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Membrane potential is involved in regulation of photosynthetic reactions in the marine diatom *Thalassiosira weissflogii*

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ABSTRACT

High-intensity Chl fluorescence transients (OJIP transients) and light-induced kinetics of the delayed light emission were measured in diatom microalga *Thalassiosira weissflogii* in the presence of various uncouplers and photosynthetic inhibitors. The I step in the OJIP transients in *T. weissflogii* was essentially reduced or completely absent but was restored in the presence of uncouplers valinomycin, FCCP, and nigericin. Moreover, valinomycin enhanced ΔpH-dependent non-photochemical fluorescence quenching following the OJIP rise. In the presence of valinomycin, the transthylakoid membrane potential was significantly inhibited as evaluated by measurements of the delayed light emission. The results suggest a membrane potential control of the fluorescence yield in *T. weissflogii*. Possible mechanisms underlying the observed effects of uncouplers are discussed.

1. Introduction

Phytoplanktonic diatoms are an extremely diverse and numerous group of unicellular algae, which play a significant role in aquatic ecosystems. They represent an important basis for the marine food chain and play a crucial role in the biochemical cycles of carbon, nitrogen, silica, and other vital elements thus causing profound effect on the global climate [1,2].

The unique properties providing the ecological and evolutionary success of diatoms are not yet fully understood. In particular, photosynthetic characteristics involve CO_2 concentrating mechanism, operation of both C_3 and C_4 carbon fixation metabolism, reduced photorespiration and elevated chlororespiration, as well as specific structure and composition of photosynthetic membranes [1,3,4]. Thus, membranes in the chloroplast are arranged in groups of three [5]. The photosystem (PS) I and PS II are homogeneously distributed in thylakoid membranes, which lack differentiation into granal and stromal regions [6]. In contrast to higher plants and green algae, the light-harvesting proteins of diatoms are not differentiated into minor and major complexes, contain chlorophyll (Chl) *c* instead of Chl *b*, carotenoid fucoxanthin instead of lutein, and diadinoxanthin and diatoxanthin as the xanthophyll cycle pigments (reviewed in [1]). Furthermore, diatoms contain cytochrome c_{553} as electron donor to PS I [7].

In aquatic ecosystems, the intensity and quality of light is highly variable that may cause photoinhibition [8]. Marine diatoms evolved various mechanisms to prevent and alleviate the harmful effects of the fluctuating light on the primary photosynthetic reactions [9,10]. The most important process enabling rapid switch into a photoprotective mode is the light-induced generation of thermal energy dissipation in the PS II antenna. In diatoms it can reach a 5-fold higher level than in plants [11]. This process is accompanied by the non-photochemical Chl fluorescence quenching (NPQ).

The high-intensity Chl fluorescence induction kinetics (OJIP transients) provides valuable information about photosynthetic processes in a time range from microseconds to seconds [12,13]. The OJIP transients have been extensively studied in green algae and plants, whereas very little information is available on diatoms demonstrating unusual shapes of kinetic curve (see e.g. [14]). In the present study we measured and analyzed OJIP transients on the diatom *Thalassiosira weissflogii* in the presence of different uncouplers and inhibitors of electron transport. The obtained results suggest for the novel membrane potential-dependent mechanism involved in the regulation of photosynthetic electron transport and proton translocation shortly after the exposure of the dark adapted cells to the strong light.

2. Materials and methods

2.1. Culture conditions

The diatoms *T. weissflogii* (Grunow) Fryxell et Hasle was grown as batch culture in a nutrient-enriched seawater medium f/2 [15] (salinity 18‰, pH 7.5) at 20 °C and constant shaking. Concentration of K^+ in medium was 5 mM. Photosynthetic photon flux density

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(PPFD) was 30 μE m^{-2} s^{-1} provided by white fluorescent lamps. Light and dark periods were 14 and 10 h, respectively. Cells from 7-day-old cultures (1.8–2.2 × 10^5 cells ml^{-1}) were used for measurements.

Chlamydomonas reinhardtii strain Dang 137 + WT was grown photoheterotrophycally in tris–acetate–phosphate medium, pH 7.0, in Erlenmeyer flasks at 30 °C under continuous illumination (PPFD = 100 μE m^{-2} s^{-1}) and constant shaking.

2.2. Measurement of Chl fluorescence, delayed light emission and $I_{810\ nm}$ – transients

The OJIP transients were recorded with an Aquapen-C (PSI, Brno, Czech Republic) fluorometer equipped with blue and red light sources and enabling measurements at low cell concentrations. Fluorescence was excited 2 s by blue actinic light peaking at 455 nm. PPFD of actinic light was 1500 μE m^{-2} s^{-1}, as proposed for measurements in diatomic algae and dinoflagellates [14]. The OJIP transients were normalized to the minimal fluorescence yield, F_O (point O), assigned to the fluorescence signal at 20 μs after the illumination onset.

The measurements of the delayed light emission kinetics (DLE) were carried out with a custom-built rotating-disc phosphoroscope under the red excitation light (PPFD = 560 μE m^{-2} s^{-1}). The excitation/dark period was 16/4 ms. A signal was recorded during 3.2 ms after 0.4 ms of dark time. Samples used for the OJIP and DLE measurements contained about 2 × 10^5 cells ml^{-1}.

P_{700} redox transitions were measured from changes in absorbance difference at 810 and 870 nm (ΔA_{810}). The measuring system consisted of a PAM-101 control unit (100 kHz modulation frequency) and ED-P700 DW dual-wavelength emitter–detector unit (Walz, Germany). Oxidation of P_{700} was induced by applying a pulse of strong white light (PPFD = 1000 μE m^{-2} s^{-1}). To increase the signal to noise ratio, cells were concentrated on a glass fiber filters (Millipore).

The stock solutions of DCMU, valinomycin, nigericin, and FCCP were prepared by dissolving them in 96% ethanol. Final ethanol concentration in samples was below 1%. The equivalent amount of ethanol was added into control. The incubation with each reagent was carried out in the dark during 3 min. Figures show typical results of at least three replicate experiments.

3. Results

The typical OJIP transients recorded in a marine diatom *T. weissflogii* and green alga *C. reinhardtii* are shown in Fig. 1. The fluorescence kinetics of *C. reinhardtii* is typical for green algae and plants showing three distinct phases of the fluorescence rise from the minimum O to the maximum P level via J and I steps, followed by the gradual decrease in the fluorescence yield. The OJIP rise has been ascribed mainly to the stepwise decrease in the photochemical fluorescence quenching, whereas the subsequent decline in the fluorescence yield is attributed to the non-photochemical processes due to generation of the high-energy-state quenching (qE) in PS II antenna (NPQ phase in a Fig. 1) [16,17]. Unlike *C. reinhardtii*, the I step was fully absent (or significantly reduced) in the fluorescence transients of *T. weissflogii*. Such a shape can be due to the elevated fluorescence yield during the JI phase and/or the reduced fluorescence yield during the IP phase.

Photosynthetic electron transport in thylakoid membranes results in the generation of transmembrane voltage, $\Delta\psi$, which can influence electrogenic reactions proceeding in PS II, PS I, and cytochrome b_6f complex (reviewed in [18]). The ionophore valinomycin functions as a potassium-specific transporter within the cell membrane, thus resulting in a 'short-circuit' with respect to the $\Delta\psi$ at the expanse of K^+. Incubation of *T. weissflogii* in the presence of this reagent caused two major effects on the OJIP transients (Fig. 2). Firstly, it led to the appearance of the distinguished I step due to the decrease in the JI yield and the concomitant increase in the IP level, whereas the OJ phase remained practically unchanged. Secondly, valinomycin increased the rate of the fluorescence declination after the peak P (NPQ phase) indicating thus to the elevated qE level. The observed effects suggest that $\Delta\psi$ gradient could be involved in the modulation Chl fluorescence yield during the JIP rise and the subsequent descending phase.

The $\Delta\psi$ gradient can be reduced at high concentrations of potassium ions. Short incubation of *T. weissflogii* cells with 20 mM KCl (initial concentration of K^+ in the medium was 5 mM) insignificantly restored the IP phase and slightly accelerated the NPQ phase (Fig. 2). However, the observed effect was negligible as compared to that one caused by valinomycin which is probably due to the limited access of potassium ions to the photosynthetic membranes in intact cells.

In order to check that the addition of valinomycin was followed by a decrease in the $\Delta\psi$, the light-induced kinetics of DLE was measured in the presence of this ionophore. The amplitude of the millisecond component of DLE is proportional to the rate of the backward reactions in PS II [19]. High $\Delta\psi$ is known to promote the backward electron flow in PS II thus increasing the DLE intensity. The DLE transients recorded between 100 ms and 2 s of *T. weissflogii* illumination with and without valinomycin are shown

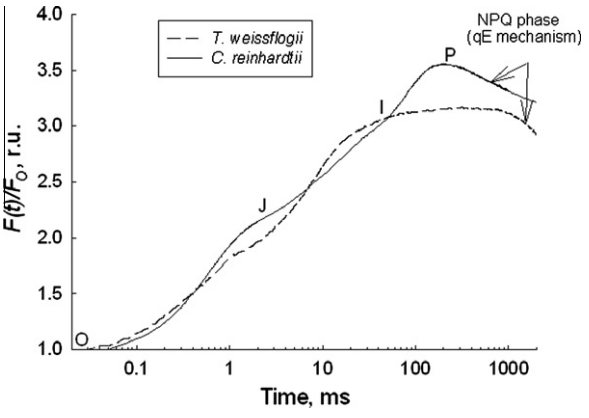

Fig. 1. OJIP transients recorded in a diatom *T. weissflogii* and in a green alga *C. reinhardtii*. Data for *C. reinhardtii* were taken from [14]. Before measurements cells were placed in the dark for 3 min.

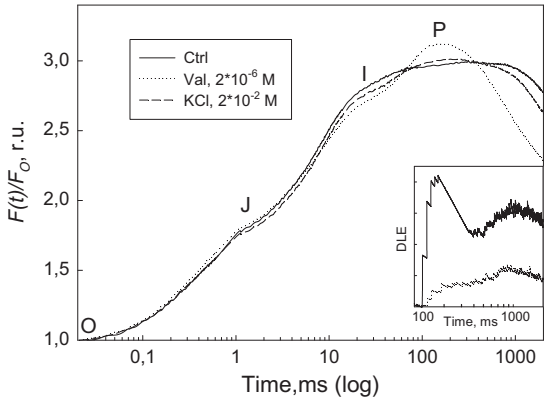

Fig. 2. OJIP transients measured in *T. weissflogii* in the presence of 2 μM valinomycin and 20 mM KCl. After addition of KCl samples were incubated 15 min in the growth light, followed by 3 min incubation in the dark. Inset demonstrates light-induced DLE kinetics recorded in *T. weissflogii* with and without 2 μM valinomycin.

in the inset of Fig. 2. As seen in a figure, the DLE signal was reduced by 60–80% in the presence of valinomycin that testifies to the inhibition of $\Delta\psi$.

Measurements of differential absorbance changes at 810–870 nm (ΔA_{810}) are used to study the photo-induced alterations in the redox state of PS I primary donor, chlorophyll P_{700}. White-light-induced redox changes of P_{700} were recorded in *T. weissflogii* in the presence of DCMU, an inhibitor of electron transport in PS II, and valinomycin (Fig. 3). Rapid increase of the absorbance signal to a steady-state level was observed in the presence of DCMU, reflecting P_{700} oxidation in the absence of PS II activity. In the absence of DCMU, P_{700}^{+} accumulated initially rapidly and then the signal amplitude decreased transiently, indicating re-reduction of P_{700}^{+} by electrons coming from PS II. Re-oxidation of P_{700} is slow during the re-reduction phase because enzymes of the Calvin–Benson cycle are inactive after dark adaptation. The transient re-reduction of P_{700}^{+} occurs simultaneously with the IP step of OJIP transients of chlorophyll fluorescence (about 40–200 ms) [20]. After this phase, P_{700} was gradually re-oxidized, apparently because of light-induced activation of stromal enzymes. Addition of valinomycin caused only a minor increase in the rate of P_{700}^{+} re-reduction, suggesting that valinomycin caused either an increase in the rate of intersystem electron transport or a decrease in the rate of P_{700} oxidation.

Furthermore, we examined effects of the ionophores FCCP and nigericin on the OJIP transients of *T. weissflogii*. FCCP is a powerful protonophore annihilating both proton gradient and membrane potential across thylakoid membranes. Nigericin promotes the exchange between K^+ and H^+ in equal portions thus dissipating ΔpH without causing an inhibitory effect on the $\Delta\psi$ value. The OJIP transients recorded on *T. weissflogii* in the presence of FCCP and nigericin are shown in Fig. 4. Both reagents altered kinetic curves in similar manner causing the elevation of the fluorescence yield during the OJ and IP phases, as well as the suppression of the subsequent NPQ stage. The alterations of the OJ and NPQ were similar to those observed in the presence of the non-saturating concentrations of DCMU (data not shown). We assumed these results indicate to the partial PS II inhibition by FCCP and nigericin. Besides the inhibitory effect, both reagents led to the pronounced demonstration of the I step as it was observed in the presence of valinomycin. The comparison between the effects of uncouplers revealed that nigericin restored the amplitude of the IP phase in a smaller extent, as compared to the FCCP and valinomycin effects. This result demonstrates that in *T. weissflogii* the fluorescence yield during the IP phase depends primarily on the thylakoid voltage whereas proton gradient *per se* shows a smaller effect. Furthermore, treatment with FCCP stimulated maximal variable fluorescence (F_V or OP amplitude in Fig. 4) that was accompanied by significant increase in the fluorescence yield including F_O (point O) and F_M (point P) values (see inset of Fig. 4).

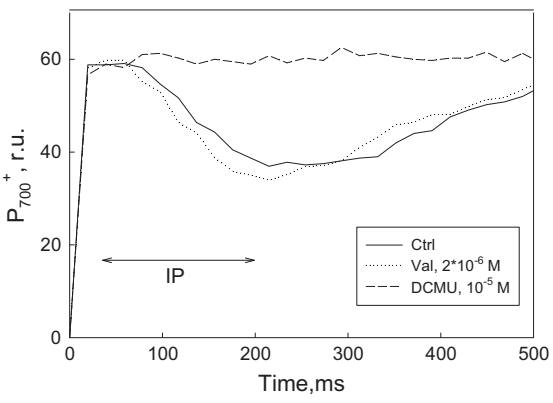

Fig. 3. Kinetics of P_{700} redox transitions measured in *T. weissflogii* control cells and in the presence of 10 μM DCMU and 2 μM valinomycin.

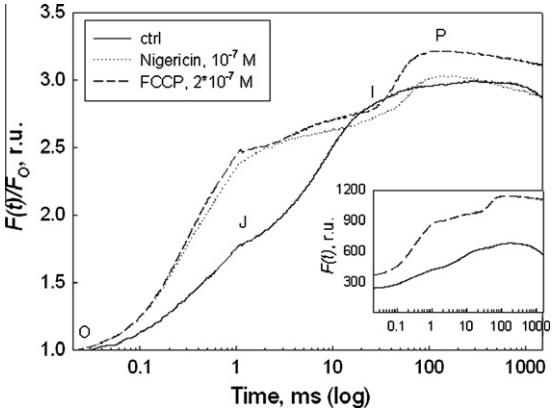

Fig. 4. Chl fluorescence induction of *T. weissflogii* cells in the presence of 0.1 μM nigericin and 0.2 μM FCCP. Inset shows original OJIP transients recorded in the presence of 0.2 μM FCCP.

4. Discussion

The fluorescence transients recorded on dark adapted plants and algae exposed to the strong light are characterized by a multiphasic rise from the minimum (O) to the maximum (P) level via two intermediate steps J and I [12]. The interpretation of the various phases of the OJIP transients is still a matter of discussions (see [17] and references therein). It has been widely accepted that the OJ phase reflects the reduction of Q_A in PS II while PQ pool remains in the oxidized state. The fluorescence rise from J to P via the I step is modulated by the redox state of the PQ pool which undergoes a transition from the fully oxidized (J) to the fully reduced (P) state, causing decrease in both photochemical and non-photochemical fluorescence quenching. Non-photochemical quenching by oxidized PQ has been suggested to maximally suppress fluorescence yield at J; thereafter the quenching decreases toward a minimum at P [21]. The unequivocal interpretation of the intermediate step I is still missing in the literature. As proposed in [22], the JI phase reflects the establishment of the equilibrium between the reduction and oxidation of the PQ pool by PS II and cytochrome b_6f, respectively. At this stage, the rate of PQs oxidation is maximal, i.e. the electron carriers beyond cytochrome b_6f are in the oxidized state. The subsequent IP phase has been attributed to the slowing down of PQ pool oxidation because PS I redox constituents turn into the reduced state. This hypothesis agrees with the experimental results demonstrating that after treatment with DBMIB, an inhibitor of PQs oxidation, the fluorescence yield increases at the I step to the maximum P level [23]. Moreover, methyl viologen, an electron acceptor from PS I, was shown to eliminate the IP phase [23] suggesting the involvement of PS I redox components in the modulation of the fluorescence yield at this stage.

Unlike plants and green algae, the OJIP transients of diatom *T. weissflogii* demonstrated significantly reduced IP phase (Fig. 1). The treatment with ionophores, which dissipate either $\Delta\psi$ (valinomycin), $\Delta\psi$ and ΔpH (FCCP), or ΔpH (nigericin) led to the distinct appearance of the I step (Figs. 2 and 4). The strongest effect was observed in the presence of valinomycin and FCCP, both being the inhibitors of $\Delta\psi$, suggesting the membrane potential is a major factor responsible for the 'hidden' I step in the fluorescence transients.

As known, the light-induced $\Delta\psi$ reaches its maximal values in the time range from 10 to 100 ms after the onset of illumination

[18,24] that is within the JIP phase (2–200 ms). As suggested in [25], high $\Delta\psi$ could affect the energy of the $[P_{680}^{+}Pheo^{-}]$ radical pair in PS II thus increasing the fluorescence yield. This mechanism has been proposed to underlie the membrane voltage-dependent stimulation of the fluorescence yield during the JI phase in thylakoid membranes of higher plants [26]. It can be considered, that in *T. weissflogii* high $\Delta\psi$ enhances fluorescence yield during the JI phase according to the proposed mechanism. An alternative explanation assumes $\Delta\psi$-dependent regulation of photosynthetic electron flow through thylakoid membranes at the level of cytochrome b_6f complex. As earlier demonstrated, the cytochrome b_6f activity is sensitive to the membrane potential which slows down re-oxidation of plastoquinol and a coupled proton translocation reaction [27,28]. It is likely that the build-up of high $\Delta\psi$ in *T. weissflogii* under illumination suppresses cytochrome b_6f activity causing increase in the fluorescence yield during the JI phase (a DBMIB-like effect [23]). Membrane potential might affect electron transfer via the cytochrome b_6f complex, and this influence might also affect redox transitions of P_{700}. However, inhibition of $\Delta\psi$ by valinomycin did not cause large alterations to P_{700} redox transitions (Fig. 3) except that P_{700} re-reduction observed between 40 and 200 ms of illumination was slightly faster in the presence than in the absence of valinomycin. The faster re-reduction may reflect faster electron flow between PS II and PS I.

The fluorescence declination observed after the P peak is believed to reflect the light-induced ΔpH-dependent NPQ process coupled to generation of thermal energy dissipation in PS II antenna (qE mechanism) [16]. Treatment with valinomycin led to an essential increase in the slope of the fluorescence decline indicating that high $\Delta\psi$ inhibits qE-related reactions. This result suggests that the high membrane potential slows down generation of transthylakoid ΔpH thus suppressing energy quenching mechanism. The down regulation of proton pumping into lumen may result in the reduced rate of plastoquinol oxidation by cytochrome b_6f complex.

The treatment of *T. weissflogii* with a protonophore FCCP led to increase in the fluorescence yield including F_O, F_M and F_V values (see Fig. 4). This effect can be explained by the capacity to maintain NPQ in the dark adapted state due to chlororespiration [29]. Indeed, in diatoms metabolic activity in dark leads to an enhanced chlororespiration accompanied by the build-up of a proton gradient high enough to drive diadinoxanthin de-epoxidation and hence NPQ [30,31]. Addition of efficient protonophore would dissipate proton gradient maintained in the dark thus inhibiting NPQ and enhancing fluorescence yield.

In conclusion, the mechanism of membrane potential action on the light-induced fluorescence transients of *T. weissflogii* is not fully clear and remains to be elucidated. It could involve changes of the charge separation/recombination reactions in PS II and/or down regulation of the PQ cycle including proton transfer into the lumen. We suppose that the peculiar characteristics of *T. weissflogii* photosynthetic apparatus such as a structure of photosynthetic membranes enable generation of high $\Delta\psi$ which influences primary photosynthetic reactions. It is worth noting that strong transmembrane electrical field could favor dissipative cycle around PS II as proposed in [32]; the latter plays an important role in diatomic algae protecting PS II from oxidative damage under high light illumination [9,33]. The $\Delta\psi$-dependent mechanism can be a part of a feedback regulation called 'photosynthetic control' [34]. Thus, in dark adapted plants and algae the Calvin–Benson cycle is inactive and sudden exposure to the strong light may cause a 'traffic jam' at the acceptor side of PS I which could result in generation of reactive oxygen species [35]. Under these conditions the down regulation of photosynthetic reactions would protect photosynthetic apparatus from oxidative damage. This mechanism together with energy-dependent quenching in PS II antenna can be important for cell adaptation to the fluctuating irradiation in extensively mixing water.

5. Abbreviations

Chl	chlorophyll
PS	photosystem
P_{680}	the primary electron donor in PS II
Pheo	pheophytin
Q_A	the primary quinone electron acceptor in PS II
PQ	plastoquinone
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
DBMIB	dibromothymoquinone
FCCP	p-trifluoromethoxyphenylhydrazone
NPQ	non-photochemical Chl fluorescence quenching
qE	energy-dependent quenching in PS II antenna
O, J, I, P	particular steps of the light-induced Chl fluorescence transients
DLE	delayed light emission
$\Delta\psi$	transmembrane electrical potential
PPFD	photosynthetic photon flux density

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