Na-Ca Exchange and the Trigger for Sarcoplasmic Reticulum Ca Release: Studies in Adult Rabbit Ventricular Myocytes

Sheldon E. Litwin,*§ Jun Li,# and John H. B. Bridge#
*Division of Cardiology, Salt Lake City Veterans Affairs Medical Center; #Nora Eccles Harrison Cardiovascular Research and Training Institute; and §Cardiology Division, University of Utah, Salt Lake City, Utah USA

ABSTRACT The importance of Na-Ca exchange as a trigger for sarcoplasmic reticulum (SR) Ca release remains controversial. Therefore, we measured whole-cell Ca currents (I_{Ca}), Na-Ca exchange currents (I_{NaCa}), cellular contractions, and intracellular Ca transients in adult rabbit cardiac myocytes. We found that changing pipette Na concentration markedly affected the relationship between cell shortening (or Ca transients) and voltage, but did not affect the Ca current-voltage relationship. We then inhibited Na-Ca exchange and varied SR content (by changing the number of conditioning pulses before each test pulse). Regardless of SR Ca content, the relationship between contraction and voltage was bell-shaped in the absence of Na-Ca exchange. Next, we rapidly and completely blocked I_{Ca} by applying nifedipine to cells. Cellular shortening was variably reduced in the presence of nifedipine. The component of shortening blocked by nifedipine had a bell-shaped relationship with voltage, whereas the "nifedipine-insensitive" component of contraction increased with voltage. With the SR disabled (ryanodine and thapsigargin pretreatment), I_{Ca} could initiate late-peaking contractions that were ~70% of control amplitude. In contrast, nifedipine-insensitive contractions could not be elicited in the presence of ryanodine and thapsigargin. Finally, we recorded reverse Na-Ca exchange currents that were activated by membrane depolarization. The estimated sarcolemmal Ca flux occurring by Na-Ca exchange (during voltage clamp steps to +30 mV) was ~10-fold less than that occurring by I_{Ca}. Therefore, Na-Ca exchange alone is unlikely to raise cytosolic Ca concentration enough to directly activate the myofilaments. We conclude that reverse Na-Ca exchange can trigger SR Ca release. Because of the sigmoidal relationship between the open probability of the SR Ca release channel and pCa, the effects of I_{Ca} and I_{NaCa} may not sum in a linear fashion. Rather, the two triggers may act synergistically in the modulation of SR release.

INTRODUCTION

A rapid rise in Ca concentration in the vicinity of the sarcoplasmic reticulum (SR) Ca release channel (ryanodine receptor) may activate or "trigger" the release of additional Ca from the SR (Fabiato and Fabiato, 1975). This is referred to as calcium-induced calcium release (CICR). There is general acceptance that Ca entering the cell through voltage-dependent L-type Ca channels (I_{Ca}) is the main stimulus for CICR (London and Krueger, 1986; Beuckelmann and Wier, 1988; Cleeman and Morad, 1991; Bouchard et al., 1993). L-type I_{Ca} has a bell-shaped relationship with voltage. When SR Ca release is triggered by an abrupt increase in Ca near the SR release channels, this release is not influenced by voltage (Nabauer and Morad, 1990; Niggli and Lederer, 1990). However, the extent of SR release is graded with the magnitude of the trigger (Fabiato, 1985). Therefore, if the sole trigger is the L-type Ca current, then one expects the triggered release (or ensuing contractions) to exhibit a bell-shaped relationship with voltage.

A number of investigators have found a bell-shaped contraction-voltage relationship (London and Krueger, 1986; Berlin et al., 1987; Beuckelmann and Wier, 1988). However, there are several reports that under appropriate circumstances, the relationship between contraction (or Ca transients) and voltage is not bell-shaped (Berlin et al., 1987; Nuss and Houser, 1991; Kohmoto et al., 1994; Vornanen et al., 1994; Wasserstrom and Vites, 1996). In these reports the amplitude of triggered contractions or Ca transients does not decline as much at positive potentials as does I_{Ca}. This is particularly the case if intracellular Na is elevated (Berlin et al., 1987; Levi et al., 1996). These results indicate that a process other than I_{Ca} may contribute to the trigger for SR Ca release under some conditions. Berlin et al. (1987) first suggested that Na-Ca exchange could trigger SR Ca release. Later, Leblanc and Hume, and subsequently Lipp and Niggli, provided evidence suggesting that Na entry during Na currents may induce Ca transients (Leblanc and Hume, 1990; Lipp and Niggli, 1994). Presumably, these are due to accumulation of Na in the vicinity of the Na-Ca exchanger, which promotes reverse Na-Ca exchange. Further evidence favoring a contribution of the Na-Ca exchanger came from several reports showing that triggered contractions or Ca transients that were largely resistant to organic Ca channel blockers could be recorded (Kohmoto et al., 1994; Levi et al., 1994; Wasserstrom and Vites, 1996). Moreover, in one study, contractions activated in the presence of nifedipine were largely inhibited in the presence of a peptide that inhibits Na-Ca exchange (Kohmoto et al., 1994).

Although the above results suggest that Na-Ca exchange may be involved in CICR, other investigators have not been

Received for publication 3 November 1997 and in final form 29 March 1998.

Address reprint requests to Dr. Sheldon E. Litwin, Cardiovascular Division (111A), Veterans Affairs Medical Center, 500 Foothill Blvd., Salt Lake City, UT 84148. Tel.: 801-584-1235; Fax: 801-581-7735; E-mail: slitwin@msscc.med.utah.edu.

© 1998 by the Biophysical Society

0006-3495/98/07/359/13 $2.00

able to find evidence of this (Bouchard et al., 1993). More recently, using confocal microscopy, two groups found that when L-type Ca currents were reduced by verapamil, local Ca transients (presumably reflecting elementary SR Ca release events) exhibited a probability of occurrence that was bell-shaped with voltage (Cannell et al., 1995; Lopez-Lopez et al., 1995). These results strongly suggest that I_{Ca} was the signal underlying the appearance of the local Ca transients. In addition, Lopez-Lopez et al. reported that the probability of local Ca transients was very low during voltage clamp steps to positive potentials when Ca entry by reverse Na-Ca exchange was known to be occurring (Lopez-Lopez et al., 1995). It is interesting that in these latter experiments, a rise in cytosolic Ca occurred, despite the fact that no (or few) elementary releases of Ca were observed.

Clearly, there is no general agreement on whether Na-Ca exchange can either enhance or directly trigger SR Ca release. The results of the present study suggest that reverse Na-Ca exchange can participate in the events of excitation-contraction coupling under a variety of conditions. The physiological importance of CICR triggered by reverse Na-Ca exchange remains uncertain.

MATERIALS AND METHODS

Male New Zealand white rabbits weighing 2.0–2.5 kg were used for all experiments. Animals were cared for according to the guidelines of the American Physiological Society, and protocols were approved by the institutional animal care committee.

Myocyte isolation

Rabbits were deeply anesthetized with pentobarbital administered via an ear vein. The heart was quickly excised and then perfused via the aorta (60 mmHg pressure) with a Ca-free HEPES-buffered saline solution that was bubbled with 100% O_2 (37°C). After 5 min the solution was changed to one containing 0.08% collagenase and 0.02% protease, and perfusion was continued for an additional 15–20 min. When the heart was soft, the right ventricle and atria were removed and the left ventricle was minced. The tissue was gently shaken in a low-Ca saline solution free of digestive enzymes and strained, and the dissociated myocytes were allowed to settle in a storage solution containing 1.0 mM Ca (23–25°C). Only cells with clear striations and no evidence of spontaneous contractions were used for voltage clamp studies.

General features of voltage clamp studies

All studies were performed within 8 h after cell dissociation. The cells were affixed with mouse laminin (Collaborative Biomedical Products, Bedford, MA) to a glass coverslip that formed the bottom of a bath. The bath was continuously perfused with a HEPES-buffered modified Tyrode's solution (concentration in mM: 138 NaCl, 1.0 $MgCl_2$, 4.4 KCl, 11.0 dextrose, 2.7 $CaCl_2$, 24.0 HEPES, pH adjusted to 7.4 with NaOH) maintained at 30°C. Cells were viewed with an inverted phase-contrast microscope (Diaphot, Nikon). Individual myocytes were voltage clamped (Axopatch-200A; Axon Instruments, Foster City, CA) with single borosilicate suction pipettes (resistance 1.0–2.0 MΩ). After whole-cell recording was established, capacitance and series resistance compensation were performed (typically 70–75% of the series resistance was compensated). During most voltage clamp episodes, cells were held at a potential of −40 mV to inactivate Na current. Between experimental protocols, cells were generally held at −80 mV to prevent run-down of Ca current (Schouten and Morad, 1989). Cell motion was monitored with a video-based edge detection system (Crescent Electronics, Salt Lake City, UT) (Bridge et al., 1988). Currents and cell motion were digitized on line with a 12-bit A/D converter (TL-1; Axon Instruments) and stored on a personal computer for later analysis with pClamp software (Axon Instruments). The peak rate of cellular shortening was used as an index of SR calcium release because the timing of peak shortening (or force) coincides fairly closely with the peak of the intracellular Ca^{2+} transient, and the rate of force development is thought to be an instantaneous function of intracellular calcium (Ca_i^{2+}) (Yue, 1987).

Pipette solutions and extracellular bathing solutions were individualized based on the particular experiment. The standard pipette solution contained (concentration in mM) 130 CsCl, 5.5 dextrose, 5.0 K_2ATP, 0.02 EGTA, 0.5 MgCl, 10 HEPES, and 0, 10, or 15 NaCl, with pH adjusted to 7.1 with KOH. This solution was chosen for a variety of reasons. Cs was used as the major cation to block outward K currents. However, some K is needed as a counterion to prevent the establishment of a Donnan equilibrium across the SR membrane. Such an equilibrium will inhibit SR Ca release. Finally, a free Mg concentration of 0.5 mM was used because we have observed that with higher concentrations, contractions are less vigorous and tend to decay with time (unpublished observations). This is compatible with known effects of Mg on the SR release channel. CsCl was substituted for NaCl in the pipette solution in experiments in which we wanted to minimize intracellular Na.

To make a Na-deficient extracellular solution, we substituted LiCl for NaCl in the normal Tyrode's solution. For experiments requiring rapid blockade of I_{Ca}, nifedipine stock solution (10 mM) was made by dissolving nifedipine in ethanol and storing it in a light-protected container at −30°C. The nifedipine stock solution was added to the normal Tyrode's solution just before each experiment to produce a final concentration of 20 μM. The nifedipine-containing Tyrode's solution was protected from light exposure during all experiments. For experiments using nickel to block Na-Ca exchange, nickel Cl was added to normal Tyrode's solution to produce a concentration of 5 mM.

Solutions superfusing cells were rapidly changed with a previously described solution switcher with modifications (Spitzer and Bridge, 1989). The modified switching device directs the solutions through two square glass tubes (200 μm) separated by a 70-μm glass septum. The bulk solution over the cell may be changed within 7 ms with this device.

Measurements of intracellular Ca transients

Cells were loaded with 10 μM Fluo-3 AM (Molecular Probes) for 30 min at room temperature. The cells were then placed in the recording chamber and washed for 20–30 min to allow deesterification of the dye. Calcium transients were measured in cells voltage clamped with pipettes containing 0, 10, or 15 mM Na. Background fluorescence was nulled after a gigaohm seal was formed between the cell and the suction pipette, but before patch rupture. Cells were illuminated with 488-nm light produced with a Hg arc lamp, and 515-nm emission was detected with a photomultiplier tube. Because each cell served as its own control, we did not attempt to calibrate the light signal into absolute [Ca]. The peak rate of rise of the Ca transient was obtained by differentiating the digitized fluorescence signal with the pClamp6 software.

Statistics

All data are shown as mean ± SEM. Statistical comparisons were performed using commercially available software (Glantz, 1992). Comparisons of cellular contractions or whole-cell currents at different pipette Na concentrations were performed using a one-way analysis of variance followed by a Student-Newman-Keuls multiple comparisons test where appropriate. When only two different conditions were being compared, a two-tailed unpaired Student's *t*-test was used. A probability of <0.05 was considered to be significant.

RESULTS

Effects of membrane potential and intracellular [Na] on cellular contractions

Previous studies have shown that the relationship between contractile force and voltage, or Ca transients and voltage, depends on intracellular [Na] (Vornanen et al., 1994; Levi et al., 1996). To confirm this finding in our preparation, we determined the relationship between I_{Ca}, cellular shortening, and membrane potential in single myocytes when patch pipettes contained 0 ($n = 7$), 10 ($n = 10$), or 15 ($n = 5$) mM Na. We applied conditioning pulses from −40 mV to +10 mV every 3 s to establish steady-state SR calcium loading. I_{Ca} and cellular shortening were then measured during 400-ms voltage clamp steps from −40 mV to potentials between −30 and +60 mV. The maximum rate of cellular shortening was determined from the contraction record. As predicted, varying pipette sodium concentration, $[Na]_p$, significantly affected the shape of the shortening-voltage relationship, but had little effect on the voltage dependence of I_{Ca} (Fig. 1). With 0 mM Na in the pipette, cellular contractions diminished at potentials greater than +10 mV. The bell-shaped relationship between contractions and voltage suggests that under these conditions, most of the contraction was triggered by I_{Ca}. In comparison, with 10 mM Na in the pipette, contractions reached a plateau at potentials greater than +10 mV. Finally, with $[Na]_p = 15$ mM, the rate of cellular shortening continued to increase at potentials greater than +10 mV. The differences were highly statistically significant.

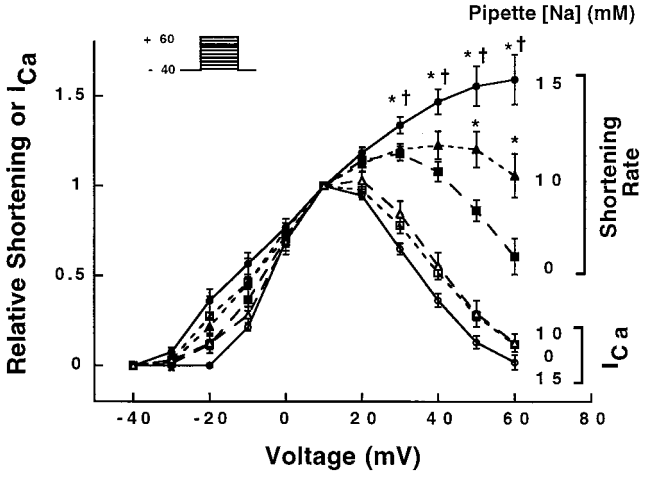

FIGURE 1 Ca currents (I_{Ca}) and shortening rates recorded in cells voltage clamped with pipettes containing 0 ($n = 7$), 10 ($n = 10$), or 15 ($n = 5$) mM Na. All data are normalized to values obtained at +10 mV and are shown as mean ± SEM. Normalization points are as follows: 1) $[Na]_p$ = zero mM; shortening rate = 0.033 μm/ms; I_{Ca} = 0.96 nA; 2) $[Na]_p$ = 10 mM; shortening rate = 0.028 μm/ms; I_{Ca} = 0.47 nA; 3) [Na]p = 15 mM; shortening rate = 0.042 μm/ms; I_{Ca} = 0.43 nA. The I_{Ca}-voltage relationship is bell-shaped at all $[Na]_p$. However, as intracellular [Na] is increased, there is a marked departure between the rate of cellular shortening and the size of Ca currents (* $p < 0.05$ versus 0 mM Na; $p < 0.05$ versus 10 mM Na). This suggests that something other than I_{Ca} is contributing to the trigger for SR Ca release. Furthermore, this additional process is more pronounced at positive membrane potentials and is highly dependent on intracellular Na concentration.

Relationship of intracellular Ca transients and cellular contractions

It is convenient to characterize excitation-contraction coupling by measuring the relationship between SR Ca release and the magnitude of the Ca current that produces that release (Wier et al., 1994). This relationship is often called the gain of the system and is defined as

$$\text{gain} = \text{rate or amplitude of SR Ca release}/I_{Ca} \quad (1)$$

To calculate gain, we measured the maximum rate of rise of the Ca transient as an indication of the SR Ca release. These values were divided by the magnitude of the Ca current. We reasoned that if both the Ca current and the Na-Ca exchanger contribute to triggering SR Ca release, then the gain should show dependence on intracellular [Na] and should reflect the voltage dependence of Na-Ca exchange currents. In particular, if the extent of triggering by Na-Ca exchange increased at positive voltages, we would expect the gain to also increase with voltage. This is because I_{Ca} is the only current measured, but the extent of SR release will be affected by the presence of the unmeasured Na-Ca exchange.

The peak rate of rise of the Ca transient was recorded during voltage clamp steps from a holding potential of −40 mV to potentials between −20 and +60 mV. Five conditioning pulses to +10 mV were applied at 3-s intervals before each test pulse. The rate of rise of the intracellular Ca transient was normalized to that recorded during the last conditioning pulse of each train. An example of these recordings is shown in Fig. 2 *A* (including the last conditioning pulse of each train). We plotted gain as a function of membrane potential (Fig. 2 *B*). In the range of 0 to +60 mV, gain was approximately constant in the nominal absence of intracellular Na. Thus, over this range of potentials, triggered release is approximately proportional to the magnitude of I_{ca} when Na-Ca exchange is inhibited. However, with 10 or 15 mM Na in the pipette, we found that gain assumed an upsloping curvilinear relationship with membrane potential when Na was included in the pipette solution. The difference in apparent gain measured in the presence and absence of Na is appreciable, particularly at more positive potentials. For example, at +40 mV, gain was approximately doubled with 10 mM Na in the pipette as compared to zero Na (Fig. 2 *B*). A concentration of 15 mM Na had a greater effect than 10 mM Na. The influence of intracellular [Na] on gain is compatible with a role for the Na-Ca exchanger in producing SR Ca release. Importantly, this effect is apparent in the very early portions of the Ca transient.

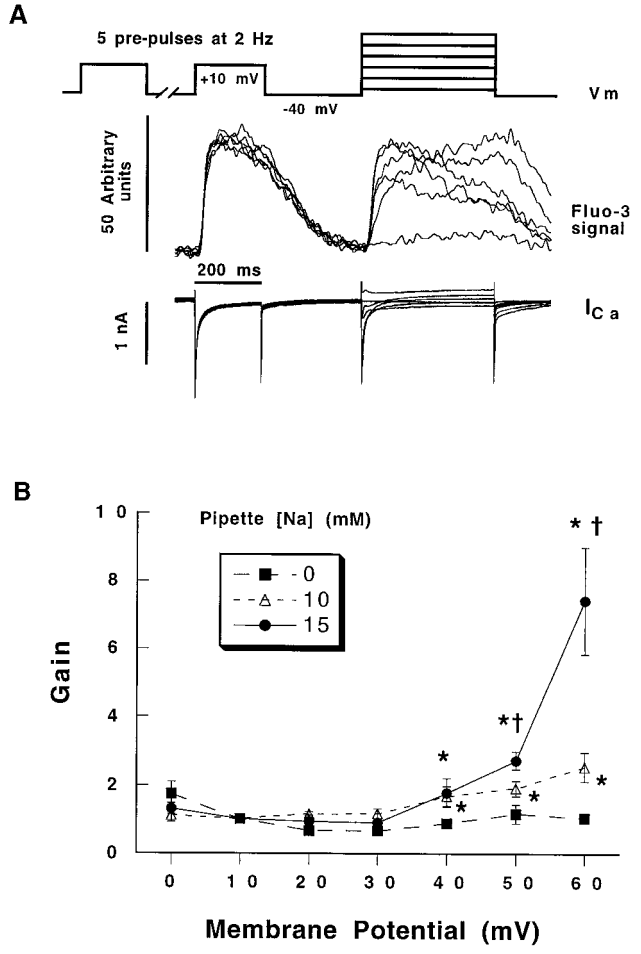

FIGURE 2 Effect of intracellular [Na] and membrane potential on intracellular Ca transients. (*A*) Example of simultaneous whole-cell currents and intracellular Ca transients in a single cardiac myocyte. To maintain stable SR loading, six conditioning pulses (from a holding potential of −40 mV to +10 mV) were applied before each test pulse (voltage clamp protocol shown above). The last conditioning pulse in each train of is shown, followed by Ca transients (*middle*) and currents (*bottom*) during voltage clamp test pulses to potentials between 0 and +60 mV. In this example, the pipette contained 10 mM Na. The gain of SR Ca release was calculated using the peak rate of rise of the Ca transient (*B*) (see Materials and Methods for further details). The relationship between gain and membrane potential was strongly affected by intracellular Na concentration. When $[Na]_p$ = 0 mM, gain showed minimal dependence on voltage. However, when $[Na]_p$ was increased, gain significantly increased at positive potentials. Data are from $n = 5$ cells and are mean ± SEM. * $p < 0.05$ versus $[Na]_p$ = zero mM; $p < 0.05$ versus $[Na]_p$ = 10 mM.

Relationship between SR calcium content and the voltage dependence of triggered calcium release

The $[Na]_i$-dependent asymmetry of the shortening-voltage relationship and the gain of SR release might be explained by a component of SR Ca release that is triggered by reverse Na-Ca exchange. However, other explanations are also plausible. For example, changes in SR Ca content could alter the voltage dependence of the release process. It is known that the fractional release of Ca from the SR increases as SR Ca content or the size of the trigger is increased (Bassani et al., 1995). To determine if changes in SR Ca content could explain the voltage-dependent divergence between I_{Ca} and shortening rate, we performed experiments in which SR Ca content was varied in the absence of a sarcolemmal Na gradient (i.e., I_{Ca} should be the only trigger for SR Ca release). In these experiments ($n = 6$ cells), Na-Ca exchange was inhibited by making $[Na]_p$ and extracellular [Na] = 0 mM. The cell was held at −40 mV, and SR Ca content was varied by changing the number of conditioning pulses (0–6) before each test pulse. Under these conditions, the Ca entering via I_{Ca} during each conditioning pulse can only be extruded very slowly from the cell and should augment SR Ca stores. The increasing SR content during sequential conditioning pulses was manifested as an increase in the extent of each subsequent contraction. A 400-ms test pulse to potentials between −30 and +70 mV was applied at the end of each series of conditioning pulses. After the test pulse, the cell was superfused with normal Tyrode's solution (containing 145 mM Na) and held at −80 mV to enhance forward Na-Ca exchange. Four voltage clamp steps to +10 mV were then applied to the cell to allow it to discharge the Ca that had accumulated in the SR, so that the SR Ca content was standardized before each conditioning train. The cell was then superfused with the zero Na solution for ~30 s (using the rapid solution switcher), and another series of 0–6 conditioning pulses followed by a test pulse was recorded. We found that with zero, three, or six conditioning pulses, cellular contractions declined at potentials greater than +10 mV when reverse Na-Ca exchange was blocked by depletion of intracellular Na (Fig. 3 *E*). The symmetry of the shortening-voltage relationship under each of these conditions implies that changes in SR Ca content alone do not cause a significant departure between the shortening-voltage and I_{Ca}-voltage relationships at positive potentials. Rather, the additional shortening at potentials greater than +10 mV is directly affected by the cytoplasmic [Na].

Contractions in the absence of Ca current

To selectively investigate the contribution of reverse Na-Ca exchange as a trigger for SR Ca release, I_{Ca} must be blocked sufficiently rapidly that SR Ca content is not significantly depleted. Therefore, we abruptly applied 20 μM nifedipine to cells using the rapid solution switcher ($n = 10$ cells). Nifedipine exposure was continued for a total of 8 s. A 1-s ascending and descending ramp from −40 mV to 0 mV was imposed during the nifedipine exposure because the Ca channel blockade is voltage-dependent. After the ramp, currents and cell shortening were recorded during voltage clamp steps to various test potentials in the presence of nifedipine. The nifedipine was then washed off by superfusing the cell with normal Tyrode's solution. A key feature of these experiments is that SR Ca content was held constant by applying six conditioning pulses to +10 mV be-

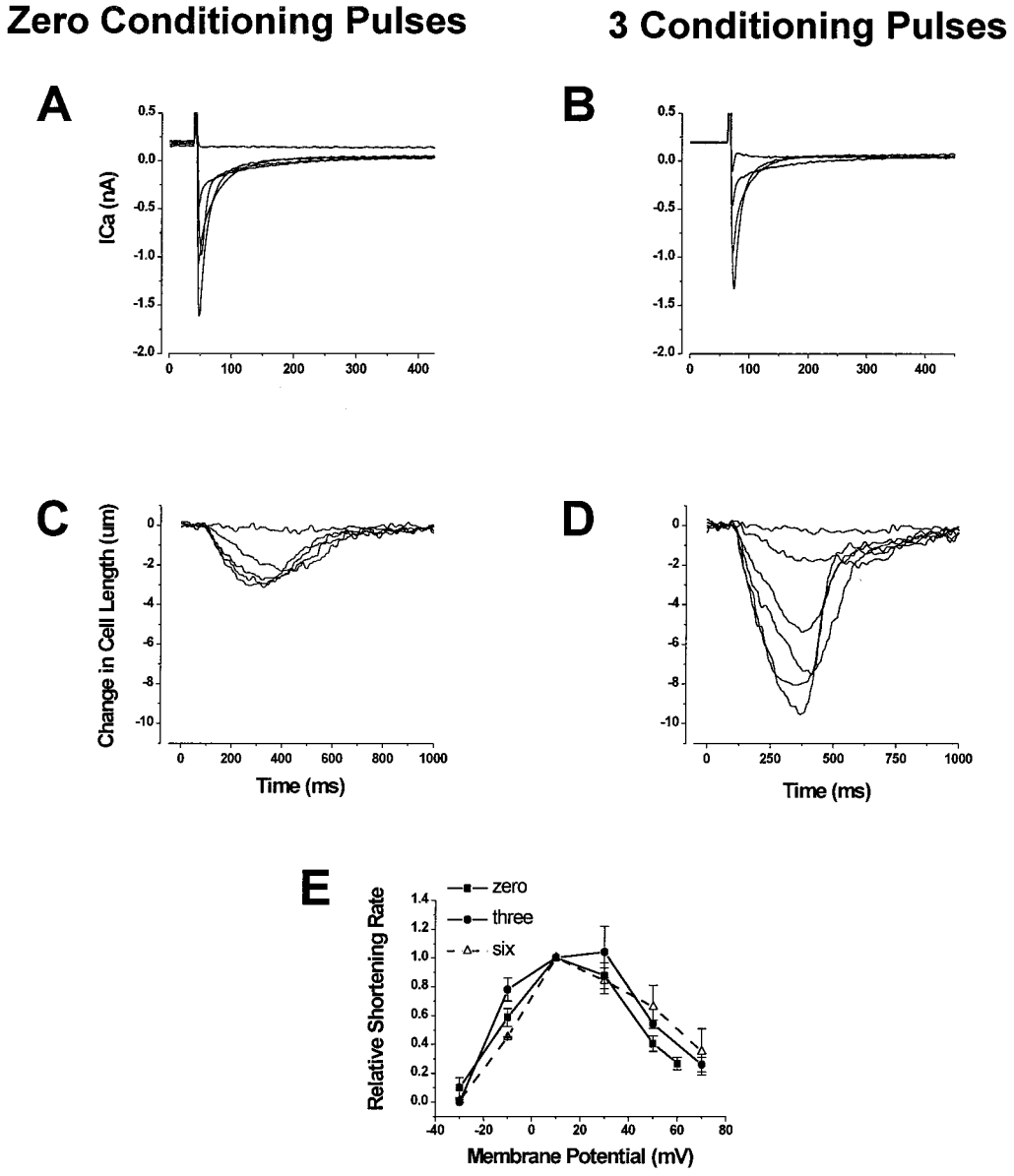

FIGURE 3 Influence of SR Ca content on the shape of the shortening-voltage relationship. Cells ($n = 6$) were voltage clamped with pipettes containing 0 mM Na and superfused with a solution containing 0 mM Na (replaced with Li^+) to eliminate both forward and reverse Na-Ca exchange (see Results). Varying numbers of conditioning pulses (0, 3, or 6) from −40 mV to +10 mV were applied before each test pulse. In the absence of a Na gradient, SR Ca content increases with each conditioning pulse. Sequential pulses (1–6) produce progressively larger contractions. After the conditioning pulses, test pulses to membrane potentials between −30 and +70 mV were applied at three different levels of SR loading (established with zero, three, or six conditioning pulses). After the test pulse, the cell was superfused with normal Tyrode's solution ([Na] = 145 mM), and four pulses to +10 mV were applied. With external Na present, some of the Ca released from the SR during each contraction could be extruded by forward Na-Ca exchange. This allowed the cell to discharge the Ca that had accumulated in the SR during the preceding series of conditioning pulses. Within four beats, a steady level of SR loading (as assessed by contraction amplitude) was achieved. Thus consistent SR loading should have been present at the beginning of each series of test pulses. (*A* and *C*) Examples of Ca currents and cellular contractions in the same cell with zero conditioning pulses or (*B* and *D*) three conditioning pulses. Clamp steps to −30, −10, +10, +30, +50, and +70 mV are shown. (*E*) Using this experimental protocol, both I_{Ca} and cellular contractions had a bell-shaped relationship with voltage, regardless of the number of prior conditioning pulses (i.e., with varying SR Ca content). Normalization points (mean peak shortening rate at a potential of +10 mV in each group) are as follows: zero conditioning pulses, shortening rate = 0.021 μm/ms; three conditioning pulses, shortening rate = 0.033 μm/ms; six conditioning pulses, shortening rate = 0.091 μm/ms. The three curves were not statistically different.

tween each application of nifedipine. In this protocol pipettes contained 10 mM Na.

Application of nifedipine produced complete block of I_{Ca}. This is important because even a small residual Ca current might trigger SR Ca release. Despite the absence of measurable I_{Ca}, a slow but substantial contraction still occurred during depolarization. We studied 10 cells with this protocol. As expected, we observed the usual bell-shaped relationship between I_{Ca} and voltage (Fig. 4 *A*). Currents recorded in the presence of nifedipine were subtracted from

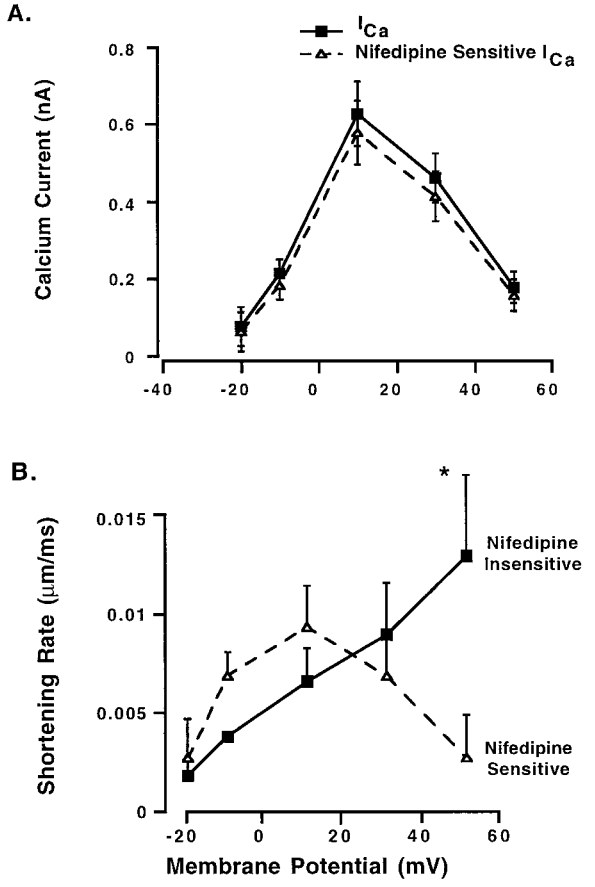

FIGURE 4 Contractions evoked during the rapid application of nifedipine (see Results for experimental protocol). Data are mean ± SEM of 10 cells. (*A*) There is a bell-shaped relationship between whole-cell I_{Ca} and voltage. Almost all of the inward current was blocked by nifedipine (nifedipine-sensitive I_{Ca}), suggesting that there was little or no Ca influx through Ca channels. (*B*) The contractions at each test potential were divided into nifedipine-sensitive (the portion of the contraction blocked by nifedipine) and nifedipine-insensitive (the portion remaining in nifedipine) components. The nifedipine-sensitive contractions have a bell-shaped relationship with voltage and are probably triggered by the Ca current. In contrast, the nifedipine-insensitive contractions increase with voltage. * $p < 0.05$ versus nifedipine-sensitive contractions. We speculate that the nifedipine-insensitive portions of the contractions are triggered by reverse Na-Ca exchange.

those in normal Tyrode's solution. This difference was referred to as the "nifedipine-sensitive current." The nifedipine-sensitive Ca current is almost identical to the unblocked *I-V* relationship, indicating that the whole-cell currents are relatively uncontaminated (Fig. 4 *A*). Cellular contractions were analyzed in a similar fashion. The difference between contractions in the presence and absence of nifedipine is designated as "nifedipine-sensitive contractions," and the contractions remaining in nifedipine are called "nifedipine-insensitive contractions." We plotted the nifedipine-sensitive and -insensitive components of contraction at each membrane potential (Fig. 4 *B*). The nifedipine-sensitive components of the contractions have a bell-shaped relationship with voltage, and presumably represent the portion of the contraction attributable to I_{Ca}. In comparison, the nifedipine-insensitive contractions, that portion of the contraction remaining in nifedipine, increased with voltage. Despite a fairly wide cell-to-cell variability in the amount of contraction remaining during exposure to nifedipine, there were significant differences between the nifedipine-sensitive and nifedipine-insensitive portions of the contraction at the more positive potentials. We hypothesize that the nifedipine-insensitive contraction is due to CICR triggered by reverse Na-Ca exchange.

Are nifedipine-insensitive contractions caused by "triggered" SR Ca release?

To confirm that the nifedipine-insensitive contractions were due to triggered SR Ca release, we pretreated cells for 1 h with ryanodine (5 μM) and thapsigargin (5 μM) to block SR Ca release and reuptake ($n = 3$ cells). Cells were then patch clamped with a pipette containing 10 mM Na. Voltage clamp steps (400 ms) from −30 to +70 mV ($V_H = -40$ mV) were applied after a series of four conditioning pulses to +10 mV. With the SR disabled, I_{Ca} could elicit late-peaking contractions that were ~70% of the amplitude seen in control cells. However, with the SR disabled, nifedipine-insensitive contractions could not be elicited (data not shown). Thus I_{Ca} may be of sufficient magnitude to directly activate the myofilaments in the absence of a functional SR; however, reverse Na-Ca exchange does not seem to be capable of directly causing contractions during 400-ms depolarizations to positive potentials less than or equal to +70 mV.

Are reverse Na-Ca exchange currents present under conditions in which nifedipine-insensitive contractions can be elicited?

If contractions are triggered by Na-Ca exchange, then the relationship between these contractions and voltage should be similar to the relationship between sodium-calcium exchange current (I_{NaCa}) and voltage. Because exchange currents responsible for triggering SR Ca release are likely to be small and obscured by larger currents that are activated at the same time, we used a series of inhibitors and subtraction methods to detect these currents. First, we voltage clamped cells with suction pipettes containing 0 ($n = 4$), 10 ($n = 8$), or 15 ($n = 4$) mM Na. Pipettes contained 130 mM CsCl to block outward K currents (Meier and Katzung, 1981). In addition, 4-aminopyridine (2 mM) was present in the bath throughout this series of experiments to specifically block the transient outward K current (I_{to}) (Kenyon and Gibbons, 1979). Ca currents were activated by clamping from a holding potential of −40 mV to potentials between −10 and +70 mV (Fig. 5 *A*). Next, we blocked I_{Ca} by exposing the cell to nifedipine (20 μM) before the voltage clamp steps. Removal of I_{Ca} revealed a family of outward currents that was dependent on voltage (Fig. 5 *B*). Removal of I_{Ca} probably also removed any Ca-dependent Cl^- cur-

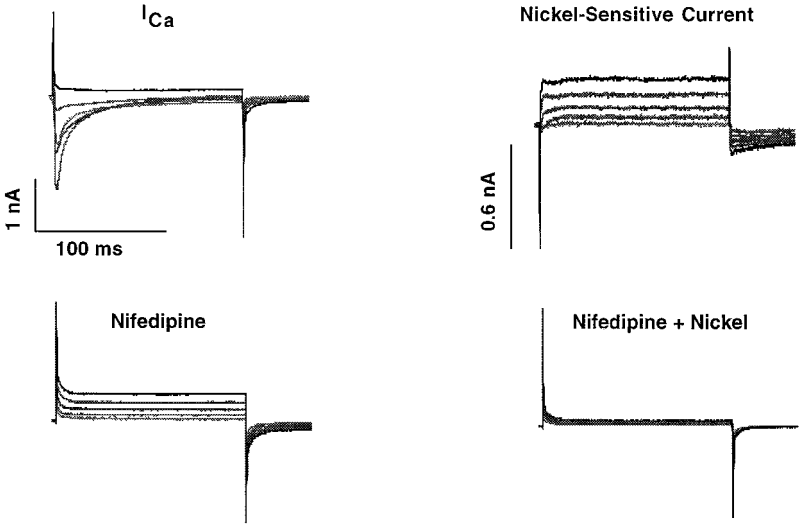

FIGURE 5 Measurement of putative Na-Ca exchange currents. In this representative example, the cell was voltage clamped with a pipette containing 15 mM Na. The cell was held at −40 mV. (*A*) The upper left panel shows Ca currents produced by voltage clamp steps between −30 and +70 mV (in 10-mV increments). (*B*) The cell was then exposed to 20 μM nifedipine, and the voltage clamp protocol was repeated. An outward current emerged when I_{Ca} was blocked. (*C*) Next, the cell was superfused with nifedipine and nickel (5 mM) (*lower right*). The outward current was reduced under these circumstances. (*D*) Finally, the currents in nickel plus nifedipine were subtracted from those in nifedipine alone. The nickel-sensitive difference current is probably due to reverse Na-Ca exchange.

rents that might have been present (Zygmunt, 1994). We assumed that this family of outward currents was either dominated by or contained outward Na-Ca exchange current. Therefore, we superfused the cells with a solution containing both nifedipine (20 μM) and nickel (5 mM). Because this concentration of nickel inhibits Na-Ca exchange, previous investigators have used nickel to identify and isolate Na-Ca exchange currents (Kimura et al., 1987; Beuckelmann and Wier, 1988; Wier, 1991). The series of voltage clamp steps was repeated in this solution. Although nickel is a nonspecific inhibitor, all major ionic currents other than Na-Ca exchange should already have been blocked before administration of nickel. It is clear that nickel reduced the amplitude of the outward currents at all potentials (Fig. 5 *C*). To detect the putative outward exchange current, we digitally subtracted the family of currents measured in the presence of both nickel and nifedipine from those in nifedipine alone:

$$\text{Putative } I_{NaCa} = I_{\text{out, nifedipine}} - I_{\text{out, nifedipine+nickel}} \qquad (2)$$

This produced a family of outward currents (Fig. 5 *D*) that increased with depolarization. We believe that these nickel-sensitive difference currents are attributable to reverse Na-Ca exchange. The nickel-sensitive currents peak in ~10 ms and then remain relatively constant for the remainder of the 150-ms voltage clamp step. The subtraction procedure also revealed a family of inward currents upon repolarization of the cell (Fig. 5 *D*). These tail currents are generally accepted to represent forward Na-Ca exchange (Eagan et al., 1989).

If the outward currents are due to reverse Na-Ca exchange, then their magnitude should depend on extracellular [Ca] as well as intracellular [Na]. Therefore, we repeated the protocol described above in cells superfused with Tyrode's solution containing 1 mM Ca instead of the usual Tyrode's solution containing 2.7 mM Ca. We found that the outward currents were significantly smaller when extracellular Ca was decreased (Fig. 6 *B*).

Finally, to determine if 4-aminopyridine-resistant chloride currents were contaminating the Na-Ca exchange currents, we repeated the above protocols using nominally chloride free pipette and superfusing solutions ($n = 5$ cells). The pipette solution included (in mM) 130 CsOH; 10 HEPES; 5 disodium adenosine 5′-triphosphate; 5.9 $MgSO_4$; 0.02 EGTA (adjusted to pH 7.1 with H_2SO_4). The superfusing solution was composed of (in mM) 137 glutamic acid (monosodium salt); 10 HEPES; 1 $MgSO_4$; 2.7 $CaSO_4$; 5.4 aspartic acid (monopotassium salt); 20 CsOH; 2 4-aminopyridine; pH was adjusted to 7.4 with NaOH. Nifedipine (20 μM) or nickel (5 μM) was added to the superfusing solution and applied to the cell with the rapid solution switching device. Under these conditions, we found that the nickel-sensitive difference currents were of a magnitude similar to those of currents described above. In addition, the shapes of the currents were similar (data not shown).

Properties of the putative reverse Na-Ca exchange current

There are several properties that the nickel-sensitive currents should exhibit if they are attributable to reverse Na-Ca exchange. Sensitivity to nickel is implicit in the method of measuring these currents. First, I_{NaCa} should have a relationship with voltage that is roughly exponential (Kimura et al., 1987; Ehara et al., 1989). In fact, the current-voltage relationships we measured were very well fitted by a monoexponential relationship (mean $r = 0.994 \pm 0.002$; Fig. 6). Second, the amplitude of outward Na-Ca exchange currents should be highly dependent on intracellular [Na] (Miura and Kimura, 1989). We found very little outward current with 0 mM Na in the pipette. The small outward currents measured under these circumstances probably represent residual Na-Ca exchange due to incomplete removal of intracellular Na with the whole-cell patch-clamp technique. There was a marked upward shift in the current-voltage relationship with

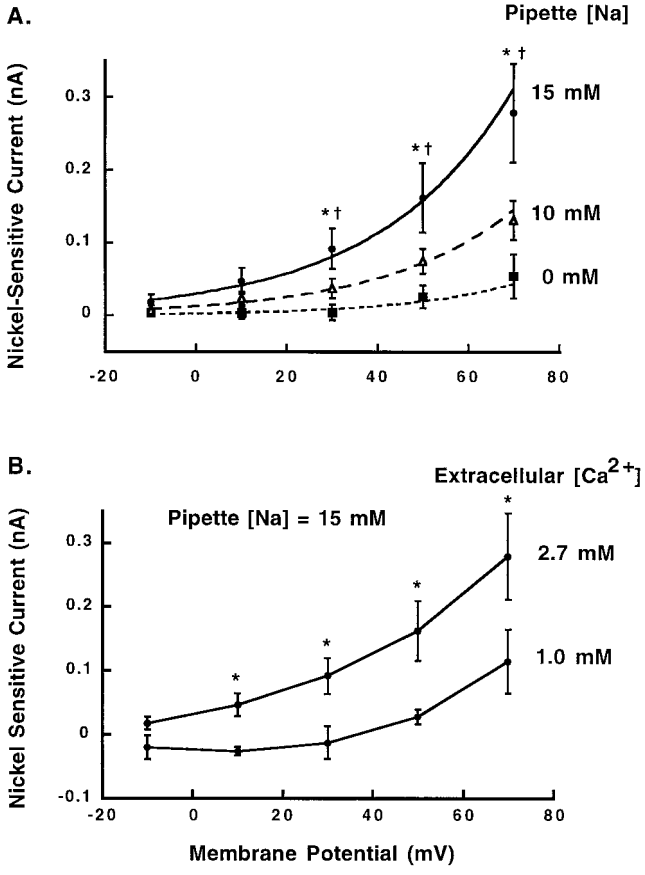

FIGURE 6 Dependence of nickel-sensitive currents on $[Na]_i$ and $[Ca^{2+}]_o$. Currents were measured as shown in Fig. 5. (*A*) Cells were voltage clamped with pipettes containing 0 ($n = 4$), 10 ($n = 8$), or 15 ($n = 4$) mM Na. All data are mean ± SEM. The currents increase monoexponentially with voltage at each pipette [Na] (mean $R = 0.994 \pm 0.002$). There is very little current with zero mM Na in the pipette, and a prominent upward shift in the *I-V* relationships with 10 and 15 mM pipette Na. * $p < 0.05$ versus 0 mM Na; $p < 0.05$ versus 10 mM Na. (*B*) The outward currents were reduced in amplitude when extracellular $[Ca^{2+}]$ was reduced from 2.7 to 1.0 mM. These characteristics are compatible with our hypothesis that these are Na-Ca exchange currents.

increased pipette [Na] (Fig. 6 *A*). Third, reduction of extracellular Ca from 2.7 to 1.0 mM resulted in a reduction in the amplitude of the outward currents (Fig. 6 *B*). Finally, the presence of inward tail currents upon repolarization supports the view that the outward currents are due to reverse exchange (Eagan et al., 1989). This is because in the absence of I_{Ca}, Ca extrusion by forward Na-Ca exchange should be comparable in magnitude to that entering by reverse exchange. The tail currents (forward Na-Ca exchange) were also smaller when pipette [Na] or extracellular [Ca] was lower. In summary, the currents demonstrate the appropriate dependence on voltage, $[Na]_i$, $[Ca]_o$, and sensitivity to nickel that are anticipated if these were reverse Na-Ca exchange currents. The fact that these currents are not initially transient introduces a difficulty that we shall discuss later.

Calcium fluxes through I_{Ca} and I_{NaCa}

To compare Ca entry via I_{Ca} and I_{NaCa}, we integrated the first 150 ms of the currents recorded during the protocols designed to isolate these two currents. The current integrals were then converted to absolute quantities of Ca influx according to the following formula:

$$\text{Ca entry} = \text{integral of current}/Z \times F \qquad (3)$$

where Ca entry is in moles, current integrals are in coulombs, Z is the charge movement per ion of Ca entry, and F is Faraday's constant (9.648×10^4 moles/coulomb). $Z = 2$ for I_{Ca} and 1 for I_{NaCa}, assuming a stoichiometry of 3 Na^+:1 Ca^{2+} (Reeves and Hale, 1984). The absolute amount of Ca entry by each mechanism is dependent on membrane potential (Fig. 7). For purposes of comparison, we can calculate net Ca entry at a membrane potential close to the plateau of the normal action potential (+30 mV) with 10 mM $[Na]_p$. Under the conditions of our experiments, Ca influx is ~10-fold greater when it occurs via L-type Ca current than by reverse Na-Ca exchange (without I_{Ca}) at this potential (Fig. 7). Thus it is unlikely that Ca entry occurring solely by the Na-Ca exchanger would produce enough change in cytosolic [Ca] to produce contractions, unless there is also a component of triggered SR Ca release.

DISCUSSION

We have obtained five main results: 1) Dialyzing [Na] strongly influenced the shape of the cellular shortening versus voltage relationship and the apparent gain of SR Ca release. 2) The divergence between the shortening-voltage and I_{Ca}-voltage relationships at higher dialyzing [Na] was not caused by increased SR Ca content. 3) Cellular contrac-

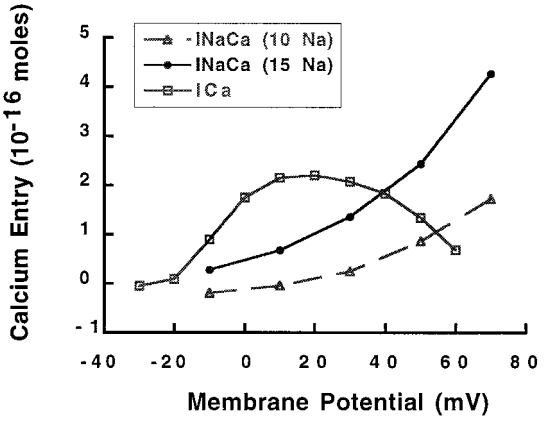

FIGURE 7 Calculated cellular Ca entry occurring via Ca current or reverse Na-Ca exchange alone. Calcium entry was calculated by integrating the first 150 ms of the whole-cell Ca currents recorded with no blockers present ($[Na]_p = 10$ mM), or the nickel-sensitive currents recorded using the method shown in Fig. 5. Current integrals were converted to absolute $[Ca^{2+}]$'s, using Faraday's constant and the known charge movements during each type of current (see Results). Calcium entry by the two pathways is voltage dependent, and Na-Ca exchange is strongly influenced by $[Na]_p$.

tions could still be elicited when I_{Ca} was completely blocked by rapid application of nifedipine. The nifedipine-sensitive component had a bell-shaped relationship with voltage, whereas the nifedipine-insensitive component increased with voltage. 4) Nifedipine-insensitive contractions could not be elicited when the SR was disabled with ryanodine and thapsigargin. 5) Nickel-sensitive outward currents (I_{NaCa}) had an *I-V* relationship that was qualitatively similar to that of the nifedipine-insensitive contractions. Together, these data support the contention that reverse Na-Ca exchange can contribute to contractions with dialyzing [Na] in the physiological range and without SR Ca overload.

Asymmetry of the shortening-voltage relationship

Our findings from unloaded cell shortening are similar to previous reports in which Ca transients or contractile force was measured (Vornanen et al., 1994; Levi et al., 1996). Our data expand on previous work (Levi et al., 1996), which also suggests that changes in SR Ca cannot explain the Na-dependent enhancement of contractility at positive potentials. We used a different protocol for varying SR content. Fig. 3 clearly demonstrates that in the absence of a sarcolemmal Na gradient, contractions were much larger after conditioning pulses were applied, even though I_{Ca} remained constant. Although the fractional SR Ca release and the rate of SR Ca release both appear to increase as SR content is increased, this should cause an upward shift in the absolute value of shortening at any given voltage, but no change in the shape or symmetry of the relationship (Bassani et al., 1995; Terraciano et al., 1995).

Measurements of gain of SR Ca release

Our measurements of gain reflect early events during the upstroke of the Ca transient (i.e., they are referrable to the rapid release of Ca from SR stores). When Na is present in the pipette solution, it should be possible for both Ca current and reverse Na-Ca exchange to participate in E-C coupling. However, only I_{Ca} was measured directly. If Na-Ca exchange contributed to the upstroke of the Ca transient, then we expected the gain of SR Ca release to increase with internal Na and voltage. This is precisely what we observed (Fig. 2). These results strengthen our conclusion that Na-Ca exchange may significantly enhance SR Ca release at positive membrane potentials. The very marked increase in gain under conditions designed to enhance reverse Na-Ca exchange could imply that there is synergy between the two triggering mechanisms.

Contractions in the absence of I_{Ca}

There was significant cell-to-cell variability in the magnitude of nifedipine-insensitive contractions. Some cells had little contraction with nifedipine present, whereas others had nearly normal contraction amplitude and rates (even though I_{Ca} appeared to be completely eliminated in all of these cells). The reasons for this variation are not clear. It is possible that relatively small differences in diastolic Ca levels in the cytoplasm might change the amount of trigger Ca required to produce opening of the release channel. Because there is a sigmoidal relationship between the probability of SR Ca release channel opening (P_o) and cytosolic [Ca^{2+}], small changes in local [Ca^{2+}] could cause a shift from a relatively flat to a relatively steep portion of the P_o versus $[Ca^{2+}]_i$ curve (Copello et al., 1997). Alternatively, the extent to which the Ca regulatory site of the exchanger was saturated could significantly effect the extent of triggering by reverse exchange (Kimura et al., 1986; Hilgemann, 1990; Noble et al., 1991). This would depend upon diastolic Ca levels, which might vary significantly from cell to cell. If $[Ca^{2+}]_i$ is less than 50 nM, Na-Ca exchange will be inhibited and Ca entry by this mechanism might not trigger SR Ca release (Kimura et al., 1987). Last, it is possible that despite intracellular dialysis with micropipettes, there was significant variation in the concentration of intracellular Na. If this were the case, then the contribution of reverse Na-Ca exchange to the trigger for SR Ca release would be expected to be rather variable, because intracellular [Na] seems to be a major determinant of this process (see Fig. 1).

The elimination of contractions by the combination of ryanodine, thapsigargin, and nifedipine suggests that the nifedipine-insensitive contractions are indeed triggered events. It is interesting that Na-Ca exchange can produce triggered contractions but not direct activation of the myofilaments. This is most likely explained by 1) differences in the length of the diffusion pathways from the Ca influx sites on the sarcolemma to the contractile elements and the ryanodine receptors; and 2) differing sensitivities of the contractile elements and the ryanodine receptors to Ca^{2+}. Ca^{2+} entry by Na-Ca exchange may simply be too slow to reach the myofilaments during a 400-ms clamp step. Indeed, our calculations suggest that it is unlikely that the exchanger could cause a large enough Ca flux across the sarcolemma in 400 ms to produce a phasic contraction or Ca transient without having a triggered component (Fig. 7). Nontriggered contractions could probably be elicited by longer clamp steps to very positive potentials. It is also possible that the Ca entering via the exchanger serves more to amplify the triggering effects of the L-type Ca current than to directly produce CICR (see below).

Demonstration of Na-Ca exchange currents

The demonstration of putative Na-Ca exchange currents that have a relationship with voltage that is similar to the nifedipine-insensitive contractions supports the idea that Na-Ca exchange may underlie the Na_i- and voltage-dependent portion of the contractions. Several issues relating to the nickel-sensitive currents should be discussed. The first question is,

why don't these currents clearly decay (i.e., show an initial transient)? Although there was a small transient component in our current records (Fig. 5 *D*), we expected the reverse Na-Ca exchange currents elicited by voltage clamp steps to rapidly increase to a maximum, and then to decline as there was collapse of the sarcolemmal Ca gradient (due to Ca influx via Na-Ca exchange or to SR Ca release). Transient behavior of reverse exchange currents has been predicted in models of cellular currents during action potentials (Luo and Rudy, 1994). However, the Na-Ca exchange currents we measured during square voltage clamp steps under conditions where most other ionic currents were blocked are likely to be quite different from exchange currents occurring during a normal action potential.

We propose several explanations for our observation that the outward currents we measured do not show a clear peak and subsequent decline. First, the transient component of the current may occur extremely early and thus may be hidden within the capacitive transient. This transient component could be lost during the subtraction procedure if there were even small changes in access resistance to the cell between recordings. Such changes occur commonly, particularly when the composition of the superfusing solution is changed. Second, it is possible that the Ca released from the SR does not diffuse freely into the "restricted space" adjacent to the exchanger (Stern, 1992). This seems reasonable because positive feedback of the CICR process is not seen under most circumstances. Third, the increased [Ca] in the vicinity of the exchanger may cause increased occupancy of both the kinetic and regulatory Ca binding sites of the exchanger. Because the first will tend to decrease the rate of exchange whereas the latter will have a stimulatory effect, there may be no net effect. Fourth, it is possible that there are contaminating currents that obscure either the peak or the decaying phase. We did not find that changing to Cl^--free solutions revealed a prominent transient component. Finally, it is possible that the exchange current was saturated under our experimental conditions, thus making it appear to be a sustained current.

Despite the uncertainties raised in the preceding discussion, we have strong evidence that the currents we measured are in fact attributable to Na-Ca exchange. We made considerable efforts to eliminate all possible contaminating currents. Outward K currents should have been blocked by the replacement of internal K with Cs (by micropipette dialysis). In addition, 4-aminopyridine was present in the superfusing solutions for these experiments. Finally, our currents are unlikely to be Ca activated outward Cl^- currents for the following reasons: 1) the shape of the nickel-sensitive current-voltage relationship is exponential, whereas Cl^- currents have a bell-shaped relationship with voltage (Zygmunt, 1994); 2) the use of nifedipine should markedly attenuate chloride currents because they are activated by a rise in intracellular Ca; 3) outward currents were still evident when nominally chloride-free internal and external solutions were used. As previously mentioned, the currents we measured show the appropriate voltage and ion dependence for Na-Ca exchange current.

We arbitrarily chose to integrate the first 150 ms of I_{Ca} and I_{NaCa}. This duration encompasses the period when SR release is expected to occur and coincides temporally with the development of contraction and the peak cellular shortening rate. Although it could be argued that CICR probably occurs over a shorter time span (10–20 ms), a slower rate of Ca^{2+} entry as might occur during I_{NaCa} alone might produce a slower more sustained Ca release. This is compatible with the slower rate of force development and more prolonged contractions we observed after exposure to nifedipine. Our estimates of calcium influx by the L-type Ca current are very comparable to other values in the literature (Isenberg and Klockner, 1982; Bers, 1991). Fabiato suggested that Ca fluxes of this magnitude would not cause direct activation of the myofilaments (Fabiato, 1983). The estimations of Ca entry by the two mechanisms are compatible with our data showing that I_{Ca} can produce relatively large contractions when the SR has been poisoned; however, I_{NaCa} does not produce contractions in the absence of SR function. If either I_{Ca} or I_{NaCa} were delivered into a restricted subsarcolemmal space, the rise in local [Ca^{2+}] would be much higher than if the Ca^{2+} had diffused freely into the entire accessible cell volume. Thus it seems reasonable that both mechanisms could produce CICR.

Comparison with previous studies

A number of studies have suggested that reverse Na-Ca exchange could directly or indirectly trigger SR Ca release under some conditions (Berlin et al., 1987; Leblanc and Hume, 1990; Kohmoto et al., 1994; Lipp and Niggli, 1994; Nuss and Houser, 1992; Levi et al., 1994; Vornanen et al., 1994; Vites and Wasserstrom, 1996; Wasserstrom and Vites, 1996). Other investigators have questioned whether Na-Ca exchange can trigger SR release (Bouchard et al., 1993; Cannell et al., 1995; Lopez-Lopez et al., 1995). Recently, two groups reported that elementary SR Ca release events visualized with confocal microscopy closely followed the behavior of sarcolemmal Ca channel openings (Cannell et al., 1995; Lopez-Lopez et al., 1995). According to one report, Ca sparks were not seen at membrane potentials more positive than the reversal potential of the Ca current unless cells showed evidence of Ca overload (Lopez-Lopez et al., 1995). However, depolarization to potentials greater than +50 mV did produce an increase in peak spatially averaged Ca concentration. Those findings imply that reverse Na-Ca exchange does not trigger SR Ca release.

How do we reconcile the results of the confocal microscopy studies with our own data and those of other authors who have seen evidence of triggering by reverse exchange? Several factors may be involved. First, most studies done in rat myocytes suggest that Na-Ca exchange has a minimal function in triggering SR release, whereas its role seems to

be substantially greater in other species (Bers et al., 1990; Cleeman and Morad, 1991; Sham et al., 1992; Bouchard et al., 1993). Thus species differences in Na-Ca exchanger density, SR function, or subcellular geometry may be important. Second, exchanger function is temperature-dependent, such that lower temperature (i.e., room temperature) tends to minimize the apparent contribution of reverse exchange, whereas the participation of the exchanger in triggering SR release is much more obvious at higher temperatures (Vornanen et al., 1994). Third, experiments using relatively low pipette Na concentrations (e.g., 0.5–5 mM) will diminish the possible portion of the trigger due to reverse exchange. Fourth, experimental protocols in which organic calcium channel blockers are applied continuously to cells may result in depletion of SR Ca stores and/or a decrease in cytosolic [Ca], leading to the erroneous conclusion that contractions cannot be triggered in the absence of I_{Ca}. Other less clearly defined variables that may affect SR Ca release (e.g., pipette [Mg]) could certainly be significant as well. Finally, in experiments in which cadmium was used to block L-type Ca currents, the contribution of the Na-Ca exchanger may be underestimated, because it is now known that cadmium inhibits Na-Ca exchange as well as Ca current (Hobai et al., 1997).

Our present data add to the case for involvement of the Na-Ca exchanger in triggering SR Ca release. A major issue is whether the exchange currents we recorded are of sufficient magnitude to induce CICR. When considering this question, it is important to recall that the magnitude of Na-Ca exchange currents will be half the size of an L-type Ca current that produces an equivalent absolute Ca flux. This follows from the stoichiometry of the Na-Ca exchange mechanism, in which three Na^+ ions are exchanged for each Ca^{2+}. Another very important consideration is that Ca entry by the exchanger or L-type Ca channels may be through a restricted space (Lederer et al., 1990). Hence a small Ca flux may cause a large local increase in [Ca]. It is difficult with our current limited knowledge of the diffusion barriers and the geometric relationship between dihydropyridine receptors, Na-Ca exchangers, and the foot processes of the ryanodine receptor to predict the concentrations of each ion in the dyadic cleft.

What is the role of Na-Ca exchange under physiological conditions?

It is relatively difficult to establish whether the Na-Ca exchanger is involved in "physiological" excitation-contraction, because physiological conditions can change abruptly. Even relatively simple parameters such as heart rate and body temperature may be substantially altered during normal activities and can vary widely during vigorous exercise. If SR Ca release is regulated by both I_{Ca} and I_{NaCa}, then it is reasonable to assume that the portion of the trigger due to the exchanger may be substantially greater at certain times. During the cardiac action potential, the Na current may cause a large subsarcolemmal accumulation of Na. If this occurs near the Na-Ca exchanger, reverse exchange may be favored much more than would occur with depolarization alone. Furthermore, increased heart rate (or stimulation frequency), as would occur during exertion, may increase intracellular Na activity (Harrison et al., 1992). This will also tend to favor reverse exchange. If reverse exchange is capable of triggering SR Ca release under some circumstances, it is not difficult to envision a case in which that function may be fairly prominent, even if this role is relatively small under normal resting conditions. If both I_{Ca} and I_{NaCa} are involved in CICR, then any circumstances that accentuate one may diminish the other. Conversely, conditions that inhibit one or the other of the triggers (e.g., low pipette Na concentrations) may cause the appearance of a single trigger for SR Ca release.

Nonlinear summation of triggers

Some investigators have suggested that Na-Ca exchange by itself triggers SR release that is too delayed to contribute to the rapidly rising Ca transients observed during normal action potentials (Sipido et al., 1997). Initially this observation seems to be at odds with our data. One potential explanation for this apparent paradox is that when both I_{Ca} and I_{NaCa} are functioning simultaneously, there may be synergy between the two triggers (i.e., they do not sum in a simple linear fashion). The open probability (P_o) of the SR Ca release channels has a sigmoid relationship with pCa (Copello et al., 1997). The ascending portion of this P_o versus pCa curve is rather steep. Depending on resting [Ca] in the region of the ryanodine receptor, a local increase in [Ca] caused by Na-Ca exchange might simply produce a shift along the flat foot of the curve toward the steeper region. Additional Ca entry via I_{Ca} would, in effect, be amplified because it is delivered on the steep portion of the relationship. Thus the net Ca entry by I_{NaCa} and I_{Ca} is unlikely to add in a simple linear fashion. Importantly, recent work by Grantham and Cannell suggests that significant reverse Na-Ca exchange is likely to occur during the early portion of an action potential (Grantham and Cannell, 1996). It is therefore entirely possible that a major effect of reverse exchange is to amplify the effect of direct triggering by I_{Ca}.

In summary, our findings suggest that both L-type Ca current and reverse Na-Ca exchange can contribute to SR Ca release and cellular contractions. The interaction between these two triggers may be quite complex. A major issue for future research will be to clarify how this interaction occurs under circumstances that recapitulate as closely as possible the conditions in an intact, contracting heart.

Presented in part at the Biophysical Society meeting, San Francisco, CA, February 1995, and the American Heart Association Meeting, Anaheim, CA, November 1995.

We are grateful for the excellent technical assistance provided by Phyllis Roberge and Gary S. Webster.

Supported by grants from the Department of Veterans Affairs, the National Institute of Health (HL 42357), the Norm Eccles Treadwell Foundation, and the University of Utah. This work was done during the tenure of a Clinician Scientist Award from the American Heart Association to SEL.

REFERENCES

Bassani, J. W. M., W. Yuan, and D. M. Bers. 1995. Fractional SR Ca release is regulated by trigger Ca and SR Ca content in cardiac myocytes. *Am. J. Physiol.* 268:C1313–C1329.

Berlin, J. R., M. B. Cannell, and W. J. Lederer. 1987. Regulation of twitch tension in sheep cardiac Purkinje fibers during calcium overload. *Am. J. Phyisol.* 253:H1540–H1547.

Bers, D. M. 1991. Control of cardiac contraction by SR Ca release and sarcolemmal Ca fluxes. *In* Excitation-Contraction Coupling and Cardiac Contractile Force. Kluwer Academic Publishers, Dordrecht, the Netherlands.

Bers, D. M., W. J. Lederer, and J. R. Berlin. 1990. Intracellular Ca transients in rat cardiac myocytes: role of Na-Ca exchange in excitation-contraction coupling. *Am. J. Physiol.* 258:C944–C954.

Beuckelmann, D. J., and W. G. Wier. 1988. Mechansim of release of calcium from sarcoplasmic reticulum of guinea-pig cardiac cells. *J. Physiol. (Lond.).* 405:233–255.

Bouchard, R. A., R. B. Clark, and W. R. Giles. 1993. Regulation of unloaded cell shortening by sarcolemmal sodium-calcium exchange in isolated rat ventricular myocytes. *J. Physiol. (Lond.).* 469:583–599.

Bridge, J. H. B., K. W. Spitzer, and P. R. Ershler. 1988. Relaxation of isolated ventricular cardiomyocytes by a voltage-dependent process. *Science.* 241:823–825.

Cannell, M. B., H. Cheng, and W. J. Lederer. 1995. The control of calcium release in heart muscle. *Science.* 268:1045–1049.

Cleeman, L., and M. Morad. 1991. Role of Ca^{2+} channel in cardiac excitation-contraction coupling in the rat: evidence from Ca^{2+} transients and contraction. *J. Physiol. (Lond.).* 432:283–312.

Copello, J. A., S. Barg, H. Onoue, and S. Fleischer. 1997. Heterogeneity of Ca^{2+} gating in skeletal muscle and cardiac ryanodine receptors. *Biophys. J.* 73:141–156.

Eagan, T. M., D. Noble, S. J. Noble, T. Powell, A. J. Spindler, and V. W. Twist. 1989. Sodium-calcium exchange during the action potential in guinea-pig ventricular cells. *J. Physiol. (Lond.).* 411:639–664.

Ehara, T., S. Matsuoka, and A. Noma. 1989. Measurement of reversal potential of Na^+-Ca^{2+} exchange current in single guinea-pig ventricular cells. *J. Physiol. (Lond.).* 410:227–249.

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

Fabiato, A. 1985. Time and calcium dependence of activation and inactivation of calcium-induced release of calcium from the sarcoplasmic reticulum of a skinned canine cardiac purkinje cell. *J. Gen. Physiol.* 85:247–289.

Fabiato, A., and F. Fabiato. 1975. Contractions induced by a calcium-triggered release of calcium from the sarcoplasmic reticulum of single skinned cardiac cells. *J. Physiol. (Lond.).* 249:469–495.

Grantham, C. J., and M. B. Cannell. 1996. Ca^{2+} influx during the cardiac action potential in guinea pig ventricular myocytes. *Circ. Res.* 79: 194–200.

Glantz, S. A. 1992. Primer of Biostatistics, 3rd ed. McGraw-Hill, New York.

Harrison, S. M., E. McCall, and M. R. Boyett. 1992. The relationship between contraction and intracellular sodium in rat and guinea pig ventricular myocytes. *J. Physiol. (Lond.).* 449:517–550.

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

Hobai, I. A., J. A. Bates, C. Howarth, and A. J. Levi. 1997. Inhibition by external Cd^{2+} of Na/Ca exchange and L-type Ca channel in rabbit ventricular myocytes. *Am. J. Physiol.* 272:H2164–H2172.

Isenberg, G., and U. Klockner. 1982. Calcium currents of isolated bovine ventricular myocytes are fast and of large amplitude. *Pflugers Arch.* 395:30–41.

Kenyon, J. L., and W. R. Gibbons. 1979. 4-Aminopyridine and the early outward current of sheep cardiac Purkinje fibers. *J. Gen. Physiol.* 73: 139–157.

Kimura, J., S. Miyame, and A. Noma. 1987. Identification of sodium-calcium exchange current in single ventricular cells of guinea pig. *J. Physiol. (Lond.).* 384:199–222.

Kimura, J., A. Noma, and H. Irisawa. 1986. Na-Ca exchange current in mammalian heart cells. *Nature.* 319:596–599.

Kohmoto, O., A. J. Levi, and J. H. B. Bridge. 1994. The relationship between reverse Na-Ca exchange and SR Ca release in guinea pig ventricular cells. *Circ. Res.* 74:550–554.

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

Lederer, W. J., E. Niggli, and R. W. Hadley. 1990. Sodium-calcium exchange in excitable cells: fuzzy space. *Science.* 248:283.

Levi, A. J., J. Li, S. E. Litwin, and K. W. Spitzer. 1996. Effect of internal sodium and cellular calcium load on voltage-dependence of the Indo-1 transient in guinea-pig ventricular myocytes. *Cardiovasc. Res.* 32: 534–550.

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

Lipp, P., and E. Niggli. 1994. Sodium current-induced calcium signals in isolated guinea-pig ventricular myocytes. *J. Physiol. (Lond.).* 474: 439–446.

London, B., and J. W. Krueger. 1986. Contraction in voltage-clamped, internally perfused single heart cells. *J. Gen. Physiol.* 88:475–505.

Lopez-Lopez, J. R., P. Shacklock, C. W. Balke, and W. G. Wier. 1995. Local calcium transients triggered by single L-type calcium channel currents in cardiac cells. *Science.* 268:1042–1045.

Luo, C. H., and Y. Rudy. 1994. A dynamic model of the cardiac ventricular action potential. I. Simulations of ionic currents and concentration changes. *Circ. Res.* 74:1071–1096.

Meier, H., and B. G. Katzung. 1981. Cesium blockade of delayed outward currents and electrically induced pacemaker activity in mammalian ventricular myocardium. *J. Gen. Physiol.* 77:531–547.

Miura, Y., and J. Kimura. 1989. Sodium-calcium exchange current. *J. Gen. Physiol.* 93:1129–1145.

Nabauer, M., and M. Morad. 1990. Ca^{2+}-induced Ca^{2+}-release as examined by photolysis of caged Ca^{2+} in single ventricular myocytes. *Am. J. Physiol.* 258:C189–C193.

Niggli, E., and W. J. Lederer. 1990. Voltage-independent calcium release in heart muscle. *Science*. 250:565–568.

Noble, D., S. J. Noble, G. Bett, Y. E. Earm, W. K. Ho, and I. K. So. 1991. The role of Na-Ca exchange during the cardiac action potential. *Ann. N.Y. Acad. Sci.* 639:334–353.

Nuss, B. H., and S. R. Houser. 1991. Voltage dependence of contraction and calcium current in severely hypertrophied feline ventricular myocytes. *J. Mol. Cell. Cardiol.* 23:717–726.

Nuss, H. B., and S. R. Houser. 1992. Sodium-calcium exchange-mediated contractions in feline ventricular myocytes. *Am. J. Physiol.* 263: H1161–H1169.

Reeves, J. P., and C. C. Hale. 1984. The stochiometry of the sodium-calcium exchange system. *J. Biol. Chem.* 259:7733–7739.

Schouten, V. J. A., and M. Morad. 1989. Regulation of Ca current in frog ventricular myocytes by holding potential, c-AMP and frequency. *Pflugers Arch.* 415:1–11.

Sham, J. K., L. Cleeman, and M. Morad. 1992. Gating of the cardiac Ca^{2+} release channel: the role of Na^+ current and Na^+-Ca^{2+} exchange. *Science.* 255:850–853.

Spitzer, K. W., and J. H. B. Bridge. 1989. A simple device for rapidly exchanging solutions surrounding a single cardiac cell. *Am. J. Physiol.* 256:C441–C447.

Sipido, K. R., M. Maes, and F. Van de Werf. 1997. Low efficiency of Ca^{2+} entry through the Na^+-Ca^{2+} exchanger as a trigger for Ca^{2+} release from the sarcoplasmic reticulum: a comparison between L-type Ca^{2+}

current and reverse-mode Na^+-Ca^{2+} exchange. *Circ. Res.* 81: 1034–1044.

Stern, M. D. 1992. Theory of excitation-contraction coupling in cardiac muscle. *Biophys. J.* 63:497–517.

Terraciano, C. M. N., R. U. Naqvi, and K. T. MacLeod. 1995. Effects of rest interval on the release of calcium from the sarcoplasmic reticulum in isolated guinea pig ventricular myocytes. *Circ. Res.* 77:354–360.

Vites, A. M., and J. A. Wasserstrom. 1996. Fast sodium influx provides an initial step to trigger contractions in cat ventricle. *Am. J. Physiol.* 271:H674–H686.

Vornanen, M., N. Shepherd, and G. Isenberg. 1994. Tension-voltage relations of single myocytes reflect Ca release triggered by Na/Ca exchange at 35°C but not 23°C. *Am. J. Physiol.* 267:C623–C632.

Wasserstrom, J. A., and A. M. Vites. 1996. The role of Na^+-Ca^{2+} exchange in activation of excitation-contraction coupling in rat ventricular myocytes. *J. Physiol. (Lond.).* 493:529–542.

Wier, W. G. 1991. Sodium-calcium exchange in intact cardiac cells. *Ann. N.Y. Acad. Sci.* 639:366–374.

Wier, W. G., T. M. Egan, J. R. Lopez-Lopez, and C. W. Balke. 1994. Local control of excitation-contraction coupling in rat heart cells. *J. Physiol. (Lond.).* 474:463–471.

Yue, D. T. 1987. Intracellular [Ca^{2+}] related to the rate of force development in twitch contraction of heart. *Am. J. Physiol.* 252: H760–H770.

Zygmunt, A. C. 1994. Intracellular calcium activates a chloride current in canine ventricular myocytes. *Am. J. Physiol.* 267:H1984–H1995.