J. Membrane Biol. 96, 263–276 (1987)

The Journal of
Membrane Biology

Demonstration and Characterization of Ca^{2+} Channel in Tonoplast-Free Cells of *Nitellopsis obtusa*

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Summary. The presence of a Ca^{2+} channel in the plasmalemma of tonoplast-free *Nitellopsis obtusa* cells was demonstrated and its characteristics were studied using current- and voltage-clamp techniques. A long-lasting inward membrane current (I_m), recorded using a step voltage clamp, consisted of a single component without time-dependent inactivation. Increasing either $[Ca^{2+}]_o$ or $[Cl^-]_o$ 1) enhanced the maximum amplitude of inward I_m ($(I_m)_p$) and 2) shifted the peak voltage ($(V_m)_p$) at $(I_m)_p$ to more positive values under ramp-shaped voltage clamping and 3) depolarized the peak value of action potentials. This behavior is consistent with predictions based on the Nernst equation for Ca^{2+} but not for Cl^-. DIDS (4,4′-diisothiocyano-2,2′-disulfonic acid stilbene) did not suppress $(I_m)_p$ in tonoplast-free cells, in contrast with its effect on normal cells. La^{3+} and nifedipine blocked $(I_m)_p$ irreversibly. On the other hand, Ca^{2+} channel agonist, BAY K 8644 irreversibly enhanced $(I_m)_p$. Both Sr^{2+} influx and K^+ efflux increased upon excitation. The charge carried by Sr^{2+} influx was compensated for by K^+ efflux. It is concluded that only the Ca^{2+} channel is activated during plasmalemma excitation in tonoplast-free cells. In terms of the magnitude of $(I_m)_p$, Sr^{2+} could replace Ca^{2+}, but Mn^{2+}, Mg^{2+} and Ba^{2+} could not. External pH affected $(I_m)_p$ and the membrane conductance (g_m) at $(I_m)_p$ ($(g_m)_p$). Increasing the external ionic strength caused increases in both $(I_m)_p$ and $(g_m)_p$, and shifted $(V_m)_p$ to positive values. At the same time, Sr^{2+} influx increased. Thus Ca^{2+} channel activation seems to be enhanced by increasing external ionic strength. The possible involvement of surface potential is discussed.

Key Words Ca^{2+} channel · *I-V* relation · membrane excitation · *Nitellopsis obtusa* · tonoplast-free cell

Introduction

Excitability has been demonstrated in plants ranging from algae (Tazawa, 1972) to higher plants (Sibaoka, 1969; Pickard, 1973). Intensive studies on the mechanism of action potentials have been carried out mainly using giant *Characean* algal cells. The action potential in *Characeae* is assumed to be due to transient increases in permeability to both Cl^- (P_{Cl^-}) and Ca^{2+} ($P_{Ca^{2+}}$) (Beilby, 1984*b*), causing the efflux and influx, respectively, of these ions down their electrochemical gradients. This inward current carried by Cl^- and Ca^{2+} is assumed to be compensated for by K^+ efflux, caused by increased membrane permeability to K^+ (P_{K^+}). Large increases in Cl^- and K^+ efflux were indeed demonstrated to accompany membrane excitation in *Chara globularis* (Gaffey & Mullins, 1958). Almost equal amounts of Cl^- and K^+ were released upon excitation in *Chara corallina* (Oda, 1976). In voltage-clamp studies, Kishimoto (1964) found using *Nitella* that the amplitude of the transient inward current decreased with increasing external Cl^- concentration. The presence of Ca^{2+} in the external medium is essential for maintaining the plasmalemma excitability (Findlay & Hope, 1964*b*). Increasing the external Ca^{2+} concentration shifted the peak of the action potential in the positive direction by the amount predicted by the Nernst equation (Hope, 1961*a,b*) and increased the inward current under voltage-clamp conditions (Findlay, 1961; 1962). Hayama et al. (1979) measured a large Ca^{2+} influx upon excitation in intact *Chara* cells. Williamson and Ashley (1982) injected the photo-protein aequorin into the cytoplasm of *Chara* and *Nitella* and demonstrated transient light emission upon membrane excitation. On the other hand, Hope and Findlay (1964) could not detect a large Ca^{2+} influx accounting for the inward current with radioactive tracer. Therefore some ambiguity remains concerning which ions carry inward current during membrane excitation in intact Characeae cells. The occurrence of both Cl^- and Ca^{2+} currents in membrane excitation was suggested by Beilby and Coster (1979) for *Chara* and by Lunevsky et al. (1983) for *Nitellopsis*.

In Characeae cells, the tonoplast can be removed by perfusing the vacuole with media containing EGTA (Williamson, 1975; Tazawa et al., 1976). Since tonoplast-free cells can generate action potentials, the plasmalemma action potential can be

Table 1. Compositions (mM) of various external solutions used

	APW							
	1	2	3	4	5	6	7	8
KCl	0.1	0.1		0.1			0.1	0.1
NaCl	0.1	0.1		0.1	0.2		0.1	0.1
$CaCl_2$	0.1	0.1					0.1	0.1
$SrCl_2$					0.5			
KNO_3			0.1					
$NaNO_3$			0.1			0.2		
$Ca(NO_3)_2$			0.1			0.1		
HEPES		2.0	2.0	2.0	2.0	2.0		
MES							1.0	
Tricine								2.0
pH	5.6	7.5	7.5	7.5	7.5	7.5	6.5	8.5

studied without interference from the tonoplast. The peak of the action potential in tonoplast-free *Chara* cells was not affected by internal Cl^- concentration (Shimmen & Tazawa, 1980). Kikuyama et al. (1984) could not detect Cl^- efflux during action potentials in tonoplast-free cells of *Chara* and *Nitellopsis*, although significant K^+ efflux was observed. The intracellular free Ca^{2+} concentration in tonoplast-free *Chara* cells increased transiently upon membrane excitation (Kikuyama & Tazawa, 1983). Thus it is reasonable to assume that only Ca^{2+} carries inward current during excitation in tonoplast-free cells.

The present study aims to test this assumption. We show that inward current under voltage-clamp conditions is carried only by Ca^{2+} in tonoplast-free cells of *Nitellopsis obtusa*. The absence of Cl^- channel activation enabled us to characterize plasmalemma Ca^{2+} channel activation, without interference from the Cl^- channel.

Materials and Methods

Plant Materials and Culture

Internodal cells of *Nitellopsis obtusa* were mainly used. Algae were cultured in large polyester buckets containing soil and tap water in an air-conditioned room (25 ± 2°C, 15-hr light/9-hr dark) or outdoors. Internodal cells were isolated from neighboring cells and kept in APW-1 containing 0.1 mM each of KCl, NaCl and $CaCl_2$. APW bathing solutions were modified in various ways (Table 1). All experiments were carried out at room temperature (20 to 25°C).

Intracellular Perfusion

Intracellular perfusion was performed according to Tazawa et al. (1976). Tonoplast-free cells were prepared by replacing the cell sap with a medium containing the Ca^{2+}-chelating agent EGTA. After ligation with polyester thread at both ends, perfused cells were kept in APW-1 until the tonoplast disintegrated. The internal perfusion medium contained (in mM): 5 EGTA, 5 or 20 PIPES, 6 $MgCl_2$, 1 ATP, 250 sorbitol and 5% (wt/vol) Ficoll-70 (pH 7.0). Ficoll-70 stabilizes the membrane potential of tonoplast-free cells (Shimmen & Tazawa, 1982). The K^+ concentration of the perfusion medium was 21.3 mM (21 K solution) when 5 mM PIPES was used and 45.4 mM (45 K solution) when 20 mM PIPES was used. 21 K solution was used in most voltage-clamp experiments. This solution was not appropriate for current-clamp experiments however, since it caused the duration of action potentials to be very long (several minutes or more). Instead 45 K solution was used for current-clamp measurements, since higher internal K^+ concentration shortened the duration of the action potential (Shimmen & Tazawa, 1980). The K^+ concentration in tonoplast-free cells perfused with 45 K solution and 21 K solution is estimated at 51 and 28 mM, respectively, assuming that the cytoplasm accounts for about 5% of the total *Nitellopsis* cell volume (Mimura & Kirino, 1984) and that the cytoplasmic K^+ concentration is 151 mM (Kikuyama et al., 1984).

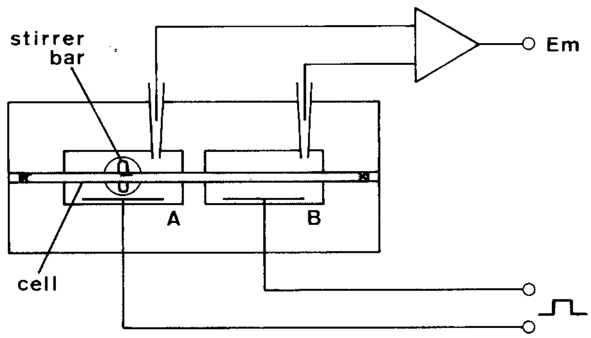

Fig. 1. Apparatus for measuring ion fluxes during single action potentials in *Nitellopsis*. An internodal cell (cell) was partitioned between two chambers, *A* and *B*. A current pulse was applied through Ag/AgCl wires between chambers *A* and *B*. The membrane potential (E_m) was measured between *A* and *B*. Chamber *B* was filled with 55 mM KCl solution. Chamber *A* was filled with the solution containing 0.5 mM $SrCl_2$ which was made isotonic to 55 mM KCl with sorbitol

Electrical Measurements

The membrane potential (E_m) was measured using the conventional microelectrode method (Shimmen & Tazawa, 1980). The cell was placed on a polyacrylate vessel with three chambers. The chambers were filled with the bathing media listed in Table 1. APW-2 was used as the external medium unless otherwise stated. E_m of the cell portion in the central chamber was measured by inserting a glass microelectrode into the cell. Electrical current was applied through Ag/AgCl wires between the central chamber and the lateral chambers. The electrical circuit for the voltage clamp was designed after Asai and Kishimoto (1975). The current (I_m) and the potential difference (V_m) between the intracellular microelectrode and the reference electrode in central chamber were measured using current-measuring and voltage-measuring circuits, respectively, and recorded on a pen recorder (National VP6527A) and an oscilloscope (Nihon Koden

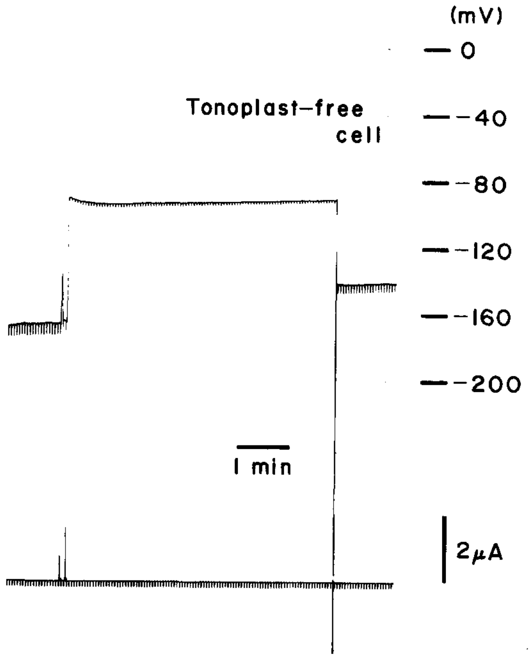

Fig. 2. Typical rectangular action potential in tonoplast-free *Nitellopsis* cells. The numbers on the right denote E_m. Depolarized E_m was repolarized by application of inward current

VC-9). Under voltage-clamp experiments, the current-voltage (*I-V*) relationship was obtained by slowly shifting V_m using a ramp-shaped depolarization (rate approx. 400 mV/min) Ohkawa & Kishimoto, 1977). Small rectangular constant-current or voltage pulses were applied to the cell for stimulation and for the measurement of membrane chord conductance (g_m).

Measurement of Ion Fluxes

The Plexiglas vessel used for the simultaneous measurement of Sr^{2+} influx, K^+ efflux and E_m during excitation is shown in Fig. 1. E_m was measured by the "K^+-anesthesia method" (Shimmen et al., 1976). Chamber B was filled with 55 mM KCl solution and chamber A with various media adjusted to be isotonic with 55 mM KCl using sorbitol. The solution in chamber A was stirred with a magnetic stirrer throughout the experiment. E_m was measured as the potential difference between chambers A and B, since E_m for the cell portion in chamber B is almost zero. For the measurement of Sr^{2+} influx and K^+ efflux the cell portion in chamber A was bathed in APW-5 containing 0.5 mM $SrCl_2$ but no K^+ for 120 sec. Thereafter the cell was placed on a Plexiglas bench. After loss of turgor pressure, both cell ends were amputated and liquid paraffin was introduced into the cell (Fujii et al., 1979). The displaced internal solution was collected in a glass capillary and diluted with distilled water. K^+ efflux was measured by analyzing the K^+ concentration in the bathing medium. Sr^{2+} and K^+ concentrations were analyzed using an atomic absorption spectrophotometer (Perkin-Elmer 370). Cl^- efflux was measured by analyzing the Cl^- concentration using an Ag-AgCl wire in bathing solutions with Cl^- substituted by NO_3^- (APW-6).

All data are shown as mean ± SEM.

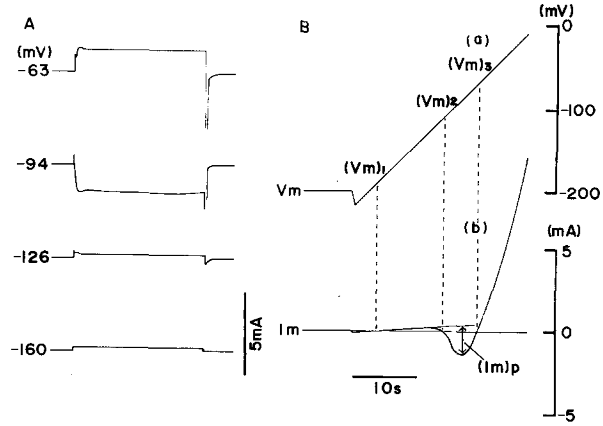

Fig. 3. (*A*) Membrane currents recorded under step voltage clamping in a tonoplast-free cell of *Nitellopsis*. The numbers on the left denote clamping potential. (*B*) Membrane current (*b*) under ramp depolarization of clamping potential (*a*) in the same cell used in (*A*). $(I_m)_p$ is the peak inward current measured by linear extrapolation of the current-voltage (*I-V*) curve

Abbreviations

A-9-C, anthracene-9-carboxylic acid; APW, artificial pond water; DIDS, 4,4'-diisothiocyano-2,2'-disulfonic acid stilbene; EGTA, ethyleneglycol-*bis*-(β-aminoethylether)N,N'-tetraacetic acid; E_m, membrane potential; g_m, membrane conductance; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; MES, 2-(N-morpholino)ethanesulfonic acid, monohydrate; PIPES, piperazine-N,N'-*bis*(2-ethanesulfonic acid)

Results

Membrane Current Under Voltage-Clamp Condition

Unlike normal cells, tonoplast-free cells generate rectangular action potentials of long duration when the intracellular K^+ concentration is low (Shimmen & Tazawa, 1980) (Fig. 2). The membrane potential remained at the depolarized level for a minute or more during which the increased g_m changed only slightly. Under voltage-clamp condition, when the clamp potential was more negative than the excitation threshold, a steady outward current, which seems to be carried by K^+ (Hope & Walker, 1975) and H^+ (Beilby, 1984*a*), was observed (Fig. 3(*A*), clamp potentials −160.0 and −126.0 mV). At a potential slightly more positive than the threshold, a steady inward current appeared which continued for the duration of the voltage clamp (Fig. 3(*A*), −94.0 mV). This inward current seems to contain only one component, unlike in normal cells where two components occur (Beilby & Coster, 1979; Lunevsky et al., 1983). The small initial inward cur-

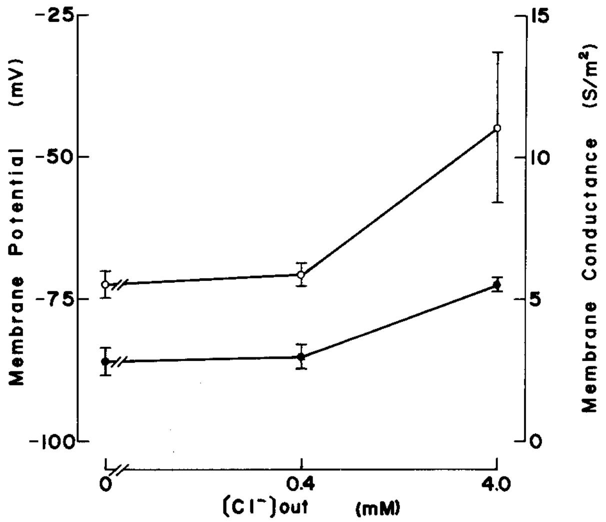

Fig. 4. E_m (open circles) and g_m (filled circles) at the action potential peak in relation to external Cl^- concentration ($[Cl^-]_o$). E_m and g_m were measured under current-clamp conditions. $[Cl^-]_o$ was varied by adding choline-Cl to APW-3. All data are shown as mean ± SEM

rent peak seen is caused by small fluctuations in the clamp voltage. At more positive potentials, a large, steady outward current was seen (Fig. 3(*A*), −63.0 mV).

Slow linear depolarization of V_m from the resting level produced the so-called N-shaped *I-V* relation characteristic of excitable membranes (Ohkawa & Kishimoto, 1977). The peak inward current in this *I-V* relation was almost identical to the largest steady inward current obtained under step voltage clamping (Fig. 3*A* and *B*). The amplitude of the peak inward current ($(I_m)_p$) was measured as shown in Fig. 3(*B*). The membrane conductance and potential difference at $(I_m)_p$ are termed $(g_m)_p$ and $(V_m)_p$, respectively, and V_m and g_m at $I_m = 0$ are termed $(V_m)_1$ ($(g_m)_1$), $(V_m)_2$ ($(g_m)_2$) and $(V_m)_3$ ($(g_m)_3$), respectively. $(V_m)_1$ and $(V_m)_3$ are regarded as E_m at rest and at the action potential peak, respectively.

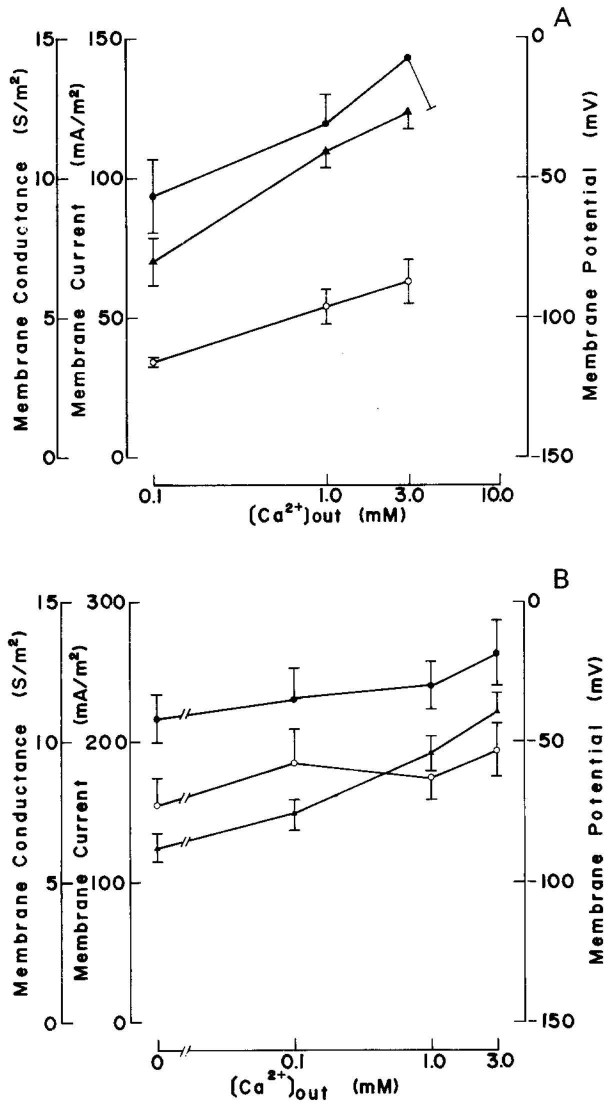

Fig. 5. $(g_m)_p$ (open circles), $(I_m)_p$ (filled circles) and $(V_m)_p$ (filled triangles) in relation to external Ca^{2+} concentration ($[Ca^{2+}]_o$) under ramp voltage clamping. (*A*) $[Ca^{2+}]_o$ was varied by adding $CaCl_2$ to APW-4. (*B*) $[Ca^{2+}]_o$ was varied by adding $CaCl_2$ to APW-4, keeping $[Cl^-]_o$ constant by adding choline-Cl. All data are shown as mean ± SEM

Dependence of E_m, g_m and I_m on External Cl^- and Ca^{2+} Concentration

To change $[Cl^-]_o$, various concentrations of choline-Cl were added to APW-3. E_m and g_m at the action potential peak are shown in Fig. 4. There was no change in E_m or g_m for $[Cl^-]_o$ in the range 0 to 0.4 mM. However, E_m depolarized and g_m increased significantly at 4.0 mM Cl^-. This change in the peak value of the action potential occurs in the opposite direction to the change in the equilibrium potential for Cl^-, calculated from the Nernst equation. The same pattern was also observed in voltage-clamp experiments. As shown in Table 4, $(V_m)_p$ (control) was shifted to more positive values by the addition of 6 mM NaCl to APW-1, with a large increase in $(I_m)_p$ and $(g_m)_p$.

When $[Ca^{2+}]_o$ was increased from 0 to 3.0 mM by the addition of $CaCl_2$ to APW-4, $(I_m)_p$ and $(g_m)_p$ increased by about 50 and 80%, respectively, and $(V_m)_p$ shifted in the positive direction by 36.1 mV for a tenfold increase in $[Ca^{2+}]_o$ (Fig. 5*A*). However, since these parameters were markedly affected by $[Cl^-]_o$ as mentioned above, we investigated the ef-

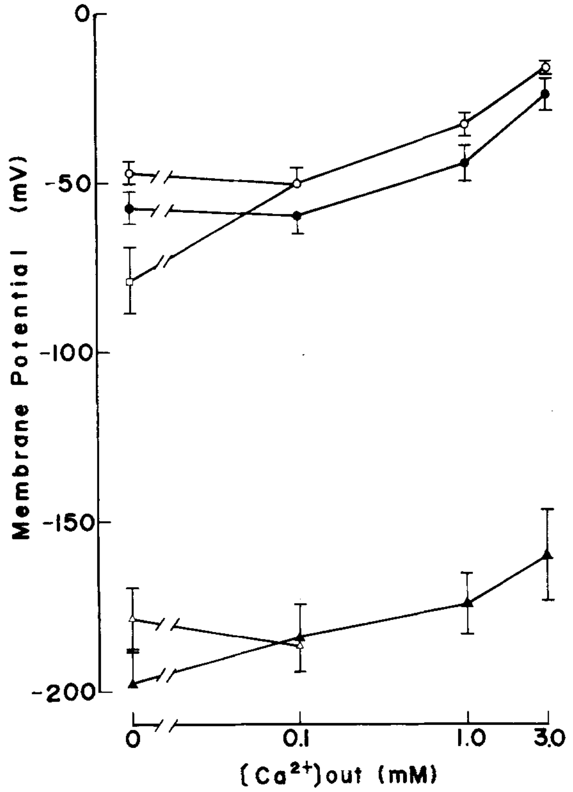

Fig. 6. E_m at the peak (open circles) and plateau (60 sec after stimulation) (filled circles) of the action potential and in the resting state (filled triangles) in relation to external $[Ca^{2+}]_o$. Open squares and triangles represent E_m at the action potential peak and in the resting state, respectively, in an external solution containing 1 mM EGTA or 0.1 mM $CaCl_2$. In the case of E_m at the action potential peak, the value of open circle and open square at 0.1 mM $[Ca^{2+}]_o$ was the same. E_m was measured under current clamping. $[Ca^{2+}]_o$ was varied by adding $CaCl_2$ to APW-4 keeping $[Cl^-]_o$ constant. All data are shown as mean ± SEM

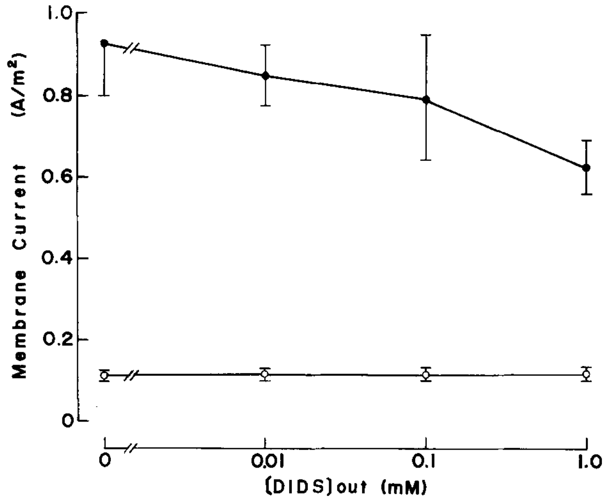

Fig. 7. $(I_m)_p$ under ramp voltage clamping in tonoplast-free (open circles) and normal (filled circles) cells of *Nitellopsis* in relation to external DIDS concentration. DIDS was added to APW-2. All data are shown as mean ± SEM

fects of $[Ca^{2+}]_o$ under constant $[Cl^-]_o$ by adding choline-Cl to APW-4. As in Fig. 5(*A*) the inward I_m increased with increasing $[Ca^{2+}]_o$ although the increase was smaller than before (Fig. 5*B*). The tendency of $(g_m)_p$ to increase disappeared when $[Cl^-]_o$ was held constant. $(V_m)_p$ shifted in the positive direction by 23.6 ± 2.5 (n = 6) mV for a tenfold increase in $[Ca^{2+}]_o$ between 0.1 and 3.0 mM. This value is close to the change in E_{Ca} calculated using the Nernst equation.

The peak and plateau (60 sec after the generation of an action potential) levels of the rectangular action potentials recorded under current-clamp conditions (*see* Fig. 12) were depolarized by 22.8 ± 3.6 (n = 5) mV and 22.1 ± 4.6 (n = 5) mV, respectively, for a tenfold increase in $[Ca^{2+}]_o$ between 0.1 and 3.0 mM at constant $[Cl^-]_o$ (Fig. 6). There was scarcely any difference in the peak and plateau potentials between 0 and 0.1 mM but the peak potential was hyperpolarized 28.7 mV by the addition of 1 mM EGTA to the external medium. This may be due to the lowering of $[Ca^{2+}]_o$ in the free space of the cell wall due to Ca^{2+} chelation by EGTA. E_m in the resting state was also depolarized by increasing $[Ca^{2+}]_o$.

Effects of DIDS

Only a small number of inhibitors have been found which block the Cl^- channel in normal Characeae cells. Ethacrynic acid was reported to partially block the Cl^- component of the action potential in *Nitellopsis* (Lunevsky et al., 1983). A-9-C reduced the Cl^- current activated by membrane hyperpolarizations and the action potential peak in *Chara inflata* and *Chara corallina* (Tyerman et al., 1986). To demonstrate the absence of functional Cl^- channels in tonoplast-free cells, we compared the effects of DIDS, which is known to block Cl^- channels in animal cells, in normal and tonoplast-free cells (Fig. 7). DIDS was added to APW-2. The $(I_m)_p$ in normal cells was about eight times larger than that in tonoplast-free cells, suggesting the presence of more functional voltage-dependent ion channels in the former. 1.0 mM DIDS applied to normal cells reduced $(I_m)_p$ by about 30%. This inhibition was reversible. In contrast, the inward I_m of tonoplast-free cells was not affected at all by DIDS. This result supports the idea that there are no functional Cl^- channels in tonoplast-free cells.

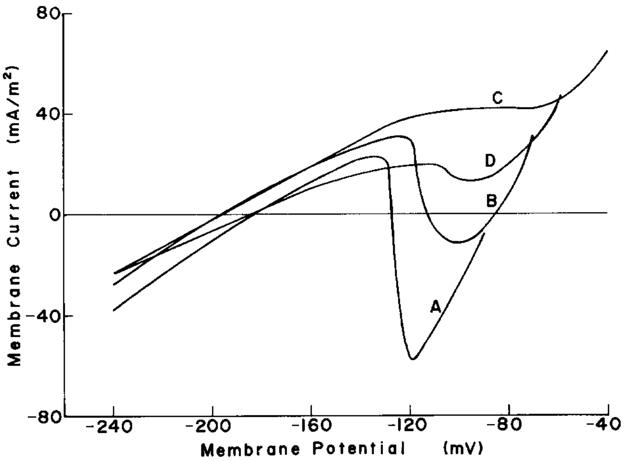

Fig. 8. Effects of external La^{3+} on the *I-V* relation recorded under ramp voltage clamping in a tonoplast-free cell of *Nitellopsis*. (*A*) control; (*B*) 20 μM La^{3+}, 15-min treatment; (*C*) 200 μM La^{3+}, 6-min treatment; and (*D*) 9-min after washing. La^{3+} was added to APW-2 as $LaCl_3$

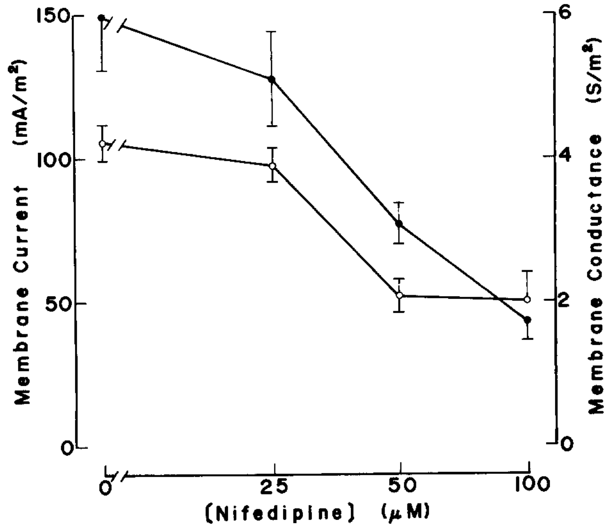

Fig. 10. $(g_m)_p$ (open circles) and $(I_m)_p$ (filled circles) in relation to external nifedipine concentration under ramp voltage clamping in tonoplast-free cells of *Nitellopsis*. Nifedipine was added to APW-2. All data are shown as mean ± SEM

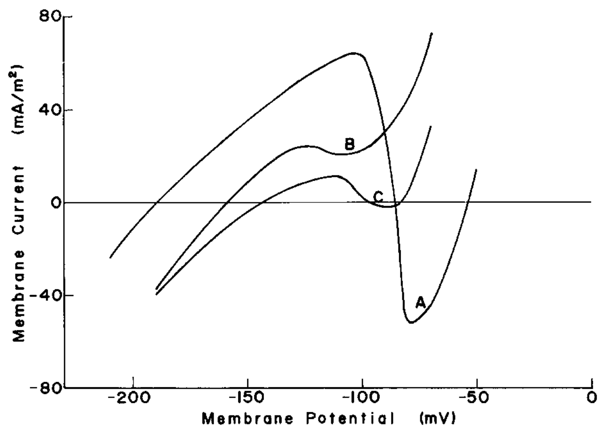

Fig. 9. Effects of nifedipine on the *I-V* relation recorded under ramp voltage clamping in a tonoplast-free cell of *Nitellopsis*. (*A*) control; (*B*) 100 μM nifedipine, 20-min treatment; and (*C*) 30-min after washing. Nifedipine was added to APW-2

Effects of Ca^{2+} Channel Antagonists and Agonists

If the Ca^{2+} channel is the sole ionic channel activated during membrane excitation in tonoplast-free cells, the inward current under voltage-clamp conditions should be inhibited by Ca^{2+} channel antagonists and enhanced by Ca^{2+} channel agonists. When a tonoplast-free cell was treated with 20 μM $LaCl_3$ for 15 min, the inward current was partially blocked (Fig. 8*B*). Treatment of a cell with 200 μM $LaCl_3$ for 6 min almost completely blocked the inward current (Fig. 8*C*). This blockage by La^{3+} was only partially reversible (Fig. 8*D*). $(g_m)_p$ decreased markedly upon La^{3+} treatment. The outward current at the less negative V_m also seemed to be reduced by La^{3+}, since the slope of the *I-V* curve in the less negative region became smaller with increasing $LaCl_3$ concentration. La^{3+} also displaced the $(V_m)_p$ to more positive values, indicating that the threshold value became larger.

We also examined the effects of several organic inhibitors of voltage-dependent Ca^{2+} channels on membrane excitation in tonoplast-free cells. The dihydropyridine derivative, nifedipine greatly reduced $(I_m)_p$ (Fig. 9*B*). $(I_m)_p$ did not recover its initial amplitude even after 30-min washing. Nifedipine seems not to affect the slope of the large outward current induced by shifting V_m to positive values. In contrast to the effects of La^{3+}, nifedipine shifted $(V_m)_p$ to more negative values. $(I_m)_p$ and $(g_m)_p$ decreased with increasing nifedipine concentration (Fig. 10). Sensitivity to nifedipine varied between culture batches. For some cells, even a saturated solution of nifedipine was without effect. Other Ca^{2+} channel antagonists such as verapamil and diltiazem were without effect in tonoplast-free cells of *Nitellopsis*.

As shown in Fig. 11, the Ca^{2+} channel agonist BAY K 8644 and Ca^{2+} channel antagonist BAY K 5552 irreversibly enhanced the $(I_m)_p$ of tonoplast-free cells by 42 and 43%, respectively. $(V_m)_p$ was scarcely affected by these agents. On the other hand, one of the Ca^{2+} channel agonists, (+)202-791 (100 μM, 15 to 20 min) inhibited $(I_m)_p$ by about 36% (*data not shown*). This inhibition was completely

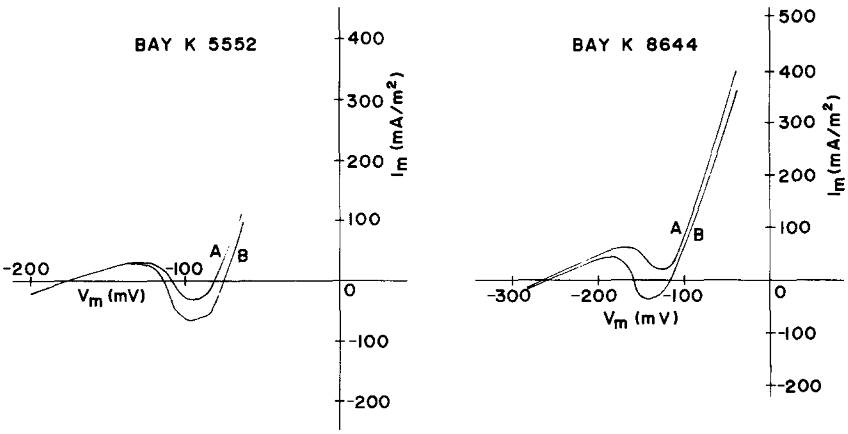

Fig. 11. Effects of BAY K 5552 (left) and 8644 (right) on the *I-V* relation recorded under ramp voltage clamping in a tonoplast-free cell of *Nitellopsis*. (*A*) control; (*B*) 15 min with 100 μM BAY Ks which were added to APW-2

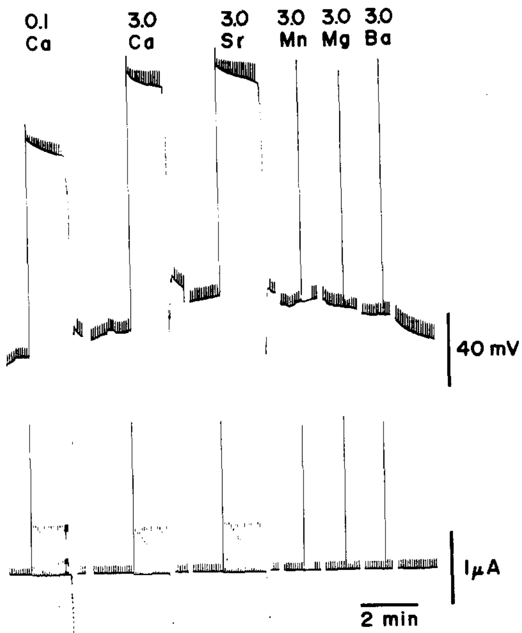

Fig. 12. Effects of external divalent cations on the action potential in a tonoplast-free cell of *Nitellopsis*. E_m was measured under current clamping. Small current pulses were applied for the measurement of g_m. Depolarized E_m was repolarized by application of inward current. The number on each action potential denotes the concentration (in mM) of divalent cation added to APW-2

reversible and $(I_m)_p$ was restored to the initial value by a 15-min wash. $(V_m)_p$ was scarcely affected by (+)202-791.

Selectivity to Divalent Cations

We compared the effects of various divalent cations added to APW-2 on membrane excitation in tonoplast-free cells under current (Fig. 12) and voltage clamping (Table 2). The peak of the action potential was scarcely changed by replacing 3 mM $CaCl_2$ with 3 mM $SrCl_2$. The generation of action potentials was blocked by replacing external $CaCl_2$ with $MnCl_2$, $MgCl_2$ or $BaCl_2$. Similarly $(I_m)_p$ and $(V_m)_p$ showed scarcely any change when $CaCl_2$ was replaced with $SrCl_2$, although $(g_m)_p$ decreased slightly (Table 2). Replacement of external $CaCl_2$ with $MnCl_2$, $MgCl_2$ or $BaCl_2$ caused large reductions in $(I_m)_p$ and $(g_m)_p$. These results clearly show that Ca^{2+} can be completely replaced with Sr^{2+} but not with Mn^{2+}, Mg^{2+} or Ba^{2+}. $(V_m)_p$ shifted in the positive direction by about 25 mV when the Ca^{2+} concentration was increased from 0.1 to 1.0 mM. This tendency was the same for all divalent cations tested (Table 2).

Table 2. Effects of external divalent cations on $(I_m)_p$, $(g_m)_p$ and $(V_m)_p$ under ramp voltage clamping in tonoplast-free cells of *Nitellopsis*[a]

	$(I_m)_p$ (mA/m²)	$(g_m)_p$ (S/m²)	$(V_m)_p$ (mV)
0.1 mM Ca^{2+}	61.0 ± 6.2	2.93 ± 0.14	−75.5 ± 6.7
1.0 mM Ca^{2+}	74.4 ± 6.8	3.01 ± 0.15	−48.7 ± 7.9
1.0 mM Sr^{2+}	69.7 ± 6.4	2.34 ± 0.24	−46.0 ± 6.8
1.0 mM Mn^{2+}	24.8 ± 2.7	1.25 ± 0.08	−51.8 ± 7.0
1.0 mM Mg^{2+}	25.2 ± 1.6	1.43 ± 0.12	−57.8 ± 7.3
1.0 mM Ba^{2+}	24.3 ± 3.5	1.21 ± 0.10	−48.0 ± 7.4

[a] Each divalent cation-Cl_2 was added to APW-2. All data are shown as mean ± SEM ($n = 4$).

External pH Effects

The effect of external pH on membrane excitation in tonoplast-free cells is shown in Fig. 13. The external solution was APW-7, -2, and -8 for pH 6.5, 7.5 and 8.5, respectively. $(I_m)_p$ and $(g_m)_p$ increased significantly as the external pH was increased from 6.5 to 8.5. Decreases in $(g_m)_p$ and $(I_m)_p$ were observed when cells were transferred to nonbuffered

Table 3. Effects of various external ions on $(I_m)_p$, $(g_m)_p$, $(V_m)_p$ and g_m at rest under ramp voltage clamping in tonoplast-free cells of *Nitellopsis*[a]

	$(I_m)_p$ (mA/m²)	$(g_m)_p$ (S/m²)	$(V_m)_p$ (mV)	At rest g_m (S/m²)
Control	184.0 ± 21.6	3.19 ± 0.45	−101.8 ± 6.6	0.62 ± 0.04
6 mM NaCl	292.8 ± 41.1	8.96 ± 1.65	−67.8 ± 4.9	0.89 ± 0.05
6 mM HEPES	275.0 ± 39.0	5.99 ± 0.78	−88.0 ± 3.1	0.79 ± 0.05
6 mM $NaNO_3$	308.7 ± 43.1	7.81 ± 1.45	−72.2 ± 4.6	0.87 ± 0.09
6 mM Na_2SO_4	412.9 ± 57.7	11.36 ± 1.26	−72.6 ± 7.2	1.07 ± 0.09

[a] 6 mM each of NaCl, HEPES-Na, $NaNO_3$ and Na_2SO_4 were added to APW-2. All data are shown as mean ± SEM ($n = 5$).

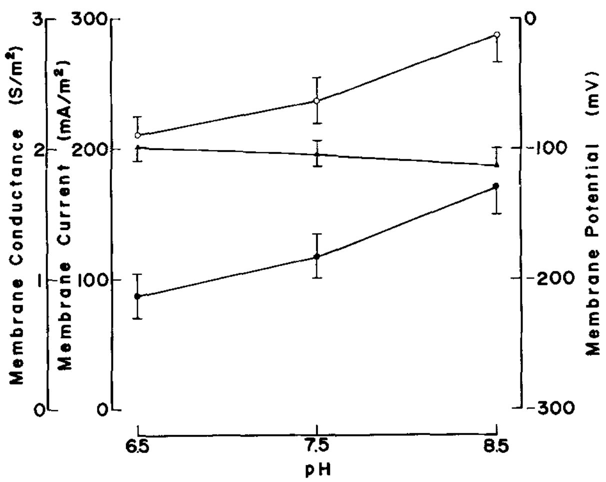

Fig. 13. $(g_m)_p$ (open circles), $(I_m)_p$ (filled circles) and $(V_m)_p$ (filled triangles) in relation to external pH under ramp voltage clamping in tonoplast-free cells of *Nitellopsis*. External solutions were APW-7, -2, and -8 for pH 6.5, 7.5 and 8.5, respectively. All data are shown as mean ± SEM

Table 4. Effects of external NaCl and choline-Cl on E_m and g_m at the action potential peak under current-clamp measurement, and $(I_m)_p$, $(g_m)_p$ and $(V_m)_p$ under ramp voltage-clamp measurement in tonoplast-free cells of *Nitellopsis*[a]

	At action potential peak	
	E_m (mV)	g_m (S/m²)
Control	−88.9 ± 1.0 (4)	4.75 ± 0.29 (4)
6 mM NaCl	−55.2 ± 1.2 (4)	11.48 ± 1.66 (4)
6 mM choline-Cl	−61.6 ± 1.2 (4)	10.96 ± 1.34 (4)

	Under voltage-clamp measurement		
	$(I_m)_p$ (mA/m²)	$(g_m)_p$ (S/m²)	$(V_m)_p$ (mV)
Control	184.0 ± 31.6 (5)	3.19 ± 0.45 (5)	−101.8 ± 6.5 (5)
6 mM NaCl	292.8 ± 41.1 (5)	8.96 ± 1.65 (5)	−67.8 ± 4.9 (5)
6 mM choline-Cl	230.7 ± 21.6 (5)	9.22 ± 1.28 (5)	−75.5 ± 6.2 (5)

[a] 6 mM NaCl and choline-Cl were added to APW-2. All data are shown as mean ± SEM. Number of cells used is shown in parentheses.

APW-1 (pH 5.6) (*data not shown*), indicating that the observed changes in $(I_m)_p$ and $(g_m)_p$ were not caused by changes in buffer species or concentration. $(V_m)_p$ was scarcely affected by pH_o.

Effects of External Ionic Strength

As shown in Fig. 4, high external concentrations of choline-Cl caused depolarization of the action potential peak and increased g_m at the action potential peak, opposite to the Nernst potential for Cl^-. We compared the effects of various anions added to APW-2 in the form of Na salts on the electrical parameters obtained under voltage-clamp conditions (Table 3). $(I_m)_p$ was increased by about 50% by the addition of 6 mM NaCl, HEPES-Na or $NaNO_3$, and 120% by the addition of 6 mM Na_2SO_4. The order of the effect on $(I_m)_p$ was Na_2SO_4 > NaCl = $NaNO_3$ = HEPES-Na. This order is consistent with the order for total ionic strength of the external solution. Increases in $(g_m)_p$ and depolarization of $(V_m)_p$ were observed for all Na salts tested. 12 mM sorbitol added to APW-2 had no effect, indicating that these effects were not a consequence of increased osmolarity of the external solution. The same tendency in $(g_m)_p$ was also seen at the resting E_m.

The action potential peak under current clamping was depolarized 33.7 mV by the addition of 6 mM NaCl, with a large increase in g_m at the action

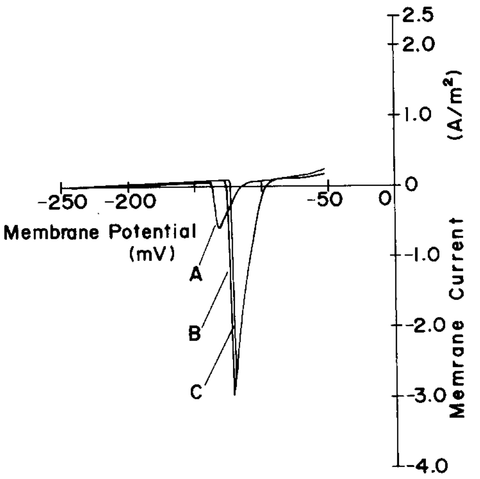

Fig. 14. Effects of external $NaNO_3$ and NaCl on the *I-V* relation recorded under ramp voltage clamping in a normal cell of *Nitellopsis*. (*A*) control; (*B*) 6 mM NaCl; (*C*) 6 mM $NaNO_3$. Na salts were added to APW-2

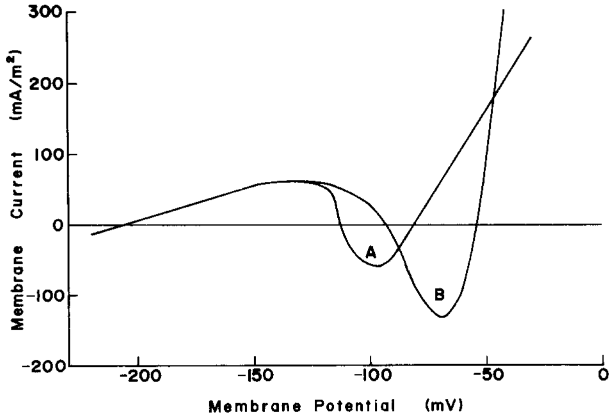

Fig. 15. Effects of external NaCl on the *I-V* relation recorded under ramp voltage clamping in a tonoplast-free *Nitellopsis* cell perfused with medium containing 7.5 mM NaCl. (*A*) control; (*B*) 6 mM NaCl. NaCl was added to APW-2

potential peak (Table 4). Substitution of Na^+ by choline$^+$ scarcely affected g_m at the action potential peak, although the action potential peak shifted to a more negative level. There was no significant difference in $(V_m)_p$, $(I_m)_p$ and $(g_m)_p$ when 6 mM NaCl or 6 mM choline-Cl was added to APW-2 (Table 4).

A marked increase in $(I_m)_p$ upon increasing external ionic strength was also observed in normal cells of *Nitellopsis* (Fig. 14) and *Chara*. $(I_m)_p$ increased about five times and the $(V_m)_p$ shifted in the positive direction by about 10 mV when 6 mM NaCl or $NaNO_3$ was added to APW-2.

Effects of High Internal Na^+ Concentration and External La^{3+} on Inward Current

Even when the internal Na^+ concentration was increased by perfusing the cell with a medium containing 7.5 mM NaCl, enhancement of $(I_m)_p$ was observed in APW-2 + 6 mM NaCl (Fig. 15). To examine the possibility that Ca^{2+} carries the enhanced inward current, we examined whether or not the enhanced inward current was blocked by La^{3+} as shown in Fig. 8. Increases in $(I_m)_p$ and $(g_m)_p$ caused by addition of 3.0 mM $NaNO_3$ to APW-2 were largely suppressed by the application of 0.2 mM $LaCl_3$ (38 and 73% of control experiment, respectively) (Table 5).

Table 5. Removal of enhancement of $(I_m)_p$ and $(g_m)_p$ by La^{3+} recorded under ramp voltage clamping in tonoplast-free cells of *Nitellopsis*[a]

	$(I_m)_p$ (mA/m²)	$(g_m)_p$ (S/m²)
Control	206.1 ± 29.5	4.83 ± 0.65
3 mM $NaNO_3$	341.8 ± 57.1	6.85 ± 1.77
3 mM $NaNO_3$ + 0.2 mM $LaCl_3$	77.9 ± 13.1	3.51 ± 0.88

[a] $NaNO_3$ and $LaCl_3$ were added to APW-2. All data are shown as mean ± SEM (n = 4).

Ion Fluxes During Excitation

Since Sr^{2+} has almost the same effects as Ca^{2+} on the electric properties of the plasmalemma (Fig. 12 and Table 2), we used Sr^{2+} as a tracer for Ca^{2+} in the measurement of Ca^{2+} influx. Sr^{2+} influx in the resting and excited states was measured by replacing the external medium with the one containing 0.5 mM Sr^{2+} but no Ca^{2+} (APW-5). The incubation time was 2 min. Although E_m and g_m at the plateau of the action potential changed slightly, we calculated the ion fluxes under the assumption that ion fluxes were constant throughout the action potential plateau.

As shown in Table 6, the Sr^{2+} influx increased about 13-fold during excitation. At the same time, K^+ efflux increased by about 19-fold. This clearly indicates the participation of Ca^{2+} as an ion carrying inward current during membrane excitation in tonoplast-free cells. Furthermore, the addition of 6 mM $NaNO_3$ to the external solution caused a 30% increase in both Sr^{2+} influx and K^+ efflux during membrane excitation. Even in the resting state, both Sr^{2+} influx and K^+ efflux increased significantly upon addition of 6 mM $NaNO_3$ to the external solution.

Table 6. Effects of high external concentrations of $NaNO_3$ on Sr^{2+} influx and K^+ efflux in the resting and excited states of plasmalemma in tonoplast-free cells of *Nitellopsis*[a]

	Resting state		Excited state	
	J_{Sr}	J_K	J_{Sr}	J_K
Control	0.13 ± 0.07 (3)	0.20 ± 0.05 (3)	1.64 ± 0.26 (5)	3.87 ± 1.25 (5)
+6 mM $NaNO_3$	0.32 ± 0.05 (3)	0.55 ± 0.17 (3)	2.48 ± 0.12 (6)	6.62 ± 1.06 (6)

[a] $NaNO_3$ was added to APW-5. All data are shown as mean ± SEM. Number of cells used is shown in parentheses. Fluxes are shown as μmol/m²/sec.

Discussion

Demonstration of Ca^{2+}-Spike Action Potential in Tonoplast-free Cells

Two transient inward currents were reported under step-voltage clamping in normal cells of *Chara* (Beilby & Coster, 1979) and *Nitellopsis* (Lunevsky et al., 1983). Beilby and Coster (1979) considered the first transient current to be carried by Cl^- and the second one by Ca^{2+}. On the other hand, Lunevsky et al. (1983) considered the former to be Ca^{2+} current and the latter Cl^- current. Superposition of Ca^{2+} and Cl^--spikes during the action potential makes analysis of the ionic processes occurring during membrane excitation complicated. The situation is simpler in tonoplast-free cells, since only Ca^{2+} channels seem to be activated during plasmalemma excitation (Kikuyama et al., 1984). This is supported by the fact that inward membrane current consisted of a single component lasting for more than 20 sec without time-dependent inactivation (Fig. 3*A*). This long-lasting inward membrane current is consistent with the rectangular action potential recorded under current clamping (Shimmen et al., 1976; Tazawa et al., 1976). The simple shape of the inward membrane current may indicate the involvement of a single ion species in plasmalemma excitation.

The absence of Cl^- channel activation in tonoplast-free cells was supported by the following results. 1) Increasing $[Cl^-]_o$ caused depolarization of E_m and an increase in g_m at the action potential peak under current clamping, and the displacement of $(V_m)_p$ to positive values and an increase in $(I_m)_p$ and $(g_m)_p$ under voltage clamping. These results cannot be explained by a shift in the Nernst potential for Cl^-. 2) Varying the intracellular Cl^- concentration in tonoplast-free cells between 0.01 and 29.0 mM scarcely affected E_m at the action potential peak (Shimmen & Tazawa, 1980). 3) $(I_m)_p$ was insensitive to DIDS, which reduces the $(I_m)_p$ in normal cells. 4) We could not detect any Cl^- efflux during excitation in tonoplast-free cells. The same results were also reported by Kikuyama et al. (1984).

The presence of Ca^{2+} channels in the plasmalemma of tonoplast-free cells is demonstrated by the following results. 1) When $[Cl^-]_o$ was kept constant, $(I_m)_p$ increased with increase in $[Ca^{2+}]_o$. This was not due to the enhancement of $(I_m)_p$ by increased external ion strength (*cf.* later discussion), since this decreased from 12.4 to 9.4 mM when $[Ca^{2+}]_o$ was increased. 2) The dependencies of $(V_m)_p$ and the action potential peak on $[Ca^{2+}]_o$ were in good agreement with Nernst equation calculations for Ca^{2+}. Thus, the plasmalemma behaves like a Ca^{2+} electrode. 3) Hyperpolarization of the action potential peak by lowering free $[Ca^{2+}]_o$ in the cell wall with EGTA is further evidence for the participation of Ca^{2+} in membrane excitation. The membrane is also permeable to Ca^{2+} during the action potential plateau, since the plateau E_m is also depolarized by the theoretically expected amount for a tenfold increase in $[Ca^{2+}]_o$. The two stable states of the membrane, the resting state and the depolarized state, found by Shimmen et al. (1976) may therefore be characterized as the closed and opened states of the Ca^{2+} channel, respectively. A similar $[Ca^{2+}]_o$ dependence has been observed in normal Characeae cells (Findlay, 1961; 1962; 1964; 1970; Hope, 1961*a,b*; Findlay & Hope, 1964*a,b*) and in higher plants such as *Aldrovanda* (Iijima & Sibaoka, 1983) and *Dionaea* (Hodick & Sievers, 1986). The resting E_m was also depolarized by increasing $[Ca^{2+}]_o$ (Fig. 6). This has been explained as being due to depolarization of the passive diffusion potential but not the active electrogenic potential (Shimmen & Tazawa, 1983).

4) La^{3+}, as a Ca^{2+} channel blocker, reduced $(I_m)_p$. However, La^{3+} is not a specific inhibitor in Characeae cells. The cation permeability in *Chara corallina* was reduced by La^{3+} (Keifer & Spanswick, 1978). The Cl^- current activated by membrane hyperpolarization in *Chara inflata* was inhibited by La^{3+} (Tyerman et al., 1986). La^{3+} also

Table 7. Effects of high external concentrations of $NaNO_3$ on I_{Ca} and I_K at the action potential peak[a]

	E_m (mV)	g_{Ca}/g_m	g_K/g_m	g_{Ca} (S/m²)	g_K (S/m²)	I_{Ca} (mA/m²)	I_K (mA/m²	$2FJ_{Sr}$ (mA/m²)	FJ_K (mA/m²)
Control	−35.6	0.43	0.57	1.71	2.26	280.7	277.9	315.6 ± 49.7	373.3 ± 120.4
+6 mM $NaNO_3$	−36.9	0.43	0.57	3.27	4.33	532.2	535.6	467.8 ± 19.8	638.7 ± 102.0

[a] Membrane currents calculated from fluxes of Sr^{2+} ($2FJ_{Sr}$) and K^+ (FJ_K) are also shown.

inhibits the large inward current during membrane excitation in normal cells of *Chara corallina* under voltage clamping (Tsutsui et al., 1986). Cl^- current inhibition may result from the blockage of Ca^{2+} entry, since Ca^{2+} is thought to activate the Cl^- channel. Both Ca^{2+} and Cl^- currents were blocked by La^{3+} (Lunevsky et al., 1983).

5) $(I_m)_p$ and $(g_m)_p$ were also reduced by the specific Ca^{2+} channel blocker nifedipine. Nifedipine effectively inhibited turgor regulation in *Lamprothamnium*, which involves Ca^{2+} channel activation (Okazaki & Tazawa, 1986*a*,*b*). Bud formation in *Funaria*, which seems to be induced via Ca^{2+} channel activation, was inhibited by nifedipine and the Ca^{2+} antagonist (−)202-791 and accelerated by the Ca^{2+} agonist (+)202-791 (P.A. Conrad & P.K. Hepler, *personal communication*). The positive shift in $(V_m)_p$ by La^{3+} may be explained using Eqs. (2) and (3) (*see below*). If the inhibitory effect of La^{3+} on g_K were larger than on g_{Ca}, g_K/g_m would decrease. Since E_{Ca} and E_K are constant, $E_m - E_{Ca}$ would decrease and E_m ($(V_m)_p$, too) depolarize. In the case of nifedipine, a decrease in g_{Ca} without a change in g_K would increase g_K/g_m. Thus, E_m would hyperpolarize in contrast to the case of La^{3+} treatment.

6) Enhancement of $(I_m)_p$ by Ca^{2+} channel agonist, BAY K 8644 strongly supports the presence of Ca^{2+} channel in plasmalemma of tonoplast-free cells. However, inhibition and enhancement of $(I_m)_p$ by Ca^{2+} channel agonist, 202-791 (+) and antagonist BAY K 5552, respectively, is contrary to our expectation. The Ca^{2+} channel in *Nitellopsis* may be different in structure from those of animal origin.

7) Ca^{2+} influx measured using Sr^{2+} as a tracer significantly increased during membrane excitation (Table 6). The Sr^{2+} influx during excitation is more than one hundred times larger than the Ca^{2+} influx measured by aequorin light emission in tonoplast-free *Chara* cells, when $[Ca^{2+}]_o$ was 1.0 mM (Kikuyama & Tazawa, 1983). This discrepancy may be due to the underestimation of Ca^{2+} flux, since only free Ca^{2+} can be detected using aequorin. The same discrepancy also exists in aequorin experiments using normal *Chara* cells, where the Ca^{2+} influx during excitation was estimated at 0.4 nmol/m²/sec (Williamson & Ashley, 1982). However, Hayama et al. (1979) reported a large Ca^{2+} influx amounting to 0.3 μmol/m²/sec during excitation using $^{45}Ca^{2+}$. This is only a little smaller than the Sr^{2+} influx measured in the present study. Our K^+ efflux is about six times larger than that measured using $^{42}K^+$ in tonoplast-free *Chara* cells (Kikuyama et al., 1984). 8) The currents carried by Sr^{2+} and K^+ during membrane excitation were calculated to be −315.6 and 373.3 mA/m²/sec, respectively (Table 7). There is no significant difference between the net Sr^{2+} and K^+ currents, and the outward current carried by K^+ efflux therefore balances the inward current carried by Sr^{2+} influx during excitation in tonoplast-free cells.

From these results, Ca^{2+} and K^+ are assumed to be the only ions which carry electric charge across the plasmalemma during excitation. Based on this assumption, g_m at the action potential peak should be the sum of the K^+ conductance (g_K) and the Ca^{2+} conductance (g_{Ca}).

$$g_m = g_K + g_{Ca}. \qquad (1)$$

The contribution of the electrogenic H^+ pump conductance (g_p) seem negligible compared with the large passive diffusion conductance during the action potential (Kishimoto et al., 1985). Thus,

$$E_m = \frac{g_K}{g_m} E_K + \frac{g_{Ca}}{g_m} E_{Ca} \qquad (2)$$

where E_K and E_{Ca} are the equilibrium potentials of K^+ and Ca^{2+}, respectively. The ratio (g_K/g_m) is given as follows:

$$\frac{g_K}{g_m} = \frac{E_m - E_{Ca}}{E_K - E_{Ca}}. \qquad (3)$$

Although in the flux measurement experiment no K^+ was included in the external medium, we took $[K^+]_o$ to be 0.1 mM, the same concentration as in normal APW. Taking the endogenous total Ca^{2+} concentration after disintegration of tonoplast to be 0.3 mM (Tazawa et al., 1976), $[Ca^{2+}]_i$ was calculated to be 2.45×10^{-8} M. $[Ca^{2+}]_o$ was taken to be 0.5 mM, the same as the $[Sr^{2+}]_o$ used in flux measurement.

From these values E_K and E_{Ca} were calculated to be −159.8 and +126.9 mV, respectively. We could not measure the exact value of g_m at the peak of action potential, because we used the "K-anesthesia" method (Shimmen et al., 1976). We used g_m at $(V_m)_3$ in voltage-clamp experiments, which is regarded as being identical to E_m at the action potential peak. The K^+ and Ca^{2+} currents (I_K and I_{Ca}) are given by

$$I_K = g_K(E_m - E_K) \quad (4)$$

$$I_{Ca} = g_{Ca}(E_m - E_{Ca}). \quad (5)$$

8) The currents carried by K^+ and Ca^{2+} calculated from the actual values of ion fluxes (J_K and J_{Ca}) were in good agreement with I_K and I_{Ca} calculated from Eqs. (4) and (5) (Table 7). Thus, K^+ and Ca^{2+} currents are well accounted for by K^+ and Ca^{2+} fluxes.

The amplitude of the Ca^{2+} current in normal *Nitellopsis* cells at low external ionic strength was about 200 mA/m² (Lunevsky et al., 1983), which is comparable to the Ca^{2+} current in tonoplast-free cells. The reversal potential is the V_m at which the current changes direction from inward to outward; this is designated as $(V_m)_3$ on the *I-V* curve. The reversal potential is −100 to −50 mV in APW-2. This value is comparable to the reversal potential of the presumptive Ca^{2+} current in normal *Chara* (−50 mV) (Beilby & Coster 1979) and *Nitellopsis* (−60 to −20 mV) (Lunevsky et al., 1983) cells. These facts imply that the activity of the Ca^{2+} channel is not suppressed by disintegration of the tonoplast. The possibility that chelation of Ca^{2+} by EGTA may inhibit Ca^{2+}-induced Cl^- channel activation in tonoplast-free cells is rejected, since an increase in $[Ca^{2+}]_i$ depolarized E_m without causing Cl^- efflux in *Nitellopsis* (Mimura & Tazawa, 1983). However, the enhancement of inward current by high external ionic strength was much larger in normal cells than in tonoplast-free cells, suggesting the activation of some ion channel other than the Ca^{2+} channel, probably the Cl^- channel, by Ca^{2+}. Thus dilution of some Ca^{2+}-sensitizing cytoplasmic factor responsible for Ca^{2+}-induced Cl^- channel activation may explain the disappearance of functional Cl^- channels. Recently, calcium-dependent anion channel was demonstrated in water mold (Caldwell et al., 1986).

Characterization of Plasmalemma Ca^{2+} Channel in Tonoplast-free Cells

We obtained the following order of selectivity for divalent cations in relation to Ca^{2+} channel activation in tonoplast-free *Nitellopsis* cells, $Ca^{2+} = Sr^{2+} > Mn^{2+} = Mg^{2+} = Ba^{2+}$. Normal cells of *Nitellopsis* became inexcitable when Ca^{2+} was replaced by Mg^{2+}, but remained excitable when Ca^{2+} was replaced with Sr^{2+}, although the duration of action potential was prolonged (Findlay, 1970). Action potentials could not be generated (Findlay & Hope, 1964*a*) and the transient inward current disappeared (Findlay & Hope, 1964*b*) in normal cells of *Chara australis* when the external divalent cation was Ba^{2+}, Cd^{2+}, Ni^{2+}, Mg^{2+} or Mn^{2+}. Sr^{2+} could replace Ca^{2+} in tonoplast-free cells although $(I_m)_p$ was less with Sr^{2+} than with Ca^{2+}. Furthermore, tonoplast-free cells of *Chara* could not produce action potentials when Ca^{2+} was replaced by Mg^{2+} (Shimmen et al., 1976). Thus *Nitellopsis* and *Chara* have the same selectivity for divalent cations with respect to Ca^{2+} channel activation. Similarly the action potential of water mold, *Blastocladiella*, requires external Ca^{2+} or Sr^{2+} (Caldwell et al., 1986). In *Aldrovanda*, Mg^{2+} could not replace Ca^{2+} (Iijima & Sibaoka, 1983). In *Dionaea*, however, Mg^{2+} could replace Ca^{2+} in generating action potentials although the duration was prolonged (Hodick & Sievers, 1986). In animal cells, not only Ca^{2+} and Sr^{2+} but also Ba^{2+} can carry inward current (Hagiwara & Byerly, 1981).

The effects of pH_o on Ca^{2+} channel activation are different from the effects of high external ionic strength, since $(V_m)_p$ was not affected by pH_o. Similar dependence of the Ca^{2+} channel on pH_o has been reported in animal cells and discussed in terms of decreased external negative surface potential induced by lowering pH_o (Iijima et al., 1986), even though displacement of the *I-V* curve was observed in this case.

Enhancement of Inward Current by Increased External Ionic Strength

The effects of increased external ionic strength on the electrophysiological properties of the plasmalemma at rest and during excitation are not due to osmotic effects since the addition of sorbitol to the external solution had no effect. Because the enhancement of $(I_m)_p$ was scarcely affected by the ionic species for both anions and cations, these effects may be attributed to an increase in the total ionic strength rather than the increase in concentration of some particular ion species.

Since the external solution usually has very low ionic strength, increased ionic strength would increase the ionic conductance and may cause the overestimation of $(I_m)_p$. However, this cannot explain the positive shift of $(V_m)_p$ and action potential peak and enhancement of Sr^{2+} influx.

There are three candidates for the ion carrying the enhanced inward I_m caused by the increased ionic strength in the external solution. The first is Na^+ influx. $[Na^+]_i$ is estimated as 0.6 mM in tonoplast-free *Nitellopsis* cells, since $[Na^+]_c$ = 12.0 mM

(Katsuhara & Tazawa, 1986). E_{Na} is calculated to be 59.4 mV when $[Na^+]_o$ is 6.1 mM. Thus the net Na^+ flux is electrochemically directed inward, when V_m is more negative than E_{Na}. However, Na^+ is not likely to carry the enhanced inward current, due to the following considerations. First, choline$^+$, which has a large ionic radius and cannot pass through the Na^+ channel in squid giant axons (Hodgkin & Huxley, 1952), was as effective as Na^+ in *Nitellopsis* (Table 5). Second, enhancement of inward current could be observed even in cells perfused with media containing Na^+ at almost the same concentration as the external solution (Fig. 15).

The second candidate is Cl^- efflux. However, there was no difference in the enhanced inward current between NaCl and $NaNO_3$ (Table 4). Furthermore, we could not detect enhanced Cl^- efflux during action potentials even when 3 mM $NaNO_3$ were added to APW-6 (*data not shown*). Thus Cl^- is eliminated as a candidate for the ion carrying enhanced inward I_m.

The last possibility is Ca^{2+} influx. The enhanced inward current was blocked by La^{3+} as was the Ca^{2+} current at low ionic strength (Fig. 7). Sr^{2+} influx induced by membrane excitation was increased significantly by the addition of 6 mM $NaNO_3$ with a large accompanying increase in K^+ efflux (Table 6). I_{Ca} and I_K calculated from Eqs. (1) to (4), can almost be accounted for by currents calculated from Sr^{2+} influx and K^+ efflux, respectively (Table 7). Thus it is concluded that the enhanced inward current under high external ionic strength is mostly carried by Ca^{2+}.

Since g_m and both Sr^{2+} and K^+ fluxes in the resting state were also increased, high ionic strength not only stimulates Ca^{2+} channel activation but also increases g_{Ca} in the resting state.

The prolongation of the action potential by increased concentrations of external monovalent cations in tonoplast-free cells of *Chara* has been explained by assuming the direct action of cations on negative charges on the membrane (Shimmen et al., 1976), based on the two stable state hypotheses (Tasaki, 1968). This prolongation may be a reflection of enhanced Ca^{2+} channel activation causing enhanced inward Ca^{2+} current. Suppression of this monovalent cation effect by external divalent cations such as Mg^{2+} and Mn^{2+} may also be explained from the reduction in Ca^{2+} current caused by these ions. However, the prolongation of the action potential was also decreased by Ca^{2+} and Sr^{2+}. Increasing $[Cl^-]_o$ greatly increased the net inward current in normal cells of *Chara australis* (Findlay & Hope, 1964*b*). The same effect was also seen with NO_3. The inward current remaining after inhibition of the Cl^- channel by ethacrinic acid in normal *Nitellopsis* cells was about 1 A/m^2 (Lunevsky et al., 1983). This large value may be due to the high external ionic strength they used. However, Kishimoto (1964) observed a decrease in the amplitude of the transient inward current by increasing $[Cl^-]_o$ in *Nitella*. The reason for this discrepancy is not known. The effect of the high external ionic strength is very similar to the effect of increased $[Ca^{2+}]_o$ with respect to the enhancement of $(I_m)_p$ and the displacement of $(V_m)_p$ to more positive values. Decrease in the surface negative potential due to increased ionic strength may be involved in the mechanism of I_{Ca} stimulation. The decrease in the surface negative potential may be caused by increase in the external ionic strength and also by decrease in pH_o. However, the former treatment enhanced but the latter inhibited $(I_m)_p$. More detailed experiments using tonoplast-free cells under voltage-clamp conditions are necessary to investigate the relationship between Ca^{2+} channel activation and surface potential. Furthermore, the possibility that protein phosphorylation and dephosphorylation may regulate Ca^{2+} channel activation was recently suggested by Shiina and Tazawa (1986) using tonoplast-free *Nitellopsis* cells.

The authors wish to thank Dr. T. Shimmen and Dr. R. Wayne for critically reading this manuscript and Dr. M. Kikuyama for technical suggestions in construction of the V-clamp apparatus. Dr. Wayne kindly shared with us BAY K 5552 and 8644 which were originally gifts from Dr. Pfizer (Gronton, CT) and 202-791(±) from Dr. H.Y.L. Tung (University of Texas). This work was supported by a Grant-in-Aid for Special Project Research (No. 61215007) from the Ministry of Education, Science and Culture of Japan, and by Special Coordination Funds for the Promotion of Science and Technology from the Science and Technology Agency of Japan.

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Received 4 November 1986; revised 29 January 1987