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THE SENSITIVITY OF *HELIX ASPERSA* NEURONES TO INJECTED CALCIUM IONS

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SUMMARY

1. When calcium chloride was injected into *Helix aspersa* neurones there was a fall in membrane resistance and the membrane potential became hyperpolarized.

2. The reversal potential of the response was dependent on the concentration of potassium in the external solution.

3. Injection of a calcium–EGTA buffer containing 9×10^{-7} M free calcium reduced the membrane resistance by 25%. When calcium chloride was injected it was necessary to increase the total intracellular calcium concentration by about 10^{-3} M to produce similar change of resistance.

4. In sodium-free (Tris) solution there was a slow fall of membrane resistance as if the intracellular calcium concentration had increased. There was a similar resistance change in the presence of 2,4-dinitrophenol and iodoacetate.

5. A series of repetitive depolarizing pulses produced a long lasting reduction in membrane resistance which was enhanced by 2,4-dinitrophenol and iodoacetate.

6. It is concluded that (*a*) injection of calcium causes an increase in potassium permeability, (*b*) the injected calcium is rapidly pumped from the cytoplasm by a sodium-dependent mechanism and by mitochondria, and (*c*) 1–2 msec depolarizing pulses stimulate an influx of calcium. This influx is rapid enough to trigger potassium activation during an action potential.

INTRODUCTION

The potassium permeability of red blood cells (Whittam, 1968), *Aplysia* neurones (Meech & Strumwasser, 1970) and cat spinal motoneurones (Krnjević & Lisiewicz, 1972) increases when the intracellular concentration of free calcium ions is increased. In the abdominal ganglion of *Aplysia*

a calcium mediated potassium permeability increase has been shown to be responsible for the post-tetanic hyperpolarization seen in cell R 15 (Meech, 1974*a*) and for the photo-response seen in cell R 2 (Brown & Brown, 1973). It has been suggested that the increase in potassium conductance induced by 2,4-dinitrophenol and other metabolic poisons in *Aplysia* (Carpenter, Snover & Barker, 1971) and by 2,4-dinitrophenol in cat cortical neurones (Godfraind, Krnjević & Pumain, 1970; Krnjević, Godfraind, Pumain & Provini, 1970) is also mediated by an increase in intracellular calcium. In addition it is possible that, in central neurones, an influx of calcium stimulates the increase in potassium conductance responsible for the recovery phase of the action potential (Meech, 1972; Krnjević & Lisiewicz, 1972). The experiments reported here were designed to test this suggestion. They confirm that *Helix aspersa* neurones respond to injected calcium chloride in the same way as those of *Aplysia*. The main new findings are (i) that the intracellular surface of the nerve membrane is sensitive to 9×10^{-7} M calcium ion and (ii) that depolarizing pulses of 1–2 msec duration can stimulate an influx of calcium.

METHODS

The suboesophageal ganglion of *H. aspersa* was dissected as described previously (Kerkut & Meech, 1966). It was bathed in a saline solution containing 5 mM-KCl; 59 mM-NaCl; 10 mM-$CaCl_2$; 16·5 mM-$MgCl_2$; 13 mM-$NaHCO_3$. Several neurones are 100–200 μm in diameter and they can be identified in every preparation. Fig. 1 is a diagram of the ganglion showing the cells used in this study. All experiments were carried out at room temperature (23° C) except where otherwise stated.

A 4–5 MΩ micropipette filled with 3 M potassium chloride was first pushed into a selected cell. The cell was allowed to recover for 5 min before a second micropipette with a tip diameter of about 2 μm was inserted. This second micropipette was used for the following operations:

(*a*) to inject small quantities of salt solution. As reported previously, a calcium chloride filled micropipette became blocked very quickly when inside the cell. It was possible to slow this blocking process by using a mixture of potassium chloride and calcium chloride in equal proportions. It was not necessary to insert the micropipette into the cell under pressure (see Meech, 1972);

(*b*) to monitor the resistance of the membrane by passing hyperpolarizing current pulses. The current pulses were about 10^{-8} A and of 0·5 sec duration. These pulses also aided the micropipette to penetrate the neurone;

(*c*) to inject current into the cell as a component of a voltage clamp circuit.

The advantage of using *Helix* instead of *Aplysia* neurones in this work is that the identifiable cells in *Helix* are smaller than those of *Aplysia*. It was therefore easier to control the membrane potential under voltage clamp. On the other hand, although 2–3 μm micropipettes could be inserted into any 200 μm diameter neurone without difficulty the maximum volume of salt which could be injected without killing the cell was about 5×10^{-11} l. (based on an estimate reported previously, Meech, 1972).

EGTA solutions

A mixture of EGTA (ethyleneglycol-bis-(β-aminoethylether)-N,N′-tetraacetic acid) and its calcium complex acts as a calcium buffer. The concentration of free calcium ions in a mixture of known composition can be calculated from the association constants given by Bjerrum, Schwarzenbach & Sillén (1957) (see Portzehl, Caldwell & Rüegg, 1964).

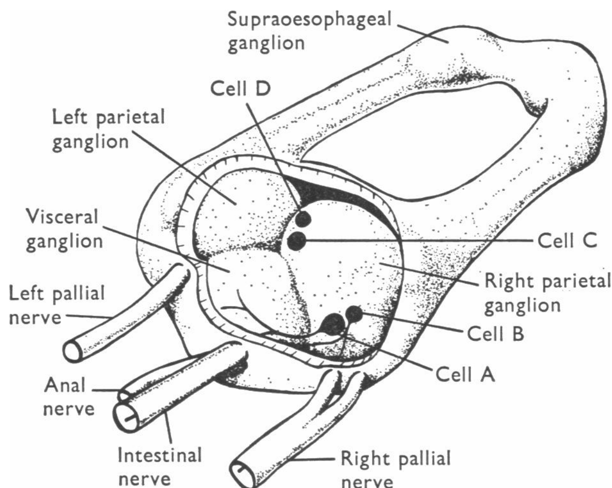

Fig. 1. Diagram of the oesophageal ganglia of *Helix aspersa*. A part of the thick outer connective tissue has been dissected to show the parietal and visceral ganglia. The positions of the cells used in this study are indicated together with the paths of the known axons (see Kerkut, French & Walker, 1970). Figure drawn by J. W. Rodford.

There are two main problems:

(i) the combined apparent association constant (K') varies with pH. Therefore since:

$$[\mathrm{Ca}^{2+}] = \frac{[\text{Ca-EGTA}]}{[\text{EGTA}]} \times \frac{1}{K'},$$

the concentration of free calcium ion in a given buffer will also depend on pH. The intracellular pH of *Helix aspersa* neurones is not known but Sorokina (1965) gives a value of 7·26 for nerve cells from *H. pomatia* (see Waddell & Bates, 1969). All the EGTA solutions were therefore made up to this pH.

(ii) EGTA binds both calcium and magnesium ions. Therefore although it binds calcium 10^5 times more effectively than it binds magnesium, the concentration of free calcium ion will depend on the level of the free magnesium ion in the cell.

For example, a calcium-EGTA buffer containing 10^{-6} M free calcium ion in magnesium-free solution will contain 3×10^{-6} M free calcium in the presence of 5 mM magnesium. The concentration of magnesium in the cytoplasm of *H. aspersa* neurones is unknown but in squid axoplasm the free magnesium concentration is about 5 mM (see Baker, Hodgkin & Ridgeway, 1971; Baker & Crawford, 1972). The magnesium concentration in *Helix* neurones is unlikely to be higher than this.

The calcium-EGTA buffers were prepared from two stock solutions, A and B. A contained 125 mM-EGTA; 250 mM-KCl; 100 mM histidine hydrochloride. The pH was adjusted to near 7·26 with potassium hydroxide. B contained 125 mM calcium-EGTA; 250 mM-KCl; 100 mM histidine hydrochloride. It was prepared by mixing equimolar solutions of calcium chloride and EGTA. The histidine hydrochloride was added and the pH adjusted to near 7·26 with potassium hydroxide. The histidine hydrochloride was used to buffer the pH.

The EGTA was tested for purity according to the method of Patton & Reeder (1956). It was anhydrous and contained no detectable impurities.

The combined apparent association constant for calcium and EGTA at pH 7·26 is $10^{7.25}$. Therefore a mixture containing 5 ml. of solution B and 1 ml. of solution A when the pH was readjusted to 7·26 would contain 2.8×10^{-7} M free calcium ion in the absence of magnesium.

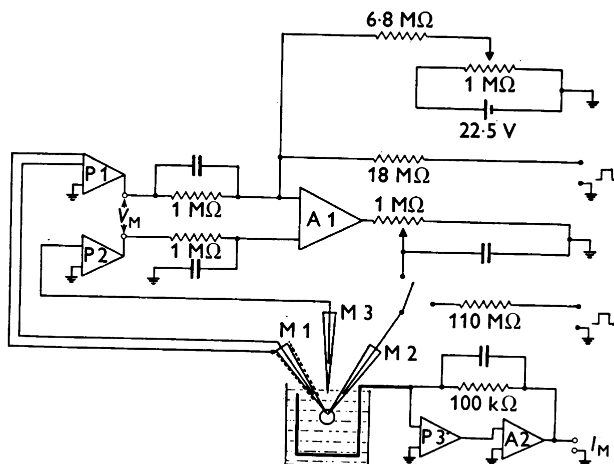

Fig. 2. Circuit diagram of the voltage-clamp apparatus. P 1, P 2 and P 3: unity gain preamplifiers; A 1: high gain DC amplifier to control membrane potential; A2: operational amplifier used to measure membrane current (I_m); M 1, M 2, M 3: micropipettes filled with 3 M-KCl each with a resistance of about 2 MΩ. The membrane potential (V_m) is recorded differentially between P 1 and P 2.

Voltage clamp experiments

For the voltage clamp experiments two micropipettes were inserted into the cell. Both were filled with 3 M potassium chloride but one (M 2) had a tip of 1–2 μm and was used to inject current. The circuit for the voltage clamp is shown in Fig. 2. It is very similar to that used by Brown *et al.* (1970). The membrane potential (V_m) was recorded on a cathode ray oscilloscope as the potential difference between a micropipette (M 1) inside the cell and another potassium chloride filled micropipette (M 3) in the external solution. The micropipette M 2 was used to supply current to the cell from the feed-back amplifier (A 1). M 1 had a driven shield consisting of aluminium foil connected to the source of the transistor in the probe unit of the preamplifier (P 1).

When a rectangular commanding voltage pulse was applied to the feed-back amplifier, the membrane potential was clamped to a final value within 1 msec.

Membrane current was displayed on the cathode ray oscilloscope as the voltage drop across the feed-back resistor of the amplifier system (P 3 and A 2) used to hold the external saline at ground potential.

RESULTS

A *Injection of calcium chloride*

Variability of response. Experiments reported previously have shown that when small quantities of calcium chloride are injected into *Aplysia* neurones the membrane potential is hyperpolarized reversibly. However, if a large quantity of calcium salt is injected the response does not readily recover. A succession of such injections causes the membrane resistance to

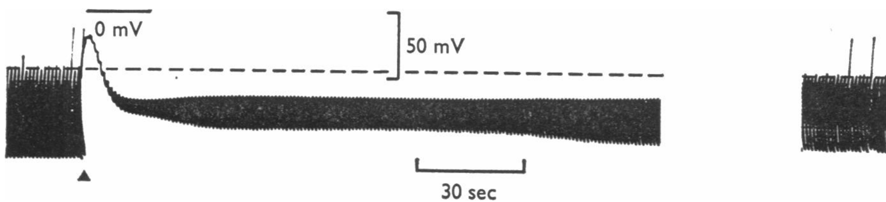

Fig. 3. The change in membrane potential and membrane resistance when a large quantity (unknown) of 100 mM calcium chloride was injected into a nerve cell at the point indicated by the filled triangle (▲). The resting potential (−46 mV) is shown by the dotted line. The membrane resistance was measured with hyperpolarizing pulses (duration 0·5 sec; frequency 1/sec) from the resting potential. The gap in the record corresponds to a period of 4 min.

decrease irreversibly (Meech, 1972). A similar response was frequently observed during injections of calcium chloride into *Helix* neurones. Fig. 3 shows a typical example. The response was biphasic. A rapid depolarization was followed by a prolonged hyperpolarization. However, if the pressure applied to the injecting electrode was carefully controlled a simple hyperpolarizing response could be obtained. Fig. 4*a* shows the response of cell D to the injection of a smaller quantity of a solution containing 100 mM calcium chloride and 100 mM potassium chloride. The micropipette used for the injection was not calibrated and consequently it is not possible to be sure exactly how much calcium chloride was injected. However, a similar micropipette under a similar pressure (about 2 lb./in.^{2}) was found to eject 10^{-11} l. in 6 sec (Meech, 1972). In Fig. 4*a* the period of injection, indicated by the filled circles (●), was 4 sec. Therefore the volume of solution injected was less than 10^{-11} l. A similar volume of 200 mM potassium chloride, when injected, had no effect on the cell.

This paper is concerned with the rapidly reversible hyperpolarizing responses illustrated in Fig. 4*a*. Similar responses were seen when barium chloride or strontium chloride was injected in place of calcium chloride. However, injection of magnesium chloride produced no effect.

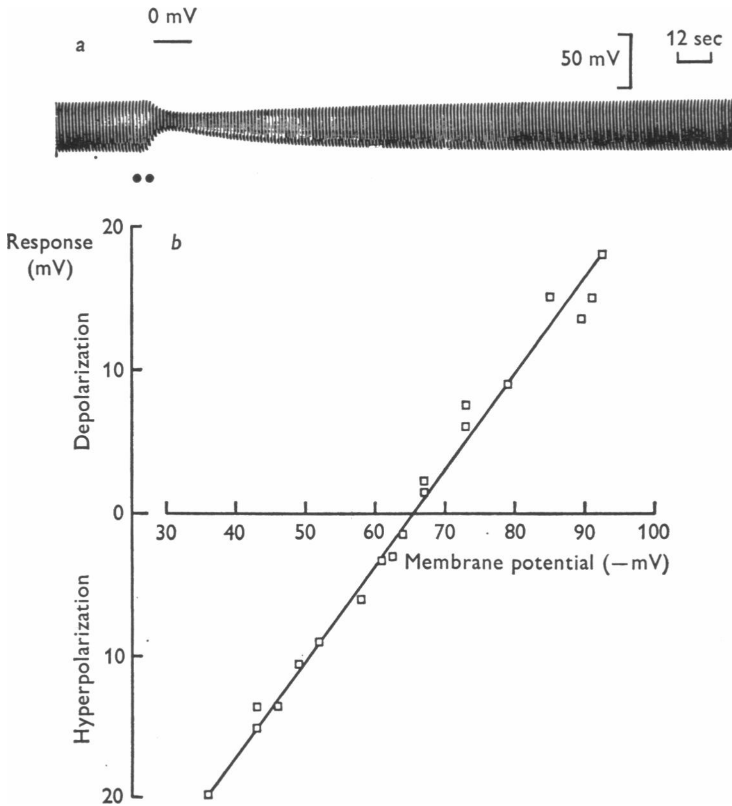

Fig. 4*a*. Effect of calcium chloride injection on the membrane potential and membrane resistance of cell D. The membrane resistance was measured with hyperpolarizing pulses (duration 0·5 sec; frequency 1/sec) from the resting potential (−57 mV). During the period indicated by the filled circles (●) a small quantity (less than 10^{-11} l.) of 100 mM calcium chloride was injected. Cell diameter about 200 μm. *b*, the relationship between the membrane potential of cell D and the size of the potential change produced by calcium chloride injection. The reversal potential was −65 mV.

The nature of the hyperpolarizing response. In *Aplysia* neurones the hyperpolarizing response to injected calcium chloride is a result of an increase in potassium conductance (Meech, 1972). Figs. 4 and 5 show this is also true of *Helix aspersa* neurones. The effect of injected calcium chloride on the membrane resistance is shown in Fig. 4*a*. Fig. 4*b* shows that the

size and polarity of the potential change depended on the membrane potential. The reversal potential of the response was −65 mV. Fig. 5 shows the effect of the concentration of potassium in the external solution on the reversal potential of the calcium response in cell A. The resting potential of cell A and the effect of a change in external potassium depends

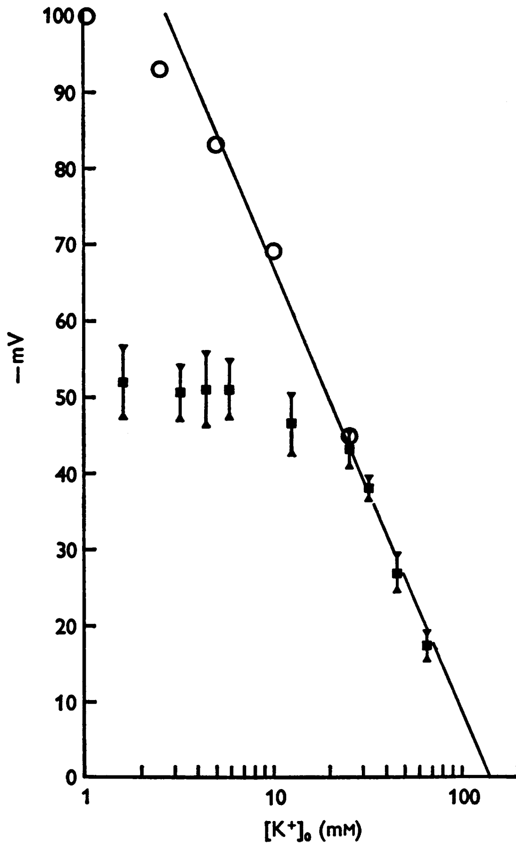

Fig. 5. The relationship between the external potassium concentration and the reversal potential of the calcium response in cell A (open circles ○). Average values (± S.E.) for the resting potential of cell A ($n = 4$) are also shown (filled squares ■). All experiments carried out during June. Resting potential values from Meech (1966).

on the time of year (Kerkut & Meech, 1967). The values for the resting potential used in Fig. 5 are taken from a survey carried out in 1965 (Meech, 1966). They are the average of observations taken from four preparations during June. The reversal potentials were taken from a single cell during June 1972. The line drawn on the graph shows a 58 mV change

in potential for a tenfold change in the external potassium concentration. The resting potential deviated from that predicted by the Nernst equation at potassium concentrations less than 20 mM. On the other hand the reversal potential plot had a 58 mV slope down to 5 mM potassium. Clearly the reversal potential depended on the concentration of potassium in the external solution over a much wider range than did the resting potential.

The quantity of calcium chloride injected. As reported previously it is possible to calibrate individual micropipettes so that the volume of a salt solution injected into a neurone can be estimated (Meech, 1972). However, this may not be an entirely satisfactory way of calculating the amount of calcium injected because the micropipette was usually partially blocked when inside the cell. In addition there are errors in calculating the cell volume. A better method is to estimate the intracellular chloride ion concentration before and after the injection of calcium chloride solution. This can be done by using neurones which hyperpolarize in response to acetylcholine. Kerkut & Thomas (1963) have shown that this hyperpolarizing response is the result of an increase in the chloride permeability of the membrane. The intracellular chloride ion concentration can be calculated from the acetylcholine reversal potential because acetylcholine produces, in these cells, an increase in chloride permeability only (Chiarandini & Gerschenfeld, 1967).

The acetylcholine reversal potentials of both cell B and cell C are dependent on the concentration of chloride in the bathing solution. The acetylcholine reversal potential of cell C changed from 50 to 81 mV when the external chloride was changed from 40 to 135 mM. That of cell B changed from 25 to 55 mV under the same conditions. The Nernst equation predicts a change of 30·5 mV. It is important to first measure the acetylcholine reversal potential in low chloride solution because the cell rapidly looses chloride in this solution. In high chloride solution the chloride moves back into the cell more slowly (Meech, 1966; see also Kerkut & Thomas, 1963).

In a series of experiments carried out on cells B and C the amount of calcium injected was calculated from the change in acetylcholine reversal potential. The membrane resistance was allowed to recover before the acetylcholine reversal potential was determined. It is possible that the cell lost chloride in this time and so the results, shown in Table 1, give the minimum change in intracellular calcium. They were somewhat variable but in summary, when sufficient calcium chloride was injected to increase the intracellular calcium concentration by $5 \times 10^{-4} - 1{\cdot}4 \times 10^{-3}$ M the membrane resistance was reduced by 8–32 %.

B *Injection of calcium-EGTA buffers*

Fig. 6 shows the effect produced when a small quantity of calcium-EGTA buffer containing 2×10^{-6} M free calcium was injected into an

unidentified cell. Apart from its duration the response was similar to that given when a similar quantity of 100 mM calcium chloride was injected. The fact that the membrane resistance recovered suggests that the pump involved in the recovery process operated in the presence of EGTA (see also Meech, 1974*a*, *b*). This means that the intracellular level of free calcium produced by a calcium-EGTA buffer may be lower than the calculated value.

TABLE 1. Changes in membrane resistance in *Helix aspersa* neurones and the concentration of injected calcium

Cell type	Diameter (μm)	Change in $[Ca^{2+}]_i$ (mM)	Resistance change (%)
B	50	0·5	32
C	130	1·3	25
C	190	(i) 1·4	24
		(ii) 0·9	8
		(iii) 1·0	25
Unidentified cell in visceral ganglion	130	0·8	24

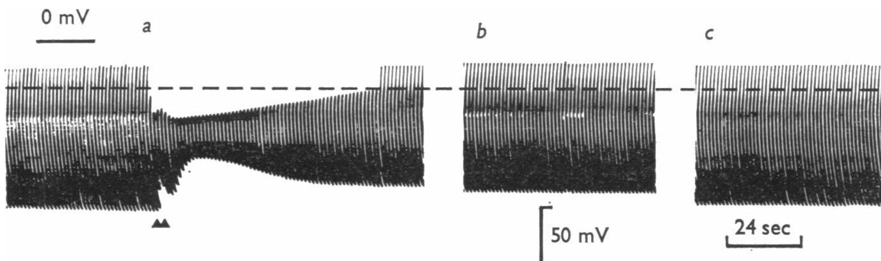

Fig. 6*a*. The change in membrane potential and membrane resistance of a nerve cell when injected with a small quantity (less than 10^{-11} l.) of calcium-EGTA buffer containing 2×10^{-6} M free calcium (i.e. Ca-EGTA: EGTA = 35·6:1 at pH 7·26). The dashed line shows the resting potential (−40 mV) which is hidden by action potentials (greatly attenuated by the pen recorder); *b*, taken 7·5 min after the injection; *c*, taken after a further 5 min. Cell diameter about 200 μM.

The effect of injecting larger quantities of different calcium-EGTA buffers is shown in Fig. 7. Record *a* shows that when a buffer containing 9×10^{-7} M free calcium ion was injected the membrane resistance rapidly fell by about 25 % and then stayed at this level for 2–3 min. At the end of this time (not shown in the record) the membrane potential declined and the cell died. The quantity of the calcium-EGTA which was injected was probably less than 5×10^{-11} l. (see Meech, 1972) which was about 0·01 of the volume of the cell. Therefore the final concentration of the buffer was

about 1 mM. If a buffer containing 10^{-7} M free calcium was injected there was no change of membrane resistance and no membrane hyperpolarization. The points plotted in Fig. 7*b* summarize the results obtained by this kind of experiment. Each point was taken from a different cell. The cells were identified in all but four cases. Clearly the inner membrane surface of *Helix* neurones was sensitive to 9×10^{-7} M calcium although the threshold level must be lower than this. Baker *et al.* (1971), report an upper limit of 3×10^{-7} M for the concentration of ionized calcium in squid exoplasm.

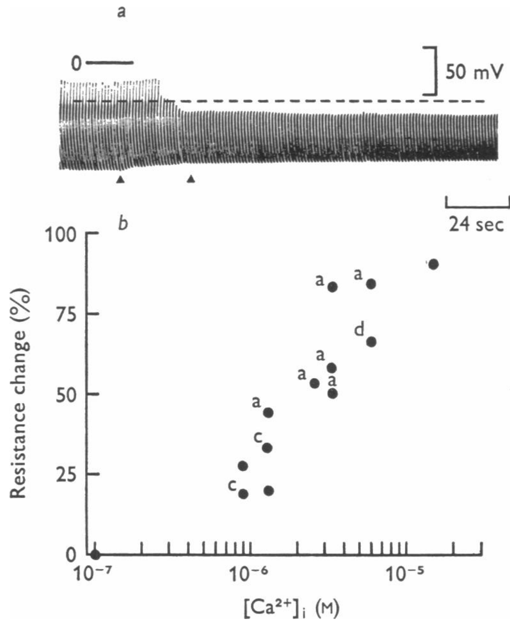

Fig. 7*a*. Effect of injecting a calcium-EGTA buffer containing 9×10^{-7} M free calcium (i.e. Ca-EGTA:EGTA = 16:1 at pH 7·26) on the membrane potential and membrane resistance of an unidentified cell. The resting potential (−43 mV), obscured by attenuated action potentials is indicated by the dashed line. The membrane resistance was measured by hyperpolarizing pulses (duration 0·5 sec; frequency 1/sec). During the period indicated by the filled triangles (▲) about 5×10^{-11} l. (exact amount unknown) of 125 mM calcium EGTA buffer was injected. Cell diameter, about 200 μm. *b*, relationship between the concentration of free calcium in the calcium-EGTA buffer injected and the change of membrane resistance. The cells used (a, c or d) are indicated where known.

C *The control of intracellular calcium concentration*

It is clear from the experiments described above that very little calcium injected into a neurone remains free. When a calcium-EGTA buffer containing about 10^{-6} M free calcium was injected into a neurone the membrane

resistance fell by about 25%. When calcium chloride was injected the intracellular calcium concentration increased about 10^{-3} M for a similar change of resistance. Much of the injected calcium must be rapidly removed from the cytoplasm. There is evidence that a sodium dependent calcium pump exists in a number of tissues (see review by Baker, 1972). A similar mechanism may exist in *Helix* neurones. Fig. 8 shows the effect of sodium-free (Tris) solution on cell A. The membrane resistance at first increased, which is to be expected if the membrane was permeable to sodium ions, and then slowly fell to almost 50% of this initial value. The membrane potential hyperpolarized by 11–17·5 mV. Both membrane potential and membrane resistance behaved as if the intracellular calcium concentration was slowly increasing. Baker *et al.* (1971) have shown that the intracellular calcium concentration in squid giant axons increases in sodium-free solution.

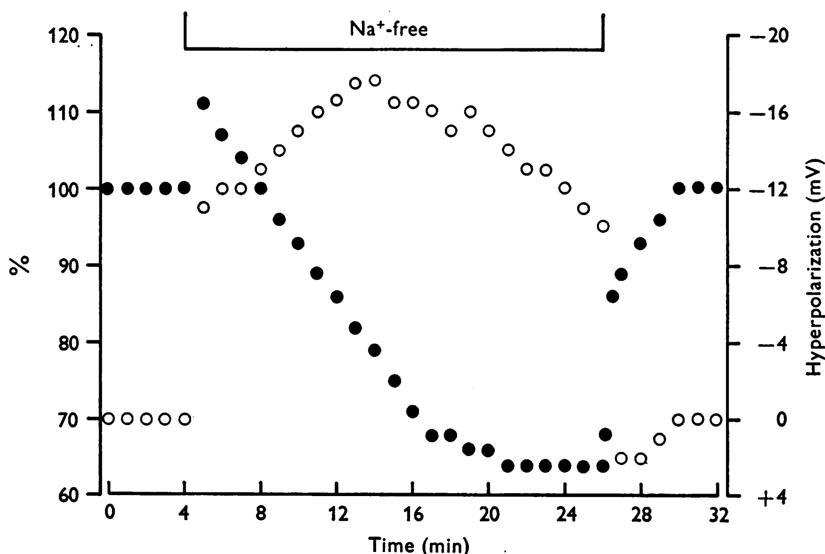

Fig. 8. Effect of sodium-free (Tris) saline on the membrane resistance (filled circles, ●) and membrane potential (open circles, ○) of cell A. The membrane potential is measured from the resting potential (−49 mV). The membrane resistance is plotted as a percentage of the resting membrane resistance. Temperature 23° C.

A sodium dependent pumping mechanism cannot be the only way in which calcium is removed from the cytoplasm, however, because the cell rapidly recovered from calcium chloride injection even in sodium-free solution. It is probable that, much of the injected calcium is taken up by

mitochondria (Vasington & Murphy, 1961; DeLuca & Engstrom, 1961). Metabolic poisons such as 2,4-dinitrophenol inhibit calcium uptake by mitochondria (Vasington & Murphy, 1962). When *Helix* neurones were bathed in saline containing 1 mM 2,4-dinitrophenol and 2 mM iodoacetate the membrane potential of cell A hyperpolarized from −48 to −61 mV. The membrane resistance was reduced to 38 % in 23 min. Metabolic poisons have the same effect on *Aplysia* neurones (Carpenter *et al.* 1971) and cat cortical neurones (Krnjević *et al.* 1970). In each case it is likely that there was a gradual increase in the intracellular calcium concentration (see also Baker *et al.* 1971). Krnjević & Lisiewicz (1972) have also argued that much of the calcium injected into cat spinal motoneurones is taken up by mitochondria.

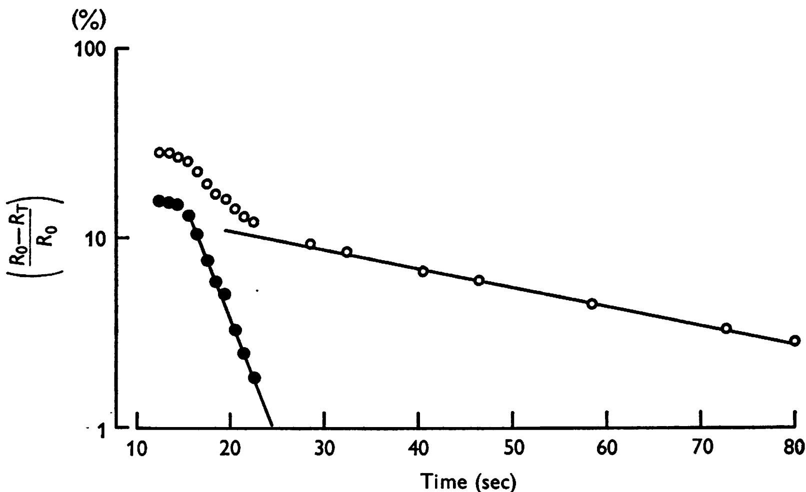

Fig. 9. The recovery of the membrane resistance of cell C after calcium chloride injection. The open circles (○) show the loss of membrane resistance ($R_0 - R_T$) expressed as a percentage of the resting resistance (R_0) and plotted on a logarithmic scale against time (T) after injection. The filled circles (●) were separated as described in the text. Cell diameter 190 μm. Temperature 24° C.

It seems probable therefore that the recovery of membrane resistance following injection of calcium chloride into a neurone depends on both the activity of a sodium dependent calcium pump and on sequestration of the calcium by the mitochondria. Fig. 9 shows that the recovery phase is indeed made up of two exponents. The open circles (○) show the resistance change induced by calcium expressed as a percentage and plotted on a logarithmic scale against time. The linear part of this graph was extrapolated and subtracted to produce the graph of the filled circles (●).

Whether these two exponents do represent two separate pump mechanisms is not yet certain. It may be that the break-down of the calcium/membrane complex is the rate limiting step.

D *Voltage clamp experiments*

In many molluscan neurones a train of action potentials is followed by a period of increased potassium conductance (*Anisodoris*, Connor & Stevens, 1971; *Aplysia*, Brodwick & Junge, 1972; *Helix*, Moreton, 1972). This post-tetanic hyperpolarization is abolished in *Aplysia* neurones by calcium-free Ringer and by the injection of EGTA which suggests that it is mediated by calcium ions (Meech, 1974*a*).

The influx of calcium during an action potential can be studied in more detail by using a voltage clamp circuit to hold the membrane potential at a defined level for brief periods of time (Baker *et al.* 1971). Fig. 10 shows that after depolarizing the membrane to +40 mV for 90 msec at a rate of ten times every second for 30 sec there was a period of increased potassium conductance which was not seen if the membrane was simply clamped to the resting potential for 30 sec. In order to enhance the effect the experiment was carried out at 6° C and the cell was bathed in a magnesium-free saline containing 26·5 mM calcium chloride.

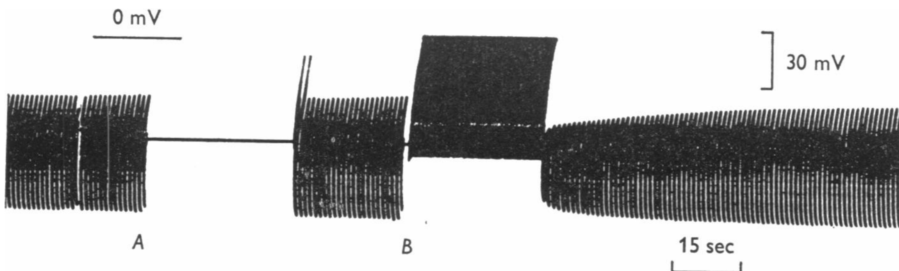

Fig. 10. Effect of repetitive depolarizing pulses on membrane resistance. The membrane resistance was measured with hyperpolarizing pulses (duration 0·5 sec; frequency 1/sec). At the point indicated *A* the membrane potential was clamped to −60 mV for 30 sec. At point *B* the membrane was depolarized from −60 to +40 mV for 90 msec, 10 times/sec for 30 sec. Saline was magnesium-free and contained 26·5 mM calcium chloride. Temperature 6° C.

The reversal potential of the response is shown in Fig. 11*a*. It was about −65 mV. This was the same as the reversal potential of the response to injected calcium. Fig. 11*b* shows that the percentage change in membrane resistance was directly proportional to the duration of the stimulating pulse up to 80 msec. Therefore although the calcium influx may have

continued after the pulse had finished the amount of calcium entering the cell was proportional to the duration of the pulse. Calcium must have entered the cell even with pulses of only 1–2 msec. This is as fast as potassium activation and therefore the influx of calcium could trigger potassium activation during an action potential in *Helix* neurones.

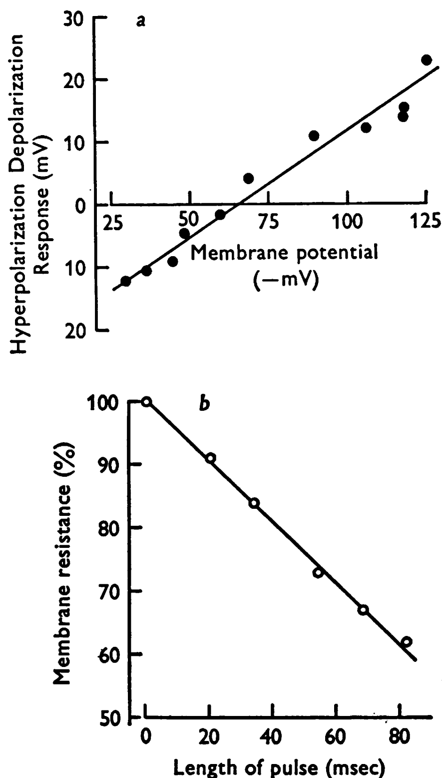

Fig. 11*a*. The relationship between membrane potential and the size of the potential change produced by a 'tetanus'. Experimental conditions were as described for Fig. 10. The reversal potential was about −65 mV. *b*, the relationship between the length of the depolarizing pulse and the membrane resistance following the 'tetanus'. Experimental conditions were the same as for Fig. 10.

The recovery of the membrane resistance shown in Fig. 10 was rapid even at 6° C. It seemed likely that the rate of recovery was governed by a metabolically driven calcium pump. To test this the cell was bathed for 20 min in a saline containing 1 mM 2,4 dinitrophenol and 2 mM iodoacetate at room temperature (23° C). The membrane was then depolarized to 0 mV for 45 msec, fourteen times each second for a period of 20 sec. This is

shown in Fig. 12. The response was different to that shown in Fig. 10; the maximum hyperpolarization was delayed by about 20 sec, the resistance change was considerably greater and the response was much longer lasting. The effect resembles the biphasic response shown in Fig. 3 which was induced by a massive injection of calcium chloride. The resistance changed by more than 80% which suggests (see Fig. 7) that the free intracellular calcium increased 10–100 times as a result of the tetanus. Although the membrane partially recovered after 6–7 min it did not completely recover until the dinitrophenol and iodoacetate had been washed away. The calcium pump must be very active in *Helix* neurones.

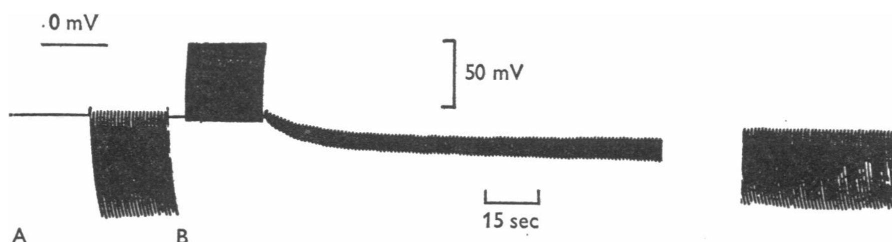

Fig. 12. Effect of repetitive depolarizing pulses on membrane resistance in the presence of 1 mM 2,4-dinitrophenol and 2 mM iodoacetate. The membrane resistance was measured with hyperpolarizing pulses (duration 0·5 sec; frequency 1/sec). At point A the membrane was clamped to −60 mV for 20 sec. At point B the membrane was depolarized from −60 to 0 mV for 45 msec, 14 times/sec for 20 sec. The gap in the record corresponds to a period of 6 min. Temperature 23° C.

DISCUSSION

Mechanism of calcium action

There are three ways in which calcium could induce the potassium permeability increase when injected into nerve cells (see Frankenhauser & Hodgkin, 1957; McLaughlin, Szabo & Eisenman, 1971).

(*a*) Divalent ions could shield negative sites on the membrane and thereby reduce the potential gradient across the membrane. The potassium conductance is potential dependent and therefore the effective depolarization would cause an increase in potassium permeability. The decrease in potential gradient across the membrane would not be seen in bulk solution. However, calcium and magnesium ions should be equally effective in this shielding process. In addition the potassium permeability increase should be blocked or partially blocked by hyperpolarizing the membrane. Fig. 4 shows that the response is linear up to −90 mV.

(*b*) Calcium ions may be adsorbed onto the membrane thereby counteracting the effect of negative charges already present. This could account for

the specificity of the process for calcium, but not for the fact that the response is linear up to -90 mV.

(*c*) The calcium ions may be involved in a chemical reaction with the membrane. If so the fact that calcium, barium and strontium ions can all induce an increase in potassium permeability, whereas magnesium cannot, suggests that the ability to form complexes with a co-ordination number of 8 is important.

The role of calcium in potassium activation

The finding that injected calcium increases the membrane potassium permeability in central neurones (Meech & Strumwasser, 1970; Krnjević & Lisiewicz, 1972) is a surprising one, because in many neurones an increase in external calcium causes an increase in membrane resistance (see Cole, 1949; Brink, 1954; Shanes, 1958). As a consequence potassium activation has often been considered to involve competition between potassium and calcium for a site on the membrane. For example, the model proposed by Goldman (1964, 1971) predicts that a high intracellular calcium concentration would decrease the potassium conductance. This is true also of the models proposed by Tobias (1958) and Tasaki. Tasaki (1968, p. 152) has proposed that the membrane changes from a low resistance excited state to a high resistance resting state when divalent cations derived from the external medium form stable complexes with fixed anionic sites on the membrane. However the fact that injected calcium causes a fall in membrane resistance suggests that, if anything, the calcium initiates potassium activation rather than the scheme Tasaki suggests.

The proposition that calcium influx stimulates potassium activation during an action potential is an attractive one because short depolarizing pulses can stimulate calcium influx in both squid giant axons (Baker *et al.* 1971) and *Helix aspersa* neurones. In squid giant axon 100–200 μsec depolarizing pulses can stimulate an increase in intracellular calcium. Although the calcium influx does not necessarily occur during the depolarizing pulse it seems probable that the calcium influx is fast enough to trigger potassium activation in both squid and *Helix*. However, evidence from the work of Baker *et al.* (1971) and Baker, Meves & Ridgway (1973) suggests that the mechanism does not operate in squid axons. Furthermore, when squid giant axons were perfused with 10 mM-EGTA there was no significant change in the potassium currents seen during voltage clamp experiments (H. Meves & R. W. Meech, unpublished observations). On the other hand, Frankenhauser & Hodgkin (1957) have suggested that there may be a loss of 'potassium carrier' from squid axons during prolonged washing with calcium-free solution, because the membrane lost

much of its normal rectifying properties. The membrane recovered when the calcium in the external solution was replaced. The potassium currents recorded from *Helix* neurones are considerably and reversibly reduced when the ganglion is washed in calcium-free saline for 3 min (Meech & Standen, 1974). It may be that calcium is involved in a potassium activation system which exists in central neurones but not in the squid axon membrane.

Is the influx of calcium during an action potential sufficient to generate a permeability change? It has not been possible to calculate the change in calcium concentration at the inner membrane surface because of the complexity of the surface of *H. aspersa* neurones (R. W. Meech & R. B. Moreton, unpublished). There are finger like invaginations running parallel to the main part of the surface (Treherne & Moreton, 1970). A major problem is to discover what effect these infoldings have on the local concentration of calcium.

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