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

Ca_i-dependent Na_i/Ca_o and Na_i/Na_o Exchange Modes

REINALDO DIPOLO and LUIS BEAUGÉ

From the Instituto Venezolano de Investigaciones Científicas (IVIC), Centro de Biofísica y Bioquímica, Caracas 1020A, Venezuela, and the División de Biofísica, Instituto de Investigación Médica M. y M. Ferreyra, 5000 Cordoba, Argentina

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 alkalinization, Mg_i, Ca_o, and the ATP analogue [γ-thio]ATP (ATPγS) on the different components of the Na efflux. The results show the following: (*a*) internal alkalinization activates both Ca_o- and Na_o-dependent Na efflux components provided that Ca_i is present; (*b*) Mg_i 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*) ATPγS 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 alkalinization 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

Address reprint requests to Dr. Reinaldo DiPolo, Centro de Biofísica y Bioquímica, IVIC, Apartado 21827, Caracas 1020A, Venezuela.

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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 Ca_o/Na_i exchange mechanism ("reverse" Na/Ca exchange) requires not only the presence of Na_i and Ca_o in order to operate, but also micromolar amounts of Ca_i (DiPolo, 1979; DiPolo and Beaugé, 1986). (In this article, Ca_i refers to the intracellular ionized calcium concentration.) This asymmetry in the activation of the exchange system by Ca_i could have important physiological implications, since the levels of Ca_i modulate the influx of Ca through the antiporter mechanism (positive feedback). This regulatory effect of Ca_i has recently been implicated in the inhibition of Ca influx via Na_i/Ca_o exchange induced by the Ca indicator quin2 (Allen and Baker, 1985), and it could also account for the 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 Ca_i activates not only a Ca_o-dependent Na efflux component (Na_i/Ca_o exchange), but also a sizable Na_o-dependent Na efflux component (Na_i/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 Na_o-dependent Na efflux in the complete absence of 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 Ca_i and ATP activate not only the "reverse" Na_i/Ca_o exchange but also a sizable Na_i/Na_o exchange, which occurs during the turnover of this mechanism. Their similarities with respect to activation by internal alkalinization, 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 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 μm o.d., 141 μm i.d., Spectrum, Los Angeles, CA). The dialysis capillary contained a 75-μm 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, 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 μM 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) (ATPγS) 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, 5×10^{-4} M ouabain and 10 μM 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}Ca]$CaCl_2$ (15–30 mCi/mg; New England Nuclear, Boston, MA) or [^{22}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}Na and ^{45}Ca. The collected radioactive samples were first counted for ^{24}Na in a gamma counter. Na efflux values were corrected for radioactive decay. After complete decay of the ^{24}Na activity, samples were counted for ^{45}Ca activity in a liquid scintillation counter.

RESULTS

Effect of Ionized Ca_i and ATP_i on the Ouabain- and Bumetanide-insensitive Na Efflux

Reversal of the Na/Ca exchange in dialyzed squid axons is best studied by measuring the 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 (Na_i/Ca_o exchange) has the advantage that any catalytic effect of Ca_i on the reversal of the Na/Ca exchange can be unambiguously ascribed to Na_i/Ca_o and not to Ca_i/Ca_o exchange. Fig. 1 shows the effect of Ca_i and ATP on the Ca_o- and Na_o-dependent Na efflux components. The Na/K pump and the Na/K/Cl cotransport were inhibited by ouabain (5×10^{-4} M) and bumetanide (10^{-5} M), respectively. When the internal medium contained nominally zero Ca_i (3 mM total EGTA)

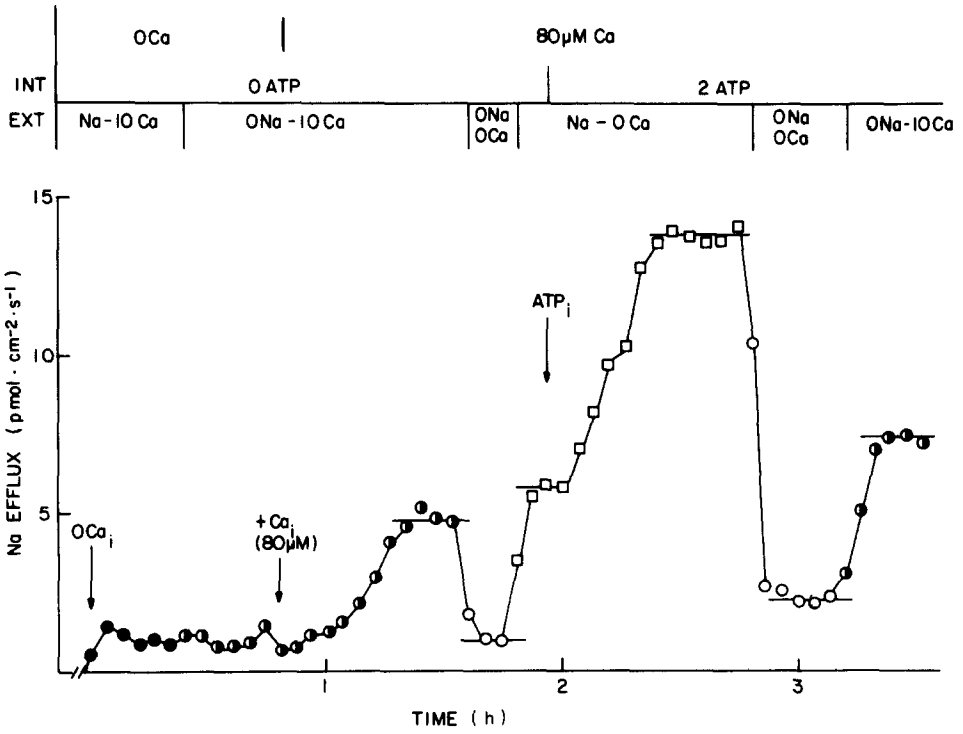

FIGURE 1. The effect of ionized Ca_i and ATP on the ouabain- and bumetanide-insensitive Na efflux. All concentrations are in millimolar except Ca_i, which is in micromolar. The arrows indicate changes in the internal medium. Different symbols are used to indicate different external solutions. All external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

and no ATP, the efflux of Na was rather small (<1 pmol·cm^{-2}·s^{-1}) and insensitive to changes in Na_o. Increasing Ca_i to 80 μM caused an increase in the Na efflux that was completely sensitive to Ca_o since removal of Ca_o in the absence of Na_o brought the efflux back to the baseline. The long latency between the addition of 80 μM Ca and the onset of the rise in the Na efflux is the consequence of the slow rise in Ca_i owing to washout of free $EGTA_i$. Addition of Na_o in the absence of Ca_o increased the Na efflux to the same level as that observed with 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 μM Ca_i. Under these conditions, the efflux of Na reached a steady value of 9 $pmol \cdot cm^{-2} \cdot s^{-1}$. Increasing the pH_i to 8.5 elicited an increase in the efflux to ~22 $pmol \cdot cm^{-2} \cdot s^{-1}$. 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 ~9 $pmol \cdot cm^{-2} \cdot s^{-1}$. 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}Ca and ^{24}Na (see Materials and Methods). This procedure allows the determination of the Na_o-dependent Ca efflux, Ca_o-dependent Ca efflux, Ca_o-dependent Na efflux, and Na_o-dependent Na efflux components. At pH_i 7.3 and in the presence of 80 μM Ca_i, the efflux of Ca and Na reached values of ~1.6 and 8 pmol·cm^{-2}·s^{-1}, respectively.

Removal of both Na_o and Ca_o dropped both fluxes to "leak" values. When Ca_o was added in the absence of Na_o, a Ca_o-dependent Na efflux of ~6 pmol·cm^{-2}·s^{-1} was obtained with little (120 fmol·cm^{-2}·s^{-1}) activation of the Ca_o-dependent

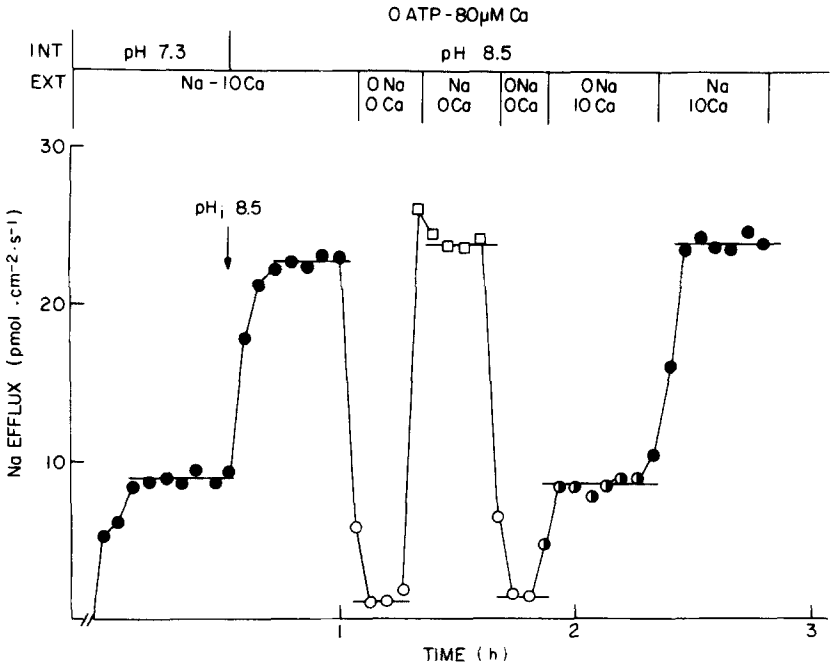

FIGURE 2. The effect of internal alkalinization on the Na_o- and Ca_o-dependent components of the Na efflux in the presence of Ca_i. The arrow indicates the change in the internal medium from pH 7.3 to 8.5. Note that the sum of the Na_o- and Ca_o-dependent Na efflux components is greater than the level of the efflux in artificial seawater. Unless otherwise stated, all concentrations are in millimolar. Axon diameter, 490 μm.

Ca efflux. Under these conditions, raising pH_i to 8.5 caused an increase in the Ca_o-dependent component of the Na efflux, with no activation of the Ca_o-dependent Ca efflux. Readmission of Na ions to the external medium caused a large increase in both the Na_o-dependent Na efflux and the Na_o-dependent Ca efflux. If the 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, Ca_o was increased up to 50 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 pmol·cm^{-2}·s^{-1}. When 2 mM ATP was introduced via the dialysis medium, a net increment of 13 pmol·cm^{-2}·s^{-1} in the Na efflux

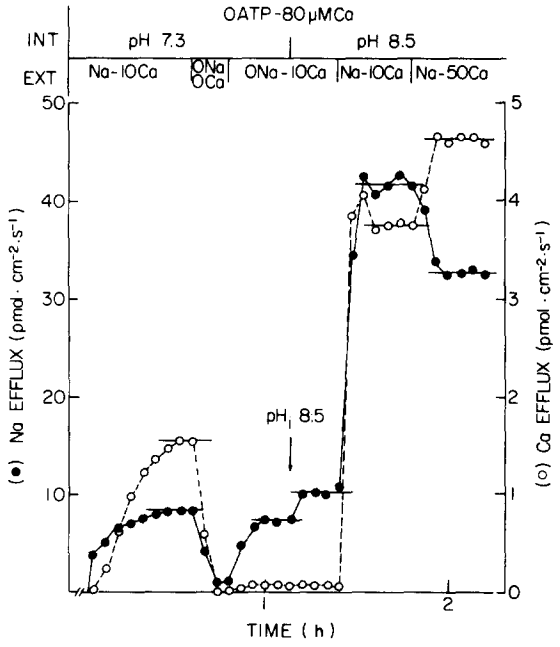

FIGURE 3. The effect of internal alkalinization on the Na_o and Ca_o components of the Na and Ca effluxes in the presence of Ca_i. The axon was predialyzed for 50 min before the addition of the radioactive medium containing both ^{24}Na and ^{45}Ca. The 4-min samples were counted immediately in a gamma counter to determine the efflux of Na. After sufficient decay of the ^{24}Na, the samples were counted for ^{45}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 pmol·cm^{-2}·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 alkalinization 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 Na_i/Ca_o exchange occurs in the absence of Ca_i (DiPolo, 1979; DiPolo and Beaugé, 1986). If the Na_o-dependent Na efflux is an operational mode of the Na/Ca exchange system, then its activation by internal alkalinization should also depend on Ca_i. Fig. 5*A* shows that in an axon dialyzed without either Ca (2 mM free EGTA) or ATP and bathed in artificial seawater, internal alkalinization caused a very small activation in the Na efflux (~0.5 pmol·cm^{-2}·s^{-1} as compared with that in the presence of Ca_i, 13 pmol·cm^{-2}·s^{-1}; see

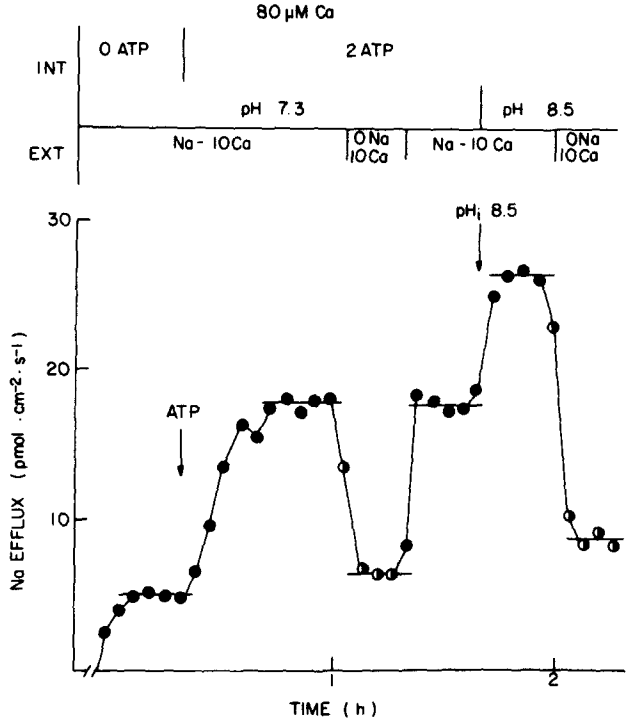

FIGURE 4. The effect of internal alkalinization on the Na_o- and Ca_o-dependent Na efflux components in the presence of Ca_i and ATP. The arrows indicate changes in the internal medium. Different symbols represent different external solutions. Axon diameter, 570 μm.

Fig. 2). Addition of 2 mM ATP in the absence of Ca_i caused an increase in the efflux of Na that was dependent on the presence of Na_o. Fig. 5*B* 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, Na_o-dependent Na efflux in the absence of 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 Ca_i. In three other axons, no significant effect of internal alkalinization was found on the ATP-activated, Ca_i-independent, 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

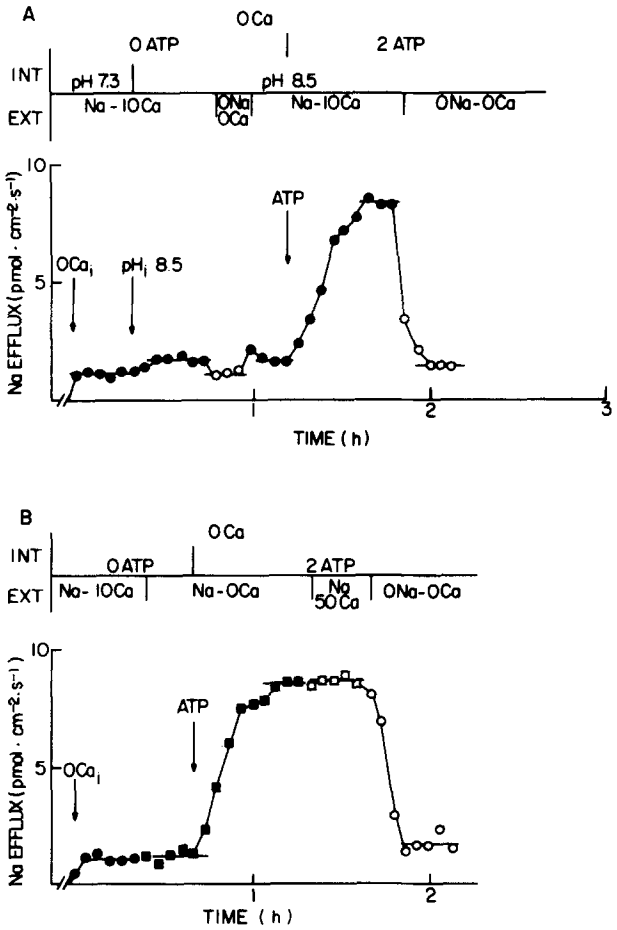

FIGURE 5. (*A*) The effect of internal alkalinization and ATP on Na efflux in an axon dialyzed without Ca_i. (*B*) The effect of Ca_o and ATP on the Na efflux in an axon dialyzed without Ca_i. The arrows indicate changes in the composition of the dialysis medium. Changes in the external medium are indicated 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 Na_i/Na_o and Na_i/Ca_o exchanges.

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

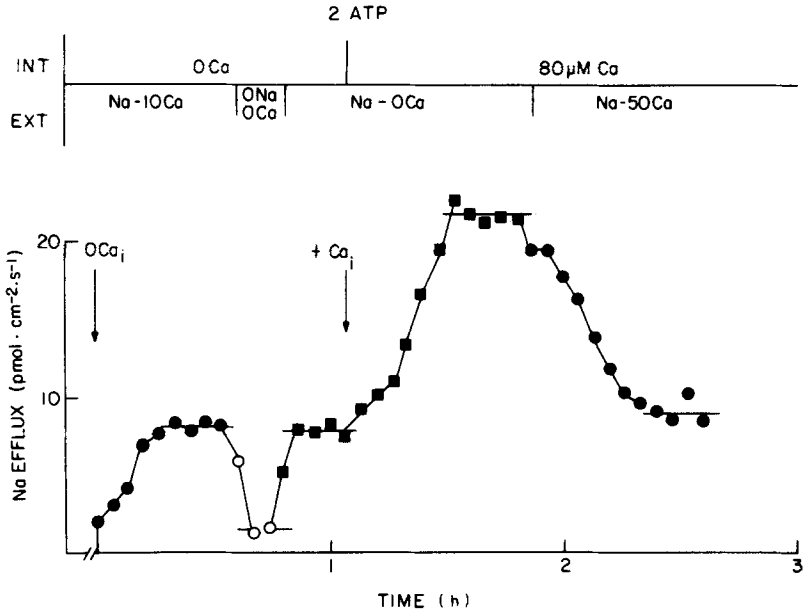

FIGURE 6. The effect of Ca_o on the Na_o-dependent Na efflux component in the present of ATP. The arrow indicates the addition of 80 μM ionized Ca to the dialysis medium. Note the large inhibition in the Na_o-dependent Na efflux upon increasing Ca_o to 50 mM. All concentrations are in millimolar except Ca_i, which is in micromolar. Axon diameter, 430 μm.

Effect of Mg_i on the Na_o- and Ca_o-dependent Na Efflux

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

the Ca_o-dependent one. Furthermore, the sum of the Na_o- and Ca_o-dependent components (13 pmol·cm^{-2}·s^{-1}) was greater than the magnitude of the Na efflux in the presence of both Na_o and Ca_o (~9.7 pmol·cm^{-2}·s^{-1}), which is in line with the hypothesis that the Na_o- and Ca_o-dependent components of the Na efflux share the same transport system.

Effect of ATPγS on the Na_o- and Ca_o-dependent Na Efflux

The preceding experiments show that in the absence of Ca_i, ATP activates an Na_o-dependent Na efflux that appears to be different from the Na_o-dependent

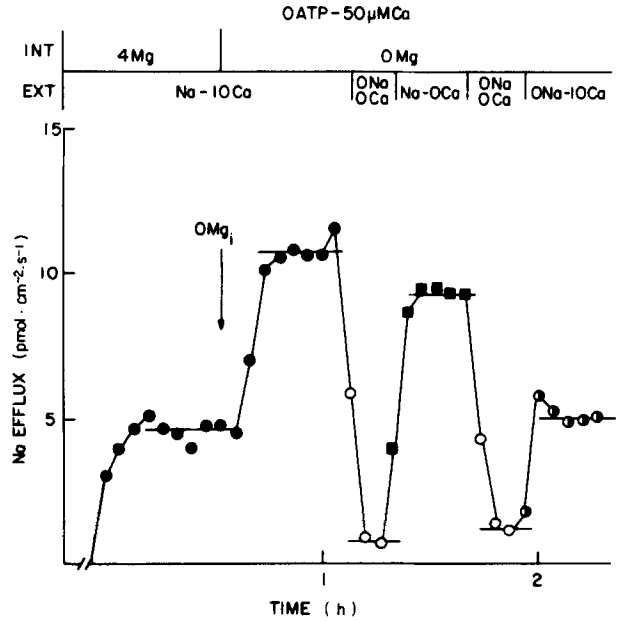

FIGURE 7. The effect of Mg_i ions on the Na_o- and Ca_o-dependent Na efflux components. The arrow indicates the removal of Mg from the dialysis medium. The axon was dialyzed from the beginning with 50 μM Ca. Unless otherwise stated, all concentrations are in millimolar. Different symbols represent different external solutions. Axon diameter, 620 μm.

Na efflux component activated by Ca_i. The former is neither activated by internal alkalinization nor inhibited by Ca_o (see Fig. 5). Another point in favor of this hypothesis comes from experiments using the ATP analogue ATPγS. In the experiment of Fig. 8*A*, 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 ~1.3 pmol·cm^{-2}·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 Ca_i to 40 μM caused an activation of the Na efflux to a steady state value of ~13 pmol·cm^{-2}·s^{-1}. This activation was totally dependent

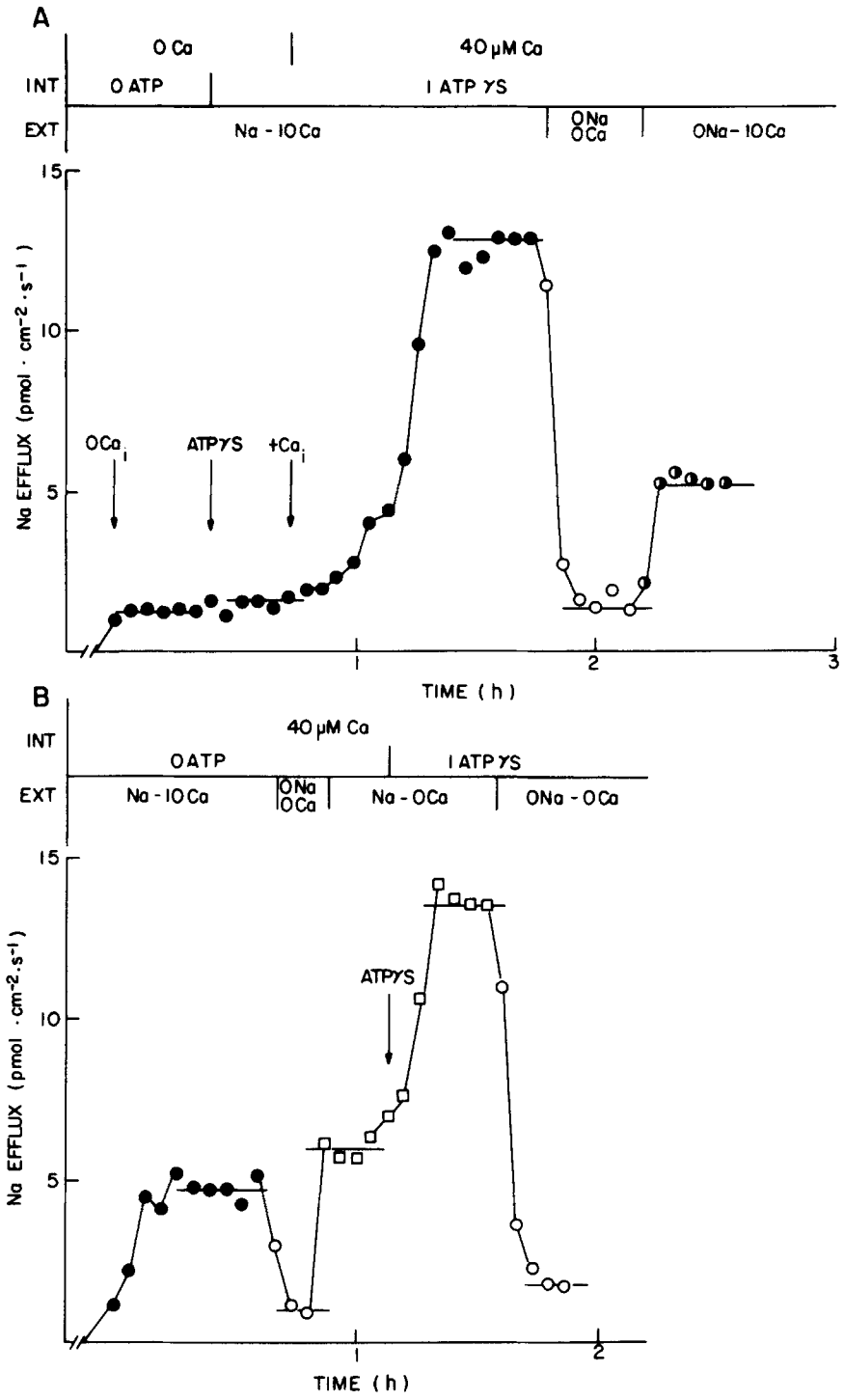

FIGURE 8.

on the presence of Na_o and Ca_o. Again, as in the case for the activation of the Na efflux by ATP in the presence of Ca_i, a large proportion of the ATP-stimulated flux corresponded to the 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 ATPγS. It should be mentioned that no activation by the analogue was observed in the absence of Mg_i (results not shown). Fig. 8*B* shows an experiment in which ATPγS was added to an axon already containing 40 μM Ca_i. Clearly, the analogue induced a large increase in the Na_o-dependent Na efflux component.

Dependence of Na_o- and Ca_o-dependent Na Efflux on Na_o and Ca_o

The Na_o and 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 Ca_i (100 μM) and ATP (2 mM) and bathed in an external medium containing no Na_o or Ca_o. In three different axons, steady state Na effluxes were measured at different values of Na_o (in the absence of Ca_o). Na_o ions activated the Na efflux with relatively low affinity ($K_{½}$ = 125 mM). Since no clear saturation was found with 440 mM 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 Ca_o and in the absence of Na_o (Fig. 9*B*). Ca_o ions activated the reversal of the exchange with a $K_{½}$ of 5 mM.

ATP Dependence of Na_o-dependent Na Efflux

Fig. 10 summarizes the results of several experiments in which the activation by ATP on the Na_o-dependent Na efflux was determined in the presence of Ca_i. In all these experiments, the axons were predialyzed for ~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 $K_{½}$ for the ATP activation is ~120 μM. Interestingly, this value is close to the activation of the Na_o-dependent Ca efflux by ATP (DiPolo and Beaugé, 1979). As is the case for the ATP-stimulated, Na_o-dependent Ca efflux (DiPolo and Beaugé, 1984), both Na_o- and Ca_o-dependent Na effluxes require Mg ions for the nucleotide effect (results not shown).

Ca_i Dependence of Na_o- and Ca_o-dependent Na Effluxes

Inasmuch as the reversal mode of the Na/Ca exchange (Na_i/Ca_o exchange) requires the presence of micromolar amounts of Ca_i for activation (DiPolo and

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FIGURE 8. (*opposite*) (*A*) The effect of ATPγS on the Na_o- and Ca_o-dependent Na efflux components in the absence and presence of Ca_i. (*B*) Effect of ATPγS on the Na_o-dependent Na efflux in an axon containing 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 Ca_i, which is in micromolar. Note the lack of activation of the ATP analogue when no Ca_i is included in the dialysis medium.

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

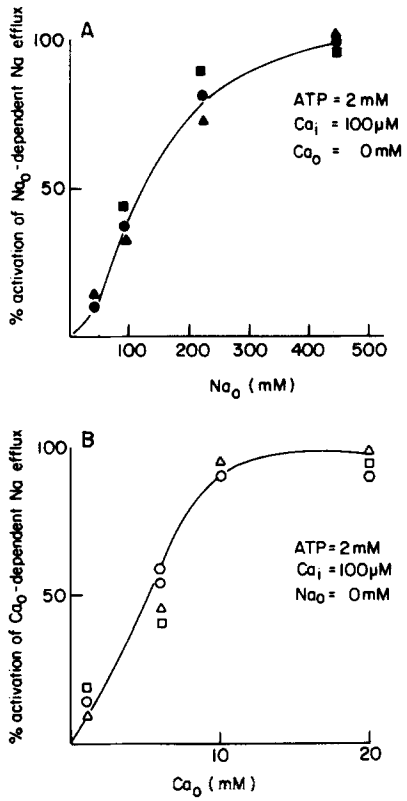

FIGURE 9. (*A*) Dependence of Na_o-dependent Na efflux on Na_o. (*B*) Dependence of Ca_o-dependent Na efflux on Ca_o. In all these experiments, the axons were dialyzed from the beginning with an internal medium containing saturating concentrations of Ca_i and ATP. Each symbol represents a different axon. The Na_o-dependent component was determined at different concentrations of Na_o in the absence of Ca_o. The Ca_o-dependent component was determined at different concentrations of Ca_o in the absence of Na_o. The mean temperature in these experiments was 17.5°C. The steady state values of the Na efflux at 440 mM Na_o and 20 mM Ca_o were taken as 100% activation of the Na_o- and 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 Ca_o-dependent component was measured in the absence of Na_o. Similarly, the Na_o-dependent Na efflux component was determined in the absence of Ca_o. In the absence of ATP, Ca_i activated the Ca_o-dependent Na efflux, with an apparent $K_{1/2}$ of 15 μM. In the presence of 2 mM ATP, the

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

DISCUSSION

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

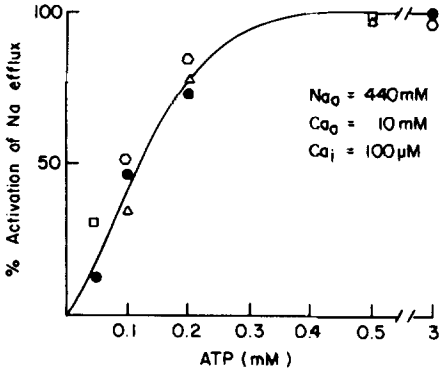

FIGURE 10. Effect of internal ATP on the ouabain- and bumetanide-insensitive Na efflux in the presence of Ca_i. Ordinate: Na efflux relative to the maximum efflux obtained in the presence of 3 mM ATP. Abscissa: ATP concentration in the dialysate in millimolar. The ATP in the axoplasm was buffered by 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 ~60 min. The external medium always contained TTX, cyanide, ouabain, and bumetanide.

with ouabain and bumetanide, Ca_i activates both Ca_o- and Na_o-dependent Na efflux components. Internal ATP stimulates both Ca_i-activated Na_o- and Ca_o-dependent Na efflux components through the Na/Ca exchange system. The nucleotide also induces a Ca_i-independent, Na_o-dependent Na efflux component.

Ca_i-activated Na_i/Na_o and Na_i/Ca_o Exchange

Although an 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 Na_o and activated at low Na_o. This finding has been interpreted as a competition between Na_o and Ca_o ions and an activation

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

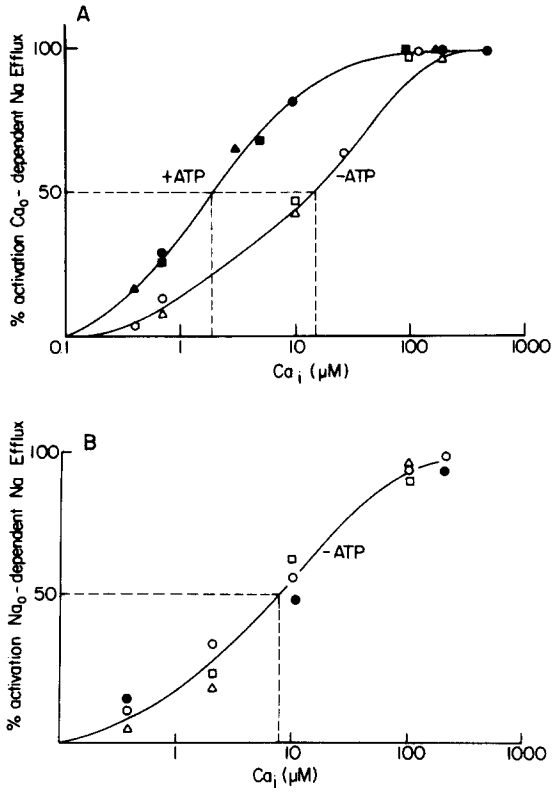

FIGURE 11. (*A*) The effect of Ca_i on the Ca_o-dependent Na efflux in the absence and in the presence of ATP. (*B*) The effect of Ca_i on the Na_o-dependent Na efflux in the absence of ATP. Each symbol represents a single axon. In these experiments, the axons were predialyzed without Ca_i and with or without ATP before the addition of the radioactive medium. The ordinate represents the percent activation of the Ca_o (*A*) or Na_o-dependent Na efflux (*B*) when taken as 100% the steady state Na efflux obtained at 200 μM Ca_i. The ionized Ca in the dialysis medium was controlled by varying the ratio (CaEGTA/free EGTA) at a constant total EGTA of 2 mM. The apparent dissociation constants for the CaEGTA and CaATP complexes were chosen as 0.15 μM and 1.4 mM, respectively (see Methods for references). Half-maximal activation for the Ca_o-dependent component was obtained at 15 μM in the absence of ATP and at 1.8 μM in the presence of ATP (2 mM). Half-maximal activation for the Na_o-dependent component was obtained at 9 μM 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 alkalinization, 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 alkalinization 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/Na 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 alkalinization. (*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 ($K_{1/2} \sim 200$ μM; 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 ($K_{1/2} \sim 130$ μM). It could be thought that the effect of ATP is somehow related to the activating effect of internal alkalinization 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 alkalinization 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. 11*A*).

The effect of ATPγS. 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 ATPγS 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 ATPγS does not promote any Na efflux component in the absence of Ca_i ions (see Fig. 8*A*) 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_i-dependent protein kinase. Since in the experiments with ATPγS, ouabain and bumetanide were not added to the external solutions and Mg_o 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_i/Mg_o 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 ATPγS substantially increases the forward (Na_o-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_i-independent, ATP-activated Na_i/Na_o Exchange

The results presented here confirm the early finding that in squid axons there is a Ca_i-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_i-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_i-independent, Na_o-dependent Na efflux (6.5 $pmol \cdot cm^{-2} \cdot s^{-1}$, average of four axons) is unaffected by internal alkalinization (see Fig. 5, *A* and *B*), in marked contrast to the Ca_i-activated Na/Na exchange system (see Fig. 4). Second, Ca_o 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_i or K_o (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 ATPγS is unable to activate this component (see Fig. 8*A*); this demonstrates a specificity of ATP in activating the Ca_i-independent Na/Na exchange compared with the Ca_i-dependent Na/Na exchange, which can be activated either by ATP or ATPγS. Unfortunately, no difference in the apparent affinity for ATP exists between these two Na/Na exchange components (see Fig. 3*A* of Beaugé and DiPolo, 1981). It is worthwhile to mention that if the Ca_i-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_i-activated Na_i/Ca_o and Na_i/Na_o 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_i (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 Bassingthwaighte, 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_i-activated Na_i/Na_o 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_i-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_i. This is in contrast to the case of the Ca/Ca exchange mode, which is only significant in the absence of Na_o and in the presence of other external alkali metal ions (Blaustein, 1977).

The physiological consequences of the effect of Ca_i 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_o-dependent Na efflux. The requirement for Ca_i, 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_i-activated outward current generated during the operation of the Na_i/Ca_o 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_i 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_i is a generalized property of the exchanger, then Ca entry via voltage-dependent Ca channels (increase in Ca_i) could modulate the activity of the exchanger. In support of this possibility is the fact that an increase in Ca_i 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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