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Cellular Distribution of Calmodulin and Calmodulin-Binding Proteins in *Vicia faba* L.[1]

Vincent Ling* and Sarah M. Assmann

Department of Organismic and Evolutionary Biology, Harvard University Cambridge, Massachusetts 02138

ABSTRACT

The distribution of calmodulin (CaM) and CaM-binding proteins within *Vicia faba* was investigated. Both CaM and CaM-binding proteins were found to be differentially distributed among organs, tissues, and protoplast types. CaM levels, on a per protein basis, were found to be the highest in leaf epidermis, containing 3-fold higher levels of CaM than in total leaf. Similarly, guard cell and epidermal cell protoplasts were also found to have higher levels of CaM than mesophyll cell protoplasts. ^{125}I-CaM blot overlay assays were performed to qualitatively examine CaM-binding proteins in these protoplast types as well as in whole tissues and organs. CaM-binding proteins with M_r 52,000, 78,000, and 115,000 were common in all metabolically active plant parts. Unique CaM-binding protein bands were detected in guard cell protoplasts (M_r 39,000, 88,000), stems (M_r 45,000, 60,000, 64,000), and roots (M_r 62,000), suggesting the presence of specialized CaM-dependent processes in these cells and organs.

In recent years, a central role of Ca^{2+} in plant cell signal transduction has become apparent (10, 20, 25, 31). CaM[2], a low mol wt, soluble, Ca^{2+}-binding protein that is known to function as an essential modulator of many Ca^{2+}-dependent processes in animal systems (16), is also found in plants. Because of the 92% amino acid sequence similarity shared between CaM of higher plants and vertebrates (25), CaM is also assumed to be an essential Ca^{2+}-dependent modulator of physiological processes in plant systems. However, only a few CaM-dependent enzymes have been isolated and characterized from plants, and very little is known about pathways that may be CaM regulated (25).

In animal systems, high levels of cellular CaM have been reported in cells that fall into two general categories: proliferating cells in regions of high mitotic activity and cells involved in specialized rapid signaling (16). By radioimmunoassay, different levels of CaM were detected in leaf, stem, root, and cotyledon of pea; the highest levels of CaM occurred in general areas of rapid cell division (21). Because of the method of sample preparation used, CaM levels in specific tissues within organs examined were not determined. Because specialized plant cells utilize Ca^{2+}-mediated signal transduction (31), important components comprising the sensory process in plants may be overlooked if examined on the organ level.

In a previous report (9), cells of etiolated pea plants were immunochemically inspected for the presence of CaM. Qualitative examination revealed uniform immunoreactive fluorescence in the nucleoplasm and background cytoplasm of all etiolated plant cells. However, subcellular localization of the immunofluorescence was not identical among cell types, with intense staining present in epidermal vacuoles and columella amyloplasts. These results indicate that cellular CaM-mediated processes may not be identical in the different cell types of plants. In addition, analysis of CaM-binding proteins in the alga *Fucus* demonstrated alterations in CaM-binding protein patterns during development, supporting the idea that CaM-mediated processes are regulated during differentiation (4). Knowledge of the distribution of CaM and CaM-binding proteins within specialized tissues and cells of higher plants would provide a focus for research in developmental processes and signal transduction. In this paper, we analytically determined CaM levels in different protoplast types, tissues, and organs of *Vicia* by gel assays (32) and by enzymic activation of CaM-dependent PDE (7). The protein targets of CaM within these samples were also surveyed by a modified ^{125}I-CaM overlay assay.

MATERIALS AND METHODS

Unless otherwise indicated, all reagents were purchased from Sigma Chemical Co.

Plant Material

Vicia faba L., cv long pod, was raised in growth chambers with light settings of 10 h day and 14 h night and day/night temperatures of 21/19°C.

Protein Preparation from Purified Protoplasts

Young *Vicia* leaves were excised from 3- to 4-week-old plants. Guard cell protoplasts were isolated according to the blender method of Kruse et al. (17). Guard cell protoplasts released by two-stage pectolytic and cellulytic digestion were suspended into 0.45% mannitol, 1 mM $CaCl_2$, and 0.5 mM ascorbic acid and then purified by centrifugation at 200*g* for 15 min onto a 100% Histopaque 1077 cushion. Healthy protoplasts were collected at the interface between the mannitol buffer and Histopaque 1077. These protoplasts were

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[2] Abbreviations: CaM, calmodulin; PDE, phosphodiesterase; PVDF, poly(vinylidene difluoride); A.U., arbitrary unit; MAP, microtubule-associated protein.

rewashed in 0.6 M mannitol, 1 mM $CaCl_2$ buffer. To obtain epidermal cell protoplasts, epidermes were peeled by hand from the abaxial surface of *Vicia* leaves and agitated in a digestion medium containing 1.0% cellulase Onozuka RS (Yakult Honsha Co., Minato-Ku, Tokyo, Japan), 0.006% pectolyase Y23 (Seishin Pharmaceutical Co. Ltd., Koamicho, Nihonbashi, Tokyo, Japan), 0.1% PVP, 0.2% BSA, 0.6 M mannitol, and 1 mM $CaCl_2$. Peels were digested for 30 min at 27°C and filtered through 100-μm mesh. The filtrate, containing epidermal protoplasts, was centrifuged at 200*g* and resuspended in 0.6 M mannitol, 1 mM $CaCl_2$, 0.5 mM ascorbic acid. Further purification was accomplished by centrifugation onto a 20% Percoll step gradient made from dilution of 100% Percoll with 0.6 M mannitol, 1 mM $CaCl_2$. Mesophyll cell protoplasts were prepared according to the method of Shimazaki et al. (27). All protoplasts were resuspended in 0.6 M mannitol, 1 mM $CaCl_2$, examined and measured by light microscopy, and quantitated with a hemocytometer. Contaminating protoplasts in preparations were clearly discernible by morphology. Enriched protoplasts were concentrated by centrifugation at 200*g*. Aliquots were removed for protein quantitation and PDE assays. The remaining protoplasts were frozen at −80°C. Protoplasts were lysed and boiled in SDS-PAGE sample buffer with a final concentration of 0.06 M Tris-Cl (pH 7.5), 2% (w/v) SDS, 0.7 M β-mercaptoethanol, 10% (v/v) glycerol, 0.1% (w/v) bromophenol blue, before SDS gel analysis.

Protein Isolation from *Vicia* Organs and Tissues

Total proteins were extracted by grinding organs and tissues into fine powder under liquid N_2, followed by suspension in 50 mM Tris (pH 7.5), 1 mM EDTA, 0.1 mM PMSF, 14 mM (v/v) β-mercaptoethanol. The suspension was clarified briefly by centrifugation at 12,000*g*. For SDS gels, aliquots of the supernatant were added to SDS sample buffer and immediately boiled for 2 min and frozen. For PDE assays, freshly made aliquots of clarified supernatant were removed, immediately boiled for 2 min, and held on ice before the PDE assay. Separate aliquots were removed for protein quantitation by the Bio-Rad Protein Assay (Bio-Rad, Richmond, CA) using 0.1 M NaOH as the suspension buffer. Lysozyme in 0.1 M NaOH was used as the standard.

CaM Purification

CaM was isolated from *Vicia* leaves according to the method of Anderson (1). Leaves were ground in liquid N_2, made into an acetone powder, and extracted in ice-cold 50 mM Tris, 1 mM EDTA, 14 mM β-mercaptoethanol. After centrifugation at 10,000*g* for 30 min, the supernatant was heated to 85°C for 5 min. The supernatant was clarified of denatured protein by recentrifugation and subjected to DEAE-cellulose column chromatography. Elution of proteins by a 0 to 400 mM NaCl gradient in 50 mM Tris-HCl (pH 7.5), 1 mM EDTA yielded fractions enriched for CaM. These fractions were pooled, dialyzed, and affinity purified by phenyl-Sepharose column chromatography. Purified CaM was dialyzed, lyophilized, and stored at −20°C. Samples of *Vicia* CaM were analyzed for total amino acid composition by phenyl isothiocyanate derivatization (Harvard Microchemistry Facility). Because of the limitations of this method, the amino acids tryptophan and cysteine were not determined.

^{125}I-Labeling of CaM

^{125}I-labeled Bolton-Hunter reagent (Dupont-NEN, Boston, MA) was used to label purified *Vicia* CaM according to the protocol of Chafouleas et al. (5). Bolton-Hunter reagent-labeled CaM has been shown to retain full activator properties in enzyme assays (5). A specific activity of 10 μCi/μg of CaM was used for the experiments described.

Electrophoresis

SDS-PAGE of samples was performed according to the method of Laemmli (18). Mobility shift assays were performed on 15% acrylamide minigels (70- × 80- × 0.75-mm format) with either 2 mM $CaCl_2$ or 2 mM EDTA in the applied sample. Minigels were used for native, discontinuous PAGE, which is diagnostic for CaM (32). Extracts analyzed by native PAGE were partially purified by heat treatment at 90°C for 3 min, followed by 50 to 80% ethanol precipitation before loading. Gradient SDS gels of 5 to 20% acrylamide (150- × 110- × 1.5-mm format) were used in fractionating proteins for overlay assays. All gels were fixed in 10% methanol and 7% acetic acid, and proteins were stained with Coomassie brilliant blue R-250. Digital imaging of stained gels for protein quantitation was performed on a MacIntosh IIsi computer equipped with a Panasonic WV-BD400 video attachment and Image 1.4 software (National Institutes of Health, Bethesda, MD).

Electroblot and ^{125}I-CaM Overlay Assays

Proteins fractionated by SDS-PAGE were electroblotted for 3 h at 30 V onto PVDF membranes (Schleicher and Schuell, Keene, NH) with 10 mM 3-[cyclohexylamino]-1-propanesulfonic acid (pH 11.0), 10% methanol as transfer buffer. After blotting, the membrane was removed and air dried. The membrane was then rewetted with methanol and equilibrated with CaM-binding buffer for 1 to 2 h. CaM-binding buffer consisted of 50 mM Tris (pH 7.5), 0.2 M NaCl, and 0.05% (v/v) polyoxyethylenesorbitan monolaurate (Tween 20). Either $CaCl_2$ or EDTA, depending on the type of overlay assay used, was added to CaM-binding buffer at a 1 mM final concentration. The blot was incubated for 30 to 40 min in buffer containing 1 μCi/mL of ^{125}I-CaM. Blots were then washed with buffer until counts, as detected by a scintillation probe (Ludlum, Sweetwater, TX), returned to background levels. Autoradiography was performed on blots with two intensifying screens for 1 week at −70°C.

In our hands, we found that efficient electrotransfer of basic proteins to a PVDF membrane required a high pH of the transfer buffer. Standard Tris-glycine electrotransfer buffer (pH 8.3) was found to be inadequate, leaving many proteins in the gel after transfer. When 10 mM [cyclohexylamino]-1-propanesulfonic acid (pH 11.0), 10% methanol was used, however, much greater efficiency of transfer was achieved. PVDF membranes were used as transfer support

because of enhanced protein retention and amenity toward protein staining by Coomassie blue. Tween-20 was included in incubation and wash buffers because of its ability to enhance CaM-binding signals of certain proteins by up to 10-fold when substituted for protein-blocking agents (12). Because of the increased ionic binding of proteins to PVDF membranes relative to nitrocellulose, problematic washoff of membrane-immobilized proteins was greatly reduced; proteins were retained on PVDF membranes despite lengthy washes of several hours in buffers containing Tween-20.

PDE Assay

PDE assays were performed based on the two-step enzymic method of Cheung (7). Bovine brain 3′:5′-cyclic nucleotide PDE and *Crotalus atrox* venom were purchased from Sigma. Heat-treated samples were resuspended in 225 μL of reaction buffer comprised of 40 mM Tris-Cl (pH 8.0), 3 mM $MgSO_4$, and 0.5 mM $CaCl_2$ at 30°C. To this was added 7 μL of 1 unit/ml of PDE and 50 μL of 20 mM cAMP, followed by incubation at 30°C for 30 min. Samples were boiled for 1 min to terminate the reaction and then cooled to 30°C for 10 min. Fifty microliters of 1 mg/mL of snake venom was added to each tube, followed by incubation at 30°C for 30 min. At the end of the incubation, 25 μL of 55% TCA, 375 μL of H_2O, and 75 μL of 2.5% ammonium molybdate in 5 N H_2SO_4 were added to each sample. Samples were vortexed and centrifuged at 16,000*g* for 10 min. The supernatants were added to 50 μL of freshly made, 0.45-μm filtered, Fiske and Subbarow reagent. After 5 min, phosphate release was determined for each sample by spectrophotometric measurement of A_{660}.

PDE assays were also performed on duplicate samples with 10 mM EGTA replacing the 0.5 mM $CaCl_2$ in the assay buffer to determine extent of background Ca^{2+}-independent phosphate release (data not shown). Values reflecting corrected Ca^{2+}-dependent phosphate release are presented. Differences in maximal PDE activity (phosphate released) from separate batches of commercially purchased PDE preparations were observed. However, the relative levels of PDE activation between samples assayed within each set of experiments (n = 3) were consistent. PDE activation expressed was in relative A.U. of CaM activity (Ca^{2+}/CaM-dependent phosphate release per gram of sample protein per 30 min), in which CaM activity for leaf samples in each experiment was normalized to 2 A.U.

RESULTS

CaM Purification and Quantitation

CaM was purified from *Vicia* leaves by standard CaM isolation procedures (1). Purified *Vicia* CaM was compared to purified spinach CaM (Sigma) by a number of assays. Fractionation by SDS-PAGE (Fig. 1) revealed that both *Vicia* and spinach CaM had an M_r = 17,000 in the presence of EDTA and shifted to an M_r = 15,000 in the presence of Ca^{2+}, typical of CaM and other Ca^{2+}-binding proteins with EF-hand structures (16). In addition, *Vicia* CaM and spinach CaM comigrated on nondenaturing PAGE. The amino acid composition of *Vicia* CaM was virtually identical with other

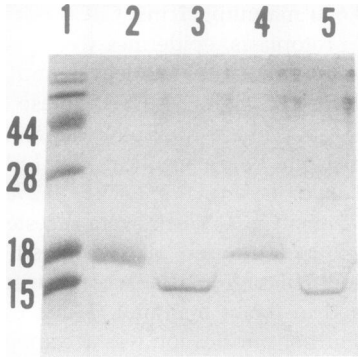

Figure 1. SDS-PAGE mobility shift assay of purified CaM. Five micrograms of purified CaM were analyzed by 15% SDS-PAGE in the presence of either 2 mM EDTA or 2 mM $CaCl_2$. Protein bands were visualized by Coomassie blue staining. Lane 1, Mol wt standards; lane 2, spinach CaM + EDTA; lane 3, spinach CaM + $CaCl_2$; lane 4, *Vicia* CaM + EDTA; lane 5, *Vicia* CaM + $CaCl_2$. M_r are expressed in thousands.

plant CaM, sharing a high percentage of acidic residues, a single tyrosine residue, and a single histidine residue (data not shown). Furthermore, *Vicia* CaM was indistinguishable from spinach CaM when used as an activator in PDE assays (data not shown).

Recently, partial-length cDNAs encoding polypeptides with sequence similarity to CaM have been isolated from plants (3). Neither the function nor the full mol wt of these proteins are known, but the similarity to CaM may constitute a source of contamination in assays of CaM in crude extracts. As a result, two standard assays for CaM quantitation were used, one based on mass and charge separation by native gel electrophoresis and the other based on Ca^{2+}/CaM-dependent PDE activation.

CaM was examined in leaf protoplasts enriched for specific cell type. Guard cell, epidermal cell, and mesophyll cell protoplasts were prepared according to enrichment procedures specific for the protoplast type. Because of the range of buoyancies exhibited by epidermal protoplasts, we were unable to attain greater than 97% purity of this protoplast type, with the remaining 3% comprised of mesophyll protoplasts. Guard cell protoplasts and epidermal cell protoplasts shared similar total protein levels per cell, whereas mesophyll cells had substantially higher levels of total protein (Table I).

Guard cell total protein levels (222.5 pg/cell) were similar to a previously published calculation (156 pg/cell), based on the dry weight of guard cell pairs (23). On a protein basis, guard cell protoplasts contained the highest levels of CaM activity (6.36×10^{-2} A.U./g of total protein), 2-fold more than mesophyll cell protoplasts (2.79×10^{-2} A.U./g of total protein). When based on volume, guard cell protoplasts contained 5-fold greater CaM activity (17.1×10^{-8} A.U./m^3) than epidermal protoplasts (2.9×10^{-8} A.U./m^3) or mesophyll protoplasts (3.34×10^{-8} A.U./m^3). On an individual protoplast basis (A.U./cell) the mesophyll protoplasts contained 2-fold greater total CaM activity than guard cell protoplasts (11.82×10^{-4} versus 6.02×10^{-4} A.U./cell), but it should be noted that mesophyll protoplasts have a 10-fold greater

Table I. *Protein and CaM Activity of Enriched Vicia Leaf Protoplasts*

	Protoplast Type		
	Guard cell	Epidermal cell	Mesophyll cell
Cell purity (%)	>99	97[a]	>99
Average vol[b] (μm^3/cell)	$3.52 \times 10^3 \pm 0.14 \times 10^3$ ($n = 60$)	$23.66 \times 10^3 \pm 2.36 \times 10^3$ ($n = 72$)	$35.29 \times 10^3 \pm 2.10 \times 10^3$ ($n = 50$)
pg Protein/cell[c]	223	287	985
pg Protein/vol (μm^3)	6.36×10^{-2}	0.86×10^{-2}	2.79×10^{-2}
CaM activity (A.U.)/μg of protein[d]	2.7	2.4	1.2
CaM activity (A.U.)/cell	6.02×10^{-4}	6.88×10^{-4}	11.82×10^{-4}
CaM activity (A.U.)/vol (μm^3)	17.10×10^{-8}	2.90×10^{-8}	3.34×10^{-8}

[a] The 3% contaminating cells in purified epidermal cell protoplast preparations were mesophyll cell protoplasts. [b] Volume ± SE. [c] Protein levels in protoplast extracts were determined by the Bio-Rad Protein Assay using lysozyme as protein standard. [d] CaM activity in protoplast extracts was determined by PDE assay.

volume than guard cell protoplasts. These calculations do not take into consideration the subcellular distribution of CaM; thus, localized concentrations of CaM cannot be assessed by the method used.

Discontinuous, nondenaturing PAGE (32) of partially purified extracts revealed bands that migrated with purified *Vicia* CaM in the guard and epidermal cell protoplast lanes (Fig. 2a); this technique was used for a second estimate of relative CaM levels. In the mesophyll cell protoplast sample, a band comigrating with CaM standard was not visible until loadings of the partially purified extracts were increased from 400-μg to 1-mg equivalents of starting protein (Fig. 2b, lane 2). These results are consistent with those of the PDE assays in indicating that CaM levels are much lower, on a per protein basis, in mesophyll cell protoplasts than either epidermal or guard cell protoplasts.

Relative levels of CaM in epidermal peels and total leaf were estimated by discontinuous PAGE (Fig. 2c). Computer-assisted densitometry of bands comigrating with CaM produced from differential loadings of epidermal peel extracts in this gel assay system revealed 3.37-fold higher levels of CaM in epidermal peels than in total leaf, on a per total protein basis. Epidermal peel extracts also showed a 3-fold higher activation of PDE than activation from equivalent protein amounts of total leaf extracts (Fig. 3). Densitometric scanning of the CaM band resolved by nondenaturing gels indicates that approximately 0.9 μg of CaM is present in 800 μg of total leaf protein, consistent with previously published CaM levels (10). Surprisingly, epidermal peel CaM activity, on a per microgram total protein basis, exceeded by 2-fold the levels of CaM found in epidermal cell or guard cell protoplasts (Fig. 3). Likewise, total leaf extracts activated PDE to a higher extent than extracts derived from mesophyll protoplasts (Fig. 3). This discrepancy in CaM levels found between intact tissue and derived protoplasts is discussed below.

A band that migrated to a slightly higher position than

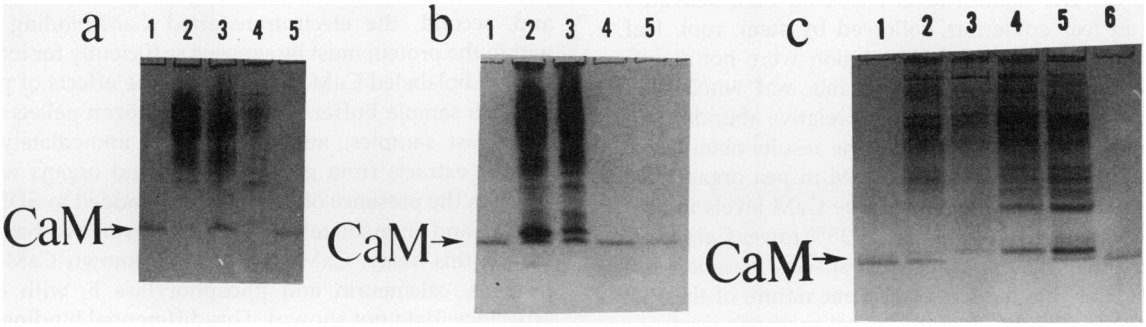

Figure 2. Native PAGE analysis of *Vicia* CaM. Protein extracts were partially purified by heat treatment and ethanol fractionation before native PAGE. The mass given in parentheses indicates the amount of protein present in each extract before partial purification. After electrophoresis, proteins were visualized with Coomassie blue staining. a, Extracts from leaf protoplasts (lanes 2–4). Lanes 1 and 5, Purified *Vicia* CaM (1 μg). Lane 2, guard cell protoplast (400 μg); lane 3, epidermal cell protoplast (400 μg); lane 4, mesophyll cell protoplast (400 μg). The band present in the mesophyll protoplast lane with mobility above CaM standard is not CaM (see text). b, Mesophyll cell protoplast and total leaf extracts (lanes 2–4). Lanes 1 and 5, Purified *Vicia* CaM (1 μg); lane 2, mesophyll cell protoplast (1 mg); lane 3, total leaf (1 mg); lane 4, phenyl-Sepharose-purified total leaf extract (3 mg). c, Epidermal peel and total leaf extracts (lanes 2–5). Lanes 1 and 6, Purified *Vicia* CaM (1 μg); lane 2, epidermal peel (200 μg); lane 3, total leaf (200 μg); lane 4, total leaf (400 μg); lane 5, total leaf (800 μg).

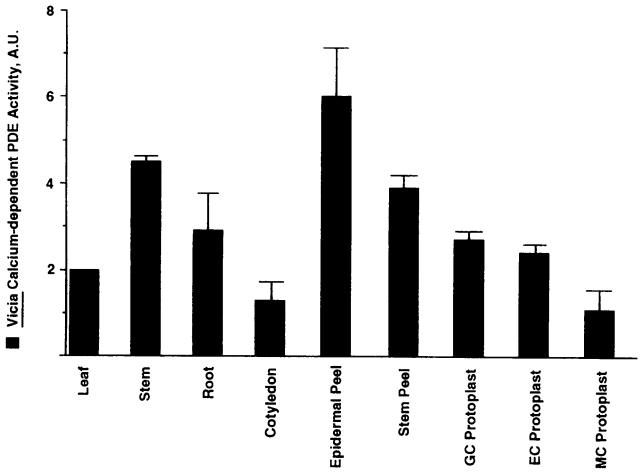

Figure 3. PDE assay of *Vicia* protoplasts, tissues, and organs. Results of PDE assays performed on each sample type. Values of CaM activity are indicated as relative A.U. of Ca^{2+}-dependent PDE activation per microgram of protein. Average values from three separate experiments for each sample are presented along with bars displaying the range of values obtained. In an experiment, specific activity in leaf samples was considered to be standard and set to 2 A.U. GC, Guard cell; EC, epidermal cell; MC, mesophyll cell.

CaM was present in both mesophyll protoplasts and total leaf extract (Fig. 2b). This band was also detected to a very minor extent in epidermal peel extract (Fig. 2c) and may be due to minor protein contamination from attached mesophyll cells and cellular debris. Multiple bands comigrating near CaM have been detected during the process of CaM purification and may be CaM-like proteins (24). However, when heat-treated, ethanol-fractionated leaf extracts containing this protein were subjected to phenyl-Sepharose affinity column chromatography, the only protein that eluted with EGTA was CaM (Fig. 2b, lane 4). This result indicated that the minor second band did not share hydrophobic interaction properties typical of CaM.

CaM levels were determined in other *Vicia* tissues and organs (Fig. 3). The highest level based on PDE activation was found in leaf epidermis, followed by stem, root, leaf, and cotyledon. Levels of CaM activation were not significantly different between stem epidermis and whole stem tissue. With the exception of stem, the relative abundance of CaM in these organs was similar to the results obtained by radioimmunoassay quantitation of CaM in pea organs (31). The slight discrepancy between relative CaM levels in stem between *Vicia* and pea (approximately 35% lower CaM levels in pea stem) may be attributable to species variation but, alternatively, may be due to the enzymic nature of the PDE assay used here and the immunological methods used previously (5, 16, 25).

Protein Profiles

Protein profiles of *Vicia* protoplasts, tissues, and organs were compared by 5 to 20% gradient SDS-PAGE followed by Coomassie blue staining (Fig. 4a). One previous report (22) indicated that guard cell protoplasts had a protein profile highly distinct from mesophyll cell protoplast and total leaf proteins and, thus, reflected the highly specialized functions of guard cells. We found that protein profiles of guard cell and epidermal cell protoplasts (Fig. 4a) were highly similar to, although not identical with, each other. Thus, it would seem that the shared origins of the cells occupying the epidermal layer might have contributed to the similarity of their protein profiles and that proteins related to specialized guard cell function are of relatively low abundance.

Although the epidermal cells in *Vicia* leaves do not contain developed chloroplasts, bands were present in epidermal cell protoplast lanes (Fig. 4a) that comigrated with the large (M_r 55,000) and small subunits (M_r 15,000) of Rubisco. Reconstruction experiments were performed in which amounts of mesophyll cells equivalent to those contaminating enriched epidermal protoplast preparations (3% contamination by cell number, 15% contamination by protein amount) were fractionated by SDS-PAGE. Results indicated that the large and small subunit proteins, as identified by mol wt, were the only proteins visible after Coomassie blue staining (data not shown). Large and small subunit bands found in the epidermal protoplast lanes, but no other bands, could, therefore, be accounted for by contaminating mesophyll proteins under SDS-PAGE.

The protein profile of epidermal peels appeared to be a composite of epidermal cell protoplast and guard cell protoplast proteins. Protein profiles of mesophyll cell protoplasts and total leaf were indistinguishable. The protein profile of whole stem was highly similar to that of epidermal peel, especially with the presence of two prominent bands with M_r = 17,000 and 19,000. Protein profiles of 1.5-week-old plumule and root were similar to, but not identical with, each other.

CaM-Binding Proteins

A modified ^{125}I-CaM overlay assay was used to qualitatively examine CaM-binding proteins present in different protoplasts, tissues, and organs of *Vicia*. The success of this assay is dependent on two factors. First, the CaM-binding protein must remain intact during the process of extraction and, second, the electrotransferred CaM-binding domain within the protein must be exposed sufficiently for interaction with radiolabeled CaM. To minimize the effects of proteolysis, SDS sample buffer was added to frozen pellets of intact protoplast samples, mixed, and then immediately boiled. Protein extracts from ground tissues and organs were prepared in the presence of 0.1 mM PMSF, added to SDS sample buffer, and immediately boiled. However, we have found that in this assay, CaM binds to two known CaM-binding proteins, calcineurin and phosphorylase b, with different efficiency (data not shown). This differential binding of CaM may be related to different affinities of CaM-binding domains to CaM or may be due to the disruption of CaM-binding domain structure in the target protein by the overlay assay. This procedure, therefore, can be used to qualitatively detect certain proteins with intact CaM-binding domains but cannot be used to gauge the relative abundances of different CaM-binding proteins based on autoradiographic density of the labeled band.

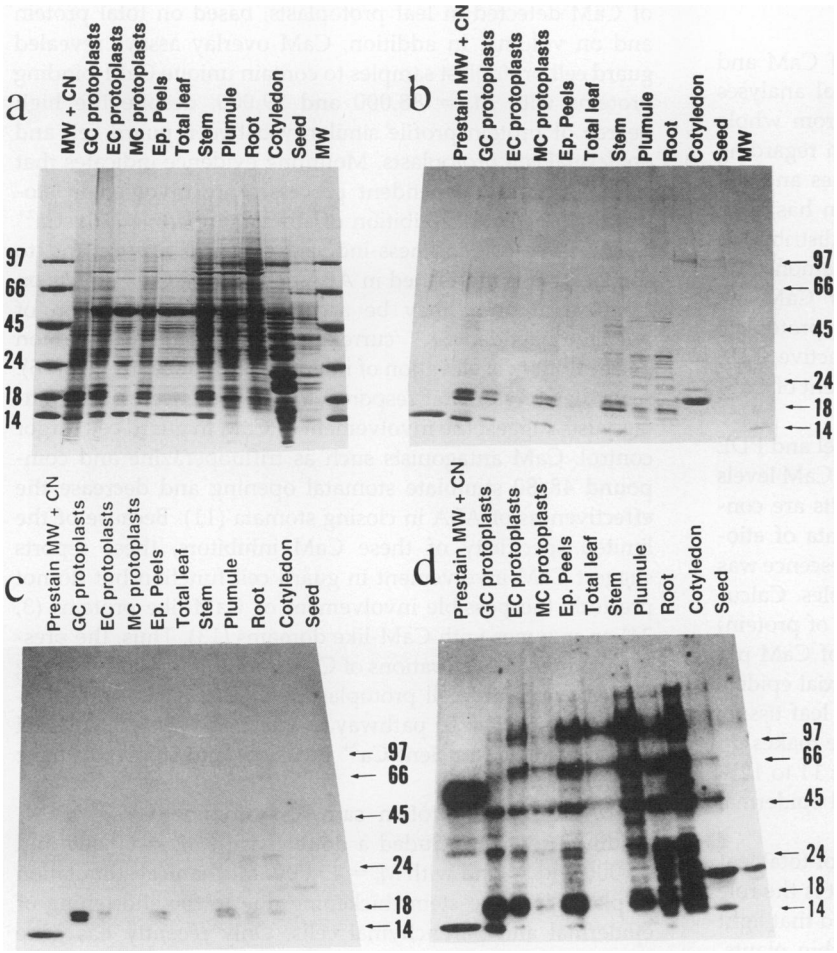

Figure 4. Results of ^{125}I-CaM overlay assays. MW + CN, Mol wt tandards (Sigma) and calcinuerin (10 ng), respectively. GC, Guard cell; EC, epidermal cell; MC, mesophyll cell; Ep. Peels, abaxial epidermal peels; M_r are expressed in thousands. a, Protein profiles of *Vicia* protoplasts, tissues, and organs. Protein extracts (20 mg) were fractionated by 5 to 20% gradient SDS-PAGE followed by Coomassie blue staining. b, Protein profiles of *Vicia* extracts in duplicate gel after electrotransfer. After electrotransfer, the gel was stained in Coomassie blue to determine retained proteins. Lane 1 contained 5 μg of prestained protein standards (Bethesda Research Laboratories) and 10 ng of calcineurin as Ca^{2+}/CaM-binding control. c, ^{125}I-CaM overlay assay + EDTA. Gel electroblot of *Vicia* proteins incubated with ^{125}I-CaM overlay buffers in the presence of 1 mM EDTA. d, ^{125}I-CaM overlay assay + $CaCl_2$. Same blot as c, washed and incubated with ^{125}I-CaM overlay buffers in the presence of 1 mM $CaCl_2$.

Protoplast, tissue, and organ extracts as well as mol wt standards were separated by 5 to 20% SDS-PAGE and electroblotted onto PVDF membranes with pH 11.0 transfer buffer. As a positive control, 10 ng of calcineurin, an M_r 61,000 Ca^{2+}-dependent CaM-binding protein, was included in the sample containing mol wt standards. Gels were stained after transfer to assess the extent of transfer (Fig. 4b). Representative results from three qualitatively similar sets of assays are presented.

When blots were incubated and processed with ^{125}I-labeled *Vicia* CaM in the presence of 1 mM EDTA, some CaM-binding proteins were detected (Fig. 4c). These include epidermal and stem-specific M_r 17,000 and 19,000 bands as well as many minor bands below M_r 30,000 in 1.5-week-old plumule and roots and a cotyledon protein of M_r 22,000. Binding of CaM to lysozyme, a highly basic protein (isoelectric point 10.5–11), was observed in lanes containing mol wt standards. Comparison of the protein pattern partially retained by the gel after high pH electrotransfer (Fig. 4b) with the +EDTA CaM-binding protein pattern (Fig. 4c) showed similarity in profile, consistent with the idea that these CaM-binding proteins are basic in nature. Nonspecific CaM-binding to highly basic proteins in the presence of EDTA has been described (28). In a few cases, CaM-binding proteins have higher affinity to CaM in the absence of Ca^{2+} than in the presence of Ca^{2+} (28). Thus, the bands present in Figure 4c may represent actual CaM-binding proteins that weakly bind to CaM in the presence of EDTA, but the possibility that these are nonspecific CaM-binding proteins cannot be excluded.

The PVDF blots were then washed and processed with ^{125}I-CaM in the presence of Ca^{2+}. An autoradiograph of Ca^{2+}-enhanced CaM-binding is shown in Figure 4d. Lane 1 shows intense binding of CaM to the M_r 61,000 calcineurin-positive control in the presence of Ca^{2+} (compare with Fig. 4c, lane 1). CaM-binding proteins present in all samples, with the exception of seed tissue, were bands with M_r = 52,000, 78,000, and 115,000. In 1.5-week-old plumule and root samples, the M_r 78,000, 52,000, and basic proteins of M_r less than 30,000, which faintly bound CaM in the presence of 1 mM EDTA, had greatly enhanced CaM-binding in the presence of 1 mM $CaCl_2$. Ca^{2+}-dependent, CaM-binding proteins specific for samples included minor M_r 39,000 and 88,000 bands present in guard cell protoplasts and an M_r 45,000 band, as well as a doublet of M_r 60,000 and 64,000, in stem extract. Most prominent was a very intense M_r 62,000 band that was only apparent in root extract. Upon completion of the assay, the PVDF blot was stained with Coomassie blue and revealed a protein pattern similar to the duplicate SDS gel of the same samples (data not shown), confirming retention of blotted proteins during the assay.

DISCUSSION

In this study, we examined the distribution of CaM and CaM-binding proteins within *V. faba*. Biochemical analyses of plant CaM most often use extracts derived from whole plant organs, and as a result, very little is known regarding the quantity of CaM within the specialized tissues and cell types of plants. To date, virtually no information has been available regarding the developmental or spatial distribution of CaM-binding proteins in higher plants. We demonstrate that neither CaM nor the protein receptors of CaM are uniformly distributed within specific tissues or protoplast types of *Vicia*. The detection of high levels of active CaM within certain samples may point to the involvement of CaM in the Ca^{2+}-mediated processes of these cells.

In extracts from abaxial epidermal peels, both gel and PDE assays revealed CaM levels that were higher than CaM levels of total leaf extracts (Figs. 2c and 3). These results are consistent with the immunocytochemical staining data of etiolated pea tissue (9) in which intense immunofluorescence was detected from the membrane of epidermal vacuoles. Calculations based on the CaM activity (A.U. per gram of protein) and density of CaM in stained gels (nanograms of CaM per gram of protein) indicated that CaM levels in abaxial epidermal peels were 3- to 3.5-fold higher than in total leaf tissue. The observation that abaxial epidermal peel tissue makes up only 3.5% of *Vicia* total leaf protein indicated that 11 to 12% of CaM in leaf tissue was associated with abaxial epidermal peels.

Extrapolating to the adaxial surface, up to 24% of total leaf CaM may be associated with leaf epidermis. What is the role of CaM in the epidermis? It has been demonstrated that light triggers electrical responses in specific tissues within plants, including the epidermis (29). Very recently, transgenic seedlings were produced expressing aequorin, a luminous reporter of Ca^{2+}. In those plants, transient increases of cytoplasmic Ca^{2+} were detected in response to treatments with touch, cold stress, and fungal elicitors (31). The authors of that report suggested that the location of the Ca^{2+} increases may include the epidermis. The above observations, combined with our observation of high levels of CaM in this tissue type, suggest that as-yet uncharacterized Ca^{2+}/CaM-mediated signal transduction processes may occur within the epidermal layer of plants.

Leaf epidermal peels were found to contain 2-fold higher levels of CaM, based on total protein, than the protoplasts derived from this cell layer. Two possible explanations may account for this discrepancy in CaM levels. Cells within the epidermal peels may initially have had high levels of intracellular CaM but may have been broken, permeablized, or biosynthetically altered by the processes of peeling, cell wall digestion, and protoplast formation. The second possibility is that much of the CaM in leaves may be loosely associated with the cell wall or may be completely extracellular. The presence of extracellular CaM in both plant (2, 30) and animal (19) systems has been documented. The exact role of extracellular CaM in plants is yet to be elucidated, but it has been hypothesized that CaM may be involved in cell wall maintenance (31).

Guard cell protoplasts contained the highest concentration of CaM detected in leaf protoplasts, based on total protein and on volume. In addition, CaM overlay assays revealed guard cell protoplast samples to contain unique CaM-binding proteins with M_r = 88,000 and 39,000, despite the high degree of protein profile similarity between guard cell and epidermal cell protoplasts. Mounting evidence indicates that specialized Ca^{2+}-dependent processes are involved in stomatal closure and inhibition of stomatal opening (26). Ca^{2+} is necessary for darkness-induced stomatal closure and to some extent is implicated in ABA action (31). Ca^{2+} effects on stomatal aperture may be accomplished by inhibition of voltage-dependent K^+ currents and activation of anion permeability via elevation of internal Ca^{2+} concentration (26).

Studies of stomatal response to the presence of CaM antagonists suggest the involvement of CaM in guard cell turgor control. CaM antagonists such as trifluoperazine and compound 48/80 stimulate stomatal opening and decrease the effectiveness of ABA in closing stomata (11). Because of the limited specificity of these CaM inhibitors, these reports suggest CaM involvement in guard cell function but do not preclude the possible involvement of CaM-like proteins (3, 34) or enzymes with CaM-like domains (13). Thus, the presence of high concentrations of CaM and unique CaM-binding proteins in guard cell protoplast samples suggests the presence of a specialized pathway in guard cells by which CaM may transduce transient Ca^{2+} increases into stomatal turgor adjustment.

In whole stem protein samples, prominent Ca^{2+}/CaM-binding proteins included a doublet with M_r = 60,000 and 64,000 and a band with M_r = 45,000. Mechanical stimulation in plants leads to stem thickening due to the shortening of epidermal and parenchymal cells. Only recently has gene expression in response to this stimulus been revealed. One of the mRNAs whose levels accumulate is that of CaM (3). Although an increase of CaM mRNA level is not necessarily reflected proportionately in CaM protein levels (15), this result does suggest that CaM and CaM-binding proteins may be involved in this response. The production of leaf protoplasts requires the mechanical removal of the epidermis, followed by prolonged incubations of leaf tissue with constant agitation in protoplast isolation medium. In our hands, this process reduces CaM levels (compare total leaf with mesophyll protoplasts, epidermal peels with guard cell and epidermal protoplasts, Fig. 3), in distinct contrast to what would be expected if mechanical stimulation increased CaM protein levels.

Interestingly, the CaM-binding protein patterns for 1.5-week-old plumule and root samples were highly similar, with many Ca^{2+}-independent CaM-binding bands occurring below M_r 30,000. These patterns bear little resemblance to the CaM-binding protein patterns present in mature leaves or stem, suggesting that plant growth and development involve expression of differentially regulated CaM-dependent processes. Developmental changes in CaM-binding protein patterns have also been reported in fucoid algae (4) and are likely to be a common theme in plant tissue differentiation.

In young primary root samples, a single M_r 62,000 CaM-binding band with a very intense Ca^{2+}-dependent signal was detected. This M_r 62,000 band was not seen in correspondingly young plumule samples. It is not currently known

whether this root-specific CaM-binding M_r 62,000 protein is related to the M_r 60,000 and 64,000 CaM-binding proteins present in stem extracts. This M_r 62,000 protein is of particular interest because of the postulated role of Ca^{2+} and CaM in root gravitropism (30). It is thought that the gravitropic signal causes Ca^{2+}/CaM activation of CaM-binding proteins in roots, leading to asymmetric growth and root bending. Elevated levels of Ca^{2+} and CaM are found at the root tips (21), an area of rapid cell proliferation and also an area from which the gravitropic signal originates (33).

With the exception of dry seed, a number of Ca^{2+}-enhanced CaM-binding proteins (M_r = 52,000, 78,000, 115,000) appeared to be present in all organs, tissues, and cell types examined. These Ca^{2+}-enhanced CaM-binding proteins were distributed throughout all metabolically active plant organs and tissues at differing intensities. The identities of these shared CaM-binding proteins are unknown. In vivo, CaM has been shown to be associated with cytoskeletal elements in cells and is known to bind to a class of MAPs in animal systems (16). A previous report described carrot MAPs to have M_r = 76,000 and 129,000. CaM affinity chromatography of protein fractions enriched for MAPs yielded proteins in which two bands had M_r = 76,000 and 129,000 (8). Thus, it is reasonable to postulate that some of the widely distributed proteins detected by the ^{125}I-CaM overlay assays may be cytoskeletal CaM-binding proteins. Other plant CaM-binding proteins with known molecular masses include the 120-kD plasma membrane Ca-ATPase (14), 50-kD cytoplasmic NAD kinase (20), and a 47-kD nuclear nucleotide triphosphatase (6). These proteins may also be candidates for the identities of the bands detected in the overlay assay.

In conclusion, we have demonstrated differential distribution of CaM and CaM-binding proteins in *Vicia*. The highest concentration of CaM is associated with the epidermis, and within this tissue, guard cell protoplasts contain the highest concentrations of CaM, based on volume. Both unique and tissue- and cell-specific CaM-binding proteins were detected, indicating spatial and developmental regulation of CaM-dependent processes. These sets of experiments represent the first steps in dissecting the pathways associated with CaM; by isolating and characterizing CaM-binding proteins at the biochemical and molecular level, the identities of these proteins may be elucidated and the role of Ca^{2+}/CaM-mediated signal transduction in these tissue and cell types may be further elaborated.

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LITERATURE CITED

1. **Anderson JM** (1983) Purification of plant calmodulin. Methods Enzymol **102:** 9–17
2. **Biro RL, Daye S, Serlin BS, Terry ME, Datta N, Sopory SK, Roux SJ** (1984) Characterization of oat calmodulin and radioimmunoassay of its subcellular distribution. Plant Physiol **75:** 382–386
3. **Braam J, Davis RW** (1990) Rain-, wind-, and touch-induced expression of calmodulin and calmodulin-related genes in *Arabidopsis*. Cell **60:** 357–364
4. **Brawley SH, Roberts DM** (1989) Calmodulin-binding proteins are developmentally regulated in gametes and embryos of fucoid algae. Dev Biol **131:** 313–320
5. **Chafouleas JG, Dedman JR, Munjall RP, Means AR** (1979) Calmodulin: development and application of a sensitive radioimmunoassay. J Biol Chem **254:** 10262–10267
6. **Chen Y-R, Datta N, Roux SJ** (1987) Purification and partial characterization of a calmodulin stimulated nucleoside triphosphatase from pea nuclei. J Biol Chem **262:** 10689–10694
7. **Cheung WY** (1971) Cyclic 3′-5′ nucleotide phosphodiesterase: evidence for and properties of a protein activator. J Biol Chem **246:** 2859–2869
8. **Cyr RJ** (1989) Calcium/calmodulin affects microtubule stability in lysed protoplasts. J Cell Sci **100:** 311–317
9. **Dauwalder M, Roux SJ, Hardison L** (1986) Distribution of calmodulin in pea seedlings: immunocytochemical localization in plumules and root apices. Planta **168:** 461–470
10. **Dieter P** (1984) Calmodulin and calmodulin-mediated procesess in plants: a review. Plant Cell Environ **7:** 371–380
11. **Donovan N, Martin S, Donkin ME** (1985) Calmodulin binding drugs trifluoperazine and compound 48/80 modify stomatal responses of *Commelina communis* L. J Plant Physiol **118:** 177–187
12. **Flanagan ST, Yost B** (1984) Calmodulin-binding proteins: visualization by ^{125}I-calmodulin overlay on blots quenched with Tween 20 or bovine serum albumin and polyethylene oxide. Anal Biochem **140:** 510–519
13. **Harper JF, Sussman MR, Schaller GE, Putnam-Evans C, Charbonneau H, Harmon AC** (1991) A calcium-dependent protein kinase with a regulatory domain similar to calmodulin. Science **252:** 951–954
14. **Hsieh W-L, Sze H** (1991) Calcium transport in vesicles from carrot cells. Stimulation by calmodulin and phosphatidyl serine (abstract No. 194). Plant Physiol **96:** S-35
15. **Ido M, Lagace L, Chafouleas JG** (1991) Increased sensitivity to *Vinca* alkaloids in cells overexpressing calmodulin by gene transfection. Cancer Res **50:** 6551–6558
16. **Klee CB, Vanaman TC** (1982) Calmodulin. Adv Prot Chem **35:** 213–321
17. **Kruse T, Tallman G, Zeiger E** (1989) Isolation of guard cell protoplasts from mechanically prepared epidermis of *Vicia faba* leaves. Plant Physiol **90:** 1382–1386
18. **Laemmli UK** (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680–685
19. **MacNeil S, Walker SW, Senior HJ, Bleehen SS, Tomlinson S** (1984) Effects of extracellular calmodulin and calmodulin antagonists on B16 melanoma cell growth. J Invest Dermatol **83:** 15–19
20. **Marme D** (1988) The role of calmodulin in plants. *In* P Cohen, CB Klee, eds, Molecular Aspects of Cellular Regulation, Vol 5: Calmodulin. Elsevier, Amsterdam, pp 313–320
21. **Muto S, Miyachi S** (1984) Production of antibody against spinach calmodulin and its application to radioimmunoassay for plant calmodulin. Z Pflanzenphysiol **114:** 421–431
22. **Ohya T, Shimazaki K-I** (1989) Profiles of proteins in guard cell and mesophyll protoplasts from *Vicia faba* L. fractionated by sodium dodecylsulfate-polyacrylamide gel electrophoresis. Plant Cell Physiol **30:** 783–787
23. **Outlaw WH, Mayne BC, Zenger VE, Manchester J** (1981) Presence of both photosystems in guard cells of *Vicia faba* L. Plant Physiol **67:** 12–16
24. **Radvanyi LG, DiCosmo F** (1988) Purification and partial characterization of a calmodulin-like protein from cell suspension cultures of *Catharanthus roseus*. FEBS Lett **234:** 432–434
25. **Roberts DM, Lukas TJ, Watterson DM** (1986) Stucture, function and mechanism of action of calmodulin. CRC Crit Rev Plant Sci **4:** 311–339
26. **Schroeder JI, Hagiwara S** (1989) Cytosolic calcium regulates ion

channels in the plasma membrane of *Vicia faba* guard cells. Nature **338:** 427–430

27. **Shimazaki K-I, Gotow K, Kondo N** (1982) Photosynthetic properties of guard cell protoplasts from *Vicia faba* L. Plant Cell Physiol **23:** 871–879
28. **Slaughter GR, Means AR** (1987) Use of the ^{125}I-labeled protein gel overlay technique to study calmodulin-binding proteins. Methods Enzymol **139:** 433–455
29. **Spaulding EP, Cosgrove DJ** (1988) Large plasma-membrane depolarization precedes rapid blue-light-induced growth inhibition in cucumber. Planta **178:** 407–410
30. **Stinemetz CL, Kuzmanoff KM, Evans ML, Jarrett HW** (1987) Correlation between calmodulin activity and gravitropic sensitivity in primary roots of maize. Plant Physiol **84:** 1337–1342
31. **Trewavas A, Gilroy S** (1991) Signal transduction in plant cells. Trends Genet **7:** 356–361
32. **Watterson DM, Harrelson WG Jr, Keller PM, Sharief F, Vanaman TC** (1976) Structural similarities between the Ca^{2+}-dependent regulatory proteins of 3′:5′-cyclic nucleotide phosphodiesterase and actomyosin ATPase. J Biol Chem **251:** 4501–4513
33. **Yang RL, Evans ML** (1990) Microsurgical removal of epidermal and cortical cells: evidence that the gravitropic signal moves through the outer cell layers in primary roots of maize. Planta **180:** 530–536
34. **Zielinski RE, Ling V, Perera I** (1990) Structure and expression of genes encoding calcium-modulated proteins in higher plants. Curr Top Plant Biochem Physiol **9:** 141–152