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The Role of Electrogenic Pump in *Chara corallina*

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Summary. The conductance, G, and the electromotive force, E, of the *Chara* membrane were determined accurately by using the current-clamp technique. The measurements at the final steady state of inhibitor poisoning give the conductance, g_1, and the electromotive force, E_1, of the passive ion conducting pathways. By knowing these values the conductance, g_2, and the electromotive force, E_2, of the electrogenic pump can be calculated from the measured G and E at each time during the progress of inhibitor poisoning. The local closed circuit current, i, which usually causes a hyperpolarization across the passive conducting pathways, can be calculated by using g_1, g_2, E_1 and E_2 thus determined. The values of g_2 and i decrease monotonically to zero with the progress of poisoning, while E_2 approaches E_1 asymptotically after a transient hyperpolarization. During excitation i increases markedly. Such an increased inward current through the passive conducting pathways may help in accelerating the inactivation of the excitatory mechanism.

Recently many experimental data have been accumulated showing the existence of the electrogenic pumps in the plasma membrane of a variety of cells. Slayman (1965*a*, *b*) showed that the membrane potential of *Neurospora* hyphae was much more hyperpolarized than that expected from the Nernst potential of any ions. The membrane potential was depolarized to the passive electrodiffusion potential by blocking the electrogenic pump with cyanide or under anoxia. Kitasato (1968) introduced experiments which suggested that the electrogenicity in the *Chara clavata* was caused by an active extrusion of H^+ and supposed that the major part of ionic conductance was due to H^+. Saito and Senda (1974) also suggested that the electrogenic ion pump in *Nitella axilliformis* and in *Nitella flexilis* were closely related to photosynthesis. Richard and Hope (1974) showed in *Chara corallina* that H^+ flux at the plasmalemma was not necessarily as large as Kitasato suggested. Keifer and Spanswick (1978) studied the effect of various types of inhibitors in *Chara corallina* and suggested that the electrogenic pump was a H^+-sensitive ATPase in the plasma membrane. They also suggested that the major part of the plasmalemma conductance was in the electrogenic pump pathway. Shimmen and Tazawa (1977) concluded from their internal perfusion experiments in *Chara corallina* that the electrogenicity was the result of Mg^{++}-dependent ATPase in the plasmalemma. With regard to the ion actively pumped out through the plasmalemma, H^+ is the most possible one. However, Na^+ extrusion, OH^-, HCO_3^-, or Cl^- uptake may also contribute to some extent to the electrogenicity, since some part of transport of these ions are known to be active processes (MacRobbie, 1970).

We intend in this report to show to what extent such an electrogenic pumping mechanism contributes to the usual electrophysiological measurements and how it will change during the process of metabolic poisoning.

Materials and Methods

The giant internodal cells of *Chara corallina* were used throughout the experiments. The whole plants have been cultured in our laboratory for years under a continuous illumination with fluorescent lamps (about 2,000 lx). The culture solution was the tap water, in which extracts of soil were added. The average diameter of the internodal cells was 0.7 mm and the length was 6 cm.

The membrane potential was measured between an internal glass pipette Ag/AgCl electrode with less than 1 μm opening which was generally placed in the central vacuole and another identical external electrode of larger opening (30 μm in diameter) with agar plug. The external current electrode was a chlorinated silver plate, additionally plated with platinum black to give both stability of the electrode potential and low electrode impedance (Cole & Kishi-

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moto, 1962). The internal current electrode was either a tungsten or a platinum-iridium alloy wire of 0.1 mm in diameter, the surface of which was coated with platinum black. This axial wire electrode was introduced into the central vacuole of the internodal cell from the nodal end (Fig. 1).

The measuring region of the internode surface was 7 mm long. This length is small enough to achieve the spatial uniformity of current clamping, since the space constant of the *Chara corallina* was about 3 cm at rest and 6 mm at the peak of excitation. The current-clamp technique was applied following Cole and Moore's method (1960) (Fig. 1).

In the present experiments only the external solution was perfused at a constant rate of about 1 liter per hr. The temperature of the external perfusing solution was controlled with a thermoelectric regulator (Sharp, TE-12K) at around 25 °C and was monitored with a thermister.

After isolation from adjacent cells, the internodal cells had been kept in the artificial pond water (APW) for at least 3 days before being used for experiments. During this period these samples were kept close to the window, avoiding direct sunshine. This gave an usual photoperiod of 12 hr light per day. APW is a mixture of 0.05 mM KCl, 0.2 mM NaCl, 0.1 mM $Ca(NO_3)_2$ and 0.1 mM $Mg(NO_3)_2$. The pH was adjusted to about 7 with 2 mM MES (2-N-morpholinoethane sulfonic acid).

Whatever the molecular mechanism of ion transport be, the current (I) and voltage (V) measured between two pairs of internal and external electrodes are related with Thevenin's theorem (Cole, 1968). That is,

$$V=I\cdot Z+E \tag{1}$$

where E is the electromotive force and Z the impedance of the *Chara* membrane. In *Chara* internodes there are two membranes, i.e., plasmalemma and tonoplast, between the internal and external electrodes. However, we treated these as a single membrane for simplicity. When some perturbation is given to this system, the relation between perturbed voltage, v, and current, i_o, is as follows:

$$v=i_o\cdot Z+I\cdot\Delta Z+\Delta E. \tag{2}$$

Under the special current-clamp condition, i.e., $I=0$, the second term in Eq. (2) can be neglected. Actually, small current pulses of constant magnitude, i_o, were applied successively under the current-clamp condition ($I=0$), and the corresponding voltage responses were recorded with a Sony Data Recorder (DFR-3515) and/or directly transferred to a digital cassette tape recorder (TEAC, MT-2) after A/D conversion (Datel, MDAS 8D). Under this condition and if the size of the voltage response was smaller than 5 mV, the shape of the voltage response could be simulated with a remarkable accuracy with the following single exponential function.

$$v(t)=r_s\cdot i_o+r_m\cdot i_o[1\text{-}\exp(-t/c_m r_m)] \tag{3}$$

where r_m and c_m are resistance and capacitance of the *Chara* membrane, respectively. The addition of a series resistance, r_s was necessary in every case for successful simulation. The simulation was carried out with a nonlinear least squares curve-fitting program by using a microcomputer (NEC, TK-80). With this method each parameter in Eq. (3) could be decided within errors of 0.1%. However, if the size of the voltage response was larger than 10 mV, we frequently found that such a simulation with a single time constant was not necessarily successful. That is, we had to assume that another exponential process was occurring in ΔE (Eq. (2)). If we were not aware of this, we would over- or underestimate the membrane conductance. Actually, we chose a current intensity small enough so that the voltage response did not exceed 5 mV. Ionic conductances were determined at rest as well as during action potential. To stimulate the *Chara* membrane, a suprathreshold current pulse of 20 msec in duration was also applied. In the following analysis the reciprocal values of r_m thus determined were used as the measure of ionic conductance (G) of the *Chara* membrane.

As far as two types of ion transporting systems – the one passive electrodiffusion and the other electrogenic pump(s) – are working at the plasmalemma, the total ionic current measured should be equal to the sum of currents which flow through these two types of transport pathways. Such a situation is equivalent to the circuit shown in Fig. 2. Possibly several ions are participating in both transport pathways. However, each transport system can be expressed simply as a series circuit of r_j ($=1/g_j$) and E_j, where $j=1$ stands for the passive pathway and $j=2$ for the electrogenic pump pathway as shown in Fig. 2. Ionic conductance of the *Chara* membrane can be determined accurately as described above. Then, adopting the model shown in Fig. 2

$$g_1+g_2=G \tag{4}$$

$$g_1E_1+g_2E_2=GE \tag{5}$$

where E is the membrane potential whenever the current through the membrane is zero.

If we can find an ideal inhibitor which blocks only the electrogenic transporting mechanism and has no influence at all on the passive ionic conduction, then the conductance and the electromo-

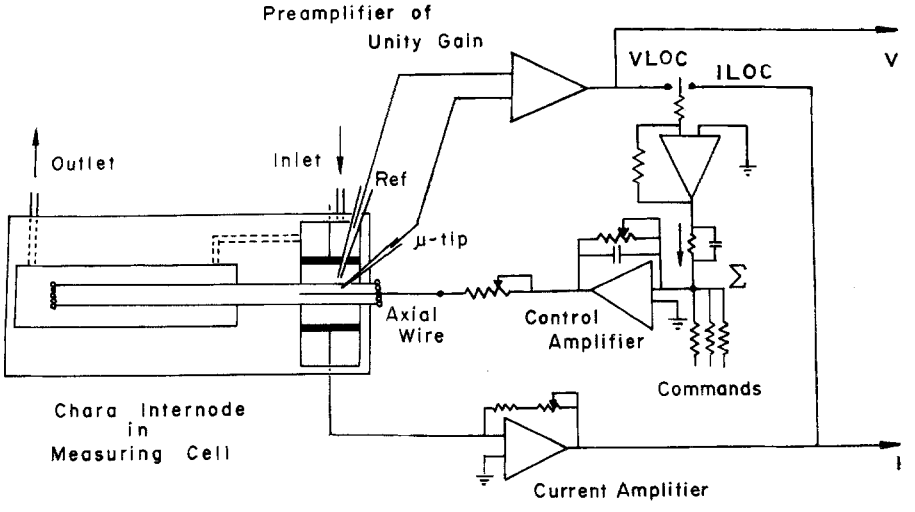

Fig. 1. Current clamp system for *Chara* internodes

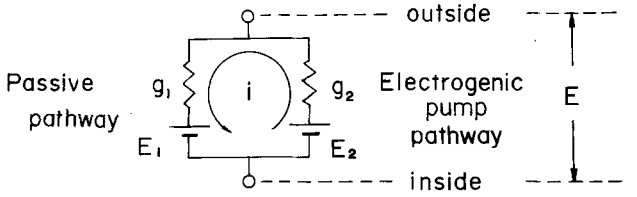

Fig. 2. Equivalent circuit model for the *Chara* membrane having the electrogenic ion pumping system

tive force measured at the final steady state after application of such an ideal inhibitor should be equal to the conductance g_1 and E_1 of the passive conducting pathway. By knowing g_1 and E_1 in this way, g_2 and E_2 of the electrogenic pump can be calculated as follows:

$$g_2 = G - g_1 \tag{6}$$

$$E_2 = (GE - g_1 E_1)/g_2. \tag{7}$$

Under the condition of zero net current, the respective currents which flow through the passive and electrogenic pump pathways are equal but in an opposite direction. Accordingly, the membrane potential is hyperpolarized by the product of i times r_1 from the passive electrodiffusion potential. This extent has been regarded as an index of the electrogenic activity.

It is worth noting here that E_2 in the electrogenic pump pathway corresponds to the potency of the electrogenic pump and g_2 to its efficiency. Moreover, the local circuit current, i in Fig. 2, which is the actual pump current, depends not only on g_2 and E_2 but also on g_1 and E_1. For instance, if E_2 happens to be equal to E_1, i is zero. In such a case the role of electrogenicity may not be noticed. On the other hand, if E_2 is less negative than E_1, the membrane potential is also less negative than the passive diffusion potential. In this case the electrogenic pump pathway, if any, may be regarded as one of the leakage pathways.

Anyway, with the parallel circuit model shown in Fig. 2 it is expected that motion of ions ordinarily pumped out electrogenically may be accelerated, decelerated, or even reverse its direction through the same active pathway, depending on the voltage applied across the membrane. This is possible thermodynamically. However, there seems to be no experimental evidence at present to support or to deny this expectation objectively.

Results

The electrogenic pump of *Characeae* internodes is believed to originate from the Mg-dependent ATP-ase in the plasmalemma. H^+ is supposed to be pumped out, being supplied energy from ATP hydrolysis. ATP is supplied mainly either by the oxidative phosphorylation in the mitochondria or by the photosynthetic phosphorylation in the chloroplasts. To avoid a possible contribution from photosynthesis, the following inhibitor experiments were carried out in complete darkness.

2,4-Dinitrophenol

First, the effect of one of the popular inhibitors, i.e., 2,4-dinitrophenol (DNP), was tested. DNP is commonly used as an uncoupler of oxidative phosphorylation. It is worth noting that DNP acts also as a proton ionophore in the inner membrane of mitochondria, causing a leakage of H^+ once carried outside by the electron transfer mechanism (Hopfer, Lehninger & Thompson, 1968).

The internodes were kept in the dark for about 30 min before the inhibitor experiments. After the control experiments in the APW the external solution was replaced with 0.2 mM DNP containing APW buffered at pH 7.0 with 2 mM MES (2-N-morpholinoethane sulfonic acid). Actually the external solution washed the external surface of the *Chara* internode at a constant rate of about 15 ml per min. The membrane potenial, E, was depolarized from -190 to -110 mV in about 40 min. The conductance, G, decreased from 200 to 85 $\mu S\ cm^{-2}$. The pump current, i, decreased from -7 to zero $\mu A\ cm^{-2}$. It is worth noting that the conductance and the membrane potential changed asymptotically to nonzero values.

It is not unreasonable to suppose that these asymptotic values correspond to the conductance, g_1, and the electromotive force, E_1, of the passive ion transport pathway. Then, it is possible to calculate g_2 and E_2 of the electrogenic pump pathway by using Eqs. (6)–(7). The values of g_2 thus calculated decreased monotonically to zero (Fig. 3*a*), while E_2 decreased asymptotically to a final value, E_1, after a transient hyperpolarization (Fig. 3*b*). Such a temporary hyperpolarization of E_2 was commonly observed among inhibitors so far tested.

It is tacitly assumed, in these calculations, that g_1 and E_1 might not be affected by DNP poisoning. Here we make an additional assumption that the excitatory mechanism also might not be affected by DNP. Whether this assumption is valid or invalid can be judged from the results based on this. Anyway, following these assumptions g_1 and E_1 at the peak of excitation during the process of DNP poisoning can be calculated with Eqs. (4)–(5), since g_2 and E_2 at each time have already been determined (Fig. 3*a* and *b*). The results are shown in Fig. 4. The large increase of conductance, G, at the peak of excitation is mainly due to g_1 of the passive ionic pathway (Fig. 4*a*). These conductances decreased markedly during the process of DNP poisoning. The pump current, i, increased markedly at the peak of excitation. However, this current decreased rapidly to almost zero by DNP poisoning (Fig. 4*c*). It should be noticed that the electromotive force, E_1, of the passive ionic pathway at the peak of excitation shifted toward a more negative level, i.e, -110 mV (Fig. 4*b*). In other words, not only a depolarization of the resting potential but also decay of the peak level of action potential occurred by DNP. Actually the excitability of *Chara* membrane

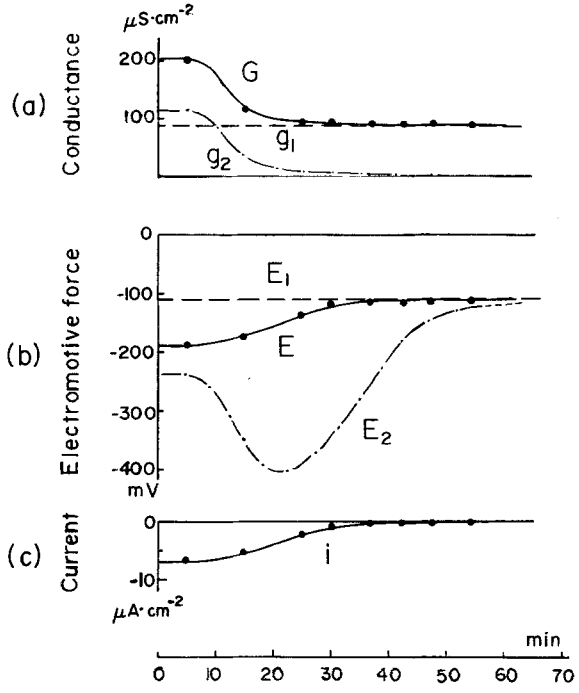

Fig. 3. Changes of conductances (*a*), electromotive forces (*b*), and pump current (*c*) of the *Chara* internode during progress of DNP (0.2 mM) poisoning. Temperature, 25 °C. The conductance, g_1 (85 μS · cm^{-2}), and electromotive force, E_1 (−110 mV), of the passive ion transport pathway are obtained from the final values of measured conductance, G, and electromotive force, E. The conductance, g_2, of the electrogenic pump decreased monotonically to zero, while the electromotive force, E_2, decreased asymptotically to E_1 after a transient hyperpolarization. The pump current, i, decreased monotonically to zero

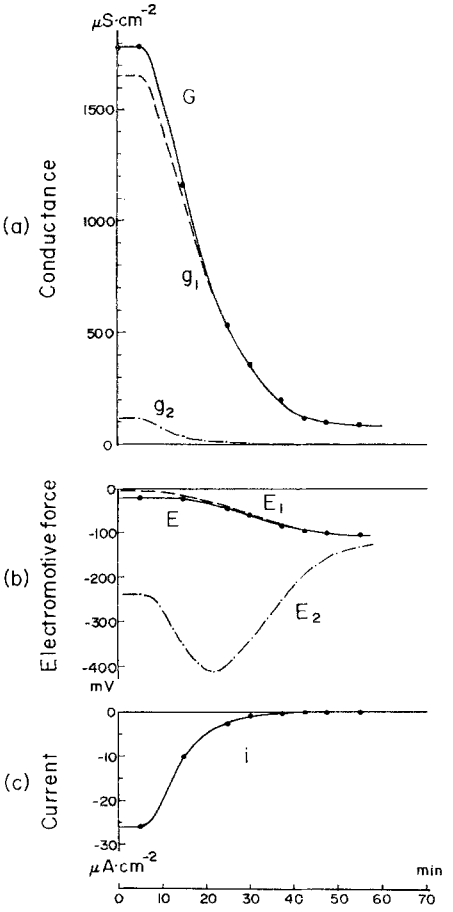

Fig. 4. Conductances (*a*), electromotive forces (*b*), and pump current (*c*) at the peak of excitation of *Chara* membrane during DNP (0.2 mM) poisoning. Temperature, 25 °C. The changes of conductance, g_2, and electromotive force, E_2, of the electrogenic pump pathway are assumed to be the same as those in Fig. 3. The rapid decrease of conductance is mainly due to the decrease of conductance, g_1, of the passive ion transport pathway. The electromotive force, E_1, at the peak of action potential shifted toward a more negative level. The large pump current, i, at the peak of excitation decreased rapidly to zero

was lost almost completely in its late period. It is generally accepted that in many excitable cells the excitation is the event which occurs at the passive ionic pathway. Since DNP had an influence on the passive ionic pathway, our assumption mentioned above should be invalid in the case of DNP experiments. We have to conclude that DNP at 0.2 mM affects not only the active electrogenic process, but also the passive ionic process. However, E, G and excitability of the *Chara* membrane recovered almost completely when the external solution was replaced with normal APW, and the internodes were illuminated (about 2,000 lx) with an incandescent lamp. Therefore, the effect of 0.2 mM DNP was reversible so long as the internode was not kept in the DNP solution for many hours.

Triphenyltin Chloride

Triphenyltin chloride (TPC) is known as a specific blocker of the function of F_1 particles at the mitochondria inner membrane and also possibly CF_1 particles at the thylakoid membrane of chloroplast, resulting in an inhibition of ATP synthesis (Stockdale, Dawson & Selwyn, 1970; Gould, 1976).

The *Chara* internodes had been kept in the dark for 20 to 30 min until the membrane potential reached a stable level. At this state the light-sensitive phosphorylation systems were not expected to be working. After a few control measurements in the APW, the external solution was replaced with 2 μM TPC-containing APW, both of which were buffered at pH 7.7 with 2 mM tricine (Tris-hydroxymethyl-methylglycine).

Similar to other inhibitors, the effects of 2 μM TPC were (i) depolarization of E from about −200 to −100 mV (Fig. 5*b*); (ii) decrease of G from about 150 to 60 μS · cm^{-2} (Fig. 5*a*); (iii) decrease of pump

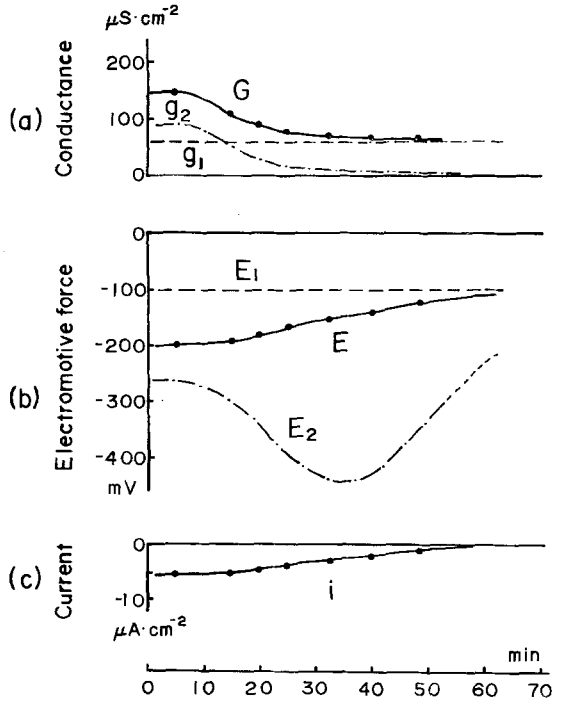

Fig. 5. Changes of conductances (*a*), electromotive forces (*b*), and pump current (*c*) of *Chara* internode during progress of TPC (2 μM) poisoning. Temperature, 25 °C. The conductance, g_1 (60 μS · cm^{-2}), and electromotive force, E_1 (−100 mV), of the passive ion transport pathway are obtained from the final values of measured conductance, G, and electromotive force, E. The conductance, g_2, of the electrogenic pump pathway decreased monotonically to zero, while the electromotive force, E_2, decreased asymptotically to E_1 after a transient hyperpolarization. The pump current decreased monotonically to zero

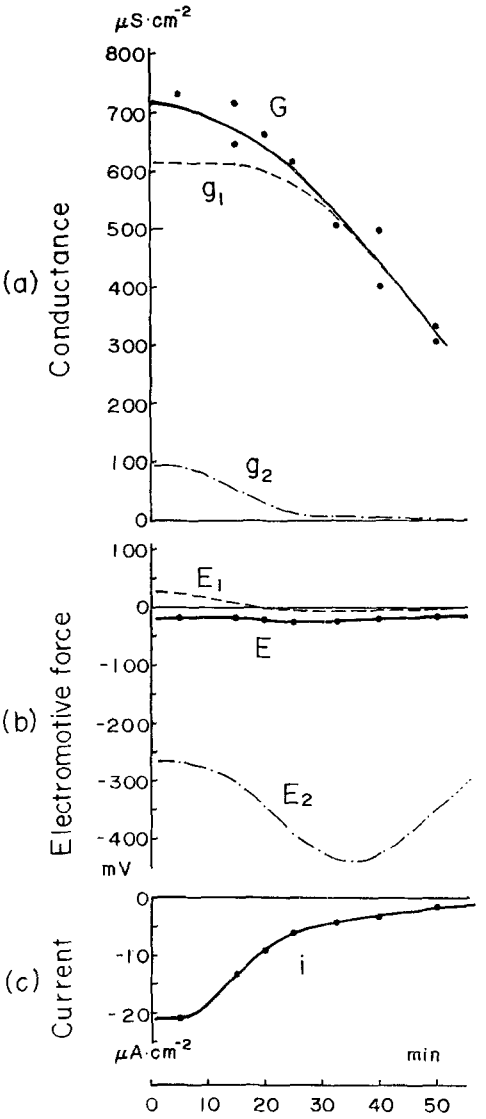

Fig. 6. Conductances (*a*), electromotive forces (*b*), and pump current (*c*) at the peak of excitation of *Chara* membrane during TPC (2 μM) poisoning. Temperature, 25 °C. The changes of conductance, g_2, and electromotive force, E_2, of the electrogenic pump pathway are assumed to be the same as those in Fig. 5. Comparatively slow decrease of conductance, G, is mainly due to the decrease of conductance, g_1, of the passive ion transport pathway. The electromotive force, E_1, at the peak of action potential did not change appreciably by TPC poisoning. The large pump current, i, at the peak of excitation decreased to zero by TPC poisoning

current, i, from 5.7 to zero μA · cm^{-2} (Fig. 5*c*). These decreases were monotonic exponential decays, like the case of the DNP experiment.

As mentioned before, if we assume that TPC attacks only the electrogenic pump without affecting the passive conducting pathway, then the final values of G and E are to be equal to g_1 and E_1 of the passive conducting pathway, respectively. Then, the changes of conductance and electromotive force of the electrogenic pumping mechanism can be calculated as in the case of the DNP experiment. The value of g_2 thus calculated decreased monotonically to zero (Fig. 5*a*), while E_2 decreased asymptotically to the level of E_1 after a transient hyperpolarization (Fig. 5*b*). These changes are quite similar to those observed in the DNP experiment.

By assuming that g_1 and E_1 might not be affected by TPC poisoning, these values were calculated at the peak of excitation during the process of poisoning. The large value of G at the peak of excitation is mainly due to g_1 of the passive ionic pathway, which decreased gradually during TPC poisoning (Fig. 6*a*). It should be noticed that E_1 of the passive ionic pathway at the peak of excitation remained almost at the zero mV level (Fig. 6*b*). This is in contrast to the DNP experiment (Fig. 4*b*). In other words, TPC depolarized the *Chara* membrane by attacking the electrogenic pumping mechanism, almost without influencing the peak level of action potential. The large pump current, i, at the peak of excitation decreased fairly rapidly to zero by TPC poisoning (Fig. 6*c*). We observed that E and G recovered almost

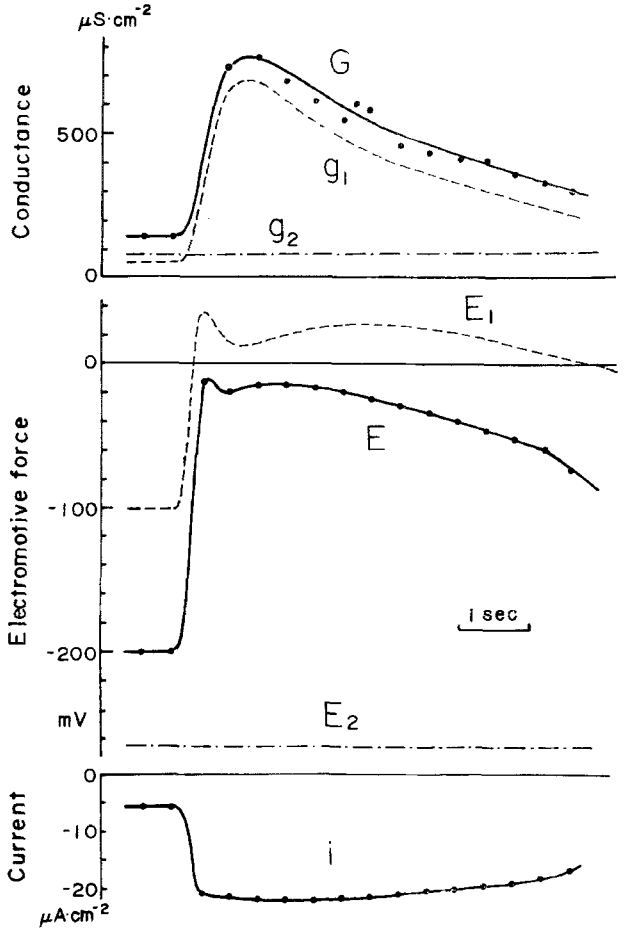

Fig. 7. Changes of conductances (*a*), electromotive forces (*b*), and pump current (*c*) during action potential of *Chara* internode before TPC (2 μM) poisoning. Temperature, 25 °C. The value of conductance, g_2, and electromotive force, E_2, of the electrogenic pump pathway are taken from those before TPC (2 μM) poisoning (Fig. 5), and it is assumed that these values were unchanged during action potential. The main part of the conductance change is due to g_1 of the passive ion transport pathway (*a*). The change of electromotive force, E_1, was almost in parallel with the measured E. E_1 was positive at around the peak of action potential (*b*). The pump current, i, increased rapidly to a large maximum at the peak of action potential and began to decrease slowly at around its falling phase

completely even without removing TPC, when the internode was illuminated with an incandescent lamp (about 2,000 lx) at this state. The size of the action potential also recovered its original value. This may suggest that the poisoning effect of TPC on CF_1 particles of the chloroplast membrane was not as large as it was on F_1 particles of the mitochondrial membrane in the *Chara* cells. However, we need more careful analysis before concluding this. Moreover, TPC at 2 μM seems to be close to an ideal inhibitor, which attacks only the active ionic process.

Taking it for granted that TPC does not attack the excitatory mechanism and that the excitation does not influence the parameters characterizing the electrogenic pump, i.e., g_2 and E_2, it is possible to calculate the changes of g_1 and E_1 during excitation at each time of TPC poisoning. One example at the beginning of TPC poisoning is shown in Fig. 7. It is worth noting that the peak level of action potential is a negative value close to zero. However, at the passive ionic pathway E_1 is 20–30 mV inside positive at this stage (Fig. 7*b*). Such an overshoot is commonly observed in many nerve and muscle cells. It is also to be emphasized here that the pump current, i, increased rapidly to a large maximum at around the peak of action potential and began to decrease slowly at around its falling phase. At the later stage of TPC poisoning the absolute value of i at rest and the amount of transient increase of i during excitation were very small. At this stage the size of action potential decreased to almost half of its original value, with a minor change in its peak level (Figs. 5*b* and 6*b*). It is worth noting that the duration of action potential frequently increased largely at this stage. These results seem to suggest that such a marked increase of inward current through the passive ionic pathway should cause a hyperpolarization of this pathway, which may help lead to an inactivation of the excitatory mechanism. This seems to be another example of negative feedback mechanism in the living excitable membranes.

Discussion

In a series of detailed experiments on the electrophysiology of *Neurospora* hyphae, Slayman and his collaborators showed that the electrogenic pump is the active extrusion of H^+ at the plasma membrane. They analyzed their data by assuming that the H^+ pump behaved as an ideal current source for hyperpolarizing the passive ionic pathway. According to their recent experiments (Slayman, Long & Lu, 1973), the extent of such a pump current depends on the ATP content in the cytoplasm. However, there seems to be no concrete evidence to show that the H^+ pumping mechanism acts as an ideal current source. So far as the extent of hyperpolarization which occurs at the passive ion pathway is an indicator of the electrogenic pumping activity, we need another parameter to express its efficiency.

Although H^+ pump is the most promising cause for the electrogenicity in *Characeae* cells, there are many evidences to show that Cl^-, Na^+, OH^-, HCO_3^-, etc., are also transported by some active processes (MacRobbie, 1970). According to Thevenin's theorem in the network analysis, these processes can be expressed simply as a circuit which is an electromotive

force in series with a resistance. Rapoport (1970) gave a similar expression for the electrogenic pump from a standpoint of thermodynamics of irreversible processes. Spanswick (1972) adopted this expression in his analysis on the electrogenicity in *Nitella translucens*. This expression was already adopted by Ussing and Zerahn (1951) for their analysis of short-circuited sodium current in isolated frog skin. It is not necessarily possible to go into the molecular mechanism with this type of simplified circuit model. It is possible, however, to determine separately the extent of contribution of each ion transporting system as described in this report. Moreover, analyses with this model seem to give us more reasonable understanding of the electrophysiological behavior of *Chara* and *Nitella* cells. Similar analyses for the data on the temperature and pH effects will be published in other reports.

Gradmann (1975) in *Acetabularia* and Gradmann et al. (1978) in *Neurospora* hyphae compared the current-voltage curves of the plasma membrane with and without inhibition of the metabolism. From these curves they were successful in demonstrating a nonlinear pump current-voltage curve. If the electrogenic pumping mechanism, which is to be characterized with g_2 and E_2, is nonlinearly dependent on the membrane potential, our analysis of the pump current during action potential would need to be modified, more or less, since we have assumed that g_2 and E_2 remained unchanged during excitation. However, the qualitative aspect of the marked increase of the pump current during excitation is still held, because the contribution of g_1 of the passive ionic process was much larger during excitation than g_2 of the electrogenic pump.

On the other hand, g_2 and E_2 changed markedly during the process of metabolic poisoning. The membrane potential also depolarized with this process. However, changes of g_2 and E_2 are not caused by the depolarization of the membrane potential, but by the metabolic poisoning as described in our analysis. Anyway, we have at present no definite data at hand to decide whether it is really nonlinear or not in *Chara*. Even if it should be so, we should need to decide carefully whether such nonlinearity comes from the change in g_2 or from that in E_2. As described in Materials and Methods, we experienced frequently that an additional term due to the change of the electromotive force was involved in the steady-state value of the voltage responses, if we did not choose test current pulses which were small enough. This also gave us an apparent nonlinearity in the current-voltage curves.

According to our preliminary experiment for measuring ATP content of the *Chara* internode during the process of TPC poisoning in complete darkness, it decreased to less than 20% of that of the normal untreated cells. For this measurement we applied Luciferin-Luciferase assay. Keifer and Spanswick (1979) gave a somewhat smaller value in their *Chara corallina* during 5 μM CCCP poisoning. We observed that the cytoplasmic streaming continued, though it was more or less at a decreased rate, at the end of the TPC poisoning. Therefore, the energy for the cytoplasmic streaming might be supplied from ATP formed, probably, by glycolysis, at least in our later stage of TPC poisoning. Anyway, there remained some ATP in the cytoplasm as a background in our inhibitor experiments. Nevertheless, it is worth noting that g_2 of the electrogenic pump decreased to almost zero and E_2 decreased to the level of E_1 which is predicted from the passive electrodiffusion theory. Accordingly, there seems to be a critical level of ATP, which is not necessarily close to zero, for activating the electrogenic pumping mechanism.

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