J. Physiol. (1983), **338**, *pp.* 295–319
With 15 text figures
Printed in Great Britain

EFFECTS OF INTERNAL SODIUM AND HYDROGEN IONS AND OF EXTERNAL CALCIUM IONS AND MEMBRANE POTENTIAL ON CALCIUM ENTRY IN SQUID AXONS

BY L. J. MULLINS, T. TIFFERT, G. VASSORT AND J. WHITTEMBURY

From the Department of Biophysics, University of Maryland School of Medicine, Baltimore, MD 21201, U.S.A., Laboratoire de Physiologie Cellulaire Cardiaque, INSERM U-241 CNRS LA 89–03, Université de Paris-Sud, Orsay, France, and Laboratorio de Biofisica, Universidad Peruana Cayetano Heredia, Lima, Peru

(*Received 11 March 1982*)

SUMMARY

1. Squid giant axons were impaled with electrodes to measure pNa_i, pH_i, E_m, and were injected with either aequorin or arsenazo III to measure $[Ca]_i$ or with phenol red to measure $[H]_i$.

2. Depolarization of such axons with elevated [K] in sea water leads to a Ca entry that is a function of $[Ca]_o$, $[Na]_i$, and $[H]_i$. With saturating $[Na]_i$ half-maximal Ca entry is produced by a $[Ca]_o$ of 0·58 mM. With saturating $[Ca]_o$, depolarization produced by 450 mM-K^+ leads to half-maximal Ca entry when $[Na]_i$ is 25 mM; entry is virtually undetectable if $[Na]_i$ is 18 mM.

3. If $[Ca]_o$ is 50 mM, Ca entry upon depolarization as measured with aequorin is phasic with a rapid phase of light emission and a plateau; Ca entry as measured with arsenazo III shows no such phasic behaviour, absorbance *vs.* time is a square wave that closely follows the depolarization *vs.* time trace. Both detectors of $[Ca]_i$ show a square-wave response if $[Ca]_o$ is 3 mM.

4. The introduction of 2 mM-CN into the sea water bathing the axon does not affect the response to depolarization nor does the destruction of most of the ATP in the axon following the injection of apyrase.

5. If axons are microinjected with phenol red rather than arsenazo, the entry of Ca produces an acidification in the peripheral parts of the axoplasm. Other experiments measuring $[Ca]_i$ show that Ca entry is strongly inhibited by a decrease in pH_i.

6. Making sea water alkaline with pH buffers scarcely affects the Ca entry induced by depolarization; making axoplasm alkaline by adding NH_4^+ to sea water greatly enhances Ca entry by Na/Ca exchange and also enhances the ability of axoplasmic buffers to absorb Ca.

INTRODUCTION

The starting point for this investigation was the observation by Mullins & Requena (1981) that the large increase in light production upon depolarizing aequorin-injected squid axons with 400 mM-K sea water could be abolished by tetanizing the axons for

30 min in Li-containing sea water. The most obvious effect of such a treatment is a reduction in $[Na]_i$ and the fact that Na_i was essential for the Ca entry effected by depolarization was confirmed by showing that raising $[Na]_i$ by tetanizing the fibre in Na-containing sea water led to an enhanced response to depolarization as judged by aequorin glow. These findings suggested that virtually all of the Ca entry resulting from the steady depolarization of a squid axon was dependent on $[Na]_i$ and hence was a manifestation of a membrane potential-dependent Na/Ca exchange.

Earlier studies (Hodgkin & Keynes, 1957) had shown that depolarization of squid axons by elevated [K] in sea water led to an increased Ca influx. This finding was extended in a study by Baker, Meves & Ridgway (1973*b*), where it was found that in aequorin-injected axons there was a phasic and tonic component of Ca entry that was thought to be Ca entry via Ca channels (the phasic component) and the possibility was mentioned that the tonic component might be via Na/Ca exchange as suggested by Baker, Blaustein, Hodgkin & Steinhardt (1969).

In another study, Mullins & Requena (1979) observed that making the axoplasm of a squid axon alkaline with NH_4^+ added to sea water in 1–10 mM concentrations had the dual effect of (*a*), decreasing the resting glow of the fibre and (*b*), of enhancing Ca entry into the fibre in response to Na-free solutions. Complementary measurements (Tiffert, Mullins, Whittembury & Brinley, 1981) of the pH of axoplasm showed that this became more acid during Ca entry as if Ca buffering were the result of an exchange of Ca for protons. These measurements suggested both that Ca entry might be influenced by pH_i and that Ca entry might change pH_i. These studies followed those of Baker & McNaughton (1977) who showed that a decrease in pH_i in squid axons reduced Ca efflux and those of Baker & Honerjäger (1978) where it was claimed that a decrease in pH_i lowered $[Ca]_i$. This claim has not been substantiated by measurements in barnacle (Lea & Ashley, 1978) nor does it agree with the study of Mullins & Requena (1979) cited above.

Two techniques that have been used to measure $[Ca]_i$ in squid axons as a function of time are: the microinjection of either aequorin (Baker, Hodgkin & Ridgway, 1971) or a Ca-sensitive dye such as arsenazo III (DiPolo, Requena, Brinley, Mullins, Scarpa & Tiffert, 1976). The aequorin technique has the advantage of great sensitivity to Ca and the disadvantage of possible non-linear responses between light emission and [Ca] while the arsenazo technique is simply less sensitive to low [Ca]. The measurements to be reported involve using several techniques known to increase Ca influx into squid axons, and compare the change in $[Ca]_i$ as measured both with aequorin and with arsenazo III. Most of the measurements were made with a Na-sensitive glass electrode in the axon to record $[Na]_i$ continuously. They also involved using a glass pH electrode inside the axon and, in some axons, injections of the pH-sensitive dye phenol red to quantify intracellular pH and its changes brought about by Ca entry. Preliminary results have been published (Vassort, Tiffert, Whittembury & Mullins, 1982).

METHODS

Experimental material. The experiments were done using living animals of the species *Loligo pealei* collected at the Marine Biological Laboratory, Woods Hole, MA U.S.A. during the months of May–June, 1981. Some preliminary experiments were done in June, 1980. The animals were rapidly

dissected and the axon cleaned so that it was ready for an injection of either aequorin or of the dyes arsenazo III or phenol red generally within 1 h of the death of the animal.

Experimental chamber. The arrangement used for the mounting of axons involved the cannulation of the fibre at each end and the mounting of the fibre in a dialysis-type chamber where it was held horizontally between end cannulae as described by Brinley & Mullins (1967). The chamber had external gold electrodes for stimulating and for the recording of action potentials passing through the bottom of the slot into which the axon was lowered after injection as shown in Fig. 1. A principal

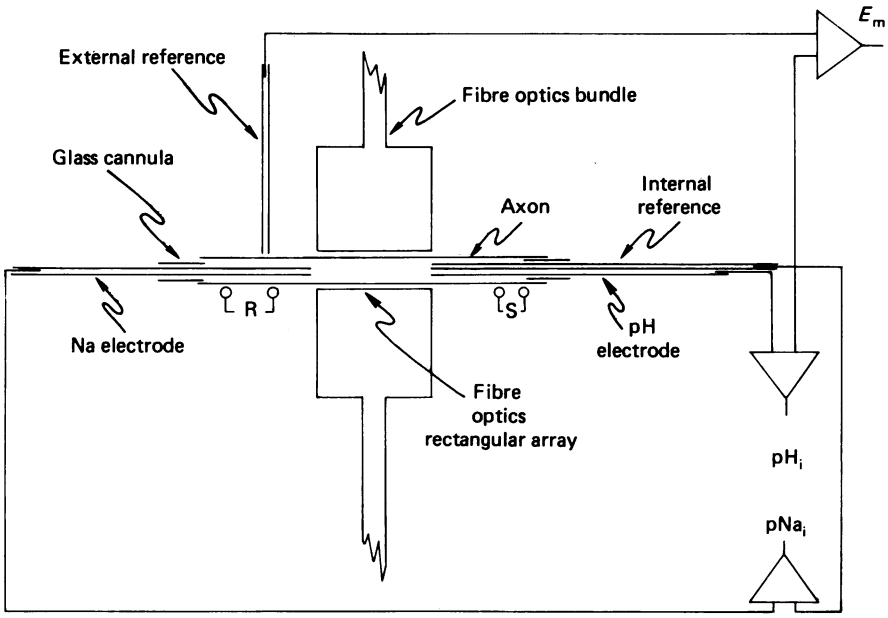

Fig. 1. This shows the arrangement of the squid giant axon, held on a glass cannula at each end and the three internal capillary electrodes used to measure membrane potential, Na_i, and pH_i. The pathway to provide either for light emission or absorption with fibre optics is shown as well as the external reference electrode and stimulating (S) and recording (R) gold wires. These were actually immediately under the axon in the slot and have been moved downward in the drawing for clarity.

feature of the chamber was the incorporation of two light guides in rectangular array (10 × 1 mm) (Dolan-Jenner, Woburn, MA, BF-824) so that for arsenazo one guide could introduce light and the other carry away light after it had passed through the fibre; for aequorin, both fibre optic light guides collected emitted light and delivered this to a photomultiplier. Flow through the chamber was maintained by LKB peristaltic pumps running at 1 ml/min. For solution changes, flow was sometimes increased to 4 ml/min for 15 s. An electrically operated Hamilton valve was positioned close to the chamber inflow so that the dead volume (Chamber slot plus flow line) was 0·2 ml.

Light measurement. The spectrophotometer for the measurement of both arsenazo III and phenol red absorbance used an air turbine rotating filter assembly and has been previously described by Brinley, Tiffert, Scarpa & Mullins (1977). In the case of arsenazo III, the wave-lengths 675, 685, and 660 nm were used and the results recorded as 685−660 and 675−685 difference spectra. For phenol red the wave-lengths 481, 504, and 525 nm were used, and the 504−525 or 481−525 differences were measured. The time constant for integrating the wave-length difference signal was usually 1 s but in the case of some especially noisy signals this was increased to 3 s.

For aequorin light, two systems of measurement were used. In the first, described by Requena, DiPolo, Brinley & Mullins (1977) a photomultiplier photometer (Aminco 7512) provided both high voltage to the photomultiplier (EMI 9824A) at −900 V and a current amplifier and dark current subtraction network. In other measurements, photon counting was employed as described by

Mullins & Requena (1981), where a Spex DPC-2 digital photometer counted the photons emitted and the usual integration time for counting was 10 s, but 1 or 2 s integration times were sometimes used.

Electrical measurements. The bath in which the axon was kept for measurements was not earthed but was surrounded by a screened cage that was earthed and all the electrode measurements were made with differential amplifiers that provided a digital display of the electrode output and an analogue signal for a chart recorder. The internal capillary and external reference electrodes were filled with 3M-KCl and the external electrode connected to an artificial-sea-water (ASW) bridge; changes in the solution flowing through the chamber necessarily changed the junction potential between the external reference electrode and the new solution. Such changes were noticed when elevated [K] in sea water or elevated [Ca] solutions were used, hence ion concentrations from the electrodes can only be read when the fibre is normally polarized. Membrane potentials are to be expected to differ from the measured value by the amount of the junction potential change. Much of the work to be reported involved prolonged stimulation of the fibres, hence preventing leakage currents from earthing was one of the reasons for keeping the bath floating.

The ion specific electrodes, described below, had in addition to the large capacitance expected from 100 μm capillaries of 75 mm length, a substantial resistance so that the expected time constant for electrode response was of the order of tens of seconds. Actual records of $[Na]_i$ and $[H]_i$ measurement show a somewhat longer initial delay in settling to a steady value probably because the potential-measuring capillary and the [Na]-measuring electrode were separated by the gap necessary for the optical measurements described. A practical result was, however, that transient deflexions of ion specific electrode outputs are artifacts and only the steady tracing is valid.

Microinjection. A horizontal microinjector as previously described for working with dialysis chambers (Requena *et al.* 1977) was used to introduce arsenazo III or phenol red into fibres for absorbance measurements, or aequorin for light emission measurements or apyrase for destruction of $[ATP]_i$. The usual final concentration for the dyes used was 300 μM; for aequorin 8 μM and for apyrase 1 u. These substances were injected over a length of 10–12 mm.

Dyes. Both arsenazo III and phenol red were purified by twice passing solutions of the dye through Chelex (K form) columns (10 cm long). The purified dye was then injected to yield appropriate final concentrations in the axoplasm.

Electrode construction. The Na electrodes used in this study were made of NAS 11–18 (Corning) glass obtained as capillaries 1 × 0·5 mm (o.d., i.d.) from Micro-electrodes Inc. These capillaries were pulled in a vertical machine to o.d. of the order of 0·1 mm and the resultant capillaries broken into lengths of *ca.* 25 mm.

Corning glass tubing (1720) was similarly drawn by the vertical puller into o.d. of the order of 0·12–0·14 mm and the NAS glass matched to the i.d. of the 1720 glass so that an assembly of 125–150 mm total length with a 5 mm projection of NAS glass at the end was achieved. The NAS glass and the 1720 glass were bonded by heating and the tip of the NAS was sealed with a micro-flame. The filling solution was anhydrous methanol saturated with NaCl and this was injected into the back of the electrode with a fine capillary tube. Heat was then applied to the tip to induce boiling and the subsequent removal of the bubble.

The filled electrode was then connected to a Ag/AgCl pellet made by mixing powdered Ag and AgCl and compressing this in a die together with a Ag wire (we are indebted to Drs F. Bezanilla and R. Taylor for suggesting the procedure). The Ag/AgCl electrode was inserted into the back end of the capillary and sealed into place with dental wax. The termination of the Ag wire was to a gold contact pin from AP Products (Painesville, OH, U.S.A.) and this connected to the measuring apparatus. The behaviour of electrodes so made was that they had a slope of 50–55 mV per decade change in [Na]. Electrodes that responded slowly to changes in [Na] could have their performance improved by immersion in 4 M-NaCl with 1 mM-EGTA.

For pH measurement, Corning 0150 glass tubing 1 mm o.d. × 0·5 mm i.d. was used and the pH electrodes were built the same way as the Na electrodes. The tubing (pH) was bonded to the supporting glass and again the usual length of the entire assembly was 125–155 mm with a 5 mm projection of pH-sensitive glass. Filling of the pH electrode was with 0·1 M-HCl saturated with AgCl, or with saturated KCl buffered with 10 mM-glycylglycine pH 8·5 depending on the asymmetry potential of the particular electrode. Electrode impedance for both the pH and Na electrodes was of the order of 10^6–10^7 Ω and, as expected, was mainly sensitive to the length of exposed ion-sensitive

glass. Measurements of potential were conveniently made with digital pH meters (Orion, model 701) which produced both a digital display and an analogue output for coupling to chart recorders.

Electrodes were inserted with a horizontal micromanipulator after the axon, cannulated at both ends, had been microinjected. The usual arrangement for electrode insertion was to have a reference capillary and the pH glass electrode inserted from one end of the axon and the Na-glass electrode from the other. Electrodes were kept out of the optical path of the rectangular array of fibre optics since they sometimes could induce optical artifacts. The result was that pH and Na electrodes did not read their respective ion activities in exactly the same point in the axoplasm.

In experiments where simultaneous measurements of pH_i, pNa, and membrane potential were made, the pH and membrane potential measuring capillary electrode were glued together using either Eastman 910 adhesive or very dilute epoxy resins. The capillary electrode used in these cases was pulled from 1 mm o.d. × 0·5 mm i.d. Pyrex glass tubing to a diameter of about 15 μm and it was filled with saturated KCl solution and connected to a tube in which a sintered Ag/AgCl pellet was present.

Electrode calibration. A glass capillary 10 cm long and 1 mm in i.d. was mounted immediately on top of the dialysis chamber and filled with either pH buffers or Na calibrating solutions. The electrodes, mounted in micromanipulators, could then be introduced into the capillary and a reading obtained and the electrode then immediately moved to the end cannula of the axon, washed, and introduced into the axoplasm and a second reading obtained. The procedure minimized the time between obtaining calibrating and axoplasmic ion activity readings. For the Na electrode a solution of NaCl 100 mM + 10 mM-TES (*N*-tris (hydroxymethyl) methyl-2-aminoethanesulphonic acid) pH 7·3 was used as a zero reference for potential and solutions 20 and 10 mM with pH buffer as above were used to obtain the response of the electrode to Na concentration change. The addition of 200 mM-KCl or of 5 mM-$MgCl_2$ to the calibrating solutions did not change the potential indicated by the Na electrode by more than 1 mV. For the pH glass electrode, the calibrating solution was 100 mM-TES pH 7·0 as a zero reference and electrode responses were also obtained at pH 7·3 and 7·5 with TES buffers. Acceptable electrode behaviour was defined as a reading between initial calibration and final calibration of $\pm 0{\cdot}004$ pH or ± 1 mV for the Na electrode or less.

To avoid the complications of having to deal with the concept of ion activity and ion activity coefficients, the Na electrode was used as a matching device so that, for example, the reading of this electrode (uncorrected for junction potential) in the axoplasm of a freshly dissected axon was very close to the reading of the electrode in a calibrating solution of 20 mM-NaCl; hence we conclude that the concentration of Na in axoplasm is close to 20 mM.

Solutions. The sea water used in the experiments to be reported had the following composition (mM): Na, 455; K, 10; Mg, 50; Ca, 3; TES, (pH 7·8) 5; Cl, 571; EDTA, 0·1 (to protect against heavy metal contamination). Solutions with 3 mM-Ca or less had 50 mM-Mg; solutions with 10 mM-Ca or higher were obtained by reducing [Mg] as Ca rose above 3 mM. Solutions with a variable [K] were obtained by substituting K for Na in sea water; choline sea water was obtained by replacing Na with choline. Cyanide sea water had 2 mM-NaCN added to normal sea water. Solutions with NH_4^+ were obtained by replacing NaCl in sea water with NH_4Cl. All solutions were adjusted to 1000 ± 10 mosmol using a Wescor dewpoint osmometer and to pH 7·8 with a pH meter. Temperature was 18 ± 2 °C.

RESULTS

Initial $[Na]_i$ of axoplasm. Substantial analytical work with axons freshly isolated from *L. pealeii* has shown that the Na content of axoplasm as measured by flame photometry, is close to 40 mM (Mullins, 1979). In the experiments to be reported it was necessary to know the value of $[Na]_i$ at the time that depolarizing pulses of sea water with an elevated [K] were applied, hence the only feasible technique for this measurement was to use a Na-sensitive glass electrode. A previous study of Na content and Na^+ ion activity a^i_{Na} (Hinke, 1961) showed that this was around 40 mM while the Na content was 95 mM in *L. forbesi* axons that were obtained from refrigerated

mantles. These findings suggested that a substantial fraction of the Na of fresh axoplasm was not participating in the Na-specific electrode reaction. At the same time, measurement of the net Na gain per impulse in axons stimulated for some time showed this to be 3·8 pmol/cm². impulse, a value identical with that measured as net analytical gain (Keynes & Lewis, 1951).

TABLE 1. Na gain with stimulation

Axon reference	$[Na]_i$ Initial (mM)	$[Na]_i$ Final (mM)	Stimulation (s)	Stimulation (frequency/s)	Na entry (pmol/cm². impulse)
052181B	13	19	330	60	3·8
	19	25	405	60	3·2
	17	44	975	60	4·7
052281	18	23	495	60	3·0
	23	42	660	110	4·8
053081A	23	47	615	120	3·9
053081B	23	55	1080	180	2·2
					3·7 ± 0·3*

* Value is expressed as the mean ± S.E. of the mean, $n = 7$.

Our measurements of the initial value of $[Na]_i$ as deduced from Na-sensitive electrode measurements of ion activity were 19·0 ± 1·6 mM (S.E. of the mean; $n = 18$) for axons injected with arsenazo III while with aequorin the value was 17·6 ± 0·5 (S.E. of the mean; $n = 5$). Note that the mean value (19 mM) is about half that for mean analytical values (40 mM) mentioned above, and hence follows the ratio observed by Hinke (1961) between analytical and free Na_i. While Hinke suggested a substantial degree of Na binding by axoplasm, it should be mentioned that an alternative idea is that the Na electrode is sampling from a very central volume of axoplasm (close to where $[Ca]_i$ is being measured) while analytical measurements require perhaps a 10 mm length of axoplasm whose [Na] may not be uniform. Since the [Na] of axoplasm at the cut end of a fibre is 455 mM (i.e. sea water) it takes a substantial distance from the cut end for $[Na]_i$ to assume a constant value.

Na gain upon stimulation. The experiments to be reported deal with a comparison of the entry of Ca with depolarization as $[Na]_i$ is varied; a most convenient way of making this change is to stimulate the axon in Na sea water at various frequencies and for varying periods of time. Since the values for $[Na]_i$ in freshly isolated axons as reported above were somewhat lower than expected, it was reassuring to find that the Na electrode reported an increase in $[Na]_i$ with stimulation that agreed well with the rather comprehensive measurements that have been made with isotopes. Table 1 shows the results with stimulation of a number of axons in terms of the gain of Na/cm². impulse and yields a mean value of 3·7 pmol/cm². impulse while Keynes & Lewis (1951) show that the net flux measured analytically is 3·8 pmol/cm². impulse in satisfactory agreement with the value obtained above.

Initial pH of axoplasm. There have been a number of studies of the pH of axoplasm (Caldwell, 1954, Boron & DeWeer, 1976) and all are in reasonable agreement that a

mean value of 7·3 is to be expected. Our measurements are in agreement with this value, the initial pH of axons was 7·32 ± 0·004 S.E. of the mean ($n = 10$). Since the studies to be reported often involved prolonged depolarization or stimulation of the fibres and these treatments have been shown to increase Ca entry which in turn has been shown to result in a lowering of pH_i, it is understandable that most axons tended

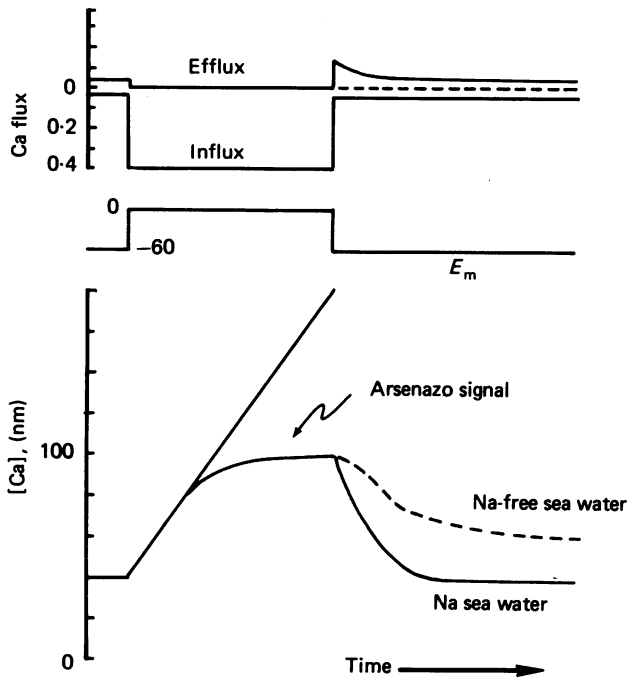

Fig. 2. A comparison is made between the expected behaviour of Ca unidirectional fluxes (top traces) in response to a depolarization of the membrane from −60 to zero (middle trace) and the arsenazo absorbance signal (bottom trace). The levelling out of the absorbance *vs.* time trace is ascribed to axoplasmic buffering as described in the text. Repolarization can be expected to yield a differing time course for the arsenazo signal depending on whether Na_o is present or not.

to become more acidic after several hours of experimental treatment. The only exception to this finding was that fibres kept in Ca-free sea water for times of the order of hours become alkaline. A point worth noting is that while $[Ca]_i$ can rise and fall rapidly in response to depolarization and repolarization, the change in $[H^+]_i$ resulting from Ca entry is very slow in returning to initial values compared with $[Ca]_i$. Since some of the measurements to be presented involve using phenol red as an indicator of $[H^+]$ it has in most cases been convenient to report values for $[H^+]_i$ rather than its negative logarithm.

An analysis of arsenazo records. The Ca complexing dye arsenazo III has been used to measure Ca entry in squid axons (Brown *et al.* 1975; DiPolo *et al.* 1976) produced by either repetitive stimulation or by Na-free solutions. Our purpose in using this indicator was both to compare its response to steady depolarization with published responses as measured with aequorin (Baker, Meves, & Ridgway, 1973*a*, *b*; Mullins

& Requena, 1981) and to make a more quantitative estimate of the level of Ca flux that is produced by depolarization. Previous studies of Ca entry (Brinley *et al.* 1977) have shown that at infinite time more than 99% of the Ca entering a nerve fibre is buffered and hence does not contribute to the increase in Ca_i that follows a variety of treatments (stimulation, Na-free solutions, depolarization) that result in enhanced Ca entry. We can expect, therefore, if depolarization produces an enhanced Ca influx, that initially the change in $[Ca]_i$ is

$$\frac{d[Ca]_i}{dt} = \frac{S}{V}\Delta m^i_{Ca},$$

where S/V is the surface: volume ratio of the axon and Δm^i_{Ca} is the increment in Ca influx produced by the depolarization. The expected relation between the Ca fluxes is shown in Fig. 2 where initially both influx and efflux are in balance when $[Ca]_o$ is 3 mM and when $E_m = -60$ mV and $[Na]_i$ has a normal value (DiPolo *et al.* 1976). Depolarization, in the example above, increases Ca influx 10-fold, decreases Ca efflux to vanishingly small values (Mullins & Brinley, 1975) and hence leads to an increase in $[Ca]_i$ as given by the straight line shown at the bottom of the Figure. Thus, a measurement of the initial slope of absorbance *vs.* time should yield a value proportional to Ca net flux inward.

As $[Ca]_i$ begins to rise, however, a greater and greater fraction of the entering Ca goes not to increase $[Ca]_i$ but is absorbed by intracellular buffers. Actual [Ca] in axoplasm will be a complex function of radial distance from the membrane (x) but arsenazo will measure a mean [Ca] which we will call $([Ca]_i)x$ so that when $[d[Ca]_i/dt) = 0$.

$$\sum_{x=o}^{x=r} \beta([Ca]_i)_x = \frac{S}{V}\Delta m^i_{Ca},$$

so the entire Ca influx is being buffered and β is the variable controlling the rate of reaction of Ca with internal buffering mechanisms in each element of axoplasm volume. The [Ca] in each element of axoplasm volume must be expected to vary greatly with distance from the surface but the equation suggests that for each value of Ca influx there exists a $[Ca]_i$ *vs.* radial distance profile that allows a match between influx and buffering. This is illustrated in Fig. 2 for the curve labelled 'arsenazo signal' as the region at 100 nM of $[Ca]_i$ where the slope of the absorbance with time is zero. Evidence will be presented later that the arsenazo signal is not changed in the presence of CN^-, implying that mitochondrial buffering is not involved in the measurements made. This, in turn, suggests that values of $[Ca]_i$ are less than 1 μM since this is a value noted by Brinley *et al.* (1977) as one above which mitochondrial buffering becomes prominent. The [Ca] just under the membrane must, however, be expected to rise to levels of the order of several hundred nanomolar (where the aequorin response becomes non-linear) since large aequorin responses have the features of an apparently transient and steady-state increase in $[Ca]_i$ which must represent a step in the setting up of a stable [Ca] *vs.* radial distance x, profile.

A comparison of aequorin and arsenazo as indicators of Ca entry. When an axon is depolarized either by raising the [K] of sea water or by passing an electric current across the membrane, the result as first described by Baker, Meves & Ridgway

(1973*a*, *b*) is an increase in aequorin glow that has both a peak value and a lower steady-state value. By contrast, we find that if an axon is injected with arsenazo III, the response to steady depolarization is an increase in absorbance of the dye without any phasic change in light absorption. A comparison of the results obtained by depolarization for axons injected with aequorin or with arsenazo is shown in Fig. 3.

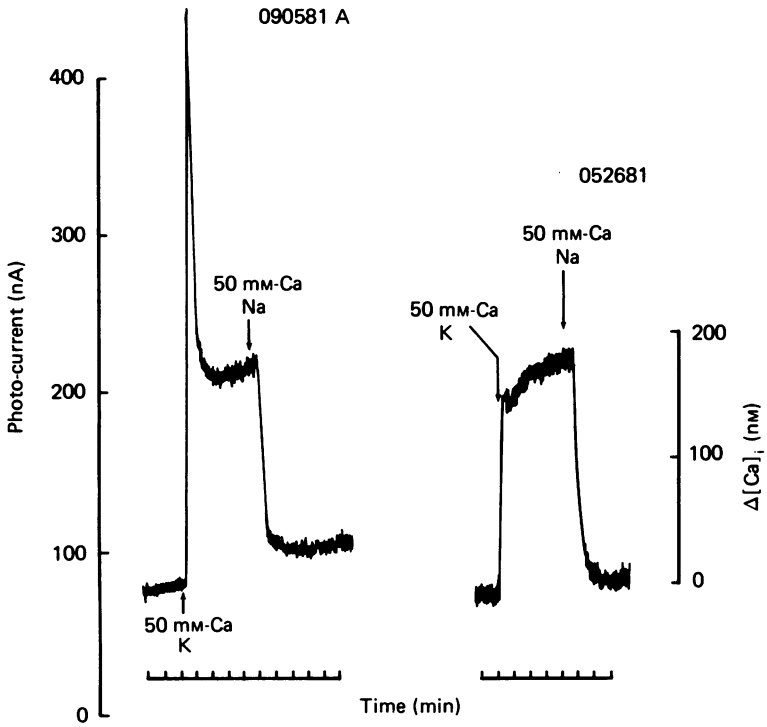

Fig. 3. A comparison is made between the response of an aequorin-injected axon (ordinate, photo-current in nA) and one injected with arsenazo III (ordinate change in $[Ca]_i$) to depolarization (a transfer from 50 mM-Ca (Na) to 50 mM-Ca (K) sea water) and to subsequent repolarization. Time marks on the abscissa are minutes. Both axons had been isolated for 4–5 h and hence could be expected to have gained substantial Na. Since the resting $[Ca]_i$ was about 60 nM with previous measurements with aequorin we can note that the initial resting glow (75 nA) was increased with depolarization and the plateau was 210 nA, a three-fold change corresponding to an increase in ionized Ca by 150 nM; this agrees reasonably well with the observation with arsenazo III.

The largest Ca entry is achieved if all the Na in sea water is replaced by K where the membrane potential changes by about 65 mV. In the example shown the response as measured by aequorin is the phasic sort where there is a transient followed by a steady-state increase in light; in the measurements with arsenazo there is only a steady elevated $[Ca]_i$ with no suggestion of a phasic response to depolarization.

The fact that the aequorin signal is substantially different from that observed for arsenazo III, and that we have never observed anything other than a square-wave response with arsenazo makes it necessary to consider the question: which indicator gives the proper response? Aequorin is known to have a highly non-linear response

to [Ca] varying between a less than linear to a linear and to a 2·5 power function in terms of light emission, and this response is relatively insensitive to pH. The actual details of Ca entry with depolarization are highly complex and are considered in the Discussion. Here it is useful to note that a phasic entry of Ca with aequorin is never seen if $[Ca]_o$ is 1–10 mM (compare figs. 1 and 4 of Mullins & Requena, 1981) and since, as we will show later in this report, these values of Ca_o are sufficient to saturate the Na/Ca exchange, it follows that the apparently phasic Ca entry observed in 50–112 mM-Ca_o is occurring via a channel mechanism. Since arsenazo III does not measure such a phasic entry, it would appear that it is the non-linearity of aequorin *vs.* [Ca] that produces the spike. Most of our measurements with aequorin-injected axons were made with a $[Ca]_o$ of 3–10 mM and hence these records could not be expected to show spikes. The few measurements in 50 mM-Ca were made with insufficient time between test depolarizations for the activation of the spike mechanism. The inactivation of the aequorin-detected spike mechanism suggests that it is at least in part mediated by some channel-controlled Ca entry; it cannot be via Na channels since tetrodotoxin (TTX) is without effect so that a K-channel mechanism is suggested. This would provide a rapid Ca entry early in depolarization and hence perhaps explain the apparently phasic entry observed when $[Ca]_o$ is high but inhibitors such as Mn, which stop Ca entry via Na/Ca, are present. The way that aequorin responds non-linearly to [Ca] is reviewed by Requena & Mullins (1979) (see their Fig. 1).

There is some controversy over whether the stoichiometry of Ca: arsenazo is 1:1 or 1:2 for concentrations of Ca involved in muscle contraction (μM) (Thomas, 1979; Ahmed, Kragie & Connor, 1980) but there is little disagreement that at concentrations of 100 nM, the interaction is 1:1. The arsenazo reaction is pH sensitive since both Ca^{2+} and H^+ can bind to the dye. Our experiments show that except for the most massive Ca entries, pH_i is stable at a level of $\pm 0{\cdot}02$ so that the reported effects of changes in pH_i on arsenazo absorbance are not applicable.

Ca entry vs. $[Na]_i$. The present experiments were undertaken to measure the quantitative relationship betwen $[Na]_i$ and Ca entry. The method was to employ a Na_i-sensitive glass electrode and to change $[Na]_i$ either in an upward or downward direction by stimulation in either a Na or Li sea water at frequencies of about 100/s for periods of time sufficient to change sensibly $[Na]_i$. In some experiments, axons were kept in Na-free, Ca-free choline sea water for several hours before being injected either with dye or aequorin. This allowed the initial $[Na]_i$ to be substantially lower than in freshly dissected axons.

An experiment using an arsenazo III-injected fibre is shown in Fig. 4; here the initial $[Na]_i$ was 21 mM and the axon was kept in 3 mM-Ca (Na) sea water and periodically tested in 3 mM-Ca (450 mM-K) sea water or in 50 mM-Ca (450 mM-K) sea water. At the initial value of $[Na]_i$, the slope of the arsenazo absorbance *vs.* time is very small for 3 mM-Ca sea water (450 mM-K) but it is substantially larger if $[Ca]_o$ is 50 mM. We suppose that this effect is in part the result of some Ca entry via channels (which is only measurable if $[Ca]_o > 10$ mM) and in part because 3 mM-Ca sea water contained 50 mM-Mg, an inhibitor of Ca entry, while 50 mM-Ca sea water did not. These conclusions result from the demonstration (below) that Ca entry via Na/Ca exchange is saturated at a $[Ca]_o$ of 3 mM and the finding that when $[Na]_i$ has been enhanced by stimulation in Na sea water, there is no difference in the response to depolarization

between 50 mM-Ca and 3 mM-Ca sea water (see tests at $[Na]_i$ of 38 and 68 mM). In this record, stimulation to raise $[Na]_i$ to 26, 38, and 68 mM was carried out and Ca entry with depolarization measured at each of these concentrations. The record supports the view that at values of $[Na]_i$ lower than 20 mM, there is no measurable Ca entry with depolarization if $[Ca]_o$ is kept at 3 mM and that beyond a $[Na]_i$ of 38 mM

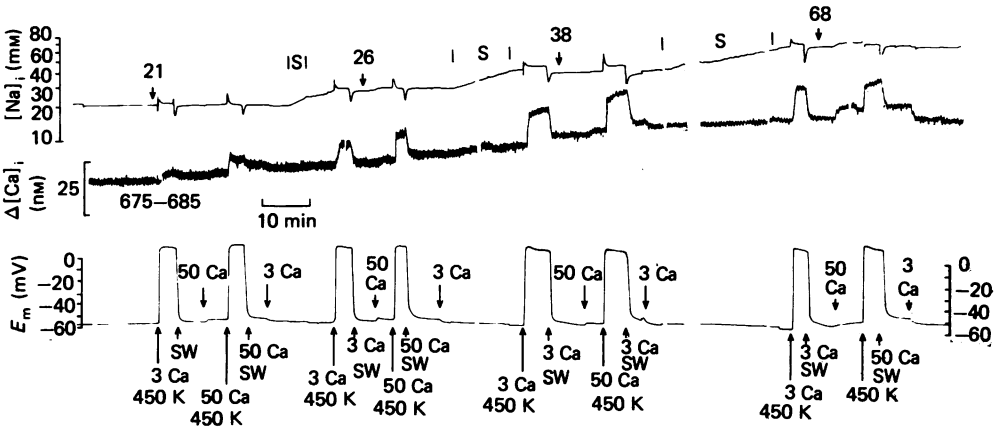

Fig. 4. The top trace gives $[Na]_i$ as a function of time. The letter |S| denotes a period of stimulation at 60/s. Numbers on the trace show $[Na]_i$ (mM) at a particular value of time. The middle trace is $\Delta[Ca]_i$ measured as the 675–685 nm arsenazo III difference signal while the bottom trace is E_m. Numbers on the trace refer to the concentration (mM) of Ca, K sea water (SW).

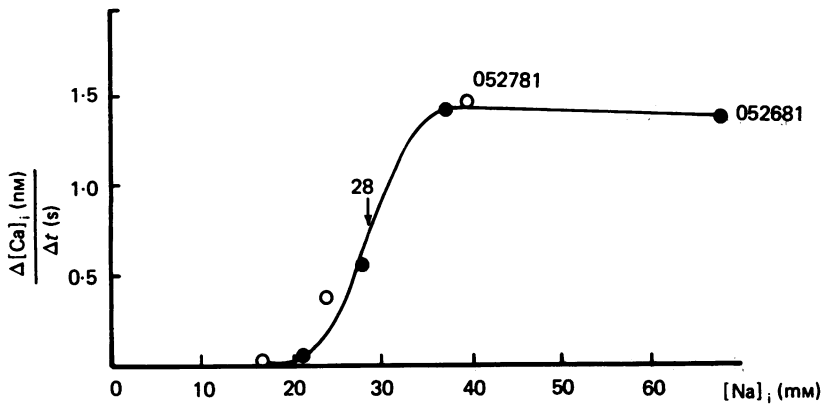

Fig. 5. The initial rate of increase in $[Ca]_i$ upon the application of 3 mM-Ca, 450 mM-K is plotted (ordinate) as a function of $[Na]_i$ for two axons injected with arsenazo III.

the process of Ca entry is saturated with respect to $[Na]_i$. One can also note that, early in the record, applying 50 mM-Ca sea water resulted in no change in $[Ca]_i$ while at the end there is a clear increase; we attribute this to an increasing passive Ca leak. The measured slopes $\Delta [Ca]_i / \Delta$ time are plotted *vs.* $[Na]_i$ for two axons as Fig. 5 and from this plot it can be noted that a mean $[Na]_i$ of 28 mM is that yielding just half-maximal Ca entry.

Similar experiments have been carried out using aequorin as an indicator of Ca

entry and the results for five measurements on three axons are that the half-activating $[Na]_i$ concentration $K_{\frac{1}{2}}$ is 22 ± 1 mM while the Hill coefficient for the experimental curves is 7. The large value for this coefficient indicates that the mechanism of Ca entry with depolarization is of a high order of kinetic complexity. The mean value of $K_{\frac{1}{2}}$ of arsenazo III axons plus aequorin axons is $[Na]_i = 25 \pm 1$ mM.

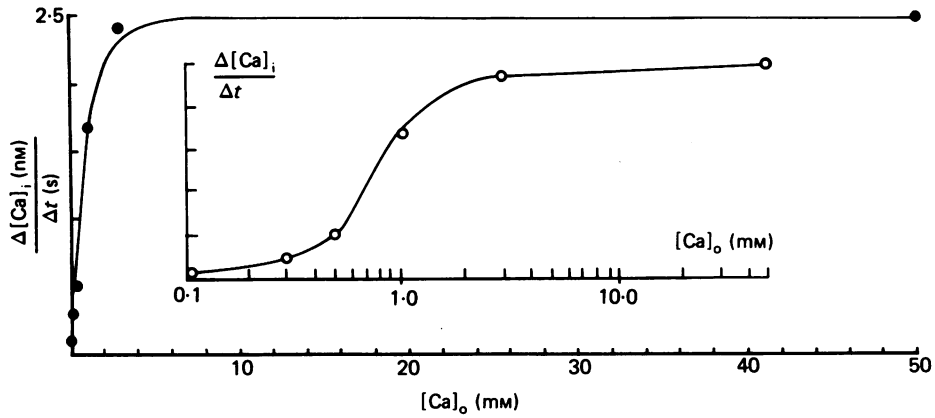

Fig. 6. The increment in Ca entry with depolarization (450 mM-K) in an axon injected with Na to yield a concentration of 156 mM is plotted as the initial slope of absorbance of arsenazo with time as a function of the [Ca] in sea water. An identical plot with $[Ca]_o$ on a semi-log scale is shown in the insert.

Ca entry vs. $[Ca]_o$. The experimental results obtained in the previous section suggested that it would be useful to determine Ca entry with depolarization as a function of $[Ca]_o$. To achieve maximal sensitivity, $[Na]_i$ was made saturating by the injection of about 0·5 μl of 1 M-NaCl solution; the axon had a Na electrode inserted in it and this read 22 mM before the injection and 156 mM after the injection. The axon was subsequently injected with arsenazo III and tested for a depolarizing response in Na sea water with 0·1, 0·3, 0·5, 1, 3, and 50 mM-Ca (the first five solutions contained 50 mM-Mg while the sixth did not). Depolarization was achieved by replacing all Na in sea water with K and the slope of the arsenazo response was measured in the usual way. The results are shown in Fig. 6 from which it can be noted that half-maximal entry of Ca with depolarization is brought about by a $[Ca]_o$ of 0·66 mM.

A different sort of experiment was carried out using an aequorin-injected axon that was freshly isolated and hence one that had a low $[Na]_i$. The axon was placed in 0·3 mM-Ca (50 mM-Mg) sea water, depolarized with 450 mM-K and the [Ca] of sea water increased in steps as shown in the left-hand part of Fig. 7. Note that for concentrations of Ca from 0·3 to 3 mM there was a constant 50 mM-Mg but that for 50 mM-Ca the Mg was removed. Since Mg has been shown to be an inhibitor of Ca entry with depolarization (Baker *et al.* 1971), our results with 50 mM-Ca can be expected to be partly due to the removal of Mg inhibition and partly any response that this Ca concentration itself may have. It is clear from all studies made, however, that at high $[Na]_i$ the Ca entry with depolarization is saturated at values of $[Ca]_o$ of 3 mM or below. Clearly, the axon responds to $[Ca]_o$ of 50 mM with an enhanced

Ca entry that is not Na/Ca exchange, (compare Fig. 4) and is most simply explained by supposing that at this high level of Ca_o there is an enhanced influx at the resting potential and that additionally there is an extra Ca entry with depolarization that may represent Ca moving through K channels. When an axon has saturating values of $[Na]_i$, the Ca movement via Na/Ca overwhelms leakage mechanisms. When $[Na]_i$ is more modest, a variety of mechanisms contribute to Ca entry. The axon of Fig. 7

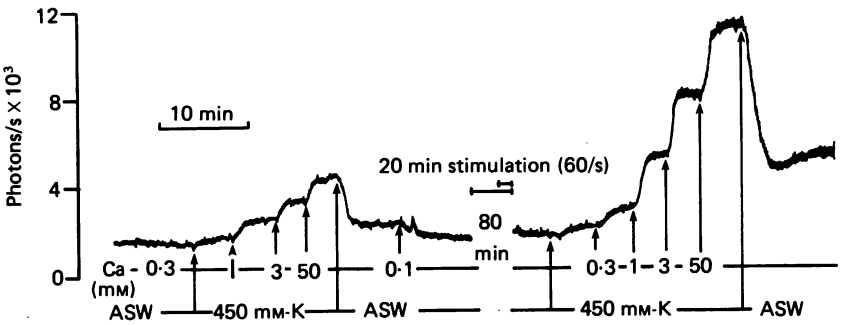

Fig. 7. An aequorin injected axon in 0·3 mM-Ca sea water was depolarized with 450 mM-K and light emission followed as $[Ca]_o$ was increased as shown on the Figure. After the first trials, the axon was stimulated and retested for light emission at an elevated $[Na]_i$. Note that in both cases the increment in light emission with 50 mM-Ca is maintained when the axon is repolarized; (leakage Ca influx); (axon 051281B).

was subjected to 20 min of stimulation of 60/s and then re-tested for a response to depolarization at various $[Ca]_o$ as shown. Note that the response to 50 mM-Ca is all a leakage response, that is, when the axon was repolarized in 50 mM-Ca (Na) sea water it showed an elevated light emission that just matched the increment of light emission found in going from 3 mM-Ca to 50 mM-Ca. Since the fibre was continuously depolarized during step changes in $[Ca]_o$, the K channels should have inactivated and hence not contributed to the Ca entries measured. Four other axons were tested using aequorin as the indicator of Ca entry; these axons were not Na-loaded by injection but may have had Na_i in a mid range. The apparent K_i^1 for all six axons examined was $[Ca]_o = 0{\cdot}58 \pm 0{\cdot}2$ mM. Some aequorin-injected axons were treated with Na_o-free media (and had a low $[Na]_i$); these showed a K_i^1 for Ca entry from low Na_o media of 1–3 mM.

The results obtained show that Ca entry in squid axons that is dependent on Ca_o/Na_i exchange is always saturated with respect of Ca_o in the intact animal where $[Ca]_o$ is estimated to be 3–4 mM. The findings of this section are also useful in analysing the response of axons to high $[Ca]_o$ since any increment in Ca entry in going from 3 to 50 mM-Ca_o must be an increase in Ca moving through channels and leakage pathways. Indeed, we have assumed that a change in [Ca] in Na sea water from 3 to 50 mM measures the leakage component of Ca entry; in very fresh axons there is no measurable change in $[Ca]_i$ when examined either with aequorin or with arsenazo, but with time the response becomes larger and ultimately limits the sorts of measurements of Ca entry that can be made.

Ca entry as a function of membrane potential. Since Mullins & Requena (1981) have shown, and the present results confirm, that more than 95 % of the Ca entry during

steady depolarization depends on $[Na]_i$, it is reasonable to ascribe this entry to Na/Ca exchange running backward, that is moving Ca inward in exchange for Na_i. The most reasonable explanation for the sensitivity of this process to membrane potential is that more charges move in one direction than in the other, specifically more Na charges move outward than Ca charges inward per translocation of the carrier. The number of excess Na charges that move ought, in principle, to be obtained from a measurement of the sensitivity of Ca influx to membrane depolarization. The conditions for the measurement of Ca influx need to be carefully defined; if one uses Na-free solutions as a base line and depolarizes in these, one goes from a large Ca influx resulting from Na-free conditions to a much larger Ca influx (providing $[Na]_i$

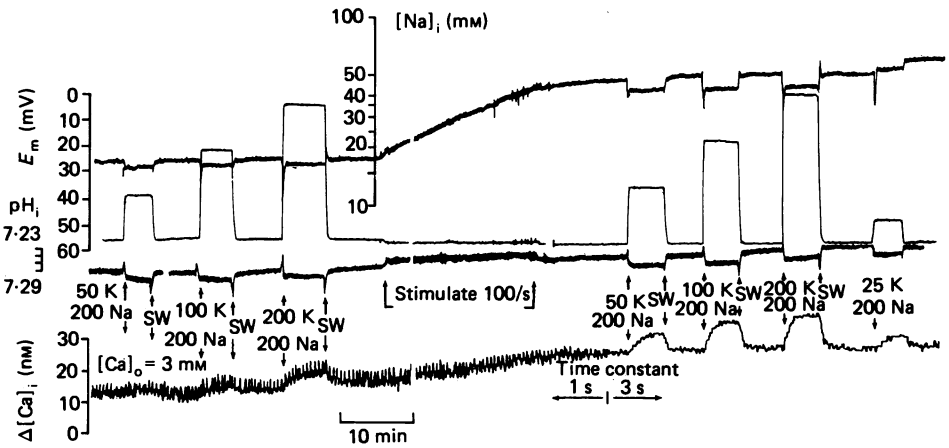

Fig. 8. The top trace is $[Na]_i$ the second is $-E_m$, the third is pH_i (measured with a glass electrode) and the fourth trace is the arsenazo absorbance signal. The initial response to membrane depolarization was obtained when $[Na]_i$ was 18 mM; stimulation at 100/s for the time indicated led to a $[Na]_i$ of 48 mM and a second series of test depolarizations was then carried out. The change in pH_i from the start of the experiment was from 7·29 to 7·23. The time constant for the arsenazo signal was changed at the point indicated on the trace from 1 to 3 s. Solutions all had a $[Ca]_o$ of 3 mM and a constant $[Na]_o$ of 200 mM. Changes in $[K]_o$ were produced by substituting K for choline. The displacement of the $[Na]_i$ and pH_i traces with depolarization is an artifact (see Methods).

is finite) that results from a reduction in membrane potential. On the other hand, using Na sea water and changing to a purely K (and divalent cation) containing sea water, yields a normal Ca influx in the polarized state and a summated effect of Na-free and depolarizing conditions in the K sea water. We have chosen, therefore, to use a sea water containing 265 mM-Na and 200 mM-choline on the basis that Requena *et al.* (1979) have shown that this solution does not produce an increase in $[Ca]_i$ as judged with aequorin in a dialysis capillary. The choline can then be replaced with KCl to the extent necessary to produce membrane potentials in the range -60 to -4 mV. An experiment is shown in Fig. 8 where an axon produced no response whatsoever to 50 mM-K sea water at a time when the $[Na]_i$ measured with the Na electrode was 18 mM, and only small responses to 100 and 200 mM-K sea water. Stimulation that raised $[Na]_i$ to 48 mM resulted in a very substantial response to 50 mM-K solution and indeed a measurable response to 25 mM-K when E_m was -50 mV.

The results of measuring the slopes ($\Delta Ca_i/\Delta t$) of this Figure are plotted in Fig. 9 for two different $[Na]_i$. The shape of the curve for the slope of absorbance with time appears an exponential function of E_m when $[Na]_i$ is 48 mM; for the lower value of $[Na]_i$ it is not possible to measure the slope of the curve over a wide enough range of E_m. What is important to note is that for $[Na]_i$ of 48 mM, the first detectable increase in Ca influx is at −40 mV while for $[Na]_i$ of 18 mM this increase does not occur until $E_m = -18$ mV. A further finding is that the slope of the curve relating Ca entry to E_m has a value of about 2·7 between $E_m = -25$ and 0 mV. This suggests

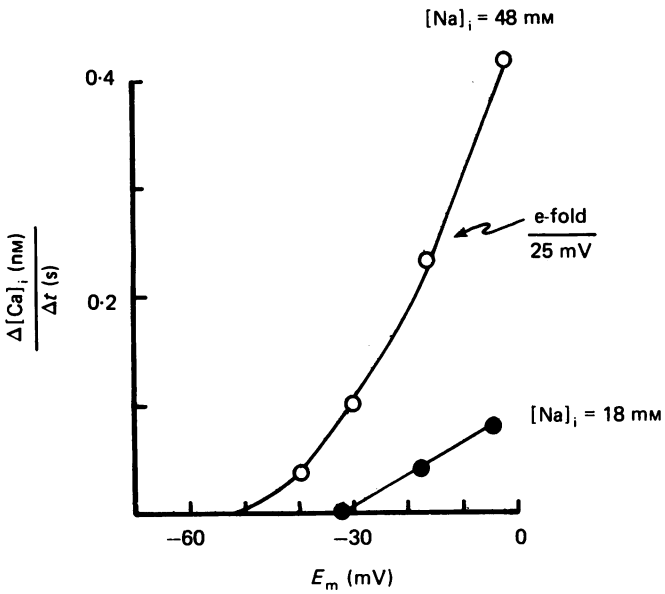

Fig. 9. The results of Fig. 8 are plotted as rate of Ca entry as a function of membrane potential for the two values of $[Na]_i$ studied; (axon 052981).

that the relationship between Ca influx and E_m is: Ca influx = $k \exp(E_m F/RT)$. A similar result was obtained with aequorin as an indicator of Ca_i in two experiments. Previous studies of Ca efflux as a function of E_m (Mullins & Brinley, 1967) showed that Ca efflux = $k' \exp(-E_m F/RT)$ (where F, R, T have their usual meanings), hence the flux ratio for Ca via a Na/Ca exchange carrier is: (influx/efflux) = $k'' \exp(2E_m F/RT)$, an equation appropriate for the movement of 2 net charges per cycle of the carrier. Alternate views of how this sensitivity to E_m might arise are considered in the Discussion.

Ca entry vs. pH. There are results in the literature that suggest that Ca movement may be influenced by changes in pH and that changes in pH may be induced by movement of Ca. Specifically, Lea & Ashley (1978) have noted that aequorin-measured resting glow in barnacle fibres is enhanced by treating the fibres with CO_2 and inhibited by the application of NH_4^+ to the fibre, treatments known to respectively acidify or alkalinize the myoplasm. A similar result has been described in squid giant axons by Mullins & Requena (1979) and in both cases it is possible to show that the result cannot be ascribed to an altered sensitivity of aequorin at different pH values.

Changes in Ca entry with $[H^+]_o$. In experiments with aequorin-injected axons, we

first measured the response to depolarization with 3 mM-Ca, 450 mM-K sea water (50 mM-Mg) at $pH_o = 7{\cdot}8$ and then changed to a sea water of pH 6·6 or to 8·5 and repeated this measurement. The results in three axons were that there was no change in the light response as a result of these changes in pH_o.

Calibration of the pH response of phenol red and of a glass electrode. Since there were substantial reasons for supposing that changes in pH_i would influence Na/Ca

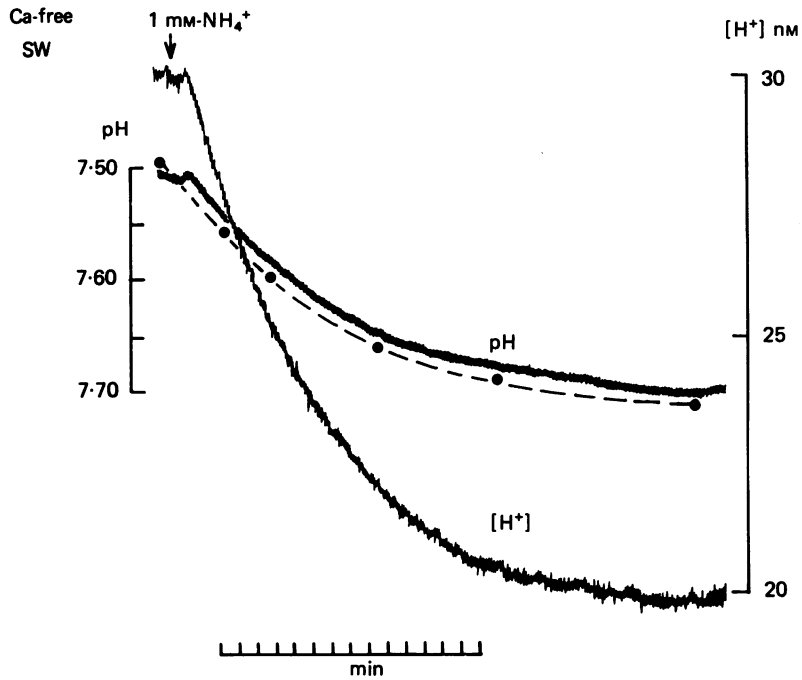

Fig. 10. The response of a phenol red injected axon kept in Ca-free (50 mM-Mg) Na sea water and treated with 1 mM-NH_4 added to the sea water at the time indicated. The curve labelled pH is the response of a glass electrode at the centre of the axon, the curve labelled $[H^+]$ is the spectrophotometer output measuring phenol red absorbance. The dashed line with filled circles is the phenol red curve expressed in pH units.

exchange (Baker & McNaughton, 1977) and that Ca entry might change pH_i (Meech & Thomas, 1977; Ahmed & Connor, 1980), it seemed useful to examine the way that axoplasm might respond to an alkaline challenge from $[NH_4]_o$ both by measuring the response of a glass electrode and that of a pH dye (phenol red).

We have made the assumption that if an axon is in 3 mM-Ca (Na) sea water then there is a reasonable steady state with respect to $[Na]_i$ and $[Ca]_i$ (Requena *et al.* 1977). We have, therefore, assumed further that a measurement of $[H^+]$ by a glass electrode that samples concentration at the centre of the axon and by phenol red that samples $[H^+]$ everywhere in the axoplasm should give the same value. If a change in $[H^+]_i$ is induced rapidly by the use of substances such as external NH_4^+, it is of some interest to know whether the glass electrode and the spectrophotometric measurement of $[H^+]$ give the same value as a function of time. An experiment is shown in Fig. 10 where the phenol red spectrophotometer tracing (labelled $[H^+]$) is compared with the

measured response of a glass pH electrode. The axon had been kept in Ca-free (Na) sea water for about an hour before this measurement was made and hence it had developed a substantially alkaline pH (presumably as a result of Ca unloading) so that when 1 mM-NH_4^+ was added to Ca-free sea water, the result was a change in pH_i of about 0·2 pH unit or a change in $[H^+]$ of about 10 nM. If the phenol red trace is replotted in pH units, the result is the series of filled circles and the dashed line

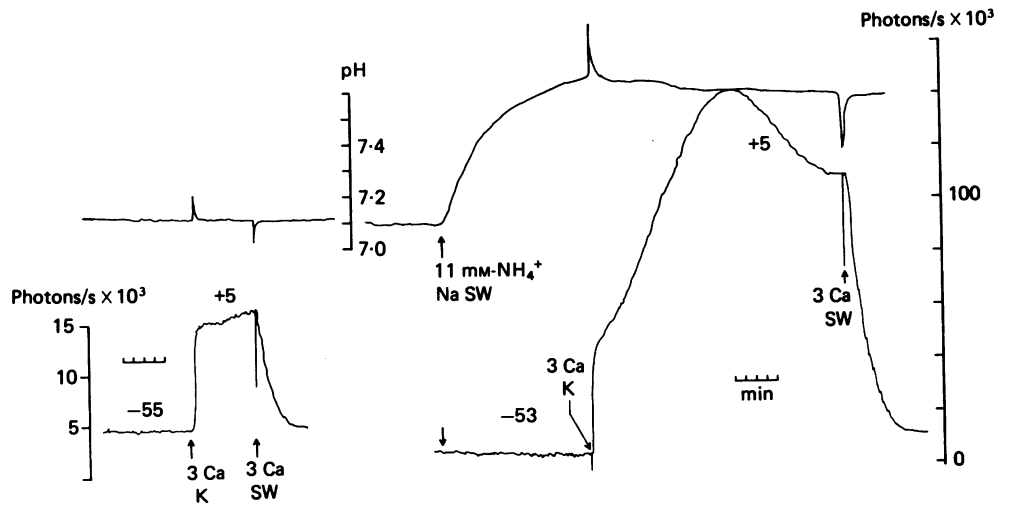

Fig. 11. An axon was injected with aequorin and kept in Ca-free (Na) SW (50 mM-Mg) for 3 h. The axon also had electrodes for the measurement of pH and membrane potential inside; the pH (upper trace) after the Ca-free treatment was 7·12. Membrane potential was not recorded on the chart but displayed digitally and values were transcribed to the record as shown. A depolarization in 3 mM-Ca (50 mM-Mg) 450 mM-K sea water changed the potential from −55 to +5 mV and led to the aequorin record shown on the lefthand lower trace. After recovery in 3 mM-Ca (Na) sea water, the axon was treated with 11 mM-NH_4^+, 3 mM-Ca, (Na) sea water and the value of pH_i changed to 7·62. A depolarization now led to the aequorin record shown on the right (lower trace). Note that the light emission scales are quite different in the two recordings so that the aequorin responses to depolarization differ by a factor of 14; (axon 060281A).

which shows clearly that both methods of measurement of $[H^+]_i$ follow the same time course. This is an important point since, by contrast, it will be demonstrated that when enhanced Ca entry is allowed to occur, the buffering of this Ca in the periphery (by exchange for H^+) results in a *local* change in $[H^+]$ that is visible to the phenol red measuring system.

Changes in Ca entry with $[H^+]_i$. The experiments of Mullins & Requena (1979) showed that making the axoplasm of squid axons alkaline with NH_4^+ added to sea water had two effects: it appeared to increase Ca buffering and to promote a more rapid entry of Ca when Na-free solutions were applied to the outside of the axon. To check on whether the response to depolarization was similarly enhanced by making the axoplasm alkaline, an aequorin injected axon was depolarized and the light response measured; the axon was then treated with 11 mM-NH_4 sea water and a second depolarization undertaken. The results are shown in Fig. 11 where it can be

noted that there is a 14-fold increase in the peak light output in response to depolarization when pH_i had been changed from 7·1 to 7·6. This finding makes it clear that either Na/Ca exchange is enhanced by raising pH_i or, alternately, that buffering of Ca is inhibited so that the same amount of Ca entry produces a larger $[Ca]_i$. In fact, making axoplasm alkaline invariably reduces the aequorin glow, a finding that is easiest to understand if one supposes that intracellular Ca buffering is increased

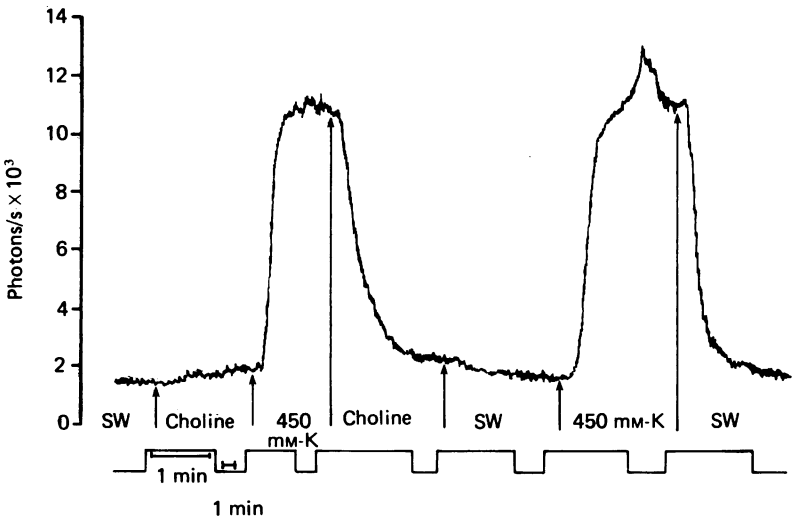

Fig. 12. Light emission as a function of time is shown for an aequorin-injected axon with pH_i is 7·3. $[Ca]_o$ was 10 mM throughout. The chart recorder was run at two speeds as indicated on the bottom trace in order that time constants for the rise and fall of light could be measured accurately. Data for this axon (060481) are given in Table 2.

by elevated pH_i. A way of examining buffering is to compare the rate at which $[Ca]_i$ declines upon repolarization in the presence and absence of Na_o. In the presence of Na_o it is to be expected that the decline of $[Ca]_i$ with time is the result of two processes: the removal of Ca via Na/Ca exchange and the buffering of Ca by intracellular buffers (see Fig. 2). In the absence of Na_o, Ca extrusion is minimized and it is principally buffering that reduces $[Ca]_i$. the comparison is not entirely exact because Ca entry under Na-free conditions is about 10-fold larger (with elevated Na_i) than in the presence of Na_o but since the entry with dpolarization is again another 10-fold larger, the effect is principally a comparison between the decline in Ca_i with or without a Ca extrusion process.

An experiment is shown in Fig. 12 where an aequorin-injected axon (pH_i = 7·3) was transferred to choline sea water, a change that led to a slow upward slope of the base line; the axon was then depolarized in 450 mM-K, 10 mM-Ca solution and when a plateau was reached, the solution changed to choline sea water (10 mM-Ca). The time constant for the decline of light was 15 s. The axon was then changed to Na sea water (10 mM-Ca) and the depolarization–repolarization repeated. The time constant for the decline of light now was 8·5 s suggesting that Na/Ca exchange (which requires Na_o) is an important contributor to the decline in $[Ca]_i$ that follows repolarization. The axon did have a pH electrode inside so that measurements of the

sort shown could be made at other values of pH_i and Table 2 shows in its ultimate and penultimate columns, a comparison of the time constants with which aequorin light declines on repolarization in Na and Choline sea water. Two points may be noted: firstly, that the time constant for light decline in Na is about half that in Na-free solution and secondly that in choline sea water the time constant is shorter at alkaline pH (implying that Ca buffering is faster).

TABLE 2. The effect of pH_i on changes in light with depolarization and repolarization of aequorin-injected axons

pH_i	Axon 060381			Axon 060481			
	Maximum rate of rise of light (photon/s²)	Peak light (photon/s × 10³)	Time constant of fall of light (s)	Maximum rate of rise of light (photon/s²)	Peak light (photon/s × 10³)	Time constant of fall of light (s)	
						Na	Choline
6·6	—	—	—	170	—	21	31
6·7	90	3·5	45	—	—	—	—
7·2	166	3·6	36	—	—	—	—
7·3	—	—	—	620	11	8·5	15
7·4	304	4·3	32	—	—	—	—
7·6	366	6	31	—	—	—	—
7·8	310	6·3	25	—	—	—	—
7·9	476	9·5	30	800	14	6·9	14

The Table also emphasizes that the maximum rate of rise of the aequorin signal (proportional to Ca entry) is more rapid as pH_i becomes more alkaline in both the axons studied and that the absolute value of peak light is also larger. The $[Na]_i$ was not measured in either axon but the relatively low values for peak light suggest a low $[Na]_i$. Values of pH less than 7·3 were obtained by measuring during the transient acidification that follows removal of $[NH_4]_o$ (Boron & DeWeer, 1976).

A freshly isolated axon was kept in 10 mM-NH_4 (Na) sea water until pH_i as measured with a glass electrode showed a value of 7·6. The axon was then transferred to NH_4-free sea water and when pH_i had fallen to 7·2, the axon was tested with 450 mM-K. These tests were repeated between pH_i 6·6 and 7·85 with the results shown in the Fig. 13. The record supports the view that Ca entry with depolarization is enhanced by an alkaline pH_i even in freshly isolated (low Na_i) axons.

Changes in $[H]_i$ upon Ca entry. Using phenol red, a previous study of the change in pH_i in squid axons produced by stimulation of the axons in 100 mM-Ca sea water (Tiffert *et al.* 1981) showed that an acidification occurred and that this could be abolished by removing Ca from sea water. An experiment of this sort is shown in Fig. 14 from which it can be noted that stimulation in 112 mM-Ca sea water increases the $[H^+]_i$ as measured by a phenol red spectrophotometer signal and that H^+ continues to increase somewhat after stimulation has ceased. By comparison, if stimulation is made in Ca-free sea water there is no pH change. This behaviour is in contrast to the behaviour of a Ca entry signal measured either with aequorin or with arsenazo where, upon terminating stimulation, the response is an immediate decline in $[Ca]_i$. Since the explanation for this effect appeared to be that the buffering of entering

Ca occurs by the release of protons in exchange for Ca^{2+} by the intracellular Ca buffering systems, it seemed useful to see whether changes in pH_i could be produced by a steady depolarization of an axon with elevated [K] in sea water. An experiment where the response of an axon to depolarization was studied while $[H]_i$ was being measured with phenol red is shown in Fig. 15. The axon had an initial $[Na]_i$ as measured by electrode of 19 mM and hence would not be expected to admit much Ca on depolarization.

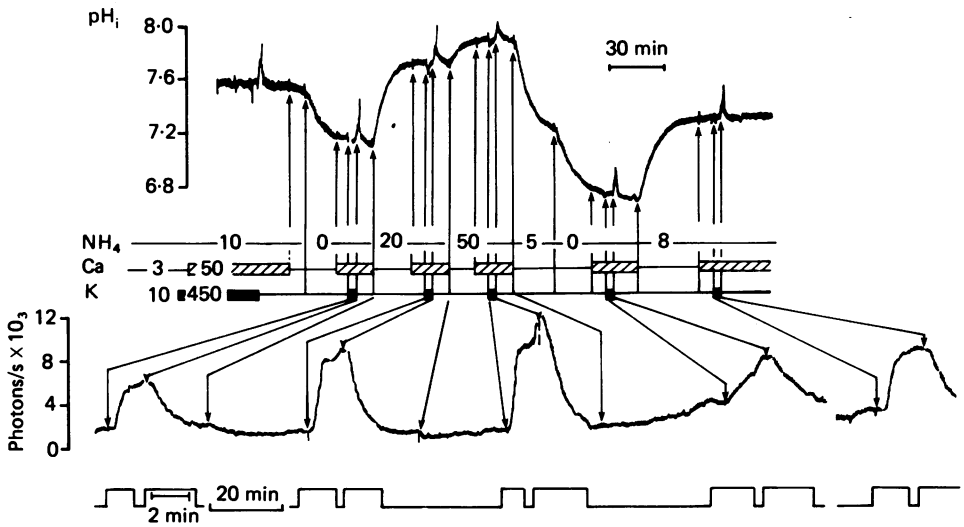

Fig. 13. An aequorin-injected axon in 10 mM-NH_4 (Na) sea water was transferred to NH_4-free sea water and to other $[NH_4]_o$. This allowed increases or decreases in pH_i to be made as transients (Boron & DeWeer, 1976). The pH_i was measured with a glass electrode and its response is shown in the top trace. The peaks in this pH electrode record are artifacts from the high impedance and capacitance of the electrode. On an expanded time scale, the light emission of the axon is shown in the lower trace as a rise and fall of light emission upon depolarization and repolarization. In this trace, two time bases were used so that the rise and fall of light emission could be measured accurately. These changed time bases are indicated by the line at the bottom of the Figure; (axon 060381).

The phenol red signal confirms that there is no measurable pH change (other than the inevitable base-line drift) even if 450 mM-K is used for depolarization. The axon was then stimulated to increase $[Na]_i$ to 30 mM and depolarization now leads to a clear change in pH_i which is not apparent for lesser degrees of membrane depolarization. The experiment emphasizes that Ca entry must be quite substantial if a measurable change in $[H]_i$ is to be produced. This is in agreement with the known strong pH buffering properties of squid axoplasm (Boron & DeWeer, 1976).

Ca entry in CN or apyrase-treated axons. Previous studies (Brinley, Tiffert & Scarpa, 1978) of squid giant axons treated with CN for periods of time less than 1 h showed that, although ATP levels are maintained close to normal, the buffering of large loads of Ca (when $[Ca]_i$ is greater than 1 μM) is much reduced if 2 mM-CN is present in the sea water. The explanation of this effect is presumably that in the presence of CN mitochondria cannot take up Ca, but as long as ATP is present in axoplasm mitochondria do not release previously accumulated Ca. In our present studies, three

axons were injected with arsenazo III, stimulated in Na sea water to raise levels of $[Na]_i$ to around 25 mM, and then depolarized with 450 mM-K in the presence and absence of CN. The presence or absence of this inhibitor did not influence the response to depolarization suggesting that mitochondrial Ca buffering is not involved in the

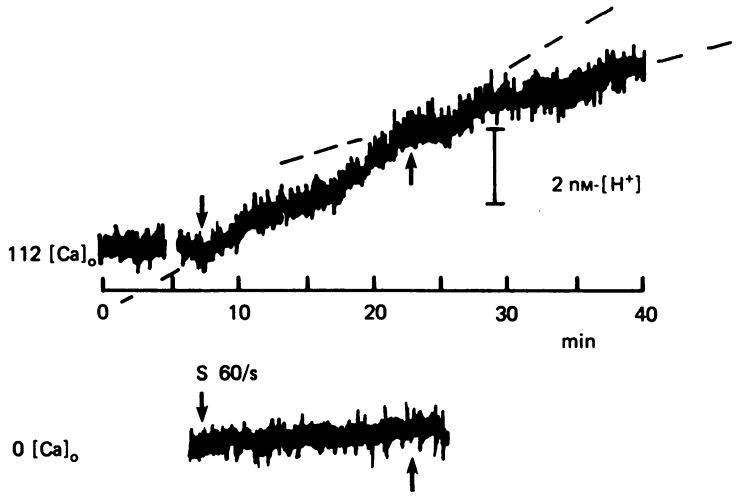

Fig. 14. The trace of phenol red absorbance with time is shown (lower trace) for stimulation of a squid axon at 60/s in Ca-free sea water and in the upper trace with $[Ca]_o$ 112 mM. An upward movement is an increase in $[H^+]_i$. A downward arrow indicates the start of stimulation (s) and an upward one a cessation of stimulation.

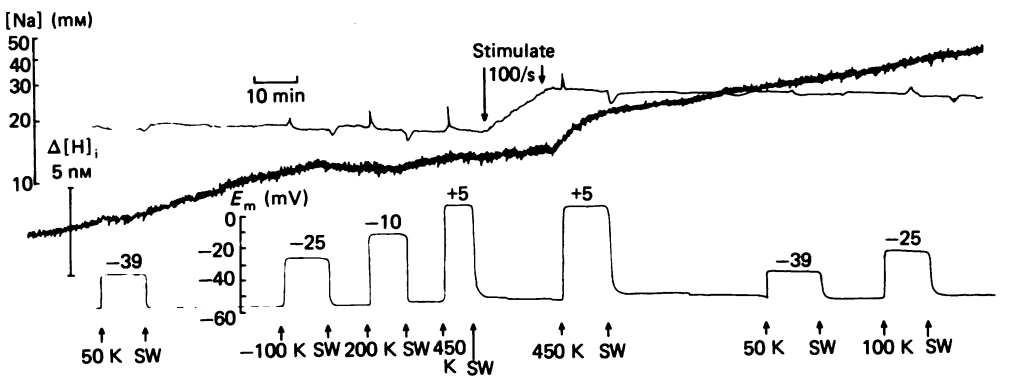

Fig. 15. An axon was injected with phenol red to yield a final concentration of 205 μM. Both $[Na]_i$ (top trace) and E_m (bottom trace) were measured as well as the phenol red signal. Test depolarizations were imposed as shown on the bottom trace.

measured change in $[Ca]_i$. Since the rate at which mitochondria accumulate Ca is very slow for values of $[Ca]_i$ less than 1 μM, these measurements suggest that Ca concentrations in axoplasm do not reach this value during prolonged depolarizations such as have been measured in this work. This value is some 30 times the resting value of $[Ca]_i$ that has been measured previously (DiPolo *et al.* 1976).

Another change in axoplasm was to introduce the enzyme apyrase (50 u. in 10 μl

of 100 mM TES buffer pH 7·3) by microinjection into the axoplasm. About 0·5 μl or 2·5 units were actually injected and since a unit is defined as the quantity of enzyme that will catalyse the hydrolysis of 1 μmol P/min, the actual destruction of most of the ATP in the fibre should take place. In other axons we have measured ATP in the range of 20–40 μM (or 1/100 of its initial value) 15 min after such an injection. Apyrase treatment seemed worth looking at because DiPolo (1979) has shown that Ca entry dependent on Na_i (as measured with ^{45}Ca) was also enhanced by ATP.

We injected two axons with arsenazo III and measured their responses to depolarization in 3 mM-Ca sea water; the axons then were injected with apyrase and this allowed to act for 15 min following which another test depolarization was made. The responses to the two depolarizations before and after the injection of apyrase were identical indicating that ATP does not influence the large Ca entry brought about by depolarization. In some respects this finding is similar to the known effects of ATP on Ca efflux that is brought about by Na/Ca exchange. If $[Ca]_i$ is high and Ca efflux large, then ATP has only a modest effect in increasing Ca efflux (*ca.* 20%) while if $[Ca]_i$ is small, there is a large (5-fold or more) enhancement of Ca efflux (DiPolo, 1977).

DISCUSSION

The experiments described in this report make it clear that Ca entry into squid axons in response to depolarization is a complex process that involves the interaction of a number of variables. The fact that Ca entry with steady depolarization can be essentially abolished by lowering $[Na]_i$ is the basis we have for assuming that it is Na/Ca exchange that controls Ca entry and that, in the course of its operation, this process involves the exchange of more Na charges than Ca charges. We have been able to measure a $[Na]_i$ where Ca entry is half maximal when $[Ca]_o$ and membrane potential are specified, but it should not be assumed that the value measured indicates solely the equilibrium constant for the reaction of Na^+ with a carrier since as Ca enters, a series of complex and not completely understood reactions are set in motion and these must be expected to affect Ca entry. The influx of Ca is followed by an absorption of the vast majority of these ions by intracellular buffering systems. For small Ca loads, Brinley *et al.* (1977) showed that 1 Ca^{2+} in 20 entering could contribute to the ionized Ca of the fibre while for larger loads, a CN- and FCCP-sensitive Ca buffering was present such that only 1 Ca^{2+} in 2000 appeared as ionized Ca. The measurements that we have made suggest strongly that Ca buffering occurs by the exchange of Ca^{2+} for protons released by the Ca buffers. Thus, most of the Ca entry goes not to produce a change in $[Ca]_i$ but rather to produce a change in intracellular H^+ release. Measurements of the equivalence between Ca entry and change in pH_i are neither easy nor extensive enough for us to specify a stoichiometry between Ca^{2+} uptake and proton release but if there were a 2 H^+:1 Ca^{2+} stoichiometry, then for the entry of 1000 Ca^{2+} at low concentrations where 20 are buffered for each one free we would have an increase of 50 Ca^{2+} intracellularly and 1900 protons would be produced. Since the measurements of Boron & DeWeer (1976) indicate that 10^4 protons are bound for each one free at normal values for pH_i, this would mean that the 1900 protons would be reduced to 0·19. We conclude, therefore, that the buffering of modest increases in $[Ca]_i$ can be accomplished with negligible changes in pH_i.

Our measurements do show, however, that changes in pH_i at the level of 0·02 to 0·05 pH unit can be measured under conditions where other experiments show very large Ca entries. We emphasize that these measurements are undoubtedly substantial underestimates of the actual pH change since (*a*), pH buffering may not be instantaneous, and (*b*), radial gradients in $[Ca]_i$ imply radial gradients in $[H]_i$ at least momentarily. Thus the rather modest estimates of changes in pH_i based on an assumption of a uniform distribution of $[H]_i$ do not rule out much larger concentrations near the membrane. Since our experiments also show that a decrease in pH_i substantially inhibits Ca entry, one can note that Ca entry leads to a lowering of pH_i, and this in turn leads to a lowering of Ca entry. The arrangement serves to limit Ca entry unless (as in our experiments with NH_4) intracellular pH buffering capacity is increased. It also could serve to steepen the relationship between $[Na]_i$ and Ca entry if the axon periphery is saturated with Ca and may in part explain the observed Hill coefficients of about 7.

A second sort of measurement that we have made indicates that when $[Ca]_o$ is about 600 μM, Ca entry with depolarization is half-maximal if $[Na]_i$ is saturating. Measurements with ^{45}Ca (Brinley, Spangler & Mullins, 1975) show that Ca efflux is half saturated at a $[Ca]_i$ of about 1 μM; this measurement was made on axons with a normal membrane polarization. But, because apparent carrier affinities must be expected to depend in a complex way on [H], [Na], and E_m the relative affinities of the Na/Ca exchange carrier might not be necessarily different at both membrane surfaces. An important practical consequence of the finding that the carrier is essentially saturated with Ca at values of $[Ca]_o$ of 2 mM or greater is that all physiological effects observed at elevated $[Ca]_o$ must be due to Ca moving via channel mechanisms.

Carrier model considerations would suggest that under the simplest circumstances Ca entry into cells should be given by (Mullins, 1977):

$$k[Ca]_o ([Na]_i)^r \exp [(r-2)\, EF/RT],$$

where k is a constant. Thus the equation predicts that depolarization *per se* will not produce a Ca entry if $[Na]_i$ is not finite and that at a fixed level of E_m, Ca entry is larger if $[Na]_i$ is larger. Both of the predictions have been confirmed and the entry of Ca with depolarization is consistent with a value r for Na/Ca coupling of 4 but higher values are not ruled out.

A possible complication in this analysis is that Baker *et al.* (1971) observed under voltage clamp that the TTX-insensitive Ca entry with depolarization had a maximum at a membrane potential close to zero. Ca entry declined to very low values both for potentials highly positive and highly negative. This sort of behaviour for a system that clearly must be Na/Ca exchange could be explained by supposing that the unloaded carrier has a fixed negative charge near its Ca binding site so that for large positive membrane potentials most of the carrier is held at the inside of the membrane and hence, unavailable for Ca transport inward. Under this arrangement, a change in membrane potential would both release unloaded carrier from its pool at the membrane interfaces as well as affect the rate of translocation of loaded carrier.

The foregoing analysis of Ca entry with depolarization is complicated by the following factors. Ca entry has been claimed to take place via the following

mechanisms: (*a*), Na channels; (*b*), K channels; (*c*), Ca channels; (*d*), Na/Ca exchange, and (*e*) leakage pathways. A definition of each of these pathways has in some instances an ambiguity but for (*a*) the Ca entry with steady depolarization is unaffected by the presence of TTX in sea water (Baker *et al.* 1973*a*,*b*) and Ca entry with depolarization can be greatly attentuated with a decrease in $[Na]_i$ (i.e. presumably (*d*)) (Mullins & Requena, 1981). This leaves Ca entry via (*b*), (*c*), and (*e*) as remaining mechanisms. It is useful to note that for (*e*) the expected effect of depolarization is a substantial decrease in Ca influx as a result of the diminished driving force of membrane potential. Thus, one is left with the conclusion that an axon poisoned with TTX and with a low $[Na]_i$ should have a Ca entry with depolarization that is enhanced only if Ca moves through either K or Ca channels and diminished by the extent to which Ca entry is via leakage. We have no basis for separating Ca entry between these two separately described entities but note that virtually all Ca entry as normally measured in Na_i-containing axons is abolished by a reduction in Na_i.

Aided by grants BNS-8006271 from the National Science Foundation, and PPG 5 PO1 NS 14800-03, and 1 R01 NS 17718-01 from the National Institutes of Health. We are indebted to the Director and staff of the Marine Biological Laboratory, Woods Hole, Massachusetts for facilities placed at our disposal. Valuable comments on the manuscript were provided by Drs D. Eisner and R. Vaughan-Jones.

REFERENCES

AHMED, Z. & CONNOR, J. A. (1980). Intracellular pH changes induced by calcium influx during electrical activity in molluscan neurons. *J. gen. Physiol.* **75**, 403–426.

AHMED, Z., KRAGIE, L. & CONNOR, J. A. (1980). Stoichiometry and apparent dissociation constant of the Ca-arsenazo III reaction under physiological conditions. *Biophys. J.* **32**, 907–920.

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

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

BAKER, P. F. & MCNAUGHTON, P. A. (1976). Kinetics and energetics of calcium efflux from intact squid giant axons. *J. Physiol.* **259**, 103–144.

BAKER, P. F. & MCNAUGHTON, P. A. (1977). Selective inhibition of the Ca-dependent Na efflux from intact squid axons by a fall in intracellular pH. *J. Physiol.* **269**, 78*P*.

BAKER, P. F. & HONERJÄGER, P. (1978). Influence of CO_2 on level of ionized Ca in squid axons. *Nature, Lond.* **273**, 160–161.

BAKER, P. F., MEVES, H. & RIDGWAY, E. G. (1973*a*). Effects of manganase and other agents on the calcium uptake that follows depolarization of squid axons. *J. Physiol.* **231**, 511–526.

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

BORON, W. F. & DEWEER, (1976). Intracellular pH transients in squid giant axons caused by CO_2, NH_3 and metabolic inhibitors. *J. gen. Physiol.* **67**, 91–112.

BRINLEY, F. J. JR & MULLINS, L. J. (1967). Sodium extrusion by internally dialyzed squid axons. *J. gen. Physiol.* **50**, 2303–2331.

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

BRINLEY, F. J. JR, TIFFERT, J. T. & SCARPA, A. (1978). Mitochondria and other calcium buffers of squid axons studied in situ. *J. gen. Physiol.* **72**, 101–127.

BRINLEY, F. J. JR, TIFFERT, T., SCARPA, A. & MULLINS, L. J. (1977). Intracellular calcium buffering capacity in isolated squid axons. *J. gen. Physiol.* **70**, 355–384.

BROWN, J. E., COHEN, L. B., DEWEER, P., PINTO, L. H., ROSS, W. N. & SALZBERG, B. M. (1975). Rapid changes of intracellular free calcium concentration. Detection by metallochromic indicator dyes in squid giant axon. *Biophys. J.* **15**, 1155–1160.

CALDWELL, P. C. (1954). An investigation of the intracellular pH of crab muscle fibres by means of micro-glass and micro-tungsten electrodes. *J. Physiol.* **126**, 169-180.

DIPOLO, R. (1977). Characterization of the ATP-dependent calcium efflux in dialyzed squid giant axons. *J. gen. Physiol.* **69**, 795–814.

DIPOLO, R., REQUENA, J., BRINLEY, F. J., MULLINS, JR, L. J., SCARPA, A. & TIFFERT, T. (1976). Ionized calcium concentrations in squid axons. *J. gen. Physiol.* **67**, 433–467.

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

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

KEYNES, R. D. & LEWIS, P. R., (1951). The sodium and potassium content of cephalopod nerve fibres *J. Physiol.* **114**, 151–182.

LEA, T. J. & ASHLEY, C. C. (1978). Increase in free Ca^{2+} in muscle after exposure to CO_2. *Nature, Lond.* **275**, 236–238.

MEECH, R. W. & THOMAS, R. C. (1977). The effect of Ca injection on the intracellular Na and pH of snail neurons. *J. Physiol.* **265**, 867–879.

MULLINS, L. J. (1977). A mechanism for Na/Ca transport. *J. gen. Physiol.* **70**, 681–695.

MULLINS, L. J. (1979). Transport across axon membranes. In *Membrane Transport in Biology*, vol. II, ed. TOSTESON, D. C., pp. 161–210. New York: Springer-Verlag.

MULLINS, L. J. & BRINLEY, F. J. Jr (1967). Some factors influencing sodium extrusion by internally dialyzed squid axons. *J. gen. Physiol.* **50**, 233–2355.

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

MULLINS, L. J. & REQUENA, J. (1979). Ca measurement in the periphery of an axon. *J. gen. Physiol.* **74**, 393–413.

MULLINS, L. J. & REQUENA, J. (1981). The late Ca channel in squid axons. *J. gen. Physiol.* **78**, 683–700.

REQUENA, J., DIPOLO, R., BRINLEY, F. J. JR & MULLINS, L. J. (1977). The control of ionized calcium in squid axons. *J. gen. Physiol.* **70**, 329–353.

REQUENA, J. & MULLINS, L. J. (1979). Ca movement in nerve fibers. *Q. Rev. Biophys.* **12**, 371–460.

REQUENA, J., MULLINS, L. J. & BRINLEY, F. J. JR (1979). Calcium content and net fluxes in squid giant axons. *J. gen. Physiol.* **73**, 327–342.

THOMAS, M. V. (1979). Arsenazo III forms 2:1 complexes with Ca and 1:1 complexes with Mg under physiological conditions. *Biophys. J.* **25**, 541–548.

TIFFERT, T., MULLINS, L. J., WHITTEMBURY, J. & BRINLEY, F. J. JR (1981). The measurement of internal H^+ in squid axons during stimulation. *Biophys. J.* **33**, 61a.

VASSORT, G., TIFFERT, T., WHITTEMBURY, J. & MULLINS, L. J. (1982). The effects of internal Na^+ and H^+, external Ca^{2+} and of membrane potential on Ca entry in squid axons. *Biophys. J.* **37**, 68a.