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A Kinetic Analysis of the Electrogenic Pump of *Chara corallina*: I. Inhibition of the Pump by DCCD

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Summary. The current-voltage curve of the *Chara* membrane was obtained by applying a slow ramp depo- and hyperpolarization by use of voltage clamp. With the progress of poisoning by DCCD (dicyclohexylcarbodiimide) the *I-V* curve moved by about 50 mV (depolarization) along the voltage axis, reducing its slope, and finally converged to the i_d-V curve of the passive diffusion channel. Changes of i_p-V curve of the electrogenic pump channel could be obtained by subtracting the latter from the former.

The sigmoidal i_p-V curve could be simulated satisfactorily by adopting a simple reaction kinetic model. Kinetic parameters of the successive changes of state of the H^+ ATPase could be evaluated. Changes of these kinetic parameters during inhibition gave useful information about the molecular mechanism of the electrogenic pump.

Depolarization of the membrane potential, decrease of membrane conductance, and decrease of pump current during inhibition of the pump with DCCD are caused mainly by the decrease of conductance of the pump channel. The decrease of this pump conductance is caused principally by a marked decrease of the rate constant for releasing H^+ to the outside.

Key Words *Chara* · electrogenic pump · chord conductance · *I-V* curve · kinetic model · computer simulation

Introduction

Slayman (1965*a*,*b*) first showed in the hyphae of the fungus *Neurospora* that the membrane potential was much more hyperpolarized than the Nernst potential of any of the ions present. This membrane potential was depolarized to the passive electrodiffusion potential by blocking the electrogenic pump with cyanide or under anoxia. He concluded that the potential generated by an electrogenic H^+-pump is closely linked to respiratory metabolism. Kitasato (1968) suggested that by using a voltage-clamp technique the electrogenicity in *Nitella clavata* was caused by an active extrusion of H^+. Saito and Senda (1973) suggested that the electrogenicity in *Nitella* was closely related to photosynthesis. Keifer and Spanswick (1978) examined the effects of various inhibitors and suggested that the electrogenic pump in *Chara corallina* is a H^+-ATPase in the plasmalemma. Inhibition of the electrogenic pump was accompanied by a decrease in membrane conductance. Shimmen and Tazawa (1977) concluded from their internal perfusion experiments in *C. australis* that the electrogenicity is due to the Mg^{2+}-dependent ATPase in the plasmalemma. An increased H^+ efflux was detected on addition of ATP into the perfusate of the tonoplast-less *Chara* internode (Shimmen & Tazawa, 1980; Lucas & Shimmen, 1981). The existence of this type of ion-translocating ATPase has recently been reported in various plants (*see* reviews by Poole, 1978; Spanswick, 1981; Harold, 1982).

It may be possible to analyze some aspects of the electrogenic pump without introducing conductance (Frumento, 1965; Goldman, 1981). On the other hand, there are theoretical grounds for introducing a conductance in the electrogenic pump (Finkelstein, 1964; Rapoport, 1970). If an enzyme catalyzes an electrogenic vectorial reaction across a membrane, the apparent rate constant of the overall process should depend on the chemical constituents as well as on the membrane potential. In other words, rate constants of at least one of the individual elementary steps in the process should be voltage dependent.

The current-voltage curve (*I-V* curve) can be obtained with an electrophysiological technique. The slope of the *I-V* curve of a cell placed in the dark or at low temperature is reduced compared with that of normal cells. This was shown in *Nitella translucens* by Spanswick (1972) and in *N. axilliformis* by Saito and Senda (1974). Similar results were obtained in *Halicystis parvula* by Graves and Gutknecht (1977) and in *Acetabularia* by Gradmann (1975, 1976). However, the electrogenicity in these two marine algae is supposed to be caused

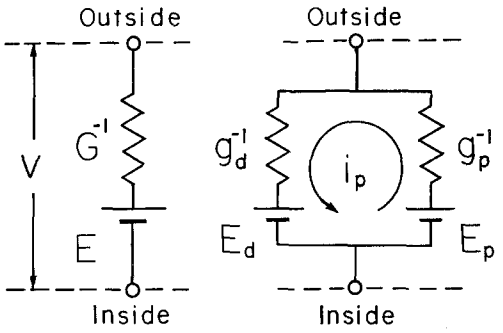

Fig. 1. Parallel circuit model for the *Chara* membrane having an electrogenic ion pump system in parallel with the passive diffusion channel. *See* text for details

by an electrogenic Cl^- pump driven by ATP hydrolysis.

The *I-V* curve at the complete inhibition of the electrogenic pump should correspond to that (i_d-*V* curve) of the passive diffusion channels. Thus, if we take successive records of the *I-V* curves during inhibition of the electrogenic pump, the change of i_p-*V* curve of the electrogenic pump can be analyzed. The i_p-*V* curve thus obtained is generally of a sigmoidal shape and has a reversal potential at the emf of the electrogenic pump (E_p). In other words, the pump current, i_p, is certainly voltage dependent, though it saturates both for large depolarization and hyperpolarization. The E_p, defined as the voltage at which $i_p = 0$, coincides with the one calculated from thermodynamics, assuming that $2H^+$ are extruded for each ATP hydrolyzed (Spanswick, 1980; Smith & Walker, 1981). E_p changes with the external pH with a slope of about 50 mV/pH unit (Kishimoto, Kami-ike & Takeuchi, 1981*a*).

In order to discuss the voltage-dependent i_p we need a kinetic analysis. Läuger (1979) proposed a kinetic model for the electrogenic ion pumps, where the essential part is an ion channel. This model was applied successfully for the light-driven H^+ pump of *Halobacterium* and redox-coupled H^+ pump in the mitochondrial respiratory chain and was suggested also to be applicable for ATP-driven ion transport. Trials of this type of kinetic analysis have begun independently by Slayman and his coworkers (Hansen, Gradmann, Sanders & Slayman, 1981; Gradmann, Hansen & Slayman, 1982) and Mummert, Hansen and Gradmann (1981) on *Neurospora, Acetabularia,* and some other systems and by Chapman, Johnson and Kootsey (1983) on Na^+-K^+-ATPase and by Kishimoto and his coworkers (1981*b*) on *Chara* membrane.

The sigmoidal i_p-*V* curve can be simulated satisfactorily with the kinetic model described in this report. From the parameters determined by the simulation program we could evaluate the rate constants of the kinetic model. Changes of these parameters under some physiological changes (i.e., metabolic inhibition, dark, low temperature, pH change, etc.) seem to give useful information about the molecular mechanism of the electrogenic pump of the *Chara* membrane.

The Model

The Electrogenic Pump with Conductance

It has been established that the plasmalemma of characeaen internodes has at least two different, i.e., passive and electrogenic, ionic pathways. This situation can be most simply and also satisfactorily illustrated with an equivalent circuit, shown in Fig. 1. The current I, which we actually measure in an *I-V* curve, is the sum of two current components, one flowing through passive diffusion channel, i_d, and another through the electrogenic pump channel i_p.

$$I = i_d + i_p \tag{1}$$

$$i_d = g_d(V - E_d) \tag{2}$$

$$i_p = g_p(V - E_p). \tag{3}$$

We suppose that the conductance g_p of the electrogenic pump channel is a part of the pumping mechanism, which is controlled by the rate constants of the kinetic model and also by the voltage.

The voltage at which I is zero in the *I-V* curve is the resting potential E of the membrane. E and the conductance G are expressed as follows:

$$E = (g_dE_d + g_pE_p)/G \tag{4}$$

$$G = g_d + g_p. \tag{5}$$

E is the weighted average of two emf's, i.e., E_d and E_p. Here the weights are the conductances of each channel, i.e., g_d and g_p. At the resting state where $I = 0$ and $V = E$

$$i_p = g_p(E - E_p). \tag{6}$$

Both conductances are generally voltage dependent, as will be shown later. Therefore, E is also voltage dependent. This fact is to be taken care of in the determination of the membrane conductance, if both channels are working (Kishimoto et al., 1982).

If we can stop the activity of the electrogenic pump without affecting the passive diffusion channel, g_p and also i_p will become practically zero. Then, the *I-V* curve will reduce to the i_d-*V* curve of

the passive diffusion channel. In this curve the voltage where $i_d = 0$ is equal to E_d and $G = g_d$. This situation can be approximately realized by treating the *Chara* membrane either with DCCD (dicyclohexylcarbodiimide) or TPC (triphenyltin chloride). Once the i_d-V curve is known, the i_p-V curve of the electrogenic pump channel can be obtained by simply subtracting the i_d-V curve from the I-V curve, so long as no qualitative changes occur during each I-V span. An excitation of the diffusion channel is induced for a large depolarization, while a breakdown phenomenon is induced for a large hyperpolarization. In both cases marked changes of g_d and E_d occur (Ohkawa & Kishimoto, 1974, 1977). In these cases qualitative changes occur not only in the diffusion channel but also possibly in the pump channel during an I-V span. If one simply subtracted the i_d-V curve from the I-V curve for a large voltage span, the resultant i_p-V curve would give misleading information in the analysis of the real i_p-V characteristics.

Consecutive Reaction Scheme for the H^+-Pump

We assume a cyclic change of five states of H^+-ATPase in the plasmalemma, i.e., $E_1 \rightleftharpoons E_2 \rightleftharpoons E_3 \rightleftharpoons E_4 \rightleftharpoons E_5 \rightleftharpoons \cdots$ (Fig. 2). E_j ($j = 1, 2, 3, 4$, or 5) means the amount of each enzyme intermediate. These reaction steps are summarized in the following:

1) Transformation of the enzyme from E_1 into E_2 is coupled with ATP hydrolysis.

$$E_2 = \varepsilon[\mathrm{ATP}]/([\mathrm{ADP}][\mathrm{P_i}])\, E_1 = ME_1 \tag{7}$$

where ε is a coupling constant with the ATP hydrolysis and M ($= \varepsilon\,[\mathrm{ATP}]/[\mathrm{ADP}]/[\mathrm{P_i}]$) is the equilibrium constant for the transition from E_1 to E_2.

2) The enzyme is then transformed into E_3 incorporating $m\mathrm{H}^+$ ions. Then,

$$E_3 = \alpha[\mathrm{H}_i]^m E_2 = \alpha M[\mathrm{H}_i]^m E_1 \tag{8}$$

where α is the ratio of the forward rate constant to the backward one for the transition, incorporating $m\mathrm{H}^+$ into the enzyme.

3) The transition of the enzyme from E_3 into E_4 occurs following the electrochemical potential gradient across the membrane. Efflux of H^+ ion, then, is written as

$$f_{34} = k_{34}E_3 - k_{43}E_4. \tag{9}$$

4) The transition from E_4 into E_5 releasing $m\mathrm{H}^+$ ions occurs with an equilibrium constant β. This parameter is the ratio of the forward rate constant to the backward one. Thus,

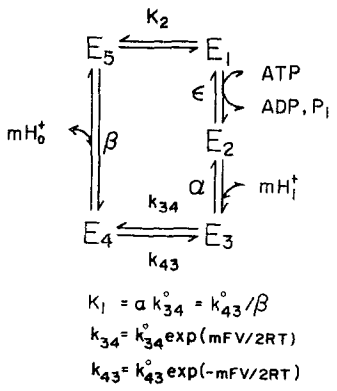

Fig. 2. A kinetic scheme for the vectorial H^+-ATPase of the electrogenic pump of *Chara* membrane. Successive changes of the state of the enzyme are assumed. *See* text for further details

$$E_4 = (1/\beta)[\mathrm{H}_o]^m E_5. \tag{10}$$

5) We assumed that only the transition $E_3 \rightleftharpoons E_4$ is a charge-carrying step and therefore its rate is voltage dependent. For simplicity, a symmetry in the energy barriers for the forward and backward transition is also assumed (Läuger, 1979). Then

$$k_{34} = k^o_{34}\exp(mFV/(2RT)); \qquad k_{43} = k^o_{43}\exp(-mFV/(2RT)). \tag{11}$$

Substituting E_3, E_4, k_{34}, and k_{43} in Eq. (9) for those in Eqs. (8), (10), and (11), respectively, the flux of H^+, i.e., f_{34}, is given with Eq. (12). That is

$$f_{34} = K_1(M[\mathrm{H}_i]^m\exp(mFV/(2RT))E_1 - [\mathrm{H}_o]^m\exp(-mFV/(2RT))E_5) \tag{12}$$

where $K_1 = \alpha k^o_{34} = k^o_{43}/\beta$, and R, T and F have their usual meanings.

6) The transition from E_5 into E_1 is assumed here as a noncharge-carrying step and is voltage independent. Then, the transition is given by

$$f_{51} = k_{51}E_5 - k_{15}E_1 = K_2(E_5 - E_1) \tag{13}$$

where k_{51} is assumed to be equal to k_{15}.

If we suppose that the efflux of H^+ is working steadily by a cyclic transition of the enzyme, i.e., $E_1 \rightleftharpoons E_2 \rightleftharpoons E_3 \rightleftharpoons E_4 \rightleftharpoons E_5 \rightleftharpoons E_1 \cdots$, then f_{34} should be equal to f_{51}. Total amount of enzyme E_0 is assumed to remain constant. Then

$$E_1 + E_2 + E_3 + E_4 + E_5 = E_0. \tag{14}$$

The current carried by H^+, i.e., i_p (=pump current), is proportional to f_{34} (=f_{51}). Then, i_p can be calculated with Eqs. (15) and (16).

$$i_p = (D - A_1/D)A_4/(D + A_2/D + A_3) \tag{15}$$

where $D = \exp(mFV/(2RT))$, $R_2 = [H_o]^m$ and $R_1 = M[H_i]^m$ and

$$\begin{aligned} A_1 &= R_2/R_1 \\ A_2 &= (R_2/R_1)(1 + R_1\alpha)/(1 + R_2/\beta) \\ A_3 &= (K_2/K_1)(1/R_1)(1 + R_1\alpha)/(1 + R_2/\beta) \\ A_4 &= mK_2E_0F/(1 + R_2/\beta). \end{aligned} \tag{16}$$

E_p can be calculated as the voltage where $i_p = 0$.

$$E_p = (1/m)(RT/F)[\ln([ADP][P_i]/[ATP]) + \ln(1/\varepsilon)] + (RT/F)\ln([H_o]/[H_i]). \tag{17}$$

On the other hand, the conductance of the pump channel, i.e., g_p (Fig. 1) is given by

$$g_p = i_p/(V - E_p). \tag{18}$$

The conductance g_p in this report is given not as a slope conductance, but as a chord conductance. The slope of the i_p-V curve has been adopted on not a few occasions for discussion of conductance of the pumping mechanism. If this were the case, such a slope conductance would be zero for a large depolarization or for a large hyperpolarization. This means that the pump is acting as a constant current source in these voltage ranges. Anyway, it is a paradox for the conductance in a finite voltage range to be zero irrespective of a nonzero value of i_p. In our model (Fig. 1) g_p should be the chord conductance [Fig. 1 and Eq. (3)] and the size of g_p is to be calculated with Eq. (18).

Materials and Methods

Giant internodes of *C. corallina* were used throughout the present experiments. The internodes, which averaged 0.7 mm in diameter and 6 cm in length, were isolated from adjacent cells. Next, the internodes were kept in artificial pond water (APW) for at least two days with a photoperiod of 12 hr light (ca. 2000 lx) and 12 hr dark. The APW contained 0.05 mM KCl, 0.2 mM NaCl, 0.1 mM $Ca(NO_3)_2$ and 0.1 mM $Mg(NO_3)_2$, and the pH was buffered at 7 with 2 mM TES (N-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid).

The details of the voltage clamp and the current measurement were almost the same as described previously (Kishimoto, Kami-ike & Takeuchi, 1980; Kishimoto et al., 1981*a*, 1982). The speed of the voltage clamp is fast enough (30 μsec) to cause no appreciable deformation in the current response of the *Chara* membrane. The space constant of the internode we used was about 3 cm at rest and 6 mm at the peak of excitation. As a compromise for accuracy in the surface area estimation and in the spacial uniformity of the voltage clamping, we chose the length of the measuring region as 6 mm.

First, we examined the steady-state current and voltage relation by giving step depolarizations and hyperpolarizations under voltage-clamp conditions (data not shown). Next, we gave a ramp depolarization or hyperpolarization under voltage-clamp condition. We found these two I-V curves to be almost the same if we chose the ramp rate as slow as 100 mV/30 sec. Therefore, in the following experiments we determined I-V curves, first, by giving a slow hyperpolarization and, second, a slow depolarization of moderate size.

After A/D conversion of the data of current, together with the ramp voltage and trigger pulse with a Data Acquisition System (MDAS 8D,Datel), they were recorded with a floppy disk system (YE DATA). The data of the current and voltage thus digitized served for later computations. Further details are described in a previous report (Kishimoto et al., 1982). The experimentally obtained i_p-V curves of the electrogenic pump could be generally simulated satisfactorily with Eq. (15), assuming the stoichiometric ratio $m = 2$. Four unknown parameters, i.e., A_1, A_2, A_3, A_4, were determined with a program of successive approximation to find the best simulation (Powell, 1965; Kotani, 1979). Actual computation was carried out through a terminal microcomputer (PC8001,NEC) by MELCOM in the Computer Center of Osaka University. Kinetic parameters in Fig. 2 were calculated from A_1, A_2, A_3 and A_4 [Eq. (16)]. By using A_1, A_2, A_3 and A_4 the reconstituted i_p-V curve (full lines) with corresponding actual data (shown with symbols) were displayed with a X-Y plotter (Watanabe, WX4671).

Results

Changes of I-V Curve during DCCD Poisoning

DCCD is known as a specific blocker of F_0,F_1-ATPase of mitochondrial inner membrane and of thylakoid in chloroplasts. It binds to one of the subunits, resulting in blockage of the H^+ flux through H^+-channel portion of the ATPase. Actually the internal ATP-level decreased to $\frac{1}{3}$ of the original level by treating the *Chara* internode with 50 μM DCCD (Keifer & Spanswick, 1979; Takeuchi & Kishimoto, 1983). DCCD was dissolved in the APW and pH was adjusted at 7 with TES. The DCCD solution was perfused externally at a constant speed of about 1 liter/hr.

Effect of DCCD on the electrogenic pump was almost the same whether it was applied in the dark or in the light. The I-V curve moved to the right (depolarization) along the voltage axis with the progress of DCCD poisoning (Fig. 3). About 100 min after application of DCCD the I-V curve reached a steady state. This steady state lasted for about an hour. At this stage the V for $I = 0$ was

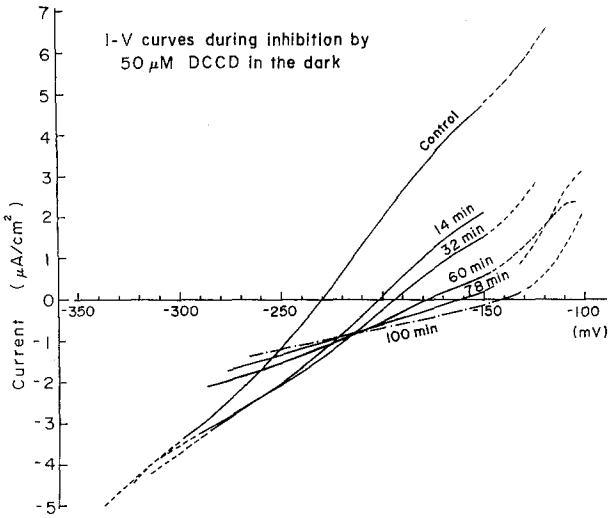

Fig. 3. The *I-V* curve of *Chara* membrane shifts the direction for depolarization, reducing its slope, on application of 50 μM DCCD. In about 100 min the *I-V* curve reaches a steady state, which corresponds to that (i_d-*V* curve) of the passive diffusion channel. Only a limited range (from about −280 to −150 mV) of the *I-V* curves (full lines) was used for the analysis. pH_o = 7. Temperature, 20°C. If we include a larger voltage span, some qualitative changes, such as breakdown for a large hyperpolarization and excitation for a larger depolarization, may be included during each voltage span. This will cause an unnecessary complexion in the analysis

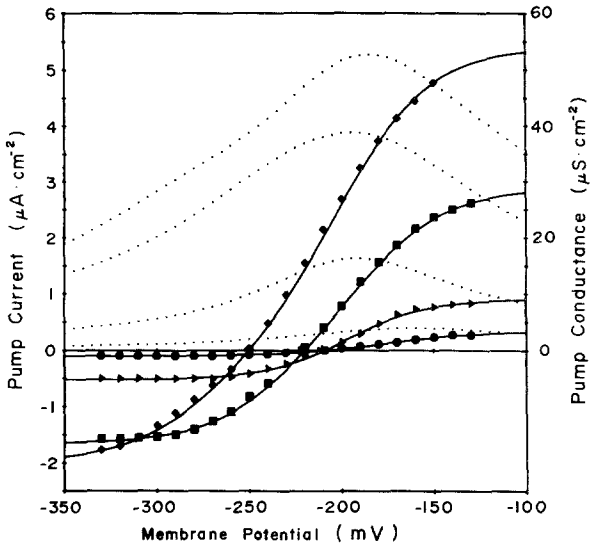

Fig. 4. Changes of i_p-*V* curve of the electrogenic pump channel during inhibition by 50 μM DCCD. The i_p-*V* curves were obtained by subtracting the i_d curve from each *I-V* curve in Fig. 3 and are shown here with symbols. (♦), before inhibition; (■), 14 min; (▶), 60 min; (●), 78 min after application of DCCD. These are of sigmoidal shape and could be simulated with Eq. (15) satisfactorily (full lines). The chord conductance of the electrogenic pump is voltage dependent. Its values along each i_p-*V* curve were calculated with Eq. (18) and are shown by dotted lines

about −130 mV, which is close to E_d of the passive diffusion channel. We adopted this *I-V* curve as the i_d-*V* curve of the passive diffusion channel.

Decay of i_p-*V* Curve during DCCD Poisoning

Once the i_d-*V* curve of the passive diffusion channel was known, the i_p-*V* curve of the electrogenic pump channel could be obtained by subtracting i_d-*V* curve from each *I-V* curve. The i_p-*V* curve of the pump channel decayed as shown in Fig. 4. Simulations of the data (symbols) with Eq. (15) (full lines) gave satisfactory results. The emf of the pump channel, E_p, shifted toward depolarization by about 50 mV. The chord conductance, g_p, of the pump channel, which was calculated with Eq. (18), decreased markedly with the progress of DCCD poisoning (dotted lines in Fig. 4). Such a marked decrease of g_p is an essential point of inhibition of the electrogenic pump.

Changes of g_p, i_p and E_p during Depolarization of E toward E_d

Once i_p and g_p are known as functions of voltage during DCCD poisoning (Fig. 4), it is possible to evaluate g_p and i_p at each level of the resting membrane potential where total $I = 0$. E_p is the voltage at which $i_p = 0$ in a series of i_p-*V* curves shown in Fig. 4. Changes of these values during DCCD poisoning are plotted in Fig. 5. It is evident that g_p and i_p decreased to almost zero in about 100 min after application of 50 μM DCCD, while E_p was depolarized by only 50 mV. The essential point of inhibition of the pump is the marked decrease of g_p and not the depolarization of E_p.

Changes of Kinetic Parameters during DCCD Poisoning

The procedure of simulation of each i_p-*V* curve with Eq. (15) gave values of A_1, A_2, A_3 and A_4. From these values kinetic parameters in Fig. 2 could be evaluated with Eq. (16). For simplicity, we assumed that K_2 in Fig. 2 remained unchanged during DCCD poisoning. This assumption may not invalidate our results, as will be described later. The results are shown in Fig. 6.

What is important in the inhibition of the electrogenic pump is that $K_1(=\alpha k_{34}^{\beta})$ increased by DCCD poisoning, while β or $K_2\beta$ decreased markedly. In other words, incorporation of $m\mathrm{H}^+$ and the transi-

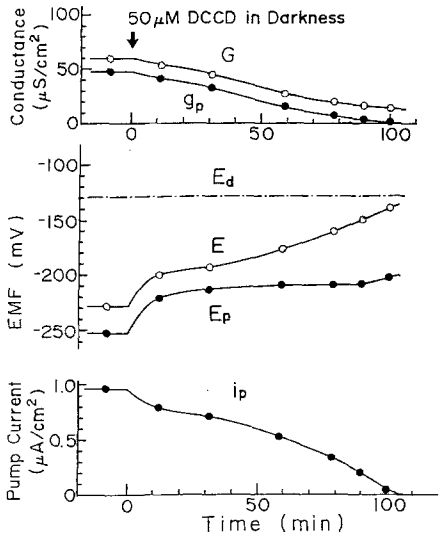

Fig. 5. Characteristics of inhibition of the electrogenic pump of the *Chara* membrane are the depolarization of the membrane potential E and the decrease of membrane conductance G. These are caused mainly by the decrease of the pump conductance g_p. The pump current i_p calculated by Eq. (6), which is another index of the electrogenic activity, reduced to almost zero by inhibition. The pump emf, E_p, was depolarized by about 50 mV, which corresponds to the decrease of internal ATP level (and increases of ADP and P_i levels)

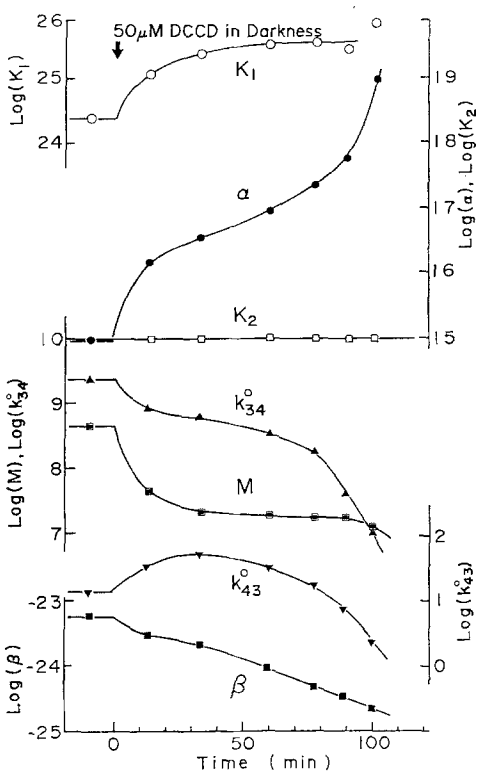

Fig. 6. Changes of kinetic parameters (shown in Fig. 2) during inhibition of the electrogenic pump by 50 μM DCCD. K_1, which is equal to αk^o_{34}, increases, while β or $K_2\beta$ decreases markedly during inhibition. The decrease of M reflects the decrease of internal ATP level (and increases of ADP and P_i levels)

tion $E_2 \rightleftharpoons E_3 \rightleftharpoons E_4$ were accelerated, while release of $m\mathrm{H}^+$ and the transition $E_4 \rightleftharpoons E_5 \rightleftharpoons E_1$ were markedly suppressed. The decrease of $M(=\varepsilon[\mathrm{ATP}]/[\mathrm{ADP}][\mathrm{P_i}])$ corresponds to the decrease of internal ATP-level during DCCD treatment (Takeuchi & Kishimoto, 1983).

Changes of Fractions of Enzyme Intermediates $[E_j]$

We assumed that the pump current is proportional to $E_5 - E_1$. According to our simulation E_5 and E_1 are both small quantities, compared with others, even before DCCD poisoning (Fig. 7). In other words, the step $E_4 \rightleftharpoons E_5$ releasing $m\mathrm{H}^+$ is a rate-limiting process in the active H^+ extrusion mechanism. Both E_5 and E_1, and hence $E_5 - E_1$, were markedly decreased by DCCD. It is worth noting that E_2 and E_3 also decreased markedly. Only E_4 increased so that it may occupy 100% of the enzyme state. This indicates that the enzyme can make a transition $E_3 \rightleftharpoons E_4$ incorporating $m\mathrm{H}^+$ with the progress of DCCD poisoning, while it is gradually frozen at the E_4 state without proceeding further.

Discussion

The effects of 50 μM DCCD on the electrogenic pump of *Chara* membrane progressed slowly at pH 7. Both g_p and i_p decreased to almost zero in about 100 min. E_p was depolarized from −250 to −200 mV (Fig. 5). DCCD solution was perfused externally at a speed of about 1 liter/hr, and therefore the supply of DCCD molecules were enough to block the electrogenic pump of the plasmalemma.

It is worth noting that β is the smallest of the five rate constants. This means a transition $E_4 \rightarrow E_5$ releasing $m\mathrm{H}^+$ or actual extrusion of H^+ is the rate-limiting step. Kinetic parameters, i.e., α, k_{34}, k_{43}, K_1 and β changed slowly until about 90 min, and changed rapidly later (Fig. 6). Fractions of enzyme intermediates except for E_4 decreased rapidly after about 90 min. At these times the fraction of E_4 reached almost 100% (Fig. 7). In other words, the rate constants for the incorporation of H^+ and transitions $E_2 \rightarrow E_3 \rightarrow E_4$ increased to some extent,

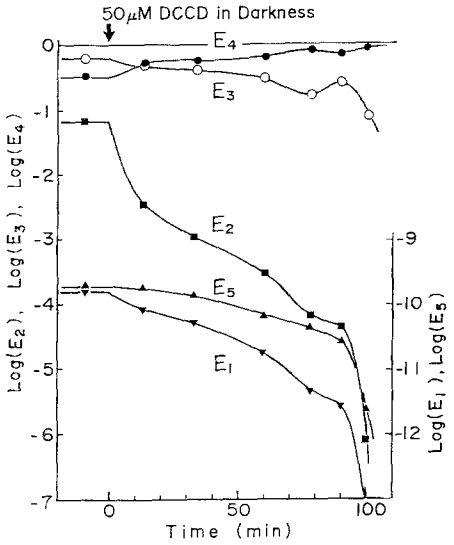

Fig. 7. Changes of fraction of each enzyme intermediate state during inhibition of the electrogenic pump by 50 μM DCCD. All states of the enzyme except E_4 decrease markedly with the progress of inhibition

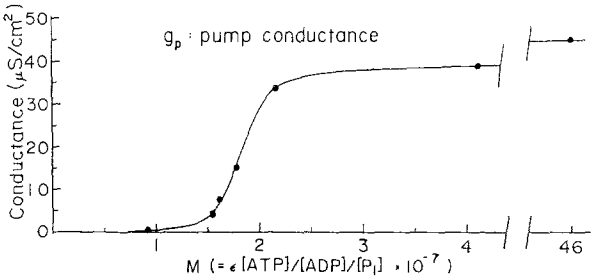

Fig. 8. The conductance of the pump channel, g_p, is plotted against M (= ε [ATP]/[ADP]/[P_i]). Note a marked change of g_p at M of about 1.5×10^7. This corresponds to [ATP] = 400 μM. *See* text for further details

while rate constants for releasing H^+ (or translocation of H^+) decreased markedly. Only the E_4 state remains among intermediate states at late stages of inhibition. The smaller the concentration of DCCD, the slower were the changes of kinetic parameters (data not shown). It is worth noting that the effect of DCCD was irreversible. If we applied a more concentrated DCCD solution (>200 μM), we noticed that the period of steady inhibited state was very short and that the passive channel was also affected. That is, the membrane became leaky and was depolarized to less than −70 mV.

Takeuchi and Kishimoto (1983) reported that the ATP-level of the cytosol of *C. corallina* was about 700 μM at the beginning, 400 μM at 70 min, and 300 μM at 90 min or later after application of 50 μM DCCD in the dark. Our present data in Figs. 4 and 5 also show that inhibition of the electrogenic pump is not complete 70 min after application of 50 μM DCCD, while it is almost complete after 90 min. In this sense g_p seems to be correlated with the ATP-level of the cytosol. If we plot the g_p against M(= ε[ATP]/[ADP]/[P_i]) or [ATP], we obtain a sigmoidal curve (Fig. 8). A similar curve was reported by Spanswick (1980). He suggested an allosteric site on the ATPase associated with the electrogenic pump, which seems quite probable. However, we need to be careful before concluding this. Shimmen and Tazawa (1977), Mimura, Shimmen and Tazawa (1983) showed on the internally perfused tonoplast-less *C. australis* and *Nitellopsis* that the plasmalemma potential and plasmalemma conductance were practically unchanged for a decrease of internal ATP-level from 1000 to 100 μM. Similar data were also found by Smith and Walker (1981). The ATP-level of *Chara* internode did not change appreciably for about 2 hr after being kept in the dark (Keifer & Spanswick, 1979; Takeuchi & Kishimoto, 1983), while g_p reduced to almost half (*unpublished*). On the other hand, in the tonoplast-less perfused internode g_p of the plasmalemma is generally somewhat lower than the normal one. This g_p was about 30 μS cm^{-2} at external pH of 7, even if the ATP-level of the perfusate was as small as 70 μM (*unpublished*). Therefore, we must conclude that DCCD blocks the electrogenic pump of the plasmalemma directly, as well as reducing the internal ATP level caused by its blocking effect on mitochondria and chloroplasts.

In the present analysis we simply assumed that only one step, i.e., $E_3 \rightleftharpoons E_4$ is charge carrying and thus voltage dependent. If we assume another step, i.e. $E_5 \rightleftharpoons E_1$, is also a charge-carrying one, then the i_p-V curve deforms from a simple sigmoidal curve (data not shown).

The H^+ that is extruded by the electrogenic pump must return to the inside of the internode. It is not likely that H^+ returns through a diffusion channel, since g_H of the diffusion channel is negligibly small compared with g_K, g_{Cl}, etc., at least between pH 6 and 8 (Kishimoto et al., 1981*a*). For this, a carrier system like cotransport or countertransport is possibly working. If this is an electroneutral one, then the i_p-V curve obtained, as in our experiments, should not deviate from a simple sigmoidal shape. In our present conditions, i.e., pH = 7 and 20°C, the i_p-V curves were in general sigmoidal and could be simulated with the $2H^+$/1ATP pump. This suggests that the possible cotransport or countertransport

system may be an electroneutral one. On the other hand, with regard to the mechanism of Cl^- entry into the characean internode Cl^-/Cl^- exchange diffusion, Cl^-/H^+ cotransport and Cl^-/OH^- countertransport have been speculated (Hope & Walker, 1975). Recently, Smith and Walker (1976), Beilby and Walker (1981), Sanders (1980), and Sanders and Hansen (1981) suggested that in *Chara* the transport system for Cl^- is not electroneutral, but probably is $2H^+/1Cl^-$ type. On the other hand, Lucas, Keifer and Sanders (1983) suggested a possibility of HCO_3^-/H^+ cotransport. We noticed that i_p-V curve could not be simulated simply with a $2H^+/1ATP$ H^+-pump alone when the external pH was lower than 6 or higher than 8. We need a careful analysis of the pH dependence of the i_p-V curve.

Previously Kishimoto et al. (1980, 1981*a*) reported that E_p of the *Chara* membrane showed a transient hyperpolarization during inhibition of the electrogenic pump with 2 μM TPC in the dark, while the decrease of g_p was monotonic. This was confirmed again during the present experiments (data not shown). However, as shown in this report, such a transient hyperpolarization of E_p was not appreciable during inhibition with DCCD. The difference in the mechanism of inhibition between these two inhibitors will be reported elsewhere.

Localized formations of acid and alkaline zones over the surface of characean internode have been noted and the physiological relations have been discussed (Lucas & Smith, 1973; Walker & Smith, 1977). Deposits of $CaCO_3$ on the cell wall surface at the alkaline zone becomes notable in the internode of an old culture under a strong illumination. The electrophysiological behavior of the *Chara* membrane of the alkaline region is certainly different qualitatively from that of the acid region. If formation of such an alkaline zone is apparent, we can exclude this zone from the measuring area (ca. 6 mm in length). We have a culture of *Chara* that shows apparently no sign of such a zone formation. In practice we selected young internodes that had clean surfaces and showed no sign of apparent zone formation. However, a careful analysis of the I-V curve of the plasmalemma in the isolated alkaline band region remains to be performed.

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