Stoichiometry of the Cardiac Na^+/Ca^{2+} Exchanger NCX1.1 Measured in Transfected HEK Cells

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ABSTRACT The stoichiometry with which the Na^+/Ca^{2+} exchanger, NCX1, binds and transports Na^+ and Ca^{2+} has dramatic consequences for ionic homeostasis and cellular function of heart mycocytes and brain neurons, where the exchanger is highly expressed. Previous studies have examined this question using native NCX1 in its endogenous environment. We describe here whole-cell voltage clamp studies using recombinant rat heart NCX1.1 expressed heterologously in HEK-293 cells. This system provides the advantages of a high level of NCX1 protein expression, very low background ion transport levels, and excellent control over clamped voltage and ionic composition. Using ionic conditions that allowed bi-directional currents, voltage ramps were employed to determine the reversal potential for NCX1.1-mediated currents. Analysis of the relation between reversal potential and external [Na^+] or [Ca^{2+}], under a variety of intracellular conditions, yielded coupling ratios for Na^+ of 1.9–2.3 ions per net charge and for Ca^{2+} of 0.45 $\pm$ 0.03 ions per net charge. These data are consistent with a stoichiometry for the NCX1.1 protein of 4 Na^+ to 1 Ca^{2+} to 2 charges moved per transport cycle.

INTRODUCTION

The cardiac Na^+/Ca^{2+} exchanger, NCX1.1, is the principal means by which adult heart cells extrude calcium that enters via voltage-gated Ca^{2+} channels to initiate sarcoplasmic reticulum release and muscle contraction (Blaustein and Lederer, 1999). Consequently, the precise activity of NCX1.1 has dramatic consequences to cardiac Ca^{2+} homeostasis and the control of muscle contractility. Not surprisingly, the exchanger is heavily regulated, both at the transcriptional level and at the protein level, through allosteric modulation (Philipson and Nicoll, 2000). Primary control of exchange activity, however, is determined by the relative occupancy of the ion transport sites on both sides of the membrane. The direction of flux through the exchanger will be determined by the electrochemical gradient of the transported ions (Ca^{2+} and Na^+) and the stoichiometry with which they bind and move through the exchanger. Alteration of the electrochemical ion gradients during systole has been suggested to switch the direction of Ca^{2+} movement through the exchanger, hence allowing Ca^{2+} entry. However, the extent to which Ca^{2+} entry through the exchanger contributes to the physiological release of sarcoplasmic reticulum Ca^{2+} has been the source of controversy in recent years (Blaustein and Lederer, 1999).

Partly due to the above considerations, the stoichiometry of the Na^+/Ca^{2+} exchanger has been of considerable interest for many years. Initial experiments suggested a coupling of 4 Na^+ to 1 Ca^{2+} (Ledvora and Hegyvary, 1983; Mullins, 1977), but subsequent studies, using a variety of cell preparations and methods, converged on a stoichiometry of 3 Na^+ to 1 Ca^{2+} (summarized by Blaustein and Lederer, 1999). This was the generally accepted model for operation of the exchanger until recently, when Matsuoka and colleagues published an electrophysiological study indicating a stoichiometry of 4 Na^+ to 1 Ca^{2+} for the exchanger in macro patches from cardiac myocytes (Fujioka et al., 2000). The Na^+/Ca^{2+} exchanger is highly expressed in the plasma membrane of excitable cells, such as cardiac myocytes and brain neurons (as well as squid axon). Such membranes, however, also express a high density of other transporters that also allow the movement of Na^+ and Ca^{2+}, such as the Na^+,K^+-ATPase and various ionic channels. As a consequence, all of the stoichiometry studies using native exchanger expressed in its endogenous environment are potentially confounded by the movement of ions through parallel pathways and uncertainty in ionic composition close to the membrane. Because the Na^+/Ca^{2+} exchanger was cloned in 1990 (Nicoll et al., 1990), it has been theoretically possible to revisit the stoichiometry issue using a heterologous cell system to express recombinant exchanger at high levels in a membrane environment largely devoid of contaminating transport pathways. Surprisingly, only one recent report has examined NCX1 stoichiometry using such a system (Szerencsei et al., 2001). In the current paper, we have expressed rat heart NCX1.1 in HEK-293 cells and used electrophysiology to measure the thermodynamic equilibrium point for Na^+/Ca^{2+} exchanger operation under different ionic conditions. These data are consistent with a transport stoichiometry of 4 Na^+ to 1 Ca^{2+}.

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METHODS

Cell culture and transfection

The human embryonic kidney cell line HEK-293 (tsA201 variant) was grown in Dulbecco's minimal essential medium and transfected by Ca^{2+}-

phosphate precipitation as described previously (Dong et al., 2001). Rat heart NCX1.1 cDNA was constructed as follows. The coding region of the kidney NCX1.7 clone, F1 (GenBank accession number U04933; Lee et al., 1994), was excised by *Mun*I and *Bst1107*I digestion, treated with Klenow fragment of DNA polymerase to make the ends blunt, and subcloned into the *Sma*I site of pBluescript II SK(−) (Stratagene, La Jolla, CA), oriented so the *Hin*dIII site of the polylinker was close to the 5′ end of the clone. A fragment corresponding to the central cytoplasmic loop of NCX1.1 was amplified from rat heart mRNA by reverse-transcription-coupled polymerase chain reaction and sequenced to confirm its identity. This fragment encompassed the site of alternative splicing and corresponded to the unique isoform, NCX1.1, observed in heart tissue (Lee et al., 1994). A *Bcl*I fragment from the polymerase chain reaction product was ligated with *Bcl*I-digested pBluescript-NCX1.7. The resultant chimeric construct corresponded in sequence to a combination of GenBank accession numbers U04933 and U04937 and encoded the rat heart NCX1.1 isoform. The coding region of this construct was excised by *Hin*dIII and *Bam*HI digestion and ligated into pcDNA3.1(+) (Invitrogen Corp., Carlsbad, CA). The pcDNA3.1-NCX1.1 cDNA was cotransfected together with a green fluorescent protein cDNA as a means to identify transfected cells for electrophysiological analysis. Control transfections employed empty vector and the green fluorescent protein cDNA. Following transfection, cells were incubated for 24 h and then replated using trypsin into 35-mm dishes for use from 3 h to 3 days later.

Electrophysiology

Whole-cell patch clamp recording of the transfected HEK-293 cells was performed essentially as described previously (Dong et al., 2001). Pipettes were prepared from borosilicate glass capillaries (Corning 8161) and fire polished to a resistance of 3–4 MΩ when filled. The membrane seal resistance was 2–10 GΩ in all cases. Voltage clamp was conducted with a patch amplifier (Axopatch 200B; Axon Instruments, Foster City, CA), using a holding potential of 0 or −20 mV for steady-state currents, and a ramp voltage protocol (dV/dt = 0.5 V/s) over the range −100 mV to +60 mV. All experiments were carried out at room temperature (22–24°C). When the pipette solution contained potassium gluconate, a liquid junction potential of −8 to −10 mV was present that was independent of the bath solution composition and was corrected for in all applied potentials. Recorded currents were low pass-filtered at 100 Hz and sampled at 1 kHz using pClamp software (Clampex, version 8.0, Axon Instruments).

Solutions

All chemicals were of analytical grade or better and were obtained from either Fisher (Nepean, ON, Canada), BDH (Toronto, ON, Canada), or Sigma (St. Louis, MO), unless indicated otherwise. The bath solution used for the recording of outward currents contained 145 mM LiCl, 1 mM $MgCl_2$, 10 mM D-glucose, 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid/tetramethylammonium (HEPES/TMA), pH 7.4, and either 0.5 mM EGTA (free $[Ca^{2+}]$ ~1 nM) or 1 mM $CaCl_2$. The pipette solution used to record outward currents contained 120 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 20 mM tetraethylammonium-chloride (TEA-Cl), 1 mM Na_2ATP, 8 mM D-glucose, 10 mM HEPES/TMA, pH 7.2, and either 5 mM EGTA (free $[Ca^{2+}]$ less than 0.5 nM), or 5 mM EGTA plus 4.28 mM $CaCl_2$, which generated a free $[Ca^{2+}]$ of 1 μM. Reversal potential experiments employed bath solutions containing various combinations of NaCl, KCl, and/or LiCl totaling 125 mM, 1 mM $MgCl_2$, 20 mM TEA-Cl, 10 mM D-glucose, 10 mM HEPES/TMA, and 0.5 mM EGTA. External $[Ca^{2+}]$ was varied from 0.3 to 30 μM using 10 mM EGTA and various amounts of $CaCl_2$, calculated using the method described by Fabiato (1988), or by unbuffered addition of $CaCl_2$ above this range. In all cases, the osmolarity was measured and maintained at 280 mOsm/kg by altering the amount of LiCl present. The pipette solution for reversal potential measurements contained 0.5 mM NaCl and 117.5 mM potassium gluconate, 18 mM NaCl, and 100 mM potassium gluconate or 58 mM NaCl and 60 mM potassium gluconate plus 20 mM TEA-Cl, 1 mM Na_2ATP, 10 mM D-glucose, 10 mM HEPES/TMA, pH 7.2, 10 mM EGTA, and either 6.40, 8.56, or 9.68 mM $CaCl_2$ (free $[Ca^{2+}]$ = 0.3, 1, or 5 μM, respectively). In some experiments, 10 mM BAPTA replaced EGTA, and the addition of 5.28 or 7.89 mM $CaCl_2$ generated free $[Ca^{2+}]$ of 0.3 or 1 μM, respectively.

Data analysis

Electrophysiological data were analyzed using pClamp software (Clampfit, version 8.0, Axon Instruments). The data were fit to various models by nonlinear regression using either MacCurveFit (Kevin Raner Software, Victoria, Australia) or Prism (GraphPad Software, San Diego, CA). Statistical comparisons between groups were conducted using ANOVA, whereas comparisons between fitted models employed an *F* test. *P* values less than 0.05 were regarded as statistically significant.

RESULTS

To study properties of the Na^+/Ca^{2+} exchanger, it is imperative that functional measurements are not contaminated by endogenous activities. To achieve this goal, we have used whole-cell patch clamp to measure currents due to the Na^+/Ca^{2+} exchange activity of rat heart NCX1.1 expressed by transfection in the tsA201 variant of HEK-293 cells. We had previously used such a system to characterize the electrophysiological properties of rat brain NCKX2 (Dong et al., 2001) and found that HEK-293 cells were electrically quiet and displayed only very small membrane currents under the conditions used in these experiments. Fig. 1 *A* illustrates the outward membrane currents, measured at a holding potential of 0 mV, elicited by a perfusion switch from EGTA- to Ca^{2+}-containing bath solution. The pipette solution contained high $[Na^+]$ in these experiments, and the measured currents were consistent with the movement of three or more Na^+ out of the cell in exchange for one Ca^{2+} entering (i.e., electrogenic transport). Cells transfected with NCX1.1 routinely exhibited currents in the range of 40–80 pA, whereas control-transfected cells showed no appreciable steady-state currents. Averaged and normalized data (Fig. 1 *B*) indicate at least a 50-fold increase in current magnitude for NCX1.1- versus control-transfected cells. NCX1.1 activity has been shown previously to require the presence of "regulatory" Ca^{2+} on the cytoplasmic face of the membrane (Hilgemann et al., 1992). Fig. 1, *A* and *B*, illustrates that this phenomenon can be observed in NCX1.1-transfected HEK-293 cells, as no significant external Ca^{2+}-induced currents could be measured when cytosolic $[Ca^{2+}]$ was reduced below 0.5 nM by the inclusion of 5 mM EGTA in the pipette. This result is further confirmation that the currents we observed in NCX1.1-transfected cells arose from the unequal movement of charge through the Na^+/Ca^{2+} exchanger. Fig. 1, *C* and *D*, also illustrates that NCX1.1 currents of reproducible magnitude could be elicited repeatedly in a single cell, hence allowing sequential solution switches to be employed in the experiments to be described below.

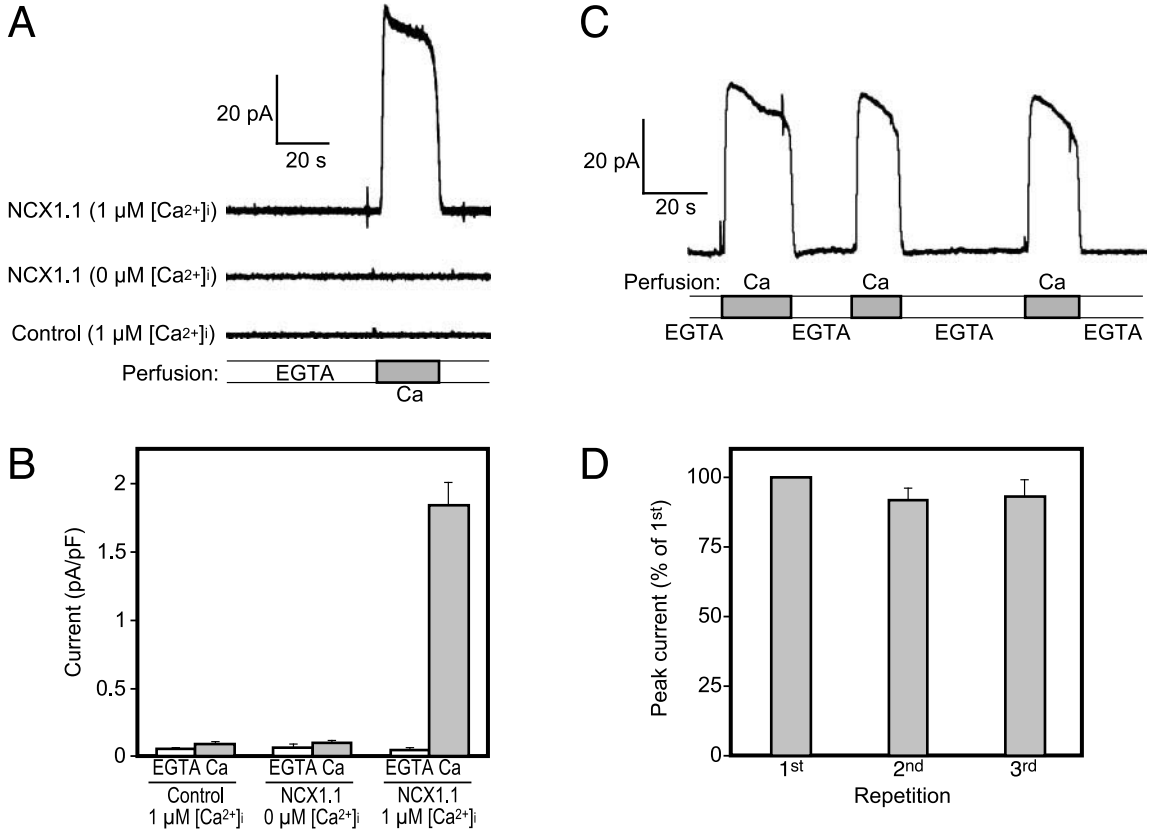

FIGURE 1 NCX1.1 outward currents in HEK-293 cells are $[Ca^{2+}]_I$-dependent and can be elicited repetitively. (*A*) Typical traces from HEK-293 cells transfected with either vector (control) or rat heart NCX1.1 cDNA (NCX1.1) and analyzed by whole-cell patch clamp at a holding potential of 0 mV. The pipette solution contained 120 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 20 mM TEA-Cl, 1 mM Na_2ATP, 8 mM D-glucose, 10 mM HEPES/TMA, pH 7.2, and either 5 mM EGTA (0 μM $[Ca^{2+}]_i$) or 5 mM EGTA plus 4.28 mM $CaCl_2$ (1 μM $[Ca^{2+}]_i$), and the bath solution contained 145 mM LiCl, 1 mM $MgCl_2$, 10 mM D-glucose, 10 mM HEPES/TMA, pH 7.4, and either 0.5 mM EGTA (EGTA) or 1 mM $CaCl_2$ (Ca). (*B*) Summary data of normalized current amplitudes obtained from transfected cells perfused with either EGTA or Ca solution as shown in *A*. The data represent the average ± SEM from 6 (control, 1 μM $[Ca^{2+}]_i$), 5 (NCX1.1, 0 μM $[Ca^{2+}]_i$), or 13 (NCX1.1, 1 μM $[Ca^{2+}]_i$) cells. (*C*) Typical trace from an NCX1.1-transfected HEK-293 cell illustrating the reproducibility of currents elicited by sequential perfusion switches, as described in *A*. (*D*) Summary data from five cells, showing the magnitude of the peak current, normalized to the first repetition, from three sequential perfusion switches each separated by 30–60 s. Error bars indicate SEM; there is no statistical difference between the three peak current values ($p > 0.3$).

The direction of the Na^+/Ca^{2+} exchanger-mediated transport (and hence the direction of current) is dependent on the transmembrane electrochemical gradients of Na^+ ($\Delta\mu_{Na}$) and Ca^{2+} ($\Delta\mu_{Ca}$) and the number of ions that bind to and are transported through the exchanger (Blaustein and Lederer, 1999). At thermodynamic equilibrium, for a transporter that couples the movement of n_{Na} Na^+ ions in exchange for n_{Ca} Ca^{2+} ions, the relation between the electrochemical gradients for Na^+ and Ca^{2+} is given by:

$$n_{Na}\Delta\mu_{Na} = n_{Ca}\Delta\mu_{Ca} \quad (1)$$

The electrochemical gradient for each ion of valence z is related to the concentration of the ion on the outside and inside of the cell ($[Ion]_o$ and $[Ion]_i$, respectively) and the membrane potential, E_m, as follows:

$$\Delta\mu_{Ion} = 2.303RT\log([Ion]_0/[Ion]_i) - zFE_m, \quad (2)$$

where R, T, and F are the gas constant, absolute temperature, and Faraday's constant, respectively. Equation 2 can be used to expand Eq. 1 by substituting for both $\Delta\mu_{Na}$ and $\Delta\mu_{Ca}$. In this special case where the same membrane potential is in equilibrium with both of the ion gradients, and the net work done through the exchanger is zero, the E_m is referred to as the reversal potential for the exchanger, and is denoted E_{NCX}:

$$2.303RTn_{Na}\log([Na^+]_0/[Na^+]_i) - n_{Na}FE_{NCX} = 2.303RTn_{Ca}\log([Ca^{2+}]_0/[Ca^{2+}]_i) - 2n_{Ca}FE_{NCX}$$

This equation can be rearranged to yield:

$$E_{NCX} = \frac{1}{(n_{Na} - 2n_{Ca})}\frac{2.303RT}{F}(n_{Na}\log([Na^+]_0/[Na^+]_i) - n_{Ca}\log([Ca^{2+}]_0/[Ca^{2+}]_i)), \quad (3)$$

where the denominator term, $n_{Na} - 2n_{Ca}$, corresponds to the net number of charges moved per transport cycle. The

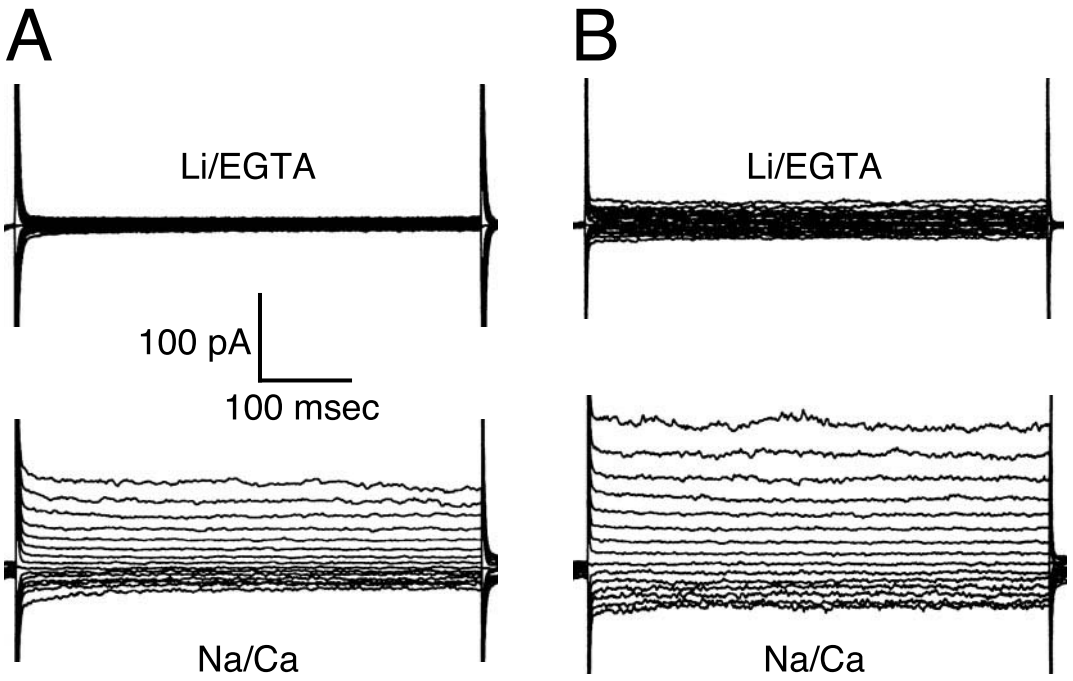

FIGURE 2 NCX1.1 currents generated using a step potential protocol. HEK-293 cells were transfected with rat heart NCX1.1 cDNA and analyzed by whole-cell patch clamp using a holding potential of 0 mV. (*A* and *B*) Each panel shows typical tracings from an individual cell. The pipette solution contained 18 mM NaCl, 100 mM potassium gluconate, 20 mM TEA-Cl, 1 mM Na_2ATP, 10 mM D-glucose, 10 mM HEPES/TMA, pH 7.2, and either 10 mM EGTA and 6.40 mM $CaCl_2$ (0.3 μM free [Ca^{2+}]) in *A* or 10 mM BAPTA and 5.28 mM $CaCl_2$ (0.3 μM free [Ca^{2+}]) in *B*. In both cases the cells were perfused alternately with solutions containing either 125 mM LiCl and 0.5 mM EGTA (Li/EGTA) or 65 mM LiCl, 60 mM NaCl and 0.5 mM $CaCl_2$ (Na/Ca), plus 1 mM $MgCl_2$, 10 mM D-glucose, 20 mM TEA-Cl, and 10 mM HEPES/TMA, pH 7.4. Once the steady-state current had stabilized following the perfusion switch, the cells were subjected to a voltage protocol that consisted of a 50-ms pre-pulse to −20 mV, followed by a 500-ms step pulse to voltages ranging from −80 to +60 mV in 10-mV intervals. These data are representative of at least 10 cells analyzed under each condition. Note that omitting the −20-mV pre-pulse, or employing a range of different [Na^+] in the perfusion solution, had no effect on the shape of currents induced by the step potentials. The capacitance transients have been truncated in this figure.

stoichiometry for either Na^+ or Ca^{2+} can then be determined by measuring E_{NCX} as the external concentration of either ion is varied, while the concentrations of all other ions on both sides of the membrane are held constant. For example, in the case where external [Na^+] is varied, Eq. 3 reduces to:

$$E_{NCX} = \frac{n_{Na}}{(n_{Na} - 2n_{Ca})} \frac{2.303RT}{F} \log([Na^+]_0) + \sum C_i, \quad (4)$$

where C_i are a set of constant terms, one for each constant ion, determined by the stoichiometry and concentration of that ion. A plot of E_{NCX} versus log([Na^+]) will have a slope that is a measure of $n_{Na}/(n_{Na} - 2n_{Ca})$, the number of Na^+ ions that move per unitary charge in each transport cycle. It is noteworthy that the precise concentration of each fixed ion does not need to be known explicitly, as these values will alter only the C_i terms. Thus, so long as the fixed ion concentrations remain constant while the varying ion is changed, the slope of the relation will not be affected. The key assumption in this approach is the notion that the exchanger operates with a fixed stoichiometry (Eq. 1).

The voltage dependence of NCX1.1 activity, measured using solutions compatible with both inward and outward current, was first tested using a series of step potentials, as illustrated in Fig. 2. Following the capacitance transients that were normally less than 20 ms in duration, the elicited currents were stable and essentially time independent over the 500-ms pulse duration. As expected, when cells were exposed to solutions containing Na^+ and Ca^{2+}, the currents elicited by step potentials were significantly larger than those observed during Li/EGTA perfusion. The *I-V* relation of these currents was noticeably outwardly rectifying (a phenomenon also observed using ramp potentials, as illustrated in Figs. 3 and 4), presumably reflecting different concentrations of transported ion and their differential interactions with the exchanger on either side of the membrane as well as the intrinsic voltage dependence of NCX1.1 (Matsuoka and Hilgemann, 1992). The character of these trace families was unaffected by the presence or absence of a prepulse to −20 mV. Having established the stability of the NCX1.1 currents, subsequent experiments employed ramp potentials of 0.5 V/s.

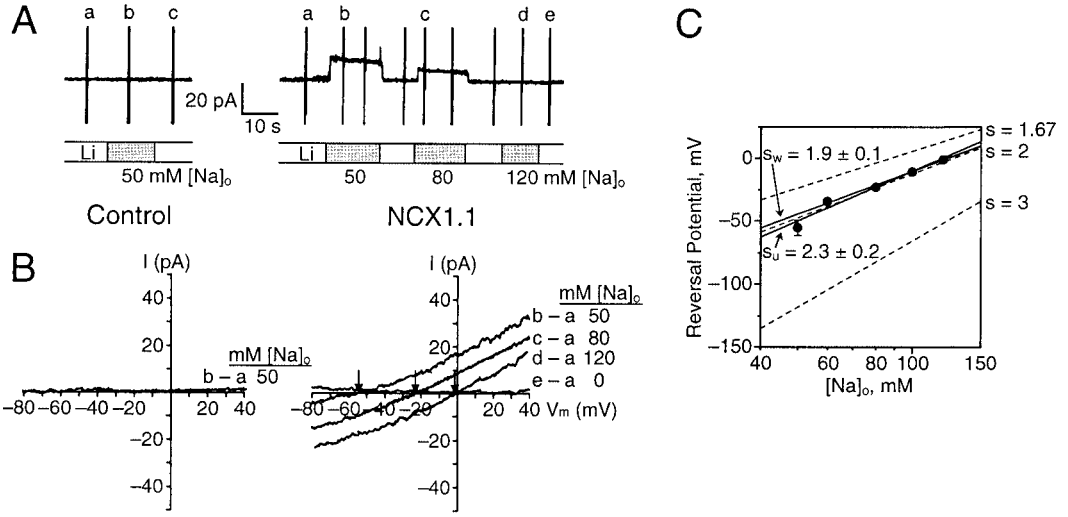

FIGURE 3 Reversal potential determinations for NCX1.1 with varying [Na^+]. HEK-293 cells transfected with either vector (control) or rat heart NCX1.1 cDNA (NCX1.1) were analyzed by whole-cell patch-clamp using a holding potential of 0 mV and a pipette solution as described in the legend for Fig. 2 *A*. Bath perfusion solutions contained 1 mM $MgCl_2$, 10 mM D-glucose, 20 mM TEA-Cl, 10 mM HEPES/TMA, pH 7.4, and either 125 mM LiCl, 0.5 mM EGTA (Li), or various combinations of NaCl (as indicated) and LiCl totaling 125 mM plus 0.5 mM $CaCl_2$. Voltage ramps from −100 to +60 mV were applied during each perfusion and in Li at the start and the end of the experiment. (*A*) Representative current traces for control or NCX1.1-transfected cells subjected to perfusion switches of varying [Na^+]. (*B*) *I-V* curves obtained from the indicated ramps during the perfusions illustrated in *A*, with the *I-V* curve obtained in Li at the start of the experiment (marked *a*) digitally subtracted. Arrows in *B* indicate the reversal potentials recorded for NCX1.1 under the three different [Na^+] perfusions. Note that the regions of the trace from the first and last 40 ms of each ramp, corresponding to the voltage ranges −100 to −80 and +40 to +60, are not shown as the currents in these regions were unstable and/or contaminated with the capacitance transient. (*C*) Summary plot of reversal potential against log of the external [Na^+], determined as illustrated in *A* and *B*. Averages ± SEM for between five and eight determinations at each data point are shown; in some cases the extent of the error is smaller than the symbol size. Solid lines show the fit of these data to Eq. 4, above, determined using either an unweighted algorithm, or one weighted according to the reciprocal of the SEM squared. The stoichiometry (s_u, unweighted, or s_w, weighted; the number of Na^+ moved per net charge, i.e., $n_{Na}/(n_{Na} - 2n_{Ca})$) was extracted from the slope of these fits using a value of 58.5 for the term $2.303(RT/F)$. For comparison, dashed lines show the theoretical relationship described by Eq. 3, above, using the indicated $n_{Na}/(n_{Na} - 2n_{Ca})$ value (*s*) shown on the right side of the panel.

Data from a typical reversal potential experiment in which external [Na^+] was varied are illustrated in Fig. 3. In this experiment, control- or NCX1.1-transfected cells were subjected to voltage-ramp protocols while perfused with bath solutions containing 0.5 mM [Ca^{2+}] and varying [Na^+] (Fig. 3 *A*). The currents observed due to voltage ramps in LiCl/EGTA bath solution (conditions preventing the exchanger from operating in either direction) at the start of the experiment were digitally subtracted from subsequent currents obtained under various conditions, and the resulting *I-V* traces are shown in Fig. 3 *B*. Under these conditions, control-transfected cells did not display any significant Ca^{2+}- and Na^+-activated currents at any potential, although clear and large currents were observed for NCX1.1-transfected cells. The NCX1.1 traces had reversal potentials that depended upon the external [Na^+], as anticipated. Furthermore, subtraction of currents measured in LiCl/EGTA solution before and after the Na^+ perfusion (traces *e–a* in Fig. 3 *B*) resulted in an *I-V* trace that was essentially flat and not different from zero.

The data from several such experiments are summarized in Fig. 3 *C*, in which E_{NCX} has been plotted against log([Na^+]). A fit of this data to Eq. 4, weighted according to the inverse of the standard error squared, yields a slope corresponding to a value for $n_{Na}/(n_{Na} - 2n_{Ca})$ of 1.9 ± 0.1, whereas an unweighted fit gives a value of 2.3 ± 0.2. If n_{Ca} is unity, this implies that n_{Na} is 4 and that 2 charges move per exchanger cycle. For comparison, the dashed lines in Fig. 3 *C* have been calculated from Eq. 3, assuming an n_{Ca} of 1 and n_{Na} values of 5, 4, or 3 (hence resulting in $n_{Na}/(n_{Na} - 2n_{Ca})$ values of 1.67, 2, or 3, respectively). The slope derived from the fit of the Na^+ data is significantly different from the slope of the $n_{Na} = 3$ function ($p < 0.05$) but not significantly different from those of $n_{Na} = 4$ or 5.

Conceptually identical reversal potential experiments were conducted using perfusion solutions in which extracellular [Ca^{2+}] was varied, and the data are presented in Fig. 4. In this case, the $n_{Ca}/(n_{Na} - 2n_{Ca})$ obtained from the fit of the data was 0.45 ± 0.03. This value is not significantly different from the theoretical relation where 1 Ca^{2+} is transported per 2 charges moved (an $n_{Ca}/(n_{Na} - 2n_{Ca})$ value of 0.5) but is significantly different from either 1 Ca^{2+} per 1 charge (an $n_{Ca}/(n_{Na} - 2n_{Ca})$ value of 1; $p < 0.001$) or 1 Ca^{2+} per 3 charges (an $n_{Ca}/(n_{Na} - 2n_{Ca})$ value of 0.33; $p < 0.03$). Both sets of reversal potential data are thus internally and uniquely consistent with a stoichiometry

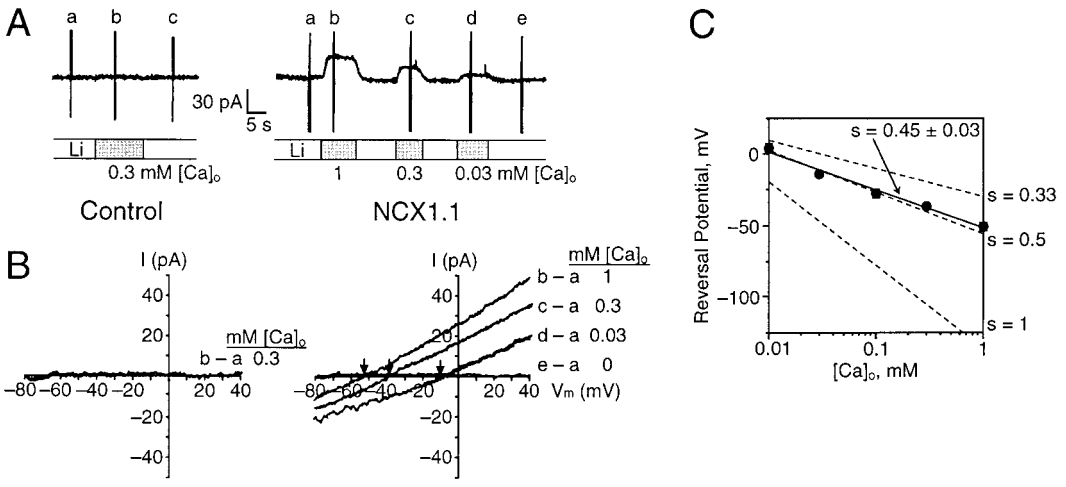

FIGURE 4 Reversal potential determinations for NCX1.1 with varying [Ca^{2+}], determined exactly as described in the legend for Fig. 3, above, except that the perfusion solutions contained 50 mM NaCl, 75 mM LiCl and varying [$CaCl_2$] (buffered with 10 mM EGTA in the range of 10–100 μM, and unbuffered above that range) in place of the varying [Na^+]. The stoichiometry (s, the number of Ca^{2+} moved per net charge, i.e., $n_{Ca}/(n_{Na} - 2n_{Ca})$) was extracted from the slope of an unweighted fit using a value of 58.5 for the term 2.303(RT/F). A weighted fit did not significantly change the calculated value for s. For comparison, dashed lines show the theoretical relationship described by Eq. 3, above, using the indicated $n_{Ca}/(n_{Na} - 2n_{Ca})$ value (s) shown on the right side of the panel.

model for NCX1.1 transport of 4 Na^+ in exchange for 1 Ca^{2+}, accompanied by the movement of 2 positive charges. Note that not only are the fitted slopes of the data indistinguishable from this model but also that the data points themselves fall very close to the theoretical relation. It is also clear that these data are significantly different from a stoichiometry model of 3 Na^+ to 1 Ca^{2+} to 1 charge transported.

Fujioka et al. (2000) suggested that NCX1.1 stoichiometry might vary depending upon the precise concentration of Na^+ or Ca^{2+} present on the cytoplasmic side of the membrane. Therefore, we repeated the reversal potential experiments varying extracellular [Na^+] at several different intracellular concentrations of both Na^+ and Ca^{2+}. The data from these experiments are summarized in Fig. 5 and indicate that neither varying [Na^+] from 2.5 to 60 mM, nor [Ca^{2+}] from 0.3 to 5 μM, had any significant effect on the calculated $n_{Na}/(n_{Na} - 2n_{Ca})$ value. Furthermore, the choice of EGTA or BAPTA as intracellular Ca^{2+} buffering agent had no impact on the calculated stoichiometry data either (Fig. 5 *B*). In all cases the $n_{Na}/(n_{Na} - 2n_{Ca})$ value was not significantly different from 2 but was significantly different from 3 ($p < 0.03$ in all cases), consistent with a stoichiometry model of 4 Na^+ per two charges transported for each enzyme cycle, independent of intracellular ionic conditions.

Several experiments have been performed to establish that control over ionic conditions, particularly close to the cytoplasmic face of the membrane, was very good during these studies. First, there was no difference in reversal potential measurements between experiments where intracellular (pipette) [Ca^{2+}] was controlled with EGTA or with BAPTA, which is a much faster chelator (Fig. 5 *B*). We have also demonstrated that no detectable NCX1.1 current was observed when intracellular (pipette) Ca^{2+} was reduced below 0.5 nM, even though the extracellular perfusion solution contained 1 mM $CaCl_2$ (Fig. 1). Together, these data support the fact that [Ca^{2+}] was well controlled, even in the sub-membrane space adjacent to the exchanger.

Second, reversal potential measurements were obtained under several different conditions from both ascending and descending ramps. Significant current, and hence ion movement, is driven by the exchanger at the extremes of voltage used in the ramps (see Figs. 3 and 4). As the direction of ion movement is different at the two extremes, one would expect to observe reversal potentials that differed with the direction of the voltage ramp if ion movement resulted in poor control of the sub-membrane ion concentrations. This was never the case. Measured at external [Ca^{2+}] of 30, 100, and 300 μM, the reversal potentials ± SEM for three determinations from ascending versus descending ramps were −6.3 ± 0.3 mV versus −10 ± 2 mV, −27 ± 4 mV versus −30 ± 2 mV, and −42 ± 3 mV versus −38 ± 1 mV, respectively.

Third, voltage ramps were performed at two different times following the activation of NCX1.1 steady-state currents by perfusion switch. Once again, if operation of the exchanger caused ion movement resulting in poor control of ionic concentration, the reversal potential would be expected to change with time. This was not observed. Voltage ramps were imposed 2 s and then again 7 s following NCX1.1 activation by perfusion switches to either 30 or 300 μM [Ca^{2+}]. The measured reversal potentials ± SEM were −14 ± 3 mV versus −14 ± 3 mV ($n = 4$) and −37 ± 2 mV versus −36 ± 1 mV ($n = 2$), respectively.

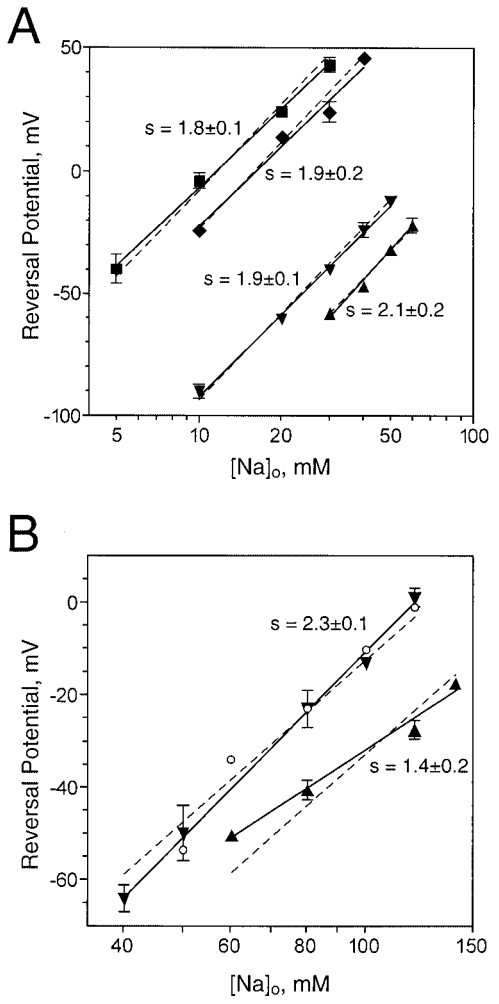

FIGURE 5 Stoichiometry determinations under differing intracellular conditions. Plots of reversal potential against log of the external $[Na^+]$, obtained as described in the legend to Fig. 3, are shown. (*A*) Perfusion solutions contained 0.5 mM $CaCl_2$ and varying [NaCl] (supplemented with LiCl to a total of 125 mM). Pipette solutions contained various $[Na^+]$ (substituted with K^+) and $[Ca^{2+}]$, prepared using a 10 mM EGTA buffering system: ■, 2.5 mM Na^+, 1 μM Ca^{2+}_{free}; ◆, 2.5 mM Na^+, 0.3 μM Ca^{2+}_{free}; ▼, 20 mM Na^+, 5 μM Ca^{2+}_{free}; ▲, 20 mM Na^+, 1 μM Ca^{2+}_{free}. Averages ± SEM for between three and six determinations at each data point are shown (except for ■ at 30 mM $[Na^+]$, where $n = 2$, and ◆ at 40 mM $[Na^+]$, where $n = 1$); in some cases the extent of the error is smaller than the symbol size. Solid lines show the fit of these data to Eq. 4, above. The stoichiometry (s; the number of ions moved per net charge, i.e., $n_{Na}/(n_{Na} - 2n_{Ca})$) ± SEM extracted from the slope of these fits using a value of 58.5 for the term $2.303(RT/F)$ is shown for each data set. For comparison, dashed lines show the theoretical relationship described by Eq. 3, above, using an $n_{Na}/(n_{Na} - 2n_{Ca})$ value of 2. (*B*) As above, except the pipette solutions used a 10 mM BAPTA buffering system to control $[Ca^{2+}]$. ▲, perfusate contained 0.1 mM $CaCl_2$, and pipette contained 60 mM Na^+ and 1 μM Ca^{2+}_{free}; ▼, perfusate contained 0.5 mM $CaCl_2$, and pipette contained 20 mM Na^+ and 0.3 μM Ca^{2+}_{free}. Averages ± SEM for either three or four determinations at each data point are shown. For comparison, the data from Fig. 3 *C* are superimposed (○).

Fourth, voltage ramps were initiated from different holding potentials, in case holding potential altered the sub-membrane ionic conditions. Using an 80 mM $[Na^+]$ perfusion, reversal potentials ± SEM of −25 ± 2 mV versus −23 ± 2 mV ($n = 2$) were obtained from holding potentials of either 0 mV or −20 mV, respectively. Because these measured potentials were not significantly different, we conclude that holding potential did not interfere with the control of ionic concentration.

Fifth, to establish that internal dialysis was adequate, voltage ramps were conducted at sequential points separated by 2 min after membrane rupture and entry into the whole-cell patch mode. The resulting reversal potentials ± SEM measured during an 80 mM $[Na^+]$ perfusion were −23 ± 3 mV and −23 ± 2 mV ($n = 3$), respectively. Once again, as the measured reversal potentials were not dependent upon intracellular dialysis time, we conclude that dialysis was adequate and that control over ionic concentration was good.

Sixth, conditions were chosen that allowed large outward currents when extracellular $[Ca^{2+}]$ was high (3 mM), and small inward currents when extracellular $[Ca^{2+}]$ was low (30 μM). During the high $[Ca^{2+}]$ perfusion, the outward currents will move a significant amount of Ca^{2+} into the cell via the exchanger. If control over the sub-membrane ionic environment was poor, this Ca^{2+} should accumulate and then drive a transient inward current larger than anticipated upon a perfusion switch to low $[Ca^{2+}]$. The expected observation would be an inward current that relaxed from a high to a low value, upon a bath perfusion switch from 3 mM to 30 μM $[Ca^{2+}]$. This phenomenon was never observed, suggesting that control over buffered Ca^{2+} was very good even close to the membrane in these experiments.

Seventh, the influence of either 1 or 10 mM KCl on reversal potentials measured in the presence of 80 mM $[Na^+]$ was determined. The values obtained (reversal potential ± SEM for four determinations) were −21 ± 2 mV versus −23 ± 4 mV. Thus, K^+ addition was without significant effect, indicating that there was no significant contamination of NCX1.1 currents with K-channel currents and confirming that NCX1.1 does not depend upon or transport K^+.

These controls indicate that we have very precise and accurate control over ionic concentration in our configuration of the whole-cell patch clamp technique using HEK-293 cells and that the measured currents were due to operation of the Na^+/Ca^{2+} exchanger in isolation of all other membrane currents. Consequently, we were able to determine an accurate stoichiometry for NCX1.1 of 4 Na^+ transported in exchange for 1 Ca^{2+}, accompanied by the movement of 2 positive charges. This result is consistent with the recent findings of Matsuoka and colleagues using membrane macro-patches from cardiac myocytes (Fujioka et al., 2000).

DISCUSSION

In this study we have used a thermodynamic approach to measure the thermodynamic property of ionic stoichiometry for the Na^+/Ca^{2+} exchanger, NCX1.1. The success of these experiments rested upon two requirements: 1) that we were able to isolate pure NCX1.1-mediated current and 2) that there was precise control (though it need not have been accurate) over ionic concentrations, particularly those close to the intracellular side of the membrane. As indicated above, NCX1.1 was expressed at high levels in HEK-293 cells and produced large currents that were absent from control-transfected cells. Under the ionic conditions used in this study, HEK-293 cells did not display significant membrane conductance. The subsequent high signal-to-noise ratio was essential for the success of the experiments. Furthermore, the HEK-293 cells are relatively small (the average capacitance of the cells used in these studies was 33 pF), which allowed excellent control over intracellular ionic composition via the patch pipette. We have performed several different control experiments to establish that the ionic conditions close to the membrane were those defined by the pipette solution and were maintained during the course of our experiments. It is also important to note that because our assay was an electrical one, we measured only operation of NCX1.1 in the Na-Ca exchange mode and thus ignored fluxes due to Na-Na exchange or Ca-Ca exchange modes, which undoubtedly occur under conditions close to equilibrium.

It is conceivable that during perfusion with solutions of differing composition, the ionic concentrations under the membrane also change, irrespective of exchanger function. For example, in the experiments of Fig. 3, changing the perfusate from zero $[Na^+]$, zero $[Ca^{2+}]$ to high $[Na^+]$, high $[Ca^{2+}]$ may induce changes in $[Na^+]$ and $[Ca^{2+}]$ under the membrane. If the sub-membrane $[Na^+]$ had increased through the range of external $[Na^+]$ employed, then a slope consistent with a stoichiometry of 4:1 would have been obtained even for an exchanger that operated with a 3:1 stoichiometry. A similar argument can be used to explain the data of Fig. 4. Indeed, a set of internally consistent numbers that satisfy the conditions of both Figs. 3 and 4 can be calculated. For Fig. 3, these values are $[Ca^{2+}] = 12\ \mu M$ and $[Na^+]$ ranging from 27 to 36 mM; for Fig. 4, the values are $[Na^+] = 27$ mM and $[Ca^{2+}]$ ranging from 1.8 to 21 μM. Although we cannot formally exclude the possibility of changing sub-membrane ionic concentrations, several pieces of data argue strongly against it. First, and as elaborated above, it is highly unlikely that sub-membrane $[Ca^{2+}]$ changes to the extent required, as we saw no current when pipette $[Ca^{2+}]$ was below 0.5 nM (Fig. 1) and no difference in reversal potential when BAPTA was used instead of EGTA as the Ca^{2+} buffering agent (Fig. 5 *B*). Second, we have also conducted the reversal potential experiments using a pipette $[Na^+]$ of 60 mM, thus reducing the gradient across the membrane needed to alter sub-membrane $[Na^+]$. This condition did not reveal a lower stoichiometry (Fig. 5 *B*). Third, if we use the values for sub-membrane concentrations calculated here and apply them to the data for NCKX2 stoichiometry obtained recently in our laboratory under virtually identical conditions (Dong et al., 2001), then a Na^+ stoichiometry of between 2 and 3 Na^+ per net charge moved is implied. As it is clear from our data as well as from those of others (Dong et al., 2001; Sheng et al., 2000; Szerencsei et al., 2001) that NCKX2 is electrogenic and transports both Ca^{2+} and K^+, such a low Na^+ stoichiometry would either not be compatible with the electrogenic nature of NCKX2 transport or would require 5 or more Na^+ to move (together with 2 or more positive charges in exchange for 1 Ca^{2+} and 1 K^+). Either of these scenarios seems highly unlikely. Consequently, we believe the idea that varying sub-membrane ionic concentrations can explain our data with a 3:1 NCX1.1 stoichiometry model to be untenable.

The conclusion from this study is that NCX1.1 operates with a stoichiometry of 4 Na^+ in exchange for 1 Ca^{2+}, resulting in the movement of 2 positive charges per transport cycle. Such a model was first proposed by Mullins (1977) to account for control of Ca^{2+} in the nanomolar range inside the squid axon in the face of known Na^+ gradients. This model was also given recent support from careful electrophysiological studies, conceptually similar to those used here, performed on membrane patches isolated from cardiac myocytes (Fujioka et al., 2000). In the more than two decades that has intervened, however, a number of careful studies have appeared that clearly support a model of 3 Na^+ to 1 Ca^{2+} to 1 charge transported. These experiments have been performed in various systems, including barnacle muscle cells, squid axon, brain synaptosomes, and cardiac myocytes, and are based on the measurement of intracellular ionic concentrations (Axelsen and Bridge, 1985; Crespo et al., 1990; Sheu and Fozzard, 1985), ion fluxes (Blaustein and Russell, 1975; Bridge and Bassingthwaighte, 1983; Pitts, 1979; Rasgado-Flores et al., 1989; Reeves and Hale, 1984; Szerencsei et al., 2001; Wakabayashi and Goshima, 1981), charge movement (Bridge et al., 1990), and reversal potential of the current mediated by NCX1.1 (Ehara et al., 1989; Kimura et al., 1987). The obvious question that needs to be addressed, if the NCX1.1 stoichiometry is really 4 Na^+ to 1 Ca^{2+}, is why did all these studies arrive at a lower estimate of 3:1?

Several potential explanations seem evident. In those studies that employed whole-cell patch clamp to measure reversal potential, as we have done, the control over intracellular sub-membrane ion concentrations is critical. Unlike HEK-293 cells, ventricular myocytes are much larger, with typical capacitances in the range of 150–250 pF. These cells additionally have a complex intracellular architecture of myofibrils, mitochondria, and sarcoplasmic reticulum. Ionic diffusion may therefore be a limiting problem, particularly

during voltage-ramp protocols that induce large transmembrane ion fluxes, as implied by the time-dependent shift in reversal potential noted by Ehara et al. (1989). Ascending voltage ramps would then be expected to increase intracellular Na^+ and decrease intracellular Ca^{2+} slightly, which would have the effect of making the reversal potential more negative than predicted, leading to the conclusion of a reduced stoichiometry. A further problem in these experiments is the possible presence of contaminating conductances that might also lead to a reduced apparent stoichiometry. Based on the control experiments we have performed, we believe it unlikely that either of these possibilities is a concern for our determinations of reversal potential in HEK-293 cells. Of note, under the conditions used in our study, possible contamination of NCX1.1 currents with Ca^{2+}-activated Cl^- currents would have had the consequence of decreasing the apparent stoichiometry, rather than increasing it.

Studies based on the measurement of intracellular ionic concentrations or quantitative ion fluxes can be seriously affected by parallel pathways for ion movement, particularly for Ca^{2+}. Thus, if a significant fraction of measured overall Ca^{2+} flux is through non-exchanger pathways, the coupling ratio will be underestimated. Such pathways are generally relatively abundant in the muscle and nerve preparations used to measure Na^+/Ca^{2+} exchanger activity. As there is no truly selective inhibitor for the Na^+/Ca^{2+} exchanger, it is often difficult to be sure that parallel pathways do not interfere with the measurements. Indeed, it has recently been suggested that the magnitude of Ca^{2+} movement through the sarcolemmal Ca^{2+}-ATPase has been underestimated (Choi and Eisner, 1999). Furthermore, estimates of stoichiometry based on steady-state ion concentrations are reliable only when they are measured at equilibrium, which is rarely the case.

Two studies stand out as requiring additional comments. The first (Reeves and Hale, 1984) used a thermodynamic equilibrium approach to $^{45}Ca^{2+}$-flux measurements in cardiac sarcolemmal vesicles. The Na^+ gradient across the vesicle membrane was varied until it balanced an electrical gradient generated by K^+ and valinomycin, so that net Ca^{2+} flux was zero. The authors demonstrated that under their conditions, the only voltage-driven Ca^{2+} flux was Na^+ dependent, thus ruling out all parallel pathways for Ca^{2+} movement. They also found that their data were stable over the time interval from 1 to 5 s, suggesting the established membrane potential was not being dissipated at a significant rate. From these experiments, they obtained a stoichiometry of 3 Na^+ to 1 Ca^{2+}. In these very carefully controlled experiments, no direct estimate of the membrane potential was made. Thus, although perhaps unlikely, it remains possible that the actual membrane potential was less than the assumed value. Such a scenario would have led to an underestimation of the Na^+:Ca^{2+} stoichiometry.

The second study was a recent one, in which bovine NCX1.1 was expressed in insect cells and stoichiometry determined by a quantitative comparison of Na^+ release and $^{45}Ca^{2+}$ uptake into Na^+-loaded cells over time (Szerencsei et al., 2001). Flux determination in a recombinant cell system that provides a high signal-to-noise ratio is a very direct method to determine stoichiometry that relies upon very few assumptions, except that only Na^+/Ca^{2+} exchange is being measured. Although perhaps unlikely, the existence of alternative pathways for Ca^{2+} flux (for example, Ca^{2+}-Ca^{2+} exchange through NCX1.1) would have led to an underestimation of the true NCX1.1 stoichiometry.

A possible explanation for these differences in the determined stoichiometry value may lie in the difference in experimental methodology. The experiments of Reeves and Hale (1984) and Szerencsei et al. (2001) both employ radioactive $^{45}Ca^{2+}$ flux to measure NCX1.1 function, and deduced a 3:1 stoichiometry. Our experiments and those of Fujioka et al. (2000) employ net electrical current to measure NCX1.1 function, and deduced a 4:1 stoichiometry. A satisfactory mechanism to account for such a methodological difference, however, is currently not clear.

Another explanation for the different measured NCX stoichiometries may be that the number of Na^+ ions required to activate transport via the exchanger is not fixed. Matsuoka and colleagues (Fujioka et al., 2000), while arguing for a stoichiometry of 4 Na^+ to 1 Ca^{2+}, noted that when intracellular [Na^+] dropped below ~10 mM, the measured stoichiometry seemed to drop toward 3:1. As these determinations were based on single data points under conditions where the power to discriminate between a Na^+ stoichiometry of 3 or 4 was low, it is difficult to be certain of the conclusions. We have also tested this idea by determining the reversal potential at a series of different extracellular [Na^+], under different intracellular conditions. The data from these experiments (Fig. 5) suggest that NCX1.1 operates with a fixed stoichiometry of 4 Na^+ to 1 Ca^{2+} regardless of the intracellular [Na^+] or [Ca^{2+}].

Nevertheless, the Na^+,K^+-ATPase has been suggested to operate with an altered stoichiometry under conditions where the Na^+ binding site is only partially occupied by its cognate substrate (i.e., when [Na^+] is well below its K_M value) and possibly occupied instead by competing cations, such as protons especially when solution pH drops (Blostein and Polvani, 1992). Such variable ion-binding-site occupancy may lead to a variety of measured stoichiometries, depending upon the conditions and the measurement technique. Although we favor a fixed stoichiometry model for NCX1.1 of 4 Na^+ to 1 Ca^{2+} to 2 charges moved under close-to-physiological conditions, our experiments were all conducted at constant pH (intracellular pH of 7.2, extracellular pH of 7.4) using Li^+ as the extracellular cationic substitute for Na^+, whereas K^+ was used intracellularly. It is possible that if other substituting cations and/or conditions of lower pH were employed with NCX1.1, then exchange

function might be measured when the Na^+ sites were (partially) occupied by other cations (Egger and Niggli, 2000), possibly resulting in an altered determination of stoichiometry, similar to observations for the Na^+,K^+-ATPase.

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