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EFFECT OF DICYCLOHEXYLCARBODIIMIDE ON THE PROTON CONDUCTANCE OF THYLAKOID MEMBRANES IN INTACT CHLOROPLAST

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Summary

The effects of dicyclohexylcarbodiimide, a potent inhibitor of chloroplast ATPase, on the light-induced electric potential changes in intact chloroplasts of *Peperomia metallica* and of a hornwort *Anthoceros* sp. were investigated by means of glass microcapillary electrodes. The characteristics of potential changes induced by flashes or continuous light in chloroplasts of both species are similar except for the phase of potential rise in continuous light, which is clearly biphasic in *Anthoceros* chloroplasts. Dicyclohexylcarbodiimide at concentration $5 \cdot 10^{-5}$ M completely abolishes the transient potential undershoot in the light-off reaction but has little effect on the peak value of the photoelectric response. The membrane conductance in the light and in the dark was tested by measuring the decay kinetics of flash-generated potential in dark-adapted and preilluminated chloroplasts. In the absence of dicyclohexylcarbodiimide, preillumination causes a significant acceleration of the potential decay. The light-induced changes in the decay kinetics of flash-induced responses were abolished in the presence of dicyclohexylcarbodiimide, whereas the rate of potential decay in dark-adapted chloroplasts was not altered by dicyclohexylcarbodiimide. The results are consistent with the notion that dicyclohexylcarbodiimide diminishes H^+ conductance of energized thylakoid membranes by interacting with the H^+ channel of ATPase. The occurrence of a lag (approx. 300 ms) on the plot of potential undershoot (diffusion potential) versus illumination time might suggest the increase in H^+ permeability coefficient of thylakoid membrane during illumination.

Abbreviation: DCCD, dicyclohexylcarbodiimide.

Introduction

The energy conversion in chloroplast membrane is closely related to a generation of the electric potential difference and the ion transport across the thylakoid membrane [1—3]. It is becoming increasingly evident that the electric potential difference across the thylakoid membrane, which is maintained in continuous light, is composed of two components. One of these components is a potential generated directly by electrogenic proton pumping and the other is a diffusion potential, which is created during illumination in conjunction with the redistribution of H^+, K^+ and other ions between stroma and thylakoids. When the light is switched off the electrogenic H^+ transport ceases and the diffusion potential is temporary established across the thylakoid membrane [3—6]. The subsequent dark decay of diffusion potential occurs concomitantly with the dissipation of ion gradients. The membrane potential recordings with microelectrodes seem to be useful for measuring the light-induced diffusion potential in chloroplasts. It was shown that the polarity of the diffusion potential in *Peperomia metallica* chloroplasts depends on the membrane permeability for H^+ and other ions [4,6]. The positive diffusion potential, which is found to arise during illumination of valinomycin-treated chloroplasts in low K^+ medium, seems to be determined by the diffusion of K^+ from the stroma into the thylakoid space [4]. It was suggested by Vredenberg and Tonk [7] that the potential undershoot which is seen in the light-off electric reaction of *P. metallica* chloroplasts after long illumination in the absence of ionophores, is a negative diffusion potential associated with a diffusion of protons from the acidified thylakoid space into the stroma of a chloroplast. This interpretation relies on the assumption that the contribution of H^+ conductance to the total conductance of the thylakoid membrane increases greatly during illumination. On the other hand, it predicts that the development of the negative diffusion potential must be related to the kinetics of building up a pH gradient. In order to verify the above interpretation it is desirable to measure the time course of the diffusion potential formation as well as to assess the contribution of H^+ conductance to the total membrane conductance of illuminated thylakoids. It has been shown that the conductance of chloroplast membranes can be estimated from the decay kinetics of membrane potential after a single flash [8].

In the present study, membrane potential recordings with microelectrodes were used both for measuring the diffusion potential and for testing the membrane conductance in the light and in the dark. It is shown that the potential undershoot in the light-off electric reaction is not only attributed to *P. metallica* chloroplasts but is also exhibited in chloroplasts of a moss *Anthoceros*. The light-induced increase in proton conductance of thylakoid membrane in *Anthoceros* chloroplasts is demonstrated and the time course of the diffusion potential is measured. It is also shown that dicyclohexylcarbodiimide (DCCD), an inhibitor of chloroplast ATPase, eliminates the potential undershoot as well as the light-induced changes of membrane conductance both in *Anthoceros* and *P. metallica* chloroplasts. The results are consistent with the concept that DCCD acts as a potent inhibitor of passive H^+ conductance in energized intact chloroplast.

Materials and Methods

Electric potential measurements were performed on single chloroplasts of the higher plant *P. metallica* and of the hornwort *Anthoceros* sp. Plants of *P. metallica* were cultivated as described before [9]. Plants of *Anthoceros* were collected from their natural habitat together with the adherent layer of soil and were kept under dim sunlight in petri dishes. Sections of *Anthoceros* plants were prepared in a way similar to that one used for preparation of leaf sections of *P. metallica* [10]. The slices were placed into the experimental solution containing KCl, NaCl and $CaCl_2$ at concentrations 0.1, 1.0 and 0.5 mM, respectively (pH 7.0). Chloroplasts in situ were impaled for electric potential measurements with glass microcapillary electrodes as described before [10,11]. Microelectrodes filled with 1 M choline chloride solution were used [8]. The microelectrode was connected to the electrometer with input resistance of $10^{10}\ \Omega$ and output was displayed on both oscilloscope and a pen recorder. The time resolution of the measuring circuit was approx. 1 ms. Chloroplasts were illuminated with a saturating white light either from an incandescent lamp or from an electronic flash (half-width 2 ms). The incident light beam was focussed through the microscope lense system on to a small area of leaf preparation (illuminated area of about 0.01 mm^2).

Results

Fig. 1 shows the light-induced potential changes of the chloroplast (granum stack) of *Anthoceros*. The peak value of the potential (+50—60 mV) and the steady level in the light (approx. +10 mV) are about the same as those in chloroplasts of *P. metallica*. The kinetics of the light-induced potential changes

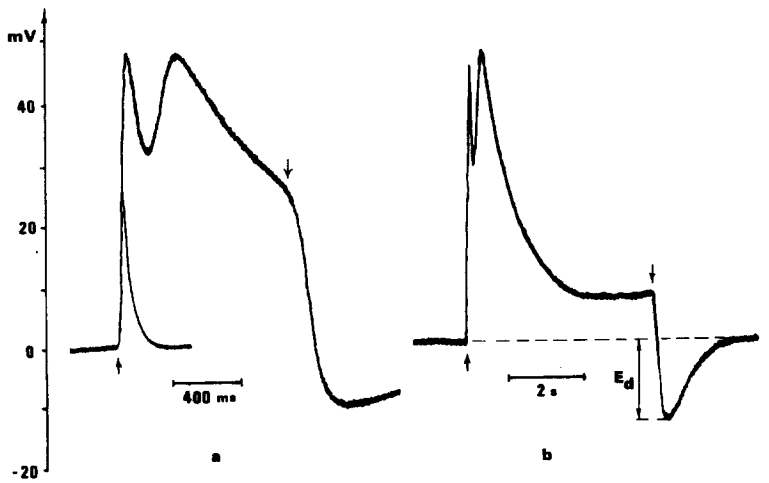

Fig. 1. Light-induced changes in membrane potential of *Anthoceros* chloroplasts. (a) The electric responses induced by a saturating 2 ms flash and by a saturating light pulse of 1 s duration; (b) The photoresponse of the same chloroplast in a different time scale. Upward and downward arrows mark the beginning and end, respectively, of illumination. The diffusion potential which is established on darkening is denoted by E_d.

is also very similar to that found in *P. metallica* except for the phase of potential rise. It is frequently observed that the potential rise is biphasic and has a transient minimum which is achieved in about 100—150 ms after beginning of illumination. In some chloroplasts these two phases were separated less distinctly and only one peak of the potential was seen. The delayed component of the potential rise is found to be more susceptible to inhibition by electron transport inhibitors (3-(3,4-dichlorophenyl)-1,1-dimethylurea and dibromothymoquinone) than the fast initial phase.

There is a transient undershoot in the rapid light-off reaction. In accord with the previous interpretation [2,4], this undershoot is believed to be associated with the diffusion potential created across the thylakoid membrane during illumination. In Fig. 2 the extent of the potential undershoot is plotted as a function of illumination time. The undershoot is not seen after light pulses of duration shorter than 300 ms but develops at longer periods of illumination. The maximal amplitude of the potential undershoot (of about 20 mV) is achieved within 3 s of illumination.

The addition of DCCD, an inhibitor of chloroplast ATPase, into the external medium up to a concentration of 50 μM brought about within a few minutes a complete inhibition of the potential undershoot in the light-off reaction (Fig. 2), although the extent of light-induced potential changes was not essentially altered. The rate of potential decay in the light is comparatively slower in chloroplasts treated with DCCD than in untreated chloroplasts. The effects of DCCD on the light-induced potential changes in chloroplasts of *Anthoceros* and *P. metallica* were similar (Fig. 3). In *Anthoceros* chloroplasts treated with DCCD the two components of the potential rise were not clearly separated and routinely appeared as a single peak. In the presence of DCCD, switching off the light is usually accompanied by a rapid return of the potential to the initial dark level. However, in some cases (on an average, in two impalements out of ten), in the presence of DCCD a positive after-potential (diffusion potential)

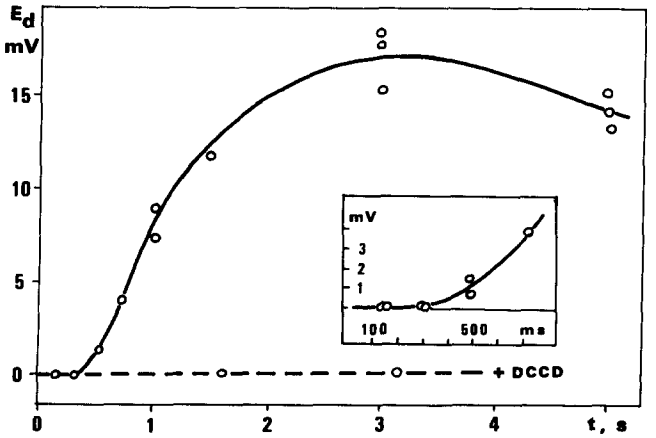

Fig. 2. The dependence of the potential undershoot E_d on illumination time in the absence and presence of 50 μM DCCD; data for the plot were obtained on one chloroplast of *Anthoceros*. The insert in the figure shows the initial part of the plot of E_d vs. illumination time.

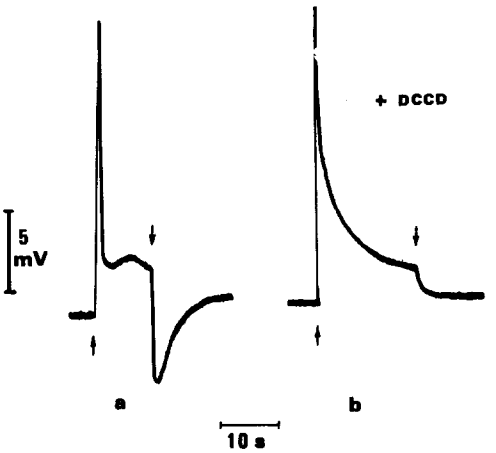

Fig. 3. Light-induced changes of the electric potential in *P. metallica* chloroplast before (a) and after addition of 50 μM DCCD (b).

with a very slow dark decay was observed. The photoresponses with a slow dark decay are believed to be characteristic for spontaneously swelled chloroplasts (cf. Ref. 9).

In order to test the effect of DCCD on the membrane conductance, the decay kinetics of the flash-induced response were measured on dark-adapted and preilluminated chloroplasts. The potential decay after a flash in *Anthoceros* chloroplasts as well as in chloroplasts of *P. metallica* is described by a single exponential with a time constant τ, where τ is determined from the

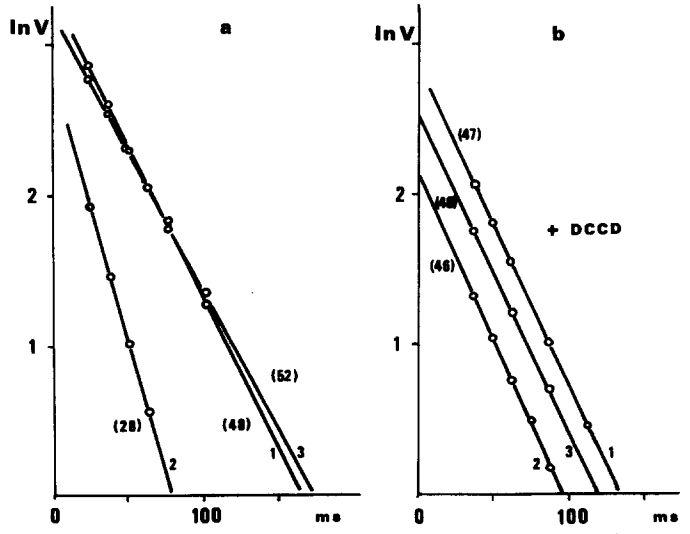

Fig. 4. Semilogarithmic plots of the decay of the flash-induced membrane potential in dark-adapted and preilluminated *Anthoceros* chloroplast under control conditions (a) and after addition of 50 μM DCCD (b). The flashes were given after dark period of 3 min (1), and 100 ms (2) and 10 s (3), respectively, after 5 s preillumination period with saturating white light. The figure in the brackets along the lines are the respective time constants τ of the potential decay in milliseconds.

relation $\ln V(t+\tau) = \ln V(t) - 1$ [8]. It was found that the dark decay of the flash-induced response is considerably accelerated after preillumination of the chloroplast (Fig. 4). As shown in Fig. 4a, the time constant of the potential decay in *Anthoceros* chloroplast decreases to one-half after 5 s preillumination with respect to that estimated in dark-adapted chloroplasts. Such dependency of the potential decay on preillumination resembles those found for chloroplasts of *P. metallica* [8]. The accelerating effect of preillumination on the rate of the potential decay suggests that the membrane conductance increases during illumination (see Ref. 8). DCCD at concentration 50 μM has no noticeable effect on the time constant τ of the potential decay in dark-adapted chloroplasts (Fig. 4). However, in the presence of DCCD the decay of the flash-induced potential occurs with the same kinetics both in dark-adapted and preilluminated chloroplasts (Fig. 4b).

Discussion

The present results show that giant chloroplasts of the hornwort *Anthoceros* can be succesfully used for measuring the photoelectric potential across the thylakoid membrane (cf. Ref. 12). The photoelectric responses in chloroplasts of *Anthoceros* and of the higher plant *P. metallica* are similar, though some subtle differences have been observed. The potential rise in *Anthoceros* chloroplasts consists of two distinct phases, the second of which is not evident in *P. metallica* chloroplasts under usual conditions but becomes evident after certain treatments (e.g. in the presence of phenazinemethosulfate [11]). The potential transients, that occur in a time range of 300 ms from the beginning of illumination, have not been studied in detail and their origin is not yet understood. These potential transients could be determined either by the change in the rate of charge separations (i.e. the rate of electron flow) or by alterations in membrane conductance. However, no appreciable diffusion potential is generated within the time range of 300 ms (Fig. 2).

The potential undershoot, which is seen in the light-off reaction after long illumination, has been interpreted as the diffusion potential determined mainly by permeation of H^+ and K^+ through the thylakoid membrane [4,6]. Under the assumption of constant electric field the diffusion potential is defined by the Goldman equation which is given in Ref. 2 (Eqn. 8b) in the specific form suitable for the thylakoid membrane. The fluxes of Cl^- across the thylakoid membrane are large in broken chloroplasts but seem to be unsignificant within the intact chloroplast [13]. Therefore the diffusion potential is given by

$$E_d = \frac{RT}{F} \ln \frac{P_K c_K^o}{P_K c_K^i + P_H c_H^i} \qquad (1)$$

where c_K^o is the concentration of K^+ in stroma, c_K^i and c_H^i are the concentrations of K^+ and H^+ inside the thylakoid, P_K and P_H are membrane permeability coefficients for K^+ and H^+, respectively, and RT/F is 25 mV. The term $P_H c_H^o$ in the numerator of the above relation is neglected, since the concentration of protons in the chloroplast stroma (pH $\approx$ 8 [3,13]) is too low as compared to the concentration of K^+ ($c_K^o \approx 10^7 \cdot c_H^o$). The concentrations of K^+ in thylakoids and stroma (approx. 0.1 M) do not change appreciably during illumination

under physiological conditions [6], whereas the internal H^+ concentration increases by three orders of magnitude (e.g. Ref. 3). It is expected therefore, that the term $P_H c_H^i$ in Eqn. 1 is negligible with respect to the term $P_K c_K^i$ in the dark but becomes of comparable size with $P_K c_K^i$ in the light, thus giving rise to a negative diffusion potential. The potential undershoot in the light-off reaction is selectively abolished in the presence of DCCD, the agent which is reported to reduce H^+ permeability of isolated thylakoids and of some reconstituted liposomal systems [14—16]. The inhibition of the potential undershoot by DCCD (Figs. 2 and 3) is consistent with the assumed mode of action of this agent and supports the suggestion that the potential undershoot reflects the diffusion potential associated with the proton gradient. As can be seen from Eqn. 1, the reduction of passive H^+ permeability (P_H) after addition of DCCD could account for the abolishment of the diffusion potential, even though a pH gradient would not be eliminated by DCCD.

Calculations of membrane conductance (g) according to the formula $g = C_m/\tau$ [8], where the membrane capacity C_m is 10^{-6} F/cm^2 and the time constants τ of the flash-induced potential are 50 ms and 25 ms for dark-adapted and preilluminated *Anthoceros* chloroplasts, respectively (Fig. 4), yield the values of 20 $\mu\Omega^{-1}$/cm^2 for dark conditions and 40 $\mu\Omega^{-1}$/cm^2 for steady light conditions. The light-induced increase in membrane conductance is thought to be associated with a 1000-fold increase in H^+ concentration inside the thylakoid. It was revealed that DCCD has no effect on the membrane conductance in the dark but eliminates completely the light-induced increase in membrane conductance (fig. 4). These results exclude possible effect of DCCD on permeability of the non-energized thylykoid membrane for the main conducting ions and indicate that the increase in membrane conductance in the light is totally due to the DCCD-sensitive H^+ conductance. The light-dependent H^+ conductance, according to our estimates, is approx. 20 $\mu\Omega^{-1}$/cm^2, which is somewhat lower than the values of 40—80 $\mu\Omega^{-1}$/cm^2 calculated on the basis of the different experimental approach for broken lettuce chloroplasts [15].

Independent estimates of H^+ permeability and H^+ conductance can be obtained from the characteristics of the light-off response after long illumination. Subtituting into Eqn. 1 $E_d = -20$ mV (Fig. 1), $c_K^o = c_K^i = 0.1$ M, $c_H^i = 10^{-4}$ M and $P_K = 4 \cdot 10^{-8}$ cm/s [2,7,8], we get that P_H is approx. $5 \cdot 10^{-5}$ cm/s. By definition, the proton conductance of the membrane g_H is (e.g. Ref. 2):

$$g_H = F \frac{d\varphi}{dE}$$

where φ is a passive flux of protons and E is a membrane potential. In continuous light, i.e. under conditions when $c_H^i >> c_H^o$ and $|E| \leqslant 25$ mV, the passive H^+ conductance is given by [2]:

$$g_H \approx \frac{F^2}{2RT} P_H c_H^i \qquad (2)$$

The H^+ conductance in the light, estimated according to Eqn. 2, is approx. 10 $\mu\Omega^{-1}$/cm^2. Thus two independent methods of calculation of passive H^+ conductance (from the kinetics of the electric responses induced either by flash or continuous light) yield the values of g_H which are in a reasonably good agree-

ment with each other. Similar calculations show that in the presence of DCCD the H^+ conductance and the H^+ permeability coefficient of chloroplast membranes are diminished by at least an order of magnitude. DCCD is supposed to interact with the counterpart of coupling factor CF_1 in the ATPase complex and thereby to block the H^+ channel of chloroplast ATPase [14]. Inhibition of the potential undershoot and of the light-induced changes in membrane conductance after addition of DCCD suggests that, at least under saturating illumination, the diffusion of protons from the thylakoid interior occurs mainly through the H^+ channel of the ATPase complex. This conclusion seems to be justified with respect to the free protons which are not bound to buffering groups at the inner surface of the thylakoid membrane [5,17]. The efflux of protons that are bound within the membrane during illumination may occur both through the ATPase complex and unspecific leakage channels.

There is a lag on the plot of the potential undershoot as a function of illumination time (Fig. 2). Since the lag period, required to create a measurable diffusion potential, is rather long (of about 300 ms) we have to assume either relatively slow initial increase in the internal H^+ concentration (due to a high buffering capacity of thylakoid interior [18]) or a substantial increase in H^+ permeability coefficient during illumination. The assumption of the delayed increase in the internal H^+ concentration is in contradiction with the fast absorption changes of the pH_{in}-indicating dye neutral red [3,17], but it would be consistent with the observations [19] that high concentrations of permeant buffers, which are sufficient to cause a lag in the acidification of the thylakoid volume by 0.3—0.5 s, were without effect on photophosphorylation. The alternative explanation (the increase in P_H during illumination) seems to be more probable. It is in line with the results of Schönfeld and Neumann [15], who showed that the H^+ conductance of thylakoid membrane in broken chloroplasts becomes 400 times higher when the pH gradient increases from 2 up to 3 pH units. Such a great increase in H^+ conductance could not be a trivial consequence of 10-fold increase in the internal H^+ concentration. Therefore, it seems probable that the sharp increase in H^+ conductance, observed in Ref. 15, is determined by the increase in permeability coefficient P_H. Such an increase in H^+ permeability might be a consequence of the conformational changes of chloroplast coupling factor, which are believed to be induced when the proton gradient or the electric potential difference exceeds a certain threshold [15,20,21]. The effect of conformational changes of a chloroplast coupling factor on the kinetics of the electric potential deserves further study.

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