J. Physiol. (1976), **259**, *pp.* 103–144
With 15 text-figures
Printed in Great Britain

KINETICS AND ENERGETICS OF CALCIUM EFFLUX FROM INTACT SQUID GIANT AXONS

BY P. F. BAKER AND P. A. MCNAUGHTON
From the Department of Physiology, King's College, Strand, London WC2R 2LS and the Department of Pharmacology, Medical School, Hills Road, Cambridge CB2 2QD

(*Received 1 October 1975*)

SUMMARY

1. The Ca efflux from intact squid axons consists of three major components: one that is activated by Ca_o, one that is activated by Na_o and a residual flux that persists in the nominal absence of both Ca_o and Na_o. The properties of these components have been investigated in unpoisoned axons and in axons poisoned with cyanide.

2. Under all conditions the shape of the curve relating Ca_o to Ca_o-activated Ca efflux approximates to a section of a rectangular hyperbola, consistent with simple Michaelis activation.

3. The external Ca concentration giving half-maximal activation of Ca_o-activated Ca efflux is about 2 μM in unpoisoned axons immersed in Na-ASW, but on poisoning changes progressively to values in the range 1–10 mM. The residual efflux from unpoisoned axons may reflect activation by traces of Ca present immediately external to the axolemma.

4. The apparent affinity for Ca_o of Ca_o-activated Ca efflux is very similar in unpoisoned axons immersed in sea waters containing Na, Li, Tris or K as major cation, whereas in poisoned axons the affinity in Na and Li is about the same but higher than that in choline and Tris.

5. In unpoisoned axons Ca influx increases linearly as Ca_o is increased from 2 μM to 110 mM. The absolute value of the Ca influx from 10 μM-Ca_o is less than 1 % of the Ca_o-activated Ca efflux at this external Ca concentration. In poisoned axons the sizes of Ca_o-activated Ca efflux and Ca influx were similar at all Ca concentrations examined.

6. The shape of the curve relating Na_o to Na_o-activated Ca efflux approximates to a section of rectangular hyperbola in unpoisoned axons but is clearly sigmoidal in axons that have been fully poisoned with cyanide. The sigmoidal shape develops progressively during poisoning.

7. The external Na concentration giving half-maximal activation of

Na_o-activated Ca efflux is about 60 mM in unpoisoned axons and often exceeds 300 mM in fully poisoned axons.

8. In both unpoisoned and poisoned axons, at constant Na_o, replacement of 200 mM-Tris by 200 mM-K reduced the Na_o-activated Ca efflux reversibly.

9. The magnitude and kinetics of both Ca_o-activated Ca efflux and Na_o-activated Ca efflux were unaffected by injection into the axon of a solution containing 220 mM-EGTA and 100 mM-$CaCl_2$. Subsequent exposure to cyanide reduced the amplitude of the Ca efflux to about one third, and the kinetics of both components of the Ca efflux changed to that characteristic of the poisoned state. Injection of ATP produced a transient increase in efflux and restored a Na_o-activation curve close to that seen in unpoisoned axons. AMP and cyclic AMP were ineffective.

10. Application of cyanide in the presence of oligomycin produced a large increase in Ca efflux. Subsequent injection of ATP had little effect on the size of the efflux but altered the kinetics of Na_o-activation so that they resembled more closely those seen in unpoisoned axons.

11. These results are discussed and shown to be consistent with a model in which the Ca pump must bind ATP for efflux to occur.

INTRODUCTION

The squid giant axon shares with all other cells so far investigated an ability to maintain a low intracellular concentration of ionized Ca by virtue of an active mechanism for extruding Ca ions across the axolemma. The energy source for this process is still something of a puzzle, as the Ca efflux increases when intracellular ATP has been reduced to a low level by poisoning with cyanide (Rojas & Hidalgo, 1968; Blaustein & Hodgkin, 1969). Under comparable conditions the ATP-dependent Na:K exchange pump is strongly inhibited. One possible source of energy to maintain the Ca efflux is the transmembrane Na concentration gradient which persists for many hours in a fully poisoned axon. An influx of Na in exchange for intracellular Ca has not been demonstrated in intact axons, but removal of external Na reduces the Ca efflux (Baker, Blaustein, Hodgkin & Steinhardt, 1967) and increases calcium influx, apparently in exchange for intracellular sodium (Baker, Blaustein, Hodgkin & Steinhardt, 1969*a*). While an electroneutral exchange of two Na ions for one Ca would not be energetically adequate to maintain the observed low level of intracellular Ca (Baker, 1972), there is evidence that the Ca efflux is depressed by depolarization (Blaustein, Russell & de Weer, 1974; Mullins & Brinley, 1975; this paper) which suggests that the exchange may be electrogenic with three or more Na ions exchanging for each Ca. An electrogenic exchange

of three or more Na ions for one Ca ion would be able to maintain the observed intracellular levels of ionized Ca without assistance from other energy sources (Blaustein & Hodgkin, 1969; Baker, 1972; Blaustein, 1974).

Despite the plausibility of a passive counter transport of Na for Ca, there is some evidence for an involvement of intracellular energy sources in maintaining or modifying the Ca efflux. Blaustein & Hodgkin (1969) occasionally observed a fall in Ca efflux on poisoning before the rise in efflux that presumably results from liberation of sequestered intracellular Ca. Baker & Glitsch (1973) have seen a similar fall, not followed by a rise, when the intracellular ionized Ca concentration is stabilized in the normal range by pre-injection of various Ca-EGTA buffers. Injection of the ATP-destroying enzyme apyrase produces a marked fall in Ca efflux, and in the poisoned state the apparent kinetics of both Na_o-activation and Ca_o-activation of Ca efflux are altered, indicating an involvement of metabolizable energy sources. More recently Dipolo (1974), Baker & Blaustein (1974) and Mullins & Brinley (1975) have obtained an enhancement of the Ca efflux when the ATP concentration inside dialysed axons is increased.

In this paper we are concerned with investigating the detailed properties of the Ca efflux from intact squid axons under conditions of high and low intracellular ATP. Preliminary reports referring to parts of this work have appeared earlier (Baker & Glitsch, 1973; Baker & Blaustein, 1974).

METHODS

Preparation. The hindmost stellar giant axons from *Loligo forbesi* and occasionally *Loligo vulgaris* were used throughout. Axons, 600–1000 μm in diameter, were mostly obtained from freshly killed animals, but some were from mantles that had been kept in refrigerated sea water for 2–4 hr. Axons required for all efflux experiments and some influx experiments were cleaned carefully to remove adhering small nerve fibres.

Measurement of Ca efflux. Axons were loaded with ^{45}Ca by micro-injection (Hodgkin & Keynes, 1956; Baker, Blaustein, Keynes, Manil, Shaw & Steinhardt, 1969*b*). About 10^6 c.p.m. ^{45}Ca were normally introduced axially over a length of 5 or 10 mm and at least 2 cm from either end of the axon. After injection, axons were left for 1–2 hr to allow the ^{45}Ca to become radially distributed within the segment injected. Injection increased $[Ca]_i$ by about 150–250 μM. Experiments with aequorin indicated that addition of this amount of Ca had little effect on the final intracellular ionized Ca. The axon was subsequently tested electrically and provided it could conduct a good diphasic action potential from end to end was transferred to a glass tube, internal diameter 3 mm, attached to a 1 cc disposable syringe. This allowed the solution bathing the axon to be changed easily and quickly whilst keeping its volume to a minimum (about 0·3 cc) to facilitate assaying the ^{45}Ca by conventional liquid scintillation counting. Samples were normally collected over 2 or 5 min periods. At the end of the experiment, the axon was digested in Nuclear Chicago Solvent and counted. All counts were corrected for quenching where necessary and the efflux expressed as that fraction of the total ^{45}Ca in the axon lost per minute. The Ca efflux can also be expressed as an absolute flux (p-mole/cm^2 sec). The absolute flux calculated on the

assumption that the Ca content of the axons averages 0·4 m-mole/kg, is shown in a number of the figures in this paper. Although there are a number of possible errors inherent in this transposition – for example the internal Ca after injection is probably higher than 0·4 m-mole/kg – expressing the flux in absolute terms both facilitates comparison with dialysed axons and simplifies comparisons between axons injected with various Ca-EGTA buffers containing different absolute amounts of Ca.

EGTA-injected axons. For some experiments it was necessary to buffer the intracellular concentration of ionized Ca. This was achieved by injecting mixtures of ethylene glycol *bis* (β-aminoethylether)-*N*,*N*′-tetra-acetic acid (EGTA) and Ca. According to Baker, Hodgkin & Ridgway (1971) injection of a mixture containing 40 mM-$CaCl_2$ and 100 mM-EGTA at pH 7·2 stabilizes an ionized Ca close to that which normally exists inside squid axons. Provided enough buffer is injected, it should be possible to buffer the ionized calcium in poisoned axons close to its value in unpoisoned axons and thus dissociate any effect of metabolism *per se* on Ca transport from its effects on intracellular Ca binding.

Two experimental regimes were adopted. (1) Unpoisoned axons were injected, usually along their whole length, with a mixture containing 220 mM-K-EGTA and 100 mM-$CaCl_2$, pH 7·2. They were subsequently injected with a short column of ^{45}Ca and the injector, loaded with the metabolite or energy source under investigation, reinserted such that when the time came to make the injection, the injected material would overlap the patch of ^{45}Ca by about 1 cm at each end. Axons prepared in this way invariably showed a fall in Ca efflux following exposure to cyanide, but after 1–3 hr in cyanide the flux often began to rise. The reason for this rise is unclear. An obvious possibility is the release of intracellular Ca, but this should not have been adequate to change the Ca content of the Ca-EGTA buffer to the extent suggested by the observed rise in efflux. The total intracellular Ca available for release is not greater than 0·5 m-mole/kg axoplasm and in a 900 μm axon this could raise the Ca content of the intracellular buffer from 2·9 mM-Ca: 6·3 mM-EGTA to 3·4 mM-Ca: 6·3 mM-EGTA which would cause a 1·5-fold increase in ionized Ca. A larger rise might be produced if Ca entered the poisoned axon; but the slow rise in efflux was also seen in Ca-free sea waters. Other possibilities include an increase in the permeability of the poisoned axon to Ca-EGTA (Brinley, Spangler & Mullins, 1975) and a change in internal pH. Ca-EGTA buffers are rather sensitive to pH and a progressive fall in pH_i would lead to a rise in Ca_i. In an attempt to avoid some of these problems, we sometimes adopted another approach. (2) Axons were prepoisoned with cyanide for 2–4 hr before carrying out the injection procedures described under (1) above.

All solutions for microinjection were buffered at pH 7·2 with Tris.

Measurement of Ca influx. Influx determinations were made in essentially the same way as previously described (Baker *et al.* 1969*a*). Where influx measurements were to be made on axons that had to be preinjected, for example with EGTA, the axons were cleaned along their whole length. Axons were also partially cleaned for experiments in which low concentrations of Ca were used.

External solutions. The main external solutions used are listed in Table 1. They were all modified artificial sea waters of the kind previously used. The only noteworthy difference from previous work is that it soon became apparent that the Ca efflux from unpoisoned axons is activated by micromolar amounts of Ca and in order to analyse this activation it was necessary to prepare external solutions of very low Ca content. For this reason 'spectroscopically pure' chemicals ('Specure', Johnson Matthey) were used whenever available. Otherwise chemicals were Analar grade. LiCl was obtained from Fisher and both choline and Tris from Sigma. Bicarbonate-free sea waters buffered with Tris were used in experiments involving La.

The Ca contents of these solutions were measured by atomic absorption spectroscopy with and without the addition of known amounts of Ca. The values obtained give the total Ca but provide no information on whether or not this Ca is ionized.

Analysis of efflux data. The efflux experiments usually lasted for many hours and over this period there were often slow changes – both up and down – in efflux into the same solution. In analysing the efflux data any drifts in flux were assumed to occur in a linear fashion.

TABLE 1. Composition of main external solutions. All concentrations are in mM. All solutions were pH 7·8. The contaminating Ca concentration in nominally zero-Ca solutions was somewhat variable, and is therefore noted where appropriate in the text

Reference name	Na	K	Li	Tris	Mg	Cl	HCO_3^-	Ca added
Na 10 Ca	402·5	10	—	—	100	630	2·5	10
Na 0 Ca	402·5*	10	—	—	100	610	2·5	0
K 0 Ca	2·5	410*	—	—	100	610	2·5	0
Li 0 Ca	2·5	10	400	—	100	610	2·5	0
Tris 0 Ca	2·5	10	—	400	100	610	2·5	0

* Denotes use of spectroscopically pure ('Specpure') chemicals.

RESULTS

General

The Ca efflux from intact squid axons can be divided operationally into three components: one that depends on Ca_o, one that depends on Na_o and a residual efflux that persists in the nominal absence of both Ca_o and Na_o. Three similar components can be recognized in fully poisoned axons, but their relative sizes are different. The Ca_o and Na_o-dependent components are usually about ten times larger, and the residual efflux somewhat smaller.

Dependence of the Ca efflux on external Ca

Several authors (Baker *et al.* 1967; Blaustein & Hodgkin, 1969; Dipolo, 1973, 1974) have noted that in the presence or absence of external Na, removal of external calcium reduces the Ca efflux from both unpoisoned and poisoned axons. The properties of the Ca_o-sensitive calcium efflux have been investigated in the experiments described below. This phenomenon has been referred to as a Ca–Ca exchange, but in the absence of conclusive evidence it is safer to adhere to the descriptive nomenclature 'Ca_o-activated Ca efflux' (see p. 120 and Discussion p. 136).

Unpoisoned axon

Experiments in the presence of Na. An investigation of the effects of external Ca on Ca efflux in the presence of Na is illustrated in Fig. 1*A*.

Removal of Ca as completely as possible from Na-ASW reduces the Ca efflux by about one third. Raising the Ca concentration by even 5 μM largely reactivates the efflux.

The high apparent affinity for Ca of the Ca_o-dependent Ca efflux raises the problem of Ca contamination in nominally Ca-free solutions. As noted in the Methods section, the Ca-free ASW made with Analar chemicals contained about 10 μM-Ca. When this sea water was made from Specpure NaCl the Ca concentration measured by atomic absorption spectroscopy was about 1 μM. For this reason Specpure chemicals were used whenever possible. The precise ionized Ca immediately external to the axo-

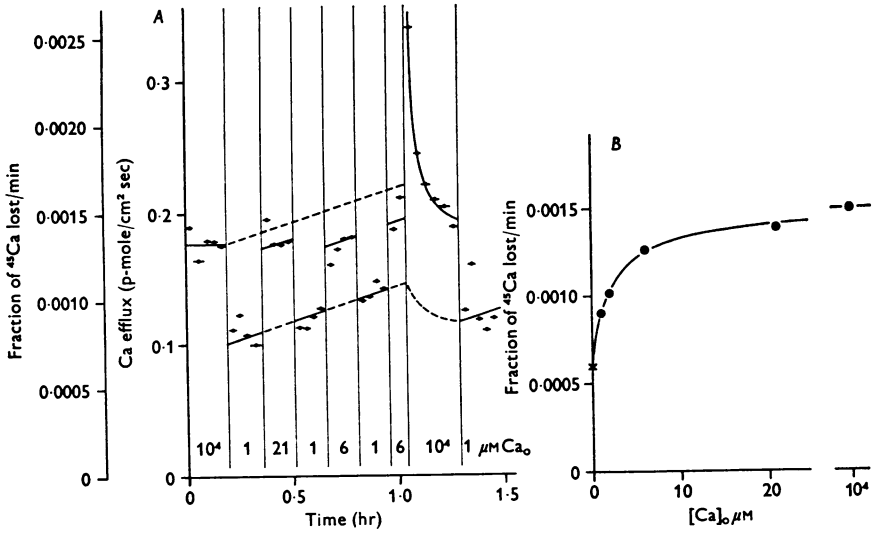

Fig. 1. The activating effect of external Ca on the Ca efflux in the presence of Na. Unpoisoned axon. Axon diameter 800 μm; temperature 20° C. *A*, the ^{45}Ca efflux as a function of time. The Ca_o concentration was varied as shown at the bottom of the graph. The Na-containing sea water was made from Specpure NaCl as described in Methods. Left-hand ordinate: fraction of ^{45}Ca lost/min (the quantity directly measured). Right-hand ordinate: absolute Ca efflux calculated from the left-hand ordinate by assuming a total internal Ca concentration of 0·4 mM and isotopic equilibrium. *B*, ^{45}Ca efflux (ordinate, same scale as in *A*) expressed as a function of the external Ca concentration. The points are taken from *A*. The sloping 'base line' efflux observed in *A* has been allowed for somewhat arbitrarily by taking the efflux in 1 μM-Ca_o to be equal to its average value during the course of the experiment (0·0009 min^{-1}). The effect of higher $[Ca]_o$ has been measured as distance above the sloping base line in 1 μM-Ca_o in *A*, and plotted here as an absolute value above 0·0009 min^{-1}. The points are well fitted (continuous line) by assuming Michaelis–Menten kinetics of binding of external Ca ions to activating sites with apparent affinity $K_m^{Ca_o} = 2$ μM and total Ca_o-sensitive efflux 0·000875 min^{-1}, and by assuming that the ^{45}Ca efflux would be deactivated to 0·0006 min^{-1} in the complete absence of external Ca.

lemma is difficult to assess: on the one hand it may be reduced because of leakage of phosphate and other Ca chelators from the axon, and on the other hand it may be raised as a result either of Ca being pumped out of the axon and Schwann cells or of Ca desorbing from non-specific binding sites that may act as short-term Ca buffers. Attempts to overcome the problem of Ca contamination by incorporating a few μmoles of EGTA in the Ca-free solutions surprisingly produced a transient enhancement of the apparent efflux, probably due to desorption of ^{45}Ca from extracellular binding sites in the presence of the strong chelator.

Two other features of Fig. 1*A* were commonly noted in similar experiments. First, the Ca efflux into nominally Ca-free Na ASW rose gradually during exposure to this solution but, after re-exposure to high Ca, dropped to its initial low level on return to nominally Ca-free Na ASW. Secondly, exposure to high Ca (in other experiments any concentration greater than about 50 μM) results in a transient elevation of the apparent ^{45}Ca efflux. Both these phenomena may be due to Ca adsorption to extracellular sites. Thus immediately on immersing the axon in nominally Ca-free Na ASW part of the ^{45}Ca leaving the axon may be adsorbed directly to extracellular sites, increasing their specific activity and reducing the apparent efflux. As these sites become more fully occupied by ^{45}Ca the apparent efflux rises slightly, presumably approaching the actual efflux, and on re-exposure to high Ca solution most of the adsorbed ^{45}Ca is displaced, generating the observed large transient. Additional support is lent to this hypothesis by a similar transient rise in apparent efflux observed on adding EGTA to a nominally Ca-free solution, and by the observation that a similar transient occurs in poisoned axons where the intracellular concentrations and mobility of Ca are much higher than in unpoisoned axons.

The Ca efflux has been plotted as a function of external Ca in Fig. 1*B*. The analysis of data such as that in Fig. 1*A* is complicated by the tendency for the efflux in low Ca solutions to drift up gradually with time (see Figs. 1, 2, 3). This has been allowed for as far as possible by measuring the activating effect from a base line interpolated between efflux values in a constant concentration of Ca (see legend of Fig. 1 for details).

The activating effect of Ca_o in Fig. 1*B* can be fitted into a Michaelis–Menten formalism by assuming one-to-one binding between Ca ions and activating sites, and by supposing that the Ca_o-dependent Ca efflux is proportional to the fractional occupation of activating sites. Points obtained from Fig. 1*A*, together with another at $[Ca]_o = 2$ μM obtained later in the same experiment, are well fitted by taking an apparent $K_m^{Ca_o}$ of 2 μM and by assuming that the efflux would be deactivated to 6×10^{-4} min^{-1} in a hypothetical Ca-free solution.

This calculation assumes that the Ca concentration in the periaxonal space is the same as that in the bulk solution. As discussed earlier, it seems likely that the Ca concentration immediately external to the axolemma could be up to 1 μM higher than in the bulk solution. The presence of any extra Ca will lead to underestimates both of the apparent affinity for Ca_o and of the over-all size of the Ca_o-activated component of Ca efflux.

Experiments in the absence of Na. The effect of Na on the Ca_o-activated Ca efflux is interesting from the point of view of possible competition

between Na and Ca for external activating sites. An unequivocal analysis of the effect of Ca_o on the Ca efflux in the absence of Na is, however, difficult for two reasons. The concentration of contaminating Ca in nominally Ca-free Na-free solution was always greater than in Ca-free Na-ASW because of the unavailability of suitable Specpure Na substitutes. Nominally Ca-free sea waters based on K, Tris and Li were found to contain total Ca concentrations of 2, 4 and 7 μM respectively, although the actual ionized Ca concentrations may be somewhat lower because of the possible presence of trace amounts of Ca chelators, such as phosphates. The second and more fundamental problem concerns the presumed reversal of Na–Ca exchange. In the absence of external Na raising the external Ca concentration produces a large influx of Ca in exchange for internal Na (Baker *et al.* 1967, 1969*a*; Baker, 1972). The changes in specific activity of intracellular ^{45}Ca consequent on this influx can make any meaningful interpretation of efflux data impossible. Fortunately the Ca concentration required for half-maximal activation of Na efflux in exchange for Ca influx from a Na-free solution is about 3 mM (Baker *et al.* 1969*a*), while that required for half-maximal activation of Ca_o-activated Ca efflux is very much lower (about 2 μM in Na-containing solution, as discussed above), and therefore, provided the external Ca is below about 50 μM, internal ^{45}Ca activity changes associated with the presumed reversal of Na:Ca exchange will be small and most of the Ca_o activation of Ca efflux can be observed. Changes in internal specific activity were checked by re-examining the efflux in nominally Ca-free Tris ASW after an exposure to Ca. If the efflux was not significantly below its value before exposure to Ca, specific activity changes were assumed to be negligible.

Fig. 2*A* shows the activating effects of several Ca concentrations in the presence of the Na substitutes Li and K (this Figure is a direct continuation of Fig. 1 and the points in Na + 1 μM-Ca are the same as those plotted at the end of Fig. 1*A*). The results are rather similar to those obtained in the presence of Na, with the exception that a constant Ca efflux due to Na activation is absent. Small concentrations of added Ca are effective in activating Ca efflux, much as in the presence of Na. The problems of contamination of nominally zero-Ca solutions by trace amounts of Ca are therefore similar to those in the presence of Na.

The activation of Ca efflux as a function of $[Ca]_o$ has been plotted in Fig. 2*B*. A sloping base line has been subtracted as in Fig. 1. The form of the activation in both K and Li are generally similar to those in the presence of Na (continuous line in the top part of the Figure, replotted from Fig. 1*B*).

A second experiment, in which higher concentrations of Ca_o were used, is illustrated in Fig. 3. In the presence of Tris small amounts of added Ca are again found to activate an appreciable Ca efflux. Application of about

50 μM-Ca results in an initial transient – probably due to desorption of ^{45}Ca from non-specific binding sites – but in the steady state the activation by 50 μM-Ca is not much greater than the activation by 8 μM-Ca, indicating a high affinity binding site for external Ca.

The situation is somewhat different in the presence of K. In the experiment of Fig. 3 changes in specific activity of internal ^{45}Ca are evidently occurring, as judged by the difference in steady levels in K + 2 μM-Ca

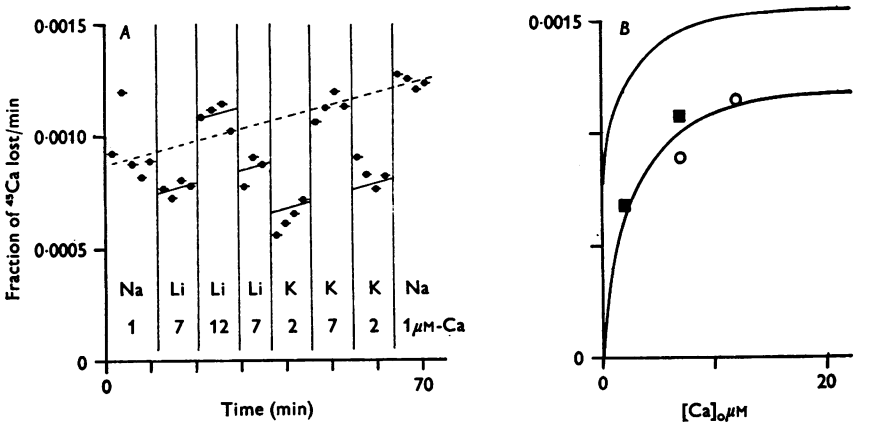

Fig. 2. The activating effect of external Ca on the ^{45}Ca efflux in the presence of Li and K. Unpoisoned axon. *A*, ^{45}Ca efflux as a function of time. Same experiment as Fig. 1. Zero-Na solutions made by substituting 400 mM-LiCl or KCl for NaCl (see Methods). Ca concentration varied as shown at the bottom of the graph. *B*, ^{45}Ca efflux as a function of $[Ca]_o$. Points are plotted as efflux activated or deactivated from the sloping base line efflux in Na + 1 μM-Ca, as in Fig. 1*B*. The average value of the base line over the range of this graph is 0·00108, slightly higher than in Fig. 1. Upper line: Ca-activated Ca efflux in the presence of Na (replotted from Fig. 1*B* about the slightly elevated base line). ○, efflux activated by Ca in Li-ASW; ■, efflux activated by Ca in K-ASW. Lower line: Michaelis binding curve drawn by taking $K_m^{Ca_o} = 2$ μM, total Ca-sensitive efflux 0·00129, and assuming no Ca efflux in the complete absence of external Ca and Na.

before and after the application of higher Ca concentrations. This activity change may be due to the enhanced permeability to Ca which occurs both transiently and, to a lesser extent, in the steady state in response to depolarization by high K (Hodgkin & Keynes, 1957; Baker, Meves & Ridgway, 1973). An attempt has been made in Fig. 3*A* to allow for the specific activity changes by extrapolating a base line between successive applications of K + 2 μM-Ca. While the precise values obtained using such a procedure are somewhat arbitrary, the form of activation of efflux by low concentrations of added Ca in the presence of K is not dissimilar from the activation occurring in the presence of Tris.

The activation of Ca efflux has been expressed as a function of Ca concentration in Fig. 3*B*. The activation in K (open squares) and Tris (filled squares) has been compared with the two points obtained in Na (filled circles), which have been fitted by a Michaelis activation curve with apparent $K_m^{Ca_o} = 2\ \mu M$.

A number of hypotheses are possible to explain the Ca_o-activation of Ca efflux in Figs. 2*B* and 3*B*, and it has unfortunately not been possible to take the Ca_o concentration to sufficiently low levels to decide between them. The simplest hypothesis is that the activation is first-order, as it is in the presence of Na. This idea has been used to draw the lower curves in Figs. 2*B* and 3*B*, with the additional assumptions that (1) the apparent

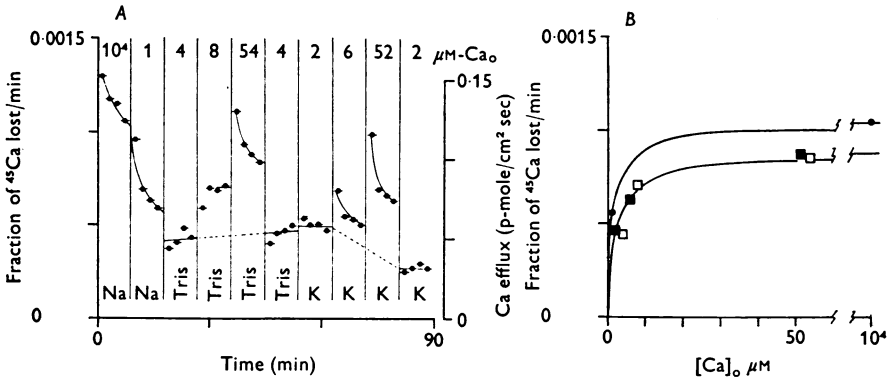

Fig. 3. The effect of Ca on the Ca efflux in the presence of Tris and K. Unpoisoned axon. Axon diameter 700 μm. Temp. 19° C. *A*, fraction of ^{45}Ca lost per min (left-hand ordinate) and Ca efflux (right-hand ordinate) as a function of time. *B*, Ca efflux as a function of $[Ca]_o$. Effluxes have been plotted relative to the slightly sloping base line in Tris + 4 μM-Ca_o shown in *A*. The two points obtained in the presence of Na (●) have been fitted by a first-order Michaelis curve with $K_m^{Ca_o} = 2\ \mu M$. The same Michaelis constant has been used for the lower curve, but the maximum Ca_o-sensitive efflux has been slightly increased and the curve has been assumed to pass through the origin. ■, efflux activated by Ca_o in Tris-ASW; □, efflux activated by Ca_o in K-ASW.

Michaelis constant, $K_m^{Ca_o}$, is 2 μM, as it is in the presence of Na, and (2) that the Ca_o-activated component accounts for *all* the Ca efflux in the absence of Na, the 'residual' efflux being that fraction of the Ca_o-activated Ca efflux that can be activated by the traces of Ca immediately external to the axolemma. A good fit to the points necessitates, on these assumptions, a slightly increased maximum value for the Ca_o-sensitive component in the absence of Na. This may reflect a small non-competitive inhibition of the Ca_o-sensitive Ca efflux by Na, or on the other hand may be due to

a slightly greater periaxonal Ca contamination (for the same added Ca_o concentration) in the presence of Na, due to the activation of Ca efflux by Na (see Discussion).

If the Ca efflux consists of only two components, one activated by Na_o and one activated by Ca_o, it follows that in the nominal absence of both external Na and Ca ions, the addition to the sea water of a Ca chelator such as EGTA should reduce the efflux to a very low level. This has never been observed. After an initial increase in efflux, probably due to desorption of extracellularly bound ^{45}Ca, the Ca efflux stabilizes at, or close to, its original level (see Discussion).

Other hypotheses can be constructed to fit the data of Figs. 2 and 3. They include:

(1) Michaelis activation of efflux in the absence of Na, but with lower affinity for Ca_o than the value of 2 μM observed in the presence of Na. A corollary to this suggestion is the necessity to postulate an additional residual efflux not affected by either external Na or Ca, since a Michaelis curve with $K_m^{Ca_o}$ greater than 2 μM cannot be made to pass through the origin in Figs. 2*B* and 3*B* and still provide a satisfactory fit to the experimental points. Dipolo (1974) has postulated such a residual efflux, and has proposed that it is abolished by the removal of Mg ions from the sea water. Replacement of both Ca and Mg by an isotonic solution of choline has, in our experiments, resulted in either a small fall, no change, or else a rise in Ca efflux. The choline was, however, always contaminated by small amounts of Ca.

(2) The points of Figs. 2*B* and 3*B* can be fitted by a number of sigmoidal curves. Again, assumption of second or higher order binding of Ca ions to activating sites introduces the necessity for postulating a residual efflux not abolished by the removal of either Ca or Na.

One observation is, however, independent of the particular hypothesis used to describe the results shown in Figs. 2 and 3. The apparent affinity for Ca in the absence of Na is clearly not greater than it is in the presence of Na. Indeed, the points of Figs. 2*B* and 3*B* indicate, if anything, a slightly reduced affinity in the absence of Na. This observation is surprising in that it does not favour the idea of a competition between external Na and Ca ions for a single site on a pump capable of operating either in a Ca:Ca or in a Na:Ca exchange mode. If there were such competition the apparent affinity for Ca in the presence of Na should be lower than it is in the absence of Na, due to competitive inhibition of Ca-sensitive efflux by the Na ions.

The *total* efflux is greater, of course, in the presence of Na because of the operation of a Na-sensitive component. This in itself is incompatible with competition between Na and Ca for activating sites, because the affinity of these sites for Ca is so high compared with that for Na (2 μM for Ca, as noted above, as opposed to about 60 mM for Na; see Baker & Glitsch, 1973; Dipolo, 1974; and this paper) that at normal concentrations of Ca all the sites should be occupied by Ca, leaving none available for activation by Na. Our results are more compatible with separate external binding sites for Na and Ca (see Discussion, p. 138). The data of Figs. 2

and 3 indicate that Ca-activated Ca efflux may be a little larger in the absence of Na than in its presence (implying non-competitive inhibition by Na) but the data are not really good enough to justify any firm conclusion, especially in view of uncertainties over the concentration of Ca in the periaxonal space.

The results of Figs. 2 and 3 are also interesting in that they indicate that K behaves like Li and Tris as a Na substitute in so far as its action on the Ca-sensitive Ca efflux is concerned. (The apparent difference between K and Li in Fig. 2 may be due to the ionized Ca concentration in the Li solution being lower than the total Ca concentration due to the presence of contaminating amounts of some Ca chelator.) The observation that a large depolarization (about 60 mV in K sea water) produces no significant change in either the form or in the over-all magnitude of the Ca-sensitive Ca efflux indicates that the presumed exchange is either electroneutral or is maintained at a constant rate by drawing to a variable extent on some other source of energy. Later evidence in this paper indicates an involvement of ATP, so it is difficult to conclude much about the electroneutrality or otherwise of the calcium fluxes which may underlie these observations.

Experiments with other divalent cations. The effectiveness of external Sr, Ba and Co at reactivating a Ca efflux into nominally Ca-free Na-ASW was examined in two experiments. Both Sr and Ba activated Ca efflux, generating a maximum rate similar to Ca, although the apparent affinity for Sr and Ba was much lower than that for Ca and the possibility cannot be excluded that the activation produced resulted from Ca contamination. Co inhibited Ca efflux to a variable extent.

Preliminary experiments with lanthanum suggest that this ion may effect preferential inhibition of Ca_o-activated Ca efflux. It is particularly interesting that in the presence of 10 μM-Ca_o, addition of 300 μM-$LaCl_3$ to the external medium has little effect on the size of the Na_o-activated component of Ca efflux, but almost completely inhibits both Ca_o-activated Ca efflux and the residual efflux that persists in the nominal absence of external Na and Ca.

Assuming that the activation by Sr and Ba is not due to Ca contamination, the apparent K_m for Sr was about 100 μM and for Ba about 10,000 μM. As the experiments were performed in the presence of 100 mM-Mg, and removal of Mg has little consistent effect on the Ca efflux, the apparent K_m for activation of Ca efflux by Mg_o must be $> 10^5$ μM. An alternative explanation for the apparent lack of effect of Mg_o on Ca efflux may be that removal of Mg increases the already very high affinity for external Ca. This possibility has not been excluded.

In a single experiment, manganous chloride was injected into an axon to give a final concentration of 1 mM. After injection the total Ca efflux was reduced to about 40 % of its value before injection. As Mn is strongly bound in axoplasm, the observation suggests that quite low concentrations of intracellular Mn can interfere with Ca extrusion.

Poisoned axon

When a squid axon is exposed to cyanide the concentration of ATP in the axoplasm falls to a low level (Caldwell, 1960; Baker & Shaw, 1965). This reduction in ATP causes Ca to be discharged from the mitochondria after a delay of 1 to 3 hr, and in consequence the Ca efflux rises approximately tenfold (Blaustein & Hodgkin, 1969).

Experiments in the presence of Na. In the poisoned axon, as in the unpoisoned axon, part of the Ca efflux is dependent on external Ca, and removal of external Ca reduces the efflux by between one third and one half. The properties of the Ca_o-activated Ca efflux are, however, very

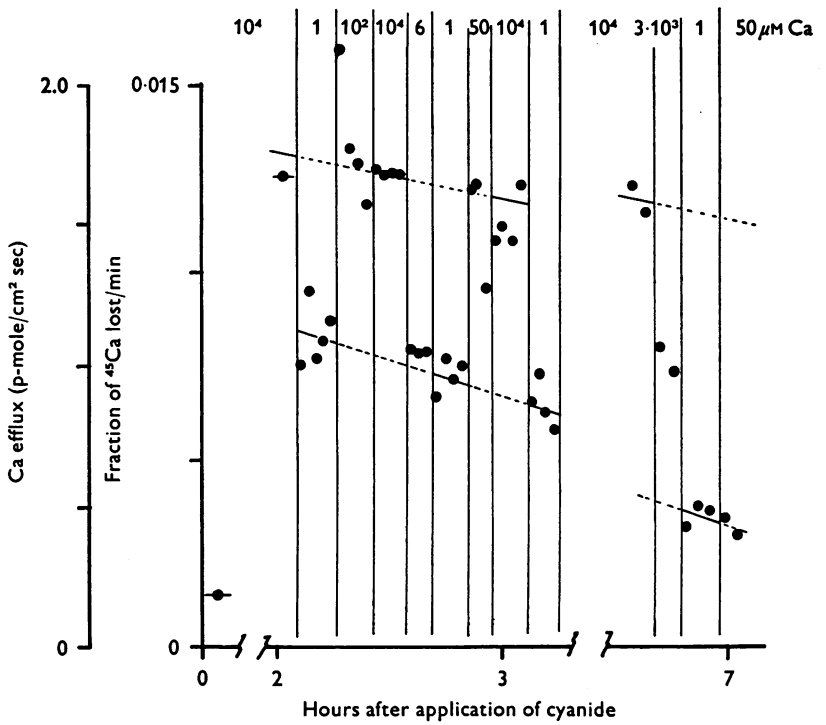

Fig. 4. The activation of Ca efflux by Ca_o in a cyanide-poisoned axon, in the presence of Na. From later in the experiment of Figs. 1 and 2. Right-hand ordinate: fraction of ^{45}Ca lost/min. Left-hand ordinate: Ca efflux in p-mole/cm² sec. Abscissa: time in hours after application of 2 mM cyanide. The rise in efflux which occurred between 1 and 2 hr after poisoning has been omitted for simplicity. The axon was maintained throughout in the Na sea water described in Methods, except that the Ca concentration was varied as noted at the top of each panel. Straight lines drawn through effluxes activated by successive applications of 10 mM and 1 μM-Ca have been used to construct Fig. 5.

different in the poisoned state. The high apparent affinity for Ca_o in the unpoisoned state changes progressively to much lower values with increasing time in cyanide.

Fig. 4 illustrates typical changes in Ca efflux on varying the Ca_o concentration in the presence of Na. This Figure is from the same experiment as used for Figs. 1 and 2. Poisoning the axon had increased the efflux in Na-ASW about tenfold in 2 hr. The efflux stayed roughly constant thereafter. Two to 3 hr after commencement of poisoning the apparent affinity

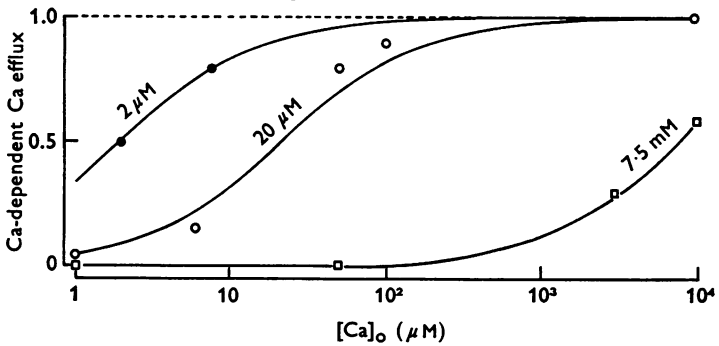

Fig. 5. The activation of Ca efflux under different metabolic conditions, expressed as a function of Ca_o concentration. Data from Fig. 4. Ordinate: Ca efflux expressed relative to the maximum efflux under each condition. Abscissa: $[Ca]_o$ on a logarithmic scale. ●, unpoisoned axon (absolute amplitude of Ca_o-sensitive efflux 0·117 p-mole/cm² sec); ○, between 2 and 3 hr after commencement of cyanide poisoning (absolute amplitude of Ca_o-sensitive efflux averaged 0·69 p-mole/cm² sec); □, between 6·5 and 7 hr after commencement of poisoning (absolute amplitude of Ca-sensitive Ca efflux calculated to be 1·85 p-mole/cm² sec). The continuous lines have been drawn on the assumption of Michaelis–Menten kinetics, with the apparent $K_m^{Ca_o}$ values shown on the graph. After 2–3 hr poisoning, the best fit to a first-order curve was obtained by assuming that the lowest Ca_o concentration (1 μM) activated a small fraction (0·05) of the total Ca_o-activated Ca efflux.

for Ca_o has evidently fallen below the value of about 2 μM obtained for the unpoisoned state. This is shown by the fact that application of 6 μM-Ca externally activates little efflux, but 50 μM and 100 μM activate it almost fully (the initial transients on applying both 100 μM and, later, 50 μM-Ca are probably due to desorption of ^{45}Ca from non-specific binding sites, as proposed in the previous section. Later points in 50 and 100 μM-Ca have therefore been taken as an estimate of efflux activated). Seven hours after poisoning 3 mM-Ca activates the efflux only partially, and 10 mM-Ca can no longer be assumed to have activated the efflux fully.

The values of Ca_o-activated Ca efflux obtained in Fig. 4 have been replotted as a function of Ca concentration in Fig. 5. The Ca_o-activation

curve in the unpoisoned axon (apparent $K_m^{Ca_o} = 2\ \mu M$) has been replotted from Fig. 1*B* for comparison with the points obtained after poisoning. Two to 3 hr after poisoning the efflux can be fitted by a Michaelis activation curve with an apparent $K_m^{Ca_o}$ of 20 μM (note that the best fit is obtained by assuming a small residual Ca_o-activated efflux in 1 μM-Ca). Seven hours after poisoning the points can again be fitted on the assumption of first-order kinetics by taking the apparent $K_m^{Ca_o}$ to be 7·5 mM, and assuming that the efflux in 10 mM-Ca was only 0·6 of that activated by a saturating concentration of Ca.

While the kinetics of activation have not been examined in sufficient detail to be identified unequivocally as first-order, the assumption of first-order binding with increasingly lower affinity at longer times in cyanide is at least consistent with the results. Another possibility might be that two populations of sites binding extracellular Ca are available during poisoning: one with high affinity for Ca_o, as in the unpoisoned state, and another lower affinity form that is produced by poisoning. The observations shown in Fig. 5 are not consistent with this hypothesis, since there is no need to use more than one affinity constant to describe the Ca_o-activated Ca efflux at short or at long times after poisoning. This is not the case in all experiments. In some (see, for example, Fig. 6*A*) poisoning seemed to result in two components of the Ca_o-dependent Ca efflux: a small component with relatively high affinity ($K_m^{Ca} \approx 50$–$100\ \mu M$) and a larger component of increasingly lower affinity ($K_m^{Ca_o} \approx 5$ mM) similar to that seen in Fig. 5.

The slow change in apparent affinity for external Ca is surprising, as the ATP concentration in the axon might be expected to be low when the efflux reaches its maximum after 3 hr poisoning (i.e. when the mitochondria have fully discharged their sequestered Ca in the absence of ATP). Either the Ca_o-activated Ca efflux is sensitive to very low levels of ATP (see Discussion), or else prolonged poisoning induces other changes in the axon – for example, the running down of the Na and K gradients across the membrane or a change in intracellular pH.

Experiments in the absence of external Na. In the unpoisoned axon various sodium substitutes (choline, Li, Tris and K) seemed all alike, so far as analysis was possible, in increasing the apparent maximum rate of the Ca_o-activated Ca efflux without affecting the affinity of the efflux mechanism for Ca_o. In the poisoned axon it is possible to investigate the characteristics of the Ca efflux as a function of Ca_o much more fully for three reasons: (1) the high affinity mode disappears, removing the problem of uncertainty about the precise Ca concentration adjacent to the axolemma at low measured Ca concentrations; (2) intracellular Ca binding is reduced, lessening the changes in specific activity that normally follow exposure

to high Ca concentrations, and (3) Ca influx from Na-free solutions is much reduced in poisoned axons. The results are very different from those already described for the unpoisoned state. Fig. 6*A* illustrates the Ca_o activation of Ca efflux observed between 4 and 7 hr after poisoning. When Li is used as a Na substitute the form of the efflux activation curve is similar to that obtained in Na, with the exception that at all Ca concentrations the efflux values in Na are elevated due to the Na-activated component of the Ca

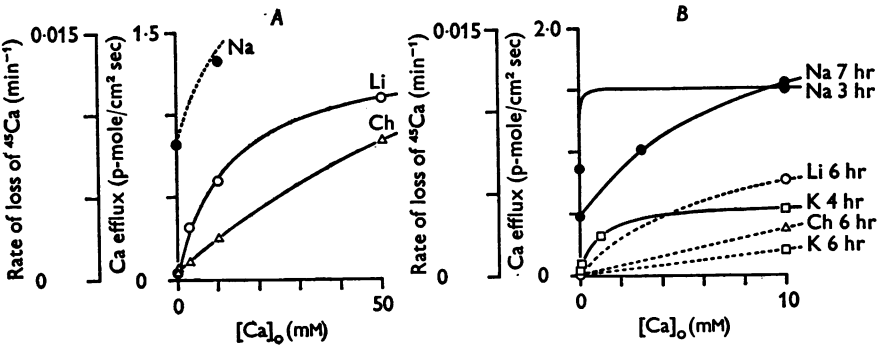

Fig. 6. The activation of Ca efflux by Ca_o in cyanide-poisoned axons in the presence of various monovalent sodium substitutes. Data from two experiments. *A*, Ca_o activation of efflux in the presence of Na, Li and choline. The points in Li and choline were obtained 5 hr, and those in Na 7 hr after commencement of poisoning. The curve through the points obtained in Li is a Michaelis function with apparent affinity K_m^{Cao} = 13·5 mM and maximum rate 0·0133 min^{-1}. The same curve has been displaced upwards and redrawn through the points in Na (interrupted line) to illustrate the similarity between Ca_o activation of efflux in Na and Li. The curve through the points obtained in choline has been drawn by eye. Axon diameter 600 μm. *B*, Ca_o activation of efflux in the presence of Na, Li, K and choline. The points were obtained at various times after the application of cyanide, as noted to the right of each set of points. From the same experiment as Figs. 1, 2, 4 and 5. Curves and points in Na redrawn from Fig. 5. The curve through the points obtained in K after 4 hr poisoning is a Michaelis function with K_m^{Cao} = 1 mM and maximum rate 0·55 p-mole/cm^2 sec.

efflux. The activation curve in Li is well fitted by assuming first order binding kinetics with an apparent Michaelis constant for Ca_o of 13·5 mM. In choline the data do not fit a Michaelis formalism, although the affinity for external Ca is clearly lower than in lithium.

A number of Na substitutes have been investigated for their effect on the Ca efflux activated by an external Ca concentration of 10 mM (Fig. 6*B*). Broadly speaking Na and Li activate the efflux to a similar extent; choline is similar to Tris, but both activate only one half to one third as much

efflux as do the alkali cations. Potassium seems to occupy an anomalous position, in that soon after poisoning it is as effective as Na and Li, but at longer times activates progressively less efflux.

At a constant Ca_o of 10 mM, progressive replacement of choline by Li increases the Ca_o-dependent Ca efflux (Fig. 7*A*). According to the kinetic analysis described above, the replacement of choline by Li increases the

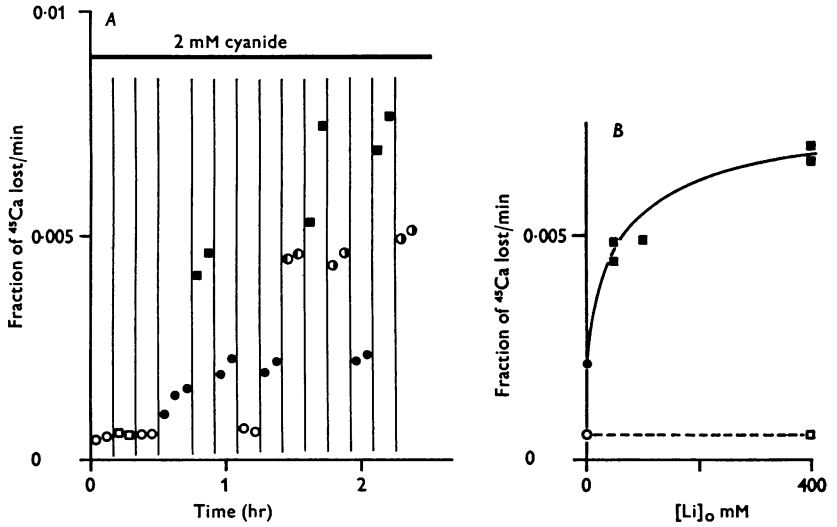

Fig. 7. Effect of replacing external choline by lithium on the Ca_o-activated Ca efflux from a fully cyanide-poisoned axon. Axon diameter 740 μm, temp. 19° C. *A*, experimental data. Axon exposed to cyanide for 2 hr, during which time the efflux increased eightfold before the zero time point. ○, nominally Ca-free choline; □, nominally Ca-free Li; ●, choline sea water containing 11 mM-Ca; ■, Li sea water containing 11 mM-Ca; ◐, mixture of choline and Li sea water containing 11 mM-Ca and 100 mM-Li; ◑, mixture of choline and Li sea water containing 11 mM-Ca and 50 mM-Li. *B*, data from *A* replotted as a function of the external Li concentration. Li was replaced isomotically by choline. Open symbols, nominally Ca-free; filled symbols; solutions containing 11 mM-Ca. Circles, choline; squares, Li. The curve through the filled points is a rectangular hyperbola with apparent K_m^{Li} of 50 mM.

affinity of the Ca_o-binding site for Ca and, provided V_{max} has not been reached, leads to an increase in Ca efflux. Fig. 7*B* shows that the site binding Li had an apparent affinity for Li of about 50 mM. These properties are strikingly similar to those of the Ca_o-dependent Na efflux first described by Baker *et al.* (1969*a*): both Ca influx and Ca_o-dependent Na efflux are activated more strongly by external Li ions than by choline, and the Li concentration required for half maximal activation of both fluxes is 50–70 mM.

Dependence of Ca influx on external Ca

A few measurements have been made of Ca influx into both unpoisoned and poisoned axons immersed in Na-ASW of different Ca content. The results are very striking and are summarized in Fig. 8. In unpoisoned axons Ca influx increases linearly from the lowest Ca_o concentration attainable (2 μM) to 100 mM and at 10 μM-Ca_o the absolute magnitude of the Ca influx

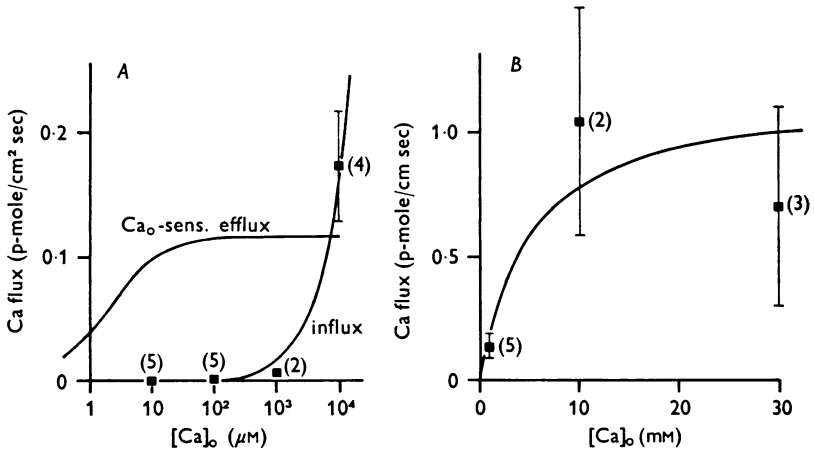

Fig. 8. Comparison of the dependence on external Ca of Ca influx and the Ca-dependent component of Ca efflux. All measurements were made in Na sea water. The number of axons used for each influx point (squares) is noted alongside the point. S.E. of mean indicated by vertical bars. Temp. 18–21° C. *A*, unpoisoned axons. Note logarithmic abscissa – the relation between Ca influx and $[Ca]_o$ does not in fact deviate significantly from linearity up to $[Ca]_o$ = 110 mM, the highest Ca_o examined. The absolute value of Ca influx at 100 μM was 0·0024 p-mole/cm² sec. The curve relating Ca_o-sensitive Ca efflux to $[Ca]_o$ is a Michaelis relation, redrawn from Fig. 1*B*. *B*, axons pre-poisoned for 3 hr in 2 mM-cyanide and exposed to cyanide sea water containing ^{45}Ca for a further hour. The dependence of Ca influx on $[Ca]_o$ is not significantly different from a typical relation between Ca-dependent Ca efflux and $[Ca]_o$ for an axon poisoned for this length of time (cf. Figs. 5, 6). The continuous curve intended to represent both Ca efflux and influx is a Michaelis function with K_m^{Cao} = 5 mM, and maximum value of Ca-dependent Ca efflux or Ca influx 1·15 p-mole/cm² sec.

(0·00048 p-mole/cm² sec) is less than 1 % of the Ca_o-activated Ca efflux. A discrepancy of this order is not seen in fully poisoned axons, where both Ca influx and Ca_o-activated Ca efflux are always roughly equal.

Dependence of the Ca efflux on external Na

The fall in Ca efflux when Na is removed from Ca-free ASW has been described by a number of authors (Baker *et al.* 1967; Blaustein & Hodgkin,

1969; Dipolo, 1973, 1974; Mullins & Brinley, 1975), and has been noted in several experiments in the previous section.

It seems likely that this effect involves an influx of Na ions in exchange for Ca ions. Indeed, reversal of a Na:Ca exchange pump is strongly suggested by the observations of Baker *et al.* (1967, 1969*a*) that in the absence of external Na, Ca influx is increased and it is possible to detect a Ca_o-dependent Na efflux. Both the extra Ca influx and a Ca_o-dependent component of the Na efflux are similarly affected under a variety of conditions. In the absence, however, of any clear demonstration of an actual influx of Na linked to the Na-dependent Ca efflux we shall avoid the prescriptive nomenclature 'Na:Ca exchange' in favour of 'Na_o-dependent Ca efflux'.

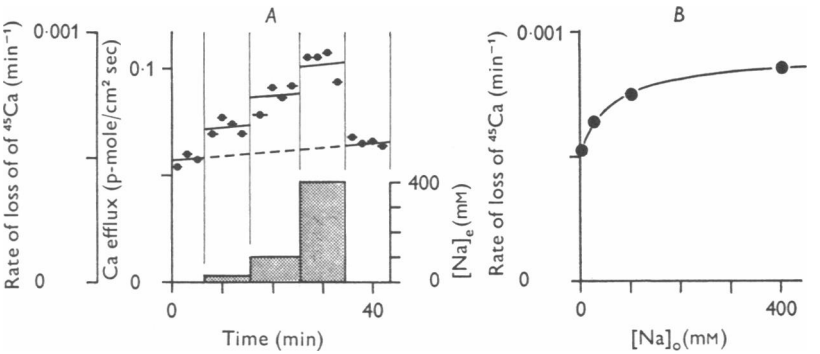

Fig. 9. The activation of Ca efflux by Na in an unpoisoned axon in the nominal absence of Ca. *A*, the effect of progressive replacement of Tris by Na, as indicated by the bars at the bottom of the graph. Axon diameter: 700 μm. Temp. 19° C. Ca contamination in Tris-ASW 4 μM, in Na-ASW 2 μM. *B*, Ca efflux as a function of Na concentration. The curve is a best-fit Michaelis function, with $K_m^{Na_o} = 61$ mM and maximum rate 0·0004 min^{-1}.

Unpoisoned axon

Baker & Glitsch (1973) have described an activation of Ca efflux by added external Na which is well approximated by assuming first-order equilibrium binding of Na ions to reactive sites, and consequent proportional activation of efflux. In their experiment Li was used as Na-substitute. In our experiments we have normally used Tris as Na substitute because Tris is less contaminated than Li by traces of Ca. The results obtained with Tris are similar to those obtained with Li (Fig. 9). A Michaelis activation curve with apparent affinity 61 mM is a good description of the activation of efflux when Tris is replaced by Na. It is important to bear in mind, though, that such an apparently simple result may be complicated by changes in the activation of Ca efflux by contaminating amounts of Ca_o.

The activation of Ca efflux by Na when K is used as a Na substitute is roughly similar to that obtained when Tris replaces Na. The most sensitive test for any difference between the effects of Na activation in a depolarized and resting axon is to compare on the same axon sea waters in which part of the Na has been replaced by either Tris or K. An experiment of this sort is illustrated in Fig. 10. The Ca efflux activated by 100 mM-Na + 300 mM Tris, when the axon should be at about its resting potential, is compared with that activated by 100 mM-Na + 300 mM-K, when the axon would have been depolarized to about −5 mV. In some experiments replacing 100 Na 300 Tris by 100 Na 300 K produced a reversible reduction in Na-activated Ca efflux of about 70 %. The absolute change in efflux was small and not enough experiments were performed to establish the statistical significance of the observation.

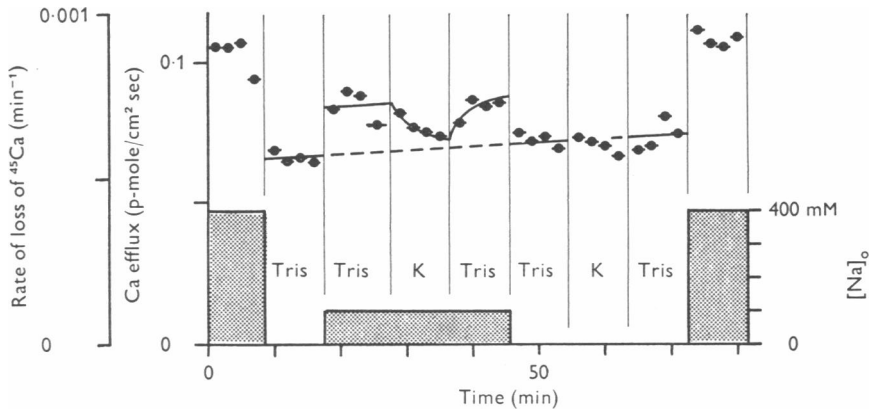

Fig. 10. The effect of K on the Na_o-activated Ca efflux in an unpoisoned axon in the nominal absence of Ca_o. The concentration of Na_o in the sea water is shown by the bars at the bottom of the graph. Tris or K were used to maintain isotonicity, as indicated above each bar. Same experiment as in Fig. 8.

Poisoned axon

The activation of Ca_o efflux by Na_o is markedly sigmoidal both in poisoned intact axons (Baker & Glitsch, 1973) and in axons dialysed in the absence of ATP (Dipolo, 1974). Fig. 11 shows a similar effect, in an axon which had been poisoned for 2 hr.

The most notable feature of the kinetics of activation of Ca efflux by external Na in poisoned axons is not that the shape of the Na_o-activation curve is sigmoidal but that it becomes progressively more sigmoidal throughout exposure to the metabolic inhibitor (up to 7 hr). In view of this progressive change we have not attempted to fit any form of theoretical

curve to the data in Fig. 11 (but see Discussion, p. 134). In those experiments where it was examined, the changes in activation kinetics seen in poisoned axons were always partially reversible on removing the cyanide.

The effect of depolarization on the Na_o activation of Ca efflux has been observed in the latter part of Fig. 11 by comparing the effects of K as a Na substitute with those of Tris. At a constant external Na_o of 200 mM, depolarization to about -15 mV in a 200 mM-K-ASW produces a marked depression of Ca efflux, an observation which is in accord with those of

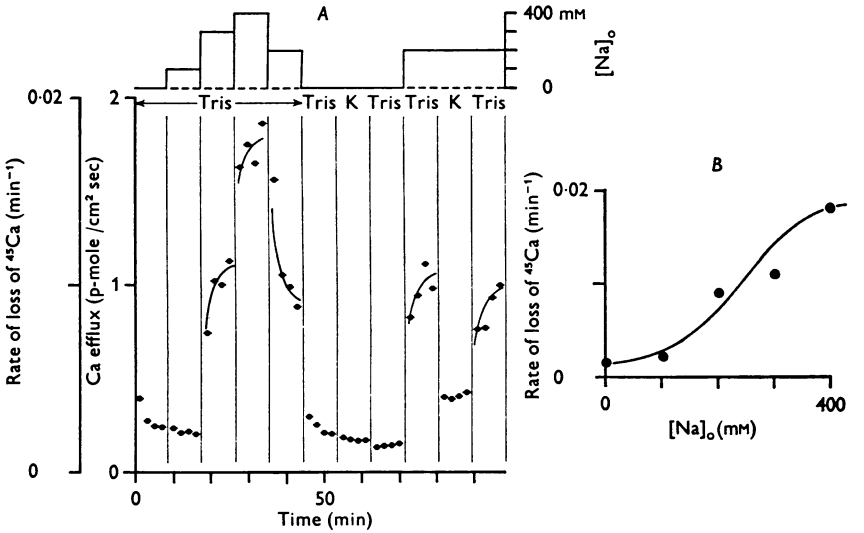

Fig. 11. Ca efflux from cyanide-poisoned axon. The activation of Ca efflux by Na_o in the nominal absence of Ca, and the effect of K on the Na-activated Ca efflux. *A*, Ca efflux as a function of time. The first half of the graph shows the effect on Ca efflux of replacing Na by Tris. The second half compares the effects on the Ca efflux of partial and complete replacement of Na by Tris and K. Na concentrations shown by the bars above the graph; Na substitutes indicated below the bars. Axon diameter 600 μm. Temp. 20° C. Poisoning (2 mM-cyanide) begun 2 hr before the time origin. *B*, the Na activation of Ca efflux as a function of external Na concentration. Continuous curve drawn by eye.

Blaustein *et al.* (1974) and of Mullins & Brinley (1975) in axons dialysed without ATP. Such an effect may indicate an electrogenic exchange of one Ca for three or more Na ions (Blaustein & Hodgkin, 1969; Baker, 1972; Blaustein, 1974) or simply reflect some sort of chemical interaction between external K ions and the external Na activation site. These possibilities can only be distinguished satisfactorily under conditions where the membrane potential can be altered at constant K_o.

The effects of ATP on the Ca efflux

Experiments at constant $[Ca^{2+}]_i$. In the previous two sections characteristics of the Ca efflux have been compared in unpoisoned and in poisoned axons. Before these changes in apparent kinetics can be linked unequivocally to the depletion of ATP in a poisoned axon it must be shown that other changes caused by poisoning – for instance the rise in intracellular ionized Ca (Blaustein & Hodgkin, 1969; Baker *et al.* 1971) are not responsible for the altered properties of the Ca efflux. The experiments described in this section attempt to circumvent the problem of changes in ionized Ca by observing the effects of cyanide poisoning and ATP injection on axons whose Ca concentration has been stabilized either by the use of EGTA buffers or by using oligomycin to prevent mitochondrial reaccumulation of Ca in the presence of ATP (Baker *et al.* 1971).

The use of EGTA is not without problems of interpretation. Injection of EGTA or Ca-EGTA buffers into unpoisoned intact axons immersed in Na-free Li sea water results in an immediate fall in the Ca_o-dependent Na efflux (Baker, 1970) and a parallel fall in the Ca influx (influx of 0·33 p-mole/cm^2 sec in EGTA-injected axon immersed in Li-ASW as compared to 1·9 p-mole/cm^2 sec in KCl-injected paired axon also in Li-ASW) – suggesting that the presence of EGTA inside the axon may be exerting an inhibitory effect on the exchange of internal Na for external Ca. This observation is not confined to axons immersed in Li-sea water as the Ca influx from Na-sea water into axons preinjected with EGTA is considerably less than into axons injected with KCl. This is a somewhat surprising observation and has yet to be explained satisfactorily. It nevertheless raises the question whether internal EGTA also affects the movement of Na and Ca in the opposite direction, i.e. Na_o-dependent Ca efflux. The data in Table 2 suggest that injection of a variety of Ca-EGTA buffers does not alter appreciably either the magnitude or properties of the Ca efflux. When allowance is made for the altered specific activity of Ca inside the axon, the mean Ca efflux (0·185 p-mole/cm^2 sec, see Table 2) is within the normal range and is reduced both by removal of external Ca and by removal of external Na.

An example of the effects of poisoning and subsequent injection of ATP in an EGTA-buffered axon is illustrated in Fig. 12. The Ca efflux was activated by small quantities of added Ca (24 and 50 μM) in the unpoisoned state, as described on p. 107). The activation of Ca efflux from the unpoisoned axon by external Na was not examined in the experiment illustrated in Fig. 12, but in other experiments closely resembled that for unpoisoned axons that had not been injected with EGTA. Poisoning produced a decline in efflux to a level about one third that in the unpoisoned state. The preparation was still sensitive to the removal of external Ca and

Na, but both components were only about one third their size in the unpoisoned state. The kinetics of activation of efflux were consistent with those described in the preceding sections for the poisoned state. Thus the Ca efflux was not activated by 135·5 mM-Na, while 402·5 mM-Na induced a significant activation; likewise in the presence of Na the Ca_o-dependent Ca efflux was activated significantly by 10 mM-Ca, but not by 0·05 mM-Ca or 0·5 mM-Ca. The kinetics of activation of the Ca efflux by Ca_o and Na_o are summarized in Fig. 12*B* and *C*, respectively.

TABLE 2. The effect of poisoning on the Ca efflux, with constant $[Ca]_i$

Experiment no.	Total added Ca / Total added EGTA	Ca efflux before poisoning (p-mole/cm² sec)	Minimum Ca efflux after poisoning (p-mole/cm² sec)	Poisoned efflux / Unpoisoned efflux
18. 10. 72	0·4	0·13	0·05	0·39
19. 10. 72	0·5	0·24	0·06	0·25
30. 11. 72	0·5	0·145	0·043	0·30
12. 10. 74	0·5	0·24	0·08	0·33
18. 10. 74	0·5	0·26	0·06	0·23
21. 10. 74	0·5	0·21	0·078	0·37
23. 10. 74	0·5	0·20	0·065	0·32
18. 11. 74	0·45	0·521	0·18	0·35
19. 11. 74	0·45	0·103	0·069	0·67
20. 11. 74	0·45	0·108	0·062	0·57
21. 11. 74	0·45	0·098	0·028	0·29
27. 11. 74	0·45	0·194	0·085	0·44
28. 11. 74	0·45	0·17	0·043	0·25
2. 12. 74	0·45	0·065	0·022	0·33
3. 12. 74	0·45	0·084	0·029	0·35
Mean ± S.E. of mean		0·185 ± 0·029	0·064 ± 0·010	0·36 ± 0·03

The efflux data were obtained from axons injected along most of their length with Ca-EGTA buffers (buffer ratio stated for each experiment) before injection with a short (~ 1 cm) patch of ^{45}Ca. Final axoplasmic EGTA concentration was about 9 mM in most experiments. The Ca efflux was tested for sensitivity to removal of external Na and Ca before poisoning. The efflux generally fell continuously for about 1·5 hr during poisoning with 2 mM cyanide and thereafter stayed constant or rose somewhat (cf. Fig. 11). The efflux data in column 4 refer to the minimum efflux reached during poisoning. Experiments 18. 11. 74 and 21. 11. 74 are illustrated in full in Figs. 12 and 13, respectively. Axon diameters range from 680 to 1030 μm, temp. from 19 to 21° C.

On removal of cyanide there was a rapid return of the efflux to the level before poisoning. Repoisoning rapidly reversed the efflux to the poisoned level, probably because the ATP-generating systems of the axon had not built up any reserves of ATP during the short recovery period (Baker *et al.* 1971). When the efflux had returned to the fully poisoned level ATP was injected to give a final concentration of 4·4 mM. The efflux rose quickly to

a level considerably above that in the unpoisoned state and then slowly declined, but at the end of the experiment was still sensitive to removal of Na and Ca.

Fig. 13 shows an investigation of the apparent kinetics of activation of Ca efflux by Na_o after an injection of ATP into a fully poisoned axon. A complete investigation is difficult because of the transient nature of the response to an injection, but a comparison of the efflux activated by 100 mM-Na with those activated in the absence of Na and in the presence of 400 mM-Na (Fig. 13*B*) indicates that shortly after injection the Ca efflux is activated by Na_o with higher apparent affinity than in the fully poisoned state (cf. Fig. 11).

Another feature of Fig. 13 worth noting is the change in residual efflux observed in the nominal absence of Ca and Na (the Ca-free choline sea water contained 11 μM-Ca and 2·5 mM $NaHCO_3$ buffer). We have suggested in the first section that this residual efflux may be caused by the activation of Ca efflux by contaminating amounts of Ca_o. This explanation is consistent with Fig. 13 when the change is apparent activation kinetics of Ca efflux by Ca_o are taken into account. Thus in the poisoned state the low apparent affinity for Ca_o would mean that the Ca contamina-

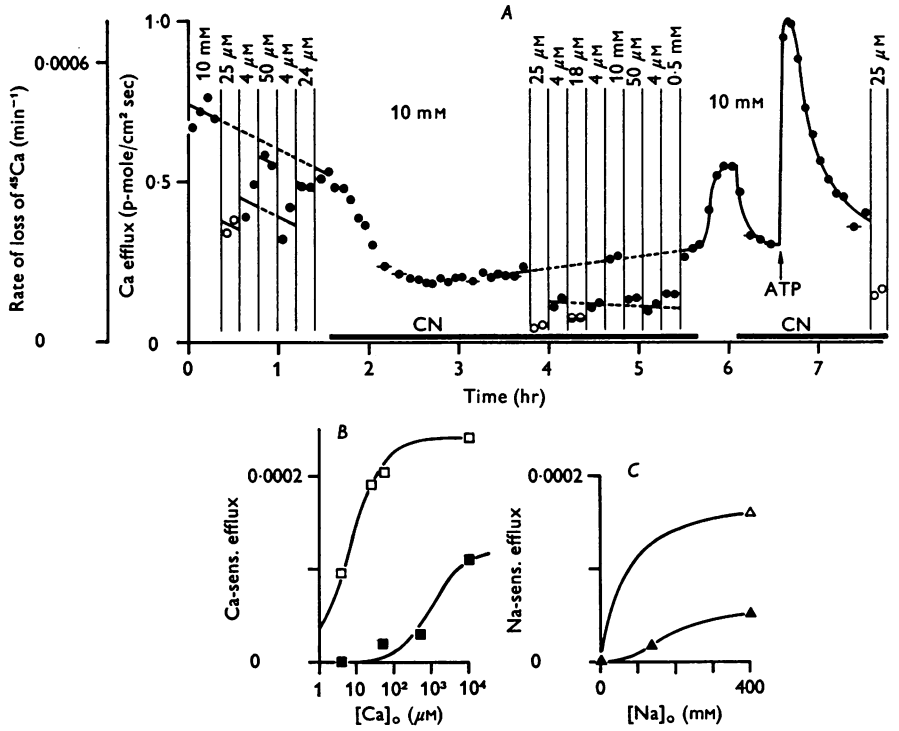

Fig. 12. For legend see facing page.

tion has little activating effect, while in the unpoisoned state, and in poisoned axons injected with ATP, the increase in apparent affinity for Ca_o considerably increases the Ca_o-sensitive Ca efflux – the residual efflux – activated by this contaminating level of Ca. A second feature also fits in with this explanation. After injection of ATP the 'residual efflux' declines from its initial high level much faster than does the efflux observed in full Ca_o and Na_o. This accords with a decline in the affinity for Ca_o, with a lesser change in the maximum value of Ca efflux activated by saturating concentrations of Ca_o.

The sensitivity of the Ca efflux to added ATP has been investigated by Dipolo (1974) in the dialysed axon, where a K_m for activation of Ca efflux by internal ATP of about 600 μM is observed. We have not attempted any systematic experiments of this nature in the intact axon, as accurate control of the ATP concentration over time is difficult. However, we have found that the injection of even quite small amounts of ATP produces a significant enhancement of Ca efflux. In one experiment injection of 1 mM-ATP, to give a final concentration of 60 μM, substantially enhanced the efflux (Table 3). Whether this apparently greater sensitivity of intact axons

---

Fig. 12. The effect on the Ca efflux of poisoning and subsequent ATP injection under conditions of constant internal [Ca]. The axon was pre-injected with a Ca-EGTA buffer (220 mM-EGTA: 100 mM-Ca) giving a final concentration of 9·6 mM-EGTA. Axon diameter 730 μm. Temp. 21·5° C. *A*, Ca efflux as a function of time. Various Ca concentrations were applied through the experiment, and are indicated above their time of application. Various Na concentrations are indicated by: filled circles (●), 402·5 mM-Na; open circles (○), 2·5 mM-Na + 400 mM-Li; half-filled circles (◒), 135·5 mM-Na + 267 mM-Li (note that Na + 4 μM-Ca and Li + 25 μM-Ca are both nominally zero-Ca solutions). Applications of cyanide are indicated by the horizontal black bars, and injection of ATP (final axoplasmic concentration 4·38 mM) by the vertical arrow. *B*, the activation of Ca efflux by external Ca (note logarithmic scale on abscissa). Open squares (□): Ca_o-dependent Ca efflux in the unpoisoned state. The total amplitude of the Ca_o-dependent efflux was deduced by assuming that the difference in efflux between applications of Li + 25 μM-Ca and Na + 24 μM-Ca represents the Na_o-dependent component, and that all the remainder is Ca_o-dependent. Allowance was made, as described in the legend to Fig. 1, for the sloping base line observed before poisoning. The continuous curve through the open squares is a best fit Michaelis function with Michaelis constant $K_m^{Ca_o} = 6{\cdot}6$ μM and maximum rate $2{\cdot}4 \times 10^{-4}$ min^{-1}. Filled squares (■): Ca_o-dependent Ca efflux measured between 3 and 4 hr after poisoning. Allowance has again been made for base line drift. The continuous curve is a Michaelis function with $K_m^{Ca_o} = 1$ mM and maximum rate $1{\cdot}2 \times 10^{-4}$ min^{-1}. *C*, the activation of Ca efflux by external Na. Open triangle (△) the amplitude of the Na-sensitive Ca efflux in the unpoisoned state, obtained as explained in *B*. The curve is a Michaelis function with $K_m^{Na_o} = 61$ mM, as obtained in Fig. 9. Filled triangles (▲) the Na-dependent Ca efflux measured 2–3 hr after poisoning. Continuous curve drawn through the points by eye to indicate sigmoidal activation kinetics after poisoning.

to internal ATP is due to a difference between dialysed and intact axons is not clear.

The experiments described so far have indicated that the change from Ca efflux kinetics of high apparent affinity to lower affinity is dependent on

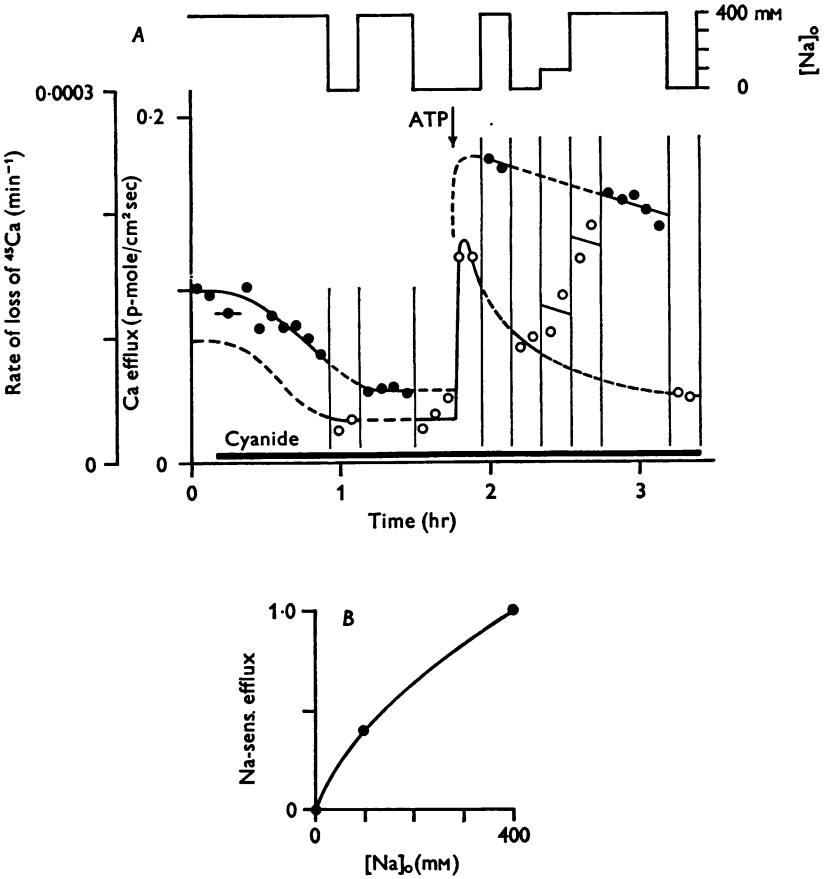

Fig. 13. The effect on the form of the Na_o-dependent Ca efflux of poisoning and subsequent injection of ATP at constant internal Ca. Axon pre-injected with a 220 mM-EGTA : 100 mM-Ca buffer to a final concentration of 9·4 mM-EGTA. Axon diameter 740 μm. Temp. 19·5° C. *A*, Ca efflux during poisoning by 2 mM cyanide (black bar) and subsequent ATP injection (arrow; final axoplasmic concentration 4·26 mM). Na concentration shown by continuous line above the graph (isotonicity maintained with choline). Ca concentration either 10 mM (filled circles, ●) or nominally zero (open circles, ○; Ca contamination in Na-ASW was 4 μM, in choline-ASW 11 μM). The interrupted curve through the points obtained in choline-ASW has been extrapolated to include data obtained before the origin of this graph. *B*, the activation of Ca efflux by Na, measured between 20 and 60 min after ATP injection.

the ATP level, or on something dependent on it, rather than on the intracellular ionized Ca concentration. A further way to investigate this point is to carry out the comparison between the kinetics, under conditions of normal and depleted ATP, when the efflux is maintained at a high level by a high concentration of intracellular Ca rather than being buffered to a low level as in the previous experiments. Fig. 14 illustrates such an experiment. The axon was pre-injected with oligomycin (500 μg/ml. in 10% alcohol to give a final concentration of about 12 μg/ml.) and oligomycin (5 μg/ml.) was added to all external solutions. The effect of oligomycin is to block ATP-dependent accumulation of Ca by mitochondria. The only other mechanism of accumulating Ca in the mitochondria requires electron transport, and if this is blocked by cyanide the mitochondria should release accumulated Ca. When ATP is injected into a cyanide-poisoned and oligomycin treated axon the ionized Ca concentration should remain at a stable high level, even in the absence of a Ca buffer, due to the inability of the mitochondria to reaccumulate the excess Ca.

In the unpoisoned state the Na_o-dependent Ca efflux was almost fully activated by 100 mM-Na. On poisoning the efflux rose rapidly, presumably due to the inability of mitochondria to use endogenous ATP for Ca accumulation in the presence of oligomycin. After 2 hr poisoning the Na_o-dependent Ca efflux exhibited typical poisoned characteristics (but note that the Ca_o-dependent efflux is still activated by 50 μM-Ca_o). Injection of ATP, to a final concentration of 2·9 mM, barely increased the efflux, but the apparent kinetics of activation by Na_o changed to those more typical of the unpoisoned state, with 100 mM added Na providing substantial activation of Ca efflux. Note that the 'residual efflux' observed in a nominally zero-Na, zero-Ca solution is also increased by the injection of ATP, as in Fig. 13.

The experiments in this section effectively rule out the possibility that the changes in apparent activation kinetics of Ca efflux observed in the poisoned state are due solely to the higher ionized Ca concentration in poisoned axons. Under conditions of approximately constant low and high levels of efflux it has been possible to show that at least the Na_o-activated moiety of the Ca efflux is affected primarily by the level of ATP. An additional effect of intracellular Ca cannot be ruled out in the present experiments, but there is little to support this possibility because at a constant ATP level there is no significant change in the form of the apparent activation kinetics of the Ca efflux over a tenfold efflux range, which probably corresponds to a much greater range of intracellular concentrations of Ca (Baker, 1972).

The absence of any observable effect of intracellular Ca concentration on the apparent kinetics of activation of Ca efflux at the extracellular face

of the membrane is important when considering possible candidates for the molecular mechanism of hypothetical Na:Ca and Ca:Ca exchange pumps. Models of the circulating-carrier type (Shaw, 1954; Caldwell, 1960) have been proposed for the Na_o-dependent Ca efflux (Blaustein, 1974; Jundt, Porzig, Reuter & Stucki, 1975; Mullins & Brinley, 1975). Unless very special assumptions are made such models have the property that

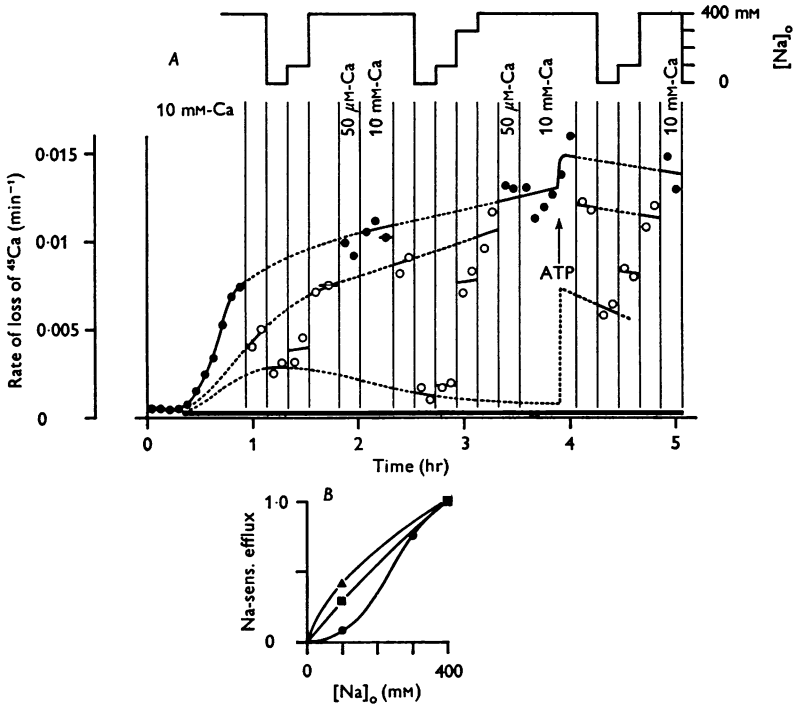

Fig. 14. The behaviour of the Na_o-dependent Ca efflux in cyanide-poisoned axons containing oligomycin. The axon was pre-injected with oligomycin 500 μg/ml. in 10% ethanol, and oligomycin 5 μg/ml. was present in all external solutions. Axon diameter 900 μm. Temp. 19° C. *A*, Ca efflux during poisoning by 2 mM cyanide (black bar) and on ATP injection (arrow; final concentration 2·88 mM). Na concentration shown by the bars above the graph. Isotonicity maintained by Tris. Nominally zero-Ca solutions are shown by open circles (○), and Ca-containing solutions by filled circles (●). The Ca concentration of Ca-containing solutions is noted where appropriate above each period of application; nominally zero-Ca solutions contained 2 μM-Ca (Na-ASW) and 4 μM-Ca (Tris-ASW). In the period preceding the time origin as drawn here, the Ca_o and Na_o dependence of the efflux were examined and found to be typical of an unpoisoned axon. *B*, the activation of Ca efflux by Na early in poisoning (■, obtained between 40 and 80 min after poisoning), later in poisoning (●, 2 hr 20 min to 3 hr 20 min after poisoning) and after injection of ATP (▲, between 10 and 60 min after ATP injection). Curves drawn through points by eye.

TABLE 3. The effect of injection of ATP and various analogues of ATP on the Ca efflux in poisoned axons with constant $[Ca]_i$

Experiment no.	Total added Ca / Total added EGTA	Ca efflux before injection (p-mole/cm² sec)	Injection (final axoplasmic concentration stated in mM)		Ca efflux after injection (p-mole/cm² sec)	Injected efflux / Poisoned efflux
Group A						
30. 10. 74	0·35	3·8	ATP	2·9	15·0	3·9
1. 11. 74	0·35	0·18	ATP	2·9	0·57	3·1
4. 11. 74	0·35	4·21	ATP	0·06	12·0	2·9
5. 11. 74	0·35	2·9	AMP	3·53	2·9	1·0
7. 11. 74	0·35	0·33	cAMP	1·54	0·33	1·0
6. 11. 74	0·35	0·34	KCl	6·8	0·34	1·0
Group B						
30. 11. 72	0·5	0·043	ATP	1·96	0·24	5·6
18. 11. 74	0·45	0·31	ATP	4·38	1·0	3·2
27. 11. 74	0·45	0·086	ATP	4·26	0·359	4·2
19. 11. 74	0·45	0·072	AMP-PCP	5·27	0·116	1·6
2. 12. 74	0·45	0·034	AMP-PCP	0·14	0·046	1·4
21. 11. 74	0·45	0·105	AMP-PNP	3	0·359	3·4
20. 11. 74	0·5	0·062	AMP-PNP	3·5	0·025	0·4

Axons in group A were pre-poisoned for 3–5 hr before injection with 10 mM-Ca, then with a Ca-EGTA buffer (final concentration of EGTA about 9 mM) and finally with a short ($\sim$ 1 cm) patch of ^{45}Ca. The efflux was tested for sensitivity for Na and Ca removal before injection. Axons in group B were injected in the unpoisoned state with a Ca-EGTA buffer (final concentration of EGTA about 9 mM) and a short ($\sim$ 1 cm) patch of ^{45}Ca, before poisoning for between 1·5 and 5 hr. During poisoning the efflux fell to the minimum values noted for the same experiments in Table 2, but often rose slightly thereafter to the values noted in column 3 and used as 'Poisoned efflux' for column 6 in this Table. Axon diameters 680–1030 μm, temp. 19–21° C.

the apparent activation kinetics at one face of the membrane should be affected by the concentrations of transported species at the opposite face (Baker & Stone, 1966). In the case of the Na:K exchange pump careful experiments (Hoffman & Tosteson, 1971; Garay & Garrahan, 1973; Garrahan & Garay, 1974) have failed to reveal any alteration in activation kinetics at one face under a variety of conditions at the other (see Glynn & Karlish, 1975). While our experiments are less definitive there is no clear evidence for an alteration of apparent kinetics of activation of Ca efflux by Ca_o or Na_o which is dependent on the intracellular Ca concentration. The present results, and earlier data on Ca_o-dependent Na efflux (Baker *et al.* 1969*a*), are more consistent with some kind of system in which transport requires the presence simultaneously of Na on one side of the membrane and Ca on the other.

The effects of ATP analogues on the Ca efflux

A number of ATP analogues were investigated for their effect on the Ca efflux. Injections of KCl (final concentration 7·1 mM), AMP (final concentration 3·53 mM) and 3′-5′ cyclic AMP (final concentration 1·54 mM) were without significant effect (Table 3). (In these experiments the simple protocol of prepoisoning the axons for 3–5 hr before injection of Ca-EGTA buffer and ^{45}Ca was used. This different protocol did not affect the action of ATP, which still produced a substantial enhancement of efflux.)

The actions of the non-metabolized ATP analogues β, γ-imido ATP (AMP-PNP) and β,γ-methylene ATP (AMP-PCP) are of interest in view of their ability to substitute for ATP in the K:K exchange mode of the Na pump (Simons, 1975). It is possible that the changes in the Ca efflux caused by the presence of ATP are independent of actual break-down of ATP, and that the non-metabolized analogues would therefore be capable of substituting for ATP. The results are very variable. In some instances in which high concentrations of analogue were used substantial enhancement of Ca efflux was seen but in others the analogue had little or no effect. It is possible that in those instances where the efflux was increased the analogue was either contaminated with ATP or by inhibiting ATP break-down in the cell caused a build-up of endogenous ATP. Experiments with dialysed axons are needed to provide a more definite answer.

DISCUSSION

The components of the Ca efflux

The experiments of Baker *et al.* (1967) showed that the Ca efflux from squid axons depends in part on the presence of external Ca ions and in

part on the presence of external Na ions. Blaustein & Hodgkin (1969) subsequently showed that these two components of the Ca_o efflux persist in cyanide-poisoned axons and they suggested that the energy for extruding Ca may be derived from the Na gradient and not directly from ATP. The more detailed analysis of Ca fluxes presented in this paper reveals a dramatic alteration in the kinetics of activation of both the Ca_o-dependent and Na_o-dependent components of the Ca efflux in fully poisoned axons. These observations are not incompatible with the Na gradient providing the energy for Ca extrusion, but they imply some form of interaction between both components of the Ca efflux and the metabolic state of the cell.

In an unpoisoned axon, removal of both external Na and Ca only reduces the Ca efflux to about one third of its initial value, whereas in a poisoned axon the residual efflux is a very much smaller percentage of the total efflux, and in absolute terms is usually smaller than the residual efflux from unpoisoned axons. The present experiments provide a simple explanation for the residual efflux in unpoisoned axons and its virtual disappearance in poisoned axons. The affinity for external Ca of Ca_o-activated Ca efflux is very high in unpoisoned axons (apparent $K_m^{Ca_o}$ about 2 μM), but much lower in poisoned axons (apparent $K_m^{Ca_o}$ about 5 mM) and the residual efflux in unpoisoned axons might simply reflect activation of Ca efflux by contaminating Ca. Even solutions made from spectroscopically pure chemicals contain about 2 μM-Ca and it is likely that the Ca concentration immediately external to the axolemma may be maintained a few μM higher by desorption and leakage of Ca from the preparation. If Ca diffuses through the Schwann cell layer at a similar rate to K, the data of Frankenhaeuser & Hodgkin (1956) suggest that a steady net Ca efflux of 0·1 p-mole/cm^2 sec would maintain a Ca concentration in the periaxonal space of at least 2 μM. Activation by these contaminating levels of Ca is unlikely in poisoned axons where the apparent affinity for external Ca is much lower.

The strongest piece of experimental evidence favouring a common origin for both Ca_o-dependent Ca efflux and the residual efflux in unpoisoned axons is the observation that low concentrations of La^{3+} ions block both components without inhibiting the Na_o-dependent Ca efflux. An observation that is difficult to reconcile with this hypothesis is that addition of a powerful Ca chelator such as EGTA to a nominally Ca-free and Na-free sea water fails to reduce the Ca efflux almost to zero. Apart from an initial transient increase in Ca efflux, external EGTA has very little effect. This topic is discussed again on p. 137.

Na_o-dependent Ca efflux

It is tempting to ascribe Na_o-activated Ca efflux to exchange of external Na for internal Ca, but no direct evidence for this has been obtained in unpoisoned axons, possibly because the expected Na influx is too small to detect in squid axons where the resting Na influx is rather large.

A particularly striking feature of this component of Ca efflux is the progressive change in the shape of the Na-activation curve during poisoning. In the unpoisoned axon it approximates a section of a rectangular hyperbola, but becomes markedly sigmoidal following poisoning.

An exchange of more than one Na_o for 1 Ca_i might be expected to lead to a sigmoidal relation between Na_o and Ca efflux such as is seen in poisoned axons. Assuming independent binding of Na ions and co-operation between n bound Na ions to effect the extrusion of 1 Ca ion, it is possible to calculate n from the shape of the Na-activation curve. But in view of the progressive changes in the shape of the Na-activation curve, values of n calculated in this way would be difficult to interpret.

It is not clear whether the sigmoidal curve seen in poisoned axons is also present in unpoisoned axons with the sigmoidal part restricted to very low Na concentrations or whether the actual form of the kinetics of activation change following poisoning. Two pieces of evidence support the first suggestion. In the presence of 0·3 mM-La, activation of Ca efflux by external Na becomes sigmoidal even in unpoisoned axons. In addition, both in poisoned and unpoisoned axons application of K-rich sea waters reversibly reduces the Na_o-dependent component of Ca efflux, an observation that is consistent with a similar stoichiometry of exchange in both conditions. It is of interest that in a number of other unpoisoned preparations including intact barnacle muscle fibres (Russell & Blaustein, 1974), synaptosomes (Blaustein & Oborn, 1975) and slices of adrenal medulla (Rink & Baker, 1975), the curve relating Na_o-activated Ca efflux to Na_o is sigmoidal. In these preparations it has not been examined whether this relation becomes more sigmoidal in poisoned cells.

On the assumption that the kinetics of Na_o-activated Ca efflux observed in unpoisoned and poisoned axons are manifestations of the same system, a model that seems compatible with the observed changes in Na_o-activation is one in which ATP, or possibly some derivative of it, must bind to the transport system *before* external Na can itself bind and activate Ca extrusion. This is illustrated in the reaction scheme below where the transport system is denoted M and the binding of internal Ca is ignored. Only one Na_o is shown binding although the experiments especially on poisoned axons suggest that more than one Na_o ion is required to activate Ca efflux. It makes no difference to the kinetic analysis whether the

activating Na is transported nor whether ATP is merely bound or subsequently phosphorylates the transport system. For simplicity only binding of ATP is shown:

$$M \underset{K_m^{ATP}}{\overset{ATP}{\rightleftharpoons}} M\cdot ATP \underset{K_m^{Na_o}}{\overset{Na_o}{\rightleftharpoons}} M\cdot ATP\cdot Na_o \longrightarrow \text{Ca efflux} + M + ATP + Na$$

Making the conventional assumption of equilibrium throughout except at the rate-limiting step we obtain:

$$\frac{Na_o\text{-dependent Ca efflux}}{\text{Maximum } Na_o\text{-dependent}} \text{Ca efflux} = \frac{1}{1+\frac{K_m^{Na_o}}{[Na]_o}\left(1+\frac{K_m^{ATP}}{[ATP]}\right)}$$

The following points emerge:

(1) The Ca efflux is activated by external Na in a Michaelis fashion.

(2) The apparent Michaelis constant for external Na is dependent on intracellular ATP, i.e. $K_m^{Na_o}{}_{(apparent)} = K_m^{Na_o}(1+K_m^{ATP}/[ATP])$. Thus as the ATP concentration declines, the apparent affinity for external Na declines progressively (i.e. $K_m^{Na_o}{}_{(apparent)}$ increases).

(3) The maximum efflux activated by saturating levels of Na_o remains unaltered even at vanishingly low levels of ATP.

These three predictions tally well with the observed progressive change in apparent affinity for Na_o of the Na_o-dependent Ca efflux during poisoning (Figs. 9 and 11) and with the lack of effect of ATP on the efflux when both internal and external binding sites might be expected to be saturated (Fig. 14).

The appearance of a sigmoidal activation curve in fully poisoned axons is not predicted by the model but could be seen if more than one Na_o ion is required for activation. If activation of Ca efflux requires the binding of ATP followed by the independent binding of n Na_o ions, the fractional activation of Ca efflux becomes

$$1\Big/\left[\left(1+\frac{K_m^{Na_o}}{[Na_o]}\right)^n+\frac{K_m^{ATP}}{[ATP]}\cdot\left(\frac{K_m^{Na_o}}{[Na_o]}\right)^n\right]$$

which predicts increasing sigmoidicity as ATP is reduced. A fully quantitative test of the model requires more knowledge of the number of Na_o binding sites and their respective infinities for Na_o. A formally similar dependence on ATP may be found in other Na_o-dependent transport systems that are sensitive to the metabolic state of the cell, for instance glutamate transport (Baker & Potashner, 1973).

This simple model does not include the binding of internal Ca, presumably essential for Ca efflux to occur, and therefore does not predict the increase in efflux when Ca_i is increased, for example by poisoning. More realistic schemes can be constructed retaining the sequential binding of ATP and Na_o, as above, and requiring in addition

that binding of Ca_i be either (i) consecutive with the binding of ATP, Ca binding either before or after ATP or (ii) independent of ATP binding. Both types of model explain satisfactorily the increase in Ca efflux when Ca_i is increased: but in the first (consecutive) class of model, the apparent K_m^{Nao} is dependent on both internal Ca and ATP whereas in the second (independent) class of model, K_m^{Nao} is independent of internal Ca. The available data (see pp. 129, 132) suggest that the apparent K_m^{Nao} is rather insensitive to quite large changes in Ca_i which favours models of the second type, with transport only occurring when the appropriate sites are occupied simultaneously by Na_o, Ca_i and ATP. If Ca_i binds in an independent fashion, the apparent K_m^{Cai} should not change in poisoned axons. The present data suggest that there may be a small, threefold reduction in affinity for Ca_i on poisoning (Table 2, and Figs. 12 and 15) but this requires confirmation under conditions of internal dialysis.

Ca_o-dependent Ca efflux

The simplest interpretation of this component of the Ca efflux is that it represents exchange of internal Ca for external Ca. At least in unpoisoned axons this seems not to be the case. At an external Ca concentration of 10 μM, a condition under which the electrochemical gradient for Ca is still inwardly directed, the Ca influx into unpoisoned axons averaged less than 1 % of the Ca efflux measured under comparable conditions. Both components were roughly equal at a Ca_o concentration close to that in squid blood. In poisoned axons Ca efflux and Ca influx were roughly equal at all Ca_o concentrations examined. These observations imply that the Ca_o-activated Ca efflux in unpoisoned axons is probably not an obligatory Ca–Ca exchange, but may reflect a net outward movement of Ca. As the external Ca is increased, Ca influx and Ca efflux become more nearly equal, but in the absence of specific inhibitors it is not possible to say whether under these conditions influx and efflux occur by the same or different systems. In fully poisoned axons the available data are consistent with the existence of Ca–Ca exchange, but definitive proof is again lacking.

The existence in unpoisoned axons of a Ca extrusion system that is activated by, but does not necessarily occur in exchange for, external Ca is particularly interesting and may be of considerable physiological significance. If Ca is not extruded in exchange for an external Ca ion, the immediate question is to determine whether it is exchanged with another extracellular cation. This seems very unlikely as the Ca-activated Ca efflux persists, apparently unchanged, in the absence of extracellular K, Na and Mg. The other obvious potential source of energy for Ca extrusion is intracellular ATP or some derivative of it, and the results described in this paper are consistent with such an involvement as seen both in the reduction in Ca efflux that occurs when an axon with Ca_i buffered to a constant level is poisoned, and in the dramatic and progressive alteration in the kinetics of activation of Ca efflux by Ca_o during poisoning. Under all conditions the curve relating Ca efflux to external Ca approximates to a section of a

rectangular hyperbola although the apparent $K_m^{Ca_o}$ changes from about 2 μM in unpoisoned axons to greater than 5 mM in fully poisoned axons. The Ca efflux from axons dialysed without ATP resembles that of fully poisoned axons (Baker & Blaustein, 1974; Blaustein, Russell & de Weer, 1974).

The progressive reduction in the apparent affinity for external Ca of the Ca_o-dependent Ca efflux can be explained in terms of a requirement for ATP, or some derivative of it, by an argument analogous to that used for the Na-dependent Ca efflux.

Making the same assumptions as for the Na_o-dependent Ca efflux, the predicted changes in $K_{m\,(apparent)}^{Ca_o}$ are quantitatively satisfactory. Thus, if $K_{m\,(apparent)}^{Ca_o} = K_m^{Ca_o}(1 + K_m^{ATP}/[ATP])$, taking the intracellular ATP as 3 mM (Caldwell, 1960; Baker & Shaw, 1965) and assuming K_m^{ATP} to be 3 mM, lowering ATP from 3 mM to 3 μM will alter tbe apparent $K_m^{Ca_o}$ by a factor of 500. The observed change in apparent $K_m^{Ca_o}$ depends on the duration of exposure to cyanide, but is usually of this order or a little larger.

However, application of this model would only be justified if the Ca_o-dependent Ca efflux from both unpoisoned and poisoned axons involves the same system. The measurements of Ca influx suggest that they may not. The nature of the Ca_o-activated Ca efflux from unpoisoned axons is clearly crucial and the observation that, in the nominal absence of both external Ca and Na, inclusion of EGTA in the external medium fails to reduce the Ca efflux seems particularly significant. Two possible explanations are (1) that the apparent activation by external Ca may reflect uptake and loss of ^{45}Ca from Ca-binding sites lying between the external solution and a Ca_o-independent Ca pump in the axolemma and (2) that EGTA may react in some way with the Ca efflux mechanism, uncoupling Ca extrusion from its external activation site. A clear distinction between these and other explanations has still to be made.

In line with the first possibility is the observation that the exterior of a cleaned squid axon binds appreciable amounts of Ca, although the properties of this extracellular binding do not parallel those of the Ca_o-dependent Ca efflux. The saturable component of binding has an apparent K_m for external Ca of about 0·1 μM and this is unaffected by prolonged exposure to cyanide (P. F. Baker & P. A. McNaughton, unpublished observations). The only observation pertinent to the second suggestion is that intracellular EGTA inhibits Ca_o-dependent Na efflux and the associated Ca influx suggesting that EGTA may in some way – perhaps through removal of a heavy metal – react directly with the Ca transport mechanism.

An attractive hypothesis is that in unpoisoned axons the Ca efflux that persists in the nominal absence of external Na may reflect an uncoupled extrusion of Ca into a Ca-binding matrix. On poisoning, this uncoupled Ca efflux disappears and is replaced by a conventional Ca-Ca exchange. The origin of the Ca-Ca exchange seen in poisoned axons and its precise relation to uncoupled Ca extrusion is not clear.

Separate systems or different modes of operation of the same system

The present data provide some new information on the interdependence of the two major components of the Ca efflux and their relation to the Ca_o-dependent Na efflux. A number of the more salient features are summarized in Fig. 15. If Ca_o-activated and Na_o-activated Ca effluxes represent two modes of operation of the same transport system, the affinity of Ca_o-activated Ca efflux for Ca_o might be expected to depend on the external Na concentration and a reciprocal relation should exist between Ca_o and the affinity for external Na of the Na_o-activated Ca efflux. The Ca_o-dependent Na efflux exhibits a relationship of this kind (Baker *et al.* 1969). As discussed on p. 110 there are experimental problems in measuring Ca efflux into Ca-rich, Na-poor solutions; but within these limitations we were unable to find any evidence for competition between external Na and Ca for activation of Ca_o-activated Ca efflux. In unpoisoned axons replacing Na_o by Li, Tris, choline or K all left the apparent affinity for external Ca unchanged at about 2 μM, and in poisoned axons, although the apparent $K_m^{Ca_o}$ in Na was very much larger (about 5 mM), it was unchanged following replacement of Na by Li, and increased when Tris or choline were used as Na substitutes. As mentioned on p. 113, if Na_o-activated Ca efflux depends on competition between external Na and Ca for an external activation site with apparent Michaelis constants for Na and Ca of 60 mM and 2 μM respectively, in the presence of the Ca concentration that exists in squid blood (4–8 mM; Blaustein, 1974) the external site will be permanently saturated with Ca and virtually no Na_o-activated flux will be possible.

These observations provide no evidence for competition between Na_o and Ca_o and as such are not obviously compatible with the widely held view that the Ca_o-activated Ca efflux and Na_o-activated Ca efflux represent two modes of operation of the same Ca efflux system, the relative amounts of Ca_o-activated and Na_o-activated Ca efflux being determined by competition between Ca_o and Na_o for the same external binding site (see for instance Blaustein, 1974). Some of these objections might be overcome if there are two quite separate external binding sites, one for Na and one for Ca, both of which can activate a common Ca efflux mechanism.

In favour of a common Ca efflux system is the observation that both Na_o-activated and Ca_o-activated components of the Ca efflux are altered in a roughly parallel fashion by the metabolic poison cyanide. When Ca_i is maintained at a constant low value by injection of an EGTA-Ca buffer, cyanide poisoning reduces both components of the Ca efflux to a similar extent and in the absence of internal chelators, poisoning increases both components of the Ca efflux by about the same amount. Despite these similarities, detailed inspection of the kinetics during onset of cyanide

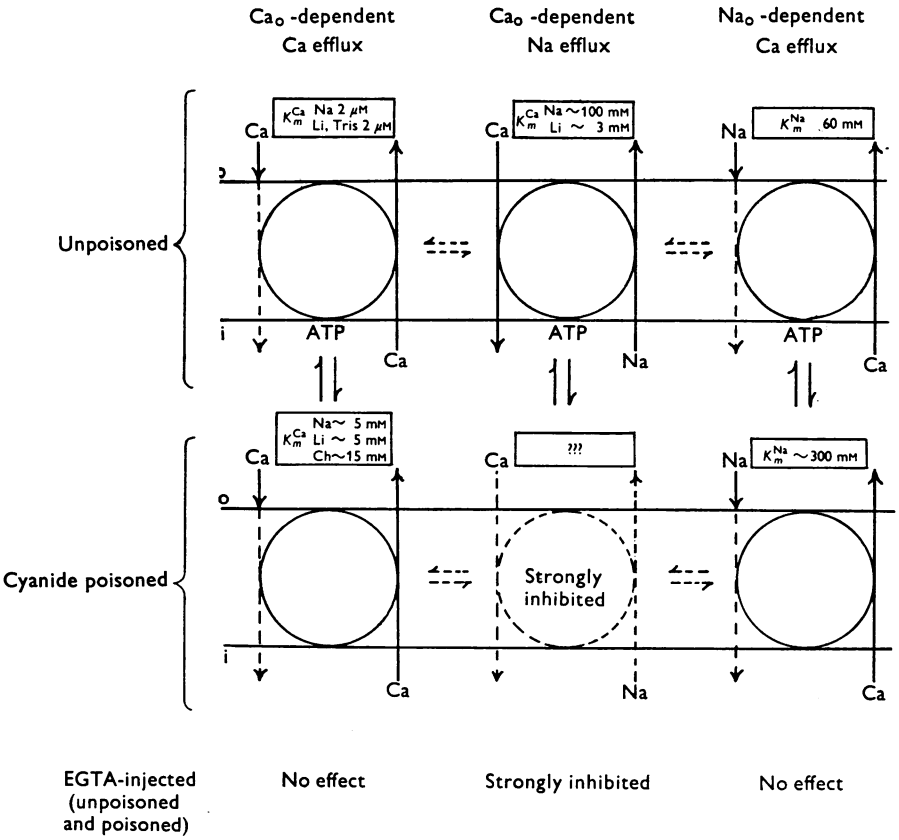

Fig. 15. Summary of Ca fluxes observed in intact axons of *Loligo* under different experimental conditions, and their possible interrelationships. The inner and outer faces of the axolemma are denoted i and o respectively. Interrupted lines indicate fluxes that have not yet been observed. Upper row: unpoisoned intact axon; middle row: axon fully poisoned with 2 mM cyanide and lower row, effect of internal injection of Ca-EGTA buffers stabilizing an ionized Ca close to that usually found in unpoisoned axons. Available information on apparent affinities at the outer face of the membrane is given for each component of Ca flux. We have no direct information for an intact axon on the apparent affinity at the internal face although it is possible to make reasoned guesses. The data of Baker *et al.* (1969) suggest that the apparent K_m for Na_i for Ca_o-dependent Na efflux may be in the region of 100 mM. Data from dialysed axons suggest that the apparent K_m for Ca_i in the absence of ATP (i.e. equivalent to a poisoned axon) is in the range 0·5–10 μM (Dipolo, 1973; Blaustein, 1974; Brinley, Spangler & Mullins, 1975), but, as yet, there are no comparable data in the presence of ATP.

poisoning occasionally reveals large changes in Na_o-activation kinetics at a time when the activation by Ca_o of Ca-activated Ca efflux shows relatively little change (see, for example, Fig. 13).

Inclusion of Ca_o-activated Ca efflux and Ca_o-activated Na efflux in a common reaction mechanism poses even greater problems. Examination of the apparent affinity for external Ca of Ca_o-activated Ca efflux and Ca_o-activated Na efflux (Fig. 15) reveals differences so large that they seem to preclude both systems sharing the same external Ca-activation site. Nevertheless, there is one striking similarity between these two systems, especially between the Ca-activated Ca efflux from poisoned axons and the Ca_o-dependent Na efflux from unpoisoned axons. Both are activated by external monovalent cations, Li being more potent than choline, and in both the activation by Li is half maximal at about 50 mM.

It is also still unclear whether Na_o-activated Ca efflux and Ca_o-activated Na efflux represent the same system operating in different directions (Baker *et al.* 1969*a*). The present data provide no conclusive evidence, but two observations are particularly pertinent.

(1) In poisoned axons Ca_o-dependent Na efflux is completely inhibited whereas Na_o-activated Ca efflux persists. This observation does not necessarily imply two separate systems as poisoning markedly reduces the affinity for external Na of Na_o-activated Ca efflux and if a similar change occurred at the internal Na-binding site or external Ca-binding site, Ca_o-dependent Na efflux might be inhibited in the observed fashion.

(2) Internal EGTA or mixtures of Ca and EGTA buffering the ionized Ca in the physiological range inhibit Ca_o-dependent Na efflux and the associated Ca influx, but have no apparent effect on Na_o-activated Ca efflux and Ca_o-activated Ca efflux. This observation is difficult to rationalize on the basis of a single system. The only explanation that seems at all viable is to postulate that EGTA, either directly or by removing a heavy metal, stabilizes the transport system in the Ca-efflux mode and does not allow Ca_o-dependent Na efflux to take place. Further examination of the effects of internal EGTA and other chelating agents is clearly desirable.

In conclusion, there is as yet no clear evidence to prove that any of the three forms of Ca transport shown in Fig. 14 represent different modes of operation of the same system. Until such evidence is forthcoming, it is perhaps simplest to assume that they represent three distinct systems; although it is possible to construct a model that can accommodate within a single system most of the known properties of these three forms of Ca transport. A clear answer will probably have to await the discovery of specific inhibitors of Ca fluxes.

Transport energetics

The role of ATP. Irrespective of the exact interrelationships of the three forms of Ca transport in squid axons, all three are affected by the metabolic state of the cell (Fig. 15). In axons injected with EGTA-Ca buffers to maintain an internal Ca concentration close to physiological, subsequent exposure to cyanide reduces to about one third both Ca_o and Na_o-activated components of the Ca efflux. Part of the residual efflux under these conditions may reflect loss of Ca-EGTA (Brinley, Spangler & Mullins, 1975). Injection of ATP, but not AMP or cyclic AMP, restores the efflux often to a value transiently higher than that existing before application of cyanide. Examination of the kinetics of activation by Ca_o and Na_o reveals a reduction in the apparent affinity for Ca_o and Na_o in poisoned axons, and these changes are qualitatively consistent with a model in which ATP or some derivative of it is an essential requirement for Ca efflux.

ATP, or some derivative of it, may participate in Ca transport in at least three ways.

(1) It may be consumed in stoichiometric fashion each time a Ca ion is transported across the axolemma.

(2) It may be an essential co-factor for transport to occur, although not itself being hydrolysed each time a Ca ion is transported.

(3) It may facilitate transport without in any way being essential for transport to occur.

A final decision between these three possibilities is very difficult and unlikely to be made solely on the basis of experiments on intact axons. It might be assumed that a strong argument against the first two possibilities is that the Ca efflux persists in axons that have been either fully poisoned with cyanide (Blaustein & Hodgkin, 1969) or dialysed for appreciable periods with ATP-free solutions (Dipolo, 1973, 1974; Blaustein *et al.* 1974); but even in these preparations there is always a residual ATP concentration of a few micromolar which, according to the model proposed in this paper, would be adequate to maintain transport.

One way of distinguishing between the first two possibilities is to examine the ability of non-metabolizable ATP analogues to reactivate the Ca efflux from cyanide-poisoned axons. Reactivation by these analogues would suggest that ATP hydrolysis is not essential. The experiments summarized in Table 3 show that AMP-PCP and AMP-PNP, neither of which are thought to be metabolized by squid axons, are both rather inconsistent in their effects on reactivating Ca efflux from fully-poisoned axons. As discussed on p. 132, there is a possibility that at least part of this reactivation may be due either to ATP contamination of the analogues or build-up of endogenous ATP in the presence of the analogues. The dependence on

ATP is an important point that should be amenable to more critical analysis in dialysed axons. If ATP is essential it will be difficult to prove in an intact axon whether the various components of Ca transport involve hydrolysis of ATP in a stoichiometric fashion. The measurements of Ca influx described in this paper make it very likely that in unpoisoned axons immersed in Na-free media net extrusion of Ca can occur against an electrochemical gradient. Under these conditions it seems very likely that the extrusion of Ca may involve hydrolysis of ATP. A more direct approach is to look for Ca-activated ATPases in the axon membrane.

In the case of the Na_o-dependent Ca efflux none of the three possible modes of dependence on ATP can be ruled out. The inwardly directed Na gradient provides an additional source of energy and there is evidence in poisoned axons that Na influx may be linked to Ca efflux (Blaustein & Russell, 1975). In order to maintain the concentration of ionized Ca found in intact axons, an exchange of three or, more probably, four Na ions for each Ca ion extruded would be required. In the absence of other ion movements, such an exchange would be electrogenic. Depolarization of axons by K-rich solutions reduces the Ca efflux in a reversible manner which is fully consistent with an electrogenic extrusion of Ca. Similar results have been obtained in dialysed axons by Blaustein *et al.* (1974) and Mullins & Brinley (1975). Nevertheless, despite the clear evidence for a dependence on, and possible exchange with external Na_o, other properties of the Na_o-dependent Ca efflux are consistent with a dependence on ATP or some derivative of it. Further studies are needed to determine whether this ATP is hydrolysed during Na_o-dependent Ca efflux and, if so, whether Ca_o-dependent Na efflux involves reversal of this reaction and resynthesis of ATP.

We wish to thank the Director and staff of the Laboratory of the Marine Biological Association, Plymouth, for providing facilities and an excellent supply of material. P.F.B. was in receipt of a grant from the Medical Research Council, and P.A.McN. of a Research Fellowship of Clare College, Cambridge, and a grant from the Dale Fund of the Physiological Society.

REFERENCES

BAKER, P. F. (1970). Sodium-calcium exchange across the nerve cell membrane. In *Calcium and Cellular Function*, ed. CUTHBERT, A. W. London: Macmillan.

BAKER, P. F. (1972). Transport and metabolism of calcium ions in nerve. *Prog. Biophys. molec. Biol.* **24**, 177–223.

BAKER, P. F. & BLAUSTEIN, M. P. (1974). Apparatus for the internal dialysis of giant axons of *Loligo forbesi*: a comparison of calcium efflux from intact and dialysed axons. *J. Physiol.* **242**, 52–54*P*.

BAKER, P. F., BLAUSTEIN, M. P., HODGKIN, A. L. & STEINHARDT, R. A. (1967). The effect of sodium concentration on calcium movements in giant axons of *Loligo forbesi*. *J. Physiol.* **192**, 43*P*.

BAKER, P. F., BLAUSTEIN, M. P., HODGKIN, A. L. & STEINHARDT, R. A. (1969*a*). The influence of calcium on sodium efflux in squid axons. *J. Physiol.* **200**, 431–458.

BAKER, P. F., BLAUSTEIN, M. P., KEYNES, R. D., MANIL, J., SHAW, T. I. & STEINHARDT, R. A. (1969*b*). The ouabain-sensitive fluxes of sodium and potassium in squid giant axons. *J. Physiol.* **200**, 459–496.

BAKER, P. F. & GLITSCH, H. G. (1973). Does metabolic energy participate directly in the Na^+-dependent extrusion of Ca^{2+} ions from squid giant axons? *J. Physiol.* **233**, 44–46*P*.

BAKER, P. F., HODGKIN, A. L. & RIDGWAY, E. B. (1971). Depolarization and calcium entry in squid giant axons. *J. Physiol.* **218**, 709–755.

BAKER, P. F., MEVES, H. & RIDGWAY, E. B. (1973). Calcium entry in response to maintained depolarization of squid axons. *J. Physiol.* **231**, 527–548.

BAKER, P. F. & POTASHNER, S. J. (1973). The role of metabolic energy in the transport of glutamate by invertebrate nerve. *Biochim. biophys. Acta* **318**, 123–139.

BAKER, P. F. & SHAW, T. I. (1965). A comparison of the phosphorus metabolism of intact squid nerve with that of the isolated axoplasm and sheath. *J. Physiol.* **180**, 424–438.

BAKER, P. F. & STONE, A. J. (1966). A kinetic method for investigating hypothetical models of the sodium pump. *Biochim. biophys. Acta* **126**, 321–329.

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

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

BLAUSTEIN, M. P. & OBORN, C. J. (1975). The influence of sodium on calcium fluxes in pinched-off nerve terminals *in vitro*. *J. Physiol.* **247**, 657–686.

BLAUSTEIN, M. P. & RUSSELL, J. M. (1975). Sodium–calcium exchange and calcium–calcium exchange in internally-dialysed squid giant axons. *J. Membrane Biol.* **22**, 285–312.

BLAUSTEIN, M. P., RUSSELL, J. M. & DE WEER, P. (1974). Calcium efflux from internally dialysed squid axons: the influence of external and internal cations. *J. Supramolecular-Structure* **2**, 558–581.

BRINLEY, F. J., JR., SPANGLER, S. G. & MULLINS, L. J. (1975). Calcium and EDTA fluxes in dialysed squid axons. *J. gen. Physiol.* **66**, 223–250.

CALDWELL, P. C. (1960). The phosphorus metabolism of squid axons and its relationship to the active transport of sodium. *J. Physiol.* **152**, 545–560.

DIPOLO, R. (1973). Calcium efflux from internally dialysed squid giant axons. *J. gen. Physiol.* **62**, 575–589.

DIPOLO, R. (1974). Effect of ATP on the calcium efflux in dialysed squid giant axons. *J. gen. Physiol.* **64**, 503–517.

FRANKENHAUSER, B. & HODGKIN, A. L. (1956). The after-effects of impulses in the giant nerve fibres of *Loligo*. *J. Physiol.* **131**, 341–376.

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

GARRAHAN, P. J. & GARAY, R. P. (1974). A kinetic study of the Na pump in red cells: its relevance to the mechanism of active transport. *Ann. N.Y. Acad. Sci.* **242**, 445–458.

GLYNN, I. M. & KARLISH, S. (1975). The sodium pump. *A. Rev. Physiol.* **37**, 13–55.

HODGKIN, A. L. & KEYNES, R. D. (1956). Experiments on the injection of substances into squid giant axons by means of a micro-syringe. *J. Physiol.* **131**, 592–616.

HODGKIN, A. L. & KEYNES, R. D. (1957). Movements of labelled calcium in squid giant axons. *J. Physiol.* **138**, 253–281.

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

JUNDT, H., PORZIG, H., REUTER, H. & STUCKI, J. W. (1975). The effect of substances releasing intracellular calcium ions on sodium-dependent calcium efflux from guinea-pig auricles. *J. Physiol.* **246**, 229–253.

MULLINS, L. J. & BRINLEY, F. J., JR. (1975). Sensitivity of calcium efflux from squid axons to change in membrane potential. *J. gen. Physiol.* **65**, 135–152.

RINK, T. J. & BAKER, P. F. (1975). The role of the plasma membrane in the regulation of intracellular calcium. In *Calcium Transport in Contraction and Secretion*, ed. CARAFOLI, E., CLEMENTI, F., DRABIKOWSKI, W. & MARGRETH, A. Amsterdam: North Holland.

ROJAS, E. & HIDALGO, C. (1968). Effect of temperature and metabolic inhibitors on ^{45}Ca outflow from squid giant axons. *Biochim. Biophys. Acta* **163**, 550–556.

RUSSELL, J. M. & BLAUSTEIN, M. P. (1974). External cation-dependent calcium efflux from barnacle muscle fibres: evidence for Na–Ca exchange. *J. gen. Physiol.* **63**, 144–167.

SCHATZMANN, H. J. (1975). Active calcium transport and Ca^{2+}-activated ATPase in human red cells. *Curr. Tops. Memb. Transport* **6**, 125–168.

SHAW, T. I. (1954). Sodium and potassium movements in red cells. Cambridge University Ph.D. Thesis.

SIMONS, T. J. B. (1975). The interaction of ATP-analogues possessing a blocked γ-phosphate group with the sodium pump in human red cells. *J. Physiol.* **244**, 731–739.