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Characteristics of the Vacuolar Membrane of *Nitella*

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Summary. Taking advantage of vacuolar perfusion, concentrations of K^+, Cl^-, and H^+ in the vacuole of *Nitella pulchella* were changed in a wide range. Both the potential difference (E_{vo}) and specific resistance (R_{vo}) between the vacuole and the external medium were scarcely affected by K^+ in the vacuole, while they responded sensitively to K^+ in the external medium.

E_{vo} also responded to Cl^- in both internal (vacuolar) and external medium. However, the sign of the response was opposite to that expected from the constant field assumption. R_{vo} was almost independent of Cl^--concentrations of both internal and external medium.

The response of E_{vo} to internal pH was similar to that of external pH. Between pH's 4 and 8, E_{vo} changed by about 10 mV for one unit change of both external and internal pH. E_{vo} responded very sensitively to internal pH in the strongly acid region (30–60 mV at pH 3–4) irrespective of the concentration of KCl in the vacuole. In the alkaline region, however, E_{vo} responded to vacuolar pH only when the KCl concentration in the vacuole was low (0.1 mM). R_{vo} increased significantly when the vacuolar pH was lowered to 4 or 3.

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

Even when the chemical potential gradient between the internal and external medium was made zero by replacing the cell sap for the same solution used for the external medium, a significant amount of E_{vo} was observed. The short-circuit current which was first outward decreased to zero or changed its direction with time. Light did not affect the current. These facts show that the possibility for the contribution of an ion pump to E_{vo} can be excluded.

The results were discussed under the assumption that responses of E_{vo} and R_{vo} to either internal or external ions reflect the passive property of either tonoplast or plasmalemma.

Most electrophysiological work on plant cells, including Characeae internodes, has 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 & Costerton, 1967; Spans-

wick, Stolarek & Williams, 1967; Findlay 1970). From such studies it was found that in *Chara corallina* and in *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 the 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 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, Nagai & Tazawa 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. However, in the preceding paper (Tazawa, Kikuyama & Nakagawa, 1975) we 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^+.

The vacuolar potential of *Nitella* responds to pH of the bathing medium (Kitasato, 1968; Lefebvre & Gillet, 1973; Saito & Senda, 1973; 1974). Kitasato (1968) concluded that the permeability of the *Nitella* membrane to H^+ is much greater than those to K^+ and Cl^-. Lucas and Smith (1973) deduced the $HCO_3^- - OH^-$ exchange mechanism in *Chara* membrane from local alkalization of the cell during illumination with white light. Many workers (Spanswick 1972, 1974; Vredenberg & Tonk, 1973; Saito & Senda, 1973; 1974) demonstrated that hyperpolarization of the vacuolar potential induced by illumination in Characeae internodes is caused by an electrogenic ion pump which may be a 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 present paper electric responses of the tonoplast not only to vacuolar K^+ and Cl^- but also to vacuolar H^+ are described and compared with those of the plasmalemma.

Materials and Methods

The main material used throughout the experiment was *Nitella pulchella*. This material has a very thick cell wall ($\simeq 50$ μm) and is therefore hardly deformed through loss of turgor by evaporation of water and by cutting the cell, the procedures necessary for perfusion

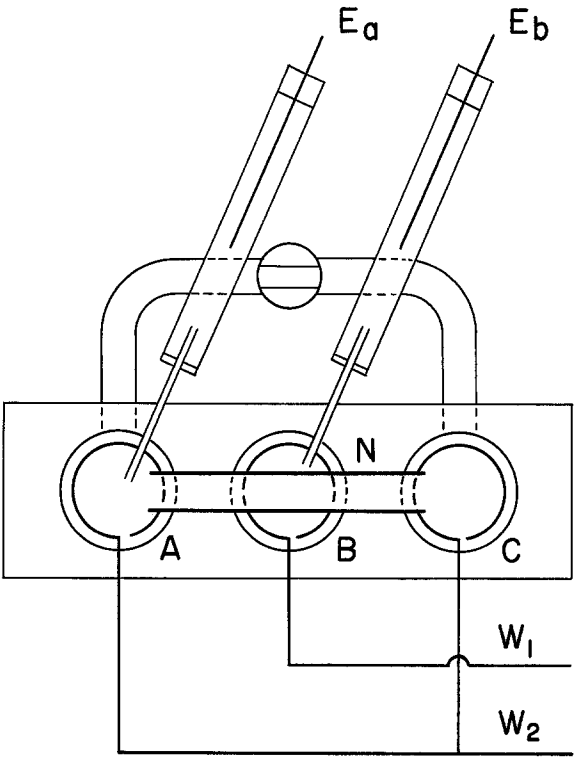


Fig. 1. Perfusion chamber with three pools (*A, B, C*) and the setup for the open-vacuole measurements of vacuolar potential and electric resistance of the cell part at *B. N: Nitella* internode. E_a, E_b: electrodes with vinyl tubing filled with 100 mM KCl-agar which is connected to Ag–AgCl wire through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

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 μm 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 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),

$$\Delta E = E_{vo} + \Delta e. \quad (1)$$

In the previous paper (Tazawa *et al.*, 1975) Δe was determined in the following manner. A chamber with two pools was prepared, one being filled with perfusion (vacuolar) medium and the other with 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 solution (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 in 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 referred to *iAPW* hereafter. The tonicities of the perfusion media listed in Table 1 were adjusted to 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, Kishimoto & Kikuyama 1974). The medium of low ionic concentrations (*iAPW*-10 Ca) 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*-10 Ca 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 hr. However, when the perfusion medium of low ion contents (*iAPW*-10 Ca in Table 1) was used, E_{vo} was stable only for the first 20 min, then it depolarized gradually (*cf.* Fig. 11). In the following only the stable values measured within 20 min 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

Table 1. Ionic compositions of vacuolar and bathing media[a]

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-10 Ca[b]	0.1	0.1	10	–	20.2
150-KCl[c]	150	10	10	–	ca. 180
0.1-KCl[c]	0.1	10	10	–	ca. 30
Bathing media					
iAPW	0.1	0.1	0.1	–	0.4
iAPW-1 Na[d]	0.1	1	0.1	–	ca. 1

[a] Osmotic value of each medium was adjusted to 300–330 mM with sorbitol.
[b] This medium was also used for the bathing medium.
[c] 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.
[d] 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.

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 to 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}).

To measure short-circuit current in the cell whose internal (vacuolar) and external media were the same the potential difference between pools *A* and *B* (Fig. 1) was clamped at zero after Kishimoto (1972).

During the measurements cells were exposed to diffused dim light (ca. 200–300 lux) and the temperature was kept at 20–24 °C. Average values of electric potentials and electric resistances are shown as mean ±SE.

Results

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

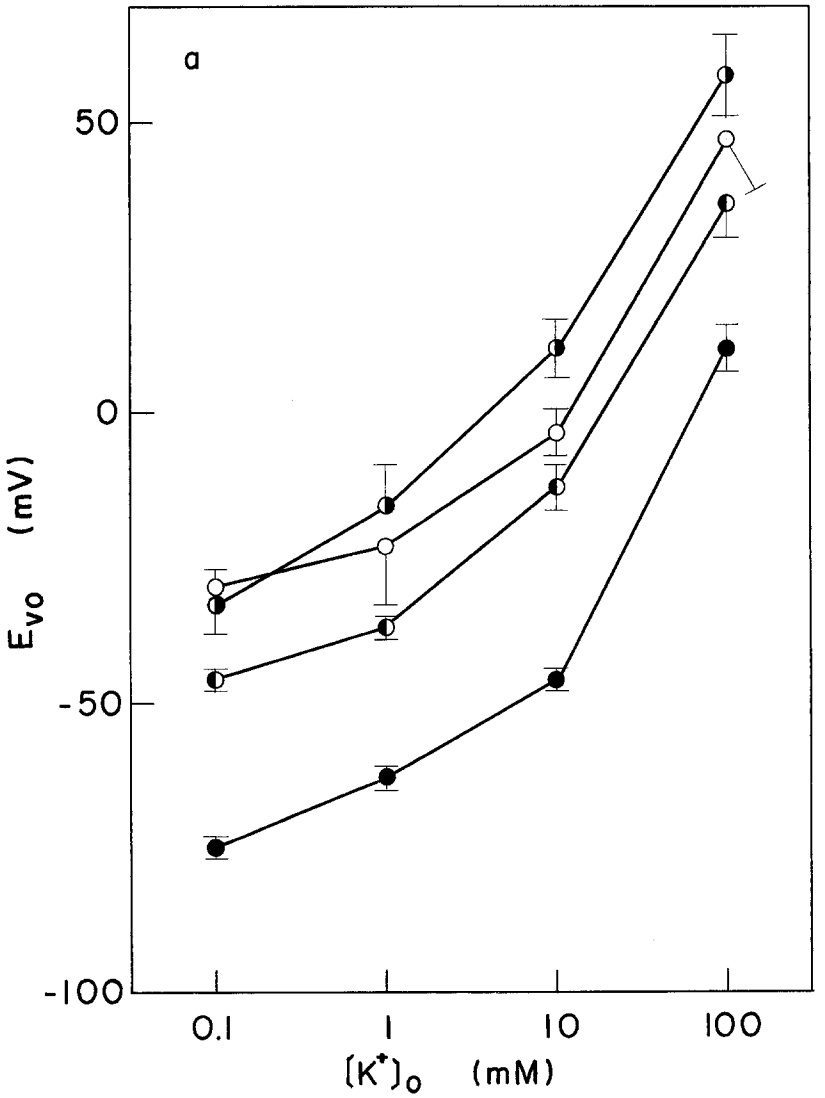
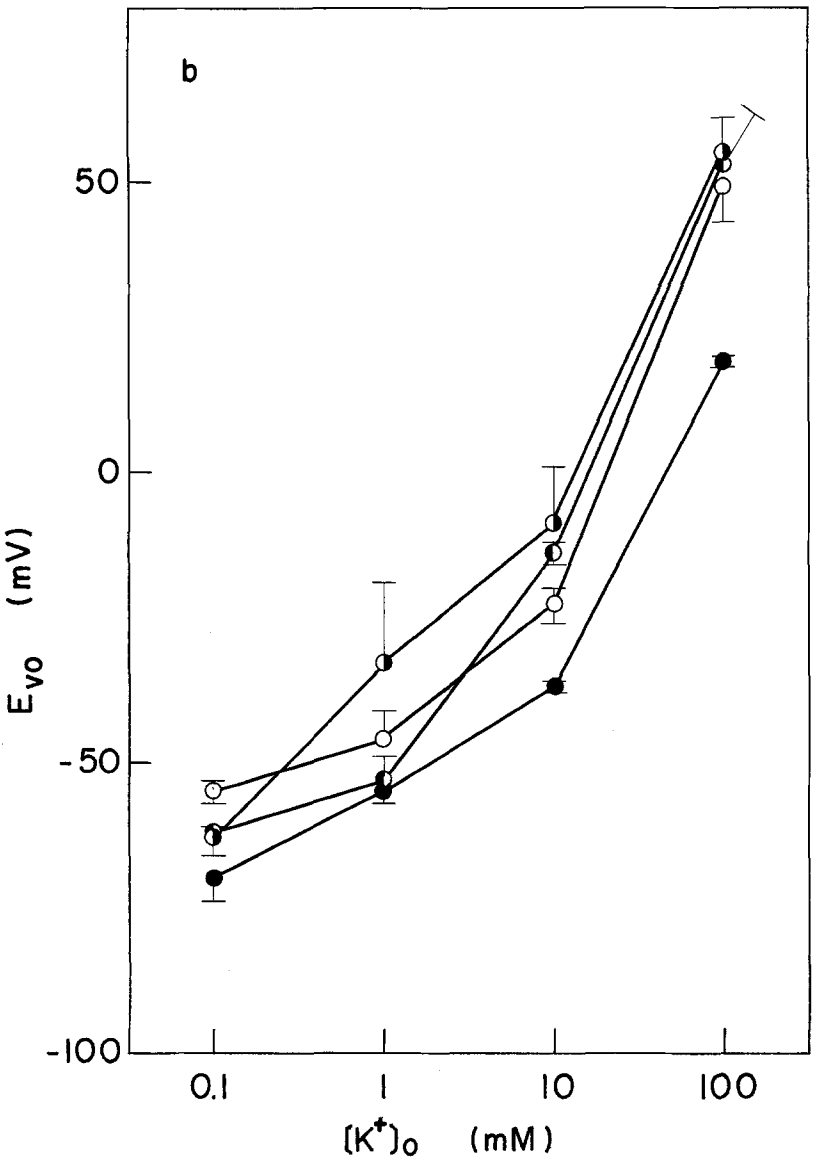


Fig. 2. Relation of vacuolar potential (E_{vo}) and concentration of K^+ in the external solution ($[K^+]_o$). All external media contained (in mM): 0.1 Na^+, 1.0 Ca^{2+} and 0.1 Cl^-. $[K^+]_o$ and $[K^+]_v$ were changed from 0.1 to 100 mM by adding K_2SO_4. Curves with different marks indicate experiments 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*)

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 chloride 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-}, 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 similar 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. 2*a*, 100 mM in Fig. 2*b*) and at various

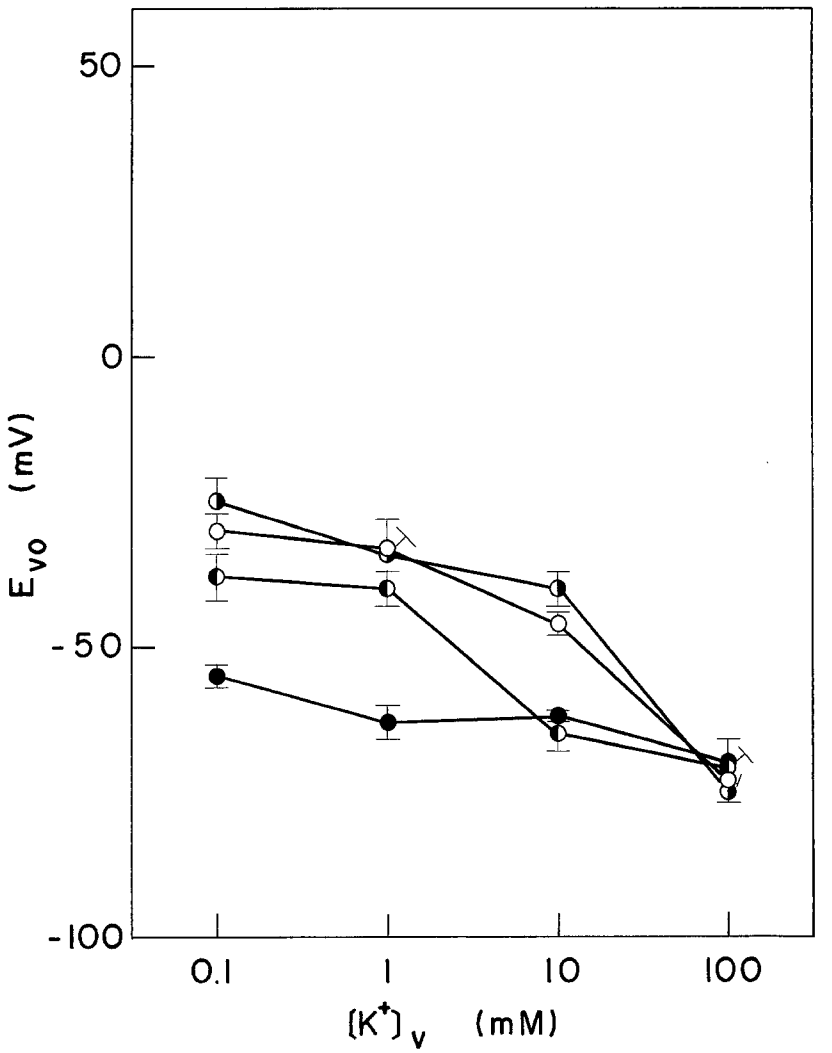


Fig. 3. Relation of vacuolar potential (E_{vo}) and concentration of K^+ in the vacuole ($[K^+]_v$). The external medium contained 0.1 mM each of K^+, Na^+, Cl^-, 1.0 mM Ca^{2+} and 1.05 mM SO_4^{2-}. Curves with different marks indicate experiments with 0.1 mM (○), 1.0 mM (◑), 10 mM (◐) and 100 mM (●)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

$[K^+]_v$ (0.1–100 mM) were plotted against $[K^+]_o$. In both Fig. 2*a* and *b*, E_{vo} became significantly less negative when $[K^+]_o$ increased. 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 against $[K^+]_v$. When $[K^+]_v$ was varied from 0.1 to 100 mM, E_{vo} changed slightly to the hyperpolarizing direction (Fig. 3), indicating that the tonoplast was less sensitive to K^+ than the plasmalemma.

In our previous paper (Tazawa *et al.*, 1975), it was reported that E_{vo} of *N. flexilis* changed to the depolarizing 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*). In the range of $[Cl^-]_o$ below 10 mM, E_{vo} was almost insensitive to $[Cl^-]_o$ or became slightly less negative as $[Cl^-]_o$ increased. When $[Cl^-]_o$ was increased from 10 to 100 mM, E_{vo} changed significantly to the depolarizing direction except when $[K^+]_v$ was 100 mM (curves with closed circles in Fig. 4*a* and *b*). On the other hand, the response of E_{vo} to $[Cl^-]_v$ (Fig. 5) occurred in the opposite direction to that of E_{vo} to $[Cl^-]_o$ (Fig. 4). 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 manner similar to the plasmalemma.

Effects of external or vacuolar ions such as K^+ and Cl^- on the electric resistance (R_{vo}) of *Nitella* internode were also studied. The electric resistance of the plasmalemma was strongly dependent on $[K^+]_o$. In Fig. 6, R_{vo} of cells whose vacuolar saps contained 100 mM K^+, 100 mM Cl^-, and 1 mM Ca^{2+} were plotted against $[K^+]_o$ (Fig. 6*a*) and against $[Cl^-]_o$ (Fig. 6*b*). In Fig. 6*a* $[Cl^-]_o$ was 0.1 mM and in Fig. 6*b* $[K^+]_o$ was 0.1 mM. It is clear that the higher the $[K^+]_o$, the smaller the R_{vo}. On the other hand, $[Cl^-]_o$ does not decrease R_{vo} but increases it slightly. In Fig. 7*a*, $[K^+]_v$ was varied from 0.1 to 100 mM, while $[Cl^-]_v$ was kept at 100 mM. In Fig. 7*b*, $[Cl^-]_v$ was 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. Fig. 7*a* and *b* show that R_{vo} is almost independent of $[K^+]_v$ and $[Cl^-]_v$. This fact does not necessarily mean that the electric resistance of the tonoplast (R_{vc}) is independent of concentrations of these ions, because one would only expect to see a change in R_{vc} if it is a significant fraction of the total resistance (R_{vo}). However, the fact that R_{vo} is greatly decreased when $[K^+]_o$ is 100 mM (Fig. 6*a*) suggests that R_{vc} is only a minor fraction of R_{vo}.

E_{vo} in Relation to Concentration of H^+ in the Vacuole

To examine the effect of concentration of H^+ in the vacuole ($[H^+]_v$), 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

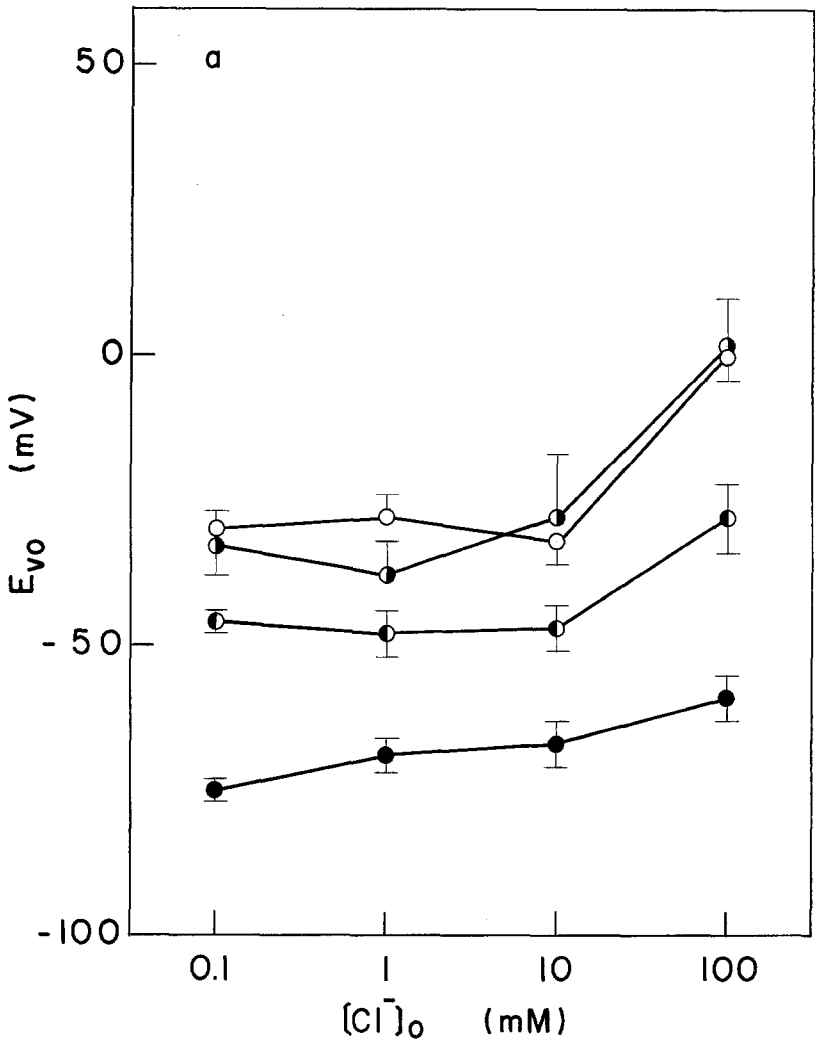
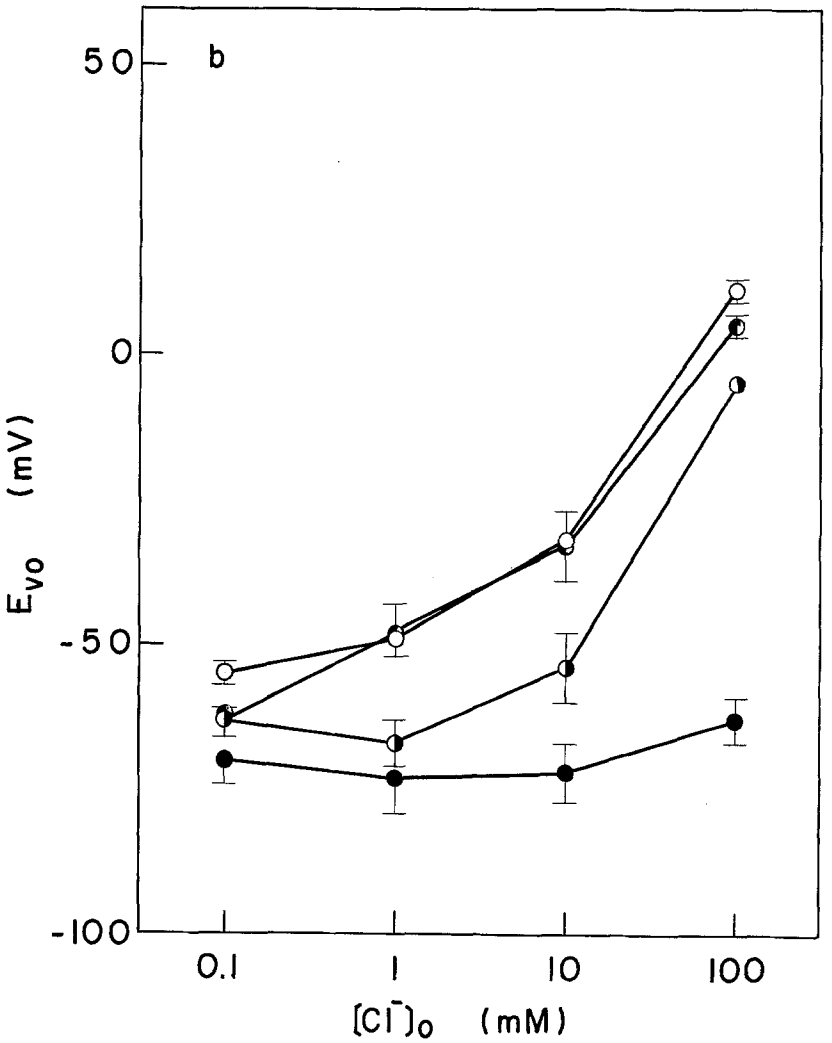


Fig. 4. Relation of vacuolar potential (E_{vo}) and concentration of Cl^- in the external medium ($[Cl^-]_o$). All external media contained (in mM): 0.1 K^+, 0.1 Na^+ and 1.0 Ca^{2+}. $[K^+]_v$ and $[Cl^-]_o$ were changed from 0.1 to 100 mM by adding K_2SO_4 and choline chloride, respectively. Curves 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*)

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 were added into each perfusion medium with HCl or NaOH. Concentration of Na^+ was always kept at 10 mM. For external media, *iAPW*-1 Na 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*-1 Na 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. 8*a*), 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

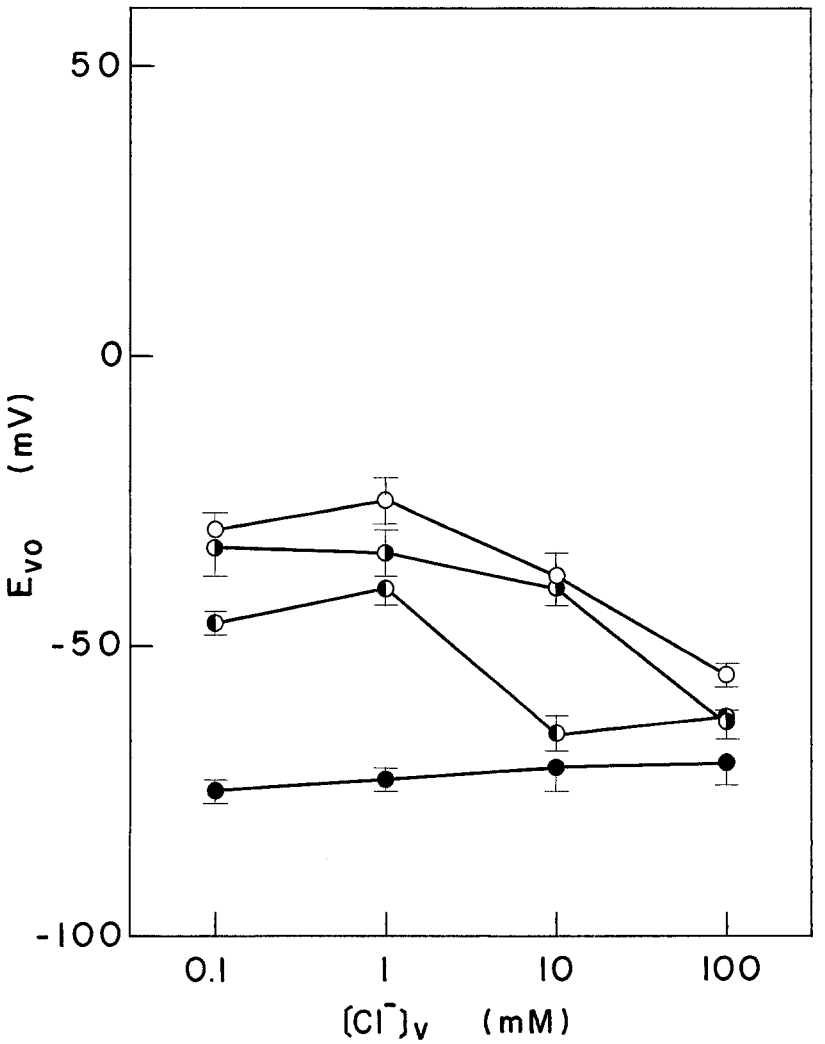


Fig.5. Relation of vacuolar potential (E_{vo}) and concentration of Cl^- in the vacuole ($[Cl^-]_v$). The external medium contained 0.1 mM each of K^+, Na^+, Cl^-, 1.0 mM Ca^{2+} and 1.05 mM SO_4^{2-}. Curves with different marks indicate the experiments with 0.1 mM (○), 1.0 mM (◑), 10 mM (◐) and 100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

the concentration of KCl in the vacuole is low (Fig. 8*b*), E_{vo} changes steadily to the hyperpolarizing direction with the increase in $[H^+]_v$ 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. 8*b*. 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 Fig. 8*a* and *b* the mode of response

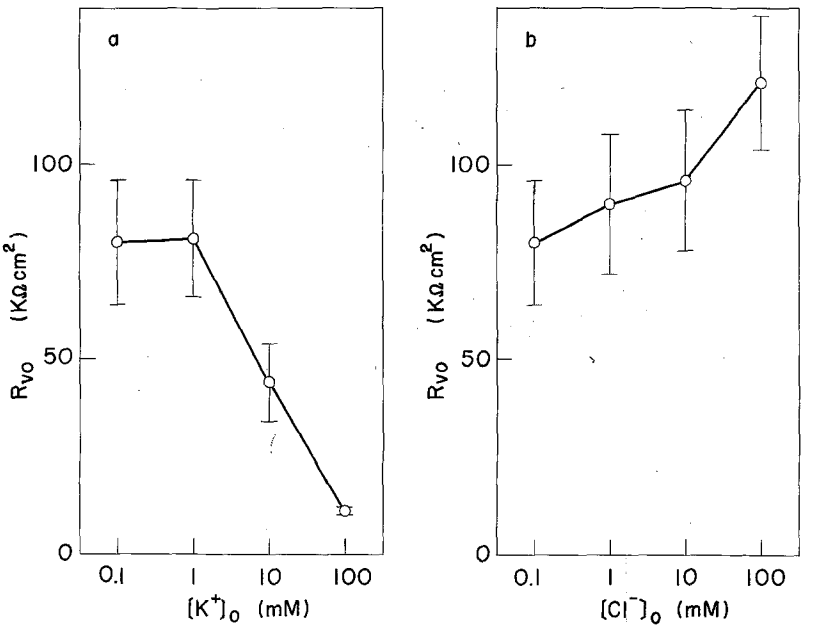


Fig. 6. Effects of concentrations of K^+ ($[K^+]_o$) and Cl^- ($[Cl^-]_o$) in the external medium on the specific resistance (R_{vo}) across the plasmalemma and the tonoplast. Vacuolar medium contained (in mM): 100 K^+, 100 Cl^-, 0.1 Na^+, 1 Ca^{2+} and 1.05 SO_4^{2-}. $[K^+]_o$ was varied from 0.1 to 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*)

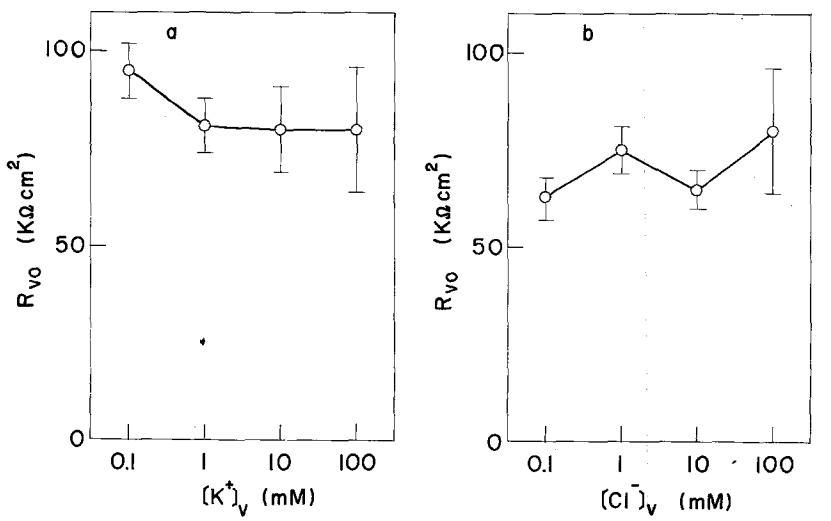


Fig. 7. Effects of concentrations of vacuolar K^+ ($[K^+]_v$) and Cl^- ($[Cl^-]_v$) on the specific resistance (R_{vo}) across the plasmalemma and the tonoplast. The external medium contained 0.1 mM each of K^+, Na^+, Cl^-, 1.0 mM Ca^{2+} and 1.05 mM SO_4^{2-}. $[K^+]_v$ was varied from 0.1 to 100 mM 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*)

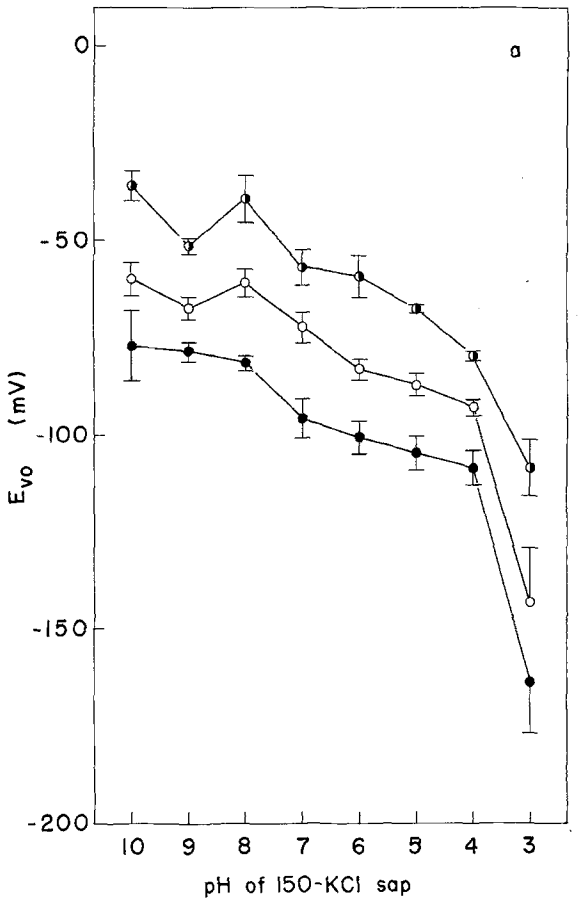
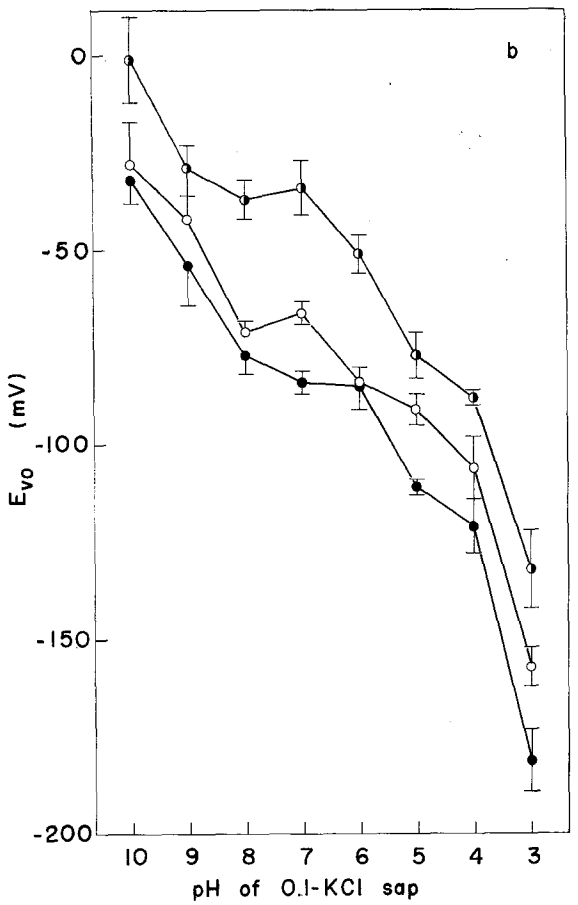


Fig. 8. Relation of vacuolar potential (E_{vo}) and pH of the vacuolar medium. Curves with different marks indicate the measurement in *iAPW*-1 Na (Table 1) of pH 4 (◑), pH 6 (○) and pH 8 (●). Vacuolar medium was either 150-KCl sap (*a*) or 0.1-KCl sap (*b*) (*cf.* Table 1)

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. Therefore, 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. 8*a* and *b* and is shown in Fig. 9 where results obtained by Kitasato (1968) on *Nitella clavata* and by Lefebvre and Gillet (1973) on *N. flexilis* are also shown. They demonstrated 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 the natural cell sap of *N. flexilis* (Hirakawa & Yoshimura 1964), irrespective of the concen-



tration of KCl in the vacuole. In cells with 150-KCl sap (curve with open circles in Fig. 9) E_{vo} at pH 8 is 41 mV more negative than that at pH 4, and in cells with 0.1-KCl sap (curve with closed circles) 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. 8*a* and 43 mV in Fig. 8*b*, it can be said that in *N. pulchella* the tonoplast is about equally sensitive to H^+ as the plasmalemma so far as potential responses are concerned.

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

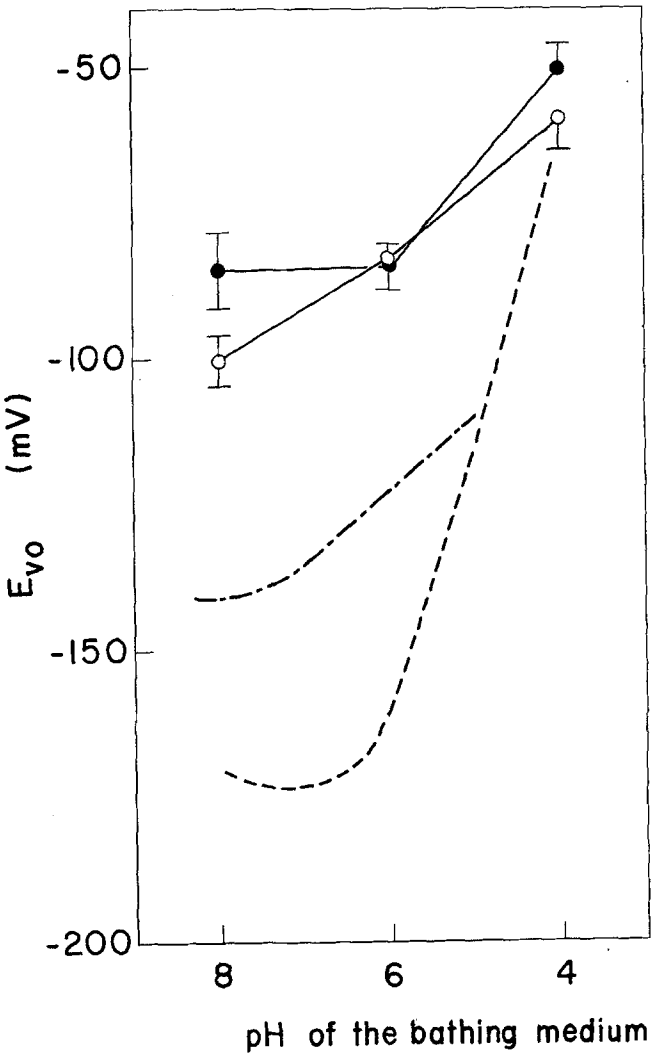


Fig. 9. Effects of external pH on vacuolar potential (E_{vo}). External media were *iAPW*-1 Na (Table 1) of pH 4, 6 and 8. Curves of different marks indicate experiments with 150-KCl sap (○) and 0.1-KCl sap (●) of pH 6. Results obtained by Kitasato (1968) on *N. clavata* (-----) and by Lefebvre and Gillet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

Passive Nature of E_{vo} Observed in the Absence of Chemical Potential Gradient between Internal and External Media

When the cell sap of *N. flexilis* or *Chara australis* was replaced with an isotonic artificial pond water (*iAPW*-10 Ca, *cf.* Table 1), where the concentrations 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.*,

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

Vacuolar medium	Bathing medium	E_{vo} (mV)	R_{vo} (KΩcm^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-10 Ca	*iAPW*	−106±6 (10)	68±3 (6)
iAPW-10 Ca	*iAPW*-10 Ca	−36±4 (7)	28±7 (6)
2-*Nf*[b]	2-*iAPW*[b]	−111±3 (22)	79±9 (20)
2-KCl[b]	2-*iAPW*	−103±4 (6)	
2-*iAPW*-10 Ca[b]	2-*iAPW*	−95±7 (3)	

[a] Data are shown as the average ±SE. Figures in parentheses indicate the number of cells used.

[b] Osmotic values of the media were 600 mM.

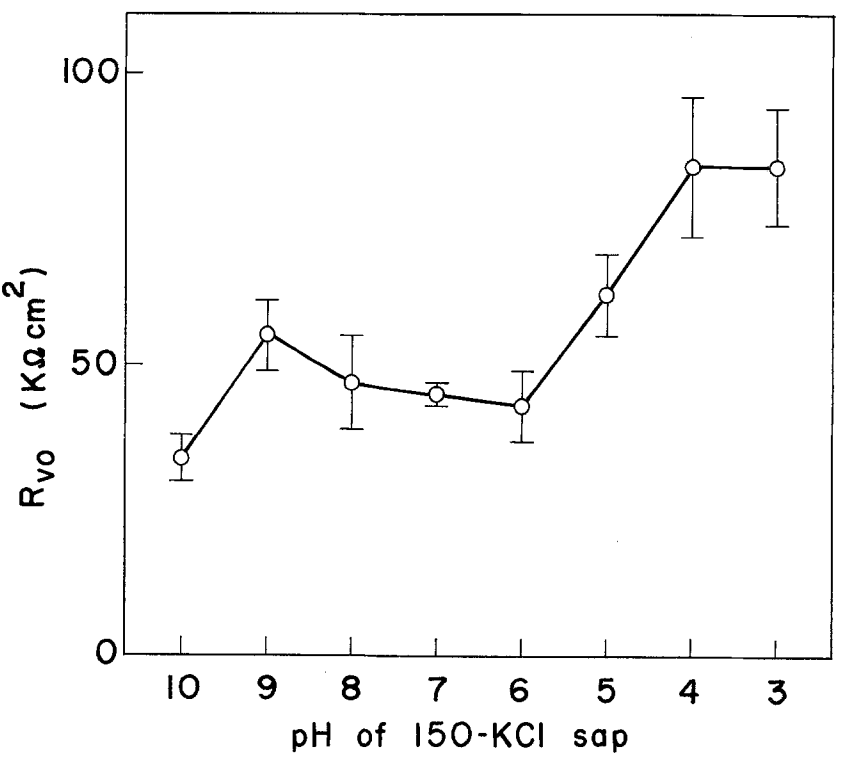


Fig. 10. Relation of pH of the vacuolar medium with 150-KCl sap (Table 1) and the specific resistance (R_{vo}) across the plasmalemma and the tonoplast. The external medium was *iAPW*-1 Na (Table 1) of pH 6

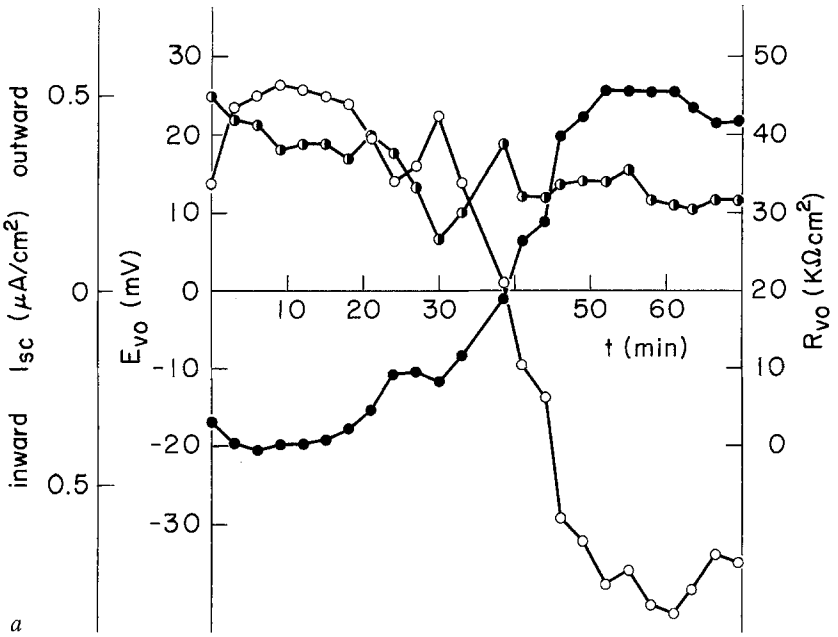
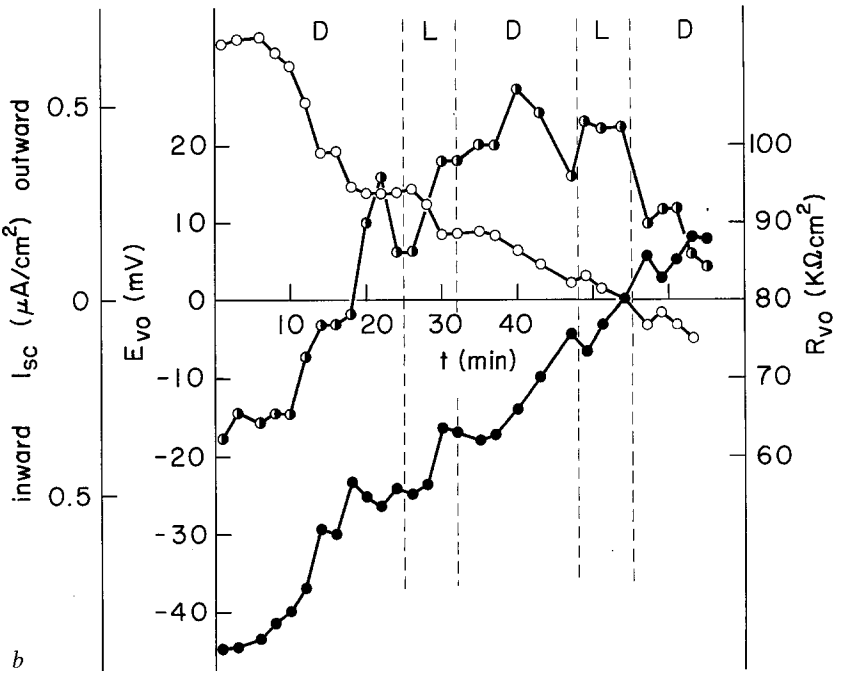


Fig. 11. Short-circuit current measured on an internode of *N. pulchella* whose external and internal (vacuolar) medium was the same one containing (in mM): 0.1 KCl, 10 NaCl, 5 $CaCl_2$ and 300 sorbitol. During the measurements, potential difference between external and vacuolar medium was clamped at 0 mV. Curves with different marks indicate short-circuit current (I_{sc}; ○), electromotive force of the cell (E_{vo}; ●), and specific resistance across the protoplasmic layer (R_{vo}; ◑). R_{vo} was measured by supplying small voltage pulses. Electromotive force, E_{vo}, was calculated from I_{sc} and R_{vo}. (*a*) I_{sc}, which was initially outward, changed its direction with time to inward. (*b*) Illumination with about 2000 lux white light did not show any significant effect on I_{sc}. *D* and *L* in the figure mean dark and light (ca. 2000 lux white light), respectively

1975). Under the same ionic conditions, cells of *N. pulchella* also possessed a significant E_{vo} amounting to −36 mV (Table 2). These values of E_{vo} may either be generated by the electrogenic ion pump or reflect only the difference between E_{co} and E_{cv} which are functions of passive permeabilities of the plasmalemma and the tonoplast to ions.

To check whether or not the electrogenic pump is working in *N. pulchella*, the short-circuit current was measured under the condition that the internal and external spaces were occupied with the same solution containing (in mM): 0.1 KCl, 10 NaCl, 5 $CaCl_2$ and 300 sorbitol. Two typical examples are shown in Fig. 11. The current, which was first outward, shifted with time to zero and, in some cases, changed direction to inward (Fig. 11*a*). The current was not affected by illumination with about 2000 lux white light (Fig. 11*b*). Furthermore, treatment of the cell with 2,4-dinitrophenol (0.3 mM) and with a low temperature



(5 °C) did not make the current zero. The short-circuit current under metabolic inhibition showed similar time courses as that without the treatments. These results seem to be against the possibility that the electrogenic ion pump is working under the experimental condition. Then, it is most probable that E_{vo} observed under the null chemical potential gradient between the internal and external media has its origin in differences in passive ionic permeabilities between the plasmalemma and the tonoplast. This idea is supported by the fact that E_{vo} of *N. pulchella* was almost indifferent to a wide variation of concentrations of K^+ and Cl^- in the cell sap. Table 2 shows that E_{vo} and R_{vo} of cells of *N. pulchella* having the sap of extremely low ionic concentrations (*iAPW*-10 Ca sap) are practically equal to those of cells 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. 7). On the other hand, E_{vo} (Fig. 2) and R_{vo} (Fig. 6*a*) are very sensitive to $[K^+]_o$. Different responses of E_{vo} and R_{vo} to internal K^+ from those to external K^+ are also observed in *N. flexilis* and *C. australis* (Tazawa *et al.*, 1975).

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 inner cytoplasmic membranes behaved. It is interesting to know, however, how the membranes respond to changes in concentrations of ions in the cytoplasm. The simplest way to realize this is to change the osmotic value of the cell sap. Since the tonoplast is assumed to be semipermeable, change in the osmotic value 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 a decrease of cytoplasmic volume to about half the original, and consequently an increase in concentrations of ions in the cytoplasm to twice the normal concentrations.

Table 2 shows that E_{vo} and R_{vo} are not affected appreciably by doubling the tonicity of the cell sap from 300 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. For example, cells with 600 mM osmolarity could produce action potential with electric stimulus of 0.87 μA per cm^2 (average of 9 cells), while cells with normal osmolarity (300 mM) could do so with 0.22 μA per cm^2 (average of 12 cells). This indicates that heightening the internal tonicity increases threshold for excitation.

Discussion

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

$$E_{vo} = E_{co} - E_{cv}. \tag{2}$$

In the present study E_{cv} was not measured directly. However, we assume that ions in the external medium affect only the plasmalemma potential (*cf.* Findlay & Hope, 1964; Findlay, 1970) and those in the vacuole affect only the tonoplast potential. After this assumption, responses of E_{vo} (ΔE_{vo}) to the external and vacuolar ions reflect those of E_{co} (ΔE_{co}) and E_{cv} (ΔE_{cv}), respectively,

$$\Delta E_{co} = \Delta E_{vo}, \tag{3}$$

$$\Delta E_{cv} = \Delta E_{vo}. \tag{4}$$

This assumption is supported by the fact shown in Fig. 2, where E_{vo} responded to $[K^+]_o$ in a similar manner irrespective of $[K^+]_v$ and $[Cl^-]_v$, and also by the fact shown in Fig. 8, where E_{vo} responded to vacuolar pH in a similar manner irrespective of the pH of the external medium. In the previous paper (Kikuyama & Tazawa, 1976) it was clearly demonstrated that the amplitude of the plasmalemma action potential is almost independent of $[Cl^-]_v$ in the range 0.1–20 mM, while the amplitude of the tonoplast action potential is strongly dependent on $[Cl^-]_v$. There were, however, some cases where the above assumption was not strictly valid. In Fig. 4, the response of E_{vo} to $[Cl^-]_o$ differed to some extent according to $[K^+]_v$ or $[Cl^-]_v$, although E_{vo} changed in all cases to the same direction.

Before discussing the results, it is to be mentioned that in *N. pulchella* responses of E_{vo} to vacuolar and external ions reflect solely passive natures of the plasmalemma and tonoplast, since it was difficult in E_{vo} to demonstrate existence of an active component driven by the electrogenic ion pump.

The plasmalemma behaved as a K^+-electrode when K^+ in the external medium was above 10 mM, because E_{vo} changed by about 60 mV for the tenfold changes in K^+, irrespective of the ionic compositions in the vacuole. On the other hand, E_{vo} responded less sensitively to $[K^+]_v$ than to $[K^+]_o$ (Fig. 3), indicating that the tonoplast is less sensitive to K^+ than the plasmalemma. This may explain the result that E_{vo} was 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*, E_{vo} changed less sensitively to the change in the vacuolar ionic composition from the artificial cell sap to *iAPW*-10 Ca than to the same change in the external one (Tazawa *et al.*, 1975).

Although response of E_{vo} to $[K^+]_o$ was much larger than to $[K^+]_v$, response of E_{vo} to $[Cl^-]_o$ was in the same order of magnitude as to $[Cl^-]_v$ so far as the concentration range from 0.1 to 10 mM is concerned. In contrast to responses of E_{vo} to K^+, those of E_{vo} to Cl^- is peculiar in that E_{vo} changed in the direction opposite to that expected from the constant field assumption. If the assumption is valid, increase in $[K^+]_o$ makes E_{vo} less negative and that of $[K^+]_v$ makes E_{vo} more negative. As for K^+, experimental results (Fig. 2 and 3) coincide with the expectations. As for Cl^-, increase in $[Cl^-]_o$ should make E_{vo} more negative and that of $[Cl^-]_v$ should make E_{vo} less negative. However, the experimental results (Fig. 4 and 5) are against the theoretical expectations. To explain this phenomenon there are at least two possibilities; one is that

increase in Cl^--concentration modifies the membrane to increase its permeability to Cl^- relative to K^+, and the other is that it modifies the phase boundary potential (Hodgkin & Chandler, 1965).

E_{vo} depends on pH of the bathing medium (Kitasato, 1968; Lefebvre & Gillet, 1973; Saito & Senda, 1973; 1974). In the present study we showed that E_{vo} is also dependent on pH of the vacuolar medium (Fig. 8*a* and *b*). When pH of the vacuolar medium was low (pH 3–4), E_{vo} was very sensitive to vacuolar pH. Since E_{vo} changed about 30–50 mV for the change in $[H^+]_v$ between 0.1 and 1 mM even when vacuolar KCl was 150 mM (Fig. 8*a*), tonoplast should be more sensitive to H^+ than to K^+ or Cl^-. When the concentration of vacuolar K^+ was low, E_{vo} also changed greatly in response to vacuolar pH change in the alkaline region (Fig. 8*b*), indicating that the tonoplast potential is also sensitive to OH^-. Relatively low sensititivity of E_{vo} to vacuolar pH at the pH range of 5–8 is explained by the fact that the concentration of H^+ or OH^- is much lower at this pH region than other ions such as K^+ or Cl^-. E_{vo} of *N. puchella* also responded to external pH (Fig. 9). The amplitude of the response of E_{vo} to vacuolar pH between 8 and 4 was the same order of magnitude as the response to vacuolar pH between 8 and 4, irrespective of the external pH values (Fig. 8*a* and *b*), suggesting that the tonoplast is equally as sensitive as the plasmalemma to H^+.

R_{vo} was affected insignificantly by a large change in concentrations of K^+ and Cl^- in the vacuole (Table 2, Fig. 7). On the other hand, it was affected by vacuolar pH. In Fig. 10, R_{vo}'s of cells with acid saps were about twice as large as those of cells with neutral or alkaline saps. If increase in R_{vo} reflects increase in R_{cv}, the above result means that the tonoplast has nearly the same resistance 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 its permeabilities to ions.

The electric current required to generate the action potential in cells with higher tonicity of the cell sap was significantly larger than that in cells with normal tonicity. The problem remains unsolved whether higher tonicity itself or higher ionic concentrations in the cytoplasm are responsible for this phenomenon. Characteristics of the tonoplast under excitation was reported in the previous paper (Kikuyama & Tazawa, 1976).

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