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Tonoplast Action Potential of Characeae

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The plasmalemma action potential was found to be indispensable to the production of the tonoplast action potential. In a solution lacking Ca^{2+} and containing other divalent cations such as Ba^{2+}, Mg^{2+} or Mn^{2+}, the plasmalemma excited in *Nitella* but did not in *Chara*. In *Nitella*, however, both the tonoplast action potential and EC-coupling were abolished due to depletion of Ca^{2+} from the external medium. Ca^{2+} ions injected into the cytoplasmic layer caused a transient change in both plasmalemma and tonoplast potentials. These results suggest that a transient rise in Ca^{2+} concentration during excitation of the plasmalemma may trigger the tonoplast action potential.

Key words: Action potential — *Chara corallina* (Characeae) — Divalent cations — *Nitella pulchella* — Tonoplast.

Excitation of Characeae cells has long been a subject of intensive studies in plant electrophysiology (Umrath 1930, Sibaoka 1958). The existence of two membranes, the plasmalemma and the tonoplast, in characean cells makes analysis of excitability more complex than in squid giant axons which have only the plasmalemma. Findlay and Hope (1964) and Findlay (1970) studied the electrical properties of the two membranes separately and found that both could generate action potential on electrical stimulation. The plasmalemma action potential was always accompanied by a slow change of the tonoplast potential or the tonoplast action potential. Kikuyama and Tazawa (1976) demonstrated that the tonoplast action potential of *Nitella pulchella* strongly depends on the Cl^- concentration in the vacuole. Although they tried to stimulate two membranes separately, the action potential of the tonoplast could be elicited only when that of the plasmalemma was generated. These reports suggest that some factor released from the plasmalemma during its excitation may diffuse to the tonoplast and induce the action potential there.

Recently, Williamson and Ashley (1982) and Kikuyama and Tazawa (1983) demonstrated that the excitation of the plasmalemma causes a transient increase in the cytoplasmic Ca^{2+} concentration. Thus Ca^{2+} seems to be the most probable candidate for the factor inducing the tonoplast action potential. This possibility was examined in this study by observing the role of divalent cations on the generation of the tonoplast action potential.

Materials and Methods

Chara corallina Klein ex Wild., em. R. D. W. (=*C. australis* R. Brown) and *Nitella axilliformis* Imahori cultured in an air-conditioned room of 23–25°C were used.

The second or third internode from the apex was visually identified and isolated from

Abbreviations: APW, artificial pond water; EC-coupling, excitation-cessation coupling.

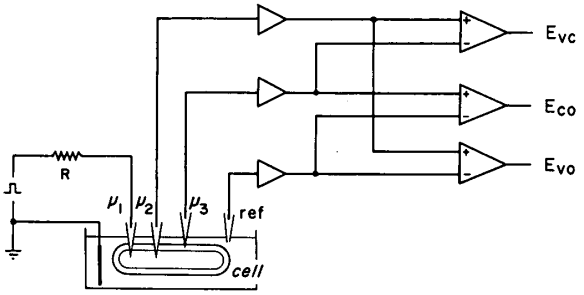

Fig. 1 Schematic diagram of the experimental setup. A cell 5–10 mm in length was placed in a chamber in which APW was perfused at about 1 ml min^{-1}. A reference electrode (ref) was placed in the APW. Three glass microelectrodes (μ_1, μ_2, μ_2) were inserted into the cell, μ_1 and μ_2 in the vacuole and μ_3 in the cytoplasm. One electrode (μ_1) was used to apply an electrical stimulation through 100 MΩ resistor (R) and the others (μ_2, μ_3) were used for leading E_{vc}, E_{co} and E_{vo}.

neighboring cells then kept in APW containing 0.1 mM each of KCl, NaCl and $CaCl_2$ or in the culture medium for at least a day. The length of the cells was 5–10 mm.

Three glass micropipette electrodes filled with 3 M KCl connected to Ag-AgCl wire were inserted into the cell (Fig. 1). The first one (μ_1) inserted into the vacuole was used to apply an electric current to the cell. The pulse generator was made a constant current source by inserting 100 MΩ resistor (R) between μ_1 and the output of the generator. The second one (μ_2) also in the vacuole was used to lead the vacuolar potential (E_{vo}) which was measured against the reference electrode (ref) in the extracellular solution. The reference electrode was constructed with a polyethylene tubing filled with 2% agar-APW connected to the Ag-AgCl wire via 3 M KCl. The third electrode (μ_3) inserted into the cytoplasm was used to measure the potential difference across the plasmalemma (E_{co}). The tonoplast potential (E_{vc}) was obtained by subtracting E_{co} from E_{vo}:

$$E_{vc}=E_{vo}-E_{co}.$$

Since a local circuit current should occur during excitation, records of action potentials obtained by this method would include some distortions. For example, the transient change of E_{vc} in the negative direction in Fig. 3 may be accounted for by the local circuit current.

The extracellular solution used was mainly APW. To study the effect of Ca^+ on excitation, $CaCl_2$ in APW was replaced with other chloride salts of divalent cations. When 0.1 mM $CaCl_2$ originally contained in APW was replaced, for example with 5 mM $BaCl_2$, the solution was expressed as 5Ba-APW. The extracellular solution was continuously perfused throughout the experiment at the rate of 1 ml min^{-1}.

Ions were injected into the cytoplasm as described by Kikuyama and Tazawa (1982). A cell was made inexcitable by immersing it in 10 mM KCl solution. A micropipette electrode filled with 1 M $CaCl_2$ was inserted into the cytoplasmic layer. Ca^{2+} or Cl^- was injected iontophoretically by making the electrode anodal or cathodal. When K^+ or Mg^{2+} was injected, the micropipette electrode filled with 1 M KCl or $MgCl_2$ was used. The current for the injection was maintained for 1 sec.

Experiments were carried out at room temperature 18–25°C.

Results

On electrical stimulation of *C. corallina*, both plasmalemma and tonoplast elicited action potentials in the positive direction (Fig. 2a). The maximum changes of E_{vo}, E_{co} and E_{vc} were 171, 157 and 24 mV, respectively. In *N. axilliformis* (Fig. 2b), however, on excitation E_{vc} shifted in the negative direction which was opposite to that in *Chara* (Fig. 2a, Findlay and Hope 1964), *N. pulchella* (Kikuyama and Tazawa 1976) or *Nitellopsis obtusa* (Findlay 1970). The maximum change in E_{vo}, E_{co} and E_{vc} was 70, 71 and −20 mV, respectively.

In the case shown in Fig. 1, the applied electric current should pass through the plasmalemma

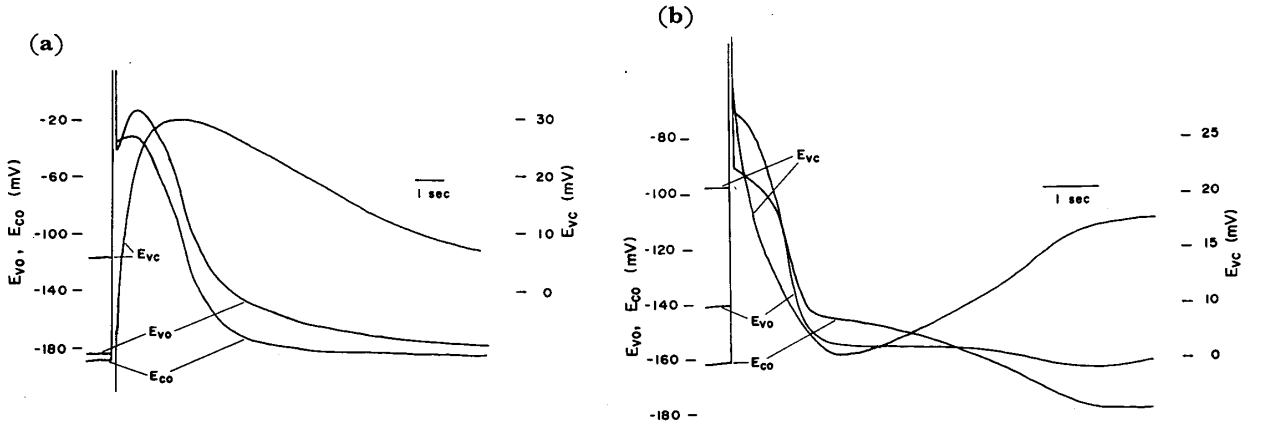

Fig. 2 Action potentials of *C. corallina* (a) and *N. axilliformis* (b) in APW. a. On electric stimulation, E_{vo}, E_{co} and E_{vc} changed to the positive direction. The maximum changes of E_{vo}, E_{co} and E_{vc} were 171, 157 and 24 mV, respectively. b. On stimulation E_{vo} and E_{co} changed to the positive direction but E_{vc} did to the negative one. The maximum changes of E_{vo}, E_{co} and E_{vc} were 70, 71 and −20 mV, respectively.

and the tonoplast. To stimulate the plasmalemma alone, Kikuyama and Tazawa (1976) placed a current-injecting electrode in the cytoplasm. In the present study, an attempt was made to stimulate only the plasmalemma. It was chemically stimulated by changing the bathing solution from APW to 10 mM KCl. When this was done, E_{co} in *Chara* showed a gradual depolarization followed by an action potential and finally reached a steady potential which was significantly more positive than E_{co} in APW (Fig. 3). The tonoplast action potential and the instantaneous cessation of cytoplasmic streaming (EC-coupling) was also observed as was the case with induction by electric stimulation (cf. Shimmen et al. 1984,) though the tonoplast action

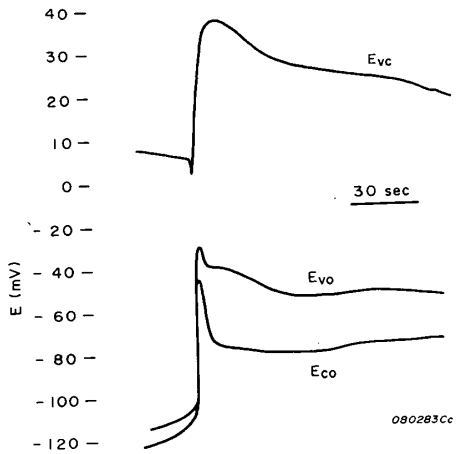

Fig. 3

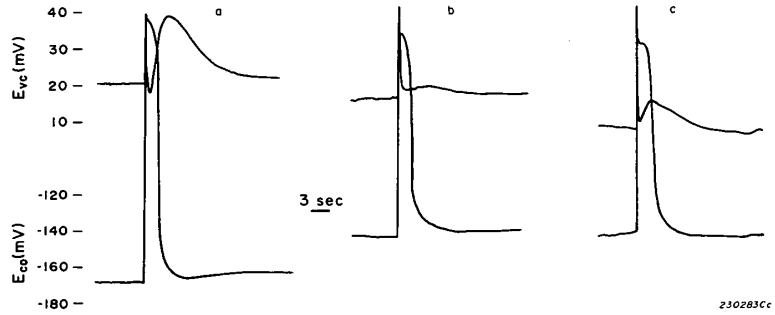

Fig. 4

Fig. 3 Chemically induced action potential of *C. corallina*. On exchanging the bathing medium from APW to 10 mM KCl, the plasmalemma depolarized, generated an action potential and finally reached a more depolarized potential. The plasmalemma action potential was followed by a tonoplast action potential of much longer duration than that elicited by electric stimulation.

Fig. 4 Action potentials as revealed by E_{co} and E_{vc} of *C. corallina*. a. Tonoplast action potential and EC-coupling were clearly observed in 5Sr-APW as in APW. b. When the cell was kept in 5Sr-APW for 16 h, the tonoplast action potential became very small while the EC-coupling was observed. c. Tonoplast action potential was recovered in 5Ca-APW.

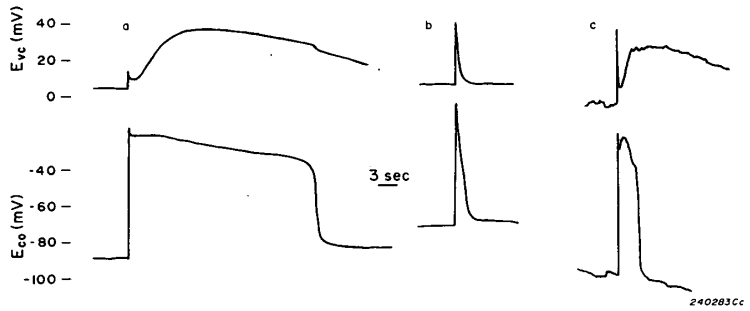

Fig. 5 Action potentials as revealed by E_{co} and E_{vc} of *C. corallina*. a. After replacing APW with 5Ba-APW, the action potentials of plasmalemma (E_{co}) and tonoplast (E_{vc}) changed to prolonged ones. EC-coupling was observed. b. On a second stimulation in 5Ba-APW, no excitation was observed. c. Excitabilities of both plasmalemma and tonoplast were recovered in 5Ca-APW. EC-coupling was also recovered.

potential lasted longer with KCl stimulation than with electrical stimulation. This was also observed in *N. axilliformis*.

As Ca^{2+} is known to play an important role in generating action potential (Barry 1969, Williamson and Ashley 1982), the action potentials of plasmalemma and tonoplast and the EC-coupling were studied in media lacking Ca^{2+} but containing other divalent cations. In 5Sr-APW, *Chara* cells generated action potentials at both the plasmalemma and the tonoplast (Fig. 4a). Cells kept in 5Sr-APW for 16 h generated the tonoplast action potential though it was very small (Fig. 4b). If the bathing medium was changed from 5Sr-APW to 5Ca-APW, the tonoplast action potential was restored (Fig. 4c). In all cases, EC-coupling was clearly observed. In Fig. 5, Ca^{2+} in APW was replaced with Ba^{2+} for *Chara*. After the bathing solution had been changed from APW to 5Ba-APW, long-lasting action potentials were observed at both the plasmalemma and the tonoplast with the first stimulation. The EC-coupling was observed as in APW (Fig. 5a). After this, no excitation was observed in 5Ba-APW at both membranes, and the cytoplasmic streaming showed no significant change on electrical stimulation (Fig. 5b).

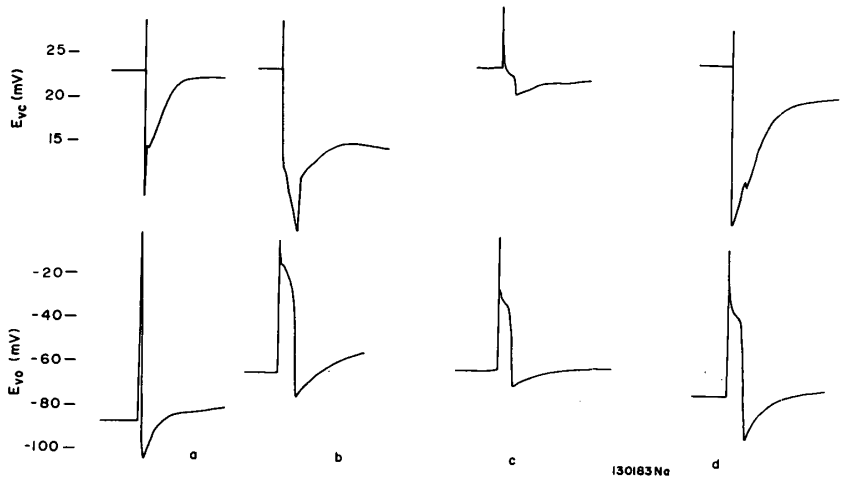

Fig. 6 Action potentials as revealed by E_{vo} and E_{vc} of *N. axilliformis* in 5Ba-APW. a. In APW, both plasmalemma and tonoplast became excited. b. On the first stimulation in 5Ba-APW, both membranes became excited though the durations of action potentials became longer. EC-coupling was not observed. c. On a second stimulation, only the plasmalemma action potential took place. A small change in E_{vc} was assumed to be a reflection of the local circuit current, not the tonoplast action potential. EC-coupling was not observed. d. Tonoplast action potential and EC-coupling were restored in 5Ca-APW.

The excitability and the EC-coupling was also restored completely in 5Ca-APW (Fig. 5c). Excitations of *Chara* in 5Mg-APW and 5Mn-APW were essentially the same as that in 5Ba-APW.

As mentioned above, *Chara* cells lost their excitability with time in solutions lacking Ca^{2+} but containing Ba^{2+}, Mg^{2+} or Mn^{2+}. On the other hand, *Nitella* cells could repeatedly elicit an action potential in 5Ba-APW or 20 Mg-APW, though its shape changed to a rectangular one and the EC-coupling was abolished (Barry 1969). To study the tonoplast action potential in solutions with various divalent cations, *N. axilliformis* was more suitable than *C. corallina* because the former cells maintain their excitabilities in such solutions. As shown in Fig. 2b, the tonoplast action potential in *N. axilliformis* was a change in the negative direction in E_{vc}.

On replacement of APW with 5Ba-APW, the action potential measured as E_{vo}, which is close to E_{co} (Fig. 2b), changed its shape from a normal (Fig. 6a) to rectangular one (Fig. 6b, c). The tonoplast action potential was significant on the first action potential in 5Ba-APW (Fig. 6b). After this, it did not take place during excitation of the plasmalemma though there was a small change in E_{vc} which may reflect a local circuit current. No EC-coupling was observed. Both the tonoplast action potential and EC-coupling were completely recovered in 5Ca-APW (Fig. 6d). As was the case in *Chara*, observations in 20Mg-APW and 5Mn-APW were essentially the same as in 5Ba-APW.

Since the replacement of Ca^{2+} with divalent cations such as Ba^{2+}, Mg^{2+} or Mn^{2+} resulted in depletion of the tonoplast action potential, Ca^{2+} is assumed to play an essential role in the generation of an action potential at the tonoplast. To test this assumption, ion injections were carried out. Since the cell was immersed in 10 mM KCl as shown in Materials and Methods, E_{co} was significantly less negative than in APW. When Ca^{2+} was injected into the *Chara* cytoplasm, both E_{co} and E_{vc} changed transiently in the positive direction (Fig. 7a). Injection of Ca^{2+} into *Nitella* cytoplasm also caused changes in E_{co} and E_{vc} which were positive and negative, respectively (Fig. 8a, b, d). These directional changes in E_{co} and E_{vc} are the same as those of the action potentials (cf. Fig. 2). Injection of Cl^- (Fig. 7b, Fig. 8c), K^+ (Fig. 7c) or Mg^{2+} (Fig. 7d) did not show any effect on potentials of both membranes except the electrotonic effect of the injection current. Since it was very difficult to estimate where the injected ion was distributed as pointed

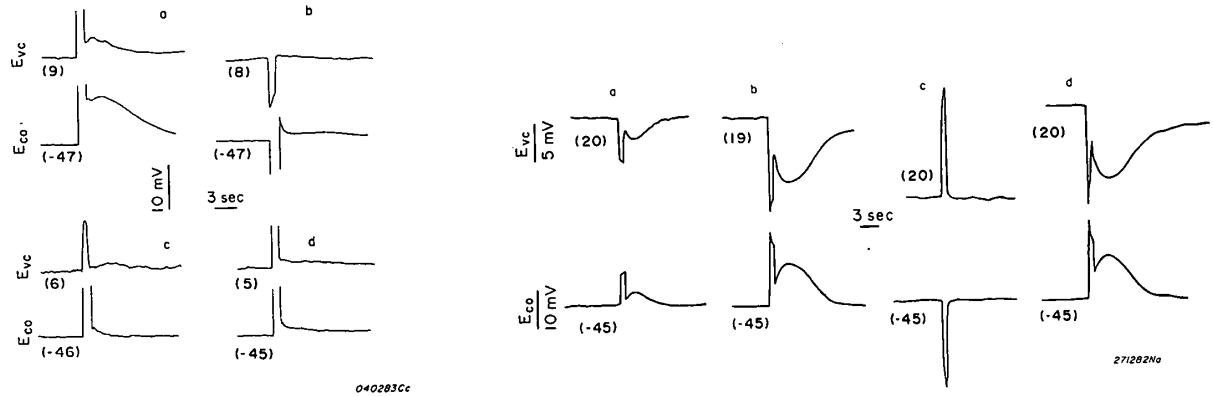

Fig. 7 Effect of ions injected iontophoretically into the cytoplasm of *C. corallina*. a. Ca^{2+} caused changes in the membrane potentials of plasmalemma (E_{co}) and tonoplast (E_{vc}). Directions of changes were the same as those during an excitation. Cl^- (b), K^+ (c) and Mg^{2+} (d) showed no effect on membrane potentials except the electrotonic responses (b,c,d). The amplitude of the electric current used for ion injection was about 0.8, 1.2, 2.5 and 0.9 μA for a, b, c and d, respectively. Figures in parentheses show values of E_{co} or E_{vc} (mV) before ion injection.

Fig. 8 Effect of ions injected into the cytoplasm of *N. axilliformis*. E_{co} and E_{vc} responded to injected Ca^{2+} (a, b, d) and changed directions as under excitation, though they did not respond to Cl^- except by electrotonic responses (c). The amplitude of the electric current used for ion injection was about 0.5, 1, 2 and 1 μA for a, b, c and d, respectively. Figures in parentheses show values of E_{co} or E_{vc} (mV) before ion injection.

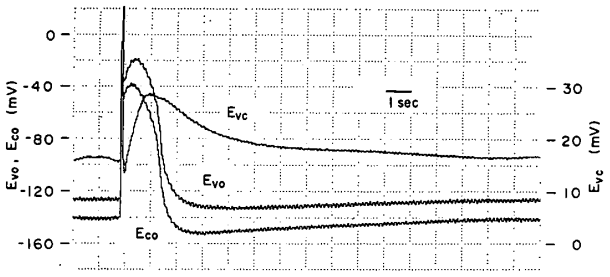

Fig. 9 Action potential of *N. axilliformis*. In contrast to Fig. 1b, not only E_{vo} and E_{co} but also E_{vc} changed to a positive direction. The maximum changes of E_{vo}, E_{co} and E_{vc} were 108, 104 and 13 mV, respectively.

out by Kikuyama and Tazawa (1982), changes in cytoplasmic ion concentration were not calculated.

Further study of the tonoplast action potential of *N. axilliformis* showed that it was in the positive direction (Fig. 9). The maximum changes of E_{vo}, E_{co} and E_{vc} were 108, 104 and 13 mV, respectively.

Discussion

Findlay and Hope (1964) and Findlay (1970) observed that the tonoplast action potential was closely linked to that of the plasmalemma in the sense that the action potential could not be initiated at the tonoplast alone. Findlay (1970) suggested two possibilities to explain the close linkage of action potentials at both membranes. The first one was that both have the same current threshold for an action potential. The second one was that the flux of a particular ion into or out of the cytoplasm, resulting from the start of the plasmalemma action potential, causes a change in the tonoplast threshold sufficient to initiate an action potential. He suggested that the latter possibility is more likely. Kikuyama and Tazawa (1976) inserted a current-injecting microelectrode into the cytoplasm of *Nitella* and tried to separately stimulate the plasmalemma and the tonoplast. The action potential at the tonoplast was always associated with that at the plasmalemma. They also claimed that an electrical coupling exits between the plasmalemma and the tonoplast. The chemical stimulation triggered action potentials not only at the plasmalemma but also at the tonoplast (Fig. 3). This result also agrees with suggestions proposed by the above investigators.

When Characeae cells become excited, the cytoplasmic streaming stops instantly (EC-coupling). Since the streaming is inhibited by an increase in the cytoplasmic Ca^{2+} concentration (Williamson 1975, Kikuyama and Tazawa 1982, Tominaga et al. 1983), EC-coupling may reflect a transient increase in this concentration. Abolishment of EC-coupling in Ca^{2+} free APW, such as 5Ba-APW, supports the above assumption. Williamson and Ashley (1982) reported that EC-coupling was observed only when the concentration of intracellular free Ca^{2+} increased.

Using tonoplast-free *Chara* cells loaded with aequorin, Kikuyama and Tazawa (1983) found two phases of light emission composed of an initial rapid rise and a subsequent slow rise. They also found that the light emission decreases with repeated stimulation of short intervals but recovers its original strength after a longer pause. From these facts they assumed that the rapid initial rise of light emission reflects a Ca^{2+} release from the plasmalemma which may be charged again with Ca^{2+} from the external medium. In *Nitellopsis obtusa* Lunevskey et al. (1983) analyzed electric current flowing across the plasmalemma and tonoplast by clamping the voltage on each membrane separately and obtained results supporting the hypothesis that Ca^{2+} ions entering the cytoplasm from both the external medium and the vacuole activates the Cl^--channel of the membranes. Kataev et al. (1984) demonstrated activation of inward transient current, probably the Cl^- current across the plasmalemma, by Ca^{2+} in tonoplast-free *Nitellopsis* cells. The Cl^- permeability of the tonoplast increases during its excitation (Findlay and Hope 1964, Kikuyama

and Tazawa 1976). Thus the Cl^--channel activation by Ca^{2+} is assumed to be responsible for generating the tonoplast action potential. Activation of Cl^--channels by divalent cations other than Ca^{2+} was studied in *Nitellopsis* by Lunevskey et al (1983). Sr^{2+} is a good substitute for Ca^{2+} but Mg^{2+}, Ba^{2+} and Mn^{2+} are much less effective than Ca^{2+}. The same result was obtained in the present study (Fig. 5, 6). On the other hand, Mimura and Tazawa (1983) found that in tonoplast-free *Nitellopsis* cells an increase in intracellular Ca^{2+} to 5 μM depolarized the plasmalemma potential to almost zero and decreased the resistance to 1/10 the original value. They found no detectable Cl^- efflux during the increase in intracellular Ca^{2+} and concluded that Cl^--channel activation is not always essential for membrane depolarization.

Replacement of Ca^{2+} with other divalent cations such as Ba^{2+}, Mg^{2+} and Mn^{2+} caused a loss of excitability in *Chara* or inhibited the tonoplast action potential and EC-coupling in *Nitella*. The occurrence of action potentials on both membranes by the first stimulation in 5Ba-APW (Fig. 5, 7) may be caused by Ca^{2+} ions which are pre-loaded in the plasmalemma in APW and released on stimulation (Kikuyama and Tazawa 1983). Since the plasmalemma could not obtain Ca^{2+} from 5Ba-APW, the *Chara* cells would become inexcitable. On the other hand, *Nitella* cells could elicit the plasmalemma action potential in 5Ba-APW though the tonoplast action potential and the EC-coupling were abolished. Ba^{2+} ions can thus replace Ca^{2+} for the plasmalemma excitation in *Nitella* but not for inducing the tonoplast action potential and stopping the streaming. Sr^{2+} ion, on the other hand, caused both plasmalemma and tonoplast action potentials and the streaming cessation. This indicates that Sr^{2+} is a good substitute for Ca^{2+} on action potentials and the EC-coupling of *Chara* (cf. Barry 1969, Hayama and Tazawa 1980).

The important role of Ca^{2+} in inducing the tonoplast action potential was demonstrated directly by injecting Ca^{2+} into the cytoplasmic layer (Fig. 7, 8). E_{co} changed to a depolarizing direction which agrees with the results of Mimura and Tazawa (1983) in which the plasmalemma potential of tonoplast-free *Nitellopsis* cell depolarized upon increases of intracellular Ca^{2+}. The direction of the change in E_{vc} agreed with that in the normal action potentials of *C. corallina* and *N. axilliformis* in APW (Fig. 2). The result may indicate that Ca^{2+} activates ion channels at the tonoplast. In *Chara*, the ion channel may be the Cl^--channels as suggested by Lunevskey et al. (1983). On the other hand, the tonoplast action potential in *N. axilliformis* cannot be accounted for by openings of Cl^--channels. If this does occur, E_{vc} should change to a more positive direction since the Cl^- concentration in the cytoplasm is much lower than that in the vacuole in many Characeae cells (Tazawa et al. 1974). If the negative-direction change of E_{vc} during excitation of *N. axilliformis* can be explained by activation of ion channels in the tonoplast, the ion may be Ca^{2+}. The Ca^{2+} concentration in the vacuole was 19 mM for *Chara* (Moriyasu et al. 1984) and that in the cytoplasm was 0.22 μM for *Chara* and 1.1 μM for *Nitella* (Williamson and Ashley 1982). Assuming that these values are acceptable for *N. axilliformis*, the equilibrium potential for Ca^{2+} across the tonoplast was calculated to be about -120 mV (vacuole negative) A recent study on *N. axilliformis* showed that the tonoplast action potential was a positive directional one (Fig. 9) as in other Characeae cells studied so far (Findlay 1970, Findlay and Hope 1964, Kikuyama and Tazawa 1976). In such cells the tonoplast action potential may be accounted for by the opening of Cl^--channels. If the tonoplast potential is controlled by both the Ca^{2+}- and the Cl^--channel and the degrees of activation of both channels change with some factor such as culture condition, the direction of tonoplast action potential may be accounted for. The Cl^--channel of the plasmalemma which is operating in normal cells was completely inactivated by making *Chara* or *Nitellopsis* cells to tonoplast-free (Kikuyama et al. 1984). In *Chara* or *Nitellopsis* the inactivation of Cl^--channel of the plasmalemma is induced artificially but it may be possible that in the tonoplast one of two kinds of ion channels becomes inactive with culture conditions or so.

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