Apoplastic Calmodulin Receptor-like Binding Proteins in Suspension-cultured Cells of Arabidopsis thaliana*

Received for publication, February 4, 2005, and in revised form, June 24, 2005
Published, JBC Papers in Press, July 6, 2005, DOI 10.1074/jbc.M501349200

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Calmodulin, a highly conserved protein family that has long been well known as an intracellular calcium sensor, was identified in the culture medium and cell walls of Arabidopsis thaliana suspension-cultured cells by immunoblotting assay. A promotion effect by applying exogenous purified calmodulin and an inhibition effect by the addition of anti-calmodulin anti-serum or calmodulin antagonist to the medium on proliferation of suspension cells were found by monitoring incorporation of [methyl-3H]thymidine into nuclear DNA. Radioligand binding analysis with 35S-labeled calmodulin indicated the presence of specific, reversible, and saturable calmodulin binding sites on the surface of both Arabidopsis suspension-cultured cells and its protoplasts; among them at least one is on the surface of Arabidopsis protoplasts, with the $K_d$ ~9.2 nM, and two are on the out-surface of Arabidopsis suspension-cultured cells, with $K_d$ values of ~47.5 and 830 nM. Chemical cross-linking of 35S-labeled calmodulin to protoplasts revealed 117- and 41-kDa plasma membrane proteins specifically bound to calmodulin, whereas cross-linking with intact suspension-cultured cells verified more calmodulin binding proteins which might be cell wall-associated in addition to membrane-localized. Taking together, our data provide first evidence for the presence of apoplastic calmodulin receptor-like binding proteins on the cell surface of Arabidopsis suspension-cultured cells, which strongly supports our previous idea that apoplastic calmodulin functions as a peptide signal involved in regulation of cell growth and development.

Because the first peptide hormone, insulin, was found in 1922 (1), hundreds of peptide hormones and their specific receptors have been verified to play important roles in animal cell communications, whereas few were found in the plant cells until last decade, when plant biologists realized that “plant cells, like animal, may make use of peptide signals” (2). From then on about 10 polypeptide hormones or putative polypeptide signals have been reported in plants (3–6), but only 4 of them are widely recognized up to date, because their receptors and the functions of each pair, including systemin-SR160, SCR-SRK, PSK-PSKR, and CLV3-CLV1/CLV2, in plant defense, growth, and development were investigated both genetically and biochemically (6). Bioinformatics analysis of Arabidopsis thaliana genome sequence, however, mined out hundreds of candidate genes which more likely encode orphan receptors and orphan apoplastic polypeptides (7). Confirmation of existence of plasma membrane receptors and their polypeptide ligands experimentally become significant and important for our understanding the molecular mechanism by which plant polypeptide hormone functions in regulation of plant physiology and development.

Calmodulin (CaM)3 has been long-described since it was discovered in late 1960s as a conserved multifunctional calcium sensor that is well known to mediate intracellular Ca2+ signal transduction in eukaryotic cells. Our previous work and that of other laboratories have provided evidence for the presence of extracellular CaM (8–18) and for their functions in regulating the proliferation of suspension-cultured cells, cell wall regeneration, and cell division of protoplasts of Angelica dahurica (15, 16), pollen germination and pollen tube growth (17, 18), and stomata movement (19). Pharmacological, biochemical, and cell biology experiments demonstrated that a heterotrimeric G protein, phospholipase C, and inositol 1,4,5 trisphosphate, calcium influx, and H2O2 may be involved in the transmembrane signal transduction of apoplastic CaM in lily pollen system or Vicia faba guard cells (18–21). Based on all the data, we proposed that extracellular CaM might act as a polypeptide signal (10, 12, 20). However, the receptor on the cell surface of apoplast CaM has not yet been identified and has become the bottleneck of this hypothesis.

In this study we first confirmed the existence of CaM in the cell wall and culture medium of Arabidopsis suspension cells by immunoblotting assay of different cell fractions and the functions of CaM in stimulating proliferation of suspension-cultured cells. Then we analyzed the binding kinetics, reversibility, specificity, and saturation of 35S-AcAM2 (Arabidopsis calmodulin isofom 2) on Arabidopsis suspension-cultured cells and protoplasts. Finally we found candidate apoplastic CaM receptor-like binding proteins on the cell surface by chemical cross-linking of 35S-AcAM2 with protoplasts and suspension-cultured cells.

* This work was supported by National Natural Science Foundation of China Grants 90208004 and 30470889, National Key Basic Research Special Funds of China Grants G19990117 and 2006CB10101, Outstanding Younger Scientist Foundation of China Grant 30220524, Natural Science Foundation of Hebei Province in China Grant C200400152, and Doctor Foundation of Hebei Normal University Grant 12004B11. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1724 solely to indicate this fact.
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3 The abbreviations used are: CaM, calmodulin; ACaM2, Arabidopsis calmodulin isoform 2; BS7, bis(sulfosuccinimidyl) suberate; SCaM1, -4, and -5, soybean calmodulin isoform 1, 4, and 5; MS, Murashige and Skoog; sulfo-EGS, ethylene glycol bis(sulfosuccinimidyl) succinate; Mes, 4-morpholineethanesulfonic acid; BSA, bovine serum albumin.
Apoplastic Calmodulin Receptor-like Binding Proteins

EXPERIMENTAL PROCEDURES

Callus and Cell Suspension Cultures—Arabidopsis Columbia (Col-0) seeds were surface-sterilized and grown on Murashige and Skoog (MS) solid medium supplemented with 1 mg/liter 2,4-dichlorophenoxyacetic acid, 0.5 mg/liter 6-benzyladenine, 3% sucrose, and 0.6% agar, pH 5.7, for 2 weeks in the dark at 25 °C. The callus was collected from germinating seeds and transferred to fresh MS solid medium the same as above except for decreasing 6-benzyladenine to 0.1 mg/liter. After 3–4 such transfers, the callus became soft and pale yellow, and approximately 3 g of callus was transferred to 100 ml of MS liquid medium supplemented with 1 mg/liter 2,4-dichlorophenoxyacetic acid, 0.1 mg/liter 6-benzyladenine, and 3% sucrose, pH 5.7, and agitated at 120 rpm at 25 °C. Cells were subcultured every 7 days. Suspension-cultured cells of 3–5 days subculture were filtered through 50-μm stainless steel meshes to remove large cell clusters. The cells were collected by centrifugation at 500 × g for 10 min at 20 °C followed by 2 washes with digestion buffer or binding buffer with 1 mM Ca2+.

Preparation of Protoplasts—About 2 g of callus was incubated with 20 ml of enzyme solution (1% cellulase, 0.3% macerozyme in digestion buffer containing 5 mM Mes-KOH, pH 5.6, 0.5 mM mannitol, 1 mM CaCl2) with shaking at 50 rpm at 25 °C for 4 h in the dark. The digestion solution was filtered through 50-μm sieves, and the protoplasts were harvested by centrifugation at 600 × g for 10 min at 20 °C followed by 2 washes with digestion buffer and then resuspended and incubated in digestion buffer at a density of 105–106 protoplasts/ml for 35S-ACaM2 binding assay and chemical cross-linking. Preparation of Recombinant CaM and 35S-ACaM2—Recombinant CaM was prepared as described by Zielinski (25), and 35S-ACaM2 was labeled as described by Cui et al. (26).

Measurement of Suspension-cultured Cell Proliferation Rate—Cell proliferation rate was measured as described by Li et al. (15) with some modifications. In brief, 1 ml of suspension cell pretreated with CaM, anti-CaM antibody, and CaM antagonist W-7-agarose (N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride conjugated to agarose, Sigma) was incubated with 1 μCi of [methyl-3H]thymidine (Amersham Biosciences) for 12 h. The cells were then filtered onto glass filters (Millipore, APWA02500), washed with 20 ml of 0.9% NaCl, dehydrated with 10 ml of 95% ethanol, and fixed with 10 ml of 5% trichloroacetic acid. The filters were dried at 80 °C for 30 min, and the radioactivity was measured with a liquid scintillation analyzer. The Excel program was used to process the data. Binding of 35S-ACaM2 to Arabidopsis Protoplasts and Suspension-cultured Cells—In the standard assay 0.5 ml of protoplasts (106/ml) or suspension-cultured cells (106/ml) were transferred to 24-well culture plates and incubated on a shaker (150 rpm) at room temperature for 4 °C. Cross-linking was initiated by adding 35S-ACaM2 in the absence (total binding) or presence (nonspecific binding) of 200-fold unlabeled ACaM2 if not indicated. In competition assays the competitor was added just before 35S-ACaM2. Incubations were for 30 min, except when measuring the time course or reversibility of binding. After incubation, the reaction mixture was filtered through a glass fiber prefilters (GF/C, Millipore). Protoplasts or cells were washed with 10 ml of 4 °C binding buffer within about 20 s. The 35S-ACaM2 bound to the protoplast- or cell-retained filters was analyzed with liquid scintillation analyzer at 95% efficiency. Specific binding was calculated by subtracting nonspecific binding from total binding. Data were analyzed with GraphPad Prism 4.0.

Chemical Cross-linking of 35S-ACaM2 to Extracellular Binding Sites—800 μl of protoplasts or cells at a density of 107/ml were transferred to 2 ml of siliconized Eppendorf tubes that contained 100 μl of digestion buffer or binding buffer with 1 mM CaCl2, 1 mM EGTA, or 200 μM trifluoperazine for each. 35S-ACaM2 was then added to each tube to a final concentration of 10−7 M. After a 30-min reaction, the cross-linking reagent bis(sulfo)succinimidyl carbonate (BS3) or sulfo-EGS (Pierce) was directly added to each tube with a final concentration of 1 mM. The cross-linking was allowed to proceed for 1 h at 4 °C. The unbound and non-cross-linked 35S-ACaM2 was removed by washing twice with digestion buffer or binding buffer, the cross-linked cell or protoplast pellet was lysed in 100 μl of 1× Laemmli sample buffer (24), boiled for 15 min, and centrifuged at 12,000 × g for 5 min, and the supernatants were separated by SDS-PAGE. The gels were then dried and exposed to a storage phosphor screen for 12 h. Images were scanned and analyzed by Typhoon 9210 imager (Amersham Biosciences).

RESULTS

Immunoblotting Identification of Apoplast CaM in Arabidopsis Suspension-cultured Cells—To demonstrate the presence of extracellular CaM in the system of Arabidopsis suspension-cultured cells, we extracted proteins from the cell wall (Fig. 1, lanes 3, 6, and 9), cultured medium (Fig. 1, lanes 4, 7, and 10), and whole cell (Fig. 1, lanes 2, 5, and 8) and probed each fraction for CaM with anti-CaM polyclonal antibody by immunoblotting (Fig. 1, lanes 5–7). Anti-tubulin antibody was also used to detect tubulin, which could be used as an intracellular protein indicator (Fig. 1, lanes 8–10). Amino black staining of the blot (Fig. 2, lanes 2–4) showed a loading reference for each fraction that has the same amount of proteins (about 15 μg of total proteins per lane) but different protein patterns one from the other in SDS-PAGE. A specific 16-kDa band corresponding to the CaM molecular mass was recognized by anti-CaM antibody in all three fractions (Fig. 1, lanes 5–7), whereas 43-kDa tubulin was only detectable in whole cell fractions (Fig. 1, lane 8). These results demonstrated the presence of extracellular CaM in Arabidopsis suspension-cultured cells, which was either bound to the cell wall or secreted into the medium, but not contaminated by intracellular CaM from leaky cells generated in the process of protein sample preparation.
Fig. 2. Effect of apoplast CaM on proliferation of Arabidopsis suspension-cultured cells. To test the effect of endogenous CaM on cell proliferation, 1 ml of Arabidopsis suspension-cultured cells subcultured 7 days were treated with different concentrations of anti-CaM antiserum (A) and W-7-agarose (B) for 24 h, then incubated with 1 μCi of [methyl-3H]thymidine for another 12 h. The radioactivity of each sample was quantified as described under “Experimental Procedures.” To test the effect of exogenous CaM on cell proliferation, 1 ml of subcultured cells was pretreated either with 5 μM W-7-agarose for 2 h (C) or 1 mM EGTA in MS medium for 15 min (D) to remove endogenous apoplast CaM, then incubated with concentrations of CaM for 24 h followed with [methyl-3H]thymidine and quantified for radioactivity in the same way as A and B. Error bars indicated the S.D. of three independent experiments.

The Effect of Apoplast CaM on Proliferation of Arabidopsis Suspension-cultured Cells—To determine the functions of apoplast CaM in Arabidopsis suspension-cultured cells, we investigated cell mitogenic activities before and after the addition of effectors or reagents as indicated in Fig. 2 by monitoring incorporation of [methyl-3H]thymidine into nuclear DNA. Anti-CaM antiserum was first used to detect endogenous apoplast CaM function in cell proliferation. We found that radioactivity of 3H labeling was substantially decreased with the increase of antiserum concentration, i.e. 50% decrease by 100 μg/ml and 82% decrease by 200 μg/ml, respectively, whereas pre-immune serum has little depression effect (Fig. 2A). Similarly, W7-agarose, another membrane-impermeable antagonist specific for CaM, also inhibited cell proliferation in a dose-dependent manner. The concentration for 50% inhibition, IC50, was 5 μM, and for 100% inhibition it was 100 μM (Fig. 2B). Based on this, we pretreated suspension cells with 5 μM W7-agarose for 2 h to inhibit endogenous apoplastic CaM, then added a series of concentrations of recombination purified ACaM2 to the medium as indicated in Fig. 2C. We found that the inhibition of cell mitogenic activities by W7-agarose was gradually recovered as CaM, increasing to 10−6 μM, at which cell proliferation CaM was almost totally restored (Fig. 2D). These results implied that endogenous apoplastic CaM is an important factor necessary for suspension cell proliferation.

To further verify this idea we tested the effect of exogenous purified CaM on cell proliferation. We first washed suspension cells with 1 mM EGTA in MS medium followed with fresh MS medium to remove endogenous apoplastic CaM. We then applied a series of concentration of purified recombinant ACaM2 to the suspension culture. An increase in 3H radioactivity was observed and was determined to be concentration-dependent. The optimal CaM concentration is 10−4 μM, at which 3H radioactivity was increased by more than 1-fold when compared with 10−7 μM BSA treatment (Fig. 2D). These results indicated that exogenous CaM functions in the same way as endogenous CaM and supported the idea that apoplast CaM plays a very important role in suspension cell proliferation.

Binding Kinetics of 35S-ACaM2 to Arabidopsis Protoplasts and Cells—Radioligand binding assays at cell level provide important information about apoplast CaM binding sites. In the initial experiments, simple separation of bound 35S-ACaM2 from free 35S-ACaM2 by quick centrifugation followed by a rapid rinse gave us an unacceptable high background. Filtering cells through a GF/C membrane and washing the membrane quickly reduced both background and variation, and this method was used for subsequent binding assays. To block non-specific binding of radioligand to glass filters, protoplasts, or cells, we preincubated glass filters with 1% BSA for 1 h and protoplasts and cells with 0.1% BSA in binding assay buffer for 0.5 h before adding 35S-ACaM2. Unless otherwise noted all the experiments were done in this way.

The time course binding was performed first. The binding of 40 nM 35S-ACaM2 to protoplasts (Fig. 3A) or to intact cells (Fig. 3B) reached the top in 10 min and remained relatively constant for next 10–40 min. The observed rate constant, koff, was determined by using the one-phase exponential association program of GraphPad Prism. The koff of 35S-ACaM2 binding to protoplasts was 0.45/min (Fig. 3A) and to intact cells was 0.21/min (Fig. 3B) at room temperature, t0.5, the time needed for half-maximum binding of 35S-ACaM2 to protoplasts, was 1.53 min (Fig. 3A) and to intact cells was 3.3 min (Fig. 3B).

Reverse binding was tested by adding an excess of unlabeled CaM to the reactions after 30 min of 35S-ACaM2 binding to protoplasts (Fig. 3C) or to intact cells (Fig. 3D). At each time point indicated, the reactions were stopped by quickly filtering protoplasts and cells onto the glass filter, and the binding of 35S-ACaM2 was determined. As time progressed, we found that binding was gradually reversed, and within 10 min for protoplasts (Fig. 3C) or 30 min for cells (Fig. 3D), the binding was almost back to the non-specific binding level. The dissociation rate constant, koff, which indicates the separation rate of the ligand from the receptor.

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complex, and \( t_{1/2} \), the time needed for dissociation of half-bound \(^{35}\text{S}-\text{ACaM2} \), were determined using the one-phase exponential decay program. The \( K_{\text{off}} \) and \( t_{1/2} \) values were 0.48/min and 2.48 min from protoplasts (Fig. 3C), and 0.31/min and 2.22 min from intact cells (Fig. 3D), respectively.

**Binding Specificity of \(^{35}\text{S}-\text{ACaM2} \) to Arabidopsis Protoplasts and Cells**—Homologous and heterologous competitive binding assays were performed to test \(^{35}\text{S}-\text{ACaM2} \) binding specificity. In homologous competitive experiments, a series of concentrations of unlabeled ACaM2 were used as competitors of 10 nM \(^{35}\text{S}-\text{ACaM2} \) for binding to protoplasts or suspension-cultured cells. Competition happened only when the competitor was more than 10\(^{-9}\) M for binding to protoplasts (Fig. 4A) or 10\(^{-8}\) M for binding to intact cells (Fig. 4B). When more than 10\(^{-5}\) M (for protoplast) or 10\(^{-7}\) M (for intact cells) unlabeled ACaM2 was used, the \(^{35}\text{S}-\text{ACaM2} \) binding was reduced almost equal to the level of nonspecific binding.

In heterologous protein competitive experiments, increasing amounts of S-100 (Ca\(^{2+}\)-binding protein from bovine brain with two EF-hand domains), BSA, rabbit IgG fragment, or unlabeled ACaM2 as a control were used as competitors of 5 nM \(^{35}\text{S}-\text{ACaM2} \) for binding to protoplasts (Fig. 4C). Neither BSA nor IgG competed with \(^{35}\text{S}-\text{ACaM2} \) for binding to protoplasts. S-100 was able to decrease \(^{35}\text{S}-\text{ACaM2} \) binding by 20% at each molar ratio; it is probably a nonspecific competition binding to protoplasts as compared with specific binding of ACaM2, which effectively decreased the \(^{35}\text{S}-\text{ACaM2} \) binding to protoplasts (Fig. 4C). To determine whether the binding sites on intact cells are CaM isoform-specific, a different source of CaM, including soybean CaM isoform 1 and 5 (SCaM1,5) and ACaM2 and S-100, a calcium-binding protein with EF domain, each at 200 M, was used to compete with \(^{35}\text{S}-\text{ACaM2} \) for binding to suspension cells (D). These analyses were repeated five times independently with three replicates in each sample. Excel was used to process data. Error bars indicate S.D.
Binding Saturation of \(^{35}\text{S-ACaM2}\) to Arabidopsis Protoplasts and Cells—Binding saturation is the minimal significant requirement for ligand binding to a receptor. To examine this, protoplasts or cells were incubated with various concentrations of \(^{35}\text{S-ACaM2}\), and the bound radioactivity was determined after 30 min of incubation (Fig. 5, A and B). The data analyzed showed that on the surface of Arabidopsis protoplasts there is one kind of binding site with a \(K_d\) of ~9.2 nM and nearly 25,000 sites per protoplast (Fig. 5A), whereas on the surface of Arabidopsis suspension-cultured cells there are at least two kinds of binding sites; one has high affinity with a \(K_d\) of ~47.5 nM, about 31,000 sites per cell, another is low affinity with a \(K_d\) of 830 nM, 280,000 sites per cell (Fig. 5B).

Characterization of the Protein Nature of Apoplastic CaM Binding Sites—To assess the nature of CaM binding sites, we treated protoplasts (Fig. 6A) and suspension-cultured cells (Fig. 6B) with proteinase E and then analyzed the \(^{35}\text{S-ACaM2}\) binding sites. Treatment with proteinase E had no visible effect on the intactness of protoplasts and cells as revealed by fluorescein diacetate staining (date not shown), but specific binding sites of \(^{35}\text{S-ACaM2}\) on the surface of both protoplasts and cells were greatly abolished. One mg/ml proteinase E treatment abolished more than 90% specific binding of \(^{35}\text{S-ACaM2}\) to protoplasts (Fig. 6A), whereas 10 mg/ml proteinase E was needed to abolish the same percentage of specific binding as that in suspension cells (Fig. 6B). These results indicated that most of the apoplastic CaM binding sites on the surface of cells and protoplasts are sensitive to proteinase, and there are more binding sites on the intact cell surface than that on the protoplast surface.

Identification of the Possible Apoplastic CaM Binding Proteins on the Surface of Protoplast and Cell—To further characterize the cell-surface CaM-binding proteins, we first chemically cross-linked the protoplast surface with 100 nM \(^{35}\text{S-ACaM2}\), then separated total proteins by 7% SDS-PAGE and analyzed protein bands with phosphorimaging. Two cross-linked complex bands with molecular masses about 130 and 58 kDa each were detected (Fig. 7A, lane 1). Both disappeared when a 200× excess of unlabeled ACaM2 and 1 mM Ca\(^{2+}\) were present in the cross-linking reaction (Fig. 7A, lane 2). Intact cells were also chemically cross-linked with \(^{35}\text{S-ACaM2}\) in the presence of 1 mM Ca\(^{2+}\), and total proteins were separated and analyzed as above. About 7 bands, with molecular masses approximately 180, 130, 96, 84, 65, 58, and 50 kDa were detected (Fig. 7B, lane 1). When a 100× (Fig. 7, lane 2) or 200× (Fig. 7, lane 3) excess of unlabeled ACaM2 was present the cross-linked complexes of 180, 130, and 58 kDa disappeared. When cross-linking was done in the presence of 1 mM EGTA (Fig. 7B, lane 4), 2 mM EGTA (Fig. 7B, lane 5), or 200 \(\mu\)M CaM antagonist trifluoperazine (Fig. 7B, lane 6), the 96-, 84-, and 50-kDa bands were still detectable, whereas the 180-, 130-, and 58-kDa bands almost disappeared.

**DISCUSSION**

CaM, traditionally an intracellular calcium binding regulatory protein that mediates signal transduction and regulatory pathways, has been found extracellularly in both animals (9, 27–31) and plants. In the plant system the presence of extracellular CaM has been verified in oat, wheat, maize, cauliflower, carrot, tobacco, tomato, and *A. dahurica* by several methods including radioimmunoassay, phosphodiesterase or NAD kinase assay, immunoelectron microscopy, etc. (8, 12, 14, 15). Moreover, in our recent works we found that extracellular localized CaM is isoform-preferable when observing green fluorescent protein fusion proteins of five CaM isoforms from soybean, *i.e.* SCAm1, -2, -3, -4, -5, that were expressed in tobacco cells under the control of double CaMV35S promoter. Among them, highly conserved SCAm1, SCAm2, and SCAm3 but not SCAm4 and SCAm5 were visualized with green fluorescent protein fluorescence in the cell wall after plasmolysis in addition to their intracellular localization. The results were further confirmed by immunogold electron microscopy with anti-SCA1, anti-SCA4 and anti-SCA5 isoform-specific antibodies.2 Based on these data, we hold the idea that the conserved CaM isoform somehow has more chance to be secreted actively into extracellular space by living cells through an unidentified pathway. To date, positive secretion of extracellular CaM was only observed in animal cells (30).

On the other hand, *A. thaliana* has become an important plant model system for identifying genes and determining their functions especially when its genomic sequence was completely available in 2000 (The *Arabidopsis* Genome Initiative, 2000). There are at least 11 CaM isoforms in *A. thaliana* genome; among them, ACaM2 is highly conserved, with only one amino acid different from SCAm1. Therefore, in the present work, we focused on the studies of ACaM2 localization, function, and binding activities in *Arabidopsis* suspension culture cells. We first confirmed the presence of CaM in the cell wall and cultured medium of *Arabidopsis* suspension cells by immunoblotting assay with anti-CaM antibody (Fig. 1), which would not be the contamination by intracellular CaM due to leakage of cells, since one of abundant intracellular protein, tubulin, was undetectable in the cell wall and culture medium (Fig. 1).

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2 S. S. Song, M. Liu, W. Guan, J. Bai, G. H. Mao, and D. Y. Sun, data not published.
proliferation in a dosage-dependent manner (Fig. 2, meable anti-CaM antibody or W-7-agarose, which inhibited cell blocking endogenous apoplastic CaM with membrane-imper-

CaM again in proliferation of (17, 18). In this study we verified the essential role of apoplastic division (15, 16) and pollen germination and pollen tube growth suspension cell proliferation, cell wall regeneration, and cell exogenous purified cauliflower CaM has a promotional effect on suspension cells. From our previous studies we knew that presence (Fig. 3, lane 1) or presence of 200 M trifluoperazine (lane 6). M, 14C-methylated protein molecular mass markers. These experiments were repeated three times independently. A representative result is presented

Next we tested the function of apoplast CaM in Arabidopsis suspension cells. From our previous studies we knew that exogenous purified cauliflower CaM has a promotional effect on suspension cell proliferation, cell wall regeneration, and cell division (15, 16) and pollen germination and pollen tube growth (17, 18). In this study we verified the essential role of apoplastic CaM again in proliferation of Arabidopsis suspension cells by blocking endogenous apoplastic CaM with membrane-impermeable anti-CaM antibody or W-7-agaroase, which inhibited cell proliferation in a dosage-dependent manner (Fig. 2, A and B), and by adding exogenous recombinant CaM, which either reversed the inhibition effect on cell proliferation by W-7-agaroase (Fig. 2C) or stimulated cell proliferation (Fig. 2D). Recently, we also provided genetic evidence for the function of apoplastic CaM in promoting pollen germination and pollen tube growth when comparing non-transgenic pollen with transgenic pollen in which ACaM2 was overexpressed in apoplastic space by introducing signal peptide to N terminus of ACaM2 to facilitate its secretion.2

Based on accumulated evidence, we proposed that apoplastic CaM might function as a polypeptide signal to regulate cell growth and development and identified some intracellular molecules involving in apoplastic CaM signal transduction includ-

ing heterotrimmeric G protein (19), phospholipase C, and inositol 1,4,5 trisphosphate, calcium flux (32), all of which specifically responded to exogenous CaM when applied to outside of protoplasts.

As a 17-kDa hydrophilic molecular, it is unlikely for extracellular CaM to permeate directly through the plasma membrane. Neither can it be internalized by the cell within 2 h because binding of 35S-ACaM2 to intact cells is the same at 25 and 4 °C (it is thought that no physiology activities in the cells happen at 4 °C); nor were degradation products of 35S-ACaM2 found after 24 h of incubation with suspension-cultured Arabidopsis thaliana cells when checking SDS-PAGE and autoradiography (data not shown). All these results implied that there should be binding sites of intact CaM at surface of cells, which are key components in mediating transmembrane signal transduction of apoplastic CaM.

In animal systems radiolabeled ligand binding assays are often used to deduce the occurrence of binding sites and to analyze receptor-like binding characteristic of primary messenger (ligand) with cells, tissue, and organ. Recently, this approach has also been successfully used to study membrane receptors for polypeptide signals in plant systems, such as PSK, Systemin, and Elicitor (33–35). In this paper we used 35S-ACaM2, which has the same activity as native ACaM2 (25, 36), to identify CaM-binding proteins and their receptor-like characteristics on the surface of suspension culture cells of Arabidopsis.

Binding kinetics assay showed the time saturation and reversibility of 35S-ACaM2 binding to Arabidopsis protoplasts and suspension-cultured cells (Fig. 3). Binding to protoplast (Fig. 3A) was more rapid than binding to suspension cells (Fig. 3B). Dissociation of bound 35S-ACaM2 from protoplasts was slower than that from suspension cells (Fig. 3, C and D). The different of K on and K off between protoplasts and suspension cells may be due to existence of cell wall in intact cells.

The binding of 35S-ACaM2 to protoplasts or suspension cells is specific as indicated by homologous competition, in which unlabeled ACaM2 effectively competed with 35S-ACaM2 for binding to protoplasts and intact cells in a dosage-dependent manner (Fig. 4, A and B), whereas as a protein control of CaM, BSA and IgG, did not (Fig. 4C). S-100, a more stringent control of CaM, which has two EF-hand domains and calcium binding capacity, had little competition with 35S-ACaM2 for binding to protoplasts but is not specific (Fig. 4C). The competitor needed to compete with 35S-ACaM2 is 10-fold concentrations for binding to intact cells as that for binding to protoplasts (Fig. 4, A and B). We concluded that there is some cell wall-associated

Y. Y. Pan, X. Wang, L. G. Ma, and D. Y. Sun, unpublished information.
CaM binding sites on intact cells in addition to the membrane-localized binding site.

When checking CaM binding sites in intact cells by using different CaM isoforms as competitor, we found that highly conserved ACaM2 and SCaM1 has more powerful competitive ability than that of non-conserved SCaM5 or S-100 control for binding to cells (Fig. 4D). This means the binding sites on the surface of cells are more likely specific for conserved CaM isoform, and this result is consistent with our previous finding that conserved CaM isoform is preferably secreted into cell walls and functions outside of cells.

The specific binding of \(^{35}\text{S}\)-ACaM2 to both protoplast and intact cells is almost saturated when the concentration of radiolabeled ligand was increased to more than 100 nM (Fig. 5). However, the binding of \(^{35}\text{S}\)-SCaM2 to protoplasts in the presence of 1 mM EGTA was highly increased, up to 5-fold that if the non-EGTA control (data not shown). It is hard to evaluate the result because EGTA changes the stability and permeability of protoplast plasma membrane and, therefore, cell physiology, and CaM is still active. Actually it needs at least 10 mM EGTA in reaction buffer to chelate Ca\(^{2+}\) to a concentration around 10\(^{-8}\) M (46), which is near the \(K_d\) of Ca\(^{2+}\) with apoplastic CaM. But it is impossible to maintain protoplasts intact in such a binding reaction because Ca\(^{2+}\) is very important for the stability of plasma membrane. 5 mM EGTA already cause some protoplasts to burst (data not shown).

Therefore, in the present studies it is still hard to tell whether the binding activity of apoplastic CaM on the cell surface is absolutely Ca\(^{2+}\)-dependent or not.

For the first time we confirmed the presence of apoplastic CaM receptor-like binding proteins on plasma membranes, which is significantly important evidence for supporting our hypothesis that CaM, in addition to its traditional role in mediating the intracellular Ca\(^{2+}\)-signaling pathway, is able to function as a polypeptide signal in the regulation of plant growth and development when secreted into apoplast space. Such a dual messenger molecule, whose extracellular function or signaling pathways are not same as their intracellular function, is not alone. Molecules such as calmodulin and Ca\(^{2+}\) (43, 44), whose functions have been verified in Dictyostelium discoideum and some animal or plant cells, act both inside and outside of cells in different pathways. We are doing gene cloning of apoplastic CaM receptor to further provide evidence for function and transmembrane signaling of apoplastic CaM.

Acknowledgments—We thank Dr. Raymond E. Zielinski from University of Illinois for providing a plasmid of pET5a-ACaM2, Dr. Jan Miernyk from the University of Missouri for critical reading of the manuscript, and Guo Yi and Zhao Junfeng for technical assistance.

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To some extent the binding of \(^{35}\text{S}\)-ACaM2 to suspension cells was Ca\(^{2+}\)-dependent, since the presence of 1 or 2 mM EGTA greatly disrupted the cross-linking complex (Fig. 7B, lanes 4 and 5). However, the binding of \(^{35}\text{S}\)-SCaM2 to protoplasts in the presence of 1 mM EGTA was highly increased, up to 5-fold that if the non-EGTA control (data not shown). It is hard to evaluate the result because EGTA changes the stability and permeability of protoplast plasma membrane and, therefore, cell physiology, and CaM is still active. Actually it needs at least 10 mM EGTA in reaction buffer to chelate Ca\(^{2+}\) to a concentration around 10\(^{-8}\) M (46), which is near the \(K_d\) of Ca\(^{2+}\) with apoplastic CaM. It is impossible to maintain protoplasts intact in such a binding reaction because Ca\(^{2+}\) is very important for the stability of plasma membrane. 5 mM EGTA already cause some protoplasts to burst (data not shown). Therefore, in the present studies it is still hard to tell whether the binding activity of apoplastic CaM on the cell surface is absolutely Ca\(^{2+}\)-dependent or not.
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