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Tonoplast Action Potential in *Nitella* in Relation to Vacuolar Chloride Concentration

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Summary. The action potential of *Nitella* internode was studied in relation to K^+ and Cl^- concentrations in the vacuole. When the vacuole of *Nitella pulchella* was filled with an artificial solution with extremely low Cl^- concentration, a diphasic action potential (*DAP*) was observed. The first phase consists of a rapid depolarization followed by a relatively rapid repolarization, and the second one consists of a strong hyperpolarization followed by a gradual return to the resting potential.

When the cell was stimulated immediately after the generation of *DAP*, a monophasic action potential which resembles an action potential of the natural cell was observed, indicating that the *DAP* consists of two components with different refractory periods. The refractory period of the component responsible for the depolarizing phase is shorter than that of a component responsible for the hyperpolarizing phase. Measuring the plasmalemma potential and vacuolar potential separately, it was demonstrated that the hyperpolarizing component of *DAP* originates from the tonoplast.

The action potential of the tonoplast, in contrast with that of the plasmalemma, could be generated independently of concentration of K^+ in the vacuole. Since the maximum amplitude of hyperpolarization decreased significantly by increasing Cl^- concentration of the vacuole, it is concluded that the tonoplast is very sensitive to Cl^- during excitation.

An internodal cell of *Characeae* has a large central vacuole surrounded by the protoplasmic layer. Most studies on ionic and electric behaviors of *Characeae* internodes have generally been carried out by measuring potential difference and resistance between the vacuole and outside, i.e., those across the tonoplast and plasmalemma. Furthermore, interests of the workers seem to have concentrated on responses of the plasmalemma to changes in ionic compositions of the external medium, probably due to lack of technique to change the vacuolar ionic compositions. The technique of exchanging the vacuolar sap of *Characeae* internode with an artificial solution (Tazawa, 1964) is a practical tool for studying responses of the tonoplast to vacuolar ions. Under such a situation, we first developed a new method, called the open-vacuole method, for measuring the electric potential and resistance across the tonoplast and

plasmalemma of *Characeae* internodes having artificial vacuolar media without inserting a glass needle into the cell (Tazawa, Kikuyama & Nakagawa, 1975). Using this method, we measured responses of the electric potential and resistance across the two membranes to either external or vacuolar ions (K^+, H^+, Cl^-) and found that the tonoplast potential of *Nitella* responds to H^+ and Cl^- in ways similar to the plasmalemma potential, but less sensitively to K^+ than the latter (Kikuyama & Tazawa, 1976).

Another way to characterize a membrane is to study its excitability. So far, excitability of the vacuolar membrane has been reported by Chang (1960) and Eckert and Sibaoka (1968) on *Noctiluca*, by Findlay and Hope (1964) on *Chara corallina*, and by Findlay (1970) on *Nitellopsis obtusa*. In the present work, conditions for excitation of the tonoplast of *Nitella pulchella* was studied, and the characteristic of the tonoplast action potential was compared with that of the plasmalemma action potential.

Materials and Methods

The material used throughout this work was *Nitella pulchella* which was collected from a pond and stored outdoors in a large pot with soil.

The open-vacuole method (Tazawa *et al.*, 1975), in which both cell ends are open to the outside and therefore the cell remains turgorless, was mainly used for measuring the vacuolar potential (E_{vo}) of the cell whose vacuolar sap was replaced with an artificial solution (Fig. 1). *Nitella* internode (*N*) was placed on a polyacrylate vessel which has three pools (*A*, *B*, *C*) and a connecting tubing (*T*) with a valve (*V*). *A*, *C* and *T* were filled with a perfusion medium, while *B* was kept empty. After both ends of the cell were amputated, *V* was closed and a small amount of the perfusion medium was added to *A*. Then, the medium in *A* was introduced into the cell vacuole (Fig. 1*a*). After checking that the cell vacuole was completely filled with the medium, *V* was opened to stop the vacuolar perfusion. To measure the vacuolar potential of the cell part in *B*, *B* was filled with an isotonic solution, e.g., isotonic artificial pond water (Fig. 1*b*). The electrode consisting of a vinyl tubing (inner diameter; ca. 1 mm) was filled with 100 mM KCl-agar (2%), and was connected to Ag-AgCl wire through 3 M KCl. Potential difference between two electrodes (E_b and E_c) whose tips were immersed in pools *B* and *C* was amplified and recorded with a pen-writing recorder and a cathode ray oscilloscope (Nihon Kohden VC-8).

To detect the action potential of the tonoplast directly, two glass microelectrodes filled with 3 M KCl were inserted into the turgid cell, one into the vacuole and the other into the cytoplasm (Fig. 2, *cf.* Findlay & Hope, 1964; Eckert & Sibaoka, 1968; and Findlay, 1970). When the turgid cell with an artificial solution was used (Fig. 2), both open ends of the cell were closed after vacuolar perfusion (Fig. 2*a*) by ligation with strips of polyester thread (Fig. 2*b*). The turgor of the cell was recovered by bringing the cell into the artificial pond water. Such a cell or a normal cell was mounted on polyacrylate vessel which had three pools (Fig. 2*c*). Two glass microelectrodes whose resistances were less than several

MΩ were inserted into the cell, one (μ_c) into the cytoplasm, the other (μ_v) into the vacuole. The plasmalemma potential (E_{co}) and the vacuolar potential (E_{vo}) were recorded separately (Fig. 2*d*). The difference between two potentials gives the tonoplast potential (E_{vc}).

Perfusion media mainly used are two kinds of artificial cell saps. The one, *Np* medium, resembles the natural cell sap of *N. pulchella* (cf. Tazawa *et al.*, 1974) and contains 120 mM KCl, 6 mM NaCl, 1 mM $CaCl_2$, and 100 mM sorbitol. Its osmolarity is equivalent to 330 mM sorbitol. The other, *Np*-SO_4 medium, which is characterized by its low Cl^- (0.1 mM) concentrations, contains 0.1 mM KCl, 60 mM K_2SO_4, 3 mM Na_2SO_4, 1 mM $CaSO_4$ and 184 mM sorbitol. Its osmolarity is also 330 mM. For varying the concentration of Cl^- in the vacuole, K_2SO_4 in *Np*-SO_4 medium was exchanged for KCl. As the bathing solution, a simplified artificial pond water (*APW*) which contained 0.1 mM each of KCl, NaCl and $CaCl_2$ was used for the microelectrode measurements, and the isotonic *APW* (*iAPW*) which was made isotonic (330 mM) to the cell sap with sorbitol was used for the open-vacuole measurements. The concentrations of K^+ and Cl^- in *iAPW* were changed by adding K_2SO_4 and choline chloride into *iAPW*, respectively.

The temperature was kept at about 20 °C except for the low temperature experiment.

Results

Occurrence of Diphasic Action Potential (DAP) in Cells with Np-SO_4 Sap

The vacuolar sap of *N. pulchella* was replaced with *Np* or *Np*-SO_4 medium and the action potential was recorded. Fig. 3*a* shows a typical action potential of a cell with *Np* sap. The shape is very similar to that of a cell with natural cell sap. This type of action potential often has two steps: first, rapid depolarization; and second, large and gradual depolarization followed by gradual return to the resting level. When the vacuole was occupied with the artificial solution of very low Cl^- concentration (*Np*-SO_4), a diphasic action potential (*DAP*) was recorded (Fig. 3*b*). It is shown that the *DAP* consists of two phases, i.e., the initial rapid depolarizing phase (*D*-phase) and the subsequent hyperpolarizing phase (*H*-phase). The same type of *DAP* was observed under very low vacuolar Cl^- concentrations (0.1 mM or less) in *Nitella flexilis*, and a similar one was also observed in *Nitella axilliformis* but not in *Chara australis*.

If a cell with *Np*-SO_4 sap was stimulated by an electric current which was large enough to elicit an action potential immediately after the *DAP* had elapsed, a monophasic action potential lacking the *H*-phase was observed (Fig. 4). This resembled the action potential of the normal cell or the cell with *Np* sap (Fig. 3*a*). Another *DAP* could be observed if the cell was stimulated again after 10 min (Fig. 4*b*). This result indicates

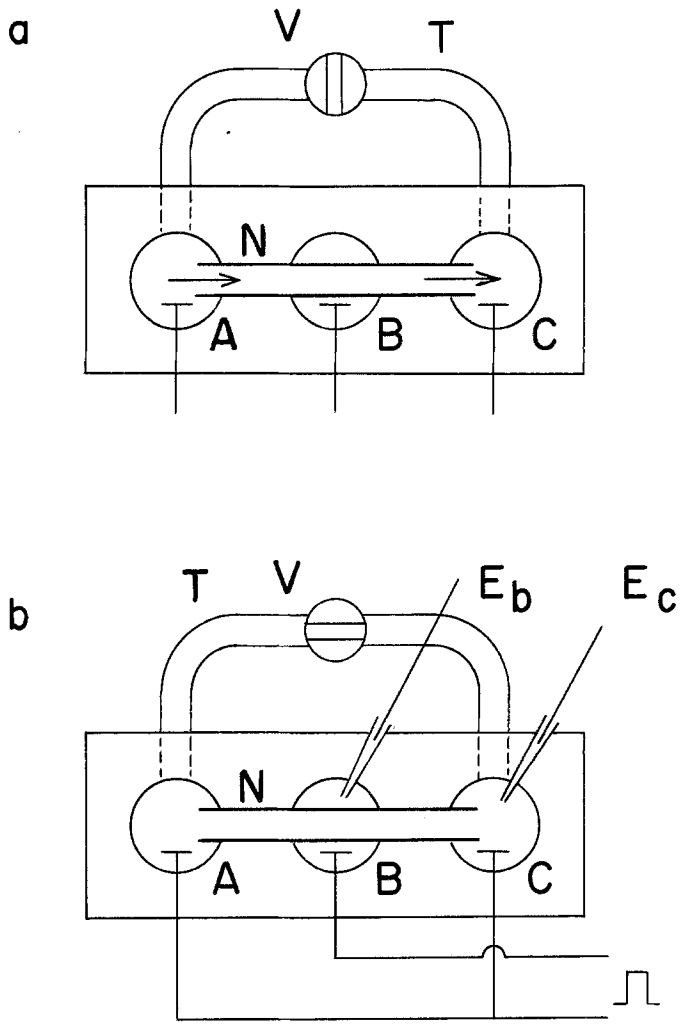


Fig. 1. (*a*) Polyacrylate vessel with an internode (*N*) during perfusion. Pools *A*, *C* and connecting tubing *T* are filled with perfusion medium. For perfusion the valve (*V*) is closed and a small amount of the perfusion medium is added to pool *A*. (*b*) After the cell sap has been completely replaced with the perfusion medium, the valve is opened and pool *B* is filled with an isotonic solution. Tips of two agar electrodes, E_b and E_c, are immersed in pool *B* and *C* to measure the vacuolar potential of the cell in *B*. Electric current is supplied through Ag-AgCl wires. For further explanation *see* text

that the *DAP* consists of two components with different refractory periods. The refractory period of one component which is responsible for the *H*-phase is longer than that of another component which is responsible for the *D*-phase.

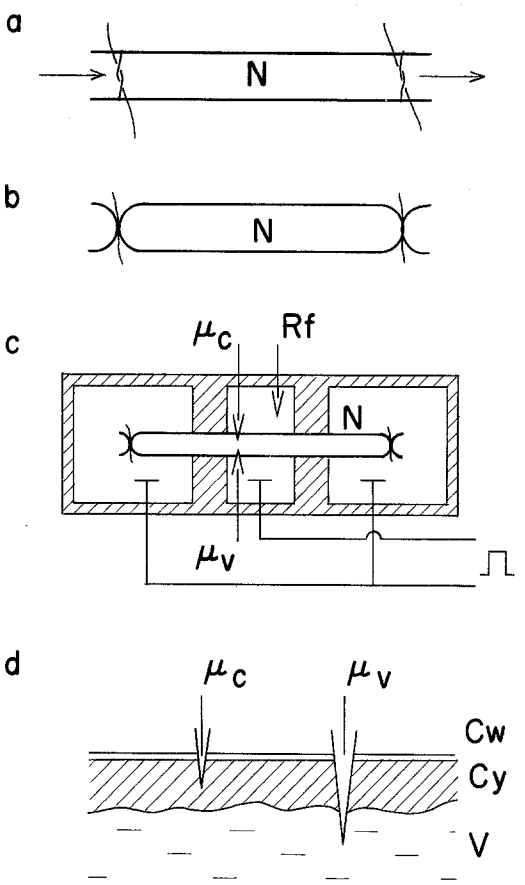


Fig. 2. Procedures for measurements of vacuolar and plasmalemma potentials in *Nitella* internode with an artificial vacuolar medium. (*a*) After replacing the natural cell sap with artificial medium, (*b*) both openings of the cell (*N*) are closed by ligation with strips of polyester thread. (*c*) The cell is mounted on a polyacrylate vessel. Two glass microelectrodes are inserted into the cell part in the central pool, one (μ_c) into the cytoplasm and the other (μ_v) into the vacuole. Plasmalemma and vacuolar potentials are measured against the reference electrode (*Rf*). Electric current was supplied through Ag-AgCl wires. (*d*) Positions of two microelectrodes are shown schematically. *Cw* cell wall; *Cy* cytoplasm; *V* vacuole

In *C. australis*, the action potential is not elicited when the cell is cooled below 10 °C (Kishimoto, 1972). On the other hand, it was observed even at 2 °C in *N. pulchella* and *N. flexilis*. Although the *DAP* was also observed at 2 °C in *N. pulchella*, the *H*-phase was significantly depressed and, in some cases, disappeared (Fig. 5*b*). In contrast to the *H*-phase, the *D*-phase was depressed only slightly and never disappeared at 2 °C. This result shows that the temperature dependency of the *H*-phase is different from that of the *D*-phase. The results shown in Figs. 4 and

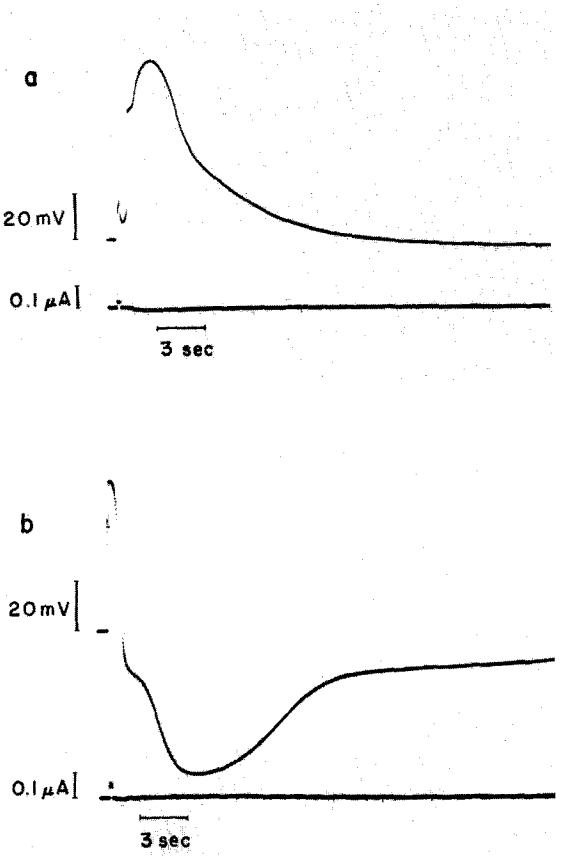


Fig. 3. Oscilloscope traces of (*a*) normal action potential of *Np* sap cell, and (*b*) diphasic action potential of *Np*-SO_4 sap cell

5 suggest that the *DAP* does not represent a single process composed of two phases, but the sum of the two different processes, i.e., depolarizing process and hyperpolarizing process.

It is well known that the action potential of the *Characeae* cell is prolonged at low temperatures (Blatt, 1974). As described above, the *H*-phase of *DAP* often disappeared at the low temperature (2 °C). In some cells, however, the *DAP* with weak but distinct hyperpolarizing component was recorded even at 2 °C (Fig. 5*c*). Since the progress of action potential is markedly slowed down, changes in electric resistance of the membranes can be followed easily at 2 °C by applying small constant inward current pulses during excitation. Fig. 5*c* shows that the electric resistance becomes minimum at the point where the *D*-phase ends and the *H*-phase begins.

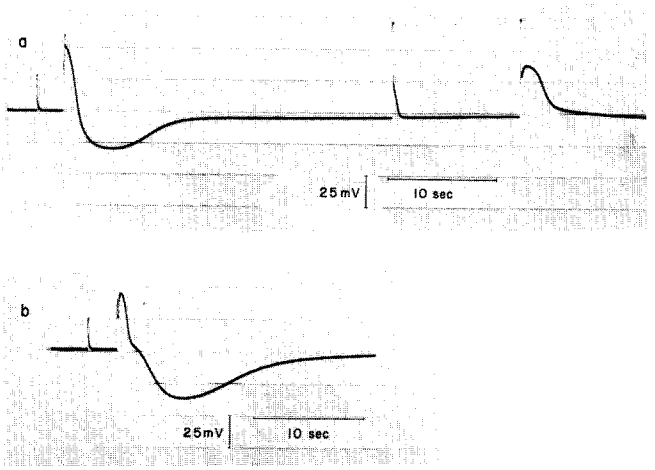


Fig. 4. Two types of action potential observed on the cell with Np-SO_4 sap. (*a*) The monophasic action potential was observed when the Np-SO_4 sap cell was stimulated immediately after the diphasic action potential had occurred. (*b*) The diphasic action potential was reobserved when the cell was stimulated 10 min after the generation of monophasic action potential

Magnitude of Each Phase of DAP vs. Concentrations of K^+ and Cl^-

The fact that the *H*-phase could never be observed in cells with the normal cell sap or with *Np* sap, but in cells with Np-SO_4 sap, suggests that one process responsible for the *H*-phase may occur at the tonoplast and another responsible for the *D*-phase at the plasmalemma. Therefore, it is expected that the *H*-phase will be affected by ions in the vacuole and the *D*-phase by those in the external medium. Experiments along this line were carried out with the open-vacuole method (*cf.* Fig. 1), and the differences between the resting potential and the peak potential of both *D*-phase (ΔE_d^p) and *H*-phase (ΔE_h^p) were determined. Action potentials showing no diphasic modes were discarded in the measurements. In Fig. 6*a*, K^+ concentration in the external solution ($[K^+]_o$) was changed from 0.1 to 3 mM by adding K_2SO_4 into *iAPW*. In Fig. 6*b*, Cl^- concentration in the bathing medium ($[Cl^-]_o$) was varied from 0.4 to 10 mM with choline chloride. In both experiments, the vacuolar medium was Np-SO_4. It is clear that both ΔE_h^p and ΔE_d^p are not affected much by K^+ and Cl^- in the external medium. When cells were bathed in the medium containing 10 mM K^+ or 30 mM Cl^-, they did not generate an action potential.

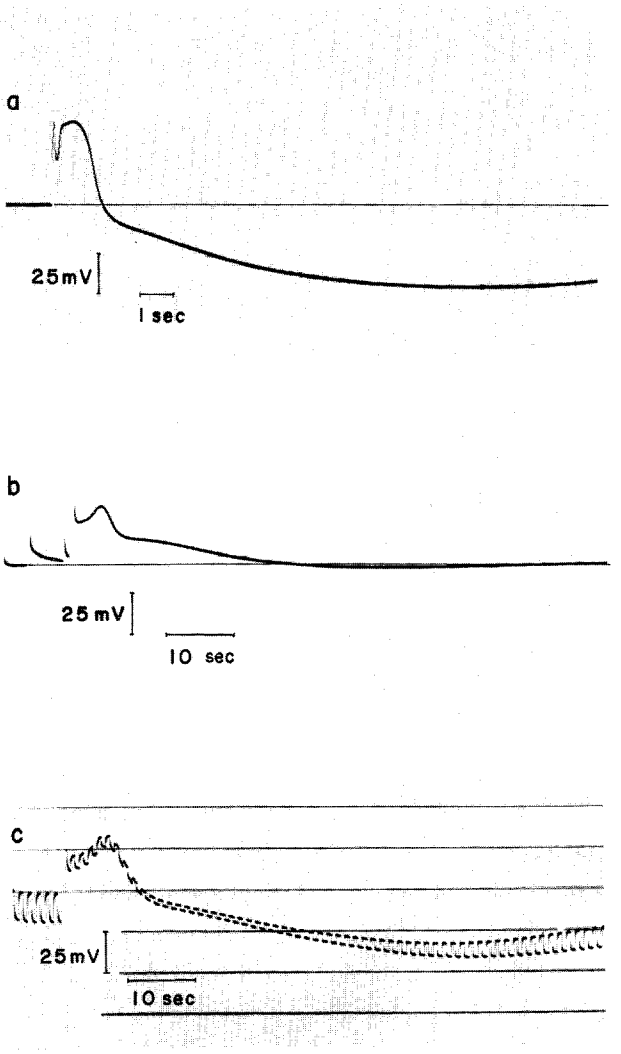


Fig. 5. Temperature dependencies of two components in the diphasic action potential. (*a*) Oscilloscope trace of the diphasic action potential recorded at 20 °C. (*b*) Action potential of the same cell recorded at 2 °C. The hyperpolarizing phase diminished completely. (*c*) Change in electric resistance of another cell during the diphasic action potential recorded at 2 °C

Next, effects of ions in the vacuolar medium were studied. The concentration of Cl^- in *Np*-SO_4 medium in the vacuole ($[Cl^-]_v$: 0.1 mM) was increased to 100 mM by exchanging K_2SO_4 for KCl. The bathing medium used was *iAPW*. Fig. 7 shows that ΔE_h^p depends on $[Cl^-]_v$ strongly, while ΔE_d^p does not. When $[Cl^-]_v$ was increased to 20 mM, the *H*-phase of the *DAP* was strongly depressed and often disappeared. Further, the

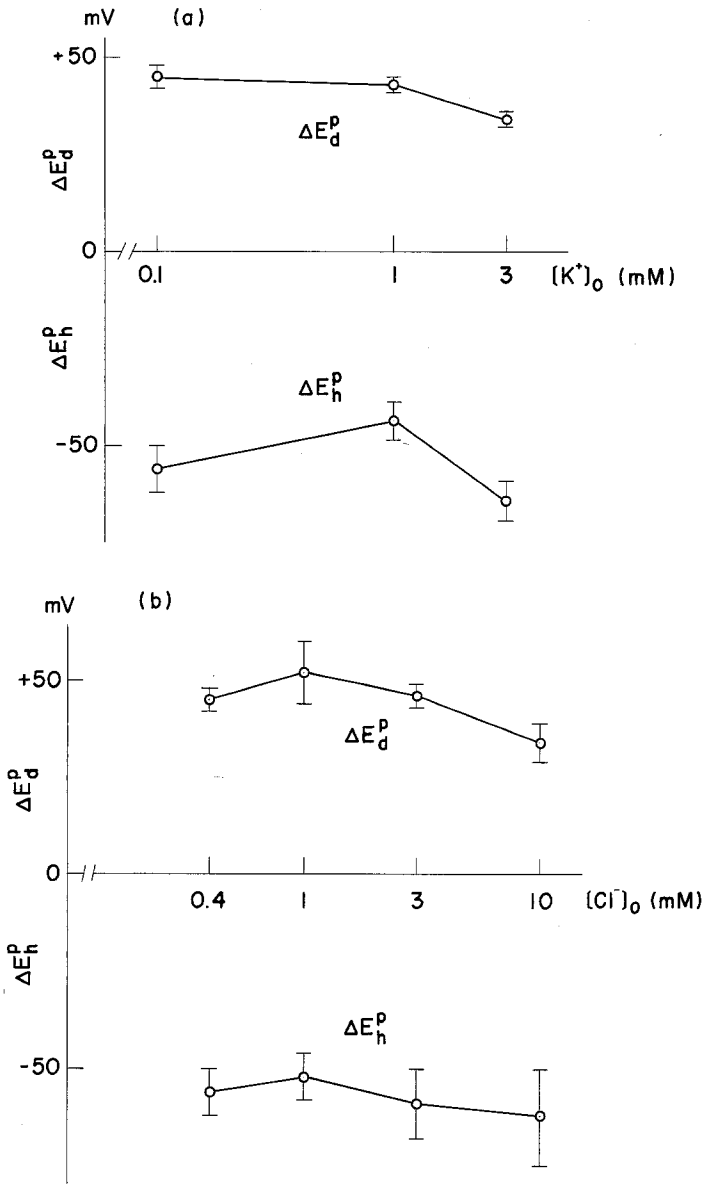


Fig. 6. Effects of external K^+ and Cl^- concentrations ($[K^+]_o$, $[Cl^-]_o$) on the amplitude at the peak of the depolarizing phase (ΔE_d^p) and that of the hyperpolarizing phase (ΔE_h^p) of diphasic action potentials. Vertical lines indicate SE. (*a*) $[K^+]_o$ was varied by adding K_2SO_4 into *iAPW*. (*b*) $[Cl^-]_o$ was varied by adding choline chloride into *iAPW*

DAP was never observed when $[Cl^-]_v$ was 100 mM. Fig. 8 shows the *DAP* of the cell whose vacuolar sap contained 5 mM $CaSO_4$ only. The amplitude, ΔE_h^p, observed in cells with no K^+ in the vacuole (vacuolar medium was 5 or 10 mM $CaSO_4$ only) was -51 mV (average of three

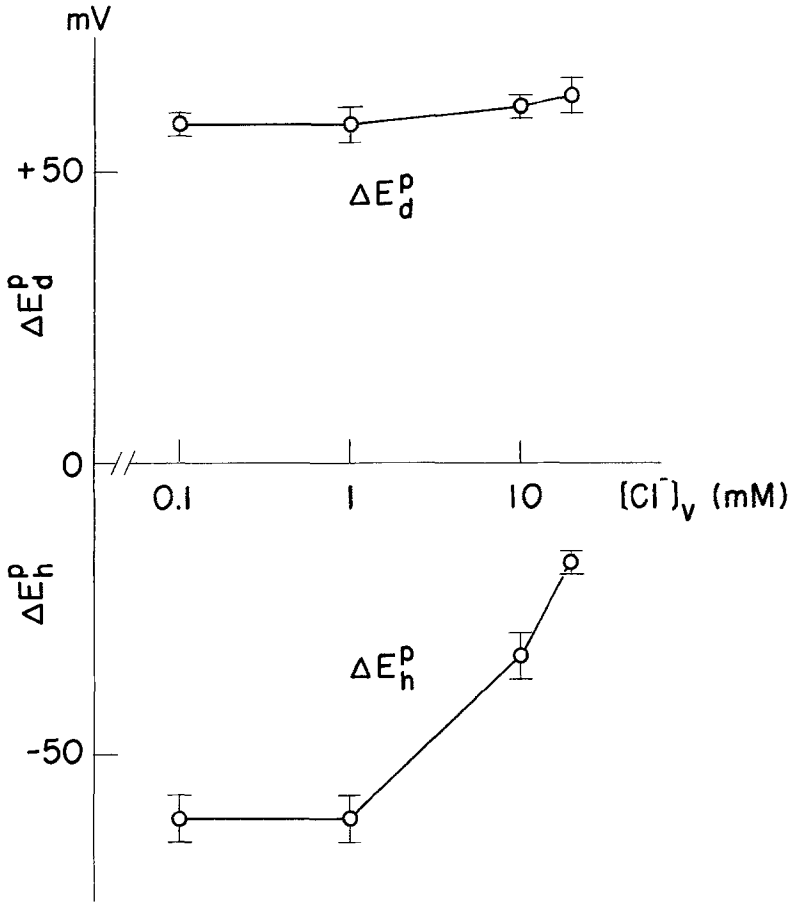


Fig. 7. Amplitudes of depolarizing and hyperpolarizing phases (ΔE_d^p, ΔE_h^p) of the diphasic action potential in relation to Cl^- concentration in the vacuole ($[Cl^-]_v$). $[Cl^-]_v$ was varied by exchanging K_2SO_4 in *Np*-SO_4 medium for KCl. Cells were bathed in *iAPW*. Vertical lines indicate SE

cells). These two results (Figs.7 and 8) indicate that generation of the action potential accompanying the *H*-phase is not dependent on $[K^+]_v$ and $[Na^+]_v$ but on $[Cl^-]_v$. Moreover, the *DAP* was observed not only when the vacuolar pH was 6 but also when it was 3 or 8.

Separate Measurements of Tonoplast Action Potential from DAP

The fact that $[Cl^-]_v$ affects the magnitude of *H*-phase makes such an assumption more reliable in that the *H*-phase of the *DAP* may be generated at the tonoplast. To confirm this, however, it is necessary

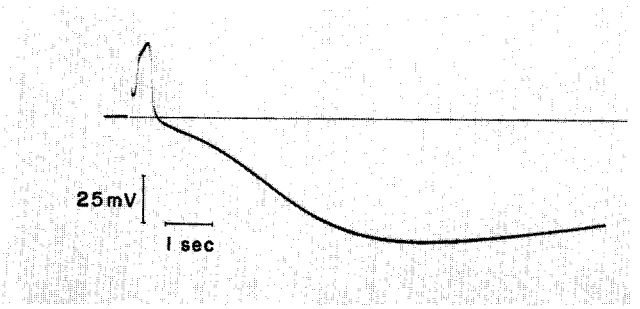
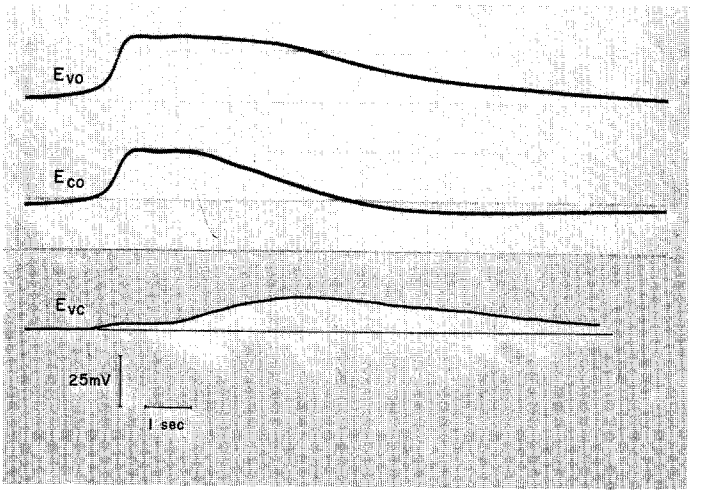
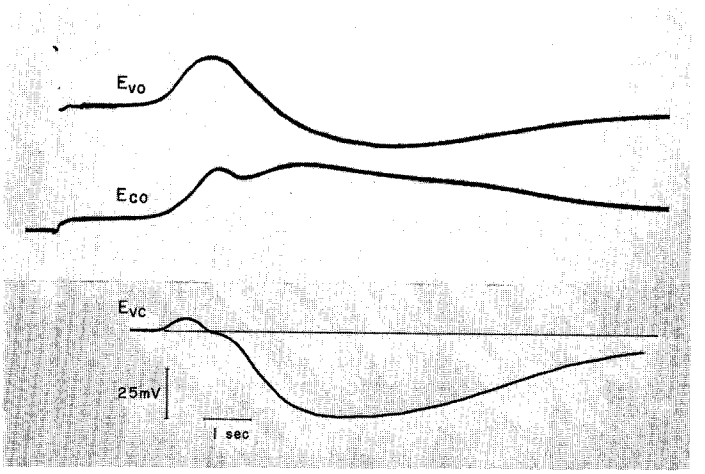


Fig. 8. Oscilloscope trace of the diphasic action potential of a cell whose vacuole contained 5 mM $CaSO_4$ only



(*a*)



(*b*)

Fig. 9. Action potentials recorded from the cytoplasm (E_{co}) and from the vacuole (E_{vo}) of the cell with (*a*) natural cell sap and (*b*) with *Np*-SO_4 sap. Tonoplast action potential (E_{vc}) was obtained by subtracting E_{co} from E_{vo}

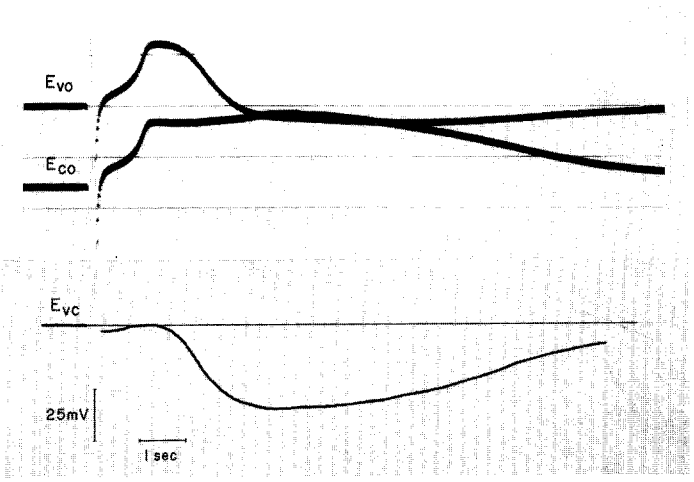


Fig. 10. Action potentials recorded from the cytoplasm (E_{co}) and the vacuole (E_{vo}) of the cell with *Np*-SO_4 sap. Tonoplast action potential (E_{vc}) was obtained by subtracting E_{co} from E_{vo}. Stimulating current was supplied only across the tonoplast from the vacuole to the cytoplasm

to measure the tonoplast potential directly. For this, two glass microelectrodes were inserted into the cell, one into the cytoplasm and the other into the vacuole, and the plasmalemma potential (E_{co}) and the vacuolar potential (E_{vo}) were recorded simultaneously (Fig. 2*c*). It is quite difficult to insert an electrode effectively into the thin layer of cytoplasm of *N. pulchella* through a very thick cell wall, at times reaching 50 μm. Only a limited number of recordings were obtained from both the cytoplasm and the vacuole on cells with exceptionally thin cell walls. Fig. 9*a* shows oscilloscope traces of the action potential of the cell with the natural cell sap recorded from two electrodes inserted into the cell. The potential change of the tonoplast during excitation, E_{vc} in Fig. 9*a*, is obtained by subtracting E_{co} from E_{vo}. Change in E_{vc} to the positive direction is in accord with the results obtained by Findlay and Hope (1964) in *Chara corallina* and by Findlay (1970) in *Nitellopsis obtusa*. When the vacuolar sap was *Np*-SO_4, the *DAP* was recorded from the microelectrode in the vacuole, while the monophasic action potential consisting of the *D*-phase was recorded from the microelectrode in the cytoplasm (Fig. 9*b*). The tonoplast potential, E_{vc} in Fig. 9*b*, did not change to the positive direction but to the negative one.

When a stimulus large enough to cause an action potential at the plasmalemma was applied between a microelectrode inserted in the cytoplasm and an Ag-AgCl wire in the bathing solution, the *DAP* was

recorded from a microelectrode in the vacuole of Np-SO_4 sap cell. Furthermore, three electrodes were inserted into Np-SO_4 sap cell, one into the cytoplasm and another two into the vacuole. Even when only the tonoplast was stimulated by supplying an electric current from the electrode in the vacuole to the electrode in the cytoplasm, the *DAP* was also recorded (Fig. 10). It is possible, however, that a part of the current passed across the plasmalemma and caused an action potential there first.

Discussion

Effluxes of K^+ and Cl^- increase significantly during excitation in *Characeae* cells (Gaffey & Mullins, 1958; Mullins, 1962; Hope & Findlay, 1964; Haapanen & Skoglund, 1967; Oda, 1975). From the voltage clamp experiment, Kishimoto (1965) reported that the peak current during excitation of *Nitella* depends on concentration of Cl^- in the external medium, i.e., the peak current decreased to about half when $[Cl^-]_o$ was increased from 0.75 to 10 mM. All these workers claimed tacitly that the plasmalemma becomes highly permeable to K^+ and Cl^- during its excitation.

The peak of *D*-phase of *DAP* approximately corresponds to the peak of action potential at the plasmalemma (Fig. 9). In the present experiment, the amplitude of the *D*-phase of *DAP* (=amplitude of the plasmalemma action potential) was not affected by changing concentrations of K^+ and Cl^- in the bathing medium in the ranges 0.1–3 mM and 0.4–10 mM, respectively (Fig. 6*a* and *b*). These results do not necessarily show that the plasmalemma action potential is independent of $[K^+]_o$ and $[Cl^-]_o$. If the Goldman equation is valid for the peak potential of *D*-phase, the peak potential (E_d^p) is expressed as follows;

$$E_d^p = 58 \log \frac{[K^+]_o + \beta[Cl^-]_c}{[K^+]_c + \beta[Cl^-]_o},$$

where $[K^+]_c$ and $[Cl^-]_c$ are the K^+ and Cl^- concentrations in the cytoplasm, respectively; β is the permeability ratio P_{Cl}/P_K, where P_{Cl} and P_K are the permeability coefficients of the plasmalemma to Cl^- and K^+. Since $[K^+]_c$ and $[Cl^-]_c$ are 100 mM and 30 mM, respectively (Tazawa,

Kishimoto & Kikuyama, 1974), changes in $[K^+]_o$ or $[Cl^-]_o$ brings about no significant change in E_d^p even if β, which is very small at resting state (*cf.* Findlay & Hope, 1964), increases to 4.0 (Findlay, 1970) at active state. Furthermore, the potential difference across the plasmalemma depends insignificantly on $[K^+]_o$ and $[Cl^-]_o$ if they are below 10 mM (Kikuyama & Tazawa, 1976). Since both the resting potential and the peak potential of the *D*-phase are scarcely affected by $[K^+]_o$ and $[Cl^-]_o$, the amplitude of the *D*-phase of *DAP* should not be affected by them.

Findlay and Hope (1964) and Findlay (1970) suggested that the transient increase in permeability to Cl^- takes place during excitation at the tonoplast as well as at the plasmalemma. Then, it is expected that the amplitude of tonoplast action potential may change according to concentration of Cl^- in the vacuole. This presumption was clearly demonstrated in the present experiment.

Although the *H*-phase of *DAP* reaches its peak nearly at the same time as the tonoplast action potential, the maximum amplitude of the former is smaller than that of the latter (Fig. 9*b*). However, changes in amplitude of the former should be equal to those of the latter. Since the maximum amplitude of *H*-phase of *DAP* observed when the vacuole contained no K^+ ($[K^+]_v=0$) was approximately equal to that observed when $[K^+]_v$ was 120 mM, it is clear that the magnitude of the tonoplast action potential is not dependent on $[K^+]_v$. On the other hand, the amplitude of the *H*-phase depends strongly on $[Cl^-]_v$ (Fig. 7), indicating a close dependence of the tonoplast action potential on $[Cl^-]_v$. The tonoplast potential, E_{vc}, changes to the positive direction during excitation, if the vacuolar solution is the natural cell sap (Fig. 9*a*, *cf.* Findlay & Hope, 1964; Findlay, 1970), and changes to the negative direction, if $[Cl^-]_v$ is below 20 mM (Figs. 7 and 9*b*). This behavior of the tonoplast action potential is well accounted for by assuming that the tonoplast behaves as a Cl^- electrode. Normally, the Nernst potential for Cl^- across the tonoplast is about 48 mV (vacuole positive) when the vacuole is filled with the natural cell sap (about 200 mM Cl^-, Tazawa *et al.*, 1974) and about 150 mV (vacuole negative) when it contained *Np*-SO_4 medium (0.1 mM Cl^-). Since in Fig. 7 ΔE_d^p almost represents the amplitude of the plasmalemma action potential, the difference between ΔE_h^p and ΔE_d^p should represent the amplitude of the tonoplast action potential (*cf.* Fig. 9*b* and 10). From Fig. 7 the differences is about -120 mV which is close to the Nernst potential for Cl^- across the tonoplast.

Besides the difference in responses to Cl^- between the tonoplast potential and plasmalemma potential, there is another essential difference

in excitability between these two membranes. The tonoplast can generate an action potential even when $[K^+]_v$ is 120 mM, while the plasmalemma does not at $[K^+]_o$ of 10 mM or more.

In our experiments, the *D*-phase of *DAP* always appeared prior to the *H*-phase, i.e., action potential at the tonoplast always followed action potential at the plasmalemma even when only the plasmalemma was stimulated with an electric current pulse. Application of stimulating current between the cytoplasm and the vacuole, too, did not elicit tonoplast action potential without accompanying plasmalemma action potential (Fig. 10). Findlay (1970) showed that in *Nitellopsis obtusa* the duration of the plateau component of the tonoplast action potential is dependent on the occurrence of the second peak of the plasmalemma action potential. Generation of *DAP* by stimulation of either plasmalemma or tonoplast suggests that some kind of electric coupling exists between the plasmalemma and the tonoplast.

It is to be noted that we could not observe *DAP* in *C. australis,* dioecious species of *C. corallina* in which Findlay and Hope (1964) recorded the tonoplast action potential.

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