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# Voltage-Dependent Changes in the Permeability of Nerve Membranes to Calcium and Other Divalent Cations [and Discussion]

P. F. Baker, H. G. Glitsch and E. Rojas

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# Voltage-dependent changes in the permeability of nerve membranes to calcium and other divalent cations

By P. F. Baker* and H. G. Glitsch†
*The Physiological Laboratory, Cambridge*

Transmitter release from depolarized nerve terminals seems to be preceded by a rise in the intracellular concentration of ionized calcium. In squid giant axons, depolarization promotes calcium entry by two routes: one that is blocked by tetrodotoxin and one that is insensitive to tetrodotoxin. The TTX-sensitive route seems to be the sodium channel of the action potential; but the TTX-insensitive route seems to be quite distinct from the sodium and potassium channels of the action potential. It is blocked by $Mg^{2+}$, $Mn^{2+}$ and $Co^{2+}$ ions and by the organic calcium antagonist D-600 and has many features in common with the mechanism that couples excitation to secretion.

## Introduction

The central role of sodium in excitable tissues stems from two factors: (1) the electrochemical gradient of sodium ions is steep and inwardly directed and (2) the membrane permeability to sodium ions is dependent on voltage and time. Sodium is not unique in these respects. In many animal cells the same two factors that underlie the importance of sodium also apply to the divalent cations calcium and magnesium. Thus in a number of excitable tissues, the permeability of the cell membrane to both calcium and magnesium seems to be sensitive to the membrane potential and for both these cations the electrochemical gradient is inwardly directed and for calcium, very steep indeed. It follows that calcium and magnesium ions are potentially capable of carrying inward current into the cell and hence of participating, like sodium ions, in the generation of action potentials.

As much more is known about calcium than magnesium, the following discussion will, of necessity, centre on calcium; but mention of magnesium will be made where information is available. There is good evidence that calcium can serve as the major carrier of inward current during the action potential of some nerve and muscle cells (see review by Reuter 1973); but in many other excitable cells the extra entry of calcium during the action potential is small compared to that of sodium (see for instance Hodgkin & Keynes 1957). In all excitable cells, irrespective of the quantitative contribution of calcium to the inward current, an important consequence of an increased entry of calcium into the cell is the change this brings about in the intracellular concentration of ionized calcium. In the few large cells where measurement has proved possible, the intracellular concentration of ionized calcium is less than 1 μM and probably close to 0.1 μM (see review by Baker 1972). Because the intracellular activity of calcium is so low, a quantitatively small increment in calcium entry can effect a large increase in the absolute concentration of ionized calcium in the cell. This assumes considerable physiological significance when coupled with the fact that many enzyme systems including those responsible for contraction and secretion are sensitive to the intracellular concentration of

* Present address: Department of Physiology, King's College, London WC2R 2LS.
† On leave from the Department of Cell Physiology, Ruhr-University, Bochum, W. Germany.

ionized calcium. It follows that changes in calcium entry may serve to couple membrane events to biochemical processes occurring inside the cell and a rise in calcium entry can be used to trigger certain specific forms of cell behaviour such as contraction and secretion.

In this paper we shall discuss some features of the voltage-sensitive changes in calcium permeability seen in nerve cells and although much of the data finds parallels in other excitable cells no systematic comparison will be attempted as this has recently been the subject of a review by Reuter (1973).

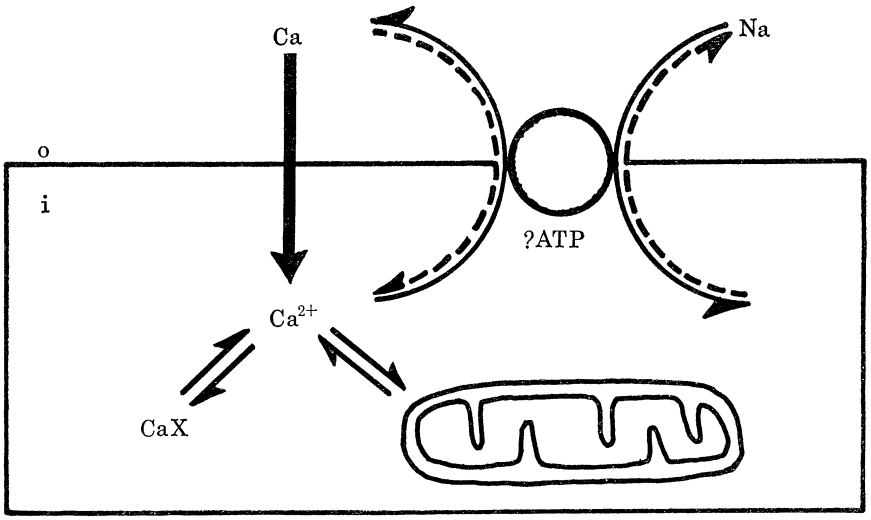


FIGURE 1. Diagrammatic summary of the reactions known to be involved in the regulation of the intracellular concentration of ionized calcium in nerve cells. The intracellular ionized calcium is maintained by the interplay of four factors: the rate of calcium entry, the rate of calcium extrusion (shown as an exchange for external sodium ions), and the effectiveness of various intracellular buffer systems including the mitochondria and a number of other calcium-binding systems designated X.

In order to obtain a more critical understanding of the available experimental results, it is necessary to consider briefly the various processes known to be involved in the regulation of the intracellular concentration of ionized calcium in nervous tissues (for further information see the review of Baker 1972). These are illustrated schematically in figure 1. A calcium ion entering a nerve may suffer one of at least four possible fates: it may remain in the cytosol as ionized calcium; it may form an undissociated complex with some component of axoplasm; it may be sequestered within intracellular structures such as mitochondria or it may immediately be expelled from the cell by some form of pump. In response to a sudden increase in calcium entry, the quantitatively most important reactions seem to be intracellular buffering of the calcium both by binding to axoplasmic constituents and by uptake into the mitochondria and rather little of the calcium that enters remains as ionized calcium. As the calcium content of the cell does not increase but stays rather constant with time, in the long term any calcium entering the cell must ultimately be removed and this seems to be achieved by calcium pumping systems in the surface membrane. Two systems for pumping calcium out of animal cells have been described: one in which the energy for the extrusion of calcium is derived directly from the splitting of ATP and a second in which calcium is exchanged for extracellular sodium by means of a counter-transport system, the energy for the extrusion of calcium apparently being derived from the inward movement of sodium ions down their electrochemical gradient. So far, only the second of these mechanisms has been clearly demonstrated in nervous tissue. Of

particular significance to the present topic is the suggestion that both of these systems may be inherently electrogenic (i.e. during calcium pumping the net movement of charge across the cell membrane is probably not zero) and hence both these calcium pumps are likely to be sensitive to changes in membrane potential (Baker 1972; Blaustein 1974; Schatzmann 1974).

## Calcium entry during the action potential

### *Experiments using radioactive tracers*

The most direct evidence for an increased entry of calcium during nervous activity is the observation that propagation of impulses in the squid giant axon is accompanied by a small net entry of calcium (Flückiger & Keynes 1955; Hodgkin & Keynes 1957). Separate measurements were made of the effects of stimulation on calcium influx and calcium efflux. Calcium influx was measured by stimulating one axon of a pair from the same animal in sea water containing $^{45}$Ca, the unstimulated axon being used to measure the resting entry of calcium from the same solution. Calcium influx was determined with considerable accuracy in these experiments by measuring the $^{45}$Ca content of axoplasm extruded from the axons. The extra calcium entry per pulse was obtained by subtracting the resting from the stimulated influx and dividing by the number of impulses carried. In separate experiments nervous activity had no effect on the efflux of $^{45}$Ca from axons previously loaded with the tracer by microinjection.

In seawater containing 10.7 mM Ca and 55 mM Mg, the extra uptake of Ca per impulse at 20 °C is about 0.006 pmol/cm$^2$ – that is 36 Ca ions per square micrometre or $\frac{1}{700}$ of the net entry of sodium. The calcium entry per impulse increases roughly linearly with the external calcium concentration and at 112 mM calcium the extra entry is about 0.08 pmol/cm$^2$ impulse. As the extra entry of calcium per pulse is very small, Hodgkin & Keynes (1957) had to use long periods of stimulation involving up to $10^5$ impulses and one possible explanation of their results is that the extra calcium entry was brought about not by a primary increase in calcium permeability but by some other change occurring in the axon for instance it may be secondary to a rise in intracellular sodium. This is quite possible because Hodgkin & Keynes (1957) used fresh axons with a low internal sodium content and there is good evidence that a rise in internal sodium can lead to increased calcium influx, apparently through partial reversal of the sodium–calcium counter-transport mechanism in the cell membrane – calcium now entering the cell in exchange for internal sodium (see Baker, Blaustein, Hodgkin & Steinhardt 1969). For technical reasons, it is difficult to rule out this possibility entirely in experiments on intact axons but it seems rather unlikely for the following reasons:

(1) The extra calcium influx in previously cold-stored axons, i.e. axons with an elevated internal sodium content, is about the same as in fresh axons, despite a much higher resting influx in the cold-stored axons (see table 2). As an increase of internal sodium enhances calcium entry (Baker *et al.* 1969), the relative constancy of the extra calcium entry per pulse at two quite different internal sodium concentrations strongly suggests that the extra entry is not secondary to changes in internal sodium.

(2) Lowering the temperature from 20 to 8 °C reduces the resting calcium influx from seawater containing 112 mM calcium. Under the same conditions the extra calcium entry per pulse is increased from 0.08 to 0.15 pmol/cm$^2$.

(3) Calcium influx is increased in axons depolarized by exposure to seawater containing




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50–200 mM potassium. This effect is unlikely to be secondary to a rise in internal sodium because raising the external potassium concentration stimulates the sodium pump and should, if anything, lead to a fall in internal sodium. If depolarization promotes calcium entry, one might expect that the calcium influx in response to a maintained potassium depolarization should be considerably greater than that during an equivalent period of repetitive stimulation. This is not the case. External K concentrations from 50 to 200 mM increase the rate of calcium influx from seawater containing 112 mM calcium from 0.61 pmol $cm^{-2}$ $s^{-1}$ to 2.9 pmol $cm^{-2}$ $s^{-1}$. In the presence of the same calcium concentration and 10 mM potassium, stimulation at 156 impulses/s gives a mean calcium uptake of 15.2 pmol $cm^{-2}$ $s^{-1}$. It follows that either depolarization is not the major factor promoting calcium entry, or that calcium entry, like that of sodium, is not maintained in response to a steady depolarization. According to the second possibility the maximum entry of calcium should be seen, as it is, in response to a series of brief (1–5 ms) depolarizations returning to the resting potential between each pulse. There is now good experimental evidence in squid axons that calcium entry in response to a maintained depolarization is only transient (see p. 402) and it is quite possible that the increased uptake of calcium seen in squid axons exposed for long periods (30–60 min) to K-rich solutions may reflect changes in calcium pump activity and not changes in calcium permeability. This possibility finds some support in the squid axon where K-rich seawaters are known to promote a component of the sodium efflux that is dependent on external Ca ions (Baker *et al.* 1969); but this same argument cannot be applied to all nerves. For instance, exposure of the walking leg nerves of the spider crab *Maia squinado* to K-rich seawaters also increases the uptake of calcium, but the properties of this uptake render unlikely the possibility that it results from an alteration in calcium pumping (see Baker 1972).

To summarize, in the squid giant axon experiments with $^{45}Ca$ provide clear evidence for an increased uptake of calcium both after a prolonged period of nervous activity and also in response to K-rich solutions. Similar results have been described in other preparations including the adrenal medulla (Douglas & Poisner 1962); sympathetic ganglia (Blaustein 1971) the neurohypophysis (Douglas & Poisner 1964; Dreifuss, Grau & Nordmann 1973) and synaptosomes (Blaustein, Johnson & Needleman 1972) but in all instances the data do not allow an entirely clear-cut distinction to be made between primary voltage-sensitive changes in calcium permeability and changes in calcium pumping that may occur in response to changes in membrane potential or secondarily to a redistribution of sodium or potassium ions.

At least part of this problem might be resolved if it were possible to monitor the calcium influx in a single axon continuously. Changes in calcium pumping that result from redistribution of sodium or potassium ions might be expected to persist for some time after stimulation ceases whereas a voltage-sensitive alteration in calcium permeability should be restricted to the period of stimulation, the rate of calcium uptake returning to its initial value after stimulation.

An apparatus for making continuous measurements of $^{45}Ca$ uptake in squid giant axons is illustrated in figure 2*a*. It is based on a technique first described by Caldwell & Lea (1973) for measuring uptake of $^{14}C$-labelled compounds in squid axons. The principle is to insert along the long axis of the fibre a rod of a cerium-activated lithium glass (Koch-Light Laboratories Ltd, Glass Scintillator Type G.S.F. 1), 100 μm in diameter, that serves as an internal scintillator. The energy of the β particles emitted by the $^{45}Ca$ is rather weak, and only a very small fraction of the β particles emitted from $^{45}Ca$ in the external solution penetrate to the internal scintillator which largely responds to radioactivity that has entered and become distributed


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within the axon. This technique allows continuous measurement of small amounts of intracellular radioactivity in the presence of very large amounts of extracellular tracer. The scintillator seems to be non-toxic and does not affect impulse conduction. The main drawback is that the internal scintillator is rather inefficient. When compared with conventional liquid scintillation techniques, the internal scintillator used in these experiments had an efficiency only a few percent of that of Brays solution.

Figure 2*b* illustrates an essential check on the performance of the internal scintillator. Complete replacement of external sodium by lithium ions produces a large and reversible

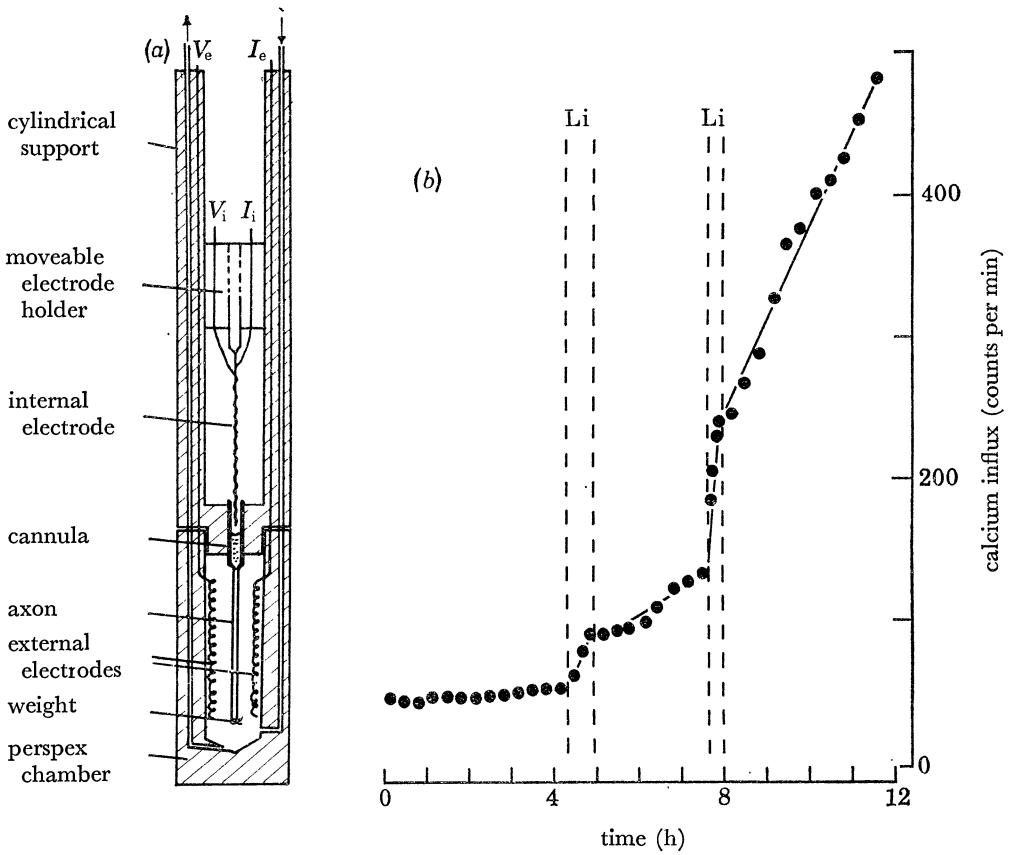


FIGURE 2. Use of an internal scintillator for the continuous measurement of calcium uptake in squid axons. (*a*) Vertical section (diagrammatic) through the device used to effect axial impalement of the axon with the internal scintillator, shown wound with a double spiral of platinum wire for voltage-clamp studies. The whole apparatus was cylindrical in cross-section and fitted into the well of an automatic liquid scintillation counter (Packard). The internal scintillator was rendered ineffective over all but 2.0 cm of its length by application of a series of coats of shellac. The exposed portion of scintillator started 0.5 cm from the tip of the probe. The current and voltage wires were insulated such that the voltage was measured over about 5–10 mm in the middle of the patch of exposed scintillator and current could be passed over a length that exceeded by 2–3 mm at each end the patch of exposed scintillator. All bare regions of platinum were platinized.

(*b*) Changes in calcium influx following isotonic replacement of external sodium by lithium. The apparatus used was that shown in figure 2*a*. The external solution was artificial seawater containing (mM): NaCl, 460; KCl, 10; $MgCl_2$, 55; $CaCl_2$, 11 and $NaHCO_3$, 2.5 except for the periods indicated when the NaCl was replaced isosmotically by LiCl (Fisher). Temperature 23 °C. Axon diameter 700 μm. By calibrating the internal scintillator in the external solution and knowing the diameter of the axon in the recording region, it is possible to express the rate of gain of counts as a flux. The first exposure to lithium increased the influx from 0.08 to 3.2 pmol $cm^{-2}$ $s^{-1}$ and the second from 0.23 to 7.6 pmol $cm^{-2}$ $s^{-1}$. In each case on return to sodium seawater the flux was reduced but not to its value before exposure to lithium.

increase in calcium influx which is in complete agreement with earlier results obtained by the conventional paired axon technique. The increased calcium influx seen in Li seawater results from reversal of the sodium–calcium counter transport process (see Baker *et al.* 1969) and the changes in calcium influx observed with the internal scintillator are within the range obtained by other methods.

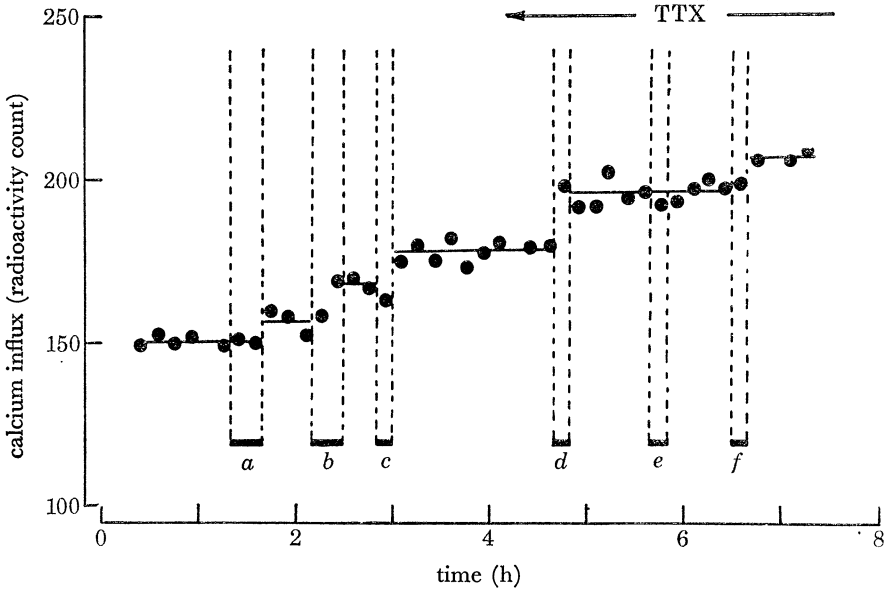


FIGURE 3. Continuous measurement of calcium influx in an axon of *Loligo forbesi* under voltage clamp conditions. External solution Mg-free artificial seawater containing (mM): $CaCl_2$, 20; NaCl, 400; choline-chloride, 140; KCl, 10; $NaHCO_3$, 2.5. The axon had been pre-injected over 4 cm with 2 M tetraethylammonium chloride to give a final intracellular TEA concentration of about 60 mM. The axon was stimulated at 160 pulses/s during the periods marked with solid bars. Details of the stimulations are: *a*, membrane action potentials; *b*, depolarizing voltage clamp pulses of 2 ms duration and amplitude −80 mV; *c*, *d* and *f*, depolarizing voltage clamp pulses of 3 ms duration and amplitude −80 mV; *e*, depolarizing voltage-clamp pulses of 3 ms duration and amplitude −30 mV. Tetrodotoxin (0.1 μg/ml) was added to the external solution at the time indicated. Axon diameter 800 μm. Temperature 20 °C. Calibration of the probe in the external solution indicated that the increase in influx in *d* was equivalent to 0.0048 pmol/cm² per pulse.

By winding a conventional double spiral of 20 μm diameter platinum wires round the internal scintillator, it is a relatively simple step to convert the bare scintillator rod used in the experiment of figure 2*b* into a combined internal scintillator and voltage-clamp electrode. This device permits continuous measurement of $^{45}Ca$ influx under voltage clamp conditions and its use is illustrated in figure 3. The following points should be noted:

(1) The resting calcium influx is very low.

(2) Repetitive stimulation (membrane action potentials at 160/s for 10 min) results, after a lag of 5–10 min, in a step increase in the calcium influx. After stimulation, calcium influx returns to its initial value.

(3) Repetitive depolarizing voltage clamp pulses of 80 mV amplitude and either 2 or 3 ms duration also produce a step increase in calcium influx after an initial lag.

(4) Inclusion of tetrodotoxin (0.1 μg/ml) in the external solution does not affect the resting rate of calcium influx or the response to repetitive depolarizing voltage clamp pulses (80 mV amplitude 3 ms duration).


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Although these results are consistent with an increased entry of calcium occurring during the period of repetitive depolarization, there are two points that need some comment. (1) The increment in calcium entry is small and can still only be demonstrated after massive stimulation. Even under these conditions it is essential for the resting entry of calcium to be as low as possible and we have not been able to perform satisfactory experiments on axons that have been cold-stored for more than 2 or 3 h. (2) The step in calcium entry has two interesting features: it occurs with a lag of 5–10 min which is most pronounced early in the experiment and the rise is often followed by an apparent fall in internal radioactivity. Although experiments have not been performed to prove the point, it seems likely that the lag reflects the time taken for $^{45}Ca$ entering at the surface to come within range of the scintillator. It is well known that calcium is bound within axoplasm and the observed lag is of the order expected for the inward diffusion of $^{45}Ca$ through a calcium-binding matrix (see Blaustein & Hodgkin 1969; Baker, Hodgkin & Ridgway 1971). The subsequent fall seen in some experiments is more difficult to explain, but it may result from preferential sequestration of $^{45}Ca$ in peripheral structures perhaps because axoplasmic binding is impaired in the immediate vicinity of the internal scintillator.

Apart from providing further evidence for calcium entry in response to repetitive depolarization, the experiment of figure 3 also shows that depolarization-induced calcium entry persists after inclusion in the external medium of enough tetrodotoxin to block the sodium channels completely. This is an important point and will be taken up later (see p. 403).

### *Experiments using aequorin*

A much more sensitive method for following the voltage-sensitive changes in calcium entry continuously is to introduce into the axon the calcium-sensitive protein aequorin (Baker *et al.* 1971). Aequorin reacts with calcium ions to generate light (Shimomura, Johnson & Saiga 1963; Shimomura & Johnson 1969). No other co-factors are required and the rate of light emission from an aequorin-loaded axon provides a continuous measure of the concentration of ionized calcium inside the axon. The reaction is extremely sensitive responding to calcium concentrations as low as $10^{-8}$ M; but the technique has some important limitations of which the main ones are:

(1) The reaction is difficult to make quantitative. This arises partly because two calcium ions are required to react with each aequorin molecule in the light generating reaction and partly because light production is sensitive to other intracellular substances such as magnesium ions.

(2) The reaction is relatively slow. Hastings *et al.* (1969) have provided evidence that at 20 °C the aequorin reaction proceeds with a half time of approximately 6 ms.

(3) The reaction measures ionized calcium and from the point of view of the present topic, an increase in light emission from intracellular aequorin does not necessarily represent an increased entry of calcium. It could also reflect a decreased rate of calcium extrusion, or a reduction in the effectiveness of the intracellular buffer systems.

Figure 4 shows the light output from an aequorin-loaded axon in response to a brief train of action potentials. After an initial lag, the light output rises steadily throughout the period of stimulation and begins to fall shortly after stimulation ceases. Using a more highly purified sample of aequorin, Hallett & Carbone (1972) were able to observe a similar increase in light output in response to a single action potential. If the period of stimulation is prolonged, the


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light output rises exponentially to a new steady level and falls with a time constant of 10–15 s when the stimulus is switched off. Recovery is probably due to uptake of the calcium by intracellular binding systems and the steady level may represent a balance between entry at the surface and uptake into the internal stores.

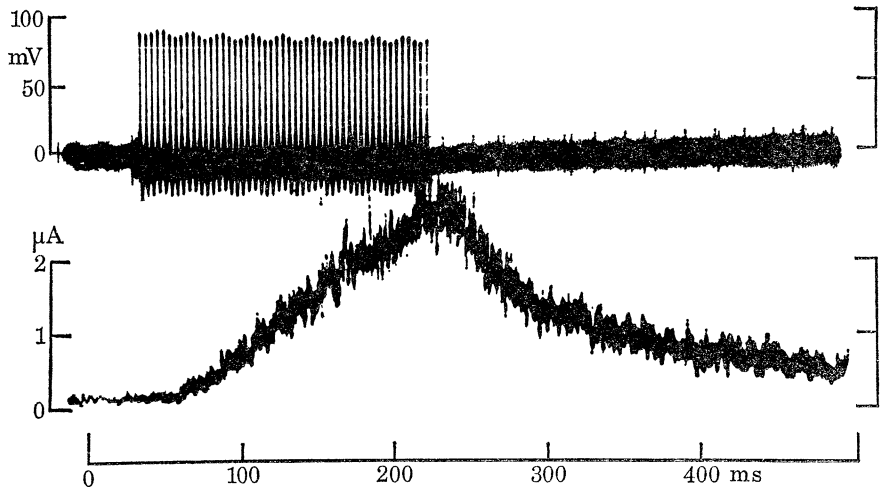


FIGURE 4. Changes in intracellular ionized calcium during a brief tetanus. Upper trace: action potentials. Lower trace: light output from aequorin injected into the axon. The light output was detected by a photomultiplier and displayed on an oscilloscope. An increase in light reflects a rise in ionized calcium. The axon was immersed in sodium seawater containing 112 mM calcium. Temperature 20 °C (unpublished data of Baker, Hodgkin & Ridgway).

The relation between the frequency of stimulation and both the rate of light production and the final steady level achieved illustrates one of the quantitative problems encountered in using aequorin. In some experiments the relation was linear; but in others both the initial rate and steady level of light emission increased as the square of the stimulus frequency. One suggestion put forward by Baker *et al.* (1971) to explain this difference is as follows. Suppose stimulation increases the intracellular ionized calcium from $Ca_R$ to $(Ca_R + \Delta Ca)$. If the rate of the aequorin reaction is proportional to the square of the ionized calcium, the increment in light emitted will be proportional to $2Ca_R\,\Delta Ca + (\Delta Ca)^2$. A linear dependence on frequency is expected when $Ca_R \gg \Delta Ca$ and a square law dependence when $\Delta Ca \gg Ca_R$.

A critical question posed by the aequorin results is to determine the source of the increase in ionized calcium. As there is no increment in light output from axons conducting action potentials in seawaters in which the calcium has been replaced entirely by magnesium, Baker *et al.* (1971) concluded that the increased light emission probably results from the entry of calcium into the cell. Although this conclusion is in accord with the tracer data that has already been discussed, it should be pointed out that other possibilities are not entirely excluded. For instance, calcium entry at the surface may promote a much larger release of calcium from intracellular sites. If this were the case, the actual contribution of calcium influx to the rise in ionized calcium might be quite small.

## Calcium entry under voltage-clamp conditions

### *Evidence for two phases of calcium entry*

On the assumption that the bulk of the rise in ionized calcium detected by aequorin reflects calcium entering at the surface, it is possible to determine the timing of calcium entry using the voltage-clamp technique. A major problem is that the aequorin reaction is relatively slow and there is a lag of a few milliseconds between a step increase in calcium entry and the emission of light. This means that aequorin cannot be used to follow directly the time course of any changes in calcium permeability. Baker *et al.* (1971) overcame this problem by comparing the calcium entry associated with trains of depolarizing pulses of different durations. If all the calcium entry occurs in very short pulses it might be associated with the increase in permeability to sodium ions, whereas if it is only seen with longer pulses it might be associated with the increase in permeability to potassium. Figure 5 shows that calcium entry occurs in two phases: an early phase that roughly parallels the increase in sodium permeability and a late phase that turns on at much the same time as the increase in potassium permeability. In many axons studied by Baker *et al.* (1971) the two phases were of about equal size; but in some the later phase predominated.

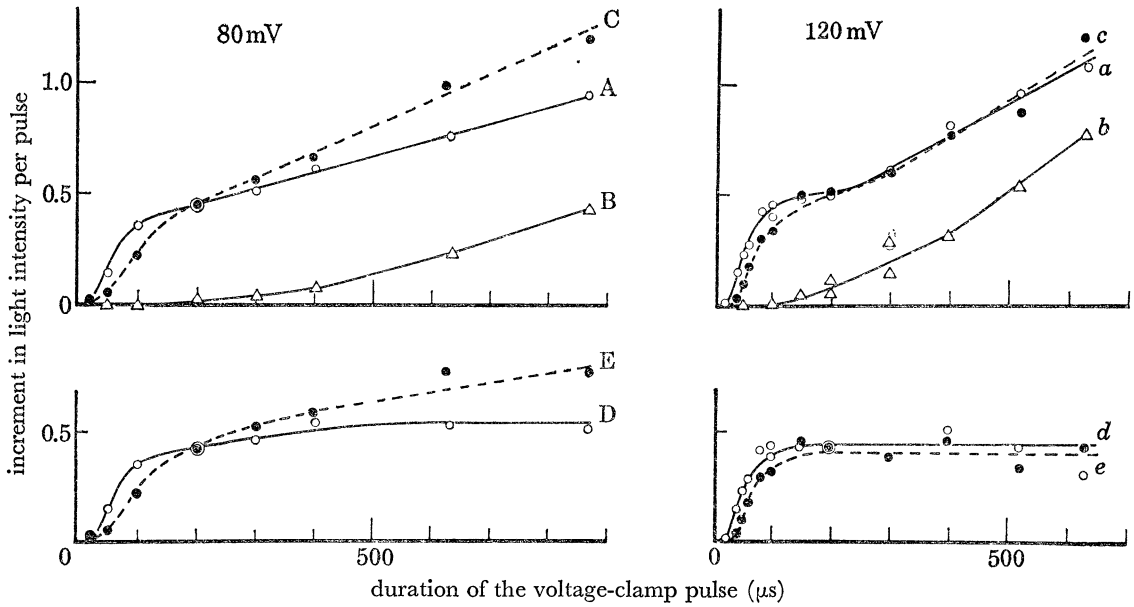


Figure 5. Evidence for two phases of calcium entry in giant axons of *Loligo forbesi*. The upper curves show the relation between the duration of the voltage-clamp pulse (abscissa) and the increment in light intensity per pulse (ordinate). A, *a*: before TTX; B, *b*: in TTX (0.8 μM); C, *c*: after removal of TTX. A, B, C: 80 mV depolarizations. *a*, *b*, *c*: 120 mV depolarizations. The ordinate was measured as the initial rate of rise of light intensity at 200 pulses/s divided by the initial rate of rise of light intensity at 200 action potentials/s. For the lower curves the ordinate is the TTX-sensitive component of the calcium entry obtained as D = A−B or *d* = *a*−*b* before TTX and E = C−B or *e* = *c*−*b* after TTX. Temperature 22 °C. (From Baker *et al.* 1971.)

### *Properties of the early phase of calcium entry*

One method of establishing whether the early phase of calcium entry reflects calcium passing through the sodium channel of the action potential is to examine its sensitivity to agents such as tetrodotoxin that are known to block the sodium channel. Figure 5 shows that the early

phase of calcium entry is abolished reversibly by tetrodotoxin at concentrations which also block the sodium conductance. The preliminary analysis of Baker, Hodgkin & Ridgway (1970) suggested that the calcium entry might precede the maximum increase in sodium permeability; but this assumed that all the calcium entered during the depolarizing pulse. In practice the sodium permeability cannot be switched off instantaneously and for a short period after repolarizing the membrane the sodium channels remain open. At the resting potential the driving force on the calcium ion is high and calcium entry continues after the pulse for as long as the sodium channels remain open. For short pulses the entry of calcium following the pulse may be an appreciable fraction of the total calcium entry. When calcium entering after the pulse is allowed for, the early phase of calcium entry exactly parallels the rise in sodium permeability (Baker *et al.* 1971). These experiments show that calcium ions can pass through the sodium channels and the ratio of estimated calcium conductance to sodium conductance is roughly 0.01 when the external Ca and Na concentrations are 112 and 400 mM respectively.

Working with internally perfused squid axons immersed in sodium-free media, Watanabe, Tasaki, Singer & Lerman (1967) observed an action potential that was dependent on external calcium ions and was blocked by tetrodotoxin. This suggests that under the conditions of their experiment calcium was carrying inward current through a channel that is sensitive to tetrodotoxin. Meves & Vogel (1973) have recently examined the properties of this calcium inward current and their experiments provide strong evidence that the tetrodotoxin-sensitive calcium inward current is passing through the sodium channel (this is discussed in detail by Meves, see page 377).

An interesting feature of the aequorin experiments is the observation that calcium entry still occurs even when sodium ions are moving out of the axon, for instance in sodium-free seawater or at internal potentials more positive than the sodium-equilibrium potential. This suggests (1) that there is little interaction between inflow and outflow of ions through the sodium channels and (2) that calcium entry is not secondary to sodium entry.

### *Properties of the late phase of calcium entry*

Since the early phase of calcium entry seems to reflect calcium ions passing through the sodium channel, it is natural to ascribe the late phase to calcium entering through the potassium channel. The voltage-dependence and timing of the late phase, as well as its insensitivity to tetrodotoxin are consistent with this idea; but other evidence points strongly against it (Baker *et al.* 1971; Baker, Meves & Ridgway 1973*a*, *b*). Thus injection of enough tetraethylammonium ions to block outward movement of potassium through the potassium channel has no effect on calcium entry; but the tetrodotoxin-insensitive calcium entry is blocked by a variety of agents including $Mg^{2+}$, $Mn^{2+}$, $Co^{2+}$ and the organic calcium antagonists iproveratril and D-600 at concentrations that have little or no effect on either inward or outward currents through the potassium channel (see table 1). These observations strongly indicate that the late phase of calcium entry does not take place through the potassium channels and suggest that, in addition to the well known sodium and potassium channels of nerve membranes, there must be a quite separate calcium channel that opens late in the action potential. This channel has been called the 'late calcium channel'. The term *late* is used to distinguish calcium entering through this channel from calcium entering through the sodium channel. Experiments in which aequorin has been injected into *Aplysia* neurons (Stinnakre & Tauc 1973) and the squid giant synapse

TABLE 1. EFFECT OF A VARIETY OF AGENTS ON THE MOVEMENTS OF Na, K AND Ca THAT OCCUR IN RESPONSE TO DEPOLARIZATION OF THE SQUID AXON MEMBRANE

| | | | Ca entry | |
|---|---|---|---|---|
| | Na current | K current | early phase | late phase |
| tetrodotoxin | blocks | no effect | blocks | no effect |
| injected tetraethylammonium | no effect | blocks | no effect | no effect |
| $Mg^{2+}$, $Mn^{2+}$, $Co^{2+}$ | little effect | little effect | little effect | blocks |
| D-600, iproveratril | reduces | little effect | reduces | blocks |

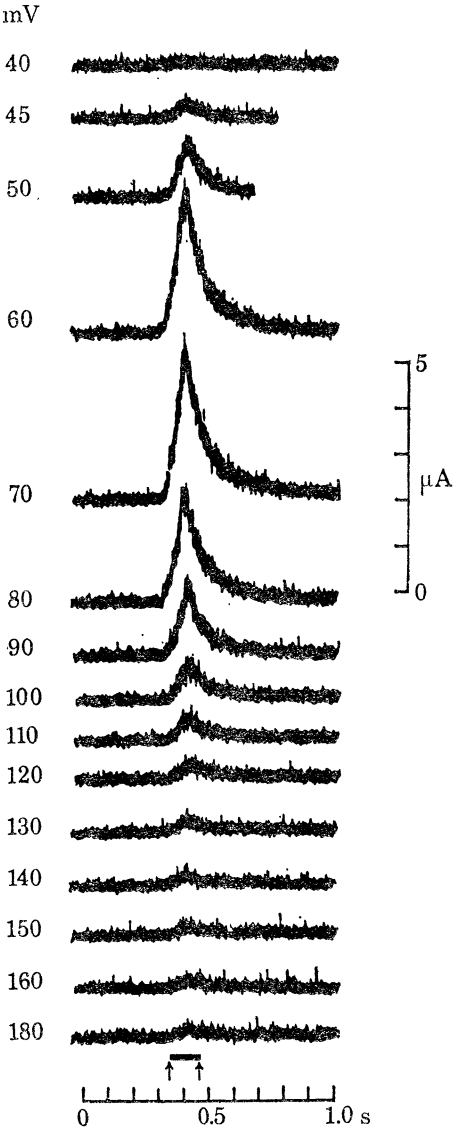


FIGURE 6. Responses of aequorin-injected squid axon to 100 ms pulses of different amplitude. Axon in magnesium-free artificial seawater containing (mM): $CaCl_2$, 22; NaCl, 400; KCl, 10; $NaHCO_3$, 2.5 with 1.6 μM tetrodotoxin. The axon had also been pre-injected with tetraethylammonium chloride to give a final TEA concentration of about 15 mmol/l of axoplasm. The numbers on the left give the amplitude of the depolarizing pulse measured from the resting potential. Temperature 22.5 °C (from Baker *et al.* 1971).


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(Llinás, Blinks & Nicholson 1972) suggest that the 'late calcium channel' may be widely distributed in nervous tissue.

The main properties of the 'late calcium channel' are summarized below and in table 1. In many respects they resemble the properties of the calcium-dependent transmitter release mechanism of the squid giant synapse (Katz & Miledi 1967*a*, *b*, 1969) and other nerve terminals (see Baker 1972).

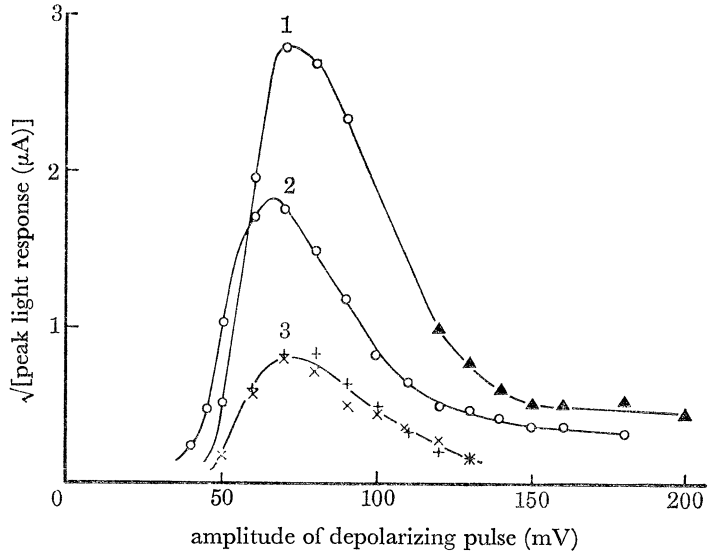


FIGURE 7. Effect of pulse amplitude on the aequorin response to single pulses of duration 100 ms. The abscissa is the amplitude of the depolarizing pulse measured from the resting potential and the ordinate is the square root of the peak light response. The composition of the external solution was: curve 1, 112 mM Ca, 0 Mg; curve 2, 22 mM Ca, 0 Mg; curve 3, 22 mM Ca; 90 mM Mg. The points were determined in the order 112 Ca (▲); 22 Ca, 90 Mg (+); 22 Ca 0 Mg (○); 22 Ca, 90 Mg (×); 112 Ca, (○). From the experiment of figure 6.

(i) *The time course of activation*

Calcium entry in response to depolarization increases along a sigmoidal time course that closely parallels the activation of the potassium conductance.

(ii) *Dependence of activation on potential*

Figures 6 and 7 show the calcium entry in response to depolarizations of different amplitude. The curve relating calcium entry to depolarization has a characteristic bell shape. It increases steeply between 35 and 50 mV, reaches a peak at about 70 mV and declines to a low value between 80 and 140 mV. For depolarizations between 35 and 50 mV, calcium entry increased e-fold in 6.3 mV. A small increase in light was seen even at very large depolarizations and there was no evidence for a true equilibrium potential at which the direction of the light response was reversed. A reversal of the response was, however, seen when the fibre was hyperpolarized. Failure to detect an equilibrium potential at large depolarizations may be because potential affects some other calcium transporting system in the cell membrane. The experimental observations could be explained if the Na–Ca counter transport system is sensitive to potential in such a way that depolarization decreases the rate of calcium extrusion and promotes calcium entry. This suggestion is supported by the observation that depolarization of squid axons with K-rich solutions reduces $^{45}$Ca efflux and increases $^{45}$Ca influx (Baker 1972;

Blaustein 1974). Hyperpolarization should have the opposite effect, increasing calcium extrusion and decreasing calcium entry which could account for the observed fall in ionized calcium. Sensitivity of the sodium–calcium counter transport system to potential may come about because the exchange of Na for Ca is not electroneutral but involves the exchange of three or more Na ions for each calcium ion.

(iii) *Dependence on external calcium and other divalent cations*

Fig. 7 shows that calcium entry is dependent on the external calcium concentration; but the relation does not seem to be linear. The entry from seawater containing 20 mM calcium is more than half of that from seawater containing 112 mM calcium which suggests that calcium entry through the late calcium channel may saturate. This clearly merits further analysis especially as it apparently contradicts the tracer experiments described on page 391 where calcium entry increases linearly with the external calcium concentration; but the latter experiments presumably measured the sum of the early and late components of calcium entry.

At a constant calcium concentration increasing the concentration of magnesium in the seawater reduces the calcium entry at all voltages. The inhibitory effect of magnesium is reversible (figure 7). A larger reduction in calcium entry is seen with other divalent cations including $Mn^{2+}$, $Co^{2+}$ and $Ni^{2+}$. Trivalent cations including $La^{3+}$ are also inhibitory.

In seawaters containing 112 mM calcium, the calcium equilibrium potential should be about +150 mV, that is about 210 mV positive to the resting potential. With such a high value of $E_{Ca}$ it is surprising that the light response, and presumably calcium entry, decline when the depolarization exceeds 80 mV. One possible explanation is that the late calcium channel may also be permeable to other divalent cations including magnesium. A deviation towards less positive potentials can be explained provided (*a*) the late calcium channels permit magnesium ions to move outwards down their electrochemical gradient and (*b*) the outward movement of magnesium impedes the entry of calcium. More quantitative data is required to test this possibility further; but there is some evidence for this suggestion. (1) Baker & Crawford (1972) have shown that the concentration of ionized magnesium inside squid axons is about 2–3 mM (that is 10000 times greater than the concentration of ionized calcium) and (2) during repetitive stimulation of squid axons there is an extra entry of magnesium amounting to 0.007 pmol/cm$^2$ impulse at 20 °C (Baker & Crawford 1972). This is roughly equal to the extra entry of calcium from seawater. As the magnesium content of seawater is five times that of calcium, these observations suggest that the increased permeability to magnesium is about one fifth of that to calcium. Measurements on a few axons of the uptake of $^{54}Mn^{2+}$ during repetitive stimulation revealed an extra uptake of manganese. The extra uptake of $^{54}Mn$ from seawater containing 25 mM $Mn^{2+}$ and 20 mM Ca was 0.007 pmol/cm$^2$ impulse at 20 °C. Yamagishi (1973) has also reported manganese-dependent action potentials in perfused squid axons. Experiments on conducted action potentials provide very little information on the route by which the extra entry takes place, but there is some evidence for magnesium implicating the late calcium channel. This is based on two observations: (1) the extra entry of magnesium during a train of action potentials is markedly reduced in the presence of 55 mM $Mn^{2+}$ which blocks calcium entry through the late channel (Baker & Crawford 1972) and (2) in perfused squid axons Rojas & Taylor (1973) have detected an extra entry of $^{28}Mg$ in response to repetitive depolarizing voltage clamp pulses and this extra entry of magnesium persists in the presence of external tetrodotoxin and internal tetraethylammonium ions.


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(iv) *Inactivation*

Calcium entry in response to depolarization is not maintained, but declines with time. This behaviour is seen both in response to maintained electrical depolarization and also to depolarization induced by application of K-rich solutions. These observations suggest that depolarization has a dual action serving both to activate and inactivate calcium entry (Baker, Meves & Ridgway 1973*b*). The rate of inactivation is dependent on potential and seems to be faster for large depolarizations; but its absolute rate is very slow when compared with the rate of inactivation of the sodium conductance.

The relation between steady-state inactivation of calcium entry and membrane potential can be examined by exposing axons to seawaters containing different potassium concentrations and then measuring calcium entry in response to 400 mM potassium. Inactivation increases along an S-shaped curve and is half complete at about $-25$ mV in seawater containing 112 mM calcium and at about $-40$ mV in seawater containing 20 mM calcium. The calcium-dependent shift in the curve relating inactivation to membrane potential is in general agreement with the findings of Frankenhaeuser & Hodgkin (1957) on the effects of external calcium ions on other electrical properties of the squid axon membrane.

Recovery from inactivation is slow. After exposure to high K for 2–3 min the response to a second exposure to the same potassium concentration is much reduced and recovery is half complete in about 3–4 min. Longer exposures to high K seem to result in even slower recovery. Although the depolarization-induced increase in potassium permeability also inactivates slowly, on repolarization it recovers much more rapidly than the late calcium channel, providing further evidence that the two channels are distinct.

(v) *Dependence on external and internal sodium ions*

In view of the evidence for Na–Ca counter-transport in squid axons it is important to know whether sodium ions exert any effect on calcium entry through the late calcium channel. This has not been examined systematically; but replacement of external sodium by lithium which should stimulate calcium entry in exchange for internal sodium does not produce any marked change in the calcium entry during a brief period of repetitive stimulation and replacement of external sodium by choline only causes a small increase in the tetrodotoxin-insensitive phase of calcium entry. The effects of internal sodium have not been examined in the squid axon although calcium entry is still seen at potentials more positive than $E_{Na}$ which rules out the possibility that calcium uptake is secondary to sodium entry. These observations provide no support for the possibility that the TTX-resistant component of calcium entry results from a direct effect of depolarization on sodium–calcium counter-transport; but suggest that the two systems are quite distinct.

(vi) *Metabolism*

The dependence on metabolism of calcium entry through the late calcium channel has not been examined. An extra uptake of $^{45}Ca$ – within the normal range – is still seen during repetitive stimulation of axons fully poisoned with cyanide; but no information is available on the relative contributions to this uptake of the early and late phases of calcium entry. Poisoning raises the concentration of ionized calcium by releasing calcium from intracellular binding


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sites. Under these conditions any injected aequorin is consumed very rapidly which renders the aequorin technique unsuitable for analysing calcium entry in poisoned cells.

This topic is of some importance because the calcium permeability system in cardiac muscle seems to be under metabolic control (Reuter 1974) and if a similar stituation exists in nerve, it might, for instance, contribute to the extreme sensitivity of synapses to anoxia.

(vii) *Changes in apparent calcium entry over long periods*

In a number of the experiments of Baker *et al.* (1971) the rate of light emission in response to a constant depolarization increased slowly over a period of many hours. In some old axons light production was increased 1000-fold. This occurred without change in the resting light and the increased response to depolarization was largely restricted to the tetrodotoxin-resistant phase of calcium entry. Under conditions where the aequorin response increased there were no appreciable changes in potassium currents.

This time-dependent increase in apparent calcium entry is rather puzzling. The term apparent is used because there is at present no evidence for a comparable increase in the entry of radioactive calcium or for a calcium current and until one or both of these have been demonstrated it is possible that the aequorin response is increased not through extra calcium entry but for some other reason. For instance, there may be a progressive decline in the effectiveness of the calcium buffering systems immediately internal to the axolemma or a progressive fall in the concentration of an inhibitor of the aequorin reaction. The main argument against a non-specific increase in sensitivity is the observation that the early and late phases of calcium entry do not increase in parallel. A different but conceivably related phenomenon has been reported by Stinnakre & Tauc (1973). Working with the aequorin-injected *Aplysia* neuron they found an apparent increase in calcium entry in response to successive impulses in a train.

These observations might have important physiological implications. If changes of the kind seen in squid axons and *Aplysia* neurons can also occur at nerve terminals they would promote the release of transmitter and increase the effectiveness of synaptic transmission.

This last observation illustrates one of the major limitations in our present understanding of the late calcium channel. Evidence for this channel relies heavily on the use of aequorin. Until the results are fully corroborated by other techniques, for instance radioactive tracers or direct measurement of a calcium current, it is necessary to exercise caution in identifying the light emitted by aequorin solely with an entry of calcium at the surface.

The results obtained with aequorin are supported by the following observations.

(1) Inclusion of cobalt in the seawater reduces the extra entry of calcium during the action potential (see table 2). Under the same conditions cobalt had no effect on the resting entry of calcium. Although this observation is consistent with the known action of cobalt on the late calcium channel, an alternative explanation is that cobalt also reduces the inward sodium current and a reduction in calcium entering through the sodium channel could also account for these results.

(2) Experiments with the combined internal scintillator and voltage-clamp electrode showed an increased uptake of $^{45}Ca$ in response to repetitive depolarizing pulses of 80 mV amplitude (figure 3). At least part of this uptake may be through the late calcium channel because the increased uptake persists in the presence of enough tetrodotoxin to block the sodium channel. The voltage-dependence of the tetrodotoxin-resistant uptake is also consistent with calcium

entering through the late calcium channel. No uptake was seen in response to repetitive hyperpolarizing pulses of 80 mV and the response to depolarizing pulses was maximal around 80 mV. The effects of other divalent cations such as $Co^{2+}$ and $Mn^{2+}$ have not yet been examined.

(3) Working with internally perfused squid axons, Rojas & Taylor (1970) have reported an extra entry of $^{45}Ca$ in response to depolarizing pulses. The extra entry has many features in common with the aequorin results. $^{45}Ca$ entry can be separated into an early tetrodotoxin-sensitive component and a late tetrodotoxin-insensitive component. The detailed properties of the latter component have not been analysed.

TABLE 2. $^{45}Ca$ INFLUXES IN PAIRED SQUID AXONS

The external solution was artificial seawater (a.s.w.) containing (mM): NaCl, 400; choline chloride, 140; KCl, 10; $NaHCO_3$, 2.5; $CaCl_2$, 20 or 112. Isotonicity was maintained by exchanging choline chloride for $CaCl_2$. Temperature 19–20 °C. All axons were from previously refrigerated mantles. Cobalt was added as solid cobaltous chloride. For comparison, the resting calcium influx in fresh axons is 0.37 pmol $cm^{-2}$ $s^{-1}$ and 0.61 pmol $cm^{-2}$ $s^{-1}$ at 22 and 112 mM Ca respectively and the extra calcium entry per pulse is 0.0098 and 0.083 pmol/$cm^2$ at the same two calcium concentrations. (Hodgkin & Keynes 1957.)

| $[Ca^{2+}]$ in a.s.w. (mM) | resting calcium influx (pmol $cm^{-2}$ $s^{-1}$) | | extra calcium influx per impulse (pmol/$cm^{-2}$) | |
|---|---|---|---|---|
| | without $Co^{2+}$ | with 25 mM $Co^{2+}$ | without $Co^{2+}$ | with 25 mM $Co^{2+}$ |
| 20 | 1.43 ± 0.16 ($n$ = 18) | 1.56 ± 0.35 ($n$ = 11) | 0.009 ± 0.0016 ($n$ = 19) | 0.0028 ± 0.0025 ($n$ = 12) |
| 112 | 3.14 ± 0.54 ($n$ = 20) | 3.31 ± 0.47 ($n$ = 24) | 0.049 ± 0.011 ($n$ = 12) | 0.026 ± 0.006 ($n$ = 16) |

The clearest evidence would be to demonstrate a calcium current with the properties of the late calcium channel; but this has not so far proved possible in the squid giant axon. The aequorin data suggest that any current associated with calcium entry through the late calcium channel should be very small and the technical problem of detecting such a small current in the presence of the much larger currents of sodium and potassium is considerable, even when the latter are reduced by tetrodotoxin and tetraethylammonium ions respectively. Meves & Vogel (1973) have examined this problem in some detail. They perfused squid axons with a solution containing caesium fluoride and immersed the axons in a calcium-rich sea-water that lacked sodium ions. These conditions minimized the possibility of seeing currents carried by sodium or potassium ions. The only calcium currents they could detect were sensitive to tetrodotoxin and probably represent calcium ions entering through the sodium channel (see page 398). Even under these conditions the calcium currents were very small and their observations do not necessarily imply that calcium currents do not flow through the late calcium channel. Although roughly equal quantities of calcium probably enter through the sodium channels and late calcium channels respectively the data shown in figure 5 suggest that the rate of calcium entry through the two channels and hence the calcium current expected is quite different. The rate of calcium entry is 5–10 times faster through the sodium channel than through the TTX-insensitive 'late calcium channel'. The rough equality in the two phases of calcium entry stems from the fact that the late calcium channel only inactivates very slowly and once opened allows calcium ions to flow into the axon for much longer than does the sodium channel. A second slower calcium current only 10 % or perhaps less of that observed by Meves & Vogel (1973) might not have been detected under the conditions of their experiments. This topic clearly merits further examination. Apart from the technical

argument outlined above, other possibilities include (1) the late calcium channel may be more labile than the sodium and potassium channels and it may not survive perfusion with caesium fluoride; (2) calcium may enter with an anion or exchange with a cation.

Perhaps the strongest argument that a calcium current should be associated with the late calcium channel is the excellent evidence for such a current at the squid giant synapse (Katz & Miledi 1969) and in a variety of other excitable cells. At the squid giant synapse, when the sodium and potassium conductances have been suppressed by tetrodotoxin and tetraethylammonium ions respectively, it is possible to detect calcium-dependent action potentials. These tetrodotoxin-resistant action potentials are blocked by $Mn^{2+}$ and $Co^{2+}$ ions and it seems very likely that the inward current of these action potentials is carried by calcium ions passing through the late calcium channel. It is of interest that such action potentials can only be detected in the pre-synaptic termination of the axon, suggesting that there is a much higher density of these channels in the regions involved in transmitter release.

There is evidence in other preparations for calcium currents that are resistant to tetrodotoxin, but blocked by $Co^{2+}$ and $Mn^{2+}$ ions and by the organic calcium antagonist D-600. Notable examples are the calcium action potentials of amphibian sympathetic ganglia (Koketsu & Nishi 1969), certain mammalian peripheral neurons (Hirst & Spence 1973), neurons of the crayfish X-organ (Iwasaki & Satow 1971), snail neurons (Gerasimov, Kostyuk & Maiskii 1965; Kerkut & Gardner 1967; Geduldig & Junge 1968; Meves 1968; Geduldig & Gruener 1970; Connor & Stevens 1971; Meech & Standen 1974), crustacean muscle (Fatt & Katz 1953; Hagiwara & Nakajima 1966; Keynes, Rojas, Taylor & Vergara 1973), smooth muscle and the calcium current during the plateau of the cardiac action potential (see the review of Reuter 1973). Many of their properties are strikingly similar to those of the 'late calcium channel' in squid axons.

## Mechanism of voltage-sensitive changes in calcium permeability

The mechanism of ion permeation through the sodium channel is discussed elsewhere in this volume. As yet, nothing is known of the structural basis of the late calcium channel. No highly specific blocking agent analogous to tetrodotoxin has yet been found, and in the absence of very specific inhibitors, isolation of the late calcium channel is likely to be a slow process. A different and very interesting approach has been suggested by work on the protozoan *Paramecium*, the locomotory behaviour of which seems to be controlled by changes in the calcium permeability of the surface membrane (Kamada 1938; Kinosita 1954; Naitoh 1968; Eckert 1972). When the anterior end of the animal is mechanically deformed, for instance when an animal bumps into an object, the membrane in that region is depolarized and calcium enters the cell. The local rise in ionized calcium causes a reversal of the direction of beat of the cilia with the result that the animal changes its direction of movement and swims away from the obstacle. The particular advantage of working with *Paramecium* is that it is possible to isolate mutants with abnormal locomotory behaviour (Kung 1971; Kung & Eckert 1972; Satow & Kung 1974) and this may provide a powerful tool for probing the calcium permeability system in this animal.


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## Conclusions

In most nerve cells depolarization leads to an increased uptake of calcium. At least four processes can be recognized.

(1) The rate of calcium pumping by the sodium–calcium counter-transport system may be sensitive to membrane potential. In squid axons potassium depolarization increases calcium entry and decreases calcium efflux.

(2) Calcium ions can enter through the sodium channel of the action potential; this route is blocked by tetrodotoxin and inactivates rapidly in response to a maintained depolarization.

(3) Calcium ions can enter through a voltage-sensitive channel that is distinct from the sodium and potassium channels of the action potential and has been called the 'late calcium channel'. This route is insensitive to tetrodotoxin but is blocked by $Mg^{2+}$, $Co^{2+}$, $Mn^{2+}$ and the organic calcium antagonists iproveratril and D-600. In squid axons it inactivates very slowly in response to a maintained depolarization.

(4) Furthermore, in secretory cells, a rise in intracellular ionized calcium brought about by (1)–(3) above may promote secretion by exocytosis and there is now strong evidence that recovery from exocytosis involves membrane retrieval by a process (endocytosis) that is functionally similar to pinocytosis (Heuser & Reese 1973; Ceccarelli, Hurlbut & Mauro 1973). It seems likely that endocytosis will lead to a further uptake of extracellular calcium into the cell (see, for example, Nordmann *et al.* 1974). The fate of this calcium is unknown, but if it remains within the vesicles, it will not contribute to the level of ionized calcium in the cytosol.

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## *Discussion*

E. Rojas (*University of Chile and Physiological Laboratory, Cambridge*)

R. E. Taylor and I made simultaneous determinations of magnesium, calcium and sodium extra influxes using $^{28}Mg$, $^{45}Ca$ and $^{22}Na$ in perfused fibres. Figure 8 shows the results obtained in a typical experiment. Part (*a*) shows three examples of total membrane current records for a depolarizing pulse of 70 mV from a holding potential of −70 mV. The numbers given in parenthesis represent the estimation of the extra sodium influx during each cycle in pmol/cm² per cycle (calculated as $1/F\int_0^\infty I_{Na}\,dt$, where $F$ is the Faraday constant). Part (*b*) shows the extra calcium influx as a function of pulse duration. Numbers in parenthesis represent the estimated extra entry of sodium during the inward currents.

If we subtract from the experimental values of the extra calcium entry an amount which is proportional to the extra sodium influx ($K1/F\int_0^\infty I_{Na}\,dt$ with $K = 9.59 \times 10^{-4}$) we get the blacked-in triangles in this figure. Part (*c*) represents the data on extra magnesium entry measured simultaneously with the extra calcium entry.

The most relevant conclusions from these experiments can be summarized as follows: (*a*) In fibres internally perfused with either 275 or 550 mM KF and with 10 mM $CaCl_2$ seawater outside the average resting calcium influx was $0.016 \pm 0.008$ pmol cm$^{-2}$ s$^{-1}$ for *Dosidicus* and $0.017 \pm 0.013$ pmol cm$^{-2}$ s$^{-1}$ for *Loligo* axons. (*b*) With 55 mM $MgCl_2$ in the seawater the average resting magnesium influx was $0.124 \pm 0.080$ pmol cm$^{-2}$ s$^{-1}$ for *Loligo* axons. (*c*) Using depolarizing pulses of various durations, we found the extra calcium influx occurred in two phases. The early phase was eliminated by external application of tetrodotoxin. The results of analysis are consistent with, but do not rigorously demonstrate, the conclusion that the TTX-sensitive calcium entry is flowing through the normal sodium channels. If it does we estimate $P_{Na}/P_{Ca}$ to be $188 \pm 80$. (*d*) Measurements of extra influxes using $^{22}Na$ and $^{45}Ca$ simultaneously indicate that the time courses of the TTX-sensitive calcium and sodium entry are similar but not identical. It is very doubtful that any significant calcium entry occurs before the sodium or is involved in the activation of the sodium system. (*e*) Using $^{28}Mg$, or mixtures of $^{45}Ca$ and $^{28}Mg$, we observed a single phase of magnesium entry which was insensitive to external TTX or internal TEA. The magnitude of the magnesium influx was considerably greater than the calcium extra entry and large enough to have been detected in the experiments reported by Meves (this volume, page 377) if it represented current.

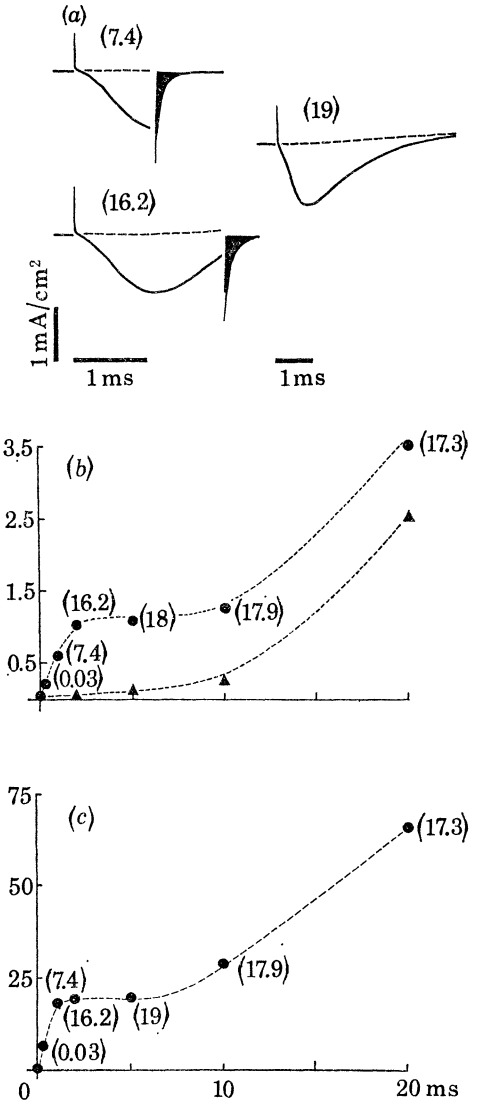


FIGURE 8

P. F. BAKER

The experiments described by Professor Rojas are most interesting and it is very satisfactory to see the results obtained with aequorin confirmed by another technique. I do, however, find figure 8*c* a little difficult to fit into the story. Professor Rojas states that he observed 'a single phase of magnesium entry insensitive to external TTX or internal TEA' yet his results (figure 8*c*) look almost identical to those for calcium entry (figure 8*b*) where two separate permeability systems are implicated. If magnesium entry really is insensitive to TTX, what is the explanation for the large entry of magnesium in response to very short depolarizing pulses? Could it be that some Mg entry is associated with opening one of the other permeability changes (Ca, Mg or even K)?