Cytosolic Concentration of Ca\textsuperscript{2+} Regulates the Plasma Membrane H\textsuperscript{+}-ATPase in Guard Cells of Fava Bean

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Opening of the stomata is driven by the light-activated plasma membrane proton pumping ATPase, although the activation and inactivation mechanism of the enzyme is not known. In this study, we show that the H\textsuperscript{+}-ATPase in guard cells is reversibly inhibited by Ca\textsuperscript{2+} at physiological concentrations. Isolated microsomal membranes of guard cell protoplasts from fava bean exhibited vanadate-sensitive, ATP-dependent proton pumping. The activity was inhibited almost completely by 1 \textmu M Ca\textsuperscript{2+} with a half-inhibitory concentration at 0.3 \textmu M and was restored immediately by the addition of 1,2-bis(2-aminophenoxy)ethane N,N,N',N'-tetraacetic acid, a calcium chelating reagent. Similar reversible inhibition by Ca\textsuperscript{2+} was shown by the generation of electrical potential in the membranes. Activity of ATP hydrolysis was inhibited similarly by Ca\textsuperscript{2+} in the same membrane preparations. The addition of 1,2-bis(2-aminophenoxy)ethane N,N,N',N'-tetraacetic acid and EGTA, Ca\textsuperscript{2+} chelators, to epidermal peels of fava bean induced stomatal opening in the dark, and the opening was suppressed by vanadate. This suggests that the lowered cytosolic Ca\textsuperscript{2+} activated the proton pump in vivo and that the activated pump elicited stomatal opening. Inhibition of H\textsuperscript{+}-ATPase by Ca\textsuperscript{2+} may depolarize the membrane potential and could be a key step in the process of stomatal closing through activation of the anion channels. Furthermore, similar inhibition of the proton pumping and ATP hydrolysis by Ca\textsuperscript{2+} was found in isolated plasma membranes of mesophyll cells of fava bean. These results suggest that Ca\textsuperscript{2+} regulates the activity of plasma membrane H\textsuperscript{+}-ATPases in higher plant cells, thereby modulating stomatal movement and other cellular processes in plants.

INTRODUCTION

Stomatal pores surrounded by guard cells regulate CO\textsubscript{2} uptake and water loss in leaves (Zeiger, 1983; Assmann, 1993). Opening of the stomata, which is induced by the accumulation of potassium salt in guard cells, is driven by the light-activated proton-pumping ATPase in the plasma membranes (Assmann et al., 1985; Shimazaki et al., 1986). The pump creates an electrochemical gradient across the plasma membrane and drives potassium uptake through the voltage-gated, inward-rectifying K\textsuperscript{+} channels (Hedrich and Schroeder, 1989; Briskin and Hanson, 1992). The pump in guard cells can be activated by various stimuli, such as red light absorbed by chlorophyll, blue light absorbed by the blue light photoreceptor, the growth regulator auxin, a second messenger 1,2-diacylglycerol, and the fungal toxin fusicoccin (Assmann, 1993). An autoinhibitory domain identified in the C terminus of the H\textsuperscript{+}-ATPase has been suggested as having an important role in such a post-translational regulation of the H\textsuperscript{+}-ATPase activity (Sze, 1985; Serrano, 1989; Palmgren, 1991). However, the mechanism by which the pump is regulated by distinct stimuli is essentially not known.

Closing of the stomata is caused by effluxes of potassium and anions from guard cells during darkness and drought stress. The efflux occurs through the simultaneous openings of outward-rectifying K\textsuperscript{+} channels and Ca\textsuperscript{2+}- and voltage-dependent anion channels in the plasma membrane. Openings of these channels require the membrane depolarization that can be achieved at least partly by Ca\textsuperscript{2+} influx into the cytosol and/or anion efflux from the cytosol (Schroeder and Hagiwara, 1990; MacRobbie, 1992). During these processes, however, a role of the plasma membrane H\textsuperscript{+}-ATPase has been overlooked, despite the fact that inhibition of this enzyme shifts the membrane to a depolarized potential (Tazawa et al., 1987).

In this study, we show that the plasma membrane H\textsuperscript{+}-ATPase from fava bean guard cell protoplasts is inhibited reversibly by Ca\textsuperscript{2+} at submicromolar concentrations and suggest that inhibition of the H\textsuperscript{+}-ATPase by Ca\textsuperscript{2+} may be a key step in initiating stomatal closing as well as inhibiting stomatal opening. We also report similar inhibition of H\textsuperscript{+}-ATPase by Ca\textsuperscript{2+} in the plasma membranes from mesophyll cells.
Figure 1. ATP-Dependent Proton Pumping in the Microsomal Membranes of Guard Cells from Fava Bean Plants.

(A) Inhibition of proton pumping by vanadate (50 μM) in the membranes of guard cells. Concentration of free Ca²⁺ was 0.01 μM. Carbonyl cyanide m-chlorophenylhydrazone (CCCP) was added at 5 μM.

(B) Inhibition of proton pumping by Ca²⁺.

(C) Sustained inhibition of proton pumping by Ca²⁺. Downward arrows indicate the addition of ATP at 2 mM. Upward arrows indicate the addition of carbonyl cyanide m-chlorophenylhydrazone. Proton pumping was measured by the absorbance decrease of acridine orange as described in Methods. Experiments repeated three times on different occasions had similar results.

RESULTS

Inhibition of the Plasma Membrane H⁺-ATPase by Ca²⁺

In microsomal membranes of guard cells, proton pumping, which was measured as the absorbance decrease of acridine orange, was inhibited by 50 μM vanadate, an inhibitor of the plasma membrane H⁺-ATPase, suggesting that the pumping was mediated by the H⁺-ATPase (Figure 1A). The proton gradient was dissipated by 5 μM carbonyl cyanide m-chlorophenylhydrazone, a protonophore. Proton pumping was inhibited markedly at low concentrations of free Ca²⁺, which was buffered with EGTA, with a half-inhibitory concentration of 0.3 μM (Figure 2A). Inhibition by Ca²⁺ was almost complete at 1 μM, and a higher concentration of Ca²⁺ (10 μM) did not enhance inhibition (Figure 1B). Pumping inhibition by Ca²⁺ at 1 μM was sustained for >30 min, but a large proton gradient was formed across the membranes at 0.01 μM Ca²⁺ (Figure 1C). Similar inhibition of proton pumping was shown when the concentration of Ca²⁺ was regulated with 1,2-bis(2-aminophenoxy)ethane N,N',N''-tetraacetic acid (BAPTA). Inhibition of proton pumping by Ca²⁺ was 50% at 0.32 μM and almost complete at 1 μM (data not shown).

The inhibition of the proton pumping by Ca²⁺ could be the result of a Ca²⁺/H⁺ antiport, which can be stimulated by the addition of Ca²⁺ and negates the proton uptake across the microsomal membranes (Rasi-Caldogno et al., 1987; Briskin, 1990; Kasai and Muto, 1990; Olbe and Sommarin, 1991). If so, the activity of ATP hydrolysis should not be suppressed by Ca²⁺. However, ATP hydrolysis was strongly inhibited by 1 μM Ca²⁺ in the membranes (Figure 2B), ruling out a significant contribution of the Ca²⁺/H⁺ antiport to the inhibition of proton pumping. The Ca²⁺ concentration required for the half inhibition of ATP hydrolysis was 0.3 μM, a value very close to that required for proton pumping. The activity of ATP hydrolysis was inhibited by vanadate (Figure 2B). These results indicate that the plasma membrane H⁺-ATPase is inhibited by free Ca²⁺ at submicromolar concentrations.

Inhibition of ATP hydrolytic activity by Ca²⁺ was clearly shown only when the reaction period was short (for example, 3 min; Figures 2B and 3A). If the reaction was allowed to proceed for 30 min, the extent of inhibition decreased. ATP hydrolysis was inhibited by only 10% at 1 μM and 50% at 10 μM Ca²⁺ (Figure 3B). In contrast, 50 μM vanadate reduced the activity by 85% under the same conditions.

Low sensitivity of ATP hydrolysis to Ca²⁺ (Figure 3B), when the measuring period was lengthened (for example, 30 min), could have been due to the fact that either Ca²⁺ inhibition of H⁺-ATPase-mediated ATP hydrolysis was transient or activation of Ca²⁺-ATPase, which caused ATP hydrolysis, was induced by Ca²⁺. However, the latter possibility is unlikely because the activity of Ca²⁺-ATPase in the membranes was blocked by 1 μM erythrosin B, which had been included in the reaction mixture, as shown in Figure 4. Hydrolysis activity of ITP, a specific substrate for Ca²⁺-ATPase (Carnelli
et al., 1992), increased with the increase of Ca\(^{2+}\) concentration, which is indicative of plasma membrane Ca\(^{2+}\)-ATPase (Carnelli et al., 1992), and this Ca\(^{2+}\)-dependent activity was inhibited almost completely by erythrosin B at 1 \(\mu\)M (Figure 4). ITP hydrolysis was inhibited by vanadate, and inhibition was greater than that by erythrosin B, probably because vanadate inhibits both Ca\(^{2+}\)- and H\(^{+}\)-ATPases and because a portion of ITP hydrolysis is catalyzed by H\(^{+}\)-ATPase (Briskin, 1990; Carnelli et al., 1992; Cocucci and Marre, 1984).

**Reversible Inhibition of Proton Pumping by Ca\(^{2+}\)**

Inhibition of proton pumping by Ca\(^{2+}\) was reversible in microsomal membranes of guard cells. Proton pumping ceased within 2 min after initiation of the reaction in the presence of 1 \(\mu\)M Ca\(^{2+}\). Pumping was restored immediately by the addition of 2.9 mM BAPTA, a Ca\(^{2+}\) chelator, and the rate after recovery was almost the same as that of the control (Figure 5A). When 1 \(\mu\)M free Ca\(^{2+}\) was added to the reaction mixture 30 sec after the initiation of the reaction, proton pumping ceased instantaneously, whereas immediate restoration occurred when BAPTA was added (Figure 5B).

We isolated plasma membranes from the microsome of fava bean guard cells by aqueous two-phase partitioning (Johansson et al., 1993) and confirmed the inhibition of proton pumping by Ca\(^{2+}\). As shown in Figure 6, proton pumping was inhibited almost completely by both vanadate at 50 \(\mu\)M and Ca\(^{2+}\) at 1 \(\mu\)M. Pump inhibition by Ca\(^{2+}\) was reversible because the pumping was restored immediately by BAPTA.

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**Figure 2.** Inhibition of Proton Pumping and ATPase Activity by Free Ca\(^{2+}\) in Microsomal Membranes of Fava Bean Guard Cells.

(A) Inhibition of proton pumping by Ca\(^{2+}\). Pumping activities are presented as the magnitude obtained 1 min after the initiation of the reaction. The open and filled circles indicate proton pumping in the presence and absence of vanadate, respectively. Vanadate was added at 50 \(\mu\)M. Proton pumping was measured by the absorbance change of acridine orange as described in Methods.

(B) Inhibition of ATP hydrolytic activity by Ca\(^{2+}\). The open and filled circles show ATPase activity with and without vanadate, respectively. Vanadate was added at 50 \(\mu\)M. ATP hydrolytic activity was determined as described in Methods. The reaction was run for 3 min. Concentrations of Ca\(^{2+}\) were added at 0.01, 0.1, 0.32, 0.1, 1, and 10 \(\mu\)M. Experiments repeated at least three times on different occasions had similar results.

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**Figure 3.** Different Sensitivities of ATPase to Free Ca\(^{2+}\) in Microsomal Membranes of Fava Bean Guard Cells.

(A) Three-minute reaction time.

(B) Thirty-minute reaction time.

Concentrations of Ca\(^{2+}\) were 0.01, 1, and 10 \(\mu\)M. Vanadate was added at 50 \(\mu\)M. The microsomal membrane was prepared as described in Methods. The assay of ATPase is as given in Methods.
We have noted the possible involvement of Ca\textsuperscript{2+}/H\textsuperscript{+} antiporter (Kasai and Muto, 1990) and/or plasma membrane Ca\textsuperscript{2+}-ATPase in the inhibition of proton pumping by Ca\textsuperscript{2+} because Ca\textsuperscript{2+}-ATPase may act as an ATP-fueled Ca\textsuperscript{2+}/H\textsuperscript{+} antiporter (Rasi-Callegno et al., 1987; Briskin, 1990; Olbe and Sommarin, 1991). If the antiporter is involved in inhibition, the generation of electrical potential would not be inhibited by Ca\textsuperscript{2+}, as has been shown in the microsomal vesicles from radish (Rasi-Callegno et al., 1987). However, similar sensitivity of proton pumping and generation of electrical potential to Ca\textsuperscript{2+} (Figures 1 and 7) again excludes the involvement of the antiporter.

Activation of H\textsuperscript{+}-ATPase by Treatment of the Epidermis with Ca\textsuperscript{2+} Chelators

When 1 mM BAPTA was added to fava bean epidermal tissue, stomata opened widely in the dark; this opening was inhibited by 0.5 mM vanadate, an inhibitor of plasma membrane H\textsuperscript{+}-ATPase in guard cells (Figure 8; Schwartz et al., 1991; Amodeo et al., 1992). The addition of the chelator EGTA, which has been shown to decrease cytosolic Ca\textsuperscript{2+} ([Ca\textsuperscript{2+}]\textsubscript{cyt}) to 0.05 μM or less in Commelina guard cells (Gilroy et al., 1991), to fava bean epidermal tissue also induced stomatal opening in the dark. This opening was inhibited by vanadate at 0.5 mM (Figure 8). These results suggest that the pump is activated by decreased [Ca\textsuperscript{2+}]\textsubscript{cyt} in vivo and that the activated pump

![Figure 4](image)

**Figure 4.** Increase of the Activity of ITP Hydrolysis by Ca\textsuperscript{2+} and Inhibition of ITP Hydrolysis by Erythrosin B in Microsomal Membranes of Fava Bean Guard Cells.

Concentrations of Ca\textsuperscript{2+} were 0.01, 1, and 10 μM. Erythrosin B (EB) concentrations were 0.1, 1, and 10 μM. Vanadate was added at 50 μM. The microsomal membrane was prepared as described in Methods. The assay of ITP hydrolysis is as given in Methods. Experiments repeated three times on different occasions had similar results.

**Inhibition of the Generation of Electrical Potential by Ca\textsuperscript{2+}**

When ATP was added to microsomal membranes, fluorescence quenching of bis(3-propyl-5-oxoisoxazol-4-yl)pentamethine oxonol (Oxonol VI) was observed, suggesting that electrical potential (inside positive) was generated by the addition of ATP (Figure 7A). Quenching was dissipated completely by 5 μM of carbonyl cyanide m-chlorophenylhydrazone, a protonophore, and inhibited by 100 μM of vanadate. The results indicate that the electrical potential was generated by proton transport that was mediated by plasma membrane H\textsuperscript{+}-ATPase. Quenching was inhibited markedly at low concentrations of free Ca\textsuperscript{2+} (Figure 7B). The Ca\textsuperscript{2+} concentration required for half inhibition of the generation of electrical potential was 0.3 μM, almost the same value as that required for half inhibition of proton pumping. In the presence of 1 μM free Ca\textsuperscript{2+}, fluorescence quenching was inhibited strongly; however, quenching was restored when BAPTA was added at 2.9 mM (Figure 7C). These results indicate that the generation of electrical potential by H\textsuperscript{+}-ATPase in the plasma membrane is reversibly inhibited by Ca\textsuperscript{2+} at submicromolar concentrations and is in agreement with those shown for proton pumping.

**Figure 5.** Inhibition of Proton Pumping by Free Ca\textsuperscript{2+} and Its Restoration by BAPTA in Microsomal Membranes of Fava Bean Guard Cells.

(A) Open circles indicate that the concentration of Ca\textsuperscript{2+} was 1 μM prior to the addition of BAPTA.

(B) Open circles indicate that the Ca\textsuperscript{2+} concentration was 0.01 μM prior to the addition of Ca\textsuperscript{2+}. Ca\textsuperscript{2+} was added at 1 μM. The concentration of Ca\textsuperscript{2+} was 0.01 μM, as indicated by filled circles. BAPTA at 2.9 mM in 10 mM Mops-KOH, pH 7.0, was added to the reaction mixture in the presence of 1 μM Ca\textsuperscript{2+}. Proton pumping was measured by the absorbance change of acridine orange as described in Methods.
elicit stomatal opening. However, vanadate inhibition of stomatal opening in the epidermis may be due in part to the inhibition of plasma membrane Ca\(^{2+}\)-ATPase by vanadate (Briskin, 1990) because inhibition of Ca\(^{2+}\)-ATPase may increase [Ca\(^{2+}\)\(_{\text{cyt}}\)]. Vanadate is unlikely to affect the viability of guard cells because fusicoccin, an activator of the H\(^{+}\)-ATPase, induced stomatal opening in the vanadate-treated epidermis that had been washed in the basal medium in the dark for 30 min. No stomatal opening was observed in the presence of 0.1 mM CaCl\(_2\).

**Inhibition of H\(^{+}\)-ATPase in the Plasma Membranes of Mesophyll Cells**

We examined the effect of Ca\(^{2+}\) on the H\(^{+}\)-ATPase in the plasma membranes from mesophyll cells of fava bean. Proton pumping in the plasma membranes of mesophyll cells was much lower than that in microsomal membranes of guard cells (Figures 2A and 9A) on the protein basis of these membranes. This could be due in part to the high density of the pump in the plasma membranes of guard cells (Villalba et al., 1991; Becker et al., 1993). Pumping in the membrane preparations from mesophyll cells was inhibited by Ca\(^{2+}\) in a manner similar to that in the microsomal membranes of guard cells (Figure 9A). The inhibition was reversible (data not shown).
Figure 8. Stomatal Opening in Fava Bean Epidermal Tissue Induced by BAPTA and EGTA in the Dark and Inhibited by Vanadate.

Means of 75 measurements with standard errors are presented. The basal incubation medium contained 10 mM 2-(N-morpholino)ethanesulfonic acid, pH 6.4, and 5 mM KCl. One of the following was added to the basal medium: 1 mM BAPTA (open squares), 1 mM EGTA (open circles), 1 mM BAPTA and 0.5 mM vanadate (closed squares), 1 mM EGTA and 0.5 mM vanadate (closed circles), and 0.1 mM CaCl₂ (x). To facilitate the uptake of vanadate, it was administered to the epidermis during the preincubation period 15 min prior to the initiation of stomatal opening. The vanadate-treated epidermal strips that showed no stomatal opening were washed in the basal medium in the dark for 30 min and then transferred to the new incubation medium containing 5 μM fusicoccin (FC). Stomatal apertures were determined after the epidermal strips had been incubated in the new medium for 1 hr. The epidermis had been treated with BAPTA and vanadate (closed triangle) or EGTA and vanadate (open triangle).

The concentration of Ca²⁺ required for half inhibition of pumping was 0.3 μM. ATP hydrolysis was inhibited by Ca²⁺ with a half-inhibition concentration of 0.3 μM (Figure 9B).

Specificity of Ca²⁺ in the Inhibition of ATPase

We investigated the inhibitory action of other divalent cations on proton pumping without buffering the cations with EGTA. Pumping was very low (<25%) when EGTA was omitted from the reaction mixture and was difficult to measure by the absorbance change of acridine orange. Thus, pumping was measured as fluorescence quenching with quinacrine, which can be measured easily. At the beginning, we confirmed that proton pumping measured as fluorescence quenching was reversibly inhibited by free Ca²⁺ at submicromolar concentrations (data not shown). The effect of cations on the pump was then investigated. As shown in Table 1, quenching was inhibited by ~80% in the presence of 10 μM Ca²⁺ but was not inhibited by Ba²⁺, Mn²⁺, and Sr²⁺ at the same concentrations, indicating that inhibition was specific to Ca²⁺.

Figure 9. Inhibition of Proton Pumping and ATPase Activity by Free Ca²⁺ in Plasma Membranes of Fava Bean Mesophyll Cells.

(A) Inhibition of proton pumping by Ca²⁺. Pumping activities are presented as the magnitude obtained 1 min after the initiation of the reaction. The open and filled circles indicate proton pumping in the presence and absence of vanadate, respectively. Vanadate was added at 50 μM. Proton pumping was measured by the absorbance decrease of acridine orange as described in Methods.

(B) Inhibition of ATP hydrolytic activity by Ca²⁺. The open and filled circles show ATP hydrolytic activity in the presence and absence of vanadate. Vanadate was added at 50 μM. ATP hydrolysis was measured as described in Methods. The reaction was run for 3 min. The concentration of Ca²⁺ was 0.01, 0.1, 0.32, 1, and 10 μM.
Table 1. Specific Inhibition of Proton Pumping by Ca²⁺

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<th>Divalent Cations</th>
<th>Rate of Quinacrine Fluorescence Quenching (%)</th>
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<td>100</td>
</tr>
<tr>
<td>Ba²⁺</td>
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</tr>
<tr>
<td>Mn²⁺</td>
<td>108</td>
</tr>
<tr>
<td>Sr²⁺</td>
<td>94</td>
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The basal reaction mixture (250 μL) contained 10 mM Mops-KOH, pH 7.0, 0.25 M mannitol, 5 mM MgCl₂, 50 mM KNO₃, 5 μg/mL oligomycin, 1 μM quinacrine, and the membrane preparation (10 μg of which was protein). All divalent cations were added at 10 pM. Two different cations were added at 10 μM. Fluorescence quenching of quinacrine was initiated by the addition of ATP and measured as described in Methods. Experiments repeated two times on different occasions had similar results.

**DISCUSSION**

Our data show that the plasma membrane H⁺-ATPase in guard cells is inhibited by Ca²⁺ at physiological concentrations. The Ca²⁺ concentration required for half inhibition was 0.3 μM for proton pumping, generation of electrical potential, and ATP hydrolysis (Figures 2 and 7). This finding is distinct from previous reports that showed the inhibition of ATP hydrolysis by Ca²⁺ (Leonard and Hotchkiss, 1976; Vara and Serrano, 1982; Nejdat et al., 1986; Shimazaki and Kondo, 1987); the concentration required for half inhibition was 150 μM in the plasma membranes of maize roots and was far from physiological levels of [Ca²⁺]cyt.

The low sensitivity of the ATP hydrolysis to Ca²⁺ was shown when the reaction time was long (for example, 30 min; Figure 3B). In contrast, complete and sustained inhibition of proton pumping was demonstrated with 1 μM Ca²⁺ under the same conditions (Figure 1C). A possible explanation for the insensitivity of ATP hydrolysis is that the addition of Ca²⁺ activates Ca²⁺-ATPase in the plasma membranes and induces ATP hydrolysis. However, the activity of Ca²⁺-ATPase was blocked by erythrosin B under our conditions, suggesting that Ca²⁺-ATPase cannot contribute to ATP hydrolysis. Thus, the discrepancy between proton pumping and ATP hydrolysis with respect to Ca²⁺ sensitivity may be explained by the fact that Ca²⁺ uncouples pumping and ATP hydrolysis, which has been suggested as a slip in vacuolar and plasma membranes of plants (Moriyama and Nelson, 1988; Ohkawa and Tsutsumi, 1988) where the pump is suppressed without affecting ATP hydrolysis. An alternative explanation is that Ca²⁺ may induce an increase in the permeability of H⁺ across the membranes.

The inhibition of the pump by Ca²⁺ is fully reversible; the addition of BAPTA immediately restored pumping, and the pumping rate after the recovery was almost the same as that of the control (Figure 5). Reversible inactivation and reactivation of the pump modulated by submicromolar Ca²⁺ may be one of the ideal regulatory mechanisms for this enzyme. In support of this model, [Ca²⁺]cyt is shown to lie between 0.05 to 0.25 μM in resting guard cells and increases quickly up to 1 μM in response to various stimuli in vivo (McAinsh et al., 1990; Schroeder and Hagiwara, 1990; Gilroy et al., 1991). The proton pump in the plasma membrane of guard cells in intact leaves seems to be in an inactivated or low-activity state in the dark. If the pumping activity is inhibited by [Ca²⁺]cyt in guard cells, the activation of the pump is expected when [Ca²⁺]cyt is lowered. Such treatment of the epidermis with Ca²⁺ chelators in the dark induced stomatal opening that was suppressed by vanadate, suggesting that the lowered [Ca²⁺]cyt in guard cells activated the proton pump in vivo (Figure 8).

There are several distinct pathways to activate the pump in guard cells: light absorbed by chlorophyll and the blue light photoreceptor (Assmann et al., 1985; Shimazaki et al., 1986; Serrano et al., 1988) and the growth regulator auxin can activate the pump (Lohse and Hedrich, 1992). It is tempting to speculate that some of the signals are mediated by the lowering of [Ca²⁺]cyt in guard cells. Investigations have indicated that photosynthesis decreases [Ca²⁺]cyt and hyperpolarizes the membrane potential in the characean alga Nitellopsis (Miller and Sanders, 1987), and photosynthesis activates the pump in guard cell protoplasts of fava bean (Serrano et al., 1988).

Recent studies, however, have revealed that auxin and fusicoccin, which activate the proton pump, increase [Ca²⁺]cyt in guard cells of Paphiopedilum (Irving et al., 1992). They have also revealed that an increase in the [Ca²⁺]cyt appears to be required for pump activation by blue light in guard cells of fava bean (Shimazaki et al., 1992). These results apparently conflict with our current interpretation. Ca²⁺ may activate the pump through the signal transduction pathway and inhibit the pump in a different manner as shown here.

The proton pump hyperpolarizes the membrane potential and activates inward-rectifying K⁺ channels, resulting in stomatal opening. Thus, there is no doubt that inhibition of the proton pump by Ca²⁺ inhibits stomatal opening because inward-rectifying K⁺ channels are also activated by Ca²⁺ (Schroeder and Hagiwara, 1989; Fairley-Grenot and Assmann, 1992). However, inhibition of the pump upon the increase of [Ca²⁺]cyt in guard cells may provide a key step for the initiation of stomatal closing. Recent investigations have suggested that Ca²⁺ triggers stomatal closing through membrane depolarization by influx of Ca²⁺ and increase of [Ca²⁺]cyt (McAinsh et al., 1990; Schroeder and Hagiwara, 1990; Gilroy et al., 1991). The depolarization and elevated levels of [Ca²⁺]cyt activate voltage-gated and Ca²⁺-dependent anion channels. Anion efflux through these channels depolarizes the membrane potential, thereby opening outward-rectifying K⁺ channels. Simultaneous openings of outward K⁺ and anion channels.
various plant tissues (Hepler and Wayne, 1985).

The possibility that the inhibition of the H+-ATPase by Ca²⁺ is greater than AHA3 (Palmgren and Christensen, 1994). We investigated similar mechanisms to be needed to depolarize the membrane potential. Thus, we conclude that the inhibition of the pump by Ca²⁺ may provide a key step for the voltage-dependent activation of the anion channels because inhibition of the proton pump induces a large membrane depolarization in plants, such as Chara cells (Tazawa et al., 1987).

Most recently, depolarization-activated Ca²⁺ channels were found in the plasma membrane of carrot suspension cells (Thuleau et al., 1994). These Ca²⁺ channels may be the path- way for Ca²⁺ influx from the external medium. Therefore, if the proton pump is inhibited by the increased cytosolic Ca²⁺, the resulting membrane depolarization may further induce the influx of Ca²⁺ through the Ca²⁺ channels, although depolarization-activated Ca²⁺ channels have not been identified in the plasma membrane of guard cells.

H⁺-ATPase provides a driving force for nutrient uptake in specialized cell types, such as root cells and companion cells of phloem and guard cells. H⁺-ATPase is encoded by a multigene family in Arabidopsis and tobacco, and expression of its isoforms in distinct cell types has been reported (Sussman and Harper, 1989; DeWitt et al., 1991; Perez et al., 1992). Thus, the activity seems to be regulated differently in these specialized cell types. Recent studies have demonstrated that, among the three isoforms of H⁺-ATPases found in Arabidopsis (AHA1, AHA2, AHA3), AHA1 and AHA2 have a 10-fold higher affinity for ATP and a threefold higher sensitivity to vanadate than AHA3 (Palmgren and Christensen, 1994). We investigated the possibility that the inhibition of the H⁺-ATPase by Ca²⁺ is unique to guard cells. However, the H⁺-ATPase is inhibited similarly by Ca²⁺ in mesophyll cells (Figure 9). This result suggests that post-translational modulation of H⁺-ATPase activity by Ca²⁺ may be a general regulatory mechanism for this enzyme.

In conclusion, our results from in vitro and in vivo experiments may provide evidence that cytosolic Ca²⁺ regulates the plasma membrane H⁺-ATPase in guard cells and that the inhibition of the enzyme by Ca²⁺ may be a key step in stomatal closing that is coordinated with the activation of anion channels, whereas the activation of H⁺-ATPase by lowering [Ca²⁺]m may provide a driving force for stomatal opening. Furthermore, inhibition of the H⁺-ATPase by Ca²⁺ also occurs in the plasma membrane of mesophyll cells. Investigation of the regulatory mechanism by Ca²⁺ for this enzyme in guard cells will increase our general understanding of the transport phenomena and signal transduction processes mediated by H⁺-ATPase in various plant tissues (Hepler and Wayne, 1985).

METHODS

Preparation of Microsomal and Plasma Membranes

Guard cell protoplasts were isolated enzymatically from the abaxial epidermis of 4- to 5-week-old leaves of fava bean (Vicia faba cv Ryosai Issun) as described previously (Shimazaki et al., 1992). The protoplast suspension (3 mg of protein per mL) in 0.4 M mannitol and 0.2 mM CaCl₂ was mixed with the same volume of solution containing 0.1 M mannitol, 20 mM 3-(N-morpholino)-propanesulfonic acid, pH 7.5, 2 mM EGTA, 4 mM EDTA, 8 mM DTT, 20 µg/mL leupeptin, and 2 mM phenylmethylsulfonyl fluoride. After standing for 2 min on ice, the protoplasts were ruptured with a hand-held Teflon (Yazawa, Tokyo, Japan) homogenizer, and the homogenate was centrifuged at 10,000 g for 10 min. The supernatant was centrifuged again at 100,000 g for 60 min. The microsomal membrane fraction was obtained as a pellet. The pellet was resuspended in 0.25 M mannitol, 10 mM 3-(N-morpholino)-propanesulfonic acid (Mops)-KOH, pH 7.0, 1 mM EGTA and stored on ice until use. Mesophyll protoplasts were isolated from 4- to 5-week-old fava bean leaves (20 g) as described previously (Sakaki and Kondo, 1985). The protoplast suspension in 0.6 M mannitol and 0.2 mM CaCl₂ was mixed with the same volume of 0.1 M mannitol, 20 mM Mops-KOH, pH 7.5, 2 mM EGTA, 4 mM EDTA, 8 mM DTT, 1% BSA, 2% PVP, 20 µg/mL leupeptin, and 2 mM phenylmethylsulfonyl fluoride. After standing for 2 min on ice, the protoplasts were ruptured with a Teflon homogenizer, and microsomal membranes from mesophyll protoplasts were prepared by the method described previously.

Plasma membranes of guard and mesophyll cells were isolated from microsomes by aqueous two-phase partitioning (Johansson et al., 1993). The resulting upper phase was mixed with the solution containing 0.25 M mannitol, 10 mM Mops-KOH, pH 7.5, 1 mM EGTA and centrifuged at 100,000 g for 60 min. The plasma membrane was obtained as a pellet. Plasma membranes were frozen in liquid nitrogen and thawed at 20°C twice to obtain inside-out membrane vesicles.

Proton Pumping and Electrical Potential

Proton pumping was measured by the absorbance decrease of acridine orange according to Becker et al. (1993) at 24°C. Absorbance at 492 and 551 nm was determined every 30 sec using a spectrophotometer (model DU-70; Beckman Instruments Inc., Fullerton, CA). The reaction mixture (100 µL) contained 10 mM Mops-KOH, pH 7.0, 0.25 M mannitol, 5 mM MgCl₂, 1 mM EGTA, 50 mM KNO₃, 5 µg/mL oligomycin, 8 µM acridine orange, membrane preparation (10 µg of which was protein), and free Ca²⁺. The Ca²⁺ concentration used was 0.01, 0.1, 0.32, 1, and 10 µM that was buffered with EGTA. The Ca²⁺ concentration was calculated using the dissociation constants of EGTA chelates of Ca²⁺ and Mg²⁺ (Silten and Martelli, 1971). The reaction was started by adding 1 µL of 200 mM ATP in 200 mM Mops-KOH, pH 7.0. To know the specificity of Ca²⁺ for the inhibition, the proton pumping was measured by fluorescence quenching of quinacrine at 22°C. Membrane preparations were excited at 424 nm, and fluorescence emission at 500 nm was measured using a fluorescence spectrophotometer (model RF-5000; Shimadzu, Kyoto, Japan). The basal reaction mixture (250 µL) contained 10 mM Mops-KOH, pH 7.0, 0.25 M mannitol, 5 mM MgCl₂, 50 mM KNO₃, 5 µg/mL oligomycin, 1 µM quinacrine, membrane preparation, and various cations at 10 µM.

The concentration of Ca²⁺ was also buffered with 1,2-bis(2-amino phenoxy)ethane N,N,N',N'-tetracetic acid (BAPTA). The reaction mixture contained the same aforementioned ingredients, except that BAPTA
was used instead of EGTA. BAPTA and ATP were added simultane-
ously in this experiment because the pumping was suppressed strongly
when microsomal membranes had been incubated in the presence
of BAPTA without Ca²⁺. The Ca²⁺ concentration was calculated using
the dissociation constants of BAPTA chelates of Ca²⁺ and Mg²⁺ (Tsien,
1980).

Generation of electrical potential in microsomal membranes was mea-
sured by fluorescence quenching of bis(3-propyl-5-oxoisoxazol-4-yl)
pentamethine oxonol (Oxonol VI; Molecular Probes, Inc., Eugene, OR)
at 22°C. Oxonol VI at 2 μM was dissolved in DMSO. Membrane prepa-
ration solutions were excited at 591 nm, and fluorescence emission at 630 nm
was measured using a fluorescence spectrophotometer (Shimadzu).

ATP and ITP Hydrolysis

H⁺-ATPase activity was measured by determining the Pi released from
ATP according to a previously described method (Shimazaki and Kondo,
1987) with modifications. The reaction mixture (100 μL) contained 10 mM Mops-KOH, pH 7.0,
0.25 M mannitol, 5 mM MgCl₂, 1 mM EGTA, 50 mM KNO₃, 5 μg/mL
oligomycin, 1 μM Oxonol VI, membrane preparation (40 μg of which was
protein), and free Ca²⁺. The concentration of Ca²⁺ used was 0.01,
0.1, 0.32, 1, and 10 μM. The reaction was initiated by adding of 2.5
μL of 200 mM ATP in 200 mM Mops-KOH, pH 7.0.

Measurement of Stomatal Apertures

Stomatal aperture was determined at 24°C as described previously
(Shimazaki et al., 1992). Epidermal strips (4 × 4 mm) from fava bean
plants were incubated in a Petri dish in 10 mM 2-(N-morpholino)
ethanesulfonic acid-KOH, pH 6.4, 0.1 mM CaCl₂ for 1 hr in the dark,
and stomatal opening was initiated by transferring the epidermis to
the basal incubation medium containing 10 mM 2-(N-morpholino)
ethanesulfonic acid-KOH, pH 6.4, and 5 mM KCl.

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