EFFECTS OF INTERNAL AND EXTERNAL CATIONS AND OF ATP ON SODIUM-CALCIUM AND CALCIUM-CALCIUM EXCHANGE IN SQUID AXONS

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ABSTRACT Calcium-45 efflux was measured in squid axons whose internal solute concentration was controlled by internal dialysis. Most of the Ca efflux requires either external Na (Na-Ca exchange) or external Ca plus an alkali metal ion (Ca-Ca exchange; cf. Blaustein & Russell, 1975). Both Na-Ca and Ca-Ca exchange are apparently mediated by a single mechanism because both are inhibited by Sr and Mn, and because addition of Na to an external medium optimal for Ca-Ca exchange inhibits Ca efflux. The transport involves simultaneous (as opposed to sequential) ion counterflow because the fractional saturation by internal Ca (Ca_i) does not affect the external Na (Na_o) activation kinetics; also, Na_o promotes Ca efflux whether or not an alkali metal ion is present inside, whereas Ca-Ca exchange requires alkali metal ions both internally and externally (i.e., internal and external sites must be appropriately loaded simultaneously). ATP increases the affinity of the transport mechanism for both Ca_i and Na_o, but it does not affect the maximal transport rate at saturating $[Ca^{2+}]_i$ and $[Na^+]_o$; this suggests that ATP may be acting as a catalyst or modulator, and not as an energy source. Hill plots of the Na_o activation data yield slopes $\simeq$ 3 for both ATP-depleted and ATP-fueled axons, compatible with a 3 Na^+-for-1 Ca^{2+} exchange. With this stoichiometry, the Na electrochemical gradient alone could provide sufficient energy to maintain ionized $[Ca^{2+}]_i$ in the physiological range (about 10^{-7}M).

INTRODUCTION

There is substantial evidence that sodium ions play an important role in the "active" (i.e., "uphill") extrusion of calcium in a large variety of tissues from both vertebrates and invertebrates (cf. Blaustein, 1974). One of the most compelling arguments, illustrated (for example) by data from squid axons (Baker et al., 1969; Blaustein and Hodgkin, 1969), is that a reduction in extracellular Na leads to net gain of Ca, as a consequence of increased Ca influx and reduced Ca efflux. Moreover, Requena et al. (1977) have employed the Ca-specific photoprotein aequorin to confirm that extracellular Na is required for net Ca extrusion from squid axons.

Recent data indicate that the sodium-dependent calcium transport (Na-Ca ex-

change) mechanism may be very similar in squid axons and in mammalian nerve terminals (Blaustein and Ector, 1976); this implies that the mechanism was evolved fairly early in the history of the animal kingdom and has been retained during the evolution of the higher animals. The detailed kinetic properties of the Na-Ca exchange system have been studied most extensively in squid axons because these large nerve fibers are admirably suited for experiments in which both the intracellular and extracellular environments are carefully controlled by internal dialysis techniques (Brinley and Mullins, 1967). Dialysis experiments carried out during the past few years indicate that a single "carrier" mechanism may mediate both Na-Ca exchange and Ca-Ca exchange (Blaustein and Russell, 1975); this view is supported by additional evidence obtained in the present study. The Na-Ca exchange is membrane potential-sensitive (Brinley and Mullins, 1974; Blaustein et al., 1974; Mullins and Brinley, 1975, Baker and McNaughton 1976*a*; but see Baker and McNaughton, 1976*b*), and may be promoted by ATP (Baker and Glitsch, 1973; DiPolo, 1974, 1976; Baker and McNaughton, 1976*a*), although the mechanism apparently does not have an absolute requirement for ATP, at least at high internal ionized Ca^{2+} concentrations (Di Polo, 1974; Blaustein and Russell, 1975; Blaustein, 1976).

The present report focuses primarily on several critical questions concerning the Ca efflux kinetics and energetics in squid axons: (*a*) Are Na-Ca and Ca-Ca exchange mediated by the same carrier mechanism? (*b*) Do the external and internal sites on the Ca carrier load sequentially or simultaneously? (*c*) What is the stoichiometry of the Na-Ca exchange? and (*d*) How does ATP affect the Na-Ca exchange system?

To answer these questions, various internal and external monovalent and divalent cations were tested for their effects on ^{45}Ca efflux from ATP-depleted axons. The effect of ATP on the ^{45}Ca efflux kinetics was studied in a second series of experiments. In all of the experiments, axoplasmic solute control was maintained by internal dialysis. Some of the data from these studies have been described in preliminary communications (Blaustein, 1976, 1977).

METHODS

Biological Material

The experiments were made at the Marine Biological Laboratory, Woods Hole, Mass., during the months of May and June, 1975 and 1976. Axons 500–800 μm in diameter from the hindmost stellar nerve of live specimens of the squid *Loligo pealei* were used for the experiments.

External Solutions

Table I shows the composition of representative external solutions and indicates the nomenclature that will be followed. In some experiments, mixtures of these solutions were employed to obtain intermediate Na or Ca concentrations; details will be given in the Results section. In several experiments the Ca or some of the Mg was replaced by Sr, Mn, or Ba, as indicated below; in all instances, the total external divalent cation concentration was maintained constant at 60 mM. The Ca concentration in the nominally Ca-free external solutions was 12 μM (in Na seawater), 30 μM (in Li seawater), or 4 μM (in choline seawater), as determined by atomic absorption spectroscopy.

TABLE I

REPRESENTATIVE EXTERNAL SOLUTIONS

Solution*	NaCl	KCl	Choline Cl	LiCl	TMA Cl‡	$CaCl_2$	$MgCl_2$	$SrCl_2$	KCN
					mmol/liter				
10 K(Na)	425	10	—	—	—	10	50	—	—
Ca-free 10 K (choline)	—	10	425	—	—	—	60	—	—
12 K(Na) + CN	425	10	—	—	—	10	50	—	2
Ca-free 12 K(Na) + CN	425	10	—	—	—	—	60	—	2
12 K(Li) + CN	—	10	—	425	—	10	50	—	2
12 K(TMA) + CN	—	10	—	—	425	10	50	—	2
Ca-free 12 K(Na) + 10 mM Sr + CN	425	10	—	—	—	—	50	10	2
12 K(Li) + 10 mM Sr + CN	—	10	—	425	—	10	40	10	2

*In addition, all solutions contained 0.1 mM Na_2EDTA and 2.5 mM HEPES. The solutions were all buffered to pH 7.8 at 20°C with Tris base.

‡TMA Cl was recrystallized from absolute ethanol.

The osmolalities of the external solutions were determined on a vapor pressure osmometer (Wescor Inc., Logan, Utah). All of the osmolalities ranged between 960 and 980 mosmol/kg.

Internal Solutions

The composition of representative internal (dialysis) fluids is listed in Table II. Most of the Ca in these fluids (0.75–1.15 mmol/liter) was directly added as $^{40}CaCl_2$. The Ca concentration in each of the dialysis fluids was determined by atomic absorption spectroscopy (before the addition of ^{45}Ca); the reported total Ca concentrations, $[Ca_t]_i$ (see figure legends), include the Ca added as $CaCl_2$, as well as the Ca contamination from other components in the dialysis fluids (usually about 10–15 μmols/liter) and the contribution of carrier ^{40}Ca from the $^{45}CaCl_2$. The ionized Ca concentration, $[Ca^{2+}]_i$, was buffered to the desired

TABLE II

REPRESENTATIVE INTERNAL (DIALYSIS) FLUIDS

Solution*	Na isethionate	Na glutamate‡	K glutamate	Li glutamate‡	TMA glutamate‡	Taurine
K-DF	5	—	395	—	—	138
Na-DF	—	400	—	—	—	138
Li-DF	—	—	—	400	—	138
TMA-DF	—	—	—	—	400	138
K-DF (75 mM Na)	75	—	395	—	—	—

*In addition, all dialysis fluids (DF) contained 5 mM $MgCl_2$, 2 mM KCN, 0.5 mM phenol red, 10 mM HEPES, and 0.75–1.15 mM $CaCl_2$; the ionized Ca^{2+} concentration was buffered to the desired level (see Results for details) with EGTA. The solutions were buffered to pH 7.30 (cf. Boron and De Weer, 1976) at 20°C with Tris base.

‡Na glutamate, Li glutamate, and TMA glutamate were prepared from the respective hydroxides by titration to pH 7.30 with L-glutamic acid.

level (see Results) by the addition of ethyleneglycol-bis[β-aminoethyl ether] N,N'-tetraacetate (EGTA).

About 60 μCi of ^{45}Ca (New England Nuclear, Boston, Mass.) was added to each 1 ml of dialysis fluid in most experiments, to give a specific activity of about 50–60 μCi/μmol Ca. In experiments involving a very low $[Ca^{2+}]_i$, however, dialysis fluids with specific activities of up to 250 μCi/μmol Ca were employed so that the small effluxes could be measured with reasonable accuracy (e.g., Fig. 7). The figure legends indicate the $[Ca_t]_i$ and $[EGTA_t]_i$ (total EGTA concentration in the dialysis fluid) values for the respective experiments. The reported nominal $[Ca^{2+}]_i$ values were based on a Ca EGTA stability constant value of $7.6 \times 10^6 M^{-1}$ (Portzehl et al., 1964). For ATP-containing dialysis fluids with $[Ca_t]_i \geq [EGTA_t]_i$, the complexation of Ca with ATP was taken into account in calculating the nominal $[Ca^{2+}]_i$; the Ca-ATP stability constant was taken as $5 \times 10^2 M^{-1}$ (Kendrick et al., 1977) for these computations.

In many experiments various other agents were added to the dialysis fluids: ATP, phosphoenol pyruvate (PEP), oligomycin, and/or carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP). The ATP and PEP (both Sigma Chemical Co., St. Louis, Mo), were added immediately before use, and the pH of each solution was readjusted to 7.30; when present, the PEP concentration was 5 mM, and the ATP concentration was 4 mM. The total Mg concentration was raised to 9 mM in the ATP-containing fluids. Oligomycin (from Sigma) and FCPP (from DuPont Company, Wilmington, Del.) were stored as stock solutions (10 mg/ml and 1 mM, respectively) in 95% ethanol; immediately before use, small portions of the ethanolic solutions were added to the dialysis fluids to give a final concentration of 100 μg/ml oligomycin or 15 μM FCCP. Control experiments indicated that the ethanol alone did not significantly affect the Ca efflux.

Procedures

CALCIUM EFFLUX MEASUREMENTS WITH AN ON-LINE FLOW CELL The internal dialysis techniques of Brinley and Mullins (1967), including their recent modifications (Brinley and Mullins, 1974), were employed in these experiments. Some details and minor alterations of the techniques, as used in our laboratory, were described in a recent article (Blaustein and Russell, 1975). In the present series of ^{45}Ca efflux experiments, a modified method of assaying ^{45}Ca was employed. Instead of counting aliquots of the superfusion fluid, as was done previously, the superfusion effluent from the experimental (axon) chamber (see Fig. 1A of Blaustein and Russell, 1975) was diverted through a flow cell (Beckman Instrument Co., Fullerton, Calif., or Packard Instrument Co., Downers Grove, Ill.) set in the well of a Beckman Beta-mate II liquid scintillation counter. The counts due to ^{45}Ca were read out directly, on the counter's NIXIE-tube read-out; the counts (per minute) were also printed digitally, each minute, on a DDP-7 panel printer (Datel Systems, Inc., Canton, Mass.). In addition, the output of the Beta-mate II was passed through a Beckman linearizer, and continuously monitored (see Figs. 1, 9, and 12) on a two-channel strip chart recorder (Houston Instrument Div., Bausch & Lomb, Inc., Austin, Texas). The second channel of the recorder was used to monitor simultaneously the membrane potential of the axon (Blaustein and Russell, 1975). The background count for the flow cell arrangement was 21–25 cpm. The count rate for ^{45}Ca samples in the flow cell system was, for the Beckman cell 18.1% and, for the Packard cell, 61.4% of the rate for 1.0-ml samples of the same labeled fluid mixed with a toluene-Triton X-100 cocktail (Nadarajah et al., 1969; Blaustein and Russell, 1975) and counted in a more conventional liquid scintillation system. Most of the low efficiency of the flow cell system could be accounted for by the small volume of the flow cells. Nevertheless, a high ^{45}Ca specific activity was employed in most experiments (see above) to ensure that the count rate during superfusion with 10 K(Na) or 12 K(Na) + CN was at least 15–30 times background; un-

fortunately, this high count rate could not always be achieved in axons with a very low intracellular ionized Ca concentration ($[Ca^{2+}]_i < 0.3\ \mu M$; e.g., Fig. 7).

Aliquots of the radioactive dialysis fluids were diluted 1:1,000, mixed with toluene-Triton X-100 cocktail, and counted conventionally (in counting vials) in a Beckman LS-300 liquid scintillation counter to determine specific activities. Appropriate corrections were made for the differences in efficiency between the two counting methods, so that the flow cell data could be reported in picomoles per square centimeters · second.

The axons were superfused with external solutions at a rate of 1.2 ml/min; 0.1 ml/min flowed through the guards (cf. Blaustein and Russell, 1975) and was discarded. The remaining 1.1 ml/min was collected, and about 80% was channeled through the flow cell. (It should be noted that the absolute rate of flow through the flow cell is important only in terms of the time required to wash out the flow cell. The count rate is only related to the concentration of ^{45}Ca (μCi/ml) in the solution flowing through the flow cell.)

The performance of the flow cell apparatus is illustrated by the data in Fig. 1. This recording was obtained with an axon in place, at the end of a ^{45}Ca efflux experiment; although the axon was being dialyzed with nonradioactive fluid, the high background may be due to efflux of residual ^{45}Ca from the axoplasm. At "*a*", the 10 K(Na) entering the chamber was replaced by a similar solution containing a small amount of ^{22}Na (sufficient to give a count rate

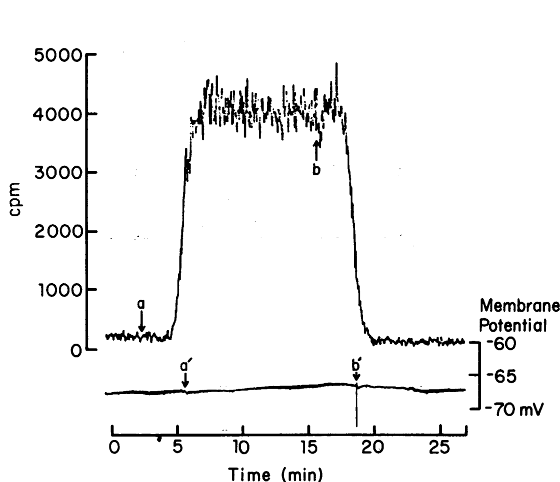


FIGURE 1

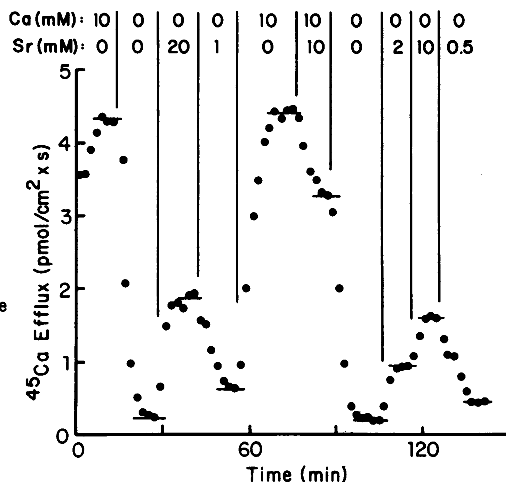


FIGURE 2

FIGURE 1 Time-course of wash-in and wash-out of squid axon chamber and flow cell with ^{22}Na-labeled 10 K(Na). Axon 6,036 was present in the chamber, and was being dialyzed with nonradioactive dialysis fluid. The data were obtained at the end of a ^{45}Ca efflux experiment. At *a*, the inflow to the axon chamber was switched from nonradioactive to ^{22}Na-labeled 10 K(Na); at *b*, the nonradioactive 10 K (Na) was reintroduced. The points, *a'* and *b'* on the voltage record correspond in time to *a* and *b*, on the liquid scintillation counter record. Temp = 15°C, axon diam = 640 μm.

FIGURE 2 Ca efflux into (Na-free) 12 K(Li) + CN solutions containing mixtures of Mg, Ca, and Sr. The data are plotted as a function of time; ^{45}Ca-containing dialysis fluid was introduced 90 min before 0 time on the abscissa. The sum of $[Mg^{2+}]_o + [Ca^{2+}]_o + [Sr^{2+}]_o$ was always 60 mM; the concentrations of Ca and Sr in the superfusion fluids are indicated at the top of the graph. Axon 5,154A; $[Ca_t]_i$ = 1.06 mM; $[EGTA_t]_i$ = 1.00 mM; free $[Ca^{2+}]_i \simeq 60\ \mu M$, diam = 610 μm, temp = 14.5°C, resting potential = −65 mV.

of about 5,000 cpm/ml with standard liquid scintillation methods); preliminary experiments indicated that both ^{45}Ca and ^{22}Na could be counted similarly in the flow cell. Note that the count rate of the Beta-mate II began to rise about 2 min after the flow of ^{22}Na solution was started, and began to fall about 2 min after the reintroduction of the nonradioactive solution (at "*b*"); these delays were due to the dead time between the solution reservoir and the chamber, and between the chamber and the flow cell. Ignoring the dead time (since this time does not reflect chamber or flow cell washout time), the data indicate that the time constants for the rise and the fall of the count rate were both about 1 min and a nearly steady count rate was reached in about 3 min. This does not take into account the washout of the space immediately surrounding the axolemma. During Ca efflux studies the time constant for the approach to a new steady Ca efflux, when the external Ca or Na concentration was changed (e.g. Figs. 2 and 9), averaged about 3 min.

MEMBRANE POTENTIALS The resting membrane potential was recorded through a pair of calomel electrodes. The reference electrode made contact, via a KCl bridge, with the fluid emerging from the axon chamber through one of the guard outflows (see Fig. 1 of Blaustein and Russell, 1975); the internal potential was then recorded through an electrode which contacted, via a KCl bridge, the dialysis fluid emerging from the distal end of the dialysis capillary. Further details of the membrane potential recording apparatus, and of procedures used to monitor action potentials during the efflux experiments, are given elsewhere (Blaustein and Russell, 1975).

MEMBRANE POTENTIAL CONTROL IN EXPERIMENTS WITH LOW $[K]_i$ In several experiments the axons were dialyzed with K-free fluids (Table II). Because replacement of internal K caused the axon to depolarize, and because the membrane potential is known to affect Ca efflux (Brinley and Mullins, 1974; Blaustein et al., 1974; Mullins and Brinley, 1975), the membrane potential in these axons was "clamped" at a value close to the original resting potential. The hollow cellulose acetate tube used to dialyze these axons was fitted with a 25-μm diameter platinized Pt-Ir (10%-90%) axial wire. The wire was connected to the anode of a Grass SD-9 stimulator (Grass Instrument Co., Quincy, Mass.). The cathode of the stimulator was connected through a 1-MΩ series resistor to an Ag-AgCl wire embedded in an agar-sea water electrode which, in turn, was in contact with the external solution bathing the dialyzed portion of the axon. Hyperpolarizing DC current could then be passed across the axon membrane to maintain the membrane potential (recorded between the external medium and the dialysis fluid effluent, as described above) at about the level observed during the control period (i.e., before lowering $[K]_i$).

PREPARATION OF ATP-DEPLETED AXONS In a number of experiments the axons were depleted of ATP by adding 2 mM CN to both the internal and external fluids, and by omitting ATP from the internal fluids. The axons were exposed to cyanide for about 1.5-2 h, and dialyzed with the ATP-free, CN-containing fluid for at least 1 h before any critical flux measurements were made (cf. Blaustein and Russell, 1975). Although the ATP concentrations in the effluent dialysis fluids were not checked, values below about 10 μM may be expected (cf. Mullins and Brinley, 1967; DiPolo, 1973).

METHOD OF EXPRESSING THE FLUX DATA All reported Ca flux values are based on the averaged digitally recorded count rates from the last 3 min of exposure to the relevant external (and internal) fluid; the fluxes are given in pmol/cm^{2}s. Rather than describing the total Ca efflux, it will often be convenient to refer to the external Na- and Ca-dependent fractions of the efflux. The term "Na_o-dependent Ca efflux" will be used to denote the increment in Ca efflux observed when an Na-free external fluid (usually choline-based) is replaced by one containing a partial or full (425 mM) complement of Na. The term "Ca_o-dependent Ca efflux" will be used to indicate the increment in Ca efflux when a Ca-free external fluid is replaced by one containing Ca. In a few experiments the stimulation of Ca efflux by Li

was also measured; the terms "Li_o-stimulated" or "Li_o-dependent Ca efflux" will be used to refer to the increment in Ca efflux observed when Li replaced choline as the predominant external monovalent cation; this increment is also Ca_o-dependent (Blaustein and Russell, 1975).

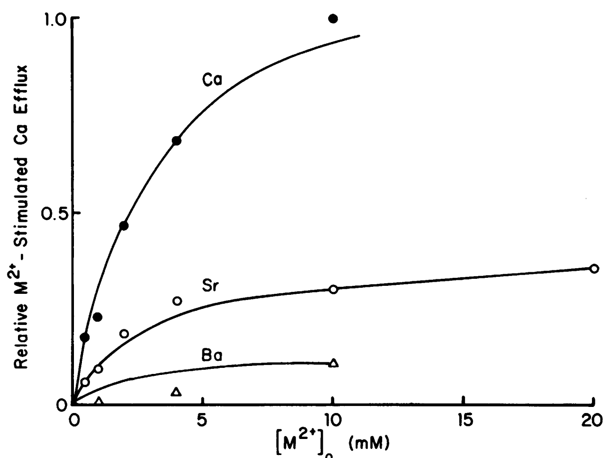
RESULTS

ATP-Depleted Axons

EXTERNAL DIVALENT CATION ACTIVATION OF CA EFFLUX As shown in several previous studies (Blaustein and Hodgkin, 1969; DiPolo, 1973; Blaustein et al., 1974; Blaustein and Russell, 1975), much of the Ca efflux from squid axons, especially from poisoned axons, depends upon the presence of alkali metal and alkaline earth cations in the bathing medium. The activation of Ca efflux by external alkali metal ions was recently explored in some detail (Blaustein and Russell, 1975); the data indicate that Na, alone, is sufficient to promote Ca efflux, while Li, K, or Rb promote Ca efflux only in the presence of external Ca. In view of these findings, it seemed appropriate to examine the influence of various external divalent cations, to obtain information about the selectivity of the divalent cation site on the presumed carrier.

The main observation made in this series of experiments on nine ATP-depleted axons was that Sr and, to a lesser extent, Ba were able to substitute for external Ca in promoting Ca efflux into Li-containing media. Data from a portion of a representative experiment, in which the activation curve for Sr was determined, are graphed in Fig. 2; activation curves for Ca, Sr, and Ba are shown in Fig. 3. The reference solution for these experiments was Ca-free 12K(Li) + CN, which contained 60 mM Mg^{2+} (Table I); complete omission of external divalent cation seemed unwise in view of the known deleterious effect of this treatment on axon stability (e.g., Frankenhaeuser and Hodgkin, 1957). Consequently, the total divalent cation concentration was maintained constant, at 60 mM, as Ca, Sr, and/or Ba (or Mn, see below) was substituted for some of the Mg. The Ca efflux data reported here are thus subject to the error that external Mg may, itself, support Ca efflux to a small extent (Blaustein and Hodgkin, 1969), or may inhibit the action of other external divalent cations on Ca efflux.

Activation of Ca efflux by external Ca appears to fit a rectangular hyperbola, with half-saturation (K_{Ca_o}) at about 3 mM Ca (Fig. 3); a nearly identical curve was obtained in an earlier series of experiments on *Loligo forbesi* (Blaustein et al., 1974). In three experiments (e.g. Fig. 2), a similar relationship between Ca efflux and external Sr concentration was observed (Fig. 3). The apparent half-saturation for Sr (K_{Sr_o}) ranged between about 3.0 and 3.6 mM, not significantly different from the value for Ca; however, the maximal Ca efflux at saturating Sr concentrations was much less than the efflux with high external Ca. These Ca and Sr data suggest that, although Sr may bind to the carrier as well as Ca (i.e. $K_{Ca_o} \simeq K_{Sr_o}$), the turnover (i.e., maximum velocity) with Sr is slower than with Ca. The kinetic aspects of activation by Ba are more difficult to evaluate, because this ion increased Ca efflux only slightly (e.g. see Fig. 3): the data from four axons indicate that the maximal Ca efflux into a Ba-con-



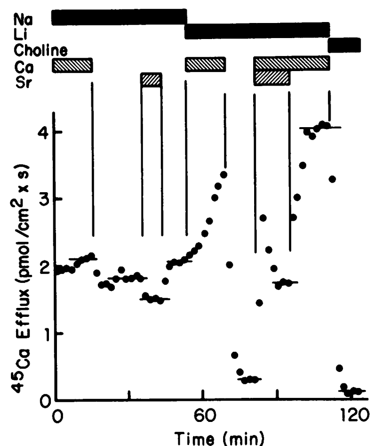


FIGURE 3

FIGURE 4

FIGURE 3 Relative external divalent cation-dependent ^{45}Ca efflux graphed as a function of the external divalent cation concentration ($[M^{2+}]_o$). The reference solution (relative Ca efflux = 0) was Ca-free 12 K(Li) + CN (see Table I); the extra Ca efflux, when $[Ca^{2+}]_o$ was raised to 10 mM, was taken as a relative efflux of 1.0. All three curves are fitted to an equation of the form: $J_{Ca} = J^*_{Ca} / (1 + K_{M_o^{2+}}/[M^{2+}]_o)$ where J^*_{Ca}, the (relative) maximal Ca efflux, has values of 1.22, 0.40, and ~ 0.14 for Ca, Sr, and Ba, respectively. The apparent half-saturation values ($K_{M_o^{2+}}$) for the three cations are, 3.1, 3.0, and ~ 3 mM for Ca, Sr, and Ba, respectively, (the constants for Ba were approximated from data from four axons). The Sr data are from axon 5,145A (see Fig. 2); the Ca and Ba data are from axon 5,145B ($[Ca_t]_i$ = 1.06 mM; $[EGTA_t]_i$ = 1.00 mM; free $[Ca^{2+}]_i$ ≃60 μM, diam 600 μm, temp = 15°C, resting potential = −59 mV). The mean Ca efflux into Ca-free 12 K(Li) + CN was 0.35 pmol/cm^{2}s for the two axons, and into 12 K (Li) + 10 mM Ca + CN, 4.17 pmol/cm^{2}s.

FIGURE 4 Effect of strontium ions on ^{45}Ca efflux into Ca-free 12 K (Na) + Ca and into 12 K (Li) + 10 mM Ca + CN. Solid bars at the top indicate the presence of 426 mM Na, Li, or choline, respectively, in the superfusion fluid; hatched bars indicate the presence of 10 mM Ca and/or 10 mM Sr. The total divalent cation concentration (sum of $[Mg^{2+}]_o$ + $[Ca^{2+}]_o$ + $[Sr^{2+}]_o$) was always 60 mM. Dialysis with ^{45}Ca-containing fluid was initiated 50 min before 0 time on the graph. Axon 5, 155B; $[Ca_t]_i$ = 1.06 mM, $[EGTA_t]_i$ = 1.00 mM, free $[Ca^{2+}]_i$ ≃60 μM, diam = 675 μm, temp = 15.5°C, resting potential = −69 mV.

taining solution may be less than that into a Ca- or Sr-containing solution, and that the affinity for Ba may be similar to that for Ca or Sr.

Another observation, consistent with the foregoing statements about turnover, is that when 10 mM Sr (Fig. 2) or Ba was added to a solution containing 10 mM Ca, the Ca efflux was inhibited, although Sr or Ba alone stimulated Ca efflux. In four experiments (Figs. 2 and 4) 10 mM Sr inhibited the Ca_o-dependent Ca efflux by an average of 30%; in a single experiment, 10 mM Ba reversibly reduced the Ca efflux by 65% (a K_{Ba_o} of about 1.6 mM is needed to account for this much inhibition). The fact that Sr and Ba inhibit the Ca_o-dependent Ca efflux also implies that the stimulation due to Sr or Ba in the Ca-free solutions (Figs. 2 and 3) cannot be explained by Ca contamination in the Sr- and Ba-containing fluids (cf. Baker and McNaughton, 1976*a*).

One possibility is that the Ca effluxes promoted by Ca and Sr (and Ba) involve, respectively, Ca-Ca exchange and Sr-Ca exchange (and Ba-Ca exchange). If external Ca and Sr compete for external binding sites and if both ions activate Ca efflux by first-order saturation kinetics (Fig. 3) then when both ions are present, the total external alkaline earth cation-dependent Ca efflux will be given by the sum of Ca-Ca exchange ($J_{\text{Ca-Ca}}$) and Sr-Ca exchange ($J_{\text{Sr-Ca}}$):

$$J_{\text{Ca-Ca}} + J_{\text{Sr-Ca}} = \frac{J^*_{\text{Ca-Ca}}}{K_{\text{Ca}_o}/[\text{Ca}]_o + ([\text{Sr}]_o/[\text{Ca}]_o)(K_{\text{Ca}_o}/K_{\text{Sr}_o}) + 1} + \frac{J^*_{\text{Sr-Ca}}}{K_{\text{Sr}_o}/[\text{Sr}]_o + ([\text{Ca}]_o/[\text{Sr}]_o)(K_{\text{Sr}_o}/K_{\text{Ca}_o}) + 1}. \quad (1)$$

The $J^*_{\text{Ca-Ca}}$ and $J^*_{\text{Sr-Ca}}$ terms are the maximal Ca-Ca and Sr-Ca exchange fluxes; the apparent half-saturation constants for external Ca and Sr are, respectively, K_{Ca_o} and K_{Sr_o}. Substituting the appropriate values for these constants, obtained from Fig. 3 (see caption), into Eq. 1 indicates that the alkaline earth-dependent Ca efflux should be about 25% less when $[\text{Ca}]_o = [\text{Sr}]_o = 10$ mM, than when $[\text{Ca}]_o = 10$ mM and $[\text{Sr}]_o = 0$. This expected inhibition is reasonably close to the mean of the four observed values, 30% (cf. Figs. 2 and 4).

The effects of Mn ions were also tested, because these ions are known to inhibit the Ca_o-dependent Ca efflux in barnacle muscle fibers (Russell and Blaustein, 1974) and in pinched-off presynaptic nerve terminals (synaptosomes) from rat brain (Blaustein and Ector, 1976). In two preliminary experiments, 10 mM Mn was observed to reduce reversibly the Ca_0-dependent Ca efflux by 37 and 47%; Mn did not significantly affect the Ca efflux into Na-free Ca-free superfusion fluids. These data are consistent with the idea that Mn ions displace Ca from the carriers, but that the Mn-load carriers do not cycle.

THE EFFECTS OF SR AND MN ON THE NA_o-DEPENDENT CA EFFLUX As noted above (and see Blaustein and Russell, 1975), Ca efflux from squid axons can be promoted by external Na, or by external Ca plus an alkali metal ion; these effluxes are presumably manifestations of Na-Ca and Ca-Ca exchange, respectively. The assumption is that both these exchanges involve a single (common) carrier mechanism, because external Na and Ca appear to compete for carrier sites (Baker et al., 1969). Additional evidence that Na and divalent cations may compete for common carriers comes from the observation that Sr (Fig. 4) and Mn both inhibit Ca efflux into Ca-free 12 K(Na) + CN; in two axons 10 mM Mn inhibited Na_o-dependent Ca efflux by 22 and 33%, while in two other axons, 10 mM Sr inhibited by 24 (Fig. 4) and 26%, respectively.

THE EFFECT OF EXTERNAL NA ON CA EFFLUX INTO LI SEAWATER The foregoing considerations support the idea that a single carrier mechanism is used to extrude Ca in exchange for Na, or for another Ca ion. That both the Na_o-dependent and the Ca_o-dependent Ca effluxes are inhibited by internal Na, and are half-maximally activated by the same internal free Ca^{2+} concentration (about 5 μM in ATP-

depleted axons; Blaustein and Russell, 1975), also lends credence to this idea. However, this evidence is certainly not conclusive.

Data from an experiment intended to help settle this point are illustrated in Fig. 5. The experiment is based on the observation that the Na_o-dependent Ca efflux is fully activated by a solution containing only half the normal concentration of Na, i.e., 212.5 mM Na (Fig. 10; and see Blaustein et al., 1974, Fig. 3), while the Ca_o-dependent Ca efflux, which requires an alkali metal ion, is also fully activated by a solution containing only 212.5 mM Li (Fig. 5; and see Blaustein et al., 1974, Fig. 9). Under these circumstances, if the Na_o-dependent and Ca_o-dependent Ca effluxes are mediated by independent mechanisms (i.e., the alternative hypothesis), the Ca efflux into a solution containing 212.5 mM Na + 212.5 mM Li should be approximately equal to the sum of the Na_o-dependent and Ca_o-dependent (Li_o-stimulated) Ca effluxes. However, as shown in Fig. 5, the Ca efflux into the Na-Li mixture is almost the same as into 12K(Na) + CN, and is much lower than the efflux into 12K(Li) + CN. In evaluating these data, it is important to note that external choline behaves neither like Na (i.e., choline does not promote Ca efflux in the absence of external Ca), nor like Li (i.e., choline has only a slight stimulatory effect on Ca efflux in the presence of external Ca): see last segment of the experiment illustrated in Fig. 5, and Blaustein et al. (1974), and Blaustein and Russell (1975). The conclusion is that the Na_o- and Ca_o-dependent Ca effluxes are not independent; the most straightforward possibility is that Na-Ca and Ca-Ca exchange are mediated by a single carrier system.

The very marked inhibition by external Na (Fig. 5) may seem somewhat surprising

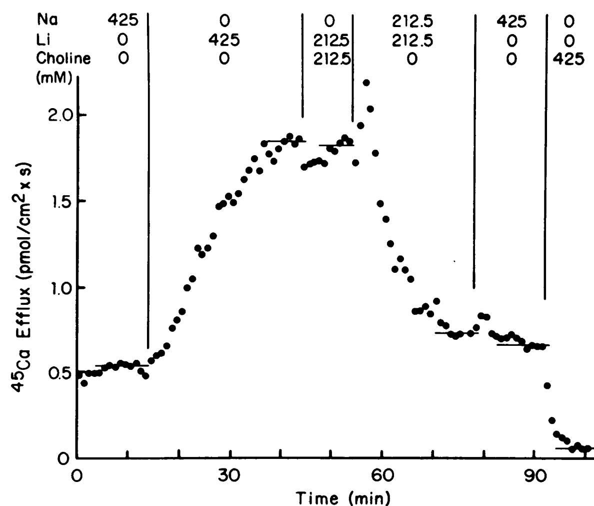


FIGURE 5 "Occlusion" of Ca efflux into Li-containing fluid by external Na. The monovalent cation content of the superfusion fluid bathing the axon during each segment of the experiment is indicated at the top of the respective time segments. All external solutions contained 10 mM Ca, 50 mM Mg, 12 mM K, and 2 mM CN. The ^{45}Ca-containing dialysis fluid flow was begun 30 min before 0 time on the graph. Axon 5,136B. $[Ca_t]_i$ = 0.90 mM, $[EGTA_t]_i$ = 1.00 mM, free $[Ca^{2+}]_i$ $\simeq$ 1 μM, diam = 560 μm, temp = 14.5°C, resting potential, −72 mV.

TABLE III

EFFECT OF INTERNAL MONOVALENT CATIONS ON ^{45}CA EFFLUX

Axon	Internal monovalent cation*	^{45}Ca efflux into: 12 K(Na)-+ CN	12 K(Li) + CN	12 K(choline) + CN	Δ_{Na}‡	Δ_{Li}§
			pmol/cm^{2}s		*pmol/cm^{2}s*	
5,195	K	1.44	1.80	0.18	1.26	1.62
	TMA	1.38	0.37	0.23	1.15	0.14
5,245	K	2.08	4.36	0.26	1.82	4.10
	TMA	1.82	0.98	0.31	1.51	0.67
	Li	3.42	3.66	0.34	3.08	3.32
5,255	K	2.04	4.47	0.07	1.97	4.40
	Na	0.27	0.26	0.08	0.19	0.18
5,265	K	3.03	4.97	0.05	2.98	4.92
	TMA	2.17	1.07	0.05	2.12	1.02
	100 mM Na +300 mM TMA	0.43	0.44	0.04	0.39	0.40
	Li	3.20	3.96	0.17	3.03	3.79
	K	2.90				
5,275B	K	2.19	3.11	<0.5	1.69	2.64
	TMA	1.47	0.72	0.30	1.17	0.42
	K	3.15	3.60	0.94	2.21	2.66
5,295A	K	2.83	4.50	0.23	2.60	4.27
	Li	2.29	3.48	0.09	2.20	3.39

*At a concentration of 400 mM (except for the Na-TMA mixture used in axon 5,265). In all experiments the dialysis fluids contained 1.13 mM Ca and 1.0 mM EGTA (nominal $[Ca^{2+}]_i \simeq 130\ \mu M$). During dialysis with K-free fluids the membrane potential was routinely clamped at −65 to −70 mV.

‡Δ_{Na} = efflux into 12 K(Na) + CN minus efflux into 12 K(choline) + CN.

§Δ_{Li} = efflux into 12 K(Li) + CN minus efflux into 12 K(choline) + CN.

because the apparent half-saturation constants for both Na_o and Li_o are about 120 mM (Blaustein et al., 1974, and see Fig. 10). However, the inhibition could be accounted for if three Na^+ ions can bind to the carrier at (three) noninteracting sites (cf. Fig. 10 and related text), and if the occupancy of any one of these sites by an Na^+ is sufficient to prevent Li from binding (at the one site that accepts Li^+). In addition (or, alternatively), external Na may displace Ca from the carrier sites (cf. Baker et al., 1969); these effects may be comparable to the competition between Na and Ca displayed at the internal binding sites (e.g. Table III and Fig. 11, and Blaustein and Russell, 1975).

EFFECTS OF INTERNAL MONOVALENT CATIONS ON CA EFFLUX The experiments described above and in two earlier papers (Blaustein et al., 1974; Blaustein and Russell, 1975) help to define the effects of internal and external Ca, and of external monovalent cations on the kinetics of Ca efflux in ATP-depleted axons. To characterize the Ca transport mechanism further, the influence of internal alkali metal ions on Ca efflux was examined in several experiments; the results are summarized in Table III. The main observation, viz. that the influence of the dialysis fluid composition on Ca efflux also depends upon the composition of the external medium, is exemplified by

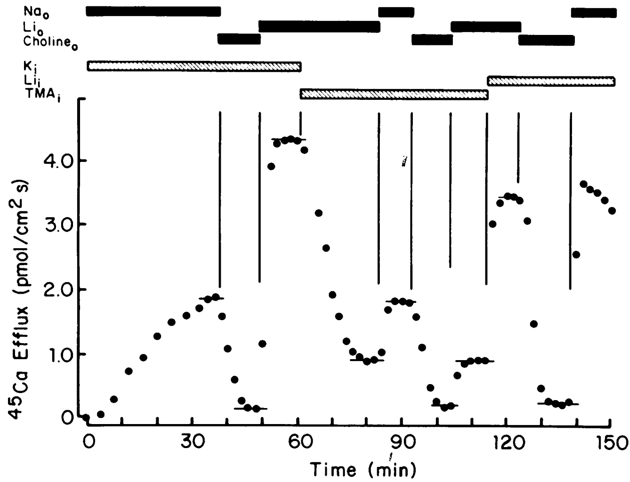


FIGURE 6 Effect of internal and external monovalent cations on Ca efflux. The efflux of ^{45}Ca is graphed as a function of time in the lower portion of the figure. Infusion of ^{45}Ca-containing dialysis fluid was started at zero-time. The solid bars at the top of the figure indicate the periods when the axon was superfused with 12 K(Na) + CN (Na_o), 12 K(Li) + CN (Li_o), or 12 K(choline) + CN($choline_o$) (see Table I); all solutions contained 10 mM Ca and 2 mM CN. The hatched bars refer to the periods of internal dialysis with K-DF (K_i), Li-DF (Li_i), or TMA-DF (TMA_i) (see Table II). All the dialysis fluids contained 1.13 mM Ca and 1.00 mM EGTA ($[Ca^{2+}]_i \simeq 130\,\mu M$). Axon 5,245, diam = 600 μm, temp = 14.5°C. The membrane potential was −66 mV during the dialysis with K-DF; during the remainder of the experiment the potential was clamped at −65 mV (see Methods).

the data in Fig. 6. When this axon was dialyzed with the standard (K-rich) solution, there was a considerable Ca efflux into 12 K(Na) + CN (presumably Na-Ca exchange, primarily) and into 12 K(Li) + CN (presumably Ca-Ca exchange). The larger efflux into the Li-rich medium has been noted previously (Blaustein et al., 1974; Blaustein and Russell, 1975). The Ca efflux into the alkali metal ion-depleted (choline-containing) medium is rather small; in two preliminary experiments, an identical result was obtained when all the external NaCl was replaced by tetramethylammonium (TMA) chloride.

In the third part of the experiment of Fig. 6, when Li was the predominant cation in the dialysis fluid, a large Ca efflux into 12 K(Na) + CN and into 12 K(Li) + CN was also observed; the efflux into 12 K(choline) + CN was, again, very small. However, when the internal alkali metal ion was replaced by TMA (middle portion of the experiment of Fig. 6), the Ca efflux into 12 K(Li) + CN was markedly reduced and, in fact, fell below the level of Ca efflux into 12 K(Na) + CN. This effect, inhibition of the Ca efflux into 12 K(Li) + CN when internal K (and Na) was replaced by TMA, was very reproducible, as shown in Table IV, where data from four axons are summarized. Clearly, the effect is not simply due to removal of internal K, because Ca efflux into 12 K(Li) + CN is also prominent when Li is the main internal monovalent cation (Fig. 6 and Table III). The fact that the Na_o-dependent Ca efflux is, on the

TABLE IV

INHIBITION OF EXTERNAL Na-DEPENDENT AND Li-DEPENDENT CALCIUM EFFLUX BY REPLACEMENT OF INTERNAL K WITH TMA

Axon		Na_o-dependent Ca efflux (Δ_{Na})*			Li_o-dependent Ca efflux (Δ_{Li})*		
		K-DF	TMA-DF	Inhibition	K-DF	TMA-DF	Inhibition
		pmol/cm²s		%	*pmol/cm²s*		%
5,195		1.26	1.15	9	1.62	0.14	91
5,245		1.82	1.51	17	4.10	0.67	84
5,265		2.98	2.12	29	4.92	1.02	79
5,275B		1.69	1.17	31	2.64	0.42	84
	Mean ± SE	1.94 ± 0.37	1.41 ± 0.16	21 ± 5	3.32 ± 0.74	0.56 ± 0.19	85 ± 3

*Increment in ^{45}Ca efflux due to replacement of external choline by Na (Δ_{Na}) or by Li (Δ_{Li}); see Table III.

average, inhibited only slightly when internal K is replaced by TMA (Table IV), indicates that Ca efflux into Na-rich media (primarily Na-Ca exchange) does not need to be activated by an internal alkali metal ion. On the other hand, Ca efflux into Li-rich media (primarily Ca-Ca exchange) requires alkali metal ions on both sides of the axolemma (although high internal concentrations of Na are inhibitory, because the carrier may then start to operate in the "backward" Na-Ca exchange mode; see below and Baker et al., 1969, and Blaustein and Russell, 1975). The slight inhibition of Ca efflux into 12 K(Na) + CN observed when TMA replaced internal K (Table IV) could be accounted for if some of the efflux ino 12 K(Na) + CN with K (but not TMA) inside, is Ca-Ca exchange (Blaustein and Russell, 1975).

One possible explanation for the observation that Li_o- and Ca_o-dependent Ca efflux, but not Na_o-dependent Ca efflux, requires an internal alkali metal ion is that the two fluxes (Na_o- and Li_o-dependent, respectively) are mediated by different mechanisms. However, the results discussed in the preceding section appear to rule out this possibility. An alternative explanation is that the Ca transport mechanism involves the simultaneous transfer of inward- and outward-moving ions. This hypothesis, also supported by data from axons dialyzed with ATP-containing fluids (see below), will be reviewed in the Discussion section.

An unusually large Na_o-dependent Ca efflux was observed when, with Li as the predominant internal cation, external choline was replaced by Na (Fig. 6). In this case, the Ca efflux was still declining and had not yet reached a steady level when the experiment was terminated. Even with K as the main internal cation, reintroduction of a superfusion fluid containing 425 mM Na was often (but not always) observed to induce a large transient Ca efflux (see Fig. 9). Moreover, in two other fibers (Table III, axons 5,245 and 5,265), the steady Ca efflux into 12 K(Na) + CN, with Li inside was comparable in magnitude to the efflux with K as the main internal cation.

The cause of the large efflux transients, observed during reintroduction of the standard (425 mM) Na superfusion fluid (Figs. 6 and 9) is uncertain. One possibility is that the Na concentration in the axoplasm, just inside the axolemma, may slowly rise as a consequence of Na influx and the inability of the dialysis system to control

$[Na^+]_i$ in this region of the axoplasm when the concentration of Na in the dialysis fluid is low (5 mM). A rise in $[Na^+]_i$ would be expected to inhibit the Ca efflux due to competition between internal Na and Ca (see Fig. 11 and Blaustein and Russell, 1975).

Axons Dialyzed with ATP-Containing Fluids

In 1973, Baker and Glitsch suggested that ATP might facilitate Ca extrusion from squid axons. Their hypothesis was based on the observation that Ca efflux from ^{45}Ca-injected axons with a low free $[Ca^{2+}]_i$ was reduced by cyanide poisoning or by injection of apyrase (which splits ATP to AMP + 2 P*i*). Furthermore, the affinity of the Na-dependent Ca extrusion mechanism for external Na appeared to be reduced by cyanide poisoning. Experiments on the effects of ATP in dialyzed squid axons (DiPolo, 1974; Blaustein et al., 1974) have helped to substantiate this hypothesis. To evaluate further the role of ATP in Ca extrusion, the influence of ATP on the internal and external cation activation of Ca efflux was investigated in the present study.

EFFECT OF ATP ON THE ACTIVATION OF CA EFFLUX BY $[Ca^{2+}]_i$ In ATP-depleted axons, both the Na_o-dependent and the Ca_o-dependent (Li_o-stimulated) Ca effluxes are half-maximally activated by a free $[Ca^{2+}]_i$ in the 3–8 μM range (Fig. 8, and Blaustein and Russell, 1975). However, the free $[Ca^{2+}]_i$ in normal, ATP-fueled axons may be only about 0.1 μM or lower (Baker, 1972; Blaustein, 1974; Di Polo et al., 1976), well below the 3–8 μM concentration range. It is difficult to imagine how a

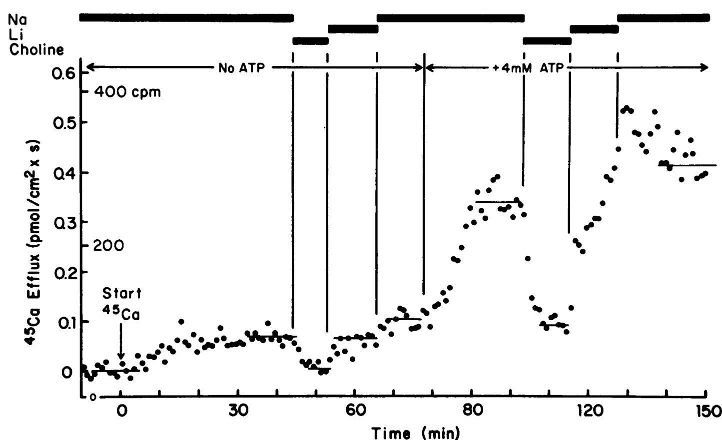


FIGURE 7 Effect of ATP on ^{45}Ca efflux into 12 K (Na) + CN, 12 K (Li) + CN, and 12 K (choline) + CN. The periods of exposure to the respective superfusion fluids are indicated by the bars at the top of the graph; all external fluids contained 2 mM CN. The axon was dialyzed initially with a fluid containing 2 mM CN, 15 μM FCCP, and no ATP. Dialysis with ^{45}Ca-labeled fluid began at 0 time; 76 min later, the ATP-free dialysis fluid was replaced by a similar fluid containing 4 mM ATP and 9 mM $MgCl_2$ (instead of the usual 5 mM $MgCl_2$). Both the actual count rate (in cpm) and the calculated Ca efflux (in pmol/cm^2s) are indicated on the ordinate. Axon 5,166, $[Ca_t]_i$ = 0.86 mM, $[EGTA_t]_i$ = 1.20 mM, free $[Ca^{2+}]_i \simeq 0.33$ μM, diam 545 μm, temp = 14.5°C, resting potential = −65 mV.

transport system with such a low affinity for Ca could maintain $[Ca^{2+}]_i$ at the normal resting level. Therefore, to determine whether or not ATP influences the kinetics of free $[Ca^{2+}]_i$ activation (as proposed by Baker and Glitsch, 1973), the Na_o-dependent ^{45}Ca efflux was measured in a number of axons dialyzed with ATP-free and then with ATP-containing solutions. Data from a representative experiment are illustrated in Fig. 7; the main observation here is that the Na_o-dependent Ca efflux increased

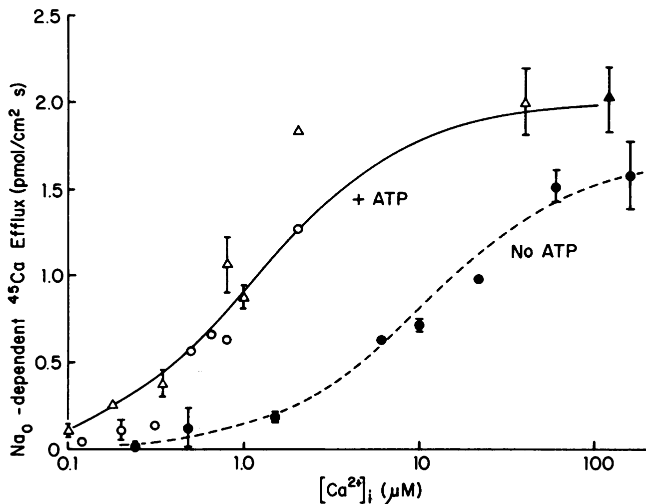


FIGURE 8 Effect of ATP on the relationship between $[Ca^{2+}]_i$ and the Na_o-dependent ^{45}Ca efflux. Circles: data obtained in the absence of external Ca; the graphed fluxes represent the difference between the efflux into Ca-free 10 K (or 12 K) (Na), and that into Ca-free 10 K (or 12 K) (choline), or Ca-free 10 K (or 12 K) (Li). Triangles: data obtained in the presence of external Ca; the graphed fluxes represent the difference between the efflux into 10 K (or 12 K) (Na) and that into 10 K (or 12 K) (choline). Solid symbols: ATP-depleted axons. Open symbols: axons fueled with 4 mM ATP. The solid circles are data from Blaustein and Russell (1975); open circles and solid and open triangles are data from the present study. The curves were drawn to fit the equation (Blaustein and Russell, 1975): $J_{Na\text{-}Ca} = J^*_{Na\text{-}Ca}/\{1 + (K_{Ca_i}/[Ca^{2+}]_i)(1 + [Na^+]_i/K_{Na_i})^2\}$, where $J_{Na\text{-}Ca}$ is the Na_o-dependent Ca efflux (Na-Ca exchange) at any $[Ca^{2+}]_i$ and $[Na^+]_i$. The maximal efflux, $J^*_{Na\text{-}Ca}$, was taken as 2.0 pmol/cm^{2}s (continuous line) or 1.7 pmol/cm^{2}s (interrupted line). The apparent half-saturation constant for internal Ca, K_{Ca_i}, was 0.73 μM (continuous line) or 8 μM (interrupted line). For both curves, the mean half-saturation constant for internal Na, $\overline{K}_{Na_i}$, was taken as 30 mM, and $[Na]_i$ was 5 mM (see Fig. 11). The figure represents a summary of the data from determinations of the Na_o-dependent Ca efflux on 71 axons (including 32 axons from Blaustein and Russell, 1975). In most experiments, two or more data points were obtained (e.g. Ca efflux $\pm$ ATP at constant $[Ca^{2+}]_i$, and/or Ca efflux at two or more levels of $[Ca^{2+}]_i$). The triangles and solid circles represent the means of three or more determinations; error bars are shown when they extend beyond the edges of the symbols. The solid triangle at $[Ca^{2+}]_i = 110$ μM (representing the Na_o-dependent Ca efflux from ATP-depleted axons in the presence of external Ca) indicates the mean efflux ($\pm$ SE) from 7 axons; the open triangle at $[Ca^{2+}]_i = 40$ μM, represents the mean efflux ($\pm$ SE) from 13 ATP-fueled axons. The latter axons were dialyzed with solutions containing the same $[Ca_t]$ and $[EGTA_t]$ as were the former (ATP-depleted) axons; the difference in the (calculated) nominal $[Ca^{2+}]_i$ in these two sets of axons is a consequence of the complexation of Ca with the ATP present in the dialysis fluids of the fueled axons.

significantly when ATP was added to the dialysis fluid at constant low (0.33 μM) $[Ca^{2+}]_i$.

A number of similar experiments were made with varying concentrations of EGTA in the dialysis fluids (to alter $[Ca^{2+}]_i$ values), both before and after addition of ATP to the dialysis fluid. Other axons were fueled with ATP throughout the experiment, while $[Ca^{2+}]_i$ was varied by altering $[EGTA_t]_i$. In a number of instances the Na_o-dependence was measured in the absence of external Ca to avoid possible ambiguity concerning the influence of ATP on the magnitude of the Ca_o-dependent Ca efflux. The data from all of these experiments (comprising 71 axons) are summarized in Fig. 8, which shows a graph of the Na_o-dependent Ca efflux plotted as a function of log $[Ca^{2+}]_i$ for both ATP-depleted and ATP-fueled axons; data from Table II (and Fig. 3) of Blaustein and Russell (1975) for ATP-depleted axons have also been included. The semi-logarithmic plot was used to expand the scale at low $[Ca^{2+}]_i$ values; the sigmoid curves here are equivalent to rectangular hyperbolas on a linear plot (see Fig. 11). Note that for the ATP-fueled axons the curve is shifted to the left, an indication that the apparent affinity of the carrier sites for internal Ca^{2+} is increased (i.e., K_{Ca_i} is decreased) by ATP. Another noteworthy observation is that at high $[Ca^{2+}]_i$ the Na_o-dependent Ca efflux is not greatly influenced by ATP; i.e., the maximal rate of Na-Ca exchange, $J^*_{Na\text{-}Ca}$, is relatively unaffected (cf. Blaustein, 1976). This behavior may be contrasted with the effect of ATP on the squid axon Na pump (Brinley and

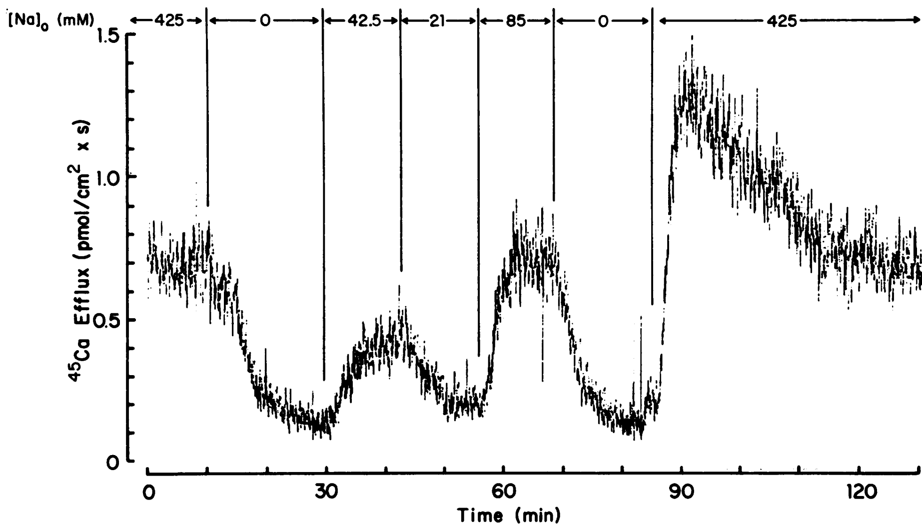


FIGURE 9 Effect of $[Na]_o$ on ^{45}Ca efflux in an ATP-fueled axon. The original output from the liquid scintillation counter is shown; an efflux of 1.0 pmol/cm^{2}s in this experiment corresponds to 2,104 cpm. At the times indicated, the composition of the fluid superfusing the axon was altered to give the Na concentration shown above the record; all external fluids were mixtures of Ca-free 10 K (Na) and Ca-free 10 K (choline). Dialysis with ^{45}Ca-labeled fluid was begun 225 min before 0 time on the graph. Axon 5,246, diam 600 μm, temp. 15°C, resting potential = −60 mV, $[Ca_t]_i$ = 1.00 mM, $[EGTA_t]_i$ = 1.20 mM, $[Ca^{2+}]_i \simeq 0.50$ μM; dialysis fluid contained 10 μM FCCP + 31 μM oligomycin + 4 mM ATP.

Mullins, 1968), and on the human erythrocyte Ca pump (Schatzmann, 1973, and personal communication): in these transport systems, the rate of transport is greatly enhanced by the nucleotide even at high concentrations of the transported ions.

EFFECT OF ATP ON THE ACTIVATION OF CA EFFLUX BY EXTERNAL NA The relationship between $[Na]_o$ and Ca efflux was determined by measuring the efflux into Ca-free Na-based solutions, Ca-free choline-based solutions, and mixtures of the two. The original recording of the Beta-mate II output from a representative experiment on an ATP-fueled axon is shown in Fig. 9. The Na_o-dependent Ca efflux data (i.e., with the efflux into the Na-free choline-based solution subtracted off) from four axons, are graphed as a function of $[Na]_o$ in Fig. 10A; these data correspond to three different conditions. One axon (solid circles) was ATP-depleted and had a relatively high $[Ca^{2+}]_i$ ($\simeq$ 100 μM). The sigmoid shape of this curve and the apparent mean half-saturation constant for external Na, $\bar{K}_{Na_o}$ (approximately 120 mM), are virtually identical to the shape and $\bar{K}_{Na_o}$, respectively, observed in ATP-depleted axons with a high $[Ca^{2+}]_i$, when mixtures of Ca-free 12 K(Na) + CN and Ca-free 12 K(Li) + CN were used to obtain the activation curve (Blaustein et al., 1974). Rather similar results have also been obtained in CN-poisoned, ^{45}Ca-injected axons (Baker and Glitsch, 1973; Baker and McNaughton, 1976*a*).

The three axons represented by the open symbols, in Fig. 10A, were all dialyzed with ATP-containing fluids. One of these axons (△) had a free $[Ca^{2+}]_i$ ($\simeq$ 2.5 μM) considerably in excess of K_{Ca_i} ($\simeq$ 0.73 μM under ATP-fueled conditions; see above and Fig. 8); the other two fueled axons (○, □) had $[Ca^{2+}]_i$ values below K_{Ca_i}(cf. Fig. 8). When normalized to the efflux into 425 mM Na-containing solution, and plotted on a relative scale (Fig. 10B), the Na_o-dependent Ca efflux curves for these three axons were nearly identical. In all three axons the calculated value for $\bar{K}_{Na_o}$ was about 50 mM, which indicates that the affinity of the Ca transport mechanism for external Na is also increased by intracellular ATP. Baker and his colleagues (Baker and Glitsch, 1973; Baker and McNaughton, 1976*a*) and Di Polo (1974) have reached a similar conclusion. However, Baker and his colleagues also concluded that ATP changes the shape of the Na activation curve from a sigmoid curve (in the ATP-depleted condition) to a section of a rectangular hyperbola; in contrast, the data described here (Figs. 9 and 10A) clearly fit a sigmoid curve even under ATP-fueled conditions. This is best illustrated by Hill plots of the data (Fig. 10C), which yield slopes ("Hill coefficients") of about three for all four axons, indicating that at least three external Na^+ ions may be required to activate the efflux of one Ca^{2+} ion even in the presence of ATP. The simplest explanation for the discrepancy between the observations reported here (Figs. 9 and 10) and those of Baker and his colleagues is that the Na_o-dependent Ca fluxes in injected axons (with a low $[Ca^{2+}]_i$) are rather small; moreover, Baker and his colleagues tested rather few external Na concentrations in the range 10–100 mM (e.g., Fig. 14 in Baker and McNaughton, 1976*a*), where the sigmoid nature of the Na activation curve is manifest when $\bar{K}_{Na_o}$ is about 50 mM.

A second important feature of the external Na activation curves (in ATP-fueled axons) is that there is no significant change in kinetics when the $[Ca^{2+}]_i$ is raised from

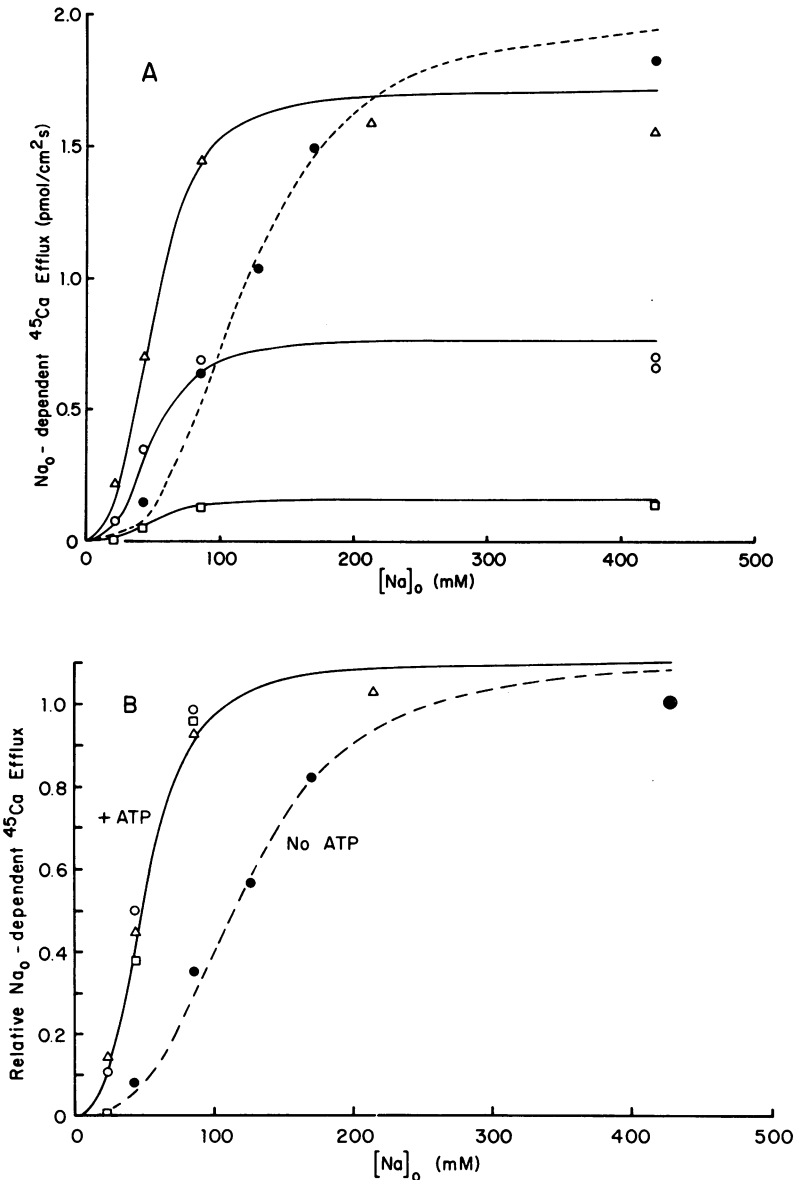
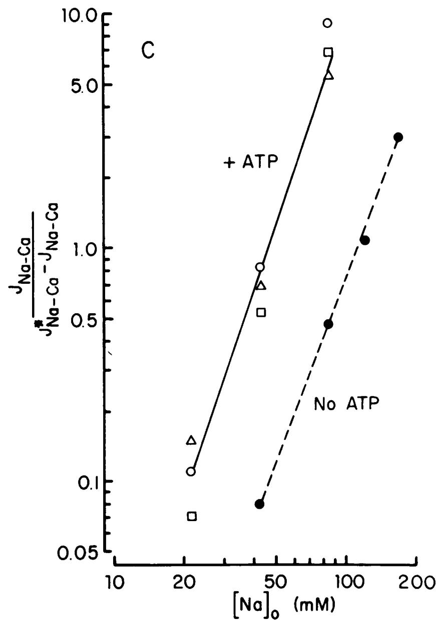


FIGURE 10 A. The Na_o-dependent ^{45}Ca efflux graphed as a function of the external Na concentration. The Ca efflux into Ca-free 10 K (choline) or Ca-free 12 K (choline) + CN was used as a reference (0). When $[Na]_o$ was reduced below 425 mM, it was replaced mole for mole by choline. Each symbol refers to a different axon; those represented by the open symbols were dialyzed with ATP-containing fluids, while the axon represented by the solid circles was ATP-depleted. (●) Axon 5,125B, diam = 575 μm, temp 14.5°C, resting potential = −65 mV, $[Ca_t]$ = 1.10 mM, $[EGTA_t]$ = 1.00 mM, $[Ca^{2+}]_i \simeq$ 100 μM, ATP-free dialysis fluid. (○) Axon 5,246, diameter = 600 μm, temp. = 15°C, resting potential = −60 mV, $[Ca_t]$ = 1.00 mM, $[EGTA_t]$ = 1.20 mM, $[Ca^{2+}]_i \simeq$ 0.50 μM, dialysis fluid with 10 μM FCCP + 31 μg/ml oligomycin + 4 mM ATP (see Fig. 9). (□) Axon 5,256B, diam = 570 μm, temp = 15°C, resting potential = −65 mV, $[Ca_t]$ =



0.95 mM, [EGTA$_t$] = 1.35 mM, [Ca^{2+}]$_i$ $\simeq$ 0.31 μM, CN-free dialysis fluid with 10 μM FCCP + 31 μg/ml oligomycin + 4 mM ATP. (△) Axon 6,046B, diam = 545 μM, temp 14.2°C, resting potential = −67 mV, [Ca$_t$] = 0.95 mM, [EGTA$_t$] = 1.00 mM, [Ca^{2+}]$_i$ $\simeq$ 2.5 μM, dialysis fluid with 2 mM CN + 31 μg/ml oligomycin + 5 mM PEP + 4 mM ATP. The curves were drawn to fit the equation: $J_{Na\text{-}Ca} = J^*_{Na\text{-}Ca}/[1 + (K_{Na}/[Na]_o)^n]$ where $J_{Na\text{-}Ca}$ is the Na$_o$-dependent Ca efflux at external Na concentration, $[Na]_o$. The maximal efflux, $J^*_{Na\text{-}Ca}$ had a value of 1.95 (●), 0.77 (○), 0.16 (□), and 1.72 (△) pmol/cm^{2}s, for the four curves the Hill coefficient, n, had a value of 3 (see Fig. 10C). The apparent mean half-saturation constant for Na, $\bar{K}_{Na}$, had value of 50 mM for the continuous curves (representing the axons dialyzed with ATP-containing fluids) and 120 mM for the broken curve (representing the ATP-depleted axon). B. Relative Na$_o$-dependent Ca efflux graphed as a function of $[Na]_o$. The data from Fig. 10A have been normalized to a value of 1.0 for the Na$_o$-dependent Ca efflux into Ca-free 10 K (Na) or Ca-free 12 K (Na) + CN. The curves were drawn to fit the equation given in the caption to Fig. 10A, with $J^*_{Na\text{-}Ca}$ = 1.1. C. Hill plot of the Na$_o$-dependent Ca efflux data from the same axons as represented in Fig. 10A. Ordinate: $\log[J_{Na\text{-}Ca}/(J^*_{Na\text{-}Ca} - J_{Na\text{-}Ca})]$. Abscissa: $\log[Na]_o$. See caption to Fig. 10A for explanation of symbols. The calculated (least squares) slope for the ATP-fueled axons (open symbols, continuous line) is 3.07 ± 0.23 (SE); the slope for the ATP-depleted axon (solid circles, broken line) is 2.74 ± 0.58 (SE).

a value below K_{Ca_i} to a value near saturation (i.e., well above K_{Ca_i}) (see also Baker and McNaughton, 1976*a*). The fact that the kinetics at the external site do not appear to be influenced by the fractional saturation of the transport mechanism at the internal site militates against a sequential (shuttle) system (Baker and Stone, 1966; Hoffman and Tosteson, 1971; Garay and Garrahan, 1973; Garrahan and Garay, 1976), and supports the idea that a simultaneous exchange mechanism underlies Na-Ca exchange.

Effect of internal Na on Ca efflux Raising the internal Na concentration inhibits both the Na$_o$-dependent and the Ca$_o$-dependent Ca effluxes from ATP-

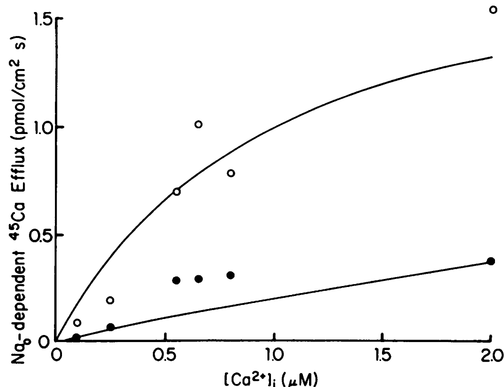


FIGURE 11 Effect of internal Na on the Na_o-dependent Ca efflux in ATP-fueled axons; the data are graphed as a function of the nominal $[Ca^{2+}]_i$ in the dialysis fluids. The axons were dialyzed with solutions containing 4 mM ATP and 5 mM Na (o), and then with solutions containing 4 mM ATP and 75 mM Na (•); Na isethionate was used as a (isosmotic) replacement for the taurine (see Table II). Pairs of data points (at low and high $[Na]_i$, respectively), at the same $[Ca^{2+}]_i$ were obtained on the same axon. Each data point indicates that Na_o-dependent Ca efflux, obtained by subtracting the ^{45}Ca efflux into 10 K (choline) from the efflux into 10 K (Na). The curves are drawn to fit the equation given in the caption to Fig. 8; the values of the constants are the same as those used to fit the continuous curve in Fig. 8.

depleted axons (Blaustein and Russell, 1975); this inhibition is presumably a consequence of the displacement of Ca from the carriers' internal sites by the intracellular Na. To determine whether or not a similar type of competition occurs in ATP-fueled axons, the Na_o-dependent Ca efflux was measured in fueled axons dialyzed first with a standard (5 mM Na) dialysis fluid, and then with a fluid containing the same ionized Ca^{2+} concentration and an elevated Na concentration (75 mM). Na_o-dependent ^{45}Ca efflux data from five axons are shown in Fig. 11. The data are graphed as a function of the nominal $[Ca^{2+}]_i$ in the dialysis fluids. Clearly, even in these ATP-fueled axons, raising $[Na]_i$ inhibits the Na_o-dependent Ca efflux. However, these data indicate that apparent mean half-saturation constant for internal Na, $\bar{K}_{Na_i}$, in the fueled axons (~ 30 mM; see Fig. 11) may not be very different from the value for ATP-depleted axons (~ 30 mM; Blaustein and Russell, 1975).

ACTIVATION OF CA EFFLUX BY EXTERNAL CA IN ATP-FUELED AXONS The relationship between $[Ca]_o$ and Ca efflux was tested in six ATP-fueled axons. When axons were exposed to Ca-free solutions [either Ca-free 10 K(Na) or Ca-free 10 K (choline)], and external Ca was then added back, a brief transient increase in Ca efflux was invariably observed (see Fig. 12); this transient occurred when the Ca-containing superfusion fluid first entered the chamber, and before a steady Ca concentration was established in the chamber (cf. Methods). Ca concentrations as low as 50 μM (the lowest tested) induced a Ca efflux transient; a similar transient was induced by the addition of EGTA to nominally Ca-free superfusion fluid (Fig. 12B). In a control experiment when no axon was present in the chamber, no transient was observed when

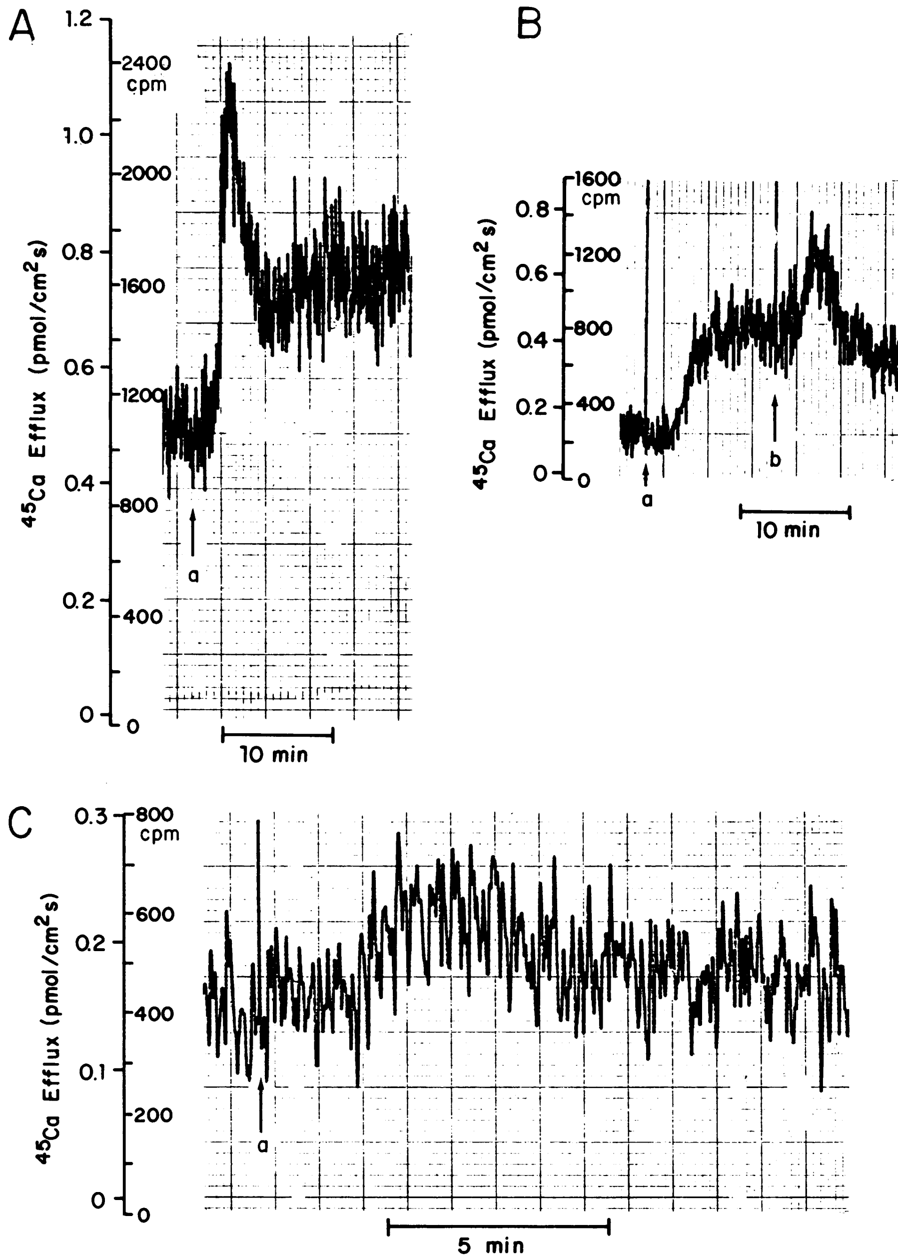
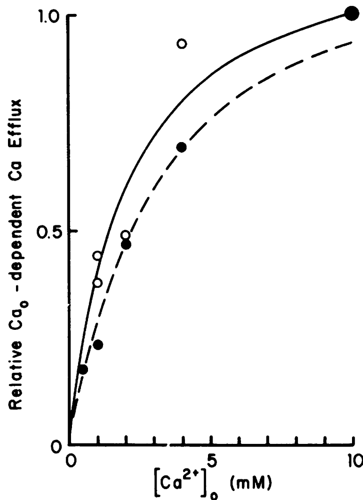


FIGURE 12 Examples of the transient increase in ^{45}Ca efflux from axons induced by replacing a Ca-free superfusion fluid with one containing Ca (A, C), or with a Ca-free fluid containing EGTA (B). A. The axon was superfused with Ca-free 10K (Na); at *a* this solution was replaced by 10 K(Na) with 2 mM Ca. Axon 6,246 (see caption to Fig. 10) dialyzed with ATP-containing fluid; $[Ca^{2+}]_i \simeq 0.56\,\mu M$. B. The axon was superfused with Ca-free 10 K(choline); at *a*, Ca-free 10 K(Na) was introduced, and at *b*, the latter solution was replaced by a similar solution which contained 0.5 mM EGTA. Axon 6,046B (see caption to Fig. 10), dialyzed with a solution containing 4 mM ATP and 75 mM Na (see Fig. 11), $[Ca^{2+}]_i \simeq 2.5\,\mu M$. C. The axon was superfused with Ca-free 10 K(Na); at *a* this solution was replaced with a similar solution containing 100 μM Ca. Axon 6,046A, diam = 575 μm, temp = 15°C, resting potential = −65 mV, $[Ca_t]$ = 1.0 mM, $[EGTA_t]$ = 2.0 mM, $[Ca^{2+}]_i \simeq 0.25\,\mu M$, dialysis fluid with 31 μg/ml oligomycin + 5 mM PEP + 4 mM ATP. The areas under the transient peaks correspond to 63 (A), 22 (B) and 23 (C) pmol Ca/cm^2.

^{45}Ca was washed out of the chamber first by Ca-free 10 K(Na) and then by 10 K(Na) containing 10 mM $CaCl_2$. Baker and McNaughton (1976*a*) observed a similar Ca_o-induced transient in the Ca efflux from ^{45}Ca injected axons. A likely explanation for this effect (cf. Baker and McNaughton, 1976a) is that, when $[Ca]_o$ is very low, some of the ^{45}Ca exiting from the axon is bound to superficial sites on the axolemma, Schwann cell membrane, and/or connective tissue; this bound ^{45}Ca can be readily removed by EGTA or displaced by ^{40}Ca from the Ca-containing superfusion fluids. Calculations based on the area under the transients and the known ^{45}Ca specific activity of the dialysis fluids indicate that, on the average (five axons), about 40 pmol of Ca are bound per cm^2 of axon surface area. (Although not directly comparable, it may be of interest to recall that Gilbert and Ehrenstein (1969) determined, from the effects of divalent cations on the potassium conductance of *Loligo pealei* axons, that the surface charge density on the external face of the axolemma is about 1 charge/120 A^2 or about 140 pmol of charge/cm^2.)

External Ca concentrations in the 1–10 mM range also induce a more sustained increase in Ca efflux (cf. Fig. 12), as was observed in ATP-depleted axons (Blaustein and Russell, 1975; and see Figs. 3 and 13). The curve relating the Ca_o-dependent increment in Ca efflux to the external Ca concentration, for both ATP-fueled and ATP-depleted axons, is illustrated in Fig. 13. These data imply that ATP may only slightly (if at all) increase the affinity of the external carrier site for Ca. Baker and McNaughton (1976*a*) have suggested that the affinity for external Ca is about the same in Na-based and Li-based superfusion fluids; however, the evidence that Na-Ca exchange and Ca-Ca exchange utilize the same carrier mechanism, and that Ca and Na compete for some common sites on the carriers (Baker et al., 1969; Blaustein and Russell, 1975; and Discussion), raises the possibility that the affinity for external Ca may be underestimated when Na is the predominant monovalent cation in the bathing medium. To this extent, the effect of ATP on the apparent K_{Ca_o} cannot be resolved by these experiments. However, the data clearly show that the apparent K_{Ca_o} for Ca efflux from dialyzed ATP-fueled axons into Na-based media is in the millimolar range. No evidence indicates that there is, in dialyzed axons, a sustained Ca efflux activated by micromolar concentrations of external Ca, such as described by Baker and McNaughton (1976*a*, *c*), in ^{45}Ca-injected axons.

The external Ca-dependent Li_o-stimulated Ca efflux (cf. Fig. 4), a manifestation of Ca-Ca exchange (Blaustein and Russell, 1975), was not studied in detail in this series of experiments. However, in several experiments (e.g., Fig. 7) the Ca efflux into 12 K(Li) + CN was observed before and after addition of ATP to the dialysis fluids. ATP greatly enhanced the efflux into the Li-based solution and had but little effect on the efflux into 12 K (choline) + CN. Moreover, data from an earlier study (Blaustein and Hodgkin, 1969) showed that ^{45}Ca influx from Li seawater was markedly reduced in intact, cyanide-poisoned (i.e., ATP-depleted) axons. These observations imply that ATP probably also increases K_{Ca_i} for Ca-Ca exchange. This, too, is consistent with the idea that Na-Ca exchange and Ca-Ca exchange are mediated by a common mechanism.



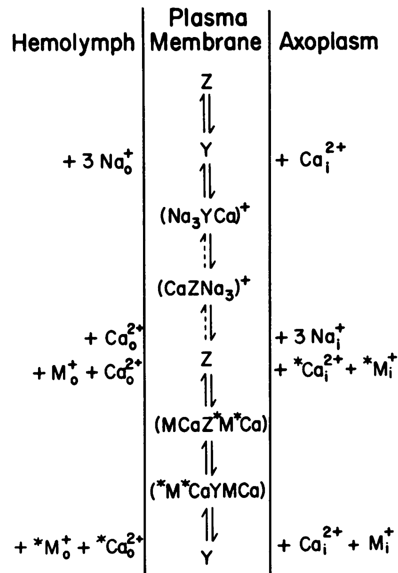


FIGURE 13

FIGURE 14

FIGURE 13 Effect of $[Ca]_o$ on Ca efflux in an ATP-depleted axon and in an ATP-fueled axon. The data are graphed on a relative scale: 0 = Ca efflux into Ca-free 10 K(Na) (fueled axon), or into Ca-free 12 K (Li) + CN (ATP-depleted axon); 1.0 is the relative Ca efflux into Ca-containing 10 K(Na) (fueled axon) or 12 K(Li) + CN (ATP-depleted axon). The curves were drawn to fit the equation:

$$J_{Ca\text{-}Ca} = J^*_{Ca\text{-}Ca}/[1 - (K_{Ca_o}/[Ca]_o)],$$

where $J_{Ca\text{-}Ca}$ is the relative Ca_o-dependent Ca efflux at any $[Ca]_o$, with a maximum value, $J^*_{Ca\text{-}Ca}$, of 1.22 at saturating $[Ca]_o$. The half-saturation constant for external Ca, K_{Ca_o}, had a value of 2 mM (solid line) or 3 mM (broken line). Solid circles represent ATP-depleted axon; data from same axon (5,145B) as solid circles of Fig. 3. Open circles represent ATP-fueled axon; data from same axon (5,246) as open circles of Fig. 10.

FIGURE 14 A model for the Na-Ca (and Ca-Ca) exchange carrier with simultaneous ion binding at the two membrane faces. Y and Z represent the two conformations of the free carrier; the free carrier is arbitrarily assumed to be an uncharged molecule. Although not shown in the diagram (which illustrates primarily the stoichiometric relationships), ATP increases the affinity for Ca at the external sites, and for Na at the internal sites. Further details are given in the text.

When external Na and Ca were both replaced (e.g., Fig. 9), there remained a small, but significant, residual ^{45}Ca efflux: 0.10 pmol/cm^2 s, on the average, in eight axons with $[Ca^{2+}]_i < 0.8$ μM. ATP appeared to have little, if any, effect on the residual efflux into Ca- and Na-free solution. About 0.02–0.04 pmol/cm^{2}s of the efflux may be attributable to buffer (Ca-EGTA) leak (cf. Brinley et al., 1975). Some (or most) of the remainder might be Mg_o-dependent (cf. Blaustein and Hodgkin, 1969), although this possibility was not tested in the present series of experiments.

DISCUSSION

In axons depleted of ATP and dialyzed with solutions containing ionized Ca^{2+} concentrations in excess of about 0.5 μM, nearly all of the Ca efflux can be accounted for by the exchange of Na for Ca, or of Ca for Ca, or by the leakage of Ca-EGTA (Blaustein and Russell, 1975). In intact, nonpoisoned axons, too, much of the Ca efflux is dependent on external Na and Ca (Blaustein and Hodgkin, 1969), which may indicate that these exchange mechanisms play an important role in maintaining Ca balance in squid axons. The present study was undertaken to further characterize the Na-Ca and Ca-Ca exchange mechanisms.

Are Na-Ca and Ca-Ca Exchange Independent, or Are They Mediated by a Common Transport Mechanism?

Clearly, one of the important problems to be resolved concerns the interrelationship, if any, between Na-Ca exchange and Ca-Ca exchange. One obvious possibility is that these two types of exchange are manifestations of two completely independent transport mechanisms. However, a variety of observations favor the alternative hypothesis, namely, that Na-Ca and Ca-Ca exchange are simply different modes of operation of a single carrier mechanism:

(*a*) In ATP-depleted axons, both Na-Ca and Ca-Ca exchange are half-maximally activated by about the same intracellular free Ca^{2+} concentration, approximately 3–8 μM Ca (Blaustein and Russell, 1975).

(*b*) The affinity of the internal Ca binding sites for both the Na_o-dependent (Fig. 8) and the Ca_o-dependent (Li_o-stimulated) Ca effluxes are increased by ATP.

(*c*) Both fluxes are inhibited by internal Na (Blaustein and Russell, 1975).

(*d*) Both fluxes are inhibited by external Sr and Mn (see Figs. 2 and 4 and related text).

(*e*) Perhaps most convincing, however, is the experiment (Fig. 5) that shows that the Na_o-dependent and Li_o-stimulated (Ca_o-dependent) Ca effluxes do not sum under conditions that should be (simultaneously) optimal for both fluxes; in fact, the total Ca efflux is much smaller than the maximal Li_o-stimulated Ca efflux (Ca-Ca exchange) alone. This "occlusion" strongly implies that the two fluxes are not independent. The apparent inhibition of Ca-Ca exchange by external Na may indicate that external Na and Ca compete for common (or mutually exclusive) binding sites on the carrier system (cf. Baker et al., 1969), and that the Na-loaded carriers have a slower turnover rate than those loaded with Ca (and Li) at the external site.

Are Na-Ca and Ca-Ca Exchange Mediated by a Simultaneous or a Sequential Mechanism?

Because Ca extrusion clearly involves a counterflow exchange with inwardly moving Na or Ca ions (Blaustein and Russell, 1975), another pertinent question is: does the exchange involve a shuttle mechanism, with sequential movement of the translocated

ions (i.e., alternating influx and efflux)? Or, do the translocations in the two directions take place simultaneously? Two sets of observations focus on this issue. One relevant set of data concerns the activation of Ca efflux by external Na in ATP-fueled axons. The main observation is that the kinetics of external Na activation are unaffected by the fractional saturation of the transport system by internal Ca; the apparent $\bar{K}_{Na_o}$ with the internal Ca binding site nearly saturated is indistinguishable from the value obtained when the Ca binding site is less than half-saturated (Fig. 10). Comparable observations have been made by Baker and McNaughton (1976*a*) in intact axons.

Baker and Stone (1966) have described a variety of theoretical schemes for coupled (counter-) transport processes involving either sequential or simultaneous binding. Hoffman and Tosteson (1971; and Garay and Garrahan, 1973; Garrahan and Garay, 1976) have elaborated upon the kinetic consequences of some of these schemes. These analyses lead to the conclusion that, when activation on one side of the membrane is independent of saturation on the other side, a mechanism involving simultaneous loading of the binding sites on the two sides of the membrane is generally implied.

The second (independent) set of observations bearing on the problem of simultaneous versus sequential mechanisms concerns the influence of internal monovalent cations on Ca efflux (Fig. 6 and Tables III and IV). It has been repeatedly stressed that Ca efflux can be activated either by external Na alone, or by external Ca in concert with an alkali metal ion (Li, K, Rb, and perhaps Na); choline and TMA are relatively ineffective in promoting the Ca_o-dependent Ca efflux. At the internal site, Ca is obviously required. Internal Na can inhibit Ca efflux, presumably by displacing Ca, because the transport mechanism also appears capable of mediating a reversed exchange (Na efflux for Ca influx; Baker et al., 1969). When Na is present in the external medium, an alkali metal ion is not needed in the axoplasm; Na_o-dependent Ca efflux proceeds whether K, Li, or TMA is the predominant internal cation. The situation is very different, however, in the absence of external Na: Ca-Ca exchange (e.g., with Li and Ca in the external medium) is promoted by internal K or Li, but is markedly reduced when all of the internal alkali metal ion is replaced by TMA (Fig. 6 and Table IV). It is apparent, nevertheless, that internal sites can be occupied by Ca in the presence of TMA, because Ca efflux can be activated by external Na. Consequently, assuming that Na-Ca and Ca-Ca exchange are mediated by a single mechanism (see above), the reduction in efflux observed when TMA replaces K or Li inside, and when Li + Ca are present outside, indicates that both the internal and external sites must be appropriately loaded (with Ca plus an alkali metal ion) simultaneously, for the Ca-Ca exchange to proceed.

In sum, the preceding considerations lead to the conclusion that a single transport system probably mediates both Na-Ca and Ca-Ca exchange, and that it does so by a mechanism that involves the simultaneous loading of internal and external carrier sites. This is, of course, consistent with the idea that the carrier is a "long molecule" with access to both the intracellular and extracellular media simultaneously, a notion first suggested by Baker et al. (1969).

What Role Does ATP Play in Ca Extrusion?

We turn next to a consideration of the energetics of Ca extrusion, and of the role of ATP in this process. The situation is rather complex because both Na and Ca ions appear to interact with the internal and with the external carrier sites, and because the carrier system apparently mediates Na-Ca exchange (Na influx-Ca efflux), Ca-Ca exchange, and reversed Na-Ca exchange (Na efflux-Ca influx; Baker et al., 1969). Consequently, we must inquire about the influence of ATP on the carrier affinities (for the ions) and transport velocities, with respect to each of the various modes of operation of the transport system.

The second half of the Results section focuses primarily on the Na influx-Ca efflux mode of operation of the carrier: the data (in Figs. 8 and 10, respectively) clearly indicate that ATP increases the affinity of the carrier for internal Ca and for external Na. Because the kinetics at the two sites (internal and external) appear to be independent (cf. Fig. 10 and related text), these observations imply that ATP affects both sides simultaneously.

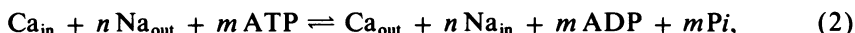
ATP also enhances Ca efflux into 10 K(Li) when $[Ca^{2+}]_i$ is low (see Fig. 7), evidence that the nucleotide increases the internal site's affinity for Ca during Ca-Ca exchange as well as during Na-Ca exchange. Whether or not ATP affects K_{Ca_o} during Ca-Ca exchange is uncertain.

The limited data available on the inhibition of Ca efflux by internal Na indicate that the apparent affinity for internal Na may not be markedly influenced by ATP. Because the same carrier system presumably also mediates reversed Na-Ca exchange, these observations might be construed as evidence that only the Na influx-Ca efflux mode of Na-Ca exchange is promoted by ATP. Baker et al. (1969) noted that cyanide poisoning abolished the Ca_o-dependent Na efflux (reversed Na-Ca exchange), and attributed this inhibition to a rise in internal Ca (with resultant displacement of Na by Ca at the internal sites). Unfortunately, Baker (1970) was thwarted in his attempt to test this hypothesis by injecting EGTA into the axoplasm (to reduce $[Ca^{2+}]_i$), because the EGTA appeared to inhibit directly the Na efflux. Clearly, more information is needed about the kinetics of Na and Ca activation (and inhibition) during reversed Na-Ca exchange.

Perhaps the most interesting and unexpected observation is that ATP has but little effect on the magnitude of the Na_o-dependent Ca efflux at high $[Ca^{2+}]_i$ (Fig. 8). This behavior may be contrasted with that of several other transport systems, where ATP enhances the rate of transport even at very high (or saturating) concentrations of the transported ion(s): e.g., Na-K exchange in squid axons (Mullins and Brinley, 1967), Ca extrusion in human erythrocytes (Schatzmann and Vincenzi, 1969; Schatzmann, 1973; Schatzmann, personal communication), and Ca sequestration in muscle sarcoplasmic reticulum (Carvalho and Leo, 1967). In these systems, of course, ATP hydrolysis is required to power the ion translocations. The fact that, in the squid axon, the maximal rate of Na_o-dependent Ca extrusion is not much affected, while the affinities for external Na and internal Ca are increased by ATP, may indicate that the

nucleotide acts as a co-factor or modulator, rather than as an energy source, in promoting Ca extrusion.

Other considerations also raise doubts that ATP hydrolysis directly powers Na_o-dependent Ca extrusion. In typical ATP-powered transport systems, such as the plasma membrane Na-K exchange (Glynn and Lew, 1970; Lew et al., 1970), and the sarcoplasmic reticulum Ca pump (Barlogie et al., 1971; Makinose, 1971; Makinose and Hasselbach, 1971), the "normal" ion gradient(s) can be reversed and, with a very low ATP/(ADP + P*i*) ratio, the pumps can be run "backwards," so as to generate ATP. This is, of course, the expected consequence of the principle of "mass action." By analogy, we may write a mass balance equation for the Na-Ca exchange system, on the assumption that ATP hydrolysis drives the reaction forward (i.e., in the direction of Ca extrusion):[1]



$$Ca_{in} + n\,Na_{out} + m\,ATP \rightleftharpoons Ca_{out} + n\,Na_{in} + m\,ADP + m\,Pi, \qquad (2)$$

where m and n represent the number of moles of ATP hydrolyzed and of Na which enter, respectively, when 1 mol of Ca is extruded. However, a number of experimental observations appear to be inconsistent with this formulation: (*a*) Na_o-dependent Ca efflux can proceed when ATP is reduced to very low levels (Blaustein and Hodgkin, 1969; Blaustein and Russell, 1975; and Fig. 8); this implies that ATP is not an obligatory substrate for the forward reaction. (*b*) Ca_o-dependent Na efflux (reversed Na-Ca exchange) can be observed in axons with a relatively high (i.e., normal) ATP/(ADP + P*i*) ratio (Baker et al., 1969). And (*c*) Ca_o-dependent Na efflux is abolished by cyanide poisoning, which lowers the ATP/(ADP + P*i*) ratio (Baker et al., 1969).

In sum, the available evidence implies that Ca extrusion via Na-Ca exchange is not powered by ATP hydrolysis. One possibility is that, at an early stage in animal evolution, energy from ATP hydrolysis was used directly to power Ca extrusion from cells. Subsequently, in many types of cells (but not in the erythrocyte; Schatzmann and Vincenzi, 1969, and Schatzmann, 1973) another form of energy became available for this process (see below). Even then, ATP retained a vestigial, but important role; namely, to lower K_{Ca_i} so that the Na-Ca exchange mechanism could operate at a reasonable rate in the physiological range of $[Ca^{2+}]_i$ (i.e., below 1 μM; see Fig. 8). The situation may be somewhat analogous to the Na-Na exchange mediated by the cardiac glycoside-sensitive Na pump (Garrahan and Glynn, 1967); but in this case, ATP is required (to form one of the reaction intermediates; Glynn and Karlish, 1975), even though the Na-Na exchange proceeds without net ATP hydrolysis.

Where Does the Energy for Ca Extrusion Come from?

If ATP does not directly power Na-Ca exchange, where, then, does the energy come from, to maintain the very large electrochemical gradient for Ca that prevails across

[1] There is scarcely reason to think that ATP is required to drive the reaction backward in squid axons because Ca entry is down a steep electrochemical gradient, and Na extrusion is mediated by the ATP-dependent Na-K pump. Furthermore, the Ca_o-dependent Na efflux accounts for only a tiny fraction of the total Na efflux from intact axons (Baker et al., 1969).

the axolemma of the squid axon (Baker, 1972; Blaustein, 1974; Di Polo et al., 1976)? It has recently been suggested (Baker, 1976; Baker and McNaughton, 1976*c*) that a fraction of the Ca efflux from normal (ATP-fueled) axons, but not from poisoned axons, may involve an uncoupled (net) extrusion of Ca activated by micromolar concentrations of external Ca. We have been unable to reproduce this finding in dialyzed, ATP-fueled *Loligo pealei* axons: for example, as illustrated in Fig. 12B, the addition of 0.5 mM EGTA to a nominally Ca-free 10 K(Na) superfusion fluid did not significantly reduce the Ca efflux (the Ca concentration in this nominally "Ca-free" 10 K(Na) solution, was 12 μM, as determined by atomic absorption spectroscopy). Moreover, even the addition of 100 μM Ca to the nominally Ca-free 10 K(Na) did not markedly enhance the steady Ca efflux from an ATP-fueled axon (Fig. 12C), indicating that the affinity of the Ca efflux mechanism for external Ca is not in the sub-millimolar range in fueled axons (Fig. 13).

Requena et al. (1977) have also concluded that external Na plays a critical role in net Ca efflux from squid axons. They monitored ionized $[Ca^{2+}]_i$ with aequorin, and found that the aequorin glow is increased when there is net Ca gain (as a consequence of electrical stimulation or reduction of $[Na]_o$); the glow did not fall to its original levels unless the axons (whether ATP-depleted or ATP-replete) were superfused with Na-rich fluids.

These observations all emphasize the importance of external Na, and imply that it may be premature to attribute net Ca efflux from the squid axon to any mechanism other than Na-Ca exchange (but see Baker, 1976; Baker and McNaughton, 1976*c*). If Ca extrusion is not powered by ATP hydrolysis, nor accounted for by an uncoupled efflux of Ca, and if Na-Ca exchange is the main (or exclusive) mechanism available for net Ca extrusion, then the question of whether or not sufficient energy can be obtained from the Na electrochemical gradient, to maintain the normal Ca gradient, becomes of prime importance.

The Role of the Na Electrochemical Gradient in Maintaining $[Ca^{2+}]_i$.

For every mole of Na^+ ions entering the axon, the amount of energy dissipated is given by the Na electrochemical potential difference across the axolemma, $\Delta\bar{\mu}_{Na}$:

$$\Delta\bar{\mu}_{Na} = RT \ln \frac{a_{Na_o}}{a_{Na_i}} - V_M F, \tag{3}$$

where V_M is the membrane potential, and R, T, and F have their usual meanings. The external/internal Na activity ratio is a_{Na_o}/a_{Na_i}; if the activity coefficients for Na are approximately equal in squid axoplasm and hemolymph, the activity ratio will be (nearly) equal to the concentration ratio.

Every mole of Ca^{2+} exiting from the axon requires the expenditure of an amount of energy given by the Ca electrochemical potential difference across the axolemma, $\Delta\bar{\mu}_{Ca}$:

$$\Delta\bar{\mu}_{Ca} = RT \ln \frac{a_{Ca_o}}{a_{Ca_i}} - 2\,V_M F, \tag{4}$$

where $a_{\mathrm{Ca}_o}/a_{\mathrm{Ca}_i}$ is the Ca activity ratio. If, during Na-Ca exchange, the entering Na^+ is tightly coupled to the net Ca^{2+} exit, then the energy dissipated by the Na^+ entry may be used to drive Ca^{2+} out, so that at equilibrium:

$$n\,\Delta\bar{\mu}_{\mathrm{Na}} - \Delta\bar{\mu}_{\mathrm{Ca}} = 0, \tag{5}$$

or (from Eqs. 3 and 4):

$$a_{\mathrm{Ca}_o}/a_{\mathrm{Ca}_i} = (a_{\mathrm{Na}_o}/a_{\mathrm{Na}_i})^n \exp\{(2 - n)\, V_M F/RT\}, \tag{6}$$

where n is the number of moles of Na^+ entering per mole of Ca^{2+} extruded. Note that, as long as the Na electrochemical gradient is maintained, and net Ca^{2+} efflux is (tightly) coupled to a fraction of the Na^+ influx, the fact that Na^+ may also enter the axon through other, parallel pathways is irrelevant (cf. Heinz and Geck, 1974).

For the Na-Ca exchange mechanism to maintain $[Ca^{2+}]_i$ (or a_{Ca_i}) close to the value expected from Eq. 6, the system must normally operate near equilibrium. That this condition may prevail is suggested by the fact that the total Ca efflux from intact squid axons, about 0.2 pmol/cm^2 s (Blaustein and Hodgkin, 1969), is only a small fraction of the maximum rate of Ca extrusion via Na-Ca exchange, about 2 pmol/cm^2 s (Fig. 8). Moreover, about 35% of the Ca efflux from intact axons is Ca_o-dependent (Ca-Ca exchange, presumably), and only about 16% (or about 0.03 pmol/cm^2 s) is Na_o-dependent (Blaustein and Hodgkin, 1969). It seems satisfactory that, with $[Ca^{2+}]_i$ = 50 nM and $[Na^+]_i$ = 30 mM (see below), Na_o-dependent Ca efflux calculated from the equation given in the caption to Fig. 8 is about 0.03 pmol/cm^2 s for an ATP-fueled axon. It is important to note that the residual Ca efflux into nominally Na- and Ca-free media (Blaustein and Hodgkin, 1969; and see Baker and McNaughton, 1976*a*) is too large to be explained by flux-ratio considerations. A fraction of this Ca efflux may depend upon external Mg (Blaustein and Hodgkin, 1969); however, the bulk of this residual efflux, also observed in poisoned axons, remains unaccounted for.

The next problem is to decide upon appropriate values for the various parameters in Eq. 6. The question of stoichiometry must first be settled. Observations in several laboratories (Brinley and Mullins, 1974; Blaustein et al., 1974; Mullins and Brinley, 1975; Baker and McNaughton, 1976*a*; but see Baker and McNaughton, 1976*c*) indicate that the Ca efflux from squid axons is voltage-sensitive, and that depolarization reduces the Na_o-dependent flux. This implies that the Na-Ca exchange cycle involves the net entry of positive charge (or net exit of negative charge), and suggests that more than two Na^+ ions enter in exchange for each Ca^{2+} leaving the axon. Measurements of the Na_o-dependent Ca efflux and Ca_i-dependent Na influx, made under comparable conditions (in ATP-depleted axons), provided direct evidence for a stoichiometry of about three Na^+ for one Ca^{2+}. This stoichiometry is consistent with the evidence, described above (Fig. 10) and in Blaustein et al. (1974), that the cooperative action of about three Na^+ ions is required to activate the efflux of one Ca^{2+} in both ATP-depleted and ATP-fueled axons with a high or low $[Ca^{2+}]_i$ (but see

Baker and McNaughton, 1976*a*). In sum, the weight of evidence is consistent with a stoichiometry of three Na^+ for one Ca^{2+}, which corresponds to a value of $n = 3$ in Eq. 6.

Then, taking Hinke's (1961) values of 10–13 for the Na activity ratio, a_{Na_o}/a_{Na_i}, in squid axons, and a resting membrane potential of -77 mV (Moore and Cole, 1960), calculated values in the range of $2\text{–}5 \times 10^4$ are obtained for the Ca activity ratio, a_{Ca_o}/a_{Ca_i}, at 15°C. The ionized Ca^{2+} concentration in squid hemolymph is about 3–4 mM (Blaustein, 1974; Di Polo et al., 1976); thus, if the activity coefficients for Ca in the axoplasm and hemolymph are equal, the $[Ca^{2+}]_i$, calculated from Eq. 6, is in the range of $0.6\text{–}2.0 \times 10^{-7}$ M (60–200 nM). If the Ca activity coefficient for axoplasm is (slightly) larger than that for hemolymph (because the ionic strength of axoplasm is lower; cf. Blaustein, 1974), the calculated range of values for $[Ca^{2+}]_i$ would be even (slightly) lower than 60–200 nM. These calculated values may be compared with recent experimental measurements that indicate that $[Ca^{2+}]_i$ in squid axoplasm is less than 300 nM (Baker, 1972), and perhaps in the range of 30–50 nM (Di Polo et al., 1976). In view of the many errors inherent in determinations of $[Ca^{2+}]_i$, and of the a_{Na_o}/a_{Na_i} ratio (e.g., Hinke studied axons from "refrigerated mantles," so that his values for a_{Na_i} were surely higher than the a_{Na_i} in the axons of freshly killed squid; cf. Baker et al., 1969), the agreement between the experimental values for $[Ca^{2+}]_i$ and those calculated from Eq. 6 is surprisingly good. The conclusion is that the Na electrochemical gradient alone may be able to provide sufficient energy for Ca extrusion, via three-for-one Na-Ca exchange coupling, to maintain $[Ca^{2+}]_i$ at about the experimentally observed level.

A Model for the Na-Ca Exchange Carrier

A model which takes into account many of the observations discussed above is diagrammed in Fig. 14. The two configurations of free carrier are represented by Y and Z; the free carriers are arbitrarily represented as uncharged molecules, because of lack of information to the contrary. The Z configuration corresponds to the situation in which the ion binding sites face opposite sides of the membrane, relative to the Y configuration. For example, in the Y configuration, Ca^{2+} can bind to the internal site and three Na^+ ions can bind to external sites; the fully loaded carrier, $(Na_3YCa)^+$, can then change to configuration $(CaZNa_3)^+$, so that the Ca^{2+} can be discharged into the extracellular fluid, and the three Na^+ into the axoplasm. To account for the net (Na_o-dependent) Ca extrusion, free carriers (Y and Z) must be capable of oscillating between configurations (as shown at the top of the diagram); partially loaded carriers are, presumably, unable to change configuration (because, for example, Ca cannot exit unless the external sites are appropriately loaded). Furthermore, the possibility that Na and Ca compete for common or interacting binding sites at both membrane faces must be considered, to explain the apparent competition between internal Na and Ca, and between external Na and Ca (Figs. 5 and 11, Table III, and see Blaustein and Russell, 1975).

The alternative mode of carrier operation, Ca-Ca exchange, is diagrammed in the

lower portion of the figure; the Ca^{2+} and alkali metal ion (M^+) loading at the internal sites, in the Z configuration, are labeled with an asterisk, to distinguish them from the Ca^{2+} and alkali metal ion loading at the external sites. Unfortunately, there is as yet no information about whether or not the alkali metal ions, which activate Ca-Ca exchange, actually are co-transported with the Ca^{2+}.

Although the diagram implies that all the reactions are fully reversible, and that "backward Na-Ca exchange" (Na efflux-Ca influx; i.e., moving up the diagram) may simply be the reverse of "forward Na-Ca exchange" (Na influx-Ca efflux; i.e., moving down the diagram), the experimental data indicate that this may not be so straightforward. Baker et al. (1969) noted that the Ca influx and Ca_o-dependent Na efflux was particularly prominent in Li-containing media, and was greatly reduced when all external alkali metal ions were removed. They suggested that, during backward Na-Ca exchange, Ca^{2+} may enter with an alkali metal ion in exchange for exiting Na^+. In view of this uncertainty, backward exchange, $(CaZNa_3)^+ \rightarrow (Na_3YCa)^+$, is indicated by a broken arrow in the diagram. Clearly, more information about the stoichiometry and kinetics of backward exchange (including the effect of ATP) is needed to complete the picture.

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REFERENCES

Baker, P. F. 1970. Sodium-calcium exchange across the nerve cell membrane. *In* Calcium and Cellular Function. A. W. Cuthbert, editor. Macmillan & Co. Ltd., London. 96–107.

Baker, P. F. 1972. Transport and metabolism of calcium ions in nerve. *Prog. Biophys. Mol. Biol.* **24:** 177–233.

Baker, P. F. 1976. Regulation of intracellular Ca and Mg in squid axons. *Fed. Proc.* **35:**2589–2595.

Baker, P. F., M. P. Blaustein, A. L. Hodgkin, and R. A. Steinhardt. 1969. The influence of calcium on sodium efflux in squid axons. *J. Physiol. (Lond.).* **200:**431–458.

Baker, P. F., and H. G. Glitsch. 1973. Does metabolic energy participate directly in the Na^+-dependent extrusion of Ca^{2+} ions from squid axons? *J. Physiol. (Lond.).* **233:**44–46P. (Abstr.).

Baker, P. F., and P. A. McNaughton. 1976*a*. Kinetics and energetics of calcium efflux from intact squid giant axons. *J. Physiol. (Lond.).* **259:**103–144.

Baker, P. F., and P. A. McNaughton. 1976*b*. The effect of membrane potential on the calcium transport systems in squid axons. *J. Physiol. (Lond.).* **260:**24–25P.

Baker, P. F., and P. A. McNaughton. 1976*c*. Calcium-dependent calcium efflux from intact squid axons: Ca-Ca exchange or net extrusion? *J. Physiol. (Lond.).* **258:**97–98P. (Abstr.).

Baker, P. F., and A. J. Stone. 1966. A kinetic method for investigating hypothetical models of the sodium pump. *Biochim. Biophys. Acta.* **126:**321–329.

BARLOGIE, B., W. HASSELBACH, and M. MAKINOSE. 1971. Activation of calcium efflux by ADP and inorganic phosphate. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* **12**:267–268.

BLAUSTEIN, M. P. 1974. The interrelationship between sodium and calcium fluxes across cell membranes. *Rev. Physiol. Biochem. Pharmacol.* **70**:33–82.

BLAUSTEIN, M. P. 1976. The ins and outs of calcium transport in squid axons: internal and external ion activation of calcium efflux. *Fed. Proc.* **35**:2574–2578.

BLAUSTEIN, M. P. 1977. Calcium transport in internally-dialyzed squid axons: effects of internal and external cations and ATP. *Biophys. J.* **17** (pt. 2):155*a*. (Abstr.).

BLAUSTEIN, M. P., and A. C. ECTOR. 1976. Carrier-mediated sodium-dependent and calcium-dependent calcium efflux from pinched-off presynaptic nerve terminals (synaptosomes) *in vitro*. *Biochim. Biophys. Acta.* **419**:295–308.

BLAUSTEIN, M. P., and A. L. HODGKIN. 1969. The effect of cyanide on the efflux of calcium from squid axons. *J. Physiol. (Lond.).* **200**:497–527.

BLAUSTEIN, M. P., and J. M. RUSSELL. 1975. Sodium-calcium exchange and calcium-calcium exchange in internally dialyzed squid giant axons. *J. Membr. Biol.* **22**:285–312.

BLAUSTEIN, M. P., J. M. RUSSELL, and P. DE WEER. 1974. Calcium efflux from internally dialyzed squid axons: the influence of external and internal cations. *J. Supramol. Struct.* **2**:558–581.

BORON, W. F., and P. DE WEER. 1976. Intracellular pH of squid giant axons and kinetics of its modification by extracellular weak electrolytes. Effect of CO_2, NH_3 and some metabolic inhibitors. *J. Gen. Physiol.* **67**:91–112.

BRINLEY, F. J., JR., and L. J. MULLINS. 1967. Sodium extrusion by internally-dialyzed squid axons. *J. Gen. Physiol.* **50**:2303–2331.

BRINLEY, F. J., JR., and L. J. MULLINS. 1968. Sodium fluxes in internally dialyzed squid axons. *J. Gen. Physiol.* **52**:181–211.

BRINLEY, F. J., JR., and L. J. MULLINS. 1974. Effects of membrane potential on sodium and potassium fluxes in squid axons. *Ann. N. Y. Acad. Sci.* **242**:406–432.

BRINLEY, F. J., JR., S. G. SPANGLER, and L. J. MULLINS. 1975. Calcium and EDTA fluxes in dialyzed squid axons. *J. Gen. Physiol.* **66**:223–250.

CARVALHO, A. P., and B. LEO. 1967. Effects of ATP on the interaction of Ca^{++}, Mg^{++}, and K^+ with fragmented sarcoplasmic reticulum isolated from rabbit skeletal muscle. *J. Gen. Physiol.* **50**:1327–1352.

DI POLO, R. 1973. Calcium efflux from internally dialyzed squid giant axons. *J. Gen. Physiol.* **62**:575–589.

DI POLO, R. 1974. Effect of ATP on the calcium efflux in dialyzed squid giant axons. *J. Gen. Physiol.* **54**: 503–517.

DI POLO, R. 1976. The influence of nucleotides upon calcium fluxes. *Fed. Proc.* **35**:2579–2582.

DI POLO, R., J. REQUENA, F. J. BRINLEY, JR., L. J. MULLINS, A. SCARPA, and T. TIFFERT. 1976. Ionized calcium concentrations in squid axons. *J. Gen. Physiol.* **67**:433–467.

FRANKENHAEUSER, B., and A. L. Hodgkin. 1957. The action of calcium on the electrical properties of squid axons. *J. Physiol. (Lond.).* **137**:218–244.

GARAY, R. P., and P. J. GARRAHAN. 1973. The interaction of sodium and potassium with the sodium pump in red cells. *J. Physiol. (Lond.).* **231**:297–325.

GARRAHAN, P. J., and R. P. GARAY. 1976. The distinction between sequential and simultaneous models for sodium and potassium transport. *Curr. Top. Membranes Transp.* **8**:29–97.

GARRAHAN, P. J., and I. M. GLYNN. 1967. Factors affecting the relative magnitudes of the sodium:potassium and sodium:sodium exchanges catalysed by the sodium pump. *J. Physiol. (Lond.).* **192**:189–216.

GILBERT, D. L., and G. EHRENSTEIN. 1969. Effect of divalent cations on potassium conductance of squid axons: determination of surface charge. *Biophys. J.* **9**:447–463.

GLYNN, I. M., and S. J. D. KARLISH. 1975. The sodium pump. *Annu. Rev. Physiol.* **37**:13–55.

GLYNN, I. M., and V. L. LEW. 1970. Synthesis of adenosine triphosphate at the expense of downhill cation movements in intact human erythrocytes. *J. Physiol. (Lond.).* **207**:393–402.

HEINZ, E., and P. GECK. 1974. The efficiency of energetic coupling between Na^+ flow and amino acid transport in Ehrlich cells—a revised assessment. *Biochim. Biophys. Acta.* **339**:426–431.

HINKE, J. A. M. 1961. The measurement of sodium and potassium activities in the squid axon by means of cation-selective glass micro-electrodes. *J. Physiol. (Lond.).* **156**:314–335.

HOFFMAN, P. G., and D. C. TOSTESON. 1971. Active sodium and potassium transport in high potassium and low potassium sheep red cells. *J. Gen. Physiol.* **58**:438–466.

KENDRICK, N. C., R. W. RATZLAFF, and M. P. BLAUSTEIN. 1977. Arsenazo III as an indicator for ionized calcium in physiological salt solutions; its use for determination of the Ca ATP dissociation constant. *Anal. Biochem.* In press.

LEW, V. L., I. M. GLYNN, and J. C. ELLORY. 1970. Net synthesis of ATP by reversal of the sodium pump. *Nature (Lond.).* **225**:865–866.

MAKINOSE, M. 1971. Calcium efflux dependent formation of ATP from ADP and orthophosphate by membranes of the sarcoplasmic reticulum vesicles. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* **12**:269–270.

MAKINOSE, M., and W. HASSELBACH. 1971. ATP synthesis by the reverse of the sarcoplasmic calcium pump. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* **12**:271–272.

MOORE, J. W., and K. S. COLE. 1960. Resting and action potentials of the squid axon in vivo. *J. Gen. Physiol.* **43**:961–970.

MULLINS, L. J., and F. J. BRINLEY, JR. 1967. Some factors influencing sodium extrusion by internally dialyzed squid axons. *J. Gen. Physiol.* **50**:2333–2355.

MULLINS, L. J., and F. J. BRINLEY, JR. 1975. Sensitivity of calcium efflux from squid axons to changes in membrane potential. *J. Gen. Physiol.* **65**:135–152.

NADARAJAH, A., B. LEESE, and G. F. JOPLIN. 1969. Triton X-100 scintillant for counting calcium-45 in biological fluids. *Int. J. Appl. Radiat. Isot.* **20**:733–735.

PORTZEHL, H., P. C. CALDWELL, and J. C. RÜEGG. 1964. The dependence of contraction and relaxation of muscle fibres from the crab *Maia squinado* on the internal concentration of free calcium ions. *Biochim. Biophys. Acta.* **79**:581–591.

REQUENA, J., R. DI POLO, F. J. BRINLEY, JR., and L. J. MULLINS. 1977. The control of ionized calcium in squid axons. *J. Gen. Physiol.* In press.

RUSSELL, J. M., and M. P. BLAUSTEIN. 1974. Calcium efflux from barnacle muscle fibers. Dependence on external cations. *J. Gen. Physiol.* **63**:144–167.

SCHATZMANN, H. J. 1973. Dependence on calcium concentration and stoichiometry of the calcium pump in human red cells. *J. Physiol. (Lond.).* **235**:551–569.

SCHATZMANN, H. J., and F. F. VINCENZI. 1969. Calcium movements across the membrane of human red cells. *J. Physiol. (Lond.).* **201**:369–395.