Transport and Regulation of the Cardiac Na\textsuperscript{+}-Ca\textsuperscript{2+} Exchanger, NCX1

Comparison between Ca\textsuperscript{2+} and Ba\textsuperscript{2+}

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ABSTRACT Cardiac muscle fails to relax upon replacement of extracellular Ca\textsuperscript{2+} with Ba\textsuperscript{2+}. Among the manifold consequences of this intervention, one major possibility is that Na\textsuperscript{+}-Ba\textsuperscript{2+} exchange is inadequate to support normal relaxation. This could occur due to reduced transport rates of Na\textsuperscript{+}-Ba\textsuperscript{2+} exchange and/or by failure of Ba\textsuperscript{2+} to activate the exchanger molecule at the high affinity regulatory Ca\textsuperscript{2+} binding site. In this study, we examined transport and regulatory properties for Na\textsuperscript{+}-Ca\textsuperscript{2+} and Na\textsuperscript{+}-Ba\textsuperscript{2+} exchange. Inward and outward Na\textsuperscript{+}-Ca\textsuperscript{2+} or Na\textsuperscript{+}-Ba\textsuperscript{2+} exchange currents were examined at 30°C in giant membrane patches excised from Xenopus oocytes expressing the cloned cardiac Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger, NCX1. When excised patches were exposed to either cytoplasmic Ca\textsuperscript{2+} or Ba\textsuperscript{2+}, robust inward Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange currents were observed, whereas Na\textsuperscript{+}-Ba\textsuperscript{2+} currents were absent or barely detectable. Similarly, outward currents were greatly reduced when pipette solutions contained Ba\textsuperscript{2+} rather than Ca\textsuperscript{2+}. However, when solution temperature was elevated from 30°C to 37°C, a substantial increase in outward Na\textsuperscript{+}-Ba\textsuperscript{2+} exchange currents was observed, but not so for inward currents. We also compared the relative abilities of Ca\textsuperscript{2+} and Ba\textsuperscript{2+} to activate outward Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange currents at the high affinity regulatory Ca\textsuperscript{2+} binding site. While Ba\textsuperscript{2+} was capable of activating the exchanger, it did so with a much lower affinity (K\textsubscript{D} ~ 10 μM) compared with Ca\textsuperscript{2+} (K\textsubscript{D} ~ 0.3 μM). Moreover, the efficiency of Ba\textsuperscript{2+} regulation of Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange is also diminished relative to Ca\textsuperscript{2+}, supporting ~60% of maximal currents obtainable with Ca\textsuperscript{2+}. Ba\textsuperscript{2+} is also much less effective at alleviating Na\textsuperscript{+}-induced inactivation of NCX1. These results indicate that the reduced ability of NCX1 to adequately exchange Na\textsuperscript{+} and Ba\textsuperscript{2+} contributes to failure of the relaxation process in cardiac muscle.

KEY WORDS: sodium-calcium exchange • transport • regulation • calcium • barium

INTRODUCTION

Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange plays a major role in Ca\textsuperscript{2+} homeostasis in cardiac muscle. Removal of myoplasmic Ca\textsuperscript{2+} by this mechanism is essential for physiological cardiac relaxation (Bers, 1991). In general, removal of Ca\textsuperscript{2+} by Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange is equivalent to Ca\textsuperscript{2+} entry through L-type Ca\textsuperscript{2+} channels on a beat-to-beat basis (Bridge et al., 1990). Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange may also serve an important role as a Ca\textsuperscript{2+} entry mechanism during cardiac excitation. Several studies have demonstrated that this “reverse mode” of Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange can trigger sarcoplasmic reticulum Ca\textsuperscript{2+} release (LeBlanc and Hume, 1990; Kohmoto et al., 1994; Levi et al., 1994; Vornanen et al., 1994; Wasserstrom and Vites, 1996). Consequently, alterations in Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange function, exemplified by digitalis treatment (Lee and Dagastino, 1982) or alterations in the intracellular Na\textsuperscript{+} concentration (Harrison and Boyett, 1995), produce major effects on cardiac contractility.

Recently, several regulatory properties have been characterized for the cardiac Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger, NCX1. Detailed characterization of these regulatory mechanisms has been accomplished for the native and cloned cardiac Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger using the giant excised patch technique. Regarding ionic regulation, both Na\textsuperscript{+} and Ca\textsuperscript{2+} regulate exchange activity in addition to serving as the transport substrates (Hilgemann, 1990). Examination of outward (reverse) Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange currents reveals a complex waveform. The application of Na\textsuperscript{+} induces an outward current which undergoes a time-dependent inactivation. The extent of this inactivation is governed by both cytoplasmic Na\textsuperscript{+} and Ca\textsuperscript{2+} levels. However, in the presence of a constant level of cytoplasmic Ca\textsuperscript{2+} (e.g., 1 μM), both outward currents and the extent of inactivation increase as Na\textsuperscript{+} levels are increased. This behavior is referred to as Na\textsuperscript{+}-induced or I\textsubscript{Na} inactivation (Hilgemann et al., 1992b).

Cytoplasmic Ca\textsuperscript{2+} levels regulate exchange activity by influencing the extent of Na\textsuperscript{+}-induced inactivation and through an apparent direct activation of the exchange molecule (Hilgemann et al., 1992a, b). This direct pathway is referred to as I\textsubscript{Ca} inactivation where removal of cytosolic Ca\textsuperscript{2+} favors entry into an inactive (I\textsubscript{s}) state. Both forward and reverse modes of Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange are
regulated by cytoplasmic Ca\textsuperscript{2+} (Matsuoka et al., 1995), and the high affinity regulatory Ca\textsuperscript{2+} binding site has been identified for the cardiac exchanger, NCX1 (Levitsky et al., 1994; Matsuoka et al., 1995). The ability of other divalent cations to substitute for Ca\textsuperscript{2+} at the regulatory site has not been examined in detail.

Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange has a strict specificity for Na\textsuperscript{+} as the transported monovalent cation (Philipson and Nicoll, 1993). Less stringency is observed for transport of other divalent cations with Ba\textsuperscript{2+} and Sr\textsuperscript{2+} being transported to varying degrees. Earlier studies of this nature have used cardiac sarcosomal vesicles to examine radioisotope fluxes for different divalent cations. From these reports, it appeared that Ca\textsuperscript{2+} and Sr\textsuperscript{2+} were transported at nearly equal rates, whereas Ba\textsuperscript{2+} transport was ~20 times slower and exhibited a two- to threefold reduction in affinity for the exchanger (Tropper and Philipson, 1983; Tibbits and Philipson, 1985). More recent electrophysiological and fluorescence measurements yield conflicting results. In one instance, outward Na\textsuperscript{+}-Ba\textsuperscript{2+} exchange currents were not detectable from whole cell patch clamp experiments using guinea-pig ventricular myocytes (Kimura et al., 1987). However, Ba\textsuperscript{2+} uptake through the exchanger was readily detected in CHO cells expressing the bovine cardiac Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger (Chernaya et al., 1996). An experimental limitation of all the above studies is the difficulty in discriminating between transport and regulatory consequences for this ionic substitution. However, this limitation can be circumvented by using the giant excised patch clamp. In this study, we have compared the effects of Ca\textsuperscript{2+} and Ba\textsuperscript{2+} on properties of the cloned, cardiac Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger, NCX1. The specific goals were to examine how Ba\textsuperscript{2+} substitution alters the transport and regulatory properties of NCX1 and to determine if these differences can provide a reasonable account for why cardiac muscle fails to relax in Ba\textsuperscript{2+}-containing media.

METHODS

Myocyte Preparation and Shortening Measurements

Canine ventricular myocytes were provided by Dr. A. Lukas (University of Manitoba, Winnipeg, Canada) and were prepared as described previously (Lukas and Antzelowitch, 1993). Myocytes were superfused with (in mM): 136 NaCl, 10 HEPES, 8.33 NaH\textsubscript{2}PO\textsubscript{4}, 5.4 KCl, 1 MgCl\textsubscript{2}, 1 CaCl\textsubscript{2} or BaCl\textsubscript{2}, pH 7.4 (using NaOH) at 30°C. Field stimulation of myocytes at 0.5 Hz (1.1–1.5 times threshold) was performed using a Grass SD-9 Stimulator (Grass Instrument Co., Warwick, RI). Shortening was monitored with a video edge detection system (Crescent Electronics, Sandy, UT) and recorded using Axon Instruments (Foster City, CA) hardware and software as described previously (Hryshko et al., 1989).

Molecular Biological Techniques

Oocytes were obtained from Xenopus laevis as described previously (Hryshko et al., 1996). Oocytes were treated with collagenase (20 mg/ml) for 1 h, washed in Barth’s solution, treated with 100 mM K\textsubscript{2}HPO\textsubscript{4} for 15 min, washed, and stored overnight in fresh Barth’s solution. NCX1 cRNA was prepared using T3 Message mMachine (Ambion Inc., Austin, TX) according to the manufacturer’s instructions. Oocytes were injected with cRNA (~5 ng/oocyte), and activity was measured 3–6 d later.

Electrophysiological Techniques

Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange activity was measured using the giant excised patch clamp technique of Hilgemann (1989) as described previously (Matsuoka et al., 1995; Hryshko et al., 1996). Pipettes were pulled from borosilicate glass and polished to a final diameter of 20–40 μm. Pipettes were coated with a paraffin/mineral oil mixture to enhance patch stability and reduce electrical noise. For seal formation, oocytes were placed in a solution containing (in mM): 100 KOH, 100 MES, 5 EGTA, 5 MgCl\textsubscript{2}, pH 7.0 with MES. Gigaohm seals were formed by gentle suction and patches were excised by progressive movements of the pipette tip. Excised patches were in the inside-out configuration. For outward Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange current measurements, pipettes were filled with (in mM): 100 NMG-MES, 50 HEPES, 30 TEA-OH, 16 sulfamic acid, 8 CaCO\textsubscript{3}, 6 KOH, 0.25 ouabain, 0.1 niflumic acid, 0.1 flufenamic acid, pH 7.0 (using MES). Outward Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange currents were elicited by switching from a Li\textsuperscript{+} to Na\textsuperscript{+}-based superfusate containing (in mM): 100 Na- or Li-aspartate, 20 MOPS, 20 TEA-OH, 20 CoOH, 10 EGTA, 0–2.30 CaCO\textsubscript{3} or 0–7.55 Ba(OH)\textsubscript{2}, 1–1.5 Mg(OH)\textsubscript{2}, pH 7.0 (using MES or LiOH). The amounts of Ca\textsuperscript{2+} and Mg\textsuperscript{2+} were adjusted to yield free Mg\textsuperscript{2+} concentrations of 1 mM and various free Ca\textsuperscript{2+} concentrations as indicated. MAXC software was used to calculate free Ca\textsuperscript{2+} and Mg\textsuperscript{2+} concentrations (Bers et al., 1994). For inward current measurements, pipettes contained (in mM): 100 Na-MES, 20 TEA-MES, 20 Cs-MES, 10 HEPES, 10 EGTA, 4 Mg(OH)\textsubscript{2}, 0.2 ouabain, 0.1 niflumic acid, 0.1 flufenamic acid, 0.002 verapamil, pH 7.0. Inward Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange currents were activated by switching to the Li\textsuperscript{+}-based Ca\textsuperscript{2+} containing superfusates described above for outward current measurements. Current data were acquired and analyzed using Axon Instruments (Foster City, CA) hardware and software. Solution changes (~200 ms) were accomplished using a custom-built 20-channel computer-controlled solution switch. All experiments were conducted at 30 ± 1°C unless indicated otherwise. The rationale and design of the different types of experiments are summarized in Fig. 1.

RESULTS

Fig. 2 illustrates the effects of replacing 1 mM extracellular Ca\textsuperscript{2+} with 1 mM Ba\textsuperscript{2+} on electrically evoked shortening in a canine ventricular myocyte. Almost immediately, contraction fails and a contracture develops. Upon restoration of Ca\textsuperscript{2+} to the superfusate, the contracture gradually wanes and near normal resting length and contractions resume. We observed this pattern of contracture in ten cells from three different myocyte preparations. Initial shortening averaged 10.9 ± 0.8% (mean ± SD, n = 10) in the Ca\textsuperscript{2+}-containing superfusate. After recovery from the Ba\textsuperscript{2+}-induced contracture, shortening averaged 8.1 ± 1.0%. While numerous cellular processes are affected by this intervention, our specific goal was to determine how Ba\textsuperscript{2+} substitution affects the operation of the Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger. In particular, we sought to distinguish be-
between the effects of Ba$^{2+}$ substitution on transport vs. regulatory aspects of Na$^+$.Ca$^{2+}$ exchange function.

Fig. 3A shows inward Na$^+$.Ca$^{2+}$ and Na$^+$.Ba$^{2+}$ exchange currents from a single excised patch. Physiologically, this “forward” mode of transport corresponds to Ca$^{2+}$ extrusion. Currents were activated by the application of Li$^+$-based Ca$^{2+}$- or Ba$^{2+}$-containing solutions to the cytoplasmic surface of the patch at the concentrations indicated. The pipette solution contained 100 mM Na$^+$ and Ca$^{2+}$- and Ba$^{2+}$-containing solutions were applied in a random order. For Ca$^{2+}$-activation, note the progressive increase of inward currents observed up to concentrations between 30–300 $\mu$M. In contrast, at Ba$^{2+}$ concentrations up to 300 $\mu$M, inward currents were barely detectable. In eight patches showing robust Ca$^{2+}$-activated inward currents at 30°C, Ba$^{2+}$-activated currents were absent or barely detectable. Three patches were also examined at 37°C and did not exhibit substantial Ba$^{2+}$-activated inward exchange currents, whereas Ca$^{2+}$-activated currents were readily observed (not shown). We did not routinely examine higher Ba$^{2+}$ concentrations as these led to a small outward current at concentrations of 1 mM and above. This small outward current was also observed using pipette solutions containing Li$^+$ rather than Na$^+$ and may represent residual Ca$^{2+}$ activated Cl$^-$ conductance (see DISCUSSION).

Fig. 3B shows pooled data from nine patches for inward current measurements activated by Ca$^{2+}$ or Ba$^{2+}$. Each point represents the average from between three and nine determinations with data normalized to the inward current obtained at 3 $\mu$M Ca$^{2+}$. $K_D$ values are
Comparison between Ca\textsuperscript{2+} and Ba\textsuperscript{2+} on Na\textsuperscript{+}-Ca\textsuperscript{2+} Exchange

not reported as not all divalent concentrations could be examined in a single patch. For comparative purposes, currents obtained at 3 \textmu M Ca\textsuperscript{2+} (n = 9) exceeded currents obtained with 100 \textmu M Ba\textsuperscript{2+} (n = 4) by 17-fold and 300 \textmu M Ba\textsuperscript{2+} (n = 6) by 10-fold. As shown below (see Fig. 5), the absence of inward current is not a consequence of failure to activate the exchanger with Ba\textsuperscript{2+}.

Fig. 4 illustrates outward currents activated by the application of 100 mM Na\textsuperscript{+} to the cytoplasmic surface of the patch. Physiologically, this “reverse” mode of transport corresponds to Ca\textsuperscript{2+} entry. Different concentrations of regulatory Ca\textsuperscript{2+} were present before and during Na\textsuperscript{+} application, as indicated. Pipettes contained either 8 mM Ba\textsuperscript{2+} (Fig. 4, traces A and B) or Ca\textsuperscript{2+} (traces C and D). Several features are noteworthy. For Ba\textsuperscript{2+}-containing pipettes, we observed very small currents at 30°C (<20 pA in six separate patches). Although regulated by Ca\textsuperscript{2+}, the extent of I\textsubscript{2} regulation appeared blunted and Na\textsuperscript{+}-induced (I\textsubscript{1}) inactivation was not observed. At 37°C, six of six patches exhibited Na\textsuperscript{+}-Ba\textsuperscript{2+} exchange activity with regulatory properties more typical of those observed with Ca\textsuperscript{2+}-containing pipette solutions. In three patches for which both temperatures could be examined, a three to fourfold increase in current magnitude was observed for this 7°C increase in temperature, as shown in Fig. 4, A and B. Recall that this striking increase in outward Na\textsuperscript{+}-Ba\textsuperscript{2+} exchange currents was not evident for inward currents at 37°C. Comparatively, for Ca\textsuperscript{2+}-containing pipettes (Fig. 4, C and D), normal I\textsubscript{1} and I\textsubscript{2} regulation were observed at both temperatures. Typical for three patches where currents were obtained at both temperatures, Fig. 4, C and D, shows less augmentation of current (30–50% increase) at 37°C than that observed for Na\textsuperscript{+}-Ba\textsuperscript{2+} exchange. For both Ba\textsuperscript{2+}- and Ca\textsuperscript{2+}-containing pipettes, it is likely that we have underestimated the true temperature sensitivity as current rundown likely occurs during the 5–10 min period required to increase perfusate temperature.

The activity of Na\textsuperscript{+}-Ca\textsuperscript{2+} exchangers is regulated by the occupancy status of a high affinity Ca\textsuperscript{2+} binding site.
on the cytoplasmic surface of the molecule (Levitsky et al., 1994; Matsuoka et al., 1995; Hryshko et al., 1996). Consequently, alterations in the activation of Na\(^{+}\)-Ca\(^{2+}\) exchange activity by Ba\(^{2+}\) could also contribute to the reduced ability of NCX1 to transport Ba\(^{2+}\). To investigate this possibility, we compared regulation of outward Na\(^{+}\)-Ca\(^{2+}\) exchange currents by Ca\(^{2+}\) and Ba\(^{2+}\). Fig. 5, A and B, shows current traces obtained from a single patch at different concentrations of regulatory Ca\(^{2+}\) or Ba\(^{2+}\), respectively. At each concentration indicated, regulatory Ca\(^{2+}\) or Ba\(^{2+}\) was present before and during the application of 100 mM Na\(^{+}\) to activate the outward current. Pipette Ca\(^{2+}\) was constant at 8 mM. Note that Ba\(^{2+}\) was considerably less effective at activating Na\(^{+}\)-Ca\(^{2+}\) exchange compared to Ca\(^{2+}\). For example, virtually no current is observed at 3 \(\mu M\) Ba\(^{2+}\) whereas this level of Ca\(^{2+}\) leads to near maximal currents for Ca\(^{2+}\). In addition, the progressive loss of I\(_1\) inactivation with increasing Ca\(^{2+}\) is much less apparent for Ba\(^{2+}\)-regulated currents.

Fig. 5, C and D, shows typical concentration dependencies for Ca\(^{2+}\) and Ba\(^{2+}\) regulation, respectively, for outward exchange currents in two separate patches. The concentration ranges examined were selected to determine \(K_D\) and illustrate differences between Ca\(^{2+}\) and Ba\(^{2+}\) on steady-state current properties. Current levels were similar in these two different patches. The \(K_D\) for Ca\(^{2+}\) regulated peak I\(_{NaCa}\) was 0.35 \(\mu M\). The complex relationship of regulatory Ca\(^{2+}\) with I\(_1\) and I\(_2\) inactivation leads to a near linear relation for steady state currents up to 10 \(\mu M\) Ca\(^{2+}\), followed by a progressive decline as Ca\(^{2+}\) begins to compete with cytoplasmic Na\(^{+}\) (as seen in Fig. 5 A). Ba\(^{2+}\) regulated peak I\(_{NaCa}\) and steady-state I\(_{NaCa}\) exhibited \(K_D\)'s of 9.3 and 10.3 \(\mu M\), respectively, and I\(_1\) inactivation was not dramatically changed (as seen in Fig. 5 B). We observed similar differences in regulation of outward currents between Ca\(^{2+}\) vs. Ba\(^{2+}\) in 12 different patches.

Pooled data from 9 patches is shown in Fig. 6 for peak outward currents regulated by Ba\(^{2+}\) or Ca\(^{2+}\). Current values were normalized to that obtained for peak outward current in the presence of 3 \(\mu M\) regulatory Ca\(^{2+}\), allowing direct comparison of all data. Two features are obvious from this graph. First, the affinity of regulatory Ba\(^{2+}\) (\(K_D = 8.7 \mu M\)) is \(\sim 30\) times lower than that for Ca\(^{2+}\) (\(K_D = 260 \mu M\)). Second, the efficiency of Ba\(^{2+}\) regulation is substantially lower than that observed with Ca\(^{2+}\). Even at the highest Ba\(^{2+}\) concentrations examined, peak current was substantially lower than that observed for Ca\(^{2+}\).

Ba\(^{2+}\) appeared to be much less effective at alleviating Na\(^{+}\)-induced inactivation compared with Ca\(^{2+}\). This behavior is illustrated in Fig. 7 for pooled results from nine patches (3–9 determinations at each concentration). As regulatory Ca\(^{2+}\) was progressively increased, the difference in concentration range between these graphs.

![Figure 5](image-url)  
**Figure 5.** The effects of different concentrations of regulatory Ca\(^{2+}\) or Ba\(^{2+}\) on outward Na\(^{+}\)-Ca\(^{2+}\) exchange currents are shown for a single patch in A and B. The pipette solution contained 8 mM Ca\(^{2+}\). The different concentrations of regulatory Ca\(^{2+}\) (A) or Ba\(^{2+}\) (B) were present before and during the application of 100 mM Na\(^{+}\) to activate the current. Typical concentration dependencies of peak and steady-state (SS) outward currents are shown for regulation by Ca\(^{2+}\) (C) and Ba\(^{2+}\) (D) from two separate patches. Note the difference in concentration range between these graphs.

![Figure 6](image-url)  
**Figure 6.** Regulation of outward Na\(^{+}\)-Ca\(^{2+}\) exchange currents by Ba\(^{2+}\) and Ca\(^{2+}\) (*inset*). Pooled results (mean ± SD) from three to nine determinations in nine separate patches. Currents were normalized to the value of current obtained at 3 \(\mu M\) regulatory Ca\(^{2+}\), (in all 9 patches).
Comparison between Ca\textsuperscript{2+} and Ba\textsuperscript{2+} on Na\textsuperscript{+}-Ca\textsuperscript{2+} Exchange

steady-state current approached the same level as peak current. In contrast, this behavior was not observed when Ba\textsuperscript{2+} served as the regulatory ion. In two patches examined at 1 mM regulatory Ba\textsuperscript{2+} (not shown), a small reduction in peak to steady state ratio was observed.

Finally, we compared the ability of Ca\textsuperscript{2+} and Ba\textsuperscript{2+} to compete for Na\textsuperscript{1+} at the intracellular transport site. To accomplish this, outward Na\textsuperscript{1+}-Ca\textsuperscript{2+} exchange currents were examined in excised patches after treatment with 1–2 mg/ml α-chymotrypsin for 1–2 min. After this treatment, both I\textsubscript{1} inactivation and divalent regulatory effects (I\textsubscript{2} inactivation) were eliminated. Thus, competition between Na\textsuperscript{1+} and divalent cations could be observed by examining outward current with different concentrations of divalents present. Divalent vs. Na\textsuperscript{1+} competition appears as a reduction of outward current due primarily to: (a) increasing electroneutral Ca\textsuperscript{2+}-Ca\textsuperscript{2+} or Ca\textsuperscript{2+}-Ba\textsuperscript{2+} exchange, (b) simple competition between Na\textsuperscript{1+} and divalent cation occupancy of the transport site, and/or (c) the progressive reduction in driving force for the exchange reaction. Typical and pooled results (mean ± SD, n = 4) are shown in Fig. 8. Outward currents were activated by 100 mM Na\textsuperscript{1+}, and pipette Ca\textsuperscript{2+} was constant at 8 mM. For Ca\textsuperscript{2+} competition, substantial inhibition of outward exchange current is evident at concentrations greater than 3 μM Ca\textsuperscript{2+}. In contrast, the inhibitory effects of Ba\textsuperscript{2+} are greatly reduced and only become evident between 100 and 300 μM Ba\textsuperscript{2+}.

**DISCUSSION**

We examined the ability of Ba\textsuperscript{2+} to substitute for Ca\textsuperscript{2+} on several aspects of Na\textsuperscript{1+}-Ca\textsuperscript{2+} exchange function. We show that Na\textsuperscript{1+}-Ba\textsuperscript{2+} exchange is substantially reduced in the forward direction (i.e., Na\textsuperscript{1+}-Ba\textsuperscript{2+} exchange), appears to be reduced in the reverse direction (Na\textsuperscript{1+}-Ba\textsuperscript{2+} exchange) and is considerably less effective as an activator of the exchanger at the high affinity regulatory Ca\textsuperscript{2+} binding site. These alterations in Na\textsuperscript{1+}-Ca\textsuperscript{2+} exchange function are likely to contribute to the failure of cardiac relaxation during superfusion with Ba\textsuperscript{2+}-containing media.

For inward current measurements, substantial Na\textsuperscript{1+}-Ca\textsuperscript{2+} exchange currents were observed at Ca\textsuperscript{2+} concentrations of 1 μM and above. In contrast, inward currents due to Na\textsuperscript{1+}-Ba\textsuperscript{2+} exchange were barely detectable even at 100 and 300 μM Ba\textsuperscript{2+} (Fig. 3). As both Na\textsuperscript{1+}-Ca\textsuperscript{2+} and Na\textsuperscript{1+}-Ba\textsuperscript{2+} currents were measured in the same patches, our results indicate that Na\textsuperscript{1+}-Ba\textsuperscript{2+} exchange is orders of magnitude less effective than Na\textsuperscript{1+}-Ca\textsuperscript{2+} exchange. Our data indicate that both the affinity...
for transport and the efficiency of transport are greatly reduced for Na\(^{+}\)-Ba\(^{2+}\) exchange. For example, less inward current was observed at 300 \(\mu\)M Ba\(^{2+}\) than was produced by 3 \(\mu\)M Ca\(^{2+}\). Comparatively, with equal concentrations of Ca\(^{2+}\) and Ba\(^{2+}\) (e.g., 100 \(\mu\)M), inward Na\(^{+}\)-Ca\(^{2+}\) exchange current exceeded Na\(^{+}\)-Ba\(^{2+}\) exchange by nearly 300-fold.

A \(K_0\) for Ba\(^{2+}\)-activation of inward Na\(^{+}\)-Ba\(^{2+}\) exchange was not determined as higher concentrations of Ba\(^{2+}\) (e.g., 1 mM) invariably led to the appearance of a small outward current. Although the induction of this unidentified outward current may partially mask inward currents at lower Ba\(^{2+}\) concentrations (e.g., 100–300 \(\mu\)M), it seems unlikely that we have grossly underestimated Na\(^{+}\)-Ba\(^{2+}\) exchange. One possibility is that the outward current represents an endogenous Ca\(^{2+}\)-activated Cl\(^{-}\) conductance in oocyte membranes. Despite using Cl\(^{-}\)-free pipette and perfusing solution, residual Cl\(^{-}\) from the sealing solution invariably contaminated the pipette. However, if present, inward Na\(^{+}\)-Ca\(^{2+}\) exchange currents would also be underestimated, presumably by a similar or greater amount. In addition, we observed this pattern of large Na\(^{+}\)-Ca\(^{2+}\) vs. small Na\(^{+}\)-Ba\(^{2+}\) exchange currents during several long recordings (>10 min) in single patches. Over this time course, nearly complete run-down of the Ca\(^{2+}\)-activated Cl\(^{-}\) conductance occurs due to diffusion of Cl\(^{-}\) from the pipette tip and genuine current rundown.

Both forward and reverse transport modes of Na\(^{+}\)-Ca\(^{2+}\) exchange are regulated by cytoplasmic Ca\(^{2+}\) (Matsuoka et al., 1995). Therefore, the possibility exists that reduced inward Na\(^{+}\)-Ba\(^{2+}\) exchange is a consequence of reduced exchanger activation by Ba\(^{2+}\). However, the large differences we observed between inward Na\(^{+}\)-Ca\(^{2+}\) and Na\(^{+}\)-Ba\(^{2+}\) exchange currents cannot be attributed to the failure of Ba\(^{2+}\) to activate the exchanger. As shown in Figs. 5 and 6, 300 \(\mu\)M Ba is sufficient to activate \(~60\%\) of the current obtainable with Ca\(^{2+}\) activation. Therefore, even though Ba\(^{2+}\) activation is less effective than Ca\(^{2+}\), substantial activation of inward Na\(^{+}\)-Ba\(^{2+}\) exchange currents would be expected at the concentrations examined. Thus, alterations of \(V_{\text{max}}\) and/or the apparent \(K_0\) for transport appear to be causal for reduced inward Na\(^{+}\)-Ba\(^{2+}\) exchange. The results obtained from patches after deregulation by \(\alpha\)-chymotrypsin (Fig. 8) showed relatively weak competition between Ba\(^{2+}\) and Na\(^{+}\), supporting the idea of lower affinity at the intracellular transport site.

It is of interest to compare our results with those obtained from other experimental systems. For example, in cardiac sarcolemmal vesicles, \(V_{\text{max}}\) is reduced by 21-fold and the \(K_m\) is 2.4-fold larger when Ba\(^{2+}\) is substituted for Ca\(^{2+}\) (Tibbitts and Philipson, 1985). As vesicular uptake is now considered to be mediated almost exclusively by inside-out oriented vesicles (Li et al., 1991), this result is analogous to inward current measurements from giant excised patches. Our results show a similar or greater reduction in transport capacity and a much larger shift in affinity for Na\(^{+}\)-Ba\(^{2+}\) exchange. As an example, a 100 pA inward current for Na\(^{+}\)-Ca\(^{2+}\) exchange would result in a comparatively small Na\(^{+}\)-Ba\(^{2+}\) exchange current (i.e., 5 pA). However, at Ba\(^{2+}\) concentrations 30 \(\mu\)M and below, inward currents were never observed. This may be due to our inability to reliably measure currents less than a few picamps. In CHO cells expressing the bovine cardiac Na\(^{+}\)-Ca\(^{2+}\) exchanger, extracellular Na\(^{+}\)-dependent \(^{133}\text{Ba}^{2+}\) efflux could be measured, consistent with forward Na\(^{+}\)-Ba\(^{2+}\) exchange. However, a reduction in Ba\(^{2+}\) concentration due to forward Na\(^{+}\)-Ba\(^{2+}\) exchange was not observed in these same cells based on fura-2 measurements (Condrescu et al., 1997). The reasons for these discrepancies remain unknown but may reflect loss of resolution by the various techniques.

Outward Na\(^{+}\)-Ba\(^{2+}\) exchange currents appear to be considerably smaller than those observed using Ca\(^{2+}\) as the transported cation. However, since we cannot measure outward Na\(^{+}\)-Ca\(^{2+}\) and Na\(^{+}\)-Ba\(^{2+}\) exchange currents in the same patch, this comparison is strictly qualitative. Notwithstanding this limitation, comparison with other published data shows that Ba\(^{2+}\) substitution for Ca\(^{2+}\) can eliminate or greatly diminish currents associated with the exchanger based on whole cell measurements in guinea pig (Kimura et al., 1987) and rabbit ventricular cells (Shimoni and Giles, 1987). In contrast, Na\(^{+}\)-Ba\(^{2+}\) exchange activity could be readily measured in CHO cells expressing the cardiac exchanger using either \(^{133}\text{Ba}^{2+}\) or fura-2 measurements under conditions analogous to our outward exchange measurements (Chernaya et al., 1996; Condrescu et al., 1997). Exchange activity was also regulated by cytosolic Ca\(^{2+}\) in this preparation (Chernaya et al., 1996; Condrescu et al., 1997).

One surprising result from the present study is the marked effects of temperature on the appearance of outward Na\(^{+}\)-Ba\(^{2+}\) exchange currents (Fig. 4). At 30°C, the small outward currents are regulated by Ca\(^{2+}\)-dependent (I\(_1\)) but do not exhibit Na\(^{+}\)-dependent (I\(_2\)) inactivation. Substantially greater currents are observed at 37°C, with near normal I\(_1\) and I\(_2\) regulation. This suggests a high energy barrier for Ba\(^{2+}\) translocation. This condition or a greatly reduced affinity for extracellular Ba\(^{2+}\) would reduce the fraction of exchangers with intracellularly oriented ion binding sites and consequently would alleviate I\(_2\) inactivation. Such behavior is analogous to lowering extracellular Ca\(^{2+}\) which reduces I\(_1\) inactivation (Hilgemann et al., 1992a). This strong temperature dependence must also be considered when comparing results from other studies examining Na\(^{+}\)-Ba\(^{2+}\) exchange.

Finally, we examined the ability of Ba\(^{2+}\) to substitute
for Ca\textsuperscript{2+} as an activator of the exchanger at the high affinity regulatory Ca\textsuperscript{2+} binding site. Three major differences are observed for Ba\textsuperscript{2+}. First, the affinity for Ba\textsuperscript{2+} regulation is reduced nearly 30-fold. Second, the efficiency of Ba\textsuperscript{2+} activation is considerably less than that observed with Ca\textsuperscript{2+} over the concentration range examined. At 300 \textmu M regulatory Ba\textsuperscript{2+}, maximal outward Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange currents are only \textasciitilde 60\% of those observed for regulatory Ca\textsuperscript{2+}. Third, unlike Ca\textsuperscript{2+}, Ba\textsuperscript{2+} does not appear to strongly influence I\textsubscript{1} inactivation. Raising cytoplasmic Ca\textsuperscript{2+} progressively alleviates I\textsubscript{1} inactivation (Hilgemann et al., 1992a) whereas differences in the profile of Ba\textsuperscript{2+} regulated currents are unremarkable. This feature may prove useful in future studies of the mechanism(s) of I\textsubscript{1} and I\textsubscript{2} regulation.

In conclusion, we have characterized the effects of substituting Ba\textsuperscript{2+} for Ca\textsuperscript{2+} on inward and outward exchange currents and on regulatory properties of the cloned canine cardiac Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger, NCX1. By all accounts, the transport and regulatory consequences of this substitution should severely impair the ability of the exchange mechanism to maintain ionic homeostasis. Other contributory factors include the reduction or absence of SR Ba\textsuperscript{2+} uptake (Palade, 1987; Chernaya et al., 1996) and prolonged depolarization due to effects on L-type Ca\textsuperscript{2+} channels (Lee et al., 1985) and K\textsuperscript{+} channels (Imoto et al., 1987). However, while Ba\textsuperscript{2+} substitution alters numerous other homeostatic processes, the demonstrated changes in Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange function alone would appear sufficient to prevent cardiac relaxation.

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