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ON THE STEADY-STATE ELECTRICAL POTENTIAL DIFFERENCE ACROSS THE THYLAKOID MEMBRANES OF CHLOROPLASTS IN ILLUMINATED PLANT CELLS

W. J. VREDENBERG and W. J. M. TONK

Center for Plant Physiological Research, Wageningen (The Netherlands)

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SUMMARY

The potential difference across the thylakoid membranes under steady-state saturating light conditions, measured with microcapillary glass electrodes, was found to be small as compared to the potential initially generated at the onset of illumination. This result is discussed to be in agreement with quantitative estimates on the approximate magnitudes of the potential generating electron flux through the photosynthetic electron transport chain and of the potential dissipating ion fluxes across the thylakoid membrane under steady-state conditions. It is concluded that a pH gradient of approx. 3–3.4 units is built up in the light across the membrane. The negative diffusion potential associated with this gradient is suggested to cause the transient negative potential observed in the dark after illumination.

INTRODUCTION

The electrical potential difference (V) across the thylakoid membrane is the electrical component of the proton motive force, which, in terms of the chemi-osmotic coupling mechanism [1], drives the synthesis of ATP in the chloroplasts. It is well established that a pH gradient which represents the chemical component of the proton motive force, is built up upon energization of chloroplasts [2–4]. The relative magnitudes of the electrical and chemical components under physiological conditions are not known with certainty. Several biochemical studies have revealed that, at least in isolated chloroplasts, the pH gradient is the major component of the proton motive force [4–6]. From indirect spectroscopic analyses it has been concluded [7, 8] that under steady-state conditions in saturating light the potential across the thylakoid membranes of *Chlorella* cells is of the order of 100 mV.

In this paper we will describe some characteristics of light-induced electrical signals, measured in intact chloroplasts of *Peperomia metallica*, which likely are of the potential across the thylakoid membranes. The results indicate that the potential

Abbreviations used: DCMU, 3,4-dichlorophenyl-*N*,*N*-dimethyl urea; $DCPIPH_2$, reduced 2,6-dichlorophenol indophenol.

under steady-state conditions in saturating light is considerably lower than the potential initially generated at the onset of illumination. A detailed paper has been presented at the recently held third International Congress on Photosynthesis in Rehovoth (Israel) [9].

MATERIALS AND METHODS

Measurements were performed on individual chloroplasts in mesophyll cells of leaf sections of the terrestial plant *P. metallica*. Experimental conditions and methods were about the same as described in previous papers [10, 11]. Potential changes were measured by means of micro-capillary glass electrodes inserted into a single chloroplast. Selected electrodes with a tip diameter of 0.2 to 0.5 μm were used. Information on the internal structure of these chloroplasts, especially with respect to the size and distribution of granum stacks, was obtained from electron micrographs of chloroplast sections. This has led us to the conclusion [9] that microelectrodes of this small size have a much higher probability of hitting and probing a granum stack than of being in the stroma phase, as originally assumed [11]. As an average approx. 20 % of our impalements resulted in a situation in which the light responses, charac-

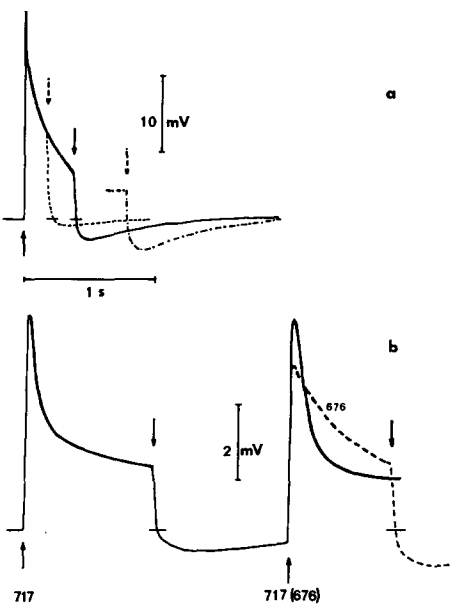

Fig. 1. (a) Potential responses of electrode inserted into a chloroplast of a mesophyll cell of *P. metallica*, in white light flashes of 0.4 (solid trace), 0.2 and 0.8 s (broken traces) duration. The potential change is attributed to the change across the membranes of thylakoids, constituting a granum stack (see text). (b) Potential change of thylakoid membrane in two consecutive 717 nm flashes (solid traces), and in a 717-nm flash (left solid curve) followed by a 676-nm flash (dotted trace). An upward deflection means an increase in potential (inside more positive). Upward and downward pointing arrows mark the beginning and end, respectively, of illumination. Approximate intensities of white, 717 or 676 nm light were 600, 20 and 6 kergs/cm^2 per s, respectively. The horizontal dashes in the light-off traces mark the level of the dark potential, and are given to mark the potential undershoot during the light-off reaction.

terized by distinct phases of the potential changes with specific rate constants (see Discussion) were reproducible and stable during the course of an experiment which lasted 10 to 30 min. These signals have been studied in some detail and are believed to reflect the potential changes of the membranes of an undamaged granum stack.

RESULTS AND INTERPRETATION

Typical potential responses of the thylakoid membrane to saturating white light flashes of different duration are reproduced in Fig. 1a. At the start of the flash a positive potential (cf. ref. 11) is generated (phase 1) which decreases within 0.5 to 0.8 s in continuing light (phase 2) towards a steady state (phase 3). Upon turning off the light a rapid change occurs to a potential level below the original dark state, and which is followed by a slower increase to the steady-state dark potential. The magnitude of the transient negative potential difference in the dark appears to depend on the duration of the light period and, at the intensities used, reaches a constant value when flashes longer than 0.5–0.8 s are applied. We have suggested that this transient potential difference is associated with the proton diffusion potential across the thylakoid membrane (inside negative), built up in the light by the electron transport coupled

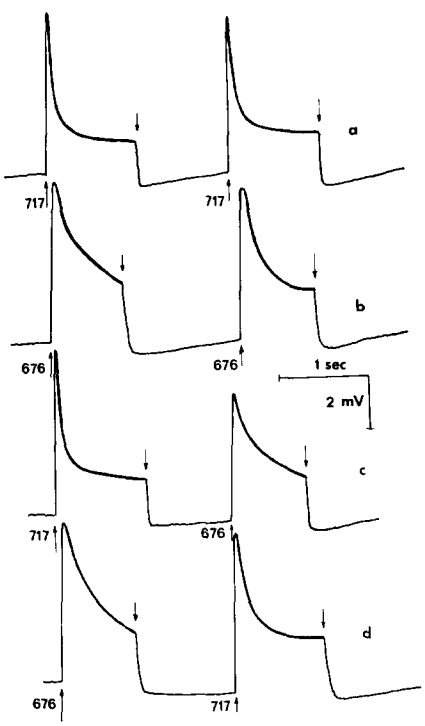

Fig. 2. Potential responses of chloroplast (thylakoid) membrane in two sequential monochromatic flashes. The sequence of wavelength of the flashes was: 717–717 (a), 676–676 (b), 717–676 (c) and 676–717 nm (d). The first flash was given after a dark period of approx. 2 min. Intensities were 20 and 6 kergs/cm^2 per s for 717 and 676 nm flashes, respectively. Further details are in the legend of Fig. 1.

proton movement into the thylakoid [9].

The kinetics of the potential decrease in 676 and 717 nm light during phase 2, and their dependence on a pre-illumination, are shown in Figs 1b and 2a–2d. The results show that the rate of the potential decrease in phase 2 is dependent on the wavelength of actinic light. It is comparatively faster in 717 than in 676 nm light, at least after dark adaptation. Moreover, the rate of the phase 2 change in 676 nm light is affected by pre-illumination with 717 and 676 nm light, 717 nm causing a decrease and 676 nm light an increase in the rate of this change. These observations suggest that the rate of the reaction causing the phase 2 potential change is controlled by the rate of electron transfer in the photosynthetic electron transport chain. The rate of electron transfer from Photosystem II to Photosystem I is known to depend on the redox state of an electron carrier pool, probably plastoquinone, between the two photochemical systems [12–14]. In the dark, or after illumination of Photosystem I, the pool will be in the oxidized state. Under these conditions the state of the pool initially allows a prolonged high turn-over rate of photochemical charge separations in Photosystem II, which will decrease as the pool becomes reduced in the light. Consequently the relatively slow phase 2 change in 676 nm illumination after darkness or 717 nm pre-illumination, as compared to the changes observed after 676 nm pre-illumination, may result from a higher rate of electron transfers through Photosystem II. This conclusion is consistent with the effect of 3,4-dichlorophenyl-*N*-,*N*-dimethyl-urea (DCMU), which is known to inhibit the electron transfer in the chain between the primary electron acceptor Q of Photosystem II and plastoquinone [15]. In the

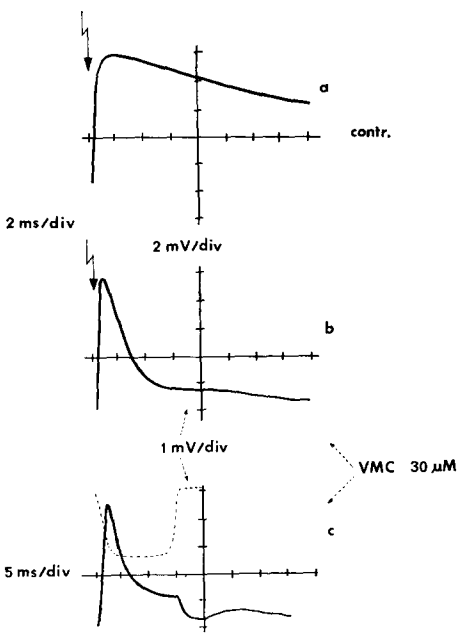

Fig. 3. Oscilloscope display of chloroplast potential change, induced by intense (50 µs halfwidth) Xenon flash (traces a and b), and by a 15-ms white flash (trace c) in the absence (a) and presence (b, c) of 30 µM valinomycin (VMC). The dashed curve in trace c marks the opening and closing of the shutter in the actinic light beam, probed by a photodiode.

presence of DCMU the reaction kinetics of phase 2 were found to be identical in 676 and 717 nm illumination, and were not affected by pre-illumination [9]. An effect of the electron transfer rate through Photosystem I on the phase 2 potential decay in 717 nm light, similar to that discussed above for Photosystem II, has been found in the presence of DCMU and reduced 2,6-dichlorophenolindophenol ($DCPIPH_2$) [9].

The potential decay in the dark after a short saturating flash (Figs 3a and 3b), as well as the phase 2 decay in the light (Fig. 3c and compare with Fig. 1a), are enhanced considerably after the addition of 30 μM valinomycin. This result suggests that the phase 2 decrease in the light is due to a discharge of the potentiated membrane by passive ion fluxes which are driven by the potential generated in the primary charge separating and ion binding acts (phase 1) [11]. The kinetics of the potential changes suggest that during phase 2 the passive ion fluxes are in excess of the electron flux, causing the decay in the potential during this phase in the light.

DISCUSSION

Light-induced potential responses were routinely observed after the impalement of a chloroplast by an electrode. However, only those signals are regarded to reflect the response of undamaged thylakoids in a granum stack, which showed a phase 2 potential decay completed in 0.2 to 0.8 s, and a potential undershoot upon darkening. In most of these situations the signals were found to be stable and reproducible during an appreciable time. In the other, most frequent, cases the phase 2 decay occurred in less than 0.1 s and no, or only a small undershoot was observed. These signals usually were lost within 1 to 2 min. These cases probably refer to situations in which the electrode has caused a partial damage of the membranes and consequently an artificial ionic leak. The fact that in the optimal situations valinomycin was found to cause an increase in the rate of potential decay in the light, as well as in the dark (cf. Fig. 3), strongly suggests that in these situations no artificial leaks were induced by the electrode.

The present results suggest reasonable good evidence that the light-induced potential changes observed are of the thylakoid membrane indeed. The following estimates on the approximate magnitudes of the electron and ion fluxes per single thylakoid are of interest to be considered in this respect. Assuming an average chlorophyll concentration of 25 mM in chloroplasts [16], a disc-shaped thylakoid of $1 \times 1 \times 1.5 \cdot 10^{-2}$ μm in size (volume approx. $1.5 \cdot 10^{-14}$ cm^3 and surface area approx. $2 \cdot 10^{-8}$ cm^2) will contain approx. $2 \cdot 10^5$ chlorophyll molecules. According to the approximate size of the photosynthetic units [17], one thylakoid will be covered by about $4 \cdot 10^2$ units (Photosystem I+Photosystem II). Junge and Witt [18] estimated an approx. number of 200 units per thylakoid. As the transfer time of an electron across the electron transport chain connecting both systems is about 10 ms [7, 19], the steady-state electron flux per thylakoid in saturating light will be approx. $4 \cdot 10^4$ electrons $\cdot s^{-1}$. According to the Goldman flux equation [20], the initial net flux $J(0)$ of a monovalent kation across the membrane, driven by an electric potential $V(0)$ created in the light will be:

$$J(0) = P \cdot V(0) \cdot F/RT[\exp(V(0) \cdot F/RT) - 1]^{-1} \cdot [c_0 - c_i \exp(V(0) \cdot F/RT)].$$

P is the permeability coefficient of the ion in the membrane, c_0 and c_i are the internal (thylakoid inner space) and external (chloroplast stroma phase) concentrations of the ion, and RT/F is 25 mV at 20 °C. Assuming passive equilibrium across the membrane in the dark at $V = 0$, i.e. $c_0 = c_i = c$, the ion flux will be:

$$J(0) = P \cdot V(0) \cdot c \cdot F/RT$$

Estimates on the ionic content of chloroplasts [21, 22] of different plants have indicated that potassium is the most abundant kation, present at a concentration of 100–300 mM. The potassium permeability coefficient has been determined with some precision for the plasmamembrane in Nitella [23], $P_K = 4 \cdot 10^{-8}$ cm $\cdot$ s^{-1}. This value is of the same order of magnitude as the one computed from the kinetics of salt-induced luminescence changes for the thylakoid membrane [24]*. Junge and Witt [18] have calculated that the initial potential generated in the light is about 50 mV. With $c_K = 300$ mM, $P_K = 4 \cdot 10^{-8}$ cm $\cdot$ s^{-1} and $V(0) = 50$ mV, the initial potassium (ef-)flux would be $J_K(0) = 2.4 \cdot 10^{-11}$ mol $\cdot$ cm^{-2} $\cdot$ s^{-1}, or $3.9 \cdot 10^5$ ions $\cdot$ s^{-1} per thylakoid. A constant flux of this size would cause a change in internal potassium concentration of about 30 mM $\cdot$ s^{-1}. It is reasonable to conclude that, according to the flux equation, the potassium flux is linearly dependent on the potential during the first second of illumination at which the concentration change is expected to be less than 10 %. Electron transfer rate from Photosystem II to Photosystem I will have reached a steady state in saturating light in the first second of illumination [3]. Because the initial (membrane-discharging) potassium efflux of $3.9 \cdot 10^5$ ions $\cdot$ s^{-1} is larger than the steady-state (charging) electron flux of $4 \cdot 10^4$ electrons $\cdot$ s^{-1}, the potential across the thylakoid membrane is expected to decrease during the first second of illumination. In the steady state the potassium flux, $J_K(ss)$ $(= c \cdot P \cdot V(ss) \cdot F/RT)$, will balance the electron flux. Thus the steady-state potential $V(ss)$ is about 5 mV. For $c_K = 100$ mM, $J_K(0)$ will be $1.3 \cdot 10^5$ ions $\cdot$ s^{-1} and $V(ss) = 15$ mV. The rate of potential decay from $V(0)$ to $V(ss)$ in the light will be slower at $c_K = 100$ mM. Alternatively $J_K(0)$ will be increased and $V(ss)$ will become smaller, when P_K is increased (e.g. in the presence of valinomycin). The increase in $J_K(0)$ will be reflected by an increase in the rate of the potential decay in the light (cf. Fig. 3), as well as in the dark (cf. ref. 18 and Fig. 3).

According to the model proposed by Grünhagen and Witt [25], the discharging cation efflux is associated with and, except for the sign, is equal to the electrogenic influx of H^+ into the thylakoid inner space. It has been shown for isolated chloroplasts suspended in a (high) salt medium containing 50 mM KCl [3], that the proton influx occurs approximately in a first-order reaction with a rate constant $k \approx 10$ s^{-1}. We may approximate that the number of protons transported electrogenically into the thylakoid during the potential decay phase, and not balanced for by a passive proton efflux, is equal to $(J_H(0) - J_H(ss)) \cdot k^{-1}$ equiv $\cdot$ cm^{-2}. With $J_H(0) = J_K(0) = 2.4 \cdot 10^{-11}$, and $J_H(ss) = J_K(ss) = 2.4 \cdot 10^{-12}$ equiv $\cdot$ cm^{-2} $\cdot$ s^{-1} this number would

* It should be noted that an apparent error has been made in the calculations on the ion fluxes across, and the P values of the thylakoid membrane in ref. 24. On basis of the experimental data and the assumptions made on the thylakoid volume and surface area, given in the referred paper, the correct calculation would yield numbers which are two orders of magnitude higher than those presented. The calculation would give fluxes of the order of 10^{-12} mol $\cdot$ cm^{-2} $\cdot$ s^{-1} and P_K values of about 10^{-8} (in the absence) and 10^{-7} cm $\cdot$ s^{-1} (in the presence of valinomycin).

be equal to $2.16 \cdot 10^{-12}$ equiv $\cdot$ cm^{-2}. Assuming that 99 % of the transported H^+ are neutralized by internal buffering groups, the entry of these protons has caused a change in the inner proton concentration of about 27 μM. With a dark pH of about 7.5–7.9 [26], this would mean that during the potential decay in the light the inner pH has decreased by approx. 3.0–3.4 units. The internal concentrations of K^+ and H^+ will reach a steady-state level which is maintained by a passive efflux of protons through the conducting channels (including the ATPase system), and by a passive influx of K^+ [7]. Other ions may be involved as well [27]. It has been shown for isolated chloroplasts in high salt medium [3] that this steady-state level is reached in approx. 1 s with a pH gradient of 2.6–3.0 units. The steady-state level will be associated with a diffusion potential, the magnitude of which can be measured in the dark immediately after illumination. In the light this diffusion potential will be superimposed on the potential maintained by the electrogenic influx of the protons.

The kinetics of the chloroplast potential changes measured with the electrode (Figs 1–3) during and after illumination are in qualitative agreement with the changes predicted for the thylakoid membrane, as discussed above. Preliminary experiments on the effect of ionophores on the kinetics of the transient negative potential after illumination (cf. Fig. 1a) have given conclusive evidence that the potential undershoot is due to the proton gradient built up in the light. These results will be presented in a forthcoming paper.

The kinetics of the potential changes in 1-s flashes are quite different from those of the 515-nm changes in intact cells and chloroplasts [28], which have been suggested [18] to be a linear indicator of the electrical potential across the thylakoid membrane. However, it must be considered (cf. refs 29 and 30) that there is as yet no experimental proof that the 515-nm absorption changes occur in response to a potential gradient independently from accompanying conformational and configurational changes in the membrane. One might for instance speculate, after comparing the kinetics of the multi-phasic changes in 515 nm absorption in 1-s flashes [28] with the changes in potential and pH gradient predicted by quantitative reasoning, or measured by our method, that the extent of the 515-nm change, associated with the potential, is strongly dependent on the pH, i.e. is increased concomitantly with a decrease in pH of the thylakoid inner space.

The estimates on the approximate electron and ion flux densities in the thylakoid membrane appear to be conclusive with the experimental observation that the steady-state electrical potential (phase 3) across the thylakoid membranes of chloroplasts in vivo is smaller than the (phase 1) potential initially generated at the onset of illumination with saturating light. Usually the steady-state potential was found to be less than 20 % of the initial potential, which under optimal conditions has been measured as high as 50 mV. A steady-state potential in the light of 100 mV, as concluded for *Chlorella* cells [8], would be difficult to reconcile with our estimates on the electron and ion fluxes. Our electrophysiological data indicate that in the intact plant cell the energized state leading to ATP formation is mainly associeted with a pH gradient across the thylakoid membrane.

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