Sodium Channel Blockade Reduces Hypoxic Sodium Loading and Sodium-Dependent Calcium Loading

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Background Studies have shown that the rise in intracellular ionized calcium, [Ca^{2+}], in hypoxic myocardium is driven by an increase in sodium, [Na^{+}], but the source of Na^{+} is not known.

Methods and Results Inhibitors of the voltage-gated Na^{+} channel were used to investigate the effect of Na^{+} channel blockade on hypoxic Na^{+} loading, Na^{+}-dependent Ca^{2+} loading, and reoxygenation hypercontracture in isolated adult rat cardiac myocytes. Single electrically stimulated (0.2 Hz) cells were loaded with either SBFI (to index [Na^{+}]) or indo-1 (to index [Ca^{2+}]) and exposed to glucose-free hypoxia (PO_{2} <0.02 mm Hg). Both [Na^{+}] and [Ca^{2+}] increased during hypoxia when cells became inexicable following ATP-depletion contracture. The hypoxic rise in [Na^{+}] and [Ca^{2+}] was significantly attenuated by 1 μmol/L R 56865. Tetrodotoxin (60 μmol/L), a selective Na^{+}-channel blocker, also markedly reduced the rise in [Ca^{2+}] during hypoxia and reoxygenation. Reoxygenation-induced cellular hypercontracture was reduced from 83% (45 of 54 cells) under control conditions to 12% (4 of 32) in the presence of R 56865 (P<.05). Lidocaine reduced hypercontracture dose dependently with 13% of cells hypercontracting in 100 μmol/L lidocaine, 42% in 50 μmol/L lidocaine, and 93% in 25 μmol/L lidocaine. The Na^{+}-H^{+} exchange blocker, ethylisoproylamiloride (10 μmol/L) was also effective, limiting hypercontracture to 12%. R 56865, lidocaine, and ethylisoproylamiloride were also effective in preventing hypercontracture in normoxic myocytes induced by 75 μmol/L veratridine, an agent that impairs Na^{+} channel inactivation. Ethylisoproylamiloride prevented the veratridine-induced rise in [Ca^{2+}] without affecting Na^{+}-Ca^{2+} exchange, suggesting that amiloride derivatives can reduce Ca^{2+} loading by blocking Na^{+} entry through Na^{+} channels, an action that may in part underlie their ability to prevent hypoxic Na^{+} and Ca^{2+} loading.

Conclusions Na^{+} influx through the voltage-gated Na^{+} channel is an important route of hypoxic Na^{+} loading, Na^{+}-dependent Ca^{2+} loading, and reoxygenation hypercontracture in isolated rat cardiac myocytes. Importantly, the Na^{+} channel appears to serve as a route for hypoxic Na^{+} influx after myocytes become inexcitable. (Circulation. 1994;90:391-399.)

Key Words • calcium • sodium • channels • hypoxia • ischemia

A consensus has emerged that Ca^{2+} overload contributes importantly to ischemic and postischemic myocardial damage. It has been demonstrated that interventions that attenuate the rise in [Ca^{2+}], are associated with improved postischemic or posthypoxic myocardial functional recovery.1,2 Importantly, Ca^{2+} channel antagonists have been effective only in certain circumstances, ie, when given before ischemia.1 Furthermore, direct blockade of the L-type Ca^{2+} channel with conventional antagonists, such as nifedipine, has been ineffective both in preventing the increase in [Ca^{2+}] and in improving posthypoxic recovery of isolated rat ventricular myocytes.3 Evidence is accumulating from studies in both tissue and cellular models that the rise in [Ca^{2+}], occurring during ischemia and hypoxia is in large part driven by an increase in [Na^{+}], and is mediated by "reverse-mode" Na^{+}-Ca^{2+} exchange.3-6

Increases in myocardial [Na^{+}] occurring during ischemia and hypoxia have been demonstrated in many different models and with various techniques ranging from Na^{+}-sensitive microelectrodes and isotopic techniques to Na^{+} magnetic resonance spectroscopy.10 While the mechanism of the increase in [Na^{+}] remains unresolved, potential avenues for the increase include a nonspecific leak of extracellular Na^{+} through the sarclemma, failure to extrude Na^{+} by the Na^{+}-K^{+} ATPase, leakage through dysfunctional Ca^{2+} channels, entry through Na^{+}-K^{+}-Cl^{-} cotransport, Na^{+}-H^{+} exchange, or via the voltage-gated Na^{+} channel. The Na^{+}-K^{+} ATPase does not function in hypoxia or ischemia when a profound reduction of ATP is achieved, and so it certainly contributes to the increase in [Na^{+}], later in ischemia by failing to maintain Na^{+} extrusion. In the isolated myocyte exposed to hypoxia, the major increase in [Na^{+}], occurs after full depletion of ATP when the Na^{+}-K^{+} ATPase would be inhibited. [Na^{+}] does not rise in this model in the absence of extracellular Na^{+}, demonstrating that Na^{+} loading must require an influx pathway in addition to failure of this efflux mechanism.5 Morphological studies do not support sarclemal disruption as the cause of a nonspecific Na^{+} leak.11,12 The Ca^{2+} channel may lose cation selectivity during hypoxia or ischemia and allow Na^{+} entry to occur; conversely, there is evidence that standard L-type channel antagonists

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have no effect on Na+-dependent Ca²⁺ loading. Little is known about the role of the Na⁺-K⁺-Cl⁻ cotransport in pathological conditions, but the Na⁺-H⁺ exchanger has been the subject of numerous investigations. Several investigators have shown that amiloride and its derivatives, Na⁺-H⁺ exchange blockers, reduce Na⁺ loading and Ca²⁺ loading and improve postischemic or posthypoxic recovery in whole hearts. Unfortunately, amiloride-type agents have many cellular effects beyond antagonizing Na⁺-H⁺ exchange, including Na⁺ channel blockade and inhibition of Na⁺-Ca²⁺ exchange. The role of the voltage-gated Na⁺ channel in hypoxic Na⁺ overload has been neglected previously. Na⁺ channels typically inactivate rapidly in depolarized tissues, and action potentials cease with the exhaustion of cellular ATP. Since the major increase in [Na⁺] in the hypoxic isolated myocyte occurs after depletion of ATP when the cell is inexcitable, it would seem unlikely that the Na⁺ channel would represent an influx pathway. Yet it should be considered that even in the absence of an action potential, Na⁺ may enter through Na⁺ channels through window currents in depolarized cells. Additionally, recent work suggests that compounds accumulating in hypoxia and ischemia may alter the activation and inactivation properties of the channel, which could result in Na⁺ influx. In this study, we test the hypothesis that Na⁺ loading occurs through the voltage-gated Na⁺ channel and specifically examine the contribution of the Na⁺ channel to hypoxic Na⁺ loading after full ATP depletion when the action potential is abolished. We used a number of Na⁺ channel blockers, including R 56865 (N-[1-[4-[4-(fluorophenoxyl) butyl]-4-piperidinyl]-N-methyl-2-benzothiazolamine; a gift of Janssen Pharmaceutica, Piscataway, NJ), a compound that has little effect on the Na⁺ current under usual circumstances but blocks the "nonactivating current" through the Na⁺ channel occurring under certain pathological conditions. R 56865 also blocks the rise in [Ca²⁺] seen during exposure to veratridine, which increases the open probability of the voltage-gated Na⁺ channel, inducing a Na⁺-dependent tetrodotoxin (TTX)-blockable Ca²⁺ overload. To see if R 56865 prevents the rise in [Na⁺], seen during exposure to hypoxia, Na⁺-dependent Ca²⁺ loading, and subsequent cellular injury at reoxygenation, we exposed single isolated adult rat myocytes to prolonged substrate-free hypoxia. Changes in [Na⁺] and [Ca²⁺] were indexed by the use of ion-sensitive fluorescent probes, Na⁺-binding benzofuran isothalate (SBFI, Na⁺ sensitive), and indo-1 (Ca²⁺ sensitive). Cell morphology was also observed. The effects of R 56865 on [Ca²⁺] during hypoxia were compared with those of the conventional Na⁺ channel blocker TTX, and its effects on cellular morphology at reoxygenation were compared with those of lidocaine, a conventional "local anesthetic"-type Na⁺ channel antagonist. (Lidocaine was substituted for TTX in these experiments because it is inexpensive and relatively nontoxic.) Similarly, we examined the effect of the amiloride derivative ethylisopropylamiloride (EIPA), an inhibitor of Na⁺-H⁺ exchange, on posthypoxic cellular injury. The potential effects of EIPA on Na⁺ entry through veratridine-modified Na⁺ channels and on Na⁺-Ca²⁺ exchange were explored. Finally, the influence of R 56865 on Na⁺-H⁺ exchange was tested.

Methods
Cardiac Myocyte Isolation
Single cardiac myocytes were isolated from the ventricle of 2- to 3-month-old rats according to a technique previously described. Studies conformed to the guiding principles of the American Physiological Society. Briefly, the heart was retrogradely perfused with a low-Ca²⁺, collagenase-containing, bicarbonate buffer (37°C, pH 7.2), and the perfusion was terminated when the tissue became soft (20 to 30 minutes). The left ventricle was then mechanically dissociated, and the myocytes were resuspended in a series of bicarbonate-based buffers with gradually increasing Ca²⁺ concentrations. Cells were finally suspended in a HEPES-based buffer with 1.0 mmol/L extracellular Ca²⁺ concentration. Cells were stored at 37°C. These buffers contained glucose as a metabolic substrate.

Exposure to Hypoxia
Single myocytes were studied in a specially developed chamber, the Laminar Counterflow Barrier Well, an open device mounted on the stage of an inverted microscope. A gas conditioning system previously described has been used to maintain a stable laminar layer of argon to prevent the back diffusion of atmospheric oxygen yielding a Po2 <0.02 mm Hg. Unless otherwise indicated, myocytes were superfused with glucose-free HEPES-based buffer (144 mmol/L NaCl, 5 mmol/L KCl, 1.2 mmol/L MgSO₄, 10 mmol/L HEPES, 1 mmol/L CaCl₂, pH 7.4, at 37°C) and 0.5 mmol/L octanoate as respiratory substrate. The buffer was equilibrated with either room air (normoxia) or 99.9995% argon (hypoxia). All cells were electrically field stimulated at 0.2 Hz. Stimulation at more rapid rates was avoided to minimize the potential for contamination with oxygen generated electrologically at the anode. The anode also served as the buffer outflow that remained downstream of the myocytes, removing oxygen with the effluent.

Measurement of Cell Fluorescence
The optical system used for cellular studies was a modified Zeiss IM-35 inverted microscope and has been described previously. Unless otherwise indicated, cells were loaded with either SBFI (to index [Na⁺]) or indo-1 (to index [Ca²⁺]) as previously described.

Myocytes were illuminated with a red light to permit visualization, and fluorescence was excited with a xenon arc. An interference filter selected a wavelength of 350±2.5 nm to excite indo-1. After excitation, the Ca²⁺-bound and -free forms of this indicator emit fluorescence, peaking at 410 and 490 nm, respectively. Fluorescent light was collected at a 5-millisecond resolution from the entire microscope field, which contained a single myocyte. Signals were acquired with two photomultiplier tubes. The ratio of light at these wavelengths provided an estimate of the ratio of bound-to-free Ca²⁺ forms of indo-1. The minimum ratio (R₉₀₈) obtained by measuring fluorescence from a Ca²⁺-free buffer to which indo-1 free acid was added was 0.2; the maximum ratio (R₆₉₃) obtained from the AM ester of indo-1 buffer was 2.8. When cardiac myocytes are loaded with indo-1 by means of the AM ester, approximately half of the fluorescence is nonspecific and is located in the mitochondria. This dye contributes a background fluorescence whose magnitude is uncertain in any given myocyte. This precludes precise quantitative calibration of the Indo-1 signal as a measure of cytosolic Ca²⁺, as slow changes in the fluorescence signal over several minutes may result from changes in [Ca²⁺] in the cytosolic or mitochondrial compartments.

SBFI is excited at two wavelengths (350 and 380 nm) and emitted light monitored at 490 nm. As Na⁺ binds to SBFI, fluorescence increases when the dye is excited at 350 nm. Binding of Na⁺ to the probe also results in a simultaneous, but less marked, decrease in emission intensity with excitation at 380 nm. This permits the use of the probe in a ratiometric
mode. The same epifluorescence microscope was used with fluorescence collected in the “490-nm” channel after manual switching of 350- and 380-nm excitation filters. The time involved in manual switching of the excitation filter limited data collection to 5-minute intervals.

Intracellular pH was measured with the pH-sensitive fluorescent probe SNARF-131 (carboxy-seminaphthorhodolfluor, Molecular Probes). This was accomplished by exposure of myocytes to the membrane-permeant acetoxy methyl ester of the dye.32 Compartmentation studies indicated that >98% of the dye was cytosolic.32 Excitation was at 530 nm, and fluorescence emission was collected simultaneously at 590±5 nm (acid form) and 640±5 nm using the same hardware used for indo-1 except for different dichroic mirrors and interference filters. An intracellular calibration was performed as described previously.32

Statistical Analysis

Data are presented as mean±SEM. Differences in fluorescence ratios are compared using a repeated-measures ANOVA with multiple comparisons made by Newman-Keuls testing. Differences in morphological data are compared using a Kruskal-Wallis one-way ANOVA on ranks with multiple comparisons made by Dunn's method. P<.05 values are considered statistically significant.

Experimental Protocols

In all hypoxia protocols, the cells were exposed to glucose-free hypoxia and reoxygenated 30 minutes after the development of rigor contracture. This time was selected based on our prior studies, which indicated that the likelihood of cellular recovery would be roughly 15% to 20%.2,5 Studies were performed in the presence of either control HEPES-based buffer or buffer containing the compound under study in that protocol, either 1 μmol/L R 56865, 10 μmol/L EIPA, 60 μmol/L TTX, or 25, 50, or 100 μmol/L lidocaine. Three groups of cells were studied in hypoxia. The first group consisted of cells loaded with SBFI to index [Na+]i. The second group consisted of cells loaded with indo-1 to monitor [Ca2+]i. In the third group, cellular morphology was observed in fields of non–dye-loaded cells exposed to hypoxia and reoxygenation. Single myocytes were studied in the first two groups and a field of cells (two to five cells) in the last group. In the last group, hypoxia was continued for a period after the development of rigor. All cells in a given field were reoxygenated following the same total hypoxic period, resulting in a distribution of post-rigor hypoxic times for the cells studied between 30 to 40 minutes (since the time to rigor development varied from cell to cell). At reoxygenation, cells either partially relengthened, retaining their rectangular shape, sarcomere pattern, and response to electrical stimulation (recovery), or hypercontracted to a rounded blebbled form with absent sarcomere pattern and disordered myofilibrar (hypercontracture).

The effects of prolonged hypoxia on contraction, morphology, and survival of individual cardiac cells are well described.3,13 Myocytes exposed to profound hypoxia continue to contract until they exhaust their stores of anaerobic substrates. Once ATP levels become profoundly depressed, there is failure of the action potential28 and cessation of contraction. This is followed within 3 to 5 minutes by rigor shortening to lengths roughly two thirds that of control. The development of rigor contracture has been demonstrated to result from profound intracellular ATP depletion that occurs as intracellular glycogen stores are depleted.24 Cells remain inexhaustible during this phase. Cells that are reoxygenated rapidly after undergoing rigor contracture invariably demonstrate partial relengthening and preservation of sarcomere pattern and resume contraction in response to electrical stimulation. Cells that are reoxygenated after longer periods following rigor contracture (>30 minutes) typically experience hypercontracture with further severe shortening and loss of sarcomere pattern and

![Image](http://circ.ahajournals.org/)

**Fig 1.** Plot of average change in Na+–binding benzofuran isophthalate (SBFI) fluorescence ratio from cells exposed to hypoxia and reoxygenation in the presence and absence of 1 μmol/L R 56865. C, Control (before hypoxia); H, 1 minute after the onset of hypoxia; H+10, 10 minutes into hypoxia; R, R+10, R+20, and R+30, 1, 10, 20, and 30 minutes after the onset of rigor, respectively; and O2+1 and O2+5, 1 and 5 minutes after reoxygenation, respectively. Six cells were exposed to hypoxia in the presence of control buffer (c) and six cells were exposed in the presence of 1 μmol/L R 56865 (e). Results are given as mean±SEM. *P<.05 vs control.

blebbing. Often, these cells lose sarcolemmal integrity and fail to exclude Trypan blue. We have found that there is no correlation between the length of time from hypoxia onset to ATP-depletion contracture and cell recovery at reoxygenation but that the probability of recovery at reoxygenation decreases with the time of hypoxia after rigor onset.

**Results**

**Effects of R 56865 on SBFI Fluorescence Ratio During Hypoxia**

Cells were loaded with SBFI and exposed to hypoxia and reoxygenation in the presence of control buffer (n=6) or 1 μmol/L R 56865 (n=6), a compound capable of blocking nonactivating components of the Na+ current. In both groups of cells there was a small initial rise in the SBFI ratio with the onset of hypoxia likely due to an autofluorescence shift.5 Autofluorescence typically increases by 50% to 60% within 2 to 3 minutes after the onset of hypoxia and remains unchanged even after the development of rigor contracture.5 Autofluorescence returns to baseline levels at reoxygenation. SBFI-loaded myocytes have fluorescence levels of threefold to fivefold that of autofluorescence, thus minimizing the effects of hypoxia-induced changes in autofluorescence on the SBFI ratio. After the onset of rigor contracture there was a rapid and progressive rise in SBFI ratio, consistent with a large increase in [Na+]i (Fig 1) in the control cells. The initiation of reoxygenation brought an immediate fall in SBFI ratio, consistent with a return to near baseline [Na+]i. The cells superfused with 1 μmol/L R 56865 demonstrated a significantly smaller rise in the SBFI ratio immediately before reoxygenation, consistent with a significant reduction in Na+ loading occurring during rigor (P<.05 at R+30). It should be noted that the ratios do not begin to diverge until the onset of rigor. This suggests that
Na\textsuperscript{+} loading is prevented by R 56865 principally after the depletion of cellular ATP. Since the action potential disappears before this point, the effects of R 56865 are not attributable to a reduction in Na\textsuperscript{+} influx during phase 0 depolarization.

**Effects of R 56865 and TTX on Indo-1 Fluorescence Ratio During Hypoxia**

Changes in [Ca\textsuperscript{2+}], during and after hypoxia were monitored in parallel experiments with cells loaded with indo-1 to test the hypothesis that a reduction in Na\textsuperscript{+} gain during hypoxia by R 56865 or the Na\textsuperscript{+} channel antagonist TTX would result in a reduction in Ca\textsuperscript{2+} gain. The rise in indo-1 fluorescence in the control cells (n=10) was significantly greater after the onset of rigor than in the cells superfused with 1 \mu mol/L R 56865 (n=5) or 60 \mu mol/L TTX (n=3), indicating that the Na\textsuperscript{+} channel blockers blunted the rise in [Ca\textsuperscript{2+}]. (see Fig 2). While there is no significant difference between the indo-1 ratios of the control (0.98±0.04) and the R 56865 cells (1.02±0.03) at the onset of rigor (the point of full ATP depletion), the values rapidly diverge, reaching a maximum difference at 1 minute after reoxygenation, when the ratios are 2.35±0.23 for control and 1.19±0.03 for the R 56865 group (P<.05). The indo-1 ratios of the cells superfused with TTX closely approximate those of the R 56865 group, suggesting a similar degree of reduction in hypoxia-induced Ca\textsuperscript{2+} overload.

**Effects of R 56865, Lidocaine, and EIPA on Cell Morphology After Reoxygenation**

To assess the significance of the reduction in Na\textsuperscript{+} and Ca\textsuperscript{2+} loading achieved during rigor by our pharmacologic interventions, we observed the effects on cell morphology at reoxygenation in the control cells (n=54) and cells exposed to 1 \mu mol/L R 56865 (n=32), 25 \mu mol/L lidocaine (n=16), 50 \mu mol/L lidocaine (n=12), and 100 \mu mol/L lidocaine (n=16). Lidocaine was used in this protocol rather than TTX because of its lower cost and toxicity. Cells were also studied in the presence of 10 \mu mol/L EIPA, a potent Na\textsuperscript{+}-H\textsuperscript{+} exchange blocker. All cells were exposed to hypoxia and reoxygenated 30 to 40 minutes after the onset of rigor. Cells superfused with R 56865 had a lower incidence of hypercontracture at reoxygenation (12% versus 83% for control, P<.05). EIPA and 100 \mu mol/L lidocaine were equally effective at preventing hypercontracture, with 12% of EIPA-exposed cells and 13% of cells exposed to 100 \mu mol/L lidocaine hypercontracting (P<.05 compared with control). Both 50 \mu mol/L and 25 \mu mol/L lidocaine were less effective in preventing hypercontracture (see Fig 3).

**Effects of R 56865, Lidocaine, and EIPA on Veratridine-Induced Hypercontracture and Ca\textsuperscript{2+} Loading**

The similarity between the effects of R 56865, lidocaine, and EIPA on hypercontracture following prolonged hypoxia suggested that the compounds shared a similar pharmacologic mechanism. Veratridine has been used to pharmacologically impair Na\textsuperscript{+} channel inactivation, resulting in cellular Na\textsuperscript{+} loading, subsequent Ca\textsuperscript{2+} loading mediated by Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange, and cellular hypercontracture.\textsuperscript{26} This effect is inhibited by R 56865.\textsuperscript{27} To determine whether EIPA and lidocaine also inhibit the veratridine-induced impairment of Na\textsuperscript{+} current inactivation, resting cells were superfused with either control HEPES-based buffer (1 mmol/L Ca\textsuperscript{2+}) or buffer with either R 56865, EIPA, or lidocaine for 5 minutes. They were then exposed to 75 \mu mol/L veratridine for 5 minutes while being stimulated at 0.2 Hz. This concentration of veratridine was chosen because it resulted in 100% hypercontracture in the control cells in this protocol and therefore represented a Na\textsuperscript{+} challenge likely as severe as that resulting from
exposure to hypoxia (as above). Veratridine induced hypercontracture in 20% of cells studied in the presence of 1 μmol/L R 56865 (P<.05 versus control, n=10) and in 10% in the presence of 10 μmol/L EIPA (P<.05 versus control, n=10); lidocaine showed a dose-dependent suppression of hypercontracture (see Fig 4).

To establish whether EIPA prevented hypercontracture by preventing veratridine-induced Na⁺ loading and consequent Ca²⁺ loading, we exposed indo-1-loaded cells (to index changes in [Ca²⁺]) to the above protocol in the presence or absence of 10 μmol/L EIPA (n=5 cells in each group). Attempts to observe a veratridine-induced increase in [Na⁺], using SBFI were unsuccessful (data not shown), probably because the rapidity of Na⁺-Ca²⁺ exchange (uninhibited by acidosis or low [ATP]) as well as extrusion of Na⁺ by the Na⁺-K⁺, ATPase prevented a sufficient increase in [Na⁺] to be detected with this method, which is relatively insensitive to small fluctuations in [Na⁺]. Veratridine induced a rapid increase in [Ca²⁺] in the cells studied in the absence of EIPA, the indo-1 ratio rising from a baseline of 0.94±0.02 to 1.90±0.14, whereas there was little change in the ratio in the cells studied in the presence of EIPA (0.90±0.03 to 0.94±0.02; P<.05 versus control; Fig 5). This experiment does not exclude the possibility that EIPA acted by inhibiting Na⁺-Ca²⁺ exchange and thus preventing Ca²⁺ gain. Cells were therefore loaded with indo-1 and equilibrated for 5 minutes in HEPES buffer containing 137 mmol/L Na⁺ and 5 mmol/L Ca²⁺ and stimulated at 2 Hz. Na⁺-free buffer (replaced by choline chloride) in the presence of 5 mmol/L Ca²⁺ (with or without 10 μmol/L EIPA) was then substituted for 15 minutes to induce Ca²⁺ loading via Na⁺-Ca²⁺ exchange through reversal of the usual intracellular-extracellular Na⁺ gradient (n=8). Ca²⁺ loading occurred in both groups with indo-1 ratios changing from a baseline of 0.74±0.01 in control cells to 2.04±0.32 after 20 minutes and 0.80±0.02 to 2.23±0.44 in the EIPA group (P=NS), demonstrating no significant inhibition of Na⁺-Ca²⁺ exchange by 10 μmol/L EIPA.

Fig 3. Bar graph of percent of cells hypercontracting at reoxygenation when studied in buffers containing 1 μmol/L R 56865 (n=32), 10 μmol/L ethylisopropylamiloride (EIPA) (n=16), 100 μmol/L lidocaine (n=16), 50 μmol/L lidocaine (n=12), 25 μmol/L lidocaine (n=16), and control (n=54). *P<.05 vs control.

Fig 4. Bar graph of percent of cells hypercontracting in the presence of 75 μmol/L veratridine and buffer containing the following compounds: 1 μmol/L R 56865 (n=10), 10 μmol/L ethylisopropylamiloride (EIPA) (n=10), 100 μmol/L lidocaine (n=8), 25 μmol/L lidocaine (n=6), and control (n=20). *P<.05 vs control.

Fig 5. Plot of change in indo-1 fluorescence ratio in cells exposed to 75 μmol/L veratridine in the presence of control buffer (n=5, ○) and 10 μmol/L ethylisopropylamiloride (EIPA) (n=5, ●). *P<.05 vs control.
Effects of R 56865 on Na⁺-H⁺ Exchange

Having established that the protective effects of R 56865 against hypoxic injury were shared with EIPA and that EIPA inhibited Na⁺ entry through the veratridine-modified Na⁺ channel, it remained necessary to confirm that the protective effect of R 56865 was not the result of Na⁺-H⁺ exchange inhibition. To assess the effect of R 56865 on Na⁺-H⁺ exchange, cells (n = 3) were loaded with the pH-sensitive fluorescent probe SNARF-1 and exposed to a series of buffers intended to alter the cytosolic pH of the cell. Each cell was first superfused with a control HEPES-based buffer. Ammonium chloride was added to the buffer and then washed out after 5 minutes. By this method a cellular alkalosis is induced during exposure and an acidosis at washout. This acidosis is ordinarily rapidly corrected by extrusion of protons through the Na⁺-H⁺ exchanger.35 In this experiment, SNARF-1 fluorescence was measured continuously while three sequential NH₄Cl pulses were administered: one with control buffer only, one with 1 μmol/L R 56865 included, and one pulse with 10 μmol/L EIPA, to compare the effects of the agents on the Na⁺-H⁺ exchange-dependent restoration of pH. In Fig 6, a tracing from an individual myocyte, one can see a rise in the pH of the cell superfused with control buffer followed by a rapid fall in pH after the completion of the NH₄Cl pulse and then a more gradual return to baseline pH over the 17-minute observation period. The next tracing shows the effect of 1 μmol/L R 56865. Note that there is no qualitative difference in the restoration of pH following the acidic phase. When the cell was exposed to EIPA, a marked inhibition in the restoration of baseline pH is evident, consistent with an inhibition of the Na⁺-H⁺ exchange-dependent restoration of pH. These results suggest that 1 μmol/L R 56865 has no effect on Na⁺-H⁺ exchange. Results were qualitatively similar in the three cells studied.

Discussion

This study uses isolated adult mammalian cardiac myocytes loaded with ion-sensitive fluorescent probes to assess the contribution of the voltage-gated Na⁺ channel to hypoxic Na⁺ loading. Direct measurements of [Na⁺]ᵢ and [Ca²⁺]ᵢ are made and correlated with changes in cell contraction and morphology. We demonstrate that (1) the hypoxic rise in [Na⁺]ᵢ occurs chiefly after full ATP depletion when cells are inexcitable; (2) pharmacological blockers of the Na⁺ channel, including R 56865, which acts predominantly on noninactivating components of the Na⁺ current, reduce hypoxic Na⁺ loading, associated Ca²⁺ loading, and subsequent reoxygenation-induced hypercontracture; and (3) EIPA, a Na⁺-H⁺ exchange blocker, reduces reoxygenation-induced hypercontracture as well as hypercontracture induced by veratridine, which causes Na⁺ loading by impairing Na⁺ channel inactivation. These findings provide new evidence that Na⁺ channels participate in hypoxic Na⁺ loading even in the absence of an action potential. They further suggest that the protective effect of amiloride derivatives in hypoxic and ischemic models may be mediated in part by their effects on Na⁺ channels.

The Na⁺ Channel

Investigators have assumed that the Na⁺ channel is closed during hypoxia because of prolonged membrane depolarization,36 but evidence is accumulating that the electrophysiological function of ion channels can be modified by acute or chronic injury. While Na⁺ channels
normally inactivate rapidly after depolarization-induced activation, certain phenomena can induce spontaneous channel openings and prolong inactivation, therefore increasing the open probability of the channel, resulting in a "noninactivating" Na⁺ current. Phospholipase A₂ is stimulated in ischemia and hypoxia, resulting in a rapid accumulation of lysophosphatidylcholine (LPC).¹⁹ a compound that partially depolarizes the resting membrane potential of cat ventricular cells.²⁰ LPC induces a noninactivating Na⁺ current²¹ and sustained channel bursting at membrane potentials positive to -70 mV. Undrovinas et al²² detected a shift in the pattern of activation of all rat and rabbit cells exposed to LPC resulting in channel opening at potentials ≥-80 mV. Furthermore, in 5% to 14% of the cells, prolonged bursting was seen at potentials negative to -70 mV with supranormal conductances, suggesting that a significant noninactivating current can occur in ischemic tissue that is not depolarized.²²

Other ischemic products, including long-chain acylcarnitines, are likely to play an important role in ischemic-hypoxic Na⁺ loading by their effects on Na⁺ channels. Long-chain acylcarnitines are amphiphilic molecules like LPC that accumulate when β-oxidation is inhibited in hypoxia. Ordinarily, fatty acids are thioesterified to coenzyme A and then transamified to carnitine to produce acylcarnitine, which can cross the inner mitochondrial membrane and be metabolized.³⁷ The metabolism of fatty acids yields NADH and FADH₂, which then enter the electron transport chain for the production of ATP. Ischemia and hypoxia inhibit electron transport, resulting in an increase in NADH and FADH₂, which through negative feedback inhibit β-oxidation and result in long-chain acylcarnitine accumulation. Recent studies bear considerably on the present study. Wu and Corr²³ showed, in single adult rabbit cardiac myocytes, that exposure to palmitoyl carnitine induced spontaneous and slowly inactivating Na⁺ currents at potentials as negative as -80 mV. These currents were reversible on washout of palmitoyl carnitine and were blocked by TTX. Another study showed that long-chain acylcarnitine content increased ninefold and sarcocellular accumulation increased 100-fold in isolated adult canine myocytes exposed to 10 minutes of hypoxia.²⁴ Thus, long-chain acylcarnitines accumulate in hypoxic isolated myocytes and would likely lead to Na⁺ influx through Na⁺ channels even when cells are inexcitable. The currents have not been examined in hypoxic myocytes, likely due to the technical challenges presented.

In a study by Mejia-Alvarez and Marban,²⁶ a reduction in Iₙa was seen in guinea pig ventricular myocytes exposed to either iodoacetate or 2,4-dinitrophenol. No steady-state current was observed. Unlike hypoxia, however, there is no evidence that long-chain acylcarnitines would accumulate under these circumstances (ie, in the absence of inhibition of electron transport).

**Effect of Na⁺ Channel Antagonists on Hypoxic Na⁺ and Ca²⁺ Overload**

In this study we found that R 56865, a known blocker of the Na⁺-driven Ca²⁺ overload induced by veratridine, prevented Na⁺ loading in hypoxia, suggesting that the Na⁺ channel plays an important role in this pathological state. SBFII, a fluorescent Na⁺ indicator, was used to index changes in [Na⁺], because it does not require micropunc-

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ture seen in cells at washout of "ischemic buffer." A 5-minute exposure to cyanide, 2-deoxyglucose, and lactate at pH 6.5 was followed by a transient increase in systolic [Ca^{2+}], and the development of hypercontracture. That TTX eliminated hypercontracture would suggest that it also blunted the increase in [Ca^{2+}] in that model. The effect of TTX on [Na^+] and [Ca^{2+}] in hypoxia was not examined.

**Effect of EIPA on Veratridine-induced Cellular Hypercontracture**

EIPA has been previously shown to effectively block the rise in [Na^+] seen in ischemia and hypoxia. In our study, its effects on reoxygenation hypercontracture were remarkably similar to those of R 56865 and lidocaine. Using 10 μmol/L EIPA, we found an inhibition of veratridine-induced hypercontracture equal to 1 μmol/L R 56865 and 100 μmol/L lidocaine and a significant reduction of Ca^{2+} gain consistent with an effect on Na^+ channels. The reported effectiveness of amiloride and related compounds in preventing hypoxic Na^+ loading may be in part due to inhibition of Na^+ currents as well as or rather than effects on Na^-H^+ exchange. Indeed, Imai et al. found that while 10 μmol/L EIPA had no tendency to increase intracellular acidification during low-flow ischemia, it achieved a significant bradycardic effect at 1 μmol/L and complete inhibition of contraction at 100 μmol/L concentration, which may be due to direct Na^+ channel antagonism. Tytgat et al. found a partial inhibition of INa in guinea pig myocytes by 1 mmol/L amiloride, and we have found that 100 μmol/L EIPA eliminates contraction in 90% of electrically stimulated rat myocytes (unpublished observations).

**Study Limitations and Conclusions**

Previous attempts to elucidate the route of Na^+ gain during ischemia or hypoxia have concentrated on Na^-H^+ exchange. In this study, we have shown that Na^+ channel blockers reduce hypoxic Na^+ and Ca^{2+} loading. Furthermore, R 56865 does not block Na^-H^+ exchange yet reduces Na^+ and Ca^{2+} loading during hypoxic rigor and reduces reoxygenation hypercontracture. Since any intervention that reduces Na^+ entry may blunt Na^-dependent Ca^{2+} loading, our study does not necessarily demonstrate that the Na^+ channel serves as the major route of hypoxic or ischemic Na^+ loading. The study does, however, suggest that the channel serves as a route for hypoxic Na^+ influx when myocytes are fully energy depleted and thus inexorable. That the channel participates under these circumstances has not been previously demonstrated. Amiloride derivatives, which have previously been found effective in preventing ischemic Na^+ gain, appear to also inhibit Na^-H^+ entry through Na^+ channels, and thus this action may account for their effectiveness in ischemic and hypoxic models. An important role for Na^-H^+ exchange in ischemia cannot be excluded by this study, of course. It may be the case that both Na^-H^+ exchange and Na^+ channel currents contribute significantly to Na^+ loading in ischemia, and further work will be necessary to dissect the relative importance of these pathways; specific inhibitors of Na^-H^+ exchange need to be developed, and confirmatory electrophysiological measurements of INa during hypoxia are required.

The major limitation of the present study is the lack of electrophysiological evidence of nonactivating Na^+ currents. The isolated cardiac myocyte is, however, a powerful model that should permit future measurements of Na^+ currents in hypoxia. The fluorescence measurements provide important data relating to the ionic derangements occurring in hypoxia. While the single myocyte model does not closely mimic ischemia and studies have been limited by the use of stimulation rates that are well below those in the physiological range, the model does allow precise control of the extracellular environment with the virtual elimination of diffusion barriers. It permits an assessment of the heterogeneity of cellular response and the opportunity to precisely time changes in intracellular ions with changes in contractility and morphology. The dependency of hypoxic Ca^{2+} loading on Na^+ loading and Na^+-Ca^{2+} exchange in this model is highly relevant as a similar reliance on the exchanger is seen in ischemia. Future studies focusing on the role of the Na^+ channel in hypoxic and ischemic Na^+ loading may help to define the importance of this pathway in Na^+ loading and subsequent Ca^{2+} loading and cell death.

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