

Hydrogen peroxide-induced changes in intracellular pH of guard cells precede stomatal closure

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ABSTRACT

Epidermal bioassay demonstrated that benzylamine, a membrane-permeable weak base, can mimic hydrogen peroxide (H_2O_2) to induce stomatal closure, and butyric acid, a membrane-permeable weak acid, can partly abolish the H_2O_2 -induced stomatal closure. Confocal pH mapping with the probe 5-(and-6)-carboxy seminaphthorhodafluor-1-acetoxymethylester (SNARF-1-AM) revealed that H_2O_2 leads to rapid changes in cytoplasmic and vacuolar pH in guard cells of *Vicia faba* L, i. e. alkalization of cytoplasmic areas occur red in parallel with a decrease of the vacuolar pH, and that butyric acid pretreatment can abolish alkalization of cytoplasmic areas and acidification of vacuolar areas of guard cells challenged with H_2O_2 . These results imply that the alkalization of cytoplasm via efflux of cytosol protons into the vacuole in guard cells challenged with H_2O_2 is important at an early stage in the signal cascade leading to stomatal closure.

Key words: H_2O_2 , pH, SNARF-1-AM, confocal, *Vicia guard cell*.

INTRODUCTION

Even under optimal conditions, many metabolic processes, including chloroplastic, mitochondrial, and plasma membrane-linked electron transport systems, produce reactive oxygen species (ROS) such as the superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl free radical ($OH\cdot$)[1, 2]. Furthermore, the imposition of biotic and abiotic stress conditions can give rise to excess concentrations of ROS, resulting in oxidative damage at the cellular level. Interestingly, ROS appear to play a crucial role in several metabolic processes of plants in a beneficial manner[3]. H_2O_2 , in particular, have been previously implicated as a second messenger in plant cells exposed to environmental stresses such as chilling[4], heat[5], and pathogens [6, 7]. The evidences that O_2^- - and H_2O_2 involved in

regulation of stomatal movement have been found [8], and also oxidative stress resulting from exposure to methyl viologen (which generates O_2^- -radicals) or H_2O_2 has a marked effect on stomatal aperture[9], and exogenous H_2O_2 ($\leq 10^{-5}M$) can induce stomatal closure and inhibit stomatal opening without any damage to cells[10]. Recent studies using recombinant aequorin techniques have demonstrated that H_2O_2 stimulates a transient increase in whole-plant calcium concentration ($[Ca^{2+}]_i$) in tobacco seedling[11]. In addition, ozone (O_3) has been shown to inhibit stomatal opening by inactivating the inward-rectifying K^+ channel in plasma membrane[12]. Moreover, our previous work using voltage clamp had demonstrated that externally applied H_2O_2 could induce stomatal closing by the inhibition of K^+ uptake and evoking K^+ release through K^+ channels in plasma membrane of guard cells[13]. It has been widely confirmed that H_2O_2 regulates stomatal movement as a stress

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signal, yet there remain considerable gaps in our knowledge and in our attempts to construct a detailed description of the events and underlying signaling chains involved in the process of stomatal opening and closing.

Intracellular pH changes have long been proposed to function in signaling and transport control in plant cells, such as plant defense responses [14], tip growth [15], [16], nodulation [17], elicitation of benzophenanthridine alkaloids [18], and response to hormone activity such as gibberellic acid [19] and abscisic acid [20]. To date, cytoplasmic alkalization is a major step in the ABA-triggered signal cascade in guard cells leading to H⁺ efflux and stomatal closure [21], [22], and internal and external ABA can induce the generation of H₂O₂ in guard cells (manuscript to be submitted). However, up to now, no conclusive evidence has been provided concerning the earliest signals for stomatal closure by H₂O₂. Therefore, in the present paper we report dynamic changes of the intracellular pH distribution observed by confocal pH topography in guard cells challenged with H₂O₂.

MATERIALS AND METHODS

Chemicals and reagents

SNARF-1-AM was purchased from Molecular Probes (Eugene, OR). Nigericin, benzylamine and butyric acid were from Sigma. Dimethyl sulfoxide (DMSO) was from Amresco. Pluronic F-127 was from Bio-Rad. Unless stated otherwise, other chemicals were of analytical grade.

Plant material

Broad bean (*Vicia faba* L.) was grown from seed in pots with culture soil (nutrient soil/vermiculite 2:1). The plants were kept in a greenhouse with a humidity of about 70%, a photon flux density of 0.20 to 0.30 mmol m⁻² s⁻¹ and day/night temperature of 25 ± 2 °C/20 ± 2 °C. There were no stresses on plants during growing period.

Epidermal strip bioassay

Stomatal bioassay experiments were performed as described by McAinsh et al. [10] with slight modifications. Immediately prior to each experiment, the epidermis was peeled carefully from the abaxial surface of the youngest and fully expanded leaves of 4-week-old plants, and cut into pieces of 5-mm length. To study promotion of stomatal closure by H₂O₂, freshly prepared abaxial epidermis was first incubated in CO₂-free 50 mM KCl/10 mM Mes, pH 6.15 (Mes-KCl) for 3 h under conditions promoting stomatal opening (at 22 °C to 25 °C, under a photon flux density of 0.20 to 0.30 mmol m⁻² s⁻¹) to open the stomata, and then transferred to CO₂-free Mes-KCl in the presence of H₂O₂ (10-

5M) or benzylamine (10 mM) or butyrate (10 mM) for 2 h under opening conditions, and stomatal apertures were determined microscopically at 30-min intervals. Values are the means of 120 measurements ± SE values.

Loading with SNARF-1-AM

The uncharged SNARF-1-AM can passively diffuse across the plasma membrane to cytosol, where it is hydrolyzed by cellular esterases to free SNARF-1. It is this form which is fluorescent, and is consequently the pH probe. The epidermal strips, which were first incubated for 3 h under conditions promoting stomatal opening, were placed in a small Petri dish containing 1 ml CO₂-free Mes-KCl buffer (pH 6.15), with 20 μM SNARF-1-AM from a 3 mM stock solution in DMSO, and were incubated for 30 min in the dark at 25-27°C. The fluorescence probe uptake was further facilitated when 0.05% pluronic F-127 was present. Loading was terminated by rinsing the strips several times in dye-free incubation buffer.

Confocal pH topography

Intracellular pH measurement and pH calibration using single excitation-dual emission fluorescence ratios were performed according to the method developed by Roos et al [18], [23]. Briefly, a confocal laser scanning microscope (Bio-Rad MicroRadiance) was used in dual-channel mode. Wavelength settings for SNARF-1 were Ex=488nm; Em=580nm (channel 1) and Em > 600 nm (channel 2). Images were obtained with a Nikon TE300 inverted microscope (objective 40×/0.60 Plan Fluor). Signals from selected frames were scanned simultaneously in both channels and the intensity ratio (channel 1 : channel 2) was calculated for each pixel. pH maps were obtained by color coding these intensity ratios according to self-defined look-up tables, as supplied by the manufacturer. To enable the comparison of changes in signal intensity, confocal images were taken using identical exposure conditions (in manual setup) for all samples. Experiments were repeated at least three times with reproducible results, and the selected confocal image was the representative of about eight pictures.

The measured intensity ratios were converted to pH by means of a calibration graph. For this purpose, SNARF-preloaded epidermal strips were incubated in Mes buffers, pH 5.5-7.5 (adjusted with Tris), containing 50 mM KCl, 10 mM Mes, 10 μM butyrate, 10 mM nigericin (a K⁺/H⁺ exchanger which results in an equilibration of external and internal pH), for another 20 min, then the ratio-versus-pH curve were obtained by scanning pH maps of above cells and averaging to yield fluorescence ratios at defined pH values (Fig 1). Changes in intracellular pH can be calculated as a linear relationship between pH and fluorescence intensity ratios over the pH ranges 5.5-7.5.

RESULTS

Changes in stomatal behavior in response to H₂O₂, benzylamine and butyric acid

To determine whether an intracellular pH change is involved in stomatal closure by H₂O₂, we

measured the effects of weak base (benzylamine) and acid (butyric acid) on stomatal movement. As shown in Fig 2 and Fig 3, 10 mM benzylamine, similar to 10^{-5} M H_2O_2 , induced stomatal closure, and 10 mM butyric acid partly abolished the H_2O_2 -induced stomatal closure. However, treatment with 10 mM butyric acid alone induced a slight stomatal opening over untreated controls. These indicated that guard cell alkalization occurred in response to H_2O_2 , and cytosolic alkalization was possibly an “upstream” factor of stomatal closure induced by H_2O_2 .

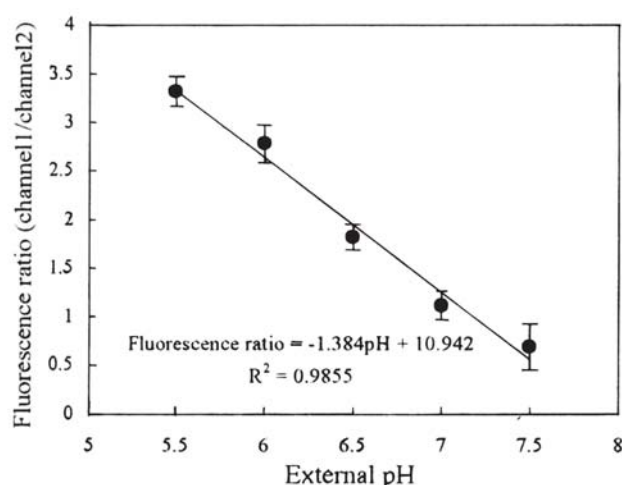


Fig 1. Calibration of pH-dependent fluorescence of carboxy SNARF-1. Fluorescence intensity ratios are averaged from at least 5 values, each representing the mean intensity ratio of an area over the whole guard cell tested.

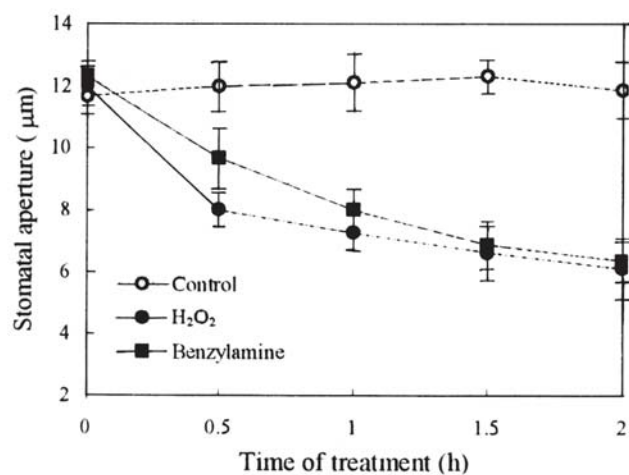


Fig 2. Effect of benzylamine (10 mM) and H_2O_2 (10^{-5} M) on the stomatal closure.

H_2O_2 -induced changes in intracellular pH of guard cells

Having established that cytosolic alkalization was involved in the regulation of stomatal closure induced by H_2O_2 in above epidermal strips bioassay experiments, we then considered whether external H_2O_2 induces changes in intracellular pH of guard cells or not. In this study, subcellular regions were delineated by using bright-field and pH-sensitive fluorescence ratio analysis to determine which cellular compartments had undergone changes in fluorescence intensity. Time-course quantitative analysis was performed in the cytosol (area 1, 3, 5) and the vacuole (area 2, 4). As shown in Fig 4, the mean pHs of the cytoplasmic and vacuolar areas stayed about 7.0 and 5.9 respectively in guard cell without treatment (Fig 4 D). After adding 10^{-5} M H_2O_2 , the mean cytoplasmic pH increased by around 0.3 unit in parallel with the mean vacuolar pH decreasing by around 0.4 unit (Fig 4 H, Fig 4 I). Following H_2O_2 treatment, an increase in intracellular pH also occurred in the surrounding epidermal cells (decreasing background fluorescence ratio as in Fig 4), which is in accordance with the effect of ABA on stomatal closure[24].

Effects of butyric acid on changes in intracellular pH of guard cells induced by H_2O_2

The data presented above raise the question of whether an increase of the cytoplasmic pH alone is sufficient to produce a signal for the induction of stomatal closure. By using a 10 min treatment with

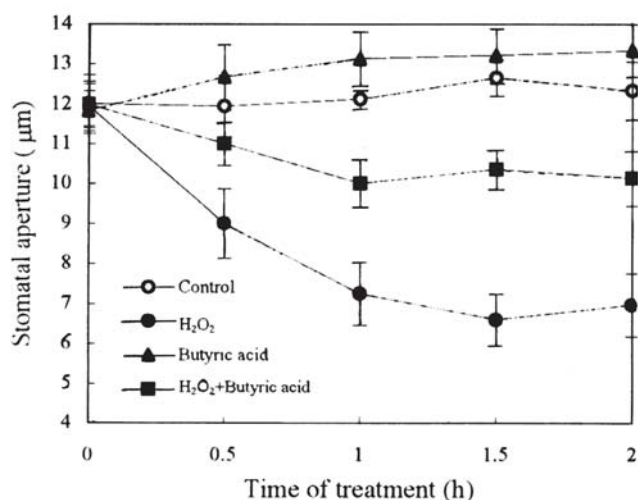


Fig 3. Effect of butyric acid (10 mM) on the promotion of stomatal closure by H_2O_2 .

butyric acid (a membrane-permeable weak acid that has frequently been used to decrease the intracellular pH of various cell types in experiments [14], [25]), we were able to evoke an acidification of the cytoplasm. As shown in Fig 5, butyric acid (in Mes-KCl buffer with pH adjusted to 6.15) pre-treatment lowered significantly the intracellular pH in both cytoplasmic and vacuolar areas, especially the latter (compare the pH maps D in Fig 4 and Fig 5 at zero min before the addition of H₂O₂). With the prolongation of H₂O₂ treatment, the mean cytoplasmic pH stayed around 6.8 and that of vacuolar pH showed no drop but a slight increase (compare Fig 5 G with Fig 4 I), both indicating that butyric treatment can abolish partly the cytosolic alkalin-

ization induced by H₂O₂.

DISCUSSION

Despite the recognition of H₂O₂ as a central signaling molecule in stress and wounding responses, pathogen defense, and regulation of cell cycle and cell death, little is known about how the signal is perceived and transduced in plant cells. Previous studies have unraveled that H₂O₂, similar to ABA, regulates stomatal movement via an increase in [Ca²⁺]_i as a second messenger [10,11]. Intracellular pH regulation is a characteristic and necessary feature of all living cells. There is increasing evidences that suggest changes in intracellular pH can act as a second messenger in plants [14,26,

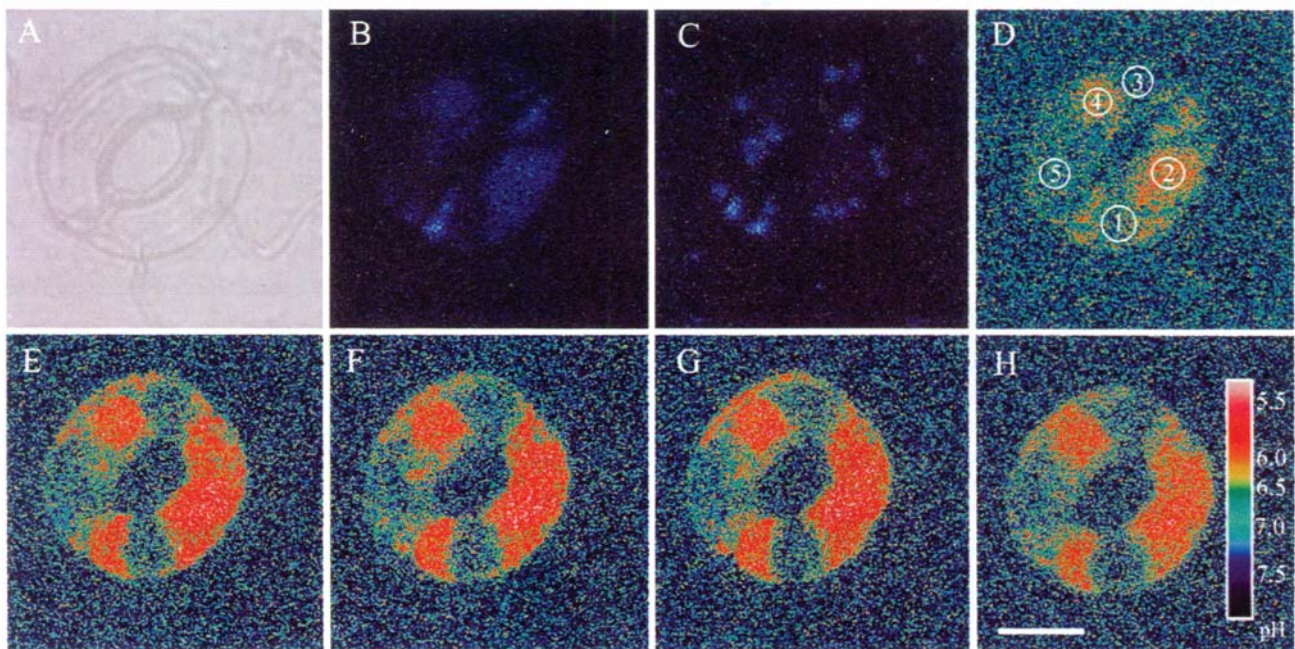
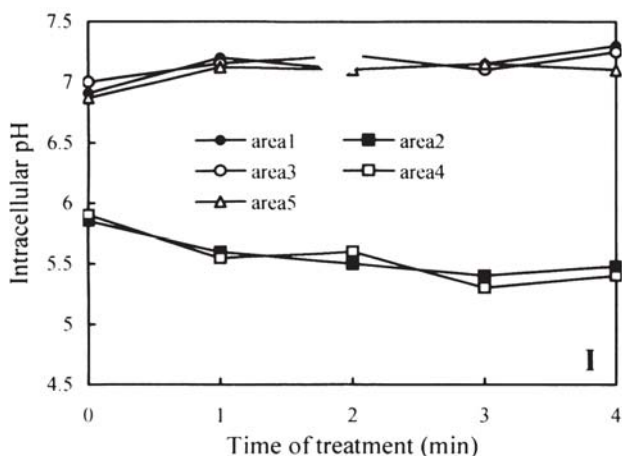


Fig 4. pH distribution of an open stomata of broad bean guard cells after the addition of 10⁻⁵ M H₂O₂.

Photograph A is the transmission light image. Photographs B and C are the fluorescence intensity images acquired from channel 1 (Em 580 nm) and channel 2 (Em >600 nm) respectively at 0 min before the addition of H₂O₂. Photograph D is a pH map derived by ratioing the image intensities (channel 1/channel 2) at 0 min before the addition of H₂O₂; circles point to analyzed areas with vacuoles (2, 4) and cytoplasm (1, 3, 5). Photograph E, F, G, H are pH maps derived by ratioing the image intensities (channel 1/channel 2) at 1 min, 2 min, 3 min and 4 min after the addition of H₂O₂ respectively. Fig I is the time-course plot of fluorescence ratios of selected areas in photograph D. The pseudocolor bar in photograph H represents a pH range from about 5.5 in vacuolar area to 7.5 in cytoplasmic area. Bar=10 μm.



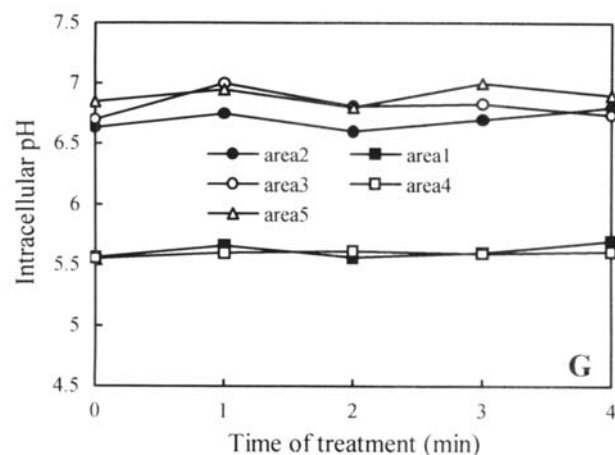
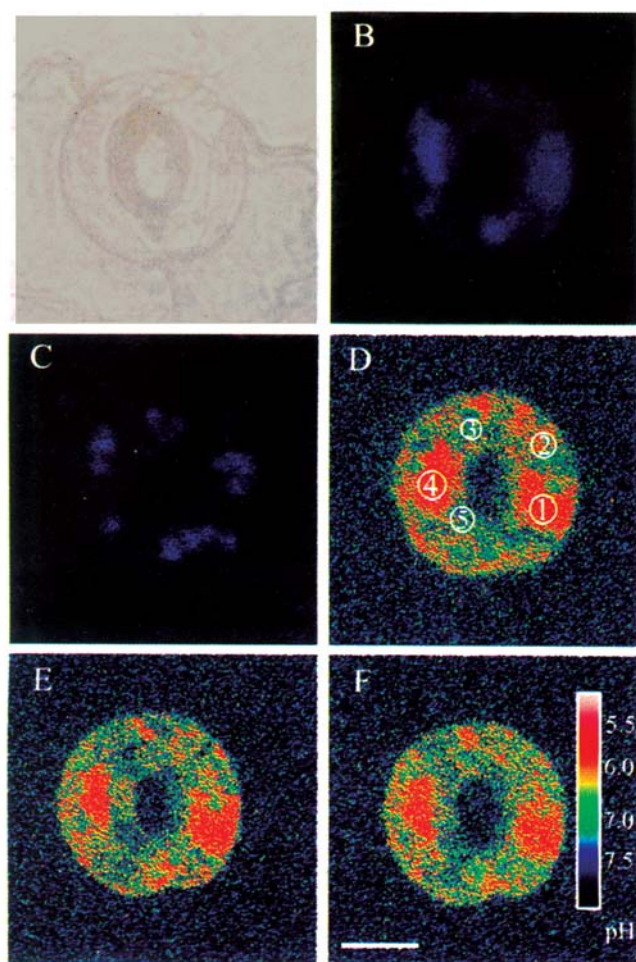


Fig 5. Effects of 10 min pretreatment with butyric acid on changes of intracellular pH in guard cells challenged with 10^{-5} M H_2O_2 . Prior to experiment, SNARF-1-loaded epidermis were incubated in Mes-KCl buffer containing 10 m M butyric acid for 10 min. Photograph A is the transmission light image. Photographs B and C are the fluorescence image intensities acquired from channel 1 (E_m 580 nm) and channel 2 ($E_m > 600$ nm) respectively at 0 min before the addition of H_2O_2 . Photograph D is a pH map derived by ratioing the image intensities (channel 1/channel 2) at 0 min before the addition of H_2O_2 ; circles point to analyzed areas with vacuoles (1, 4) and cytoplasm (2, 3, 5). Photographs E, F, are pH maps derived by ratioing the image intensities (channel 1/channel 2) at 1 min, 4 min after the addition of H_2O_2 respectively. Fig G is the time-course plot of fluorescence ratios of selected areas in photograph D. The pseudocolor bar in photograph F represents a pH range from about 5.5 to 7.5 as in Fig 4, Bar = 10 μ m.

27]. Unlike intracellular free Ca^{2+} concentrations, which can rapidly change by perhaps 100-fold, the pH inside a cell varies by only fractions of a pH unit, but even with such small change, it still exerts many physiological roles[15].

The data presented here, for the first time as far as our knowledge, is concerned that intracellular pH changes are involved in H_2O_2 -induced stomatal closure. Epidermal strip bioassay has shown that the cytoplasmic alkalization possibly mediated H_2O_2 -induced stomatal closure (Fig 2, Fig 3), which is similar to results seen in guard cells in response to ABA[21,24]. To provide further evidence for the specific involvement of cytoplasmic alkalization in H_2O_2 signaling leading to stomatal closure and to investigate the shifts of intracellular pH distribution, we used pH-sensitive SNARF-1-AM to directly measure the fast intracellular pH changes. SNARF-1 enters cells in the diacetate form (SNARF-1-AM), and is then hydrolyzed by cellular

esterases and trapped as SNARF-1, which is a single-excitation dual-emission fluorescent probe and its optical properties make it amenable to ratiometric imaging by confocal laser scanning microscopy to measure quantitatively intracellular pH changes. Additionally, ratiometric measurements reduce or eliminate variations of several determining factors in measuring fluorescence intensity, including indicator (dye or probe) concentration, excitation pathlength, excitation intensity and detection efficiency, and many artifacts are also eliminated including photobleaching and leakage of the indicator, variable cell thickness, and nonuniform indicator distribution within cells (due to compartmentalization). A single cell assay illustrated that H_2O_2 induces rapid changes in cytoplasmic and vacuolar pH in guard cells of *Vicia faba*, i.e. alkalization of cytoplasmic areas occurred in parallel with a decrease of the vacuolar pH, and that butyric acid

pretreatment partly abolished alkalization of cytoplasmic areas and acidification of vacuolar areas of guard cells challenged with H₂O₂ (Fig 4 and Fig 5), which are coincident with the epidermal bioassay (Fig 2 and Fig 3). These results imply that the alkalization of the cytosol may be accomplished via a H⁺ efflux from cytosol to vacuole through vacuolar H⁺-ATPase. Proton gradient across tonoplast is possible a necessary factor in H₂O₂-induced stomatal closure.

In addition, ratiometric fluorescence of the edges of guard cell challenged with H₂O₂ is more significant than that in the same cell with H₂O₂-free treatment (Fig 4), and this implies that H₂O₂ possibly activates plasma membrane H⁺-ATPase, which will cause a more acidification of cell wall. In plant cells, plasma membrane H⁺-ATPase is primarily responsible for generating membrane potential [28,29]. Therefore, changes in membrane potential may cause or may be a reflection of changes in cytosolic pH that are involved in signaling leading to stomatal closure. Taken together with other recent data [10,12,13,21,24], we postulate that H₂O₂-induced stomatal closure is probably mediated by the alkalization of cytoplasm, intracellular Ca²⁺ increase and depolarization of plasma membrane leading to K⁺ channel changes. However the spatio-temporal relationship between pH and Ca²⁺ as well as plasma membrane K⁺ channel in H₂O₂ signaling leading to stomatal closure remains unknown.

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