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INTERACTION OF CALMODULIN AND OPHIOBOLIN A, A FUNGAL PHYTOTOXIN

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Interaction of calmodulin and ophiobolin A, a fungal phytotoxin

by

Pak Chow Leung

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of the
Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Department: Biochemistry and Biophysics
Major: Biochemistry

Approved:

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Iowa State University
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1986
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ABBREVIATIONS

A  Absorbance
ATP  Adenosine triphosphate
ATPase  Adenosine triphosphatase
BSA  Bovine serum albumin
CaM  Calmodulin
cAMP  cyclic Adenosine 3',5'-monophosphate
d  day
DEAE  Diethylaminoethyl
ε  Molar extinction
EDTA  Ethylenediamine tetraacetic acid
EGTA  Ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid
IR  Infra-red
LD\textsubscript{50}  Lethal dosage killing 50%
lit.  literature
MW, M\textsubscript{r}  Molecular weight
M.p.  Melting point
NAD  Nicotinamide adenine dinucleotide
NADP  Nicotinamide adenine dinucleotide phosphate
NMR  Nuclear magnetic resonance
OAc  Acetate
PAGE  Polyacrylamide gel electrophoresis
PDE  Bovine brain cyclic nucleotide phosphodiesterase
Pi  Inorganic phosphate
. \[ \text{pI} \quad \text{pH of isoelectric point} \]

\[ \text{pm} \quad \text{picomole} \]

\[ \text{PMSF} \quad \text{Phenylmethysulfonyl-fluoride} \]

\[ \text{R}_f \quad \text{Relative distance to the solvent front} \]

\[ \text{SDS} \quad \text{Sodium dodecyl sulfate} \]

\[ \text{TFP} \quad \text{Trifluoperazine} \]

\[ \text{TLC} \quad \text{Thin layer chromatography} \]

\[ \text{UV} \quad \text{Ultra-violet} \]
GENERAL INTRODUCTION

Our laboratory has been involved with the study of plant toxins for several years. First, the host-specific T-toxin produced by the plant pathogen *Helminthosporium maydis* race T was studied. The T-toxin affects corn that contains the Texas male sterile cytoplasm and is believed to be responsible for the enhanced pathogenicity of *H. maydis* race T on T-cytoplasm corn (1, 2). Widespread use of T-cytoplasm corn led to the occurrence, in 1970, of an epidemic of southern corn leaf blight disease caused by *H. maydis* race T, which destroyed a large portion of the entire U.S. corn crop (1, 2). However, a study by Halloin et al. (3) showed that the extract from partially purified T-toxin is not host-specific. The extract induced carbohydrate leakage from both normal corn roots and corn roots which contained T-cytoplasm. Later, Betts discovered that besides producing the T-toxin, *H. maydis* also produces ophiobolin A (4). Tipton et al. (5) then showed that ophiobolin A induces carbohydrate leakage from both normal corn root and corn roots which contain T-cytoplasm. They suggested that the lack of specificity in the extract of partially purified T-toxin extract used by Halloin et al. (3) might have been due to the presence of ophiobolin A. We set out, then, with the aim of finding the cellular component that would bind to ophiobolin A. So far, we have (i) found that the protein calmodulin can be inhibited by ophiobolin A, (ii) tried to establish a correlation between the calmodulin-ophiobolin A interaction and the physiological effect of the toxin on corn roots, and (iii) characterized the interaction between calmodulin and ophiobolin A.
Ophiobolin A

Ophiobolin A is a sesterterpene of the ophiobolane structure, a tricyclic 5-8-5 ring system (structure I, p. 25). Ophiobolin A, first member discovered in the ophiobolin family, was isolated by Mario Orsenigo in Italy in 1957 from the culture medium of the plant pathogen *H. oryzae* while he was studying a disease of the rice plant *Oryzae sativa* (6). A year later, Nakamura and Ishibashi (7) in Japan also reported the isolation of ophiobolin A from the growth medium of the plant pathogen *Ophiobolus myiabeanus*. The genus name of the fungus is the origin of the toxin name ophiobolin. *O. myiabeanus* and *H. oryzae* were the names for the sexual and asexual stages, respectively, of the same fungus. The toxin affects plants, microorganisms and animal cells (8). Ophiobolin A inhibits the growth of rice root and coleoptile, causes chlorotic brown spots on rice leaves, and inhibits the growth of bacteria, fungi and protozoa. The toxin is identified as an "aversion factor" produced by *Cochliobolus setarie* strain 6387, which inhibits the growth of strain 6635 of the same fungus (9). Ophiobolin A is lethal to mice (LD$_{50}$ of 4.4 mg/kg, intraperitoneally). It depresses the insulin stimulation of glucose uptake in rat adipose tissue (10). Ophiobolin A affects the integrity of the cell membrane, causing a net leakage of cellular materials to the outside of the cells. For reviews on ophiobolin A, see references 8, 11, 12.
Calmodulin

Calmodulin is a calcium-binding protein with about 150 amino acids and a MW of about 17,000. The exact composition of amino acids varies slightly from species to species. Calmodulin is an acidic protein with a pI about 4.0 and is stable in boiling water. It is found in virtually all eukaryotic cells. Proteins similar to calmodulin exist in the procaryotes, *Escherichia coli* (13, 14), *Bacillus subtilis* (15), and the cyanobacterium *Oscillatoria limnetica* (16). The calmodulin-like proteins have some of the properties of the eukaryotic calmodulin, such as the activation of the bovine brain calmodulin-dependent cyclic nucleotide phosphodiesterase in the presence of Ca$^{2+}$, binding to the phenothiazine-coupled chromatographic column, or cross-reactivity with antibody to the animal calmodulin. However, no sequence information is available for the calmodulin-like proteins from prokaryotes.

Calmodulin was first discovered by W. Y. Cheung in 1967 to 1971 in the study of cyclic nucleotide phosphodiesterase from bovine brain (17, 18). Independently, Kakiuchi and Yamazaki in 1970 also found the activator in the same system (19). In 1973, Teo and Wang (20) reported that the activator is a Ca$^{2+}$-binding protein. They showed that after binding four Ca$^{2+}$, the activator binds to and activates the enzyme. When the Ca$^{2+}$ is removed from the calmodulin, the calmodulin dissociates from the enzyme and the enzyme returns to its less active form. The mechanism of Figure 1 is the general mode of action of calmodulin.
Figure 1. Mode of action of calmodulin and the possible sites of inhibition (I-VI) in the enzyme reaction. Calmodulin antagonists act on sites II, III, and IV.
Investigations of structure and function of calmodulin has become a major branch of research in the last decade (see reviews (21-45), monograph series (46-50) and symposia (51-55)). A volume of Methods in Enzymology (56) is devoted to the study of calmodulin. The protein interacts with many cellular and metabolic processes.

Plant calmodulin

The research on plant calmodulin started about ten years later than that of the animal calmodulin. Muto and Miyachi in 1977 isolated a protein in a pea which can activate NAD kinase (57). They found similar activators in spinach, rice, corn, Chinese cabbage, and the green alga Chlorella. They recognized the possibility of the protein to be a calmodulin. Preliminary observations on the presence of calmodulin in plants were also made by Waisman et al. in 1978 (58). Anderson and Cormier (59) showed that the activator of NAD kinase is indeed a calmodulin. They also showed that the plant calmodulin can activate the phosphodiesterase of bovine brain, thus showing similar functions for the plant and animal calmodulins. Calmodulin is widely distributed in plants and that the protein is present in many tissues (Table 1).

Calmodulin is a soluble protein and is found, therefore, mostly in the cytoplasm. However, calmodulin exists in many subcellular organelles. Calmodulin is in the cell wall of oat cells (85), the plasma membrane of a pea (87), the chloroplast of a pea (88), and the flagella of Chlamydomonas (73). Matsumoto et al. (89) have demonstrated
Table 1. Calmodulin in plants

<table>
<thead>
<tr>
<th>Fungi:</th>
</tr>
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<tbody>
<tr>
<td>Dictyostelium discoideum (60-63)</td>
</tr>
<tr>
<td>Physarum polycephalum (64)</td>
</tr>
<tr>
<td>Ceratocystis ulmi (65, 66)</td>
</tr>
<tr>
<td>Neurospora crassa (67)</td>
</tr>
<tr>
<td>Blastocladiella emersonii (68)</td>
</tr>
<tr>
<td>Candida albicans (69)</td>
</tr>
<tr>
<td>Saccharomyces cerevisiae (69, 70)</td>
</tr>
<tr>
<td>Basidiomycetes:</td>
</tr>
<tr>
<td>Russula sp. (71)</td>
</tr>
<tr>
<td>Cortinaries sp. (71)</td>
</tr>
<tr>
<td>Agaricus bisporus (26, 71, 72)</td>
</tr>
<tr>
<td>Agaricus campestris (70)</td>
</tr>
<tr>
<td>Coprinus lagopus (70)</td>
</tr>
</tbody>
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<table>
<thead>
<tr>
<th>Algae:</th>
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<tbody>
<tr>
<td>Chlamydomonas reinhardtii (73, 74)</td>
</tr>
<tr>
<td>Euglena gracilis (64)</td>
</tr>
<tr>
<td>Chlorella sp. (57)</td>
</tr>
<tr>
<td>Volvox sp. (75)</td>
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<table>
<thead>
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<th>Higher plants:</th>
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<tbody>
<tr>
<td>Spinach leaf (76)</td>
</tr>
<tr>
<td>Barley leaf, seedling shoot and root (71, 77, 78)</td>
</tr>
<tr>
<td>Pea seedling (57, 59, 79)</td>
</tr>
<tr>
<td>Peanut seeds (59, 79)</td>
</tr>
<tr>
<td>Zucchini hypocotyl (80)</td>
</tr>
<tr>
<td>Corn (78, 81)</td>
</tr>
<tr>
<td>Wheat leaf (82)</td>
</tr>
<tr>
<td>Cotton seedling (83)</td>
</tr>
<tr>
<td>Apple (84)</td>
</tr>
<tr>
<td>Oat (85)</td>
</tr>
<tr>
<td>Chinese cabbage (57)</td>
</tr>
<tr>
<td>Rice seedling (57)</td>
</tr>
<tr>
<td>Mungbean (59)</td>
</tr>
<tr>
<td>Wild carrot (59)</td>
</tr>
<tr>
<td>Broccoli (44, 86)</td>
</tr>
<tr>
<td>Turnip (44, 86)</td>
</tr>
<tr>
<td>Beet (44, 86)</td>
</tr>
<tr>
<td>Potato (44, 86)</td>
</tr>
<tr>
<td>Cucumber (44, 86)</td>
</tr>
<tr>
<td>Alfalfa (86)</td>
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</table>
the presence of a calmodulin-like protein associated with chromatin from the pea bud. The yield was about 5.7 μg calmodulin-like protein per mg DNA. The subcellular distributions of calmodulin in two plants appear in Table 2.

The cellular content of calmodulin varies among cell types. For example, the brain usually has a higher concentration of calmodulin than other tissues. The reported yields of calmodulin from many plant sources are usually 4 to 50 mg compared to up to 500 mg calmodulin in bovine brain per kg tissue (21, 26, 36). In animals and possibly for plants, the concentration of calmodulin in a cell changes with the cell cycle stage (31).

**Characteristics of plant calmodulin**

Generally, the MWs of plant calmodulins are smaller than that of bovine brain calmodulin and usually range from 15,000 to 19,000 daltons by SDS-PAGE analysis. The characteristics common to plant and bovine brain calmodulins are binding of four Ca$^{2+}$, activation of the bovine brain phosphodiesterase in the presence of Ca$^{2+}$, heat stability, pI~4.0, no tryptophan residue in the amino acid sequence and multiple absorption maxima from 250 to 280 nm.

Some differences from the bovine brain calmodulin are as follows. Most plant calmodulins have only one tyrosine residue instead of two in the brain calmodulin. As a result, the molar extinction at 276 nm is half of that of the brain calmodulin. Calmodulins from higher plants
Table 2. Subcellular distribution of plant calmodulin

Wheat (*Triticum aestivum* L.) leaf mesophyll cells (82):

<table>
<thead>
<tr>
<th>% of total cellular calmodulin</th>
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<tbody>
<tr>
<td>cytosol</td>
</tr>
<tr>
<td>mitochondria</td>
</tr>
<tr>
<td>chloroplast</td>
</tr>
<tr>
<td>microsomal fraction</td>
</tr>
</tbody>
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Oat (*Avena sativa* var. Garry) coleoptile and leaf (85):

<table>
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<tr>
<th>µg / ml extracted protein</th>
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<tbody>
<tr>
<td>cell wall</td>
</tr>
<tr>
<td>mitochondria</td>
</tr>
<tr>
<td>outer membrane of</td>
</tr>
<tr>
<td>mitochondria</td>
</tr>
<tr>
<td>isolated nuclei</td>
</tr>
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usually have one cysteine residue, for example, peanut and zucchini calmodulins (90, 91). Animal calmodulins do not usually have cysteine. For reviews on plant calmodulin, see references 24, 25, 26, 33, 34, 43, 92, 93, 94.

**Calmodulin-dependent enzymes and processes in plants**

NAD kinase, the Ca\(^{2+}\)-transport ATPase, aspartate kinase, and some protein kinases are enzymes in plants which are dependent upon calmodulin. A quinate:NAD oxidoreductase is indirectly controlled by calmodulin. Also, many metabolic processes involve calmodulin. Calmodulin is recognized as a mediator between Ca\(^{2+}\) and many Ca\(^{2+}\)-regulated enzymes and processes. The advent of calmodulin-Sepharose affinity chromatography has facilitated the isolation of calmodulin-dependent enzymes. A report by Dieter and Marme showed at least seven proteins in an extract of zucchini squash bind to the calmodulin-Sepharose column (95). Grand *et al.* (71) also reported calmodulin-binding proteins from barley and *Basidiomycetes* cellular extracts. The following paragraphs describe some of these calmodulin-dependent enzymes and processes.

1. **NAD kinase**

NAD kinase catalyzes the phosphorylation of NAD to NADP in the presence of ATP. NAD and NADP are important co-factors of many enzymes. The calmodulin-dependent form of the enzymes has been purified partially...
from pea seedling by Muto and Miyachi (57), and has been purified further by Jarrett et al. (25). NAD kinase has been isolated also from corn (24) and zucchini (95). The enzyme has an absolute dependence on calmodulin (25). It has a MW of about 50,000, estimated by gel filtration (96) and about 57,000 by SDS-PAGE (25). NAD kinase is present in the cytoplasm and chloroplast (82, 88) and there are reports that its activity is affected by light (37, 88, 97). Muto et al. (98) has reported a light-induced uptake of Ca\(^{2+}\) by intact wheat and spinach leaf chloroplasts. They suggested the influx of Ca\(^{2+}\) may be enough to activate the calmodulin in the chloroplasts which in turn activates the calmodulin-dependent NAD kinase. In contrast Simon et al. (99) show that almost no calmodulin was in spinach chloroplast and that NAD kinase in the chloroplast was not calmodulin-dependent. However, Marme and Dieter (24) suggested that the calmodulin-dependent NAD kinase might have been lost during their preparation.

2. Ca\(^{2+}\)-transport ATPase

In 1980, Dieter and Marme reported the simulation by calmodulin of Ca\(^{2+}\) uptake by the enriched microsomal fraction of plasma membrane from zucchini-squash. This calmodulin-stimulation of Ca\(^{2+}\) uptake was abolished by the calmodulin antagonist fluphenazine or by EGTA (100). In 1981, they reported the isolation of a partially purified Ca\(^{2+}\),Mg\(^{2+}\)-ATPase from the microsomal fraction of corn coleoptile (101). The microsomal Ca\(^{2+}\),Mg\(^{2+}\)-ATPase can be activated by calmodulin. Half-maximal stimulation occurred at about 0.16 \(\mu\)M calmodulin. Stimulation
by calmodulin could also be abolished by fluphenazine and EGTA. Dieter and Marme suggested that the Ca\(^{2+}\),Mg\(^{2+}\)-ATPase is responsible for the extrusion of cellular Ca\(^{2+}\) to provide a low Ca\(^{2+}\) concentration environment inside the cell (for reviews, see 24, 102).

3. Aspartate kinase

Aspartate kinase catalyzes the conversion of aspartic acid to β-aspartylphosphate, the precursor of lysine, methionine, and threonine in cells. Sane et al. in 1984 (103) showed that aspartate kinase, partially purified from seeds of Dolichos lablab, spinach leaves, or winged beans, is stimulated by calmodulin. Sane et al. suggest that the loss of activity of the enzyme in many isolations previously reported might result from the dissociation of the endogenous calmodulin from the kinase during the preparations.

4. Protein kinases

Protein kinases are enzymes that phosphorylate proteins in the presence of ATP. The result is usually a change in the properties of the protein, such as activation or deactivation of an enzyme. Protein kinases have been reported in plants for a long time (104). In 1982, Hetherington and Trewavas showed the presence of protein kinases activated by Ca\(^{2+}\) in the membrane fraction of pea bud. The protein kinases of Hetherington and Trewavas could be activated by calmodulin, but the extent of activation by calmodulin was low (105). A subsequent report by Hetherington and Trewavas (87) has not demonstrated the involvement of calmodulin in the kinase activity.
Polya and Davis in 1982 (106) partially purified a soluble protein kinase from the chromatin extract of wheat germ. Calmodulin activates the kinase 16-fold by calmodulin in the phosphorylation of histone. The soluble protein kinase has a MW of 90,000 (107). The kinase also catalyzes the phosphorylation of endogenous proteins. The enzyme is inhibited by the calmodulin antagonist chlorpromazine. However, the concentration of chlorpromazine required for half-maximal inhibition (320 μM) is higher than usual (10-50 μM). Polya and Davis suggest that the complex of calmodulin with kinase is not accessible to the antagonist.

Salimath and Marme in 1983 (108) demonstrated phosphorylation of protein dependent upon Ca$^{2+}$- and calmodulin in a membrane fraction from zucchini hypocotyl. The phosphorylation is enhanced by the addition of 1 μM calmodulin. The calmodulin stimulation is inhibited by fluphenazine. The kinase was not purified.

A Ca$^{2+}$, calmodulin-dependent protein kinase is also reported in *Neurospora crassa* (109).

A distinct example in which calmodulin-dependent phosphorylation affects the activity of an enzyme is that of the quinate:NAD oxidoreductase (quinate dehydrogenase). The enzyme catalyzes the conversion of quinate into dehydroquinate, an intermediate in the shikimate pathway. The enzyme has been found in carrot cells and corn seedling (110), in suspension cells of *Phaseolus aureus* (111), in mungbean seedlings (112), and potato tuber. The activity of the enzyme
from carrot cell is controlled indirectly by reversible phosphorylation
(113). The enzyme is activated when phosphorylated. Ranjeva et al. in
1983 (114) showed that this phosphorylation is calmodulin dependent,
although the protein kinase that phosphorylates the oxidoreductase was
not isolated. The oxidoreductase contains a calcium-binding protein as
a regulatory subunit (115). The regulatory subunit is 60 to 63
kilodalton in size. Ranjeva et al. suggested that this calcium-binding
protein might be representative of a class of calciprotein that regulate
phosphorylation.

Calmodulin behaves in an inhibitory, rather than an activating
manner in only a few cases. Maruta et al. (116) showed that calmodulin
inactivates the phosphorylation activity of the kinase of myosin heavy
chain from Dictyostelium discoideum. Albert et al. (117) found that
calmodulin inhibits the in vitro phosphorylation of an 87-kilodalton
protein by a calcium/phospholipid-dependent protein kinase from bovine
brain.

Other physiological processes which depend on calmodulin are listed
below. They are mostly based on the finding that calmodulin antagonists
interfere with these processes.

i. Electron transport and photosynthesis:
   a. transplasmalemma electron transport in carrot cells (118),
   b. electron transport in photosystem II of spinach chloroplasts
      (119),
   c. regulation of proton secretion and electrogensis in maize
      root and pea stem (120, 121),
d. inhibition of photosynthetic oxygen evolution in spinach chloroplast membrane vesicles (122),
e. inhibition of photosynthesis of the alga *Chlorococcum oleofaciens* (123).

ii. Hormone response:
   a. expression of cytokinin, auxin, and gibberellic acid responses in *Amaranthus tricolor* seedlings (124, 125),
   b. secretion of α-amylase in aleurone cells of barley seeds and of rice seed scutellar epithelium (126, 127)
   c. gravitropic growth in oat coleoptile (128).

iii. Cytoskeleton:
   a. chloroplast movement in the alga *Mougeotia* (129, 130),
   b. cell shape regulation in *Euglena gracilis* (131),
   c. regulation of fungal dimorphism in *Ceratocystis ulmi* (65, 66),

iv. Senescence:
   a. in apple (84), in pea foliage (132, 133).

v. Growth and development:
   a. peroxidase secretion in relation to habituation in sugar beet cell suspension (134, 135),
b. osmoregulation in the flagellate *Poterioochromonas malhemsis* (136),
c. morphogenesis and development in *Volvox carteri* (75).

**Calmodulin antagonists**

Many of the calmodulin-dependent processes and enzymes have been identified with the help of calmodulin antagonists. The perturbation of a process or an enzyme reaction by a calmodulin antagonist is a preliminary indication of the involvement of calmodulin in the process or enzyme reaction. A calmodulin antagonist binds to and prevents the calmodulin from carrying out its function, e.g., prevents the activation of the calmodulin-dependent phosphodiesterase.

Calmodulin antagonists can be conveniently divided into natural and synthetic ones. Synthetic antagonists include a variety of pharmacological agents such as antipsychotics, smooth muscle relaxants, antidepressants, antihistamines, antithrombins, antianginal agents, local anesthetics, α-adrenergic and β-adrenergic antagonists, antitumor agents, antihypertensives, antidiarrheals, and antimalarials (137). The broad range of pharmacological activities exhibited by calmodulin antagonists are not due entirely to their effect on calmodulin in vivo. In many cases calmodulin antagonists do not have a pharmacological effect. Also, many compounds that have the pharmacological effects above, are not antagonists of calmodulin. For reviews of synthetic calmodulin antagonists, see references 137-144.
The study of natural antagonists of calmodulin postdates studies of synthetic antagonists. Natural antagonists of calmodulin fall into two groups, namely, exogenous and endogenous ones. Exogenous antagonists are substances such as secondary metabolites and peptides secreted by organisms, and metal ions. Endogenous antagonists are those produced by the organism itself. Examples are some hormones and some small neuropeptides. Endogenous antagonists of calmodulin probably regulate the activity of calmodulin in the cell. A list of naturally occurring antagonists of calmodulin is in Table 3.

Mechanisms and specificity of calmodulin antagonists

According to the mode of action of calmodulin (Figure 1, p. 4), antagonists have six opportunities to inhibit the enzyme reaction (sites I through VI). Calmodulin antagonists act at sites II, III, IV (Table 4).

Most of the antagonists that bind to calmodulin in the absence of Ca\(^{2+}\) are metal ions (site II, Fig. 1). Metal ions can interact with calmodulin by binding (i) to the Ca\(^{2+}\)-binding sites, and/or (ii) to sites other than the Ca\(^{2+}\)-binding sites. Metal ions can replace Ca\(^{2+}\) at the Ca\(^{2+}\)-binding sites of the calmodulin. Only ions with sizes close to that of Ca\(^{2+}\) (0.099 nm) can fit into the Ca\(^{2+}\)-binding site, e.g., Mn\(^{2+}\) (0.080 nm), Tb\(^{3+}\) (0.092 nm) (171). The binding of a different ion at the Ca\(^{2+}\) sites usually reduces the activating ability of calmodulin. Heavy metal ions such as Cd\(^{2+}\), Hg\(^{2+}\), and Pb\(^{2+}\) can also replace Ca\(^{2+}\) in
Table 3. Calmodulin antagonists

I. Natural:

A. endogenous:

1. peptides:
   - secretin (145-147)
   - vasoactive intestinal peptide (145-147)
   - substance P (145, 146)
   - glucagon (145, 146)
   - dynorphin (145, 146)
   - corticotropin (148)
   - histone (148)
   - β-endorphin (146, 148-151)

2. calmodulin-binding proteins:
   - calcineurin (152)
   - caldesmon (153)

3. secondary metabolites:
   - alkaloids: vinblastine (154-155)
     - desacetylvinblastine amide (154)
     - vincristine (154, 155)
     - vindoline (154)
     - catharanthine (154)
     - adriamycin (156)
     - maytansine (155)
     - papaverine (148)
   - phenolic: caffeic acid (157)
   - flavonoid: catechin (157)
     - naringenin (157)
     - epicatechin (157)
     - quercetin (158)
   - alloxan (159)
Table 3. (continued)

B. exogenous:

1. peptides:
   - melittin (160, 161)
   - mastoparans (162)
   - insect venom: apamin, granuliberin R (163)
   - tertiapin (from bee venom) (164)
   - δ-hemolysin (165)
   - cyclosporin (166)

2. terpene:
   - ophiobolin A (81, 167)

3. metal ions:
   - Al(III) (168, 169)
   - Pb(II) (170, 171)

II. Synthetic:
   e.g., trifluoperazine, calmidazolium, W7, etc., over 50 have been reported, and have been well-reviewed. See references 137-144.

Table 4. Mechanisms of calmodulin antagonists (137)

<table>
<thead>
<tr>
<th>Sites (Fig. 1)</th>
<th>Mechanisms</th>
<th>examples</th>
</tr>
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<tbody>
<tr>
<td>II</td>
<td>binding to CaM</td>
<td>metal ions</td>
</tr>
<tr>
<td>III</td>
<td>binding to Ca(^{2+})-CaM complex</td>
<td>most antagonists</td>
</tr>
<tr>
<td>IV</td>
<td>competing with Ca(^{2+})-CaM complex</td>
<td>chlorpromazine-CaM</td>
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<td>for the binding site on the enzyme</td>
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calmodulin and inhibit its activity (170, 171). The relationship of these effects to the bad effects of environmental pollutants is unclear. A report that relates toxicity of some metal ions and calmodulin inhibition shows a tentative correlation (172).

Siegel and Haug (168) and Siegel et al. (169) reported that Al\(^{3+}\) binds to calmodulin in the absence or presence of Ca\(^{2+}\) and changes the conformation of the calmodulin to one with more hydrophobic surfaces exposed than when Ca\(^{2+}\) is bound. The Al\(^{3+}\)-calmodulin does not activate phosphodiesterase. Zn\(^{2+}\) has also been reported to bind to calmodulin (173), and to inhibit the calmodulin-dependent Ca\(^{2+}\)-transport ATPase in the red blood cells (174). However, since the dissociation constant for Zn\(^{2+}\) is much higher than that for Ca\(^{2+}\), the inhibition may be on the enzyme itself rather than on calmodulin (173).

The majority of antagonists of calmodulin, natural and synthetic, bind to the Ca\(^{2+}\)-calmodulin complex (site III, Fig. 1), and as a result, the complex cannot activate the enzyme. LaPorte et al. (175) and Follenius and Gerard (176) showed that hydrophobic regions are exposed when Ca\(^{2+}\) is bound to the calmodulin. Various antagonists may bind to this hydrophobic site and prevent enzymes from binding calmodulin. This agrees with the hydrophobic nature of most calmodulin antagonists.

The diversity of compounds that can affect calmodulin suggests that it is difficult to generalize on the structural requirement for successful binding. However, the requirement for structural compatibility is evident when different enantiomers or epimers behave
differently in inhibiting calmodulin activity (137, 139, 167).
Furthermore, the specific interaction between enzymes and calmodulin
must require structural specificity. These considerations suggest that
structural requirements are present in the interaction between
calmodulin and its antagonists for the most effective complex formation.

Calmodulin antagonists as probes for calmodulin-dependent processes

When using antagonists of calmodulin in order to probe for a
calmodulin dependency, some caution should be exercised in interpreting
results. Commonly used calmodulin antagonists will interact with other
cellular components as well. For example, chlorpromazine can interact
with phospholipid membranes (177, 178, 179). Chlorpromazine can bind
directly to the phosphodiesterase (180). Trifluoperazine, W7, and
calmidazolium inhibits the activity of the kinase of myosine light chain
independently of calmodulin (181). Pimozide, penfluridol,
chlorpromazine, and haloperidol block $\text{Ca}^{2+}$ entry, but also inhibit
calmodulin (137, 182). Thus, the above antagonists are not specific for
calmodulin.

In order to reliably interpret data using antagonists as probes for
calmodulin involvement, controls are necessary. (i) Determine whether
the antagonist of calmodulin affects other components in the system
under study. This is easier for a well-defined in vitro biochemical
reaction but is more difficult for complicated systems like cells and
tissues. (ii) Determine whether the enzyme under study is affected by the antagonist independently. (iii) Use one or more inactive, or less potent, derivatives of the calmodulin antagonist, and correlate the physiological results with those of inhibition study of calmodulin in vitro.

By using calmodulin antagonists, a lot has been learned about the involvement of calmodulin in physiological processes, the mechanism of regulation of cellular activities, the structure of the calmodulin molecule, the putative binding site on the calmodulin for enzymes, and the possible mechanisms of some toxins and drugs. Calmodulin antagonists have been bound covalently to Sepharose. The antagonists-Sepharose then is used to purify calmodulin from tissue extracts. Calmodulin antagonists also induce mutant cell lines that are resistant to the calmodulin antagonists (75, 183, 184).
Explanation of Dissertation Format

In this dissertation the Alternate Format is used. Three papers are included. Paper 1 is published in the Journal of Biological Chemistry, 1984, 259, 2742-2747. Paper 2 is published in Plant Physiology, 1985, 77, 303-308. Paper 3 has been submitted to the Journal of Biological Chemistry for consideration of publication. Papers 1 and 2 are collaborative work with professor Jerry H. Wang and his student Mr. William A. Taylor. Some of the experiments in both papers were done in professor Wang's laboratory at the University of Manitoba, Winnipeg, Manitoba, Canada, while I was there for two months. Mr. Lee M. Graves was an undergraduate research student in our laboratory. He worked on the reactions of ophiobolin A and amino acids and amino-acid derivatives.
SECTION I.

OPHIOBOLIN A. A NATURAL PRODUCT INHIBITOR OF CALMODULIN\textsuperscript{a}

Pak C. Leung\textsuperscript{b}, William A. Taylor\textsuperscript{c, d}, Jerry H. Wang\textsuperscript{c, d}
and Carl L. Tipton\textsuperscript{b}

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\textsuperscript{b}Department of Biochemistry and Biophysics, Iowa State University, Ames, Iowa, USA 50011.

\textsuperscript{c}Department of Biochemistry, Faculty of Medicine, University of Manitoba, Winnipeg, Manitoba, Canada R3E 0W3.

\textsuperscript{d}Supported in part by the Medical Research Council of Canada.
SUMMARY

Ophiobolin A, a fungal metabolite and a phytotoxin which can stimulate the net leakage of electrolytes of glucose from maize seedling roots (Tipton, C. L.; Paulsen, P. V.; Betts, R. E. Plant Physiol. 1977, 59, 907-910) was found to be a potent inhibitor of calmodulin-activated cyclic nucleotide phosphodiesterase. The physiologically less active analogue, 3-anhydro-ophiobolin A, was found to be less inhibitory than ophiobolin A in the phosphodiesterase assay. The direct interaction between ophiobolin A and calmodulin could be demonstrated by changes in fluorescence of the protein and by the effect of ophiobolin A on calmodulin activity upon preincubation. Addition of ophiobolin A to calmodulin solutions resulted in an instantaneous quenching of the intrinsic tyrosine fluorescence followed by a time-dependent quenching. The instantaneous quenching is probably due to the inner filtering effect of ophiobolin A. The time-dependent fluorescence quenching could be correlated with a time-dependent inhibition of calmodulin upon preincubation with ophiobolin A. The inhibition of calmodulin by ophiobolin A could not be reversed by dialysis, dilution, nor denaturation by urea in the presence of methanol followed by renaturation, and was much more pronounced in solutions containing Ca$^{2+}$ than in those containing EGTA. Ophiobolin A also inhibits spinach calmodulin. The results of the present study suggest that calmodulin may be one of the target proteins of the phytotoxic action of ophiobolin A and that the interaction of ophiobolin A with calmodulin may involve a covalent modification of the protein by the fungal metabolite.
INTRODUCTION

Ophiobolin A (I) is a phytotoxin produced by the plant pathogen *Helminthosporium maydis* Nisikado and Miyake and by other members of the same genus of fungi (1). Its structure has been determined (2, 3). It causes ion leakage, inhibition of hexose transport, and other effects in higher plants (4, 5). It and the closely related ophiobolin B also produce toxic effects in animals (6, 7). The mechanism of toxicity is not known for either plants or animals.

![Chemical structure of Ophiobolin A](image)

The Ca$^{2+}$-binding protein calmodulin is ubiquitously distributed in eukaryotes. It has been implicated in the Ca$^{2+}$-dependent regulation of many biological processes. (For reviews, see references 8-11.) In plants, calmodulin has been shown to function in the Ca$^{2+}$-dependent activation of NAD kinase and in ATP-dependent Ca$^{2+}$-uptake by microsomal vesicles. Calmodulin inhibitors are reported to stimulate acid secretion by plant tissues (12).

Various hydrophobic drugs have been shown to bind calmodulin in a Ca$^{2+}$-dependent process and inhibit activation of calmodulin target enzymes (13). In view of the hydrophobic nature of ophiobolin A and its
possible relationship to auxin-mediated Ca$^{2+}$-dependent processes in plants, we have initiated a study on the effect of ophiobolin A on calmodulin. This study has demonstrated that ophiobolin A is a potent inhibitor of calmodulin. The inhibition seems to involve covalent interaction with calmodulin and the effect seems specific since the analogue, 3-anhydro-ophiobolin A, is much less effective in inhibiting calmodulin activity.
MATERIALS AND METHODS

Preparation of ophiobolin A and derivatives

Ophiobolin A was isolated from culture filtrates of Helminthosporium maydis. The culture medium was modified Fries medium (14) supplemented with 0.4% (w/v) potato-dextrose extract (Difco Laboratories). The fungus was grown as a mycelial mat on 500 ml of autoclaved culture medium in a Fernbach flask at 25 to 28°C for 11 days. The mycelium was removed by filtration through cheesecloth and then Whatman No. 1 filter paper. The filtrate was extracted for 48 hours with diethyl ether in a continuous extractor. The orange-yellow ether extract was dried overnight over anhydrous Na₂SO₄, then reduced to a small volume by rotary evaporation, and left in a fume hood for crystallization. Usually 200 to 500 mg of impure crystals could be obtained from 4 l of culture filtrate. The crude crystals were purified by silica gel column chromatography. Approximately 200 mg crude crystals were dissolved in methylene chloride (1 ml) and applied to a 2.2 cm x 30 cm silica gel column (Brinkman MN silica gel, 40-63 μm) prewashed with methylene chloride. Ophiobolin A was then eluted with 8% (v/v) acetone in methylene chloride. The purity of the ophiobolin A fractions was checked by TLC on SiO₂ plates (Brinkman Silica Gel G UV 254, 250 μm) using 7% (v/v) acetone in methylene chloride as the developing solvent. Pure ophiobolin A fractions were pooled together and allowed to crystallize. Typically, a yield of 100 to 200 mg pure ophiobolin A was obtained from 4 l of culture filtrate. M.p. 181-2; lit. 182.
Anhydro-ophiobolin A was obtained as a by-product from the ophiobolin A preparation. The two substances were separated on the silica gel column with anhydro-ophiobolin A eluted ahead of ophiobolin A. The sample of anhydro-ophiobolin A was pooled, dried, and then re-chromatographed on silica gel columns developed with 20% acetone in hexane. Anhydro-ophiobolin A is the first material eluted, and it crystallizes upon evaporation of solvent. M.p. 134-135; lit. 135.

The purity of ophiobolin A and anhydro-ophiobolin A was also established by TLC in 7% (v/v) acetone in methylene chloride and 20% (v/v) acetone in hexane. Their identities were confirmed by 1H-NMR, UV and IR spectra, and exact mass determinations, which agree very well with published data (2,3).

**Protein preparation and assay procedure**

Bovine brain calmodulin was purified to homogeneity by a modification of the method described by Sharma and Wang (15). The modification was the inclusion of fluphenazine-Sepharose 4B affinity chromatography in the isolation of the calmodulin. The fluphenazine-Sepharose 4B was prepared as described by Charbonneau and Cormier (16). Calmodulin-deficient calmodulin-dependent phosphodiesterase was prepared from bovine brain according to Ho et al. (17). The assay of cyclic nucleotide phosphodiesterase and calmodulin was performed as described in (15) and is outlined in the legend of Fig. 1. Essentially, the phosphodiesterase reaction was coupled to the 5'-nucleotidase (*Crotalus atrox* venom, from Sigma) reaction, and the amount of inorganic phosphate
released represented the activity of the calmodulin or that of the phosphodiesterase. One unit of calmodulin was defined as that amount giving 50% of the maximal activation of the phosphodiesterase.

Trypsin-activated phosphodiesterase was obtained by incubating the enzyme with trypsin (37 μg/ml) in 20 mM Tris-HCl, 1 mM magnesium acetate, 1 mM imidazole, pH 7.0 (buffer A) prior to the enzyme assay at 30°C for 1 min, followed by the addition of lima bean trypsin inhibitor (60 μg/ml). The calmodulin-independent form of the phosphodiesterase was isolated from bovine heart according to the method of Ho et al. (17).

Fluorescence spectrophotometry

Fluorescence experiments were performed on Perkin-Elmer MPF-44 fluorescence spectrophotometer. Excitation and emission spectra were first obtained on calmodulin and the maximum wavelengths chosen as 280 nm and 307 nm, respectively. The calmodulin and Ca^{2+} (or EGTA) was first incubated in 20 mM Tris-HCl, 5 mM magnesium acetate and 100 mM sodium chloride, pH 7.0 (buffer B) for 5 to 10 min in the cuvette until the fluorescence reading was stable. The temperature of the cuvette chamber was controlled at 25.0°C. Ophiobolin A and anhydro-ophiobolin A, as 10 mM solutions in methanol, were added to the calmodulin solution in the cuvette using a 10-μl Hamilton glass syringe. Controls with equivalent amounts of methanol added were also carried out. The fluorescence spectra and measurements have not been corrected for phototube response or grating transmission. Only changes in fluorescence in the samples were of interest and there was no shifting of the emission maximum. The UV absorbances of 100 μM ophiobolin A at
280 nm and 307 nm were 0.050 and 0.026, respectively, and those of 100 μM anhydro-ophiobolin A were 0.046 and 0.026, respectively.

Protein determinations were carried out by Bradford's Coomassie Blue dye-binding method (18) with bovine serum albumin as a standard.

**Isolation and purification of spinach calmodulin**

Spinach leaves were chopped and then homogenized in a Waring blender in 50 mM Tris-HCl, 2 mM EDTA, 1 mM 2-mercaptoethanol, 0.15 M sodium chloride, pH 7.0, in the cold and was strained through cheesecloth. The filtrate was centrifuged at 10,000 xg for 20 minutes at 4°C. The supernatant was then passed through a DEAE-cellulose column previously equilibrated with 20 mM Tris-HCl, 0.1 mM EGTA, 1 mM 2-mercaptoethanol, 0.15 M sodium chloride, pH 7.0 (buffer C). The column was then washed with buffer C until no protein was eluted. Then a gradient of sodium chloride between 0.15 M and 0.7 M in buffer C was applied. The fractions containing calmodulin activity were pooled and dialyzed in the cold against buffer A with 0.5 mM calcium chloride (buffer D). The dialyzed sample was then applied to fluphenazine-Sepharose 4B column pre-equilibrated with buffer D. The column was then washed with buffer D containing 0.5 M sodium chloride until no protein eluted. Calmodulin was eluted with buffer A containing 5 mM EGTA. The fractions containing calmodulin were pooled, dialyzed against buffer D and then lyophilized. The calmodulin isolated was essentially pure in sodium dodecyl sulfate polyacrylamide gel electrophoresis and it co-migrated with bovine brain calmodulin in the presence of Ca^{2+}.
RESULTS

**Inhibition of bovine brain calmodulin-dependent phosphodiesterase by ophiobolin A and anhydro-ophiobolin A**

The inhibition of the calmodulin-dependent cyclic nucleotide phosphodiesterase by various concentrations of ophiobolin A is shown in Fig. 1. The half-maximal inhibition of the enzyme occurred at about 9 μM ophiobolin A. With longer preincubation times, the concentration required for half-maximal inhibition decreased to as low as 4 μM (data not shown). The time dependence of the reaction of ophiobolin A and calmodulin is described in more detail later. Anhydro-ophiobolin A, an analogue obtained as a by-product in the purification ophiobolin A, also inhibits the activity of the calmodulin-dependent phosphodiesterase. The inhibition potency of this analogue, however, is much lower. The concentration required for half-maximal inhibition, about 77 μM, is much higher than that for ophiobolin A. It should also be noted that the phytotoxic activity of anhydro-ophiobolin A has been found to be lower than that of ophiobolin A.

The inhibitory effect of ophiobolin A seems to be directed at the calmodulin-activated activity of the enzyme. When the enzyme was treated briefly with trypsin to eliminate its response to calmodulin, there was no change in activity in the presence of ophiobolin A (Fig. 2). Furthermore, neither the basal activity of the enzyme, nor the calmodulin-independent form of the enzyme was inhibited by the fungal metabolite (Fig. 2).
Figure 1. Effect of ophiobolin A and anhydro-ophiobolin A on calmodulin-dependent cyclic nucleotide phosphodiesterase

Four units (10 pmol) of calmodulin (M_r 16,700) were incubated with about 0.015 unit of calmodulin-deficient, calmodulin-dependent phosphodiesterase for 3 min in an 0.8 ml assay solution containing 0.3 units 5'-nucleotidase, 45 mM Tris-HCl, 5.6 mM magnesium acetate, 45 mM imidazole, and 2.5 mM calcium chloride, pH 7.0. Ophiobolin A (●) and anhydro-ophiobolin A (○) were then added to the assay mixtures as a 4.5 mM solution in methanol, using a 10-μl Hamilton glass syringe, and the samples were incubated for 30 min. Then 0.1 ml 10.8 mM cAMP, pH 7.0, was added to start the assay. After 30 min the assay was stopped by the addition of 0.1 ml 55% (w/v) trichloroacetic acid. All the above steps were carried out at 30.0°C. The phosphate produced in the assay was measured by the method of Fiske and Subbarow (19) as used in (15). The wavelength used for the phosphate assay was 660 nm. Controls showed that the amount of methanol in the ophiobolin A and anhydro-ophiobolin A samples did not affect the assay. The basal activity of the enzyme in the absence of Ca^{2+} was 0.135.
Figure 2. Effect of ophiobolin A on various forms of phosphodiesterase

Basal activity of calmodulin-dependent, calmodulin-deficient phosphodiesterase (●), calmodulin-independent phosphodiesterase (○), and trypsin-activated phosphodiesterase (□) were assayed with ophiobolin A from 10 to 100 μM. Assay procedures and conditions were as in Fig. 1, omitting the calmodulin. The basal activity of the enzyme was measured with EGTA instead of calcium chloride in the buffer. The amount of phosphodiesterase in each case was about 0.015 unit.
Demonstration of direct interaction between ophiobolin A and calmodulin

The inhibition studies suggest that ophiobolin A may interact with calmodulin to render it inactive. To test this suggestion, an attempt was made to demonstrate the direct interaction between calmodulin and ophiobolin A. The intrinsic tyrosine fluorescence of calmodulin has previously been shown to be enhanced upon Ca\(^{2+}\)-binding to the protein, thus suggesting that the microenvironment of the tyrosine residue is sensitive to the protein conformation (20). Chemical modification and other spectroscopic studies (21-23) have led to the same conclusion. Therefore, the tyrosine fluorescence of calmodulin was used in an attempt to probe the interaction between the protein and the phytotoxin.

Fig. 3 demonstrates that the fluorescence of calmodulin in the presence of Ca\(^{2+}\) was quenched by the addition of 50 \(\mu\)M ophiobolin A, showing that ophiobolin A can interact with calmodulin. There was no shift in the emission maximum. The quenching of the fluorescence was time-dependent and continued, at a slower rate, even after 30 min. The decrease in fluorescence cannot be attributed to photodestruction of the tyrosine residue in calmodulin because the extent of fluorescence quenching for samples incubated in the dark and for those in the cuvette holder with constant illumination was identical (data not shown).

The time-course of the fluorescence quenching of calmodulin by ophiobolin A was examined at several concentrations of the effector over the range of 10 \(\mu\)M to 100 \(\mu\)M. The results in Fig. 4 indicate that there were two phases in these time-courses: an instantaneous quenching
Figure 3. Effect of ophiobolin A on the intrinsic tyrosine fluorescence of calmodulin. Emission spectra of 2.4 μM calmodulin in 2 ml buffer B with 0.1 mM calcium chloride following the addition of ophiobolin A to a concentration of 50 μM. Spectra a, b, c, d, e, f, g were recorded at 0, 5, 10, 30, 50, 60, and 70 min, respectively. Scanning speed: 15 nm/min. Excitation: 280 nm, bandpass 5 nm. Emission: 307 nm, bandpass 10 nm. The spectrum of the control sample without ophiobolin A, but with an equivalent amount of methanol, coincided with spectrum a and did not change with time. Other experimental details are described under "Materials and Methods."
Figure 4. Quenching of intrinsic tyrosine fluorescence of calmodulin by ophiobolin A and anhydro-ophiobolin A. Changes in fluorescence with time at 307 nm (emission, bandpass 6 nm) of 4 μM calmodulin in 2 ml buffer B with 0.1 mM calcium chloride in the presence of various concentrations of ophiobolin A (●): a. 0 μM, b. 10 μM, c. 50 μM, d. 100 μM, or anhydro-ophiobolin A (○): 100 μM. Excitation: 280 nm, bandpass 5 nm. The percent fluorescence at each time is calculated by comparison with the fluorescence of the same sample before addition of ophiobolins. Other experimental details are described under "Materials and Methods."
followed by a slowly increasing quenching. The second phase was not completed even after incubation of the protein with ophiobolin A for over 1 hour. In addition to ophiobolin A, anhydro-ophiobolin A was also tested for its ability to influence the calmodulin fluorescence (Fig. 4). There was only a pronounced rapid fluorescence decrease with the analogue, the magnitude of this fluorescence quenching being similar to that of the first phase in ophiobolin A quenching. There could indeed be a second phase in the kinetics of the calmodulin fluorescence change induced by anhydro-ophiobolin A, but it was so slow that over the 70 min observation, there was only a slight fluorescence decrease following the first phase. The results from Fig. 4 suggest that the difference in the actions of these effectors resides in the second phase of the reaction with calmodulin.

To test if the binding of ophiobolin A to calmodulin is \(\text{Ca}^{2+}\)-dependent, as is the phenothiazine-calmodulin interaction, the quenching of calmodulin fluorescence by ophiobolin A was examined in solutions containing EGTA (Fig. 5). It can be seen that, in the presence of EGTA, ophiobolin A caused a rapid quenching of calmodulin with a magnitude similar to that observed in the presence of \(\text{Ca}^{2+}\). However, there was practically no further fluorescence change. The results suggest that the second phase of fluorescence quenching is highly \(\text{Ca}^{2+}\)-dependent.
Figure 5. Effect of Ca$^{2+}$ on the quenching of calmodulin fluorescence by ophiobolin A. Samples with 4 μM calmodulin in 2 ml buffer B with, a. 0 μM, b. 100 μM ophiobolin A, both in 0.1 mM EGTA, or c. 100 μM ophiobolin A in 0.1 mM Ca$^{2+}$. Conditions and procedures for the measurements are as in Fig. 4.
Nature of interaction between ophiobolin A and calmodulin

Initial fluorescence drop  To investigate the implication of the initial rapid fluorescence quenching, the fluorescence after incubation with ophiobolin A for 15 s was studied. This time interval was chosen because it was the earliest time manageable for the fluorescence measurements. The results are shown in Fig. 6. There was no sign of saturation even up to 200 μM ophiobolin A. When the calmodulin activities of these samples were analyzed there was not a noticeable decrease for up to 100 μM ophiobolin A (data not shown). At higher ophiobolin A concentrations, noticeable inhibition could be detected. This general lack of inhibition at this initial phase of fluorescence quenching suggests that the fluorescence quenching may not be due to a calmodulin-ophiobolin A interaction. Simple calculations show that the initial rapid quenching is caused by the inner filtering effect of ophiobolin A. In these calculations, the factor by which the fluorescence is decreased is $10^{-A d}$, where $A$ is the absorbance of ophiobolin A and $d$ is 1/2 cm, half of the length of the cuvette (24). As an example, at 100 μM ophiobolin A, the calculated intensity of fluorescence after the absorbance of light at both excitation and emission wavelengths by ophiobolin A are taken into account is 92%, in close agreement with the measured 93% (Fig. 6). Similar agreement was also obtained with other ophiobolin A concentrations.

Slow interaction  To study the nature of the slow interaction between ophiobolin A and calmodulin, as indicated by the second phase of
Figure 6. **Initial instantaneous quenching of calmodulin fluorescence by ophiobolin A** The fluorescence of the sample was read before and 15 s after the addition of ophiobolin A. The latter was then expressed as a percentage of the former. Conditions and procedures for the measurements are as in Fig. 4.
fluorescence quenching, the reversibility of the interaction was examined. If the slow interaction involves covalent modification of calmodulin by the effector, the protein sample preincubated with ophiobolin A would be expected to be irreversibly inhibited. In this experiment, the samples which were used in the fluorescence studies were dialyzed thoroughly and were then analyzed for calmodulin activity in the phosphodiesterase activation system. Fig. 7 shows the essential findings. The ophiobolin A treated samples had much less calmodulin activity after dialysis (curve c). The presence of EGTA in the dialyzing solution did not affect the observed results. The results suggest that the inhibition of calmodulin by ophiobolin A is irreversible. However, if the incubation of calmodulin with ophiobolin A was performed in the presence of EGTA, and then the sample was dialyzed, the dialyzed protein showed almost the same activity as the control sample, suggesting that ophiobolin A does not interact with calmodulin in the absence of Ca$^{2+}$. From the data we cannot determine whether the residual activity after ophiobolin A treatment and dialysis (curve c) is due to unmodified calmodulin or is due to partial activity of modified calmodulin.

Because the time-dependent calmodulin-ophiobolin A interaction is not readily reversible, samples treated with ophiobolin A can be diluted and assayed for calmodulin activity. The results of such an experiment (Fig. 8) show that the decrease in calmodulin activity was similar in pattern to that of the second phase of fluorescence change in Fig. 4. In addition, incubation of calmodulin and ophiobolin A in the presence
Figure 7. **Calmodulin activity after fluorescence studies and subsequent dialysis** After the fluorescence studies of Figs. 4 and 5, the samples were dialyzed in the cold for 24 h against 1 liter (per sample) buffer A with 0.1 mM Ca$^{2+}$ or EGTA. The dialysis buffers were replaced twice. After dialysis, each sample was diluted with water to 1.5 μg protein/ml and assayed for calmodulin activity as outlined in Fig. 1, omitting the addition of ophiobolins. a. control sample in which calmodulin had been incubated with 0.1 mM Ca$^{2+}$ in the fluorescence studies; b. sample in which calmodulin had been incubated with 0.1 mM EGTA and 100 μM ophiobolin A; c. sample in which calmodulin had been incubated with 0.1 mM Ca$^{2+}$ and 100 μM ophiobolin A in the fluorescence studies.
Figure 8. Time-course studies of calmodulin interaction with ophiobolin A and anhydro-ophiobolin A. Samples with 4 μM calmodulin in buffer A with 0.1 mM calcium chloride were incubated under conditions similar to those in Figs. 4 and 5. An aliquot of 50 μl was taken from each sample after various times of incubation with ophiobolin A and was diluted immediately 500-fold with buffer A to stop the reaction. The amount of calmodulin activity in the diluted solution was then assayed as in Fig. 1, omitting the addition of ophiobolins. The final concentration of ophiobolin A in the assay mixture was 0.1 μM or less with 2 units (4 pmol) of calmodulin protein, enough to give a good calmodulin assay in the absence of excess calmodulin. Incubation with ophiobolin A (●) at a. 0 μM, b. 10 μM, c. 50 μM, and d. 100 μM. Incubation with 100 μM anhydro-ophiobolin A (○). Incubation with 200 μM ophiobolin A with 0.1 mM EGTA instead of calcium chloride in the buffer (□).
of EGTA, or incubation with anhydro-ophiobolin A, caused little loss in calmodulin activity during this time period. These results in conjunction with those of Fig. 4 show that the amount of quenching of the fluorescence of calmodulin and the loss of calmodulin activity are directly related and that the interaction between ophiobolin A and calmodulin leads to an irreversible loss of calmodulin activity.

To further substantiate the possibility of a covalent binding between ophiobolin A and calmodulin, the ophiobolin A-calmodulin complex was denatured by 8 M urea in the presence of 25% (v/v) methanol. It has been reported that calmodulin is essentially completely unfolded in 8 M urea (25). If the binding between ophiobolin A and calmodulin were of a non-covalent nature, the unfolding of the calmodulin-ophiobolin A complex would release the ophiobolin A into the solution. This release would be facilitated in the presence of methanol because ophiobolin A is much more soluble in methanol than in water. It was previously found that the calmodulin assay was not affected by urea and methanol up to 0.5 M and 1.6% (v/v), respectively. Fig. 9 shows that in the absence of ophiobolin A, the urea and methanol treatment does not affect the calmodulin activity, indicating that the calmodulin can re-nature and regain its activity when the concentrations of urea and methanol were reduced. When ophiobolin A-inactivated calmodulin was treated by urea and methanol and then renatured, it did not regain its activity.
Figure 9. **Reversibility of calmodulin-ophiobolin A interaction by urea and methanol treatment**

Samples with 4 μM calmodulin in 2 ml buffer A with 0.1 mM Ca$^{2+}$ were incubated with or without 200 μM ophiobolin A for 24 h at 25°C. The samples were then divided into two identical sets and were diluted 37.5-fold, one set with urea and methanol in the above buffer, and the other set with buffer only. The final concentrations of urea, methanol, and ophiobolin A in the samples were 8.0 M, 25% (v/v) and 5.3 μM, respectively. After 2 h in the urea and methanol at 25°C, all samples were diluted 20-fold with the above buffer and assayed for calmodulin activity as in Fig. 1, omitting the addition of ophiobolins, on a scale 1/10 of that described. The phosphate was measured by the malachite green method (26). No ophiobolin A or urea and methanol treatments (●); ophiobolin A treatment but no urea and methanol treatment (○); no ophiobolin A treatment but with urea and methanol treatment (□); both ophiobolin A and urea and methanol treatments (■).
Effect of ophiobolin A on calmodulin-phosphodiesterase complex

Although the preceding results indicate that ophiobolin A reacts with calmodulin to result in the inactivation of the protein, the possibility existed that the metabolite could also inactivate the enzyme-calmodulin complex. To test this possibility, ophiobolin A (100 μM) was incubated with a phosphodiesterase-calmodulin mixture (1:2 molar ratio) in buffer A for 1 h at room temperature. Controls without ophiobolin A were also performed. After incubation, the solutions were diluted 300-fold for phosphodiesterase activity measurement in the presence of added calmodulin. The ophiobolin A had no inhibitory effect on the phosphodiesterase-calmodulin complex (data not shown).

Effect of ophiobolin A on plant calmodulin

Since ophiobolin A is a phytotoxin, its effect on a plant calmodulin was also investigated. Calmodulin isolated from spinach was also found to be inhibited by ophiobolin A in the bovine phosphodiesterase assay (Fig. 10). The half-maximal inhibition is at about 10 μM ophiobolin A, of the same magnitude as that with bovine brain calmodulin.
Figure 10. Effect of ophiobolin A on the activation of cyclic nucleotide phosphodiesterase by spinach calmodulin. The phosphodiesterase assay was as in Fig. 1, using approximately 4 units of spinach calmodulin instead of bovine brain calmodulin.
DISCUSSION

A variety of pharmacological agents including phenothiazine antipsychotic drugs (13), local anesthetics (27,28), muscle relaxants (29), and drugs for treatment of neoplasms (30), and other chemical agents such as 2-p-toluidinylnaphthaline-ô-sulfonate (31) and Triton X-100 (32) have been reported to inhibit calmodulin action mostly by undergoing a Ca$^{2+}$-dependent association with the protein. All these inhibitory compounds are hydrophobic and it seems likely that Ca$^{2+}$-binding to calmodulin exposes a hydrophobic region that binds these compounds. The results of the present study indicate that ophiobolin A, a hydrophobic fungal metabolite, is also a calmodulin antagonist. Its effective concentration in the inhibition of calmodulin activity is in the micromolar range in the cyclic nucleotide phosphodiesterase assay system. It shows high specificity for calmodulin since it does not inhibit the basal phosphodiesterase activity, the trypsin-activated or calmodulin-independent phosphodiesterase, or the phosphodiesterase-calmodulin complex. This specificity is higher than that of most pharmacological agents because these agents usually also affect the basal phosphodiesterase activity under comparable conditions, e.g., trifluoperazine (33).

As with other calmodulin inhibitors, the interaction between ophiobolin A and calmodulin is influenced by Ca$^{2+}$, but the mechanism of interaction between ophiobolin A and calmodulin is apparently different from those of other inhibitory agents. Based on the studies in this report, it appears reasonable to suggest that ophiobolin A reacts with
calmodulin via a slow reaction which coincides with a slow decrease in the protein fluorescence and a loss of activity. The recorded initial instantaneous fluorescence quenching does not seem to be related to a calmodulin-ophiobolin A interaction. It does not seem to inhibit calmodulin. It is \( \text{Ca}^{2+} \)-independent and non-specific, since anhydro-ophiobolin A also causes initial fluorescence quenching similar to that of ophiobolin A, although it does not participate very much in the slower quenching and loss of activity. This initial instantaneous fluorescence quenching can be accounted for by the inner filtering effect of ophiobolin A. On the other hand, the slower interaction between ophiobolin A and calmodulin is dependent on time and \( \text{Ca}^{2+} \). It is not reversible by dialysis, dilution or urea denaturation in the presence of methanol, followed by renaturation. The irreversibility suggests that the inhibition involves covalent modification of the protein. If this were the case, it may be feasible to use ophiobolin A as an affinity label for the hydrophobic site of calmodulin. Further experimentation is needed, however, to establish the occurrence of the covalent modification of the protein.

The differential calmodulin inhibitory potencies of ophiobolin A and anhydro-ophiobolin A also suggest that there is some specificity in the interaction between calmodulin and ophiobolin A.

Ophiobolin A has long been known to possess phytotoxic activity but the mechanism of action and its target molecules in plants are not known. The concentration of the metabolite for calmodulin inhibition is approximately the same as that required for its phytotoxic activity (4).
Although most of the present study was performed by using bovine brain calmodulin, calmodulin from spinach is equally susceptible to the inhibitory actions of this compound, suggesting that ophiobolin A reacts with spinach calmodulin in the same way as with bovine brain calmodulin. Anhydro-ophiobolin A, which possesses weaker phytotoxic activity, has been found in this study to demonstrate less calmodulin inhibitory activity. In view of the ubiquitous distribution and the diverse regulatory activities of calmodulin, it seems reasonable that ophiobolin A acts on calmodulin to exert its phytotoxic activity. This suggestion, however, does not exclude the possibility that the metabolite could also affect other molecules in plant cells. Because ophiobolin A seems to covalently modify calmodulin in vitro, it may be possible to correlate the phytotoxic action and the extent of ophiobolin A modification of calmodulin in plant tissues so as to evaluate the physiological relevance of the present results. Work is now progressing along this line.
REFERENCES

SECTION II.

ROLE OF CALMODULIN INHIBITION IN THE MODE OF ACTION OF OPHIOBOLIN A

Pak C. Leung, William A. Taylor, Jerry H. Wang, and Carl L. Tipton

Department of Biochemistry and Biophysics, Iowa State University,
Ames, Iowa, USA 50011

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2Department of Biochemistry, Faculty of Medicine, University of
Manitoba, Winnipeg, Manitoba, Canada R3E OW3, and supported
by the Medical Research Council of Canada

3Present address: Department of Medical Biochemistry, University
of Calgary, Calgary, Alberta, Canada T2N 4N1
ABSTRACT

Calmodulin has been isolated from the root of *Zea mays*. It activates the bovine brain calmodulin-dependent cyclic nucleotide phosphodiesterase and has electrophoretic mobility very similar to that of bovine brain calmodulin. Ophiobolin A, a fungal toxin, interacts with the maize calmodulin. The interaction is not reversed by dilution or denaturation in SDS and results in the loss of ability of the calmodulin to activate the phosphodiesterase. The inhibition is much faster in the presence than in the absence of Ca$^{2+}$. The electrophoretic mobility of ophiobolin A-treated calmodulin is less than that of untreated calmodulin. Several similarities are found between the inhibition of maize calmodulin by ophiobolin A *in vitro* and the effects of ophiobolin A on excised roots. Both are irreversible and time-dependent. The concentration of ophiobolin A for half-maximal inhibition of calmodulin in the phosphodiesterase assay is similar to that for phytotoxicity. In both cases, ophiobolin A derivatives behave similarly, i.e., 18-bromo-19-methoxyophiobolin A is as potent as ophiobolin A, while 3-anhydro-ophiobolin A and 6-epi-ophiobolin A are less potent. A smaller amount of active calmodulin was measured in the extract from ophiobolin A-treated roots than in those from untreated roots. The present study suggests that calmodulin is a target molecule in the root for the toxicity of ophiobolin A.
INTRODUCTION

Ophiobolin A is non-host-specific phytotoxin first isolated from Helminthosporium oryzae, the fungus that causes brown spot disease of rice (1, 2). It is one of a series of closely related sesterterpenes, whose occurrence and properties have been reviewed (3, 4). During studies of the host-specific toxin produced by race T of Helminthosporium maydis Nisikado and Miyake (Cochliobolus heterostrophus), it was found that some of the toxic effects of impure preparations of the host-specific toxin could be accounted for by ophiobolin A, which is also produced by this organism (5). Evidence has been reported for a role for ophiobolin A in the production of disease symptoms during the infection of rice by H. oryzae (6). Ophiobolin A has also been identified as an "aversion factor" produced by Cochliobolus setariae (7).

The mode of action of ophiobolin A has not been established. Cocucci et al. (8) reported rapid effects on membrane potential and $K^+$ efflux from maize roots. We have shown (9) that ophiobolin A reacts in vitro with bovine brain and spinach calmodulin so they are unable to activate PDE. The time course of the inhibition of calmodulin was much slower than the responses reported by Cocucci et al. (8). In this report, we present evidence that the toxic effects ophiobolin A on excised maize roots can be accounted for by its effects on calmodulin.
MATERIALS AND METHODS

Materials and equipment

*H. maydis* Nisikado and Miyake race T was a gift from Charles J. Arntzen (Michigan State University). Corn (*Zea mays*) seed was obtained from Black Seed Co., Ames, Iowa (B73Ht x Mo17Ht) and from Dr. C. A. Martinson, Iowa State University (W64A N and T cytoplasm). Fluphenazine-2HC1 was a gift from E. R. Squibb and Sons, Inc. DEAE-cellulose, 5'-nucleotidase (*Crotalus atrox* venom), and cAMP were from Sigma. Sepharose-4B was from Pharmacia. TLC plates (Silica Gel G UV254, 250 μm) and silica gel (MN Silica Gel, 40-63 μm) were from Brinkman Instruments, Inc. All other chemicals were of reagent grade. Some of the equipment used were FTIR spectrometer IR/98 IBM Instruments Inc.; WM-300 Bruker Aspect 2000 NMR; MS 902 AEI (Kratos Co.) mass spectrometer; conductivity bridge, Model 31, Yellow Spring Inst. Co., Yellow Spring, Ohio; and Willems Polyton Model PT10 from Brinkman Instruments, Inc.

Isolation of maize root calmodulin

Corn seeds were germinated and the seedlings grown in the dark for 6 d at 29°C on paper towels soaked with 0.1 mM CaCl₂ and 0.5 mM KCl. The roots were excised and rinsed 5 times with cold deionized, distilled water and then homogenized in a Waring blender in 50 mM Tris-HCl, 1 mM EDTA, 1 mM 2-mercaptoethanol, 0.6 mM PMSF, pH 7.0, (1 ml/g tissue). The homogenate was filtered through 4 layers of cheesecloth on ice. The
filtrate was centrifuged at 10,000 g for 0.5 h at 4°C. The resulting supernatant was applied to a DEAE-cellulose column pre-equilibrated with buffer A (20 mM Tris-HCl, 1 mM Mg(OAc)$_2$, 1 mM imidazole, 1 mM EGTA, 1 mM 2-mercaptoethanol, pH 7.0). The column was then washed with 3 bed volumes of buffer A and then with buffer A containing 0.15 M NaCl until no more protein was eluted. Calmodulin was then eluted with buffer A containing 0.6 M NaCl. The fractions containing calmodulin were pooled and dialyzed overnight in the cold against buffer B (20 mM Tris-HCl, 1 mM Mg(OAc)$_2$, 1 mM imidazole, 0.5 mM Ca$^{2+}$, pH 7.0). The dialyzed calmodulin solution was then applied to fluphenazine-Sepharose 4B column pre-equilibrated with buffer B. The column was then washed with buffer B and then with buffer B containing 0.5 M NaCl until no more protein was eluted. Calmodulin was then eluted from the column with 20 mM Tris-HCl, 1 mM Mg(OAc)$_2$, 1 mM imidazole, 0.5 mM EGTA, pH 7.0. The fractions with calmodulin were pooled and dialyzed overnight against buffer B in the cold, and then against 0.1 mM CaCl$_2$. The dialyzed calmodulin was then lyophilized and stored at -20°C.

The fluphenazine-Sepharose 4B was prepared as described in (10). Protein determination was by Bradford's Coomassie Blue dye-binding method (11) using BSA as a standard. SDS-PAGE was done according to the method of Laemmli (12), using 12% separation slab gel.

Protein preparation

Bovine brain calmodulin was isolated according to Sharma and Wang (13), and the use of fluphenazine-Sepharose 4B affinity chromatography
was included. PDE was prepared from bovine brain as described by Ho et al. (14).

**Calmodulin assay**

Calmodulin was assayed according to Sharma and Wang (13) and is described in the legend of Figure 2. Briefly, the activity of the PDE was coupled to that of the 5'-nucleotidase. The amount of phosphate released represented the amount of phosphodiesterase activity.

**Preparation of ophiobolin A and derivatives**

Ophiobolin A and 3-anhydro-ophiobolin A were isolated and purified as described by Leung *et al.* (9). Purified ophiobolin A (m.p. 181-2, lit. 181 (15)) gave a single spot upon TLC in two solvent systems, 8% v/v acetone in methylene chloride and 20% v/v acetone in hexane. 3-Anhydroophiobolin A (m.p. 134-5, lit. 135 (15)) when examined by TLC in 8% v/v acetone in methylene chloride showed one major spot and a very small amount of material streaking up from the starting point.

The preparation of 18-bromo-19-methoxyophiobolin A was described by Morisaki *et al.* (16). One equivalent of Br\textsubscript{2} in methanol was added in small portions to a stirring solution of ophiobolin A in methanol, containing a slight excess of sodium acetate. The extent of the reaction was followed by TLC. The solvent for the TLC was 8% v/v acetone in methylene chloride. To extract the product, water was added to the reaction mixture and the mixture was then extracted 4 times with an equal volume of methylene chloride. The combined methylene chloride
extract was dried with anhydrous \( \text{Na}_2\text{SO}_4 \) and then concentrated by a stream of \( \text{N}_2 \). The clear oil obtained was chromatographed on a silica gel column prewashed and equilibrated with methylene chloride. The bromomethoxy ophiobolin A was eluted with 5% acetone, 0.75% methanol, 0.25% \( \text{H}_2\text{O} \) in methylene chloride (v/v). The fractions containing bromomethoxy ophiobolin A were pooled, and dried, and then re-chromatographed twice on silica gel columns prewashed and equilibrated with \( \text{n-hexane} \). The elution solvent was 20% v/v acetone in \( \text{n-hexane} \). Pure bromomethoxy ophiobolin A solidified on evaporation of the solvent. M.p. 155; lit. (16) 155-157.

The preparation of 6-epi-ophiobolin A was as suggested by Canonica and Fiecchi (3). Sodium methoxide in methanol was added in small aliquots to a stirred solution of ophiobolin A in methanol. After the reaction, methylene chloride was added to the mixture and the resulting mixture was extracted 3 times with deionized distilled water. The methylene chloride extract was then dried and concentrated. The resulting pale yellow oil was chromatographed twice on silica gel columns prewashed and equilibrated with methylene chloride. Epi-ophiobolin A was eluted with 8% v/v acetone in methylene chloride. Evaporation of the solvent yielded a waxy solid (m.p., decomposition, 110-119). TLC in 8% acetone in methylene chloride yields a single major spot and several very small spots at lower \( R_f \).

The identities of all four compounds were confirmed by \(^1\text{H-NMR}, \text{UV} \) and IR spectra, and exact mass determinations, which agree very well with published data (3, 15, 16).
Ion-leakage measurement

Corn seeds were grown in arrays to avoid tangling of roots. After 2.5 to 3 d, the tip (about 3 cm) of each first root was excised. Fifty of these root tips were distributed to each 250-ml beaker and were gently rinsed 5 times with deionized distilled water and incubated for 1 h at 30°C before the addition of ophiobolin A and derivatives. The beakers were covered to reduced evaporation. Ophiobolin A or derivatives were applied as 0.1 ml solutions in methanol. Ionic leakage was estimated by measuring increases in conductivity of the bathing solution. In experiments comparing N and T cytoplasm roots, 25 roots tips were suspended in 25 ml water for the ion-leakage measurements.

Inhibition of calmodulin in maize root

After incubation for 8 h in the ion-leakage experiment, the roots were thoroughly rinsed with deionized distilled water and then homogenized in 15 ml of 0.1 M Tris-HCl, 1 mM EDTA, 1 mg PMSF/sample, pH 8.0, using the Polytron. The homogenates were filtered through 4 layers of cheesecloth. The filtrates were centrifuged at 13,000 g for 15 min at 4°C. The supernatants were then dialyzed, to get rid of Pi, against buffer A for 36 h at 4°C, with 1 change of the buffer. After dialysis, the extracts were heated in a boiling water bath for 1.5 min and then centrifuged in a desk-top centrifuge. The supernatants were then assayed for calmodulin.
RESULTS

Isolation and characterization of maize calmodulin

Electrophoretically pure calmodulin has been isolated from maize root extract by this simple procedure of DEAE-cellulose ion-exchange chromatography followed by fluphenazine-Sepharose affinity chromatography. The yield is about 4 mg per kg tissue. The isolated calmodulin appears as one band in SDS-PAGE with a slight smearing behind it (Fig. 1). The smearing is present even after the calmodulin has been further purified by gel filtration chromatography or by heating for 5 min in boiling water. Figure 1 also shows that in the presence of Ca\(^{2+}\), the maize calmodulin has the same electrophoretic mobility as that of bovine brain calmodulin. It has generally been found that calmodulin usually moves more slowly in the absence of Ca\(^{2+}\) in SDS-PAGE (17). Figure 1 shows that this is also true for the maize calmodulin. However, the change in mobility for the maize calmodulin is smaller than that for bovine brain calmodulin.

The activation of PDE is usually used to demonstrate the activity of calmodulin. Figure 2 shows that the maize calmodulin can activate the phosphodiesterase, although the activation is not as efficient as that of bovine brain calmodulin. When Ca\(^{2+}\) is removed from the medium by EGTA, the activation is abolished, thus showing the necessity of Ca\(^{2+}\)-binding to the maize calmodulin for its stimulatory activity.
Figure 1. SDS-PAGE of calmodulin isolated from maize seedling roots
Left: Electrophoresis in the presence of 1 mM Ca$^{2+}$; Right: Electrophoresis in the presence of 1 mM EGTA. Lane B: 6 µg bovine calmodulin; C: 7 µg maize root calmodulin; D: 5 µg bovine brain calmodulin; E: 3 µg maize root calmodulin; A & F: mol wt standards, lysozyme (14,300), myoglobin (17,500), soybean trypsin inhibitor (20,100), carbonic anhydrase (29,000), glyceraldehyde-3-phosphate dehydrogenase (36,000).
Figure 2. Activation of PDE by calmodulin from maize root and bovine brain

The assay procedure was as in (13) with the ingredients scaled down 1/10 of those described. The final reaction mixture is 0.090 ml and contains 0.0015 unit PDE, 40 mM Tris-HCl, 40 mM Mg(OAc)$_2$, 2.2 mM CaCl$_2$, 0.003 unit 5'-nucleotidase, 1.2 mM cAMP, pH 7.0, and various amounts of calmodulin. The cAMP was added last to start the reaction. The reaction mixture was incubated at 30°C for 30 min. Then, 10 µl 55% TCA were added to stop the reaction. The amount of Pi released was measured by the malachite green method (18).

Maize calmodulin (●); bovine brain calmodulin (○); maize calmodulin in the presence of 2.2 mM EGTA instead of Ca$^{2+}$ (■).
Inhibition of maize calmodulin by ophiobolin A

Figure 3 shows that ophiobolin A can inhibit maize calmodulin in the PDE assay. The effect is on the calmodulin because it has been shown that ophiobolin A does not affect the phosphodiesterase (9). From the Figure, the concentration of ophiobolin A required for half-maximal inhibition is about 12 μM. This concentration for half-maximal inhibition is higher when the time of preincubation with ophiobolin A is shorter than 1 h (data not shown), suggesting the inhibition is time-dependent.

Characteristics of the ophiobolin A-calmodulin interaction

To see if the interaction between ophiobolin A and maize calmodulin is irreversible, as is the case with bovine brain calmodulin (9), the maize calmodulin was first reacted with ophiobolin A and then subjected to SDS-PAGE. The results are shown in Figure 4. Calmodulin that has been treated with ophiobolin A has lower electrophoretic mobility than untreated calmodulin, even though the denaturing condition would be expected to release any ophiobolin A bound noncovalently.

The inhibition of maize calmodulin by ophiobolin A is strongly dependent on Ca\(^{2+}\) (Table I). Ophiobolin A in the presence of 0.1 mM Ca\(^{2+}\) completely inhibits calmodulin activity, while the same treatment in the presence of 6 mM EGTA has no effect.
Figure 3. **Inhibition of maize calmodulin by ophiobolin A and derivatives, measured by the loss of the ability to stimulate PDE**

The assay procedure was exactly as in (13). Each assay mixture contained 4 units of maize calmodulin, with a unit defined as the amount necessary for half-maximal activation of PDE. To each reaction mixture, ophiobolin A or a derivative as a 4.5 mM solution in methanol was added and the mixture was incubated for 1 h before starting the assay by addition of cAMP. Methanol, in the amounts used, has no effect on the assay. 3-Anhydro-ophiobolin A (●), 6-epi-ophiobolin A (○), 18-bromo-19-methoxyophiobolin A (■), ophiobolin A (▲).
Figure 4. SDS-PAGE of maize calmodulin after treatment with ophiobolin A
Electrophoresis of 10 μg samples of maize calmodulin in the presence 0.1 mM CaCl$_2$. Pretreatments of the calmodulin samples, for 1.5 h at 30°C in the presence of 0.1 mM CaCl$_2$: A, no additions; B, 2.5% methanol; C, 0.25 mM ophiobolin A in 2.5% methanol. Lane D: mol wt standards lysozyme, myoglobin, carbonic anhydrase, pepsin (34,700).
Table I. Ca\(^{2+}\) dependence of the inhibition of maize calmodulin by ophiobolin A

Maize calmodulin, 200 units per sample, in buffer A was incubated with the additions shown, total vol 50 μl, for 1 h at 30°C. The samples were then diluted 100-fold with buffer A to stop the reaction and the calmodulin activity in each sample was assayed. When added, CaCl\(_2\) was 0.1 mM, methanol 1% v/v, ophiobolin A 0.1 mM and EGTA 6 mM.

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In vivo inhibition of maize calmodulin by ophiobolin A

The inhibition and the electrophoresis studies demonstrate quite well that ophiobolin A can interact with and inhibit the activity of maize calmodulin in vitro. To see if this is the basis for the toxicity of ophiobolin A in vivo, maize roots that had been treated with ophiobolin A were extracted, and the amount of active calmodulin was estimated. The results of such an experiment (Fig. 5) show that the extract from ophiobolin A-treated roots has less active calmodulin than that of the untreated roots. These results seem to suggest that ophiobolin A can interact with calmodulin in situ.

We also used ophiobolin A derivatives to probe the involvement of calmodulin in the toxicity of ophiobolin A. 18-Bromo-19-methoxyophiobolin A, 6-epi-ophiobolin A, and 3-anhydro-ophiobolin A were prepared. Their potencies in inhibiting maize calmodulin in the PDE assay are shown in Figure 3. Bromomethoxyophiobolin A is as potent as ophiobolin A, whereas epi- and anhydro- ophiobolin A are less potent than ophiobolin A. The concentrations required for half-maximal inhibition are 10, 60 and >100 μM for bromomethoxy-, epi-, and anhydro- ophiobolin A, respectively. When the derivatives were used to induce ion-leakage in roots, the pattern of potencies, shown in Figure 6, is similar to that for calmodulin inhibition in Figure 3. The bromomethoxy derivative is as potent as ophiobolin A and the epi- and anhydro-derivatives are less potent. These results suggest a relation exists between calmodulin inhibition and the physiological effect of ophiobolin.
Figure 5. Inhibition of calmodulin in excised maize seedling roots by ophiobolin A. Control roots soaked in deionized water with equivalent amount of methanol (●); Ophiobolin A-treated roots (○); PDE assay with the above two root extracts plus EGTA instead of Ca$^{2+}$ (■, □).
Figure 6. **Stimulation of ion-leakage from maize seedlings by ophiobolin**

A and derivatives:  
- A: control with 0.1 ml methanol;  
- B: 6-epi-ophiobolin A;  
- C: 3-anhydro-ophiobolin A;  
- D: 18-bromo-19-methoxyophiobolin A;  
- E: ophiobolin A.
A. Similar experiments measuring the effects of ophiobolin A and the 
epi- and anhydro- derivatives on ion leakage from roots of W64A N and T 
cytoplasm plants were performed. No difference in the effects of the 
compounds on N and T cytoplasm roots was seen (Table II).
Table II. **Stimulation of ion leakage from N and T cytoplasm maize seedling roots by ophiobolin A and derivatives**

Ion-leakage measurements were made as described in Material and Methods. The concentrations of the test substances were 50 µM. Controls showed that methanol in the amount introduced with the test substances has no effect on ion leakage.

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DISCUSSION

The ubiquitous Ca$^{2+}$-binding protein calmodulin has been isolated from many plants, including Zea mays (19, for review see 17). In this study, we have shown the calmodulin from maize root shares some characteristics with calmodulins from other plants and animals. The maize calmodulin can bind Ca$^{2+}$, activate PDE, and shows a Ca$^{2+}$-dependent electrophoretic mobility shift in SDS-PAGE. Like other plant calmodulins, the maize calmodulin has a greater electrophoretic mobility in SDS-PAGE than bovine brain calmodulin, and is less efficient than brain calmodulin in the activation of PDE. Aside from these slight differences, the maize calmodulin seems to be functionally quite similar to other calmodulins, revealing the conserved nature of the molecule.

The inhibition of maize calmodulin by ophiobolin A is similar to that of brain calmodulin (9). It is not reversed by dilution or denaturation by SDS and is much faster in the presence than in the absence of Ca$^{2+}$, suggesting the inhibition is related to the biological function of calmodulin.

In this study, we have found some correlations between the inhibition by ophiobolin A of maize calmodulin and the physiological effect of ophiobolin A on the root. Firstly, the irreversibility of the interaction between ophiobolin A and calmodulin agrees with the previous observation that the effect of ophiobolin A on roots was irreversible (5). Secondly, the rate of calmodulin inhibition agrees with that of ion-leakage. Calmodulin inhibition in vitro is nearly complete in an
hour. For ion-leakage, it takes 1 to 2 h for the increase in rate due
to ophiobolin A to be obvious. The slower rate in the ion-leakage
induction may be due to a lower Ca\(^{2+}\) concentration in the cell or other
cellular factors that may interfere with the interaction between
calmodulin and ophiobolin A \textit{in situ}. Thirdly, the concentrations of
ophiobolin A required for the inhibition of calmodulin \textit{in vitro} are
similar to those inducing physiological effects in roots. The
concentration of ophiobolin A for half-maximal inhibition of the root
calmodulin in the phosphodiesterase assay is about 12 \textmu M. In
physiological measurements, 25 \textmu M ophiobolin A inhibited by 85\% the
uptake of 0.5 mM 2-deoxyglucose by maize root (5). Fourthly, there is a
parallel between calmodulin inhibition and ion-leakage by ophiobolin A
derivatives. The derivatives that are less inhibitory in the calmodulin
assay are also less effective in inducing ion-leakage in root. The
derivative 18-bromo-19-methoxyophiobolin A is at least as potent as
ophiobolin A both in inhibiting calmodulin in the PDE assay and in
inducing ion-leakage in roots. The isolation of a smaller amount of
active calmodulin from roots after \textit{in situ} treatment with ophiobolin A
is also a strong support for the involvement of calmodulin in the
ophiobolin A toxicity, although this loss of calmodulin in the
ophiobolin A-treated roots is partial even after 8 h of treatment.
Because the ophiobolins used are derived from cultures of the same
organism that produced the host-specific effect HM-T toxin, we tested
three of the compounds for host-specific effects on ion leakage using N
and T cytoplasm seedling. No host-specific effect was seen.
Considering these correlations, it seems very likely that calmodulin is a target molecule for the toxicity of ophiobolin A in root cells. An implication of this suggestion is that enzymes or metabolic processes dependent on calmodulin will be affected. Transport enzymes such as calmodulin-dependent Ca-ATPase may thus be affected and lead to transport defect in the cell, explaining the physiological effect of the toxin. Although the inhibition of calmodulin offers a reasonable explanation for the mode of action of ophiobolin A, we have not excluded the possibility that the toxin may also interact with other cellular components to carry out its full spectrum of activity.
REFERENCES


SECTION III.
CHARACTERIZATION OF INTERACTION OF OPHIOBOLIN A AND CALMODULIN

Pak C. Leung, Lee M. Graves and Carl L. Tipton

Department of Biochemistry and Biophysics, Iowa State University,
Ames, Iowa, USA 50011

Journal Paper No. J-11916 of the Iowa Agriculture and Home Economics
Experiment Station. Project No. 2560.

Present address: Department of Biochemistry, University of Illinois,
Urbana, Ill, 61801.
SUMMARY

The fungal toxin ophiobolin A has been shown to be a potent calmodulin inhibitor. Here, we present evidence that it reacts with the ε-amino group of lysine residues in the calmodulin molecule. Ophiobolin A reacts with primary amino groups to give conjugated enamine products with λmax from 270-280 nm. With the ε-amino group of N-α-acetyl-L-lysine, the λmax is 272 nm. The same λmax is also observed when bovine brain calmodulin is reacted with ophiobolin A, suggesting that a conjugated enamine product is formed between the ε-amino group of lysine residues and ophiobolin A. Ophiobolin A-treated calmodulin is resistant to tryptic digestion at lysine 77, suggesting that ophiobolin A binds at this region of the molecule. This suggestion is consistent with the finding that trifluoperazine interferes with the calmodulin-ophiobolin A interaction since trifluoperazine binds at this region of the calmodulin molecule. Dictyostelium discoideum calmodulin, which has glutamine instead of lysine at residue 77, is shown to be equally inhibited by ophiobolin A in the phosphodiesterase assay. This suggests that lysine 77 in the brain calmodulin is not essential for the ophiobolin A interaction and that lysine 75 is the more probable reaction site. By the use of UV spectroscopy we have estimated that there are two binding sites on the brain calmodulin for ophiobolin A.
INTRODUCTION

The ubiquitous calcium-binding protein calmodulin has been shown to regulate many Ca$^{2+}$-dependent enzymes and metabolic processes (for reviews, see 1, 2). In 1976, Levin and Weiss (3) showed that the calmodulin activation of a PDE could be inhibited by certain phenothiazines. It was thought that a phenothiazine such as TFP binds to the site on the calmodulin that binds enzyme and prevents the enzyme from binding to calmodulin. The location of this binding site will therefore give the location of the binding site for the PDE.

Since the finding of the phenothiazine antagonists, many synthetic and natural calmodulin antagonists have been reported (for reviews, see 4,5). Reaction of many of these antagonists with calmodulin is Ca$^{2+}$-dependent. It is believed that upon binding Ca$^{2+}$, a hydrophobic site on the calmodulin is exposed (6) and the antagonist binds to this hydrophobic site. When the Ca$^{2+}$ is removed, the calmodulin resumes a conformation in which this hydrophobic site is not available. It is now realized that some charge interaction may also be involved in an effective binding of the antagonist and calmodulin (4). Proton NMR studies by Dalgarno et al. (7) suggest that there is one binding site for the drug on each end of the calmodulin and that each site is made up of an assembly of hydrophobic side groups of the residues, brought together by the change in conformation upon Ca$^{2+}$ binding to the calmodulin. By using the synthetic irreversible antagonist 10-(3-propionyloxy)succinimide-2-trifluoromethylphenothiazine to affinity-label the calmodulin, Jarrett obtained results that suggest
that the ε-amino group of lysine may react with the antagonist (8).

Previous $^1$H-NMR studies by Klevit et al. (9) have shown that proton resonances of lysine residues are affected by the binding of TFP.

Earlier, Walsh et al. (10) suggested that lysine 77 is exposed when Ca$^{2+}$ is bound to calmodulin because the calmodulin is cleaved by trypsin at lysine 77 in the presence of Ca$^{2+}$ but not in its absence. A report by Walsh and Stevens (11) showed that chemical modification of 1 or 2 lysine residues in the calmodulin by isocyanate in the presence of Ca$^{2+}$ resulted in 60-70% loss of the calmodulin activity. A preliminary report by Mann and Vanaman (12) suggests the lysine-75 region of calmodulin is modified by the drug 1-(2-chloroethyl)-3-[trans-4-methylcyclohexyl]-1-nitrosourea. Another preliminary report by Giedroc et al. (13) suggests that lysine 75 and 77 are affected by TFP and that lysine 75 is a more reactive residue in the presence of Ca$^{2+}$.

All these observations suggest that there is a binding site for drugs, and probably for the enzyme phosphodiesterase, near lysine 75 and that this lysine could be an essential residue in the binding of drugs and enzymes to calmodulin.

Recently, we have reported that the fungal toxin ophiobolin A is a potent irreversible calmodulin antagonist (14) and that the inhibition of calmodulin could be a mode of action of the toxin in plants (15). In this report, we present evidence that ophiobolin A reacts with the ε-amino group of lysine 75 of bovine brain calmodulin. By the use of UV spectrosocopy, we have estimated the number of binding sites on calmodulin for ophiobolin A.
EXPERIMENTAL PROCEDURES

Dictyostelium discoideum Ax-3 ATCC 28236 was purchased from American Type Culture Collection. Fresh bovine brains were obtained from the Meat Laboratory, Animal Science Department, Iowa State University. Amino acids and acetylated amino acids, phenyl-sepharose 4B, Affi-gel blue, TPCK-trypsin, and TFP were obtained from Sigma. Bradford protein assay dye solution was obtained from Bio-Rad. Ophiobolin A was isolated from the growth medium of Helminthosporium maydis as previously described (14). All other chemicals were of reagent grade.

Protein preparation and assay procedures

Bovine brain calmodulin was purified to homogeneity according to the method described by Sharma and Wang (16) with the inclusion of phenyl-Sepharose affinity chromatography as described by Dedman and Kaetzel (17). Calmodulin-deficient calmodulin-dependent cyclic nucleotide phosphodiesterase was partially purified by the method described by Sharma et al. (18) with the omission of the calmodulin-Sepharose 4B affinity chromatography.

Dictyostelium discoideum Ax-3 was grown as described by Clark et al. (19). The calmodulin from this slime mold was isolated essentially according to the method described by Marshak et al. and then further purified by high pressure liquid chromatography as follows. Calmodulin fractions from a phenyl-Sepharose column were pooled together and dialyzed against 20 mM Tris-HCl, 1 mM Ca\(^{2+}\), pH 7.0, and then against
distilled deionized water in the cold. The dialyzed calmodulin was lyophilized and re-dissolved in 20 mM Tris-HCl, 1 mM imidazole, 1 mM Mg acetate, 5 mM CaCl₂, 0.1 M NaCl, pH 7.0, and then applied to a Bio-Rad TSK-DEAE-5-PW ion exchange column (75 x 7.5 mm), which was equilibrated with 10 mM imidazole, 1 mM EDTA, 0.3 M Na acetate, pH 7.0. The column was then eluted with 10 mM imidazole, 1 mM EDTA, 1.0 M Na acetate, pH 7.0, with a 30-min linear Na acetate gradient at a flow rate of 0.5 ml/min. The calmodulin was eluted at 26 min (0.9 M Na acetate) as a single peak. SDS-PAGE of the calmodulin gave a single band.

Calmodulin was assayed by the activation of the bovine brain PDE as described by Sharma and Wang (16) with modifications as described in (14). In this modification the ingredients were scaled down 1/10 of those in (16). The final reaction mixture was 0.090 ml and contained 0.0015 unit PDE, 40 mM Tris-HCl, 40 mM imidazole, 5 mM Mg acetate, 1.2 mM cAMP and various amounts of calmodulin. The cAMP was added last to start the reaction. After 30 min at 30°C, the reaction was stopped by dilution to 1 ml with cold water. The amount of Pi released was measured by the malachite green method as described by Carter and Karl (21). The wavelength used for the Pi assay was 625 nm. One unit of calmodulin is defined as that amount of calmodulin giving 50% of the maximal activation of the phosphodiesterase.

SDS-PAGE was done according to the method of Laemmli (22). Protein determinations were carried out by Bradford's Coomassie blue dye-binding method (23) with bovine serum albumin as a standard. This protein
determination agrees within 10% with UV absorbance and amino acid analysis measurements of the calmodulin concentration.

**Binding measurements**

UV measurements were performed on a Perkin-Elmer UV/Vis Model 552 spectrophotometer with a Model 561 recorder. The molar extinction coefficient for the product of reaction of the ε-amino group N-α-acetyl-L-lysine and ophiobolin A was first obtained as follows. The reaction was carried out in a quartz cuvette in 1 ml 0.1 M NaHCO₃, pH 8.1, with 53 mM N-α-acetyl-L-lysine. Ophiobolin A was added as a 20 mM solution in methanol to various final concentrations (10-50 μM). Under these conditions the reaction was completed in 15 min, as indicated by the lack of further change of the absorbance at the λmax (272 nm) of the new absorption band. Reaction with higher concentrations of N-α-acetyl-L-lysine did not increase the extent of the reaction, suggesting that all the ophiobolin A had reacted to form the product. The slope of the plot of A₂₇₂nm versus ophiobolin A concentration gave the molar extinction coefficient of the product. This molar extinction was used to calculate the amount of product formed when calmodulin was reacted with ophiobolin A. In these reactions the calmodulin protein was in 0.30 ml 20 mM Tris-HCl, 1 mM Mg acetate, 1 mM Ca²⁺, pH 7.0. Various concentrations of ophiobolin A were added as 2 mM or 20 mM solutions in methanol. The reaction mixtures were incubated at about 23°C for 9 h to let the reaction go to completion. Then the absorbance at 272 nm was measured. This absorbance was corrected for the absorbance of the calmodulin
itself and that of ophiobolin A to obtain the absorbance due to the reaction product.

**Trypsin digestion**

The procedure used was as described by Drabikowski et al. (24) and by Walsh et al. (10) except that the electrophoresis of the digested peptide mixture was performed according to the method of Laemmli (22) with 15% separation slab gel in the presence of 1 mM Ca\(^{2+}\). The ophiobolin A-treated calmodulin was prepared by treating 2 ml 4.2 μM bovine brain calmodulin with 400 μM ophiobolin A for 2.5 h at 23°C. After this treatment, the calmodulin had no activity in the PDE assay. The reacted calmodulin was then dialyzed thoroughly against distilled deionized water at 23°C. The calmodulin was lyophilized and then re-dissolved in 20 mM \((NH_4)_2CO_3\), 0.1 mM Ca\(^{2+}\), pH 8.2 for the trypsin digestion. A control calmodulin sample was treated in the same way without ophiobolin A.
RESULTS

Ophiobolin A reaction with amino acids

A reaction that is likely to happen with ophiobolin A is the formation of a Schiff base between the aldehyde group and the amino group of amino acids. To test this possibility, various amino acids were mixed with ophiobolin A at pH 8. The reaction was monitored by following the UV spectra of the reaction mixtures. The results are summarized in Table I. Ophiobolin A reacted with compounds that had free amino groups. The products had absorption maxima between 272 and 285 nm. When the amino groups of the amino acids were protected by an acetyl group as in N-α-acetyl-L-arginine, N-acetyl-DL-serine and N-acetyl-L-cysteine, there was no reaction with the guanidino, hydroxyl or sulfhydryl side-chain groups. Ophiobolin A reacts with cysteine readily. However, the product did not have a UV absorption maximum, suggesting a reaction different from Schiff base formation. This reaction required the presence of both -SH and -NH₂ groups because there was no reaction when the -NH₂ group was acetylated in the N-acetyl-L-cysteine. The formation of substituted aldimine products has been described by Schubert (25) for the condensation of cysteine and aldehydes. Bovine brain calmodulin does not have a cysteine residue, and the cysteine residue in the plant calmodulin does not have a free amino group, so the reaction between ophiobolin A and cysteine is not relevant to the reaction with calmodulin.
Table I. **UV absorbance of ophiobolin A and amino acid reactions**

Reactions were carried out with 2 volumes of 0.1 M amino acid in water, pH 8.0 with NaOH, and 1 volume 10 mM ophiobolin A in methanol. After about 1 h at 23-25°C, aliquots of the reaction mixtures were diluted 66-fold with methanol for measurement of the UV spectrum from 210 to 400 nm.

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<td>L-valine</td>
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<tr>
<td>N-α-acetyl-L-arginine</td>
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<td>no product</td>
</tr>
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<td>N-acetyl-DL-serine</td>
<td>no change</td>
<td>no product</td>
</tr>
<tr>
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<td>280 nm</td>
</tr>
<tr>
<td>N-α-acetyl-L-lysine</td>
<td>disappeared</td>
<td>272 nm</td>
</tr>
<tr>
<td>L-cysteine</td>
<td>disappeared</td>
<td>no peak in UV</td>
</tr>
<tr>
<td>N-acetyl-L-cysteine</td>
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<td>no product</td>
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The product of reaction of ophiobolin A with amino-acid amino groups is evidently a conjugated enamine, formed by tautomeric rearrangement of an initial Schiff base:

\[
R-\text{NH}_2 + O=C- \rightarrow R-N=C \rightarrow R-N-C=\]

This reaction is analogous to the reaction of amines with \( \Delta^4\)-3-ketosteroids, which produces conjugated enamines with absorption maxima near 280 nm and molar extinctions near 20,000 (26). Figure 1 shows that, at pH 7.0, 6.7 mM N-\( \alpha \)-acetyl-L-lysine reacted with 1.7 mM ophiobolin A to give a product with an absorption maximum at 272 nm, while the absorption of ophiobolin A at 238 nm decreased accordingly. In aqueous solution, the rate of this reaction is lower at lower pH (data not shown). The reaction is also faster in pure methanol than in water (data not shown). When sodium borohydride was added to the reaction mixture, the absorbance at 272 nm disappeared rapidly (Fig. 1). The reaction between ophiobolin A and N-\( \alpha \)-acetyl-L-lysine could be reversed by dilution in aqueous buffer as shown in Figure 2. The total concentrations of N-\( \alpha \)-acetyl-L-lysine and ophiobolin A after the 30-fold dilution were 622 \( \mu \)M and 57 \( \mu \)M, respectively. The reversal of the reaction can be seen by the gradual decrease of the 272 nm absorption of the product and a corresponding increase in the 238 nm absorption of ophiobolin A. This behavior is consistent with the conjugated enamine structure proposed.
Figure 1. Reaction of N-α-acetyl-L-lysine with ophiobolin A  Two ml of 0.01 M N-α-acetyl-L-lysine in 0.01 M NaHCO₃ pH 7.0 was reacted with 1 ml 5 mM ophiobolin A in methanol. At various time intervals, aliquots of the reaction mixtures were diluted 26-fold with methanol and the spectrum was taken: a) control sample, spectrum of ophiobolin A; b) 4 min; c) 10 min; d) 35 min; e) spectrum after excess 0.26 M NaBH₄ was added to the reaction mixture.
Figure 2. Reversal of reaction of ophiobolin A and N-\alpha-acetyl-
lysine methyl-ester-HCl by dilution in water
Two ml of 28 mM N-\alpha-acetyl-lysine methyl-ester-HCl in 0.1 M NaHCO₃
pH 7.0 were reacted with 1 ml 5 mM ophiobolin A in
methanol. After 4.5 h at 23°C, an aliquot of the
reaction mixture was diluted 30 times with 0.1 M NaHCO₃,
pH 7.0, and the UV spectra of the diluted reaction
mixtures were taken at: a) 5 min; b) 10 min; c) 30 min.
Reaction of calmodulin with ophiobolin A

When 33 \, \mu\text{M} ophiobolin A was added to a solution of 21 \, \mu\text{M} bovine brain calmodulin in the presence of \text{Ca}^{2+} at pH 7.0, there was a gradual increase in the UV absorbance at 272 nm (Fig. 3), very much like the reaction of ophiobolin A with the \varepsilon\text{-amino group of lysine in Figure 1. This suggested that ophiobolin A was reacting with the \varepsilon\text{-amino group of the lysyl residues in the calmodulin. This new absorption at 272 nm is dependent on the presence of \text{Ca}^{2+}. Figure 4 shows that in the absence of \text{Ca}^{2+} (in the presence of EGTA), there was only a slight initial increase at 272 nm. Part of this slight increase in absorbance was due to the absorbance by ophiobolin A. There was no further increase even after more than 1 h of incubation, suggesting that no reaction occurs in the absence of \text{Ca}^{2+}. This lack of reaction in the absence of \text{Ca}^{2+} coincides with the lack of interaction and inhibition of calmodulin by ophiobolin A as shown previously (14).

Binding experiments with calmodulin and ophiobolin A

First the molar extinction at 272 nm, \( \varepsilon_{272\text{nm}} \), was measured with N-\alpha\text{-acetyl-L-lysine as described under "Experimental Procedures." The \( \varepsilon_{272\text{nm}} \) obtained was 19,200 M\(^{-1}\) cm\(^{-1}\). This value is very similar to the molar extinction of the pyrrolidinyl enamine of progesterone, \( \varepsilon_{277\text{nm}} = 20,475 \, \text{M}^{-1} \, \text{cm}^{-1} \) (26). By using \( \varepsilon_{272\text{nm}} = 19,200 \, \text{M}^{-1} \, \text{cm}^{-1} \), the number of moles of ophiobolin A bound per mole of calmodulin is 2 (Fig. 5). When the calmodulin activity in the reaction mixture was assayed, it was found that binding of 1 mole of ophiobolin A to calmodulin was enough to eliminate all the calmodulin activity (Fig. 5).
Figure 3. **UV spectra of the reaction product of ophiobolin A and brain calmodulin**  
Bovine brain calmodulin (21 µM) was incubated at 23°C in 1 mM NaHCO$_3$, 1 mM Ca$^{2+}$, pH 7.0. One µl of 10 mM ophiobolin A in methanol was added by means of a Hamilton glass syringe to make 33 µM ophiobolin A. UV spectra were then taken: a) spectrum before the addition of ophiobolin A; b,c,d,e,f) spectra at 3.5, 10.5, 30.5, 67, and 114 min, respectively, after the addition of 33 µM ophiobolin A. Scanning speed was 60 nm/min.
Figure 4. **UV spectra of the reaction of ophiobolin A and calmodulin in the absence of Ca\(^{2+}\).** The conditions were the same as in Figure 3 except 1 mM EGTA was used instead of 1 mM Ca\(^{2+}\).  

a) Spectrum before the addition of ophiobolin A; b,c) spectra at 3.5 and 69 min, respectively, after the addition of ophiobolin A.
Figure 5. Binding and activity profiles of calmodulin and ophiobolin A. Samples with 12 μM bovine brain calmodulin in 20 mM Tris-HCl, 1 mM imidazole, 0.1 mM Ca^{2+}, pH 7.0 were incubated with various concentrations of ophiobolin A. The control sample had no ophiobolin A. The absorbance at 272 nm was measured. The concentrations of the reaction product formed were calculated and the calmodulin activity in the reacted solution was assayed as described under "Experimental Procedures." Before the calmodulin assay, aliquots from the reaction solutions were diluted 200 times. Two units of calmodulin protein were used per assay. • mole of ophiobolin A/mole of calmodulin, ○ enzyme activity, ● basal activity (the enzyme activity in the absence of calmodulin).
Ophiobolin (A)
Location of the binding site for ophiobolin A in calmodulin

The UV spectra suggest that ophiobolin A reacts with the ε-amino group of lysine residues of calmodulin. The possibility that lysine 77 may be one of the residues involved was considered because Walsh et al. (10) have reported that lysine 77 is exposed for tryptic digestion in the presence of Ca²⁺ but not in the absence of Ca²⁺. In this limited tryptic digestion, the calmodulin molecule was cleaved by trypsin at lysine 77 into two peptides. To test this possibility, we carried out the limited tryptic digestion as described by Drabikowski et al. (24) and by Walsh et al. (10). The result is shown in Figure 6. The calmodulin in the control was cleaved very readily into essentially two main peptides as shown by Walsh et al. There seems to be a third peptide present. This third peptide might be due to cleavage at lysine 75. On the other hand, the ophiobolin A-treated calmodulin is very much resistant to the tryptic cleavage. Only slight cleavage is seen after longer incubation.

TFP has been suggested to bind to calmodulin near lysine 75 (9,13). If ophiobolin A binds to the same region, the presence of TFP might interfere with the interaction between ophiobolin A and calmodulin. The results in Figure 7 show that in the presence of a 10-fold excess of TFP, the rate of ophiobolin A inhibition of the activity of calmodulin is reduced. At longer times of incubation, however, the irreversible binding by ophiobolin A prevailed over the reversible binding by TFP. The effect of fluphenazine on the action of ophiobolin A is repeatable.
Figure 6. SDS-PAGE of limited tryptic digestion of ophiobolin A-treated calmodulin. The digestion was carried out as described under "Experimental Procedures" with a concentration of 1 μg bovine brain calmodulin/μl. The numbers in the figure represent min after the addition of the trypsin. The 0-min sample was the aliquot taken just before the addition of trypsin. The digestion was stopped by putting the sample aliquot in SDS-PAGE sample buffer and heating for 3 min in a 100°C water bath. The samples were then electrophoresed. A) Control calmodulin; B) ophiobolin A-treated calmodulin. Band a is trypsin.
Figure 7. **Effect of TFP on the inhibition of calmodulin by ophiobolin A** Bovine brain calmodulin (20 μM) in 20 mM Tris-HCl, 1 mM Mg acetate, 1 mM imidazole, 0.1 mM Ca^{2+}, pH 7.0, was incubated with 1 mM TFP for 5 min at 25°C. Then, ophiobolin A was added to give a final concentration of 100 μM. Aliquots were taken from the samples before and after the addition of ophiobolin A and diluted 1000 times with 20 mM Tris-HCl, 1 mM Mg acetate, 1 mM imidazole, pH 7.0. Calmodulin assay was then done on these diluted samples with 4 units calmodulin protein used per assay.

- ●, control, no TFP, no ophiobolin A;
- ○, + TFP, no ophiobolin A;
- ▲, no TFP, + ophiobolin A;
- △, + TFP, + ophiobolin A;
- ■, basal activity.
The reversibility of TFP binding can be seen by the sample treated with 1 mM TFP only. Upon dilution, no inhibition by TFP is seen.

**Inhibition of Dictyostelium calmodulin by ophiobolin A**

To distinguish between possible reactions of ophiobolin A with lysines 75 and 77 of calmodulin, we made use of the calmodulin from the slime mold *Dictyostelium discoideum*. The *Dictyostelium* calmodulin can activate bovine brain calmodulin-dependent phosphodiesterase and shows great homology in amino acid sequence with that of bovine brain calmodulin (20). However, residue 77 in the *Dictyostelium* calmodulin is glutamine instead of lysine as in the brain calmodulin. If reaction with lysine 77 is essential for the inactivation of calmodulin by ophiobolin A, then *Dictyostelium* calmodulin should be unaffected.

Figure 8 shows that *Dictyostelium* calmodulin is also inhibited by ophiobolin A in the PDE assay. The half-maximal inhibition is at 28 μM ophiobolin A. For bovine brain calmodulin, it is at 10 μM (14). The difference between the two values might have been due to the purity of the PDE used or to the calmodulins themselves. A time course study of the inhibition by ophiobolin A of both *Dictyostelium* and bovine brain calmodulins showed that the rate of inhibition of the *Dictyostelium* calmodulin is similar to that of bovine brain calmodulin and that the inhibition of the *Dictyostelium* calmodulin is also not reversed by dilution (Fig. 9).
Figure 8. Inhibition of Dictyostelium calmodulin by ophiobolin A

Four units (22 ng) of Dictyostelium calmodulin were incubated with the PDE assay mixture with various concentrations of ophiobolin A for 30 min at 30°C. Then, cAMP was added to start the assay as described under "Experimental Procedures." ■, basal activity.
Figure 9. Time course studies of the inhibition of calmodulin by ophiobolin A. Dictyostelium or bovine brain calmodulin (4.9 µg) was incubated in 0.1 ml 20 mM Tris-HCl, 1 mM imidazole, 1 mM Mg acetate, 1 mM Ca$^{2+}$, pH 7.0, with 100 µM ophiobolin A at 25°C. Aliquots (10 µl) were taken at various times and diluted more than 200 times immediately in the buffer, with no Ca$^{2+}$, to stop the reaction. Calmodulin was then assayed in the diluted reaction mixtures, using 3 units of calmodulin protein for each assay (13.2 ng for bovine brain calmodulin, 15.0 ng for Dictyostelium calmodulin).

Dictyostelium calmodulin: •, + methanol (control), ○, + ophiobolin A.

Bovine brain calmodulin: ▲, + methanol, △, + ophiobolin A.

■, basal activity.
Hours of incubation
DISCUSSION

The UV spectra of the products of reaction of ophiobolin A and amino acids or amino acid derivatives suggest that the products are conjugated enamines formed by rearrangement of Schiff bases. This conclusion is supported by the observation that the reaction is reversed by dilution in water, is faster at higher pH or in methanol, and the product can be reduced by sodium borohydride. The reaction of ophiobolin A with calmodulin forms a product with a similar UV spectrum, suggesting that the reaction occurs at the ε-amino group of one or more lysine residues. Assuming the molar extinction of the product of reaction with calmodulin is the same as that of the product of reaction with N-α-acetyl-L-lysine, bovine brain calmodulin reacts with two moles of ophiobolin A per mole of protein but is inactivated after reaction with one mole (Fig. 5).

It seems likely that ophiobolin A reacts with bovine brain calmodulin at lysines 75 and 77, with reaction at 75 being sufficient to inactivate the protein. This suggestion is based on several pieces of evidence. TFP has been suggested to bind in the region of lysines 75 and 77 (9, 13), and the interference by TFP in the inhibition of calmodulin by ophiobolin A (Fig.7) suggests that ophiobolin A reacts in the same region. The resistance of ophiobolin A-treated calmodulin to tryptic cleavage (Fig. 6) suggests that the lysine residue(s) accessible to trypsin is(are) blocked by ophiobolin A. Tryptic cleavage of the native protein occurs at lysine 77 (10).
Dictyostelium discoideum calmodulin, in which lysine 77 is replaced by glutamine, also is inactivated by ophiobolin A. In mammalian calmodulins, lysine 75 seems more reactive than lysine 77 with various antagonists (12, 13, 27). Lysine 75 is adjacent to an arginine residue, which may have the effect of lowering the pKa of the lysine (28) and thus increasing its reactivity at neutral or slightly acidic pH. Calmodulin reacts readily with ophiobolin A both at lower concentrations and at lower pH than required for rapid reaction with N-α-acetyl-L-lysine. In the experiments reported here, calmodulin was reacted at pH 7.0, but it reacts readily in cacodylate buffer at pH 5.2 (Leung and Tipton, unpublished experiments). The rate of reaction with N-α-acetyl-L-lysine drops rapidly below pH 7.0.

Another possible location for reaction of the second mole of ophiobolin A with calmodulin is lysine 148, the C-terminus. A preliminary report by Jackson and Puett (27) shows that an acylating spin label reagent reacts with lysines 75 and 148 of bovine brain calmodulin. The two residues at the C-terminus of Dictyostelium calmodulin are arginine and asparagine, which will not react with ophiobolin A. This further supports the suggestion that the site for the first mole of ophiobolin A is lysine 75.

The reaction of ophiobolin A with N-α-acetyl-L-lysine is readily reversible (Fig. 2) whereas the reaction with calmodulin is not reversed, even under denaturing conditions, such as treatment with SDS (15) or dilution in 8M urea (14). This suggests that, in addition to
formation of an enamine by reaction of the aldehyde with a protein amino group, ophiobolin A undergoes further interaction with the protein. Lysine 75 and 77 are both in the central helix of calmodulin, a region exposed to solvent and making few contacts with the rest of the molecule, according to the structure determined by X-ray crystallography (29). It is plausible to expect these residues to react with reagents such as ophiobolin A, but an understanding of why this leads to an irreversible inactivation of calmodulin will require a determination of how the remainder of the ophiobolin A molecule interacts with the protein.
REFERENCES


SUMMARY AND DISCUSSION

In nature, organisms have developed a number of ways to get food for their own survival. The green plant and the autotrophic bacteria make their own food from simple inorganic compounds and sunlight. All other organisms depend on other organisms. Animals usually get their food by capturing other organisms. Among pathogenic organisms, some get their food by invading the cells of the host and absorb nutrients inside the host. Some produce toxins to kill the host. The fungus H. maydis produces toxins to make the cells of the host leak and then feeds on the cellular materials leaked out of the cells. Therefore, the toxins are the tools the fungus uses to get food, but from another point of view, these toxins are poisons to the plants.

In this study on the mode of action of ophiobolin A, we have found evidence that suggests that the physiological effect of the toxin may be due to its effect on calmodulin. Several correlations are found. First, both the inhibition of calmodulin in vitro and the induction of ion-leakage from corn roots by ophiobolin A are time-dependent. Second, both events are irreversible. Third, the effective concentration of ophiobolin A for calmodulin inhibition and for leakage induction are comparable. Both are in the micromolar range. Fourth, the similar behavior of the analogues of ophiobolin A in both calmodulin inhibition and ion-leakage induction also suggests a relation exists. Fifth, a smaller amount of active corn calmodulin is isolated from ophiobolin A-treated roots than from untreated roots. Furthermore, the inhibition of
Calmodulin can explain the physiological effect of ophiobolin A. A plausible consequence of the inhibition of calmodulin by ophiobolin A in vivo is that calmodulin-dependent enzymes such as the calmodulin-dependent Ca^{2+}-transport ATPase will be affected. This will lead to transport defects and therefore may account for the leakage observed in ophiobolin A-treated roots. The inhibition of calmodulin also explains the non-host specificity of the toxin.

The question as to whether ophiobolin A affects calmodulin only has not been fully investigated. We have shown that it is quite specific for calmodulin, as ophiobolin A inhibits calmodulin at a low concentration and does not inhibit the phosphodiesterase or the 5'-nucleotidase. However, tests with more proteins and enzymes are required to give a better evaluation. The specificity of ophiobolin A for calmodulin is weakened by the initial observations by Orsenigo (6) and Nakamura and Ishibashi (7) that ophiobolin A can inhibit the growth of bacteria, such as E. coli and B. subtilis. Although calmodulin-like proteins have been reported in the two bacteria, the presence of calmodulin in bacteria has not yet been confirmed. This suggests that ophiobolin A acts on other cellular component(s) than calmodulin in bacteria.

The interaction of calmodulin and ophiobolin A was further characterized by in vitro studies. Using bovine brain calmodulin as the model calmodulin, the interaction of calmodulin and ophiobolin A can be shown by the inhibition of the calmodulin-activation of the
phosphodiesterase, the quenching of the intrinsic tyrosine fluorescence of calmodulin and the appearance of a new absorption maximum at 272 nm for the reaction product. This UV absorption maximum is the same as that of the conjugated enamine product between the aldehyde group of ophiobolin A and the ε-amino group of N-α-acetyl-L-lysine. It is therefore concluded that a conjugated enamine product is also formed in the ophiobolin A-calmodulin reaction. However, the reaction to form the conjugated enamine product of ophiobolin A and N-α-acetyl-L-lysine is reversible whereas that between ophiobolin A and calmodulin is irreversible. This seems to suggest that there is further reaction than the conjugated enamine formation in the protein. What this further reaction is is not clear yet.

The molar extinction of the enamine product between ophiobolin A and N-α-acetyl-L-lysine is found to be 19,200 M$^{-1}$ cm$^{-1}$. Applying this to the calmodulin-ophiobolin A reaction, we found that 2 moles of ophiobolin A reacts with one mole of calmodulin and that the binding of 1 mole of ophiobolin A will abolish the calmodulin activity. The binding site on the calmodulin for the first molecule of ophiobolin A is probably lysine 75 because lysine 77 is shown to be not essential for the interaction by the use of Dictyostelium calmodulin. Dictyostelium calmodulin has glutamine instead of lysine at residue 77 and yet is inhibited by ophiobolin A. Also, the physical situation of lysine 75 suggests that lysine 75 is more reactive than lysine 77. The charge on arginine 74 would very likely decrease the pK of the ε-amino group of
lysine 75, making lysine 75 more reactive at lower pH than a free lysyl ε-amino group. Further experiments are needed to confirm the location of this binding site. This can be done by peptide mapping. The ophiobolin A-treated calmodulin is first digested into peptide fragments. These peptide fragments are then isolated by high performance liquid chromatography. The molar extinction at 272 nm may be used as the chromophore in identifying the ophiobolin A-peptide. The ophiobolin A-peptide can then be sequenced or its amino acid composition analyzed. The location on the protein can then be found.

There are some interesting consequences for the reaction between ophiobolin A and the ε-amino group of lysine residue. They are as follows.

(1) The reaction seems to suggest that other compounds with aldehyde groups will also react with calmodulin. However, we have found that this is not quite the case. We have tried to react calmodulin with all-trans retinal and pyridoxal. All-trans retinal reacted at high concentration, with a half-maximal inhibition at about 800 µM retinal in the phosphodiesterase assay. Pyridoxal does not inhibit the calmodulin activation in the phosphodiesterase assay even at 1.25 mM pyridoxal. Therefore, it seems the reaction between the ε-amino group of the lysine and aldehyde requires some more factors than the presence of the reactive groups.

(2) Proton NMR experiments by Klevit et al. (185) and Dalgarno et al. (186) showed that the binding site for trifluoperazine is made up of
methyl groups of methionine and aromatic side chains. Walsh and Stevens (187) have shown that modification of methionines 71, 72, 76 and possibly 109 of calmodulin abolished the calmodulin activity. Lysine 75 is surrounded by methionines 71, 72, and 76. It seems these three methionines are important and that the trifluoperazine binding site may have them as components. It would be interesting to see how an ophiobolin A molecule interacts with both lysine 75 and the putative hydrophobic binding site. Work is being done along this line by means of X-ray crystallography of the ophiobolin A-calmodulin complex.

(3) A relation may exist between the quenching of the intrinsic tyrosine fluorescence by ophiobolin A and the appearance of a UV absorption maximum at 272 nm in the calmodulin-ophiobolin A product. The quenching might have been due to the absorption by the new chromophore. The excitation wavelength in the fluorescence experiments was 278 nm, very close to the UV maximum of the new chromophore.

It will also be interesting to find out how the fungus H. maydis copes with the toxicity of ophiobolin A. Calmodulin activity has been detected in a partially purified extract of H. maydis by the phosphodiesterase assay. This activation of the phosphodiesterase by the fungal extract is abolished by the addition of 50 μM ophiobolin A, suggesting that the calmodulin in the fungus is also susceptible to ophiobolin A and that other mechanisms must be present to protect the fungus from the toxicity of ophiobolin A. Ophiobolin A is probably synthesized in the cell and then secreted out of the cell. Therefore,
the fungal cell must have a means of preventing ophiobolin A coming into contact with the cellular calmodulin. Once outside the cell, there must also be another mechanism to prevent the ophiobolin A from re-entering the cell. These mechanisms are not known yet.
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