Asymmetrical Properties of the Na-Ca Exchanger in Voltage-clamped, Internally Dialyzed Squid Axons Under Symmetrical Ionic Conditions

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ABSTRACT   In this work we have investigated whether the asymmetrical properties of the Na/Ca exchange process found in intact preparations are intrinsic to the exchange protein(s) or the result of the asymmetric ionic environment normally prevailing in living cells. The activation of the Na/Ca exchanger by Ca^{2+} ions, monovalent cations, ATPγS and the effect of membrane potential on the different operational modes of the exchanger (Na_o/Ca_i, Ca_o/Na_i, Ca_o/Ca_i, and Na_o/Na_i) was studied in voltage-clamped squid giant axons externally perfused and internally dialyzed with symmetrical ionic solutions. Under these conditions: (a) Ca ions activate with higher affinity from the inside ($K_{1/2}$ = 22 μM) than from the outside ($K_{1/2}$ = 300 μM); (b) experiments measuring the Ca_o-dependent Ca efflux in the conditions Li_o-$Tris_i$, Li_o-Li_i, $Tris_o$-$Tris_i$, and $Tris_o$-Li_i, show that the activating monovalent cation site on the exchanger faces the external surface; (c) ATPγS activates the Ca_o-dependent Ca efflux (Ca_o/Ca_i exchange) only at nonsaturating $[Ca^{2+}]_i$. Its effect appears to be on the Ca transport site since no alteration in the apparent affinity of the activating monovalent cation site was observed. The above results show that the Na/Ca exchange process is indeed a highly asymmetric transport mechanism. Finally, the voltage dependence of the components of the different exchange modes was measured over the range of +20 to −40 mV. The voltage dependence (~26% change/25 mV) was found to be similar for all modes of operation of the exchanger except Na_o/Na_i exchange, which was found to be voltage insensitive. The sensitivity of the Ca_o/Ca_i exchange to voltage was found to be the same in the presence and in the complete absence of monovalent cations. This finding does not support the proposition that the voltage sensitivity of the Ca_o/Ca_o exchange is induced by the binding and transport of an external monovalent cation.

INTRODUCTION

Because of the physiological implications of the Na/Ca exchanger a vast amount of literature has evolved in recent years on the characterization of this plasma mem-

brane Ca transport mechanism (Mullins, 1981; Reeves, 1985; Baker and Allen, 1986; Rasgado-Flores and Blaustein, 1986; DiPolo and Beaugé, 1988). Studies in large cells such as squid axons and barnacle muscle fibers and in membrane vesicle preparations have demonstrated that the Na/Ca exchange is a complex carrier mechanism subjected to regulatory processes including modulation by Ca_i^{2+}, ATP, and acidic phospholipids (Caroni and Carafoli, 1983; Philipson et al., 1983; DiPolo and Beaugé, 1986).

An interesting problem that has become apparent from comparison studies is that membrane vesicle preparations do not seem to exhibit properties similar to those found in intact cells. First, Na/Ca exchange in vesicles appears to be symmetrical with respect to affinity for Ca^{2+} ions at both sides of the membrane (Reeves, 1985), which is in marked contrast to the high degree of kinetic asymmetry for Ca^{2+} found in intact cells (Wakabayashi and Goshima, 1981; Baker and Allen, 1986; DiPolo and Beaugé, 1988), and second, the large increase in the affinity of the Na/Ca exchanger towards Na and Ca ions induced by ATP in living cells has been difficult to demonstrate in membrane vesicles (Reeves, 1985; however, see Caroni and Carafoli, 1983). In fact, based on kinetic measurements of Ca transport in a single subpopulation of vesicles (inside-out), Philipson and Nishimoto (1982) conclude that the Na/Ca exchange mechanism is indeed asymmetric with respect to its affinity for Ca^{2+} ions. They also found that hydrogen ions had identical effects on this system both in inside-out and mixed populations of sarcolemmal vesicles, an observation they consider to support the proposal that the Ca-binding site on the carrier is similar at the two faces of the membrane. Although the nature of this discrepancy (symmetry vs. asymmetry) is still uncertain, Baker and Allen (1986) have suggested that the apparent symmetry of the exchanger in vesicles may reflect a component that is either missing or modified (proteolysis?) during membrane isolation. As an alternative to this explanation, Philipson and Nishimoto (1982) have pointed out that during an in vivo experiment the extra- and intracellular sides of the plasma membrane are exposed to different ionic environments, which could determine the asymmetric behavior of the Na/Ca exchanger that is intrinsically symmetric.

In the present work we have investigated (a) whether asymmetrical features of the Na/Ca exchanger that prevail in intact squid axons (different affinity for Ca_i and Ca_o, activation by intracellular ATP) are preserved under symmetrical ionic conditions, and (b) using similar intra- and extracellular solutions we have explored the location of the monovalent cation site and the potential dependence of all the different modes of operation of the Na/Ca exchange. Our results provide conclusive evidence that the Na/Ca exchanger is a highly asymmetric carrier system whose properties, as found in intact cells, are inherent to the exchange protein(s) and that they are not the consequence of manipulations of the ionic environments.

MATERIALS AND METHODS

The experiments were carried out with live specimens of the tropical squid *Loligo plei* at the Instituto Venezolano de Investigaciones Científicas, Caracas, Venezuela, and of the squid *Loligo pealei* at the Marine Biological Laboratory, Woods Hole, MA. After cleaning, the axon was mounted in a chamber designed for dialyzing and voltage clamping the axons (see DiPolo et al., 1985 for details). Dialysis capillaries were of hollow regenerated cellulose fibers with a

molecular cutoff of 9,000 D (150 μm o.d., 141 μm i.d.; Spector, Los Angeles, CA). For axons smaller than 400 μm, cellulose acetate capillaries (130 μm o.d., 100 μm i.d.; Fabric Research, MA) were used (DiPolo, 1977). The dialysis capillary contained a 75-μm platinized platinum wire (20% iridium) to pass current. In all experiments the axons were predialyzed for at least 45 min with an isotope-free internal dialysis medium containing no ATP or Ca (see Table I).

Solutions

The external and internal solutions used in these experiments are listed in Table I. External solutions always contained 1 mM cyanide and 200 nM tetrodotoxin (TTX). For the Na efflux experiments, ouabain (5 × 10⁻⁴ M) and bumetanide (10 μM, kindly supplied by Hoffmann La Roche Inc., Nutley, NJ) were also added to the external medium to block the Na/K pump and the Na/K/Cl cotransport. As shown in Table I, Na, Li, or Tris (or N-methyl-D-glucamine, NMG) solutions were used both as internal and external media. To obtain different monovalent cation concentrations, Na and Li solutions were mixed with Tris (or NMG). Similar results were obtained with Tris or NMG as cation substitutes. In some experiments, 50 mM of internal and external Tris (NMG) was replaced by tetraethylammonium ion (TEA). When Mg ions were removed from any of the experimental solutions they were replaced isosmotically

TABLE I
Composition of Experimental Solutions

External and internal solutions	NaCl	LiCl	TrisCl	TrisMOPS	MgCl₂	EGTA-Tris	Glycine
Na	100	—	100	185	20	1-2	285
Li	—	200	—	185	20	1-2	285
Tris	—	—	200	185	20	1-2	285

Ca²⁺ was added as CaCl₂ salt, replacing isosmolar amounts of glycine. The pH was 7.5 at 17°C for both internal and external solution. Osmolarity was 1,000 mosmol/kg. All concentrations are in millimolar.

with glycine. When submicromolar concentration of Ca²⁺ were used, a CaEGTA dissociation constant of 0.15 μM was used (DiPolo et al., 1976). The pH of both internal and external solutions was matched at 7.5 (17°C) and the osmolarity at 1,000 mosmol/kg.

After the membrane potential stabilized between −10 and +5 mV, the axons were voltage clamped at 0 mV. The leakage current was measured with 20-mV hyperpolarizing pulses. Membrane resistance of axons bathed in these solutions ranged from 2,000 to 4,000 Ω.cm². Since in some experimental protocols Ca fluxes were measured during steady (6-min) changes in the membrane potential (from −40 to +40 mV), it was important to eliminate the possibility that the holding current during the steady membrane depolarization was damaging the axons. For this purpose, axons were internally dialyzed and externally perfused with symmetrical ionic solutions, voltage clamped at 0 mV, and their membrane resistances were measured with a 20-mV hyperpolarizing pulse. Subsequently, the holding potential was changed first to −40 and then to +40 mV and the membrane resistance was measured again. In five different axons the mean membrane resistance was 2,750 Ω.cm² and did not change significantly over the range between −40 and +40 mV, thus indicating that under our experimental conditions no rectification of the current-voltage relationship is observed. This behavior is what should be expected on the basis of (a) the absence of K ions in our experimental solutions and (b) the loss of K conductance after prolonged exposure to K-free media (Bezanilla et al., 1986).

All reagents used were of analytical grade. Adenosine-5'-0-(3-thiophosphate) (ATPγS) was purchased from Boehringer Mannheim (GmbH, Mannheim, FRG). Radioactive solutions were made by adding solid $^{45}CaCl_2$ or $^{22}NaCl$ (New England Nuclear, Boston, MA). In most experiments radioactive samples were collected at either 2- or 3-min periods, mixed with 5 ml of scintillation liquid and counted in a liquid scintillation counter.

RESULTS

Kinetics of Activation of Ca_o-dependent Ca Efflux by $[Ca^{2+}]_i$ and $[Ca^{2+}]_o$ under Symmetrical Ionic Conditions

To measure the apparent affinity of the Ca site(s) from both extra- and intracellular faces of the membrane each axon was used as its own control. In the experiment illustrated in Fig. 1, the axon was predialyzed for 40 min with a Li solution (see
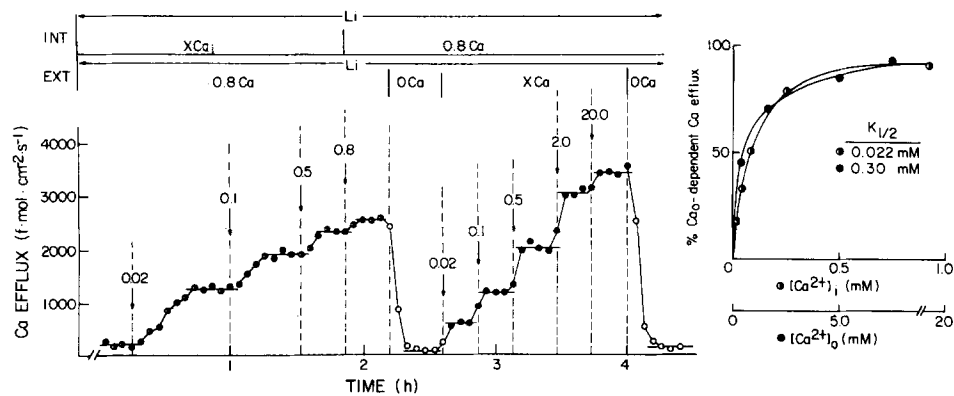

FIGURE 1. The effect of $[Ca^{2+}]_i$ and $[Ca^{2+}]_o$ on Ca efflux under symmetrical ionic conditions. (*Ordinate*) Ca efflux in fmol·cm^{-2} · s^{-1}. (*Abscissa*) Time in hours. All concentrations are in millimolar. The correspondence between symbols and ionic composition is the following: ◒, variable $[Ca^{2+}]_i$ and constant $[Ca^{2+}]_o$ (0.8 mM); ●, variable $[Ca^{2+}]_o$ and constant $[Ca^{2+}]_i$ (0.8 mM); ○, in the absence of Ca_o. The arrows indicate additions of ionized Ca to the internal or external medium. The time periods between the dashed lines correspond to changes in the ionized Ca_i or Ca_o concentrations. (*Inset*) Activation of Ca_o-dependent Ca efflux by Ca_o and Ca_i in this particular experiment. The lines through the points were drawn by eye. To obtain the $K_{1/2}$ values the Ca effluxes at 0.8 mM Ca_i and 20 mM Ca_o were taken as 100%.

Table I) without added Ca, and bathed in a similar medium containing 0.8 mM $[Ca^{2+}]_o$ (1 mM total EGTA + 1.8 mM total Ca). Additions of different amounts of $[Ca^{2+}]_i$ cause progressive increases in Ca efflux reaching saturation at a value near 1 mM. That the increase in Ca efflux is Ca_o-dependent is clearly demonstrated by the large drop in the efflux level upon removal of Ca_o. In the second part of the experiment a nearly identical protocol was used to measure the activation of Ca efflux by $[Ca^{2+}]_o$ at a constant internal ionized Ca of 0.8 mM. Clearly, larger $[Ca^{2+}]$ are required from the outside than from the inside to fully activate the Ca_o-dependent component. The inset of Fig. 1 shows a plot of the activation of the Ca/Ca exchange component by Ca_i and Ca_o ions for this particular experiment. The $K_{1/2}$ for internal

Ca (~0.022 mM) is an order of magnitude smaller than that for external Ca (0.3 mM).

Fig. 2 summarizes the results of different experiments in which the apparent affinity of the Na/Ca exchange (operating in its Ca/Ca exchange mode) towards Ca_i and Ca_o was determined by saturating $[Ca^{2+}]$ at both sides of the membrane ($Ca_i = 0.8$ mM, $Ca_o = 20$ mM).

Effect of Li_o and Li_i on the Ca_o-dependent Ca Efflux

In intact cells, Ca_o-dependent Ca efflux (Ca/Ca exchange) and Ca_o-dependent Na efflux (reverse exchange) are activated by several external monovalent cations including Li, K, and Cs (Baker and McNaughton, 1976; Blaustein, 1977; Philipson
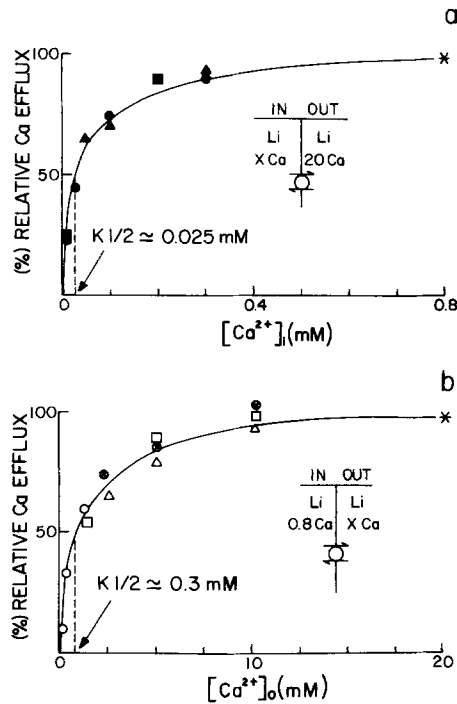

FIGURE 2. Activation of Ca_o-dependent Ca efflux by internal and external ionized Ca in symmetrical Li solutions. Each symbol represents a different axon. In these experiments steady-state efflux was measured at different Ca_i and Ca_o while the *trans* Ca^{2+} concentration was kept constant at a saturating concentration (0.8 mM for the internal and 20 mM for the external side). A mean value of 25 μM was obtained for the activation of Ca efflux by $[Ca^{2+}]_i$, and 300 μM was found for the activation by $[Ca^{2+}]_o$. The mean maximum level of Ca efflux attained under saturating conditions was 2,872 fmol·cm^{-2} · s^{-1}. Temperature was 17°C.

and Nishimoto, 1981; Slaughter et al., 1983). Whether this activating site is present on both sides (symmetric) of the carrier or only on the external surface (asymmetric) remains to be explored. In isolated membrane preparations this question is difficult to explore due to the presence of mixed vesicle populations (inside out and right side out).

The differential effects of Li_o, Li_i, $Tris_o$, and $Tris_i$ on the Ca_o-dependent Ca efflux were studied in several axons at nonlimiting internal and external $[Ca^{2+}]$ and with the *trans* membrane potential held at 0 mV (see Table II). Fig. 3 shows a typical experiment whereas Table II summarizes the results of several experiments. With Tris on the inside and Li on the outside, Ca efflux reached a steady value of ~1,300 fmol·cm^{-2}·s^{-1}. Replacing external Li with Tris causes a marked drop in the efflux

The Effect of Internal and External Cations on Ca Efflux in Dialyzed and Voltage-clamped Squid Axons

Axon reference	Internal Tris$^+$	Li$^+$	Ca^{2+}	External Tris$^+$	Li$^+$	Ca^{2+}	Ca efflux
		mM			*mM*		*fmol·cm^{-2}·s^{-1}*
070587A	—	200	0.2	—	200	20	2016
080587A	—	200	0.2	—	200	20	2450
080587B	—	200	0.2	—	200	20	2520
110587A	—	200	0.2	—	200	20	2670
110587B	—	200	0.2	—	200	20	2360
120587B	—	200	0.2	—	200	20	2300
130587A	—	200	0.2	—	200	20	1910
140587A	—	200	0.2	—	200	20	3150
180587A	—	200	0.2	—	200	20	3480
180587B	—	200	0.2	—	200	20	2680
200587A	—	200	0.2	—	200	20	2530
Mean ± SEM							2551 ± 139
070587A	200	—	0.2	—	200	20	1250
080587A	200	—	0.2	—	200	20	1500
110587A	200	—	0.2	—	200	20	1550
180587B	200	—	0.2	—	200	20	1560
Mean ± SEM							1465 ± 73
130587C	—	200	0.2	200	—	20	170
210587A	—	200	0.2	200	—	20	160
Mean							165
070587A	200	—	0.2	200	—	20	163
130587B	200	—	0.2	200	—	20	138
140587B	200	—	0.2	200	—	20	145
Mean ± SEM							149 ± 7
070587A	—	200	0.2	—	200	0	30
110587A	—	200	0.2	—	200	0	32
110587B	—	200	0.2	—	200	0	34
120587B	—	200	0.2	—	200	0	25
130587A	—	200	0.2	—	200	0	40
180587A	—	200	0.2	—	200	0	20
180587B	—	200	0.2	—	200	0	28
200587A	—	200	0.2	—	200	0	26
Mean ± SEM							29.4 ± 2
110587A	—	200	0.2	200	—	0	28
130587C	—	200	0.2	200	—	0	20
Mean							24
070587A	—	200	0.2	—	200	0	35
130587B	—	200	0.2	—	200	0	30
140587B	—	200	0.2	—	200	0	27
Mean ± SEM							30.6 ± 2.34

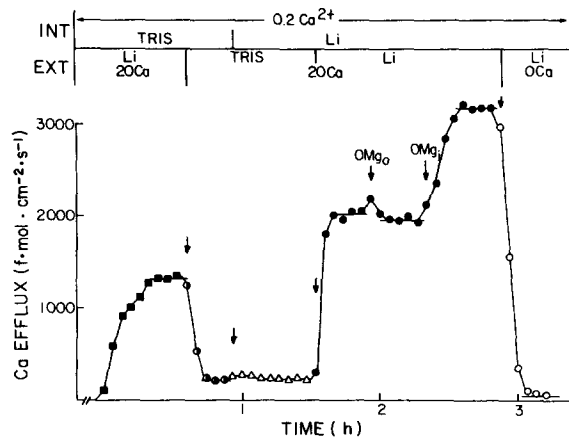All external solutions contained 200 nM TTX and 1 mM cyanide. Mean temperature was 17.1°C.

FIGURE 3. The effects of Li_o and Li_i on the Ca_o-dependent Ca efflux. All concentrations are in millimolar. The arrows indicate changes in the internal or external medium. The correspondence between symbols and ionic composition is the following: ■, $Tris_i$-Li_o; ◓, $Tris_i$-$Tris_o$; △, Li_i-$Tris_o$; and ●, Li_i-Li_o in the presence of 20 mM Ca_o; ○, the external solution is Ca free. Notice that external Mg has no effect on the level of Ca efflux while internal Mg is inhibitory.

of Ca to a value close to 160 $fmol \cdot cm^{-2} \cdot s^{-1}$. Under these conditions addition of internal Li (in the absence of external Li) had no effect on the level of Ca efflux. Readdition of Li_o increased the Ca efflux to 2,000 $fmol \cdot cm^{-2} \cdot s^{-1}$. At the end of the experiment, upon complete removal of Ca_o, the efflux dropped to leak values (5–10 $fmol \cdot cm^{-2} \cdot s^{-1}$). Two important points should be mentioned in these experiments: first, in the absence of external Li internal Li does not activate Ca_o-dependent Ca efflux, and second, nevertheless, Ca_o-dependent Ca efflux is higher in the presence of Li on both sides of the membrane.

Fig. 4 illustrates a case in which the effects of internal and external Li were explored in more detail. In $Tris_i$-Li_o solutions Ca efflux is ~1,500 $fmol \cdot cm^{-2} \cdot s^{-1}$ and increases to 2,450 $fmol \cdot cm^{-2} \cdot s^{-1}$ in Li_i-Li_o solutions. In the second part of the experiment four different Li_o concentrations were used to activate Ca efflux. No sign of saturation was observed up to 200 mM. As shown at the end of the run, under the present conditions all Ca efflux is dependent on Ca_o. Another point worth mentioning is that the level of Ca efflux attained in the absence of Ca_o (see
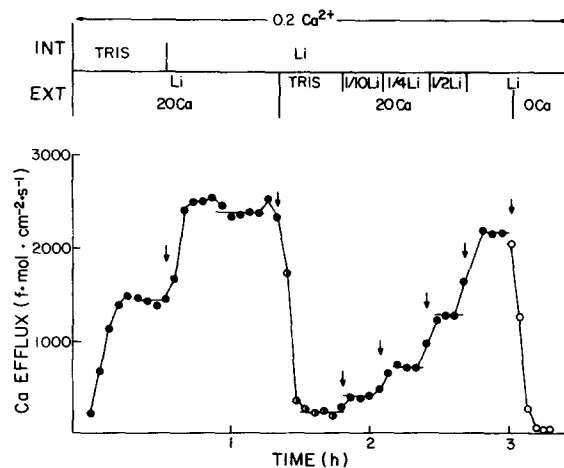

FIGURE 4. The effects of different Li_o concentrations on the Ca_o-dependent Ca efflux. All concentrations are in millimolar. The arrow indicate changes in the external or internal medium. Axon diameter, 525 μm.

Table II) is significantly smaller than that observed at saturating Ca_o without any internal or external monovalent cation ($Tris_i$-$Tris_o$ configuration). This indicates that the Ca/Ca exchange mode has no absolute requirement for an external monovalent cation, although its magnitude is considerably reduced in its absence. Fig. 5 summarizes the activation of Ca_o-dependent Ca efflux (Ca/Ca exchange) by external Li in four different axons. Clearly, Li_o activates the external cation site with low apparent affinity ($K_{1/2}$ of ~100 mM). This value is quite similar to that reported in intact axons, indicating that neither the asymmetric ionic composition of intact axons nor the value of the membrane potential seem to affect the affinity of the external monovalent cation site.

Effect of ATPγS on Both Divalent and Monovalent External Cation Sites

Allen and Baker (1986a) have shown that the apparent affinity of the Na/Ca exchanger for Ca_o increases dramatically in the presence of a monovalent cation (Li, K). In the following experiments we have explored whether ATP affects (a) the monovalent external cation site and (b) the Ca_o/Ca_i exchange mode. To avoid activa-
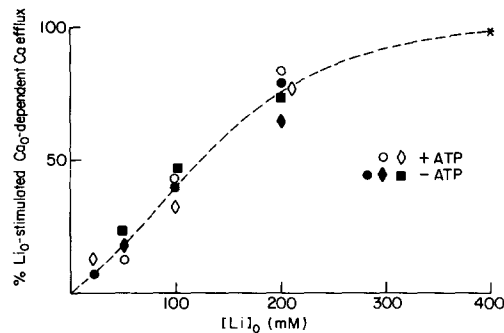

FIGURE 5. Activation of Ca_o-dependent Ca efflux by external Li ions in the presence and absence of ATP. Different symbols represent different axons. Notice the absence of ATP effect on the apparent affinity of the activating monovalent cation site.

tion of the Ca pump by ATP, the ATP analogue ATPγS was used (DiPolo and Beaugé 1987a). In these experiments axons were dialyzed from the beginning with the standard Li solution (see Methods) free of ATP, and bathed in a similar medium containing 20 mM Ca_o. Fig. 6 shows two such experiments in which the effect of 1 mM ATPγS was explored at two different $[Ca^{2+}]_i$. At submicromolar Ca_i (0.5 μM, see Fig. 6 a), Ca_o-dependent Ca efflux amounts to ~28 fmol·cm^{-2} · s^{-1}, which increased to 60 fmol·cm^{-2} · s^{-1} when 1 mM ATPγS was added to the dialysis medium. In contrast, at a saturating $[Ca^{2+}]_i$ of 200 μM (see Fig. 6 b), Ca_o-dependent Ca efflux was not affected by the addition of 1 mM ATPγS. This lack of effect of ATPγS on the Ca_o/Ca_i exchange component agrees with a similarly reduced effect of ATP on the Na_o-dependent Ca efflux (forward Na/Ca exchange) at saturating $[Ca^{2+}]_i$ (Blaustein, 1977).

Internal ATP is known to increase the affinity of the exchanger towards Na_o and Ca_i ions (Baker and Glitsch, 1973; DiPolo, 1974; Blaustein, 1977; DiPolo and Beaugé, 1987b). To determine whether or not a similar effect exists for the external monovalent cation site of the exchanger, Li_o activation curves of the Ca_o-dependent Ca efflux component were carried out with and without ATP. The results shown in

Fig. 5 demonstrate that the nucleotide (ATP or ATPγS) does not seem to modify the affinity of the external monovalent site towards the alkali metal ion.

Effect of Membrane Potential on the Ca and Na Efflux Components of the Na/Ca Exchange in Symmetrical Li, Tris, and Na Solutions

Early work in injected squid axons (Baker and McNaughton, 1976) and in dialyzed axons (Blaustein et al., 1974; Mullins and Brinley, 1975), showed that Na_o-dependent Ca efflux is voltage sensitive in accord with the idea that the Na/Ca exchange is an electrogenic process. In the next series of experiments we have explored (a) what partial reactions of the Na/Ca exchanger are voltage sensitive, and (b) how they are affected by the membrane potential. To assure identical monovalent cation concen-
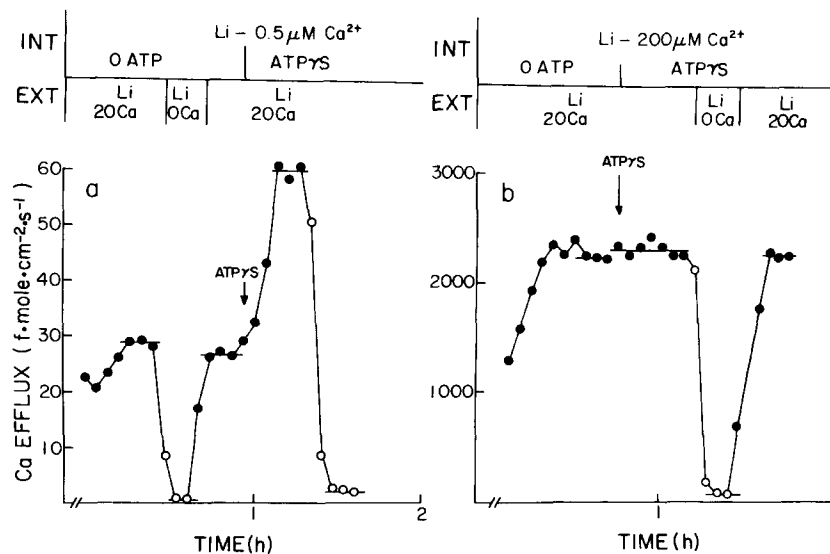

FIGURE 6. Effect of ATPγS on Ca_o-dependent Ca efflux in the presence of symmetrical Li solutions (a) at 0.5 μM Ca_i and (b) at 200 μM Ca_i. All concentrations are in millimolar except Ca_i, which is in micromolar. Axon diameter, 450 μm.

trations at each side of the membrane we have used symmetrical Na, Li, and Tris solutions (see Methods).

Fig. 7 illustrates two different experiments in which Ca_o-dependent (in Li and Tris solutions) Ca effluxes were measured in the range between −40 and +40 mV (axons were initially clamped at 0 mV). It is evident (Fig. 7, a and b) that although the magnitudes of the Ca_o-dependent Ca efflux in Li_o-Li_i or $Tris_o$-$Tris_i$ media are quite different, their sensitivity to changes in membrane potential are similar. That the levels of Ca efflux observed under these conditions reflect an exchange for external Ca is confirmed by the low levels of Ca efflux reached upon removal of Ca_o. This is what should be expected since no ATP was included in the dialysis medium and therefore no ATP-dependent uncoupled Ca pump is present (DiPolo and Beaugé, 1979). Fig. 8 shows a similar experimental protocol, but one in which
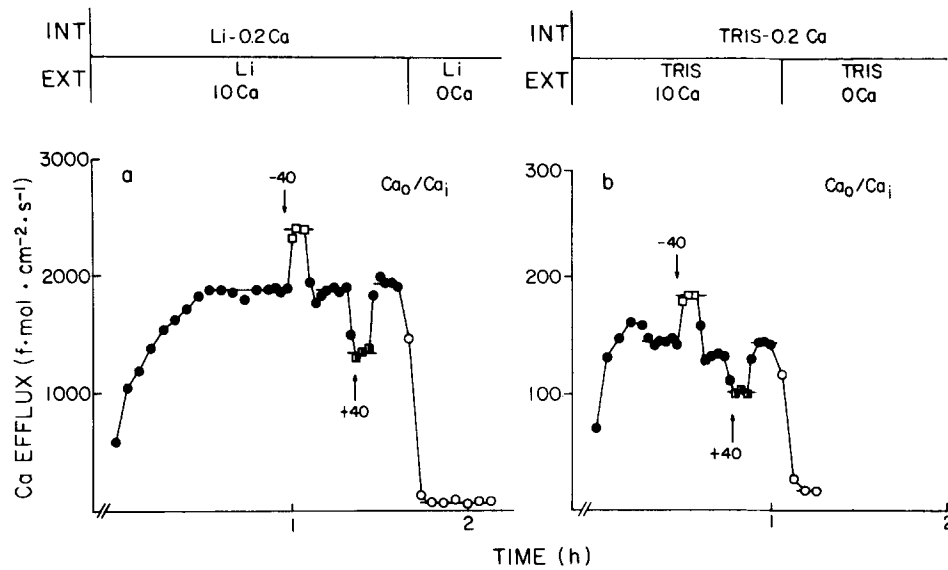
FIGURE 7.   Effects of steady membrane potential changes on Ca efflux in (a) symmetrical Li and (b) symmetrical Tris solutions. The arrows indicate the step changes in membrane potential from a steady value of 0 mV. Notice the difference in the flux scales in Li and Tris solutions.

the Na_o-dependent component of the Ca efflux was measured (in Na solutions) at different steady values of membrane potential and under identical internal and external ionic conditions (including $[Ca^{2+}]$). In the last portion of Fig. 8 it is shown that substitution of external Na for Li brings the Ca efflux to "leak" values, which indicates that, under these conditions all efflux is Na_o-dependent. The relatively low levels of Na_o-dependent Ca efflux, even at a saturating internal Ca_i, reflects the
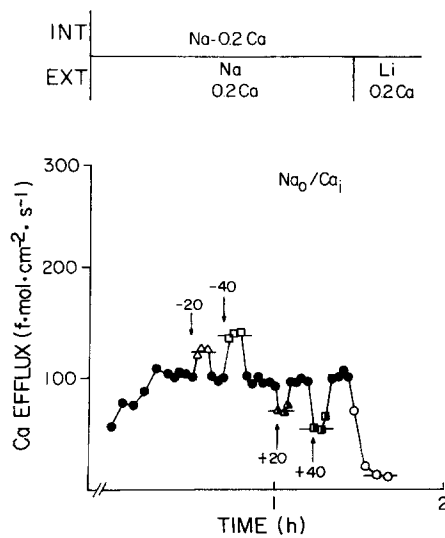

FIGURE 8.   Effects of steady membrane potential changes on Ca efflux in symmetrical Na solutions. The arrows indicate the step changes in membrane potential from a steady value of 0 mV.

inhibitory effect of internal Na (200 mM) on this exchange component since it increased to the expected values when Na_i was removed (not shown).

In another series of experiments we have used Na efflux to study the voltage dependence of the Ca_o/Na_i (reverse) and Na_o/Na_i exchanges. Fig. 9 shows that in an axon dialyzed from the beginning with ATP-free 50 mM Na, 50 mM TEA, 0.3 mM Ca, and in the absence of Na_o and Ca_o, Na efflux reaches "leak" values (<1 pmol·cm^{-2}·s^{-1}, indicating that under the above conditions Na ions are not being transported through the exchanger. The addition of 2 mM Ca_o produces a significant rise in Na efflux (reverse Na/Ca exchange). As expected (Allen and Baker, 1986a), a hyperpolarization of 40 mV reversibly decreases the Ca_o-dependent Na efflux. At zero membrane potential a further addition of 50 mM Na_o causes a much
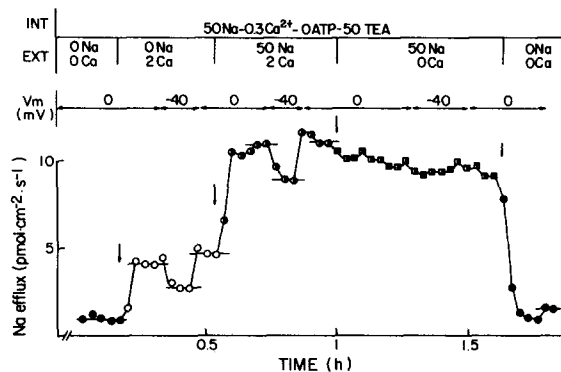

FIGURE 9. Effect of membrane potential on Ca_o- and Na_o-dependent Na efflux. (*Ordinate*) Na efflux in pmol·cm^{-2}·s^{-1}. (*Abscissa*) Time in hours. All external solutions contained TTX, ouabain, cyanide, and bumetanide. Notice the low level ("leak") of Na efflux in the absence of both external Na and Ca. The arrows indicate changes in the external medium. The axon was voltage clamped at zero membrane potential. A voltage test pulse of −40 mV was maintained long enough (6 min) to attain a steady flux. Different symbols are used to indicate different external solutions. All concentrations are in millimolar. Notice that only the Ca_o-dependent component is affected by changes in membrane potential. Axon diameter, 600 μm.

larger increase in Na efflux (Na/Na exchange through the exchanger; see DiPolo and Beaugè, 1987b). Interestingly, hyperpolarization to −40 mV causes a similar drop in Na efflux as in the absence of Na_o. The lack of effect of membrane potential on the Na_o-dependent Na efflux becomes evident in the last part of the experiment in which Na efflux in the absence of Ca_o is unaffected by hyperpolarization. Finally, at the end of the experiment it is shown that upon substitution of Li_o for Na_o, Na efflux returns back to "leak" values.

Fig. 10 *a* summarizes the results of several experiments on the voltage dependence of the Na_o/Ca_i and Ca_o/Ca_i exchanges. Over the range of membrane potential examined the voltage sensitivity of Ca efflux is the same for the Na_o/Ca_i and

Ca_o/Ca_i exchange modes. The curve relating to Ca_o- (reverse) and Na_o-dependent Na efflux (Na_o/Na_i exchange) components to changes in membrane potential is illustrated in Fig. 10 b.

During the course of these present experiments it was interesting to explore whether the apparent affinity of the Na/Ca exchanger for Na_o and Ca_o could be influenced by membrane potential. One of the advantages of the dialysis technique is that these experiments can be performed using each axon as its own control. Consequently, we measured the steady-state levels of Ca efflux at each Na_o or Ca_o concentrations in an axon voltage clamped at 0 mV and after a steady hyperpolarization to -50 mV. Fig. 11 shows two such experiments. These and three other similar ones
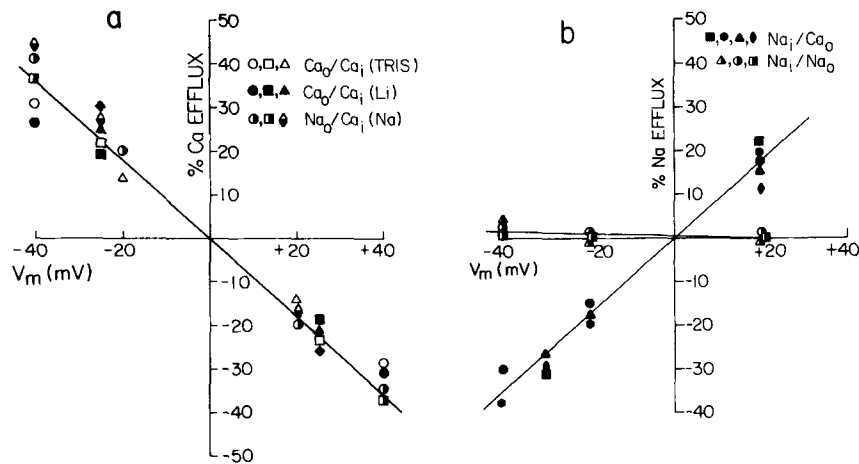

FIGURE 10. Influence of membrane potential on the different components of the Na/Ca exchange from 16 different axons. (a) Na_o/Ca_i and Ca_o/Ca_i exchanges; (b) Na_i/Ca_o and Na_i/Na_o exchanges.(●, ■, ▲) Ca_o-dependent Ca efflux (Ca_o/Ca_i exchange in symmetrical Li media; (O, □, △) Ca_o-dependent Ca efflux in symmetrical Tris solutions. (◓, ◨, ◈) Na_o-dependent Ca efflux (forward exchange) in symmetrical Na solutions (■, ●, ▲, ♦) Ca_o-dependent Na efflux (reverse exchange) in symmetrical Li solutions; (▲, ◓, ◨) Na_o-dependent Na efflux (Na_o/Na_i exchange) in symmetrical Na solutions. Each symbol represents a different axon. Notice that all modes of operation of the Na/Ca exchange except the Na_o/Na_i exchange are voltage sensitive.

indicate that the $K_{1/2}$ for the activation of Ca efflux by Na_o or Ca_o does not change significantly over the range of membrane potential investigated.

DISCUSSION

It has been recently stressed that vesicles and reconstituted membrane preparations do not display some of the properties of the Na/Ca exchange present in intact cells, in particular its asymmetric characteristics (Baker and Allen, 1986; DiPolo and Beaugé, 1988). The main purpose of this work was to clarify whether the asymmetric properties of the Na/Ca exchange are intrinsic to the exchange protein(s), or secondary to differences in the ionic conditions normally prevailing across the

plasma membrane. In order to do so, it was necessary to set up experimental protocols that allow one to measure the activity of the Na/Ca exchanger under conditions resembling experiments in isolated membrane vesicles. We have investigated the different operational modes of the Na/Ca exchanger (Na_o/Ca_i, Ca_o/Na_i, Ca_o/Ca_i, and Na_o/Na_i) in axons externally superfused and internally dialyzed with identical ionic solutions and subject to membrane potential control. Under these particular conditions it has been possible to examine: (a) the apparent affinity for $[Ca^{2+}]_o$ and $[Ca^{2+}]_i$ ions, (b) the sidedness of the activating monovalent cation site, and (c) the voltage dependence of the operational modes of the Na/Ca exchange.

Affinity of the Exchanger for Ca²⁺ Ions under Symmetrical Ionic Conditions

The data presented in this paper provide clear evidence that the marked difference in the affinity of the Na/Ca exchanger for Ca_i and Ca_o observed in living cells is an
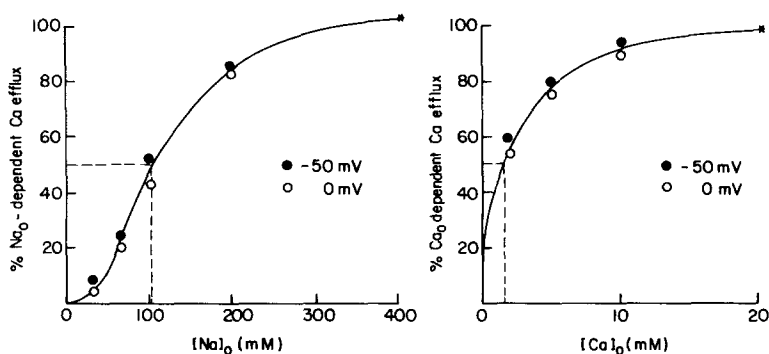

FIGURE 11. Na_o and Ca_o activation curves of the Na_o and Ca_o-dependent Ca efflux at two different membrane potentials in two different axons. In these experiments the axons were dialyzed from the beginning with an internal dialysis medium containing 50 mM Na, 300 μM ionized Ca, and no ATP. With the axon voltage clamped at zero membrane potential the external medium was changed to one containing only 10 mM Na. After a steady Ca efflux was attained, a long enough test pulse of -50 mV was applied to reach a new steady flux. This procedure was repeated with 20, 50, 100, 220, and 440 mM Na_o. A similar general protocol was used for the activation of Ca efflux by Ca_o. Notice that the apparent affinity of the Na/Ca exchanger towards Na_o and Ca_o ions is the same at -50 and 0 mV.

intrinsic property of the exchanger. In fact, the experiments of Figs. 1 and 2 show that under symmetrical ionic conditions the exchanger displays a relatively high affinity Ca activation ($K_{1/2} = 25$ μM) at the cytoplasmic face and a relatively low affinity site ($K_{1/2} = 300$ μM) at the external surface. This finding rules out that differences in the ionic environment to which both membrane faces are expected in vivo are responsible for the asymmetry of the exchanger. Although the exact nature of the discrepancy between intact cells and isolated membrane preparations is still uncertain, Baker and Allen (1986) have proposed that this alteration in Ca affinity could reflect a component that is either lost or modified during membrane isolation.

Activation of the Na/Ca Exchange by Monovalent Cations

It is well known that external Li^+, K^+, or Rb^+ cannot substitute for Na in promoting Na_o/Ca_i or Na_o/Na_i exchange through the Na/Ca exchanger (Blaustein, 1977; Baker, 1978; DiPolo and Beaugé, 1988), in contrast with their marked stimulation of Na_i/Ca_o and Ca_o/Ca_i exchanges (Allen and Baker, 1986). This suggests that the activating monovalent cation binding site is only generated when the carrier binds Ca from the outside (Reeves, 1986). An interesting question is whether there is a similar activating site at the inner side of the Na/Ca exchanger. Blaustein (1977) found that replacing internal K with tetramethylammonium ions inhibits Ca_o/Ca_i exchange, and suggested that both external and internal cation sites must be simultaneously loaded in order for Ca_o/Ca_i exchange to function (Blaustein, 1977, 1984). On the other hand, in dialyzed and voltage-clamped squid axons internal K ions per se activate all the modes of operation of the Na/Ca exchange (DiPolo and Rojas, 1984; DiPolo and Beaugé, 1988). This generalized effect of K_i^+ contrasts with that of K_o^+, which only activates the Ca_o-dependent fluxes.

TABLE III

The Effect of ATPγS on Ca_o-dependent Ca Efflux in Squid Axons Dialyzed under Symmetrical Li Solutions

Axon No.	$[Ca^{2+}]_i$	$[Ca^{2+}]_o$	Ca efflux	
			$-ATP\gamma S$	$+ATP\gamma S$
	μm	mM	$fmol \cdot cm^{-2} \cdot s^{-1}$	
120588A	0.7	20	48	86
	0.7	0	5	7
140588B	0.5	20	28	60
	0.5	0	4	5
120588B	200	20	2300	2420
	200	0	30	40
130588A	200	20	1910	2006
	200	0	45	38

Axons were dialyzed and externally superfused with the standard Li solution. All external solutions contained 200 nM TTX and 1 nM cyanide. The ATPγS concentration was 1 mM. Mean temperature, 17.5°C.

The results presented in Table II and Fig. 3 demonstrate that the activating monovalent cation site is present only at the external side of the membrane, thus constituting an additional asymmetrical feature of the Na/Ca exchange. The arguments proving this point are as follows. (a) In the absence of an internal alkali metal ion Ca_o/Ca_i exchange can be strongly activated by an external alkali metal ion (in $Tris_i$-Li_o solutions; Ca_o-dependent Ca efflux is 1,435 $fmol \cdot cm^{-2} \cdot s^{-1}$). (b) In the absence of an external alkali metal ion, Ca_o/Ca_i exchange can not be activated by an internal alkali metal ion (in Li_i-$Tris_o$ solutions; Ca_o-dependent Ca efflux is 165 $fmol \cdot cm^{-2} \cdot s^{-1}$). (c) Ca_o-dependent Ca efflux is the same in $Tris_i$-$Tris_o$ or Li_i-$Tris_o$ solutions, again showing that internal alkali metal ions do not activate Ca_o/Ca_i exchange unless the external monovalent cation site is loaded (Fig. 4 and Table II). Most probably this effect corresponds to an internal potassium-like effect of Li ions

on the Na/Ca exchanger. In addition, our results under symmetrical conditions agree well with those in intact injected squid axons, which show that Li_o activates Ca/Ca exchange with low affinity (see Fig. 5).

We have previously demonstrated that ATPγS, under physiological conditions, greatly activates Na_o/Ca_i, Na_i/Ca_o, and Na_o/Na_i modes of the Na/Ca exchange (DiPolo and Beaugé, 1986, 1987a). The results presented in Fig. 6 and Table III indicate that this is also the case for Ca_o/Ca_i exchange. The Na_o-dependent Ca efflux shows a small activation at saturating $[Ca^{2+}]_i$ (Blaustein, 1977). Likewise ATPγS has little effect on the Ca_o-dependent Ca efflux at high $[Ca^{2+}]_i$. The finding that ATPγS also activates the Ca_o/Ca_i exchange mode indicates that in squid axons phosphorylation of the exchanger always increases its turn over whatever mode of operation it is engaged in. An interesting finding related to the effect of ATP on the Ca_o/Ca_i exchange mode is that although ATP increases the turnover rate, it does not affect the affinity of the activating external monovalent cation site (Fig. 5). A possible explanation is that monovalent cations (K, Li, Rb) exert their effect at a different site than that which supports Na_o/Ca_i exchange.

Voltage Dependence of the Different Operational Modes of the Na/Ca Exchange

The flux-voltage relationship shown in Fig. 10 *a* for both the Na_o-dependent and Ca_o-dependent Ca efflux (-40 to $+40$ mV) can be fitted by a single straight line with a slope of ~26% increase in flux per 25 mV membrane potential hyperpolarization. On the other hand, the voltage dependence of the Ca_o-dependent Na efflux (reverse is almost identical in size but opposite in direction (see Fig. 10 *b*). This might be just a coincidence, but it could also mean that Na_o/Ca_i, Ca_o/Na_i, and Ca_o/Ca_i exchanges sense the potential changes with equal efficiency, suggesting a similar voltage-dependent mechanism for these exchange modes. The voltage sensitivities reported here are not different from those previously reported in injected (Allen and Baker 1986a, b) and dialyzed squid axons (DiPolo et al., 1985) under nonsymmetrical ionic conditions. An unexpected finding is that the Na_o-dependent Na efflux component (Na_o/Na_i exchange) is rather voltage insensitive between -40 and $+20$ mV (Figs. 9 and 10 *b*). At present we have no explanation for this observation.

Allen and Baker (1986b) have suggested that if the externally activating cation (Li, K, Rb) is also transported during Ca_o/Ca_i exchange it would add an extra charge to the carrier and therefore make it sensitive to changes in membrane potential. By the same line of thought a voltage-insensitive Ca/Ca exchange is to be expected in Tris or NMG where the monovalent cation site is unoccupied. Although measurements of rubidium fluxes in cardiac sarcolemmal vesicles indicate that alkali metal ions are not transported during the operation of the exchanger (Slaughter et al., 1983), data obtained in squid axons suggest that they might be transported (Allen and Baker, 1986a, b). More critical experiments are needed in squid axons to clarify this important point. Nevertheless, the experiments reported in this paper suggest that the voltage dependence of the Ca_o/Ca_i exchange (and probably of the other operational modes) is more likely to be an electric field effect on the exchanger itself (which affects the translocation rate) than an unbalance of charges induced by the binding and transport of an external alkali cation (see Figs. 7 and 10 *a*).

Although small in magnitude, Ca_o/Ca_i exchange in the complete absence of external and internal alkali metal ions is still voltage sensitive and shows a voltage dependence similar to that in symmetrical Li solutions. In addition, a similar voltage dependency was found in the absence of monovalent cations and in the presence of an inwardly directed Li gradient $(Tris_i-Li_o)$. Furthermore, the apparent affinities of the exchanger for the external transported site(s) are not affected by changes in the electric field across the membrane (Fig. 11). The simplest model that could explain the present voltage-clamp data is the existence of a voltage-dependent energy barrier that controls the translocation rate of the carrier and which is not greatly affected by the occupation of the external activating monovalent cation site.

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