Electrical Signals at the Plasma Membrane and Their Influence on Chlorophyll Fluorescence of *Chara* Chloroplasts *in vivo*

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Abstract—Action potentials of plant cells are engaged in the regulation of many cell processes, including photosynthesis and cytoplasmic streaming. Excitable cells of characean algae submerged in a medium with an elevated K^+ content are capable of generating hyperpolarizing electrical responses. These active responses of plasma membrane originate upon the passage of inward electric current comparable in strength to natural currents circulating in illuminated *Chara* internodes. So far, it remained unknown whether the hyperpolarizing electrical signals in *Chara* affect the photosynthetic activity. Here, we showed that the negative shift of cell membrane potential, which drives K^+ influx into the cytoplasm, is accompanied by a delayed decrease in the actual yield of chlorophyll fluorescence F' and the maximal fluorescence yield F_m' under low background light (12.5 µmol m^{-2} s^{-1}). The transient changes in F' and F_m' were evident only under illumination, which suggests their close relation to the photosynthetic energy conversion in chloroplasts. Passing the inward current caused an increase in pH at the cell surface (pH$_o$), which reflected high H^+/OH^- conductance of the plasmalemma and indicated a decrease in cytoplasmic pH due to the H^+ entry into the cell. The shifts in pH$_o$ arising in response to the first hyperpolarizing pulse disappeared upon repeated stimulation, thus indicating the long-term inactivation of plasmalemmal H^+/OH^- conductance. Suppression of plasmalemmal H^+ fluxes did not abolish the hyperpolarizing responses and the analyzed changes in chlorophyll fluorescence. These results suggest that K^+ fluxes between the extracellular medium, cytoplasm, and stroma are involved in the functional changes of chloroplasts reflected by transients of F' and F_m'.

INTRODUCTION

Many processes in plant cells, such as photosynthesis, signal transduction, and intracellular regulation, are associated with changes in the electrical potentials across the plasmalemma and organellar membranes. Excitable membranes generate action potentials (APs) in response to mechanical, chemical, and electrical stimuli. Thylakoid membranes perform photosynthetic electron transport (PET) coupled with the H^+ transfer and electric potential generation. Drachev et al. [1] have proposed an effective method for studying electrogenesis of phototrophic bacteria using polymer films and adsorbed pigment−protein complexes and chromatophores. The use of short flashes allowed to overcome some kinetic limitations, such as dark inactivation of the photosystem I (PSI) acceptor side and slowing of PET by the increasing thylakoid ΔpH. At the same time, such limiting factors have a pronounced effect on PET in isolated plastids and chloroplasts of whole cells,

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Abbreviations: AP, action potential; APW, artificial pond water; Chl, chlorophyll; HR, hyperpolarizing response; NPQ, non-photochemical quenching; pH$_o$, pH in water layers adjacent to the cell surface; PET, photosynthetic electron transport; PS, photosystem; PSA, photosynthetic apparatus; YII, quantum yield of electron transfer.

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thus affecting the induction curves of membrane potential and chlorophyll (Chl) fluorescence [2, 3]. This explains a significant interest in the *in vivo* studies of PET regulation mechanisms, which are much more diverse than in the preparations of thylakoids and isolated intact plastids. One of these mechanisms involves regulation of photosynthesis by electrical signals of the plasma membrane.

Pulse-amplitude-modulated microfluorometry provides the means to monitor the effective quantum yield of PSII photoreaction (YII) and relative rates of linear PET on microscopic cell areas, as well as to follow rapid transients in non-photochemical quenching (NPQ) that reflect ΔpH in the thylakoids. The studies on the excitable cells of the alga *Chara* have revealed the involvement of plasma membrane electrogenesis in the regulation of photosynthetic electron flow. The generation of AP in illuminated cells caused a rapid (~30 s) decrease in YII by nearly 50% and increased NPQ for a period of up to 15 min [4, 5]. Apparently, the composition of the cytoplasmic constituents (ions and metabolites) is labile and depends on the state of plasma membrane ion channels. Experiments with alternating local and total illumination of the internodal cell demonstrated that the spatial heterogeneity of the cytoplasm, which is attributed to the presence of photosynthetically active and inactive zones, remains hidden under stationary conditions, when fluorescence is recorded in a given cell area, but becomes apparent after excitation-induced reversible arrest of cyclosis [6].

Changes in the cytoplasm composition are only one factor in the transformation of plasmalemmal signals to the functional manifestations in the chloroplasts. An important stage in this process is the cytoplasm-to-stroma transfer of ions, first of all, H^+ and Ca^{2+} [7, 8]. The influence of Ca^{2+} on Chl fluorescence is mediated by its effect on the activity of Calvin cycle enzymes and by accompanying pH shifts in the stroma and thylakoid lumen. During AP generation, the cytoplasmic content of Ca^{2+} in plant cells increases from 0.1 to 10-40 μM [9], which promotes Ca^{2+} entry into illuminated plastids due to the negative electric potential of the stroma [10]. The increase in stromal $[Ca^{2+}]$ suppresses the Calvin cycle and decreases consumption of ATP and NADPH in its reactions [11]. Consequently, ΔpH in the thylakoids increases, which promotes NPQ and slows down linear electron flow [12].

The inner membrane of the chloroplast envelope contains ion channels permeable to mono- and divalent cations, as well as K^+/H^+ antiporters (KEA1/2) [13-15]. An important role of ion exchange mediated by KEA1/2 becomes evident as photosynthesis disorders, e.g., in mutant plants grown on media with different salt compositions. In experiments with isolated chloroplasts, K^+ removal from the external medium lowered stromal pH and inhibited photosynthetic O_2 evolution due to

the K^+/H^+ exchange [16]. At the same time, it remains unclear whether the treatments that elevate the cytoplasmic and stromal K^+ levels affect the functional parameters of chloroplasts in short-term experiments *in vivo*.

The electrical activity of characean cells is not limited to the AP generation. Stimulation of internodal cells by inward electric current in the presence of elevated K^+ concentrations in the medium induces active hyperpolarizing response (HR) manifested as nonmonotonic changes in the membrane potential [17, 18]. It should be noted that electric currents inducing HRs are comparable in their strength to natural electric currents circulating between different parts of illuminated cell. The increasing shift in the membrane potential during HR results from partial suppression of K^+ conductance and activation of other yet poorly studied electrogenic mechanisms. So far, there are no data on a possible influence of HR on Chl fluorescence in charophytes. However, this information is important because ion fluxes associated with the plasmalemmal HR differ principally from the AP-triggered fluxes. During HR measurements, the studied part of the cell is bathed with a K^+-rich medium (K^+ concentration, ~100 mM) and is completely depolarized. The negative shift of the cytoplasm electric potential during the passage of inward current drives K^+ into the cell in amounts that exceed the fluxes of other ions due to the increased membrane permeability for K^+ and high K^+ concentration in the medium. Because of the presence of envelope transporters, accumulation of K^+ in the cytoplasm should induce K^+ entry into the chloroplast stroma in exchange for H^+ leaving the stroma. In this case, the HR can modulate chloroplast fluorescence via the K^+/H^+ exchange across the plastid membrane.

In this work, we studied the influence of plasma membrane electrical signals in *Chara* and *Nitella* algal cells on Chl fluorescence that served as an indicator of photosynthetic apparatus (PSA) performance. We found that hyperpolarization affected the actual fluorescence yield F' and maximum fluorescence yield F_m' in the cells exposed to continuous background illumination. The results revealed the relationship between the plasmalemmal HR, H^+ influx into the cytoplasm, and Chl fluorescence parameters. These findings expand current knowledge on the regulation of photosynthesis by the plasma membrane electrical activity.

MATERIALS AND METHODS

Experiments were performed on the internodal cells of *Chara australis* R. Br. and *Nitella translucens* (Pers.) C. Agardh obtained from the laboratory of Professor Ilse Foissner (University of Salzburg). The algae were grown in glass vessels under room lighting. The internodal cells 40-50 mm in length and 0.9 mm (*Chara*) and 0.3 mm (*Nitella*) in diameter were excised from the

thalli and placed in artificial pond water (APW) containing 0.1 mM KCl, 1 mM NaCl, and 0.1 mM $CaCl_2$. $NaHCO_3$ was added to adjust the solution pH to 7.0. Isolated cells were kept in APW for at least one day after their excision from adjacent internodes.

Chara internodes that have completed their growth are structurally heterogeneous and contain cytoplasmic regions with a high and low content of charasomes that alternate along the internode length. Charasomes are special organelles enriched with H^+-ATPase that facilitate the entry of photosynthetic substrate CO_2 into the cells [19]. The spatial heterogeneity of internodes is also manifested as an alternation along the cell axis of calcified and crystal-free areas on the cell surface. The calcified areas are typically located in the regions with low photosynthetic activity and high pH in the perimembrane layer of the outer medium. Chl fluorescence and plasma membrane potential were measured in noncalcified or weakly calcified cell areas.

An internodal cell was mounted in a three-section transparent chamber (Fig. 1) and placed on a stage of an Axiovert 25-CFL inverted microscope (Zeiss, Germany) equipped with a Microscopy-PAM fluorometer (Walz, Germany). The sections of the experimental chamber were separated with air gaps 4 mm in diameter to ensure their electrical insulation. The surface area of a *Chara* cell segment occupying the central section was approximately 0.1 cm². The solution in the central section was in contact with an Ag/AgCl reference electrode (surface area, ~15 cm²) via a flexible salt bridge filled with 1 M KCl solution and 2% agar. The electric potential difference across the plasma membrane was measured using Pyrex glass capillary micropipettes in the cell segment occupying the central compartment of the chamber and recorded with a U5-9 electrometric amplifier (Russia). The voltage from the amplifier was fed to a PCI-6024E analog-to-digital converter (National Instruments, USA) and a computer monitor.

The side compartments of the chamber contained connected Ag/AgCl electrodes to which a command voltage from the ADC/DAC (PCI-6024E) was applied through a load resistance of 1.2 MΩ; these electrodes served for passing stepwise pulses of electric current. The current strength varied from 3 to 8 μA, which corresponded to the current density from 30 to 80 μA cm⁻² in the central part of *Chara* cell.

Chl fluorescence was measured on cell regions with a diameter of ~100 μm. The actual yield of Chl fluorescence F' was recorded under background illumination of the entire cell with modulated blue light emitted by a LED of a Microscopy-PAM instrument (Walz, Germany). Measuring light flashes with a duration of 5 μs were emitted at a frequency of 24 Hz. The duration of the saturating flashes produced by the same LED was 800 ms. A 2 mm-thick red light filter RG645 (Schott, Germany) was placed in front of the photomultiplier input window
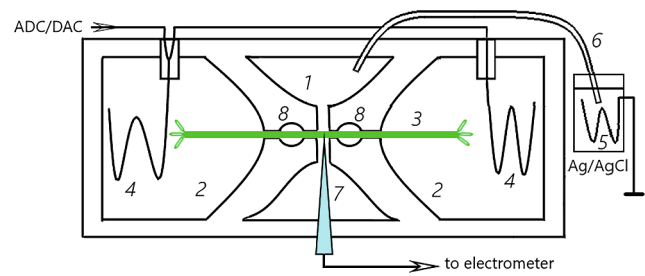

Fig. 1. Scheme of the three-section experimental chamber with the internodal cell and electrodes: *1*) central compartment filled with KCl-enriched medium; *2*) side compartments filled with APW; *3*) internodal cell of *Chara* (*Nitella*) alga; *4*) Ag/AgCl electrodes used for passing electric current; *5*) Ag/AgCl reference electrode common for the measuring circuit and current pathway; *6*) flexible salt bridge; *7*) electrode microsensor (intracellular capillary electrode for measuring plasma membrane potential or pH-sensitive extracellular microelectrode for measuring local pH shifts on the cell surface); *8*) air gaps providing electrical insulation of the central and side compartments.

(see description of a Microscopy-PAM fluorometer on the Walz website https://www.walz.com/downloads.html). Chl fluorescence of microscopic cell regions was measured as described by Goh et al. [20]. Background illumination of the whole cell was provided by the microscope upper light source through a blue-green (SZS-22, λ < 580 nm) and neutral density (NS-3) glass filters at the intensity of 12.5 μmol quanta m⁻² s⁻¹. The effective quantum yield of the electron transfer in PSII was estimated using saturating light pulses and WinControl-3 program (Walz, Germany). The PSII quantum yield (YII) at background irradiance under control conditions ranged from 0.7 to 0.745. The output signal from the photomultiplier was processed with the WinControl-3 and WinWCP programs (Strathclyde Electrophysiology, United Kingdom) and displayed on a computer monitor.

The influence of HRs on the maximum fluorescence yield F_m' was studied using saturating light pulses separated by 10-s intervals. Initiation of a series of saturating pulses was usually accompanied by a noticeable decrease in F_m'. For this reason, the cells were stimulated with a stepwise current pulse after the reduction in F_m' caused by saturating light pulses had slowed down.

pH at the cell surface (pH_o) was measured with glass-insulated H^+-specific antimony microelectrodes and a U5-9 electrometric amplifier [4]. The raw readings of the pH microsensor were processed by subtracting the background voltage drop created by the electric current on a series resistance of the medium, salt bridge, and reference electrode.

Cytochalasin D (Sigma-Aldrich, USA) was dissolved in dimethyl sulfoxide (DMSO) to obtain a stock solution that was then diluted to the final concentration of 10-20 μM. The final concentration of DMSO did not exceed 0.2%. At this concentration, DMSO did not affect the measured parameters of Chl fluorescence, membrane potential, and cytoplasmic streaming.

a

b

c

d
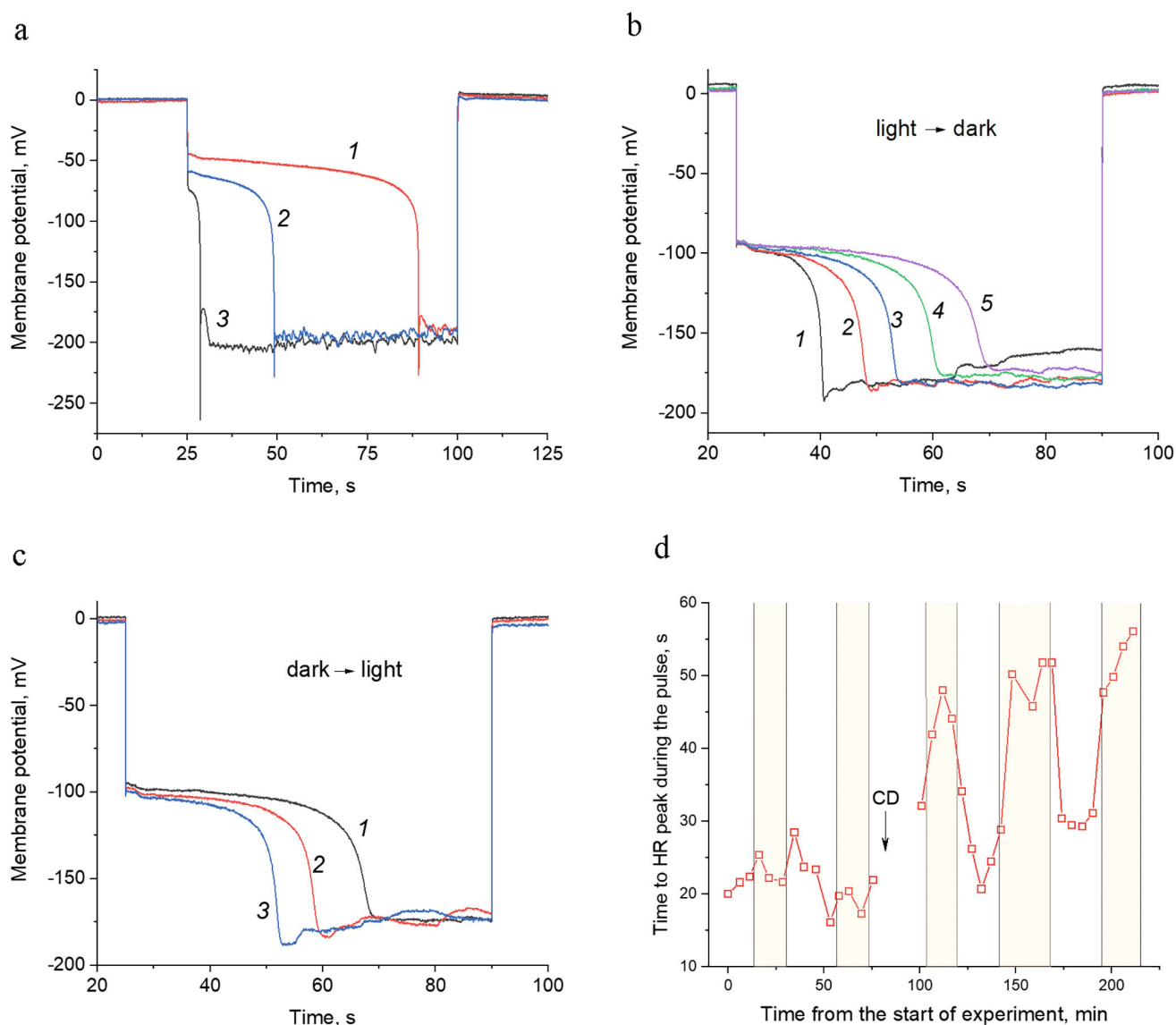
Fig. 2. HRs of *Chara* cells caused by passing an inward electric current in the presence of 0.1 M KCl in the medium. a) HR kinetic curves at various current densities: *1*) 33 µA cm^{-2}, *2*) 45 µA cm^{-2}, and *3*) 50 µA cm^{-2}. b) HR kinetic curves in the presence of 20 µM cytochalasin D after transferring the cell to darkness: *1*) in the background light (photon flux density, 12.5 µmol m^{-2} s^{-1}); *2-5*) 0.5, 5, 10, and 15 min, respectively, after switching off the background light. c) HR kinetic curves for the same cell in the presence of cytochalasin D after transition from the dark to the light: *1-3*) 0.5, 5, and 10 min after transferring the cell to the light. Current density during electric pulses in (b) and (c) was 33 µA cm^{-2}. d) Effect of light conditions on the time of attaining the HR peak (counted from the onset of inward current) in the absence of inhibitors and after arrest of cyclosis with 20 µM cytochalasin D (CD). The moment of CD addition is marked with an arrow. Vertical light and shaded areas mark the light and dark periods, respectively; current density during the pulse was 58 µA cm^{-2}.

The results presented in the figures were obtained from at least four cells. The averaged kinetic curves for F' and standard errors are presented with indication of the total number of measurements (n) on different cells (N). For averaging the F' values obtained on different cells, the curves were normalized to the average fluorescence level within the time interval of 0-25 s recorded before the pulse application. The average F_m' kinetics for different cells was obtained after normalizing F_m' values recorded in response to the first saturating pulse in a series of 30 flashes.

RESULTS

Under standard experimental conditions (cells bathed in APW), the membrane potential of illuminated *Chara* cells ranged from −170 to −210 mV. Depolarizing current pulse (2 µA, 150 ms) elicited an AP with the amplitude of up to 200 mV, which was accompanied by a short-term stoppage of cytoplasmic streaming followed by its recovery within ~5 min at 25°C.

Effects of light and cytoplasmic streaming on the HR. At the beginning of each experiment, we verified that

the membrane potential at rest and during excitation fell into the characteristic ranges and that the effective quantum yield of the PSII-driven electron transfer was 0.7-0.745 to ensure an adequate physiological state of the cell. After APW in the central section of the chamber was replaced with APW containing 0.1 M KCl, the passage of a standard supra-threshold current pulse caused a stepwise cell depolarization to ~0 mV. This state is characterized by a high K^+ conductance of the plasmalemma [21] and the membrane potential corresponding to zero level of equilibrium Nernst potential.

Subsequent passage of a rectangular inward current pulse (30-80 μA cm^{-2}; pulse duration, 50-75 s) was accompanied by the generation of an active HR (Fig. 2). During the HR development, the negative membrane potential increases at an accelerating rate towards a sharp spike that is followed by partial depolarization [22]. At equal concentrations of K^+ in the medium and in the cytoplasm (~100 mM), the negative shift in the membrane potential causes K^+ ions to enter the cell due to the high K^+ conductivity of the membrane.

The time required to achieve the peak hyperpolarization reduced upon the increase in the density of inward current (Fig. 2a). At the same time, the stationary potential established after the spike was nearly independent of the current density. Both peak hyperpolarization and subsequent rapid depolarization were markedly smoothed over after cell treatment with cytochalasin D, an inhibitor of actin polymerization (Fig. 2, b and c). Similar suppression was previously observed for the action of cytochalasin B on the surface membrane of isolated chloroplast-free cytoplasmic droplets ([22] and references therein). In our experiments, addition of 20 μM cytochalasin D to the medium caused cessation of cytoplasmic streaming. In the presence of this inhibitor, the HR curves at a constant current density strongly depended on the illumination conditions (Fig. 2, b and c).

In the dark, the time toward the peak hyperpolarization increased within approximately 20 min (Fig. 2b), but then restored back to shorter times after the cells were illuminated again (Fig. 2c). Interestingly, the effect of illumination on the HR was observed only in the presence of cytochalasin D but not under the control conditions. As can be seen from Fig. 2d, the time required to achieve the HR peak in the light and in the dark was almost the same if cytoplasmic streaming remained undisturbed, but differed significantly when cyclosis was blocked by cytochalasin D.

Thus, chloroplasts were able to affect plasmalemmal ion channels when the actin cytoskeleton was disturbed. It is possible that the light−dark transitions altered the composition of the cytoplasm, which modified plasma membrane channels involved in the HR generation. However, this effect was prevented by active cytoplasmic streaming that smoothed over the differences in the cytoplasmic composition between cell parts with different photosynthetic activity.

Influence of hyperpolarizing response on the Chl fluorescence yield F'. We have focused our attention mainly on a possible impact of HR on Chl fluorescence parameters indicative of the redox state of the PET chain and energy-dependent dissipation of Chl excited states (NPQ). Emergence of the driving force for the K^+ influx from the medium to the cytoplasm upon the negative shift in the plasma membrane potential should be accompanied by K^+ accumulation in the cytoplasm, which, in turn, can induce the K^+/H^+ exchange across the chloroplast envelope membranes and affect Chl fluorescence.

Figure 3a and b shows changes in the fluorescence yield F' induced by the pulse of an inward current in *C. australis* and *N. translucens* internodes exposed to low-intensity background light (12.5 μmol m^{-2} s^{-1}). In both characean species, the F' changes comprised a delayed stage of fluorescence yield decrease that started approximately 25 s after the onset of the current pulse and continued for ~50 s after the end of HR.

The decrease in F' in *N. translucens* cells was preceded by the increase in fluorescence that started at the first seconds after the current pulse application. In *C. australis* cells, the increase in F' preceding F' reduction was observed occasionally, but not in the averaged recordings. The opposite changes in F' associated with the HR indicate the occurrence of several processes of different origin. Among them, only slow changes in F' can result from the K^+/H^+ exchange across the chloroplast envelope membranes, since high cytoplasmic K^+ concentration ($[K^+]_{cyt}$, ~100 mM) excludes its rapid change. The reasons for the rapid increase in fluorescence in *N. translucens* cells remain unclear. It is possible that the inward electric current is associated with the electroosmotic water inflow and visually inconspicuous swelling of the plastids. This is indirectly indicated by the fact that the increase in F' was clearly evident in small-diameter cells (*N. translucens*) featuring a comparatively large surface to volume ratio.

The stage of delayed fluorescence decrease during the HR generation was observed only in illuminated *C. australis* and *N. translucens* cells, but not in the cells placed in the dark (Fig. 3, c and d). In contrast, the stage of F' increase clearly visible in *N. translucens* cells was independent of illumination conditions. This confirms the assumption that the described opposite changes in fluorescence have different nature.

Influence of HR on the fluorescence yield F_m'. In *Chara* internodes placed into standard APW with 0.1 mM K^+, F_m' in low light approached the highest values, which indicated the absence of NPQ. Replacement of APW with the medium containing 0.1 M KCl led to a noticeable decrease in F_m', i.e., to the NPQ development. In this connection, we examined the effect of plasma
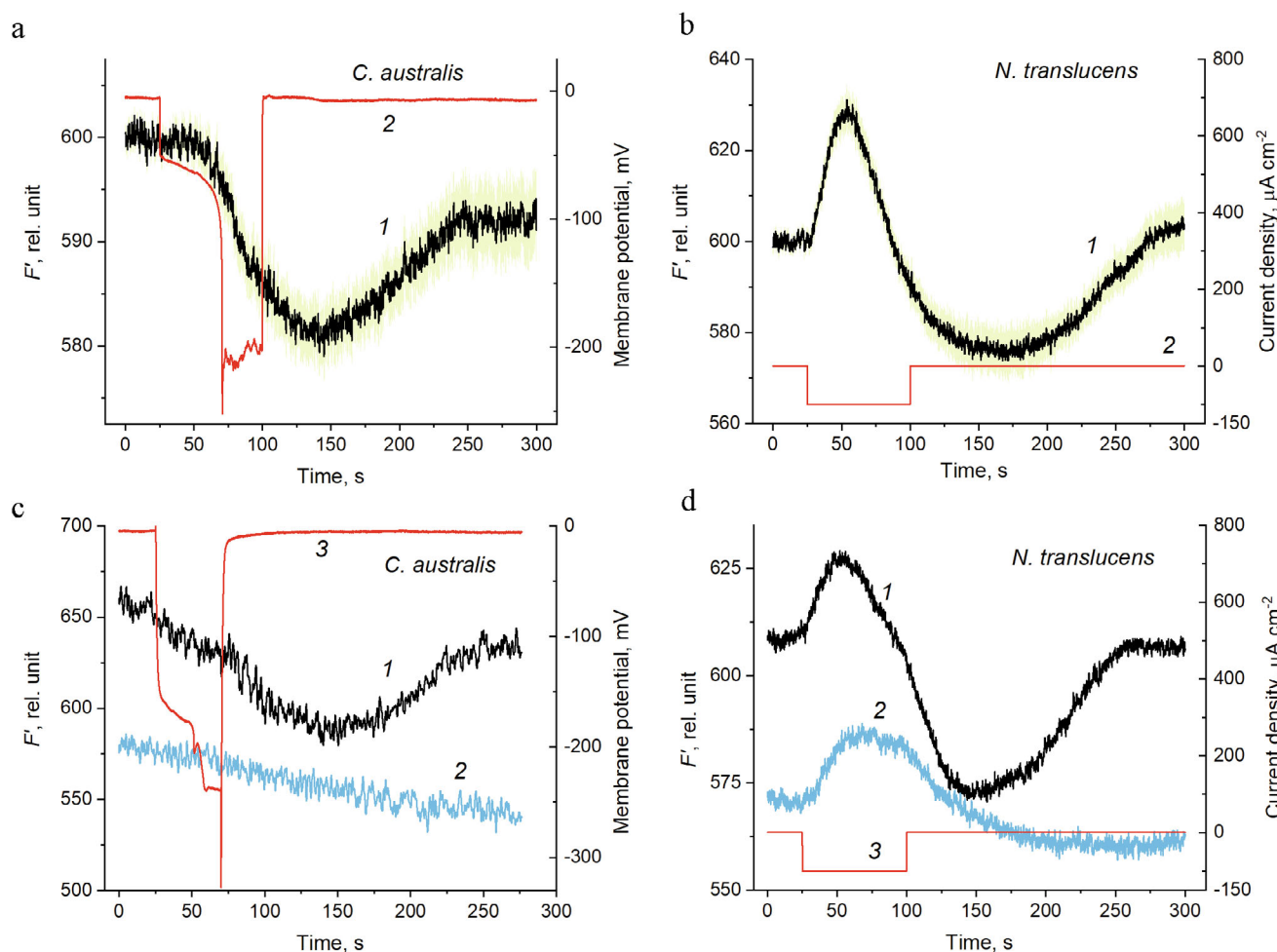
Fig. 3. Changes in Chl fluorescence yield F' caused by the inward current in *C. australis* (a, c) and *N. translucens* (b, d) cells. a) Changes in (*1*) F' and (*2*) membrane potential caused by the inward current. Curve *1* (black line) represent an averaged recording of F' ($n = 45$, $N = 9$) on the background of standard error of the mean (SEM). Curve *2* shows changes in the membrane potential caused by a pulse of inward current with a density of 35 µA cm^{-2}. b) Changes in Chl fluorescence in *N. translucens* cells (curve *1*) caused by a pulse of inward current with a density of 100 µA cm^{-2} (curve *2*). The averaged recording of F' is presented on the background of SEM ($n = 11$, $N = 4$). c, d) Light dependency of hyperpolarization-induced decrease in F' for *C. australis* and *N. translucens* cells ($n = 9$-11) in the (*1*) light and (*2*) dark. Curves *3* show voltage changes during HR in panel (c) and current density in the central cell region in panel (d).

membrane hyperpolarization on F_m' as an NPQ indicator. Figure 4 shows the results of a representative experiment in which saturating light pulses were applied either with or without electrical stimulation. The cell was alternately subjected to several cycles of such treatment and the recordings with the HR induction were alternated with the recordings during stationary depolarization. The onset of saturating pulses released at 10-s intervals was often accompanied by the initial decrease in F_m' (Fig. 4, curves *3* and *4*, segments at $t \leqslant 75$ s). After the F_m' decline slowed down (within 75 s after the start of recording), a step-like current pulse was applied to induce the HR in the cell area under study (curve *1*). Under the same conditions but in the absence of current, the plasmalemma remained in the depolarized state (curve *2*).

As can be seen in Fig. 4, the curves *3* and *4* almost coincided in their initial segments ($t \leqslant 75$ s), i.e., before

the HR induction, but diverged significantly at $t > 100$ s depending on the occurrence or absence of HR. When the plasmalemma remained steadily depolarized, the F_m' values reached a constant level after a smoothed maximum (curve *4*), while after plasma membrane hyperpolarization, a slight increase (or shoulder) in F_m' was transiently observed that was replaced by a strong decrease in F_m' (curve *3*). Subsequent recovery of F_m' took ~10 min. The kinetic curves of F_m' showed some similarity with the F' changes during the HR. Under a low-intensity background illumination, the quantum efficiency of the PSII-driven electron transfer (YII) slightly decreased ($\leqslant 0.03$). At the same time, at the light intensity of 25 µmol m^{-2} s^{-1}, the HR-induced decrease in the PSII quantum yield reached substantial values (~0.15 units). In cells placed in the dark, the HR had no effect on the PSII quantum yield.
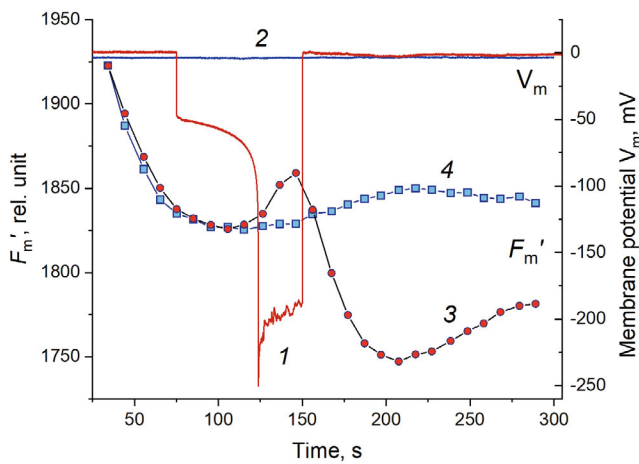
Fig. 4. HR of the plasma membrane in the K^+-conductive state and its effect on F_m'. Hyperpolarization of the plasma membrane (curve *1*) was induced by an inward current pulse (42 μA cm^{-2}, duration 75 s) under weak background irradiance (12.5 μmol m^{-2} s^{-1}). In the absence of electrical stimulation, the membrane remained steadily depolarized (curve *2*). Curves *3* and *4* represent F_m' changes observed with or without HR generation (curves *3* and *4*, respectively). The monotonic decrease in F_m' over $t \leqslant 75$-100 s time interval was caused by a series of saturating light pulses. Curves *3* and *4* were obtained in a representative experiment by averaging the results of five measurements per treatment.

As shown in Fig. 5, the influence of HR on F_m' was only evident in the light but disappeared after the light was switched off. In the illuminated cell, the kinetics of F_m' changes differed clearly in the presence and absence of the hyperpolarizing stimulus (Fig. 5a). However, after the cell was placed in darkness, changes in F_m' observed upon the current passage and in the absence of electrical stimulation were almost the same

(Fig. 5b). Thus, PET coupled with the transmembrane H^+ flows is a necessary condition for the signal transmission from the plasma membrane to chloroplasts.

Transmembrane H^+ fluxes during hyperpolarization and their inactivation. When the plasmalemma is in a K^+-conductive state, the transmembrane K^+ fluxes are predominant. At the same time, the negative shift of cytoplasmic electric potential during HR may in principle cause the redistribution of H^+ between the cytoplasm and outer medium, as the plasma membrane of illuminated *Chara* cells is permeable to H^+ and/or OH^- ions [4, 5]. We tested this possibility by measuring pH values near the cell surface (pH$_o$) during the current-induced hyperpolarization of *Chara* plasmalemma.

Curve *1* in Fig. 6a shows the pH$_o$ shift induced by passing a stepwise inward current through the plasmalemma of *Chara* cell. The hyperpolarizing pulse caused an increase in pH$_o$ by approximately 0.5 units, which in some cases, but not always, continued for a while after the pulse termination. The rate of pH$_o$ shift increased during the stimulus, which was presumably due to progressive hyperpolarization and the increasing driving force for H^+ entry into the cytoplasm. After pulse termination, the pH$_o$ values usually fell below the initial level (Fig. 6a, curve *1*); however, the pH$_o$ depression was released with time (Fig. 6a, curve *2*).

Unlike the impact of the first electrical stimulus on pH$_o$, the second or third current pulses did not cause any discernible pH$_o$ changes (Fig. 6a, curve *2*), which indicated that the first hyperpolarizing stimulus caused an almost complete inactivation of the plasmalemma passive conductance for H^+. In some cells, the H^+ conductance was restored after a resting period of ~1 h. However, in many cases, no restoration of the H^+
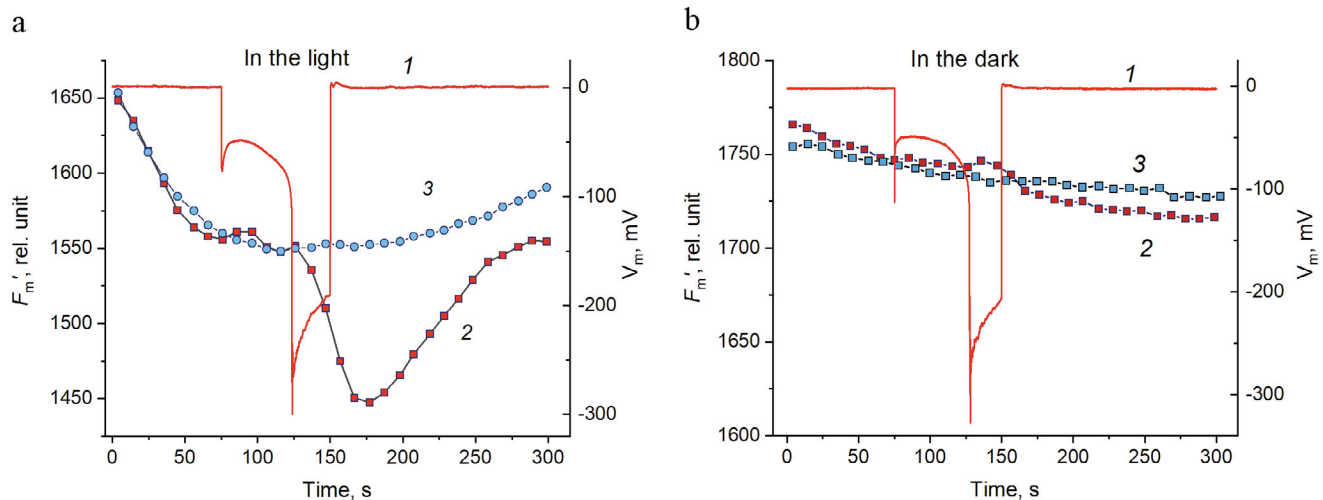
Fig. 5. Light dependency of hyperpolarization-induced changes in F_m'. a) Plasma membrane hyperpolarization in illuminated cells causes a delayed decrease in F_m'. b) Effect of hyperpolarization on F_m' disappears after transferring the cell to darkness. *1*) Changes in the membrane potential caused by a rectangular current pulse (83 μA cm^{-2}); *2*) F_m' changes caused by saturating light pulses and HR generation: (a) in the light and (b) in the dark; *3*) F_m' changes caused by saturating light pulses in the resting cell in the absence of electrical stimulation and HR induction. Traces *2* and *3* represent average values obtained from two measurements per treatment.
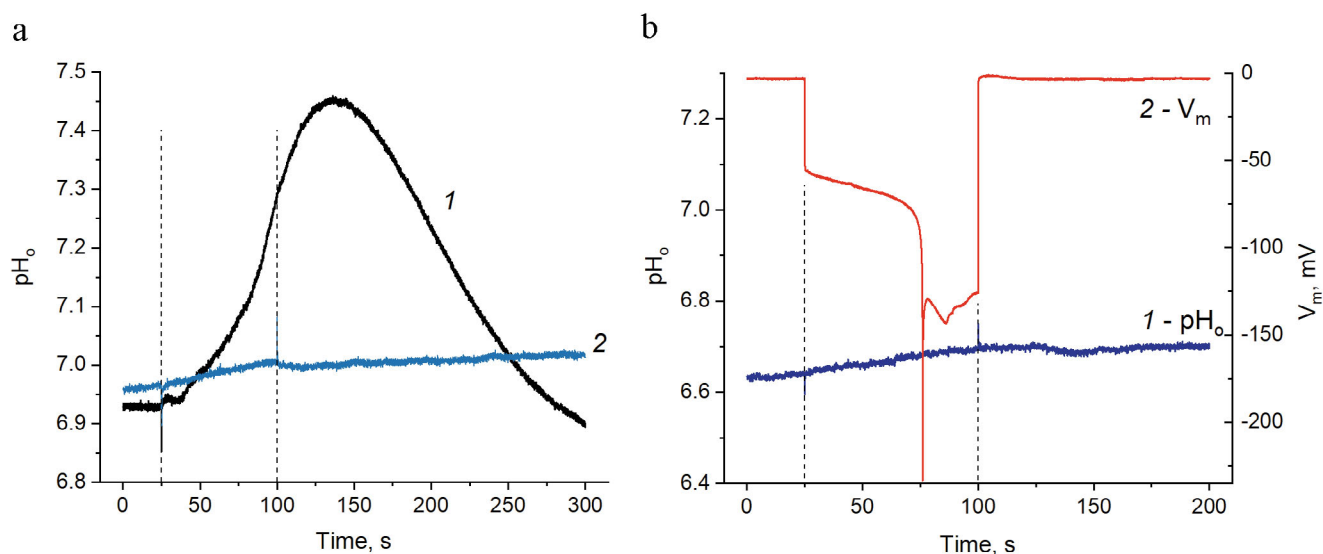
Fig. 6. Changes in the cell surface pH during induced hyperpolarization. a) Changes in pH at the *Chara* cell surface (pH$_o$) caused by passing a hyperpolarizing current pulse through the plasmalemma: *1*) changes in pH$_o$ in response to the first pulse with a density of 58 μA cm^{-2}; *2*) disappearance of pH$_o$ shift upon subsequent application of current pulses of the same strength. b) Suppression of pH$_o$ changes in response to the repeated electrical stimulation of plasmalemma (curve *1*, pH$_o$) did not eliminate hyperpolarizing shifts of the membrane potential (curve *2*, V$_m$). Results presented in (a) and (b) were obtained on different cells. Dashed vertical lines mark the moments when the inward electric current was switched on and off.
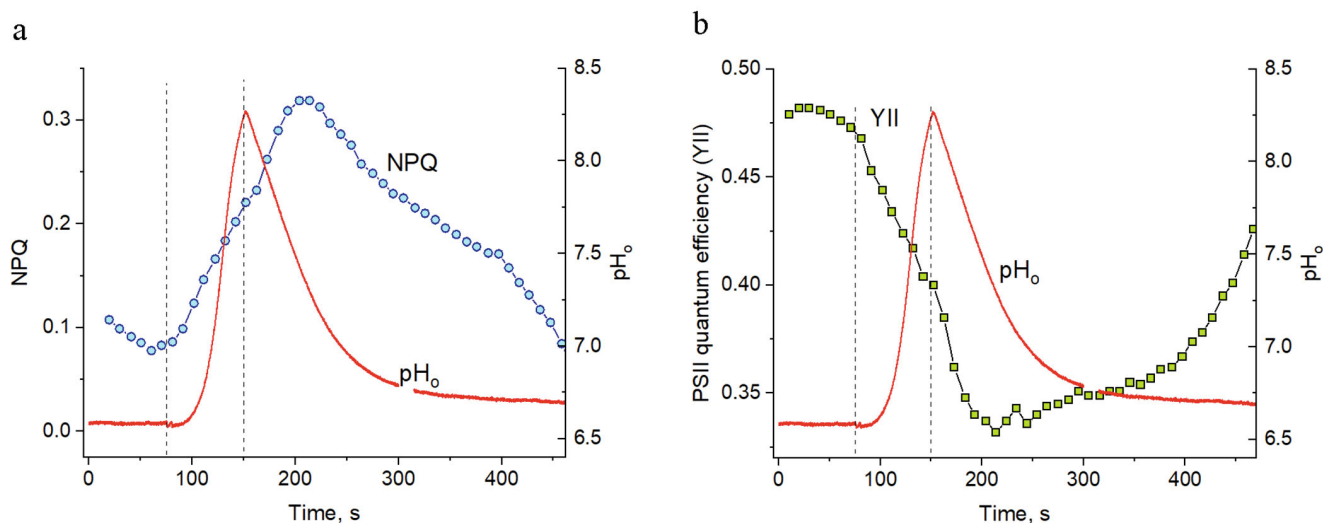
Fig. 7. Changes in NPQ (a), effective quantum yield of PSII reaction (YII) (b), and pH on the *Chara* cell surface (pH$_o$) caused by a hyperpolarizing pulse with a current density of 33 μA cm^{-2}. The medium (APW) in the central compartment of the chamber was supplemented with 30 mM KCl; background light intensity was 25 μmol m^{-2} s^{-1}. Vertical dashed lines mark the moments when the electric current was switched on and off.

conductance was observed in continuous experiments. Nevertheless, pH$_o$ changes in response to the current pulse restored on the next day provided the cells were kept overnight in standard APW. The data presented in Fig. 6b show that suppression of plasmalemmal H$^+$ conductance (curve *1*) had no inhibitory effect on the HR development (curve *2*).

Figure 7 shows that the pH$_o$ shifts associated with the hyperpolarizing pulses could be as high as 1.5 units and were accompanied by a significant increase in NPQ (Fig. 7a) and a transient decrease in the effective quan-

tum yield of PSII reaction (Fig. 7b). The emergence of HR under these particular conditions was verified in parallel experiments. The decrease in YII indicated transient deceleration of the photosynthetic linear electron flow.

DISCUSSION

Cells of many plants respond to physiological and damaging stimuli by generating electrical signals (action

potentials, variable potentials) that influence photosynthesis and Chl fluorescence [4, 5, 23]. The mechanisms of signal transduction from the plasma membrane to chloroplast membranes have been only partly clarified. The effects of the electrical activity on the PSA performance can be studied on characean internodes since they combine excitability, cytoplasmic streaming, and spatially nonuniform distribution of H^+ fluxes and photosynthesis.

The ability of plasmalemma to generate active HRs has been long known [17, 24], but the relation of HRs to other cellular events and their effect on PSA remained unexplored. We found that the time from the onset of electric pulse to the HR peak was altered during the light−dark transitions, provided cyclosis was arrested by cytochalasin D (inhibitor of actin cytoskeleton polymerization). The effect of dark−light transitions on the rate of HR development (Fig. 2) was probably due to changes in the cytoplasm composition during the onset and cessation of the light-dependent transport across the chloroplast envelope membranes. The distribution along the cell length of cytoplasmic components transported through the plastid membrane seems to become more heterogeneous in the absence of cyclosis, because intense streaming smooths down the concentration profiles of these components. This explains the lack of a visible effect of illumination on the HR development during active cytoplasmic streaming, as well as a marked HR dependence on light conditions after cessation of cyclosis.

According to the earlier study of isolated cytoplasmic droplets, the disturbance of actin cytoskeleton by cytochalasin B modified the HR shape by eliminating the sharp peak tentatively ascribed to the activity of the electrogenic pump ([22] and references therein). The effect of cytochalasin D on whole cells also smoothed down the hyperpolarization peak and the depolarization stage of HR (Fig. 2). These observations suggest that the state of actin cytoskeleton controls the conductance of channels involved in the HR formation. The mechanisms of interaction between ion channels and actin cytoskeleton have been discussed in [25]. These mechanisms can be applied to explain the effect of cortical actin on ion channels in the plasma membrane of *Chara*. HR modulation by light conditions might be based on changes in the cytoplasmic $[Ca^{2+}]$ and $[H^+]$ that arise from the photoinduced fluxes of these ions across the plastid membrane and exert direct or indirect action on the activity of ion channels. The mechanisms of Ca^{2+} and H^+ influence on the cytoskeleton of plant cell have been described in the review by Hepler [26].

We investigated the effects of HR on the PSA by assessing changes in F' and F_m'. Here possible relations between HR and cell physiological events are worth noting. In illuminated *Chara* and *Nitella* cells, natural electric currents circulate between the cell parts that produce external acidic and alkaline zones [27]. The density of light-dependent inward currents in the alkaline zones of *Chara* cells is 20-60 $\mu A\ cm^{-2}$ [28]. In our study, the HR of the plasma membrane was induced by electric current with a density of 30-80 $\mu A\ cm^{-2}$, i.e., in the physiological range. Hence, the observed interactions between the plasma membrane and chloroplasts can, in principle, arise upon the increase in the external K^+ level. When an illuminated cell is challenged by a solution with a high K^+ concentration (10-100 mM), the inward current might lead to a protective decrease in the K^+ conductance due to the HR generation and also block the H^+ conductance (Fig. 6) to prevent operation of the H^+ pump in a short-circuited mode.

The HRs of charophytes show some similarities with the hyperpolarization signals in higher plants, which arise when a leaf is heat-treated or damaged with an open flame. In particular, moderate heating of wheat leaves resulted in gradual cell hyperpolarization by 10-20 mV for 20 min, which was accompanied by the increase in NPQ [29]. The high-amplitude HRs in *Chara* cells (~250 mV) developing within 10-75 s caused a marked increase in the regulated energy dissipation in PSII (Fig. 7a), as well as a decline in the quantum yield of PSII reaction and slowing of the linear electron flow (Fig. 7b).

The effects of HRs on cellular processes attract a particular interest because the negative voltage arising during HR creates a driving force for the K^+ entry into the cytoplasm through the plasma membrane pre-converted to the K^+-conductive state. Therefore, HR can be used as a tool to elevate the cytoplasmic K^+ level and to examine the outcomes of the increased $[K^+]_{cyt}$ on the PSA condition.

We used relatively long intervals between the current pulses (600 s) to facilitate the recovery of $[K^+]_{cyt}$ during transitions from the HR to the resting state. The cells subjected to recurrent HR generation remained viable on the next day after the experiment and all measured parameters (cytoplasmic streaming velocity, effective quantum yield of PSII, and resting potential) remained within their normal ranges. The redistribution of ions due to the HR generation did not usually lead to the irreversible cell damage. Nevertheless, the HR generation was followed by a long-term inactivation of H^+/OH^- fluxes. Under physiological conditions, the H^+/OH^- conductance depends on illumination. The H^+/OH^- fluxes caused by the HR generation occurred both in the light and, occasionally, in the dark. Presently, it remains unclear whether the potential-driven H^+/OH^- fluxes are equally pronounced in photosynthetically active and inactive cell areas.

An elevation in $[K^+]_{cyt}$ by itself is insufficient to affect PSA. However, it can influence the ionic composition of the stroma via carriers located in the plastid envelope.

Thus, the inner envelope membrane contains K^+/H^+ antiporters [13, 14, 30]. When $[K^+]_{cyt}$ increases, these transporters should transfer K^+ to the stroma in exchange for H^+ from the plastid stroma. Importantly, the activity of KEA1/2 is retained in the dark [31]. An increase in the stromal pH is known to stimulate the reactions of carbon dioxide fixation, which can shift the redox states of plastoquinone and Q_A acceptor towards oxidation, thus decreasing the actual yield of Chl fluorescence F' (Fig. 3). According to the proposed scheme, delayed changes in F' and F_m' in response to the plasma membrane hyperpolarization are due to the induction of K^+/H^+ exchange across the chloroplast envelope.

At the same time, hyperpolarizing electric pulses can potentially affect the water balance of the cell and chloroplasts. If the pore of the plasma membrane K^+ channel contains fixed negative charges, electroosmotic entry of water into the cell and corresponding swelling of chloroplasts cannot be ruled out. The redistribution of granal system in a larger volume might underlie rapid F' increase in *N. translucens* cells that feature an increased surface to volume ratio. In addition, K^+ entry into the stroma of plastids in exchange for H^+ efflux might be accompanied by the osmotic regulation of chloroplast volume. Taking into account various factors occurring simultaneously during the current flow will help to clarify the mechanistic relations between the electrical processes at the plasmalemma and chloroplast activity.

Hyperpolarization creates a driving force not only for the K^+ entry but also for the H^+ influx into the cytoplasm. The dependence of cytosolic pH on the shift in the membrane potential seems likely, because the plasmalemma of *Chara* cells contains passive conducting pathways for H^+ or OH^- [5, 27]. Previously, the existence of plasmalemmal H^+/OH^- conductance was deduced from the formation of narrow alkaline zones on the surface of illuminated internodal cells bathed in a low-K^+ medium (i.e., under conditions ensuring sustained circulation of natural electric currents at low K^+ conductance of the plasma membrane). Here, we demonstrated the existence of H^+ fluxes caused by hyperpolarization at a high K^+ conductance of the plasmalemma (Figs. 6 and 7).

The results presented in Figs. 6 and 7 reveal two principally different types of pH_o changes taking place after switching off the inward current. The instant reversal of the sign of pH changes at the peak in Fig. 7 and the ongoing pH rise after termination of the current pulse in Fig. 6 indicate that the hyperpolarization-induced pH changes comprise the stages of different origin. The changes in pH reverted after the shift of the membrane potential to zero apparently reflect the voltage-dependent transfer of H^+ (or OH^-) along the plasmalemmal conducting pathways. In contrast, pH changes proceeding at almost the same rate after removal of the driving force (Fig. 6) indicate a different nature of the

post-hyperpolarization pH shift. Such increase in pH_o is similar to the formation of a local alkaline zone within approximately 30 s after microperforation of *Chara* cell [32]. Parallel measurements of local shifts in pH and O_2 concentration in the area of cell microinjury in the absence and presence of inhibitors indicated that the pH increase concurrent with the sharp drop in O_2 level was due to the enzymatic transfer of electrons through the plasmalemma from NAD(P)H to O_2 and subsequent consumption of H^+ in the course of $O_2^{\cdot}$ reduction to H_2O_2 [33]. Based on these data, it is reasonable to conclude that the pH_o rise during plasma membrane hyperpolarization can involve different mechanisms, i.e., transmembrane transport of H^+ under the influence of electrochemical proton gradient, as well as a combined mechanism that includes biochemical processes insensitive to the transmembrane H^+ gradient.

Interestingly, pH_o changes occurred only in response to the first and, rarely, to the second current pulse, while subsequent stimulation had almost no effect on pH_o. Since the effect of HR on F' and F_m' remained after application of multiple electrical stimuli, it appears that H^+ fluxes caused by hyperpolarization did not play a critical role in the signal transmission from the plasmalemma to chloroplasts upon repeated stimulation. However, considering possible changes in the cytoplasmic pH during HR development, it seems important to examine the role of cytoplasmic pH in PSA rearrangements in more detail.

The HR-induced F_m' transients are somewhat similar to the AP-induced F_m' changes that take place at a moderate light intensity. It is known that hyperpolarization can activate Ca^{2+}-permeable channels [34], thus allowing Ca^{2+} entry to the cytoplasm. According to one of the hypotheses, the depolarization stage occurring after the HR peak in cytoplasmic droplets of charophytes is similar to the front of the AP in intact cells and is associated with an increase in the cytoplasmic Ca^{2+} [22]. However, this hypothesis is not entirely convincing with respect to intact cells, as cytoplasmic streaming does not stop at the peak of HR, even though cyclosis is extremely sensitive to the increase in cytoplasmic Ca^{2+} [35]. The role of thylakoid ΔpH in the regulation of F' and F_m' is evidenced by the fact that the impact of HR on these parameters was manifested only in illuminated cells and disappeared in the dark along with the PET (Figs. 3 and 5).

The long lag period preceding the HR-induced changes in F' and F_m' reflects the multistep transformation of plasmalemmal electrical signal into fluorescence response in the thylakoid membranes. The proposed intermediate steps include changes in $[K^+]_{cyt}$, K^+/H^+ exchange across the chloroplast envelope, modulation of activity of stromal enzymes, and pH effect on the electron transport and fluorescence quenching in the antenna.

In conclusion, this study revealed new interactions between the plasma membrane and chloroplasts, which are mediated by ion fluxes mobilized due to the electrical cell signaling. Hyperpolarization of the plasmalemma in the K$^+$-conductive state leads to noticeable changes in F' and F_m' of Chl fluorescence pointing to the existence of regulatory mechanisms that differ from previously discovered pathways triggered by the AP generation. Our experimental observations are consistent with the notion that the K$^+$/H$^+$ exchange on the inner envelope membrane has a regulatory impact not only in the long-term experiments (e.g., in plants grown at elevated salinity), but also in the case of relatively short-term treatments. Further studies are needed for deeper understanding of the intracellular interactions underlying the effect of electrical events at the plasmalemma on the photosynthetic performance of chloroplasts.

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