Transmembrane Na^+ and Ca^{2+} Electrochemical Gradients in Cardiac Muscle and Their Relationship to Force Development

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ABSTRACT Na^+- and Ca^{2+}-sensitive microelectrodes were used to measure intracellular Na^+ and Ca^{2+} activities (a_{Na}^i and a_{Ca}^i) of sheep ventricular muscle and Purkinje strands to study the interrelationship between Na^+ and Ca^{2+} electrochemical gradients ($\Delta\tilde{\mu}_{Na}$ and $\Delta\tilde{\mu}_{Ca}$) under various conditions. In ventricular muscle, a_{Na}^i was 6.4 ± 1.2 mM and a_{Ca}^i was 87 ± 20 nM ($[Ca^{2+}] = 272$ nM). A graded decrease of external Na^+ activity (a_{Na}^o) resulted in decrease of a_{Na}^i, and increase of a_{Ca}^i. There was increase of twitch tension in low-a_{Na}^o solutions, and occasional increase of resting tension in 40% a_{Na}^o. Increase of external Ca^{2+} (a_{Ca}^o) resulted in increase of a_{Ca}^i and decrease of a_{Na}^i. Decrease of a_{Ca}^o resulted in decrease of a_{Ca}^i and increase of a_{Na}^i. The apparent resting Na-Ca energy ratio ($\Delta\tilde{\mu}_{Ca}/\Delta\tilde{\mu}_{Na}$) was between 2.43 and 2.63. When the membrane potential (V_m) was depolarized by 50 mM K^+ in ventricular muscle, V_m depolarized by 50 mV, a_{Na}^i decreased, and a_{Ca}^i increased, with the development of a contracture. The apparent energy coupling ratio did not change with depolarization. 5×10^{-6} M ouabain induced a large increase in a_{Na}^i and a_{Ca}^i, accompanied by an increase in twitch and resting tension. Under the conditions we have studied, $\Delta\tilde{\mu}_{Na}$ and $\Delta\tilde{\mu}_{Ca}$ appeared to be coupled and n was nearly constant at 2.5, as would be expected if the Na-Ca exchange system was able to set the steady level of a_{Ca}^i. Tension threshold was about 230 nM a_{Ca}^i. The magnitude of twitch tension was directly related to a_{Ca}^i.

INTRODUCTION

Cytoplasmic free Ca^{2+} activity (a_{Ca}^i) appears to be regulated by transport mechanisms in the surface membrane, the sarcoplasmic reticulum, and the mitochondria (Carafoli and Crompton, 1978). The surface membrane has two modalities for outward Ca^{2+} movement, a Na-Ca countertransport or exchange system that uses the energy of the Na^+ electrochemical gradient, and

a Ca^{2+} pump that uses ATP in a fashion analogous to the Na-K pump. (For Na-Ca countertransport, see Baker, 1972; Blaustein, 1974; Mullins, 1981; and Reuter, 1982. For Ca^{2+} pump, see Sulakhe and St. Louis, 1980.) Contraction in heart muscle is strongly influenced by extracellular sodium and calcium, which suggests that the Na-Ca exchange system may have a special role in the regulation of cardiac contraction (Chapman, 1979).

Na-Ca exchange uses the energy of the Na^+ electrochemical gradient to transport Ca^{2+} out of the cell. This mechanism could be studied by measuring the magnitudes of the Na^+ and Ca^{2+} electrochemical gradients ($\Delta\tilde{\mu}_{Na}$ and $\Delta\tilde{\mu}_{Ca}$) under normal conditions and after interventions that change the gradients. This would permit us to test the role of Na-Ca exchange in determining a_{Ca}^i by comparing the change in $\Delta\tilde{\mu}_{Ca}$ in response to a change in $\Delta\tilde{\mu}_{Na}$. The gradient ratio might also provide some insight into the coupling of the two ion movements. Finally, measurements of tension under these conditions may provide information about the relation between sarcolemmal regulation of a_{Ca}^i and contraction.

We have used ion-sensitive microelectrodes to monitor a_{Na}^i and a_{Ca}^i in sheep ventricular muscle and Purkinje strands. The results show that the gradients were changed proportionally when extracellular Na^+ activity (a_{Na}^o) was varied between 40 and 100%, a_{Ca}^o was varied between 20 and 267%, V_m was depolarized by 50 mM $[K^+]_o$, and a_{Na}^i was increased by applying 5×10^{-6} M ouabain. These observations support the suggestion that Na-Ca exchange has a direct effect on a_{Ca}^i and thereby influences muscle contraction.

Some preliminary results have been published in abstract form (Sheu and Fozzard, 1981).

METHODS

Ventricular trabecular muscles (200-900 μm in diameter) and free-running Purkinje strands (200-500 μm in diameter) from sheep hearts were used. The hearts were obtained from a nearby slaughterhouse and transported to the laboratory in cool Tyrode solution, containing (in mM): 127 NaCl, 4 KCl, 1.8 $CaCl_2$, 1.05 $MgCl_2$, 22 $NaHCO_3$, 2.5 NaH_2PO_4, and 5.5 glucose. Reduced a_{Na}^o solutions were made by equiosmolar substitution of sucrose for NaCl (usually 1.71 M sucrose was used for 1 M NaCl). Because the ionic strength was reduced in these solutions, the ionic concentrations of K^+ and Ca^{2+} were adjusted according to the calculated activity coefficients to keep the K^+ and Ca^{2+} activities constant, and the concentration of Na^+ was changed to yield solutions with 80, 60, and 40% a_{Na}^o. The change in Ca^{2+} concentration to 4.8 or 0.36 mM was made by addition or subtraction of $CaCl_2$ from the Tyrode solution. For the solution containing 50 mM K^+, the Na^+ was reduced to 40% and the necessary amounts of KCl and sucrose were added. Ouabain was added from a stock solution that contained 1 mM ouabain. The osmolalities of the solutions were in the range of 295-305 mosmol, as measured with an osmometer (G-66, Fiske, Oxbridge, MA). All solutions were gassed with 95% O_2 and 5% CO_2 at 34 ± 1°C, pH = 7.2 ± 0.1.

V_m was measured with conventional microelectrodes made from thick-walled borosilicate tubing (WPI-1B 150 F4; WP Instruments, New Haven, CT) that had resistances of 8-15 MΩ when filled with 3 M KCl. The same micropipettes were used to fabricate the Na^+-sensitive microelectrodes. The Ca^{2+}-sensitive microelectrodes were made from micropipettes pulled from thin-walled borosilicate tubing (WPI-TW 150 F4; WP Instruments, Inc.) that gave resistances of 3-10 MΩ if filled with 3 M

KCl. These thin-walled micropipettes, although they had lower resistances than those made with thick walls, gave the same V_m when filled with KCl. The micropipettes for ion activity measurements were siliconized with vapors of dimethyldichlorosilane or trimethylchlorosilane and baked at 200°C in the oven for 1 h. The Na^+-sensitive microelectrodes, after 10–150 μm of ligand (ETH 227; Steiner et al., 1979) was introduced by suction into the tip, were backfilled with 300 mM NaCl. The Ca^{2+}-sensitive microelectrodes, after 300–1,000 μm of ligand (ETH 1001; Oehme et al., 1976) was introduced into the tip, were backfilled with pCa 2 or pCa 3 calibrating solutions (pCa $= -\log [Ca^{2+}]$). To obtain a stable electrode response, all the ion-sensitive microelectrodes were stored for at least 4 h before they were calibrated. Na^+-sensitive microelectrodes were calibrated with 1, 3, 10, 30, 100, and 150 mM of NaCl and mixed NaCl-KCl solutions containing 1, 3, 10, 30, 100, and 150 mM NaCl added with KCl to have a total concentration of 150 mM. In pure NaCl calibration, no adjustment was made for any alteration in reference potential induced by a change in Cl^- concentration. All KCl and NaCl solutions were made by mixing equal amounts of 1 N KOH and NaOH solution with 1 N HCl solution (Fisher Scientific Co., Pittsburgh, PA). Then the desired calibration solution was made from these two standard solutions by dilution with double-distilled water. To ensure that there was no Ca^{2+} in the calibrating solution, 0.1 mM EGTA was added. The activity coefficient for Na^+ in the mixed solutions was 0.76. This was calculated from equations described by Pitzer and Mayorga (1973), and the MacInnes convention (MacInnes, 1961) was used for obtaining single-ion activity. Ca^{2+}-sensitive microelectrodes were calibrated in different pCa solutions. The solutions contained background interfering ions in an attempt to mimic the intracellular ionic environment. These were 140 mM KCl, 10 mM NaCl, and 1 mM $MgCl_2$, buffered by 1 mM imidazole to have a pH of 7.0. pCa 5–pCa 8 solutions were made by mixing various amounts of Ca-EGTA and EGTA solutions, using an apparent stability constant of 3.26×10^6 M^{-1} (6.513). Note that pCa 5 and pCa 8 solutions are outside the well-buffered range; thus there may have been some error in the calibrating potential in these two solutions. The stability constant was obtained from those of Boyd et al. (1965) and corrected for temperature and ionic strength (Scharff, 1979). The stability constant, when adjusted for temperature and ionic strength effects, is consistent with the Schwarzenbach constant (Martell and Smith, 1974) that has been used by several other groups who measured intracellular Ca^{2+} activity (Marban et al., 1980; Coray and McGuigan, 1981), as well as that determined experimentally (Bers, 1982). Because the ion-sensitive microelectrode measured activity, the Ca^{2+} activity of the calibrating solutions was calculated using the activity coefficient of 0.32, which was obtained from the modified Debye-Huckel equation (Robinson and Stokes, 1959). Every electrode was calibrated at 34°C immediately after the experiments and in most cases before the experiments.

The experimental procedure was to make several impalements (two to seven) with the KCl-filled microelectrode and one or both of the ion-sensitive microelectrodes while the tissue was in the control solution. The potential recorded by the ion-sensitive microelectrode, after subtraction of the resting membrane potential, was used for obtaining intracellular ion activity from the calibration curve. After control V_m and ionic activities were determined, the electrode impalements were maintained during changes to solutions of different composition. The solution was flowing at a rate of 10 ml/min and the tissue chamber volume was <2 ml. When the ion-sensitive microelectrode was calibrated in the tissue chamber with the same flowing speed of calibrating solution, the potential response to 90% of the steady level took 10–60 s because of the dead space from the switching point to the chamber. After 10–15 min of exposure, which in each instance appeared to be adequate to achieve a new steady level, the solution was switched back to the Tyrode solution. Occasionally, the

electrode signals did not return to their previous control values, requiring that the measurements be discarded. After one successful sequence of Tyrode–test solution–Tyrode, another test solution was used, so that most of the experiments were performed sequentially in the same fibers. Because of the difficulty in maintaining the impalements for long periods, the experiments were accepted if one or the other ion activity measurement were successful. However, in each type of experiment, some of the observations were made with simultaneous recording by both ion-sensitive microelectrodes. In the ouabain experiments we did not require return to control values after drug exposure, because recovery was quite slow. The ion-sensitive microelectrodes measured similar potentials in Tyrode solution and Tyrode solution containing 5 × 10^{-6} M ouabain, which suggested that ouabain has an insignificant effect on the electrodes. Where appropriate, the data are reported as means ± SEM.

Tension was measured with a photoelectric force transducer (TIL 138; Texas Instruments Inc., Houston, TX) similar to that used by Gibbons and Fozzard (1971). The preparations were pinned to the bottom of the chamber at one end, attached to the transducer at the other, and stretched 10–15% over rest length. Twitch tension was measured at a stimulation rate of 0.2 Hz.

RESULTS

Sensitivity and Selectivity of Ion-sensitive Microelectrodes

The Na^+-sensitive microelectrodes had Nernstian responses in pure NaCl calibrating solutions between 150 and 1 mM (Fig. 1). In mixed NaCl-KCl solutions, the electrodes began to deviate from Nernstian response at 30 mM NaCl plus 120 mM KCl. From 13 electrodes that were used for the intracellular measurements, the average potential difference was 22.9 ± 0.8 mV between 30 mM NaCl plus 120 mM KCl and 10 mM NaCl plus 140 mM KCl; 17.8 ± 1.8 mV between 10 mM NaCl plus 140 mM KCl and 3 mM NaCl plus 147 mM KCl; and 10.0 ± 1.6 mV between 3 mM NaCl plus 147 mM KCl and 1 mM NaCl plus 149 mM KCl. Thus, in the range of a_{Na}^i we were studying, 1 mM change of Na^+ gave a voltage change of 2–3 mV. The selectivity coefficient was calculated from the Nicolski equation:

$$E = E_o + \frac{RT}{Z_x F} \ln \left[a_x + \sum_y k_{xy} (a_y)^{Z_x/Z_y} \right]$$

where E_o is the standard potential, Z is the ionic valance, R, T, and F have their usual meaning, a_x is the principal x ion activity, a_y is the interfering y ion activity, and k_{xy} is the selectivity coefficient. The average selectivity coefficient between Na^+ and K^+ (k_{NaK}) was 0.024 at 10 mM NaCl plus 140 mM KCl. Therefore, any modest change of intracellular K^+ activity in this study would have an insignificant effect on the measured a_{Na}^i. The electrode had rather poor Na^+/Ca^{2+} selectivity (k_{NaCa}) (Steiner et al., 1979; Bers and Ellis, 1982). The average k_{NaCa} was ~2.5 in Tyrode solution for these 13 electrodes. This implies that 1.8 mM Ca^{2+} in Tyrode solution would give an apparent Na^+ activity ($k_{NaCa} \cdot [a_{Ca}]^{1/2}$) of ~60 mM. In another three electrodes, when calibrating in solutions of 10 mM NaCl and 140 mM KCl containing different pCa, the k_{NaCa} decreased slightly as Ca^{2+} concentration was reduced. The average apparent Na^+ activities resulting from pCa 3, 4, 5, 6, and 7 were 44.7,

13.0, 4.4, 1.2, and 0.3 mM, respectively. These numbers were used for correcting the a^i_{Ca} influence on a^i_{Na} measurements. There is some variation of selectivity coefficient from electrode to electrode, so that use of an averaged selectivity coefficient for correcting a^i_{Ca} interference on a^i_{Na} is bound to give some errors. We only used those electrodes that had slopes >35 mV between 30 mM NaCl plus 120 mM KCl and 3 mM NaCl plus 147 mM KCl, which
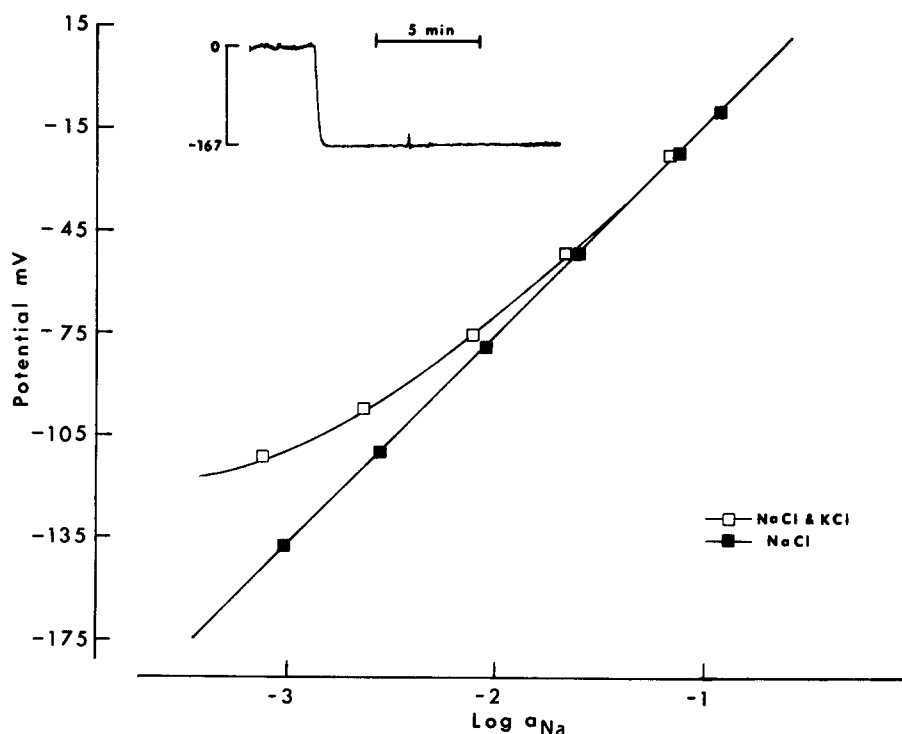

FIGURE 1.   Calibration of a Na$^+$-sensitive microelectrode. The measured potential is plotted against Na$^+$ activity. Pure NaCl solutions (■), and mixed NaCl and KCl solutions (□) to achieve a total concentration of 150 mM were used. The inset shows a recording of an impalement in a ventricular muscle. This recorded potential, after subtraction of resting membrane potential, was used for obtaining a^i_{Na} from the curve (□). Ca^{2+} interference on a^i_{Na} was then corrected (see text). The reference potential before impalement is that in the Tyrode solution. It is different from the signal in the 150 mM NaCl calibrating solution because of the effect of Ca^{2+} in the Tyrode solution.

indicates a good selectivity for Na$^+$ over Ca^{2+}. Therefore, the variation was probably small.

The Ca^{2+}-sensitive microelectrode had a Nernstian response from Ca^{2+} activity of 3.2×10^{-3}–3.2×10^{-7} M (pCa 2–pCa 6) (Fig. 2). Most of the electrodes showed greater than Nernstian slope between 3.2×10^{-5} M–3.2×10^{-7} M (pCa 4–pCa 6) of Ca^{2+} activity if they were calibrated immediately

after filling. However, when they were stored for several hours or overnight, the calibration became approximately linear. From 18 electrodes used for intracellular measurements, the average potential was 14.9 ± 1.4 mV between Ca^{2+} activity of 3.2×10^{-7} M–3.2×10^{-8} M (pCa 6–pCa 7). Thus, in the range of a_{Ca}^{i} we studied, 100 nM of Ca^{2+} change would give a 1–3-mV signal.
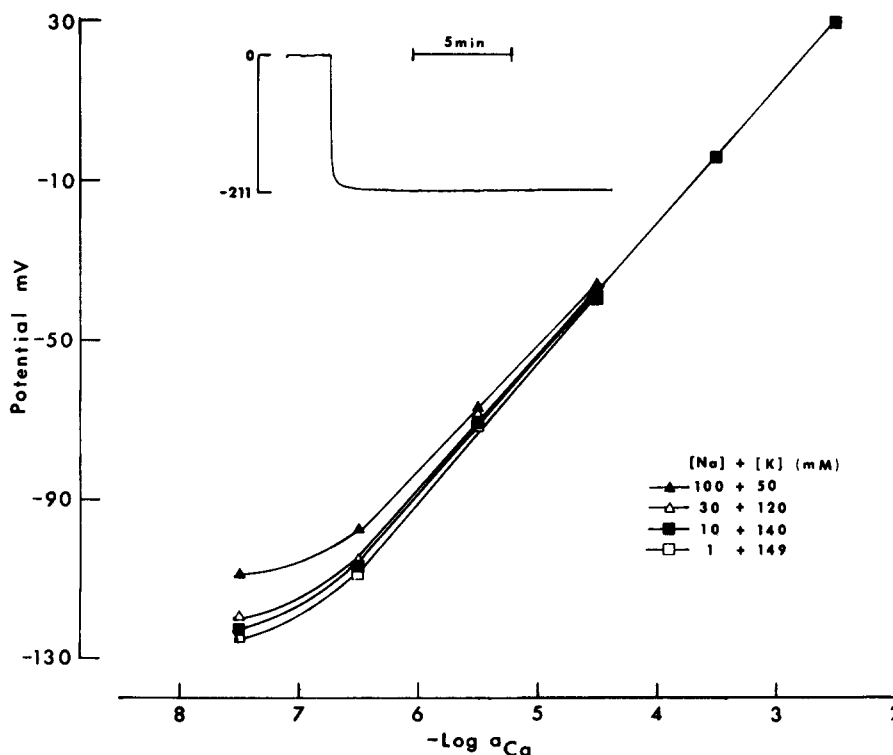

FIGURE 2.   Calibration curve of a Ca^{2+}-sensitive microelectrode. Different Ca^{2+} solutions were used containing 1 mM NaCl and 149 mM KCl (□), 10 mM NaCl and 140 mM KCl (■), 30 mM NaCl and 120 mM KCl (△), and 100 mM NaCl and 50 mM KCl (▲). The inset shows a recording of an impalement in a ventricular muscle. This recorded potential, after subtraction of resting membrane potential, was used for obtaining a_{Ca}^{i} from the curve (■). a_{Ca}^{i} can be transformed to free Ca^{2+} concentration $[Ca^{2+}]_i$ by dividing by 0.32.

The average selectivity coefficient between Ca^{2+} and K^{+} (k_{CaK}) of these 18 electrodes calculated from the Nicolski equation was $\sim 10^{-5}$ (range 5×10^{-5}–5×10^{-6}) and k_{CaNa} was $\sim 5 \times 10^{-4}$ (range 8×10^{-4}–10^{-4}) in pCa-7 solution. Thus, a 10-mM change of K^{+} would give an apparent Ca^{2+} activity change ($k_{CaK} \cdot a_K^2$) of 1 nM, and a 10-mM change of Na^{+} would give an apparent Ca^{2+} activity change ($k_{CaNa} \cdot a_{Na}^2$) of 50 nM. a_K^{i} was measured in four ventricular muscles and was 103.8 ± 5.7 mM ($[K^{+}]_i = 138.4$ mM). Variation of Mg^{2+}

from 1 to 5 mM gave no detectable change of electrode response. We used the calibration curve obtained from solutions containing 10 mM NaCl, except for the experiments with ouabain. In those cases, the a_{Na}^i rose substantially, requiring correction for interference on a_{Ca}^i measurements.

a_{Na}^i and a_{Ca}^i in Tyrode Solution

The results of a_{Na}^i and a_{Ca}^i of ventricular muscle and Purkinje strands are summarized in Table I. Comparing the measurements between the two tissues, there was a significant difference of the resting membrane potential ($P > 0.01$, Student's t test); the differences between the two tissues for a_{Na}^i and a_{Ca}^i were not significant ($P > 0.2$). Using the Na^+ activity of Tyrode solution (a_{Na}^o) of 115.1 mM and the Ca^{2+} activity of 0.576 mM, it is possible to calculate the Na^+ equilibrium potential (V_{Na}) to be 80.2 ± 4.9 mV and the Ca^{2+} equilibrium potential (V_{Ca}) to be 118.9 ± 3.4 mV in ventricular muscle. $\Delta\tilde{\mu}_{Na}$ is then

TABLE I

INTRACELLULAR Na^+ AND Ca^{2+} ACTIVITIES IN TYRODE SOLUTION

	Ventricular muscle	Purkinje strands
V_m (mV)	-88.5 ± 1.1 (10)*	-76.6 ± 1.2 (11)
a_{Na}^i (mM)	6.4 ± 1.2 (10)	8.0 ± 0.6 (11)
$[Na]_i$ (mM)‡	8.4	10.5
V_m (mV)	-90.1 ± 0.8 (8)	-76.3 ± 0.8 (10)
a_{Ca}^i (nM)	87 ± 20 (8)	92 ± 9 (10)
$[Ca]_i$ (nM)§	272	288

* Number of preparations.
‡ $[Na]_i = a_{Na}^i/0.76$.
§ $[Ca]_i = a_{Ca}^i/0.32$.
Data are presented as mean ± SEM.

estimated to be ($V_{Na} - V_m$) = 168.7 mV and $\Delta\tilde{\mu}_{Ca}$ is $2(V_{Ca} - V_m)$ = 418 mV, yielding a $\Delta\tilde{\mu}_{Ca}/\Delta\tilde{\mu}_{Na}$ of 2.48. The possible meaning of this ratio will be discussed later. In Purkinje strands, V_{Na} was calculated to be 70.9 ± 1.8 mV and V_{Ca} was calculated to be 116.2 ± 1.8 mV. $\Delta\tilde{\mu}_{Na}$ was 147.5 mV and $\Delta\tilde{\mu}_{Ca}$ was 385 mV, for a $\Delta\tilde{\mu}_{Ca}/\Delta\tilde{\mu}_{Na}$ = 2.61.

Each measurement of a_{Na}^i and a_{Ca}^i was the result of multiple stable impalements, with some being maintained for hours without drift. Nevertheless, the measurements could be in error because of local cell injury, unrecognized interference of other ions, or problems with the electrode calibration. Response of the measurements to interventions, reported next, offers some encouragement that these values were not greatly in error.

Effects of Changing a_{Na}^o on a_{Na}^i and a_{Ca}^i

The intent of these experiments changing a_{Na}^o was simple. Lowering the a_{Na}^o should reduce the Na^+ gradient, thereby lowering the energy available for efflux of Ca^{2+} via the Na-Ca exchange system and resulting in an increased

a_{Ca}^i. This would allow the cells to be loaded with Ca^{2+} and lead to the well-known effect of low a_{Na}^o to produce increased contraction strength.

In preliminary experiments, Na^+ was replaced by choline or Li^+, but the results were not consistent. There was often a depolarization with the Na^+ substitution by those agents, which suggests complex effects. Sucrose substi-
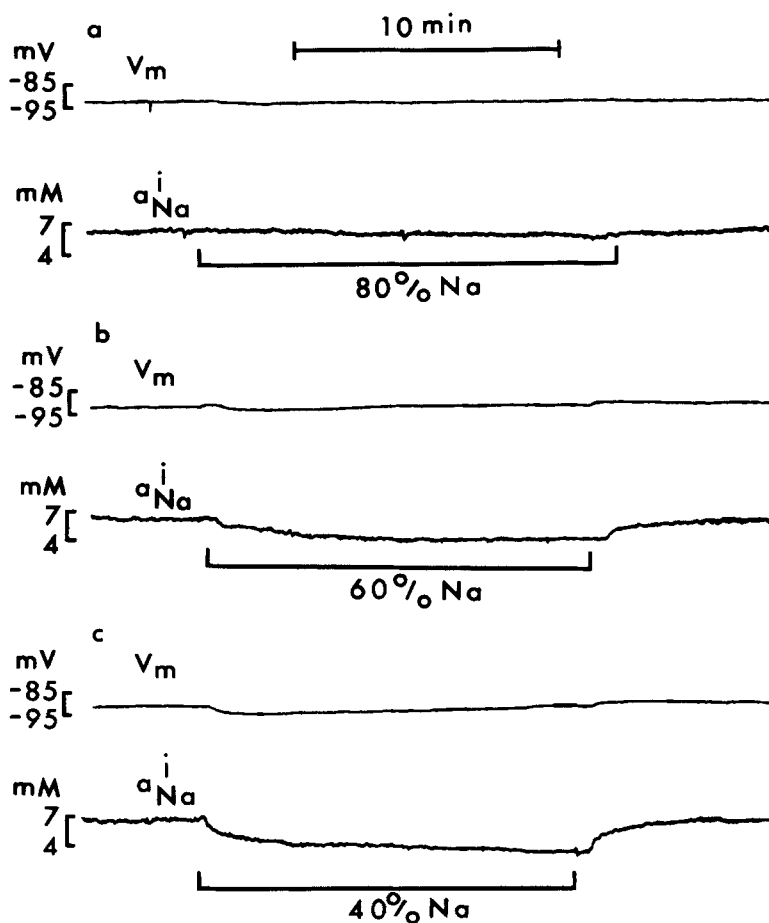

FIGURE 3. Continuous recording of membrane potential and intracellular Na^+ activity in a ventricular muscle during change to various external Na^+ solutions. The external Na^+ activity was reduced to 80 (a), 60 (b), and 40% (c) of normal by sucrose substitution.

tution provided consistent responses if ionic concentrations were adjusted for the change in ionic strength that resulted. Although the change of ionic strength may cause changes of junction potential of the 3 M KCl-agar reference electrode, calculation from Henderson's equation (MacInnes, 1961) indicates that this change is <1 mV. In addition, this had no effect on ion

activity determination, because the ion-selective microelectrode was electronically referred to the conventional microelectrode.

a^o_{Na} was reduced to 80, 60, and 40% of its normal value. The experiments reported were performed in ventricular muscle, but the same results were obtained in less complete experiments in Purkinje strands. Responses of V_m and a^i_{Na} to reductions in a^o_{Na} are shown in Fig. 3. For example, with reduction of a^o_{Na} to 40% of normal, a^i_{Na} decreased rapidly from 6.1 mM, then fell slowly to its steady level of 4.0 mM by 15 min. After return to normal Tyrode solution, the a^i_{Na} recovered rapidly. V_m hyperpolarized 3–4 mV in the low-a^o_{Na} solutions, then depolarized a little, to remain somewhat hyperpolarized over control by 15 min. After return to normal solution there was often a small depolarization before the prior control level was achieved.

Fig. 4 illustrates the response of a^i_{Ca} to the a^o_{Na} reduction. For example, upon
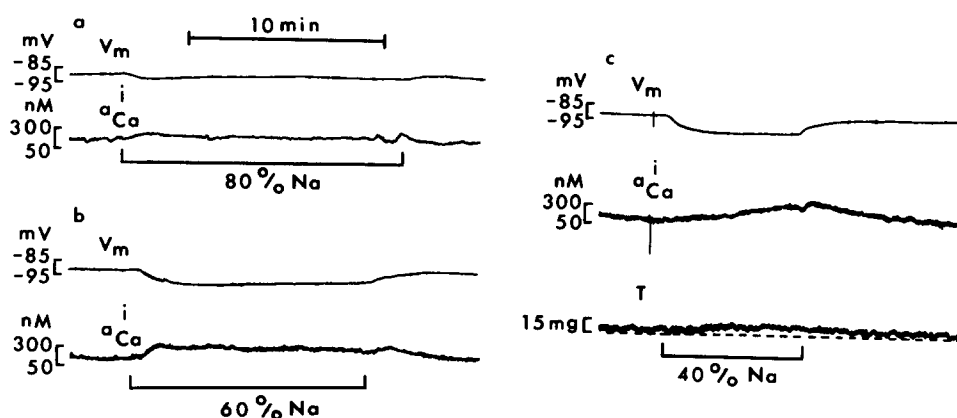

FIGURE 4.   Continuous recording of membrane potential and intracellular Ca^{2+} activity in a ventricular muscle during change to various external Na$^+$ solutions. In 40% Na$^+$, resting tension was also recorded.

change to 40% a^o_{Na}, it rose from 70 to 260 nM. The time courses of changes may reflect underlying cellular mechanisms, but they could also be simply the result of slow equilibrium of the solutions in the extracellular spaces of the muscles. Because of these concerns, we avoid attempting to interpret the transients and choose to report only the values of a^i_{Ca} at the end of exposure to low-a^o_{Na} solutions.

In five experiments we were able to obtain a^i_{Na} measurements in all four solutions without change in control a^i_{Na}. In four experiments we were able to measure a^i_{Ca} in all four solutions without change in control a^i_{Ca}. One of these experiments was made with simultaneous a^i_{Na} and a^i_{Ca} measurements. There were also additional partial experiments that gave similar values. The average values from the complete experiments are shown in Table II and Fig. 5. With a reduction in a^o_{Na} to 40%, the a^i_{Na} fell from 6.4 to 3.9 mM and a^i_{Ca} rose from 113 to 230 nM. The fall in a^i_{Na} was not quite sufficient to restore the activity gradient, so V_{Na} and $\Delta\bar{\mu}_{Na}$ fell. The reduction in $\Delta\bar{\mu}_{Na}$ was accompanied by a

rise in a_{Ca}^i, as would be expected if the Na-Ca exchange system were influencing a_{Ca}^i. The size of the a_{Ca}^i rise that would be predicted depends on the details of the model of Na-Ca exchange. One way of comparing the 40% fall in a_{Na}^i with the 100% rise in a_{Ca}^i is to compare the electrochemical energy ratio $\Delta\tilde{\mu}_{Ca}/\Delta\tilde{\mu}_{Na}$.

TABLE II

CHANGES IN a_{Na}^i, a_{Ca}^i, AND TENSION IN VENTRICULAR MUSCLE WITH DIFFERENT a_{Na}^o SOLUTIONS

a_{Na}^o	a_{Na}^i $n = 5$	V_m	V_{Na}	$\Delta\tilde{\mu}_{Na}$	a_{Ca}^i $n = 4$	V_m	V_{Ca}	$\Delta\tilde{\mu}_{Ca}$	T_x/T_c	$\Delta\tilde{\mu}_{Ca}/\Delta\tilde{\mu}_{Na}$
%	mM	mV	mV	mV	nM	mV	mV	mV		
100	6.4±0.9	−90.7±1.1	77.8±4.6	168.5	113±36	−89.8±1.0	115.2±4.8	410.0	1	2.43
80	5.6±0.8	−90.9±0.8	75.6±4.7	166.5	132±40	−90.7±0.9	112.9±4.7	407.2	2.8±0.44	2.45
60	4.7±0.7	−91.9±0.0	72.4±4.6	164.3	163±46	−91.6±0.8	109.6±3.9	402.4	4.9±1.26	2.45
40	3.9±0.7	−93.6±0.8	67.2±5.5	160.8	230±75	−92.7±0.6	105.5±4.3	396.4	8.4±2.13	2.47

$r = 0.997$
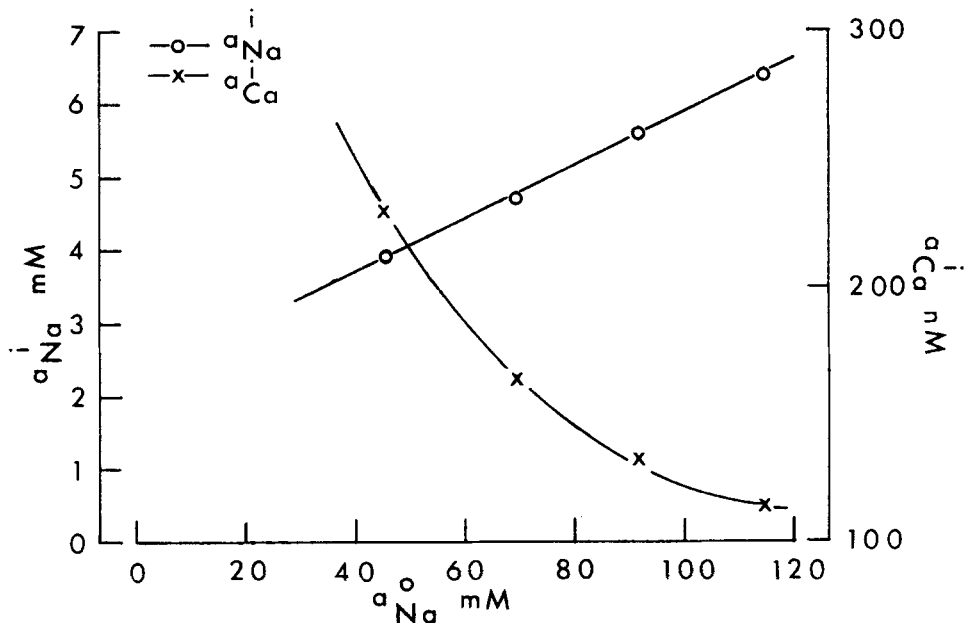$P > 0.001$

$r = 0.977$
$P > 0.05$

FIGURE 5. Intracellular Na$^+$ and Ca^{2+} activities in ventricular muscle as a function of extracellular Na$^+$ activity. Intracellular Na$^+$ (O) decreases linearly with the reduction of extracellular Na$^+$. Intracellular Ca^{2+} (X) increases as the extracellular Na$^+$ is reduced.

This ratio was 2.43 in the control solution, and it remained approximately the same in the low-a_{Na}^o solutions.

Also shown in Fig. 4 is a recording of tension during exposure to 40% a_{Na}^o solution. The resting tension increased slightly in that experiment, which suggests that the a_{Ca}^i level achieved was just above the tension threshold.

Tension was seen in 4 of 11 muscles during exposure to 40% a_{Na}^o, and it was never seen during exposure to 60 or 80% a_{Na}^o solutions. This would place contraction threshold near 230 nM a_{Ca}^i, or 720 nM [Ca^{2+}]$_i$. In separate experiments, twitch tension was measured in the different solutions. Because of variability in size of the preparations, the responses (T_x) were normalized by the control twitch size (T_c) to yield the ratio T_x/T_c. The relationship of the twitch size in 17 muscles to a_{Na}^o is listed in Table II. Change in twitch size seems to be proportional to a_{Ca}^i.

Effects of Changing a_{Ca}^o on a_{Na}^i and a_{Ca}^i

If the resting a_{Ca}^i is controlled by Na-Ca exchange, then alteration in a_{Ca}^o should produce a proportionate change in a_{Ca}^i, re-establishing the $\Delta\tilde{\mu}_{Ca}$ to
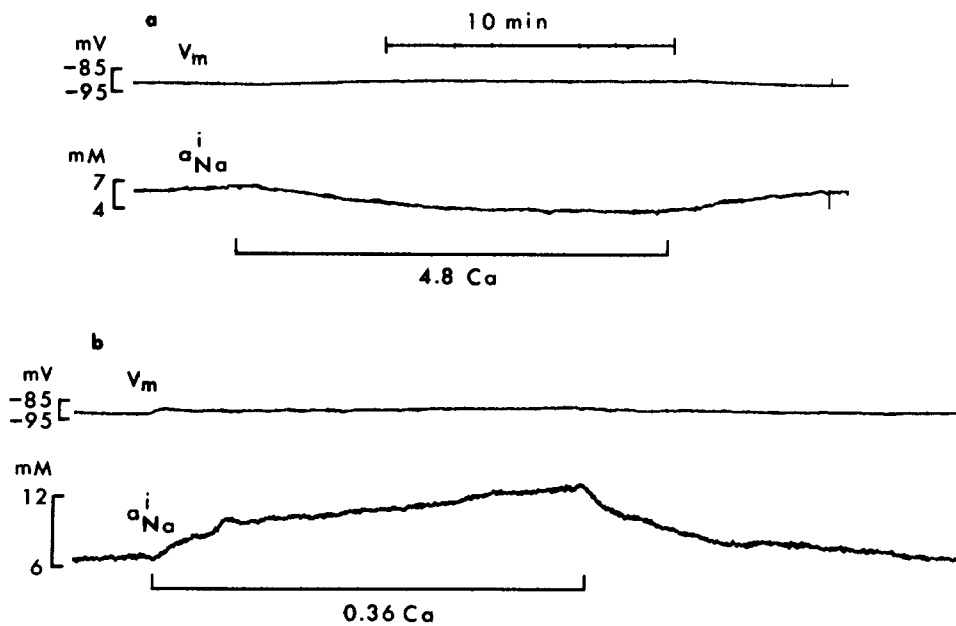

Figure 6.   Recording of membrane potential and intracellular Na$^+$ activity in ventricular muscle during change to extracellular Ca^{2+} concentrations: 4.8 mM (a), 0.36 mM (b).

match the energy available in the Na$^+$ gradient. If, however, some other factor should control a_{Ca}^i, then the changes might differ from this prediction. This is the same as saying that the electrochemical energy gradient ratio should not change if the Ca^{2+} gradient is controlled by the Na$^+$ gradient.

Ventricular muscle fibers were exposed to Tyrode solution containing 0.36 or 4.8 mM [Ca^{2+}]$_o$. The response of a_{Na}^i is illustrated in Figs. 6 and 8. Exposure to increased [Ca^{2+}]$_o$ caused a small fall in a_{Na}^i from 6.9 ± 0.5 mM (four muscles) to 6.0 ± 0.4 mM ($P < 0.001$), and exposure to low [Ca^{2+}]$_o$ caused a rise in a_{Na}^i to 12.5 ± 2.4 mM ($P < 0.001$). A few experiments using tetrodotoxin (TTX) with the low-[Ca^{2+}]$_o$ solutions suggested that the a_{Na}^i increase results

partially from a change in passive Na^+ permeability (H. A. Fozzard, unpublished observations). V_m in the control solution was -90.9 ± 1.2 mV and it did not change significantly in the solutions with altered $[Ca^{2+}]_o$. Fig. 7 shows a record of a^i_{Ca} and tension during exposure to high- and low-$[Ca^{2+}]_o$ solutions. In 4.8 mM $[Ca^{2+}]_o$ solution, a^i_{Ca} rose from 93 ± 3 nM (two muscles) to 164 ± 29 nM, and in 0.36 mM $[Ca^{2+}]_o$ solution, it fell to 38
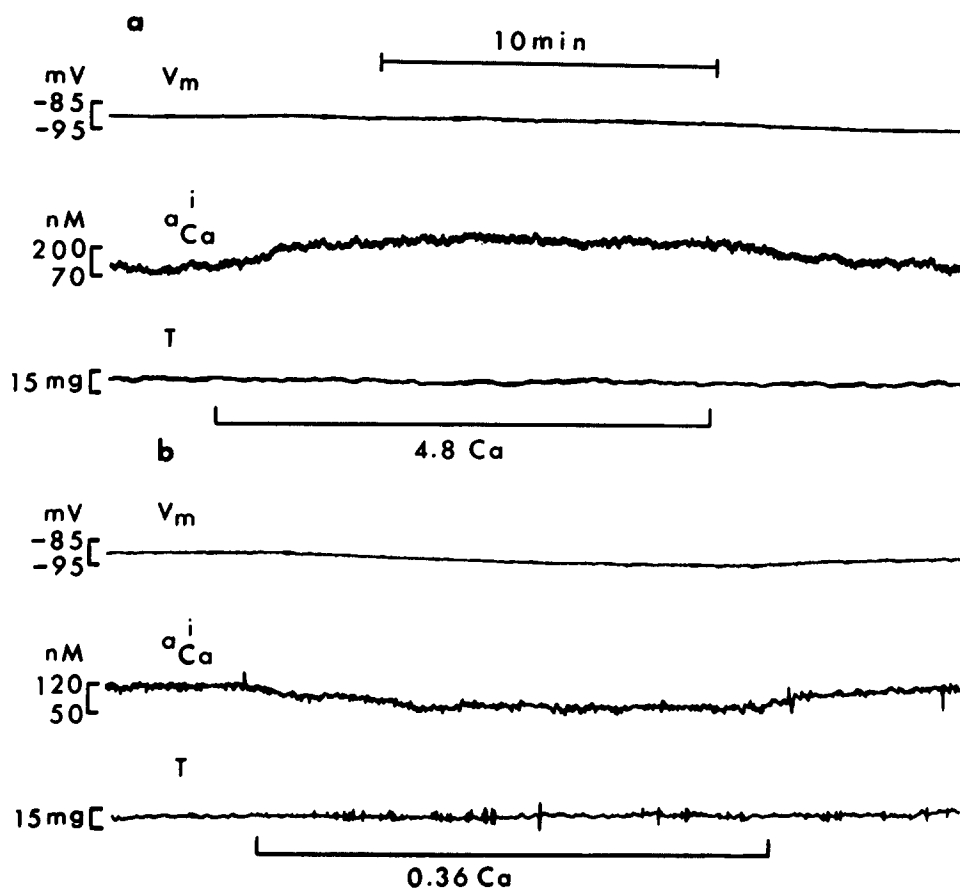

FIGURE 7.   Recording of membrane potential, intracellular Ca^{2+} activity, and resting tension in ventricular muscle during change to 4.8 mM external Ca^{2+} (a), and 0.36 mM external Ca^{2+} (b).

± 15 nM (Fig. 8). No change in resting tension was seen in these solutions. The value of $\Delta\tilde{\mu}_{Ca}/\Delta\tilde{\mu}_{Na}$ in the control solution was 2.54, and in the low-$[Ca^{2+}]_o$ solution it was 2.63. The relation between normalized twitch magnitude and $[Ca^{2+}]_o$ was measured in four separate experiments. As expected, twitches were larger in 4.8 mM $[Ca^{2+}]_o$ ($T_x/T_c = 3.7 \pm 1.1$, $n = 4$), and they were smaller in 0.36 mM $[Ca^{2+}]_o$ ($T_x/T_c = 0.38 \pm 0.06$).

Effects of K^+ Depolarization on a^i_{Na} and a^i_{Ca}

If the Na-Ca exchange is not neutral, it will respond to changes in V_m. In these experiments we changed V_m by exposure of the fibers to 50 mM $[K^+]_o$, which is expected to depolarize the cells by ~40–50 mV, thereby changing the Na$^+$ electrochemical gradient directly. A prediction of the magnitude of the effect of depolarization on a^i_{Ca} is difficult to make because depolarization
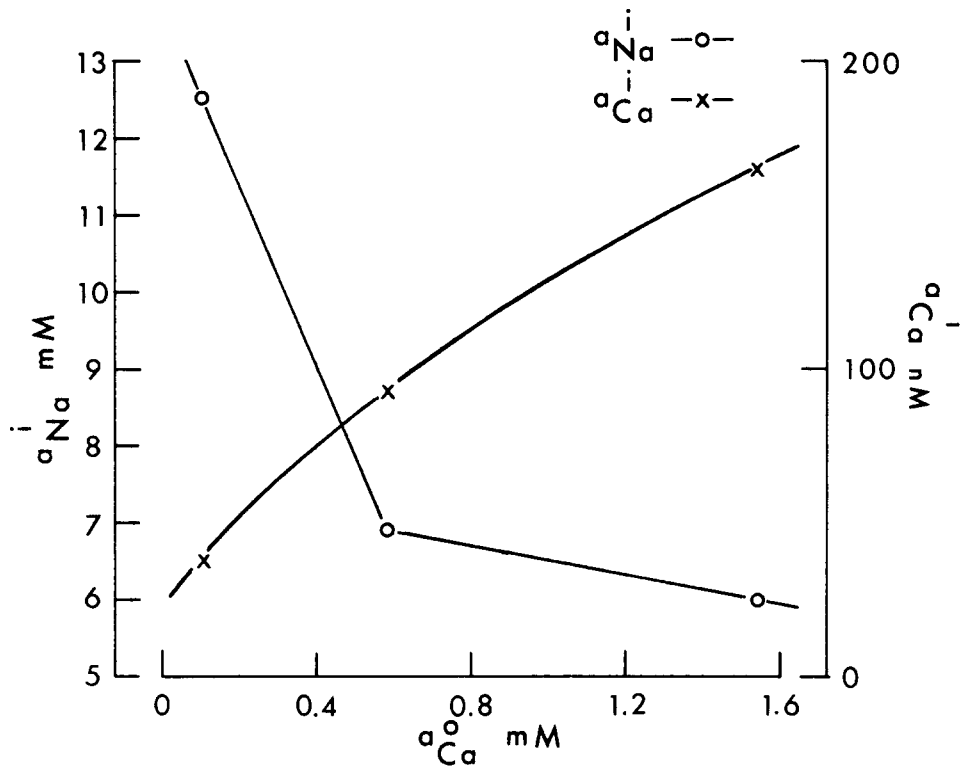

FIGURE 8. Intracellular Na$^+$ and Ca^{2+} activities in ventricular muscle as a function of extracellular Ca^{2+} activity. Intracellular Na$^+$ (O) decreases when the extracellular Ca^{2+} activity is increased. Intracellular Ca^{2+} (X) increases when the extracellular Ca^{2+} activity is increased.

decreases a^i_{Na} (Eisner et al., 1981), releases Ca^{2+} from the sarcoplasmic reticulum, and perhaps increases Ca^{2+} permeability. However, the steady levels of a^i_{Ca} should maintain a fixed relationship to the Na$^+$ energy gradient if Na-Ca exchange controls a^i_{Ca} under these conditions.

Two kinds of experiments in ventricular muscle are illustrated in Fig. 9. In the upper panel the normal Tyrode solution was replaced with one containing 40% a^o_{Na} and 50 mM $[K^+]_o$. a^i_{Na} fell from 8.7 to 3.2 mM and recovered upon return to normal solution. The lower panel illustrates the effects of a^o_{Na}

reduction on a_{Na}^i separately from the K^+ depolarization. Upon reduction of a_{Na}^o to 40% of normal without change in K^+, the a_{Na}^i fell from 12.7 to 7.7 mM. The subsequent depolarization with 50 mM $[K^+]_o$ caused a further decrease to 3.5 mM, consistent with the idea that V_m can have a direct effect on a_{Na}^i. In two separate experiments, a_{Ca}^i rose upon depolarization from 82 ± 41 nM to 310 ± 41 nM, often with an early transient to a higher level (Fig. 10). When normal solution was restored, a_{Ca}^i sometimes fell to below the control
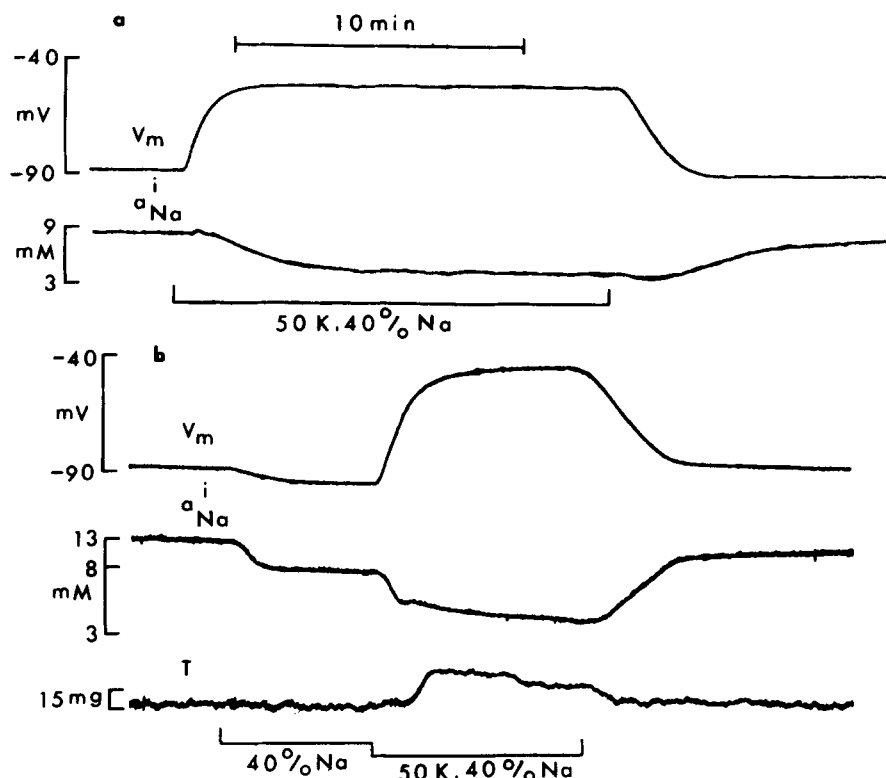

FIGURE 9. Membrane potential and intracellular Na^+ activity in ventricular muscle at 50 mM external K^+ and 40% external Na^+ solution. Panel a shows the decrease of a_{Na}^i in this solution. Panel b shows the separate effect of lowering Na^+ and K depolarization on extracellular Na^+ activity. In the second experiment resting tension was measured simultaneously.

level transiently. Tension was recorded in these experiments (Figs. 9 and 10), showing development of a contracture upon depolarization and prompt relaxation when normal solution was restored. The tonic tension would be expected, because a_{Ca}^i was considerably above the tension threshold of ~230 nM found in low-a_{Na}^o solutions.

Most of the experiments in ventricular muscle were not usable because the large contracture displaced the impalements. Consequently, we decided to

pursue these studies with Purkinje strands. Fig. 11 illustrates an experiment in a Purkinje strand with simultaneous measurements of V_m, a_{Na}^i, and a_{Ca}^i. Intracellular activities changed with a complex time course, leading to a lower a_{Na}^i and a higher a_{Ca}^i. In six such strands, a_{Na}^i fell from a 8.0 ± 0.4 mM to 3.0 $\pm$ 1.7 mM in association with reduced a_{Na}^o and depolarization (Table III). This resulted in a change in $\Delta\tilde{\mu}_{Na}$ from 157.9 to 109.3 mV. In six strands, a_{Ca}^i rose from 90 $\pm$ 14 nM to 332 $\pm$ 51 nM with depolarization from -76.5 ± 1.1 mV to -37.7 ± 2.1 mV. $\Delta\tilde{\mu}_{Ca}$ changed from 387 to 274.4 mV. In the normal solutions, $\Delta\tilde{\mu}_{Ca}/\Delta\tilde{\mu}_{Na}$ was 2.62 and in the high-$[K^+]_o$ solutions it was 2.51.

One of the difficulties in these experiments was the technical requirement to subtract V_m from the ion-sensitive microelectrode signal. If the solutions
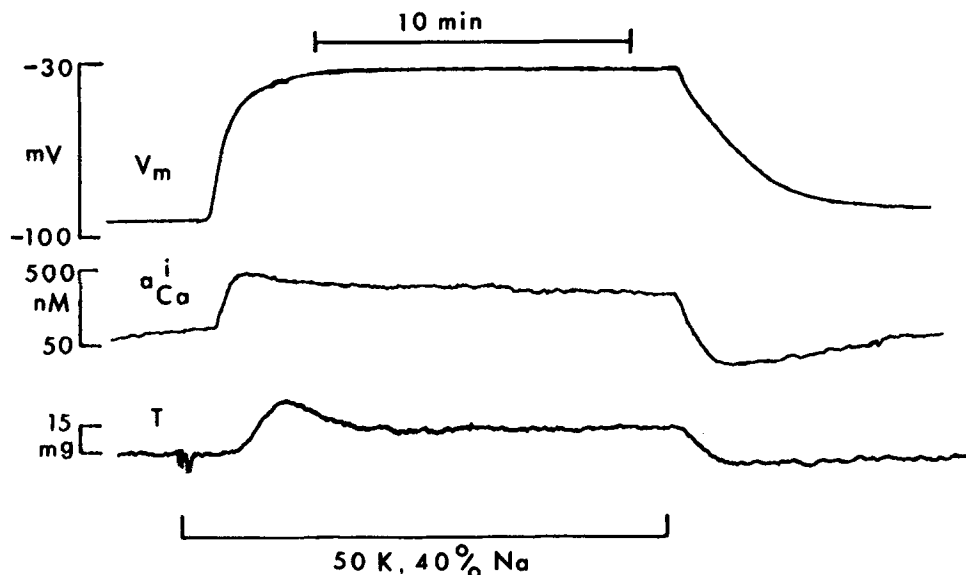

FIGURE 10.   Simultaneous measurements of membrane potential, intracellular Ca^{2+} activity, and resting tension in a ventricular muscle in 40% a_{Na}^o and 50 mM $[K^+]_o$ solution.

reached the two recording electrodes at different times, artifactual transient signals could result. In addition, any shunt through the membrane parallel to the ion-sensitive microelectrode would be changed by depolarization (Eisner et al., 1981). In these experiments this shunt problem was examined by exposure of the Purkinje strands to 10 mM $[K^+]_o$, as illustrated in the inset to Fig. 11. Although this maneuver produces ~8 mV depolarization, the change is also registered in the ion-sensitive microelectrode signal, so that minimal change was apparent in a_{Na}^i or a_{Ca}^i. If the electrode impalements passed this test, the experiment proceeded to the 50 mM $[K^+]_o$ solution.

Effect of Ouabain on a_{Na}^i, a_{Ca}^i, and Tension

Up to this point, alteration in $\Delta\tilde{\mu}_{Na}$ and $\Delta\tilde{\mu}_{Ca}$ was achieved by changing extracellular ionic compositions. Another way to alter $\Delta\tilde{\mu}_{Na}$ is to apply ouabain

to the muscle and cause an increase of a_{Na}^i. Fig. 12 shows an experiment in which V_m, a_{Na}^i, and a_{Ca}^i were measured simultaneously in a ventricular muscle. After 25 min exposure to 5×10^{-6} M ouabain, V_m depolarized only slightly from −88.7 to −84.6 mV. Both a_{Na}^i and a_{Ca}^i increased gradually and appeared to reach plateau values. a_{Na}^i increased from 8.5 to 30 mM, and a_{Ca}^i increased
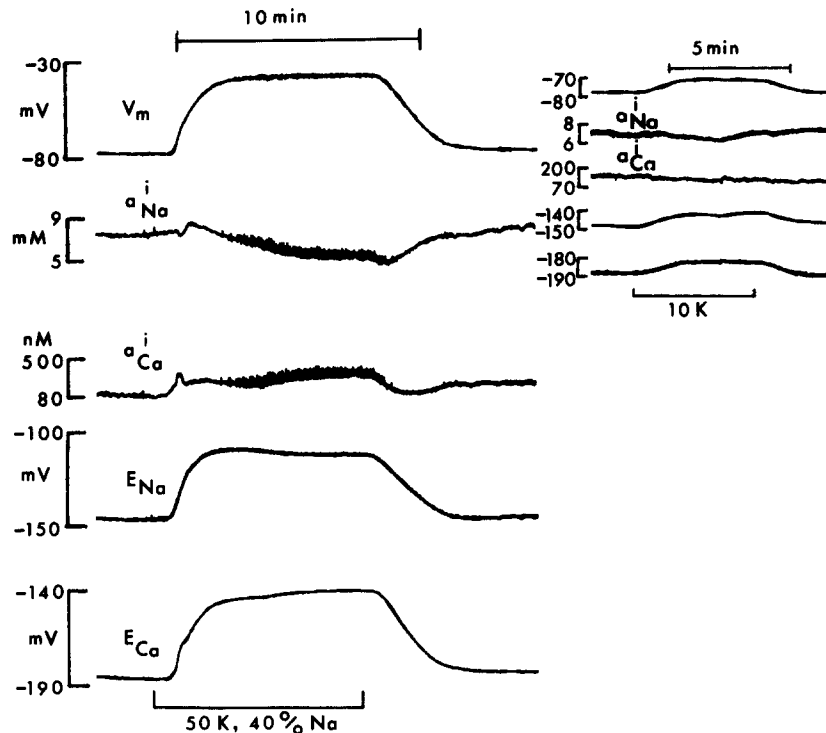

FIGURE 11. Simultaneous measurements of membrane potential, intracellular Na+, and intracellular Ca^{2+} in a cardiac Purkinje strand exposed to 50 mM [K+]o and 40% a_{Na}^o. The inset shows all three impalements during exposure to 10 mM [K+]o, with insignificant change in a_{Na}^i and a_{Ca}^i traces.

TABLE III

EFFECT OF K+ DEPOLARIZATION ON a_{Na}^i AND a_{Ca}^i IN PURKINJE STRANDS

	a_{Na}^i $n = 6$	V_m	V_{Na}	$\Delta\tilde{\mu}_{Na}$	a_{Ca}^i $n = 6$	V_m	V_{Ca}	$\Delta\tilde{\mu}_{Ca}$	$\Delta\tilde{\mu}_{Ca}/\Delta\tilde{\mu}_{Na}$
	mM	mV	mV	mV	nM	mV	mV	mV	
4K	8.0±0.4	−77.3±1.5	70.6±1.5	147.9	90±14	−76.5±1.1	117±2.8	387	2.62
50K	3.0±0.6	−35±1.7	74.3±5.0	109.3	332±51	−37.7±2.1	99.5±2.5	274.4	2.51

from 51 to 320 nM. In another muscle, tension was measured under similar conditions. The twitch tension increased gradually and reached a maximum value at ~15 min. At this time, there was a slight increase of resting tension, the twitch size began to decline, and the resting tension began to increase more dramatically. In addition, oscillations of contraction appeared. The

maximum tension was about 14 times larger than that of the control. Similar responses of tension were found in four other muscles. Again, because of the difficulty in maintaining stable impalements in ventricular muscle, most of the experiments were carried out in Purkinje strands. Exposure to 5×10^{-6} M ouabain for 25 min in another preparation caused a depolarization from -78.3 to -75.0 mV. a^i_{Na} increased from 8.3 to 34.7 mM, and a^i_{Ca} increased from 80 to 279 nM. Three other experiments showed the similar results. In one experiment, we were able to maintain the three electrode impalements while the fiber was stimulated at 0.2 Hz. In this case, a^i_{Na} and a^i_{Ca} responses were quite similar to those of the quiescent strands except that V_m depolarized more. The summarized results of these five preparations are: before ouabain, the average $V_m = -76.2 + 1.0$ mV, $a^i_{Na} = 7.7 \pm 0.8$ mM, and $a^i_{Ca} = 67 \pm 5$ nM; at the end of ouabain exposure, $V_m = -69.2 \pm 3.5$ mV, $a^i_{Na} = 25.0 \pm 3.5$
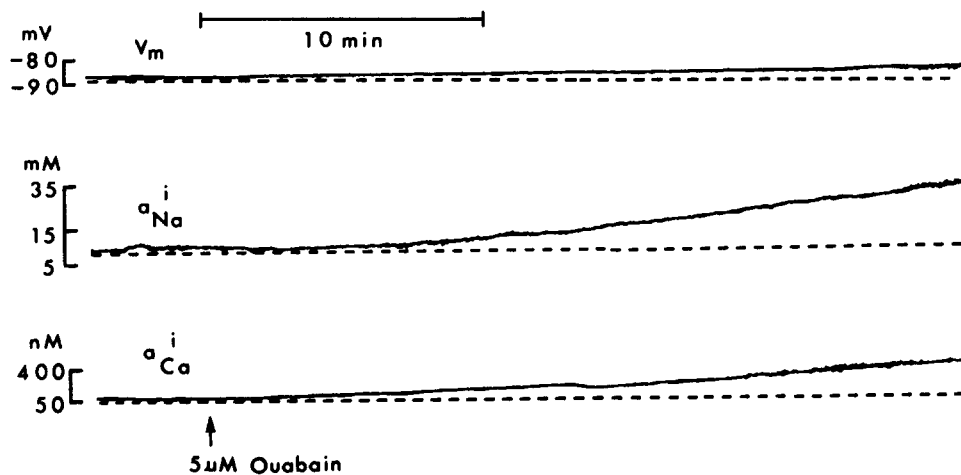

Figure 12.   Simultaneous measurements of membrane potential, intracellular Na$^+$, and intracellular Ca^{2+} in a ventricular muscle exposed to 5×10^{-6} M ouabain.

mM, and $a^i_{Ca} = 491 \pm 106$ nM. The coupling ratio n was 2.65 in control, and after 20–30 min of exposure to ouabain it was 3.03.

Relationship between $\Delta\tilde{\mu}_{Na}$ and $\Delta\tilde{\mu}_{Ca}$

The underlying concept of the Na-Ca exchange mechanism is the coupled countertransport of two ions driven by their electrochemical gradients. If the exchange system is at equilibrium, then the relationship between the two energy gradients can be expressed in the following manner:

$$2(V_{Ca} - V_m) = n(V_{Na} - V_m)$$

where the term to the left is the electrochemical gradient for Ca^{2+} ($\Delta\tilde{\mu}_{Ca}$) and the term to the right is the electrochemical gradient for Na$^+$ multiplied by the energetic coupling ratio of the exchange process (Baker, 1972; Blaustein, 1974; Mullins, 1981). Under normal conditions the Na$^+$ transmembrane gradient is

the result of a balance between the Na-K pump and the passive Na^+ leak into the cell. This establishes the energy gradient to drive Ca^{2+} movement. If $\Delta\tilde{\mu}_{Ca}$ is less than that found in $(n\Delta\tilde{\mu}_{Na})$, then the Na-Ca exchange would drive Ca^{2+} out of the cell in exchange for Na^+. On the other hand, if $\Delta\tilde{\mu}_{Ca}$ is more than
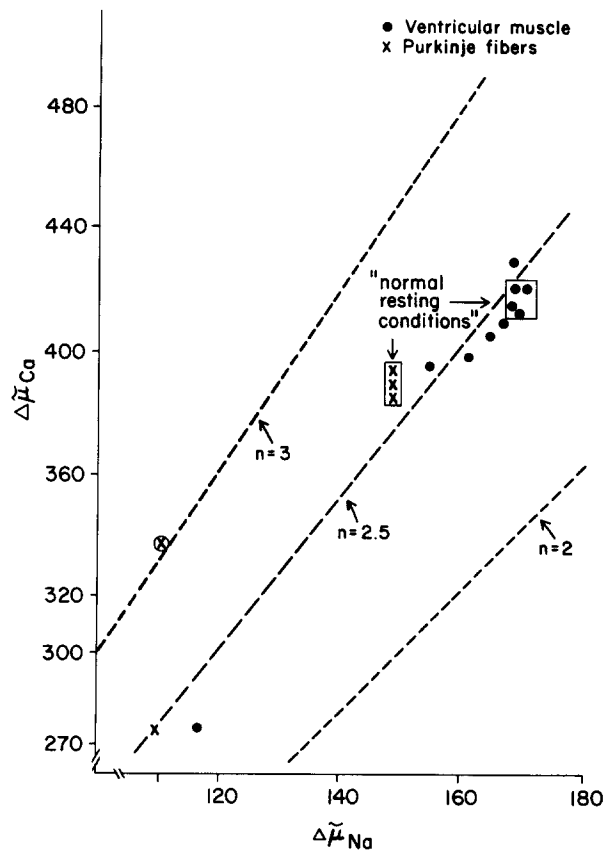

FIGURE 13.   Interrelationship between Na^+ electrochemical gradient and Ca^{2+} electrochemical gradient. Each symbol represents one set of experiments reported in the Results. The values enclosed in boxes were obtained under normal conditions. The broken lines represent the predicted relationships of the coupling ratios (n) between Ca^{2+} electrochemical gradients and Na^+ electrochemical gradients of 2, 2.5, and 3. The closed circles represent the values obtained in studies of ventricular muscles and the crosses represent values obtained in studies of Purkinje strands. One cross is circled and it is the value obtained at the end of exposure of Purkinje strands to 5×10^{-6} M ouabain.

$(n\Delta\tilde{\mu}_{Na})$, then the exchange system would transport Ca^{2+} into the cell. If the exchange is not neutral ($n \neq 2$), then the voltage terms do not cancel, and the equilibrium is influenced by V_m.

In these experiments the measurements of a_{Na}^i, a_{Ca}^i, and V_m were used to calculate the energy coupling ratio under a wide variety of conditions. Fig. 13

illustrates the resulting relationships between $2(V_{Ca} - V_m)$ and $(V_{Na} - V_m)$. Theoretical relationships are indicated for n of 2, 2.5, and 3. Data for ventricular muscle are denoted by filled circles. The four sets of data from resting ventricular muscle in Tyrode solution are enclosed in the box. Data for Purkinje strands are denoted by crosses and three sets of data for resting strands in Tyrode solution are also enclosed in the box. The novelty of this figure is that when $\Delta\tilde{\mu}_{Na}$ was changed by altering a_{Na}^o, a_{Ca}^o, a_{Na}^i, and V_m, $\Delta\tilde{\mu}_{Ca}$ followed the change. Calculation of their relationship under these conditions yielded a linear correlation coefficient of 0.965 and the least-squares-fit line was $\Delta\tilde{\mu}_{Ca} = 2.44\ \Delta\tilde{\mu}_{Na} + 11$. It should be noted that this calculation is heavily influenced by the values obtained in high-$[K^+]_o$ solutions.

DISCUSSION

Ion-sensitive Microelectrodes

Although several types of ion-sensitive microelectrodes have been used successfully in a variety of tissues (Walker and Brown, 1977; Thomas, 1978; Lee, 1981), there remain problems of accurate calibration and artifact-free impalement. Before interpretation of the results of these experiments is possible, it is necessary to assure ourselves that the measurements are reasonably accurate.

The electrochemical properties of the neutral Na$^+$-sensitive carrier ETH 227 and microelectrodes made from it have been carefully studied by Steiner et al. (1979). They reported that stable microelectrodes could be made with drift of <1 mV/day. Our electrodes were usually stable for at least 1 wk. It has been suggested (Lewis and Wills, 1980) that these microelectrodes have shunts across their walls, yielding falsely high values of a_{Na}^i. However, our microelectrodes were made of thick-walled glass and had resistances (when filled with 3 M KCl) of 8–15 MΩ, compared with the higher resistances and thinner walls of the microelectrodes used by Lewis and Wills (1980). Although a small shunt is difficult to exclude, the values of a_{Na}^i obtained were as low as those reported by others and those we have obtained using other types of microelectrodes. The typical selectivity of the electrode for Na over K was 40:1. As we have already discussed in some detail, these microelectrodes are also sensitive to Ca^{2+}. We were able to estimate the selectivity for Ca^{2+} and to correct our results accordingly, and the corrections were <1 mM a_{Na}^i in almost all cases.

The neutral Ca^{2+} carrier ETH 1001 used in these experiments has been studied by Oehme et al. (1976). Microelectrodes made with this resin have been criticized because of drift and instability, greater slope than predicted by the Nernst equation, and poor response below 1 μM (Tsien and Rink, 1980). We found that electrodes stored for >4 h were stable. When filled with pCa-2 or -3 solution, rather than pCa-7 solution, the slopes did not exceed the Nernst prediction. When the column of carrier in the microelectrode tip was >500 μM in height, the electrodes were stable for several days with fair sensitivity. Lee (1981) has reported similar microelectrode characteristics. The microelectrodes were sensitive to Na$^+$, and correction was required when a_{Na}^i changes significantly.

Resting Levels of a^i_{Na} and a^i_{Ca}

The resting levels of a^i_{Na} in heart muscle are surprisingly similar in all reports of measurements with ion-sensitive microelectrodes, in spite of considerable differences in measurement techniques (Lee and Fozzard, 1975; Ellis, 1977; Fozzard and Sheu, 1980; Sheu et al., 1980; Glitsch and Pusch, 1980; Lee et al., 1980a; Eisner et al., 1981). Therefore, it appears quite likely that a^i_{Na} in ventricular muscle is near our value of 6.4 mM. The 95% confidence limits for a^i_{Na} estimated from our measurements were 4.0 and 8.8 mM. V_{Na} is 70–80 mV, and the energy gradient for Na^+ is 160–180 mV. The small difference between ventricular muscle and Purkinje strands may be real, but its demonstration will require direct study.

The resting levels of a^i_{Ca} of 87 and 92 nM ([Ca]$_i$ of 272 and 288 nM) in heart muscle are also surprisingly similar to those reported by others. In ventricular muscle Lee et al. (1980b) found a^i_{Ca} to be 38 nM and Marban et al. (1980) reported [Ca^{2+}]$_i$ to be 0.26 μM (a^i_{Ca} of 83 nM). Interestingly, when the difference in stability constant was adjusted, their results turned out to be exactly the same. In Purkinje strands at room temperature Coray et al. (1980) found [Ca^{2+}]$_i$ levels of 0.36 μM (a^i_{Ca} of 115 nM). Coray and McGuigan (1980) also measured a [Ca]$_i$ of 270 nM in guinea pig ventricular muscle (again, surprisingly close to our 272-nM measurement). These investigators used different calibration techniques and somewhat different values for the Ca^{2+} EGTA apparent stability constant. The level of uncertainty of our measurements of a^i_{Ca} in muscle can be expressed in the 95% confidence limits of 55 and 119 nM. The response of the measurement to various interventions also supports the accuracy of the values. Using the method of resting aequorin luminescense in cardiac Purkinje fibers, Wier and Hess (personal communication) have estimated [Ca^{2+}]$_i$ to be 280 nM, which would be 90 nM a^i_{Ca} using the activity coefficient of 0.32. The aequorin method is also subject to calibration problems, but it appears to provide results in the same range as the ion-sensitive microelectrode measurements.

The ratio of $\Delta\tilde{\mu}_{Ca}/\Delta\tilde{\mu}_{Na}$ in resting ventricular muscle was 2.48, and in Purkinje strands it was 2.61. Using a propagation of error calculation and Feiller's method, 95% confidence limits of the ratio are estimated to be 2.86 and 2.15 for ventricular muscle, and 2.77 and 2.43 for Purkinje fibers.

Response to Change of a^o_{Na} and a^o_{Ca}

The fall in a^i_{Na} upon reduction of a^o_{Na} is in agreement with the report of Ellis (1977). Because the level of a^i_{Na} is set by a balance between the passive inward leak of Na^+ and active efflux via the Na-K pump, a reduced leak leads directly to a fall in a^i_{Na}. The level achieved represents a new balance between the leak and the pump. This behavior tends to restore $\Delta\tilde{\mu}_{Na}$ toward its value in normal a^o_{Na} solutions, so that the fall in the sodium electrochemical gradient was not large.

Reduction in a^o_{Na} caused an increase in the steady level of a^i_{Ca}. Because it was sustained, the rise in a^i_{Ca} cannot be explained simply by release of Ca^{2+} from the sarcoplasmic reticulum or the mitochondria. Consequently, there

must have been some change in the balance between leak of Ca^{2+} into the cell and its efflux. If the ATP-dependent Ca^{2+} pump is responsible for this change, then it must be sensitive to a_{Na}^o, contrary to present biochemical evidence (Caroni and Carofoli, 1980). The rise in a_{Ca}^i would be expected if it were controlled by the Na^+ electrochemical gradient through a Na-Ca exchange system. In these experiments the ratio $\Delta\tilde{\mu}_{Ca}/\Delta\tilde{\mu}_{Na}$ did not change upon reducing a_{Na}^o, with the apparent coupling ratio remaining near 2.5.

Experimental change in a_{Ca}^o resulted in a concomitant change in a_{Ca}^i, so that $\Delta\tilde{\mu}_{Ca}$ remained proportional to $\Delta\tilde{\mu}_{Na}$. The change in a_{Ca}^o had effects on a_{Na}^i also. This may be partly caused by Ca^{2+} effects on Na^+ permeability, and preliminary experiments using TTX to block passive Na^+ entry supported this idea. a_{Ca}^o may also independently affect the Na-K pump activity (Lindenmayer and Schwartz, 1975). It seems unlikely that all the Na^+ efflux to lower a_{Na}^i occurred via the Na-Ca exchange, since even with a high coupling ratio, the quantity transferred must be micromolar.

Depolarization from Elevated $[K^+]_o$

The change in the Na^+ electrochemical gradient resulting from a reduction in a_{Na}^o was not large because of the accompanying fall in a_{Na}^i. A more direct way to reduce $\Delta\tilde{\mu}_{Na}$ is to change V_m. Depolarization with elevated $[K^+]_o$ also may cause release of Ca^{2+} from the sarcoplasmic reticulum and/or Ca^{2+} entry via the calcium channel. Indeed, a transient a_{Ca}^i elevation was often seen during the first few minutes of $[K^+]$ depolarization. However, the steady levels after 5–10 min should reflect the balance between the Ca^{2+} leak, its outward transport, and intracellular buffering systems. The Na-Ca exchange model predicts a large increase in a_{Ca}^i with a large reduction in $\Delta\tilde{\mu}_{Na}$. The experimentally observed increase was such that the ratio $\Delta\tilde{\mu}_{Ca}/\Delta\tilde{\mu}_{Na}$ was restored. Depolarization also reduced a_{Na}^i directly, as would be expected for a reduction in the electrochemical gradient for passive entry of Na^+ (Eisner et al., 1981; January et al., 1981).

Increase in a_{Na}^i by Inhibition of the Na-K Pump

Exposure of the cells to ouabain produced a substantial rise in a_{Na}^i and a concomitant rise in a_{Ca}^i. The levels of a_{Na}^i obtained are similar to those found by Ellis (1977); Deitmer and Ellis (1978), and Lee et al. (1980a). The relationship between $\Delta\tilde{\mu}_{Ca}$ and $\Delta\tilde{\mu}_{Na}$ was maintained initially, but as a_{Ca}^i approached 400–500 nM, its increase did not keep pace with the continued increase in a_{Na}^i. One possible explanation for the failure of a_{Ca}^i to continue to increase is that mitochondria began to take up Ca^{2+} when a_{Ca}^i reached high levels (McMillin-Wood et al., 1980).

Control of a_{Ca}^i

The purpose of these experiments was to measure as carefully as possible the sodium and calcium electrochemical gradients in resting heart muscle and to determine whether changes in the sodium gradient would alter the calcium gradient. A relationship between the gradients is predicted by the Na-Ca

exchange model and its predictive accuracy presents a partial test of the model. Thermodynamic description of the gradient relationship predicts a_{Ca}^i from measurements of a_{Na}^i and V_m (and known a_{Na}^o and a_{Ca}^o) with the energy coupling ratio as a variable. The form of the equation is:

$$a_{Ca}^i = \frac{a_{Ca}^o (a_{Na}^i)^n}{(a_{Na}^o)^n \exp - [(n-2)V_m F/RT]}.$$

Using the values for V_m and a_{Na}^i obtained in these studies for resting ventricular muscle we predict a_{Ca}^i to be 1,600 nM for $n = 2$ and 3 nM for $n = 3$. Introducing our measured average value for a_{Ca}^i of 87 nM, we can compute n to be 2.48. Estimating the variability of our measurements by summing their variances provides us 95% confidence limits that exclude n of 2 or 3. We are left with a remarkably constant value for n of ~2.5. It should be noted that the confidence limit estimates do not include the possibility of an undiscovered systematic error present in every experiment.

As we described briefly earlier, the energy coupling ratio is the molecular coupling ratio (stoichiometry) of the Na-Ca exchange process only if the following two assumptions hold: (a) Ca^{2+} pump contributes insignificant amounts of outward Ca^{2+} movement; (b) Na-Ca exchange system is very near equilibrium.

An ATP-dependent Ca pump has been demonstrated in cardiac sarcolemmal vesicles (Caroni and Carafoli, 1980), similar to that seen in red blood cells (Schatzmann, 1973) and squid axon (DiPolo and Beaugé, 1979). Such a pump might lower a_{Ca}^i below that expected for the Na-Ca exchange system. If the Ca pump is important for control of resting a_{Ca}^i in heart muscle, then it would imply that the stoichiometry of Na-Ca exchange is lower than the energy coupling ratio we have measured. The response of the muscles to depolarization and the consistency of n under different conditions argue against this idea, because it would require a very complex relationship between Na^+ and the Ca^{2+} pump.

Leakage of Ca^{2+} into the cell requires that Na-Ca exchange operate in its Ca^{2+} efflux mode, so that the steady level is determined by a balance between exchange and leak, analogous to the Na-K pump-leak balance (Mullins, 1981). It seems unlikely that in resting muscle the Ca^{2+} leak should be high enough to bias the exchange far from its equilibrium, since the V_{max} of Na-Ca exchange appears to be quite high (Caroni et al., 1980). In these experiments conditions varied to alter the Ca^{2+} leak over a substantial range failed to alter the relationship between the sodium and calcium gradients. Recently, sarcolemmal vesicles prepared from heart tissue have been shown to have a Na-Ca exchange system (Reeves and Sutko, 1979). Pitts (1979) estimated the coupling ratio in these vesicles to be ~3. The Hill coefficient for Na^+ stimulation of Ca^{2+} flux was 2.53 (Kadoma et al., 1981), similar to that in barnacle muscle fibers (Nelson and Blaustein, 1981). Our estimate of the energy coupling ratio of 2.5 could indicate a molecular coupling of 5:2 if Na-Ca exchange indeed is very close to equilibrium state. Alternative possibilities are that the molecular

coupling is 3:1 and there is slippage in the transport process, the carrier is charged itself, or other ions are coupled to the transport. The one clear exception to the constancy of n was the failure of a_{Ca}^i to continue to rise despite progressive increase in a_{Na}^i upon inhibition of the Na-K pump, thus leading n to increase toward 3. Although we suggest that mitochondria may begin to buffer a_{Ca}^i at these high levels, it is also possible that Na-K pump activity has in some way fortuitously caused the sodium and calcium gradient ratio to be 2.5.

An implication of n values >2 is that the exchange is voltage dependent. In their early isotopic flux experiments Reuter and Seitz (1968) suggested a neutral exchange, but the experiments were not exact enough to exclude some voltage dependency. In the squid axon, careful studies of Na-Ca exchange demonstrate clearly that it is voltage dependent (Mullins and Brinley, 1975; Baker and McNaughton, 1976). The exact value of the coupling ratio remains unclear, partly because of uncertainties in isotopic flux methods. Our measurements of sodium and calcium gradients strongly support the conclusion that Na-Ca exchange is voltage dependent in heart muscle. Recently, several studies in cardiac muscle have supported the electrogenicity of Na-Ca exchange (Horackova and Vassort, 1979; Chapman and Tunstall, 1980; Coraboeuf et al., 1981).

In summary, the values of the steady state sodium and calcium gradients found in heart muscle and their responses to changes in intracellular Na^+ and Ca^{2+} and to changes in $[K^+]_o$ can be explained by a voltage-dependent Na-Ca exchange system. Under all conditions tested except that of digitalis poisoning, the value of a_{Ca}^i could be predicted from a Na-Ca exchange model with a fixed energetic coupling ratio of ~ 2.5.

Relationship of a_{Ca}^i to Tension

The steady level of a_{Ca}^i could influence tension in three ways. It could directly activate the cross-bridges to produce tonic tension, it could influence the loading of the sarcoplasmic reticulum by Ca^{2+} (Feher and Briggs, 1981), and/or it could influence the magnitude of the slow inward current (Isenberg, 1977).

Tonic tension could be seen in solutions containing 40% a_{Na}^o, where a_{Ca}^i was 230 nM. This value of threshold for tonic tension is similar to that found by Fabiato and Fabiato (1975) in skinned cardiac fibers (a_{Ca}^i of 230 nM = $[Ca^{2+}]$ of 720 nM). The a_{Ca}^i of the resting cell under our experimental conditions is far below this level, so that no activation of contraction would be expected. In all experiments with low a_{Na}^o or high a_{Ca}^o, no tonic tension was seen unless the level of a_{Ca}^i exceeded 200 nM. During depolarization induced by increasing $[K^+]_o$ to 50 mM, contractures were easily produced and a_{Ca}^i was far above the threshold of 230 nM. These contractures have been shown to be sensitive to a_{Ca}^o (Gibbons and Fozzard, 1971), and in frog muscle they can be analyzed quantitatively in terms of a Na-Ca exchange.

It was more difficult to correlate a_{Ca}^i with twitch tension in these experiments since they were measured in different preparations. The most plausible

explanation for the change of twitch tension is that a_{Ca}^i influences loading of sarcoplasmic reticulum with Ca^{2+}, so that the action potential trigger may release different amounts of Ca^{2+} with excitation. Feher and Briggs (1981) have observed increased loading in sarcoplasmic reticulum vesicles exposed to higher levels of Ca^{2+}. Although the Ca pump of the sarcoplasmic reticulum is no doubt activated by a_{Ca}^i, the Ca^{2+} content of the sarcoplasmic reticulum would also be influenced by the volume, the buffering with calsequestrin, and the availability of other ions to dissipate the voltage developed across the sarcoplasmic reticulum membrane.

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