Functional Adult Myocardium in the Absence of Na\textsuperscript{+}-Ca\textsuperscript{2+} Exchange

Cardiac-Specific Knockout of NCX1


Abstract—The excitation–contraction coupling cycle in cardiac muscle is initiated by an influx of Ca\textsuperscript{2+} through voltage-dependent Ca\textsuperscript{2+} channels. Ca\textsuperscript{2+} influx induces a release of Ca\textsuperscript{2+} from the sarcoplasmic reticulum and myocyte contraction. To maintain Ca\textsuperscript{2+} homeostasis, Ca\textsuperscript{2+} entry is balanced by efflux mediated by the sarcolemmal Na\textsuperscript{-}Ca\textsuperscript{2+} exchanger. In the absence of Na\textsuperscript{-}Ca\textsuperscript{2+} exchange, it would be expected that cardiac myocytes would overload with Ca\textsuperscript{2+}. Using Cre/loxP technology, we generated mice with a cardiac-specific knockout of the Na\textsuperscript{-}Ca\textsuperscript{2+} exchanger, NCX1. The exchanger is completely ablated in 80% to 90% of the cardiomyocytes as determined by immunoblot, immunofluorescence, and exchange function. Surprisingly, the NCX1 knockout mice live to adulthood with only modestly reduced cardiac function as assessed by echocardiography. At 7.5 weeks of age, measures of contractility are decreased by 20% to 30%. We detect no adaptation of the myocardium to the absence of the Na\textsuperscript{-}Ca\textsuperscript{2+} exchanger as measured by both immunoblots and microarray analysis. Ca\textsuperscript{2+} transients of isolated myocytes from knockout mice display normal magnitudes and relaxation kinetics and normal responses to isoproterenol. Under voltage clamp conditions, the current through L-type Ca\textsuperscript{2+} channels is reduced by 50%, although the number of channels is unchanged. An abbreviated action potential may further reduce Ca\textsuperscript{2+} influx. Rather than upregulate other Ca\textsuperscript{2+} efflux mechanisms, the myocardium appears to functionally adapt to the absence of the Na\textsuperscript{-}Ca\textsuperscript{2+} exchanger by limiting Ca\textsuperscript{2+} influx. The magnitude of Ca\textsuperscript{2+} transients appears to be maintained by an increased gain of sarcoplasmic reticular Ca\textsuperscript{2+} release. The myocardium of the NCX1 knockout mice undergoes a remarkable adaptation to maintain near normal cardiac function. (Circ Res. 2004;95:604-611.)

Key Words: Na\textsuperscript{-}Ca\textsuperscript{2+} exchange ■ excitation–contraction coupling ■ genetically altered mice

Cardiac excitation–contraction coupling is initiated by the influx of Ca\textsuperscript{2+} through voltage-dependent Ca\textsuperscript{2+} channels that triggers release of Ca\textsuperscript{2+} from the sarcoplasmic reticulum (SR). Ca\textsuperscript{2+} influx must, of course, be balanced by Ca\textsuperscript{2+} efflux, and the sarcolemmal Na\textsuperscript{-}Ca\textsuperscript{2+} exchanger and ATP-dependent Ca\textsuperscript{2+} pump are the two mechanisms that mediate myocardial Ca\textsuperscript{2+} efflux. A voluminous literature has documented the dominant role of Na\textsuperscript{-}Ca\textsuperscript{2+} exchange in the Ca\textsuperscript{2+} efflux process.\textsuperscript{1,2} The sarcolemmal Ca\textsuperscript{2+} pump appears to have little role in excitation–contraction coupling. By regulating intracellular Ca\textsuperscript{2+} levels, Na\textsuperscript{-}Ca\textsuperscript{2+} exchange is a determinant of cardiac contractility, and acute alterations in exchange activity have major effects on contractile strength. For example, small changes in intracellular Na\textsuperscript{+} in response to digitalis produce positive inotropy.

With our current understanding of excitation–contraction coupling, it is almost inconceivable that myocardium could survive in the absence of Na\textsuperscript{-}Ca\textsuperscript{2+} exchange activity. Without a vigorous Ca\textsuperscript{2+} efflux mechanism, cardiac myocytes should Ca\textsuperscript{2+} overload, leading to a nonfunctional myocardium. Four laboratories, including ours, have reported that global knockout of the Na\textsuperscript{-}Ca\textsuperscript{2+} exchanger, NCX1, is embryonic lethal.\textsuperscript{3-6} The result is not surprising and the lethality has been attributed to a cardiac phenotype.\textsuperscript{3,5} One study, however, indicated that the lethality had an extracardiac origin.\textsuperscript{7}

Unexpectedly, heart tubes from NCX knockout embryos, dissected at day 9.5, had almost normal Ca\textsuperscript{2+} transients in response to electrical stimulation.\textsuperscript{5,8} Excitation–contraction coupling was still dependent on Ca\textsuperscript{2+} influx and, apparently,
the sarcolemmal Ca\textsuperscript{2+} pump was sufficient to maintain Ca\textsuperscript{2+} homeostasis. The heart tubes were operating under low stress conditions (1 Hz stimulation at 26°C), however, and did not tolerate interventions (eg, increased stimulation rate) that increased the need for Ca\textsuperscript{2+} efflux. Additionally, day 9.5 NCX knockout embryos are within 1 day of death and other adaptations of the myocardium have occurred.\textsuperscript{8} Thus, embryonic heart tubes are a difficult preparation and not ideal for analysis of NCX ablation.

We have now generated, using Cre/loxP technology, mice with a cardiac-specific knockout of NCX1. The exchanger is completely ablated in at least 80% of the myocytes. Strikingly, these mice survive to adulthood with diminished, yet surprisingly good, cardiac function. We report our initial characterization of these mice.

**Methods**

**Cardiac-Specific Knockout of NCX1**

To ablate NCX1 in the ventricular myocardium, a mouse line was produced such that exon 11 was flanked by loxP sites (floxed). Typically, male and female breeders were homozygous for the NCX1 floxed allele (NCX1\textsuperscript{lox/lox}), and male breeders were additionally hemizygous for Cre expression under transcriptional control of the endogenous ventricular myosin light chain-2 (MLC2v) promoter.\textsuperscript{9} Offspring were genotyped by polymerase chain reaction (primer sequences provided on request) of DNA from tail biopsy specimens. All experiments were performed under approved institutional recombinant DNA and animal protocols.

For a description of other methods, see the expanded Methods available in the online data supplement at http://circres.ahajournals.org.

**Results**

**Knockout of NCX1**

We generated mice with a loxP site inserted into each of the two introns flanking exon 11 of the Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger, NCX1. These mice were mated with mice expressing Cre recombinase under the control of the cardiac-specific MLC2v promoter.\textsuperscript{9} Cre recombinase excises DNA between loxP sites from the chromosomes of ventricular myocytes beginning during embryonic development.\textsuperscript{9} No other Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange activity besides NCX1 is known to be present in myocardium.

We chose to excise exon 11 because we had previous evidence that amino acids encoded by this exon are critical for exchange activity. Single site mutations within this region eliminate exchange activity.\textsuperscript{10} Exon 11 codes for amino acids 722 to 813 (Figure 1A), encompassing two transmembrane segments including part of the α-2 region essential for Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange activity.\textsuperscript{11} Excision of exon 11 leaves the reading frame intact. To rigorously test the assumption that an exchanger with amino acids 722 to 813 deleted is inactive, we constructed NCX1 cDNA with this deletion (Δ722–813). Function of Δ722–813 was tested in three different expression systems: after transient transfection of BHK and HEK cells and after cRNA injection into Xenopus oocytes. Using a Na\textsuperscript{+}-gradient-dependent \textsuperscript{45}Ca\textsuperscript{2+} uptake assay, the deletion mutant displayed no detectable exchange activity in any expression system (Figure 1B). Expression of Δ722–813 protein was confirmed by immunoblot (not shown).

Homozygous NCX1 knockout mice survive to adulthood, and all experiments described used homozygous knockout mice. We performed no experiments with heterozygous knockout mice. In initial experiments, we compared hearts from wild-type mice and mice with exon 11 of NCX1 flanked from wild-type mice and mice with exon 11 of NCX1 floxed (Δ722–813). A, Topological model of the Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger, NCX1. The 9 cylinders represent transmembrane segments with the extracellular surface at the top. The α-1 and α-2 regions (shaded) are homologous and are essential for exchange function. The approximate locations of amino acids 722 and 813 are indicated (arrows). The region between the arrows is deleted in Δ722–813. B, Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange activity of wild-type (WT) and Δ722–813 (Δ) exchangers expressed in BHK or HEK cells or in Xenopus oocytes. Exchange activity is indicated as the difference of \textsuperscript{45}Ca\textsuperscript{2+} uptake of Na\textsuperscript{+}-loaded cells in K\textsuperscript{+} and Na\textsuperscript{+} media. No difference in uptake in K\textsuperscript{+} and Na\textsuperscript{+} media, and hence no exchange activity, is detected for the deletion mutant. See Methods for details.

**NCX1 Expression**

We directly assessed the level of Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger protein in the hearts of homozygous NCX1 knockout mice by immunoblot (Figure 2A). As shown, the level of NCX1 protein is greatly reduced. A typical pattern of bands at 120 and 160 kDa is seen with homogenate of control hearts. With the knockout hearts, an additional band arises at ≈110 kDa (arrow in Figure 2A), representing exchangers with the Δ722–813 deletion. The intensity of this band is diminished, suggesting that the abnormal and nonfunctional protein gets degraded more rapidly. The level of decrease in functional exchanger protein is 88% (Figure 2A and Table 1).
Why does any NCX1 protein remain in knockout mice? The extent of ablation depends on the efficacy of the Cre recombinase, and it has been a common finding that cardiac-specific knockouts do not occur with 100% efficiency. Those cells in which excision occurs will have a complete absence of NCX1 and the remaining cells will have a normal expression level; however, there is also the possibility that in some cells excision will occur on only one allele. The immunoblot data indicate that 88% of the myocytes have no NCX1. We further addressed this issue by using immunofluorescence with isolated myocytes.

Figure 2C shows the immunofluorescence of myocytes isolated from a NCX1 knockout heart. When stained using a monoclonal antibody to NCX1, a majority of myocytes showed only a weak diffuse background staining (left panel of Figure 2C). This staining is equivalent to that obtained when primary antibody is omitted (not shown). A minority of myocytes from a knockout heart showed staining similar to that observed in myocytes from control hearts (right panel in Figure 2C). In this representative experiment, 35 of 39 cells showed the weak background staining pattern, whereas 4 of 39 cells displayed a staining pattern similar to that of wild-type cells. One hundred percent of control cells had a staining pattern similar to that displayed in the right panel of Figure 2C (not shown). The results are consistent with the immunoblot data. The ultrastructure of knockout hearts was normal as assessed by thin-section electron microscopy (not shown). Myofibrils showed normal alignment, and structures involved in excitation–contraction coupling (transverse tubules; subsarcolemmal and junctional SR) also appeared normal.

Expression of Other Myocardial Proteins
It might be expected that the myocardium would respond to the absence of NCX1 by altering the expression of other proteins involved in excitation–contraction coupling. We could find no evidence for any adaptations at either the protein or the transcript level. By immunoblot, there were no changes in the levels of the plasma membrane Ca$^{2+}$ pump (PMCA), the sarcoplasmic reticular Ca$^{2+}$ pump (SERCA2), the $\alpha_2$ subunit of the dihydropyridine receptor (DHPR$\alpha_2$), and calsequestrin. Molecular weight markers (kDa) are shown on the right. Quantification of all data is shown in Table 1. C, Immunofluorescence of isolated myocytes from a NCX1 knockout heart. Myocytes were immunolabeled using the monoclonal antibody R3F1 to the cardiac Na$^+-$Ca$^{2+}$ exchanger. In this representative experiment, ~90% (35/39) of the knockout myocytes displayed only a diffuse background staining (left panel). The other 10% (4/39) of the myocytes showed a staining pattern indistinguishable from that of wild-type myocytes (right panel).
transcript levels of several proteins involved in the handling of Ca$^{2+}$. Notably, the transcript levels of other known Na$^{+}$-Ca$^{2+}$ exchangers (NCX2 and NCX3) remained at nondetectable levels after knockout of NCX1. NCX2 and NCX3 were also nondetectable by immunoblot (not shown). Transcript levels of NCX1 were unchanged in the myocardium of NCX1 knockouts and were also nondetectable by immunoblot (not shown). Treatment of NCX1 knockout mice with caffeine showed the Ca$^{2+}$ concentration to be reduced to 368 nmol/L (n=7) and 329±22 nmol/L (n=5) for control and knockout cells, respectively (P<0.001 as compared with control).

Cardiac Function

We assessed the cardiac function of the NCX1 knockout mice by echocardiography (Table 2). At 7 to 8 weeks of age, a relatively modest diminution of cardiac function is apparent. Fractatial shortening, velocity of circumferential fiber shortening, and ejection fraction (three measures of contractility) are decreased by 30%, 25%, and 21%, respectively. Wall thicknesses and left ventricular chamber size are unchanged, demonstrating absence of hypertrophy. The increase in end systolic dimension reflects the decrease in contractility.

Although at 7 to 8 weeks of age the NCX1 knockout mice have myocardium without a large decrease of function (Table 2), the animals are not normal and cannot withstand stress. Thus, homozygous knockout females could not survive the stress of breeding. Of 6 knockout females that gave birth, all died within 9±2 days of delivery. Another 4 knockout females died after mating but before delivery. Cardiac function displayed further decline with age as assessed by echocardiography (not shown), and lifespan was reduced. Nineteen knockout animals died at 103±7 days, representing ~10% of the animals. No animals in the control group died within this time span. Postmortem inspection indicated enlarged hearts, and death was probably associated with heart failure. Mice beyond this age were either euthanized or used for experiments, so average life span was not determined. In this report, we focus on younger knockout mice (6 to 8 weeks of age) with minimal pathology.

Excitation-Contraction Coupling

We were unable to detect any difference in the appearance or yield of enzymatically isolated ventricular myocytes from control and knockout hearts. We assessed Na$^{+}$-Ca$^{2+}$ exchange activity in patch-clamped myocytes by rapidly applying 5 mmol/L caffeine to the bath solution while recording membrane current at a constant holding potential of ~40 mV. To ensure constant SR loading, cells were prepulsed from −80 to 0 mV (100 ms duration) at 1 Hz. Caffeine elicited Ca$^{2+}$ transients of similar amplitude in control and knockout myocytes (Figure 3), suggesting similar SR Ca$^{2+}$ contents. The increases in the [Ca$^{2+}$] induced by caffeine were 368±34 (n=7) and 329±22 nmol/L (n=5) for control and knockout cells, respectively (P=0.4). The Ca$^{2+}$ transient declined in control cells, even in the presence of caffeine, as Ca$^{2+}$ was

### TABLE 1. Lack of Adaptation to the Absence of the Na$^{+}$-Ca$^{2+}$ Exchanger

<table>
<thead>
<tr>
<th>Protein</th>
<th>Control</th>
<th>Knockout</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quantitation of immunoblot data (normalized to control; n=8 [NCX1] or n=4 [others])</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na$^{+}$-Ca$^{2+}$ exchanger (NCX1)</td>
<td>1.00±0.14</td>
<td>0.12±0.02*</td>
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<tr>
<td>Sarcolemmal Ca$^{2+}$ pump (PMCA)</td>
<td>1.00±0.07</td>
<td>0.93±0.09</td>
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<tr>
<td>L-type Ca$^{2+}$ channel (α2)</td>
<td>1.00±0.17</td>
<td>0.83±0.13</td>
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<tr>
<td>Calsequestrin</td>
<td>1.00±0.23</td>
<td>0.93±0.08</td>
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</tbody>
</table>

Quantitation of microarray analysis (relative abundance, arbitrary units; n=3 for knockouts and n=4 for controls)

<table>
<thead>
<tr>
<th>Protein</th>
<th>Control</th>
<th>Knockout</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sarcolemmal Ca$^{2+}$ pump (PMCA4)</td>
<td>1.3±0.5</td>
<td>1.3±0.3</td>
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<tr>
<td>SR Ca$^{2+}$ pump (SERCA2)</td>
<td>0.5±0.1</td>
<td>0.5±0.2</td>
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<tr>
<td>Ryanodine receptor (RyR2)</td>
<td>79.0±11.9</td>
<td>88.9±5.6</td>
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<tr>
<td>Phospholamban</td>
<td>14.9±0.3</td>
<td>14.2±0.3</td>
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<tr>
<td>L-type Ca$^{2+}$ channel α1C</td>
<td>74.4±7.7</td>
<td>78.6±5.3</td>
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<tr>
<td>L-type Ca$^{2+}$ channel α2O1</td>
<td>20.8±1.9</td>
<td>17.1±0.9</td>
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<tr>
<td>β2</td>
<td>61.2±4.4</td>
<td>61.7±8.6</td>
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<tr>
<td>Calsequestrin</td>
<td>70.0±5.8</td>
<td>74.7±6.2</td>
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<tr>
<td>Na$^{+}$-Ca$^{2+}$ exchanger</td>
<td>45.7±4.6</td>
<td>51.8±3.0</td>
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<tr>
<td>NCX1</td>
<td>18.6±1.4</td>
<td>21.9±2.0</td>
</tr>
<tr>
<td>NCX2</td>
<td>9.9±4.2</td>
<td>14.6±3.4</td>
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<tr>
<td>NCX3</td>
<td>3.2±0.1</td>
<td>2.7±0.3</td>
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<tr>
<td>Na$^{+}$-Ca$^{2+}$ exchanger</td>
<td>1.6±0.4</td>
<td>1.4±0.2</td>
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<tr>
<td>NCX1</td>
<td>1.2±0.4</td>
<td>0.8±0.1</td>
</tr>
<tr>
<td>NCX2</td>
<td>0.7±0.4</td>
<td>0.7±0.3</td>
</tr>
<tr>
<td>NCX3</td>
<td>0.1±0.1</td>
<td>0.1±0.1</td>
</tr>
</tbody>
</table>

Multiple values for a protein indicate that the transcript was represented more than once on the microarray. All values are means±SEM. *P<0.001 as compared with control.

### TABLE 2. Cardiac Function in the Absence of the Na$^{+}$-Ca$^{2+}$ Exchanger from Echocardiography

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control (n=11)</th>
<th>Knockout (n=11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, wk</td>
<td>7.5±0.3</td>
<td>7.5±0.3</td>
</tr>
<tr>
<td>Ventricular septal thickness, mm</td>
<td>0.70±0.03</td>
<td>0.68±0.03</td>
</tr>
<tr>
<td>Posterior wall thickness, mm</td>
<td>0.72±0.03</td>
<td>0.67±0.03</td>
</tr>
<tr>
<td>End diastolic dimension, mm</td>
<td>3.64±0.12</td>
<td>3.82±0.11</td>
</tr>
<tr>
<td>End systolic dimension, mm</td>
<td>2.49±0.12</td>
<td>2.97±0.10*</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>581±37</td>
<td>602±26</td>
</tr>
<tr>
<td>Left ventricular fractional shortening, %</td>
<td>39.7±2.3</td>
<td>22.3±1.6*</td>
</tr>
<tr>
<td>Velocity of circumferential fiber shortening, mm/s</td>
<td>6.0±0.5</td>
<td>4.5±0.4*</td>
</tr>
<tr>
<td>Left ventricular ejection fraction, %</td>
<td>66.3±2.7</td>
<td>52.1±2.5*</td>
</tr>
</tbody>
</table>

All values are means±SEM. *P<0.05 as compared with control.
extruded by Na\(^+\)-Ca\(^{2+}\) exchange. The decline in the Ca\(^{2+}\) transient in knockout cells in the presence of caffeine was always slower. Based on the initial decline in the Ca\(^{2+}\) transient in the presence of caffeine, we estimated the half-time for the decline in the transient to baseline. These values were 1.2±0.1 (n=7) and 6.3±2.2 s (n=5) for control and knockout cells, respectively (P<0.02).

In control cells, application of caffeine always induced an inward Na\(^+\)-Ca\(^{2+}\) exchange current. However, in 11 of 12 knockout cells, caffeine-induced Na\(^+\)-Ca\(^{2+}\) exchange current was completely absent (Figure 3, middle trace). Thus, the wild-type phenotype was present in <10% of randomly chosen knockout myocytes. These functional data are consistent with the immunoblot and immunofluorescence data. We estimate that we would detect any residual Na\(^+\)-Ca\(^{2+}\) exchange current in knockout myocytes that was >1% of that in control myocytes.

We examined Ca\(^{2+}\) transients in externally paced isolated ventricular myocytes loaded with fluo-3 acetoxymethyl ester (fluo-3 AM) (Figure 4). Ca\(^{2+}\) transients from NCX1 knockout myocytes were of similar magnitude to those in wild-type myocytes and had no relaxation deficit. Diastolic Ca\(^{2+}\) levels were similar. Both the wild-type and knockout myocytes displayed a negative staircase typical of mouse myocytes. In an attempt to expose consequences of NCX1 ablation, we stimulated isolated myocytes with the β agonist, isoproterenol (1 µmol/L for 3 minutes). Robust and identical inotropic responses to isoproterenol were induced in both wild-type and knockout myocytes (Figure 5). Remarkably, we could detect no effects of the NCX1 knockout on cellular Ca\(^{2+}\) dynamics. Cells from knockout myocardium retained dependence on external Ca\(^{2+}\); on removal of external Ca\(^{2+}\), transients were rapidly eliminated (not shown).

A difference between control and NCX1 knockout myocytes did become manifest in measurements of voltage-dependent L-type Ca\(^{2+}\) currents (I\(_{Ca}\)) under whole-cell clamp conditions. Current–voltage (I–V) relationships are shown in Figure 6A and 6B. I\(_{Ca}\) in knockout cells was substantially decreased compared with control cells. The percentage decreases were 48%, 49%, 47%, and 32% at −10, 0, +10, and +20 mV, respectively. Both I–V curves peaked at 0 mV. The rates of inactivation of I\(_{Ca}\) were similar in the two cases. It is notable that all four measures of L-type Ca\(^{2+}\) channel expression shown in Table 1 show a modest reduction in the knockout hearts, although in none of the cases is statistical significance achieved. We have no other data on the mechanism of decreased Ca\(^{2+}\) current, although perhaps posttranslational modification is involved.

There was also a substantial difference in the morphology of action potentials between wild-type and NCX1 knockout myocytes. Whereas action potentials from control myocytes always exhibited a distinct plateau after the upstroke and rapid repolarization, action potentials from NCX1 knockout myocytes completely lacked a plateau in 6 of 8 myocytes (Figure 6C). At 85 ms after onset of the stimulus, the plateau level in control myocytes was −48±3 mV, whereas the voltage in the 6 knockout myocytes was −67±2 mV (P<0.001). These data are consistent with the absence of NCX1 in the majority of isolated myocytes as expected. Resting membrane potentials were indistinguishable in control and knockout myocytes (−68.1±1.0 [n=10] and −68.0±1.7 mV [n=5], respectively). At 0 mV, the width of the action potential spike was 3.6±0.5 ms for control and 1.5±0.3 ms for knockout myocytes, respectively (P<0.01).
Discussion

We generated mice in which the Na\(^+\)-Ca\(^{2+}\) exchanger, NCX1, was completely removed from \(\approx 8\%\) to 90\% of the cardiac myocytes as assessed by immunoblot, immunofluorescence, and function. In vivo, mouse myocardium contracts 600 times per minute, and Ca\(^{2+}\) influx accompanies each excitation. The expectation was that excitation–contraction coupling would fail in the absence of the dominant Ca\(^{2+}\) efflux mechanism. Whole animal knockout of NCX1 is embryonic lethal,\(^3\)–\(^6\) although the cause may not be cardiac in origin.\(^7\) Also, in our cardiac-specific knockout, disruption of the NCX1 gene will begin during embryonic development and is limited to ven- tricular myocytes.\(^8\) The presence of NCX1 in the atria and early ventricular cells may prevent the lethality that occurs in the global knockout. In any case, the survival of the cardiac-specific knockout mice to adulthood is surprising.

There are several issues to consider. Mice, like rats, are known to have quantitatively distinct Ca\(^{2+}\) flux pathways. Ca\(^{2+}\) influx is required to initiate excitation–contraction coupling in all myocardium, but the contribution of transsarcolemmal pathways is smaller in mice and rats than in other mammalian species. For example, \(\approx 25\%\) of activator Ca\(^{2+}\) crosses the sarcolemma during each contraction–relaxation cycle in rabbit cardiac myocytes.\(^14\)–\(^16\) The remaining 75\% of the Ca\(^{2+}\) is released from and then sequestered by the SR. In contrast, mice and rats are much more SR-dependent. Only \(\approx 8\%\) of activator Ca\(^{2+}\) traverses the sarcolemma.\(^14\)–\(^16\) Thus, the role of Ca\(^{2+}\) efflux mechanisms, although still essential, is reduced in magnitude in adult mouse myocardium. Nevertheless, the mouse heart undergoes excitation 600 times per minute. Ca\(^{2+}\) enters myocytes with each excitation, and over time the magnitude of Ca\(^{2+}\) that must be extruded may equal that of other species.

Although the Na\(^+\)-Ca\(^{2+}\) exchanger is absent in most myo- cytes from knockout myocardium, Ca\(^{2+}\) efflux must occur after each excitation. An alternative efflux mechanism must function, and the only known viable candidate is the sarcolemmal Ca\(^{2+}\) pump. The Ca\(^{2+}\) pump is thought to have minor significance in excitation–contraction coupling, but this contention may need to be reexamined. Some studies using rat and mouse myocytes find that the capacity of the Ca\(^{2+}\) pump is only a small fraction of that of the Na\(^+\)-Ca\(^{2+}\) exchanger,\(^14\)–\(^17\) although other studies using rat myocytes indicate that the Ca\(^{2+}\) pump can remove Ca\(^{2+}\) at a rate of 30\% of that of the exchanger.\(^18\)–\(^20\) We see no increase in the expression level of the sarcolemmal Ca\(^{2+}\) pump (Table 1) in response to knockout of NCX1. Nevertheless, the sarcolemmal Ca\(^{2+}\) pump is highly regulated and may be activated by phosphorylation or calmodulin. This possibility is difficult to assess. The only direct measure of Ca\(^{2+}\) efflux in our experiments is the decline of the Ca\(^{2+}\) transient in the presence of caffeine (Figure 3). The relationship between the diminished Ca\(^{2+}\) efflux of the knockout myocytes in caffeine experiments and the need for Ca\(^{2+}\) balance during excitation– contraction coupling is not quantitatively clear. The sarcolemmal Ca\(^{2+}\) pump may seem an unlikely candidate to completely compensate for the absence of NCX1 but, nevertheless, appears to permit survival of mouse myocytes.

The myocardium is a syncitium and the 20\% of myocytes with normal NCX1 levels may compensate for the absence of exchanger in adjoining cells as Ca\(^{2+}\) diffuses from cell to cell. We expect that this mechanism is unlikely to make a substantial contribution to Ca\(^{2+}\) homeostasis for two reasons. First, diffusion is sufficiently slow that cells with no exchanger would experience elevated Ca\(^{2+}\) levels for prolonged times, leading to contractile abnormalities and altered gene expression. Second, and more cogently, we see unaltered Ca\(^{2+}\) transients in isolated myocytes. In this case, the syncitial nature of myocardium has been eliminated, and NCX1 knockout cells cannot rely on adjoining cells for Ca\(^{2+}\) extrusion.

The expression in heart of a member of the K\(^{-}\)-dependent Na\(^+\)-Ca\(^{2+}\) exchanger family (NCKX6) has recently been described.\(^21\) These exchangers countertransport 4 Na\(^+\) for 1 Ca\(^{2+}\) plus 1 K\(^+\). It is unlikely that this exchanger, or other members of the NCKX family, contribute significantly to Ca\(^{2+}\) efflux in myocardium. First, no Na\(^+\)-Ca\(^{2+}\) exchange activity with a high-affinity K\(^{-}\)-dependence has ever been observed in cardiac sarcolemma.\(^22\) Second, by immunofluorescence, this exchanger was absent from T tubules.\(^21\) Third, exchangers of the NCKX family are electrogenic, and we can detect no exchanger-associated currents in NCX1 knockout myocytes. In short, no other known Na\(^+\)-Ca\(^{2+}\) exchangers
have been shown to be active in cardiac myocytes other than NCX1.

As mentioned, cardiac pathology develops in the knockout mice as they age, but it is quite remarkable that isolated myocytes from younger knockout mice have Ca\textsuperscript{2+} transients indistinguishable from those in wild-type myocytes. In addition, responses to changes in stimulation frequency or isoproterenol were normal. These results are completely consistent with our inability to detect any adaptation of the myocardiun to the absence of NCX1, as indicated by immunoblots and microarray analysis. Ca\textsuperscript{2+} regulates transcription as well as contraction; chronic abnormalities in Ca\textsuperscript{2+} handling would induce alterations in protein expression. We conclude that mouse myocytes lacking the Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger display normal Ca\textsuperscript{2+} dynamics.

How is Ca\textsuperscript{2+} homeostasis maintained in the absence of Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange? Rather than upregulate other Ca\textsuperscript{2+} efflux mechanisms, it appears that the cell limits Ca\textsuperscript{2+} influx. Thus, the need for robust Ca\textsuperscript{2+} efflux is lessened and the sarcoplasmal ATP-dependent Ca\textsuperscript{2+} pump suffices. Ca\textsuperscript{2+} influx through the L-type Ca\textsuperscript{2+} channel is substantially diminished (Figure 6), supporting this hypothesis, although the mechanism of decreased I\textsubscript{Ca} is unknown. Interestingly, we have recently reported that overexpression of NCX1 results in an increased I\textsubscript{Ca} again by an unknown mechanism.\textsuperscript{23} In addition, the action potential of knockout myocytes is markedly abbreviated. The plateau phase is eliminated and the duration of the initial spike of depolarization is also shortened. The more rapid repolarization of the cell in the absence of the exchanger may induce a more rapid closing of Ca\textsuperscript{2+} channels, further limiting Ca\textsuperscript{2+} influx beyond that seen under voltage clamp conditions.

Inhibition of Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange has previously been observed to abbreviate the action potential.\textsuperscript{24,25} whereas overexpression of the exchanger prolongs action potential duration.\textsuperscript{26} Shortening the action potential, especially at positive potentials, can limit Ca\textsuperscript{2+} influx without compromising subsequent Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release.\textsuperscript{27} That is, the initial phase of I\textsubscript{Ca} appears to be most important as the trigger for SR Ca\textsuperscript{2+} release.

If Ca\textsuperscript{2+} influx into NCX1 knockout myocytes is diminished, then the presence of normal Ca\textsuperscript{2+} transients must be explained. We hypothesize that the gain of Ca\textsuperscript{2+} release is increased. That is, the trigger Ca\textsuperscript{2+} (I\textsubscript{trig}) more efficiently induces SR Ca\textsuperscript{2+} release than in wild-type mice. Thus, an equivalent Ca\textsuperscript{2+} transient could be evoked with a reduced I\textsubscript{trig}. Our observations of decreased I\textsubscript{trig} and normal Ca\textsuperscript{2+} transients directly imply that gain in the knockout myocytes is increased, although we have not directly assessed these two properties in the same myocytes. We have recently described that overexpression of the cardiac Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger results in a decreased gain of Ca\textsuperscript{2+} release.\textsuperscript{23} It would be an interesting parallel if underexpression of the exchanger produces an increase in gain. In any case, the cardiac-specific NCX1 knockout mice provide a remarkable example of the ability of the myocardiun to functionally adapt to altered Ca\textsuperscript{2+} handling.

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Functional Adult Myocardium in the Absence of Na\(^+\)-Ca\(^{2+}\) Exchange: Cardiac-Specific Knockout of NCX1


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DATA SUPPLEMENT

METHODS

Expression of Na\(^+\)-Ca\(^{2+}\) exchange activity

cDNAs encoding wild type and deletion mutant Na\(^+\)-Ca\(^{2+}\) exchangers were expressed in HEK cells, BHK cells, or Xenopus oocytes, and Na\(^+\)-Ca\(^{2+}\) exchange activity was measured as Na\(^+\) gradient-dependent \(^{45}\)Ca\(^{2+}\) uptake into intact cells as described previously.\(^1,2\)

Immunoblots

Mouse heart homogenate (20 µg) was dissolved in SDS reducing buffer and heated in boiling water for 5 min. Proteins were separated on 8 % SDS-PAGE and transferred to nitrocellulose. Blots were probed with antibodies specific for the Na\(^+\)-Ca\(^{2+}\) exchanger (NCX1; R3F1)\(^3\) or with antibodies (Affinity Bioreagents) to the plasma membrane Ca\(^{2+}\) pump, the SR Ca\(^{2+}\) pump, the \(\alpha2\) subunit of the dihydropyridine receptor, or calsequestrin. Blots were developed using chemiluminescence (NEN Life Science) and quantitated by densitometry.

Microarray analysis

Cardiac ventricles from 5-6 week old mice were placed in RNAlater (Ambion) to preserve RNA. Total RNA was isolated using TRIzol (Gibco BRL) and further purified with RNeasy (Qiagen). Integrity of the RNA was verified prior to reverse transcription by visualization of the 28S and 18S ribosomal RNA bands on an agarose gel. cDNA was \textit{in vitro} transcribed with incorporation of labeled ribonucleotides. cRNA was fragmented and hybridized to Affymetrix Murine Genome MOE430A arrays. Hybridization quality was checked by measuring the ratio of hybridization intensities of the 3' to 5' regions of control genes. Data analysis was performed using Genespring software (Silicon
Genetics). Normalization allowed chip to chip comparisons by dividing the expression values for each gene by the median gene expression value for each chip.

**Immunofluorescence**

Isolated mouse myocytes were fixed with 2% formaldehyde (15 min), quenched in Na\(^+\) borohydride, treated with Triton X-100, and exposed to blocking solution and antibody to NCX1 (R3F1; 1/500 dilution) as previously described.\(^4\) For visualization, the cells were incubated with fluorescein-labeled goat anti-mouse secondary antibody for 45 min, rinsed, and mounted on glass slides with 90% glycerol plus a photobleaching inhibitor.

**Echocardiography**

Mice were sedated with tribromoethanol (0.25 mg/g, i.p.) for ultrasound echocardiographic evaluation with an Acuson Sequoia C256 equipped with a 15L8, 15 MHz probe. ECG needle electrodes were attached to the extremities and the mouse was positioned in the left lateral decubitus position for 2-D, M-mode and Doppler imaging. Acuson and AccessPoint software (Freeland Systems) was used for data analysis. Mice were kept warm via a circulating water blanket system and supplemental oxygen was given via a miniature nose cone.

**Isolation of Ventricular Myocytes**

Adult mice were anesthetized with intraperitoneal sodium pentobarbital (300 U/kg) and hearts were quickly removed via thoracotomy. Single ventricular myocytes were isolated enzymatically using collagenase (2 mg/ml, Type II collagenase; Gibco BRL, Life
Technologies, Gaithersburg, MD) and protease (0.166 mg/ml, Type XIV protease; Sigma-Aldrich, St. Louis, MO) digestion according to the method of Mitra and Morad.\(^5\)

The dissociated cells were stored for up to 6 hours at room temperature in modified Tyrode solution, containing (in mmol/L): 136 NaCl, 5.4 KCl, 10 HEPES, 1.0 MgCl\(_2\), 0.33 NaH\(_2\)PO\(_4\), 1.0 CaCl\(_2\), 10 glucose, pH 7.4 with NaOH. This solution was also used, with modifications described below, as the standard bath for electrophysiological recordings.

**Electrophysiology**

To record whole cell membrane currents and action potentials, we placed isolated myocytes in an experimental chamber (0.5 ml) mounted on the stage of a Zeiss Axiovert 100 inverted microscope (Carl Zeiss Microimaging, Thornwood, NY). Patch electrodes were pulled from borosilicate glass (TW150F-3; World Precision Instruments, Sarasota, FL) on a Sutter P-97 horizontal puller (Sutter Instruments, Novato, CA). The fire-polished electrodes had a tip diameter of 2-3 µm and a resistance of 1-2 M\(\Omega\) when filled with patch electrode solutions (described below). Whole cell membrane current and membrane voltage were recorded at 22\(^0\) C using an Axopatch 200A patch clamp amplifier (Axon Instruments, Foster City, CA) and a Digidata 1200 (Axon Instruments) data acquisition system controlled by pCLAMP 8 software (Axon Instruments). Action potential recordings were performed in the standard bath solution described above, using the amphotericin perforated patch technique\(^6\) with the amplifier in current clamp mode after correcting for electrode capacitance. For some experiments, a Nikon Diaphot (Nikon Instruments, Melville, NY) inverted microscope was used and membrane current
was recorded with an Axopatch 200 patch clamp amplifier and a Digidata 1320 data acquisition system (Axon Instruments) controlled by pCLAMP 9 software. We applied series resistance compensation to all voltage clamp recordings.

**Fluorescence measurements**

To simultaneously record \([\text{Ca}^{2+}]_i\) during voltage clamping, we employed a custom designed photometric epifluorescence detection system, previously described in detail.\(^7\) Cells were loaded with the \(\text{Ca}^{2+}\) indicator fura-2 via the pipette solution, which contained (in mmol/L): 110 CsCl, 30 TEA Cl, 10 NaCl, 10 HEPES, 5 MgATP, 0.1 cAMP, 0.1 K$_5$Fura-2, pH 7.3 with CsOH. We replaced \(\text{K}^+\) in the standard bath Tyrode’s solution with Cs$^+$ to block \(\text{K}^+\) currents. Tetrodotoxin (10 \(\mu\)mol/L; Calbiochem, La Jolla, CA) was included in the bath to block \(\text{Na}^+\) currents during depolarization. \([\text{Ca}^{2+}]_i\) was calculated from the ratio (R) of the fluorescence intensities at the two excitation wavelengths (ratios at 600 Hz) using the method of Grynkiewicz et al.,\(^8\) according to the equation: 

\[
[\text{Ca}^{2+}]_i = K_{d-Fura}(\beta)(R - R_{\text{Min}})(R_{\text{Max}} - R). 
\]

\(R_{\text{Max}}\), \(R_{\text{Min}}\), and \(\beta\) were determined by measuring the fluorescence intensity of drops of internal solution containing fura-2 and either high or low \(\text{Ca}^{2+}\); \(K_{d-Fura}\) was determined using drops of fura-2 at different concentrations in standard bath solution.

For experiments using the \([\text{Ca}^{2+}]_i\) indicator fluo-3 AM, we paced cells externally using a Grass S9 stimulator (Grass-Telefactor, West Warwick, RI) via bipolar platinum electrodes in the bath at 22° C. Cells were loaded with fluo-3 AM (10 \(\mu\)mol/L) for 20 minutes, followed by two washes in standard bath solution for 10 minutes each. We
recorded fluo-3 fluorescence using a Zeiss Pascal 5 laser scanning confocal microscope, with an excitation wavelength of 488 nm and an emission wavelength of 505 nm. Background fluorescence of unloaded cells was negligible. Fluorescence transients were normalized to diastolic fluorescence and are expressed as F/F₀. Miniature solenoid valves (The Lee Co., Westbrook, CT) controlled by pCLAMP’s digital outputs controlled the bath solution flow through a micromanifold (ALA Scientific Instruments, Westbury, NY). This enabled precise timing of caffeine application. The solution surrounding the cell exchanges with a half-time less than 100 ms.

REFERENCES
