

Chapter 8

CALMODULIN IN PLANTS

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I. INTRODUCTION

Since the discovery of calmodulin (CaM) in plants,¹⁻³ the role of this calcium-binding protein in plant metabolism has been under active investigation. cAMP is not a second messenger in the plant systems so far studied,⁴ and Ca²⁺ is known to be an important mediator of extracellular signals. The specificity of the Ca²⁺-CaM complex toward the activation of enzymes allows extracellular signals to direct the activation of specific metabolic processes. This review will cover processes in plants for which there is a known or implied role for CaM. As will become apparent to the reader, what physiological advantage there is in the CaM mediation of Ca²⁺ signals in plants, as opposed to direct Ca²⁺ activation, is not well understood. There are available a number of good reviews of CaM-mediated processes in plants.⁵⁻⁸

II. CHARACTERIZATION

A. Identification

To date CaM has been identified in a number of photosynthetic algae and higher plants (leaves, seeds, and endosperm): asparagus,⁹ barley,¹⁰ Chinese cabbage,² *Chlamydomonas*,^{11,12} *Chlorella*,² corn,^{2,13,14} *Euphorbia lathyris*,¹⁵ *Mougeotia*,¹⁶ mungbean,^{9,17} oat,¹⁸ onion,¹⁹ peanut,^{9,20,21} pea,^{1,2,9,19,20} rice,² soybean,^{9,22} spinach,^{2,23,24} wheat,^{9,25} and zucchini.²⁶

B. Amino Acid Composition and Sequence

Often the only evidence for the presence of CaM in a plant is the stimulation of CaM-dependent enzymatic activity by a crude extract. However, the CaMs of barley,^{10,27} *Chlamydomonas*,¹² corn,⁶ *Euphorbia lathyris*,¹⁵ oat,¹⁸ peanut,²⁰ spinach,^{24,27} soybean,²² and zucchini⁶ have been purified to homogeneity, and their amino acid compositions determined (Table 1). The CaMs of peanut, soybean, spinach, zucchini, and probably barley contain one cysteine residue in contrast to animal CaM which contains no cysteine or cystine. The cysteine content of corn and oat CaM was not determined. Barley CaM was reported to contain no trimethyllysine,¹⁰ but recently this claim has been refuted.²⁷ The CaM of *Chlamydomonas* contains no cysteine nor trimethyllysine.^{12,28} It also contains three histidine residues rather than the one residue usually found in plant and mammalian CaMs. The CaM of *Euphorbia lathyris* has four prolines per molecule instead of the usual two residues.

Table 1
COMPARISON OF AMINO ACID COMPOSITION OF SELECTED
CALMODULINS (RESIDUES/MOLECULE)

Amino acid	Bovine brain	Spinach ^{24,27}	Zucchini ⁶	Peanut ²⁹	<i>Euphorbia lathyris</i> ¹⁵
Aspartic acid	23	24	26	27	27
Threonine	12	9	9	9	7
Serine	4	4	5	5	7
Glutamic acid	27	27	26	28	30
Proline	2	2	2	2	4
Glycine	11	10	10	11	11
Alanine	11	11	11	11	10
1/2 Cystine	0	1	1	1	1
Valine	7	8	7	6	7
Methionine	9	8	7	7	5
Isoleucine	8	7	7	6	6
Leucine	9	11	12	11	9
Tyrosine	2	1	1	1	1-2
Phenylalanine	8	9	9	8	7
Histidine	1	1	1	1	1
Trimethyllysine	1	1	1	1-2	1
Lysine	7	9	10	8	9
Tryptophan	0	0	0	0	0
Arginine	6	5	5	4	4

The amino acid sequences of the CaMs from *Chlamydomonas*²⁸ and spinach²⁹ have been determined. *Chlamydomonas* CaM has both an amino- and carboxy-terminal extension, in contrast to the CaM of spinach which is the same length as bovine brain CaM. The unique cysteine residue to spinach CaM (cysteine-26) is located in one of the potential calcium-binding sites.³⁰ Comparison of all CaMs for which sequence data are available shows that the greatest length of amino acid sequence identity is from positions 27 to 52.²⁸

C. Comparison of Plant and Animal CaM

In many respects mammalian and plant CaMs are very similar. Indeed, plant CaM can activate mammalian cyclic nucleotide phosphodiesterase^{1,10-12,16,20,23,27} and myosin light chain kinase.³¹ Plant CaMs show a Ca²⁺-dependent enhancement of tyrosine fluorescence,²⁰ and bind amounts of Ca²⁺ (at fixed levels of free Ca²⁺) similar to those bound by the mammalian protein.^{32,33} The electrophoretic behaviors of plant and mammalian CaMs are similar and show a calcium-dependent shift in mobility.^{10,11,16,18,20,23,27} Also, plant CaM action is inhibited by phenothiazine antagonists.^{11,20,26}

It is possible to demonstrate differences among mammalian, higher plant, and algal CaMs without reliance on amino acid sequence analysis. For example, antisera have been prepared which can differentiate between mammalian and higher plant or algal CaM and between algal and higher plant CaMs.^{12,27,34} Mammalian phosphodiesterase binds mammalian-derived CaM tighter than plant-derived CaM. In contrast, NAD kinase from peas binds plant-derived CaM tighter than mammalian-derived CaM.¹⁷ A saturating concentration of CaM from either spinach or *Chlamydomonas* has been reported to enhance pea NAD kinase activity (V_{max}) above that given by mammalian CaM.³¹

A saturating concentration of *Chlamydomonas* CaM is a particularly effective activator of NAD kinase, although its affinity for this enzyme is low. Since *Chlamydomonas* CaM lacks trimethyllysine, this suggests that the presence of this amino acid in CaM, although not a prerequisite to activity, modulates activity. In support of this conclusion it has recently been shown that upon in vitro methylation,³⁵ *Chlamydomonas* CaM activated NAD kinase

to a level that was about threefold lower than the level of activation obtained with the unmethylated protein. Methylation did not affect the ability of this CaM to activate mammalian phosphodiesterase. A genetically engineered CaM containing arginine, instead of lysine, at position 115 was a superior activator of NAD kinase, and the activator properties of this CaM were unaffected by the action of methyltransferase.

III. DISTRIBUTION

Three methods have been used to determine the distribution or concentration of CaM in plants: enzymatic assay, radioimmunoassay, and fluorescent microscopy. All three techniques suffer limitations. Plants often contain inhibitors of CaM-stimulated enzyme activity.³⁶⁻³⁸ The presence of such inhibitors can result in an underestimation of CaM levels by enzymatic assay. Antibodies prepared for radioimmunoassay have been reported to bind to a protein from *Chlamydomonas* which by other criteria is not CaM.³⁹ Although CaM is a highly conserved protein, antibodies prepared against CaM from one source do not necessarily have equal affinity toward CaM from other sources. The specificity of various antibody preparations to plant CaM has been noted.^{22,34,38,40} Fluorescent microscopy employs either the photo-oxidation of phenothiazine antagonists to fluorescent derivatives or antibodies to CaM which are tagged with a second fluorescent antibody. CaM antagonists are hydrophobic molecules which may bind to a variety of cellular components in plants: membranes,⁴¹ the terpene fraction of plant latex,⁴² and CaM-independent protein kinases.⁴³ In addition to the problem of antibody specificity mentioned above, another problem is that penetration of the antibody to the interior of the cell requires either the physical or chemical disruption of cellular barriers. In studies involving subcellular fractionation, endogenous cytosolic CaM can bind to the outer membrane of organelles. However, a correction for this artifact can be made by fractionating the cell in the presence of radiolabeled CaM.⁴⁴

Summaries of CaM distribution and localization studies conducted to date in plants are presented below. Also included is one study of CaM-binding proteins. These types of studies that yield important information will provide an insight into the physiological function of CaM. However, the reservations expressed above apply here, and confirmation of these observations is necessary in establishing their validity.

A. Growing Cells

CaM antagonists inhibit pollen tube growth,⁴⁵ and the induced fluorescence of an antagonist revealed that CaM was concentrated in the tip of younger stages of the pollen tubes of *Lilium longiflorum* and the tip of root hairs of *Lepidium sativum*.⁴⁶ Older stages of either pollen tubes or root hairs showed more uniform, nonlocalized fluorescence. Immunofluorescent localization of tubulin in *Lilium* pollen tubes showed it to be localized in the same area as CaM.⁴⁷

B. Dividing Cells

CaM was localized during cell division using immunofluorescence in *Haemanthus* endosperm cells¹⁴ and onion and pea root meristematic cells.¹⁹ In interphase all cells showed diffuse cytoplasmic fluorescence. In prophase CaM-derived fluorescence was associated with the mitotic apparatus. *Haemanthus* CaM was found to be associated with the polar microtubule-conveying centers and kinetochore microtubules, although in a pattern distinct from that of tubulin. In addition, the CaM distribution corresponded closely to the calcium distribution. Pea or onion CaM was associated with the microtubules of the spindle. During anaphase in *Haemanthus*, pea, and onion, the CaM distribution contracted and became concentrated at the shortening spindles. In *Haemanthus* little or no CaM was seen in the interzone region. In peas or onions, however, the entire width of the phragmoplast cell plate reacted with anti-CaM.

CaM levels in etiolated and green-pea seedlings were determined by radioimmunoassay.³⁴ They were expressed on the basis of fresh weight, total protein, or total DNA. In the etiolated plant the highest CaM concentration (as expressed all three ways) was in the stem segment just below the shoot apex. In green seedlings CaM levels were lower than in the etiolated plant; on a protein basis the CaM concentration was highest in a lateral root. Expressed on the basis of total DNA, the highest CaM concentration occurred in a young but fully expanded leaf.

C. Guard Cells

Using immunofluorescence CaM was localized in the area surrounding the aperture of stomata in guard cells from *Lilium*.⁴⁸ Immunofluorescent localization of tubulin showed it to be localized adjacent to the aperture and at the poles of the aperture.⁴⁸ Fluorescence attributable to CaM was diffuse, but tubulin antibodies exhibited definite immunofluorescence.

D. Subcellular Fractions

The subcellular distribution of CaM was determined in mesophyll-cell protoplasts from wheat by enzymatic assay and radioimmunoassay.⁴⁴ Nearly 90% of CaM was found in the cytosol. However, the microsomes, mitochondria, and chloroplasts contained measurable amounts of CaM. The majority of mitochondrial and chloroplastic CaM was found in the matrix and stroma, respectively. The amount of CaM detected by radioimmunoassay was four- to sixfold lower than indicated by enzymatic assay. The addition of subcellular fractions from wheat did not reduce or alter the radioimmunoassay of purified spinach CaM, eliminating the possibility that interfering substances were responsible for low radioimmunoassay values.

CaM levels were measured in the nuclei, mitochondria, and cell wall fractions of *Avena sativa* using radioimmunoassay,¹⁸ and expressed on a total-protein basis. The cell wall fractions contained the largest amount of CaM. The nuclei lost about 80% of CaM when extracted in EGTA. Trypsin digestion of whole mitochondria reduced CaM levels by about a factor of two, whereas removal of the outer mitochondrial membrane caused the loss of nearly all CaM; the outer membrane contained no CaM. Thus it was concluded that CaM is located between the inner and outer membranes of mitochondria.

The association of CaM and purified chromatin from pea buds has been reported.⁴⁹ The derived CaM was an activator of CaM-dependent enzyme activity and was purified by affinity chromatography.

CaM was detected in pea chloroplasts.⁵⁰ The majority of CaM was not accessible to trypsin, but was released when chloroplasts were lysed by osmotic shock. The CaM content of chloroplasts was measured in pea plants using radioimmunoassay and was found to decrease steadily as the plants aged from 9 to 18 days.⁵¹ Over the same time period the Ca²⁺ content of the chloroplasts increased.

The CaM-binding proteins of spinach and pea chloroplasts were detected using a radio-labeled CaM gel overlay assay.⁵² The major CaM-binding protein had a molecular weight of 33 kdaltons and was found in the envelope fractions, but not in the stroma or thylakoid fractions. The binding of CaM to this protein did not require Ca²⁺.

IV. REGULATION OF PROCESSES AND ENZYMES

This section will discuss biological processes and enzyme activities for which there is direct evidence (except as noted) for participation of CaM.

A. Ca²⁺ Transport

All known CaM-dependent Ca²⁺ transport activities are examples of active transport that

require the addition of ATP. Thus it is possible that in some cases CaM-dependent ATPase is linked in vivo to CaM-dependent Ca^{2+} transport. In apple, a correlation between inhibition of mitochondrial Ca^{2+} uptake and ATPase activity by the CaM antagonist, W-7, was taken as support for the link between these two processes.⁵³ Unfortunately, it has never been demonstrated by direct methods that mitochondrial Ca^{2+} uptake is dependent on CaM.

CaM stimulated ATP-dependent Ca^{2+} transport into microsomes prepared from etiolated zucchini hypocotyl hooks.⁵⁴ The observed stimulation over the basal rate was one- to three-fold. Since fluphenazine, a CaM antagonist, did not inhibit basal Ca^{2+} uptake as much as CaM-dependent uptake, it was suggested that both CaM-dependent and independent Ca^{2+} transport systems exist.

ATP-dependent Ca^{2+} transport into microsomes derived from coleoptiles of dark-grown corn was stimulated by CaM.⁵⁵ However, CaM did not stimulate Ca^{2+} transport in far-red light-grown corn. Evidence suggests that the far-red light-induced inhibition of transport is mediated by the nonphototransformable proto-chlorophyll (lide) 630. It was suggested that the microsomes are derived from inside-out plasma membrane. Thus, in vivo CaM would stimulate Ca^{2+} transport out of the cell, and the application of far-red light would inhibit transport.

The addition of CaM to corn coleoptile microsomes caused K_m for Ca^{2+} transport to decrease and V_{max} to increase.⁵⁶ Mitochondrial Ca^{2+} uptake was not CaM-dependent, and V_{max} and K_m for mitochondrial Ca^{2+} uptake were over ten times larger than for microsomal uptake. Thus, at the low concentration of Ca^{2+} normally found in the cell, microsomal transport is more important in controlling the cytoplasmic Ca^{2+} concentration.

CaM enhanced Ca^{2+} uptake by microsomes derived from green spinach leaves.⁵⁷ Uptake was enhanced in microsomes derived from the lower epidermis, lamina, petiole, and midrib. It was suggested that an $\text{H}^+/\text{Ca}^{2+}$ antiporter is not operative since m-chlorophenylhydrazine (CCCCP) is not an inhibitor.

In other plant systems, evidence suggests that microsomal preparations are primarily derived from tonoplast or endoplasmic reticulum.⁵⁸ It is therefore unclear whether the CaM-stimulated Ca^{2+} transport activities discussed above are associated exclusively with the plasma membrane, as has been suggested,⁵⁵ or with other membrane systems as well.

B. ATPase

An ATPase solubilized from a microsomal fraction of corn coleoptiles was stimulated 7% by CaM.⁵⁹ After partial purification of the ATPase using CaM-Sepharose® affinity chromatography, ATPase activity was stimulated more than twofold by CaM. The activity of a microsomal CaM-dependent ATPase from the roots of wheat seedlings was stimulated by the application of a synthetic cytokinin that also caused a decrease in K_m for Ca^{2+} .²⁵ It was suggested that the time period needed for cytokinin-induced changes in the properties of the ATPase are consistent with an action on the gene or ribosomal level.

The envelopes of spinach chloroplasts contained an ATPase that was stimulated as much as 63% by CaM in the presence of Mg^{2+} and Ca^{2+} .⁶⁰ The ATPase was partially purified by affinity chromatography. In an extension of this work the solubilized ATPase was found to have a molecular weight of 65 kdaltons using SDS-polyacrylamide electrophoresis and 260 kdaltons on native gels.⁶¹ The protein was resolved into two bands using isoelectric focusing.

Isolated nuclei from pea buds contained a CaM-stimulated ATPase activity.⁶² The CaM-stimulated activity was inhibited by the antagonist, trifluoperazine; the concentration giving 50% inhibition (I_{50}) = 50 μM .

C. NAD Kinase

1. Physiological Significance

The light-induced conversion of NAD to NADP, catalyzed by NAD kinase, was noted

in *Chlorella* and a variety of higher plants.⁶³ The phosphorylation of NAD was associated with photophosphorylation,⁶³ and it would therefore seem obvious that increased ATP levels are responsible for the light-induced rise in NADP levels. However, upon the application of light, ATP levels rose before NADP levels,⁶⁴ indicating that NAD kinase activity is regulated in some manner. The discovery of Ca^{2+} -CaM-stimulation of NAD kinase^{1,2,20} provided an explanation for its regulation in vivo. In support of this explanation is the observation that the CaM antagonist, trifluoperazine, inhibited the light-induced conversion of NAD to NADP in pea protoplasts.⁵⁰

2. Purification

The method of choice for the purification of NAD kinase is via CaM-Sepharose®.^{65,66} Although CaM-Sepharose® retains several proteins, NAD kinase was purified to homogeneity by one additional step using ion-exchange chromatography.⁶⁷ Rapid purification of NAD kinase is necessary to obtain enzyme which is differentially sensitive to spinach and *Chlamydomonas* CaMs.³⁰ An enzymatic assay for plant CaM based on the use of NAD kinase has been devised.¹⁷

3. CaM Independent

The elution profiles of pea and zucchini NAD kinases from CaM-Sepharose® show that greater than 95% of these enzymes bind to CaM.^{65,66} Nonetheless, as discussed below, there is evidence for forms of NAD kinase that are not activated by CaM. It is not clear whether CaM-independent NAD kinase is of physiological significance or whether its detection is an artifact of partial proteolysis,⁶⁸ as with the CaM-independent form of cyclic nucleotide phosphodiesterase.⁶⁹

4. Chloroplastic

Chloroplasts and the post-chloroplastic supernatant from protoplasts were tested for NAD kinase activity.⁶³ To correct for the contribution of broken chloroplasts in the supernatant, the activity of NADP-triose phosphate dehydrogenase was also measured. The results indicate that in wheat, pea, sunflower, crabgrass, and foxtail, NAD kinase is found mostly in the chloroplast.

The NAD kinase activity in a spinach homogenate was found to be predominantly in a 39,000g supernatant.⁷⁰ A small amount of activity was found in the stroma of the chloroplast, although its activity was not stimulated by Ca^{2+} -CaM. In contrast to other results, a minority of the NAD kinase activity in the supernatant or chloroplasts was retained on CaM-Sepharose®.

The majority of recoverable NAD kinase activity from protoplasts of pea was found to be associated with chloroplasts.⁵⁰ Activity could be measured only after the chloroplasts were lysed by osmotic shock.

About half of the CaM-dependent NAD kinase activity of pea seedlings and most of the CaM-independent activity were found to be associated with the chloroplasts.⁷¹ CaM-dependent activity could be measured using intact chloroplasts, and fractionation experiments indicated that this activity was localized at the envelope.

5. Mitochondrial

NAD kinase activity from dark-grown corn coleoptiles was found to be almost totally CaM dependent and to be associated with intact mitochondria.⁷² Upon rupture of the mitochondria by osmotic shock and sonication, activity was associated with the outer mitochondrial membrane.

NAD kinase activity from maize roots was found to be associated with both inner and outer membrane fractions of mitochondria.⁷³ The mitochondria were lysed by osmotic shock and homogenization. Only a fraction of NAD kinase activity could be measured using intact mitochondria.

D. Protein Kinases

1. Pea

Membrane-bound kinase activity was measured in a centrifuge-derived (80,000g) fraction of homogenized etiolated pea shoots.⁷⁴ The phosphorylation of endogenous proteins was increased five- to sevenfold in the presence of Ca^{2+} . Exogenous CaM stimulated activity about 30%, and a CaM antagonist inhibited phosphorylation. The stimulation of phosphorylation by Ca^{2+} was particularly prominent in three proteins (36, 50, and 110 kdaltons).

Isopycnic separation of crude microsomal preparations from etiolated pea buds indicated that the majority of Ca^{2+} -stimulated kinase activity (endogenous protein substrates) occurred in a light membrane fraction similar in density to the plasma membrane.⁷⁵ Enriched plasma membrane preparations also showed Ca^{2+} -stimulated kinase activity. Calf histone H1 was a substrate for Ca^{2+} -dependent kinase. Inhibition of Ca^{2+} -dependent kinase by a CaM antagonist was increased if the membranes were prepared in EGTA containing buffers. Solubilized protein kinase activity was very sensitive to added antagonist. Endogenous CaM was detected in the membrane preparations, and EGTA treatments did not remove all CaM from the membranes.

A protein kinase of etiolated pea buds was separated from other proteins by electrophoresis and transferred to nitrocellulose.⁷⁶ The transferred kinase retained the capacity for autophosphorylation primarily on serine which was stimulated threefold by CaM. Kinase activity was localized in plasma membrane-enriched preparations. Sodium dodecyl sulfate gels indicated that the autophosphorylating kinase is a mixture of two proteins of size 18 and 19 kdaltons with the 18-kdalton protein containing the predominant activity.

2. Wheat Germ

Phosphorylation of calf thymus histones was stimulated by Ca^{2+} and CaM after passage of the protein kinase through DEAE cellulose.⁷⁷ Significant CaM stimulation of protein kinase activity toward other substrates was not initially observed. After two gel filtrations in the presence of EGTA, CaM stimulation of histone phosphorylation was increased, and the CaM stimulation of casein phosphorylation was observed. A significant fraction of protein kinase was retained on a column of CaM-Sepharose[®] and eluted by buffer containing EGTA.

A Ca^{2+} -independent protein kinase was partially purified which showed CaM-stimulated activity toward histones.⁷⁸ This protein kinase is identical to the protein that phosphorylates wheat germ cytokinin-binding protein and is distinct from Ca^{2+} -dependent protein kinase. Ca^{2+} -dependent, CaM-stimulated protein kinase (protein kinase I) was separated from Ca^{2+} -independent protein kinase using gel filtration.⁷⁹ The molecular weight of the Ca^{2+} -dependent kinase was estimated to be 90 kdaltons. Histone and casein Ca^{2+} -dependent phosphorylation required Mg^{2+} , while Mn^{2+} was a less effective substitute for both Mg^{2+} and Ca^{2+} . Phosphorylation of casein was inhibited by various tri- and diphosphonucleotides.

Another Ca^{2+} -dependent protein kinase (protein kinase II) was isolated from wheat germ.⁸⁰ Protein kinase II was shown to be distinct from protein kinase I because its activity is insensitive to inhibition by tri- and dinucleotides. Protein kinase II activity toward histone, but not casein or phosvitin, was stimulated by CaM. Relatively high concentrations of CaM antagonists were required to inhibit protein kinase II activity toward casein.

A variety of inhibitors were tested on protein kinase I and II activities.⁴³ The assays were performed without CaM, using casein as the substrate. Therefore, inhibition did not depend upon CaM antagonism. The phenothiazines were inhibitors of kinase activity. However, IC_{50} values for kinase inhibition were larger than those usually obtained for CaM antagonism with in vitro assays. Nonphenothiazine, neuroleptic compounds (haloperidol, pimozide, and perfluridol) did not inhibit kinase activity, although they are CaM antagonists. Nor were the anesthetics, tetracaine and dibucaine, good inhibitors of kinase activity, although they are inhibitors of CaM-independent protein kinase activity.

3. *Corn and Zucchini*

The effect of Ca^{2+} , CaM, and CaM antagonists on the phosphorylation of soluble and membrane-bound proteins was observed in fractions from etiolated corn coleoptiles.⁸¹ Addition of the phosphatase inhibitor MnO_4^{-2} resulted in increased phosphorylation. Protein phosphorylation was stimulated by the addition of Ca^{2+} , and it was stimulated by CaM in both the presence and the absence of Ca^{2+} . The phosphorylation of two proteins was reduced in the presence of CaM.

Protein phosphorylation was observed in membrane fractions prepared from etiolated zucchini hypocotyl hooks using endogenous proteins as substrates.⁸² Low concentrations of Na^+ , Mg^{2+} , Ca^{2+} , and CaM enhanced total phosphorylation. Mg^{2+} and Ca^{2+} selectively stimulated the phosphorylation of only certain proteins, and Ca^{2+} strongly inhibited the phosphorylation of one protein. CaM stimulated the phosphorylation of about one half of the endogenous proteins. The dephosphorylation of some phosphoproteins indicated that phosphatases were also present in membrane fractions.

4. *Sycamore Tonoplast*

Purified tonoplast fractions were prepared from isolated sycamore vacuoles.⁸³ The rate of phosphorylation of endogenous tonoplast proteins was strongly stimulated by the addition of Ca^{2+} -CaM. However, relatively rapid dephosphorylation was observed after about 2 min. The use of a sulfur-containing analog of ATP as the phosphorylation substrate stabilized the phosphoproteins for at least 5 min.

E. Quinate: NAD 3-Oxidoreductase

Quinate: NAD 3-oxidoreductase (QORase) from carrot cells was found to be activated by covalent phosphorylation.⁸⁴ An endogenous protein kinase activity that can restore the activity of dephosphorylated QORase was shown to be Ca^{2+} -CaM dependent.⁸⁵ An endogenous protein which is not identical to CaM also activated the endogenous protein kinase in the presence of Ca^{2+} .⁸⁶

F. Isofloridoside Phosphate Synthetase

The activation of a crude preparation of isofloridoside phosphate synthetase from *Pteriochromonas* was enhanced by the addition of Ca^{2+} and CaM.⁸⁷ At least one other endogenous component aided in the enhancement of activity by Ca^{2+} -CaM, as did phosphatase inhibitors. The enhancement (rather than inhibition) of enzyme activity by CaM antagonists was suggested to be the result of their detergent-like qualities, since certain detergents also caused activity enhancement.

G. Phospholipase

The addition of CaM antagonists inhibited phospholipase action toward phosphatidylcholine in homogenates of potato leaves,⁸⁸ while addition of Ca^{2+} -CaM stimulated activity about twofold. Phospholipase activity from potato tubers was inhibited by low concentrations of CaM antagonists and stimulated by high concentrations.⁸⁹ The detergent deoxycholate also inhibited lipase activity at low concentrations and stimulated it at higher concentrations. Thus it was concluded that tuber phospholipase activity, in contrast to the activity of potato leaves, is not regulated by CaM. Consistent with this conclusion was the observation that added CaM provided no enhancement of activity.

H. Aspartate Kinase

Aspartate kinase activity isolated from the seeds of *Dolichos lablab*, spinach, and winged beans was stimulated by either plant or bovine brain CaM.⁹⁰ The stimulation by CaM was dependent on Ca^{2+} , and activity was inhibited by CaM antagonists. The lysine-sensitive

aspartate kinase activity purified from carrot cell suspension culture was found to be insensitive to added CaM or antagonists to CaM (except 500 μ M trifluoperazine).⁹¹ Crude preparations of aspartate kinase activities from barley and pea leaves were also not sensitive to the addition of EGTA or a CaM antagonist. Aspartate kinase from spinach leaves was resolved by column chromatography into lysine- and threonine-inhibited activities.⁹² Only the activity of the threonine-inhibited aspartate kinase was stimulated by Ca^{2+} -CaM.

V. ANTAGONISTS

A. Uses and Limitations of Antagonists

Commonly used CaM antagonists are synthetically prepared compounds, often prepared originally for purposes other than CaM antagonism, that interfere with Ca^{2+} -CaM-stimulated enzyme activity *in vitro*. Inhibition of complex, multistep biological processes by antagonists has been taken as evidence for the involvement of CaM in these processes. Unfortunately, CaM antagonists have been shown to inhibit a number of other processes. In animal-derived systems, CaM antagonists have been shown to interfere with CaM-independent ATPase activity,⁹³ phosphodiesterase activity,⁹⁴ and phospholipid-sensitive protein kinase activity.⁹⁵⁻⁹⁷ CaM antagonists also interact with animal membranes.^{98,99} A CaM antagonist has been shown to bind to a plant membrane system,⁴¹ and CaM antagonists have been shown to interfere with CaM-independent protein kinase activity from plants.⁴³

One strategy for sorting out specific and nonspecific effects by CaM antagonists has been to use slightly modified, ineffective (or more precisely, less effective) antagonists as controls, e.g., chlorpromazine sulfoxide, trifluoperazine sulfoxide, and W-5, W-12, A-3, A-4, and A-5. Unfortunately there is a lack of studies on the effects of these compounds in processes which are inhibited by CaM antagonists, but are not actually CaM-regulated processes. An indication that these compounds may not always be able to differentiate between CaM and nonCaM-regulated processes come from the results of one study of a structural series of CaM antagonists.⁹⁴ As antagonism toward CaM stimulation decreased in the series, the inhibition of trypsin-treated phosphodiesterase activity (CaM independent) also decreased.

Another strategy used to differentiate between specific and nonspecific effects of CaM antagonists is to measure inhibition over a range of antagonist concentrations and determine I_{50} (IC_{50}), the concentration of antagonist required to give 50% inhibition. *In vitro* I_{50} values for CaM antagonism are generally lower than I_{50} values for nonspecific inhibition. The pitfall in this approach is that the concentration of the CaM antagonist at its site of action in a complex system is not usually known and may be very different than the bulk solution concentration of the antagonist. For example, a membrane-bound enzyme may be severely inhibited due to concentration of the antagonist in the membrane. At the other extreme, CaM antagonists were rendered ineffective by their absorption into a hydrophobic terpene fraction of plant latex.⁴²

Even if the weight of evidence suggests involvement of CaM in a biological process, there is no assurance that CaM's major role is in the process of interest, rather than in primary energy-supplying metabolism or enzyme synthesis. Dissection of the process of interest into simpler parts may be the only way to substantiate direct CaM participation.

Finally, it should be noted that CaM has been reported to bind or to stimulate the activity of some target proteins in a Ca^{2+} -independent manner.^{52,78} Since antagonist action requires Ca^{2+} , it follows that antagonists cannot be used as probes for Ca^{2+} -independent CaM processes. Thus the importance of Ca^{2+} -independent CaM processes (if any) in plant metabolism remains obscure.

B. Natural Antagonists

It has been recognized for some time that crude plant extracts containing CaM often do

not stimulate CaM-dependent enzyme activity as much as expected. Thus, plants may contain inhibitors of CaM as well as general enzyme inhibitors. As shown below, in a few cases, natural inhibitors of CaM have been studied in a systematic fashion.

Small volumes of extracts of cold-stored apples stimulated phosphodiesterase activity, but larger volumes inhibited activity.³⁶ Pretreatment of the apples with Ca^{2+} reduced the inhibitory action. The inhibitor(s) was a heat-stable, dialyzable, low molecular weight component. The concentration of the inhibitor increased in apples stored at room temperature. Apple-derived CaM inhibitors were found to be neutral, phenolic compounds.³⁷ The identity of one inhibitor, catechin (a flavanoid), was determined using a combined gas chromatographic-mass spectrometric analysis. Other flavanoids and the simple phenolic, caffeic acid, were shown to be inhibitors of calmodulin-promoted phosphodiesterase activity. Caffeic acid and catechin were inhibitors of CaM-stimulated protein phosphorylation. The degree and type of inhibition of protein phosphorylation induced by these compounds was similar to that seen with synthetic CaM antagonists.

Ophiobolin A, a fungal metabolite and phytotoxin in maize roots was found to be a potent inhibitor of CaM-stimulated phosphodiesterase activity.¹⁰⁰ Ophiobolin A binding to CaM could not be reversed by dialysis or urea-methanol treatment indicating that binding involves the covalent modification of CaM.

The interaction of ophiobolin A and corn root CaM was studied.¹³ The binding of ophiobolin A to CaM was not reversed by dilution or denaturation in SDS. I_{50} values for inhibition of CaM-stimulated phosphodiesterase activity and phytotoxicity were similar. Treatment of roots with ophiobolin A resulted in reduced CaM levels (determined by phosphodiesterase activity).

C. Processes Inhibited by Antagonists

1. Photosynthesis and Electron Transport

Phenothiazine CaM antagonists were found to inhibit the photosynthetic evolution of oxygen by coral, algae, sea anemone,¹⁰¹ and pea protoplasts.⁵⁰ The sulfoxide of chlorpromazine was a poor inhibitor of photosynthesis in sea anemone and pea protoplasts.^{50,101} Photosynthesis in the green alga *Nannochloris bacillaris* and isolated mesophyll cells of asparagus was inhibited by phenothiazine CaM antagonists as well as propranol and dibucaine.¹⁰² Inhibition of whole-chain Hill reaction activity required higher chlorpromazine concentrations than were needed for inhibition of photosynthesis, suggesting more than one site of inhibition.

Electron transport in photosystem II of isolated spinach chloroplasts was inhibited by phenothiazine antagonists.¹⁰³ The inhibition site was located on the diphenyl carbazide to indophenol pathway, and Ca^{2+} ions could partially protect against inhibition.

Transmembrane ferricyanide reduction of cultured carrot cells was inhibited by phenothiazine antagonists and calmidazolium.¹⁰⁴ The calcium chelator, TMB-8, was also a good inhibitor.

2. Movements

a. Stomatal

In *Commelina communis*, Ca^{2+} accelerated dark-induced stomatal closure and reduced stomatal apertures in the light.¹⁰⁵ CaM antagonists, trifluoperazine and compound 48/80, were able to promote stomatal opening at low Ca^{2+} concentrations.¹⁰⁶ High Ca^{2+} concentrations, however, overcame the effects of CaM antagonists. Inhibition of stomatal opening by abscisic acid is enhanced by Ca^{2+} and is blocked by calcium-channel blockers.¹⁰⁷ The addition of CaM antagonists also blocked the action of abscisic acid. These experimental results suggest that abscisic acid promotes Ca^{2+} entry into stomata, and the resulting Ca^{2+} -CaM complex participates in the biochemical events leading to stomatal closure. As discussed previously CaM has been detected in guard cells using immunofluorescence.⁴⁸

b. Chloroplastic

The addition of Ca^{2+} and ionophore induce chloroplast rotation in the alga *Mougeotia*.¹⁰⁸ and application of the CaM antagonists W-7 and W-13 diminished rotation. The antagonist analogs W-5 and W-12 were less effective as inhibitors of rotation.

c. Algal

At moderate light intensities untreated cells of *Chlamydomonas reinhardtii* swam toward the light source (phototaxis), while cells treated with the CaM antagonist, chlorpromazine, swam away from the light source.¹⁰⁹ Since high light intensity can also induce reverse phototaxis, it was suggested that light and chlorpromazine both affect an intracellular mediator of the phototactic response.

3. Hormonal Action, Gravitropism, and Enzyme Secretion

The CaM antagonist, chlorpromazine, inhibited the gravitropic response of intact or decapitated coleoptiles of oat seedlings.¹¹⁰ The overall growth rate was not affected.

A number of CaM antagonists inhibited the naphthaleneacetic acid (auxin)-induced growth of coleoptile segments from oat.¹¹¹ The antagonists had little effect on the basal growth rate. Antagonist-induced amino acid release was not related to inhibition of auxin-induced growth.

Several CaM antagonists interfered with cytokinin-induced betacyanin synthesis in *Amaranthus tricolor*,¹¹² the auxin-dependent increase in wheat coleoptile segments, and gibberellic acid-dependent induction of α -amylase synthesis in barley aleurone layers.¹¹³

The CaM antagonist W-7 inhibited secretion of α -amylase from scutellar epithelial cells of rice.¹¹⁴ The synthesis of α -amylase was less sensitive to W-7 at low Ca^{2+} concentrations. Calcium ion promoted the release of peroxidases from sugar beet cells in liquid cultures.¹¹⁵ Treatment of the cells with the CaM antagonist, 2-chloro-10-(3-aminopropyl)-phenothiazine, inhibited the Ca^{2+} -stimulated release of peroxidases.

4. Miscellaneous

a. Protoplast Fusion

The addition of Ca^{2+} , particularly with the addition of the ionophore A 23187, increased the fusion potential of protoplasts from *Daucus carota*.¹¹⁶ Several CaM antagonists were potent inhibitors of protoplast fusion.

b. Senescence

Treatment of cut pea foliage with Ca^{2+} promoted lateral phase separation of the bulk lipids in microsomal membranes.¹¹⁷ Addition of the CaM antagonist, fluphenazine, delayed this process. It was suggested that senescence is mediated by the activation of phospholipase by Ca^{2+} -CaM. This suggestion is consistent with the observed stimulation by Ca^{2+} -CaM of phospholipase activity in potato leaves.⁸⁸

c. Budding and Germination

The CaM antagonists, trifluoperazine and chlorpromazine, inhibited bud formation in *Funaria hygrometrica*.¹¹⁹ CaM antagonists did not affect nuclear migration, but inhibited mitosis in the target cell.

Externally added Ca^{2+} was required for the light-induced germination of the spores of *Onoclea sensibilis*.¹²⁰ CaM antagonists reversibly inhibited light-induced germination but had no effect upon dark germination. In contrast to the above results, CaM antagonists inhibited the nuclear migration that occurs about 1 day after irradiation.

d. Triterpenoid Biosynthesis

A number of CaM antagonists inhibited the biosynthesis of triterpenols and their fatty

acid esters in a centrifuged pellet fraction of tapped latex.¹¹⁸ Inhibition was observed in 3-hr assays, probably ruling out effects on enzyme biosynthesis or degradation. Various chlorinated phenoxyamines were also tested as inhibitors of triterpenoid biosynthesis. The best inhibitor, the pentachlorinated compound, was also the best CaM antagonist.

A latex supernatant fraction containing endogenous triterpenes could absorb and thereby block the action of CaM antagonists.⁴² Terpenoid biosynthesis in a chlorpromazine-inhibited pellet fraction could be partially restored by the addition of latex supernatant.

VI. CONCLUSION

It is now certain that CaM is an important component of plant metabolism. Surely, future studies will reveal additional CaM-regulated enzymes, and this knowledge will enhance our understanding of the function of this protein. CaM directs Ca^{2+} signals to promote specific biochemical events. The importance of these biochemical events to the plant cell is not constant, but varies depending on cell age and environmental factors. Modulation of CaM levels in various cellular compartments during growth, development, and in response to a changing environment undoubtedly provides a component of metabolic regulation. However, the difficulties involved in determining CaM levels or in determining where CaM is localized in the plant cell retard progress in our understanding of this important area. The development of better techniques for detecting and quantitating CaM would be of great benefit.

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REFERENCES

1. Anderson, J. M. and Cormier, M. J., Calcium-dependent regulator of NAD kinase in higher plants. *Biochem. Biophys. Res. Commun.* 84, 595. 1978.
2. Muto, S. and Miyachi, S., Properties of a protein activator of NAD kinase from plants. *Plant Physiol.* 59, 55. 1977.
3. Wang, J. H. and Waisman, D. M., Calmodulin and its role in the second-messenger system. in *Current Topics in Cellular Regulation*, Vol. 15. Horecker, B. L. and Stadtman, E. R., Eds., Academic Press, New York, 1978, 47.
4. Marmé, D., The role of Ca^{2+} and calmodulin plants. *What's New in Plant Physiology*, 13, 37. 1982.
5. Cormier, M. J., Jarrett, H. W., and Charbonneau, H., Role of Ca^{2+} -calmodulin in metabolic regulation in plants. in *Calmodulin and Intracellular Ca^{2+} Receptors*. Kakiachi, S., Hidaha, H., and Means, A. R., Eds., Plenum Press, New York, 1982, 125.
6. Dieter, P., Calmodulin and calmodulin-mediated processes in plants. *Plant Cell Environ.* 7, 371. 1984.
7. Marmé, D. and Dieter, P., Role of Ca^{2+} and calmodulin plants. in *Calcium and Cell Function*, Vol. 4. Cheung, W. Y., Ed., Academic Press, New York, 1983, 263.
8. Poovaiah, B. W., Role of calcium and calmodulin in plant growth and development. *Hortic. Sci.* 20, 347. 1985.
9. Anderson, J. M., Purification of plant calmodulin. *Methods Enzymol.* 102, 9. 1983.
10. Grand, R. J. A., Nairn, A. C., and Perry, S. V., The preparation of calmodulins from barley (*Hordeum* sp.) and basidiomycete fungi. *Biochem. J.* 185, 755. 1980.
11. Gitelman, S. E. and Witman, G. B., Purification of calmodulin from *Chlamydomonas*: calmodulin occurs in cell bodies and flagella. *J. Cell Biol.* 87, 764. 1980.
12. Schleicher, M., Lukas, T. J., and Watterson, D. M., Isolation and characterization of calmodulin from the motile green alga *Chlamydomonas reinhardtii*. *Arch. Biochem. Biophys.* 229, 33. 1984.
13. Leung, P. C., Taylor, W. A., Wang, J. H., and Tipton, C. L., Role of calmodulin inhibition in the mode of action of ophiobolin A. *Plant Physiol.* 77, 303. 1985.

14. Vantard M., Lambert, A.-M., De Mey, J., Picquot, P., and Van Eldik, L. J., Characterization and immunocytochemical distribution of calmodulin in higher plant endosperm cells: localization in the mitotic apparatus, *J. Cell Biol.*, 101, 488, 1985.
15. Piazza, G. J., Saggese, E. J., and Thompson, M. P., Regulation of terpenoid biosynthesis in tapped latex, in *The Metabolism, Structure, and Function of Plant Lipids*. Stumpf, P. K., Mudd, J. B., and Ness, W. D., Eds., Plenum Press, New York, 1987, 111.
16. Wagner, G., Valentin, P., Dieter, P., and Marmé, D., Identification of calmodulin in the green alga *Mougeotia* and its possible function in chloroplast reorientation movement, *Planta*, 162, 62, 1984.
17. Harmon, A. C., Jarrett, H. W., and Cormier, M. J., An enzymatic assay for calmodulins based on plant NAD kinase activity, *Anal. Biochem.*, 141, 168, 1984.
18. Biro, R. L., Daye, S., Serlin, B. S., Terry, M. E., Datta, N., Sopory, S. K., and Roux, S. J., Characterization of oat calmodulin and radioimmunoassay of its subcellular distribution, *Plant Physiol.*, 75, 382, 1984.
19. Wick, S. M., Muto, S., and Duniec, J., Double immunofluorescence labeling of calmodulin and tubulin in dividing plant cells, *Protoplasma*, 126, 198, 1985.
20. Anderson, J. M., Charbonneau, H., Jones, H. P., McCann, R. O., and Cormier, M. J., Characterization of the plant nicotinamide adenine dinucleotide kinase activator protein and its identification as calmodulin, *Biochemistry*, 19, 3113, 1980.
21. Charbonneau, H. and Cormier, M. J., Purification of plant calmodulin by fluphenazine-Sepharose affinity chromatography, *Biochem. Biophys. Res. Commun.*, 90, 1039, 1979.
22. Harper, J. F., Antigenic structure of calmodulin: production and characterization of antisera specific for plant calmodulins of Ca^{2+} replete vs. Ca^{2+} -free calmodulins, *J. Cyclic Nucleotide Protein Phosphorylation Res.*, 9, 3, 1983.
23. Van Eldik, L. J., Grossman, A. R., Iverson, D. B., and Watterson, D. M., Isolation and characterization of calmodulin from spinach leaves and *in vitro* translation mixtures, *Proc. Natl. Acad. Sci. U.S.A.*, 77, 1912, 1980.
24. Watterson, D. M., Iverson, D. B., and Van Eldik, L. J., Spinach calmodulin: isolation, characterization, and comparison with vertebrate calmodulins, *Biochemistry*, 19, 5762, 1980.
25. Oláh, Z., Bérczi, A., and Erdei, L., Benzylaminopurine-induced coupling between calmodulin and Ca^{2+} -ATPase in wheat root microsomal membranes, *FEBS Lett.*, 154, 395, 1983.
26. Dieter, P. and Marmé, D., Calmodulin-activated plant microsomal Ca^{2+} uptake and purification of plant NAD kinase and other proteins by calmodulin-sepharose chromatography, *Ann. N.Y. Acad. Sci.*, 356, 371, 1980.
27. Schleicher, M., Lukas, T. J., and Watterson, D. M., Further characterization of calmodulin from the monocotyledon barley (*Hordeum vulgare*), *Plant Physiol.*, 73, 666, 1983.
28. Lukas, T. J., Wiggins, M. E., and Watterson, D. M., Amino acid sequence of a novel calmodulin from the unicellular alga *Chlamydomonas*, *Plant Physiol.*, 78, 477, 1985.
29. Lukas, T. J., Iverson, D. B., Schleicher, M., and Watterson, D. M., Structural characterization of a higher plant calmodulin, *Plant Physiol.*, 75, 788, 1984.
30. Roberts, D. M., Crea, R., Malecha, M., Alvarado-Urbina, G., Chiarello, R. H., and Watterson, D. M., Chemical synthesis and expression of a calmodulin gene designed for site-specific mutagenesis, *Biochemistry*, 23, 440, 1984.
31. Roberts, D. M., Burgess, W. H., and Watterson, D. M., Comparison of the NAD kinase and myosin light chain kinase activities of vertebrate, higher plant, and algal calmodulins, *Plant Physiol.*, 75, 796, 1984.
32. Dieter, P., Cox, J. A., and Marmé, D., Calcium-binding and its effect on circular dichroism of plant calmodulin, *Planta*, 160, 210, 1985.
33. Yoshida, M., Minoura, O., and Yagi, K., Divalent cation binding to wheat germ calmodulin, *J. Biochem.*, 94, 1925, 1983.
34. Muto, M. and Miyachi, S., Production of antibody against spinach calmodulin and its application to radioimmunoassay for plant calmodulin, *Z. Pflanzenphysiol.*, 114, 421, 1984.
35. Roberts, D. M., Rowe, P. M., Siegel, F. L., Lukas, T. J., and Watterson, D. M., Trimethyllysine and protein function. Effect of methylation and mutagenesis of lysine 115 of calmodulin and NAD kinase activation, *J. Biol. Chem.*, 261, 1491, 1986.
36. Paliyath, G. and Poovaiah, B. W., Calmodulin inhibition in senescing apples and its physiological and pharmacological significance, *Proc. Natl. Acad. Sci. U.S.A.*, 81, 2065, 1984.
37. Paliyath, G. and Poovaiah, B. W., Identification of naturally occurring calmodulin inhibitors in plants and their effects on calcium- and calmodulin-promoted protein phosphorylation, *Plant Cell Physiol.*, 26, 201, 1985.
38. Chafouleas, J. G., Dedman, J. R., Mungaal, R. P., and Means, A. R., Calmodulin, development and application of a sensitive radioimmunoassay, *J. Biol. Chem.*, 254, 10262, 1979.

39. Van Eldik, L. J., Piperno, G., and Watterson, D. M., Similarities and dissimilarities between calmodulin and a *Chlamydomonas* flagellar protein. *Proc. Natl. Acad. Sci. U.S.A.*, 77, 4779, 1980.
40. Schleicher, M., Iverson, D. B., Van Eldik, L. J., and Watterson, D. M., Calmodulin. in *The Cytoskeleton in Plant Growth and Development*, Lloyd, C. W., Ed., Academic Press, New York, 1982, 85.
41. Cerana, R., Bonetti, A., and Lado, P., Uptake and accumulation at membrane level of the calmodulin antagonist chlorpromazine in higher plants. *Plant Sci. Lett.*, 30, 267, 1983.
42. Piazza, G. J., Saggese, E. J., and Spletzer, K. M., Triterpene biosynthesis in the latex of *Euphorbia luthyris*: calmodulin antagonists are ineffective in whole latex. *Plant Physiol.*, 83, 181, 1987.
43. Polya, G. M. and Micucci, V., Interaction of wheat germ Ca^{2+} -dependent protein kinases with calmodulin antagonists and polyamines. *Plant Physiol.*, 79, 968, 1985.
44. Muto, S., Distribution of calmodulin within wheat leaf cells. *FEBS. Lett.*, 147, 161, 1982.
45. Picton, J. M. and Steer, M. W., The effects of ruthenium red, lanthanum, fluorescein isothiocyanate and trifluoperazine on vesicle transport, vesicle fusion and tip extension in pollen tubes. *Planta*, 163, 20, 1985.
46. Hausser, I., Herth, W., and Reiss, H.-D., Calmodulin in tip-growing plant cells visualized by fluorescing calmodulin-binding phenothiazines. *Planta*, 162, 33, 1984.
47. Thompson, M. P. and Brower, D. P., Immunofluorescent localization of microtubules in pollen tubes of *Lilium*. *Fed. Proc. Fed. Am. Soc. Exp. Biol.*, 44, 6076, 1985.
48. Thompson, M. P. and Brower, D. P., Immunofluorescent localization of calmodulin and tubulin in guard cells of higher plants. *Fed. Proc. Fed. Am. Soc. Exp. Biol.*, 43, 3477, 1984.
49. Matsumoto, H., Tanigawa, M., and Yamaya, T., Calmodulin-like activity associated with chromatin from pea buds. *Plant Cell Physiol.*, 24, 593, 1983.
50. Jarrett, H. W., Brown, C. J., Black, C. C., and Cormier, M. J., Evidence that calmodulin is in the chloroplast of peas and serves a regulatory role in photosynthesis. *J. Biol. Chem.*, 257, 13795, 1982.
51. Jones, H. and Halliwell, B., Calcium ion and calmodulin in pea chloroplasts as a function of plant age. *Photochem. Photobiophys.*, 7, 293, 1984.
52. Roberts, D. M., Zielinski, R. E., Schleicher, M., and Watterson, D. M., Analysis of suborganellar fractions from spinach and pea chloroplasts for calmodulin-binding proteins. *J. Cell Biol.*, 97, 1644, 1983.
53. Fukumoto, M. and Nagai, K., Effects of calmodulin antagonists on the mitochondrial and microsomal Ca^{2+} uptake in apple fruit. *Plant Cell Physiol.*, 23, 1435, 1982.
54. Dieter, P. and Marmé D., Calmodulin activation of plant microsomal Ca^{2+} uptake. *Proc. Natl. Acad. Sci. U.S.A.*, 77, 7311, 1980.
55. Dieter, P. and Marmé D., Far-red light irradiation of intact corn seedlings affects mitochondrial and calmodulin-dependent microsomal Ca^{2+} transport. *Biochem. Biophys. Res. Commun.*, 101, 749, 1981.
56. Dieter, P. and Marmé D., The effect of calmodulin and far-red light on the kinetic properties of the mitochondrial and microsomal calcium-ion transport systems from corn. *Planta*, 159, 277, 1983.
57. Stosic, V., Penel, C., Marmé D., and Greppin, H., Distribution of calmodulin-stimulated Ca^{2+} -transport into membrane vesicles from green spinach leaves. *Plant Physiol.*, 72, 1136, 1983.
58. Schumaker, K. S. and Sze, H., A Ca^{2+}/H^{+} antiport system driven by the proton electrochemical gradient of a tonoplast H^{+} -ATPase from oat roots. *Plant Physiol.*, 79, 1111, 1985.
59. Dieter, P. and Marmé D., A calmodulin-dependent, microsomal ATPase from corn (*Zea mays* L.). *FEBS. Lett.*, 125, 245, 1981.
60. Nguyen, T. D. and Siegenthaler, P.-A., Proteins and polypeptides of envelope membranes from spinach chloroplasts. Properties of a membrane-bound ATPase. *FEBS. Lett.*, 164, 67, 1983.
61. Nguyen, T. D. and Siegenthaler, P.-A., Purification and some properties of Mg^{2+} -, Ca^{2+} - and calmodulin-stimulated ATPase from spinach chloroplast envelope membranes. *Biochim. Biophys. Acta*, 840, 99, 1985.
62. Matsumoto, H., Yamaya, T., and Tanigawa, M., Activation of ATPase activity in the chromatin fraction of pea nuclei by calmodulin. *Plant Cell Physiol.*, 25, 191, 1984.
63. Muto, S., Miyachi, S., Usuda, H., Edwards, G. E., and Bassham, J. A., Light-induced conversion of nicotinamide adenine dinucleotide to nicotinamide adenine dinucleotide phosphate in higher plant leaves. *Plant Physiol.*, 68, 324, 1981.
64. Muto, S., Kinetic nature of calmodulin-dependent NAD kinase from pea seedlings. *Z. Pflanzenphysiol.*, 109, 385, 1983.
65. Dieter, P. and Marmé D., Partial purification of plant NAD kinase by calmodulin-Sepharose affinity chromatography. *Cell Calcium*, 1, 279, 1980.
66. Jarrett, H. W., Charbonneau, H., Anderson, J. M., McCann, R. O., and Cormier, M. J., Plant calmodulin and the regulation of NAD kinase. *Ann. N.Y. Acad. Sci.*, 356, 119, 1980.
67. Jarrett, H. W., Da Silva, T., and Cormier, M. J., Calmodulin activation of NAD kinase and its role in the metabolic regulation of plants, in *Metals and Micronutrients: Uptake and Utilization of Metals by Plants*, Robb, D. A. and Pierpoint, W. S., Eds., Academic Press, London, 1983, 205.
68. Cormier, M. J., Charbonneau, H., and Jarrett, J. W., Plant and fungal calmodulin: Ca^{2+} -dependent regulation of plant NAD kinase. *Cell Calcium*, 2, 313, 1981.

69. Kincaid, R. L., Stith-Coleman, I. E., and Vaughan, M., Proteolytic activation of calmodulin-dependent cyclic nucleotide phosphodiesterase. *J. Biol. Chem.*, 260, 9009, 1985.
70. Simon, P., Dieter, P., Bonzon, M., Greppin, H., and Marmé, D., Calmodulin-dependent and independent NAD kinase activities from cytoplasmic and chloroplastic fractions of spinach (*Spinacia oleracea* L.). *Plant Cell Rep.*, 1, 119, 1982.
71. Simon, P., Bonzon, M., Greppin, H., and Marmé, D., Subchloroplastic localization of NAD kinase activity: evidence for a Ca^{2+} -calmodulin-dependent activity at the envelope and for a Ca^{2+} -calmodulin-independent activity in the stroma of pea chloroplasts. *FEBS. Lett.*, 167, 332, 1984.
72. Dieter, P. and Marmé, D., A Ca^{2+} -calmodulin-dependent NAD kinase from corn is located in the outer mitochondrial membrane. *J. Biol. Chem.*, 259, 184, 1984.
73. Sauer, A. and Robinson, D. G., Calmodulin dependent NAD-kinase is associated with both the outer and inner mitochondrial membranes in maize roots. *Planta*, 166, 227, 1985.
74. Hetherington, A and Trewavas, A., Calcium-dependent protein kinase in pea shoot membranes. *FEBS. Lett.*, 145, 67, 1982.
75. Hetherington, A. M. and Trewavas, A. J., Activation of a pea membrane protein kinase by calcium ions. *Planta*, 161, 409, 1984.
76. Blowers, D. P., Hetherington, A., and Trewavas, A., Isolation of plasma-membrane-bound calcium/calmodulin-regulated protein kinase from pea using western blotting. *Planta*, 166, 208, 1985.
77. Polya, G. M. and Davies, J. R., Resolution of Ca^{2+} -calmodulin-activated protein kinase from wheat germ. *FEBS. Lett.*, 150, 167, 1982.
78. Polya, G. M., Calmodulin stimulates a Ca^{2+} -independent plant protein kinase. *Biochem. Int.*, 7, 339, 1983.
79. Polya, G. M., Davies, J. R., and Micucci, V., Properties of a calmodulin-activated Ca^{2+} -dependent protein kinase from wheat germ. *Biochim. Biophys. Acta*, 761, 1, 1983.
80. Polya, G. M. and Micucci, V., Partial purification and characterization of a second calmodulin-activated Ca^{2+} -dependent protein kinase from wheat germ. *Biochim. Biophys. Acta*, 785, 68, 1984.
81. Veluthambi, K. and Poovaiah, B. W., Calcium- and calmodulin-regulated phosphorylation of soluble and membrane proteins from corn coleoptiles. *Plant Physiol.*, 76, 359, 1984.
82. Salimath, B. P. and Marmé D., Protein phosphorylation and its regulation by calcium and calmodulin in membrane fractions from zucchini hypocotyls. *Planta*, 158, 560, 1983.
83. Teulieres, C., Alibert, G., and Ranjeva, R., Reversible phosphorylation of tonoplast proteins involves tonoplast-bound calcium-calmodulin-dependent protein kinases(s) and protein phosphatase(s). *Plant Cell Rep.*, 4, 199, 1985.
84. Refeno, G., Ranjeva, R., and Boudet, A. M., Modulation of quinate: NAD⁺ oxidoreductase activity through reversible phosphorylation in carrot cell suspension. *Planta*, 154, 193, 1982.
85. Ranjeva, R., Refeno, G., Boudet, A. M., and Marmé, D., Activation of plant quinate: NAD⁺ 3-oxidoreductase by Ca^{2+} and calmodulin. *Proc. Natl. Acad. Sci. U.S.A.*, 80, 5222, 1983.
86. Ranjeva, R., Graziana, A., Ranty, B., Cavalie, G., and Boudet, A. M., Phosphorylation of proteins in plants: a step in the integration of extra and intracellular stimuli. *Physiol. Veg.*, 22, 365, 1984.
87. Kauss, H., Volume regulation in *Pterioochromonas*, involvement of calmodulin in the Ca^{2+} -stimulated activation of isofluidoside-phosphate synthase. *Plant Physiol.*, 71, 169, 1983.
88. Moreau, R. A. and Isett, T. F., Autolysis of membrane lipids in potato leaf homogenates: effect of calmodulin and calmodulin antagonists. *Plant Sci.*, 40, 95, 1985.
89. Moreau, R. A., Isett, T. F., and Piazza, G. J., Dibucaine, chlorpromazine, and detergents mediate membrane breakdown in potato tuber homogenates. *Phytochemistry*, 24, 2555, 1985.
90. Sane, P. V., Kochhar, S., Kumar, N., and Kochhar, V. K., Activation of plant aspartate kinase by calcium and calmodulin-like factor from plants. *FEBS. Lett.*, 175, 238, 1984.
91. Bonner, P. L. R., Hetherington, A. M., and Lea, P. J., Lysine-sensitive plant aspartate kinase is not regulated by calcium or calmodulin. *FEBS. Lett.*, 195, 119, 1986.
92. Kochhar, S., Kochhar, V. K., and Sane, P. V., Isolation, characterization and regulation of isoenzymes of aspartate kinase differentially sensitive to calmodulin from spinach leaves. *Biochim. Biophys. Acta*, 880, 220, 1986.
93. Anderson, K. W. and Call, R. J., Inhibition of skeletal muscle sarcoplasmic reticulum CaATPase activity by calmidazolium. *J. Biol. Chem.*, 259, 11487, 1984.
94. Itoh, H. and Hidaka, H., Direct interaction of calmodulin antagonists with Ca^{2+} /calmodulin-dependent cyclic nucleotide phosphodiesterase. *J. Biochem.*, 96, 1721, 1984.
95. Wise, B. C., Glass, D. B., Jen Chou, C.-H., Raynor, R. L., Kato, N., Schatzman, R. C., Turner, R. S., Kibler, R. F., and Kuo, J. F., Phospholipid-sensitive Ca^{2+} -dependent protein kinase from heart. II. Substrate specificity and inhibition by various agents. *J. Biol. Chem.*, 257, 8489, 1982.
96. Schatzman, R. C., Wise, B. C., and Kuo, J. F., Phospholipid-sensitive calcium-dependent protein kinase: inhibition by antipsychotic drugs. *Biochem. Biophys. Res. Commun.*, 98, 669, 1981.

97. Mori, T., Takai, Y., Minakuchi, R., Yu, B., and Nishizuka, Y., Inhibitory action of chlorpromazine, dibucaine, and other phospholipid-interacting drugs on calcium-activated phospholipid-dependent protein kinase. *J. Biol. Chem.*, 255, 8378, 1980.
98. Luxnat, M., Müller, H.-J., and Galla, H.-J., Membrane solubility of chlorpromazine. *Biochem. J.*, 244, 1023, 1984.
99. Landry, Y., Amellal, M., and Ruckstuhl, M., Can calmodulin inhibitors be used to probe calmodulin effects?. *Biochem. Pharmacol.*, 30, 2031, 1981.
100. Leung, P. C., Taylor, W. A., Wang, J. H., and Tipton, C. L., Ophiobolin A: a natural product inhibitor of calmodulin. *J. Biol. Chem.*, 259, 2742, 1984.
101. Burris, J. E. and Black, C. C., Jr., Inhibition of coral and algal photosynthesis by Ca^{2+} -antagonist phenothiazine drugs. *Plant Physiol.*, 71, 712, 1983.
102. Brown, L. M. and Vanlerberghe, G. C., Structure-activity relations for inhibition of photosynthesis by calmodulin antagonists. *Plant Sci.*, 41, 199, 1985.
103. Barr, R., Troxel, K. S., and Crane, F. L., Calmodulin antagonists inhibit electron transport in photosystem II of spinach chloroplasts. *Biochem. Biophys. Res. Commun.*, 104, 1182, 1982.
104. Barr, R., Stone, B., Craig, T. A., and Crane, F. L., Evidence for Ca^{2+} -calmodulin control of trans-plasmalemma electron transport in carrot cells. *Biochem. Biophys. Res. Commun.*, 126, 262, 1985.
105. Schwartz, A., Role of Ca^{2+} and EGTA on stomatal movements in *Commelina communis* L. *Plant Physiol.*, 79, 1003, 1985.
106. Donovan, N., Martin, S., and Donkin, M. E., Calmodulin binding drugs trifluoperazine and compound 48/80 modify stomatal responses of *Commelina communis* L. *J. Plant Physiol.*, 118, 177, 1985.
107. De Silva, D. L. R., Cox, R. C., Hetherington, A. M., and Mansfield, T. A., Suggested involvement of calcium and calmodulin in the responses of stomata to abscisic acid. *New Phytol.*, 101, 555, 1985.
108. Serlin, B. S. and Roux, S. J., Modulation of chloroplast movement in the green alga *Mougeotia* by the Ca^{2+} ionophore A23187 and by calmodulin antagonists. *Proc. Natl. Acad. Sci. U.S.A.*, 81, 6368, 1984.
109. Hirschberg, R. and Hutchinson, W., Effect of chlorpromazine on phototactic behavior in *Chlamydomonas*. *Can. J. Microbiol.*, 26, 265, 1980.
110. Biro, R. L., Hale, C. C., II, Wiegand, O. F., and Roux, S. J., Effects of chlorpromazine on gravitropism in *Avana* coleoptiles. *Ann. Bot. (London)*, 50, 735, 1982.
111. Raghothama, K. G., Mizrahi, Y., and Poovaiah, B. W., Effect of calmodulin antagonists on auxin-induced elongation. *Plant Physiol.*, 79, 28, 1985.
112. Elliott, D. C., Inhibition of cytokinin-regulated responses by calmodulin-binding compounds. *Plant Physiol.*, 72, 215, 1983.
113. Elliott, D. C., Batchelor, S. M., Cassar, R. A., and Marinos, N. G., Calmodulin-binding drugs affect responses to cytokinin, auxin and gibberellic acid. *Plant Physiol.*, 72, 219, 1983.
114. Mitsui, T., Christeller, J. T., Hara-Nishimura, I., and Akazawa, T., Possible roles of calcium and calmodulin in the biosynthesis and secretion of α -amylase in rice seed scutellar epithelium. *Plant Physiol.*, 75, 21, 1984.
115. Kevers, C., Sticher, L., Penel, C., Greppin, H., and Gaspar, Th., Calcium-controlled peroxidase secretion by sugarbeet cell suspensions in relation to habituation. *Plant Growth Reg.*, 1, 61, 1982.
116. Grimes, H. D. and Boss, W. F., Intracellular calcium and calmodulin involvement in protoplast fusion. *Plant Physiol.*, 79, 253, 1985.
117. Leshem, Y. Y., Skidhara, S., and Thompson, J. E., Involvement of calcium and calmodulin in membrane deterioration during senescence in pea foliage. *Plant Physiol.*, 75, 329, 1984.
118. Piazza, G. J., Saggese, E. J., and Spletzer, K. M., Triterpene biosyntheses in the latex of *Euphorbia lathyris*: effect of calmodulin antagonists and chlorinated phenoxy compounds. *Plant Physiol.*, 83, 177, 1987.
119. Saunders, M. J. and Hepler, P. K., Calcium antagonists and calcium inhibitors block cytokinin-induced bud formation in *Funaria*. *Dev. Biol.*, 99, 41, 1983.
120. Wayne, R. and Hepler, P. K., The role of calcium ions in phytochrome-mediated germination of spores of *Onoclea sensibilis* L. *Planta*, 160, 12, 1984.