

CALCIUM IONS AND THE ACTION POTENTIAL IN *NITELLA*

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[Manuscript received July 20, 1961]

Summary

Experiments are described in which a "voltage-clamping" technique has been applied to large ecorticate internodal cells of the freshwater alga *Nitella*. In this technique, a feedback circuit is used to change the potential difference between the vacuole of the cell and the external medium to some predetermined level and maintain it as close as possible to this level during the electrical activity of the cell. It is shown that the main factor in the phenomena of potential change and current flow, during the initial stages of the action potential in *Nitella*, is a transient increase in the permeability of the cell membrane to calcium ions, and a consequent flow of these ions into the cell from the external medium.

I. INTRODUCTION

Recent hypotheses to account for the action potential in the internodal cells of the Characeae have been put forward by Gaffey and Mullins (1958) and Hope (1961a, 1961b).

Gaffey and Mullins, working with a corticated species *Chara globularis*, have put forward the hypothesis that the action potential is caused by an increase in the permeability of the membrane to chloride ions. Because the electrochemical equilibrium potential of the chloride ion is more positive than the resting potential, the membrane potential would be expected to rise when the cell is stimulated. The recovery of the potential is considered to be caused by an efflux of potassium ions. However, Gaffey and Mullin's interpretation of their results has been questioned by Hope (1961a, 1961b).

Hope has shown that in cells of *Chara australis*, the depolarization of the plasmalemma during an action potential may be accounted for by a sudden transient increase in the permeability of this membrane to calcium ions, and a consequent inward movement of these ions. This conclusion followed from an investigation of the effects of changes in concentration of various ions on the action potential in single *Chara* cells.

Recently, in this Laboratory, it has been possible to examine in more detail the action potential process by applying a "voltage-clamp" technique to the ecorticate internodal cells of a species of *Nitella* (Findlay 1961). This technique was first applied by Marmont (1949) to the study of the electrical activity of the squid giant axon. Later, Hodgkin, Huxley, and Katz (1952) used the method to give a detailed analysis of the movement of sodium and potassium ions across the membrane of the squid giant axon. The results of experiments described in

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this paper in which the *Nitella* cell membrane has been subjected to "voltage-clamping" give considerable information about the flow of ions across the membrane, and, in particular, confirm Hope's (1961*a*, 1961*b*) suggestion that calcium ions play a major role in the action potential in the internodal cells of at least two members of the Characeae.

II. MATERIALS AND METHODS

The cells of *Nitella* used in the experiments described in this paper were taken from a tap water-river gravel culture grown under low light intensity. Experiments were performed on the ecorticate internodal cells (about 1 cm long and 150 μ in diameter) which were separated from the culture and soaked for about 16 hr in an artificial pond water (A.P.W.) before use. The ionic constituents of the A.P.W. are shown in Table 1.

TABLE 1
IONIC CONSTITUENTS OF ARTIFICIAL POND WATER

Ion	Concentration (μ -equiv/l)	Ion	Concentration (μ -equiv/l)
Na ⁺	690	Cl ⁻	300
K ⁺	70	NO ₃ ⁻	500
Ca ²⁺	90	SO ₄ ²⁻	90
Mg ²⁺	80	HPO ₄ ²⁻	40

The experimental set-up was similar to that used by Findlay (1959). The cell, mounted on a "Perspex" slide, was kept in a continuously flowing solution to maintain a constant external environment. Two "Pyrex" glass microelectrodes were inserted into the vacuole of the cell. One, filled with 0.3*N* KCl solution, recorded the vacuole potential, the other, filled with saturated KCl solution (to obtain the minimum electrical resistance), was used to pass electric currents from the vacuole to the exterior of the cell. Two external reference electrodes were employed, a large distant indifferent electrode and a small near electrode. The near electrode was a glass microelectrode with a tip diameter of 10–15 μ and filled with 3*N* KCl solution in 3% agar, and was placed immediately adjacent to the intracellular potential recording microelectrode. The bathing solution was connected by a platinum plate through a 50 k Ω resistance to earth.

The resting resistance of the cell membrane was measured either by passing rectangular pulses of constant current through the membrane, or a linearly increasing current, and observing the corresponding change in membrane potential. Action potentials were usually initiated with rectangular current pulses of 0.1 sec duration. In most experiments cells were stimulated every 10 min. It was possible with this frequency of stimulation to obtain reproducible action potentials over a period of many hours. More frequent stimulation often caused changes in the form of the action potential. Action potentials were measured between the intracellular microelectrode and the distant electrode. The electric current

flowing in the external solution during the action potential is so small that any potential difference that arises in the external solution is negligible, and thus the potential difference as measured above represented the action potential across the cell membrane.

The experimental arrangement for voltage clamping is shown in Figure 1. In this technique, a feedback current is used to change the membrane potential to some predetermined level and maintain it as close as possible to this level by means of negative feedback during the electrical activity of the cell. The current flowing across the membrane during the voltage clamp is recorded. The operation of the system is as follows: The membrane potential (measured between *A* and *B*)

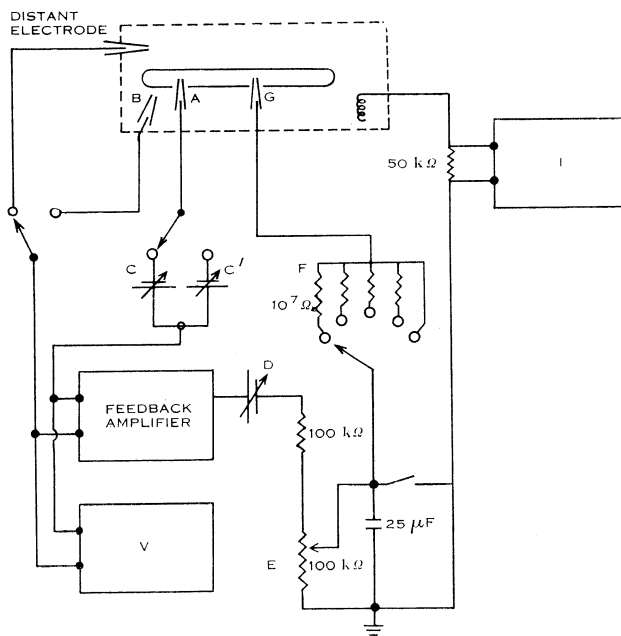


Fig. 1.—Block diagram of the voltage-clamping circuit. The details of its operation are given in the text.

is fed into the feedback amplifier (gain 3000), through a backing-off potential source *C*. With the aid of *C*, the input is made zero. The output from the feedback amplifier is +105 V from earth, and this is backed off by the potentiometer *D*. The amount of feedback is controlled by the potential divider *E*. This is first set at zero, with a large resistance *F* in series with the current electrode *G*. The feedback is then increased to maximum, and finally the series resistance *F* decreased to zero. The cell membrane is then “clamped” at the resting level, set by *C*, since any tendency for the potential in the cell to change is counteracted by a current from the output of the feedback amplifier. To clamp the membrane potential at some other level, *C* is switched to *C'* (which is set at the required new potential). The current flowing during the voltage clamp is recorded by electrometer *I*, and the membrane potential by electrometer *V*.

Considerable difficulty has been experienced in keeping the feedback system stable. It was generally not possible to utilize the maximum gain available, which itself was often not sufficient to hold the membrane potential steady at the required clamp level. Figure 2(a) shows a series of voltage clamps. It can be seen that for voltage clamps nearest the resting level there was a considerable excursion of the membrane potential during the electrical activity of the cell.

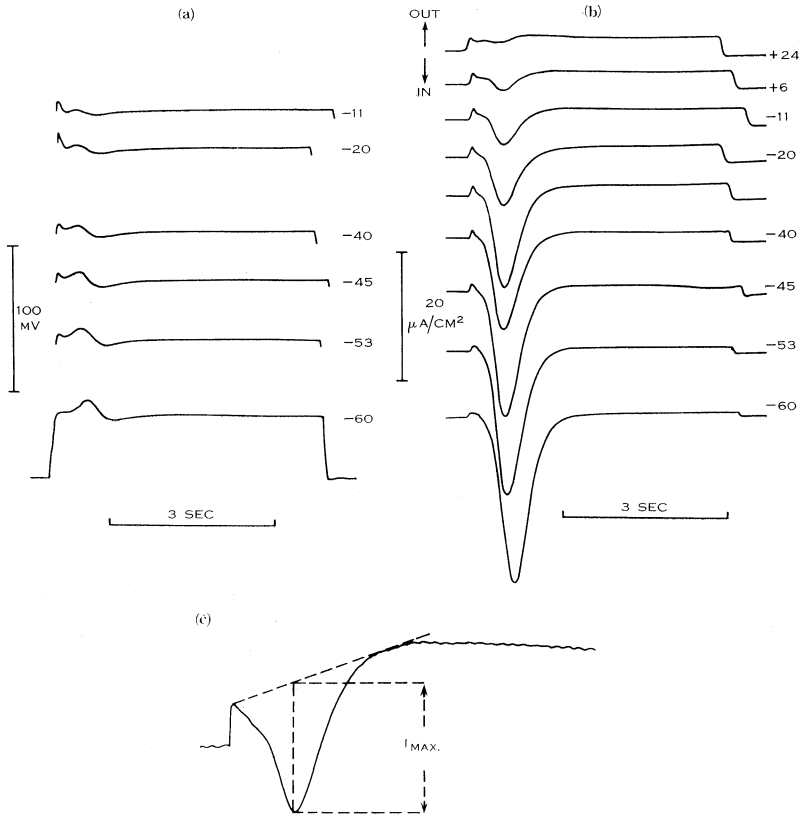


Fig. 2.—(a) Membrane potential during a voltage clamp with the *Nitella* cell in A.P.W. The final potential levels are shown at the right of each trace. Note that the traces have been spread out on the vertical scale and thus do not start from a common base line. (b) Corresponding membrane current during the voltage clamp as shown in (a). The clamp potential is shown at the right of each curve. (c) The extrapolation used to estimate the outward clamp current and the peak inward current, I_{max} .

It was found that the feedback circuit was often more stable if, instead of the membrane potential, the potential between the microelectrode in the vacuole and the distant external electrode was clamped. In most of the experiments described in this paper, the latter potential was clamped, but the actual membrane potential was always recorded.

The feedback amplifier, and amplifiers used to measure membrane potential and membrane current were chopper-stabilized to reduce zero drift, and have been described previously by Findlay (1959). Their outputs were fed into high-speed Evershed and Vignoles amperometric pen recorders, with a response time of 0.1 sec for 95% of the full deflection of 3.5 in.

The voltage-clamping technique has also been used by Cole and Moore (1960). For their work with squid axon they used an intracellular metallic current electrode, whereas, in the present work with the *Nitella* cell, a KCl-filled micro-electrode is used as the current electrode.

III. RESULTS

(a) Cell in A.P.W.

Determination of the resting resistance of the membrane either by passing a series of constant current pulses of increasing magnitude and 1 sec duration, or a linearly increasing current across the cell membrane, gave closely similar results. The mean resting membrane resistance, determined by the former method and measured in 25 cells was 54 ± 6 k Ω cm². These values were obtained after corrections were made for the vacuolar resistance following Walker (1960) who has shown that for *Nitella* cells, the membrane resistance per unit area is given by

$$r = (R - R_L/12)A,$$

where R is the total resistance measured between a probe in the vacuole and one external but near the cell surface, R_L is the resistance of the sap, and A the surface area of the cell.

The relationship between the applied current and the change in membrane potential was similar to that described by Findlay (1959). However, a close examination of the region near the resting potential shows a steady transition of the membrane resistance with the change from hyperpolarizing to depolarizing current. Previously, it had been considered (Findlay 1959) that an abrupt change of slope of the curve of applied current *v.* change in membrane potential occurred at the resting potential. The relation between membrane resistance and potential has also been discussed by Hope and Walker (1961) for *Chara australis*.

The mean resting potential in the above 25 cells was -115 ± 3.5 (standard error of the mean) mV. The threshold for stimulation, -76 ± 2.5 mV (21 cells), was determined by continuing the application of the linearly increasing current to the membrane until an action potential was initiated. The rate of increase of the applied current does not affect the value of the threshold potential to any appreciable extent, i.e. there is very little "accommodation" in *Nitella* cells. The form of the action potential which has been described previously by Findlay (1959) generally showed a single rising phase, to a peak of $+2 \pm 1.7$ mV (25 cells). The electric current flowing across the membrane during the voltage clamp was recorded. Figure 2(b) shows the relationship between membrane current and time, for different membrane potentials. In these experiments, the membrane potential was first clamped at the resting level, then at the new level for 5 sec, and finally clamped back at the original resting level.

The clamping current may be divided into three components: a small outward current beginning at the time of application of the voltage clamp, followed within 0.25 sec by a transient inward current lasting up to 2.0 sec, and finally an outward current continuing to the end of the voltage clamp. As the membrane potential is changed in the positive direction the transient inward current decreases, and eventually becomes zero, while the outward current components increase in magnitude. When the membrane potential is clamped back to the resting level, there is often a small inward current flow which decreases approximately exponentially with a time constant of 1–2 sec.

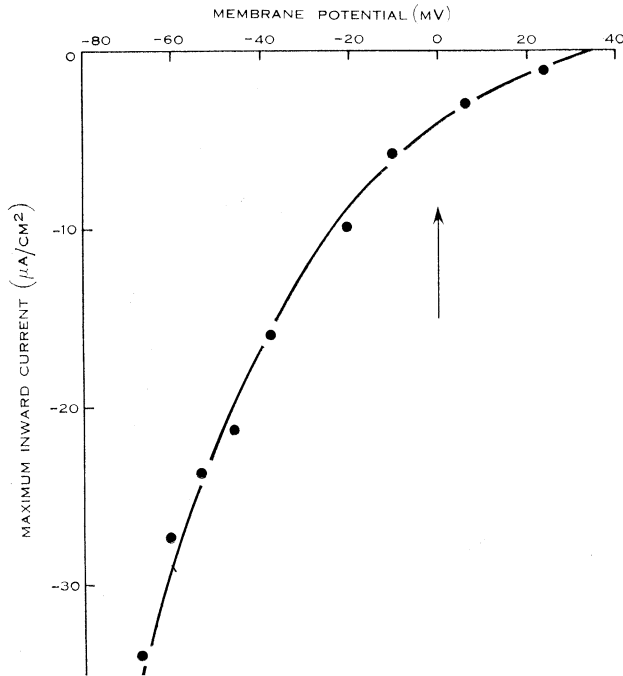


Fig. 3.—Relation between maximum inward current and membrane potential during a voltage clamp with the *Nitella* cell in A.P.W. This graph has been obtained from the curves shown in Figure 2(b). The arrow indicates the peak of an ordinary action potential.

For potential clamps not far removed from the resting potential, the two outward components are approximately equal in magnitude. However, as the membrane potential is clamped at increasingly more positive values, the second outward component increases at a greater rate than the first, and begins to vary with time. The problem arises as to how to measure the magnitude of the transient inward current in the presence of a more slowly changing outward current. Figure 2(c) shows how the maximum inward current ($I_{max.}$) has been determined. This method of measuring $I_{max.}$ is used in all the results presented in this paper. The justification of the extrapolation is as follows. The magnitude

of the initial outward current flow following a change in membrane potential indicates that the membrane resistance is at its resting value when the clamp is first applied. After the transient inward current has occurred, the membrane resistance is somewhat lower than the resting level. It seems a reasonable assumption that the outward current flows throughout the clamp with the inward current superimposed on it. The simplest estimation of the time course of the outward current that can be made is a straight line as indicated in the figure.

From the curves shown in Figure 2(b) the maximum inward current during a voltage clamp is plotted against the corresponding membrane potential (Fig. 3). Because the membrane potential is changing, there will be an extra capacitive current flow across the membrane, but this is negligible compared to the ionic

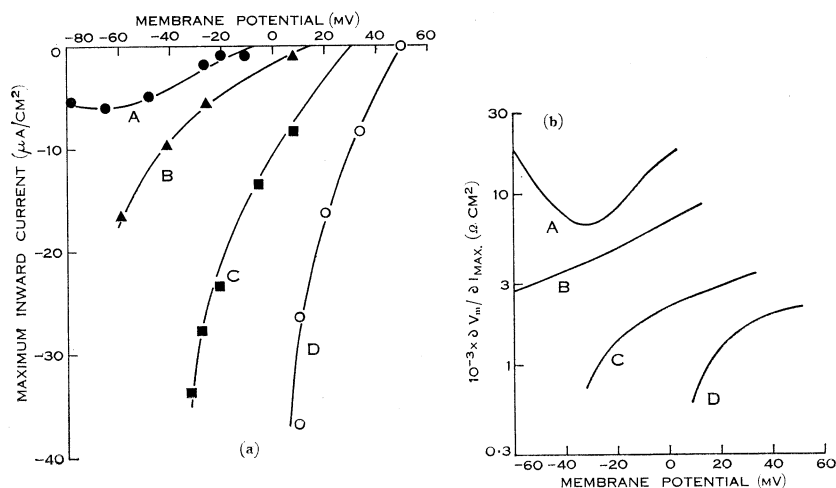


Fig. 4.—(a) Relationship between maximum inward current and membrane potential for different Ca_o : ● 0.1 mM; ▲ 0.3 mM; ■ 1.0 mM; ○ 3.0 mM. (b) The differential resistance, $\partial V_m / \partial I_{max}$, as a function of potential, for different Ca_o : A, 0.1 mM; B, 0.3 mM; C, 1.0 mM; D, 3.0 mM. The curves have been calculated by taking, at successive points, the slopes of the curves A, B, C, and D respectively in Figure 4(a).

current flow. This is discussed further in Section IV. The arrow indicates the potential of the peak of the action potential itself. It can be seen that the membrane potential for which the transient inward clamp current is zero is above the action potential peak.

(b) Effect of Calcium Ions

To examine the effects of external calcium concentration (Ca_o) on the action potential process, cells were first soaked overnight in A.P.W. and action potentials were measured with the cell in this solution to check that it behaved normally. The external solution was then changed to one of calcium and magnesium

chlorides. The cell was left in this solution for 30–40 min and then two action potentials with a 10-min interval between them were usually recorded. Following this, a series of voltage clamps were applied to the cell. Finally, another action potential was measured. This was compared with action potentials, measured before clamping, to detect any changes in the membrane characteristics. The calcium concentration was then varied at the expense of the magnesium, and the clamp series repeated. The total ionic concentration used was 1.1 mN, and in a few experiments 3.1 mN, and was kept constant throughout the experiment to

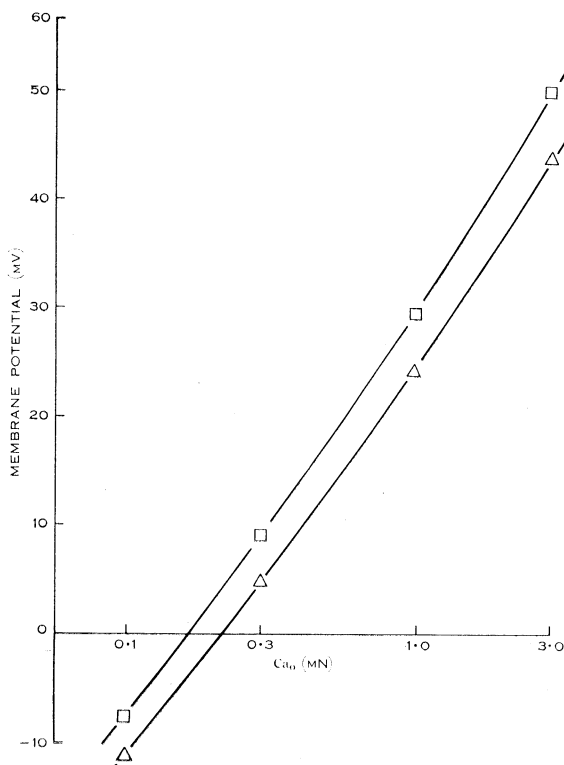


Fig. 5.—Peak of the action potential (Δ), and the membrane potential for zero I_{max} . (\square) as functions of Ca_o .

eliminate changes in potential difference across the cell wall Donnan system (Hope and Walker 1961). The concentrations of calcium used were 0.1, 0.3, 1.0, and 3.0 mN.

The relationship I_{max} , *v.* membrane potential (V_m) for different Ca_o is shown in Figure 4(a). It can be seen that the slope of the curves increases as Ca_o increases, and the value of V_m for which I_{max} is zero becomes more positive as Ca_o increases. In Figure 4(b), $\partial V_m / \partial I_{max}$ is shown as a function of V_m for differing Ca_o . Figure 5 shows both V_m for zero I_{max} , and the action potential peak as a function of Ca_o .

(c) *Effect of Chloride Ions*

To examine the effect of chloride ions on the action process, the relationship between V_m and I_{max} , for different Cl_o and fixed Ca_o was determined. As in the case of the variation of calcium, the total ionic concentration was kept constant. The external chloride concentration was varied at the expense of the benzenesulphonate ion. Other ions present were potassium (0.3 mN), sodium (0.6 mN), and calcium at either 0.1 mN or 1.0 mN. The results are shown in Figure 6. The curve A was obtained with $Ca_o=0.1$ mN, and $Cl_o=0.1, 0.3$, and 1.0 mN, and curve B with $Ca_o=1.0$ mN. It is seen that the concentration of chloride has practically no effect on the I_{max} - V_m relationship, in contrast to the calcium effect which separates the graphs A and B.

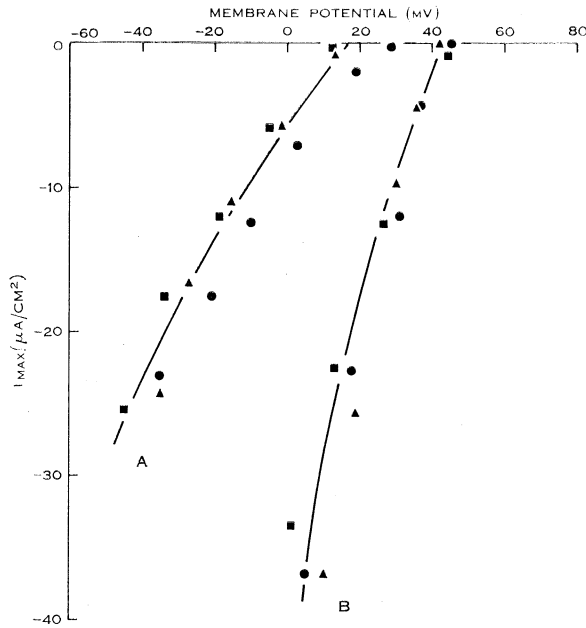


Fig. 6.—Relationship between maximum inward current and membrane potential for varying Cl_o and fixed Ca_o . A, 0.1 mN Ca_o ; B, 1.0 mN Ca_o with $Cl_o=0.1$ (●), 0.3 (▲), and 1.0 (■) mN.

(d) *Outward Currents during a Voltage Clamp*

When the membrane potential was clamped just above the stimulation threshold, there was often a delay of up to 2.0 sec before the start of the large inward current flow. During this initial stage a steady outward current flowed. As the clamped potential was raised the inward current commenced more quickly, and for potential clamps more than about 20 mV above the threshold potential the inward current commenced within 0.1 sec of the start of the clamp. Any outward current flow was then obscured until the inward current flow had ceased. Measurements of the outward clamp current 4.0 sec after the clamp was

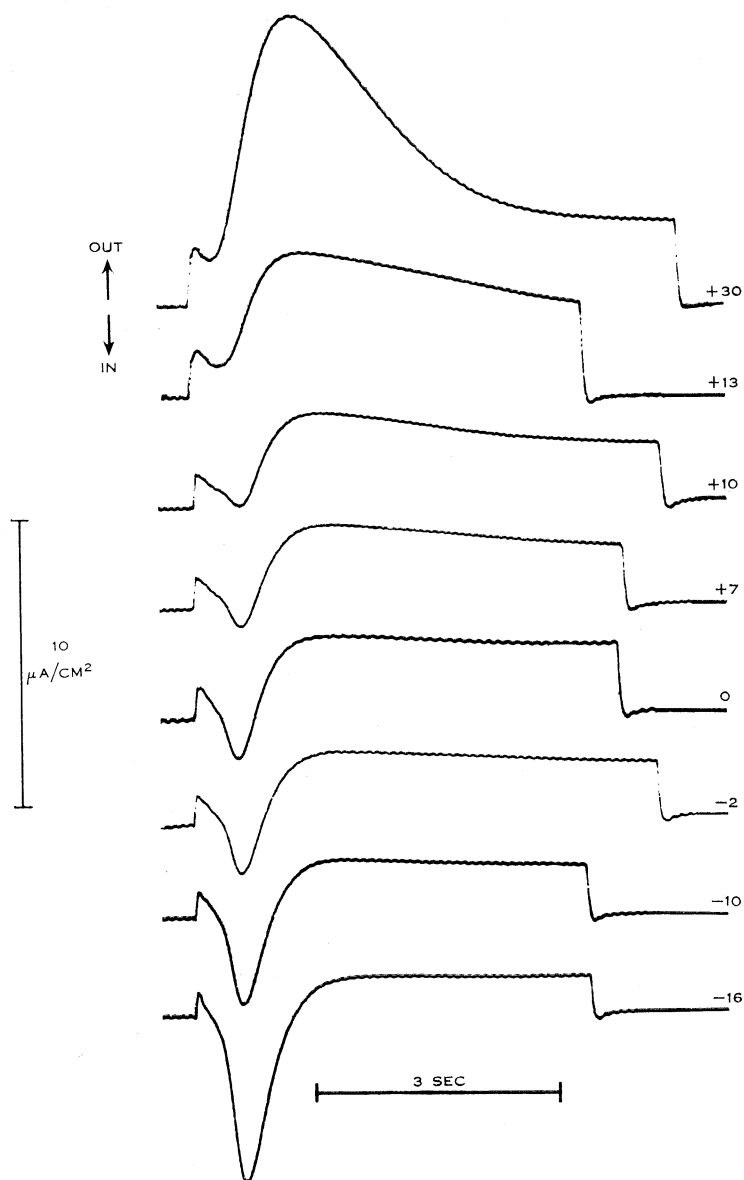


Fig. 7.—Membrane currents during a voltage clamp showing transient behaviour of the outward current phase for clamped potentials above -20 mV. Note that this transient behaviour has commenced before the maximum inward current flow has become zero. The cell was bathed in 1.0 mN CaCl_2 .

applied showed that the membrane resistance had returned almost to the resting level for clamp potentials just above threshold, but became less as the clamp potential was changed in the positive direction. For a voltage clamp of +20 mV the membrane resistance was often as low as 50% of the resting value.

Furthermore, the outward current also shows some transient behaviour for large depolarizations of the membrane. Figure 7 shows an extreme example where the membrane resistance for V_m clamped at +30 mV decreased to one-third its resting value. It is also apparent in this cell that the transient behaviour of the outward current phase has occurred before the inward current phase has disappeared.

IV. DISCUSSION

The results of the experiments described in this paper are clearly consistent with the view that the *Nitella* cell membrane, during its active state, is highly permeable to calcium ions. This conclusion is based on evidence obtained first from the experiments on voltage clamping, and, secondly, from the experiments in which the peak of the action potential was considered as a function of the concentration of external calcium.

In the voltage-clamp experiments, the time course of the membrane current was examined at fixed membrane potentials. By keeping the membrane potential constant throughout the activity of the cell, it is possible to measure the ionic current flow, the capacitive current being too small to be important by comparison. That this is the case is shown as follows. Assuming that the total membrane current can be expressed as

$$I = C(\partial V_m / \partial t) + I_i,$$

where C is the membrane capacitance, V_m the membrane potential, and I_i the ionic current, it is clear that the transient inward component of the clamp current, which occurs at a time when V_m is not constant, will consist of both ionic and capacitive components. However, $\partial V_m / \partial t$ is usually no greater than 100 mV sec⁻¹. The membrane capacitance is of the order of 1 μ F cm⁻² (Cole and Curtis 1938). Thus the capacitive component of the clamp current will be no greater than 0.1 μ A cm⁻². This value is small compared with the total measured current of up to 40 μ A cm⁻².

When the clamp is first applied, the membrane behaves initially as an ohmic resistance, but very shortly the current flow reverses, the membrane exhibits a transient negative resistance, and then returns to its "ohmic" behaviour. Such behaviour is characteristic of most excitable tissue, and has been examined in considerable detail in the squid giant axon by Hodgkin, Huxley, and Katz (1952). In the experiments described here on *Nitella*, the relationship between the inward current flow (during this negative resistance phase of the membrane) and the concentration of various ions in the external solution has been investigated.

The relationship between the maximum inward current and the membrane potential during the voltage clamp indicates clearly the dependence of the membrane characteristics on the external calcium concentration. In Figure 4(b) it can be seen that although the minimum resistance of the membrane varies with

membrane potential, there is an overall decrease in the resistance (over the range of membrane potentials investigated) as the concentration of external calcium increases. However, such an effect is not found for variation of external chloride ions (see Fig. 6). Furthermore, it is also shown in Figure 6 that the presence of chloride ions does not influence the effect of calcium ions. The decrease of minimum membrane resistance with increasing Ca_o is the expected behaviour, if the cell membrane increases in permeability specifically to calcium. The greater is Ca_o , the more calcium ions are available to move inwardly across the cell membrane, and thus the greater the ionic current, and the smaller the measured membrane resistance.

Some caution is needed in using Figure 4(b) to determine the minimum membrane resistance. In the resting state of the cell, the resistance of the vacuolar sap can be taken into account to calculate the membrane resistance (Walker 1960). However, when the membrane resistance decreases during activity the current tends to flow less uniformly across the cell surface, and a simple calculation can no longer be made to determine the vacuolar resistance. Consequently, with the experimental arrangement used here to clamp the *Nitella* cell, it is not possible to obtain an accurate value of the minimum membrane resistance when Ca_o is greater than about 0.3 mN. The correction for curves for $Ca_o=0.1$ mN, is approximately $1500 \Omega \text{ cm}^2$, but will be less than this value for $Ca_o=0.3, 1.0$, and 3.0 mN. Hence, it is only possible at this stage to conclude that the minimum resistance of the cell membrane for $Ca_o=3.0$ mN is less than $600 \Omega \text{ cm}^2$.

It would be expected that the membrane potential for which there is no inward transient current should be closely related to the electrochemical equilibrium potential for calcium, and should vary with Ca_o according to the Nernst equation, viz. a change of +29 mV for each tenfold increase in Ca_o . A change with concentration is found, but usually amounts to 35–40 mV.

One possible explanation of this larger value is that calcium is partly in the form of a monovalent complex, and both this and the divalent calcium together control the action potential. The 35–40 mV change for tenfold change in Ca_o would represent the combined behaviour of the two forms of calcium. Furthermore, it is possible, as Hope (1961a, 1961b) suggests, that other ions besides calcium may be involved to some extent in the action potential, and that the membrane potential for zero inward current is not the electrochemical equilibrium potential for calcium alone.

Further evidence for an increase in conductance to calcium is afforded by the result (unpublished) from an experiment in which the cell was clamped periodically at the same potential, while Ca_o was changed from 0.1 mN to zero. The maximum transient current declined within 30 minutes to approximately 20% of its value in A.P.W. The clamp current remained constant for another hour, but was of complex form. When the external calcium was restored, the clamping current increased to its original value in about 40 min. The delay in the decline of the peak current is presumably caused by the slow exchange of wall calcium with external magnesium (Hope and Walker 1961), but it is seen, allowing for this exchange, that the transient clamp current depends on Ca_o .

From the curve of electrochemical equilibrium potential *v.* Ca_o (Fig. 5) it is possible to calculate the activity of calcium on the inner side of the cell membrane. It is likely that such a value will represent the activity of calcium in the cytoplasm. If the Nernst equation holds, then

$$E = (RT/zF) \ln (\text{Ca}_o/\text{Ca}_i),$$

where the activities of calcium in the cytoplasm (Ca_i) and outside solution (Ca_o) are used in the equation. When $E=0$, $\text{Ca}_i=\text{Ca}_o$. From Figure 5, $E=0$ when $\text{Ca}_o=\text{Ca}_i=0.18 \text{ mN}$. Hope (1961*b*) has calculated for *Chara australis* a value for the apparent internal activity of calcium ions of 1.5 mN , assuming that when the peak of the action potential is zero, the internal and external activities are equal. If other ions are involved to any extent in the action potential of *Nitella* the value of 0.18 mN for Ca_i will be incorrect, but the value obtained is probably not grossly inaccurate because measurements of action potential of *Nitella* (unpublished) in solutions containing magnesium, strontium, and barium chlorides and nitrates show that these ions have little effect, whereas the action potential shows strong specific dependence on calcium ions.

From the above, it is now possible to describe the electrical activity of the *Nitella* membrane as follows: when the membrane is in its resting state, calcium ions are not in electrochemical equilibrium. In fact, the resting potential difference and resistance may be largely a function of permeability to sodium and potassium (Hope and Walker 1961). The electrochemical equilibrium potential for calcium is usually more positive than the resting potential, and thus there is a tendency for calcium ions to move inwardly across the cell membrane. To prevent any considerable net inward movement of calcium ions, the cell membrane, in its resting state, presumably has a low permeability to this ion (cf. Walker 1957). Some type of ion pump may also operate.

When the cell is stimulated, and the membrane potential raised above the threshold, the permeability to calcium rapidly increases, and the membrane potential moves towards the calcium electrochemical equilibrium potential. Before this potential is reached however (see Fig. 5), the calcium permeability has decreased, and the membrane potential returns to the resting level. The recovery of the membrane potential is probably the result of an outward movement of potassium ions. Some measurements made on *Chara australis*, using radioactive potassium, have shown an increased efflux of this ion from the cell during the action potential (Findlay and Hope, unpublished data), in agreement with Gaffey and Mullins (1958).

The results described in this paper clearly indicate that calcium ions are the chief factor in the phenomena of potential change and current flow during the action potential in *Nitella*. The same conclusion has been reached for *C. australis* by Hope (1961*a*, 1961*b*), and thus it seems unlikely that chloride ions are involved in the action potential of *C. globularis* as claimed by Gaffey and Mullins (1958).

It is hoped, by refinements in the clamping technique and by flux measurements with radioactive calcium, to compare the transient inward currents with influx of calcium in the larger cells of *C. australis*.

V. ACKNOWLEDGMENTS

Thanks are due to Dr. B. I. H. Scott for guidance and criticism during the experimental work and the preparation of this paper, and to Dr. A. B. Hope for many helpful discussions and advice. Mr. D. E. Millwood gave invaluable assistance in the design and operation of the voltage-clamping circuit.

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