Transient removal of alkaline zones after excitation of Chara cells is associated with inactivation of high conductance in the plasmalemma

Alexander A. Bulychev and Natalia A. Krupenina

Department of Biophysics; Faculty of Biology; Moscow State University; Moscow, Russia

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The action potential (AP) of excitable plant cells is a multifunctional physiological signal. Its generation in characean algae suppresses the pH banding for 15–30 min and enhances the heterogeneity of spatial distribution of photosynthetic activity. This suppression is largely due to the cessation of H⁺ influx (OH⁻ efflux) in the alkaline cell regions. Measurements of local pH and membrane conductance in individual space-clamped alkaline zones (small cell areas bathed in an isolated pool of external medium) showed that the AP generation is followed by the transient disappearance of alkaline zone in parallel with a large decrease in membrane conductance. These changes, specific to alkaline zones, were only observed under continuous illumination following a relaxation period of at least 15 min after previous excitation. The excitation of dark-adapted cells produced no conductance changes in the post-excitation period. The results indicate that the origin of alkaline zones in characean cells is not due to operation of electroneutral H⁺/HCO₃⁻ symport or OH⁻/HCO₃⁻ antiport. It is concluded that the membrane excitation is associated with inactivation of plasmalemma high conductance in the alkaline cell regions.

Introduction

The action potential (AP) of excitable plant cells is a physiological signal involved in regulation of osmotic balance, gene expression and other cell functions. It also affects the plasmemembrane proton transport that plays a crucial role in regulation of cytoplasmic pH, electrogenesis, mineral nutrition and cell turgor of plant cells. The electrochemical gradient $\Delta \mu_{\text{H}^+}$ created by the plasma membrane H⁺ pump provides the driving force for accumulation of mineral nutrients, such as K⁺ and NO₃⁻, and benefits to photosynthesis of aquatic plants inhabiting weakly alkaline stagnant waters. Local acidification of the apoplast near the cell surface to pH 6.3–6.7 increases the content of a neutral species CO₂, which readily passes through the membranes unlike poorly permeable charged species HCO₃⁻ and CO₃²⁻. In characean algae, which are close relatives of higher plants, the zones of H⁺ extrusion (acid zones) alternate with spatially separated alkaline regions. The alternated pattern of broad acidic and narrow alkaline zones, known as the pH banding phenomenon, is mirrored by the spatial pattern of photosynthetic activity in a single layer of immobile chloroplasts. The pH banding pattern vanishes in darkness and is smoothed transiently after the propagation of action potential along the cell, while the photosynthetic pattern becomes temporarily enhanced.

The long-lasting impact of AP on H⁺ transport (pH banding) and photosynthesis (chlorophyll fluorescence) in characean algae is consistent with its possible regulatory role in these processes. The AP propagation inhibits pH banding and selectively suppresses photosynthesis in alkaline cell areas for the period of 15–30 min, while the signal itself (AP) lasts typically few seconds. It is not yet known whether the suppression of pH banding after AP results primarily from inhibition of the electrogenic H⁺ pump operating in acid regions or from inhibition of passive H⁺ influx (OH⁻ efflux) in alkaline regions, or from combination of both causes. The development of pH pattern is associated with local electric currents circulating between the acid and alkaline zones. In the H⁺ extrusion zones, the electric current is directed outward and is supposedly due to operation of the plasma membrane H⁺ pump. The mechanism of H⁺ (OH⁻) transport in the alkaline cell regions is not determined with certainty. Based on the idea of H⁺/HCO₃⁻ symport coupled to H⁺ pump operation, Shimmen et al. suppose that alkaline regions arise from the electroneutral OH⁻/HCO₃⁻ antiport. However, the occurrence of a large inward current in the alkaline zones might indicate that at least a part of this current is carried by electrogenic H⁺ influx without
HCO₃⁻ as a cotransported ion.⁵,¹² According to this view, the high pH in the alkaline zones is due to H⁺ (OH⁻) uniport through the "high pH channels".¹⁵-¹⁷ These channels are thought to account for a very high area-specific membrane conductance in the alkaline cell regions.

The questions of whether the transmembrane H⁺ transport in the alkaline zones is electroneutral or electrogenic and whether this transport is affected by AP directly or indirectly (through inhibition of the H⁺ pump) can be clarified by measurements of membrane conductance and local pH in electrically isolated alkaline cell regions before and after cell excitation. The rapid cessation of H⁺ influx after AP should not affect the membrane conductance in the case of electroneutral transport (H⁺/HCO₃⁻ symport or OH⁻/HCO₃⁻ antiport) but the conductance changes are likely to occur in the case of uniport.

Membrane conductances in specific cell regions can be assessed under space clamp conditions that assure uniform membrane potential on a small area of cell surface.¹⁶ With these precautions, it was thus found that the plasmalemma conductance in illuminated characean algae is higher in alkaline zones than in acid zones and reduces greatly upon darkening.¹⁶,¹⁸ The goal of this study was to examine the effect of AP on membrane conductances in alkaline and acid areas of illuminated Chara cells and in the same regions of darkened cells. We show that the cessation of transmembrane H⁺ flows upon AP generation is paralleled by several-fold decrease in membrane conductance in alkaline regions with much smaller changes in acid regions. It becomes evident that the H⁺ influx in these cell regions is electrogenic and not coupled to the cotransport of anion. The results indicate that the cessation of inward H⁺ flow in alkaline cell regions is closely associated with area-specific inactivation of high membrane conductance.

Results
When the cell was placed in the chamber divided into separate pools (see diagram in Fig. 1), the isolated cell regions retained the capacity of producing alkaline or acid areas, although formation of high and low pH zones took longer time and the extent of alkaline peaks was smaller than in unconstrained cells. Figure 2 shows pHo changes in spatially isolated alkaline and acid cell regions following stimulation with a short excitatory pulse of electric current. It is seen that the AP generation was followed by a considerable pH drop in the alkaline zone and a small pH increase in the acid zone. These changes were reversible and developed synchronously, which indicates that both active H⁺ extrusion and the passive H⁺ leak in spatially isolated areas were arrested after cell excitation. Thus, the influence of AP on area-specific H⁺ fluxes is not only observed on unconstrained cells but is also evident on cells placed in a sectioned chamber with insulating partitions. This similarity of cell responses in space-clamped and free-lying cells proves the suitability of the model system employed.
Post-excitation conductance changes in Chara of inward current pulses appeared in figures as a wavy band whose width is proportional to $R_m$ values. The upper edge of this band is a measure of membrane potential $V_m$. The $R_m$ values in the alkaline region of a resting cell were low, yielding after recalculation a high membrane conductance ($8.32 \pm 1.06$ S/m$^2$, $n=19$). This value is consistent with the range of 5–15 S/m$^2$ determined by Smith and Walker$^{16,18}$ for alkaline zones of illuminated Chara cells. After the AP generation, the membrane resistance increased several fold within about 60 s. The membrane conductance decreased correspondingly to the average value of $1.79 \pm 0.58$ S/m$^2$ ($n=19$). The increase in $R_m$ was paralleled by a large (50–60 mV) hyperpolarization of the plasmalemma $V_m$. It should be noted that a similar, though delayed hyperpolarization was also induced by darkening.$^{19}$ The AP-induced changes in $R_m$ and $V_m$ were fully reversible: the initial values of both parameters were restored after at least 15 min from the moment of cell excitation. This period is much longer than the refractory period of membrane excitation.

It can be seen also in Figure 3 that the second AP triggered in 30 s after the first one induced the AP of a different shape and extent. The AP amplitude was higher and there was no additional post-excitation hyperpolarization. Furthermore, the pattern of $R_m$ changes was also different: $R_m$ decreased during AP and returned rapidly to values observed in the absence of the second excitation. There was no additional increase in $R_m$ after the second AP. Different patterns of $V_m$ and $R_m$ changes associated with the first and repeated cell excitation were also evident after triggering the second AP in 1–3 min after the first one.

Figure 4 shows a series of events associated with the AP-induced $R_m$ increase in space-clamped alkaline cell regions. Based on amplitudes of hyperpolarizing $V_m$ shifts induced by periodic pulses of constant current, we calculated resistance values and plotted $R_m$ changes (open circles, curves 1) with other parameters measured (curves 2–4). It is seen that the post-excitation increase in $R_m$ (inverse of membrane conductance, $G_m$) developed in parallel with large hyperpolarization (curve 2). The excitation-induced increase in $R_m$ was also accompanied by a strong decrease in external pH near the cell surface (curve 3). The peak of $R_m$ was attained long before the minimum of external pH. Furthermore, the slow reversal of $R_m$ started 1–2 min after cell excitation, when the pH continued to decrease. Thus, the $R_m$ kinetics did not follow the pH change, indicating that the conductance change is not a trivial consequence of pH decrease but, most probably, is the cause of H$^+$ flux cessation. The lower panel shows that the increase in $R_m$ was also accompanied with a pronounced decline in maximal chlorophyll fluorescence $F_m'$ (curve 4), like it was observed in unconstrained cells.$^{20}$ This strong suppression of $F_m'$ fluorescence, together with the decrease in actual fluorescence $F$, and the effective quantum yield of photosystem II primary reaction, $\Delta F/F_m' = (F_m' - F)/F_m'$ (data not shown), suggest that the $R_m$ increase after AP was concurrent with changes in cytoplasmic composition that elevated thermal losses of...
chlorophyll excited states and suppressed noncyclic electron flow in chloroplasts. All changes described above—membrane conductance, plasma membrane potential, pH, chlorophyll fluorescence, and photochemical efficiency of PSII—were reversible and returned to their initial values within about 15 min at maximal irradiance used. The recovery took longer time at lower irradiance.

In experiments shown in Figure 5, we monitored changes in the “alkaline” cell region during and after AP generation under continuous light (curve 1) and following a 30-min dark adaptation (curve 2). The dark period of such length is sufficient for almost complete flattening of the pH banding profile. The R_m and V_m changes induced by AP in illuminated cell were similar to those in Figure 3: a large increase in R_m and a concomitant hyperpolarization were observed. After a 30-min dark exposure, the R_m value in a resting cell increased several fold, indicating, accordingly, a strong decrease in membrane conductance G_m. The mean value of G_m in darkened cell was 0.58 ± 0.09 (n = 4), more than 14 times lower than the mean G_m value for the alkaline areas in illuminated cells. The triggering of AP in the darkened cell was not accompanied by the post-excitation increase in R_m and did not lead to cell hyperpolarization. The membrane resistance in the darkened cell decreased during AP but returned to the initial level immediately after the end of AP. The ratio of G_m values measured before AP (resting cell) and 50–100 s after AP cell was 0.98.

The results of G_m measurements before and after AP generation in the acid region of illuminated and darkened cell are listed in Table 1. Under resting conditions in continuous light, the membrane conductance in acid cell areas was about twice lower than that in the alkaline regions under similar conditions. In the time range of 1–2 min after AP generation, the membrane conductance decreased slightly, by about 30% of the initial value. The post-excitation hyperpolarization was either small (<20 mV) or absent. In dark-adapted cells the membrane conductance of “acid regions” was low and close to that of “alkaline regions.” The R_m changes associated with AP in darkened “acid regions” were similar to those shown in Figure 5 (curve 2) for darkened “alkaline regions”. After 30-min dark adaptation, the AP generation induced neither the post-excitation hyperpolarization nor the increase in R_m. In the darkened cell, the surface pH and chlorophyll fluorescence parameters were insensitive to cell excitation.

Discussion

This study is based on the ability of space-clamped cell areas to produce alkaline and acid zones in similarity to pH bands of unconstrained cells. This feature, helpful for the purpose of experiments, was not a priori expected considering that the pH band formation is related to extracellular electric currents. The possible explanation is that insulating partitions between chamber compartments suppress extracellular currents but do not remove them completely. Residual currents might circulate within the isolated cell area.
Post-excitation conductance changes in Chara

In cell regions producing acid zones, the AP generation was followed by a small increase in pHo, concomitant with a slight decrease in membrane conductance and insignificant hyperpolarization.

The results suggest that the smoothing of pH pattern after AP in whole cells involves the suppression of passive H⁺ (OH⁻) transport in the alkaline regions, which coincides with and might result from the rapid inactivation of "high pH channels". At the same time, the H⁺ extrusion in the acidic zones was also blocked after AP. Since local acidification is a direct indicator of the H⁺ pump activity, it is evident that the increase in pHo after AP in the acid zones originates from the H⁺ pump inhibition. Although the H⁺ pump is a driving source of extracellular currents, the suppression of passive H⁺ flux in the alkaline regions after AP is clearly caused by the Gm decrease, not by the change in the driving force. The driving force, ΔμH⁺ increased actually along with the drop in pHo and membrane hyperpolarization. Thus, the Gm reduction cannot be a secondary event arising from the inhibition of the H⁺ pump.

On the other hand, the AP-induced Gm decrease in the alkaline cell regions of unconstrained cells might contribute to inhibition of H⁺ pump activity. According to some reviews and research studies, 2,21 the conductance of electrogenic H⁺ pump is comparable to the conductance of passive diffusion pathways. Then, the pump current should be sensitive to the conductance of ion channels. In this case, the conductance of alkaline cell region might effectively influence the H⁺ pump activity. Specifically, the inactivation of high pH channels would diminish the pump current.

The membrane excitation in characean cells was usually analyzed under low light conditions securing spatially uniform distribution of plasmalemmal properties. 22-24 This may explain why the large inactivation of plasma membrane conductance in alkaline regions remained undiscovered until now. The pH band formation in characean internodes requires irradiance above the threshold level ~5 \( \mu \text{E m}^{-2} \text{s}^{-1} \).25 Smith and Beilby26 examined the membrane conductance in unconstrained Chara cells exposed to sufficiently high irradiance (50 \( \mu \text{E m}^{-2} \text{s}^{-1} \)) and observed the AP-induced decrease in Gm by 30–40% within about 30 s. They attributed these changes to inactivation of the H⁺ pump conductance.

The conductance changes revealed in the present study are almost an order of magnitude higher than in experiments without selection of alkaline zones. Nevertheless, the kinetics of the conductance changes in our experiments was similar to those described for the whole cell in the absence of space-clamped conditions.26 Apparently, these changes have a common origin and reflect primarily the inactivation of channel conductance in the alkaline cell regions. Remarkably, we observed a 30% decrease in membrane conductance in acid regions, which is in line with earlier reports and with the supposed attribution of these changes to inhibition of H⁺ pump conductance.

The effect of cell excitation on membrane conductance in the alkaline cell regions was found to mimic the influence of prolonged conductance. By contrast, in cell regions producing acid zones, the AP generation was followed by a small increase in pHo, concomitant with a slight decrease in membrane conductance and insignificant hyperpolarization.

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The effect of cell excitation on membrane conductance in the alkaline cell regions was found to mimic the influence of prolonged
darkness. Both the AP and dark incubation caused a large reduction of Gm, although the effect of a single AP on Gm in illuminated cells was not as strong as the influence of 30-min darkness. The conductances of formerly acid and alkaline cell regions became almost even in darkness (Table 1), while longer dark incubation (1–2 h) might be needed to attain completely uniform Gm distribution. The possible reason for similar effects of excitatory stimulation and darkening on membrane conductance is that both treatments elevate the cytoplasmic Ca2+ level, which might affect ion permeabilities. The combined influence of AP-associated cytosolic Ca2+ increase and cytoplasm alkalinization upon the blockade of Gm and H+ influx in the alkaline cell areas might underlie the effect of AP on chloroplast functioning (Fig. 4, curve 4).

The AP-induced hyperpolarization was found to correlate with the decrease in Gm; it was absent or small in the acidic zones. We suppose that Vm of alkaline cell regions under rest conditions in continuous light is shifted toward the equilibrium potential for H+ (EHi), in similarity with the original hypothesis put forward by Kitasato.28,29 The inactivation of H+-conducting channels after AP is expected to shift Vm toward the Nerst potential for K+ (EK), which is more negative than EHi. Furthermore, an abrupt decrease in Gm after AP might elevate the electrogenic component of Vm (the product of pump current and passive membrane resistance), provided the resistance increase is larger than the decline in pump current. The post-excitation hyperpolarization was previously observed in a particular characean species and was regarded as a unique property of Chara globularis.21 Our results indicate that the post-excitation hyperpolarization is not attributed to individual species. The main requirement for its appearance in C. corallina is heterogeneous spatial distribution of pHo and Gm. This heterogeneity is restored in continuous light during prolonged rest periods (≥15 min) after previous cell excitation. At shorter periods between stimuli, the profound effect of excitation on Gm is damped and can be overlooked.

In higher plant leaves, the propagation of electric signals along the plant also induced transient hyperpolarization.30 In contrast to our conclusions derived with Chara, the hyperpolarization in higher plants was supposedly due to activation, rather than inhibition of the electrogenic H+ pump, because the hyperpolarization was prevented by the pretreatment with orthovanadate, an inhibitor of plasma membrane H+-ATPase. However, it is reasonable to expect that after-hyperpolarization in Chara is also sensitive to H+-pump inhibitors since the effects of AP-induced H+-pump inhibition can be only observed on the background of active H+ pump.

The results of this study led us to conclude that the origin of high pHo in alkaline zones of Chara cells is not related to functioning of H+/HCO3− symport or OH−/HCO3− antiport. In the case of electroneutral symport or antiport, the cessation of H+ (OH−) transport should have affected the pH without simultaneous decrease in Gm. Thus, the idea is substantiated that the active H+ transport and counter-directed passive H+ flux in whole cells are spatially separated but coordinated by means of extracellular currents carried in the bulk solution by dominant ions.31

Considering the functional role of spatially separated zones with active H+ extrusion and passively conducting zones, the passive H+ transport noncoupled to symport or antiport of nutrient ions might seem wasteful. However, the large distance between zones of H+ extrusion and passive H+ leak can be favorable, because it allows the cell to lower pH over a large areas (broad acid zones) close to pK ~ 6.3 for CO2/HCO3− equilibrium. This shift enriches the apoplast with dissolved CO2 and facilitates permeation of this photosynthetic substrate into the cytoplasm. Such lowering of pH would have been hampered or weakened if the proton leak channels were distributed homogeneously over the cell membrane.

The supposed functional significance of membrane areas with passive H+ leak (high pH channels) is related to the high conductance of these areas (Gm values more than tenfold higher than the Gm of plasma membrane in darkened cells). As already mentioned above, the Gm changes in alkaline zones might regulate the H+ pump activity by providing the adjustable load for this electromotive source. From this point of view, the minimal width and number of alkaline zones are determined by the required pump current satisfying the cell demand in photosynthetic CO2 acquisition. It is interesting to note in this context that the outward pump current in acid zones (~10 μA/cm2)10 corresponding to H+ efflux -100 pmol cm−2 s−1 is comparable with the average rate of CO2 fixation by characean internodes in saturating light (40 pmol cm−2 s−1).32 This implies that a considerable portion of extruded protons is used for HCO3− to CO2 conversion and subsequent utilization of carbon dioxide in photosynthesis. Thus, the role of H+ pump in algal cells inhabiting weakly alkaline environments is not restricted to accumulation of mineral nutrients but also includes the supply of membrane-permeable substrate of photosynthesis. It is more important that this plasma membrane function is sensitive to electric signals (action potential) propagating along the cell under stressful treatments.

### Material and Methods

Chara corallina Klein ex Willd. was grown in an aquarium at room temperature under scattered daylight illumination. Individual internodes measuring about 6 cm in length and 1 mm
in diameter were excised from the strand and placed in artificial pond water (APW) containing 0.1 mM KCl, 1.0 mM NaCl and 0.1 mM CaCl\(_2\) (pH 7.0 adjusted with NaHCO\(_3\)). Isolated cells were allowed to stay in APW for at least 1 day prior to experiments. We used young noncalcified cells and mature cells with depositions of calcium crystals on the cell wall. Visible zones of calcification served as a preliminary indicator for the localization of alkaline zones. In the absence of calcium depositions, the zones of different pH were detected with phenol red, a pH indicator that turns yellow and red in acid and alkaline zones, respectively.

The cell was placed into a three-compartment transparent chamber in such a way that the selected region was in the narrow middle compartment between insulating partitions. This arrangement was used to ensure space clamp conditions for the selected cell region. The chamber with outer dimensions 100, 80 and 10 mm (length, width and height) was mounted on a stage of an Axiovert 25 CFL inverted microscope (Zeiss, Germany). The side compartments were large, accommodating 20 ml bathing solution each. The central compartment was narrow (a 3-mm gap between transverse partitions) with a volume of 1.2 ml. The medium in this pool was changed periodically but was stagnant during measurements. The slits in 5-mm thick partitions fixed the cell position. The interstices between the cell and partition edges were filled with insulating silicone grease. The width of the central compartment (3 mm) was less than the cable length values, which are 3–5 and 10–15 mm for alkaline and acid regions, respectively, in Chara cells. The transcellular current was passed through Ag/AgCl electrodes. Two interconnected Ag/AgCl wires were placed in the side compartments. The other half-cell was connected to the middle pool via a flexible agar salt bridge put close to the cell. This electrode was also used as a reference electrode for potential measurements.

The resistance of the selected cell region was measured by passing square pulses of current (0.5–1 \(\mu\)A, pulse duration 180 or 360 ms) at a frequency of 2.5 or 1.25 Hz; these pulses induced hyperpolarizing shifts of the membrane potential in the central compartment. Periodic pulses of current were obtained from an electronic stimulator connected to the electrodes through a 10 \(\Omega\) load resistor. The plasmalemma membrane potential (\(V_{\text{m}}\)) and its changes induced by transcellular current were measured by means of capillary microelectrodes (Pyrex glass) filled with 2 M KCl using an EPC-5 microelectrode amplifier (List-Medical, Germany). Prior to micropipette insertion into the cell and after the micropipette withdrawal in the end of experiment, voltage drops on a series resistance (the resistance of external medium, salt bridge, and the reference electrode) were measured. These small voltage drops (about 4 mV) were taken into account in calculations of the membrane resistance \(R_m\). Records in figures were obtained after digital subtraction of these voltage drops. The \(R_m\) values, when presented in figures, are expressed in k\(\Omega\)-cm\(^2\). For convenient comparison with previously published conductance values (\(G_m\)) in alkaline and acidic regions, we calculated \(G_m^\text{m,M}\), an inverse of \(R_m\), and listed these values (in S/m\(^2\)) in Table 1. The AP generation was evoked by single pulses of current (4–6 \(\mu\)A, 100–200 ms).

The pH in unstirred layers near the cell surface was measured with antimony pH microelectrodes. The microelectrodes having tip diameter of about 20 \(\mu\)m were pulled from a Pyrex capillary filled with molten antimony. The potential difference between the pH electrode and the reference electrode was measured with an electrometric amplifier VAJ-51 (RFT, Germany). The signals from the output of EPC-5 and VAJ-51 amplifiers were recorded by means of ADC-DAC converter (PCI-6024E, National Instruments, United States) and WinWCP software (Strathclyde Electrophysiology Software).

During experiments the cell was continuously illuminated from the upper light source of the microscope through a SZS-22 blue glass filter (photon flux density at the cell level -100 \(\mu\)E m\(^{-2}\) s\(^{-1}\)). In some cases the cell was illuminated with white light (500 \(\mu\)E m\(^{-2}\) s\(^{-1}\)) in order to accelerate formation of high and low pH zones. The saturation pulse method and its application to chlorophyll fluorescence measurements on microscopic cell regions (100 \(\mu\)m in diameter) were described previously. During fluorescence measurements the actinic light was attenuated with neutral density glass filters.

In each experiment, pH near the outer cell surface (pH\(_o\)) was first measured to verify the formation of an alkaline or acid zone in an isolated cell area. The H\(^+\) transporting activity was additionally checked by measuring pH changes evoked by AP generation. Next, the membrane potential and plasmalemma resistance were measured under rest conditions and following cell excitation. After AP generation the cell was allowed to relax for 15–20 min for restoration of the initial membrane potential and pH\(_i\). After few cycles of stimulation and relaxation, the cell was repositioned in the chamber with an aim to accommodate the counterpart pH zone in the central compartment, and similar measurements were carried out. Figures represent the results of typical experiments performed in at least four replicates. The membrane conductance (\(G_m^\text{m,M}\)) values are presented as mean values ± standard errors.

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### References
Post-excitation conductance changes in Chara