

# Characterization of the Reverse Na/Ca Exchange in Squid Axons and Its Modulation by $Ca_i$ and ATP

## *Ca<sub>i</sub>-dependent Na<sub>i</sub>/Ca<sub>o</sub> and Na<sub>i</sub>/Na<sub>o</sub> Exchange Modes*

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**ABSTRACT** We have used dialyzed squid axons to characterize the ouabain- and bumetanide-insensitive Na efflux components and their relation to the operation of the Na/Ca exchange mechanism. In axons dialyzed with solutions containing nearly physiological concentrations of K, Na, and Mg, three components of the Na efflux can be distinguished:  $Ca_i$ -activated,  $Ca_o$ -dependent Na efflux ("reverse" Na/Ca exchange);  $Ca_i$ -activated,  $Na_o$ -dependent Na efflux; and  $Ca_i$ -independent, ATP-activated,  $Na_o$ -dependent Na efflux. We have studied the effects of internal alkalization, Mg,  $Ca_o$ , and the ATP analogue [ $\gamma$ -thio]ATP (ATP $\gamma$ S) on the different components of the Na efflux. The results show the following: (a) internal alkalization activates both  $Ca_o$ - and  $Na_o$ -dependent Na efflux components provided that  $Ca_i$  is present; (b) Mg<sub>i</sub> inhibits both the  $Ca_i$ -activated,  $Ca_o$ - and  $Na_o$ -dependent Na efflux components; (c)  $Ca_o$  inhibits the  $Na_o$ -dependent component by competition for a common site; (d) ATP $\gamma$ S activates both  $Na_o$ - and  $Ca_o$ -dependent Na efflux components only in the presence of  $Ca_i$ ; and (e) ATP activates the  $Na_i/Na_o$  and  $Na_i/Ca_o$  exchanges, causing a 10-fold increase in the affinity of the reverse Na/Ca exchange toward  $Ca_i$ . In the absence of  $Ca_i$ , ATP stimulates an  $Na_o$ -dependent Na efflux that is not affected either by internal alkalization or high  $Ca_o$ . The ATP analogue does not activate the  $Ca_i$ -independent Na/Na exchange system. These experiments demonstrate that the  $Ca_i$ -activated Na/Na exchange is a mode of operation of the Na/Ca exchange mechanism that substantially contributes to Na movement during the activation of the Na/Ca antiporter. The experimental evidence obtained on the  $Ca_i$ -independent Na/Na exchange component shows that this system is not part of the Na/Ca exchange.

### INTRODUCTION

The Na/Ca exchange mechanism is generally considered to be a carrier-mediated transport system in which the movement of Ca ions is coupled to reciprocal

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movement of Na ions. Furthermore, it is thought that, depending on the magnitude and direction of the electrochemical Na gradient, the Na/Ca exchange mechanism can induce net movements of Ca ions in or out of the cell (Mullins, 1977). Recent experiments in dialyzed squid axons have demonstrated that Ca entry through the  $\text{Ca}_o/\text{Na}_i$  exchange mechanism ("reverse" Na/Ca exchange) requires not only the presence of  $\text{Na}_i$  and  $\text{Ca}_o$  in order to operate, but also micromolar amounts of  $\text{Ca}_i$  (DiPolo, 1979; DiPolo and Beaugé, 1986). (In this article,  $\text{Ca}_i$  refers to the intracellular ionized calcium concentration.) This asymmetry in the activation of the exchange system by  $\text{Ca}_i$  could have important physiological implications, since the levels of  $\text{Ca}_i$  modulate the influx of Ca through the antiporter mechanism (positive feedback). This regulatory effect of  $\text{Ca}_i$  has recently been implicated in the inhibition of Ca influx via  $\text{Na}_i/\text{Ca}_o$  exchange induced by the Ca indicator quin2 (Allen and Baker, 1985), and it could also account for the  $\text{Ca}_i$  requirement of the outward current generated by the Na/Ca exchange in ventricular cells (Kimura et al., 1986).

Previous evidence obtained in dialyzed squid axons indicates that  $\text{Ca}_i$  activates not only a  $\text{Ca}_o$ -dependent Na efflux component ( $\text{Na}_i/\text{Ca}_o$  exchange), but also a sizable  $\text{Na}_o$ -dependent Na efflux component ( $\text{Na}_i/\text{Na}_o$  exchange) (DiPolo and Beaugé, 1986). When the possible modes of operation of the Na/Ca exchange system are studied, a further complication arises from the fact that ATP is able not only to activate these two components, but also to promote an  $\text{Na}_o$ -dependent Na efflux in the complete absence of  $\text{Ca}_i$  (Beaugé and DiPolo, 1981; DiPolo and Beaugé, 1986). Whether these ouabain-insensitive components of the Na efflux are modes of operation of the Na/Ca exchange system remains an open question. An analysis of the possible modes of operation of the exchange system and their magnitude under different experimental conditions is of critical importance when determining the number of Na ions exchanged for Ca (stoichiometry) during the operation of the exchange system.

In the present work, we have used different experimental procedures that are known to affect the Na/Ca exchange mechanism in squid axons, in order to characterize kinetically the ouabain-insensitive Na efflux components and to explore whether they are indeed modes of operation of the Na/Ca exchange system. Our results provide conclusive evidence that  $\text{Ca}_i$  and ATP activate not only the "reverse"  $\text{Na}_i/\text{Ca}_o$  exchange but also a sizable  $\text{Na}_i/\text{Na}_o$  exchange, which occurs during the turnover of this mechanism. Their similarities with respect to activation by internal alkalinization,  $\text{Mg}_i$  inhibition, and competition for a common external site are indicative of their similar origin. Interestingly, the Na/Ca exchange component activated by ATP in the absence of  $\text{Ca}_i$  (Beaugé and DiPolo, 1981) appears to be a different mechanism operating in parallel with the Na/Ca exchange.

#### MATERIALS AND METHODS

The experiments were carried out with live specimens of *Loligo pealei* at the Marine Biological Laboratory, Woods Hole, MA, and with the tropical squid *Loligo plei* at the Instituto Venezolano de Investigaciones Científicas, Caracas, Venezuela. After careful cleaning, an axon was mounted in a modified chamber for dialyzing and voltage-clamping the axons (DiPolo et al., 1985). An important modification is that 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 cut ends of the axon are separated from the central pool by two air gaps (DiPolo et al., 1985). Dialysis capillaries were from hollow regenerated cellulose fibers with a nominal molecular weight cutoff of 9,000 (150  $\mu\text{m}$  o.d., 141  $\mu\text{m}$  i.d., Spectrum, Los Angeles, CA). The dialysis capillary contained a 75- $\mu\text{m}$  platinized platinum wire (5% iridium). In most of the experiments, the axons were predialyzed for 1 h with an isotope-free standard dialysis medium containing no ATP and a given nominal concentration of ionized Ca. Since the axons in the present work do not need to be voltage-clamped during flux measurements, most of the axons were voltage-clamped for a short time (5–10 min) during the predialysis period and their leakage currents were measured with a 20-mV depolarizing pulse.

### *Solutions*

*Dialysis medium.* The standard dialysis solution had the following composition (millimolar): 310 K, 40 (*L. plei*) or 50 (*L. pealei*) Na, 4 Mg, in excess with respect to the ATP concentration, 30 Tris, 98 Cl, 310 aspartate, 1–3 EGTA, and 330 glycine, pH 7.3 (17.5°C). The osmolarity was adjusted to 998 mosmol/kg water. Removal of Na or Mg was compensated with equiosmolar amounts of Tris. In the experiments designed to measure the reversal of the Na/Ca exchange,  $\text{Na}_i$  was increased to a saturating value of 100 mM. The estimation of the ionized Ca was based on a dissociation constant of 0.15  $\mu\text{M}$  for CaEGTA (0.3 ionic strength; DiPolo et al., 1976) and 1.4 mM for CaATP (De Weer, P., personal communication). The estimation of the ionized Mg was based on a dissociation constant of 0.7 mM for MgATP (De Weer, P., personal communication) and 30 mM for MgEGTA (DiPolo et al., 1976). ATP (vanadium-free) was obtained from Sigma Chemical Co. (St. Louis, MO). Phosphoarginine at a concentration of 5 mM was usually added to the ATP-containing solution. Adenosine-5'-O-(3-thiophosphate) (ATP $\gamma$ S) was purchased from Boehringer Mannheim GmbH, Federal Republic of Germany. Na orthovanadate (from Fisher Scientific Co., Pittsburgh, PA) was prepared as a 100 mM solution.

*Artificial seawater.* The standard artificial seawater had the following composition (millimolar): 10 K, 440 Na, 10 Ca, 50 Mg, 10 Tris, 590 Cl, and 0.1 EGTA, pH 7.6 (17.5°C). The osmolarity was 1,000 mosmol/kg water. Removal of Na, Ca, or Mg ions was compensated with equiosmolar amounts of Tris. All external solutions contained 1 mM cyanide and 300 nM tetrodotoxin (TTX). In most of the experiments,  $5 \times 10^{-4}$  M ouabain and 10  $\mu\text{M}$  bumetanide (a gift of Dr. J. Russell) were added to the external medium to block the Na/K pump and the Na/K/Cl cotransport.

All reagents used were of analytical grade. Radioactive solutions were made by adding solid [ $^{45}\text{Ca}$ ]CaCl<sub>2</sub> (15–30 mCi/mg; New England Nuclear, Boston, MA) or [ $^{22}\text{Na}$ ]NaCl (641 mCi/mg). Radioactive samples collected at 4-min periods were mixed with 5 ml of scintillation liquid and counted in a liquid scintillation counter. Double-label experiments were carried out by dialyzing the axons with an internal medium containing  $^{24}\text{Na}$  and  $^{45}\text{Ca}$ . The collected radioactive samples were first counted for  $^{24}\text{Na}$  in a gamma counter. Na efflux values were corrected for radioactive decay. After complete decay of the  $^{24}\text{Na}$  activity, samples were counted for  $^{45}\text{Ca}$  activity in a liquid scintillation counter.

## RESULTS

### *Effect of Ionized Ca, and ATP, on the Ouabain- and Bumetanide-insensitive Na Efflux*

Reversal of the Na/Ca exchange in dialyzed squid axons is best studied by measuring the  $\text{Ca}_o$ -dependent Na efflux. Measuring the efflux of Na instead of

the influx of Ca to investigate the reverse mode of the Na/Ca exchange ( $\text{Na}_i/\text{Ca}_o$  exchange) has the advantage that any catalytic effect of  $\text{Ca}_i$  on the reversal of the Na/Ca exchange can be unambiguously ascribed to  $\text{Na}_i/\text{Ca}_o$  and not to  $\text{Ca}_i/\text{Ca}_o$  exchange. Fig. 1 shows the effect of  $\text{Ca}_i$  and ATP on the  $\text{Ca}_o$ - and  $\text{Na}_o$ -dependent Na efflux components. The Na/K pump and the Na/K/Cl cotransport were inhibited by ouabain ( $5 \times 10^{-4}$  M) and bumetanide ( $10^{-5}$  M), respectively. When the internal medium contained nominally zero  $\text{Ca}_i$  (3 mM total EGTA)

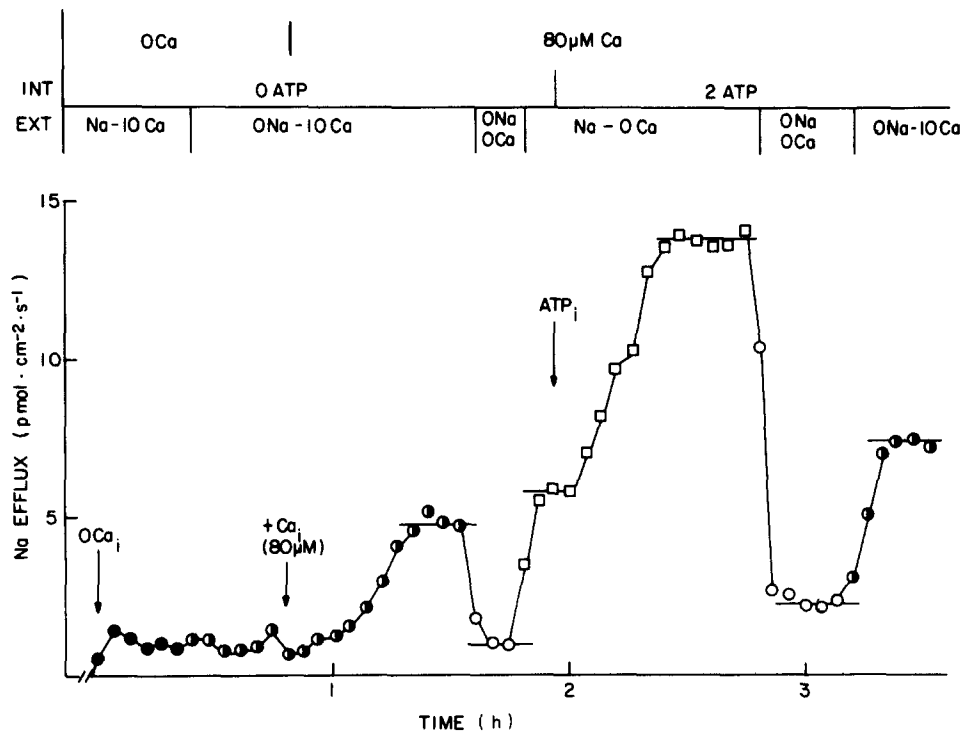


FIGURE 1. The effect of ionized  $\text{Ca}_i$  and ATP on the ouabain- and bumetanide-insensitive Na efflux. All concentrations are in millimolar except  $\text{Ca}_i$ , which is in micromolar. The arrows indicate changes in the internal medium. Different symbols are used to indicate different external solutions. All external solutions contained TTX, cyanide ouabain, and bumetanide. Axon diameter, 550  $\mu\text{m}$ .

and no ATP, the efflux of Na was rather small ( $<1 \text{ pmol} \cdot \text{cm}^{-2} \cdot \text{s}^{-1}$ ) and insensitive to changes in  $\text{Na}_o$ . Increasing  $\text{Ca}_i$  to 80  $\mu\text{M}$  caused an increase in the Na efflux that was completely sensitive to  $\text{Ca}_o$  since removal of  $\text{Ca}_o$  in the absence of  $\text{Na}_o$  brought the efflux back to the baseline. The long latency between the addition of 80  $\mu\text{M}$  Ca and the onset of the rise in the Na efflux is the consequence of the slow rise in  $\text{Ca}_i$  owing to washout of free  $\text{EGTA}_i$ . Addition of  $\text{Na}_o$  in the absence of  $\text{Ca}_o$  increased the Na efflux to the same level as that observed with  $\text{Ca}_o$  alone. This confirms previous findings that, in the absence of ATP and in the presence

of  $Ca_i$ , the  $Ca_o$ - and  $Na_o$ -dependent components of the Na efflux are about the same in magnitude (DiPolo and Beaugé, 1986). Under the above experimental conditions (full  $Na_o$ , no  $Ca_o$ ), addition of ATP further increased the  $Na_o$ -sensitive Na efflux. In this regard, it is of interest that the  $Ca_o$ -dependent Na efflux component was also increased by ATP. The increase in the  $Ca_o$ -dependent Na efflux induced by ATP was small compared with that of the  $Na_o$ -dependent component. Nevertheless, in a total of six different experiments, an ATP-stimulated,  $Ca_o$ -dependent Na efflux was always observed when ATP was added in the presence of  $Ca_o$  and in the absence of  $Na_o$ . These data support the idea that both  $Ca_i$  and ATP modulate the reversal of the Na/Ca exchange (see Fig. 11). Although the  $Ca_o$ -dependent Na efflux component observed in the presence of  $Ca_i$  (with or without ATP) is clearly a part of the Na/Ca exchange mechanism (reverse mode), it is unclear whether the  $Na_o$ -dependent Na efflux component observed in the presence of  $Ca_i$  is also a mode of operation of the Na/Ca exchange (Na/Na exchange mode) or a different parallel system. This is also true for the  $Na_o$ -dependent Na efflux component stimulated by ATP. The results presented in the following sections deal mainly with the analysis of these Na efflux components and their relation to the Na/Ca antiporter.

#### *Effect of Internal Alkalinization on the $Na_o$ - and $Ca_o$ -dependent Na Efflux*

*In the presence of  $Ca_i$  and in the absence of ATP.* We have previously demonstrated (DiPolo and Beaugé, 1984) that the forward Na/Ca exchange ( $Na_o$ -dependent Ca efflux) in squid axons is affected by intracellular ligands other than the transported ions (Na and Ca). One such ligand that has profound effects on the forward Na/Ca exchange is the ion  $H^+$ . Internal alkalinization (from pH 7.3 to 8.5) increases the  $Na_o$ -dependent Ca efflux by a factor of 3 (DiPolo and Beaugé, 1982). In principle, it would be expected that Na ions exiting through the Na/Ca exchange mechanism, whether as  $Na_i/Ca_o$  or  $Na_i/Na_o$  exchange, would exhibit a dependence on  $pH_i$ .

In the experiment illustrated in Fig. 2, the axon was dialyzed with an internal ATP-free medium that was buffered at  $pH_i$  7.3 and contained  $80 \mu M Ca_i$ . Under these conditions, the efflux of Na reached a steady value of  $9 \text{ pmol} \cdot \text{cm}^{-2} \cdot \text{s}^{-1}$ . Increasing the  $pH_i$  to 8.5 elicited an increase in the efflux to  $\sim 22 \text{ pmol} \cdot \text{cm}^{-2} \cdot \text{s}^{-1}$ . Subsequent removal of  $Na_o$  and  $Ca_o$  caused the efflux to drop to "leak" values. Returning the  $Na_o$  to 440 mM in the absence of  $Ca_o$  increased the Na efflux to a value similar to that obtained in artificial seawater. In order to measure the  $Ca_o$ -dependent component in the same experiment, a new baseline ("leak") was obtained in the absence of  $Na_o$  and  $Ca_o$ . Addition of 10 mM  $Ca_o$  increased the Na efflux to  $\sim 9 \text{ pmol} \cdot \text{cm}^{-2} \cdot \text{s}^{-1}$ . Finally, the addition of  $Na_o$  increased the Na efflux to a value identical to that found originally in an artificial seawater. The results of this and similar experiments show that internal alkalinization causes an increase in both the  $Na_o$ - and  $Ca_o$ -dependent Na efflux components.

Most of the  $pH_i$  effects on Na efflux take place on the  $Na_o$ -dependent rather than the  $Ca_o$ -dependent component. In the experiment of Fig. 3, both Ca and Na effluxes were measured simultaneously by dialyzing an axon with a standard

internal medium containing both  $^{45}\text{Ca}$  and  $^{24}\text{Na}$  (see Materials and Methods). This procedure allows the determination of the  $\text{Na}_o$ -dependent Ca efflux,  $\text{Ca}_o$ -dependent Ca efflux,  $\text{Ca}_o$ -dependent Na efflux, and  $\text{Na}_o$ -dependent Na efflux components. At  $\text{pH}_i$  7.3 and in the presence of  $80 \mu\text{M Ca}_i$ , the efflux of Ca and Na reached values of  $\sim 1.6$  and  $8 \text{ pmol}\cdot\text{cm}^{-2}\cdot\text{s}^{-1}$ , respectively.

Removal of both  $\text{Na}_o$  and  $\text{Ca}_o$  dropped both fluxes to "leak" values. When  $\text{Ca}_o$  was added in the absence of  $\text{Na}_o$ , a  $\text{Ca}_o$ -dependent Na efflux of  $\sim 6 \text{ pmol}\cdot\text{cm}^{-2}\cdot\text{s}^{-1}$  was obtained with little ( $120 \text{ fmol}\cdot\text{cm}^{-2}\cdot\text{s}^{-1}$ ) activation of the  $\text{Ca}_o$ -dependent

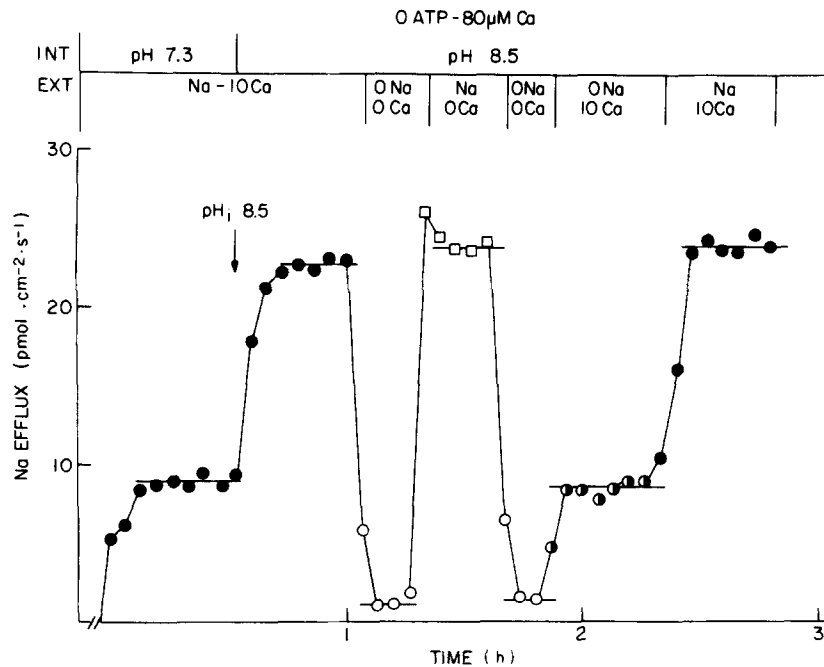


FIGURE 2. The effect of internal alkalization on the  $\text{Na}_o$ - and  $\text{Ca}_o$ -dependent components of the Na efflux in the presence of  $\text{Ca}_i$ . The arrow indicates the change in the internal medium from  $\text{pH}_i$  7.3 to 8.5. Note that the sum of the  $\text{Na}_o$ - and  $\text{Ca}_o$ -dependent Na efflux components is greater than the level of the efflux in artificial seawater. Unless otherwise stated, all concentrations are in millimolar. Axon diameter,  $490 \mu\text{m}$ .

Ca efflux. Under these conditions, raising  $\text{pH}_i$  to 8.5 caused an increase in the  $\text{Ca}_o$ -dependent component of the Na efflux, with no activation of the  $\text{Ca}_o$ -dependent Ca efflux. Readmission of Na ions to the external medium caused a large increase in both the  $\text{Na}_o$ -dependent Na efflux and the  $\text{Na}_o$ -dependent Ca efflux. If the  $\text{Na}_o$ -dependent Na efflux component is indeed part of the Na/Ca exchange system (see Discussion), this experiment implies that during the operation of the forward Na/Ca exchange, there is a large Na/Na exchange taking place. At the end of the experiment,  $\text{Ca}_o$  was increased up to  $50 \text{ mM}$ ; this point will be discussed later.

*In the presence of  $Ca_i$  and ATP.* In a second series of experiments, we measured the effect of increasing  $pH_i$  on the  $Na_i/Ca_o$  and the  $Na_i/Na_o$  exchange components in axons containing both  $Ca_i$  and ATP. Ouabain and bumetanide were present in the external medium during the experiment. Fig. 4 illustrates one such experiment. After 1 h of predialysis to remove the ATP, the efflux of Na reached a steady value of  $5 \text{ pmol} \cdot \text{cm}^{-2} \cdot \text{s}^{-1}$ . When 2 mM ATP was introduced via the dialysis medium, a net increment of  $13 \text{ pmol} \cdot \text{cm}^{-2} \cdot \text{s}^{-1}$  in the Na efflux

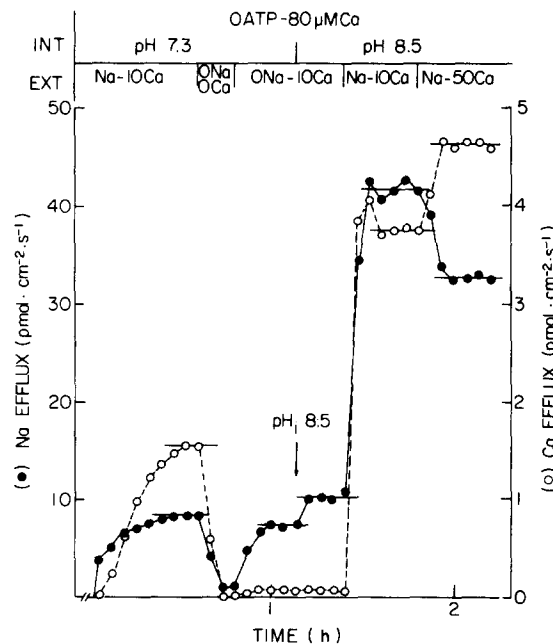


FIGURE 3. The effect of internal alkalization on the  $Na_o$  and  $Ca_o$  components of the Na and Ca effluxes in the presence of  $Ca_i$ . The axon was predialyzed for 50 min before the addition of the radioactive medium containing both  $^{24}\text{Na}$  and  $^{45}\text{Ca}$ . The 4-min samples were counted immediately in a gamma counter to determine the efflux of Na. After sufficient decay of the  $^{24}\text{Na}$ , the samples were counted for  $^{45}\text{Ca}$  in a liquid scintillation counter. All concentrations are in millimolar except  $Ca_i$ , which is in micromolar.

was obtained. Clearly, most of this increment is on the  $Na_o$ -dependent component, as can be seen upon removal of  $Na_o$ . Increasing  $pH_i$  from 7.3 to 8.5 activated the Na efflux ( $8 \text{ pmol} \cdot \text{cm}^{-2} \cdot \text{s}^{-1}$  beyond the level activated by ATP). Again, most of the activation caused by raising  $pH_i$  is on the  $Na_o$ -dependent component. Since in the presence of 2 mM ATP the nucleotide effect is completely saturated (see Fig. 11), the extra increment in the  $Na_o$ - and  $Ca_o$ -dependent Na efflux components implies that internal alkalization does not simply mimic the ATP effect.

*In the absence of  $Ca_i$ , with or without ATP.* It is already known, and confirmed

in the experiment of Fig. 1, that no  $\text{Na}_i/\text{Ca}_o$  exchange occurs in the absence of  $\text{Ca}_i$  (DiPolo, 1979; DiPolo and Beaugé, 1986). If the  $\text{Na}_o$ -dependent Na efflux is an operational mode of the Na/Ca exchange system, then its activation by internal alkalization should also depend on  $\text{Ca}_i$ . Fig. 5A shows that in an axon dialyzed without either Ca (2 mM free EGTA) or ATP and bathed in artificial seawater, internal alkalization caused a very small activation in the Na efflux ( $\sim 0.5 \text{ pmol} \cdot \text{cm}^{-2} \cdot \text{s}^{-1}$ ) as compared with that in the presence of  $\text{Ca}_i$ ,  $13 \text{ pmol} \cdot \text{cm}^{-2} \cdot \text{s}^{-1}$ ; see

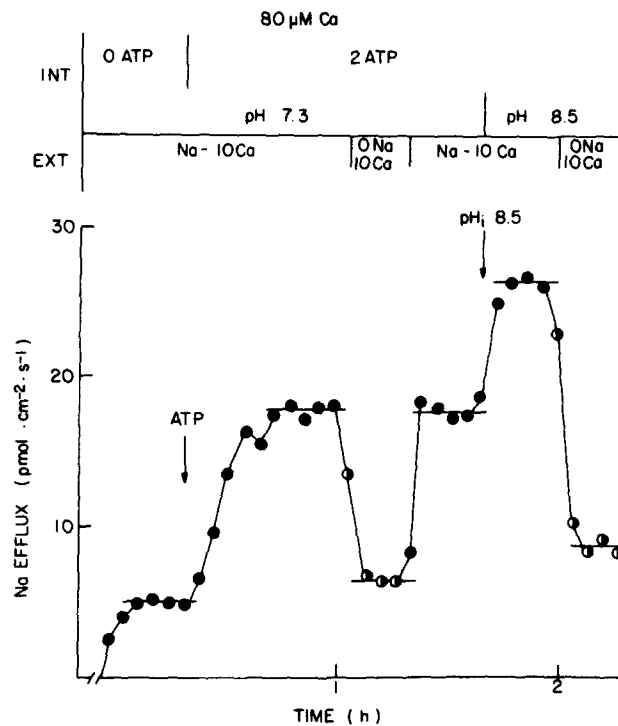


FIGURE 4. The effect of internal alkalization on the  $\text{Na}_o$ - and  $\text{Ca}_o$ -dependent Na efflux components in the presence of  $\text{Ca}_i$  and ATP. The arrows indicate changes in the internal medium. Different symbols represent different external solutions. Axon diameter, 570  $\mu\text{m}$ .

Fig. 2). Addition of 2 mM ATP in the absence of  $\text{Ca}_i$  caused an increase in the efflux of Na that was dependent on the presence of  $\text{Na}_o$ . Fig. 5B shows an experiment similar to that in A but carried out at pH 7.3 instead of 8.5. The magnitude of the ATP-stimulated,  $\text{Na}_o$ -dependent Na efflux in the absence of  $\text{Ca}_i$  was the same at pH 7.3 or 8.5, which suggests an Na/Na exchange system different from that found in the presence of  $\text{Ca}_i$ . In three other axons, no significant effect of internal alkalization was found on the ATP-activated,  $\text{Ca}_i$ -independent,  $\text{Na}_o$ -dependent Na efflux component.



*Effect of  $Ca_o$  on the  $Na_o$ -dependent Na Efflux*

*In the presence of  $Ca_i$ .* Fig. 6 shows that the  $Ca_i$ -activated,  $Na_o$ -dependent Na efflux was greatly reduced by raising the external Ca to 50 mM. This was also evident in the double-label experiment (see Fig. 3), which showed that raising

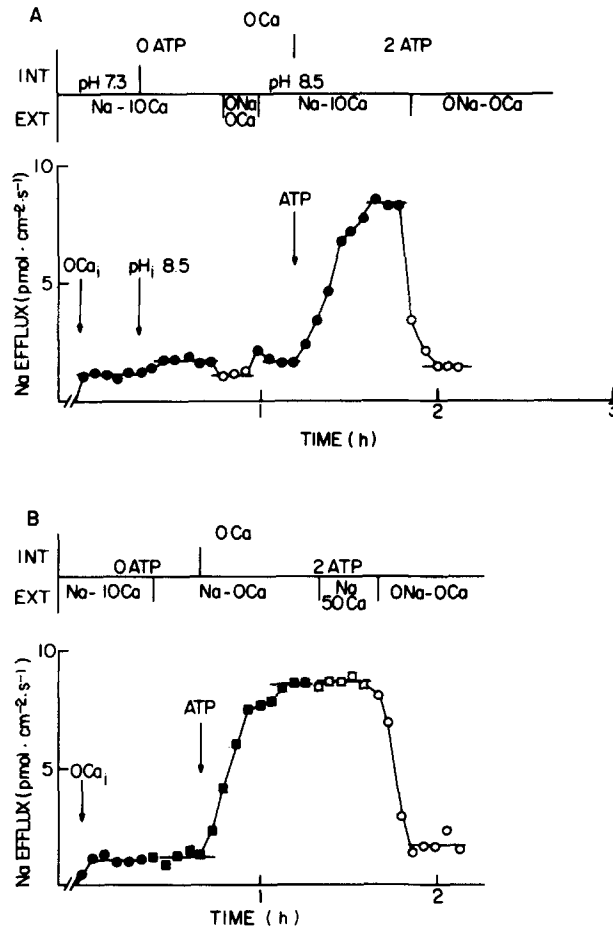


FIGURE 5. (A) The effect of internal alkalinization and ATP on Na efflux in an axon dialyzed without  $Ca_i$ . (B) The effect of  $Ca_o$  and ATP on the Na efflux in an axon dialyzed without  $Ca_i$ . The arrows indicate changes in the composition of the dialysis medium. Changes in the external medium are indicated by different symbols. Note the lack of effect of internal alkalinization (A) and  $Ca_o$  (B) on the Na efflux in the absence of  $Ca_i$ .

$Ca_o$  from 10 to 50 mM caused a drop in the Na efflux. Although no kinetic data exist from these experiments that would imply that inhibition by  $Ca_o$  is competitive, such inhibition is in line with the notion that  $Na_o$  and  $Ca_o$  ions compete for a cation-binding site on the exchange carrier (Baker et al., 1969; Blaustein and

Russell, 1975; DiPolo and Beaugé, 1986; Reeves, 1986); this suggests a common mechanism for the  $\text{Na}_i/\text{Na}_o$  and  $\text{Na}_i/\text{Ca}_o$  exchanges.

*In the absence of  $\text{Ca}_i$ .* In the experiment of Fig. 5B, the effect of increasing  $\text{Ca}_o$  was explored in an axon dialyzed without  $\text{Ca}_i$ . After the Na efflux had reached a steady state level of  $\sim 1 \text{ pmol} \cdot \text{cm}^{-2} \cdot \text{s}^{-1}$  in the nominal absence of  $\text{Ca}_i$ , addition of ATP (in the presence of  $\text{Na}_o$  and in the absence of  $\text{Ca}_o$ ) caused the efflux to increase to  $\sim 8 \text{ pmol} \cdot \text{cm}^{-2} \cdot \text{s}^{-1}$ . Increasing  $\text{Ca}_o$  to 50 mM had no effect on the level of Na efflux, in contrast to experiments performed in the presence of  $\text{Ca}_i$  (see Figs. 3 and 6).

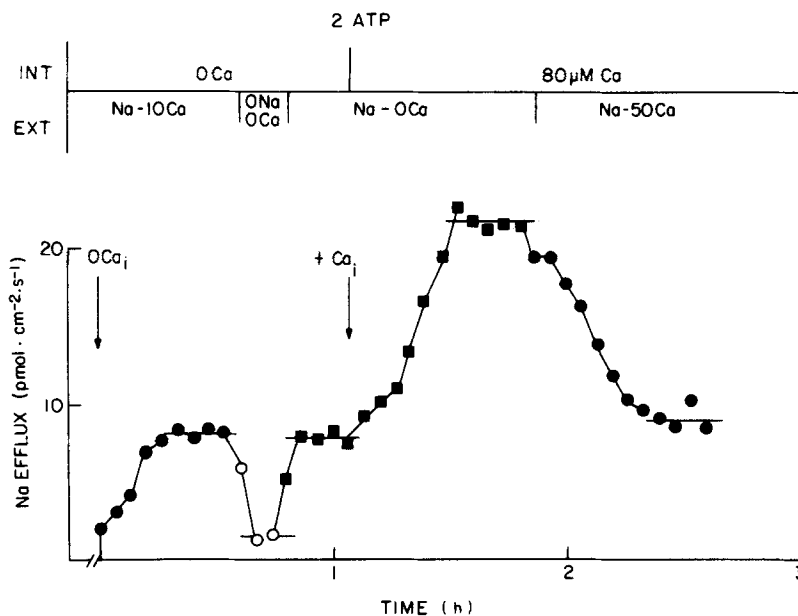


FIGURE 6. The effect of  $\text{Ca}_o$  on the  $\text{Na}_o$ -dependent Na efflux component in the present of ATP. The arrow indicates the addition of  $80 \mu\text{M}$  ionized Ca to the dialysis medium. Note the large inhibition in the  $\text{Na}_o$ -dependent Na efflux upon increasing  $\text{Ca}_o$  to 50 mM. All concentrations are in millimolar except  $\text{Ca}_i$ , which is in micromolar. Axon diameter,  $430 \mu\text{m}$ .

#### *Effect of $\text{Mg}_i$ on the $\text{Na}_o$ - and $\text{Ca}_o$ -dependent Na Efflux*

It has previously been shown (DiPolo and Beaugé, 1984) that  $\text{Mg}_i$  is an inhibitor of the  $\text{Na}_o$ -dependent Ca efflux in squid axons. At physiological levels of  $\text{Mg}_i$  (2–3 mM), the forward Na/Ca exchange is inhibited by 50%. The experiment of Fig. 7 shows that removal of  $\text{Mg}_i$  from the dialysis medium caused an increase in the Na efflux from a steady level of  $4.6 \text{ pmol} \cdot \text{cm}^{-2} \cdot \text{s}^{-1}$  to a level of  $10.5 \text{ pmol} \cdot \text{cm}^{-2} \cdot \text{s}^{-1}$  in the presence of both  $\text{Na}_o$  and  $\text{Ca}_o$ . The  $\text{Na}_o$ - and  $\text{Ca}_o$ -dependent components of the Na efflux in the absence of  $\text{Mg}_i$  were measured by adding  $\text{Na}_o$  in the absence of  $\text{Ca}_o$  and vice versa. Clearly, the  $\text{Na}_o$ -dependent component of the Na efflux in the absence of  $\text{Mg}_i$  ions (“leak subtracted”) was greater than

the  $\text{Ca}_o$ -dependent one. Furthermore, the sum of the  $\text{Na}_o$ - and  $\text{Ca}_o$ -dependent components ( $13 \text{ pmol} \cdot \text{cm}^{-2} \cdot \text{s}^{-1}$ ) was greater than the magnitude of the Na efflux in the presence of both  $\text{Na}_o$  and  $\text{Ca}_o$  ( $\sim 9.7 \text{ pmol} \cdot \text{cm}^{-2} \cdot \text{s}^{-1}$ ), which is in line with the hypothesis that the  $\text{Na}_o$ - and  $\text{Ca}_o$ -dependent components of the Na efflux share the same transport system.

*Effect of ATP $\gamma$ S on the  $\text{Na}_o$ - and  $\text{Ca}_o$ -dependent Na Efflux*

The preceding experiments show that in the absence of  $\text{Ca}_i$ , ATP activates an  $\text{Na}_o$ -dependent Na efflux that appears to be different from the  $\text{Na}_o$ -dependent

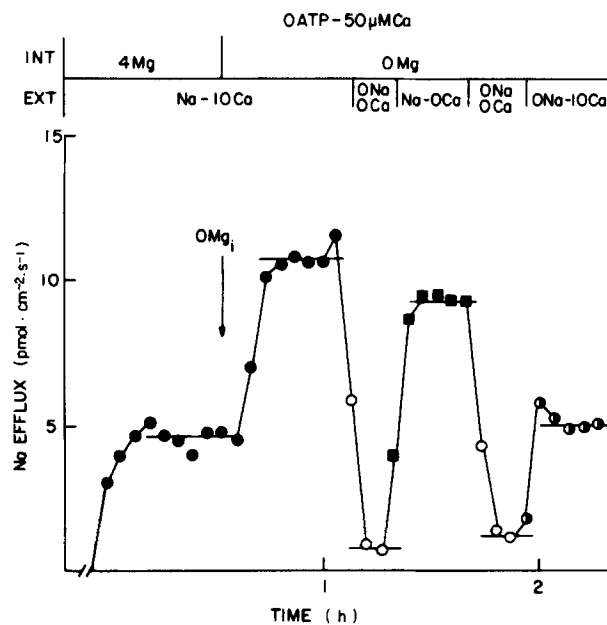


FIGURE 7. The effect of  $\text{Mg}_i$  ions on the  $\text{Na}_o$ - and  $\text{Ca}_o$ -dependent Na efflux components. The arrow indicates the removal of Mg from the dialysis medium. The axon was dialyzed from the beginning with  $50 \mu\text{M}$  Ca. Unless otherwise stated, all concentrations are in millimolar. Different symbols represent different external solutions. Axon diameter,  $620 \mu\text{m}$ .

Na efflux component activated by  $\text{Ca}_i$ . The former is neither activated by internal alkalization nor inhibited by  $\text{Ca}_o$  (see Fig. 5). Another point in favor of this hypothesis comes from experiments using the ATP analogue ATP $\gamma$ S. In the experiment of Fig. 8A, Na efflux was measured in an axon dialyzed with an internal medium lacking both Ca and ATP and bathed in artificial seawater containing no ouabain or bumetanide. Under these conditions, the efflux of Na reached a steady value of  $\sim 1.3 \text{ pmol} \cdot \text{cm}^{-2} \cdot \text{s}^{-1}$ . The addition of 1 mM of the ATP analogue to the internal dialysis medium had no effect on the Na efflux level. Raising ionized  $\text{Ca}_i$  to  $40 \mu\text{M}$  caused an activation of the Na efflux to a steady state value of  $\sim 13 \text{ pmol} \cdot \text{cm}^{-2} \cdot \text{s}^{-1}$ . This activation was totally dependent

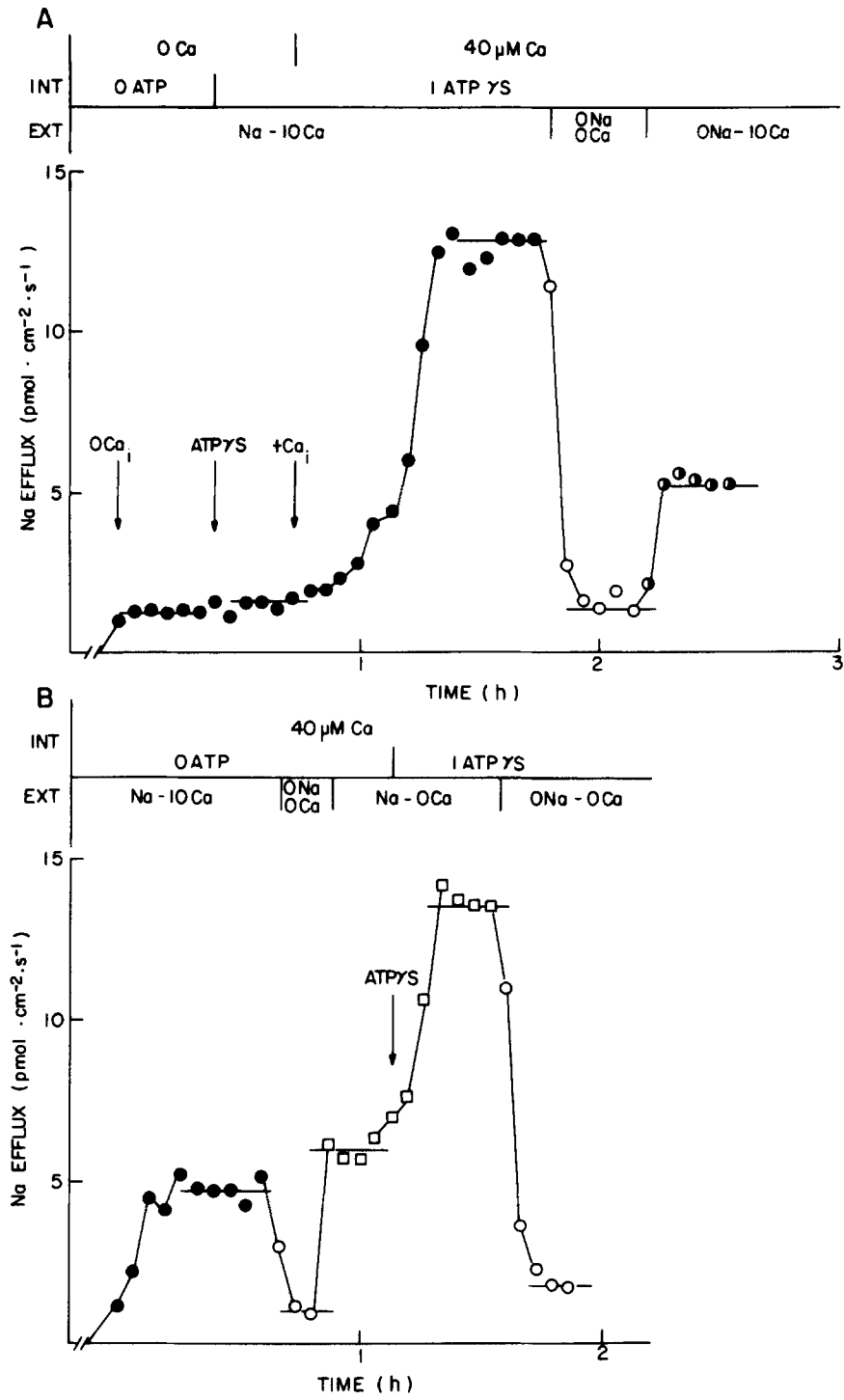


FIGURE 8.

on the presence of  $\text{Na}_o$  and  $\text{Ca}_o$ . Again, as in the case for the activation of the Na efflux by ATP in the presence of  $\text{Ca}_i$ , a large proportion of the ATP-stimulated flux corresponded to the  $\text{Na}_o$ -dependent component. An interesting observation is that neither the Na/K pump nor any other transport system, including Na/K/Cl co-transport and Na/Mg exchange, appeared to be activated by ATP $\gamma$ S. It should be mentioned that no activation by the analogue was observed in the absence of  $\text{Mg}_i$  (results not shown). Fig. 8B shows an experiment in which ATP $\gamma$ S was added to an axon already containing 40  $\mu\text{M}$   $\text{Ca}_i$ . Clearly, the analogue induced a large increase in the  $\text{Na}_o$ -dependent Na efflux component.

#### *Dependence of $\text{Na}_o$ - and $\text{Ca}_o$ -dependent Na Efflux on $\text{Na}_o$ and $\text{Ca}_o$*

The  $\text{Na}_o$  and  $\text{Ca}_o$  dependence of the Na efflux is examined in Fig. 9. In these experiments, axons were dialyzed from the beginning with an internal medium containing saturating concentrations of both  $\text{Ca}_i$  (100  $\mu\text{M}$ ) and ATP (2 mM) and bathed in an external medium containing no  $\text{Na}_o$  or  $\text{Ca}_o$ . In three different axons, steady state Na effluxes were measured at different values of  $\text{Na}_o$  (in the absence of  $\text{Ca}_o$ ).  $\text{Na}_o$  ions activated the Na efflux with relatively low affinity ( $K_{1/2} = 125$  mM). Since no clear saturation was found with 440 mM  $\text{Na}_o$ , an apparent affinity of 125 mM should be taken as a lower limit. We measured the steady state Na efflux in four axons at different values of  $\text{Ca}_o$  and in the absence of  $\text{Na}_o$  (Fig. 9B).  $\text{Ca}_o$  ions activated the reversal of the exchange with a  $K_{1/2}$  of 5 mM.

#### *ATP Dependence of $\text{Na}_o$ -dependent Na Efflux*

Fig. 10 summarizes the results of several experiments in which the activation by ATP on the  $\text{Na}_o$ -dependent Na efflux was determined in the presence of  $\text{Ca}_i$ . In all these experiments, the axons were predialyzed for  $\sim 1$  h without ATP before testing a given nucleotide concentration. Phosphoarginine (5 mM) was added to buffer the concentration of ATP in the axoplasm (Brinley and Mullins, 1968). The  $K_{1/2}$  for the ATP activation is  $\sim 120$   $\mu\text{M}$ . Interestingly, this value is close to the activation of the  $\text{Na}_o$ -dependent Ca efflux by ATP (DiPolo and Beaugé, 1979). As is the case for the ATP-stimulated,  $\text{Na}_o$ -dependent Ca efflux (DiPolo and Beaugé, 1984), both  $\text{Na}_o$ - and  $\text{Ca}_o$ -dependent Na effluxes require Mg ions for the nucleotide effect (results not shown).

#### *$\text{Ca}_i$ Dependence of $\text{Na}_o$ - and $\text{Ca}_o$ -dependent Na Effluxes*

Inasmuch as the reversal mode of the Na/Ca exchange ( $\text{Na}_i/\text{Ca}_o$  exchange) requires the presence of micromolar amounts of  $\text{Ca}_i$  for activation (DiPolo and

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FIGURE 8. (*opposite*) (A) The effect of ATP $\gamma$ S on the  $\text{Na}_o$ - and  $\text{Ca}_o$ -dependent Na efflux components in the absence and presence of  $\text{Ca}_i$ . (B) Effect of ATP $\gamma$ S on the  $\text{Na}_o$ -dependent Na efflux in an axon containing  $\text{Ca}_i$ . In these experiments, neither ouabain nor bumetanide was added to the external medium. The arrows indicate changes in the composition of the dialysis fluid. Different symbols correspond to different external solutions. All concentrations are in millimolar except  $\text{Ca}_i$ , which is in micromolar. Note the lack of activation of the ATP analogue when no  $\text{Ca}_i$  is included in the dialysis medium.

Beaugé, 1986), it is important to determine the dependence of the  $\text{Na}_o$ - and  $\text{Ca}_o$ -dependent Na efflux components on  $\text{Ca}_i$ . In the experiments of Fig. 11, A and B, the axons were predialyzed with nominally zero  $\text{Ca}_i$  (2–3 mM total EGTA) before the addition of different concentrations of Ca to the dialysis medium.

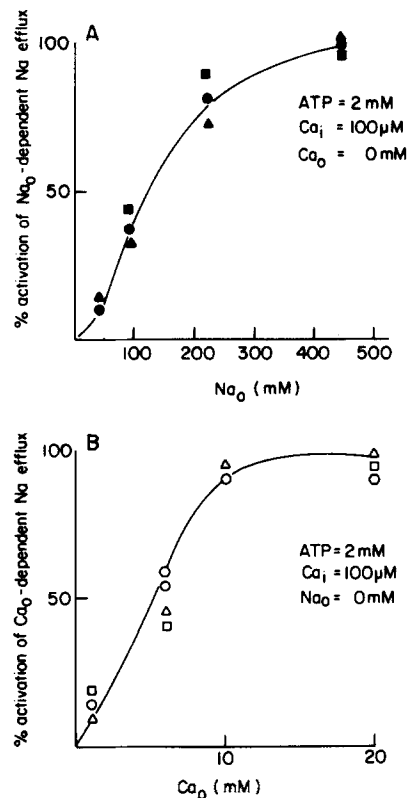


FIGURE 9. (A) Dependence of  $\text{Na}_o$ -dependent Na efflux on  $\text{Na}_o$ . (B) Dependence of  $\text{Ca}_o$ -dependent Na efflux on  $\text{Ca}_o$ . In all these experiments, the axons were dialyzed from the beginning with an internal medium containing saturating concentrations of  $\text{Ca}_i$  and ATP. Each symbol represents a different axon. The  $\text{Na}_o$ -dependent component was determined at different concentrations of  $\text{Na}_o$  in the absence of  $\text{Ca}_o$ . The  $\text{Ca}_o$ -dependent component was determined at different concentrations of  $\text{Ca}_o$  in the absence of  $\text{Na}_o$ . The mean temperature in these experiments was 17.5°C. The steady state values of the Na efflux at 440 mM  $\text{Na}_o$  and 20 mM  $\text{Ca}_o$  were taken as 100% activation of the  $\text{Na}_o$ - and  $\text{Ca}_o$ -dependent components, respectively. In all these experiments, the external medium contained TTX, cyanide, ouabain, and bumetanide.

The data represent the steady state Na efflux at different internal ionized Ca concentrations. The  $\text{Ca}_o$ -dependent component was measured in the absence of  $\text{Na}_o$ . Similarly, the  $\text{Na}_o$ -dependent Na efflux component was determined in the absence of  $\text{Ca}_o$ . In the absence of ATP,  $\text{Ca}_i$  activated the  $\text{Ca}_o$ -dependent Na efflux, with an apparent  $K_{1/2}$  of 15  $\mu\text{M}$ . In the presence of 2 mM ATP, the

apparent  $K_{1/2}$  was reduced to  $1.8 \mu\text{M}$ . Since in the presence of ATP the  $\text{Na}_o$ -dependent Na efflux component that occurs through the Na/Ca exchange system is complicated by the presence of an ATP-activated,  $\text{Ca}_i$ -independent,  $\text{Na}_o$ -dependent Na efflux component (see Discussion), its dependence on  $\text{Ca}_i$  was determined in the absence of ATP. Fig. 11B shows that ionized  $\text{Ca}_i$  activated the  $\text{Na}_o$ -dependent Na efflux along a sigmoidal curve, with an apparent  $K_{1/2}$  of  $8 \mu\text{M}$ .

#### DISCUSSION

The results of the present study confirm and extend earlier work (DiPolo, 1979; DiPolo and Beaugé, 1986) showing that the level of ionized  $\text{Ca}_i$  modulates the velocity of the Na/Ca exchange working in the reverse mode. In axons treated

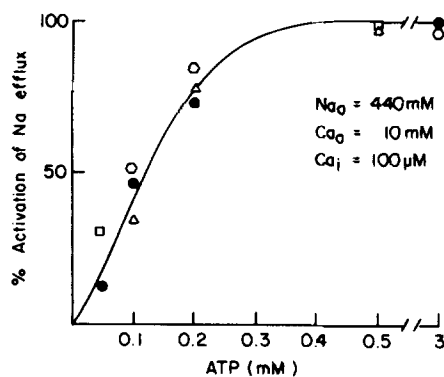


FIGURE 10. Effect of internal ATP on the ouabain- and bumetanide-insensitive Na efflux in the presence of  $\text{Ca}_i$ . Ordinate: Na efflux relative to the maximum efflux obtained in the presence of 3 mM ATP. Abscissa: ATP concentration in the dialysate in millimolar. The ATP in the axoplasm was buffered by adding 5 mM phosphoarginine to the dialysis medium. Each symbol represents a different axon. In all these experiments, the axon was predialyzed without ATP for  $\sim 60$  min. The external medium always contained TTX, cyanide, ouabain, and bumetanide.

with ouabain and bumetanide,  $\text{Ca}_i$  activates both  $\text{Ca}_o$ - and  $\text{Na}_o$ -dependent Na efflux components. Internal ATP stimulates both  $\text{Ca}_i$ -activated  $\text{Na}_o$ - and  $\text{Ca}_o$ -dependent Na efflux components through the Na/Ca exchange system. The nucleotide also induces a  $\text{Ca}_i$ -independent,  $\text{Na}_o$ -dependent Na efflux component.

#### *Ca<sub>i</sub>-activated Na<sub>i</sub>/Na<sub>o</sub> and Na<sub>i</sub>/Ca<sub>o</sub> Exchange*

Although an  $\text{Na}_o$ -dependent Na efflux has been demonstrated in cardiac membrane vesicles exhibiting several properties similar to the Na/Ca exchange system operating in an Na/Na exchange mode (Reeves and Sutko, 1979), Na/Na exchange as part of the Na/Ca exchange mechanism has not yet been demonstrated in an intact preparation. In squid axons, the efflux of Na in the presence of ouabain is inhibited at high  $\text{Na}_o$  and activated at low  $\text{Na}_o$ . This finding has been interpreted as a competition between  $\text{Na}_o$  and  $\text{Ca}_o$  ions and an activation

of the  $\text{Na}_i/\text{Ca}_o$  exchange by a monovalent cation (Baker et al., 1969). Although these experiments may suggest that the ouabain-insensitive  $\text{Na}/\text{Na}$  exchange does not exist in squid axons or that it occurs at a slower rate than the  $\text{Na}/\text{Ca}$  exchange, no systematic studies were carried out on the  $\text{Na}_o$ -dependent  $\text{Na}$  efflux component when the  $\text{Na}/\text{K}$  pump was fully inhibited by ouabain and the  $\text{Na}/\text{Ca}$  exchange was completely activated with high  $\text{Ca}_i$ .

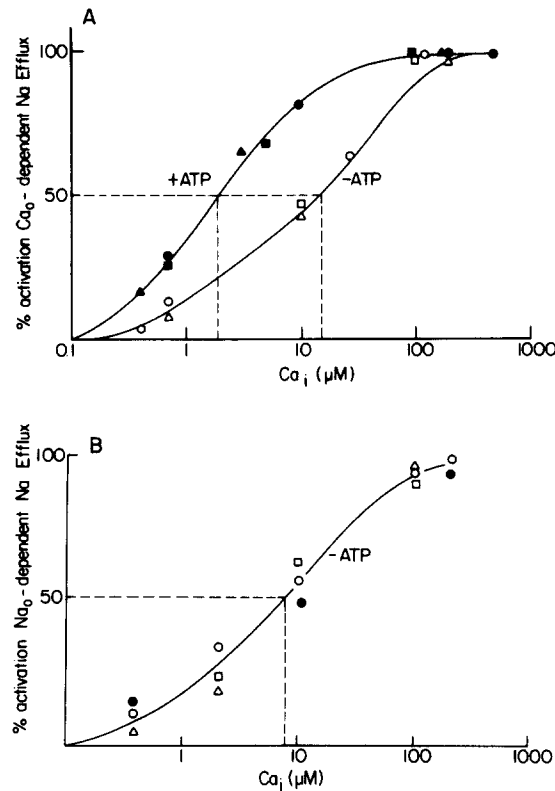


FIGURE 11. (A) The effect of  $\text{Ca}_i$  on the  $\text{Ca}_o$ -dependent Na efflux in the absence and in the presence of ATP. (B) The effect of  $\text{Ca}_i$  on the  $\text{Na}_o$ -dependent Na efflux in the absence of ATP. Each symbol represents a single axon. In these experiments, the axons were predialyzed without  $\text{Ca}_i$  and with or without ATP before the addition of the radioactive medium. The ordinate represents the percent activation of the  $\text{Ca}_o$  (A) or  $\text{Na}_o$ -dependent Na efflux (B) when taken as 100% the steady state Na efflux obtained at 200  $\mu\text{M}$   $\text{Ca}_i$ . The ionized Ca in the dialysis medium was controlled by varying the ratio ( $\text{CaEGTA}/\text{free EGTA}$ ) at a constant total EGTA of 2 mM. The apparent dissociation constants for the  $\text{CaEGTA}$  and  $\text{CaATP}$  complexes were chosen as 0.15  $\mu\text{M}$  and 1.4 mM, respectively (see Methods for references). Half-maximal activation for the  $\text{Ca}_o$ -dependent component was obtained at 15  $\mu\text{M}$  in the absence of ATP and at 1.8  $\mu\text{M}$  in the presence of ATP (2 mM). Half-maximal activation for the  $\text{Na}_o$ -dependent component was obtained at 9  $\mu\text{M}$  in the absence of ATP.



*Effects of  $pH_i$ ,  $Mg_i$ , and  $Ca_o$ .* One of the major findings reported here is that the  $Na_o$ -dependent Na efflux component activated by  $Ca_i$  corresponds to a mode of operation of the Na/Ca exchange. Several arguments favor a common origin of the  $Ca_i$ -activated,  $Na_o$ -dependent Na efflux and the Na/Ca exchange mechanism. (a) Internal alkalization, which is known to increase the forward Na/Ca exchange, also activates the  $Na_o$ - and  $Ca_o$ -dependent Na efflux components. (b) The sum of the  $Na_o$ -dependent and  $Ca_o$ -dependent components is always greater than the Na efflux in the presence of both  $Na_o$  and  $Ca_o$  ions, whether at  $pH_i$  7.3 or 8.5; this suggests that both components are a manifestation of the same exchange system. (c) Internal alkalization fails to activate the Na efflux in the absence of  $Ca_i$ , which indicates that the  $pH_i$  effect is on the  $Ca_i$ -activated Na/Ca exchange component. (d)  $Ca_i$ -activated,  $Na_o$ -dependent Na efflux can be largely inhibited by raising  $Ca_o$ , in line with the hypothesis that Na and Ca compete for a common external site on the exchanger. (e)  $Mg_i$  inhibits the  $Na_o$ -dependent Na efflux as well as the  $Na_o$ -dependent Ca efflux (see Fig. 7 and DiPolo and Beaugé, 1984). The activation of the Na efflux by removal of  $Mg_i$  is preferentially on the  $Na_o$ -dependent component, a result qualitatively similar to the effect of internal alkalization. (f) The kinetics of activation of the  $Na_o$ -dependent Na efflux by  $Na_o$  ( $K_{1/2} = 140$  mM) are in agreement with the activation of the  $Na_o$ -dependent Ca efflux by  $Na_o$  (DiPolo, 1974; Blaustein, 1977). (g) Finally, in the absence of ATP, both the  $Na_o$ - and  $Ca_o$ -dependent components are activated by  $Ca_i$  with a low apparent affinity (half-maximal activation in the micromolar range) (see Fig. 11, A and B).

*Effect of ATP on the kinetics of activation.* Experiments investigating the ATP dependence of the forward Na/Ca exchange show that the nucleotide stimulates the  $Na_o$ -dependent Ca efflux with low affinity ( $K_{1/2} \sim 200$   $\mu$ M; DiPolo and Beaugé, 1979). This also appears to be the case for the activation of the  $Na_o$ -dependent Na efflux through the Na/Ca exchange system. In the presence of saturating concentrations of  $Na_o$ ,  $Ca_o$ , and  $Ca_i$ , ATP stimulates the efflux of Na with low affinity ( $K_{1/2} \sim 130$   $\mu$ M). It could be thought that the effect of ATP is somehow related to the activating effect of internal alkalization since both treatments affect the  $Na_o$ -dependent component more than the  $Ca_o$ -dependent one. However, the experiment of Fig. 4 shows that even at saturating concentrations of ATP, internal alkalization still induces an increase in the  $Na_o$ -dependent Na efflux; this suggests that there are different mechanisms for the activation of the Na/Ca exchange by ATP and by the removal of  $H_i$  ions.

From studies of the  $Na_o$ -dependent Ca efflux in injected (Baker and Glitsch, 1973) and dialyzed squid axons (DiPolo, 1974; Blaustein, 1977), it has been possible to demonstrate that ATP markedly increases the affinity of the Na/Ca exchange system toward  $Ca_o$  and  $Ca_i$ . One of the interesting findings of the present work is that ATP affects the reverse mode of the exchange system by markedly reducing the  $Ca_i$  for half-maximal activation of the  $Ca_o$ -dependent Na efflux component (see Fig. 11A).

*The effect of ATP  $\gamma$ S.* The evidence accumulated in squid axons that only hydrolyzable ATP analogues activate the Na/Ca exchange (nonhydrolyzable ATP analogues inhibit it; DiPolo, 1977), and that  $Mg_i$  ions are strictly required

for the ATP effect (DiPolo, 1977; DiPolo and Beaugé, 1984), suggest that the effect of ATP involves a phosphorylation of the Na/Ca exchanger. The experiments reported here with the analogue ATP $\gamma$ S are of interest because this analogue is known to act as a substrate of kinases but not of ATPases (Gratecos and Fischer, 1974; Cassidy et al., 1979). The finding that ATP $\gamma$ S does not promote any Na efflux component in the absence of Ca<sub>i</sub> ions (see Fig. 8A) is in agreement with recent evidence obtained in cardiac sarcolemma vesicles (Caroni and Carafoli, 1983), which suggested a phosphorylation of the Na/Ca exchanger by ATP mediated by a Ca<sub>i</sub>-dependent protein kinase. Since in the experiments with ATP $\gamma$ S, ouabain and bumetanide were not added to the external solutions and Mg<sub>o</sub> was always present, one can conclude that the analogue is unable to activate the Na/K pump, the Na/K/Cl co-transport, or the Na<sub>i</sub>/Mg<sub>o</sub> exchange. As in the case of ATP, the analogue preferentially activates the Na/Na exchange mode of operation. Results not shown (DiPolo, R., and L. Beaugé, unpublished results) indicate that ATP $\gamma$ S substantially increases the forward (Na<sub>o</sub>-dependent Ca efflux) Na/Ca exchange without affecting the Ca pump component of the Ca efflux, an indication of a remarkable selectivity of the ATP analogue in activating only the Na and Ca fluxes through the Na/Ca exchange system.

#### *Ca<sub>i</sub>-independent, ATP-activated Na<sub>i</sub>/Na<sub>o</sub> Exchange*

The results presented here confirm the early finding that in squid axons there is a Ca<sub>i</sub>-independent, ATP-activated Na/Na exchange (Beaugé and DiPolo, 1981). This Na/Na exchange has been related to a glycoside-poisoned Na pump (Brinley and Mullins, 1968), and although some evidence exists that this might be the case (Beaugé and DiPolo, 1981), at present it is not clear that this component is really induced by the glycoside. On the basis of theoretical considerations, it is also possible that the Ca<sub>i</sub>-independent Na/Na exchange component is an operational mode of the Na/Ca exchange system (DiPolo and Beaugé, 1986). However, the experimental data presented in this work strongly argue against this possibility and show that it might represent a system different from the Na/Ca exchange. Four main arguments favor this proposal. First, the magnitude of the Ca<sub>i</sub>-independent, Na<sub>o</sub>-dependent Na efflux ( $6.5 \text{ pmol} \cdot \text{cm}^{-2} \cdot \text{s}^{-1}$ , average of four axons) is unaffected by internal alkalinization (see Fig. 5, A and B), in marked contrast to the Ca<sub>i</sub>-activated Na/Na exchange system (see Fig. 4). Second, Ca<sub>o</sub> up to 50 mM does not inhibit the level of the Na efflux, which suggests that Na and Ca do not compete for a common external site. Third, this component is not modified by the removal of K<sub>i</sub> or K<sub>o</sub> (Beaugé and DiPolo, 1982), an experimental treatment that is known to affect the Na/Ca exchange mechanism (DiPolo and Rojas, 1984). A final important argument is that the ATP analogue ATP $\gamma$ S is unable to activate this component (see Fig. 8A); this demonstrates a specificity of ATP in activating the Ca<sub>i</sub>-independent Na/Na exchange compared with the Ca<sub>i</sub>-dependent Na/Na exchange, which can be activated either by ATP or ATP $\gamma$ S. Unfortunately, no difference in the apparent affinity for ATP exists between these two Na/Na exchange components (see Fig. 3A of Beaugé and DiPolo, 1981). It is worthwhile to mention that if the Ca<sub>i</sub>-independent Na/Na exchange is an induced ion flux through a glycoside-poisoned Na pump, it would not exist under physiological conditions.

*Implications of Ca<sub>i</sub>-activated Na<sub>i</sub>/Ca<sub>o</sub> and Na<sub>i</sub>/Na<sub>o</sub> Exchange Systems*

As reported here (see also DiPolo and Beaugé, 1986), the dependence of every mode of operation of the Na/Ca exchange system on Ca<sub>i</sub> (the "essential activator") should be taken into account in future kinetic models of the Na/Ca exchange. This is particularly important for calculating net Ca movements from traditional symmetric models (Mullins, 1977; Wong and Bassingthwaight, 1981; Johnson and Kootsey, 1985) since the electrochemical ionic gradients of Na and Ca exclusively will not predict Ca entry (or Na exit) in an asymmetric system. The presence of a Ca<sub>i</sub>-activated Na<sub>i</sub>/Na<sub>o</sub> exchange as part of the Na/Ca exchange mechanism should also be considered when calculating the stoichiometry of the exchange process from unidirectional Na and Ca isotope flux measurements. Otherwise, in the presence of Na ions at both sides of the membrane, the number of Na ions exchanged for Ca will be overestimated by the presence of the Ca<sub>i</sub>-activated Na/Na exchange component. A point that is worth stressing is that the Na/Na exchange mode of operation of the Na/Ca exchange will occur under physiological conditions whenever the exchanger is activated by a rise in the ionized Ca<sub>i</sub>. This is in contrast to the case of the Ca/Ca exchange mode, which is only significant in the absence of Na<sub>o</sub> and in the presence of other external alkali metal ions (Blaustein, 1977).

The physiological consequences of the effect of Ca<sub>i</sub> as an essential activator of the Na/Ca exchange are under investigation. Nevertheless, it is consistent with earlier experiments reported by Baker (1972) and Baker and McNaughton (1976) showing that injection of EGTA into squid axons inhibits the Ca<sub>o</sub>-dependent Na efflux. The requirement for Ca<sub>i</sub>, but not a direct pharmacological effect of EGTA (DiPolo, 1979), would certainly explain the inhibition of the "reverse" Na/Ca exchange observed in squid axons injected with the Ca chelating agent quin2, as well as the presence in the same preparation of a Ca<sub>i</sub>-activated outward current generated during the operation of the Na<sub>i</sub>/Ca<sub>o</sub> exchange (DiPolo et al., 1987). This interpretation also agrees with the observation of an outward current caused by the Na/Ca exchange in Na-loaded myocytes, which disappears in the absence of Ca<sub>i</sub> ions (Kimura et al., 1986). Recent experiments with membrane vesicles from squid optic nerves have shown that in vesicles loaded with different CaEGTA concentrations, the rate of Ca uptake in exchange for Na is dependent on micromolar quantities of Ca present inside the vesicles, in agreement with an asymmetric Na/Ca exchange system (Condrescu et al., 1987). Finally, if the dependence of the reverse mode on Ca<sub>i</sub> is a generalized property of the exchanger, then Ca entry via voltage-dependent Ca channels (increase in Ca<sub>i</sub>) could modulate the activity of the exchanger. In support of this possibility is the fact that an increase in Ca<sub>i</sub> from 0.1 to 0.6 μM led to a 10-fold increase in Ca entry through the Na/Ca exchange system induced by membrane depolarization (DiPolo et al., 1982).

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