Cellular Biology

Upregulation of Na\(^+/\)Ca\(^{2+}\) Exchanger Expression and Function in an Arrhythmogenic Rabbit Model of Heart Failure

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Abstract—Three-dimensional cardiac mapping in rabbits with nonischemic cardiomyopathy has shown that ventricular arrhythmias initiate by a nonreentrant mechanism that may be due to triggered activity from delayed afterdepolarizations. Delayed afterdepolarizations are thought to be due to spontaneous release of Ca\(^{2+}\) from the sarcoplasmic reticulum (SR) and consequent activation of an inward Na\(^+/\)Ca\(^{2+}\) exchange (NaCaX) current. The goal of this study was to determine whether there is enhanced NaCaX gene expression and functional activity that may contribute to nonreentrant activation. Heart failure (HF) was induced in rabbits by combined aortic insufficiency and aortic constriction. HF rabbits had left ventricular enlargement (left ventricular end-diastolic dimension increased from 1.43±0.03 to 1.97±0.05 cm) and severely depressed function (fractional shortening reduced from 37% to 26%, P<0.02). Heart-to-body weight was increased by 79% in HF. Western blots showed a 93% increase in NaCaX protein in HF (P<0.04). NaCaX mRNA (7-kb transcript) was increased by 104% relative to the 18S rRNA in HF. A 14-kb NaCaX transcript was also seen in the HF rabbits, raising total NaCaX mRNA to 2.7-fold compared with controls. The amplitude of caffeine-induced contractures, used to assess SR Ca\(^{2+}\) load, was not significantly different in HF. Relaxation and [Ca\(^{2+}\)]\(_i\) decline during caffeine-induced contractures is attributable to Ca\(^{2+}\) transport by NaCaX and was 61% and 45% faster in HF (P<0.05), respectively. NaCaX current measured under controlled voltage clamp conditions was also 2-fold higher in HF cells. SR Ca\(^{2+}\)-ATPase mRNA and protein levels and Ca\(^{2+}\) current density were not significantly altered in HF. Twitch amplitudes from HF myocytes were 26% smaller compared with control (P<0.02), but twitch relaxation and [Ca\(^{2+}\)]\(_i\) decline (due largely to SR Ca\(^{2+}\)-ATPase) were not altered. Thus myocytes and myocardium from HF rabbits exhibit enhanced NaCaX expression and function. The enhanced NaCaX activity may contribute to depressed contractions, increased transient inward current (for a given SR Ca\(^{2+}\) release), delayed afterdepolarizations, and nonreentrant initiation of ventricular tachycardia in this arrhythmogenic model of HF. (Circ Res. 1999;85:1009-1019.)

Key Words: heart failure \(\bullet\) Na\(^+/\)Ca\(^{2+}\) exchange \(\bullet\) ventricular tachycardia \(\bullet\) delayed afterdepolarization \(\bullet\) Ca\(^{2+}\)

Sudden death in patients with congestive heart failure (HF) is commonly due to lethal ventricular arrhythmias, including ventricular tachycardia (VT) and ventricular fibrillation. However, very little is known about the electrophysiologic and molecular mechanisms underlying arrhythmogenesis in HF. An understanding will require 1) development of arrhythmogenic animal models of HF, 2) delineation of the arrhythmic mechanisms in these animal models, 3) validation of these mechanisms by studies in the human heart, and 4) assessment of the function and expression of the channels and carrier proteins that may underlie these arrhythmic mechanisms.

There are a considerable number of experimental animal models of HF, but very few are arrhythmogenic. An arrhythmogenic experimental model of nonischemic cardiomyopathy has recently been developed in rabbits, combining aortic insufficiency and aortic constriction. These HF rabbits develop severe depression of left ventricular (LV) function, pathologic alterations similar to that in patients with nonischemic cardiomyopathy, and spontaneously-occurring VT. Using 3D cardiac mapping, Pogwizd has demonstrated that the spontaneously-occurring VT in these HF rabbits initiates by a nonreentrant mechanism. Recent 3D mapping studies in failing human hearts at the time of heart transplantation in patients with idiopathic dilated cardiomyopathy have demonstrated that spontaneous and induced VT in these patients also initiates by a focal nonreentrant mechanism.

The nature of this nonreentrant mechanism in the myopathic heart is unknown. Vermeulen et al have demonstrated that failing myocardium from rabbits with aortic insufficiency and aortic stenosis demonstrate delayed afterdepolarizations (DADs) when exposed to catecholamines and rapid pacing. These findings suggest that DADs, due to a transient inward current (\(I_\text{ti}\))...
mediated by Na\textsuperscript+/Ca\textsuperscript{2+} exchange (NaCaX) or a nonselective cationic current,\textsuperscript{8,9} may underlie the nonreentrant mechanism in the setting of nonischemic cardiomyopathy.

The goal of the present study was to test the hypothesis that this arrhythmogenic model of HF demonstrates upregulation of NaCaX (as in failing human heart\textsuperscript{10,11}) and enhanced functional NaCaX activity and current, which could underlie development of \(I_N\). We therefore quantitated the levels of NaCaX mRNA and protein in myocardial tissue from failing and control rabbits by Northern blotting and immunoblotting. Decreased expression of the sarcoplasmic reticulum (SR) Ca\textsuperscript{2+}-ATPase (SERCA2) has been noted in some experimental models of HF\textsuperscript{12} and in the failing human heart.\textsuperscript{13-15} Those changes could also contribute to arrhythmias by increasing levels of cytoplasmic Ca\textsuperscript{2+}. Thus we also assessed the levels of SERCA2 mRNA and protein. Studies were also performed in isolated myocytes from control and HF rabbits to assess the functional activity of NaCaX and SERCA2 (during twitch and caffeine-induced contractures [CafC]) and the levels of NaCaX current (\(I_{\text{NaCaX}}\)) and Ca\textsuperscript{2+} current (\(I_{\text{Ca}^+}\)).

**Materials and Methods**

**Animal Preparations and Induction of HF**

Studies were performed on healthy adult New Zealand White rabbits of either sex (2.9 to 3.5 kg; Doe Valley Farms, Inc, Bentonville, Ark). HF was produced by combined aortic insufficiency (AI) and aortic constriction. Induction of AI was performed by inserting a beveled catheter via the left carotid artery and repeatedly pushing it through the aortic valve to increase pulse pressure by at least 50%, as previously described.\textsuperscript{4} The severity of AI was assessed by 2D echocardiography with color flow mapping.\textsuperscript{16} After 14 to 28 days, a left thoracotomy was performed, allowing aortic constriction (to 10% loss of NaCaX (as in failing human heart\textsuperscript{10,11}) and enhanced this arrhythmogenic model of HF demonstrates upregulation of cardiac NaCaX mRNA used 1.3 kb cDNA of guinea pig NaCaX (from Dr K.D. Philipson, University of California, Los Angeles) or with rabbit anti-rat SERCA2 antiserum (from Drs R. Hartong and W. Dillmann, University of California, San Diego). Primary antibody binding was detected with an ECL Western blotting kit (Amersham) and quantified by laser densitometry.

**Myocyte Isolation**

Left ventricular myocytes were isolated via Langendorff perfusion with 0.5 mg/mL collagenase as described,\textsuperscript{22} except that back flow across the incompetent aortic valve in HF rabbits was blocked by a balloon-tipped catheter inserted across the aortic valve and inflated in the LV outflow tract. Also, additional incubations with 0.4 mg/mL collagenase plus 0.02 mg/mL pronase were done at 37°C for up to 12 minutes. Cells were studied at 23°C or 37°C in superfusion chambers on glass coverslip bases pretreated with laminin.

**Contraction and Ca\textsuperscript{2+} Transients**

Along with cell shortening, [Ca\textsuperscript{2+}]\textit{c} was measured using indo 1 fluorescence (excited at 365 nm and measured at 405 and 485 nm, \(K_{d}=441 \text{ mmol/L}\)) as described.\textsuperscript{23} Normal Tyrode’s (NT) solution contained (in mmol/L): NaCl 140, KCl 4, MgCl\textsubscript{2} 1, CaCl\textsubscript{2} 2, glucose 10, and HEPES 5, pH 7.4 at 23°C. After steady-state twitches (0.5 Hz), stimulation was stopped and NT+10 mmol/L caffeine solution (NTCafi) was rapidly applied for 10 seconds (causing SR Ca\textsuperscript{2+} release and extrusion via NaCaX\textsuperscript{22,23}).

**Whole-Cell Voltage Clamp**

Ion currents were measured with the whole cell ruptured-patch technique (pipette tip resistance=0.8 to 1.2 M\textOmega) with membrane capacitance measured by 5 mV voltage steps from −80 mV, \(I_{\text{NaCaX}}\) was measured as described by Hobai et al.\textsuperscript{24} Pipettes were filled with (in mmol/L) 45 CsCl, 55 Cs methanesulfonic acid, 10 ATP-tris, 0.3 GTP-tris, 20 HEPES, 10.8 MgCl\textsubscript{2} (1 mmol/L free Mg), 5 1,2-bis(2aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid, 5 Dib 1,2-bis(2aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid, 2.21 CaCl\textsubscript{2} (100 mmol/L free Ca), and 14 NaCl (pH 7.3 with CsOH). To isolate \(I_{\text{Ca}^+}\), the NT solution \((37^\circ\text{C})\) was modified (6 CsCl replaced KCl and 10 \(\mu\text{mol/L}\) strophanthidin, 30 mmol/L BDM and sometimes 10 mmol/L nifedipine were added). Ni (5 mmol/L) was added to block \(I_{\text{Ca}^+}\) and \(I_{\text{NaCaX}}\).

**Data Analysis**

All results were expressed as means±SE and unpaired \(t\) tests were used to compare 2 groups (significance at \(P<0.05\)).

An expanded Materials and Methods section is available online at http://www.circresaha.org.

**Results**

**Development of Hypertrophy and Failure**

HF was induced in 13 rabbits. Severe aortic insufficiency, confirmed by 2D color flow Doppler echocardiography, was first produced. Two to 6 weeks later, aortic constriction was performed. Over the subsequent 1 to 24 months, the rabbits developed progressive cardiac enlargement and decreased left ventricular systolic function. LVESD increased by 40% \((P<0.001)\), and LVESD increased by 68% \((P<0.0001)\), Table 1). Mean fractional shortening decreased from 36±2% to 23±1% \((P<0.0001)\). The 6 age-matched control rabbits demonstrated LVEDD, LVESD, and fractional shortening that were comparable with baseline values for the rabbits that underwent induction of HF (Table 1). There were significant increases in heart weight and heart-to-body weight ratio (77% and 79%, respectively), whereas body weight was not different between control and HF rabbits (Table 1). There was also a corresponding significant increase in resting myocyte length (31%) and width (45%) in cells isolated from control.
and HF rabbits (Table 1) resulting in an 89% increase in the length×width product.

Spontaneously Occurring Ventricular Arrhythmias
Holter monitoring in the conscious state was performed in 11 rabbits at baseline and, on average, every 1 to 2 months after the induction of HF in 10 of the 11 rabbits. At baseline, none of the rabbits exhibited any ventricular arrhythmias. However, over the course of the 1 to 24 months after the induction of HF, all of the HF rabbits exhibited spontaneously-occurring ventricular arrhythmias, with up to 13,339 premature ventricular complexes, 402 couplets, and 32 runs of nonsustained VT over the course of 24 hours. Nine of the 10 HF rabbits demonstrated nonsustained VT during serial Holter monitoring. The longest run of VT was 31 beats and the fastest was a 15 beat run of VT at a rate of 360 beats per minute (Figure 1).

Arrhythmogenic Effects of Isoproterenol
To determine whether HF myocytes also exhibit spontaneous activity (as in the whole heart in vivo), the appearance of aftercontractions in isolated myocytes was also studied. Myocytes were field-stimulated at increasing frequency (0.2 to 1.8 Hz) in the absence and presence of 10 μmol/L isoproterenol. After the 20th stimulated twitch, there was a 10-second interval with no stimulation to observe for the presence of aftercontractions. At baseline (without isoproterenol), no aftercontractions occurred in either control or HF myocytes (Figure 2). After the addition of 10 μmol/L isoproterenol, aftercontractions were induced in 4 of 5 HF myocytes but in 0 out of 5 control myocytes studied with this protocol. Thus HF myocytes exposed to β-adrenergic agonist demonstrate spontaneous SR Ca\(^2+\) release and the induction of aftercontractions.

NaCaX and SERCA2 mRNA and Protein Levels
Figure 3 shows Northern blot analysis of NaCaX mRNA in LV myocardium from control and HF rabbits. All control and HF rabbits demonstrated a 7-kb hybridization band, corresponding to the expected position for the NaCaX. Four of the 8 HF rabbits also demonstrated a high molecular weight transcript at ~14 kb that was not evident in any of the controls. However, on prolonged exposure, the control rabbit with the highest NaCaX mRNA expression showed very low levels of the 14-kb transcript. Expression of total NaCaX mRNA, normalized to 18S

### Table 1. Cardiac Hypertrophy and Failure

<table>
<thead>
<tr>
<th></th>
<th>Age-Matched Control</th>
<th>Baseline</th>
<th>HF</th>
<th>n</th>
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</thead>
<tbody>
<tr>
<td>Heart weight, g</td>
<td>10.2±0.7</td>
<td>18.1±1.2 §</td>
<td>16</td>
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<tr>
<td>Body weight, kg</td>
<td>4.20±0.18</td>
<td>4.21±0.40</td>
<td>16</td>
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</tr>
<tr>
<td>HW/BW, ×10(^{-3})</td>
<td>2.43±0.12</td>
<td>4.36±0.31§</td>
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<tr>
<td>LVEDD, cm</td>
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<td>1.40±0.05</td>
<td>1.97±0.05§</td>
<td>18</td>
</tr>
<tr>
<td>LVESD, cm</td>
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<td>0.90±0.04</td>
<td>1.51±0.05§</td>
<td>18</td>
</tr>
<tr>
<td>Fractional short, %</td>
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<td>36±2</td>
<td>23±1†</td>
<td>18</td>
</tr>
<tr>
<td>Cell length, μm</td>
<td>127±6</td>
<td>166±22*</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>Cell width, μm</td>
<td>20±2</td>
<td>29±3*</td>
<td>26</td>
<td></td>
</tr>
</tbody>
</table>

HW/BW is heart weight-to-body weight ratio. *P<0.02 vs control, †P<0.001 vs control and baseline, ‡P<0.0001 vs control and baseline, and §P<0.0001 vs control.
rRNA, was increased 2.7-fold in the HF rabbits compared with controls (P<0.01). If only the 7-kb transcript is considered, there was still a 2.04-fold increase in NaCaX mRNA expression in the HF group (P<0.05, see Figure 9). Similar values were obtained when NaCaX mRNA expression was normalized to GAPDH mRNA (eg, 2.9-fold increase in HF over controls for 7+14 kb).

Western blots revealed a single band for NaCaX at 120 kDa for both control and HF rabbits. Quantification indicated a 93% increase of NaCaX protein expression, consistent with the mRNA data (see Figure 9).

Quantification of the levels of SERCA2 mRNA in LV was performed by Northern blot analysis using a 2.3-kb cDNA fragment specific for rat cardiac SERCA2. All control and HF rabbits exhibited a single hybridization band at 4.2 kb. SERCA2 expression of HF rabbits normalized to 18S rRNA or to GAPDH was not significantly different from that of control rabbits (P≠0.05).

Figure 4 shows a Western blot for SERCA2, revealing a single band at 110 kDa for both control and HF rabbits. When normalized to the mean level of the control rabbits, the SERCA2 protein levels of the HF rabbits was not significantly different (see Figure 10). However, as shown in Figure 4, the level of SERCA2 protein did vary among the HF rabbits. Of note, the lowest level of SERCA2 protein in the HF rabbits (lanes 1 and 3 of HF) was evident in the 2 rabbits that had the most marked degree of LV enlargement and the most severe LV dysfunction. There was no significant relationship between the levels of NaCaX and SERCA2 protein levels.

There was good correlation between NaCaX mRNA and protein expression, especially among the HF group (Figure 5A). We also examined the relationship of NaCaX (and SERCA) expression to LV systolic function in individual whole hearts. Figure 5B shows that the level of NaCaX mRNA and LV fractional shortening (FS) were inversely correlated (P=0.01). A similar relationship between NaCaX protein and FS was seen, but it did not attain statistical significance (P=0.067). There was no significant relationship between FS and SERCA2 mRNA (Figure 5C) or protein (data not shown).

Biochemical measurements (above) and isolated myocyte studies (below) were performed in 2 different groups of rabbits. However, in each case, HF rabbits demonstrated comparable levels of LV dilatation, depression of LV systolic function, and incidence of spontaneously-occurring ventricular arrhythmias (measured in both groups). In this regard, they were also comparable with the HF rabbits studied by 3D cardiac mapping. NaCaX and SERCA2 expression was assessed in left ventricular homogenates, rather than isolated myocytes. Because SERCA2 and NaCaX are so concentrated in ventricular myocytes, contamination from nonmyocytes in the homogenate were considered inconsequential. In previous studies in hypertrophied rat heart, changes in SERCA2 expression were comparable whether measured in left ventricular homogeneate or isolated myocytes.

**Relaxation and [Ca^{2+}], Decline in Isolated Cardiac Myocytes**

The rate of myocyte relaxation and [Ca^{2+}] decline during sustained application of 10 mmol/L caffeine has been shown to depend mainly on cytosolic Ca^{2+} extrusion by NaCaX in rabbit ventricular myocytes. This is because net SR Ca^{2+} re-uptake is prevented by caffeine, such that NaCaX only competes with the much slower sarcolemmal Ca^{2+-}ATPase and mitochondrial unipporter. Thus relaxation and [Ca^{2+}], decline during CafC provide functional data in the intact cell concerning Ca^{2+} transport by NaCaX.

Figure 6C shows CafC in control and HF myocytes and the normalized relaxation (Figure 6D) is much faster in HF. Mean data (Table 2) show that the half-time (t_{1/2}) of relaxation was 546±70 ms in control and 340±40 ms in HF. Considering relaxation rate to be inversely proportional to t_{1/2}, this constitutes a 61% increase in NaCaX-dependent relaxation in HF. Figure 7C shows comparable data for [Ca^{2+}], during a CafC. In this case, [Ca^{2+}], decline was approximately twice as fast in HF as in control. On average, [Ca^{2+}], decline rate during CafC was 45% faster in HF than control (Table 2).

Twitch relaxation in normal rabbit ventricular myocytes is dominated by the SERCA2 but only by a factor of 2 to 3 over the NaCaX. Thus the time course of relaxation and [Ca^{2+}], decline of twitches should provide functional information about the SERCA2 in control versus HF. Figures 6B and 7B...
show normalized twitch relaxation and \([\text{Ca}^{2+}]\), decline in control and HF. The \(t_{1/2}\) values were not significantly different between these groups (Table 2). Superficially, this could be construed as evidence of unchanged SERCA2 function. However, some additional quantitative analysis may be warranted.

The issue here is that the SERCA2 and NaCaX are competing during the twitch. Thus, if NaCaX is upregulated and SERCA2 is unchanged (as the above data indicate), we would expect twitch relaxation to be accelerated in HF. If we consider simply that relaxation is attributed to parallel function of SERCA2 and NaCaX working at rates given by pseudo-rate constants (\(\lambda_{SR}\) and \(\lambda_{NCX}\) where \(\lambda = \ln 2/t_{1/2}\)), then during a twitch, \(\lambda_{\text{twitch}} = \lambda_{SR} + \lambda_{NCX}\) and during a CafC, \(\lambda_{\text{CafC}} = \lambda_{NCX}\). Applying this simple scheme to control contractions, \(\lambda_{NCX} = 1.41 \pm 0.49\ s^{-1}\) and \(\lambda_{\text{twitch}} = 4.77 \pm 0.18\ s^{-1}\), giving \(\lambda_{SR} = 3.36\ s^{-1}\). This would suggest 29% of \(\text{Ca}^{2+}\) removal during a twitch in control rabbit myocytes is by NaCaX (in close agreement with more detailed quantitative analysis\(^2\)). In HF cells, \(\lambda_{NCX}\) is increased to \(2.34 \pm 0.26\ s^{-1}\) and \(\lambda_{\text{twitch}}\) is comparable at \(4.9 \pm 0.3\ s^{-1}\), giving a reduced \(\lambda_{SR}\) of \(2.54\ s^{-1}\). This change in \(\lambda_{SR}\) (from 3.36 to 2.54) would imply a 24% reduction in SERCA2 function. Furthermore, the balance between the SERCA2 and the NaCaX is changed in HF (so \(\lambda_{NCX} < \lambda_{SR}\) such that they contribute about equally to twitch relaxation (ie, NaCaX component increases from control of 29% to 48% in HF). Analysis of the \(\text{Ca}^{2+}\) transient data yields a similar conclusion (17% reduction in SERCA2 function). Thus we cannot rule out a small reduction in SERCA2 function in the HF versus control rabbits.

**Twitch Amplitude and SR Ca\(^{2+}\) Content**

Twitches from HF myocytes were 26% smaller in amplitude compared with control (12.9 ± 1.2% versus 17.4 ± 1.3% of resting cell length, \(P, 0.02\); Table 2). The amplitude of CafC provides an index of the SR \(\text{Ca}^{2+}\) content, and the mean value was only 8% smaller in HF (24.6% versus 26.5% of resting cell length, \(P = \text{not significant}\)). This raises the possibility that there is a reduction in fractional SR \(\text{Ca}^{2+}\) release in HF. However, using the ratio of twitch:CafC amplitude as a crude

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**Figure 4.** SERCA2 protein in control and HF rabbits. Immunoblot of myocardial SERCA2 protein for 6 age-matched controls (left) and 8 HF rabbits (right). Although there were no significant differences between HF and control (see Figure 10), the lowest levels of SERCA2 protein among the HF were found in the first and third HF lanes. These same 2 animals had the most severe degree of LV enlargement and LV dysfunction.

**Figure 5.** Correlations with NaCaX and SERCA2 expression from individual hearts. A, Relationship between NaCaX mRNA (normalized to the levels of 18S rRNA) and NaCaX protein levels (normalized to total protein). B and C, Correlations between LV fractional shortening and NaCaX (B) and SERCA2 (C) mRNA expression (normalized to controls) for control and HF rabbits. Each point in these graphs is data from an individual heart.
index of fractional SR Ca\(^{2+}\) release, we found no significant difference between HF and control.

There was no difference in resting \([\text{Ca}^{2+}]_i\) between the groups. The amplitude of twitch Ca\(^{2+}\) transients was 30% smaller in HF versus control cells (Table 2). This is comparable with the depression of twitch contractions and LV fractional shortening but was not significant (possibly due to greater variance in \([\text{Ca}^{2+}]_i\) measurements). The 11% decrease in CafC \(\Delta[\text{Ca}^{2+}]_i\), was not significantly different (but again comparable with contraction results). The twitch:CafC ratio for \(\Delta[\text{Ca}^{2+}]_i\), was not significantly different between control and HF. Because the results for contraction and Ca\(^{2+}\) transients are relatively similar, we have no evidence of altered myofilament Ca\(^{2+}\) sensitivity in HF (although not measured directly). These results suggest that the reduction of twitch amplitude in HF is likely to be due to decreased Ca\(^{2+}\) transients. Reduced Ca\(^{2+}\) transients could, in principle, be due to reduced \(I_{\text{Ca}}\) trigger, lower SR Ca\(^{2+}\) load (possible based on the CafC data), or a depression of the E-C coupling process.

### Measurement of \(I_{\text{Ca}}\) and NaCaX Current in Isolated Myocytes

Voltage clamp experiments were carried out to evaluate \(I_{\text{Ca}}\) (with respect to the issue of E-C coupling above) and also to measure \(I_{\text{NaCaX}}\) under well controlled experimental conditions (to further verify the expression and functional results above). Figure 8A shows the central protocol used, where \([\text{Ca}^{2+}]_i\), was buffered to 100 nmol/L to minimize alterations in \([\text{Ca}^{2+}]_i\), during protocols. The conditions were also selected so that Ca, Na, and NaCaX currents should be the only ones available. From an initial holding potential of −90 mV, the cell was depolarized to −45 mV to activate and inactivate Na current. Then a step from −45 to 0 mV was used to activate and inactivate \(I_{\text{Ca}}\). Finally, the voltage was stepped to 80 mV and ramped down to −140 mV to assess \(I_{\text{NaCaX}}\). The protocol was repeated in the presence of 5 mmol/L Ni (Figure 8A) to obtain Ni-sensitive \(I_{\text{Ca}}\) and \(I_{\text{NaCaX}}\) (Figure 8B).

Figures 8C and 8D show that both inward and outward \(I_{\text{NaCaX}}\) are increased by 2.2-fold in HF. The apparent reversal potential is unchanged and close to the predicted value based on the pipette and extracellular solutions. This is consistent with the above data based on mRNA, protein, \([\text{Ca}^{2+}]_i\), decline, and relaxation of CafC (see Figure 9).

The amplitude of \(I_{\text{Ca}}\) was unchanged during the pulses to 0 mV (Figure 8E). Some additional cells were also studied with standard \(I_{\text{Ca}}\) protocols to determine the voltage dependence of \(I_{\text{Ca}}\) (Figure 8F). There was no difference in either \(I_{\text{Ca}}\) amplitude or voltage dependence. The \(I_{\text{Ca}}\) results suggest that depressed Ca\(^{2+}\) transients and contractions in HF were not attributable to altered \(I_{\text{Ca}}\). Moreover, \(I_{\text{Ca}}\) density stayed remarkably constant despite the cellular hypertrophy in the HF cells.

Figures 9 and 10 summarize results from the different approaches used here to assess NaCaX and SERCA2 expression and function in control and HF. All of the data are consistent with an approximate doubling of NaCaX expression and function. In contrast, the results do not indicate any significant change in SERCA2 expression, although there might be a small decrease in function.
Correlation of NaCaX Functional Expression and SR Ca\(^{2+}\) Content

An increase in NaCaX expression (with all other things remaining the same) might be expected to reduce the steady-state SR Ca\(^{2+}\) content (because NaCaX would compete better with the SERCA2 during [Ca\(^{2+}\)]\(_{i}\) decline and diastole). Although hearts used for cell isolation were not used for Western blotting, we can use the \(t_{1/2}\) of relaxation of CafC as a functional index of NaCaX level in a given cell. Figure 11 shows that the greater the functional expression of NaCaX (smaller \(t_{1/2}\) CafC values), the lower the SR Ca\(^{2+}\) content. Despite substantial cellular heterogeneity, the correlation was significant \((P=0.015)\). Thus increased NaCaX expression may contribute to unloading the SR of Ca\(^{2+}\) in the steady state. There was no comparable correlation between twitch amplitude and functional expression of NaCaX.

Based on the results above, the depression of twitch amplitude in HF myocytes does not seem to be due to alteration of \(I_{Na/Ca}\). There does seem to be a tendency toward lower SR Ca\(^{2+}\) content in HF. This by itself might explain the reduced twitch amplitude. However, altered E-C coupling cannot be completely ruled out because an altered SR Ca\(^{2+}\) load makes it more difficult to assess E-C coupling directly (see Discussion).

Discussion

The results of the present study demonstrate that this arrhythmogenic rabbit model of nonischemic cardiomyopathy is associated with an upregulation of NaCaX both on an mRNA and a protein level. The results in this model are in agreement with findings of NaCaX upregulation in myocardium from patients with end-stage HF.\(^{10,11}\) This concurrent upregulation of NaCaX on both an mRNA and a protein level and the strong association between mRNA and protein levels all suggest that this upregulation likely occurs at a pretranslational level. This NaCaX upregulation is carried forward in isolated myocytes as faster \([Ca^{2+}]_{i}\) decline during CafC and greater \(I_{Na/Ca}\). This enhanced NaCaX function may be involved in both the mechanical dysfunction and arrhythmogenic characteristics in this model of HF.

Functional Increase in NaCaX Activity in Failing Myocytes

In normal rabbit ventricular myocytes, relaxation and \([Ca^{2+}]_{i}\) decline are attributable to the following 4 competing Ca\(^{2+}\) removal systems: 1) SERCA2, 2) NaCaX, 3) sarcolemmal Ca\(^{2+}\)-ATPase, and 4) mitochondria Ca\(^{2+}\) uniporter.\(^{22,23}\) Bassani et al\(^{23}\) analyzed the relative contributions quantitatively, concluding that the SERCA2 removed 70%, NaCaX 28%, and the others totaled only 2%. The data from control hearts in the present study (29% NaCaX) agree well with those conclusions. However, the functional upregulation of NaCaX in HF shown here shifts this balance such that Ca\(^{2+}\) removal is closer to 50% SERCA2 and 50% NaCaX during twitch relaxation. The increase in NaCaX may well contribute to mechanical dysfunction and a larger inward \(I_{Na/Ca}\) could also contribute directly to arrhythmogenesis (see below).

Altered NaCaX expression and activity has been noted in experimental models of HF and in the failing human heart. Dogs with pacing-induced HF exhibit an increase in NaCaX protein and function (during \([Ca^{2+}]_{i}\) decline).\(^{25}\) Rabbits with pacing-induced HF had a 44% increase in NaCaX protein but normal \(I_{Na/Ca}\).\(^{26}\) In contrast, Yao et al\(^{27}\) found decreased NaCaX mRNA and \(I_{Ca}\) in a similar model.\(^{27}\) Increased \(I_{Na/Ca}\) has been demonstrated in myocytes from cardiomyopathic Syrian hamsters\(^{28}\) and infarcted rabbits.\(^{29}\) Several studies have shown an increase in both NaCaX mRNA and protein in myocardium from patients with end-stage HF obtained at the time of cardiac transplantation.\(^{10,11}\) Although little human functional data are available, Pieske et al\(^{30}\) recently showed a similar shift in NaCaX:SERCA2 function in human HF (from 25%:75% to \(\sim 50%:50\%)\).

Hasenfuss et al\(^{31}\) recently showed that in human HF, upregulation of NaCaX protein correlated with a relative lack of LV diastolic dysfunction. This is consistent with our results in which twitch relaxation and \([Ca^{2+}]_{i}\) decline were maintained and NaCaX was functionally increased in HF.
rabbits, but we did not directly evaluate diastolic function in vivo.

**High Molecular Weight Transcript of NaCaX mRNA**

In the present study, 4 of the 8 HF rabbits demonstrated a high molecular weight (~14 kb) transcript of the NaCaX mRNA using a cDNA probe that recognizes cardiac NaCaX mRNA. We found this transcript at very low levels in only one of the controls, and in rabbit brain and kidney, but not in skeletal muscle (data not shown). A similar 14-kb transcript was detected in mouse heart and kidney; rabbit heart, kidney, and brain; and human brain but not in myocardium from patients with end-stage HF. It remains to be determined as to whether this 14-kb transcript is due to alternative splicing of NCX1 (the gene for NaCaX) or a long poly-A tail on some NaCaX mRNA and as to what its role is.

However, the 2-fold increase in the 7-kb NaCaX transcript in the HF rabbits provides evidence that enhanced functional NaCaX activity is due to a generalized increase in NaCaX expression and not solely to increased expression of the high molecular weight transcript.

**SERCA2 Expression**

In this rabbit model of nonischemic HF, we found no change in SERCA2 mRNA or protein expression. Function of the SERCA2 based directly on the rate of twitch relaxation and [Ca\(^{2+}\)] decline was apparently unaltered in HF versus control myocytes. However, more detailed analysis (see Results) indicated that there might be a modest functional depression of SERCA2 in the HF myocytes (but smaller than the change in NaCaX). Although downregulation of SERCA2 had been reported in several experimental models of HF and in myocardium from patients with HF, other studies have failed to demonstrate this effect.

Of course reduced SERCA2 function alone would tend to reduce SR Ca\(^{2+}\) content, lower SR Ca\(^{2+}\) release, and slow

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**Figure 8.** NaCaX expression, activity, and current. Values are shown for NaCaX mRNA (7- and 14-kb transcripts) from Northern blotting, NaCaX protein from immunoblotting, pseudo-rate constant of relaxation of the caffeine contraction (\(\lambda_{NCX}=ln2/\tau_{1/2}\)), and \(I_{Na/Ca}\) (inward and outward, excluding values near the reversal potential, ~70 to ~50 mV). Results are normalized to values from control myocytes. Mean control values for \(\lambda_{NCX}\) was 1.41±0.49 s\(^{-1}\) and for \(I_{Na/Ca}\) at 60 mV was 2.96±0.54 pA/pF. The ratio of \(I_{Na/Ca}\) for HF:control was 2.13 at 70 mV and 2.18 at ~120 mV. *P<0.05 versus controls.

**Figure 9.** SERCA2 expression and activity. Plots of SERCA2 mRNA from Northern blotting, SERCA2 protein from immunoblotting, and pseudo rate constant of twitch relaxation (\(\lambda_{twitch}=ln2/\tau_{1/2}\)). Results are normalized to values from control myocytes. Mean control \(\lambda_{twitch}\) was 4.77±0.18 s\(^{-1}\). No statistical differences were seen between HF and control.
relaxation of twitches. However, the dynamic interplay between the SERCA2 and NaCaX compels one to consider Ca\sup{2+} balance in a more integrated context.

**Mechanical Dysfunction**

Diastolic dysfunction usually refers to either a slowing of relaxation or elevation of diastolic pressure. In isolated ventricular myocytes, there was no change in diastolic [Ca\sup{2+}]. Neither twitch relaxation nor [Ca\sup{2+}]\sub{d, rest} declined in this model of HF. On one hand, this unaltered relaxation could be simply ascribed to an unaltered expression level of SERCA2. On the other hand, the large and compelling increase in NaCaX function would have been expected to accelerate [Ca\sup{2+}]\sub{d, rest} decline if SERCA2 did not change. Thus it is possible that the large increase in NaCaX expression compensates for a small depression of SR Ca\sup{2+} transport function (which could be hard to detect and could also be due to factors other than protein levels, such as phosphorylation state).

Systolic dysfunction can also result from alterations in Ca\sup{2+} transport mechanisms. In the HF cells, there was no change in I\sub{Ca}, which is expected to be the central trigger for SR Ca\sup{2+} release. However, there have been reports of depressed response of SR Ca\sup{2+} release to a given I\sub{Ca, trigger} in certain models of hypertrophy and HF.\cite{40,41} The shift in balance between NaCaX and SERCA2 function would also mean that for a given Ca\sup{2+} transient, there would be greater extrusion from the cell and less refilling of the SR. This would be expected to result in a lower steady state level of SR Ca\sup{2+} loading, consistent with our results from CafC amplitudes. Although the decreases in CafC and Δ[Ca\sup{2+}] in Table 2 were not significant, there was a significant inverse correlation between NaCaX function and CafC amplitude (Figure 11). A lower SR Ca\sup{2+} load would decrease SR Ca\sup{2+} release, and this could contribute to the lower twitch contractions and Ca\sup{2+} transients observed. Indeed, because fractional SR Ca\sup{2+} release depends steeply on SR Ca\sup{2+} content, a modest depression of SR Ca\sup{2+} content could decrease fractional release during E-C coupling to a greater extent.\cite{42} Although the twitch/CafC ratios did not uncover a significant E-C coupling defect, this issue should be more directly addressed in controlled voltage clamp experiments.\cite{40,41} Greater extrusion of Ca\sup{2+} via NaCaX during the twitch could also directly lower the twitch Ca\sup{2+} transient amplitude.\cite{23}

Thus, although numerous possibilities cannot be explicitly ruled out, our preferred working hypothesis is the following: systolic function may be depressed simply because of reduced Ca\sup{2+} transients that result from reduced SR Ca\sup{2+} load, which is due to shifts in the balance between NaCaX and SERCA2 (with unaltered I\sub{Ca, intrinsic}}, intrinsic E-C coupling gain, or myofilament Ca\sup{2+} sensitivity). Causality is difficult to prove, and it may be that increased NaCaX expression is secondary to other factors in the failing heart, accounting for heterogeneity in NaCaX expression (Figure 5 and Reference 31). Thus further tests of this hypothesis will be required.

**Spontaneous SR Ca\sup{2+} Release and Arrhythmogenesis**

In the intact animal, the lowered Ca\sup{2+} transients (and SR Ca\sup{2+} load) could be partially offset by β-adrenergic activation of the SERCA2. This may be sufficient for the SR to reach an adequate Ca\sup{2+} load to produce relatively frequent spontaneous SR Ca\sup{2+} release events (Ca\sup{2+} sparks or waves).\cite{43,44} Indeed, we found in HF myocytes that treatment with isoproterenol increased the propensity for spontaneous aftercontractions (Figure 2). Thus β-adrenergic agonists may offset a modest reduction in SR Ca\sup{2+} load in the myocytes and aftercontractions may be the cellular manifestation of the enhanced arrhythmias in the intact HF animals (Figure 1).\cite{5}

Increased NaCaX in HF could also allow more Ca\sup{2+} influx, as an outward current early in the action potential, which could even contribute to triggering of SR Ca\sup{2+} release.\cite{29,45,46} More importantly, for a given SR Ca\sup{2+} release during an aftercontraction, the elevated NaCaX activity in HF myocytes would result in greater inward I\sub{Na,Ca}, contributing to I\sub{i, DADs}, and ultimately, nonreentrant VT as we see in the whole heart setting using 3D mapping.\cite{5} The specific role of upregulated NaCaX in the development of I\sub{i} remains to be determined, but our present findings support the hypothesis that an upregulated NaCaX may itself mediate enhanced I\sub{i} and the nonreentrant mechanism in this HF model.

Figure 12 illustrates our working hypothesis based on increased arrhythmias and NaCaX in HF. Local SR Ca\sup{2+} release elevates local [Ca\sup{2+}]\sub{i}, which stimulates Ca\sup{2+} extrusion via I\sub{Na,Ca}. This Ca\sup{2+} extrusion produces an inward, depolarizing current (I\sub{i}) that can produce a DAD, bringing the diastolic membrane potential closer to threshold to trigger an inappropriately timed action potential. With higher NaCaX activity (as shown in the present study), any given amount of SR Ca\sup{2+} release will result in greater inward I\sub{i} and increased probability that a triggered arrhythmia will result. It would be of interest to explore whether arrhythmias are more prevalent in the transgenic mouse that overexpresses NaCaX.\cite{45,47}

**Relevance of the Model of Nonischemic HF**

In the present study, all of the HF rabbits had severe depression of LV function, and 9 of 10 rabbits with serial Holter monitoring demonstrated nonsustained VT. Additionally, we have found that overall, ~10% of these HF rabbits have sudden death. These findings reflect what is observed in patients with end-stage nonischemic cardiomyopathy where 60% to 80% of patients exhibit nonsustained VT, and up to 40% to 45% will die suddenly.\cite{1} This validates the use of this
model to study the cellular and molecular mechanisms underlying arrhythmogenesis in the failing human heart. Indeed, this rabbit HF model seems to combine aspects of both mechanical dysfunction and arrhythmogenic potential that may be especially relevant to the failing human heart.

Implications
The results of the present study suggest that an upregulation of NaCaX expression and functional activity could contribute to both mechanical dysfunction and arrhythmogenesis in the failing heart. Upregulation of NaCaX could have an adaptive role in enhancing Ca\(^{2+}\) efflux from myocytes (or possibly increasing Ca\(^{2+}\) entry early in the development of HF). However, with the progression of HF, this enhanced NaCaX activity may limit SR Ca\(^{2+}\) loading and cellular Ca\(^{2+}\) transients and also play a direct role in the nonreentrant mechanisms underlying VT, which have been demonstrated by 3D cardiac mapping. In the setting of enhanced NaCaX activity, approaches to the treatment of HF in patients with drugs that either increase intracellular [Na] (such as digitalis glycosides) or agents that directly enhance NaCaX activity could have proarrhythmic effects in the failing heart that would greatly limit their efficacy.

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References


Upregulation of Na\textsuperscript{+}/Ca\textsuperscript{2+} Exchanger Expression and Function in an Arrhythmogenic Rabbit Model of Heart Failure

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