Sodium/calcium exchange modulates intracellular calcium overload during posthypoxic reoxygenation in mammalian working myocardium. Evidence from aequorin-loaded ferret ventricular muscles.

Y Kihara, … , M Inoko, J P Morgan

Research Article

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Sodium/Calcium Exchange Modulates Intracellular Calcium Overload during Posthypoxic Reoxygenation in Mammalian Working Myocardium

Evidence from Aequorin-loaded Ferret Ventricular Muscles

Yasuki Kihara, Shigetake Sasayama, Moriaki Inoko, and James P. Morgan

The Second Department of Internal Medicine, Toyama Medical and Pharmaceutical University, School of Medicine, Toyama 930, Japan; and Harvard-Thorndike Laboratory, Beth Israel Hospital and Harvard Medical School, Boston, Massachusetts 02215

Abstract

We tested the hypothesis that the intracellular Ca^{2+} overload of ventricular myocardium during the period of posthypoxic reoxygenation is mediated by transsarcolemmal Ca^{2+} influx via Na^+/Ca^{2+} exchange. In aequorin-loaded, ferret right ventricular papillary muscles, blockers of the sarcolemmal and the sarcoplasmic reticulum Ca^{2+} channels, slowed the Ca_i^{2+} transient, producing a convex ascent during membrane depolarization, followed by a concave descent during repolarization. The magnitude of the Ca_i^{2+} transient was affected by changes in the membrane potential, Na_i^+, Na_o^+, and Ca_o^{2+}, and was blocked by Ni^{2+}, or dichlorbenzamil. The calculated Na^+/Ca^{2+} exchange current was in the reverse mode (Ca^{2+} influx) during the ascending phase of the Ca_i^{2+} transient, and was abruptly switched to the forward mode (Ca^{2+} efflux) at repolarization, matching the time course of the Ca_i^{2+} transient. During hypoxic superfusion, the Ca_i^{2+} transient was abbreviated, which was associated with a shorter action potential duration. In contrast, immediately after reoxygenation, the Ca_i^{2+} transient increased to a level greater than that of the control, even though the action potential remained abbreviated. This is the first demonstration on a beat-to-beat basis that, during reoxygenation, Ca^{2+} influx via Na^+/Ca^{2+} exchange is augmented and transports a significant amount of Ca^{2+} into the ventricular myocardial cell. The activation of the exchanger at the time of reoxygenation appears to be mediated by Na_i^+ accumulation, which occurs during hypoxia. (*J. Clin. Invest.* 1994. 93:1275–1284.) Key words: aequorin • calcium • ferret • Na^+/Ca^{2+} exchange • reperfusion

Introduction

Calcium might play a crucial role in the myocardial cell injury that occurs during postischemic reperfusion. Previous work has shown that intracellular calcium (Ca_i^{2+}) of the myocardial cell rapidly increased during the early phase of global ischemia (1–4). The Ca_i^{2+} subsequently decreased during reperfusion if the duration of ischemia was limited to several minutes (1–4).

Dr. Kihara's and Dr. Sasayama's current address is Third Division, Department of Internal Medicine, Faculty of Medicine, Kyoto University, Kyoto 606, Japan.

Address correspondence to Dr. James P. Morgan, Cardiovascular Division, Beth Israel Hospital, 330 Brookline Avenue, Boston, MA 02215.

By contrast, when the ischemic period lasted longer than 20 min, the elevated level of Ca_i^{2+} further increased during the reperfusion period (5, 6), and was associated with delayed afterdepolarizations and arrhythmogenicity (5, 7). These findings suggested that (a) the cellular mechanisms responsible for Ca_i^{2+} accumulation during reperfusion are qualitatively different from those in acute ischemia and, (b) the duration of the preceding ischemia is a crucial factor which determines the level of Ca_i^{2+} loading when the tissue is reperfused. The Na^+/Ca^{2+} exchange mechanism may be responsible for the Ca_i^{2+} loading during reperfusion (8, 9) or reoxygenation (10, 11). However, there is no concrete evidence to support this hypothesis because of the difficulty of demonstrating the functional state of this exchanger in actively contracting preparations. Using aequorin-loaded, isolated myocardium from the ferret ventricle, we demonstrated that the Ca_i^{2+} transient, under experimental conditions where it is governed primarily by transsarcolemmal Ca^{2+} influx via Na^+/Ca^{2+} exchange, increased when the preparation was reoxygenated after hypoxia. This increase appears to be caused by time-dependent Na_i^+ accumulation during hypoxia. Our results suggest that reperfusion injury could be minimized by interventions that prevent intracellular Na^+ (Na_i^+) loading during ischemia.

Methods

Muscle preparation. Right ventricular papillary muscles (< 0.7 mm in external diameter) were isolated from 6–8-wk-old male ferrets and mounted as described previously (12). In the tissue bath, each muscle was electrically stimulated to contract isometrically at 0.20 Hz; 30.0±0.2°C. The superfusate was a bicarbonate-buffered physiological salt solution with the following composition (in mM): 118 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 1.2 $MgCl_2$, 1.0 $CaCl_2$, 23 $NaHCO_3$, and 5.5 dextrose. The solution was continuously bubbled with a gaseous mixture of 95% O_2/5% CO_2, and the pH was adjusted to 7.40±0.02. During the run-in period, each muscle was gradually stretched to a length that produced the maximal tension development. To record the Ca_i^{2+} transients, a bioluminescent protein, aequorin, was loaded into surface myocytes by means of the modified chemical procedure (macroinjection [1, 13]) while the electrical stimulation was temporarily terminated and the superfusate replaced with a nominally Ca^{2+}-free solution composed of (in mM): 118 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 5.0 $MgCl_2$, 23 $NaHCO_3$, 20 dextrose, and 5.0 pyruvate; pH 7.30±0.02 at 20°C (1). Subsequently, Ca^{2+} concentrations and temperature were increased in a stepwise fashion, and finally, the superfusate was replaced with the control solution. As reported previously, the loading procedure did not affect the pattern of tension traces either in amplitude or in time course in these muscles (1, 5).

Signal acquisition and analysis. The tissue bath was shielded from external light, and the aequorin luminescence was detected with a photomultiplier tube (R464, Hamamatsu Photonics, Hamamatsu, Japan). The membrane potential of the surface myocyte was recorded with microelectrodes. The glass micropipettes used for electrical recording were filled with 3 M KCl and had tip resistances of 15–25 MΩ, as

measured in the solution described above. During experiments, the position of the pipette was remotely controlled from outside the light shield by a three-dimensional aqua-purificate micromanipulator (WR-88, Narishige Scientifics, Tokyo, Japan) while the action potential was continuously monitored on an oscilloscope (COM7061A, Kikusui Co., Yokohama, Japan). The aequorin light signal (anode current of the photomultiplier tube), isometric tension (measured by a BG-10 load cell, Kulite Semiconductor Products, Leonia, NJ), and the action potential were simultaneously recorded onto strip-chart paper and magnetic tape. At the end of the experiment, all aequorin activity remaining in the muscle was discharged by 4% Triton X-100 (Sigma Chemical Co., St. Louis, MO) perfusion in the presence of 20 mM $CaCl_2$. A rate constant for aequorin consumption of 2.11/s was used for this integration (1). The normalized luminescence was referred to a calibration equation at 30°C (1) to estimate the absolute Ca_i^{2+}. For presentation and subsequent analyses, the signal-to-noise ratio of aequorin signals was improved by a stimulus-triggered average process for 16–64 serial events with an off-line computer (7T18A, NEC-Sanei, Tokyo, Japan).

Isolation of Na^+/Ca^{2+} exchanger-operated Ca_i^{2+} transients. After control records were obtained, the following pharmacological agents were added to the superfusate in a cumulative manner: 1×10^{-5} M DPI 201-106 (Sandoz Ltd., Basel, Switzerland), 1×10^{-5} M ryanodine (Calbiochem Corp., La Jolla, CA), 3×10^{-6} M verapamil (Eisai Co., Tokyo), 1×10^{-5} M acetylstrophanthidin (Sigma Chemical Co.), and the Ca^{2+} concentration (Ca_o^{2+}) increased to 4 mM. DPI 201-106 is a tetrodotoxin (TTX)[1]-sensitive Na^+ channel agonist that blocks the channel inactivation process and prolongs action potentials of the mammalian myocyte (12, 14, 15). With this combination of drugs, Ca^{2+} release from the sarcoplasmic reticulum (SR), which predominantly regulates the Ca_i^{2+} transient in normal ferret myocardium, and the transsarcolemmal Ca^{2+} influx via the Ca^{2+} channels are inhibited (16, 17). In contrast, with increases in Na^+ influx, Ca_o^{2+}, and action potential duration, the Ca^{2+} influx through the reverse mode of the Na^+/Ca^{2+} exchanger should be enhanced (16, 17). Under these pharmacological interventions, the preparation continued to contract with punctate electrical stimulation at the base (square wave pulse with 1 ms duration; amplitude 10% above the threshold). In the presence of TTX (Sigma Chemical Co.), $NiCl_2 \cdot 6H_2O$ (Wako Pure Chemical Industries, Ltd., Osaka, Japan), or dichlorobenzamil (DCB; Merck Sharpe & Dohme, Rahway, NJ), the electrical stimulation was maintained by increasing the amplitude of the pulses by 100%.

Hypoxia and reoxygenation. Twenty minutes before induction of hypoxia, 5.5 mM dextrose in the solution was replaced with equimolar sucrose (18). Then, hypoxia was induced by an abrupt change of solutions from one bubbled with a gaseous mixture of 95% O_2/5% CO_2 to one bubbled with 95% N_2/5% CO_2 (18, 19). No other conditions were altered, including the temperature and the pH, during this exchange. After 20 min of hypoxia, reoxygenation was accomplished by switching these solutions back to the control.

Statistics. Data are presented as mean±SD. Comparisons were made between parameters under control, hypoxia, and reoxygenation by Student's t test or by analysis of variance for repeated measures. Differences are considered significant at $P < 0.05$.

Results

Evidence for Na^+/Ca^{2+} exchange-dependent Ca_i^{2+} transient and contraction. The combination of pharmacological interventions substantially altered the pattern of the membrane potential, Ca_i^{2+} transient, and isometric tension traces, as shown in Fig. 1 (*top*). The rapid, spike-like rise in Ca_i^{2+} observed under control conditions (*left panel*) disappeared. Instead, after the stimulus, Ca_i^{2+} slowly increased with an ascending

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1. *Abbreviations used in this paper:* DCB, dichlorobenzamil; SR, sarcomplasmic reticulum; TTX, tetrodotoxin.

convex pattern, which then abruptly began to descend with a downward convex pattern (*right panel*). The timing of the shift between the ascending and descending phases was coincidental with the initiation of membrane repolarization. The tension trace followed the Ca_i^{2+} transient with a delay of 60–80 ms. The effects of cumulative drug applications on these signals were shown in Fig. 1 (*bottom*). With 1×10^{-5} M DPI 201-106, the tension development was increased with its time course prolonged. The Ca_i^{2+} transient showed a modest increase while maintaining its spikelike pattern (12). The action potential was also prolonged (14, 15). The addition of 1×10^{-5} M ryanodine significantly depressed the peak levels of tension and Ca_i^{2+}. In addition, it delayed their time course. The Ca_i^{2+} transient lost its spike-like pattern (20, 21), and the tension and membrane potential showed marked prolongations. With 3×10^{-6} M verapamil, the tension and Ca_i^{2+} transient were further depressed (12, 22). In five of eight preparations, Ca_i^{2+} transients were no longer detected during depolarization. An additional increase in the verapamil concentration to 1×10^{-5} M did not affect these signals ($n = 2$ [17]). Subsequently, however, by addition of 1×10^{-5} M acetylstrophanthidin and 4 mM Ca_o^{2+}, the slow biphasic Ca_i^{2+} transient reappeared, and was associated with increased tension development. The time courses of membrane potential and tension were unchanged when compared to those with ryanodine and verapamil. The final pattern of signals produced by these cumulative drug additions was stable for over 20 min, unless the level of Ca_o^{2+} was increased to higher concentrations (> 8 mM). In this case, relaxation of tension became progressively delayed.

To characterize the Ca_i^{2+} transient and the corresponding action potential and tension trace, the preparation was challenged with changes in Ca_o^{2+}, extracellular Na^+ concentration (Na_o^+), supplementation with TTX, Ni^{2+} (17, 23), or DCB (24, 25). In Fig. 2 (*left*), an increase in Ca_o^{2+} from 4 to 8 mM augmented the amplitude of the Ca_i^{2+} transient, while the action potential did not significantly change (*top row*). In contrast, TTX at a concentration of 1×10^{-6} M (17) decreased the amplitude of the Ca_i^{2+} transient, which was accompanied by an abbreviated time course (*bottom, left*). This condition was also associated with a shorter duration of the action potential, and the timing of repolarization was coincidental with the end of the ascending phase of Ca_i^{2+} as observed in Fig. 1. The effects of Ca_o^{2+} or TTX reached the steady-state within 3 min. The data were consistent among seven preparations and suggested that the Ca_i^{2+} transient depends on Ca_o^{2+}, and the shape of action potential. Of note, TTX, which blocks the DPI 201-106-activated Na^+ influx (12, 15), not only shortened the Ca_i^{2+} transient, but also decreased its amplitude during membrane depolarization. This indicated that the Ca_i^{2+} transient also depends on the level of intracellular Na^+ (Na_i^+) even under the condition that the SR function was pharmacologically deleted (26). Ni^{2+} (5 mM), which is a divalent cation that blocks the Na^+/Ca^{2+} exchanger (17, 23), inhibited the remaining part of the Ca_i^{2+} transient in the presence of 1×10^{-6} M TTX (*bottom, right*). The suppression of the entire time course of Ca_i^{2+} transients (~ 1 s) by Ni^{2+} could not be attributed solely to its effect on the T-type Ca^{2+} channel because duration of this channel activation is at most 100 ms in various ventricular cells (27–30). In Fig. 2 (*right*), the Na^+ concentration of the solution was reduced by replacing 100 mM NaCl with the equimolar LiCl (Sigma Chemical Co.) With Li^+, the Ca_i^{2+} transients were rapidly depressed to below the minimally detectable level in
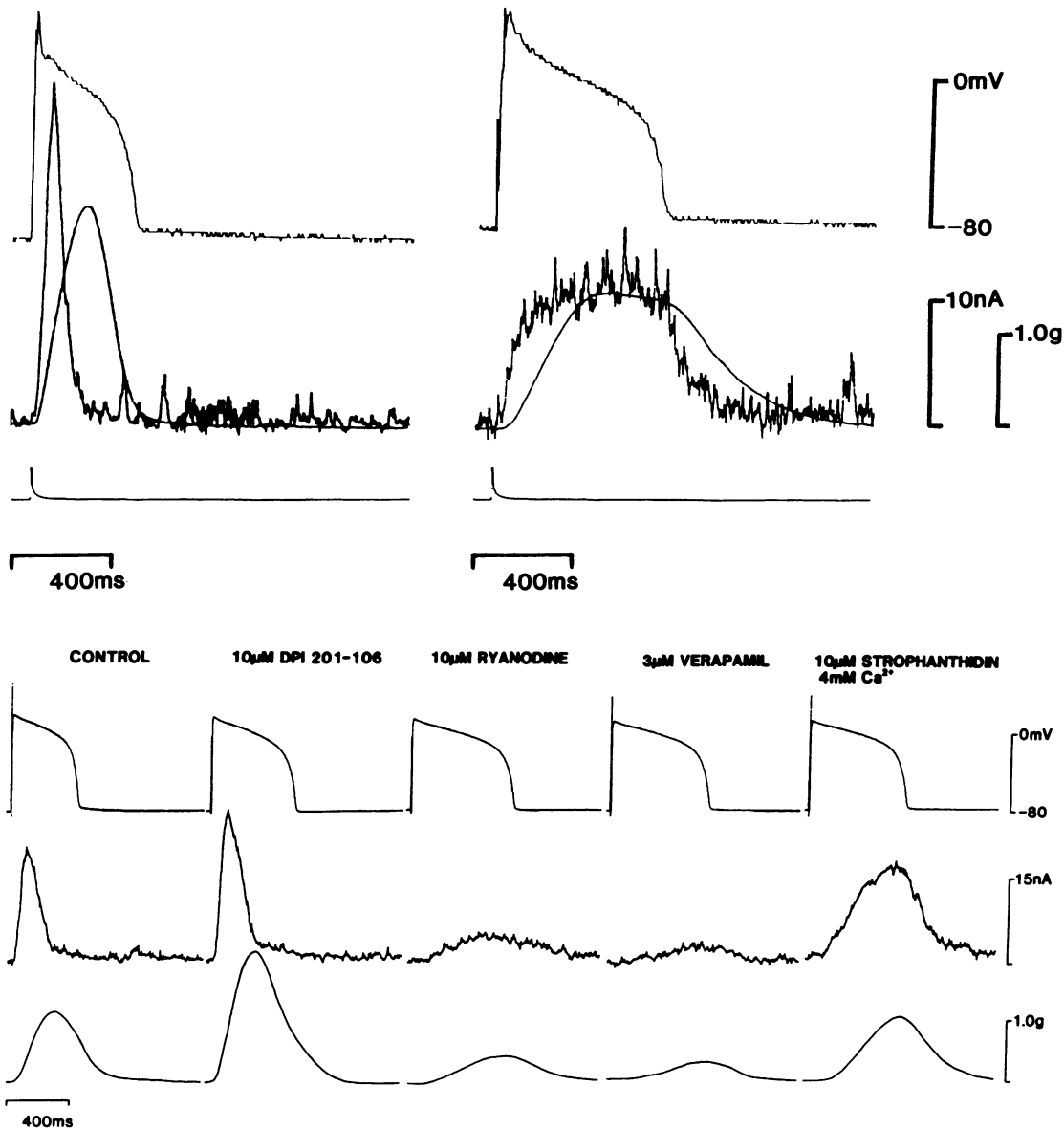
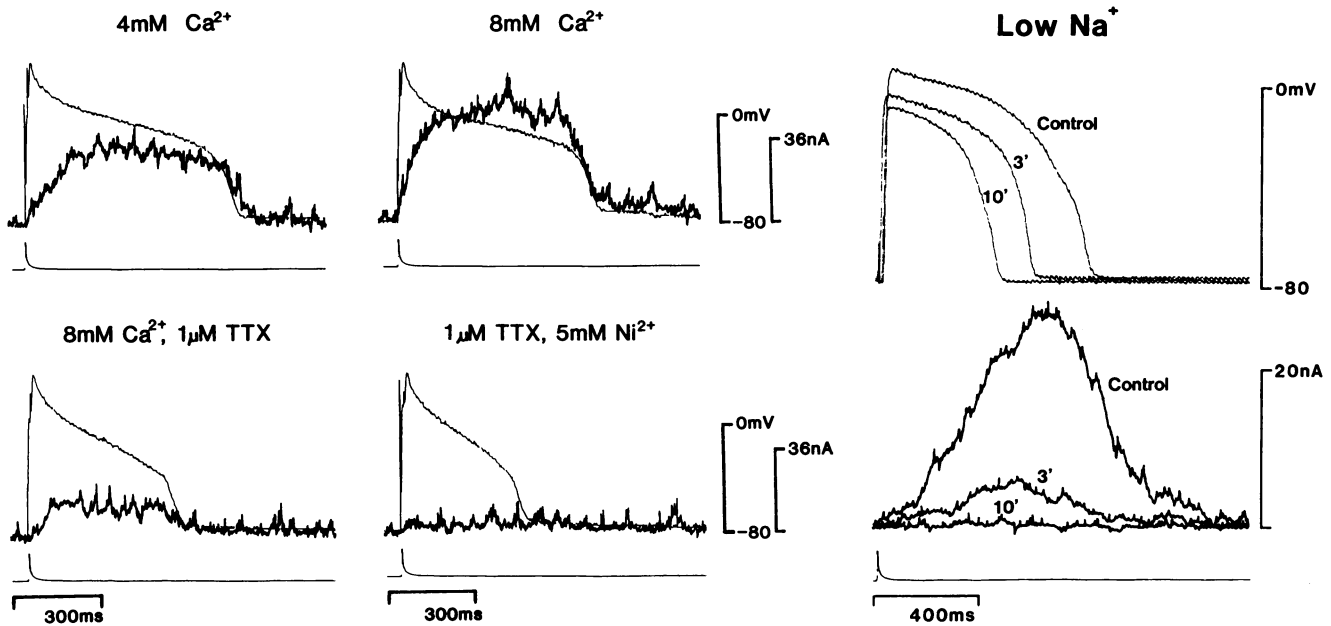*Figure 1. Top panel:* Simultaneous recordings of action potential (*top*), Ca_i^{2+} transient (notched signal, *middle*), tension trace (smooth signal, *middle*), and the stimulus artifact (*bottom*), before (*left*) and after (*right*) exposure to a combination of 1×10^{-5} M DPI 201-106, 1×10^{-5} M ryanodine, 3×10^{-6} M verapamil, 1×10^{-5} M acetylstrophanthidin, and 4 mM Ca^{2+}. The Ca_i^{2+} signals were averaged for 16 cycles. The calibration bar (10 nA) corresponds to 0.82 μM of Ca^{2+} on the left and to 0.84 μM on the right, respectively. 1 mM Ca_o^{2+} in the control (*right*); 30°C, 0.2 Hz. *Bottom panel:* Modulations of the signals during each step of the cumulative drug additions. Drugs were added in the order from the left corner (control) to the right panels. In each stage, records were obtained 5 min after the drug application. The Ca_i^{2+} signals were averaged for eight cycles.

our system; therefore, the Ca_i^{2+} transients were also affected by the level of Na_o^+ in a manner that Li^+ could not replace the role of Na_o^+ (9). We further tested the effects of DCB, an amiloride derivative known to block the Na^+/Ca^{2+} exchange site relatively selectively (24, 25). After verifying in vitro that DCB does not affect aequorin luminescence, we found that DCB (5 mM) also blocked the Ca_i^{2+} transient in a similar manner as observed during the Ni^{2+} protocol (data not shown). Taken together, results from all these pharmacological interventions on this preparation supported the hypothesis that the observed Ca_i^{2+} transient and contraction were primarily mediated by the transsarcolemmal Ca^{2+} influx through Na^+/Ca^{2+} exchange during membrane depolarization.

Alteration of Ca_i^{2+}, tension, and action potential during hypoxia and reoxygenation. The muscle was subjected to acute hypoxia for 20 min, and then returned to control conditions for reoxygenation. The composition of the superfusate other than the gaseous mixtures was unchanged. During the initial half of the hypoxic period, the Ca_i^{2+} transient, action potentials, and tension traces did not change detectably. However, during the second half of the hypoxic period, the action potential gradually shortened, which was accompanied by an abbreviation of the Ca_i^{2+} transient and a decrease in peak tension development (19). The resting levels of Ca_i^{2+} and tension started to increase during the last quarter of the hypoxic period (Fig. 3). The changes in these measurements at the end of a 20-min hypoxic

Figure 2. Left panel: In each panel, simultaneous records of action potential, Ca^{2+} transient, and the stimulus artifact are shown in a muscle treated with a combination of DPI 201-106, ryanodine, and verapamil as described in Fig. 1. The Ca^{2+} concentration of the solution was increased from 4 mM (*top, left*) to 8 mM (*top, right*). Then, 1 μM TTX (*bottom, left*) and 5 mM Ni^{2+} (*bottom, right*) were cumulatively added to the solution. Data were obtained from the same muscle. The Ca_i^{2+} signals were averaged for 32 cycles. The calibration bar (36 nA) corresponds to 0.60 μM of Ca^{2+} in all panels. 30°C, 0.2 Hz. **Right panel:** Effects of lowering Na_o^+ by 100 mM Li^+ substitution on action potentials (*top*) and the Ca_i^{2+} transients (*middle*) are shown. Signals were obtained during the process (3 min; *3'*) and at the steady state (10 min; *10'*) after the Li^+ substitution. The Ca_i^{2+} transients were averaged for 16 cycles. The calibration bar (20 nA) corresponds to 0.52 μM of Ca^{2+}. 30°C, 0.2 Hz.

period are summarized in Table I. During reoxygenation, the data acquisition was interrupted for 1 min during switching the superfusates. When the measurements were re-started, we consistently observed that the Ca_i^{2+} signals increased in peak amplitude and exceeded the control level before hypoxia (Fig. 3). Thus, there was a substantial increase in the peak Ca_i^{2+} after reoxygenation. Despite this increase in Ca_i^{2+}, the biphasic pattern of the Ca_i^{2+} signals was unchanged. That is, they consisted of ascending convex and descending concave curves and, although the time course was abbreviated by the shorter action potential, the peak was associated with the timing of repolarization. Data from 3 min after reoxygenation are summarized in Table I. The shortened action potential duration was again prolonged, but did not recover to the control value. The depressed tension trace did not show a corresponding increase or recovery in response to the increased Ca_i^{2+} level, which is consistent with previous reports in the reperfused or reoxygenated myocardium (19, 21, 31, 32). The increase in Ca_i^{2+} gradually declined, but remained elevated at 5 min of reoxygenation when the measurement was terminated for the Triton X-100 perfusion. In order to further test the hypothesis that this increase in Ca_i^{2+} with reoxygenation was mediated by mechanisms other than Na^+/Ca^{2+} exchange (such as nonspecific Ca^{2+} leak, Ca^{2+} pump inhibition, or release of Ca^{2+} from the internal buffers), the hypoxia/reoxygenation protocol was repeated in the presence of 5 mM Ni^{2+} ($n = 3$). When Ni^{2+} was added to the preparation 10 min before hypoxia, the Ca_i^{2+} signals became flat at the base line, as shown in Fig. 2 and there was no detectable transient or rise throughout the hypoxia/reoxygenation period (data not shown). This means that the increase in Ca_i^{2+} at the point of reoxygenation was facilitated (during hypoxia) and/or mediated (at reoxygenation) by Ni^{2+}-sensitive mechanisms.

Discussion

Results from an increasing number of studies have suggested a role for Na^+/Ca^{2+} exchange in producing Ca_i^{2+} overload during reoxygenation (8, 9, 11, 33–35). However, the present study provides the first direct evidence of Ca_i^{2+} loading by the exchanger into a reoxygenated working myocardial preparation that is actively contracting and relaxing. This study also demonstrated that (a) a putative increase in the cytosolic Ca^{2+} level by sarcolemmal Ca^{2+} influx occurs immediately after a period of hypoxia in mammalian ventricular myocardium and (b) because the SR function and the sarcolemmal Ca^{2+} channels are pharmacologically blocked in this preparation, these subcellular functions are not necessary for Ca_i^{2+} loading during reoxygenation. Furthermore, the results suggest that Na_i^+ loading during the hypoxic period is the driving force behind excessive Ca^{2+} influx via Na^+/Ca^{2+} exchange during this period, which will be discussed later.

In the presence of ryanodine and verapamil (16, 17, 36), the Ca_i^{2+} transients we observed were dependent on Ca_o^{2+}, and on the membrane potential. The Ca_i^{2+} transients were enhanced by interventions that increased Na_i^+ activity, and were blocked by lowering Na_o^+, TTX, Ni^{2+}, and DCB. These observations are consistent with the notion that the primary determinant of the Ca_i^{2+} transient in these experiments is Na^+/Ca^{2+} exchange. We further tested whether the SR Ca^{2+} handling was sufficiently inhibited in our preparations by using a short-term force-interval response ($n = 2$ [37]). By varying the coupling interval of the extra stimuli from 2 to 5 s, we found no alteration of the levels of Ca_i^{2+} nor tension of the postextrastimulation beats (37). This was also true even when a train of conditioning stimuli (three beats with a 2-s interval) was applied to the muscle (data not shown). Therefore, the Ca_i^{2+} transients

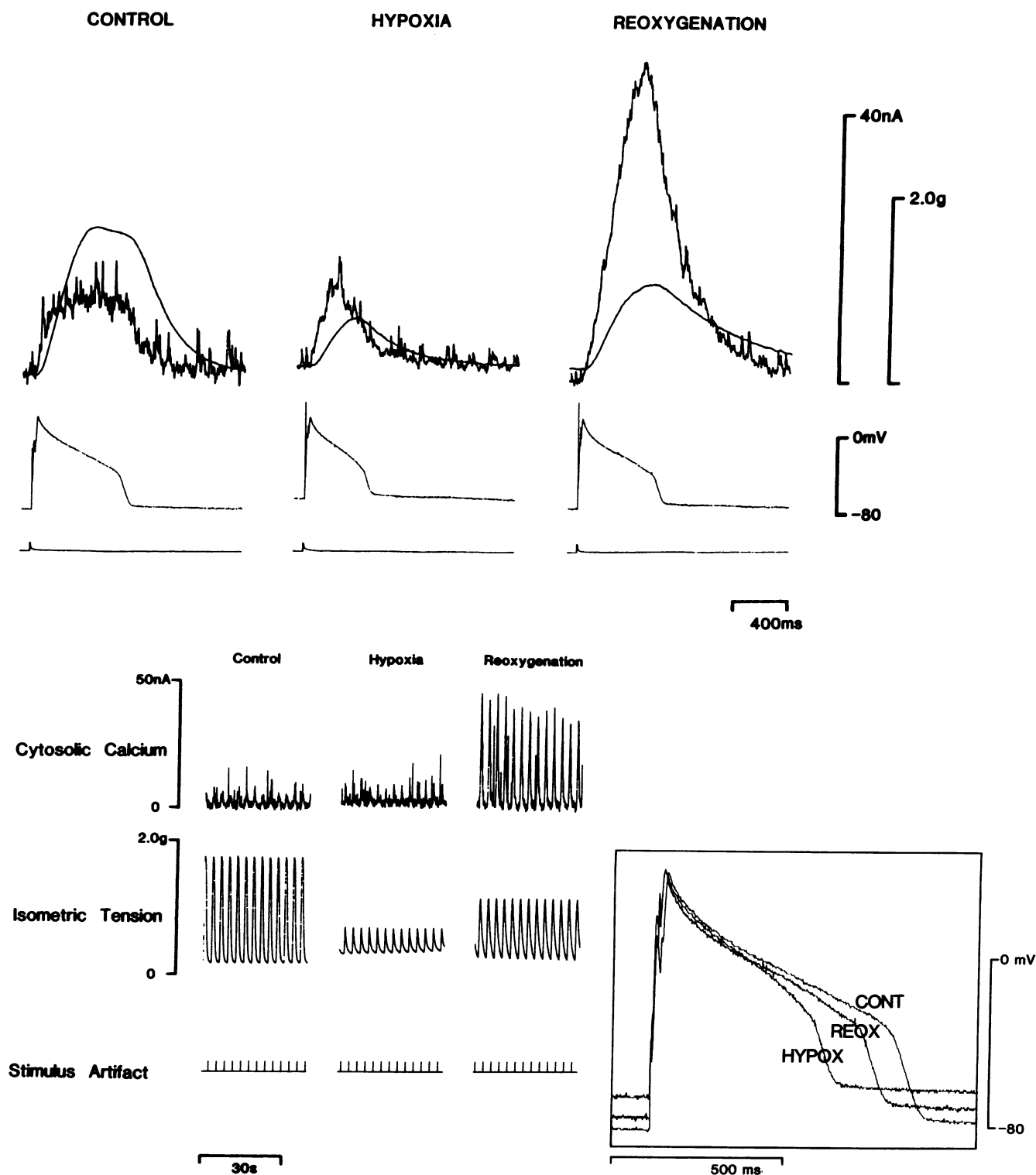CONTROL   HYPOXIA   REOXYGENATION

Figure 3. Top panel: Simultaneous records of the Ca_i^{2+} transient, tension trace (top), action potential (middle), and the stimulus artifact (bottom), during control conditions (left), during 20-min hypoxia (middle), and during 3-min reoxygenation (right). Data were serially obtained from the same muscle. The Ca_i^{2+} signals were averaged for 16 cycles. The calibration bar (40 nA) corresponds to 1.10 μM of Ca^{2+} in control, 1.14 μM in hypoxia, and 1.20 μM in reoxygenation, respectively. 30°C, 0.33 Hz. Bottom left panel: The slow tracings of Ca_i^{2+} transients, tension, and the stimulus artifacts in these conditions are shown. Bottom right panel: Action potentials are superimposed for comparison. CONT, control; HYPOX, hypoxia; REOX, reoxygenation.

we observed did not contain an interval-dependent component, which is attributable to released Ca^{2+} from the SR (37, 38). During the low Na_o^+ protocol (replaced with Li^+), the Ca_i^{2+} transient was markedly depressed with relaxed tension.

Since Li^+ blocks the forward mode as well as the reverse mode of the exchanger, this observation also suggested that the cytosolic Ca^{2+} supplied through the SR or the sarcolemmal Ca^{2+} channels were sufficiently blocked so that the remaining Ca^{2+}

	Control	Hypoxia	Reoxygenation
Developed tension (g/mm^2)	2.6±0.7	0.5±0.4‡	1.2±0.6*‖
Time to the end of tension ascent (ms)	880±192	454±170*	823±250§
Time to 80% tension regression (ms)	392±84	256±102	681±334‡‖
Peak Ca_i^{2+} (μM)	0.8±0.3	0.5±0.2*	1.2±0.5*‖
Resting Ca_i^{2+} (μM)	0.2	0.3	0.4
Time to the end of Ca_i^{2+} ascent (ms)	805±150	380±172*	711±286§
Time to 80% Ca_i^{2+} regression (ms)	110±22	106±34	143±66
APD_{80} (ms)	866±131	404±153‡	720±232§
Resting membrane potential (mV)	−76±6	−56±10*	−70±10

Data are mean±SD ($n = 7$). Because of relative insensitivity of aequorin to the lower level of Ca^{2+} and the basal noise from the photomultiplier tube, quantitative estimation of resting Ca_i^{2+} was performed in two muscles during control, four muscles during hypoxia, and five muscles during reoxygenation, in which detectable rises of the signal level were observed. The mean values of this measurement are presented. APD_{80}, action potential duration to the 80% regression; *‡ $P < 0.05$ and $P < 0.01$ to the control values; §‖ $P < 0.05$ and $P < 0.01$ to the hypoxia values, respectively.

leaks were able to be handled by the sarcolemmal Ca pump (39, 40).

We did not directly measure the Na^+/Ca^{2+} exchange current (I_{NaCa}) in our multicellular preparation; however, the results suggested that I_{NaCa} might operate in the reverse mode during the ascending phase of the Ca_i^{2+} transient (hence, during membrane depolarization) and it might switch to the forward mode at the initiation of the membrane repolarization. Such a sustantation of the reverse mode of I_{NaCa} throughout depolarization may not be consistent with reported patterns of I_{NaCa} in the working myocardium, where I_{NaCa} was estimated to be predominantly directed inwards, except for the very early phase of the action potential (41, 42). Therefore, we tried to estimate the instantaneous direction and relative scales of I_{NaCa} at every 10-ms interval in our preparation using a thermodynamic equation initially proposed by DiFrancesco and Noble (16, 17, 24, 43), which is given as follows:

$$I_{NaCa} = k_{NaCa}[\exp(rEF/RT)[Ca_o^{2+}][Na_i^+]^3$$
$$- \exp(-(1 - r)EF/RT)[Ca_i^{2+}][Na_o^+]^3],$$

where k_{NaCa} is a scaling factor which includes the rate constant of exchanger translocation at $E_m = 0$; r is the fractional distance through the membrane of the limiting energy barrier. A value of 0.35 was used in accordance with previous studies with guinea-pig myocytes (17, 23, 24); E is the membrane potential; R, T, and F have the usual thermodynamic meanings. The activity coefficients of Na_o^+ and Na_i^+ were assumed to be 0.7 (44). See footnote 2 for more assumptions and discussion. As shown in Fig. 4, we found that under our experimental conditions, the Na^+/Ca^{2+} exchange current might be outwardly directed during depolarization. This implies that the exchanger

acts in the reverse mode for Ca^{2+} loading during this period. With repolarization, the current abruptly shifted to the inward direction; the forward mode of the exchanger acting to exclude Ca^{2+} from the cytoplasm. The dip in the current immediately after repolarization appears to be consistent with the transient inward currents that have been reported in systems using voltage-clamped single myocytes (17, 45, 46), although the shape was somewhat smoothed by gradual membrane repolarization under our conditions (e.g., compare with Fig. 2 in Beuckelmann and Wier [17]). Although the Na_i^+ level in the range of 12 to 18 mM is higher than the reported value in ferret ventricular myocardium (18, 44), this appears to be within the expected range under our experimental conditions using acetyl-strophanthidin and DPI 201-106 (18, 47). As suggested by Leblanc and Hume (26), the Na_i^+ value around the exchanger could be much higher, especially during the early phase of depolarization: however, this possibility does not affect our conclusion obtained from this estimation that the exchanger operates in reverse mode during this period.

Besides Na^+/Ca^{2+} exchange, the Ca_i^{2+} transient might be modulated by other Ca^{2+}-related subcellular functional units. Those include the sarcolemmal Ca^{2+} pump (48), sarcolemmal Ca^{2+} leak currents (17) and internal Ca^{2+} buffers (49, 50). Especially, during hypoxia and reoxygenation, these subcellular functions may be altered, and, could cause a Ca_i^{2+} rise with

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2. To estimate I_{NaCa} in the working muscle, we made the following assumptions: (a) Na^+ versus Ca^{2+} exchange is carried with 3:1 stoichiometry in ferret myocardium as shown in other species (17, 23, 45, 55); (b) none of the effects of changes in Ca_i^{2+} or membrane potential on the mode and activity of the exchanger are delayed over the order of milliseconds (40, 42); (c) Na_i^+ at the internal site of the exchanger is kept constant during a cardiac cycle (17, 38); (d) the instantaneous Ca_i^{2+} as measured by aequorin represents the average level through the cytosolic space (63), which might not significantly differ from the level at the exchangers' translocation site inside the sarcolemma (17, 20). Except for the very early phase of depolarization when the action potential did still not reach the plateau, change in the level of Ca_i^{2+} was very slow and smooth (as shown in Fig. 5, the time constant of the descending phase was around 1 s, which is consistent with a recent report [39]). Homogeneous (no oscillatory) distributions of Ca_i^{2+} through the cytosolic space (64) and their gradual change during depolarization under similar pharmacological interventions were also shown in several studies using the isolated single myocyte (20, 65). Accordingly, during each 10-ms interval, we assumed the level of Ca_i^{2+} to be constant; hence, the rapidly-accessible Ca^{2+} buffers, such as troponin, calmodulin, or phosphocreatine (17, 37), are equilibrated. Then, the current during that period was estimated for a given set of Ca_i^{2+}, Na_i^+, and the membrane voltage. It should be noted that such estimations could include substantial errors in a manner dependent on the rate of Ca_i^{2+} change, and should not be applied to the control condition such as the left panel of Fig. 1, in which Ca_i^{2+} transients and their buffers underwent more dynamic changes. In addition, our assumption of the constancy of the Na_i^+ level during a cardiac cycle may not be consistent with a recent theory that Na^+/Ca^{2+} exchange may predominantly access a discrete cation pool around the inner sarcolemma, in which the Na_i^+ level could be transiently increased by Na^+ influx from the fast Na^+ channels (26, 38, 66). However, at least under the conditions in which the Ca^{2+} influxes via the SR and sarcolemmal Ca^{2+} channels were blocked, I_{NaCa} was reported to be directly correlated with the level of space-averaged Ca_i^{2+} and the membrane potential, but not with Na_i^+ (16, 17). In the same experimental conditions, Ca_i^{2+} and I_{NaCa} measurements with (17) or without (16) Na^+ channel blockade showed undetectable changes. Moreover, in the presence of caffeine, Bridge et al. clearly showed that the Na^+/Ca^{2+} exchanger keeps free access to the entire pool of cytoplasmic Ca^{2+} (40, 48).
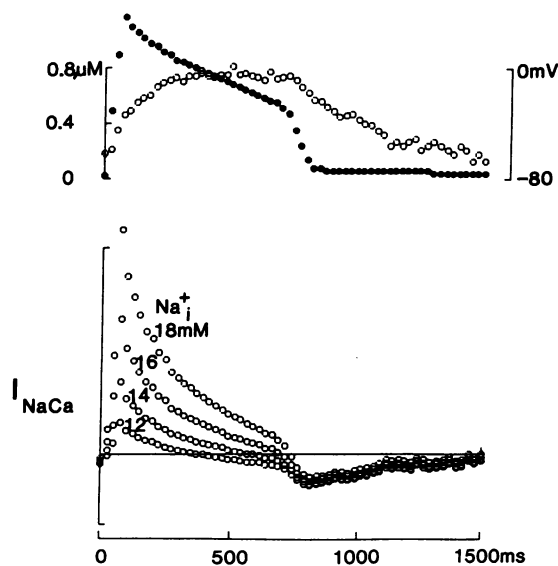
Figure 4. The calibrated Ca_i^{2+} transient (○) and the corresponding action potential (●) are shown in the top row. Data were sampled at 10-ms intervals. By adapting these data to the DiFrancesco and Noble equation, relative magnitudes and time courses of the transsarcolemmal current via Na^+/Ca^{2+} exchange were estimated (I_{NaCa}; *bottom row*). Na_i^+ level was assumed to be 12–18 mM. An original trace of this figure is presented in the left panel of Figure 3 (*top*). For details, see the discussion and footnote 2.

reoxygenation. The following discussions, however, do not support any role for these functions on the observed pattern of Ca_i^{2+} during reoxygenation. Even after the blockade of SR function, most of the Ca^{2+} that entered the intracellular space would be captured by the buffering systems, which include the internal sarcolemmal surface, calmodulin, troponin, and phosphocreatine. If the buffer capacity were suddenly reduced at reoxygenation, the Ca^{2+} released from the binding sites might cause an increase in Ca_i^{2+}. In this case, the altered profile of Ca_i^{2+} after reoxygenation would consist of a primary increase in the diastolic or resting level of Ca_i^{2+}, since the released Ca^{2+} from the internal sites would remain in the cytosolic space until removed by the secondarily activated Ca^{2+} pump or the exchanger. Ca^{2+} influx during depolarization would instead be inhibited by the persistent rise of Ca_i^{2+}. Such alterations in the Ca_i^{2+} pattern by internal Ca^{2+} release have been observed with acidotic interventions (51, 52) or in studies of acute global ischemia in mammalian ventricular myocardium (1, 53). In these studies, the descending phases of the Ca_i^{2+} transients were delayed, which were also attributed to effects of the decreased buffer capability. These expected patterns of change in the Ca_i^{2+} signal by the reduction of internal buffer capacity are however, inconsistent with our observations during reoxygenation. In our study, the Ca_i^{2+} transients were primarily augmented by an increase in the systolic amplitude, which was associated with a relatively small increase in basal Ca_i^{2+} levels. This implies that the increase in Ca_i^{2+} occurred during depolarization and the increase was dynamic rather than static. The bottom row of Fig. 5 shows a comparison of the descending slope of Ca_i^{2+} signals under three conditions, by adjusting their peak Ca_i^{2+} values to equivalent levels. The slope during reoxygenation was lower than that of the control, but was higher than the slope during hypoxia. This observation contrasted with the expected delay in the Ca_i^{2+} descent, which should be

apparent if a reduced buffer capacity plays the primary role in the Ca_i^{2+} increase during reoxygenation. Instead, the descending slopes of Ca_i^{2+} appear to be dependent on the resting membrane potential, which is the chief determinant of slope when the Ca_i^{2+} levels are primarily regulated by Na^+/Ca^{2+} exchange (17, 39, 48, 54).

The ohmic leak of Ca^{2+} may also increase at the time of reperfusion, which might shift the resting Ca_i^{2+} to higher levels. However, this increase does not appear to explain the observed dynamic increase in Ca_i^{2+}, as discussed above.

Impaired Ca^{2+} pump activity possibly causes an increase in Ca_i^{2+} after reoxygenation. This possibility also does not fit the observed Ca_i^{2+} pattern because Ca^{2+} pump inhibition should further delay the Ca_i^{2+} descent when it causes an increase in peak Ca_i^{2+} (48). Taken together, none of these subcellular functions appear to modulate the Ca_i^{2+} transient towards the profile observed during reoxygenation. Besides, if these modulatory effects were activated by reoxygenation, they might increase the Ca_i^{2+} levels even in the presence of Ni^{2+}. However, this was not the case in our Ni^{2+} study.

Recent studies have revealed evidence consistent with the concept that the Na^+/Ca^{2+} exchange carries ions via specific ion binding sites that operate in an asymmetrical manner between inside and outside the membrane (23, 55). The thermodynamic equation proposed by DiFrancesco and Noble (43) may still be adaptable to these series of chemical reactions because the Na^+/Ca^{2+} exchange current is under dynamic control primarily by Ca_i^{2+} (high affinity of the internal binding sites to Ca^{2+}) and the membrane potential (electronegicity) (16, 17, 23, 24, 55). From this equation, the changes in these two parameters that were shown during reoxygenation appear to favor Ca^{2+} extrusion by the exchanger, since both increases in Ca_i^{2+} and in the level of negativity of the resting potential increase the second term of the equation (inward current, thus, for Ca^{2+} efflux) while decreasing the first term (outward current for Ca^{2+} influx). Therefore, changes in Ca_i^{2+} and membrane potentials are not the direct driving forces for Ca^{2+} loading during reoxygenation. Instead, they appear to activate the exchanger against the Ca_i^{2+} excess in order to maintain the intracellular environment. The calculated exchange current and the net Ca^{2+} influx would be smaller during reoxygenation than those in the control state before hypoxia, if other parameters in the equation remain unchanged (Fig. 6, *top*). However, there are several lines of evidence suggesting that Na_i^+, which is one of the parameters assumed to be a constant in the above discussion, might change during the course of ischemia/reper-
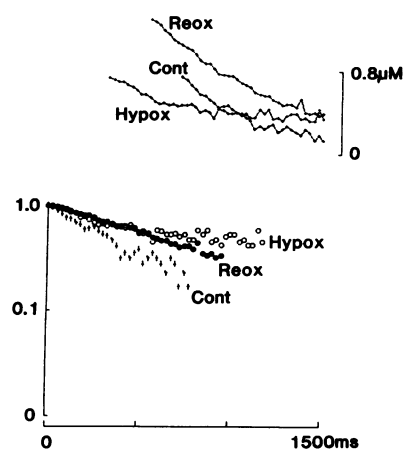
Figure 5. The descending phases of the Ca_i^{2+} transients shown in Fig. 3 (*top*) were superimposed after the calibration process, as shown in the top row. The time courses of the Ca_i^{2+} decline are presented in a semilogarithmic scale in the bottom row. The abbreviations are the same as in Fig. 3 (*bottom right*).

fusion as well as during hypoxia/reoxygenation. Recent studies using NMR spectroscopy showed a three- to five-fold increase in Na_i^+ at the end of 20-min global ischemia in Langendorff preparations (33, 56). In these studies, at the time of reperfusion, the Na_i^+ accumulation was abruptly shifted to a monoexponential decline towards the control level for over 10 min, demonstrating the reversal of the net Na_i^+ balance at the time of reperfusion. These observations not only provide supportive evidence for Na_i^+ excess during ischemia but also suggest a role of Na^+/Ca^{2+} exchange during reperfusion, because the Ca_i^{2+} accumulation and Na_i^+ regression appear to be coincidental during this period. In studies of hypoxia, a time-dependent increase in Na_i^+ was shown using ion-selective microelectrodes (18, 47). The level of increase was reported from 10 mM in controls to 19 mM during 25 min of hypoxia in the ferret ventricular muscle (18). If a similar level of Na_i^+ loading were assumed to occur at reoxygenation in our study, the net Ca^{2+}

influx by the exchanger would be tremendously augmented even in the presence of a high Ca_i^{2+} and a low membrane potential (Fig. 6, *bottom*). This may not be surprising from the dynamic characteristics of Na^+/Ca^{2+} exchange, since the increase in Na_i^+ affects the outward current by a factor of 3, while changes in Ca_i^{2+} modulate the inward current by a factor of 1. Thus, when there was an equivalent increase in Na_i^+ at the time of reoxygenation as demonstrated by other investigators, the observed increase in Ca_i^{2+} transient after reoxygenation could be attributed to the Na^+/Ca^{2+} exchange process which operates according to its known characteristics.

There are a large number of factors that may modulate the exchanger activity (corresponding to k_{NaCa} in the equation [57, 58]). Among them, redox agents increase the exchange rate up to 10-fold by promoting thiol-disulfate interchange in the protein (57). Because the production of reduced oxygen species might occur during reoxygenation (59–61), an abrupt activa-
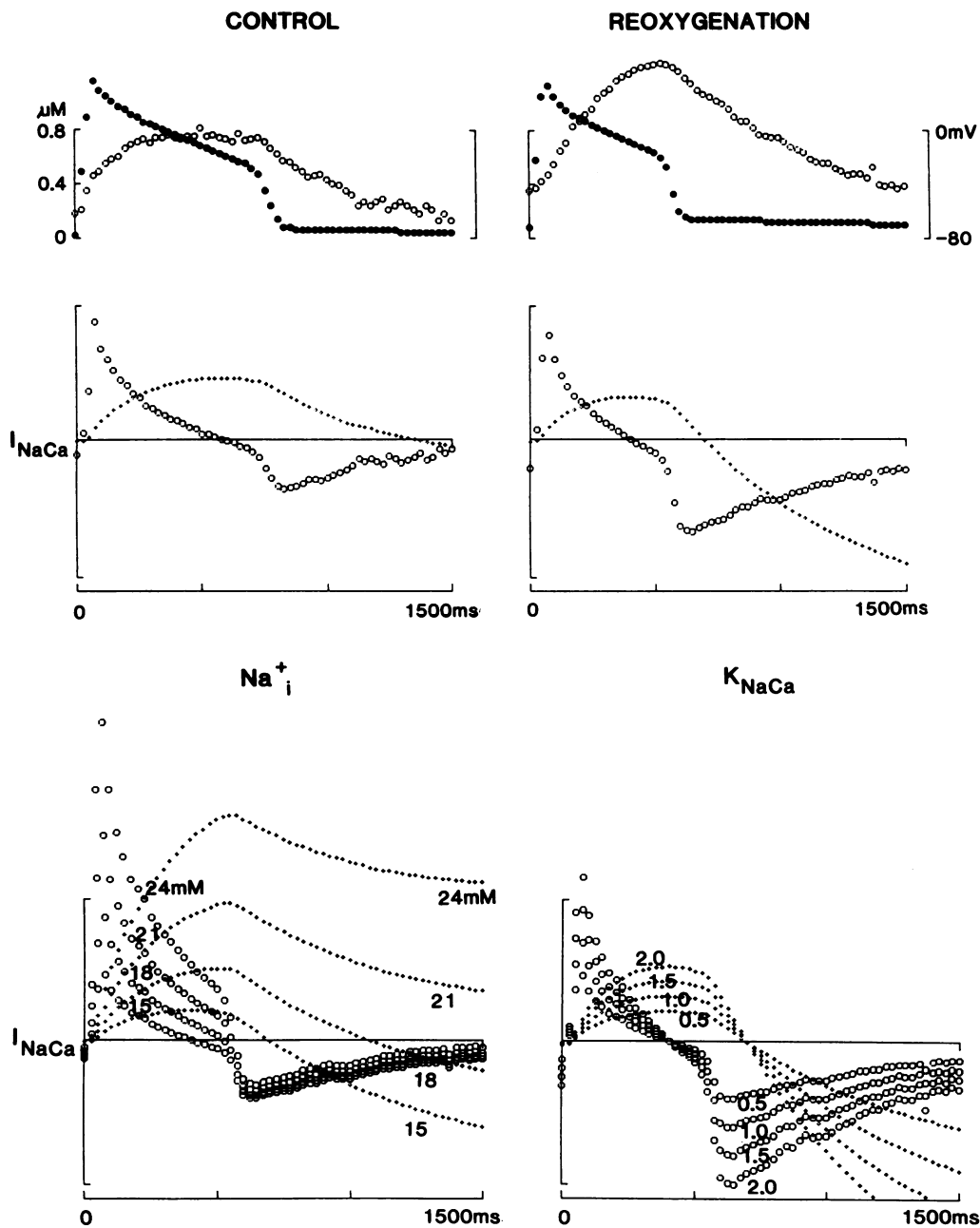

Figure 6. Top panel: The calibrated Ca_i^{2+} transient (o) and the corresponding action potential (●) during control conditions (*left*) and during reoxygenation (*right*) are shown in the top row. In the bottom row, the transsarcolemmal current via Na^+/Ca^{2+} exchange (o) and the time integral (small dots) are estimated in the same manner as presented in Fig. 4. The level of Na_i^+ was assumed to be 15 mM in both conditions. Note that the outward current (Ca^{2+} influx) is smaller during reoxygenation than during control unless their Na_i^+ and k_{NaCa} are altered. *Bottom panel:* The transsarcolemmal current via Na^+/Ca^{2+} exchange (o) and the time integral (·····) during reoxygenation were reassessed while the Na_i^+ values are varied from 15 to 24 mM (*left*) or the k_{NaCa} values are modulated over the range of 0.5 to 2.0-times of the control (*right*). For the definition of the scaling factor, k_{NaCa}, see the text.

tion of Na^+/Ca^{2+} exchange as detected by the sudden increase in the Ca^{2+} influx during this period may be due to modulatory effects by these free radicals (34). Of importance, the more rapid rate of the exchanger's translocation facilitates Ca^{2+} efflux as well as Ca^{2+} influx, and the net effect might be determined by the combination of Ca_i^{2+}, Na_i^+, and the membrane potential (Fig. 6, *bottom*). Hence, under a normal transmembrane Na^+ gradient and resting potential, this activation may lead to an increased efflux of Ca^{2+}. Instead, with Na_i^+ accumulation, which might occur during hypoxia, the activation would further facilitate Ca_i^{2+} loading into the myocardial cell. Tissue pH is another factor that affects the translocation rate of the exchanger (58). The sudden reversal from the acidotic condition to the transient alkalinization at reoxygenation may synergistically support the role of free radicals. Although such a pH change in hypoxia/reoxygenation studies might be modest, this factor could play a much more significant role in the setting of ischemia and reperfusion.

Accumulated studies indicated that the stoichiometry of Na^+/Ca^{2+} exchange in cardiac and nervous tissues in various species is most probably 3 Na^+:1 Ca^{2+} (17, 23, 45, 55). We think it is reasonable to apply this stoichiometry to the analyses of our results from the ferret myocardium. However, two parameters, k_{NaCa} and r, in the equation may vary among species. As discussed above, our analyses did not require knowledge of the absolute scale of the exchange current. Accordingly, the value of k_{NaCa} was assessed as a ratio for the control level. The values of r were reported as 0.28–0.36 in studies of guinea-pig myocytes (17, 23, 24). We tested the effect of varying r value in a wider range of 0.10–0.50 (23); however, the shift of r in this range did not alter our findings presented in Fig. 4 (data not shown). Furthermore, using the right ventricular papillary muscles from the guinea-pig ($n = 5$), we confirmed that the similar pattern of biphasic Ca_i^{2+} transient was reproducibly observed by the same combination of pharmacological treatment, although the Ca_i^{2+} transient (peak Ca_i^{2+}, 0.8±0.2 µM; time to the end of Ca_i^{2+} ascent, 984±64 ms) and the action potential (duration to 80% regression, 1,032±91 ms) appeared to be more prolonged than those observed in the ferret preparation (Table I). Taken together, quantitative characteristics of the Na^+/Ca^{2+} exchange current in the ferret ventricle should await future studies using the isolated single cell preparation; however, the difference between the ferret versus guinea-pig, if any, will probably not affect our conclusions.

By a combination of pharmacological interventions, we modulated the subcellular functions of isolated ferret papillary muscle, in which the SR and the slow Ca^{2+} channels were blocked and Na^+/Ca^{2+} exchange was made to operate in the direction of Ca^{2+} loading throughout the depolarization period. Despite these modulations, all measurements of Ca_i^{2+} transients, tension traces, and the action potentials, showed a pattern of change during hypoxia and reoxygenation that was consistent with control preparations (19, 32). The tension trace especially, which represents the final outcome of the E-C coupling process, showed a significant decrease in peak values with an abbreviated time course during hypoxia. These changes were followed by a slow recovery with a prolonged relaxation during reoxygenation (62). These findings suggest not only that our preparations were affected by hypoxia and reoxygenation but also that the subcellular functions remaining after drug intervention were much more essential as targets of hypoxia/reoxygenation than the functions deleted by it. Hence, the role of Na^+/Ca^{2+} exchange for Ca^{2+} influx and its

modulation during reoxygenation presented in this study could not be interpreted as unusual findings under a specific pharmacological condition. Of interest, while the durations of Ca_i^{2+} transients were abbreviated by the transition from hypoxia to reoxygenation, tension relaxation was prolonged after reoxygenation (19, 32, 62). This discrepancy between Ca_i^{2+} and tension time courses, however, is consistent with observations in previous hypoxia/reoxygenation studies in the intact ferret papillary muscle using aequorin (19, 21) or in the isolated rat single myocyte loaded with indo-1 (31). Our data also provide evidence that the impaired relaxation that occurs during reoxygenation develops without SR dysfunction or other Ca_i^{2+} handling disturbances. A reasonable conclusion from these studies is that the cause of tension prolongation during reoxygenation and reperfusion resides down-stream from Ca_i^{2+} handling; namely, in the process of cross-bridge cycling (31).

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