Calcium Efflux from Squid Axons under Constant Sodium Electrochemical Gradient

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ABSTRACT The effect of varying Na_o and Na_i on Ca efflux while maintaining the ratio Na_o/Na_i constant was explored in squid giant axons dialyzed with and without ATP. In the absence of ATP, the Ca efflux increased 3.4 ± 0.2-fold when the Na_o/Na_i concentrations were reduced from 440/80 to 110/20 mM. In the presence of ATP a similar change did not have an appreciable effect. The inhibition of Ca efflux produced by Na_i was studied in the presence and in the absence of ATP. In the absence of ATP, inhibition is very marked and is reminiscent of a unimolecular noncompetitive reaction (inactivation constant $[K_I]$ of 34 ± 5 mM of Na_i) whereas in the presence of ATP, the slight inhibition observed indicates that ATP probably increases the K_I to 200 mM. From the inhibition of the Ca efflux produced by Na_i in the presence or absence of ATP a curve describing the dependence of Na_i of the ATP-promoted fraction of Ca efflux was constructed. The effect of Na_o on Ca efflux was studied as a function of $[Na]_i$: at low Na_i, an activation constant (K_A) of 41 mM for Na_o was obtained either in the presence or in the absence of ATP. As the intracellular Na is increased in the presence of ATP, Na_i seems to have no effect on the apparent half-activation constant. However, in the absence of ATP, the K_A for activation increases along a sigmoid curve reaching a value of 112 mM at 100 mM Na_i. It is concluded that the Ca efflux system uses the energy of the Na electrochemical gradient. The action of Na_i appears to be such that the interaction of a single Na^+ is sufficient to block Ca extrusion whereas several Naps externally are necessary to activate Ca extrusion.

INTRODUCTION

The mechanism responsible for maintaining the very low concentration of ionized intracellular Ca observed in nerve cells (DiPolo et al., 1976) must reside in the cell membrane (Hodgkin and Keynes, 1957). Much work has been done in attempting to characterize this mechanism and to determine the nature of its energy source which is capable of supporting the extrusion of Ca against a large and inwardly directed electrochemical gradient (Baker, 1972, 1976; Blaustein, 1974, 1976; Mullins, 1976). Only three possibilities are thought to be feasible for the energy source of this transport process: the inwardly directed Na electrochemical gradient; the hydrolysis of a high energy nucleotide such as ATP; or a combination of both. The idea that the energy for extruding Ca out of a cell is derived from the coupled inward movement of several Na ions down their electrochemical gradient was developed by Reuter and Seitz (1968) in their study of Ca efflux in cardiac muscle, and by Blaustein and Hodgkin (1969) in their

J. GEN. PHYSIOL. © The Rockefeller University Press · 0022-1295/78/1001-0443$1.00

Volume 72 October 1978 443-470

demonstration that most of the Ca efflux from squid axon depended on external Na (Na_o). The intracellular dialysis technique, developed by Brinley and Mullins (1967), made possible the study of the effect of highly labile compounds such as ATP on transport mechanisms. DiPolo (1974) clearly showed that the addition of ATP to the internal media in a dialyzed squid axon caused a trebling of the Ca efflux level observed in the virtual absence of the nucleotide. This fraction of Ca efflux stimulated by ATP was shown also to be dependent upon Na_0 and Ca_0. In injected axons a similar effect of ATP has been inferred (Baker and Glitsch, 1973). Although the above-mentioned evidence and the specificity of the mechanism for ATP (DiPolo, 1976, 1977) would appear to strengthen the hypothesis that ATP can energize the Ca efflux mechanism, the hydrolysis of ATP as a direct result of Ca extrusion has not been observed.

In the present study, the effect on Ca efflux of varying the concentrations of external and internal Na, while maintaining a constant electrochemical gradient for Na, was studied in squid giant axons dialyzed with and without ATP. This study of the effect of the absolute concentrations of Na on Ca efflux is complemented by separate observations, in the presence and in the absence of ATP, of the effect on Ca efflux of the concentration of internal Na (Na_i) at constant Na_0 and of the concentration of external Na at constant Na_i. In the discussion an explanation is developed for the effect on the Ca efflux of the absolute concentrations of Na, at a constant electrochemical gradient for Na.

A preliminary report of these findings has been communicated to the AsoVAC (Asociacíon Venezolana para el Avance de la Ciencia) (Requena, 1976), and to the Biophysical Society (Requena, 1978).

METHODS

The experiments reported here were performed on giant axons isolated from living specimens of the tropical squid *Dorytheutis plei*. The hindmost axon from the stellate ganglion was dissected and carefully cleaned of connective tissue under a dissecting microscope. Axon diameters were measured using a calibrated eyepiece and usually were of the order of 400 μm.

The dialysis chamber had provisions for stimulation and extracellular recording of action potentials. In all the experiments reported here, isotope was collected as long as the axon showed signs of electrical activity, provided the external solution permitted such a response. The axon was kept in the dialysis chamber at 18 ± 1°C under continuous solution flow. The apparatus and basic technique for internal dialysis of giant excitable cells have been described previously and were used in this study with minor modifications (Brinley and Mullins, 1967; Brinley et al., 1975; DiPolo, 1977). Plastic tubing, specially manufactured by Fabric Research, Ltd. (Needham, Mass.) and kindly supplied by Professors F. J. Brinley and L. J. Mullins, was used for dialysis capillaries. The tubing was 145 μm OD × 95 μm ID. It was stretched (DiPolo, 1977) and rendered porous by soaking for 24 h in 0.05 M NaOH, 0.005 M EDTA (ethylenediaminetetraacetic acid). The porosity of the capillaries was occasionally checked by measuring the amount of ^{45}Ca which permeated through the porous wall. It should be mentioned that although some internal dialysis media contained no Na, this concentration is probably not the actual one at the inner side of the axolemma. For this reason the experimental condition of zero Na_i is referred throughout as nominally zero Na_i. Most probably the actual concentration of internal Na is in the neighborhood of 1–4 mM under the condition Na_o/Na_i = 440/0.

Solutions

The composition of the external and internal solutions used in the experiments is listed in Table I. Solutions which required a nonstandard concentration of an electrolyte were prepared by mixing appropriate amounts of stock solutions. All of the inorganic chemicals used in the preparation of the solutions were reagent grade and when possible they were chosen so as to have minimum quantities of contaminant Ca, Na, and Mg. Those solutions described as free of a given cation, usually showed a contamination in the micromolar range for that particular ion as determined with emission (Na) or atomic absorption spectrometry (Ca, Mg). The external solutions were prepared $SO_4^=$-free to avoid complexation of Ca and (or) Mg. The pH of these solutions was buffered to 7.8 with 10 mM of Tris (tris (hydroxymethyl) aminomethane).

The biochemical reagents used in this work were purchased from Sigma Chemical Co. (St. Louis, Mo.) with the exception of Hepes (*N*-2-hydroxyethylpiperazine-*N*'2-ethane-

TABLE I

SOLUTIONS

	External				Internal			
Constituents	ASW	O Na	O Na O Ca	O Ca	ANa	ACh	HNa	HCh
				mM				
K	10	10	10	10	340	340	240	240
Na	440	0	0	440	100	0	200	0
Mg	50	50	50	60	5	5	5	5
Ca	10	10	0	0	*	*	1.01	1.01
Choline	0	440	460	0	0	100	0	200
TRIS	10	10	10	10	0	0	0	0
Cl	575	575	575	575	110	110	210	210
CN^-	1	1	1	1	0	0	0	0
EDTA	0.1	0.1	0.1	0.1	0	0	0	0
EGTA	0	0	0	0.1	1	1	1	1
Aspartate	0	0	0	–	330	330	230	230
Glycine	0	0	0	–	300	300	300	300
Hepes	0	0	0	–	10	10	10	10
pH	7.8	7.8	7.8	7.8	7.3	7.3	7.3	7.3
mosmol/kg	1,010	1,010	1,010	1,010	990	990	990	990

* Variable (see text).

sulfonic acid) which was purchased from Calbiochem (San Diego, Calif.). The pH of the internal solutions was set to 7.3 with 10 mM of Hepes-KOH buffer mixture. Stock solutions of 100 mM Tris or Mg ATP neutralized to pH 7.2 were stored at −20°C. When necessary, aliquots of this work solution were added, before the experimental run, to the radioactive dialysis solutions.

To destroy the functional capacity of mitochondria to produce ATP and (or) sequester calcium from the axoplasm, the external media was always made to contain 1 mM CN^- by addition of an aliquot of freshly made stock CN^- solution (Na^+ or K^+ as required), while to the internal solution 0.1 μl/ml of a dimethylsulfoxide solution containing 2 and 5 μg/ml of the uncoupler FCCP and oligomycin was added. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) was a kind gift from Dr. A. Scarpa.

Although isethionate is a normal constituent of the axoplasm, it was excluded from the dialysis media because the commercially available K salt (Eastman Organic Chemicals

Div., Eastman Kodak Co., Rochester, N. Y.) is heavily contaminated with Na and Ca. The ionized calcium concentration of the internal solution was set at will by using EGTA (ethyleneglycol-bis-(β-aminoethyl ether) *N,N'* tetraacetic acid) as a calcium buffer system. The total calcium concentration required to give a desired ionized fraction was produced by adding given amounts of $CaCl_2$ from stock solutions to the internal dialysis solution which always contained 1 mM EGTA. In the computation of the fraction of the total calcium which is ionized, the apparent dissociation constant of 0.15 μM was taken for the Ca-EGTA complex; this is the value computed by DiPolo et al. (1976) for the complex at physiological pH and ionic strength.

The dialysis solution was made radioactive by the addition of a desired amount of $^{45}CaCL_2$ of the highest specific activity available (usually 20 mCi/mg) obtained from New England Nuclear (Boston, Mass.). As a visual tracer of the radioactive dialysis solution, an aliquot of stock solution of phenol red was added such as to give a final concentration of 0.5 mM. This solution had its final pH corrected to 7.3 $\pm$ 0.1 because this is the value that has been obtained for the axoplasm (Boron and De Weer, 1976). The osmolality of the internal dialysis solutions was set to 990 mosmol/kg using a commercial psychrometer which compared the dewpoint of the solution with that of a standard solution of NaCl (5700A osmometer, Wescor Inc., Logan, Utah). The external solutions were adjusted to 1010 mosmol/kg by similar means.

RESULTS

The Effect on Ca Efflux of the Concentration of Sodium at Constant Electrochemical Gradient for Na in the Absence of ATP

During the past few years the effect on Ca efflux of varying the concentration of Na bathing one face of the axolemma while the Na concentration is kept constant in the other side of it has been studied in some detail (Blaustein et al., 1974; DiPolo, 1974; Brinley et al., 1975). The interpretation of the results obtained, in terms of a model for the energetics of Ca transport, in which the Na electrochemical gradient is thought to be the energy source, is not unequivocal, however, inasmuch as the effect of a chemical interaction of Na with the transport mechanism could not be easily separated from that of changes in the energy supplied by the Na electrochemical gradient. In view of this, it was thought to be of interest to study the effect on Ca efflux of various Na concentrations at constant electrochemical gradient for Na. Fig. 1 shows a typical experiment. The time-course for the Ca efflux from an axon dialyzed with a medium free of ATP and an ionized calcium level buffered to 0.5 μM was observed under several concentrations of Na_o and Na_i, maintaining the ratio Na_o/Na_i constant. At the beginning of the experiment the external medium contained 440 mM Na_o and 80 mM Na_i. At this level of Na concentrations, called the control condition, this axon showed a steady-state Ca efflux of 0.08 $pmol \cdot cm^{-2} \cdot s^{-1}$. When the Na concentrations were halved to 220 mM Na_o and 40 mM Na_i the Ca efflux level increased to 0.19 $pmol \cdot cm^{-2} \cdot s^{-1}$, even though these new concentrations of Na had the same ratio as in the control condition. Further reduction of the absolute concentration of Na to 165/30 mM Na_o/Na_i raised the Ca efflux to a value of 0.30 $pmol \cdot cm^{-2} \cdot s^{-1}$. When the Na concentrations were made one-fourth that of the control condition, 110 and 20 mM of Na_o/Na_i, respectively, the calcium efflux reached a peak value of 0.43 $pmol \cdot cm^{-2} \cdot s^{-1}$. This level for the Ca efflux represents an almost fourfold increase when

compared with 0.12 pmol·cm^{-2}·s^{-1} observed when the Na concentrations were returned to the control levels. Finally, a large fraction of the Ca efflux level was dependent on the presence of external Na and Ca, as shown at the end of the experiment.

Table II summarizes the results of all the experiments done in a fashion

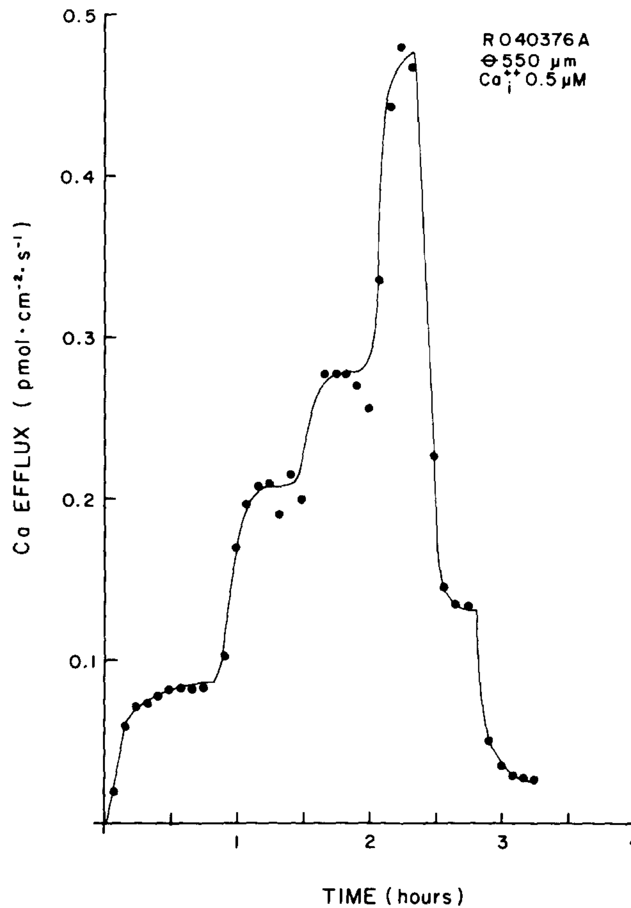


FIGURE 1. The time-course of the Ca efflux under various concentrations of Na_o/Na_i at a constant electrochemical gradient for Na in an ATP-free dialyzed squid axon.

similar to the one just described. In all of these experiments the intracellular ionized calcium concentration was buffered to 0.5 μM while the extracellular Ca was kept constant at 10 mM. This table lists the observed Ca efflux level for each test condition, the ratio of external/internal Na concentrations always being the same. In all cases listed, the Ca efflux level increases above that of the control condition when both concentrations of Na were proportionally reduced.

Fig. 2 shows relative Ca efflux values plotted as a function of the external and internal concentrations of Na at a constant electrochemical gradient for this cation. For each experiment listed in Table II, Ca efflux values were taken and normalized with respect to the level observed at 440/80 mM Na_o/Na_i, to which the value of 1.0 was assigned. The line drawn in the figure is the smooth curve that joins the mean for all of the experimental points for a given set of Na concentrations. It can be seen that the reduction of the absolute concentrations of sodium at constant electrochemical gradient for Na and in the absence of ATP slowly increases the Ca efflux level to a maximum of 3.4 ± 0.2 (mean ± SEM) at 110/20 mM of Na_o/Na_i. Further reduction of the absolute concentrations of sodium rapidly decreases the Ca efflux level as Na_o and Na_i approach zero.

TABLE II

SENSITIVITY OF Ca EFFLUX TO THE CONCENTRATION OF SODIUM AT CONSTANT ELECTROCHEMICAL GRADIENT FOR THIS CATION IN THE ABSENCE OF ATP

Axon reference	External/internal sodium concentration (mM/mM)									
	440/80	396/72	330/60	275/50	220/40	165/30	110/20	66/12	44/8	22/4
	Ca efflux ($pmol \cdot cm^{-2} \cdot s^{-1}$)									
R 290176	0.29	–	–	–	0.59	–	–	–	–	–
R 030276 A	0.14	–	–	–	0.39	–	–	–	–	–
R 030276 B	0.19	–	–	–	0.45	–	0.56	–	–	0.21
R 190276	0.16	0.17	0.17	–	0.35	–	–	–	–	–
R 240276 B	0.11	–	–	0.20	0.37	–	–	–	–	–
R 040376 B	0.08	–	–	–	0.19	0.30		–	–	–
R 230376 B	0.12						0.43			
R 240376	0.22	–	–	–	–	–	0.87	–	–	–
R 260376 A	0.20	–	–	–	–	–	0.89	–	–	–
R 260376 B	0.29	–	–	–	–	–	0.92	–	–	–
R 270376	0.30	–	–	–	–	–	0.88	–	–	–
R 010476	0.40	–	–	–	–	–	1.00	–	–	–
R 020476	0.30	–	–	0.43	–	0.67	–	–	–	–
	0.18	–	–	–	–	–	0.61	0.48	0.48	–

Ca_i buffered to 0.5 μM.

The observed behavior of the Ca efflux level as a function of the absolute concentrations of Na, at constant electrochemical gradient for Na, cannot be explained in terms of changes in the resting membrane potential, because the threshold for excitation scarcely changed throughout the experiment, nor in terms of the electrochemical gradient for Ca because that was kept constant. Therefore, the effect described in Fig. 2 must be associated with the absolute concentrations of Na. The biphasic nature of the curve seen in this figure indicates that two processes may be occurring concomitantly with the removal of Na from the extra- and intracellular media. The increase in Ca efflux level from 0 up to the maximum of 3.4 (at 110/20 mM Na_o/Na_i) argues in favor of the appearance of an activating factor, probably the external Na, that energizes the

transport mechanism, whereas the decrease in the Ca efflux level from 3.4 toward the reference value of 1.0 (at 440/80 mM Na_o/Na_i), argues in favor of the action of an inhibitory factor, probably internal Na.

The Effect on Ca Efflux of the Concentration of Sodium at Constant Electrochemical Gradient for Na in the Presence of ATP

Fig. 3 shows that time course of the Ca efflux from an axon dialyzed with Na_o/Na_i = 440/80 and Na_o/Na_i = 110/20, both of which exhibit the ratio 5.5. The effects of these Na concentrations were tested in the absence and in the presence of ATP. The intracellular Ca concentration was buffered to 0.5 μM while Ca_o

FIGURE 2. The effect on Ca efflux of the absolute concentrations of Na_o/Na_i at constant electrochemical gradient for Na in the absence of ATP. Ca efflux values were normalized with respect to the level observed at 440/80 mM Na_o/Na_i. See text for further details.

was kept constant at 10 mM. In the first half of the experiment the axon was exposed, in the absence of internal ATP, to the control concentrations of Na_o/Na_i, i.e., 440/80 mM. Once the Ca efflux level reached a steady-state value of 0.30 $pmol \cdot cm^{-2} \cdot s^{-1}$, both concentrations of Na were reduced to one-fourth that of the control value. As shown previously, this experimental condition produces an increase in Ca efflux compared to that level at the control concentrations of Na. At this point the introduction of 1 mM (Tris) ATP into the dialysis media increased Ca efflux from 0.88 $pmol \cdot cm^{-2} \cdot s^{-1}$ to 1.48 $pmol \cdot cm^{-2} \cdot s^{-1}$. Finally, the restoration of the control concentrations of Na in the presence of ATP, produced a reduction in the Ca efflux to a new steady state value of 1.29 $pmol \cdot cm^{-2} \cdot s^{-1}$. It should be noticed that the change in Ca efflux due to the variation of the concentrations of Na from 110/20 to 440/80 mM of Na_o/Na_i

represented a 1.15-fold increase in the level of Ca efflux in the presence of ATP, whereas a similar treatment done at the beginning of the experiment, but in the absence of ATP, caused roughly a threefold increase.

Table III summarizes the results of all the experiments carried out following the protocol described above. Ca efflux values are given in the absence and in the presence of ATP at each set of test concentrations of Na_o/Na_i. It can be concluded from the data presented in this table that a reduction of the concentrations of extra- and intracellular sodium from the control condition of

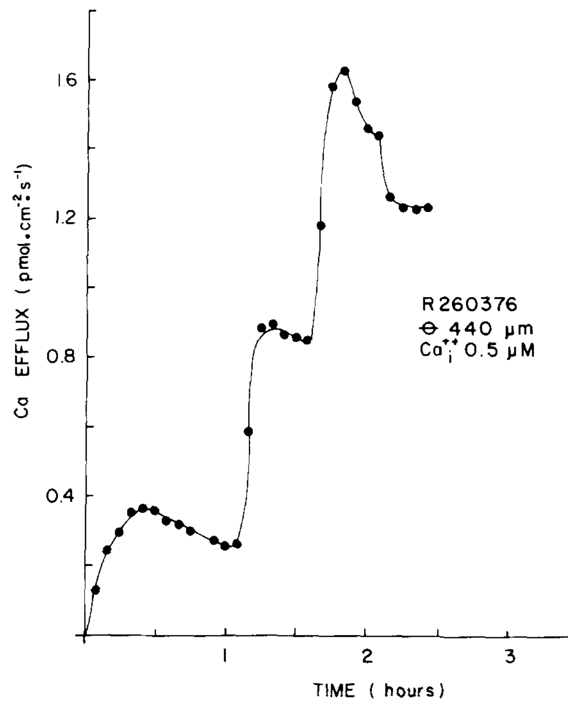


FIGURE 3. The time-course of Ca efflux at two concentrations of Na_o/Na_i which exhibit the same ratio, in the presence and in the absence of ATP.

440/80 mM to 110/20 mM produced an insignificant increase (5%) in the level of Ca efflux in the presence of ATP.

The Effect on Ca Efflux of Internal Na in the Absence of ATP

To understand the effect on Ca efflux of the concentrations of external and internal Na, at constant electrochemical gradient for Na, a series of experiments was carried out in which the effect of internal Na on the Ca efflux was studied at constant external Na (440 mM). The effect on Ca efflux of Na_i has previously

been examined by Blaustein and Russel (1975) and by Brinley et al., (1975) who observed an inhibition of Ca efflux with increasing concentration of Na_i.

In the experiment plotted in Fig. 4 *a*, the time-course for the Ca efflux is shown for an axon dialyzed with various concentrations of Na_i in a medium free of ATP and with an ionized Ca concentration set to 250 μM. At the beginning of the experiment, the axon was dialyzed with a solution containing 0 mM Na, all of the internal Na having been replaced by choline. The external medium, which was kept constant almost up to the end of the experiment, contained the normal concentration of Na (440 mM) and Ca (10 mM). When the Na_i concentration was made 80 mM, the Ca efflux level decreased from 3.1 $pmol \cdot cm^{-2} \cdot s^{-1}$ to 0.6 $pmol \cdot cm^{-2} \cdot s^{-1}$. This fivefold drop in Ca efflux was partially

TABLE III

SENSITIVITY OF Ca EFFLUX TO THE CONCENTRATION OF SODIUM AT CONSTANT ELECTROCHEMICAL GRADIENT FOR THIS CATION IN THE PRESENCE OF ATP

	External/internal sodium concentrations mM/mM					
	0 ATP			1 mM ATP		
Axon reference	$\frac{440}{80}$	$\frac{110}{20}$	Efflux ratio $\frac{\phi \frac{110}{20}}{\phi \frac{440}{80}}$	$\frac{440}{80}$	$\frac{110}{20}$	Efflux ratio $\frac{\phi \frac{110}{20}}{\phi \frac{440}{80}}$
	Ca efflux (ϕ) $pmol \cdot cm^{-2} \cdot s^{-1}$			Ca efflux (ϕ) $pmol \cdot cm^{-2} \cdot s^{-1}$		
R 260376 B	0.30	0.88	2.93	1.29	1.48	1.15
R 270376	0.40	1.00	2.50	1.40	1.34	0.95
R 280376	0.30	–	–	1.63	1.70	1.04
Mean			2.72			1.05 ± 0.05

Ca_i buffered to 0.5 μM.

reversed by lowering Na_i from 80 mM to 40 mM as shown by the next step of solution change. A Ca efflux level of 1.3 $pmol \cdot cm^{-2} \cdot s^{-1}$ was increased to 2.3 $pmol \cdot cm^{-2} \cdot s^{-1}$ when Na_i in the dialysis fluid was lowered 20 mM. At 10 mM Na_i, the Ca efflux was 2.5 $pmol \cdot cm^{-2} \cdot s^{-1}$ and, with the removal of the remaining Na_i, reached a value of 3 $pmol \cdot cm^{-2} \cdot s^{-1}$. The experiment ended with the replacement of all the external Na and Ca by choline, a procedure which decreased the Ca efflux to a negligible value (20 $fmol \cdot cm^{-2} \cdot s^{-1}$). This change demonstrates the existence of a sodium-calcium counter-transport mechanism in the nominal absence of internal Na.

In Fig. 4 *b* a similar experiment is shown. Here the internal ionized Ca concentration was buffered to 100 nM. One observes that the addition of 20 mM Na_i reduces the steady state Ca efflux level of 13.1 $fmol \cdot cm^{-2} \cdot s^{-1}$ seen under the condition of 0 Na_i to 8.2 $fmol \cdot cm^{-2} \cdot s^{-1}$. Subsequent addition of another 20 mM of internal Na reduced the level of Ca efflux to 6.7 $fmol \cdot cm^{-2} \cdot s^{-1}$. When the Na_i

concentration was made 100 mM, the Ca efflux reached a value of 2.4 $fmol \cdot cm^{-2} \cdot s^{-1}$. This level was raised to 5.4 $fmol \cdot cm^{-2} \cdot s^{-1}$, when 40 mM Na_i was removed from the dialysis media. Finally, replacement of the remaining internal Na by choline brought the Ca efflux level to 13 $fmol \cdot cm^{-2} \cdot s^{-1}$.

Table IV lists the results obtained for the Ca efflux as a function of the internal concentrations of Na in axons dialyzed with two concentrations of

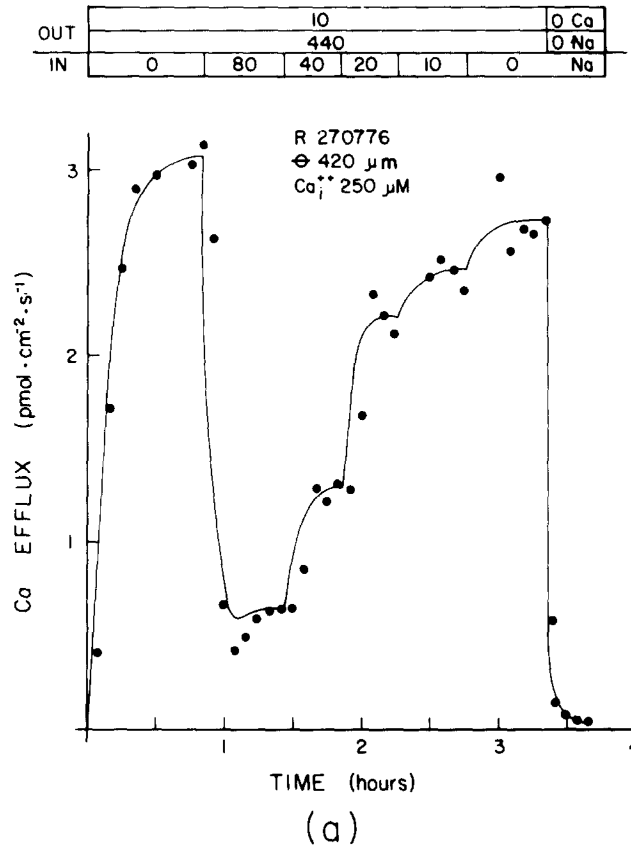
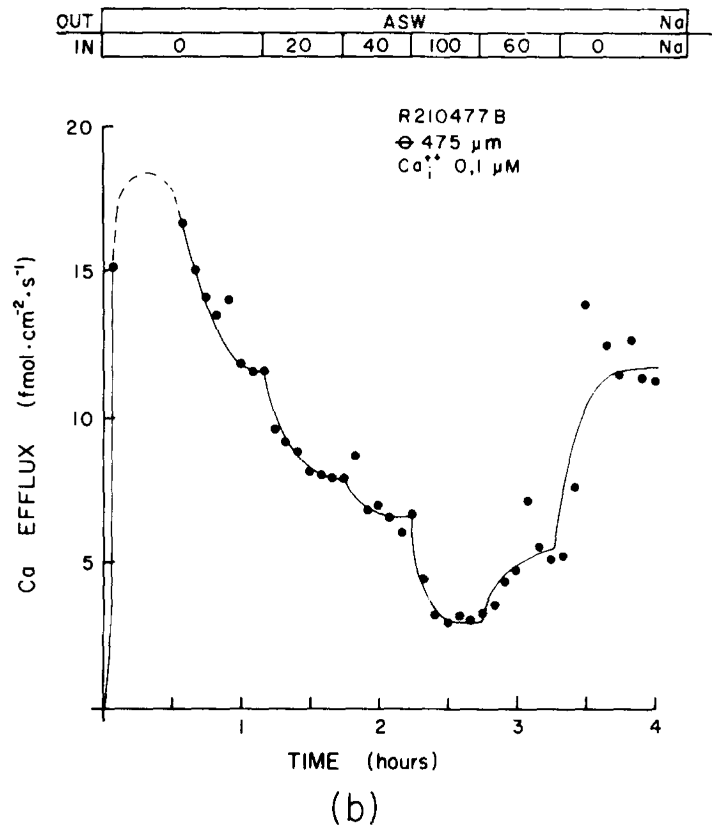


FIGURE 4. The time-course of Ca efflux at various concentrations of internal Na in axons dialyzed without ATP. (a) Ionized axoplasmic Ca concentration set at 250 μM. (b) Ionized axoplasmic Ca concentration buffered to 0.1 μM.

ionized intracellular Ca, a high level of 300 μM and a lower level of 100 nM which is a more physiological figure for the ionized axoplasmic calcium. It can be noted in this table that at either ionized Ca concentrations, the addition of internal Na always reduced the Ca efflux level below that observed under the experimental condition of nominally zero Na_i. The pattern for the inhibition produced on the Ca efflux by Na_i is more clearly shown in Fig. 5. In this figure, normalized Ca efflux levels are plotted as a function of the concentration of internal Na. For each experiment, the Ca efflux value obtained under the

condition of nominally zero Na_i is taken as 1.0, while the other Na_i-dependent Ca efflux levels are normalized accordingly. Examination of the figure and a statistical analysis of the data shows that there is no significant difference between the extent of the inhibition produced by Na_i at high or low concentrations of ionized intracellular calcium. This fact strongly argues in favor of the existence of a noncompetitive interaction in which a specific site in the transport



(b)

FIGURE 4

mechanism binds exclusively internal Na, a binding which results in an inhibition of the outward transport of Ca ions.

The data summarized in Table IV, normalized as in Fig. 5 can be fitted, by the least squares method, to an Eadie-Haldane linear transformation (Eq. 1) in an attempt to describe the molecular nature of the inhibitory phenomena of Na_i on Ca efflux.

$$\bar{I} = 1 - \frac{1 - \bar{I}_{\min}}{1 + \left(\frac{K_I}{Na_i}\right)^n} \qquad (1)$$

In Eq. 1, $\bar{I}$ represents the noninhibited fraction of Ca efflux at a given

concentration of Na_i, $\bar{I}_{min}$ is the minimum level of this noninhibited fraction to be observed. at high Na_i, K_I is the apparent dissociation constant and n is the molecularity of the inhibition process.The continuous line drawn in Fig. 5 represents the curve best fitted to the data using 1 for the molecularity of the reaction, while the broken line corresponds to a similar curve in which a value of 2 was chosen for n.

Table V lists the kinetic parameters (K_I and $\bar{I}_{min}$) obtained by fitting the data to Eq. 1, for both uni- and bimolecular reactions. It also lists the predicted noninhibited fractions of the Ca efflux for internal Na concentrations of 100 and 200 mM for uni- and bimolecular reactions. As can be seen in the table, the magnitude of the predicted fraction at 200 mM of Na_i is different for each type

TABLE IV

EFFECT OF Na_i ON Ca EFFLUX IN THE ABSENCE OF ATP

Axon reference	Ca_i^{++}	Internal sodium concentration									Flux units
		0	10	20	30	40 Ca efflux	50	60	80	100	
	μM					mM					
R 190876 A	0.10	18.1	–	–	–	11.6	–	–	–	–	$\frac{fmol}{cm^2 \cdot s}$
R 190876 B	0.10	18.0	–	–	–	–	–	6.4	2.5	–	"
R 120477	0.10	17.9	–	11.7	–	–	5.6	–	6.3	4.5	"
R 150477	0.10	18.3	–	11.3	–	–	7.4	–	5.5	5.4	"
R 210477 B	0.10	13.1	–	8.2	–	6.7	–	5.4	–	2.4	"
R 120477 A	0.25	142.0	–	–	–	–	–	–	–	26.3	"
R 270776	250	3.1	2.5	2.3	–	1.3	–	–	0.6	–	$\frac{pmol}{cm^2 \cdot s}$
R 030876	300	7.5	–	–	4.5	–	–	3.0	2.5	–	"
R 050876	300	11.6	–	–	6.9	–	–	–	3.0	–	"
R 120876	300	8.7	6.7	6.5	–	4.4	–	3.3	–	–	"
R 260477	300	5.8	–	3.4	–	2.8	–	–	–	1.4	"
R 270477	300	6.6	–	–	–	–	–	–	–	2.7	"
R 120577	300	6.0	–	–	–	–	–	–	–	1.7	"
R 130577	300	6.3	–	–	–	–	–	–	–	1.7	"
R 070677 B	300	11.2	–	–	–	–	–	–	–	3.9	"
R 090677	300	11.0	–	–	–	–	–	–	–	2.0	"

of reaction. The ratio of the estimated noninhibited fraction present at 100 Na_i to that observed at 200 Na_i is calculated to be 1.60 if the reaction is unimolecular. If the reaction is bimolecular the predicted ratio is 1.05. These ratios differ enough to permit an experimental distinction between the two processes.

To test this hypothesis, axons were dialyzed with media which contained up to 200 mM Na_i. To do this, about 100 mM of K_i must be replaced by choline or Na. Under these conditions the axons were depolarized by some 10 mV from their normal resting potential. An ionized Ca concentration of 10 μM was chosen for this experiment. Fig. 6 shows the time-course for the Ca efflux in one of the experiments in which the effect of very high internal Na was tested. At the beginning of the experiment, in the nominal absence of ATP and Na_i, a Ca efflux which increased and reached a steady value of 6.1 pmol·cm^{-2}·s^{-1} was

observed. This efflux level dropped to 0.65 pmol·cm^{-2}·s^{-1} when Na_i was made 200 mM. The replacement is the dialysis medium of 100 mM Na by choline, increased the Ca efflux value to 1.3 pmol·cm^{-2}·s^{-1}, a level which represents a doubling of the level previously observed under the condition of 200 Na_i. Further reduction of the internal Na to 30 mM brought the Ca efflux level to 3.2 pmol·cm^{-2}·s^{-1}, whereas return to the initial experimental condition of nominally zero Na_i, raised the CA efflux to its initial value. Table VI lists the Ca

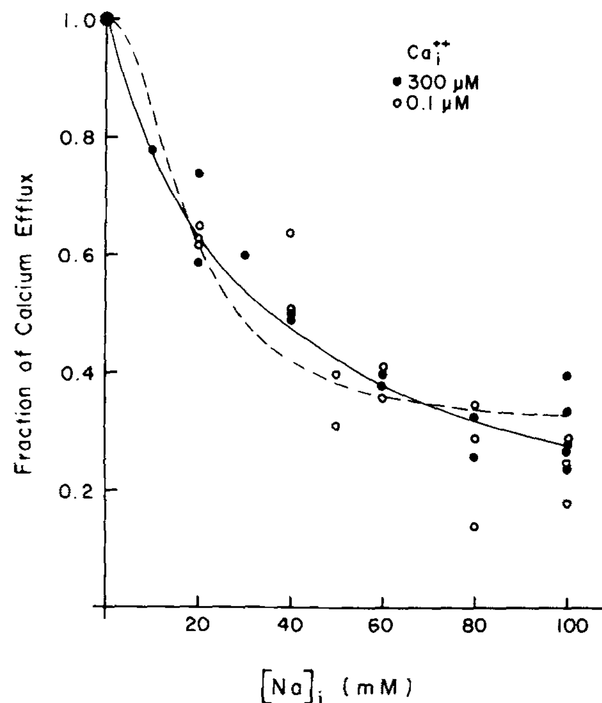


FIGURE 5. The effect on Ca efflux of internal Na at constant Na_o (440 mM) in the absence of ATP. Ca efflux values were normalized in each experiment with reference to the Ca efflux level observed at zero Na_i to which the value of 1.0 was assigned. (●) Axoplasmic ionized Ca set at 300 μM; (○) ionized Ca buffered to 0.1 μM. The continuous line corresponds to a unimolecular inhibition reaction; the broken line corresponds to a bimolecular reaction as given by Eq. 1. The kinetic parameters of the two curves are those given in Table V. For further details see text.

efflux values obtained at concentrations of 200, 100, 30, and 0 mM Na_i for the two experiments performed. As can be seen in the last column of this table, the ratio of the observed Ca efflux level at 100 mM Na_i to that observed at 200 mM Na_i was 2.0 in one experiment and 1.6 in the other. The agreement between these ratios and the predicted ratio of 1.6 for the unimolecular reaction, strongly suggests that the inhibitory effect of internal Na on the mechanism that translocates Ca outward is unimolecular in nature.

The Effect on Ca Efflux of Internal Na in the Presence of ATP

The effect on Ca efflux of the internal concentration of Na, at a constant external Na concentration of 440 mM, was explored in several axons dialyzed with ATP. Fig. 7 shows two records of the time-course of the Ca efflux under various levels of internal Na. In Fig. 7 *a* the intracellular ionized Ca level was set to 300 μM whereas in Fig. 7 *b* it was buffered to 100 nM. In Fig. 7 *a* a Ca efflux level of 11.0 $pmol \cdot cm^{-2} \cdot s^{-1}$ was observed at the beginning of the experiment in the nominal absence of internal Na and ATP. This level dropped to 2.0 $pmol \cdot cm^{-2} \cdot s^{-1}$ when the concentration of Na in the axoplasm was made 100 mM. At that point, the addition of 2 mM (Mg) ATP to the dialysis media stimulated Ca efflux which increased to a level of 7.2 $pmol \cdot cm^{-2} \cdot s^{-1}$. It should be noted that this level of Ca efflux observed in the presence of ATP and 100 mM of Na_i is considerably lower than that of 11.0 $pmol \cdot cm^{-2} \cdot s^{-1}$ observed at the beginning of the experiment in the absence of Na_i and ATP. The removal of all of the internal Na returned the Ca efflux to its initial level of 11.0 $pmol \cdot cm^{-2} \cdot s^{-1}$ even though the ATP content of the fiber was unchanged. The

TABLE V

KINETICS PARAMETERS OF THE CURVES RELATING Ca EFFLUX AND Na_i INHIBITION AND EXTENT OF THE PREDICTED INHIBITION AT HIGH Na_i

Molecularity	K_i	Minimal noninhibited fraction $\bar{I}_{min}$	Noninhibited fraction $[\bar{I}]_{Na_i}$		Ratio
	mM		100 mM	200 mM	$\frac{[\bar{I}]_{100\ Na_i}}{[\bar{I}]_{200\ Na_i}}$
			%		
$n=1$	34.0	3.5	28.0	17.6	1.60
	±5.0	±6.1	±0.4	±0.7	±0.07
$n=2$	17.6	30.8	32.9	31.4	1.05
	±6.3	±2.5	±6.4	±3.1	±0.23

addition of 50 mM Na_i brought the Ca efflux down to 9.5 $pmol \cdot cm^{-2} \cdot s^{-1}$, when the concentration of Na in the dialysis media was again made 100 mM. In this last solution change, the concentration of ATP was doubled to 4 mM. This increase in the concentration of intracellular ATP was accomplished in order to see whether the concentration of 2 mM of ATP used during most of the experiments was supramaximal. This point is considered proved because the Ca efflux level of 7.2 $pmol \cdot cm^{-2} \cdot s^{-1}$ obtained in the middle of the experiment, under the conditions of 100 mM Na_i and 2 mM ATP, was very similar to the Ca efflux value of 6.6 $pmol \cdot cm^{-2} \cdot s^{-1}$ obtained at the end of the experiment at an identical level of Na_i but at 4 mM ATP.

Fig. 7 *b* shows an experiment similar to the one just described except that the ionized intracellular calcium level was buffered to 100 nM. At the beginning of the experiment 3 mM (Mg) ATP was added to the dialysis fluid which contained 100 mM choline instead of Na_i. A steady state Ca efflux of 36.4 $fmol \cdot cm^{-2} \cdot s^{-1}$ was observed under these conditions. The addition of 50 mM Na to the internal media brought Ca efflux down to 26.1 $fmol \cdot cm^{-2} \cdot s^{-1}$; the level had risen to 33

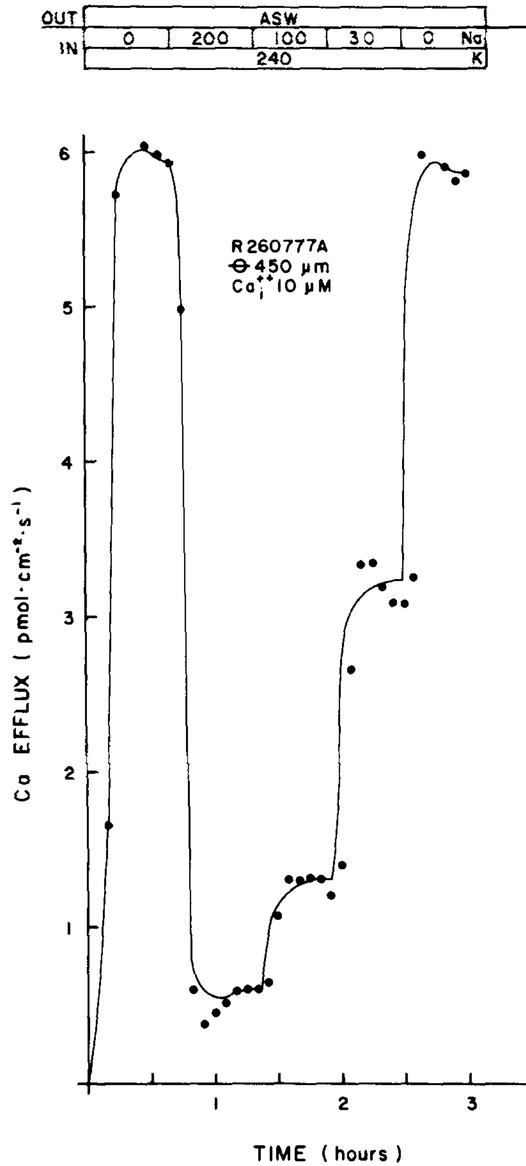


FIGURE 6. The time-course of Ca efflux at high concentrations of internal Na in an axon dialyzed with 240 mM of K_i and zero ATP.

$fmol \cdot cm^{-2} \cdot s^{-1}$ when 30 mM Na_i was removed from the dialysis fluid. The initial value of 36.4 $fmol \cdot cm^{-2} \cdot s^{-1}$ was restored when the remaining 20 mM of Na_i were replaced by choline. At this point in the experiment, all of the choline and the ATP were removed from the dialysis medium and 100 mM of Na was introduced into the fiber. This treatment produced a large drop in the Ca efflux level which was partially reversed, however, when ATP was reintroduced into the dialysis fluid.

Fig. 8 shows normalized Ca efflux values, obtained from all the experiments performed following the above described protocol, plotted as a function of the concentration of Na in the dialysis fluid. The value of 1.0 was assigned to the level of Ca efflux seen under the condition of nominally zero Na_i. As observed in axons depleted of ATP, there was no significant difference between the inhibition produced by Na_i at low or high levels of intracellular ionized Ca. However, the extent of the inhibition of Ca efflux seen in the presence of ATP is markedly different from that observed in axons dialyzed without ATP. At 100 mM Na_i some 73% of the Ca efflux mechanism is inhibited in axons with no ATP whereas only a 25% inhibition is observed in ATP-fueled axons. It should be noted that, in axons fueled with ATP, Ca efflux was independent of the absolute concentration of Na if the Na_i/Na_o ratio was held constant in the range of 440/80 to 110/20 mM, whereas Na_i had little inhibitory action in that range. In axons depleted of ATP, however, raising Na_i caused a large inhibition of Ca efflux, while the peak level of Ca efflux observed at 110/20 mM of Na_o/Na_i was

TABLE VI

EFFECT OF HIGH INTERNAL Na ON THE Ca EFFLUX IN THE ABSENCE OF ATP

Axon reference	CA_i^{++}	Internal sodium concentrations				Ratio
	μM	mM				
		0	30	100	200	$\frac{(Efflux)_{100\ Na_i}}{(Efflux)_{200\ Na_i}}$
		Ca efflux $pmol \cdot cm^{-2} \cdot s^{-1}$				
R 26077 A	10	6.1	3.2	1.3	0.65	2.0
R 26077 B	10	5.0	2.0	0.6	0.38	1.6
						Mean 1.8

Axons dialyzed with K_i = 240 mM.

significantly reduced by raising the absolute concentration of Na, the electrochemical gradient for Na being kept constant.

Table VII summarizes the absolute values for Ca efflux observed at various internal concentrations of Na in the presence of ATP and at two concentrations of ionized axoplasmic Ca; Fig. 8 was constructed from these values.

The Effect of Internal Na on the ATP-Sensitive Fraction of the Ca Efflux

There is an aspect of Fig. 7 *a* which should be emphasized. It concerns the effect of ATP on the Ca efflux level observed in the nominal absence of internal Na. At the beginning of the experiment a Ca efflux level of 11.0 $pmol \cdot cm^{-2} \cdot s^{-1}$ was observed in the absence of ATP and internal Na. 3 h later a Ca efflux level of 11.0 $pmol \cdot cm^{-2} \cdot s^{-1}$ was seen, this time in the presence of ATP and, as earlier, in the absence of internal Na. In two other experiments, a similar result was obtained. Table VIII lists the Ca efflux values observed in the presence or absence of ATP in axons dialyzed with a high internal level of ionized calcium and nominally zero Na_i. From these values and from a similar observation made by DiPolo (1976) in an axon dialyzed with an ionized Ca level buffered to 0.6

μM, it can be concluded that in axons in which the internal Na has been removed by dialysis, the level of Ca efflux observed in the absence of ATP is not affected by the addition of ATP, at least within the specified range of internal calcium concentrations of 0.6–300 μM.

In Fig. 9 curve *c* (taken from Fig. 8) describes the inhibitory action of Na_i on Ca efflux in the presence of ATP, and curve *b* (taken from Fig. 5) describes the inhibitory action of Na_i on Ca efflux in the absence of ATP. Subtracting curve *b* from curve *a* we obtain curve *a* which represents the dependence on internal Na of the fraction of Ca efflux promoted by ATP. To construct this figure, curve *c*, which relates Ca efflux to internal Na in the presence of ATP, was extrapolated linearly from 100 mM, the highest concentration of Na_i used in the presence of ATP, to 200 mM, the highest concentration of Na_i used in the absence of ATP. Fig. 9 shows that ATP is capable of relieving part of the inhibition of Ca efflux produced by Na_i in the physiological range of Na concentration. Fig. 9 can be also interpreted as if the effect of Na_i on Ca efflux in the presence of ATP is the result of two simultaneous processes: (*a*) a component of Ca efflux present in the absence of ATP; and (*b*) an ATP-dependent component of Ca efflux.

The Effect of External Na on Ca Efflux at Low Internal Na

The activating effect of external Na on Ca efflux has been extensively studied. In axons subjected to internal dialysis, DiPolo (1974) showed that the apparent half-dissociation constant (K_A) for the process is 144 mM in axons dialyzed with out ATP and is 80 mM Na_o in axons dialyzed with ATP. In those experiments, the internal Na concentration was kept at 72 mM while the internal ionized Ca was buffered at 0.3 μM. Blaustein et al. (1974) observed, in the absence of ATP, a strong dependence of Ca efflux on Na_o with a K_A of 125 mM at 50 mM Na_i. More recently, Blaustein (1977) obtained values of 50 and 120 mM of Na_o for axons dialyzed with and without ATP.

A similar shift of the K_A for Na_o has been observed in injected axons. Baker and Glitsch (1973) showed that ATP shifts the curve relating Ca efflux to Na_o. More recently, Baker and McNaughton (1976) observed that in intact axons the value of Na_o that causes half-maximal activation of Ca efflux changes from 50 mM to 300 mM as cyanide poisoning proceeds. This change in K_A was accompanied by a change in the curve relating Ca efflux to the concentration of Na_o from a rectangular hyperbola to a clearly sigmoid curve. In some of the experiments reported elsewhere, especially those done on injected axons, the concentration of internal Na is undetermined. Inasmuch as the effect of ATP on Ca efflux seems to be related to Na_i, we studied the effect of Na_o on calcium efflux in axons dialyzed with various levels on Na_i with or without ATP.

Fig. 10 shows a typical experiment. An axon, in which the ionized Ca concentration was buffered with 2 mM of EGTA to 0.33 μM, was dialyzed with a solution containing 5 mM of internal Na and 95 mM choline. Throughout this experiment the external solution was Ca-free and its concentration of Na was varied. At the beginning of the experiment Ca efflux was about 0.7 $pmol \cdot cm^2 \cdot {}^{-1}$ with a Na_o of 441 mM. Replacement of all of Na_o by 441 mM of choline dropped the Ca efflux to 0.033 $pmol \cdot cm^{-2} \cdot s^{-1}$. This level went up to 0.436 $pmol \cdot cm^{-2} \cdot s^{-1}$

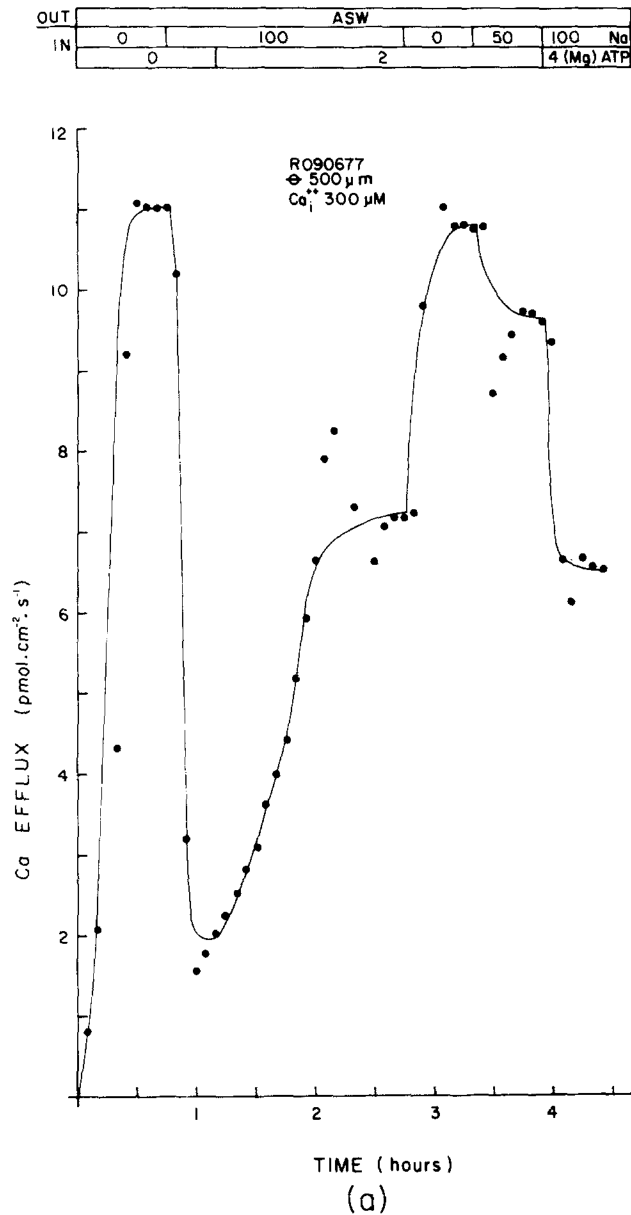


FIGURE 7. The time-course of Ca efflux at various concentrations of internal Na in axons dialyzed with ATP. (a) Ionized axoplasmic Ca concentration set to 300 μM. (b) Ionized axoplasmic Ca concentration buffered to 0.1 μM.

when Na_0 was raised to 45 mM. At 89 mM Na_0 the Ca efflux was 0.586 whereas at the normal concentration of Na_0 the efflux rose to 0.601 $pmol \cdot cm^{-2} \cdot s^{-1}$. When Na_0 was lowered to 23 mM the Ca efflux fell to 0.221 $pmol \cdot cm^{-2} \cdot s^{-1}$. At this point 2 mM of (Mg) ATP was added to the dialysis fluid, which still

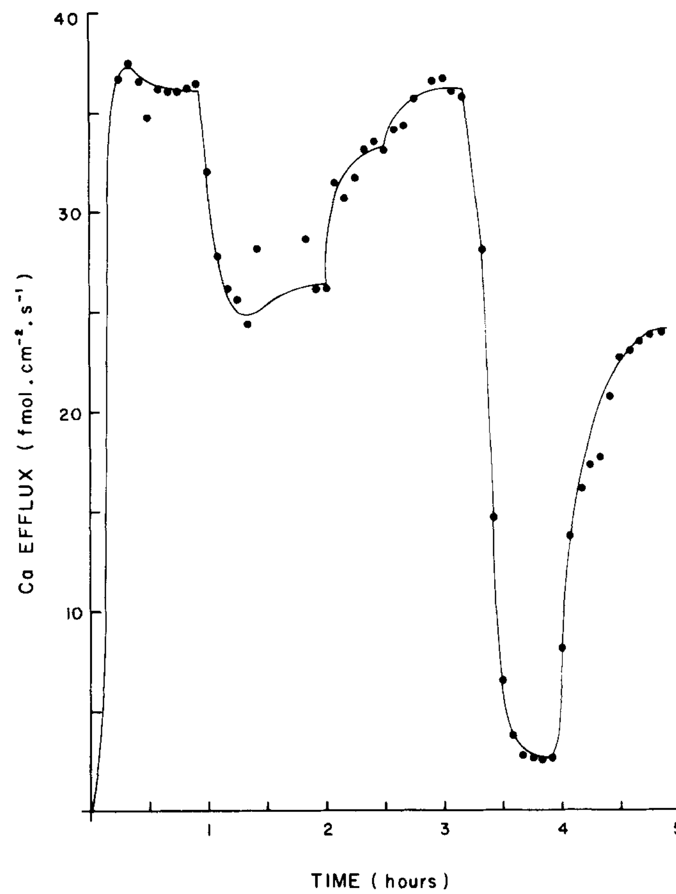


FIGURE 7

contained 5 mM of internal Na and Ca efflux rose to 0.350 pmol·cm^{-2}·s^{-1}. Raising Na_o from 23 to 45 mM increased the efflux to 0.686 pmol·cm^{-2}·s^{-1}. A return to the normal Na concentration of 441 in millimolar produced a transient rebound in Ca efflux which eventually stabilized at 1.06 pmol·cm^{-2}·s^{-1}. At this point all of the external Na was replaced by 441 mM choline and the Ca efflux fell to 0.270 pmol·cm^{-2}·s^{-1}. Reintroduction of 89 mM of Na_o raised the efflux to 0.888 pmol·cm^{-2}·s^{-1}. Finally, Na_o was returned to the level at which ATP was originally added, i.e., 23 mM and the Ca efflux returned to the same value

seen when the axon was first exposed to 23 mM Na_o and ATP, i.e., 0.22 $pmol \cdot cm^{-2} \cdot s^{-1}$.

From this kind of experiment, the apparent half-activation constant for the effect of Na_o on Ca efflux can be calculated: K_A values[1] were computed by interpolation of that Na_o concentration at which the net Na-dependent Ca efflux (defined as the Ca efflux level observed at 441 mM of Na_o minus that seen at 0 Na_o) is reduced to half. For the experiment shown in Fig. 10, a value of 37 + 7 mM of Na_o was calculated for the ATP-free condition while 43 ± 5 mM of Na_o

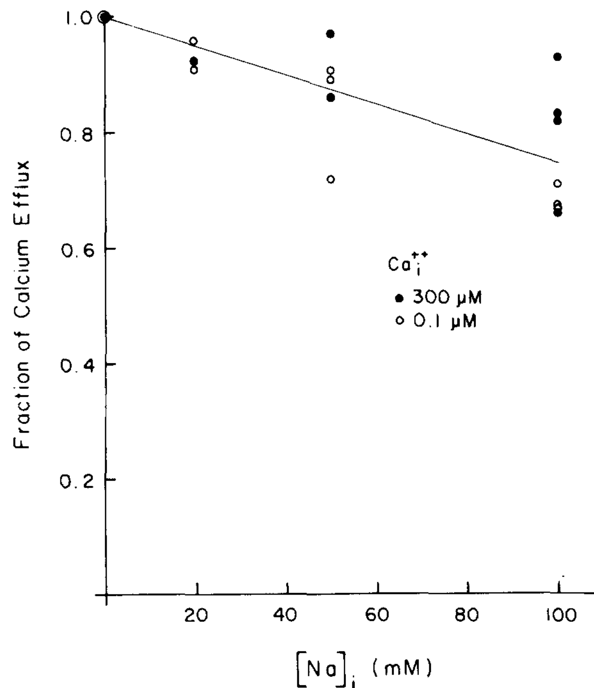


FIGURE 8. The effect on Ca efflux of internal Na at constant Na_o (440 mM) in the presence of ATP. The straight line was fitted by least squares method to the experimental data. The slope of the line is $-0.0025\ mM^{-1}$. Ca efflux levels were normalized in each experiment with reference to the Ca efflux level observed at zero Na_i to which the value of 1.0 was assigned. (●) Axoplasmic ionized Ca set at 300 μM; (○) ionized Ca buffered to 0.1 μM.

was calculated for that part of the experiment in which the axon was fueled with ATP. It is clear that if Na_i is very low (5 mM) ATP has no effect on the activation produced by external Na. This conclusion is supported by experiments done at various levels of Na_i, the results of which are summarized in Table IX. It can be seen that with Na_i nominally zero the half-activation constant for external Na of axons dialyzed without ATP is indistinguishable from that of axons dialyzed with ATP. This is also true for axons dialyzed with 30 mM of Na_i. However, at higher concentrations of internal Na the apparent half-activation constant for

[1] The uncertainty in determining the K_A by this procedure is reflected as the upper and lower limits set for the K_A reported here.

Na_o becomes dependent upon ATP. Specifically, one axon dialyzed with 100 mM Na_i showed a K_A of 112 mM in the absence of ATP while in the presence of ATP, the same axon showed a K_A of 39 mM Na_o.

The relationship between the apparent half-activation constant for Na_o and the concentration of internal Na is better seen in Fig. 11. Although it is evident that in the presence of ATP the K_A for Na_o is independent of Na_i, in the absence of ATP the apparent half-activation constant for Na_o depends upon the concentration of internal Na. The pronounced sigmoidal shape of the curve relating K_A for Na_o to Na_i, observed in the absence of ATP, varies from 41 mM

TABLE VII

EFFECT OF Na_i ON Ca EFFLUX IN THE PRESENCE OF ATP

Axon reference	Ca_i^{++}	Internal sodium concentration				Flux units
		μM			mM	
					0	
		0	20	50	100	
		Ca efflux				
R 210677	0.10	36.0		32.5	24.3	fmol/cm²·s
		40.0	38.5			
R 220677	0.10	31.7	–	28.7	22.5	"
R 230677	0.10	36.4	33.0	26.1	25.0	"
R 130577	300	6.5	–	–	6.0	pmol/cm²·s
R 090677	300	11.0	–	9.5	7.3	"
R 140677	300	10.3	9.5	8.9	8.4	"
R 270477	300	6.8	–	6.6	5.7	"

TABLE VIII

EFFECT OF ATP ON Ca EFFLUX IN THE ABSENCE OF INTERNAL Na

Axon reference	Ca_i^{++}	Ca efflux: No ATP	Ca efflux: ATP	Ratio $\frac{(\text{Ca efflux})_{ATP}}{(\text{Ca efflux})_{No\ ATP}}$
	μM	$pmol \cdot cm^{-2} \cdot s^{-1}$		
R 130577	300	6.4	6.5	1.02
R 090677	300	11.2	11.0	0.98
R 270677	300	6.6	6.8	1.03
			Mean	1.01

Na_o at low Na_i (<30 mM) to 140 mM Na_o at high Na_i (≃200). This sigmoidal curve is characterized by a high power dependence on Na_i (≃3) and a half constant of some 81 mM Na_i for the process. It should be noted in Fig. 11 that at low levels of Na_i, the K_A for Na_o for the Ca efflux mechanism has a limiting value of 41 mM Na_o which does not seem to depend upon either the presence of ATP or on small variations in Na_i.

DISCUSSION

Ca Efflux at a Constant Na Electrochemical gradient

In discussing the experiments in which Ca efflux was measured at a constant Na_o/Na_i ratio, it is important to recall studies which suggest that the Ca transport

system is fully activated when Na_o is 180 mM or higher. First, Requena et al. (1977) showed that in an intact axon with or without ATP, a steady-state Ca_i is maintained in the range of 180–440 mM of Na_o. Secondly, Blaustein et al. (1974) showed that Ca efflux is almost fully activated by an external concentration of 180 mM of Na_i, if the axon contains no ATP. In the presence of the nucleotide this concentration should be lower, because the half-activation value for Na_o shifts towards smaller concentration when ATP is added to the dialysis fluid (DiPolo, 1974; Blaustein, 1977). Finally, the experiments of Baker et al. (1969),

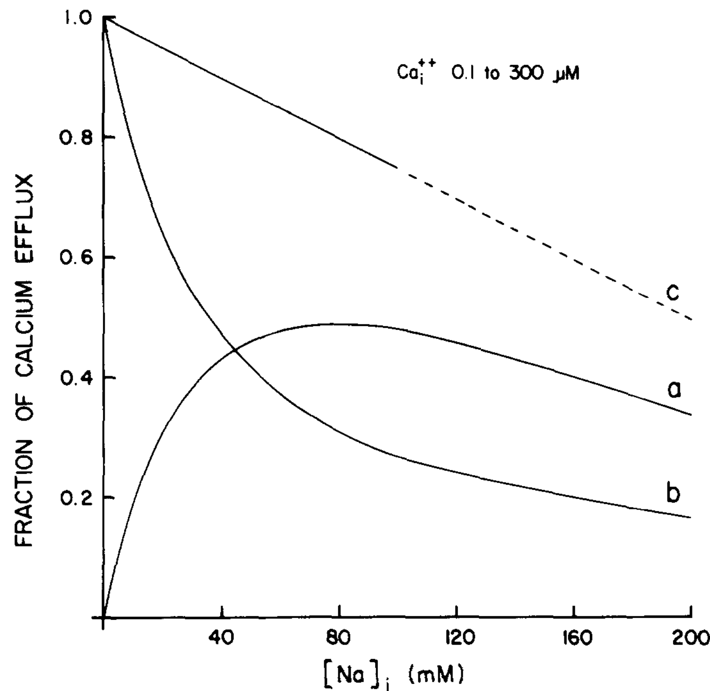


FIGURE 9. The effect of internal Na on the ATP-promoted fraction of Ca efflux. Curve *a* was obtained by subtracting the solid curve of Fig. 5 (curve *b*) from the straight line of Fig. 8 (extrapolated in the range 100–200 mM of Na_i) (curve *c*). See text for further details.

relating Ca influx and external Na, clearly demonstrated that Ca influx is at a minimal value in the range of 200–460 mM of Na_o.

The experimental findings presented in this paper showed that at a constant electrochemical gradient for Na, increase in Na_o/Na_i from 110/20 to 440/80 mM did not produce a significant effect on Ca efflux provided the internal dialysis media contained ATP (2 mM). However, a similar increase in Na_o/Na_i carried out in the absence of ATP causes a threefold reduction in Ca efflux. An increase in the absolute concentration of Na_i appears to inhibit Ca efflux. This inhibition, which is observed in the absence of ATP, was obtained by raising both Na_o and Na_i. Since increases in Na_o are known not to affect Ca efflux in the range of 180–440 mM of Na_o, it is clear that it must be the increase in Na_i that is responsible for the inhibition of Ca efflux.

Examination of the dependence of Ca efflux on Na_i revealed that, in the absence of ATP, there is a substantial decline in Ca efflux as Na_i is raised from 1 to 80 mM when Na_o remains at 440 mM. It must be admitted that the data summarized in Fig. 5 can be fitted to an expression involving log (Na_o/Na_i) as was done by Brinley et al. (1975), but an equally good fit can be obtaned on the assumption that the interaction of a single Na with the carrier is sufficient to inhibit Ca efflux. The reason for selecting the latter hypothesis is that the data obtained at constant electrochemical gradient for Na, in the absence of ATP,

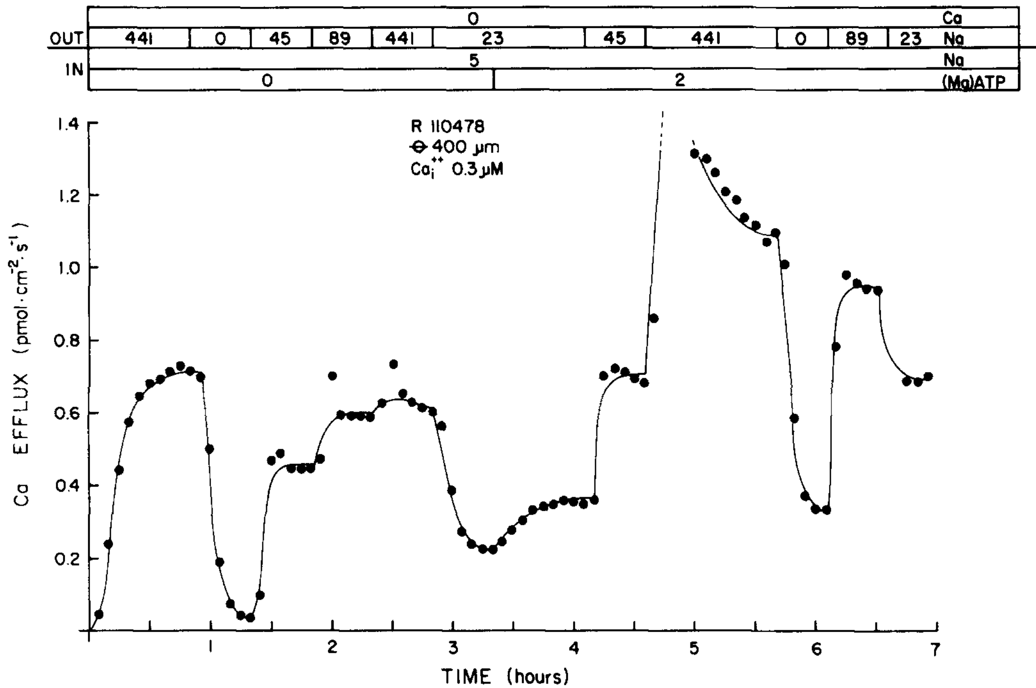


FIGURE 10. The time-course of Ca efflux under various concentrations of external Na and zero Ca_o at constant low Na_i (5 mM) in an axon dialyzed in the presence and in the absence of ATP.

show the same sort of strong inhibition, which means that the inhibition cannot be explained by changes in the energy supply available to the transport mechanism. Similarly, if the weak dependence of Ca efflux on internal Na, observed in the presence of ATP, is assumed to result from a purely inhibitory effect of Na_i, then Ca efflux in ATP-fueled axons should not vary significantly with changes in the Na concentrations if Na_o is in the range in which the carrier is fully activated. This is what was found in the range 180–440 mM Na when Na_o/Na_i was held constant.

Empirically, the Ca-outward transport reaction can be reconstructed from the summation of two processes, activation and inhibition. For instance, the increase in Ca efflux seen in Fig. 2 when Na_i and Na_o are increased from zero to 20 mM and to 110 mM, respectively, corresponds to the onset of the activation caused by external Na, whereas the decline of the peak Ca efflux value seen as the

concentrations Na_o/Na_i exceed 110/20 mM corresponds to the inhibition caused by Na_i dominating the activation caused by Na_o. Such as exercise is, however, a fairly complicated matter, since as shown herein, the activation by Na_o depends upon the concentration of the inhibiting ion on the other side of the axolemma. This state of affairs questions most carrier models described in the literature which involve independent binding sites for Na (Baker and McNaughton, 1977; Blaustein, 1977; Mullins, 1977).

In the present context one may note that other workers have found it necessary to have the term containing the inhibiting Na concentration squared in order to reconstruct, via kinetic models, Ca flux data. Specifically, Blaustein and Russell (1975) in their study in squid axons of Ca efflux as a function of the internal concentration of Ca and Na, squared the term containing Na_i, whereas

TABLE IX

EFFECT OF Na_o ON Ca EFFLUX* AT VARIOUS CONCENTRATIONS OF Na_i AND ATP IN THE ABSENCE OF Ca_o

Axon reference	Na_i	(Mg) ATP	External sodium concentration (mM) 0	23	45	89	133	441	K_A‡
	mM	mM	Ca efflux (pmol·cm^{-2}·s^{-1})						
R 100478	0	0	0.030	–	0.457	0.586	0.665	0.746	43±5
R 130478	0	0	0.038	0.194	0.422	–	–	0.701	44±6
R 100478	5	0	0.033	0.221	0.436	0.586	–	0.601	39±7
R 180478	30	0	0.027	0.057	0.100	0.132	–	0.139	42±6
R 170478	60	0	0.024	–	0.058	0.095	0.114	0.127	70±6
R 120478	100	0	0.014	–	–	0.036	0.042	0.065	112±9
R 160478	0	2	0.093	0.258	0.572	0.720	–	1.026	43±5
R 110478	5	2	0.270	0.350	0.686	0.898	–	1.060	43±5
R 120478	100	2	0.014	–	0.115	0.142	–	0.150	39±8

* Ca_i^{++} buffered to 0.3 μM with 2 mM EGTA.
‡ Apparent half-saturation constant.

Baker and Blaustein (1968) in their study of Ca uptake by crab nerve, had to square the term which contained the dependency on Na_o. Although this power dependence of the transport reaction on a function of the concentration of the inhibiting Na ion could represent a particular property of the kinetic model employed, the similarity between the influx and the efflux transport reaction suggest that there is only one carrier for Ca translocation. This hypothesis is further supported by the evidence presented herein which shows that the apparent affinity constant for external Na (41 mM) is not significantly different from that observed for internal Na (34 mM). In other words, (*a*) the carrier mechanism binds Na equally well on either side of the membrane; (*b*) if Ca occupies a site on the carrier, the Na sites opposite when occupied become activators, whereas (*c*) if a single Na ion occupies a site that is similar but is on the same side of the bound Ca the result is inhibition of the translocating process. The symmetry of the affinity of the carrier is not unique to Na,

however, Blaustein (1977) and Baker and McNaughton (1976) showed that the apparent half-affinity constant of the carrier for internal and external Ca is similar and in the micromolar range.

The single carrier hypothesis for Ca transport implies that the inhibition of one of the unidirectional fluxes should be reflected in an activation of the other unidirectional flux. Indeed, it can be shown that an experimental condition

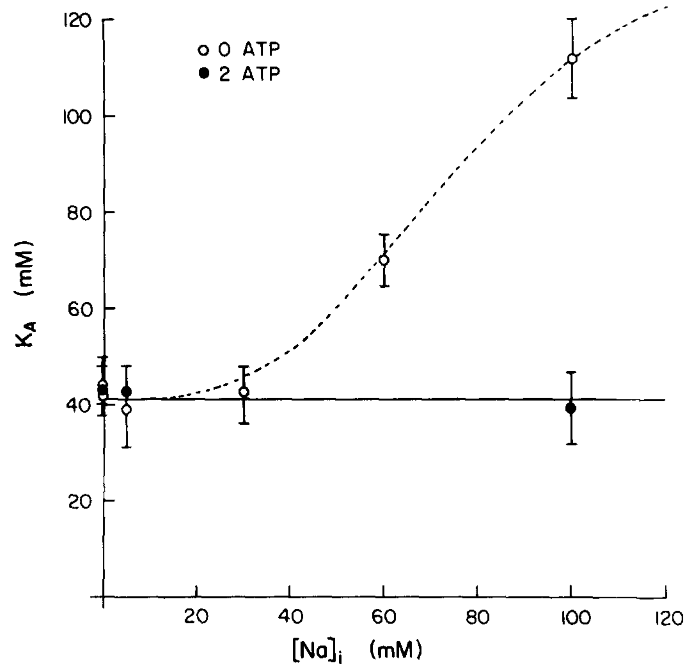


FIGURE 11. The effect of internal Na on the calculated apparent half-activation constant (K_A) for external Na. (●) Data obtained in axons dialyzed with 2 mM (Mg) ATP; (○) ATP-free axons. The solid line joins the experimental points obtained in the presence of ATP and is drawn as the zero slope regression line K_A (ATP) = 41 mM Na_o + 0 Na_i. The broken line is the best fitted sigmoid curve to the experimental points obtained in the absence of ATP. It is described by an equation of the form:

$$K_A = 41 + \frac{107}{1 + \left(\frac{81}{Na_i}\right)^3}.$$

which reduced Ca efflux such as raising the internal Na from 40 to 80 mM, also produces an enhancement of the Ca influx (see Baker et al. [1969]). Similarly, the addition of some 200 mM of Na_o to a medium containing no Na_o causes an activation of the Ca efflux system, and as described by Baker et al. (1969), an inhibition of the maximal Ca influx level observed in the absence of Na_o.

Internal Na and the Mode of Action of ATP

An increase in concentration of internal Na in the absence of ATP reduces Ca efflux in a fashion reminiscent of the unimolecular noncompetitive inhibition of

enzyme kinetics. The noncompetitiveness of the penomenon is substantiated by the observation that the extent of the inhibition at a given concentration of Na_i was found not to depend upon the concentration of internal ionized Ca. This point is in agreement with a similar observation of Brinley et al. (1975). The unimolecular scheme for the inhibition caused by Na_i on Ca efflux is based upon data obtained in a much wider range of concentrations of Na_i than previously used, and this could account for the contradiction of this conclusion with that of Blaustein and Russell (1975) who postulated a competition of two Na ions with one Ca ion for an allosterically related binding site of the transport system. With regard to the ATP-stimulated fraction of Ca efflux, the data presented herein leave no doubt of the existence of a small but measurable and apparently nonsaturable, noncompetitive inhibition by internal Na. This conclusion disagrees with that of DiPolo (1976) who did not observe such a phenomenon in the one experiment reported. Although it is not possible at present to decide whether the inhibition caused by Na_i on Ca efflux in the presence of ATP reflects a different mechanism than that observed in the absence of ATP, it should be noted that an ATP-induced modification of the binding constant of the carrier for Na_i, such as the sixfold increase in the K_I observed in the absence of ATP, would be reflected in a pattern similar to the one presented in Fig. 8. Blaustein (1977) did not observe an effect of ATP on the apparent affinity constant for the inhibitory effect of Na_i, although an inhibition of Ca efflux by Na_i in the presence of ATP was reported.

An important experimental finding in this study is that maximal Ca efflux occurs in the absence of Na_i whether or not ATP is present. Current notions about the role of ATP in affecting Ca efflux are (*a*) it enhances Ca efflux by some catalytic non-energy-yielding reaction, or (*b*) it fuels a pump that operates to extrude Ca independent of the Na electrochemical gradient, using the free energy of ATP hydrolysis. The absence of an effect of ATP in the absence of Na_i renders the metabolically driven Ca pump hypothesis unlikely. Moreover, the observation that ATP does not affect the apparent half-activation value for Na_o in axons dialyzed with low Na_i and the similarities of Ca efflux parameters, such as K_A, observed under the condition of very low Na_i and those seen at high Na_i and ATP, strongly suggest that ATP acts on the Ca efflux system by removing the inhibition caused by Na_i.

Two points should be commented upon in connection with the conclusion above. The first concerns the assignment of a regulatory role of the Ca efflux mechanism to internal Na and (or) ATP. Inasmuch the evidence presented here shows that both act as antagonists on the same regulatory site, and because ATP is normally present in the axoplasm while Na_i is kept as low as possible via the Na-K pump , there is no possibility at present to decide which factor controls the rate of Ca efflux from an axon. The second point concerns ATP. Although ATP could interact catalytically with the Ca efflux system with no energy expenditure, it could also be hydrolyzed by the efflux system, in which case energy would be transferred to the carrier. Inasmuch as it has been shown herein that ATP is not necessary to produce maximal Ca efflux observable under the condition of 0 Na_i and 440 mM of Na_o, it is not necessary to postulate hydrolysis of ATP in the step which allows Na ions to move down their maximal

electrochemical gradient causing an outward translocation of Ca. Therefore, in the overall process leading to the exchange reaction, the involvement of ATP and its possible hydrolysis had to be related to the process which removes the inhibiting Na ions from the transport mechanism. An estimate of the free energy required for this process is of the order of 2 kcal/mol.[2] This value is more consistent with a reaction involving the breakage of weak bond than with one involving the hydrolysis of a high energy phosphate bond.

The findings reported here support the notion that the Ca efflux system uses the energy stored in the Na gradient to support the active extrusion of Ca ions from the axoplasm. The optimal condition for the transport process occurs when internal Na is not present at the transport site, i.e., at the largest possible electrochemical gradient for Na across the axolemma. This condition can also be obtained in the presence of ATP_i, by means of a specific interaction of ATP with the transport mechanism. This effect of ATP presumably could occur at a result of lowering the affinity of the carrier for Na_i.

The author would like to thank Drs. L. J. Mullins and F. J. Brinley, Jr. for their helpful advice and comments, Drs. P. Laüger and R. Villegas for their critical reading of the manuscript, Drs. Carlo Caputo and R. DiPolo for their suggestions during the progress of the work, and Dr. Carlos Sevcik for his help with the computations. I would like to thank Mr. Héctor Rojas for his technical assistance, Mr. José Mora and Pedro V. Pérez for the construction of the experimental setup, Don Amalio Sánchez and Mr. Angel Cazorla for maintaining the electronic equipment, Mr. Nehemias Mujica for the adequate supply of squid, and Mrs. Pura de Bolaños for her excellent assistance in drawing the figures. Finally, my special recognition to Mrs. Margarita Kondracki and Miss Isabel Otaegui for their invaluable secretarial help.
This work was partially supported by a grant from the Consejo Nacional de Investigaciones Cientficas (CONICIT), no. 3126-S-0602.

Received for publication 19 October 1977.

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[2] $\Delta G = RT \ln K_i$, in which K_i is the apparent inhibition constant of internal Na on Ca, determined to be 34 mM.

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