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/Ca²⁺ entry (Na/Ca exchange operating in the Ca²⁺ influx mode) was studied in giant barnacle muscle cells by measuring ⁴²Na⁺ efflux and ⁴⁰Ca²⁺ influx in internally perfused, ATP-fueled cells in which the Na⁺ pump was poisoned by 0.1 mM ouabain. Internal free Ca²⁺, [Ca²⁺]i, was controlled with a Ca-EGTA buffering system containing 8 mM EGTA and varying amounts of Ca²⁺. Ca²⁺ sequestration in internal stores was inhibited with caffeine and a mitochondrial uncoupler (FCCP). To maximize conditions for Ca²⁺ 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 Ca²⁺ (Ca₀)-dependent Na⁺ efflux was observed when [Ca²⁺]i was increased above 10⁻⁸ M; this efflux was half-maximally activated by [Ca²⁺]₀ = 0.3 μM (LiSW) to 0.7 μM (Tris-SW). The Ca₀-dependent Na⁺ efflux was half-maximally activated by [Ca²⁺]₀ = 2.0 mM in LiSW and 7.2 mM in Tris-SW; at saturating [Ca²⁺]₀, [Ca²⁺]i, and [Na⁺]i the maximal (calculated) Ca₀-dependent Na⁺ efflux was ~75 pmol/cm².s. This efflux was inhibited by external Na⁺ and La³⁺ with IC₅₀'s of ~125 and 0.4 mM, respectively.

A Na⁺-dependent Ca²⁺ influx was also observed in Tris-SW. This Ca²⁺ influx also required [Ca²⁺]₀ > 10⁻⁸ M. Internal Ca²⁺ activated a Na⁺-independent Ca²⁺ influx from LiSW (tracer Ca/Ca exchange), but in Tris-SW virtually all of the Ca₀-activated Ca²⁺ influx was Na₀-dependent (Na/Ca exchange). Half-maximal activation was observed with [Na⁺]₀ = 30 mM. The fact that internal Ca²⁺ activates both a Ca₀-dependent Na⁺ efflux and a Na₀-dependent Ca²⁺ influx in Tris-SW implies that these two fluxes are coupled; the activating (intracellular) Ca²⁺ does not appear to be transported by the exchanger. The maximal (calculated) Na₀-dependent Ca²⁺ influx was ~25 pmol/cm².s. At various [Na⁺]₀ between 6 and 106 mM, the ratio of the Ca₀-dependent Na⁺ efflux to the Na₀-dependent Ca²⁺ 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 [Ca²⁺]i; much of the Ca²⁺ extrusion at rest

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(≈1 pmol/cm²·s) is thus mediated by an ATP-driven Ca²⁺ pump. When the cells are activated and depolarized, and [Ca²⁺], begins to rise, the exchanger is activated and moves Ca²⁺ into the cells; then, during repolarization and recovery, the exchanger moves Ca²⁺ out of the cells, thereby providing a negative feedback to slow itself down.

INTRODUCTION

Changes in the intracellular concentration of free Ca²⁺ ([Ca²⁺]) 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 Ca²⁺ in intracellular stores (endoplasmic reticulum and mitochondria) and/or by an increase in Ca²⁺ influx and efflux across the plasmalemma. Ca²⁺ influx can be induced by the activation of voltage-gated and/or receptor-operated Ca²⁺ channels; Ca²⁺ efflux can be induced by the activation of an ATP-driven Ca²⁺ pump.

In parallel with these latter mechanisms, the plasmalemma of most types of animal cells contains another Ca²⁺ transport system, the Na/Ca exchanger, that can transport Ca²⁺ bidirectionally across the membrane in exchange for Na⁺ (cf. Blaustein, 1974). It can move Ca²⁺ out of the cells in exchange for entering Na⁺ ("forward mode" or "Ca²⁺ efflux mode"), and it can move Ca²⁺ into the cells in exchange for exiting Na⁺ ("reverse mode" or "Ca²⁺ influx mode").

An understanding of how this exchange system functions in parallel with the Ca²⁺ channels and the ATP-driven Ca²⁺ pump is of fundamental concern. To address this issue we need to determine the contribution of the Na/Ca exchange system to Ca²⁺ fluxes under various physiological conditions. Therefore, we require information about the thermodynamic factors that regulate the direction of net Ca²⁺ 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 Ca²⁺ (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 Ca²⁺ 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 Ca²⁺-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 Ca²⁺ movement is determined by changes in the Na⁺ electrochemical gradient. To avoid misinterpretation, the terms "Ca²⁺ influx mode" and "Ca²⁺ 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 Ca²⁺ 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 Ca²⁺, have been extended in the present study. In addition, we have examined several kinetic parameters of the Na/Ca system operating in the Ca²⁺ influx mode (Na⁺ exit/Ca²⁺ entry). These data provide new insight into the relationship between the Na/Ca exchanger and the other systems involved in Ca²⁺ movement across the plasmalemma.

**MATERIALS AND METHODS**

**Reagents and Solutions**

The chlorides of Na⁺, K⁺, Ca²⁺, and Mg²⁺ 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 MgCl₂; 11 CaCl₂; 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 CaCl₂; in these instances, the CaCl₂ was replaced by equimolar MgCl₂ to keep the ionic strength constant.

**Internal (perfusion) solutions.** The low Na⁺ (6 mM Na⁺) internal perfusion solution contained (in millimolar): 3 Na₂ATP, 38 KCl, 210 K aspartate, 340 sucrose, 10 MgCl₂, 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-(β-aminoethylether)-N,N'-tetraacetic acid (EGTA) plus varying amounts of CaCl₂ (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 osmometer: Wescor, Inc., Logan, UT).

Various [Ca²⁺], were obtained by using a Ca-EGTA buffer calculated on the basis of a Ca-EGTA stability constant of 7.54 × 10⁶ M⁻¹ (Blinks et al., 1982). The total EGTA concentration was 8 mM; in the 10⁻⁸, 10⁻⁷, and 10⁻⁶ M [Ca²⁺], solutions, the CaCl₂ 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 Ca²⁺ (Hagiwara and Nakajima, 1966), and cells contracted when they were initially perfused with fluids that contained [Ca²⁺], in this range. However, barnacle cells that were perfused for ~2 h or more with solutions containing [Ca²⁺], > 10⁻⁶ M did not contract when [Ca²⁺], 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 \([\text{Ca}^{2+}]_i\)
was varied over the entire dynamic physiological range (10^{-8} to 10^{-3} 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 myoplasmic space near the tendon end of the cell; it then flowed back through the myoplasmic 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 \(^{22}\text{Na}\) and influx of \(^{45}\text{Ca}\). The relevant fluxes were defined, operationally, as the extracellular Ca\(^{2+}\) (Ca\(_o\))-dependent \(^{22}\text{Na}\) efflux and the intracellular Na\(^+\) (Na\(_i\))-dependent \(^{45}\text{Ca}\) influx. To promote Ca\(^{2+}\) influx mode exchange, a large outwardly-directed Na\(^+\) gradient was established by increasing the [Na\(^+\)] on 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, \(^{22}\text{Na}\) (New England Nuclear, Boston, MA) was added to the perfused fluid (0.6 mCi/mmole Na). The appearance of \(^{22}\text{Na}\) 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/cm\(^2\) 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 Ca²⁺ influx experiments.

**Ca²⁺ influx.** To measure Ca²⁺ influx, ⁴⁵Ca (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 ⁴⁵Ca in the intracellular fluid was determined by collecting, at fixed time intervals, 5–20-μl 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 ⁴⁵Ca influx was determined from samples of the internal perfusion fluid, it was important to insure that virtually all of the ⁴⁵Ca 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 ⁴⁵Ca with the ⁴⁵Ca in the myoplasmic space. For example, with an influx of 1 pmol/cm²·s, the entering ⁴⁵Ca was diluted approximately 500-fold with the ⁴⁵Ca in the perfusion fluid at [Ca²⁺] = 10⁻⁷ M, and about 1,000-fold at [Ca²⁺] = 10⁻⁶ M; even with an influx of 20 pmol/cm²·s, the dilution was about 50-fold at [Ca²⁺] = 10⁻⁶ M. Second, the myoplasmic fluid was continuously exchanged at a rate of 5 μl/min. Since the volume of the barnacle muscle cells was ~50 μl, 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 Ca²⁺ sequestration by the sarcoplasmic reticulum (SR) and the mitochondria, respectively. ⁴⁵Ca might, nevertheless, exchange for unlabeled Ca²⁺ in intracellular compartments during the rising phase of ⁴⁵Ca uptake. In the steady-state (i.e., when Ca²⁺ influx reaches a constant level), however, such exchanges should be in equilibrium and should not interfere with quantitation of the ⁴⁵Ca 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_{i/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_{i/2} values was determined using the t test for unpaired data.

**RESULTS**

**Ca²⁺-dependent Na⁺ Efflux**

**Inhibition by extracellular Na⁺.** The component of the Na⁺ efflux mediated by the Na/Ca exchanger during Ca²⁺ influx mode operation should be manifested as a Ca²⁺-dependent Na⁺ efflux. Indeed, as illustrated in Fig. 1 A, with [Na⁺] = 45 mM (~2.5 times normal; Brinley, 1968) and [Ca²⁺] = 10⁻⁷ M, replacement of external Ca²⁺ by equimolar Mg²⁺ produced a small reduction in the Na⁺ efflux into NaSW (~2 pmol/cm²·s). The Ca²⁺-dependent Na⁺ efflux into NaSW was considerably
larger when $[\text{Ca}^{2+}]_{i}$ was increased to 1 $\mu$M, under otherwise identical conditions (~9 pmol/cm$^2$/s in the experiment of Fig. 1 B). Conversely, if $[\text{Ca}^{2+}]_{i}$ was maintained constant, replacement of external Na$^+$ by Tris or Li$^+$ was associated with a larger Ca$^+$-dependent Na$^+$ efflux (Figs. 2-4). These data are consistent with the view that the Ca$^+$-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$^+$-dependent Na$^+$ efflux) under normal resting conditions (i.e., with low $[\text{Ca}^{2+}]_{i}$ and normal $[\text{Na}^+]_{o}$).

Fig. 2 shows the relationship between $[\text{Na}^+]_{o}$ and the Ca$^+$-dependent Na$^+$ efflux when external Na$^+$ was replaced by Tris. It is unclear whether the apparent inhibition of the Ca$^+$-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\(_{\text{a}}\)-dependent efflux of Na\(^+\).

**Activation by intracellular Ca\(^{2+}\).** The Ca\(_{\text{a}}\)-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 Beaug\(\mathring{\text{e}}\), 1986, 1987). Fig. 3A shows data from a representative experiment in which the Ca\(_{\text{a}}\)-dependent Na\(^+\) efflux was measured at several different \([\text{Ca}^{2+}]_i\) with Li\(^+\) as the main external monovalent cation. There was no detectable Ca\(_{\text{a}}\)-dependent Na\(^+\) efflux with \([\text{Ca}^{2+}]_i = 10^{-8}\) M (not shown). A small Ca\(_{\text{a}}\)-dependent Na\(^+\) efflux was observed with \([\text{Ca}^{2+}]_i = 10^{-7}\), and a much larger Ca\(_{\text{a}}\)-dependent Na\(^+\) efflux at \([\text{Ca}^{2+}]_i = 10^{-6}\); there was not much more increase in this efflux when \([\text{Ca}^{2+}]_i\) was raised to \(10^{-5}\) M, which indicates that the efflux saturates at high \([\text{Ca}^{2+}]_i\).

**FIGURE 2.** Inhibition of the Ca\(_{\text{a}}\)-dependent Na\(^+\) efflux by external Na\(^+\) in mixtures of NaSW and Tris-SW. These data were compiled from three cells with \([\text{Na}^{+}]_i = 46\) mM and \([\text{Ca}^{2+}]_i = 1\) mM, and were normalized to "0" inhibition in Tris-SW; bars indicate the range of values. All solutions contained 0.1 mM ouabain. The Ca\(_{\text{a}}\)-dependent Na\(^+\) efflux averaged 56 pmol/cm\(^2\)·s in (Na-free) Tris-SW, and 5.3 pmol/cm\(^2\)·s in NaSW. The curve is the best-fit solution to the Hill equation, the calculated parameters are \(K_{\text{Na}} = 126 \pm 12\) mM, \(J_{\text{Na}} = 98 \pm 6\) pmol/cm\(^2\)·s, and the Hill coefficient = 2.6 ± 0.6.

The activation curve for the Ca\(_{\text{a}}\)-dependent Na\(^+\) efflux by internal Ca\(^{2+}\) is shown in Fig. 3B; the efflux was half-maximally activated at \([\text{Ca}^{2+}]_i = 0.3\) µM. The time course of the decline in the Na\(^+\) efflux when external Ca\(^{2+}\) was removed (Fig. 3A) 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\(_{\text{a}}\)-dependent Na\(^+\) efflux was measured at several different \([\text{Ca}^{2+}]_i\) in Tris-SW are shown in Fig. 4A; similar results were obtained in four other experiments of this type. The activation curve for the Ca\(_{\text{a}}\)-dependent Na\(^+\) efflux into Tris-SW by internal Ca\(^{2+}\) is shown in Fig. 4B, which includes data from all five experiments; the efflux was half-maximally activated at \([\text{Ca}^{2+}]_i = 0.7\) µM. The difference between the \(K_{\text{Ca}}\) values obtained in LiSW (0.3 µM) and Tris-SW (0.7 µM) is significant (\(P < 0.01\)).

Recently, it was reported that in squid axons the \([\text{Ca}^{2+}]_i\) required for half-maxi-
mal activation of the $\text{Ca}_\text{o}$-activated $\text{Na}^+$ efflux in Tris-SW is $\sim 1.4 \ \mu\text{M}$ (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 $\text{Na}^+$ and $\text{Ca}^{2+}$ during $\text{Ca}^{2+}$ entry mode exchange comparable to that observed during $\text{Ca}^{2+}$ exit mode exchange (Blaustein, 1977) because $[\text{Na}^+]_i$ was 46 mM in the barnacle muscle and 100 mM in the squid axons. Alternatively, as discussed below, $[\text{Ca}^{2+}]_i$ just inside the sarcolemma may have been poorly controlled in the barnacle muscle cells when there was a large $\text{Ca}^{2+}$ influx.

The relationship between the $\text{Na}^+$ efflux and $[\text{Ca}^{2+}]_i$ 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 $\text{Ca}^{2+}$ involves more than one $\text{Ca}^{2+}$ ion on each carrier molecule.

Figs. 3 A and 4 A also show that there was a large increase in the $\text{Ca}_\text{o}$-independent

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**Figure 3.** $\text{Ca}_\text{o}$-dependent $\text{Na}^+$ efflux measured at various $[\text{Ca}^{2+}]_i$. The external medium was (Na-free) LiSW containing 0.1 mM ouabain. The internal perfusion fluid contained 46 mM $\text{Na}^+$; $[\text{Ca}^{2+}]_i$ was altered by varying the total $\text{CaCl}_2$ concentration in a CaEGTA buffer system with $[\text{EGTA}]_\text{total} = 8 \ \text{mM}$. (A) The downward arrowheads above the efflux data indicate where external $\text{Ca}^{2+}$ (normally 11 mM) was replaced by $\text{Mg}^{2+}$; the $\text{Ca}^{2+}$ was restored at the times denoted by the upward arrowheads below the efflux data. The $[\text{Ca}^{2+}]_i$ values are shown at the top of the figure. Note the change in ordinate scale above 50 pmol/cm$^2$-s. (B) $\text{Ca}_\text{o}$-dependent $\text{Na}^+$ efflux into LiSW (data from A and one other cell) graphed as a function of $[\text{Ca}^{2+}]_i$ (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, $K_{\text{Ca}} = 0.3 \pm 0.1 \ \mu\text{M}$ $J_{\text{Na}(\text{max})} = 65 \pm 8$ pmol/cm$^2$-s; Hill coefficient $= 2.1 \pm 1.4$. For the Michaelis-Menten equation, $K_{\text{Ca}} = 0.3 \pm 0.2 \ \mu\text{M}$ and $J_{\text{Na}(\text{max})} = 70 \pm 10$ pmol/cm$^2$-s.
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\(^{2+}\)-dependent Na\(^+\) efflux. The presence of this large Ca\(^{2+}\)-activated increase in Na\(^+\) conductance and (nonexchange mediated) Na\(^+\) influx in

![Figure 4](image-url)

**Figure 4.** Ca\(^{2+}\)-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\(_{out}\). (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\(^{2+}\)-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 \(K_{Ca} = 0.7 ± 0.1 \mu M, J_{Na\text{out}} = 72 ± 3 \text{ pmol/cm}^2\text{s},\) Hill coefficient = 2.2 ± 0.5, and for the Michaelis-Menten equation, \(K_{Ca} = 0.7 ± 0.2 \mu M\) and \(J_{Na\text{out}} = 81 ± 6 \text{ pmol/cm}^2\text{s}.\) Note that if the [Ca\(^{2+}\)], 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 \(K_{Ca}\) 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\textsuperscript{2+}, inhibition of Ca\textsuperscript{2+} influx mode exchange should depolarize the membrane. Indeed, a reversible depolarization was usually observed when the Na\textsuperscript{+} efflux was reduced by removing extracellular Ca\textsuperscript{2+} (Figs. 3 A and 4 A). However, some depolarization was also observed when external Ca\textsuperscript{2+} was removed and [Na\textsuperscript{+}] 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\textsuperscript{2+}**. The preceding data indicate that the Ca\textsubscript{o}-dependent Na\textsuperscript{+} efflux (Na/Ca exchange) is activated by [Ca\textsuperscript{2+}]\textsubscript{i} in the normal physiological range (10\textsuperscript{-7}-10\textsuperscript{-5} M). Since there is negligible Ca\textsubscript{o}-dependent Ca efflux in Tris-SW (cf. Rasgado-Flores and Blaustein, 1987) and, thus, no Ca/Ca exchange, the internal Ca\textsuperscript{2+} must be acting at a “nontransporting” site on the exchanger. To measure the affinity of the carrier for external Ca\textsuperscript{2+}, and to determine whether there is also a “nontransporting” Ca\textsuperscript{2+} activation site at the external surface of the plasmalemma, we compared the external Ca\textsuperscript{2+} dependence of the Ca\textsubscript{o}-activated Na\textsuperscript{+} efflux into LiSW and Tris-SW. Fig. 5 A shows that a LiSW, with [Na\textsuperscript{+}] = 31 mM and [Ca\textsuperscript{2+}] = 0.5 \mu M (triangles) or 1.0 \mu M (circles), activation by external Ca\textsuperscript{2+} followed Michaelis-Menten kinetics with Ca\textsuperscript{2+} concentrations of 2.0 mM (at [Ca\textsuperscript{2+}] = 1.0 \mu M) to 2.9 mM (at [Ca\textsuperscript{2+}] = 0.5 \mu M) required for half-maximal activation (K\textsubscript{Ca}). At [Ca\textsuperscript{2+}] = 11 mM (the normal concentration), the fluxes reached 80-85% of the exchanger-mediated Na\textsuperscript{+} efflux, J\textsubscript{Na\textsubscript{m}}. In Tris-SW, with [Na\textsuperscript{+}] = 46 mM and [Ca\textsuperscript{2+}] = 1.0 \mu M, the Ca\textsubscript{o}-dependent Na\textsuperscript{+} efflux also increased with increasing [Ca\textsuperscript{2+}]\textsubscript{o}, following Michaelis-Menten kinetics with a K\textsubscript{Ca} of 7.2 mM (Fig. 5 B), but the efflux only appeared to reach 64% of J\textsubscript{Na\textsubscript{m}} at [Ca\textsuperscript{2+}] = 11 mM. Higher Ca\textsuperscript{2+} concentrations caused problems because the controls required higher Mg\textsuperscript{2+} levels to maintain constant ionic strength. These high Mg\textsuperscript{2+} levels directly affect the Na\textsuperscript{+} 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\textsuperscript{2+} inhibits Na/Ca exchange (Smith et al., 1987).

The Ca\textsubscript{o}-dependent Na\textsuperscript{+} efflux into LiSW, with [Ca\textsuperscript{2+}] = 11 mM and [Ca\textsuperscript{2+}] = 1 \mu M (Fig. 5 A), was substantially closer to J\textsubscript{Na\textsubscript{m}} 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\textsuperscript{2+} in the two sea waters (compare Figs. 3 B and 5 A with Figs. 4 B and 5 B). The respective K\textsubscript{Ca} 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\textsuperscript{2+} binding site (a “transporting site”) on the exchanger, facing the extracellular fluid. This site has an apparent affinity for Ca\textsuperscript{2+} about 4 orders of magnitude lower than the affinity for Ca\textsuperscript{2+} at the intracellular site (see Figs. 3 B and 4 B).

**Inhibition by external La\textsuperscript{3+}**. There is no specific blocker available for the Na/Ca exchanger at present (Bielefeld et al., 1986). However, La\textsuperscript{3+} at least partially blocks both the Ca\textsuperscript{2+} exit and Ca\textsuperscript{2+} entry modes of Na/Ca exchange in barnacle muscle.
cells: i.e., it inhibits the Na⁺-dependent Ca²⁺ efflux (Ashley et al., 1974; but see Ledderer and Nelson, 1983), although not as effectively as it inhibits the Ca²⁺ influx from low Na⁺ media (Ashley and Lea, 1978). In squid axons, La³⁺ also blocks the Ca⁰⁺-dependent Na⁺ efflux (i.e., Ca²⁺ influx mode exchange) (Baker et al., 1969). Fig. 6 shows that the Ca⁰⁺-dependent Na⁺ efflux from internally perfused barnacle muscle cells ([Na⁺]ᵢ = 46 mM; [Ca²⁺]ᵢ = 1 μM) into either LiSW or Tris-SW was inhibited by 50% in the presence of 0.4 mM external La³⁺.

![Figure 5](https://example.com/Figure5.png)

**Figure 5.** The [Ca²⁺]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⁺]₀ was 31 mM in A, and 46 mM in B; [Ca²⁺]ᵢ was 0.5 μM (open triangles) or 1.0 μM (closed circles) in A, and 1.0 μM 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), KᵦCa = 2.0 ± 0.1 and 2.9 ± 1.0 mM, and Jₑ(Na)max = 66.2 ± 1.3 and 45.8 ± 6.0 pmol/cm²·s for the closed circles and open triangles, respectively; in B (Tris-SW), KᵦCa = 7.2 ± 2.8 mM and Jₑ(Na)max = 78.4 ± 15.8 pmol/cm²·s.

**Na⁺-dependent Ca²⁺ Influx**

*Activation by intracellular Ca²⁺.* Figs. 3 and 4 show that the Ca⁰⁺-dependent Na⁺ efflux is activated by intracellular Ca²⁺. If this fraction of the Na⁺ efflux is coupled to Ca²⁺ influx, then a Na⁺-dependent Ca²⁺ influx that is activated by intracellular Ca²⁺ should be demonstrable. The Ca²⁺ influx was therefore measured at various
[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\(^{-}\)-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\(_i\)-dependent Na\(^{-}\) effluxes and the Na\(^{-}\)-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. 7A, the Ca\(^{2+}\) influx increased by ~1.5 pmol/cm\(^2\)s when [Na\(^{+}\)]_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\(^2\)s. To

**Figure 6.** The effect of extracellular LaCl\(_3\) on the Ca\(_i\)-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\(_{50\,(La)} = 0.38 \pm 0.07\) mM.

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

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\textsuperscript{2+} influx mode Na/Ca exchange coupling ratio directly by measuring the coupled, Ca\textsuperscript{2+}-activated fluxes of Na\textsuperscript{+} (Ca\textsuperscript{2+}-dependent Na\textsuperscript{+} efflux) and Ca\textsuperscript{2+} (Na\textsuperscript{+}-dependent Ca\textsuperscript{2+} influx) under essentially identical conditions. Fig. 8 shows the mean Ca\textsuperscript{2+} influx data from 12 cells (circles), and the mean Na\textsuperscript{+} efflux data from 21 cells (triangles) in which these fluxes were measured at various [Na\textsuperscript{+}]\textsubscript{i} between 6 and 106 mM, and at [Ca\textsuperscript{2+}]\textsubscript{i} = 10\textsuperscript{-8} M (open symbols) and [Ca\textsuperscript{2+}]\textsubscript{i} = 10\textsuperscript{-6} M (closed symbols). Note that the Ca\textsuperscript{2+} influx and Na\textsuperscript{+} efflux data at [Ca\textsuperscript{2+}]\textsubscript{i} = 10\textsuperscript{-6} M are virtually superimposable with the Na\textsuperscript{+} efflux scale set one-third as large as the Ca\textsuperscript{2+} influx scale. The ratio of the Ca\textsuperscript{2+}-dependent Na\textsuperscript{+} efflux to the Na\textsuperscript{+}-dependent Ca\textsuperscript{2+} influx, over this entire range of [Na\textsuperscript{+}]\textsubscript{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\textsuperscript{+}]\textsubscript{i} = 30 mM. When [Ca\textsuperscript{2+}]\textsubscript{i} was 10\textsuperscript{-8} M, however, there was no measureable Ca\textsuperscript{2+}-dependent Na\textsuperscript{+} efflux or Na\textsuperscript{+}-dependent Ca\textsuperscript{2+} influx at any of the [Na\textsuperscript{+}]\textsubscript{i} tested. These results indicate that the coupling ratio of the Na/Ca exchanger in barnacle muscle cells is 3 Na\textsuperscript{+}:1 Ca\textsuperscript{2+}, and the intracellular Na\textsuperscript{+} and Ca\textsuperscript{2+} are both required to activate Ca\textsuperscript{2+} influx mode exchange.

If this exchange results in the uncompensated net extrusion of 1 positive charge (in the form of a Na\textsuperscript{+} ion) during each carrier cycle, activation of the Ca\textsuperscript{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 kΩ/cm\textsuperscript{2} (Lederer and Nelson, 1984; Rasgado-Flores and Rakowski, unpublished observations). With this \(R_M\), the uncompensated efflux of 20 pmol/cm\textsuperscript{2} 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. 3A and 4A, 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$_2^+$-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$_{2+}$]$\_1 = 1 \mu$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.

**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 stoichiometry 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$_{2+}$-dependence of the Na$^+$-dependent Na$^+$ efflux and the Na$_{2+}$-dependent Ca$_{2+}$ influx).
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\textsubscript{Ca}-dependent Na\textsuperscript{+} efflux and Na\textsubscript{Na}-dependent Ca\textsuperscript{2+} influx appear to be mediated exclusively by the Na/Ca exchanger operating in the Ca\textsuperscript{2+} influx mode. Moreover, both of these fluxes are activated by (non-transported) internal Ca\textsuperscript{2+}; this is direct evidence that these two fluxes are coupled. Evidence that the activating internal Ca\textsuperscript{2+} is not transported comes from our observation (unpublished) that there is no detectable Ca\textsubscript{Ca}-activated Ca\textsuperscript{2+} efflux into Tris-SW even with relatively high [Na\textsuperscript{+}]\textsubscript{i} (46 mM) and [Ca\textsuperscript{2+}]\textsubscript{i} (10^{-6} M).

Reliability of the Na\textsuperscript{+} Efflux and Ca\textsuperscript{2+} Influx Measurements, and Determination of the Na/Ca Exchange Coupling Ratio

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

A possible source of error for the Ca\textsubscript{Na}-dependent Na\textsuperscript{+} efflux is a “leak” Ca\textsuperscript{2+} influx that might activate a monovalent cation channel through which 22Na could exit (Sheu and Blaustein, 1983). Unidirectional 22Na efflux via this pathway would therefore appear as a Ca\textsubscript{Na}-dependent Na\textsuperscript{+} 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\textsuperscript{2+} channels in barnacle muscle under our experimental conditions ([Mg\textsuperscript{2+}]\textsubscript{o} = 25 mM and [Ca\textsuperscript{2+}]\textsubscript{o} = 11 mM; Hagiwara and Naka, 1964; Hagiwara and Takahashi, 1967). Second, Ca\textsubscript{Na}-dependent Na\textsuperscript{+} efflux was not observed when [Ca\textsuperscript{2+}]\textsubscript{i} was low (Fig. 8), which is direct evidence that a “leak” Ca\textsuperscript{2+} influx did not contribute to the activation of Na\textsuperscript{+} efflux. Indeed, if the Ca\textsubscript{Na}-dependent Na\textsuperscript{+} efflux was overestimated, or the Na\textsubscript{Na}-dependent Ca\textsuperscript{2+} influx was underestimated (see below and Methods), the corrected coupling ratio would be less than 3 Na\textsuperscript{+} per Ca\textsuperscript{2+}, which seems unlikely.

A critical problem for the Ca\textsuperscript{2+} influx measurements is that some of the entering Ca\textsuperscript{2+} may be sequestered in intracellular organelles. This would result in underestimation of the true Ca\textsuperscript{2+} influxes. To overcome this potential drawback, the internal perfusion fluids all contained caffeine to inhibit Ca\textsuperscript{2+} accumulation in the SR, and FCCP to inhibit Ca\textsuperscript{2+} sequestration in the mitochondria. Most important, a substantial concentration of Ca-EGTA buffer (8 mM EGTA) was used to “trap” the entering 45Ca 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\textsubscript{Na}-dependent Na\textsuperscript{+} effluxes and Na\textsubscript{Na}-dependent Ca\textsuperscript{2+} influxes provide a reliable measure of the Na/Ca exchange coupling ratio in barnacle muscle, viz. 3 Na\textsuperscript{+}:1 Ca\textsuperscript{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 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$^+$] 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 |
|-------------------|-------|-----------|
| $K_{Ca_{in}}$     | 7.2 mM (Tris-SW) | This report |
|                   | 2.5 mM (LiSW) | This report |
| $K_{Ca_{ex}}$     | 0.7 µM (Tris-SW) | This report |
|                   | 0.3 µM (LiSW) | This report |
| $K_{Na_{in}}$     | 30 mM (Tris-SW) | This report |
| Coupling ratio ($n$) | 3 | This report |
| $J_{Ca_{in}}$     | 25 pmol/cm²·s | This report |
| $J_{Na_{in}}$     | 75 pmol/cm²·s | This report |
| $IC_{0_{Na}}$     | 126 mM | This report |
| $IC_{0_{Ca}}$     | 0.4 mM | This report |

Ca$^{2+}$ efflux mode Na/Ca exchange

| Kinetic parameter | Value | Reference |
|-------------------|-------|-----------|
| $K_{Ca_{ex}}$     | < 1 µM | Nelson and Blaustein, 1981 |
| $K_{Ca_{in}}$     | ? | See text |
| $K_{Na_{in}}$     | 60 mM | Russell and Blaustein, 1974 |
| $n$               | 3 | Russell and Blaustein, 1974 |
| $J_{Ca_{in}}$     | > 10–12 pmol/cm²·s | Lederer and Nelson, 1983 |
| $J_{Na_{in}}$     | ? | ? |
| $IC_{0_{Na}}$     | > 1 mM | Ashley et al., 1974 |

* $K_{Ca_{in}}$ and $K_{Ca_{ex}}$ are, respectively, the extracellular and intracellular carrier binding sites for transported Ca$^{2+}$; $K_{Na_{in}}$ is the intracellular "catalytic" site for nontransported Ca$^{2+}$; $K_{Na_{in}}$ and $K_{Na_{ex}}$ are, respectively, the intracellular and extracellular binding sites for transported Na$^+$; $J_{Na_{in}}$ and $J_{Ca_{in}}$ are, respectively, the maximal exchanger-mediated fluxes of Na$^+$ and Ca$^{2+}$; $IC_{0_{Na}}$ and $IC_{0_{Ca}}$ are the external Na$^+$ and Ca$^{2+}$ concentrations, respectively, required for half-maximal inhibition of Na/Ca exchange.

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

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

$^t$This is an underestimate because the maximal Na$^+$-dependent Ca$^{2+}$ efflux was not determined in these ATP-depleted cells perfused with fluids containing [Ca$^{2+}$], ≈ 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): $K_{Ca_{in}}$ at the catalytic site decreased from 0.7 to 0.3 µM, and $K_{Ca_{ex}}$ at the transport site decreased from 7.2 to 2.0 mM (at 1 µM [Ca$^{2+}$]) in LiSW. Also, $K_{Na_{in}}$ 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.
Na/Ca Exchange Kinetics and Stoichiometry

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. 3B and 4B) 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 μM, because of the large net Ca$^{2+}$ influx under these conditions. Thus, the true relationship between the Na$^+$ efflux and [Ca$^{2+}$]$_o$, as [Ca$^{2+}$]$_i$ increases, may be less steep than is apparent in Figs. 3B and 4B (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 of 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, ΔV$_{Na/Ca}$, 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., $ΔV_{Na/Ca} = V_M - E_{Na/Ca}$), 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$^2$·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 \([\text{Ca}^{2+}]_o, [\text{Ca}^{2+}]_i, \text{and [Na}^{+}]_i\), is expected to be \(~25\) pmol \(\text{Ca}^{2+}/\text{cm}^2\cdot\text{s}\) (see Figs. 7 A and 8, Table I, and related text).

When the cells are activated, however, and \([\text{Ca}^{2+}]_i, \text{and [Na}^{+}]_i\), 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 \(\Delta V_{\text{Na/Ca}}\). 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 \([\text{Ca}^{2+}]_i\) 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 \([\text{Ca}^{2+}]_i\). During the period of depolarization and elevated \([\text{Ca}^{2+}]_i\), \(E_{\text{Na/Ca}}\) 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” \([\text{Ca}^{2+}]_i\) 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, \(E_{\text{Na/Ca}}\), 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 \([\text{K}^{+}]_o\), only minimally inhibits the Na\(^{-}\)-dependent Ca\(^{2+}\) efflux (Blaustein, 1977).

This article is dedicated to the memory of our friend and colleague, Peter F. Baker, a pioneer in the study of Na/Ca exchange.

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