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RESEARCH PAPERS

Induction of Alkaline Bands in *Chara* by External Electric Current Reveals the Influence of Plasma Membrane H^+ Fluxes on Cyclosis-Mediated Signaling

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Abstract—Long-distant communications in plants and giant plant cells are essential for optimal cell functioning under variable environmental conditions. In characean internodal cells exposed to spotted or flickering light, the redox balance in dimly lit chloroplasts is sensitive to the reducing power produced in brightly illuminated chloroplasts of a distal cell region located upstream in the directed cytoplasmic flow. The distant communication is mediated by excessive production in the high-light region of reducing metabolites that are exported into the streaming cytoplasm and delivered by cytoplasmic flow to light-starving chloroplasts. These target chloroplasts perceive the transportable metabolic signal, which is reflected in the transient increase of modulated chlorophyll fluorescence *F*'. Previous studies of cyclosis-mediated *F*' transients revealed that they are sensitive to natural H^+ fluxes across the plasma membrane and that the signal transduction is suppressed in cell areas with massive H^+ influx. In this study with *Chara australis* R. Br., we show that the injection of inward electric current is accompanied by proton influx sufficient for the creation of artificial alkaline band. The combined application of PAM chlorophyll microfluorometry, local light stimuli, and measurements of pericellular pH showed that the microfluidic signal transduction is notably suppressed under artificially produced alkaline band where H^+ influx acidifies the cytoplasm. It is hypothesized that changes in cytoplasmic pH regulate the rates of electron flows directed to NADP reduction and O_2 reduction, which may disturb long-distance redox control system involving production and consumption of reducing equivalents.

Keywords: *Chara australis*, chlorophyll microfluorometry, microfluidic signaling, hyperpolarization, plasmalemmal H^+ flows, electrically induced alkaline band

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INTRODUCTION

The long-range signaling in plants is highly important for proper responses to biotic and abiotic challenges under variable environmental conditions [1–3]. In giant cells of characean algae, the long-distance communication relies mainly on two mechanisms: the propagation of action potentials (APs) and the cyclosis-mediated transmission of metabolites [4–6]. Although the rate of cytoplasmic streaming is almost 100 times lower than the velocity of AP transmission along the internode, it might still be adequate for the delivery of metabolic signals to remote cell parts under slowly changing environment. Pulse amplitude-modulated (PAM) chlorophyll microfluorometry provides an effective means to track the cyclosis-mediated transmission of photometabolites having regulatory action on photosynthesis [7]. Application of an intense 30-s local light (LL) pulse produces a transient increase in chlorophyll fluorescence yield *F*' at various distances (up to 10 mm) from the illuminated cell site after a lag period from 10 up to 120 s [6]. A primary step in the complex mechanism underlying *F*' transient is presumably the export of excessive NADPH from brightly illuminated chloroplasts into the streaming cytoplasm by "malate valve" [8, 9]. The next steps comprise the transfer of reducing power with the cytoplasmic flow and the entry of reducing equivalents into shaded chloroplasts via the envelope membranes along the concentration gradient [10]. The elevation of stromal NADPH concentration in sink plastids leads finally to plastoquinone reduction by means of chloroplast NADH dehydrogenase-like (NDH) complex and the antimycin A-sensitive pathway [11, 12].

Because the transduction of signals arising in source chloroplasts exposed to bright light involves many stages, the recorded *F*' fluorescence transients are sensitive to multiple factors, such as the rate of cytoplasmic streaming, functional condition of membrane transporters, gradients of metabolite concentrations between the cytosol and stroma at the source and sink sites, and electron transport rate in chloroplasts of shaded cell areas. The *F*' transients caused by local

light ($\Delta F'_{LL}$) were large in cell regions adjacent to external acidic zones where the plasma membrane H^+-pump activity is high and were low under external alkaline zones featuring high passive H^+/OH^- conductance [10, 12, 13]. Apparently, the microfluidic signal transduction is controlled by site-specific H^+ flows across the plasma membrane (PM). The direction and magnitude of H^+ fluxes might affect chloroplast functions by influencing the cytoplasmic pH (pH_{cyt}). There is evidence that pH_{cyt} is acid-shifted in the regions of H^+ influx (under external alkaline zones) and is alkaline-shifted in regions where H^+ is expelled by the PM H^+-pump [14, 15].

The cytoplasmic pH may affect microfluidic interactions of spatially remote chloroplasts by directing the photosynthetic electron transport along either the CO_2-dependent route or the alternative pathway associated with O_2 reduction [16, 17]. It is known that the lowered pH in the suspension medium of isolated intact chloroplasts facilitates the photoreduction of oxygen [18]. A similar stimulation of O_2 reduction by lowered pH_{cyt} is likely to occur in vivo. If linear electron flow is diverted to O_2 reduction, this may suppress the microfluidic signaling mediated by NADPH transport and metabolism.

Experiments on manipulating cytosolic pH indicated that the suppression of microfluidic signaling (LL-induced F' transients, $\Delta F'_{LL}$) under external alkaline zones is due to the acid-shifted pH_{cyt}. One way to elevate pH_{cyt} is based on the inhibition of H^+ influx in alkaline cell regions after the AP generation [13]. Triggering a single AP greatly diminishes the passive H^+/OH^- conductance of PM [19], which restricts the H^+ influx and transiently eliminates the external alkaline zones. The cessation of H^+ influx in the formerly alkaline zones was indeed accompanied by several-fold increase in the amplitude of $\Delta F'_{LL}$ [13]. The second way to increase the cytoplasmic pH under the alkaline bands consists in the addition of ammonium salts to the bath medium [20, 21]. When the membrane-permeable deprotonated form NH_3 enters the cytoplasm from the medium enriched in NH_4Cl concentration, it binds protons with the production of NH_4^+, thus elevating pH_{cyt}. The addition of 5 mM NH_4Cl to the external medium reduced the pH in the initially alkaline zone from 10 to 6.7, indicating the cessation of inward H^+ flux across the PM. This treatment was followed by a large stimulation of cyclosis-mediated F' changes [13].

The hypothesis that the lowered pH_{cyt} impairs microfluidic signaling between distant chloroplasts needs further validation. In addition to treatments elevating pH_{cyt}, it is useful to apply the treatments that diminish pH_{cyt}. The most direct way of affecting pH_{cyt} is to induce proton flows by applying the electric potential difference across the PM. The negative shift of the electric potential on the cytoplasmic side should drive H^+ inflow (or OH^- outflow) provided the membrane conductance for H^+/OH^- is high. Such an induction of H^+ influx has been recently observed on internodal *Chara* cells that were preconverted to the K^+-conducting state by high external K^+ concentration (100 mM KCl) [22, 23]. The passage of inward electric current across the electrically insulated cell portion of the internode in these conditions is known to inactivate the high K^+-conductance of PM. This inactivation was associated with the appearance of so-called "hyperpolarizing response" (nonlinear increase of the negative electric potential on the cytoplasmic side) [24, 25] and with the formation of external alkaline zone indicative of H^+ influx into the cytoplasm [22]. It is not yet known whether inward H^+ fluxes associated with the formation of external alkaline zones can be induced by injecting electric current at physiologically low K^+ concentrations in the medium.

In this study we aimed to elucidate if the development of H^+ influx during the PM hyperpolarization has an influence on microfluidic signaling, i.e., on F' fluorescence transients induced by local illumination of a distal cell region. For this purpose, recordings of LL-induced F' changes were performed under alternating conditions with and without the injection of hyperpolarizing electric current at elevated (30 mM) and low (0.1 mM) K^+ concentrations in the medium. The results indicate that the inward H^+ fluxes induced by the hyperpolarizing current pulses suppress the cyclosis-mediated signaling. These findings support the previously observed inhibition of $\Delta F'_{LL}$ transients under natural alkaline zones. They are tentatively ascribed as a result of acidic shift of pH_{cyt} that redirects linear electron transport from NADP reduction to O_2 reduction.

MATERIALS AND METHODS

Plant material. The alga *Chara australis* R. Br. was grown in the laboratory under scattered daylight at room temperature. Individual internodal cells (the third internode from the apex, measuring approximately 50 mm in length and 0.9 mm in diameter) were excised from thalli and kept in artificial pond water (APW) containing 0.1 mM KCl, 1 mM NaCl, and 0.1 mM $CaCl_2$. The pH 7 was adjusted with $NaHCO_3$. The cells were kept in APW for at least one day prior to measurements. The majority of fluorometric measurements were performed on noncalcified cells or cell regions. In some cases, calcified areas capable of forming alkaline bands in the light were examined.

The isolated internodal cell was mounted in a multi-compartment chamber in which a small (2 mm wide) central cell segment was electrically insulated from the rest of the internode. The electrical insula-

tion was achieved by means of 4-mm wide air gaps. The construction of the chamber and the electrode arrangement for measuring the membrane potential and passing electric current were described in [23]. The central and side pools were first filled with APW, and later the central pool was refilled in many experiments with APW containing 30 mM KCl.

Electric stimulation and membrane potential measurements. Electric stimulation was accomplished with extracellular Ag/AgCl electrodes placed in the central and side compartments of the chamber. In order to trigger the action potential (AP), square voltage pulses (3 V, 150 ms) were applied from a digital–analog converter (PCI-6024E, National Instruments, United States) through a load resistance of 1.2 MΩ; the polarity of the voltage ensured the plasma membrane depolarization above the threshold value in the central compartment (current density, 25 μA/cm^2). Hyperpolarizing responses (HRs) were induced by passing inward current pulses with the duration of 20–75 s.

The membrane potential changes were measured with Pyrex glass microcapillary electrodes filled with 1 M KCl [17]. The electric current injection and the monitoring of membrane potential were accomplished with electrode circuits that were configured as independent or had a common reference electrode. The electric potential difference between the microelectrode and the reference electrode connected to bath solution via a salt bridge was measured with a U5-9 electrometric amplifier (USSR). The density of hyperpolarizing current pulses varied depending on the plasma membrane conductance and the ionic strength of the medium. When APW contained 30 mM KCl (PM in the K^+-conducting state), the current density varied from 50 to 83 μA/cm^2; at 0.1 mM KCl the current densities were 8.3–23.5 μA/cm^2.

Chlorophyll fluorescence measurements. Chlorophyll fluorescence parameters were measured using an Axiovert 25-CFL inverted microscope (Zeiss, Germany) equipped with a Microscopy-PAM fluorometer (Walz, Germany) as previously described [26, 27]. The analyzed area of interest (AOI) had a diameter of ~100 μm. The internodal cell was illuminated with continuous background light provided with a tungsten lamp of the microscope upper light source (photon flux density 12.5 μmol/m^2 s). This light passed through a blue-green (SZS-22, $\lambda < 580$ nm) and neutral density (NS-3) glass filters. The AOI was additionally illuminated with modulated weak measuring light and saturation pulses emitted by a blue LED of a Microscopy-PAM instrument (Walz, Germany). The primary measured parameters of modulated chlorophyll fluorescence (F' and F'_m) allowed the calculations of the effective quantum yield of photosystem II (PSII) photochemical reaction and non-photochemical quenching (NPQ). These parameters served as indicators of adequate physiological condition of analyzed cells.

Local illumination. The propagation of F' fluorescence wave along the internode with the streaming cytoplasm was induced by the application of a local pulse of white light (~500 μmol quanta/m^2 s, 30 s duration) through a quartz optic fiber (400 μm in diameter). The source of local light (LL) was a neutral-white Luxeon LXK2-PWN2-S00 light-emitting diode (Lumileds, United States). The emission spectrum included wavelengths from 420 to 750 nm. The optic fiber directing the light beam from LED to the cell was positioned normally to the longitudinal cell axis. The distance between the centers of the optic fiber and AOI was 1 mm in most cases. This distance was optimal with account for the restrictions imposed by cable properties of the internode and the necessity to minimize stray light in the vicinity of fiber optic tip. The application of LL pulse induced F' transients in downstream cell regions ($\Delta F'_{LL}$) where light conditions remained constant.

Local measurements of pH on cell surface. pH in the outer medium near cell surface (pH_o) was measured with glass-insulated H^+-specific antimony microelectrodes and a U5-9 electrometric amplifier as previously described [22]. The output of the amplifier was fed to a PCI-6024E analog-to-digital converter (National Instruments) and a computer monitor. The raw readings of the pH microsensor were processed by subtracting the background voltage drop created by electric current on a series resistance of the medium, salt bridge, and the reference electrode.

Figures illustrate the results of representative experiments performed in at least four replicates on different cells. Traces in figures are either individual or averaged curves calculated from several records, with n indicating the number of replicate measurements. The curve with error bars shows the averaged trace and standard errors obtained from the records on different cells.

RESULTS

Effect of Local Light on F' at Rest and After Cell Excitation in Low- and High-[K^+] Media

Since the hyperpolarizing response is observed at elevated external KCl concentrations, it was first necessary to check the effects of high-[K^+] solution (30 mM) on $\Delta F'_{LL}$ transients and find out if elevated K^+ levels in the medium affect cyclosis-mediated signaling. Figure 1a shows that the replacement of standard APW (0.1 mM K^+) with the medium containing 30 mM KCl had no discernible influence on the shape of LL-induced F' changes after prolonged incubation. The kinetics of LL-induced F' changes (n = 13 and n = 4 in the presence of 0.1 and 30 mM K^+, respectively) were similar, even though the plasma membrane (PM) was normally polarized under control

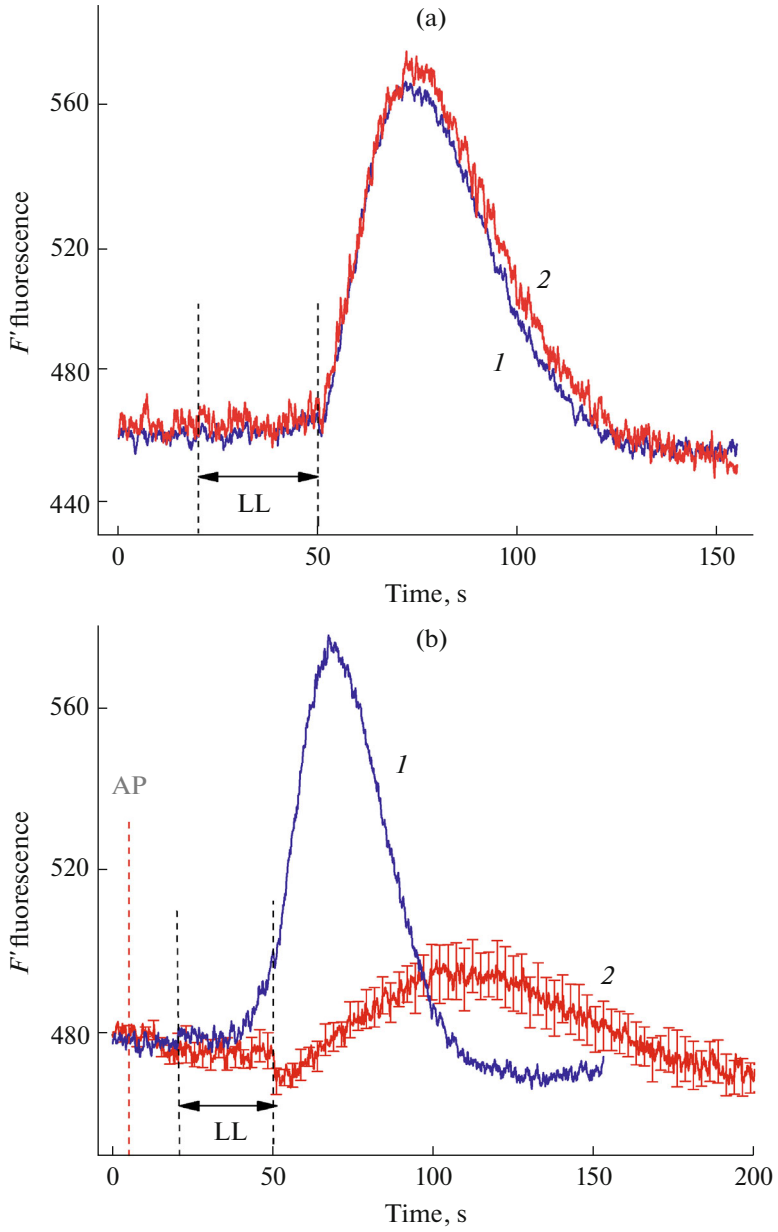


Fig. 1. Transient changes in actual fluorescence yield (F') induced by 30-s local illumination of an upstream cell area. (a) Identical kinetics of $\Delta F'_{LL}$ in the central part of *Chara* internode bathed with APW containing 0.1 mM KCl (curve *1*) and 30 mM KCl (curve *2*) under stationary conditions. (b) Different kinetics of F' changes induced by local light (LL) at resting state (curve *1*) and after triggering the AP in cells bathed with low-[K^+] APW (curve *2*). The curve with error bars represents the averaged kinetics of $\Delta F'_{LL}$ and standard errors obtained from experiments on six cells. Averaged data for individual cells were obtained in 3–5 replicates. The control curve *1* is the average trace obtained on five cells; error bars for this curve were similar to those in electrically stimulated cells. The double headed arrows labeled LL mark the beginning and end of local illumination. The dashed line labeled AP in Fig. 1b indicates the moment of triggering the action potential.

conditions and was depolarized in the medium containing 30 mM KCl. This observation indicates that, under steady-state conditions, the velocities of cytoplasmic streaming were unaffected by K^+ concentration and cell membrane potential in the central compartment. By contrast, the transient depolarization caused obvious changes in the kinetics of $\Delta F'_{LL}$. As can be seen in Fig. 1b for cells bathed with standard APW, the triggering of AP 15 s before the onset of LL reduced the amplitude of $\Delta F'_{LL}$ and delayed the achievement of $\Delta F'_{LL}$ peak by 40 s. The extended lag-period of $\Delta F'_{LL}$ transients stems from the temporal cessation of cytoplasmic streaming and its gradual recovery after the AP generation [5].

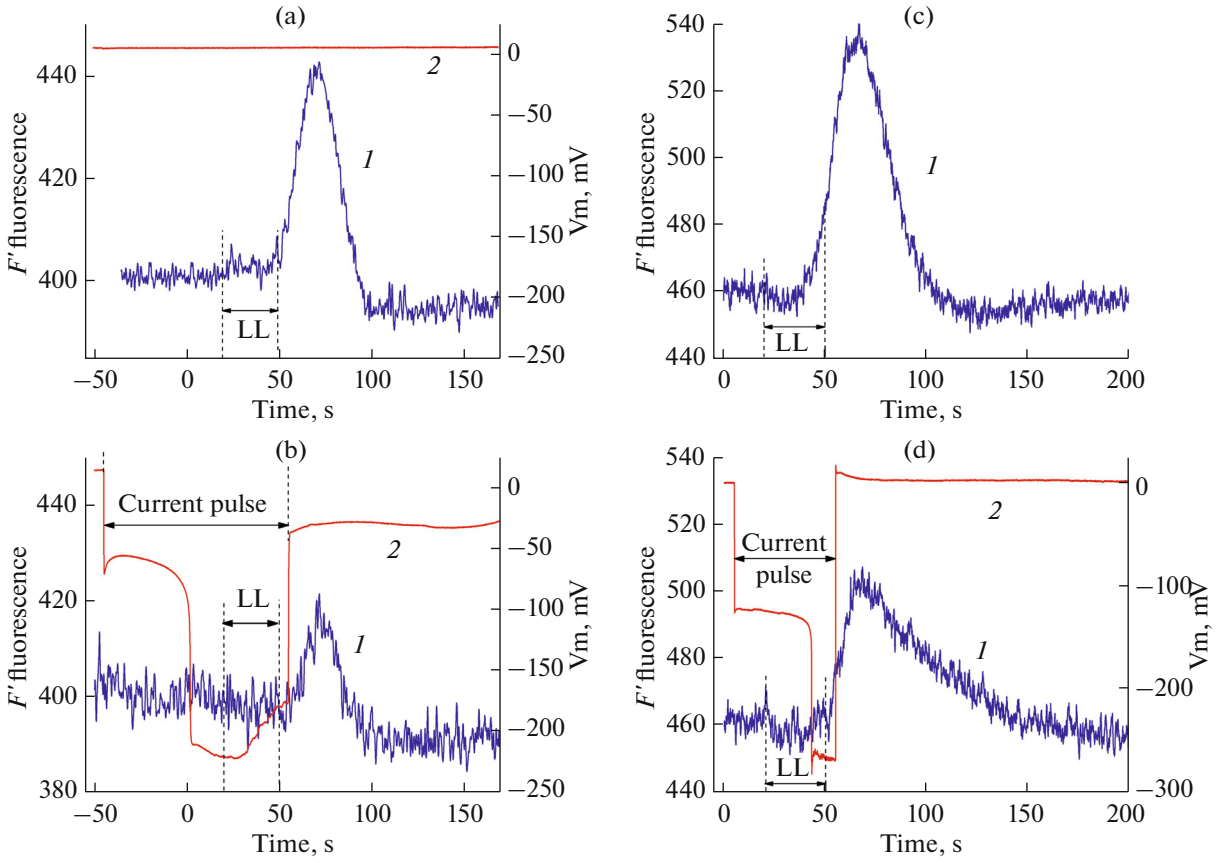


Fig. 2. Suppression of cyclosis-mediated *F'* fluorescence changes ($\Delta F'_{LL}$) and the development of hyperpolarizing response (HR) of the membrane potential Vm under electric current injection in the presence of 30 mM K^+ in the central compartment: (a, b) and (c, d) are examples obtained with different cells. Fluorescence changes $\Delta F'_{LL}$ are shown by curves *1*; membrane potential Vm, by curves *2*. (a) Depolarized Vm and the $\Delta F'_{LL}$ transients in the resting cell ($n = 6$) exposed to local light (LL) treatment. (b) Suppression of $\Delta F'_{LL}$ transients in the same cell as in (a) after eliciting the HR (curve *2*) by inward current with the density of 83 μA/cm^2 ($n = 2$). (c) $\Delta F'_{LL}$ transients for another group of cells in the absence of electric stimulation ($n = 6$). (d) Suppression of $\Delta F'_{LL}$ in the same cells after eliciting the HR by inward current with the density of 67 μA/cm^2 ($n = 6$). Note that the *F'* decline phase in (d) is decelerated. Double-headed arrows mark the moments of electric pulse and local light (LL) application.

Changes in Microfluidic Signaling Related to the Hyperpolarizing Response

The left and right panels in Fig. 2 illustrate the influence of hyperpolarizing response (HR) on microfluidic signaling in various cells. Solid curves labeled Vm in Figs. 2b and 2d show a nonlinear increase of negative electric potential difference across the PM upon the passage of an inward current pulse. The HR consists in a sharp hyperpolarization of PM after a long lag period [24, 28]. This transition results from the inactivation of high K^+ conductance of PM in the presence of elevated K^+ concentrations in the medium [25]. The origin of tail potentials established after the termination of current pulse is not yet clarified. It should be noted that the high negative potential produced during the electric pulse in the cytoplasm provides a strong driving force for the entry of cations, specifically protons. In the absence of current pulse, the PM remained permanently depolarized (Fig. 2a, trace Vm).

The generation of HR under the injection of inward current modified the kinetics of $\Delta F'_{LL}$ in a way that differed from the AP-induced modification of $\Delta F'_{LL}$. In the majority of experiments, the development of HR in cells bathed with APW containing 30 mM KCl was accompanied by the suppression of $\Delta F'_{LL}$. The position of the peak emission remained either unchanged or shifted to longer time, but the time shift was shorter than after triggering the AP. The return of *F'* after the peak to the steady level proceeded at usual rate in some cells (Fig. 2b) or was markedly decelerated in others (Fig. 2d). The delayed relaxation of *F'*, together with visual observation in transparent light, indicates that cytoplasmic streaming in these cells was retarded by the end of current injection. Using microfluorometry,

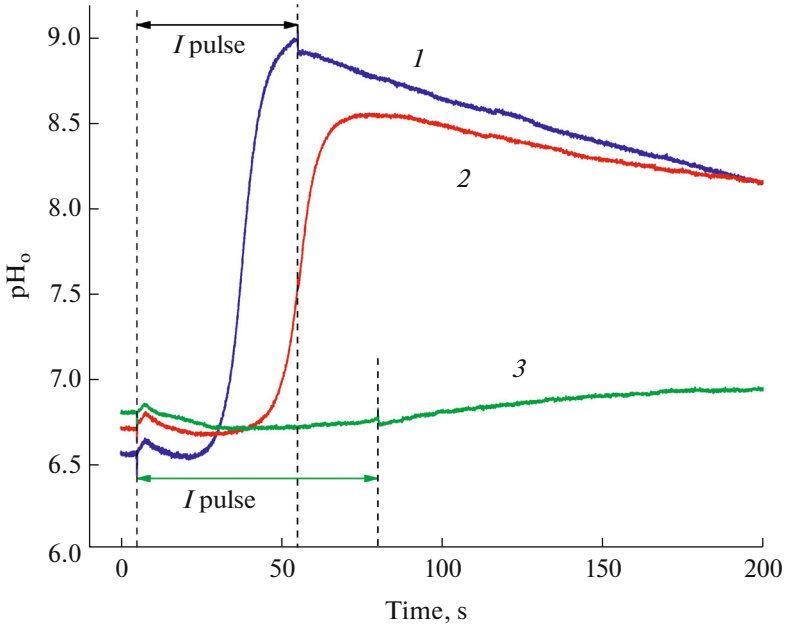


Fig. 3. Kinetics of cell surface pH in the central compartment of experimental chamber filled with standard (low-[K^+]) APW during and after the injection of inward electric current: (*1*) pH response to the first pulse of inward current (23 μA/cm^2); (*2*) pH response to the third pulse of inward current applied 25 min after the first one; (*3*) inactivation of electric field-driven H^+ influx observed in response to the fifth current pulse applied 1 h after the first one.

the exact moment of streaming deceleration cannot be detected and remains uncertain.

Alkaline Band Formation under Injection of Hyperpolarizing Electric Current

Having found out that the HR suppressed the development of cyclosis-mediated increase in *F*' fluorescence, we attempted to clarify a possible role of H^+ fluxes during hyperpolarization in the inhibition of $\Delta F'_{LL}$. Therefore, we examined the influence of inward electric current on pH near cell surface. The injection of inward electric current was found to increase the apoplastic pH (pH$_o$) in many internodal cells, not only at elevated KCl concentrations in the medium [22, 23] but also at low K^+ content in standard APW.

Figure 3 shows changes in pH on cell surface that were elicited by hyperpolarization of the PM in the cell bathed with standard APW (0.1 mM KCl) under injection of an inward current pulse (23 μA/cm^2). It is seen that the pH change followed the sigmoid kinetics with the lag period of 25–30 s. The major part of the pH change (from 10 to 90% of the total amplitude) was completed within ~15 s. The amplitude of pH changes varied in different cells from several tenths of pH unit to 2.5–3 units. The results show that the alkaline zones can be produced by the passage of electric current from the external source, similarly to their generation by natural currents circulating between different cell regions. The current density sufficient for the induction of artificial pH zone fell into the range of natural currents (20–60 μA/cm^2) observed in alkaline bands of illuminated cells [29].

Sequential records of current-induced pH changes were made at 10-min intervals; these intervals enabled relaxation of pH$_o$ and its return to nearly the initial level. Comparison of traces *1* and *2* in Fig. 3 reveals a peculiar feature of electrically induced pH$_o$ changes. In trace *1*, the end of hyperpolarizing pulse was followed by the reversal of the pH change. This reversal is expected if the membrane potential represents the main driving force for H^+ (or OH^-) transport. However, in record *2* the pH$_o$ continued growing after the end of the current pulse (at $t = 55$ s). This pH transient is reminiscent of the pH rise evolving in 20–30 s after the perforation of the cell wall with a glass microneedle [30, 31]. The results suggest that, in general case, there is an additional driving force for H^+ entry.

Traces *1–3* in Fig. 3 demonstrate the use-dependent inactivation of electrically induced pH changes. After applying several current pulses of equal strength, the pH responses disappeared, even under the prolongation of pulse length to 80 s. In the given cell the inactivation was observed in 1 h after the first pH$_o$ response to the hyperpolarizing pulse.

The current-induced pH transients were usually observed under background illumination of the whole cell but they could be observed in the dark as well. Figure 4a shows the formation of external alkaline band induced by inward current after 5 min incubation of

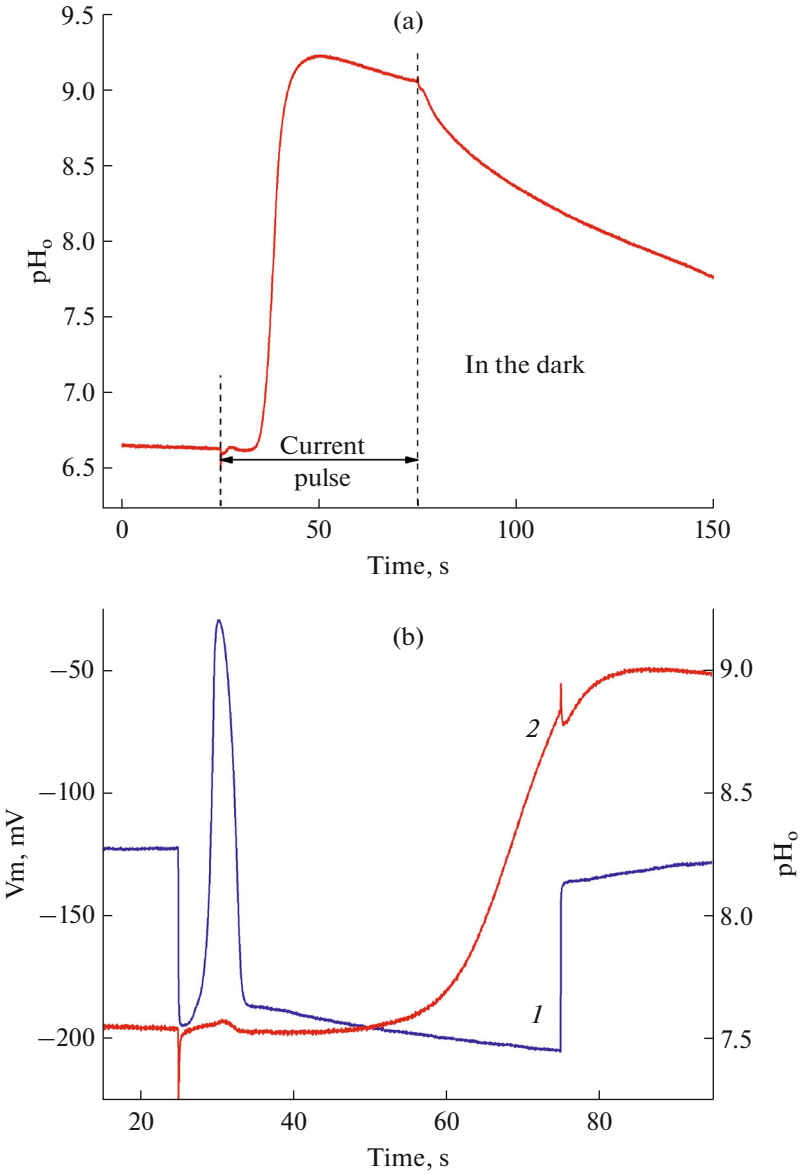


Fig. 4. The alkaline band formation upon the injection of inward electric current in *Chara* internodes bathed with standard APW can proceed (a) in darkness and (b) in the post-excitation period. (a) Local pH_o changes caused by injection of inward current (21 μA/cm^2) in a cell kept for 5 min in the dark. The dashed lines mark the interval of electric pulse application. (b) The AP generation (curve *1*) occurring in some cells under the passage of inward electric current (21 μA/cm^2) does not prevent the formation of high pH zone (curve *2*). The beginning and end of the current pulse (at t = 25 and 75 s, respectively) are marked by instant shifts of membrane potential Vm in curve *1*.

the internode in darkness. Clearly, photosynthetic electron transport in chloroplasts is not required for the origin of pH rise. It should be noted, however, that the current-induced pH transients were reduced or abolished after transferring the cell to darkness in some experiments. Apparently, the electrically induced pH changes depend on the presence of some photoreaction product that accumulates to a larger or smaller extent. Under sufficient resources of this metabolite, the high pH may develop both in the light and in the dark.

Microfluidic Signaling and H^+ Transport under Injection of Inward Current

After establishing that pH_o changes can be induced by passing current at normal low K^+ concentration in the medium, we used standard APW in further experiments to examine the influence of hyperpolarizing pulses on microfluidic signaling, as assessed from $\Delta F'_{LL}$ transients. The main obstacle in such experiments is that many cells are easily excited during or after the hyperpolarizing pulses under low-[K^+] medium.

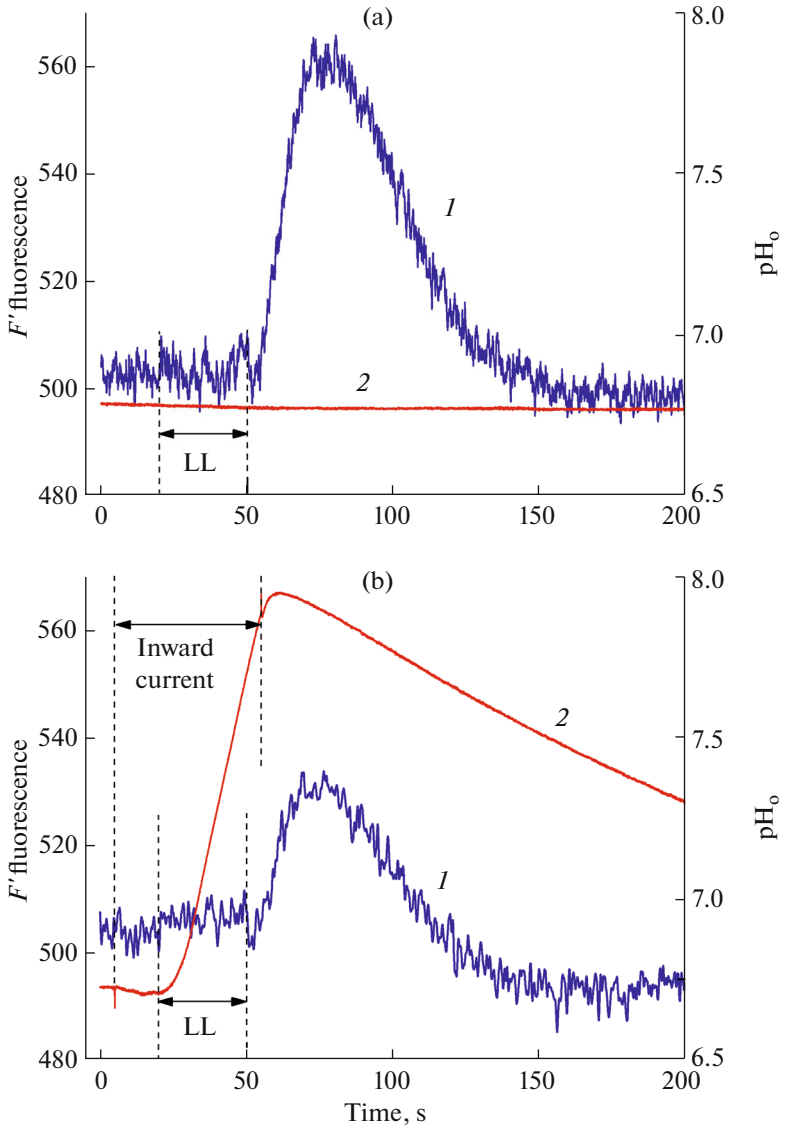


Fig. 5. Simultaneous measurements of cyclosis-mediated *F′* transients (*1*) and cell surface pH (*2*) in: (a) resting cell bathed with a standard APW ($n = 7$) and (b) the same cell stimulated with an inward electric current pulse ($n = 5$). Double-headed arrows designate the period of local light (LL) application and the interval of injecting the inward electric current (23 μA/cm^2).

Figure 4b shows that the hyperpolarizing current pulse induced the AP generation in some cells. In the case of cell excitation, the cytoplasmic streaming was transiently arrested and the response of *F'* to local illumination of an upstream cell region ($\Delta F'_{LL}$) was either abolished or delayed. As can be seen in Fig. 4b, the cell excitation did not prevent the formation of high pH band during the passage of hyperpolarizing current. However, the cessation of cytoplasmic streaming impaired microfluidic signaling, which made such cells unsuitable for the purpose of this study. Only the cells showing no excitation under the action of hyperpolarizing current pulses were taken for analysis.

Figure 5 shows the influence of injecting the inward electric current across the plasma membrane on $\Delta F'_{LL}$ transients (*1*) and cell surface pH (*2*) in *Chara* internode bathed with standard (low-[K^+]) APW. It is seen that the H^+ influx initiated by hyperpolarization of PM was accompanied by the inhibition of *F'* fluorescence changes arising in response to local illumination of a distal (upstream) cell site. It is noteworthy that the position of $\Delta F'_{LL}$ peak was unaffected by passing the inward electric current (Figs. 5a and 5b). Hence, the suppression of *F'* response to local illumination of a remote cell region under the action of electric current was not caused by the deceleration of cytoplasmic streaming.

Finally, we checked if the H^+ influx and the inhibition of $\Delta F'_{LL}$ can be observed in calcified cell

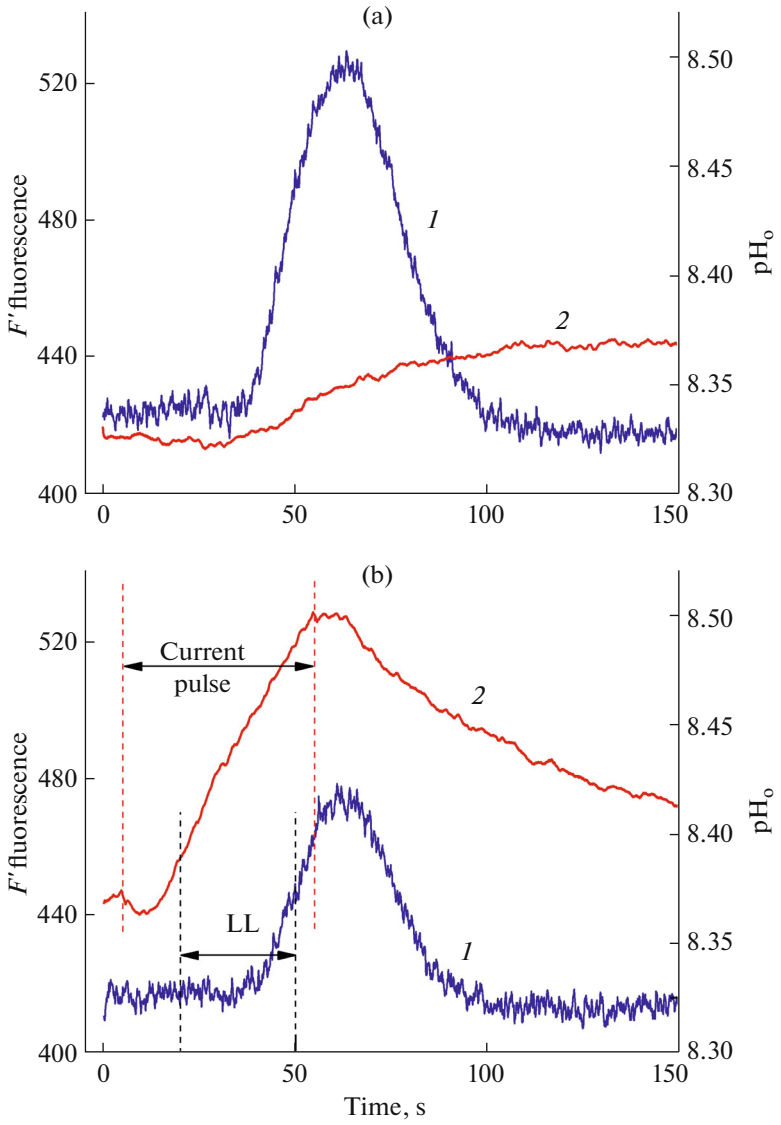


Fig. 6. Effects of hyperpolarizing current pulses on cell surface pH (pH$_o$) and cyclosis-mediated change in chlorophyll fluorescence yield ($\Delta F'_{LL}$) in the cell region located close to calcium depositions (latent alkaline zone). (a) Changes in F' (*1*) and pH$_o$ (*2*) induced by local light (LL) pulse in the resting cell ($n = 6$). (b) Changes in F' (*1*) and pH$_o$ (*2*) induced in the same cell area under combined application of an inward electric current (8.3 μA/cm^2) and the LL pulse ($n = 5$). Double-headed arrows designate the length of LL stimulus and the interval of passing the inward electric current.

regions that are able to generate external alkaline zones at sufficient irradiance. Under low intensity light (12.5 μmol/m^2 s), the cell surface pH in calcified cell regions was higher than in noncalcified areas. The application of LL pulse in the absence of electrical stimulation led to a slight increase in pH$_o$ (Fig. 6a), which is in line with the previously reported facilitation of alkaline band formation by local illumination of an upstream cell region [32, 33]. The passage of an inward current (Fig. 6b) induced the pH increase that surpassed the pH shift caused by local light. Under the action of inward current pulse, the increase in cell surface pH in calcified areas started after a comparatively short lag period, earlier than in noncalcified cell regions (cf. Fig. 6b and Figs. 3–5). This could be attributed to a shorter time required for opening H^+-channels, provided that their state was near to the threshold determined by elevated external pH [34]. On the whole, the suppression of $\Delta F'_{LL}$ transients concurrent with the alkaline shift in external pH occurred both in crystal-free and calcified cell areas.

DISCUSSION

Previous studies revealed that cyclosis-mediated changes of F' chlorophyll fluorescence are strongly

suppressed in chloroplasts underlying the alkaline cell regions of illuminated *Chara* cells [10, 12, 13]. The results of this study show that the external alkaline zones can be artificially created by passing an inward electrical current across the plasma membrane. Moreover, such mimicking of natural alkaline zone was accompanied by the suppression of microfluidic signaling in an apparent similarity with the inhibition of the signal transduction under original high pH bands.

Alkaline bands induced by injection of electric current exhibit some distinctions from the natural high pH bands. One difference is that the artificial alkaline bands can be generated without direct relation to photosynthetic electron transport, whereas natural pH bands disappear after the inhibition of electron transport with diuron [35]. Another difference is that the H^+ transport driven by externally imposed hyperpolarization is subject to long-lasting or irreversible inactivation after several repeated treatments with inward electric current (Fig. 3), whereas natural H^+ transport is associated with durable maintenance of alkaline bands in illuminated cells. It should be noted that natural H^+ flows are inactivated transiently after the AP generation [19], but the H^+-conductance is fully restored within 15 min after cell excitation. The long-lasting inactivation of artificially imposed H^+ flows suggests that these flows are susceptible to some unidentified side factors of passing current. This vulnerability of H^+ conductance might be relevant to the fact that the PM proton flows were observed not in all cells subjected to current injection.

Remarkably, H^+ flows induced by hyperpolarization were driven not only by the membrane potential but also by metabolic reactions that consume or produce protons. This can be seen from pH_o records in Fig. 3. In the beginning of experiment (trace *1*) the termination of current pulse and the respective drop of the plasma membrane voltage were followed by the immediate reversal in the direction of H^+ flow, but later on (trace *2*) the pH_o became insensitive to switching off the current and rose steadily for 20 s after the end of the hyperpolarizing pulse. A known example of the pH_o increase in *Chara* not caused by the membrane potential shifts is the creation of alkaline spot on cell surface after the microperforation of cell wall [30, 31]. It was assumed that the locally damaged cell wall loses its strength and cannot ensure a strong mechanical support for the plasmalemma. The plasma membrane is pressed into the cell wall cavity by high intracellular pressure and the membrane area is locally expanded. The extension of PM opens stretch-activated Ca^{2+} channels and eventually activates the plasma membrane NADPH oxidase [31]. The transmembrane electron transfer to oxygen is associated with the production of O_2^- and with the subsequent H^+ consumption during the dismutation of superoxide. The above example shows that the transmembrane redox reactions associated with H^+ consumption and release may proceed under a driving force other than the membrane voltage.

Nevertheless, the plasma membrane hyperpolarization represents the primary driving force for the influx of cations, protons in particular. The pH rise induced by hyperpolarizing electric current was accompanied by the reduction in amplitude of cyclosis-mediated *F'* fluorescence changes (Figs. 5, 6). While the amplitude of $\Delta F'_{LL}$ transients was diminished, the position of the *F'* fluorescence peak on a time scale remained either non-shifted or shifted slightly, which rules out the possibility that the suppression of $\Delta F'_{LL}$ was caused by deceleration of cytoplasmic streaming.

Based on the literature data, the deceleration of cyclosis after HR and H^+ inflow might seem likely for several reasons. A possible influence on cytoplasmic streaming is inferred by the existence in plant cells of hyperpolarization-activated Ca^{2+}-permeable channels [2, 36]. The activation of these channels can lead to $[Ca^{2+}]_{cyt}$ elevation and cyclosis retardation. Recent studies revealed the release of Ca^{2+} from cytoplasmic stores upon the activation of H^+ influx through H^+-selective channels [37]. Experiments with the internally perfused characean cells revealed that lowering of cytopasmic pH from 7 to 6.5 reduced the velocity of cytoplasmic streaming by nearly 35% [38]. However, the conclusion in [38] was based on manipulation of pH_{cyt} over the whole cell. In our experimental system pH_{cyt} was lowered on a small (2-mm wide) segment of the internode. The local and whole-cell displacements of pH_{cyt} may have different influence on cytoplasmic streaming. At the same time, the AP generation and the cessation of cytoplasmic streaming were frequently observed under the passage of inward current at low K^+ in the medium (Fig. 4b). If the cell was excited during the hyperpolarizing pulse, the *F'* responses to local light were strongly suppressed and became indiscernible. By selecting the cells in which cytoplasmic streaming was insensitive to hyperpolarizing stimuli, we found consistent inhibition of $\Delta F'_{LL}$ both in non-calcified and calcified cell regions.

The results of this study raise the question of how the H^+ influx affects the microfluidic signaling. The inhibition might be the consequence of switching the pathways of electron transport under variations of cytoplasmic pH [17]. It is known that slightly alkaline pH values in suspensions of intact chloroplasts favor CO_2-dependent electron flow, whereas lowering the pH redirects electron flow from PSI toward oxygen reduction [18]. It is possible that the redirection of linear electron flow from $NADP^+$ reduction to the reduction of O_2 after the acidic shift of cytoplasmic pH reduces the amount of NADPH that is produced under intense local illumination and exported from

chloroplasts into the streaming cytoplasm. The diminished export of reducing power would explain the restricted NADPH delivery to chloroplasts in AOI and the smaller amplitude of $\Delta F'_{LL}$ response. The above hypothetical view foresees that the influence of acidic cytoplasm is realized at the stage of export of reducing equivalent by envelope transporters. However, it is quite possible that the acidified cytoplasm hampers the delivery of reducing power to recipient chloroplasts rather than its export from the source chloroplasts [12].

It should be also noted that, apart from the retardation of cyclosis after the injection hyperpolarizing current in some cells, there are other factors potentially contributing to the slow decline of F' in response to LL (Fig. 2d). The switching of photosynthetic electron transport from CO_2-dependent NADPH reduction to the pathway of O_2 reduction leads to energization of the thylakoid membrane, i.e., increases the thylakoid pH gradient, which may restrict the electron flow at the stage of plastoquinone oxidation (photosynthetic control). When electron flow to PSI is suppressed, the elevated F' fluorescence related to the level of stromal reductants would decline slower than in the case of unrestricted electron flow to PSI [12]. Elucidation of complex relations between the plasma membrane H^+ fluxes and the role of microfluidic mass flow in the regulation of cell redox balance would require further studies.

ABBREVIATIONS AND NOTATION

AOI	area of interest
AP	action potential
APW	artificial pond water
F'	actual yield of modulated chlorophyll fluorescence under actinic light
HR	hyperpolarizing response
LL	local light (treatment)
PAM	pulse amplitude-modulated (fluorometry)
pH_{cyt}	cytoplasmic pH
pH_o	pH in pericellular layers of outer medium
PM	plasma membrane
PSI and PSII	photosystems I and II
$\Delta F'_{LL}$	transient changes in modulated chlorophyll fluorescence F' caused by local lighting of a distal cell region

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This work does not contain any studies involving human and animal subjects.

CONFLICT OF INTEREST

The authors of this work declare that they have no conflicts of interest.

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