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# Role of Calmodulin Inhibition in the Mode of Action of Ophiobolin A[1]

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## 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.**

Ophiobolin A is a non-host-specific phytotoxin first isolated from *Helminthosporium oryzae*, the fungus that causes brown spot disease of rice (15, 17). It is one of a series of closely related sesterterpenes, whose occurrence and properties have been reviewed (2, 8). 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 (19). 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* (16).

The mode of action of ophiobolin A has not been established. Cocucci *et al.* (7) reported rapid effects on membrane potential and $K^+$ efflux from maize roots. We have shown (12) that ophiobolin A reacts *in vitro* with bovine brain and spinach calmodulins so they are unable to activate PDE[4]. The time course of the inhibition of calmodulin was much slower than the responses reported by Cocucci *et al.* (7). In this report, we present evidence that the toxic effects of 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 × Mo17Ht) and from Dr. C. A. Martinson, Iowa State University (W64A N and T cytoplasms). Fluphenazine-2 HCl 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 Springs Instrument Co.; and Willems Polytron 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 $CaCl_2$ and 0.5 mM KCl. The roots were excised and rinsed five times with cold deionized, distilled $H_2O$ and then homogenized in a Waring Blendor 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 four 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 $Mg(acetate)_2$, 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 $Mg(acetate)_2$, 1 mM imidazole, 0.5 mM $Ca^{2+}$, pH 7.0). The dialyzed calmodulin solution was then applied to a 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. Cal-

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[4] Abbreviations: PDE, bovine brain calmodulin-dependent cAMP phosphodiesterase; PMSF, phenylmethylsulfonyl-fluoride.

modulin was then eluted from the column with 20 mM Tris-HCl, 1 mM $Mg(acetate)_2$, 1 mM imidazole, 0.5 M NaCl, 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 Charbonneau and Cormier (5). Protein determination was by Bradford's Coomassie Blue dye-binding method (1) using BSA as a standard. SDS-PAGE was done according to the method of Laemmli (11) using 12% separation slab gel.

**Protein Preparation.** Bovine brain calmodulin was isolated according to Sharma and Wang (18), and the use of fluphenazine-Sepharose 4B affinity chromatography was included. PDE was prepared from bovine brain as described by Ho *et al.* (9).

**Calmodulin Assay.** Calmodulin was assayed according to Sharma and Wang (18) 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.* (12). Purified ophiobolin A (Tm 181–182°C, lit. 181°C [3]) 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-Anhydro-ophiobolin A (Tm, 134–135°C, lit. 135°C [3]) 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 as described by Morisaki *et al.* (14). One equivalent of $Br_2$ in methanol was added in small portions to a stirred 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 four times with an equal volume of methylene chloride. The combined methylene chloride extract was dried with anhydrous $Na_2SO_4$ and then concentrated by a stream of $N_2$. 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% $H_2O$ in methylene chloride (v/v). The fractions containing bromomethoxy ophiobolin A were pooled, and dried, and then rechromatographed twice on silica gel columns prewashed and equilibrated with *n*-hexane. The elution solvent was 20% (v/v) acetone in *n*-hexane. Pure bromomethoxy ophiobolin A solidified on evaporation of the solvent (Tm 155°C; lit. [14] 155–157°C.

The preparation of 6-epi-ophiobolin A was as suggested by Canonica and Fiecchi (2). 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 three times with deionized distilled $H_2O$. 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 (Tm, decomposition, 110–119°C). 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 $^1H$ NMR, UV, and IR spectra, and exact mass determinations, which agree very well with published data (2, 3, 14).

**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 five times with deionized distilled $H_2O$. They were then suspended in 50 ml deionized distilled $H_2O$ and incubated for 1 h at 30°C before the addition of ophiobolin A or derivatives. The beakers were covered to reduce 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 root 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 $H_2O$ 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 four 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 one 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/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 (13). 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.

**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 (12). 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 (12), 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 a lower electrophoretic mobility than untreated calmodulin, even though the denaturing conditions 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

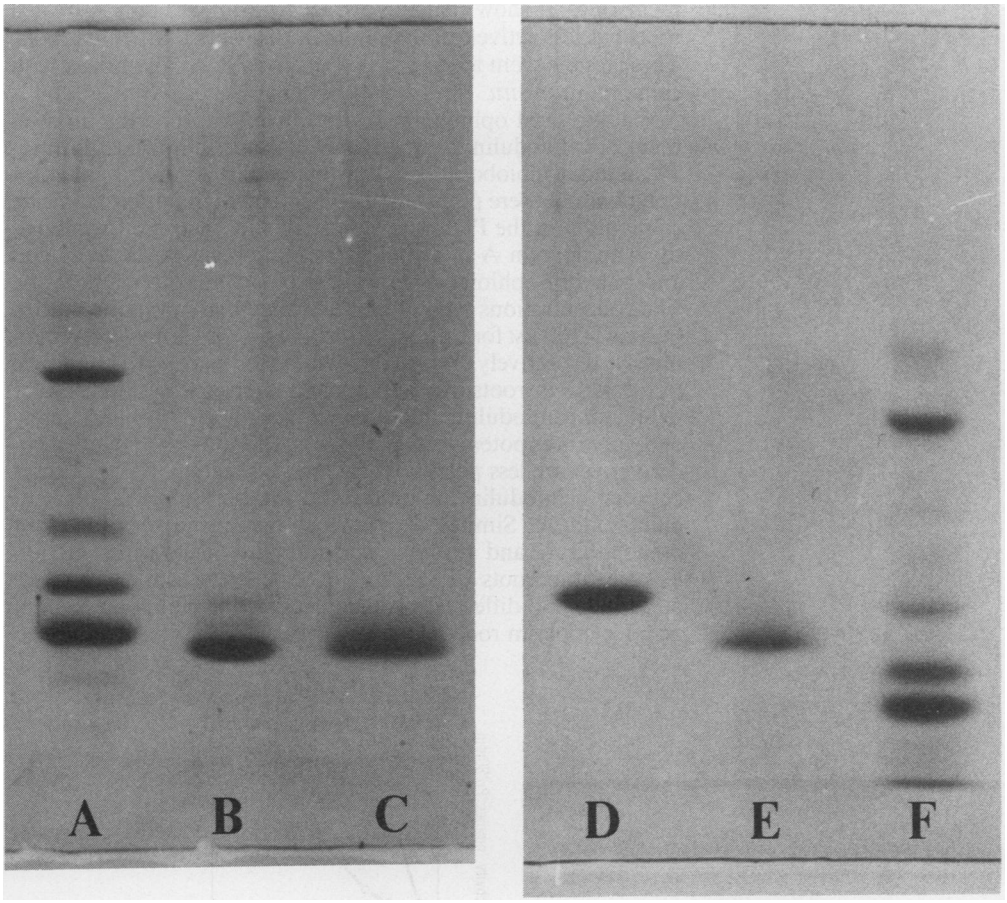


FIG. 1. SDS-PAGE of calmodulin isolated from maize seedling roots. Left, Electrophoresis in the presence of 1 mM $Ca^{2+}$; right, Electrophoresis in the presence of 1 mM EGTA. Lane B, 6 μg bovine brain calmodulin; C, 7 μg maize root calmodulin; D, 5 μg bovine brain calmodulin; E, 3 μg maize root calmodulin; A and 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).

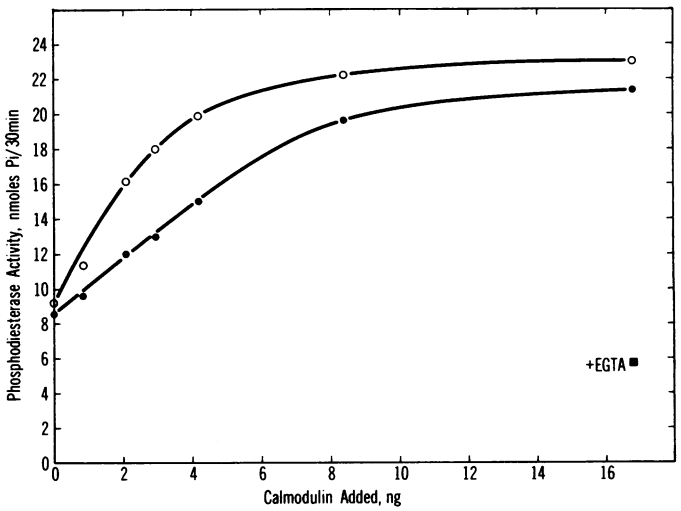


FIG. 2. Activation of PDE by calmodulins from maize root and bovine brain. The assay procedure was as in Sharma and Wang (18) with the ingredients scaled down 1/10 of those described. The final reaction mixture is 0.090 ml and contains 0.0015 unit PDE, 40 mM Tris-HCl, 40 mM imidazole, 5 mM $Mg(acetate)_2$, 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% w/v TCA were added to stop the reaction. The amount of Pi released was measured by the malachite green method (4). Maize calmodulin (●); bovine brain calmodulin (○); maize calmodulin in the presence of 2.2 mM EGTA instead of $Ca^{2+}$ (■).

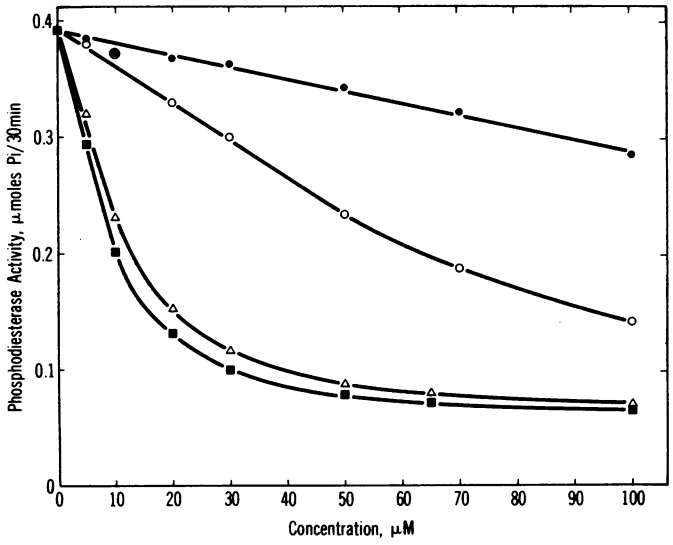


FIG. 3. Inhibition of maize calmodulin by ophiobolin A and derivatives, measured by the loss of the ability to stimulate PDE. The assay procedure was exactly as in Sharma and Wang (18). Each assay mixture contained 4 units of maize calmodulin, with 1 unit defined as the amount necessary for half-maximal 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 (○), ophiobolin A (△), 18-bromo-19-methoxyophiobolin A (■).

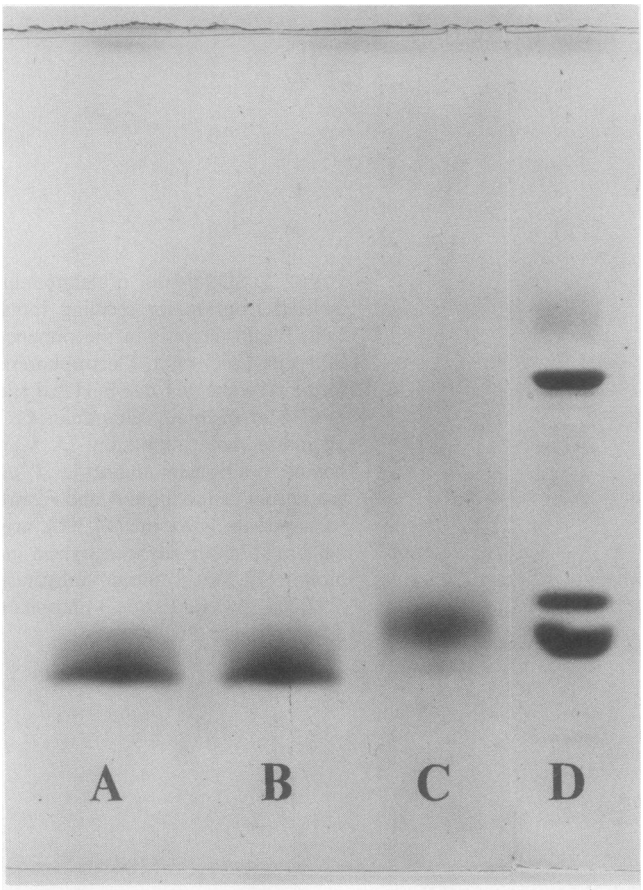


FIG. 4. SDS-PAGE of maize calmodulin after treatment with ophiobolin A. Electrophoresis of 10 μg samples of maize calmodulin in the presence of 0.1 mM $CaCl_2$. Pretreatments of the calmodulin samples, for 1.5 h at 30°C in the presence of 0.1 mM $CaCl_2$: A, no additions; B, 2.5% methanol; C, 0.25 mM 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, 2 units per sample, in buffer A was incubated with the additions shown, total volume of 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 was 1% (v/v), ophiobolin A was 0.1 mM, and EGTA was 6 mM.

| Additions | PDE Activity | |
|---|---|---|
| | *nmol Pi/30 min* | % |
| None (control) | 10.3 | 100 |
| $CaCl_2$ | 22.8 | 221 |
| $CaCl_2$, methanol | 23.4 | 227 |
| $CaCl_2$, methanol, ophiobolin A | 10.8 | 105 |
| EGTA, methanol, ophiobolin A | 20.6 | 200 |

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.

***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 (Fig. 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 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).

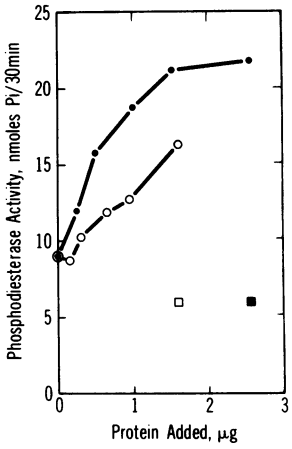


FIG. 5. Inhibition of calmodulin in excised maize seedling roots by ophiobolin A. Control roots soaked in deionized water with equivalent amount of methanol (●); ophiobolin A-treated roots (○); PDE assay with the above two root extracts plus EGTA instead of $Ca^{2+}$ (■, □).

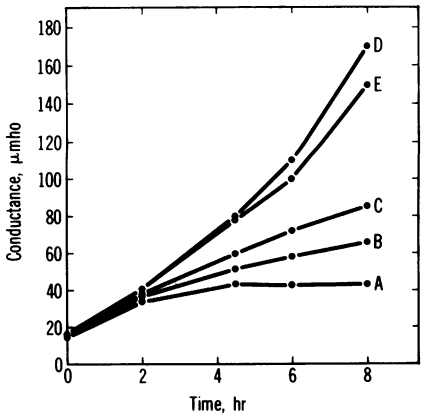


FIG. 6. Stimulation of ion leakage from maize seedlings by ophiobolin A and derivatives. A, Control with 0.1 ml methanol; B, 6-epi-ophiobolin A; C, 3-anhydro-ophiobolin A; D, 18-bromo-19-methoxyophiobolin A; E, ophiobolin A.

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 "Materials 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.

| | Conductivity at Following Times (h) | | | | | |
|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 4 | 6 | 8 |
| | *μmho* | | | | | |
| Control | | | | | | |
| N cytoplasm | 8 | 24 | 30 | 46 | 58 | 69 |
| T cytoplasm | 10 | 29 | 39 | 63 | 75 | 85 |
| Ophiobolin A | | | | | | |
| N cytoplasm | 8 | 26 | 41 | 90 | 130 | 160 |
| T cytoplasm | 6 | 29 | 45 | 95 | 120 | 136 |
| Anhydroophiobolin A | | | | | | |
| N cytoplasm | 9 | 28 | 42 | 72 | 97 | 125 |
| T cytoplasm | 7 | 30 | 41 | 74 | 97 | 125 |
| Epi-ophiobolin A | | | | | | |
| N cytoplasm | 8 | 26 | 34 | 48 | 56 | 68 |
| T cytoplasm | 6 | 21 | 30 | 48 | 56 | 66 |

## DISCUSSION

The ubiquitous $Ca^{2+}$-binding protein calmodulin has been isolated from many plants, including *Zea mays* (10; for review, see 13). In this study we have shown that 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 (12). 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. First, 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 (19). Second, the rate of calmodulin inhibition agrees with that of ion leakage. Calmodulin inhibition *in vitro* is nearly complete in 1 h. 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 *in situ*. Third, the concentrations of ophiobolin A required for the inhibition of calmodulin *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 μM. In physiological measurements, 25 μM ophiobolin A inhibited by 85% the uptake of 0.5 mM 2-deoxyglucose by maize root (19). Fourth, 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 *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 produces the host-specific HM-T toxin, we tested three of the compounds for host-specific effects on ion leakage using N and T cytoplasm seedlings. 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.

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