Sodium-Calcium Exchange Current

Dependence on Internal Ca and Na and Competitive Binding of External Na and Ca

YASUHIKO MIURA and JUNKO KIMURA

From the National Institute for Physiological Sciences, Myodaiji, Okazaki, 444 Japan

ABSTRACT   Na-Ca exchange current was measured at various concentrations of internal Na ($[Na]_i$) and Ca ($[Ca]_i$) using intracellular perfusion technique and whole-cell voltage clamp in single cardiac ventricular cells of guinea pig. Internal Ca has an activating effect on Na_i-Ca_o exchange beginning at ~10 nM and saturating at ~50 nM with a half maximum $[Ca]_i$ ($K_m[Ca]_i$) of 22 nM (Hill coefficient, 3.7). Measurement of Na_i-Ca_o exchange current at various concentrations of $[Na]_i$ revealed an apparent $K_m[Na]_i$ of 20.7 ± 6.9 mM ($n = 14$) with i_{max} of 3.5 ± 1.2 $\mu A/\mu F$. For $[Ca]_i$ transported by the exchange, a $K_m[Ca]_i$ of 0.60 ± 0.24 μM ($n = 8$) with an i_{max} of 3.0 ± 0.54 $\mu A/\mu F$ was obtained by measuring Na_o-Ca_i exchange current. These values are apparently different from the values for the external binding site which have been reported previously. Whether Na and Ca compete for the external binding site, and if so, how it affects the binding constants was then investigated. Outward Na_i-Ca_o exchange current became larger by reducing $[Na]_o$. The double reciprocal plot of the current magnitude and $[Ca]_o$ at different $[Na]_o$ revealed a competitive interaction between Na and Ca. In the absence of competitor $[Na]_o$, an apparent $K_m[Ca]_o$ of 0.14 mM was obtained. When comparing internal and external K_m values, the external value is markedly larger than the internal one and thus we conclude that binding sites of the Na-Ca exchange molecule are at least apparently asymmetrical between the inside and outside of the membrane.

INTRODUCTION

The electrogenic Na-Ca exchange mechanism has been known to play an important role in extruding Ca from the sarcolemma to keep the intracellular Ca concentration ($[Ca]_i$) 10^3–10^4 times lower than the extracellular concentration in cardiac cells (Mullins, 1981; Carafoli, 1987). Recently a membrane current generated by this exchange system was identified by several groups in single cardiac cells; e.g., in guinea pig ventricle (Kimura et al., 1986), guinea pig atria (Mechmann and Pott, 1986) and in frog atria (Hume and Uehara, 1986). In guinea pig ventricular cells,

various properties of the Na-Ca exchange current were investigated including the apparent K_m values for [Na]$_o$ and [Ca]$_o$ (Kimura et al., 1987). In the present study we have used the same technique of intracellular perfusion combined with whole-cell voltage clamp and carried out further quantitative analysis of the Na-Ca exchange current.

We first investigated the effect of [Ca]$_i$ as an activator of the exchange system. It has been known that Na$_i$-Ca$_o$ exchange does not operate in the absence of [Ca]$_i$ (Baker, 1972; Dipolo, 1979; Allen and Baker, 1985; Kimura et al., 1987). This evidence suggests that [Ca]$_i$ is not only transported by the exchanger but also plays the role of an activator of the carrier molecule. We have thus measured Na$_i$-Ca$_o$ exchange current while perfusing the patch pipette with various concentrations of Ca to obtain a dose-response relation. Secondly, we obtained apparent K_m values for [Na]$_i$ and [Ca]$_i$. Thirdly, we investigated whether [Na]$_o$ and [Ca]$_o$ interact competitively for the binding site of the exchange molecule. The present results will provide further information on the Na-Ca exchange current in intact cardiac cells.

METHODS

Cell Preparation

Single ventricular cells were dissociated from the guinea pig heart. Animals of either sex weighing 0.2–0.4 kg were anesthetized with sodium pentobarbital 30 mg/kg. The chest was opened under artificial ventilation and the aorta was cannulated in situ. The heart was then dissected out and perfused with normal Tyrode solution on the Langendorff apparatus. After the blood was washed out, the solution was changed to Ca-free Tyrode solution. When the spontaneous heart beat ceased, the perfusate was changed to low-Ca (30–60 μM) Tyrode solution containing 0.4 mg/ml collagenase (Type I; Sigma Chemical Co., St. Louis, MO) for ~20 min. The collagenase solution was washed out by high-K, low-Cl solution and then the heart was dissected into the same solution and stored at 4°C. The temperature of all solutions was maintained at 35–37°C using the water jacket.

Voltage-Clamp Technique

Whole-cell voltage-clamp method was essentially the same as those described by Hamill et al. (1981). Patch electrodes with a tip diameter of 4–5 μm and a resistance of 1–2 MΩ were used. The feedback resistance in the clamp circuit was 100 MΩ containing series resistance compensation. The shape of a ramp pulse of 200 mV/500 ms is shown in Fig. 1 A. The current-voltage (I-V) relation was obtained at the second hyperpolarizing phase of the pulse. Fig. 1 B illustrates the original I-V curve of Fig. 1 A and C after compensating for the capacitive current. All the I-V curves shown in the following are after the capacitive current compensation. The data were stored on line at 330 Hz, ~1 kHz, with a personal computer (NEC PC-9801 Vm) for further analysis.

Change of Solutions in the Pipette

Intracellular perfusion technique has been described in detail in other papers (Soejima and Noma, 1984; Sato et al., 1985). Briefly, the pipette solution was changed by applying suction in the pipette whereby a new solution was introduced through thin polyethylene tubing, which was inserted in the pipette, to the tip of the pipette from small containers located near the head of the pipette (see Fig. 1 of Sato et al., 1985). The time required to change the solution was ~3 min.

Solutions

External solution: normal Tyrode solution contained (in millimolar): 136.9 NaCl, 5.4 KCl, 1.8 CaCl$_2$, 0.5 MgCl$_2$, 0.33 NaH$_2$PO$_4$, 5 glucose, 5 HEPES, (pH 7.4). High-K, low-Cl solution contained (in millimolar): 70 KOH, 40 KCl, 50 l-glutamic acid, 20 taurine, 20 KH$_2$PO$_4$, 3 MgCl$_2$, 10 glucose, 0.5, EGTA, 10 HEPES (pH 7.4). Na-free Li external solution contained (in millimolar) 150 LiCl, 2 MgCl$_2$, 0.1 EGTA, 5 HEPES-LiOH (pH 7.4). The external solution contained 2 mM CsCl to block the inward rectifying K current, and nifedipine (2 μM) for the Ca current, and ouabain (20 μM) to block the Na-K pump. In some of the experiments shown in Fig. 7, 1 mM BaCl$_2$ was used to block the inward rectifier K channel instead of CsCl. The data were not significantly different between Ba and Cs. To induce Na$_i$-Ca$_o$ exchange current, 0.2 mM CaCl$_2$ was added to the test solution. To elicit Na$_o$-Ca$_i$ exchange current, 150 mM LiCl was replaced for NaCl in the presence of 0.5 mM CaCl$_2$.
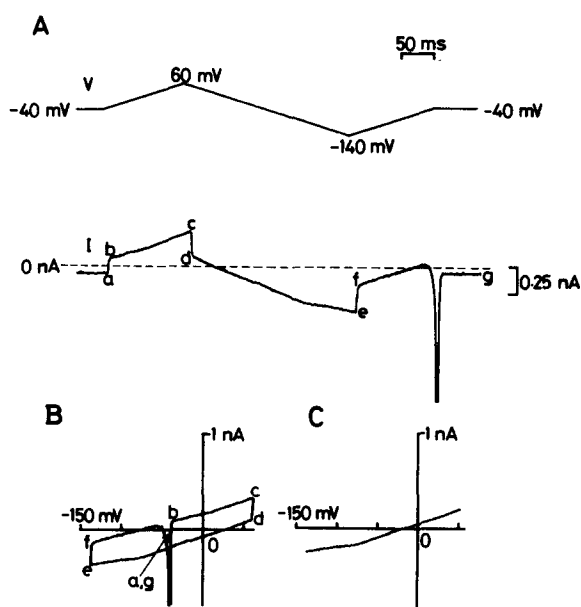

FIGURE 1. (A) Configuration of the ramp voltage pulse (V) and the corresponding current (I) with respect to the time. The holding potential is -40 mV. The maximum voltage is 60 mV and the minimum potential is -140 mV. The parts of the current ab, cd, and ef represent the capacitive current transients. (B) I-V relation plotted from A. The ascending and descending limbs of the current are almost parallel except at the Na current (just before g). (C) The descending limb of de from B is plotted after capacitive current compensation. A similar treatment has been used for all the I-V curves presented in the following figures.

Internal solutions: three types of internal solutions were used. Li-internal solution contained (in millimolar): 70 LiOH, 30 LiCl, 20 BAPTA, 5 Na$_2$ATP, 5 Na$_2$CrP, 30 aspartic acid, 5 MgCl$_2$, 20 HEPES (pH 7.4). Free Ca concentrations used were 0, 5, 10, 20, 30, 50, 100, and 300 nM, which were obtained by adding, respectively, 0, 0.88, 1.7, 3.1, 4.3, 6.3, 9.6, and 14.7 mM CaCl$_2$ to 20 mM BAPTA solution according to the calculation with binding constants of BAPTA (Tsien, 1980) and Fabiato and Fabiato's equations (1979) with a correction by Tsien and Rink (1980).

Cs-rich internal solution contained (in millimolar): 150 CsOH, 5 MgATP, 5 K$_2$CrP, 25 CaCl$_2$, 40 EGTA, 50 MES (2-[N-morpholino] ethanesulfonic acid) (pH 7.4). The Na-containing internal solution was prepared by mixing the equimolar NaOH instead of CsOH and the test solution was made by mixing the two internal solutions to obtain the desired concentration of Na$_i$. For the experiment of Na$_o$-Ca$_i$ exchange, the internal solution contained (in millimolar): 70 CsOH, 30 CsCl, 20 BAPTA, 5 MgATP, 5 Tris$_2$CrP, 30 aspartic acid, 20 MES (pH

7.4). Concentrations of free Ca were 50, 100, 300, and 500 nM by adding 6.3, 9.6, 14.7, and 16.4 mM $CaCl_2$, respectively.

No significant difference was observed between the numerical data obtained in 40 mM EGTA and 20 mM BAPTA, MES and HEPES, or Cs^+ and Li^+ internal solution. When the internal solution was changed from one solution to the other, we waited at least 10 min for the cell interior to be fully equilibrated with the pipette solution, although it has been reported that 2 min is enough to equilibrate the cell once the solution reaches the pipette tip. (Imanaga et al., 1987). In the present study $[Ca]_i$ or $[Na]_i$ indicates the concentration of the pipette solution.

RESULTS

[Ca]$_i$ As an Activator of Na-Ca Exchange Mechanism

As mentioned in the Introduction, Na_i-Ca_o exchange system requires intracellular Ca for its operation. Quantitative investigation on this effect, however, had not been sufficiently carried out. We studied the relation between $[Ca]_i$ and the Na_i-Ca_o exchange current by perfusing the pipette with various Ca concentrations and measuring the Na_i-Ca_o exchange current that was elicited by the fixed concentrations of $[Na]_i$ (20 mM) and $[Ca]_o$ (0.2 mM).

Fig. 2 A shows the representative current traces at the holding potential of −40 mV. The control bath solution contains 150 mM LiCl and 0.1 mM EGTA without Ca and the pipette solution contains 20 mM Na and 5 nM free Ca^{2+}, which is obtained by mixing 20 mM BAPTA and 0.88 mM $CaCl_2$. When the external Ca^{2+} concentration is changed from Ca free to 0.2 mM during a brief period (indicated by the bar above the current record) to induce Na_i-Ca_o exchange current, the holding current shifted outward by ~30 pA at the peak (Fig. 2 A, *top*). The current returned to the control level upon changing the external solution to the original Ca-free solution. When the Ca concentration in the pipette ($[Ca]_i$) was then raised to 20 nM in the same cell, the brief superfusion of 0.2 mM $[Ca]_o$ induced a significantly larger outward current of 120 nA at the peak. The current became even larger as $[Ca]_i$ was elevated to 50 nM. A similar experiment was repeated in the presence of various concentrations of Ca between 0 and 300 nM in the pipette solution. Two to four different Ca concentrations were tested in the same cell.

The lower part of Fig. 2 A shows the I-V relations obtained by the ramp pulse before (*a, c*, and *e*) and at the peak shift during the superfusion of 0.2 mM $[Ca]_o$ (*b, d*, and *f*). Increasing $[Ca]_i$ increased the control current conductance at very negative potentials while the zero-current level remained almost the same. This effect may be attributed to the Ca-sensitive nonspecific cation current (Colquhoun et al., 1981; Ehara et al., 1988). The difference currents between the peak and the control of Fig. 2 A are illustrated in Fig. 2 B. The currents elicited by 0.2 mM $[Ca]_o$ are outward at all potentials, and at $[Ca]_i$ higher than 10 nM the currents become progressively larger at more positive potentials. This result is compatible with the voltage-dependent Na_i-Ca_o exchange current reported by Kimura et al. (1987). The outward Na-Ca exchange current becomes larger as $[Ca]_i$ becomes higher, indicating that the activity of the exchanger increases at higher $[Ca]_i$.

An outward current at 5 nM $[Ca]_i$ does not show a characteristic voltage dependency of the Na-Ca exchange current but remains constant at all potentials. This

current cannot be explained by the surface charge effect of [Ca]$_o$ because the surface charge effect should shift the control current in the opposite direction. Since the similar effect was observed in the absence of [Na]$_i$ (see Fig. 2 A, Kimura et al., 1987), this voltage-independent current at low concentrations of [Ca]$_i$ may not be a Na-Ca exchange current.

The outward current induced by [Ca]$_o$ did not sustain a peak steady state but decayed slowly when the current was small and decayed more rapidly as the current became larger. The most likely reason for this is the depletion of Na and/or the
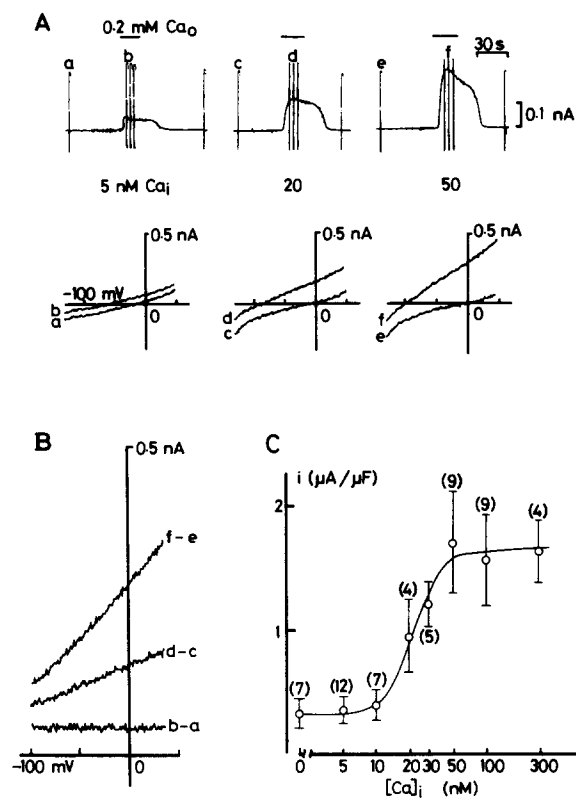

FIGURE 2. (*A, Top*) An outward current is evoked in response to superfusion of 0.2 mM [Ca]$_o$ in the presence of 20 mM [Na]$_i$ at different [Ca]$_i$. The outward current is larger at higher [Ca]$_i$. The holding potential is −40 mV. (*Bottom*) I-V curves of the control (a, c, e) and the peak (b, d, f) obtained above. (*B*) I-V curves of the difference between the peak and the corresponding control current shown in A as indicated on the right of each trace. (*C*) Dose-response relation between the mean current desnity and [Ca]$_i$. The current density was obtained by measuring the difference current at 0 mV and dividing it by the membrane capacitance. The current starts to become larger at ~10 nM and saturates at ~50 nM. The half-maximum [Ca]$_i$ is 22 nM and the Hill coefficient is 3.7. The bars indicate the standard deviation of the mean and the numbers in the parentheses are the number of original data.

accumulation of Ca under the membrane. To minimize the error of measurement induced by this effect, the external solution was changed as quickly as possible so that the peak could be reached before the perturbation of the internal ions became significant.

To investigate a dose-response relation, the magnitude of the peak current was measured at 0 mV, where the contamination of the Ca-sensitive background current should be at a minimum, and the current density was calculated in order to compare the results from different cells. As shown in Fig. 2 C, a sigmoid relation is obtained

between the current density and $[Ca]_i$. The minimum $[Ca]_i$ that could elicit the Na-Ca exchange current is ~10 nM and saturation is seen at concentrations higher than 50 nM with a half-maximum response at 22 nM. The Hill plot between the current density and $[Ca]_i$ (not shown) is linear with a Hill coefficient of 3.7.

These findings indicate that $[Ca]_i$ indeed activates the exchanger in an extremely low concentration range and that the effect is saturated at physiological $[Ca]_i$, which is usually higher than 50 nM. The Hill coefficient of 3.7 indicates that more than one Ca binds to the activation site cooperatively.

The absence of Ca (0.1 mM EGTA present) in the external solution did not inhibit Na_o-Ca_i exchange current. Thus, external Ca is not required for the activation of the exchanger. In this sense the exchange molecule is not symmetrical between the inside and outside of the membrane.

Relation between [Na]_i and Na_i-Ca_o Exchange Current

The affinity of the internal binding sites for Na has not been investigated in intact cardiac preparations. We studied the relation between $[Na]_i$ and the outward Na_i-Ca_o exchange current by varying $[Na]_i$ in the pipette solution. Fig. 3 A shows representative current traces at the holding potential of −40 mV. The control external solution contained 150 mM LiCl without Na and $[Ca]_o$ was minimized by adding 0.1 mM EGTA to nominally free Ca. The internal solution contained 100 nM free Ca, which was obtained by mixing 40 mM EGTA and 25 mM Ca. In the presence of 4.7 mM $[Na]_i$, superfusion of 0.2 mM $[Ca]_o$ (indicated by a bar) shifted the holding current outward by ~40 pA. This outward Na-Ca exchange current increased progressively as $[Na]_i$ increased from 4.7 to 9.4, 18.8, 37.5, or to 75 mM. The I-V relations obtained at the peak response at various $[Na]_i$ are shown in Fig. 3 B. The control I-V curve in the absence of $[Ca]_o$ is approximately linear and passes 0 mV. The currents induced by the superfusion of 0.2 mM $[Ca]_o$ (Fig. 4 B, b, d, f, and h) are outward at almost all the potentials and became progressively larger at more positive potentials, as was expected for the exchange current. To isolate the exchange current, the control was subtracted from the peak current of $[Ca]_o$ superfusion at each $[Na]_i$ as shown in Fig. 3 C. The difference currents increased as $[Na]_i$ increased.

Each difference current (b-a, d-c, f-e, and h-g) was plotted on a semilogarithmic scale (Fig. 3 D) to compare the slope of the I-V curve. It was possible for the current to be fitted by an exponential function when $[Na]_i$ was relatively low. Even at higher $[Na]_i$, however, the slope became less steep and not exponential, particularly at positive potentials. At $[Na]_i$ from 4.7 and 18.8 mM, the lines fitted by computation were almost parallel with the slope factor (r) of 0.36 ± 0.04 (n = 29), which is calculated from the following equation (Noble, 1986):

$$i = a \exp (rEF/RT) \tag{1}$$

where F, R, and T have their usual meanings, i is the current magnitude, and a is a scaling factor that determines the magnitude of the current. At high $[Na]_i$, however, the slope at the negative potentials could often be fitted with the same slope factor, thus indicating that the voltage dependency is not affected by $[Na]_i$. The slope factor r represents the position of the energy barrier in the electrical field in the rate

theory. The above value of r is very similar to that obtained by Kimura et al. (1987).

The most likely reason for the apparent saturation of the I-V curve at positive potentials at high [Na]$_i$ is that [Ca]$_o$ of 0.2 mM is not high enough to induce the exponentially increasing current. Our preliminary experiment supported this hypothesis since the I-V curve became exponential when higher [Ca]$_o$ was superfused.

To obtain the dose-response relation, the current density was calculated from the current magnitude measured at 0 mV and plotted against [Na]$_i$. In one experiment (figure not shown), the exchange current elicited by 0.05 mM [Ca]$_o$ did not differ in
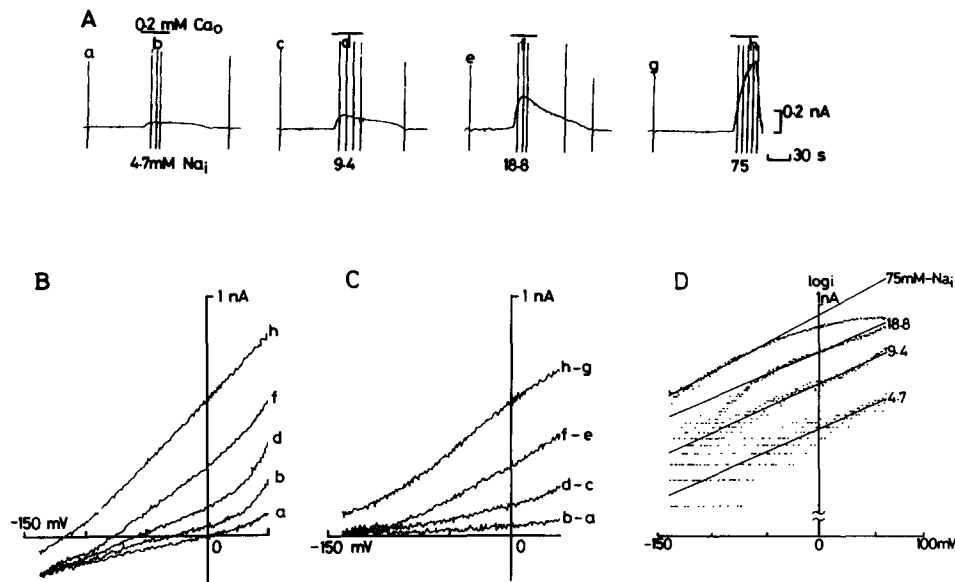

FIGURE 3. (A) An outward current of various magnitude was elicited at various [Na]$_i$ by superfusing 0.2 mM [Ca]$_o$ in the same cell. [Ca]$_i$ was 0.1 μM throughout. Increasing [Na]$_i$ induces a larger outward current. (B) I-V curves obtained at the control (a) and at the peak of each [Na]$_i$ indicated by the corresponding labels in A. (C) Difference currents between the peak and the control as indicated on the right of each curve. (D) Semilogarithmic plot of the difference currents shown in C. At low [Na]$_i$ the slope is similar but at higher [Na]$_i$ the slope is small particularly at positive potentials.

the magntiude between 75 and 150 mM [Na]$_i$. We therefore assumed that 75 mM [Na]$_i$ gives almost the maximum exchange current. As shown in Fig. 4 A, the relation between [Na]$_i$ and the current density is sigmoid and the apparent half-maximum [Na]$_i$ is ~20 mM with the maximum current density of 3.5–4 μA/μF. The apparent half-maximum [Na]$_i$ and the maximum current density were also obtained by the Lineweaver-Burk double reciprocal plot (figure not shown). The plot of $1/[Na]^2$ against the reciprocal of the current density can be fitted with a line that yields the half-maximum response at 20.7 ± 6.9 mM [Na]$_i$ and a maximum current density of 3.5 ± 1.2 μA/μF (n = 14). These values are very similar to those estimated

from Fig. 4 A, which suggests that the outward Na-Ca exchange current saturates near 75 mM $[Na]_i$ at 0.2 mM $[Ca]_o$. The plot of $1/[Na]$ nor $1/[Na]^3$ did not fit to a linear relation as well as $1/[Na]_i^2$ did. Fig. 4 B illustrates the Hill plot obtained by using the mean current magnitudes of Fig. 4 A, which yields a Hill coefficient of 1.93. The average Hill coefficient was 2.03 ± 0.2 ($n = 14$).

Relation between $[Ca]_i$ on Na_o-Ca_i Exchange Current

We then investigated the affinity of $[Ca]_i$, which is transported by the carrier in exchange for external Na by using inward exchange current. Since the above results indicated that the exchanger was fully activated above 50 nM $[Ca]_i$, various $[Ca]_i$ (50–500 nM) were tested. Fig. 5 A illustrates the representative current trace at the holding potential of -40 mV. The bath solution contained 150 mM LiCl (Na free) and 0.5 mM $CaCl_2$. The internal solution contained 150 mM Cs^+ without Na and 50 nM free $[Ca]_i$. When the external solution was suddenly changed from 150 mM Li^+ to 150 mM Na^+, as indicated by the bar above the current trace, an inward Na_o-Ca_i exchange current was elicited, which declined after the peak. The holding current returned to the control level when the external solution is returned to the Na-free
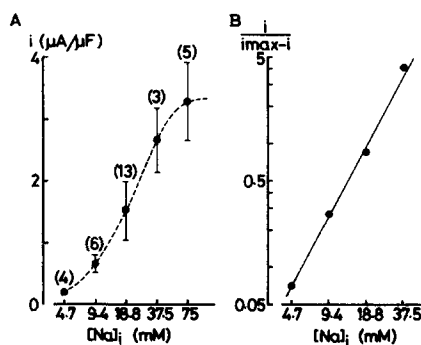

FIGURE 4. (A) Semilogarithmic dose-response curve between the current density and $[Na]_i$ obtained from the date in Fig. 3. The $K_m[Na]_i$ is ~ 20 mM. (B) Hill plot of the mean value of the current magnitude of A gives a Hill coefficient of 1.9.

solution. Increasing $[Ca]_i$ from 50 to 100, 300, or to 500 nM increases the magnitude of the inward current progressively. $[Ca]_i$ higher than 500 nM was not tested because of the fear of contracture of the cell. The I-V relation at each $[Ca]_i$ is shown in the lower row of Fig. 5 A. Increasing $[Ca]_i$ always increased the conductance of the control current as mentioned before. Fig. 5 B shows the difference between the I-V curves before and during the $[Na]_o$ superfusion. The difference currents are identified as the Na-Ca exchange current and not the Ca-activated nonselective cation channel current because the currents are (a) evoked by the superfusion of NaCl but not by LiCl, (b) inward at most of the potentials as expected for the exchange current in the absence of $[Na]_i$, and (c) larger at more negative potentials with an almost exponential I-V relation, unlike a linear Ca-sensitive cation current.

The decline of the inward current after the peak could be explained by the depletion of $[Ca]_i$ and/or accumulation of $[Na]_i$. Upon washing out the $[Na]_o$, the inward current rapidly decayed and then outward "rebound" current appeared before returning to the original holding current level. This "rebound" was larger as the preceding inward current was larger, as shown in Fig. 5 A (see for example 300 nM $[Ca]_i$). This result indicates that Na accumulates under the membrane during the

flow of the inward exchange current, and upon washing out [Na]$_o$, the accumulated [Na]$_i$ is transported by the exchanger in the presence of [Ca]$_o$ (0.5 mM). To minimize the error induced by the perturbation of [Na]$_i$ and [Ca]$_i$, as in the experiment for the outward exchange current, the external solution was changed as quickly as possible and the I-V curve was measured at the early part of the peak of the inward current.

The dose-response relation between [Ca]$_i$ and the inward exchange current was obtained by measuring the current amplitude at -50 mV, which was converted to a
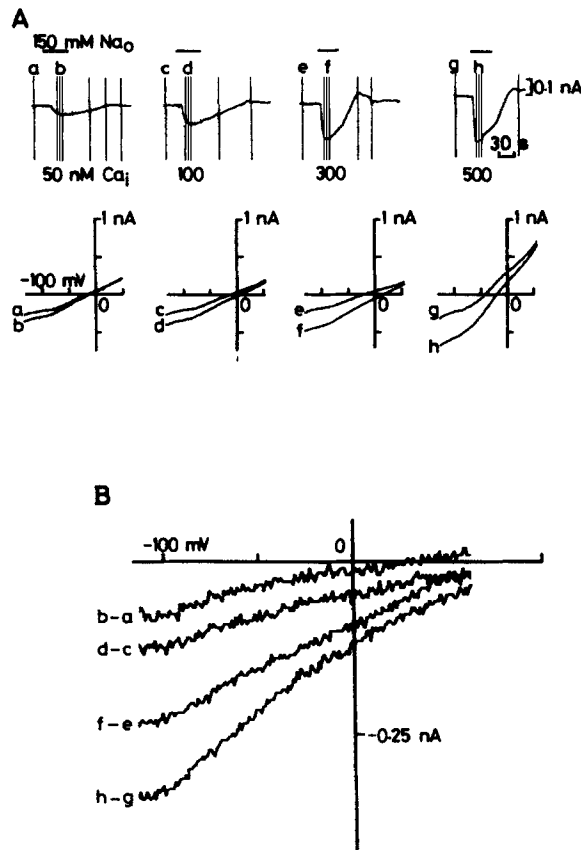

FIGURE 5. (*A*, *Top*) Inward exchange current induced by 150 mM [Na]$_o$ superfusion during the period indicated by the bar above in the presence of different [Ca]$_i$ indicated below. The holding potential is -40 mV. (*Bottom*) I-V curves obtained at the corresponding labels in the control and the peak response. (*B*) The difference currents between the control and the peak inward current as indicated on the left of each trace. As [Ca]$_i$ becomes higher, the inward exchange current becomes larger.

current density to compare the values from different cells (Fig. 6 *A*). The potential of -50 mV was chosen because the inward current was too small to measure at 0 mV and the current deviated from the exponential I-V relation at more negative potentials.

The K_m[Ca]$_i$ and the maximum current density were estimated by the double reciprocal plot (Fig. 6 *B*). The regression line obtained by the mean current magnitudes at 0.05, 0.1, and 0.3 μM [Ca]$_i$ and the two values at 0.5 μM yielded a K_m[Ca]$_i$ of 0.77 μM and a maximum current density of 3.6 μA/μF. The K_m values estimated from each cell ranged between 0.30 and 1.17 μM, the average K_m was 0.60 $\pm$ 0.24

μM, and the maximum current density was 3.0 ± 0.54 μA/μF ($n = 8$). Over the range of concentrations examined, there was no evidence for cooperativity.

To examine the voltage-dependence of the Ca binding, the current density was also measured at -25 and -75 mV. The $K_m[Ca]_i$ was 0.52 ± 0.27 μM at -25 mV and 0.45 ± 0.25 μM ($n = 8$) at -75 mV. These values do not suggest a significant difference among the $K_m[Ca]_i$ values at different membrane potentials.

Competition between [Ca]$_o$ and [Na]$_o$ at the External Binding Site

Reuter and Seitz (1968), Baker et al. (1969), and Reeves and Sutko (1983) have suggested in the flux studies that there is a competitive interaction between Na and Ca at the same site of the carrier. We investigated whether this is true in our preparation and if so, how it affects the K_m values. Two types of experiments were designed. Firstly, the outward exchange current was induced by 0.5 mM [Ca]$_o$ and 20 mM [Na]$_i$ in the presence of various [Na]$_o$ in the same cell. Secondly, the apparent K_m of [Ca]$_o$ was obtained by measuring outward exchange current at various [Na]$_o$. Different cells were used for each [Na]$_o$ in the latter case. Fig. 7 A illustrates the dose-response relation between [Na]$_o$ and the density of the outward Na-Ca
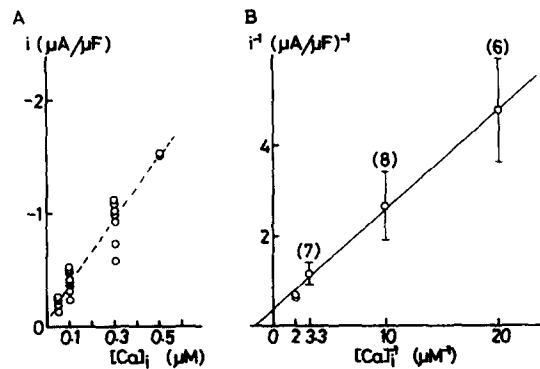

FIGURE 6. (A) Dose-response curve of the inward current obtained in the method shown in Fig. 5. (B) Lineweaver-Burk double reciprocal plot between the current density and [Ca]$_i$. The plot yields $K_m[Ca]_i$ of 0.77 μM and i_{max} of 3.6 μA/μF. The mean $K_m[Ca]_i$ obtained from eight cells is 0.60 ± 0.24 μM with an i_{max} of 3.0 ± 3.54 μA/μF ($n = 8$).

exchange current at $+25$ mV evoked by 20 mM [Na]$_i$ and 0.5 mM [Ca]$_o$. As [Na]$_o$ was decreased from 150 to 100, 75, or 50 mM, the outward exchange current became progressively larger, suggesting that there is indeed an interaction between [Na]$_o$ and [Ca]$_o$.

Upon decreasing [Na]$_o$ further to 25 mM, however, the current became smaller than that at 50 mV. Baker et al. (1969) have observed the similar phenomenon in squid axon when using dextrose to replace Na but not when using Li. We tested whether this effect was caused by the Na-Ca exchange mode changing into the electroneutral Ca-Ca exchange mode in the presence of external Li. Blaustein et al. (1974, 1975) reported that external monovalent cation facilitates Ca-Ca exchange but not if the external ion has a large molecular weight, e.g., choline. We therefore tested N-methyl-D-glucamine (NMG) or choline instead of Li to replace Na in the above-mentioned experiment. The results, however, did not differ significantly between Li and NMG or choline, and the outward exchange current was always smaller at 25 mM [Na]$_o$ than at 50 mM [Na]$_o$.

To investigate the type of interaction between Na and Ca, we carried out a double

reciprocal plot of the current densities obtained at $+25$ mV against $[Ca]_o$ at four differenct $[Na]_o$ as shown in Fig. 7 B. Each point is an average of two to eight experiments. The reciprocals of the current densities obtained at each $[Na]_o$ could be fitted with a line that crosses each other on the ordinate, which indicates the competitive type of inhibition between $[Na]_o$ and $[Ca]_o$. The reciprocal of the ordinate value gives a maximum current density of ~ 4.3 $\mu A/\mu F$.

The apparent $K_m[Ca]_o$ were 0.34, 0.46, 0.87, and 1.78 mM at 50, 75, 100, and 150 mM $[Na]_o$, respectively. The apparent $K_m[Ca]_o$ at 140 mM $[Na]_o$ was ~ 1.4 mM
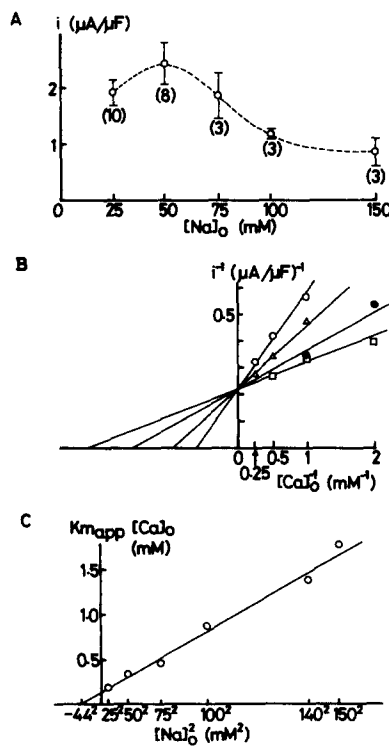

FIGURE 7. (A) To see an interaction between $[Na]_o$ and $[Ca]_o$, the outward exchange current was induced by 0.5 mM $[Ca]_o$ and 20 mM $[Na]_i$ while $[Na]_o$ was changed. Magnitude of the current density is plotted against various $[Na]_o$. As $[Na]_o$ decreases the current density increases. At 25 mM $[Na]_o$, however, the current becomes smaller than at 50 mM $[Na]_o$. (B) Double reciprocal plots of the mean current magnitude against $[Ca]_o$ at four different $[Na]_i$. Different symbols indicate different $[Na]_o$; i.e., 150 mM (O, $n = 2$, 4, and 6 at 4, 2, and 1 mM $[Ca]_o$, respectively), 100 mM $[Na]_o$ ($\triangle$, $n = 3$ at all $[Ca]_o$), 75 mM $[Na]_o$ ($\bullet$, $n = 3$ at all $[Ca]_o$), and 50 mM $[Na]_o$ ($\square$, $n = 5$, 7, and 8 at 2, 1, and 0.5 mM $[Ca]_o$, respectively). The lines fitted by eye at each $[Na]_o$ crossed at the same point on the ordinate, indicating the competitive inhibition of Na against Ca. The crossing point indicates an i_{max} of 4.3 $\mu A/\mu F$. (C) Apparent $K_m[Ca]_o$ at different $[Na]_o$ are plotted against the square of $[Na]_o$. A regression line drawn by the least-squares method produced a $K_m[Ca]_o$ of 0.14 mM in the absence of $[Na]_o$ and a $K_i[Na]_o$ of 44 mM.

(Kimura et al., 1987). Fig. 7 C shows a linear relation between the apparent K_m values for $[Ca]_o$ and the square of $[Na]_o$ which was plotted according to the following equation:

$$K_m \, app = K_m[1 + ([I]/K_i)^2] \qquad (2)$$

where [I] indicates the concentration of an inhibitor and K_i indicates the inhibition constant.

At 25 mM $[Na]_o$, although the current became smaller than that at 50 mM $[Na]_o$, the $K_m[Ca]_o$ of 0.19 mM (obtained from the $[Ca]/i$ vs. $[Ca]$ plot [Hanes-Woolf plot; see Segel, 1976] of 31 data points between 0.1 and 2 mM $[Ca]_o$) lies on the regression line, suggesting that the diminishing effect on the current of low $[Na]_o$ is independent of the competitive interaction of Na at the Ca-binding site.

The apparent K_m is reduced as the concentration of competitor is reduced. Thus the apparent $K_m[Ca]_o$ of 1.78 mM at 150 mM $[Na]_o$ became progressively smaller with the reduction of $[Na]_o$, and the regression line gave a $K_m[Ca]_o$ of 0.14 mM in the absence of $[Na]_o$ and a K_i value for $[Na]_o$ of ~44 mM.

From the above results we conclude that the external Na and Ca compete for the same binding site and that the apparent $K_m[Ca]_o$ is 0.14 mM at 0 $[Na]_o$. This value is still larger than the $K_m[Ca]_i$ of 0.6 μM, indicating asymmetry of the external and internal binding sites of Ca.

DISCUSSION

Ca_i As an Activator of Na-Ca Exchange

As has been described in the Introduction, the inhibitory effect of Ca chelator on Na-Ca exchange has been reported qualitatively by various workers in squid axon (Baker, 1972; Baker and McNaughton, 1976; Allen and Baker, 1985), single cardiac ventricular cells (Kimura et al., 1986), and more recently in dog sarcolemma vesicles (Reeves and Poronnik, 1987). Systematic investigation, however, has been carried out only by DiPolo (1979) in squid axon, where he found the activation threshold was at 40 nM but that the Na_i-dependent Ca influx did not saturate even at 0.8 μM. In our result, a sigmoid curve obtained between Na_i-Ca_o exchange current and $[Ca]_i$ gave a threshold concentration of ~10 nM and a half-maximum activation at ~22 nM $[Ca]_i$. The maximum activation was reached at ~50 nM, indicating that the exchanger is fully activated in the physiological Ca concentration range where the minimum is the resting Ca concentration of 100–260 nM (Marban et al., 1980, 1987; Sheu and Fozzard, 1982; Allen et al., 1983; Cobbold and Bourne, 1984; Wier et al., 1987). Our results also indicate that the activation site has an extremely high affinity to Ca and that more than one Ca ion binds to the site cooperatively. If the assumption underlying the Hill equation is applicable (Segel, 1976), at least four Ca ions bind to the site. Recent findings of various Ca-binding proteins have revealed that binding of four Ca ions is a prototype of Ca-binding protein (calmodulin is one example and troponin C of the skeletal muscle is another). These proteins, however, have relatively low K_d values, on the order of micromolars (Carafoli, 1987). Caroni and Carafoli (1981) reported that trifluoperazine, a calmodulin inhibitor, did not affect Na-Ca exchange activity. Thus it is unlikely that either of these Ca-binding proteins are involved. As far as the K_d value is concerned, parvalbumin has a low K_d value of ~30 nM, which is close to our value. This protein, however, binds only two Ca ions and not four. Therefore there is a possibility that some as yet unknown Ca-binding protein is involved as a part of the structure of the exchanger molecule.

The exchanger does not require external Ca, since the Na_o-Ca_i exchange current develops in the absence of $[Ca]_o$. This evidence was also mentioned in squid nerve

vesicles by DiPolo and Beauge (1986). As they have described, internal Ca seems an "essential activator" of the exchanger but not of the external Ca, and at least on this point the exchanger is not a symmetrical molecule between inside and outside the membrane.

The Half-Maximum Concentration of Na$_i$

The apparent half-maximum Na$_i$ concentration obtained in the present study is 20.7 ± 6.9 mM. Using the same technique, Gadsby and his co-workers have recently reported a similar K_m of 18.3 ± 3 mM with a Hill coefficient of 1.6 (Gadsby et al., 1988). Similar values have also been obtained in inside-out vesicles of the bovine cardiac vesicles (22 mM, Kadoma et al., 1982; 26 mM, Reeves and Sutko, 1983), in internally perfused squid axon (~30 mM, Blaustein and Santiago, 1977), and in human neutrophils (~26 mM, Simchowitz and Cragoe, 1988). Although methods and preparations were different, all these values are in a surprisingly small range between about 20 and 30 mM, which is close to experimentally elevated [Na]$_i$ by the Na-K pump block (Deitmer and Ellis, 1978).

Eisner and Lederer (1980) have shown that blocking Na-K pump elevates [Na]$_i$ and tension in Purkinje fibers. This evidence can be explained by the effective K_m[Na]$_i$ of the Na-Ca exchanger being close to the physiologically changeable level of [Na]$_i$ which is determined by the Na-K pump, Thus, blocking of the Na-K pump increases internal Na which in turn traps Ca in the cell via Na-Ca exchange by either reducing Ca extrusion or increasing Ca influx. Recently Lee and Clusin (1987) have succeeded in measuring the increase in the steady state [Ca]$_i$ from ~60 to 300 nM by blocking the Na-K pump in chick embryonic myocardial cell aggregates.

The Half-Maximum Concentration of Ca$_i$

Recently Barcenas-Ruiz et al. (1988) and Berlin et al. (1988) have measured [Ca]$_i$ by fura-2 or by indo-1, and the corresponding magnitude of inward tail current or putative Na-Ca exchange current induced at the offset of depolarizing pulses. Both showed a linear relation between [Ca]$_i$ and the current up to 1.6 or 2 μM, which suggests that the K_m[Ca]$_i$ is higher than our value of 0.6–0.8 μM.

Our data, however, were measured between a [Ca]$_i$ of 50 and 500 nM, which was limited to a smaller concentration range than the K_m value. Ideally, for the linear plot analysis, both larger and smaller concentrations of [Ca]$_i$ near the K_m value should be tested, otherwise the obtained K_m value tends to become smaller if the range of substrate concentrations is too low. In the present study, however, we were unable to extend the study to higher [Ca]$_i$ because of myofibrillar contraction. Therefore, in view of the uncertainty of the value of our K_m[Ca]$_i$, the results of Barcenas-Ruiz et al. (1988) and Berlin et al. (1988), and that of our study are not necessarily in conflict.

So far the K_m[Ca]$_i$ of 0.73 μM, which is as low as our value, was reported in squid axons in the presence of ATP; while in the absence of ATP, it increases to 8 μM (Blaustein and Santiago, 1977) or 10 μM (DiPolo and Beauge, 1983). In cardiac vesicles, Philipson and Nishimoto (1982) measured 33 μM. Reeves and Poronnik (1987) recently reported that variability of K_m values in vesicle studies (15–40 μM, see review by Philipson, 1985) may be attributed to the change in [Ca] at the activa-

tor site (not transported). The overall values of $K_m[Ca]_i$, however, are considerably lower than $K_m[Ca]_o$.

The Hill Coefficient for Internal and External Na

The Hill coefficient for $[Na]_o$ was always 3 (Kimura et al., 1987), while that for $[Na]_i$ obtained in the present study was 2. This difference may indicate asymmetrical cooperativity between inside and outside. Thus, binding of three Na ions may occur cooperatively at the external sites while in the internal sites binding of only two Na ions may be cooperative, and the sites can bind either two Na ions or one Ca, which are distinct from another internal site where only Na can bind independently. The difference in the Hill coefficient may therefore be another piece of evidence that external and internal binding sites are asymmetrical.

Decrease of Exchange Current at Very Low [Na]_o

Reuter and Seitz (1968) and Baker et al. (1969) proposed a hypothesis that Na and Ca bind to the same sites of the exchanger. Reeves and Sutko (1983) demonstrated

TABLE I

| | Apparent K_m | | Counter-ion |
	Physiological	Without competitor	
	mM	*mM*	
$[Na]_o$	87.5 ± 10.7 $(n = 6)$		0.43 μM $[Ca]_i$
$[Na]_i$		20.7 ± 6.9 $(n = 14)$	0.2 mM $[Ca]_o$
$[Ca]_o$	1.4 $(n = 5)$	0.14	20 mM $[Na]_i$
$[Ca]_i$		0.0006 ± 0.00024 $(n = 8)$	150 mM $[Na]_o$

the competitive inhibition between the flux of Na and Ca ions in cardiac vesicles and supported the above theory. Our present results also indicate the competitive inhibition of Na and Ca. In the flux studies, Na-dependent Ca uptake progressively increased as the concentration of Na decreased even to 0 mM (Reeves and Sutko, 1983). In our study, however, the outward Na-Ca exchange current initially became larger as $[Na]_o$ decreased, but reducing $[Na]_o$ further than 50 mM made the outward exchange current smaller than before. Baker et al. (1969) reported a similar decrease in Ca influx when replacing external Na with dextrose, but the decrease was not so significant when using Li in squid axons. In our study, Li, NMG, or choline did not show a significant difference. A possible explanation for this phenomenon is either that NMG or choline also activates Ca-Ca exchange despite their substantially large molecular weight, or more likely, that Na has an activating effect of the exchanger from outside the membrane.

Comparison between Internal and External K_m Values

Our data obtained in the presence (near physiological conditions) and absence of each competitor ion are shown in Table I. (Since Na-Ca exchange does not operate in the absence of $[Ca]_i$, 0.1 μM $[Ca]_i$ is present in the experiment to determine $[Na]_i$.) It should be noted that the above K_m's are "apparent" values and not the

"true K_m" values. The values of K_m, V_{max}, and also the dissociation constant of competitive inhibitors (K_i) vary depending on the substrate concentrations or, in other words, on the fraction of enzyme in the effective forms. The true K_m is the value obtained when all the other substrates are at saturating concentrations. In Table I the $K_m[Ca]_i$ may be close to the true K_m and the other K_m values do not represent the real affinities and are thus called "apparent" values.

Kadoma et al. (1982) suggested that in the vesicle, $K_m[Na]$ was different inside and outside the vesicle (22 mM for inside-out vesicles and 31 mM for the total vesicles including both inside-out and right-side-out vesicles). Meanwhile Philipson (1985) reported that the $K_m[Na]$ was similar on the two sides (30 mM). Furthermore he observed that external Na inhibited Na_i-dependent Ca uptake much more potently than that of the inside-out vesicles, and so concluded that external and internal binding sites are symmetrical, but that because of the different interacting property of the two ions it appears as if the exchanger molecule is asymmetrical.

In our present study, $K_m[Na]_o$ in the absence of $[Ca]_o$ was not studied, while a $K_i[Na]_o$ of 44 mM was obtained. $K_i[Na]_o$ is not the same as $K_m[Na]_o$ except under certain circumstances, but it is likely to be similar at least in the order of the value. Thus our $K_i[Na]_o$ value is not so different from the $K_m[Na]_o$ reported in the flux studies nor from our $K_m[Na]_i$ of 21 mM. These results indicate that the internal and external binding sites for Na may not be very different. The apparent value of $K_m[Na]_o$ under physiological conditions becomes about threefold that of $K_m[Na]_i$ because of the competitive effect of $[Ca]_o$.

As for the Ca ion, $K_m[Ca]_o$ is roughly 1,000 times $[Ca]_i$ in the presence of 140 mM $[Na]_o$. Elimination of the $[Na]_o$ decreases $K_m[Ca]_o$ by one tenth to 0.14 mM. This value is still about 100 times higher than the $K_m[Ca]_i$, indicating the asymmetry of Ca binding between inside and outside the membrane. Moreover, the effect of Ca as an activator occurs only inside the membrane. Thus we conclude that the binding property of the exchange molecule is at least apparently asymmetrical between the inside and outside of the cell.

REFERENCES

Allen, T. J. A., and P. F. Baker. 1985. Intracellular Ca indicator quin-2 inhibits Ca^{2+} inflow via Na_i/Ca_o exchange in squid axon. *Nature.* 315:755–756.

Allen, D. G., D. A. Eisner, M. J. Lab, and C. H. Orchard. 1983. The effects of low sodium solutions on intracellular calcium concentration and tension in ferret ventricular muscle. *Journal of Physiology.* 345:391–407.

Baker, P. F. 1972. Transport and metabolism of calcium ions in nerve. *Progress in Biophysics and Molecular Biology.* 24:177–223.

Baker, P. F., M. P. Blaustein, A. L. Hodgkin, and R. A. Steinhardt. 1969. The influence of calcium on sodium efflux in squid axons. *Journal of Physiology*. 200:431–458.

Baker, P. F., and P. A. McNaughton. 1976. Kinetics and energetics of calcium efflux from intact squid giant axons. *Journal of Physiology*. 259:103–144.

Barcenas-Ruiz, L., D. J. Beuckelmann, and W. G. Wier. 1988. Sodium-calcium exchange in heart: membrane currents and changes in [Ca^{2+}]$_i$. *Science*. 238:1720–1722.

Berlin, J. R., J. R. Hume, and W. J. Lederer. 1988. [Ca^{2+}]$_i$-activated "creep" currents in guinea-pig ventricular myocytes. *Journal of Physiology*. 407:128P. (Abstr.)

Blaustein, M. P., and E. M. Santiago. 1977. Effects of internal and external cations and of ATP on sodium-calcium and calcium-calcium exchange in squid axons. *Biophysical Journal*. 20:79–111.

Blaustein, M. P., and J. M. Russell. 1975. Sodium-calcium exchange and calcium-calcium exchange in internally dialyzed squid giant axons. *Journal of Membrane Biology*. 22:285–312.

Blaustein, M. P., J. M. Russell, and P. De Weer. 1974. Calcium efflux from internally dialyzed squid axons: the influence of external and internal cations. *Journal of Supramolecular Structure*. 2:558–581.

Carafoli, E. 1987. Intracellular calcium homeostasis. *Annual Review of Biochemistry*. 56:395–433.

Caroni, P., and E. Carafoli. 1981. The Ca^{2+}-pumping ATPase of heart sarcolemma. *Journal of Biological Chemistry*. 256:3263–3270.

Cobbold, P. H., and P. K. Bourne. 1984. Aequorin measurements of free calcium in single heart cells. *Nature*. 312:444–446.

Colquhoun, D., E. Neher, H. Reuter, and C. S. Stevens. 1981. Inward current channels activated by intracellular Ca in cultured heart cells. *Nature*. 29:752–754.

Deitmer, J. W., and D. Ellis. 1978. The intracellular sodium activity of cardiac Purkinje fibres during inhibition and reactivation of the Na-K pump. *Journal of Physiology*. 284:241–259.

DiPolo, R. 1979. Calcium influx in internally dialyzed squid giant axons. *Journal of General Physiology*. 73:91–113.

DiPolo, R., and L. Beauge. 1983. The calcium pump and sodium-calcium exchange in squid axons. *Annual Review of Physiology*. 45:313–324.

DiPolo, R., and L. Beauge. 1986. Reverse Na-Ca exchange requires internal Ca and/or ATP in squid axons. *Biochimica et Biophysica Acta*. 854:298–306.

Ehara, T., A. Noma, and K. Ono. 1988. Calcium-activated nonselective cation channel in ventricular cells isolated from adult guinea-pig hearts. *Journal of Physiology*. 403:117–133.

Eisner, D. A., and W. J. Lederer. 1980. The relationship between sodium pump activity and twitch tension in cardiac Purkinje fibres. *Journal of Physiology*. 303:475–496.

Fabiato, A., and F. Fabiato. 1979. Calculator programs for computing the composition of the solutions containing multiple metals and ligands used for experiments in skinned muscle cells. *Journal de Physiologie*. 75:463–505.

Gadsby, D. C., M. Nakao, M. Noda, and R. N. Shepherd. 1988. [Na] dependence of outward Na/Ca exchange current in guinea-pig ventricular myocytes. *Journal of Physiology*. 407:135P. (Abstr.)

Hamill, O. P., A. Marty, E. Neher, B. Sakmann, and J. F. Sigworth. 1981. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Archiv*. 395:6–18.

Hume, R., and A. Uehara. 1986. "Creep currents" in single frog atrial cells may be generated by electrogenic Na/Ca exchange. *Journal of General Physiology*. 87:857–884.

Imanaga, I., M. Kameyama, and H. Irisawa. 1987. Cell-to-cell diffusion of fluorescent dyes in paired ventricular cells. *American Journal of Physiology*. 252:H223–232.

Kadoma, M., J. Froehlich, J. Reeves, and J. Sutko. 1982. Kinetics of sodium ion induced calcium ion release in calcium ion loaded cardiac sarcolemmal vesicles: determination of initial velocities by stopped-flow spectrophotometry. *Biochemistry.* 21:1914–1918.

Kimura, J., S. Miyamae, and A. Noma. 1987. Identification of sodium-calcium exchange current in single ventricular cells of guinea-pig. *Journal of Physiology.* 384:199–222.

Kimura, J., A. Noma, and H. Irisawa. 1986. Na-Ca exchange current in mammalian heart cells. *Nature.* 319:596–597.

Lee, H., and W. T. Clusin. 1987. Na$^+$/Ca^{2+} exchange in cardiac myocytes. *Biophysics Journal.* 51:169–176.

Marban, E., M. Kitakaze, H. Kusuoka, J. K. Porterfield, D. T. Yue, and V. P. Chacko. 1987. Intracellular free calcium concentration measured with ^{19}F NMR spectroscopy in intact ferret hearts. *Proceedings of National Academy of Sciences.* 84:6005–6009.

Marban, E., T. J. Rink, R. W. Tsien, and R. Y. Tsien. 1980. Free calcium in heart muscle at rest and during contraction measured with Ca^{2+}-sensitive microelectrodes. *Nature.* 286:845–850.

Mechmann, S., and L. Pott. 1986. Identification of Na-Ca exchange current in single cardiac myocytes. *Nature.* 319:597–599.

Mullins, L. J. 1981. Ion Transport in Heart. Raven Press, New York. 20–43.

Noble, D. 1986. Sodium-calcium exchange and its role in generating electric current. *In* Cardiac Muscle: The Regulation of Excitation and Contraction. R. D. Nathan, editor. Academic Press, Inc., Orlando, FL. 171–200.

Philipson, K. D. 1985. Symmetry properties of the Na-Ca exchange mechanism in cardiac sarcolemmal vesicles. *Biochimica et Biophysica Acta.* 821:367–376.

Philipson, K. D., and A. Nishimoto. 1982. Na$^+$-Ca^{2+} exchange in inside-out cardiac sarcolemmal vesicles. *Journal of Biological Chemistry.* 257:5111–5117.

Reeves, J. P., and P. Poronnik. 1987. Modulation of Na$^+$-Ca^{2+} exchange in sarcolemmal vesicles by intravesicular Ca^{2+}. *American Journal of Physiology.* 252:C17–C23.

Reeves, J. P., and J. L. Sutko. 1983. Competitive interactions of sodium and calcium with the sodium calcium exchange system of cardiac sarcolemmal vesicles. *Journal of Biological Chemistry.* 258:3178–3182.

Reuter, H., and N. Seitz. 1968. The dependence of calcium efflux from cardiac muscle on temperature and external ion composition. *Journal of Physiology.* 195:451–470.

Sato, R., A. Noma, Y. Kurachi, and H. Irisawa. 1985. Effects of intracellular acidification on membrane currents in ventricular cells of the guinea pig. *Circulation Research.* 57:553–561.

Segel, I. H. 1976. Biochemical Calculations. 2nd ed. John Wiley & Sons, Inc., New York. 441 pp.

Sheu, S., and H. Fozzard, 1982. Transmembrane Na and Ca electrochemical gradients in cardiac muscle and their relationship to force development. *Journal of General Physiology.* 80:325–351.

Simchowitz, L., and E. J. Cragoe, Jr. 1988. Na$^+$-Ca^{2+} exchange in human neutrophils. *American Journal of Physiology* 254:C150–164.

Soejima, M., and A. Noma. 1984. Mode of regulation of the ACh-sensitive K-channel by the muscarinic receptor in rabbit atrial cells. *Pflügers Archiv.* 400:424–431.

Tsien, R. Y. 1980. New calcium indicators and buffers with high selectivity against magnesium and protons: design, synthesis, and properties of prototype structures. *Biochemistry.* 19:2396–2404.

Tsien, R. Y., and T. J. Rink. 1980. Neutral carrier ion-selective microelectrodes for measurement of intracellular free calcium *Biochimica et Biophysica Acta.* 599:623–638.

Wier, W. G., M. B. Cannell, J. R. Berlin, E. Marban, and W. J. Lederer. 1987. Cellular and subcellular heterogeneity of [Ca^{2+}]$_i$ in single heart cells revealed by fura-2. *Science.* 235:325–328.