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Intracellular Chloride and Potassium Ions in Relation to Excitability of *Chara* Membrane

Teruo Shimmen* and Masashi Tazawa*
Department of Biology, Faculty of Science, Osaka University, Toyonaka, Osaka 560, Japan

Summary. Internodal cells of *Chara australis* were made tonoplast-free by replacing the cell sap with EGTA-containing media; then the involvement of internal Cl^- and K^+ in the excitation of the plasmalemma was studied.

$[Cl^-]_i$ was drastically decreased by perfusing the cell interior twice with a medium lacking Cl^-. The lowered $[Cl^-]_i$ was about 0.01 mM. Cells with this low $[Cl^-]_i$ generated action potential and showed an *N*-shaped *V-I* curve under voltage clamped depolarization like Cl^--rich cells containing 13 or 29 mM Cl^-. E_m at the peak of the action potential was constant at $[Cl^-]_i$ between 0.01 and 29 mM. The possibility that the plasmalemma becomes as permeable to other anions as to Cl^- during excitation is discussed.

At $[Cl^-]_i$ higher than 48 mM, cells were inexcitable. When anions were added to the perfusion medium to bring the K^+ concentration to 100 mM, NO_3^-, F^-, SO_4^{2-}, acetate, and propionate inhibited the generation of action potentials like Cl^-, while methane sulfonate, PIPES, and phosphate did not inhibit excitability.

The duration of the action potential depended strongly on the intracellular K^+ concentration. It decreased as $[K^+]_i$ (K-methane sulfonate) increased. Increase in $[Na^+]_i$ (Na-methane sulfonate) also caused its decrease, although this effect was weaker than that of K^+. The action of these monovalent cations on the duration of the action potential is the opposite of their action on the membrane from the outside (*cf.* Shimmen, Kikuyama & Tazawa, 1976, *J. Membrane Biol.* **30**:249).

In Characeae cells, permeability of the plasmalemma to Cl^- increases enormously upon excitation (Gaffey & Mullins, 1958; Mullins, 1962; Hope & Findlay, 1964; Mailman & Mullins, 1966; Haapanen & Skoglund, 1967; Findlay, 1970; Oda, 1976). This is comparable to the enormous increase in Na^+ permeability of squid axon membrane during excitation (Hodgkin & Huxley, 1952).

Like the Na^+ hypothesis in nerve cells, an analogous hypothesis arises that in Characeae cells an increase in Cl^- permeability and an outrush of Cl^- across the membrane might be essential for excitation (p. 114 in Hope & Walker, 1975). This problem can be resolved by testing the excitability of cells having very low intracellular Cl^- concentrations. For this purpose, the Cl^- concentration in the cytoplasm that the inner surface of the plasmalemma faces must be modified. The chemical composition of the cytoplasm of Characeae cells can be controlled by making the cell tonoplast-free by replacing the cell sap with a medium containing EGTA [1], a Ca^{2+} chelator, by vacuolar perfusion (Williamson, 1975; Tazawa, Kikuyama & Shimmen, 1976). The tonoplast-free cell can generate action potentials as can the normal cell (Tazawa et al., 1976; Shimmen et al., 1976). One purpose of the present study was to clarify the role of internal Cl^- in membrane excitation by controlling its concentration.

Our previous work (Shimmen et al., 1976) showed that tonoplast-free cells generate rectangular action potentials of long durations. In the experiment, the internal K^+ concentration of the tonoplast-free cells was lowered to about $^1/_4$ the K^+ concentration in normal cytoplasm, since the perfusion medium (Cl in Table 1) contained only 18 mM K^+. To test the possibility that the internal K^+ concentration might have affected the duration of the action potentials,

* *Present address:* Department of Botany, Faculty of Science, University of Tokyo, Hongo, Tokyo 113, Japan

[1] *Abbreviations:* EGTA, ethyleneglycol-bis-(β-aminoethylether) N, N′-tetraacetic acid; ATP, adenosine-5′-triphosphoric acid; PIPES, piperazine-N, N′-bis- (2-ethanesulfonic acid)

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Table 1. Compositions of various perfusion media containing EGTA

	Medium					
	Cl	SO_4	CH_3COO	TM	P	PIPES
EGTA	5	5	5	5	5	5
$MgCl_2$	6	0	0	0	0	0
$MgSO_4$	0	6	0	0	0	0
$Mg(CH_3COO)_2$	0	0	6	0	0	0
ATP	0	1	1	0	0	0
Mg·ATP[a]	0	0	0	1	1	1
Tris-maleate	5	5	5	5	0	0
Orthophosphate	0	0	0	0	5	0
PIPES	0	0	0	0	0	5
KOH	18	22	22	18	18	21
Sorbitol	290	290	290	305	305	305
pH	7	7	7	7	7	7

Concentration is shown in mM.
[a] 1 mM Mg·ATP contained 1 mM ATP and 1.5 mM Mg^{2+}.

effects of intracellular concentrations of monovalent cations on the duration of the action potential were studied.

Materials and Methods

Chara australis, cultured outdoors, was used. In winter, cells were harvested and stored in the laboratory, as *Chara australis* can not withstand winter cold. Internodal cells were isolated from neighboring cells and kept in pond water at least for one day before use. As the external medium in electrical measurements, artificial pond water (APW) containing 0.1 mM each of KCl, NaCl and $CaCl_2$ was used. Sometimes the SO_4-type APW was used, which contained 0.05 mM K_2SO_4, 0.05 mM Na_2SO_4 and 0.1 mM $CaSO_4$. The pH of APW after measurement of the membrane potential (E_m) for 30 min was 5.63 ± 0.02 (mean $\pm$ SE, $n=4$), which was approximately equal to the original pH of APW, 5.58. Since tonoplast-free cells had low resting membrane potential in winter and sometimes failed to generate the typical rectangular-shaped action potential, the pH of APW (5.6) was increased to 7.5 by adding 5 mM Tris-H_2SO_4 buffer.

Tonoplast-free cells were prepared by replacing the cell sap with an EGTA-containing medium by vacuolar perfusion (Tazawa et al., 1976). The perfusion media containing EGTA are listed in Table 1. The Cl^- concentration of the perfusion medium was increased by adding KCl to Cl medium. To increase the K^+-concentration of the perfusion medium up to 100 mM, KCl, KF, KNO_3, K-acetate, K-propionate, K-methanesulfonate, K-PIPES, or K-phosphate was added to Cl medium. Cells with cell saps replaced with EGTA-containing media were ligated with strips of polyester thread at both ends and stored in APW for about 30 min, during which most cells lost their tonoplast. Loss of the tonoplast was judged by microscopic observation and by increases in membrane potential and membrane resistance (Tazawa et al., 1976).

The membrane potential (E_m) was measured either by the conventional glass-microelectrode method or by the K^+-anesthesia method described in the previous paper (Shimmen et al., 1976). In the K^+-anesthesia method, an internode was partitioned into two chambers, one filled with 110 mM KCl solution and other with the isotonic APW-sorbitol solution. The potential difference between the chambers, which was approximately equal to E_m of the cell bathed in APW-sorbitol solution, was measured. E_m was either the potential of the cell inside against the outside in the microelectrode method or the potential of the 110 mM KCl solution against that of APW-sorbitol solution in the K^+-anesthesia method.

To completely exclude Cl^- from the electric measuring system, a modified K^+-anesthesia method was employed. One chamber was filled with the 43 mM K_2SO_4 solution and the other with the isotonic SO_4-type APW supplemented with 100 mM sorbi-

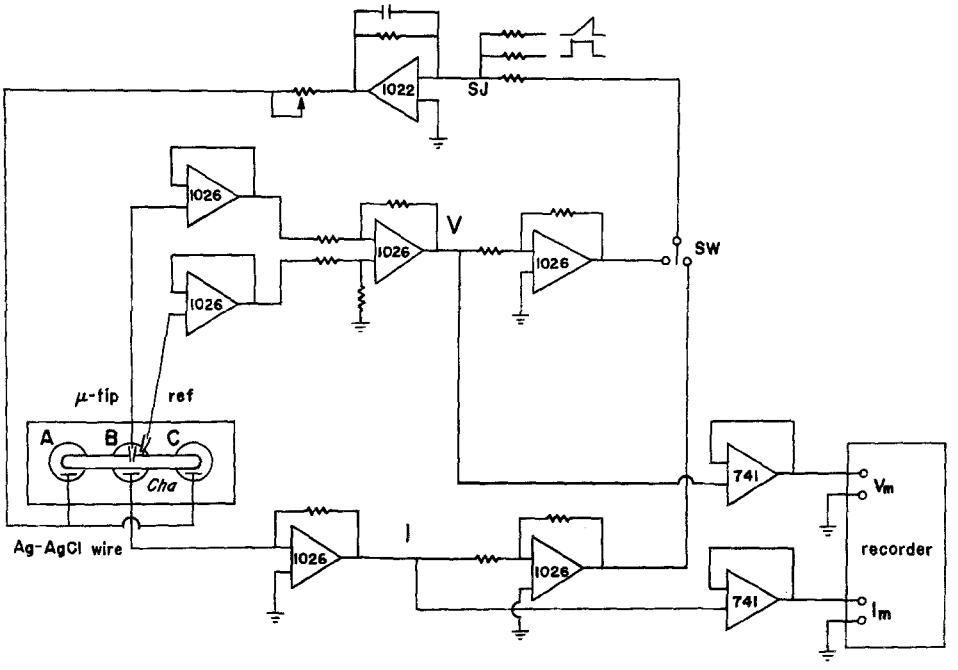

Fig. 1. A schematic diagram of the electrical circuit for voltage and current clamping. The internodal cell (*Cha*) was partitioned into three chambers, *A*, *B* and *C*. The membrane potential of the cell part in *B* was determined by measuring the potential difference between the microelectrode (*μ-tip*) whose tip had been inserted into the cell and the reference electrode (*ref*) whose tip had been placed in chamber *B*. Current was applied through Ag-AgCl wire. The current or voltage clamp was applied by connecting the current-measuring circuit (*I*) or voltage-measuring circuit (*V*) to the summing junction (*SJ*) of the control amplifier (*1022*) through the buffer amplifier (*1026*). The *I* or *V* clamp was selected with a switch (*SW*). The transmembrane voltage (V_m) and the membrane current (I_m) was recorded on the pen recorder (*recorder*)

tol. To measure the potential difference between the two chambers, 1,5% agar bridge containing SO_4-type APW was used. Since the large liquid junction potential was generated between 43 mM K_2SO_4 solution and the agar bridge, it was corrected as reported previously (Kikuyama & Tazawa, 1976).

The membrane resistance (R_m) was measured by applying small constant current pulses or constant voltage pulses to the cell across the plasmalemma.

A schematic diagram of the electrical measuring system is shown in Fig. 1 where the internodal cell (*Cha*) was partitioned into three parts, *A*, *B* and *C*, and the chambers were filled with APW. E_m of the cell part in *B* was found by measuring the potential difference between the reference electrode (*ref*) whose tip had been placed in chamber *B* and the microelectrode (*μ-tip*) whose tip had been inserted into the cell. Electrical current was applied between chamber *B* and chambers *A* and *C* through Ag-AgCl wire. The electrical circuit for the current clamp and the voltage clamp was constructed after Asai and Kishimoto (1975). The membrane current (I_m) and the potential difference between the two electrodes (V_m) were measured with the *I* circuit (*I*) and *V* circuit (*V*), respectively. They were recorded on a pen recorder (National VP-652B). The voltage or current clamp was applied by connecting the *V* or *I* circuit through the buffer amplifier (Teledyne Philblick 1026) with the control amplifier (Teledyne Philiblick 1022) at *SW*. Current or voltage pulses of rectangular or ramp shape for stimulation and measurement of the membrane resistance were applied to the cell through the summing junction (*SJ*) of the control amplifier.

The Cl^- concentration was determined by using a Ag-AgCl electrode (Tazawa, Kishimoto & Kikuyama, 1974). E_m and R_m are shown as means ± SEM with the number of cells used (n). Experiments were done at room temperature (20–25 °C) and under dim light (about 100 lux).

Results

Effects of Internal Cl^- Concentration ($[Cl^-]_i$) on E_m, R_m and Excitability

To prepare tonoplast-free cells with various $[Cl^-]_i$, the cell sap was replaced with Cl medium (Table 1) supplemented with KCl, keeping the osmotic value at 330 mM sorbitol equivalent by decreasing the sorbitol concentration. Cl^- concentrations of perfusion media thus prepared were 12, 30, 50, 70 and 100 mM, and $[Cl^-]_i$ after loss of the tonoplast are calculated to be 13, 29, 48, 66 and 93 mM, respectively, if all Cl^- ions disperse homogeneously in the tonoplast-free cell. For this calculation it was postulated that the cytoplasm volume was about $^1/_{10}$ the total cell volume (Tazawa et al., 1974) and that the Cl^- concentration in the cytoplasm was 21 mM (Tazawa et al., 1974). The intracellular fluid, composed of protoplasm and the perfusion medium (pH 7.0), was isolated after disintegration of the tonoplast, and its pH value was measured to be 6.82 ± 0.03 (mean ± SEM; $n = 10$).

To lower $[Cl^-]_i$ the cell sap was replaced with SO_4 medium instead of Cl medium (Table 1) and the cell was kept in APW. After disintegration of the tonoplast, cells were perfused again with SO_4 medium of three times the cell volume. ATP was added to SO_4 medium because the second perfusion with a medium lacking ATP would have inhibited maintenance of the normal resting potential and excitability (Shimmen & Tazawa, 1977). To check the effect of the second perfusion itself on E_m, R_m, and excitability, cells were perfused with Cl medium (12 mM Cl^-) supplemented with 1 mM ATP after disintegration of the tonoplast with SO_4 medium. It is to be noted that the second perfusion brought about a slight depolarization and lowering of R_m. To measure $[Cl^-]_i$, the intracellular medium was transferred to a glass capillary by perfusing the cell interior with a medium containing no Cl^-, which was actually a 330 mM sorbitol solution. The internal medium thus taken out was analyzed for Cl^- by means of the Ag-AgCl electrode method. Since it was found that EGTA interfered with the measurement, use of SO_4 medium was avoided. Instead, a medium containing 10 mM potassium phosphate and 330 mM sorbitol was used for the first perfusion, since phosphate disintegrated the tonoplast as EGTA and scarcely interfered with the Cl^- measurement. After disintegration of the tonoplast, the cell interior was perfused again with 330 mM sorbitol solution of three times the cell volume. To measure $[Cl^-]_i$ after the second perfusion, fluid in the cell was collected by perfusing the cell interior with 330 mM sorbitol solution. The Cl^- concentration in 330 mM sorbitol solution was measured by the Ag-AgCl electrode method. The $[Cl^-]_i$ after the second

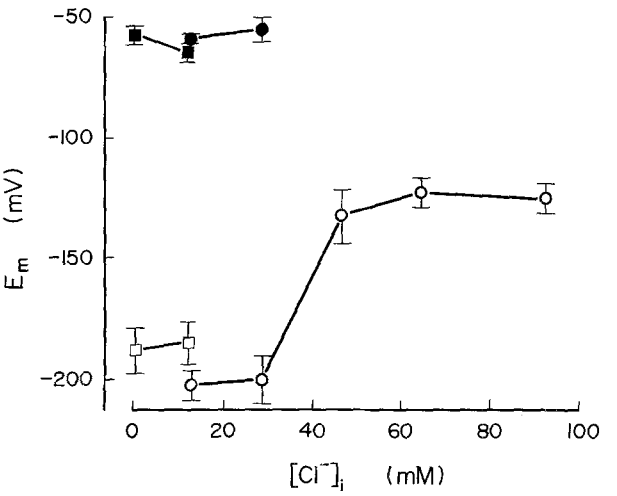

Fig. 2. Membrane potentials (E_m) in the resting state and at the peak of the action potential in relation to intracellular Cl^- concentration ($[Cl^-]_i$). Circles and squares represent E_m of cells perfused once and twice, respectively. Open and closed symbols represent E_m at the resting state and at the peak of action potential, respectively. E_m was measured by the microelectrode method. The Cl^--concentration of the perfusion medium was controlled by adding KCl to Cl medium. The $[Cl]_i$ was decreased to 0.01 mM by perfusing the cell twice with SO_4 medium. Values are means ± SEM

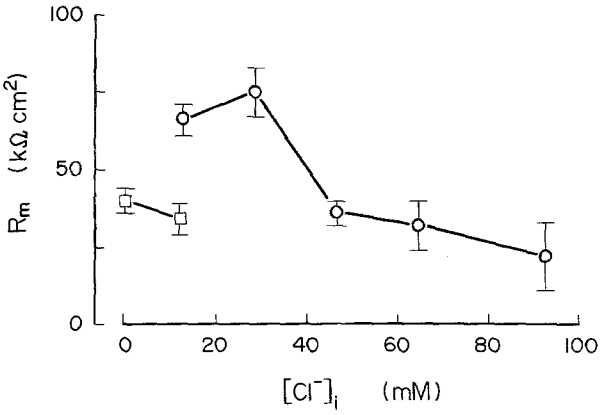

Fig. 3. Membrane resistance (R_m) in the resting state in relation to $[Cl^-]_i$. Circles and squares represent R_m of cells perfused once and twice, respectively. The Cl^- concentration of the perfusion medium was controlled by adding KCl to Cl medium. The $[Cl^-]_i$ was decreased to 0.01 mM by perfusing the cell twice with SO_4 medium. Values were plotted ± SEM

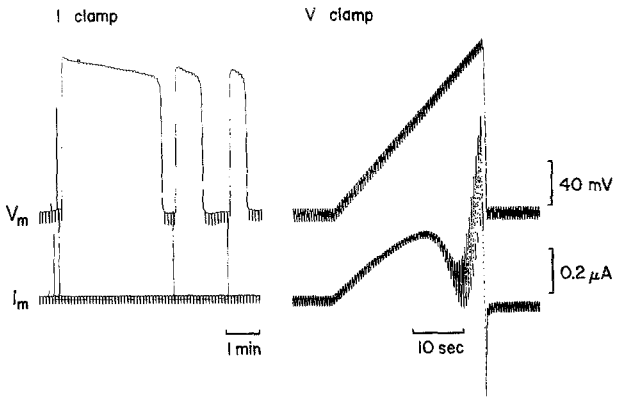

Fig. 4. Excitation of a tonoplast-free cell containing 13 mM $[Cl^-]_i$. E_m was measured by the microelectrode method. *Left:* Rectangular action potentials were elicited by outward current pulses (*I clamp*). The duration of action potential decreased with repeated excitations. *Right:* V_m was clamped at the resting potential (−202 mV) and changed in the positive direction. *N*-shaped *V-I* relation was observed (*V clamp*)

perfusion with the medium lacking Cl^- was found to be 0.01 ± 0.005 mM ($n=4$).

The membrane potential of cells with various $[Cl^-]_i$ was measured by the microelectrode method in APW. E_m at the resting state was constant at $[Cl^-]_i$ between 0.01 and 29 mM and decreased at $[Cl^-]_i$ higher than 48 mM (Fig. 2). R_m was high at $[Cl^-]_i$ of 13 and 29 mM and decreased when $[Cl^-]_i$ was made either extremely low or higher than 48 mM (Fig. 3). Low R_m at 0.01 mM $[Cl^-]_i$ is supposed to result from the second perfusion.

Action potentials were observed at $[Cl^-]_i$ lower than 29 mM. The value of E_m at the peak of the action potential was independent of $[Cl^-]_i$ (Fig. 2). A typical result of the experiment carried out on the tonoplast-free cell containing 13 mM Cl^-, which is similar to the concentration in normal cytoplasm (21 mM, Tazawa et al., 1974), is shown in Fig. 4. As reported in the previous paper (Tazawa et al., 1976; Shimmen et al., 1976), tonoplast-free cells generated rectangular shaped action potentials. The duration of the action potential decreased with repeated excitations (Shimmen et al., 1976). When V_m was shifted from the resting level (−202 mV) linearly with time in the depolarizing (or positive) direction by using the voltage-clamp circuit, the curve showed a so-called *N*-shaped *V-I* relation characteristic of excitable membranes (Ohkawa & Kishimoto, 1977). It must be noticed that even in cells containing extremely low $[Cl^-]_i$ (0.01 mM), action potentials could be elicited by electric stimulation (Fig. 5, left). When V_m was shifted linearly with time from the resting level (−144 mV) in the positive direction using the *V*-clamp, the *N*-shaped *V-I* relation was observed (Fig. 5, right). At

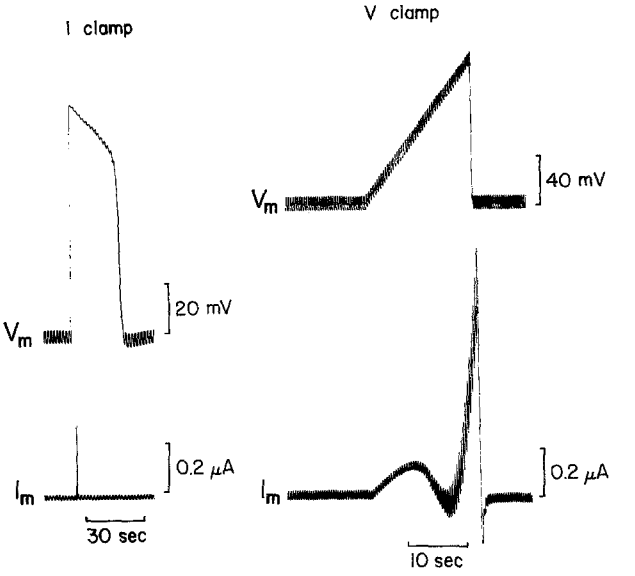

Fig. 5. Excitation of a cell containing very low $[Cl^-]_i$ (0.01 mM). E_m was measured by the microelectrode method. *Left:* The rectangular action potential was elicited by the outward current pulse (*I clamp*). *Right:* V_m was clamped at the resting potential (−144 mV) and changed in the positive direction. An *N*-shaped *V-I* relation was observed. The relatively lower resting potential may be caused by the second perfusion which was necessary to lower $[Cl^-]_i$

$[Cl^-]_i$ higher than 48 mM, no action potential could be elicited by electric stimulation and no *N*-shaped *V-I* relation was observed (data not shown). Action potentials were also observed when Cl^- ions were replaced with CH_3COO^- ions by perfusing the cell interior twice with the CH_3COO^- medium. The level of E_m at the peak of the action potential was −58 mV,

nearly equal to that of the action potential of cells with SO_4 medium (Table 2).

To see whether or not Cl^-, SO_4^{2-} or CH_3COO^- ions are involved in membrane excitation, these anions were removed from the perfusion media by using Mg·ATP instead of $MgCl_2$, $MgSO_4$ or Mg·$(CH_3COO)_2$ (Table 1). Cells perfused twice with TM, P or PIPES medium could also elicit action potentials. In these cells, E_m at the excited state was almost the same (−48 to −57 mV), irrespective of buffer species in the perfusion medium, although E_m at the resting state deviated to some extent (−176 to −193 mV) according to buffer species (Table 2). Values of R_m of cells perfused with TM, P and PIPES media were small compared to those of cells perfused with Cl, SO_4 and CH_3COO media. The difference in R_m between the former and latter cells may result from the difference in culture batches.

Since $[Cl^-]_i$ might increase during the measurement, due to Cl^- leaking from the microelectrode or coming from APW, E_m of Cl^--free cells prepared by perfusing twice with SO_4 medium was measured by the modified K-anesthesia method, where Cl^- ions were completely excluded (*see* Methods). E_m and R_m at the resting state were -169 ± 6 mV ($n=5$) and 40 ± 10 kΩ cm^2 ($n=5$), respectively. These cells also generated action potentials. E_m at the peak of the action potential was 52 ± 1 mV ($n=5$), nearly equal to that in Fig. 2 and Table 2.

Table 2. E_m and R_m of cells containing various kinds of anions at the resting and excited states

Perfusion medium	Resting state		Excited state
	E_m (mV)	R_m (kΩ cm^2)	E_m (mV)
Cl	$-201\pm7(18)$	$68\pm5(16)$	$-60\pm3(14)$
SO_4	$-189\pm8(16)$	$40\pm4(16)$	$-58\pm5(9)$
CH_3COO	$-169\pm7(5)$	$44\pm5(5)$	$-58\pm5(5)$
TM	$-193\pm13(6)$	$27\pm3(6)$	$-52\pm4(6)$
P	$-176\pm6(6)$	$28\pm4(6)$	$-48\pm5(6)$
PIPES	$-190\pm6(7)$	$34\pm4(7)$	$-57\pm5(7)$

Number of cells used is shown in parentheses.

Effect of Internal K^+ Concentration ($[K^+]_i$)

Before beginning the main experiment to see the effect of $[K^+]_i$ on membrane excitability, two problems had to be solved. First, suitable internal anions for membrane excitation and, second, a suitable external pH had to be found.

1) Internal anions favorable for excitation. Since an increase in $[K^+]_i$ in the form of KCl resulted in decreases in both E_m and R_m and also loss of excitability (Fig. 2), we tried to find anions favorable to maintain excitability. K^+ salts with various anions were added to the Cl medium to raise the K^+ concentration up to 100 mM. The osmotic value of the perfusion medium was controlled at about 330 mM sorbitol equivalent by decreasing the sorbitol concentration.

The E_m was measured in APW by the microelectrode method. When 82 mM KF, K-acetate, or K-propionate were added, both E_m and R_m decreased markedly and action potential was not elicited by the outward electrical current (Table 3). On the other hand, addition of 41 mM K_2SO_4 supported high values of E_m. R_m was extremely high for an unknown reason. The action potential was not elicited by electric stimulation. When ramp depolarization was applied to the plasmalemma by the voltage-clamp method, no *N*-shaped *V-I* relation was observed (Fig. 6). In contrast to sulfate, methane sulfonate (82 mM) could maintain not only high E_m and R_m but also excitability. Therefore, in the following experiments $[K^+]_i$ was changed by adding K-methane sulfonate to Cl medium. PIPES and orthophosphate had the same effect as methane sulfonate.

2) Effect of external pH on the excitability. Normally, tonoplast-free cells of *Chara australis* which contain

Table 3. E_m and R_m at the resting state, and excitability of cells containing various kinds of K-salts at higher concentrations

	Anion species added[a]								
	Control[b]	Methane-sulfonate	P_i	PIPES	SO_4	Acetate	Propionate	NO_3	F
E_m (mV)	$-200\pm6(6)$	$-184\pm10(5)$	-155 ± 8 (7)	$-169\pm11(5)$	$-176\pm12(5)$	$-119\pm4(4)$	$-136\pm6(7)$	$-141\pm5(5)$	$-131\pm6(5)$
R_m (kΩ cm^2)	$54\pm6(6)$	$98\pm17(5)$	$64\pm12(5)$	$56\pm18(5)$	$107\pm32(5)$	$25\pm3(4)$	$29\pm1(7)$	$33\pm7(5)$	$41\pm7(5)$
Excitability	+	+	+	+	−	−	−	−	−

Cell number used is shown in parentheses.

[a] Various K-salts were added to Cl medium (Table 1) to increase K^+-concentration up to 100 mM.

[b] Cl medium.

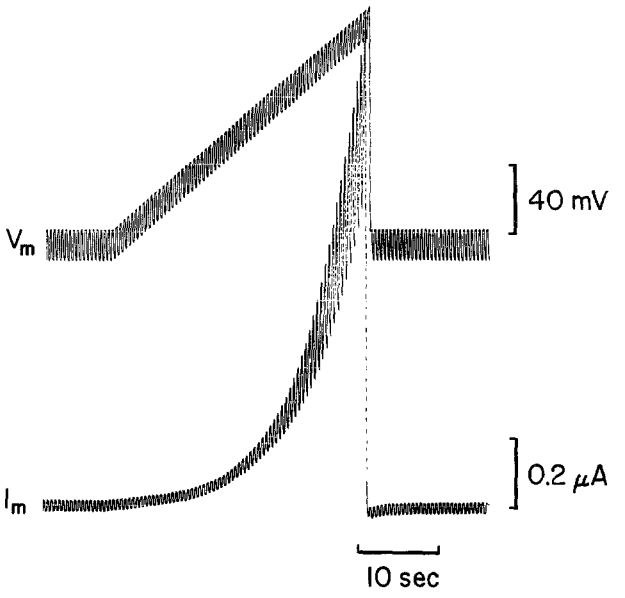

Fig. 6. Voltage-current relation obtained for a cell containing high concentration of SO_4^{2-}. E_m was measured by the microelectrode method. The cell sap was replaced with Cl medium, the K^+ concentration of which had been increased to 100 mM by adding 41 mM K_2SO_4. After disintegration of the tonoplast, V_m was first clamped at the resting level (−164 mV) with the voltage clamp and then ramp depolarization was applied to the cell. *N*-shaped *V-I* relation was not observed

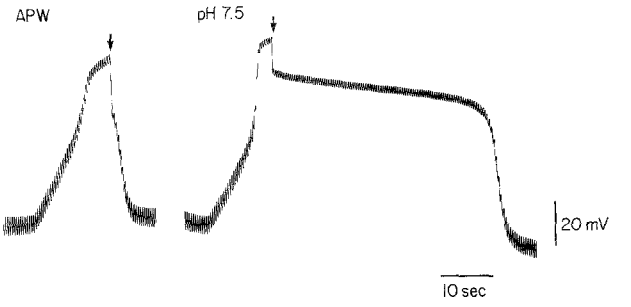

Fig. 7. Duration of action potential influenced by external pH. E_m was measured by the K^+-anesthesia method. E_m in the resting state was −126 mV in APW (pH 5.6). The action potential was elicited by the outward ramp current. E_m returned to the resting potential as soon as the current was withdrawn (first arrow). The external pH was increased from 5.6 to 7.5 by replacing APW with APW whose pH was adjusted to 7.5 with 5 mM Tris-H_2SO_4 buffer. The rectangular action potential lasted for about 45 sec after withdrawal of the current (second arrow). Constant inward current pulses of 0.01 μA were applied during the measurement

Cl medium generate action potentials of longer duration. However, when experiments on the effect of $[K^+]_i$ were conducted in winter, the cell bathed in APW often did not generate typical action potentials of longer duration. In the Fig. 7 experiment, the cell sap was first replaced with Cl medium and E_m was measured by the K^+-anesthesia method, where one half of the cell was bathed in 110 mM KCl solution and the other in APW containing 200 mM sorbitol. After disintegration of the tonoplast, E_m in the resting state was −126 mV. The cell was stimulated by the outward ramp current, which was withdrawn when the action potential was elicited (first arrow in Fig. 7). E_m returned to the original resting level soon after withdrawal of the current. APW at pH 5.6 was exchanged for APW at pH adjusted to 7.5 by 5 mM Tris-H_2SO_4 buffer. Alkalization of the bathing medium allowed generation of the typical rectangular action potential, which lasted for about 45 sec even after removal of the outward current (second arrow in Fig. 7). On the average, duration of the action potential was increased 2 ± 0.3-fold ($n=8$) by increasing pH_o from 5.6 to 7.5. In the following experiments to see the effect of $[K^+]_i$ on the duration of action potentials, APW at pH 7.5 was used as the bathing medium.

3) $[K^+]_i$ vs. duration of the action potential. The membrane potential of cells containing Cl medium with various concentrations of K-methanesulfonate was measured by the K^+-anesthesia method, where one half of the cell was bathed in APW at pH 7.5 supplemented with 200 mM sorbitol solution, another half in 110 mM KCl solution. After disintegration of the tonoplast, action potentials were elicited by electric stimuli. Duration of action potentials in tonoplast-free cells was fairly variable even among those in the same cell. The general tendency is that the duration decreases with repeated excitations and the once shortened duration tends to lengthen after a longer pause (Shimmen et al., 1976). In the present study, the longest duration, observed mostly for the first action potential, was adopted as the duration of the action potential of the cell. Since it was not easy to determine the time of termination of the action potential, the time interval between the half heights of the rising and the falling phases of the action potential was adopted as the duration of the action potential. The relation between $[K^+]_i$ and the duration of the action potential (τ) is shown in Fig. 8. When the cell vacuole was perfused with Cl medium containing 18, 60, and 100 mM K^+, $[K^+]_i$ after disintegration of the tonoplast was estimated to be 27, 65, and 101 mM, respectively, since K^+ concentration in the cytoplasm is 110 mM (Tazawa et al., 1974). The average durations of action potentials in cells containing K^+ of 27, 65, and 101 mM were 231 ± 36 sec ($n=19$), 73 ± 2 sec ($n=19$), and 6 ± 1 sec ($n=11$), respectively (Fig. 8). The typical action potential of the tonoplast-free cells containing 101 mM K^+ did not exhibit a rectangular shape but a triangular one (Fig. 9), which

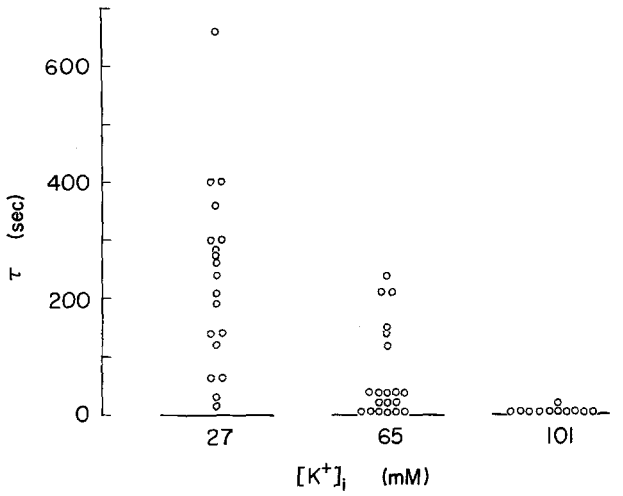

Fig. 8. Distribution of duration of action potentials (τ) of *Chara* cells in relation to $[K^+]_i$. τ was defined as the time interval between the half heights of the rising and falling phases of the action potential

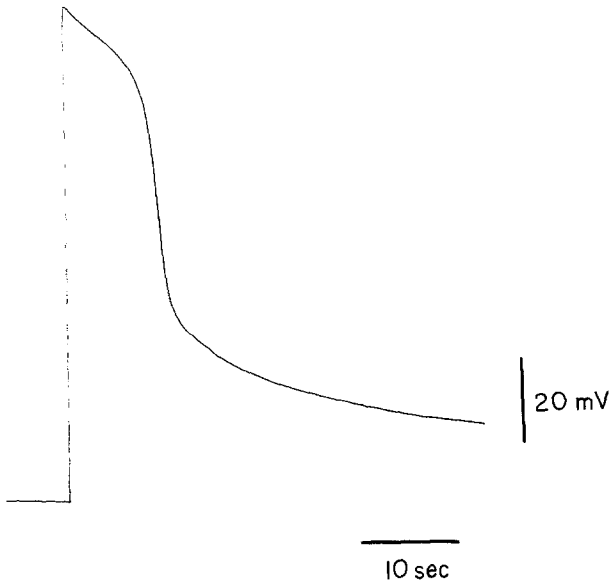

Fig. 9. Action potential generated by a cell containing about 100 mM K^+. Methane sulfonate was used as anion. E_m was measured by the K^+-anesthesia method. E_m in the resting state was −192 mV

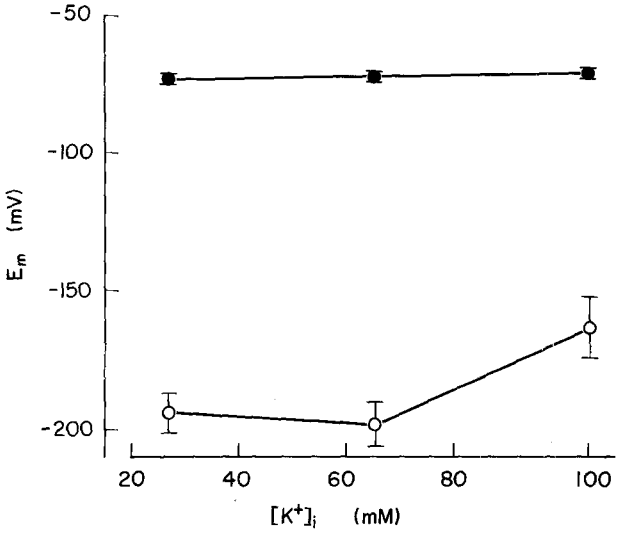

Fig. 10. Membrane potential (E_m) in the resting state (○) and at the peak of action potential (●) in relation to $[K^+]_i$. Values are means ± SEM

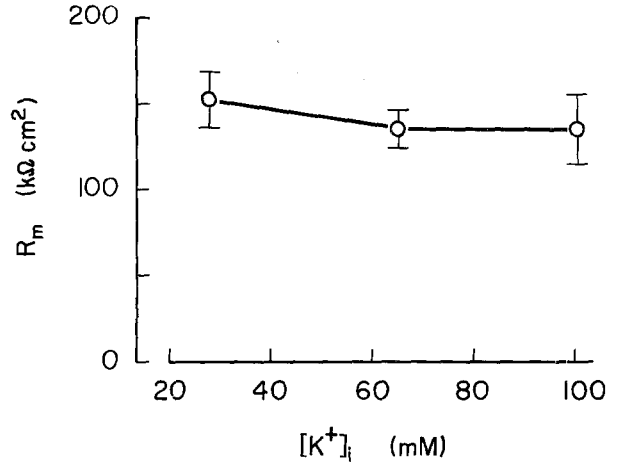

Fig. 11. Membrane resistance (R_m) in the resting state in relation to $[K^+]_i$. Values are means ± SEM

was very similar to the shape of normal action potentials of shorter duration.

E_m in the resting state and at the peak of the action potential is shown in relation to $[K^+]_i$ in Fig. 10. E_m at the peak of the action potential was constant between 27 and 101 mM of $[K^+]_i$. In the resting state, it was constant between 27 and 65 mM of $[K^+]_i$ but decreased at 101 mM.

R_m in the resting state *vs.* $[K^+]_i$ is shown in Fig. 11. R_m was nearly constant in this range of $[K^+]_i$ between 27 and 101 mM.

When the cell sap was replaced with Cl medium containing 100 mM Na^+ instead of 100 mM K^+, the cell also generated action potential. A typical example is shown in Fig. 12. The duration of the action potential was 90 ± 82 sec ($n=5$), significantly longer than that of cells with 101 mM $[K^+]_i$. Responses of E_m to current pulses at the plateau of the action potential in Figs. 6 and 12 were not significantly smaller than those in the resting state. This is contradictory to the well-known fact that R_m decreases drastically during excitation. Deviation of E_m due to small current pulses should represent the sum of the IR drop across the membrane and that across other components including the external medium, the cell wall, and the

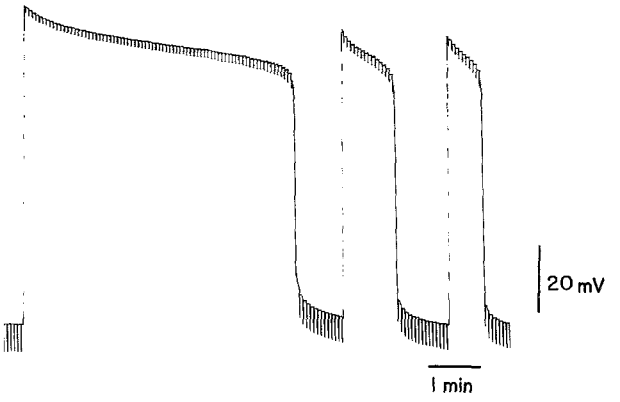

Fig. 12. Action potentials generated by a cell containing about 100 mM Na^+. Methane sulfonate was used as anion. E_m was measured by the K^+-anesthesia method. E_m in the resting state was −168 mV. Constant outward current pulses of 0.02 μA were applied during the measurement

cell sap (*cf.* Ohkawa & Kishimoto, 1974). In the K^+-anesthesia method, the deflection of E_m in the depolarized state represents mostly the latter components of the *IR* drop.

Discussion

Membrane Excitability in Relation to Internal Anions

It was found that the plasmalemma of *Chara* takes either of the two electrically different states in response to $[Cl^-]_i$. At $[Cl^-]_i$ lower than 29 mM, the plasmalemma kept a high (more negative) E_m, high R_m, and excitability. At $[Cl^-]_i$ higher than 48 mM, both E_m and R_m were low and excitability was lost. At higher concentrations, effects of other anions including F^-, NO_3^-, acetate and propionate on E_m, R_m, and excitability were same as those of Cl^-. SO_4^{2-} also suppressed excitability at 41 mM, although high E_m and R_m were maintained. Phosphate, methane sulfonate, and PIPES could maintain E_m, R_m, and excitability. In squid giant axons, the order of the favorableness of anions for stabilizing the membrane obeys the lyotropic series (Tasaki, Singer & Watanabe, 1965). As shown above, essentially the same tendency was observed in *Chara*. In *Chara*, however, F^-, which occupies a higher position in the lyotropic series, was unfavorable for maintaining high E_m, high R_m, and the excitability at higher concentrations.

At lower concentrations, however, all anions maintained E_m, R_m, and excitability, i.e., Cl^- lower than 29 mM, SO_4^{2-} at 6 mM, acetate at 6 mM, and F^- lower than 10 mM (data not shown). It is interesting to note the fact that the concentration of Cl^- in the cytoplasm of Characeae cells is 20–30 mM (Tazawa et al., 1974), almost the critical concentration for the membrane to maintain normal physiological activities. Since the concentration of K^+, which is the main cation in the cytoplasm, is around 100 mM, favorable anions other than Cl^- should exist in the cytoplasm to keep electroneutrality. In a squid giant axon the main anion is isethionate, which has SO_3 group (Hodgkin, 1964). The organic anions, methane sulfonate and PIPES, which supported excitability of *Chara* membrane, even added to the perfusion medium at higher concentrations as a counter ion for 100 mM K^+, also have SO_3 group as isethionate.

Assuming that an enormous increase in Cl^- permeability of the membrane is a cause of action potential in normal cells, generation of the action potential by the cell whose $[Cl^-]_i$ was extremely lowered by replacing $MgCl_2$ by $MgSO_4$ or $Mg(CH_3COO)_2$ (Table 2) shows that Cl^- can be functionally replaced by another anion such as SO_4^{2-} or CH_3COO^-. Similarly, the plasmalemma of *Chara* does not distinguish between Cl^-, Br^-, and NO_3^- in the external medium (Findlay and Hope, 1964). Furthermore, the fact that action potentials were observed even in cells perfused twice with the TM, P, or PIPES medium lacking Cl^-, SO_4^{2-}, and CH_3COO^- (Table 2) suggests that the plasmalemma becomes permeable to EGTA or anions used for buffering the perfusion media, maleate, orthophosphate, and PIPES. Equal permeability of these anions assumed in the above discussion should be examined by tracer experiment.

When the transmembrane voltage was changed to the positive direction linearly by using the voltage-clamp technique, the *V-I* curve showed a so-called negative resistance even when $[Cl^-]_i$ was decreased extremely (*cf.* Fig. 5). The appearance of the negative resistance region in the *V-I* curve can well be accounted for in terms of the change in the electromotive force of the membrane (Kishimoto, 1966, 1968). It has been postulated that the electric current flowing in the inward direction under the step depolarization is carried by Cl^-, since increase in $[Cl^-]_o$ depressed the magnitude of the inward current (Kishimoto, 1964). A question arises as to which ion or ions carry current when $[Cl^-]_i$ was extremely lowered. One possibility is that sulfate, acetate, EGTA, or anions used for buffering of media (maleate, phosphate, and PIPES) carry current when the Characeae membrane becomes very permeable to these anions as well as to Cl^-.

In squid giant axon it was reported that Ca^{2+} carries inward current under a bi-ionic condition (Meves & Vogel, 1973) where Na^+ was absent in the external medium. Voltage-dependent influx of Ca^{2+} was also reported in normal giant axon (Hodgkin & Keynes, 1957; Baker, Hodgkin & Ridgway, 1967). Recently, partial participation of Ca^{2+} in the

excitation of *C. corallina* was suggested by Beilby and Coster (1979). We observed the increase in Ca^{2+}-influx of ca. 100-fold in excitation of *C. australis* (Hayama, Shimmen & Tazawa, 1979). The Ca^{2+}-influx in the resting state and during excitation were 4×10^{-14} mole/cm^2/sec and 2×10^{-12} mole/cm^2/sec, respectively. Influx of Ca^{2+} amounting to 2×10^{-12} mole/cm^2/sec corresponds to inward current of 0.4 μA/cm^2. Since this value is considerably smaller than that of inward curent under voltage clamped depolarization (several tenth μA, Beilby & Coster, 1979), it is improbable that Ca^{2+} plays the primary role in membrane excitation in *Chara*.

Functional Asymmetry of the Plasmalemma as Revealed by Different Actions of K^+

The duration of the action potential of normal cells, which contain 112 mM K^+ in the cytoplasm (Tazawa et al., 1974), was 5 ± 0.4 sec ($n=8$) in APW at pH 7.5. The duration of 6 sec observed for tonoplast-free cells containing 101 mM K^+ agrees well with the duration of normal cells. Thus the duration of the action potential is determined or controlled by $[K^+]_i$.

Interestingly, monovalent cations act oppositely on the membrane, according to whether they act from the outside or from the inside of the cell. Monovalent cations in the external medium prolong the action potential (Shimmen et al., 1976), while they shorten it when they are inside the cell. This may be a reflection of an asymmetrical structure of the biological membrane (Zwaal, Roelofsen & Colley, 1973; Steck, 1974). In squid axon, effects of monovalent or divalent cations on membrane excitability from the inside were different from those from the outside (*cf.* Tasaki, 1968). Also, the functional asymmetry of the surface membrane of protoplasmic droplets of *Nitella* was revealed by effects of photooxidizing treatment from the inside and the outside of the membrane on E_m and the tension at the surface (Ueda, Muratsugu & Kobatake, 1976).

Na^+ could shorten the duration of the action potential (90 sec), although the effect was weaker than that of K^+ (6 sec). This indicates that the plasmalemma has selectivity for internal cations in determining duration of action potential. Differences in duration of the action potential between K^+ salts and salts of Na^+, choline$^+$, or TEA$^+$ are known to exist in squid giant axons (Tasaki, Lerman & Watanabe, 1969).

E_m in the Two Stable States in Relation to $[K^+]_i$

E_m in the resting state was independent of $[K^+]_i$ between 27 and 65 mM, and it decreased or became less negative when $[K^+]_i$ was increased to 101 mM (Fig. 10). This decrease is not understandable if E_m is determined by the ratio of K^+ concentrations between the inside and outside of the cell. As reported in the previous paper (Shimmen & Tazawa, 1977), E_m of *Chara* cells is composed of two components, a passive one independent of intracellular ATP concentration and an electrogenic one dependent on ATP concentration. The decrease in E_m at 101 mM $[K^+]_i$ may reflect a decrease in the electrogenic component.

E_m at the peak of the action potential in tonoplast-free cells was insensitive to change in $[K^+]_i$ (Fig. 10). It is also insensitive to change in K^+ concentration in the external medium (Shimmen & Tazawa, 1977). Insensitivity of E_m at the peak of action potential to changes of K^+ concentrations in both the inside and the outside of the cell can be explained by assuming that the plasmalemma behaves as a Cl^--electrode rather than a K^+-electrode in the depolarized state.

Implication of $[H^+]_o$ in Membrane Excitability

It must be mentioned that there were seasonal fluctuations in the effect of pH_o on the duration of action potential. Heightening of pH_o often failed to prolong action potential in another season. The mechanism of prolongation of action potential by alkalization of the external medium remains obscure.

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