

Antisense inhibition of $\text{Na}^+/\text{Ca}^{2+}$ exchange during anoxia/reoxygenation in ventricular myocytes

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Eigel, B. N., and R. W. Hadley. Antisense inhibition of $\text{Na}^+/\text{Ca}^{2+}$ exchange during anoxia/reoxygenation in ventricular myocytes. *Am J Physiol Heart Circ Physiol* 281: H2184–H2190, 2001.—This study investigated the role of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX) in regulating cytosolic intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) during anoxia/reoxygenation in guinea pig ventricular myocytes. The hypothesis that the NCX is the predominant mechanism mediating $[\text{Ca}^{2+}]_i$ overload in this model was tested through inhibition of NCX expression by an antisense oligonucleotide. Immunocytochemistry revealed that this antisense oligonucleotide, directed at the area around the start site of the guinea pig NCX1, specifically reduced NCX expression in cultured adult myocytes by $90 \pm 4\%$. Antisense treatment inhibited evoked NCX activity by $94 \pm 3\%$ and decreased the rise in $[\text{Ca}^{2+}]_i$ during anoxia/reoxygenation by $95 \pm 3\%$. These data suggest that NCX is the predominant mechanism mediating Ca^{2+} overload during anoxia/reoxygenation in guinea-pig ventricular myocytes.

hypoxia; calcium; heart; ischemia; sodium/calcium exchanger

PROLONGED MYOCARDIAL ischemia, followed by reperfusion, can produce cytosolic acidification as well as elevations in intracellular sodium concentration ($[\text{Na}^+]_i$) and intracellular calcium concentration ($[\text{Ca}^{2+}]_i$) (18, 27, 38). It is believed that the pathophysiological accumulation of $[\text{Ca}^{2+}]_i$ contributes to myocardial injury and cell death (38). However, the characterization of mechanisms by which $[\text{Ca}^{2+}]_i$ overload develops is still incomplete.

The progression of injury in cellular models of hypoxia or metabolic inhibition is similar to what occurs during ischemia-reperfusion. Intracellular acidification is commonly observed in cardiac myocytes during either hypoxia or metabolic poisoning (31, 34) and could be coupled to the rise in $[\text{Na}^+]_i$ through the Na^+/H^+ exchanger (NHE) (2, 27, 43). Elevated $[\text{Na}^+]_i$ occurs in both myocardial ischemia (12) and hypoxic cardiac myocytes (11, 16, 31) and usually precedes the rise in $[\text{Ca}^{2+}]_i$ (15, 28, 43). These observations led to the hypothesis that the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX), which can couple a rise in $[\text{Na}^+]_i$ to a rise in $[\text{Ca}^{2+}]_i$, could be responsible for excessive Ca^{2+} entry in these

studies (16, 21, 25, 43). However, a complete understanding of NCX activity during ischemia or hypoxia is complicated, because the NCX would likely be affected by changes in intracellular ATP concentration (5, 6, 17), intracellular pH (9, 29), or anoxia (36). In addition, the lack of a specific NCX inhibitor has made it difficult to quantify what contribution the NCX makes to $[\text{Ca}^{2+}]_i$ accumulation during models of ischemia or hypoxia.

Previous demonstrations of NCX involvement in $[\text{Ca}^{2+}]_i$ overload in whole organ and cellular models have altered plasmalemmal Na^+ gradients (16, 25), blocked ischemic or hypoxic Na^+ entry (12), or used less specific NCX inhibitors such as dichlorobenzamil (26) or dimethylthiourea (47). Recently, KB-R7943 was shown to be cardioprotective in hypoxic myocytes (21), most likely through inhibition of the NCX (20), although KB-R7943 can inhibit other ion transport proteins (45). The lack of adequate pharmacological tools has led to an emphasis on molecular biology techniques to study the NCX. Overexpression of the NCX in a transgenic mouse model demonstrated that the NCX could mediate ischemia-reperfusion injury (7). However, this model cannot demonstrate the extent to which the NCX normally contributes to tissue injury during ischemia or hypoxia. An alternative approach used antisense oligonucleotides directed to the NCX to successfully reduce both NCX expression and function in rat myocytes (22, 40, 42); however, this approach has not been applied to models of ischemia or hypoxia.

The chief aim of this study was to explicitly determine the contribution of the NCX to $[\text{Ca}^{2+}]_i$ accumulation during anoxia/reoxygenation in guinea pig ventricular myocytes. Qualitatively, NCX has been established as a significant route for Ca^{2+} entry; however, the quantitative contribution NCX makes to $[\text{Ca}^{2+}]_i$ accumulation, and thus its importance during anoxia/reoxygenation, is not known. Our primary experimental approach toward this aim was to largely suppress NCX expression in adult cultured myocytes with an antisense oligonucleotide. This approach was supplemented by experiments using either direct NCX inhibition with NiCl_2 or indirect NCX inhibition by

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suppressing Na^+ entry during anoxia. We (10) have previously published some of the data in an abstract.

MATERIALS AND METHODS

Cell Preparation

Experiments were conducted in single adult guinea pig ventricular myocytes. Female Hartley guinea pigs were anesthetized with an intraperitoneal injection of pentobarbital sodium before the heart was excised. Cells were isolated using an established technique (8). This method of euthanasia was approved by the University of Kentucky Institutional Animal Care and Use Committee.

Freshly isolated myocytes were used immediately. Cultured adult myocytes were isolated using a similar procedure with common sterile techniques (24). Isolated myocytes were then suspended in serum-free medium 199 supplemented with (in mM) 25 HEPES, 5 creatine, 2 L-carnitine, 5 taurine, and 10^{-4} insulin. Also included were the following: 0.002% wt/vol BSA, 100 IU penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin. Corning tissue-grade culture dishes were treated with mouse laminin (2 $\mu\text{g}/\text{cm}^2$). Myocytes were plated at a density of 10^4 cells/ cm^2 and allowed to attach for 4 h, after which the medium was removed and replaced with fresh medium. Myocytes were kept under sterile conditions in a 5% CO_2 incubator at 37°C.

Experimental Protocols

Anoxia/reoxygenation experiments used a Tyrode's solution containing (in mM) 140 NaCl, 4 KCl, 2.5 CaCl_2 , 1 MgCl_2 , and 10 HEPES (pH 7.2, 22°C). Myocytes were made anoxic in a gas-tight, glass petri dish (31). Anoxia was maintained for 20-min posttriggor contracture, at which time the anoxic Tyrode's solution was removed and replaced with an oxygenated Tyrode's solution. The intent of this anoxia/reoxygenation protocol was to place each myocyte under substantial metabolic stress, such that each myocyte had a significant risk for cell injury and death. In this protocol, most control myocytes underwent hypercontracture during reoxygenation, which is the single cell equivalent of irreversible injury (1). This likely represents the initial stages of necrotic injury, because experiments with calcein-AM showed a slight decrease in cytosolic fluorescence (10–20%) after 10 min of reoxygenation followed by a substantial decrease in cytosolic fluorescence (85–90%) after 4 h of reoxygenation. This result is consistent with previous reports showing hypercontracture is followed by membrane disruption and necrosis (4, 41). In the latter study, adult isolated rat myocytes underwent necrosis but not apoptosis in response to anoxia/reoxygenation. Only quiescent, rod-shaped myocytes were chosen to undergo the anoxia/reoxygenation protocol. Myocytes were not electrically paced.

TTX (30 μM) was used to inhibit TTX-sensitive Na^+ channels and 10 μM 4-isopropyl-3-methylsulfonylbenzoyl guanidine methanesulfonate (HOE-642) was used to inhibit NHE (35). NiCl_2 (10 mM) was added during reoxygenation in some experiments to inhibit the NCX.

$[\text{Ca}^{2+}]_i$ was measured using 2.5 μM indo 1-AM. The loading of indo 1 and the use of microfluorimetry has been described previously (31). Briefly, indo 1 measurements were made on a conventional, inverted fluorescence microscope with a xenon arc lamp for an excitation source. Ratiometric measurements were made by using an excitation filter centered at 355 nm, and emission filters were centered at 405 and 485 nm. Fluorescence was measured by using a photomultiplier detection system (Photon Technology Interna-

tional; South Brunswick, NJ). Although the indo 1 ratio changed during the time course of anoxia/reoxygenation, there was no significant decrease in absolute indo 1 fluorescence at any time during this protocol as judged by the individual measurements of the 405- and 485-nm emission fluorescence. A severe disruption in membrane integrity would be expected to result in a gross loss of absolute indo 1 fluorescence irrespective of the indo 1 ratio. The lack of any significant change in absolute indo 1 fluorescence indicated that membrane integrity remained largely intact during the early phase of reoxygenation. These observations are supported by additional experiments conducted using calcein-AM to also measure membrane integrity during the anoxia/reoxygenation protocol. Calcein fluorescence only slightly decreased during the initial 10 min of reoxygenation, which indicated that membrane integrity remained largely intact, even as many myocytes underwent hypercontracture. However, calcein-AM fluorescence was reduced by roughly 90% of its initial value by 4-h postreoxygenation. These data support the idea that early measurements during reoxygenation are not confounded by a gross loss of membrane integrity (37), and, furthermore, it does appear that hypercontracture seen during reoxygenation is an irreversible injury that can lead to necrotic death (41) similar to previous reports in the isolated rat heart (1, 4).

Immunocytochemistry

Myocytes were fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. A monoclonal mouse anti-NCX antibody using purified canine cardiac NCX as an antigen (Research Diagnostics) was used in conjunction with a goat anti-mouse fluorescein isothiocyanate-conjugated secondary antibody (Santa Cruz Biotechnology) to visualize NCX immunofluorescence. Images were obtained using a confocal microscope (Nikon RCM 8000), stored on an optical disk recorder, and analyzed using Metamorph (Universal Imaging).

Oligonucleotides

Oligonucleotides were synthesized at the University of Kentucky Macromolecular Structure Analysis Facility. An antisense oligonucleotide (5'-TCGCAGCATGTTGTACAA-TG-3') was targeted to a region around the start codon of the cardiac guinea pig NCX (-11 to +9) (44). A nonsense oligonucleotide (5'-TCTCGAACGTGTTCAAGATG-3') was used to control for any nonspecific effects of the antisense oligonucleotide. Both oligonucleotides had eight phosphorothioate-modified nucleotides (boldfaced). Cultured myocytes were treated with antisense (2 μM), nonsense (2 μM), or no oligonucleotide as appropriate. Fresh oligonucleotides and medium 199 were added every 48 h. Myocytes were maintained in culture for 6 days.

RESULTS

Anoxia/Reoxygenation in Freshly Isolated Myocytes

Pharmacological sensitivity of cytosolic Ca^{2+} overload. The intent of this study was to determine whether the NCX is the predominant route for excessive Ca^{2+} entry during anoxia/reoxygenation in adult guinea pig ventricular myocytes. Figure 1A shows $[\text{Ca}^{2+}]_i$ measurements made in a quiescent ventricular myocyte loaded with indo 1-AM and then subjected to an anoxia/reoxygenation procedure consisting of 20-min posttriggor anoxia followed by 20 min of reoxy-

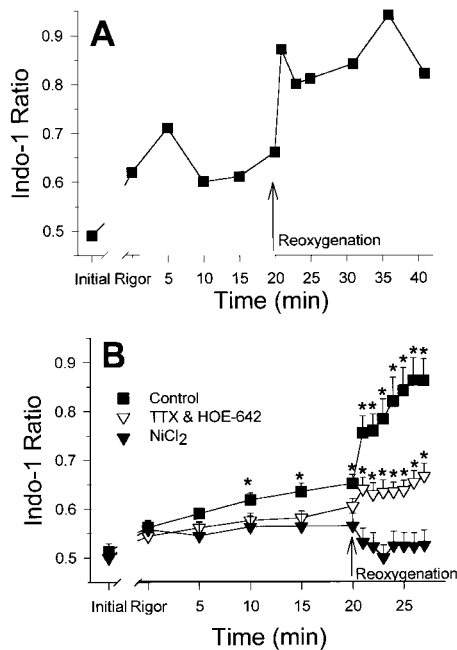


Fig. 1. Inhibition of intracellular Na^+ concentration ($[\text{Na}^+]_i$) accumulation or $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX) activity significantly reduces intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) accumulation during anoxia/reoxygenation. **A:** $[\text{Ca}^{2+}]_i$ was measured in a representative myocyte, loaded with the fluorescent Ca^{2+} indicator indo 1-AM, which was subjected to 20 min of postrigor anoxia followed by 20 min of reoxygenation. The myocyte hypercontracted after 8 min of reoxygenation. **B:** freshly isolated myocytes were exposed to 20 min of postrigor anoxia followed by 8 min of reoxygenation. TTX ($30 \mu\text{M}$) and HOE-642 ($10 \mu\text{M}$) were used to suppress $[\text{Na}^+]_i$ accumulation during anoxia, whereas 10 mM NiCl_2 was used during reoxygenation to directly inhibit NCX activity. Higher indo 1 ratios represent higher $[\text{Ca}^{2+}]_i$. Data were not converted to $[\text{Ca}^{2+}]_i$, because intracellular pH during anoxia/reoxygenation is constantly changing, which affects the calibration of indo 1 (31). In separate experiments, 10 mM NiCl_2 was demonstrated to inhibit evoked NCX activity by $95 \pm 3\%$ in the same assay described in Fig. 4. The break in the x-axis is due to variability between cells in the time to onset of rigor (mean of $25 \pm 10 \text{ min}$); therefore, groups were statistically compared after this time point. $*P < 0.05$ vs. same treatment at the initial time point. Each data point represents the mean \pm SE of 8 cells.

ation. This protocol was designed to provide a severe enough insult to put the myocyte at significant risk of cell injury and death. As shown in Fig. 1A, the most profound increase in $[\text{Ca}^{2+}]_i$ occurred immediately after reoxygenation. $[\text{Ca}^{2+}]_i$ failed to recover to preanoxia levels despite a prolonged period of reoxygenation, presumably because the rise in $[\text{Ca}^{2+}]_i$ was severe enough to induce irreversible injury. The myocyte hypercontracted after 8 min of reoxygenation, after which it is likely that the cell Ca^{2+} transport processes were compromised (38).

Figure 1B summarizes the results from a more extensive series of experiments where $[\text{Ca}^{2+}]_i$ was measured in freshly isolated myocytes subjected to 20 min of postrigor anoxia followed by 8 min of reoxygenation. These experiments were designed to test the sensitivity of NCX inhibitors on the rise in $[\text{Ca}^{2+}]_i$ during the period of reoxygenation that precedes irreversible injury. Similar to the experiment shown in Fig. 1A,

$[\text{Ca}^{2+}]_i$ in untreated myocytes increased slightly during anoxia in these cells; however, the most prominent increase in $[\text{Ca}^{2+}]_i$ was during reoxygenation. The addition of 10 mM NiCl_2 , a NCX inhibitor, to the bathing solution during reoxygenation completely suppressed this rise in $[\text{Ca}^{2+}]_i$ during reoxygenation. NiCl_2 was chosen for its ability to strongly inhibit NCX activity, despite the fact that NiCl_2 could also inhibit additional Ca^{2+} entry pathways (30) such as Ca^{2+} channels (46). Similar experiments were also done with a combination of drugs ($30 \mu\text{M}$ TTX and $10 \mu\text{M}$ HOE-642) that would be expected to indirectly inhibit NCX activity by reducing the rise in $[\text{Na}^+]_i$ during anoxia (11, 33, 35). Figure 1B shows that TTX/HOE-642 also significantly reduced $[\text{Ca}^{2+}]_i$ throughout reoxygenation compared with untreated myocytes. Overall, these data suggest that the majority of the increase in $[\text{Ca}^{2+}]_i$ during reoxygenation is mediated by the NCX, although the limitations of the available inhibitors emphasize the need for a novel approach that would both directly and specifically inhibit NCX activity.

Antisense Oligonucleotides in Cultured Myocytes

An antisense oligonucleotide can inhibit NCX expression and activity. Given the limitations of the available pharmacological approaches, we then attempted to use antisense oligonucleotides to specifically inhibit NCX activity in cultured adult guinea pig ventricular myocytes. We screened eight oligonucleotides for activity and found the most effective oligonucleotide was a 20-mer that encompassed the NCX1 start site. Figure 2 shows that NCX immunofluorescence decreased significantly starting at day 2 and was significantly reduced at every time point compared with untreated control myocytes. The greatest effect was at days 5 and 6; therefore, all cultured myocytes in subsequent experiments were assayed after 6 days of treatment in culture.

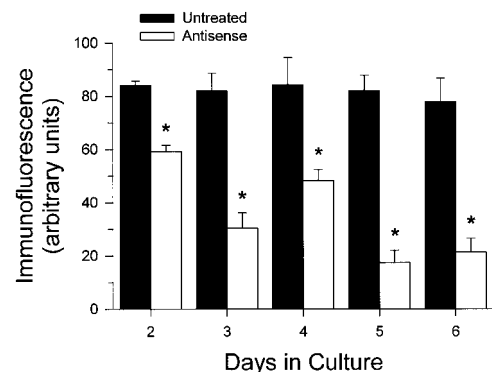


Fig. 2. An antisense oligonucleotide directed against the cardiac NCX can significantly reduce NCX expression in adult cultured myocytes. Myocytes were incubated for the indicated number of days in medium 199 that either contained $2 \mu\text{M}$ antisense oligonucleotide or no oligonucleotide (untreated). The medium was changed every 48 h. Standard immunocytochemistry techniques were used to determine the effectiveness of the antisense oligonucleotide (see MATERIALS AND METHODS). $*P < 0.05$ vs. untreated myocytes at the same time point. Each bar represents the mean \pm SE of 6 cells.

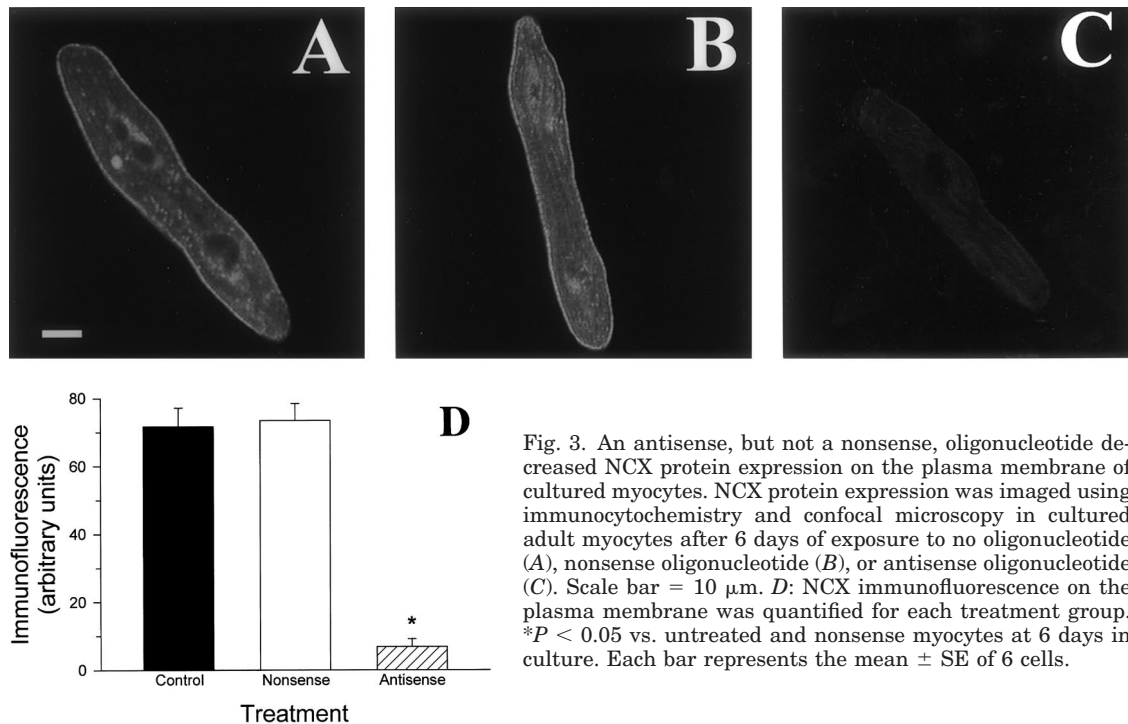


Fig. 3. An antisense, but not a nonsense, oligonucleotide decreased NCX protein expression on the plasma membrane of cultured myocytes. NCX protein expression was imaged using immunocytochemistry and confocal microscopy in cultured adult myocytes after 6 days of exposure to no oligonucleotide (A), nonsense oligonucleotide (B), or antisense oligonucleotide (C). Scale bar = 10 μm . D: NCX immunofluorescence on the plasma membrane was quantified for each treatment group. * $P < 0.05$ vs. untreated and nonsense myocytes at 6 days in culture. Each bar represents the mean \pm SE of 6 cells.

To characterize the effect of the antisense probe, a nonsense oligonucleotide was used to control for any nonspecific effects of the antisense oligonucleotide. Untreated, nonsense-, and antisense-treated myocytes were cultured in parallel and then assessed for NCX-mediated immunofluorescence after 6 days in culture (Fig. 3). In both untreated (Fig. 3A) and nonsense-treated (Fig. 3B) myocytes, NCX immunofluorescence was concentrated on the sarcolemma with some punctuate fluorescence that most likely represents NCX expression in transverse tubules (13). There also appeared to be some additional perinuclear fluorescence that could represent de novo NCX synthesis. There were no significant qualitative or quantitative differences in NCX immunofluorescence between untreated and nonsense-treated myocytes. However, there was a marked decrease in sarcolemmal immunofluorescence in antisense-treated myocytes (Fig. 3C). Also, note the lack of any perinuclear immunofluorescence. On average, NCX immunofluorescence decreased by $90 \pm 4\%$ in antisense-treated myocytes (Fig. 3D).

To confirm the immunocytochemistry data, a functional NCX assay was used to induce an increase in $[\text{Ca}^{2+}]_i$. The assay consisted of first exposing a myocyte to a 10-min period of K^+ -free Tyrode's solution to increase $[\text{Na}^+]_i$, followed by 8 min of exposure to Na^+ -free Tyrode's solution to evoke a NCX-mediated $[\text{Ca}^{2+}]_i$ increase, and finally, 8 min of exposure to normal Tyrode's solution. NCX activity, as judged by peak indo 1 fluorescence during the Na^+ -free period, was not significantly different in untreated fresh or untreated cultured myocytes or in cultured myocytes treated with the nonsense oligonucleotide (Fig. 4). However, the evoked increase in indo 1 fluorescence was decreased by $94 \pm 3\%$ in antisense-treated myocytes compared

with cultured untreated or nonsense-treated myocytes. These data suggest $[\text{Ca}^{2+}]_i$ response to this functional assay is not significantly different in fresh and cultured myocytes and the loss of this $[\text{Ca}^{2+}]_i$ response is due to a specific antisense effect.

An antisense oligonucleotide suppresses $[\text{Ca}^{2+}]_i$ during reoxygenation. The development of an effective, specific antisense probe made a quantitative test of the contribution of NCX activity to the rise of $[\text{Ca}^{2+}]_i$ during anoxia/reoxygenation possible. After being treated in culture for 6 days, myocytes underwent the standard anoxia/reoxygenation protocol. As shown in Fig. 5, $[\text{Ca}^{2+}]_i$ in both untreated and nonsense-treated myo-

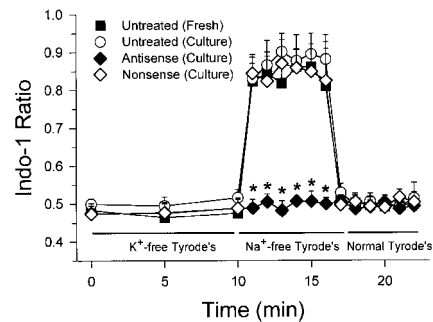


Fig. 4. Antisense oligonucleotides can suppress reverse-mode NCX activity in cultured adult guinea pig ventricular myocytes. $[\text{Ca}^{2+}]_i$ was monitored in either freshly isolated or cultured myocytes during reverse-mode NCX activity. Reverse-mode NCX activity was evoked by exposing cells to K^+ -free Tyrode's solution, followed by exposure to Na^+ -free Tyrode's solution. Cell culture and oligonucleotide treatment protocols were carried out as previously described (Figs. 2 and 3). * $P < 0.05$ vs. untreated and nonsense-treated cultured myocytes. Indo 1 ratios were not significantly different between untreated fresh and untreated cultured myocytes. Each data point represents the mean \pm SE of 6 cells.

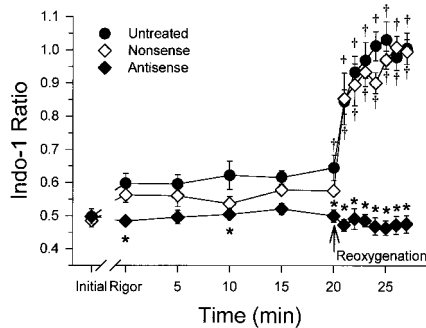


Fig. 5. Reoxygenation-induced $[\text{Ca}^{2+}]_i$ overload in adult cultured myocytes is suppressed by antisense oligonucleotide treatment. Fluorescence measurements, culture protocols, and the anoxia/reoxygenation procedure were the same as previously described (Figs. 1–3). * $P < 0.05$ vs. untreated indo 1 ratio at the same time point. † $P < 0.05$ vs. same treatment at initial time point. Each data point represents the mean \pm SE of 6 cells.

cytes rose significantly during reoxygenation, similar to what was observed in fresh myocytes (Fig. 1B). In addition, there was no significant difference in $[\text{Na}^+]_i$ in control, nonsense-, or antisense-treated adult myocytes maintained in culture for 6 days (data not shown). There was no significant difference in $[\text{Ca}^{2+}]_i$ between untreated and nonsense-treated myocytes at any time point (Fig. 5). However, $[\text{Ca}^{2+}]_i$ in the antisense-treated myocytes was significantly lower than $[\text{Ca}^{2+}]_i$ in both untreated and nonsense-treated myocytes at every time point during reoxygenation. In fact, the rise in $[\text{Ca}^{2+}]_i$ during anoxia/reoxygenation was completely suppressed in the antisense-treated myocytes, because at no time point was $[\text{Ca}^{2+}]_i$ significantly different from the initial $[\text{Ca}^{2+}]_i$. These data support the hypothesis that the NCX is the predominant source of Ca^{2+} entry in this experimental model.

DISCUSSION

Inhibition of NCX Expression and Function Using Antisense Oligonucleotides

Antisense oligonucleotides have been previously used to reduce NCX activity, although not with myocardial ischemia or hypoxia. An antisense oligonucleotide targeted to the 3' region of rat NCX1 achieved partial inhibition of NCX activity in primary cultured embryonic rat myocytes (42). In a different study, another antisense probe targeted to the 3' region achieved extensive inhibition of NCX activity within 48 h in primary cultured neonatal rat myocytes (22). Both studies used a control oligonucleotide, which supports a specific effect of the antisense oligonucleotide; however, neither study directly measured changes in NCX protein expression. Another set of studies used two antisense oligonucleotides targeted to regions around the rat NCX1 start site (22, 40). This approach led to a significant decrease in both NCX function and expression in primary cultured neonatal rat myocytes (39). However, in contrast to earlier studies, it took at least 4 days for these effects to develop, which was

attributed to a slow turnover rate for the NCX protein (39).

In this study, eight different oligonucleotides were tested for efficacy. A 20-mer oligonucleotide targeted to a region encompassing the guinea pig NCX start site showed the greatest inhibitory effect. Immunocytochemistry demonstrated a marked decrease in expression of the NCX protein on the sarcolemma (Fig. 3), which was confirmed by a functional assay of reverse-mode NCX activity (Fig. 4). This antisense effect developed slowly and reached a maximal effect after 5–6 days in culture (Fig. 2), similar to a previous report in neonatal rat myocytes (39). The specific action of the antisense oligonucleotide was confirmed through the use of a nonsense oligonucleotide. These data establish the usefulness of this antisense probe, because extensive, specific NCX inhibition was achieved in this preparation.

The NCX is the Predominant Route for Ca^{2+} Entry during Anoxia/Reoxygenation

In this study, we tested the hypothesis that reverse-mode NCX activity is responsible for essentially all of the Ca^{2+} overload seen during anoxia/reoxygenation. Three approaches were used to assess this hypothesis: 1) direct inhibition of NCX activity by NiCl_2 , 2) decreasing the accumulation of $[\text{Na}^+]_i$ with TTX/HOE-642 (11), and 3) decreasing NCX protein expression with an antisense oligonucleotide. NiCl_2 is probably the least selective approach, as NiCl_2 is known to block Ca^{2+} channels (46) and possibly other transporters (30). Both TTX and HOE-642 (35) are regarded as selective for Na^+ channel and NHE1 inhibition, respectively. However, these drugs can only inhibit reverse-mode NCX activity indirectly by reducing $[\text{Na}^+]_i$ accumulation during anoxia (11). In contrast, antisense oligonucleotides, in theory, act through a very selective and direct mechanism, and this was verified through the use of a nonsense oligonucleotide to control for any nonspecific oligonucleotide effects. Overall, the antisense data are the most compelling and novel feature of this study. The NiCl_2 and TTX/HOE-642 data do provide additional support for the antisense data, because three distinct mechanisms of suppressing NCX activity substantially inhibited Ca^{2+} overload during reoxygenation.

We cannot preclude the possibility that cell culture could alter the relative importance of some ion transport mechanisms. However, the similarity of the $[\text{Ca}^{2+}]_i$ response to anoxia/reoxygenation in freshly isolated (Fig. 1) and cultured adult ventricular myocytes (Fig. 5) is notable, and in addition, there was no significant difference in the $[\text{Ca}^{2+}]_i$ response when reverse-mode NCX activity was deliberately evoked (Fig. 4). In addition, the accumulation of $[\text{Na}^+]_i$ during anoxia in both freshly isolated and cultured myocytes was measured using the Na^+ -sensitive fluorescent indicator SBFI. Myocytes were subjected to 20 min of post-rigor anoxia and at no time point was there any significant difference in $[\text{Na}^+]_i$ between freshly isolated or

6-day-old cultured myocytes (data not shown). Therefore, a conservative interpretation of our data is that the NCX is the predominant mechanism for Ca²⁺ entry during anoxia/reoxygenation in isolated adult guinea pig ventricular myocytes. It is also possible that NCX may be the only significant source for Ca²⁺ entry in this experimental model, although we did not explicitly test other possible routes of Ca²⁺ entry.

It is evident that extensive NCX inhibition occurs in this model during anoxia and that this inhibition is rapidly relieved during reoxygenation. Previous reports demonstrated there is a profound increase in [Na⁺]_i during anoxia in this model (11, 31), capable of driving substantial Ca²⁺ entry via reverse-mode NCX activity. However, the data presented in Figs. 1 and 5 clearly show that most of the increase in [Ca²⁺]_i does not occur until reoxygenation even in the presence of elevated [Na⁺]_i during anoxia. Other investigators have also reported strong inhibition of reverse-mode NCX activity in guinea pig ventricular myocytes during hypoxia (25, 36). We have not yet carried out extensive studies of potential mechanisms that could explain the apparent NCX inhibition during anoxia and subsequent recovery during reoxygenation. Decreased cytosolic ATP levels (5, 6, 17) or cytosolic acidification (9, 29) could potentially contribute to NCX inhibition during anoxia. NCX recovery during reoxygenation seems relatively rapid (Fig. 1) and could simply reflect the removal of such inhibitory influences or could also be a result of the activation of certain processes known to stimulate NCX such as protein kinase C (3, 19) or reactive oxygen species (14, 32).

Limitations of this Study

There are several potential limitations of this study. It is obvious that some differences in myocyte morphology and perhaps function should be expected between freshly isolated myocytes and myocytes maintained in culture for 6 days. Cultured myocytes were maintained in a serum-free environment, thus slowing potential changes over time (24). A loss of transverse tubules has been reported during cell culture (23) and would be consistent with our observations of a gradual loss of the organized, punctuate distribution of NCX immunofluorescence (data not shown), which is representative of NCX in transverse tubules (13). Altered expression of ion channels has also been reported, although these differences were more quantitative than qualitative (23, 24). [Ca²⁺]_i responses in cultured myocytes to anoxia/reoxygenation and evoked reverse-mode NCX activity closely resemble those of freshly isolated myocytes, although small differences between the two preparations do exist. The adult culture model was needed to carry out the antisense experiments due to the prolonged period of time necessary to achieve sufficient NCX inhibition. The anoxia/reoxygenation protocol itself is meant to mimic key features of ischemia-reperfusion, particularly the metabolic stress of ischemia, but there are obvious differences between this model and true ischemia (11). Overall, the cul-

tured myocyte model, with pharmacological inhibition in fresh myocytes, allowed us to make the most rigorous and comprehensive test to date of NCX activity during anoxia/reoxygenation. This study clearly supports previous work done in both single cell models and whole organs and extends previous conclusions concerning the role of the NCX during hypoxia or ischemia through the use of an antisense oligonucleotide to specifically inhibit the NCX.

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