Transcellular $\Delta\bar{\mu}_{H^+}$ in *Valonia ventricosa* and Its Effect on Delayed Fluorescence

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Abstract. The possible effect of the illumination-induced transcellular H^+-gradient between the central vacuole and the external medium, on both the intensity and the kinetics of delayed fluorescence was studied by measuring both the membrane potentials and H^+ fluxes across the plasmatic membranes, and the millisecond component of delayed fluorescence in single cells of the marine alga *Valonia ventricosa*. The kinetics of the formation of transcellular $\Delta\bar{\mu}_{H^+}$ was shown to correlate with the induction kinetics of the millisecond component of delayed fluorescence. Disturbances of transcellular $\Delta\bar{\mu}_{H^+}$ by electrical breakdown of the cell or by acidification of the external medium resulted in a decrease of the steady-state level of the light emission and in a decline of the minute oscillations observed in intact cells. The possible involvement of the membrane connections between the chloroplasts and the plasmatic membranes in the formation of transcellular $\Delta\bar{\mu}_{H^+}$ is discussed.

Key words: Transcellular $\Delta\bar{\mu}_{H^+}$ — Photosynthetic electron transport — Delayed fluorescence — Microelectrodes — *Valonia ventricosa*

Introduction

The transport and distribution of H^+ ions in plant cells have been intensively studied in the past few years in relation to the hypothesis about the involvement of protons in cell electrogenesis and metabolism (Kitasato 1968; Spanswick 1972; Saito and Senda 1973; Vredenberg 1974; Richards and Hope 1974; Raven 1976; Gyenes et al. 1978a, b; 1981). Most plant cells have an acidic cell sap and

Abbreviations used: PD — potential difference; DCCD — dicyclohexylcarbodiimide; $\Delta\bar{\mu}_H$ — proton electrochemical gradient.

their central vacuole serves as a store for proton accumulation during illumination (Gyenes et al. 1978a, b, 1981; Moriyasu et al. 1984; Davis 1974). In this respect, the photosynthetically active giant algal cells, e.g. the spherical cells of the marine alga *Valonia ventricosa*, are of a particular interest since they can be used to study electrical characteristics of their plasmatic membranes and ion fluxes across them with microelectrodes; also, single cells of this species can be used to study photosynthetic activity with fluorimetric methods. In recent works (Gyenes et al. 1978a, b, 1981) carried out on the freshwater alga *Nitellopsis obtusa* and the marine alga *Valonia ventricosa* we studied the photoelectric characteristics of the plasmatic membranes and the light-induced H^+ fluxes across them using glass microelectrodes to measure transmembrane electric potential differences (PD), and antimony microelectrodes to measure vacuolar pH changes and pH of the external medium near the cell surface (pH_v and pH_o, respectively) during intermittent illumination. In these experiments, we could show that illumination of the cells induced accumulation of H^+ ions in the central vacuole and alkalinization of the medium which corresponded to proton transport from the external medium into the vacuole (or to equivalent OH^- transport in opposite direction). With respect to PD and ΔpH ($\Delta\bar{\mu}_{H^+}$) across the tonoplasts of these cells it was concluded that H^+ must be transported into the vacuole actively. However, the onset of the photoelectric response was so fast (within 100 ms) and the kinetics of the light-induced changes in PD, pH_o and pH_v in *Valonia ventricosa* were so similar that a possible involvement of direct membrane connections between the chloroplasts and the plasmatic membranes in the photo-induced proton translocation from the external medium into the central vacuole could not be ruled out either (Gyenes et al. 1981; Gyenes 1985).

Several papers (De Grooth and Van Gorkom 1981; Crofts et al. 1971; Vierke 1980; Arnold and Azzi 1971) have reported that the intensity of delayed fluorescence of the chloroplasts, originating from the recombination of primary separated charges in the reaction center of photosystem II (Lavorel 1975), depends on the electric potential difference and proton concentration gradient across the thylakoid membranes. Assuming the existence of direct membrane connections between the chloroplasts and the plasmatic membranes one could expect that the formation of (and change in) transcellular electrochemical gradient for protons measured between the central vacuole and the external medium may affect the value of $\Delta\bar{\mu}_{H^+}$ across the thylakoid membranes of the chloroplasts and, as a result, the intensity of delayed fluorescence. In the present work, we tried to examine this assumption.

Materials and Methods

Green spherical cells of the marine alga *Valonia ventricosa* with a diameter of 2 mm, grown from aplanospores were used. Young aplanospores were formed spontaneously from the material of the

protoplasm when the maternal coenocyte (collected at the shores of Cuba) was damaged (see Steward 1939). Artificial seawater, containing 3.6 % sea salt was used for cultivation and in the experiments. The pH of the medium in the corresponding experiments was changed with Tris buffer and HCl within a range from 8 to 5.

PD between the central vacuole and the external medium was measured by means of a glass microelectrode inserted into the vacuole and a reference electrode in contact with the external medium through an agar-agar bridge. The time resolution of both the measuring amplifier VA-J-51 and the fast operating recorder was below 10 ms, allowing to observe the initial rapid photoelectric responses. In experiments with light flash the pen recorder was replaced for a storage oscilloscope S8-1. For current injection from an electronic stimulator ESL-2, a second glass microelectrode was inserted into the vacuole allowing to perform resistance measurements as well as to initiate electrical breakdown. The pH of the vacuolar sap was measured using antimony microelectrodes with a diameter of the measuring tip of about 3 μm, constructed and tested in our laboratory (Gyenes et al. 1978a). The same pH-microelectrodes (in separate experiments carried out under control conditions) were used to measure changes in pH of the external medium in the vicinity of the cell surface. During simultaneous measurements of PD and pH_v, the glass microelectrode served as a common reference electrode (Fig. 1.). In the microelectrode measurements, cells were illuminated from an incandescent lamp with an intensity of 2000 lx at the level of the measuring chamber.

The induction kinetics of the millisecond component of delayed fluorescence was recorded in single cells with the use of a conventional rotating cylinder phosphoroscope. The interval between actinic light illumination and luminescence measurement was 1.25 ms. The light from the incandescent lamp was led through a red filter ($\lambda > 620$ nm). The experimental arrangements used for delayed fluorescence measurements in our department were described in a previous paper (Matorin et al. 1978).

Electron microphotographs of cross sections of the Valonia cells were obtained with conventional methods (Roland 1978) in an electron microscope JEM 100B with the kind help of Dr. E. B. Kurkova, Institute of Plant Physiology, Moscow.

Results

Electrical PD measured in the dark between the central vacuole and the external medium (average from 20 cells) was about +40 mV (the vacuole positive — a peculiarity of these cells). On illumination, PD in the dark-adapted cells increased within 10 min up to $+(80 \pm 15)$ mV. The initial phases of the light-induced potential changes were very complex and typical of these cells (Fig. 2.). Within 100 ms after the start of illumination the potential first shifted towards positive values; in 1—2 s the potential shift changed sign and turned negative. Within a few subsequent seconds this negative shift was completed and the potential turned again positive. During the subsequent 2—3 min there was one more oscillation of the potential. The kinetics of the light-induced changes in pH_o measured with a pH microelectrode near the cell surface followed exactly the kinetics of the potential changes (with a delay of about 1 s). Simultaneous measurements of PD and pH_v showed that the kinetics of the H^+ transport into the vacuole also followed the kinetics of the potential changes (Fig. 2).

In special experiments we tried to establish which one of the two plasmatic membranes, and to what extent, contributes to the light-induced PD changes. For this purpose a glass microelectrode was inserted into the cytoplasm (with a motor-driven device, high-precision, 1 μm, constructed in our laboratory). The value and the sign of PD across the plasmalemma served as control for the localization of the microelectrode tip. PD across the plasmalemma was always negative on the inside and its value ranged from $-(30-40)$ mV to $-(70-80)$ mV. It should be noted that (to our surprise), upon stepwise insertion of the microelectrode, PD across the plasmalemma increased in several jumps as if the electrode were penetrating several membranes. At the last step, the potential reversed instantaneously from $-(70-80)$ mV to $+(30-40)$ mV; this might correspond to the penetration of the tonoplast. Further insertion of the microelectrode was associated with no more potential jumps. Measurable light-induced potential changes could be recorded only after the electrode had penetrated the tonoplast. This was rather unexpected (see Discussion) since considerable changes in pH were observed near the cell surface during intermittent illumination.

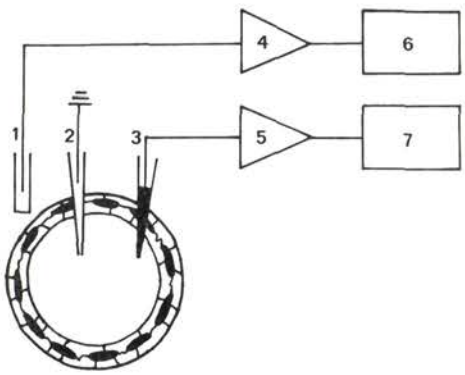

Fig. 1. Experimental arrangement for simultaneous measurements of both potential differences between the vacuole and the external medium and the vacuolar pH in *V. ventricosa*. 1 — calomel macroelectrode; 2 — glass microelectrode; 3 — antimony microelectrode; 4, 5 — electrometric amplifiers; 6, 7 — fast operating recorders.

In some experiments, we measured the light-induced changes in PD (between the vacuole and the external medium) at a reduced light pulse duration. Even at short light flashes (10 μs), measurable (0.5—1 mV) positive potential shifts occurred with a delay of about 100 ms. The flash-induced potential shifts displayed no oscillations.

Fig. 3-1. shows a typical induction kinetics curve of the millisecond component of delayed fluorescence recorded from a single *V. ventricosa* cell at $pH_o = 8$; the cell had been adapted to dark during 15 min. It can be seen that the induction kinetics of delayed fluorescence of these cells is also complex: the first luminescence maximum occurs within 1—1.5 s after the start of illumination through the rotating cylinder of the phosphoroscope; another maximum

occurred within 15—20 s. In addition to these two maxima, there was a third one occurring after 2—3 min of illumination. Thereafter, the fluorescence remained at a steady-state level which was relatively higher in small cells and lower in large ones. It should be noted that the maxima of delayed fluorescence seem to coincide with the maxima of the light-induced potential shifts shown in Fig. 2. Fig. 3-2. shows the induction kinetics on the same cell adapted to dark for only 1 min after 15 min preillumination.

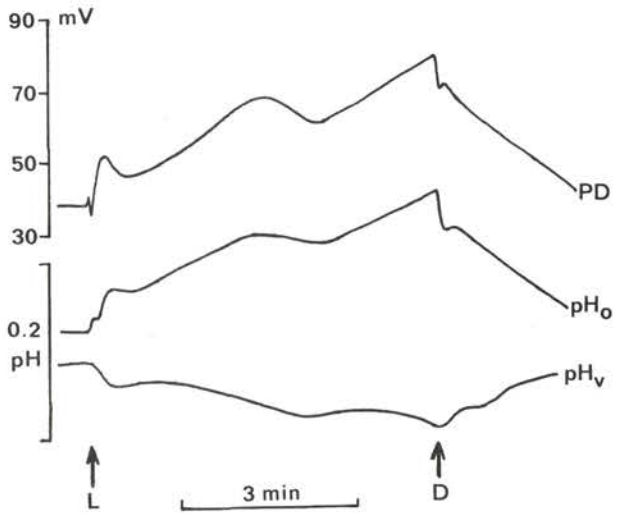

Fig. 2. Light-induced changes in the potential difference between the vacuole and the external medium (PD), in the external pH near to the cell surface (pH_o) and in the vacuolar pH (pH_v). Simultaneous PD—pH_o and PD—pH_v measurements were carried out in separate experiments under identical conditions.

Fig. 4. shows the induction kinetics of delayed fluorescence from a single cell recorded in control conditions (1) and after electrical breakdown of the same cell (2). The electrical breakdown was initiated with a 10 ms current pulse shifting PD between the vacuole and the external medium to about 1 V (Coster and Zimmermann 1975). As confirmed in resistance measurements, after this current pulse the cell remained in the low resistance state for at least 10 min, a time enough to record the induction kinetics of delayed fluorescence. It can be seen that after the reversible breakdown, the steady-state level of delayed fluorescence was lower and that there were no minute oscillations on the induction curve. After full recovery from the breakdown (30—40 min) the cell showed a normal fluorescence induction kinetics.

Fig. 5. shows induction kinetics of delayed fluorescence recorded from a single cell at $pH_o = 8$ (1) and at $pH_o = 5$ (2). In more acidic external medium, the initial luminescence maximum was higher than that in control conditions.

measurements carried out with in situ Anthoceros chloroplasts (Bulychev et al. 1980) showed that the photoinduction kinetics of the membrane potential in intact chloroplasts was more complicated than that recorded in isolated chloroplasts. As pointed out by Bulychev (1984), the oscillations of the thylakoid electrical potential after the start of illumination seem to be typical and physiologically important for intact chloroplasts.

The above hypothetic scheme of intracellular membrane organization in *V. ventricosa* agrees also with the finding that the considerable light-induced changes in pH_o are not accompanied by corresponding changes in potential across the plasmalemma; they however are accompanied by changes in potential across the tonoplast. The observed delay of 100 ms between the start of illumination and the onset of measurable photoelectric response can likely be explained by an increased capacitance of the mutually linked membrane system including the functional membranes of the chloroplasts. The first oscillation occurs within 1 s, a time required for the thylakoid ATPase to be activated (Junge 1977; Inoue et al. 1978).

The existence of such a membrane organization between the chloroplasts and the plasmatic membranes may explain the influence of transcellular $\Delta\bar{\mu}_{H^+}$ on $\Delta\bar{\mu}_{H^+}$ across the thylakoid membranes, the delayed fluorescence intensity being mainly determined by the latter. The association between the two $\Delta\bar{\mu}_{H^+}$ seems to be supported by the results shown in Figures 3, 4 and 5. The maxima of delayed fluorescence coincide with the maxima of transcellular $\Delta\bar{\mu}_{H^+}$, and the induction kinetics of the light emission correlates with that of the photoresponses of PD and pH measured transcellularly. Disruption of transcellular $\Delta\bar{\mu}_{H^+}$ lowers the steady-state level of delayed fluorescence and abolishes its minute oscillations (Fig. 4). At $pH_o = 5$ of the external medium (Fig. 5), the thylakoid ATPase seems to be activated more slowly. This can be explained by the assumed more acidic chloroplast stroma in these conditions (see also Mills et al. 1980). As a result an increased initial level of delayed fluorescence occurs. On the other hand, no considerable transcellular $\Delta\bar{\mu}_{H^+}$ may be formed in acidic external pH within the first minutes of illumination; this seem to explain the lowered steady-state level of light emission and the absence of minute oscillations on the induction curves. The differences in the induction kinetics of delayed fluorescence recorded in the presence and absence of transcellular $\Delta\bar{\mu}_{H^+}$ can be explained by the formation of an elevated $\Delta\bar{\mu}_{H^+}$ across the thylakoid membranes in intact living cells compared with that in isolated chloroplasts; this may be due to the intactness of the membrane continuities between the chloroplasts and the plasmatic membranes. Recently, Joliot and Joliot (1980) have pointed to the existence of these differences, claiming that "living cells differ from chloroplasts isolated either from algae or from higher plants with respect to the rates of the back reaction and of the intensity of delayed luminescence. It is possible to

interprete these differences by assuming that in living cells exists, even in dark adapted material, a permanent proton gradient and membrane potential which could stimulate charge recombination" and delayed light emission. This seems to explain the differences in induction kinetics of delayed fluorescence between the long-dark-adapted and short-dark-adapted Valonia cells shown in (Fig. 3). Keeping in mind that short-dark-adapted cells did not display minute potential and pH oscillations (data not shown), it may be concluded that the absence of minute oscillations of delayed fluorescence and the rapid establishment of the control steady-state level are due to an elevated transcellular $\Delta\bar{\mu}_{H^+}$ which does not fully dissipate during the 1 min lasting adaptation to dark. It was shown recently (Bulychev 1984) that the oscillations of photo-induced electrical potential in intact chloroplasts measured in situ disappeared in preilluminated short-dark-adapted material and reappeared after long dark adaptation.

Similar membrane organization in the cells of *V. ventricosa* may have certain functional role: the excess amounts of protons produced during illumination may be transported from the thylakoids into the vacuole without changing cytoplasic pH, utilizing directly the energy of light quanta. With this membrane organization, the transcellular H^+ gradient, which may become considerable during a few hours of illumination, could be realized in the dark through the same H^+-ATPase of the thylakoid membranes. This assumption seems to be supported by the observation that switching off the light induced an opposite H^+ flux eventually directed from the central vacuole to the external medium. The kinetics of this H^+ flux also correlates with the simultaneously measured kinetics of the corresponding potential changes (Fig. 2).

In conclusion, based on our experiments it can be suggested that transcellular $\Delta\bar{\mu}_{H^+}$ in *V. ventricosa* affects the energy conversion system in the chloroplasts through direct membrane connections between these organelles and the plasmatic membranes. Additional electron-microscopic studies are required to disclose the nature of these membrane continuities.

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