ROS are required for rapid reactivation of Na\(^+\)/Ca\(^{2+}\) exchanger in hypoxic reoxygenated guinea pig ventricular myocytes

B. N. Eigel, H. Gursahani, and R. W. Hadley

Department of Molecular and Biomedical Pharmacology, College of Medicine, University of Kentucky, Lexington, Kentucky 40536-0298

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Eigel, B. N., H. Gursahani, and R. W. Hadley. ROS are required for rapid reactivation of Na\(^+\)/Ca\(^{2+}\) exchanger in hypoxic reoxygenated guinea pig ventricular myocytes. Am J Physiol Heart Circ Physiol 286: H955–H963, 2004. First published October 30, 2003; 10.1152/ajpheart.00721.2003.—The cardiac Na\(^+\)/Ca\(^{2+}\) exchanger (NCX) contributes to cellular injury during hypoxia, as its altered function is largely responsible for a rise in cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)). In addition, the NCX in guinea pig ventricular myocytes undergoes profound inhibition during hypoxia and rapid reactivation during reoxygenation. The mechanisms underlying these changes in NCX activity are likely complex due to the participation of multiple inhibitory factors including altered cytosolic Na\(^+\) concentration, pH, and ATP. Our main hypothesis is that oxidative stress is an essential trigger for rapid NCX reactivation in guinea pig ventricular myocytes and is thus a critical factor in determining the timing and magnitude of Ca\(^{2+}\) overload. This hypothesis was evaluated in cardiac myocytes using fluorescent indicators to measure [Ca\(^{2+}\)]\(_i\) and oxidative stress. An NCX antisense oligonucleotide was used to decrease NCX protein expression in some experiments. Our results indicate that NCX activity is profoundly inhibited in hypoxic guinea pig ventricular myocytes but is reactivated within 1–2 min of reoxygenation at a time of rising oxidative stress. We also found that several interventions to decrease oxidative stress including antioxidants and diazoxide prevented NCX reactivation and Ca\(^{2+}\) overload during reoxygenation. Furthermore, application of exogenous H\(_2\)O\(_2\) was sufficient by itself to reactivate the NCX during sustained hypoxia and could reverse the suppression of reoxygenation-mediated NCX reactivation by diazoxide. These data suggest that elevated oxidative stress in reoxygenated guinea pig ventricular myocytes is required for rapid NCX reactivation, and thus reactivation should be viewed as an active process rather than being due to the simple decline of NCX inhibition.

sodium-calcium exchanger; antioxidants; diazoxide; heart; hypoxia; ischemia

DANGEROUSLY ELEVATED LEVELS of cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)), or Ca\(^{2+}\) overload, occur during ischemia or hypoxia in both intact heart and isolated cardiac myocytes (32). The important contribution of Ca\(^{2+}\) overload to rapid necrotic death and arrhythmogenesis following ischemia has long been appreciated (24, 32), and it is also likely that Ca\(^{2+}\) overload contributes to the induction of myocardial apoptosis (9, 35). A number of studies have shown that the Na\(^+\)/Ca\(^{2+}\) exchanger (NCX) is involved in mediating Ca\(^{2+}\) overload after ischemia or hypoxia (11, 21, 30, 33). It is thought that under hypoxic conditions, there is a substantial rise in cytosolic Na\(^+\) concentration ([Na\(^+\)]) that can promote reverse-mode NCX transport whereby the NCX mediates Na\(^+\) efflux and Ca\(^{2+}\) influx. We previously reported that the NCX is the predominant route of Ca\(^{2+}\) entry after hypoxiareoxygenation in adult guinea pig ventricular myocytes, as cultured myocytes pretreated with an NCX antisense oligonucleotide showed little rise in [Ca\(^{2+}\)]\(_i\) when subjected to hypoxiareoxygenation (7). Therefore, a more complete understanding of the factors that regulate NCX activity and thus influence Ca\(^{2+}\) overload during hypoxiareoxygenation would be of great interest.

Previous studies reported evidence that the NCX is likely inhibited to some degree during hypoxia and becomes reactivated at some point during reoxygenation (20, 31). However, a full understanding of NCX activity during hypoxia is complicated by the potential involvement of several factors known to regulate NCX activity including decreased ATP levels (4, 13), cytosolic acidification (5), and increased [Na\(^+\)]\(_i\) (13). Na\(^+\)-dependent inactivation may be of special interest, as it could serve as a nexus for several NCX regulatory signaling pathways (5, 12).

Reactive oxygen species (ROS) could also modulate NCX activity during ischemia or hypoxia. ROS can be generated in cardiac myocytes during hypoxia, although a much larger burst of ROS often occurs during reoxygenation (34). The effects of ROS on NCX activity seem complex and may vary with cell type, species of ROS, and experimental model (1, 10, 27). Perhaps the most interesting effects of ROS on NCX were reported by Santacruz-Tolosa et al. (29), who concluded that oxidizing conditions could stimulate NCX activity through removal of Na\(^+\)-dependent inactivation. This suggests the possibility that increased ROS levels might be able to overcome some of the inhibitory effects of hypoxia.

We hypothesize that increased oxidative stress during reoxygenation is crucial to initiating both NCX reactivation and the subsequent appearance of Ca\(^{2+}\) overload in guinea pig ventricular myocytes. Thus NCX reactivation would be viewed as a result of the rapid stimulation of NCX activity during reoxygenation rather than simply due to the slow waning of inhibition as the cell restores more physiological levels of Na\(^+\), pH, and ATP. This hypothesis also implies that NCX reactivation could be induced even during sustained hypoxia by the application of ROS to the cardiac myocyte. Here we present data in support of this hypothesis, obtained from guinea pig ventricular myocyte preparations where we can largely suppress NCX protein expression by using measurements of [Ca\(^{2+}\)]\(_i\), oxidative stress, and reverse-mode NCX activity.
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MATERIALS AND METHODS

Cell preparations. Experiments were conducted on single adult guinea pig ventricular myocytes. Experiments that involved long-term treatment of the myocytes with antisense oligonucleotides (see Figs. 2 and 9) required the use of cultured adult guinea pig ventricular myocytes, whereas the other experiments used freshly isolated myocytes. Female Hartley guinea pigs were anesthetized with an intra-peritoneal injection of pentobarbital sodium before the hearts were excised. Cells were isolated using an established collagenase dispersion technique (26). A total of 40 animals were used to prepare myocytes for this study; each figure (except Fig. 1) contains data obtained using 3–9 isolations. All procedures using animals were approved by the University of Kentucky Institutional Animal Care and Use Committee.

Cultured adult myocytes were prepared using a sterile technique described previously (7). Isolated myocytes were suspended in serum-free medium 199 that was supplemented with 25 mmol/l HEPES, 5 mmol/l creatine, 2 mmol/l t-carnitine, 5 mmol/l taurine, 10−7 mol/l insulin, 0.2% BSA, 100 IU penicillin, and 100 μg/ml streptomycin. MatTek glass-bottom, 35-mm microwell dishes were coated with 6 μg of Cell-Tak cell adhesive (BD Biosciences; Bedford, MA). Myocytes were plated at a density of 104 cells/cm2 and were 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% CO2 incubator at 37°C.

The responses of freshly isolated or cultured guinea pig ventricular myocytes to either the reverse-mode NCX activity assay (7) or hypoxia-reoxygenation (see Fig. 2B) were very similar. However, to account for potential qualitative differences between the two types of myocyte preparations, statistical comparisons were only made between treatment groups within a given preparation.

Experimental protocols. For hypoxia-reoxygenation experiments, a nitrogen-bubbled extracellular solution was used that contained the following (in mmol/l): 144 NaCl, 2.5 CaCl2, 1 MgCl2, 10 HEPES (pH 7.2, 22°C). Experiments were carried out at 22°C to ensure compatibility with earlier studies (6, 7). Quiescent myocytes were made hypoxic in a glass, gas-tight petri dish (6, 26). Hypoxia was maintained for 20 min postgrir at which time the hypoxic solution was removed and replaced with oxygenated solution that contained 11 mmol/l glucose. This protocol produces a robust rise in [Ca2+]i that is almost entirely due to reverse-mode NCX activity (7). In experiments where a shorter hypoxic interval was used, a smaller rise in [Ca2+]i was produced; however, this increase was also NCX dependent (see also Fig. 2B).

The K+–free solution used in the reverse-mode NCX activity assay contained the following (in mmol/l): 144 NaCl, 2.5 CaCl2, 1 MgCl2, and 10 HEPES (pH 7.2). The Na+–free solution contained (in mmol/l) 140 LiCl, 4 KCl, 2.5 CaCl2, 1 MgCl2, and 10 HEPES (pH 7.2). Glucose (11 mmol/l) was also added unless the solutions were made hypoxic.

Fluorescence measurements of [Ca2+]i, and oxidative stress. Oxidative stress was measured in ventricular myocytes that were loaded with a calcium indicator (CM-DCFDA) for 30 min at 22°C. Indicator fluorescence was measured on a Nikon RCM 8000 laser-scanning confocal microscope using either the 543-nm output of a HeNe laser or the 488-nm output filtered with a time constant of 50 ms and was then digitized on a microcomputer at 1–2 kHz. Standard background subtraction and ratiometric analysis methods were used.

Inhibition of NCX protein expression by an antisense oligonucleotide. An antisense oligonucleotide (5′-TTCGCGACGATGTTGTA-CATG-3′) was targeted to a region around the start codon of the cardiac guinea pig NCX (−11 to −9). The oligonucleotide had eight phosphorothioate-modified nucleotides (shown in boldface). A non-sense oligonucleotide (5′-TCTCGAAGCTTGTTCAAGATG-3′) was used to control for nonspecific effects of the antisense oligonucleotide. Cultured myocytes were treated with antisense (2 μmol/l), nonsense (2 μmol/l), or no oligonucleotide as needed. Fresh oligonucleotide and medium 199 were added every 48 h. All cultured myocytes were maintained in culture for 5–7 days. This protocol is very effective at inhibiting NCX protein expression, as there is almost complete suppression of both evoked reverse-mode NCX activity and sarcenemal NCX immunofluorescence (7). Oligonucleotides were synthesized at the University of Kentucky Macromolecular Structure Analysis Facility or Integrated DNA Technologies (Corvalle, IA).

Statistical analysis. Differences between means were analyzed using paired t-tests when two groups were under comparison or a one-way ANOVA for more than two groups. Student-Newman-Keuls multiple comparison testing was used for post hoc significance testing between appropriate groups if warranted after ANOVA testing. Variance is described as ± SE.

RESULTS

We have previously shown that in guinea pig ventricular myocytes, Ca2+ overload during hypoxia-reoxygenation is primarily due to reverse-mode NCX activity (7). Figure 1 shows an example of the response of [Ca2+]i to hypoxia-reoxygenation in this model. [Ca2+]i was measured in a freshly isolated guinea pig ventricular myocyte loaded with the fluo-
rescent Ca$^{2+}$ indicator indo-1 AM. The myocyte was exposed to hypoxic conditions (see methods) for 20 min postrigor when it was then reoxygenated. It can be seen from the data that the majority of the rise in [Ca$^{2+}$]i in this model occurs after reoxygenation (also see Figs. 3, 4, 7, and 8 and Ref. 7) despite the presence of substantially elevated [Na$^+$]i during the prolonged hypoxic period (6, 26).

The possibility that cardiac NCX is profoundly inhibited during hypoxia and rapidly reactivated by reoxygenation of these cells was evaluated in experiments summarized in Fig. 2. Figure 2A shows typical results from an indo-1 AM-loaded isolated myocyte undergoing a functional assay of reverse-mode NCX activity. The myocyte was first exposed to a K+-free solution, which is known to inhibit the Na$^+$-K$^+$-ATPase and elevate [Na$^+$]i. The solution was then switched to a Na$^+$-free one, which stimulates the NCX to exchange intracellular Na$^+$ for extracellular Ca$^{2+}$. The result of this reverse-mode NCX activity is seen as an increase in the indo-1 fluorescence ratio and is not seen in guinea pig ventricular myocytes pretreated with an NCX antisense oligonucleotide (7).

Figure 2B shows a similar protocol except that either freshly isolated or cultured myocytes were made hypoxic for 15 min before the K+-free period, and hypoxia was maintained until the final 5 min of Na$^+$-free conditions. The responses of both isolated and cultured myocytes were very similar. In either set of control myocytes, there was no significant increase in [Ca$^{2+}$]i during Na$^+$-free hypoxia, which is in contrast to the large rise in [Ca$^{2+}$]i, that occurred under normoxic conditions (Fig. 2A). However, when control myocytes were subjected to Na$^+$-free reoxygenation, there was a significant increase in [Ca$^{2+}$]i. These data suggest that NCX activity was strongly inhibited during hypoxia and this inhibition was overcome during reoxygenation. Furthermore, the inability of reoxygenation to induce a significant rise in [Ca$^{2+}$]i, in cultured myocytes pretreated with 2 μmol/l NCX antisense oligonucleotide is evidence that the rise in [Ca$^{2+}$]i can be attributed to reverse-mode NCX activity. The maximum indo-1 ratio of nonsense-treated myocytes during reoxygenation was indistinguishable from control cultured myocytes (1.32 ± 0.09, n = 3 vs. 1.36 ± 0.09, n = 6, respectively).

Figure 2B also shows our first data that evaluate whether elevated ROS levels are important to NCX reactivation during reoxygenation. It can be seen that either isolated or cultured myocytes treated with 40 μmol/l manganese tetrakis(4-benzoic acid)porphyrin chloride (MnTBAP), which is a mimetic of superoxide dismutase, also failed to show a significant rise in [Ca$^{2+}$]i during Na$^+$-free reoxygenation.

To further examine the role of ROS in our model, three different antioxidants were used to test whether reduced oxidative stress altered [Ca$^{2+}$]i overload during reoxygenation (Fig. 3). As shown, [Ca$^{2+}$]i, rose significantly during reoxygenation in control myocytes but not when either MnTBAP, resveratrol, or trolox was present throughout hypoxia-reoxygenation. These data suggest that reducing ROS during hypoxia-reoxygenation can largely prevent Ca$^{2+}$ overload during reoxygenation. Furthermore, because the NCX is the predominant route of Ca$^{2+}$ entry during reoxygenation in this model (7), these data also suggest that ROS are involved in the reactivation of NCX activity during reoxygenation.

The results shown in Fig. 3 demonstrate that three chemically diverse antioxidants could inhibit NCX-mediated Ca$^{2+}$ overload when present throughout hypoxia-reoxygenation. We also carried out additional experiments to see whether antioxidants were still effective when applied right at reoxygenation,
confirm that diazoxide decreased oxidative stress in guinea pig ventricular myocytes when the drug was present throughout hypoxia-reoxygenation. The data show the change in either DHR-123 or CM-DCFDA fluorescence measured at 8 min post-reoxygenation relative to a measurement taken in the same cell just before reoxygenation. The measurement is thus relevant to reoxygenation-induced oxidative stress. Diazoxide (30–100 μmol/l) had the expected result of strongly inhibiting the increase in both DHR-123 and CM-DCFDA fluorescence. This effect of diazoxide could be prevented by administering 5-hydroxycarboxic acid (5-HD), a drug frequently used to block diazoxide-sensitive ATP-dependent K⁺ channels (16). These measurements were taken at 8 min postreoxygenation to ensure that any drug effect on oxidative stress had to be sustained over the entire period of time that intracellular Ca²⁺ was accumulating during reoxygenation (see Figs. 1 and 3). However, increased oxidative stress in this model is evident as early as 2 min after reoxygenation, as reoxygenation significantly increased DHR-123 fluorescence by 87 ± 22% (n = 6).

Fig. 3. Antioxidants applied throughout hypoxia-reoxygenation significantly reduce NCX-mediated [Ca²⁺] overload during reoxygenation. Isolated myocytes loaded with calcium orange AM were subjected to the standard hypoxia-reoxygenation protocol. One of three different antioxidants (40 μmol/l MnTBAP, 10 μmol/l resveratrol, or 1 mmol/l trolox) were used throughout hypoxia-reoxygenation to reduce oxidative stress. *P < 0.05 compared with initial Ca²⁺ indicator fluorescence (1.0). Each data point represents mean ± SE of 6 myocytes.

Figs. 6 and 7 show data from a complimentary set of experiments that were also designed to test whether decreasing oxidative stress inhibits NCX reactivation by reoxygenation, but these used an independent pharmacological approach distinct from antioxidants.

Diazoxide, which is an activator of some subtypes of ATP-dependent K⁺ channels, has been consistently reported to decrease ROS levels in cardiac myocytes when the drug is present throughout ischemia-reperfusion (22, 34). The data in Fig. 6, A and B, show the results of experiments where we used either the fluorescent indicator DHR-123 or CM-DCFDA to...
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Fig. 5. Resveratrol does not affect either cytosolic acidification or the rise in cytosolic Na\(^+\) concentration ([Na\(^+\)]) during hypoxia. Cytosolic pH or [Na\(^+\)], were measured in freshly isolated myocytes loaded with either 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF)-AM or sodium-binding benzofuran isophthalate (SBFI)-AM. Measurements were made in either untreated control myocytes or in myocytes exposed to 10 \mu mol/l resveratrol. Data are plotted as changes in either cytosolic pH or the SBFI fluorescence ratio. An increase in [Na\(^+\)], is seen as an increase in the SBFI ratio. Each data point represents mean ± SE of 4 myocytes.

Figure 6C shows data from additional CM-DCFDA experiments that confirm that in this model, essentially all of the increase in oxidative stress occurs after reoxygenation. CM-DCFDA fluorescence was measured in untreated myocytes at four time points during hypoxia-reoxygenation: at the start of hypoxia, at the time of onset of rigor, immediately before reoxygenation (end hypoxia), and 8 min postreoxygenation. In this model, hypoxia induced no increase in CM-DCFDA fluorescence (relative to the initial measurement), but a significant increase was seen upon reoxygenation. This suggests that the inhibitory effects of the antioxidants on NCX reactivation must have manifested during reoxygenation, as that is the only time that there was increased oxidative stress in this model.

The experiments summarized in Fig. 7 demonstrate that diazoxide, at concentrations that decrease oxidative stress in guinea pig ventricular myocytes during reoxygenation, also inhibits NCX-mediated Ca\(^{2+}\) overload. Again, [Ca\(^{2+}\)], was measured using a fluorescent indicator while freshly isolated guinea pig ventricular myocytes were subjected to 20 min of postrigor hypoxia followed by reoxygenation.

Figure 7A plots the average change in [Ca\(^{2+}\)] indicator fluorescence under control (drug-free) conditions or in the presence of 100 \mu mol/l diazoxide, diazoxide plus 100 \mu mol/l 5-HD, or diazoxide plus 2.5 \mu mol/l glibenclamide, which is another ATP-dependent K\(^+\) channel blocker. It can be seen that 100 \mu mol/l diazoxide profoundly inhibited the NCX-mediated rise in [Ca\(^{2+}\)], which suggests that diazoxide interferes with NCX reactivation during reoxygenation, likely by keeping ROS levels low.

It is of course possible that the inhibitory effects of diazoxide on the rise in [Ca\(^{2+}\)], during reoxygenation could be due to a direct inhibitory effect on the NCX rather than an effect mediated through altered oxidative stress. Figure 7A also shows our first data that evaluate this possibility. Glibenclamide and 5-HD often can prevent or reverse the effects of diazoxide on cardiac muscle (8, 16), and it can be seen in Fig. 7A that both drugs could also prevent the inhibitory effects of diazoxide on the rise in [Ca\(^{2+}\)], in reoxygenated myocytes. These observations argue against diazoxide having any direct inhibitory effects on the NCX and are consistent with a previous report that high concentrations of diazoxide did not alter NCX activity in rat ventricular myocytes (18). It is notable that 5-HD could suppress the inhibitory effects of diazoxide on both oxidative stress (Fig. 6) and [Ca\(^{2+}\)], during reoxygenation.

Figure 7B summarizes [Ca\(^{2+}\)], data obtained at a single time point 10 min after reoxygenation using a wider range of diazoxide concentrations. Either 30 or 100 \mu mol/l was found to prevent the rise in [Ca\(^{2+}\)], during reoxygenation.

Figures 6 and 7 demonstrate that diazoxide is a pharmacological tool that could decrease both oxidative stress and the NCX-mediated rise in [Ca\(^{2+}\)], during hypoxia-reoxygenation in guinea pig ventricular myocytes. These observations are consistent with our hypothesis that NCX reactivation during reoxygenation is linked to the generation of ROS. However, an additional implication of our hypothesis is that if diazoxide is...
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Fig. 7. Diazoxide inhibits the NCX-mediated rise in \([\text{Ca}^{2+}]_i\), during reoxygenation. A: \([\text{Ca}^{2+}]_i\) was measured in isolated myocytes loaded with calcium orange AM. Treatment with either 100 \(\mu\text{mol/l}\) 5-hydroxydecanoic acid (5-HD) or 2.5 \(\mu\text{mol/l}\) glibenclamide (Glib) prevented the effects of diazoxide. Drugs were present throughout hypoxia-reoxygenation. B: dose response of three concentrations of diazoxide present throughout hypoxia-reoxygenation. \([\text{Ca}^{2+}]_i\) was significantly increased at the end of reoxygenation in all treatment groups except when myocytes were treated with either 30 or 100 \(\mu\text{mol/l}\) diazoxide. *P < 0.05 vs. initial \([\text{Ca}^{2+}]_i\) indicator fluorescence (1.0). Each data point represents the mean ± SE of 6 myocytes.

Fig. 8. Application of \(\text{H}_2\text{O}_2\) reverses the inhibitory effects of diazoxide on the reoxygenation-induced rise in \([\text{Ca}^{2+}]_i\). We measured \([\text{Ca}^{2+}]_i\) using isolated myocytes loaded with calcium green AM. Diazoxide (100 \(\mu\text{mol/l}\)) significantly reduced \([\text{Ca}^{2+}]_i\), as seen in Fig. 6A. However, when 20 \(\mu\text{mol/l}\) \(\text{H}_2\text{O}_2\) was added to diazoxide-treated myocytes after 7 min of reoxygenation, \([\text{Ca}^{2+}]_i\), significantly increased. *P < 0.05 vs. initial \([\text{Ca}^{2+}]_i\) indicator fluorescence (1.0). Each data point represents mean ± SE of 6 myocytes.

Fig. 9. \(\text{H}_2\text{O}_2\) increases \([\text{Ca}^{2+}]_i\) in hypoxic control myocytes but not in NCX antisense-treated myocytes. Adult guinea pig ventriculmyocytes were maintained in culture for 5–7 days; \([\text{Ca}^{2+}]_i\), was measured in myocytes loaded with calcium green AM. Myocytes were subjected to hypoxia, and 20 \(\mu\text{mol/l}\) \(\text{H}_2\text{O}_2\) was added 15 min later if required. After application of \(\text{H}_2\text{O}_2\), \([\text{Ca}^{2+}]_i\) increased in control myocytes but not in antisense-treated myocytes. *P < 0.05 vs. initial \([\text{Ca}^{2+}]_i\) indicator fluorescence (1.0). Each data point represents mean ± SE of 6 myocytes.

In this study, we evaluated the hypothesis that oxidative stress is an essential trigger for rapid NCX reactivation in hypoxic guinea pig ventricular myocytes and is thus a critical factor in determining the timing and magnitude of \([\text{Ca}^{2+}]_i\) overload during hypoxia-reoxygenation.

The experiments described here have provided considerable support for this hypothesis including the following new observations: 1) hypoxia inhibited, whereas reoxygenation reacti-
vated, evoked reverse-mode NCX activity in both cultured and isolated myocytes; 2) antioxidants could prevent reoxygenation from reactivating NCX activity in either the evoked reverse-mode assay or our standard hypoxia-reoxygenation protocol; 3) diazoxide inhibited both the generation of ROS and NCX-dependent Ca\textsuperscript{2+} accumulation in the hypoxia-reoxygenation protocol; 4) application of exogenous ROS during reoxygenation rapidly reversed the inhibitory effects of diazoxide on NCX activity; and 5) application of exogenous ROS caused immediate NCX reactivation even in the normally inhibitory condition of sustained hypoxia. These results support our view of reoxygenation-induced NCX reactivation as resulting from active stimulation of cardiac NCX rather than simply being due to the slow removal of inhibitory influences such as altered [Na\textsuperscript{+}], or cytosolic pH or ATP.

**NCX regulation in hypoxic cardiac myocytes.** Other investigators have reported evidence that the NCX is substantially inhibited in guinea pig cardiac myocytes during hypoxia and becomes reactivated during reoxygenation (20, 31). However, a full understanding of the role of the NCX during hypoxia is complicated by the potential participation of several known modulators of NCX. Decreased levels of ATP could potentially inhibit NCX in cardiac myocytes (4, 13). Cytosolic acidification is known to inhibit cardiac NCX (5), and cytosolic pH falls by nearly 0.4 pH units in hypoxic guinea pig ventricular myocytes (see Fig. 5). Finally, elevation of [Na\textsuperscript{+}]\textsubscript{i} at the cytoplasmic surface of the cardiac NCX to levels known to occur during hypoxia (6, 26) induces an inactivated state of the NCX (15).

A large burst of ROS generation also seems to occur during reoxygenation in this (see Fig. 6) and similar cardiac myocyte models (34). ROS are another potential modulator of NCX function during hypoxia-reoxygenation, but much less is known about their role, and so much of this study has focused on ROS modulation of cardiac NCX. The effects of ROS on NCX have been previously studied, but the effects seem to vary both with cell type and type of oxidative stress. Nitric oxide has been reported to stimulate NCX activity in a glioma-derived cell line (1), whereas exogenous H\textsubscript{2}O\textsubscript{2} has been reported to either stimulate (10) or have little effect (27) on NCX. ATP-dependent K\textsuperscript{+} channel expression with antisense oligonucleotides and the robust response of the NCX to hypoxia-reoxygenation. However, the primary limitation of the model is its relative simplicity. We expect that NCX regulation in intact myocardium during ischemia-reperfusion is further complicated by additional factors such as extracellular acidification as well as a more graded and variable degree of hypoxia and substrate depletion.

NCX regulation in guinea pig ventricular myocytes during hypoxia-reoxygenation is a particularly dramatic model with a profound inhibition of NCX during hypoxia and rapid NCX reactivation during reoxygenation that leads to most Ca\textsuperscript{2+} overload occurring during reoxygenation. However, as described above, multiple factors may be involved in NCX modulation during hypoxia or ischemia, and quantitative differences in these modulatory factors could produce different temporal patterns of NCX inhibition-reactivation and Ca\textsuperscript{2+} overload. We would still expect all the qualitative elements involved in NCX regulation during hypoxia-reoxygenation in guinea pig ventricular myocytes to be involved in other cardiac models, but model-specific quantitative differences may cause differences in the timing and magnitude of the onset of Ca\textsuperscript{2+} overload. A possible example may be rat ventricular myocytes, where NCX-mediated Ca\textsuperscript{2+} accumulation can occur earlier, during hypoxia (25). In future experiments, it would be interesting to evaluate whether model-specific differences in NCX-mediated Ca\textsuperscript{2+} accumulation can be explained by differences in the timing and magnitude of ROS generation.

The identification of oxidative stress as a critical to NCX reactivation and Ca\textsuperscript{2+} overload during reoxygenation implies that drugs or other treatments that decrease oxidative stress would be effective at decreasing Ca\textsuperscript{2+}-mediated injury in hypoxic or ischemic myocytes. We used antioxidants and diazoxide to decrease oxidative stress and NCX reactivation in our studies, and previous studies have demonstrated that trolox (28), resveratrol (17), and diazoxide (8) are cardioprotective.

We would like to point out that diazoxide has also been reported to increase ROS levels in cardiac myocytes (3, 23); however, those studies are not directly comparable to our experimental conditions, as diazoxide was used in pharmacological preconditioning protocols, where the drug was applied and washed out before the onset of hypoxia. Therefore, it seems likely that the inhibitory effects of diazoxide on NCX reactivation and NCX-mediated Ca\textsuperscript{2+} overload could contribute to the cardioprotective effects of diazoxide, but most likely only when diazoxide is present throughout hypoxia and reoxygenation.

There is an ongoing controversy concerning whether the cardioprotective effects of diazoxide (such as altered ROS generation) are initiated through ATP-dependent K\textsuperscript{+} channel activation or through other mechanisms (19). Here we simply want to point out that the usefulness of diazoxide to our study of NCX reactivation depends only on the ability of diazoxide to decrease oxidative stress (see Fig. 6) and not on the specific mechanism by which it does this. It is clear that the ability of diazoxide to inhibit NCX activity in these experiments depended on the drug’s ability to decrease oxidative stress rather than on other mechanisms such as direct NCX inhibition, or...
alteration of the diastolic membrane potential (2), because the drug’s effects were mimicked by antioxidants and reversed by H2O2, glibenclamide, and 5-HD.

Finally, we would like to point out that the conclusions that NCX reactivation during reoxygenation and the subsequent rise in \([\text{Ca}^{2+}]_i\) are mediated by oxidative stress are supported by multiple, parallel experimental approaches that both increased and decreased ROS levels. Although some of the experiments used cytoprotective agents such as diazoxide or antioxidants, it is not at all likely that their ability to decrease \([\text{Ca}^{2+}]_i\) during reoxygenation could be attributed to a nonspecific reduction of hypoxic injury. The \([\text{Na}^+]_i\) measurements (see Fig. 9) and the immediate reversibility of the inhibitory effects of diazoxide (see Fig. 9) demonstrate that hypoxic injury still occurred in myocytes treated with these agents, but that the agents had uncoupled hypoxic injury from NCX-mediated Ca2+ entry. Furthermore, the data shown in Fig. 9 also support our conclusion, and no cytoprotective agents were used in the experiment.

These studies have contributed valuable evidence supporting a critical role for oxidative stress in modulating NCX activity during hypoxia-reoxygenation in guinea pig ventricular myocytes. Consideration of these results also identifies possible new experimental directions. These include identifying the specific source of the oxidative stress (e.g., H2O2 generated secondary to mitochondrial dysfunction) most relevant to NCX reactivation during reoxygenation. Another likely future direction is identifying the mechanism by which oxidative stress such as H2O2 induces NCX reactivation in hypoxic myocytes.

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DISCLOSURE

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