*J. Physiol.* (1968), **199**, *pp.* 347–365
*With 9 text-figures*
*Printed in Great Britain*



# SODIUM AND CALCIUM COMPONENTS OF ACTION POTENTIALS IN THE *APLYSIA* GIANT NEURONE

BY D. GEDULDIG* AND D. JUNGE†

*From the Marine Neurobiology Facility (of the Brain Research Institute, U.C.L.A.), Scripps Institution of Oceanography, University of California, San Diego, La Jolla, California, U.S.A.*

(*Received* 29 *May* 1968)

SUMMARY

1. Action potentials resulting from direct stimulation can be recorded from the soma of the *Aplysia* giant neurone (located in the visceral ganglion) in sodium-free and in calcium-free external solutions. The neurones were impaled by internal micro-electrodes throughout the change of external solutions.

2. Complete replacement of either sodium or calcium in the bathing medium with Tris results in only a partial reduction of spike overshoot. Simultaneous replacement of both sodium and calcium reversibly and quickly abolishes the spike.

3. The sodium component of the spike in a calcium-free medium is blocked by tetrodotoxin; the drug has no effect on the calcium-dependent spike in sodium-free medium. Externally applied cobalt chloride blocks only the calcium-dependent component.

4. In calcium-free media, the overshoot value varies with sodium concentration in the manner predicted for a sodium electrode. In sodium-free media, the membrane behaves like a calcium electrode.

5. These results suggest that, during the normal action potential, both sodium and calcium act as carriers of the inward-directed current.

## INTRODUCTION

Some excitable membranes can produce action potentials in sodium-free solutions, and in these cases calcium seems to carry the inward-going current (crab muscle: Fatt & Katz, 1953; crayfish muscle: Fatt & Ginsborg, 1958; Abbott & Parnas, 1965; Takeda, 1967; barnacle muscle:

* Present address: Department of Biophysics, University of Maryland School of Medicine, Baltimore, Md.

† Present address: School of Dentistry, University of California at Los Angeles.

Hagiwara & Naka, 1964; *Helix* neurones: Gerasimov, Kostyuk & Maiskii, 1964, 1965; Meves, 1966; Kerkut & Gardner, 1967). In addition to these calcium-dependent action potentials, in other cells *both* sodium and calcium are required for the maintenance of action potentials having normal overshoots (smooth muscle: Bülbring & Kuriyama, 1963; frog heart: Niedergerke & Orkand, 1966*a*, *b*; snail neurones: Kerkut & Gardner, 1967). Previously it has been reported (Junge, 1967) that the spikes of the *Aplysia* giant neurone could persist when either the sodium or calcium in the bathing medium was replaced with sucrose. The attenuated spike which occurred in the absence of either ion was immediately abolished when sucrose replaced both sodium and calcium. For these systems which have spike overshoots sensitive to external calcium and sodium, it is possible that both ions enter the cell during activity.

Alternatively, the sodium and calcium sensitivity of the overshoot might be due to a regulation of the sodium activation by the external calcium concentration. In this case a variation of the overshoot in solutions containing a constant sodium concentration would result from a variation of external calcium, and the inward-going current could still be entirely carried by sodium ions (cf. fig. 5 in Frankenhaeuser, 1957 and also Niedergerke & Orkand, 1966*b*). Equally plausible, sodium might regulate an inward-going calcium current. Thus, either or both of these ions may (1) act directly by carrying the inward-going current of the action potential, or (2) act indirectly by regulating the membrane-permeability activation for the other ion, or (3) be subject to both kinds of action.

One way to examine the possibility of transmembrane current-carrying capabilities for each ion would be to test the overshoot sensitivity in solutions which were free of the other presumptive current carrier. If both ions can carry a transmembrane current, then in calcium-free solutions an inward sodium current should occur during the action potential, while in sodium-free solutions an inward calcium current should occur during the action potential. In these test solutions (18° C) an overshoot sensitivity of 58 mV/tenfold change of sodium and 29 mV/tenfold change of calcium could be expected. Alternatively, if both Na and Ca ions are present in this kind of system, smaller sensitivities to changes of either of the individual ion concentrations might be expected than in solutions where either Na or Ca was absent.

The present paper is concerned with further experiments on the *Aplysia* giant neurone to test the possibility that both sodium and calcium actually carry the inward-going current of the action potential. Substances were applied which selectively interfere with the sodium activation (tetrodotoxin) and with calcium activation (cobaltous ions). In addition, the behaviour of spike overshoot was examined with changes in external sodium

in the absence of calcium and vice versa. A preliminary report of these results has already been presented (Junge & Geduldig, 1967; Geduldig & Junge, 1967).

## METHODS

The visceral ganglion with four of its connectives was removed from *Aplysia californica* (length *ca.* 5 in., 12·7 cm) and mounted (dorsal surface upward) in a measurement chamber filled with the normal *Aplysia* saline. The connective tissue covering of the ganglion was snipped just over the giant cell ($R_2$ cell, see Coggeshall, Kandel, Kupfermann & Waziri, 1966), which allowed a portion of the 400 $\mu$ giant soma to protrude through the opening.

*Electrical measurements.* Two micro-electrodes (filled with 3 M-KCl, with tip potentials less negative than $-3$ mV and resistances between 5 and 12 MΩ in sea water) were inserted into the exposed soma and remained there throughout the solution changes. Two agar–sea-water/AgCl bridge electrodes were used externally in the bath solution. The membrane potential was recorded differentially between one internal and one external electrode by means of two conventional d.c. pre-amplifiers ($10^{-10}$ A grid current) and an oscilloscope which drove a pen-recorder. Records of the action potentials were photographed from the oscilloscope and the resting potentials were recorded on the pen recorder. Current pulses (for stimulation of the soma) were applied to the second internal micro-electrode through a 30 MΩ series resistor, and passed out of the solution through the second external electrode, and then through a 10 kΩ current-meter resistor to ground. In some cases the stimulus current (potential across the current-meter resistor) was observed on the second beam of the oscilloscope.

*Solutions.* The normal *Aplysia* saline (hereafter called normal saline, N.S.) was based on the body-fluid ion composition given by Hayes & Pelluet (1947). The composition of the normal saline is shown in Table 1 with the compositions of the master solutions which were used to prepare test solutions with varying Na or Ca. The compositions of the sucrose-substituted test solutions have been given previously (Junge, 1967). The master solutions (from Reagent Grade salts) were designed to be either Ca-free, Na-free, or both Ca- and Na-free, with HCl-neutralized Tris substituted for the missing chloride salts. (Tris is Tris-(hydroxymethyl)aminomethane obtained as Trizma Base from Sigma Chemical Co., St Louis, Mo. Although Tris is chemically uncharged, in this report 'Tris' is used to signify the cation form of the HCl-neutralized Tris (e.g. Na-free (Tris substituted) test solution).) The Tris was neutralized with HCl to the desired pH (*ca.* 7·7), and the other ingredients were then added to obtain the desired master solutions. At pH 7·7 (assumed pK of 8·1 for Trizma Base) about 73 % of the Tris was ionized. The total Tris which was added to make a given master solution was determined from the pK, pH, and activity coefficient of monovalent ions in sea water. (The activity coefficient was obtained from a computation of ionic strength in sea water and from Tables 10 and 13, Appendix 8.10 in Robinson & Stokes, 1965.) Test solutions with variable sodium and calcium compositions were made up by appropriate mixtures of two of the master solutions, and the pH was readjusted on the morning of use to 7·6–7·8.

For some experiments tetrodotoxin (TTX) (Sankyo Co., Tokyo) was used with the bath solutions. These solutions were made by addition of an appropriate volume of a TTX stock solution to the bath solution just before use, and an uncorrected dilution of about 1 % resulted. (The 3 mM stock solution of TTX was made by dissolving the drug in $10^{-4}$ N-HCl.) Although the TTX-stock solution was stored in the refrigerator (for up to several weeks) it was added to the test solutions only on the day of use. Bath solutions containing $Co^{2+}$ were also mixed just before use. For these solutions solid $CoCl_2$ was added directly to the test solution. When the external solution was changed, the 1 ml. chamber was perfused with 50 ml. (5 min) of the new solution before taking measurements.

*Liquid junction potentials of the external voltage electrode.* In the previous report (Junge,

1967), resting potential measurements were not constant in solutions containing different amounts of sucrose. This may have been due to changes of the external electrode tip potential. In the present study, additional experiments were done to determine the range of variation of the liquid junction potentials of the external agar–sea water/AgCl electrode which resulted from changes of the solutions having substitutions of either sucrose or Tris. Two agar/AgCl electrodes (a *test electrode* made up with sea water and a *reference electrode* with 3 M-KCl) were set several centimetres apart in a narrow perfusion channel. The perfusate first passed the test electrode and then was rapidly removed by suction on passing the reference electrode. In this way the liquid junction potential of the test electrode (sea water agar) should be affected by the composition of the perfusate alone, and not by any contamination from the 3 M-KCl reference electrode. The usual assumption is that a *large* 3 M-KCl electrode will not be subject to a variation of liquid junction potential when its bathing solution is changed. In this situation, as the perfusate is switched the resulting alterations of interelectrode potential should equal the variation of liquid junction potential for the test electrode alone.

TABLE 1. Composition of the normal saline and master solutions used to prepare test solutions with variable Na or Ca. Trizma Base was neutralized with HCl to pH 7·7 before other components were added

| | NaCl (mM) | KCl (mM) | $CaCl_2$ (mM) | $MgCl_2$ (mM) | $MgSO^4$ (mM) | Total Trizma base (mM) |
|---|---|---|---|---|---|---|
| Normal saline | 494 | 11 | 11 | 19 | 30 | 10 |
| Master solutions | | | | | | |
| Ca-free (normal Na) | 494 | 11 | — | 19 | 30 | 26 |
| Na-free (high Ca) | — | 11 | 88 | 19 | 30 | 429 |
| Na-free (Ca-free) | — | 11 | — | 19 | 30 | 557 |

On switching from normal saline to the Na-free (Tris) test solution, the potential of the test electrode decreased about 1 mV and this effect was essentially complete in half a minute. On switching from the normal saline to the Ca-free (Tris) solution, the same result was observed; and switching between the Ca-free (Tris) and Na-free (Tris) solutions produced no change in the potential. However, on switching from normal saline to the Na-free (*sucrose*) test solution, the potential of the test electrode increased by about 5 mV and the time required to reach this potential was usually more than 5 min. These results indicated the advisability of using neutralized Tris in preference to sucrose for substitutions of sodium and calcium chlorides. Except for a few experiments which were done to confirm the earlier studies, sucrose was not used in the present work.

*Stimulation.* The usual stimulus was a 50 msec depolarizing current pulse, and depending upon the solution used and the size of the soma, from $2 \times 10^{-8}$ to $10^{-6}$ A was required to elicit a spike after the stimulus. In some of the solutions (notably those which were Na-free) the membrane did not always produce an action potential even with as much as 1 $\mu$A of stimulating current. A 'conditioning' seemed to be required. As the stimulating pulses were applied, the voltage response increased, and activation (during the stimulus pulse) occurred with decreasing delay.

After about a dozen of these 'conditioning' pulses, the strength of the stimulus could be considerably reduced, and the spike would then fire after the stimulus pulse. Without this conditioning process our early studies indicated that the neurone (in Na-free solutions at room temperature) was often incapable of *immediate* activation by direct stimulation. After conditioning, the spike could be readily obtained as long as the train of stimuli (0·5/sec) was continued. After a period of 1–2 min with no stimuli, conditioning was again required to obtain the spike. When the stimulus intensity was increased, the spike occurred earlier. The peak of the overshoot potential was greater if the spike was fired during the stimulus. Since it seemed possible that an additional IR-drop in the membrane might result from the

stimulus current, we decided to limit our data to those records which showed a successful spike *after* stimulus, at a time when there could be no component of the potential due to externally imposed currents.

## RESULTS

*Sufficiency of external sodium or calcium for spike generation in the soma.* Figure 1 shows the effects of replacing sodium, calcium, or both ions with Tris. The complete replacement of external sodium typically caused a

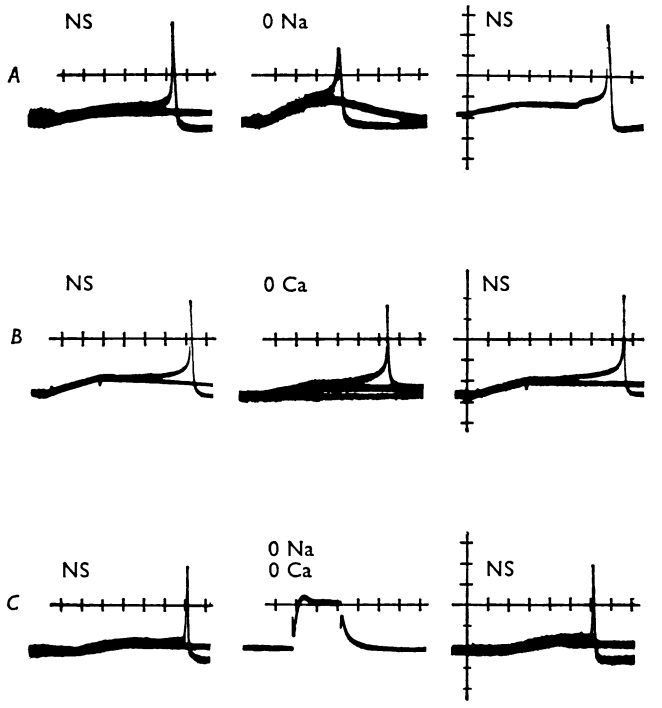


Fig. 1. Row *A*, effect of complete replacement of sodium with Tris. In all rows, the left record shows an action potential in the normal saline (NS). The middle record shows the response after about 5 min in a test solution (0 Na and 0 Ca are sodium free and calcium-free test solutions respectively). The right record shows recovery about 5 min after return to the normal saline. Row *B*, effect of complete replacement of calcium with Tris. Row *C*, effect of simultaneous replacement of sodium and calcium with Tris. In some records a subthreshold response is shown with a spike. Calibrations: 20 mV/division, 0·1 $\mu$A/division (vertical); 20 msec/division (horizontal). Rows *A* to *C*, zero membrane potential is indicated by the graticulated horizontal line.

reduction in overshoot of about 18 mV (Fig. 1, row *A*). A replacement of external calcium (Fig. 1, row *B*) produced a smaller reduction, typically 3 mV. Both effects were rapid (within 5 min), and action potentials could be obtained in the absence of either sodium or calcium even after several

hours. If, however, *both* ions were replaced with Tris, the spike was completely blocked and at most only a small oscillatory response remained (Fig. 1, row *C*). On replacement with the normal saline (Fig. 1, right hand column), after a previous application of these test solutions, the spike overshoots always returned to near-normal values.

The presence of Tris at high concentrations, neither blocked the spike (Fig. 2, row *A*) nor permitted the spike (Fig. 1, row *C*), and this indicates that Tris may be used as an inactive substitute for sodium and calcium. Nevertheless, Tris might possibly augment or attenuate the spike, when it is produced. (Magnesium at 49 mM, which was also present in all of these solutions, did not effectively permit spike generation. However, 11 mM-Ba in the absence of sodium and calcium permitted spike production (Fig. 2).) The presence of either Na or Ca was sufficient to permit spike formation, and in the normal saline both ions could contribute to the action potential.

In addition to the differential reductions of the overshoot potential produced by the Na-free and by the Ca-free solutions, the maximum rate of rise of the action potential was also diminished by these two test solutions. In eight cells, the average reduction in maximum rate of rise due to sodium removal was about twenty times that due to calcium removal. Both the observed change of overshoot and the observed change of rate of rise of the action potential might suggest that for action potentials in the normal saline, sodium plays a stronger role than calcium. However, this suggestion is still open to question as neither the effects of sodium removal on calcium activation nor the effects of calcium on sodium activation have been examined.

A few experiments in Na-free (sucrose) solution were repeated (this time with an external agar–sea water/AgCl electrode). These observations were substantially the same as those found previously (Junge, 1967). As in the case of the Tris-substituted (Na-free) solutions, a removal of external sodium ions results in a decrease of the overshoot potential. Since comparable reductions in the overshoot potentials were observed in the two Na-free test solutions containing great differences in the chloride content, it seems unlikely that the effect seen in the sucrose solutions was due to a chloride replacement.

*Sensitivity of the spike to tetrodotoxin.* For a number of excitable membranes (those having early inward action currents carried by sodium ions), voltage-clamp studies have shown that externally applied tetrodotoxin (TTX) specifically blocks the early conductance increase, and has no effect on the late component of the action current. (TTX blocks the sodium current activation: lobster axon at 15 nM (Narahashi, Moore & Scott, 1964), squid axon at about 300 nM (Nakamura, Nakajima & Grundfest, 1965) frog ranvier node at about 10 nM (Hille, 1966).) When these mem-

branes are not voltage-clamped, TTX can block the production of action potentials. However, for other excitable membrane systems (those having an early inward current carried by *calcium*), relatively high concentrations of TTX have no effect on the action potentials (barnacle muscle, 12 $\mu$M (Hagiwara & Nakajima, 1966); guinea-pig taenia coli, 15 $\mu$M (Kuriyama, Osa & Toida, 1966); procaine-treated crayfish muscle, 30 $\mu$M (Ozeki, Freeman & Grundfest 1966); snail neurones, 3 $\mu$M (Meves, 1966)). One exception to the TTX-insensitivity of calcium-dependent spikes should be noted.

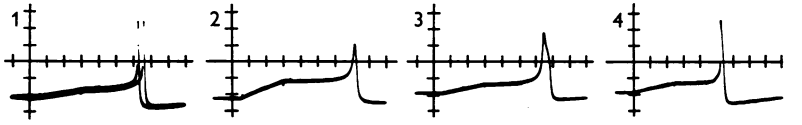


Fig. 2. Effect of substitution of barium for calcium in sodium-free solution. External solutions left to right: normal saline; 494 mM sodium replaced with Tris; 11 mM barium substituted for normal (11 mM) calcium in sodium-free solution; normal saline. In all figures of this report, all records of a given *row* were taken from the same cell, the horizontal graticule indicates zero membrane potential with a scale of 20 msec/division and the graticule in the vertical direction indicates 20 mV/division.

In the squid giant axon, whose axoplasm has been removed by enzymic action and replaced with c.s.f., a spike can be obtained in Na-free solution, and this spike requires the presence of external divalent cations. In this case, the 'divalent spike' is suppressed by 60 nM–1·2 $\mu$M TTX (Watanabe, Tasaki, Singer & Lerman, 1967).

In our first experiments, we observed a variable effect of TTX when applied at 3 $\mu$M in normal saline. In some cases the spike overshoots were reduced by about 20 mV; in other cases only 2 or 3 mV. A similar variability of TTX potency has also been reported by other authors (Nakamura *et al.* 1965; Benolken & Russell, 1967). Consequently, we increased the standard dose of TTX to 30 $\mu$M, and this resulted in reproducible attenuations of the overshoots.

In Fig. 3, row *A*, the effect of 30 $\mu$M TTX in normal saline is shown, and the reduction of the overshoot is quite close to that seen in Na-free (Tris) solution without TTX. In Ca-free solution, the Na-dependent spike (Fig. 4, row *B*) was further reduced to a small transient depolarization by the addition of 30 $\mu$M TTX. In Na-free solution, the Ca-dependent residual spike (Fig. 4, row *C*) was not affected by application of 30 $\mu$M TTX. The full effects of TTX were established within 5 min after application of the drug. Subsequent wash-out with TTX-free solutions demonstrated almost complete reversibility of the drug effects, although in a few cases the recovery was not very rapid and required about 15 min. The above results indicate that TTX (i) acts to block the sodium component of the spike (in the presence or absence of external calcium) and (ii), has no effect on the calcium

component. If TTX specifically blocks sodium activation, then these results provide additional support for the requirements of both sodium and calcium for normal spike overshoots.

*Sensitivity of the spike to cobalt.* Hagiwara & Takahashi (1967) have shown that cobaltous ion can effectively inhibit the calcium spike of the barnacle giant muscle fibre. In this system, cobalt appears to compete with calcium

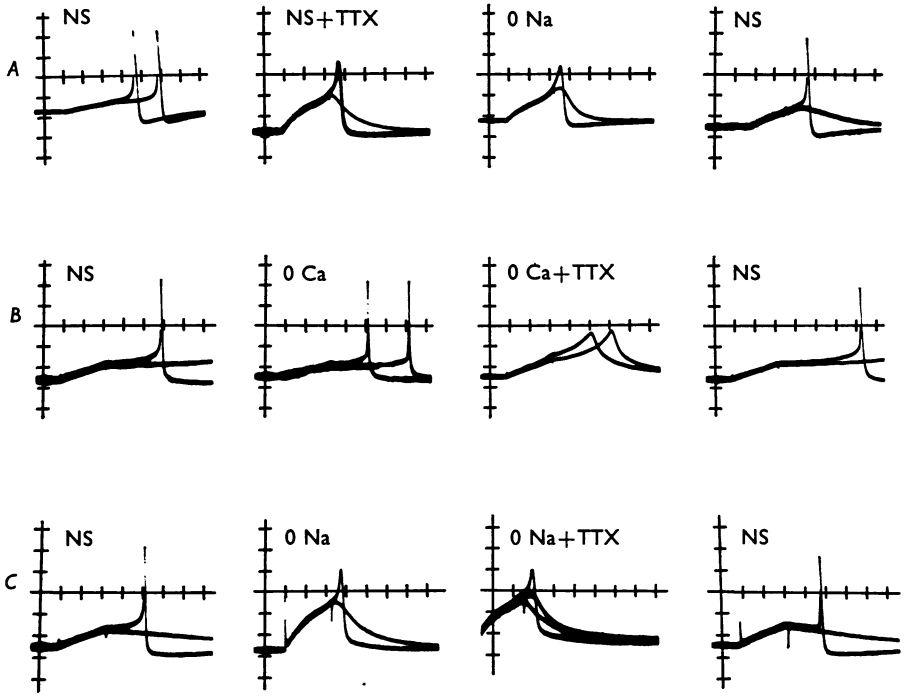


Fig. 3. Blockage of the sodium component of the spike by tetrodotoxin (TTX). Row *A*, effect of 30 $\mu$M TTX applied in normal saline, compared to the effect of complete replacement of sodium with Tris. For all rows the left column records show the action potentials in normal saline, the middle two columns show responses in the test solutions, and the right column records show the recoveries of the action potential in normal saline. Row *B*, effect of 30 $\mu$M TTX on the sodium-dependent spike in calcium-free medium. Row *C*, lack of effect of 30 $\mu$M TTX on the calcium-dependent spike in sodium-free medium.

for membrane sites, and thereby reduces the surface density of calcium on the membrane. Cobalt has one of the smallest dissociation constants for the barnacle membrane of all of the divalent inhibitor ions studied by these authors ($Co^{2+}$, $Mn^{2+}$, $Ni^{2+}$, $Mg^{2+}$, $Zn^{2+}$, $Fe^{2+}$, $UO_2^{2+}$) and has a strong inhibitory action.

Although the *Aplysia* giant neurone has both sodium- and calcium-dependent spikes, it seemed possible that in this system the effect of

cobalt might be similar to that found in the barnacle giant muscle fibre system. It would be interesting to see if the *Aplysia* giant neurone (in Ca-containing solutions) could mimic a Ca-free situation in both the presence and absence of sodium. Thus, one might reasonably ask: (i) does cobalt block a calcium activation and (ii) can a sodium-activation occur in the presence of cobalt?

The decrease in spike overshoot which results from the addition of 30 mM-$CoCl_2$ to the normal saline is comparable to the decrease which results from a replacement of the external calcium (Ca-free (Tris) solution)

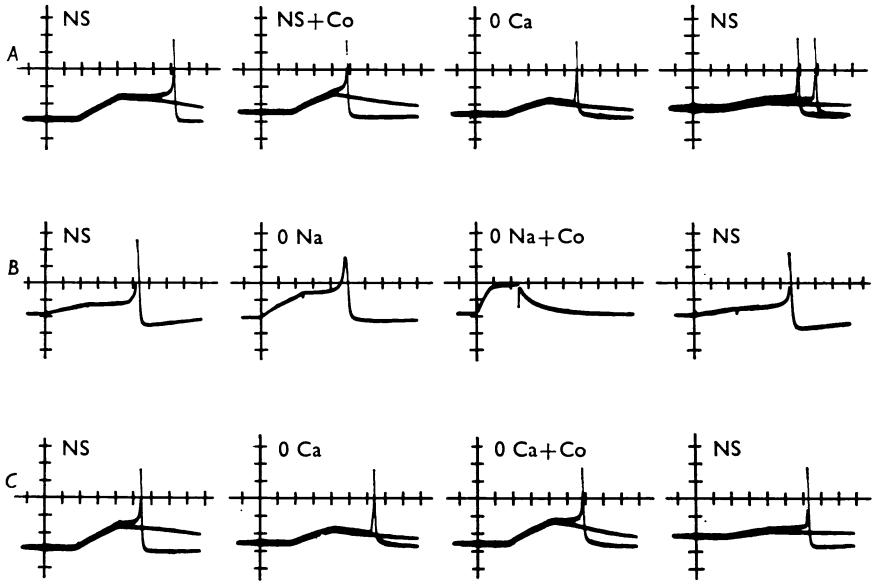


Fig. 4. Blockage of calcium component of the spike by cobalt (Co). Row *A*, effect of 30 mM $CoCl_2$ applied in normal saline, compared to effect of replacement of calcium with Tris. For all rows, the left hand column records show the action potentials in normal saline, middle two in test solutions, and the right hand column records show recoveries of the action potential in normal saline. Row *B*, effect of 30 mM cobalt on the calcium-dependent spike in sodium-free medium. Row *C*, lack of effect of 30 mM cobalt on the sodium-dependent spike in calcium-free medium.

when no cobalt is added (Fig. 4, row *A*). The Ca-dependent spike in Na-free solution (Fig. 4, row *B*) was completely blocked by the addition of 30 mM-$CoCl_2$. (In the Na-free (Tris) solution, some of the $Co^{2+}$ may have been oxidized to $Co^{3+}$. This reaction was suggested by a yellowing of the initially lavender-coloured solution which occurred 5–10 min after cobalt was added to the Tris-containing solution. Dr D. Hafeman pointed out that $Co^{3+}$ is strongly complexed by substituted amines (Cotton & Wilkinson, 1962), which would favour oxidation of additional $Co^{2+}$ by the $O_2$ normally

present. Nevertheless, the remaining $Co^{2+}$ was sufficient to block the calcium-dependent spike.) However, cobalt does not reduce the size of the Na-dependent spike in Ca-free solution (Fig. 4, row *C*). The effects of cobalt were rapid (within 5 min) and reversible. Recovery in normal saline (Co-free) following a treatment with cobalt was more rapid (also within about 5 min) than that with TTX. These results indicate that cobalt (i) acts to block the calcium component of the spike (in the presence or absence of external sodium) and (ii), has no effect on the sodium component. These results also support the sodium and calcium requirements for normal spike overshoots.

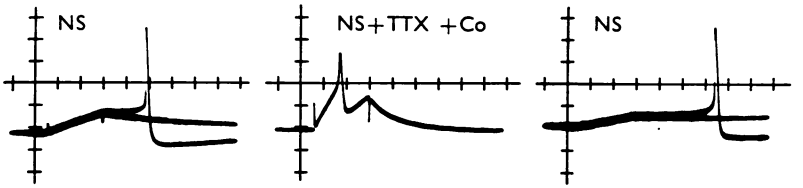


Fig. 5. Inability of 30 $\mu$M TTX and 30 mM-$CoCl_2$ to block the spike when applied together in normal saline. First and last records show the action potential in normal saline before and after application of the blocking agents.

*Non-additive effect of tetrodotoxin and cobalt when applied together.* Although 30 $\mu$M TTX in Ca-free solution and also 30 mM-$CoCl_2$ in Na-free solutions could block spike activation, simultaneous application of these two blocking agents in a normal saline did not completely abolish the spike. A reduced spike could be produced during (but not after) a 50 msec. stimulating pulse (Fig. 5). However, if the stimulating pulse duration was decreased to 20 msec, the spike could be produced after the pulse.

One explanation for this finding could have been that cobalt inhibited the action of TTX or vice versa. To test for a possible trans-inhibition, 30 $\mu$M TTX was first applied in a Ca-free solution, and then applied in a Ca-free test solution containing 30 mM-$CoCl_2$ (Fig. 6, upper row). The addition of cobalt caused no clear increase in the response, and indicated that cobalt causes no substantial inhibition of the usual sodium-spike blocking action of TTX. Likewise, the blocked response in Na-free solution containing 30 mM cobalt was not changed by the addition of 30 $\mu$M TTX (Fig. 6, lower row). Thus neither of the blocking agents appeared to suppress the potency of the other in either sodium-free or calcium-free test solutions. Nevertheless, when both sodium and calcium are present and both blocking agents are applied, the potency of one or both agents is reduced. This result would indicate that the two channels for early current activation (Ca and Na) are not entirely independent and some interaction probably exists.

*Sodium dependency in calcium-free solutions.* In an earlier study (Junge, 1967) it was reported that (in the presence of 11 mM calcium) the giant neurone overshoot potential varied by about 10 mV for a tenfold change of the external sodium concentration. For an exclusively Na-dependent spike system, this sodium sensitivity would be unexpectedly low. Such a

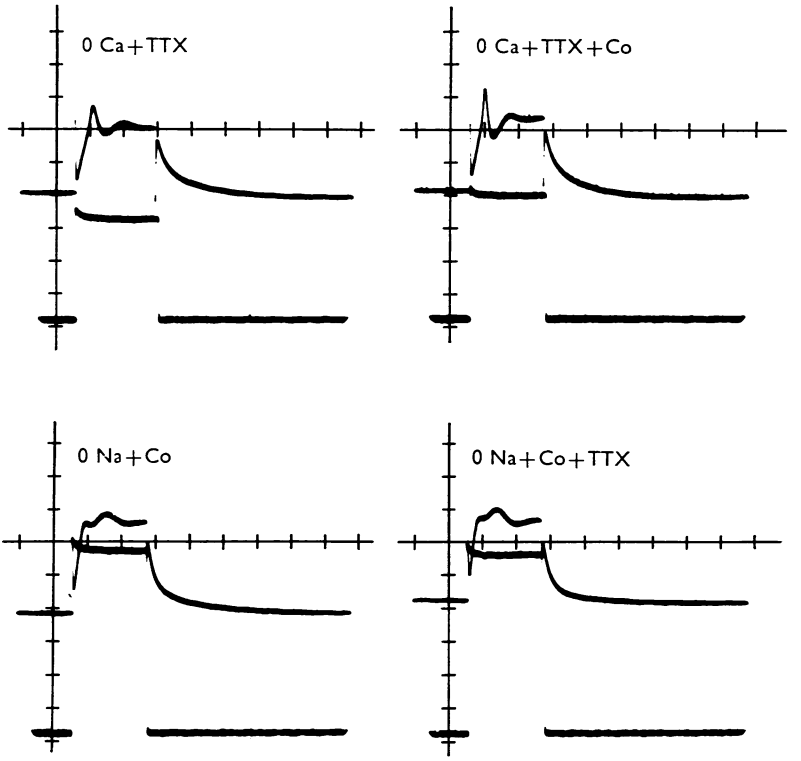


Fig. 6. Upper row: left, blockage of spike in calcium-free solutions by 30 $\mu$M TTX; right, spike remains essentially blocked upon addition of 30 mM-$CoCl_2$. Cobalt does not appear to inhibit the effect of TTX. Lower row: left, blockage of spike in sodium-free solution by 30 mM-$CoCl_2$; right, spike remains blocked upon addition of 30 $\mu$M TTX. TTX does not inhibit the action of cobalt. The lower trace of the four records shows large stimulating currents used. Calibrations: 20 mV and 0·1 $\mu$A per division (vertical), 20 msec per division (horizontal).

low sodium sensitivity could occur in a system where the early inward current was carried entirely by the calcium, but where the calcium activation was dependent on the external sodium concentration. Alternatively, this observation could also result from a combined sodium and calcium activation which would necessarily lead to a reduced over-all sodium sensitivity. The preliminary study showed that the overshoot sensitivity for sodium decreased in the presence of higher external calcium

concentrations, and this would support a model based on a dual early-current activation for both calcium and sodium. In order to extend these earlier results and to emphasize the sodium sensitivity of the somatic spike in the *Aplysia* giant neurone, the overshoot potential variation was measured in calcium-free solutions.

An exposed soma was first stimulated in the normal saline, and then records were obtained in the five Ca-free test solutions (Na concentration in mM: 165, 218, 286, 376, 494). For each of the somata tested in this way,

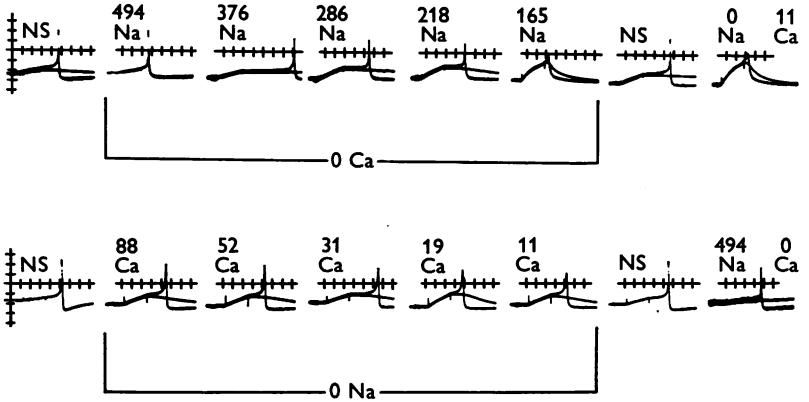


Fig. 7. Upper row, effect of varying sodium concentration in calcium-free solutions. Lower row, effect of varying calcium concentrations in sodium-free solutions. Concentrations indicated in mM.

the order of application of the test solutions was varied to eliminate a bias which might otherwise arise from a constant procedure. After this the recovery of the overshoot was tested in a normal saline, and finally the calcium dependency of the spike was tested in a Na-free solution containing normal calcium (11 mM). The electrodes remained implanted throughout all solution changes. Typical records taken from a single soma are shown in Fig. 7, upper row. The results, averaged from four somata, are shown in Fig. 8. The average overshoot potential, threshold potential and resting potential found in each of the test solutions are plotted at each of the external sodium ion concentrations. In these Ca-free test solutions, the behaviour of the action potential resembles the sodium dependent spikes of the squid giant axon (Hodgkin & Katz, 1949). The values of the average overshoot potentials closely follow a theoretical 59 mV slope line (23° C), with a zero potential intercept of 134 mM external sodium concentration. The resting potential did not vary significantly with changes in the external sodium. Although a study of the threshold potentials was not central to the present set of experiments, it is interesting to note the variation of threshold level in these Ca-free solutions. Unlike the squid giant axon,

where the threshold seems to be largely dependent upon the external calcium concentration (Frankenhaeuser & Hodgkin, 1957), for the giant neurone of the *Aplysia* (in calcium-free solutions) the threshold is strongly controlled by the external sodium concentration. (The threshold potential seems to decrease by about 55 mV for a tenfold increase of external sodium.)

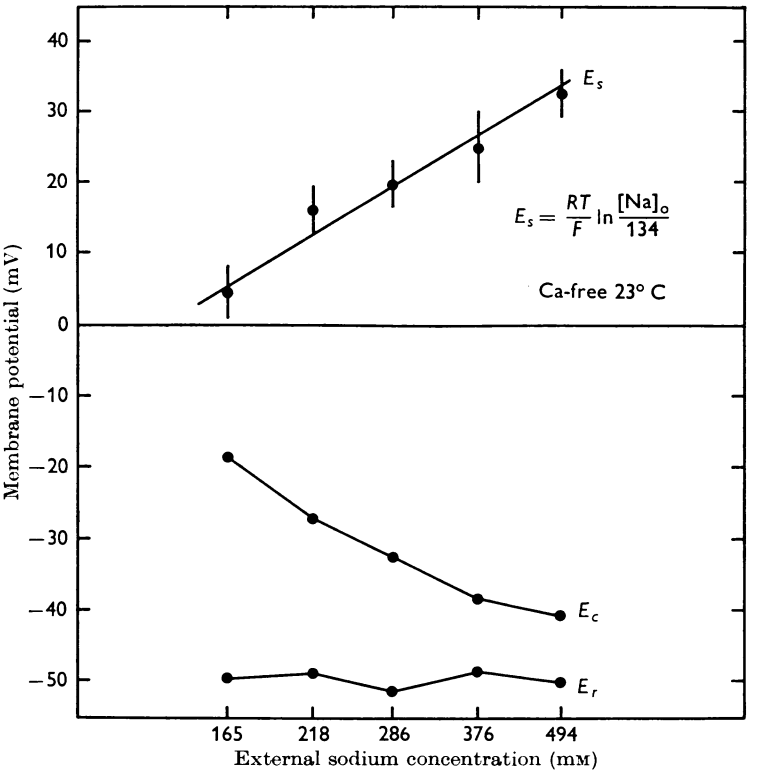


Fig. 8. Averaged results for four cells in which the sodium concentration was varied in calcium-free media. $E_s$, overshoot potential; $E_c$, critical or threshold potential; $E_r$, resting potential. The theoretical Nernst relation is plotted as the line passing through the overshoot points. The vertical bars are 2 times the S.E. of mean potentials.

*Calcium dependency in sodium-free solutions.* In the preceding section the main results showed that a Nernst-like potential variation for the overshoot potential could be obtained for alterations of the external sodium concentration in the absence of calcium. For a number of reasons, it seemed possible that, with appropriate conditions, the membrane of the *Aplysia* giant neurone could also be made to respond to changes in external calcium concentration in a Nernst-like manner. Although Junge (1967) had shown that the overshoot sensitivity to external calcium was con-

siderably less than the 29 mV change for a tenfold change of the external calcium concentration, in that study a substantial amount of external sodium was present. When, however, the external sodium concentration was reduced, it had been found that the calcium-sensitivity of the overshoot increased. In the present studies a set of Na-free test solutions were used to provide further evidence that calcium ions are actively engaged in carrying the inward-going current during the action potential.

An exposed soma was initially stimulated in the normal saline. Then five Na-free test solutions (Ca concentration in mM: 11, 19, 31, 52 and 88) were sequentially perfused into the measurement chamber (a different sequence for each soma), and in each solution records were taken of the action potentials. Recovery of the action potential was tested in a normal saline, and the sodium dependency was tested in a Ca-free solution containing a normal sodium concentration (494 mM). Typical records taken from a single soma are shown in Fig. 7, lower row. The results averaged from four somata are shown in Fig. 9. In these Na-free test solutions, the variation of the spike overshoot potential resembles the calcium dependent spikes of the barnacle giant muscle fibre (Hagiwara, Chichibu & Naka, 1964). The values of the average overshoot potentials follow a theoretical 29·5 mV slope line (23° C) with a zero-potential intercept of about 3 mM external calcium concentration. In this figure some deviation of the averaged data from the 29·5 mV line was observed at high and at low values of external calcium.

It is interesting to note that in this case the threshold potential does not vary with the external calcium concentration (compare Figs. 8 and 9). However, in the case of sodium-free solutions, one must consider the significance of threshold potential with an element of caution. In the presence of external sodium (Ca-free) there was never any doubt that the spikes were of an 'all or none' nature. Increase of stimulus strength resulted in less delay for the onset of spike, but so long as an activation or spike occurred, in any given solution the overshoot potential was independent of the stimulus strength. The potential at which the spike would 'break away' and become regenerative might vary slightly, but the minimum depolarization required to produce a spike was always the same for any given soma in a particular test solution.

The situation was somewhat different in Na-free Ca-containing solutions. Most of the time it was quite obvious that in Na-free solutions (especially at the lower Ca concentrations) the spike did not have an 'all or none' character. Small stimulus strengths (if sufficient to produce an 'activation' of the membrane) would produce a spike which was small and somewhat delayed; larger stimulus strengths produced larger spikes occurring with less delay. For any cell in a given solution there is a greatest overshoot

value which can be obtained after the stimulus, and this value can be obtained reproducibly. (If the stimulus strength is further increased, then the spike advances to appear during the stimulus duration.)

From this description of the variation of the spike with the stimulus strength in a Na-free solution it might seem that attempts to correlate the overshoot potentials with calcium concentrations could be somewhat hazardous. It also seems that for systems which do not give pure all or none responses, the term 'threshold' requires additional qualification. The membrane activation process or excitation process has not been a major concern of the present investigation. Nevertheless, we have tentatively taken the threshold potential as the potential of the greatest depolarization which could be given to the soma which would not lead to the production of a still greater depolarization at any time after the stimulus.

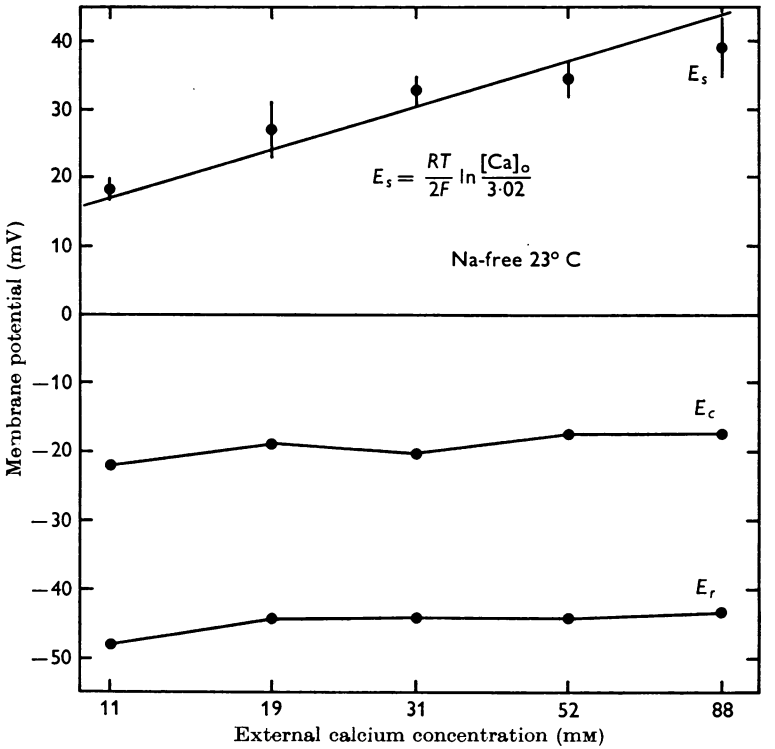


Fig. 9. Averaged results for four cells in which the calcium concentration was varied in sodium-free media. $E_s$, overshoot potential; $E_c$, approximate critical or threshold potential; $E_r$, resting potential. The theoretical Nernst relation is plotted as the line passing through the overshoot points. The vertical bars are 2 times the S.E. of mean potentials.

In the low calcium Na-free solutions the response of the *Aplysia* giant neurone to depolarizing pulses does not seem to fit into the usual class of all or none excitation. However, any possible ambiguity of the calcium sensitivity of the overshoot potential and also of its interpretation can be

removed on recognition of a 29 mV decrement (such as shown in Fig. 9). This kind of consistency could be observed so long as only the greatest overshoots (occurring after stimulus) were used in figuring the averages.

## DISCUSSION

The over-all evidence which has been presented makes it difficult not to accept a dual activation (for both sodium and calcium) of the *Aplysia* giant neurone membrane. (1) The presence of one of these ions is necessary, but either ion is sufficient, for the production of action potentials. (2) The action potential in Ca-free solutions (presumably due to sodium inflow) is blocked by tetrodotoxin. The action potential in Na-free solutions, the calcium spike, is blocked by cobaltous ions. These interfering actions are consistent with the usual properties of tetrodotoxin and cobalt. (3) In the absence of either ion, the behaviour of the excitable membrane of the *Aplysia* giant neurone (during the peak of the action potential) resembles the expected behaviour of a membrane which is selectively permeable to the ion (Ca or Na) present in the external solution. This being the case, the inward-going current which is associated with the action potentials produced in the Ca-free solutions ought to be carried by sodium, while in the Na-free solutions, the inward-going current ought to be carried by calcium. Then in solutions containing both sodium and calcium it would seem probable that the inward-going current will be carried by both of these ions. Only if the activation of the membrane for one of the ions were entirely blocked by the activation for the other ion could this not be the case. The combined evidence presented in this report and earlier (Junge, 1967) would argue strongly against the presence of this kind of blockage.

It is important to consider the possibility that the residual spike which remains after the normal saline has been replaced with a Na-free solution might be due to an incomplete wash-out of the external sodium, and that a substantial sodium diffusion space might exist just outside of the somal membrane (cf. Chamberlain & Kerkut, 1967). Several lines of evidence would seem to argue against this. The existence of such a diffusion space of any importance would be hard to reconcile with the Nernst dependencies exhibited by Figs. 8 and 9. But the following two observations are perhaps more crucial: (i) the attenuated overshoot (minimal latency) which results when a normal saline is replaced with the Na-free test solution remains quite constant, even for several hours, (ii), when a normal saline is replaced with a test solution containing neither sodium nor calcium, the spike is blocked at some time during the 5 min required for a perfusion-change of external solutions. (Both of these are completely reversible within about 5 min.)

It has been suggested that excitation of a membrane may involve the momentary release of membrane-associated calcium by depolarization currents which then could lead to a sudden increase of sodium permeability mediated by a temporary saturation of the membrane by monovalent cations (Gordon & Welsh, 1948; Hodgkin, Huxley & Katz, 1949; Frankenhaeuser & Hodgkin, 1957; Tasaki & Singer, 1966). The termination of the excitation would then be associated with a recombination of external divalent cations with the fixed negative-charged membrane. Although the above model may not be essential for the *Aplysia* giant neurone system, it is nevertheless interesting to note that the data we have obtained can fit within such a framework. When the preparation is taken from a normal saline to a Ca-free solution, the threshold potential does become more negative (cf. Austin, Yai & Sato, 1967); in Ca-free solutions, the threshold potentials become more negative as the external sodium concentration is increased. When the preparation is transferred from normal saline to Na-free solutions, it becomes much more difficult to activate the membrane; strong conditioning depolarizing currents are necessary.

Although the differences in the activation characteristics for sodium and calcium (in *Aplysia*) might at least suggest the possible existence of separate operational channels for the sodium and calcium inward-going currents, the experiments involving the use of tetrodotoxin and cobalt as specific blocking agents provide a more compelling argument for a dual channel system. It is recalled that the spikes which are produced in a normal saline solution containing TTX mimic those produced in a Na-free (normal Ca-containing) solution; but also that TTX does not appear to have any effect on the spikes produced in a Na-free solution. Likewise, cobalt appears to have no effect on the sodium spikes, but effectively blocks the calcium spikes. Certainly, these experiments do not exclude the possibility of a *topographically* single channel for both inward-going currents, but at least in an *operational* sense, they must be considered as distinct. This contrasts to the recent findings of Watanabe *et al.* (1967) for the perfused squid giant axon preparation. In their case, the toxicity of TTX for both sodium and calcium action potentials would indicate a single operational channel for both ions.

The authors wish to thank Professor S. Hagiwara for his advice and encouragement. Our thanks are also due to Dr A. Brady for a discussion of the manuscript. This work was supported by grants from the USPHS and AFOSR to Professor Hagiwara.

## REFERENCES


ABBOTT, B. C. & PARNAS, I. (1965). Electrical and mechanical responses in deep abdominal extensor muscles of crayfish and lobster. *J. gen. Physiol.* **48**, 919–931.

AUSTIN, G., YAI, H. & SATO, M. (1967). Calcium ion effects on *Aplysia* membrane potentials. In *Invertebrate Nervous Systems*, ed. WIERSMA, C. A. G. Chicago: University of Chicago Press.

BENOLKEN, R. M. & RUSSELL, C. J. (1967). Tetrodotoxin blocks a graded sensory response in the eye of *Limulus*. *Science, N.Y.* **155**, 1576–1577.

BÜLBRING, E. & KURIYAMA, H. (1963). Effects of changes in the external sodium and calcium concentrations on spontaneous electrical activity in smooth muscle of guinea-pig taenia coli. *J. Physiol.* **166**, 29–58.

CHAMBERLAIN, S. G. & KERKUT, G. A. (1967). Voltage clamp studies on snail (*Helix aspersa*) neurones. *Nature, Lond.* **216**, 89.

COGGESHALL, R. E., KANDEL, E. R., KUPFERMANN, I. & WAZIRI, R. (1966). A morphological and functional study on a cluster of identifiable neurosecretory cells in the abdominal ganglion of *Aplysia californica*. *J. cell Biol.* **31**, 363–368.

COTTON, F. A. & WILKINSON, G. (1962). *Advances Inorganic Chemistry*. New York: Interscience.

FATT, P. & GINSBORG, B. L. (1958). The ionic requirements for the production of action potentials in crustacean muscle fibres. *J. Physiol.* **142**, 516–543.

FATT, P. & KATZ, B. (1953). The electrical properties of crustacean muscle fibres. *J. Physiol.* **120**, 171–204.

FRANKENHAEUSER, B. (1957). The effect of calcium on the myelinated nerve fibre. *J. Physiol.* **137**, 245–260.

FRANKENHAEUSER, B. & HODGKIN, A. L. (1957). The action of calcium on the electrical properties of squid axons. *J. Physiol.* **137**, 218–244.

GEDULDIG, D. & JUNGE, D. (1967). Sodium and calcium dependency of action potential of *Aplysia* neurone. *Physiologist, Bethesda* **10**, 179.

GERASIMOV, V. D., KOSTYUK, P. G. & MAISKII, V. A. (1964). Excitability of the giant nerve cells of various pulmonate molluscs. *Byulletin' Eksperimental' noi Biologii i Meditsiny* **58**, 3–7. (Translated from Russian in FASEB Translation no. S65-4.)

GERASIMOV, V. D., KOSTYUK, P. G. & MAISKII, V. A. (1965). The influence of divalent cations on the electrical characteristics of membranes of giant neurones. *Biofizika* **10**, 447–453.

GORDON, H. T. & WELSH, J. H. (1948). The role of ions in axon surface reactions to toxic organic compounds. *J. cell. comp. Physiol.* **31**, 395–419.

HAGIWARA, S., CHICHIBU, S. & NAKA, K. (1964). The effects of various ions on resting and spike potentials of barnacle. *J. gen. Physiol.* **48**, 163–179.

HAGIWARA, S. & NAKA, K. (1964). The initiation of spike potential in barnacle muscle fibers under low intracellular $Ca^{++}$. *J. gen. Physiol.* **48**, 141–162.

HAGIWARA, S. & NAKAJIMA, S. (1966). Differences in Na and Ca spikes as examined by application of tetrodotoxin, procaine and manganese ions. *J. gen. Physiol.* **49**, 793–806.

HAGIWARA, S. & TAKAHASHI, K. (1967). Surface density of calcium ions and calcium spikes in the barnacle muscle fiber membrane. *J. gen. Physiol.* **50**, 583–601.

HAYES, F. R. & PELLUET, D. (1947). The inorganic constitution of molluscan blood and muscle. *J. mar. biol. Ass. U.K.* **26**, 580–589.

HILLE, B. (1966). Common mode of action of three agents that decrease the transient change in sodium permeability in nerves. *Nature, Lond.* **210**, 1220–1222.

HODGKIN, A. L., HUXLEY, A. F. & KATZ, B. (1949). Ionic currents underlying activity in the giant axon of the squid. *Archs Sci. physiol.* **3**, 129–150.

HODGKIN, A. L. & KATZ, B. (1949). The effect of sodium ions on the electrical activity of the giant axon of the squid. *J. Physiol.* **108**, 37–77.

JUNGE, D. (1967). Multi-ionic action potentials in molluscan giant neurones. *Nature, Lond.* **215**, 546–548.

JUNGE, D. & GEDULDIG, D. (1967). Sensitivity of spike overshoot to tetrodotoxin, cobalt and TEA. *Physiologist, Bethesda* **10**, 215.

KERKUT, G. A. & GARDNER, D. R. (1967). The role of calcium ions in the action potentials of *Helix aspersa* neurones. *Comp. Biochem. Physiol.* **20**, 147–162.

KURIYAMA, H., OSA, T. & TOIDA, H. (1966). Effect of tetrodotoxin on smooth muscle cells of the guinea-pig taenia coli. *Br. J. Pharmac. Chemother.* **27**, 366–376.

MEVES, H. (1966). Das Aktionspotential der Riesennervenzellen der Weinbergschnecke *Helix pomatia*. *Pflügers Arch. ges. Physiol.* **289**, R10.

NAKAMURA, Y., NAKAJIMA, S. & GRUNDFEST, H. (1965). The action of tetrodotoxin on electrogenic components of squid giant axons. *J. gen. Physiol.* **48**, 985–996.

NARAHASHI, T., MOORE, J. W. & SCOTT, W. R. (1964). Tetrodotoxin blockage of sodium conductance increase in lobster giant axons. *J. gen. Physiol.* **47**, 965–974.

NIEDERGERKE, R. & ORKAND, R. K. (1966*a*). The dual effect of calcium on the action potential of the frog's heart. *J. Physiol.* **184**, 291–311.

NIEDERGERKE, R. & ORKAND, R. K. (1966*b*). The dependence of the action potential of the frog's heart on the external and intracellular sodium concentration. *J. Physiol.* **184**, 312–334.

OZEKI, M., FREEMAN, A. R. & GRUNDFEST, H. (1966). The membrane components of crustacean neuromuscular systems. 1. Immunity of different electrogenic components to tetrodotoxin and saxitoxin. *J. gen. Physiol.* **49**, 1319–1334.

ROBINSON, R. A. & STOKES, R. H. (1965). *Electrolyte solutions*, 2nd edn. revised. London: Butterworths.

TAKEDA, K. (1967). Permeability changes associated with the action potential in procain-treated crayfish abdominal muscle fibers. *J. gen. Physiol.* **50**, 1049–1074.

TASAKI, I. & SINGER, I. (1966). Membrane macromolecules and nerve excitability: a physico-chemical interpretation of excitation in squid giant axons. *Ann. N.Y. Acad. Sci.* **137**, 792–806.

WATANABE, A., TASAKI, I., SINGER, I. & LERMAN, L. (1967). Effects of tetrodotoxin on excitability of squid giant axons in sodium-free media. *Science, N.Y.* **155**, 95–97.