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Anomalous Regulation of the *Drosophila* Na^+–Ca^{2+} Exchanger by Ca^{2+}

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ABSTRACT The Na^+–Ca^{2+} exchanger from *Drosophila* was expressed in *Xenopus* oocytes and characterized electrophysiologically using the giant excised patch technique. This protein, termed Calx, shares 49% amino acid identity to the canine cardiac Na^+–Ca^{2+} exchanger, NCX1. Calx exhibits properties similar to previously characterized Na^+–Ca^{2+} exchangers including intracellular Na^+ affinities, current–voltage relationships, and sensitivity to the peptide inhibitor, XIP. However, the *Drosophila* Na^+–Ca^{2+} exchanger shows a completely opposite response to cytoplasmic Ca^{2+}. Previously cloned Na^+–Ca^{2+} exchangers (NCX1 and NCX2) are stimulated by cytoplasmic Ca^{2+} in the micromolar range (0.1–10 μM). This stimulation of exchange current is mediated by occupancy of a regulatory Ca^{2+} binding site separate from the Ca^{2+} transport site. In contrast, Calx is inhibited by cytoplasmic Ca^{2+} over this same concentration range. The inhibition of exchange current is evident for both forward and reverse modes of transport. The characteristics of the inhibition are consistent with the binding of Ca^{2+} at a regulatory site distinct from the transport site. These data provide a rational basis for subsequent structure–function studies targeting the intracellular Ca^{2+} regulatory mechanism. Key words: sodium–calcium exchange • calcium regulation • *Drosophila melanogaster*

INTRODUCTION

Na^+–Ca^{2+} exchange plays an important role in intracellular Ca^{2+} (Ca_i^{2+})[1] homeostasis in diverse tissues. This role is best characterized in cardiac muscle where the exchanger is the primary mechanism for *trans*-sarcolemmal Ca^{2+} efflux (Bers, 1991; Blaustein et al., 1991; Philipson and Nicoll, 1993). Ca^{2+} removal is accomplished by coupling the energy in the Na^+ electrochemical gradient to the uphill movement of Ca^{2+}. In cardiac tissue, the exchanger may also be involved in Ca^{2+} entry during the action potential and may contribute to the Ca^{2+}-induced Ca^{2+} release mechanism (Leblanc and Hume, 1990; Levi et al., 1993; Kohmoto et al., 1994; Levesque et al., 1994; Levi et al., 1994). The exact role for Na^+–Ca^{2+} exchange in other tissues is less well characterized, but, in general, the exchanger likely serves as a mechanism for Ca^{2+} extrusion (Blaustein et al., 1991; Philipson and Nicoll, 1993).

The application of molecular biological and electrophysiological techniques has greatly accelerated our understanding of the Na^+–Ca^{2+} exchange protein. Recent studies indicate that the exchanger operates via a consecutive mechanism, whereby Na^+ and Ca^{2+} are transported during separate steps (Hilgemann et al., 1991; Niggli and Lederer, 1991; Powell et al., 1993). Furthermore, a number of regulatory properties have been characterized in detail. Two major autoregulatory properties are intracellular Na^+ (Na_i^+)–induced inactivation (termed I_1 inactivation) and an inactivation relieved by Ca_i^{2+} (termed I_2 inactivation) (Hilgemann, 1990; Hilgemann et al., 1992*a*; Hilgemann et al., 1992*b*). These properties have been characterized extensively for the native Na^+–Ca^{2+} exchanger in excised cardiac sarcolemmal membrane patches and for the cloned Na^+–Ca^{2+} exchanger (NCX1) in oocytes. Outward Na^+–Ca^{2+} exchange currents, where cytoplasmic (bath) Na^+ exchanges for extracellular (pipette) Ca^{2+}, have been examined in most detail. Under these conditions, opposite membrane surfaces are exposed to transported and regulatory Ca^{2+}. Na_i^+-induced inactivation is apparent as an Na_i^+-mediated reduction in outward exchange current (Hilgemann et al., 1992*a*). That is, Na^+ application that induces the outward current also leads to a partial inactivation of the current. At lower concentrations of activating Na_i^+, I_1 inactivation is reduced or not observed. A second type of inactiva-

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[1] *Abbreviations used in this paper:* Ca_i^{2+}, intracellular Ca^{2+}; *IV*, current–voltage; MES, morpholino ethanesulfonic acid; Na_i^+, intracellular Na^+; TEA, tetraethyl ammonium; XIP, exchanger inhibitory peptide.

tion is Na_i^+ independent and is relieved by Ca_i^{2+}. This stimulatory effect of cytoplasmic Ca^{2+} is observed for both inward and outward exchange currents (Hilgemann et al., 1992*b*; Matsuoka et al., 1995). In the absence of cytoplasmic Ca^{2+}, the exchanger is largely inactivated (I_2 inactivation). Ca_i^{2+} regulation of Na^+–Ca^{2+} exchange was first noted by DiPolo (1979) in the squid giant axon.

The cardiac Na^+–Ca^{2+} exchanger is modelled to have 11 transmembrane segments and a large cytoplasmic domain constituting roughly half of the protein between transmembrane segments 5 and 6 (Nicoll et al., 1990; Philipson and Nicoll, 1993). Transport and regulatory properties appear to be mediated by distinct portions of the Na^+–Ca^{2+} exchange molecule. Transport functions are associated with the transmembrane segments, whereas regulation is mediated by the cytoplasmic domain (Matsuoka et al., 1993). Support for this model has been derived largely from studies on mutant Na^+–Ca^{2+} exchange proteins. For example, in a mutant Na^+–Ca^{2+} exchanger in which the majority of the cytoplasmic domain had been deleted, both Na_i^+-induced inactivation and Ca_i^{2+}-induced activation were absent, whereas transport remained intact (Matsuoka et al., 1993). Both regulatory processes are also eliminated by proteolysis of the cytoplasmic surface of excised patches (Hilgemann, 1990; Matsuoka et al., 1993). A high affinity Ca^{2+} binding site has been localized to a portion of the cytoplasmic domain (between amino acids 371 and 508), and mutations in this region alter both Ca^{2+} binding and Ca_i^{2+} regulation of exchange current in a parallel manner (Levitsky et al., 1994; Matsuoka et al., 1995).

A variety of Na^+–Ca^{2+} exchange proteins have been identified from several species and tissue types (Nicoll et al., 1990; Komuro et al., 1992; Reilly and Shugrue, 1992; Furman et al., 1993; Low et al., 1993; Iwata et al., 1995; Valdivia et al., 1995; E.M. Schwarz and S. Benzer, manuscript submitted for publication). These exchangers exhibit considerable identity to the canine cardiac Na^+–Ca^{2+} exchanger (NCX1), which was first cloned in 1990 (Nicoll et al., 1990). Many recently identified exchangers are splice variants of NCX1 (Kofuji et al., 1994; Lee et al., 1994). Factors controlling the tissue specificity and the functional differences between particular isoforms are currently unknown. More recently, a second isoform of the Na^+–Ca^{2+} exchanger was identified by screening a rat brain cDNA library (Li et al., 1994). This isoform, NCX2, is 65% identical to the NCX1 exchanger at the amino acid level and is the product of a different gene. Functionally, the NCX2 exchanger appears quite similar to NCX1 (Li et al., 1994).

cDNA subtraction techniques were used to identify genes preferentially expressed in the *Drosophila* visual system (Hyde et al., 1990). Subsequently, one of these clones, Calx, was found to have 49% amino acid identity to NCX1. Hydropathy analysis predicts nearly identical transmembrane topology for the two proteins. When injected into *Xenopus* oocytes, Calx cRNA yielded Na_i-dependent $^{45}Ca^{2+}$ uptake similar to that observed for NCX1 (E.M. Schwarz and S. Benzer, manuscript submitted for publication). Valdivia et al. (1995) have also cloned the *Drosophila* Na^+–Ca^{2+} exchanger and have identified two splicing isoforms.

In this study, the *Drosophila* Na^+–Ca^{2+} exchanger was expressed in *Xenopus* oocytes and characterized using the giant excised patch technique (Hilgemann, 1989). Calx exhibits many similarities to previously characterized Na^+–Ca^{2+} exchange proteins. One striking difference, however, is that outward Na^+–Ca^{2+} exchange currents for Calx are inhibited by cytoplasmic Ca^{2+}. This occurs over the same Ca^{2+} concentration range previously demonstrated to enhance Na^+–Ca^{2+} exchange currents for both NCX1 and NCX2 exchange proteins (Matsuoka et al., 1993; Li et al., 1994; Matsuoka et al., 1995). Here, we characterize the fundamental transport properties of Calx and report the novel observation of negative Ca_i^{2+} regulation for an Na^+–Ca^{2+} exchanger. A preliminary report of this work has appeared in abstract form (Hryshko et al., 1995).

METHODS

Molecular Biological Techniques

Na^+–Ca^{2+} exchange activity from the original Calx clone was relatively low when cRNA was injected into *Xenopus* oocytes. To enhance expression and activity, the 3′-untranslated region from the Na-glucose transporter (kindly provided by Dr. E. Wright, University of California, Los Angeles) was added to the 3′-untranslated region of Calx cDNA. The addition of this polyadenylated sequence has previously been found to enhance activity for both the NCX1 and NCX2 exchangers (Li et al., 1994; Matsuoka et al., 1995). The resultant plasmid was linearized with HindIII, and capped cRNA was prepared using T3 mMessage mMachine (Ambion Inc., Austin, TX). Transcripts were further purified using ChromaSpin-100 DEPC-H_2O columns (CLONTECH; Palo Alto, CA).

Oocytes were obtained from *Xenopus laevis* as previously described (Hryshko et al., 1993; Matsuoka et al., 1993). Calx cRNA was injected (5 ng/oocyte), and activity was examined 3–5 d later.

Electrophysiological Techniques

The giant excised patch clamp technique of Hilgemann (1989) was used as previously described (Hryshko et al., 1993; Matsuoka et al., 1993). Inside-out patches were studied in all cases. Briefly, pipettes were pulled from borosilicate glass and polished to a final diameter of 15–30 μm. Pipettes were coated with a Parafilm and mineral oil mixture to enhance patch stability. Gigaohm seals were formed by gentle suction and patches were excised by progressive movements of the pipette tip. For outward Na^+–Ca^{2+} exchange current measurements, pipettes were filled with the following (in mM): 100 *N*-methyl-D-glucamine–morpholino ethanesulfonic acid (NMG:MES), 30 HEPES, 30 tetraethyl ammonium (TEA)-OH, 8 $CaCO_3$, 2 $Ba(OH)_2$, 2 $Mg(OH)_2$, 0.25 ouabain, 0.1 niflumic acid, 0.1 flufenamic acid, pH 7.0 (using MES). Outward

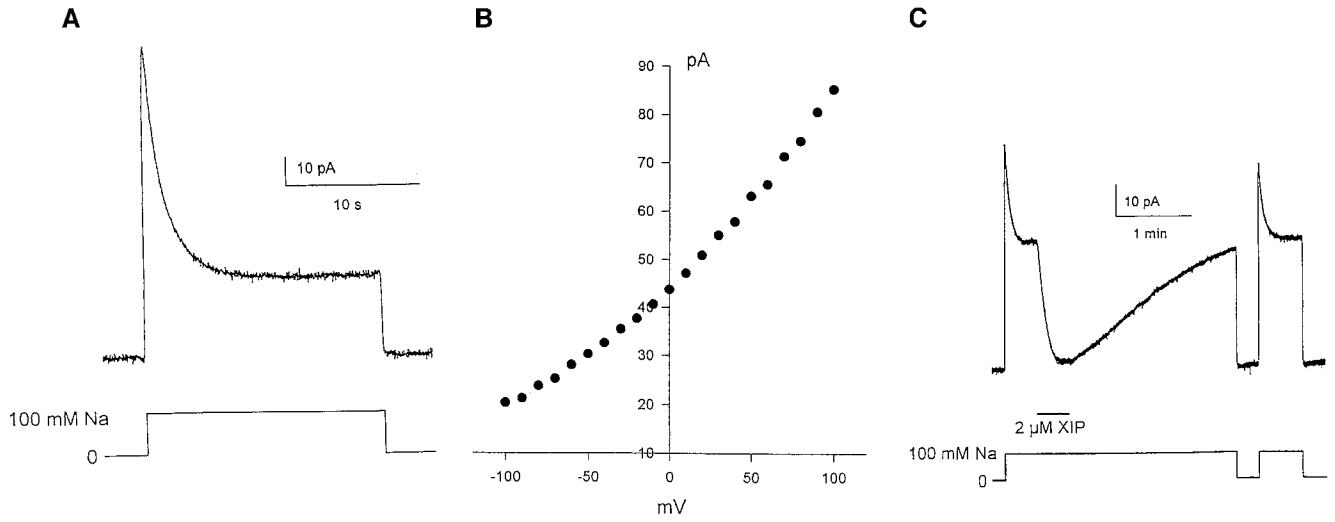

FIGURE 1. (*A*) Outward Na^+–Ca^{2+} exchange current from a giant excised patch of membrane expressing the *Drosophila* Na^+–Ca^{2+} exchanger. The current was activated by changing from a Cs^+- to Na^+-based superfusate. (*B*) *IV* relationship of steady state outward Na^+–Ca^{2+} exchange current activated by 100 mM Na^+ as in *A*. (*C*) Effect of 2 μM exchanger inhibitory peptide, XIP, on outward Na^+–Ca^{2+} exchange current. Washout of XIP led to near complete recovery of exchange current.

Na^+–Ca^{2+} exchange currents were elicited by switching from a Cs^+- to Na^+-based superfusate containing (in mM): 100 Na- or Cs-MES, 20 HEPES, 20 TEA-OH, 20 CsOH, 10 EGTA, 0–11 $CaCO_3$, 1–1.5 $Mg(OH)_2$, pH 7.0 (using MES). Ca^{2+} and Mg^{2+} amounts were adjusted to yield a free Mg^{2+} concentration of 1 mM and various Ca^{2+} concentrations as indicated. Ca^{2+} and Mg^{2+} concentrations were calculated using MAX-C software (Bers et al., 1994). For inward current measurements, pipettes contained (in mM): 100 Na-MES, 20 TEA-MES, 20 Cs-MES, 10 HEPES, 10 EGTA, 4 $Mg(OH)_2$, 0.2 ouabain, 0.1 niflumic acid, 0.1 flufenamic acid, 0.002 verapamil, pH 7.0. Inward Na^+–Ca^{2+} exchange currents were activated by switching to the Cs^+-based Ca^{2+} containing superfusates described above for outward current measurements. Current–voltage (*IV*) relationships were obtained by ramp or step protocols using Axon Instruments, Inc. (Foster City, CA) hardware and software. Solution switches were accomplished using a custom-built 20-channel computer-controlled solution switcher. All experiments were conducted at 34 ± 1°C.

RESULTS

Na^+–Ca^{2+} exchange currents were measured in giant excised patches from oocytes expressing the *Drosophila* Na^+–Ca^{2+} exchanger. In most experiments, outward currents were examined where extracellular (pipette) Ca^{2+} exchanges for cytoplasmic (bath) Na^+. Na^+–Ca^{2+} exchange currents were activated by rapidly replacing cytoplasmic Cs^+ with Na^+. Fig. 1 *A* illustrates a typical outward Na^+–Ca^{2+} exchange current from a giant excised patch. The large initial current gradually decays to a lower steady state level over several seconds. A leak-subtracted *IV* relationship for steady state currents in 100 mM Na^+ is shown in *B*. The *Drosophila* exchanger exhibits a relatively linear *IV* relationship similar to that observed for NCX1 and NCX2 proteins (Hryshko et al., 1993; Li et al., 1994). Fig. 1 *C* illustrates the inhibitory effect of 2 μM of the canine exchanger inhibitory peptide (XIP) on outward Na^+–Ca^{2+} exchange current. Outward exchange currents were almost completely inhibited (>90%, $n = 3$) by this concentration of XIP. All results in Fig. 1 were obtained in the nominal absence of regulatory cytoplasmic Ca^{2+} (10 mM EGTA without added Ca^{2+} in bath). Under these conditions, little steady state outward exchange current would be observed for either NCX1 or NCX2 (Hilgemann, 1990; Matsuoka et al., 1993; Li et al., 1994; Matsuoka et al., 1995).

Fig. 2 *A* shows outward currents for the *Drosophila* Na^+–Ca^{2+} exchanger as a function of the Na^+ concentration applied to the cytoplasmic surface of the patch. Pooled results are shown in Fig. 2, *B* and *C*, for both peak and steady state currents. Unlike NCX1, Calx shows a difference (~10–15 mM) in Na_i affinity between peak and steady state currents (Hilgemann, 1990; Hilgemann et al., 1992*a*). A clear saturation of peak current is not obvious in Fig. 2 *B*. However, Na_i^+ concentrations >100 mM were not examined to avoid osmotic gradients between the superfusate and pipette solutions. All current records were obtained in the absence of regulatory Ca^{2+} and with 8 mM Ca^{2+} in the pipette. Note that both steady state Na^+–Ca^{2+} exchange currents and the extent of current inactivation increased as intracellular Na^+ was increased. As described previously for NCX1, the Na_i^+-dependent inactivation is less pronounced at lower Na_i^+ levels (Hilgemann et al., 1992*a*). Thus, Calx shows I_1 inactivation similar to that of NCX1. The estimated Na_i^+ affinity for Calx ($K_{1/2} \sim 22$ mM) is similar to that reported for NCX1 and NCX2 ($K_{1/2} \sim 20$–25 mM) (Hilgemann et al., 1992*a*; Li et al., 1994).

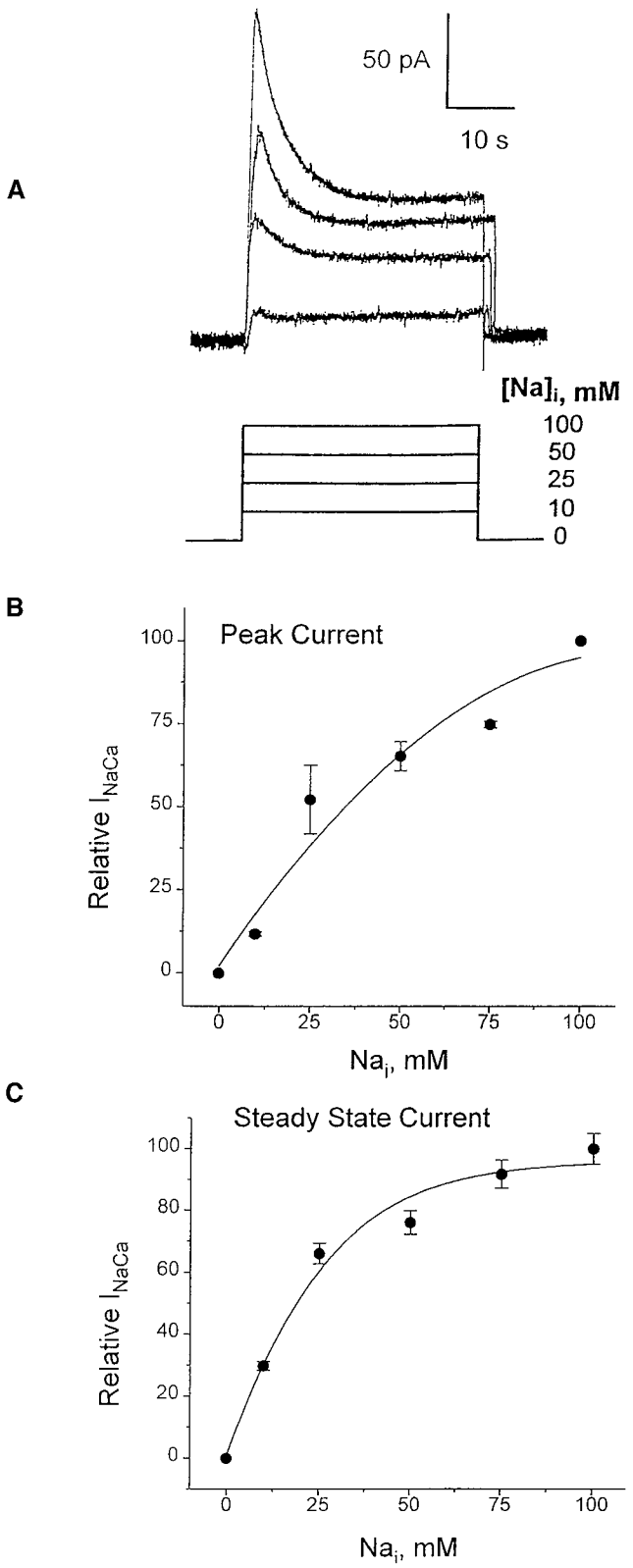

FIGURE 2. (*A*) Outward Na^+–Ca^{2+} exchange currents activated by different concentrations of cytoplasmic Na^+. Pipette Ca^{2+} was constant at 8 mM. Peak (*B*) and steady state (*C*) Na^+–Ca^{2+} exchange currents as a function of cytoplasmic Na^+ are shown from three to six different patches (means ± SE).

Fig. 3 compares the effects of 1 μM cytoplasmic Ca^{2+} on outward Na^+–Ca^{2+} exchange currents for Calx and NCX1. Outward currents were evoked by replacing 100 mM Cs^+ with 100 mM Na^+ in the perfusing solution. The presence of Ca_i^{2+} elicits completely opposite responses for the two Na^+–Ca^{2+} exchangers. For NCX1, 1 μM cytoplasmic Ca^{2+} had a pronounced stimulatory effect. Removing Ca^{2+} reduced the magnitude of exchange current, whereas introducing Ca^{2+} increased current size. For the *Drosophila* exchanger, 1 μM cytoplasmic Ca^{2+} markedly inhibits exchange current, whereas large currents were observed in its absence. The effects of regulatory Ca^{2+} were apparent for both exchangers when present before the application of Na^+ or if applied after the Na^+ addition. Thus, cytoplasmic Ca^{2+} exerts a negative effect on outward exchange current for the *Drosophila* Na^+–Ca^{2+} exchanger.

Fig. 4 *A* illustrates the effects of a range of cytoplasmic Ca^{2+} concentrations on outward exchange currents in a single excised patch. Currents were activated by switching from 100 mM Cs^+ to 100 mM Na^+. For each current activation, the same regulatory Ca^{2+} concentration was present in both Cs^+- and Na^+-containing solutions. The inhibitory effects of cytoplasmic Ca^{2+} are apparent with as little as 100 nM Ca^{2+} and ap-

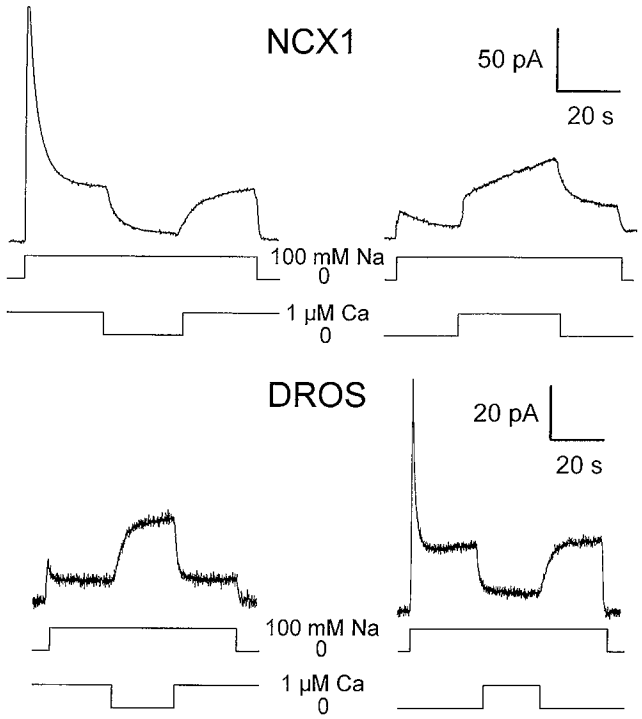

FIGURE 3. Effects of cytoplasmic Ca^{2+} on outward Na^+–Ca^{2+} exchange currents for the canine cardiac (NCX1) and *Drosophila* (DROS) Na^+–Ca^{2+} exchangers. All outward currents were activated by replacing 100 mM cytoplasmic Cs^+ with Na^+; transported Ca^{2+} (pipette) remained constant at 8 mM. Regulatory cytoplasmic Ca^{2+} (1 μM) was added or removed as indicated under the current traces.

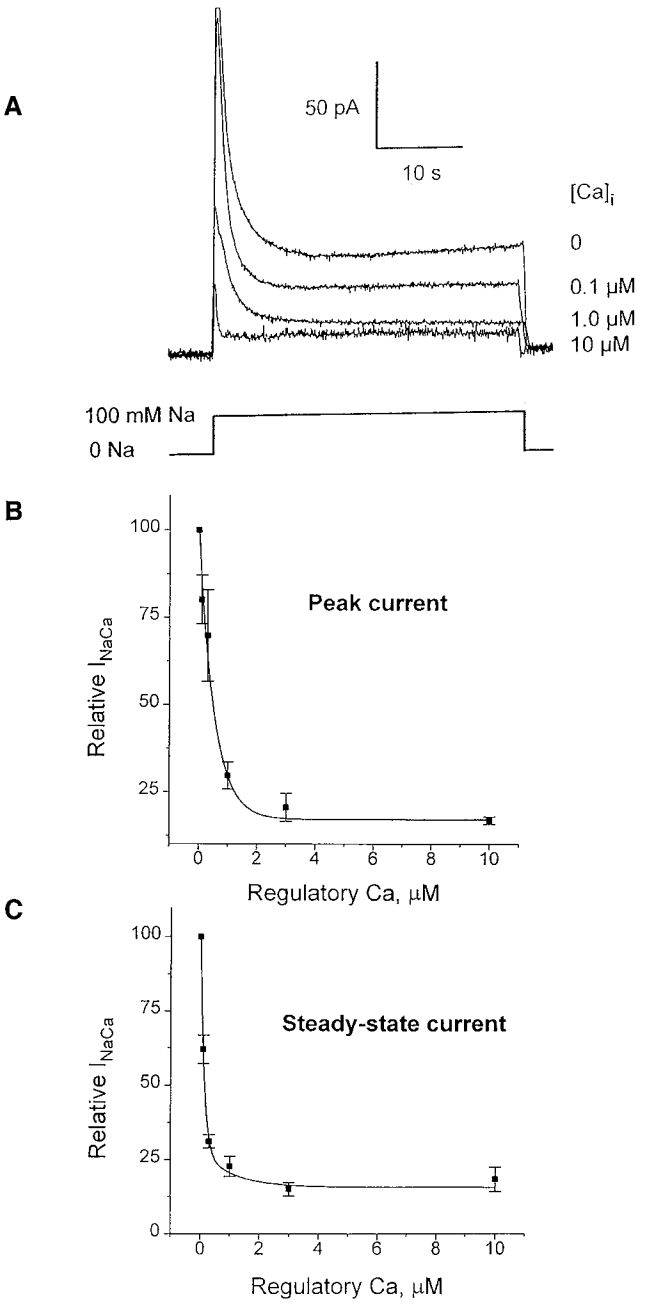

FIGURE 4. (*A*) Effects of different cytoplasmic Ca^{2+} concentrations on outward Na^+–Ca^{2+} exchange currents for *Drosophila*. Pipette Ca^{2+} was constant at 8 mM, and currents were activated by replacing 100 mM cytoplasmic Cs^+ with Na^+. The same concentration of regulatory Ca^{2+} was present before and during current activation. The inhibitory effects of cytoplasmic Ca^{2+} on peak and steady state outward Na^+–Ca^{2+} exchange currents are shown in *B* and *C* for results obtained from three to eight different patches (means ± SE).

pear to be near maximal at ~3 μM Ca^{2+}. Pooled results are illustrated in Fig. 4, *B* and *C*. Cytoplasmic Ca^{2+} reduces the peak and steady state components of outward Na^+–Ca^{2+} exchange current for the *Drosophila* exchanger. These results are completely opposite to those observed for both NCX1 and NCX2 exchangers (Hilgemann et al., 1992*b*; Li et al., 1994).

It appears that Calx is negatively regulated by Ca_i^{2+}. However, an alternative interpretation is that Ca_i^{2+} can compete with Na^+ at the intracellular transport site producing the observed inhibition. This seemed unlikely considering that inhibition was still observed when Na^+ levels (100 mM) exceeded Ca^{2+} levels (100 nM) by 10^6. However, to resolve these possibilities, the influence of preincubating patches with or without regulatory Ca^{2+} was studied. Currents were then activated by 100 mM Na^+ with or without regulatory Ca^{2+}. We reasoned that if Ca^{2+} exerts regulatory effects by binding to a regulatory site and altering the availability or activity of exchangers, then these effects might be apparent at the onset of current activation. That is, regulatory Ca^{2+} might exert its effects independently of whether or not transport was occurring. This supposition seems reasonable, given the relatively slow response of currents to changes in regulatory Ca^{2+} (e.g., Fig. 3). In contrast, competitive effects between Na^+ and Ca^{2+} would be rapid and would only be observed during transport. That is, transport activity would be a function of the instantaneous levels of Na^+ and Ca^{2+} and would not be sensitive to preincubation conditions.

The results obtained from preincubation experiments are shown in Fig. 5 for all possible permutations in a single patch. Preincubations in Cs^+-based solutions for ~30 s were conducted with (1 μM) or without (zero added Ca^{2+} plus 10 mM EGTA) regulatory Ca^{2+}. Transported Ca^{2+} within the pipette remained constant at 8 mM. Current activation by 100 mM Na^+ was then evoked with or without regulatory Ca^{2+}. The results are typical of those observed in eight different patches. Note that the initial Na^+–Ca^{2+} currents were large and nearly identical if the preincubation was conducted without regulatory Ca^{2+}, irrespective of the activating solution (Fig. 5, *A* and *B*). Similarly, initial currents were small if patches were preincubated with 1 μM cytoplasmic Ca^{2+} (Fig. 5, *C* and *D*). Steady state currents then developed to a level appropriate for the presence or absence of regulatory Ca^{2+} in the activating solution. The time course for steady state current development was similar to that shown in Fig. 3 for the application or removal of regulatory Ca^{2+}. These data indicate that cytoplasmic Ca^{2+} levels regulate Na^+–Ca^{2+} exchange activity independently of transport.

Inward currents for Calx and NCX1 exchangers were also compared (Fig. 6). The extracellular (pipette) solution contained 100 mM Na^+, and inward current was initiated by the application of 1 or 3 μM cytoplasmic Ca^{2+}. In this transport mode, three extracellular (pipette) Na^+ ions exchange for one cytoplasmic (bath) Ca^{2+} ion leading to an inward current. Note that a tran-

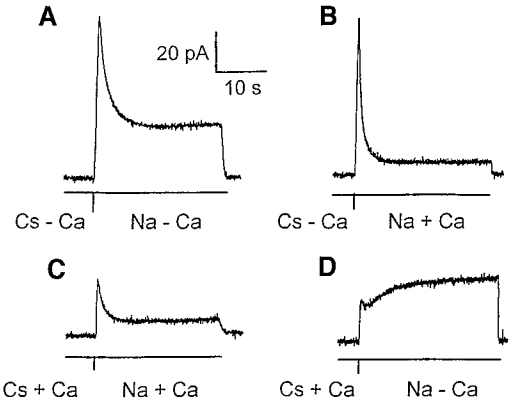

FIGURE 5. Effects of preincubating a single patch with or without 1 μM regulatory Ca^{2+} on subsequent Na^+–Ca^{2+} exchange currents. Currents were activated by replacing 100 mM cytoplasmic Cs^+ with Na^+ with or without regulatory Ca^{2+}. Pipette Ca^{2+} was 8 mM.

sient component is evident for inward Na^+–Ca^{2+} exchange currents obtained from Calx, whereas no similar component appears for the NCX1 currents. This inactivation was observed in five different patches expressing the *Drosophila* exchanger, although the magnitude was variable. The transient component likely reflects negative Ca_i^{2+} regulation of the *Drosophila* exchanger. That is, Ca^{2+} application rapidly induces Na^+–Ca^{2+} exchange transport and the regulatory inhibition develops over seconds. In contrast, for NCX1 where positive Ca_i^{2+} regulation is observed, current records appear relatively flat. Little delay in activation is apparent for NCX1. Some delay might have been expected because of a gradual increase in exchanger activation by cytoplasmic Ca^{2+}. However, this observation of rapid current activation is similar to that reported in giant patches of cardiac sarcolemmal membranes where inward current activation also occurs very rapidly (Hilgemann et al., 1992*b*). The rapid onset of inward current is also consistent with modeling in which the response to regulatory Ca^{2+} is rapid for NCX1 in the absence of intracellular Na^+ (Hilgemann et al., 1992*b*).

DISCUSSION

In this study, we have characterized a new Na^+–Ca^{2+} exchange protein, Calx, cloned from *Drosophila*. This homologue exhibits many properties similar to previously characterized Na^+–Ca^{2+} exchangers. In particular, the *Drosophila* exchanger exhibits a similar *IV* relationship, Na_i^+ affinity, sensitivity to the exchanger inhibitory peptide XIP, and slow Na_i^+-induced (I_1) inactivation. However, a striking difference was observed with regard to the effects of Ca_i^{2+}. For Calx, Ca_i^{2+} inhibits Na^+–Ca^{2+} exchange currents, whereas other cloned exchangers are markedly stimulated by Ca_i^{2+}. The inhibition reflects a regulatory mechanism unique among identified Na^+–Ca^{2+} exchange proteins.

Na^+–Ca^{2+} Exchange Proteins

The Na^+–Ca^{2+} exchanger (NCX1) was initially cloned from a canine cardiac library (Nicoll et al., 1990), and, subsequently, several splice variants have been identified (Kofuji et al., 1994; Lee et al., 1994). The presence of NCX1 can be detected in several tissues. A recently identified second isoform of the Na^+–Ca^{2+} exchanger, NCX2, is prominent only in brain and skeletal muscle by Northern blot analysis (Li et al., 1994; Quednau et al., 1995). Finally, a distantly related protein to the NCX-type exchangers has been cloned from rod outer segments of bovine retina (Reilander et al., 1992). This protein, the Na^+–Ca^{2+},K^+ exchanger, shows little sequence or functional similarity to the NCX-type exchangers (Schnetkamp et al., 1989; Perry and McNaughton, 1993). While Calx was originally postulated to represent a transcript expressed in the visual system of *Drosophila* (Hyde et al., 1990), subsequent studies indicate that the transcript exhibits a broader tissue specificity (Schwarz, E.M., and S. Benzer, manuscript submitted for publication). Calx is clearly a member of the NCX family and is functionally dissimilar to the rod outer segment exchanger.

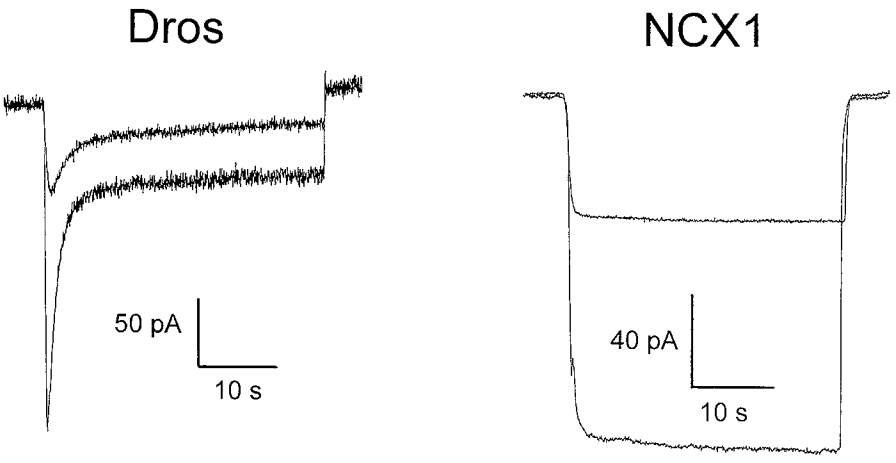

FIGURE 6. Inward Na^+–Ca^{2+} exchange currents for the *Drosophila* and NCX1 Na^+–Ca^{2+} exchangers. Currents were activated by switching from 0 Ca^{2+} superfusate to 1 or 3 μM Ca^{2+} containing superfusate. The pipette solution contained 100 mM Na^+.

The results presented in this study differ from previous reports on Ca_i^{2+} regulation (I_2 inactivation) of Na^+–Ca^{2+} exchange proteins in that cytoplasmic Ca^{2+} exerts a negative effect on Na^+–Ca^{2+} exchange current for the *Drosophila* exchanger. This negative effect occurs over the same Ca_i^{2+} concentration range that has previously been demonstrated to stimulate both NCX1 and NCX2 (Hilgemann et al., 1992*a*; Hilgemann et al., 1992*b*; Li et al., 1994). The negative effects of Ca_i^{2+} on exchange currents for Calx appear to represent a regulatory mechanism and not competition of cytoplasmic Ca^{2+} with Na^+ for intracellular transport sites.

As shown in Fig. 4, as little as 100 nM cytoplasmic Ca^{2+} is sufficient to inhibit outward Na^+–Ca^{2+} exchange currents in the *Drosophila* Na^+–Ca^{2+} exchanger. Near maximal inhibition is observed at ~3 μM Ca_i^{2+}. In contrast, inward Na^+–Ca^{2+} exchange currents were barely detectable at 100 and 300 nM Ca^{2+} (not shown). Thus, 100 nM Ca^{2+} is insufficient to bind significant Ca^{2+} to the intracellular transport site. This result is not compatible with the notion that the negative effect of Ca_i^{2+} on outward current is due to competition with Na^+ at the transport site.

The effects of preincubating patches with or without regulatory Ca^{2+} (Fig. 5) indicated that the initial current transient was determined by the preexisting Ca_i^{2+} concentration. That is, if regulatory (inhibitory) Ca_i^{2+} was present during the preincubation period (in the Cs^+ superfusate), then the initial currents were small regardless of whether Ca^{2+} was present or absent during Na^+ application. Conversely, if regulatory Ca^{2+} was absent during the preincubation, initial currents were large regardless of whether regulatory Ca^{2+} was present or absent during Na^+ application. In all cases, steady state currents then slowly developed to a level appropriate for the presence or absence of regulatory Ca^{2+}. The result indicates that the status of the Na^+–Ca^{2+} exchanger is determined by the cytoplasmic Ca^{2+} level.

It is of interest to compare the putative regulatory Ca^{2+} binding region of the *Drosophila* Na^+–Ca^{2+} exchanger with that of NCX1. In NCX1, a high affinity Ca^{2+} binding site comprising amino acids 371 to 508 has been identified by the $^{45}Ca^{2+}$ overlay technique (Levitsky et al., 1994). This portion of the cytoplasmic loop contains two highly acidic regions postulated to be involved in Ca^{2+} binding. Several site-specific mutations of this region were examined to determine the effects on $^{45}Ca^{2+}$ binding (Levitsky et al., 1994) and Ca^{2+} regulation as assessed electrophysiologically (Matsuoka et al., 1995). Mutations that reduced the $^{45}Ca^{2+}$ binding affinity also exhibited a reduced affinity for Ca^{2+} regulation of Na^+–Ca^{2+} exchange currents. When the equivalent Calx sequence for the Ca^{2+} binding region is compared with NCX1, only 40% identity (55/138) is observed for NCX1 amino acids 371–508. However, when the two acidic regions postulated to be part of the high affinity regulatory Ca^{2+} binding site are compared (amino acids 446–454 and 498–508 in NCX1), the *Drosophila* exchanger sequences are 89% (8/9) and 82% (9/11) identical (Fig. 7). Thus, this region may also represent the regulatory Ca^{2+} binding site for the *Drosophila* exchanger. The fact that completely opposite responses to Ca_i^{2+} are observed suggests that transduction of the Ca^{2+} binding signal to modulation of exchange activity is different for these two exchangers.

NCX1	446	**D**	**D**	**D**	i	**F**	**E**	**E**	**D**	**E** 454
DROS	515	**D**	**D**	**D**	v	**F**	**E**	**E**	**D**	**E** 523
NXC1	498	**D**	**D**	**D**	**H**	**A**	**G**	**I**	**F** t	**F** e 508
DROS	544	**D**	**D**	**D**	**H**	**A**	**G**	**I**	**F** a	**F** t 554

FIGURE 7. Amino acid sequence comparison between the two acidic sequences within the regulatory Ca^{2+} binding domain of NCX1 and the equivalent sites for the *Drosophila* Na^+–Ca^{2+} exchanger. Amino acid identity is indicated by upper case letters.

The results demonstrate a novel regulatory mechanism for the *Drosophila* Na^+–Ca^{2+} exchanger, Calx. Our observations should help in the design of experiments for identifying protein regions involved in Ca_i^{2+} regulation and for understanding the mechanism(s) by which Ca_i^{2+} regulation occurs. In addition, this major difference in Ca_i^{2+} regulation may have interesting functional consequences and may provide insight towards understanding the functional role of Calx in *Drosophila*. Interestingly, splice variants of the *Drosophila* exchanger exist (Valdivia et al., 1995). Perhaps the negative Ca^{2+} regulation we have observed is splice variant specific, and other *Drosophila* exchangers will display "normal" Ca^{2+} regulation. Functional consequences of alternative splicing for NCX1 have not yet been investigated in detail. Perhaps, some of the alternatively spliced variants of vertebrate exchangers will also show the "anomalous" Ca^{2+} regulation observed for the *Drosophila* Na^+–Ca^{2+} exchanger.

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REFERENCES

Bers, D.M. 1991. *In* Excitation-Contraction Coupling and Cardiac Contractile Force. Kluwer Academic Press, Dordrecht. 71–92.

Bers, D.M., C.W. Patton, and R. Nuccitelli. 1994. A practical guide to the preparation of Ca^{2+} buffers. *Methods Cell Biol.* 40:3–29.

Blaustein, M.P., R. DiPolo, and J.P. Reeves. 1991. *In* Sodium-Calcium Exchange: Proceedings of the Second International Conference. The New York Academy of Sciences, New York.

DiPolo, R. 1979. Calcium influx in internally dialyzed squid giant axons. *J. Gen. Physiol.* 73:91–113.

Furman, I., O. Cook, J. Kasir, and H. Rahamimoff. 1993. Cloning of two isoforms of the rat brain Na^+–Ca^{2+} exchanger gene and their functional expression in HeLa cells. *Fed. Eur. Biochem. Soc. Lett.* 319:105–109.

Hilgemann, D.W. 1989. Giant excised cardiac sarcolemmal membrane patches: sodium and sodium-calcium exchange currents. *Pflüg. Archiv. Eur. J. Physiol.* 415:247–249.

Hilgemann, D.W. 1990. Regulation and deregulation of cardiac Na^+–Ca^{2+} exchange in giant excised sarcolemmal membrane patches. *Nature (Lond.).* 344:242–245.

Hilgemann, D.W., D.A. Nicoll, and K.D. Philipson. 1991. Charge movement during Na^+ translocation by native and cloned Na^+/Ca^{2+} exchanger. *Nature (Lond.).* 352:715–718.

Hilgemann, D.W., S. Matsuoka, G.A. Nagel, and A. Collins. 1992*a*. Steady-state and dynamic properties of cardiac sodium-calcium exchange: sodium-dependent inactivation. *J. Gen. Physiol.* 100: 905–932.

Hilgemann, D.W., A. Collins, and S. Matsuoka. 1992*b*. Steady-state and dynamic properties of cardiac sodium-calcium exchange: secondary modulation by cytoplasmic calcium and ATP. *J. Gen. Physiol.* 100:933–961.

Hryshko, L.V., D.A. Nicoll, J.N. Weiss, and K.D. Philipson. 1993. Biosynthesis and initial processing of the cardiac Na^+–Ca^{2+} exchanger. *Biochim. Biophys. Acta.* 1151:35–42.

Hryshko, L.V., D.A. Nicoll, S. Matsuoka, J.N. Weiss, E. Schwarz, S. Benzer, and K.D. Philipson. 1995. Anomalous regulation of the Na^+–Ca^{2+} exchanger from *Drosophila*. *Biophys. J.* 68:410*a*. (Abstr.)

Hyde, D.R., K.L. Mecklenburg, J.A. Pollock, T.S. Vihtelic, and S. Benzer. 1990. Twenty *Drosophila* visual system cDNA clones: one is a homolog of human arrestin. *Proc. Natl. Acad. Sci. USA.* 87: 1008–1012.

Iwata, T., A. Kraev, M. Morad, and E. Carafoli. 1995. Cloning of the cDNA of the frog heart sarcolemmal Na^+–Ca^{2+} exchanger. *Biophys. J.* 68:136*a*. (Abstr.)

Kofuji, P., W.J. Lederer, and D.H. Schulze. 1994. Mutually exclusive and cassette exons underlie alternatively spliced isoforms of the Na/Ca exchanger. *J. Biol. Chem.* 269:5145–5149.

Kohmoto, O., A.J. Levi, and J.H.B. Bridge. 1994. Relation between reverse sodium-calcium exchange and sarcoplasmic reticulum calcium release in guinea pig ventricular cells. *Circ. Res.* 74:550–554.

Komuro, I., K.E. Wenninger, K.D. Philipson, and S. Izumo. 1992. Molecular cloning and characterization of the human cardiac Na^+–Ca^{2+} exchanger cDNA. *Proc. Natl. Acad. Sci. USA.* 89:4769–4773.

Leblanc, N., and J.R. Hume. 1990. Sodium current-induced release of calcium from cardiac sarcoplasmic reticulum. *Science (Wash. DC).* 248:372–376.

Lee, S.L., A.S.L. Yu, and J. Lytton. 1994. Tissue-specific expression of Na^+–Ca^{2+} exchanger isoforms. *J. Biol. Chem.* 269:14849–14852.

Levesque, P.C., N. Leblanc, and J.R. Hume. 1994. Release of calcium from guinea pig cardiac sarcoplasmic reticulum induced by sodium-calcium exchange. *Cardiovasc. Res.* 28:370–378.

Levi, A.J., P. Brooksby, and J.C. Handcox. 1993. One hump or two? The triggering of calcium release from the sarcoplasmic reticulum and the voltage dependence of contraction in mammalian cardiac muscle. *Cardiovasc. Res.* 27:1743–1757.

Levi, A.J., K.W. Spitzer, O. Kohmoto, and J.H.B. Bridge. 1994. Depolarization-induced Ca entry via Na-Ca exchange triggers SR release in guinea pig cardiac myocytes. *Am. J. Physiol.* 266:H1422–H1433.

Levitsky, D.O., D.A. Nicoll, and K.D. Philipson. 1994. Identification of the high affinity Ca^{2+}-binding domain of the cardiac Na^+–Ca^{2+} exchanger. *J. Biol. Chem.* 269:22847–22852.

Li, Z., S. Matsuoka, L.V. Hryshko, D.A. Nicoll, M.M. Bersohn, E.P. Burke, R.P. Lifton, and K.D. Philipson. 1994. Cloning of the NCX2 isoform of the plasma membrane Na^+–Ca^{2+} exchanger. *J. Biol. Chem.* 269:17434–17439.

Low, W., J. Kasir, and H. Rahamimoff. 1993. Cloning of the rat heart Na^+–Ca^{2+} exchanger and its functional expression in HeLa cells. *Fed. Eur. Biochem. Soc. Lett.* 316:63–67.

Matsuoka, S., D.A. Nicoll, R.F. Reilly, D.W. Hilgemann, and K.D. Philipson. 1993. Initial localization of regulatory regions of the cardiac sarcolemmal Na^+–Ca^{2+} exchanger. *Proc. Natl. Acad. Sci. USA.* 90:3870–3874.

Matsuoka, S., D.A. Nicoll, L.V. Hryshko, D.O. Levitsky, J.N. Weiss, and K.D. Philipson. 1995. Regulation of the cardiac Na^+–Ca^{2+} exchanger by Ca^{2+}: mutational analysis of the Ca^{2+} binding domain. *J. Gen. Physiol.* 105:403–420.

Nicoll, D.A., S. Longoni, and K.D. Philipson. 1990. Molecular cloning and functional expression of the cardiac sarcolemmal Na^+–Ca^{2+} exchanger. *Science (Wash. DC).* 250:561–565.

Niggli, E., and W.J. Lederer. 1991. Molecular operations of the sodium-calcium exchanger revealed by conformation currents. *Nature (Lond.).* 349:621–624.

Perry, R.J., and P.A. McNaughton. 1993. The mechanism of ion transport by the Na^+–Ca^{2+},K^+ exchange in rods isolated from the salamander retina. *J. Physiol. (Camb.).* 466:443–480.

Philipson, K.D., and D.A. Nicoll. 1993. Molecular and kinetic aspects of sodium-calcium exchange. *Int. Rev. Cytol.* 137C:199–227.

Powell, T., A. Noma, T. Shioya, and R.Z. Kozlowski. 1993. Turnover rate of the cardiac Na^+–Ca^{2+} exchanger in guinea-pig ventricular myocytes. *J. Physiol. (Camb.).* 472:45–53.

Quednau, B.D., N. Brecha, and K.D. Philipson. 1995. Tissue-specific expression of the second isoform of the Na/Ca exchanger, NCX2. *Biophys. J.* 68:410*a*. (Abstr.)

Reilander, H., A. Achilles, U. Friedel, G. Maul, F. Lottspeich, and N.J. Cook. 1992. Primary structure and functional expression of the Na/Ca,K-exchanger from bovine rod photoreceptors. *EMBO (Eur. Mol. Biol. Organ.) J.* 11:1689–1695.

Reilly, R.F., and C.A. Shugrue. 1992. cDNA cloning of a renal Na^+–Ca^{2+} exchanger. *Am. J. Physiol.* 262:F1105–F1109.

Schnetkamp, P.P.M., D.K. Basu, and R.T. Szerencsei. 1989. Na^+–Ca^{2+} exchange in bovine rod outer segments requires and transports K^+. *Am. J. Physiol.* 257:C153–C517.

Valdivia, C., P. Kofuji, W.J. Lederer, and D.H. Schulze. 1995. Characterization of the Na/Ca exchanger cDNA in *Drosophila*. *Biophys. J.* 68:410*a*. (Abstr.)