Overexpression of Na\(^{+}\)/Ca\(^{2+}\) exchanger alters contractility and SR Ca\(^{2+}\) content in adult rat myocytes

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THE NA\(^{+}\)/CA\(^{2+}\) EXCHANGER (NCX1) is an important plasma membrane transport protein that regulates cellular Ca\(^{2+}\) homeostasis in many tissues. In cardiac muscle, by exchanging three extracellular Na\(^{+}\) for one intracellular Ca\(^{2+}\) (forward mode), NCX1 is universally accepted as the dominant Ca\(^{2+}\) efflux mechanism during diastole. The reverse-mode operation of NCX1 (3 Na\(^{+}\) out:1 Ca\(^{2+}\) in) has been proposed to load the sarcoplasmic reticulum (SR) with Ca\(^{2+}\) (17), trigger SR Ca\(^{2+}\) release (16), and directly activate the contractile elements (17).

The cytosolic free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) occupies a key role in cardiac excitation-contraction coupling. During an action potential, the influx of extracellular Ca\(^{2+}\) via L-type Ca\(^{2+}\) channels and possibly reverse NCX1 (16, 17) trigger SR Ca\(^{2+}\) release, resulting in increase in [Ca\(^{2+}\)]\(_i\) and myofilament activation. During diastole, most of the myoplasmic Ca\(^{2+}\) (70–92%) is resequestered in the SR by sarco(end)plasmic reticulum Ca\(^{2+}\)-ATPase (SERCA2), with a small amount of Ca\(^{2+}\) extruded by forward NCX1 (7–28%) and, to a minor extent, by sarcolemmal Ca\(^{2+}\)-ATPase (2, 3, 26). Thus the principal cardiac Ca\(^{2+}\) extrusion mechanism is mediated by NCX1. In addition, forward Na\(^{+}\)/Ca\(^{2+}\) exchange contributed importantly to the rate of [Ca\(^{2+}\)]\(_i\) decline from the midportion of the [Ca\(^{2+}\)]\(_i\) transient and was necessary to reach end-diastolic [Ca\(^{2+}\)]\(_i\) levels (26). Whereas there is unanimous agreement on the important role of forward Na\(^{+}\)/Ca\(^{2+}\) exchange on Ca\(^{2+}\) extrusion during diastole, the physiological role of reverse Na\(^{+}\)/Ca\(^{2+}\) exchange in mediating SR Ca\(^{2+}\) release and loading the SR with Ca\(^{2+}\) is highly controversial (1, 16, 17, 20, 23, 24, 27).

The recent recognition that adult cardiac myocytes, when placed in culture and subjected to continual electrical field stimulation, retained normal contractile function (4), and the advent of high-efficiency gene

The functional consequences of overexpression of rat heart Na\(^{+}\)/Ca\(^{2+}\) exchanger (NCX1) were investigated in adult rat myocytes in primary culture. When maintained under continued electrical field stimulation conditions, cultured adult rat myocytes retained normal contractile function compared with freshly isolated myocytes for at least 95% infection as assessed by GFP fluorescence, but contraction amplitude at 6-, 24-, and 48-h postinfection was not affected. When they were examined 48 h after infection, myocytes infected by adenovirus expressing both GFP and NCX1 had similar cell sizes but exhibited significantly altered contraction amplitudes and intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) transients, and lower resting and diastolic [Ca\(^{2+}\)]\(_o\), when compared with myocytes infected by the adenovirus expressing GFP alone. The effects of NCX1 overexpression on sarcoplasmic reticulum (SR) Ca\(^{2+}\) content depended on extracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_o\)), with a decrease at low [Ca\(^{2+}\)]\(_o\) and an increase at high [Ca\(^{2+}\)]\(_o\). The half-times for [Ca\(^{2+}\)]\(_i\) transient decline were similar, suggesting little to no changes in SR Ca\(^{2+}\)-ATPase activity. Western blots demonstrated a significant (P ≤ 0.02) threefold increase in NCX1 but no changes in SR Ca\(^{2+}\)-ATPase and calserein abundance in myocytes 48 h after infection by adenovirus expressing both GFP and NCX1 compared with those infected by adenovirus expressing GFP alone. We conclude that overexpression of NCX1 in adult rat myocytes incubated at high [Ca\(^{2+}\)]\(_o\), resulted in enhanced Ca\(^{2+}\) influx via reverse NCX1 function, as evidenced by greater SR Ca\(^{2+}\) content, larger twitch, and [Ca\(^{2+}\)]\(_i\) transient amplitudes. Forward NCX1 function was also increased, as indicated by lower resting and diastolic [Ca\(^{2+}\)]\(_o\).

excitation-contraction coupling; fura 2; cardiac myocyte culture; video imaging
transfer into adult cardiac myocytes by recombinant adenovirus (13, 15) together provide the investigator with useful tools to introduce exogenous genes into cultured adult cardiac myocytes and examine the functional consequences. This approach compliments the transgenic mouse model with the additional theoretical advantage that compensatory responses to overexpression of an exogenous gene are less likely to occur in a short-term cell culture model than in a transgenic animal. The present study was undertaken to test the hypothesis that, in adult rat cardiac myocytes, NCX1 functions in both the forward (Ca\(^{2+}\) influx) and reverse (Ca\(^{2+}\) efflux) modes of operation.

**METHODS**

**Myocyte isolation and culture.** Cardiac myocytes were isolated from the septum and left ventricular free wall of male Sprague-Dawley rats (~300 g) by successive perfusion with collagenase and hyaluronidase (6). Portions of freshly isolated myocytes were seeded on laminin-coated four-well trays (Nuclone), or plated directly on laminin-coated four-well trays. Culture medium consisted of serum-free medium 199 (Earle’s balanced salt solution without l-glutamine and NaHCO\(_3\)) supplemented with (in mM) 25 NaHCO\(_3\), 5 creatine, 2 carnitine, 5 taurine, and 0.1 ascorbic acid. Insulin (100 U/ml), 5-bromo-2-deoxyuridine (31 \(\mu\)g/ml), bovine serum albumin (0.2%), penicillin (500 U/ml), and gentamicin (4 \(\mu\)g/ml) were also added to the culture medium. After 2 h, media were then changed to remove nonadherent myocytes. The myocytes were incubated for an additional 3–4 h before initiation of electrical stimulation.

Myocytes were electrically paced in culture (extracellular Ca\(^{2+}\) concentration (\([\text{Ca}^{2+}]_o\)) 1.8 mM as described by Ivester et al. (12) except that carbon electrodes (0.25-in. diameter) rather than platinum electrodes were used (4). Briefly, carbon electrodes were submersed at opposite ends of each well of the four-well culture tray. Platinum wire (22 gauge) was inserted into bored holes in the carbon to couple electrode pairs in adjacent wells and to connect the terminal electrodes to the stimulator leads. Myocytes were paced at 1 Hz, 5-ms pulses, and field strength of 4 V/cm, using a custom-built stimulator capable of delivering pulses of alternating polarity to minimize electrolysis. Culture media were changed daily over the course of the experiments.

**Construction of recombinant replication-deficient adenovirus.** The basic protocol is described by He et al. (11). Briefly, the coding sequence of rat heart NCX1 was released from pcDNAs 1.1+ by sequential digestion with HindIII and XbaI. The NCX1 sequence (3,067 bp) was inserted into the shuttle vector pAdTrack-CMV, using the same HindIII and XbaI restriction sites on the shuttle vector. The resulting shuttle vector plasmid was linearized with PmeI, mixed with supercoiled pAdEasy-1, and used to electroporate BJ-5183 cells. The recombinant plasmids usually generated smaller colones, which were selected and screened by restriction endonuclease mapping (HindIII/XbaI and BglII/ClaI). Once recombination was confirmed, supercoiled plasmid DNA (isolated from minipreps) was transformed into DH10B cells for large-scale amplification. The recombinant construct was linearized with PacI and used to transfect HEK-293 cells. Recombinant adenovirus containing both green fluorescent protein (GFP) and NCX1 (each under a separate cytomegavirus promoter) (Adv-GFP-NCX1) was harvested in 7 days and stored at −20°C in 5 mM Tris (pH 8.0), 50 mM NaCl, 0.05% bovine serum albumin, and 25% glycerol.

**Adenoviral infection of cardiac myocytes.** Two hours after isolation, myocytes seeded in four-well trays were infected with either Adv-GFP-NCX1 or adenovirus expressing GFP alone (Adv-GFP) at a multiplicity of infection of 1 for 3 h. Media were then changed and myocytes were studied after 6, 24, and 48 h in continued pacing culture. Over 95% of myocytes fluoresced green (excitation 478 nm, emission 535 nm) within 6 h, indicating successful adenoviral infection and GFP expression. For brevity, myocytes infected with Adv-GFP and Adv-GFP-NCX1 are referred to as GFP and NCX1 myocytes, respectively.

**Myocyte shortening measurements.** Myocytes adherent to cover slips were bathed in 0.6 ml of air and temperature-equilibrated (37°C), HEPES-buffered (20 mM, pH 7.4) medium containing either 0.6, 1.8, or 5.0 mM (\([\text{Ca}^{2+}]_i\)) and were placed on a temperature-controlled stage (37°C) of a Zeiss IM35 microscope (29). Fields of myocytes were chosen at random, and myocytes were field stimulated to contract (1 Hz) between platinum electrodes spaced 2 mm apart, as previously described (29–31). Myocytes were viewed through an Olympus (DApo UV ×40, 1.30 numerical aperture (NA)) oil objective and were imaged by a charge-coupled device video camera (Ionoptix; Milton, MA). Myocyte motion measurements were acquired with the use of a personal computer with interface and software that were purchased from Ionoptix. Data were permanently stored on a zip drive (Iomega; Roy, UT) and analyzed off-line by Ionoptix software. For calibration of pixels versus micrometer, a high-resolution test target (model 22-8635, Ealing Electro-Optics; Natick, MA) was used.

**\([\text{Ca}^{2+}]_o\) transient measurements.** Myocytes were exposed to 0.67 \(\mu\)M fura 2-AM for 15 min at 37°C (7). Fura 2-loaded myocytes mounted in a Dvorak-Stotel chamber situated in a temperature-controlled stage (37°C) of a Zeiss IM35 inverted microscope were field stimulated to contract at 1 Hz between platinum wire electrodes, as previously described (31). (\([\text{Ca}^{2+}]_i\)) was varied between 0.6 and 5.0 mM. Excitation light (360 and 380 nm, ±10 nm band pass, Ionoptix) was directed to individual myocytes only during data acquisition to minimize photobleaching. Epifluorescence (510 ± 18 nm) collected by a Olympus DAPo UV ×40/1.30 NA oil objective was passed through a pinhole (1.6 mm) and captured by a photomultiplier (model R928-07; Hamamatsu). Output from the photomultiplier was routed through an amplifier/discriminator (model C609, Thorn EMI; Middlesex, UK) before arrival at a counter/timer board (model C660, Thorn EMI).

Epifluorescence from a myocyte collected at 360-nm excitation was divided by that collected at 380-nm excitation to obtain the fluorescence intensity ratio (R) (31), from which \([\text{Ca}^{2+}]_i\) was calculated according to Grynkiewicz et al. (9) by using 224 nM as the \([\text{Ca}^{2+}]_o\) fura 2 dissociation constant (\(K_d\)). Background and cellular autofluorescence measured in GFP or NCX1 myocytes not loaded with fura 2 accounted for <10% of the fura 2 signal. Intracellular fura 2 fluorescence was calibrated daily for each batch of myocytes, as previously described (7). \([\text{Ca}^{2+}]_i\) transient data were analyzed with custom-written software (Ionoptix).

**Measurement of SR \([\text{Ca}^{2+}]_o\) content.** SR-releasable \([\text{Ca}^{2+}]_o\) content was estimated by measuring the time integral of forward Na\(^+\)/Ca\(^{2+}\) exchange current induced by caffeine exposure, as described by us previously (32, 33). Briefly, whole cell patch clamp recordings were performed at 30°C. Pipette solution consisted of (in mM) 100 Cs\(^+\) glutamate, 1 MgCl\(_2\), 30 HEPES, and 2.5 MgATP; pH 7.2. External solution was (in mM) 130 Na\(^+\).
NaCl, 5 CaCl, 1.2 MgSO4, 1.2 NaH2PO4, 20 HEPES, and 10 glucose; pH 7.4. [Ca2+]o was either 0.6 or 5.0 mM. Holding potential was –80 mV. At 200 ms after the 11th conditioning pulse (from –80 to 0 mV, 300 ms, 1 Hz), with membrane potential held at –80 mV, caffeine (5 mM; 2.4 s) was applied by puff superfusion (30, 33). Currents were digitized at 0.5 kHz and collected for ~4 s.

NCX1, SERCA2, and calsequestrin immunoblotting. Cultured myocytes from a four-well tray were rinsed three times with ice-cold phosphate-buffered saline and then scraped with a rubber policeman into a 1 ml of ice-cold lysis buffer containing 20 mM Tris (pH 8.1), 137 mM NaCl, 2 mM EDTA, 10% glycerol, 0.3% sodium dodecyl sulfate (SDS), 0.4% deoxycholate, 100 µg/ml phenylmethylsulfonyl fluoride, and 1 µl/ml protease inhibitor cocktail (Sigma). The cell lysate was snap-frozen with dry ice-ethanol and stored at –80°C. The amount of myocyte protein recovered from a 4-well culture tray was 887.6 ± 117.8 µg (n = 19).

Myocyte lysates in SDS sample buffer (containing either 10 mM N-ethylmaleimide (for NCX1) or 5% 2-mercaptoethanol (for SERCA2)) were applied to 7.5% polyacrylamide gel, and proteins were separated by electrophoresis (32). Proteins from SDS-polyacrylamide gel electrophoresis (PAGE) were transferred onto Trans-Blot membranes (Bio-Rad; Hercules, CA). To detect NCX1, rabbit anti-NCX1 antibody (1:500 dilution; π11–13, Swant; Bellinzona, Switzerland) was used with donkey anti-rabbit IgG (1:5,000; Amersham; Buckinghamshire, UK) as the secondary antibody. For SERCA2 detection, we used a monoclonal antibody (1:2,500; MA3–919, Affinity Bioreagents; Golden, CO), and sheep anti-mouse antibody (1:5,000; Amersham) was used as the secondary antibody. For calsequestrin immunoblotting, membranes stripped of NCX1 or SERCA2 were sequentially exposed to rabbit anti-calsequestrin antibody (1:2,500; Swant) and donkey anti-rabbit IgG (1:5,000; Amersham). Immunoreactive proteins were detected with the enhanced chemiluminescence-Western blotting system (Amersham). We quantitated the protein band signal intensities by scanning autoradiograms of the blots with a phosphorimager (Molecular Dynamics; Sunnyvale, CA).

Statistics. All results are expressed as means ± SE. In experiments in which maximal contraction amplitudes were measured as functions of experimental group (–Adv-GFP vs. +Adv-GFP), [Ca2+]o, and days in culture, three-way ANOVA was performed to determine significance of difference. A linear model fitted standard-least squares (JMP version 4, SAS Institutes; Cary, NC) was used. For analysis of a parameter (e.g., systolic [Ca2+]o, and maximal contraction amplitude) as a function of group (GFP vs. NCX1) and [Ca2+]o, two-way analysis of variance (ANOVA) was used to determine statistical significance. In all analyses, P ≤ 0.05 was taken to be statistically significant.

RESULTS

Effects of continual pacing on cultured myocyte contractility. We have previously demonstrated that myocytes cultured under quiescent conditions for as little as 18 h exhibited significantly less cell shortening compared with freshly isolated myocytes (28). When placed under continual electrical stimulation conditions, myocytes retained similar contraction amplitudes at 24 and 48 h in culture when compared with freshly isolated cells (Table 1) whether cells were studied at 0.6, 1.8, or 5.0 mM [Ca2+]o. This conclusion is supported by two-way ANOVA ([Ca2+]o, day in culture), which indicated significant [Ca2+]o effect (P < 0.0001) but insignificant day in culture (P = 0.6331) and [Ca2+]o × day interaction (P = 0.5070) effects.

Effects of adenoviral infection on cultured myocyte contractility. Infection with recombinant, replication-deficient adenovirus has been shown to be highly efficient for gene delivery to adult cardiac myocytes (13, 15). Compared with uninfected myocytes, adenoviral-infected myocytes at 6 (day 0), 24, and 48 h of continual pacing in culture had similar cell shortening amplitudes regardless of whether cell shortening was measured at 0.6, 1.8, or 5.0 mM [Ca2+]o (Table 1). Three-way ANOVA (group, [Ca2+]o, and day) indicated insignificant group (–Adv-GFP vs. +Adv-GFP; P = 0.1341) and day (P = 0.4577) but significant [Ca2+]o (P < 0.0001) effects. In addition, the group × [Ca2+]o × day interaction effect was also insignificant (P = 0.6844).

Effects of Adv-GFP-NCX1 infection on NCX1 abundance and cell size. Forty-eight hours after infection with either Adv-GFP or Adv-GFP-NCX1, cardiac myocyte lysates were collected to analyze for NCX1 abundance by immunoblotting. Under nonreducing gel conditions, NCX1 was detected as a band of apparent molecular weight of 160 kDa (18, 32). Compared with GFP myocytes, NCX1 myocytes had significantly (P < 0.02) more NCX1 protein (558 ± 97 vs. 199 ± 29 arbitrary units) (Fig. 1). In contrast, there were no (P < 0.24) differences in SERCA2 amounts between NCX1 (111 ± 4) and GFP (102 ± 6 arbitrary units) myocytes (Fig. 1). As an additional control for protein loading, we measured calsequestrin in each of the myocyte lysates (Fig. 1). Calsequestrin expression has been shown to be unchanged during ontogenic development, aging, cardiac hypertrophy, and failing human myocardium (10) and this can be used as an internal control. There were no significant (P = 0.9884) differences in calsequestrin protein amounts between GFP and NCX1 (91.3 ± 0.7 vs. 91.3 ± 0.6 arbitrary units, respectively) myocytes.
Effects of NCX1 overexpression on myocyte contractile function. At 0.6 mM [Ca\(^{2+}\)]\(_o\), GFP myocytes shortened 456% more than NCX1 myocytes (Table 2). This is because of the 21 NCX1 myocytes examined, 15 exhibited contraction amplitudes that were <1% of resting cell length (Fig. 2). Indeed, 11 of the 21 NCX1 myocytes had no discernible contractions when paced in media containing 0.6 mM [Ca\(^{2+}\)]\(_o\). In contrast, at 5.0 mM [Ca\(^{2+}\)]\(_o\), GFP myocytes shortened 30% less than the NCX1 myocytes did (Fig. 2 and Table 2). The differences in twitch amplitudes were no longer apparent at intermediate [Ca\(^{2+}\)]\(_o\) levels (Fig. 2, Table 2). These conclusions are supported by highly significant (P < 0.0001) [Ca\(^{2+}\)]\(_o\) and group × [Ca\(^{2+}\)]\(_o\) interaction effects, indicating that the magnitude and/or direction of the effects of [Ca\(^{2+}\)]\(_o\) on cell shortening was different across experimental groups (GFP vs. NCX1).

To further analyze contraction dynamics, we measured maximal shortening velocity and half-time of relaxation (t\(_{1/2}\)) (Table 2). It was not possible to compare contraction dynamics data collected at 0.6 mM [Ca\(^{2+}\)]\(_o\) because >70% of NCX1 myocytes exhibited little to no contraction when stimulated at that [Ca\(^{2+}\)]\(_o\) (Fig. 2). Maximal shortening velocity was significantly (P = 0.0108, group effect) higher in NCX1 myocytes. Raising [Ca\(^{2+}\)]\(_o\) increased maximal shortening velocity (significant [Ca\(^{2+}\)]\(_o\) effect, P = 0.05) but did not affect the inherent differences in maximal shortening velocity between the two groups (group × [Ca\(^{2+}\)]\(_o\) effect, P = 0.0736).

Compared with GFP myocytes, NCX1 myocytes had shorter t\(_{1/2}\) (Table 2; significant group effect P = 0.0034); the differences were larger at 1.8 mM [Ca\(^{2+}\)]\(_o\) (significant group × [Ca\(^{2+}\)]\(_o\) interaction effects, P = 0.0046).

Effects of NCX1 overexpression on [Ca\(^{2+}\)]\(_i\) transient. [Ca\(^{2+}\)]\(_i\) occupies a central role in cardiac myocyte excitation-contraction coupling. Thus the differences in contractile behavior between GFP and NCX1 myocytes may be related to differences in [Ca\(^{2+}\)]\(_i\) homeostasis brought about by NCX1 overexpression in cardiac myocytes. Indeed, resting [Ca\(^{2+}\)]\(_i\) was significantly lower in NCX1 myocytes (Table 3; group effect, P < 0.0001). Changing [Ca\(^{2+}\)]\(_i\) apparently had no significant effect on resting [Ca\(^{2+}\)]\(_i\), (Table 3; [Ca\(^{2+}\)]\(_i\), effect, P = 0.0596). Diastolic [Ca\(^{2+}\)]\(_i\) comparisons between GFP and NCX1 myocytes essentially mirrored the results of resting [Ca\(^{2+}\)]\(_i\), comparisons in Table 3; significant group (P < 0.0001) but insignificant [Ca\(^{2+}\)]\(_i\), (P = 0.4338) effects.

With respect to systolic [Ca\(^{2+}\)]\(_i\), at low (0.6 mM) [Ca\(^{2+}\)]\(_o\), measured values for GFP myocytes were higher than those found for NCX1 myocytes (Fig. 3 and Table 3). In addition, the magnitude of the [Ca\(^{2+}\)]\(_i\) transient (systolic-diastolic [Ca\(^{2+}\)]\(_i\)) or the percent increase in fura 2 fluorescence intensity ratio measured in NCX1 myocytes at 0.6 mM [Ca\(^{2+}\)]\(_o\) was barely discernible (Fig. 3, Table 3). Indeed, of the 26 NCX1 myocytes examined at 0.6 mM [Ca\(^{2+}\)]\(_o\), only 4 cells displayed [Ca\(^{2+}\)]\(_i\), increases of ≥50 nM when paced, and 14 myocytes had [Ca\(^{2+}\)]\(_i\), transient magnitudes of ≤20 nM. In contrast, at high (5.0 mM) [Ca\(^{2+}\)]\(_o\), systolic [Ca\(^{2+}\)]\(_i\), was higher in NCX1 myocytes (Fig. 3 and Table 3).
Table 3. Effects of NCX1 overexpression on cardiac myocytes [Ca\(^{2+}\)]\(_o\) transients

<table>
<thead>
<tr>
<th>[Ca(^{2+})](_o), nM</th>
<th>GFP</th>
<th>NCX1</th>
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<tr>
<td><strong>Resting</strong> [Ca(^{2+})](_o)</td>
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<tr>
<td>0.6</td>
<td>130 ± 10 (24)</td>
<td>88 ± 5 (26)</td>
</tr>
<tr>
<td>1.8</td>
<td>129 ± 10 (24)</td>
<td>84 ± 5 (31)</td>
</tr>
<tr>
<td>5.0</td>
<td>105 ± 7 (21)</td>
<td>81 ± 6 (27)</td>
</tr>
<tr>
<td><strong>Systolic</strong> [Ca(^{2+})](_o)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.6</td>
<td>218 ± 14</td>
<td>132 ± 7</td>
</tr>
<tr>
<td>1.8</td>
<td>344 ± 15</td>
<td>362 ± 16</td>
</tr>
<tr>
<td>5.0</td>
<td>368 ± 29</td>
<td>464 ± 21</td>
</tr>
<tr>
<td><strong>Diastolic</strong> [Ca(^{2+})](_o)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.6</td>
<td>148 ± 10</td>
<td>104 ± 5</td>
</tr>
<tr>
<td>1.8</td>
<td>146 ± 9</td>
<td>108 ± 4</td>
</tr>
<tr>
<td>5.0</td>
<td>121 ± 7</td>
<td>117 ± 4</td>
</tr>
<tr>
<td><strong>t(_{\Delta})</strong> of [Ca(^{2+})] decline, s</td>
<td></td>
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<tr>
<td>0.6</td>
<td>0.155 ± 0.009</td>
<td>0.147 ± 0.008</td>
</tr>
<tr>
<td>1.8</td>
<td>0.130 ± 0.006</td>
<td>0.130 ± 0.007</td>
</tr>
<tr>
<td>5.0</td>
<td>44.7 ± 3.0</td>
<td>53.0 ± 2.6†</td>
</tr>
<tr>
<td><strong>%Increase in fluorescence intensity ratio</strong></td>
<td></td>
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<tr>
<td>0.6</td>
<td>13.6 ± 1.6</td>
<td>5.7 ± 1.0†</td>
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<tr>
<td>1.8</td>
<td>34.0 ± 2.5</td>
<td>40.8 ± 2.5</td>
</tr>
<tr>
<td>5.0</td>
<td>44.7 ± 3.0</td>
<td>53.0 ± 2.6†</td>
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Values are means ± SE. Numbers in parentheses are numbers of myocytes, without regard to number of cells contributed by each heart. [Ca\(^{2+}\)]\(_o\), cytosolic Ca\(^{2+}\) concentration, measured 48 h after infection. *P < 0.0001; †P < 0.001, NCX1 vs. GFP.

Fig. 2. Overexpression of NCX1 alters contractile function in rat myocytes. Isolated myocytes were infected with recombinant adenoaviruses that express either GFP or GFP and NCX1 and then cultured for 48 h under continuous electrical stimulation (1 Hz) conditions. For contraction studies, cultured myocytes were paced (1 Hz) to contract at 37°C and extracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_o\)) of 0.6, 1.8, or 5.0 mM. Shown are steady-state paced twitches from myocyte overexpressing either GFP (A, C, and E) or NCX1 (B, D, and F). Note that at 0.6 mM [Ca\(^{2+}\)]\(_o\) (A and B), contraction amplitude was larger in GFP myocytes, whereas at 5.0 mM [Ca\(^{2+}\)]\(_o\) (E and F), NCX1 myocytes contracted more than GFP myocytes. At 1.8 mM [Ca\(^{2+}\)]\(_o\) (C and D), the contraction amplitudes were similar between GFP and NCX1 myocytes. Results are summarized in Table 2.

3. At intermediate (1.8 mM) [Ca\(^{2+}\)]\(_o\), differences in systolic [Ca\(^{2+}\)]\(_o\), between GFP and NCX1 myocytes were no longer apparent (Fig. 3, Table 3). This interpretation is supported by the results of two-way ANOVA: insignificant group (P = 0.5457) but significant [Ca\(^{2+}\)]\(_o\) (P < 0.0001) and group × [Ca\(^{2+}\)]\(_o\) interaction (P < 0.0001) effects, indicating the magnitude and/or direction of the effects of [Ca\(^{2+}\)]\(_o\) on systolic [Ca\(^{2+}\)]\(_o\), was different across GFP and NCX1 myocytes.

The magnitude of the [Ca\(^{2+}\)] transient is reflected by the % increase in fura 2 fluorescence intensity R, which has the advantage of being free from fluorescence signal calibration errors and uncertainties in intracellular fura 2 K\(_d\). Similar to the results observed with systolic [Ca\(^{2+}\)]\(_o\), when GFP and NCX1 myocytes were stimulated at 0.6 mM [Ca\(^{2+}\)]\(_o\), the percent increase in R was higher in GFP compared with NCX1 myocytes (Fig. 3 and Table 3). Conversely, when myocytes were paced at 5.0 mM [Ca\(^{2+}\)]\(_o\), NCX1 myocytes exhibited greater percent increase in R than GFP myocytes (Fig. 3, Table 3). This conclusion is supported by an insignificant group (P = 0.2009) but highly significant [Ca\(^{2+}\)]\(_o\) (P < 0.0001) and group × [Ca\(^{2+}\)]\(_o\) interaction (P = 0.0009) effects.

Estimates of t\(_{\Delta}\) of [Ca\(^{2+}\)] decline at 0.6 mM [Ca\(^{2+}\)]\(_o\) were imprecise because of the small magnitude of the [Ca\(^{2+}\)]\(_o\) transients, especially in NCX1 myocytes. Comparisons of t\(_{\Delta}\) of [Ca\(^{2+}\)] decline measured at 1.8 and 5.0 mM [Ca\(^{2+}\)]\(_o\), indicated no significant difference between GFP and NCX1 myocytes (Table 3; group effect, P = 0.5903). However, elevating [Ca\(^{2+}\)]\(_o\), which in-
creased amplitudes of $[Ca^{2+}]_i$ transient, significantly lowers the $t_{1/2}$ of $[Ca^{2+}]_i$ transient decline in both GFP and NCX1 myocytes (Table 3; $[Ca^{2+}]_o$ effect, $P = 0.0099$), consistent with the finding reported by Bers and Berlin (5) that the kinetics of $[Ca^{2+}]_i$ decline were dependent on peak $[Ca^{2+}]_i$. There were no significant group $\times [Ca^{2+}]_o$ interaction effects ($P = 0.6196$).

**DISCUSSION**

Adult cardiac myocytes placed in primary culture may provide a useful in vitro model to study gene expression in the heart. Unlike neonatal cardiomyocytes, healthy isolated adult cardiac myocytes are rod-shaped, striated, and do not exhibit spontaneous contractions. Early studies on primary cultures of adult cardiac myocytes demonstrated that the myocytes rapidly lost their rod-shaped morphology, spread on the culture substratum, and acquired spontaneous contractile activity (22). The loss of the normal rod-shaped morphology was prevented by exclusion of fetal calf serum and addition of creatine, carnitine, and taurine (CCT medium) to the culture media (25). Despite maintenance of rod-shaped morphology, quiescent cultured adult cardiac myocytes suffered a rapid decline in contractile function such that maximal contraction amplitudes were lower in NCX1 than GFP (Fig. 3B and D) myocytes. At 5.0 mM $[Ca^{2+}]_o$, intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) transient amplitudes were larger in NCX1 (F) than GFP (E) myocytes. At 1.8 mM $[Ca^{2+}]_o$, the $[Ca^{2+}]_i$ transient amplitudes were similar between GFP (C) and NCX1 (D) myocytes. Note also the rates of $[Ca^{2+}]_i$ decline during the first half of the $[Ca^{2+}]_i$ transient were similar between the two groups. In another 2 groups of myocytes expressing either GFP or GFP and NCX1, but not loaded with fura 2, it was determined that autofluorescence from GFP accounted for <10% of the fura 2 signal obtained either at 360- or 380-nm excitation. Composite results are summarized in Table 3.

**Effects of NCX1 overexpression on SR-releasable $Ca^{2+}$ contents.** Application of 5 mM caffeine for 2.4 s on cardiac myocytes at -80 mV caused a large inward current that rapidly returned to baseline (Fig. 4). This inward current represented forward $Na^+/Ca^{2+}$ exchange ($3 Na^+ in:1 Ca^{2+} out$ current ($I_{NaCa}$) operating to extrude the SR $Ca^{2+}$ released by caffeine. Under our experimental conditions, the time integral of the caffeine-induced inward current was an estimate of SR-releasable $Ca^{2+}$ of the myocyte (32, 33). At 0.6 mM $[Ca^{2+}]_o$, both $I_{NaCa}$ time integral (pC/cell) and SR-releasable $Ca^{2+}$ (normalized to cell size; fmol/ff) were smaller in NCX1 myocytes (Fig. 4B) than GFP myocytes (Fig. 4A) (Table 4). In contrast, at 5.0 mM $[Ca^{2+}]_o$, SR-releasable $Ca^{2+}$ was larger in NCX1 (Fig. 4D) than GFP (Fig. 4C) myocytes (Table 4). For both $I_{NaCa}$ time integral and SR-releasable $Ca^{2+}$, two-way ANOVA indicated significant group ($P < 0.03$), $[Ca^{2+}]_o$ ($P < 0.0001$), and group $\times [Ca^{2+}]_o$ interaction ($P < 0.001$) effects, indicating that changing $[Ca^{2+}]_o$ affected the inherent differences in SR $Ca^{2+}$ contents between GFP and NCX1 myocytes.
tude decreased by 20% within 18 h (28) and 30–50% within 6–24 h of plating (8). In addition, quiescent cultured myocytes gradually atrophied as indicated by loss of one-third of cellular protein content after 15 days of culture (25). These shortcomings were mitigated by the adoption of continual electrical stimulation of adult cardiac myocytes in culture, which resulted in preservation of normal contractile activity for at least 72 h (4) and maintenance of cellular protein content after 7 days (14). Thus the first major finding of our study is that we confirm and extend Berger et al.’s (4) observation that continuous pacing of adult cardiac myocytes, whether measured at low, intermediate, or high [Ca^{2+}]_o (Table 1). This important result allows the use of cultured adult cardiac myocytes in studies of the effects of various gene manipulations on excitation-contraction coupling.

A second technical difficulty encountered when using adult cardiac myocytes in gene manipulation studies relates to the relative inefficiency (compared with natal cardiomyocytes) of introduction of foreign genes by conventional, plasmid-based procedures for gene transfer. With the advent of recombinant adenovirus-mediated gene delivery methodology, high-efficiency gene transfer into adult cardiac myocytes can be accomplished (13, 15). Thus a second major finding of our present study is that there were no differences in contractile function between uninfected and adenovirus-infected adult cardiac myocytes, whether examined at low, intermediate, and high [Ca^{2+}]_o, or at 6-, 24-, and 48-h postadenovirus exposure (Table 1). Our observations provide the foundation for using continuously paced cultured adult cardiac myocytes in studies of effects of adenovirus-mediated gene transfer on cardiac excitation-contraction coupling.

To determine the physiological role of the cardiac NCX1, we studied adult rat cardiac myocytes, in which overexpression of the rat heart NCX1 was achieved by recombinant adenovirus-mediated gene delivery (Fig. 1). At “physiological” [Ca^{2+}]_o of 1.8 mM, there were no differences in twitch amplitudes between NCX1 and GFP myocytes, although maximal shortening velocity was greater and t_1/2 was shorter in NCX1 myocytes (Table 2). Our observations are similar to the findings in ventricular myocytes from transgenic mice overexpressing NCX1, in which twitches were of similar size between transgenic and wild-type myocytes but the rate of contraction and relaxation were significantly faster in transgenic than in wild-type myocytes (24). This subtle change in contractile behavior in myocytes overexpressing the NCX1 can be made more apparent by manipulating the experimental conditions. For example, under conditions favoring Ca^{2+} influx (5.0 mM [Ca^{2+}]_o) of 1.8 mM, there were no differences in twitch amplitudes between NCX1 and GFP myocytes, although maximal shortening velocity was greater and t_1/2 was shorter in NCX1 myocytes (Table 2). Conversely, under conditions favoring Ca^{2+} efflux (0.6 mM [Ca^{2+}]_o) (29, 30), NCX1 myocytes contracted significantly less than GFP myocytes (Table 2). The differences in twitch amplitudes between GFP and NCX1 myocytes were no longer apparent at intermediate [Ca^{2+}]_o. Assuming similar myofilament Ca^{2+} sensitivity between GFP and NCX1 myocytes, this complex pattern of contractile responses (Fig. 2) is consistent with the hypothesis

<table>
<thead>
<tr>
<th>[Ca^{2+}]_o</th>
<th>GFP</th>
<th>NCX1</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.6</td>
<td>67.4 ± 5.0 (6)</td>
<td>48.2 ± 2.3 (8)*</td>
</tr>
<tr>
<td>5.0</td>
<td>174.8 ± 9.3 (6)</td>
<td>287.9 ± 34.2 (6)*</td>
</tr>
<tr>
<td>SR-releasable Ca^{2+}, fmol/fF</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.6</td>
<td>3.89 ± 0.35 (6)</td>
<td>2.70 ± 0.13 (8)*</td>
</tr>
<tr>
<td>5.0</td>
<td>9.98 ± 0.45 (6)</td>
<td>16.23 ± 1.97 (6)*</td>
</tr>
</tbody>
</table>

Values are means ± SE. Numbers in parentheses are numbers of myocytes. SR, sarcoplasmic reticulum. To account for variability in cell sizes, SR-releasable Ca^{2+} values are normalized to myocyte capacitance. To convert coulombs to moles, charge was divided by Faraday’s constant of 96,487 coulombs/equivalent, based on 3 Na^+ being exchanged for each Ca^{2+}. *P < 0.03, NCX1 vs. GFP.

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Fig. 4. Estimation of sarcoplasmic reticulum (SR)-releasable Ca^{2+} in myocytes overexpressing NCX1. Myocytes were incubated with either 0.6 (A and B) or 5.0 mM (C and D) [Ca^{2+}]_o, at 30°C and were voltage clamped at −80 mV. At 200 ms after the 11th conditioning pulse (from −80 to 0 mV, 300 ms, and 1 Hz), caffeine (5 mM) was puffed onto the myocyte for 2.4 s. Large transient inward current caused by caffeine-induced SR Ca^{2+} release was observed. This current represents Na^+ entry caused by Ca^{2+} extrusion by the NCX1, and the time integral of this current provides an estimate of SR-releasable Ca^{2+} (32, 33). At 0.6 mM [Ca^{2+}]_o, SR-releasable Ca^{2+} was higher in GFP (A) than NCX1 (B) myocytes. At 5.0 mM [Ca^{2+}]_o, NCX1 current time integral was higher in NCX1 (D) than GFP (C) myocytes. Composite results are summarized in Table 4.
that alterations in both Ca\(^{2+}\) influx and efflux pathways contributed to the different contractile behavior in NCX1 myocytes. Because NCX1 can affect both Ca\(^{2+}\) influx and efflux pathways, and because there is no a priori reason to suspect L-type Ca\(^{2+}\) channel expression in cardiac myocytes is affected by NCX1 overexpression (especially in view of the observation in transgenic myocytes overexpressing NCX1) (27), our findings are supportive of our hypothesis that the overexpressed NCX1 was functional in both Ca\(^{2+}\) influx and efflux modes during stimulated twitches.

If the differences in contractile behavior between GFP and NCX1 myocytes were brought about by the overexpressed NCX1 perturbing Ca\(^{2+}\) fluxes, then one would expect corresponding changes in [Ca\(^{2+}\)]\(_i\) transient during a twitch. At 0.6 mM [Ca\(^{2+}\)]\(_o\), conditions which favored Ca\(^{2+}\) influx, the magnitude of [Ca\(^{2+}\)]\(_i\) transient was higher in the control GFP myocytes (Table 3). At 5.0 mM [Ca\(^{2+}\)]\(_o\), conditions which favored Ca\(^{2+}\) influx, [Ca\(^{2+}\)]\(_i\) transient amplitude was higher in NCX1 myocytes. At intermediate [Ca\(^{2+}\)]\(_o\) levels of 1.8 mM, differences in [Ca\(^{2+}\)]\(_i\) transient amplitude or systolic [Ca\(^{2+}\)]\(_i\) were narrowed (Table 3). This pattern of [Ca\(^{2+}\)]\(_i\) transient amplitude differences between NCX1 and GFP myocytes mirrors the pattern of contractile amplitude differences and indicates that the overexpressed NCX1 was functional in both modes of operation. It should be noted that the lack of significant differences in [Ca\(^{2+}\)]\(_i\) transient amplitudes measured in GFP and NCX1 myocytes stimulated at physiological [Ca\(^{2+}\)]\(_o\) of 1.8 mM agrees with the observation in transgenic murine myocytes overexpressing NCX1 (1, 24, 27). It is also interesting to note that both resting and diastolic [Ca\(^{2+}\)]\(_i\) were significantly lower in NCX1 myocytes (Table 3), consistent with the notion that forward NCX1 was necessary in maintaining diastolic [Ca\(^{2+}\)]\(_i\) levels (26), enhanced forward NCX1 function in NCX1 myocytes resulted in a lower resting and diastolic [Ca\(^{2+}\)]\(_i\) levels.

Despite no changes in SERCA2 expression in transgenic murine myocytes overexpressing NCX1 (24), SR Ca\(^{2+}\) uptake activity was reported to be increased (24) or unchanged (27). In our rat myocytes overexpressing NCX1, the \(t_{1/2}\) of [Ca\(^{2+}\)]\(_i\) decline (a measure of in situ SR Ca\(^{2+}\) uptake activity; Ref. 31), was not different compared with control GFP myocytes (Table 3). Thus SR Ca\(^{2+}\) uptake activity was not affected by overexpression of NCX1. This is also consistent with the observation that SERCA2 expression was unaffected by NCX1 overexpression in cultured adult rat myocytes (Fig. 1).

At high [Ca\(^{2+}\)]\(_o\), SR Ca\(^{2+}\) content was significantly higher in NCX1 myocytes (Fig. 4D and Table 4). Because neither SERCA2 activity (Table 3) nor abundance (Fig. 1) was affected by NCX1 overexpression, and in the absence of evidence suggesting L-type Ca\(^{2+}\) channel function or abundance and SR Ca\(^{2+}\)-leak were altered by NCX1 overexpression, increased SR Ca\(^{2+}\) content in NCX1 myocytes was likely due to enhanced reverse Na\(^+\)/Ca\(^{2+}\) exchange during depolarization, with the resultant increase in [Ca\(^{2+}\)]\(_i\) transient (Fig. 3F) and twitch amplitudes (Fig. 2F). Higher SR Ca\(^{2+}\) content was also observed in transgenic myocytes overexpressing NCX1 (24), although this finding is inconsistent among investigators (1, 27). Increased SR Ca\(^{2+}\) loading during diastole, rather than increased Ca\(^{2+}\) influx on the exchanger during a twitch, was proposed by Terracciano et al. (24) as the cause of enhanced contraction in transgenic myocytes overexpressing NCX1. This appears unlikely, as both resting and diastolic [Ca\(^{2+}\)]\(_i\) were significantly lower in NCX1 myocytes (Table 3), indicating increased extrusion of Ca\(^{2+}\) during rest and in diastole by the exchanger. Yao et al. (27) also reported a somewhat lower diastolic [Ca\(^{2+}\)]\(_i\) in transgenic myocytes, although this difference did not reach statistical significance.

Under conditions that promote Ca\(^{2+}\) efflux ([Ca\(^{2+}\)]\(_o\) = 0.6 mM), SR Ca\(^{2+}\) content was significantly lower in NCX1 myocytes (Fig. 4B and Table 4). The lower SR Ca\(^{2+}\) content in NCX1 myocytes at 0.6 mM [Ca\(^{2+}\)]\(_o\), explained very well the lower [Ca\(^{2+}\)]\(_i\) transient and twitch amplitudes. Thus overexpressing NCX1 in adult rat cardiac myocytes can either load or deplete the SR of Ca\(^{2+}\), depending on the experimental conditions. When one considers in total the patterns of changes in twitch amplitudes, [Ca\(^{2+}\)]\(_i\) transients, and SR Ca\(^{2+}\) contents observed in NCX1 myocytes, the most logical explanation is that the overexpressed NCX1 is functional in both forward and reverse modes. Enhanced Ca\(^{2+}\) influx during depolarization associated with the action potential was a major effect of NCX1 overexpression, and increased Ca\(^{2+}\) extrusion during diastole and at rest by the overexpressed exchanger resulted in a lower resting and diastolic [Ca\(^{2+}\)]\(_i\). Our conclusions are in agreement with the results obtained from transgenic myocytes overexpressing NCX1 (24, 27), although Adachi-Akahane et al. (1) reported that the overexpressed NCX1 in transgenic murine myocytes only produced functional consequences in the Ca\(^{2+}\) efflux mode. The discrepancy is most likely due to artifactual lowering of cytosolic Na\(^+\) by patch pipette dialysis in the study of Adachi-Akahane et al. (1).

A recent study (21) employing adenovirus-mediated gene transfer in cultured adult rabbit myocytes suggested that contractile performance was impaired, rather than enhanced, by NCX1 overexpression (21). In addition, SR Ca\(^{2+}\) content, as measured by caffeine-induced contracture, was decreased in rabbit myocytes overexpressing NCX1. The apparent discrepancy between our data and those of Schillinger et al. (21) can be reconciled if the following factors are taken into account. First, there is the well-known species difference in NCX1 function between adult rat and rabbit myocytes (2). For example, during a twitch, NCX1 of rabbit myocytes may either load or deplete the SR of Ca\(^{2+}\), depending on the experimental conditions. When one considers in total the patterns of changes in twitch amplitudes, [Ca\(^{2+}\)]\(_i\) transients, and SR Ca\(^{2+}\) contents observed in NCX1 myocytes, the most logical explanation is that the overexpressed NCX1 is functional in both forward and reverse modes. Enhanced Ca\(^{2+}\) influx during depolarization associated with the action potential was a major effect of NCX1 overexpression, and increased Ca\(^{2+}\) extrusion during diastole and at rest by the overexpressed exchanger resulted in a lower resting and diastolic [Ca\(^{2+}\)]\(_i\). Our conclusions are in agreement with the results obtained from transgenic myocytes overexpressing NCX1 (24, 27), although Adachi-Akahane et al. (1) reported that the overexpressed NCX1 in transgenic murine myocytes only produced functional consequences in the Ca\(^{2+}\) efflux mode. The discrepancy is most likely due to artifactual lowering of cytosolic Na\(^+\) by patch pipette dialysis in the study of Adachi-Akahane et al. (1).
that may be altered by continuous pacing culture and emphasized that our present culture conditions were cyte culture (4). Despite these caveats, it has to be density by another 33% compared with quiescent myocytes by 72 h. Continuous pacing for 48 h increased 55% at 24–48 h of quiescent rat myocyte culture, but not all, of the known Ca2
this study. The first is that we have measured some, application.
during 5 min of electrical pacing at 2 Hz before caffeine myocytes contracted more vigorously than GFP myocytes (2). In contrast to normal rabbit myocytes, which exhibited positive staircase phenomenon, in rabbit myocytes overexpressing NCX1, increasing pacing frequency resulted in no increases in twitch amplitudes (21), suggesting that Ca2
efflux was enhanced at high beating rates. This conclusion is consistent with our observations that contractions in NCX1 rat myocytes were “impaired” compared with GFP myocytes when studied under conditions that favored Ca2
1, rat NCX1 myocytes contracted more vigorously than GFP myocytes due to enhanced SR Ca2
loading via reverse Na+/Ca2
exchange during depolarization. Fifth, canine NCX1 was overexpressed in rabbit myocytes in the study of Schillinger et al. (21), whereas we overexpressed rat NCX1 in myocytes of the same species. Whether there are functional differences when an important membrane transport protein is overexpressed in cells from a different species is at present unknown. Finally, the lower caffeine contracture amplitude in rabbit myocytes overexpressing NCX1 (21), in contrast to the findings of Terracciano et al. (24), can be explained by SR Ca2
content depletion due to enhanced Ca2
efflux in rabbit myocytes overexpressing NCX1 during 5 min of electrical pacing at 2 Hz before caffeine application.

Finally, there are several important limitations to this study. The first is that we have measured some, but not all, of the known Ca2
regulatory pathways that may be altered by continuous pacing culture and adenosiral infection. For example, Ellingsen et al. (8) reported that L-type Ca2
 current (Ica) increased ~55% at 24–48 h of quiescent rat myocyte culture, returning to levels observed in freshly isolated myocytes by 72 h. Continuous pacing for 48 h increased Ica density by another 33% compared with quiescent myocyte culture (4). Despite these caveats, it has to be emphasized that our present culture conditions were effective in maintaining normal contractile activity compared with freshly isolated myocytes (Table 1). In addition, comparisons between GFP and NCX1 myocytes in the present study were performed under similar conditions, i.e., 48 h after adenoviral infection of continual electrically stimulated myocytes in culture, and thus should provide identical background for our measurements. A second limitation relates to the concern that NCX1 overexpression may affect other Ca2
regulatory pathways in rat cardiac myocytes. We have provided direct (SERCA2 and calsequestrin expression; Fig. 1) and indirect (SR Ca2
uptake activity; Table 3) evidence that some of the Ca2
homeostatic pathways were unaffected by NCX1 overexpression in our short-term (48 h) culture. In addition, in transgenic myocytes, in which compensatory responses are more likely to occur, NCX1 overexpression had no affect on peak Ica density (27), phospholamban, SERCA2, and calsequestrin levels (24). The weight of current evidence suggests that NCX1 overexpression was unlikely to significantly affect other important Ca2
homeostatic (Ica, and SERCA2) pathways. The third limitation is that we have not measured action potentials in either GFP or NCX1 myocytes. Potential differences in action potential waveforms between GFP and NCX1 myocytes may have major effects on the energetics of the exchanger. For example, if the action potential duration were prolonged in NCX1 myocytes, as was the case in transgenic murine myocytes overexpressing NCX1 (27), thermodynamic considerations would argue for enhanced reverse NCX1 during depolarization in NCX1 myocytes. The fourth limitation is that the overexpressed NCX1 may occupy a membrane domain that enables it to load the SR with Ca2
or trigger SR Ca2
release more readily than the endogenous native exchanger. This may account for the higher SR Ca2
content (Table 4) and [Ca2
]i transient magnitude (Table 2) observed in NCX1 myocytes. Viewed in this context, it can be argued that Ca2
influx via reverse NCX1 to trigger SR Ca2
release may not occur at all in normal myocytes. The present technology, however, does not allow one to target the NCX1 to a specific membrane domain (e.g., the Ca2
microdomains of the Ca2
channel/ryanodine receptor complex) to definitively settle this question.

In summary, we have established an in vitro myocyte culture model system in which normal contractile function was maintained with continual electrical stimulation. Infection with adenovirus to deliver exogenous genes had no effect on myocyte contractile function. By using our cultured myocyte system, which is fundamentally different than transgenic approach, we found that overexpression of NCX1 resulted in altered patterns of contraction and [Ca2
]; transients, changed SR Ca2
contents, and lower resting and diastolic [Ca2
]. Calsequestrin and SERCA2 expression, SR Ca2
uptake activity, and cell size were unaffected by overexpression of NCX1. We conclude that the most consistent explanation of our observations is that the overexpressed NCX1 was functional in both Ca2
influx and efflux modes. We speculate that changes in
NCX1 expression or activity in pathological conditions may have profound effects on cardiac contractile function.

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REFERENCES
24. Terracciano CMN, DeSouza AI, Philipson KD, and MacLeod KT. Na\(^{+}/\)Ca\(^{2+}\) exchange and sarcoplasmic reticular Ca\(^{2+}\) regulation in ventricular myocytes from transgenic mice overexpressing the Na\(^{+}/\)Ca\(^{2+}\) exchanger. J Physiol (Lond) 512: 651–667, 1998.