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The effects of extracellular calmodulin on initiation of *Hippeastrum rutilum* pollen germination and tube growth

Ligeng Ma, Daye Sun

Laboratory of Molecular Cell Biology, Hebei Normal University, Shijiazhuang, Hebei Province (050016), Peoples Republic of China

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Abstract. The effects of anti-calmodulin (CaM) serum, the CaM antagonist W7-agarose, the Ca^{2+} chelator ethyleneglycol-bis-(β-aminoethyl)-N,N,N′,N′-tetraacetic acid (EGTA) and exogenous pure CaM on pollen germination and tube growth of *Hippeastrum rutilum* Herb were studied. Pollen germination and tube growth were inhibited or completely stopped by anti-CaM serum in a dose-dependent manner, while the same amount of preimmune serum had no effect on either process. Pollen germination and tube growth were also inhibited or completely stopped by the CaM antagonist W7-agarose and the Ca^{2+} chelator EGTA. The addition of exogenous pure CaM enhanced pollen germination and tube growth, whereas the same amount of bovine serum albumin had no effect. The inhibitory effects caused by anti-CaM serum, W7-agarose and EGTA-washing could be reversed completely by the addition of exogenous pure CaM. These results indicate that extracellular CaM initiates pollen germination and tube growth, whereas exogenous CaM enhances the above processes, and may provide a novel view for understanding the control of pollen germination and tube growth.

Key words: Extracellular calmodulin – *Hippeastrum* (pollen) – Pollen germination – Pollen tube growth

Introduction

In recent years, calmodulin (CaM), an important constituent of cellular signal transduction pathways, has also been found extracellularly and may therefore have some functions outside the cell (Sun et al. 1995). In plant systems, Biro and Sun, and co-workers in S. Roux's laboratory, first detected CaM in oat coleoptile cell walls by radioimmunoassay (Biro et al. 1984). In our laboratory, a series of experiments also indicated the existence of extracellular CaM: these included purification of extracellular CaM from the extracellular area of wheat coleoptile cells (Ye et al. 1988, 1989), observation of CaM-antibody gold particles outside the plasma membrane of corn root tips by electron-microscopy (Li et al. 1993) and detection of CaM in the culture medium of *Angelica dahurica* suspension-cultured cells and that of their protoplasts (Sun et al. 1994, 1995). Recently, further work in our laboratory indicated that CaM could extracellularly stimulate not only the proliferation of several kinds of plant suspension-cultured cells but also cell wall regeneration and division of their protoplasts, e.g. *A. dahurica* (Li et al. 1992; Sun et al. 1994, 1995), *Tennisetum typhoides* and *Setaria italica* (Sun et al. 1995), which indicated that extracellular CaM had some biological significance. More recently, extracellular CaM-binding proteins were detected in suspension-cultured cells of *A. dahurica* and *Daucus carota*, and a major 21-kDa extracellular CaM-binding protein of *A. dahurica* was purified and partially characterized in our laboratory (Tang et al. 1996), indicating that extracellular CaM might achieve its role through binding to extracellular CaM-binding proteins.

In 1983, Polito reported that *Pyrus communis* pollen germination and tube growth was enhanced by the addition of purified exogenous bovine brain CaM and inhibited by trifluoperazine (TFP), but in this report, it is still difficult to distinguish whether exogenous CaM functioned intracellularly or extracellularly. In the present study, we provide evidence that the intrinsic extracellular CaM of pollen initiates *Hippeastrum rutilum* pollen germination and tube growth, whereas exogenous CaM accelerates the above processes.

Materials and methods

Plant materials. Pollen of *Hippeastrum rutilum* Herb was used in this study. The pollen grains were collected from freshly opened anthers for each experiment.

Abbreviations: CaM = calmodulin; W7 = N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide

Correspondence to: Sun Daye; Fax: 86 (311) 5804451;
E- mail: hbsd@ pku. edu. cn

Pollen culture conditions. The standard medium for pollen germination and tube growth contained 0.32 M sucrose, 1 mM $CaCl_2$, 1 mM H_3BO_3, 1 mM KNO_3, 1 mM $MgSO_4$ and 2 mM citrate-phosphate buffer (pH 6.8) . Anti-calmodulin serum, W7-agarose (Sigma, St. Louis, Mo., USA), EGTA (Sigma) or CaM was added at various concentrations as indicated in the figures. For germination, pollen was incubated in small culture dishes at 25 ± 1 °C in a saturated atmosphere (100 relative humidity) for 12 h . At the end of each experiment, pollen material was transferred to −30 °C for quick freezing to stop pollen germination and tube growth. After thawing the frozen pollen, the percentage germination was determined and the lengths of pollen tubes were measured microscopically. Three- to five-hundred pollen grains were counted for calculation of germination percentage and 10–60 tubes were measured for pollen tube growth.

Preparation of CaM and anti-CaM antibody. Cauliflower and wheat CaMs were purified by Phenyl-Sepharose 4B affinity chromatography as described by Biro et al. (1984). The final CaM preparation was homogeneous, as judged by SDS-PAGE, which was performed on a 12.5% slab gel according to the procedure of Laemmli (1970). Anti-wheat CaM antibody was raised by immunizing rabbits with purified native wheat CaM (Cheng et al. 1991). The anti-wheat CaM antibody cross-reacted with various plant CaMs (Cheng et al. 1991; Li et al. 1992) and was used in the experiments in which pollen germination and tube growth were inhibited.

Results

Effects of anti-CaM serum on pollen germination and tube growth. Pollen germination and tube growth were inhibited significantly by anti-CaM serum, the degree of inhibition increasing with the concentration of anti-CaM serum. When the concentration of anti-CaM serum was 200 μg/ml, pollen germination and tube growth were completely stopped, whereas the same amount of preimmune serum had no effect (Fig. 1). The results indicate that CaM might be involved extracellularly in pollen germination and tube growth.

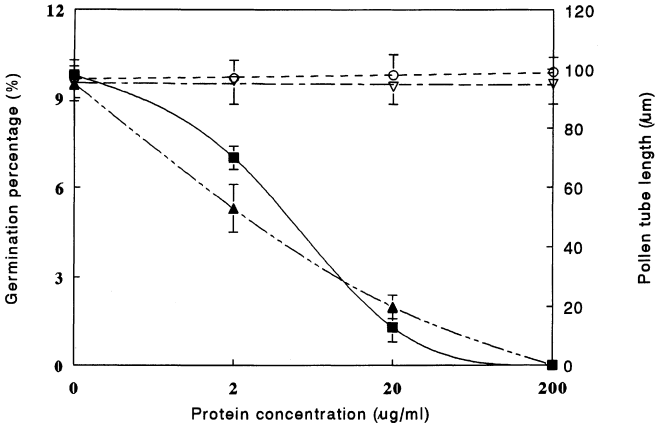

Fig. 1. The effects of anti-CaM serum (■, ▲) or preimmune serum (○, ▽) on pollen germination (■, ○) and pollen tube growth (▲, ▽). The pollen of *Hippeastrum rutilum* was incubated with the indicated amount of anti-CaM or preimmune serum in the medium. The pollen germination percentage was counted, and pollen tube length was measured microscopically at 12 h of incubation

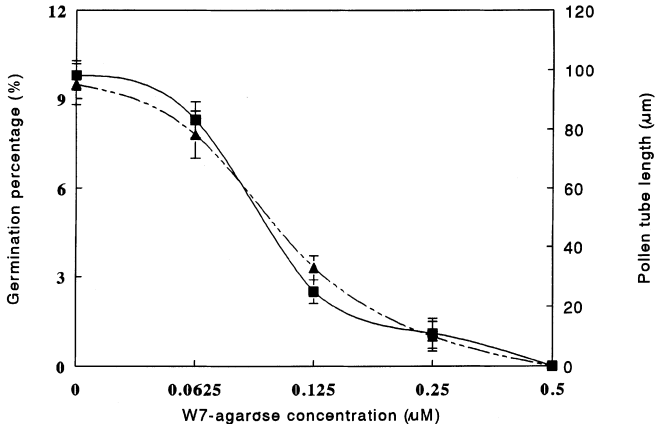

Fig. 2. The effects of the CaM antagonist W7-agarose on pollen germination (■) and pollen tube growth (▲). The pollen of *H. rutilum* was incubated with the indicated amount of W7-agarose in the medium. The pollen germination percentage was counted, and pollen tube length was measured microscopically at 12 h of incubation

Effects of W7-agarose on pollen germination and tube growth. Pollen germination and tube growth were inhibited by the CaM antagonist, W7-agarose, in a dose-dependent manner. The IC_{50} value (50% of maximum inhibition) for W7-agarose was below 0.1 mM, and pollen germination and tube growth were completely stopped by 0.5 mM of W7-agarose (Fig. 2), whereas the same concentration of W5 had no obvious effect on pollen germination and tube growth (data not shown).

Effects of exogenous CaM on pollen germination and tube growth. When various concentrations of purified cauliflower CaM were added to the culture medium directly, as shown in Fig. 3, pollen germination and tube growth were enhanced by exogenous CaM. Pollen germination and tube growth were increased approximately 100% by the addition of 0.1–1 μM CaM, but when the concentration of exogenous CaM was above 1 μM, the degree

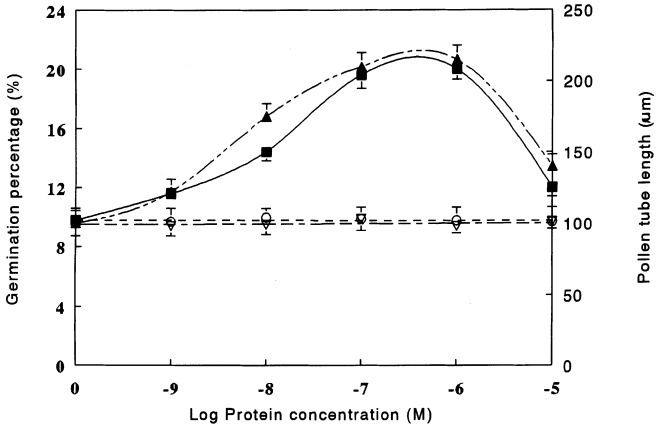

Fig. 3. The effects of exogenous CaM (■, ▲) or bovine serum albumin (○, ▽) on pollen germination (■, ○) and pollen tube growth (▲, ▽). The pollen of *H. rutilum* was incubated with the indicated amount of CaM or BSA in the medium. The pollen germination percentage was counted, and pollen tube length was measured microscopically at 12 h of incubation

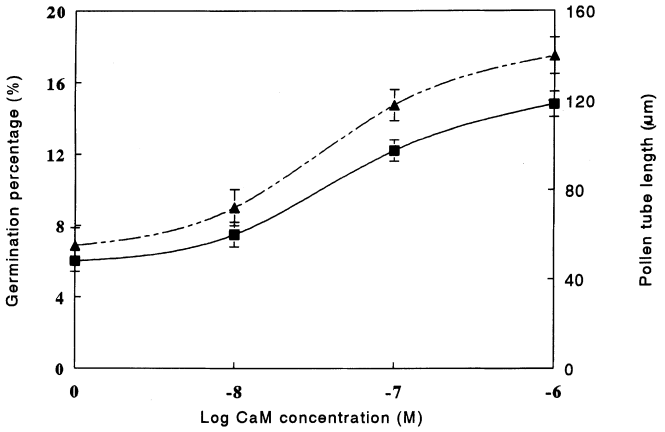

Fig. 4. The effects of exogenous CaM on the inhibitory effects of anti-CaM serum on pollen germination (■) and pollen tube growth (▲). The pollen of *H. rutilum* was incubated with 2 μg/ml of anti-CaM serum and the indicated amounts of CaM in the medium. The pollen germination percentage was counted, and pollen tube length was measured microscopically at 12 h of incubation

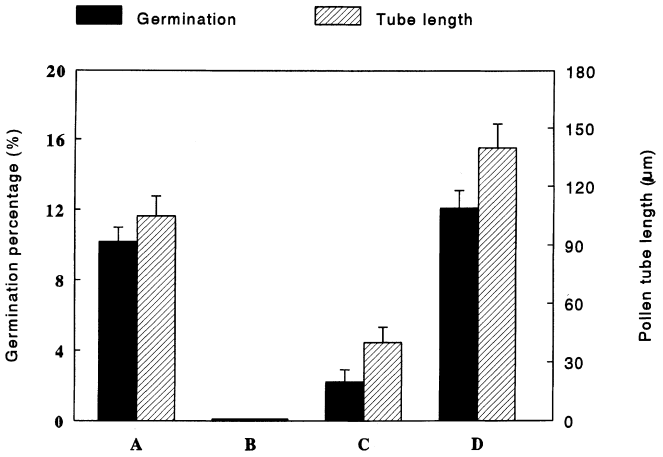

Fig. 6. The effects of EGTA on pollen germination and pollen tube growth. *A* Pollen of *H. rutilum* was cultured in standard medium. *B* Pollen grains were cultured in medium containing 1 mM EGTA. *C* After the pollen grains were washed with the medium containing 1 mM of EGTA for 10 min, they were transferred to the standard medium. *D* After the pollen grains were washed with the medium containing 1 mM of EGTA for 10 min, they were transferred to medium containing 0.1 μM of purified CaM. The pollen germination percentage was counted, and pollen tube length was measured microscopically at 12 h of incubation

of increase declined. However, the same amounts of bovine serum albumin had no effect on pollen germination and tube growth, indicating that the stimulation is specific to exogenous CaM .

In order to further verify that anti-CaM serum and W7-agarose functioned extracellularly, the effects of exogenous CaM on the inhibitory effects of anti-CaM serum and W7-agarose were examined. The inhibitory effects on pollen germination and tube growth caused by anti-CaM serum and W7-agarose were reversed completely by the addition of CaM (Figs. 4, 5).

Effects of extracellular Ca^{2+} on pollen germination and tube growth. Pollen germination and tube growth were completely stopped when the pollen was cultured in the standard medium containing 1 mM EGTA. When pollen grains were washed with the above medium for 10 min, and then transferred to the standard medium, the percentage of pollen germination and length of the tube were only partly reversed, whereas when transferred to the medium containing 0.1 μM of pure CaM, the effects on germination percentage and tube length caused by EGTA-washing were reversed completely (Fig. 6). These results indicate that some of the extracellular CaM had been removed by the EGTA.

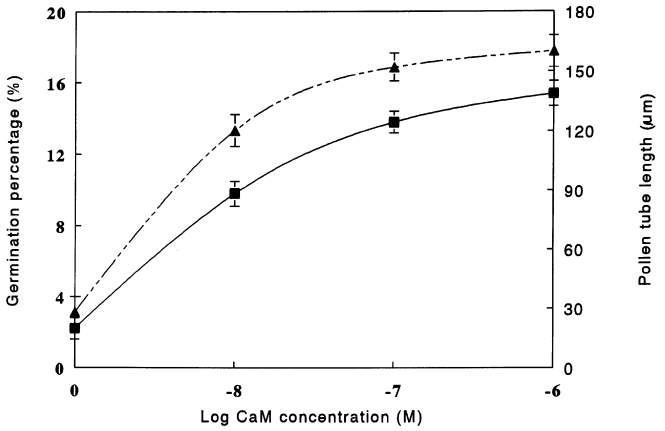

Fig. 5. The effects of exogenous CaM on the inhibitory effects of W7-agarose on pollen germination (■) and pollen tube growth (▲). The pollen of *H. rutilum* was incubated with 0.1 mM of W7-agarose and the indicated amounts of CaM in the medium. The pollen germination percentage was counted, and pollen tube length was measured microscopically at 12 h of incubation

Discussion

Polito (1983) has reported that *Pyrus communis* pollen germination and tube growth were enhanced by the addition of purified exogenous CaM, and inhibited by the CaM antagonists trifluoperazine (TFP) and chlorpromazine (CPZ). However, TFP and CPZ can penetrate the cell wall and plasma membrane freely, so it is difficult to distinguish whether CaM is involved extracellularly, intracellularly or both. In the present work with *Hippeastrum*, the observation that anti-CaM serum and the CaM antagonist W7-agarose could inhibit pollen germination and tube growth indicates that CaM is involved in the above processes and acts extracellularly since both the macromolecular antibody and the agarose bead are too large to pass through the plasma membrane; the latter is even bigger than the pollen grain.

In our experiments, the effects of anti-CaM serum, W7-agarose, exogenous CaM, and EGTA on the percentage germination of pollen and on tube length were closely correlated. It was also found that the pollen tube growth rate could be inhibited or completely stopped by anti-CaM serum and W7-agarose and enhanced by the

addition of pure CaM, after the pollen grains had germinated (data not shown). This indicates that both pollen germination and tube growth are directly and independently affected by extracellular CaM.

Besides *Hippeastrum rutilum* pollen, we found that anti-CaM serum and W7-agarose could stop the germination and tube growth of pollen from a number of other species, including *Fragaria ananassa*, *Duchesnea indica*, *Rosa xanthina*, *Robinia pseudoacacia*, *Forsythia suspensa*, *Lilium longiflorum*, *Nicotiana tabacum*, and that exogenous CaM enhanced these processes (data not shown). The percentage pollen germination for these species varied between 10 and 90% (data not shown) and these results indicate that the effects of extracellular CaM on pollen germination and tube growth have common significance in the plant kingdom.

In our previous studies, CaM was found to exist extracellularly in all species investigated, including oat (Biro et al. 1984), wheat (Ye et al. 1988, 1989), corn (Li et al. 1993), *Angelica dahurica* (Li et al. 1992; Sun et al. 1994) and *Daucus carota* (Sun et al. 1995). In animal systems, CaM also exists extracellularly in the medium of K582 human leukemic lymphocytes and human body fluids, including blood, saliva, urine and milk (MacNeil et al. 1984, 1988; Crocker et al. 1988). So it might have been predicted that CaM also exists extracellularly in *H. rutilum* pollen. The results in which anti-CaM serum and W7-agarose inhibited pollen germination and tube growth support this idea, and also indicate that the intrinsic extracellular CaM is involved in pollen germination and tube growth.

In this paper, we found that CaM antibody and W7-agarose, both of them membrane-impermeable CaM inhibitors, could completely stop pollen germination and tube growth, indicating that intrinsic extracellular CaM is not only involved in pollen germination and tube growth, but also functions as a major factor in the initiation of these processes. We also found that exogenous CaM accelerated pollen germination and tube growth. This indicates another biological role for extracellular CaM and provides a novel view for understanding the control of pollen germination and tube growth.

The importance of Ca^{2+} in pollen germination and tube growth has long been obvious from previous reports, and inward Ca^{2+} flux has been shown to be essential for pollen germination and tube growth (Miller et al. 1992; Pierson et al. 1993, 1994, 1996; Malho et al. 1994, 1995; Feijo et al.1995), but most of these reports were focused on the existence and function of a gradient of cytosolic free Ca^{2+} in germinating pollen and growing pollen tubes (Miller et al. 1992; Pierson et al. 1993, 1996; Malho et al. 1995), while the mechanism of Ca^{2+} influx was still unknown. Figure 6 indicates that the activation of extracellular CaM may be an early step in the above processes, making the relationship between extracellular CaM activity and Ca^{2+} influx interesting. We have preliminary data showing that extracellular CaM may mediate Ca^{2+} influx by controlling the G-protein-coupled calcium-channel activity (Ma et al. 1997), and further studies are being under taken to investigate the signal transduction pathways for extracellular CaM in the above processes.

We have shown in preliminary experiments that pollen germination and tube growth are dependent on exogenous calcium, with the turning point at 10 μM. When the concentration of calcium in the standard medium was below 10 μM, pollen germination and tube growth were inhibited significantly and exogenous CaM could not exert its effects on these processes. The percentage pollen germination and tube growth increased with increasing calcium concentration when it was above 10 μM; at these calcium concentrations, exogenous CaM had also shown its effects on pollen germination and tube growth (data not shown). Similar results have been reported by our laboratory for the effects of exogenous calcium on the proliferation of *A. dahurica* cells and cell protoplasts (Zhao et al. 1996), which was also promoted by extracellular CaM (Sun et al. 1994, 1995). The higher calcium required for these responses indicates that calcium might have functions other than the activation of CaM. The mechanism of ion regulation of extracellular CaM by calcium might be complicated. On the one hand, our preliminary data (not shown) indicate that the Ca^{2+} regulation of extracellular CaM might not be entirely similar to its regulation of intracellular CaM due to the different pH environment, and the pH of the medium might be one of the factors to affect the affinity between calcium and CaM. On the other hand, although the total calcium in the extracellular area of the plant cell might be 1 mM or more, the concentration of free calcium in vivo is not really known. Cleland et al. (1990) and Evans et al. (1991) have estimated that it is 10–100 μM, or even more than that, whereas Trewavas and Gilroy (1991) have estimated that it is 1 μM or less than 1 μM. So, the mechanism for ion regulation of extracellular CaM is still not clear, and further study is needed.

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