

SYMPOSIUM: CALCIUM-BINDING PROTEINS

Calcium-Binding Proteins in Fungi and Higher Plants

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ABSTRACT

Calcium has long been known to be required for many vital processes in fungi and plants. High levels of calcium are found in cell walls, vacuoles, and most organelles. In contrast, very low levels of calcium are present in the cytosol of fungal and plant cells. The most recent evidence indicates that calcium is a true second messenger in fungi and plants. Because cyclic AMP does not appear to be a second messenger in plants, calcium is the only known second messenger. Calcium-binding proteins are involved in the events that accompany the action of calcium as a second messenger; three types have been identified in fungi and plants. The first group includes several proteins that bind $^{45}\text{Ca}^{2+}$ and are not known to have any enzymatic activity. A second type includes the many enzymes from fungi and plants stimulated by millimolar levels of calcium. The third type of calcium-binding protein, calmodulin, responds to micromolar levels of Ca^{2+} by binding to certain enzymes and stimulating them. Calmodulin has been detected in every eukaryote thus far examined. The amino acid composition of several fungal and plant calmodulins have been elucidated and found to be very similar to calmodulin from animals. Eight enzymes from fungi and plants have been reported to be regulated either directly or indirectly by calmodulin. Calmodulin antagonists have been used to study the possible involvement of calmodulin in many cellular processes in fungi and plants. Recently, several endogenous calmodulin antagonists have

been identified in fungi and plants. The physiological significance of these compounds is currently being evaluated.

INTRODUCTION

Calcium is required for the growth of all higher plants and almost all fungi. Strontium satisfies the calcium requirement in *Allomyces arbuscula* and certain other fungi (46). Some of the processes in fungi that require Ca^{2+} include germination and conidiation of fungal spores, sexual reproduction, and nutrient uptake (46). In higher plants, Ca^{2+} is required for seed germination, root growth, protoplasmic streaming, pollen tip growth, secretion of cell wall material, and many other processes (10). Total calcium concentrations in fungal and plant tissues range from 3 to 26 mM (10, 46). Normal plant growth requires 1 to 5 mM Ca^{2+} in the soil or in hydroponic nutrient solution. Lower concentrations cause serious deficiency symptoms such as chlorosis (yellowing) and malformation of younger leaves, browning of roots and inhibition of their growth, and excessive rigidity of cell walls (10). Calcium concentrations of .1 to 5 mM are required in most fungal growth media (46). Much of the calcium (1 to 10 mM) in fungi and plants is associated with the extracellular milieu (mostly in cell walls) (18). Within plant and fungal cells the highest levels (>10 mM) of Ca^{2+} usually occur in the vacuole. In mature plant cells the central vacuole can take up more than 90% of the cell volume, representing the major proportion of the total intracellular Ca^{2+} . Most other organelles contain 1 to 10 mM Ca^{2+} . In contrast, the cytosol contains very low concentrations of Ca^{2+} (about .1 μM).

The purpose of this paper is to discuss recent experimental evidence for the involvement of various calcium-binding proteins (CaBP) during the growth and development of fungi and plants. To do this it will first be necessary to describe the events involved in the regulation of

extracellular and intracellular calcium. The most exciting aspect of this research involves the recent discovery that calcium serves as a second messenger in fungi and plants, as it also does in animals. Because most readers are likely to be more familiar with calcium metabolism in animals, this paper will strive to identify those aspects of calcium metabolism that are similar in fungi and plants and to describe in more detail those that are unique to fungi and plants. Due to space limitations and the fact that some excellent reviews of certain aspects of this topic are already available, a comprehensive literature review will not be attempted. Instead, a more general description of the properties of CaBP in fungi and plants will be presented with the reader being referred to appropriate review articles for in-depth coverage of specific topics. Several excellent reviews on the role of calcium and calmodulin (CaM) in higher plants have recently been published (11, 18, 30, 48, 53). Two recent reviews (31, G. J. Piazza, 1986, unpublished) provide a more comprehensive description of the properties of plant calmodulin. Because no previous review has dealt with the topic of plant CaBP other than CaM, this paper will attempt to fill that void. Pitt and Ugalde (46) have published a good review of calcium metabolism in fungi. Unfortunately, there are not yet any reviews that cover the topic of CaBP in fungi.

Regulation of Cellular Calcium

Because the concentration of Ca^{2+} in the cytosol is very low (about $.1 \mu\text{M}$) and the concentrations in the organelles and cell wall are high (1 to 10 mM), cells must have efficient mechanisms to enable them to maintain these huge concentration gradients. Indeed, some cellular membranes have a 100,000-fold higher concentration of Ca^{2+} on one side than on the other. In recent years the ATP-dependent translocation of Ca^{2+} has been demonstrated to occur in the membranes of several organelles (30). A plasma membrane Ca^{2+} -translocating ATPase has been shown to pump Ca^{2+} out of the plant cell (13). Calcium transport (influx) has been demonstrated with isolated plastids (i.e., chloroplasts), mitochondria, and vacuoles (30). Calmodulin stimulates the rate of calcium uptake by plant microsomal membranes (30) and will be discussed in more detail in a later section.

In 1969, Sutherland (59) first postulated that cyclic AMP (cAMP) served as a second messenger in mammalian cells. Subsequent studies have verified that cAMP indeed is a second messenger in animals, fungi, and protozoans. However, despite repeated experimental attempts, no evidence has yet been found that cAMP serves as a second messenger in plants. In recent years another second messenger, calcium, has been suggested to have an important regulatory role in animals, fungi, protozoans, and also plants. Experimental evidence from many types of observations have supported this model.

Primary signal \rightarrow rapid increase in cytosolic calcium concentration \rightarrow response

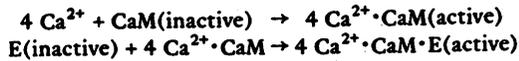
In animals, hormones and prostaglandins are thought to be primary signals that can evoke rapid changes in cytosolic calcium concentration (31). In plants there is now evidence for five unique primary signals. These include light, gravity, growth regulators, inositol triphosphate, and fungal elicitors (14, 31).

Types of Calcium-Binding Proteins in Fungi and Plants

The first group of CaBP to be discovered contained the many plant enzymes that are stimulated by Ca^{2+} . These include phospholipase D, ATPase, α -amylase, arginine kinase, adenyl kinase, apyrase, alkaline pyrophosphatase, fructose biphosphatase, glutamate dehydrogenase, glyoxysomal alkaline lipase, alkaline lipoxygenase, and several phospholipid-synthesizing enzymes (10, 25). Although all these enzymes are stimulated by Ca^{2+} , the extent of stimulation is quite variable. Some of them (such as phospholipase D) can actually be called Ca^{2+} -dependent enzymes but others are only slightly stimulated by Ca^{2+} . Most of these enzymes are stimulated to maximal levels by millimolar concentrations of Ca^{2+} . With some of these enzymes other divalent cations can replace Ca^{2+} and cause comparable stimulation.

The second group of CaBP consists of those sometimes designated "calcium-modulated proteins". This group includes only CaM in fungi and plants but in animals includes CaM plus several other CaM-like proteins such as S-100,

parvalbumin, and troponin-C (L. J. Van Eldik and D. M. Roberts, 1986, unpublished). These proteins have a very high affinity for Ca^{2+} and are often activated by micromolar levels of Ca^{2+} . Small increases in calcium concentration can cause these CaBP to stimulate specific target enzymes (E) by the following scheme:



This mechanism allows an organism to respond rapidly to changes in the concentrations of Ca^{2+} and thus explains more clearly how Ca^{2+} can serve as a second messenger. The molecular properties of plant and fungal calmodulin will be covered in detail in a later section.

The third group of CaBP include those that bind Ca^{2+} but do not have known enzyme activities. Most of these studies have been conducted by measuring the binding of $^{45}\text{Ca}^{2+}$. The plant lectin, Concanavalin A, has separate binding sites for Ca^{2+} and Mn^{2+} (25, 31). A CaBP with a molecular weight of 16,600 has been purified from wheat flour (15). Although this wheat protein is about the same size as CaM, the amino acid composition is clearly different (15). A light-harvesting pigment protein with a molecular weight of 33,000 was identified in isolated chloroplasts (9). When carrot suspension cells were grown in the dark, the regulatory subunit (M_r 60,000) of quinate oxidoreductase was reported to bind Ca^{2+} (16). A CaBP of M_r 100,000 was reported in sieve tubes of *Cucurbita* and it was proposed that this protein may be involved in the transport of Ca^{2+} in the phloem (34). Several membrane-bound CaBP were identified in various organelles of corn coleoptiles (5).

Calmodulin in Fungi and Plants

The first experimental evidence of CaM in plants appeared in 1978 (which was also the year that the name CaM was used to describe the protein activator of phosphodiesterase in animals), when Anderson and Cormier (2) demonstrated that the NAD kinase activity of peas could be regulated either by an endogenous protein activator or by the previously identified phosphodiesterase activator from animal tissue. They demonstrated that the activator proteins

from plant and animal tissues appeared to have identical isoelectric pH, heat stability, Stokes radii, and ability to bind troponin I. In 1980, convincing evidence was presented to confirm that the protein activator of NAD kinase was, in fact, CaM (1).

Calmodulin has been purified and analyzed from a number of fungal and plant tissues (G. J. Piazza, 1986, unpublished). Although the amino acid composition of CaM is remarkably similar among all of the eukaryotes so far examined, there are several interesting chemical differences between CaM from fungal and plant tissues and those from animal sources (Table 1). The common features of all CaM examined so far are the absence of tryptophan and the presence of very high levels of glutamic and aspartic acids. Although all CaM thus far examined from animal sources have each contained one trimethyllysine, one fungal CaM and one plant CaM (those from *Neurospora* and *Chlamydomonas*, as shown in Table 1) have been reported to lack this posttranslationally modified amino acid. Although cysteine is absent from the CaM in animals, fungi, and algae, the CaM in each of the higher (vascular) plant species thus far examined contains one cysteine (G. J. Piazza, 1986, unpublished). The levels of tyrosine and proline are variable among the fungal and plant CaM reported to date (Table 1).

The amino acid sequences of spinach and *Chlamydomonas* CaM have been reported (28, 29). Spinach CaM has the same number of amino acids (148 residues) as that from bovine brain. The unique cysteine in spinach CaM is located at position 26, which is in one of the four calcium binding sites (28). In contrast, *Chlamydomonas* CaM has 14 more amino acid residues with a 3 amino acid extension at the amino terminal end and an 11 amino acid extension at the carboxy terminal end (29). From amino acid analysis CaM from *Achlya* has been estimated to be even larger (181 residues) (58).

In addition to the structural differences in the calmodulins from fungi, plants, and animals the degree of stimulation of certain enzymes has been shown to vary with calmodulins obtained from different organisms (50). Calmodulins from spinach and *Chlamydomonas* were about 30% less effective than chicken CaM in activating chicken gizzard myosin light chain kinase (50). Peanut CaM was several times

TABLE 1. The amino acid composition of selected calmodulins from fungi and plants compared with bovine brain calmodulin.

Amino acid	Residues per molecule				
	Bovine brain (8)	Fungi		Plantae	
		<i>Acblya</i> (58)	<i>Neurospora</i> (8)	Spinach (66)	<i>Cblamydomonas</i> (29)
Aspartic acid	23	26	25	24	24
Threonine	12	13	9	9	12
Serine	4	9	11	4	5
Glutamic acid	27	32	26	27	28
Proline	2	4	3	2	2
Glycine	11	17	10	10	13
Alanine	11	13	8	11	13
Cysteine	0	?	0	1	0
Valine	7	7	6-7	8	8
Methionine	9	8	7	8	9
Isoleucine	8	11	7	7	6
Leucine	9	12	9	11	11
Tyrosine	2	2	1	1	1
Phenylalanine	8	11	8	9	9
Histidine	1	2	1	1	3
Trimethyllysine	1	1	0	1	0
Lysine	7	7	7-8	9	12
Tryptophan	0	?	0	0	0
Arginine	6	6	6-7	5	6
Total residues	148	181	144-147	148	162

more active than vertebrate CaM in the activation of plant NAD kinase (7). Methylation of *Cblamydomonas* CaM caused a three-fold increase in its ability to stimulate plant NAD kinase but had no effect on the stimulation of mammalian phosphodiesterase (51). Various antisera have been prepared that can distinguish between animal, plant, and algal CaM (40).

Several types of studies have been used to determine the localization of calmodulin within various types of plant cells. These include enzyme assays, radioimmunoassays, and fluorescent microscopy. One shortcoming of these studies is the widespread occurrence of endogenous CaM antagonists in fungal and plant tissues (see later section). Nevertheless, they have provided much valuable information. Immunofluorescent techniques have been used to visualize CaM in the tips of pollen tubes of *Lilium* (62); in the mitotic apparatus of dividing cells of *Haemantbus*, pea, and onion (64, 67); and in the inner aperture of guard cells of the stomata of *Lilium* (61). When radioimmunological techniques were used with pea seed-

lings, the highest levels of CaM in green seedlings were found in lateral roots and in young fully expanded leaves (40). However, when the same types of studies were performed on etiolated (dark-grown) plants the highest CaM concentration was in the part of the stem just below the shoot apex (40).

The CaM concentrations in various subcellular and extracellular fractions have also been measured in several types of plant tissues. Poovaiah (48) has estimated that the average concentration of calmodulin in most plant cells is 1 to 10 μM . In the cells of wheat leaves, 90% of CaM was in the cytosol, although the microsomes, mitochondria, and chloroplasts also contained measureable levels (38). Other studies have reported appreciable CaM in chloroplasts (22, 52) and chromatin (32). The cell-wall fraction of oat coleoptiles was reported (3) to contain the highest concentration of CaM when expressed on a total protein basis (18 to 60 μg calmodulin/mg protein). Calmodulin was also reported to be one of several proteins released from maize roots by osmotic shock

(26). In order for calcium to serve as a second messenger it would seem that CaM would need to be localized in the part of the cell where calcium concentrations would be expected to be most highly regulated, namely the cytosol. Its occurrence in other subcellular locations raises many interesting questions that have hardly begun to be considered. Only for the chloroplasts, where CaM may be involved in regulating the rates of photosynthesis (via NAD kinase) has this topic been addressed (21).

In 1980, Van Eldik et al. (63) purified a CaBP (from *Chlamydomonas* flagella) that was very similar to CaM but did not stimulate phosphodiesterase. This important paper cautioned other researchers to employ both structural and functional criteria when identifying calmodulin from new sources.

Enzymes and Processes Regulated by Calmodulin

Eight plant enzymes have been reported to be stimulated by CaM. There is good experimental evidence that CaM binds directly to the first four enzymes, which are presented below. For the other four enzymes it is not yet clear whether the stimulation is caused by direct binding of CaM or in an indirect manner (such as via protein phosphorylation by a CaM-dependent protein kinase).

Nicotinamide Adenine Dinucleotide Kinase

Nicotinamide Adenine Dinucleotide kinase converts NAD to NADP, the terminal electron acceptor of photosynthesis. This enzyme is probably one of the key regulatory enzymes in photosynthesis. In 1977, Muto and Miyachi (39) separated a protein activator of NAD kinase from plants, and the following year, Anderson and Cormier (1, 2) demonstrated that this activator was actually CaM. In addition to being regulated by CaM, NAD kinase is regulated by light. Nicotinamide Adenine Dinucleotide kinase has been reported to be localized in the cytosol, in the outer mitochondrial membrane, and in the chloroplast envelope. The specific localization of NAD kinase within these various subcompartments varies within different plant tissues (G. J. Piazza, 1986, unpublished). Fungal NAD kinase has also been shown to be regulated by CaM (7). Calmodulin is usually analyzed quantitatively by measurement of the stimulation of phosphodiesterase, but plant

NAD kinase also has been recently used as the basis of a new CaM assay (17).

Calcium Uptake

Calmodulin stimulates the ATP-dependent transport (uptake) of Ca^{2+} by several types of plant organelles (12). The current experimental evidence suggests that the plasma membrane, the endoplasmic reticulum, and the tonoplast (the vacuole membrane) contain CaM-stimulated Ca^{2+} transport activities (56). Although the mitochondria also contain ATP-dependent Ca^{2+} transport activity, they do not appear to be affected by CaM (13).

Adenosine Triphosphatase

Several plant ATPase are stimulated by CaM. These include ATPase localized in the microsomal fraction (a mixture of organelle membranes) of corn coleoptiles (43), the envelopes (outer membrane) of spinach chloroplasts (42), and nuclei isolated from pea buds (33). A CaM-stimulated ATPase has also been reported in the plasma membrane of barley roots (57). This ATPase is electrogenic and is thought to be involved in maintaining the membrane potential across the plasma membrane. Recent evidence indicates that aluminum toxicity in plants is caused by Al^{3+} binding to CaM and preventing it from being able to regulate this plasma membrane ATPase (57). Although it is likely that some of these CaM-dependent ATPase are also capable of translocating Ca^{2+} , it is not yet possible to conclude whether CaM-stimulated ATP hydrolysis is actually catalyzed by the same Ca^{2+} -translocating enzymes described in the previous paragraph.

Protein Kinases

Protein kinases catalyze the ATP-dependent phosphorylation of certain proteins. Phosphorylation stimulates some enzymes and inhibits others (65). Several plant protein kinases are stimulated by CaM. These include a protein kinase that appears to be associated with the plasma membranes of pea buds (20), a soluble protein kinase from wheat germ (47), membrane-associated protein kinases in corn coleoptiles (65) and zucchini hypocotyls (54), and a protein that occurs in the vacuoles of sycamore cells (60). In addition, there are also reports of other plant protein kinases stimulated by

calcium (65) or calcium and phospholipids (55). However, no cAMP-dependent protein kinases have yet been reported in plants; this is consistent with the absence of other evidence that cAMP serves as a second messenger in plants (48).

Quinate Oxidoreductase

Quinate:NAD-3-oxidoreductase from carrot cells catalyzes the conversion of quinate to dehydroquinate and is part of the shikimic acid pathway. This enzyme was reported to be stimulated by CaM or by protein kinase (+ATP) (49). It is not yet clear whether the CaM binds directly to the enzyme or stimulates indirectly via a CaM-dependent protein phosphorylation.

Isofloridoside Phosphate Synthase

Isofloridoside phosphate (IFP) synthase is involved in osmoregulation in certain algae. Recent experimental evidence indicates that the enzyme activity is stimulated by CaM, by proteolytic activation, and perhaps by protein phosphorylation (23). The authors think that this CaM effect is indirect and caused by the stimulation of an endogenous protease, which in turn, proteolytically activates the IFP synthase (23). Furthermore, the involvement of an endogenous protein kinase in this mechanism is implied by the stimulation of enzyme activity by NaF (an inhibitor of dephosphorylation) (23).

Aspartate Kinase

Aspartate kinase catalyzes the conversion of aspartic acid to β -aspartylphosphate, which is an intermediate in the synthesis of several amino acids in plants and bacteria. A threonine-sensitive aspartate kinase was recently reported to be stimulated by CaM (24). However, the lysine-sensitive form of the enzyme is not affected by CaM (4).

Phospholipase B

Our laboratory has recently reported that a B-type phospholipase in potato leaves is regulated by CaM (36), by protein kinase (+ATP) (35), and by proteolysis (unpublished data). This phospholipase catalyzes the hydrolysis of the 2 acyl ester bonds found in each phospholipid molecule. Phospholipase B is thought to

be involved in the degradation of membrane phospholipids and galactolipids during senescence and also perhaps functions as a defense mechanism against pathogens (36). We are currently conducting experiments to determine whether this CaM effect is direct or indirect.

Calmodulin Antagonists

Calmodulin antagonists (anticalmodulin drugs) are compounds that bind to the calcium-CaM complex and prevent its binding to target enzymes. These compounds have been very useful in identification of enzymes and processes regulated by CaM. Unfortunately, none of the known antagonists specifically inhibits CaM interactions; each has been shown to inhibit other types of enzymes and processes (G. J. Piazza, 1986, unpublished). We have recently reported an extreme case where several common CaM antagonists actually caused a five-fold stimulation of phospholipase activity in potato tubers (37). Despite the problems involved with use of these compounds, CaM antagonists are still very useful for the study of CaM interactions. One simply needs to understand their limitations and realize that inhibition of an enzyme or process by CaM antagonists is not enough evidence to implicate the involvement of CaM. Some common CaM antagonists include calmidazolium; w_7 ; tranquilizers such as fluphenazine, trifluoperazine; and the local anesthetics dibucaine and tetracaine. The herbicide dichlofopmethyl, the fungicide chloraniformethan, and the immunosuppressive agent cyclosporin A have also been shown to be CaM antagonists (6, 19). Each CaM antagonist thus far tested appears equally effective in inhibition of CaM interactions in plants, fungi, and animals. Some processes reported to be inhibited by CaM antagonists include photosynthesis, stomatal movement, phototaxis in algae, gravitropism, hormonal action, enzyme secretion, protoplast fusion, senescence, membrane lipid autolysis, germination of fungal spores, budding, and triterpenoid biosynthesis (G. J. Piazza, 1986, unpublished).

Several naturally occurring CaM antagonists have been reported in fungi and plants. Three of these compounds are common secondary metabolites that occur in many types of plant tissues. These include catechin, a flavanoid, caffeic acid, a phenolic compound, and querci-

tin, a flavanol (41, 44, 45). Two studies have indicated that the presence of CaM antagonists interferes with quantitative analysis of CaM (44, 45), and this should be considered as a common problem that may be encountered in other plant materials. The phytopathogenic fungi, *Helminthosporium maydis*, produces a phytotoxin called ophiobolin A, which is a potent CaM antagonist (27). Unlike other CaM antagonists, which interact reversibly with the calcium-CaM complex, ophiobolin A has been reported to covalently modify CaM (27). It is not yet known whether these endogenous CaM antagonists interact with CaM in the cell or whether they are sequestered in subcompartments (organelles) where they may never encounter CaM. In the case of ophiobolin A, it is interesting to speculate whether it may be involved in the infection and pathogenesis of the host plant by the fungal pathogen.

CONCLUSIONS

Calcium is required for many vital processes in plants and animals. The recent discovery that calcium is a true second messenger in all eukaryotes has revolutionized our understanding of this most abundant divalent cation. Although more than 20 CaBP have been identified in fungi and plants, only one, CaM, has been rigorously studied with modern biochemical techniques. During the 8 yr since the discovery of CaM in plants, it has been reported to be involved in regulating eight enzymes and processes. Many of the other processes that have been shown to be inhibited by CaM antagonists are currently being investigated to determine whether they represent true CaM-regulated processes.

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calcium (65) or calcium and phospholipids (55). However, no cAMP-dependent protein kinases have yet been reported in plants; this is consistent with the absence of other evidence that cAMP serves as a second messenger in plants (48).

Quinate Oxidoreductase

Quinate:NAD-3-oxidoreductase from carrot cells catalyzes the conversion of quinate to dehydroquinate and is part of the shikimic acid pathway. This enzyme was reported to be stimulated by CaM or by protein kinase (+ATP) (49). It is not yet clear whether the CaM binds directly to the enzyme or stimulates indirectly via a CaM-dependent protein phosphorylation.

Isofloridoside Phosphate Synthase

Isofloridoside phosphate (IFP) synthase is involved in osmoregulation in certain algae. Recent experimental evidence indicates that the enzyme activity is stimulated by CaM, by proteolytic activation, and perhaps by protein phosphorylation (23). The authors think that this CaM effect is indirect and caused by the stimulation of an endogenous protease, which in turn, proteolytically activates the IFP synthase (23). Furthermore, the involvement of an endogenous protein kinase in this mechanism is implied by the stimulation of enzyme activity by NaF (an inhibitor of dephosphorylation) (23).

Aspartate Kinase

Aspartate kinase catalyzes the conversion of aspartic acid to β -aspartylphosphate, which is an intermediate in the synthesis of several amino acids in plants and bacteria. A threonine-sensitive aspartate kinase was recently reported to be stimulated by CaM (24). However, the lysine-sensitive form of the enzyme is not affected by CaM (4).

Phospholipase B

Our laboratory has recently reported that a B-type phospholipase in potato leaves is regulated by CaM (36), by protein kinase (+ATP) (35), and by proteolysis (unpublished data). This phospholipase catalyzes the hydrolysis of the 2 acyl ester bonds found in each phospholipid molecule. Phospholipase B is thought to

be involved in the degradation of membrane phospholipids and galactolipids during senescence and also perhaps functions as a defense mechanism against pathogens (36). We are currently conducting experiments to determine whether this CaM effect is direct or indirect.

Calmodulin Antagonists

Calmodulin antagonists (anticalmodulin drugs) are compounds that bind to the calcium-CaM complex and prevent its binding to target enzymes. These compounds have been very useful in identification of enzymes and processes regulated by CaM. Unfortunately, none of the known antagonists specifically inhibits CaM interactions; each has been shown to inhibit other types of enzymes and processes (G. J. Piazza, 1986, unpublished). We have recently reported an extreme case where several common CaM antagonists actually caused a five-fold stimulation of phospholipase activity in potato tubers (37). Despite the problems involved with use of these compounds, CaM antagonists are still very useful for the study of CaM interactions. One simply needs to understand their limitations and realize that inhibition of an enzyme or process by CaM antagonists is not enough evidence to implicate the involvement of CaM. Some common CaM antagonists include calmidazolium; w_7 ; tranquilizers such as fluphenazine, trifluoperazine; and the local anesthetics dibucaine and tetracaine. The herbicide dichlofopmethyl, the fungicide chloraniformethan, and the immunosuppressive agent cyclosporin A have also been shown to be CaM antagonists (6, 19). Each CaM antagonist thus far tested appears equally effective in inhibition of CaM interactions in plants, fungi, and animals. Some processes reported to be inhibited by CaM antagonists include photosynthesis, stomatal movement, phototaxis in algae, gravitropism, hormonal action, enzyme secretion, protoplast fusion, senescence, membrane lipid autolysis, germination of fungal spores, budding, and triterpenoid biosynthesis (G. J. Piazza, 1986, unpublished).

Several naturally occurring CaM antagonists have been reported in fungi and plants. Three of these compounds are common secondary metabolites that occur in many types of plant tissues. These include catechin, a flavanoid, caffeic acid, a phenolic compound, and querci-

tin, a flavanol (41, 44, 45). Two studies have indicated that the presence of CaM antagonists interferes with quantitative analysis of CaM (44, 45), and this should be considered as a common problem that may be encountered in other plant materials. The phytopathogenic fungi, *Helminthosporium maydis*, produces a phytotoxin called ophiobolin A, which is a potent CaM antagonist (27). Unlike other CaM antagonists, which interact reversibly with the calcium-CaM complex, ophiobolin A has been reported to covalently modify CaM (27). It is not yet known whether these endogenous CaM antagonists interact with CaM in the cell or whether they are sequestered in subcompartments (organelles) where they may never encounter CaM. In the case of ophiobolin A, it is interesting to speculate whether it may be involved in the infection and pathogenesis of the host plant by the fungal pathogen.

CONCLUSIONS

Calcium is required for many vital processes in plants and animals. The recent discovery that calcium is a true second messenger in all eukaryotes has revolutionized our understanding of this most abundant divalent cation. Although more than 20 CaBP have been identified in fungi and plants, only one, CaM, has been rigorously studied with modern biochemical techniques. During the 8 yr since the discovery of CaM in plants, it has been reported to be involved in regulating eight enzymes and processes. Many of the other processes that have been shown to be inhibited by CaM antagonists are currently being investigated to determine whether they represent true CaM-regulated processes.

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