Characteristics of vacuolar membrane of *Nitella*

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Introduction

Most electrophysiological works on plant cells including Characeae internodes have dealt with properties of the membrane complex composed of the plasmalemma and the tonoplast. To study electrical properties of each of the two cytoplasmic membranes, two microelectrodes should be inserted into the cell, one into the vacuole and the other into the cytoplasm (Findlay & Hope 1964, Spanswick et al. 1967, Spanswick & Costerton 1967, Findlay 1970). From such studies it was found that in Chara australis and Nitellopsis obtusa only the plasmalemma responds to the change in ionic concentrations of the external medium (Findlay & Hope 1964, Findlay 1970). Under the assumption that it is only the tonoplast potential that is affected by ion species and concentration in the vacuolar medium, we can know response solely of the tonoplast to ions from the change in the vacuolar potential. Experiments along this line in Characeae cells have become possible since the success in replacing the natural cell sap with artificial solutions of various compositions (Tazawa 1964). It was found that the vacuolar potential of Nitella flexilis was scarcely affected by replacing K^+ in the vacuole with Na^+, Rb^+ and Ca^{2+} (Tazawa & Kishimoto 1964, Kishimoto et al. 1965). Kishimoto (1965) reported that the tonoplast potential of N. flexilis changed by about 100 mV for the change in vacuolar K^+-concentration from 1 mM to 50 mM. On the other hand, Tazawa et al. (1975) reported that the vacuolar potential of N. flexilis changed only about 50 mV for still greater changes in vacuolar K^+-concentration (0.1 - 90 mM) and supposed that the tonoplast is not very sensitive to K^+.

Kitasato (1968) observed that the vacuolar potential of N. clavata responds to pH of the bathing medium and concluded that the

permeability of Nitella membrane to H^+ is much greater than those to K^+ and Cl^- and that an electrogenic H^+ pump is operating in the membrane. Many workers (Spanswick 1972, 1974, Vredenberg & Tonk 1973, Saito & Senda 1973, 1974) also claimed that the change of vacuolar potential to the hyperpolarizing direction induced by illumination in Characeae internodes is caused by an electrogenic H^+ pump. Since implications of H^+ in the membrane physiology of plant cells have recently been discussed intensively, it is desirable to know how the tonoplast responds to the vacuolar pH. In the first chapter electric responses of the tonoplast not only to vacuolar K^+ and Cl^- but also to vacuolar pH are described and compared with those of the plasmalemma.

Another way to charcterize a membrane is to study its excitability. So far, excitability of the vacuolar membrane has been reported by Chang (1960) and Eckert and Sibaoka (1968) on Noctiluca, by Findlay and Hope (1964) on Chara corallina, by Findlay (1970) on Nitellopsis obtusa. In the second chapter, conditions for excitation of the tonoplast of Nitella pulchella was studied and the characteristic of the tonoplast action potential was compared with that of the plasmalemma action potential.

Chapter one: Resting potential of tonoplast in relation to concentrations of K^+, Cl^-, H^+ and OH^- in the vacuole.

Material and method

The main material used throughout the experiment was Nitella pulchella. This material has a very thick cell wall (≈ 50 μ) and is therefore hardly deformed through loss of turgor by evaporation of water and by cutting the cell, the procedures necessary for perfusion experiments. Due probably to this characteristic of N. pulchella the vacuolar perfusion can be carried out more successfully in this material than in other species such as N. flexilis.

Internodes, about 400 μ in diameter and several cm in length, were isolated from adjacent internodes and kept before use for more than a day in dechlorinated tap water or in the pond water where the material had been cultured. In the experiment an internodal cell (N) was placed on the polyacrylate vessel which has three pools (Fig. 1). Two end pools (A, C) were connected to each other with a piece of rubber tubing. The two ends of the cell were amputated and the cell sap was replaced with an artificial medium by vacuolar perfusion (Tazawa 1964). After that the potential difference and the electric resistance between the vacuole and the external medium were measured with the "open-vacuole method" (Tazawa et al. 1975) and recorded with a pen-writing recorder (Fig. 1).

The potential difference measured between the electrodes E_a and E_b was corrected for junction potentials in the following way. The electrode (E_a) whose tip is dipped into

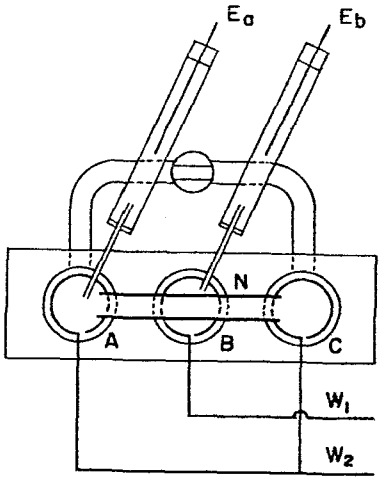

Fig. 1 Perfusion chamber with three pools (A, B, C) and the setup for the open vacuole measurements of vacuolar potential and electric resistance of the cell part at B. N: Nitella internode. E_a, E_b: electrodes with vinyl tubing filled with 100 mM KCl-agar which is connected to Ag-AgCl wire through 3 M KCl. Electric pulse are given between the wires W_1 and W_2.

the perfusion medium has two kinds of junction potentials, one between the tip of the electrode (vinyl tubing filled with 2% agar containing 100 mM KCl) and the vacuolar medium (e_a) and the other between Ag-AgCl wire and 3 M KCl solution (e'_a). The reference electrode (E_b) the tip of which is bathed in the external medium also has similar junction potentials (e_b and e'_b). Since the potential difference measured with two electrodes (ΔE) is the sum of the vacuolar potential of the cell part in B (E_{vo}) and the difference of the junction potentials of two electrodes (Δe),

$$\Delta E = E_{vo} + \Delta e.$$

In the previous paper (Tazawa et al. 1975) Δe was determined in the following manner. Namely, a chamber with two pools were prepared, one being filled with the perfusion (vacuolar) medium and another with the external medium. The pools were connected with a rubber tubing in which both media had a diffusion boundary. Then, the tip of one electrode E_a was dipped in the perfusion medium in one pool and the other electrode E_b in the external medium in the other pool. The potential difference between the two electrodes was taken as Δe, since the liquid junction potential in the rubber tubing was proved to be negligible when both media were composed of mainly K^+ and Cl^- which have nearly the same mobilities. The perfusion media used for the present study contained SO_4^{2-} which has a mobility quite different from Cl^-. In such a case the liquid junction potential between SO_4^{2-}-medium and Cl^--medium is not negligible.

Therefore, correction of the electrode potential was carried out in another way.

First, E_a was dipped into the vacuolar medium and the potential was measured against a 1 M KCl-agar salt bridge which was connected to Ag-AgCl wire electrode through 3 M KCl. The potential of the reference electrode, E_b, was also measured in the same way. The aforementioned potential difference, Δe, was obtained by subtracting the potential of E_b from that of E_a, because the electrode potential having an agar salt bridge with a very concentrated KCl such as 1 M should be nearly constant irrespective of the compositions of the medium in which the electrode was dipped. Vacuolar potential, E_{vo}, was obtained by subtracting Δe from the measured potential difference (ΔE).

The bathing and vacuolar media with definite compositions are listed on Table 1. The simplified artificial pond water (APW) contained 0.1 mM each of KCl, NaCl and $CaCl_2$. Since both cell ends are open to the outside in experiments using the open-vacuole method, the external medium of the central cell part where E_{vo} was measured (B in Fig. 1) was made isotonic or slightly hypotonic to the perfusion media with sorbitol. The isotonic APW is refered to iAPW hereafter. The tonicities of the perfusion media listed on Table 1 were adjusted either equal to or slightly larger than that of the natural cell sap which varies to some extent according to the season. Np medium was similar to the natural cell sap of N. pulchella and Nf medium to that of N. flexilis in concentrations of K^+, Na^+ and Cl^- (Tazawa et al. 1974). The medium of low ionic concentrations (iAPW-10Ca) was similar

to APW in concentrations of KCl and NaCl (each 0.1 mM) but contained more $CaCl_2$ (10 mM). To examine the effect of the tonicity on E_{vo}, Nf medium and KCl medium were concentrated simply by raising the concentration of each salt equally. The tonicity of iAPW-10Ca was also adjusted to 600 mM with addition of sorbitol. To see effects of K^+ and Cl^- in the vacuole on E_{vo} separately from each other, the concentration of K^+ was varied with K_2SO_4 and that of Cl^- with choline chloride. Since Ca^{2+} in the vacuole is essential to maintain the cell at a healthy state after vacuolar perfusion (Tazawa 1964, Tazawa & Kishimoto 1964), our perfusion media contained a small amount of either $CaCl_2$ or $CaSO_4$.

When the cell was perfused with a solution of high ion contents (e.g. Np, Nf, KCl in Table 1), E_{vo} remained stable for more than one hour. However, when the perfusion medium of low ion contents (iAPW-10Ca in Table 1) was used, E_{vo} was stable only for the first 20 minutes, then it depolarized gradually. In the followings only the stable values measured within 20 minutes are listed.

The electric resistance across the protoplasmic layer was obtained in the following way. In the case of the open-vacuole method the change in potential difference (p.d.) caused by the electric current pulse (ΔI) was the sum of the p.d. change (ΔE_{vo}) due to the resistance across the protoplasmic layer in B and that due to the resistance of the cell sap and the external media. The latter fraction of p.d. change was obtained by applying current on the cell which was killed either by a rapid perfusion or by cutting the

cell in B at the two loci near the partitions. Subtraction of this fraction from the total p.d. change gives ΔE_{VO}. The electric resistance was then calculated to be $\Delta E_{VO}/\Delta I$. Multiplying $\Delta E_{VO}/\Delta I$ with the surface area of the cell part in B gives the specific resistance across the protoplasmic layer (R_{VO}).

The temperature was kept at 20-24°C. Data are shown as the average ±SE.

Results

(1) E_{vo} in relation to K^+- and Cl^--concentrations in the vacuole

To see whether or not the characteristics of the two membranes differ from each other, responses of E_{vo} to changes in concentrations of vacuolar K^+ ($[K^+]_v$) and Cl^- ($[Cl^-]_v$) as well as to those of external K^+ ($[K^+]_o$) and Cl^- ($[Cl^-]_o$) were studied. The concentration of K^+ of the test medium, which served not only as the vacuolar medium but also as the external one, was varied with K_2SO_4 under a constant Cl^- concentration and that of Cl^- was varied with choline choride under a constant K^+ concentration. All test media contained 1 mM Ca^{2+} and 0.1 mM Na^+.

After the vacuolar sap was replaced with an artificial solution with a definite composition, e.g. 0.1 mM each of K^+, Na^+, Cl^-, 1 mM Ca^{2+} and 1.05 mM SO_4^{2-} (Table 1), E_{vo} of the cell was measured in bathing media with various K^+-concentrations under constant $[Cl^-]_o$ (0.1 mM) or with various Cl^--concentrations under constant $[K^+]_o$ (0.1 mM). In such experiments $[K^+]_v$ was maintained at 0.1, 1.0, 10 and 100 mM and $[Cl^-]_v$ at 0.1, 1.0, 10 and 100 mM. In Fig. 2, values of E_{vo} measured at a constant $[Cl^-]_v$ (0.1 mM in Fig. 2a, 100 mM in Fig. 2b) and at various $[K^+]_v$ (0.1-100 mM) were plotted against $[K^+]_o$. In both Fig. 2a and b, E_{vo} becomes significantly less negative when $[K^+]_o$ increases. Next, E_{vo} which was measured in the same bathing medium (0.1 mM each of K^+, Na^+, Cl^-, 1 mM Ca^{2+} and 1.05 mM SO_4^{2-}) was plotted aginst $[K^+]_v$. When $[K^+]_v$ is varied from 0.1 mM to 100 mM, E_{vo} changes slightly to the hyperpolarizing direction (Fig. 3), indicating that the tonoplast is less sensitive to K^+ than the plasmalemma.

Table 1. Ionic compositions of vacuolar and bathing media.

Medium	Concentrations of ions in mM				
	K^+	Na^+	Ca^{2+}	Mg^{2+}	Cl^-
Vacuolar media					
Np	120	6	1	—	128
Nf	90	40	15	5	180
KCl	150	—	10	—	170
iAPW-10Ca[a)]	0.1	0.1	10	—	20.2
150-KCl[b)]	150	10	10	—	ca.180
0.1-KCl[b)]	0.1	10	10	—	ca. 30
Bathing media					
iAPW	0.1	0.1	0.1	—	0.4
iAPW-1Na[c)]	0.1	1	0.1	—	ca. 1

Osmotic value of each medium was adjusted to 300-330 mM with sorbitol.

a): This medium was also used for the bathing medium.

b): These media were used only for the experiments to see the effects of vacuolar pH on membrane potential and resistance. Values of pH of the media were adjusted by adding glycine or Tris-maleate.

c): This medium was used in the experiments to see the effect of external pH on membrane potential and resistance. Values of pH were adjusted by adding Tris-maleate.

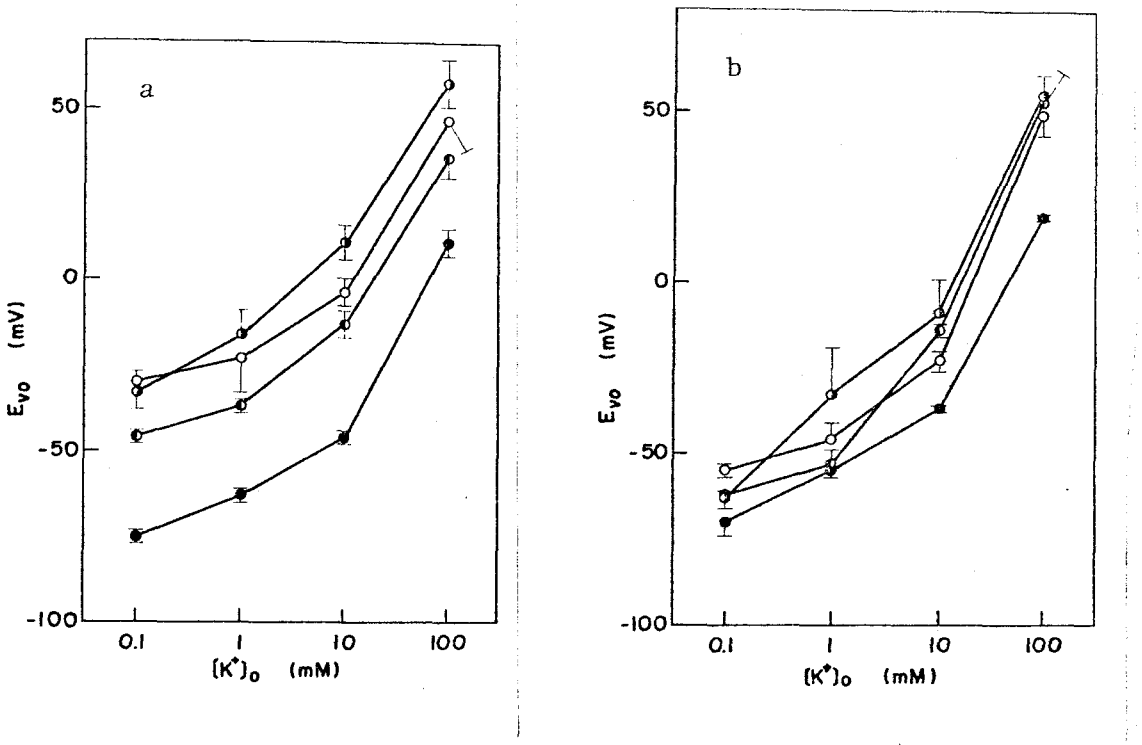

Fig. 2 Relation between vacuolar potential (E_{vo}) and concentration of K^+ in the external solution ($[K^+]_o$). All external media contained 0.1 mM Na^+, 1.0 mM Ca^{2+} and 0.1 mM Cl^-. $[K^+]_o$ and $[K^+]_v$ were changed from 0.1 mM to 100 mM (Table 1) by adding K_2SO_4. Curves with different marks indicate experiments with 0.1 mM (○), 1.0 mM (◑), 10 mM (◐) and 100 mM (●) K^+ in the vacuole. Cl^- concentration in the vacuole was kept either at 0.1 mM (a) or at 100 mM (b).

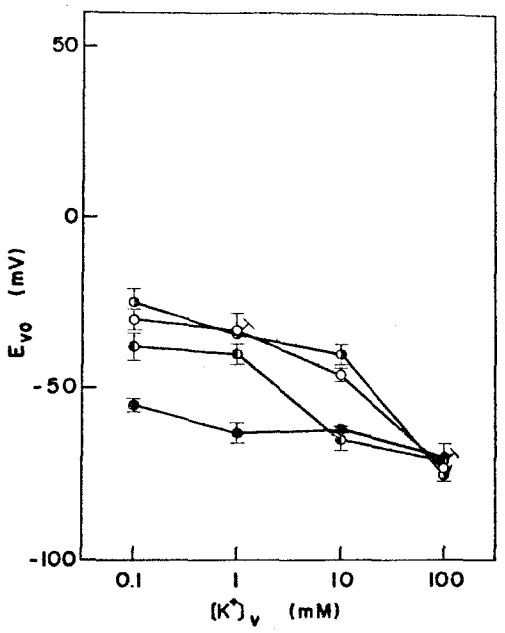

Fig. 3 Relation between vacuolar potential (E_{VO}) and concentration of K^+ in the vacuole ($[K^+]_V$). The external medium contained 0.1 mM each of K^+, Na^+, Cl^-, 1.0 mM Ca^{2+} and 1.05 mM SO_4^{2-}. Curves with different marks indicate experiments with 0.1 mM (○), 1.0 mM (◑), 10 mM (◐) and 100 mM (●) Cl^- in the vacuole.

In our previous paper (Tazawa et al. 1975), it was reported that E_{vo} of N. flexilis changed to the deplorizing direction when $[Cl^-]_o$ increased. A similar dependency of E_{vo} on $[Cl^-]_o$ was also observed in N. pulchella whatever $[K^+]_v$ and $[Cl^-]_v$ (Fig. 4 a and b). Namely, in the range of $[Cl^-]_o$ below 10 mM, E_{vo} becomes slightly less negative as $[Cl^-]_o$ increases. When $[Cl^-]_o$ is increased from 10 mM to 100 mM, E_{vo} changes significantly to the depolarizing direction. However, when the vacuolar solution contains 100 mM K^+, E_{vo} is not sensitive to $[Cl^-]_o$. On the other hand, the response of E_{vo} to $[Cl^-]_v$ occurs in the opposite direction to that of E_{vo} to $[Cl^-]_o$ (Fig. 5). Since the topographical relation of the cytoplasm to the vacuole (cytoplasm is outside the vacuole) is inverse to that of the cytoplasm to the external medium, it is concluded that the tonoplast responds to Cl^- in a similar manner as the plasmalemma.

The electirc conductance of the tonoplast seems to be almost independent of $[K^+]_v$ and $[Cl^-]_v$, since R_{vo} was not affected appreciably by changing $[K^+]_v$ and $[Cl^-]_v$. In Fig. 6a $[K^+]_v$ is varied from 0.1 to 100 mM, while $[Cl^-]_v$ is kept at 100 mM. In Fig. 6b, $[Cl^-]_v$ is varied under constant $[K^+]_v$ (100 mM). In each case, cells were bathed in the medium containing K^+, Na^+ and Cl^- each of 0.1 mM and Ca^{2+} of 1 mM. On the other hand, the electric conductance of the plasmalemma was strongly dependent on $[K^+]_o$. In Fig. 7, R_{vo} of cells whose vacuolar saps contained 100 mM K^+, 100 mM Cl^-, 0.1 mM Ca^{2+} were plotted against $[K^+]_o$ (Fig. 7a) and against $[Cl^-]_o$ (Fig. 7b). It is clear that the higher the concentration of K^+ in the bathing medium the larger is the electric conductance

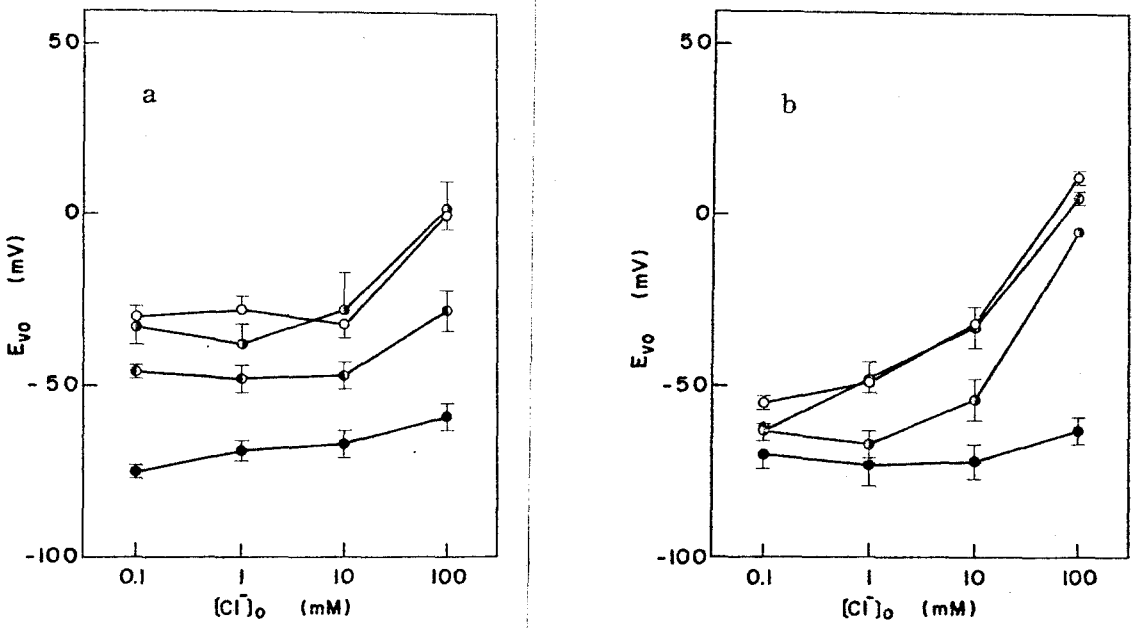

Fig. 4 Relation between vacuolar potential (E_{vo}) and concentration of Cl^- in the external medium ($[Cl^-]_o$). All external media contained 0.1 mM K^+, 0.1 mM Na^+ and 1.0 mM Ca^{2+}. $[K^+]_v$ and $[Cl^-]_o$ were changed from 0.1 mM to 100 mM by adding K_2SO_4 and choline chloride, respectively. Curves with different marks indicate experiments with 0.1 mM (○), 1.0 mM (◎), 10 mM (◫) and 100 mM (●) K^+ in the vacuole. Concentration of Cl^- in the vacuole was kept either at 0.1 mM (a) or at 100 mM (b).

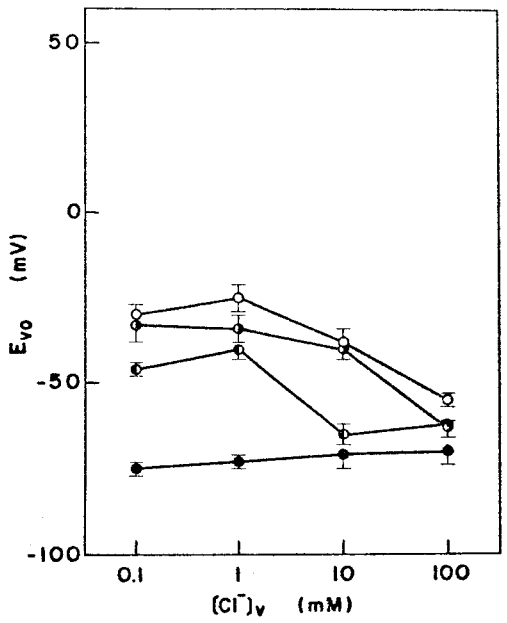

Fig. 5 Relation between vacuolar potential (E_{vo}) and concentration of Cl^- in the vacuole ($[Cl^-]_v$). The external medium contained 0.1 mM each of K^+, Na^+, Cl^-, 1.0 mM Ca^{2+} and 1.05 mM SO_4^{2-}. Curves with different marks indicate the experiments with 0.1 mM (○), 1.0 mM (◑), 10 mM (◐) and 100 mM (●) K^+ in the vacuole.

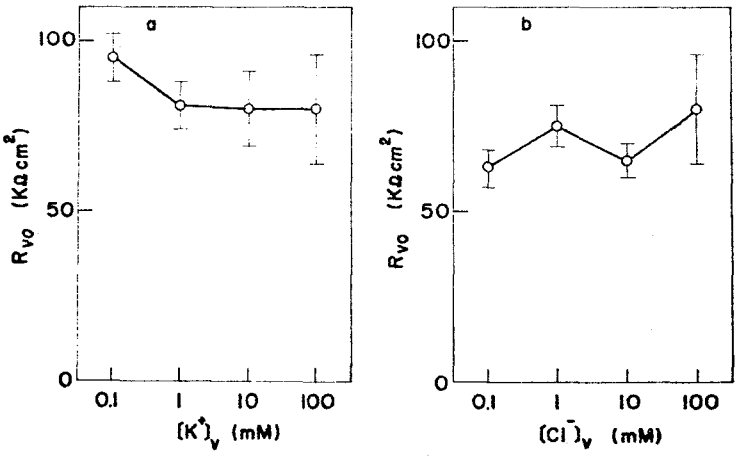

Fig. 6 Effects of concentrations of vacuolar K^+ ($[K^+]_V$) and Cl^- ($[Cl^-]_V$) on the specific resistance (R_{VO}) across the plasmalemma and the tonoplast. The external medium contained 0.1 mM each of K^+, Na^+, Cl^-, 1.0 mM Ca^{2+} and 1.05 mM SO_4^{2-}. $[K^+]_V$ was varied from 0.1 mM to 100 mM under a constant $[Cl^-]_V$ (100 mM) with K_2SO_4 (a) and $[Cl^-]_V$ was varied under a constant $[K^+]_V$ (100 mM) with choline chloride (b).

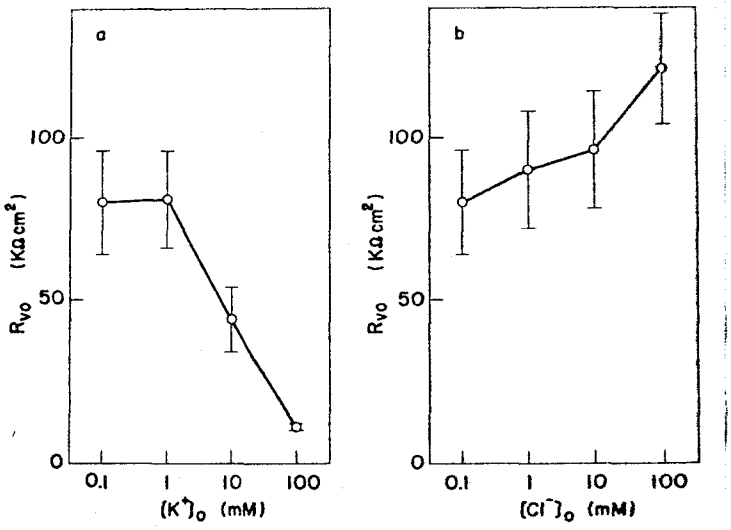

Fig. 7 Effects of concentrations of K^+ ($[K^+]_o$) and Cl^- ($[Cl^-]_o$) in the external medium on the specific resistance (R_{VO}) across the plasmalemma and the tonoplast. Vacuolar medium contained 100 mM K^+, 100 mM Cl^-, 0.1 mM Na^+, 1 mM Ca^{2+} and 1.05 mM SO_4^{2-}. $[K^+]_o$ was varied from 0.1 to 100 mM under a constant $[Cl^-]_o$ (0.1 mM) with K_2SO_4 (a) and $[Cl^-]_o$ was varied under a constant $[K^+]_o$ (0.1 mM) with choline chloride (b).

of the cell. On the other hand, $[Cl^-]_o$ does not increase the conductance but decreases it slightly.

(2) E_{vo} in relation to the concentration of H^+ in the vacuole

To examine the effect of concentration of H^+ in the vacuole, perfusion media with different pH were prepared. Two basal media were used, one with a high KCl concentration and the other with a low KCl concentration. The former had 150 mM K^+, 10 mM Na^+, 10 mM Ca^{2+} and about 180 mM Cl^-. The latter had 0.1 mM K^+, 10 mM Na^+, 10 mM Ca^{2+} and about 30 mM Cl^- (Table 1). To adjust pH, Tris-maleate (pH lower than 8) or glycine (pH higher than 9) in concentrations less than 10 mM was added into each perfusion medium with HCl or NaOH. The concentration of Na^+ was always kept at 10 mM. For external media, iAPW-1Na of pH 4, 6 and 8 were used which contained 0.1 mM K^+, 1 mM Na^+, 0.1 mM Ca^{2+}, about 1 mM Cl^- and Tris-maleate in concentrations less than 1 mM (Table 1). Again the pH was adjusted with HCl or NaOH only. After the natural cell sap was replaced with an artificial solution with known pH, E_{vo} was measured in iAPW-1Na media with various pH (pH 4, 6, 8). In each case, the pH of each medium was checked before and after the experiment.

Fig. 8 shows how E_{vo} behaves when pH of the vacuole (pH_v) is changed from 3 to 10. When the concentration of KCl is high (Fig. 8a), E_{vo} responds to pH_v sensitively (30-50 mV/pH) only in a strongly acid region (pH 4-3). Above pH 4, E_{vo} changes only about 5 mV/pH. When the concentration of KCl in the vacuole is low (Fig. 8b), E_{vo} changes steadily to the

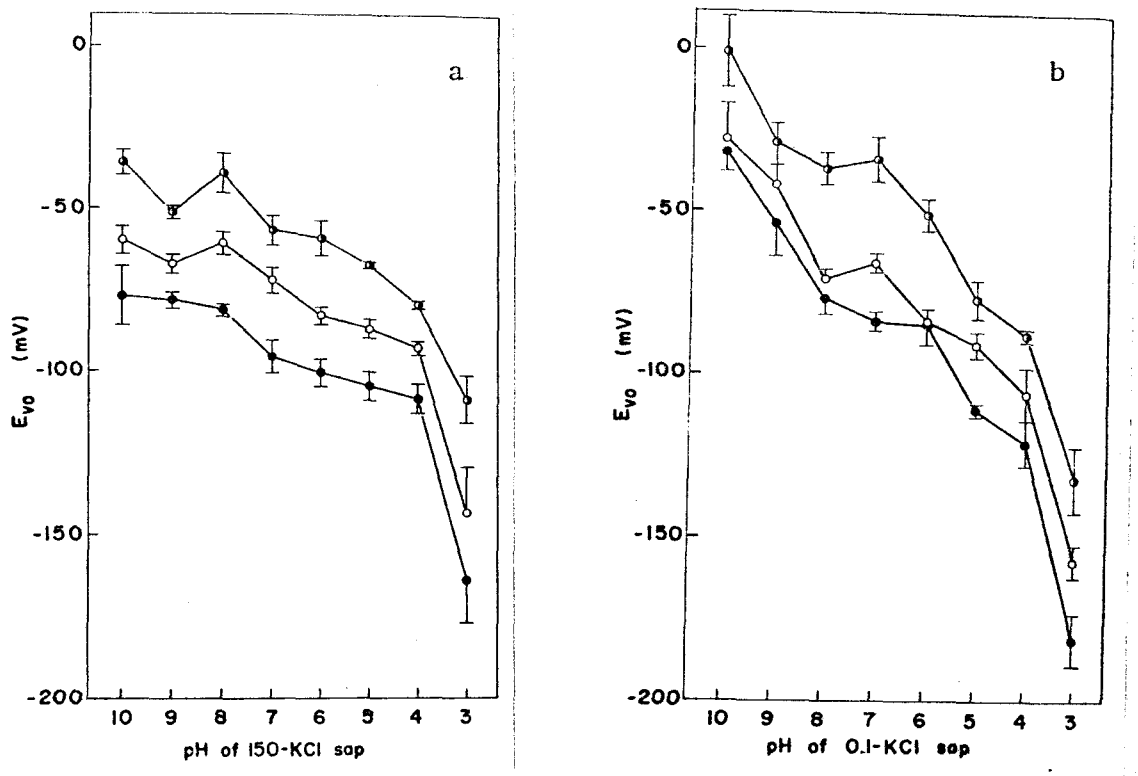

Fig. 8. Relation between vacuolar potential (E_{VO}) and pH of the vacuolar medium. Curves with different marks indicate the measurement in iAPW-1Na (Table 1) of pH 4 (○), pH 6 (◎) and pH 8 (●). Vacuolar medium was either 150-KCl sap (a) or 0.1-KCl sap (b) (cf. Table 1).

hyperpolarizing direction with the increase in H^+ concentration, although it is relatively insensitive to pH_v around 6. Average of the changes in E_{vo} between pH 4 and 10 are about 15 mV/pH for all the curves in Fig. 8b. E_{vo} in the acid region is more sensitive to pH_v (44-60 mV/pH between pH 3 and 4) than in the alkaline region (14-28 mV/pH between pH 9 and 10). As shown in both in Fig. 8a and b the mode of response of E_{vo} to pH_v is not influenced much by the change in pH of the external medium (pH_o), though the absolute values of E_{vo} depend on pH_o. Namely, the plasmalemma and the tonoplast respond to H^+ independently of each other.

The effect of pH_o on E_{vo} of the cell with the vacuolar medium of pH 6 is reconstructed from Fig. 8a and b and is shown in Fig. 9. In Fig. 9, results obtained by Kitasato (1968) on N. clavata and by Lefebvre and Gillet (1975) on N. flexilis are also shown. They demosntrated that E_{vo} becomes more negative in the alkaline region of the bathing medium. Similar tendency is also observed in N. pulchella with the cell sap of pH 6, which is nearly equal to the pH-value of natural cell sap of N. flexilis (Hirakawa & Yoshimura 1964), irrespective of the concentration of KCl in the vacuole. In cells with 150-KCl sap E_{vo} at pH 8 is 41 mV more negative than that at pH 4, and in cells with 0.1-KCl sap the former is 34 mV more negative than the latter. Since the average changes in E_{vo} for the change in pH_v between 4 and 8 are 33 mV in Fig. 8a and 43 mV in Fig. 8b, it can be said that in N. pulchella the tonoplast responds to H^+ equally sensitive as the plasmalemma. However, it is to be noticed that cells of N. pulchella survived for a day when the vacuolar pH was 3,

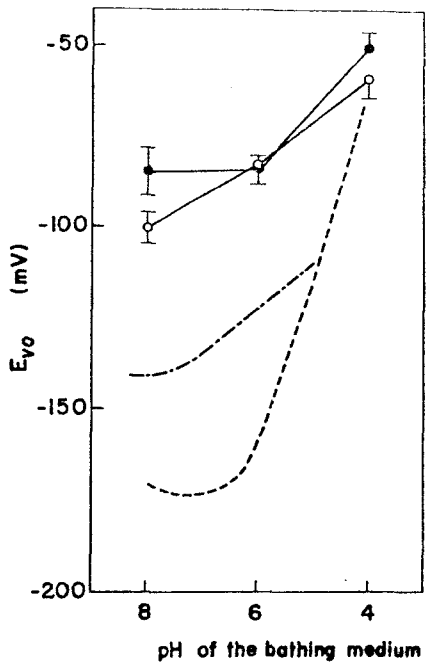

Fig. 9 Effects of external pH on vacuolar potential (E_{vo}). External media were iAPW-1Na (Table 1) of pH 4, 6 and 8. Curves of different marks indicate experiments with 150-KCl sap (○) and 0.1-KCl sap (●) of pH 6. Results obtained by Kitasato (1968) on N. Clavata (---) and by Lefebvre and Gillet (1973) on N. flexilis (—·—) are also shown.

but they died after an hour when the external medium had the same pH.

Fig. 10 shows the relation between pH of 150-KCl sap (Table 1) and R_{VO} in the cells bathed in iAPW-1Na of pH 6. Values of R_{VO} measured when pH of the cell sap is 3 and 4 are about twice as high as those measured when it is above 6.

(3) E_{VO} in relation to tonicity of the cell sap

So far we changed the ionic concentrations of the internal and external media and observed how the outer and internal cytoplasmic membranes behaved. It is interesting to know, however, how the membranes responds to changes in concentrations of ions in the cytoplasm. The simplest way to realize this is to change the osmotic pressure of the cell sap. Since the tonoplast is assumed to be semipermeable, change in the osmotic pressure of the cell sap (π_V) will bring forth a change in the volume of the cytoplasm according to van't Hoff's law (cf. Yoneda & Kamiya 1969). For instance, doubling of π_V causes the decrease of the cytoplasmic volume to half and consequently the increase in the concentrations of the ions in the cytoplasm to twice the normal ones.

Table 2 shows that E_{VO} and R_{VO} are not affected appreciably by doubling the tonicity of the cell sap from 300 mM to 600 mM. In this case, the tonicity of the bathing solution (APW) was also adjusted to 600 mM by sorbitol. The electric current required for eliciting the action potential was increased when the vacuolar osmolarity was increased. Namely, cells with 600 mM osmolarity could produce the action potential with the electric stimulus of 0.87 μA cm^{-2} (average

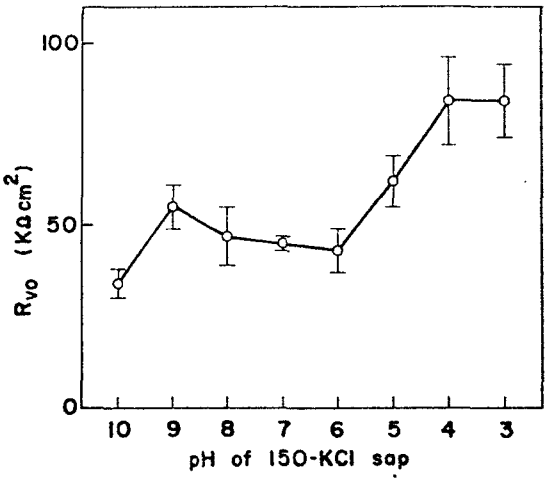

Fig. 10 Relation between pH of the vacuolar medium with 150-mM KCl sap (Table 1) and the specific resistance (R_{vo}) across the plasmalemma and the tonoplast. The external medium was iAPW-1Na (Table 1) of pH 6.

Table.2. Potential difference (E_{vo}) and resistance (R_{vo}) between the vacuole and the external medium of N. pulchella with various vacuolar media.

Vacuolar medium	Bathing medium	E_{vo} (mV)	R_{vo} ($K\Omega cm^2$)
Np	iAPW	-110 ± 3 (7)	95 ± 13 (4)
Nf	iAPW	-107 ± 2 (42)	70 ± 3 (41)
KCl	iAPW	-106 ± 2 (7)	55 ± 6 (7)
iAPW-10Ca	iAPW	-106 ± 6 (10)	68 ± 3 (6)
iAPW-10Ca	iAPW-10Ca	-36 ± 4 (7)	28 ± 7 (6)
2-Nf[a)]	2- iAPW[a)]	-111 ± 3 (22)	79 ± 9 (20)
2-KCl[a)]	2- iAPW	-103 ± 4 (6)	
2-iAPW-10Ca [a)]	2- iAPW	-95 ± 7 (3)	

Data are shown as the average ± S.E. Figures in parenthese indicate the number of cells used.
a): Osmotic values of the media were 600 mM.

of 9 cells), while cells with normal osmolarity (300 mM) could with 0.22 μA cm^{-2} (average of 12 cells). This indicates that the high tonicity of the cell increases the threshold for excitation, because it does not affect R_{vo}.

It is remarkable that E_{vo} of N. pulchella was almost indifferent to the concentrations of K^+ and Cl^- in the cell sap (Table 2). Namely, values of E_{vo} and R_{vo} of cells of N. pulchella having the sap of extremely low ionic concentrations (iAPW-10Ca sap) are practically equal to those having saps of high ionic concentrations (Np-, Nf-, KCl-sap). The insensitivity of both E_{vo} and R_{vo} of N. pulchella for the profound changes in ionic concentrations is consistent with the fact that E_{vo} is affected only slightly by large variations of $[K^+]_v$ (Fig. 3) and $[Cl^-]_v$ (Fig. 5) and also with the facts that R_{vo} is almost indifferent to changes in $[K^+]_v$ and $[Cl^-]_v$ (Fig. 6).

When the cell sap of N. flexilis or Chara australis was replaced with an isotonic artificial pond water (iAPW-10Ca), where the concentration of K^+ and Na^+ are as low as that of the artificial pond water, E_{vo} amounting to ca. -40 mV was observed even when the cells were bathed in the same isotonic artificial pond water (Tazawa et al. 1975). Under the same ionic conditions cells of N. pulchella also showed a significant E_{vo} (-36 mV).

Discussion

The vacuolar potential, E_{vo}, is the difference between the plasmalemma potential (E_{co}, potential of cytoplasm against that of outside) and the tonoplast potential (E_{cv}, potential of cytoplasm against vacuole),

$$E_{vo} = E_{co} - E_{cv}. \qquad (1)$$

In the present studies E_{cv} was not measured directly. However, it is reasonable to assume that responses of E_{vo} (ΔE_{vo}) to the change in composition of the vacuolar medium is equal to change in E_{cv} (ΔE_{cv}). Namely,

$$\Delta E_{cv} = -\Delta E_{vo}. \qquad (2)$$

This assumption is supported by the fact that E_{vo} responded to vacuolar pH in a similar manner irrespective of the pH of the external medium (Fig. 8a, b). Similarily, response of E_{co} (ΔE_{co}) to the change in ionic composition of the external medium is represented as follows,

$$\Delta E_{co} = \Delta E_{vo}. \qquad (3)$$

Even when both internal and external media of the internode of N. pulchella were the same (iAPW-10Ca), E_{vo} as large as about -40 mV was observed (Table 2). Similar results were also obtained in N. flexilis and Chara australis (Tazawa et al. 1975). This value of E_{vo} may be generated by the electrogenic ion pump, or may reflect only the difference in E_{co} and E_{cv} which are functions of passive permeabilities of the membranes to ions.

To check whether the electrogenic pump is working or not in N. pulchella, the short-circuit current was measured with the open-vacuole method on cells whose cell sap was

replaced with iAPW-10Ca. The current which was first outward shifted with time to zero and, in some cases, changed its direction to the inward one. The current was not affected by illumination with white light. Treatment of the cell with 2,4-dinitrophenol (0.3 mM) or with low temperature (5°C) did not make the short-circuit current zero. All these results are against the electrogenic ion pump. Thus, we believe that E_{vo} observed under the null chemical potential gradient between the vacuole and the external medium has its origin in difference in passive permeability characteristics between these two membranes.

The characteristics of the two membranes are summarized from the responses of E_{vo} to ions in the following. The tonoplast responds to $[H^+]$ and $[Cl^-]$ similary to the plasmalemma but differently to $[K^+]$. The plasmalemma behaves as a K^+-electrode when $[K^+]$ in the external medium is above 10 mM, because E_{vo} changes by about 60 mV for the tenfold changes in $[K^+]_o$ (Fig. 2). On the other hand, E_{vo} responds less sensitively to $[K^+]_v$ than to $[K^+]_o$ (Fig. 3). This may explain the result that E_{vo} is scarcely affected by profound changes in ionic concentrations of the vacuolar medium (Table 2) and also may explain the fact that, in N. flexilis and C. australis, the tonoplast potential changes less than the plasmalemma potential when ionic compositions of the facing media were changed from the artificial cell sap to iAPW-10Ca (Tazawa et al. 1975).

Effect of $[Cl^-]$ on E_{vo} is very strange. If the Goldman

equation is valid for E_{vo}, increase in $[Cl^-]_o$ should make E_{vo} more negative and increase in $[Cl^-]_v$ does E_{vo} less negative. Actually, E_{vo} changed, however, to the direction opposite to the expected one (Fig. 4 and 5, cf. Tazawa et al. 1975). Baker et al. (1964) reported that P_{Cl}/P_K of the squid axon changed from 0.02 to 0.1 when concentration of KCl in the internal solution was changed from 50 mM to 100 mM or more. Tazawa (1972) suggested the similar possibility that the increase in Cl^--concentration in the bathing medium of Nitella causes the increase in P_{Cl}/P_K. Assuming that increase in P_{Cl}/P_K of the membrane in response to increase in Cl^--concentration of the facing medium occurs both at the plasmalemma and at the tonoplast, the present results can be accounted for well by use of Goldman equation.

In the experiments to see the effects of Cl^-- and K^+-concentrations of the external and internal media on E_{vo} (Fig. 2, 3, 4 and 5), Na^+-concentrations of both media were only 0.1 mM. Because of this and also of the fact that Na^+-concentration in the cytoplasm of N. pulchella is also low (9 mM; Tazawa et al. 1974), the potential difference across the plasmalemma (E_{co}) and that across the tonoplast (E_{cv}) can be expressed approximately by the following equations without the terms for Na^+:

$$E_{co} = \frac{RT}{F} \ln \frac{[K^+]_o + \beta[Cl^-]_c}{[K^+]_c + \beta[Cl^-]_o} \qquad (4)$$

$$E_{cv} = \frac{RT}{F} \ln \frac{[K^+]_v + \beta'[Cl^-]_c}{[K^+]_c + \beta'[Cl^-]_v} \qquad (5)$$

where suffixes o, c and v mean outside, cytoplasm and vacuole, respectively; β and β' are ratios of permeability coefficients for K^+ and Cl^- (P_{Cl}/P_K) of plasmalemma and tonoplast, respectively; and R, T and F have usual meanings. In N. pulchella, $[K^+]_c$ and $[Cl^-]_c$ are about 100 mM and 30 mM, respectively (Tazawa et al. 1974). At a constant $[Cl^-]_o$, for example at 0.1 mM, E_{co} is a funciton of $[K^+]_o$ and β. Assuming that β is independent of $[K^+]_o$, β can be calculated from the changes in E_{co} for the tenfold increase in $[K^+]_o$. Since $[K^+]_o$ was changed in four steps (Fig. 2), three values of β were obtained. The average value amounting to 0.060 was adopted for β at 0.1 mM $[Cl^-]_o$. Next, $[K^+]_o$ was kept constant at 0.1 mM. After the value of β for 0.1 mM $[Cl^-]_o$ has been determined, values of β for 1.0, 10 and 100 mM of $[Cl^-]_o$ can be calculated from changes in E_{co} observed for tenfold increases in $[Cl^-]_o$ (Fig. 4). Values of β estimated thus are 0.064, 0.083 and 0.27 for 1.0, 10 and 100 mM of $[Cl^-]_o$, respectively. In Fig. 11, E_{co} calculated with Eqn. 4 is plotted against $[K^+]_o$ ($[Cl^-]_o$ = 0.1 mM) and also against $[Cl^-]_o$ ($[K^+]_o$ = 0.1 mM). Values of β at 1.0 mM and 100 mM of $[Cl^-]_o$ are in the same order of magnitude as that obtained at about 4 mM of $[Cl^-]_o$ in N. clavata (0.025; Kitasato 1968) and that obtained at 180 mM of $[Cl^-]_o$ in N. flexilis (0.7; Tazawa 1975).

If P_{Cl}/P_K of the tonoplast, β', also depends on $[Cl^-]_v$ irrespective of $[K^+]_v$, it can be calculated from data shown in Fig. 3 and 5 through similar processes used for estimation of β. Values thus estimated are 0.6, 0.7, 1.6 and 7.8 for

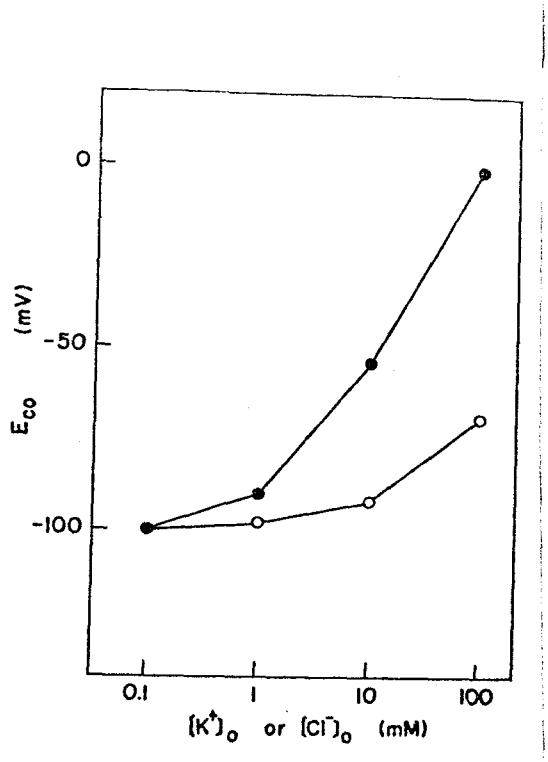

Fig. 11 Relation between plasmalemma potential (E_{co}) calculated from Eqn. 4 and concentrations of K^+ ($[K^+]_o$) or Cl^- ($[Cl^-]_o$) in the bathing medium. Ions in the bathing medium are assumed to be 0.1, 1.0, 10 and 100 mM $[K^+]_o$ while $[Cl^-]_o$ is 0.1 mM to be 0.1, -100 mM $[Cl^-]_o$ while $[K^+]_o$ is 0.1 mM. Values of β in Eqn. 4 are estimated to be 0.060, 0.064, 0.08 and 0.27 for 0.1, 10 and 100 mM $[Cl^-]_o$. ●—● : E_{co} against $[K^+]_o$ ○—○: E_{co} against $[Cl^-]_o$

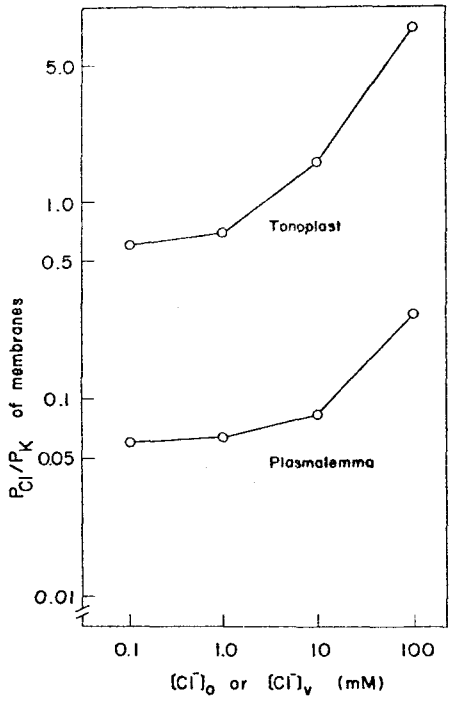

Fig. 12 Relation between Cl^--concentration in the facing medium and relative permeability to Cl^- (P_{Cl}/P_K) of the plasmalemma and that of the tonoplast. P_{Cl}/P_K of plasmalemma (β) and that of tonoplast (β') developed in Eqn 4 and 5, respectively, are plotted against Cl^--concentration of bathing medium ($[Cl^-]_o$) and that of vacuolar medium ($[Cl^-]_v$), respectively.

0.1, 1.0, 10 and 100 mM of $[Cl^-]_v$, respectively (Fig. 12). Then, E_{vc} can be obtained through use of Eqn. 5 for different $[K^+]_v$ and $[Cl^-]_v$. Since E_{vo} is the difference between E_{co} and E_{cv} (Eqn. 1), addition of E_{co}, which is -100 mV for 0.1 mM each of $[K^+]_o$ and $[Cl^-]_o$((Fig. 11), to $-E_{cv}$ gives E_{vo}. When $[Cl^-]_v$ is 100 mM, values of calculated E_{vo} are similar to those of measured one. When $[Cl^-]_v$ are 10 mM or below it, the mode of response of calculated E_{vo} to $[K^+]_v$ is similar to that of measured E_{vo} to $[K^+]_v$, while absolute values of the former are about 25 mV more negative then those of the latter. Then, the values of calculated E_{vo} except the values for 100 mM $[Cl^-]_v$ were shifted by 25 mV to positive direction. In Fig. 13, E_{vo} thus obtained are plotted against $[K^+]_v$ (a) and $[Cl^-]_v$ (b). Curves in Fig. 13 are similar to corresponding curves in Fig. 3 and 5 in the magnitude and in the shape. The difference of 25 mV between measured and calculated E_{vo} of cells with 0.1-10 mM Cl^--sap remains unexplained. Development of a phase boundary potential at the surface of the tonoplast may be a candidate for the discrepancy.

The plasmalemma potential depends on pH of the bathing medium (Kitasato 1968, Lefebvre & Gillet 1973, Saito & Senda 1973, 1974). In the present study we could show that the tonoplast potential is also dependent on the pH of the facing medium (Fig. 8a and b). When pH of the vacuolar medium is low (pH 3-4), the tonoplast potential is very sensitive to H^+. When the concentration of KCl in the vacuole was low (0.1 mM),

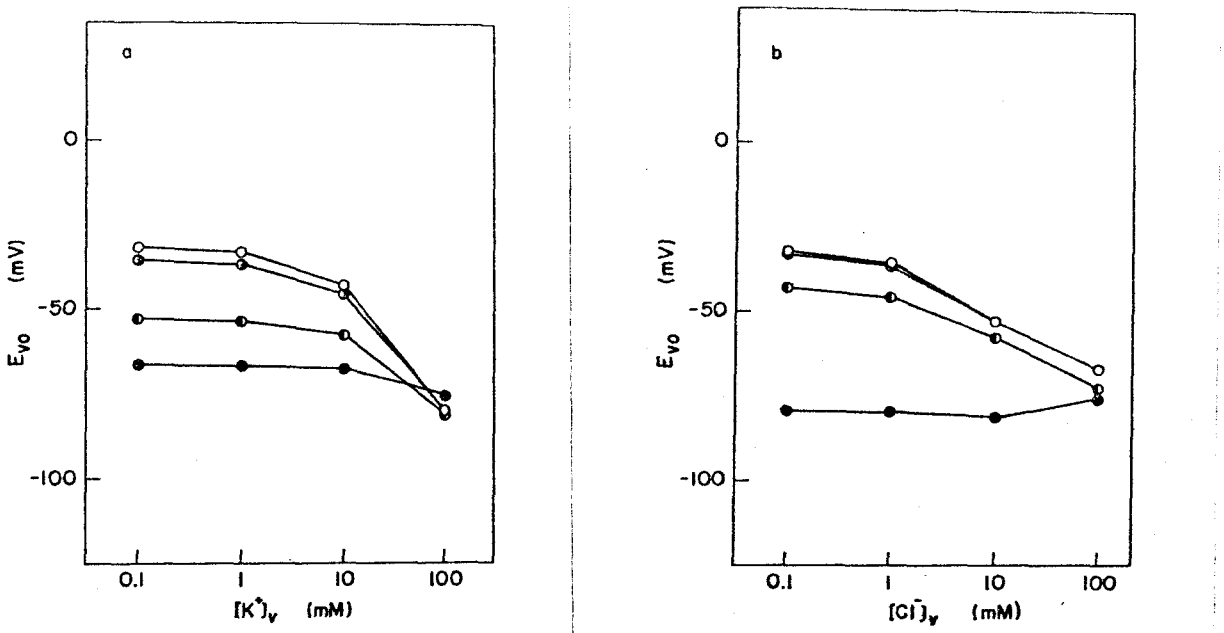

Fig. 13 Relation between vacuolar potential (E_{VO}) calculated from Eqn. 3 and concentrations of K^+ ($[K^+]_V$) and Cl^- ($[Cl^-]_V$) in the vacuole. Composition of external medium was assumed to be same as these in Fig. 3 and 5. Curves with different marks indicate the same meanings in Fig. 3 (a) and in Fig. 5 (b). The calculated E_{VO} for 0.1-10 mM Cl^- sap cell are shifted by 25 mV to the positive direction.

E_{VO} changed much also for pH change in the alkaline region (Fig. 8b). This indicates that the tonoplast potential is also sensitive to OH^- especially when KCl concentration in the vacuole is low. Then, the Goldman equation for the tonoplast potential (E_{CV}) should have terms for H^+ and OH^-, and E_{VO} is written in the following formula;

$$E_{VO} = E_{CO} - E_{CV}$$

$$= E_{CO} - \frac{RT}{F} \ln \frac{[K^+]_V + \beta'[Cl^-]_C + \gamma'[H^+]_V + \delta'[OH^-]_C}{[K^+]_C + \beta'[Cl^-]_V + \gamma'[H^+]_C + \delta'[OH^-]_V} \qquad (6)$$

where γ' and δ' mean P_H/P_K and P_{OH}/P_K of the tonoplast, respectively. The plasmalemma potential, E_{CO}, is assumed to be -98 mV (Fig. 12) because, in all pH-experimemnts, cells were bathed in iAPW-1Na with 1 mM [Cl^-] (Tabel 1). So far as we know, values of cytoplasmic pH in plant cells have never been reported. Values of cytoplasmic pH in animal cells are around 7 (Caldwell 1954, 1958, Spyropoulos 1960). We assume tentatively that the cytoplasmic pH of Nitella was also to be 7. Then, concentrations of all ions involved in Eqn. 6 are known, while relative permeabilities (β', γ', δ') are unknown. However, it is reasonable, from Fig. 12, to adopt 7.8 as β' for the cells with 150 mM KCl sap (180 mM $[Cl^-]_V$) and 4.5 as β' for the cells with 0.1 mM KCl sap (30 mM $[Cl^-]_V$). The remaining unknown parameters, γ' and δ', were determined in the following way. First, E_{VO} for different pH_V was calculated by introducing arbitrary values of γ' and δ' into Eqn. 6. Plotting E_{VO} thus calculated against pH_V, we get many curves as shown in Fig. 14a and b. In this case, calculated E_{VO} of 0.1 mM KCl sap cell (Fig. 14a) are

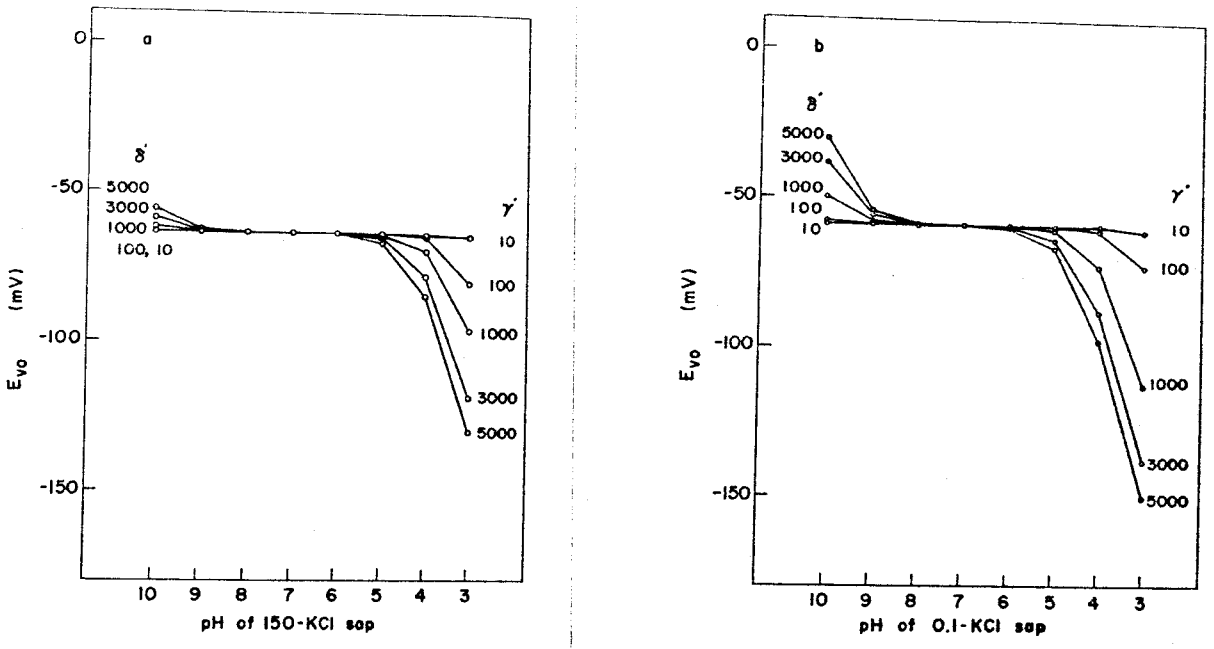

Fig. 14 Relation between pH of the vacuole and the vacuolar potential (E_{vo}) calculated by introducing arbitrary values of P_H/P_K (γ') and P_{OH}/P_K (δ') into Eqn. 6. Bathing medium is assumed to iAPW-1Na (Table 1). The value of β' in Eqn. 6 are adopted 7.8 for 150-KCl sap cell (a) and 4.5 for 0.1-KCl sap cell (b). Calculated E_{vo} of 0.1-KCl sap cell are shifted by 25 mV to the positive direction.

shifted by 25 mV to positive direction (cf. Fig. 13). When both γ' and δ' are 5000, the theoretical curves fit best to the curves obtained experimentally, irrespective of concentrations of KCl in the vacuole. The value of P_H/P_K (5000) of the tonoplast of N. pulchella is of a similar order of magnitude to that (about 10000) suggested by Kitasato (1968) in the cell of N. clavata.

R_{VO} is affected insignificantly by a large change in ionic concentrations of the vacuole (Table 2, Fig. 6). The ratio of the specific resistance of the tonoplast to that of the plasmalemma (R_{CV}/R_{CO}) is 1/10 in C. australis (Findlay & Hope 1964) and 1/8 in Nitellopsis obtusa (Findlay 1970). If N. pulchella R_{CV} is much smaller than R_{CO}, change in R_{CV} brings about only an insignificant changes in R_{VO}. On the other hand, values of R_{VO} of cells with acid saps are about twice as large as those of cells with neutral or alkaline saps (Fig. 10). If increase in R_{VO} reflects increase in R_{CV}, the above result means that the tonoplast has nearly the same conductance as the plasmalemma at the acid region of the vacuolar sap. High $[H^+]_V$ may change the characteristics of the tonoplast so as to lessen the permeabilities of the tonoplast to ions.

Kishimoto (1957) reported that R_{VO} of N. flexilis decreased at the exosmosis side where the osmotic pressure of the cell sap was made higher than that of the normal one. The result is understandable, because the increase in osmotic pressure of the vacuolar medium causes osmotic contraction of the cytoplasm and consequently an increase in ionic concentrations

of the cytoplasm, even though ionic permeabilities of the membranes remain constant. However, R_{VO} of N. pulchella does not change even when the osmotic pressure of the cell increased to twice that of the normal one (Table 2). This may be explained by assuming a decrease in permeability of the membrane to ions.

Since the electric current required to generate the action potential in the cells of high tonicity was significantly larger than that in the cells of normal tonicity, high tonicity of the vacuolar medium seems to affect the plasmalemma to make it more stable. Characteristics of the tonoplast under excitation will be reported in the subsequent paper.

Summary

Potential difference (E_{vo}) and specific resistance (R_{vo}) between the vacuole and the external medium were studied in relation to concentrations of K^+, Cl^- and H^+ in the vacuole. The results were discussed under the assumption that changes in ionic compositions of the vacuolar medium affect the potential difference (E_{cv}) and specific resistance (R_{cv}) across the tonoplast only. It was assumed that changes in E_{vo} and R_{vo} brought about when concentrations of ions in the external medium were modified reflect variations in potential difference (E_{co}) and specific resistance (R_{co}) across the plasmalemma only.

1. Both E_{co} and R_{co} respond sensitively to K^+ in the external medium, while E_{cv} and R_{cv} are scarcely affected by K^+ in the vacuole.

2. Both E_{co} and E_{cv} respond to Cl^-. However, the sign of the response is opposite to that expected from Goldman equation. Simulation of the measured E_{vo} by Goldman equation through application of adequate permeability parameters made it clear that the permeability ratio between Cl^- and K^+ (P_{Cl}/P_K) at the plasmalemma as well as at the tonoplast increases when Cl^--concentration of the facing medium increases. In both membranes the membrane resistance is almost independent of concentration of Cl^-.

3. Response of E_{co} to external pH is similar to that of E_{cv} to vacuolar pH. Both E_{co} and E_{cv} change by 10 mV for one unit change of pH in the range of pH 4-8 of the facing media. E_{cv} responds very sensitively to vacuolar pH in the strongly acid region (pH 3-4) irrespective of the concentration of

KCl in the vacuole. In the alkaline region, however, E_{cv} responds to vacuolar pH only when KCl concentration in the vacuole is low (0.1 mM). The pH-dependency of E_{vo} can be well explained if the tonoplast is assumed to be 5000 times as permeable to both H^+ and OH^- as to K^+. R_{vo} increases significantly when the vacuolar pH is lowered to 4 or 3.

4. Increase in tonicity of the vacuolar medium to twice the normal one causes no significant change in both E_{vo} and R_{vo}, while it raises the threshold for excitation.

A significant amount of E_{vo} remains even when the external and internal media of the cell are the same one. This may be accounted for by the difference in passive membrane properties between the plasmalemma and the tonoplast.

Chapter two: Tonoplast action potential in Nitella in relation to vacuolar chloride concentration.

Material and method

The material used throughout this work was Nitella pulchella which was collected from a pond and stored outdoors in a large pot with soil.

The open-vacuole method (Tazawa et al. 1975), in which both cell ends are open to the outside and therefore the cell remains turgorless, was mainly used for measuring the vacuolar potential (E_{VC}) of the cell whose vacuolar sap was replaced with an artificial solution (Fig. 1). Nitella interode (N) was placed on a polyacrylate vessel which has three pools (A, B, C) and a connecting tubing (T) with a valve (V). A, B and T were filled with a perfusion medium, while B was kept empty. After both ends of the cell were amputated, V was closed and a small amount of the perfusion medium was added to A. Then, the medium in A was introduced into the cell vacuole (Fig. 1a). After checking that the cell vacuole was completely occupied with the medium, V was opened to stop the vacuolar perfusion. To measure the vacuolar potential of the cell part in B, B was filled with an isotonic solution, e.g. isotonic artificial pond water (Fig. 1b). The electrode consisting of a vinyl tubing (inner diameter; ca. 1 mm) was filled with 100 mM KCl-agar (2%), and was connected to Ag-AgCl wire through 3 M KCl. Potential difference between two electrodes (E_b and E_c) whose tips were immersed in pools B and C was amplified and recorded with a pen-writing recorder and a cathod ray oscilloscope (Nihon Kohden VC-8).

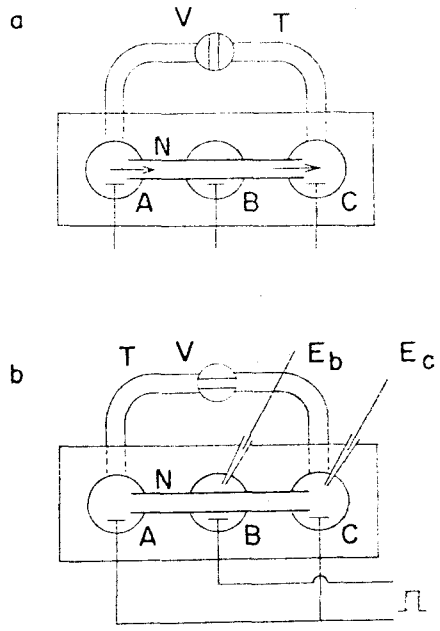

Fig. 1. a: Polyacrylate vessel with an internode (N) during perfusion. Pools A, C and connecting tubing T are filled with perfusion medium. For perfusion the valve (V) is closed and a small amount of the perfusion medium is added to pool A. b: After the cell sap has been completely replaced with the perfusion medium, the valve is opened and pool B is filled with an isotonic solution. Tips of two agar electrodes, E_b and E_c, are immersed in pool B and C to measure the vacuolar potential of the cell in B. Electric current is supplied through Ag-AgCl wires. For further explanation see the text.

To detect the action potential of the tonoplast directly, two glass microelectrodes filled with 3 M KCl were inserted into the turgid cell, one into the vacuole and the other into the cytoplasm (Fig. 2, cf. Findlay & Hope 1964, Eckert & Sibaoka 1968, Findlay 1970). When the cell with an artificial solution was used (Fig. 2), both open ends of the cell were closed after vacuolar perfusion (Fig. 2a) by ligation with strips of polyester thread (Fig. 2b). The turgor of the cell was recovered by bringing the cell into the artificial pond water. Such a cell or a normal cell was mounted on polyacrylate vessel which had three pools (Fig. 2c). Two glass microelectrodes whose resistances were less than several MΩ were inserted into the cell, one (μ_c) into the cytoplasm, the other (μ_v) into the vacuole. The plasmalemma potential (E_{co}) and the vacuolar potential (E_{vo}) were recorded separately (Fig. 2d). The difference between two potentials gives the tonoplast potential (E_{vc}).

Perfusion media mainly used are two kinds of artificial cell saps. The one, Np medium, resembles to the natural cell sap of N. pulchella (cf. Tazawa et al. 1974) and contains 120 mM KCl, 6 mM NaCl, 1 mM $CaCl_2$ and 100 mM sorbitol. Its osmorality is equivalent to 330 mM sorbitol. The other, Np-SO_4 medium, which is characterized by its low Cl^- (0.1 mM) concentrations, contains 0.1 mM KCl, 60 mM K_2SO_4, 3 mM Na_2SO_4, 1 mM $CaSO_4$ and 184 mM sorbitol. Its osmolarity is also 330 mM. For varying the concentration of Cl^- in the vacuole, K_2SO_4 in Np-SO_4 medium was exchanged for KCl. As the bathing solution, a simplified artificial pond water (APW) which

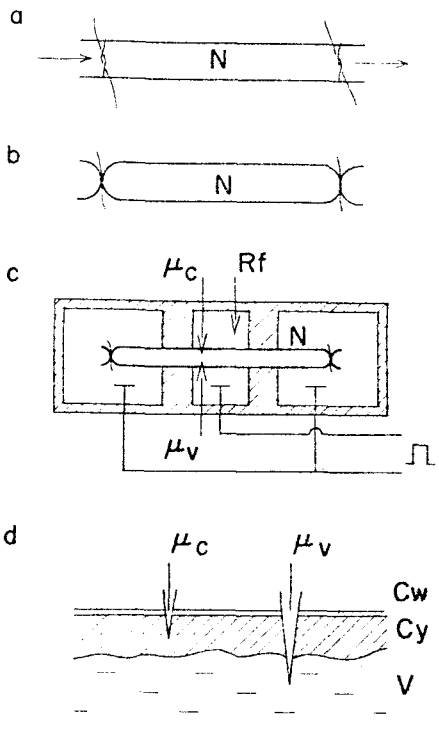

Fig. 2. Procedures for measurements of vacuolar and plasmalemma potentials in Nitella internode with an artificial vacuolar medium. After replacing the natural cell sap with artificial medium (a), both openings of the cell (N) are closed by ligation with strips of polyester thread (b). The cell is mounted on a polyacrylate vessel (c). Two glass microelectrodes are inserted into the cell part in the central pool, one (μ_c) into the cytoplasm and the other (μ_v) into the vacuole. Plasmalemma and vacuolar potentials are measured against the reference electrode (Rf). Electric current was supplied through Ag-AgCl wires. Positions of two microelectrodes are shown schematically in d. Cw: cell wall. Cy: cytoplasm. V: vacuole.

contained 0.1 mM each of KCl, NaCl and $CaCl_2$ was used for the microelectrode measurements and the isotonic APW (iAPW) which was made isotonic (330 mM) to the cell sap with sorbitol was used for the open-vacuole measurements. The concentrations of K^+ and Cl^- in iAPW were changed by adding K_2SO_4 and choline chloride into iAPW, respectively.

Temperature was kept at about 20°C except for the low temperature experiment.

Results

1. Occurrence of diphasic action potential (DAP) in cells with Np-SO_4 sap.

The vacuolar sap of Nitella pulchella was replaced with Np or Np-SO_4 medium and the action potential was recorded. Fig. 3a shows a typical action potential of a cell with Np sap. The shape is very similar to that of a cell with the natural cell sap. This type of the action potential often has two steps, the first one is rapid depolarization and the second one is large and gradual depolarization followed by the gradual return to the resting level. When the vacuole is occupied with the artificial solution of very low Cl^--concentration (Np-SO_4), diphasic action potential (DPA) is recorded (Fig. 3b). It is shown that DPA consists of two phases, i.e. the initial rapid depolarizing phase (D-phase) and the subsequent hyperpolarizing phase (H-phase). The same type of DPA was observed under very low vacuolar Cl^--concentrations (0.1 mM or less) in N. flexilis and similar one was also observed in N. axilliformis but never in Chara australis.

If a cell with Np-SO_4 sap was stimulated by an electric current which was large enough to elicit an action potential immediately after the DPA had elapsed, a monophasic action potential lacking the H-phase was observed (Fig. 4). Namely, it resembles to the action potential of the normal cell or the cell with Np sap (Fig. 3a). Another DPA could be observed if the cell was stimulated again after 10 min (Fig. 4b). This result indicates that the DPA consists of two components with different

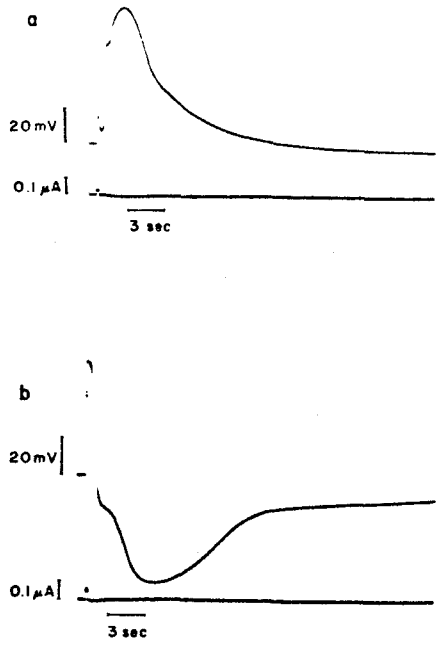

Fig. 3. Oscilloscope traces of normal action potential of Np sap cell (a) and diphasic action potential of Np-SO_4 sap cell (b).

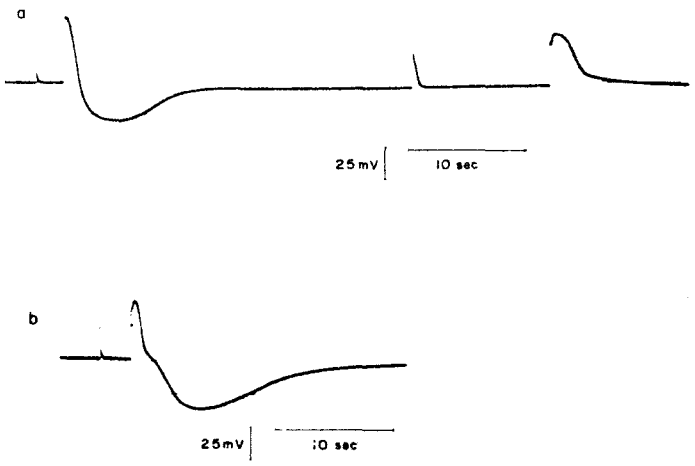

Fig. 4. Two types of action potential observed on the cell with Np-SO_4 sap. a: The monophasic action potential was observed when the Np-SO_4 sap cell was stimulated immediately after the diphasic action potential had occured. b: The diphasic action potential was reobserved when the cell was stimulated 10 minutes after the generation of monophasic one.

refractory periods. The refractory period of one component which is responsible for the H-phase is longer than that of the other component which is responsible for the D-phase.

In Chara australis, the action potential is not elicited when the cell is cooled below 10°C (Kishimoto 1972). On the other hand, it was observed even at 2°C in N. pulchella and N. flexilis. Although the DPA was also observed at 2°C in N. pulchella, the H-phase was significantly depressed and, in some cases, disappeared (Fig. 5b). In contrast to the H-phase, the D-phase was depressed only slightly and never disappeared at 2°C. This result shows that the temperature dependency of the H-phase is different from that of the D-phase. The results shown in Figs. 4 and 5 suggest that the DPA does not represent a single process composed of two phases but the sum of the different two processes, i.e. depolarizing process and hyperpolarizing one.

It is well known that the action potential of the Characeae cell is prolonged at low temperatures (Blatt 1974). As described above, the H-phase of DPA often disappeared at the low temperature (2°C). In some cells, however, the DPA with weak but distinct hyperpolarizing component was recorded even at 2°C (Fig. 5c). Since the progress of action potential is markedly slowed down, changes in electric resistance of the membranes can be followed easily at 2°C by applying small constant inward current pulses during excitation. Fig. 5c shows that the electric resistance becomes minimum at the point where the D-phase ends and the H-phase begins.

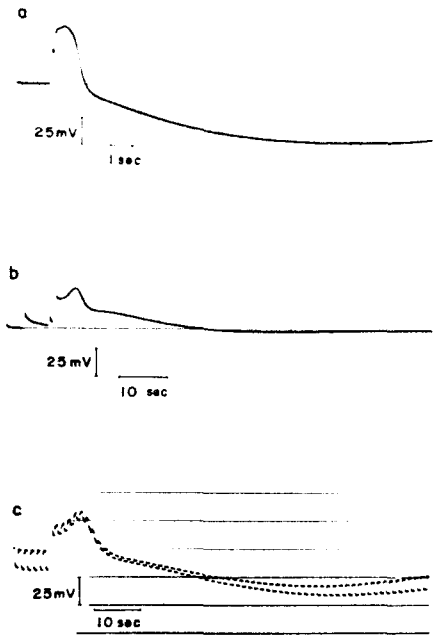

Fig. 5. Temperature dependencies of two components in the diphasic action potential. a: Oscilloscope trace of the diphasic action potential recorded at 20°C. b: Action potential of the same cell recorded at 2°C. The hyperpolarizing phase diminished completely. c: Change in electric resistance of another cell during the diphasic action potential recorded at 2°C.

2. Magnitude of each phase of DPA versus concentrations of K^+ and Cl^-.

The fact that the H-phase could be never observed in cells with the normal cell sap or with Np sap, but in cells with Np-SO_4 sap suggests that one process responsible for the H-phase may occur at the tonoplast and another responsible for D-phase at the plasmalemma. Therefore, it is expected that the H-phase will be affected by ions in the vacuole and the D-phase by those in the external medium. Experiments along this line were carried out with the open-vacuole method (cf. Fig. 1) and the differences between the resting potential and the peak potential of both D-phase (ΔE_d^p) and H-phase (ΔE_h^p) were determined. Action potentials showing no diphasic modes were discarded in the measurements. In Fig. 6a, K^+-concentration in the external solution ($[K^+]_o$) was changed from 0.1 to 3 mM by adding K_2SO_4 into iAPW. In Fig. 6b, Cl^- concentration in the bathing medium ($[Cl^-]_o$) was varied from 0.4 mM to 10 mM with choline chloride. In both experiments, the vacuolar medium was Np-SO_4. It is clear that both ΔE_h^p and ΔE_d^p are not affected much by K^+ and Cl^- in the external medium. When cells were bathed in the medium containing 10 mM K^+ or 30 mM Cl^-, they did not generate action potential.

Next, effects of ions in the vacuolar medium were studied. The concentration of Cl^- in Np-SO_4 medium in the vacuole ($[Cl^-]_v$:0.1 mM) was increased to 100 mM by exchanging K_2SO_4 for KCl. The bathing medium used was iAPW. Fig. 7 shows that ΔE_h^p depends on $[Cl^-]_v$ strongly, while ΔE_d^p does not. When $[Cl^-]_v$ was increased to 20 mM, the H-phase of the DPA was strongly depressed and often disappeared. Further, the

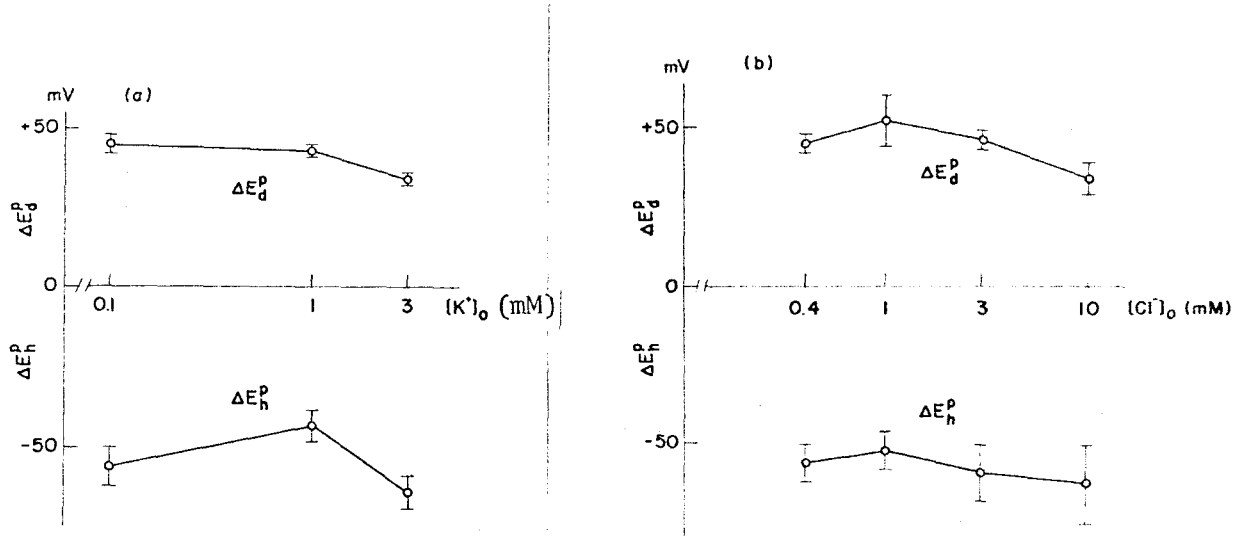

Fig. 6. Effects of external K^+- and Cl^--concentrations ($[K^+]_o$, $[Cl^-]_o$) on the amplitude at the peak of the depolarizing phase (ΔE_d^p) and that of the hyperpolarizing phase (ΔE_h^p) of diphasic action potentials. Vertical lines indicate standard errors. a: $[K^+]_o$ was varied by adding K_2SO_4 into iAPW. b: $[Cl^-]_o$ was varied by adding choline chloride into iAPW.

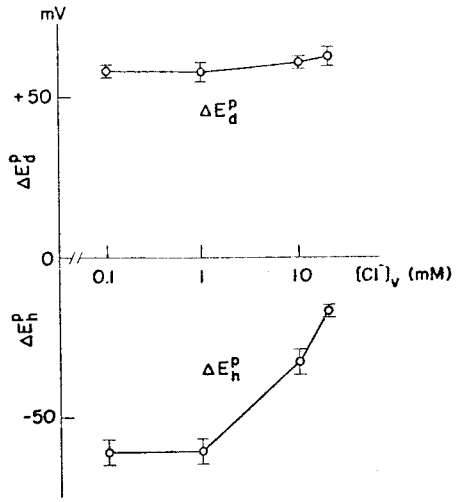

Fig. 7. Amplitudes of depolarizing and hyperpolarizing phases (ΔE_d^p, ΔE_h^p) of the diphasic action potential in relation to Cl^--concentration in the vacuole ($[Cl^-]_v$). $[Cl^-]_v$ was varied by exchanging K_2SO_4 in Np-SO_4 medium for KCl. Cells were bathed in iAPW. Vertical lines indicate standard errors.

DPA was never observed when $[Cl^-]_v$ was 100 mM. Fig. 8 shows the DPA of the cell whose vacuolar sap contained 5 mM $CaSO_4$ only. The amplitude, ΔE_h^p, observed in cells with no K^+ in the vacuole (vacuolar medium was 5 mM or 10 mM $CaSO_4$ only) was -51 mV (average of three cells). These two results (Figs. 7 and 8) indicate that generation of the action potential accompanying H-phase is not dependent on $[K^+]_v$ and $[Na^+]_v$ but on $[Cl^-]_v$. Moreover, the DPA was observed not only when the vacuolar pH was 6 but also when it was 3 or 8.

3. Separate measurements of tonoplast action potential from DPA.

The fact that $[Cl^-]_v$ affects the magnitude of H-phase makes such an assumption more reliable as that the H-phase of the DPA may be generated at the tonoplast. To confirm this, however, it is necessary to measure the tonoplast potential directly. For this, two glass microelectrodes were inserted into the cell, one into the cytoplasm and the other into the vacuole, and the plasmalemma potential (E_{co}) and the vacuolar potential (E_{vo}) were recorded simultaneously (Fig. 2c). It is quite difficult to insert an electrode effectively into the thin layer of cytoplasm of N. pulchella through a very thick cell wall reaching sometimes 50 μm. Only a limited number of recordings were obtained from both the cytoplasm and the vacuole on cells with exceptionally thin cell walls. Fig. 9a shows oscilloscope traces of the action potential of the cell with the natural cell sap recorded from two electrodes inserted into the cell. The potential change of

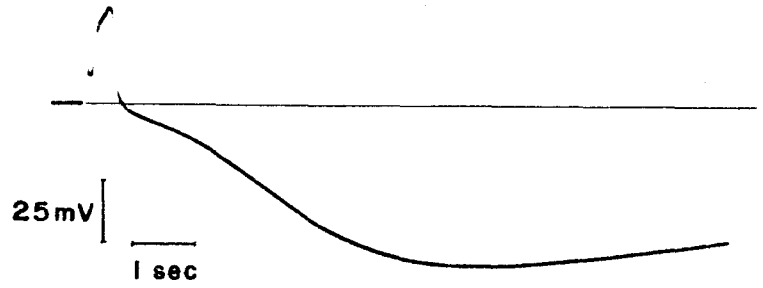

Fig. 8. Oscilloscope trace of the diphasic action potential of a cell whose vacuole contained 5 mM $CaSO_4$ only.

the tonoplast during excitation, E_{VC} in Fig. 9a, is obtained by subtracting E_{CO} from E_{VO}. Change in E_{VC} to the positive direction is in accord with the results obtained by Findlay and Hope (1964) in Chara corallina and by Findlay (1970) in Nitellopsis obtusa. When the vacuolar sap was Np-SO_4, the DPA was recorded from the microelectrode in the vacuole, while the monophasic action potential consisting of the D-phase was recorded from the microelectrode in the cytoplasm (Fig. 9b). The tonoplast potential, E_{VC} in Fig. 9b, did not change to the positive direction but to the negative one.

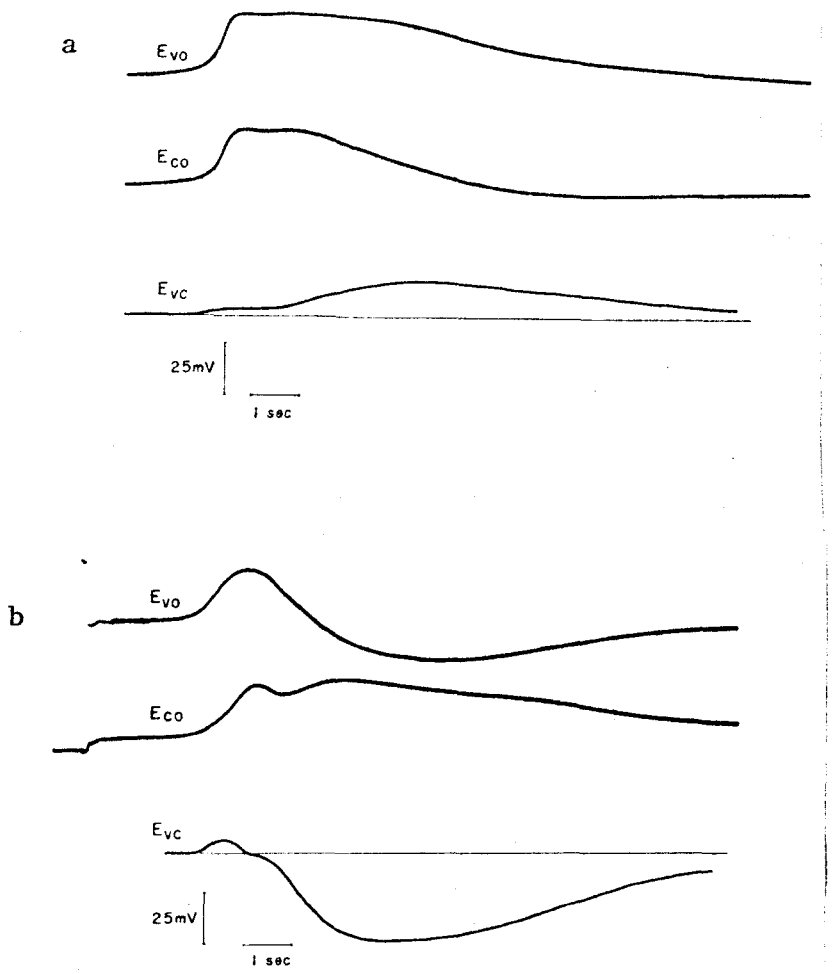

Fig. 9. Action potentials recorded from the cytoplasm (E_{co}) and from the vacuole (E_{vo}) of the cell with natural cell sap (a) and with Np-SO_4 sap (b). Tonoplast action potential (E_{vc}) was obtained by subtracting E_{co} from E_{vo}.

Discussion

Effluxes of K^+ and Cl^- increase significantly during excitation in Characeae cells (Gaffey & Mullins 1958, Mullins 1962, Hope & Findlay 1964, Haapanen & Skoglund 1967). From the voltage clamp experiment, Kishimoto (1965) reported that the peak current during excitation of Nitella depends on concentration of Cl^- in the external medium, i.e. the peak current decreased to about half when $[Cl^-]_o$ was increased from 0.75 mM to 10 mM. All these workers claimed tacitly that the plasmalemma becomes much permeable to K^+ and Cl^- during its excitation.

The peak of D-phase of DPA approximately corresponds to the peak of action potential at the plasmalemma (Fig. 9). In the present experiment, the amplitude of the D-phase of DPA (=amplitude of the plasmalemma action potential) was not affected by changing concentrations of K^+ and Cl^- in the bathing medium in the ranges 0.1-3 mM and 0.4-10 mM respectively (Fig. 6a and b). These results do not necessarily show that the plasmalemma action potential is independent of $[K^+]_o$ and $[Cl^-]_o$. If Goldman equation is valid for the peak potential of D-phase, the peak potential (E_d^p) is expressed as follows;

$$E_d^p = 58 \log \frac{[K^+]_o + \beta[Cl^-]_c}{[K^+]_c + \beta[Cl^-]_o} \qquad (1)$$

where $[K^+]_c$ and $[Cl^-]_c$ are the K^+ and Cl^- concentrations in the cytoplasm respectively; β is the permeability ratio P_{Cl}/P_K, where P_{Cl} and P_K are the permeability coefficients of the plasmalemma to Cl^- and K^+. Since $[K^+]_c$ and $[Cl^-]_c$ are 100 mM and 30 mM, respectively (Tazawa et al. 1974), changes

in $[K^+]_o$ or $[Cl^-]_o$ brings about no significant change in E_d^p even if β, which is about 0.06 at resting state as shown in chapter one, increases to 4.0 (Findlay 1970) at active state. Furthermore, potential difference across the plasmalemma depends insignificantly on $[K^+]_o$ and $[Cl^-]_o$ if they are below 10 mM (Figs. 2 and 4 in Chap. 1). Since both the resting potential and the peak potential of the D-phase are scarcely affected by $[K^+]_o$ and $[Cl^-]_o$, the amplitude of the D-phase of DPA should not be affected by them.

Findlay and Hope (1964) and Findlay (1970) suggested that the transient increase in permeability to Cl^- takes place during excitation at the tonoplast as well as at the plasmalemma. Then, it is expected that the amplitude of tonoplast action potential may change according to concentration of Cl^- in the vacuole. This presumption was clearly demosntrated in the present experiment.

Although the H-phase of DPA reaches its peak nearly at the same time as the tonoplast action potential, the maximum amplitude of the former is smaller than that of the latter (Fig. 9b). However, changes in amplitude of the former should be equal to those of the latter. Since the maximum amplitude of H-phase of DPA observed when the vacuole contained no K^+ ($[K^+]_v = 0$) was approximately equal to that observed when $[K^+]_v$ was 120 mM, it is clear that the magnitude of the tonoplast action potential is not dependent on $[K^+]_v$. On the other hand, the amplitude of the H-phase depends strongly on $[Cl^-]_v$ (Fig. 7), indicating a close dependence of the tonoplast action potential on $[Cl^-]_v$. The tonoplast potential, E_{VC}, changes to the positive direction during excitation, if the vacuolar solution is the natural cell sap (Fig. 9a, cf.

Findlay & Hope 1964, Findlay 1970) and it does to the negative one, if $[Cl^-]_v$ is below 20 mM (Figs. 7 and 9b). These behaviors of the tonoplast action potential are well accounted for by assuming that the tonoplast behaves as a Cl^--electrode. Namely, the Nernst potential for Cl^- across the tonoplast is about 48 mV (vacuole positive) when the vacuole is filled with the natural cell sap (about 200 mM Cl^-, Tazawa et al. 1974) and about 150 mV (vacuole negative) when it contained Np-SO_4 medium (0.1 mM Cl^-). From the slope of ΔE_h^p between 1 and 10 mM $[Cl^-]_v$ in Fig. 7 we can estimate the permeability ratio P_{Cl}/P_K (= β') for the tonoplast under the constant field assumption. Namely,

$$E_h^p = 58 \log \frac{[K^+]_c + \beta'[Cl^-]_v}{[K^+]_v + \beta'[Cl^-]_c} \qquad (2)$$

where $[K^+]_c$ and $[Cl^-]_c$ in N. pulchella are 100 and 30 mM, respectively (Tazawa et al. 1974). In the present experiment, $[K^+]_v$ was fixed at 120 mM. Then, the difference of ΔE_h^p between 10 mM $[Cl^-]_v$ and 1 mM $[Cl^-]_v$, which is 28 mV in Fig. 7, is expressed as follows;

$$28 = 58 \log \frac{100 + 10\beta'}{100 + \beta'} \qquad (3)$$

Thus, β' is calculated to be 29 at the peak of action potential which is somewhat larger than the value (15) obtained in Nitellopsis tonoplast (Findlay 1970). Since β' at the resting state for the range of $[Cl^-]_v$ between 1 and 10 mM was estimated to be about 1 (Fig. 12 in Chap. 1), above calculation infers that relative permeability of Cl^- to K^+ of the tonoplast

increases by a factor of thirty during excitation.

Besides the difference in responses to Cl^- between the tonoplast potential and plasmalemma potential, there are another essential difference in excitability between these two membranes. The tonoplast can generate action potential even when $[K^+]_v$ is 120 mM, while the plasmalemma does not when $[K^+]_o$ is 10 mM or more.

It is to be noted that we could not observe DPA in C. australis, dioecious species of C. corallina in which Findlay and Hope (1964) recorded the tonoplast action potential.

In our experiments, the D-phase of DPA always appeared prior to the H-phase, i.e. action potential at the tonoplast always follows that at the plasmalemma (cf. Findlay & Hope 1964, Findlay 1970). When a stimulus large enough to cause an action potential at the plasmalemma was applied between microelectrode inserted in the cytoplasm and Ag-AgCl wire in the bathing solution, the DPA was recorded from a micro-electrode in the vacuole of Np-SO_4 sap cell. Furthermore, three electrodes were inserted into Np-SO_4 sap cell, one into the cytoplasm and the other two into the vauole. Even when only the tonoplast was stimulated by supplying an electric current from the electrode in the vacuole to the electrode in the cytoplasm, the DPA was also recorded (Fig. 10). Findlay (1970) showed that the duration of the plateau component of the tonoplast action potential is dependent on the occurrence of the second peak of the plasmalemma action potential. Generation of DPA by stimulation of either plasmalemma or tonoplast suggests that some kind of electric coupling exists between the plasmalemma and the tonoplast.

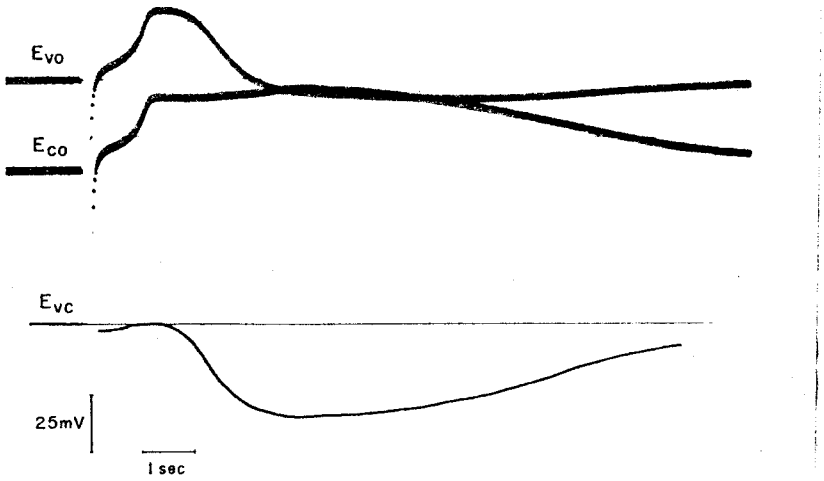

Fig. 10. Action potentials recorded from the cytoplasm (E_{co}) and the vacuole (E_{vo}) of the cell with Np-SO_4 sap. Tonoplast action potential (E_{vc}) was obtained by subtracting E_{co} from E_{vo}. Stimulating current was supplied only across the tonoplast from the vacuole to the cytoplasm.

Summary

Action potential of Nitella internode was studied in relation to K^+- and Cl^--concentrations in the vacuole. When the vacuole of Nitella pulchella was occupied with an artificial solution with extremely low Cl^-- concentration, a diphasic action potential (DPA) was observed. The first phase consists of a rapid depolarization followed by a relatively rapid repolarization and the second one consists of a strong hyperpolarization followed by a gradual return to the resting potential.

When the cell was stimulated immediately after the generation of DPA, monophasic action potential which resembles to an action potential of the natural cell was observed, indicating that the DPA consists of two components with different refractory poriods. Namely, the refractory period of a component responsible for the depolarizing phase is shorter than that of a component responsible for the hyperpolarizing phase. Measuring the plasmalemma potential and vacuolar potential separately, it was demonstrated that the hyperpolarizing component of DPA originates from the tonoplast.

The action potential of the tonoplast, in contrast with that of the plasmalemma, could be generated independently of concentration of K^+ in the vacuole. Since the maximum amplitude of hyperpolarization decreased significantly by increasing Cl^--concentration of the vacuole, it is concluded that the tonoplast is very sensitive to Cl^- during excitation. The ratio between the permeability coefficient for Cl^- and that for K^+, i.e. P_{Cl}/P_K, of the tonoplast at the peak of

its action potential is estimated to be about 30 which is the same order of magnitude as that estimated by Findlay (1970) on Nitellopsis obtusa.

Concluding remarks

In this work it was made clear tht in Nitella the electric characteristics of the tonoplast is significantly different from that of the plasmalemma. The difference in the electric characteristics between the two membranes may be responsible for a significant amount of vacuolar potential existing even when the composition of vacuolar medium of Nitella is completely equal to that of the external one.

Measuing the short-circuit current is certainly a powerful tool to study the active transport of ions when it is measured across a single membrane. However, the method can not be applied simply to the active transport of ions in plant cells occurring between the vacuole and the outside, since nullification of electrochemical potential gradient across the protoplasmic layer does not necessarily mean that across each membrane. In view of the facts obtained in the present study, it is important to consider that the short-circuit current measured in plant cells such as Nitella in which the electric characteristics of the tonoplast differes from that of the plasmalemma does not always reflects the active ion fluxes transported by an electrogenic ion pump or pumps. Namely, a certain amount of electric current should be required to cancel the electrochemical potential difference between the vacuole and the external medium even though an electrogenic ion pump is not operating.

About the tonoplast action potential it should be added that the tonoplast is not the same as the surface membrane of the endoplasmic drop isolated from Nitella internode, so far as the excitability is concerned. When the vacuole of Nitella was occupied with the test solution which can elicit an action potential in isolated

Nitella endoplasmic drops (Inoue et al. 1973), the tonoplast has never been produced action potential even 1 hr after the replacement of the sap.

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