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Electrical Tolerance (Breakdown) of the *Chara corallina* Plasmalemma: I. Necessity of Ca^{2+}

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Summary. The relationship between the external Ca^{2+} concentrations $[Ca^{2+}]_o$ and the electrical tolerance (breakdown) in the *Chara* plasmalemma was investigated. When the membrane potential was negative beyond $-350 \sim -400$ mV (breakdown potential, BP), a marked inward current was observed, which corresponds to the so-called "punch-through" (H.G.L. Coster, *Biophys. J.* **5**:669–686, 1965). The electrical tolerance of the *Chara* plasmalemma depended highly on $[Ca^{2+}]_o$. Increasing $[Ca^{2+}]_o$ caused a more negative and decreasing it caused a more positive shift of BP. BP was at about -700 mV in 200 μM La^{3+} solution. $[Mg^{2+}]_o$ depressed the membrane electrical tolerance which was supposed to be due to competition with Ca^{2+} at the Ca^{2+} binding site of the membrane. Such a depressive effect of Mg^{2+} was almost masked when the $[Ca^{2+}]_o/[Mg^{2+}]_o$ ratio was roughly beyond 2.

Key Words *Chara corallina* · Ca^{2+} · Mg^{2+} · $[Ca^{2+}]_o/[Mg^{2+}]_o$ ratio · electrical tolerance · breakdown · breakdown potential · H^+ pump

Introduction

There are various cells which show the inward-going rectification upon large hyperpolarization (Reuben, Werman & Grundfest, 1961; Coster, 1965; Candia, 1970; Saddler, 1971; Hagiwara & Takahashi, 1974; Felle, 1981; Köhler et al. 1986). Coster (1965) named this inward-going rectification in the *Chara* plasmalemma "punch-through" effect, because the current-voltage (I-V_m) relationship upon large hyperpolarization resembles the punch-through occurring in the solid state P-N junction. The "punch-through" effect can be expected from the double fixed charge membrane (DFCM) model which has been theoretically developed by Coster (1965, 1973*a,b*). Zimmermann, Pilwat and Riemann (1974), Riemann, Zimmermann & Pilwat (1975), and Coster and Zimmermann (1975) found the dielectric breakdown in the membranes of red cells, *Escherichia coli* and *Valonia*, the mechanism of which is different from that of the "punch-through." They concluded that the dielectric breakdown results from electro-mechanical instability in the membrane caused by high electric field, while the punch-through" results from ionic redistribution in the membrane introduced by high electric field (Coster & Zimmermann, 1975).

It is indeed true that the "punch-through" can be repeatedly observed at almost the same potential within fairly large hyperpolarization (Coster, 1965) but it is also true that the "punch-through" causes the membrane depolarization (Ohkawa & Kishimoto, 1977), that the membrane does not necessarily repolarize to usual large resting potential (about -200 mV), and that almost all cells used for examining this inward-going rectification cannot survive overnight. In this sense, the inward-going rectification in the *Chara* plasmalemma, the "punch-through," is sure to be related to the breakdown and, in other words, the electrical tolerance of the membrane.

In the breakdown region (the region where the I-V_m relationship on the hyperpolarization side descends) of the *Chara* plasmalemma the Cl^- efflux increases markedly (Coster & Hope, 1968) and the membrane electromotive force shifts toward large depolarization (i.e., toward the equilibrium potential for Cl^-, E_{Cl}) (Ohkawa & Kishimoto, 1977). It has been reported that the increase of external pH with NaOH causes a shift of the breakdown region toward more negative potential level (Coster, 1969), which means that the electrical tolerance increases with alkalizing the external medium. Investigations also show that the addition of moderate concentration of Ca^{2+} tends to increase the electrical tolerance of the membrane (Ohkawa & Kishimoto, 1977). However, very few reports on the relation between the electrical tolerance (breakdown) of the *Chara* plasmalemma and $[Ca^{2+}]_o$ are available. The

present experiments are intended to examine the effect of $[Ca^{2+}]_o$ on the electrical tolerance (or breakdown) of the membrane in more detail.

Materials and Methods

Internodal cells of *Chara corallina*, which have been cultured in our laboratory at 20°C under illumination (12 hr light/12 hr dark) with fluorescent lamps, were used throughout the experiments.

Three basal solutions, 2-Ca^{2+}-APW (artificial pond water), 2-Mg^{2+}-APW, and 3-Ch^{+}-APW, were used. All contained in common 0.5 mM KCl, 0.2 mM NaCl, and the pH was adjusted to 7 with 5 mM TES (N-Tris(hydroxymethyl)-methyl-2-aminoethane sulfonic acid) and NaOH. The 2-Ca^{2+}-APW contained 2 mM $CaCl_2$ but neither Mg^{2+} nor $choline^{+}$ and the other two basal solutions (2-Mg^{2+}-APW and 3-Ch^{+}-APW) have similar meanings. $[Ca^{2+}]_o$ was varied under a constant ionic strength by mixing the three basal solutions and effect of change in $choline^{+}$ (Ch^{+}) concentration could be almost neglected (Ohkawa, Tsutsui & Kishimoto, 1986). Three different $[Ca^{2+}]_o$ concentrations were prepared: 1) keeping the total concentration of both $[Ca^{2+}]_o$ and $[Mg^{2+}]_o$ at 2.0 mM; 2) keeping $[Mg^{2+}]_o$ at a constant level (0.5 or 0.1 mM); 3) keeping the ratio of $[Ca^{2+}]_o$ to $[Mg^{2+}]_o$ at 1.0. The concentration of both $[Ca^{2+}]_o$ and $[Mg^{2+}]_o$ in the $[Ca^{2+}]_o/[Mg^{2+}]_o$ ratio is expressed in mM.

The traditional glass microelectrode filled with 3 M KCl (+0 ~ 20) mM EGTA (glycol ether diaminetetraacetic acid) was used to measure the membrane potential V_m. A platinum-blacked Wt-wire of 100 μm in diameter, the tip of which had been electrolytically tapered with 0.5 M NaOH, was inserted into the central vacuole of the internodal cell through the nodal end as the current supplying electrode. The external current-measuring electrode was a pair of platinum-blacked silver chlorinated silver plates (Cole & Kishimoto, 1962). These internal and external current electrodes were positioned parallel to the axis of the cell so that the current I might flow uniformly across the part of the membrane where the measurement was carried out. The length of the measuring cell part was 6 mm.

V_m was changed by using a programmable arbitrary waveform generator (NF Electronic Instrument, Intelligent/Arbitrary Function Synthesizer 1731) under voltage-clamp conditions. Since this waveform generator can store in its memory, 12-bit, 2048-point, the extremely wide range of V_m such as shown in Fig. 1 could be spanned. V_m was linearly shifted toward 1000 mV with a rate of 100 mV/60 sec. However, in order to avoid an irreversible membrane depolarization as much as possible and to shorten the experimental time, V_m was usually released from voltage-clamp conditions at the instance when the inward current flowed more inwardly beyond about 3.0 μA (which corresponded to the order of 20 $\mu A/cm^2$ of the current density). Other detailed electrical arrangements for the measuring system were described previously (Kishimoto et al., 1982, 1984).

Judging from the results of preliminary experiments on various $[Ca^{2+}]_o$ (*see* Discussion), the present experiments were carried out at first in APW containing 0.1 mM $[Ca^{2+}]_o$, in which the internodal cell had been kept at least overnight. Then, the effect of the lowest $[Ca^{2+}]_o$ was examined, and subsequently $[Ca^{2+}]_o$ was increased. Thus, we got two data on 0.1 mM of $[Ca^{2+}]_o$; one from 0.1 mM at the starting point, and the other, after raising $[Ca^{2+}]_o$ from the lowest to 0.1 mM serially. Both data on 0.1 mM of $[Ca^{2+}]_o$ were actually different from each other. In the present paper all the data on 0.1 mM of $[Ca^{2+}]_o$ were from the latter results.

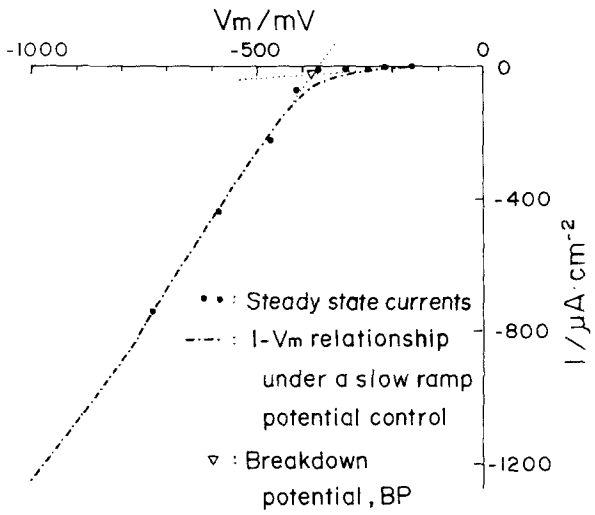

Fig. 1. Definition of the breakdown potential, BP. The steady-state currents (●) were obtained by applying a series of stepwise hyperpolarizing pulses from the resting level and they were compared to the I-V_m relationship (-·-·-·-) which was obtained by changing V_m with a rate of 100 mV/60 sec. BP is defined as V_m (▽, −380 mV) at which both low and high slopes of such an I-V_m relationship under a slow ramp potential control cross each other. The experiments were carried out in APW containing 0.1/0.1 of $[Ca^{2+}]_o/[Mg^{2+}]_o$ in mM ratio

As will be described, the membrane frequently depolarized irreversibly. When the irreversible depolarization occurred, the experiment was stopped and the data were discarded if at least three different $[Ca^{2+}]_o$ had not been examined.

All experiments were carried out under a light intensity of 2000 lux and the solution was controlled with a thermoregulator (Sharp, TE-12K) at 20 ± 0.5°C.

Definition of Breakdown Potential, BP

The I-V_m relationship which was obtained under a slow ramp potential control is shown in Fig. 1, and this is compared to the steady-state currents (●) obtained by a series of stepwise potential changes. The steady-state currents flowed 10 ~ 50 sec after the application of various stepwise potentials which commenced at the resting potential levels (about −210 mV) without applying any preholding potential pulse. When voltage-clamp conditions were released at the steady state of the current to a large negative stepwise potential change, V_m moved rapidly from a clamped voltage to either almost the peak level of the action potential or a more positive potential and then the membrane repolarized gradually to the original resting level. The time necessary for this recovery of V_m took about 20 ~ 30 min at about V_m = −400 mV and it increased by about 30 min for each increase in 50 mV hyperpolarization beyond −400 mV.

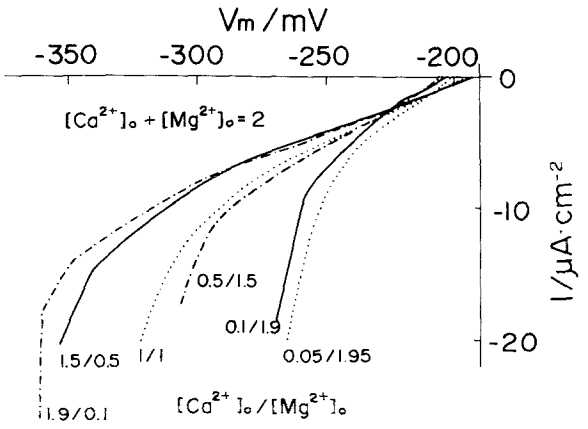

Fig. 2. I-V_m relationships at different $[Ca^{2+}]_o$, keeping the total concentration of both $[Ca^{2+}]_o$ and $[Mg^{2+}]_o$ at 2.0 mM

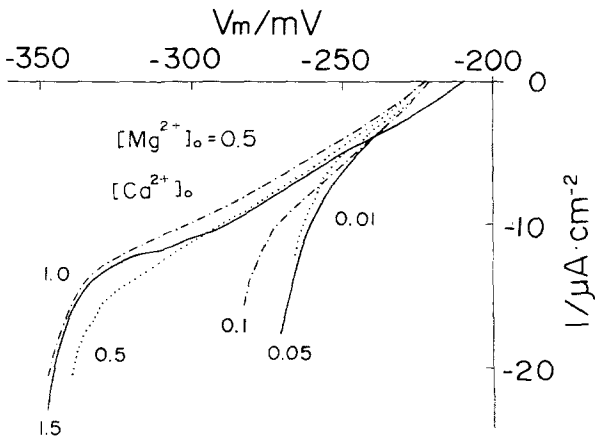

Fig. 3. I-V_m relationships at different $[Ca^{2+}]_o$ concentrations, keeping $[Mg^{2+}]_o$ at 0.5 mM

Both I-V_m relationships (i.e., the I-V_m relationship under a slow ramp potential control and the steady-state I-V_m relationship) are very similar to each other and have almost the same large slope conductance (2140 μS/cm^2) when V_m is more negative beyond about -400 mV. Thus, the I-V_m relationship under a slow ramp potential control could be considered almost the same as the steady-state I-V_m relationship. Such an adoption of the I-V_m curve under a slow ramp potential control as an approximation of the steady-state I-V_m relationship was usually reasonable as long as the rate of ramp potential change was as slow as -100 mV/30 sec. In the present paper the rate of potential change was programmed so as to be -100 mV/60 sec. In addition to such a coincidence of the two I-V_m relationships, the critical potential beyond which the inward currents were marked almost coincided with each other. This is another reason why we adopted a slow ramp potential control. In this paper, the breakdown potential (BP) is simply defined as such a critical potential ($\triangledown$) as shown in Fig. 1, at which both low and high slopes of I-V_m relationship cross each other (-380 mV). Other related membrane properties will be discussed in a subsequent paper (Ohkawa & Tsutsui, *in preparation*).

Results

Increasing $[Ca^{2+}]_o$ under $[Ca^{2+}]_o + [Mg^{2+}]_o$ = 2.0 mM

Figure 2 shows a series of I-V_m relationships which was obtained when $[Ca^{2+}]_o$ was increased from 0.05 to 1.90 mM, keeping the total concentration of both $[Ca^{2+}]_o$ and $[Mg^{2+}]_o$ at 2.0 mM (i.e., $[Ca^{2+}]_o + [Mg^{2+}]_o = 2.0$). The increase of $[Ca^{2+}]_o$ under this condition has the meanings of both the decrease of $[Mg^{2+}]_o$ and the increase of the $[Ca^{2+}]_o/[Mg^{2+}]_o$ ratio, too.

Altering $[Ca^{2+}]_o$ and $[Mg^{2+}]_o$ does not cause any appreciable change in the resting potential, while the conductance near the resting potential decreases when $[Ca^{2+}]_o$ increases. Besides, the breakdown region shifts largely toward a more negative level with the increase in $[Ca^{2+}]_o$. In other words, the increase in $[Ca^{2+}]_o$ causes an increase in the electrical tolerance. However, under this experimental condition the increase in the electrical tolerance may not be due to the increase in $[Ca^{2+}]_o$, but to the decrease in $[Mg^{2+}]_o$ or to the increase in the $[Ca^{2+}]_o/[Mg^{2+}]_o$ ratio.

In Fig. 2 the BP's of the membrane in APW's containing 0.05/1.95, 0.1/1.9, 0.5/1.5, 1.0/1.0, 1.5/0.5 and 1.9/0.1 of $[Ca^{2+}]_o/[Mg^{2+}]_o$ in mM ratio are -250.0, -258.0, -293.0, -302.5, -336.0 and -360.0 mV, respectively. BP's of different cells at different $[Ca^{2+}]_o$ are listed in part A of the Table.

Increasing $[Ca^{2+}]_o$, Keeping $[Mg^{2+}]_o$ Constant

Figure 3 shows a series of I-V_m relationships which was obtained by varying $[Ca^{2+}]_o$ from 0.01 to 1.5 mM, while keeping $[Mg^{2+}]_o$ at 0.5 mM (in this case, the increase in $[Ca^{2+}]_o$ means also the increase in the $[Ca^{2+}]_o/[Mg^{2+}]_o$ ratio). Similar to Fig. 2, raising $[Ca^{2+}]_o$ causes the decrease of conductance near the resting potential without an appreciable change in the resting potential. The breakdown region shifts towards a more negative level with the increase of $[Ca^{2+}]_o$. Since $[Mg^{2+}]_o$ is kept constant in this experiment, the effect of $[Ca^{2+}]_o$ on the electrical tolerance should be stressed more than that of $[Mg^{2+}]_o$.

Table. Breakdown potentials (BP's) under $[Ca^{2+}]_o + [Mg^{2+}]_o = 2.0$ mM

A. Breakdown potentials at different $[Ca^{2+}]_o$

	BP (mV)								
	$[Ca^{2+}]_o$ (mM)								
Cell no.	0.02	0.05	0.10	0.20	0.50	1.00	1.50	1.90	1.95
86BR01		−250.0	−258.0		−293.0	−302.5	−336.0	−360.0	
86BR02		−288.7	−275.0		−295.2	−339.2	−375.6	−395.2	
86BR03		−170.7	−203.6		−274.4	−331.6	−350.0	−368.7	
86BR04		−276.7	−275.9		−266.2	−346.1	−345.5	−375.5	
86BR05	−250.0	−252.2	−270.0	−275.0	−295.0	−346.1			−361.5
86BR06	−256.2		−264.9	−293.4		−341.0			−371.6
86BR07	−235.0		−250.0	−256.5		−350.5			−373.2
Average	−247.1	−247.7	−256.8	−275.0	−284.8	−336.6	−351.8	−374.9	−368.8

B. $BP/BP_{0.1}$ ratios at different $[Ca^{2+}]_o$[a]

	$BP/BP_{0.1}$								
	$[Ca^{2+}]_o$ (mM)								
Cell no.	0.02	0.05	0.10	0.20	0.50	1.00	1.50	1.90	1.95
86BR01		0.969	1.00		1.136	1.172	1.302	1.395	
86BR02		1.050	1.00		1.073	1.233	1.366	1.437	
86BR03		0.838	1.00		1.348	1.629	1.719	1.811	
86BR04		1.003	1.00		0.965	1.254	1.252	1.361	
86BR05	0.926	0.934	1.00	1.019	1.093	1.282			1.339
86BR06	0.967		1.00	1.108		1.287			1.403
86BR07	0.940		1.00	1.026		1.402			1.493
Average	0.944	0.959	1.00	1.051	1.123	1.323	1.410	1.501	1.412

C. $BP/BP^{1.0}$ ratios at different $[Ca^{2+}]_o/[Mg^{2+}]_o$[b]

	$BP/BP^{1.0}$								
	$[Ca^{2+}]_o/[Mg^{2+}]_o$								
Cell no.	0.010	0.026	0.053	0.111	0.333	1.00	3.000	19.00	39.00
86BR01		0.826	0.853		0.969	1.00	1.111	1.190	
86BR02		0.851	0.811		0.870	1.00	1.107	1.165	
86BR03		0.515	0.614		0.828	1.00	1.055	1.112	
86BR04		0.799	0.797		0.769	1.00	0.998	1.085	
86BR05	0.722	0.729	0.780	0.795	0.852	1.00			1.043
86BR06	0.751		0.777	0.860		1.00			1.090
86BR07	0.670		0.713	0.732		1.00			1.065
Average	0.714	0.744	0.764	0.796	0.858	1.00	1.068	1.138	1.066

[a] $BP_{0.1}$: Breakdown potential at 0.1 mM $[Ca^{2+}]_o$.
[b] $BP^{1.0}$: Breakdown potential at 1.0 of the $[Ca^{2+}]_o/[Mg^{2+}]_o$ ratio.

The main difference between Fig. 2 and Fig. 3 is the difference in the $[Ca^{2+}]_o$ range which changes the breakdown region greatly. In Fig. 3 this $[Ca^{2+}]_o$ range is restricted to only 0.1 to 0.5 mM, while in Fig. 2 it expands from 0.1 to 1.5 mM. These properties are shown more clearly in Fig. 5. In Fig. 3 the BP values of the membrane in APW's containing 0.01, 0.05, 0.1, 0.5, 1.0 and 1.5 mM $[Ca^{2+}]_o$ are −258.4, −260.2, −277.0, −333.5, −336.2 and −343.4 mV, respectively.

When $[Mg^{2+}]_o$ was kept at 0.1 mM, the dependence of the shift pattern of the I-V_m relationship on $[Ca^{2+}]_o$ was again very similar to that of Fig. 3 (*data not shown*), except that the absolute value of BP at

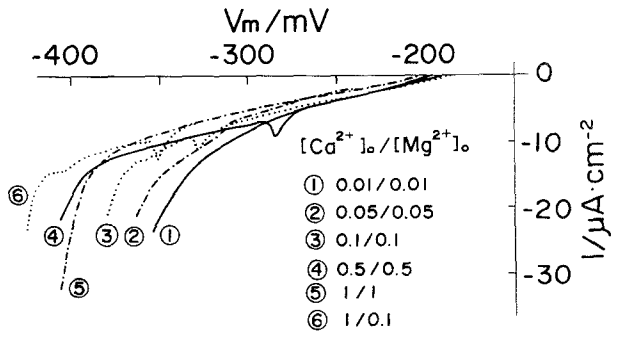

Fig. 4. I-V_m relationships at different $[Ca^{2+}]_o$, keeping the $[Ca^{2+}]_o/[Mg^{2+}]_o$ ratio at 1.0. $[Ca^{2+}]_o$ increases from 0.01 to 1.0 stepwise (Curve ① ~ ⑤). $[Mg^{2+}]_o$ is finally decreased from 1.0 to 0.1 mM, keeping $[Ca^{2+}]_o$ at 1.0 mM (Curve ⑥)

a constant $[Ca^{2+}]_o$ was more negative and a rapid shift of BP from less to more negative occurred at slightly lower $[Ca^{2+}]_o$ (*cf.* Fig. 5).

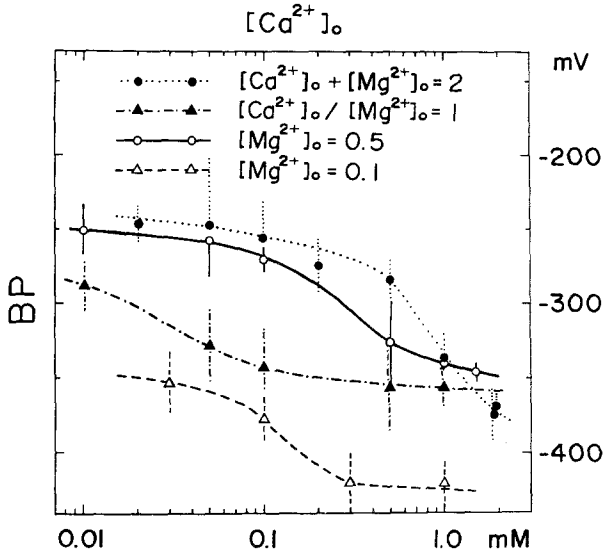

Fig. 5. Relationships between the average BP's and the logarithm of $[Ca^{2+}]_o$ under four different experimental conditions. All values are expressed as mean + SD (three to seven cells per treatment)

Increasing $[Ca^{2+}]_o$, While Keeping the Ratio of $[Ca^{2+}]_o$ to $[Mg^{2+}]_o$ at 1.0

Figure 4 shows a series of I-V_m relationships which was obtained by varying $[Ca^{2+}]_o$ from 0.01 to 1.0 mM, while keeping the ratio of $[Ca^{2+}]_o$ to $[Mg^{2+}]_o$ at 1.0. In this case, neither the resting potential nor the conductance around the resting potential are as largely affected by varying $[Ca^{2+}]_o$ and $[Mg^{2+}]_o$ as shown in the case of Figs. 2 or 3. The breakdown region shifts gradually toward a more negative level with the increase of $[Ca^{2+}]_o$ in the range less than 0.1 mM and is almost independent of $[Ca^{2+}]_o$ in the range beyond 0.5 mM. This tendency is shown more clearly in Fig. 5 (*see* Discussion).

In this cell $[Mg^{2+}]_o$ is finally decreased from 1.0 to 0.1 mM, while keeping $[Ca^{2+}]_o$ at 1.0 mM. Then, the breakdown region shifts slightly toward more negative (Curve ⑥); in other words, the increase of $[Mg^{2+}]_o$ depresses the electrical tolerance to some extent.

In Fig. 4 the BP's of the membranes in APW's containing 0.01/0.01, 0.05/0.05, 0.1/0.1, 0.5/0.5, 1.0/1.0 and 1.0/0.1 of $[Ca^{2+}]_o/[Mg^{2+}]_o$ in mM ratio are −330.0, −357.2, −371.7, −391.2, −391.9 and −419.0 mV, respectively.

Average BP's and Relative Ratio of BP's at Different $[Ca^{2+}]_o$

Actually, the absolute BP value differs in each individual internode even at the same $[Ca^{2+}]_o$ under the same experimental condition. However, when the average BP's are plotted against the logarithm of $[Ca^{2+}]_o$ (Fig. 5), the patterns of the four BP-log$[Ca^{2+}]_o$ curves are very similar. In the above event if only $[Ca^{2+}]_o$ is considered, at low $[Ca^{2+}]_o$ the BP tends to take a less negative constant value and at high $[Ca^{2+}]_o$ a more negative under respective experimental conditions. On the other hand, if both $[Ca^{2+}]_o$ and $[Ca^{2+}]_o/[Mg^{2+}]_o$ ratio are considered, then it can be supposed that the absolute level of average BP at a constant $[Ca^{2+}]_o$ is highly dependent on $[Mg^{2+}]_o$. At low $[Ca^{2+}]_o$ the average BP is about −250 mV when $[Mg^{2+}]_o$ is beyond 0.5 mM, while it is about −350 mV when $[Mg^{2+}]_o$ is 0.1 mM. At high $[Ca^{2+}]_o$ the average BP is about −350 mV when $[Mg^{2+}]_o$ is relatively high (0.5 mM), while it tends to be beyond −400 mV when $[Mg^{2+}]_o$ is relatively low (i.e., below 0.1 mM).

When the $[Ca^{2+}]_o/[Mg^{2+}]_o$ ratio is kept constant at 1.0, the average BP is almost constant in the $[Ca^{2+}]_o$ range beyond 0.1 mM and it gradually shifts toward a less negative level in the $[Ca^{2+}]_o$ range less than 0.1 mM.

In Fig. 5 it should be noticed that when the $[Ca^{2+}]_o/[Mg^{2+}]_o$ ratio is kept constant at 1.0, the $[Ca^{2+}]_o$ range which causes a marked shift of BP is lower than that when it is changed. It should also be noticed that when the $[Ca^{2+}]_o/[Mg^{2+}]_o$ ratio varies, the aforesaid $[Ca^{2+}]_o$ range is always slightly lower than $[Mg^{2+}]_o$ of the coexistent divalent cation.

To know such a $[Ca^{2+}]_o/[Mg^{2+}]_o$ effect on BP, BP's at various $[Ca^{2+}]_o$ were at first compared to

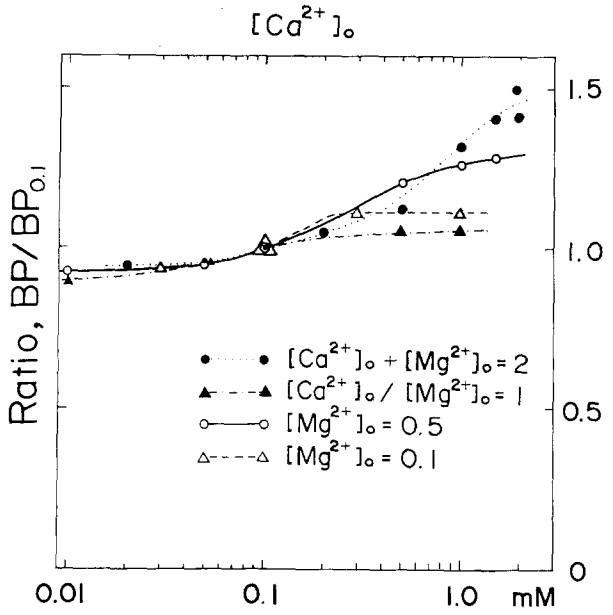

Fig. 6. Relationships between the average ratios of BP's at various $[Ca^{2+}]_o$ to $BP_{0.1}$ (BP at 0.1 mM $[Ca^{2+}]_o$) and logarithm of $[Ca^{2+}]_o$

$BP_{0.1}$ (BP at 0.1 mM $[Ca^{2+}]_o$).[1] The $BP/BP_{0.1}$ ratios were calculated from the Table and from similar data on the BP under three other different experimental conditions. One series of such calculated $BP/BP_{0.1}$ ratios is listed in part B of the Table and the average $BP/BP_{0.1}$ ratio under different experimental conditions are plotted against the logarithm of $[Ca^{2+}]_o$ in Fig. 6. At a constant $[Ca^{2+}]_o$ less than 0.1 mM the deviation in the average $BP/BP_{0.1}$ ratios among four different experimental conditions is not marked. In contrast, the marked deviation in the average $BP/BP_{0.1}$, which appears in the $[Ca^{2+}]_o$ range beyond 1.0 mM, depends on the change in the $[Ca^{2+}]_o/[Mg^{2+}]_o$ ratio during experiments. The $[Ca^{2+}]_o/[Mg^{2+}]_o$ ratio for the maximum deviation is 0.02/1.98 to 1.95/0.95, while it is at 1.0 for minimum deviation. This effect suggests that the increase of $[Ca^{2+}]_o$ causes the increase of the electrical tolerance, which is accelerated by the decrease of $[Mg^{2+}]_o$.

To find out the relationship between the $[Ca^{2+}]_o/[Mg^{2+}]_o$ ratio and the BP in more detail, BP's at various $[Ca^{2+}]_o/[Mg^{2+}]_o$ ratios under three different experimental conditions (i.e., $[Ca^{2+}]_o + [Mg^{2+}]_o = 2.0$ mM, $[Mg^{2+}]_o = 0.5$ mM, and $[Mg^{2+}]_o = 0.1$ mM) were compared with $BP^{1.0}$ (BP at 1.0 of the $[Ca^{2+}]_o/[Mg^{2+}]_o$ ratio; one example of such comparisons is listed in part C of the Table). The average $BP/BP^{1.0}$ ratios are plotted against the logarithm of the $[Ca^{2+}]_o/[Mg^{2+}]_o$ ratio in Fig. 7. Surprisingly, all data from different experimental conditions are almost on the same curve as long as the total concentration of both $[Ca^{2+}]_o$ and $[Mg^{2+}]_o$ is slightly higher than 0.10 mM. When the $[Ca^{2+}]_o/[Mg^{2+}]_o$ ratio is roughly beyond 2, the $BP/BP^{1.0}$ ratio reaches almost a constant value (about 1.11). On the other hand, when the $[Ca^{2+}]_o/[Mg^{2+}]_o$ ratio is reduced to less than 0.1, the $BP/BP^{1.0}$ ratio gradually reaches another constant value which is slightly smaller than 0.7. Thus, the $BP/BP^{1.0}$-$\log([Ca^{2+}]_o/[Mg^{2+}]_o)$ relationship shows a deformed sigmoid shape.

Effects of EGTA and La^{3+} on Electrical Tolerance

Since the decrease in Ca^{2+} in the membrane surface was thought to be the main cause of decrease in the electrical tolerance, EGTA was also tested to ascertain whether it causes the decrease in the electrical tolerance by removing Ca^{2+} from the membrane. It was found that the addition of EGTA causes the decrease in the membrane electrical tolerance as well as depolarization of the membrane at rest (Fig. 8). Such a concentration-dependent membrane depolarization at rest as shown in Fig. 8 was usually not marked when $[Mg^{2+}]_o$ was varied (Fig. 2 and Curves ⑤ and ⑥ in Fig. 4), though $[Mg^{2+}]_o$ is considered to remove Ca^{2+} from the membrane surface.

On the contrary, La^{3+}, which is expected to be bound to the Ca^{2+} binding site of the membrane more tightly than Ca^{2+}, causes a marked increase of the membrane electrical tolerance (Fig. 9). The addition of 0.1 mM $LaCl_3$ to APW containing 0.1/0.1 of $[Ca^{2+}]_o/[Mg^{2+}]_o$ in mM ratio causes a BP shift from −427 mV (Curve I) to −653 mV (Curve II) in about 30 min. However, it should be kept in mind that the I-V_m relationship has a queer bending point, the level of which corresponds almost to BP in the La^{3+}-free APW. Keeping the cell in 0.2 mM La^{3+}-APW without $[Ca^{2+}]_o$ for about 2 hr causes a much larger shift of BP (−700 mV) (Curve III).

Discussion

To avoid damage of the membrane and to shorten the experimental time, voltage-clamp conditions were usually released at the instance when a marked inward current flowed beyond about 3 μA (which corresponded to about 20 $\mu A/cm^2$). However, in the sense that V_m recovered to the original

[1] The reason why BP at 0.1 mM $[Ca^{2+}]_o$ was chosen was simply because we have many data on the electrical properties of the *Chara* plasmalemma at 0.1 mM $[Ca^{2+}]_o$ (for example, Kishimoto et al., 1982, 1984, 1985).

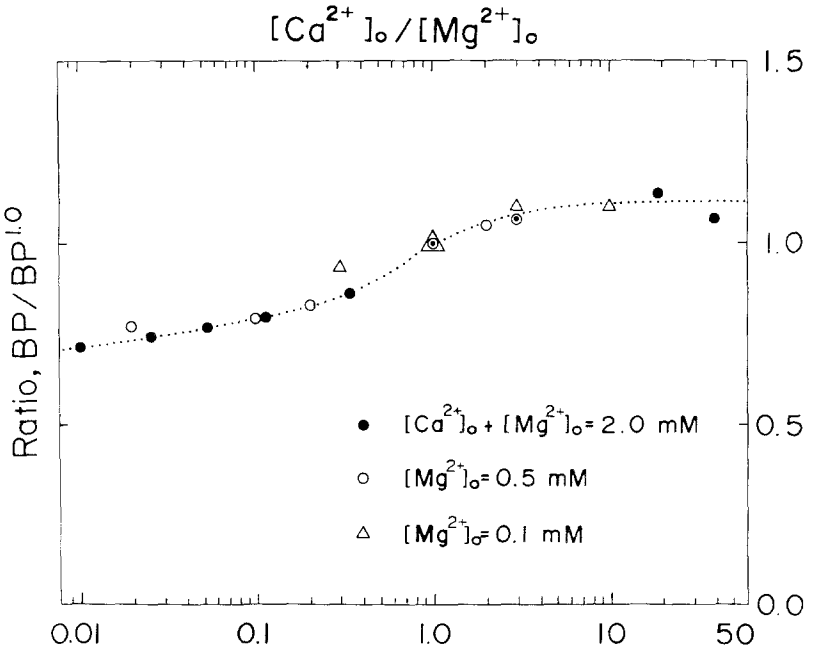

Fig. 7. Relationships between the average ratios of BP's at various $[Ca^{2+}]_o$ to $BP^{1.0}$ (BP at 1.0 of the $[Ca^{2+}]_o/[Mg^{2+}]_o$ ratio) and the logarithm of $[Ca^{2+}]_o/[Mg^{2+}]_o$

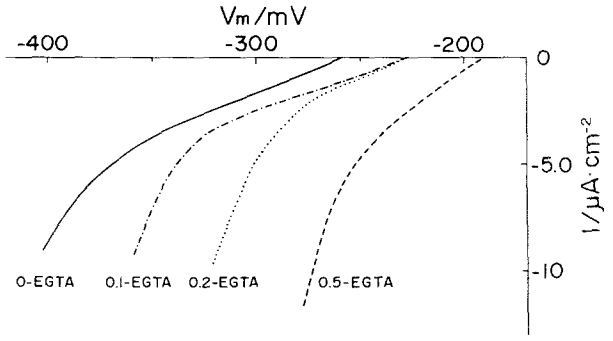

Fig. 8. Effect of EGTA on the electrical tolerance. The experiment was carried out in APW containing 0.1/0.1 of $[Ca^{2+}]_o/[Mg^{2+}]_o$ in mM ratio

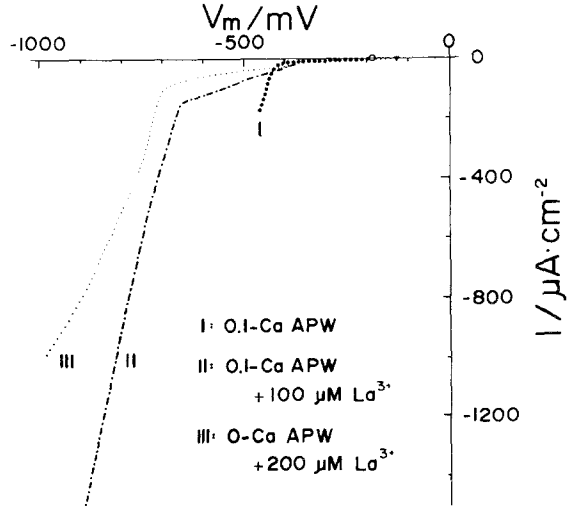

Fig. 9. Effect of La^{3+} on the electrical tolerance. I: Without $[La^{3+}]_o$. II: With 100 μM $[La^{3+}]_o$. Both experiments were carried out on the same cell in APW containing 0.1/0.1 of $[Ca^{2+}]_o/[Mg^{2+}]_o$ in mM ratio. III: The cell had been kept in Ca^{2+}-free APW containing 200 μM La^{3+} about 2 hr before the experiment. The cell was different from that of Curves I and II

resting value after the release of voltage clamp, some cells could tolerate much larger negative potential which caused much larger inward current (Fig. 1) than those shown in Figs. 2, 3 and 4. On the other hand, in some cells the membrane did not repolarize beyond −90 to −130 mV about 1 hr after the release of voltage clamp, in spite of the fact that the amount of inward current had been almost the same as that shown in Figs. 2, 3 or 4. The above potential level apparently corresponds to the so-called K-state (Beilby, 1985). However, the membrane almost lost the voltage dependence of conductance without having a negative slope conductance region on the hyperpolarization side, the latter being peculiar to the K-state (Moore, 1959; Ohkawa & Kishimoto, 1974; Beilby, 1985, 1986; Köhler et al., 1986). In this sense, the deficiency of the repolarization potency may be related to partial denaturation of the closing mechanism of voltage-dependent K^+ channel (Ohkawa et al., 1986).

It should be also kept in mind that the H^+ pump, the conductance (G_p) of which normally has a peak around −200 mV (*cf.* Fig. 10) and is essentially small in the V_m range near the K-state at the steady-state (Beilby, 1984; Kishimoto et al., 1984;

Takeuchi et al., 1985), might be structurally and functionally damaged during the breakdown. Thereby, the pump current could not flow efficiently to repolarize the membrane. The pump current flows so that the membrane may repolarize during an action potential (Kishimoto et al., 1985). However, even if the deficiency of the repolarization potency might be mainly due to the denaturation of the H^+ pump, this denaturation would be quite different from the inhibition of the H^+-pump activity by a poison such as DES (diethyl stilbestrol) or DCCD (dicyclohexylcarbodiimide). The membrane depolarized by the breakdown was generally conductive in the whole V_m range, while the one depolarized by a pump inhibitor was much less conductive (Beilby, 1984; Kishimoto et al., 1984; Takeuchi et al., 1985; Kami-ike et al., 1986).

Such an irreversible membrane depolarization[2] easily occurred after the release of voltage clamp, when $[Ca^{2+}]_o$ was less then 0.05 mM. Besides, if $[Mg^{2+}]_o$ was less than 0.05 mM, the irreversible depolarization was most frequently observed. Moreover, in about half of the cells which had been kept overnight in such a low $[Ca^{2+}]_o$ and $[Mg^{2+}]_o$ medium the membrane was almost at the potential level of the K-state already at the beginning. In contrast, in the cells which had been kept overnight in a low $[Ca^{2+}]_o$ but high $[Mg^{2+}]_o$ (beyond 0.5 mM) medium, this situation also caused the irreversible membrane depolarization, but not as frequently as in the former conditions, though the resting potential was usually more negative then E_K ($-100 \sim -150$ mV) at the beginning.[3]

Judging from such preliminary experiments and the present results examined at various $[Ca^{2+}]_o$, it is concluded that Ca^{2+} in the medium are essentially necessary for maintaining the electrical tolerance of the membrane, while Mg^{2+} in the medium depress the electrical tolerance (*cf.* Curves ⑤ and ⑥ in Fig. 4) either by dislodging Ca^{2+} or by dislodging and taking the place of Ca^{2+} in the membrane. Thus, when the $[Ca^{2+}]_o/[Mg^{2+}]_o$ ratio changes largely, the change in the $BP/BP_{0.1}$ ratio is large, too (Fig. 6). However, such a depressive effect of $[Mg^{2+}]_o$ is almost masked when the $[Ca^{2+}]_o/[Mg^{2+}]_o$ ratio is roughly beyond 2 (Fig. 7). It is also reasonable to suppose that the electrical tolerance is increased by La^{3+} which bind with the Ca^{2+} binding site more tightly than Ca^{2+} (Fig. 9), while it is decreased by EGTA which remove Ca^{2+} from the membrane (Fig. 8).

On the other hand, it should also be noticed that when the $[Ca^{2+}]_o/[Mg^{2+}]_o$ ratio is kept constant at 1.0, in the $[Ca^{2+}]_o$ range less than 0.1 mM, BP gradually shifts toward a less negative level without becoming stable (Curve (–▲·–·▲–) in Fig. 5). This should be considered as the effect of low concentration of divalent cations in addition to the effect of low $[Ca^{2+}]_o$ at a moderate $[Mg^{2+}]_o$. Such an effect of low divalent cations is marked when both $[Ca^{2+}]_o$ and $[Mg^{2+}]_o$ are less than 0.05 mM at the ratio of 1.0 (*see* Figs. 4 and 5).

The depression of the electrical tolerance by $[Mg^{2+}]_o$ can be qualitatively expected by assuming a simple competitive reaction of Mg^{2+} with Ca^{2+} at the same Ca^{2+} binding site in the membrane, which is in line with the explanation for the overshoot of Ca spike in the barnacle muscle fiber competing with other divalent cations by Hagiwara and Takahashi (1967, 1974) and Hagiwara (1973). Besides, when the Ca^{2+} binding sites are occupied by Ca^{2+}, both electrical field strength and distribution of ions in the membrane would be different from those when they are free from Ca^{2+}. Between these two membrane states the thickness of depletion layer and its voltage dependence, which have been discussed by Coster (1965, 1969, 1973*b*), might be different. According to his DFCM model, the "punch-through" occurs when the fixed-charge layer in the membrane is compressed to zero by the increase in the thickness of the depletion layer.

At the present stage, we have at least two reasons that make us hesitate from further theoretical discussion of the competition of Mg^{2+} with Ca^{2+} at the Ca^{2+} binding site. Firstly, it is difficult to determine the concentration and position of Ca^{2+} and Mg^{2+} in the cell wall layer of the *Chara* internode. For instance, if the internode had been kept in high $[Ca^{2+}]_o$ (2 mM) overnight or in contrast to our regular procedure of the present experiments, or if high $[Ca^{2+}]_o$ was used at the beginning and $[Ca^{2+}]_o$ was subsequently decreased, BP was not always dependent on $[Ca^{2+}]_o$. This was supposed to be due to Ca^{2+} accumulation effect in the cell wall layer. In fact, according to Abe and Takeda (1986), the difference in the resting potential between the intact cells and protoplasts from *Nitella expansa* can be neither explained simply by a diffusion potential, nor by Donnan potential, nor by surface potential. Moreover, replacement of $[Ca^{2+}]_o$ with $[Mg^{2+}]_o$ causes a deleterious membrane depolarization in the protoplast membrane, while in the intact cell no

[2] The total number of this series of experiments for $[Ca^{2+}]_o/[Mg^{2+}]_o$ effect was 51. The data from 24 samples were discarded due to the irreversible depolarization during or at the beginning of respective experiments. The data from eight other samples were out of the present experiment conditions. The data from 19 samples were analyzed in this paper.

[3] If the *Chara* internodes were kept in pure 2 mM $[Ca^{2+}]_o$, almost all of them showed the normal cytoplasmic streaming over seven days, while in pure 2 mM $[Mg^{2+}]_o$ some internodes lost the cytoplasmic streaming in a few days.

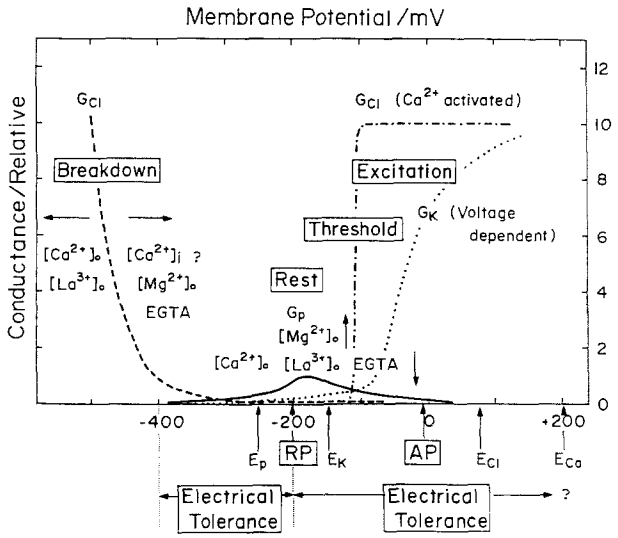

Fig. 10. Voltage dependencies of various channels in the *Chara* plasmalemma. G_{Cl}: Cl^- conductance (Ohkawa & Kishimoto, 1977; Tsutsui et al., 1987*a,b*). G_K: K^+ conductance (Ohkawa et al., 1986). G_p: H^+-pump conductance (Kishimoto et al., 1984). E_p: electromotive force of the H^+ pump. E_K, E_{Cl} and E_{Ca}: equilibrium potentials for K^+, Cl^- and Ca^{2+}, respectively. *RP:* resting potential. *AP:* peak of action potential. Each arrow represents the direction of the action of each agent. For simplicity, conductance of the leakage channel is not shown

appreciable change in the resting potential occurs. According to the present results, $[Mg^{2+}]_o$, the range of which was less then 2.0 mM, causes an increase of the conductance at rest without a marked change in the resting potential. Such a difference in the behavior of membrane to $[Mg^{2+}]_o$ between the protoplast and intact cell membrane is due to the presence of cell wall in the case of intact cell membrane.

Secondly, voltage dependencies of various channels in the *Chara* plasmalemma, schematically shown in Fig. 10, are well known. As described in the present paper, the breakdown phenomenon in the *Chara* plasmalemma is observed when the membrane cannot electrically tolerate a large negative potential. In the breakdown region, the V_m range of which is controlled mainly by $[Ca^{2+}]_o$, the Cl^- conductance, G_{Cl}, increases (Coster & Hope, 1968; Ohkawa & Kishimoto, 1977); in other words, the Cl^- channel is stabilized by $[Ca^{2+}]_o$. Thus, the relationship between electrical tolerance and breakdown mediated by $[Ca^{2+}]_o$, is very similar to that between threshold for excitation and inward currents caused by the activation of Ca^{2+}-activated Cl^- channel mediated by $[Ca^{2+}]_o$ (Lunevsky et al., 1983; Tsutsui et al., 1986; 1987*a,b*). Thus, on the basis of the generalized concept of the $[Ca^{2+}]_o$ effect, i.e., stabilization effect (Frankenhaeuser & Hodgkin, 1957), we believe, the competition mechanism between Ca^{2+} and Mg^{2+} will be more clearly explained by knowing the kinetics of voltage- and time-dependent Cl^- channel. This will be introduced in a subsequent paper (Ohkawa & Tsutsui, *in preparation*).

It is worthwhile that $[Ca^{2+}]_o$ decreases and $[Mg^{2+}]_o$ increases the membrane conductance at rest without a marked change in the resting potential (Figs. 2, 3, 4), while both 0.1 mM EGTA and La^{3+} cause both membrane depolarization and decrease of the membrane conductance at rest (Figs. 8, 9). The depolarization accompanying decrease of the membrane conductance is characteristic of the H^+-pump inhibitors (Beilby, 1984; Kishimoto et al., 1984). On the other hand, $[Ca^{2+}]_o$ and $[Mg^{2+}]_o$ can competitively change G_p without an appreciable change in the ratio of G_p to other conductances of ionic channels. Thus, the activity of the H^+ pump at rest should be controlled by both $[Ca^{2+}]_o$ and $[Mg^{2+}]_o$ (Ohkawa & Tsutsui, 1988).

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References

Abe, S., Takeda, J. 1986. The membrane potential of enzymatically isolated *Nitella expansa* protoplasts as compared with their intact cells. *J. Exp. Bot.* **37:**238–252

Beilby, M.J. 1984. Current-voltage characteristics of the proton pump at *Chara* plasmalemma. I. pH Dependence. *J. Membrane Biol.* **81:**113–125

Beilby, M.J. 1985. Potassium channels at *Chara* plasmalemma. *J. Exp. Bot.* **36:**228–239

Beilby, M.J. 1986. Factors controlling the K^+ conductance in *Chara*. *J. Membrane Biol.* **93:**187–193

Candia, O.A. 1970. The hyperpolarizing region of the current-voltage curve in frog skin. *Biophys. J.* **10:**250–260

Cole, K.S., Kishimoto, U. 1962. Platinized silver chloride electrode. *Science* **136:**381–382

Coster, H.G.L. 1965. A qualitative analysis of the voltage-current relationships of fixed charge membranes and the associated property of "punch-through." *Biophys. J.* **5:**669–686

Coster, H.G.L. 1969. The role of pH in the punch-through effect in the electrical characteristics of *Chara australis*. *Aust. J. Biol. Sci.* **22:**365–374

Coster, H.G.L. 1973*a*. The double fixed charge membrane. Low frequency dielectric dispersion. *Biophys. J.* **13:**118–132

Coster, H.G.L. 1973*b*. The double fixed charge membrane. Solution-membrane ion partition effects and membrane potentials. *Biophys. J.* **13:**133–142

Coster, H.G.L., Hope, A.B. 1968. Ionic relations of cells of *Chara australis*. XI. Chloride fluxes. *Aust. J. Biol. Sci.* **21:**243–254

Coster, H.G.L., Zimmermann, U. 1975. The mechanism of electric breakdown in the membranes of *Valonia utricularis*. *J. Membrane Biol.* **22:**73–90

Felle, H. 1981. A study of the current-voltage relationships of electrogenic active and passive membrane elements in *Riccia fluitans*. *Biochim. Biophys. Acta* **646:**151–160

Frankenhaeuser, B., Hodgkin, A.L. 1957. The action of calcium on the electrical properties of squid axons. *J. Physiol. (London)* **137:**217–244

Hagiwara, S. 1973. Ca spike. *Adv. Biophys.* **4:**71–102

Hagiwara, S., Takahashi, K. 1967. Surface density of calcium ions and calcium spikes in the barnacle muscle fiber membrane. *J. Gen. Physiol.* **50:**583–601

Hagiwara, S., Takahashi, K. 1974. The anomalous rectification and cation selectivity of the membrane of a starfish egg cell. *J. Membrane Biol.* **18:**61–80

Kami-ike, N., Ohkawa, T., Kishimoto, U., Takeuchi, Y. 1986. Kinetic analysis of the electrogenic pump of *Chara corallina:* IV. Temperature dependence of the pump activity. *J. Membrane Biol.* **94:**163–171

Kishimoto, U., Kami-ike, N., Takeuchi, Y., Ohkawa, T. 1982. An improved method for determining the ionic conductance and capacitance of the membrane of *Chara corallina*. *Plant Cell Physiol.* **23:**1041–1054

Kishimoto, U., Kami-ike, N., Takeuchi, Y., Ohkawa, T. 1984. A kinetic analysis of the electrogenic pump of *Chara corallina*. I. Inhibition of the pump by DCCD. *J. Membrane Biol.* **80:**175–183

Kishimoto, U., Takeuchi, Y., Ohkawa, T., Kami-ike, N. 1985. A kinetic analysis of the electrogenic pump of *Chara corallina:* III. Pump activity during action potential. *J. Membrane Biol.* **86:**27–36

Köhler, K., Steigner, W., Kolbowski, J., Hansen, U.P., Simonis, W., Urbach, W. 1986. Potassium channels in *Eremosphaera viridis*. *Planta* **167:**66–75

Lunevsky, V.Z., Zherelova, O.M., Vostrikov, I.Y., Berestovsky, G.N. 1983. Excitation of *Characeae* cell membranes as a result of activation of calcium and chloride channels. *J. Membrane Biol.* **72:**43–58

Moore, J.W. 1959. Excitation of the squid axon membrane in isosmotic potassium chloride. *Nature* **183:**265–266

Ohkawa, T., Kishimoto, U. 1974. The electromotive force of the *Chara* membrane during the hyperpolarizing response. *Plant Cell Physiol.* **15:**1039–1054

Ohkawa, T., Kishimoto, U. 1977. Breakdown phenomena in the *Chara* membrane. *Plant Cell Physiol.* **18:**67–80

Ohkawa, T., Tsutsui, I. 1988. Ion pumps: Structure, function, and regulation. *In:* Electrical Tolerance of the *Chara corallina* Plasmalemma. W.D. Stein, editor. Alan R. Liss, New York (*in press*)

Ohkawa, T., Tsutsui, I., Kishimoto, U. 1986. K^- channel in the *Chara* plasmalemma: Estimations of K^+ channel density and K^+ channel conductance. *Plant Cell Physiol.* **27:**1429–1438

Reuben, J.P., Werman, R., Grundfest, H. 1961. The ionic mechanism of hyperpolarizing responses in lobster muscle fibers. *J. Gen. Physiol.* **45:**243–265

Riemann, F., Zimmermann, U., Pilwat, G. 1975. Release and uptake of haemoglobin and ions in red blood cells induced dielectric breakdown. *Biochim. Biophys. Acta* **394:**449–462

Saddler, H.D.W. 1971. Spontaneous and induced changes in the membrane potential and resistance of *Acetabularia mediterranea*. *J. Membrane Biol.* **5:**250–260

Takeuchi, Y., Kishimoto, U., Ohkawa, T., Kami-ike, N. 1985. A kinetic analysis of the electrogenic pump of *Chara corallina:* II. Dependence of the pump activity on external pH. *J. Membrane Biol.* **86:**17–26

Tsutsui, I., Ohkawa, T., Nagai, R., Kishimoto, U. 1986. Inhibition of Cl^- channel activation in *Chara corallina* membrane by lanthanum ion. *Plant Cell Physiol.* **27:**1197–1200

Tsutsui, I., Ohkawa, T., Nagai, R., Kishimoto, U. 1987*a*. Role of calcium ion in the excitability and electrogenic pump activity of the *Chara corallina* membrane: I. Effects of La^{3+}, Verapamil, EGTA, W-7, and TFP on the action potential. *J. Membrane Biol.* **96:**65–73

Tsutsui, I., Ohkawa, T., Nagai, R., Kishimoto, U. 1987*b*. Role of calcium ion in the excitability and electrogenic pump activity of the *Chara corallina*. II. Effects of La^{3+}, EGTA, and calmodulin antagonists on the current-voltage relation. *J. Membrane Biol.* **96:**75–84

Zimmerman, U., Pilwat, G., Riemann, F. 1974. Dielectric breakdown of cell membranes. *Biophys. J.* **14:**881–899

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