Ca\textsuperscript{2+} Influx into Lily Pollen Grains Through a Hyperpolarization-activated Ca\textsuperscript{2+}-permeable Channel Which Can be Regulated by Extracellular CaM

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Confocal laser scanning microscopy (CLSM) and whole-cell patch-clamp were used to investigate the role of Ca\textsuperscript{2+} influx in maintaining the cytosolic Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{c}) and the features of the Ca\textsuperscript{2+} influx pathway in germinating pollen grains of \textit{Lilium davidii} D. [Ca\textsuperscript{2+}]\textsubscript{c} decreased when Ca\textsuperscript{2+} influx was inhibited by EGTA or Ca\textsuperscript{2+} channel blockers. A hyperpolarization-activated Ca\textsuperscript{2+}-permeable channel, which can be suppressed by trivalent cations, verapamil, nifedipine or diltiazem, was identified on the plasma membrane of pollen protoplasts with whole-cell patch-clamp recording. Calmodulin (CaM) antiserum on the plasma membrane of pollen protoplasts with whole-cell patch-clamp were used to investigate the role of extracellular CaM. Calmodulin (CaM) antiserum and BSA, bovine serum albumin; BK solution, Brewbaker and Kwack’s solution; CLSM, confocal laser scanning microscopy; DTT, dithiothreitol; ECM, extracellular matrix; PDE, phosphodiesterase; HACC, hyperpolarization-activated calcium channel.

Introduction

As an important secondary messenger, Ca\textsuperscript{2+} is involved in various physiological processes in plant cells (Bush 1995). Ca\textsuperscript{2+} plays a key role in sexual plant reproduction, especially in pollen germination and tube growth. It had been well established that sufficient Ca\textsuperscript{2+} in the medium is necessary for in vitro germination of pollen grains; reducing Ca\textsuperscript{2+} concentration or adding Ca\textsuperscript{2+} channel blockers to the germination medium may inhibit pollen germination and tube growth (Brewbaker and Kwack 1963, Bednarska 1989). Germinating pollen grains in vivo may adsorb Ca\textsuperscript{2+} from stillets (Wilhemi and Preuss 1997, Malhó and Trewavas 1996) or Ca\textsuperscript{2+} crystals which adhered to the wall of pollen cells (Iwano et al. 2004). In growing pollen tubes, a tip-focused Ca\textsuperscript{2+} gradient which has a close relationship with pollen tube growth was found; this gradient may result from localized Ca\textsuperscript{2+} influx (Obermeyer and Weisenseel 1991, Malhó et al. 1994, Pierson et al. 1994, Malhó et al. 1995, Franklin-Tong et al. 1997, Holdaway-Clarke et al. 1997, Franklin-Tong et al. 2002, Camacho and Malhó 2003, Holdaway-Clarke et al. 2003). Further results suggested that some calcium-binding proteins, such as calmodulin (CaM), calcium-dependent protein kinase and cytoskeleton proteins, might be a target of a cytosolic Ca\textsuperscript{2+} signal, which will then modulate downstream processes correlated with pollen germination and tube growth (Moutinho et al. 1998, Snowman et al. 2002, Golovkin and Reddy 2003, Rato et al. 2004). Pollen germination and tube growth can be inhibited by Ca\textsuperscript{2+} channel blockers (such as verapamil and nifedipine) and trivalent cations (La\textsuperscript{3+}, Al\textsuperscript{3+} and Gd\textsuperscript{3+}); it was suggested that Ca\textsuperscript{2+} channels which were sensitive to these antagonists may be the main Ca\textsuperscript{2+} influx pathways in germinating pollen grains (Reiss and Herth 1985, Bednarska 1989, Geitmann and Cresti 1998). However, to date, channels with similar features have not been identified either in pollen grains or in pollen tubes.

CaM is classically known as a multifunctional receptor protein of the intracellular Ca\textsuperscript{2+} signal (Zielinski 1998). However, CaM had also been found in the extracellular space in both plant (Biro et al. 1984) and animal kingdoms (MacNeil et al. 1984). In plant systems, the presence of extracellular CaM was first detected in soluble extracts from oat coleoptile cell wall using a radioimmunoassay (Biro et al. 1984). Further evidence revealed the wide distribution and various physiological functions of extracellular CaM (Ye et al. 1988, Li et al. 1993, Sun et al. 1994, Sun et al. 1995, Zhu et al. 1998, Ma et al. 2000). Ma and Sun (1997) reported that CaM antiserum and
W7-agarose markedly inhibited pollen germination and tube growth of *Hippeastrum rutilum*, while exogenous purified CaM could significantly accelerate these processes. After pollination and/or pollen tube growth, CaM can be detected in the extracellular matrix (ECM) of transmitting tissue, especially in the ECM surrounding the tips of the growing pollen tubes, suggesting that sufficient extracellular CaM is needed for pollen germination and tube growth (Lenartowska et al. 2001). Extracellular CaM-accelerated pollen germination and tube growth were highly suppressed by EGTA and Ca$^{2+}$ channel blockers (verapamil and nifedipine) (Ma and Sun 1997, Ma et al. 1998), showing that Ca$^{2+}$ influx may be involved in the signaling pathway of extracellular CaM in pollen cells. However, there is no direct evidence to confirm this yet.

To sum up, the feature of the Ca$^{2+}$ influx pathway through which germinating pollen grains take Ca$^{2+}$ from the extracellular medium and how extracellular CaM could modulate Ca$^{2+}$ influx is still unclear. Herein, we performed confocal laser scanning microscopy (CLSM) and whole-cell patch-clamp recording to identify the features of the Ca$^{2+}$ influx channels and to find the way in which extracellular CaM modulates [Ca$^{2+}]_c$ homeostasis in germinating pollen grains of *Lilium davidii*.

## Results

### Effect of EGTA and Ca$^{2+}$ channel blockers on resting [Ca$^{2+}]_c$ in lily pollen cells

To measure [Ca$^{2+}]_c$ in pollen cells, fluo-3 AM (acetoxymethyl ester) was loaded to pollen grains. The fluorescence intensity in pollen cells was measured with CLSM and then converted into [Ca$^{2+}]_c$. To confirm the role of Ca$^{2+}$ influx in maintaining resting [Ca$^{2+}]_c$, the effect of EGTA and Ca$^{2+}$ channel blockers on [Ca$^{2+}]_c$ was investigated. The mean [Ca$^{2+}]_c$ in germinating pollen grains was about 270 ± 30 nM, and slight oscillation was recorded (Fig. 1A, n = 30). After adding 10 mM EGTA to the bath solution, [Ca$^{2+}]_c$ began to decrease within 8–10 min, and reached the lowest level during the following 9–12 min (Fig. 1B, n = 33). After adding 50 µM verapamil (n = 35), [Ca$^{2+}]_c$ decreased about 50%; a similar decrement of [Ca$^{2+}]_c$ was found in pollen grains which had been treated with nifedipine (n = 33) or diltiazem (n = 34), although nifedipine was less effective (Fig. 1C). In pollen cells which had been treated with 25 µM trivalent cations, a similar decline of [Ca$^{2+}]_c$ was recorded (Fig. 1D; La$^{3+}$, n = 33; Al$^{3+}$, n = 34; Gd$^{3+}$, n = 34).

### Identification of an inward-rectifying Ca$^{2+}$ conductance at the plasma membrane of lily pollen protoplast

The above results indicated that Ca$^{2+}$ influx plays a key role in holding the resting [Ca$^{2+}]_c$ during pollen germination; therefore, Ca$^{2+}$ channels on plasma membrane may be an important component in Ca$^{2+}$ mobilization. To study the nature of these channels, whole-cell patch-clamp was used to detect the transmembrane Ca$^{2+}$ current in pollen protoplasts. Whole-cell recordings from protoplasts in bathing media containing 10 mM external CaCl$_2$ showed a large, time-dependent, inward-rectifying conductance at hyperpolarized potentials (more negative than approximately −80 mV), and almost no outward conductance was found when the membrane was depolarized (more positive than 0 mV) (Fig. 2A, B, n = 7). In tail current analysis, it was found that the reversal potential of inward current ranged from +4 to +20 mV (mean ± SEM: +8.5 ± 3.5 mV, n = 7) (Fig. 2C).
Ca\textsuperscript{2+}-permeable channel in lily pollens

**Fig. 2** Hyperpolarization-activated calcium conductance at the plasma membrane in pollen protoplast of *Lilium davidii*. (A) Whole-cell patch-clamp, with voltage-clamp protocol shown on the left of current traces. (B) Mean ± SEM I–V relationships of total current in (A) (n = 7). (C) Evidence for a Ca\textsuperscript{2+} component in the inward current by tail current analysis; the arrow marks current reversal (n = 7). (D) Conductance when extracellular Ca\textsuperscript{2+} was replaced by Ba\textsuperscript{2+} (n = 7). (E) Effect of CsCl on calcium conductance (n = 7). The base bath solution comprised 10 mM CaCl\textsubscript{2} and 5 mM MES/Tris, pH 5.8. The pipet solution comprised 0.5 mM CaCl\textsubscript{2}, 4 mM Ca(OH)\textsubscript{2}, 2 mM MgATP, 0.5 mM Tris ATP, 10 mM EGTA, 15 mM HEPES/Tris, pH 7.0.

**Fig. 3** The effect of trivalent cations and Ca\textsuperscript{2+} channel blockers on inward Ca\textsuperscript{2+} current. (A–F) Ca\textsuperscript{2+} conductance in pollen protoplasts after applying 25 µM trivalent cations [(A) LaCl\textsubscript{3}, (B) AlCl\textsubscript{3}, (C) GdCl\textsubscript{3}; n = 7] or 50 µM Ca\textsuperscript{2+} channel blockers [(D) verapamil, (E) diltiazem, (F) nifedipine; n = 8]. (G) and (H) Mean ± SEM I–V relationships of total current recorded in pollen protoplasts being treated by three trivalent cations (G) (n = 7) or Ca\textsuperscript{2+} channel blockers (H) (n = 8). The base bath solution comprised 10 mM CaCl\textsubscript{2} and 5 mM MES/Tris, pH 5.8. The pipet solution comprised 0.5 mM CaCl\textsubscript{2}, 4 mM Ca(OH)\textsubscript{2}, 2 mM MgATP, 0.5 mM Tris ATP, 10 mM EGTA, 15 mM HEPES/Tris, pH 7.0.
To exclude the possibility that K⁺-permeable channels participated in this conductance, the effect of Ba²⁺ and Cs⁺ on the conductance was measured. When 10 mM BaCl₂ replaced external CaCl₂, the inward-rectifying conductance was also observed; the current can be detected at hyperpolarized potentials (Fig. 2D, n = 7). Adding 10 mM CsCl had almost no effect on the inward current (Fig. 2E, n = 7).

To identify further the features of Ca²⁺ conductance, the effects of trivalent cations and Ca²⁺ channel blockers on the Ca²⁺ conductance were measured. The results showed that trivalent cations strongly inhibit the conductance (Fig. 3A–C). In the presence of 25 µM La³⁺, Al³⁺ or Gd³⁺, the current inhibition at −200 mV was 83 ± 7% (n = 8), 70 ± 11% (n = 8) or 75 ± 9% (n = 8), respectively (Fig. 3G). The conductance was also suppressed by Ca²⁺ channel blockers (Fig. 3D–F); inhibition by 50 µM verapamil, diltiazem or nifedipine at −200 mV was 65 ± 13% (n = 7), 61 ± 9% (n = 7) or 55 ± 8% (n = 7), respectively (Fig. 3H).

The isolation of plant CaM and preparation of anti-CaM serum
After isolation and purification, the purity of plant CaM isolated from inflorescences of cauliflower was detected by SDS–PAGE. As shown in Fig. 4A, only a single 17 kDa band, which was visualized by Coomassie brilliant blue staining. (B) The activation of purified CaM on bovine brain PDE. (C) Western blot analysis of the specificity of anti-CaM serum. Only one 17 kDa band was found, showing the specific binding of anti-CaM serum with calmodulin.

Fig. 4 The detection of purity and activity of extracted cauliflower calmodulin and the binding of calmodulin to its antiserum. (A) Detecting the purity of CaM isolated from cauliflower by SDS–PAGE. Note the single 17 kDa band, which was visualized by Coomassie brilliant blue staining. (B) The activation of purified CaM on bovine brain PDE. (C) Western blot analysis of the specificity of anti-CaM serum. Only one 17 kDa band was found, showing the specific binding of anti-CaM serum with calmodulin.

Fig. 5 Effect of cell-impermeable CaM antagonists and purified plant CaM on resting [Ca²⁺]c in pollen cells. The fluorescent intensity in hydrated pollen cells was measured with CLSM and then converted into [Ca²⁺]. (A) [Ca²⁺], dynamics in pollen cells after applying CaM antagonists. (B) [Ca²⁺], dynamics in pollen cells after applying BK solution (a), 10⁻⁸ M (b), 10⁻⁷ M (c) and 10⁻⁶ M (d) CaM to extracellular medium. The arrow marks the point of application time. (C) Peak [Ca²⁺], values in pollen cells after applying BK solution (a); 10⁻⁸ M (b), 10⁻⁷ M (c) and 10⁻⁶ M (d) CaM or BSA to the extracellular medium. (D) Peak [Ca²⁺], values in pollen cells which had been pre-treated with BK solution containing 10 mM EGTA or 50 µM verapamil after applying 10⁻⁷ M CaM to the extracellular medium. In (C) and (D), each data point shows the mean ± SEM peak [Ca²⁺], value in 30–35 cells from five replicate experiments.

Effects of cell-impermeable CaM antagonists and purified plant CaM on resting [Ca²⁺]c in pollen cells.
After 2% anti-CaM serum or 200 nM W₇-agarose was added to the bath solution, [Ca²⁺]c in pollen grains decreased within 5–8 min, and reached the lowest level during the follow-
ing 12–15 min; 2% pre-immune serum led to a slight \([\text{Ca}^{2+}]_c\) decrease (Fig. 5A). When purified CaM was added to the bath solution, \([\text{Ca}^{2+}]_c\) increased within 2–3 min, reached a peak in the following 13–17 min, stayed at the same level for 15–20 min, and then declined until the levels were close to basal (Fig. 5B). Added CaM evoked a \([\text{Ca}^{2+}]_c\) increase in a dose-dependent manner. The peak value of \([\text{Ca}^{2+}]_c\) stimulated by \(10^{-8}\), \(10^{-7}\) and \(10^{-6}\) M CaM was 504 ± 86, 834 ± 178 and 340 ± 67 nM, respectively (Fig. 5B, C). In 35 pollen cells which had been treated by addition of CaM, a \([\text{Ca}^{2+}]_c\) increase was detected in 26 cells. In contrast, in pollen cells which had been treated by addition of bovine serum albumin (BSA; 35 cells) or Brewbaker and Kwack’s solution (BK solution; 30 cells), a slight \([\text{Ca}^{2+}]_c\) increase was detected only in three and two cells, respectively. In pollen cells which had been pre-treated by 10 mM EGTA or 50 µM verapamil, adding \(10^{-7}\) M CaM evoked a transient \([\text{Ca}^{2+}]_c\) increase; however, the amplitude of the \([\text{Ca}^{2+}]_c\) increase in pollen cells became very weak (Fig. 5D).

In germinating lily pollen grains, the cytosolic \(\text{Ca}^{2+}\) distribution was polarized. After adding \(10^{-7}\) M CaM, \([\text{Ca}^{2+}]_c\) started to increase first in the area that had the highest \(\text{Ca}^{2+}\) concentration, and then in nearby areas, and finally \([\text{Ca}^{2+}]_c\) in the whole cell increased and the polarized \(\text{Ca}^{2+}\) distribution was strengthened (Fig. 6a). After anti-CaM serum or W7agarose application, the polarity of \([\text{Ca}^{2+}]_c\) distribution became very weak (Fig. 6b, c); pre-immune serum had almost no effect on \([\text{Ca}^{2+}]_c\) distribution (data not shown).

The effect of exogenous CaM on hyperpolarization-activated calcium current

To examine whether extracellular CaM could regulate the activity of the \(\text{Ca}^{2+}\)-permeable channel in germinating pollen

**Fig. 6** Pseudocolor image of \([\text{Ca}^{2+}]_c\) dynamics in pollen cells being treated with exogenous CaM or cell-impermeable CaM antagonists. The time at which the photograph was taken (min) is showed in the corner of each photo. A pseudocolor bar is shown at the base, with figures beside the bar showing the \(\text{Ca}^{2+}\) concentration (µM) represented by the corresponding color. Series (a–c) show pollen cells being treated with (a) \(10^{-7}\) M CaM (note the increasing \([\text{Ca}^{2+}]_c\), and strengthened polar distribution of \([\text{Ca}^{2+}]_c\)), (b) 2% anti-CaM serum and (c) 200 nM W7-agarose (note decreasing \([\text{Ca}^{2+}]_c\), and weakened polar distribution of \([\text{Ca}^{2+}]_c\)). Each representative cell was selected from 30–35 cells which had been investigated in five replicate experiments.

**Fig. 7** Effect of exogenous CaM on the inward \(\text{Ca}^{2+}\) conductance. (A and B) \(\text{Ca}^{2+}\) conductance in pollen cells with (B; \(n = 7\)) or without (A; \(n = 7\)) 50 µM verapamil pre-treatment after adding \(10^{-7}\) M CaM. (C) Mean ± SEM \((n = 7)\) I–V relationships of the \(\text{Ca}^{2+}\) current recorded in pollen protoplasts being treated by BK solution (a), \(10^{-8}\) M (b), \(10^{-7}\) M (c) and \(10^{-6}\) M (d) CaM. (D) Mean ± SEM I–V relationships for CaM-activated \(\text{Ca}^{2+}\) current recorded in pollen protoplasts with \((n = 7)\) or without \((n = 7)\) 50 µM verapamil pre-treatment. The base bath solution comprised 10 mM CaCl\(_2\) and 5 mM MES/Tris, pH 5.8. The pipet solution comprised 0.5 mM CaCl\(_2\), 4 mM Ca(OH)\(_2\), 2 mM MgATP, 0.5 mM Tris ATP, 10 mM EGTA, 15 mM HEPES/Tris, pH 7.0.
grains, the effect of exogenous CaM on the hyperpolarization-activated Ca\(^{2+}\) conductance was measured. When CaM was added to the bathing medium, Ca\(^{2+}\) current intensity increased (Fig. 7A); the increase amplitude of Ca\(^{2+}\) conductance at –200 mV after adding 10\(^{-6}\), 10\(^{-7}\) and 10\(^{-8}\) M CaM was 150 ± 43% (n = 7), 240 ± 25% (n = 7) and 45 ± 14% (n = 7), respectively (Fig. 7C). In contrast, no current increase occurred in pollen protoplasts without pre-treatment (Fig. 7D).

To confirm that the increase in calcium current results from CaM-induced Ca\(^{2+}\) channel activation, 10\(^{-7}\) M CaM was applied to the bathing medium of protoplasts which had been pre-treated by 50 µM verapamil; this time, CaM only led to a slight increase in current intensity (Fig. 7B). The Ca\(^{2+}\) current intensity at –200 mV was only 28 ± 11% (n = 7) compared with current intensity in pollen protoplasts without pre-treatment (Fig. 7D).

**Discussion**

*Extracellular Ca\(^{2+}\) influx into germinating pollen grains through a hyperpolarization-activated Ca\(^{2+}\)-permeable channel*

As we discussed in the Introduction, it had been addressed that the Ca\(^{2+}\) influx pathway in plasma membrane might be an important component for modulating [Ca\(^{2+}\)]\(_{i}\) in pollen cells. As shown in Fig. 1, EGTA or Ca\(^{2+}\) channel blockers (verapamil, nifidipine, diltiazem and trivalent cations) led to a [Ca\(^{2+}\)]\(_{i}\) decrease, indicating that sustained Ca\(^{2+}\) influx was necessary for maintenance of the resting [Ca\(^{2+}\)]\(_{i}\) in pollen cells. It was also indicated that Ca\(^{2+}\) channels which are sensitive to these reagents might be present in the plasma membrane of pollen cells. Since the main target of these blockers is voltage-dependent Ca\(^{2+}\) channels on the plasma membrane (Bush 1995, Píneros and Tester 1997, Véry and Davis 2000), the above results revealed that voltage-dependent Ca\(^{2+}\) channels might be involved in Ca\(^{2+}\) influx in pollen cells.

The suggestion was confirmed further by patch-clamp recording, in which a hyperpolarization-activated Ca\(^{2+}\) channel was indeed detected on the plasma membrane of pollen protoplasts. We described a hyperpolarization-activated, inward-rectified Ca\(^{2+}\) conductance (Fig. 2, 3). Three main lines of evidence supported that the current was a Ca\(^{2+}\) conductance. (i) Since only Ca\(^{2+}\) exists as a cation in the bathing medium, Ca\(^{2+}\) current should be involved in the inward-rectifying cation current. (ii) The reversal potential of the conductance ranged from +4 to +20 mV, which was positive to equilibrium potentials of Cl\(^{-}\) (ECl = –88 mV), suggesting the presence of a Ca\(^{2+}\) component (ECa = +129 mV) in this inward current. (iii) Inward currents were also observed when BaCl\(_2\) replaced external CaCl\(_2\), which confirmed its mainly cationic nature and demonstrated that this conductance does not involve the classical K\(^{+}\) inward rectifier, which is blocked by barium (Gassmann and Schroeder 1994). This was also confirmed by the observation that this current was not blocked by 20 mM Cs\(^{+}\), another K\(^{+}\) inward rectifier blocker. Therefore, it could be concluded that a Ca\(^{2+}\)-permeable, inward-rectifying conductance was observed.

Two main lines of evidence supported the inherent involvement of the hyperpolarization-activated Ca\(^{2+}\) conductance in Ca\(^{2+}\) influx into pollen cells. First, the conductance was active at resting membrane potentials of germinating pollen cells. As reported by Weisenseel and Wenisch (1980) who measured the resting potentials of germinating pollen, that of lily pollen protoplasts was in the range of –100 to –110 mV (data not shown). The Ca\(^{2+}\) conductance was most likely to be active at this potential. Secondly, trivalent cations and Ca\(^{2+}\) channel blockers had a remarkably inhibitory effect on pollen germination and tube growth, indicating that voltage-dependent Ca\(^{2+}\) channels might be involved in these physiological processes (Reiss and Herth 1985, Bednarska 1989, Obermeyer and Weisenseel 1991, Miller et al. 1992, Geitmann and Cresti 1998). In this study, the character of hyperpolarization-activated Ca\(^{2+}\) conductance showed that it was one of the optimal candidates in charge of Ca\(^{2+}\) influx in germinating pollen grains.

During the past years, the activity of Ca\(^{2+}\) influx channels had been investigated by ratiometric imaging and measurement of extracellular Ca\(^{2+}\) influx (Pierson et al. 1996). It had been speculated that stretch-activated Ca\(^{2+}\) channels were involved in pollen germination (Pierson et al. 1994, Feijó et al. 1995, Malhò et al. 1995). Recently, the speculation was confirmed by the identification of a stretch-activated Ca\(^{2+}\) channel in pollen grains and pollen tubes (Dutra and Robinson 2004). As an important member of voltage-dependent Ca\(^{2+}\) channels, hyperpolarization-activated calcium channels (HACCs) have been identified in root cells (Kiegle et al. 2000, Véry and Davis 2000, Foreman et al. 2003), onion epidermal cells (Pickard and Ding 1993), suspension-cultured tomato cells (Gelli and Blumwald 1997, Blumwald et al. 1998), leaf mesophyll cells (Stoelzie et al. 2003) and stomatal guard cells (Hamilton et al. 2000, Pei et al. 2000, Murata et al. 2001). These channels, which have a similar voltage-dependent mechanism of action, activate at voltages more negative than about –100 to –150 mV, are required for cell expansion and elongation (White 1998, Véry and Davis 2000, White 2000, Miedema et al. 2001, Demidchik et al. 2002) and participate in transducing signals of reactive oxygen (Pei et al. 2000, Klüssner et al. 2002, Foreman et al. 2003, Köhler et al. 2003) and elicitors (Blumwald et al. 1998). In guard cells, HACCs have a central role in regulating stomatal movements related to cytosolic Ca\(^{2+}\) (Blatt 2000, White 2000, Murata et al. 2001, Schroeder et al. 2001, Köhler and Blatt 2002). In this study, a Ca\(^{2+}\)-permeable channel with similar features was also found in pollen cells. Therefore, we suggest that hyperpolarization-activated Ca\(^{2+}\)-permeable channels, along with some other type of Ca\(^{2+}\) channels, might also be present in germinating pollen cells.
**Extracellular CaM stimulates Ca$^{2+}$ influx through the hyperpolarization-activated Ca$^{2+}$-permeable channel**

During the past few years, indirect evidence from pharmacological experiments had proved that cytoplasmic Ca$^{2+}$ is involved in extracellular CaM-accelerated pollen germination (Ma and Sun 1997, Ma et al. 1998). In this study, new evidence directly proved that extracellular CaM stimulated Ca$^{2+}$ mobilization in pollen cells. Cell-impermeable CaM antagonists (anti-CaM serum and W7-agarose) led to a [Ca$^{2+}$]$_{c}$ decrease, indicating the existence of some active CaM molecules in extracellular spaces of pollen grains, which are involved in maintaining [Ca$^{2+}$]$_{c}$ homeostasis. The role of extracellular CaM in modulating [Ca$^{2+}$]$_{c}$ was proved further by investigating CaM-stimulated [Ca$^{2+}$]$_{c}$ elevation. The effect of 10$^{-7}$ M CaM was significant, while higher or lower concentrations of CaM were less effective. A similar dose-dependent mechanism had been found in extracellular CaM-accelerated physiological functions (Sun et al. 1994, Sun et al. 1995, Ma and Sun 1997, Ma et al. 1998, Ma et al. 2000). EGTA or verapamil strongly inhibited exogenous CaM-induced [Ca$^{2+}$]$_{c}$ elevation, indicating that Ca$^{2+}$ influx through Ca$^{2+}$-permeable channels which was sensitive to verapamil may be involved in extracellular CaM-induced Ca$^{2+}$ mobilization; the activation of hyperpolarization-activated Ca$^{2+}$ conductance stimulated by extracellular CaM directly revealed that such Ca$^{2+}$-permeable channels may be the main pathway for extracellular CaM-induced Ca$^{2+}$ influx.

A polarized distribution of [Ca$^{2+}$]$_{c}$ had been found in plant cells and was related to growth and development of plant cells, such as root hair development (Wymer et al. 1997), pollen germination (Polito 1983, Tirlapur and Cresti 1992) and tube growth (Reiss and Herth 1978, Pierson et al. 1994). In developing root hair cells of Arabidopsis thaliana, a localized change of cytosolic Ca$^{2+}$ was found firstly in cytoplasm at the site at which root hair would initiate, then in emerging root hairs, and finally in the tip of growing root hairs (Wymer et al. 1997). In our previous work, a localized [Ca$^{2+}$]$_{c}$ change in germinating pollen cells was found firstly in the germinal furrow and germinaperture, then in emerging pollen tubes and finally in the tip of growing pollen tubes (Shang et al. 2001); a similar heterogeneous distribution of [Ca$^{2+}$]$_{c}$ had been found in different pollen using different methods (Reiss and Herth 1978, Polito 1983, Tirlapur and Cresti 1992, Pierson et al. 1994). Furthermore, Ca$^{2+}$ distribution might affect the growth rate and direction of the pollen tube in different plant species (Reiss and Herth 1985, Malhó et al. 1994, Malhó and Trewavas 1996, Franklin-Tong et al. 1997, Hepler 1997, Geitmann and Cresti 1998, Franklin-Tong 1999). Results in this study revealed that the activity of extracellular CaM had a close relationship with the polar distribution of [Ca$^{2+}$]$_{c}$ in pollen cells: anti-CaM serum and W7-agarose weakened the polarity, while added CaM strengthened it. The effect of extracellular CaM on the polar distribution of [Ca$^{2+}$]$_{c}$ might also lead to some physiological processes in pollen cells.

Ca$^{2+}$ dynamics have been found in plant cells stimulated by various stimuli, such as low temperature (Knight et al. 1991), heat shock (Gong et al. 1998), red light (Shacklock et al. 1992) and apoplast peptide signal (systemin) (Moyen et al. 1998). It had been suggested that transient or sustained elevation of cytosolic calcium may be the early step in transducing some important signals which can modulate growth and development of plant cells through some downstream target proteins, although the direct correlation between cellular calcium signal and some physiological processes was still unclear (reviewed by Bush 1995, McAinsh and Hetherington 1998, Sanders et al. 1999, White and Broadley 2003). In our experiment, a prolonged calcium elevation was found; similar sustained calcium signals had been found in guard cells stimulated by oxidative stress (McAinsh et al. 1996) or added abscisic acid (Allen et al. 1999), and heat-shock-induced Ca$^{2+}$ transients also show a sustained Ca$^{2+}$ elevation for 15–20 min (Gong et al. 1998). These sustained Ca$^{2+}$ elevations lead to stomatal closure or thermostolerance events. Since extracellular CaM remarkably affected the concentration and distribution of cytosolic Ca$^{2+}$, which play an important role in modulating pollen germination and tube growth, the effect of extracellular CaM on pollen germination and tube growth (Ma and Sun 1997) possibly resulted from their effect on Ca$^{2+}$ mobilization in germinating pollen grains.

During the past few years, molecular characteristics of three major families of calcium-permeable channels in plant cells had been revealed: TPC1 (Furuichi et al. 2001, Hashimoto et al. 2004, Kurusu et al. 2004), CNGC (cyclic nucleotide-gated calcium channel) (Köhler and Neuhaus 2000, Talke et al. 2003) and GLR (glutamate receptor) (Davenport 2002). Previous evidence revealed that these newly identified calcium-permeable channels could be gated by voltage (depolarization) (Furuichi et al. 2001, Hashimoto et al. 2004, Kurusu et al. 2004) or ligand (cyclic nucleotide or cytoplasmic glutamate) (Köhler and Neuhaus 2000, Davenport 2002, Talke et al. 2003). Compared with these channels, the gating mechanism of the HACC which was detected in this work may be different. In our former work, it had been found that heterotrimeric G protein might act as a membrane transducer in the signal transduction pathway of extracellular CaM (Ma et al. 1999). Recently, a receptor-like CaM-binding site (protein) has been detected in the outer surface of suspension-cultured cells of A. thaliana (our unpublished result). Taken together with the above results, it can be suggested that extracellular CaM possibly binds to the putative receptor and activates the HACC in the plasma membrane of pollen cells through heterotrimeric G protein.

**Extracellular CaM may be a polypeptide primary signal**

In past decades, more attention had been paid to extracellular peptide hormones in plants. To date, several peptide signal molecules had been reported, such as systemin (Pearce et al. 1991), phytoalexins (PSKs) (Matsubayashi and Sakagami 1996), CLV3 (Clark et al. 1995), SCR (Schopfer et
which were quite similar to the systemin-induced \[\text{Ca}^{2+}\] involved in signal transduction of extracellular CaM, confirming a \[\text{Ca}^{2+}\] elevation, generating a temporalspecific \[\text{Ca}^{2+}\] signal in tomato mesophyll cells (Moyen et al. 1998). Results in this study indicated that extracellular CaM stimulated \[\text{Ca}^{2+}\] elevation in germinating pollen cells, generating a \[\text{Ca}^{2+}\] signal with specific spatial-temporal characters which were quite similar to the systemin-induced \[\text{Ca}^{2+}\] elevation. Moreover, it was revealed with patch-clamp analysis that extracellular CaM is an apoplastic peptide signal. Using aequorin as a fluorescent indicator of \[\text{Ca}^{2+}\], it had been found that systemin stimulates \[\text{Ca}^{2+}\], elevation, generating a temporalspecific \[\text{Ca}^{2+}\] signal in tomato mesophyll cells (Moyen et al. 1998). Results in this study indicated that extracellular CaM stimulated \[\text{Ca}^{2+}\] elevation in germinating pollen cells, generating a \[\text{Ca}^{2+}\] signal with specific spatial-temporal characters which were quite similar to the systemin-induced \[\text{Ca}^{2+}\] elevation. Moreover, it was revealed with patch-clamp analysis that a hyperpolarization-activated \[\text{Ca}^{2+}\]-permeable channel was involved in signal transduction of extracellular CaM, confirming that the \[\text{Ca}^{2+}\] increase might be the early step and act as the secondary messenger in extracellular CaM-induced physiological reactions, strongly supporting the idea that extracellular CaM might act as an extracellular peptide signal in the plant kingdom.

Materials and Methods

Plant material

*Lilium davidii* D pollen grains were collected from newly opened flowers; air-dried pollen grains were then stored at −70°C. In experiments, pollen grains were first hydrated with BK solution containing 1 mM Ca(NO₃)₂, 1 mM KNO₃, 1 mM MgSO₄, 1 mM boric acid and 0.5 M sucrose, pH 5.8.

Purification of cauliflower calmodulin

Plant CAm was extracted from inflorescences of cauliflower according to Biro’s method (Biro et al. 1984). First, soluble proteins were extracted from inflorescences by homogenization with 50 mM Tris–HCl buffer containing 0.5 mM dithiothreitol (DTT), 0.1 mM CaCl₂, pH 7.5, after heating above 90°C for 3 min to denature the thermolabile proteins, then the extract was centrifuged. CAm in the suspension was then separated from other proteins by phenyl-Sepharose 4B affinity chromatography and protein was purified further with Sephadex G-75 chromatography. To determine the purity of isolated CAm, at least 200 µg of purified protein was analyzed by SDS-PAGE; the protein lane was visualized by Coomassie brilliant blue R-250 staining. To detect the activity of isolated CAm, the activation of PDE by purified protein was measured using the method of Butcher and Sutherland (1962). PDE was isolated from bovine brain using the method of Ho et al. (1976). First, purified plant CAm was added to reaction buffer consisting of 40 mM Tris, 40 mM imidazole, 5 mM magnesium acetate, 0.1 mM CaCl₂, and 50 µg of PDE, then 1.5 mM cAMP was added to trigger the reaction. The buffer was incubated at 30°C for 30 min and then boiled for 2 min to terminate the reaction. After the reaction buffer recovered to room temperature, 0.1 ml of 1 mg ml⁻¹ venine was added and incubated further at 30°C for 10 min, adding 55% trichloroacetic acid to terminate the reaction. Finally, the buffer was centrifuged (3,000 rpm, 20 min) and the phosphate in the supernatant was measured using the method of Van Veldhoven and Mannaerts 1987; as a parameter to represent the activity of PDE, A₄₅₀ was measured with a spectrophotometer.

In the experiments, CAm was diluted in BK solution.

Preparation of anti-CAM serum

Anti-CAm anti-serum was raised in a rabbit against CAm purified from cauliflower using the method of Li et al. (1993). The specific binding of antibody and CAm was detected with Western blotting. First, soybean seedlings grown for 4–5 days was ground under a pestle in liquid nitrogen, protein extract buffer consisting of 0.15 mol l⁻¹ Tris–HCl (pH 7.0), 25% glycol and 2% polyvinylpolypyrrolidone was added and the homogenate was centrifuged (17,000 rpm) at 4°C for 1 h. Then >300 µg of crude proteins in the supernatant was separated by SDS–PAGE and further transblotted to a nitrocellulose membrane, and the membrane was incubated with phosphate-buffered saline (PBS, pH 7.4) consisting of 3% BSA and 0.03% Tween-20. After that, the membrane was incubated with anti-CAm serum diluted with washing buffer (PBS with 0.03% Tween-20, pH 7.4) at 37°C for 2 h. As a control, another nitrocellulose membrane was incubated with washing buffer-diluted pre-immune serum. After the membrane was washed with washing buffer three times (10 min each time), it was incubated further with washing buffer-diluted secondary antibody at 37°C for 2 h and then washed with washing buffer three times. Then the membrane was visualized with 0.1 M NaCl, 5 mM MgCl₂, 0.165 mg ml⁻¹ 5-bromo-4-chloro-3-indolyl phosphates (BCIP) and 1.65 mg ml⁻¹ nitro blue tetrazolium (NBT) for 5–10 min. In the experiment, antisera and pre-immune serum were diluted in BK solution.

Measurement of cytoplasmic calcium dynamics

Calcium fluorescent indicator was loaded into pollen grains using the method of Zhang et al. (1998). It had been shown that cold loading only slightly affected the viability of pollen grains (Shang et al. 2001). BK solution containing 20 µM fluo-3 AM was used as the incubating solution. Fluo-3 AM (Molecular Probes, Inc., Eugene, OR, USA) was added from a stock solution of 1 mM fluo-3 AM in dimethylsulfoxide (DMSO). After 2 h incubation at 4°C, pollen cells were incubated in the dark at 25°C for 1 h. Fluorescence in pollen cells was measured with a Nikon E600 upright microscope equipped with a confocal laser scanning system (Bio-Rad, MR/A-2). fluorescent probes were excited with a 488 nm laser, and emission fluorescence was filtered by a 530/40 nm filter to eliminate the auto-fluorescence of pollen grains. During the scanning period, any reagents were added to trace cytoplasmic fluorescence dynamics before and after treatments. All reagents were dissolved in BK solution. \[\text{Ca}^{2+}\] concentration was calibrated by in vitro and in vivo calibration. For in vitro calibration, a \[\text{Ca}^{2+}\] calibration buffer kit (Molecular Probes, Inc.) was used; 20 µM fluo-3 potassium salt was added (Molecular Probes, Inc.) to calibra-
tion buffers. The fluorescence intensity in standard buffers was measured and converted into the corresponding Ca$^{2+}$ concentration, then a standard curve was plotted. For in vivo calibration, pollen cells which had been loaded with fluo-3 AM were put into BK solution containing 10 mM CaCl$_2$ and 1 mM A23187; the fluorescence intensity in these cells was recorded as $F_{\text{max}}$. In a parallel experiment, pollen cells were put into BK solution in which CaCl$_2$ was replaced by 10 mM EGTA and 1 mM A23187; the fluorescence intensity in these cells was recorded as $F_{\text{min}}$. The fluorescence intensity in cells ($F$) was converted into [Ca$^{2+}$]$_i$ using the formula [Ca$^{2+}$]$_i = K_d (F - F_{\text{min}})/(F_{\text{max}} - F)$. Similar results were obtained from two calibrations.

**Protoplast isolation.**

Pollen protoplasts were isolated using the method of Tanaka et al. (1987). The cell wall was digested in BK solution which contained 1.5% cellulase (Onazuka R-10, Yakult Honsha Co., Ltd., Tokyo, Japan) and 0.5% pectlyase (Y-23, Yakult Honsha Co., Ltd.). Isolated protoplasts were washed twice with bath solution comprising 10 mM CaCl$_2$, 5 mM MES/Tris, pH 5.8. In channel blocker experiments, trivalent cations were added as chloride salts. In experiments with AlCl$_3$, 2 mM MgATP, 0.5 mM Tris ATP, 10 mM EGTA, 15 mM HEPES/Tris, pH 7.0.

**Patch-clamp solutions.**

All patch-clamp solutions were adjusted to 300 mOsm with sucrose. The basal external (bath) solution comprised 10 mM CaCl$_2$ and 5 mM MES/Tris, pH 5.8. In channel blocker experiments, trivalent cations were added as chloride salts. In experiments with AlCl$_3$, pH was reduced to 4.5 for both control and test solutions. The basal internal (pipet) medium comprised 0.5 mM CaCl$_2$, 4 mM Ca(OH)$_2$, 2 mM MgATP, 0.5 mM Tris ATP, 10 mM EGTA, 15 mM HEPES/Tris, pH 7.0.

**Patch-clamp recording.**

Classical patch-clamp methods were used according to the method of Véty and Davis (2000). An Axon 200B amplifier controlled by pclamp8.01 software was used to record the current signal. Then data were processed with Origin.e.0 software. A glass capillary (1.5/1.1 mm diameter; from WPI Inc.) was used to make microelectrodes with a microelectrode puller (PB-7 from Narishige Co., Ltd., Tokyo, Japan). The resistance of the electrode was about 20 MΩ. In experiments, the current signal was recorded when the seal resistance between the electrode and cell membrane rose to >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 were used for investigation of membrane conductance. A series of depolarizing and/or hyperpolarizing steps of 4 s duration were used from a holding potential in the range of +100 to −200 mV. Current–voltage relationships (I–V curves) then were constructed with total whole-cell currents measured after 4 s of voltage clamp. Total currents were recorded at least 20 min after attainment of the whole-cell mode to ensure that equilibration of the pipet solution with the cytoplasm was as complete as possible.

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**References**


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