Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange in neonatal rat heart cells: antisense inhibition and protein half-life

MARTIN K. SLODZINSKI\textsuperscript{1,3} AND MORDECAI P. BLAUSTEIN\textsuperscript{1,2,3}

Departments of \textsuperscript{1}Physiology and \textsuperscript{2,3}Medicine and \textsuperscript{3}Center for Vascular Biology and Hypertension, University of Maryland School of Medicine, Baltimore, Maryland 21201

Slodzinski, Martin K., and Mordecai P. Blaustein. Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange in neonatal rat heart cells: antisense inhibition and protein half-life. Am. J. Physiol. 275 (Cell Physiol. 44): C459–C467, 1998.—Cardiac Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger (NCX) protein half-life \(t_\text{1/2}\) and antisense knockdown were studied in primary cultured neonatal rat cardiomyocytes. Protein \(t_\text{1/2}\) was determined using \[^{35}\text{S}\text{]methionine with a pulse-chase protocol. The \[^{35}\text{S}\text{]signal in NCX was identified by immunoprecipitation and Western blotting. The \(t_\text{1/2}\) of NCX protein was 33 h. Low concentrations (0.5 µM) of chimeric, phosphorothioated antisense oligodeoxynucleotides (AS-oligos) targeted to the region around the start codon of NCX1 transcript were used to knock down NCX protein and activity. Control myocytes (no oligos or scrambled oligos for at least 4 days) exhibited spontaneous Ca\textsuperscript{2+} transients (measured with fura 2). The sustained (“diastolic”) Ca\textsuperscript{2+} concentration in the cytosol \(([\text{Ca}^{2+}]_{\text{cyt}})\) of control cells was unaffected by cyclopiazonic acid (CPA) plus caffeine (Caf), which promote depletion of sarcoplasmic reticular Ca\textsuperscript{2+} stores, but [Ca\textsuperscript{2+}]\text{cyt} rose in control cells when external Na\textsuperscript{+} was removed. In contrast, \(~60\%\) of cells treated with AS-oligos for at least 4 days did not exhibit spontaneous Ca\textsuperscript{2+} transients or respond to Na\textsuperscript{+}-free medium; however, CPA + Caf did induce a prolonged elevation in \([\text{Ca}^{2+}]_{\text{cyt}}\) in these cells. In all cells, 50 mM K\textsuperscript{+} increased \([\text{Ca}^{2+}]_{\text{cyt}}\). NCX protein was reduced by \(~50\%\) in cells treated with AS-oligos for 7 days but was not reduced after only 2 days. These biochemical data are consistent with the physiological evidence of NCX knockdown in \(~60\%\) of cells.

oligodeoxynucleotides; sarcoplasmic reticular; caffeine; cyclopiazonic acid; potassium depolarization

THE CONTRACTION of cardiac myocytes is triggered by a rise in the cytosolic free Ca\textsuperscript{2+} concentration \([\text{Ca}^{2+}]_{\text{cyt}}\) \((1, 36)\). During membrane depolarization, Ca\textsuperscript{2+} enters from the extracellular fluid via L-type Ca\textsuperscript{2+} channels \((16)\). A sufficient local (subsarcomemmal) increase in \([\text{Ca}^{2+}]_{\text{ot}}\) can activate Ca\textsuperscript{2+} release from functional sarcoplasmic reticulum (SR) via ryanodine receptors (i.e., Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release; Refs. 6, 8, 16). Removal of Ca\textsuperscript{2+} from the contractile apparatus (i.e., from cytosol) is required to promote relaxation. Reduction of \([\text{Ca}^{2+}]_{\text{ot}}\) to resting levels is mediated by Ca\textsuperscript{2+} sequestration into the SR and/or extrusion to the extracellular space \((32)\). In heart cells, the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger (NCX) and, to a much smaller extent, the plasma membrane Ca\textsuperscript{2+} (PMCA) pump are responsible for extruding Ca\textsuperscript{2+} to the extracellular space \((32)\).

The physiological role of NCX in Ca\textsuperscript{2+} extrusion has been better characterized in the heart than in most other tissues \((2, 4, 11, 32, 33)\). Yet, even in the heart, the contributions of NCX to physiological activities are usually identified with acute changes in the concentrations of ions in the bathing medium (i.e., Na\textsuperscript{+} or Ca\textsuperscript{2+}-dependent fluxes or currents; e.g., see Ref. 32), and controversies about the physiological role of NCX remain. For example, the contribution of NCX to depolarization-induced Ca\textsuperscript{2+} entry (i.e., Ca\textsuperscript{2+} entry/Na\textsuperscript{+} exit mode exchange via NCX) and excitation-contraction coupling under physiological conditions is still controversial \((15, 24)\). Also unresolved is the contribution of NCX to posthypoxic reoxygenation injury \((19)\). One reason such controversies and uncertainties remain is that no sufficiently sensitive inhibitors of NCX have yet been characterized. Removal of extracellular Na\textsuperscript{+} (Na\textsubscript{ext}) can inhibit NCX, but this manipulation also affects the cytosolic Na\textsuperscript{+} concentration as well as many other Na\textsuperscript{+}-dependent processes (such as Na\textsuperscript{+}/H\textsuperscript{+} exchange). Amiloride analogs (e.g., 3,4-dichlorobenzamil) are not very selective inhibitors of NCX and may inhibit Na\textsuperscript{+}/H\textsuperscript{+} exchange or L-type Ca\textsuperscript{2+} channels \((22)\). A recently described isothiourea derivative that is reported to inhibit Ca\textsuperscript{2+} transport via NCX \((20, 40)\) is also nonspecific and blocks several types of ion channels \((40)\). The exchanger inhibitory peptide \((26)\) targets NCX putative calmodulin binding domain at the cytoplasmic face (requiring injection in intact cells) and also affects other calmodulin-dependent transport systems including, for example, the PMCA pump \((14)\). Because of these limitations, we explored the possibility that antisense oligodeoxynucleotides (AS-oligos) targeted to NCX could be used to inhibit NCX gene expression and thereby help to evaluate the functional consequences of NCX loss.

AS-oligo-induced knockdown of NCX function in heart cells has been recently reported \((3, 27)\). In both studies, relatively high concentrations \((3–10 \mu M)\) of phosphorothioated AS-oligos were targeted to the 3'-untranslated region of NCX1 gene (product of this gene is predominant isoform in heart). Such high concentrations of fully phosphorothioated oligos may have nonspecific or toxic effects \((5, 7, 18)\). These AS-oligos were reported to inhibit NCX function by 30% \((4)\) to 80% \((27)\) within 24 h, but knockdown of NCX protein was not measured. This rate of reported knockdown implies that NCX protein has a very short \((<12 \text{ h})\) half-life \((t_\text{1/2})\), whereas several other plasmalemmal transport proteins have a \(t_\text{1/2}\) of 20–40 h \((23, 34, 38)\).

For these reasons, we first measured the cardiac NCX protein \(t_\text{1/2}\), since there are no published data. Our results show that the cardiac NCX protein has a \(t_\text{1/2}\) of \(~33\) h. This raises questions about the previously published studies of cardiac NCX knockdown by AS-oligos \((3, 27)\).

The AS-oligos employed in the present study were therefore designed somewhat differently from those used previously \((3, 27)\): two short, tandem AS-oligo...
probes were targeted to the region preceding and surrounding the start codon of NCX1 gene. These probes were chimeric phosphorothioated (natural nucleotides capped with 4 phosphorothioated nucleotides at both ends) applied at a low concentration (0.5 µM) to minimize nongenetic effects. These chimeric AS-oligos were previously shown to knock down NCX function selectively in arterial myocytes: the oligos did not affect cell proliferation or cell morphology, nor did they prevent the responses to a vasoconstrictor (37). We now report that the chimeric AS-oligos selectively knock down NCX protein expression and inhibit NCX function in primary cultured rat cardiac myocytes. The long t½ (~33 h) of the cardiac NCX protein correlates with this biochemical and physiological evidence of NCX knockdown.

METHODS

Primary cell culture The cell culture technique was modified from published methods (13, 28). Neonatal rat ventricular myocytes were prepared from 1- to 2-day-old Sprague-Dawley rats. Hearts were removed and placed in ice-cold digestion buffer (10 mM HEPES, 10 mM pyruvate sodium salt, 5 mM L-glutamine, 1 mM nicotinamide, 0.4 mM L-ascorbate, 1 mM adenosine, 1 mM d-ribose, 1 mM MgCl2, 1 mM taurine, 2 mM β-carnitine, 26 mM KH2CO3, and 10 µg/ml gentamicin in Joklik MEM). The great vessels, atria, and pericardium were dissected away and discarded. The ventricles were minced and washed in ice-cold digestion buffer to remove blood. The minced ventricle were incubated (1 g/10 ml) in digestion buffer containing collagenase (0.5 mg/ml) and CaCl2 (50 µM) for 15 min at 37°C. After gentle trituration, the supernatants from the first three digestions were discarded. The next two supernatants were retained and centrifuged at 500 g. Cells were collected from the resulting pellets and were plated at 105 cells/well in DMEM containing heat-inactivated 10% FBS (55°C for 4 h to minimize exonuclease activity; Ref. 35) and appropriate oligos (see below). After 20–24 h, the cells were exposed to 4,000 rad of γ-radiation from a 137Cs radiation source (J. L. Shepherd and Associates, Glendale, CA). In preliminary experiments, nonirradiated cardiac myocytes were preplated and cultured in either serum-free or serum-containing media, but this did not prevent fibroblast overgrowth. In contrast, irradiation of the cells prevented fibroblast overgrowth (28) even when the cells were cultured in serum-containing medium. The medium was changed immediately after the irradiation, and thrice weekly thereafter.

The purity of the heart cell cultures was determined immediately after physiological experiments. Cells were fixed in 95% cold ethanol and labeled with a nuclear stain, 4′,6-diamidino-2-phenylindole (DAPI), followed by mouse anti-rat troponin C antibodies (Sigma, St. Louis, MO; Ref. 21). Rhodamine-conjugated donkey anti-mouse antibodies (J. Jackson ImmunoResearch, West Grove, PA) were used as the secondary stain. Rat heart cells were also labeled with polyclonal antibodies to the canine cardiac NCX1 (31), which cross-reacts with the rat antigen; these antibodies were a gift from Dr. G. E. Lindenmayer (see Ref. 21). FITC-conjugated goat anti-rabbit antibodies (J. Jackson ImmunoResearch) were used as a secondary stain. Virtually all cells in the cultures were identified as cardiomyocytes on the basis of positive staining for DAPI, troponin C, and NCX.

Antisense oligos. Others have reported the use of AS-oligos in cardiomyocytes targeted to the 3′-untranslated region of NCX1 mRNA (3, 27). In contrast, as described previously (37), we designed a pair of chimeric AS-oligos (5′-TGAGACTTCCAAATTTGT-3′ and 5′-AAGCATGTGGTACAA-3′) targeted to contiguous regions of NCX1 mRNA around the start codon (nucleotides –26 to –10 and –6 to +6, respectively) (29). Two short, tandem AS-oligos usually are more effective than a single longer probe (29); additionally, the efficacy of oligo uptake decreases with increasing oligo length (39). These chimeric AS-oligos have four phosphorothioated-modified nucleotides (boldface) surrounding phosphodiester nucleotides. Chimeric phosphorothioated oligos are stable (exonuclease resistant) and are transported into cells better than unmodified oligos (19). Furthermore, in our initial studies (not shown), chimeras had fewer sequence-independent toxic effects than full-length phosphorothioated oligos (see Refs. 5, 7, 17, 18). In parallel cultures, a second set of chimeric oligos, with the identical base composition but in a scrambled (nonsense, NS-oligos) sequence (5′-TAGTACCTTTCTATGAGT-3′ and 5′-CAGATATACACAGATG-3′), were used to control for the nonspecific and toxic effects of the oligos. All probes were compared with known sequences in GenBank and EMBL using the "Wisconsin Package" sequence analysis program; no significant homologies to other sequences were found.

Control cells were grown without oligos. In parallel, cells were grown in medium containing either the AS- or NS-oligo pair. The final concentration of the oligos was maintained at 0.5 µM, from the time of initial plating until experimentation, to minimize sequence-independent effects. The medium was changed thrice weekly.

Cytoplasmic Ca2+ determination using fura 2. Cells were incubated (30 min, 20°C 22°C, 5% CO2-95% O2) in culture medium containing 4.0 µM fura 2-AM, the membrane-permeable acetoxymethyl ester of fura 2. The coverslips were transferred to a tissue chamber mounted on the stage of a microscope. The cells were superfused with standard physiological salt solution (PSS; 30 min, 32°C 34°C) to wash away extracellular dye and to allow time for cellular esterases to hydrolyze the fura 2-AM.

Digital imaging, rather than high temporal resolution photometry, was employed for the physiological studies described here because the spatial resolution obtained with digital imaging enabled us to study a relatively large number of cells and to assess function in each individual cell. The imaging system was designed around a Nikon Diaphot microscope optimized for ultraviolet (UV) transmission. Fura 2 fluorescence (510-nm light emission; 380- and 360-nm excitation) and background fluorescence were imaged using a Nikon UV-Fluor objective (×40, NA 1.3). Fluorescent images were obtained with a microchannel plate image intensifier (Amperex XX1381, O pelco, Washington, DC) coupled by fiber optics to a Pulnix charge-coupled device videocamera (Stanford Photonics, Stanford, CA).

Images were acquired, background subtracted, and transformed to Ca2+ images by using the MetaFluor Imaging System (Universal Imaging, W. Chester, PA). Video frames were digitized at a resolution of 512 horizontal × 480 vertical pixels and 8 bits with a Matrox LC Imaging board (Universal Imaging). To improve the signal-to-noise ratio, 16 consecutive video frames were averaged. Images were acquired at a rate of one averaged image every 3 s to one every 60 s, dependent on experimental protocol requirements. [Ca2+]cyt was calculated from fura 2 fluorescent emission excited at 380 and 360 nm by the ratio method; 360 nm is the fura 2 isosbestic point. [Ca2+]cyt measurements were limited to small (~2 µm2) peripheral cytosolic "areas of interest" that contained relatively few organelles (e.g., Golgi, mitochondria, and SR). One
EXCHANGE IN HEART CELLS

Antisense Inhibition of Na\(^+\)/Ca\(^{2+}\) Exchange in Heart Cells

A-Sepharose bead pellet was resuspended in immunoprecipitation buffer and centrifuged; the resulting supernatant was discarded. This “washing” step was repeated three times to remove proteins that were not bound specifically to the protein A-Sepharose beads. To elute the antibodies and immunoprecipitated proteins from the protein A-Sepharose beads, the beads were incubated in 1 ml of 3× concentrated SDS sample buffer with 1% 2-mercaptoethanol at 70°C for 20 min. After centrifugation as above, the final supernatant, containing NCX protein and anti-NCX IgG, was analyzed by SDS-PAGE. The immunoprecipitated NCX protein was detected with immunoblotting. \[^{35}\text{S}\]methionine labeling of immunoprecipitated protein was detected with phosphorimaging. These experiments were limited to two replicates by the limited supply of the well-characterized polyclonal anti-NCX serum (it is no longer available).

Materials. Chimeric phosphorothioated oligos were obtained from Oligos, Etc. (Wilsonville, OR). FBS was obtained from HyClone (Logan, UT). Furca 2-AM was obtained from Molecular Probes (Eugene, OR). N-ethyl-m-glucamine (NMDG), DAPI, cyclopiazonic acid (CPA), and caffeine (Caf) were purchased from Sigma. DMEM and Joklik MEM were obtained from GIBCO BRL (Grand Island, NY). Collagenase type I was obtained from Worfington Biochemical (Freehold, NJ). All other reagents were analytical grade or the highest purity available.

RESULTS

Solubilization buffer contained 150 mM NaCl, 50 mM Tris, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 0.01% sodium azide, and protease inhibitor solution at pH 8.0. Immunoprecipitation buffer contained 150 mM NaCl, 25 mM HEPES, and 0.01% sodium azide at pH 7.4. PSS contained 140 mM NaCl, 5.9 mM KCl, 1.2 mM NaH\(_2\)PO\(_4\), 5 mM NaHCO\(_3\), 1.4 mM MgCl\(_2\), 1.8 mM CaCl\(_2\), 11.5 mM glucose, and 10 mM HEPES (titrated to pH 7.4 with NaOH). For Ca\(^{2+}\)-free solutions, CaCl\(_2\) was replaced by 1.8 mM MgCl\(_2\) (total 3.2 mM), and 0.05 mM EGTA was added. In Na\(^{+}\)-free solutions, the NaCl and NaHCO\(_3\) were isosmotically replaced by NMDG, and pH was adjusted with HCl. CPA (5 µM; an inhibitor of SR Ca\(^{2+}\) pump) and Caf (10 mM) were added to the bathing solutions (PSS) to increase [Ca\(^{2+}\)]\(_{cyt} \). A 1 mM stock solution of fura 2-AM was prepared in DMEM.

Statistical analysis and data presentation. Original fluorescence data are reported as [Ca\(^{2+}\)]\(_{cyt}\) values. Values are reported as means ± SE. Microcal Origin software (Northampton, MA) was used for calculations and graphics.

RESULTS

AS-oligos inhibit NCX activity selectively. Functional evidence of NCX knockdown in primary cultured neonatal heart cells was determined with Ca\(^{2+}\) imaging using fura 2. In these physiological experiments, cells were plated at a very low density to facilitate assessment of individual cells. After 7 days in culture, all control cells (no oligos added) and cells treated with scrambled oligos (NS-oligos) exhibited spontaneous, transient increases in [Ca\(^{2+}\)]\(_{cyt}\) ("Ca\(^{2+}\) transients", Fig. 1A, Table 1). This is consistent with previous studies demonstrating that healthy, cultured cardiomycocytes from neonatal rats undergo spontaneous contractions (13, 28). The seemingly small amplitudes of the spontaneous Ca\(^{2+}\) transients detected in these cells (Fig. 1A) is attributable to image averaging and the low frequency of image capture due to alternation of wavelengths; thus the...
Fig. 1. Antisense (AS) oligo knockdown of Na\(^+\)/Ca\(^{2+}\) exchanger (NCX) function in primary cultured rat cardiomyocytes. A: representative original cytosolic free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)\(_{cyt}\)]) data from individual cells after 7 days in culture. Control cells (no oligos added) and scrambled (NS) oligo-treated cells exhibited spontaneous transient increases in [Ca\(^{2+}\)\(_{cyt}\)], as indicated at left (before application of cyclopiazonic acid [CPA] and caffeine [Caf]). Most antisense (AS) oligo-treated cells did not exhibit Ca\(^{2+}\) transients, as illustrated in this example (see also Table 1). CPA (10 µM) and Caf (5 mM) were added to physiological salt solution (PSS) to inhibit sarcoplasmic reticular (SR) sequestration of [Ca\(^{2+}\)\(_{cyt}\)], CPA and Caf increased “resting” [Ca\(^{2+}\)\(_{cyt}\)] in AS-oligo-treated cells but not in controls and NS-oligo-treated cells. Extracellular Na\(^+\) was removed (Na\(^+\)-free) in presence of CPA + Caf to promote NCX-mediated net Ca\(^{2+}\) entry. High-K\(^+\) (50 mM) solution was superfused to evoke depolarization-induced increases in [Ca\(^{2+}\)\(_{cyt}\)] as a measure of cell viability. Bars at bottom indicate periods of exposure to CPA + Caf, Na\(^+\)-free, and high-K\(^+\)-solutions. Temperature = 30–32°C. B: percentage of AS-oligo-treated cells that exhibited a >25 nM rise in [Ca\(^{2+}\)\(_{cyt}\)] in Na\(^+\)-free media (2 min). Data are shown as a function of number of days of AS-oligo treatment. Note that suppression of NCX activity (indicated by absence of a rise in [Ca\(^{2+}\)\(_{cyt}\)]) was first observed in cells treated for 4 days. Shown are pooled data from experiments in which protocol illustrated in A was employed. Each bar represents 6–18 cells.

Peaks are substantially underestimated. Nevertheless, at continuous 380-nm excitation, these Ca\(^{2+}\) transients were readily visualized (not recorded). In contrast to these controls, 59% of the cells treated with AS-oligos did not exhibit any spontaneous Ca\(^{2+}\) transients (Fig. 1A, Table 1). Moreover, “resting” [Ca\(^{2+}\)\(_{cyt}\)] in these quiescent AS-oligo-treated cells was not significantly different from “diastolic” [Ca\(^{2+}\)\(_{cyt}\)] in the spontaneously active cells (Table 1).

To test for the ability of NCX to extrude Ca\(^{2+}\), CPA and Caf were added to the PSS to inhibit SR Ca\(^{2+}\) buffering and to unload Ca\(^{2+}\) from the SR stores. This treatment induced a large, prolonged rise in [Ca\(^{2+}\)\(_{cyt}\)] in the AS-oligo-treated cells. The rate of rise of Ca\(^{2+}\) was slow; the maximal [Ca\(^{2+}\)\(_{cyt}\)] was usually reached within ~2 min, presumably because of the underdeveloped SR (10). In a few cells (e.g., AS-oligo-treated cell in Fig. 1A), the [Ca\(^{2+}\)\(_{cyt}\)] continued to rise for several minutes. After ~10–15 min, however, [Ca\(^{2+}\)\(_{cyt}\)] began to decline despite the continued presence of CPA and Caf. This implies that Ca\(^{2+}\) was being sequestered in other organelles, or extruded from the cells. Most important is the fact that 59% of the AS-oligo-treated cells exhibited a substantial CPA + Caf-evoked increase of [Ca\(^{2+}\)\(_{cyt}\)] that dissipated slowly (Fig. 1A, Table 1); this was observed in the same cells that did not exhibit spontaneous Ca\(^{2+}\) transients.

In contrast to these effects, CPA + Caf may have evoked a brief transient elevation of [Ca\(^{2+}\)\(_{cyt}\)] in the NS-oligo-treated cells and controls (cf. Ref. 32), but this

Table 1. Effects of AS-oligos on [Ca\(^{2+}\)\(_{cyt}\)] in cardiac myocytes under a variety of conditions

<table>
<thead>
<tr>
<th></th>
<th>No Oligos</th>
<th>AS-Oligos</th>
<th>NS-Oligos</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spontaneous Ca(^{2+}) transients (n)</td>
<td>47</td>
<td>51</td>
<td>42</td>
</tr>
<tr>
<td>Resting (“diastolic”) [Ca(^{2+})(_{cyt}), nM]</td>
<td>82 ± 19</td>
<td>98 ± 23</td>
<td>92 ± 21</td>
</tr>
<tr>
<td>CPA + Caf treatment* Δ[Ca(^{2+})(_{cyt})] &gt; 25 nM (n)</td>
<td>47</td>
<td>20</td>
<td>31</td>
</tr>
<tr>
<td>Maximal Δ[Ca(^{2+})(_{cyt})], nM</td>
<td>ND</td>
<td>ND</td>
<td>176 ± 48</td>
</tr>
<tr>
<td>Na(^+)-free PSS (2 min)† Δ[Ca(^{2+})(_{cyt})] &gt; 25 nM (n)</td>
<td>47</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>Peak Δ[Ca(^{2+})(_{cyt})], nM</td>
<td>466 ± 137</td>
<td>392 ± 84</td>
<td>11 ± 32</td>
</tr>
<tr>
<td>50 mM K(^+) + PSS (2 min)† Δ[Ca(^{2+})(_{cyt})] &gt; 50 nM (n)</td>
<td>47</td>
<td>20</td>
<td>31</td>
</tr>
<tr>
<td>Peak Δ[Ca(^{2+})(_{cyt})], nM</td>
<td>91 ± 34</td>
<td>97 ± 35</td>
<td>112 ± 25</td>
</tr>
</tbody>
</table>

Data are means ± SE from 3 different preparations. Cells were cultured for 4–7 days without oligos or with antisense or nonsense oligodeoxynucleotides (AS- or NS-oligos, respectively). In all experiments, 3 groups were cultured in parallel. Data from cells that did not exhibit at least a 50 nM elevation in cytosolic free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)\(_{cyt}\)]) in response to 50 mM K\(^+\)-physiological salt solution (PSS) were regarded as “nonfunctional” and are not included in analysis (29% of control cells, 23% of AS-oligo-treated cells, and 33% of NS-oligo-treated cells did not respond). *Maximal Δ[Ca\(^{2+}\)\(_{cyt}\)] is average sustained increase in [Ca\(^{2+}\)\(_{cyt}\)], above resting or diastolic level, evoked by cyclopiazonic acid (CPA) + caffeine (Caf) in AS-oligo-treated cells. n Values refer to number of cells in each group that exhibited changes in [Ca\(^{2+}\)\(_{cyt}\)] > 25 nM. ND refers to fact that CPA + Caf-evoked transients in these cells could not be distinguished from spontaneous transients at slow image acquisition rate used for these experiments. †Peak Δ[Ca\(^{2+}\)\(_{cyt}\)] is average increase in [Ca\(^{2+}\)\(_{cyt}\)], above resting or diastolic level, evoked by each treatment. AS-oligo-treated cells have been divided into 2 groups: those that exhibited spontaneous Ca\(^{2+}\) transients and those that did not. n Values refer to numbers of cells in each of the groups that exhibited changes in [Ca\(^{2+}\)\(_{cyt}\)] greater than indicated values.

peaks are substantially underestimated. Nevertheless, at continuous 380-nm excitation, these Ca\(^{2+}\) transients were readily visualized (not recorded). In contrast to these controls, 59% of the cells treated with AS-oligos did not exhibit any spontaneous Ca\(^{2+}\) transients (Fig. 1A, Table 1). Moreover, “resting” [Ca\(^{2+}\)\(_{cyt}\)] in these quiescent AS-oligo-treated cells was not significantly different from “diastolic” [Ca\(^{2+}\)\(_{cyt}\)] in the spontaneously active cells (Table 1).
transient could not be clearly distinguished from spontaneous Ca\(^{2+}\) transients (Fig. 1A). A large CPA + Caf-evoked Ca\(^{2+}\) transient is not expected in rat neonatal cardiac myocytes; however, because the T tubules and SR are not well developed in rat neonatal cardiac myocytes (10). The absence of a sustained elevation of (diastolic) [Ca\(^{2+}\)]\(_{\text{cyt}}\) in these cells during prolonged exposure to CPA + Caf (Fig. 1A, Table 1) may be attributed to rapid extrusion of Ca\(^{2+}\) via NCX (32).

With SR Ca\(^{2+}\) sequestration blocked by CPA + Caf, external Na\(^+\) was removed to activate NCX-mediated Ca\(^{2+}\) entry. Control and NS-oligo-treated cells consistently responded with large increases in [Ca\(^{2+}\)]\(_{\text{cyt}}\) (Fig. 1A, Table 1). In 59% of the AS-oligo-treated cells, however (again, specifically, those cells not exhibiting spontaneous Ca\(^{2+}\) transients or CPA + Caf-evoked increases [Ca\(^{2+}\)]\(_{\text{cyt}}\)), removal of Na\(^+\) did not induce an increase in [Ca\(^{2+}\)]\(_{\text{cyt}}\) (Fig. 1A, Table 1). The implication is that NCX function was markedly knocked down in these cells because reversal of the Na\(^+\)/Ca\(^{2+}\) gradient did not appear to drive Ca\(^{2+}\) into the cells (via NCX).

At the end of each of these experiments, the cells were briefly exposed to 50 mM K\(^+\)/PSS (Fig. 1A). About 70% of the cells in each of the groups (AS-oligo-treated as well as NS-oligo-treated and control cells; see Table 1 legend) responded with a K\(^+\)-stimulated increase in [Ca\(^{2+}\)]\(_{\text{cyt}}\) (Fig. 1A). This was regarded as the essential criterion for viable, healthy cells, regardless of whether they were treated with oligos.

Figure 1B shows the time course of knockdown of NCX activity (measured as absence of a rise in [Ca\(^{2+}\)]\(_{\text{cyt}}\) in response to Na\(^+\) removal; see Fig. 1A) as a function of the duration of incubation with AS-oligos. Suppression of NCX activity was first observed in cells treated with AS-oligos for 4 days. Nevertheless, as indicated in Fig. 1B (and see Table 1), a significant fraction (40% on average) of the cardiomyocytes cultured with AS-oligos for 4–8 days continued to exhibit NCX activity. Those that did exhibit this activity behaved as did the control and NS-oligo-treated cells in experiments such as those illustrated in Fig. 1A.

Immunological evidence of antisense knockdown of NCX. An immunoblot assay was used to determine whether NCX protein expression was knocked down by the AS-oligos. Parallel groups of cardiomyocytes cultured in petri dishes were incubated with AS-oligos, NS-oligos, or no oligos (control). Cells were plated on day 0 and irradiated on day 1. On days 2 and 7, cells were harvested in solubilization buffer containing the protease inhibitor cocktail (cells were not harvested on day 0 or 1 so that dead cells from original plating or after irradiation could be washed away). The solubilized proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. NCX protein was immunolabeled with polyclonal rabbit anti-NCX antibodies and secondarily stained with horseradish peroxidase-conjugated mouse anti-rabbit IgG. ECL and densitometry were used to quantify NCX protein bands.

Figure 2 (top) shows that a 120-kDa band was strongly labeled by the anti-NCX antibodies; a 70-kDa band (presumably a cleavage product; see Ref. 21) was less strongly labeled. There was no detectable decrease of the 120-kDa protein in the cells treated with AS-oligos for 2 days, but there was an ~57% knockdown of this protein in cells incubated with the AS-oligos for 7 days (compared with control; 52% knockdown compared with NS-oligo-treated cells). Nearly identical results were obtained in another, similar experiment (47% knockdown compared with control; 54% compared with NS-oligo-treated cells). In these experiments, NCX protein was normalized to the troponin C concentration determined by restaining the immunoblots.

Note that the density of the 70-kDa band from the cells treated with AS-oligos for 7 days was also greatly reduced compared with the 70-kDa bands from control and NS-oligo-treated cells. Thus the reduced intensity of the AS-oligo 120-kDa band cannot be attributed to cleavage of the protein.

\[^{35}\text{S}]\text{methionine-labeled protein}\) determination. The biochemical data described above are consistent with the results of the functional studies illustrated in Fig. 1B. These data indicate that there is little knockdown of NCX protein or NCX function after 2 days of incubation with AS-oligos but ~50–60% knockdown by day 7. These results also imply that the \(t_{1/2}\) of the expressed and functional NCX protein is relatively long. To test this possibility, we measured the \(t_{1/2}\) of NCX protein in cultured rat cardiomyocytes. The cells were cultured in petri dishes in media without oligos for 3 days, and the proteins were then pulse labeled with \[^{35}\text{S}]\text{methionine. The isotopically labeled amino acid was included in the culture medium for 24 h and then replaced by isotope-free medium (see METHODS). Cells were harvested in solubilization buffer at the times indicated in Fig. 3.}
The cell proteins were immunoprecipitated using rabbit anti-NCX antibodies and protein A-Sepharose beads. The antibody-protein complex was eluted from the beads and subjected to SDS-PAGE, transferred to a nitrocellulose membrane, and immunostained for NCX using polyclonal anti-NCX antiserum and secondary staining with mouse anti-rat IgG and ECL. Arrows, position of 170-kDa bands. Samples were heated to 70°C for 20 min in these experiments; thus NCX protein ran on SDS-PAGE at an apparently higher molecular mass (170 kDa) than in experiment of Fig. 2. Numbers below gel lanes correspond to hours after a pulse chase with [35S]methionine (negative values indicate samples taken 4.5 or 3 h before removal of [35S]methionine). PI, preimmune serum (used for immunoprecipitation); C, control lane (no [35S] labeling); B, blank lane (no cell extract); M, molecular mass marker lane. B: time course of decay of [35S]methionine labeling in NCX band. For each time point, density of background-subtracted [35S]methionine-labeled NCX band (A, top) was divided by density of background-subtracted NCX immunoblot band (A, bottom); values were normalized to a value of 1.0 at time 0 for best-fit exponential decay curve. For both [35S]methionine and NCX immunoblot data, backgrounds used were comparable regions in lane labeled B (blank) because this lane did not contain cell extract. Data from 2 experiments are shown (●, corresponding to data from experiment illustrated in A; ▲, results obtained from duplicate experiment). Data were fitted with a 1st-order exponential decay curve to estimate protein t½. Calculated t½ for decay of [35S] labeling in NCX band is 33 h.

The immunoblots revealed 170-kDa bands that cross-reacted strongly with anti-NCX antibodies (Fig. 3A, bottom). The higher apparent molecular mass of these NCX bands (in contrast to 120-kDa band in Fig. 2) is due to the fact that these immunoprecipitated proteins were heated before SDS-PAGE (12). A band at the same molecular mass was labeled by the [35S]methionine (Fig. 3A, top). Although a few other bands were also labeled (see Fig. 3A, bottom), this is not surprising because small amounts of some very prevalent proteins such as myosin and actin may coprecipitate as may those proteins that appear to be directly associated with NCX, such as ankyrin (25).

Visual inspection of the original data from the PhosphorImager (Fig. 3A, top) could not be used to estimate the loss of radioactivity from NCX band directly because different amounts of protein were applied to each lane (Fig. 3A, bottom); this was the result of variable protein loss during the harvesting, immunoprecipitation, and washing steps. Thus, to estimate NCX protein t½, we needed to compensate for differences in the amount of protein in each of the gel lanes. The density of each background-subtracted [35S]methionine band that comigrated with the 170-kDa NCX (Fig. 3A) was therefore divided by the background-subtracted NCX immunoblot band density (see Fig. 3 legend) to control for the amount of protein (relative to [35S] signal); these data are graphed in Fig. 3B. Over a period of 168 h, the [35S] bands that corresponded to NCX immunoblot bands on the same nitrocellulose membranes had a first-order exponential decay t½ of 33 h (Fig. 3B, filled squares). Virtually identical results were obtained in a duplicate experiment (Fig. 3B, filled triangles).

**DISCUSSION**

We have previously described the design and use of a pair of short AS-oligos targeted to the region preceding and surrounding the start codon of the rat NCX1 mRNA (37). These AS-oligos were observed to knock down NCX activity in vascular smooth muscle cells (37). This report describes the effects of these same AS-oligos on neonatal rat ventricular cardiomyocytes. The results demonstrate that these AS-oligos selectively knock down NCX function as well as NCX protein in the primary cultured heart cells.

Design of AS-oligos targeted to NCX1. Carefully designed AS-oligos can selectively and effectively inhibit expression of their target proteins provided that these oligos are used at low concentrations (<2 µM) and applications are well controlled to avoid nonspecific effects and other artifacts (37). We targeted a highly conserved portion of NCX1 mRNA surrounding the ATG start codon. Two short (17- and 15-mer), adjacent (sequential), chimeric AS-oligos were employed, with four phosphorothioated deoxynucleotides at each end and normal phosphodiester deoxynucleotides in the middle regions. This arrangement tends to maximize oligo uptake and interference with mRNA expression while minimizing toxicity (29). These AS-oligos, at concentrations of 0.5 µM, were not lethal.

While this work was in progress, Lipp et al. (27) described the use of an AS-oligo to knock down NCX function in cultured neonatal rat cardiac myocytes. They targeted their single 19-mer nonchimeric, phosphorothioated oligo to the conserved 3′-untranslated...

---

**Fig. 3.** Protein half-life (t½) of NCX in cardiomyocytes. A: top, phosphorimage of [35S]methionine-labeled NCX; bottom, immunolabeling of same blots as at top with polyclonal anti-NCX antiserum and secondary staining with mouse anti-rat IgG and ECL. Arrows, position of 170-kDa bands. Samples were heated to 70°C for 20 min in these experiments; thus NCX protein ran on SDS-PAGE at an apparently higher molecular mass (170 kDa) than in experiment of Fig. 2. Numbers below gel lanes correspond to hours after a pulse chase with [35S]methionine (negative values indicate samples taken 4.5 or 3 h before removal of [35S]methionine). PI, preimmune serum (used for immunoprecipitation); C, control lane (no [35S] labeling); B, blank lane (no cell extract); M, molecular mass marker lane. B: time course of decay of [35S]methionine labeling in NCX band. For each time point, density of background-subtracted [35S]methionine-labeled NCX band (A, top) was divided by density of background-subtracted NCX immunoblot band (A, bottom); values were normalized to a value of 1.0 at time 0 for best-fit exponential decay curve. For both [35S]methionine and NCX immunoblot data, backgrounds used were comparable regions in lane labeled B (blank) because this lane did not contain cell extract. Data from 2 experiments are shown (●, corresponding to data from experiment illustrated in A; ▲, results obtained from duplicate experiment). Data were fitted with a 1st-order exponential decay curve to estimate protein t½. Calculated t½ for decay of [35S] labeling in NCX band is 33 h.
region of the mRNA. This AS-oligo, at a concentration of 3 μM, was reported to knock down NCX function completely in 80% of the cardiac myocytes (which do not proliferate in culture) within 24 h. These authors did not, however, show the preservation of other functions or biochemical evidence of protein knockdown in these cells. Bland et al. (3) also described AS-oligo inhibition of NCX in embryonic heart cells. They also used a relatively high concentration (10 μM) of a completely phosphorothioated AS-oligo targeted to the untranslated region of NCX1 mRNA. Within 24 h, the $^{45}$Ca$^{2+}$ influx normally evoked by reduction of Na$_{o}$ was abolished in 30% of cells, and the beating rate increased (3). In both studies (3, 27), the rapid loss of NCX function seems surprising because oligo uptake and knockdown of mRNA expression are both inefficient processes. Moreover, such rapid inhibition cannot be explained by knockdown of the expression of an integral membrane transport protein with a $t_{1/2}$ of ~33 h. Thus, in the absence of verification of selective protein knockdown, it seems possible that at least part of the inhibition of NCX function observed by Bland et al. (3) and Lipp et al. (27) might have been due to the non-sequence-specific and/or toxic effects of the relatively high concentrations of fully phosphorothioated oligos (5, 7).

Biochemical and functional knockdown of NCX by AS-oligos in cardiomyocytes. We used digital imaging methods with the Ca$^{2+}$-sensitive dye, fura 2, to obtain physiological evidence of knockdown of NCX in primary cultured neonatal rat cardiomyocytes. All control (no oligo) and NS-oligo-treated cells exhibited repetitive spontaneous Ca$^{2+}$ transients that could readily be visualized in the microscope; these Ca$^{2+}$ transients may correspond to “beating.” In contrast, a large fraction of the cells treated with AS-oligos for at least 4 days did not have Ca$^{2+}$ transients (Fig. 1A, Table 1). This observation, which differs from that of Bland et al. (3), suggests that heart cells cannot beat spontaneously when NCX is knocked down. Whether this is a consequence of the reduced ability to extrude Ca$^{2+}$ after activation or is evidence that NCX plays an important role in excitation-contraction coupling (24) is unclear. There is, however, considerable controversy on the latter topic (15, 24). The cells without Ca$^{2+}$ transients had, on the average, resting [Ca$^{2+}$]$_{cyt}$ levels that were not significantly different from the diastolic [Ca$^{2+}$]$_{cyt}$ levels in the beating cells from all three treatment groups (Table 1).

When Ca$^{2+}$ was released into the cytosol from the SR by Caf and resequestration was inhibited with CPA, a rapid, transient rise in [Ca$^{2+}$]$_{cyt}$ in control and NS-oligo-treated could not be distinguished from the spontaneous transients with our slow temporal resolution. Under these circumstances, any released Ca$^{2+}$ and/or Ca$^{2+}$ entering as a result of store depletion was probably extruded from the cytosol via NCX almost as fast as it was released from the SR; in adult cardiac myocytes, the half-time of such transients is only ~0.3 s (32). It is important to note, however, that cardiac myocytes from neonatal rats have a poorly developed SR, and large CPA + Caf-evoked Ca$^{2+}$ transients are not necessarily expected (10). Nevertheless, much of the Ca$^{2+}$ released from the SR should normally be rapidly extruded by NCX (9, 32). In contrast, in a large fraction of the AS-oligo-treated cells, application of CPA and Caf caused [Ca$^{2+}$]$_{cyt}$ to rise and remain elevated for many minutes (Fig. 1A, Table 1). The implication is that some Ca$^{2+}$ was indeed released from the SR and/or entered from the extracellular fluid and that Ca$^{2+}$ efflux via NCX was markedly reduced in the AS-oligo-treated cells, presumably because of NCX knockdown. This is consistent with the observations of Chiesi et al. (9), who electrically evoked transient elevations of [Ca$^{2+}$]$_{cyt}$ in neonatal rat cardiomyocytes. They found that the elevation of [Ca$^{2+}$]$_{cyt}$ was somewhat slowed and greatly prolonged in cells treated with thapsigargin (to block SR Ca$^{2+}$ pump) when external Na$^{+}$ was replaced by Li$^{+}$ (to inhibit NCX).

After the CPA + Caf-evoked rise in [Ca$^{2+}$]$_{cyt}$ in the AS-oligo-treated cells, there was a slow decline of [Ca$^{2+}$]$_{cyt}$ despite the continued presence of these agents (Fig. 1A). This suggests that not all of NCX was knocked down in these cells and/or that other, “slow” mechanisms (a sarcolemmal ATP-driven Ca$^{2+}$ pump or mitochondrial Ca$^{2+}$ sequestration; see Ref. 32) were eventually able to remove the cytosolic Ca$^{2+}$. Lipp et al. (27) reported that [Ca$^{2+}$]$_{cyt}$ did not decline after Ca$^{2+}$ was released from photolyzed, intracellular “caged” Ca$^{2+}$ when SR Ca$^{2+}$ sequestration was inhibited. This implies that NCX was completely inhibited and that no other Ca$^{2+}$ removal mechanisms were operating in their AS-oligo-treated cardiomyocytes in contrast to the situation in our cells (see also Refs. 32, 36). Because our AS-oligo-treated cells survived for many days with a resting [Ca$^{2+}$]$_{cyt}$ that was not significantly different from controls, it is apparent that other mechanisms were able to maintain Ca$^{2+}$ homeostasis in these quiescent cells.

NCX mediates both Ca$^{2+}$ influx and Ca$^{2+}$ efflux, thus AS-oligo-induced knockdown should inhibit both modes of net Ca$^{2+}$ transport. To assess the effect of AS-oligos on NCX-mediated Ca$^{2+}$ influx, we determined the effect of external Na$^{+}$ removal on [Ca$^{2+}$]$_{cyt}$. When control and NS-oligo-treated cells were exposed to Na$^{+}$-free media, [Ca$^{2+}$]$_{cyt}$ increased substantially, which implies that the reversal of the Na$^{+}$ gradient across the sarcolemma drove Ca$^{2+}$ into the cells via NCX. In sharp contrast, [Ca$^{2+}$]$_{cyt}$ did not rise in many AS-oligo-treated cells when external Na$^{+}$ was removed (Fig. 1A, Table 1). The prolonged elevation of [Ca$^{2+}$]$_{cyt}$ following introduction of CPA + Caf, and the absence of a rise in [Ca$^{2+}$]$_{cyt}$ after external Na$^{+}$ depletion (AS-oligo cells, Fig. 1A) indicate that NCX-mediated Ca$^{2+}$ efflux and influx are both knocked down in these cells. Nevertheless, these AS-oligo-treated cells, like all control and NS-oligo-treated cells, responded to 50 mM K$^{+}$ PSS with a large, transient rise in [Ca$^{2+}$]$_{cyt}$ (Fig. 1A, Table 1), suggesting that the voltage-gated Ca$^{2+}$ channels were operating normally in these cells.

Knockdown of NCX activity was first observed after 96 h of incubation with the AS-oligos (Fig. 1B). Further-
more, NCX activity was not knocked down in all of the AS-oligo-treated cells (Table 1). About one-third to one-half of the cells treated with AS-oligos for 4–7 days exhibited spontaneous Ca\(^{2+}\) transients and had a normal diastolic [Ca\(^{2+}\)]\(_{cyt}\); removal of external Na\(^+\) elevated [Ca\(^{2+}\)]\(_{cyt}\); these cells, but application of CPA + Caf did not. In other words, these cells were indistinguishable physiologically from control cardiomyocytes and those treated with NS-oligos. This heterogeneity in the response to AS-oligos is not surprising; similar behavior has also been seen in other preparations and has been attributed to heterogeneity in oligo uptake(30).

Biochemical evidence of NCX knockdown was obtained with immunoblotting. Figure 2 shows that AS-oligos reduced NCX protein expression by ~50–60% in cells treated for 7 days. There was, however, negligible effect of the AS-oligos on cells treated for only 48 h. These data are consistent with our physiological results both temporally and quantitatively. Physiological evidence of knockdown was first observed after 4 days of incubation with the AS-oligos, and only about one-half to two-thirds of NCX activity was inhibited even after 7 days of treatment. The physiological data suggest that the AS-oligos knocked down all or most of NCX in some cells and did not markedly affect NCX in others (those that continued to exhibit normal physiological responses). The combined results of the biochemical and physiological experiments seem inconsistent with the alternative possibility, namely, that NCX was partially inhibited in all of the cells treated with AS-oligos for 7 days. Our results might be explained if, for example, some of the cells failed to accumulate sufficient amounts of the AS-oligos to inhibit NCX expression.

NCX protein t\(_{50}\). The reason for the delayed onset of AS-oligo knockdown of NCX is unknown. One possibility is that NCX protein has a relatively long t\(_{50}\). Lipp et al. (27), however, reported virtually complete AS-oligo-induced knockdown of NCX activity in 80% of cardiomyocytes within 24 h, possibly suggesting that NCX has a t\(_{50}\) of ~6 h. On the other hand, unpublished data based on cycloheximide inhibition of protein synthesis (T. Rogers and W. J. Lederer, personal communication) suggested that the cardiac sarcolemmal NCX has a t\(_{50}\) of >36 h. To determine the t\(_{50}\) of NCX in our cells, we used a pulse chase with \(^{35}\)S)methionine to label the proteins, followed by immunoprecipitation, SDS-PAGE, and immunoblotting. The phosphorimages of the \(^{35}\)S)methionine signals revealed several \(^{35}\)S-labeled bands. Nevertheless, densitometric methods enabled us to identify the \(^{35}\)S-labeled band that comigrated with NCX (i.e., 170-kDa band), which was identified by immunolabeling. The \(^{35}\)S label in NCX bands decayed with a t\(_{50}\) of 33 h. This value is consistent with the t\(_{50}\) of NCX obtained by Rogers and Lederer (>36 h) and is comparable to the t\(_{50}\) of two other integral membrane transport proteins, the Na\(^+\)/Ca\(^{2+}\) pump and the Na\(^+\) channel (t\(_{50}\) = 20–40 h; Refs. 23, 34, 38). The t\(_{50}\) of NCX is shorter than expected from the biochemical and physiological evidence that AS-oligo-induced NCX knockdown takes longer than 48 h (Figs. 1B and 2). A likely explanation, however, is that, in part because of the low extracellular AS-oligo concentration employed (to minimize non-specific effects), it may have taken several hours to a day or more for the intracellular AS-oligo concentration to reach a level sufficient to interfere with NCX protein expression. Thus the t\(_{50}\) provides only an estimate of the minimal time required for AS-oligos to knock down NCX protein expression and function.

We thank Dr. M. Juhaszova and R. S. Rogowski for sharing knowledge and experience concerning immunoprecipitation, Dr. M. M. McCarthy for advice on the use of antisense oligos, and Drs. T. B. Rogers and W. J. Lederer for permission to cite a personal communication. We are grateful to Drs. R. J. Bloch, M. M. McCarthy, and G. R. Monteith for their critical comments on an early version of this manuscript.

This work was supported by National Institutes of Health Grant HL-45215 and Training Grant GM-02521580 and by funds from the University of Maryland, Baltimore, Graduate School.

Address for reprint requests: M. P. Blaustein, Dept. of Physiology, University of Maryland School of Medicine, 655 W. Baltimore St., Baltimore, MD 21201.

Received 22 December 1997; accepted in final form 13 May 1998.

REFERENCES


