Ophiobolin A

A NATURAL PRODUCT INHIBITOR OF CALMODULIN

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Ophiobolin A, a fungal metabolite and a phytotoxin which can stimulate the net leakage of electrolytes and glucose from maize seedling roots (Tipton, C. L., Paulsen, P. V., and Betts, R. E. (1977) Plant Physiol. 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 has been 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 was 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 Ca2+ than in those containing EGTA. Ophiobolin A also was shown to inhibit 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.

Ophiobolin A (Structure 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.

The Ca2+-binding protein calmodulin is ubiquitously distributed in eukaryotes. It has been implicated in the Ca2+-dependent regulation of many biological processes. (For reviews see Refs. 8–11.) In plants, calmodulin has been shown to function in the Ca2+-dependent activation of NAD kinase and in ATP-dependent Ca2+ 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 Ca2+-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 Ca2+-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 H. 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 h 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 liters of culture filtrate. The crude crystals were purified by silica gel column chromatography. Approximately 200 mg of crude crystals was dissolved in methylene chloride (1 ml) and applied to a silica gel column (2.2 × 30 cm) (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 of pure ophiobolin A was obtained from 4 liters of culture filtrate (m.p. 181–182 °C).

Anhydro-ophiobolin A was obtained as a by-product from the
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Ophiobolin A preparation. The two substances were separated on the silica gel column, anhydro-ophiobolin A eluted ahead of ophiobolin A. The sample of anhydro-ophiobolin A was pooled, dried, and then rechromatographed on silica gel columns developed with 20% (v/v) acetone in hexane. Anhydro-ophiobolin A is the first material eluted, and it crystallizes upon evaporation of the solvent (m.p. 134-135°C). 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 fluorescein-labeled Sepharose 4B affinity chromatography in the isolation of the calmodulin. The fluorescein-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 activity was coupled to the 5'-nucleotidase (Crotalus atrox venom) reaction, and the amount of inorganic phosphate released represented the activity of the phosphodiesterase. One unit of calmodulin was defined as that amount giving 60% 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 mMimidazole, pH 7.0 (buffer A), prior to the enzyme assay at 30°C for 1 min, followed by the addition of lina 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 a Perkin-Elmer MPF-44 fluorescence spectrophotometer. Excitation and emission spectra were first obtained and the maximum wavelengths chosen as 280 and 307 nm, respectively. The calmodulin and Ca2+ (or EGTA³⁻) 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, at 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 maxima. The UV absorbances of 100 μM ophiobolin A and 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—Whole spinach plants 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, and the cold, and was strained through cheese-cloth. The filtrate was centrifuged at 10,000 X g for 20 min 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 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 a fluorescein-Sepharose 4B column preequilibrated with buffer D. The column was then washed with buffer D containing 0.5 M sodium chloride until no protein was 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 Ca²⁺.

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 1 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 of 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 phytotoxie 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).

Fig. 1. Effect of ophiobolin A and anhydro-ophiobolin A on calmodulin-dependent cyclic nucleotide phosphodiesterase.

Four units (10 pmol) of calmodulin (Mr = 16,200) was incubated with about 0.015 unit of calmodulin-deficient, calmodulin-dependent phosphodiesterase for 5 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 (1) and anhydro-ophiobolin A (O) 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 of 10.8 mM 3-AMP, pH 7.0, was added to start the assay. After 30 min the assay was stopped by the addition of 0.1 ml of 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 Ca²⁺ was 0.135.

1 The abbreviation used is: EGTA, ethylene glycol bis(P-aminoethyl ether)-N,N',N''-tetraacetic acid.

2 P. C. Leung and C. L. Tipson, unpublished observations.
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 residues are sensitive to the protein conformation (20). Chemical modification and other spectroscopic studies (21–23) have led to suggestion, an attempt was made to demonstrate the direct 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 were 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 to 100 \mu M. The results in Fig. 4 indicate that there were two phases in these time courses: an instantaneous quenching followed by a slowly increasing quenching. The second phase was not completed even after incubation of the protein with ophiobolin A for over 1 h. 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 very 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 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 Ca^{2+}. However, there was practically no further fluorescence change. The results suggest that the second phase of fluorescence quenching is highly Ca^{2+}-dependent.

**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^{-44}, 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. 5). Similar agreement was also obtained with 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 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 observed in the presence of Ca^{2+}. However, there was practically no further fluorescence change. The results suggest that the second phase of fluorescence quenching is highly Ca^{2+}-dependent.

**Fig. 5. Effect of Ca^{2+} on the quenching of calmodulin fluorescence by ophiobolin A.** Samples with 4 μM calmodulin in 2 ml of buffer B with, 0 μM (a), 100 μM (b), ophiobolin A, in 0.1 mM EGTA, or 100 μM (c) ophiobolin A in 0.1 mM Ca^{2+}. Conditions and procedures for the measurements are as described in the legend to Fig. 4.

**Fig. 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 described in the legend to Fig. 4.

**Fig. 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) of buffer B 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 of protein/ml and assayed for calmodulin activity as outlined in Fig. 1, omitting the addition of ophiobilins. 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.
of the second phase of fluorescence change in Fig. 4. In addition, incubation of calmodulin and ophiobolin A in the presence 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 and methanol (25). If the binding between ophiobolin A and calmodulin were of a noncovalent 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 renature 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.

**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.
Ophiobolin A Inhibition of Calmodulin

DISCUSSION

A variety of pharmacological agents including phenothiazine antipsychotic drugs (15), local anesthetics (27, 29), muscle relaxants (29), and drugs for treatment of neoplasms (30), and other chemical agents such as Triton X-100 (31) and 2-p-toluidinylnaphthalene-6-sulfonate (32) have been reported to inhibit calmodulin action mostly by undergoing 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 but 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 Ca\(^{2+}\). It is not reversible by dialysis, dilution, or urea denaturation in the presence of methanol, followed by renaturation. This irreversibility suggests that the inhibition involves covalent modification of the protein. If this is 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.

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