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Ca^{2+}-Activated Cl^- Channel in Plasmalemma of *Nitellopsis obtusa*

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Summary. The mechanism of Cl^--channel activation in the plasmalemma of *Nitellopsis obtusa* was studied by measuring both the transient inward current under voltage clamp and Cl^- efflux during the action potential. 9-anthracenecarboxylic acid (A-9-C) at 1.0 mM inhibited both the transient inward current and the Cl^- efflux, but did not uncouple the sudden cessation of the cytoplasmic streaming. Since this excitation-cessation coupling is caused by a transient increase in the cytoplasmic Ca^{2+} concentration, these results suggest that A-9-C inhibited not the Ca^{2+} channel but specifically the Cl^- channel. The following results were found between the Ca^{2+}-channel activation and the Cl^--channel activation: (1) The Ca^{2+}-channel blocker La^{3+} uncoupled the excitation-cessation coupling and inhibited both the transient inward current and the Cl^- efflux, although the Cl^--channel blocker A-9-C did not affect the excitation-cessation coupling. (2) The Cl^- efflux was greatly reduced by depletion of Ca^{2+} from the external solution and restored by an increase in the external Ca^{2+} concentration. (3) An increase in the external ionic strength which increases Ca^{2+} entry (T. Shiina & M. Tazawa, *J. Membrane Biol.* **96**:263–276, 1987) enhanced the Cl^- efflux. (4) Mg^{2+}, which cannot pass through the Ca^{2+} channel, reduced both the transient inward current and the Cl^- efflux. (5) Although Sr^{2+} can pass through the plasmalemma Ca^{2+} channel, Cl^--channel activation by Sr^{2+} was only partial. These findings support the hypothesis that voltage-dependent Ca^{2+}-channel activation, which increases the free Ca^{2+} concentration in the cytoplasm, is necessary for the subsequent Cl^--channel activation.

Key Words Ca^{2+}-activated Cl^- channel · Ca^{2+} channel · Characeae · Cl^- efflux · membrane excitation · *Nitellopsis obtusa*

Introduction

The ionic relationships of the membrane excitation in Characeae cells have been studied for about 30 years since the discovery of the marked Cl^- efflux from *Chara* cells on membrane excitation by Gaffey and Mullins (1958). The same phenomenon was also observed in other species of Characeae (Mullins, 1962; Hope & Findlay, 1964; Mailman & Mullins, 1966; Findlay, 1970; Oda, 1976; Kikuyama et al., 1984). Suppression of the inward current under the voltage clamp condition by an increase in the external Cl^- concentration ($[Cl^-]_o$) suggests that the inward current is carried mainly by Cl^- efflux (Kishimoto, 1964). However, there have been electrophysiological results suggesting that Ca^{2+} is also involved in the membrane excitation of Characeae (Hope, 1961; Findlay, 1961). In fact, cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_c$) measured by light emission of the photoprotein aequorin (Williamson & Ashely, 1982; Kikuyama & Tazawa, 1983) and Ca^{2+} influx measured with radioactive tracer (Hayama, Shimmen & Tazawa, 1979) increased greatly on membrane excitation.

The contribution of both Cl^- and Ca^{2+} currents to the transient inward current in membrane excitation has been suggested for *Chara* (Beilby & Coster, 1979) and *Nitellopsis* (Lunevsky et al., 1983). Findlay and Hope (1964) hypothesized the role of Ca^{2+} as a mediator for activation of the anion channel in *Chara*. This Ca^{2+} hypothesis has further been advocated by Lunevesky et al. (1983) and Tsutsui et al. (1986, 1987*a,b*). The Ca^{2+}-activated anion channel has been reported in *Xenopus* oocytes (Robinson, 1979; Miledi, 1982; Barish, 1983; Miledi & Parker, 1984), salamander retina rod inner segments (Bader, Bertrand & Achwarts, 1982), mouse spinal cord neurons (Owen, Segal & Barker, 1984), rat lacrimal gland cells (Marty, Tan & Trautmann, 1984; Evans & Marty, 1986), mouse lacrimal gland cells (Findlay & Petersen, 1985), rat sensory neurons in cell culture (Mayer, 1985) and a water mold (Caldwell, Van Brunt & Harold, 1986).

To analyze the causal relationship between the Ca^{2+}-channel activation and the Cl^--channel activation during membrane excitation, we measured both the transient inward current under voltage-clamp condition and the Cl^- efflux. By measuring Cl^- efflux, we could examine the direct effects of various treatments on activation of the plasmalemma Cl^- channel. All the results supported the Ca^{2+}-induced Cl^--channel activation hypothesis.

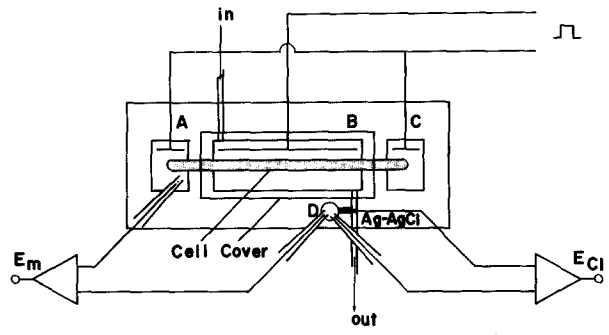

Fig. 1. Experimental setup for measuring Cl^- efflux during membrane excitation. An internode (cell) was placed in a measuring vessel having three chambers, *A*, *B* and *C*. The cell part in *B* was perfused with the bathing medium. Cl^- concentration in the medium was measured with a Ag-AgCl electrode. The generation of an action potential was monitored with a pair of electrodes immersed in *A* and *D*. The stimulating current pulse was applied through the Ag-AgCl wire placed in each chamber

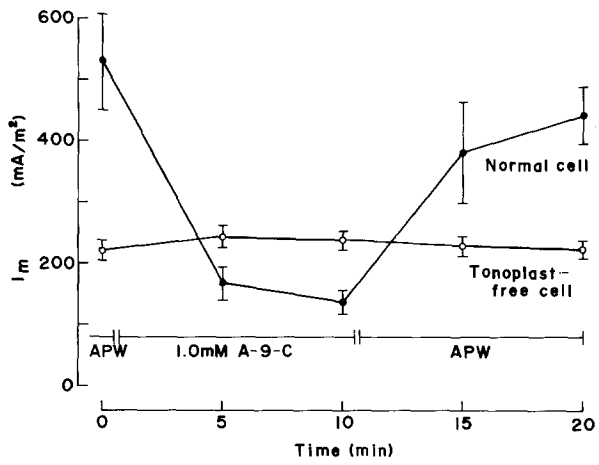

Fig. 2. Effects of A-9-C on the inward peak current under ramp voltage clamping in both normal (–●–) and tonoplast-free (–○–) *Nitellopsis* cells. The compositions of the external solutions are described in the text. All data are shown as mean ± SEM

Materials and Methods

Culture and Preparation

Nitellopsis obtusa was cultured in the laboratory as described in our previous paper (Shiina & Tazawa, 1987). Internodal cells were isolated from neighboring cells and kept in APW[1] containing 0.1 mM each of KCl, NaCl and $CaCl_2$..

Intracellular Perfusion

Intracellular perfusion was performed according to Tazawa, Kikuyama and Shimmen (1976). Tonoplast-free cells were prepared by replacing the cell sap with a medium containing 5 mM EGTA (K-salt), 5 mM PIPES, 6 mM $MgCl_2$, 1 mM ATP, 250 mM sorbitol and 5% Ficoll-70 (pH 7.0). The Ficoll-70 was dialyzed before use. After ligation with polyester thread at both cell ends, perfused cells were kept in APW until the tonoplast disintegrated.

Electrical Measurement

Membrane potential was measured using the conventional microelectrode method. Details of the current and voltage-clamp measurements were described in our previous paper (Shiina & Tazawa, 1987). A cell was placed on a polyacrylate vessel with three chambers. The membrane current and the membrane potential difference between the intracellular microelectrode and the reference electrode were measured using the current-measuring and voltage-measuring circuits, respectively, and recorded with a pen-writing recorder (National VP6521A) and an oscilloscope (Nihon Koden VC-9). In the case of normal cells, the intracellular microelectrode was in the vacuole. Therefore, the values of the membrane potential and the membrane current are summations of those for both the plasmalemma and the tonoplast. In voltage-clamp experiments, the current-voltage (*I-V*) relationship was obtained by slowly depolarizing the membrane potential (V_m) from the resting value in a ramp-shaped manner (rate approx. 400 mV/min) (Ohkawa & Kishimoto, 1977). Time-dependent inactivation of the inward current was also involved in this *I-V* relationship of normal cells, because inactivation of the inward current in normal cells is considerably faster than that of tonoplast-free cells and the amplitude of the inward current changes during the comparatively slow ramp depolarization. The bathing solution used was APW-7.5 which had its pH adjusted to 7.5 with 2.0 mM HEPES-Na buffer.

Cl^- Efflux Measurement

Cl^- efflux during the excitation was measured after Kikuyama et al. (1984). The cell was set on a Plexiglas® vessel having three chambers (Fig. 1). The central chamber (*B*) was perfused with various bathing media. The Cl^- concentration of the outgoing medium was measured with the Ag-AgCl electrode. The flow rate of the bathing medium was about 1 ml/min. The basic bathing medium (APW-NO_3) contained 0.1 mM KNO_3, 0.1 mM $NaNO_3$ and 0.1 mM $Ca(NO_3)_2$, and Ca^{2+}-free APW-NO_3 contained 0.1 mM KNO_3 and 0.1 mM $NaNO_3$.

Results

Effects of a Cl^- Channel Blocker, A-9-C

A-9-C at 0.1 and 0.5 mM reversibly reduced the inward Cl^- current which was activated by hyperpolarizing the membrane of *Chara inflata* (Tyerman,

[1] *Abbreviations:* APW, artificial pond water; A-9-C, 9-anthracenecarboxylic acid; DIDS, 4,4′-diisothiocyano-2,2′-disulfonic acid stilbene; E-C coupling, excitation-cessation coupling; EGTA, ethyleneglycol-bis-(β-aminoethylether)N,N′-tetraacetic acid; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethane-sulfonic acid; PIPES, piperazine-N,N′-bis(2-ethanesulfonic acid).

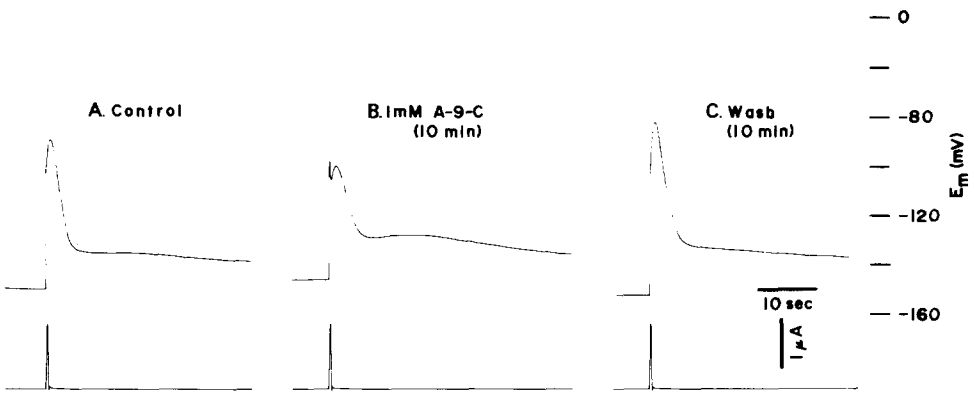

Fig. 3. Effects of A-9-C on the action potential in a normal cell of *Nitellopsis*

Findlay & Paterson, 1986*a*,*b*). To identify ions carrying the inward current during membrane excitation, we tested the effects of A-9-C on the transient inward current induced by ramp-shaped depolarization. A stock solution of A-9-C (10 to 100 mg/ml in 1.0 M NaOH solution) was diluted with APW-7.5. As the pH of APW-7.5 containing 1.0 mM A-9-C shifted to about 8.4, the pH of the control APW-7.5 was also raised to 8.4 by adding 1.0 M NaOH.

In normal cells, 1.0 mM A-9-C drastically reduced the transient inward current during membrane excitation after 5.0 min of treatment (Fig. 2). The action of A-9-C at concentrations below 1.0 mM was slow and small. The effect of A-9-C was reversed after its removal. The inward current almost returned to its initial level after 10 min. However, the inward current in tonoplast-free cells was not affected by 1.0 mM A-9-C. Since the inward current during membrane excitation of tonoplast-free cells is carried by Ca^{2+} (Shiina & Tazawa, 1987), this fact means that A-9-C does not influence the Ca^{2+}-channel activation. The threshold membrane voltage of the transient inward current in normal cells slightly shifted to a positive value with 1.0 mM A-9-C [6.4 ± 2.0 mV (n = 5)]. This positive shift was also almost restored by removal of A-9-C. The amplitude of the inward current in normal cells treated with 1.0 mM A-9-C was somewhat smaller than that in tonoplast-free cells. This may be due to a difference in cytoplasmic ionic concentrations between the normal and the tonoplast-free cells. The transient inward current of normal cells recorded with a step voltage clamp had one component. Neither the shape nor the duration of the transient inward current was affected by A-9-C, although the amplitude decreased (*data not shown*).

The shape of a typical action potential in normal *Nitellopsis* cells was a single peak with the duration of 3 to 4 sec (Fig. 3*A*). A slight depolarization was observed in the recovery phase but it returned to the initial resting level within a few min. A-9-C at 1.0 mM considerably reduced the amplitude of the action potential (Fig. 3*B*). The fast depolarization seen in the initial phase of the action potential was the IR drop caused by the stimulating current. A-9-C also caused a slight membrane depolarization. The duration of the action potential was not affected by A-9-C. The reduced amplitude and the depolarized membrane were almost restored after the removal of A-9-C (Fig. 3*C*).

The cytoplasmic streaming of normal Characeae cells suddenly stops at the time of membrane excitation (E-C coupling) (e.g., Tazawa & Kishimoto, 1968). This is assumed to be caused by a transient increase in the cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_c$) (Hayama et al., 1979). The threshold value of $[Ca^{2+}]_c$ necessary for streaming cessation is about 10^{-6} M or more (Williamson & Ashley, 1982; Kikuyama & Tazawa, 1983; Tominaga, Shimmen & Tazawa, 1983).

In the present study, changes in $[Ca^{2+}]_c$ of normal cells during excitation were monitored by measuring the time elapsed until the cytoplasmic streaming stopped and also the time needed for its recovery. The recovery time was determined by measuring the time required for the restreaming of large particles using a stereomicroscope (×80). The results are shown in Fig. 4. A-9-C had no effect on the stopping time or the recovery time. Thus, we concluded that the increase in $[Ca^{2+}]_c$ caused by the membrane excitation or the Ca^{2+}-channel activation is not influenced by A-9-C.

We measured the Cl^- efflux during membrane excitation in order to see whether or not the Cl^- channel in the plasmalemma of normal cells is actually blocked by A-9-C treatment. A stock solution of A-9-C which was dissolved in 1.0 mM NaOH was diluted with APW-NO_3 (*see* Materials and Meth-

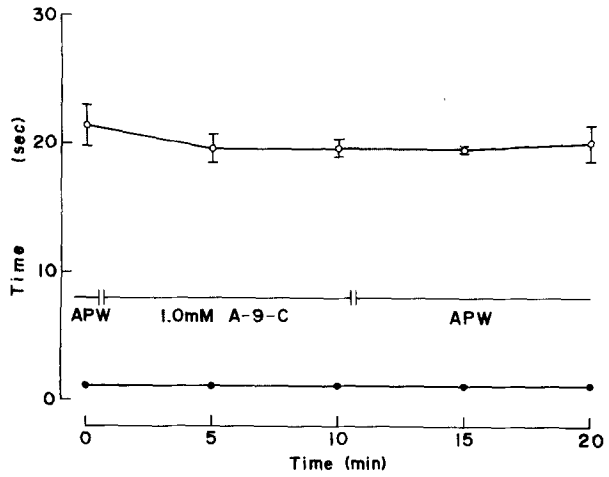

Fig. 4. Effects of A-9-C on time required for cessation of cytoplasmic streaming (–●–) and that for its recovery (–○–) after generation of an action potential in normal cells of *Nitellopsis*. All data are shown as mean ± SEM

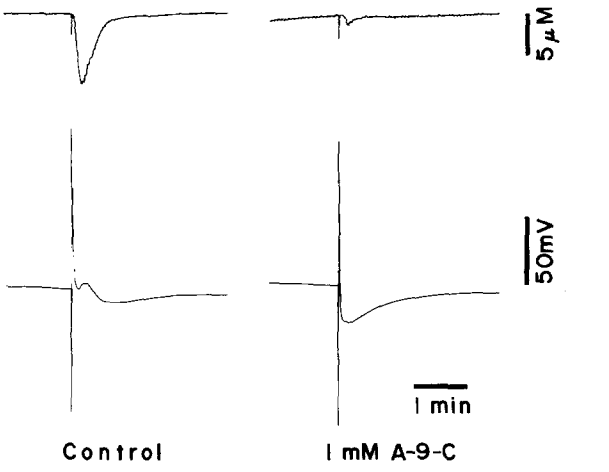

Fig. 5. Effects of A-9-C on transient Cl^- efflux (upper trace) during membrane excitation of a normal cell of *Nitellopsis*. Action potential (lower trace) was recorded externally. The vertical bars represent Cl^- concentration of 5 μM. The external perfusion media used are described in the text

ods). The pH of the solution was titrated with 1.0 M H_2SO_4 to about 8.4. The pH of the control solution which did not contain A-9-C was also adjusted to the same pH with NaOH and H_2SO_4. Figure 5 shows that the transient Cl^- efflux on membrane excitation was greatly suppressed by 1.0 mM A-9-C. Unlike the transient inward current, the inhibition of the Cl^- efflux was reversed only partially in some cells when A-9-C was removed (*data not shown*).

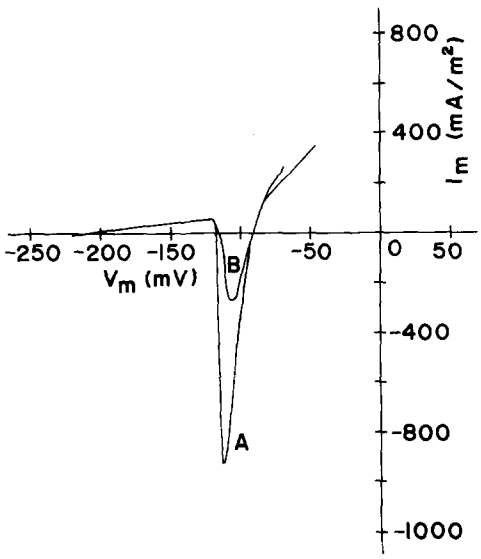

Fig. 6. Effects of external La^{3+} on the *I-V* relation recorded under ramp voltage clamping in a normal cell of *Nitellopsis*. (*A*) APW-7.5. (*B*) 0.5 mM $LaCl_3$, 10 min treatment. $LaCl_3$ was added to APW-7.5

Inhibition of the Inward Current and Cl^- Efflux by La^{3+}

Normal cells were treated with APW-7.5 containing 0.5 mM $LaCl_3$, a Ca^{2+}-channel blocker. The transient inward current was considerably reduced after 10 min (Fig. 6). Even when the $LaCl_3$ concentration was increased up to 2.0 mM, complete inhibition was not achieved. The inhibition was irreversible.

The Cl^- efflux induced by membrane excitation of normal cells was first (5 min after) enhanced by addition of 0.5 mM $La(NO_3)_3$ to APW-NO_3 (Fig. 7). This may be caused by an increase in the Ca^{2+} influx enhanced by increased ionic strength (Shiina & Tazawa, 1987), which will be discussed later. Further treatment of the cells with La^{3+} considerably reduced the Cl^- efflux. However, complete inhibition of the transient Cl^- efflux was not observed even after 30 min.

The E-C coupling was not observed in normal cells treated with 0.5 mM La^{3+} for more than 10 min, even though a small Cl^- efflux remained. This suggests that the Ca^{2+} influx in the presence of La^{3+} was too small to inhibit the cytoplasmic streaming.

Effect of External Ca^{2+} Concentration on Cl^- Efflux

To decrease the external Ca^{2+} concentration ($[Ca^{2+}]_o$), we added 1.0 mM EGTA to Ca^{2+}-free

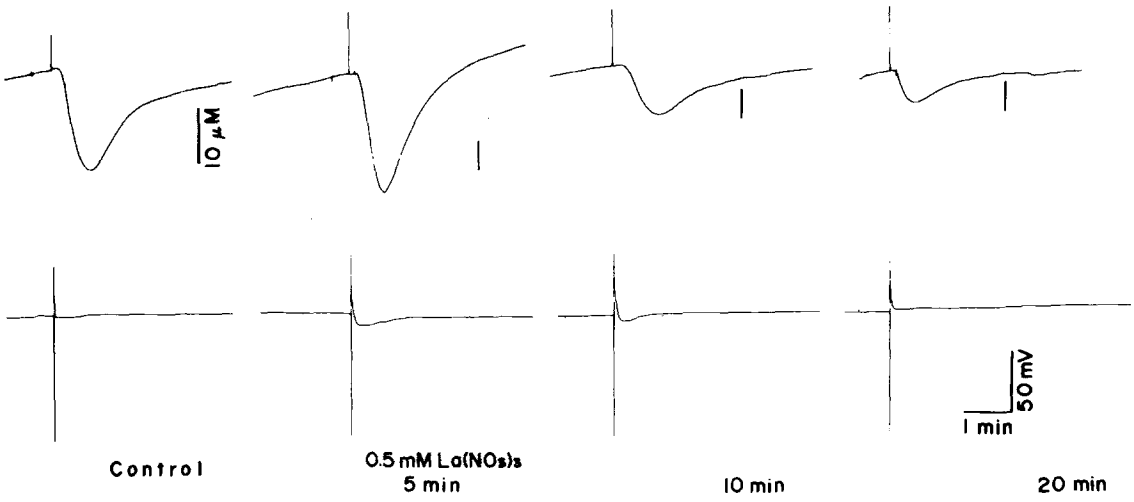

Fig. 7. Effects of external La^{3+} on transient Cl^- efflux (upper trace) during membrane excitation of a normal cell of *Nitellopsis*. Action potential (lower trace) was recorded externally. The vertical bars represent Cl^- concentration of 10 μM. The external perfusion media used are described in the text

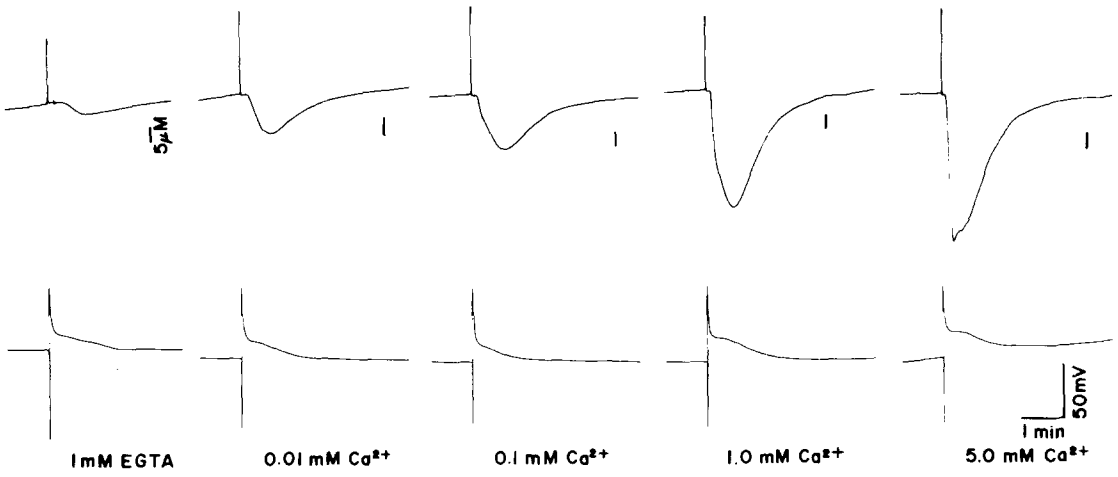

Fig. 8. Effects of $[Ca^{2+}]_o$ on transient Cl^- efflux (upper trace) during membrane excitation of a normal cell of *Nitellopsis*. Action potential (lower trace) was recorded externally. The vertical bars represent Cl^- concentration of 5 μM. The external perfusion media used are described in the text

APW-NO_3. The Cl^- efflux in normal cells was considerably reduced 5 min after EGTA treatment (*data not shown*). Even after 30 min of EGTA treatment, a small Cl^- efflux was observed. The cytoplasmic streaming did not stop on membrane excitation in the solution containing 1.0 mM EGTA.

The transient Cl^- efflux induced by membrane excitation of normal cells greatly increased when the $[Ca^{2+}]_o$ was increased to various levels with addition of $Ca(NO_3)_2$ to Ca^{2+}-free APW-NO_3, keeping the external ionic strength constant by addition of $NaNO_3$ (Fig. 8). The E-C coupling was also restored by an increase in $[Ca^{2+}]_o$, although incomplete cessation of cytoplasmic streaming was sometimes observed in the solution containing 0.01 mM Ca^{2+}.

Effects of Enhanced Ca^{2+} Entry

One method for enhancing Ca^{2+} entry into tonoplast-free cells is to raise the ionic strength of the bathing solution. Transient inward current in nor-

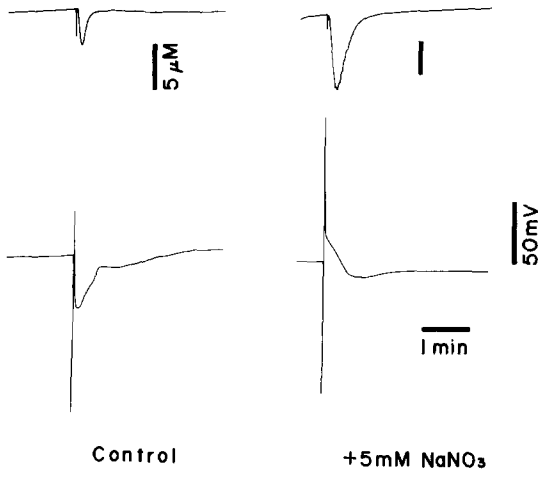

Fig. 9. Effects of external ionic strength on transient Cl^- efflux (upper trace) during membrane excitation of a normal cell of *Nitellopsis*. Action potential (lower trace) was recorded externally. The vertical bars represent Cl^- concentration of 5 μM. The external perfusion media are described in the text

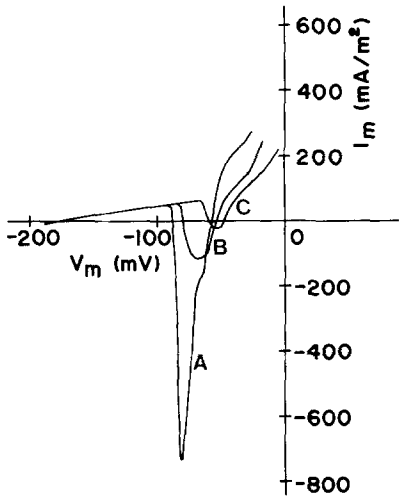

Fig. 11. Effects of external Sr^{2+} on transient inward current recorded under ramp voltage clamping in a normal cell of *Nitellopsis*. (*A*) APW-7.5 containing 1.0 mM Ca^{2+}. (*B*) Ca^{2+}-free APW-7.5 containing 1.0 mM Sr^{2+}, 10 min after the replacement of the external solution. (*C*) 20 min after the replacement

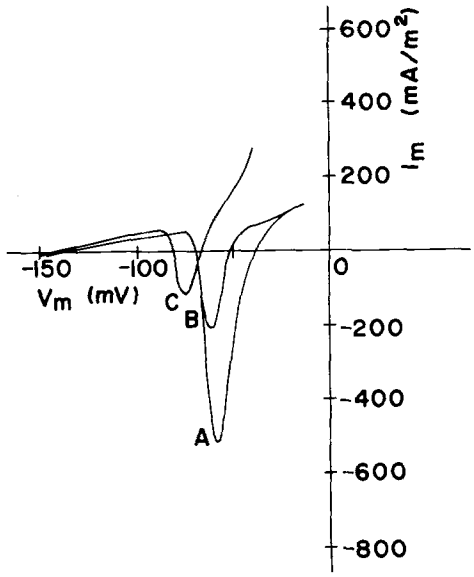

Fig. 10. Effects of external Mg^{2+} on transient inward current recorded under ramp voltage clamping in a normal cell of *Nitellopsis*. (*A*) APW-7.5 containing 1.0 mM Ca^{2+}. (*B*) Ca^{2+}-free APW-7.5 (0.1 mM KNO_3, 0.1 mM $NaNO_3$, 2 mM HEPES-Na) containing 1.0 mM Mg^{2+}, 10 min after the replacement of the external solution. (*C*) 20 min after the replacement

mal cells can be enhanced by addition of either 6.0 mM NaCl or $NaNO_3$ to APW-7.5 (Shiina & Tazawa, 1987, Fig. 14). The transient Cl^- efflux during membrane excitation of normal cells was also greatly enhanced by addition of 5.0 mM $NaNO_3$ to APW-NO_3 (Fig. 9).

Effects of External Mg^{2+} or Sr^{2+} on Transient Inward Current and Cl^- Efflux

The inward current in tonoplast-free cells measured under voltage-clamp conditions was observed in the presence of Ca^{2+} or Sr^{2+} but not Mg^{2+} (Shiina & Tazawa, 1987). As shown in Fig. 10, the transient inward current in normal cells was reduced by replacement of external Ca^{2+} with Mg^{2+}. The threshold membrane voltage, which produces the transient inward current, shifted to a more negative value with longer Mg^{2+} treatment (Fig. 10*C*). Inhibition of the transient inward current in normal cells was also observed by replacement of external Ca^{2+} with Sr^{2+} (Fig. 11). However, Sr^{2+} caused a positive shift of the threshold voltage of the inward current, in contrast to the effect of Mg^{2+}.

In normal cells, the reduced Cl^- efflux caused by EGTA could not be recovered by removing EGTA and by adding 1.0 mM Mg^{2+} (Fig. 12). The Cl^- efflux increased significantly upon replacement of Mg^{2+} with Sr^{2+}, and markedly upon replacement of Sr^{2+} with Ca^{2+}. E-C coupling was never observed in a solution containing EGTA or Mg^{2+}, and sometimes in a solution containing Sr^{2+}, but always in a solution containing Ca^{2+}.

Discussion

Inhibition of the Cl^- Channel by A-9-C

Both transient inward current and Cl^- efflux were observed during membrane excitation in normal

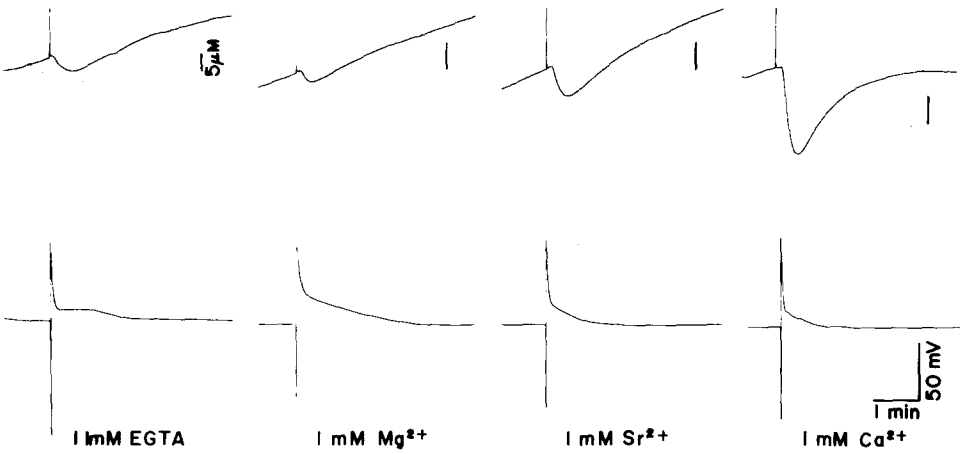

Fig. 12. Effects of external Mg^{2+}, Sr^{2+} and Ca^{2+} on transient Cl^- efflux (upper trace) during membrane excitation of a normal cell of *Nitellopsis*. Action potential (lower trace) was recorded externally. The vertical bars represent Cl^- concentration of 5 μM. The external perfusion media used are described in the text

cells of *Nitellopsis*. They were considerably reduced by external application of A-9-C, known as a Cl^--channel blocker in animal cells (Bryant & Molares-Aguilera, 1971). Another Cl^--channel blocker, DIDS, also inhibited the inward current only partially (Shiina & Tazawa, 1987). The amplitude of the action potential in normal cells of *Chara* was also reduced by A-9-C as reported by Tyerman et al. (1986*a*,*b*). In tonoplast-free cells of *Nitellopsis*, however, neither blocker affected the amplitude of the inward current, which is carried only by Ca^{2+} (Shiina & Tazawa, 1987). Furthermore, the E-C coupling, which is caused by a transient increase in $[Ca^{2+}]_c$, was not inhibited by A-9-C (Fig. 4); evidence that A-9-C acts as a specific Cl^--channel blocker in *Nitellopsis*.

The remaining inward current observed in A-9-C-treated normal cells is mostly composed of Ca^{2+} current, because the Cl^- component would be practically abolished by A-9-C treatment (Fig. 5). The inward current in treated and untreated normal cells relaxed with time, although the Ca^{2+} current in tonoplast-free cells showed no time-dependent relaxation (Shiina & Tazawa, 1987). Some components which control the time-dependent inactivation process of the Ca^{2+} channel may be lost in tonoplast-free cells.

Ca^{2+} Hypothesis

External Ca^{2+} is assumed to be essential for a transient increase in anion permeability on membrane excitation in *Chara* (Findlay & Hope, 1964). Activation of the Cl^- channels in both the plasmalemma and the tonoplast caused by increased $[Ca^{2+}]_c$ was suggested by Lunevsky et al. (1983) in *Nitellopsis*. Recently, Kikuyama (1986) injected Ca^{2+} into *Chara* cytoplasm and observed a tonoplast potential change similar to the tonoplast action potential which is assumed to be the Cl^- spike. A Ca^{2+}-dependent anion channel is activated during membrane excitation in a water mold, *Blastocladiella* (Caldwell et al., 1986). From these observations, it has been hypothesized that activation of the Cl^- channel in the plant plasmalemma may be caused by an increase in $[Ca^{2+}]_c$ (Ca^{2+} hypothesis). External Ca^{2+} also plays a key role in turgor regulation of internodal cells of *Lamprothamnium*. Hypotonic treatment of cells causes Ca^{2+}-channel activation and increases $[Ca^{2+}]_c$ which leads to passive leakage of K^+ and Cl^- (Okazaki & Tazawa, 1986*a*,*b*).

La^{3+}, which is known as a Ca^{2+}-channel blocker, inhibited both the transient inward current and the Cl^- efflux on membrane excitation of normal *Nitellopsis* cells (Figs. 6 and 7), although A-9-C, a specific Cl^--channel blocker, did not affect the Ca^{2+} entry during membrane excitation (Fig. 4). Lunevsky et al. (1983) also reported that La^{3+} from 0.5 mM to a saturated concentration inhibited both Ca^{2+} and Cl^- currents by three- to 10-fold in *Nitellopsis*. The transient inward current of *Chara* was abolished by external application of 0.02 mM La^{3+} (Tsutsui et al., 1986).

A drastic decrease in $[Ca^{2+}]_o$ by EGTA strongly inhibited the transient Cl^- efflux in normal cells. The decreased Cl^- efflux was restored by an increase in $[Ca^{2+}]_o$ (Fig. 8). However, we observed no explicit dependence of the transient inward current in normal cells on $[Ca^{2+}]_o$. Contrary to expectation, a slight decrease in the transient inward current was sometimes observed when $[Ca^{2+}]_o$ was increased (*data not shown*). The possible involvement of a voltage- or Ca^{2+}-activated K^+ channel (Shimmen &

Tazawa, 1983) may complicate the dependence of the inward current on $[Ca^{2+}]_o$. The dependence of the transient inward current on $[Ca^{2+}]_o$ has been observed in various Characeae cells (Findlay, 1961; Findlay & Hope, 1964; Beilby & Coster, 1979; Lunevsky et al., 1983; Beilby, 1984), although the effects of external ionic strength on both Ca^{2+} and Cl^- permeabilities were not sufficiently considered. Recently, steps of inward current, probably the Cl^- current, were measured with a patch-clamp technique (cell-attached type) from the plasmalemma of *Chara* (Coleman, 1986). However, no increase in the channel-opening probability induced by depolarization was observed when the solution in the patch pipette contained no Ca^{2+}.

Mg^{2+}, which cannot substitute for Ca^{2+} in membrane excitation of tonoplast-free *Nitellopsis* cells (Shiina & Tazawa, 1987), inhibited both the transient inward current and the Cl^- efflux in normal cells (Figs. 10 and 12). The reduction of the inward current in normal cells by Mg^{2+} was also observed in *Chara* (Findlay & Hope, 1964) and *Nitellopsis* (Findlay, 1970; Lunevsky et al., 1983). The efflux rate of ions from *Nitella* cells on membrane excitation was lower in a medium containing 0.1 mM Mg^{2+} than that in a medium containing 0.1 mM Ca^{2+} (Kikuyama, 1987).

If activation of the Ca^{2+} channel controls the activity of the Cl^- channel, enhancement of Ca^{2+}-channel activity should increase both the transient inward current and the Cl^- efflux. An increase in the external ionic strength also activates the Ca^{2+} channel in the plasmalemma of tonoplast-free *Nitellopsis* and *Chara* cells (Shiina & Tazawa, 1987) for unknown reasons. Both the transient inward current (Shiina & Tazawa, 1987) and the Cl^- efflux (Fig. 9) in normal cells were enhanced by this increase.

These results strongly support the Ca^{2+} hypothesis that a transient increase in $[Ca^{2+}]_c$ caused by activation of the Ca^{2+} channel opens the plasmalemma Cl^- channel. The transient increase in $[Ca^{2+}]_c$ on membrane excitation was demonstrated in both normal (Williamson & Ashley, 1982) and tonoplast-free cells (Kikuyama & Tazawa, 1983). Recently, a transient depolarization of the plasmalemma membrane potential was observed by iontophoretical injection of Ca^{2+} into the cytoplasm of *Chara* and *Nitella* (Kikuyama, 1986).

Sr^{2+} can pass through the Ca^{2+} channel in the plasmalemma of tonoplast-free *Nitellopsis* cells to the same extent as Ca^{2+} (Shiina & Tazawa, 1987). However, both the transient inward current (Fig. 11) and the Cl^- efflux (Fig. 12) in normal cells were greatly reduced by replacement of external Ca^{2+} with Sr^{2+}. This shows that Sr^{2+} activates the Cl^- channel to a lesser extent than Ca^{2+}. Although Sr^{2+} can pass through the Ca^{2+} channel of a water mold, *Blastocladiella*, it can not activate the delayed anion channel (Caldwell et al., 1986). In *Nitella* cells, excitability remains even when external Ca^{2+} is substituted with Mg^{2+}, Ba^{2+} or Sr^{2+}, although the E-C coupling can not be observed in a solution containing Mg^{2+} or Ba^{2+} (Barry, 1968).

Various treatments, such as with La^{3+}, EGTA, Mg^{2+} and Sr^{2+}, inhibited partially both the transient inward current and Cl^- efflux in normal cells. But E-C coupling was completely inhibited by these treatments. Stoppage of the cytoplasmic streaming is thought to be caused by an increase in $[Ca^{2+}]_c$, and the critical $[Ca^{2+}]_c$ necessary for streaming cessation is estimated to be about 10^{-6} M (Tominaga et al., 1983). Then the remaining Cl^- efflux may be accounted for in terms of voltage-dependent Cl^--channel activation which requires no Ca^{2+} influx. However, if there is no contribution from the voltage-dependent Cl^- channel, it is reasonable to assume that the Cl^- channel is much more sensitive to Ca^{2+} than the motile system of the cytoplasmic streaming. This channel may be activated by a very small increase in $[Ca^{2+}]_c$ to a value lower than 10^{-6} M. Williamson and Ashley (1982) found that in *Chara*, generation of the action potential, which had been inhibited by replacement of Ca^{2+} with Mg^{2+}, was recovered by reapplying Ca^{2+} before the excitation-induced aequorin light emission reappeared.

There are two possible explanations for this high sensitivity of the Cl^--channel activation to Ca^{2+}. First, the Cl^- channel is very sensitive to Ca^{2+} and can be activated by a slight increase in $[Ca^{2+}]_c$. A slight increase in $[Ca^{2+}]_c$ may be caused by release of Ca^{2+} from the intracellular stores, as suggested by Lunevsky et al. (1983) and Beilby (1984). The second possibility is a geometrical gradient of $[Ca^{2+}]_c$. Blockage of the Ca^{2+} channel with Mg^{2+} or La^{3+} will impede the Ca^{2+} influx. This would create a slow concentration gradient of free Ca^{2+} across the cytoplasmic layer. $[Ca^{2+}]_c$ inside the plasmalemma may become high enough to active the Cl^- channel, while $[Ca^{2+}]_c$ at the inner sol-gel interface, where the motile filaments are, may not increase sufficiently to stop the cytoplasmic streaming.

The Cl^- channel is not activated in tonoplast-free cells (Kikuyama et al., 1984; Shiina & Tazawa, 1987). An increase in $[Ca^{2+}]_c$ of up to 5×10^{-6} M in tonoplast-free cells should be enough to activate the plasmalemma Cl^- channel (Kikuyama & Tazawa, 1983). Increase in intracellular $[Ca^{2+}]$ in tonoplast-free cells caused depolarization of the membrane potential and decrease in membrane resistance

without significant Cl^- efflux in *Nitellopsis* (Mimura & Tazawa, 1983). However, we recently found that the Cl^- efflux was greatly stimulated by raising intracellular $[Ca^{2+}]$ in tonoplast-free *Nitellopsis* cells.

The present results strongly support the idea that the Cl^- channel of the *Nitellopsis* plasmalemma is opened by a small increase in $[Ca^{2+}]_c$ which is caused by a preceding voltage-dependent Ca^{2+}-channel activation of the plasmalemma. To establish the Ca^{2+} hypothesis, the Cl^- efflux must be measured simultaneously with the change in free $[Ca^{2+}]_c$ in the same cell.

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