Influence of prior Na\(^+\) pump activity on pump and Na\(^+\)/Ca\(^{2+}\) exchange currents in mouse ventricular myocytes

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Su, Zhi, Anruo Zou, Akihiko Nonaka, Iram Zubair, Michael C. Sanguinetti, and William H. Barry. Influence of prior Na\(^+\) pump activity on pump and Na\(^+\)/Ca\(^{2+}\) exchange currents in mouse ventricular myocytes. Am. J. Physiol. 275 (Heart Circ. Physiol. 44): H1808-H1817, 1998.—We examined the dependence of peak Na\(^+\) pump and Na\(^+\)/Ca\(^{2+}\) exchange currents on prior Na\(^+\) pump inhibition induced by exposure to zero extracellular K\(^+\) in voltage-clamped adult murine ventricular myocytes. Abrupt activation of the Na\(^+\) pump by reexposure of myocytes to extracellular K\(^+\) with a rapid solution switcher resulted in the development of a transient peak current at -500 ms, followed by a decline over 1-2 min to a steady-state level. The magnitudes of both the peak Na\(^+\) pump current \(I_p\) and the peak outward Na\(^+\)/Ca\(^{2+}\) exchange current, activated by rapidly reducing extracellular Na\(^+\) to zero with the solution switcher, were dependent on previous Na\(^+\) pump activity. [Na\(^-\)] gradients (Na\(^-\)-binding benzofuran isothiopyran fluorescence) between the patch pipette and the bulk cytosol were relatively small and could not account for the large differences between peak and steady-state \(I_p\) and reverse Na\(^+\)/Ca\(^{2+}\) exchange currents. Our results are consistent with the presence of a subsarcolemmal Na\(^+\) concentration gradient, which is similar for the Na\(^+\) pump and the Na\(^+\)/Ca\(^{2+}\) exchanger. These findings also support the hypothesis that the Na\(^+\) pump and the Na\(^+\)/Ca\(^{2+}\) exchanger are colocalized in the sarcolemma.

The sarcolemmal Na\(^-\)-K\(^+\)-ATPase, the "Na\(^+\) pump," utilizes energy derived from the hydrolysis of ATP to extrude three Na\(^+\) in exchange for two K\(^+\) (31). The Na\(^+\) pump thus generates an outward current that can be quantified by voltage-clamp techniques. In vitro studies of isolated canine cardiac sarcolemmal vesicles (25) and of partially purified cardiac Na\(^-\)-K\(^+\)-ATPase (1) have demonstrated a marked sensitivity to Na\(^+\) concentration at the internal surface of the transport enzyme with a dissociation constant \(K_d\) for activation of the Na\(^-\)-K\(^+\)-ATPase by Na\(^+\) of 10-20 mM. As pointed out by Semb and Sejersted (28), some studies in intact voltage-clamped guinea pig ventricular myocytes in which the intracellular Na\(^-\) concentration ([Na\(^-\)]) has been varied by changing the concentration of Na\(^+\) in the patch pipette have shown a considerably lower apparent sensitivity of the steady-state Na\(^+\) pump current \(I_p\) to [Na\(^-\)] (see Refs. 21 and 30). Although a theoretical analysis suggests that the steady-state cytosolic [Na\(^-\)] is determined by the pipette [Na\(^-\)] (21), this discrepancy could be due to a failure of the pipette [Na\(^-\)] to adequately control the subsarcolemmal [Na\(^-\)]. Indeed, Bielen et al. (4) have shown that in voltage-clamped ventricular myocytes when the Na\(^+\) pump is inhibited by exposure to zero extracellular K\(^+\) (K\(_o\)) and then abruptly reactivated by a resupply of K\(^+\), a transient peak in \(I_p\) can be observed, which then decays over 1-2 min to a steady-state level. This transient increase in Na\(^+\) pump activity has been interpreted as possibly reflecting the presence of a [Na\(^-\)] gradient between the bulk cytosol, where the [Na\(^-\)] is controlled by the patch pipette, and a subsarcolemmal space, the so-called Na\(^+\) "fuzzy space" (14), where the abrupt activation of the Na\(^+\) pump could cause local depletion of intracellular Na\(^+\) with a resulting fall in the [Na\(^-\)] to a level considerably below that present in the patch pipette (5). However, other factors could cause this type of behavior of \(I_p\) on abrupt reactivation of the Na\(^+\) pump, including the development of a substantial gradient in [Na\(^-\)] between the pipette and the bulk cytosol due to limited diffusion of Na\(^+\) from the tip of the patch pipette and depletion of K\(^+\) at the extracellular pump site due to either diffusion limitations in T tubules or adjacent to the K\(^-\) binding site on the Na\(^+\) pump (24).

We have recently demonstrated that isolated mouse adult ventricular myocytes have a high resting [Na\(^-\)] (15-16 mM; Ref. 33). This relatively high resting [Na\(^-\)] permits examination of \(I_p\) at a pipette [Na\(^-\)] equal to and below the resting [Na\(^-\)], conditions in which a gradient between the pipette and the bulk cytosol would not be expected to be prominent and better control of [Na\(^-\)], by pipette [Na\(^-\)] can be achieved. In the present work, we have therefore studied murine ventricular myocytes under voltage-clamp conditions to examine the relationships among pipette [Na\(^-\)], prior Na\(^+\) pump activity, and currents generated by the Na\(^+\) pump and the Na\(^+\)/Ca\(^{2+}\) exchanger, another electrogenic sarcolemmal cation transport system that is sensitive to subsarcolemmal [Na\(^-\)] (18). We have also determined the changes in [Na\(^-\)], at a variety of pipette [Na\(^-\)] with and without Na\(^+\) pump inhibition. Our results demonstrate that neither the development of a substantial gradient between the pipette [Na\(^-\)] and the [Na\(^-\)] nor depletion of K\(_o\) at the surface of the Na\(^+\) pump or in the T tubule system is likely to account for the observed peak and decay of \(I_p\) after activation of the pump. In addition, we find a similar dependence of the peak Na\(^+\)/Ca\(^{2+}\) exchanger currents (\(I_{Na/Ca}\)) on Na\(^+\) pump activity in voltage-clamped murine ventricular myocytes. Our results support the hypothesis that there is a subsarcolemmal [Na\(^-\)] gradient generated by the activity of the Na\(^+\) pump, which also affects the [Na\(^-\)] adjacent to the Na\(^+\)/Ca\(^{2+}\) exchanger.
METHODS

Dissociation of adult mouse ventricular myocytes. Mouse ventricular myocytes were dissociated as previously described (33). In brief, hearts were removed from anesthetized mice and immediately mounted on a perfusion system. After perfusion with modified Tyrode solution (Ca\(^{2+}\) free) for 5 min, the heart was digested for 7–12 min with 0.90 mg/ml collagenase D (Boehringer Mannheim Biochemicals) in modified Tyrode solution containing 25 \(\mu\)M CaCl\(_2\). The modified Tyrode solution (pH 7.4) contained the following (in mM): 126 NaCl, 4.4 KCl, 1.0 MgCl\(_2\), 18 NaHCO\(_3\), 11 glucose, 4 HEPES, 30 butanedione monoxime, and 0.13 U/ml insulin, and the solution was gassed with 5% CO\(_2\)-95% O\(_2\). The digested heart was removed from the cannula, and the left ventricle was cut into small pieces in a modified Tyrode solution containing 100 \(\mu\)M Ca\(^{2+}\). These pieces were gently agitated and then incubated in the same solution containing 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 solution containing 200 \(\mu\)M 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% heat-inactivated fetal bovine serum (HyClone), 47.5% MEM (GIBCO Laboratories), 47.5% modified Tyrode 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. Isolated cells were used for experiments within 6 h after isolation. All measurements in this study were performed at 25–27°C. In some experiments murine atrial myocytes were isolated with similar techniques.

Measurement of \(I_p\). The \(I_p\) was measured using a combination of the method described by Nakao and Gadsby (24) and a rapid solution-switcher technique (29, 34). Cells were voltage-clamped (Axopatch 200A, Axon Instruments, Foster City, CA) with single-suction pipettes, which were made from borosilicate glass tubing (Corning 7052, 1.65-mm OD, 1.2-mm ID, A-M Systems, Everett, WA) and had initial resistances of 15–25 MΩ when filled with pipette solution. Solutions were designed to minimize all other components of membrane currents (K\(^+\) currents were blocked by replacing intracellular (pipette) K\(^+\) with Cs\(^+\) and tetraethylammonium (TEA) and adding Ba\(^{2+}\) to extracellular solution; \(I_{NaCa}\) were inhibited by including 5 mM NiCl\(_2\) in the bathing solution; the inward Ca\(^{2+}\) current was blocked by using a nominal zero-Ca\(^{2+}\) solution and NiCl\(_2\)). The standard pipette solution contained (in mM) 20 NaCl, 110 CsOH, 110 aspartic acid, 20 TEA-Cl, 2.0 MgCl\(_2\), 5.0 EGTA, 5.0 MgATP, 10 HEPES, 10 glucose, and 5.0 creatine phosphate. The pH was adjusted to 7.2 with CsOH. Pipette [Na\(^+\)] was varied (5–100 mM) by equimolar adjustment of Cs\(^+\) and Na\(^+\) concentrations. The holding potential was set to –40 mV throughout the experiments to inactivate sarcollemal Na\(^+\) channels. We voltage-damped the myocytes to 0 mV when the \(I_p\) was measured. Rapid change of the extracellular solution was accomplished with a rapid solution swicher. This device changes the bulk solution surrounding a myocyte within 4 ms and the \(K^+\) adjacent to the sarcotlemma with a 90% decay time value (\(t_{90}\)) of 147 ms for an \(K^+\) increase and 260 ms for a \(K^+\) decrease in rat myocytes (34). \(I_p\) was activated by resupplying K\(^+\) in extracellular solution containing (in mM) 140 NaCl or 140 Tris-HCl, 5 KCl, 1.0 MgCl\(_2\), 2.0 BaCl\(_2\), 5.0 NiCl\(_2\), 5.0 HEPES, and 5.5 glucose (pH 7.4 adjusted with NaOH). \(I_p\) was also evaluated directly by measuring the loss of outward pump current on rapidly removing K\(^+\). To observe the influence of Na\(^+\) influx through Na\(^+\) channels on \(I_p\), in some experiments voltage pulses (from –80 to –10 mV, 50 ms, 5 Hz) were applied before peak \(I_p\) at each time point was recorded. The interval between the last pulse and each measurement of \(I_p\) was 200 ms. Peak \(I_p\) was again measured by a voltage-clamp technique (held at 0 mV) and activated by resupplying external K\(^+\) for 5 s using the rapid solution switcher.

To normalize the measured membrane currents to cell surface area, capacity current transients in response to a 10-mV depolarization pulse applied from a holding potential of −40 mV were recorded. These transients were integrated to give total membrane capacitance (pF). Thus membrane currents were expressed as current density (pA/pF).

Measurement of \(I_{NaCa}\). The reverse-exchange current (Ca\(^{2+}\) in, Na\(^+\) out) was measured by means of a whole cell voltage-clamp technique (6). Myocytes were voltage-clamped as described for measurement of \(I_p\). The cells were held at a potential of −40 mV. To measure outward exchange current, the pipette contained (mM) 0.3 MgCl\(_2\), 14.0 EGTA, 3.0 MgATP, 5.5 dextrose, and 10 HEPES. Calcium (3.9 mM) was added as H\(_2\)CaEGTA 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. Pipette [Na\(^+\)] was varied from 5 to 40 mM by adjusting the amount of NaCl added to the pipette solution. To activate outward reverse-exchange currents, voltage-clamped cells were superfused in a microstream containing (mM) 138 NaCl, 1.0 MgCl\(_2\), 1.0 CaCl\(_2\), 11.0 dextrose, and 12 HEPES. The pH was adjusted to 7.4 with NaOH, and then NaCl was added to give a final Na\(^+\) concentration of 145 mM. Outward exchange current was activated when the cell was abruptly immersed in an adjacent microstream of solution containing Li\(^+\) instead of Na\(^+\), using the solution-switching device. Preliminary experiments showed that currents elicited with Tris or Li\(^+\) substitution were similar.

To examine the effect of Na\(^+\) influx on outward \(I_{NaCa}\), we also applied 12 voltage pulses (from –80 to –10 mV, 50 ms, 5 Hz) to activate Na\(^+\) current (\(I_{Na}\)) 200 ms before recording \(I_{NaCa}\) at each time point, as described previously. For these experiments, nifedipine (10 µM) was added to the bathing solution to block Ca\(^{2+}\) current.

The effects of Na\(^+\) pump inhibition on the forward \(I_{NaCa}\) (Na\(^+\) in, Ca\(^{2+}\) out) were assessed using a modification of a previously described method (33). Myocytes were loaded with fluo 3 by exposure to 1 µM fluo 3-AM at 30°C for 30 min. Myocytes were then voltage-clamped at –80 mV with a single suction pipette filled with a solution composed of (in mM) 15 NaCl, 100 CsCl, 30 TEA-Cl, 5 MgATP, 10 HEPES, and 5.5 dextrose (pH 7.1 adjusted with CsOH). The voltage-clamped cell was superfused in a microstream containing (mM) 138 NaCl, 1.0 MgCl\(_2\), 4.4 KCl, 1.08 CaCl\(_2\), 2 CsCl, 0.1 BaCl\(_2\), 11 dextrose, and 24 HEPES (pH 7.4 adjusted with NaOH to give a final extracellular [Na\(^+\)]([Na\(^+\)]) of 145 mM). After a train of steady-state conditioning pulses (8 200-ms pulses to 0 mV, 0.25 Hz), the cell was abruptly superfused for 6 s in an adjacent switcher microstream of solution in which 10 mmol/l of caffeine was added to release sarcoplasmic reticulum Ca\(^{2+}\) and activate the electrogenic forward Na\(^+\)/Ca\(^{2+}\) exchange (3:1 stoichiometry). Intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]) was estimated by assuming that the diastolic [Ca\(^{2+}\)] under these experimental conditions was 80 nM (33). The cell was then exposed for 5 min to 0 K\(^+\) solution to inhibit the Na\(^+\) pump, and the protocol was repeated. The effects of Na\(^+\) pump inhibition on the magnitudes of the peak forward exchange current, the sarcoplasmic reticulum Ca\(^{2+}\) content (33), and the magnitude and rate of decline of the caffeine-induced Ca\(^{2+}\) transient were then measured.

Measurement of [Na\(^+\)], [Na\(^+\)], was estimated using the Na\(^+\)-sensitive fluorescent dye, Na\(^+\)-binding benzofuran isoph-
thallate fluorescence (SBFI), by modification of previously described methods (33). Extracellular solution (with or without K−) and pipette solution (except for the added SBFI) were the same as that used for the measurements of Ip. Ventricular myocytes were dialyzed with 0.5 mM SBFI salt via patch pipettes that had initial tip resistances of 1.5–3 MΩ. The myocytes were clamped at −40 mV and illuminated alternatively at 120 Hz by 340- and 380-nm excitation light (bandpass filters P10–340 and P10–380, Corion, Holliston, MA) using an optical switcher (DX-1000, Solamere Technology Group, Salt Lake City, UT), and the resulting fluorescence signals at 510 nm (P10–510 Corion) were detected with a photomultiplier (SFX-2, Solamere Technology Group). The ratio of two signals corresponding to excitation wavelengths at 340 and 380 nm was used as an indicator of [Na+]i.

Background fluorescence measured after a gigaseal was obtained just before the rupture of the membrane was zeroed at excitation wavelengths of 340 and 380 nm, respectively. All recordings started 10 min after establishment of the whole cell configuration to allow sufficient time for diffusion of SBFI from pipettes into myocytes.

When the Na+ pump and Na+ /Ca2+ exchange were inhibited by 0 K+ and Ni2+, [Na+]i was approximately equal to the pipette [Na+]i. For calibration, myocytes were bathed in zero-K+ solution for 10 min and dialyzed with pipette solutions containing 3.75, 7.5, 10, and 20 mM Na. The fluorescence ratio was then recorded before and 5 min after exposure of a cell to 5 mM K+ to reactivate the Na+ pump. The relationship between the fluorescence ratio (R) and pipette [Na+]i (with the Na+ pump inhibited) was fitted to the equation: 

\[ R = K_a \times (R - R_{\text{min}})/(R_{\text{max}} - R) \]

With the resulting calibration curve, the fluorescence ratios were then converted to [Na+]i values.

Statistics. Physiological recordings were digitized online with a DigiData 1200 Interface (Axon Instruments) and stored on disk. The digitized data were analyzed with pCLAMP6 (Axon Instruments) and ORIGIN (Micorcal Software). All curve fitting was performed using ORIGIN. Results are presented as means ± SE, and statistical differences were determined by ANOVA and unpaired or paired t-tests where appropriate. Differences were considered significant at P < 0.05.

RESULTS

Transient and steady-state Ip. In isolated mouse ventricular myocytes incubated in zero-K+ solution, Ip was activated by resupplying K+ with the rapid solution switcher. As shown in Fig. 1A, mouse ventricular myocytes exhibited a transient peak current within <1 s, followed by a decline to a near steady-state level, which plateaued 2 min after the pump was reactivated. The steady-state Ip could also be measured by abruptly exposing a clamped myocyte to 0 K+, as shown in Fig. 1B. Note that Ip decayed to 0 within ~400 ms of reducing K+ to 0. This time course is consistent with the rate of diffusion of K+ from the external sarcoplasmic surface to the bulk solution (34) and the nonlinear dependence of pump rate on [K+]o.

Both peak Ip and steady-state Ip were eliminated in the presence of 2 mM ouabain (not shown). Peak Ip increased in a time-dependent manner over 4–5 min after exposure to zero-K+ solution following establishment of a gigaseal. However, the steady-state Ip did not show any time-dependent change when the pipette [Na+]i was similar to the resting [Na+]i (Fig. 2). The integral of the area under the current transient as it decayed over a 2-min recording period from peak to steady-state Ip was 3.9 ± 0.3 nC (n = 16) when the pipette [Na+]i was 20 mM. On the basis of the stoichiometry of the electrogenic Na+ -K+ pump (3 Na+ exchanged for 2 K+), the amount of Na+ pumped out during the Ip transient was 0.12 ± 0.01 pmol or ~12% of the initial total intracellular Na+, assuming that the initial [Na+]i is equal to the pipette [Na+]i and the average cell volume is 50 pl (cell length = 125 µm, width = 20 µm, thickness = 20 µm).

Both peak Ip and steady-state Ip were dependent on the pipette [Na+]i (Fig. 3). The Kd5 values (concentration of pipette [Na+]i required for 50% stimulation of pump activity estimated from a Hill plot) for peak Ip and steady-state Ip were 14 and 49 mM, respectively.
The mean value of steady-state I_p at a pipette [Na⁺] of 20 mM (0.36 ± 0.06 pA/pF, n = 5) is quite close to that obtained directly by measuring the loss of outward pump current on exposure to zero-[K⁺]o solution (Fig. 1B). An important point is that peak I_p was substantially greater than steady-state I_p, even at a pipette [Na⁺] similar to resting cytoplasmic [Na⁺] (~16 mM) in undamped cells when no gradient in [Na⁺] between cytosol and pipette would be expected. For example, the magnitude of steady-state I_p at a pipette [Na⁺] of 20 mM is only 24% of the corresponding peak I_p, and lower than peak I_p at a pipette [Na⁺] of 5 mM, suggesting that [Na⁺] in the subsarcolemmal space (close to intracellular Na⁺-binding sites of pump) in resting mouse ventricular myocytes is <5 mM.

Changes in [Na⁺]i during activation of the pump. Several explanations could account for the finding that peak I_p is greater than steady-state I_p. One explanation mentioned above is that there is a substantial [Na⁺] gradient between subsarcolemmal space and bulk cytoplasm in mouse ventricular myocytes. The other is that a large [Na⁺] gradient develops between the pipette solution and cytoplasm of the clamped myocytes when the Na⁺ pump is activated. To examine the second possibility, we estimated the changes in cytosolic [Na⁺] during the inhibition and activation of the Na⁺ pump at various pipette [Na⁺]. The results are shown in Fig. 4. The fluorescence ratio (F₃₄₀/F₃₈₀) increased with increase in the pipette [Na⁺] over the range of 3.75–20 mM (Fig. 4A). With the Na⁺ pump inhibited by zero [K⁺]o and Na⁺/Ca²⁺ exchange inhibited by Ni²⁺, we assumed that [Na⁺] was equal to pipette [Na⁺]. The resulting calibration curve yielded values for K_d, R_min, and R_max (Fig. 4A) consistent with previous reports of in vivo characteristics of SBFI (8, 27). The [Na⁺] was then estimated in zero [K⁺]o, and in normal [K⁺]o by the observed F₃₄₀/F₃₈₀ before and 5 min after resupply of [K⁺]o (Fig. 4B). The cytosolic [Na⁺] was similar before and after activation of the Na⁺ pump, with a maximum difference of ~3 mM noted at a pipette [Na⁺] of 20 mM. These differences in [Na⁺] between pipette and bulk cytoplasm cannot account for the large difference between peak I_p and steady-state I_p (Fig. 3). This finding is consistent with the presence of a substantial [Na⁺] gradient between the bulk cytoplasm and subsarcolemmal space.

Fig. 3. Dependence of I_p on pipette Na⁺ in mouse ventricular myocytes. Current transient was activated by resupplying [K⁺]o after exposure to 0-K⁺ solution for 5 min and measured at 0 mV by means of whole cell recording. A: representative current tracings. Numbers on right indicate Na⁺ concentrations (mM) in pipettes. B: currents normalized by cell capacitance (pA/pF) and presented as means ± SE of 6–10 cells. K_0.5 represents the concentration of Na⁺ required for half-maximal activation of the I_p, determined from Hill equation.

Fig. 4. Intracellular Na⁺ measurements in mouse ventricular myocytes. Myocytes were voltage-clamped at 0 mV and dialyzed with pipettes containing 500 µM Na⁺-binding benzofuran isophthalate fluorescence (SBFI). A: mean fluorescence ratio (F₃₄₀/F₃₈₀) of SBFI in clamped cell as a function of pipette Na⁺ 5 min after cell was immersed in 0-K⁺ solution. We assume that in 0-[K⁺]o, the pipette [Na⁺] equals the cytosolic [Na⁺], allowing calibration of F₃₄₀/F₃₈₀ ratio. Ratio of pipette [Na⁺] was fit to the equation [Na⁺] = K_d (R - R_min)/(R_max - R). Derived apparent R_min, R_max, and K_d are shown in A. B: relationships between pipette [Na⁺] and cytosolic [Na⁺] when the Na⁺ pump is reactivated for 5 min by reexposure to 5 mM [K⁺]o. Results for each concentration point are presented as mean ± SE of 5–7 cells for each pipette [Na⁺]. **P < 0.01, zero [K⁺]o vs. normal [K⁺]o by paired t-test.
Effects of $[\text{Na}^+]_o$ and $[\text{K}^+]_o$ on pump current. To explore the possibility that extracellular $\text{Na}^+$ influx (leak) from the bath solution may influence $[\text{Na}^+]$ in the subsarcolemmal space, $I_p$ was also measured in the absence of extracellular $\text{Na}^+$. Removal of extracellular $\text{Na}^+$ during exposure to zero $[\text{K}^+]_o$ did not reduce $I_p$, magnitudes induced by subsequent exposure to 5 mM $[\text{K}^+]_o$ (Fig. 5A). In fact, peak $I_p$ was higher in zero extracellular $\text{Na}^+$ than in normal extracellular $\text{Na}^+$. The increased peak $I_p$ in the absence of extracellular $\text{Na}^+$ is probably due to an increase in the affinity of the pump for external $\text{K}^+$ on removal of external $\text{Na}^+$ (24). With zero $\text{Na}^+$ in the pipette and normal extracellular $\text{Na}^+$, small pump currents could be detected (relative peak $I_p$ 8.3%, relative steady-state $I_p$ 2.6%), which is consistent with the results of Nakao and Gadsby (24) and consistent with the conclusion that $\text{Na}^+$ influx has little effect on subsarcolemmal $[\text{Na}^+]$ in resting cells.

It has been reported that $\text{Na}^+$ pumps are located in both peripheral sarcolemma and T tubules of cardiomyocytes (19). Accordingly, the current transient could be caused in part by the depletion of external $\text{K}^+$ in the T tubules. To examine this possibility, we also measured $I_p$ at an $K_o^+$ of 10 mM. Figure 5B shows that 10 mM $[\text{K}^+]_o$ did not affect the $I_p$ amplitudes. A transient peak $I_p$ was also observed with a $K_o^+$ = 100 mM (data not shown). Furthermore, we observed a transient peak $I_p$ with a subsequent decline to a steady-state $I_p$ in mouse atrial myocytes, which lack T tubules (data not shown). These results suggest that an inward $\text{Na}^+$ leak does not contribute significantly to the maintenance of subsarcolemmal $[\text{Na}^+]$ in cells clamped in zero $K_o^+$ and that depletion of $\text{K}^+$ in T tubules is not a factor responsible for the observed decay of the pump current. $\text{Na}^+$ influx through $\text{Na}^+$ channels might be expected to influence subsarcolemmal $[\text{Na}^+]$ (13, 14). As shown in Fig. 6, induction of a series of $I_{\text{Na}^+}$ significantly increased the magnitude of the pump current early after exposure to zero $K_o^+$ but did not influence the plateau value after more prolonged pump inhibition.
peak \( I_p \). Reexposure of the cell to normal [K\(^+\)]\(_o\) caused \( I_{NaCa} \) to decrease to 15% of the plateau value in 2 min after [K\(^+\)]\(_o\) was reapplied (Fig. 8B). Induction of a series of \( I_{Na} \) increased the magnitude of \( I_{NaCa} \) early after exposure to zero [K\(^+\)]\(_o\), but did not affect its magnitude at plateau, as was observed with the effects of \( I_{Na} \) on \( I_p \) (Fig. 8C).

In Fig. 9 it is shown that the magnitude of outward \( I_{NaCa} \) is highly Na\(^+\) dependent and that \( I_{NaCa} \) amplitudes measured with the activated Na\(^+\) pump (in normal [K\(^+\)]\(_o\)) were significantly lower than with the inhibited Na\(^+\) pump (in zero [K\(^+\)]\(_o\)). The pattern of alteration in outward \( I_{NaCa} \) in normal [K\(^+\)]\(_o\) and zero [K\(^+\)]\(_o\) is consistent with that observed in our \( I_p \) studies, further supporting the existence of a subsarcolemmal [Na] gradient.

Inward \( I_{NaCa} \). Figure 10 shows an example of the effects of Na\(^+\) pump inhibition on the inward \( I_{NaCa} \) resulting from forward Na\(^+\)/Ca\(^{2+}\) exchange, activated by abrupt release of Ca\(^{2+}\) from the sarcoplasmic reticulum by caffeine. Activation of \( I_{NaCa} \) slightly preceded the rise in cytosolic Ca\(^{2+}\) due to caffeine-induced release of Ca\(^{2+}\) from the sarcoplasmic reticulum, and analysis of unfiltered \( I_{NaCa} \) and [Ca\(^{2+}\)]\(_i\) transients showed that the peak \( I_{NaCa} \) consistently occurred ~70 ms before the peak [Ca\(^{2+}\)]\(_i\). The [Ca\(^{2+}\)]\(_i\), as monitored by fluo 3 fluorescence, then declined as \( I_{NaCa} \) declined from a peak

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**Fig. 8.** Time course of effects of extracellular K on outward \( I_{NaCa} \) in mouse ventricular myocytes. Myocytes were voltage-clamped at -40 mV with suction pipettes containing 20 mM Na\(^+\). A: cell was clamped for 10 min in normal [K\(^+\)]\(_o\) to dialyze with EGTA and then exposed to 0 [K\(^+\)]\(_o\). \( I_{NaCa} \) rose to a plateau within 5 min of incubation in 0 [K\(^+\)]\(_o\). B: after the cell was clamped in 0 [K\(^+\)]\(_o\) solution for 10 min to reach a plateau, \( I_{NaCa} \) was measured after resupply of [K\(^+\)]\(_o\) or in the continued presence of 0 [K\(^+\)]\(_o\) (dotted line). Reactivation of Na\(^+\) pump by resupply of [K\(^+\)]\(_o\) caused a decay of \( I_{NaCa} \) within 1–2 min. C: effect of activating \( I_{Na} \) before measurement of \( I_{NaCa} \). Protocol was as described in Fig. 6. Currents were normalized by cell capacitance (pA/pF). Data were expressed as means ± SE of 7 cells (A) and 5 cells (B and C).

**Fig. 9.** Dependence of \( I_{NaCa} \) on pipette [Na\(^+\)] in presence and absence of Na\(^+\) pump inhibition. Currents were normalized by cell capacitance (pA/pF). Means ± SE are plotted for 5–7 cells in 0 [K\(^+\)]\(_o\) and 5 cells for each point in normal [K\(^+\)]\(_o\). Dependence of \( I_{NaCa} \) on pipette [Na\(^+\)] with and without prior inhibition of Na\(^+\) pump is similar to that noted for \( I_p \) at pipette [Na\(^+\)] from 5 to 40 mM (compare with Fig. 3B).

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**Fig. 10.** Examples of effects of pump inhibition on forward \( I_{NaCa} \). A: the last of a series of 8 voltage-clamp pulses from -80 to +0 mV to load the sarcoplasmic reticulum with Ca\(^{2+}\). Cell was then abruptly exposed to 10 mM caffeine with rapid switcher (top trace) causing a large [Ca\(^{2+}\)]\(_i\) transient (fluo 3 fluorescence, arbitrary units) and corresponding inward \( I_{NaCa} \). B: same sequence was repeated after exposure of the same cell to 0 K\(^+\)\(_o\) for 5 min. Note that the intracellular [Ca\(^{2+}\)]\(_i\) transient amplitude was substantially increased after pump inhibition with 0 K\(^+\)\(_o\), but peak forward \( I_{NaCa} \) was reduced.
value. After inhibition of the Na\(^+\) pump by exposure to zero K\(^+_o\) for 5 min, a greater [Ca\(^{2+}\)] was observed after caffeine exposure (Fig. 10B), consistent with increased loading of the sarcoplasmic reticulum via reverse Na\(^+\)/Ca\(^{2+}\) exchange during the preceding voltage-clamp pulses. However, the peak I\(_{NaCa}\) observed during the caffeine-induced Ca\(^{2+}\) transient was diminished. Average results are shown in Table 1. The peak [Ca\(^{2+}\)] of the caffeine-induced transient and the sarcoplasmic reticulum Ca\(^{2+}\) content were increased by ~40% after pump inhibition, yet the magnitude of the peak I\(_{NaCa}\) was decreased significantly. In addition, the \(r\) of I\(_{NaCa}\) decay and [Ca\(^{2+}\)] decline were increased. These findings are consistent with a rise in the subsarcolemmal Na\(^+\) caused by Na\(^+\) pump inhibition and a resulting reduction in forward Na\(^+\)/Ca\(^{2+}\) exchange despite an increase in [Ca\(^{2+}\)].

**DISCUSSION**

Factors contributing to the transient nature of abruptly activated I\(_p\) in intact ventricular myocytes. Our findings clearly indicate that in voltage-clamped adult murine ventricular myocytes, abrupt reactivation of the Na\(^+\) pump by rapid resupply of K\(^+_o\) results in a \(I_p\) that peaks and then declines to a steady-state value. This phenomenon has not been previously characterized in mouse ventricular myocytes, although it has been previously documented in other isolated cardiac myocyte preparations, including rabbit Purkinje and ventricular cells (4) and cultured sheep Purkinje cardioballs (10). Assuming the cytosolic Na\(^+\) is controlled by the pipette [Na\(^+\)], this would imply a nonlinear relationship between pump rate and cytosolic [Na\(^+\)] after abrupt activation of the pump. However, Eisner et al. (9) reported a linear relation between intracellular Na\(^+\) activity (a\(_{Na}^i\)) and I\(_p\) after activation of pump current by resupply of Rb\(^+\) in intact Purkinje fibers. This difference may indicate that a slower rate of pump reactivation is achieved in intact tissue as contrasted with isolated cells due to the time required to exchange the bath and for diffusion of activating cation through the tissue.

The magnitudes of the steady-state I\(_p\) that we measured in mouse myocytes at 26°C are similar to those reported by Nakao and Gadsby (24) in guinea pig myocytes at 35°C, at comparable [Na\(^+\)]. These investigators did not measure effects of abrupt reactivation of I\(_p\). In our experiments, we have examined the relationship between peak and steady-state I\(_p\) at a variety of different pipette [Na\(^+\)], and we have clearly documented that the peak I\(_p\) is substantially greater than the steady-state pump current, even at pipette [Na\(^+\)] of 100 mM. The peak current measured at a pipette [Na\(^+\)] of 100 mM was 2.1 pA/pF, a value similar to that predicted based on the pump density and maximal turnover rate (−2.8 pA/pF, see Ref. 22). A pipette [Na\(^+\)] of 25–30 mM was required to produce a steady-state I\(_p\) equal to a peak I\(_p\) produced at a pipette [Na\(^+\)] of 5 mM, a value well below the normal resting [Na\(^+\)] of 16 mM in these cells. These findings imply that there is a substantial gradient between the pipette [Na\(^+\)] and the subsarcolemmal [Na\(^+\)], which is sensed by the Na\(^+\) pump.

Our results indicate that only a small component of this gradient could be due to a gradient between the pipette and the bulk cytosol. Measurements of cytosolic [Na\(^+\)] by means of the fluorescent Na\(^+\)-sensitive dye SBFI indicate that when the [Na\(^+\)] in the pipette is higher than the resting [Na\(^+\)] in the cell (20 vs. 16 mM), a small [Na\(^+\)] gradient is apparent. However, it is clear that this is too small to account for the apparent differences in the peak and steady-state I\(_p\) at pipette [Na\(^+\)] <20 mM consistent with the analysis of Carmeliet (5). The apparent difference in peak and steady-state I\(_p\) also does not appear to be accounted for by a leak of Na\(^+\) into the subsarcolemmal space during exposure to zero [K\(^-_o\)], because it was not affected by elimination of extracellular Na\(^+\). Furthermore, depletion of K\(^+\) in the T tubules or at the external surface of the Na\(^+\) pump site appears unlikely. In our experiments, peak and steady-state I\(_p\) were not affected by increasing the K\(^+_o\) concentration used to activate the Na\(^+\) pump, and we also observed peak transients under similar experimental conditions in atrial myocytes that lack T tubules. The conclusion that T tubules are not necessary to explain this phenomenon is also supported by the results of Bielen et al. (4), who noted transient I\(_p\) in Purkinje cells that also lack T tubules. Thus our results provide further support for the existence of a [Na\(^+\)] gradient between the bulk cytoplasm and the inner surface of the Na\(^+\) pump transport site.

**Table 1. Effects of Na\(^+\) pump inhibition on caffeine-induced [Ca\(^{2+}\)] transients, inward Na\(^+\)/Ca\(^{2+}\) exchange currents, and SR Ca\(^{2+}\) content**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Peak [Ca(^{2+})] (nM)</th>
<th>Peak I(_{NaCa}) (pA/pF)</th>
<th>SR Ca(^{2+}) content (pA ms/pF)</th>
<th>% of Decline [Ca(^{2+})]</th>
<th>I(_{NaCa}) (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1,399 ± 322</td>
<td>1.06 ± 0.05</td>
<td>604 ± 28</td>
<td>925 ± 88</td>
<td>587 ± 12</td>
</tr>
<tr>
<td>Pump inhibited</td>
<td>1,903 ± 435</td>
<td>0.96 ± 0.03</td>
<td>831 ± 94</td>
<td>1,444 ± 237</td>
<td>966 ± 111</td>
</tr>
<tr>
<td>% Change</td>
<td>+45 ± 11</td>
<td>−8 ± 2</td>
<td>+38 ± 21</td>
<td>+55 ± 165</td>
<td>+64 ± 27</td>
</tr>
<tr>
<td>P values</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 5 hearts/group. [Ca\(^{2+}\)], intracellular Ca\(^{2+}\) concentration; I\(_{NaCa}\), Na\(^+\)/Ca\(^{2+}\) exchange current; SR, sarcoplasmic reticulum.

Based on the experiments of Lederer et al. (14), Lederer et al. (14) have speculated about the size of the Na\(^+\)-restricted space (Na\(^+\) fuzzy space). In our experiments, integration of the I\(_p\) transient allowed an estimation that the total amount of Na\(^+\) extruded from the cell during the transient was equivalent to ~12% of the initial Na\(^+\), at a pipette [Na\(^+\)] = 20 mM. However, it is clear that a substantial component of this total current transient reflects movement of Na\(^+\) from the pipette to the cytosol and from the cytosol to the subsarcolemmal space. Therefore, it does not appear to be possible to estimate the absolute size of the Na\(^+\) "fuzzy space" from examination of the integral of the I\(_p\). It is worth noting that in our experiments, activation of the I\(_{Na}\) before measurement of the peak Na\(^+\) pump and exchange
currents (Figs. 6 and 8C) increased these currents substantially early after exposure to zero \( K^+ \), when the subsarcolemmal \( [Na^+] \) was presumably still low. However, after more prolonged inhibition of the pump, with presumed equilibration between subsarcolemmal \( [Na^+] \) and cytosolic \( [Na^+] \), no effect of \( I_{Na^+} \) on pump and exchanger currents was apparent. This finding suggests that \( Na^+ \) entering the cell via the \( Na^+ \) channel may increase \( Na^+ \) concentration in the fuzzy space when the pump is not inhibited, as suggested by LeBlanc and Hume (13). However, it is not clear that \( Na^+ \) entering the cell by other \( Na^+ \) influx pathways gains immediate access to the restricted space sensed by the \( Na^+ \) pump, since the resting cytosolic \( [Na^+] \) (16 mM) in nonclamped cells appears higher than the \( [Na^+] \) sensed by the \( Na^+ \) pump (<5 mM). This would not be expected if \( Na^+ \) entering the resting cell across the sarcolemma had to traverse this space to gain access to the cytosol.

The apparent dependence of the \( I_p \) on \( [Na^+]_i \) in our experiments allowed an estimation of the \( K_d \) for pump activation by \( Na^+ \) of 14.3 mM when the peak current was used as an estimate of the pump activity versus 49 mM when the steady-state current was used as an indicator of pump activity. The former value is much closer to the \( K_d \) estimated in the isolated vesicle or partially purified enzyme studies of the \( Na^+-K^+ \)-ATPase. This observation also provides further support for the idea that the \( Na^+ \) activity sensed by the \( Na^+ \) pump is more accurately reflected by peak \( I_p \) than by the steady-state current. This finding emphasizes the importance of using the peak \( I_p \) to estimate \( Na^+ \) pump activity when voltage-clamp techniques are being applied to assess \( Na^+ \) pump density in ventricular myocytes (4).

Influence of \( Na^+ \) pump activity on \( I_{Na^+Ca} \). Previous investigators have used voltage-clamp techniques to assess \( Na^+ /Ca^{2+} \) exchanger activity. In work by Miura and Kimura (20), voltage-clamped myocytes were exposed to zero-\( Ca^{2+} \) and \( Na^+ \) extracellular solutions, and then extracellular \( Ca^{2+} \) was abruptly resupplied, resulting in the generation of an outward current. This approach has been used to determine the influence of intracellular \( Na^+ \) on \( I_{Na^+Ca} \) magnitude, and in all these experiments, the \( Na^+ \) pump was completely inhibited by exposure to zero \( K^+ \) during the current measurements.

Chin et al. (6) developed an alternative approach. In their experiments, voltage-clamped myocytes were abruptly exposed to zero extracellular \( Na^+ \) in the presence of extracellular \( Ca^{2+} \), resulting in the generation of an outward current due to reverse \( Na^+ /Ca^{2+} \) exchange. Chin et al. (6) observed that \( I_{Na^+Ca} \) quickly decayed if the \( Na^+ \) pump was reactivated by a resupply of \( K^+ \). In these experiments, which used voltage-clamped guinea pig ventricular myocytes, the pipette \( [Na^+] \) was considerably higher than the resting \( [Na^+] \), and a dependence of the exchanger current on \( Na^+ \) pump activity was attributed to an inability to control bulk cytosol \( [Na^+] \) with the pipette \( [Na^+] \) in the presence of \( Na^+ \) pump activity.

In our experiments, a marked dependence of \( I_{Na^+Ca} \) on the previous activity of the \( Na^+ \) pump was noted even at a pipette \( [Na^+] \) equal to or even below the normal resting cytosolic \( [Na^+] \) in mouse ventricular myocytes. Indeed, the relationship between the peak \( I_{Na^+Ca} \) measured after a period of inhibition of the \( Na^+ \) pump and in the absence of pump inhibition was similar to that observed between the peak and steady-state \( I_p \) measurements (compare Figs. 3B and 9). Furthermore, the time required to reach a plateau of \( I_{Na^+Ca} \) and peak \( I_p \) after inhibition of the \( Na^+ \) pump was similar when adequate time for diffusion of EGTA to buffer \( Ca^{2+} \) was allowed before \( Na^+ \) pump inhibition was instituted. (If \( Ca^{2+} \) is not adequately buffered with EGTA during measurement of \( I_{Na^+Ca} \), \( Ca^{2+} \) influx raises \([Ca^{2+}]_c \), reducing the \( Ca^{2+} \)-gradient and causing contraction). The \( I_{Na^+Ca} \) also demonstrated a similar time course of decay to a steady-state value after reactivation of the \( Na^+ \) pump.

The experiments shown in Fig. 10 and Table 1 also provide evidence that the magnitude of forward \( Na^+ /Ca^{2+} \) exchange is influenced by \( Na^+ \) pump activity. It is of interest that the peak \( I_{Na^+Ca} \) is similar to the peak \( I_p \) (Fig. 9 and Fig. 3B). This finding is consistent with work suggesting that the site density and turnover rate for the \( Na^+ /Ca^{2+} \) exchanger and the \( Na^+ /K^+ \)-ATPase are similar (26). Taken together, the similar dependence of \( I_p \) and the \( I_{Na^+Ca} \) on prior \( Na^+ \) pump activity is very suggestive that both transporters are sensing a similar subsarcolemmal \( [Na^+] \), which is lower than the bulk cytosolic \( [Na^+] \). Bielen et al. (4) have also reported a relationship between \( I_p \) and \( I_{Na^+Ca} \) during the decline of \( I_p \) after reactivation of the pump in Purkinje cells, and Main et al. (18) have shown an influence of pump activity on \( Na^+ /Ca^{2+} \) exchange in voltage-clamped guinea pig ventricular myocytes. In addition, recent evidence suggests that there may be colocalization of the \( Na^+ \) pump and \( Na^+ /Ca^{2+} \) exchanger in the membrane of cardiac and smooth muscle myocytes (19, 23). This colocalization may result from the binding of these ion transporters to the cytoskeletal protein ankyrin (15, 17).

Physiological implications of our findings. A number of investigators have noted a dissociation among \( Na^+ \) pump activity, changes in bulk \( [Na^+] \), and alteration of \( Ca^{2+} \) homeostasis during recovery from \( Na^+ \) pump inhibition (2). We previously reported that during recovery from \( Na^+ \) pump inhibition in cultured chick embryo myocytes, intracellular \( Na^+ \) measured with \( ^{24}Na^+ \) returned to normal levels more slowly than \( Na^+ /Ca^{2+} \) exchange and contractility (3). Eisner et al. (9) also noted a more rapid decay in twitch tension than \( a_{Na} \) measured with a \( Na^+ \)-sensitive microelectrode in voltage-clamped Purkinje fibers after reactivation of the \( Na^+ \) pump. Levi et al. (16) have also noted a dissociation between changes in bulk \( [Na^+] \) and contractility during washout of pump inhibition in isolated ventricular myocytes. The presence of a subsarcolemmal space in which \( Na^+ /Ca^{2+} \) exchanger activity is more dependent on \( Na^+ \) pump activity than on bulk cytosolic \( Na^+ \) concentration could account for these findings (2, 3). This concept could also explain why
inotropic effects of cardiac glycosides may be seen in tissues at a time when apparent inhibition of the Na pump manifested by change in cellular [Na] is very minor or nondetectable (1, 16).

Whereas our results do support the functional presence of so-called Na “fuzzy space,” there are still some issues regarding the possible existence of this space that remain unresolved. For example, pump, exchanger, and ion channel transmembrane currents that have time constants of the order of milliseconds may be measured by voltage-clamp, yet the rate of decay of the I Na, and the I NaCa, allegedly attributable to limitation of diffusion in the subsarcolemmal space, is of the order of seconds to minutes. Several factors account for this apparent discrepancy. First, it is possible that the limitation of diffusion of Na adjacent to the Na pump or the Na/Ca exchange transporter site is limited to a very small area, and that a subsarcolemmal diffusion barrier does not exist over the major portion of the sarcolemma. It also appears that the restriction of diffusion is primarily for Na, possibly due to binding of Na by intracellular buffers (11), and that restriction of movement of other intracellular ions that can carry currents such as H+, Cl−, Ca2+, and K+ is less marked. Certainly, based on the results shown in Fig. 10 and the findings of Delbridge et al. (7) and Trafford et al. (32), the movement of Ca2+ from the cytosol to the Na/Ca2+ exchanger can occur very rapidly. Consistent with the findings of Trafford et al. (32), we noted a slight delay between the activation of forward NaCa, and the rise in [Ca2+] during a caffeine transient, consistent with some diffusion limitation from the cytosol to the subsarcolemmal space for Ca2+. However, the access of cytosolic Ca2+ to the exchanger sites appears much less restricted than that of Na+. This apparent limitation of diffusion of Na+ to (and perhaps from) this site is paradoxical in view of the reported more rapid diffusion of Na+ than Ca2+ in bulk muscle cell cytoplasm (12). This paradox has been emphasized previously (2, 14) and may reflect microdomain complexity of the Na+/Ca2+ exchanger and Na+ pump sites. Resolution of these issues will require further experimentation, including possible measurement of subcellular Na+ gradients by physical techniques.

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