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H^+-ATPase in the plasma membrane of *Arabidopsis* pollen cells is involved in extracellular calmodulin-promoted pollen germination

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Abstract

In this paper, the role of the plasma membrane (PM) H^+-ATPase in extracellular calmodulin (CaM)-promoted pollen germination and in tube growth of *Arabidopsis thaliana* was investigated. Pollen germination, pollen tube growth rate, free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) and Ca^{2+} channel activity in the PM of pollen cells were measured. In response to fusicoccin or CaM treatment, *in vitro* pollen germination and pollen tube growth rate, $[Ca^{2+}]_{cyt}$ and activity of a hyperpolarization-activated Ca^{2+}-permeable channel increased. Sodium vanadate inhibited the promotion of these parameters by extracellular CaM. The results suggest that H^+-ATPase may be involved in extracellular CaM-regulated pollen germination and in tube growth by modulation of the hyperpolarization-activated Ca^{2+} channel in the PM of *A. thaliana* pollen cells.

Keywords: *Arabidopsis thaliana*; H^+-ATPase; Pollen; Extracellular calmodulin; Hyperpolarization-activated calcium channel

1. Introduction

As an important intracellular signal transduction component and metabolism regulator, Ca^{2+} plays a key role in sexual plant reproduction, especially in pollen germination and tube growth. It is well established that extracellular Ca^{2+} is necessary for *in vitro* pollen germination and tube growth [1]. In germinating pollen grains, the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) at the germination aperture is higher than any other areas. A tip-focused $[Ca^{2+}]_{cyt}$ gradient exists in growing pollen tubes [2–5], indicating that localized Ca^{2+} influx may be closely related to pollen germination and tube growth [3,6–8].

Calcium channels are the main gates governing calcium influx in the plasma membrane (PM). A voltage-dependent Ca^{2+} channel is an important member of the calcium channel family. In recent years, hyperpolarization-activated calcium channels have been identified in various plant cells. These channels are required for cell expansion, and elongation, stomatal movement and to participate in transducing signals of reactive oxygen and elicitors [9–13]. Similar Ca^{2+}-permeable channels have been identified in *Arabidopsis* [14,15], lily [16] and pear pollen protoplasts [17]. It was suggested that these channels, which are activated by hyperpolarization of membrane potentials, may be involved in modulating Ca^{2+} influx in germinating pollen grains [14–17].

As one of the most important enzymes in plant cells, the PM H^+-ATPase is involved in numerous physiological processes, such as phloem loading, regulation of stomatal movement and solute uptake. Most of these physiological functions of H^+-ATPase result from its modulation on membrane potential and the intracellular and extracellular pH. H^+-ATPase exists in the PM of pollen grains [18,19], and is closely associated with pollen development [20,21],

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pollen germination and tube growth [20,22–29]. The calcium channel blocker Gd^{3+} remarkably inhibited pollen germination and tube growth promoted by H^+-ATPase, suggesting that H^+-ATPase might stimulate pollen germination and tube growth through modulating Ca^{2+} dynamics in pollen grains [22]. However, the specific calcium channel modulated by H^+-ATPase is still unclear.

Although calmodulin (CaM) is generally recognized as a multifunctional receptor protein for intracellular Ca^{2+}, CaM molecules have also been found in the extracellular space of plant [30] and animal cells [31]. The presence of apoplastic CaM was first detected in soluble extracts from the oat coleoptile cell wall [30]. Further evidence proved that apoplastic CaM existed in various plant species and participated in many physiological processes, such as cell reproduction, protoplast cell wall regeneration and gene expression [32–36]. Ma et al. reported that apoplastic CaM positively regulated pollen germination and tube growth of *Hippeastrum rutilum* [37]. Lenartowska et al. reported that sufficient apoplastic CaM was needed for pollen germination and tube growth [38]. Pollen germination and tube growth promoted by extracellular CaM were highly suppressed by Ca^{2+} channel blockers [37,39], suggesting that calcium influx participated in this process. Shang et al. reported that a hyperpolarization-activated calcium permeable channel existed in the PM of lily pollen, and this channel could be remarkably activated by CaM. This suggests that this channel might be involved in apoplastic CaM-accelerating pollen germination, and tube growth through stimulation of the generation of the intracellular calcium signal [16]. However, the mechanism by which calcium channel activation is stimulated by apoplastic CaM is still unclear. As an important enzyme modulating transmembrane potential, H^+-ATPase may be involved in modulating voltage-dependent ion channels in the PM. In this study, the roles of H^+-ATPase in CaM-promoted pollen germination and in tube growth, cytosolic calcium dynamics and activation of calcium channels were investigated. This investigation identifies the role of H^+-ATPase in the apoplastic CaM signal transduction mechanism using an agonist (fusicoccin; FC) and antagonist (sodium vanadate) of H^+-ATPase.

2. Materials and methods

2.1. Plant materials

Plants of *Arabidopsis thaliana* (ecotype Wassilewskija) were grown in vermiculite in a growth chamber under a 15 h light/9 h dark photoperiod (120–130 μmol/m^2 s) and a 22 °C day/18 °C night temperature regime. Relative humidity was maintained at 70%.

2.2. Purification of cauliflower calmodulin

Calmodulin was extracted from a cauliflower inflorescence following Shang et al. [16]. First, soluble proteins were extracted by homogenization of the inflorescence with 50 mM Tris–HCl buffer containing 0.5 mM dithiothreitol (DTT) and 0.1 mM $CaCl_2$, pH 7.5. This was followed by heating above 90 °C for 3 min to denaturalize thermolabile proteins, and then the extract was centrifuged. Calmodulin in the suspension was separated from other proteins with a Phenyl-Sepharose 4B affinity column, and was further purified with Sephadex G-75.

2.3. In vitro pollen germination assay

Newly opened flowers were placed into a centrifuge tube and 1 ml of germination medium (1 mM KCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mM boric acid, 16% (w/v) sucrose, pH 5.8 adjusted with Mes–Tris) was added. The tube was shaken gently to suspend the pollen grains. The pollen grain suspension was filtered through nylon filter cloth (100 μm pore), then centrifuged (800*g*, 1 min) and washed with the germination medium twice. The sediment was collected as purified pollen grains.

In vitro pollen germination experiments were conducted following Fan et al. [40]. Pollen grains were sown onto the semi-solid germination medium containing 1% (w/v) agarose (reagents were added to the germination buffer followed by agarose to solidify the medium). The pollen grains were incubated in a climatic chamber (continuous light, 25 °C, 100% relative humidity). After 9 h incubation, by which time the pollen germination percentage had peaked, the germinated pollen grains were counted and the pollen tube length was measured. All experiments were repeated three times and each treatment included four replicates. For each replicate, at least 300 pollen grains were counted to determine the germination percentage, and the length of at least 100 pollen tubes was measured. Pollen grains were considered to have germinated if the emerging tubes were longer than their diameter (20–30 μm).

2.4. Measurement of cytoplasmic calcium in pollen cells

The fluorescent calcium indicator Fura-2 AM was cold loaded into pollen grains following Wu et al. [15]. Pollen germination medium containing 20 μM Fura-2 AM (Invitrogen Ltd.) was used as the incubating solution. After 2 h incubation at 4 °C, the pollen cells were incubated in the dark at 25 °C for 1 h. Fluorescence in the pollen cells was detected with an inverted microscope (DM IRB, Leica Co.) equipped with a high-sensitivity CCD (Cool-SNAP cf, Roper Scientific, Inc.). Fura-2 in pollen cells was excited by 340 and 380 nm ultraviolet illuminations using a high-speed wavelength switcher (Lambda DG-4, Sutter Instrument Co.) equipped with a 300 W Xenon lamp. The ratio $R = F340/F380$ of mean fluorescence intensity in whole pollen cells was recorded. For calibration, the ratio of fluorescence intensity in standard Ca^{2+} buffers (Molecular Probes Co.) was measured. For each genotype, $[Ca^{2+}]_{cyt}$ in more than 100 pollen cells was recorded per experiment and each experiment was repeated four times.

2.5. Protoplast isolation

Pollen protoplasts were isolated following Tanaka et al. [41]. The cell wall was digested in germination medium containing 1.5% (w/v) cellulase (Onazuka R-10, Yakult Honsha Co.) and 0.5% (w/v) pectolyase (Y-23, Yakult Honsha Co.) at 25 °C for 2 h. Isolated protoplasts were washed twice with bath solution (10 mM $CaCl_2$, 5 mM Mes/Tris, adjusted to 1.6 OsM with sorbitol, pH 6.0).

2.6. Patch-clamp solutions

All patch-clamp solutions were adjusted to 1.6 OsM with sorbitol. The basal external (bath) solution comprised 10 mM $CaCl_2$ and 5 mM Mes/Tris, pH 5.8. The basal internal (pipette) medium was composed of 0.5 mM $CaCl_2$, 4 mM $Ca(OH)_2$, 2 mM Mg·ATP, 0.5 mM Tris·ATP, 10 mM EGTA, 15 mM Hepes/Tris, pH 7.0, with free Ca^{2+} of 100 nM.

2.7. Patch-clamp recording

The patch-clamp experiment was performed following Véry and Davies [10]. An Axon 200B amplifier (Axon Instruments, Inc.) controlled by pclamp 9.0 software (Axon Instruments, Inc.) was used to record the current signal. Borosilicate glass capillaries (1.5/1.1 mm diameter; WPI, Inc.) were used to make microelectrodes using a microelectrode puller (PB-7; Narishige Co.). The resistance of the electrode in bath solution (10 mM $CaCl_2$, 5 mM Mes/Tris, pH 5.8) was about 20 MΩ. Seal resistances were greater than 2 GΩ. Data were sampled at 1 kHz and filtered at 200 Hz. Membrane potentials were corrected for liquid junction potentials and series resistance. Voltage-clamp protocols comprised a series of depolarizing and/or hyperpolarizing steps of 4 s duration from a holding potential in the range from 0 to −200 mV. Current–voltage relationships (*I–V* curves) were then constructed with total whole-cell currents measured after 4 s of voltage clamp. For slow-ramp voltage-clamping, voltage was changed from −200 to 0 mV in 4 s. Currents were recorded at least 20 min after attainment of the whole-cell mode to ensure that equilibration of the pipette solution with the cytoplasm was as complete as possible.

3. Results

3.1. The role of the H^+-ATPase in extracellular calmodulin-promoted pollen germination and tube growth

Nine hours after hydration, the germination percentages for control pollen grains, and pollen grains treated with 1 μM FC and 0.2 μM CaM were 37.2 ± 6.5% ($n = 324$), 48.1 ± 6.8% ($n = 331$) and 56.3 ± 8.2% ($n = 317$), respectively. In medium containing 300 μM sodium vanadate, pollen germination was markedly inhibited and the germination percentage was only 22.9 ± 3.6% ($n = 320$). In medium containing both sodium vanadate and CaM, CaM promotion of pollen germination was remarkably inhibited, with a germination percentage of 23.4 ± 2.7% ($n = 345$) (Fig. 1a).

Results for pollen tube growth were consistent with those for pollen germination. The control length of pollen tubes at 9 h was 167 ± 18 μm ($n = 131$). Results for pollen tubes treated with FC and CaM were 280 ± 33 μm ($n = 145$) and 299 ± 25 μm ($n = 139$), respectively. Sodium vanadate remarkably suppressed pollen tube growth, with a pollen tube length of only 124 ± 12 μm ($n = 114$)

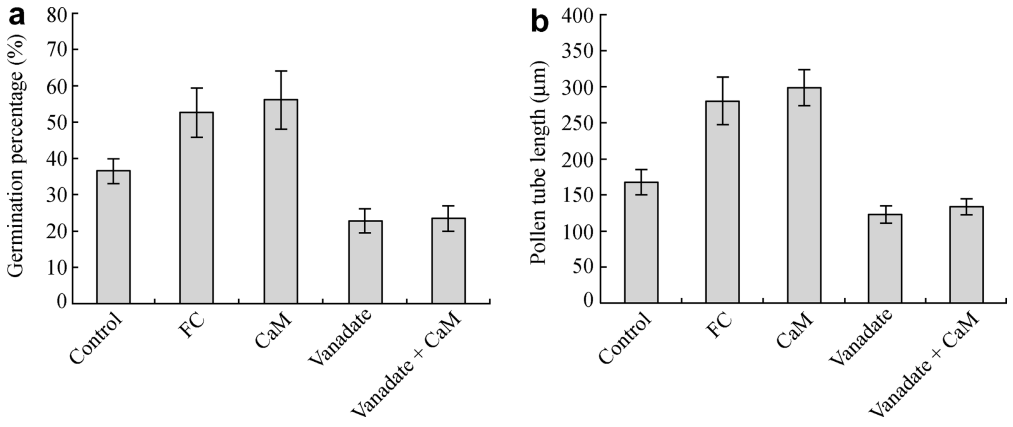

Fig. 1. The effect of H^+-ATPase agonist and antagonist compounds on calmodulin-promoted pollen germination and pollen tube growth rate in *Arabidopsis thaliana*. Pollen grains were germinated on a medium containing 1 μM fusicoccin (FC), 0.2 μM purified plant calmodulin (CaM) only, 300 μM sodium vanadate only (vanadate) or 0.2 μM purified plant CaM + 300 μM sodium vanadate (vanadate + CaM). After 9 h incubation, the pollen germination percentage was counted and pollen tube length was measured. To measure the germination percentage, at least 300 pollen grains were observed and the mean ± SE calculated is shown. To measure the pollen tube length, at least 100 pollen tubes were measured and the mean ± SE calculated is shown. The data were analyzed statistically with Student's *t*-test. (a and b) Note germination percentage and pollen tube length, respectively, of pollen grains in medium containing different reagents. The pollen germination percentage and pollen tube growth rate in response to FC, CaM treatment was significantly higher than the control (Student's *t*-test; $P \leqslant 0.05$). There was no difference between vanadate and vanadate + CaM treatments (Student's *t*-test; $P > 0.05$).

recorded. In medium containing sodium vanadate and CaM, the pollen tube length was only 135 ± 11 μm ($n = 135$) (Fig. 1b).

3.2. The effect of gadolinium on fusicoccin- or calmodulin-promoted pollen germination and tube growth

As shown in Fig. 2, both normal and FC- or CaM-promoted pollen germination and pollen tube growth were remarkably suppressed by gadolinium chloride (Gd). In medium containing Gd, the pollen germination percentage was only $19.4 \pm 2.4\%$ ($n = 315$). Results for Gd + FC and Gd + CaM treatment were $20.9 \pm 3.5\%$ ($n = 320$) and $22.1 \pm 2.1\%$ ($n = 337$), respectively (Fig. 2a). The pollen tube length in the Gd, Gd + FC and Gd + CaM were 96.1 ± 11.6 μm, 106.3 ± 9.5 μm and 102.1 ± 8.3 μm, respectively (Fig. 2b).

3.3. The role of H^+-ATPase in extracellular calmodulin-modulated $[Ca^{2+}]_{cyt}$ dynamics

To investigate the possible role of H^+-ATPase in extracellular CaM-regulated $[Ca^{2+}]_{cyt}$ dynamics in pollen grains, the Ca^{2+} fluorescent indicator Fura-2 AM was loaded into pollen grains and the dynamics of cytosolic calcium concentration was measured. The resting $[Ca^{2+}]_{cyt}$ was about 100–150 nM (mean ± SE: 132 ± 23 nM, $n = 113$, Fig. 3c, control), and oscillation of the calcium concentration occurred (Fig. 3a, b, and control). In response to 1 μM FC treatment, $[Ca^{2+}]_{cyt}$ increased quickly and reached a peak level of 267 ± 39 nM ($n = 135$, Fig. 3c, FC) in 4–5 min (Fig. 3a, FC). Treatment with 300 μM sodium vanadate induced $[Ca^{2+}]_{cyt}$ to decrease quickly and it reached a minimum concentration of 78 ± 12 nM (Fig. 3c) in 2–3 min (Fig. 3a, vanadate). In response to 0.2 μM CaM treatment, $[Ca^{2+}]_{cyt}$ started to increase within 2–3 min, reaching a maximum concentration of 316 ± 53 nM ($n = 117$, Fig. 3c, CaM) within 2–3 min, thereafter it decreased slowly (Fig. 3b, CaM). In pollen grains pretreated with vanadate, CaM stimulated only a weak elevation in $[Ca^{2+}]_{cyt}$, which peaked at 95 ± 16 nM ($n = 137$, Fig. 3c, vanadate + CaM).

3.4. The role of H^+-ATPase in modulating the Ca^{2+} conductance in the plasma membrane of pollen cells

In the whole-cell configuration, hyperpolarization-activating Ca^{2+} current (elicited by a step voltage clamp protocol), which has similar voltage dependence with the reported calcium channel [15], was recorded (Fig. 4a). The control current intensity at −200 mV was 132 ± 28 pA (Fig. 4d). Addition of 1 μM FC to the bath solution activated the calcium current remarkably (Fig. 4b). The current at −200 mV was 265 ± 56 pA (Fig. 4d). Activation of calcium current by FC was further confirmed by results from the slow-ramp voltage-clamping (Fig. 4e). Addition of 300 μM sodium vanadate to the bath solution significantly inhibited the calcium current (Fig. 4c). The current at −200 mV was only 65 ± 17 pA (Fig. 4d). Inhibition of the calcium current by sodium vanadate was further confirmed by slow-ramp voltage-clamping (Fig. 4e).

3.5. The role of H^+-ATPase in extracellular calmodulin-induced activation of Ca^{2+} channels in the plasma membrane of pollen cells

As shown in Fig. 5, addition of CaM to the bath solution activated the calcium current remarkably (Fig. 5b); the mean ± SE current intensity at −200 mV was

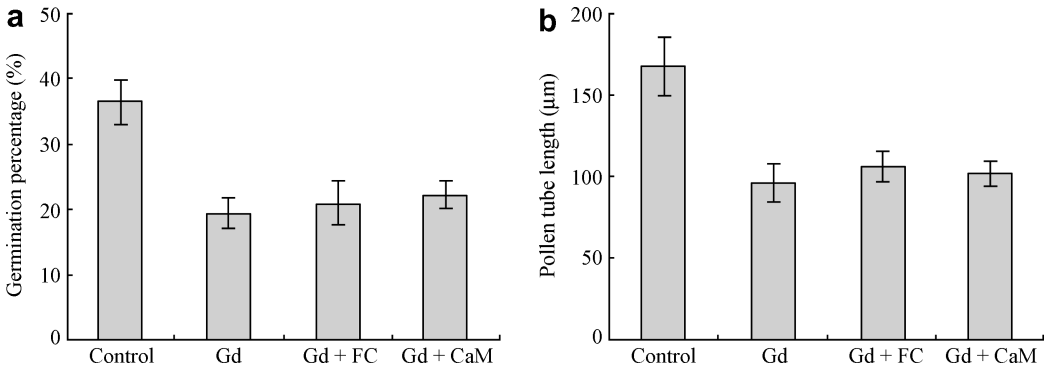

Fig. 2. The effect of the calcium channel blocker gadolinium on fusicoccin- and calmodulin-promoted pollen germination and pollen tube growth rate in *Arabidopsis thaliana*. Pollen grains were germinated on a medium containing 50 μM gadolinium chloride (Gd), 50 μM gadolinium chloride + 1 μM fusicoccin (Gd + FC) or 50 μM gadolinium chloride + 0.2 μM purified plant calmodulin (Gd + CaM). After 9 h incubation, the pollen germination percentage was counted and pollen tube length was measured. To measure the germination percentage, at least 300 pollen grains were observed and the mean ± SE calculated is shown. To measure the pollen tube length, at least 100 pollen tubes were measured and the mean ± SE is shown. The data was analyzed statistically with Student's *t*-test. (a and b) Note germination percentage and pollen tube length, respectively, of pollen grains in medium containing different reagents. Pollen germination percentage and pollen tube growth rate after Gd, Gd + FC and Gd + CaM treatment was significantly lower than the control (Students's *t*-test; $P \leqslant 0.05$). There was no significant difference between the Gd and Gd + CaM treatments or between the Gd and Gd + CaM treatments (Students's *t*-test; $P > 0.05$).

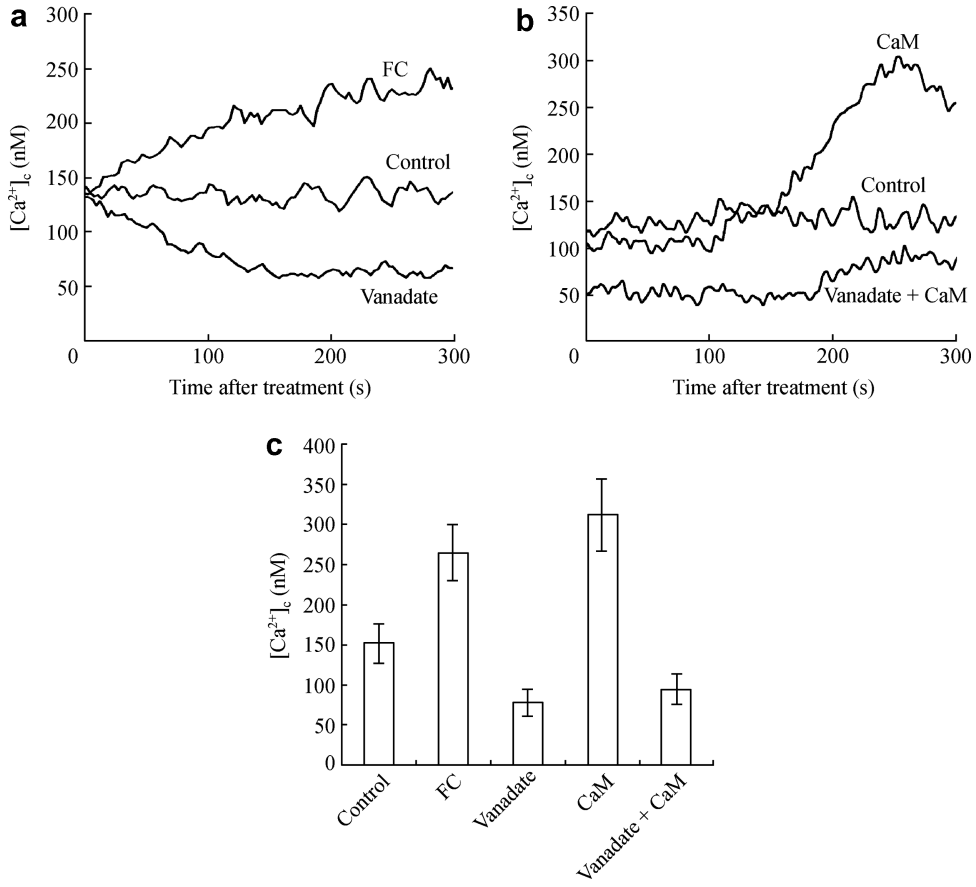

Fig. 3. The effect of calmodulin, H^+-ATPase agonist and antagonist compounds on cytosolic Ca^{2+} concentration in *Arabidopsis thaliana* pollen grains. (a) $[Ca^{2+}]_{cyt}$ dynamics in representative pollen grains in response to 1 μM fusicoccin (FC) or 300 μM sodium vanadate (vanadate) treatment. (b) $[Ca^{2+}]_{cyt}$ dynamics in representative pollen grains with 0.2 μM calmodulin (CaM) or 300 μM sodium vanadate + 0.2 μM calmodulin (vanadate + CaM) treatment. (c) $[Ca^{2+}]_{cyt}$ in pollen grains in response to CaM, FC and sodium vanadate. The data show mean ± SE of maximum $[Ca^{2+}]_{cyt}$ in at least 100 pollen grains. The results after different treatments were significantly different to the control (Student's *t*-test; $P \leqslant 0.05$). There was no significant difference between the vanadate and vanadate + CaM treatments (Student's *t*-test; $P > 0.05$).

272 ± 34 pA (Fig. 5e). In pollen protoplasts pretreated with 300 μM sodium vanadate, CaM-induced Ca^{2+} channel activation was markedly inhibited (Fig. 5c); the mean ± SE current at −200 mV was only 67.5 ± 22 pA (Fig. 5e), and there was no difference before (Fig. 4c) and after (Fig. 5c) CaM treatment. The result from slow-ramp voltage-clamping further confirmed the remarkable inhibition of sodium vanadate on CaM-induced Ca^{2+} channel activation (Fig. 5d).

4. Discussion

4.1. H^+-ATPase is involved in extracellular calmodulin-promoted pollen germination

Results from *in vitro* pollen germination showed that both FC and sodium vanadate had a marked effect on pollen germination and pollen tube growth. Since these reagents have been proved to be an agonist and antagonist, respectively, for P-type H^+-ATPase in the PM, the current results might indicate that H^+-ATPase was involved in modulation of these physiological processes. The inhibitory effect of sodium vanadate suggested that H^+-ATPase might be involved in pollen germination and tube growth in a positive manner, which was further confirmed by the effect of FC upon pollen germination and tube growth. It has been reported that H^+-ATPase in the PM participates in pollen germination and tube growth through modulating the intracellular and extracellular pH and dynamics of cytoskeleton or membrane trafficking [18–28]. This was further confirmed by results in the present study.

It has been reported that extracellular CaM participated in pollen germination and tube growth as a positive regulator [37,42], which was also demonstrated in this study. Both FC and CaM can significantly promote pollen germination and tube growth, and the amplitudes for elevated germination percentage and pollen tube length were quite similar. When H^+-ATPase was inhibited by sodium vanadate, pollen germination and pollen tube growth promoted by extracellular CaM were blocked. The results indicated that H^+-ATPase in the PM of pollen grains might be an important downstream target protein. Heterotrimeric G protein [42] and receptor-like kinase [43] were reportedly involved in the signal transduction pathway of extracellular

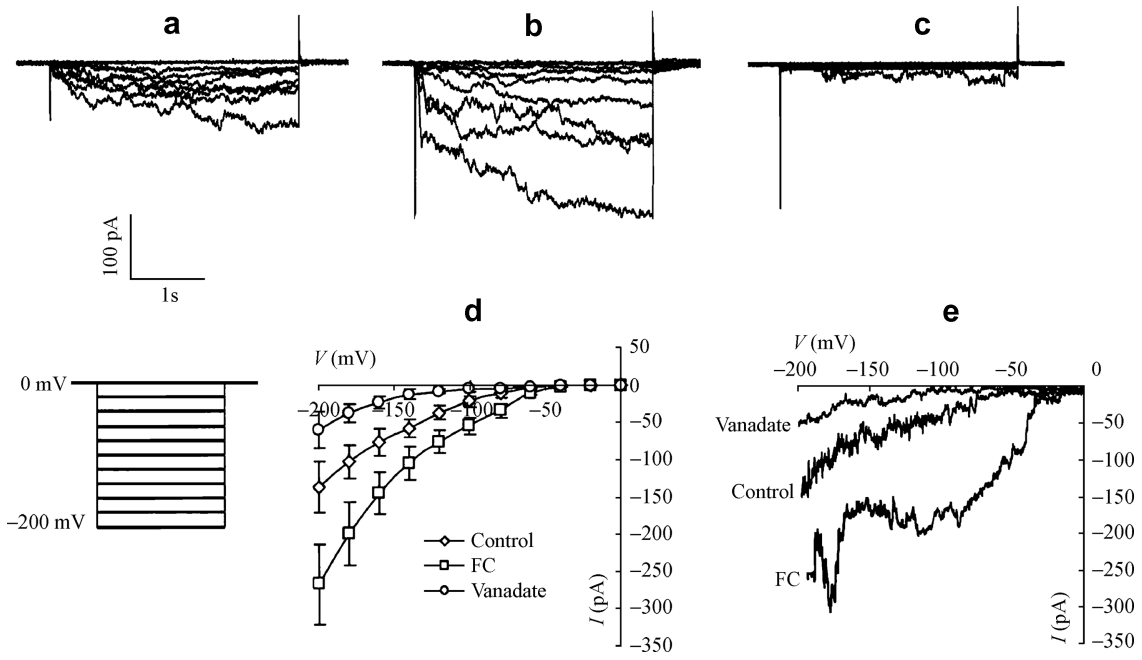

Fig. 4. The effect of H^+-ATPase agonist and antagonist compounds on Ca^{2+} conductance in the plasma membrane of *Arabidopsis thaliana* pollen protoplasts. (a–c) Ca^{2+} current of the control (a), 1 μM fusicoccin (FC) treatment (b) and 300 μM sodium vanadate treatment (c) recorded by step voltage-clamping. Traces are representative currents from six replicate protoplasts. (d) The mean ± SE *I–V* relationships of Ca^{2+} currents in (a–c) ($n = 6$ for each series of data, both FC treatment and sodium vanadate treatment results were significantly different to the control; Student's *t*-test, $P \leqslant 0.05$). (e) Ca^{2+} current after different treatments recorded by slow-ramp voltage-clamping. Traces are representative currents from six replicate protoplasts.

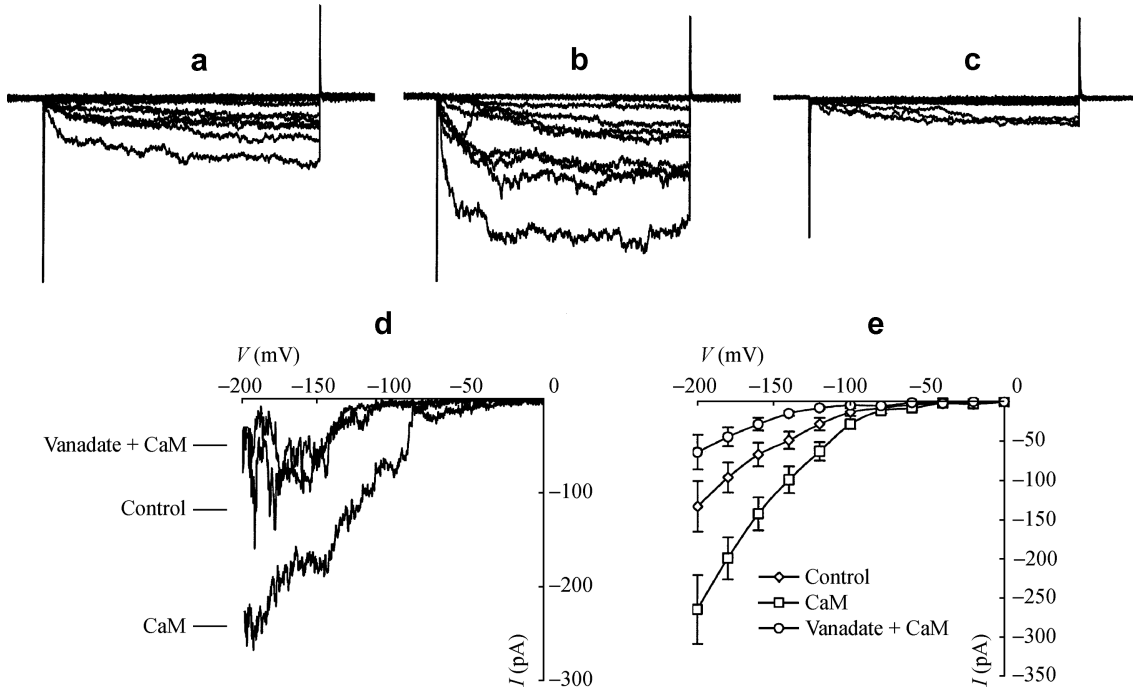

Fig. 5. The effect of sodium vanadate (300 μM) on calmodulin-promoted Ca^{2+} conductance in the plasma membrane of *Arabidopsis thaliana* pollen protoplasts. (a–c) Ca^{2+} current of the control (a), 0.2 μM calmodulin (CaM) treatment (b) and 300 μM sodium vanadate + 0.2 μM treatment (c) recorded by step voltage-clamping. Traces are representative currents from six replicate protoplasts. (d) Ca^{2+} current after different treatments recorded by slow-ramp voltage-clamping. Traces are representative currents from six replicate protoplasts. (e) The mean ± SE *I–V* relationships of Ca^{2+} currents in (a–c) ($n = 6$ for each series of data, both CaM treatment and vanadate + CaM treatment results were significantly different to the control; Student's *t*-test, $P \leqslant 0.05$).

CaM. Results in the present study suggested that H^+-ATPase in the PM might be another component in transmembrane signal transduction of extracellular CaM. As a key enzyme in the PM, H^+-ATPase plays an important role

in regulating membrane potential, ion positive transport, etc. Results in this paper revealed that H^+-ATPase in the PM of *Arabidopsis* pollen grains might be involved in transmembrane signal transduction of some extracellular stimulators.

4.2. Calcium influx is involved in H^+-ATPase- and CaM-promoted pollen germination

Ca^{2+} plays a key role in sexual plant reproduction, Ca^{2+} influx from extracellular medium is the main resource of cytoplasmic calcium [1]. To verify the role of the calcium signal in H^+-ATPase- and CaM-promoted pollen germination and tube growth, gadolinium was used to block calcium influx. H^+-ATPase- or CaM-promoted pollen germination and tube growth were strongly inhibited by gadolinium, indicating that calcium influx may play a key role in the signal transduction of the two signal molecules. Results from $[Ca^{2+}]_{cyt}$ measurement further revealed that calcium dynamics was involved in the signal transduction pathway featured by H^+-ATPase or CaM. Although $[Ca^{2+}]_{cyt}$ elevation amplitudes were quite similar after stimulation by FC and CaM, the features of calcium dynamics evoked by FC and CaM were different. As shown in Fig. 3a and b, $[Ca^{2+}]_{cyt}$ was elevated rapidly in response to FC, whereas it took 2–3 min for the $[Ca^{2+}]_{cyt}$ level to increase after addition of CaM. The lag in the reaction indicated that H^+-ATPase might be located downstream of extracellular CaM. Compared with the direct stimulation of FC on H^+-ATPase, extracellular CaM might stimulate H^+-ATPase indirectly. Some signal transduction events may occur during this time period. CaM-induced $[Ca^{2+}]_{cyt}$ elevation was strongly suppressed by sodium vanadate, which further revealed that H^+-ATPase was an important downstream target in CaM-stimulated Ca^{2+} mobilization.

4.3. H^+-ATPase is involved in extracellular calmodulin-promoted Ca^{2+} channel activation

The $[Ca^{2+}]_{cyt}$ dynamics resulting from activated Ca^{2+} influx is closely related to H^+-ATPase- and CaM-promoted pollen germination and tube growth. Therefore, the pathway mediating Ca^{2+} influx into the cytoplasm may play a key role in these processes. A hyperpolarization-activated Ca^{2+} channel is an important calcium influx channel in pollen grains [14–17]. Such a channel in the PM of lily pollen can be activated by extracellular CaM [16]. In the present study, the effects of FC and CaM on the activity of a similar channel in *Arabidopsis* were investigated.

FC-induced activation and sodium vanadate-induced inhibition of the Ca^{2+} channel indicated that the activation of the Ca^{2+} channel by hyperpolarization was closely related to H^+-ATPase activity. The collective results from *in vitro* pollen germination, $[Ca^{2+}]_{cyt}$ measurement and patch clamping, suggest that H^+-ATPase may be involved in pollen germination and tube growth. The mechanism for this is stimulating Ca^{2+} influx through a hyperpolarization-activated Ca^{2+} channel in the PM of pollen cells. H^+-ATPase is the key enzyme in maintaining the resting membrane potential, and increased H^+-ATPase activity may result in hyperpolarization of the PM potential, which will markedly stimulate the voltage-dependent ion channels. The modulation of the hyperpolarization-activated Ca^{2+} channel has been investigated in plant cells. It has been reported that the cytoskeleton [14], the heterotrimeric G protein [15] and pH [17] might be important factors involved in modulating its activity. Results in the present study indicated that H^+-ATPase might also participate in modulation of this channel.

The hyperpolarization-activated Ca^{2+} channel has been found to be activated by extracellular CaM in pollen cells of *Lilium davidii* [16]. In this study, a similar result was observed in *Arabidopsis* pollen grains. The activation of Ca^{2+} conductance induced by FC and CaM was quite similar. Sodium vanadate greatly inhibited the effect of extracellular CaM on Ca^{2+} channel activity, indicating that H^+-ATPase participated in the regulation of Ca^{2+} channel activation by extracellular CaM. The mechanism of transmembrane signal transduction of extracellular CaM has been reported during the past decade. Heterotrimeric G protein [42] and receptor-like protein kinase [43] in the PM have been demonstrated to be important components in the signal transduction of extracellular CaM. Results in the present study further revealed that another important component, H^+-ATPase, may also participate in signal transduction of extracellular CaM.

Based on results in the present study, we suggest that H^+-ATPase may be involved in extracellular CaM-promoted pollen germination and pollen tube growth. We suggest it does this through activating the hyperpolarization-activated Ca^{2+} channels in the PM of *Arabidopsis* pollen cells.

Acknowledgements

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