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ARTICLES

Lateral Transport of Photosynthetically Active Intermediate at Rest and after Excitation of *Chara* Cells

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Abstract—Cytoplasmic streaming is vital for plant cells; however, its relation to cell functions remains largely undisclosed. Microfluorometry of chloroplasts in vivo and measurements of cell surface pH under localized illumination of cell regions located upstream the cytoplasmic flow, at a distance of few millimeters from the analyzed area, is a new means to reveal the role of liquid flow for signal transmission in large cells, such as internodes of characean algae. Properties of photoinduced signals transmitted along the cell can be clarified by comparing the effects of pointed illumination under conditions of continuous and briefly arrested cytoplasmic flow. Chlorophyll fluorescence measurements with the use of saturation pulse method showed that excitation-induced cessation of cytoplasmic streaming, concomitant with the period of localized illumination, caused a significant delay and deceleration of the lateral transmission of the photoinduced signal and, in addition, diminished the peak of maximal fluorescence F'_m in the cell response to propagated signals. The relative extent of the peak suppression was small in cell regions producing light-dependent external alkaline zones and increased substantially for cell regions with slightly acidic external pH. These and other results indicate the possible role of cytoplasmic pH in controlling chlorophyll fluorescence and photosynthetic activity in vivo. When the period of streaming cessation coincided with localized illumination, the velocity of cytoplasmic flow recovered slower than after arrest of the flow without additional illumination. The results are promising for further analysis of regulatory and protective functions of cytoplasmic streaming in photosynthesizing plant cells.

Keywords: Characeae, cytoplasmic streaming, action potential, chlorophyll fluorescence, alkaline and acid zones, proton transport

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INTRODUCTION

Intracellular motility and excitability are basic properties of living organisms. Continuous cytoplasmic streaming integrates the metabolism of various cell parts [1], which is of crucial importance for giant cells, such as internodes of characean algae, whose length can exceed 10 cm [2, 3]. Physiological manifestations of the cyclosis-mediated lateral transport and the nature of transmitted signals are scarcely known. Recent studies revealed the hydrodynamic transmission of signals induced by the localized illumination of *Chara* internodes [4–6]. Continuous outflow of components exchanging between immobile chloroplasts and the flowing cytoplasm at various instants of illumination results in the spatial separation of these metabolites in the liquid flow.

The localized illumination was found to induce successive increase and decrease of F'_m fluorescence in chloroplasts residing downstream from the site of photostimulus application [7, 8]. These data indicate the existence of two functionally active laterally transported intermediates. One of them is characterized by a fast exchange between illuminated chloroplasts and the cytosol; it accounts for the stage of fluorescence increase. The second agent, featuring slow exchange across the chloroplast envelope, gives rise to the delayed non-photochemical quenching of chlorophyll fluorescence. Illumination induces rapid exchange of H^+ and Ca^{2+} between the chloroplasts and the cytoplasm [9–11] and results in the flow of hydrogen peroxide with the streaming cytoplasm after the release of H_2O_2 from photosynthesizing plastids [6, 12]. Protons and Ca^{2+}, as well as H_2O_2, can perform signaling functions in plants and might participate in the transmission of photoinduced signals along the cell. In experiments with intracellular perfusion of *Chara* internodes, chlorophyll fluorescence increased notably with the increase in cytoplasmic pH (pH_c) [7, 8]. Therefore, the upturn of fluorescence after illumination of a distant cell region might be also due to a temporary pH increase in the cytoplasm arriving from the illuminated zone.

The liquid flow transforms dynamic changes of metabolites in the vicinity of immobile chloroplasts to spatially separated fractions. In the flow system, labile

intermediate substances can be preserved by virtue of their continuous removal from the reaction zone. For example, a short-term (~15 s) increase in pH around the illuminated chloroplasts is followed by the pH decrease [11, 13], but the flowing liquid will contain separate fractions with elevated and lowered pH values. The lability (dynamic changes) of the intermediates transported with the streaming fluid can be assessed by comparing fluorescence changes arising in response to localized illumination under conditions of continuous and arrested streaming. It is known that rotational streaming of cytoplasm in excitable plant cells ceases temporally after triggering the action potential (AP) by chemical, mechanical, and electrical stimuli [14]. However, it is not yet known how cyclosis cessation during cell excitation affects the lateral transfer of signaling molecules.

Lateral transport in excitable cells can be conveniently studied with internodes of green characean algae that are close relatives of higher terrestrial plants. The rapid velocity of cytoplasmic streaming in these cells (up to 100 μm/s) is paralleled by electric excitability of the plasma membranes. Unlike muscle fibers contracting on elevation of cytosolic Ca^{2+} concentration, the cytoplasmic streaming in plant cells is temporally arrested upon the increase in intracellular Ca^{2+} level. Parallel measurements of the membrane potential and aequorin fluorescence in *Chara* cells revealed a sharp increase in $[Ca^{2+}]_c$ on the moment of AP generation, which was followed by a gradual decrease and restoration of the initial level within about 30 s [15]. During this period the cytoplasm remains immobile; full recovery of the cytoplasmic streaming takes up to 10 min. Although the inhibitory impact of Ca^{2+} on cytoplasmic motility and myosin activity is beyond any doubt [16, 17], the molecular mechanism of this influence is not yet elucidated. It is supposed that the cessation of streaming after AP generation is caused by Ca^{2+}-dependent phosphorylation of myosin [18, 19].

Following bright pointed illumination of cell regions located at a distance of 1–5 mm upstream the cytoplasmic flow from the observation area, the photoinduced signal emerges in the cytoplasm and propagates with the streaming fluid. This is evident from fluorescence changes of chloroplasts and from the shifts of cell surface pH [4, 5, 7].

The aim of this study was to clarify how fluorescence changes reflecting lateral transport of photosynthetically active metabolite are affected by the arrest of cytoplasmic streaming during localized illumination. The attention was focused on the stage of fluorescence increase prevailing at comparatively dim irradiance of background illumination. Under these conditions, the generation of an AP has no its own strong impact on chlorophyll emission. In order to check the supposed role of protons in the transmission of photoinduced signals, we conducted measurements in cell areas of H^+ extrusion and H^+ inflow. The results imply the lability of the factor responsible for the increase in F'_m fluorescence during lateral propagation of the photoinduced signal. During application of a localized photostimulus, the level of physiologically active metabolite in the irradiated zone undergoes a transient increase, which is in line with the notion that regulation of chloroplast fluorescence and photosynthetic activity involves photoinduced changes in cytoplasmic pH.

MATERIALS AND METHODS

Chara corallina algae were grown in an aquarium at room temperature at scattered daylight. Isolated internodal cells measuring 6–8 cm in length and about 0.9 mm in diameter were placed into artificial pond water containing 0.1 mM KCl, 1.0 mM NaCl, and 0.1 mM $CaCl_2$. The medium was supplemented with $NaHCO_3$ to adjust the pH at 7.0. Prior to experiments the cell was mounted in a transparent organic glass chamber and placed on a stage of an inverted fluorescence microscope Axiovert 25-CFL (Zeiss, Germany).

Parameters of chlorophyll fluorescence in vivo were measured on microscopic cell regions (diameter ~100 μm) with the saturation pulse method using a Microscopy-PAM fluorometer (Walz, Germany) equipped with a ×32/0.4 objective lens. Data represent changes in maximal fluorescence F'_m induced by saturating light pulses and changes in actual fluorescence F_t emitted under background illumination of the whole cell.

The counter-directed cytoplasmic flows in *Chara* internodes occur on diametrically opposite cell sides that are separated by distances up to 900–1000 μm. The cytoplasmic layer, about 10 μm thick, comprises the immobile ectoplasm with tightly packed rows of chloroplasts and the streaming endoplasm. At a sufficiently high numerical aperture of the objective lens, the depth of field does not exceed few micrometers. Therefore, one can selectively measure fluorescence of chloroplasts residing on the lower cell side where the direction of streaming is well defined.

The signal from a photomultiplier was fed into the PAM Control Unit of pulse-amplitude modulation system, digitized by means of an AD converter PCI-6024E (National Instruments, USA), and displayed on a computer monitor using WinWCP program (Strathclyde Electrophysiology Software).

The background illumination of the whole cell was provided from the microscope upper light source through a blue glass filter SZS-22 ($\lambda < 580$ nm, the maximal photon flux density 100 μmol/(m^2 s). The intensity of background illumination was attenuated by neutral density glass filters. The localized photostimulation of a cell region at a distance $d = 1.5$ mm from the point of measurements was performed with a quartz light guide (optic fiber diameter 400 μm) that was connected to a source of white light (a light-emitting diode Luxeon LXK2-PWN2-S00, Lumileds,

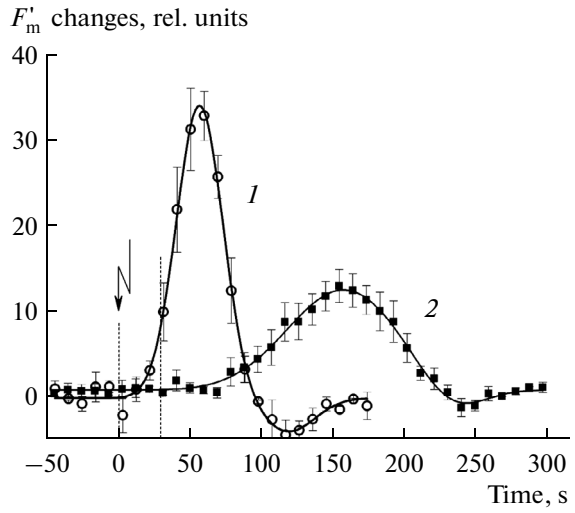

Fig. 1. Changes in F'_m fluorescence on microscopic regions of *C. corallina* internodal cell (regions with pH_o ~ 7.0) caused by transmission of the photoinduced signal under continuous and interrupted cytoplasmic streaming. (*1*) Under continuous streaming; (*2*) after synchronous arrest of cytoplasmic streaming and turning on the localized light at time $t = 0$ (*arrow* in this and other figures marks the moment of an AP elicitation by a pulse of electric current). The cell was exposed to localized illumination for 30 s (white light, 500 μM photons/(m^2 s), optic fiber diameter 400 μm, the beginning of light pulse at $t = 0$) at a distance of $d = 1.5$ mm upstream from the point of fluorescence measurements. The intensity of whole-cell background illumination was 35 μmol/(m^2 s). Data are mean values and standard errors ($n = 3$). Trace *2* was plotted from individual subtracted curves, as obtained by taking off the AP-induced F'_m changes from the F'_m fluorescence signals induced by simultaneous application of the AP and localized lighting. Vertical *dashed lines* here and in Figs. 2, 3 mark the period of localized lighting.

USA). The photon flux density at the output of the light guide was 500 μmol/(m^2 s) [4]. In order to reduce the number of intermediates traded between the chloroplasts and cytoplasm in the intense light, we used comparatively short pulses of localized illumination (30 s). In this case the cytoplasmic composition is modulated by rapidly exchanging components. For the purpose of comparison we used also longer light pulses (60 s). The free end of the light guide was fixed in the holder of a KM-1 micromanipulator under the angle of 30°–45° to a horizontal plane. After adjusting the light guide position in the view field, the optic fiber was displaced to a 1.5-mm distance upstream the cytoplasmic flow with respect to the analyzed region.

The local pH on the outer cell surface (pH_o) was measured with glass-insulated antimony pH-microelectrodes having tip diameters of 5–15 μm. The slope of the electrode function was about 54 mV/pH unit. Measurements of pH_o allowed us to identify the cell regions with active H^+ extrusion (pH_o 6.2–6.7), neutral regions (pH_o ~ 7.0), and the areas of passive H^+ inflow (pH_o 8–10).

The action potential (AP) generation and the arrest of cytoplasmic streaming were triggered by passing a short pulse of transcellular electric current (10 μA, 150 ms). The electrodes used for this purpose were isolated from the pH-measuring circuit; they were placed in electrically insulated compartments of the experimental chamber. The streaming in *Chara* cells reappears in about 30 s after the AP generation and attains its maximal velocity within about 10 min [20]. The velocity of cyclosis was determined in the transmitted light before the onset of fluorescence measurements.

The chloroplast fluorescence is sensitive to the AP generation, with the response amplitude depending on irradiance and the direction of H^+ transport in analyzed areas [21, 22]. Considering this circumstance, low intensities of background illumination were used to minimize the AP-induced fluorescence shifts. In order to exclude these shifts entirely and to extract the response attributed to the arrest of cytoplasmic streaming, we subtracted the fluorescence signals caused by the AP generation in the absence of localized lighting from the signals induced by simultaneous application of localized lighting and the AP generation.

Data in figures show results obtained at least in triplicate with different cells. Experimental curves with error bars are averaged data and standard errors of the means.

RESULTS

Lateral transmission of photoinduced signals under continuous and interrupted cytoplasmic flows. Figure 1 shows changes of chloroplast fluorescence in an intact *C. corallina* cell in response to localized 30-s illumination of a distant cell region positioned 1.5 mm upstream from the analyzed cell area. Measurements were performed on cell regions where pH in the adjacent outer medium (pH_o) was near to pH 7.0 of the bulk solution. Under continuous cytoplasmic streaming (resting state) at photon flux density of 35 μmol/(m^2 s), the F'_m, changes induced by illumination of a distant region were evident as a sharp peak at a time around $t = 50$ s from the onset of the light pulse (curve *1*). These changes were approximated by Gaussian curves [4, 7] corresponding to the movement of one or two active components with the liquid flow.

Curve *2* in Fig. 1 displays fluorescence change in response to a similar light pulse for the case when cytoplasmic streaming was arrested immediately on the onset of localized illumination. The cessation of cyclosis was elicited by the AP generation triggered with a suprathreshold pulse of electric current. Nearly equal durations of localized illumination (30 s) and full stoppage of streaming imply that the cytoplasm

stayed unmoving throughout the period of localized illumination.

The detection of fluorescence shifts following the recovery of streaming after illumination of unmoving cytoplasm is complicated by the fact that the AP generation exerts its own influence on fluorescence and photosynthetic activity [21, 22]. It is known that chlorophyll fluorescence transients after cell excitation are comparatively small in cell regions with neutral and slightly acidic pH_o. The fluorescence responses to electric excitation are almost fully eliminated by subtracting the AP-induced F'_m changes from the F'_m changes caused by simultaneous application of localized illumination and the AP. Curve *2* in Fig. 1 shows the subtracted F'_m kinetics illustrating the propagation of a photosynthetically active intermediate from the illuminated spot during recovery of cytoplasmic streaming after the temporal arrest of flow for a period of photostimulus application.

It is seen that the localized illumination combined with cell excitation induced the increase in F'_m, with a delay period lasting longer than in the case of continuous flow. The shape of the curve was fitted with a sum of two Gaussian curves. The peak of F'_m after the period of arrested movement was shifted approximately by 100 s (maxima on Gaussian curves at $t = 56.5 \pm 0.4$ and 158.7 ± 2.3 from the onset of localized lighting under resting state and after AP generation, respectively). Assuming that the AP generation arrested the flow for 30 s and that the development of F'_m changes after arrival of regulatory signal takes 20 s [4], we calculated that the intermediate crossed the distance of 1500 μm within 108 s; i.e., it moved at an average velocity of 14 μm/s, which is in line with reported velocities of cytoplasmic streaming in *Chara* at various periods after AP generation [14].

When the light guide was positioned at an equal distance on the opposite side from the point of fluorescence measurement, the localized lighting produced no appreciable fluorescence changes, both under continuous and the AP-interrupted streaming. The results provide clear evidence for the transfer of photosynthetically active intermediate with the cytoplasmic flow.

The width of the F'_m signal observed after temporal cessation of cyclosis was 80.1 ± 4.4 s on the average, exceeding almost 2.5-fold the width of the Gaussian band under continuous streaming (32.8 ± 0.9 s). Such broadening of the band indicates that the "irradiated" cytoplasm passed the site of fluorescence measurement at a slower velocity after the AP generation than in the resting state. Remarkably, the amplitude of F'_m signal was almost 2.5 times lower after irradiation of chloroplasts under stopped flow compared to control conditions. Apparently, the content of the effector substance decreased after illumination in the stopped flow, which indicates the lability (dynamic changes) of the metabolite responsible for the increase in F'_m. It appears that the factor elevating F'_m and releasing nonphotochemical quenching is partly lost during illumination of chloroplasts in unmoving cytoplasm.

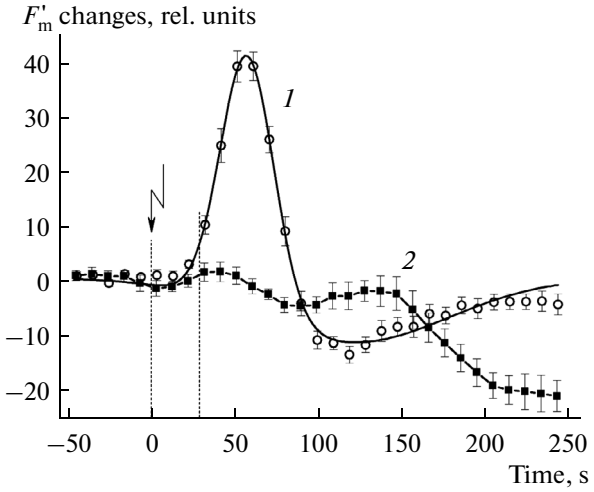

Fig. 2. Changes in F'_m reflecting the passage of photosynthetically active metabolite across the site of fluorescence measurements in cell regions underlying external acidic zones (pH_o 6.2–6.5). Conditions of localized illumination and distance *d* between the light guide and the center of analyzed area are indicated in the caption to Fig. 1; intensity of background illumination was 18–20 μmol photons/(m^2 s). (*1*) Under continuous cytoplasmic streaming; (*2*) after simultaneous arrest of streaming and the onset of localized 30-s illumination (*arrow* marks the moment of the AP generation). Data are mean values and standard errors ($n = 15$ and 8 for curves *1* and *2*, respectively). Trace *2* was obtained by averaging the F'_m transients measured directly, without calculating the subtracted curves; the trace comprises weak waves caused by the AP generation (at $t \sim 40$ s) and by restoration of cytoplasmic streaming (at $t \sim 150$ s).

Different losses of the transported component during cessation of cytoplasmic flow in the regions of H^+ extrusion and H^+ inflow. At low intensity of background illumination, the AP generation does not induce substantial F'_m changes in chloroplasts, irrespective of their location under the acid or alkaline zones [22]. In this case the influence of cyclosis cessation on the lateral transport of active intermediate is clearly seen even without plotting the subtracted curves. Figure 2 shows cyclosis-mediated F'_m changes in cell regions with active extrusion of cytoplasmic protons to the medium (pH_o 6.2–6.5) for the resting cells (curve *1*) and after the arrest of the flow by triggering an AP at the beginning of localized photostimulation (curve *2*). It is seen that temporal cessation of cyclosis caused a stronger suppression of F'_m peak in zones with low pH_o than in cell regions with neutral pH_o.

In zones of external alkalinization under dim background light, localized photostimulation of a distant

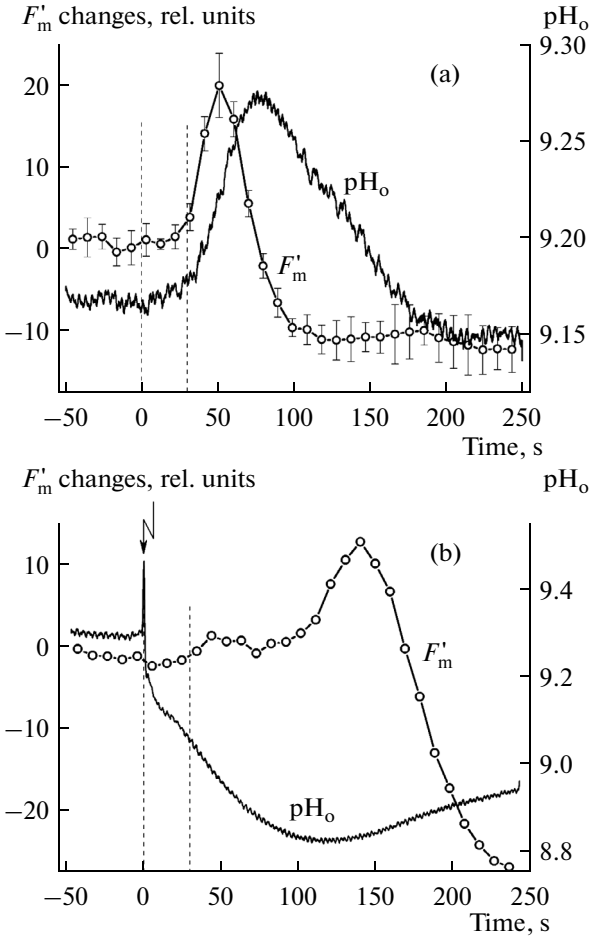

Fig. 3. Changes in F'_m and cell surface pH (pH_o) associated with the passage of photosynthetically and membrane-active metabolite across the area of fluorescence measurement in cell regions underlying the alkaline zones (pH_o 9.0–9.5). Conditions of localized illumination and distance *d* between the light guide tip and the center of analyzed area are indicated in the caption to Fig. 1; intensity of background illumination was 20 μmol photons/(m^2 s). (a) Parallel increase in F'_m and pH_o following localized illumination under continuous cytoplasmic streaming ($n = 4$); (b) Transients of F'_m and pH_o induced by synchronous elicitation of AP and the onset of localized illumination. The trace F'_m in (b) is an unprocessed record; it comprises a small wave caused by the AP generation (at $t \sim 40$ s) and the increase upon restoration of cytoplasmic flow (at $t \sim 140$ s).

region in resting cells led to the increase in pH_o at the measurement site (Fig. 3a). The alkaline shift of pH_o did not attain its maximal level because of comparatively short duration (30 s) of localized lighting. The flow-assisted increase in F'_m occurred synchronously with the pH_o increase, which indicates the common nature of processes underlying these events. In the case of localized lighting of chloroplasts under arrested cytoplasmic flow (Fig. 3b), the F'_m peak was delayed but its amplitude remained rather high. The AP generation was accompanied by a transient pH_o lowering, as previously described [23, 24].

Thus, the amplitudes of F'_m peaks after illumination of chloroplasts in the unmoving cytoplasm differed substantially for H^+-extruding and H^+-absorbing cell regions. In Fig. 4 the normalized F'_m shifts induced by localized lighting under arrest of cytoplasmic streaming are plotted as a function of pH_o in various cell regions. It is seen that the ratios of F'_m shifts induced by localized lighting under arrested and undisturbed cytoplasmic streaming were substantially lower in cell areas with slightly acidic pH_o, than in cell regions with high external pH.

The above results illustrate the influence of localized lighting of chloroplasts in streaming and unmoving cytoplasm on the ascending wave of F'_m changes. Analysis of oppositely directed F'_m changes at high-intensity background light encounters complications due to enlargement of the AP-induced F'_m responses. A strong long-lasting decrease in F'_m following the AP generation at elevated light intensities indicates that non-photochemical quenching turns saturated. The

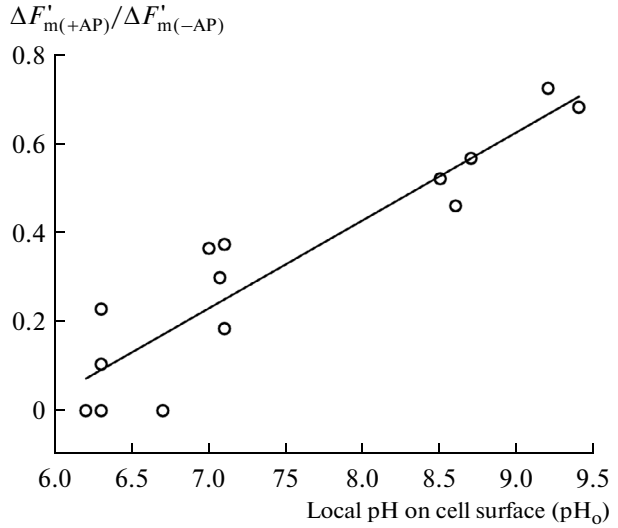

Fig. 4. Correlation between local pH on the cell surface (pH_o) and the extent of F'_m increase in response to the passage of photosynthetically active metabolite after the AP-induced cessation of streaming. The ordinate axis shows F'_m changes observed after simultaneous application of AP and localized light ($\Delta F'_{m(+AP)}$) normalized to F'_m changes following localized illumination under continuous streaming in the absence of AP ($\Delta F'_{m(-AP)}$).

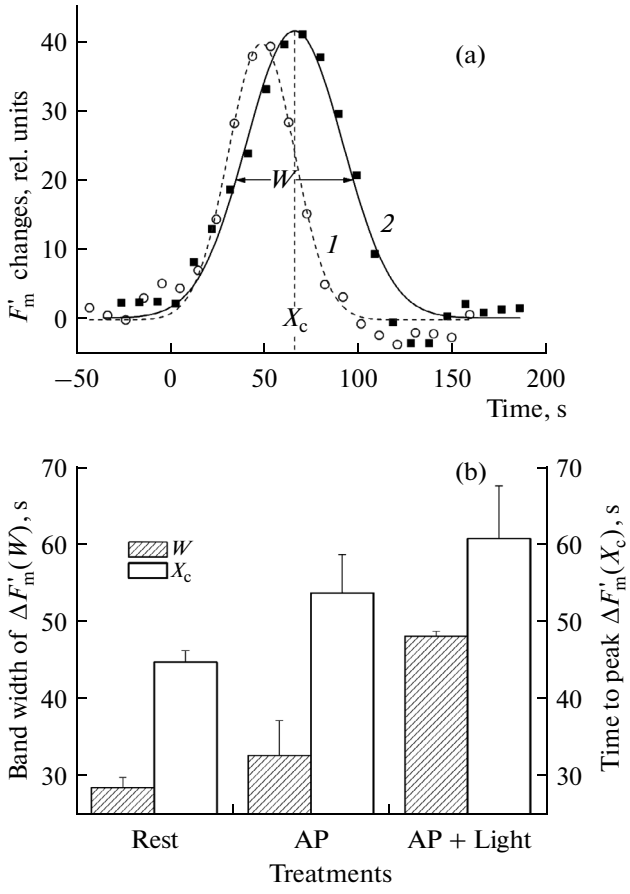

Fig. 5. Effect of preliminary conditions on parameters of F'_m changes induced by a 30-s localized lighting of a cell region upstream from the area of measurements ($d = 1.5$ mm). Measurements of F'_m were performed under continuous cytoplasmic streaming ("Rest"), in 300 s after temporal arrest of streaming ("AP"), and 300 s after the arrest of streaming synchronized with localized illumination ("AP + Light"). (a) Profiles of F'_m distribution during the passage of photosynthetically active metabolite across the measurement area in the resting cell (*1*) and in 300 s after elicitation of the AP synchronized with the onset of 30-s localized illumination (*2*). Marking lines illustrate the peak position of the Gaussian curve (X_c) and the band width at the half height level (W). (b) Dependencies of X_c and W characterizing the F'_m profiles on the preliminary conditions specified above and designated as "Rest" ($n = 9$), "AP" ($n = 4$), and "AP + Light" ($n = 4$).

photoinduced and the AP-induced F'_m signals are not additive at saturation, which makes their decomposition a complicated problem. Properties of the mediator agent, responsible for the F'_m quenching, could be a task for a separate study.

Cytoplasmic streaming recovers slower after the AP generation combined with bright localized lighting than after AP without photostimulation. Temporal stoppage of cytoplasmic streaming had an effect on the position and width of F'_m changes induced by localized illumination (Fig. 1), indicating that the kinetic curves of F'_m changes are a sensitive indicator for the average velocity and recovery of the liquid flow after its interruption. This circumstance can be used for detecting possible disturbance of the liquid flow under excess light. It was supposed earlier that high-intensity light is more injurious to cells in the absence of streaming than under continuous removal of reaction products from the zone of excessive illumination [4, 25]. Slight disturbances might be detected by comparing peak positions and bandwidths of cyclosis-mediated F'_m changes after cell excitation under background illumination alone or in combination with localized lighting.

Therefore, we measured cyclosis-mediated F'_m changes in 300 s after eliciting an AP separately or in combination with localized lighting (Fig. 5). The preliminary treatment "AP + localized lighting" was found to delay the appearance and widen the band of cyclosis-mediated F'_m changes (the increase in X_c and W, Fig. 5a), and such changes were more prominent than after an AP generation in the absence of localized lighting. Changes in parameters X_c and W were fully reversible: when the resting period after AP elicitation was prolonged to 600 s, both parameters returned to the initial values ("Rest" in Fig. 5b). Considering that the position and width of the F'_m peak under standard experimental conditions are determined by velocity of cytoplasmic streaming, we suppose that the streaming

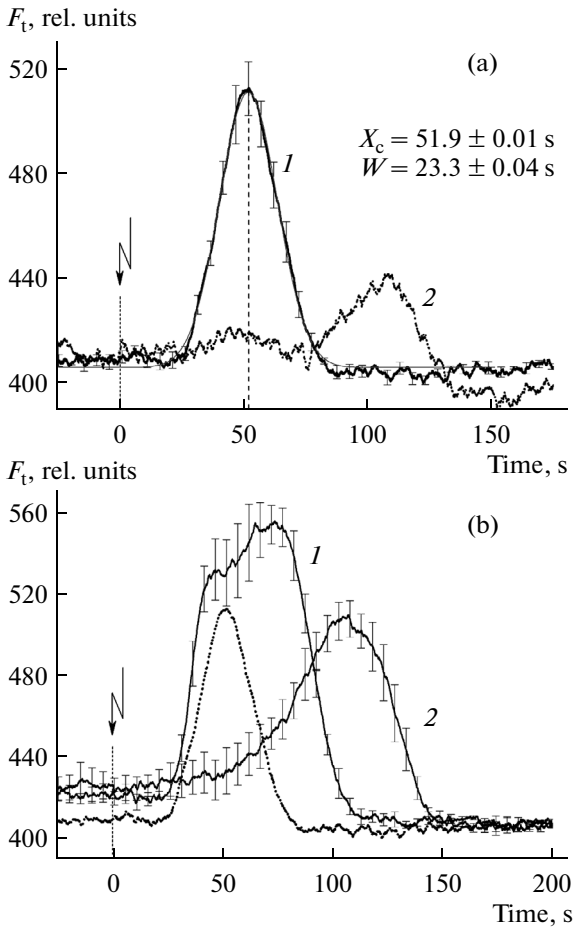

Fig. 6. Transients of F_t fluorescence on microscopic regions of *C. corallina* internode (zone with $pH_o \sim 6.3$) resembling the transmission of photoinduced signal under continuous cytoplasmic flow (curves *1*) and after temporal arrest of streaming by the AP elicitation on the moment of applying the photostimulus at $t = 0$ (curves *2*).

(a) Localized lighting of 30 s duration. A thin solid line shows the approximation of experimental data with the curve of normal distribution; X_c and W designate the position of F_t peak from the beginning of photostimulus and the band width at a half height, respectively. (b) Localized lighting of 60 s duration. For comparison, the F_t changes in response to 30-s illumination are shown with a dotted line. Intensity of background illumination 12.5 μmol photons/(m^2 s), the distance between the centers of locally illuminated and analyzed regions $d = 1.5$ mm. Curves with error bars are averaged records and standard errors of the means ($n = 3$).

velocity recovers slower after the AP generation combined with localized lighting than after AP elicitation in the absence of localized photostimulus.

Lateral transmission of the photoinduced signal as measured by changes of actual fluorescence F_t in background light. Saturating light pulses ensuring full reduction of the primary quinone acceptor of photosystem II Q_a exclude photochemical quenching (qP) during F_m' measurements, which allows the estimation of non-photochemical quenching (NPQ). However, the F_m' measurements are characterized by comparatively low temporal resolution. The resolution is limited by the minimal period of firing saturation pulses, which is 10 s in the Microscopy-PAM system. Measurements of modulated fluorescence F_t under background illumination of the whole cell without saturating light pulses improve the temporal resolution considerably, but it should be noted that the F_t level depends on both NPQ and qP. It was found in previous studies that the passage of "irradiated" cytoplasm across the area of measurement is accompanied by the increase in F_t concurrent with the peak of F_m'; furthermore, the increase in F_t was observed at low and rather high intensities of the background illumination [8]. Since the release of thermal losses in the antenna (lowering of NPQ, the increase in F_m') is accompanied by the increase in F_t, the lateral transmission of photoinduced signals can be traced as F_t with an improved temporal resolution.

Figure 6 shows the profiles of F_t changes arising in response to localized illumination at a distance $d =$ 1.5 mm from the area of measurements under photostimulus durations of 30 and 60 s. In the case of 30-s illumination (Fig. 6a) the profile of the F_t response (curve *1*) fits well to the normal distribution curve ($R^2 = 0.99$). Owing to the large number of points in the records, the position of the peak and the width of the Gaussian curve are determined with an accuracy of 0.1 s (labels X_c and W in Fig. 6a). When the onset of localized illumination at $t = 0$ coincided with the arrest of cytoplasmic streaming (curve *2*), the F_t rise appeared with a delay, peaking at about 110 s, and the F_t peak was diminished; the F_t and F_m' profiles were reshaped in a comparable manner under similar experimental conditions. The earlier development of F_t peak in Fig. 6, compared to fluorescence peaks in Figs. 1–3, was due to a comparatively fast recovery of cytoplasmic streaming. The profile *2* in Fig. 6a, unlike the respective profile in Fig. 1, was not widened after localized illumination of unmoving cytoplasm, which was presumably caused by rapid restoration of streaming velocity after the AP generation.

The F_t changes induced in the resting cell by a 60-s light pulse (Fig. 6b, curve *1*) had a trapezoid shape, with the length at half height comparable to the duration of photostimulus. When the onset of localized photostimulation coincided with an AP elicitation and arrest of cyclosis, the profile of the F_t response shifted its peak to later times and acquired a clearly asymmetric shape (curve *2*). The slope of the rare front was similar to that in the resting cell both for 60- and 30-s light pulses, whereas the steepness of the leading slope increased gradually and was on the whole much lower than in an unexcited cell. The gradual increase in steepness of the rising front reflects the restoration and

acceleration of cytoplasmic streaming. Similar slopes of F_t decline after 60- and 30-s pulses indicate that the cytoplasmic streaming velocities approached the initial values by this period. Comparison of curves *1* and *2* in Fig. 6b did not show widening of the F_t profile, in accordance with Fig. 6a.

DISCUSSION

Our study shows that cyclosis cessation for the period of localized photostimulation delays the occurrence of F'_m wave (Fig. 1). This provides further evidence for the lateral transmission of photoinduced signals affecting chlorophyll fluorescence in vivo.

Alternatively, diffusion of the active intermediate could play the role in its lateral propagation during the arrest of streaming. The diffusion-mediated origin for the AP-induced delay of fluorescence peak cannot be excluded a priory, if protons featuring high coefficient of diffusion in aqueous media ($\sim 10^{-4}$ cm^2/s) are involved as signal carriers. In this case the delayed peak of fluorescence should be evident irrespective of the direction of streaming between the locally irradiated region and the area of measurements. However, when the light guide position was displaced symmetrically with respect to unchangeable area of fluorescence assay (positioning of the optic fiber at a 1.5 mm distance downstream from the point of fluorescence detection), the AP generation at the onset of localized lighting had no discernible effect on fluorescence in the time range up to 250 s (data not shown). Estimates of the mean square displacement for free protons, based on the equation of one-dimensional diffusion, show that the period of streaming cessation (30 s) constitutes about one-fourth of the time required for diffusion to a 1.5-mm distance. Thus, the primary role of diffusion in long-distance signal transmission can be definitely excluded.

At the same time, there is no doubt that diffusion modulates the shape of the transmitted signals. This is because the length of a longitudinal cytoplasmic segment containing the signaling intermediate extends by virtue of diffusion in the period of arrested streaming beyond the limits of illuminated zone. The illuminated zone was 0.4 mm wide; according to estimates, the length of the cell segment containing the signaling agent increases by the moment of streaming recovery to 1.9 mm. This distance is notably lower than the length of cytoplasmic signal expansion achieved under continuous passage of cytoplasm across the zone of localized illumination. At a streaming velocity of 80 μm/s and 30-s illumination period, the length of the cytoplasmic segment contacting with illuminated anchored chloroplasts would be 2.4 + 0.4 = 2.8 mm.

Thus, the delayed formation and the band widening of the analyzed F'_m changes are clear manifestations of the retarded cytoplasmic streaming. The band width (W) is determined by the length of cytoplasmic segment containing the active metabolite and by the average velocity for the passage of this segment across the analyzed area. For comparison, the time X_c of achieving the F'_m peak depends on the average velocity of cytoplasmic movement over the whole period from the onset of localized illumination (from the moment of streaming arrest) to the peak of F'_m. Therefore, the parameters X_c and W are not strictly linked even though their changes in Fig. 5 are largely similar. Measurements of the F'_m and F_t profiles add to the constantly extending list of methods employed in the studies of cyclosis dynamics [26].

Studying the profiles of F'_m and F_t fluorescence allowed us to distinguish the following regimes of cytoplasmic streaming with regard to localized illumination: (1) stationary flow (curves *1* in Figs. 1, 6); (2) non-stationary flow after localized illumination coinciding in time with the period of streaming cessation (curves *2* in Figs. 1, 6); and (3) non-stationary flow when the period of localized illumination was longer than the period of streaming cessation (curve *2* in Fig. 6b). The difference between (1) and (2) is that the length of cytoplasmic segment L exposed in case (1) to direct metabolic exchange with illuminated chloroplasts equals $L = D + V \times t_L$, where D is an optic fiber diameter (diameter of the light spot), V = const is the velocity of cytoplasmic streaming, and t_L is duration of localized photostimulation, whereas in case (2) at $V = 0$ the quantity L is reduced to the diameter D. Non-stationary regime (3) differs from other regimes in that the extent of cytoplasmic segment passed across the illuminated region includes the product of variable velocity V and duration of light stimulus t_L. In general, the width of F'_m and F_t changes depends not only on the velocity of streaming in the time span of fluorescence changes but also on the length L_1 of cytoplasmic segment containing the products of metabolic exchange with illuminated chloroplasts. In the case of streaming arrest over the whole illumination period, the parameter L_1 differs from $L_0 = D$ by the extent of diffusion-mediated expansion of the active intermediate beyond the limits of illuminated region.

The results show that the content of photosynthetically active mediator in the flowing cytoplasm does not reach its maximal level if the localized illumination is applied under arrested streaming (Figs. 1, 6). These observations point to the transient kinetics of the mediator level during the illumination period. It is known, for example, that the light-induced pH shifts near the surface of isolated intact chloroplasts attain a sharp peak in 10–20 s of illumination [11]. When the chloroplast are illuminated for 30 s under arrested flow of the surrounding fluid, the pH level in the fraction of flowing cytoplasm after restoration of streaming would be lower than the transient peak value. Given the cytoplasmic alkalinization relieves non-photochemical

fluorescence quenching, the lowering of pH_c may account for the observed decline in F'_m peak after the arrest of streaming. A sharp pH peak near the surface of intact chloroplasts occurring in the light and the suppression of F'_m peak upon illumination of chloroplasts under arrested cytoplasmic flow seem to be different manifestations of pH_c dynamics as a fluorescence-controlling factor.

Additional evidence in favor of NPQ regulation by means of pH_c changes comes from observations of different lability of the acting factor in cell regions with counter-directed H^+ transport across the plasma membrane (Figs. 2–4). Correlation between the surface pH and the extent of F'_m peak suppression after irradiation of unmoving cytoplasm can be explained in view that the AP elicitation produces opposite shifts of cytoplasmic pH in areas of active H^+ extrusion and passive H^+inflow [27]. The AP generation is known to inactivate the plasma-membrane H^+-conductance in the external alkaline areas [28], which blocks the H^+ influx and should elevate pH_c, whereas the AP-induced stoppage of active H^+ extrusion in the acid areas disturbs the H^+ flux balance toward the decrease in pH_c. We assume that the acidic shift of pH_c in the post-excitation period under external acid zones neutralizes the light-induced increase in cytoplasmic pH, whereas the alkaline shift of pH_c after the AP under external alkaline zones has no such an effect. Speaking differently, the pH dependence shown in Fig. 4 might be based on divergent kinetics of pH_c resulting from superposition of light-induced cytoplasmic alkalinization and excitation-induced pH changes of opposite signs in cell regions underlying the acid and alkaline zones.

Metabolic exchange between illuminated chloroplasts and their environment involves not only labile intermediates whose level undergoes transient changes but also comparatively stable products of photosynthetic metabolism. Accumulation of such products in immobile cytoplasm may disturb interactions of myosin with actin filaments, thereby affecting the dynamics of cyclosis recovery after the AP generation. There are many factors having influence on the rate of streaming. These include changes in cytoplasmic Ca^{2+} concentration, as well as the content of ATP, ADP, phosphate, and some other metabolites. Furthermore, intense illumination is accompanied by generation of reactive oxygen species (ROS), whose local concentration is lowered by virtue of their continuous removal from the reaction site by cytoplasmic flow [6]. Under stoppage of cytoplasmic flow the local concentrations of ROS or other metabolites may increase appreciably, thus causing negative influence on the mobility of cytoplasm. Data presented in Fig. 5 suggest that even rather short (30 s) localized illumination of the cell with unmoving cytoplasm decelerates the recovery of cytoplasmic streaming. Many authors emphasized close relationships between cyclosis and cell viability [1, 2]. These and our data suggest that incidence of high-intensity light on cells with arrested cytoplasmic streaming exerts stronger injuring action than in the cells with active longitudinal flow.

The lateral propagation of photoinduced signals with the cytoplasmic streaming is a useful tool for studying the H^+ (OH^-) plasma-membrane channels whose opening results in light-induced formation of alkaline zones on the cell surface (Fig. 3a). Synchronous increases in pH_o and F'_m during the passage of the photoinduced signals indicate the kinetic link between the pH_o and F'_m changes in cell regions underlying the external alkaline zones. In internally perfused *Chara* internodes, the shift of cytoplasmic pH from neutral to alkaline range relieved the F'_m quenching, whereas the acidic shift enhanced fluorescence quenching [8]. Under dim background illumination, localized lighting induced parallel increases in pH_o and F'_m in cell regions capable of alkaline zone formation. In our view, the opening of $H^+(OH^-)$ plasmalemma channels and the increase in F'_m fluorescence following localized illumination of a distant cell region were caused by the arrival of alkaline cytoplasm from a brightly lightened cell region. At high intensities of background illumination, the increase in F'_m is hidden by large fluorescence changes of opposite polarity; under these conditions the kinetic resemblance of F'_m and pH_o changes cannot be unveiled.

The results of this study open new possibilities for elucidating the regulatory and protective roles of cytoplasmic streaming in photosynthesizing plant cell.

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REFERENCES

1. Pickard W.F. 2003. The role of cytoplasmic streaming in symplastic transport. *Plant, Cell Environ*. **26**, 1–15.
2. Verchot-Lubicz J., Goldstein R.E. 2010. Cytoplasmic streaming enables the distribution of molecules and vesicles in large plant cells. *Protoplasma*. **240**, 99–107.
3. Goldstein R.E., Tuval I., van de Meent J.W. 2008. Microfluidics of cytoplasmic streaming and its implications for intracellular transport. *Proc. Natl. Acad. Sci. USA*. **105**, 3663–3667.
4. Bulychev A.A., Dodonova S.O. 2011. Effects of cyclosis on chloroplast–cytoplasm interactions revealed with localized lighting in Characean cells at rest and after electrical excitation. *Biochim. Biophys. Acta*. **1807**, 1221–1230.
5. Dodonova S.O., Bulychev A.A. 2011. Cyclosis-related asymmetry of chloroplast–plasma membrane interactions at the margins of illuminated area in *Chara corallina* cells. *Protoplasma*. **248**, 737–749.

6. Eremin A., Bulychev A.A., Hauser M.J.B. 2013. Cyclosis-mediated transfer of H_2O_2 elicited by localized illumination of *Chara* cells and its relevance to the formation of pH bands. *Protoplasma*. **250**, 1339–1349.
7. Bulychev A.A., Alova A.V., Rubin A.B. 2013. Propagation of photoinduced signals with the cytoplasmic flow along Characean internodes: Evidence from changes in chloroplast fluorescence and surface pH. *Eur. Biophys. J.* **42**, 441–453.
8. Bulychev A.A., Alova A.V., Rubin A.B. 2013. Fluorescence transients in chloroplasts of *Chara corallina* cells during transmission of photoinduced signal with the streaming cytoplasm. *Russ. J. Plant Physiol*. **60**, 33–40.
9. Harada A., Shimazaki K. 2009. Measurement of changes in cytosolic Ca^{2+} in *Arabidopsis* guard cells and mesophyll cells in response to blue light. *Plant Cell Physiol*. **50**, 360–373.
10. Miller A.J., Sanders D. 1987. Depletion of cytosolic free calcium induced by photosynthesis. *Nature*. **326**, 397–400.
11. Remiš D., Bulychev A.A., Kurella G.A. 1988. Photoinduced pH changes in the vicinity of isolated *Peperomia metallica* chloroplasts. *J. Exp. Bot*. **39**, 633–640.
12. Naydov I.A., Mubarakshina M.M., Ivanov B.N. 2012. Formation kinetics and H_2O_2 distribution in chloroplasts and protoplasts of photosynthetic leaf cells of higher plants under illumination. *Biochemistry* (Moscow). **77**, 143–151.
13. Felle H., Bertl A. 1986. Light-induced cytoplasmic pH changes and their interrelation to the activity of the electrogenic proton pump in *Riccia fluitans*. *Biochim. Biophys. Acta*. **848**, 176–182.
14. Kamiya N. 1959. *Protoplasmic streaming*. Wien: Springer.
15. Williamson R.E., Ashley C.C. 1982. Free Ca^{2+} and cytoplasmic streaming in the alga *Chara*. *Nature*. **296**, 647–651.
16. Tominaga Y., Shimmen T., Tazawa M. 1983. Control of cytoplasmic streaming by extracellular Ca^{2+} in permeabilized *Nitella* cells. *Protoplasma*. **116**, 75–77.
17. Yokota E., Muto S., Shimmen T. 1999. Inhibitory regulation of higher-plant myosin by Ca^{2+} ions. *Plant Physiol*. **119**, 231–239.
18. Shimmen T. 2007. The sliding theory of cytoplasmic streaming: Fifty years of progress. *J. Plant Res*. **120**, 31–43.
19. Awata J., Saitoh K., Shimada K., Kashiyama T., Yamamoto K. 2001. Effects of Ca^{2+} and calmodulin on the motile activity of characean myosin in vitro. *Plant Cell Physiol*. **42**, 828–834.
20. Tsuchiya Y., Yamazaki H., Aoki T. 1991. Steady and transient behaviors of protoplasmic streaming in *Nitella* internodal cell. *Biophys. J*. **59**, 249–251.
21. Bulychev A.A., Kamzolkina N.A. 2006. Differential effects of plasma membrane electric excitation on H^+ fluxes and photosynthesis in characean cells. *Bioelectrochemistry*. **69**, 209–215.
22. Bulychev A.A., Kamzolkina N.A. 2006. Effect of action potential on photosynthesis and spatially distributed H^+ fluxes in cells and chloroplasts of *Chara corallina*. *Russ. J. Plant Physiol*. **53**, 1–9.
23. Bulychev A.A., Kamzolkina N.A., Luengviriya J., Rubin A.B., Mueller S.C. 2004. Effect of a single excitation stimulus on photosynthetic activity and light-dependent pH banding in *Chara* cells. *J. Membr. Biol*. **202**, 11–19.
24. Krupenina N.A., Bulychev A.A. 2007. Action potential in a plant cell lowers the light requirement for non-photochemical energy-dependent quenching of chlorophyll fluorescence. *Biochim. Biophys. Acta*. **1767**, 781–788.
25. Bulychev A.A. 2012. Membrane excitation and cytoplasmic streaming as modulators of photosynthesis and proton flows in Characean cells. In: *Plant electrophysiology: Methods and cell electrophysiology*. Ed. Volkov A.G. Berlin: Springer, p. 273–300.
26. van de Meent J.W., Sederman A.J., Gladden L.F., Goldstein R.E. 2010. Measurement of cytoplasmic streaming in single plant cells by magnetic resonance velocimetry. *J. Fluid Mech*. **642**, 5–14.
27. Krupenina N.A., Bulychev A.A., Roelfsema M.B.G., Schreiber U. 2008. Action potential in *Chara* cells intensifies spatial patterns of photosynthetic electron flow and non-photochemical quenching in parallel with inhibition of pH banding. *Photochem. Photobiol. Sci*. **7**, 681–688.
28. Bulychev A.A., Krupenina N.A. 2009. Transient removal of alkaline zones after excitation of *Chara* cells is associated with inactivation of high conductance in the plasmalemma. *Plant Signal. Behav*. **4**, 24–31.

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