CALX1.1, the addition of regulatory Ca^{2+}_i at steady-state current levels inhibits exchange activity (*bottom right*). This difference is further illustrated in the *top right* and *bottom left panels* using other permutations of this protocol.

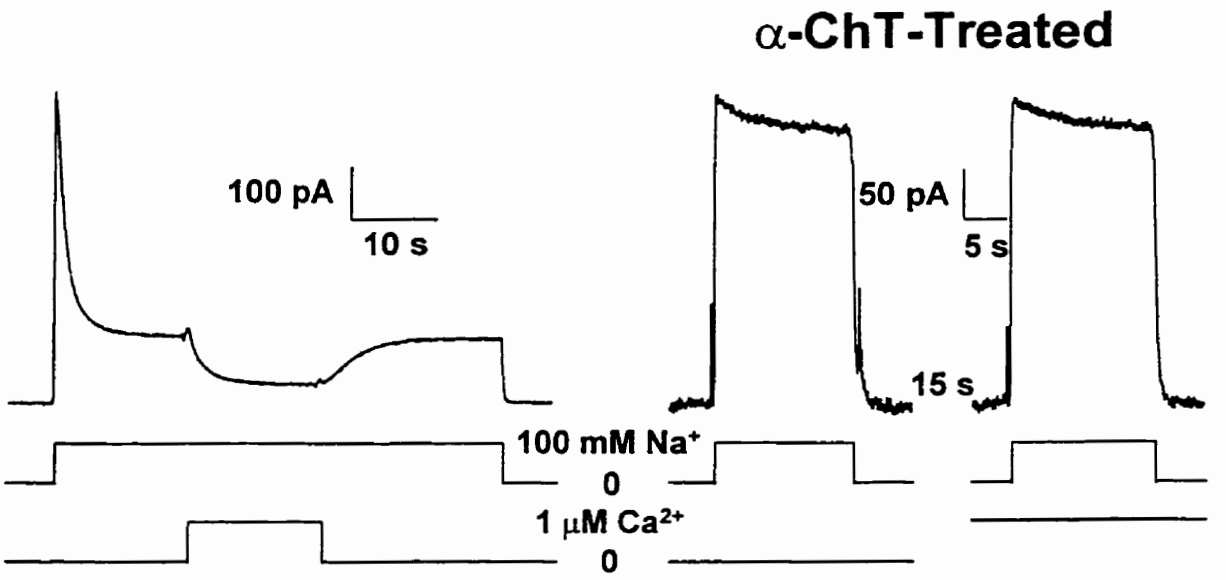

Figure 5. The Effects of Chymotrypsin Treatment on Na^+-Ca^{2+} Exchange Currents. Typical Na^+-Ca^{2+} exchange current illustrating negative Ca^{2+}_i regulation for CALX1.1 is shown in the *left panel*. However, when CALX1.1 is treated with 2 mg/ml of chymotrypsin for ≈ 1 minute, regulation is no longer observed (*right panel*). That is, regulatory Ca^{2+}_i no longer influenced the waveform of Na^+-Ca^{2+} exchange activity.

6) The Putative Regulatory Ca^{2+}_i Binding Site

The regulatory Ca^{2+}_i binding site is localized to amino acids 371-508 of NCX1.1 (Levitsky et al. 1994; Matsuoka et al. 1995; Philipson et al. 1996). This sequence in NCX1.1 appears to be highly conserved for all cloned Na^+-Ca^{2+} exchangers as shown from the alignment in Figure 6. Within this protein domain, two acidic clusters of functional importance have been identified. Despite the results supporting a role for this region in mediating Ca^{2+}_i-dependent regulation (Levitsky et al. 1994; Matsuoka et al. 1995) and the observation of a high degree of sequence similarity observed for this region between all cloned exchangers, it is unknown whether there is a conserved Ca^{2+}_i binding function associated with this region for other Na^+-Ca^{2+} exchangers. Thus, to determine whether this region may also function as the Ca^{2+}_i binding site for CALX1.1, residues of CALX1.1 within these acidic clusters analogous to those of NCX1.1 were targets for mutagenesis studies.

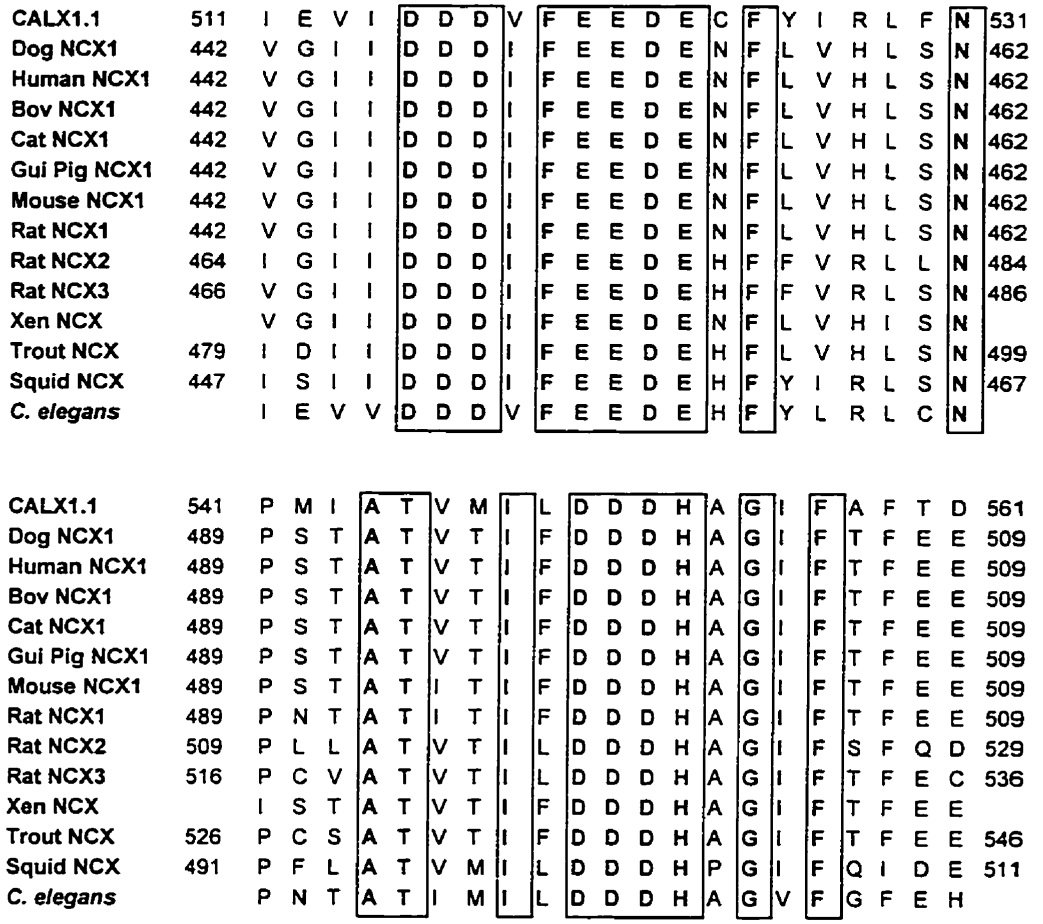

Figure 6. Regions of Sequence Similarity within the Putative Regulatory Ca^{2+}_i Binding Site. In NCX1.1, two acidic amino acid clusters have been identified within the putative regulatory Ca^{2+}_i binding site. These regions are highly conserved for all characterized Na^+-Ca^{2+} exchangers as illustrated in the above figure.

7) Mutations in the Regulatory Ca^{2+} Binding Site

Figure 7 compares the Ca^{2+}_i dependence of outward Na^+-Ca^{2+} exchange currents for CALX1.1 and 3 regulatory Ca^{2+}_i binding site mutants. Substantial differences in the affinity for Ca^{2+}_i-dependent regulation were observed as a result of point mutations targeting highly conserved amino acid residues within the regulatory Ca^{2+}_i binding site. For CALX1.1, Ca^{2+}_i-dependent inhibition of exchange currents is shown (*top left*). That is, as Ca^{2+}_i concentrations are increased (1 or 3 μM), exchange currents become less. In contrast, the analogous point mutations within the regulatory Ca^{2+}_i binding site, G555P (CALX1.1) and G503P (NCX1.1), yielded Ca^{2+}_i insensitive Na^+-Ca^{2+} exchange currents (Matsuoka et al. 1995). Thus, similar exchange currents were elicited in the presence or absence of 10 μM regulatory Ca^{2+}_i (*top right*). This blunted response to regulatory Ca^{2+}_i is also seen for the following mutant pairs, G558P and Δ680-685 in NCX1.1 and G609P and Δ691-696 in CALX1.1. (data not shown). In addition to observing Ca^{2+}_i insensitive responses, regulatory Ca^{2+}_i binding site mutations also lead to reduced affinities for Ca^{2+}_i-dependent regulation. The CALX1.1 mutants, D516V and D550I, corresponding to D447V and D498I in NCX1.1 (Matsuoka et al. 1995), are shown in Figure 7 (*bottom*). For both mutant exchangers, a reduced inhibitory effect of Ca^{2+}_i occurs following neutralization of these conserved acidic clusters within the regulatory Ca^{2+}_i binding site. To illustrate this difference in affinity, substantial inhibition of exchange current was observed at 3 μM Ca^{2+}_i for CALX1.1, whereas for D516V and D550I, only minimal inhibition occurred at 3 μM Ca^{2+}_i when compared to current

levels produced in the absence of regulatory Ca^{2+}_i. Similarly, a reduced affinity for Ca^{2+}_i-dependent regulation was also observed for the NCX1.1 mutants, D447V and D498I (Matsuoka et al. 1995).

8) The Putative XIP Region

The eXchange Inhibitory Peptide (XIP) region, initially thought to be a calmodulin binding site, is located within the large cytoplasmic loop and influences both the rate and extent of Na^+_i-induced, or I_1 inactivation. Mutations within this region for NCX1.1 resulted in the acceleration or elimination of the Na^+_i-induced inactivation process (Matsuoka et al. 1997). Although this region of NCX1.1 shows sequence similarity compared to the analogous regions of other cloned exchangers (Figure 8), it is less well conserved than the regulatory Ca^{2+}_i binding site. To determine whether this region may play a similar functional role for the *Drosophila* exchanger, CALX1.1, we examined two XIP region mutations of CALX1.1 that were analogous to those characterized in NCX1.1. We anticipated that a conserved functional role would be observed for this protein region and that XIP mutations in CALX1.1 would produce similar phenotypic changes to Na^+_i-induced inactivation as those observed for the analogous XIP mutations in NCX1.1.

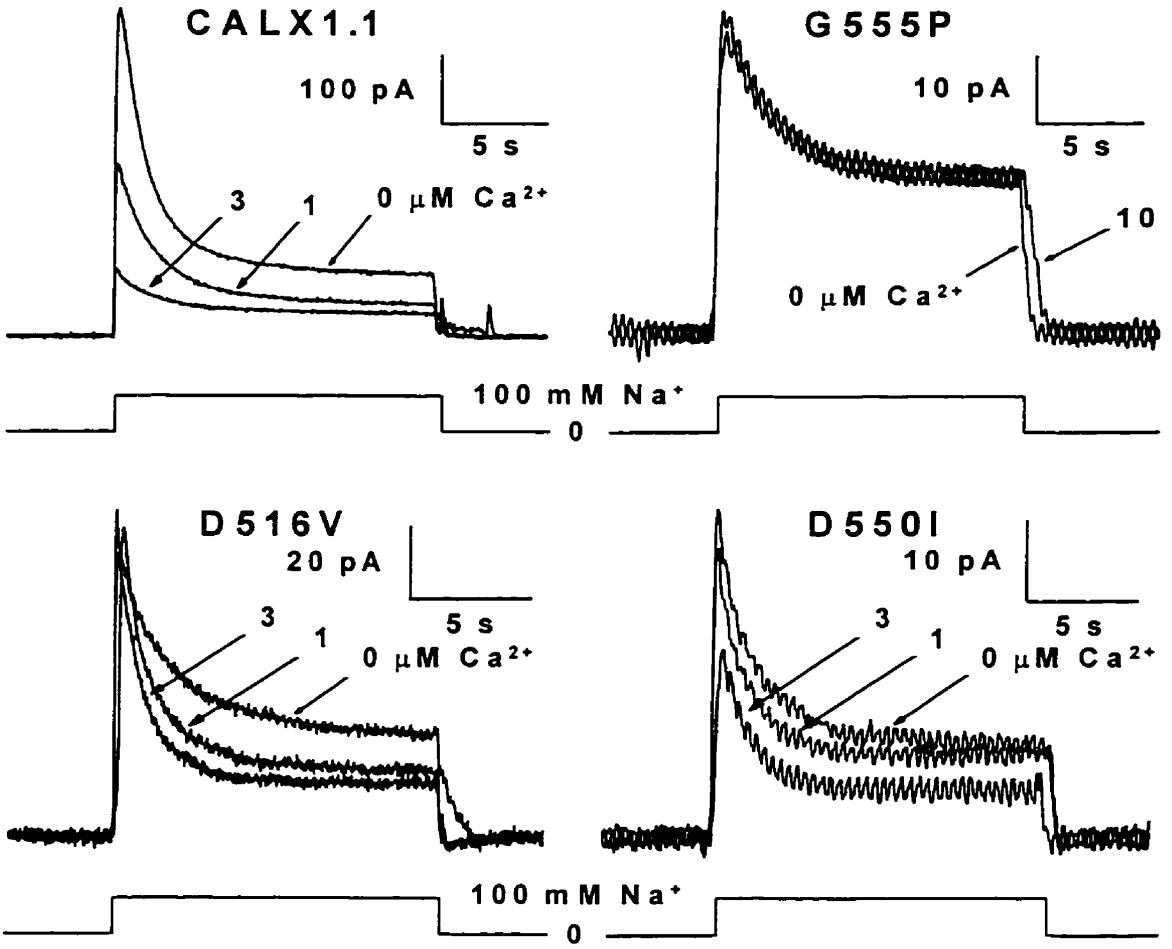

Figure 7. Mutations Targeting Residues Within the Regulatory Ca^{2+}_i Binding Site. The effects of Ca^{2+}_i on outward exchange currents for CALX1.1 and 3 regulatory Ca^{2+}_i binding site mutants are shown. These mutations, within the highly conserved acidic clusters, alter the affinity for Ca^{2+}_i regulation. Compared with CALX1.1, G555P appears insensitive to regulatory Ca^{2+}_i (n = 3), whereas D516V and D550I display a lower affinity for negative Ca^{2+}_i regulation.

CALX1.1	300	R	R	L	L	V	Y	K	Y	M	D	K	N	Y	R	V	N	K	-	R	G	T	319
Dog NCX1	219	R	R	L	L	F	Y	K	Y	V	Y	K	R	Y	R	A	G	K	Q	R	G	M	239
Human NCX1	219	R	R	L	L	F	Y	K	Y	V	Y	K	R	Y	R	A	G	K	Q	R	G	M	239
Bov NCX1	219	R	R	L	L	F	Y	K	Y	V	Y	K	R	Y	R	A	G	K	Q	R	G	M	239
Cat NCX1	219	R	R	L	L	F	Y	K	Y	V	Y	K	R	Y	R	A	G	K	Q	R	G	M	239
Gui Pig NCX1	219	R	R	L	L	F	Y	K	Y	V	Y	K	R	Y	R	A	G	K	Q	R	G	M	239
Mouse NCX1	219	R	R	L	L	F	Y	K	Y	V	Y	K	R	Y	R	A	G	K	Q	R	G	M	239
Rat NCX1	219	R	R	L	L	F	Y	K	Y	V	Y	K	R	Y	R	A	G	K	Q	R	G	M	239
Rat NCX2	248	K	R	L	L	F	Y	K	Y	V	Y	K	R	Y	R	T	D	P	R	S	G	I	268
Rat NCX3	253	K	R	L	L	F	Y	K	Y	M	H	K	R	Y	R	T	D	K	H	R	G	I	273
Xen NCX		R	R	L	L	F	Y	K	Y	V	Y	K	R	Y	R	A	G	K	Q	R	G	M	
Trout NCX	259	R	R	L	L	V	Y	K	Y	M	Y	K	R	Y	R	A	G	K	R	R	G	V	279
Squid NCX	241	R	R	L	L	-	N	K	Y	L	S	K	K	Y	R	A	S	K	Q	K	G	V	260
C. elegans		I	K	L	I	Q	N	K	F	L	P	H	R	Y	R	R	G	S	H	G	G	M	

Figure 8. Sequence Similarity of Residues Mediating Na^+_i-induced Inactivation. For NCX1.1, Na^+_i-dependent regulation is mediated by the eXchange Inhibitory Peptide (XIP) region. The analogous amino acid sequences from other identified exchangers is shown to illustrate the sequence similarity within this region.

9) Mutations in the XIP Region

Figure 9 shows outward Na^+-Ca^{2+} exchange currents for CALX1.1 and two XIP region mutants. A range of Na^+_i concentrations in the absence of regulatory Ca^{2+}_i were used to activate exchange activity. For CALX1.1 and the point mutation K306Q, increasing Na^+_i concentrations resulted in augmentation of peak current and the extent of Na^+_i-induced inactivation. However, the rate of entry into the I_1 inactive state was much greater for K306Q as compared with CALX1.1. For example, the rate of inactivation for K306Q in response to a pulse of 100 mM Na^+_i was 1.27 ± 0.06 sec^{-1} (n = 10) as compared with 0.56 ± 0.04 sec^{-1} (n = 25) for CALX1.1 ($P < 0.05$). Similarly, the analogous XIP region mutant for NCX1.1, K225Q, exhibited an accelarated rate of entry into the I_1 inactive state as compared with NCX1.1 (Matsuoka et al. 1997). In addition to an accelerated rate of entry into the I_1 inactive state, K306Q also differed from CALX1.1 by exhibiting a slightly smaller value for F_{ss}. This value, F_{ss}, represents the ratio of steady-state to peak current. Therefore, it appears a greater proportion of the K306Q exchanger population occupies this I_1 inactive state at steady-state current levels. In contrast, for all Na^+_i concentrations examined, no current decay was observed for the CALX1.1 deletion mutant Δ310-313 following stimulation of exchange currents. Thus, it appears Na^+_i-induced inactivation was eliminated for the CALX1.1 deletion mutant. Similarly, the analogous mutation in NCX1.1, Δ229-232, resulted in the elimination of Na^+_i-induced inactivation (Matsuoka et al. 1997).

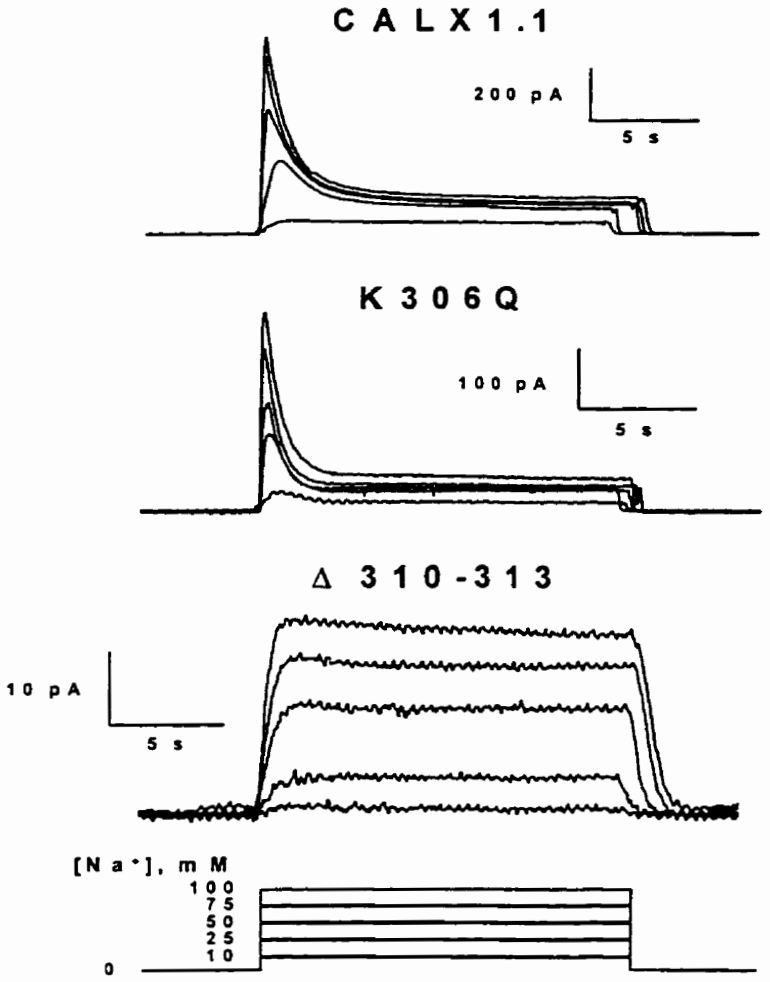

Figure 9. Mutations Targeting Residues Within the XIP Region. The Na^+_i-dependence of outward exchange currents for CALX1.1 and two XIP region mutants are shown. Significant differences with respect to Na^+_i-induced inactivation were observed between wild-type and mutant exchangers. The K306Q mutant responds in a similar fashion to CALX1.1, except the rate and extent of current decay are greater. In contrast, no Na^+_i-dependent inactivation is observed for the deletion mutant Δ310-313.

Paired-pulse experiments were used to isolate the rate of recovery from Na^+_i-induced inactivation for CALX1.1 and two XIP region mutants. Here, exchange currents were first activated by 100 mM Na^+_i in the absence of regulatory Ca^{2+}_i and allowed to reach steady-state current levels (Pulse 1), followed by the turning off of exchange current by replacing bath Na^+_i with a Li^+_i based superfusate (recovery interval), then once again activated by bath Na^+_i (Pulse 2). During the recovery interval, exchangers may only recover from Na^+_i-induced inactivation since cytoplasmic Na^+_i is absent, thereby preventing entry into the I_1 inactive configuration. In Figure 10, recovery intervals of 16, 4 and 0.5 s are shown for CALX1.1, K306Q, and Δ310-313. For all recovery intervals examined, the peak of the second pulse is much greater for K306Q as compared with CALX1.1. On average, the rate of recovery from I_1 inactivation occurred about twice as fast for K306Q. In contrast, for the deletion mutant Δ310-313, the peak of the second pulse was essentially the same as the initial pulse irrespective of the recovery interval length. These results suggest that I_1 inactivation has been eliminated for Δ310-313.

Figure 11 summarizes pooled results showing a greater recovery rate for K306Q as compared with CALX1.1. The data are from 5 patches for K306Q and 8 patches for CALX1.1. The data for Δ310-313 are not plotted as recoveries since it appears that this deletion mutant does not enter the I_1 inactive state ($P < 0.05$).

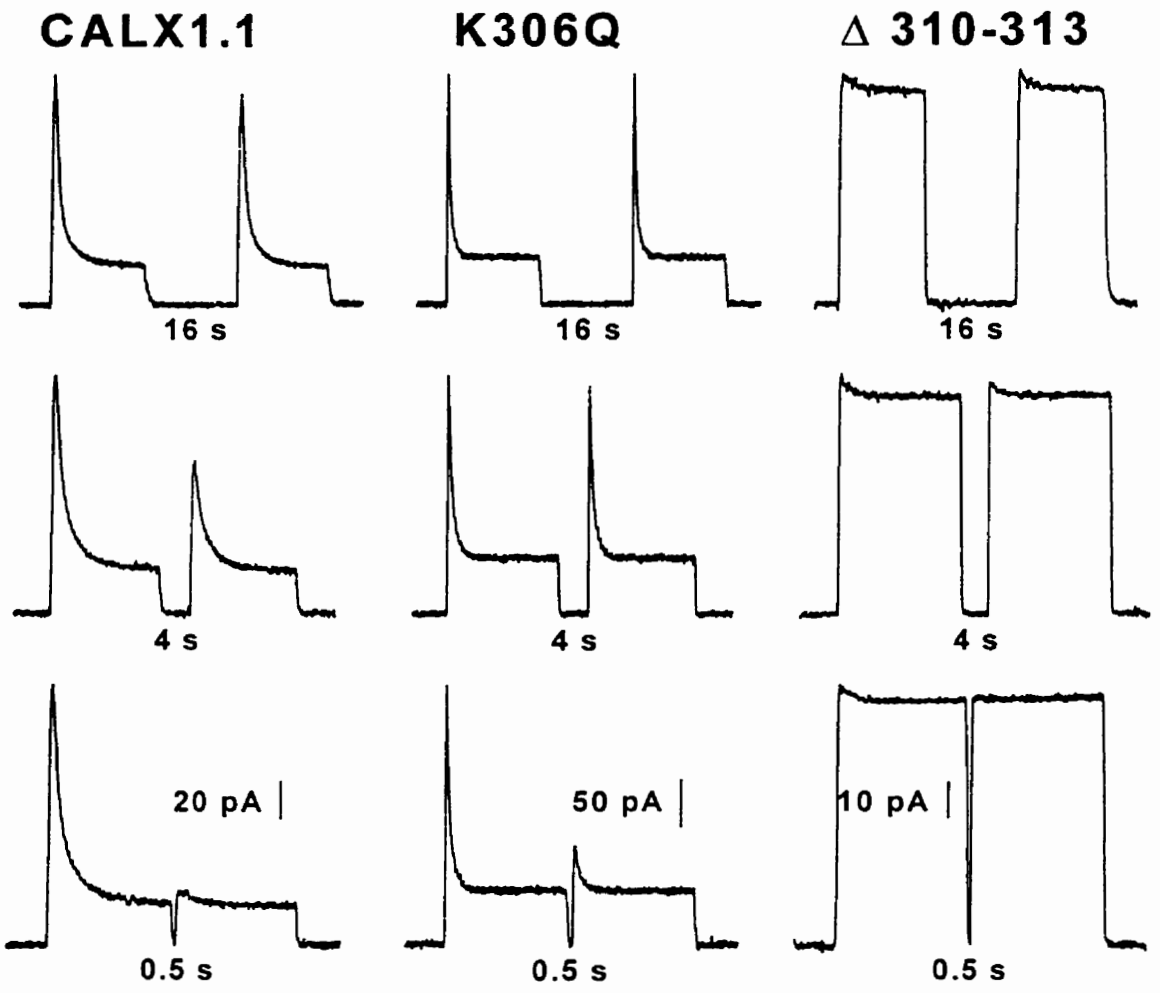

Figure 10. Paired-Pulse Experiments Showing Kinetic Differences in the Recovery from Na^+_i-induced Inactivation. Paired-pulse experiments were conducted for CALX1.1 and and two XIP region mutants. Traces for recovery intervals 16, 4 and 0.5 s are shown. Note that peak current of the second pulse recovers slower for CALX1.1 as compared to K306Q for all intervals. In contrast, it appears the Δ310-313 mutant does not enter the I_1 inactive state.

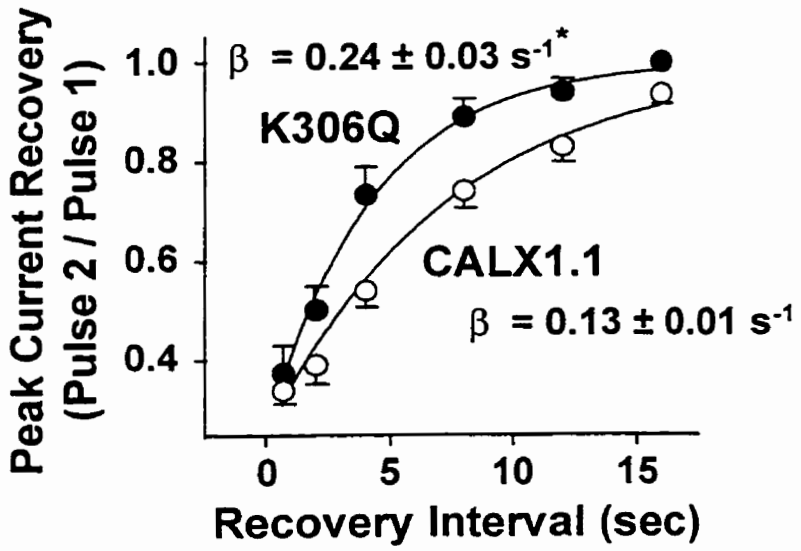

Figure 11. The Effect of XIP Mutations on the Rate of Recovery from Na^+_I-induced inactivation. Pooled results from 5 patches for K306Q show an ≈ 2 fold reduction in the rate of recovery from the I_I inactive state compared to CALX1.1 (pooled results from 8 patches). *P < 0.05 for β's of K306Q *vs* CALX1.1.

10) Ca^{2+}_i-Dependent Regulation of Chimaeric Exchangers

Despite observations suggesting a conserved functional role for the regulatory Ca^{2+}_i binding site and XIP region in NCX1.1 and CALX1.1, these exchangers exhibited opposite responses to regulatory Ca^{2+}_i. To investigate this striking difference, we constructed six CALX1.1:NCX1.1 chimaeric exchangers and examined their responses to Ca^{2+}_i. Here, silent restriction sites (Bst 1107I and Bss SI) were introduced into the large cytoplasmic loop of the exchangers and used as splice sites for substituting portions of NCX1.1 for the equivalent region of CALX1.1, and *vice versa*. Figure 12 illustrates this procedure. From these studies, we hoped to identify protein domains that are responsible for imparting a particular regulatory Ca^{2+}_i phenotype.

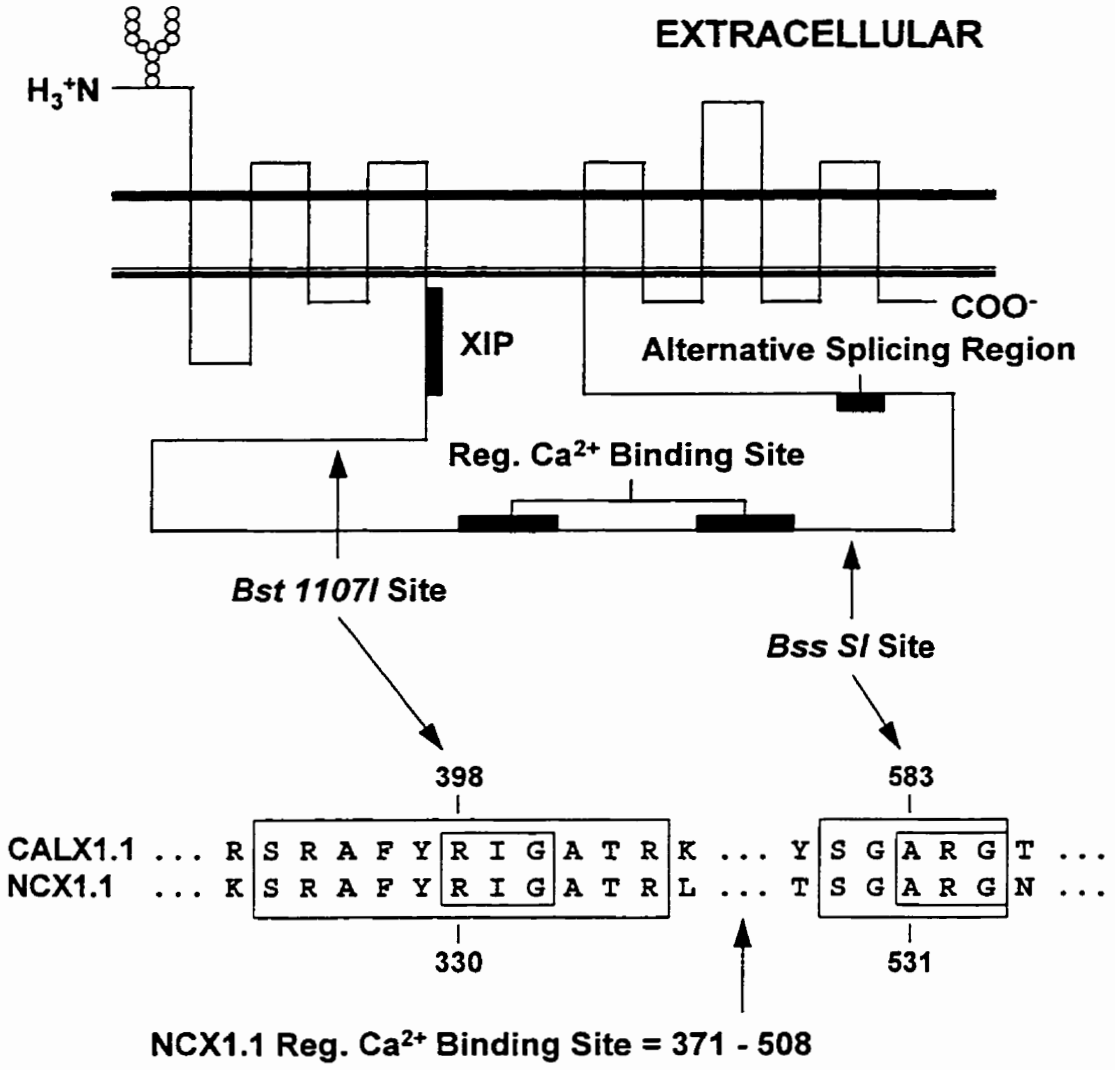

Figure 12. The Incorporation of Silent Restriction Sites for Production of Chimaeric Exchangers . Silent restriction sites were incorporated into regions of amino acid similarity between NCX1.1 and CALX1.1 (*bottom panel*). These restriction sites, located within the large cytoplasmic loop, were used to construct NCX1.1:CALX1.1 chimaeric exchangers.

Figure 13 illustrates the NCX1.1:CALX1.1 chimaeric possibilities. If the regulatory Ca^{2+}_i binding site and XIP region perform similar functions for CALX1.1 and NCX1.1, chimaeric exchangers were predicted to retain both Na^{+}_i- and Ca^{2+}_i-dependent regulatory processes. That is, normal Ca^{2+}_i binding and I_t inactivation should be observed. Furthermore, phenotypic alterations as a consequence of chimaeric production may identify the approximate location of other protein domains involved in transduction of the Ca^{2+}_i binding signal.

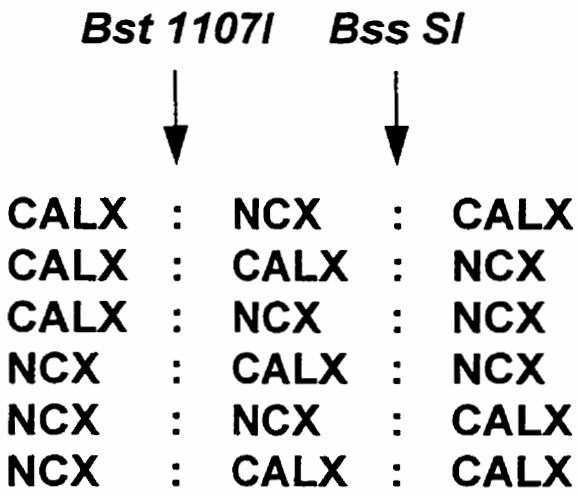

Figure 13. Construction of NCX1.1:CALX1.1 Chimaeric Exchangers. Splicing resulted in the following components: 1) the N-terminal transmembrane-spanning segments and XIP region, 2) the regulatory Ca^{2+}_i binding site and flanking sequences and 3) the alternative splicing region and C-terminal transmembrane-spanning segments. All chimaeric possibilities were constructed as shown and assayed for response to regulatory Ca^{2+}_i.

Figure 14 shows differences in the response to 1 μM regulatory Ca^{2+}_i for NCX1.1, CALX1.1 and the CALX:NCX:CALX chimaeric exchanger. For the chimaera, comprised mainly of the CALX1.1 parent transporter (Figure 12) with a portion of the cytoplasmic loop from NCX1.1 substituted into CALX1.1 for the equivalent region, regulatory Ca^{2+}_i stimulated both peak and steady-state Na^+-Ca^{2+} exchange current. The substituted sequence included the regulatory Ca^{2+}_i binding site and adjacent sequences. The graph in Figure 14 shows inhibition and stimulation of peak current over a range of Ca^{2+}_i concentrations for CALX1.1 and CALX:NCX:CALX, respectively. For all concentrations examined, peak current of CALX1.1 was inhibited by Ca^{2+}_i whereas the chimaera is stimulated. Although similar to NCX1.1, the chimaeras response to regulatory Ca^{2+}_i is not identical to that observed for the cardiac Na^+-Ca^{2+} exchanger. Therefore, only partial interconversion of regulatory phenotypes was evident for CALX:NCX:CALX. Of the remaining five chimaeras, only NCX:CALX:NCX produced measurable levels of Na^+-Ca^{2+} exchange current. Although Na^+_i-induced activation was retained, the chimaera appeared insensitive to regulatory Ca^{2+}_i up to 30 μM (data not shown) like G555P (see Figure 7).

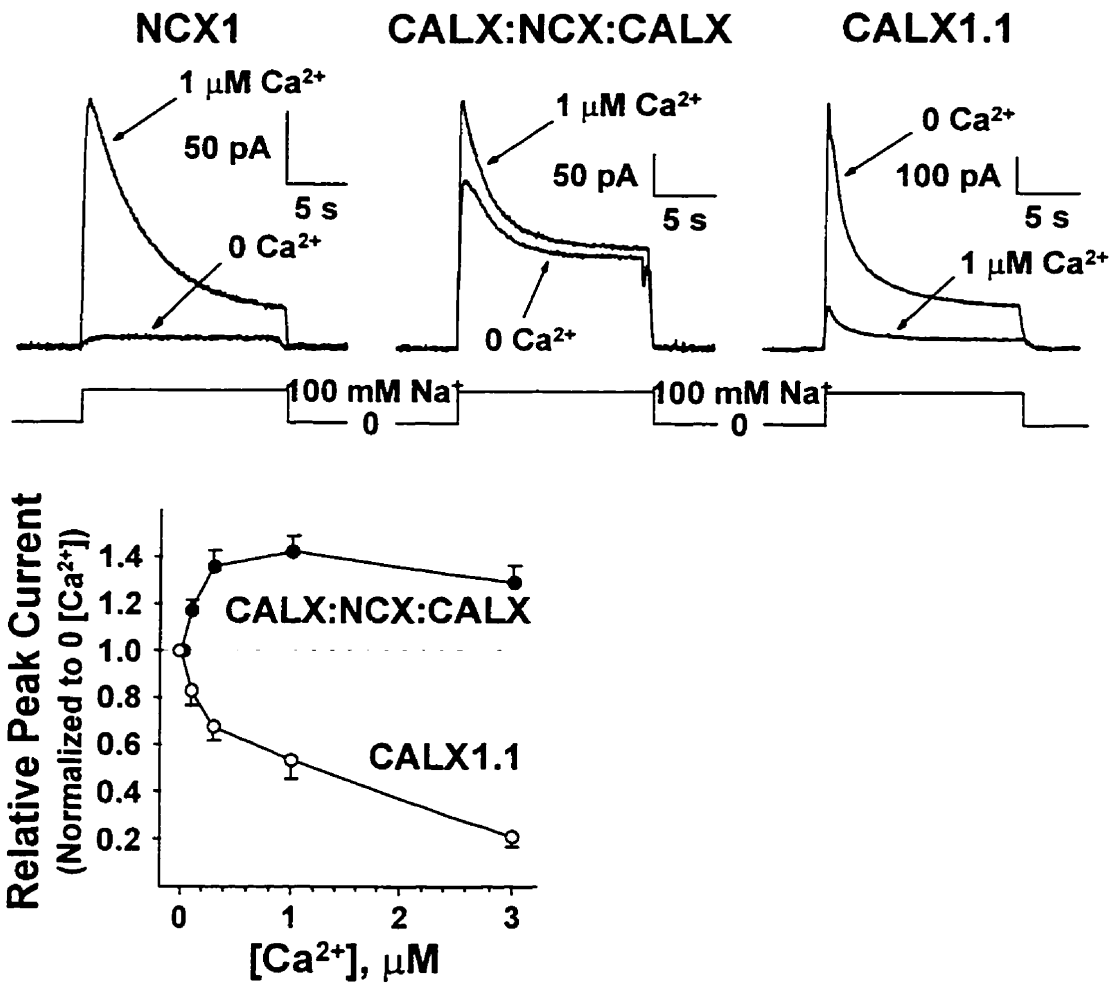

Figure 14. Partial Interconversion of Regulatory Ca^{2+}_i Phenotypes. The stimulatory and inhibitory effect of Ca^{2+}_i is shown for NCX1.1 and CALX1.1 respectively. The chimaera, CALX:NCX:CALX, is stimulated by regulatory Ca^{2+}_i. The graph in the *lower left* shows pooled data for 10 patches of CALX1.1 and 9 patches for CALX:NCX:CALX for a range of Ca^{2+}_i concentrations.

Part B: Isoform Studies

Recently, alternatively-spliced isoforms have been identified for the *Drosophila* Na^+-Ca^{2+} exchanger. In addition to showing the regulatory Ca^{2+}_i binding site and XIP region which mediate Ca^{2+}_i- and Na^+_i-dependent regulation for NCX1.1, Figure 15 also shows the location and amino acid sequences of the alternative splice site for the *Drosophila* isoforms. The analogous region in mammalian exchangers undergoes alternative splicing to give tissue-specific isoforms of the Na^+-Ca^{2+} exchanger (Ruknudin et al. 1997). These sequences for the *Drosophila* isoforms conform to the results reported by Schwarz and Benzer (1997) and differ from those of Ruknudin et al. (1997). The amino acid sequences of the alternative splice variants reported by Schwarz and Benzer (1997) are DELAA and STHYP for CALX1.1 and CALX1.2, respectively, whereas the amino acid sequences for these splice variants as reported by Ruknudin et al. (1997) are DGLAA and STHYR, respectively. Although uncertain, the explanation for these reported differences is more likely attributed to strain differences, rather than the presence of additional splice variants.

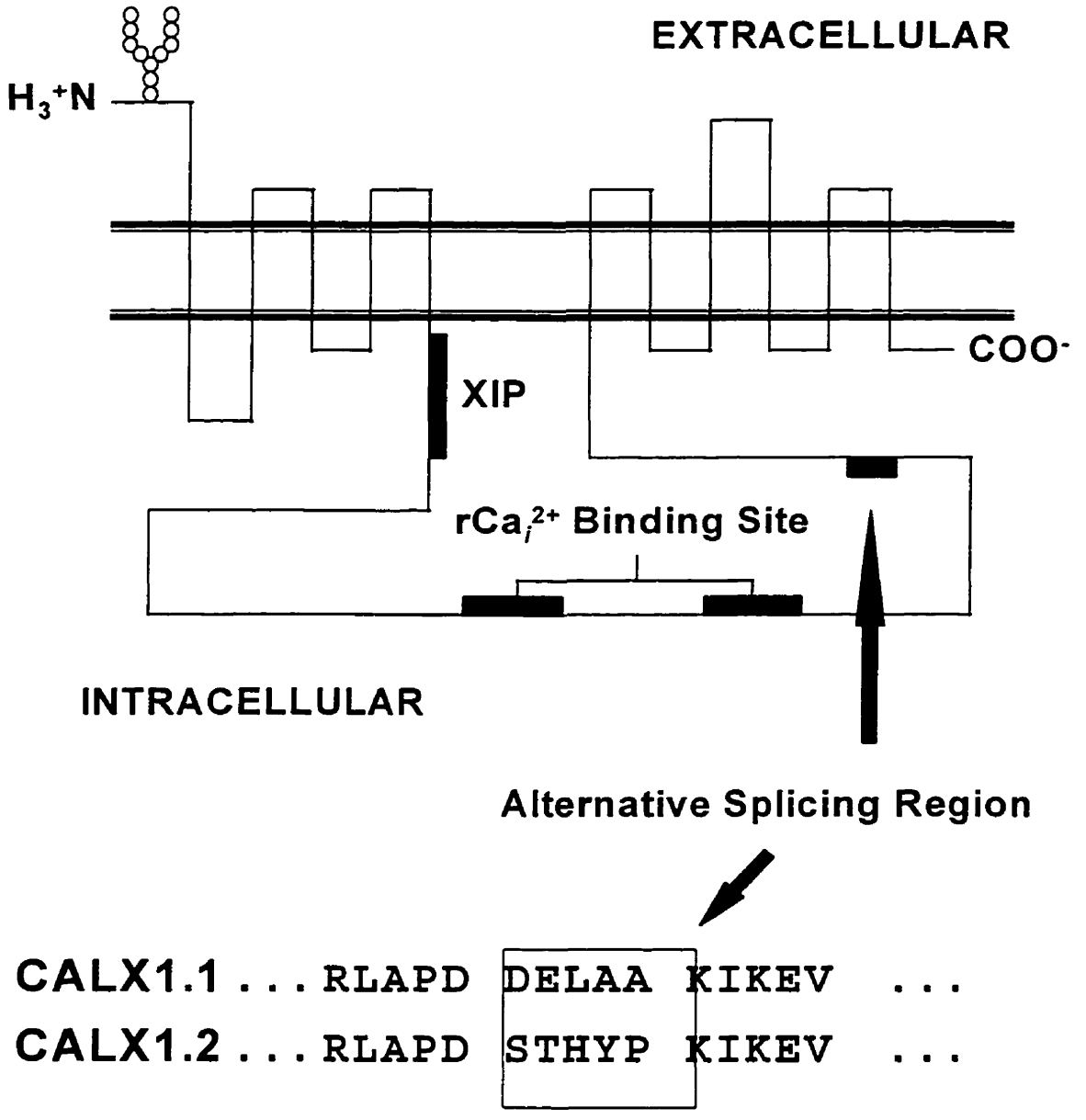

Figure 15. The Drosophila Na^+-Ca^{2+} exchanger isoforms. The putative topological organization of the Na^+-Ca^{2+} exchanger is shown in the *top panel*. The alternative splice site showing a five amino acid difference between the *Drosophila* isoforms, CALX1.1 and CALX1.2, is illustrated in the *bottom panel*.

1) Na^+_i-Dependence of Na^+-Ca^{2+} exchange

Figure 16 shows the activation of outward Na^+-Ca^{2+} exchange currents with various Na^+_i concentrations in the absence of regulatory Ca^{2+}_i for both CALX isoforms. As Na^+_i concentrations were increased for CALX1.1 and CALX1.2, we observed an accelerated decay rate of exchange current reflecting the Na^+_i-induced, or I_1, inactivation process (Hilgemann et al. 1992b). Although both exchangers exhibited Na^+_i-dependent regulation, differences in the extent and rate of inactivation were seen between the *Drosophila* isoforms. For example, whereas the CALX1.2 isoform inactivated to a greater extent than CALX1.1, the rate of inactivation of CALX1.1 was greater than that observed for CALX1.2. The decay rate constants, λ, for CALX1.1 and CALX1.2 activated by 100 mM Na^+_i were 0.49 ± 0.19 s^{-1} (mean ± S.D., n = 74) and 0.22 ± 0.05 s^{-1} (mean ± S.D., n = 38), respectively ($P < 0.05$).

The Na^+_i dependence of peak and steady-state current levels for the *Drosophila* isoforms is shown in Figure 17. Averaged data from seven patches from each exchanger were normalized to the peak (Fig. 17A) and steady-state (Fig. 17B) values produced in response to 100 mM Na^+_i. Similar values for peak K_d, the Na^+_i concentration producing half-maximal peak current, were observed for both exchangers (35.2 ± 2.3 mM and 34.2 ± 1.1 mM for CALX1.1 and CALX1.2, respectively). On the other hand, steady-state K_d, the Na^+_i concentration producing half-maximal steady-state currents, was higher for CALX1.1 as compared with CALX1.2 (16.6 ± 1.0 mM and 11.6 ± 0.7 mM for CALX1.1 and CALX1.2, respectively, $P < 0.05$).

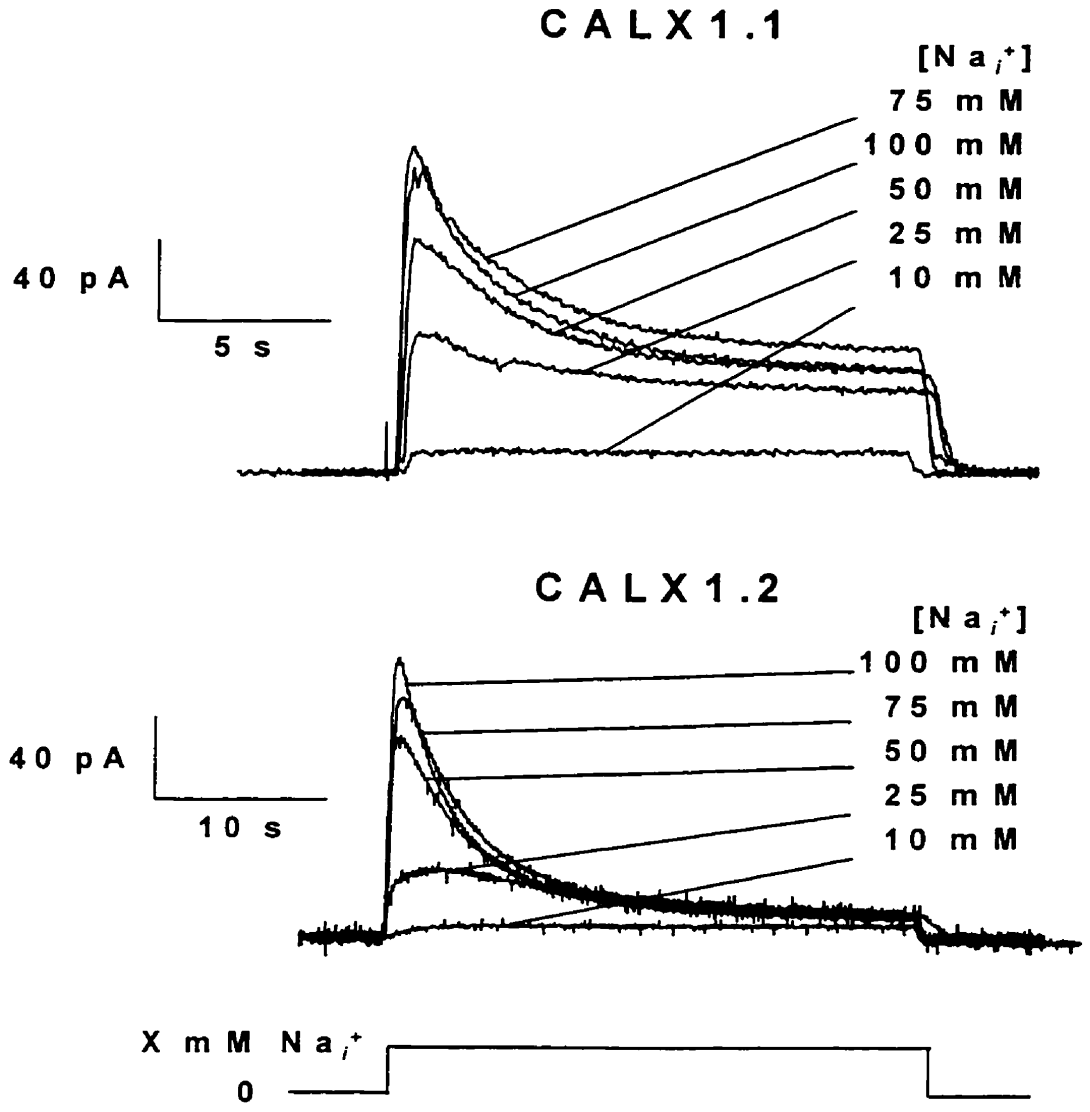

Figure 16. ***Na^+_i-dependent Regulation of CALX isoforms.*** Various Na^+_i concentrations were used to activate outward exchange currents for both CALX isoforms in the absence of regulatory Ca^{2+}_i. Significant differences in the extent of Na^+_i-induced inactivation were observed between CALX1.1 and CALX1.2.

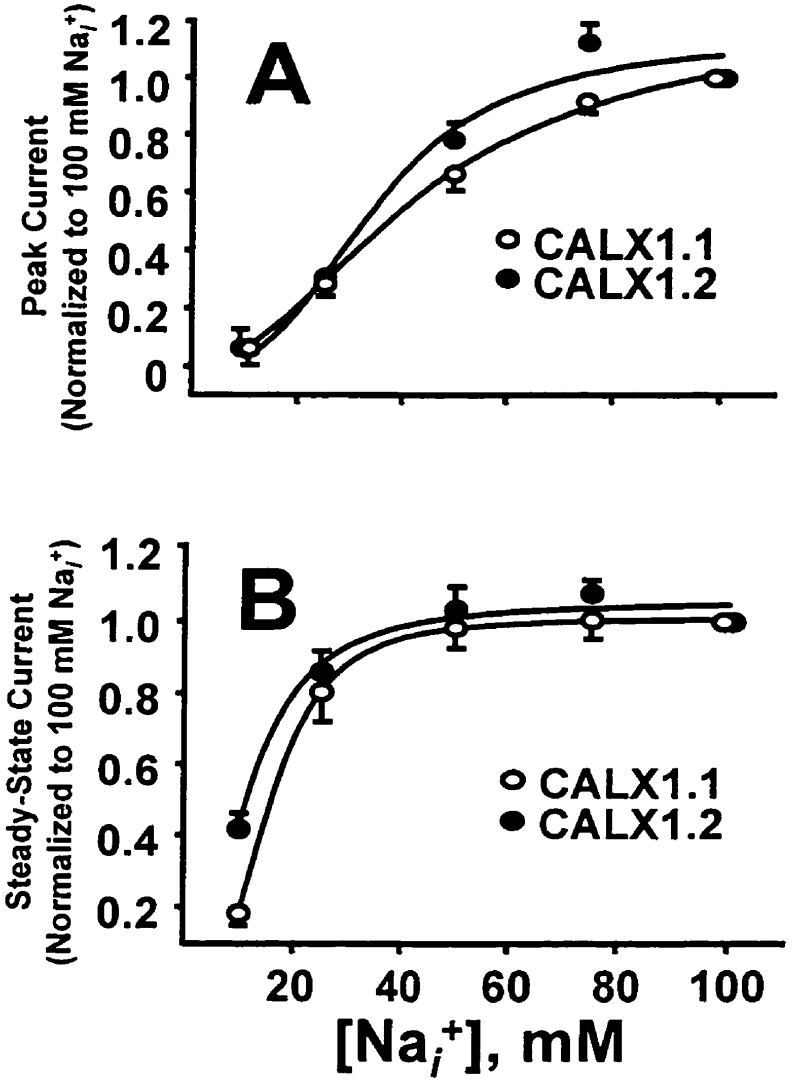

Figure 17. The Na^+_i-dependence of peak and steady-state exchange currents for CALX isoforms. Data for each exchanger was normalized to the peak (**A**) and steady-state (**B**) currents produced by the application of 100 mM Na^+_i in the absence of regulatory Ca^{2+}_i. Similar K_d values were observed for CALX1.1 and CALX1.2 with respect to peak and steady-state current.

A significant difference was observed in the extent to which Na^+_i-induced inactivation influences exchange activity for CALX1.1 and CALX1.2. This difference is shown in Figure 18, where F*ss*, the ratio of steady-state to peak exchange current is plotted against Na^+_i. Since lower F_{ss} values are observed for CALX1.2 at all Na^+_i concentrations above 10 mM, CALX1.2 undergoes a greater extent of Na^+_i-dependent inactivation as compared with CALX1.1. For example, F_{ss} values for CALX1.1 and CALX1.2 in response to 100 mM Na^+_i application were 0.26 ± 0.06 (mean ± S.D., n = 74) and 0.14 ± 0.04 (mean ± S.D., n = 38) respectively ($P < 0.05$). These differences may result from either an accelerated entry into, or hindered exit from the Na^+_i-induced inactive state for CALX1.2 as compared with CALX1.1.

In addition to the extent of inactivation being altered between these splice variants, we observed differences in the rate of recovery from the I_1 inactive state for CALX1.1 and CALX1.2. To examine the difference in recovery rates, paired-pulse experiments were conducted for both CALX isoforms as shown in Figure 19. Here, the extent of recovery from I_1 inactivation is reflected by the magnitude of the second pulse elicited upon reapplication of Na^+_i following the recovery intervals. That is, with greater recovery from the I_1 inactive state, more exchangers participate in current production for the second pulse resulting in larger current values. Note that at longer intervals (*e.g.*, 48 s), complete recovery is observed, whereas at shorter intervals (*e.g.*, 2 s), little to no recovery is seen for both *Drosophila* isoforms.

Furthermore, larger currents were observed for CALX1.1 as compared with CALX1.2 at the 2 and 12 s recovery intervals.

Data obtained for recovery intervals between 0.5 -48 s from 13 patches of CALX1.1 and 5 of CALX1.2 are summarized in Figure 20. The recovery rate constants, β, were determined as $0.13 \pm 0.01\ s^{-1}$ and $0.05 \pm 0.01\ s^{-1}$ for CALX1.1 and CALX1.2, respectively ($P < 0.05$). Our results indicate an $\approx$ 3-fold reduction in the rate of recovery from I_t inactivation for CALX1.2 as compared with CALX1.1. Thus, the difference in recovery rates between the *Drosophila* isoforms suggests a more stable I_t inactive state for CALX1.2.

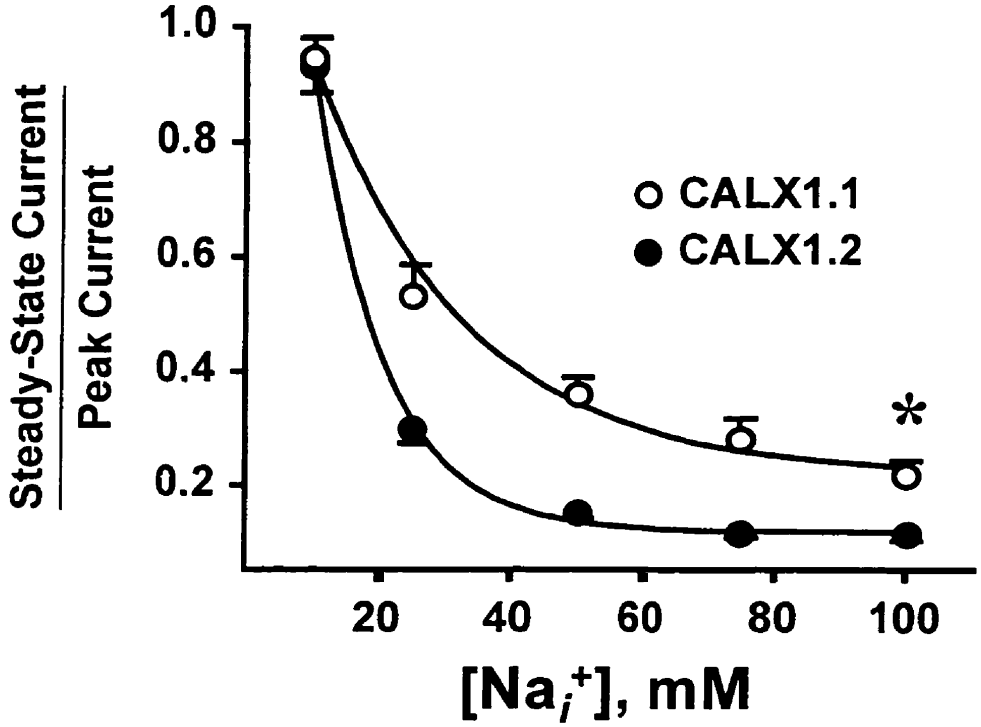

Figure 18. The Na^+_i-dependence of F_{ss}, the ratio of steady-state to peak currents, for CALX isoforms. The Na^+_i-dependence of Fss, the ratio of steady-state to peak outward currents, is shown for CALX1.1 and CALX1.2. At all Na^+_i concentrations, F_{ss} is smaller for CALX1.2. Values are mean ± SEM of 6 - 7 determinations for CALX1.1 and 4 - 6 determinations for CALX1.2. $^*P < 0.05$ for F_{ss} values at 100mM Na^+_i for CALX1.1 and CALX1.2.

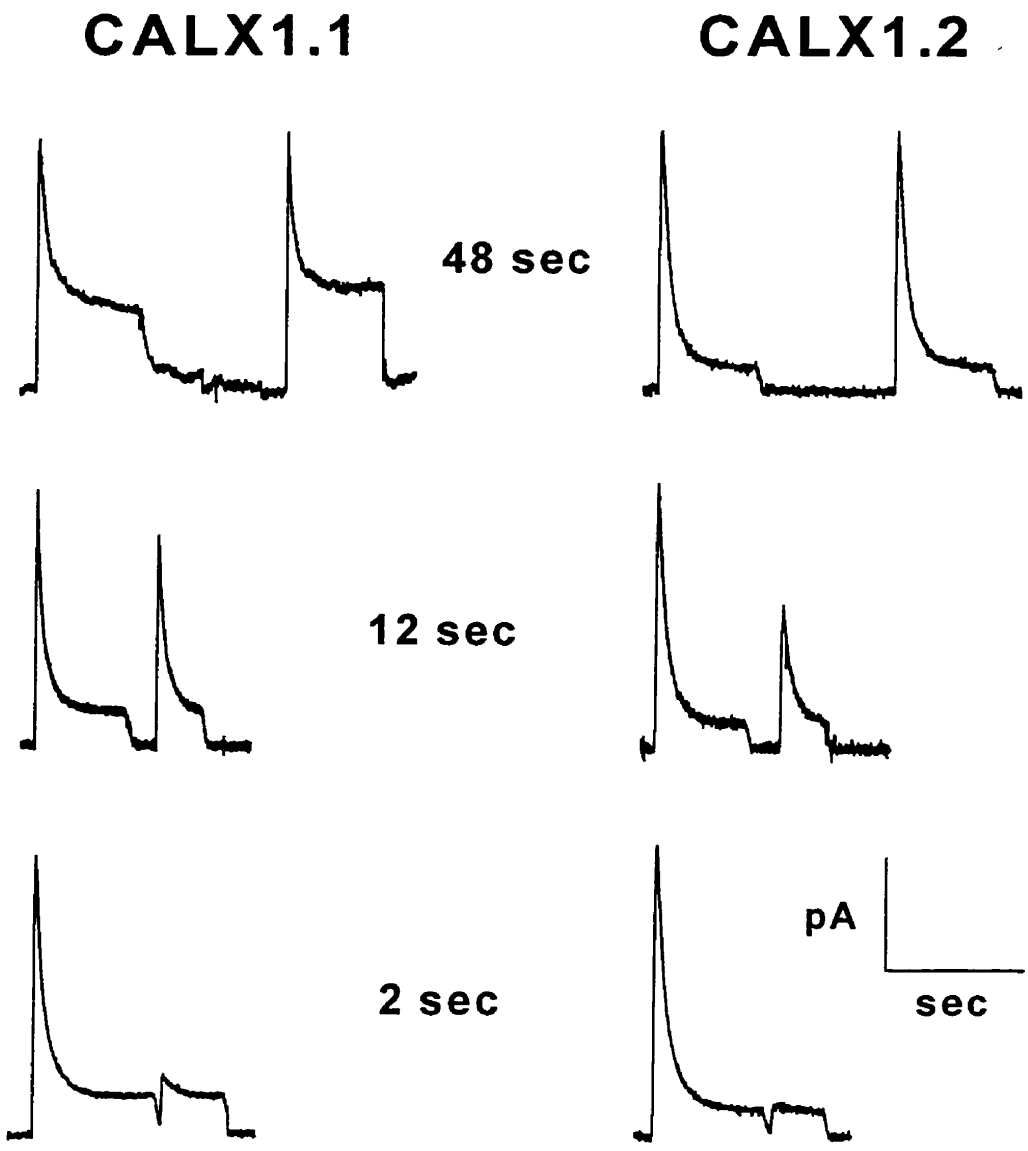

Figure 19. Paired Pulse Experiments Showing Kinetic Differences in the Rate of Recovery from Na^+_i-induced inactivation. In the absence of regulatory Ca^{2+}_i, exchange currents were activated, allowed to reach steady-state current levels, followed by recovery from Na^+_i-induced inactivation for CALX1.1 and CALX1.2. Traces for recovery intervals 48, 12 and 2 s are shown. Note that peak current of Pulse 2 recovers slower for CALX1.2.

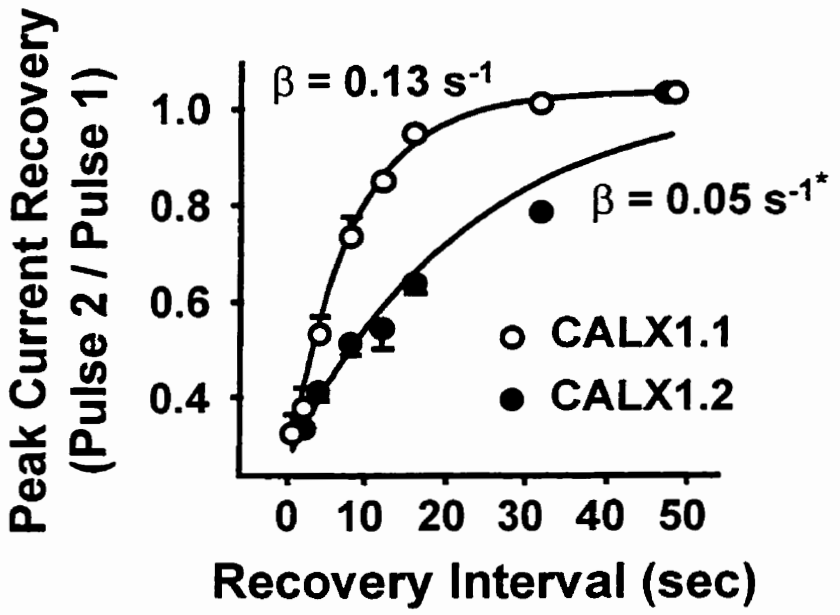

Figure 20. Differences in the Rate of Recovery from Na^+_i-induced Inactivation for the Drosophila Isoforms. An approximately 3 fold reduction in the rate of recovery from Na^+_i-induced inactivation is observed for CALX1.2 as compared to CALX1.1. Data obtained from 13 patches of CALX1.1 and 5 of CALX1.2 spanning recovery intervals of 0.5 - 48 s are summarized. $^*P < 0.05$ for β's of CALX1.1 *vs* CALX1.2.

2) Ca^{2+}_i-Regulation of Na^+-Ca^{2+} Exchange Currents

With the exception of the *Drosophila* isoforms, CALX1.1 and CALX1.2, regulatory Ca^{2+}_i stimulates exchange activity for all cloned Na^+-Ca^{2+} exchangers. Although inhibitory for both CALX1.1 and CALX1.2, the nature of this inhibition is significantly different between these isoforms. For example, in Figure 21, we show a substantial difference in response to the application of regulatory Ca^{2+}_i between the splice variants at steady-state current levels. Here, currents were activated in the absence of regulatory $Ca^{2+}i$ by 100 mM Na^+_i. After reaching steady-state current levels, 1 μM regulatory Ca^{2+}_i was applied for a brief period and then removed. Whereas regulatory Ca^{2+}_i substantially inhibited exchange current for CALX1.1, steady-state current levels remained constant in the presence or absence of Ca^{2+}_i for CALX1.2. Thus, for CALX1.2, steady-state current appeared insensitive to regulatory Ca^{2+}_i.

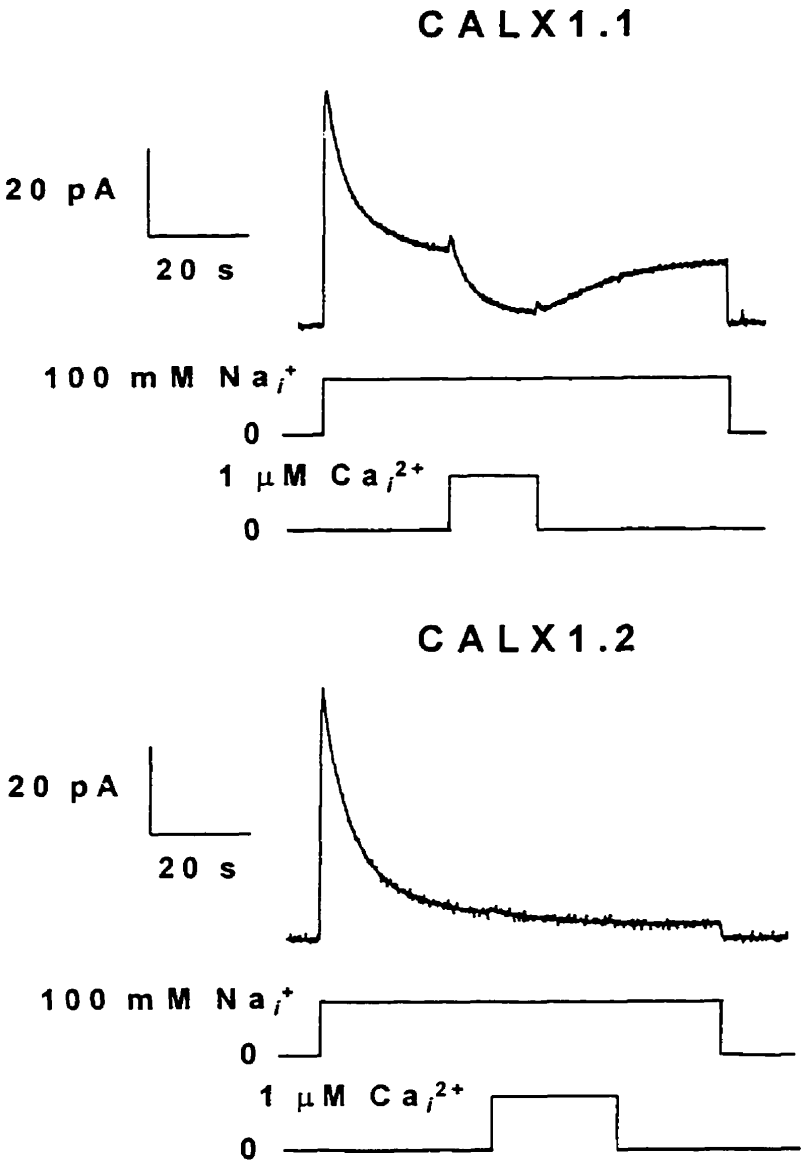

Figure 21. The effect of regulatory Ca^{2+}_i on steady-state Na^+-Ca^{2+} exchange currents for CALX isoforms. At steady-state current levels, 1 μM regulatory Ca^{2+}_i was applied to both CALX isoforms and then removed as indicated. Note that CALX1.1 is substantially inhibited by regulatory Ca^{2+}_i whereas exchange current for CALX1.2 remains relatively constant in the presence or absence of Ca^{2+}_i.

In Figure 22, outward Na^+-Ca^{2+} exchange currents were activated by 100 mM Na^+_i in the presence or absence of regulatory Ca^{2+}_i for CALX1.1 and CALX1.2. When present, regulatory Ca^{2+}_i was maintained for the entire protocol (*i.e.* before and during current activation). For both isoforms, we observed a progressive decrease in peak current with increasing Ca^{2+}_i concentrations, showing the inhibitory effect of regulatory Ca^{2+}_i on peak currents for CALX1.1 and CALX1.2. Although negative Ca^{2+}_i regulation was observed for both splice variants, two major differences in the nature of this inhibition was apparent between CALX1.1 and CALX1.2. First, whereas Ca^{2+}_i significantly inhibited peak and steady-state currents for CALX1.1 (Hryshko et al. 1996), regulatory Ca^{2+}_i only inhibited peak currents for CALX1.2. The steady-state current levels for CALX1.2 were insensitive to regulatory Ca^{2+}_i. Though less obvious, the second difference is the observation that much higher concentrations of regulatory Ca^{2+}_i are required to exert effects on CALX1.1 compared to CALX1.2 (*i.e.* the affinity for negative Ca^{2+}_i regulation is much higher for CALX1.2 as compared with CALX1.1).

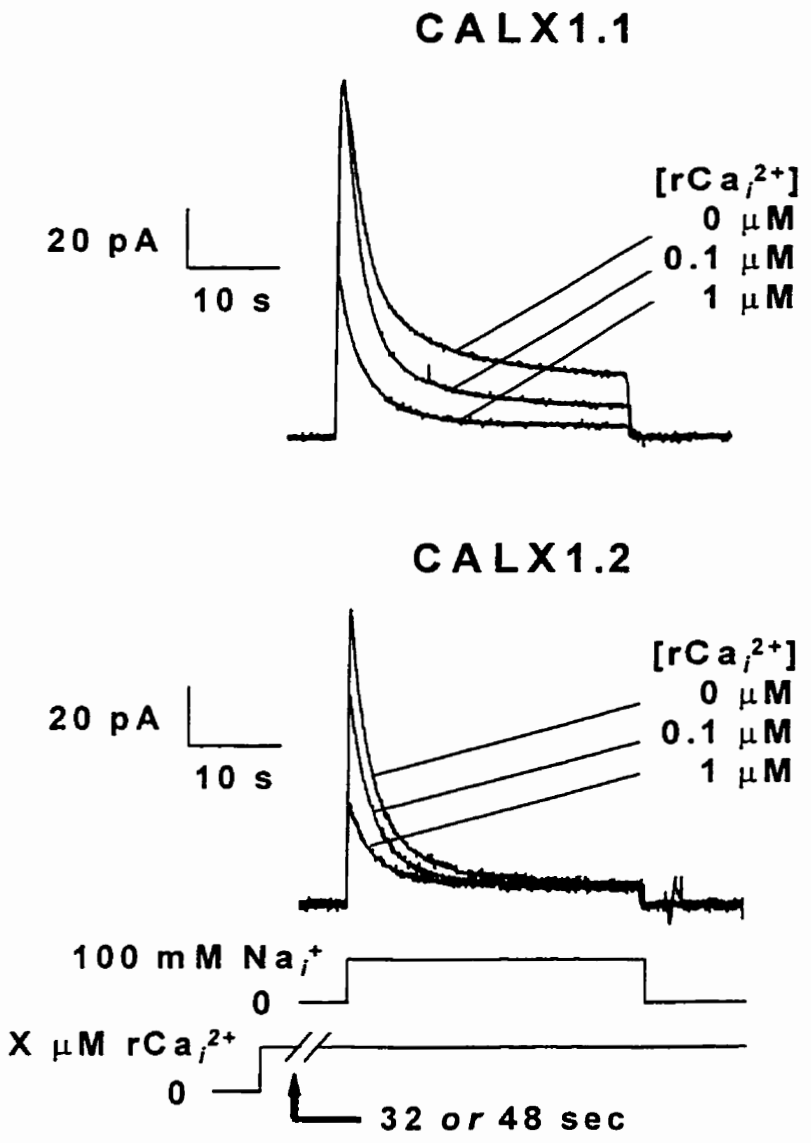

Figure 22. The effect of pre-incubation with regulatory Ca^{2+}_i on Na^+-Ca^{2+} exchange current transients for CALX isoforms. For all traces, regulatory Ca^{2+}_i was present before and during current activation. The affinity for inhibition of peak current by regulatory Ca^{2+}_i was greater for CALX1.2 as compared to CALX1.1.

Figure 23 illustrates the differences in affinity for negative Ca^{2+}_i regulation with respect to peak and steady-state currents for CALX1.1 and CALX1.2. Results are from 10 patches of CALX1.1 and 8 for CALX1.2. Both peak and steady-state currents were normalized to values obtained in the absence of regulatory Ca^{2+}_i. As shown, the affinity and extent to which inactivation occurs is considerably different between these isoforms. For example, although regulatory Ca^{2+}_i inhibits peak current of CALX1.2 with a greater affinity than peak current of CALX1.1, the extent of this inactivation is considerably less for CALX1.2. In other words, regulatory Ca^{2+}_i inhibits peak current of CALX1.1 with a lower affinity but to a greater extent as compared with CALX1.2. For steady-state currents, substantial differences in the extent of negative Ca^{2+}_i regulation were also observed between CALX1.1 and CALX1.2. For CALX1.2, we see a greater affinity but lower efficiency for Ca^{2+}_i regulation on steady-state current levels. In contrast, Figure 23 (*lower panel*) illustrates this lower affinity, higher efficiency effects of regulatory Ca^{2+}_i on CALX1.1. That is, regulatory Ca^{2+}_i inhibits steady-state currents of CALX1.1 with a lower affinity than that observed for CALX1.2, but the extent of this inactivation is greater for CALX1.1.

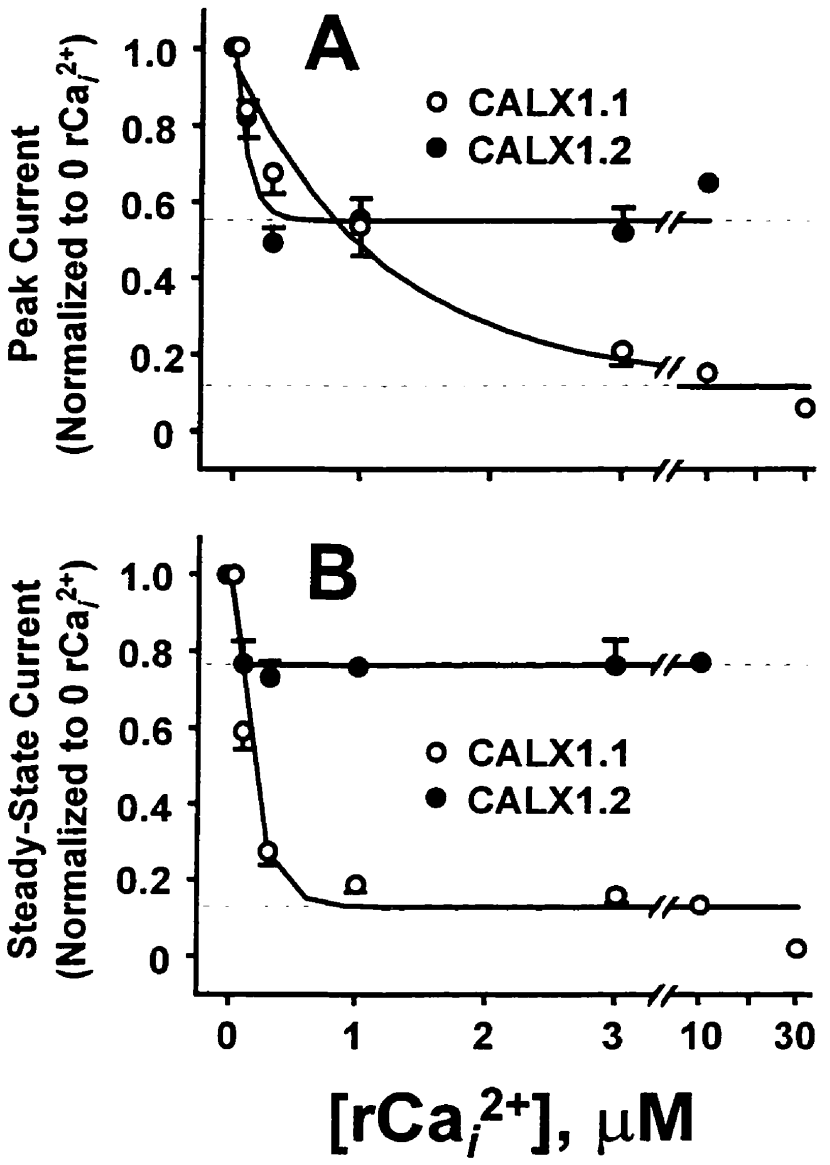

Figure 23. ***The effect of regulatory $Ca^{2+}{}_i$ on peak and steady state Na^+-Ca^{2+} exchange currents for CALX isoforms.*** Peak (**A**) and steady-state (**B**) currents for CALX1.1 and CALX1.2 were normalized to the peak or steady-state currents obtained in the absence of regulatory $Ca^{2+}{}_i$. Regulatory $Ca^{2+}{}_i$ inhibited peak and steady-state currents of CALX1.2 with a greater affinity compared to CALX1.1, but the extent of this inhibition was less. The values shown are means ± SEM.

To further examine the differences in response to regulatory Ca^{2+}_i for CALX1.1 and CALX1.2, two additional protocols were used. First, to determine the rate at which negative Ca^{2+}_i regulation occurs, Ca^{2+}_i was absent prior to current activation by 100 mM Na^+_i (*i.e.* regulatory Ca^{2+}_i was applied simultaneously with cytoplasmic Na^+_i). Upon stimulation, the inhibitory influences of both Na^+_i and Ca^{2+}_i should be absent, and thus, yield the maximal number of exchangers for generation of exchange current. Under these conditions, independent of regulatory Ca^{2+}_i concentration applied during exchanger activation, both CALX1.1 and CALX1.2 showed similar peak currents (Figure 24). That is, prior to inhibition by regulatory Ca^{2+}_i, the maximal number of exchangers were available to participate in the generation of peak current. After reaching peak levels, exchange currents decayed slowly to steady-state values appropriate for the concentration of regulatory Ca^{2+}_i present. Therefore, this slow, time-dependent inactivation following peak values to lower steady-state current levels for CALX1.1 and CALX1.2, shows that negative Ca^{2+}_i regulation for both isoforms is a relatively slow process. In contrast, for the cardiac exchanger, current activation by regulatory Ca^{2+}_i is extremely rapid (Hilgemann et al. 1992a). Furthermore, the difference of regulatory Ca^{2+}_i on steady-state currents for CALX1.1 and CALX1.2 can be seen. For CALX1.1, Ca^{2+}_i inhibited steady-state currents, whereas steady-state levels of CALX1.2 appeared insensitive to regulatory Ca^{2+}_i.

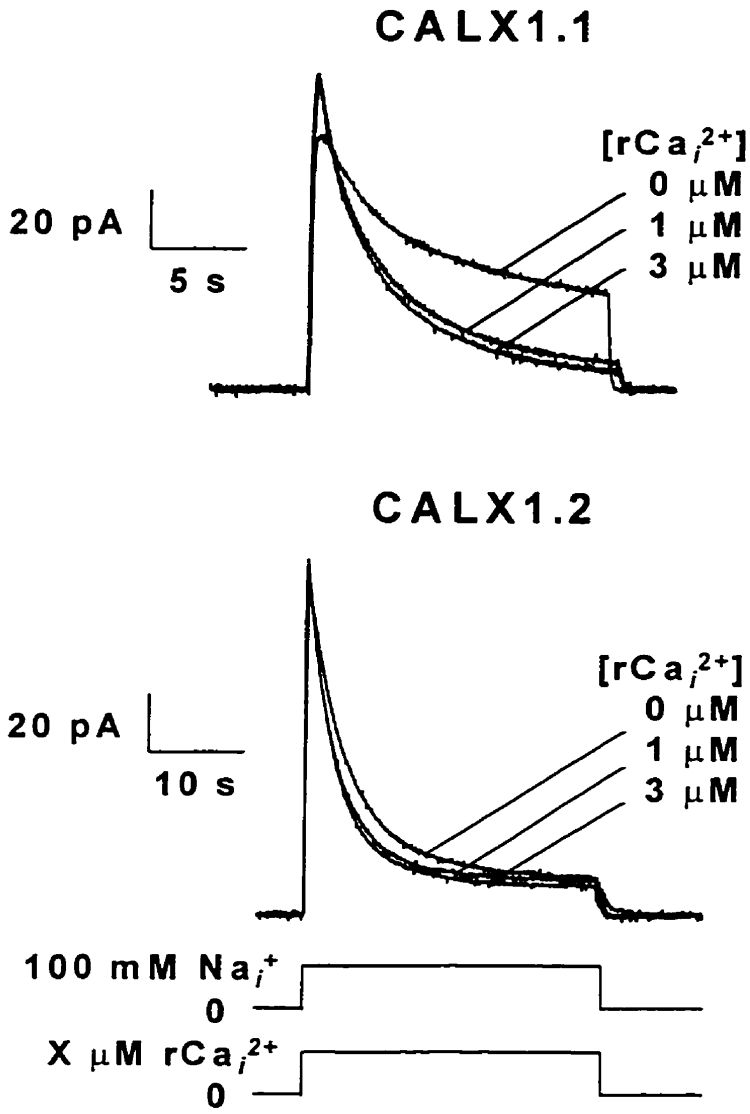

Figure 24. The effect of regulatory Ca^{2+}_i on Na^+-Ca^{2+} exchange current transients for CALX isoforms. For all traces, regulatory Ca^{2+}_i was present only during current activation. A slow inhibition by regulatory Ca^{2+}_i is observed for both *Drosophila* isoforms.

To further investigate the role of regulatory Ca^{2+}_i, our second protocol examined how recovery from Na^+_i-induced inactivation was influenced by Ca^{2+}_i. Paired-pulse experiments of 4 s recovery intervals were used to determine the recovery rate from the I_1 inactive state for CALX1.1 and CALX1.2. Regulatory Ca^{2+}_i was either absent or present (300 nM) for the duration of the experiment, including the recovery interval. For CALX1.1, the peak of the second pulse with 300 nM Ca^{2+}_i was less than that observed in the absence of Ca^{2+}_i. For NCX1, the exact opposite response is observed. That is, accelerated recovery from I_1 inactivation is seen with increasing regulatory Ca^{2+}_i concentrations (Hilgemann et al. 1992a). In contrast to CALX1.1, the presence of regulatory Ca^{2+}_i increases the rate of recovery from Na^+_i-induced inactivation for CALX1.2. Thus, CALX1.2 behaves similar to NCX1, and opposite to CALX1.1 in terms of the influence regulatory Ca^{2+}_i has on the rate of recovery from Na^+_i-induced inactivation.

Figure 25 (*right panel*) illustrates this regulatory Ca^{2+}_i effect for a range of recovery intervals for both *Drosophila* isoforms. These results are summarized from five patches for CALX1.2 and three for CALX1.1. For CALX1.2, we consistently observed an increase in the recovery rate from Na^+_i-induced inactivation, whereas for CALX1.1 we observed a decrease.

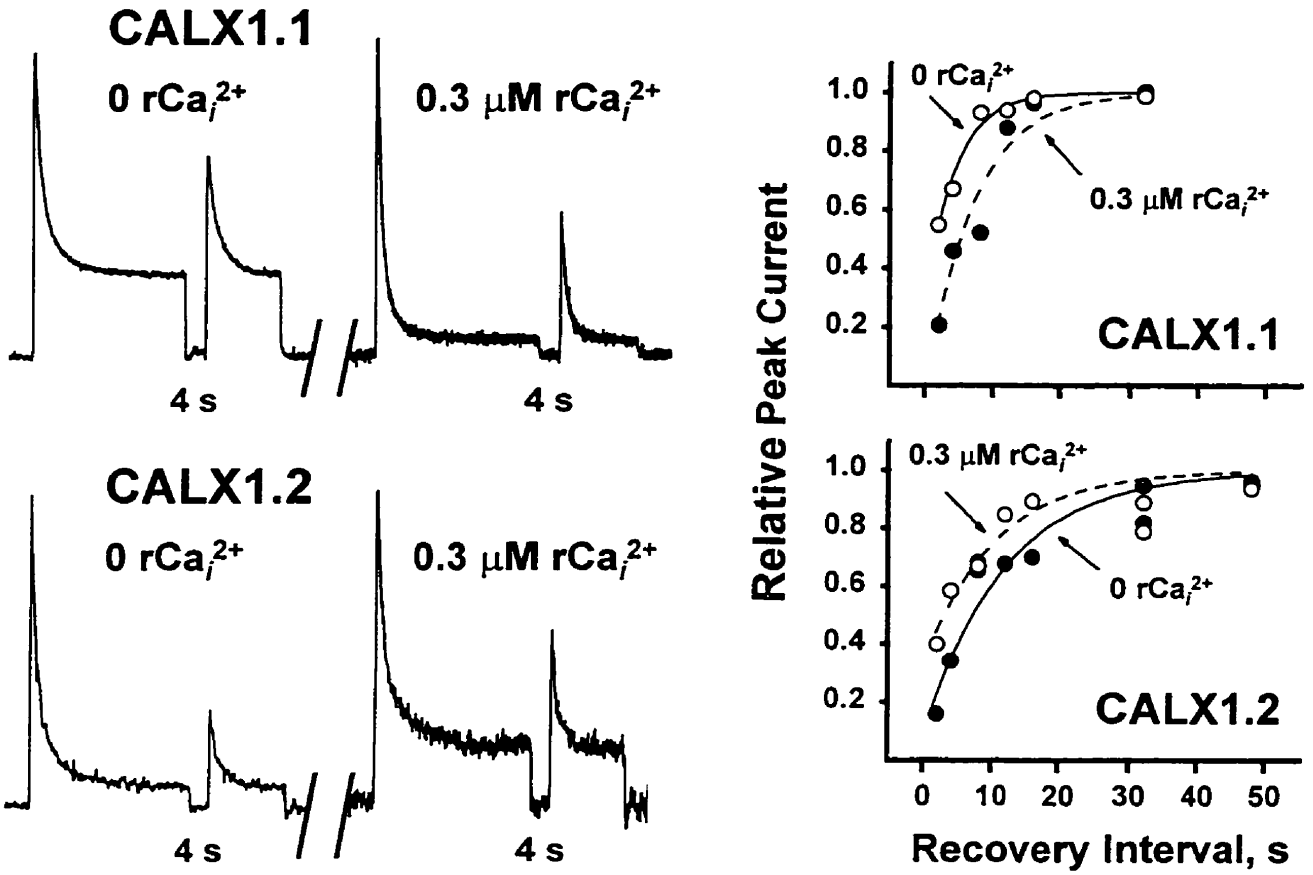

Figure 25. The effect of regulatory Ca^{2+}_i on rates of recovery from Na^+_i-induced inactivation for CALX isoforms. Paired-pulse experiments (4 s recovery intervals) were used to assess the effects of zero and 300 nM Ca^{2+}_i on recovery from I_1 inactivation for both CALX isoforms. The absence or presence of Ca^{2+}_i (300 nM) was maintained through-out the entire protocol. Whereas the rate of recovery from I_1 inactivation is reduced in the presence of Ca^{2+}_i for CALX1.1, regulatory Ca^{2+}_i augmented the the rate of recovery for CALX1.2 A full range of recovery intervals (*i.e.*, 0.5 - 48 s) in the absence and presence of 300 nM Ca^{2+}_i is summarized in the graph.

DISCUSSION

Part A: Structure-function Studies

1) Ca^{2+}_i-Dependent Regulation of CALX1.1

The stimulation of Na^+-Ca^{2+} exchange current by cytoplasmic Ca^{2+}_i has been observed for all mammalian exchangers characterized to date, including the unique gene products NCX1 (Matsuoka et al. 1995), NCX2 (Li et al. 1994) and NCX3 (Linck et al. 1998). Although regulatory Ca^{2+}_i influences exchange activity over the same concentration range for the *Drosophila* Na^+-Ca^{2+} exchanger as compared with NCX1.1, CALX1.1 is unique among all identified exchangers in that Ca^{2+}_i inhibits exchange activity (Hryshko et al. 1996). To investigate the possibility of a conserved functional role for the regulatory Ca^{2+}_i binding site which exhibits a high degree of sequence similarity between NCX1.1 and CALX1.1, structure-function studies were conducted for CALX1.1. The *Drosophila* exchanger was selected for two reasons. First, if there is a conserved functional role associated with this region, the affinity for Ca^{2+}_i regulation should be altered in a similar fashion when analogous residues are mutated within this amino acid sequence for NCX1.1 and CALX1.1. Second, if the protein region involved in the transduction of the Ca^{2+}_i binding signal is localized to this segment of NCX1.1, then substituting this region between the cardiac and *Drosophila* exchanger presented the greatest possibility of interchanging regulatory Ca^{2+}_i phenotypes.

Figure 6 illustrates amino acid residues within the regulatory Ca^{2+}_i binding site that are highly conserved for all identified Na^+-Ca^{2+} exchangers. When

analogous mutations targeting these residues were introduced into NCX1.1 and CALX1.1, similar changes in the ability of Ca^{2+}_i to regulate exchange activity were observed (Matsuoka et al. 1995). That is, whether simulatory (NCX1.1) or inhibitory (CALX1.1), the affinity for regulatory Ca^{2+}_i was reduced. Thus, our findings suggest that the regulatory Ca^{2+}_i binding site may serve a similar functional role in both NCX1.1 and CALX1.1. Furthermore, the high degree of sequence similarity for acidic clusters within the Ca^{2+}_i binding site for all cloned exchangers, together with our results showing a conserved functional role for this site between two unique transporters, suggests that this region may perform a similar function for all other exchanger subtypes and alternatively spliced isoforms.

2) Na^+_i-Dependent Regulation of CALX1.1

Figure 8 shows the amino acid residues comprising the eXchange Inhibitory Peptide (XIP) region for NCX1.1 compared to the corresponding regions for other cloned exchangers. Similar to the findings for the regulatory Ca^{2+}_i binding site, our results suggest that the protein region mediating Na^+_i-dependent regulation serves a similar functional role for CALX1.1 and NCX1.1. The observations that analogous XIP mutations alter Na^+_i-induced inactivation in a similar fashion for both exchangers supports this notion. For example, the CALX1.1 mutant K306Q, analogous to K225Q in NCX1.1, showed an $\approx$ 2 fold increase in the rate of entry and recovery from I_1 inactivation (Matsuoka et al. 1997). Similarly, the analogous deletion mutations Δ310-313 (CALX1.1) and Δ229-232 (NCX1.1) resulted in elimination of Na^+_i-induced inactivation for both exchangers (Matsuoka et al. 1997).

Although the sequence similarity for the XIP region amongst all identified exchangers is substantially less than that observed for the Ca^{2+}_i binding site, our results support the hypothesis for a conserved functional role of this region with respect to Na^{+}_i-induced inactivation.

3) Ca^{2+}_i-Insensitive and Chimaeric Exchangers

Although our results suggest a conserved role for the regions mediating Na^{+}_i- and Ca^{2+}_i-dependent regulation, an explanation for the opposite response to regulatory Ca^{2+}_i observed between NCX1.1 and CALX1.1 remains unknown. With Ca^{2+}_i binding appearing to occur at a similar site for both exchangers, the striking difference in response to regulatory Ca^{2+}_i may be a consequence of different transduction mechanisms mediated by sequences distinct or remote from the binding site. Analogous point mutations within the Ca^{2+}_i binding site, G555P (CALX1.1) and G503P (NCX1.1), resulted in exchangers that were insensitive to regulatory Ca^{2+}_i (Matsuoka et al. 1995). This eliminated response may be the result of an inability to transduce the Ca^{2+}_i signal, whereas Ca^{2+}_i binding remains unaffected. The observation of normal Ca^{2+}_i binding for a fusion protein containing a G503P equivalent mutation within the Ca^{2+}_i binding site of NCX1.1 supports this notion (Levitsky et al. 1994). These results, in addition to our initial chimaeric studies, suggest that Ca^{2+}_i binding is restricted to acidic clusters within the Ca^{2+}_i binding site. For the CALX:NCX:CALX chimaera, where a portion of the cytoplasmic loop of NCX1.1 including the regulatory Ca^{2+}_i binding site was substituted for the equivalent sequence in CALX1.1, normal Ca^{2+}_i binding function was conserved as the

exchanger retained sensitivity to regulatory Ca^{2+}_i. However, a partial interconversion of the regulatory Ca^{2+}_i phenotypes was also observed for this chimaera. If Ca^{2+}_i binding is localized to acidic clusters within the Ca^{2+}_i binding site, this result suggests that additional protein domains are involved in transducing the Ca^{2+}_i binding signal. Therefore, our results support the possibility that Ca^{2+}_i binding and signal transduction are mediated by discrete regions. Additional studies involving chimaeric exchangers may allow further identification and delineation of the regions involved in transducing the Ca^{2+}_i binding signal.

4) Significance

The Na^+-Ca^{2+} exchanger performs diverse physiological functions in a wide array of tissues (McDaniel et al. 1993; Kofuji et al. 1992; Komuro et al. 1992). In the heart, for example, the Na^+-Ca^{2+} exchanger is capable of functioning over a dynamic range where the Ca^{2+} equivalent entering the myocyte on a beat-to-beat basis must be removed *via* Na^+-Ca^{2+} exchange to maintain Ca^{2+} homeostasis (Bridge et al. 1990). At present, the mechanisms responsible for coupling Na^+-Ca^{2+} exchange activity to Ca^{2+} influx remain unknown. Although two forms of ionic regulation modulating exchanger function have been identified using the giant excised patch clamp technique, it is uncertain whether Na^+_i- and Ca^{2+}_i-dependent regulation have physiological significance. Our ability to alter properties at the molecular level provides the tools to investigate the importance of regulation using transgenic mice. Specifically, we can ablate or accelerate Na^+_i- and Ca^{2+}_i regulatory

properties in transgenic mouse hearts and examine the physiological consequences. Studies of this nature are currently underway.

Part B: Isoform Studies

To date, numerous alternatively spliced isoforms of the Na^+-Ca^{2+} exchanger have been identified in mammalian tissues (Nakasaki et al. 1993; Lee et al. 1994; Quednau et al. 1997; Kofuji et al. 1994). Like NCX1.1, Na^+_i- and Ca^{2+}_i-dependent regulation have been shown to influence exchange activity for these mammalian isoforms (unpublished data). The observation that splice variants are expressed in a tissue-specific fashion, suggests a role for alternative splicing in tailoring ionic regulatory properties to alter exchange function according to tissue demands. Recently, alternatively spliced isoforms of the Na^+-Ca^{2+} exchanger have also been observed for *Drosophila melanogaster* (Schwarz and Benzer, 1997; Ruknudin et al. 1997). Although both isoforms, CALX1.1 and CALX1.2, exhibit Na^+_i- and Ca^{2+}_i-dependent regulation, substantial differences in the nature of these regulatory processes have been observed. These results examining the regulatory properties of these splice variants is the first report of functional differences between two such isoforms.

1) Na^+_i-Induced Inactivation

The current decay, termed Na^+_i-induced, or I_1, inactivation, may reflect either a decreased efficiency of ion transport for each exchanger within the population, or the removal of a fraction of exchangers within the population available for transport. However, since evidence supporting altered transport properties for the entire exchanger population is lacking, it is therefore thought that Na^+-Ca^{2+} exchangers partition between a fully-active or fully-inactive state (Hilgemann et al. 1992b). The

equilibrium between these states is influenced by the Na^+_i-dependent regulatory process and is reflected by steady-state currents levels. Following Na^+_i application and the binding of 3 Na^+_i ions, the Na^+-Ca^{2+} exchanger can either contribute to current production or enter the I_1 inactive state (Hilgemann et al. 1992b). With increasing Na^+_i concentrations, a greater fraction of exchangers are found in the 3 Na^+_i bound configuration, thus augmenting both current production and Na^+_i-induced inactivation.

Our results show substantial differences in the Na^+_i-induced inactivation process for the *Drosophila* isoforms, CALX1.1 and CALX1.2. For example, the extent of I_1 inactivation is much greater for CALX1.2 as compared with CALX1.1. The ratio of steady-state to peak current, F_{ss}, was used to measure the extent of this inactivation. Therefore, the greater extent of inactivation for CALX1.2 results in lower steady-state current levels and F_{ss} values as compared with CALX1.1. To determine whether an increased rate of entry into Na^+_i-induced inactivation, or a decreased recovery rate from I_1 was responsible for these changes in F_{ss}, paired pulse experiments that allow for the isolation of recovery rates were conducted. The recovery interval required to alleviate I_1 inactivation completely, and thus yield the same number of exchangers available for the subsequent activation as were available for the initial stimulation, reflects the the rate of recovery from Na^+_i-induced inactivation. From these experiments, we observed an $\approx$ 3 fold reduction in the rate of recovery from the I_1 inactive state for CALX1.2 as compared with CALX1.1,

indicating that the I_1 inactive configuration for CALX1.2 is more stable than it is for CALX1.1.

The existence of two ionic regulatory processes affecting exchange activity have been known for a number of years (Hilgemann et al. 1992a; Hilgemann et al. 1992a; Hilgemann, 1990a). An understanding of the mechanism of function for the XIP region in mediating the Na^+_i-dependent regulatory process is only now being addressed. For NCX1.1, mutations within the XIP region resulted in either elimination or acceleration of the Na^+_i-induced inactivation process (Matsuoka et al. 1997). Subsequently, we have shown that corresponding mutations in CALX1.1 produced phenotypes analogous to those for NCX1.1 (Dyck et al. 1998). When an exogenous peptide, based on the 20 amino acid sequence of the endogenous XIP region was applied to NCX1.1 and CALX1.1, exchange activity was potently inhibited (Li et al. 1991; Hryshko et al. 1996). We speculate that the exogenous XIP peptide and endogenous XIP region inhibit exchange activity *via* the same mechanism. Here, the XIP region may function as an inhibitory domain mediating the transition of the Na^+-Ca^{2+} exchanger from an active to inactive state. The plausibility of this transition between active and inactive states to reflect the mechanism for the Na^+_i-induced inactivation process is supported by previous electrophysiological studies (Hilgemann et al. 1992a). With substantial differences observed for the rate and extent of Na^+_i-induced inactivation between these isoforms, our findings suggest that alternative splicing may play a role in tailoring ionic

regulatory properties between these splice variants. However, it remains unknown as to how this region influences Na^+_i-dependent inactivation.

2) Ca^{2+}_i-Dependent Regulation

In addition to being transported by the Na^+-Ca^{2+} exchanger, Ca^{2+}_i has also been shown to regulate exchange activity (Hilgemann et al. 1992a; Hilgemann, 1990a; Matsuoka et al. 1995a). Two processes are affected by regulatory Ca^{2+}_i. First, as Ca^{2+}_i concentrations are increased, the extent of Na^+_i-induced inactivation is reduced resulting in larger steady-state current values. That is, we observe less decay of exchange current with increasing Ca^{2+}_i concentrations. The second effect of Ca^{2+}_i is observed as current decay following the removal of regulatory Ca^{2+}_i at steady-state current levels. This current decay represents a slow entry of exchangers into another inactive state. However, rapid recovery from this inactive state occurs upon stimulation with regulatory Ca^{2+}_i. Therefore, the rates of entry into and exit from this inactive state are markedly different for NCX1.1.

Within the large cytoplasmic loop of NCX1.1, the regulatory Ca^{2+}_i binding site mediates Ca^{2+}_i-dependent regulation (Levitsky et al. 1994; Matsuoka et al. 1995). This region, spanning amino acids 371-508, appears to be highly conserved for all cloned Na^+-Ca^{2+} exchangers. Furthermore, the affinity for Ca^{2+}_i regulation was significantly altered for NCX1.1 and CALX1.1 when mutations were introduced into acidic clusters within the Ca^{2+}_i binding domain (Matsuoka et al. 1995).

In contrast to all mammalian Na^+-Ca^{2+} exchangers, the alternatively spliced isoforms from *Drosophila melanogaster*, CALX1.1 and CALX1.2, exhibit negative

regulation by cytoplasmic Ca^{2+}_i (Hryshko et al. 1996). Although the regulatory Ca^{2+}_i binding site is highly conserved between these isoforms, substantial differences in Ca^{2+}_i-dependent regulation are observed. Thus, our results suggest that alternative splicing may play a role in modifying Ca^{2+}_i-dependent regulatory properties. For CALX1.1, Ca^{2+}_i inhibits exchange current to a greater extent as compared to CALX1.2. As a result, lower steady-state current levels are observed for CALX1.1. However, the affinity for Ca^{2+}_i inhibition is much lower for CALX1.1 as compared to CALX1.2. Thus, although a greater extent of inhibition is observed for CALX1.1, much higher concentrations of regulatory Ca^{2+}_i are required to exert this inhibitory effect. For CALX1.2, maximal inhibition for peak and steady-state currents is only $\approx$ 20 - 40 %, whereas for CALX1.1, this inhibition is > 80 %.

3) Interactions Between Na^+_i- and Ca^{2+}_i-Dependent Regulation

Interactions between the Na^+_i- and Ca^{2+}_i-dependent regulatory processes have been shown with the use of electrophysiological analysis (Hilgemann et al. 1992a; Matsuoka et al. 1995a; Trac et al. 1997). For NCX1.1, regulatory Ca^{2+}_i reduces the fraction of exchangers entering the I_1 inactive state. In other words, as regulatory Ca^{2+}_i concentrations increase, less Na^+_i-induced inactivation occurs resulting in larger steady-state current levels. For NCX1.1, an increased recovery from, or decreased entry into the I_1 inactive state may account for the observed reduction in Na^+_i-induced inactivation. Thus, it appears regulatory Ca^{2+}_i destabilizes the I_1 inactive configuration for NCX1.1. These interactions between Na^+_i- and Ca^{2+}_i-dependent regulation have also been observed for the two *Drosophila* isoforms,

CALX1.1 and CALX1..2. In contrast to NCX1.1, regulatory Ca^{2+}_i stabilized the I_1 inactive configuration for CALX1.1. This result is consistent with the observation that regulatory Ca^{2+}_i inhibits exchange current for CALX1.1. Furthermore, in contrast to CALX1.1, where Ca^{2+}_i stabilized the I_1 inactive configuration, we observed an alleviation of Na^+_i-induced inactivation by regulatory Ca^{2+}_i for CALX1.2. This observation suggests that Ca^{2+}_i destabilizes the I_1 inactive configuration for CALX1.2. Therefore, differences in the two ionic regulatory processes and the interactions between them were observed for the two alternatively spliced isoforms of the *Drosophila* Na^+-Ca^{2+} exchanger.

4) Significance

Although protein regions mediating ionic regulation are highly conserved between the *Drosophila* isoforms, substantial differences were observed in Na^+_i- and Ca^{2+}_i-dependent regulation and the interactions between these two processes for CALX1.1 and CALX1.2. Thus, our results showing differences between these processes suggest alternative splicing may play a role in modifying these regulatory mechanisms. These data are the first report of alterations in regulatory properties for splice variants of the Na^+-Ca^{2+} exchanger. Recently, we have also observed alterations of regulatory processes for tissue-specific alternatively spliced mammalian isoforms of the Na^+-Ca^{2+} exchanger (unpublished data). Together, these findings suggest that alternative splicing may modify regualtory properties in such a way as to tailor exchanger function accordingly to meet tissue-specific demands.

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IMAGE EVALUATION
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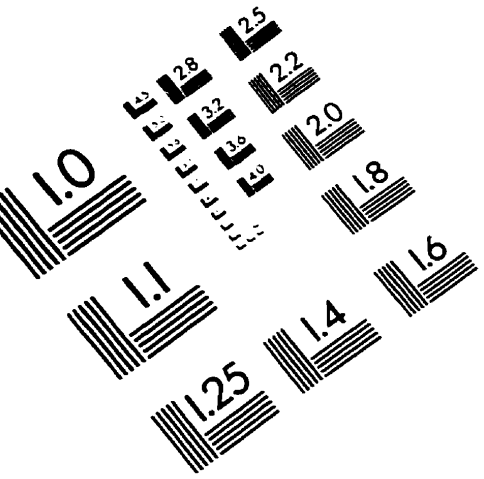

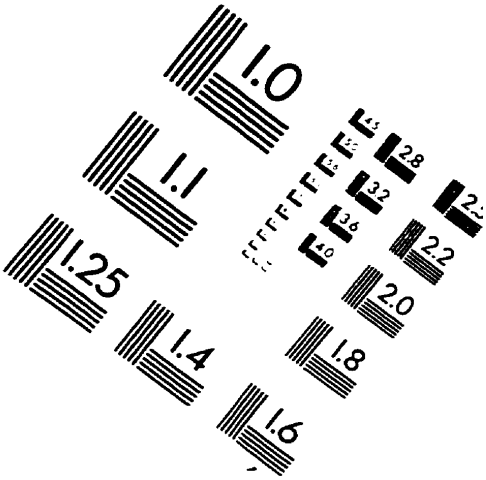

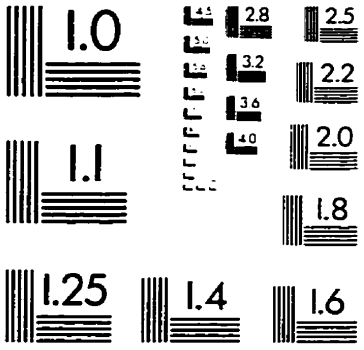

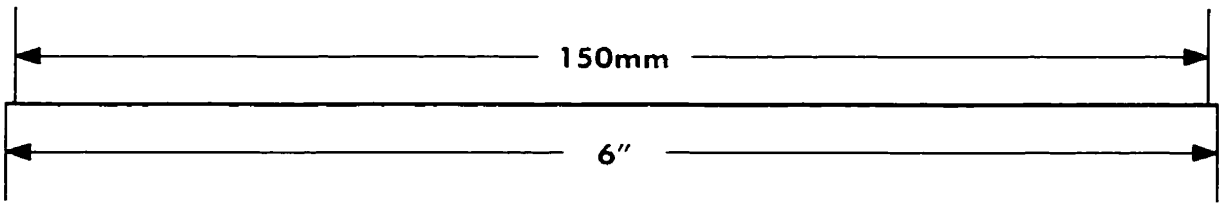

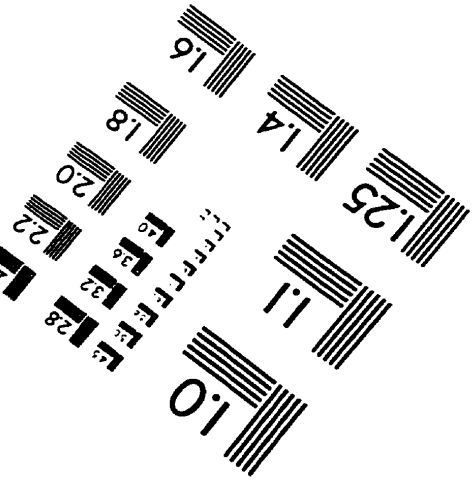

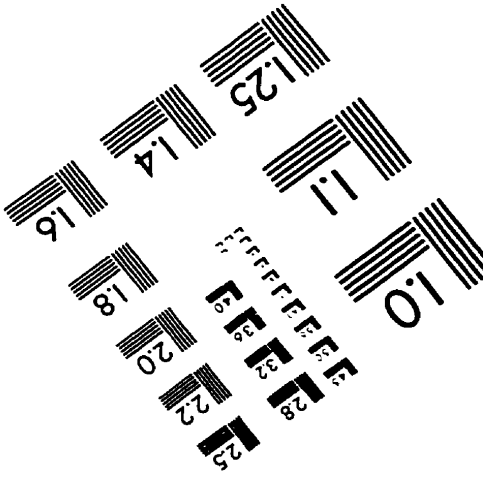

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