The Sodium Pump Modulates the Influence of I_{Na} on $[Ca^{2+}]_i$ Transients in Mouse Ventricular Myocytes

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ABSTRACT To investigate whether activity of the sarcolemmal Na pump modulates the influence of sodium current on excitation-contraction (E-C) coupling, we measured $[Ca^{2+}]_i$ transients (fluo-3) in single voltage-clamped mouse ventricular myocytes ($[Na^+]_{pip}$ = 15 or 0 mM) when the Na pump was activated (4.4 mM K_o^+) and during abrupt inhibition of the pump by exposure to 0 K_o^+ with a rapid solution-switcher device. After induction of steady state $[Ca^{2+}]_i$ transients by conditioning voltage pulses (0.25 Hz), inhibition of the Na pump for 1.5 s immediately before and continuing during a voltage pulse (200 ms, −80 to 0 mV) caused a significant increase (15 ± 2%; $n = 16$; $p < 0.01$) in peak systolic $[Ca^{2+}]_i$ when $[Na^+]_{pip}$ was 15 mM. In the absence of sodium current (I_{Na}, which was blocked by 60 μM tetrodotoxin (TTX)), inhibition of the Na pump immediately before and during a voltage pulse did not result in an increase in peak systolic $[Ca^{2+}]_i$. Abrupt blockade of I_{Na} during a single test pulse with TTX caused a slight decrease in peak $[Ca^{2+}]_i$, whether the pump was active (9%) or inhibited (10%). With the reverse-mode Na/Ca exchange inhibited by KB-R 7943, inhibition of the Na pump failed to increase the magnitude of the peak systolic $[Ca^{2+}]_i$ (4 ± 1%; p = NS) when $[Na^+]_{pip}$ was 15 mM. When $[Na^+]_{pip}$ was 0 mM, the amplitude of the peak systolic $[Ca^{2+}]_i$ was not altered by abrupt inhibition of the Na pump immediately before and during a voltage pulse. These findings in adult mouse ventricular myocytes indicate the Na pump can modulate the influence of I_{Na} on E-C coupling in a single beat and provide additional evidence for the existence of Na fuzzy space, where $[Na^+]$ can significantly modulate Ca^{2+} influx via reverse Na/Ca exchange.

INTRODUCTION

Voltage-gated sodium channels mediate the voltage-dependent increase in sodium ion permeability that is responsible for the upstroke of the action potential. In cardiac muscle Ca^{2+} influx via voltage-gated sarcolemma Ca^{2+} channels triggers a rapid release of Ca^{2+} from sarcoplasmic reticulum (SR), causing an increase in the concentration of intracellular Ca^{2+} ($[Ca^{2+}]_i$) that leads to contraction (Fabiato, 1985; for review see Bers, 1991). Leblanc and Hume (1990) have found that excitation-contraction (E-C) coupling can also occur in the absence of Ca^{2+} current and proposed that it is triggered by influx of Ca^{2+} via reverse-mode Na/Ca exchange, presumably stimulated by influx of Na^+ through voltage-gated Na^+ channels (I_{Na}). Based on these experiments and the fact that it is the subsarcolemmal Na^+ concentration ($[Na^+]$) that directly controls Ca^{2+} influx via Na/Ca exchange, Lederer et al. (1990) have speculated that the presence of a restricted subsarcolemmal space (fuzzy space) for Na^+ accumulation may be necessary to explain these findings.

Recent studies have provided evidence for the presence of a subsarcolemmal Na^+ concentration gradient in arterial smooth muscle (Arnon et al., 2000) and in cardiac myocytes (Bielen et al., 1991; Semb and Sejersted, 1996; for a review see Carmeliet, 1992). Lipp and Niggli (1994) have also observed that during I_{Na} $[Na^+]$ increases underneath the cell membrane and activates the Ca^{2+} influx mode of the Na/Ca exchange. Our recent results obtained from mouse ventricular myocytes also support the hypothesis that there is a subsarcolemmal $[Na^+]$ gradient generated by the activity of the Na pump that also affects the $[Na^+]$ adjacent to the Na/Ca exchanger (Su et al., 1998). The sarcolemmal Na pump, the Na-K ATPase, which utilizes energy derived from the hydrolysis of ATP to extrude three Na^+ in exchange for two K^+, appears to play an important role in maintaining this $[Na^+]$ gradient between the cytosol and subsarcolemmal space. Na/Ca exchange (including forward and reverse modes) was shown to be dramatically influenced by the functional states of the Na pump. As an example, outward Na/Ca exchange current measured with the Na pump active was only 20% of that measured with the Na pump inhibited (Su et al., 1998). This is consistent with the idea that the Na/Ca exchanger can be used as a sensitive indicator of changes in $[Na^+]$ in the subsarcolemmal space and the Na pump activity (Main et al., 1997). In addition, it has been well documented that the prolonged inhibition of the Na pump with cardiac glycoside or by removing extracellular K^+ significantly alters the activity of the Na/Ca exchange, increases SR Ca^{2+} content, and enhances $[Ca^{2+}]_i$ transient magnitude (Barry et al., 1985; Bers and Bridge, 1988; Su et al., 1998). However, it has not been clear whether this effect was due to an alteration of forward or reverse exchange.

We previously observed that induction of a series of I_{Na} increased the magnitude of outward Na/Ca exchange cur-

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rent early after exposure to zero K_o^+ (Su et al., 1998). Therefore, we hypothesized that during E-C coupling the activity of the Na pump could be of importance in modulating the influence of I_{Na} on subsarcolemmal $[Na^+]$, and the function of the Na/Ca exchanger. To examine these possibilities, we measured $[Ca^{2+}]_i$ transients in single, voltage-clamped mouse ventricular myocytes when the Na pump was fully activated and during abrupt inhibition of the pump by exposure to zero K_o^+ with a rapid solution switcher. The rapid solution-switching technique (Yao et al., 1997) makes it possible to abruptly inhibit the Na pump for 1.5 s immediately before and during E-C coupling, to examine the functional importance of the Na pump on a beat-to-beat basis.

MATERIALS AND METHODS

Dissociation of adult mouse ventricular myocytes

Single mouse ventricular myocytes were isolated as described previously (Su et al., 1998). After retrograde perfusion with modified Tyrode's solution (Ca^{2+}-free) for 5 min, the heart was digested for 7–12 min with 0.9 mg/ml collagenase D (Boehringer Mannheim Biochemicals, Indianapolis, IN) in modified Tyrode's solution containing 25 μM $CaCl_2$. The modified Tyrode's solution (pH 7.4) contained the following (mM): 126 NaCl, 4.4 KCl, 1.0 $MgCl_2$, 18 $NaHCO_3$, 11 glucose, 4 HEPES, 30 butanedione monoxime (BDM), and 0.13 U/ml insulin, and was gassed with 5% CO_2/95% O_2. The digested left ventricle was cut into small pieces in modified Tyrode's solution containing 100 μM Ca^{2+}. These pieces were gently agitated to release single myocytes and then incubated in the same solution with 2% albumin at 30°C for 20 min. The cell suspension was centrifuged at 300 rpm for 3 min, and the pellet of cells was resuspended in modified Tyrode's solution containing 200 μM Ca^{2+} and 2% albumin and allowed to settle for another 20 min at 30°C. Cells were then suspended in culture medium composed of 5% fetal bovine serum, 47.5% MEM (Gibco Laboratories, Bethesda, MD), 47.5% modified Tyrode's solution, 10 mM pyruvic acid, 4.0 mM HEPES, and 6.1 mM glucose and finally maintained in a 5% CO_2 atmosphere at 30°C until use. All experiments in this study were performed at 25–27°C.

Measurement of $[Ca^{2+}]_i$ and voltage clamp

The $[Ca^{2+}]_i$ was measured as previously described (Yao et al., 1998; Su et al., 1998). Myocytes were loaded with fluo-3 by exposure to 1 μM fluo-3 AM (Molecular Probes, Eugene, OR) at 30°C for 30 min. Fluo-3-loaded myocytes were placed in a chamber mounted on an inverted microscope. Once myocytes had settled to the bottom, they were superfused with a HEPES-buffered solution containing (in mM): 126 NaCl, 4.4 KCl, 1.0 $MgCl_2$, 1.08 $CaCl_2$, 11 dextrose, 0.5 probenecid, 24 HEPES (pH 7.4 adjusted with NaOH to give a final external Na^+ concentration of 140 mM).

The set-up for voltage clamp has been previously described in detail (Su et al., 1998). Cells were voltage clamped with single suction pipettes that were made from borosilicate glass tubing (Corning 7052, 1.65 mm o.d., 1.2 mm i.d., A-M System, Everett, WA) and had initial resistances of 1.5–2.5 MΩ when filled with pipette solution containing (in mM): 15 or 0 NaCl, 100 CsCl, 30 tetraethylammonium chloride, 5 MgATP, 10 HEPES, 5.5 dextrose (pH 7.1 adjusted with CsOH). Myocytes were held at −80 mV and clamped to 0 mV for 200 ms to trigger $[Ca^{2+}]_i$ transients. Unless mentioned otherwise, eight conditioning pulses (200 ms, −80 to 0 mV, 0.25 Hz) were applied before the test pulse to provide a steady-state loading of SR with Ca^{2+}.

Fluo-3-loaded myocytes were illuminated by a 485-nm excitation light (a mercury-arc lamp system) through an epifluorescence attachment (510-nm dichroic mirror, Omega, Brattleboro, VT) and a 40× Fluor oil objective lens. The resulting fluorescence signals at 530 nm (DF30, Omega) were detected with a photomultiplier (SFX-2, Solamere Technology Group, Salt Lake City, UT). The intensity of the fluorescence at 530 nm increases with an increase in $[Ca^{2+}]_i$. Fluo-3 fluorescence was transformed to $[Ca^{2+}]_i$ by a pseudo-ratio method (Cheng et al., 1993). $[Ca^{2+}]_i = Kd(F/Fo)/(Kd/[Ca^{2+}]_{irest} + 1 - (F/Fo))$, where Kd is the dissociation constant for fluo-3 (493 nmol/L at 25°C), F the fluorescence intensity, Fo the intensity at rest, and $[Ca^{2+}]_{irest}$ the $[Ca^{2+}]_i$ at rest, assumed to be 80 nM under our experimental conditions.

Abrupt inhibition of Na^+ pump

Rapid change of the extracellular solution was accomplished with a fast solution switcher (Yao et al., 1997). After whole-cell access was obtained, the voltage-clamped myocyte was superfused in a switcher microstream containing all components in the HEPES-buffered solution with additional 0.2 mM $BaCl_2$ to inhibit K^+ currents. To institute the abrupt inhibition of the Na pump immediately before and during a voltage pulse, the extracellular K^+ was removed by perfusing the myocyte in a zero K_o^+ HEPES solution. This rapid switcher device can change univalent cation concentration at the external surface of the sarcolemma with a 90% decay time value of 260 ms for a K_o^+ decrease in rat myocytes (Yao et al., 1997).

Measurement of Na/Ca exchange current ($I_{Na/Ca}$)

The exchange current was measured by means of a whole-cell voltage clamp technique (Su et al., 1999). Myocytes were voltage clamped as described above and held at a potential of −40 mV. To measure outward exchange current, the pipette contained (mM) 20 NaCl, 0.3 $MgCl_2$, 14.0 EGTA, 3.0 MgATP, 5.5 dextrose, and 10 HEPES. Calcium (3.9 mM) was added as H_2Ca-EGTA to obtain an estimated free Ca^{2+} of 100 nM. The solution pH was adjusted to 7.1 with CsOH, and then CsCl was added to give a final Cs^+ concentration of 130 mM. Voltage-clamped cells were superfused in a microstream containing (mM) 126 NaCl, 1.0 $MgCl_2$, 1.08 $CaCl_2$, 11 dextrose, and 24 HEPES. The pH was adjusted to 7.4 with NaOH, which gives a final Na^+ concentration of 140 mM. Outward exchange current was activated when the cell was abruptly exposed to an adjacent microstream of solution in which Li^+ replaced Na^+, using the solution-switching device. Currents were measured in the presence and absence of KB-R 7943 (5 μM).

Data analysis

All recordings were digitized online with a DigiData 1200 Interface (Axon Instruments, Foster City, CA) and stored on disk. The digitized data were analyzed with pCLAMP6 (Axon Instruments) and ORIGIN (Microcal Software, Northampton, MA). Results were presented as means ± SEM, and statistical differences were determined by unpaired or paired t-tests. Differences were considered significant at $p < 0.05$.

RESULTS

Effects of abrupt pump inhibition on $[Ca^{2+}]_i$ transients and membrane currents

To induce a stable $[Ca^{2+}]_i$ transient magnitude, a pre-pulse protocol (eight pulses at 0.25 Hz, −80 to 0 mV, for 200 ms) was applied before each test pulse. The left panel of Fig. 1 shows the last of a series of eight conditioning voltage-

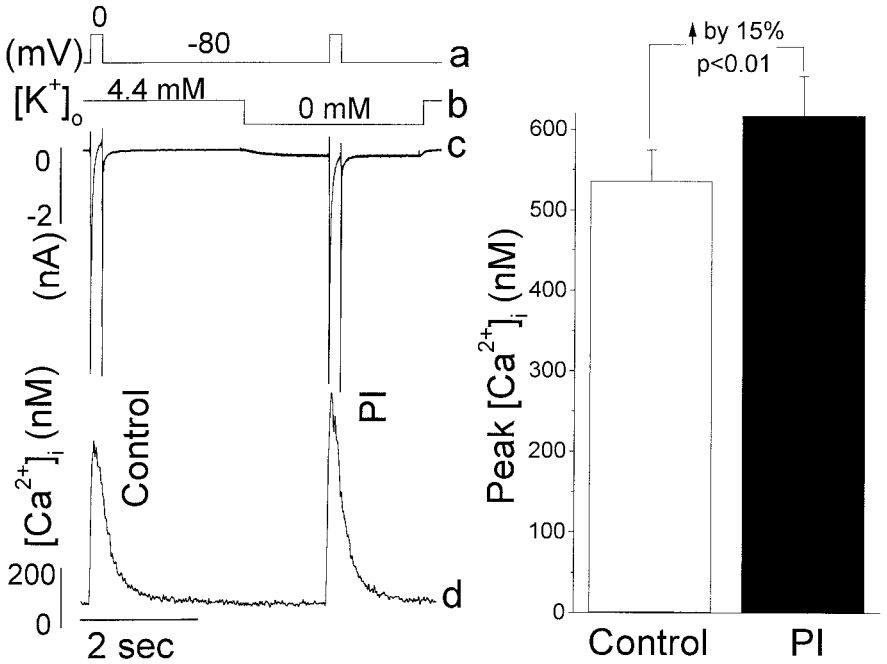

FIGURE 1 Effects of abrupt inhibition of the Na pump (PI) on $[Ca^{2+}]_i$ transients and membrane currents. The left panel shows the last of a series of eight voltage-clamp protocols to induce a stable $[Ca^{2+}]_i$ transient magnitude (*trace a*) in which the cell was held at −80 mV and depolarized to 0 mV to trigger membrane currents (I_{Na} and I_{Ca}, *trace c*) and $[Ca^{2+}]_i$ transients (*trace d*). Abrupt inhibition (by removing extracellular K^+ using a rapid solution switcher, *trace b*) of the Na pump for 1.5 s immediately before and continuing for 1.5 s during the test pulse was evidenced by the loss of the outward pump current (*trace c*). Pump inhibition (PI) caused a significant increase in peak $[Ca^{2+}]_i$ (*trace d*), and the statistics are shown in the bar graph on the right. Results are presented as mean ± SEM of 16 cells. Pipette Na^+ concentration was 15 mM.

clamp pulses (a) and its corresponding membrane currents (c) as well as the triggered $[Ca^{2+}]_i$ transient (d). The test pulse was initiated 4 s after the start of the last conditioning pulse. To determine the effects of abrupt inhibition of the Na pump on $[Ca^{2+}]_i$ transients, we inhibited the Na pump for 1.5 s immediately before and continuing during the test pulse by removing extracellular K^+ using a rapid solution switcher (*trace b* in Fig. 1). It should be noted that K^+ currents were blocked by replacing intracellular (pipette) K^+ with Cs^+ and tetraethylammonium (TEA) and adding Ba^{2+} to extracellular solution. Abrupt inhibition of the Na pump was evidenced by the loss of the outward pump current (*trace c*). Pump inhibition (PI) for 1.5 s caused an increase in peak $[Ca^{2+}]_i$ (*trace d*). Average results in 16 cells are shown in the right panel and indicate that the 15% increase was statistically significant.

The results shown in Fig. 1 indicate that the Na pump activity can acutely modulate the $[Ca^{2+}]_i$ transients in mouse ventricular myocytes when I_{Na} is present. However, it should be noted that in our experimental protocol there is a 1.5-s inhibition of the Na pump before the test pulse. This prior inhibition of the Na pump might lead to an increase in SR Ca^{2+} load, resulting in more Ca^{2+} release and then a greater $[Ca^{2+}]_i$ transient during the following test pulse. To examine this possibility, we have performed experiments in which after the 1.5-s inhibition of the Na pump, reactivation of the pump was instituted 150 ms before the test pulse. As shown in Fig. 2, the peak $[Ca^{2+}]_i$ was not enhanced under these conditions. The absence of an increase in the magnitude of $[Ca^{2+}]_i$ transients in this protocol suggests that altered pump activity rather than SR Ca^{2+} content is causing the changes in the $[Ca^{2+}]_i$ transients.

Effects of abrupt blockade of I_{Na}

To determine the importance of I_{Na} in PI-induced increase in the magnitude of $[Ca^{2+}]_i$ transients, we examined the change in $[Ca^{2+}]_i$ transient magnitude when I_{Na} was abruptly blocked coincident with the inhibition of the Na pump. In this subset of experiments, we first made sure that TTX at 60 μM was able to rapidly block I_{Na} and that it did not inhibit the L-type calcium currents (data not shown). In Fig. 3, the effects of zero K_o^+ and simultaneous TTX applications are shown. The zero-K_o^+-induced increase in peak $[Ca^{2+}]_i$ disappeared, and in contrast, peak $[Ca^{2+}]_i$ was decreased by 10% in the test pulse if TTX was also applied to block the I_{Na} while the Na pump was abruptly inhibited for 1.5 s immediately before and during the test pulse. We next examined the change of $[Ca^{2+}]_i$ transient when I_{Na} was blocked alone. Fig. 4 illustrates that abrupt blockade of I_{Na} with TTX before a single voltage pulse also resulted in a slight decrease in peak systolic $[Ca^{2+}]_i$. It is important to note that the magnitude of the decrease in $[Ca^{2+}]_i$ transient induced by TTX was similar with the pump active (Fig. 4, 9%) and the pump inhibited (Fig. 3, 10%). These results indicate that the presence of I_{Na} is required for PI-induced increase in the magnitude of $[Ca^{2+}]_i$ transients and that I_{Na} is an important determinant for the generation of a normal $[Ca^{2+}]_i$ transient, which is consistent with the results obtained from guinea pig ventricular myocytes (Lipp and Niggli, 1994; Levesque et al., 1994).

In a separate series of experiments, myocytes were clamped to the Na^+ reversal potential (+60 mV) for 200 ms with $[Na^+]_i$ = 15 mM. This caused a small, slow rising $[Ca^{2+}]_i$ transient that was not altered by abrupt exposure to

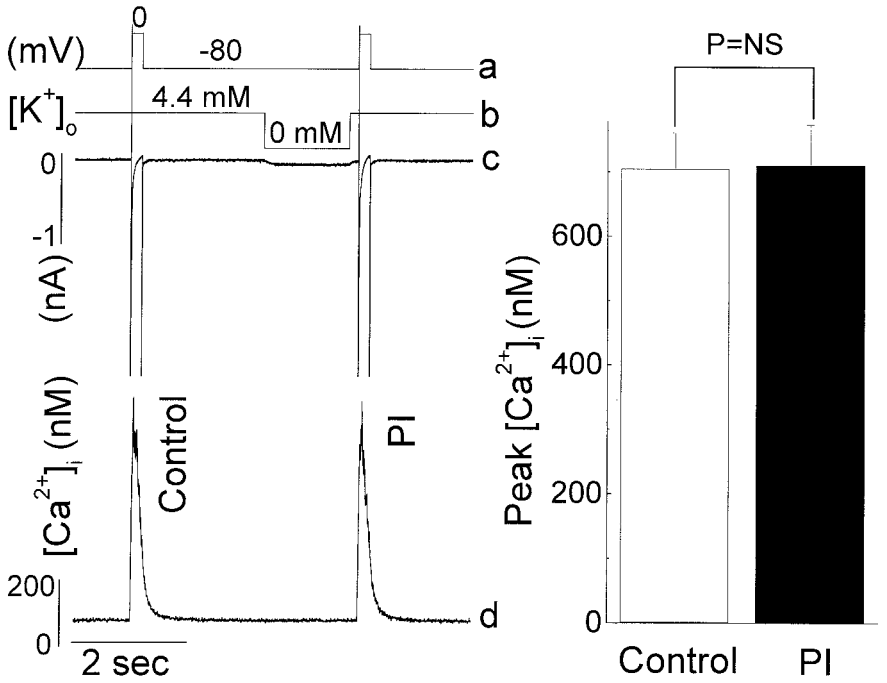

FIGURE 2 Effects of 1.5-s pump inhibition followed by pump reactivation 150 ms before the test pulse on $[Ca^{2+}]_i$ transient and membrane currents. The peak $[Ca^{2+}]_i$ was not altered in the test pulse (*trace d* and *bar graph* on the right) when the Na^+ pump was reactivated immediately before the voltage-clamp pulse. Results are mean ± SEM of six cells. Pipette Na^+ concentration was 15 mM.

TTX ($n = 5$, data not shown). This finding indicates that the effects of TTX are likely mediated by an alteration in Na^+ influx via the fast Na^+ channel.

Effects of abrupt pump inhibition on $[Ca^{2+}]_i$ transients in the presence of KB-R 7943

To understand the role of the reverse-mode Na/Ca exchange in the changes of $[Ca^{2+}]_i$ transient amplitudes induced by Na pump inhibition, we examined the effects of the Na pump inhibition on the amplitudes of $[Ca^{2+}]_i$ transients in the presence of KB-R 7943, which has been described as a selective antagonist of the reverse Na/Ca exchange (Iwamoto et al., 1996; Watano et al., 1996; Satoh et al., 2000). In this subset of experiments, we first examined the effects of KB-R 7943 on the reverse Na/Ca exchange current and $[Ca^{2+}]_i$ transients in mouse ventricular myocytes. KB-R 7943 at 5 μM was found to inhibit the reverse Na/Ca

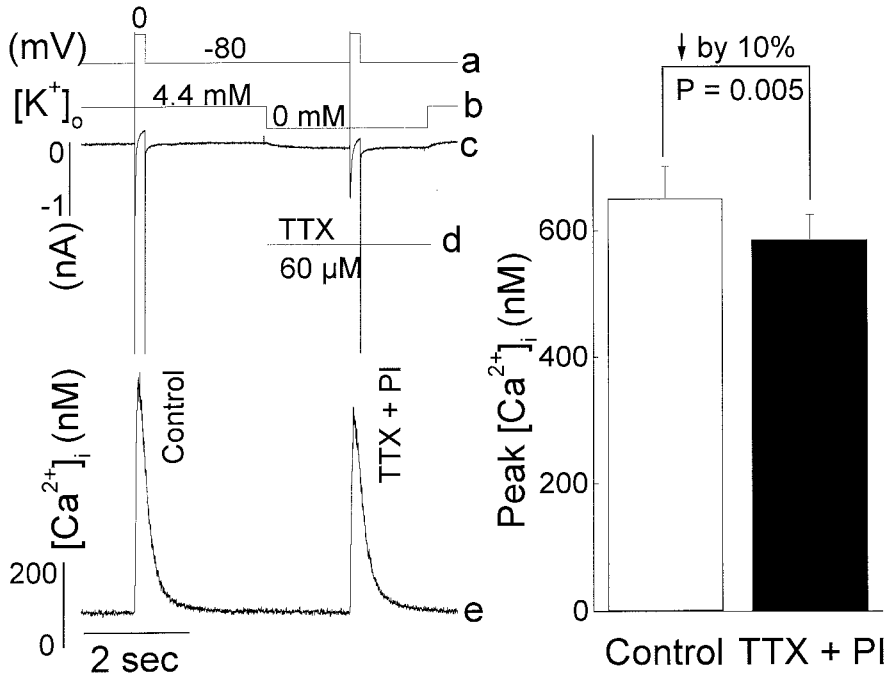

FIGURE 3 Effects of abrupt pump inhibition and TTX application on $[Ca^{2+}]_i$ transients and membrane currents. In the left panel, trace a shows the same voltage protocol as in Fig. 1. While the Na pump was abruptly inhibited (removing extracellular K^+ using a rapid solution switcher, *trace b*) for 1.5 s immediately prior to and during the test pulse, TTX (60 μM) was also applied (*trace d*) to block the I_{Na}. The loss of the outward pump current and the blockade of I_{Na} are shown in trace c. Peak $[Ca^{2+}]_i$ was slightly decreased in the test pulse (see example *trace e* and average values in *bar graph* on the right). Results are mean ± SEM of five cells. Pipette Na^+ concentration was 15 mM.

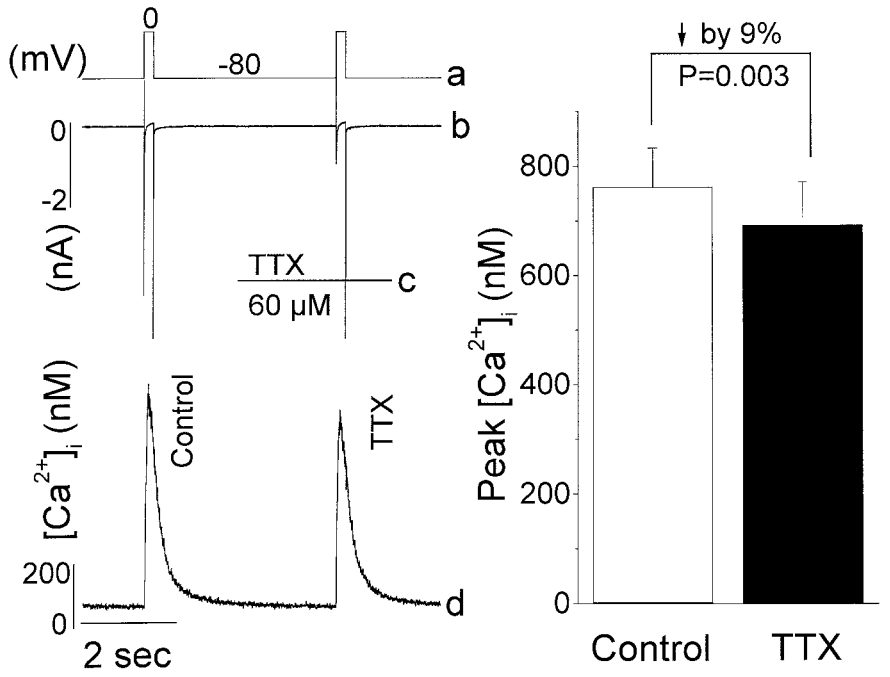

FIGURE 4 Influence of TTX alone on $[Ca^{2+}]_i$ transients and membrane currents. Trace a on the left shows the same voltage protocol as in Fig. 1. Rapid application of TTX (60 μM) was accomplished by a double-barreled solution switcher, which resulted in a complete blockade of I_{Na} (*trace b*) and a concomitant decrease in peak $[Ca^{2+}]_i$ (*trace d*). Average values of the peak $[Ca^{2+}]_i$ are presented as mean ± SEM of seven cells and shown in the bar graph on the right. Pipette Na^+ concentration was 15 mM. Note that the magnitude of the decrease in $[Ca^{2+}]_i$ transient induced by TTX was similar with the pump active (Fig. 4) and the pump inhibited (Fig. 3).

exchange by 64% (1.54 ± 0.23 vs. 0.55 ± 0.27 pA/pF; n = 6; $p < 0.01$) and to reduce the amplitudes of $[Ca^{2+}]_i$ transients by 26% (457 ± 78 to 310 ± 43 nM; $n = 8$; $p < 0.05$) in field-stimulated (0.5 Hz) myocytes and by 30% (864 ± 66 ($n = 32$) vs. 606 ± 98 nM ($n = 13$), $p < 0.05$) in myocytes stimulated by 200-ms voltage pulses from −80 to 0 mV at 0.25 Hz. Fig. 5 shows that in the presence of KB-R 7943 (5 μM) abrupt pump inhibition enhances the peak amplitudes of the $[Ca^{2+}]_i$ transients only by 4%, which is not statistically significant. These results suggest that the presence of a functionally intact Na/Ca exchanger is required for PI-induced increase in the magnitude of $[Ca^{2+}]_i$ transients.

Effects of abrupt pump inhibition on $[Ca^{2+}]_i$ transients and membrane currents when pipette Na concentration is zero

All results described in the foregoing experiments were obtained with a patch pipette Na^+ concentration of 15 mM,

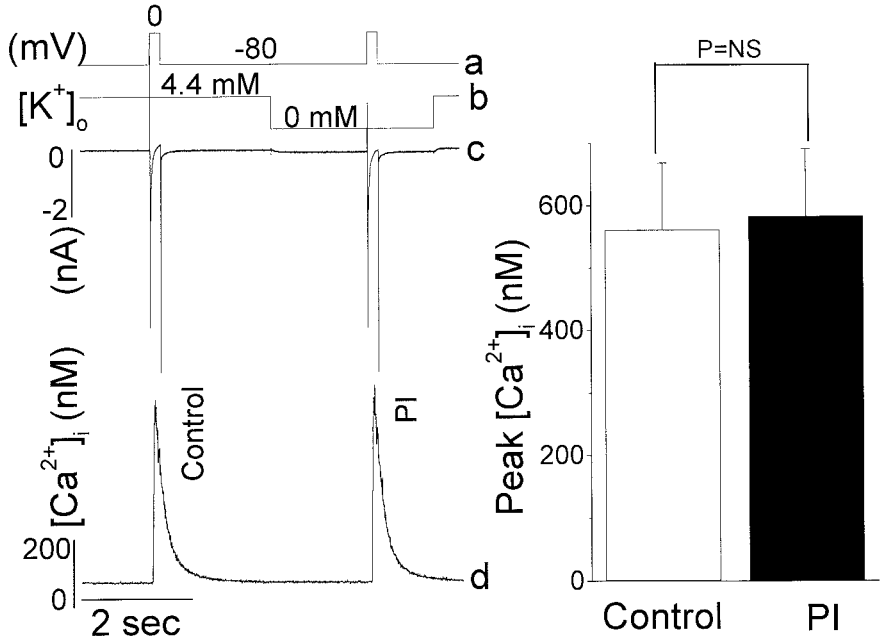

FIGURE 5 Effects of abrupt inhibition of the Na pump on $[Ca^{2+}]_i$ transients and membrane currents in the presence of KB-R 7943. Myocytes were pretreated with KB-R 7943 (5 μM) for 5 min to inhibit the reverse Na-Ca exchange. KB-R 7943 remained present during the conditioning pulses and the test pulse. The same experimental protocol as in Fig. 1 was employed to trigger membrane currents (I_{Na} and I_{Ca}, *trace c*) and $[Ca^{2+}]_i$ transients (*trace d*). Abrupt inhibition (by removing extracellular K^+ using a rapid solution switcher, *trace b*) of the Na pump for 1.5 s immediately before and during the test pulse was evidenced by the loss of the outward pump current (*trace c*). In the presence of KB-R 7943, pump inhibition (PI) did not significantly increase the amplitude of the peak $[Ca^{2+}]_i$ (*trace d* and the *bar graph* on the right). Results are presented as mean ± SEM of five cells. Pipette Na^+ concentration was 15 mM.

which is very close to the resting cytoplasmic $[Na^+]$ (Yao et al., 1998). To examine whether resting cytoplasmic $[Na^+]$ influences the effects of Na pump activity plus I_{Na} on $[Ca^{2+}]_i$ transients in mouse ventricular myocytes, we observed the effects of abrupt PI on $[Ca^{2+}]_i$ transients when pipette Na concentration was zero. As shown in Fig. 6, the voltage-clamp pulses (*trace a*) and the solution change protocol (*trace b*) are identical to those in Fig. 1. With 0 mM Na^+ in the patch pipette, outward pump current could no longer be observed (no downward shift of the baseline current, see *trace c*) during abrupt inhibition of the Na pump and pump inhibition did not result in any increase in the peak $[Ca^{2+}]_i$ transients. These results indicate the functional interaction between the Na pump and the Na channels during E-C coupling is influenced by cytosolic $[Na^+]$.

DISCUSSION

The importance of the Na/Ca exchange system (three Na^+ exchanged for one Ca^{2+}) is well established in cardiac muscle (Barry and Bridge, 1993; Hryshko and Philipson, 1997; Yao et al. 1998). The Na-Ca exchanger mainly operates in the forward mode extruding Ca^{2+} out of the cell during relaxation. It can also operate in a reverse mode bringing Ca^{2+} into the cell during the initial upstroke of the action potential of cardiac myocytes. This Ca^{2+} influx via the reverse-mode Na/Ca exchange has been proposed to increase the content of Ca^{2+} in SR (Barry et al., 1985; Bers, 1987; Nuss and Houser, 1992), directly trigger Ca^{2+}-induced Ca^{2+} release (CICR) (Nuss and Houser, 1992; Levi et al., 1993; Kohmoto et al., 1994), or to act in concert with I_{Ca} to activate CICR from SR (Yao et al. 1998; Litwin et al., 1998).

The $[Na^+]_i$, particularly the subsarcolemmal $[Na^+]$, has a profound impact on the function of the sarcolemmal Na/Ca exchanger because the transmembrane electrochemical gradient of Na^+ is an important driving force for the exchanger. Recently, results from several groups have indicated that activation of I_{Na} appears to promote Ca^{2+} entry into cardiac cells by stimulation of reverse-mode Na/Ca exchange, enhancing Ca^{2+} release from the SR (Leblanc and Hume, 1990; Levesque et al. 1994; Lipp and Niggli, 1994; Vites and Wasserstrom, 1996). A growing body of evidence supports the existence of a subsarcolemmal Na fuzzy space in which the Na diffusion is limited (Lederer et al., 1990; Bielen et al., 1991; Semb and Sejersted, 1996; Su et al., 1998). Accordingly, it is possible that $[Na^+]$ increases transiently underneath the sarcolemma during I_{Na}. In response to a transient rise of subsarcolemmal $[Na^+]$, the Na/Ca exchanger is able to promote Ca^{2+} entry into cardiac cells.

This idea is further supported by the results presented in this study. We have observed an increase in the magnitude of $[Ca^{2+}]_i$ transient in mouse ventricular myocytes during abrupt inhibition of the Na pump immediately before and during a voltage pulse by removing K_o^+ using a rapid solution switcher. This increase in the magnitude of the $[Ca^{2+}]_i$ transients during abrupt inhibition of the Na pump (Fig. 1) appears to be dependent on the presence of I_{Na} and the presence of cytosolic Na^+ as it was not increased when I_{Na} was blocked, or when $[Na^+]_i$ was zero. Presumably when the $[Na^+]$ is close to zero in both the cytosol and the

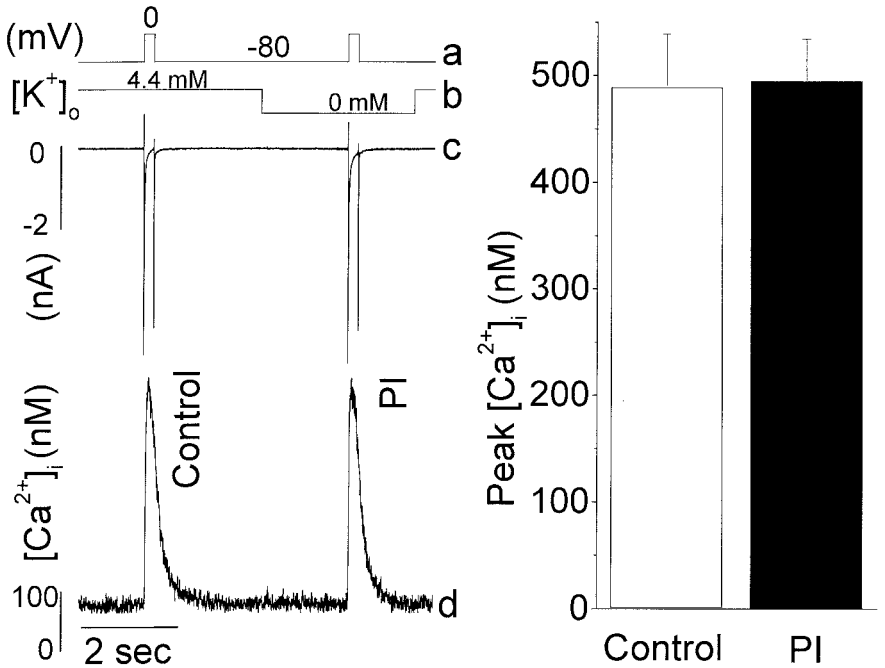

FIGURE 6 Effects of abrupt inhibition of the Na pump on $[Ca^{2+}]_i$ transients when $[Na^+]_{pip}$ is 0 mM. The same voltage protocol (*trace a*) and solution change protocol (*trace b*) as in Fig. 1 were used. Pipette Na^+ concentration was 0 mM. Outward pump current could not be observed (no downward shift of the baseline current, see *trace c*) during abrupt inhibition of the Na pump for 1.5 s immediately before and continuing during the test pulse. Pump inhibition (PI) did not result in any increase in peak $[Ca^{2+}]_i$ (*trace d*). The average values are shown in the bar graph on the right. Results are presented as mean ± SEM of five cells.

subsarcolemmal space, local accumulation of Na^+ during brief pump inhibition is not sufficient to cause enough enhancement of Na/Ca exchange to influence the $[Ca^{2+}]_i$ transient.

Modulation of reverse Na/Ca exchange appears to account for the effect of pump inhibition because the magnitude of the $[Ca^{2+}]_i$ transient was not increased when the reverse-mode Na/Ca exchange was largely inhibited by KB-R 7943 (Fig. 5). At the beginning of a step membrane depolarization to 0 mV, when membrane potential becomes more positive than the reversal potential of the Na/Ca exchange, the voltage-dependent sarcolemmal Na/Ca exchange functions in a reverse mode to generate a transient Ca^{2+} entry. This reverse-mode Na/Ca exchange is expected to be enhanced by the increased $[Na^+]$ in the subsarcolemmal space, which is produced by inhibiting the sarcolemmal Na pump during I_{Na}. Our results also suggest that exposure to zero K_o^+ is not altering SR Ca^{2+} loading by influencing Ca^{2+} extrusion via Na/Ca exchange just before the test pulse (Fig. 2). If this were the case, an enhanced Ca^{2+} release induced by I_{Ca} alone (i.e., in the presence of TTX) would be expected but was not observed. Also, as shown in Fig. 2, abrupt reactivation of the pump only 150 ms before a clamp pulse eliminated the increase in $[Ca^{2+}]_i$ transient.

Satoh et al. (2000) have recently reported that KB-R 7943 does not alter contractility of rat ventricular myocytes and have suggested that Ca^{2+} influx via reverse Na/Ca exchange plays no role in E-C coupling. However, our results in mouse myocytes show that both KB-R 7943 and TTX cause a significant reduction of the $[Ca^{2+}]_i$ transient in mouse ventricular myocytes. Because mouse ventricular myocytes have a relatively high $[Na^+]_i$ (Yao et al., 1998) and a relatively high density of the sarcolemmal Na/Ca exchanger (Su et al., 1999), the importance of reverse Na/Ca exchange in E-C coupling and effects of abrupt Na pump inhibition may be more marked in this species.

Although the above evidence supports the idea that activation of I_{Na} appears to promote Ca^{2+} entry into cardiac cells by stimulation of reverse-mode Na/Ca exchange, enhancing Ca^{2+} release from the SR, mechanisms by which I_{Na} eventually lead to an increase in SR Ca^{2+} release are still controversial. As discussed by Hancox and Levi (1995), there are three possible mechanisms by which activation of I_{Na} might influence CICR. First, I_{Na} might lead to an accumulation of subsarcolemmal Na and indirectly activate reverse Na/Ca exchange (Leblanc and Hume, 1990; Lipp and Niggli, 1994; Levesque et al. 1994; Vites and Wasserstrom, 1996). Results in the present study also support this idea (see above discussion). Second, voltage escape during I_{Na} might activate $I_{Ca,L}$, and this, in turn, can activate CICR (Bouchard et al., 1993; Sham et al., 1992; Vites and Wasserstrom, 1996). This could be the case when the cell membrane is depolarized to test potentials (from -70 to -40 mV) to activate I_{Na}, although Hancox and Levi (1995) have observed no significant activation of $I_{Ca,L}$ during a very brief voltage escape. In our study, however, I_{Na} and $I_{Ca,L}$ were activated simultaneously by a step membrane potential depolarization to 0 mV. Third, a brief membrane potential escape during I_{Na} might directly activate reverse-mode Na/Ca exchange to trigger SR release (Hancox and Levi, 1995). Even though voltage escape during I_{Na} will always be an issue in these types of experiments, it does not alter the interpretation of our results. If voltage escape occurs in some of our experiments (Figs. 1, 2, 5, and 6), it occurs in both control pulses and test pulses in the same cell. It should be noted that abrupt removal of K_o^+ should not alter transmembrane K^+ currents, and thus affect the degree of voltage escape, because K^+ channels were blocked with Cs^+, TEA^+, and Ba^{2+}.

Results presented in this study are also consistent with our previous observation that activation of I_{Na} increased reverse-mode Na/Ca exchange early after exposure of mouse ventricular myocytes to zero K_o^+ (Su et al. 1998) and the idea that Na/Ca exchange can be used as a sensitive indicator of changes in $[Na^+]$ in the subsarcolemmal space and the Na pump activity in guinea pig ventricular myocytes (Main et al., 1997). Functional interactions among Na/Ca exchange, Na pump, and Na channel are also supported by the work of James et al. (1999) who found different functional effects of genetically reduced levels of α1 and α2 Na/K-ATPase isoforms in mice, and proposed that the pump units containing the α2 isoform are associated with Na/Ca exchangers involved in Ca^{2+} signaling during E-C coupling. Such a co-localization may result from the association of these ion transporters or channels with the cytoskeletal protein ankyrin (Lee et al., 1996; Li et al., 1993; Srinivasan et al., 1992). Given that the Na/Ca exchange system plays prominent roles in cardiac calcium homeostasis and/or E-C coupling, the Na pump seems to be an important indirect modulator of cardiac calcium homeostasis and E-C coupling by influencing Ca^{2+} influx via reverse Na/Ca exchange.

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