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CALCIUM AND SODIUM IONS AS CHARGE CARRIERS IN THE ACTION POTENTIAL OF AN IDENTIFIED SNAIL NEURONE

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SUMMARY

1. The soma of cell A in *Helix aspersa* produced action potentials in sodium-free or calcium-free saline, but not in saline with neither sodium nor calcium.

2. The axon had a sodium-dependent action potential.

3. Tetrodotoxin (5×10^{-6} M) had no effect on the overshoot except at low external divalent ion concentrations.

4. The action potential in sodium-free saline was blocked by cobalt.

5. The slope of action potential overshoot against sodium concentration in the presence of 10 mM calcium was 10·5 mV/tenfold change. That of overshoot against calcium concentration in the presence of 75 mM sodium was 22 mV/tenfold change.

6. In sodium-free saline the slope of overshoot versus calcium concentration was 27 mV/tenfold change.

7. It is concluded that calcium is an important charge carrier in the action potential of cell A.

INTRODUCTION

Calcium ions have been shown to be charge carriers during the inward current of the action potential in many excitable cells, for example barnacle muscle fibres (Hagiwara & Naka, 1964), mammalian cardiac muscle fibres (Reuter, 1965, 1966), some vertebrate smooth muscle fibres (Brading, Bülbring & Tomita, 1969), and several nerve cells (cf. Reuter, 1973). In the nerve cell bodies of several species of gastropod molluscs calcium ions have been implicated in spike production, e.g. *Aplysia californica* (Geduldig & Junge, 1968), *Helix pomatia* (Meves, 1968) and *Limnaea stagnalis* (Sattelle, 1974). In electrophysiological studies of most molluscan ganglion preparations problems of cell recognition necessitate

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pooling data from several unidentified nerve cell bodies. Nevertheless, considerable variations are observed in the membrane properties of cells within a single ganglion. Kerkut & Gardner (1967), for example, divided neurones of *Helix aspersa* into three classes according to the ionic requirements of their action potentials: (1) mainly sodium-dependent cells; (2) cells dependent on both sodium and calcium ions; (3) mainly calcium-dependent cells. Moreton (1972) also found some neurones in *H. aspersa* which were sodium-independent. Using *Aplysia* Geduldig & Junge (1968) and Geduldig & Gruener (1970) obtained consistent results from the identifiable giant neurone and concluded that both sodium and calcium ions were charge carriers during the action potential.

This paper describes experiments on an identified nerve cell body in *Helix aspersa*, cell A (Kerkut & Meech, 1966). Evidence is presented from studies on the action potential of cell A that calcium is the major charge carrier of inward current in normal saline and is the only carrier of inward current in sodium-free saline. The cell provides a convenient preparation for the investigation of some properties of potential-dependent changes in membrane calcium permeability. The subsequent paper describes voltage-clamp studies of the calcium inward current.

METHODS

Garden snails, *H. aspersa*, were kept in the dry in tins without food. Under these conditions the snail sealed up the mouth of its shell and became dormant. This eliminated the seasonal variations in the resting potential of cell A described by Kerkut & Meech (1967) and snails kept in this way survived for about a year.

Cell A is situated in the right parietal ganglion of the snail brain and has an axon which runs in the visceral nerve. The cell can be identified by its large size (200 μm), position and electrical behaviour. Cell A is normally spontaneously active, and has a depolarizing response to applied acetylcholine (Kerkut & Meech, 1966). The dissection of the snail ganglion and the position of cell A have been fully described by Kerkut & Meech (1966) and Meech (1974). In the present work the inner connective tissue sheath was always removed in the region of cell A to allow free access of salines to the membrane and easy impalement with electrodes.

The cell was impaled with two 3 M potassium chloride filled micro-electrodes (resistance 4–8 MΩ, tip potentials 0 to -2 mV). One of these was used to record membrane potential and the second to pass current. Steady current to de- or hyperpolarize the membrane from a battery and brief pulses from a Farnell pulse generating system were passed to this electrode via a 100 MΩ resistor. Traces were photographed from a Tektronix 502A oscilloscope and the membrane potential monitored on a Servoscribe pen recorder.

The experimental bath had a volume of 1 ml. and 20–40 ml. of saline was run through in 90 sec for solution changes. The basic physiological saline was as follows: (mM) NaCl 75, KCl 5, $CaCl_2$ 10, $MgCl_2$ 15, Tris/HCl 5 (pH 7·5). In sodium-free saline sodium chloride was replaced by 83 mM Tris/HCl. In calcium-free saline calcium chloride was replaced by magnesium chloride and in (Na, Ca)-free saline both substitutions were performed. Cobalt was either substituted for magnesium or simply added to the saline; it was added on the day of the experiment to reduce oxidation

to the trivalent form. Other salines are described in the text. Experiments were done at room temperature, 18–24° C. The variation in the course of a single experiment was less than 1° C.

RESULTS

The resting potential

Kerkut & Meech (1967) found that the resting potential of cell A showed seasonal variations. For example, cell A showed a mean resting potential of 61 mV (S.E. ± 2 mV) in July, whereas in April a mean resting potential of 43 mV (S.E. ± 1 mV) was noted. In the present study it was found that starvation for 2–3 weeks abolished this variation. The July resting potential was 44·6 mV (S.D. ± 4·5 mV) (eight cells) and the April value was 45·3 mV (S.D. ± 4·0 mV) (eight cells). The mean resting potential for seventy-five cells was 45·1 mV (S.D. ± 4·1 mV).

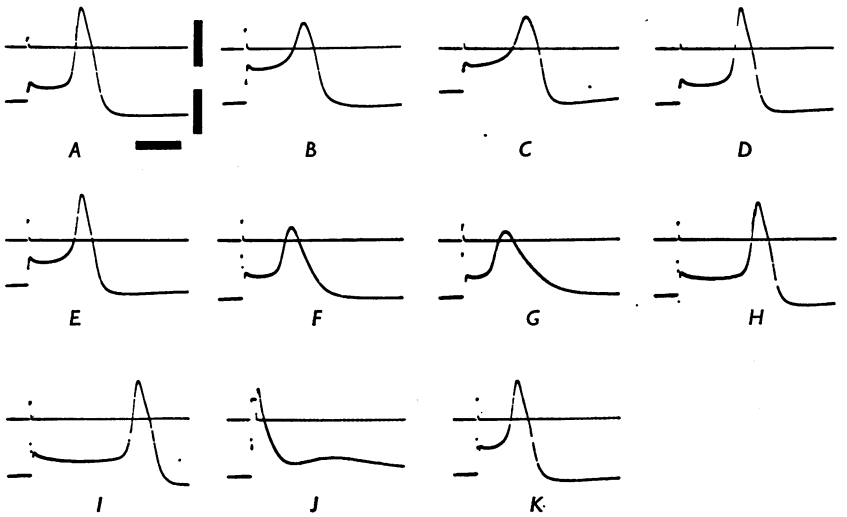

Fig. 1. Effects of sodium-free and calcium-free salines on cell A. *A*, normal saline; *B*, after 2 min in sodium-free saline; *C*, after 30 min in sodium-free saline; *D*, 5 min after return to normal saline; *E*, normal saline; *F*, after 3 min in calcium-free saline; *G*, 10 min in calcium-free saline; *H*, 5 min after return to normal saline; *I*, normal saline; *J*, (Na, Ca)-free saline; *K*, 4 min after return to normal saline. The upper trace marks the zero level and the stimulating current pulse. Calibrations 4×10^{-8} A, 40 mV, 20 msec. Cell diam. 205 μm. Temp. 20° C.

Ionic requirements for the action potential

The action potential overshoot in normal saline was 39·5 (S.E. ± 1·0) mV (Fig. 1). When the normal saline was changed to sodium-free (tris) saline the membrane potential hyperpolarized by around 10 mV and the cell ceased to fire spontaneously. Action potentials could still be elicited by

depolarizing current pulses, though it was sometimes necessary to depolarize the cell back to its normal resting potential of around −45 mV with steady current in order to get a maximal action potential. The overshoot was 22 (S.E. ± 1·5) mV after 5 min in sodium-free saline (Fig. 1*B*) and declined by less than 2–3 mV more during the subsequent hour. Moreton (1972), using other cells of *H. aspersa*, reported a steady decline of the action potential overshoot in sodium-free saline, which he attributed to calcium-loading of the cell interior caused by reduced function of a sodium-calcium exchange pump which extrudes calcium. The very slow decline in action potential overshoot suggests that calcium-loading is slow in cell A.

On replacement of normal saline by calcium-free saline a depolarization of about 5 mV occurred, coupled with an increase in the rate of spontaneous firing. The overshoot declined to 10–15 mV in 3 min (Fig. 1*F*) and then declined more slowly. Calcium-free saline caused irreversible effects on the cell after 30–40 min at which time the action potential peak was near 0 mV.

When normal saline was replaced by saline with neither sodium nor calcium ions, the action potential was blocked completely within 2 min (Fig. 1*J*).

These results show that either sodium or calcium ions are sufficient for action potential production in cell A. One or other of these ions must be present.

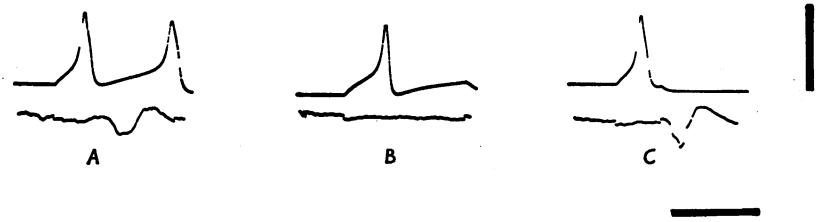

Fig. 2. Effects of sodium-free saline on somatic and axonal action potential. *A*, in normal saline; *B*, after 5 min in sodium-free saline; *C*, 3 min after return to normal saline. Upper trace: intracellular record from cell body. Lower trace: record from visceral nerve obtained using a suction electrode. Calibrations: upper trace 100 mV, lower trace 1 mV, 50 msec. Cell diam. not measured. Temp. 22° C.

The axonal action potential

An extracellular record was obtained from the visceral nerve using a suction electrode (Fig. 2). The action potential from the axon of cell A could be identified by its large size and time relation to the somatic spike. The axonal action potential was blocked within 30 sec in sodium-free

saline and recovered within 1 min of return to sodium-containing saline. Thus the axon differs from the soma in that calcium cannot support the action potential. Similar axon–soma differences have been shown in *Cryptomphallus aspersa* (Wald, 1972) and *Aplysia* (Kado, 1973).

Effects of tetrodotoxin

Moreton (1968) reported that high concentrations of tetrodotoxin (5×10^{-6}–10^{-5} M) abolished the action potentials of *H. aspersa* neurones, but was later unable to repeat this effect (Moreton, 1972). In five experiments on cell A, 5×10^{-6} M tetrodotoxin in normal saline had no measurable effect on the overshoot. Colquhoun, Henderson & Ritchie (1972) have shown that there is some competition between calcium ions and tetrodotoxin in vertebrate unmyelinated nerve. I tested the effect of

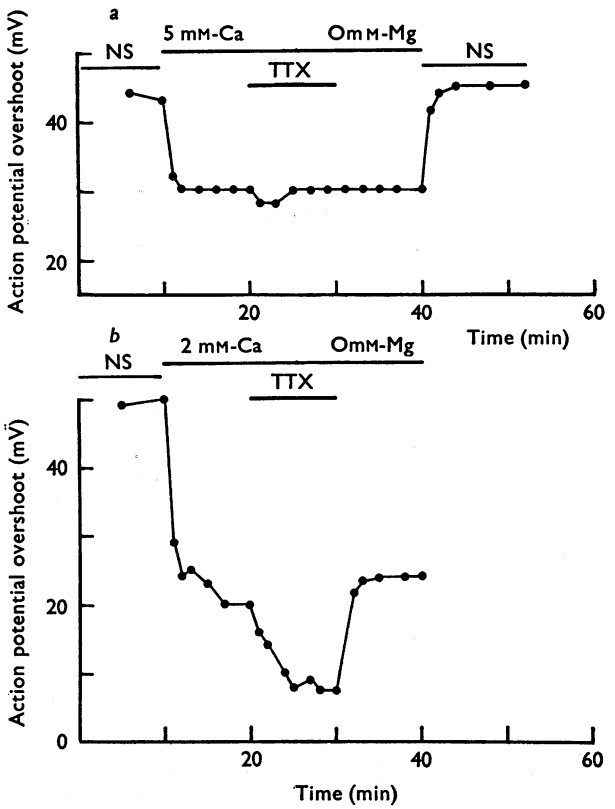

Fig. 3*a*, effect of 5×10^{-6} M tetrodotoxin (TTX) in magnesium-free saline with 5 mM calcium. Ordinate: action potential overshoot. Abscissa: time. Diam. 195 μm. Temp. 19·5° C. *b*, effect of 5×10^{-6} M tetrodotoxin in magnesium-free saline with 2 mM calcium. Ordinate and abscissa as in *a* above. Temp. 19·5° C. Diam. not measured.

5×10^{-6} M tetrodotoxin in magnesium-free saline containing 5 mM calcium or 2 mM calcium (Fig. 3). Three cells tested with tetrodotoxin in 5 mM calcium gave overshoot reductions of 2, 1 and 0 mV. In 2 mM calcium reductions of 15, 12 and 11 mV were observed with tetrodotoxin.

The tentative conclusion from these experiments, which need extending, is that the sodium component of the action potential of cell A is tetrodotoxin-sensitive to some extent, but that the divalent ion concentration in normal saline may be too high for 5×10^{-6} M tetrodotoxin to be effective. This could be due to Ca-tetrodotoxin competition or alternatively the calcium inward current in normal saline may be large enough to mask the effect of removal of a small component of the sodium current by tetrodotoxin.

Effects of cobaltous ions

Cobaltous ions block the calcium action potential of barnacle giant muscle fibres (Hagiwara & Takahashi, 1967), the calcium component of the action potential in *Aplysia* (Geduldig & Junge, 1968) and other tissues

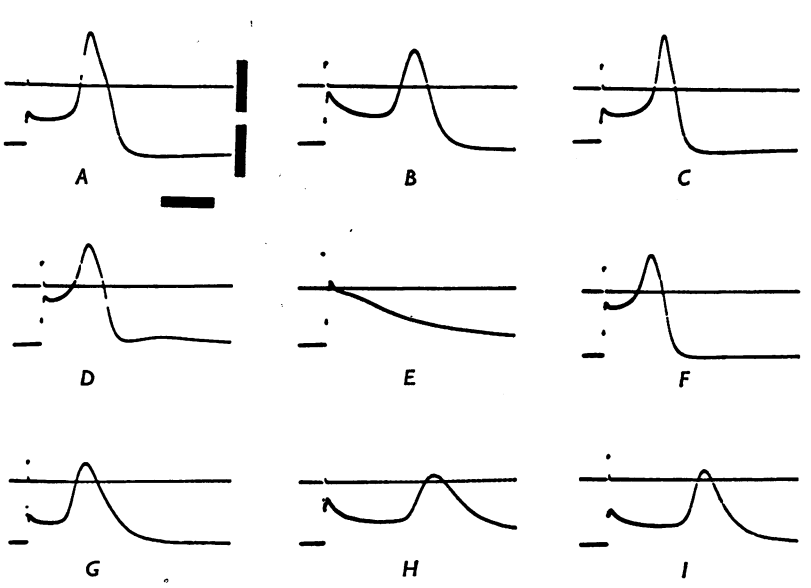

Fig. 4. Effects of cobalt. *A*, normal saline; *B*, normal saline + 10 mM cobaltous chloride after 10 min; *C*, 5 min after return to normal saline; *D*, sodium-free saline after 10 min; *E*, sodium-free saline + 10 mM cobaltous chloride after 2 min; *F*, 4 min after return to sodium-free saline; *G*, calcium-free saline, 10 min; *H*, calcium-free saline + 10 mM cobaltous chloride, 8 min; *I*, 5 min after return to calcium-free saline. The upper trace marks the zero level and the stimulating current. Calibrations 4×10^{-4} A, 40 mV, 20 msec. Cell diam. 205 μm. Temp. 20° C.

(cf. Reuter, 1973). Baker, Meves & Ridgway (1973) showed that cobaltous and manganous ions blocked the tetrodotoxin-insensitive component of the calcium entry into squid axons.

The effects of cobaltous ions on cell A are shown in Fig. 4 (four other preparations gave similar results). In normal saline 10 mM cobaltous chloride caused a reversible reduction in overshoot of about 10 mV and a slight lengthening of the action potential (Fig. 4*B*). In sodium-free saline 10 mM cobaltous chloride blocked the action potential completely and reversibly within 3 min (Fig. 4*E*). In calcium-free saline cobalt caused a 5 mV reduction in overshoot (Fig. 4*H*) which was not completely reversible and so may be partly caused by the decline in the condition of the cell seen in calcium-free saline.

Effects of different sodium and calcium concentrations on the action potential

Fig. 5 shows the effect of different sodium concentrations on the overshoot in the presence of calcium ions and the effects of different calcium concentrations on the overshoot in the presence of sodium. Normal saline (75 mM sodium, 10 mM calcium) was used as control and results were linearly corrected for changes between controls. The effects of saline changes on the overshoot were complete within 2–3 min.

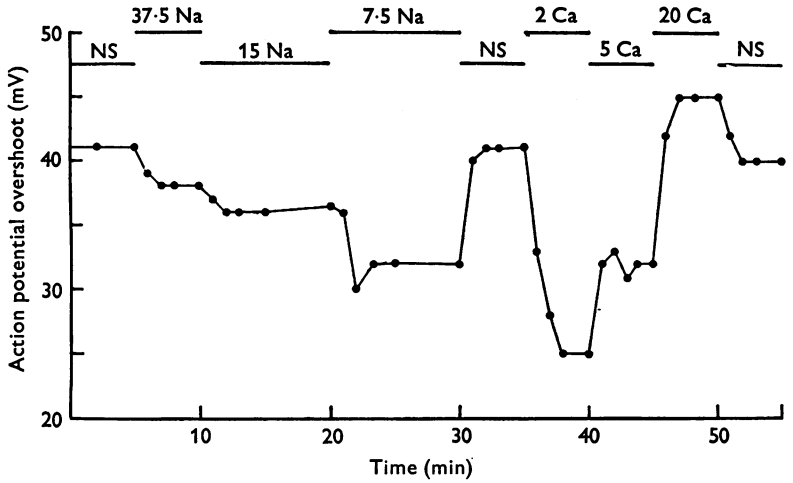

Fig. 5. Effects of different sodium and calcium concentrations on the action potential overshoot in cell A. Ordinate: overshoot. Abscissa: time. Cell diam. 220 μm. Temp. 21° C.

Fig. 6*a* shows a plot of overshoot against log external sodium concentration in the presence of 10 mM calcium. The slope is 10·5 mM for a tenfold change in $[Na]_0$. Fig. 6*b* shows a plot of overshoot against log external

calcium concentration in the presence of 75 mM sodium. As the external magnesium concentration was constant (15 mM) changes in $[Ca]_o$ will change the total external divalent ion concentration and this may affect the threshold and the degree of activation of the conductance mechanisms (cf. Frankenhaeuser & Hodgkin, 1957; Hille, 1968). For this reason the resting potential was varied by passing steady current and the maximum action potential obtainable at each calcium concentration was recorded. In 2 mM calcium it was necessary to hyperpolarize the membrane by about 10 mV to obtain a maximal action potential. The slope was 22 mV for a tenfold change in external calcium concentration.

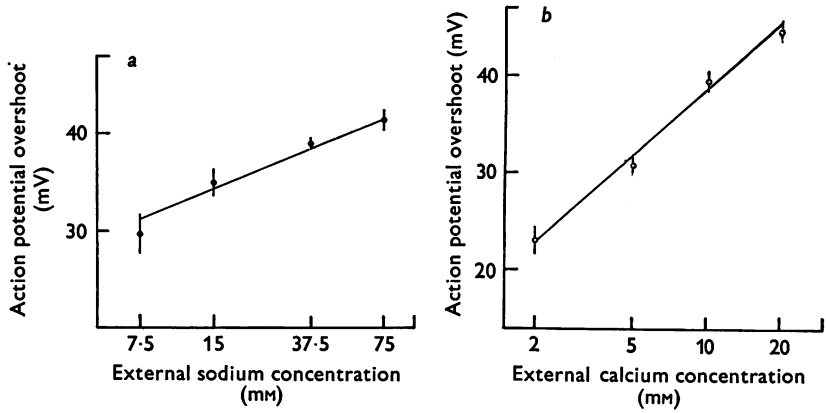

Fig. 6*a*, results for four cells showing the effect of different sodium concentrations on the overshoot (calcium 10 mM). Vertical bars are 2 × S.E. of mean. Diam. 200–220 μm. Temp. 20·5–21° C. *b*, results for the same four cells as in *a* above showing the effect of different calcium concentrations on the overshoot (sodium 75 mM). Bars 2 × S.E. of mean.

The dependence of overshoot on external calcium concentration was also tested in sodium-free saline. Under these conditions the intracellular sodium should be reduced to about 1·5 mM in 10 min (Thomas, 1972) and so apart from a small outward sodium current the active membrane should approximate to a calcium electrode.

In the experiment shown in Fig. 7*a* calcium was replaced by magnesium in order to keep the total divalent ion concentration constant at 25 mM. This was to reduce the effect of increased $[Ca]_o$ in shifting the activation curves along the voltage axis. The slope of overshoot against $[Ca]_o$ was 27 mV/tenfold change. A possible disadvantage of this procedure was that it involved changing the magnesium:calcium ratio. In squid axon (Baker, Hodgkin & Ridgway, 1971) and frog neuromuscular junction (del Castillo & Katz, 1954) a raised Mg:Ca ratio reduces calcium influx. For this reason a second experiment in which different calcium concentrations

were tested in the absence of magnesium was performed (Fig. 7*b*). The resting potential was varied to obtain a maximal action potential as described previously. The cells tolerated magnesium-free salines rather poorly, especially at low $[Ca]_o$, and one cell become irreversibly inexcitable in 2 mM calcium. The slope was 26·5 mV/tenfold change in $[Ca]_o$.

It was not possible to examine the dependence of overshoot on external sodium concentration in a calcium-free saline owing to the progressive decline in the condition of the cells in this saline.

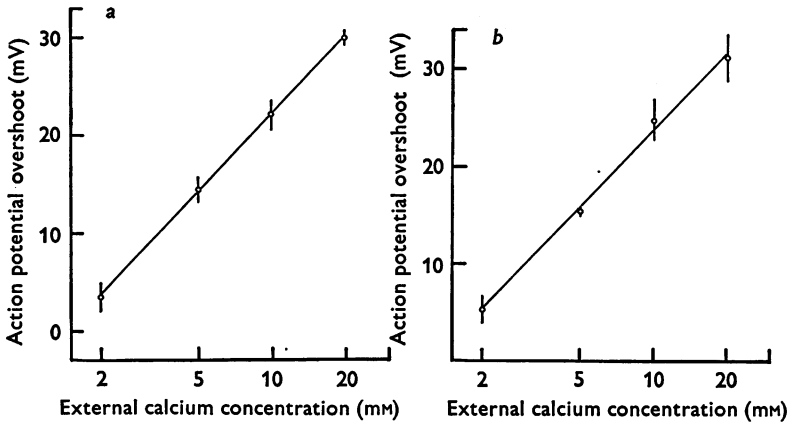

Fig. 7*a*, results for five cells showing the effect of different external calcium concentrations on the overshoot in sodium-free saline. Calcium + magnesium = 25 mM. Bars 2 + S.E. of mean. Diam. 180–210 μm. Temp. 23–25° C. *b*, results for four cells (2 mM calcium point for three cells) showing effect of different external calcium concentrations in sodium-free, magnesium-free saline. Bars 2 × S.E. of mean. Diam. 195–220 μm. Temp. 19·5–21° C.

DISCUSSION

It is clear from these experiments using an identified cell that much of the variability in ionic requirements for the action potential in *H. aspersa* (Kerkut & Gardner, 1967; Moreton, 1972) is caused by differences between cells. Using the same cell the results were consistent from preparation to preparation.

In common with many other molluscan neurones cell A of *Helix aspersa* produces action potentials in sodium-free saline. It is unlikely that sodium ions remain in the intercellular spaces since Moreton (1972) has shown that potassium ions exchange freely between the intercellular spaces and the bathing medium by diffusion. In addition, the change in overshoot which occurs on changing the saline to sodium-free saline was complete within 2–3 min. Moreton (1972) showed, using ouabain, that little or no sodium-loading of the cell interior occurred in sodium-free saline and pointed out

that this suggests that the sodium concentration immediately outside the cell membrane was considerably below normal in sodium-free saline. This argues against the proposal that a store of sodium ions maintains the action potential in sodium-free saline (Chamberlain & Kerkut, 1969).

The present study provides further evidence for a calcium inward current.

(*a*) In sodium-free saline the action potential overshoot fell rapidly to around +22 mV and this level declined little over the next hour.

(*b*) A change in overshoot of 27 mV/tenfold change in external calcium concentration was observed in sodium-free saline. This is close to the 29 mV slope predicted for a calcium electrode.

(*c*) Cobaltous ions, which are known to block calcium action potentials in other preparations (Reuter, 1973), blocked the action potential in sodium-free saline.

(*d*) Saline with neither sodium nor calcium ions rapidly and reversibly abolished the action potential.

In order to explain these results on the basis of a sodium store it would be necessary to assume that external calcium concentration affected the availability of stored sodium ions and that the store was unavailable in the absence of external calcium or the presence of 10 mM cobalt. There is no experimental precedent for any of these assumptions from other preparations.

Meves (1968) pointed out that the increased overshoot in raised $[Ca]_o$ in *Helix pomatia* neurones might be explained by removal of inactivation from the sodium-carrying system. In the present case this explanation does not apply since it was not possible to mimic the effect of increased $[Ca]_o$ on the overshoot by hyperpolarization, which should also remove inactivation.

It is likely, therefore, that the inward current in sodium-free saline is carried by calcium ions. The results also suggest that sodium ions are involved in the action potential in normal saline since sodium-free saline reduces the overshoot. The action potential of cell A, therefore, appears to be a mixed action potential similar to that of the *Aplysia* giant cell (Geduldig & Junge, 1968; Geduldig & Gruener, 1970), though in cell A calcium ions may be more important charge carriers than sodium ions.

In common with calcium action potentials in other preparations the overshoot in cell A does not reach the calculated calcium equilibrium potential. Calcium injections (Meech, 1974) and calcium-dependent potassium currents (Meech & Standen, 1974) suggest that the intracellular calcium concentration is rather lower than 10^{-6} M. With $[Ca]_o = 10$ mM and $[Ca]_i = 10^{-6}$ M the calcium equilibrium potential would be +116 mV. The membrane is thus not completely calcium-selective at the peak of the

action potential. The constant field theory of Goldman (1943) has been used by Fatt & Ginsborg (1958) and Meves (1968) to calculate the action potential peak in other preparations which have a calcium spike. The constant field theory can also be used to predict overshoot values similar to those observed experimentally in cell A, by assuming a small permeability to potassium ions at the peak of the spike. It must be added, however, that there is no experimental evidence for this assumption at present.

The equations given by Meves (1968) were used to calculate the overshoot for a given ration of $P_{Na}:P_K:P_{Ca}$ where these are the permeabilities to sodium, potassium and calcium respectively at the peak of the action potential. Taking $[Na]_o$ = 75 mM, $[Na]_i$ = 4 mM (Thomas, 1972), $[K]_o$ = 5 mM, $[K]_i$ = 100 mM and $[Ca]_i$ as negligibly small the permeability ratio which was found to fit the results most closely was $P_{Na}:P_K:P_{Ca}$ = 1:0·3:10. With $[Ca]_o$ = 10 mM the predicted overshoot was 36·9 mV and the calculated slope of overshoot against $[Ca]_o$ in the presence of 75 mM sodium was 21 mV/tenfold change in $[Ca]_o$. Experimentally the observed overshoot was 39·5 mV and the slope 22 mV/tenfold change in $[Ca]_o$.

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