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Ca^{2+}-Dependent Cl^- Efflux in Tonoplast-Free Cells of *Nitellopsis Obtusa*

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Summary. In order to demonstrate the presence of a Ca^{2+}-activated Cl^--channel in the *Nitellopsis* plasmalemma, tonoplast-free cells were prepared and their intracellular Ca^{2+} concentration was modified by internal perfusion. An increase in the Ca^{2+} concentration caused a large Cl^- efflux with a concomitant depolarization of the membrane potential. These changes were for the most part reversible. The critical Ca^{2+} concentration was about 4.0 μM. Neither the Cl^- efflux nor the membrane depolarization showed a time-dependent inactivation. A Cl^--channel blocker, A-9-C (9-anthracenecarboxylic acid) reduced both the Cl^- efflux and the magnitude of the membrane potential depolarization. A small increase in the intracellular Ca^{2+} concentration, which is caused by membrane excitation of tonoplast-free cells is not sufficient to activate this Ca^{2+}-dependent Cl^--channel.

Key Words Ca^{2+}-dependent Cl^--channel · Characeae · Cl^- efflux · membrane excitation · *Nitellopsis obtusa* · tonoplast-free cell

Introduction

Involvement of the Cl^--channel activation in the membrane excitation of characean cells has been demonstrated by measuring the Cl^--dependent transient inward current under voltage clamp (Kishimoto, 1964) and a large Cl^- efflux (Gaffey & Mullins, 1958; Mullins, 1962; Hope & Findlay, 1964; Mailman & Mullins, 1966; Haapanen & Scoglund, 1967; Findlay, 1970; Oda, 1976; Kikuyama et al., 1984). Presence of Ca^{2+} in the external solution is essential for maintaining the plasmalemma excitability (Findlay & Hope, 1964). The occurrence of both Cl^- and Ca^{2+} currents during membrane excitation was suggested by Beilby and Coster (1979*a*) for *Chara* and by Lunevsky et al. (1983) for *Nitellopsis*. Tsutsui et al. (1987*a,b*) observed inhibitory effects of both Ca^{2+} depletion and Ca^{2+}-channel blockers on the membrane excitability of *Chara* cells. Thus the idea that the Cl^- channel in characean cells is activated by Ca^{2+} has been hypothesized. Recently, Shiina and Tazawa (1987*b*) presented results, which strongly support this idea. The transient Cl^- efflux during membrane excitation was influenced by various treatments, which influence the Ca^{2+} influx through the Ca^{2+} channel in *Nitellopsis* cells.

In this paper, we will directly show that Cl^- efflux and E_m depolarization[1] is dependent on $[Ca^{2+}]_i$, which can be modified in tonoplast-free *Nitellopsis* cells using the open vacuole method (Tazawa, Kikuyama & Nakagawa, 1975; Tazawa & Shimmen, 1980).

Materials and Methods

Plant Materials and Culture

Internodal cells of *Nitellopsis obtusa* were used. The alga was cultured in large polyester buckets in an air conditioned room (25 ± 2°C, 15 hr light/9 hr dark). Internodal cells isolated from neighboring cells were kept in APW containing 0.1 mM each of KCl, NaCl and $CaCl_2$. All experiments were carried out at room temperature (20–25°C).

Open-Vacuole Method

A Plexiglas vessel (Fig. 1) composed of three chambers was used to measure E_m, R_m and Cl^- efflux simultaneously (Mimura & Tazawa, 1983; Shiina & Tazawa, 1987*b*). Measurements of E_m

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[1] *Abbreviations:* A-9-C, 9-anthracenecarboxylic acid; α-NP, α-naphthylphosphate; APW, artificial pond water; EGTA, ethyleneglycol-bis-(β-aminoethylether)N,N'-tetraacetic acid; E_m, membrane potential; $[Ca^{2+}]_c$, cytoplasmic Ca^{2+} concentration; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; $[Ca^{2+}]_o$, extracellular Ca^{2+} concentration; PIPES, piperazine-N,N'-bis(2-ethanesulfonic acid); R_m, membrane resistance; W-7, N-(6,aminohexyl)-5-chloro-1-naphthalenesulfonamide.

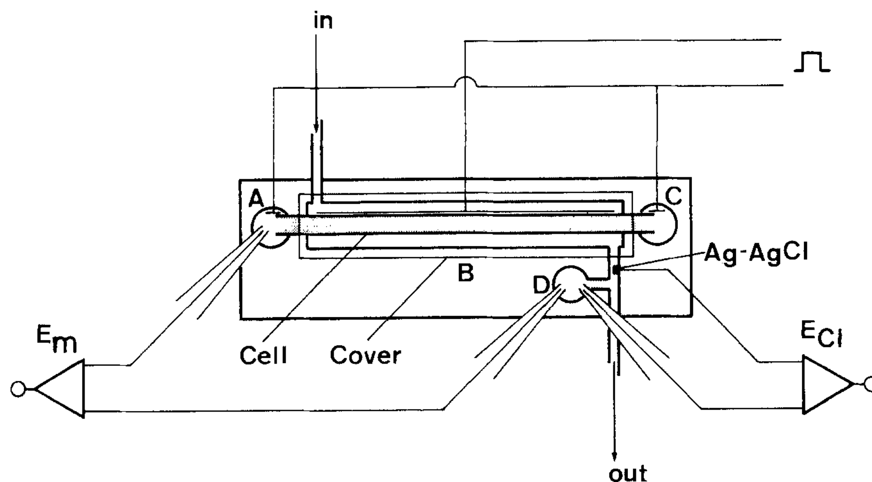

Fig. 1. Experimental setup for measuring Cl^- efflux using the open-vacuole method. Details are described in the text

Table 1. Concentrations of $CaCl_2$ added to the perfusion medium to adjust different values of *p*Ca

*p*Ca	$CaCl_2$ (mM)
<7.00	0
6.96	1.04
5.95	3.60
5.40	4.46
4.76	4.82

and R_m in vacuole-opened cells were done following Tazawa et al. (1975), and Tazawa and Shimmen (1980). The lateral chambers (*A* and *C*) were filled with the perfusion medium. Both cell ends in the lateral chambers were cut off, and the central chamber (*B*) was filled with isotonic APW containing 0.1 mM each of KNO_3, $NaNO_3$ $Ca(NO_3)_2$ and 4 mM $Pb(NO_3)_2$. Pb^{2+} was used to suppress the spontaneous membrane excitation (Kamitsubo, 1976; Shimmen & Tazawa, 1977). Pb^{2+} does not interfere with the dependence of the electrogenic H^+ pump activity on the intracellular ATP concentration (Mimura, Shimmen & Tazawa, 1983).

The basic perfusion medium contained 20 mM PIPES, 5 mM EGTA, 6 mM $MgCl_2$, 250 mM sorbitol, 5% (wt/vol) Ficoll-70, 1 mM ATP and 0–4.83 mM $CaCl_2$. The pH of the medium was adjusted to 7.0 by KOH. Ficoll-70 was dialyzed against distilled water before use. The perfusion media containing various concentrations of Ca^{2+} were prepared by using Ca^{2+}-EGTA buffer and listed in Table 1. The free $[Ca^{2+}]$ of the perfusion media were calculated from the apparent association constants of EGTA for Ca^{2+} and Mg^{2+}, and of ATP for these ions. The purity of EGTA (98.32%) was taken into account for the calculation.

E_m was measured as the potential difference between the central chamber (*B*) and the lateral chambers (*A* and *C*). Small constant current pulses were applied to the cell for the measurement of R_m.

The Cl^- efflux was measured as described in our previous paper (Shiina & Tazawa, 1987*b*). The central chamber was continuously perfused with isotonic APW. The Cl^- concentration of the outgoing medium was measured with a Ag-AgCl electrode. The flow rate of the medium was about 1 ml/min.

W-7 and α-NP were purchased from Sigma. ATP-γ-S was purchased from Boehringer Mannheim. Protein phosphatase-1 was a gift from Dr. H. Y. Lim Tung (Cornell University).

Results

We showed a typical example of Ca^{2+}-dependent Cl^- efflux in Fig. 2. An increase in $[Ca^{2+}]_i$ depolarized E_m, decreased R_m and increased the Cl^- efflux. These changes caused by $[Ca^{2+}]_i$ were for the most part reversible. Neither the Cl^- efflux nor the E_m depolarization showed a time-dependent inactivation. Table 2 shows the average values of 8 experiments of 7 cells. The Ca^{2+}-dependent Cl^- efflux was observed in almost all cells tested, although magnitude of the Ca^{2+}-induced Cl^- efflux varied from cell to cell.

The Ca^{2+}-dependent depolarization of E_m was always observed. However, an increase in the $[Ca^{2+}]_i$ from *p*Ca 5.4 to *p*Ca 4.8 caused a large increase in the Cl^- efflux without a further depolarization of E_m. Furthermore, the Ca^{2+}-induced depolarization usually reached the value of about +10 mV. Because we used Cl^--free solution for the external medium, the equilibrium potential of Cl^- (E_{Cl^-}) would be more positive than +10 mV. We cannot understand this discrepancy between the measured E_m and the calculated E_{Cl^-}. The same discrepancy was also observed in Ca^{2+} channel of Characean plasmalemma (Lunevsky et al., 1983; Beilby, 1984; Shiina & Tazawa, 1987*a*). Since there is a considerably large resistance along the cell (R_s), the exact value of R_m could not be measured. Thus, we could not know the relationship between the amount of Cl^- efflux and the decrease of R_m. Ca^{2+}-dependent Cl^- efflux was also seen in tonoplast-free *Chara* cells (*data not shown*).

A-9-C, which inhibits the Cl^--channel activation during membrane excitation (Tyerman, Findlay

Table 2. Average values of Ca^{2+}-dependent E_m depolarization and Cl^- efflux

*p*Ca	E_m (mV)	No.[a] (>50 mV)	J_{Cl^-} (μmol/m²/sec)	No.[b] (>1 μmol/m²/sec)
>7	—	0/8	—	0/8
7.0	−0.9 ± 0.4	0/8	0	0/8
6.0	6.4 ± 4.9	0/8	0.05 ± 0.04 (0–0.27)	0/8
5.4	107.9 ± 5.9	8/8	5.83 ± 2.53 (0–20.81)	5/8
4.8	114.2 ± 5.2	8/8	12.78 ± 4.64 (0.51–37.50)	7/8

[a] Number of experiments in which a large E_m depolarization was seen.
[b] Number of experiments in which a large Cl^- efflux was seen.

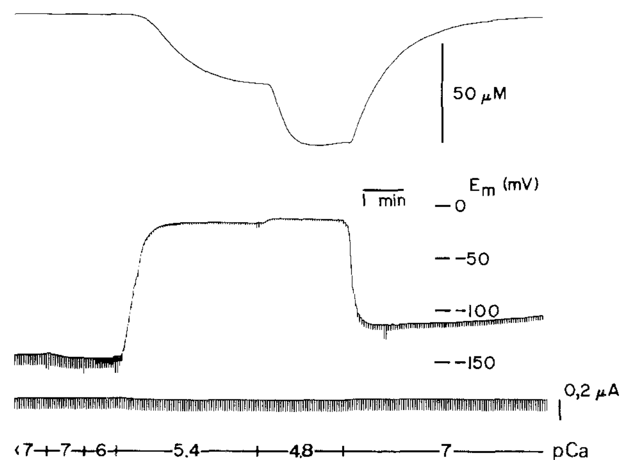

Fig. 2. E_m and R_m (lower trace), and Cl^- efflux (upper trace) in response to changes in $[Ca^{2+}]_i$ in a tonoplast-free cell of *Nitellopsis*. Downward reflection of the upper trace means the increase in the Cl^- efflux. Small current pulses were applied to the cell for the measurement of R_m

& Paterson, 1986; Shiina & Tazawa, 1987*b*), also reduced both the Cl^- efflux and the E_m depolarization, which were caused by the $[Ca^{2+}]_i$ increase (Fig. 3). Ca^{2+}-activated Cl^- efflux was reduced from 16.8–8.4 μmol/m²sec to 5.5–0 μmol/m²sec by 1 mM A-9-C treatment (4 experiments). Reduced Cl^- efflux was partially reversed by removal of A-9-C. E_m depolarization by $[Ca^{2+}]_i$ increase was also seen in all cells tested (8 experiments of 4 cells) (Table 2). However, their reversibilities could not be seen.

In our preliminary results, Ca^{2+}-dependent Cl^- efflux was not affected by the intracellular application of calmodulin antagonist, W-7 (100 μM) (2 experiments of 2 cells) nor protein phosphatase-1 (2 U/ml) (2 experiments of 2 cells) nor ATP-γ-S (1 mM) (4 experiments of 2 cells) nor protein phosphatase inhibitor, α-NP (1 mM) (10 experiments of 4 cells) (*data not shown*). Depletion of $[ATP]_i$ did not change the amount of Ca^{2+}-dependent Cl^- efflux (5 experiments of 3 cells) (*data not shown*).

Discussion

Although a large Cl^- efflux is observed during membrane excitation of normal cells, it has been reported that Cl^- efflux can not be detected in tonoplast-free cells (Kikuyama et al., 1984). Linear depolarization of E_m under voltage-clamp conditions caused only the voltage-dependent activation of Ca^{2+} channel in tonoplast-free cells (Shiina & Tazawa, 1987*a*). These facts clearly demonstrate that Cl^- channel in tonoplast-free cells can not be activated only by E_m depolarization.

However, a large Cl^- efflux was induced by an increase in $[Ca^{2+}]_i$ using the open-vacuole method (Fig. 2). This result clearly demonstrates the presence of a Ca^{2+}-activated Cl^--channel in the plasmalemma of *Nitellopsis* cells. The critical $[Ca^{2+}]_i$ for Cl^--channel activation was about 4.0 μM (*p*Ca = 5.4) in *Nitellopsis* (Fig. 2). This value is almost the same order of magnitude as the maximum increase in $[Ca^{2+}]_c$ during the action potential, which was measured in normal cells of *Chara* (6.7 μM) and *Nitella* (43 μM). Depolarization of E_m during the action potential was almost coincident with an increase in $[Ca^{2+}]_c$ (Williamson & Ashley, 1982). Thus, the transient increase in $[Ca^{2+}]_c$ during the action potential seems to be enough to activate the Ca^{2+}-activated Cl^--channel in normal Characean cells. However, the Ca^{2+}-activated Cl^- efflux was not strictly coupled with the E_m depolarization. The Cl^- efflux increased much without a significant change in E_m depolarization as shown in Fig. 2. Furthermore, the peak value of E_m depolarization by $[Ca^{2+}]_i$ increase was not consistent with the calculated E_{Cl^-}. We could not understand these discrepancies. Some other ion channels may also be activated by either $[Ca^{2+}]_i$ increase or E_m depolarization and make the behavior of E_m complicated.

Beilby and Coster (1979*a,b*) observed two time-separated, transient inward currents under voltage clamp, and analyzed them based on the Hodgkin-Huxley equations. They assumed the initial current

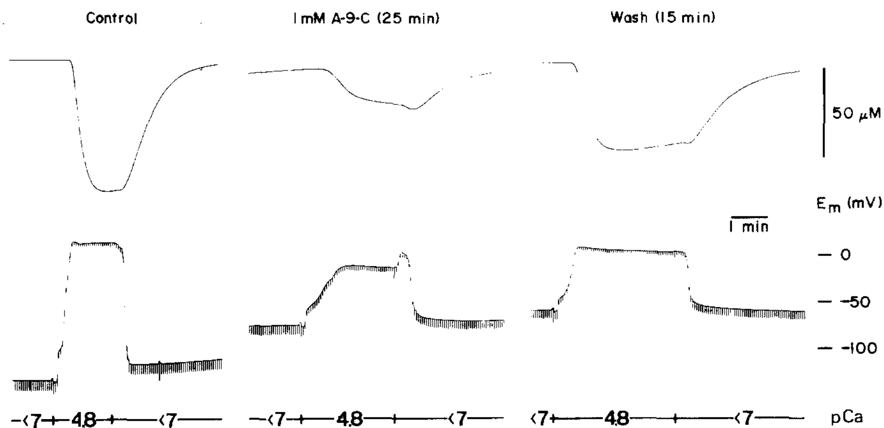

Fig. 3. Effects of A-9-C on E_m and R_m (lower trace), and Cl^- efflux (upper trace) induced by an increase in $[Ca^{2+}]_i$. Downward reflection of the upper trace means the increase in the Cl^- efflux. Small current pulses were applied to the cell for the measurement of R_m

as I_{Cl^-} and the subsequent one as an unknown cation current (probably $I_{Ca^{2+}}$). Based on the channel-inhibitor experiments, however, Lunevsky et al. (1983) assumed that $I_{Ca^{2+}}$ precedes I_{Cl^-} in *Nitellopsis*. We previously reported that the Cl^--channel activation was strongly dependent on the Ca^{2+}-channel activation (Shiina & Tazawa, 1987*b*). Furthermore, our present experiment clearly demonstrated that a large Cl^- efflux is induced by elevating the intracellular Ca^{2+} concentration. These results strongly support the hypothesis of Ca^{2+}-induced Cl^--channel activation.

In our preliminary results, any agent that would affect the calmodulin action or protein phosphorylation did not interfere with the Ca^{2+}-activated Cl^- efflux. Thus, the Cl^- channel in *Nitellopsis* plasmalemma may be directly activated by Ca^{2+}. Further detailed experiments should be necessary to know the activation mechanism of the Cl^- channel.

The free $[Ca^{2+}]_i$ at the peak of the initial light emission of aequorin observed in the tonoplast-free cells were estimated to be 3.2 μM when cells were bathed in an external solution containing 0.1 mM Ca^{2+} (Kikuyama & Tazawa, 1983). Thus, the Cl^- channel in the tonoplast-free cells is also expected to be activated by an increase in $[Ca^{2+}]_i$ during action potential. However, the Cl^- channel is not activated in tonoplast-free cells (Kikuyama et al., 1984; Shiina & Tazawa, 1987*a*). This discrepancy is accounted for in the following way. Kikuyama and Tazawa (1983) used a perfusion medium containing 1.0 mM EGTA, because they could not measure the transient Ca^{2+} increase by using a medium containing 5.0 mM EGTA (M. Kikuyama & M. Tazawa, *personal communication*). Thus, chelation of Ca^{2+} by excessive EGTA may restrain the increase in $[Ca^{2+}]_i$ in tonoplast-free cells. In fact, the $[Ca^{2+}]_i$ increase estimated in the presence of 5 mM EGTA is 10^{-9} M, when the Ca^{2+} influx of tonoplast-free cells during the action potential is 1.51 μmol/m²/sec (Shiina & Tazawa, 1987*a*). This value is too small to activate the Cl^- channel. Thus we conclude that the disappearance of the Cl^--channel activation during an action potential in tonoplast-free cells is not due to an impairment of the Cl^- channel but by an inhibition by EGTA of the $[Ca^{2+}]_i$ increase.

The transient increase in Cl^- efflux during an action potential is suppressed by A-9-C (Shiina & Tazawa, 1987*b*). Thus, the inhibitory effect of A-9-C on the Ca^{2+}-activated Cl^- efflux suggests that both Cl^- effluxes are induced by the same Cl^- channel. The Cl^- efflux measured using the open-vacuole method was about 3 μmol/m²/sec, when the $[Ca^{2+}]_i$ was 4 μM. This value is the same order of magnitude as the Cl^- efflux measured during an action potential in the normal *Nitellopsis* cells (3 μmol/m²/sec or more). Thus the $[Ca^{2+}]_c$ increase during the action potential of normal cells is estimated to be around 4 μM or more, which is similar to the values (6.7 μM) measured by Williamson and Ashley (1982) with aequorin light emission using *Chara* cells.

Mimura and Tazawa (1983) reported that an increase in $[Ca^{2+}]_i$ in tonoplast-free cells caused depolarization of E_m and a decrease in R_m without a significant Cl^- efflux in *Nitellopsis*. We do not know the reason for the discrepancy between these data and ours. Other Ca^{2+}-dependent processes such as Ca^{2+}-activated Ca^{2+}-channel may also be involved in this phenomenon and they may change seasonally.

We conclude that there is a Ca^{2+}-dependent Cl^--channel in the *Nitellopsis* plasmalemma. This channel seems to be opened during membrane excitation by a small increase in $[Ca^{2+}]_c$, which is caused by a preceding voltage-dependent Ca^{2+}-channel activation in the plasmalemma.

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