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Demonstration of Two Stable Potential States of Plasmalemma of *Chara* without Tonoplast

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Summary. The tonoplast of cells of *Chara australis* was removed by replacement of the cell sap with a medium containing 5 mM EGTA (ethyleneglycol-bis-(β-aminoethyl ether) N, N′-tetraacetic acid). Such cells without tonoplast could generate an action potential of rectangular shape. In the present paper characteristics of the action potential were studied under various external ionic conditions.

Action potentials could be elicited without refractory period and the peak of the action potential was constant among action potentials.

Duration of the action potential decreased under repeated excitations, but recovered after pause. Increase in concentrations of alkali metal cations, Li^+, Na^+, K^+, Rb^+ and Cs^+, resulted in prolongation of the action potential.

At proper concentrations of monovalent cations the membrane potential could stay either at the resting level or at the depolarized level and could be shifted reversibly from the former level to the latter one or *vice versa* by applying outward or inward current. Further increase in concentrations of monovalent cations resulted in arrest of the membrane potential at the depolarized level. The critical concentrations of the monovalent cations to hold the membrane potential at the depolarized level were about 10 mM irrespective of the cation species.

Divalent cations, Ca^{2+}, Mg^{2+}, Sr^{2+}, Ni^{2+} and Mn^{2+}, added to the bathing medium suppressed the effect of monovalent cations to prolong the action potential.

Ca^{2+} and Mg^{2+} added to the bathing medium caused repolarization of the plasmalemma which had been depolarized by application of high concentrations of K^+ to the bathing medium. The antagonism between monovalent and divalent cations on the state of the plasmalemma of *Chara* cells was discussed based on the two stable states hypothesis proposed by Tasaki (Tasaki, I. 1968. Nerve Excitation. Charles C. Thomas, Springfield, Illinois).

One of the most useful ways to study characteristics of a biological membrane is to control its surrounding environments. The external environment can be modified by changing the composition of the bathing medium of the cell. The internal environment can be modified by intracellular perfusion with media of known compositions. For example, the internal space of a squid giant axon can be perfused with artificial salt solutions, thus enabling us to control the internal environment which

the plasmalemma faces (Baker, Hodgkin & Shaw, 1961; Tasaki, Teorell & Spyropoulos, 1961). The large central vacuole of an internodal cell of Characeae can be perfused with artificial solutions, thus enabling us to control the composition of the fluid occupying the innermost space of the plant cell, the vacuole (Tazawa, 1964). Characeae cells have two membranes, the plasmalemma and the tonoplast. The vacuolar perfusion, therefore, can only modify the internal environment which the tonoplast faces. Responses of the tonoplast to the modification of the ionic composition of the vacuolar medium of *Nitella pulchella* were studied in detail by Kikuyama and Tazawa (1976*a*, *b*). More detailed information on properties of Characeae membranes will be obtained, if chemical compositions of the media on both sides of each membrane are controlled. This can be realized if the cytoplasm is replaced with artificial media of known compositions, as is the case in squid giant axons. To study the electric characteristics of the plasmalemma alone and also to modify the cytoplasmic environment just inside the plasmalemma, a method was developed to prepare the internodal cell without tonoplast (Tazawa, Kikuyama & Shimmen, 1976). Such cells showed not only active cytoplasmic streaming but also membrane excitability. The cell without the tonoplast generates an action potential of rectangular shape (Tazawa *et al.*, 1976). It seems that the membrane of such a cell has two stable levels of the membrane potential, the presence of which were clearly demonstrated in the squid giant axon (Tasaki, Takenaka & Yamagishi, 1968). The present work was undertaken to demonstrate definitely the presence of the two stable membrane potentials in *Chara australis* and to find factors controlling transitions between the two levels.

Materials and Methods

Chara australis, diaecious species of *Chara corallina*, used throughout this work was cultured outdoors in large pots. Internodal cells were isolated from neighboring cells. Cells without tonoplast were prepared by replacing the cell sap with an artificial solution containing EGTA (Tazawa *et al.*, 1976). Replacement of the cell sap with an artificial medium was carried out by the vacuolar perfusion (Tazawa, 1964) where both cell ends were cut and the artificial medium was perfused through the vacuole by establishing a slight difference in the water level between the cell ends. The perfusion medium contained (in mM): 5, EGTA; 5, Tris-maleate; 17, KOH; 6, $MgCl_2$; and 290, sorbitol (pH 7.0). For convenience, this perfusion medium will be called EGTA-sap. Both cell openings were closed by ligating the cell ends with strips of polyester thread. The ligated cell (*Cha*) was placed on the polyacrylate vessel composed of double chambers as illustrated in Fig. 1. Chamber *A* was filled with artificial pond water (*APW*; 0.1 mM each of KCl, NaCl and

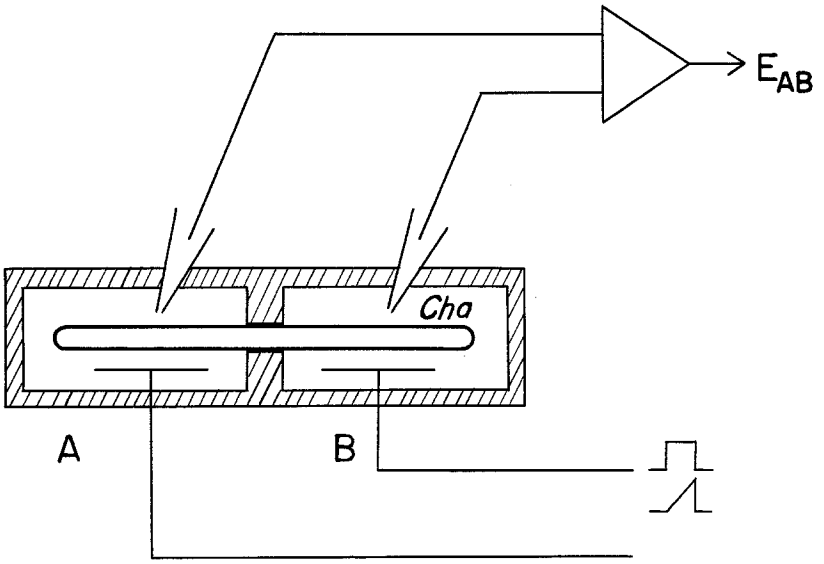

Fig. 1. Diagram of apparatus to measure the membrane potential and resistance by "K-anesthesia method". Internodal cell (*Cha*) was placed on the polyacrylate vessel composed of two chambers. Ramp and rectangular electric current for the stimulation and measurement of the membrane resistance was applied between chamber *A* and chamber *B* through Ag-AgCl wire. The potential difference between two chambers (E_{AB}) was recorded on a pen-writing recorder

$CaCl_2$) supplemented with 200 mM sorbitol to adjust the osmotic value to that of 110 mM KCl solution in chamber *B*. Modification of the bathing medium was done by adding salts to *APW* and the osmotic value was also adjusted to 200 mM with sorbitol.

The potential difference across the plasmalemma of the cell half in *APW* (E_m) is obtained by the following equation,

$$E_m = E_{AB} + (E_m)_B . \tag{1}$$

E_{AB} is the potential of chamber *A* against chamber *B* and can be measured with two electrodes made of polyethylene tubing filled with 100 mM KCl−2% agar which was connected to Ag-AgCl wire through 3 M KCl. Since the plasmalemma potential in 110 mM KCl which was measured with the microelectrode method [$(E_m)_B$ in Eq. (1)] was found to be very small (*cf. Results*), E_{AB} is approximately equal to E_m.

Electric current pulses for measuring the resistance between the two chambers were applied through Ag-AgCl wire. For the measurement of the membrane resistance small inward current pulses lasting about 1 sec were applied throughout the electric measurements. The electric resistance of the membrane (R_m) of the half bathed in *A* is given by the following equation:

$$R_m = R_{AB} - R_B - R_s , \tag{2}$$

where R_{AB} shows the electric resistance between the two chambers, R_B the membrane resistance of the cell half bathed in 110 mM KCl and R_s the sum of electric resistances of the external media, the cell wall and the cell sap. Since in the cell bathed in 110 mM KCl the membrane resistance measured by the microelectrode method was found to be

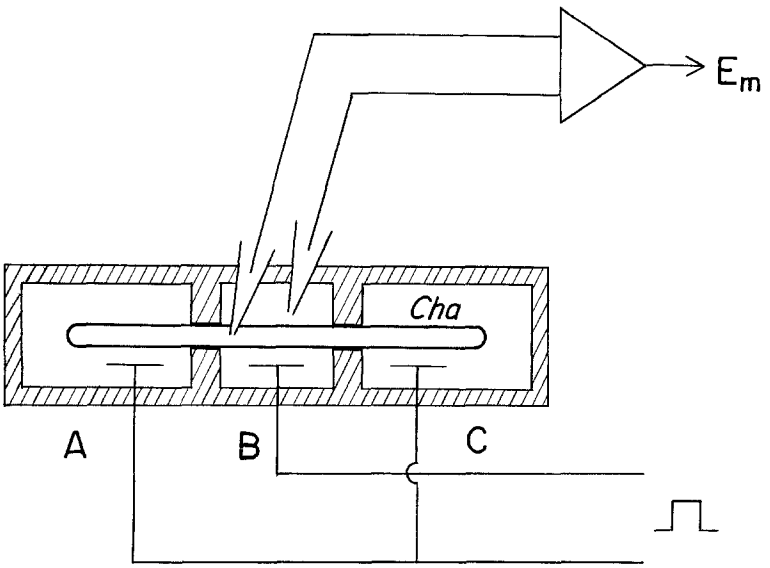

Fig. 2. Diagram of apparatus to measure the membrane potential and resistance by "microelectrode method". Internodal cell (*Cha*) was placed on the polyacrylate vessel composed of three chambers. Rectangular electric current for the stimulation and measurement of the membrane resistance was applied to the cell part in chamber *B* by passing the current between chamber *B* and chambers *A* and *C* through Ag-AgCl wire. Membrane potential (E_m), represented by the potential difference between reference electrode and microelectrode inserted into the cell, was recorded on the pen-writing recorder

negligibly small (about 0.1 kΩcm^2), R_B in Eq. (2) can be neglected. Then

$$R_m = R_{AB} - R_s. \tag{3}$$

R_s was measured by filling both chambers with 110 mM KCl solution. For convenience, we call the method described above "K-anesthesia method".

In some cases the potential difference between the cell interior and the exterior was measured by inserting a glass microelectrode into the cell. The ligated cell whose cell sap had been replaced with the EGTA-sap was partitioned into three parts (Fig. 2). Three chambers were filled with the same solution. The glass microelectrode filled with 3 M KCl was introduced into the cell segment in *B*, and the potential difference across the plasmalemma of the cell was measured between the microelectrode and the reference electrode placed in the chamber *B*. Electric current was applied to the cell between chamber *B* and chambers *A* and *C* through Ag-AgCl wire. The electric potential and the electric current applied were recorded simultaneously with a pen-writing recorder.

Since the K-anesthesia method is simpler than the microelectrode method and is sufficient for the purpose of the present study to demonstrate the presence of the two stable membrane potentials, most of the records represented in this paper were obtained by the former method except that shown in Fig. 6.

Experiments were done at room temperature (20–25 °C) and under dim light. The light intensity was kept at about 100 lux which was too low to induce membrane potential change due to light.

Average values of the electric potentials, the electric resistances and the concentrations of monovalent cations to arrest the membrane potential at the depolarized level are shown with ± SEM and with the number (n) of cells used.

Results

Electric Properties of the Plasmalemma at the Resting State

The value of E_{AB} of normal cells of *C. australis* was -180 ± 6 mV ($n = 17$) and the membrane resistance was 40 ± 3 kΩcm^2 ($n = 6$). Fig. 3 shows changes in E_{AB} and R_{AB} of the cell whose cell sap was replaced with the EGTA-sap. The cell was mounted in the chamber (Fig. 1) immediately after the replacement of the cell sap with the EGTA-sap. E_{AB}, which is roughly equal to E_m of the cell half in *A*, was low for the initial 10 min and then increased up to -170 mV. Simultaneously with the changes in E_{AB}, R_{AB} increased conspicuously. These characteristic changes in electric potential and resistance are symptoms of disintegration of the tonoplast (Tazawa *et al.*, 1976). As already reported in the previous

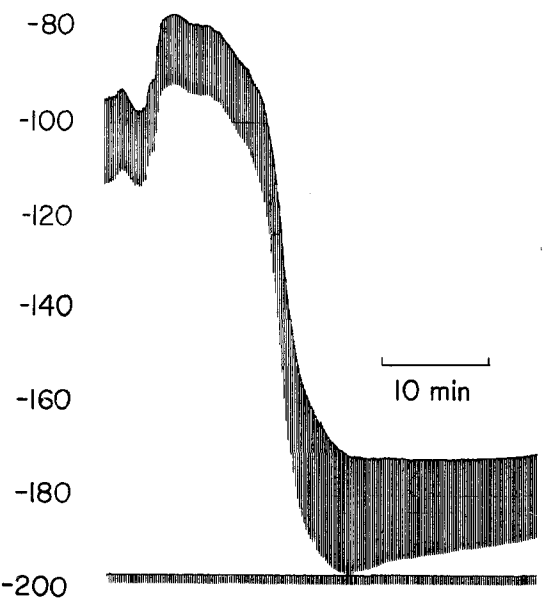

Fig. 3. Increase in membrane potential and membrane resistance after the cell sap was replaced with EGTA-sap. The cell was placed on the chamber (Fig. 2) within a few min after replacement of cell sap and *APW* was used as the bathing medium. Rectangular electric current pulses of 0.02 μA were applied during the measurement (lower trace). The value of E_{AB} is shown in mV. R_s in Eq. (3) was 150 kΩ

paper (Tazawa *et al.*, 1976), increases in E_{AB} and R_{AB} were observed within 30 min after the perfusion. The average value of E_{AB} after the loss of the tonoplast was -173 ± 4 mV (n=48). This value is nearly equal to E_{AB} of the normal cell. The membrane potential of the cell measured by the microelectrode method was 8 ± 2 mV (n=10), when the cell had been bathed in 110 mM KCl for 30 min during which the tonoplast was disintegrated. Since this value should be equal to $(E_m)_B$ in Eq. 1, E_m can be calculated by adding 8 mV to -173 mV. This value (-165 mV) is nearly equal to the membrane potential of the normal cell. The average value of R_m of the cells lacking tonoplast is calculated to be 116 ± 9 kΩcm^2 (*cf.* Eq. (2)) which is about three times as high as that of normal cells.

Excitability of the Plasmalemma

After the tonoplast was removed, action potentials of rectangular shape were generated by electric stimuli. In Figs. 4 and 5 the outward current pulses were applied to the cell after the appearance of the electric symptoms of disintegration of the tonoplast. The current was withdrawn immediately after the action potential was induced. The first action potential lasted for 2 min in Fig. 4 and 7 min in Fig. 5. Both in Figs. 4 and 5 the deflections of the membrane potential due to constant current pulses reflect R_{AB} of Eq. 3. It decreased significantly during the depolarized state of the action potential. At the depolarized state R_{AB} was

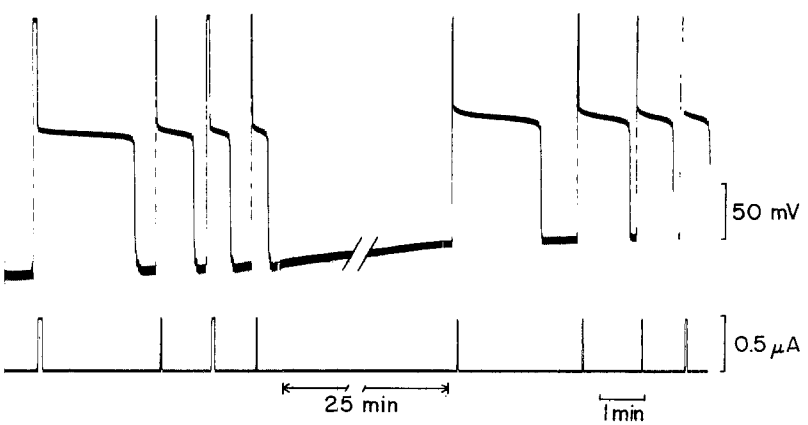

Fig. 4. Decrease in duration of the action potential by repeated excitations and its recovery after pause. Four action potentials were repeatedly elicited by outward current and four action potentials were elicited again after 25 min of pause in *APW*. Rectangular electric current pulses of 0.02 μA were applied during the measurement. R_s in Eq. (3) was 150 kΩ

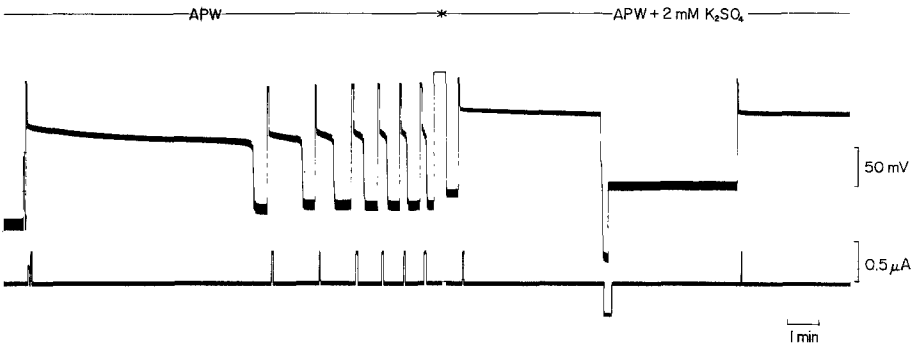

Fig. 5. Decrease in duration of the action potential in *APW* and reversible transition of the membrane potential by electric current in *APW* containing 2 mM K_2SO_4. Seven action potentials were elicited in *APW*, and the duration of the action potential decreased. Reversible transitions of the membrane potential between two levels by electric current were observed after the bathing medium was changed for *APW* containing 2 mM K_2SO_4. Rectangular electric current pulses of 0.05 μA were applied during the measurement. R_s in Eq. (3) was 100 kΩ

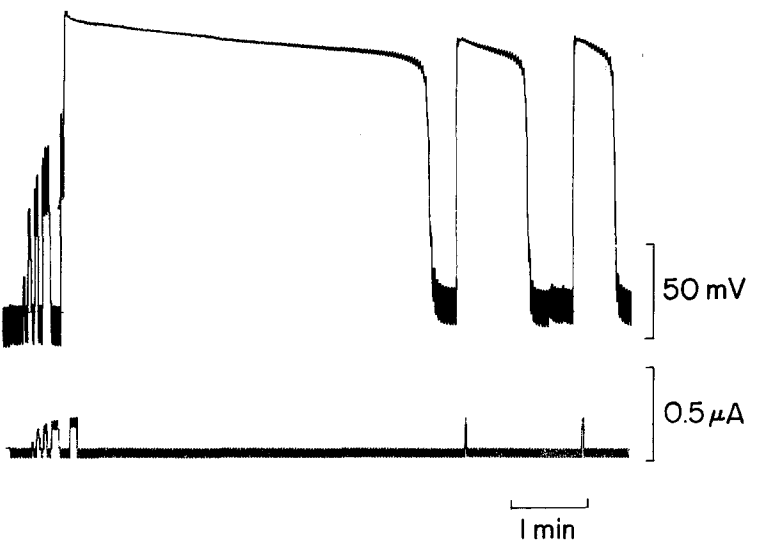

Fig. 6. Action potentials and membrane resistance measured by the microelectrode method. Action potentials were elicited in *APW*. Duration of the action potential decreased with numbers of the action potential, and the membrane resistance decreased significantly during the plateau of action potentials. Rectangular electric current pulses of 0.05 μA were applied during the measurement

found to be nearly equal to R_s, indicating that R_m is very small (Eq. 3). When E_m was measured with the microelectrode method (Fig. 2), current pulses of the same intensity brought about very small deflections of the potential at the state of depolarization (Fig. 6).

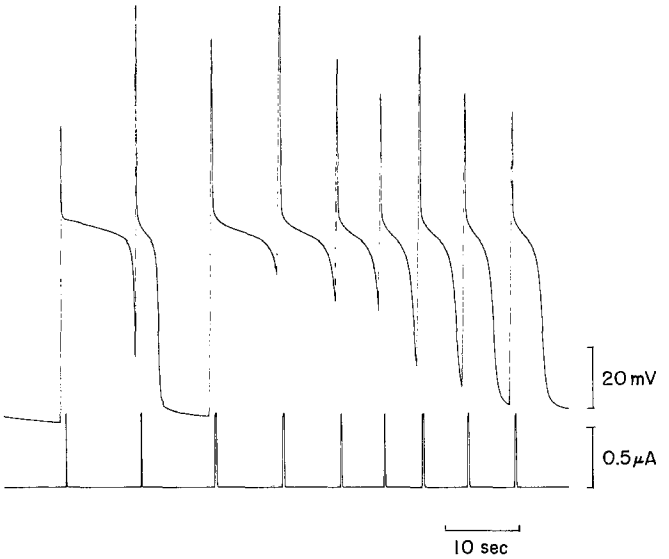

Fig. 7. Generation of action potentials without refractory period. The cell was bathed in *APW* and stimulated repeatedly. Action potentials could be elicited just after the preceeding action potential or even in its falling phase

Besides a very long duration of the action potential there are, in addition, three characteristics of the action potential in the tonoplast-free *Chara* cell. (1) When the cell was repeatedly stimulated, it could generate action potentials without refractory periods. Even when the stimulus was given in the falling phase of the action potential, the potential shifted back promptly to the excited depolarized level (Fig. 7). (2) The peak of the action potential was constant on repeated excitations (Figs. 4 and 5). (3) The duration of the action potential decreased with the number of stimuli applied consecutively with short intervals (Figs. 4 and 5). The duration of the action potential which had decreased much on repeated excitations increased again after a long pause. In Fig. 4 the action potential lasted for 135 sec in the first excitation and for only 24 sec in the fourth excitation. Keeping the cell at the resting state for 25 min, the duration of the action potential increased to 120 sec and was decreased again by repeated excitations.

Prolongation of the Action Potential

There are at least two ways to prolong the duration of the action potential which has been decreased by repeated excitations. One way

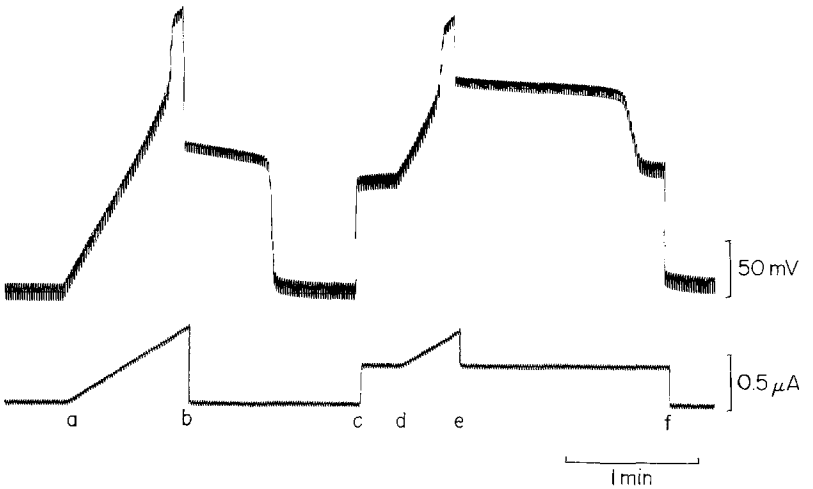

Fig. 8. Prolongation of the action potential by application of outward electric current to the cell. Two action potentials were elicited by outward ramp current under the absence (*a–b*) or presence (*d–e*) of constant outward current in *APW*. Rectangular electric current pulses of 0.05 μA were applied during the measurement. R_s in Eq. (3) was 150 kΩ

is to apply an outward current to the cell. In Fig. 8 an action potential was induced by the outward ramp current applied during the interval between *a* and *b*. The action potential lasted for 37 sec after the current had been withdrawn at *b*. When the same cell was stimulated again with a ramp current (*d*−*e*) which was superposed on a subthreshold constant outward current (*c*−*f*), the duration (80 sec) of the action potential was markedly prolonged.

The other way to prolong the action potential is to add a few mM of alkali metal ions to the bathing medium. Prolongation of the action potential by addition of alkali metal ions was observed in all cells tested, although extent of prolongation varied from cell to cell. Fig. 9 shows one typical example.

The duration of the action potential was decreased to 7 sec by repeated excitations of the cell in *APW*. When the concentration of K^+ in the bathing medium was increased from 0.1 mM to 1.1 mM by adding 0.5 mM K_2SO_4 to *APW*, the duration of the action potential was prolonged to 22 sec. A further prolongation was observed by increasing the concentration of K_2SO_4 to 1 mM. Other alkali metal ions, Li^+, Na^+, Rb^+ and Cs^+, could also prolong the action potential. Comparisons among abilities of alkali metal ions to prolong the action potential were not done.

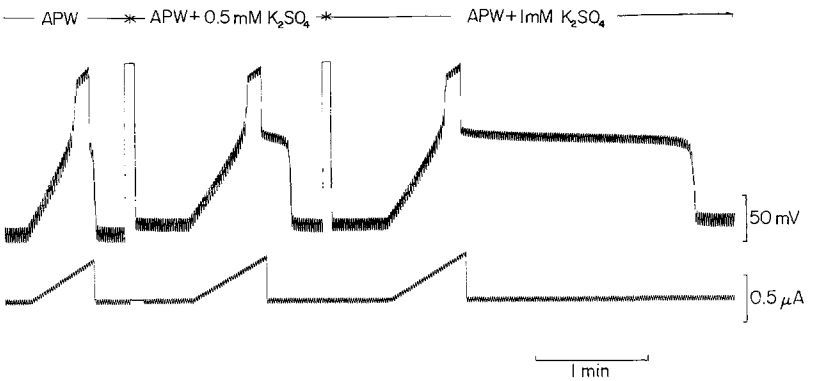

Fig. 9. Prolongation of the action potential by application of K_2SO_4 to the external medium. After the duration of the action potential had been decreased by repeated excitations in *APW*, prolonged action potentials were elicited under the presence of 0.5 or 1 mM K_2SO_4 in the bathing medium. Rectangular electric current pulses of 0.05 μA were applied during the measurement. R_s in Eq. (3) was 130 kΩ

Transitions of the Membrane Potential between Two Levels

When the concentration of K^+ in the bathing medium was further increased, the effect of K^+ to prolong the action potential was strengthened. For instance, in Fig. 5 the duration of the action potential decreased from 7 min to 10 sec due to successive stimulations at short intervals. After the end of the seventh action potential 2 mM K_2SO_4 was added to the bathing medium (*APW*), and the cell was stimulated electrically to cause an action potential. When the current was withdrawn, however, the plasmalemma potential did not come back spontaneously to the original resting level but stayed at the depolarized level for a long time. When the inward current was applied to the cell at this prolonged excited state, the membrane potential showed a hyperpolarizing response accompanying an increase in R_m. The membrane potential shifted to a value which was more negative than that expected by ohmic change due to the inward current, indicating an increase in the electromotive force of the membrane (*cf.*Ohkawa & Kishimoto, 1974). Even when the current was removed the membrane potential did not come back to the depolarized level but stayed at the resting level. Such transitions between the two levels triggered by electric stimuli could be repeated many times. The critical concentration of external K^+ at which the membrane potential exhibits reversible transitions between the two stable levels by electric stimuli was variable between 3–10 mM. When the

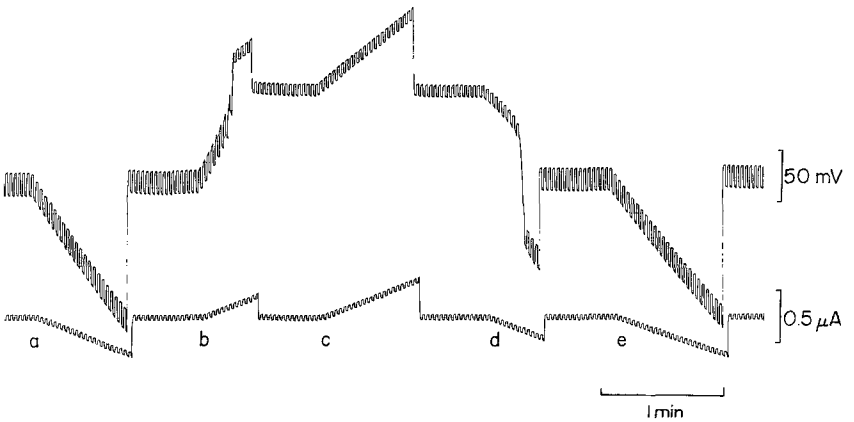

Fig. 10. Responses of the plasmalemma to the outward and inward ramp currents in resting and depolarized states. Outward and inward ramp currentely were applied alternately to the cell bathed in *APW* containing 2 mM K_2SO_4. The cell responded to outward current (*b*) but not to inward current (*a* and *e*) in the resting state, and to inward current (*d*) but not to outward current (*c*) in the depolarized state. Rectangular electric current pulses of 0.05 μA were applied during the measurement. R_s in Eq. (3) was 150 kΩ

concentration of K^+ was further increased, the plasmalemma showed a hyperpolarizing response to the inward current, and the membrane potential returned to the depolarized level on removal of the current. A similar behavior of the membrane potential in response to the increase in ionic concentrations was also observed in other alkali metal ions. The critical concentrations of alkali metal ions to arrest the membrane potential at the depolarized level were obtained by increasing the concentrations at steps of 2 mM. The critical concentrations of Li^+, Na^+, K^+, Rb^+ and Cs^+ were 14 ± 2 mM (n = 5), 13 ± 2 mM (n = 5), 9 ± 0.4 mM (n = 12), 10 ± 2 mM (n = 4), and 10 ± 3 mM (n = 4), respectively.

Next, responses of the plasmalemma at each state to the outward and inward electric currents were studied. In Fig. 10, the cell was bathed in *APW* containing 2 mM K_2SO_4 and the membrane potential was at first at the resting level. When the inward ramp current was applied to the cell at *a*, the membrane potential showed only an ohmic change. It returned to the original resting level on withdrawal of the current. When the ramp outward current was applied to the cell at *b*, a characteristic change in the potential showing excitation was observed and the membrane resistance decreased. The membrane potential stayed at the depolarized level after withdrawal of the current. Applying the outward ramp current to the cell at this state (*c*), only an ohmic change of the membrane potential was observed. To the inward current, however,

the cell showed a hyperpolarizing response with an increase in the membrane resistance (*d*). The membrane potential stayed at the resting level after withdrawal of the current. Thus, the plasmalemma responds differently to the direction of the current according to whether the membrane is at the resting state or at the excited state.

Effect of Divalent Cations in the Bathing Medium

When the divalent cations such as Ca^{2+}, Mg^{2+}, Sr^{2+}, Ni^{2+} and Mn^{2+} were added to the bathing medium, they suppressed the effect of monovalent cations to prolong the action potential. One example is shown in Fig. 11. At the start of the record, the cell was bathed in *APW* with 2 mM K_2SO_4. As described in the foregoing section, the membrane potential stayed for a long time either at the resting level, or at the depolarized level. Transitions of the membrane potential between the two stable levels could be controlled by the electric current. Next, the bathing medium was replaced with *APW* containing 2 mM K_2SO_4 and 1 mM $CaSO_4$. Though the outward current caused excitation of the membrane as before, the membrane potential did not stay at

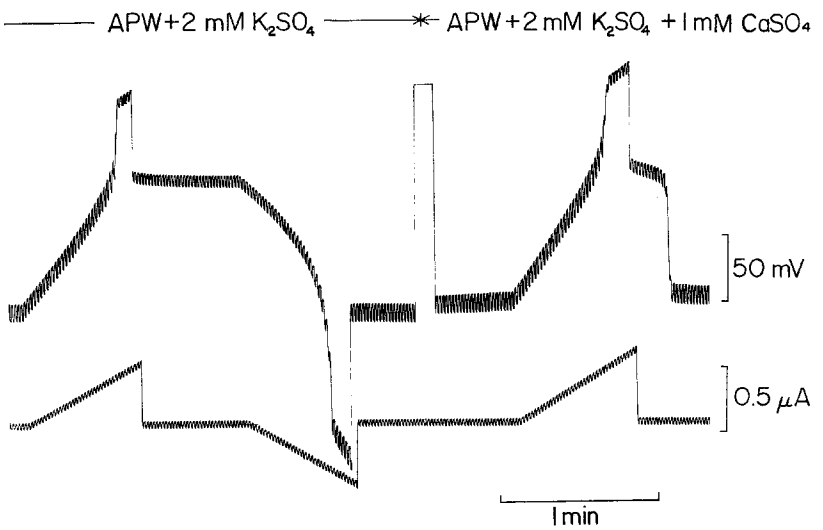

Fig. 11. Suppression of the effect of K^+ to prolong the duration of the action potential by Ca^{2+}. The prolonged action potential was induced by outward ramp current in *APW* containing 2 mM K_2SO_4, and the action potential was abolished by inward ramp current. The action potential was elicited again in *APW* containing 2 mM K_2SO_4 and 1 mM $CaSO_4$, and the duration was shortened. Rectangular electric current pulses of 0.05 μA were applied during the measurement. R_s in Eq. (3) was 130 kΩ

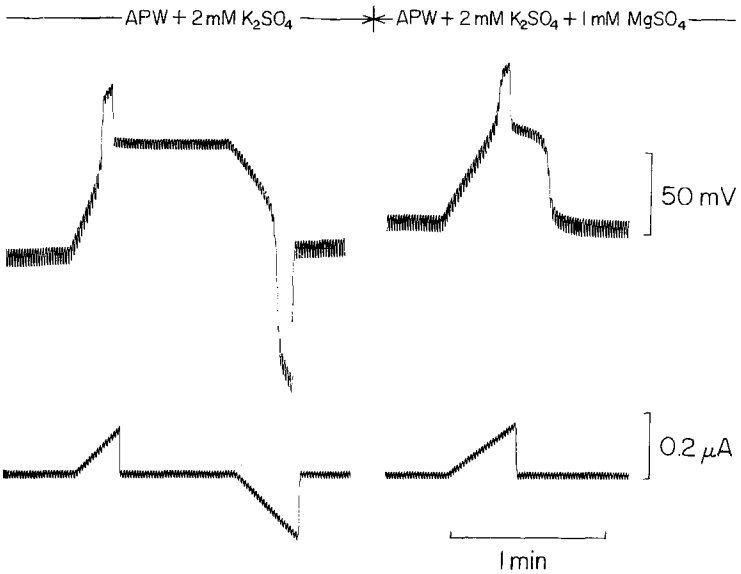

Fig. 12. Suppression of the effect of K^+ to prolong the duration of the action potential by Mg^{2+}. The prolonged action potential was induced by outward ramp current in *APW* containing 2 mM K_2SO_4, and the action potential was abolished by inward ramp current. The action potential was elicited again in *APW* containing 2 mM K_2SO_4 and 1 mM $MgSO_4$, and its duration was shortened. Rectangular electric current pulses of 0.024 μA were applied during the measurement. R_s in Eq. (3) was 240 kΩ

the depolarized level but returned to the original resting level soon after withdrawal of the current. The effective concentration of external Ca^{2+} which brings the depolarized membrane potential back to the resting level changed to a higher value with time during experiment and varied from cell to cell. Essentially the same result was obtained in the experiment using Mg^{2+} instead of Ca^{2+} (Fig. 12). The other divalent cations, Sr^{2+}, Ni^{2+}, and Mn^{2+}, were also effective in suppressing the effect of monovalent cations to prolong the action potential. When the bathing medium contained 2 mM K_2SO_4, the lowest effective concentrations of divalent cations to reduce the duration of the action potential to less than 1 min were 0.5 mM irrespective of ion species (Ca^{2+}, Mg^{2+}, Sr^{2+}, Ni^{2+}, Mn^{2+}).

Transitions of Membrane Potential by Chemical Stimuli

Results obtained so far indicate that the tendency of the membrane potential to stay at the depolarized level is strengthened by increasing concentrations of the monovalent cations in the bathing medium and

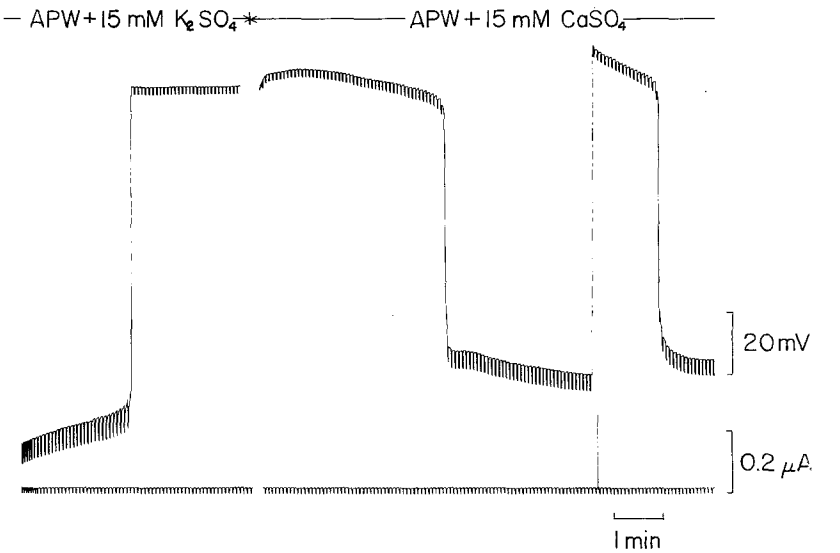

Fig. 13. Chemical stimulation of *Chara*. The bathing medium was first *APW* containing 15 mM K_2SO_4 and next *APW* containing 15 mM $CaSO_4$. The action potential of rectangular shape was elicited by electric outward current, when the plasmalemma repolarized in 15 mM $CaSO_4$. Rectangular electric current pulses of 0.02 μA were applied during the measurement. R_s in Eq. (3) was 100 kΩ

is weakened by adding the divalent cations to the bathing medium. Therefore, it is assumed that the monovalent cations are preferrable to bring the membrane from the resting state to the excited state, and the divalent cations from the excited state to the resting state. The assumption was verified by experiments shown in Fig. 13, where the bathing medium of the cell was first *APW* containing 15 mM K_2SO_4 which was later changed for *APW* containing 15 mM $CaSO_4$. Transferring the cell from *APW* to the K_2SO_4 medium, the membrane potential shifted spontaneously to the depolarized level and stayed there, and the membrane resistance decreased. When the K_2SO_4 medium was replaced with the $CaSO_4$ medium, the depolarized membrane potential abruptly shifted, after a short lag, to the polarized resting level and the membrane resistance increased. Addition of Mg^{2+} instead of Ca^{2+} to *APW* also brought back the depolarized membrane potential to the resting level (Fig. 14). When the cell at this state was stimulated electrically, the action potential of rectangular shape was not observed in Mg^{2+}-enriched *APW* (Fig. 14), but in Ca^{2+}-enriched *APW* (Figs. 13 and 14). It is to be noted that the transitions of the membrane potential between the two levels without electric stimuli could be repeated many times, indicating that monovalent

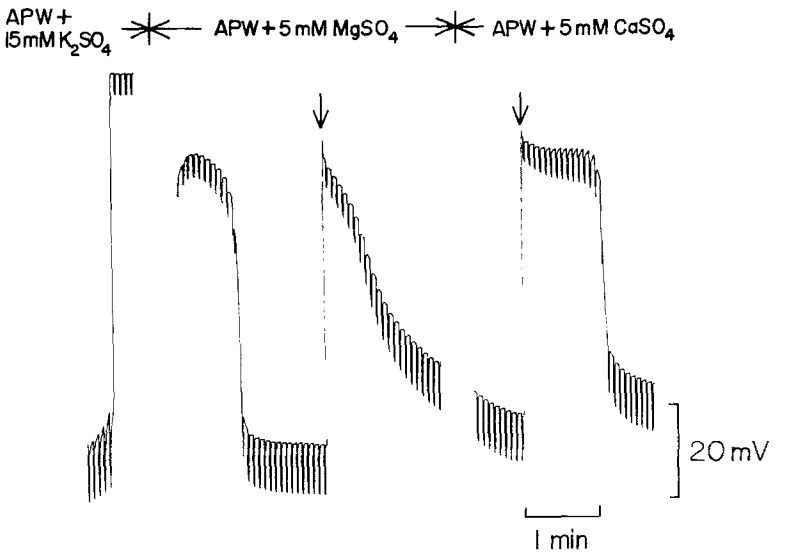

Fig. 14. Chemical stimulation of *Chara*. The bathing medium was first *APW* containing 15 mM K_2SO_4, then *APW* containing 5 mM $MgSO_4$, and finally *APW* containing 5 mM $CaSO_4$. Action potentials were induced in both Mg^{2+} and Ca^{2+}-medium by electric current (downward arrows). Rectangular electric current pulses of 0.02 μA were applied during the measurement. R_s in Eq. (3) was 200 kΩ

and divalent cations are essential factors responsible for determining the state of the membrane.

Discussion

Intactness of the Plasmalemma of Tonoplast-free Cell

The action potential of the tonoplast-free cell showed peculiar phenomena which were not observed in the normal cell: a very long duration, absence of the refractory period, decrease in duration on repetitive firings at short intervals and recovery of shortened duration under a long pause. These abnormal behaviors suggest that the membrane is modified to some extent by changes in cytoplasmic environments due to loss of tonoplast. However, the modification may be a moderate one which can be reversed by restoring normal cytoplasmic conditions. This presumption was supported by the fact that the duration of the action potential of the tonoplast-free cell became as short as that of the normal *Chara* cell, when the concentration of K^+ in the EGTA-sap was raised

to that of the normal cytoplasm (Shimmen, Kikuyama & Tazawa, 1976). Furthermore, the following fact indicates that the plasmalemma remains almost intact even after loss of the tonoplast. First, the hydraulic conductivity of the tonoplast-free cell was practically the same as that of the normal cell (Kiyosawa & Tazawa, 1975). Second, light-induced changes in the membrane potential of the tonoplast-free cell were very similar to those of the normal cell (Kikuyama, Fujii & Tazawa, 1976).

Electric Potential and Resistance before and after Tonoplast Disintegration

When the cell sap was replaced with the EGTA-sap, the electric potential (E_m) between the interior and the exterior of the cell was first low and increased to that of the normal cell when the tonoplast was removed (Fig. 3). Before removal of the tonoplast E_m represents the sum of the plasmalemma potential (E_{co}) and the tonoplast potential (E_{vc}),

$$E_m = E_{vc} + E_{co}. \tag{4}$$

In *Nitella pulchella* decreases in concentrations of K^+ and H^+ in the vacuole made E_m less negative by shifting E_{vc} to the positive direction (Kikuyama & Tazawa, 1976*a*). Replacement of the normal cell sap of *C. australis* with the EGTA-sap decreased the vacuolar concentration of K^+ from 112 mM (Tazawa, Kishimoto & Kikuyama, 1974) to 24 mM and that of H^+ from $10^{-5.5}$ M (Hirakawa & Yoshimura, 1964) to 10^{-7} M. Therefore, the low value of E_m before removal of the tonoplast may be due to low concentrations of K^+ and H^+ in the EGTA-sap.

After removal of the tonoplast E_m should be equal to E_{co}. Since E_{AB} (*cf.* Eq. (1)) of the normal cell (−180 mV) was nearly equal to that of the tonoplast-free cell (−173 mV), E_{co} seems insensitive to the change in K^+-concentration of the cytoplasm which was brought about by the loss of the tonoplast. The concentration of K^+ in the cell after loss of the tonoplast is estimated to be 24 mM, since the contribution of the cytoplasmic K^+ to it is about 7 mM (Tazawa *et al.*, 1976). Since the equilibrium potential for K^+ across the plasmalemma in this case is calculated to be −138 mV which is 27 mV more positive than observed E_m (−165 mV), the observed E_m cannot be explained simply as the diffusion potential of K^+ across the plasmalemma. For further discussions systematic study of the relationship between the plasmalemma potential and the internal concentrations of ions is necessary.

To explain the increase in the membrane resistance observed after replacement of the cell sap with the EGTA-sap, it was postulated in the previous paper (Tazawa *et al.*, 1976) that the plasmalemma of tonoplast-free cells becomes less permeable to H^+ or K^+, or to both ions.

Two Stable Potential Levels

The most significant result to be stressed in the present study is that the duration of the action potential can be controlled by changing concentrations of monovalent cations and divalent cations in the external medium. Addition of monovalent cations to the external medium prolonged the duration of the action potential. A prolonged action potential was also reported in the normal cell of *Chara braunii* treated with 20–30 mM of K^+ for 30 min or more (Oda, 1961). At proper concentrations (a few mM) of monovalent cations, the membrane potential of the tonoplast-free cells stayed at the excited level for several tens of minutes or more. Under this condition the membrane potential could be easily shifted by the electric current pulse between the resting and excited levels and could stay at each potential level for a long time.

The fact that there are two stable membrane potentials under the constant internal and external ionic conditions suggests that the plasmalemma has two stable states; one characterized with less negative membrane potential and high membrane conductance, and the other characterized with more negative membrane potential and low membrane conductance. The former state corresponds to the excited state of the membrane and the other to its resting state.

When the concentration of the monovalent cations was increased further, the membrane potential seemed to settle indefinitely at the depolarized level. Such a membrane showed a hyperpolarizing response by applying an inward current. At removal of the current, the membrane potential returned instantly to the depolarized level, as was previously reported in Characeae cells (Ohkawa & Kishimoto, 1974) and squid giant axons (Tasaki, 1959). The critical concentrations of monovalent cations to arrest the membrane potential at the depolarized level were not significantly different among cation species. They were about 10 mM. The fact that in *Chara* membrane no differences were observed among monovalent cations in the ability to arrest the membrane potential at the depolarized level is in striking contrast to the fact that the characeaen membrane shows different permeabilities to these ions (Nakagawa, Ka-

taoka & Tazawa, 1974). The lack of selectivity may be explained by assuming first that the cations act directly on the negatively charged sites of the membrane which are exposed to the external medium and second that the affinities of the sites of the membrane with the cations do not differ much among the cations.

In the squid giant axon effective concentrations of monovalent cations to depolarize the membrane differ much among cation species (Tasaki *et al.*, 1968). The negatively charged sites of the axon membrane may have a higher selectivity to monovalent cations than those of the *Chara* membrane. An alternative explanation is that the negatively charged sites of the membrane are not directly exposed to the external medium and therefore that accesses of the cations to them are limited by the permeabilities of the membrane to these ions.

Monovalent and divalent cations seem to act on the plasmalemma competitively and determine its state. No detectable differences were observed among divalent cations in their ability to suppress the effect of monovalent cations to prolong the action potential (Figs. 11 and 12). However, there is a significant difference between Ca^{2+} and Mg^{2+} in the shape of the action potential elicited, when their concentrations are high (Fig. 14).

Transitions of the membrane potential between the two levels by chemical stimuli were also reported in the squid giant axon (Tasaki *et al.*, 1968) and the Ranvier node of the toad (Spyropoulos, 1961). In the squid giant axon the critical concentrations of monovalent cations in the bathing medium to induce the abrupt depolarization of the membrane increase with increasing the concentration of $CaCl_2$ in the bathing medium (Tasaki *et al.*, 1968).

Results obtained in the present study on the effect of monovalent and divalent cations in the external medium on the excitation of the plasmalemma of *Chara* cells can well be accounted for on the basis of the two stable states hypothesis proposed by Tasaki on squid giant axons (Tasaki, 1968). According to the hypothesis, the membrane takes the resting state when the fixed negative charges at the external layer of the membrane are occupied by divalent cations and takes the excited state when the divalent cations are exchanged for monovalent cations. Since cations in the external medium act directly on the external layer of the membrane, the monovalent/divalent cation ratio in the external medium can modify the same cation ratio in the membrane. When the action potential was elicited by the electric stimulus in the presence

of a few mM of monovalent cations and 0.1 mM Ca^{2+} in the external medium, the membrane stayed at the excited state for a longer period (Fig. 9). On the basis of the two stable states hypothesis this fact implies that the exchange of monovalent cations occupying the negatively charged sites in the membrane for divalent cations from the external medium takes a longer time. The fact that the duration of excitation was shortened by raising the divalent/monovalent cation ratio (Figs. 11 and 12) is explained by the acceleration of the exchange of monovalent cations for divalent cations.

That the essential process of membrane excitation is a chemical one was clearly demonstrated by the fact that both excitation and repolarization were induced only by changing the external monovalent/divalent cation ratio without applying electric current (Figs. 13 and 14). The action of the outward electric current in inducing the action potential can be explained as the processes of expelling Ca^{2+} from the membrane into the bathing medium and of carriage of K^+ into the membrane from the cell interior (Tasaki, 1968). Then the negatively charged sites in the membrane are occupied by K^+ and the membrane is brought into the excited state. When the outward current is put off, exchange of K^+ for Ca^{2+} from the external medium may proceed and the membrane returns to the original resting state. Inhibition of the exchange by a continuous supply of K^+ into the membrane and/or continuous repelling of Ca^{2+} from the membrane by the outward electric current will result in a prolongation of the action potential (Fig. 8).

The presence of the two stable membrane potential states suggests that the action potential is a reversible transition of the plasmalemma between the two stable states. During the action potential the membrane first shifts from the resting state to the excited state and then from the excited state to the resting state. In the normal cell it is difficult to characterize the excited state of the membrane since the duration of the peak of the action potential is not long enough to allow the exact measurements of the electric and ionic parameters. In the tonoplast-free cell, however, the excited state is extended to several or sometimes to several tens of minutes by raising external K^+ concentration. In the tonoplast-free cell, therefore, it is easy to characterize the excited state of the membrane in comparison with the resting state. There are many reports that both K^+- and Cl^--effluxes increase during the action potential of Characeae (Mullins, 1962; Hope & Findlay, 1964; Mailman & Mullins, 1966; Oda, 1975). Our preliminary experiments using tonoplast-free cells showed that K^+-efflux at the excited state was 84 pmoles per

cm^2 per sec which is about 30 times as high as the resting efflux (3 pmoles per cm^2 per sec).

Constancy of the Peak Potential

In tonoplast-free cells the potential at the peak of the action potential remained constant on repeated excitations with short intervals (Figs. 4–7), while in normal cells it fluctuates to some extent. The action potential observed in normal cells is the sum of the action potential generated at the tonoplast and that at the plasmalemma (*cf.* Eq. (4)). In *N. puchella* the action potential at the tonoplast always accompanies the action potential at the plasmalemma with a short lag and has a longer refractory period than that of the plasmalemma (Kikuyama & Tazawa, 1976*b*). Difference in refractory periods and variations of the lag between the two action potentials should cause fluctuations of the magnitude and duration of the action potential in normal cells. After disintegration of the tonoplast the action potential represents only the action potential of the plasmalemma. Absences of both tonoplast action potential and refractory period of the plasmalemma may be responsible for the constancy of the peak levels of the repeated action potentials in tonoplast-free cells.

Internal Ca^{2+}- and K^+-concentrations

In all experiments shown in this paper, intracellular perfusion was conducted only once. Concentrations of the ions in the cell can be estimated from the contents of the ions in the cytoplasm and in the perfusion medium under the assumption that the ions in the cytoplasm are distributed homogeneously in the cell after the disappearance of the tonoplast (Tazawa *et al.*, 1976). When the present EGTA-sap was used, the internal concentrations of K^+, Cl^- and Ca^{2+} after the loss of the tonoplast were estimated to be 24 mM, 14 mM and 2.9×10^{-8} M, respectively. Generation of the action potential under the extremely low internal Ca^{2+} concentration indicates that the presence of Ca^{2+} inside the plasmalemma is not necessary for excitation of the plasmalemma. This coincides with the result obtained on squid giant axons that the presence of Ca^{2+} in the cell is unfavorable for excitation (Tasaki, Watanabe & Takenaka, 1962).

In squid giant axons the internal conditions necessary for generation of the action potential of longer duration is not lowering of the ionic strength, but lowering of concentration of a special cation species, K^+ (Tasaki, Lerman & Watanabe, 1969). Implications of internal ionic strength and ion species in the duration of action potential of *C. australis* will be dealt with in subsequent papers.

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