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Studies on the Tonoplast Action Potential of *Nitella flexilis*

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Summary. The origins of the two peaks of the action potential in *Nitella flexilis* were analyzed by inserting two microelectrodes, one into the vacuole and the other into the cytoplasm. It was unequivocally demonstrated that the rapid first peak was generated at the plasmalemma and the slow second peak at the tonoplast. $MnCl_2$ applied in the external medium abolished the second, tonoplast, peak but not the first, plasmalemma, peak. $MnCl_2$ also inhibited the cessation of the cytoplasmic streaming accompanying the action potential. $CaCl_2$ added in $MnCl_2$-containing medium recovered generation of the tonoplast action potential and the streaming cessation. Since it has been established that the cessation of cytoplasmic streaming on membrane excitation is caused by an increase in cytoplasmic free Ca^{2+} (Williamson, R.E., Ashley, C.C., 1982. *Nature (London)* **296**:647–651; Tominaga, Y., Shimmen, T., Tazawa, M., 1983. *Protoplasma* **116**:75–77), it is suggested that the tonoplast action potential is also induced by an increase in cytoplasmic Ca^{2+} resulting from the plasmalemma excitation. When vacuolar Cl^- was replaced with SO_4^{2-} by vacuolar perfusion, the polarity of the second, slow peak was reversed from vacuolar positive to vacuolar negative with respect to the cytoplasm, supporting the previous report that the tonoplast action potential is caused by increase in Cl^- permeability (Kikuyama, M., Tazawa, M., 1976. *J. Membrane Biol.* **29**:95–110).

Key Words action potential · Ca^{2+} · chloride channel · *Nitella* · plasmalemma · tonoplast

Introduction

Action potentials are involved in the transmission of signals, not only in animal nerve cells but also in plant cells (Sibaoka, 1958). Internodal cells of ecorticate members of Characeae are suitable materials for studying plant action potential (Hope & Walker, 1975) because of their large cell size. It has been reported that the action potential is generated not only at the plasmalemma but also at the tonoplast in *Chara australis* (Findlay & Hope, 1964*a*), *Nitellopsis obtusa* (Findlay, 1970), *Nitella pulchella* (Kikuyama & Tazawa, 1976), *Nitella syncarpa* (Denesh & Kurella, 1978) and *Nitella axilliformis* (Kikuyama, 1986). The two components can be measured separately by inserting two microelectrodes, one into the cytoplasm and the other into the vacuole. The involvement of the Cl^- channel in the tonoplast action potential has been suggested, since the membrane potential across the tonoplast approached the equilibrium potential for Cl^- across the tonoplast (Coster, 1966; Findlay, 1970; Findlay & Hope, 1984*a*). When the natural cell sap was replaced with an artificial cell sap with a low Cl^- concentration by vacuolar perfusion, the polarity of the action potential was reversed from vacuolar positive to negative (Kikuyama & Tazawa, 1976). This result unequivocally supported the Cl^- hypothesis for tonoplast action potential.

Beilby and Coster (1979) found two components in the membrane current across the plasmalemma under voltage-clamp measurement in *Chara*, indicating that two ion channels are involved in the action potential at the plasmalemma. It has been suggested that both Cl^- and Ca^{2+} channels are involved in the plasmalemma action potential of Characeae (Beilby & Coster, 1979; Lunevsky et al., 1983; Kikuyama et al., 1984; Shiina & Tazawa 1987*a*,*b*).

The action potential of *Nitella flexilis* is composed of two components, the first rapid peak and the second slow peak (Osterhout, 1934; Oda, 1960). Two possibilities are suggested for generation of two peaks. (i) Two peaks are generated by two different ion channels of the plasmalemma. (ii) One peak is generated at the plasmalemma and the other at the tonoplast. The present study aims to elucidate the origin of these two peaks.

Material and Methods

Nitella flexilis was cultured outdoors. Internodal cells were isolated from neighboring cells and kept in artificial pond water (APW) containing 0.1 mM K_2SO_4, 0.1 mM $CaCl_2$ and 1 mM NaCl. Ca^{2+} concentration of APW for incubation of cells was increased to 1 mM by $CaSO_4$.

Membrane potential was measured by the microelectrode method according to Shimmen, Kikuyama and Tazawa (1976)

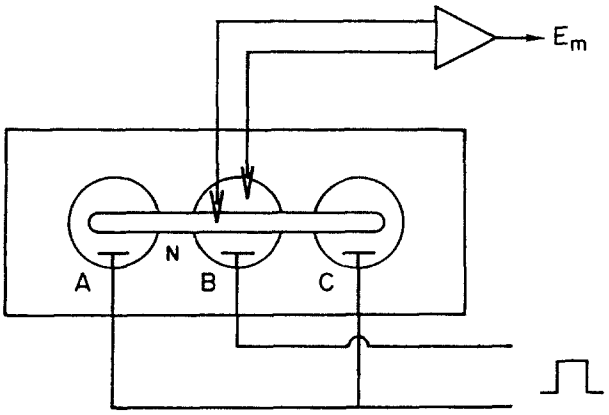

Fig. 1. Microelectrode method. *N:* internodal cell of *N. flexilis*. *A, B, C:* pools. E_m: membrane potential. For further explanation, *see* text

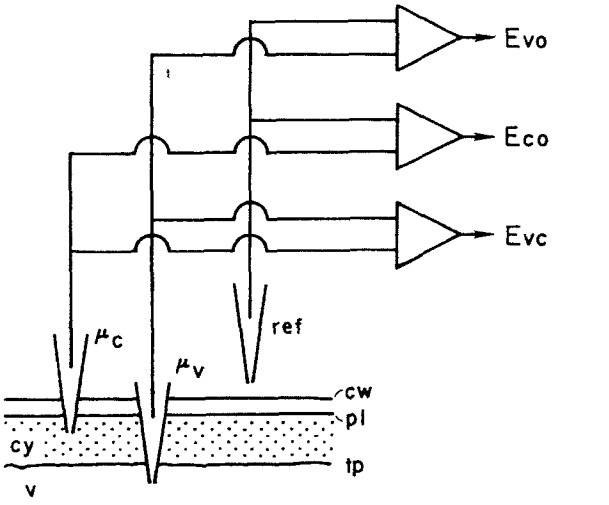

Fig. 2. Separate measurement of E_{co} and E_{vc} by microelectrode method. μ_c: microelectrode inserted into the cytoplasm: μ_v: microelectrode inserted into the vacuole; *ref:* reference electrode; *cy:* cytoplasm: *cw:* cell wall; *pl:* plasmalemma; *tp:* tonoplast; *v:* vacuole

(Fig. 1). An internodal cell was separated into three parts (*A, B* and *C*) with white Vaseline. APW, whose pH had been adjusted at 7.5 with 2 mM HEPES-Tris buffer, was used for the external medium in pools *A, B* and *C*. The microelectrode was inserted into the cell part in *B* and the membrane potential of the cell part in *B* was measured by placing the reference electrode in pool *B*. The cell part in *B* was stimulated by applying electric current pulses between pool *B*, and pools *A* and *C* through Ag-AgCl wire. For stimulation, an electric stimulator (Nihon Kohden, MSE-3R) was used. The membrane potential was recorded by a pen-writing recorder (National VP6527A) after amplification. To measure rapid changes exactly, the membrane potential was recorded, stored in a transient memory (Autonics, Autodigitizer Model S 210), and later replayed on the pen-writing recorder.

To measure separately the membrane potential across the plasmalemma (E_{co}) and that across the tonoplast (E_{vc}), two microelectrodes were inserted, one into the cytoplasm and the other into the vacuole (Fig. 2).

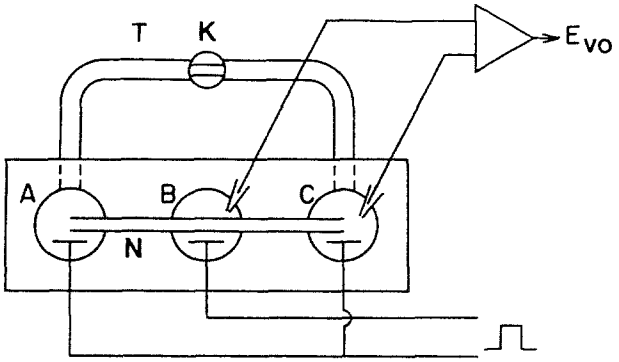

Fig. 3. Open-vacuole method. *N:* internodal cell of *N. flexilis; A, B, C:* pools; *T:* rubber tubing; *K:* cock. For further explanation, *see* text

To induce water influx at the cell part in *B* (Fig. 1) by transcellular osmosis (TCO) (Hayama, Nakagawa & Tazawa, 1979*a*), APW in pools *A* and *C* was replaced with APW supplemented with 200 mM sorbitol.

To measure E_{vo} of a vacuolar-perfused cell, the open-vacuole method (Tazawa, Kikuyama & Nakagawa, 1975) was used. An internodal cell was separated into three parts, *A, B* and *C* (Fig. 3). Pools *A* and *C* and the tubing (*T*) were filled with a vacuolar perfusion medium, and pool *B* was filled with APW which had been made isotonic to the vacuolar perfusion medium by adding sorbitol. The cell was cut open with scissors at both ends in pools *A* and *C*. After closing *T* with a cock (*K*), the vacuole was perfused by applying a difference in water level between pools *A* and *C*. After the natural cell sap had been completely replaced with the perfusion medium, vacuolar perfusion was stopped by opening *K*. E_{vo} of the part of the cell in *B* was measured as the potential difference between pools *B* and *C*. The cell part in *B* was stimulated by applying current pulses between pools *A* and *C*, and pool *B* through Ag-AgCl wire. Experiments were carried out at room temperature (20–25°C).

Abbreviations

APW, artificial pond water; $[Ca^{2+}]_c$, free Ca^{2+} concentration in cytoplasm; $[Cl^-]_c$, Cl^- concentration in cytoplasm; $[Cl^-]_v$, Cl^- concentration in vacuole; E_{co}, potential difference across plasmalemma; $(E_{Cl})_{vc}$, equilibrium potential for Cl^- across tonoplast; E_{vc}, potential difference across tonoplast; E_{vo}, potential difference between external medium and vacuole; IR-drop, Ohmic potential change due to current pulse; $[K^+]_c$, K^+ concentration in cytoplasm; $[K^+]_v$, K^+ concentration in vacuole; TCO, transcellular osmosis.

Results

A typical action potential of *N. flexilis* with two peaks recorded with a single microelectrode inserted into the vacuole (E_{vo}) is shown in Fig. 4*a*. To separate the first rapid component from the Ohmic potential change due to the current pulse for stimu-

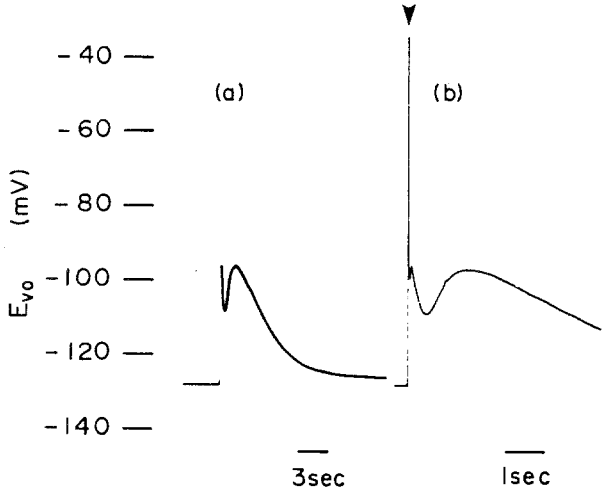

Fig. 4. Action potential of *N. flexilis* recorded by pen-writing recorder (*a*) and that by transient memory (*b*). Arrowhead shows *IR*-drop

Table 1. Values of E_{vo}, E_{co}, and E_{vc} at the resting state and at the peak of the action potential

	E_{co}	E_{vc}	E_{vo}
Resting state	−131.0 ± 6.7	10.9 ± 4.3	−120.0 ± 8.6
First peak	−85.3 ± 4.8	—	−74.4 ± 4.2
Second peak	−126.0 ± 10.5	41.6 ± 3.5	−84.8 ± 14.0

Average values of three cells are shown with SD.

lation (*IR* drop), an action potential was induced by outward current pulse with a short duration of less than 30 msec. When the action potential was directly recorded with the pen-writing recorder, the movement of the pen could not follow the change of the membrane potential during the *IR* drop and the first peak of the action potential. To record the first rapid peak exactly, the action potential was first stored in the transient memory and then replayed slowly on the pen-writing recorder (Fig. 4*b*). The *IR* drop and the first peak of the action potential could be clearly separated.

Separate measurement of E_{co} and E_{vc} were carried out by inserting two microelectrodes, one into the cytoplasm and the other into the vacuole (Fig. 5). Values of E_{vo}, E_{co}, and E_{vc} are summarized in Table 1. Upon stimulation, E_{vo} showed a typical action potential of two peaks (Fig. 5*a*). On the other hand, E_{co} showed only a single rapid peak (Fig. 5*b*). After the initial rapid depolarization, E_{co} repolarized with an exponential time course. The *IR*-drop and the peak of the action potential were clearly separated (Fig. 5*d*). E_{co} at the peak was −85 mV (Table 1). The time required to return to the half

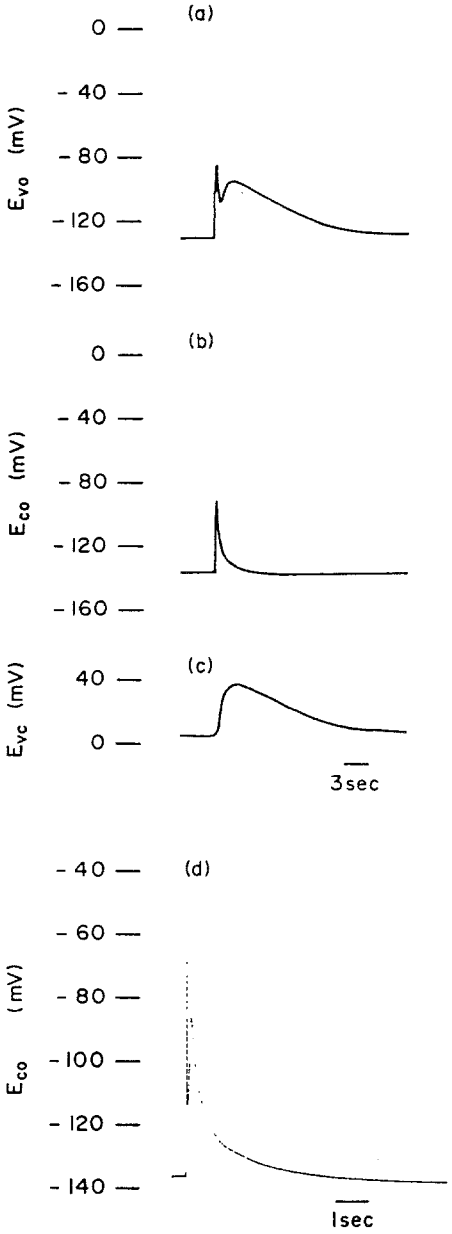

Fig. 5. Separate measurement of E_{vo} (*a*), E_{co} (*b*) and E_{vc} (*c*). E_{co} was measured by transient memory (*d*)

maximum potential during the recovery phase was 330 msec (Fig. 5*d*). E_{vc} showed a single slow peak (Fig. 5*c*). The peak value of E_{vc} was 41 mV (vacuolar positive) and E_{co} repolarized to −126 mV at the peak of E_{vc} (Table 1). The time required to return to the half maximum potential of E_{vc} during the recovery phase was 9 sec (Fig. 5*c*).

The effect of 5 mM $MnCl_2$ on the action potential was studied (Fig. 6). In APW, E_{co} and E_{vc} showed a rapid and a slow transient, respectively (Fig. 6, *APW*). When 5 mM $MnCl_2$ was added to APW, the shape of the E_{vo} action potential signifi-

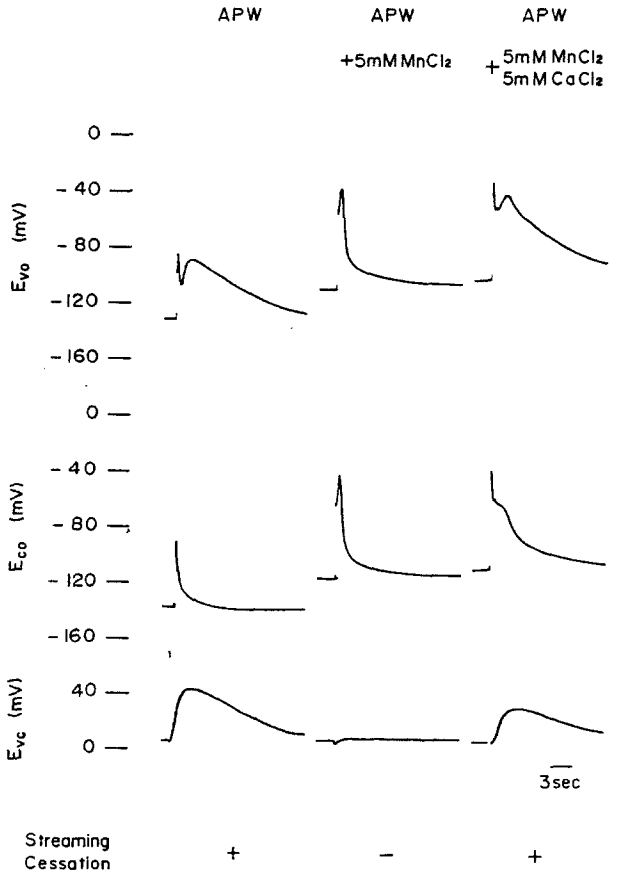

Fig. 6. Effect of 5 mM $MnCl_2$ on action potential and streaming cessation. During the experiment, E_{vo}, E_{co}, and E_{vc} were measured separately. Cessation of cytoplasmic streaming was monitored with microscope objective ×10. +: Streaming stopped upon excitation. −: Streaming did not stop upon excitation

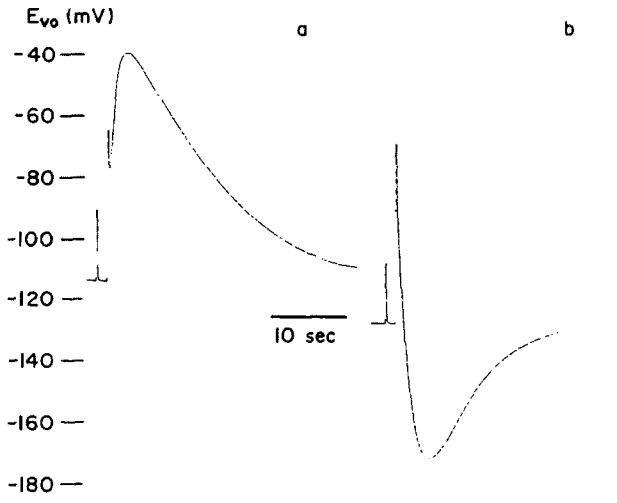

Fig. 7. Effect of removal of Cl^- in the vacuole on polarity of the tonoplast action potential. Membrane potential was measured by the open-vacuole method. Vacuole was perfused with an artificial cell sap containing Cl^- (*a*) or lacking Cl^- (*b*). For further explanation, *see* text

cantly changed (Fig. 6, *APW + 5 mM $MnCl_2$*), showing only a single peak. The shape of the E_{co} action potential was exactly the same as that of E_{vo}, indicating that the action potential at the tonoplast (E_{vc}) was completely inhibited. The amplitude of the plasmalemma action potential increased following $MnCl_2$ treatment. When 5 mM $CaCl_2$ was added to APW containing 5 mM $MnCl_2$, the slow component reappeared in E_{vc} (Fig. 6, *APW + 5 mM $MnCl_2$ + 5mM $CaCl_2$*). E_{co} showed a rapid peak and the following plateau. $MnCl_2$ alone sometimes induced a plateau in the plasmalemma action potential (*data not shown*).

In APW, cytoplasmic streaming stopped upon membrane excitation and recovered slowly (Fig. 6, *APW*). It has been reported that the cytoplasmic streaming stops on membrane excitation due to loss of the motive force (Tazawa & Kishimoto, 1968). In the presence of 5 mM $MnCl_2$, however, cessation of streaming did not occur (Fig. 6, *APW + 5mM $MnCl_2$*). In the presence of both $MnCl_2$ and $CaCl_2$, the streaming cessation accompanying the action potential was recovered (Fig. 6, *APW + 5 mM $MnCl_2$ + 5 mM $CaCl_2$*). Thus, generation of the tonoplast action potential and occurrence of the streaming cessation showed a good correlation.

Kikuyama and Tazawa (1976) reported in *N. pulchella* that the polarity of the tonoplast action potential was reversed by lowering $[Cl^-]_v$, clearly demonstrating that the tonoplast action potential was generated by activation of the Cl^- channel. This was also examined in the present material by the open-vacuole method (Tazawa et al., 1975). Using this method, the membrane potential measured necessarily represents E_{vo}. The natural cell sap was replaced with an artificial cell sap whose Cl^- concentration was similar to that of natural cell sap. It contained 120 mM KCl, 5 mM $CaCl_2$ and 100 mM sorbitol (Tazawa et al., 1974). A typical action potential with two peaks was generated (Fig. 7*a*), indicating that the vacuolar perfusion itself did not change the action potential. When the natural cell sap was replaced with a Cl^--free artificial cell sap containing 60 mM K_2SO_4, 5 mM $CaSO_4$ and 150 mM sorbitol, the shape of the action potential changed significantly. There was first a rapid depolarizing component and a second slow hyperpolarizing component (Fig. 7*b*).

During TCO in *N. flexilis*, membrane depolarization accompanied by an action potential(s) is induced in the cell part where water influx occurs (Hayama et al., 1979*a*). To induce TCO, APW in pools *A* and *C* (Fig. 1) was replaced with APW supplemented with 200 mM sorbitol. Under such condi-

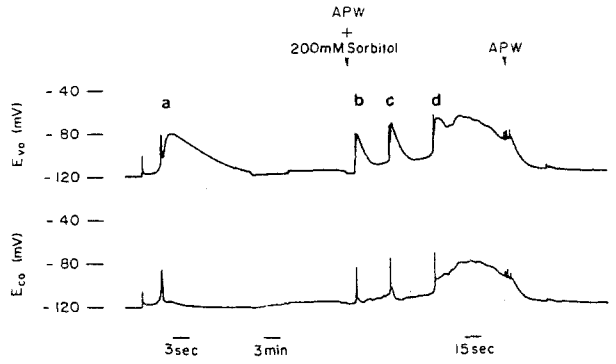

Fig. 8. Membrane depolarization by transcellular osmosis. E_{vo} and E_{co} were measured separately. Before TCO, an action potential was generated electrically (*a*). When TCO was started by applying 200 mM sorbitol in pools *A* and *C* of Fig. 1, a slow depolarization accompanying three action potentials (*b*, *c*, and *d*) was induced

tion, endosmosis occurs in the cell part in *B* and exosmosis in the cell parts in *A* and *C*. During the experiment, E_{vo} and E_{co} of the cell part in *B* were measured separately (Fig. 8). Before the induction of TCO, an action potential was generated by electric stimulation (Fig. 8*a*). When TCO was started by 200 mM sorbitol, a slow depolarization accompanying three action potentials was induced (Fig. 8*b*, *c* and *d*). Slow peaks of the action potentials were not seen in E_{co} as in the case of the action potential generated electrically. During the third action potential, a large slow depolarization related to TCO began. The greater part of this slow depolarization occurred at the plasmalemma. However, the amplitude of depolarization at E_{vo} is larger than that at E_{co}, indicating that the TCO-induced potential change also occurred at the tonoplast. This result is consistent with the previous report by Hayama et al. (1979*a*). By removing sorbitol, both E_{vo} and E_{co} repolarized.

Discussion

The present study unequivocally showed that the first rapid peak was generated at the plasmalemma and the second slow peak at the tonoplast (Fig. 5). $MnCl_2$ at 5 mM completely uncoupled the excitation of the tonoplast from that of the plasmalemma. In the present study, a good correlation was observed between the generation of the tonoplast action potential and occurrence of the cessation of streaming upon plasmalemma excitation (Fig. 6). Kikuyama (1986) also reported in *N. axilliformis* that $MnCl_2$ inhibited both tonoplast action potential and the streaming cessation. In *N. syncarpa*, an action potential of two peaks was also generated (Denesh & Kurella, 1978). In this material, however, both peaks were generated at the plasmalemma. E_{vc} changed simultaneously to the negative direction, resulting in a smaller second peak. In *N. syncarpa*, the plasmalemma second peak, the tonoplast action potential and the streaming cessation were all inhibited by $MnCl_2$ (Denesh & Kurella, 1978). Kikuyama (1986) proposed that the tonoplast action potential is caused by an increase in free Ca^{2+} in the cytoplasm, based on the fact that microinjection of Ca^{2+} into the cytoplasm induced a change in E_{vc}. Recently, Kikuyama (1988) demonstrated Ca^{2+}-induced Cl^- efflux across the tonoplast in plasmalemma-permeabilized cells (Shimmen & Tazawa, 1983*a*). The concentration of free Ca^{2+} in the cytoplasm ($[Ca^{2+}]_c$) increases upon membrane excitation (Williamson & Ashley, 1982; Kikuyama & Tazawa 1983). It has been almost established that the streaming cessation upon membrane excitation is caused by increased cytoplasmic Ca^{2+} (*cf.* Tominaga et al., 1983). A good correlation between the generation of the tonoplast action potential and the occurrence of the streaming cessation (Fig. 6) supports the Ca^{2+} hypothesis proposed for generation of the tonoplast action potential by Kikuyama (1986). Recovery from Mn^{2+} inhibition by Ca^{2+} (Fig. 6) also supports the hypothesis. External Ca^{2+} seems to play an essential role in coupling between plasmalemma excitation, the tonoplast excitation and the cessation of streaming. A significant increase in Ca^{2+} influx on membrane excitation has been reported in *C. australis* (Hayama, Shimmen & Tazawa, 1979*b*). Mn^{2+} might inhibit Ca^{2+} influx across the plasmalemma.

The Cl^- channel hypothesis has been proposed for the tonoplast action potential based on the fact that E_{vc} approached the equilibrium potential for Cl^- across the tonoplast upon excitation (Findlay & Hope, 1964*a*; Coster, 1966; Findlay, 1970). By replacing Cl^- in the vacuole with SO_4^{2-}, the polarity of the slow second peak (E_{vc}) changed from positive to negative (Fig. 7). Before the vacuolar perfusion, $[Cl^-]_v$ and $[Cl^-]_c$ of *N. flexilis* are 179 and 27 mM, respectively (Table 2). The equilibrium potential for Cl^- across the tonoplast ($(E_{Cl})_{vc}$) is calculated to be 47 mV (vacuolar positive). By drastically decreasing vacuolar Cl^-, $(E_{Cl})_{vc}$ changes from positive to negative. The reversal of polarity of the slow peak by removal of vacuolar Cl^- is consistent with the change of $(E_{Cl})_{vc}$, indicating that the tonoplast action potential is caused by activation of the Cl^- channel. Kikuyama and Tazawa (1976) had reported the reversal of polarity of the tonoplast action potential by lowering $[Cl^-]_v$ in *N. pulchella*. E_{vc} at the peak of the action potential in unperfused cell was +41 mV (Table 1), which is similar but not

Table 2. Concentrations of ions in the cytoplasm and the vacuole, and the equilibrium potential across the tonoplast

Ions		Concentrations		Equilibrium potential (mv)
		Cytoplasm	Vacuole (mM)	
K^+		78 mM	73	1
Cl^-		27 mM	179	47
Ca^{2+}	Resting state	1.1 μM	12	−117
	Excited state	43 μM		−70

Data for K^+ and Cl^- were cited from Tazawa, Kishimoto and Kikuyama (1974). The datum for Ca^{2+} in the cytoplasm and in the vacuole was cited from Williamson and Ashley (1982) and Spanswick and Williams (1965), respectively.
The equilibrium potential is shown as a value in the vacuole with respect to the cytoplasm.

identical to $(E_{Cl})_{vc}$ (Table 2). E_{vc} should be dependent on both K^+ and Cl^-, since K^+ channels are abundant in the characean tonoplast (Lühring, 1986).

$$E_{vc} = 58 \log \frac{[K^+]_c + \alpha[Cl^-]_v}{[K^+]_v + \alpha[Cl^-]_c} \quad (1)$$

where α represents ratio of Cl^- permeability to K^+ permeability. Using values of Table 2, α is calculated to be 7.06. Kikuyama (1986) suggested the involvement of Ca^{2+} channel in the tonoplast action potential of *N. axilliformis*. However, it is improbable in *N. flexilis*, since the polarity of the tonoplast action potential in unperfused cell (vacuolar positive) is opposite to that of the equilibrium potential for Ca^{2+} across the tonoplast (vacuolar negative, Table 2).

Thus, the Cl^- channel of the tonoplast seems to be activated by an increase in $[Ca^{2+}]_c$ resulting from the plasmalemma excitation. Ion movements during excitation of characean cells are schematically shown in Fig. 9. When the action potential is generated at the plasmalemma, Ca^{2+} flows into the cytoplasm (Hayama et al., 1979*b*; Shiina & Tazawa, 1987*a*), which results in an increase in $[Ca^{2+}]_c$ (Williamson & Ashley, 1982). The release of Ca^{2+} from organelle(s) may be also responsible for the increase in $[Ca^{2+}]_c$, although it has not been demonstrated yet. The increased $[Ca^{2+}]_c$ (Williamson & Ashlay, 1982) induces cessation of cytoplasmic streaming due to the loss of the motive force (Tazawa & Kishimoto, 1968) generated at the inner surface of the chloroplasts (Kamiya & Kuroda, 1956). In addition, Ca^{2+} causes an opening of the tonoplast Cl^- channel. The Ca^{2+} wave must travel through the endoplasmic layer to open the tonoplast Cl^- channel.

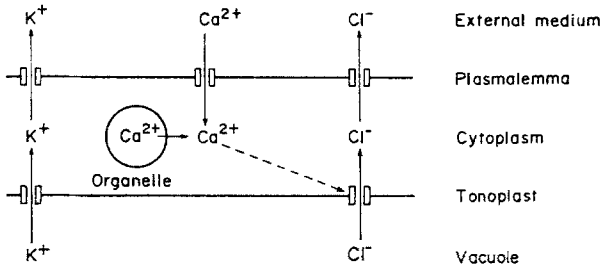

Fig. 9. Flux of ions during excitation of characean cells. Arrows of solid lines show flux of ions, and arrows of broken line show activation of Cl^- channel by Ca^{2+}

Movement of a Ca^{2+} wave across the endoplasm has been suggested from a study on motility (Hayama et al., 1979*b*). Chloroplasts detached from the cortical gel layer sometimes rotate in the flowing endoplasm due to sliding between actin bundles around the chloroplasts and the endoplasm (myosin). The rotation also stops the moment an action potential is generated, presumably due to an increased $[Ca^{2+}]_c$. When the thickness of the endoplasm was increased by centrifuging and ligating internodal cells of *C. australis*, the cessation of chloroplast rotation occurred following a time lag after excitation. From the time lag between the rotation cessation and the electrical stimulation. Hayama et al. (1979*b*) calculated the speed of the Ca^{2+} wave to be 15 μm/sec. The rotation of chloroplasts located about 20 μm from the gel layer stopped about 1.5 sec after the electrical stimulation. Since the thickness of the flowing endoplasm of *N. flexilis* ranges between 4.5–13 μm (Kamiya & Kuroda, 1958), the above value may be a reasonable explanation for a delay of the tonoplast action potential in *N. flexilis*. Efflux of K^+ across the tonoplast during the excitation has not been demonstrated. However, it is reasonable to postulate that there is sufficient K^+ efflux to balance the charge movement due to Cl^- efflux, since the presence of K^+ channel has been demonstrated in the characean tonoplast (Lühring, 1986).

The involvement of the Cl^- channel (Gaffey & Mullins, 1958; Mullins, 1962; Findlay & Hope, 1964*b*; Hope & Findlay, 1964; Kishimoto, 1964; Mailman & Mullins, 1966; Haapanen & Skouglund, 1967; Oda, 1976) and the Ca^{2+} channel (Beilby & Coster, 1979; Shiina & Tazawa, 1987*a*) in plasmalemma excitation has been reported. Lunevsky et al. (1983) assumed that Ca^{2+} flows into the cytoplasm from both the cell exterior and the cell sap and activates the Cl^- channel of the plasmalemma and that of the tonoplast in *Nitellopsis*. Tsutsui et al. (1987*a*,*b*), and Shiina and Tazawa (1987*b*) also postulated a Ca^{2+}-activated Cl^- channel in the plasmalemma. On the other hand, Beilby and Coster

(1979) reported that Cl^- channel activation precedes Ca^{2+} channel activation in *C. corallina*. Efflux of K^+ across the plasmalemma occurs upon excitation (Kikuyama et al., 1984). In *N. axilliformis*, Shimmen and Tazawa (1983*b*) suggested the possible involvement of a Ca^{2+}-activated K^+ channel in the repolarizing process of the plasmalemma.

It is concluded that the slow second peak of the action potential of *N. flexilis* is generated by activation of Cl^- channels of the tonoplast presumably due to an increase in cytoplasmic Ca^{2+}. The possibility remains that the cessation of cytoplasmic streaming causes the activation of the tonoplast Cl^- channel. The abrupt cessation of cytoplasmic streaming may mechanically stimulate the tonoplast. In other characean species, the tonoplast action potential overlap with the plasmalemma action potential. Therefore, they can be separately measured by inserting two microelectrodes or by replacing the cell sap with a medium lacking Cl^- (*cf.* Kikuyama & Tazawa, 1976). In *N. flexilis*, however, two components are temporally separated and can be discriminated as two peaks using a single microelectrode in the vacuole. Therefore, this species provides suitable material for studying the tonoplast action potential.

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