Allosteric Regulation of Na/Ca Exchange Current by Cytosolic Ca in Intact Cardiac Myocytes

Christopher R. Weber,* Kenneth S. Ginsburg,* Kenneth D. Philipson,†
Thomas R. Shannon,* and Donald M. Bers*

From the *Department of Physiology, Loyola University Chicago, Stritch School of Medicine, Maywood, Illinois 60153; and †Cardiovascular Research Lab, University of California Los Angeles School of Medicine, Los Angeles, California 90095

ABSTRACT The cardiac sarcolemmal Na-Ca exchanger (NCX) is allosterically regulated by [Ca], such that when [Ca] is low, NCX current (I_{NCX}) deactivates. In this study, we used membrane potential (E_m) and I_{NCX} to control Ca entry into and Ca efflux from intact cardiac myocytes to investigate whether this allosteric regulation (Ca activation) occurs with [Ca], in the physiological range. In the absence of Ca activation, the electrochemical effect of increasing [Ca] would be to increase inward I_{NCX} (Ca efflux) and to decrease outward I_{NCX}. On the other hand, Ca activation would increase I_{NCX} in both directions. Thus, we attributed [Ca]-dependent increases in outward I_{NCX} to allosteric regulation. Ca activation of I_{NCX} was observed in ferret myocytes but not in wild-type mouse myocytes, suggesting that Ca regulation of NCX may be species dependent. We also studied transgenic mouse myocytes overexpressing either normal canine NCX or this same canine NCX lacking Ca regulation (Δ680–685). Animals with the normal canine NCX transgene showed Ca activation, whereas animals with the mutant transgene did not, confirming the role of this region in the process. In native ferret cells and in mice with expressed canine NCX, allosteric regulation by Ca occurs under physiological conditions (K_{CaAct} = 125 ± 16 nM SEM ≈ resting [Ca])]$. This, along with the observation that no delay was observed between measured [Ca] and activation of I_{NCX} under our conditions, suggests that beat to beat changes in NCX function can occur in vivo. These changes in the I_{NCX} activation state may influence SR Ca load and resting [Ca]$, helping to fine tune Ca influx and efflux from cells under both normal and pathophysiological conditions. Our failure to observe Ca activation in mouse myocytes may be due to either the extent of Ca regulation or to a difference in K_{CaAct} from other species. Model predictions for Ca activation, on which our estimates of K_{CaAct} are based, confirm that Ca activation strongly influences outward I_{NCX}, explaining why it increases rather than declines with increasing [Ca].

KEY WORDS: Na/Ca exchanger • cardiac electrophysiology • ferret • mouse • dog

INTRODUCTION

The cardiac sarcolemmal Na/Ca exchanger (NCX) is a reversible transporter that exchanges three (Reeves and Hale, 1984; Kimura et al., 1987) or possibly four (Fujikawa et al., 2000) Na for one Ca. Outward Na/Ca exchange current (I_{NCX}), or Ca influx, is electrochemically favored by low [Ca]$, high [Na]$, and depolarized membrane potentials. Inward I_{NCX}, or Ca efflux, is favored by the opposite conditions (Mullins, 1979; Bers, 1991). In addition to the electrochemical potential, which sets the direction of I_{NCX}, NCX is allosterically regulated by [Ca], such that when [Ca] is low, I_{NCX} deactivates (Miura and Kimura, 1989). NCX proteins in this state do not contribute to I_{NCX}. Increasing [Ca] can activate NCX, allowing I_{NCX} to flow.

Allosteric Ca binds at two acidic regions in the large cytoplasmic loop of the NCX, each containing three consecutive aspartic acid residues (D446–D448 and D498–D500; Matsuoka et al., 1995). The loop has been shown to bind Ca cooperatively (n_{Hill} ≈ 2) with a K_{Ca} ranging from 0.3 to 3 μM in vitro, depending on the concentration of Mg (0.2–5 mM, respectively; Levstik et al., 1994). α-Chymotrypsin treatment eliminates Ca activation for outward (Hilgemann, 1990; Hilgemann et al., 1992; Matsuoka et al., 1993) and inward I_{NCX} (Kappl and Hartung, 1996) in giant membrane patches. Attempts to measure the affinity for Ca to the activating site of the NCX have given disparate results in different experimental conditions.

Miura and Kimura (1989), using intracellular perfusion to control [Ca], showed Ca-dependent activation of outward I_{NCX} in guinea pig myocytes. Outward I_{NCX} was fully activated at [Ca] ≈ 50 nM (below the normal resting [Ca] of ~100 nM). Fang et al. (1998) measured Ca activation (K_{CaAct} = 44 nM, n_{Hill} = 1.6) of Na-dependent Ba influx in Chinese hamster ovary cells expressing bovine NCX, to assay I_{NCX}. Deletion of a large portion of the cytoplasmic loop (Δ241–680), which contains the putative Ca regulatory domain, eliminated...
variations in Ba influx with changing [Ca], supporting the study by Matsuoka et al. (1993). In contrast, Haworth and Goknur (1991) showed apparent activation of I_{NCX} at physiological [Ca]. Field stimulation of ^{22}Na-loaded rat myocyte suspensions resulted in increased Na pump-independent Na efflux. This suggests that the entry of physiologically relevant amounts of Ca through Ca channels can activate the NCX.

All measurements in giant excised patches so far have shown Ca-dependent regulation at physiological [Ca]. In giant patches from guinea pig myocytes, Ca activation of outward I_{NCX} was seen with K_{mCaAct} = 300–600 nM (Hilgemann et al., 1992). In patches from Xenopus oocytes expressing canine cardiac NCX, mutations of various residues within and near to the two acidic Ca regulatory regions increase K_{mCaAct} from 0.4 μM to between 1.1 and 1.8 μM (Matsuoka et al., 1995). Approximately 180 amino acids downstream of the two acidic DDD segments, amino acids 680–685 (IIEESY in canine NCX1) are also important for Ca activation. Giant patches from transgenic mouse myocytes overexpressing a deletion of these six residues (Δ680-685) show outward I_{NCX}, which is nearly fully activated at all [Ca], (Maxwell et al., 1999).

In this study, we took advantage of the electrogenicity of the NCX to control Ca entry into and Ca efflux from intact cardiac myocytes to measure the effects of changing [Ca] on I_{NCX}. Using solutions that were selective for I_{NCX}, we voltage-clamped single cells at +100 mV to bring Ca into the cell through outward I_{NCX} and at −100 mV to bring Ca out of the cell through inward I_{NCX}. By varying the depolarization duty factor (t_{100 mV}/total t), we were able to directly control [Ca]_i, and at the same time observe the effects of changing [Ca] on I_{NCX}. This study differs from earlier studies in that [Ca]_i was dynamically controlled by a physiological process rather than being artificially clamped to particular values by exogenous buffering. Advantages of our study are as follows: measurements were made in the normal cellular environment; and dynamic changes in [Ca]_i in the physiological range were examined.

We observed clear Ca activation of I_{NCX} in ferret myocytes, but did not detect appreciable regulation in wild-type (WT) mouse myocytes using the same protocols (in contrast to Maxwell et al., 1999). No delay was observed between the rise in [Ca]_i and activation of I_{NCX}. We also studied mouse myocytes overexpressing normal canine NCX (Adachi-Akahane et al., 1997) and mouse myocytes overexpressing this same canine NCX lacking the Ca regulatory domain Δ680-685 (Maxwell et al., 1999). Animals with the normal canine NCX transgene showed Ca activation, whereas animals with the mutant transgene did not. Some of the results shown in this article were presented in abstract form (Weber et al., 1999, 2000).

MATERIALS AND METHODS

Cell Preparation

Cells were isolated using approved methods. Ferrets were anesthetized with sodium pentobarbital (50 mg/kg IP). Hearts were excised, rinsed in ice-cold 0 mM Ca Tyrode’s solution, and placed on a Langendorf apparatus for retrograde constant flow (30–36 ml/min) perfusion of the coronary vasculature via the aorta at 37°C. The heart was perfused for 5 min with 0-mM Ca Tyrode’s solution, after which an otherwise identical solution containing filtered collagenase (Yakult Corp. or Boehringer) as well as 10 μM Ca was perfused. When the heart was slightly flaccid to touch, enzyme activity was stopped by perfusion with 0-mM Ca Tyrode’s solution containing BSA. Ventricles were removed from the apparatus, cut into ~2-mm² size pieces, and gently triturated. After washing in Ca-free solution at room temperature, cells were placed on laminin-coated perfusion chambers for recording. Cells were used within 6 h of isolation.

The preparation of mouse myocytes was modified to reduce the risk of ischemic damage to the hearts. Mice were anesthetized by inhalation with a mixture of 5% isoflurane and 95% O2. Each mouse was tracheotomized and mechanically ventilated. Upon opening the chest cavity, we injected cold high K cardioplegia solution into the vena cava and quickly excised the heart, which was subsequently transferred to a gravity-driven (80 mmHg) apparatus for perfusion at 37°C (flow rate ~ 3 ml/min) and treated as above.

Fluorescent Measurement of [Ca]

Perfusion chambers were mounted on a Nikon TMD epifluorescence microscope. Cells were loaded with 10 μM indo-1 AM, applied externally for 22 min at 23°C, or 100 μM K indo-1, applied through the pipet, in perforated patch and ruptured patch experiments, respectively. We allowed indo-1 AM to deesterify for 20 min before proceeding with [Ca]_i measurements. Cells were excited at 565 ± 5 nm using a 75-W xenon arc lamp via epillumination, and emitted fluorescence (405 ± 10 nm and 485 ± 10 nm) was recorded using photometers (PTI Corp.). For indo-1 AM experiments, a linear regression of autofluorescence at each wavelength versus two-dimensionally projected cell surface area for 10 unloaded cells of the same preparation was used to predict the background to be subtracted from the loaded signal for each cell studied, according to the particular cell’s size. For K indo-1 experiments, the autofluorescence of the cell being studied was always recorded before patch rupture. The ratio (R) of emitted fluorescence at 405 nm to emitted fluorescence at 485 nm was converted to [Ca] using the Grynkiewicz et al. (1985) equation:

\[ [Ca] = K_d \frac{R - R_{min}}{R_{max} - R} \]

where R_{min} (0.24), R_{max} (2.91), and β (4.17) were determined, and K_d was taken as 844 nm as described by Bassani et al. (1995). Cell contraction was apparent under visible light >650 nm provided by transillumination (which was deflected from the fluorescence detector pathway by a short pass dichroic mirror), and was simultaneously recorded using a video edge detection system. This served as an additional indicator of changes in [Ca].

In most mouse myocyte experiments, we used fluo-3 AM, since we were able to attain better Ca signals than with indo-1 AM or K indo-1. 5 μM fluo-3 AM was loaded until fluorescence was three or more times background (up to ~45 min), and a comparable time was allowed for deesterification. In experiments using fluo-3, cells were excited at 480 ± 5 nm, and emission was recorded at 535 ± 20 nm. To convert nonratiometric signals into [Ca], the following pseudoratio equation was used (Cheng et al., 1993):

\[ [Ca] = K_d \cdot R/(K_d/[Ca]_{act} + 1 - R) \]

R was taken as the emitted fluorescence divided by the resting emitted fluorescence, each
after background subtraction, \([Ca]_{rest}\) was taken as 100 nM, and \(K_a = 1,100 \text{ nM} \) was used to calculate \([Ca]_i\). In several experiments with indo-1 AM, when an ideal calibration was not available, we also treated background subtracted indo-1 ratios using the pseudoratio method. There was no apparent qualitative or quantitative difference in results which could be attributed to the different indicator methods used.

**Solutions for Electrophysiological Recording**

To eliminate any contribution of the SR to Ca handling, cells were pretreated for 10 min with 0Ca/0Na Tyrode’s solution containing (in mM): 0.001 thapsigargin, 10 caffeine, 140 LiCl, 6 KCl, 1 MgCl₂, 10 glucose, and 5 HEPES, pH 7.4 with LiOH. Patch electrodes (1–2 MΩ) were tip-dipped and backfilled with solution containing (in mM): 40 CsCl, 80 tetraethylammonium chloride (TEACl), 0.92 MgCl₂, 10 HEPES, 10 NaCl, 5 MgATP, 0.3 LiGTP, and 0.1 K indo-1; pH was set to 7.2 with CsOH and free [Mg] was \(\sim 0.5 \text{ mM}\). In some cases, we used aspartic acid and equimolar CsCl instead of the TEACl. For perforated patch experiments on cells loaded with indo-1 AM or fluo-3 KCl. To secure the best possible voltage control, series resis-
tance was compensated to the extent possible. Additionally, we switched to Tyrode’s solution containing 4 mM CsCl instead of KCl. To secure the best possible voltage control, series resistance was compensated to the extent possible. Additionally, we applied capacitance transient cancellation either during the re-
tention or specifically and to observe correspond-
ing changes in inward and outward INCX magnitudes. For example, with 100 ms at +100 mV and 200 ms at -100 mV in Fig. 2 C (bottom) the duty factor was 0.33. In the four myocyte types studied, INCX was recorded with increasing [Ca], using alternating protocols like that shown in Fig. 2 C (bottom). The duty factor of the voltage protocol was chosen for each cell (0.25–0.67) to adjust net Ca entry into the cell in a range which would vary activation of INCX from minimal to maximal.

**Qualification of Recorded Currents**

To minimize any influence of leakage current, cells were selected where the resting current after initialization to low [Ca], (above) was as low as possible. Under this condition, the state of activation for INCX and the driving force for inward INCX would both be minimized, and any residual inward current would be largely due to leak. INCX was taken as the current blocked by 10 mM Ni. In some cases, when it was not possible to apply Ni to the cell, the records were leak subtracted. For this purpose, we used the linear leak conductance (\(g_{leak}\)) determined from the inward resting current of the initialized cell to determine \(g_{leak} = \frac{I_{leak}}{E_{leak}}\). This method of leak subtraction would define the initial INCX at the lowest resting [Ca], to be zero. This is reasonable, since as cells rest at -100 mV during initialization to low [Ca], INCX would be expected to approach equivalence (where \(E_m = E_{Nax} + 2E_{ca}\)). Some inward INCX will flow to oppose any slow Ca entry into cells (Coulombe et al., 1989), so that in steady state it is not likely to reach zero. Despite this, the actual INCX we measured by Ni subtraction in cells initialized at resting [Ca], was quite close to zero (see example in Fig. 2).

**Simulations and Calculations**

INCX was represented as the product of an electrochemical (\(\Delta E\)) and an allosteric (Allo) factor. The Allo factor is based on Equation IX-141 in Segel (1993), which describes the net reaction velocity (forward – backward) of the ping pong bi bi cyclic reaction scheme. We described the voltage dependence of the velocity in each direction using exponentials as defined by Mullins et al. (1979) and by Luo and Rudy (1994). Segel’s equation was further simplified by assuming that the maximal flux is the same in both directions (\(V_{max} = V_{max} = V_{max}\)) and by making the approximation that the NCX dissociation constants for Cai and Naι from catalytic sites are equivalent to the apparent \(K_a\) for Caι and Naι transport by the NCX when other substrates are present at saturating concentrations (\(K_{sat\,Ca}\) and \(K_{sat\,Na}\)). An instantaneous Hill equation for Ca binding to the regulatory site, and thus availability of the NCX, was used for the Allo factor as shown below:

\[
I_{INCX} = \text{(Allo)}(\Delta E)
\]

\[
\text{Allo} = \frac{1}{1 + \left(\frac{[Ca]_i}{K_{Ca\text{Allo}}}\right)^{h_{\text{hill}}}}
\]

\[
\Delta E = \frac{1}{1 + \left(\frac{[Ca]_i}{K_{Ca\text{Allo}}}\right)^{h_{\text{hill}}}} \left\{ \left[\frac{K_{Na\text{Allo}}[Na]_i}{[Ca]_i}\right]^{\frac{5(V-F)}{RT}} \right\} \rightbrack - [Na]_i^{3}[Ca]_i^{\left(\frac{0-1(V-F)}{RT}\right)}
\]

\[
\frac{\left\{K_{Ca\text{Allo}}[Na]_i + K_{Na\text{Allo}}[Ca]_i + K_{Na\text{Allo}}[Na]_i \right\} \left\{ 1 + \frac{[Ca]_i}{K_{Ca\text{Allo}}} \right\}^{5(V-F)} + \left\{K_{Ca\text{Allo}}[Na]_i^{3} + K_{Na\text{Allo}}[Ca]_i^{3} \right\} \left\{ 1 + \frac{[Ca]_i}{K_{Ca\text{Allo}}} \right\}^{5(V-F)}}
\]

\[
\frac{\left\{1 + \frac{[Ca]_i}{K_{Ca\text{Allo}}} \right\}^{5(V-F)}}{\left\{1 + \frac{[Ca]_i}{K_{Ca\text{Allo}}} \right\}^{5(V-F)}}
\]

\[
(1)
\]
The $K_m$ values are the Na and Ca dissociation constants for intracellular (i) and extracellular (o) Na and Ca.  $K_m$ is a factor that controls the saturation of $I_{NCX}$ at negative potentials. $K_{CaAct}$ for each experiment was determined from curve fits of the $I_{NCX}$ versus $[Ca]_i$ relationship better than the same equation with $K_{CaAct}$ not determined (ND) in cell types when including it did not improve the fits. Fixed parameters are listed in MATERIALS AND METHODS (see Simulations and Calculations).

$[Ca]_t$ was converted to $[Ca]_i$ for every time step taking into account intrinsic cellular buffering (Hove-Madsen and Bers, 1993) as well as buffering by indo-1:

$$[Ca]_t^{2+} = [Ca]_i^{2+} + \frac{215}{1 + \frac{[Ca]_i^{2+}}{79}} + \frac{705}{1 + \frac{[Ca]_i^{2+}}{0.75}} - \frac{50}{1 + \frac{[Ca]_i^{2+}}{0.036}}.$$

The $[Na]_i$ increment for each $\Delta\tau$ was defined by the following equation:

$$\Delta[Na]_i = \frac{-\int_{t-f}^{t+\Delta\tau} I_{NCX}}{3F \cdot \text{Volume}},$$

in which Volume was the myoplasmic volume.

**Data Acquisition and Analysis**

Currents, fluorescence signals, and contraction data were recorded using PClamp6.4 and PClamp8 software (Axon Instruments). Data were analyzed using customized software written in Microsoft Visual Basic 6 and Microsoft Visual Basic for Applications, using GigaSof ProEssentials Scientific Graphing Routines (www.gigasoft.com). Curve fits of the data to the equation for $I_{NCX}$ were performed using the MajesticTM (Logix Consulting, Inc.) add-in for Microsoft Excel. Means and standard deviations of curve fit parameters are presented.

**R E S U L T S**

As described in MATERIALS AND METHODS, we blocked all currents other than $I_{NCX}$ (including $I_{Ca}(L)$, $I_{Na}(L)$, and the Na/K pump) as well as SR function. Thereafter, we regarded Ni-sensitive currents like that in Fig. 2 C as $I_{NCX}$. Thus, we have the fortunate situation that only $I_{NCX}$ has the ability to bring Ca into and out of the cell. We control $I_{NCX}$ directly using our voltage protocols.

*Figure 1.* Computer model of cardiac myocyte containing nonblocked sources of Ca flux, used in Fig. 8. Ca background leak current is defined by constant conductance and electrochemical driving force for Ca. Ca pump follows Michaelis-Menten kinetics, and $I_{NCX}$ is defined by Eq. 1.
Fig. 2 shows a representative record where the response to the same pulse protocol was recorded once in the absence (Fig. 2 A) and once in the presence (Fig. 2 B) of 10 mM Ni to reveal Ni-sensitive difference current, INCX (Fig. 2 C). With the Ni present, the current was relatively small and constant; correspondingly, the cell failed to contract with Ni present (compare bottom panels of Fig. 2, A and B).

Fig. 2 C shows how we established that Ca activation occurred. In the absence of allosteric regulation, the electrochemical effect of increasing $[\text{Ca}]_i$ would be to increase inward INCX but to decrease outward INCX. Ca activation, on the other hand, would increase INCX in both directions. Thus, we looked closely for changes in outward INCX since allosteric regulation opposes electrochemical regulation. Increases in outward $I_{\text{NCX}}$ can only be attributed to allosteric regulation.

Fig. 3 (top, Measured) shows an example of the fluo-3 $[\text{Ca}]_i$ signal measured from a mouse myocyte overexpressing canine NCX1 superimposed with a prediction of $[\text{Ca}]_i$ based on integration of the recorded INCX (Fig. 3 top, Predicted). In this example, the duty factor of outward versus inward $I_{\text{NCX}}$ was 25% (Fig. 3, bottom). As represented by the first term of Eq. 2, we calculated cumulative $[\text{Ca}]_{\text{total}}$ from the integrated $I_{\text{NCX}}$, using the fixed 3:1 Na/Ca $I_{\text{NCX}}$ stoichiometry, and estimated myoplasmic volume (34 pl, based on 13.0 pF/pl cytosol for rat; Satoh et al., 1996). We used a standard cytosolic Ca buffering relationship (Hove-Madsen and Bers, 1993; Eq. 3) to solve for $[\text{Ca}]_i$ at each time point (Fig. 3 top, Predicted). The prediction corresponds reasonably well with $[\text{Ca}]_i$ measured using fluo-3. This provides additional support that the currents we were measuring are indeed $I_{\text{NCX}}$.

Fig. 4 A is an example record from a ferret myocyte ($n = 22$) that shows direct evidence of Ca activation at physiological $[\text{Ca}]_i$. Notice that the magnitude of outward and inward $I_{\text{NCX}}$ both increase with rising $[\text{Ca}]_i$. The increase in outward $I_{\text{NCX}}$ represents Ca activation. It is not due to the change in electrochemical potential because increasing $[\text{Ca}]_i$ decreases the driving force for allosteric Ca influx via outward $I_{\text{NCX}}$. The increase in inward $I_{\text{NCX}}$ represents the combined electrochemical and allosteric effects of increasing $[\text{Ca}]_i$.

**TABLE II**

<table>
<thead>
<tr>
<th>Simulation Parameters Used in Fig. 8</th>
<th>Ca activation</th>
<th>No Ca activation</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_{mCaAct}$</td>
<td>125 NA</td>
<td>NA</td>
</tr>
<tr>
<td>$V_{max} (A/F)$</td>
<td>22.6</td>
<td>13.7</td>
</tr>
<tr>
<td>$[\text{Na}]_i$</td>
<td>7.16</td>
<td>7.10</td>
</tr>
<tr>
<td>$G_{CaKbg} (S/F)$</td>
<td>3.2</td>
<td>5.1</td>
</tr>
</tbody>
</table>

Parameters used in the computer simulation (Fig. 8) NA, not applicable. $K_{mCaAct}$, $V_{max}$ and $[\text{Na}]_i$ were based on fits to ferret data with and without the allosteric factor (Allo). $G_{CaKbg}$ adjusted to give resting $[\text{Ca}]_i = 100$ nM. Fixed parameters are listed in Materials and Methods (see Simulations and Calculations).

Values are measured in nanomolar.

Values are measured in millimolar.

Fig. 2 shows a representative record where the response to the same pulse protocol was recorded once in the absence (Fig. 2 A) and once in the presence (Fig. 2 B) of 10 mM Ni to reveal Ni-sensitive difference current, INCX (Fig. 2 C). With the Ni present, the current was relatively small and constant; correspondingly, the cell failed to contract with Ni present (compare bottom panels of Fig. 2, A and B).

Fig. 2 C shows how we established that Ca activation occurred. In the absence of allosteric regulation, the electrochemical effect of increasing $[\text{Ca}]_i$ would be to increase inward $I_{\text{NCX}}$ but to decrease outward $I_{\text{NCX}}$. Ca activation, on the other hand, would increase INCX in both directions. Thus, we looked closely for changes in outward $I_{\text{NCX}}$ since allosteric regulation opposes electrochemical regulation. Increases in outward $I_{\text{NCX}}$ can only be attributed to allosteric regulation.

Fig. 3 (top, Measured) shows an example of the fluo-3 $[\text{Ca}]_i$ signal measured from a mouse myocyte overexpressing canine NCX1 superimposed with a prediction of $[\text{Ca}]_i$ based on integration of the recorded INCX (Fig. 3 top, Predicted). In this example, the duty factor of outward versus inward $I_{\text{NCX}}$ was 25% (Fig. 3, bottom). As represented by the first term of Eq. 2, we calculated cumulative $[\text{Ca}]_{\text{total}}$ from the integrated $I_{\text{NCX}}$, using the fixed 3:1 Na/Ca $I_{\text{NCX}}$ stoichiometry, and estimated myoplasmic volume (34 pl, based on 13.0 pF/pl cytosol for rat; Satoh et al., 1996). We used a standard cytosolic Ca buffering relationship (Hove-Madsen and Bers, 1993; Eq. 3) to solve for $[\text{Ca}]_i$ at each time point (Fig. 3 top, Predicted). The prediction corresponds reasonably well with $[\text{Ca}]_i$, measured using fluo-3. This provides additional support that the currents we were measuring are indeed $I_{\text{NCX}}$.

Fig. 4 A is an example record from a ferret myocyte ($n = 22$) that shows direct evidence of Ca activation at physiological $[\text{Ca}]_i$. Notice that the magnitude of outward and inward $I_{\text{NCX}}$ both increase with rising $[\text{Ca}]_i$. The increase in outward $I_{\text{NCX}}$ represents Ca activation. It is not due to the change in electrochemical potential because increasing $[\text{Ca}]_i$ decreases the driving force for allosteric Ca influx via outward $I_{\text{NCX}}$. The increase in inward $I_{\text{NCX}}$ represents the combined electrochemical and allosteric effects of increasing $[\text{Ca}]_i$.  

**FIGURE 2.** Protocol used to show regulation of $I_{\text{NCX}}$ by $[\text{Ca}]_i$. K currents, $I_{\text{K}}(\text{Ca})$, $I_{\text{Cl}}(\text{Ca})$, Na/K pump, SR Ca pump blocked. (A) Current and contraction with application of alternating voltage clamp protocol in transgenic mouse myocyte overexpressing canine NCX1. B is the same as A, but with 10 mM Ni present. (C, top) Current in A minus current in B, which represents $I_{\text{NCX}}$. (C, bottom) Alternating voltage clamp protocol applied to the cell: +100 mV for 100 ms and −100 mV for 200 ms.
Fig. 4 B shows an example record made with the same protocol from a WT mouse myocyte \((n = 10)\). Of the 10 records obtained from WT mouse myocytes, only one appeared to show any sign of allosteric Ca regulation (an increase of outward \(I_{\text{NCX}}\)). In 9/10 experiments, the outward \(I_{\text{NCX}}\) decreased. As expected from the purely electrochemical effects of increasing \([\text{Ca}]_i\), the inward \(I_{\text{NCX}}\) increased, although not dramatically in mice (see Fig. 7). Notably, mouse myocytes generally showed less inward \(I_{\text{NCX}}\) when compared with ferret (see Fig. 6). Fig. 4 C shows the response of a mouse myocyte overexpressing canine NCX1 to the alternating pulse protocol \((n = 7)\). There were dramatic increases in both outward and inward \(I_{\text{NCX}}\), which, as we noted above, is strong evidence of allosteric Ca regulation. Fig. 4 D illustrates the same protocol applied to a mouse myocyte overexpressing the \(\Delta 680-685\) mutant of canine NCX1 \((n = 8)\). Deleting these amino acids clearly eliminated the increase in outward \(I_{\text{NCX}}\) we attribute to allosteric Ca regulation. In every experiment, outward \(I_{\text{NCX}}\) decreased similarly as in WT mouse myocytes. Again, inward \(I_{\text{NCX}}\) still increased because of electrochemical considerations.

In Fig. 3, the outward and inward currents decline considerably during respective individual pulses. Such declines in the outward direction were minimal in most of our data (see examples in Fig. 4), especially in ferret.
myocytes. We attribute these declines to transient changes in [Ca\textsubscript{i}] and [Na\textsubscript{i}] during the overall increase in [Ca\textsubscript{i}] in a protocol (see Fig. 8). Declines in inward \(I_{\text{NCX}}\) were also pronounced in mouse myocytes expressing canine NCX, and this may be attributable to the combined effects of [Ca\textsubscript{i}] acting at allosteric and transport sites.

Data like those of Fig. 4 of each experiment where [Ca\textsubscript{i}] was measured were fit to Eq. 1 with and without the allosteric factor. Fig. 5 A shows fits of \(I_{\text{NCX}}\) versus [Ca\textsubscript{i}] for all the ferret myocytes where [Ca\textsubscript{i}] was measured (14/22). As noted in MATERIALS AND METHODS, each of the individual data points for the fit (symbols) was defined by \(I_{\text{NCX}}\) and [Ca\textsubscript{i}] time averaged over the duration of a particular voltage step. For the purpose of displaying the data only, \(I_{\text{NCX}}\) is shown normalized to its values at high [Ca\textsubscript{i}]. For ferret (and mouse + canine NCX1), the fit to Eq. 1 with the allosteric term was better in every case \((P < 0.05\) by F test described in MATERIALS AND METHODS). \(K_m\) for allosteric Ca regulation was close to [Ca\textsubscript{rest}] (Ferret \(K_m^\text{Act} = 125 \pm 16\) nM SEM). In contrast, data from the 6 (out of 10) WT mouse myocytes where [Ca\textsubscript{i}] was available were not fit better (or even as well) with the allosteric factor, with the exception of one cell, in which \(K_m^\text{Act}\) was 49 nM, well below physiological [Ca\textsubscript{i}] (data not shown).

Both fits of \(I_{\text{NCX}}\) versus [Ca\textsubscript{i}] in mice expressing canine NCX1 were better with the allosteric term (\(K_m^\text{Act} = 158\) or 418 nM, \(n = 2\); data not shown). Since WT mouse myocytes do not show Ca activation over the range of [Ca\textsubscript{i}] studied (Fig. 4), the situation is simplified, and we can attribute the observed allosteric regulation to purely canine NCX1. Fig. 5 B shows fits of \(I_{\text{NCX}}\) versus [Ca\textsubscript{i}] in the \(\Delta680-685\) mice where [Ca\textsubscript{i}] was available (3/8). None of these fits was improved by including the allosteric factor, verifying that amino acids 680–685 play a role in Ca regulation of canine NCX1 expressed in mouse myocytes. Fit parameters for the four cell types studied are shown in Table I.

Fig. 6 shows the magnitudes of \(I_{\text{NCX}}\) at high [Ca\textsubscript{i}] for the four myocyte types studied. \(I_{\text{NCX}}\) at \(+100\) mV was larger in ferret than WT mouse (2.47 vs. 1.48 A/F), but was increased three- to fourfold in transgenic mice (6.90 and 5.23 A/F). Where measured directly, the maximum [Ca\textsubscript{i}] attained was (mean \(\pm\) SEM) 257 \(\pm\) 73 in WT mouse \((n = 6)\); 254 \(\pm\) 36 in ferret \((n = 14)\); and, in accordance with the larger \(I_{\text{NCX}}\), 861 and 765 in two mouse cells overexpressing canine NCX1, and 1,357 \(\pm\) 613 in mouse overexpressing canine NCX1 \(\Delta680-685\) \((n = 3)\). The severalfold larger outward \(I_{\text{NCX}}\) seen for both transgenic mouse models in this figure confirm the expected overexpression of canine NCX in comparison to NCX of WT mouse and ferret. Allosteric activation is nearly complete at these high [Ca\textsubscript{i}], and so this factor has no influence here. The change of the electrochemical factor with increasing [Ca\textsubscript{i}] does not contribute to the increase of outward \(I_{\text{NCX}}\) (in fact, this factor decreases slightly; see Fig. 10). Thus, the large outward \(I_{\text{NCX}}\) in the transgenic mice is evidence of up-regulation of \(I_{\text{NCX}}\).

Fig. 7 shows summary data for the increment of \(I_{\text{NCX}}\) with increasing [Ca\textsubscript{i}] in all four NCX types studied. In the outward direction, \(I_{\text{NCX}}\) increased in ferret and in mouse myocytes expressing canine NCX1, but decreased in WT mouse and in mouse myocytes expressing the \(\Delta680-685\) deletion mutant of canine NCX1. Inward \(I_{\text{NCX}}\) always increased with increasing [Ca\textsubscript{i}], as would be expected even in the absence of Ca activation because of the increased inward electrochemical driving force, but the largest increases were in the ferret and the mouse overexpressing normal canine NCX1. These are the two preparations where Ca activation
would be expected to cause additional increase in $I_{\text{NCX}}$ beyond the electrochemical component.

We have shown in Fig. 7 the absolute increment in $I_{\text{NCX}}$ density without normalization to the initial $I_{\text{NCX}}$ density. In all cases, we attempted to choose a voltage protocol that brought $I_{\text{NCX}}$ to a maximally activated state. Accordingly, we did not adjust these data for the particular amount of $[\text{Ca}]_i$ increase. Thus, in this summary figure, we were able to include experiments where we did not record $[\text{Ca}]_i$, as long as we had contraction records consistent with large increases in $[\text{Ca}]_i$.

Fig. 8 A shows simulated $I_{\text{NCX}}$ in response to our alternating voltage protocol (Fig. 8 G) as predicted using Eq. 1, with allosteric Ca regulation included ($K_{\text{mCaAct}} = 125$ nM). Fig. 8 E (bold trace) shows $[\text{Ca}]$, as defined by Eq. 2. $V_{\text{max}}$ and $[\text{Na}]_i$ were determined from fits of the ferret data (Fig. 5 A) with the Ca regulatory term (Table II). Fig. 8 (B, D, F, and H) shows $I_{\text{NCX}}, [\text{Na}]_i, [\text{Ca}]_i$, and $E_m$, respectively, for an early and late pulse of Fig. 8 A. Note that during the first few pulses, the outward $I_{\text{NCX}}$ increased during 100 ms at $+100$ mV (because of allosteric activation; Fig. 8 B, a). However during later pulses, outward $I_{\text{NCX}}$ was relatively flat, and even started to decline slightly (Fig. 8 B, b). This is explained by a 71-$\mu$M decrease in $[\text{Na}]_i$ (Fig. 8 D) and a corresponding 132-nM increase in $[\text{Ca}]_i$ (Fig. 8 F), resulting in decreases in $\Delta E$ of 4 and 3%, respectively, while this decline in $\Delta E$ was countered almost exactly by a 7% increase in allosteric factor over the same increase in $[\text{Ca}]_i$. This spectrum of behavior reflects reasonably well the behavior of $I_{\text{NCX}}$ recorded experimentally where allosteric regulation was apparent (Fig. 2 C and Fig. 4, A and C).

Fig. 8 C shows the same simulation for $I_{\text{NCX}}$ without Ca regulation, and the corresponding $[\text{Ca}]$, is shown in Fig. 8 E (thin trace). $V_{\text{max}}$ and $[\text{Na}]_i$ were determined from fits of the ferret data without the Ca regulatory term (Table II). There is a decline in the outward $I_{\text{NCX}}$ envelope when no allosteric regulation is considered (by 8.8%) and this matches the observations in WT mouse and mouse overexpressing $\Delta 680-685$ canine NCX1 (Fig. 4, B and D). This decline is due to the electrochemical effect of the rising $[\text{Ca}]_i$, and declining $[\text{Na}]_i$, as described above, which in this case (unlike Fig. 8 A), are not offset by an increasing allosteric factor. Thus, the simple model provides a reasonable description of both the current envelope, and also describes changes within individual pulses.

To test how rapidly Ca activation of outward $I_{\text{NCX}}$ occurs, we induced a rapid and steady-state increase in $[\text{Ca}]_i$, from $\sim 100$ nM to $\sim 1$ $\mu$M (Fig. 9). In this case, thapsigargin was omitted and the SR was preloaded.
Weber et al.

With Ca by outward I_{NCX}. Depolarization from −100 to +100 mV caused an initial slow rise in [Ca], and outward I_{NCX}. Abrupt exposure to 10 mM caffeine induced a rapid rise in [Ca] (τ = 61 ms), and this caused a nearly parallel increase in outward I_{NCX} (τ = 40 ms) without any appreciable time lag. Although this does not define an explicit time constant for I_{NCX} activation, it makes our simplifying assumption of instantaneous regulation reasonable. Moreover, the rapidity of activation of Na/Ca exchange suggests that regulation may occur on a beat to beat basis.

**DISCUSSION**

We have examined intact cardiac myocytes from four different sources (WT ferret and WT mouse, as well as two transgenic mouse models) for allosteric Ca regulation of I_{NCX} at physiological [Ca]. We used a protocol in which the activating Ca was alternately brought into the cell via outward I_{NCX} at +100 mV, and extruded via inward I_{NCX} at −100 mV (Fig. 2). By varying the periods at ±100 mV (in the range of 100–300 ms), we were able to control and measure the rate of growth in [Ca], while simultaneously measuring both inward and outward I_{NCX}. In ferret myocytes, the outward and inward I_{NCX} both increased with increasing [Ca], (Figs. 4 and 7). We attribute the increasing outward I_{NCX} to Ca activation, which has predominance over the reduction in electrochemical gradient for Ca to enter the cell. The electrochemical gradient would otherwise decrease outward I_{NCX}. In contrast to the increase in outward I_{NCX} that we always saw in ferret myocytes, WT mouse outward I_{NCX} decreased in all but 1 out of 10 experiments (although inward I_{NCX} still increased; Figs. 4 B and 7). Additionally, in the absence of Ca activation, inward I_{NCX} increased to a smaller extent (Fig. 7, mouse versus ferret).

We also investigated allosteric Ca regulation in mouse myocytes overexpressing canine NCX1 (Fig. 4 B). The rationale behind experiments with transgenic mice was twofold. First, we wanted to determine if canine NCX overexpressed in mice would show allosteric regulation like the ferret NCX. Figs. 4 B and 7 illustrate that outward I_{NCX} did increase with increasing [Ca], indicating that canine NCX has similar Ca activation properties to ferret NCX (for outward I_{NCX}, ΔI_{NCX}/final I_{NCX} represented an increase of 38 ± 12% SEM [n = 7] in canine versus 41 ± 4% SEM [n = 22] in ferret). Second, since mutant canine NCX lacked Ca-dependent regulation of outward I_{NCX} in giant membrane patch studies (Maxwell et al., 1999), a comparison of I_{NCX} between these two types of transgenic myocytes overexpressing canine NCX should help verify that the increase in outward I_{NCX} was due to allosteric regulation and not some other process.
Figs. 4 D, 5 B, and 7 show that deletion of this putative Ca regulatory region eliminated the increase in outward $I_{\text{NCX}}$ in our protocol. This deletion made the canine $I_{\text{NCX}}$ look very much like WT mouse $I_{\text{NCX}}$, with the exception that its magnitude was severalfold larger (Fig. 6).

Fig. 10 A shows how the predicted allosteric activation (Allo) and electrochemical factors ($\Delta E$) in Eq. 1 would contribute to increasing outward and inward $I_{\text{NCX}}$ (Allo $\cdot \Delta E$) as $[\text{Ca}]_i$ increases up to 500 nM. In the outward direction, $I_{\text{NCX}}$ is largely dominated by the Allo factor, whereas in the inward direction, the Allo factor and $\Delta E$ factors both contribute. For this figure, the $[\text{Ca}]_i$-dependent allosteric and electrochemical factors in Eq. 1 were predicted using the averages of the respective parameters fit to the 14 sets of ferret data, and are shown normalized to their values at 500 nM $[\text{Ca}]_i$. From the predictions, we can infer the respective experimentally observed final values of $[\text{Ca}]_i$, the relative contributions of allosteric activation and electrochemical driving force. In ferrets, outward $I_{\text{NCX}}$ increased by 67% from its initial value as $[\text{Ca}]_i$ rose from 115 ± 9 nM (SEM) to 254 ± 36 nM (SEM). This was mainly due to a 75% increase in the allosteric factor, offset slightly by a 4% decrease in the electrochemical factor ($1.75 \times 0.96 = 1.67$). Inward $I_{\text{NCX}}$ in ferret increased by 283% with this $[\text{Ca}]_i$ increase. The allosteric factor increased by the same 75%, but in this case it was complemented by a 119% increase in the electrochemical factor, resulting in the final effect ($1.75 \times 2.19 = 3.83$). Thus, the allosteric and electrochemical factors contribute nearly equally to the increase in inward $I_{\text{NCX}}$ recorded in ferret.

Fig. 10 B shows the effects of $[\text{Na}]_i$ on the electrochemical factor of Eq. 1 at $[\text{Ca}]_i = 100$ and 500 nM. Changes in $[\text{Na}]_i$ (in the millimolar range) are not expected to occur rapidly, but species-dependent variations in $[\text{Na}]_i$ do occur. $[\text{Na}]_i$ appears to be substantially elevated in mouse myocytes relative to nonmammals (7.2 in rabbit and 12.7 in rat [Shattock and Bers, 1989]; 14-17 mM in mouse [Yao et al., 1998]). This is also suggested by the results of our fits (Table I), and this may account for the small inward $I_{\text{NCX}}$ in mouse (Fig. 4, B–D, and Fig. 6).

Our simulations (Fig. 8) also predict that $I_{\text{NCX}}$ drives micromolar changes in $[\text{Na}]_i$ within pulses during our oscillatory protocols. Referring to outward $I_{\text{NCX}}$, during the final 100-ms pulse to +100 mV in Fig. 8 B, b, declining $[\text{Na}]_i$ (Fig. 8 D) and increasing $[\text{Ca}]_i$ (Fig. 8 F) contribute about equally to declines in $\Delta E$ (see results), and this, combined with allosteric activation by Ca, results in the relatively flat current during the pulse. However, the relatively flat relationship between $\Delta E$ and $[\text{Na}]_i$ for inward $I_{\text{NCX}}$ (Fig. 10 B), coupled with the steep dependence of inward $I_{\text{NCX}}$ on $[\text{Ca}]_i$, at $-100$ mV makes inward $I_{\text{NCX}}$ relatively insensitive to changing $[\text{Na}]_i$. Changing $[\text{Na}]_i$ alone, within the range observed in Fig. 8, causes less than a 1% decline in inward $I_{\text{NCX}}$ and almost all of the within-pulse changes in inward $I_{\text{NCX}}$ are due to changing $[\text{Ca}]_i$, acting either electrochemically or allosterically.

**Measurement of $I_{\text{NCX}}$**

There is no inhibitor that is selective for $I_{\text{NCX}}$ without affecting other currents; accordingly, we approached this problem by blocking all currents other than $I_{\text{NCX}}$ with their appropriate inhibitors (as well as by ion substitution). We took $I_{\text{NCX}}$ as either the difference current before versus after applying Ni or the current remaining after analytical leak subtraction. Two additional observations support the idea that the currents we recorded are $I_{\text{NCX}}$. Fig. 6 shows that $I_{\text{NCX}}$ is indeed larger in the NCX overexpressing myocytes compared with...
Comparison to Earlier Studies

We have shown for the first time allosteric regulation in intact myocytes at physiological \([\text{Ca}]_i\), and under conditions where the NCX is able to dynamically control \([\text{Ca}]_i\). Miura and Kimura (1989), in intact guinea pig myocytes, showed Ca regulation with \([\text{Ca}]_i\), artificially held well below the normal resting \([\text{Ca}]_i\) (\(K_{\text{mCaAct}} = 22 \text{nM}\)). They found a steep Ca dependence (\(n_{\text{Hill}} = 3.7\)) and concluded that \(I_{\text{NCX}}\) was maximally activated when \([\text{Ca}]_i\) reached 50 nM. A similar low \(K_{\text{mCaAct}}\), 44 nM (\(n_{\text{Hill}} = 1.6\)), was found by Fang et al. (1998) for the bovine cardiac NCX expressed in Chinese hamster ovary cells. Maxwell et al. (1999) showed Ca activation of WT mouse NCX in giant membrane patches between 0 and 3 \(\mu\text{M}\) (but no values were obtained between 0 and 1 \(\mu\text{M}\) \([\text{Ca}]_i\)). It is conceivable that our failure to observe Ca regulation of WT mouse \(I_{\text{NCX}}\) (as described by Maxwell et al., 1999) is due to a low intrinsic \(K_{\text{mCaAct}}\) in mouse (comparable to the 22–44 nM values above), and lower than we observe in the ferret or dog NCX; i.e., mouse NCX may be fully activated at diastolic \([\text{Ca}]_i\) of 100 nM. Indeed, the only WT mouse myocyte whose \(I_{\text{NCX}}\) versus \([\text{Ca}]_i\) fit was actually improved with the Ca regulatory term, showed activation with an apparent \(K_{\text{mCaAct}}\) of 49 nM. At 100 nM resting \([\text{Ca}]_i\), this would imply 82% activation (for \(n_{\text{Hill}} = 2\) or 95% for \(n_{\text{Hill}} = 4\)), in contrast to 39% activation at 100 nM \([\text{Ca}]_i\) in the ferret (for \(n_{\text{Hill}} = 2\)). Thus, since we did not explore subphysiological \([\text{Ca}]_i\), intrinsic Ca regulation in the mouse could have escaped our detection. On the other hand, this made mouse myocytes a convenient background in which to study transgenic overexpression of canine NCX with respect to Ca regulation at physiological \([\text{Ca}]_i\), since the endogenous mouse NCX may always be fully activated.

Our \(K_{\text{mCaAct}}\), 125 nM in the ferret, although only somewhat larger numerically, would result in maximal sensitivity of \(I_{\text{NCX}}\) to changes in \([\text{Ca}]_i\), near \([\text{Ca}]_{\text{i,rest}}\). The disparity between previous results and ours, as well as our finding of a functional difference between the ferret and WT mouse, indicate that there are species and/or preparation-dependent differences in the regulation of \(I_{\text{NCX}}\) by \([\text{Ca}]_i\). For example, EGTA can increase the apparent \([\text{Ca}]_i\) dependence of NCX in sarcolemmal vesicles (Trooper and Philipson, 1984), which could have produced underestimates of \(K_{\text{mCaAct}}\) in earlier work. An interesting side point is that the NCX of Drosophila melanogaster, calx, although regulated by \([\text{Ca}]_i\), in the 100-nM range, shows inactivation, not activation, with increasing \([\text{Ca}]_i\) (Hryshko et al., 1996).

Species dependency does not explain why Hilgemann et al. (1992) observed \(K_{\text{mCaAct}}\) to be 0.3–0.6 \(\mu\text{M}\) in giant excised patches, 10 times higher than Miura and Kimura (1989) reported, and 2 to 4 times as high as our value. Both studies used guinea pig myocytes. Either preparation, Ca buffering, or washout of other factors might explain these differences. Our study differs from all previous approaches, not only in using intact myocytes, which probably retain key regulatory components, but also in allowing dynamic \([\text{Ca}]_i\) regulation.

We have noted (Fig. 8) that \([\text{Na}]_i\) and \([\text{Ca}]_i\) can vary transiently during the overall increase of \([\text{Ca}]_i\) in our protocol, possibly explaining the declines in \(I_{\text{NCX}}\) within pulses (Fig. 3). In connection with these variations, subsarcolemmal \([\text{Ca}]_i\) may have reached higher values than our fluorescence-based \([\text{Ca}]_i\) measurements indicated. Then, we would have underestimated \(K_{\text{mCaAct}}\). However, we fit \(K_{\text{mCaAct}}\) using only ferret data, and these data had very little within-pulse \(I_{\text{NCX}}\) variation.

Physiological Relevance

A reduction in \(I_{\text{NCX}}\) at low \([\text{Ca}]_i\), may limit the ability of outward \(I_{\text{NCX}}\) to trigger SR Ca release during the upstroke of an action potential (Noble et al., 1991). During diastole, deactivation of inward \(I_{\text{NCX}}\) may help maintain the SR Ca load by shifting the Ca flux balance away from sarcolemmal Ca extrusion and towards the SR. Deactivation of \(I_{\text{NCX}}\) may help prevent resting \([\text{Ca}]_i\), from going much below threshold for myofilament activation (Fabiato, 1983). Additionally, the probability of \([\text{Ca}]_i\) to trigger SR Ca release is a steep function that depends on the square of local \([\text{Ca}]_i\) near the ryanodine receptors (Santana et al., 1996; Cheng et al., 1996). Thus, small variations in NCX activity may fine tune resting \([\text{Ca}]_i\), and, in doing so, may help to define the threshold necessary for Ca to trigger the opening of the SR release channels (Litwin et al., 1998). Finally, since NCX normally works to extrude Ca as \([\text{Ca}]_i\) increases, Ca activation may reduce the likelihood of Ca overload (unless \([\text{Na}]_i\) gets very high).

We have assumed a stoichiometry of 3:1 \text{Na}/Ca in this paper (Reeves and Hale, 1984; Kimura et al., 1987), however, Fujioka et al., (2000) recently reported a stoichiometry closer to 4:1 \text{Na}/Ca, so that for every net charge
transferred by the flow of \( I_{\text{NCX}} \), 1/2 Ca ion rather than 1 Ca ion is transferred in the opposite direction. A 4:1 NCX would tend to draw \([Ca]\), to 130 pM at \( E_{\text{rest}} \), rather than 38 nM for a 3:1 NCX (~80 mV, 10 mM \([Na]\), 140 mM \([Na]\), 2 mM \([Ca]\), 37°C). In the prediction of Fig. 3, a 4:1 stoichiometry would also reduce the Ca flux inferred from \( I_{\text{NCX}} \) by a factor of 2 and leave it insufficient to explain \( \Delta [Ca] \). Although further evaluation of the proposed 4:1 stoichiometry is needed, if true, then deactivation of \( I_{\text{NCX}} \) at low \([Ca]\) would be even more crucial to prevent diastolic \([Ca]\) from falling far too low.

If mean \([Ca]\) is elevated, as may occur at high heart rates or during heart failure (Gwathmey et al., 1987), the average state of NCX activity may be elevated. If this is the case, and the NCX functions predominantly in the Ca efflux mode, Ca activation may serve as a negative feedback mechanism that helps to prevent Ca overload and spontaneous SR Ca release. On the other hand, since \( I_{\text{NCX}} \) is thought to be a major component of the transient inward current (Fedida et al., 1987; Lipp and Pott, 1988; Schlothauer and Bers, 2000), Ca activation may actually promote delayed afterdepolarizations with spontaneous SR release. Furthermore, if \([Na]\) rises to high enough levels (e.g., submembrane space or digitalis toxicity), Ca influx is favored electrochemically and, in this case, Ca activation could worsen Ca overload.

**Model**

We chose to fit an instantaneous model because experiments in intact cells do not allow the fast homogenous cytoplasmic solution changes needed to study the dynamics of Ca activation precisely. There may be a lag between the \([Ca]\) in the vicinity of the NCX and the bulk \([Ca]\) measured by fluorescent indicators (Trafford et al., 1995; Niell et al., 1999), which is not included in our model. Since we cannot distinguish spatial and temporal \([Ca]\), heterogeneities from activation kinetics, we have chosen to model Ca activation as an instantaneous process. Our simulation of the ferret data (Fig. 8) with allosteric Ca regulation modeled in this manner matched well with the characteristic growing outward envelope of \( I_{\text{NCX}} \) and also with changes during individual pulses. Furthermore, we found no delay in activation of \( I_{\text{NCX}} \) when a rapid release of SR Ca was used to cause \([Ca]\), to increase from 100 to >1 \( \mu \text{M} \) (Fig. 9). This might be related to Baazov et al. (1999), who found very rapid activation, although Kapp and Hartung (1996) indicated an activation time constant of 620 ms. Rapid activation, along with \( K_{\text{Ca}^{2+}} \) = 125 nM, suggests that variation in Ca activation might be relevant in beat-to-beat regulation of \( I_{\text{NCX}} \). Indeed, the \([Ca]\) dependence of inward \( I_{\text{NCX}} \) reflects a composite of both \( K_{\text{Ca}^{2+}} \) and \( K_{\text{Ca}^{2+}} \) and the apparent linearity often reported may be serendipitous.

The primary aim of this study was to determine if allosteric regulation of the NCX played a role in the intact cell under conditions where \([Ca]\), changed within a physiological range. We conclude that allosteric Ca-dependent activation of \( I_{\text{NCX}} \) occurs in ferret myocytes and in mouse myocytes overexpressing dog NCX1. We also confirm that amino acids 680–685 are important for Ca regulation, since regulation did not occur in mouse myocytes overexpressing canine NCX1 lacking this region. Under our experimental conditions and \([Ca]\), range, Ca activation did not occur in WT mouse myocytes (contrast Maxwell et al., 1999). Although this lack of Ca regulation in the WT mouse may be condition-dependent, we speculate that it may be due to species differences in amino acid sequences within NCX1. Although NCX1 is ~99% identical between mouse and dog, a difference does occur within the 680–685 region (IIQESY in dog versus IIEESY in mouse; Nicoll et al., 1990; Nicoll and Philipson, 1991).

We thank Dr. Donald W. Hilgemann for stimulating discussions and Drs. Jeanne Nerbonne, Weimong Guo, and Haodong Xu for their help with our development of mouse myocyte preparations. We also thank Steve Scaglione and Sarah Wimbiscus for their help with the ferret myocyte isolations.

This work was supported by the National Institutes of Health grants R01HL-30077DMB, R01-HL64908DMB, R01-HL48509KDP, and AHA Fellowship 0010180ZCRW.

Submitted: 3 August 2000
Revised: 17 November 2000
Accepted: 13 December 2000

**References**


Fabiani, A. 1983. Calcium-induced release of calcium from the cardiac sarcoplasmic reticulum. Am. J. Physiol. 245:C1–C14.