Voltage Dependence of the Na/Ca Exchange in Voltage-clamped, Dialyzed Squid Axons

Na-dependent Ca Efflux

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ABSTRACT A combination of the voltage-clamp and the intracellular dialysis techniques has been used to study the membrane potential dependence of the Na+-dependent Ca efflux in squid giant axons. In order to improve axon survival, experiments were carried out using internal solutions prepared with large impermeant organic anions and cations, which did not affect the operation of the Na/Ca exchange mechanism. In axons dialyzed with solutions prepared without internal Na, the Na+-dependent Ca efflux had a small sensitivity to membrane potential changes. For a 25-mV membrane displacement in the hyperpolarizing direction, the basal Ca efflux increased by only 7.4% (n = 13). When the dialysis medium contained Na (from 20 to 55 mM), the efflux increased 32.3% (n = 25) for the same membrane potential change. The $K_{1/2}$ for this effect is $\sim$5 mM Na, and saturation appears to occur at a Na concentration above 20 mM. Adding ATP to the dialysis medium increased the magnitude of the Na+-dependent Ca efflux without changing its voltage sensitivity. Wide changes in the intracellular ionized Ca concentration (from 0.1 to 230 $\mu$M) did not modify the voltage sensitivity of the exchange system. Elimination of the reversal of Na/Ca exchange (Na+-dependent Ca influx) by removing Ca$_o$ did not modify the voltage sensitivity of the Na+-dependent Ca efflux. When the axon membrane potential was submitted to prolonged changes, the corresponding changes in the Ca efflux were not sustained, but declined exponentially to intermediate values. This effect may indicate a slow inactivation process in the Na/Ca exchange mechanism. Voltage-clamp pulse experiments revealed: (a) the absence of a fast inactivation process in the Na/Ca exchange, and (b) that the activation of the carrier for hyperpolarizing pulses occurs as rapidly as 1 ms.

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INTRODUCTION

The long-term regulation of intracellular Ca in animal cells is achieved by constant extrusion against the electrochemical Ca gradient, for which energy expenditure is necessary. There are two mechanisms for this uphill Ca extrusion: one is through the functioning of an ATP-driven Ca pump, which was described in detail in red cells (Schatzmann, 1973) and later found in many other preparations, including squid axon (DiPolo, 1978) and cardiac muscle (Caroni and Carafoli, 1980); the other is a mechanism through which internal Ca is exchanged for external Na, and is thought to derive its energy from the Na electrochemical gradient. This mechanism, which may also operate in the reverse mode, i.e., exchanging internal Na for external Ca, was first described in heart muscle (Reuter and Seitz, 1968) and studied in detail in squid axon (Baker et al., 1969; DiPolo, 1974; Blaustein, 1977; Mullins, 1977), in barnacle muscle fiber (Russell and Blaustein, 1974; Lederer and Nelson, 1983), and in cardiac muscle preparations (Pitts, 1979; Reeves and Sutko, 1980; Eisner et al., 1983).

In spite of much work, the mechanism by which this Na/Ca exchange system operates is not yet well understood. It is thought that a carrier with definite affinities for Na and Ca at both sides of the membrane operates the exchange mechanism (Blaustein, 1977; Mullins, 1977). The mode of operation, however, appears to be dependent not only on the ions being transported but also on the presence of several physiological ligands (K\(^+\), ATP, H\(^+\)), thus adding to the complexity of the system (DiPolo and Beaugé, 1984). There is evidence that the Na/Ca exchange is electrogenic: Ca movements through the carrier can generate a membrane current (Pitts, 1979; Reeves and Sutko, 1980; Mentrard et al., 1984) and be influenced by membrane potential changes (Baker and Glitsch, 1975; Mullins and Brinley, 1975; Blaustein et al., 1974; Lederer and Nelson, 1983). However, the stoichiometry with which the carrier operates, and its membrane potential dependence, are important aspects of Na/Ca exchange about which there is still no information. The knowledge about the membrane potential dependence of the Na/Ca exchange carrier has been obtained using different approaches, such as changing the membrane potential with different external K concentrations or with veratridine, or by passing electrical current through an internal axial wire (Brinley and Mullins, 1975; Blaustein et al., 1974). Although these approaches have clearly demonstrated the voltage dependence of the exchange mechanism, there is still little information about the factors that may affect it.

In the present work, we have combined the voltage-clamp with the dialysis technique to measure the potential dependence of Na/Ca exchange in squid axons under a variety of experimental conditions. Our results show that the sensitivity of the Na\(_o\)-dependent Ca efflux to membrane potential is dependent on Na\(_o\), and is not affected by wide variations in Ca\(_i\) or by removal of Ca\(_o\). ATP increases the magnitude of the Na\(_o\)-dependent Ca efflux without affecting its voltage sensitivity. Finally, the results show fast (<1 ms) activation kinetics and a slow inactivation process of the exchange mechanism in response to membrane potential changes.
Some of these results have been communicated in preliminary form elsewhere (DiPolo et al., 1984; Bezanilla et al., 1985).

**METHODS**

The experiments were carried out with giant axons from *Loligo plei* at IVIC, Caracas, Venezuela, or *Loligo pealei* at the Marine Biological Laboratory, Woods Hole, MA. Immediately after decapitation of the squid, the giant axon was dissected from the mantle in artificial seawater (ASW).

**Experimental Chamber**

The experimental chamber contained the essential features for the voltage-clamp and dialysis techniques, for which some compromises were necessary to meet adequately their most important requirements. For instance, for efflux measurements under standard internal dialysis conditions, it is desirable to maximize the length of the axon segment from which the isotope has been collected to reduce the end effects derived from Ca efflux from the guard regions. For voltage-clamp experiments, it is necessary to minimize the length of the current-measuring segment in order to assure homogeneous longitudinal control, which is otherwise jeopardized by the possibility of polarization of the axial wire current electrode. Fig. 1 shows a diagram of the chamber used in this work. It consists basically of a dialysis chamber (Brinley and Mullins, 1967) modified to reduce the length (to 1 cm) of the dialyzed axon segment, from which efflux is collected. Platinum plates are included in the chamber to measure the current from the clamped collecting region and to ground the guard compartments. Another modification is the elimination of the end cannulae and the Lucite partitions that held them. In the chamber we used, the axon is not cannulated; instead, its ends lie on pedestals and slits are opened in them for the insertion of the electrode and the dialysis capillary. The axon cut ends are separated from the central chamber pool by two air gaps. The porous cellulose acetate dialysis capillary of 150 μm o.d., containing a 38-μm platinized platinum wire (20% iridium) for passing current, was introduced into one end of the axon and advanced longitudinally until its tip
protruded from the other (distal) end. The porous region of the capillary extended through the whole chamber length. The internal voltage electrode consisted of a glass cannula of ~40-60 μm o.d., containing a 25-μm floating platinum wire, and was filled with 0.5 M KCl in electrical contact with a Ag/AgCl pellet electrode. This cannula was inserted into the axon from the distal end and its tip was positioned in the middle of the collecting chamber. In some experiments, a second voltage electrode was introduced from the same end into the axon and positioned at different points in the axon to test for voltage uniformity along the clamped and dialyzed axon segment (see Fig. 1). The voltage reference electrode, made with a pulled glass pipette, was filled with 3 M KCl, connected to a Ag/AgCl pellet, and positioned in the chamber central compartment. The isotope guards were covered with thin plastic coverslips and their content was continuously removed by suction with a withdrawal pump, thus preventing isotope flow from the guard regions to the central compartment.

Solutions

DIALYSIS SOLUTIONS
The standard dialysis medium had the following composition (mM): 310 K+, 40 Na+, 4 Mg2+, 30 Tris+, 98 Cl-, 310 aspartate, 1 EGTA, 350 glycine, pH 7.3, at 17-18°C. The ionized Ca activity was estimated using a dissociation constant of 0.15 μM for Ca-EGTA and 1.4 mM for Ca-ATP (DiPolo et al., 1984). Removal of K+ and Na+ was compensated with equiosmolar amounts of N-methylglucamine (NMG+). The osmolarity of all solutions was determined using a commercial osmometer (Wescor Inc., Logan, UT) and adjusted to 980 mosmol/kg water. ATP (vanadium-free) was obtained from Sigma Chemical Co. (St. Louis, MO) as Trissalt, neutralized with TrisOH, and stored at −20°C as a 0.25-M solution.

ASW The standard ASW had the following composition (mM): 10 K+, 440 Na+, 50 Mg2+, 10 Ca2+, 580 Cl−, 0.1 EDTA, pH 7.7, at 17-18°C. Removal of Na and Ca ions was compensated with equiosmolar amounts of Tris+. When external chloride was substituted, the impermeable anion methanesulfonate was used. The osmolality was 1,000 mosmol. All external solutions contained 1 mM cyanide (CN) and 300 nM tetrodotoxin (TTX). Radioactive solutions were made by adding solid 46CaCl2 (15-30 mCi/mg; New England Nuclear, Boston, MA) to the internal solutions. Radioactive samples were collected at either 1- or 4-min intervals, mixed with 5 ml of scintillation solution, and counted in a liquid scintillation counter for times long enough to give standard errors of ~1%.

RESULTS

Membrane Potential Dependence
A series of experiments was carried out to explore the effects of solutions of altered ionic composition on the voltage dependence of the Na+-dependent Ca efflux (forward Na/Ca exchange). In order to improve axon survival under voltage-clamp and dialysis conditions, it was necessary to use internal and external solutions prepared with large, nonpermeating anions and cations to increase the membrane resistance. This procedure minimizes the amount of current passing through the axon membrane.

Fig. 2A shows the results obtained with an axon dialyzed with an internal solution that contained 280 mM K, 30 mM tetraethylammonium (TEA), 4 mM Mg, 40 mM Na, and 100 μM Ca2+, and was initially bathed in ASW containing CN and TTX. Under these conditions, and with the membrane clamped at a holding potential value of ~60 mV (near the axon’s own resting potential), a...
steady state Ca efflux of ~2.1 pmol·cm⁻²·s⁻¹ was obtained. Hyperpolarization to ~85 mV caused a rapid increase in Ca efflux to 3.5 pmol·cm⁻²·s⁻¹, which was largely reversible upon returning the membrane potential to ~60 mV. After changing the external medium to one with 0 Na, 0 Ca, the efflux fell to ~0.1 pmol·cm⁻²·s⁻¹. Under these conditions, membrane hyperpolarization to ~85 mV was without effect, whereas the addition of 10 mM Ca_o caused a small increase in the efflux of Ca, probably because of Ca/Ca exchange. These results,

confirmed in several other experiments (see Fig. 6), indicate that under these conditions most of the total Ca efflux and its voltage-sensitive component can be attributed to Na-dependent Na/Ca. Fig. 2B shows a similar experiment in which the axon was dialyzed with a “high-resistance” internal solution (K⁺ replaced by NMG⁺) and bathed in a medium prepared with methanesulfonate as the main anion. Under these conditions and before voltage-clamping, the axon resting potential rapidly (15 min) decreased to ~0 mV. For this reason, the membrane was clamped at this value. The steady Ca efflux of 2.7 pmol·cm⁻²·s⁻¹ obtained at this potential was rapidly increased to 4.3 pmol·cm⁻²·s⁻¹ after a step membrane change to ~40 mV. A membrane potential change of the same magnitude but in the opposite direction caused a decrease in the efflux to ~2.0 pmol·cm⁻².
The results indicate that these solution changes and, perhaps more importantly, the change in the basal membrane potential level do not appear to modify substantially the membrane potential sensitivity of the exchange mechanism. It should be mentioned that the apparent rectification of the system observed in the positive potential region could be due in part to the current-passing electrode behavior (polarization).

Fig. 3 shows a graph obtained with six different axons, which demonstrates that in the membrane potential range between 0 and −80 mV, the Ca efflux measured during the first 5 min after the potential change (and expressed relative to the efflux value at zero membrane potential) increases by 37% per each 25-mV membrane hyperpolarization. Table I summarizes the voltage dependence of the Na$_{\text{v}}$-dependent Ca efflux obtained with 23 different axons under several experimental conditions.

**Effect of Internal Na and ATP**

Fig. 4 shows an experiment in which an axon was dialyzed first with a solution prepared without Na and ATP. Under this condition, a steady Ca efflux of 3.0 pmol·cm$^{-2}$·s$^{-1}$ was obtained at a holding potential of 0 mV. Hyperpolarization to −40 mV produced a small increase in the Ca efflux to 3.4 pmol·cm$^{-2}$·s$^{-1}$, which amounts to a change of −8% per 25 mV. After reversing this effect by returning the membrane potential to 0 mV, the internal medium was changed to one containing 40 mM Na. This caused a transient decrease in the Ca efflux, which later increased to a value of −2.6 pmol·cm$^{-2}$·s$^{-1}$. A subsequent hyperpo-
**TABLE I**

*Effect of Membrane Potential Changes on the Na-dependent Ca Efflux: Influence of Na, Ca, and ATP*

| Axon   | Na  | Ca  | ATP | $V_1$ | $V_2$ | $F(V_1)/F(V_2)$ | Change/25 mV |
|--------|-----|-----|-----|-------|-------|-----------------|--------------|
| 081184 | 90  | 0.1 | 0   | 0     | -40   | 1.5             | 32           |
| 121184 | 40  | 0.1 | 0   | 1     | -40   | 2.0             | 42           |
| 270384 | 40  | 0.21| 0   | 0     | -40   | 1.73            | 36           |
| 240384 | 40  | 0.68| 0   | 0     | -40   | 1.35            | 21           |
| 230384 | 40  | 0.89| 0   | 0     | -40   | 1.19            | 12           |
| 250385A| 25  | 5   | 0   | -55   | -85   | 1.22            | 18           |
| 250385B| 55  | 5   | 0   | -55   | -85   | 1.53            | 44           |
| 240385 | 55  | 5   | 0   | -55   | -10   | 0.65            | -19          |
| 090385 | 40  | 10  | 0   | -60   | -20   | 0.63            | -25          |
| 090385 | 40  | 10  | 0   | -60   | -80   | 1.42            | 53           |
| 070385 | 40  | 20  | 0   | -60   | 0     | 0.52            | -20          |
| 220385 | 40  | 20  | 0   | -60   | -20   | 0.41            | -37          |
| 101185 | 40  | 60  | 0   | -58   | -80   | 1.32            | 36           |
| 281185 | 40  | 60  | 0   | -54   | -75   | 1.38            | 45           |
| 210385 | 40  | 100 | 0   | -60   | -85   | 1.65            | 65           |
| 011283A| 30  | 200 | 0   | 0     | -40   | 1.69            | 43           |
| 011283B| 30  | 200 | 0   | 0     | -25   | 1.25            | 25           |
| 071283 | 30  | 200 | 0   | -10   | -50   | 1.52            | 33           |
| 301185 | 50  | 200 | 0   | -52   | 0     | 0.68            | -16          |
| 081283 | 40  | 200 | 0   | 0     | -40   | 1.73            | 45           |
| 081283 | 40  | 200 | 2   | 0     | -40   | 1.54            | 34           |
| 270384 | 40  | 200 | 0   | 0     | -40   | 1.58            | 36           |
| 280284 | 40  | 230 | 0   | 0     | -40   | 1.56            | 35           |
| 280284 | 40  | 230 | 0   | 0     | -40   | 0.82            | -11          |
| 290284 | 40  | 230 | 1   | 0     | -40   | 1.60            | 38           |
| 290284 | 40  | 230 | 1   | 0     | -70   | 2.17            | 42           |
| 250384 | 40  | 230 | 0   | 0     | -40   | 1.48            | 30           |
| 141084 | 40  | 200 | 1   | 0     | -40   | 1.70            | 44           |
| 161084 | 90  | 200 | 0   | 0     | -40   | 1.65            | 41           |
| 181084 | 33  | 200 | 0   | 0     | -40   | 1.60            | 38           |

Mean ± SEM = 36.8±3*  
Mean ± SEM = 7.4±1*  

* The mean was computed with the absolute value of the percent change.
Polarization to -40 mV caused the Ca efflux to increase to 5.0 pmol·cm⁻²·s⁻¹, which amounts to a 42% change per 25 mV. A further hyperpolarization to -75 mV caused a still larger increase in Ca efflux, which was not sustained but appeared to decay with time. This "inactivation" effect will be discussed in more detail later.

Fig. 5 shows the effect of different internal Na concentrations on the voltage sensitivity of the Na⁺-dependent Ca efflux. Because of the well-known inhibitory effect of Na⁺ on the forward Na/Ca exchange, it is important to obtain stable baselines of Ca efflux at each Na⁺ for a proper analysis of the fractional change in Ca efflux induced by a constant membrane hyperpolarization. In the absence of Na⁺ and with the axon clamped at 0 mV, a basal efflux of 2.4 pmol·cm⁻²·s⁻¹ was obtained. A 40-mV hyperpolarization caused the efflux to rise to 2.7 pmol·cm⁻²·s⁻¹ (5% change per 25 mV). Raising the internal sodium to 10 mM caused a decrease in the efflux to 1.6 pmol·cm⁻²·s⁻¹, which later increased to 1.9 pmol·cm⁻²·s⁻¹. The same hyperpolarization caused the efflux to rise to 2.6 pmol·cm⁻²·s⁻¹ (23% per 25 mV change). A further increase in internal Na to 90 mM caused the efflux to drop to a steady level of 0.6 pmol·cm⁻²·s⁻¹ (31% change

![Figure 4](image-url)
per 25 mV). Finally, lowering internal Na to 33 mM stimulated the efflux to 1.3 pmol·cm⁻²·s⁻¹, which was further increased to 1.83 pmol·cm⁻²·s⁻¹ with membrane hyperpolarization (27% change per 25 mV). The observation that internal Na markedly influences the fraction of the Na⁺-dependent Ca efflux that senses a membrane potential change is exemplified in Fig. 6. The graph presents the percent increment in Ca efflux caused by a 40-mV hyperpolarization as a function of Naᵢ. The continuous line fits the results obtained with six different axons, each represented by a different symbol. The broken line shows the inhibition of Ca efflux caused by internal Na in a single axon.

The results of several experiments of this type are summarized in Table 1. In agreement with the results shown in Figs. 4–6, it appears that the membrane potential sensitivity of the Na⁺-dependent Ca efflux is greatly dependent on the presence of internal Na since, in its absence, a 25-mV step potential change increases the Ca efflux by only 7%. On the other hand, in the presence of Naᵢ (20–40 mM), the relative voltage sensitivity of the Ca efflux increases up to 40% (per 25 mV potential change), showing a $K_{1/2}$ for Naᵢ of 5 mM and reaching saturating levels at ~30–40 mM. This indicates that the maximal internal Na effect is achieved near physiological concentrations of this cation (20–30 mM).

It is well known that ATP activates the Na/Ca exchange by modifying the affinity of the carrier for Na and Ca (Baker and Glitsch, 1975; DiPolo, 1974). The possibility that ATP may affect the voltage sensitivity of the Na⁺-dependent Ca efflux was also investigated. Fig. 7 shows an experiment in which step changes in membrane potential were tested in the absence and presence of both internal Na and ATP. In the absence of internal Na and ATP, membrane potential

![Figure 5](image-url)
**FIGURE 6.** Percent increment in Na-dependent Ca efflux induced by a test hyperpolarizing pulse (-40 mV) as a function of the Na concentration. Each symbol represents a different axon. The open circles show the inhibition in the Ca efflux by Na in a single axon. The $K_0$ for the Na effect is 5 mM.

**FIGURE 7.** The effect of ATP on the voltage sensitivity of the Na-dependent Ca efflux. Notice the stimulation of the Ca efflux caused by ATP, and the large increment in the magnitude of the Na-dependent Ca efflux component induced by the hyperpolarizing test pulse.
changes to $-40$ and $+30$ mV from a holding value of 0 mV caused only minor changes in the Ca efflux from its basal value of 1.5 pmol·cm$^{-2}$·s$^{-1}$ (<7% per 25 mV potential change). The addition of 30 mM Na to the internal medium caused an inhibition in the efflux of Ca to 0.5 pmol·cm$^{-2}$·s$^{-1}$, and a hyperpolarization to $-40$ mV caused the efflux of Ca to increase to 0.8 pmol·cm$^{-2}$·s$^{-1}$ (38% change per 25 mV). After reversing the membrane potential effect, the addition of 2 mM ATP caused an increase in the basal Ca efflux to 2.0 pmol·cm$^{-2}$·s$^{-1}$ as a result of the activation of both the Na$_{v}$-dependent Ca efflux and the uncoupled Ca efflux (Ca pump; DiPolo and Beaugé, 1984). Under these new conditions, hyperpolarization to $-40$ mV increased the Ca efflux to 3.0 pmol·cm$^{-2}$·s$^{-1}$ (31% per 25 mV, with a pump flux of 0.2 pmol·cm$^{-2}$·s$^{-1}$ subtracted; DiPolo and Beaugé, 1979). Note that the level of Ca efflux obtained in the presence of ATP (pump flux subtracted) is similar to that in the absence of Na$_{v}$, which confirms the finding that ATP has little effect on Na/Ca exchange in the absence of Na$_{v}$ (DiPolo, 1976).

The fact that the level of Ca efflux reached in the presence of ATP and during membrane hyperpolarization surpasses by almost 1 pmol·cm$^{-2}$·s$^{-1}$ the level of the flux in 0 Na$_{v}$ and 0 ATP indicates that the efflux of Ca is not saturated under the present conditions (high internal Ca and zero membrane potential). The same conclusion can be derived from the experiment of Fig. 4, in which, starting from similar baselines of Ca efflux, hyperpolarization in the presence of Na$_{v}$ increased the efflux above the value obtained in 0 Na$_{v}$. Another important feature of the voltage sensitivity of the Ca efflux is the effect of ATP. Fig. 7 shows that, in the presence of the nucleotide, the absolute increase in Ca efflux upon hyperpolarization is much greater than in its absence. Nevertheless, the relative voltage sensitivity fraction of the Na$_{v}$-dependent Ca efflux is the same in the presence and in the absence of ATP. The data indicate that, contrary to the effect of Na$_{v}$, ATP only affects the rate of carrier turnover, without changing its voltage sensitivity.

**Effect of Internal and External Ca**

The data of Table I and Fig. 8 show the effect of varying the internal ionized Ca upon the voltage sensitivity of the Na$_{v}$-dependent Ca efflux. The experiment shown in Fig. 8A was carried out to explore the effects of membrane potential changes in the presence of a physiological internal Ca concentration (0.06 μM). At this low Ca concentration, and with 40 mM Na and 2 mM ATP, a basal Ca efflux of $\sim$20 pmol·cm$^{-2}$·s$^{-1}$ is obtained. Hyperpolarization is practically without effect on the level of the Ca efflux. Under these conditions, external Na has little effect on the efflux level, which is expected to be due mainly to the operation of the ATP-driven Ca pump (DiPolo and Beaugé, 1979). This expectation is fulfilled by the effect of internal vanadate, which significantly reduces the efflux to a leakage level. In other experiments (not shown), in which the Ca$^{2+}$ was close to 0.1 μM, and with a measurable Na$_{v}$-dependent Ca efflux ($\sim$20% of total Ca efflux), the fractional increase in Ca efflux upon hyperpolarization was not different from that at high Ca$^{2+}$. In the experiment of Fig. 8B, the effect of membrane potential was explored at two different values of Ca$^{2+}$. In the presence of 0.2 μM Ca$^{2+}$, hyperpolarizing the membrane to $-40$ mV caused the
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Efflux to increase reversibly to a value equivalent to 39% per 25 mV change. In the second part of the experiment, the ionized Ca was increased to 200 µM, which caused the Ca efflux to increase to ~1,800 fmol·cm⁻²·s⁻¹, and membrane hyperpolarization to -40 mV increased the Ca efflux to 2,900 fmol·cm⁻²·s⁻¹, which is equivalent to 42% per 25 mV change in membrane potential, the same as that obtained with 1,000-fold less Ca²⁺. Fig. 8B also shows that the removal of external Ca caused only a slight change in Ca efflux, which indicates a small contribution of Ca/Ca exchange to the total Ca efflux. This point is confirmed by the large drop in Ca efflux that follows the removal of external Na. Finally, the experiment shows that in the absence of external Na and Ca, membrane hyperpolarization to -40 mV has practically no effect on the “leak” level of Ca efflux.

It is known that the forward and reverse modes of the Na/Ca exchange are affected in opposite directions by membrane potential changes (Mullins, 1977). If the two unidirectional fluxes were electrically coupled, it might be thought that the elimination of the reverse Na/Ca exchange could be responsible for the internal Na effect. This point was explored by measuring the voltage sensitivity of the Ca efflux in the absence of external Ca. Fig. 9 shows an experiment in

**Figure 9.** The effect of external Ca on the sensitivity of the Ca efflux to membrane potential. Unless otherwise stated, all concentrations are in millimolar.

**Figure 8.** (opposite) The effect of internal ionized Ca concentration on the voltage sensitivity of the Na,-dependent Ca efflux component. (A) Ca efflux at a low 0.06 µM Ca²⁺. (B) Ca efflux at 0.2 and 200 µM Ca²⁺. Notice the change in the efflux scale after changing internal Ca from 0.2 to 200 µM. Filled circles: efflux into ASW; open squares: efflux into Na⁻–0 Ca; open triangles: efflux into 0 Ca⁻–0 Na; filled squares: efflux into 0 Na⁻–10 Ca. The experiment shows that practically all the Ca efflux is dependent on the presence of external Na.
which an axon was dialyzed with a medium containing 40 mM Na, 200 μM Ca, and no ATP. At a membrane potential of 0 mV, a Ca efflux of 1.8 pmol·cm⁻²·s⁻¹ was obtained in the presence of 10 mM external Ca, which was not substantially modified after washing out the external Ca. Under this condition, a 40-mV hyperpolarization raised the efflux to 2.2 pmol·cm⁻²·s⁻¹. The same increase was obtained after restoring the external Ca, which indicates that the voltage sensitivity of the carrier is not affected by eliminating the reversal mode of the Na/Ca carrier mechanism.

**INT**

**OK-40Na-4Mg-230μM Ca²⁺-OATP**

**EXT**

**No-SW-CN-TTX**

**FIGURE 10.** The effect of prolonged membrane potential changes on calcium efflux. The axon was predialyzed for 50 min before the isotope was added to the dialysis medium. In this experiment, the isotope collection period was 3 min, and a second voltage electrode was inserted in the axon to check for spatial and temporal potential uniformity along the axon. The axon was initially clamped at 0 mV (filled circles), and then hyperpolarized to -50 and -60 mV (open circles). During the decay of the Ca efflux observed while the axon was hyperpolarized, the membrane potential did not suffer temporal modifications along the segment of the axon lying in the collecting chamber.

**Inactivation of the Potential Effect**

It was mentioned above that after a step potential change, the corresponding change in Ca efflux was not sustained but decreased slowly toward a new steady level in spite of the maintained membrane potential change. An indication of this behavior is given in Fig. 4, and a clearer demonstration of it is given in Fig. 10. In this type of experiment, a second internal potential electrode was posi-
tioned in the axon at the border of the guard region and moved along the collecting compartment to explore whether longitudinal potential nonuniformities, after a step potential change, could be associated with the decline in the potential-stimulated Ca efflux. As mentioned in the Methods, the dimensions of the chamber did not allow perfect longitudinal control of the membrane potential; thus, the membrane potential at the ends of the chamber differed by a few millivolts from the potential at its center. For hyperpolarizing voltages, the end of the collecting region was within 5% of the center, but for depolarizing potentials, it could deviate as much as 20%. When the potential was stepped to a new value, the voltage recorded with the second electrode changed to a lower level but thereafter remained constant during the pulse. From this and similar measurements, it can be safely concluded that the decline in the Ca efflux during a step potential change is not due to potential nonuniformities derived from the current-passing electrode behavior. Fig. 10 demonstrates that after a step potential change to -50 or -60 mV, Ca efflux is not sustained but declines roughly in an exponential fashion to a new steady level. Both the peak and the steady state level appear to be potential-dependent. The decay from one level to another occurs with a time constant on the order of minutes. The rate constant of decay is apparently also potential-dependent. In the experiment of Fig. 10, the rate constant of decay is 0.09 min\(^{-1}\) for the pulse to -50 mV and 0.17 min\(^{-1}\) for the pulse to -60 mV. In other similar experiments, rate constants of the same magnitude were obtained; this indicates a wide scatter, which could explain the absence of inactivation behavior when relatively small potential changes were applied, as in the experiment of Fig. 2.

Kinetics of Voltage Activation

A series of experiments was carried out to study the kinetics of activation of the potential-dependent component of the exchange system, which is responsible for Ca extrusion. In order to compare the relative effectiveness of hyperpolarizing pulses of different durations, it is necessary to consider the interval between pulses, which, together with the pulse duration, determines the pulsing duty cycle. Fig. 11 shows some examples of experiments of this type. Part A shows the results obtained with one axon, dialyzed with an internal solution containing 0 K, 40 mM Na, 0 ATP, and 230 \(\mu\)M Ca. The axon was clamped at a holding potential of 0 mV and pulsed to -60 mV, first with a train of 3-ms pulses at 15-ms intervals (duty cycle, 0.17), and later, after a resting period, with a train of 30-ms pulses at 150-ms intervals (duty cycle, 0.17). It is clear that the stimulation of the Ca efflux is very similar in spite of the 10-fold difference in pulse duration. This similarity was confirmed in the last part of the experiment, in which the axon was pulsed continuously with the two different pulse patterns. Part B shows the results obtained with another axon, under the same experimental conditions, pulsed to -40 mV, first with 6-ms, then with 30-ms, and finally with 10-ms pulses, using the same duty cycle (0.5). In spite of the smaller hyperpolarization, the efflux stimulation is larger than that shown in A, because of the larger duty cycle value. Again, at constant duty cycle, there appeared to be no difference in the effectiveness of pulses between 6 and 30 ms duration. Finally, part C shows the results of one experiment in which the axon was pulsed from 0 to -40 mV,
with 20-ms pulses at 20-ms intervals (duty cycle, 0.5), and immediately after held steady at −40 mV, which is equivalent to a duty cycle of 1. The results demonstrate that the steady hyperpolarization is almost twice as effective as the 0.5 duty cycle pulsing. The small discrepancy could be due to the slow inactivation process, previously described, that appears during the steady but not during the pulsed hyperpolarization.

The results shown above indicate that voltage activation occurs at least as rapidly as 3 ms, and that the important parameter during pulsing experiments of this type is the duty cycle. Fig. 12 demonstrates more clearly the effect of
Figure 12. Effect of hyperpolarizing pulses of different duty cycle and similar duration on the Na<sub>x</sub>-dependent Ca efflux. Inset: the increment in the Na<sub>x</sub>-dependent Ca efflux as a function of the duty cycle. Each symbol represents a different axon.
duty cycle variation using a constant pulse duration, of 1 ms in this case. For this experiment, the axon was dialyzed with a solution containing 90 mM Na, 230 μM Ca, and 0 ATP, and was tested successively with trains of 1-ms pulses at intervals of 1 (duty cycle, 0.5), 19 (duty cycle, 0.05), 9 (duty cycle, 0.1), and, again, 1 ms. The efflux stimulation appears to be proportional to the duty cycle value. This conclusion is better demonstrated in the inset of Fig. 12, which shows a graph in which the increase in Ca efflux is plotted against the duty cycle value. The ordinate on the left shows the increase in Ca efflux expressed in pico-moles per square centimeter per second, and the ordinate on the right shows the fractional increase in Ca efflux normalized for a duty cycle of 0.5. The black circles represent the results of the experiment shown in the main part of the figure, and the triangles and the squares show the results obtained with two other axons. Similar results were obtained using the same protocol but increasing the test pulse to 10 and 30 ms.

**DISCUSSION**

The purpose of the present work was to measure the voltage dependence of the Na-dependent Ca efflux under a variety of experimental conditions, using a combination of two techniques: intracellular dialysis and the voltage clamp. In order to increase axon survival, and therefore obtain reliable steady state isotope effluxes, it was necessary to substitute all the internal K+ with large organic cationic species. This procedure causes a large increase in membrane resistance, thus decreasing the amount of current supplied by the electronic system during a maintained potential change. Recently, it has been reported that K ions from the cytoplasmic side are partially required for the activation of the Na-dependent Ca efflux in squid axons (DiPolo and Rojas, 1984). In these experiments, the decrease in Ca efflux caused by the lowering of K was compensated by using larger Ca²⁺ concentrations. In any case, the observations reported in this work concerning the voltage dependence of Na/Ca exchange were found not to depend on the presence of internal K ions (see Fig. 1).

In agreement with previous findings in squid axons (Blaustein et al., 1974; Mullins and Brinley, 1975; Baker and McNaughton, 1976), the Na-dependent Ca efflux was found to be voltage-dependent. In the range between −80 and 0 mV, the steady state Ca efflux measured during the first 5 min is potential-dependent (see Fig. 3) and changes by ~40% per 25 mV potential change relative to the flux at 0 mV. This value is not different from that reported in axons dialyzed with Na and high Ca (≥100 μM) and hyperpolarized with an axial current-passing wire (Mullins and Brinley, 1975) or depolarized either with high K or externally applied veratridine (Blaustein et al., 1974). In the positive region of potential (+40 mV), our results show less voltage sensitivity, which could be due in part to inadequate space clamp at positive potentials (see Methods). From the simple relationship between Na-dependent Ca efflux and membrane potential, little can be inferred about the exact stoichiometry of the Na/Ca exchange system. In fact, depending on the assumptions of the model (location within the membrane of the energy barrier for translocation; presence of an electroneutral fraction of the Na-dependent Ca efflux), different coupling ratios can be
obtained. Experiments in which Na influx and Ca efflux are measured in the same axon are in progress in our laboratory to clarify this point.

We have reported a marked dependence of the voltage sensitivity of the Na\textsubscript{0}-dependent Ca efflux on internal Na. In the complete absence of Na\textsubscript{0}, the Ca efflux changes by 7% when the membrane potential is changed by 25 mV as compared with 40% in the presence of Na\textsubscript{0}. This reduction in the potential dependence occurs in spite of the fact that, in the absence of Na\textsubscript{0}, a much larger Na\textsubscript{0}-dependent Ca efflux occurs (see Figs. 5 and 7). The amount of internal Na needed to promote half of the effect is only 5–8 mM, which indicates that under normal physiological conditions for Na\textsubscript{0}, the voltage-dependent fraction of the Ca efflux is fully activated. Furthermore, the “site” at which Na ions are promoting this effect has a much higher affinity than that at which Na inhibits the Na\textsubscript{0}-dependent Ca efflux (\(K_{Na} = 35\) mM; Requena, 1978). The way in which internal Na ions modulate the voltage sensitivity of the Ca efflux is not clear yet. Possible explanations for this finding are that either the stoichiometry of the exchange is affected by the binding of Na to the carrier (making it nearly electroneutral in the absence of Na\textsubscript{0}), or that the removal of Na\textsubscript{0} promotes an electroneutral Na\textsubscript{0}-dependent Ca efflux component, without affecting the potential-dependent fraction. The latter possibility would imply the existence of two different populations of carriers. If this were the case, one would expect a constant potential-dependent fraction of the Na\textsubscript{0}-dependent Ca efflux independent of the level of Na\textsubscript{0}. The experiments of Figs. 4, 5, and 7 seem to rule out this latter hypothesis. In fact, in the presence of internal Na, the Na\textsubscript{0}-dependent Ca efflux that senses the membrane potential increases not only in its fractional value, but also in its absolute magnitude. This suggests that the effect of Na ions is probably due to a direct effect on the voltage sensitivity of the Na/Ca carrier rather than to the appearance of new carriers working in an electroneutral fashion when Na\textsubscript{0} is removed.

Since in exploring the effect of Na\textsubscript{0}, there is a substantial variation in the reversal of the exchange (Na\textsubscript{0}-dependent Ca influx), an important question to clarify is whether this situation affects the voltage sensitivity of the forward exchange. This could be so, since in the presence of Na\textsubscript{0}, together with the voltage-sensitive component of the Na\textsubscript{0}-dependent Ca efflux, there is an opposite (Na\textsubscript{0}-dependent Ca influx) electrogenic component (Mullins and Requena, 1981). The results of experiments on the voltage sensitivity of the Na\textsubscript{0}-dependent Ca efflux carried out in the virtual absence of external Ca strongly argue against this hypothesis. In fact, in the absence of Ca\textsubscript{0} (no reversal of the Na/Ca exchange), the voltage sensitivity of the forward Na/Ca exchange remains unaffected.

It is well known that in the absence of Na\textsubscript{0}, ATP has little if any effect on the Na\textsubscript{0}-dependent Ca efflux, which suggests that ATP activates the exchange by releasing the Na\textsubscript{0} inhibition (change in affinity of Na\textsubscript{0} site; DiPolo, 1976; Requena 1978; DiPolo and Beaugé, 1984). On the other hand, the effect of ATP on the voltage sensitivity of the exchange system shows that the nucleotide increases the rate of the forward Na/Ca exchange without affecting its voltage dependence. The “site” at which Na\textsubscript{0} modifies the potential sensitivity of the exchange is not affected by ATP, as is the case for the inhibition of Ca efflux by Na\textsubscript{0}, which suggests that there are two apparently different Na sites.
The other obvious parameter that could influence the voltage sensitivity of the \( \text{Na}_c \)-dependent Ca efflux is the level of the internal ionized Ca. Our observation that varying the intracellular Ca from nearly physiological values (0.1 \( \mu \)M; see Table 1) up to 200 \( \mu \)M apparently does not affect the relative voltage sensitivity of the forward Na/Ca exchange suggests that, at least in this range, the stoichiometry of the exchange is independent of the work to be performed during Ca transport. In this regard, \( \text{Ca}_i \) acts like ATP by increasing the rate of the carrier, apparently without affecting its voltage sensitivity. In conclusion, the present experiments indicate that neither the levels of \( \text{Ca}_i \) nor ATP nor \( \text{K}_i \) affect the relative voltage sensitivity of the \( \text{Na}_c \)-dependent Ca efflux.

The effect of potential changes upon the \( \text{Na}_c \)-dependent Ca efflux is not sustained, but decays slowly and exponentially with time. As already pointed out, this effect cannot be due to a failure of the space clamp far from the center of the chamber, which is derived from polarization properties of the current-passing wire (see Results). A most attractive explanation for this behavior is that Na/Ca exchange undergoes a very slow (minutes), inactivation-like process after a prolonged step potential change. At this stage, we can only speculate about this mechanism. For instance, the conformational change of a charged macromolecule induced by a change in the electric field across the membrane could expose groups that may interact with other sites on one side of the membrane to stabilize the new conformation. This in turn would decrease the number of carriers available to exchange Na for Ca.

The demonstration that the Na/Ca exchange mechanism can undergo a slow inactivation process brings about an important question: is there also a fast inactivation of the Na/Ca carrier? This point is crucial, since otherwise the magnitude of the \( \text{Na}_c \)-dependent Ca efflux measured during a prolonged potential change would be underestimated and therefore the measured voltage sensitivity of the process would appear smaller. This matter was examined using trains of voltage-clamp pulses. The following results were obtained: (a) no inactivation in the voltage-dependent, \( \text{Na}_c \)-dependent Ca efflux was observed during the first 30 ms; (b) the Na/Ca exchange system activates with hyperpolarizing pulses as fast as 1 ms, and is completely activated after 3 ms. The first result indicates that the increase in Ca efflux during a train of hyperpolarizing pulses is only a function of the duty cycle time and is not related to the interval between the pulses. The lack of a fast inactivation of the exchange system will increase the possibility of measuring the current associated with the electrogenic exchange since voltage-clamp pulses of hundreds of milliseconds' duration can be given without signs of inactivation. The second result is interesting because it shows that activation of the Na/Ca exchange can occur during the time scale of most voltage signals present in excitable tissues. These two experimental findings can be taken as evidence that Na/Ca exchange, like the gated Ca channel, can contribute to Ca entry during periods of time comparable to the duration of the action potential.

To summarize, we have found that the voltage sensitivity of the \( \text{Na}_c \)-dependent Ca efflux is affected by the level of intracellular Na ions and is independent of the level of Ca\(^{2+}\) and ATP. The studies on the kinetic parameters of the Na/Ca
exchange system indicate a fast activation followed by a slow inactivation process. While the origins of this behavior remain uncertain, its study will contribute to the further characterization of the Na/Ca countertransport system.

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