# Kinetics and Stoichiometry of Coupled Na Efflux and Ca Influx (Na/Ca Exchange) in Barnacle Muscle Cells

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ABSTRACT    Coupled $Na^+$ exit/$Ca^{2+}$ entry (Na/Ca exchange operating in the $Ca^{2+}$ influx mode) was studied in giant barnacle muscle cells by measuring $^{22}Na^+$ efflux and $^{45}Ca^{2+}$ influx in internally perfused, ATP-fueled cells in which the $Na^+$ pump was poisoned by 0.1 mM ouabain. Internal free $Ca^{2+}$, $[Ca^{2+}]_i$, was controlled with a Ca-EGTA buffering system containing 8 mM EGTA and varying amounts of $Ca^{2+}$. $Ca^{2+}$ sequestration in internal stores was inhibited with caffeine and a mitochondrial uncoupler (FCCP). To maximize conditions for $Ca^{2+}$ influx mode Na/Ca exchange, and to eliminate tracer Na/Na exchange, all of the external $Na^+$ in the standard $Na^+$ sea water (NaSW) was replaced by Tris or $Li^+$ (Tris-SW or LiSW, respectively). In both Na-free solutions an external $Ca^{2+}$ ($Ca_o$)-dependent $Na^+$ efflux was observed when $[Ca^{2+}]_i$ was increased above $10^{-8}$ M; this efflux was half-maximally activated by $[Ca^{2+}]_i$ = 0.3 $\mu$M (LiSW) to 0.7 $\mu$M (Tris-SW). The $Ca_o$-dependent $Na^+$ efflux was half-maximally activated by $[Ca^{2+}]_o$ = 2.0 mM in LiSW and 7.2 mM in Tris-SW; at saturating $[Ca^{2+}]_o$, $[Ca^{2+}]_i$, and $[Na^+]_i$ the maximal (calculated) $Ca_o$-dependent $Na^+$ efflux was ~75 pmol/$cm^2 \cdot$s. This efflux was inhibited by external $Na^+$ and $La^{3+}$ with $IC_{50}$'s of ~125 and 0.4 mM, respectively.

A $Na_i$-dependent $Ca^{2+}$ influx was also observed in Tris-SW. This $Ca^{2+}$ influx also required $[Ca^{2+}]_i > 10^{-8}$ M. Internal $Ca^{2+}$ activated a $Na_i$-independent $Ca^{2+}$ influx from LiSW (tracer Ca/Ca exchange), but in Tris-SW virtually all of the $Ca_i$-activated $Ca^{2+}$ influx was $Na_i$-dependent (Na/Ca exchange). Half-maximal activation was observed with $[Na^+]_i$ = 30 mM. The fact that internal $Ca^{2+}$ activates both a $Ca_o$-dependent $Na^+$ efflux and a $Na_i$-dependent $Ca^{2+}$ influx in Tris-SW implies that these two fluxes are coupled; the activating (intracellular) $Ca^{2+}$ does not appear to be transported by the exchanger. The maximal (calculated) $Na_i$-dependent $Ca^{2+}$ influx was -25 pmol/$cm^2 \cdot$s. At various $[Na^+]_i$ between 6 and 106 mM. the ratio of the $Ca_o$-dependent $Na^+$ efflux to the $Na_i$-dependent $Ca^{2+}$ influx was 2.8–3.2:1 (mean = 3.1:1); this directly demonstrates that the stoichiometry (coupling ratio) of the Na/Ca exchange is 3:1. These observations on the coupling ratio and kinetics of the Na/Ca exchanger imply that in resting cells the exchanger turns over at a low rate because of the low $[Ca^{2+}]_i$; much of the $Ca^{2+}$ extrusion at rest

($\sim$1 pmol/cm$^2$·s) is thus mediated by an ATP-driven Ca$^{2+}$ pump. When the cells are activated and depolarized, and [Ca$^{2+}$]$_i$ begins to rise, the exchanger is activated and moves Ca$^{2+}$ into the cells; then, during repolarization and recovery, the exchanger moves Ca$^{2+}$ out of the cells, thereby providing a negative feedback to slow itself down.

## INTRODUCTION

Changes in the intracellular concentration of free Ca$^{2+}$ ([Ca$^{2+}$]$_i$) plays a critical role in the physiology of most types of animal cells. These changes can be achieved either by the release and sequestration of Ca$^{2+}$ in intracellular stores (endoplasmic reticulum and mitochondria) and/or by an increase in Ca$^{2+}$ influx and efflux across the plasmalemma. Ca$^{2+}$ influx can be induced by the activation of voltage-gated and/or receptor-operated Ca$^{2+}$ channels; Ca$^{2+}$ efflux can be induced by the activation of an ATP-driven Ca$^{2+}$ pump.

In parallel with these latter mechanisms, the plasmalemma of most types of animal cells contains another Ca$^{2+}$ transport system, the Na/Ca exchanger, that can transport Ca$^{2+}$ bidirectionally across the membrane in exchange for Na$^+$ (cf. Blaustein, 1974). It can move Ca$^{2+}$ out of the cells in exchange for entering Na$^+$ ("forward mode" or "Ca$^{2+}$ efflux mode"), and it can move Ca$^{2+}$ into the cells in exchange for exiting Na$^+$ ("reverse mode" or "Ca$^{2+}$ influx mode").[1]

An understanding of how this exchange system functions in parallel with the Ca$^{2+}$ channels and the ATP-driven Ca$^{2+}$ pump is of fundamental concern. To address this issue we need to determine the contribution of the Na/Ca exchange system to Ca$^{2+}$ fluxes under various physiological conditions. Therefore, we require information about the thermodynamic factors that regulate the direction of net Ca$^{2+}$ transport mediated by the Na/Ca exchanger, and about the kinetic factors that control the rate of exchange during the cell activity cycle.

The properties of the Na/Ca exchanger appear to be very similar in most cells where such comparisons are possible. For example, indirect evidence from various preparations indicates that the stoichiometry (coupling ratio) is about 3 Na$^+$:1 Ca$^{2+}$ (e.g., Reeves and Hale, 1984; Yau and Nakatani, 1984; and see Sheu and Blaustein, 1986). Many observations indicate that the exchange is voltage sensitive (e.g., Blaustein et al., 1974; Mullins and Brinley, 1975; DiPolo et al., 1985; Allen and Baker, 1986a, b; Lagnado et al., 1988; Caputo et al., 1988; Noda et al., 1988) and electrogenic (e.g., Eisner and Lederer, 1979; Yau and Nakatani, 1984; Hume and Uehara, 1986a, b; Kimura et al., 1986, 1987; Mechmann and Pott, 1986). Direct determinations of the coupling ratio for unidirectional Na$^+$ and Ca$^{2+}$ measurements have been difficult because of uncertainties about initial transport rates (cf. Pitts, 1979) and/or because of possible parallel Ca/Ca and Na/Na exchanges and Na$^+$ flux through Ca$^{2+}$-activate cation channels (Sheu and Blaustein, 1983). Indirect esti-

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[1] The term "reverse mode," which has often been used in the past, is subject to misinterpretation. It may erroneously imply that the "forward mode" is the (only) normal mode of operation of the Na/Ca exchanger, and the "reverse mode" is a "backward" operation. This is incorrect because the direction of net Ca$^{2+}$ movement is determined by changes in the Na$^+$ electrochemical gradient. To avoid misinterpretation, the terms "Ca$^{2+}$ influx mode" and "Ca$^{2+}$ efflux mode" have been adopted here.

mates of the coupling ratio may be model-dependent (Eisner and Lederer, 1985), and are also subject to a variety of other pitfalls (Sheu and Blaustein, 1986).

Recently, we showed that it is possible to determine the coupling ratio of the exchange directly from measurements of $Ca^{2+}$ influx and $Na^+$ efflux in internally perfused giant barnacle muscle cells (Rasgado-Flores and Blaustein, 1987). These results, which indicate that 3 $Na^+$ are exchanged for 1 $Ca^{2+}$, have been extended in the present study. In addition, we have examined several kinetic parameters of the Na/Ca system operating in the $Ca^{2+}$ influx mode ($Na^+$ exit/$Ca^{2+}$ entry). These data provide new insight into the relationship between the Na/Ca exchanger and the other systems involved in $Ca^{2+}$ movement across the plasmalemma.

## MATERIALS AND METHODS

### Reagents and Solutions

The chlorides of $Na^+$, $K^+$, $Ca^{2+}$, and $Mg^{2+}$ were all "Baker Analyzed" reagents. Ouabain was obtained from Aldrich Chemical Co., Milwaukee, WI; phenol red and HCl were from Fisher Scientific Co., Fairlawn, NJ; sucrose was from Schwartz/Mann Biotechnology, Cleveland, OH; FCCP (carbonylcyanide *p*-trifluoromethoxyphenyl-hydrazone) was from Dupont Chemical Co., Wilmington, DL. All other reagents were purchased from the Sigma Chemical Co., St. Louis, MO.

*External (superfusion) solutions.* The standard external superfusion solution (artificial sea water, NaSW) contained (in millimolar): 456 NaCl; 10 KCl; 25 $MgCl_2$; 11 $CaCl_2$; 6 tris(hydroxy-methyl)aminomethane (Tris) base (pH = 7.8, adjusted at room temperature with maleic acid). As described in Results, in many instances the NaCl was completely replaced by 519 mM Tris (buffered to pH 7.8 at room temperature with concentrated HCl; Tris-SW) or by 456 mM LiCl (LiSW). Some solutions were also Ca-free (e.g., Ca-free NaSW), or contained reduced $CaCl_2$; in these instances, the $CaCl_2$ was replaced by equimolar $MgCl_2$ to keep the ionic strength constant.

*Internal (perfusion) solutions.* The low $Na^+$ (6 mM $Na^+$) internal perfusion solution contained (in millimolar): 3 $Na_2ATP$, 38 KCl, 210 K aspartate, 340 sucrose, 10 $MgCl_2$, 60 *N*-2-hydroxyethyl-piperazine-*N'*-2 -ethanesulfonic acid (HEPES) buffered to pH 7.3 at room temperature with Tris, 0.2 phenol red, 3.5 caffeine, 0.03 FCCP, 8 ethyleneglycol-bis-($\beta$-aminoethylether)-*N,N'*-tetraacetic acid (EGTA) plus varying amounts of $CaCl_2$ (see below), and an ATP-regenerating system (1.5 mM phosphoenol pyruvate and 0.08 mg/ml pyruvate kinase). The composition of the high $Na^+$ (106 mM $Na^+$) perfusion solution was similar, except that this solution also contained 100 mM $Na^+$ aspartate, and only 220 mM sucrose. Intermediate concentrations of $Na^+$ were obtained by mixing the 6 mM $Na^+$ and 106 mM $Na^+$ solutions in appropriate proportions.

The osmolarity of all the internal and external solutions was 960 ± 10 mosmol (determined with a vapor pressure osometer: Wescor, Inc., Logan, UT).

Various $[Ca^{2+}]_i$ were obtained by using a Ca-EGTA buffer calculated on the basis of a Ca-EGTA stability constant of $7.54 \times 10^6 \, M^{-1}$ (Blinks et al., 1982). The total EGTA concentration was 8 mM; in the $10^{-8}$, $10^{-7}$, and $10^{-6}$ M $[Ca^{2+}]_i$ solutions, the $CaCl_2$ concentrations were 0.56, 3.44, and 7.06 mM, respectively. The normal contraction threshold of barnacle muscle is between $10^{-7}$ and $10^{-6}$ M $Ca^{2+}$ (Hagiwara and Nakajima, 1966), and cells contracted when they were initially perfused with fluids that contained $[Ca^{2+}]_i$ in this range. However, barnacle cells that were perfused for ~2 h or more with solutions containing $[Ca^{2+}]_i = 10^{-8}$ M did not contract when $[Ca^{2+}]_i$ was subsequently raised above the contraction threshold (Nelson and Blaustein, 1981), presumably because a critical factor was washed

out of the myoplasm. This fortuitous situation enabled us to study ion transport when $[Ca^{2+}]_i$ was varied over the entire dynamic physiological range ($10^{-8}$ to $10^{-5}$ M) in these cells.

### Experimental Procedures

Internally perfused single muscle cells from the giant barnacle *Balanus nubilus* were used. The methods for the perfusion of these cells have been described (Nelson and Blaustein, 1980). In brief, single muscle cells were dissected in NaSW and then incubated for 90 min in Ca-free NaSW (to prevent contractions when the cells were cut at their bases). Single cells were mounted in the tissue chamber (in Ca-free NaSW) by cannulating the cut (basal) end and by tying the tendon end to a hook. Subsequently, a double-barrel capillary tube was inserted axially through the cut basal end of the cell. The open tip of the longer barrel was guided, under microscope observation, to a position close to the tendon end of the fiber; this barrel was used to perfuse the myoplasmic space with the desired intracellular solutions. The tip of the shorter barrel opened about midway along the length of the cell; this barrel was filled with 3 M KCl and was used to monitor the membrane potential. With this configuration, the internal perfusion fluid flowed out the end of the longer barrel, into the myoplastic space near the tendon end of the cell; it then flowed back through the myoplastic space until it reached the cut base, where it exited through the glass end-cannula. As this fluid emerged from the cannula, aliquots could be collected and assayed for radiolabeled ions using standard liquid scintillation procedures.

The tendon and basal ends of the cell were isolated by vaseline seals, and the 1.3-cm-long central segment was superfused. The transport of tracer-labeled $Na^+$ and $Ca^{2+}$ across the plasmalemma in this central segment was measured. The barnacle muscle plasmalemma is invaginated by deep, branching clefts, so that the determination of true surface area is difficult. All flux measurements are therefore reported in terms of a simple circular cylinder approximation, although this represents an underestimate of the true plasmalemma surface area by a factor of ~15–20 (cf. Nelson and Blaustein, 1980).

The membrane potential of the cell, $V_M$, was monitored with a pair of calomel half-cell electrodes. One half-cell was in contact with the intracellular space via the 3-M-KCl capillary; the other half-cell was in contact with the extracellular (superfusion) fluid. All experiments were carried out at 16°C.

$Ca^{2+}$ influx mode Na/Ca exchange was determined by measuring the coupled efflux of $^{22}Na$ and influx of $^{45}Ca$. The relevant fluxes were defined, operationally, as the extracellular $Ca^{2+}$ ($Ca_o$)-dependent $^{22}Na$ efflux and the intracellular $Na^+$ ($Na_i$)-dependent $^{45}Ca$ influx. To promote $Ca^{2+}$ influx mode exchange, a large outwardly-directed $Na^+$ gradient was established by increasing the $[Na^+]_i$ and by replacing some or all of the external $Na^+$ either by Tris or by $Li^+$. The use of the Na-Free solutions eliminated the possible contribution of Na/Na exchange to the measured $Na^+$ efflux.

*Na$^+$ efflux.* To measure $Na^+$ efflux, $^{22}Na$ (New England Nuclear, Boston, MA) was added to the perfused fluid (0.6 mCi/mmol Na). The appearance of $^{22}Na$ in the extracellular solution that superfused the central region of the cell was determined with an in-line liquid scintillation counter (cf. Nelson and Blaustein, 1980). The superfusion rate was 6.4 ml/min. The counts per minute were determined each minute; because of statistical variation in the individual counts, the last four to six counts for each condition were averaged (i.e., when the flux reached a steady level: see broken lines in Fig. 1 A) to obtain reliable flux values for kinetic analysis.

Specific activities of the perfusion fluids was determined by averaging the counts in triplicate 20-μl samples of the perfusion fluids diluted into 20 ml of NaSW, LiSW, or Tris-SW, as determined with the in-line counter. The background was 30 cpm; this count rate was equivalent to an $Na^+$ efflux of 1–2 pmol/cm² s.

To minimize disturbance to the preparation, in some experiments the perfusion fluid was not flushed through the polyethylene tube that connected the perfusion syringe to the capillary tube in the muscle cell. This resulted in long dead times between the change of the solutions in the syringe and the change in the myoplasmic space (see Results). In other experiments, these dead times were reduced by 30 min or more by flushing the tube with the new solutions.

To prevent $Na^+$ efflux through the $Na^+$ pump, 0.1 mM ouabain was added to all external solutions in the $Na^+$ efflux experiments (Nelson and Blaustein, 1980). For consistency, ouabain was also used in all of the $Ca^{2+}$ influx experiments.

*Ca$^{2+}$ influx.* To measure $Ca^{2+}$ influx, $^{45}Ca$ (New England Nuclear, Boston, MA), 0.45 mCi/mmol Ca, was added to the superfusion solution that bathed the central segment of the cell. The appearance of $^{45}Ca$ in the intracellular fluid was determined by collecting, at fixed time intervals, 5–20-$\mu$l aliquots of the perfusion fluid as it emerged from the cannula at the basal end of the cell. The radioactivity in these aliquots was assayed with standard liquid scintillation spectroscopy methods using "Redi-Solve" scintillation cocktail (Beckman Instruments, Inc., Fullerton, CA).

Because unidirectional $^{45}Ca$ influx was determined from samples of the internal perfusion fluid, it was important to insure that virtually all of the $^{45}Ca$ that entered the cell remained in the perfusion fluid, and that none of it was sequestered in the cell. Several precautions were therefore taken. First, a large concentration of Ca-EGTA buffer (see above) was added to the perfusion fluid to provide a large dilution of entering $^{45}Ca$ with the $^{40}Ca$ in the myoplasmic space. For example, with an influx of 1 pmol/cm$^2\cdot$s, the entering $^{45}Ca$ was diluted approximately 500-fold with the $^{40}Ca$ in the perfusion fluid at $[Ca^{2+}]_i = 10^{-7}$ M, and about 1,000-fold at $[Ca^{2+}]_i = 10^{-6}$ M; even with an influx of 20 pmol/cm$^2\cdot$s, the dilution was about 50-fold at $[Ca^{2+}]_i = 10^{-6}$ M. Second, the myoplasmic fluid was continuously exchanged at a rate of 5 $\mu$l/min. Since the volume of the barnacle muscle cells was ~50 $\mu$l, the intracellular fluid was renewed about once every 10 min. Third, 3.5 mM caffeine and 0.03 mM FCCP (a mitochondrial uncoupler) were included in the perfusion fluid to inhibit $Ca^{2+}$ sequestration by the sarcoplasmic reticulum (SR) and the mitochondria, respectively.$^{45}Ca$ might, nevertheless, exchange for unlabeled $Ca^{2+}$ in intracellular compartments during the rising phase of $^{45}Ca$ uptake. In the steady-state (i.e., when $Ca^{2+}$ influx reaches a constant level), however, such exchanges should be in equilibrium and should not interfere with quantitation of the $^{45}Ca$ influx.

*Statistical analysis.* The data for the activation and inhibition curves were analyzed by a computerized least-squares method (Maple Grove Software, St. Louis, MO) to obtain "best-fit" curves and the kinetic parameters (maximum flux, $J_{max}$; ion concentration for half-maximal activation or inhibition, $K_{1/2}$; and Hill coefficient). The kinetic parameters included in the figure legends include the standard errors of the means, where appropriate. Significance of the difference between $K_{1/2}$ values was determined using the $t$ test for unpaired data.

## RESULTS

### Ca$_o$-dependent Na$^+$ Efflux

*Inhibition by extracellular Na$^+$.* The component of the $Na^+$ efflux mediated by the Na/Ca exchanger during $Ca^{2+}$ influx mode operation should be manifested as a Ca$_o$-dependent $Na^+$ efflux. Indeed, as illustrated in Fig. 1 A, with $[Na^+]_i = 45$ mM (~2.5 times normal; Brinley, 1968) and $[Ca^{2+}]_i = 10^{-7}$ M, replacement of external $Ca^{2+}$ by equimolar $Mg^{2+}$ produced a small reduction in the $Na^+$ efflux into NaSW (~2 pmol/cm$^2\cdot$s). The Ca$_o$-dependent $Na^+$ efflux into NaSW was considerably

larger when $[Ca^{2+}]_i$ was increased to 1 $\mu$M, under otherwise identical conditions ($\sim$9 pmol/cm$^2 \cdot$s in the experiment of Fig. 1 $B$). Conversely, if $[Ca^{2+}]_i$ was maintained constant, replacement of external Na$^+$ by Tris or Li$^+$ was associated with a larger Ca$_o$-dependent Na$^+$ efflux (Figs. 2–4). These data are consistent with the view that the Ca$_o$-dependent Na$^+$ efflux is activated by intracellular Ca$^{2+}$ and inhibited by extracellular Na$^+$. Apparently, there is relatively little Na/Ca exchange (manifested by a Ca$_o$-dependent Na$^+$ efflux) under normal resting conditions (i.e., with low $[Ca^{2+}]_i$ and normal $[Na^+]_o$.
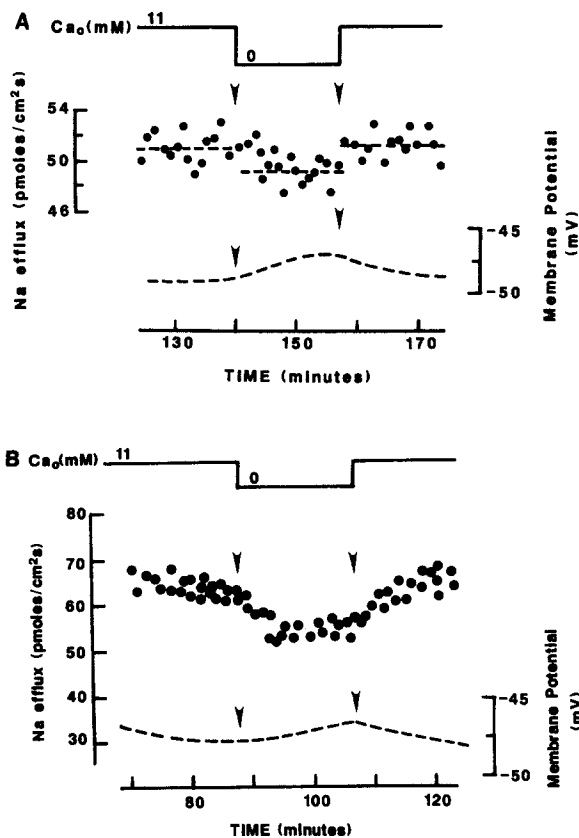


FIGURE 1. Effect of removing external Ca$^{2+}$ on Na$^+$ efflux into NaSW in internally perfused barnacle muscle cells. ($A$) Ca$_o$-dependent Na$^+$ efflux measured at low $[Ca^{2+}]_i$. $[Na^+]_o$ = 456 mM, $[Na^+]_i$ = 46 mM, and $[Ca^{2+}]_i$ = 0.1 $\mu$M; 0.1 mM ouabain was present throughout the experiment. At 140 min, external Ca$^{2+}$ was replaced, mole-for-mole by Mg$^{2+}$. At 157 min, the normal concentration of Ca$^{2+}$ (11 mM) was restored. The broken lines indicate the means of the Na$^+$ effluxes from the last 14 min of each of the test periods. The abscissa indicates the time after starting perfusion with tracer $^{22}$Na; during the first hour, the perfusion fluid $[Ca^{2+}]_i$ was 10$^{-8}$ M and the external solution was NaSW. The temperature was 16°C in this and all subsequent experiments. ($B$) Ca$_o$ dependent Na$^+$ efflux measured at high $[Ca^{2+}]_i$. $[Na^+]_o$ = 456 mM and $[Ca^{2+}]_i$ = 1.0 $\mu$M; other conditions similar to those in $A$.

Fig. 2 shows the relationship between $[Na^+]_o$ and the Ca$_o$-dependent Na$^+$ efflux when external Na$^+$ was replaced by Tris. It is unclear whether the apparent inhibition of the Ca$_o$-dependent Na$^+$ efflux by external Na$^+$ represents competition between Na$^+$ and Ca$^{2+}$ for the same binding sites, or whether the external Na$^+$ simply stabilizes the exchange carriers in a configuration in which most of the Na$^+$ sites remain at the external surface. Nevertheless, the curve is sigmoid, with a Hill

coefficient of 2.6. This implies that two or more external $Na^+$ ions act cooperatively to inhibit $Ca_o$-dependent efflux of $Na^+$.

*Activation by intracellular $Ca^{2+}$.* The $Ca_o$-dependent $Na^+$ efflux into $Na^+$-free, Tris-SW or LiSW in barnacle muscle was activated by intracellular $Ca^{2+}$ (Figs. 3 and 4), as observed in squid axons (DiPolo and Beaugé, 1986, 1987). Fig. 3 A shows data from a representative experiment in which the $Ca_o$-dependent $Na^+$ efflux was measured at several different $[Ca^{2+}]_i$ with $Li^+$ as the main external monovalent cation. There was no detectable $Ca_o$-dependent $Na^+$ efflux with $[Ca^{2+}]_i = 10^{-8}$ M (not shown). A small $Ca_o$-dependent $Na^+$ efflux was observed with $[Ca^{2+}]_i = 10^{-7}$, and a much larger $Ca_o$-dependent $Na^+$ efflux at $[Ca^{2+}]_i = 10^{-6}$; there was not much more increase in this efflux when $[Ca^{2+}]_i$ was raised to $10^{-5}$ M, which indicates that the efflux saturates at high $[Ca^{2+}]_i$. The activation curve for the $Ca_o$-dependent $Na^+$
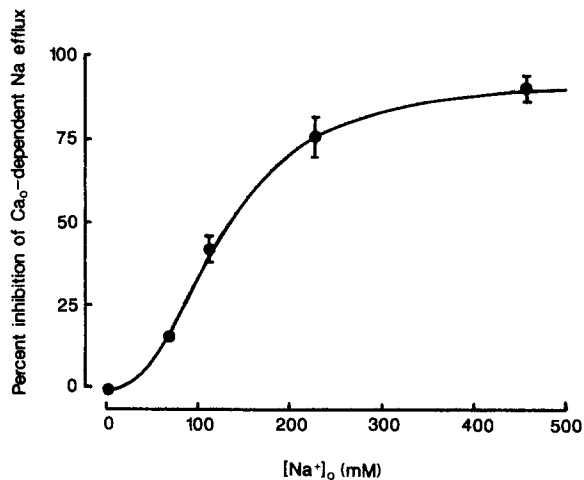


FIGURE 2. Inhibition of the $Ca_o$-dependent $Na^+$ efflux by external $Na^+$ in mixtures of NaSW and Tris-SW. These data were compiled from three cells with $[Na^+]_i = 46$ mM and $[Ca^{2+}]_i = 1$ $\mu$M, and were normalized to "0" inhibition in Tris-SW; bars indicate the range of values. All solutions contained 0.1 mM ouabain. The $Ca_o$-dependent $Na^+$ efflux averaged 56 pmol/cm$^2 \cdot$s in (Na-free) Tris-SW, and 5.3 pmol/cm$^2 \cdot$s in NaSW. The curve is the best-fit solution to the Hill equation, the calculated parameters are $K_{Na_o} = 126 \pm 12$ mM, $J_{Na(max)} = 93 \pm 6$ pmol/cm$^2$ s, and the Hill coefficient. $= 2.6 \pm 0.6$.

efflux by internal $Ca^{2+}$ is shown in Fig. 3 B; the efflux was half-maximally activated at $[Ca^{2+}]_i = 0.3$ $\mu$M. The time course of the decline in the $Na^+$ efflux when external $Ca^{2+}$ was removed (Fig. 3 A) indicates that the half-time for washout of the extracellular space was on the order of 4–5 min.

Data from an analogous experiment in which the $Ca_o$-dependent $Na^+$ efflux was measured at several different $[Ca^{2+}]_i$ in Tris-SW are shown in Fig. 4 A; similar results were obtained in four other experiments of this type. The activation curve for the $Ca_o$-dependent $Na^+$ efflux into Tris-SW by internal $Ca^{2+}$ is shown in Fig. 4 B, which includes data from all five experiments; the efflux was half-maximally activated at $[Ca^{2+}]_i = 0.7$ $\mu$M. The difference between the $K_{Ca_i}$ values obtained in LiSW (0.3 $\mu$M) and Tris-SW (0.7 $\mu$M) is significant $(P < 0.01)$.

Recently, it was reported that in squid axons the $[Ca^{2+}]_i$ required for half-maxi-

mal activation of the $Ca_o$-activated $Na^+$ efflux in Tris-SW is ~1.4 $\mu M$ (DiPolo and Beaugé, 1987). This value is about double the value we obtained in barnacle muscle. A possible explanation for this difference, other than a species difference, is that there may be competition between internal $Na^+$ and $Ca^{2+}$ during $Ca^{2+}$ entry mode exchange comparable to that observed during $Ca^{2+}$ exit mode exchange (Blaustein, 1977) because $[Na^+]_i$ was 46 mM in the barnacle muscle and 100 mM in the squid axons. Alternatively, as discussed below, $[Ca^{2+}]_i$ just inside the sarcolemma may
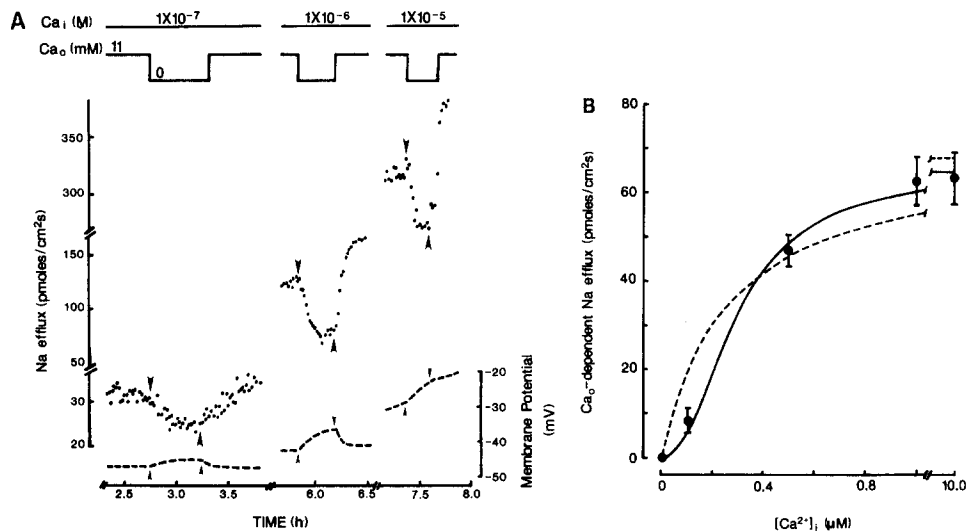


FIGURE 3. $Ca_o$-dependent $Na^+$ efflux measured at various $[Ca^{2+}]_i$. The external medium was (Na-free) LiSW containing 0.1 mM ouabain. The internal perfusion fluid contained 46 mM $Na^+$; $[Ca^{2+}]_i$ was altered by varying the total $CaCl_2$ concentration in a CaEGTA buffer system with $[EGTA]_{total}$ = 8 mM. (A) The downward arrowheads above the efflux data indicate where external $Ca^{2+}$ (normally 11 mM) was replaced by $Mg^{2+}$; the $Ca^{2+}$ was restored at the times denoted by the upward arrowheads below the efflux data. The $[Ca^{2+}]_i$ values are shown at the top of the figure. Note the change in ordinate scale above 50 $pmol/cm^2 \cdot s$. (B) $Ca_o$-dependent $Na^+$ efflux into LiSW (data from A and one other cell) graphed as a function of $[Ca^{2+}]_i$ (the error bars indicate the range of values). The continuous and discontinuous lines are the solutions to the Hill equation and the Michaelis-Menten equation, respectively. The calculated parameters are: for the Hill equation, $K_{Ca_i}$ = 0.3 ± 0.1 $\mu M$ $J_{Na(max)}$ = 65 ± 8 $pmol/cm^2 \cdot s$; Hill coefficient = 2.1 ± 1.4. For the Michaelis-Menten equation, $K_{Ca_i}$ = 0.3 ± 0.2 $\mu M$ and $J_{Na(max)}$ = 70 ± 10 $pmol/cm^2 \cdot s$.

have been poorly controlled in the barnacle muscle cells when there was a large $Ca^{2+}$ influx.

The relationship between the $Na^+$ efflux and $[Ca^{2+}]_i$ illustrated in Figs. 3 B and 4 B is apparently sigmoid; the best fits to the data, using the Hill equation, were obtained with Hill coefficients of 1.2 and 2.2, respectively. This may indicate that the activation by intracellular $Ca^{2+}$ involves more than one $Ca^{2+}$ ion on each carrier molecule.

Figs. 3 A and 4 A also show that there was a large increase in the $Ca_o$-independent

$Na^+$ efflux when $[Ca^{2+}]_i$ was increased. This latter flux is most likely due to the activation of a cation-selective conductance by intracellular $Ca^{2+}$ (Sheu and Blaustein, 1983). Care must therefore be taken to define any $Na^+$ efflux attributable to Na/Ca exchange as the $Ca_o$-dependent $Na^+$ efflux. The presence of this large $Ca_i$-activated increase in $Na^+$ conductance and (nonexchange mediated) $Na^+$ influx in
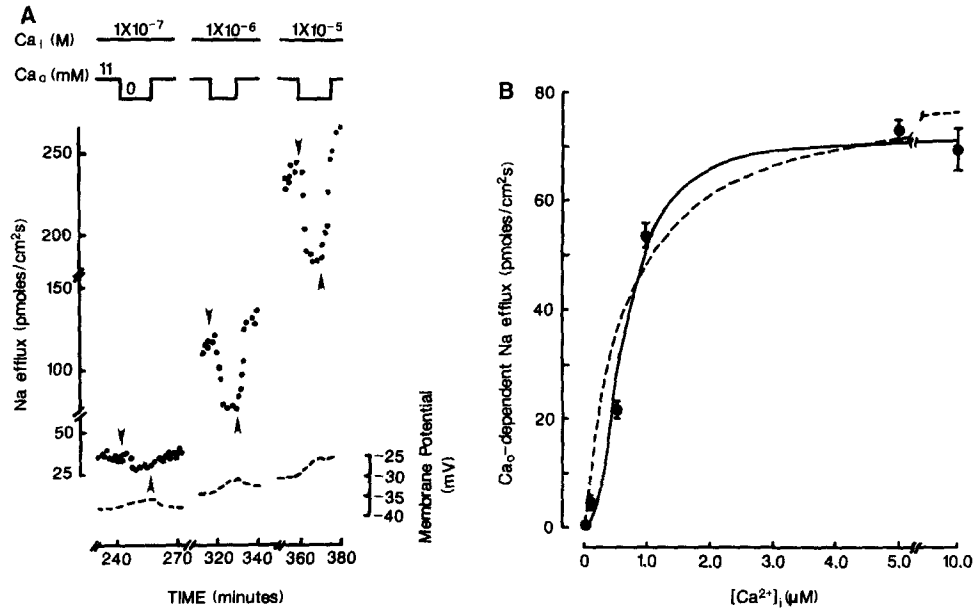


FIGURE 4. $Ca_o$-dependent $Na^+$ efflux measured at various $[Ca^{2+}]_i$. The external solution was (Na-free) Tris-SW containing 0.1 mM ouabain; the internal perfusion fluid contained 46 mM $Na^+$ and various $[Ca^{2+}]_i$ obtained with a CaEGTA buffer containing 8 mM $EGTA_{total}$. (A) Downward arrowheads denote times when external $Ca^{2+}$ (11 mM) was replaced by $Mg^{2+}$; upward arrowheads indicate when $Ca^{2+}$ was restored. The various $[Ca^{2+}]_i$ levels are shown at the top of the figure. The abscissa indicates the time after starting perfusion with tracer $^{22}Na$ (see Fig. 1 legend). (B) $Ca_o$-dependent $Na^+$ efflux graphed as a function of $[Ca^{2+}]_i$, with Tris as the predominant external cation. These data are from the experiment in A and four similar experiments; closed circles indicate mean ± SEM. The continuous and discontinuous lines are the best-fit solutions to the Hill equation and the Michaelis-Menten equation, respectively. The calculated parameters are: for the Hill equation $K_{Ca_i} = 0.7 \pm 0.1$ $\mu$M, $J_{Na(max)} = 72 \pm 3$ pmol/cm$^2 \cdot$s, Hill coefficient $= 2.2 \pm 0.5$, and for the Michaelis-Menten equation, $K_{Ca_i} = 0.7 \pm 0.2$ $\mu$M and $J_{Na(max)} = 81 \pm 6$ pmol/cm$^2 \cdot$s. Note that if the $[Ca^{2+}]_i$ just under the sarcolemma was underestimated at nominally 1.0 $\mu$M, and was actually 1.5 or 2.0 $\mu$M because of the large $Ca^{2+}$ influx (see Discussion), the $K_{Ca_i}$ would be increased to 0.8 to 1.0 $\mu$M, and the Hill coefficient would be reduced to 1.6 or 1.3, respectively.

Ca-free media (Sheu and Blaustein, 1983) makes it difficult to determine the stoichiometry of the Na/Ca exchanger directly from flux measurements during $Na^+$ influx/$Ca^{2+}$ efflux mode operation in the barnacle muscle (cf. Lederer and Nelson, 1984).

If the Na/Ca exchanger is electrogenic, and mediates the exchange of 3 $Na^+$ for 1

$Ca^{2+}$, inhibition of $Ca^{2+}$ influx mode exchange should depolarize the membrane. Indeed, a reversible depolarization was usually observed when the $Na^+$ efflux was reduced by removing extracellular $Ca^{2+}$ (Figs. 3 A and 4 A). However, some depolarization was also observed when external $Ca^{2+}$ was removed and $[Na^+]_i$ was very low (i.e., in the absence of Na/Ca exchange; cf. Rasgado-Flores and Blaustein, 1987). The component of the membrane potential change attributable to Na/Ca exchange therefore needs to be carefully defined (see below).

*Dependence on extracellular $Ca^{2+}$.* The preceding data indicate that the $Ca_o$-dependent $Na^+$ efflux (Na/Ca exchange) is activated by $[Ca^{2+}]_i$ in the normal physiological range ($10^{-7}$–$10^{-5}$ M). Since there is negligible $Ca_o$-dependent Ca efflux in Tris-SW (cf. Rasgado-Flores and Blaustein, 1987) and, thus, no Ca/Ca exchange, the internal $Ca^{2+}$ must be acting at a "nontransporting" site on the exchanger. To measure the affinity of the carrier for external $Ca^{2+}$, and to determine whether there is also a "nontransporting" $Ca^{2+}$ activation site at the external surface of the plasmalemma, we compared the external $Ca^{2+}$ dependence of the $Ca_o$-activated $Na^+$ efflux into LiSW and Tris-SW. Fig. 5 A shows that a LiSW, with $[Na^+]_i$ = 31 mM and $[Ca^{2+}]_i$ = 0.5 $\mu$M (triangles) or 1.0 $\mu$M (circles), activation by external $Ca^{2+}$ followed Michaelis-Menten kinetics with $Ca^{2+}$ concentrations of 2.0 mM (at $[Ca^{2+}]_i$ = 1.0 $\mu$M) to 2.9 mM (at $[Ca^{2+}]_i$ = 0.5 $\mu$M) required for half-maximal activation ($K_{Ca_o}$). At $[Ca^{2+}]_o$ = 11 mM (the normal concentration), the fluxes reached 80–85% of the exchanger-mediated $Na^+$ efflux, $J_{Na(max)}$. In Tris-SW, with $[Na^+]_i$ = 46 mM and $[Ca^{2+}]_i$ = 1.0 $\mu$M, the $Ca_o$-dependent $Na^+$ efflux also increased with increasing $[Ca^{2+}]_o$, following Michaelis-Menten kinetics with a $K_{Ca_o}$ of 7.2 mM (Fig. 5 B), but the efflux only appeared to reach 64% of $J_{Na(max)}$ at $[Ca^{2+}]_o$ = 11 mM. Higher $Ca^{2+}$ concentrations caused problems because the controls required higher $Mg^{2+}$ levels to maintain constant ionic strength. These high $Mg^{2+}$ levels directly affect the $Na^+$ efflux (unpublished observations), perhaps because barnacle muscle also appears to have a Na/Mg exchange transport system (Ashley and Ellory, 1972), and/or because $Mg^{2+}$ inhibits Na/Ca exchange (Smith et al., 1987).

The $Ca_o$-dependent $Na^+$ efflux into LiSW, with $[Ca^{2+}]_o$ = 11 mM and $[Ca^{2+}]_i$ = 1 $\mu$M (Fig. 5 A), was substantially closer to $J_{Na(max)}$ than the efflux into Tris-SW (Fig. 5 B), perhaps because Tris partially inhibits the flux. This difference in fractional activation may account for the differences in the apparent affinities for internal and external $Ca^{2+}$ in the two sea waters (compare Figs. 3 B and 5 A with Figs. 4 B and 5 B). The respective $K_{Ca_o}$ values obtained in barnacle muscle in LiSW (2.0 mM) and Tris-SW (7.2 mM) were very similar to those reported for squid axons under comparable conditions (Baker et al., 1969; Allen and Baker, 1986a; DiPolo and Beaugé, 1987).

The fact that the activation curves in LiSW and Tris-SW corresponded to sections of rectangular hyperbolae, and were not sigmoid, implies that there is only a single $Ca^{2+}$ binding site (a "transporting site") on the exchanger, facing the extracellular fluid. This site has an apparent affinity for $Ca^{2+}$ about 4 orders of magnitude lower than the affinity for $Ca^{2+}$ at the intracellular site (see Figs. 3 B and 4 B).

*Inhibition by external $La^{3+}$.* There is no specific blocker available for the Na/Ca exchanger at present (Bielefeld et al., 1986). However, $La^{3+}$ at least partially blocks both the $Ca^{2+}$ exit and $Ca^{2+}$ entry modes of Na/Ca exchange in barnacle muscle

cells: i.e., it inhibits the $Na_o$-dependent $Ca^{2+}$ efflux (Ashley et al., 1974; but see Lederer and Nelson, 1983), although not as effectively as it inhibits the $Ca^{2+}$ influx from low $Na^+$ media (Ashley and Lea, 1978). In squid axons, $La^{3+}$ also blocks the $Ca_o$-dependent $Na^+$ efflux (i.e., $Ca^{2+}$ influx mode exchange) (Baker et al., 1969). Fig. 6 shows that the $Ca_o$-dependent $Na^+$ efflux from internally perfused barnacle muscle cells ([$Na^+$]$_i$ = 46 mM; [$Ca^{2+}$]$_i$ = 1 $\mu$M) into either LiSW or Tris-SW was inhibited by 50% in the presence of 0.4 mM external $La^{3+}$.
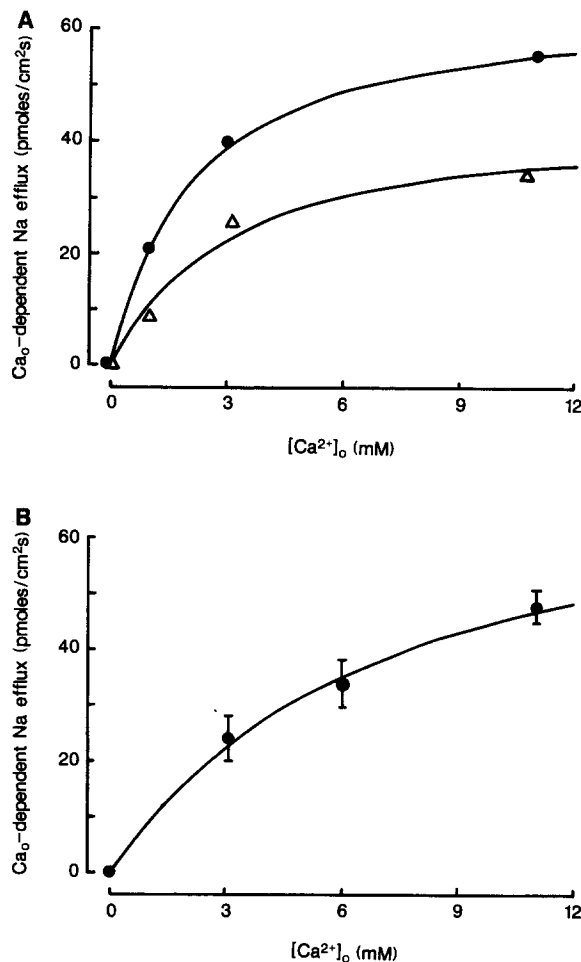


FIGURE 5. The [$Ca^{2+}$]$_i$ dependence of the $Na^+$ efflux in barnacle muscle fibers superfused with (Na-free) LiSW (*A*) or Tris-SW (*B*) containing 0.1 mM ouabain. [$Na^+$]$_o$ was 31 mM in *A*, and 46 mM in *B*; [$Ca^{2+}$]$_i$ was 0.5 $\mu$M (open triangles) or 1.0 $\mu$M (closed circles) in *A*, and 1.0 $\mu$M in *B*. The two curves in *A* are from different single muscle cells; the data in *B* are the means ± SE of the fluxes from four different muscle cells. The curves are the best fits to the Michaelis-Menten equation. The calculated parameters are: in *A* (LiSW), $K_{Ca_o}$ = 2.0 ± 0.1 and 2.9 ± 1.0 mM, and $J_{Na(max)}$ = 66.2 ± 1.3 and 45.8 ± 6.0 pmol/cm$^2\cdot$s for the closed circles and open triangles, respectively; in *B* (Tris-SW), $K_{Ca_o}$ = 7.2 ± 2.8 mM and $J_{Na(max)}$ = 78.4 ± 15.8 pmol/cm$^2\cdot$s.

## $Na_i$-dependent $Ca^{2+}$ Influx

*Activation by intracellular $Ca^{2+}$.* Figs. 3 and 4 show that the $Ca_o$-dependent $Na^+$ efflux is activated by intracellular $Ca^{2+}$. If this fraction of the $Na^+$ efflux is coupled to $Ca^{2+}$ influx, then a $Na_i$-dependent $Ca^{2+}$ influx that is activated by intracellular $Ca^{2+}$ should be demonstrable. The $Ca^{2+}$ influx was therefore measured at various
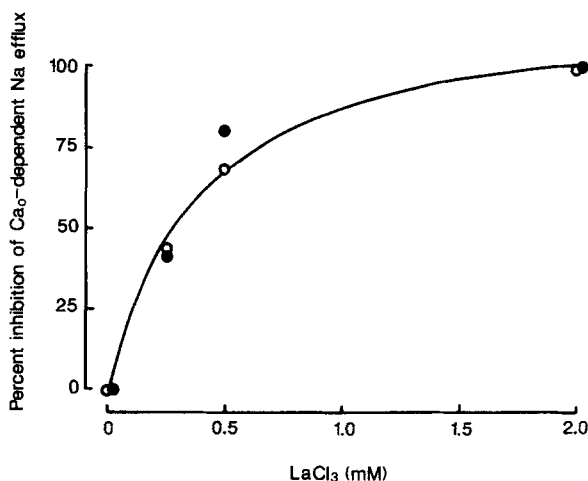
FIGURE 6. The effect of extracellular $LaCl_3$ on the $Ca_o$-dependent $Na^+$ efflux in two barnacle muscle cells (*open* and *closed circles*, respectively). The internal perfusion fluid contained 46 mM $Na^+$; $[Ca^{2+}]_i$ was 1.0 $\mu M$. The external solution was either (Na-free) LiSW (*open circles*) or (Na-free) Tris-SW (*closed circles*); all solutions contained 0.1 mM ouabain. The line represents the best fit to the data; the calculated $IC_{50(La)}$ = 0.38 ± 0.07 mM.

$[Ca^{2+}]_i$ and $[Na^+]$ under conditions identical to those used for the experiments described in Figs. 3 and 4. When $Li^+$ was used as the predominant external cation, however, an increase in $[Ca^{2+}]_i$ produced a $Na_i$-independent $Ca^{2+}$ influx (not shown). This $Ca^{2+}$ influx was mediated not by Na/Ca exchange, but by Ca/Ca (tracer) exchange (Lederer et al., 1982). A similar Ca/Ca exchange has been observed in squid axons superfused with LiSW (Blaustein and Russell, 1975; Blaustein, 1977). Clearly, the contribution of Ca/Ca exchange to the $Ca^{2+}$ influx must be eliminated to enable determination of the Na/Ca exchange stoichiometry.

Experiments on squid axons indicate that there is little Ca/Ca exchange when external $Na^+$ is replaced by nonalkali metal cations (Blaustein and Russell, 1975; Blaustein, 1977). With this in mind, we compared the $Ca_o$-dependent $Na^+$ effluxes and the $Na_i$-dependent $Ca^{2+}$ influxes in Tris-SW. Fig. 7 shows data from two representative examples of a series of 12 experiments of this kind. In the experiment of Fig. 7 A, the $Ca^{2+}$ influx increased by ~1.5 $pmol/cm^2 \cdot s$ when $[Na^{2+}]_i$ was raised from 6 to 106 mM with $[Ca^{2+}]_i$ = $10^{-8}$ M (at $a$). However, when $[Ca^{2+}]_i$ was subsequently raised to $10^{-6}$ M (at $b$), the $Ca^{2+}$ influx increased by ~21 $pmol/cm^2 \cdot s$. To

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FIGURE 7. (*Opposite*) Effects of intracellular $Ca^{2+}$ and $Na^+$ on $Ca^{2+}$ in two barnacle muscle cells (*A* and *B*, respectively). The external medium in both experiments was (Na-free) Tris-SW containing 0.1 mM ouabain. In these experiments the polyethylene tube between the perfusion fluid syringe and intracellular capillary tube was not rapidly flushed with new fluid when the fluid in the syringe was changed and the pump was restarted. This accounts for most of the delay between the time when the fluid in the syringe was changed (indicated by the bars at the top and the arrowheads) and the time when the flux actually began to change (see Methods). (*A*) The initial internal perfusion fluid contained 6 mM $Na^+$; $[Ca^{2+}]_i$ was 0.01 $\mu M$. At $a$, $[Na^+]_i$ was raised to 106 mM and the internal sucrose concentration was reduced to maintain osmolarity; at $b$, $[Ca^{2+}]_i$ was increased to 1.0 $\mu M$. (*B*) The initial internal perfusion fluid contained 6 mM $Na^+$; $[Ca^{2+}]_i$ was 0.01 $\mu M$. At $a$, $[Ca^{2+}]_i$ was raised to 1.0 $\mu M$; at $b$, $[Na^+]_i$ was raised to 30 mM and the internal sucrose concentration was reduced to maintain osmolarity.

FIGURE 7 (continued)
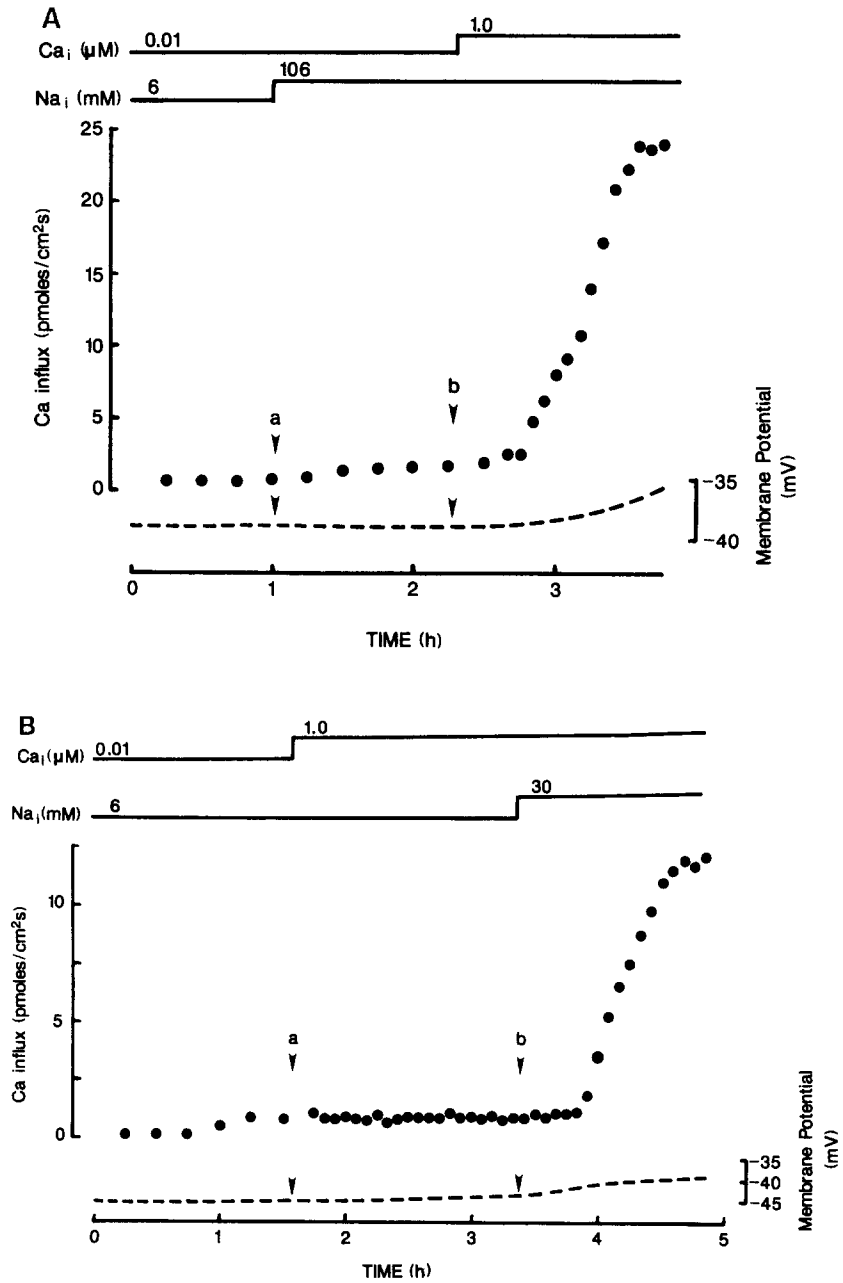
determine whether this $Ca_i$-activated $Ca^{2+}$ influx was a manifestation of Ca/Ca exchange or activation of $Ca^{2+}$ influx mode Na/Ca exchange, experiments such as those of Fig. 7 $B$ were performed. In this case, $[Na^+]_i$ was maintained at 6 mM when $[Ca^{2+}]_i$ was raised from $10^{-8}$ to $10^{-6}$ M (at $a$): no significant increment in $Ca^{2+}$ influx was observed under these circumstances, indicating that there was no Ca/Ca exchange. However, when $[Na^+]_i$ was subsequently raised to 30 mM (at $b$), the $Ca^{2+}$ influx increased by ~11 $pmol/cm^2 \cdot s$. These results indicate that in Tris-SW there was negligible Ca/Ca exchange, and that the stimulation of the $Ca^{2+}$ influx was dependent upon *both* intracellular $Na^+$ and intracellular $Ca^{2+}$. We did not explicitly determine the relationship between this $Ca^{2+}$ influx and $[Ca^{2+}]_i$. However, it seems evident from the data in Fig. 7 that this influx is activated by $[Ca^{2+}]_i$ in the same range as is the $Ca_o$-dependent $Na^+$ efflux, i.e., from $10^{-8}$ to $10^{-6}$ M $Ca^{2+}$ (Figs. 3 $B$ and 4 $B$).

*The coupling ratio of Na/Ca exchange.* In the absence of Na/Na exchange and Ca/Ca exchange, it is possible to calculate the $Ca^{2+}$ influx mode Na/Ca exchange coupling ratio directly by measuring the coupled, $Ca_i$-activated fluxes of $Na^+$ ($Ca_o$-dependent $Na^+$ efflux) and $Ca^{2+}$ ($Na_i$-dependent $Ca^{2+}$ influx) under essentially identical conditions. Fig. 8 shows the mean $Ca^{2+}$ influx data from 12 cells (circles), and the mean $Na^+$ efflux data from 21 cells (triangles) in which these fluxes were measured at various $[Na^+]_i$ between 6 and 106 mM, and at $[Ca^{2+}]_i = 10^{-8}$ M (open symbols) and $[Ca^{2+}]_i = 10^{-6}$ M (closed symbols). Note that the $Ca^{2+}$ influx and $Na^+$ efflux data at $[Ca^{2+}]_i = 10^{-6}$ M are virtually superimposable with the $Na^+$ efflux scale set one-third as large as the $Ca^{2+}$ influx scale. The ratio of the $Ca_o$-dependent $Na^+$ efflux to the $Na_i$-dependent $Ca^{2+}$ influx, over this entire range of $[Na^+]_i$, was 2.8–3.2:1 (average = 3.1 ± 0.5:1, with 95% confidence limit for variance of the ratio of independent means). The activation curve for the two fluxes fitted the Hill equation with a Hill coefficient of 3.7 (the broken line in the figure; the continuous line is drawn to fit the Hill equation with a Hill coefficient of 3.0). Both fluxes were half-maximally activated at $[Na^+]_i = 30$ mM. When $[Ca^{2+}]_i$ was $10^{-8}$ M, however, there was no measureable $Ca_o$-dependent $Na^+$ efflux or $Na_i$-dependent $Ca^{2+}$ influx at any of the $[Na^+]_i$ tested. These results indicate that the coupling ratio of the Na/Ca exchanger in barnacle muscle cells is 3 $Na^+:1$ $Ca^{2+}$, and the intracellular $Na^+$ and $Ca^{2+}$ are both required to activate $Ca^{2+}$ influx mode exchange.

If this exchange results in the uncompensated net extrusion of 1 positive charge (in the form of a $Na^+$ ion) during each carrier cycle, activation of the $Ca^{2+}$ influx mode should cause the cells to hyperpolarize, while blocking the exchange should depolarize the cells. Internally perfused barnacle muscle cells exposed to solutions similar to those used in the present experiments have resting membrane resistances, $R_M$, of ~0.5–1.5 $k\Omega/cm^2$ (Lederer and Nelson, 1984; Rasgado-Flores and Rakowski, unpublished observations). With this $R_M$, the uncompensated efflux of 20 $pmol/cm^2 \cdot s$ of charge (see Fig. 7) should produce a voltage change ($\Delta V_M = \Delta I_{Na/Ca} \times R_M$, where $\Delta I_{Na/Ca}$ is the uncompensated current flow) of ~1–3 mV. Appropriate changes in $V_M$ of this magnitude were often observed when the exchange was turned on and off (e.g., see Figs. 3 $A$ and 4 $A$, and Rasgado-Flores and Blaustein, 1987). However, these changes were usually attenuated or obscured because of a decline in

$R_M$ due to a $Ca_i$-activated cation conductance increase (see Figs. 3 *A*, 4 *A*, and 7, and Sheu and Blaustein, 1983).

Na/Ca exchange is also voltage-sensitive (see Introduction). Therefore, it is important to note that the data at $[Ca^{2+}]_i = 1 \mu M$ in Fig. 8 were all obtained within a relatively narrow range of $V_M$ ($-33$ to $-43$ mV). More than 90% of the data were obtained with $V_M = -37 \pm 3$ mV.
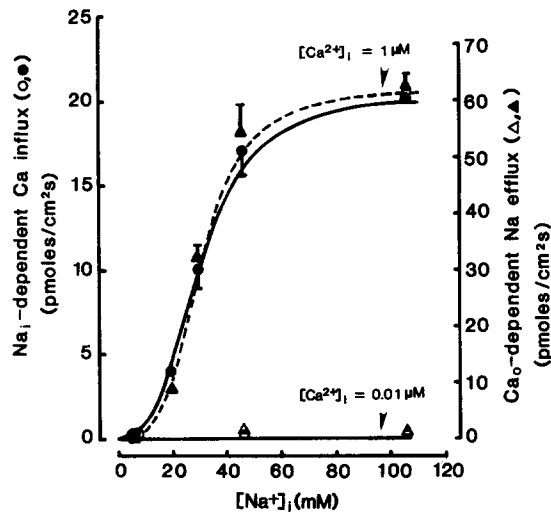


FIGURE 8. Effects of $[Na^+]_i$ on the $Ca_o$-dependent $Na^+$ efflux ($\triangle$, $\blacktriangle$; right-hand ordinate scale) and the $Na_i$-dependent $Ca^{2+}$ influx (O, ●; left-hand ordinate scale) in internally perfused barnacle muscle cells. Data from 27 cells are summarized in the figure; data for at least two different $[Ca^{2+}]_i$ and/or $[Na^+]_i$ were obtained in each cell. Each symbol represents the mean of at least three flux measurements; the bars indicate ± SEM for each of the data points where the errors extend beyond the symbols. The external solution in all experiments was (Na-free) Tris-SW containing 0.1 mM ouabain; $[Ca^{2+}]_i$ was either 0.01 $\mu M$ (*open symbols*) or 1.0 $\mu M$ (*solid symbols*). Note that the ordinate scale for the $Ca^{2+}$ influx is expanded threefold, relative to the scale for the $Na^+$ efflux. The solid line fits the Hill equation with a Hill coefficient of 3.0, a $K_{Na_i}$ of 30 mM, and maximal fluxes of 20.5 pmol/cm²·s for $Na_i$-dependent $Ca^{2+}$ influx and 61.5 pmol/cm²·s for $Ca_o$-dependent $Na^+$ efflux. The discontinuous line is the best fit to the data and has a Hill coefficient of 3.7 ± 0.4 and calculated $J_{Na(max)}$ = 62.4 ± 1.8 pmol/cm²·s and $K_{Na_i}$ = 30.0 ± 0.8 mM. All the data in this figure were obtained with $V_M$ between $-33$ and $-43$ mV; >90% of the data were obtained with $V_M = -37 \pm 3$ mV. These data indicate that the coupling ratio of the $Ca^{2+}$ influx mode Na/Ca exchange is 3 $Na^+$:1 $Ca^{2+}$.

## DISCUSSION

### *Separation of Na/Ca Exchange-mediated and Exchange-independent $Na^+$ and $Ca^{2+}$ Fluxes*

It is essential to separate the ion fluxes mediated by the exchanger from those mediated by other mechanisms to determine the stoichoimetry of the Na/Ca exchange system from tracer flux measurements. Ideally, a selective blocker would be very helpful, but none are known (Bielefeld et al., 1986). We must therefore rely on the counterion dependence of the fluxes (e.g., the $Ca_o$-dependence of the $Na^+$

efflux) to define the exchange-mediated transport (cf. Baker et al., 1969; Blaustein and Hodgkin, 1969).

As described in the Results section, Na/Na and Ca/Ca exchanges are eliminated in Tris-SW. Under these conditions, the $Ca_o$-dependent $Na^+$ efflux and $Na_i$-dependent $Ca^{2+}$ influx appear to be mediated exclusively by the Na/Ca exchanger operating in the $Ca^{2+}$ influx mode. Moreover, both of these fluxes are activated by (non-transported) internal $Ca^{2+}$; this is direct evidence that these two fluxes are coupled. Evidence that the activating internal $Ca^{2+}$ is not transported comes from our observation (unpublished) that there is no detectable $Ca_o$-activated $Ca^{2+}$ efflux into Tris-SW even with relatively high $[Na^+]_i$ (46 mM) and $[Ca^{2+}]_i$ ($10^{-6}$ M).

### Reliability of the $Na^+$ Efflux and $Ca^{2+}$ Influx Measurements, and Determination of the Na/Ca Exchange Coupling Ratio

In these experiments the $Ca^{2+}$ influx mode exchange was measured in $Na_o$-free media after the cells had been perfused for long periods of time with $^{22}$Na-labeled solutions. The specific activity of the $^{22}$Na in the myoplasmic space was presumed constant. The specific activity of the $^{22}$Na appearing in superfusion fluids as a result of $Na^+$ efflux was assumed to be equal to that in the myoplasmic space. Futhermore, there is no reason to suspect that a portion of the exiting $Na^+$ was bound to the extracellular matrix in these high ionic strength solutions.

A possible source of error for the $Ca_o$-dependent $Na^+$ efflux is a "leak" $Ca^{2+}$ influx that might activate a monovalent cation channel through which $^{22}$Na could exit (Sheu and Blaustein, 1983). Unidirectional $^{22}$Na efflux via this pathway would therefore appear as a $Ca_o$-dependent $Na^+$ efflux. This possibility is unlikely for two reasons. First, all of our measurements of stoichiometry were made at membrane potentials more negative ($-33$ to $-43$ mV; see Fig. 8 legend) than the threshold for voltage-gated $Ca^{2+}$ channels in barnacle muscle under our experimental conditions ($[Mg^{2+}]_o = 25$ mM and $[Ca^{2+}]_o = 11$ mM; Hagiwara and Naka, 1964; Hagiwara and Takahashi, 1967). Second, $Ca_o$-dependent $Na^+$ efflux was not observed when $[Ca^{2+}]_i$ was low (Fig. 8), which is direct evidence that a "leak" $Ca^{2+}$ influx did not contribute to the activation of $Na^+$ efflux. Indeed, if the $Ca_o$-dependent $Na^+$ efflux was overestimated, or the $Na_i$-dependent $Ca^{2+}$ influx was underestimated (see below and Methods), the corrected coupling ratio would be less than 3 $Na^+$ per $Ca^{2+}$, which seems unlikely.

A critical problem for the $Ca^{2+}$ influx measurements is that some of the entering $Ca^{2+}$ may be sequestered in intracellular organelles. This would result in underestimation of the true $Ca^{2+}$ influxes. To overcome this potential drawback, the internal perfusion fluids all contained caffeine to inhibit $Ca^{2+}$ accumulation in the SR, and FCCP to inhibit $Ca^{2+}$ sequestration in the mitochondria. Most important, a substantial concentration of Ca-EGTA buffer (8 mM EGTA) was used to "trap" the entering $^{45}$Ca in the perfusing fluid so that it could be washed through and sampled when it emerged from the end-cannula. These considerations indicate that our direct determinations of $Ca_o$-dependent $Na^+$ effluxes and $Na_i$-dependent $Ca^{2+}$ influxes provide a reliable measure of the Na/Ca exchange coupling ratio in barnacle muscle, viz. 3 $Na^+$:1 $Ca^{2+}$.

*Kinetics of Na/Ca Exchange in Barnacle Muscle*

Fig. 9 shows a diagram of the Na/Ca exchanger operating in both the $Ca^{2+}$ efflux and $Ca^{2+}$ influx modes. Several kinetic parameters for the barnacle muscle Na/Ca exchanger operating in the $Ca^{2+}$ influx mode was determined in this study. The values for these parameters, as well as values for some of the kinetic parameters for the $Ca^{2+}$ efflux mode of operation that were determined or estimated in prior studies, are summarized in Table I. These values were all measured in ATP-fueled cells (with the exception of the $J_{Ca(max)}$ for $Ca^{2+}$ efflux; see Table I); removal of ATP appears to alter the values of some of the kinetic parameters (Nelson and Blaustein, 1981; Blaustein, 1977).

Under the asymmetric ion distribution conditions in which these data were obtained, the Na/Ca exchanger is apparently asymmetric. For example, activation of the $Ca^{2+}$ influx mode exchange by extracellular $Ca^{2+}$ follows Michaelis-Menten
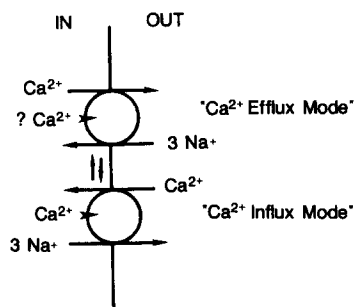


FIGURE 9. Diagram illustrating the $Ca^{2+}$ efflux mode (coupled $Na^+$ entry/$Ca^{2+}$ exit) and $Ca^{2+}$ influx mode (coupled $Na^+$ exit/$Ca^{2+}$ entry) operations of the Na/Ca exchanger. The coupling ratio of the exchange is, 3 $Na^+$:1 $Ca^{2+}$; thus, the reversal potential for the exchanger, $E_{Na/Ca}$ = 3 $E_{Na}$ − 2; $E_{Ca}$. The presence of an internal "catalytic" ("nontransporting") site occupied by $Ca^{2+}$ is indicated by the arrowhead in the lower part of the diagram; whether or not such a site is involved in the lower part of the diagram; whether or not such a site is involved in $Ca^{2+}$ efflux mode exchange is not known.

kinetics (Fig. 5 and related text) without evidence of cooperativity; this implies that there is no catalytic Ca-binding site on the outside, analogous to the one on the inside. The $Ca^{2+}$ efflux mode of exchange may be activated by the binding of intracellular $Ca^{2+}$ to a catalytic site, but this has not yet been explored. If this is, indeed, the case, the activation of the $Na_o$-dependent $Ca^{2+}$ efflux should be a sigmoid function of $[Ca^{2+}]_i$.

The apparent affinities of the exchanger for extracellular $Na^+$ during $Ca^{2+}$ efflux mode operation, and for intracellular $Na^+$ during $Ca^{2+}$ influx mode operation, are similar (but see footnotes to Table I). These affinities, and those for intra- and extracellular $Ca^{2+}$, do not take into account the apparent competition between $Na^+$ and $Ca^{2+}$ at either side of the membrane (Fig. 2; and see, for example, Blaustein et al., 1974); Blaustein and Russell, 1975; Blaustein, 1977). This may be a functional competition, based upon the expectation that, for example, a rise in $[Ca^{2+}]_i$ will tend to

shift more carriers into $Ca^{2+}$ efflux mode operation, and thus promote a carrier-mediated net $Ca^{2+}$ efflux. Conversely, a rise in $[Na^+]_i$ should shift more carriers into $Ca^{2+}$ influx mode operation, and thus promote net $Ca^{2+}$ influx. In other words, $Na^+$ and $Ca^{2+}$ may not necessarily have to compete for the same binding sites on the carrier to explain the apparent mutual inhibition. Unfortunately, insufficient data are available to resolve this uncertainty.

TABLE I

*Kinetic Parameters for the Na/Ca Exchanger in ATP-fueled Barnacle Muscle Cells*

| Kinetic parameter[*] | Value | Reference |
|---|---|---|
| $Ca^{2+}$ influx mode Na/Ca exchange | | |
| $K_{Ca_{o(t)}}$ | 7.2 mM (Tris-SW) | This report |
| | 2.5 mM (LiSW) | This report |
| $K_{Ca_{i(c)}}$ | 0.7 μM (Tris-SW) | This report |
| | 0.3 μM (LiSW) | This report |
| $K_{Na_i}$ | 30 mM (Tris-SW)[‡] | This report |
| Coupling ratio (n) | 3 | This report |
| $J_{Ca(max)}$ | 25 pmol/cm²·s | This report |
| $J_{Na(max)}$ | 75 pmol/cm²·s | This report |
| $IC_{50(Na)}$ | 126 mM | This report |
| $IC_{50(La)}$ | 0.4 mM | This report |
| $Ca^{2+}$ efflux mode Na/Ca exchange | | |
| $K_{Ca_{i(t)}}$ | < 1 μM | Nelson and Blaustein, 1981 |
| $K_{Ca_{i(c)}}$ | ? | See text |
| $K_{Na_o}$ | 60 mM | Russell and Blaustein, 1974 |
| $n$ | 3[§] | Russell and Blaustein, 1974 |
| $J_{Ca(max)}$ | > 10–12 pmol/cm²·s[l] | Lederer and Nelson, 1983 |
| $J_{Na(max)}$ | ? | ? |
| $IC_{50(La)}$ | > 1 mM | Ashley et al., 1974 |

[*]$K_{Ca_{o(t)}}$ and $K_{Ca_{i(t)}}$ are, respectively, the extracellular and intracellular carrier binding sites for transported $Ca^{2+}$; $K_{Ca_{i(c)}}$ is the intracellular "catalytic" site for nontransported $Ca^{2+}$; $K_{Na_i}$ and $K_{Na_o}$ are, respectively, the intracellular and extracellular binding sites for transported $Na^+$; $J_{Na(max)}$ and $J_{Ca(max)}$ are, respectively, the maximal exchanger-mediated fluxes of $Na^+$ and $Ca^{2+}$; $IC_{50(Na)}$ and $IC_{50(La)}$ are the external $Na^+$ and $La^{3+}$ concentrations, respectively, required for half-maximal inhibition of Na/Ca exchange.

[‡]Although it was not systematically measured in LiSW, available data indicate that $K_{Na_i}$ in this solution was substantially smaller than in Tris-SW, perhaps ~20 mM, which is the normal value of $[Na^+]_i$ in these cells (Brinley, 1968).

[§]Estimated from the kinetics of activation of $Ca^{2+}$ efflux by external $Na^+$.

[l]This is an underestimate because the maximal $Na_o$-dependent $Ca^{2+}$ efflux was not determined in these ATP-depleted cells perfused with fluids containing $[Ca^{2+}]_i = 10$ μM.

The apparent affinities of all the ionic binding sites were higher when $Li^+$ was used as the $Na^+$ replacement, as compared with the values obtained when Tris was used (Table I): $K_{Ca_i}$ at the catalytic site decreased from 0.7 to 0.3 μM, and $K_{Ca_o}$ at the transport site decreased from 7.2 to 2.0 mM (at 1 μM $[Ca^{2+}]_i$) in LiSW. Also, $K_{Na_i}$ appeared to be smaller in LiSW than in Tris-SW (see Table I footnotes). These data raise the possibility that either Tris has a slight inhibitory action on the exchanger

kinetics, or $Li^+$ has an activating effect. This needs to be investigated further with other $Na^+$ substitutes.

The observation that the activation of the $Ca_o$-dependent $Na^+$ efflux fits a sigmoid curve with a Hill coefficient of about 2 (Figs. 3 B and 4 B) may indicate that two or more $Ca^{2+}$ ions act cooperatively at the internal catalytic site. However, an alternative possibility is that the true $[Ca^{2+}]_i$ immediately under the sarcolemma may be a little higher than the nominal $[Ca^{2+}]_i$, with nominal $[Ca^{2+}]_i$'s of 0.5–1.0 $\mu$M, because of the large net $Ca^{2+}$ influx under these conditions. Thus, the true relationship between the $Na^+$ efflux and $[Ca^{2+}]_i$, as $[Ca^{2+}]_i$ increases, may be less steep than is apparent in Figs. 3 B and 4 B (see Fig. 4 legend). This possibility is supported by preliminary observations (Rasgado-Flores, H., E. M. Santiago, and M. P. Blaustein, unpublished observations) which indicate that the apparent affinity of the exchanger for internal $Ca^{2+}$, during $Ca^{2+}$ efflux mode exchange, is a little lower in Ca-free than in Ca-containing media, perhaps because $Ca^{2+}$ influx is abolished when external $Ca^{2+}$ is removed. We have calculated that the activation of $Ca_o$-dependent $Na^+$ efflux by internal $Ca^{2+}$ in squid axons (data from Fig. 1 of DiPolo and Beaugé, 1987) fits the Hill equation with a Hill coefficient of ~1.0. Also, in mammalian cardiac muscle internal $Ca^{2+}$ activates the Na/Ca exchanger with a Hill coefficient of 1, which implies that there is no cooperativity (Noda et al., 1988).

### Physiological Role of the Na/Ca Exchanger

Our data provide new insight into the physiological role in the Na/Ca exchanger, and its operation in parallel with $Ca^{2+}$ channels and the ATP-driven $Ca^{2+}$ pump. The rate of $Ca^{2+}$ transport mediated by the exchanger is governed by the driving force on the exchanger, $\Delta V_{Na/Ca}$, which also determines the direction of net transport, and by a number of kinetic parameters. The driving force is equal to the difference between $V_M$ and the exchanger reversal potential, $E_{Na/Ca}$ (i.e., $\Delta V_{Na/Ca} = V_M - E_{Na/Ca}$), where $E_{Na/Ca} = 3E_{Na} - 2E_{Ca}$ when the coupling ratio = 3 $Na^+$:1 $Ca^{2+}$. The kinetic factors pertain to the relative saturation of the transport and catalytic sites by their respective ions, and to the influence of ATP and $K^+$ on the affinities for those ions (e.g., Blaustein, 1977; DiPolo and Beaugé, 1984, 1986; Allen and Baker, 1986a, b). $V_M$ may also influence the kinetics of the transport process (Lagnado et al., 1988; Noda et al., 1988).

Normally, the rate-limiting factors may be the relative saturation by internal $Ca^{2+}$ at the internal catalytic site, for $Ca^{2+}$ influx mode exchange (and, perhaps, for $Ca^{2+}$ efflux mode exchange also), and at the internal $Ca^{2+}$ transport site, for $Ca^{2+}$ efflux mode exchange (cf. Nelson and Blaustein, 1981; Blaustein, 1977). Under resting conditions, when $[Ca^{2+}]_i$ and $[Na^+]_i$ are low, the exchanger operates at a slow rate, and much (but not all) of the $Ca^{2+}$ extrusion is mediated by the ATP-driven $Ca^{2+}$ pump (Rasgado-Flores and Blaustein, 1987). This is consistent with observations that (total) $Ca^{2+}$ influx (Blaustein, M. P., unpublished) and efflux (Ashley et al., 1972; Russell and Blaustein, 1974) in intact, resting barnacle muscle cells are both ~1 $pmol/cm^2 \cdot s$, and are usually only slightly influenced by marked reductions in $[Na^+]_o$. Removal of external $Na^+$ also does not induce contractions in these cells (Blaustein, 1976), probably because even the relatively sparse SR (Hoyle et al., 1973) can sequester most of the entering $Ca^{2+}$ under these circumstances (cf.

Ashida and Blaustein, 1987). Although only small fractions of the unidirectional $Ca^{2+}$ fluxes in resting cells are mediated by the Na/Ca exchanger (Russell and Blaustein, 1974; and see Fig. 1 and related text), this may be sufficient to regulate the size of the SR $Ca^{2+}$ store. This also indicates that the Na/Ca exchanger has a large "reserve" capacity, unlike the $Na^+$ pump which normally operates near its half-maximal capacity, unlike the $Na^+$ pump which normally operates near its half-maximal transport rate (cf. Nelson and Blaustein, 1980): The maximal transport rate of the exchanger, with saturating $[Ca^{2+}]_o$, $[Ca^{2+}]_i$, and $[Na^+]_i$, is expected to be ~25 pmol $Ca^{2+}/cm^2 \cdot s$ (see Figs. 7 A and 8, Table I, and related text).

When the cells are activated, however, and $[Ca^{2+}]_i$ and $[Na^+]_i$ rise as a result of activation of voltage-gated channels and release of $Ca^{2+}$ from the SR, the internal $Ca^{2+}$ transport sites (for $Ca^{2+}$ efflux mode exchange) and catalytic sites (for $Ca^{2+}$ influx mode and, perhaps, $Ca^{2+}$ efflux mode exchange) will then become increasingly saturated with $Ca^{2+}$, so that the turnover of the exchanger (i.e., the rate of $Ca^{2+}$ transport in both directions) will then increase markedly. The direction of net $Ca^{2+}$ transport will be determined by the sign of $\Delta V_{Na/Ca}$. During depolarization, $Ca^{2+}$ will tend to move into the cells in association with an *outward* current; during repolarization, $Ca^{2+}$ will be extruded via the exchanger in association with an *inward* current (cf. Hume, 1987; Kenyon and Sutko, 1987). Then as $[Ca^{2+}]_i$ declines, the exchanger will slow down as the saturation of the transport and catalytic sites by internal $Ca^{2+}$ is reduced. Some evidence for such effects have recently been obtained in cardiac muscle (Hume, 1987; Kenyon and Sutko, 1987) and crustacean "skeletal" muscle (Mounier and Goblet, 1987).

This behavior of the Na/Ca exchanger may have another consequence in barnacle muscle cells. These cells give graded electrical and mechanical responses (Hoyle and Smyth, 1963; Edwards et al., 1964) with prolonged elevation of $[Ca^{2+}]_i$ (Ashley and Caldwell, 1974; Dubyak and Scarpa, 1982). During the period of depolarization and elevated $[Ca^{2+}]_i$, $E_{Na/Ca}$ is likely to approach $V_M$. Thus, despite activation of the exchanger by intracellular $Ca^{2+}$, there may be relatively little net $Ca^{2+}$ movement mediated by the exchanger during the depolarization "plateau." In fact, the exchanger may help to "clamp" $[Ca^{2+}]_i$ at an elevated level by serving as a "low resistance" pathway for bidirectional $Ca^{2+}$ movements across the sarcolemma.

*Note added in proof:* While this article was in press, two reports on the stoichiometry of the Na/Ca exchanger in other tissues were published. Measurements of the exchanger reversal potential, $E_{Na/Ca}$, indicated that the coupling ratio was 3 $Na^+$:1 $Ca^{2+}$ in mammalian cardiac muscle (Ehara et al., 1989), and 4 $Na^+$:1 $Ca^{2+}$ + 1 $K^+$ in amphibian rod outer segments (Cervetto et al., 1989). The influence of $K^+$ on the Na/Ca exchanger in barnacle muscle has not been systematically explored; in squid axons marked reduction of $[K^+]_i$ only minimally inhibits the $Na_o$-dependent $Ca^{2+}$ efflux (Blaustein, 1977).

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