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Sodium–Calcium Exchange and Calcium–Calcium Exchange in Internally Dialyzed Squid Giant Axons

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Summary. The influx and efflux of calcium (as ^{45}Ca) and influx of sodium (as ^{24}Na) were studied in internally dialyzed squid giant axons. The axons were poisoned with cyanide and ATP was omitted from the dialysis fluid. The internal ionized Ca^{2+} concentration ($[Ca^{2+}]_i$) was controlled with Ca-EGTA buffers. With $[Ca^{2+}]_i > 0.5$ μM, ^{45}Ca efflux was largely dependent upon external Na and Ca. The Na_o-dependent Ca efflux into Ca-free media appeared to saturate as $[Ca^{2+}]_i$ was increased to 160 μM; the half-saturation concentration was about 8 μM Ca^{2+}. In two experiments ^{24}Na influx was measured; when $[Ca^{2+}]_i$ was decreased from 160 μM to less than 0.5 μM, Na influx declined by about 5 pmoles/cm^2 sec. The Na_o-dependent Ca efflux averaged 1.6 pmoles/cm^2 sec in axons with a $[Ca^{2+}]_i$ of 160 μM, and was negligible in axons with a $[Ca^{2+}]_i$ of less than 0.5 μM. Taken together, the Na influx and Ca efflux data may indicate that the fluxes are coupled with a stoichiometry of about $3Na^+$-to-1 Ca^{2+}. Ca efflux into Na-free media required the presence of both Ca and an alkali metal ion (but not Cs) in the external medium. Ca influx from Li-containing media was greatly reduced when $[Ca^{2+}]_i$ was decreased from 160 to 0.23 μM, or when external Li was replaced by choline. These data provide evidence for a Ca–Ca exchange mechanism which is activated by certain alkali metal ions. The observations are consistent with a mobile carrier mechanism which can exchange Ca^{2+} ions from the axoplasm for either $3Na^+$ ions, or one Ca^{2+} and an alkali metal ion (but not Cs) from the external medium. This mechanism may utilize energy from the Na electrochemical gradient to help extrude Ca against an electrochemical gradient.

The extrusion of calcium ions from squid giant axons depends in part upon external sodium, an observation which may implicate an Na–Ca exchange mechanism (Blaustein & Hodgkin, 1969). This idea is strengthened by the evidence that, under certain conditions, the mechanism may operate in reverse: Ca ions may enter the axon in exchange for exiting Na (Baker, Blaustein, Hodgkin & Steinhardt, 1969*a*). Since Na and Ca must

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both be extruded from the axon against large electrochemical gradients (*see* reviews by Baker, 1972; and Blaustein, 1974), the coupled counter-flow mechanism might be used to harness energy released by the ("downhill") entry of Na, in order to drive Ca out. Presumably, the ATP-dependent Na–K exchange pump (*see* review by De Weer, 1974) could then be used to re-extrude the entering Na.

If the Na–Ca exchange does indeed couple the "downhill" and "uphill" processes, the amount of energy which could be made available (in the absence of exogenous sources) for Ca extrusion would depend upon the stoichiometry of the exchange. This is an important consideration because the electrochemical gradient for Ca is much larger than the electrochemical gradient for Na (*cf.* Blaustein, 1974), and a 1-for-1 or 2-for-1 (Na^+-for-Ca^{2+}) exchange would not, alone, be sufficient to power Ca extrusion in normal, intact axons (*see* Discussion). The present report describes some experiments designed to explore the coupling by measuring the Ca_i-dependent Na influx and the Na_o-dependent Ca efflux in internally dialyzed squid axons. The axons were poisoned with cyanide and dialyzed with ATP-free and metabolic substrate-free solutions to minimize the energy contribution from metabolic sources. The results are consistent with previous (indirect) estimates that the Na–Ca exchange stoichiometry may be about 3-for-1 (Blaustein, Russell & De Weer, 1974).

Available data suggest that the Na–Ca exchange mechanism may, under some circumstances, participate in Ca–Ca exchange, since the external monovalent cation activation of Ca_o-dependent Ca efflux (Blaustein *et al.*, 1974) and Ca_o-dependent Na efflux (Baker *et al.*, 1969*a*) are similar. The stoichiometry and cation activation of Ca–Ca exchange has therefore also been investigated in dialyzed axons in an effort to obtain additional information about the properties of the Ca transport mechanism.

A preliminary report of these findings has been communicated to the Biophysical Society (Blaustein & Russell, 1975).

Materials and Methods

Biological Material

Axons, 500–800 μ in diameter, were dissected from the first stellar nerve of live specimens of *Loligo pealii*. The experiments were made at the Marine Biological Laboratory, Woods Hole, Massachusetts.

Solutions

Table 1 shows the composition of some representative external solutions and indicates the nomenclature which will be followed. The osmolality of the 12 K(Na)+CN was 945 ± 5 milliosmoles/kg.

Table 1. Representative external solutions

Solution[a]	NaCl	LiCl	Choline Cl	KCl	$CaCl_2$	$MgCl_2$	KCN
			(all in mmoles/liter)				
12 K(Na)+CN	425	–	–	10	10	50	2
12 K(Li)+CN	–	425	–	10	10	50	2
12 K(choline)+CN	–	–	425	10	10	50	2
Ca-free 12 K(Na)+CN	425	–	–	10	–	60	2
116 K(choline)+CN	–	–	321	114	10	50	2

[a] In addition, all solutions contained 0.1 mM EDTA and 2.5 mM HEPES (N-2-hydroxyethyl-piperazine-N′-2-ethanesulfonic acid). The solutions were all buffered to pH 7.8, at 20 °C, with tris base (tris [hydroxymethyl] aminomethane).

All of the experiments in this study were made on axons poisoned with cyanide (2 mM in both the external and internal fluids) to minimize ATP-dependent Ca sequestration in the axoplasm (Blaustein & Hodgkin, 1969; DiPolo, 1973). Details regarding the addition of other drugs to the external media will be given in the Results section.

The standard (5 mM Na) dialysis fluid contained (all in mmoles/liter): K glutamate, 395; Na isethionate, 5; taurine, 200; $MgCl_2$, 10; $CaCl_2$, 1.056; KCN, 2; phenol red, 0.5; HEPES (N-2-hydroxyethyl-piperazine-N′-2-ethanesulfonic acid), 5; and EGTA, 0.5–2.0. The concentration of EGTA used in each experiment will be given in the Results section. All dialysis fluids were buffered at pH 7.10 at 20 °C with Tris base. In some experiments (where specifically noted) the Na concentration in the dialysis fluid was altered; in these cases, some of the taurine was replaced isosmotically by Na isethionate. Preliminary experiments indicated that neither the change in ionic strength nor the alteration of anion concentration had any significant effect on the Ca fluxes.

The Ca concentration in the dialysis fluid (1.056 mM) includes the contribution from the $^{45}CaCl_2$ and from Ca contamination in the other reagents (determined by atomic absorption spectroscopy). The nominal concentrations of ionized Ca^{2+} in the dialysis fluids, mentioned in the Results section, were calculated with a CaEGTA stability constant value of 7.6×10^6 M^{-1} (Portzehl, Caldwell & Rüegg, 1964). The dialysis fluids had osmolalities of 920±5 millios-moles/kg, as determined by dewpoint depression.

The radioactive tracers used for these experiments were obtained from New England Nuclear (Boston, Mass). The ^{24}Na was prepared in H_2O (5 mCi/ml), and small aliquots were used directly. The external solutions labeled with ^{24}Na were made up to contain 0.14–0.2 cpm/pmole Na.

The $^{45}CaCl_2$ was originally dissolved in 0.5 N HCl, this solution was evaporated to dryness and redissolved in glass-distilled H_2O to give a concentration of 2 mCi/ml. Small aliquots of this solution were added to the dialysis fluids to give a specific activity of about 55 cpm/pmole Ca. For ^{45}Ca influx studies, the ^{45}Ca specific activity in the external media ranged from 1 to 3.5 cpm/pmole Ca, but was constant in any given experiment.

Procedures

The dialysis techniques of Brinley and Mullins (1967), including their recent modifications (Brinley & Mullins, 1974) were employed for this study. The experimental chamber (*see* Fig. 1 and Brinley & Mullins, 1967) was filled with Ca-free 12 K(Na)+CN. A cleaned giant axon

was mounted horizontally, with its two cut ends cannulated onto thin-walled glass "end-cannulae"; the cannulae had been previously filled with a solution containing 300 mM EGTA + 50 mM $MgSO_4$, buffered to pH 7.2 with Tris base. A hollow cellulose acetate tube (195 μ O.D. × 145 μ I.D. from Fabric Research Laboratories, Dedham, Mass.), stiffened with an axial 75 μ diameter tungsten wire, was inserted horizontally through the end-cannulae and axis of the squid axon under direct visual control. When the cellulose acetate tube and tungsten wire emerged from the distal (left-hand, in Fig. 1) end-cannula, and the tube was properly positioned, the wire was removed and the flow of dialysis fluid commenced. A 250 μliter gas-tight Hamilton syringe (Hamilton Co., Reno, Nevada) served as the dialysis fluid reservoir; the fluid was perfused through the hollow cellulose acetate tube at a rate of 1 μliter/min by means of a Harvard model 1100 syringe pump (Harvard Apparatus Co., Millis, Mass.).

Prior to each experiment, a selected region (usually 18 mm long) of the hollow cellulose acetate tube was rendered porous by soaking it for 18 hr in 0.1 N NaOH. This porous region had to be positioned so that its middle was centered in the middle compartment (*B* of Fig. 1) of the dialysis chamber. The positioning could be checked at the beginning of the experiment because the phenol red in the dialysis fluid rapidly dyed the axoplasm surrounding the porous region. The dye had no apparent adverse effects on the viability of the axons.

Efflux experiments. The axon was positioned in the chamber with the two cut ends in compartments *A* and *C* (Fig. 1*a*). These regions were then sealed off from the central compartment (*B*) by Vaseline seals and greased blocks. The portion of the axon in compartment *B* was superfused with external fluids of desired composition. The flow rate was 1.4 ml/min (or, in later experiments, 2.8 ml/min); most of this fluid exited directly, and was collected so that samples could be assayed for radioactivity (when the dialysis fluid was labeled with ^{45}Ca). However, about 3% of the inflow to the central compartment (1.3 cm wide) also superfused the lateral reaches of the dialyzed portion of the axon, and then exited through the guard outflows and was discarded. The inflow and outflow in the central compartment was controlled by a set of three LKB 10200 Perpex (LKB Instruments Inc., Rockville, Md.) peristaltic pumps, two for inflow and one for outflow; a Harvard Apparatus Co. (Millis, Mass.) model 940 infusion/withdrawal syringe pump, set with two syringes in the withdrawal mode, was used to control the guard outflows. A refrigerated waterbath (Lauda, model K-2/R, Brinkmann Instruments, Westbury, N.Y.) circulated a cooled methanol-water mixture through a separate compartment which jacketed compartment *B*, so that the temperature in compartment *B* could be maintained at 15 ± 1 °C.

Influx experiments. As shown in Fig. 1*b*, during influx experiments the "guard" regions were sealed off from the central compartment with Vaseline. External superfusing fluid, labeled with either ^{24}Na or ^{45}Ca, entered compartment *B* through the inflow port at one end of the compartment; the flow rate was 20 μliters/min. The fluid exited at the opposite end of the compartment after circulating through the compartment, around the axon. The amount of isotope which entered the axon could be determined by collecting consecutive 5-min samples of effluent dialysis fluid (*cf.* Brinley & Mullins, 1967).

Radioactive tracer counting procedures. For ^{45}Ca efflux experiments, 2.8 ml aliquots of the central chamber effluent were collected directly into liquid scintillation counting vials. Ten ml of 2:1 toluene/Triton X-100 counting cocktail (Nadarajah, Leese & Joplin, 1969) containing 4 g of Omnifluor (New England Nuclear, Boston, Mass.) per liter were added, and the samples were counted in a Beckman liquid scintillation counter. Small aliquots of diluted (1:100), labeled dialysis fluids were added to 2.8 ml of 10 K(Na) and were counted in a similar manner following the addition of counting cocktail.

Consecutive 5-min samples of the dialysis fluid effluent were collected during influx experiments. The distal tip of the dialysis tube was rinsed with 2.8 ml of distilled water at the end of each collection period, and both the effluent and wash were collected in plastic counting

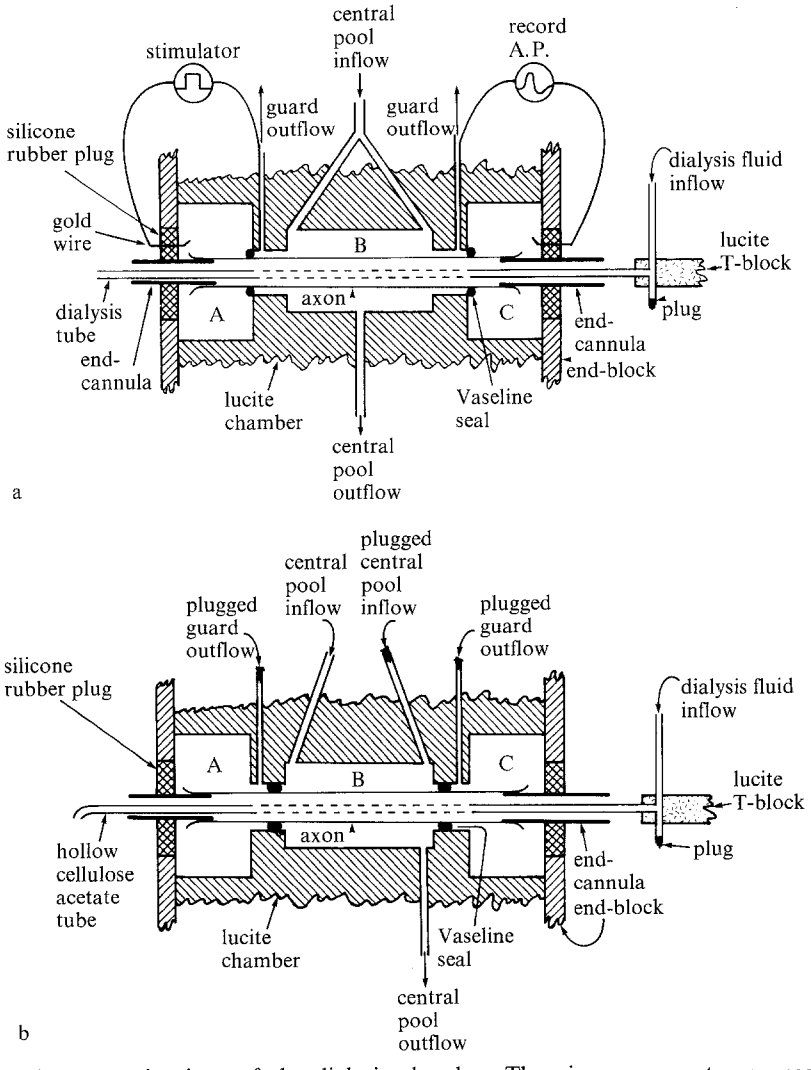

Fig. 1. Diagrammatic views of the dialysis chamber. The views represent cross-sections through the lucite chamber, parallel to the upper surface. They are not drawn to scale and are meant primarily to illustrate the relative positions of the axon, hollow cellulose acetate fiber, chamber compartments and electrodes. The porous region of the dialysis tube is indicated by the interrupted lines. The article by Brinley and Mullins (1967) should be consulted for details of chamber design. (*a*) Chamber arrangement for efflux experiments (from Blaustein *et al.*, 1974). The narrow regions of the chamber between compartment *B* and compartments *A* and *C* are the "guard" regions, i.e., the regions surrounding the lateral reaches of the dialyzed portion of the axon. About 3 % (or 0.04–0.08 ml/min) of the fluid entering the central pool (compartment *B*) superfused these portions of the axon and then exited through the guard outflows. Only fluid leaving through the central pool outflow (1.4–2.8 ml/min) was sampled for ^{45}Ca activity. The calomel half-cells used to measure resting membrane potentials have been omitted from the figure; the reference electrode was in contact with the fluid entering the central pool, and the "intracellular" electrode made contact, via a salt bridge, with the dialysis fluid emerging from the left-hand end of the hollow cellulose acetate tube. (*b*) Chamber arrangement for influx experiments. Note that the Vaseline seals exclude the "guard" regions from communication with the central compartment

vials. For the ^{24}Na influx experiments, these samples without counting cocktail were counted directly in the liquid scintillation counter (set in the manual mode, so that only one sample was in the counter at a time—to reduce background cross-talk), to determine the Cerenkov radiation (Haberer, 1965) from the ^{24}Na. When ^{45}Ca influx was measured, 10 ml of counting cocktail was added to each sample, to prepare the sample for counting in the liquid scintillation counter. The specific activity of the isotope (^{24}Na or ^{45}Ca) in the extracellular fluid was determined on appropriate dilutions of this fluid.

Membrane potentials. The resting membrane potential was recorded by means of a pair of calomel electrodes. One electrode made contact, by means of a KCl bridge, with the dialysis fluid emerging from the distal tip of the hollow cellulose acetate fiber. The second (reference) electrode made contact, also via a KCl bridge, with the superfusion (external) fluid as it entered the central compartment of the experimental chamber. The potential difference between the electrodes was read through a W-P model VF-1 voltage follower (W-P Instruments, Hamden, Conn.) and recorded on an Omniscribe strip chart recorder (Houston Instrument Co., Bellaire, Texas). The resting potential was recorded continuously during efflux experiments but only at the beginning and end of influx experiments.

The action potential was also monitored in efflux experiments. Stimulating current was passed between the guard region and the end-pool at one end of the axon, and the recording leads were arranged to monitor the potential difference between the guard and end-pool at the other end of the axon (Fig. 1*a*). In all of the experiments described here, the axons were excitable during the initial exposure to 12 K(Na) + CN, when dialyzed with ^{45}Ca-labeled fluids. As noted previously (Blaustein *et al*, 1974), the CN-poisoned axons became inexcitable during prolonged exposure to Ca-free external media despite maintenance of the resting potential.

Results

The External Na-dependent Ca Efflux

Fig. 2 illustrates the results of a representative experiment in which the effects of external Ca and Na on Ca efflux were monitored. As in intact axons (Blaustein & Hodgkin, 1969), the Ca efflux from poisoned, dialyzed squid axons is partially dependent upon these two external cations. In the experiment of Fig. 2, the dialysis fluid had a nominal ionized Ca^{2+} concentration ($[Ca^{2+}]_i$) of 1.5 μM; the Ca efflux into 12 K(Na) + CN was about 0.2 pmoles/cm^2 sec. The efflux fell to 0.03 pmoles/cm^2 sec when the external Ca was replaced by Mg, and the Na by Li; when the Na, but not Ca, was re-introduced, the efflux recovered to about 0.15 pmoles/cm^2 sec. It will be convenient to refer to the difference between the efflux into 12 K(Na) + CN and into Ca-free 12 K(Na) + CN (about 0.05 pmoles/cm^2 sec in the experiment of Fig. 2) as the "Ca_o-dependent Ca efflux"; the term "Na_o-dependent Ca efflux" will be used to indicate the difference between the efflux into Ca-free 12 K(Na) + CN and into Ca-free Na-free solution (about 0.12 pmoles/cm^2 sec in this experiment).

Calcium efflux data from a number of experiments with $[Na]_i = 5$ mM are summarized in Table 2; the data are grouped according to the nominal

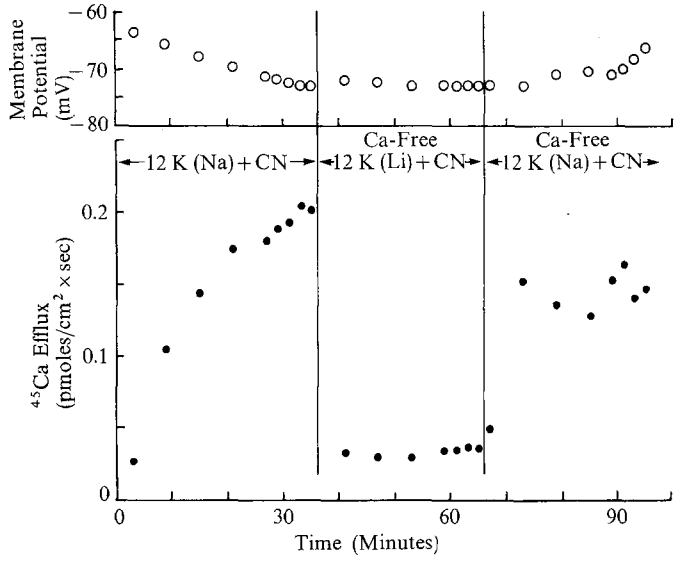

Fig. 2. Effect of external Ca and Na on ^{45}Ca efflux from a dialyzed squid axon. The upper part of the Figure shows the resting membrane potential, and the lower part shows the Ca efflux as a function of time. The axon was dialyzed with a fluid containing 5 mM Na, 1.2 mM EGTA and 1.056 mM $CaCl_2$ (nominal $[Ca^{2+}]_i = 1.5$ μM). The superfusion fluid was initially 12K(Na)+CN. Forty-two minutes after the ^{45}Ca-labeled dialysis fluid was introduced, both Ca and Na were removed from the external medium; 30 min later, Na alone was added back. The axon was excitable for the first 80 min of the experiment. It was frequently noted that cyanide-poisoned axons bathed in Ca-free media became inexcitable after a prolonged exposure to Na-free solutions; the cause of this effect is uncertain. Axon 5204B; diameter, 500 μ; temperature, 15 °C

$[Ca^{2+}]_i$. Di Polo (1973) also examined the effect of $[Ca^{2+}]_i$ (between 0.05 and 3 μM) on Ca efflux from dialyzed axons. Although his axons were fueled with ATP (*see* Fig. 1 in Di Polo, 1973), his values for Ca efflux into Na+Ca-containing media are comparable to our values for poisoned, ATP-depleted axons shown in column 2 of Table 2. This may indicate that ATP does not play a direct role in the Ca extrusion mechanism (but *see* Di Polo, 1974).

The Na_o-dependent ^{45}Ca efflux data from Table 2 are graphed as a function of $[Ca^{2+}]_i$ in Fig. 3. Note that the efflux appears to saturate as $[Ca^{2+}]_i$ is increased from 0.23 to 160 μM (also compare Fig. 10); the apparent half-saturation value for Ca_i^{2+}, K_{Ca_i}, is about 8 μM.

In two experiments (axons 5254B and 5274 of Table 2) the $[Ca^{2+}]_i$ was increased to 560 μM so that data from the present study could be directly compared with those from a previous study (Blaustein *et al.*, 1974). Table 2 shows that the Na_o-dependent efflux increased in proportion to

the increase in $[Ca^{2+}]_i$. The large Na_o-dependent efflux at $[Ca^{2+}]_i \simeq$ 560 μM, 4.88–5.86 pmoles/cm² sec, is close to the value obtained in the earlier study, 6.0 ± 0.2 pmoles/cm² sec (mean ± SE of data from three axons) when $[Ca^{2+}]_i$ was 540 μM and $[Na]_i$ was 5 mM. These observations may

Table 2. Calcium efflux from cyanide-poisoned axons into 12K(Na), Ca-free 12K(Na) and Ca-free 12K(Li)

Axon	Calcium efflux into:			Na_o-dependent Ca efflux[a]	Resting potential[b]	Diameter[b]
	12K(Na)	Ca-free 12K(Na)	Ca-free 12K(Li)	(pmoles/cm² sec)	(mV)	(μ)
1. $[EGTA]_i = 2.00$ mM; $[Ca^{2+}]_i \simeq 0.23$ μM						
5084	—	0.10	0.10	0	−66	575
5094	–	0.06	0.04	0.02	−56	525
5204A	–	0.09	–	–	−55	510
5214A	0.26	0.13	0.08	0.05	−63	550
5214B	–	0.19	0.20	−0.01	−70	640
5224B	0.05	0.05	0.07	−0.02	−65	500
Mean ± SE	0.16	0.10 ± 0.02	0.10 ± 0.03	0.01 ± 0.01		
2. $[EGTA]_i = 1.50$ mM; $[Ca^{2+}]_i \simeq 0.47$ μM						
5104A	–	0.08	0.06	0.02	−60	580
5234A	–	0.30	0.06	0.24	−67	615
Mean		0.19	0.06	0.13		
3. $[EGTA]_i = 1.20$ mM; $[Ca^{2+}]_i \simeq 1.5$ μM						
5084	–	1.22	0.95	0.27		
5094	–	1.12	0.92	0.20		
5104A	–	0.18	0.03	0.15		
5104B	–	0.77	0.60	0.17	−60	600
5204A	0.32	0.14	–	–		
5204B	0.21	0.15	0.03	0.12	−73	500
5214A	0.31	0.25	0.08	0.17		
5214B	0.32	0.28	0.06	0.22		
5224A	–	0.63	0.48	0.15	−67	625
6014	0.26	0.26	0.05	0.21	−54	520
6054A	0.45	–	0.05[c]	–	−76	575
Mean ± SE	0.31 ± 0.03	0.50 ± 0.13	0.33 ± 0.12	0.19 ± 0.01		
4. $[EGTA]_i = 1.10$ mM; $[Ca^{2+}]_i \simeq 6$ μM						
5304A	1.05	0.76	0.13	0.63	−60	590
5314A	0.75	–	0.05[c]	–	−72	540
5314B	0.73	0.68	0.05	0.63	−66	550
Mean ± SE	0.84 ± 0.10	0.72	0.08 ± 0.03	0.63		

Table 2 (continued)

Axon	Calcium efflux into:			Na_o-dependent Ca efflux[a]	Resting potential[b]	Diameter[b]
	12 K(Na)	Ca-free 12 K(Na)	Ca-free 12 K(Li)			
	(pmoles/cm^2 sec)				(mV)	(μ)
5. $[EGTA]_i = 1.05$ mM; $[Ca^{2+}]_i \simeq 10$ μM						
5104A	–	0.85	0.25	0.60		
5134	–	0.68	0.07	0.61	−68	575
5234A	–	1.00	0.19	0.81		
5234B	0.95	0.79	0.09	0.70	−65	510
5244A	–	1.01	0.24	0.76	−63	500
5244B	–	0.86	0.16	0.70	−58	600
5254A	0.83	0.80	0.12	0.68	−68	530
5254B	–	0.78	0.12	0.66	−70	560
5284A	2.10	–	–	–	−63	560
5284B	1.16	–	–	–	−70	530
5294A	1.04	–	–	–	−70	540
5294B	1.25	–	–	–	−64	560
5294C	0.85	–	–	–	−63	665
5294D	1.25	1.05	0.22	0.83	–	590
6054B	1.80	–	0.14[c]	–	−73	560
Mean ± SE	1.25 ± 0.14	0.87 ± 0.04	0.16 ± 0.02	0.71 ± 0.03		
6. $[EGTA]_i = 1.00$ mM; $[Ca^{2+}]_i \simeq 60$ μM						
5304B	1.85	1.53	0.10	1.43	−68	590
5304B′	–	1.80	0.19	1.61		
Mean	1.85	1.67	0.15	1.52		
7. $[EGTA]_i = 0.90$ mM; $[Ca^{2+}]_i \simeq 160$ μM						
5224A	(> 1.10)	1.04	0.09	0.95		
5224B	–	2.12	0.42	1.70		
5234B	–	2.16	0.41	1.75		
5254B	–	1.75	0.51	1.24		
5274	–	1.90	0.83	1.07	−79	590
6034A	2.75	2.31	0.30	2.01	−66	550
6034B	2.65	–	0.15	–	–	550
6044A	2.75	2.50	0.15	2.35	−66	550
Mean ± SE	2.72 ± 0.03	1.97 ± 0.18	0.36 ± 0.09	1.58 ± 0.19		
8. $[EGTA]_i = 0.50$ mM; $[Ca^{2+}]_i \simeq 560$ μM						
5254B	–	5.98	0.12	5.86		
5274	–	5.34	0.46	4.88		
Mean		5.66	0.29	5.37		

[a] The Na_o-dependent Ca efflux is equal to the difference between the efflux into Ca-free 12 K(Na) and Ca-free 12 K(Li).

[b] In many axons Ca efflux was measured at more than one $[Ca^{2+}]_i$. The resting potential and diameter are shown only for the first entry in the Table.

[c] Efflux was measured in Ca-free 12 K(choline) + CN.

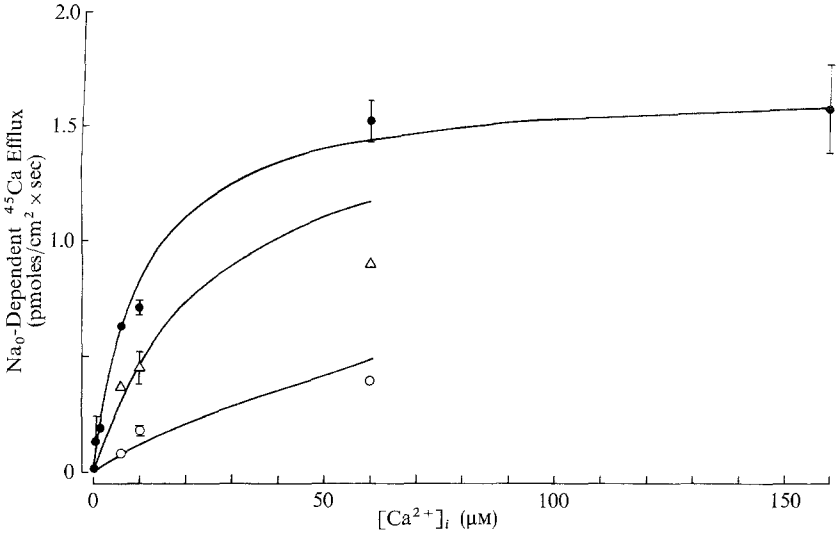

Fig. 3. Effect of internal ionized Ca^{2+} concentration on external Na-dependent ^{45}Ca efflux. The graph shows the mean values for the $[Na]_i = 5$ mM data (●) from Table 2. Also shown are data for $[Na]_i = 25$ mM (△) and $[Na]_i = 100$ mM (○) from Table 3. The vertical bars indicate the standard errors of the means (of more than two values) or the range of a pair of values (where the range extends beyond the symbol). The curves were drawn to fit the equation (e.g. *see* Baker & Blaustein, 1968):

$$J = \frac{J^{Max}}{1 + \frac{K_{Ca_i}}{[Ca^{2+}]_i}\left(1 + \frac{[Na]_i}{K_{Na}}\right)^2}$$

where J is the Na_o-dependent Ca efflux at any $[Ca^{2+}]_i$. The maximal Na_o-dependent Ca efflux, J^{Max}, had a value of 1.7 pmoles/cm^2 sec. The half-saturation constant for Ca_i, K_{Ca_i}, had a value of 8 μM; the apparent mean inhibitory constant for Na_i, K_{Na}, had a value of 30 mM

indicate that there is something deceptive about the apparent saturation seen in the upper curve of Fig. 3. One possible explanation is that a separate ("second") Ca flux mechanism is called into play at high $[Ca^{2+}]_i$. However, this possibility seems unlikely because the large Ca efflux with $[Ca^{2+}]_i > 500$ μM, and the smaller efflux with $[Ca^{2+}]_i \leqq 160$ μM have many similar properties (*see below* and Blaustein *et al.*, 1974): activation by external cations, apparent stoichiometry, and sensitivity to membrane potential. An alternative explanation for these observations at high $[Ca^{2+}]_i$ is that the kinetic parameters of the Ca transport mechanism may be influenced by the ionic composition of the axoplasm (*see* Discussion).

A Ca influx of 5–6 pmoles/cm^2 sec has also been observed in squid axons (Baker *et al.*, 1969*a*), and may be related to the aforementioned findings (*see* Discussion). The large influx occurs in axons with a high

$[Na]_i$ when external Na is replaced by Li. This may be further evidence that the Ca flux does not really saturate at a maximal value of only about 2 pmoles/cm^2 sec.

The other two fractions of the Ca efflux, the Ca_o-dependent Ca efflux and the residual efflux into Ca- and Na-free solutions (*see* Table 2), are also of considerable interest and will be discussed below.

Internal Ca-dependent Na Influx; the Evidence for Na–Ca Exchange

The observation that a fraction of the Ca efflux is dependent upon external Na led to the suggestion that the Ca efflux from squid axons may, in part, involve a Na-coupled counterflow-exchange mechanism (Blaustein & Hodgkin, 1969). Additional circumstantial evidence for such a mechanism comes from the observation that Ca influx partially depends upon internal Na, and that a fraction of the sodium pump-independent Na efflux depends upon external Ca (Baker *et al.*, 1969*a*). This raises the possibility that there is a carrier-mediated mechanism capable of moving Ca and/or Na in either direction across the axolemma; the prevalent fluxes may then be determined, at least in part, by the relative and absolute concentrations of ionized Ca^{2+} and Na^+ on the two sides of the membrane (*cf.* Blaustein, 1974). Unfortunately, one important piece of evidence has been lacking: the direct evidence that a fraction of the Na entry is internal Ca-dependent. The sodium influx in dialyzed squid axons was therefore monitored to determine whether or not this influx is influenced by $[Ca^{2+}]_i$. The results of one such experiment are shown in Fig. 4.

Even if all of the Na_o-dependent Ca efflux is tightly-coupled to Na influx (for example with 2-to-1 or 3-to-1, Na-to-Ca stoichiometry), this would, under normal circumstances (with $[Ca^{2+}]_i < 1$ μM), account for only a minute (*see* Table 2) and probably undetectable fraction of the total resting Na influx (about 30–40 pmoles/cm^2 sec in poisoned, dialyzed squid axons; Brinley & Mullins, 1968). An effort was therefore made to maximize the presumed Na–Ca exchange component of the Na influx, while minimizing other fractions of the Na influx. In the first place, axons were dialyzed with fluid containing a high nominal $[Ca^{2+}]$ (160 μM) because previous experiments (Table 2 and Fig. 3) had established that the Na_o-dependent Ca efflux component was quite sizeable under these circumstances. The internal Na concentration was 5 mM, to minimize possible Na–Na exchange (*cf.* Brinley & Mullins, 1968; Baker, Blaustein, Keynes, Manil, Shaw & Steinhardt, 1969*b*). The external fluid was Ca-free, to avoid Ca–Ca exchange (*see below*) and possible competition between

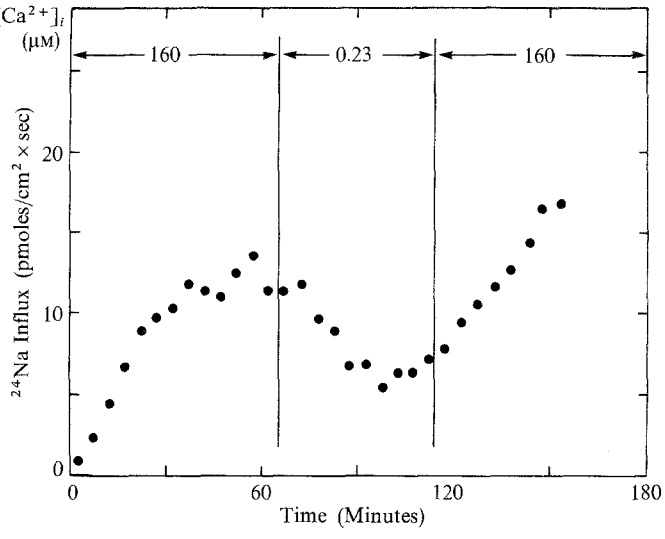

Fig. 4. Effect of the internal ionized Ca^{2+} concentration on ^{24}Na influx. The axon was superfused with ^{24}Na-labeled Ca-free 12K(200 mM Na+225 mM Li)+CN containing 10^{-5} M strophanthidin and 10^{-6} M tetrodotoxin. It was dialyzed initially with a fluid containing 1.056 mM $CaCl_2$ and 0.90 mM EGTA (nominal $[Ca^{2+}]_i \simeq 160$ μM). During the middle segment of the experiment the EGTA concentration was transiently raised to 2.0 mM (nominal $[Ca^{2+}]_i = 0.23$ μM), as indicated at the top of the graph. Axon 5154B; diameter, 700 μ; resting potential, −55 mV; temperature, 15 °C

Ca and Na for sites on the presumed carrier (*cf.* Baker *et al.*, 1969*a*; Blaustein *et al.*, 1974). The external fluid also contained 225 mM Li and only 200 mM Na to limit passive Na entry or any other Na influx which was a direct function of $[Na]_o$; this external Na concentration should suffice to nearly saturate the Na_o-dependent component of the Ca efflux (Blaustein *et al.*, 1974). Two drugs were also added to the external medium: 2×10^{-7} M tetrodotoxin, to inhibit passive Na entry through Na conductance channels (Baker *et al.*, 1969*b*), and 10^{-5} M strophanthin, to inhibit possible Na influx through the Na pump (e.g. Baker *et al.*, 1969*b*). Neither drug has any apparent effect on the Ca efflux from squid axons (Blaustein & Hodgkin, 1969; Blaustein *et al.*, 1974).

In the experiment of Fig. 4, the resting Na influx was about 12 pmoles/cm^2 sec. When the nominal $[Ca^{2+}]_i$ was decreased from 160 to 0.23 μM, the Na influx declined by about 5 pmoles/cm^2 sec. A similar result was observed in one other experiment, when $[Ca^{2+}]_i$ was reduced from 160 to 0.47 μM. These observations provide strong circumstantial evidence that the Ca efflux and Na influx are coupled.

The Influence of $[Na]_i$ on Ca Efflux

There is evidence that external Na and Ca compete for sites on the carrier which moves Na out in exchange for Ca (Baker *et al.*, 1969*a*). If the same carrier mechanism is involved in the coupled entry of Na and exit of Ca, competition between Na and Ca may also be expected at the inner surface of the membrane. Preliminary data from a previous study (Blaustein *et al.*, 1974) gave evidence of such behavior (but *see* Baker & Glitsch, 1973); however, the effect of $[Na]_i$ was tested only at very high $[Ca^{2+}]_i$ (540 μM). The effect of $[Na]_i$ was re-examined at lower $[Ca^{2+}]_i$ in the present study; the complete data from one experiment are illustrated in Fig. 5, and the results of all the relevant experiments are summarized in Table 3 and Fig. 3.

The main observation is that the Na_o-dependent Ca efflux is reduced when $[Na]_i$ is increased. In the experiment of Fig. 5, the nominal $[Ca^{2+}]_i$ was 60 μM and, with $[Na]_i = 5$ mM, the Ca efflux into Ca-free 12 K(Na)+CN was about 1.5 pmoles/cm^2 sec, nearly all of which was Na_o-dependent. The Na_o-dependent Ca efflux declined to about 0.4 pmoles/cm^2 sec when

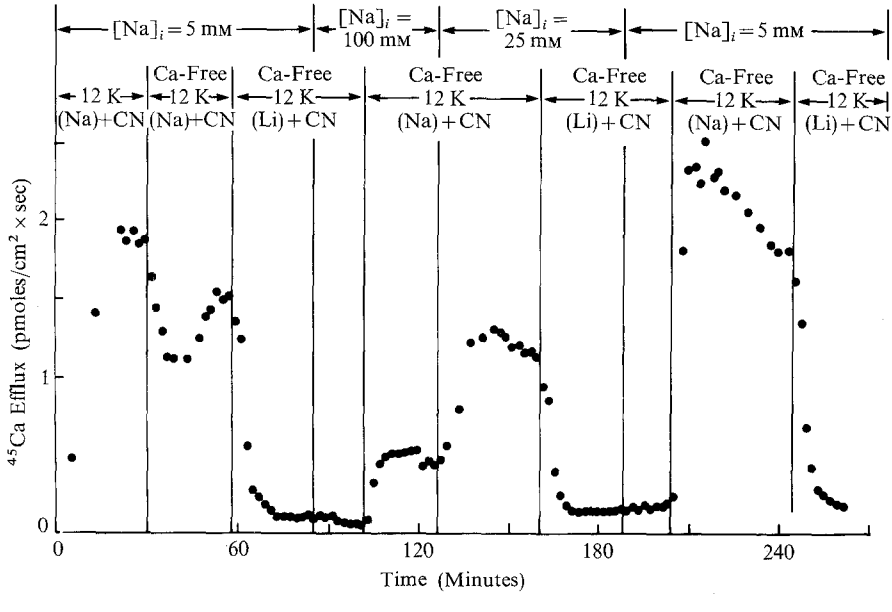

Fig. 5. Effect of internal Na concentration on ^{45}Ca efflux into Na- and Li-containing media. The axon was dialyzed with ^{45}Ca-labeled fluids containing 1.056 mM $CaCl_2$ and 1.00 mM EGTA (nominal $[Ca^{2+}]_i = 60$ μM); the internal Na concentration present during each time period is indicated at the top of the Figure. The sequence of the superfusion fluids is indicated below the internal Na concentrations. Axon 5304B; diameter, 590 μ; resting potential, −68 mV; temperature, 15 °C

Table 3. Effect of $[Na]_i$ on the Na_o-dependent Ca efflux

Axon	Na_o-dependent ^{45}Ca efflux (pmoles/cm^2 sec)			
$[Na]_i$ (mM)	1[a]	5	25	100
1. $[Ca^{2+}]_i = 6$ μM				
5304A	–	0.63	0.36	0.08
2. $[Ca^{2+}]_i = 10$ μM				
5244A	1.00	0.76	0.40	0.18
5244B	–	0.71	0.58	0.20
5254A	–	0.68	0.36	0.17
Mean ± SE	1.00	0.71 ± 0.02	0.45 ± 0.07	0.18 ± 0.01
3. $[Ca^{2+}]_i = 60$ μM				
5304B	–	1.43	0.90	0.40

[a] Nominally Na-free.

$[Na]_i$ was increased to 100 mM, and recovered to about 0.9 pmole/cm^2 sec when $[Na]_i$ was lowered to 25 mM. The fit of the pooled data to the square-law relationship between $[Na]_i$ and Ca efflux (Fig. 3) suggests that two Na^+ ions may compete with one Ca^{2+} for identical or allosterically-related binding sites at the inner surface of the axolemma.

Effects of $[Ca^{2+}]_i$ and $[Li]_o$ on Ca Influx; Evidence for Ca–Ca Exchange

The data in Fig. 2 and Table 2 (and *see* Blaustein & Hodgkin, 1969; Blaustein *et al.*, 1974) indicate that removal of Ca from the external medium *reduces* the Ca efflux. On the face of it, this observation suggests that some of the Ca efflux may be a manifestation of a Ca–Ca exchange diffusion process (*cf.* Ussing, 1947). A Ca_o-dependent Ca efflux of about 0.5 pmole/cm^2 sec or less is observed when $[Ca^{2+}]_i \leqq 160$ μM and Na is the predominant external cation (Table 2); the properties of this small efflux have not been examined in detail primarily because of possible confusion between Na–Ca and Ca–Ca exchange. The Ca_o-dependent efflux is markedly influenced by external monovalent cations; it is increased when Li_o is substituted for Na_o, and reduced when the Li_o or Na_o is replaced by choline (Blaustein *et al.*, 1974; and *see* Figs. 8–11). However, in the absence of external Ca there is little difference between the efflux into Li-containing, and choline-containing media (Blaustein *et al.*, 1974). Thus, Li_o appears to stimulate Ca–Ca exchange.

If this Li-stimulated Ca_o-dependent Ca efflux is indeed a manifestation of Ca–Ca exchange, then Ca influx and Ca_o-dependent Ca efflux should

have similar properties. Therefore, since the Ca_o-dependent Ca efflux is inhibited when $[Ca^{2+}]_i$ is reduced, or when external Li is replaced by Na or choline, similar results may be expected for Ca influx. In two experiments, of which the data in Fig. 6 are representative, the influx from 12 K(Li)+CN decreased significantly when $[Ca^{2+}]_i$ was lowered from 160 to 0.23 μM. A similar experiment was not performed with 12 K(Na) as the external medium because, as mentioned above, the Ca–Ca exchange component may be less than 0.5 pmole/cm^2 sec, and therefore rather difficult to detect in an influx experiment.

Fig. 7 illustrates the effect of replacing external Li by choline on the Ca influx. Again, as was the case for the Ca_o-dependent Ca efflux, this treatment (in the two axons tested) reduced Ca influx considerably, thereby providing further evidence that much of the Ca influx from 12 K(Li)+CN is probably Ca–Ca exchange. Although the effect on Ca influx of substituting Na for Li was not tested, previous experiments in intact axons (Baker *et al.*, 1969*a*) have shown that replacement of Na by Li increases Ca influx. However, in those experiments most of the Ca entry was presumably involved in Na–Ca exchange since the axons had a relatively high $[Na]_i$ and a low $[Ca^{2+}]_i$ (*see* Discussion).

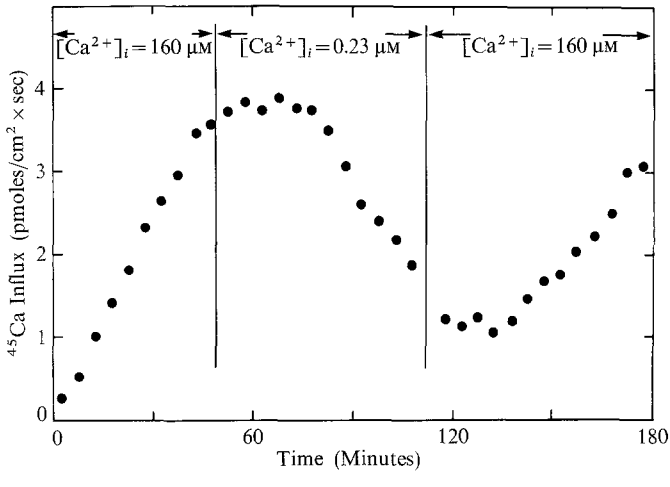

Fig. 6. Effect of the internal ionized Ca^{2+} concentration on ^{45}Ca influx. The axon was superfused with ^{45}Ca-labeled 12 K(Li)+CN. At the beginning of the experiment the dialysis fluid contained 1.056 mM $CaCl_2$ and 0.90 mM EGTA (nominal $[Ca^{2+}]_i$=160 μM). After 49 min, the EGTA concentration was raised to 2.0 mM (nominal $[Ca^{2+}]_i$=0.23 μM); it was returned to its initial level 63 min later. Axon 5174A; diameter, 520 μ; resting potential, −61 mV; temperature, 15 °C

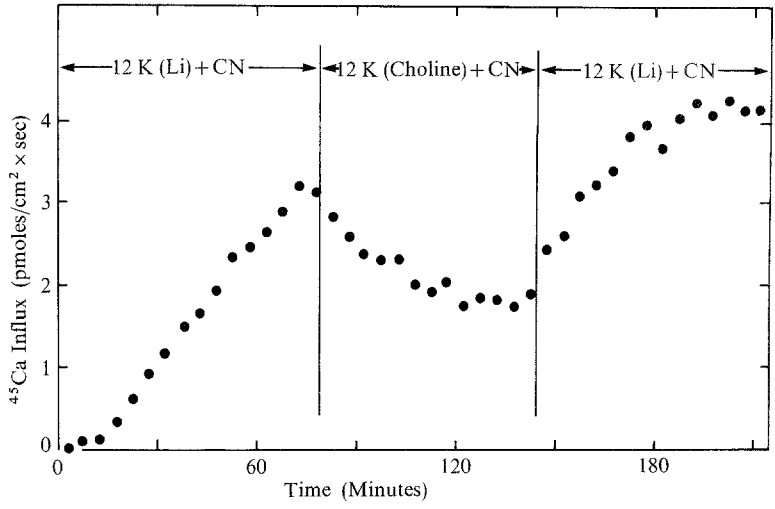

Fig. 7. Effect of external monovalent cations on ^{45}Ca influx. The axon was dialyzed with a fluid containing 1.056 mM $CaCl_2$ and 0.90 mM EGTA (nominal $[Ca^{2+}]_i = 160\ \mu M$). The initial superfusion fluid was ^{45}Ca-labeled 12 K(Li) + CN. This was replaced by ^{45}Ca-labeled 12 K(choline) + CN after 79 min; the original external medium was re-introduced 65 min later. At the time of each change the central compartment of the experimental chamber was flushed several times with the new solution to ensure rapid and complete exchange. Axon 5184 A; diameter, 625 μ; resting potential, −58 mV at start and −55 mV at end of experiment; temperature, 15 °C

Activation of Ca–Ca Exchange by Alkali Metal Ions

The foregoing observations provide strong evidence that Ca–Ca exchange occurs when the external medium is 12 K(Li), and is a prominent feature of the Ca efflux if $[Ca^{2+}]_i$ is relatively high. The Ca–Ca exchange component is markedly reduced if the external Li is replaced by choline, but the fact that a Ca_o-dependent Ca efflux is observed when the axon is superfused with 12 K(Na) + CN (*see above*) may indicate that Ca–Ca exchange is not activated exclusively by Li. Moreover, as illustrated by the experiment of Fig. 8, Ca efflux was enhanced when 104 mM choline of the 12 K(choline) + CN was replaced by K (*see* Table 1). Although the Ca efflux increased only by about 0.2 pmoles/cm^2 sec (in one other experiment, with $[Ca^{2+}]_i = 10\ \mu M$, an increase of 0.6 pmoles/cm^2 sec was observed), all of this increment was abolished when external Ca was removed; this suggests that the 116 mM K-stimulated increment involved Ca–Ca exchange.

The experiment of Fig. 9 compares the effects of Rb, Cs and Li on Ca efflux into Na-free media. While not as effective as 100 mM Li, 100 mM Rb clearly stimulated the Ca efflux. Cesium had little, if any, effect on the Ca

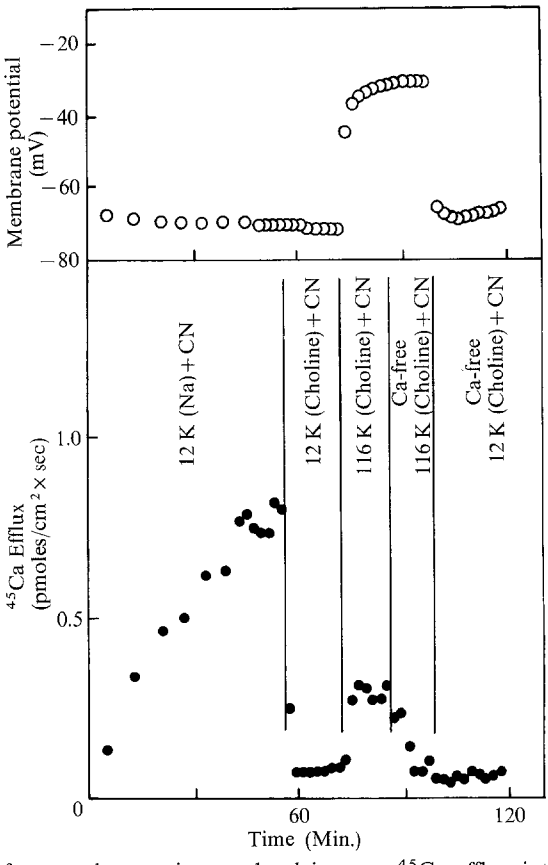

Fig. 8. Effect of external potassium and calcium on ^{45}Ca efflux into Na-free media. The axon was dialyzed with a fluid containing 1.056 mM $CaCl_2$ and 1.10 mM EGTA; the nominal $[Ca^{2+}]_i$ was 6 μM. The standard superfusion fluid, 12 K(Na)+CN, was replaced by 12 K(choline) +CN 56 min after starting dialysis with ^{45}Ca. At the times indicated in the Figure, external K was increased to 116 mM (by isomotic substitution for choline), external Ca was then removed and, finally, the K was reduced to its original level. The resting membrane potential is given, as a function of time, at the top of the Figure. Axon 5314A; diameter, 540 μ; temperature, 15 °C

efflux; unfortunately, for an unknown reason, the axon depolarized progressively following exposure to the Cs-containing solution.

The effect of $[Ca^{2+}]_i$ on the Li_o-stimulated (Ca_o-dependent) Ca efflux is illustrated in Fig. 10. The data indicate that this efflux saturates as $[Ca^{2+}]_i$ is increased; the apparent half-saturation constant (K_{Ca_i}) is of the order of 2.5 μM.

As noted above, one of the characteristics of Na–Ca exchange is an apparent competition between Na and Ca for carrier binding sites, since Na_o-dependent Ca efflux is inhibited by internal Na (Figs. 3 and 5) and

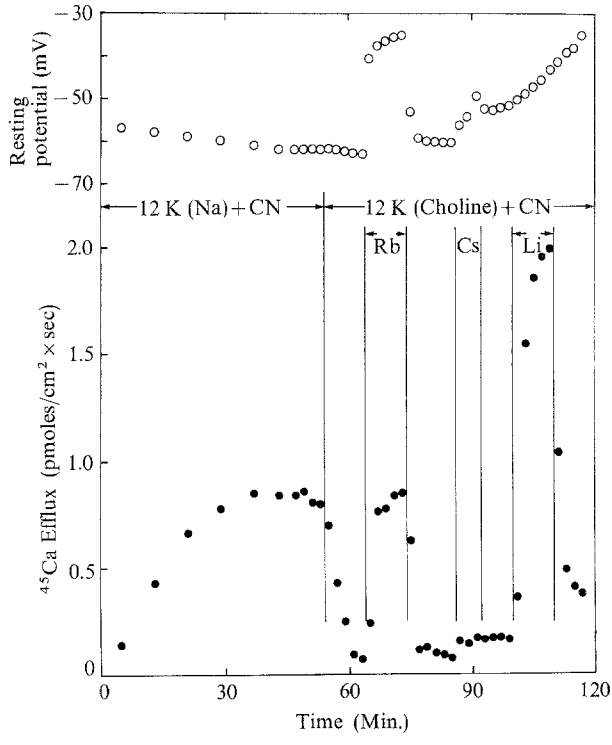

Fig. 9. Effect of external alkali metal ions on ^{45}Ca efflux into Ca-containing 12 K(choline)+CN solutions. The axon was dialyzed with a fluid containing 1.056 mM $CaCl_2$ and 1.050 mM EGTA; nominal $[Ca^{2+}]_i$ was 10 μM. Fifty-four minutes after starting dialysis with ^{45}Ca, all the external Na was replaced by choline; then, at the times indicated, 100 mM choline was replaced by 100 mM Rb, Cs or Li, for brief intervals. The resting membrane potential is given, as a function of time, at the top of the Figure. Axon 5294C; diameter, 665 μ; temperature, 15 °C

Na_i-dependent Ca influx is inhibited by external Na (Baker *et al.*, 1969*a*). The experiment of Fig. 11 shows that the (Ca_o-dependent) Ca efflux into 12 K(Li)+CN is also inhibited when the internal Na concentration is increased. This observation seems consistent with the idea that a single transport mechanism is involved in both Na–Ca exchange and Ca–Ca exchange.

Discussion

Before reviewing the properties of the Na–Ca exchange mechanism, it seems appropriate to consider several critical aspects of the Ca efflux which were neglected in the Results section. The first of these is the "re-

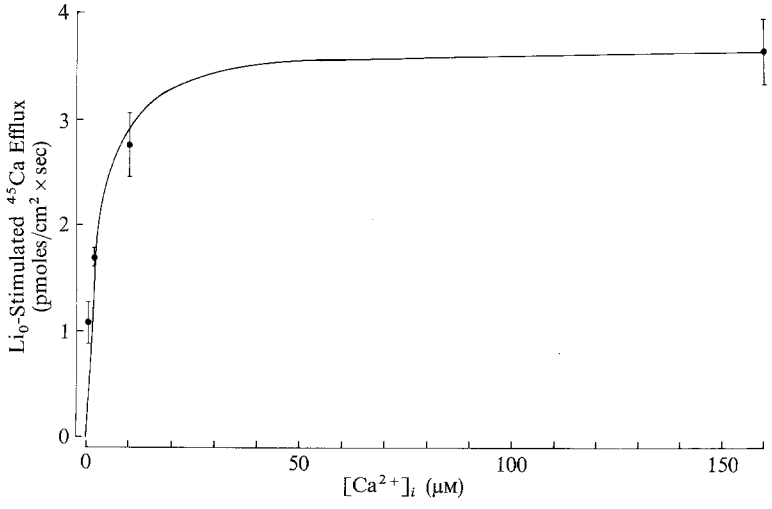

Fig. 10. Effect of $[Ca^{2+}]_i$ in the Li_o-stimulated ^{45}Ca efflux. The data represent the differences between ^{45}Ca efflux into 12K(Li)+CN and 12K(choline)+CN. Each symbol on the graph is the mean of three difference determinations; the vertical bars indicate ±1 SE. The curve was drawn to fit the equation:

$$J = \frac{J^{Max}}{1 + \frac{K_{Ca_i}}{[Ca^{2+}]_i}}$$

where J is the Li_o-stimulated Ca efflux at any $[Ca^{2+}]_i$. The maximal Li_o-stimulated Ca efflux, J^{Max}, had a value of 3.7 pmoles/cm^2 sec. The apparent half-saturation constant for Ca_i^{2+}, K_{Ca_i}, had a value of 2.5 μM. The data for this Figure were obtained from a total of seven axons, in most axons the Li_o-stimulated Ca efflux was measured at two different internal Ca^{2+} concentrations

sidual" Ca efflux: namely, that Ca efflux which remains after removal of external Ca and Na. The magnitude of the Ca_o- and Na_o-independent efflux appeared to vary somewhat from fiber to fiber at constant $[Ca^{2+}]_i$, but on the average, did not increase much when $[Ca^{2+}]_i$ was increased from 0.23 to 560 μM (*see* Table 2). Some of this residual efflux may be external Mg-dependent (perhaps a Mg–Ca exchange; *see* Blaustein & Hodgkin, 1969); unfortunately, this hypothesis was not tested in the present series of experiments. However, recent experiments of Brinley & Mullins (*personal communication*) indicate that efflux of ^{14}C-EDTA from dialyzed squid axons varies directly with the EDTA concentration in the dialysis fluid and amounts to about 0.03 pmoles/cm^2 sec per mM EDTA. Assuming that these EDTA data can be directly extrapolated to EGTA, most of the residual efflux from the axons with a low residual efflux, even at relatively high $[Ca^{2+}]_i$, might be accounted for by "leak" of complexed CaEGTA. In some axons the Ca_o- and Na_o-independent Ca efflux was

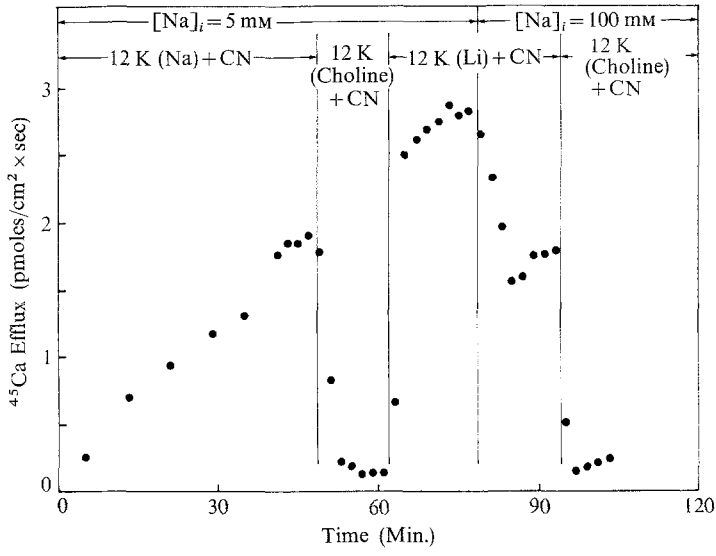

Fig. 11. Effect of the internal Na concentration on the ^{45}Ca efflux into Na-free media. The axon was dialyzed initially with a fluid containing 5 mM Na isethionate, 1.056 mM $CaCl_2$, and 1.050 mM EGTA; the nominal $[Ca^{2+}]_i$ was 10 μM. The graph indicates the periods during which the axon was superfused with the Na-, choline-, and Li-containing fluids; all the external solutions contained 10 mM $CaCl_2$. Also shown is the period when the original dialysis fluid was replaced with one containing 100 mM Na isethionate and only 20 mM taurine; this dialysis fluid also had a nominal $[Ca^{2+}]$ of 10 μM. Axon 6054 B; diameter, 560 μ; resting potential, −73 mV; temperature, 15 °C

observed to increase during the course of the experiments, while the magnitudes of the Na_o-dependent and Ca_o-dependent Ca effluxes remained constant. These considerations suggest that most of the residual Ca efflux can probably be attributed to Ca or CaEGTA "leak". This fraction of the Ca efflux will therefore be ignored in the discussion of the Na_o-dependent and Ca_o-dependent Ca effluxes which follows.

A second important point is that all of the experiments reported here were made on axons which were poisoned with cyanide and dialyzed with ATP-free and metabolic substrate-free solutions. These axons may be expected to have very low concentrations of high-energy metabolic intermediates in the axoplasm (*cf.* Mullins & Brinley, 1967). Since Ca must be extruded against a large electrochemical gradient (*cf.* Blaustein, 1974), it is pertinent to question whether or not we have disregarded a potentially important ATP-dependent Ca efflux mechanism. While the resolution of this question must await future study, available evidence (Di Polo, 1973; and *see* Results) suggests that Ca extrusion does not have an absolute requirement for ATP.

More directly related to the present experiments is the question of whether or not ATP influences the Na–Ca exchange mechanism. The observations of Baker and Glitsch (1973) and Baker and Blaustein (1974) suggest that ATP does, indeed, influence the affinity of the Ca transport mechanism for external Na and Ca in intact axons. Although Di Polo's (1973) data, from dialyzed axons in which the internal environment can be more precisely controlled, indicated that ATP had no influence on Na–Ca exchange, his more recent experiments (Di Polo, 1974) suggest that ATP may indeed affect the kinetics of Na–Ca exchange. Clearly, this in an important, unresolved problem, beyond the scope of the present investigation, and will require careful study.

Finally, since the $[Ca^{2+}]_i$ of normal, intact axons is probably less than 1 μM (*cf.* Blaustein, 1974), it is fair to ask whether or not the Ca fluxes studied at high $[Ca^{2+}]_i$ are relevant to the intact axon. Although a definitive answer cannot be provided, the fact that Na_o-dependent Ca fluxes are observed in intact axons (Blaustein & Hodgkin, 1969) and in axons dialyzed with a relatively low $[Ca^{2+}]_i$ (Table 2 and Fig. 3) suggests that very similar Na-dependent Ca transport mechanisms operate in axons with a low $[Ca^{2+}]_i$ and in those with a high $[Ca^{2+}]_i$. The principle reason for working at a high $[Ca^{2+}]_i$ was to magnify these fluxes so that they could be more readily studied, on the assumption that the main effect of increasing $[Ca^{2+}]_i$ is to bring the Na_o-dependent Ca efflux closer to saturation (*see* Fig. 3). There is, at present, no evidence that the kinetics or stoichiometry of Na–Ca or Ca–Ca exchange alters when $[Ca^{2+}]_i$ is increased from about 0.2 to about 160 μM; however, the possibility that the kinetics of Na–Ca exchange may be altered at $[Ca^{2+}]_i > 160$ μM (*see* p. 311) certainly requires more detailed exploration.

In view of the fact that EGTA was used to buffer $[Ca^{2+}]_i$ in all of the experiments reported here, a word of caution may be in order. Baker (1970) has reported that in intact squid axons, injection of EGTA inhibits the (Ca_o-dependent) Na efflux into Li sea water. This was a somewhat surprising result because previous observations led to the prediction that a reduction of $[Ca^{2+}]_i$ (by injection of EGTA) would stimulate the efflux of Na in exchange for entering Ca by reducing the competition between internal Ca and Na (*cf.* Figs. 3, 5 and 11). Until an alternative explanation is offered, we must at least consider the possibility that EGTA may directly affect the Na–Ca exchange mechanism. However, in dialysis experiments the composition of the internal environment, including the concentrations of Na^+, Mg^{2+} and H^+, can be much more carefully controlled than in injected axons; a significant alteration in the concentration of one or more

of the aforementioned ions, as a consequence of EGTA injection, could have contributed to the inhibition of Na efflux observed by Baker. In most of the experiments described here (*see* Table 2) at least 0.9 mM EGTA was present in the dialysis fluids, and small changes in EGTA concentration (e.g., from 1.00 to 1.05 mM), at constant total $[Ca]_i$, resulted in large changes in the Na_o-dependent Ca efflux (Table 2). The most straightforward explanation is that this is simply a consequence of $[Ca^{2+}]_i$ buffering.

Na–Ca Exchange

A prominent feature of the Ca efflux from squid axons (Blaustein & Hodgkin, 1969; Blaustein *et al.*, 1974), is the dependence of this efflux upon external Na. This dependence has been attributed to a hypothetical Na–Ca exchange mechanism (Blaustein & Hodgkin, 1969). The present report provides direct evidence that there is a concomitant Ca_i-dependent Na entry, thereby filling a major missing link in the proposed mechanism. The available data clearly indicate that more than one Na^+ ion enters in exchange for each exiting Ca^{2+}:

(1) Na influx is reduced by about 5 pmoles/cm^2 sec, when $[Ca^{2+}]_i$ is decreased from 160 to 0.23 μM (Fig. 4; or to 0.47 μM in one other axon); the Na_o-dependent efflux, with $[Ca^{2+}]_i = 160$ μM, is about 1.6 pmoles/cm^2 sec (Table 2). These limited data are compatible with a ratio of $3\,Na^+$-to-$1\,Ca^{2+}$, although the ratio may be underestimated if the Na_o-dependent Ca efflux was not saturated at $[Na]_o = 200$ mM (the concentration used for the Na influx experiments; *see* p. 296).

(2) the relationship between the Na_o-dependent Ca efflux and the external Na concentration fits best to a cubic function of $[Na]_o$ (Blaustein *et al.*, 1974). This may indicate that $3\,Na^+$ ions are required to activate the efflux of one Ca^{2+} ion.

(3) the (Na_o-dependent) Ca efflux is reduced *e*-fold, or a little less, by a 25 mV depolarization (Blaustein *et al.*, 1974; Brinley & Mullins, 1974). This may imply that the exit of each Ca^{2+} ion is associated with the net exit of one negative charge or net entry of one positive charge (perhaps a third Na^+).

These observations are all compatible with a Ca transport mechanism in which the exit of one Ca^{2+} may be coupled to the entry of three Na^+ ions.

A Model for the Na–Ca Exchange Carrier

A mobile carrier[1] model which can accommodate this stoichiometry ($3\,Na^+$-for-$1\,Ca^{2+}$) for Ca transport in squid axons has been described previously (Blaustein, 1974; Blaustein *et al.*, 1974; and *see* Baker *et al.*, 1969*a*). Fig. 12 illustrates the expected reactions and transmembrane fluxes of the hypothetical carrier model, and may provide a useful focus for discussion of data described in the preceding sections. Although the free carrier ($R^{2-}S^-$) is arbitrarily shown as a charged molecule with a net valence of -3, it should be obvious that the magnitude of this charge is irrelevant to the ensuing discussion.

To account for the coupled counterflow exchange of Na-for-Ca, the carriers must have binding sites for Na and for Ca. Furthermore, since Na appears to compete with Ca (perhaps in the ratio, 2Na-to-1Ca) at both the internal (*see* Fig. 3) and external (Baker *et al.*, 1969*a*) surfaces of the axolemma, we assume that the Na and Ca binding sites (R^{2-}) are identical or allosterically related and mutually exclusive. The evidence that Ca efflux into Ca-free media is not promoted by other alkali metal ions or by choline (*see* Results), indicates that these sites are highly selective for Na^+ ions. The divalent cation selectivity has not been extensively explored; however, the fact that Ca is extruded into Ca-free Na-containing media from axons dialyzed with fluids having a nominal $[Mg^{2+}]/[Ca^{2+}]$ ratio of $>10^3$ (*see* Table 2 and Fig. 3), implies that the selectivity for Ca over Mg is rather high.

If the R^{2-} sites can accommodate only two Na^+ ions (as inferred from the Na–Ca competition data), the carrier must have an additional Na^+ binding site (denoted by S^- in Fig. 12) to account for the apparent $3\,Na^+$-for-$1\,Ca^{2+}$ stoichiometry.

Ca–Ca Exchange

Even more compelling evidence for the presence of this additional alkali metal ion site comes from the Ca_o-dependent Ca efflux and Ca_i-dependent Ca influx data. Fig. 7 clearly shows that Ca influx from Na-free media is promoted by Li, confirming an earlier observation made on intact axons (Baker *et al.*, 1969*a*). In the latter experiments, the Ca influx appeared to be coupled to a cardiac glycoside-insensitive Na extrusion (i.e., Na–Ca exchange). However, in the dialysis experiments described

1 The transport mechanism will be referred to as a "carrier" because counterflow exchange such as Na–Ca exchange and Ca–Ca exchange provides strong evidence that mobile carriers are involved (Wilbrandt & Rosenberg, 1961).

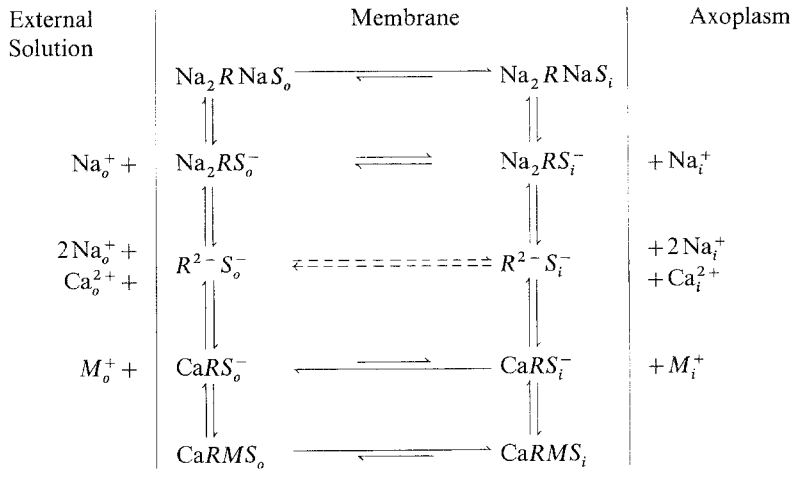

Fig. 12. A carrier model for Na–Ca and Ca–Ca exchange. The free carrier, $R^{2-}S^-$, is assumed to have two types of cation-binding sites. One site is monovalent (S^-), and can bind a single alkali metal ion (M^+); the second site is divalent (R^{2-}), with specificity for either Na (two Na^+ ions can bind) or Ca. The chemical reactions between carrier and counter-ions are assumed to be very rapid, while the diffusion of the carrier complexes across the membrane are rate-limiting. The diffusible carrier-cation complexes are: Na_2RNaS (or Na_2RMS), $CaRMS$, Na_2RS^- and $CaRS^-$ (the latter two are driven by the membrane potential and therefore move preferentially in the outward direction). Free carrier is assumed to diffuse slowly, if at all. The long, solid arrows show the direction the reactions would tend to go in if the steady-state were disturbed by a slight increase in $[Ca^{2+}]_i$. The subscripts, *i* and *o*, refer to the axoplasm and hemolymph, or internal and external surfaces of the membrane, respectively

above, with $[Na]_i = 5$ mM, much of the Ca entry from Li-containing media depended upon internal Ca^{2+} (*see* Fig. 6), which suggests that this entry is a manifestation of Ca–Ca exchange.

Ca_o-dependent Ca efflux into Na-free media is also promoted by external Li and K (Fig. 8), and perhaps Rb (Fig. 9; unfortunately, the Ca_o-dependence was not tested in this case), further supporting the view that these alkali metal ions activate Ca–Ca exchange. The fact that addition of Ca to Na-containing external media also stimulates Ca efflux (Fig. 2, Table 2, and Blaustein *et al.*, 1974), suggests that Ca–Ca exchange is also promoted by external Na; however, under these conditions, the Ca efflux may be a mixture of Na–Ca and Ca–Ca exchange.

The selectivity of the alkali metal ion activation site (S^-) is somewhat difficult to assess. Lithium appeared to stimulate Ca efflux more effectively than either K or Rb (Figs. 8 and 9). However, it seems possible that the K and Rb may both exert two opposing effects: they may promote Ca–Ca exchange by binding to the S^- site, and they may inhibit Ca efflux by

depolarizing the axons if Ca–Ca exchange, like Na–Ca exchange (Blaustein *et al.*, 1974; Brinley & Mullins, 1974), is inhibited by depolarization. Consequently, the selectivity sequence obtained from the flux data, $Li > Na, K, Rb \gg Cs$, choline, must be viewed with considerable caution.

The Ca influx and efflux data may also provide a tentative estimate of the Ca–Ca exchange stoichiometry. As shown in Fig. 10, the Li_o-stimulated Ca efflux, with $[Ca^{2+}]_i = 160$ μM, averaged 3.7 ± 0.3 pmoles/cm^2 sec in three axons. The decrement in ^{45}Ca influx, upon changing the external solution from 12 K(Li) + CN to 12 K(choline) + CN, was 2.4 and 3.3 pmoles/cm^2 sec, respectively, in two axons dialyzed with fluid containing 160 μM Ca^{2+} (the former influx value is from the experiment of Fig. 7). Although rather limited, these data may indicate that the stoichiometry of Ca–Ca exchange, with respect to Ca, is approximately 1-for-1. A somewhat less reliable estimate may be obtained from experiments on the effect of $[Ca^{2+}]_i$: as shown in Fig. 10, the Li_o-stimulated ^{45}Ca efflux declined, by about 2.6 pmoles/cm^2 sec, when $[Ca^{2+}]_i$ was reduced from 160 to 0.23 μM. By way of comparison, ^{45}Ca influx from 12 K(Li) + CN fell by 2.7 (*see* Fig. 6) and 3.2 pmoles/cm^2 sec, respectively, in two axons, when $[Ca^{2+}]_i$ was decreased from 160 to 0.23 μM; however, these Ca_i-dependent influxes may be underestimated because the $[Ca^{2+}]_i$ was changed before the fluxes reached steady levels.

It would be of interest to know whether or not the Ca–Ca exchange (diagrammed in the lower portion of Fig. 12) is accompanied by the flux of an alkali metal ion. Unfortunately, lack of a convenient radioactive isotope for the most likely candidate, Li, precludes direct measurement of such a flux.

The Energetics of Na–Ca Exchange

All of the reactions in which the Ca carrier of Fig. 12 participates are assumed to be reversible. [For example, the same Na–Ca exchange carrier can presumably move Ca in and Na out (Baker *et al.*, 1969*a*), or Ca out and Na in (*see* Results).] The broken lines in the diagram (Fig. 12) indicate that free carrier probably cannot readily traverse the membrane. This behavior is suggested by the observation (*see* Results) that Ca efflux is markedly curtailed if Na, or Ca plus an appropriate alkali metal ion, is not available at the external surface. This may imply that the influx and efflux legs of the cycle are rather tightly coupled, so that most of the energy liberated by the inward movement of Na, down its electrochemical gradient, may be used to drive Ca out. If the stoichiometry is 3-for-1, as

suggested above, the carrier could maintain a Ca concentration gradient given by (Blaustein & Hodgkin, 1969):

$$\frac{[Ca^{2+}]_o}{[Ca^{2+}]_i} = \frac{[Na^+]_o^3}{[Na^+]_i^3} e^{-V_M F/RT}$$

where V_M is the membrane potential and F, R and T have their usual meanings. With a 60 mV resting potential and a 10:1 Na gradient ($[Na^+]_o/[Na^+]_i$), this mechanism could, in principle, maintain a Ca gradient of $10^4:1$, which is close to the value observed experimentally in squid axons (*cf.* Blaustein, 1974). Although a possible role for ATP in the Ca extrusion mechanism (*cf.* Baker & Glitsch, 1973; Di Polo, 1974) is not excluded, these considerations are consistent with Di Polo's (1973) observation that Ca efflux from squid axons does not have an absolute requirement for ATP.

Kinetics of Na–Ca and Ca–Ca Exchange; Some Unsolved Problems

Although the kinetic properties of the Na–Ca and Ca–Ca exchange carrier have been ignored in the foregoing discussion, the limited data available are certainly noteworthy. The most interesting kinetic feature is the discrepancy between the apparent half-saturation constants for Ca^{2+} at the inner and outer surfaces of the axolemma, K_{Ca_i} and K_{Ca_o}, respectively. In the poisoned fuel-free axon, with $[Ca^{2+}]_i = 1.3$ μM, K_{Ca_o} for the Li_o-stimulated (Ca_o-dependent) Ca efflux was found to be about 3 mM (Blaustein *et al.*, 1974). In the present series of experiments, under comparable conditions (with $[Ca^{2+}]_i \leqq 160$ μM), K_{Ca_i} was found to be about 8 μM for the Na_o-dependent Ca efflux (Fig. 3) and about 2.5 μM for the Li_o-stimulated Ca efflux (Fig. 10). Clearly, if the same carrier can move Ca in and out across the axolemma, this three order-of-magnitude difference in apparent affinity for Ca^{2+} at the inner and outer membrane surfaces will have to be explained. There is some evidence (Baker & Blaustein, 1974) that the apparent K_{Ca_o} may be reduced by internal ATP. However, in the aforementioned experiments on poisoned, dialyzed axons, $[ATP]_i$ was maintained at a very low, presumably constant level, so that the change in apparent K_{Ca} as the carrier moves from the outside to the inside cannot be explained by an ATP-dependent reaction.

By way of contrast, the available data indicate that there is only a small difference between the apparent mean half-saturation constants for Na ($\bar{K}_{Na}$) at the inner and outer membrane surfaces of poisoned axons, 30 mM (*see* caption to Fig. 3) and 125 mM (Blaustein *et al.*, 1974), respectively.

An additional unexplained observation is that raising $[Ca^{2+}]_i$ from 160 to 560 μM increases the apparent K_{Ca_i} and the maximal efflux. This raises the possibility that the ionic environment may also influence the kinetic parameters of the Ca carriers. More experiments on the effects of internal (and external) ionic conditions will certainly be required to clarify this situation.

The Possible Role of Na–Ca Exchange in Cellular Ca Regulation

Several previous studies (Luxoro & Yañez, 1968; Blaustein & Hodgkin, 1969; Baker, Hodgkin & Ridgeway, 1971; Di Polo, 1973) have shown that squid axoplasm contains ATP-dependent Ca storage sites (possibly mitochondria). If Na–Ca exchange plays a role in trans-axolemmal Ca transport, it would be of interest to know how this mechanism interacts with the axoplasmic Ca storage mechanism in terms of $[Ca^{2+}]_i$ regulation. Although no direct information is available on the subject, Ca efflux recovers very rapidly from cyanide poisoning, presumably as a consequence of a change in $[Ca^{2+}]_i$ (Blaustein & Hodgkin, 1969), and ATP injection very rapidly lowers ionized Ca^{2+} in cyanide-poisoned axons (Baker *et al.*, 1971). These observations suggest that the ATP-dependent Ca sequestration system may serve as an "instantaneous" regulator (i.e., in the millisecond to second range) of $[Ca^{2+}]_i$. Sodium-calcium exchange, on the other hand, may be involved in the long-term regulation of Ca homeostasis in the axon.

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