CURRENT VOLTAGE RELATIONS IN *NITELLA**

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An ionic theory of membrane behavior was introduced by HODGKIN and KATZ (1949) and greatly elaborated by HODGKIN and HUXLEY (1952) for the squid giant axon. It explained normal excitation as a rapid inflow of sodium ions and recovery as a slower outflow of potassium ions. Similar explanations have been made on the basis of similar data for several other excitable animal cell membranes and there has been at least the possibility that the theory might also apply to irritable plant cells such as *Nitella*.

In a corticated *Chara* internode, GAFFEY and MULLINS (1958) found that the effluxes of K^+ and Cl^- of 6.7 and 5.8 pmole/cm² sec at rest were increased to 211 and 131 pmole/cm² sec during excitation and they explained the excitation process as an initial outflow of Cl^- followed by the later outflow of K^+ . During the present further investigation of the phenomena in *Nitella* with voltage clamp, HOPE (1961) and FINDLAY (1961) reported that the size of the action potential in *Chara* and that of the peak inward current during the voltage clamp in *Nitella* were dependent on the external calcium concentration. They claimed that the inward current during excitation was due to inflow of Ca ion across the plasmalemma. Also TASAKI *et al.*, (1961) found no appreciable increase of Cl efflux during excitation and that the time course of K efflux was almost parallel to that of the action potential. But then a marked increase of Cl efflux was found for *Nitella* by MULLINS (1963).

In the present investigation the current flows are measured during excitation by applying an improved voltage clamp technique between the vacuolar sap and the external solutions. These are referred to as "membrane" characteristics without attempting to identify their origin among the cell wall, plasmalemma, outer gel layer, inner sol endoplasm or tonoplast. The changes of the membrane currents with increase of the external Cl or Ca concentration lead to the conclusion that the early inward current flow in the voltage clamp

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and the normal excitation process are probably due to a Cl outflow rather than a Ca inflow.

MATERIALS AND METHODS

The internodal cells of the fresh water Characeae, *Nitella*, (probably *N. axillaris* A. Braun) were used throughout. They were maintained in the artificial pond water (APW) as given in TABLE 1. The growth was normal and the velocity of the protoplasmic streaming was about 60 micron/sec at 18°C which is in the normal range. The average diameter of the internodes used was 0.5 mm. The ionic composition of the vacuolar sap, also given in TABLE 1, is about the same as for other Characeae and the concentration of each ion is higher than in APW. This produces a high internal osmotic pressure which is balanced by the tension of the cell. All of the equilibrium potentials, calculated by the Nernst equation in TABLE 1, are negative inside except for Cl. The resting potentials of the internodes used were between -90 and -130 mv, inside negative and somewhat less than the K potential (OSTERHOUT, 1955).

TABLE 1
Ionic Composition of the Vacuolar Sap is Compared
with that of External Artificial Pond Water.

	Concentration (Molar)		Equilibrium Potential (mv)
	Inside	Outside	
K ⁺	10 ⁻¹	7.5×10 ⁻⁵	-181
Na ⁺	5×10 ⁻²	2×10 ⁻⁴	-140
Cl ⁻	10 ⁻¹	1.2×10 ⁻³	+111
Ca ⁺⁺	10 ⁻²	5×10 ⁻⁴	-38
Mg ⁺⁺	10 ⁻³	10 ⁻⁴	-29

The composition of the artificial pond water, APW, is as follows: 2.5×10⁻⁵ M KCl, 5×10⁻⁵ M KNO₃, 10⁻⁵ M NaH₂PO₄, 2×10⁻⁴ M NaCl, 5×10⁻⁴ M CaCl₂, and 10⁻⁴ M MgSO₄. These were dissolved in distilled deionized water. pH was adjusted to about 6.5. The concentrations of K, Na, and Cl of the vacuolar sap of this material were measured by Dr. L. J. MULLINS as 114 mM, 52.5 mM, and 94 mM respectively. These values are of the same order as those obtained by HOAGLAND and DAVIS (1923) on *Nitella clavata*, by COLLANDER (1936) on *Nitella flexilis* and on *Nitella clavata*, and by GAFFEY and MULLINS (1958) on *Chara globularis*. The vacuolar concentrations of Ca and Mg in this TABLE are taken from the measurements by these authors.

The ionic currents flowing across the membrane in a few seconds following a step change of the membrane potential were recorded with some modification of the previous techniques of COLE and MOORE (1960) and MOORE and COLE (1963). An internal current electrode along the axis of the cell as used for the squid axon (MARMONT, 1949; COLE, 1949; HODGKIN, HUXLEY and KATZ, 1952), should provide an adequate "space clamp" with a reasonably uniform potential and current density over a considerable length of internode. This has, however, not yet been done in *Nitella* without an excessive loss of the vacuolar sap and a micropipette inserted

through the membrane has been used to supply current to the interior. This arrangement has also been used by FINDLAY (1961) who measured the current over the entire internode on the assumption that the variation of the membrane potential was not significant. Although the space constant is calculated to be more than 4 cm at rest, it is reduced to nearly a centimeter during excitation. The current and potential variation can be more extreme and more complicated for a quasi steady state negative resistance and the current is better measured over a short length of internode (COLE, 1961).

The measuring chamber is a horizontal square trough with the external current electrodes set in each side as shown in FIG. 1. These three pairs of current electrodes are of platinized silver-silver chloride which are used to give a stable electrode potential and a low electrical resistance (COLE and KISHIMOTO, 1962). The current measurement is restricted to the length of the internode covered by the short central pair of electrodes while the two outer pairs serve as guard electrodes. The internodal cell, preferably less than 2 cm long is held straight in the center of the trough by cotton threads tied to the adjacent internodes and fastened at the ends of the chamber. The external solution flows in through the hole indicated by the dotted circle at the center of the trough and is removed through the holes indicated by the dotted circles at each end. The circulation of the external solution is about 1 cc/min.

FIG. 1. Diagram of electrode system for the control of the membrane potential of *Nitella* internode. An internodal cell of preferably about 2 cm long is selected. Two adjacent internodes are tied with cotton threads and far ends are cut off. These two threads are hooked at both ends of the vessel, keeping the internodal cell straight at the center of the electrode system. Two microtips are inserted into the vacuole. One is used for measuring the vacuolar potential against a small outside reference electrode, at the opposite side of the membrane, and the other is used for supplying current across the membrane to the external current electrodes (three pairs of heavy black blocks). The circulating solution flows in at the center hole (dotted circle in the middle) and out into two holes at both ends (dotted circles at the ends).

The membrane potential is measured between an outside reference electrode (Ref.) and a micropipette electrode inserted into the vacuole (μ -Tip) and the current is supplied by a similar internal micropipette as also shown in FIG. 1. The reference electrode is silver-silver chloride in a small glass capillary filled with 3 M KCl. The tip diameter is about 150μ and has a 1 M KCl agar plug to prevent a rapid flow of

KCl into the external solution. The resistance is about 15-20 K Ω . The two micro-electrodes were 3 M KCl to calomel with a tip diameter less than 1 μ and had a resistance usually between 3 and 5 meg Ω .

The potential difference across the membrane, V_m , is measured with μ -Tip and Ref. in FIG. 2. The "Current Supply" from the "Control Amplifier" then flows to the external current electrodes to correct any difference between V_m and the Command Potentials and clamps V_m . The vacuolar potential is measured with a cathode follower of high input impedance and the potential of the reference electrode is obtained from an operational amplifier of unity gain with 180° phase change (FIG. 2).

FIG. 2. Schematic circuit of the measurement and control system. The system which has been used by COLE and MOORE (1960), MOORE and COLE (1963), on squid giant axon is applied for *Nitella* experiment with minor modifications. See text for further explanation.

These two potentials are added together and amplified with another operational amplifier of unity gain to yield a continuous automatic recording of the resting potential ($-V_m$ in FIG. 2). The sign of the output is then reversed (V_m in the figure) and connected to a high gain control amplifier (USA-3, Philbrick). The holding potential, which is used for maintaining the membrane potential usually at the resting level, positive and negative experimental pulses of various sizes and pulses for the calibration of the amplifier system are also introduced at this point (Command Potentials in FIG. 2). The output of the control amplifier is then connected to the current supplying microelectrode with its tip in the vacuole.

The magnitude of the current I_c which flows across the membrane at the clamped membrane potential is estimated from the potential drop across a resistor (10 k Ω) which connects the center current electrodes to ground. The time course is monitored with a cathode ray oscilloscope (Tektronix 561) and was photographed.

The membrane potentials were also monitored with an oscilloscope. Any preparations in which the voltage clamp pulse was not a perfect step were discarded.

RESULTS

Ionic current at various levels of membrane potential. The typical patterns of the ionic current density in APW after a series of depolarizations and hyperpolarizations are shown in FIG. 3. In this figure the initial capacity charging currents, which are proportional to the applied potential changes, are omitted.

FIG. 3. Pattern of ionic current during various levels of clamped membrane potential. Upper figure is for depolarizations and lower one is for hyperpolarizations. The number attached to each figure indicates the change of depolarization or hyperpolarization from the resting membrane potential.

The resting potential and the height of the action potential of this example are -90 mV and 70 mV respectively. After small depolarizations, with the vacuolar sap less than 25 mV positive with respect to the resting potential, only small constant outward currents are found. Moderate depolarizations, from 25 mV to 80 mV produce an initial outward current which is followed first by a transient inward current and later by a steady outward current. As the depolarization is increased in this range, the initial and the steady state outward currents increase steadily. The net inward currents decrease to become zero for about $+70$ mV which is then the limit for the height of the action potential in this case.

For further depolarizations the initial current and the final steady outward current continue to increase as the transient inward component is

reduced and is not apparent beyond 160 mv or $V_m=70$ mv, inside positive to outside. In contrast to the squid membrane, where the transient sodium current is considerably faster than the rise of the outward potassium current, the outward current component here appears to increase appreciably faster than the transient component for depolarizations larger than 90 mv.

The inward current after hyperpolarization increase monotonically with the potential change at all times, as shown in FIG. 3. It should be noted that the time scale for the duration of the action potential in *Nitella* is in seconds, rather than in milliseconds as for axons.

Current-voltage relations. The initial current, outward for depolarization and inward for hyperpolarization, has not been interpreted and, as has been found for the squid membrane (ADELMAN and TAYLOR (1961)), it may be difficult to do so. In the frog node, DODGE (1963) has identified it as a K current. Here we will refer to this initial current in *Nitella* only as the "leakage" current as has been done for the squid membrane.

The leakage current, the steady current and the transient peak inward current in APW from FIG. 3 are plotted in FIG. 4 as functions of the change of membrane potential. The steady outward current drawn in FIG. 4 is one from which the initial leakage current is subtracted assuming that it is constant during a step potential change. The curves for both the leakage and the steady currents show a considerable rectification in the same direction as

FIG. 4. Current-voltage relations in normal artificial pond water. Peak of the transient inward current, steady outward and inward currents in FIG. 3 are plotted against step membrane potential changes. Initial outward or inward current, indicated as "Leakage" current, is also plotted.

for normal axon membranes. The curve for the peak transient inward current appears more abruptly, in the region of 25 mv depolarization, than is usually found in axon membranes but represents a region of negative resistance similar to that of the semi-conductor tunnel diode.

The potential at which the early transient current reversed from inward to outward was identified as the sodium potential for the normal squid axon membrane and is a particularly important parameter in the ionic theory for the interpretation of the data of voltage clamp experiments. The gradual fading away of the peak inward current component without any clear appearance of a corresponding outward current as shown in FIGS. 3 and 4 for *Nitella* is in marked contrast to the results of squid axon and considerably increases the difficulty of interpretation of the present results.

The transmembrane resistances estimated from the slopes for the leakage current curves show a maximum of 70 k Ω . cm² in hyperpolarization, a minimum of 10 k Ω . cm² in depolarization and 40 k Ω . cm² at the resting potential. Similarly at steady state the resistances are 250 k Ω . cm² in hyperpolarization, 20 k Ω . cm² in depolarization and 60 k Ω . cm² at rest, when calculated on an assumption of no leakage currents. The minimum positive resistance from the slope of the peak inward current curve is about 4 k Ω . cm². Thus we can expect the membrane resistance of about 50 k Ω . cm² at rest to decrease to about 4 k Ω . cm² during excitation.

High Chloride Solution. The external chlorine ion concentration was increased by adding 10 mM NaCl to the artificial pond water. This is about a tenfold increase of chloride concentration. An increase of the osmolarity of the external solution by the addition of 10 mM sucrose usually had no appreciable influence on the resting and action potentials. The recovery after replacing the high chloride solution with normal pond water is usually quite complete but only after more than an hour. The measurements given here were taken only twenty minutes after return to APW and may show only partial recovery.

The resting potential was -105 mv at the beginning, -90 mv in the high chloride solution, and returned to -106 mv in APW. The action potentials in each case were 80 mv, 70 mv and 78 mv respectively. The current voltage relations in this high chloride solution are shown in FIG. 5. The steady state rectification is considerably less in high chloride and has not recovered after the twenty minutes in APW. The most conspicuous points are that the negative resistance region was almost unchanged and the peak inward current was decreased by 40 per cent. Again there can be found no clear indication of a potential at which the inward transient turns to become outward but it seems likely to be more than 150 mv positive to the resting potential for both APW, and the high chloride media.

FIG. 5. Current-voltage relations in high chloride solution (10 mM NaCl is added to normal artificial pond water). APW(1) are the curves for *Nitella* in the artificial pond water at the beginning, High Cl are the ones in high chloride solution which are indicated with crossed points, and APW(2) the ones 20 minutes after replacing the solution with normal artificial pond water. "Leakage" currents are shown with dotted lines.

FIG. 6. Current-voltage relations in high calcium solution (10 mM CaSO_4 added to the normal artificial pond water). APW(1) and APW(2) are the curves for *Nitella* in the artificial pond water before and after the high calcium experiment, (High Ca), of which data are indicated by crossed points. Dotted lines are the curves for leakage current in each solution.

High Calcium Solution. The external calcium ion concentration is increased by the addition of 10 mM CaSO_4 to the artificial pond water. This is about a twenty fold increase of the external Ca concentration, with the Cl concentration remaining the same. The resting potential was -94 mv in APW, -94 mv in the high calcium and -88 mv later in APW. The action potentials in the three cases were 69–72 mv, 68–71 mv and 57 mv respectively. The threshold for excitation and the duration of the action potential were both increased in the high calcium.

The current voltage relations for the increase of calcium are shown in FIG. 6. The leakage current decreases markedly and the recovery is relatively prompt. The steady state rectification is not much changed. The negative resistance region appears at higher depolarization potentials in high calcium than in APW and during recovery it is moved even farther from the resting potential. The maximum inward current is not significantly changed and again there cannot be found any clear indication of the change of a potential for the reversal of the transient components except that it appears to be more than 150 mv positive to the resting potential in all cases.

DISCUSSION

The resistances of the "membrane", are calculated from the leakage, or initial, rectification curve as $62.5 \text{ k}\Omega \cdot \text{cm}^2$ for hyperpolarization and $10 \text{ k}\Omega \cdot \text{cm}^2$ for depolarization. These values are of the same order as those obtained by WALKER (1957) and FINDLAY (1959) with an internal microelectrode. On the other hand, from the additional current flowing in the steady state the calculated resistances are $250 \text{ k}\Omega \cdot \text{cm}^2$ for hyperpolarization and $20 \text{ k}\Omega \cdot \text{cm}^2$ for depolarization. These are rather comparable to the resistances of 100–700 $\text{k}\Omega \cdot \text{cm}^2$ given by BLINKS (1930) and of $100 \text{ k}\Omega \cdot \text{cm}^2$ given by COLE and CURTIS (1938) for cells without internal electrodes. There is thus some reason to believe that the electrode insertion may increase the leakage of the *Nitella* membrane. The minimum resistance from the slope of the peak inward current against membrane potential change is $4 \text{ k}\Omega \cdot \text{cm}^2$ and this corresponds quite closely to the value obtained during activity by COLE and CURTIS (1938).

The interpretation of the ionic currents in *Nitella* is difficult and probably they are complex. The structure of the so-called membrane is obviously complicated. But there is reason to expect that the internal protoplasmic surface is relatively passive and that the phenomena of activity are largely those of the external surface—as shown by WALKER (1955), FINDLAY (1959) and KISHIMOTO (1959). The cell wall, although almost certainly passive, may give rise to considerable problems because of its ion exchange properties that have been noted by COLE and CURTIS (1937), GAFFEY and MULLINS (1958), NAGAI and KISHIMOTO (1964) and TASAKI (personal communication). The ex-

perimental data themselves are reasonably reproducible and appear to be reliable. One reservation is that the sudden rise of the transient inward current over a small range of depolarizing potential indicates that the margin for controlling the membrane potential in space and time is small at best although there is no indication that the controls are not adequate. The presence of the large initial or leakage current and the early rise of the steady state current and the absence of an obvious early transient outward current component all contribute an uncertainty to the quantitative explanation of these three currents. The first two seem relatively unimportant for understanding of excitation while the transient inward current component is principal for it. In fact the action potential cannot go beyond the clamp potential at which the net inward current becomes zero, (COLE, 1949).

The initial leakage current has not been identified and there are no helpful suggestions on its nature from the present work. The steady state current seems to be due to a K ion current both from the view of the equilibrium potential for this ion between the vacuolar sap and APW and from the tracer K outflow data of GAFFEY and MULLINS (1958) during excitation. The resting potential is particularly sensitive to external K ion concentration and the tracer outflow corresponds to the current of $20 \mu\text{a}/\text{cm}^2$ which is reasonably expected from the data of voltage clamp experiments as shown in FIG. 3. By analogy with the squid membrane it would be expected that the K ion current would lag behind the action potential, but TASAKI *et al.*, (1961) found for *Nitella* that the time course of K-efflux was almost parallel to the action potential. However the present data show that the steady current rises relatively more rapidly in *Nitella* than in the squid axon which would account for much of the apparent discrepancy.

That an outflow of Cl ion is responsible for the inward current during excitation was proposed by GAFFEY and MULLINS (1958) for *Chara* and supported by the fact that the efflux of tracer Cl increases during excitation. This conclusion was also reached by KISHIMOTO (1959).

The explanation is not only attractive but necessary if the inward current is a passive flow under an electrochemical potential gradient. As only the equilibrium potential of Cl is positive, as is shown in TABLE I, the passive inward currents may appear by clamping the voltage. It has not, however, been possible to demonstrate a decrease of chloride potential with an increase of external chloride concentration. On the other hand, the data of the present experiment are not inconsistent with such a change of chloride potential and the fact indicating a reduction of the maximum inward current in high chloride in the present experiment supports this view.

The decrease of the resting potential, *ca* 15 mv, in high chloride is somewhat disturbing because the sharp cutoff of a chloride inward current at about 25 mv positive to the resting potential would lead one to expect a

negligible chloride permeability at the resting potential.* There is also but little evidence of an appreciable sodium permeability in this range. Even if a potassium permeability is primarily responsible for the resting potential, either chloride or sodium or both must contribute to reduce the membrane potential from the potassium equilibrium of -181 mv to the resting values in the neighborhood of -90 mv. Then small permeability changes would be sufficient to bring the resting potential to -90 mv. Another objection against this view is that of TASAKI *et al.*, (1961) who found no appreciable increase of Cl efflux from *Nitella* during excitation. It could be suggested, however, that this may be explained as due to a species difference or that Cl which had been excreted during excitation was reabsorbed during the period between successive stimuli. MULLINS (1963) criticized their experiments on the basis of low specific activity and, repeating the experiments on *Nitella*, essentially confirmed the results for *Chara*.

Thus the passive outflow of Cl down the electrochemical gradient during excitation remains a possible basis of excitation, which is ascertained by the voltage clamp results.

HOPE (1961) and FINDLAY (1961) rejected the chloride explanation in favor of a calcium inflow on the basis of their experiments with changes of external calcium concentration. It has been shown by FATT and GINSBORG (1958) and by HAGIWARA *et al.*, (1964) that divalent ions can be responsible for excitation in crustacean muscle. HODGKIN and KEYNES (1957) reported a slight increase of Ca^{45} influx during stimulation of the squid axon and concluded that almost all calcium in the axoplasm was in bound form. A similar enhancement of Ca^{45} influx was observed on sartorius muscle of *Rana pipiens* by BIANCHI and SHANES (1959), on heart muscle of *Rana pipiens* by THOMAS (1960), and on guinea pig atria by WINEGRAD and SHANES (1962). In these examples it has seemed rather improbable that Ca^{++} was a major ion in the transient inward current during excitation. The well known effect of Ca^{++} on a triggering mechanism, presumably by its release from binding sites is a more plausible explanation in those cases (HEILBRUNN, 1952, 1956; FRANKENHAEUSER and HODGKIN, 1957; TOBIAS, AGIN, and PAWLOWSKI, 1962; HUXLEY, 1959; and MULLINS, 1963).

In *Chara* and *Nitella* there is as yet no isotope evidence to show that an inflow of Ca^{++} could account for the inward current either in excitation or in a voltage clamp. A passive inflow would require a positive calcium potential and since this is not shown in TABLE 1, it in turn would require that the calcium in the vacuolar sap be largely bound. Since this is most unlikely, the Ca would have to move in by some active transport mechanism during

* The reduction of the resting potential by such amount in this solution is to be explained by the decrease of the cell wall potential reported by NAGAI and KISHIMOTO (1964).

excitation, which is a unique but not attractive alternative at the present time. On the other hand the relatively slight effect of calcium on the positive equilibrium potential and on the maximum inward current suggests that it is not the major ion in excitation. Also the shift of the negative resistance to a more positive potential by calcium increase is similar to the effect on the squid membrane and is consistent with the powerful but secondary role of this ion. Some of the experimental results of HOPE and of FINDLAY are to be explained on this basis.

Thus it appears that the excitation and voltage clamp inward currents of *Nitella* are now more difficult to explain by calcium ion flow. Therefore, it is concluded that the inward current is most probably due to an outward flow of chlorine ions.

SUMMARY

This paper deals with voltage clamp experiments on the single plant cell. *Nitella axillaris* give membrane currents (after step changes of membrane potential) that are very similar to those of the squid giant axon except that they are much slower and smaller. For depolarizations of 20-60 mv a transient inward current is followed by a steady outward current. For larger depolarizations, the steady outward current sets in during the flow of the transient inward current. The vacuolar potential at which the transient inward current does not appear, or turns to become outward, is 40-60 mv positive to outside. For increasing hyperpolarizations only increasing inward currents are observed. The resistance of the membrane at rest is estimated to be 250 k Ω . cm² for hyperpolarization and 20 k Ω . cm² for depolarization. The minimum resistance calculated from the peak transient inward current is 4 k Ω . cm².

An increase of outside chloride concentration to 10 mM reduces the maximum transient inward current by 40 per cent. An increase of outside calcium concentration to 10 mM, with constant Cl concentration, produces only a small decrease of the transient inward current, while the durations of the inward current and the action potential are usually increased. It is concluded that the ion responsible for the normal electrophysiological activity of the cell may be mainly chlorine ion rather than calcium ion.

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