**Na\(^+\)-Ca\(^{2+}\) Exchange Current and Submembrane [Ca\(^{2+}\)] During the Cardiac Action Potential**

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**Abstract**—Na\(^+\)-Ca\(^{2+}\) exchange (NCX) is crucial in the regulation of [Ca\(^{2+}\)], and cardiac contractility, but key details of its dynamic function during the heartbeat are not known. In the present study, we assess how NCX current (\(I_{\text{NCX}}\)) varies during a rabbit ventricular action potential (AP). First, we measured the steady-state voltage and [Ca\(^{2+}\)], dependence of \(I_{\text{NCX}}\) under conditions when [Ca\(^{2+}\)] was heavily buffered. We then used this relationship to infer the submembrane [Ca\(^{2+}\)], \([\text{Ca}^{2+}]_{\text{sm}}\), sensed by NCX during a normal AP and [Ca\(^{2+}\)], transient (when the AP was interrupted to produce an \(I_{\text{NCX}}\) tail current). The [Ca\(^{2+}\)], dependence of \(I_{\text{NCX}}\) at \(-90\) mV allowed us to convert the peak inward \(I_{\text{NCX}}\) tail currents to \([\text{Ca}^{2+}]_{\text{sm}}\). Peak \([\text{Ca}^{2+}]_{\text{sm}}\) measured via this technique was >3.2 μmol/L within <32 ms of the AP upstroke (versus peak [Ca\(^{2+}\)], of 1.1 μmol/L at 81 ms measured with the global Ca\(^{2+}\) indicator indo-1). The voltage and [Ca\(^{2+}\)], dependence of \(I_{\text{NCX}}\) allowed us to infer \(I_{\text{NCX}}\) during the normal AP and Ca\(^{2+}\) transient. The early rise in \([\text{Ca}^{2+}]_{\text{sm}}\) causes \(I_{\text{NCX}}\) to be inward for the majority of the AP. Thus, little Ca\(^{2+}\) influx via NCX is expected under physiological conditions, but this can differ among species and in pathophysiological conditions. (Circ Res. 2002;90:103940)

**Key Words:** Na\(^+\)-Ca\(^{2+}\) exchanger ■ action potential ■ rabbit ■ calcium

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The Na\(^+\)-Ca\(^{2+}\) exchanger (NCX) of cardiac sarcolemma, transports 3 Na\(^+\) for 1 Ca\(^{2+}\), is the main mechanism of Ca\(^{2+}\) extrusion from ventricular myocytes, and contributes to relaxation.\(^1\) NCX exhibits a thermodynamically defined reversal potential (E\(_{\text{NCX}}\)) analogous to those of ion channels (eg, E\(_{\text{K}}\) and E\(_{\text{Cl}}\)). Based on the transport stoichiometry, E\(_{\text{NCX}}\)=3E\(_{\text{Na}}\)-2E\(_{\text{Ca}}\). Thus, when the membrane potential (E\(_{\text{m}}\)) exceeds E\(_{\text{NCX}}\), Ca\(^{2+}\) entry (outward \(I_{\text{NCX}}\)) is favored. The exact role of Ca\(^{2+}\) influx via outward \(I_{\text{NCX}}\) during the cardiac action potential (AP) is controversial, but it can contribute to sarcoplasmic reticulum (SR) Ca\(^{2+}\) loading and Ca\(^{2+}\)-induced Ca\(^{2+}\) release from the SR.\(^2\) NCX increases quickly, even before fluorescent [Ca\(^{2+}\)] indicators sense a change in bulk [Ca\(^{2+}\)]. During spontaneous SR release at \(-80\) mV, Trafford et al observed a 133-ms lag between the submembrane compartment containing \(I_{\text{NCX}}\) and bulk [Ca\(^{2+}\)].

Our goals were to measure the \([\text{Ca}^{2+}]_{\text{sm}}\) sensed by NCX during a normal AP and Ca\(^{2+}\) transient and to determine the time course and amplitude of inward and outward \(I_{\text{NCX}}\) during the AP. We hypothesize that local elevations in \([\text{Ca}^{2+}]_{\text{sm}}\) due to \(I_{\text{NCX}}\) or SR Ca\(^{2+}\) release occurring near NCX proteins, will limit Ca\(^{2+}\) influx via outward \(I_{\text{NCX}}\) during the majority of the AP, such that \(I_{\text{NCX}}\) is primarily a Ca\(^{2+}\) extrusion mechanism in the cardiac myocyte.

Our method combined both experimental data and modeling. First, we measured the steady-state dependence of \(I_{\text{NCX}}\) on [Ca\(^{2+}\)], and E\(_{\text{m}}\) under conditions when [Ca\(^{2+}\)], was heavily buffered (ie, \([\text{Ca}^{2+}]_{\text{sm}}=[\text{Ca}^{2+}]\)). We then used this relationship and the fact that \(I_{\text{NCX}}\) can be measured at a fixed E\(_{\text{m}}\) as a bioassay for \([\text{Ca}^{2+}]_{\text{sm}}\) during a normal Ca\(^{2+}\) transient and AP at 37°C. Cells were voltage-clamped with an AP waveform,
which was interrupted at different times by hyperpolarization to reveal \( I_{\text{NCX}} \) tail currents.\(^{11}\) The time course of \([\text{Ca}^{2+}]_{\text{im}}\) during an AP, combined with our knowledge of the \( E_{\text{Na}} \) and \([\text{Ca}^{2+}]_{\text{im}}\) dependence of \( I_{\text{NCX}} \), allowed us to infer \( I_{\text{NCX}} \) during an AP.

Our results suggest that \([\text{Ca}^{2+}]_{\text{im}}\) reaches \( >3.2 \) mmol/L, peaking \( <32 \) ms after the AP upstroke. This limits outward \( I_{\text{NCX}} \) to the first \( \approx 19 \) ms of the AP, and it is largely driven by depolarization before \([\text{Ca}^{2+}]_{\text{im}}\) has reached its peak. These data indicate that during almost the entire rabbit cardiac cycle \( I_{\text{NCX}} \) extrudes \([\text{Ca}^{2+}]_{\text{im}}\).

Materials and Methods

Cell Isolation, Voltage Clamping, and \([\text{Ca}^{2+}]_{\text{im}}\) Measurement

Rabbit myocytes were isolated, currents (whole-cell patch clamp), and \([\text{Ca}^{2+}]_{\text{im}}\) (using indo-1 \(-K^+\)-salt) were measured as previously described.\(^{6}\)

Steady-State \( I_{\text{NCX}} \) Dependence on \([\text{Ca}^{2+}]_{\text{im}}\) and \( E_{\text{m}} \)

Cells were pretreated for 12 minutes with Tyrode’s solution containing (mmol/L) CsCl 40, cesium glutamate 80, MgCl 1, glucose 10, and HEPES 5, pH 7.4 with NaOH. Bath Tyrode’s solution (after patch rupture) contained 30 mmol/L KCl. Unlike the experiments above, SR function was not blocked in these experiments.

A typical rabbit AP, recorded in current clamp with physiological solutions at 1 Hz and 37°C, was driven by hyperpolarization to reveal \( I_{\text{NCX}} \) tail currents. \(^{10}\) The AP clamp was interrupted at 10, 25, 50, 100, 200, 300, and 400 ms by hyperpolarization to \(-90 \) mV, allowing uncomplicated \( I_{\text{NCX}} \) tail current recordings. The interrupted AP templates were also inverted for P8 capacitance subtraction. The AP clamp interrupted at 10 ms was always recorded before loading the SR with \([\text{Ca}^{2+}]_{\text{i}}\) by conditioning pulses (below). This gave the tail current component that was independent of SR \([\text{Ca}^{2+}]_{\text{i}}\) load. This current, attributable to \( I_{\text{Ca,deact}} \) deactivation at \(-90 \) mV, decayed monexponentially with \( \tau_{\text{deact}} \approx 1 \) ms, and was not apparent at interruptions \( \approx 25 \) ms. Subsequently, cells were conditioned with ten 200-ms depolarizations to \(+40 \) mV at 1 Hz and then either a full or interrupted AP was applied, followed by the respective P8 protocol.

Data Acquisition and Analysis

Currents and fluorescence signals were recorded using PClamp6.4 and PClamp8 software (Axon Instruments). All experiments were performed at 37°C.

\( I_{\text{NCX}} \) Steady-State Relationship

\( I_{\text{NCX}} \) was represented as described previously.\(^{6}\)

\[
I_{\text{NCX}}(t) = \frac{V_{\text{max}}(\text{Na}^{+})[\text{Ca}^{2+}]_{\text{i}}e^{0.5t} - [\text{Na}^{+}]_{\text{i}}[\text{Ca}^{2+}]_{\text{i}}e^{-0.5t}}{1 + (K_{\text{NaNCX}}/[\text{Ca}^{2+}]_{\text{i}}^2)}
\]

\[
K_{\text{NaNCX}}[\text{Na}^{+}] + K_{\text{NaNCX}}[\text{Ca}^{2+}]_i + K_{\text{NaNCX}}[\text{Ca}^{2+}]_i/[1 + (\text{Ca}^{2+})/K_{\text{Ca}^{2+}}(1 + (\text{Na}^{+})/K_{\text{Na}^{+}})]
\]

\(
1 + K_{\text{Ca}^{2+}}e^{-0.5t}
\)

\( K_{\text{NaNCX}} \), values are the \( \text{Na}^{+} \) and \( \text{Ca}^{2+} \) dissociation constants for intracellular (i) and extracellular (o) \( \text{Na}^{+} \) and \( \text{Ca}^{2+} \). \( \eta \) is the position of the energy barrier of NCX in the membrane electric field, \( k = \text{F/RT} \), and \( k_{\text{sat}} \) is a factor that controls the saturation of \( I_{\text{NCX}} \) at negative \( E_{\text{m}} \). Fixed parameters were as follows: \( K_{\text{NaNCX}} = 125 \) mmol/L, \( K_{\text{Na}} = 87.5 \) mmol/L, \( K_{\text{NaNCX}} = 12.3 \) mmol/L, \( K_{\text{Ca}} = 3.6 \) mmol/L, \( K_{\text{Ca}} = 3.6 \) mmol/L, \( K_{\text{Mo}} = 130 \) mmol/L, \( k_{\text{max}} = 0.32 \), \( \eta = 0.27 \), and \( T=37^\circ\text{C} \). \( V_{\text{max}}(\text{in A/F}) \) varied and was determined for each cell. When \( \text{Ni}^{2+} \) block was not recorded, a linear leak component was also included in \( I_{\text{NCX}} \) fits. Equation 1 was used in steady-state conditions \( ([\text{Ca}^{2+}]_{\text{i}} = [\text{Ca}^{2+}]_{\text{i}}) \) to solve for \( V_{\text{max}} \) and \([\text{Na}^{+}]_{\text{i}} \) for measured \( I_{\text{NCX}} \) and \( E_{\text{m}} \). In the interrupted AP clamp experiments, it was used to predict \([\text{Ca}^{2+}]_{\text{i}}\) at each AP interruption, from the measured \( I_{\text{NCX}} \).

Results

\( I_{\text{NCX}} \) Steady-State Relationship

Figure 1 shows the steady-state \( I_{\text{NCX}} \) dependence on \([\text{Ca}^{2+}]_{\text{i}}\), and \( E_{\text{m}} \) using voltage ramps where \([\text{Ca}^{2+}]_{\text{i}}\), was heavily buffered. \([\text{Ca}^{2+}]_{\text{i}}\), was gradually driven up by outward \( I_{\text{NCX}} \) at \( E_{\text{m}} = -440 \) mV between ramps (Figure 1A) but did not change during a single ramp (Figure 1D). Figures 1B through 1D show the first, fifth, and tenth ramps recorded from a single myocyte using this protocol. Figures 1E and 1F show that the \( \text{Ni}^{2+} \)-sensitive \( I_{\text{NCX}} \) data are well fit by Equation 1, either as the \( E_{\text{m}} \) dependence of \( I_{\text{NCX}} \) at different \([\text{Ca}^{2+}]_{\text{i}}\), or as the \([\text{Ca}^{2+}]_{\text{i}}\), dependence of \( I_{\text{NCX}} \) at different \( E_{\text{m}} \). We repeated this protocol on cells loaded with SBFI and found that \([\text{Na}^{+}]_{\text{i}}\), also decreased by several mmol/L over a similar time course as \([\text{Ca}^{2+}]_{\text{i}}\), rose to \( >1 \) mmol/L (data not shown). In Figure 1, integration of \( I_{\text{NCX}} \) over the entire 46 seconds indicates net influx of 645 mmol/L total \( \text{Ca}^{2+} \) into the cell (and extrusion of 2 mmol/L \( \text{Na}^{+} \)), as \([\text{Ca}^{2+}]_{\text{i}}\), rises by 852 mmol/L. Although this strong \( \text{Ca}^{2+} \) buffering is somewhat less than expected for the pipette solution, it allows characterization of \( I_{\text{NCX}} \) under relatively static conditions. It is also in reasonable agreement with the measured \([\text{Na}^{+}]_{\text{i}}\), decline (above) and the inferred \([\text{Na}^{+}]_{\text{i}}\), changes based on \( E_{\text{m}} \) and \([\text{Ca}^{2+}]_{\text{i}}\), measurements at successive ramps. There was large cell-to-cell variation in \( V_{\text{m}} \) \( V_{\text{m}} = 17.8 \) A/F±4.8 SEM; range: 3.6 to 33.7; n=14), but other parameters of Equation 1 did not need to be varied. Thus, Equation 1 can be used with confidence in subsequent experiments to describe the \([\text{Ca}^{2+}]_{\text{i}}\), and \( E_{\text{m}} \) dependence of \( I_{\text{NCX}} \).
Determination of \([\text{Ca}^{2+}]_{\text{sm}}\) During an AP

Equation 1 describes \(I_{\text{NCX}}\) as a function of static \([\text{Ca}^{2+}]_{\text{i}}\) but also allows us to infer the \([\text{Ca}^{2+}]_{\text{sm}}\) that drives \(I_{\text{NCX}}\) during a normal \(\text{Ca}^{2+}\) transient. Note that fluorescent indicators cannot detect \([\text{Ca}^{2+}]_{\text{i}}\) elevations local to the NCX molecules and that \([\text{Ca}^{2+}]_{\text{sm}}\) does not refer to a physical volume that we can describe. Rather, it is the average \([\text{Ca}^{2+}]_{\text{i}}\), sensed by all NCX molecules in the cell (see Discussion). Figure 2 shows the voltage protocol, \(I_{\text{NCX}}\) tail currents, and \([\text{Ca}^{2+}]_{\text{i}}\) for determining \([\text{Ca}^{2+}]_{\text{sm}}\) during an AP. The AP clamp interruptions produce different inward \(I_{\text{NCX}}\) tails. For clarity, and to illustrate how hyperpolarization accelerates \([\text{Ca}^{2+}]_{\text{i}}\) decline, only three \([\text{Ca}^{2+}]_{\text{i}}\) transients are shown for AP interruptions (Figure 2C).

Figure 3A shows \(I_{\text{NCX}}\) as a function of \([\text{Ca}^{2+}]_{\text{i}}\), for the record in Figure 2. At early time points, \(I_{\text{NCX}}\) far exceeds the value that would be expected from the global \([\text{Ca}^{2+}]_{\text{i}}\) (which is shown as the steady-state [SS] curve for this cell). This reflects the fact that \([\text{Ca}^{2+}]_{\text{sm}}\) sensed by NCX is much higher than bulk \([\text{Ca}^{2+}]_{\text{i}}\). We used the peak value of \(I_{\text{NCX}}\) immediately upon hyperpolarization to indicate \([\text{Ca}^{2+}]_{\text{sm}}\) at the moment of hyperpolarization. Inward \(I_{\text{NCX}}\) usually rises during the initial few milliseconds, probably reflecting the noninstantaneous transition to \(-90\,\text{mV}\). For earlier interruptions, however, it may also represent an actual rise in \([\text{Ca}^{2+}]_{\text{sm}}\) during continued SR \(\text{Ca}^{2+}\) release. Because we cannot differentiate between these two possibilities, we recorded both the time and the value of \(I_{\text{NCX}}\) at its peak (see Discussion: Sources of Error). The steady-state relationship for \(I_{\text{NCX}}\) versus \([\text{Ca}^{2+}]_{\text{i}}\), for this cell (SS curve) is defined by the region where all traces of \(I_{\text{NCX}}\) versus \([\text{Ca}^{2+}]_{\text{i}}\) converge (at 300 ms) and is fit by Equation 1 with a leak offset.

\[ V_{\text{max}} \] values obtained in this manner (\(V_{\text{max}} = 10.6\,\text{A/F} \pm 2.5\,\text{SEM} \); range = 3.0 to 23.1; \(n = 8\)) were not significantly different from values obtained under buffered conditions. For each
AP interruption, the [Ca\(^{2+}\)]\(_{\text{sm}}\) is inferred by extending the peak \(I_{\text{NCX}}\) value to the SS curve (arrows in Figure 3A). Figure 3B shows [Ca\(^{2+}\)]\(_{\text{sm}}\) together with [Ca\(^{2+}\)]\(_{\text{i}}\). Variations in [Ca\(^{2+}\)]\(_{\text{sm}}\) were not correlated with variations in \(V_{\text{max}}\).

**Determination of \(I_{\text{NCX}}\) During an AP**

Figure 4B shows pooled [Ca\(^{2+}\)]\(_{\text{sm}}\) values (n=8 cells) calculated as in Figure 3 and an average [Ca\(^{2+}\)]\(_{\text{i}}\) transient, during the representative full-length AP (Figure 4A). Peak [Ca\(^{2+}\)]\(_{\text{sm}}\) is 3.2 \(\mu\text{mol/L}\) at 32 ms. Figure 4C shows \(I_{\text{NCX}}\) based on [Ca\(^{2+}\)]\(_{\text{i}}\) and \(E_{\text{m}}\) as predicted by Equation 1, using the average \(V_{\text{max}}\) value of these same cells and 10 mmol/L \([\text{Na}^{+}]\) if we use [Ca\(^{2+}\)], to define \(I_{\text{NCX}}\). We predict outward \(I_{\text{NCX}}\) for 142 ms of the AP. If we use [Ca\(^{2+}\)]\(_{\text{sm}}\), \(I_{\text{NCX}}\) is inward after only 19 ms. The dotted lines show \(I_{\text{NCX}}\) calculated using a linear extrapolation of [Ca\(^{2+}\)]\(_{\text{sm}}\) to [Ca\(^{2+}\)]\(_{\text{rest}}\) (at 2 ms). Figure 4D shows currents from Figure 4C integrated and converted to cumulative \([\text{Ca}^{2+}]\) (using 6.4 \(\text{pF}/\text{pL}\) cytosol).\(^{13}\) When [Ca\(^{2+}\)], is used to calculate \(I_{\text{NCX}}\), 285 ms are needed to extrude the 4.4 \(\mu\text{mol/L}\) Ca\(^{2+}\) that entered via outward \(I_{\text{NCX}}\). When [Ca\(^{2+}\)]\(_{\text{sm}}\) is used to calculate \(I_{\text{NCX}}\), only 0.6 \(\mu\text{mol/L}\) Ca\(^{2+}\) enters the cell, and all of this Ca\(^{2+}\) has been extruded by 90 ms. Therefore, at 1 Hz, >98% of the cardiac cycle is spent with \(I_{\text{NCX}}\) functioning to extrude Ca\(^{2+}\), which enters predominantly through \(I_{\text{Ca}}\).

**Simple Method to Transform [Ca\(^{2+}\)]\(_{\text{i}}\) to [Ca\(^{2+}\)]\(_{\text{sm}}\)**

It is not always possible to measure [Ca\(^{2+}\)]\(_{\text{sm}}\) as we have done here. Therefore it may be useful to predict how [Ca\(^{2+}\)]\(_{\text{sm}}\) may change during cellular Ca\(^{2+}\) transients. Trafford et al.\(^{10}\) using spontaneous [Ca\(^{2+}\)] transients, transformed [Ca\(^{2+}\)], to [Ca\(^{2+}\)]\(_{\text{sm}}\).

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**Figure 2.** Protocol to determine [Ca\(^{2+}\)]\(_{\text{sm}}\) during an AP. A, AP was interrupted and clamped to −90 mV at the indicated times in ms. B, \(h_{\text{NCX}}\) tails at −90 for the protocol in panel A. C, [Ca\(^{2+}\)], for 10-, 100-, and 400-ms AP interruptions. Data were smoothed using empirical fits.

**Figure 3.** \(I_{\text{NCX}}\) is driven by [Ca\(^{2+}\)]\(_{\text{i}}\). A, \(I_{\text{NCX}}\) versus [Ca\(^{2+}\)]\(_{\text{i}}\) from Figure 2. Multiple traces intersect the steady-state (SS) \(I_{\text{NCX}}\) versus [Ca\(^{2+}\)] relationship for this cell (Equation 1: \(V_{\text{max}}=17.3 \text{ A/F}, [\text{Na}^{+}]=10 \text{ mmol/L}, E_{\text{m}}=-90 \text{ mV}, \) including a leak offset of 0.59 A/F). [Ca\(^{2+}\)], was smoothed. Arrows illustrate extrapolation from peak inward \(I_{\text{NCX}}\) to [Ca\(^{2+}\)]\(_{\text{sm}}\) (superimposed with [Ca\(^{2+}\)]\(_{\text{i}}\) in panel B).
Figure 4. Pooled data. A, $E_m$ during an AP. B, Mean $[Ca^{2+}]_{sm}$ calculated as in Figure 3 (n=8); $[Ca^{2+}]_{sm}$ represents an average of 5 cells. Broken lines in panels B and C represent linear extrapolations of $[Ca^{2+}]_{sm}$ to $[Ca^{2+}]_{rest}$ (at 2 ms). Data empirically smoothed (see Materials and Methods). C, $I_{NCX}$ for $[Ca^{2+}]_i$ (thin trace) or $[Ca^{2+}]_{sm}$ (thick trace) and $E_m$, using Equation 1 ($V_{max}=10.6$ A/F, $[Na^+]=10.0$ mmol/L). D, Integrated $I_{NCX}$ in panel C converted to $\Delta[Ca^{2+}]_{tot}$.13
using a single time constant for diffusion between cytosol and submembrane space:

\[ [\text{Ca}^{2+}]_{\text{sm}} = [\text{Ca}^{2+}]_i \cdot \gamma \frac{d[\text{Ca}^{2+}]/dt}{t^2} \]

where \( \gamma = 133 \pm 47 \) ms. When applied to our data, Equation 2 described the rising phase of \([\text{Ca}^{2+}]_{\text{sm}}\) adequately (in our case with \( \gamma = 110 \) ms), but the \([\text{Ca}^{2+}]_{\text{sm}}\) declined too rapidly (Figure 5A). More comprehensive mathematical compartment models are surely needed to describe \([\text{Ca}^{2+}]_{\text{sm}}\) (Figure 5A). More comprehensive mathematical compartment models are surely needed to describe \([\text{Ca}^{2+}]_{\text{sm}}\) (after careful characterization of the \([\text{Ca}^{2+}]_{\text{sm}}\), and \( \text{E}_m \) dependence of \( I_{\text{NCX}} \)).

**Figure 5. Transfer function expressing \([\text{Ca}^{2+}]_{\text{sm}}\) in terms of \([\text{Ca}^{2+}]_i\).** A. Thick trace: \([\text{Ca}^{2+}]_i + \gamma \frac{d[\text{Ca}^{2+}]/dt}{t^2}\); thin trace: decay of \([\text{Ca}^{2+}]_{\text{sm}}\) after peak \( d[\text{Ca}^{2+}]/dt \) (\( t = 24.5 \) ms) represented empirically as \([\text{Ca}^{2+}]_{\text{sm}} = [\text{Ca}^{2+}]_i + \text{Aexp}(-t/\tau)\). B. Thick trace: \( \gamma \frac{d[\text{Ca}^{2+}]/dt}{\tau} \) (\( \gamma = 110 \) ms); thin trace: \( \text{Aexp}(-t/\tau) \), where \( A = 2770 \) nmol/L, \( \tau = 92 \) ms, and \( t_0 = 24.5 \) ms.

**Discussion**

Our results assess rather directly the actual \( I_{\text{NCX}} \) that flows during a normal rabbit ventricular myocyte AP. Because NCX can produce both \( \text{Ca}^{2+} \) influx and \( \text{Ca}^{2+} \) efflux, this type of information is essential in the understanding of \( \text{Ca}^{2+} \) and force regulation in cardiac muscle. This importance is reinforced by recent work suggesting that increased NCX expression and altered NCX function contribute to contractile dysfunction and arrhythmias in pathophysiological conditions.15–18

\( I_{\text{NCX}} \) is inward during the majority of the normal rabbit ventricular AP. In demonstrating this, we also showed how \( I_{\text{NCX}} \) can be used as a bioassay for local \([\text{Ca}^{2+}]_{\text{sm}}\) (after careful characterization of the \([\text{Ca}^{2+}]_{\text{sm}}\), and \( \text{E}_m \) dependence of \( I_{\text{NCX}} \)). During the AP, \([\text{Ca}^{2+}]_{\text{sm}}\) peaks earlier and higher than \([\text{Ca}^{2+}]_i\). This high \([\text{Ca}^{2+}]_{\text{sm}}\) drives \( I_{\text{NCX}} \) inward by 19 ms into the AP. Before this time, outward \( I_{\text{NCX}} \) may be driven by membrane depolarization as \([\text{Ca}^{2+}]_{\text{sm}}\) rises.

**Steady-State Relationship for \( I_{\text{NCX}} \)**

We used the \( I_{\text{NCX}} \) equation published by Weber et al6 to describe \( \text{E}_{\text{m}} \), \([\text{Na}^{+}]\), and \([\text{Ca}^{2+}]\) dependence of \( I_{\text{NCX}} \) under conditions when \([\text{Ca}^{2+}]_i\) is not changing appreciably. The \( \text{E}_m \) dependence of \( I_{\text{NCX}} \) in Equation 1 is the same as in the Luo and Rudy1 equation for \( I_{\text{NCX}} \), but the electrochemical component of our equation considers both intracellular and extracellular ion dependencies at the transport sites as well as an allosteric \( \text{Ca}^{2+} \) activation factor. In our previous study,8 we characterized allosteric \( \text{Ca}^{2+} \) activation in ferret myocytes (\( K_{\text{CaAl}} = 125 \) nmol/L), and we see evidence of similar \( \text{Ca}^{2+} \) activation in rabbit myocytes using similar protocols (not shown). The present study illustrates that Equation 1 (including both the allosteric \( K_{\text{CaAl}} = 125 \) nmol/L and \( \text{Ca}^{2+} \) transport \( K_{\text{CaTransport}} = 3.6 \) \( \mu \)mol/L) describes very well the \( \text{E}_m \) and \([\text{Ca}^{2+}]\) dependence of \( I_{\text{NCX}} \) (Figure 1).

**Comparison to Earlier Studies**

Our peak inward \( I_{\text{NCX}} \) was 1.2 A/F, occurring roughly at the end of AP phase 3 repolarization (Figure 4). This peak value is close to that predicted by Luo and Rudy9 in guinea pig at a similar degree of AP repolarization. However, their published model does not consider \([\text{Ca}^{2+}]_{\text{sm}}\) as sensed by the NCX or binding of \( \text{Ca}^{2+} \) to internal transport or allosteric sites. The Luo and Rudy model has outward \( I_{\text{NCX}} \) lasting \( \approx 100 \) ms. We believe \( I_{\text{NCX}} \) should be driven inward much
earlier (≈19 ms), as a result of elevated \([\text{Ca}^{2+}]_{\text{sm}}\). If we use \([\text{Ca}^{2+}]_{\text{sm}}\) instead of \([\text{Ca}^{2+}]_{\text{im}}\), we predict \(I_{\text{NCX}}\) to turn inward at 143 ms, comparable with Luo and Rudy.\(^9\)

Several studies have used pharmacological techniques to characterize \(I_{\text{NCX}}\). Grantham and Cannell\(^7\) measured \(I_{\text{NCX}}\) during AP clamp by applying nifedipine rapidly after conditioning the cell with a train of APs with \(I_{\text{Ca}}\) unblocked. They recorded outward \(I_{\text{NCX}}\) through most of the AP, but the rate of rise and peak of the \([\text{Ca}^{2+}]_{\text{im}}\) transient were markedly decreased in the presence of nifedipine. It follows that \([\text{Ca}^{2+}]_{\text{im}}\) would also be much less than normal, and this would greatly favor more outward \(I_{\text{NCX}}\) during most of the AP. Thus, this type of experiment does not provide data on \(I_{\text{NCX}}\) during a normal AP and \([\text{Ca}^{2+}]_{\text{im}}\) transient. In ferret ventricular APs, Janvier et al\(^8\) showed that AP duration was reduced when \(I_{\text{NCX}}\) was blocked by rapid substitution of external Na\(^+\) with Li\(^+\) or buffering \([\text{Ca}^{2+}]_{\text{im}}\) with BAPTA-AM. While these data might reflect mainly inward \(I_{\text{NCX}}\) during an AP (as we find), it is not easy to quantify \(I_{\text{NCX}}\) in absolute terms using their techniques.

Egan et al\(^1^\) interrupted APs by repolarizing back to resting \(E_m\) and usually found that inward \(I_{\text{NCX}}\) tail currents were largest at interruptions between 50 and 100 ms after the start of the AP. This is later than the average time (32 ms) at which we observed maximal inward \(I_{\text{NCX}}\) tails. They did not measure \([\text{Ca}^{2+}]_{\text{sm}}\), but used an indirect conversion factor (1 μmol · L\(^{-1}\) · nA\(^{-1}\)) based on data of Kimura et al\(^3\) to predict \([\text{Ca}^{2+}]_{\text{sm}}\). Their reported \([\text{Ca}^{2+}]_{\text{sm}}\) peaked at ≈100 ms and ≈1.6 μmol/L, similar to \([\text{Ca}^{2+}]_{\text{im}}\), as we measured with fluorescent indicators (Figure 4). Our data indicate that \([\text{Ca}^{2+}]_{\text{im}}\) peaks earlier (32 ms) and is much higher.

**Sources of Error**

Up to this point, we have assumed that \([\text{Na}^+]\) is constant and equal to pipette \([\text{Na}^+]\) (10 mmol/L). In reality, submembrane \([\text{Na}^+]\), \([\text{Na}^+]_{\text{sm}}\) may be elevated transiently by \(I_{\text{Na}}\) and \(I_{\text{NCX}}\). For example, a 10-ms triangular Na\(^+\) current, peaking at 50 nA, flowing into a submembrane volume equivalent to 2% of cytosolic volume (≈0.6 pL)\(^1\) could increase local \([\text{Na}^+]\), by a maximum of 4 mmol/L (ignoring diffusion from the space). To assess the possible effect of elevated \([\text{Na}^+]_{\text{sm}}\) on our calculated \([\text{Ca}^{2+}]_{\text{sm}}\), we assumed that \([\text{Na}^+]_{\text{im}}\) peaks at >14 mmol/L, 2 ms into the AP, and declines to 10 mmol/L with a tau of 8 ms (Figure 6B). This is an upper limit for \([\text{Na}^+]_{\text{im}}\) because the NCX is not well colocalized with Na\(^+\) channels.\(^1^\) Transient elevations in \([\text{Na}^+]_{\text{im}}\) could reduce inward \(I_{\text{NCX}}\), which would lead us to underestimate \([\text{Ca}^{2+}]_{\text{sm}}\), based on the steady-state relationship for \(I_{\text{NCX}}\), which was always measured after submembrane elevations would have dissipated. Using Equation 1, our first value for \([\text{Ca}^{2+}]_{\text{sm}}\) in Figure 3 (at \(t=17.9\) ms) may be underestimated by 8.1% ((\([\text{Ca}^{2+}]_{\text{sm}}\) = 2021 μmol/L versus 1858 μmol/L using \([\text{Na}^+]_{\text{sm}}\) = 10.0 mmol/L), but subsequent points will not be greatly affected (Figure 6C). Thus, our technique used to determine \([\text{Ca}^{2+}]_{\text{sm}}\) should be relatively insensitive to these \([\text{Na}^+]_{\text{im}}\) changes. This is partly because the electrochemical determinants for inward \(I_{\text{NCX}}\) at ≈90 mV are driven largely by \([\text{Ca}^{2+}]_{\text{im}}\).\(^5\)

On the other hand, elevated \([\text{Na}^+]_{\text{im}}\) can substantially impact predicted outward \(I_{\text{NCX}}\) during an AP. It has been suggested that \(I_{\text{Na}}\) may augment the ability for \(I_{\text{NCX}}\) to trigger SR \([\text{Ca}^{2+}]\) release.\(^2\) Using \([\text{Ca}^{2+}]_{\text{im}}\), \(E_m\), and \([\text{Na}^+]_{\text{im}}\), Figure 6A shows increased outward \(I_{\text{NCX}}\) by 86% for our first data point (Figure 6D). Extrapolating \([\text{Ca}^{2+}]_{\text{im}}\) and \(I_{\text{NCX}}\) to earlier times (dotted traces) would predict even greater outward \(I_{\text{NCX}}\). This, however, does not confirm the ability of \(I_{\text{NCX}}\) to trigger SR \([\text{Ca}^{2+}]\) release (see Physiological Implications).

Our protocol for measuring \([\text{Ca}^{2+}]_{\text{sm}}\) involves large voltage steps to ≈90 mV from \(E_m\) during an AP that cannot be instantaneous. We usually observe about a 7-ms delay between our desired hyperpolarization and the measured peak

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**Figure 6.** Elevated \([\text{Na}^+]_{\text{im}}\) raises both predicted \([\text{Ca}^{2+}]_{\text{sm}}\) and \(I_{\text{NCX}}\) during an AP. A, \(E_m\) during an AP. B, Hypothetical \([\text{Na}^+]_{\text{im}}\) and \([\text{Na}^+]\). C, \([\text{Ca}^{2+}]_{\text{sm}}\) using \([\text{Na}^+]_{\text{im}}\) or \([\text{Na}^+]\). D, \(I_{\text{NCX}}\) for \([\text{Na}^+]_{\text{im}}\) or \([\text{Na}^+]\), and \(E_m\) using Equation 1 (\(V_{\text{max}}=10.6\) A/F). Broken lines in panels C and D represent linear extrapolations of \([\text{Ca}^{2+}]_{\text{sm}}\) to \([\text{Ca}^{2+}]_{\text{im}}\) at 2 ms.
inward $I_{\text{NCX}}$. During hyperpolarization, the driving force ($E_m - E_{\text{NCX}}$) for inward $I_{\text{NCX}}$ will increase as the true $E_m$ reaches the commanded value ($\sim 90$ mV). Because inward $I_{\text{NCX}}$ flows during this transition, $[\text{Ca}^{2+}]_{\text{sm}}$ is necessarily different from that at the instant hyperpolarization started, and therefore, our values of $[\text{Ca}^{2+}]_{\text{sm}}$ are likely to be underestimated. To estimate this possible error, we linearly extrapolated $I_{\text{NCX}}$ from its measured inward peak back to the exact time of hyperpolarization. For the record in Figure 2B, $[\text{Ca}^{2+}]_{\text{sm}}$ based on extrapolated $I_{\text{NCX}}$ (using 40 ms of data after the peak), was on average 14% higher for interruptions up to 200 ms, but not much different for later interruptions, where the voltage steps were smaller and $[\text{Ca}^{2+}]_{\text{sm}}$ was lower. Thus, a 7-ms delay and an underestimation of $[\text{Ca}^{2+}]_{\text{sm}}$ imply that the real $I_{\text{NCX}}$ during an AP is less outward than indicated in Figures 4C and 6D.

Allosteric Ca$^{2+}$ activation is taken as an instantaneous process in Equation 1. However, delayed activation could contribute to the slow rise in $I_{\text{NCX}}$ tail currents. This will be particularly true for the earlier values of $I_{\text{NCX}}$ recorded immediately after SR release, when $I_{\text{NCX}}$ is changing from about half activation to full activation. This could have caused us to underestimate the rate of rise and peak $[\text{Ca}^{2+}]_{\text{sm}}$ and could also explain why the curves in Figure 3A diverge from one another. However, the steepness (supralinear) of the early interruption curves (versus SS) cannot be attributed to allosteric regulation.

Taking elevated $[\text{Na}^{+}]_{\text{sm}}$ noninstantaneous hyperpolarization, and noninstantaneous kinetics of allosteric activation together, all could cause us to underestimate the peak $[\text{Ca}^{2+}]_{\text{sm}}$ reported in Figure 4 and overestimate the time at which it is achieved. Thus $[\text{Ca}^{2+}]_{\text{sm}}$ probably reaches $\geq 3.5$ mmol/L in $< 25$ ms during an AP in rabbit ventricular myocytes. This would shift $I_{\text{NCX}}$ inward even earlier than indicated in Figure 4.

**Physiological Implications**

Our results suggest that submembrane elevations in $[\text{Ca}^{2+}]_{\text{sm}}$ drive $I_{\text{NCX}}$ to function predominantly as a Ca$^{2+}$ efflux mechanism throughout the cardiac cycle in rabbit (Figure 4). If $[\text{Ca}^{2+}]_{\text{sm}}$ is used to predict $I_{\text{NCX}}$, outward $I_{\text{NCX}}$ flows for 142 ms, bringing 4.4 mmol/L Ca$^{2+}$ into the cell. An additional 152 ms is required just to extrude the portion of Ca$^{2+}$ that entered on $I_{\text{NCX}}$. At a physiological frequency of 2 Hz, this would leave only $\approx 200$ ms for $I_{\text{NCX}}$ to extrude the $\approx 10$ mmol/L Ca$^{2+}$ entry that occurs on $I_{\text{Ca}}$. This seems implausible, even with the help of the sarcolemmal Ca$^{2+}$ pump. When $[\text{Ca}^{2+}]_{\text{sm}}$ is used in Equation 1 to predict $I_{\text{NCX}}$, all of the Ca$^{2+}$ that enters via outward $I_{\text{NCX}}$ is extruded by 90 ms, leaving plenty of time for inward $I_{\text{NCX}}$ to extrude the additional Ca$^{2+}$ that entered via $I_{\text{Ca}}$.

With AP depolarization, outward $I_{\text{NCX}}$ may bring Ca$^{2+}$ into the cell for up to $\approx 15$ ms. It is important to realize, however, that these data are based on average $[\text{Ca}^{2+}]_{\text{sm}}$ as sensed by all of the NCX molecules in the sarcolemma. These data do not directly determine $[\text{Ca}^{2+}]_{\text{sm}}$ within a physical compartment, nor does our data directly address the role of $I_{\text{NCX}}$ in triggering SR release. However, the NCX molecules most likely to trigger SR Ca$^{2+}$ release (those closest to the dyadic cleft) would sense even higher $[\text{Ca}^{2+}]_{\text{sm}}$ earlier during the AP, causing $I_{\text{NCX}}$ to become inward even earlier at that location. For example, as soon as an L-type Ca$^{2+}$ channel opens, cleft $[\text{Ca}^{2+}]_{\text{sm}}$ may rise to $> 10$ mmol/L in $< 1$ ms. Furthermore, SR Ca$^{2+}$ release may be activated rapidly ($< 1$ ms) by local $I_{\text{Ca}}$ and $[\text{Ca}^{2+}]_{\text{sm}}$ in the junctional cleft may rise to $\geq 42$ mmol/L in 5 to 10 ms based on computer simulations. Thus, cleft $[\text{Ca}^{2+}]_{\text{sm}}$ may greatly exceed $[\text{Ca}^{2+}]_{\text{sm}}$ and limit the functional ability for outward $I_{\text{NCX}}$ to trigger SR Ca$^{2+}$ release. There are two important counterpoints. First, outward $I_{\text{NCX}}$ carried by exchanger molecules outside the cleft may raise $[\text{Ca}^{2+}]_{\text{sm}}$ enough to slow diffusion of high cleft $[\text{Ca}^{2+}]_{\text{sm}}$, which would enhance the efficacy of an $I_{\text{Ca}}$ trigger for SR Ca$^{2+}$ release. Second, in the latent period before opening of any L-type Ca$^{2+}$ channel at a junction (or if $I_{\text{Ca}}$ fails locally or is blocked), outward $I_{\text{NCX}}$ may become an alternative means to trigger SR release. However, L-type Ca$^{2+}$ channel latency is very brief under normal conditions at positive $E_m$. Moreover, L-type channels are believed to be located close to ryanodine receptors, while NCX molecules may not be.

**Pathophysiological Implications**

Alterations in $[\text{Na}^{+}]_{\text{sm}}$ can profoundly affect $I_{\text{NCX}}$, and species-dependent differences in $[\text{Na}^{+}]_{\text{sm}}$ exist. For example, $[\text{Na}^{+}]_{\text{sm}}$ may be as high as 16 mmol/L in rat and mouse versus 5 to 10 mmol/L in rabbit or guinea pig ventricle. $[\text{Na}^{+}]_{\text{sm}}$ is elevated during hypothyroidism, heart failure (HF) (Despa S, Mohammed AI, Weber CR, Pogwizd SM, Bers DM, unpublished data, 2001), and glycoside therapy. In HF, Ca$^{2+}$ transient amplitude is also decreased and AP duration is prolonged. All three of these changes in HF (higher $[\text{Na}^{+}]_{\text{sm}}$, lower peak $[\text{Ca}^{2+}]_{\text{sm}}$ and longer AP duration) would increase Ca$^{2+}$ influx via $I_{\text{NCX}}$ during the AP. Thus, in HF, Ca$^{2+}$ entry via NCX may be more important than under normal physiological conditions.

Greater Ca$^{2+}$ influx during the AP via $I_{\text{NCX}}$ in HF would leave less time for net Ca$^{2+}$ extrusion via $I_{\text{Ca}}$, which is required to balance the Ca$^{2+}$ influx via $I_{\text{Ca}}$ (and NCX). However, increased NCX expression in HF may act compensatorily to enhance Ca$^{2+}$ extrusion during diastole. In fact, basal SR Ca$^{2+}$ content is reduced in HF (as a result of increased NCX and reduced SR Ca$^{2+}$-ATPase function). Although elevated NCX expression may be compensatory (in limiting Ca$^{2+}$ overload), when Ca$^{2+}$ overload and spontaneous SR release do occur (eg, during $\beta$-adrenergic activation), NCX can also produce more transient inward current, thereby promoting triggered arrhythmias.

**Conclusions**

We used inward $I_{\text{NCX}}$ to predict the time course of $[\text{Ca}^{2+}]_{\text{sm}}$ sensed by the NCX, during a normal rabbit ventricular AP under physiological conditions. Elevated $[\text{Ca}^{2+}]_{\text{sm}}$ (peaking at $> 3.2$ mmol/L) should prevent outward $I_{\text{NCX}}$ within $< 19$ ms of the AP upstroke. Before that time, some outward $I_{\text{NCX}}$ might flow, but much less outward $I_{\text{NCX}}$ will flow on NCX mole-
cules located closer to the dyadic cleft (where local \([\text{Ca}^{2+}]\), would be higher). Although these results suggest limited outward \(I_{\text{NCX}}\) during the majority of the cardiac cycle, it is not yet possible to define the role of NCX in excitation-contraction coupling before SR release. These results emphasize the importance of spatial \([\text{Ca}^{2+}]\), heterogeneities in myocyte \(\text{Ca}^{2+}\) fluxes. Furthermore, the balance of \(\text{Ca}^{2+}\) fluxes on NCX may differ among species and under pathological conditions.

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Na⁺-Ca²⁺ Exchange Current and Submembrane [Ca²⁺] During the Cardiac Action Potential

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