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Inhibition of Phytoalexin Biosynthesis in Elicitor-Treated Tobacco Cell-Suspension Cultures by Calcium/Calmodulin Antagonists[1]

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ABSTRACT

A role for calcium/calcium-binding proteins in a mechanism of signaling elicitor-inducible phytoalexin biosynthesis was investigated. Two classes of calcium/calmodulin antagonists, phenothiazines and naphthalenesulfonamides, inhibited sesquiterpene phytoalexin accumulation in tobacco (*Nicotiana tabacum*) cell-suspension cultures when added 1 h before elicitor. The antagonists also inhibited the induction of sesquiterpene cyclase enzyme activity, a key regulatory enzyme for sesquiterpene biosynthesis. The antagonists suppressed the induction of sesquiterpene cyclase only if added before or simultaneously with elicitor. Additionally, the antagonists inhibited (a) accumulation of the cyclase protein as measured in immunoblots; (b) the in vivo synthesis rate of the cyclase protein, measured as the incorporation of [^{35}S]methionine into immunoprecipitable cyclase protein; and (c) the cyclase mRNA translational activity, measured as the incorporation of [^{35}S]-methionine into immunoprecipitable cyclase protein synthesized by in vitro translation of RNA isolated from antagonist-treated, elicitor-induced cells. In contrast, elicitor-inducible phenylalanine ammonia lyase enzyme activity, the level of the enzyme protein, the in vivo synthesis rate, and the mRNA translational activity were not affected by any of the antagonist treatments. Uptake and incorporation of [^{35}S]methionine into total cellular proteins and total in vitro translation products were also not indiscriminately altered by the antagonist treatments. The current results suggest that calcium and/or calmodulin-like proteins may be elements of a signal transduction pathway mediating elicitor-induced accumulation of phytoalexins in tobacco.

Plants can respond to pathogen challenge by the induction of a number of biochemical responses (3), including phytoalexin accumulation (11, 13), synthesis and secretion of hydrolytic enzymes (4), and the putative reinforcement of their cell walls with lignin and/or structural glycoproteins (32). The induction of such responses purportedly involves recognition between pathogen and plant, and the subsequent transduction of that recognition phenomenon into an activation of the defensive response. Evidence for recognition specificity has recently been obtained in experiments demonstrating specific elicitors (11), factors of pathogen or host origin that elicit specific defensive responses, and receptors, host factors that bind a specific elicitor (10, 30). However, how such recognition events might be transduced into an intracellular signal eventually leading to the activation of an inducible defense response is currently unknown (12).

Calcium has been implicated as a second messenger in a number of signal transduction pathways. In animal systems, recognition events can lead to transient increases in the intracellular levels of calcium, and the additional calcium can subsequently bind to and activate calcium-binding proteins, including calmodulin (1). The calcium/calmodulin complexes can, in turn, activate other proteins, including protein kinases, which are important for transcriptional regulation (19). No comparable evidence for calcium and calmodulin in a signal transduction pathway in plants is currently available. Nonetheless, calcium and calmodulin are abundant in plants and have been implicated in a number of signal transduction pathways (14, 17, 25, 27, 28).

Particularly relevant to our investigation, Kurosaki et al. (22) observed an inhibition of phytoalexin accumulation in pectic elicitor-treated carrot (*Daucus carota*) cells if calcium antagonists were used. Zook et al. (40) and Stäb and Ebel (33) provided evidence for the necessity of calcium in the elicitation of phytoalexins in potato (*Solanum tuberosum*) and soybean (*Glycine max*), respectively. In contrast, Kendra and Hadwiger (20) did not find compelling evidence for an involvement of calcium or calmodulin in the elicitation of pisatin accumulation in pea (*Pisum sativum*). More recently, Knight et al. (21) demonstrated that elicitation of tobacco (*Nicotiana tabacum*) resulted in a rapid but transient increase in intracellular calcium levels. None of these studies, however, investigated whether calcium/calcium-binding proteins interacted directly with enzymes for phytoalexin biosynthesis or possibly affected the expression of the genes coding for these enzymes.

Tobacco plants challenged with a variety of pathogens accumulate large amounts of sesquiterpene phytoalexins (8). Similar sesquiterpenoids are synthesized and secreted into the media of tobacco cell-suspension cultures upon addition of cellulase (9) or fungal cell wall fragments prepared from

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Phytophthora parasitica (8). Previous work correlated the induction of sesquiterpenoid accumulation with an induction of a sesquiterpene cyclase, 5-epi-aristolochene synthase (34, 36). Regulation of the cyclase enzyme activity was recently demonstrated to be controlled primarily by the transcription rate of the respective gene (37). With calcium/calmodulin antagonists, the results presented herein suggest that calcium and/or calmodulin-like proteins may be elements of a signal transduction chain mediating elicitor-induced accumulation of sesquiterpenoids. Specifically, evidence for calcium and/or calcium-binding proteins regulating the level of the cyclase mRNA translational activity is presented.

MATERIALS AND METHODS

Materials

[^{35}S]Methionine (106 Ci/mmol) was purchased from ICN. *trans,trans*-[1-^{3}H]Farnesyl diphosphate (87 mCi/mmol) was synthesized according to the method of Schechter and Bloch (29) and Popjak et al. (26). W7[4], W5, TFP, and CP were purchased from Sigma Chemical Co.

Cell Cultures and Treatment with Elicitor and Calcium/Calmodulin Antagonists

Cell-suspension cultures of *Nicotiana tabacum* cv KY 14 were maintained in Murashige-Skoog medium as previously described (8). Cell cultures in their rapid phase of growth, corresponding to 3 d after subculturing, were used for all the experiments presented. Elicitor treatment was initiated by the addition of 2 to 2.5 μg of Glc equivalents of fungal cell wall hydrolysate per mL of cell culture. In preliminary experiments, the amount of elicitor used was optimized for maximal capsidiol production and induction of cyclase enzyme activity. The elicitor was prepared from the cell walls of *Phytophthora parasitica* according to the method of Ayers et al. (2). The calmodulin antagonists were added to the cell cultures 1 h before the initiation of the elicitor treatment, except when otherwise indicated. W7 and W5 were dissolved in water and used as a 20 mM stock solution; TFP and CP were prepared in DMSO, resulting in concentrations of the stock solutions of 100 and 40 mM, respectively. In some experiments, mock treatment of the cell cultures included the addition of DMSO to measure any effects of this solvent on the cell culture responses to elicitor treatment and the antagonists.

During the course of this work, Dr. Tom Vanaman (Biochemistry Department, University of Kentucky) also cautioned us that the calmodulin antagonists were thought to undergo chemical modifications in their crystalline state as well as in solution. Hence, the antagonists were routinely obtained from Sigma Chemical Co., and the latter experiments were designed to maximize comparison between one another.

[4] Abbreviations: W7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide; W5, *N*-(6-aminohexyl)-1-naphthalenesulfonamide; TFP, trifluoperazine; CP, chlorpromazine; PAL, phenylalanine ammonia lyase.

Capsidiol Determinations

Capsidiol was extracted from the cell culture media with chloroform and was evaluated qualitatively by TLC (8). The level of capsidiol was quantified by GC (8) for only those samples positive for capsidiol in the TLC assay.

Enzyme Assays

Cyclase enzyme activity was measured as previously described in detail (37). PAL enzyme activity was determined according to published methods (39). Protein concentrations were determined by the Bradford method (6) with BSA as the standard.

In Vivo Synthesis of Proteins

[^{35}S]Methionine (50 μCi) was added to 10 mL of appropriately treated cell cultures. After a 2-h incubation, cells were harvested. Cyclase and PAL enzyme activities and protein content were measured in the freshly prepared extracts. The amount of radioactivity incorporated was measured by scintillation spectrophotometry of TCA precipitates. Equal amounts of incorporated radioactivity (100,000 dpm) were analyzed directly by SDS-PAGE/fluorography (5,23). Aliquots of 300,000 dpm were used to determine the amount of radioactivity incorporated into immunoprecipitable cyclase and PAL protein.

RNA Isolation and in Vitro Translation

Total RNA was isolated according to the method of Vögeli-Lange et al. (38). Total RNA (12 μg) was used in a 15-μL in vitro translation assay containing 10 μCi of [^{35}S]methionine. The incorporation of radioactivity into TCA-precipitable material was determined in 1-μL aliquots. Samples containing equal amounts of TCA-precipitable radioactivity (160,000 dpm) were used for immunoprecipitations, and samples containing 60,000 dpm were analyzed for the total in vitro translation products by SDS-PAGE/fluorography (5, 23).

Immunological Techniques

Immunoblot analysis of proteins separated by SDS-PAGE and specific immunoprecipitation of the radioactive cyclase and PAL proteins produced in vivo or in vitro have been described previously (37). The PAL antibodies were kindly provided by Dr. Lee Hadwiger (Washington State University, Pullman, WA). Optimal amounts of PAL and cyclase antibody required for quantitative precipitations were determined empirically. The amount of radioactivity associated with an immunoprecipitate was determined by scintillation spectrophotometry of appropriately excised regions of the SDS-polyacrylamide gels.

RESULTS

Elicitor-Induced Capsidiol Accumulation Is Suppressed by Calcium/Calmodulin Antagonists

The accumulation of extracellular capsidiol was determined in tobacco cell-suspension cultures treated for 11 h with

elicitor and compared with the capsidiol content of cell cultures that had received different calcium-calmodulin antagonists 1 h before the elicitor (Table I). Representatives of the naphthalenesulfonamide and phenothiazine class of calcium/calmodulin antagonists, W7 and TFP, respectively, suppressed elicitor-induced capsidiol accumulation in a concentration-dependent manner. W7 or TFP at 200 μM resulted in a 68 and 98% reduction of capsidiol in the media of the elicitor-treated cell cultures, respectively. W5, a structural analog of W7 possessing a much lower affinity for calmodulin (18), did not interfere with elicitor-induced capsidiol accumulation at these concentrations. W7 and TFP also reduced the intracellular capsidiol content, which normally represents 5 to 10% of the total extra- and intracellular capsidiol found in elicitor-treated cultures (8), paralleling the effect observed on the capsidiol content in the media (data not shown). The antagonists by themselves (without elicitor) did not induce capsidiol accumulation under any experimental conditions.

Calcium/Calmodulin Antagonists Suppress Elicitor-Inducible Sesquiterpene Cyclase Activity

The effect of the antagonists on sesquiterpene cyclase activity was determined to test the possibility that the calcium/calmodulin antagonists interfered with the elicitor-mediated induction of sesquiterpene biosynthetic enzymes. Sesquiterpene cyclase enzyme activity is not detectable in control cell cultures, but is induced in elicitor-treated cell cultures and is considered a key branch point enzyme for the diversion of carbon into the synthesis of sesquiterpenes (36). In Table II, the cyclase enzyme activity of elicitor-treated cells is compared with that of cells that received different calcium/calmodulin antagonists 1 h before the addition of elicitor. All three antagonists, W7, TFP, and CP suppressed the elicitor inducibility of sesquiterpene cyclase enzyme activity in a concentration-dependent manner. The induction of the cyclase enzyme activity was reduced approximately 50% by 250 μM W7, 150 μM TFP, and 50 μM CP. At similar concentrations, W5 was ineffective in suppressing sesquiterpene cyclase activity. The antagonists by themselves did not induce sesquiterpene cyclase activity (Figs. 4 and 6).

To discern whether the effect of the calcium/calmodulin antagonists on elicitor-inducible enzyme activities was a specific or general response, it was desirable to measure another inducible enzyme unrelated to sesquiterpenoid metabolism. PAL is a key regulatory enzyme of phenylpropanoid metabolism and is induced by elicitor with a similar time course to the sesquiterpene cyclase (Fig. 1). The calcium/calmodulin antagonists did not suppress the inducibility of PAL enzyme activity (Table II). In some cases, W7, as well as W5, increased elicitor-induced PAL activity by 30 to 65% (also compare Figs. 4 and 6). Addition of the antagonists to control cell cultures also caused a slight induction of PAL activity (Table II, Figs. 4 and 6).

Table I. *Effects of Calcium/Calmodulin Antagonists on the Elicitor-Inducible Accumulation of Capsidiol in Tobacco Cell-Suspension Cultures*

Calcium/calmodulin antagonists were added 1 h before elicitor (2.5 μg of Glc equivalent/mL of culture) was added. After an additional 11 h of incubation, the extracellular capsidiol content was determined by GC.

Treatment	Capsidiol Content	
	μg/mL of culture	Relative amount
		%
Control	0	0
Elicitor	10.3	100
Elicitor + 200 μM W5	11.5	112
Elicitor + 100 μM W7	5.9	57
Elicitor + 200 μM W7	3.3	32
Elicitor + 100 μM TFP	1.6	16
Elicitor + 200 μM TFP	0.2	2

Table II. *Effects of Calcium/Calmodulin Antagonists on the Induction of Sesquiterpene Cyclase and PAL Enzyme Activities in Elicitor-Treated Cell Cultures*

Antagonists were added 1 h before the elicitor (2.5 μg of Glc equivalent/mL) was added. In experiment 1, cells were incubated for 11 h in the presence of elicitor, and in experiment 2, the incubation was for 8 h before the enzyme activities were measured. Enzyme activities are expressed relative to their level in elicitor-treated cells; experiment 1, cyclase activity was 5.3 nmol product/h·mg of protein and PAL activity was 199 nmol product/h·mg of protein; experiment 2, the corresponding values were 13.5 and 737.

Treatment	Sesquiterpene Cyclase (relative activity)		PAL (relative activity)	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2
	%		%	
Control	0	0	12	35
Elicitor	100	100	100	100
Elicitor + 100 μM W5	85	86	120	132
Elicitor + 200 μM W5	106	103	116	155
Elicitor + 100 μM W7	79	74	97	132
Elicitor + 200 μM W7	57	61	107	118
Elicitor + 100 μM TFP	90	81	89	95
Elicitor + 200 μM TFP	31	15	90	105
Elicitor + 30 μM CP		78		96
Elicitor + 60 μM CP		33		96

Addition of a Calcium/Calmodulin Antagonist to the Cell Cultures after Elicitor Does Not Suppress the Induction of Sesquiterpene Cyclase

If we assume that elicitor binds to a receptor that activates a transient signal transduction pathway, then addition of a second-messenger antagonist after elicitor should have little or no effect on the subsequent induction of sesquiterpene cyclase enzyme activity. The calcium/calmodulin antagonist CP was administered 1 to 4 h before or after the addition of

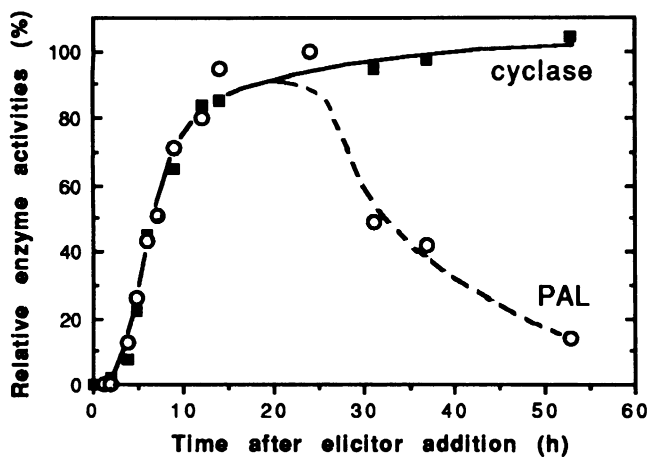

Figure 1. Induction time courses for sesquiterpene cyclase and PAL enzyme activities in elicitor-induced tobacco cell-suspension cultures. Enzyme activities were determined in cell free extracts of cell cultures treated with 3.0 μg of Glc equivalents of elicitor per mL of culture for the times indicated. Enzyme activities are expressed as percentages of their maxima of 14.8 nmol/h·mg of protein for cyclase and 590 nmol/h·mg of protein for PAL.

elicitor (Fig. 2). Cells were then collected after a total of 9 h of incubation with elicitor, and sesquiterpene cyclase and PAL enzyme activities were measured.

Addition of CP 1 to 4 h before elicitor suppressed the induction of sesquiterpene cyclase activity by 70 to 75% (Fig. 2). When CP and elicitor were added to the cell cultures simultaneously, the inducible cyclase enzyme activity was reduced by 50%. When the calcium/calmodulin antagonist was added 1 to 4 h after elicitor, no suppression of the ensuing cyclase activity was observed. The elicitor-inducible PAL activity was completely insensitive to the antagonist.

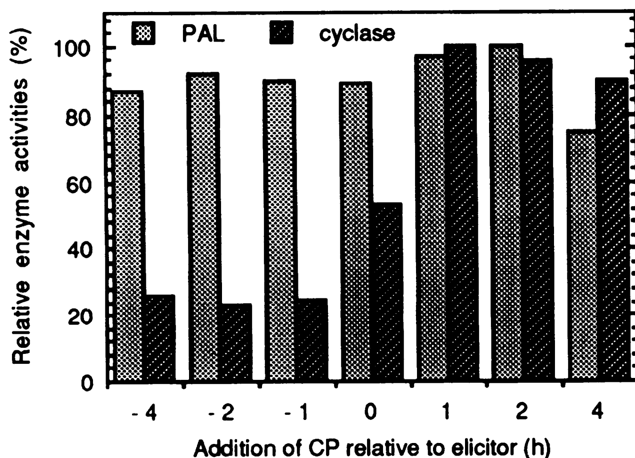

Figure 2. Effects of a calcium/calmodulin antagonist added before and after elicitor on the induction of sesquiterpene cyclase and enzyme activities. CP, 60 μM, was added to the cell cultures at different times relative to 2.5 μg of Glc equivalents of elicitor per mL cell culture as indicated. Nine hours after initiation of the elicitor treatment, cell extracts were prepared and sesquiterpene cyclase and PAL activities were determined. Enzyme activities are expressed as percentages of their maxima of 31.4 nmol/h·mg of protein for cyclase and 473 nmol/h·mg of protein for PAL.

Elicitor-Inducible Synthesis of the Sesquiterpene Cyclase Protein Is Suppressed by Calcium/Calmodulin Antagonists

The differential response of the cell cultures to the antagonists with respect to the inducible enzyme activities could represent a differential interaction of the antagonists directly with the cyclase polypeptide (thus inhibiting its activity) but not with the PAL polypeptide. However, the antagonists did not affect the extractable cyclase enzyme activity when given to cells 1 h after elicitor. Alternatively, the antagonists could suppress the induced de novo synthesis of the cyclase polypeptide (37) but not that of the PAL enzyme. Consistent with this possibility was that the absolute amount of cyclase protein, but not the PAL protein, was indeed sensitive to the calcium/calmodulin antagonists (Fig. 3). Sesquiterpene cyclase polypeptide was not present in control cells but accumulated in elicitor-treated cells as previously described (36). However, when calcium/calmodulin antagonists were added to the cell cultures before the addition of elicitor, the accumulation of cyclase polypeptide was significantly suppressed.

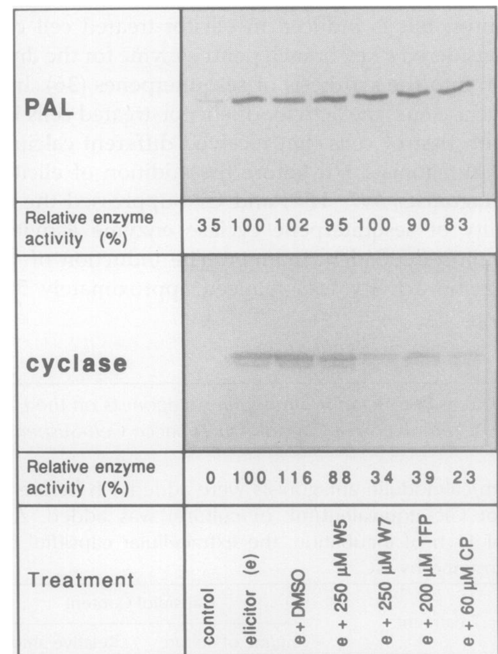

Figure 3. Effects of calcium/calmodulin antagonists on the level of inducible sesquiterpene cyclase and PAL enzyme proteins. Antagonists were added at the indicated concentrations to the cell cultures 1 h before 2.5 μg of Glc equivalents of elicitor per mL of cell culture were added. Eight hours after initiation of the elicitor treatment, cells were collected and extracts were prepared. Enzyme activities are expressed relative to their maxima of 19.8 nmol/h·mg of protein for cyclase and 713 nmol/h·mg of protein for PAL. Aliquots of 40 μg of protein from each extract were also separated by SDS-PAGE and probed for either the cyclase or PAL protein by immunoblotting.

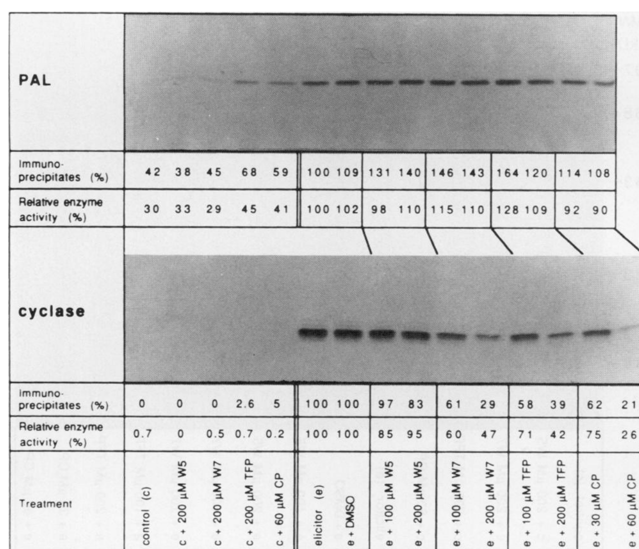

Figure 4. Effects of calcium/calmodulin antagonists on the in vivo synthesis rate of the sesquiterpene cyclase and PAL proteins. The antagonists were added at the indicated concentrations to the cell cultures 1 h before elicitor (2.5 μg of Glc equivalents/mL) was added. Four hours after the initiation of the elicitor treatment, the cells were pulse labeled with [^{35}S]methionine for an additional 2 h. Extracts were prepared from the cells, and aliquots containing equal amounts of TCA-precipitable radioactivity were challenged with either cyclase or PAL antibodies. The radioactive, immunoprecipitable cyclase and PAL proteins were detected by SDS-PAGE/fluorography. The immunoprecipitates were also quantified by excising the appropriate bands from the gel, followed by counting in a scintillation counter, and are presented as percentages of the maxima in elicitor-treated cells. Cyclase and PAL enzyme activities were measured in the same extracts. The activities are expressed relative to their maxima of 16.1 nmol/h·mg of protein for cyclase and 405 nmol/h·mg of protein for PAL.

W5, the structural analog of W7 having reduced binding affinity for calcium/calmodulin complex, did not alter the level of elicitor-induced cyclase polypeptide. In all cases, the level of the cyclase polypeptide was proportional to the cyclase enzyme activity measured in the same extracts. In contrast to the cyclase polypeptide, accumulation of the PAL polypeptide in elicitor-treated cell cultures was insensitive to the calcium/calmodulin antagonists.

These results suggested that the antagonists interfered with the synthesis or degradation of sesquiterpene cyclase protein in elicitor-treated cells but not with that of the PAL polypeptide. Therefore, the de novo synthesis rate of each enzyme was determined. Four hours after initiation of the elicitor treatment, when the synthesis rate of the cyclase polypeptide was near maximum (37), cells treated with antagonists and/or elicitor were incubated for another 2 h in the presence of [^{34}S]methionine, and extracts of the in vivo synthesized proteins were prepared. Aliquots of the in vivo labeled proteins were incubated with cyclase and PAL antibodies, and the immunoprecipitates were evaluated by SDS-PAGE/fluorography to determine the synthesis rate of the sesquiterpene cyclase and PAL proteins (Fig. 4). Incorporation of radioactivity into aliquots of the immunoprecipitates was also quantified, and those results are presented for comparison along with the changes in enzyme activities. In elicitor/antagonist-treated cells, the amount of immunoprecipitable, radioactive cyclase polypeptide was significantly reduced as compared with that from elicitor-treated cell cultures. For example, 200 μM TFP reduced the apparent synthesis rate of the elicitor-inducible cyclase polypeptide by 61%, which was also reflected in a reduced enzyme activity of 58%. W5 caused much smaller changes in the synthesis rate and enzyme activity. In contrast to the sesquiterpene cyclase, the synthesis rate of the PAL polypeptide was either enhanced or not affected by the calcium/calmodulin antagonists.

Addition of the antagonists alone to the tobacco cells had little effect on the uptake and incorporation of radioactive methionine into total cellular protein (Fig. 5B). The pattern of total de novo synthesized proteins was also examined by SDS-PAGE/fluorography to evaluate what other effects the antagonists alone or in combination with elicitor might have on protein synthesis (Fig. 5A). Several distinct changes in the pattern of proteins synthesized by elicitor-treated versus control cells have already been noted (37). However, when antagonist-treated cells were compared with control cells or antagonist/elicitor-treated cells were compared with elicitor-treated cells, only minor differences in the de novo synthesized protein patterns were detectable. For example, TFP and CP induced the synthesis of an 18- to 20-kD polypeptide in control as well as in elicitor-treated cells. Furthermore, there was no significant inhibition or stimulation of [^{35}S]methionine

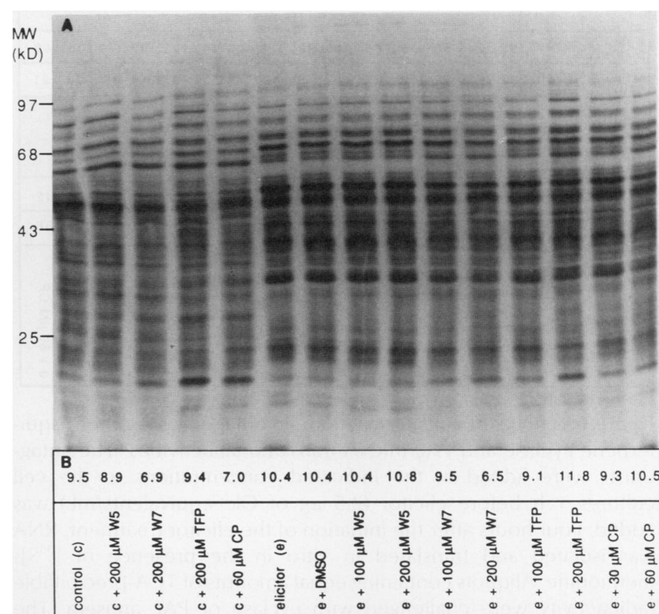

Figure 5. Effects of calcium/calmodulin antagonists on total in vivo protein synthesis capacity of control and elicitor-treated cell suspension cultures. A, Aliquots of equal TCA-precipitable radioactivity from cell extracts described in Figure 4 were separated by SDS-PAGE and the radioactive proteins detected by fluorography. B, As a measure of the [^{35}S]methionine uptake and incorporation rate, the specific radioactivity (dpm per mg of protein) is also presented.

incorporation into protein measured in the variously treated cell cultures.

Calcium/Calmodulin Antagonists Suppress the Elicitor-Inducible Sesquiterpene Cyclase mRNA Translational Activity

Control cell cultures and those pretreated with calcium/calmodulin antagonists were incubated for 5 h in the presence or absence of elicitor before their total RNA was isolated and translated in an in vitro system containing [^{35}S]methionine. Incorporation of radioactivity into in vitro translation products immunoprecipitable with the cyclase and the PAL antibodies was quantified as a measure of the cyclase and PAL mRNA translational activities and was compared with the enzyme activities measured in the respectively treated cells (Fig. 6). The specificity of the immunoprecipitations was further evaluated by SDS-PAGE/fluorography (Fig. 6).

Cyclase mRNA translational activity was not detectable in RNA isolated from control or antagonist-treated cell cultures. However, the level of cyclase mRNA translational activity in elicitor-treated cells was similar to that previously described (37). The cyclase translational activity was substantially reduced when elicitor-treated cell cultures were exposed to the calmodulin antagonists, 15 to 72% depending on antagonist and concentration used. The decreased cyclase mRNA translational activity was also paralleled by a suppression in the extractable cyclase enzyme activity. The level of the PAL mRNA translational activity was, likewise, induced in elicitor-treated cell cultures as compared with control cell cultures. However, the PAL translational activity was not affected by the calcium/calmodulin antagonists.

The patterns of the total in vitro translation products were also examined to further evaluate nonspecific effects of the antagonists. Total proteins synthesized in vitro by RNA isolated and translated as described above were compared by SDS-PAGE/fluorography (Fig. 7). Other than a modest induction of mRNAs coding for a 15- and a 20-kD polypeptide, the mRNA populations from cell cultures treated with the calcium/calmodulin antagonists did not exhibit any other obvious differences relative to their respective controls.

Treatment	control (c)	c + 200 µM W5	c + 200 µM W7	c + 200 µM TFP	c + 60 µM CP	elicitor (e)	e + DMSO	e + 100 µM W5	e + 200 µM W5	e + 100 µM W7	e + 200 µM W7	e + 100 µM TFP	e + 200 µM TFP	e + 30 µM CP	e + 60 µM CP
PAL Immunoprecipitates (%)	65	64	58	76	65	100	99	128	104	143	102	97	113	88	88
PAL Relative enzyme activity (%)	34	14	52	66	48	100	102	99	122	127	121	115	96	98	91
cyclase Immunoprecipitates (%)	0	0	0	2	3	100	98	90	95	68	46	70	28	85	42
cyclase Relative enzyme activity (%)	0	0	0	0	0	100	106	91	90	68	30	78	46	65	36

Figure 6. Effects of calcium/calmodulin antagonists on the sesquiterpene cyclase and PAL mRNA translational activities. The antagonists were added at the indicated concentrations to the cell cultures 1 h before elicitor (2.5 μg of Glc equivalents/mL) was added. Four hours after the initiation of the elicitor treatment, RNA was isolated and translated in vitro in the presence of [^{35}S]-methionine. Aliquots containing equal amounts of TCA-precipitable radioactivity were challenged with cyclase or PAL antisera. The radioactive, immunoprecipitable cyclase and PAL proteins were detected by SDS-PAGE/fluorography. The immunoprecipitates were also quantified by excising the appropriate bands from the gel, followed by counting in a scintillation counter, and are presented as percentages of that found for elicitor-treated cells. Cyclase and PAL enzyme activities were measured in the same extracts. The activities are expressed relative to their maxima of 4.3 nmol/h·mg of protein for cyclase and 485 nmol/h· mg of protein for PAL.

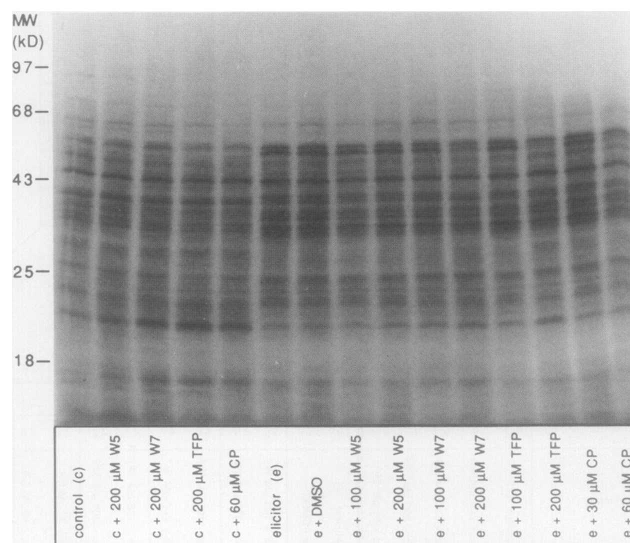

Figure 7. Effects of calcium/calmodulin antagonists on the total in vitro translation products produced by RNAs isolated from control or elicitor-treated cell-suspension cultures. Aliquots of equal TCA-precipitable radioactivity from in vitro translations described in Figure 6 were separated by SDS-PAGE and the radioactive proteins were detected by fluorography.

DISCUSSION

The use of metabolic inhibitors or antagonists to block biochemical reactions must be interpreted with caution, and any conclusions based on their use must address questions relating to the specificity of the inhibitor(s). In fact, the calcium/calmodulin antagonists used in the current study are known to have secondary effects. For example, TFP has been reported to inhibit a calmodulin-insensitive Mg^{2+} ATPase in mammalian cells (35). The antagonists are also capable of accumulating in the membranes of plant cells, thus potentially disrupting general structure-function relationships (7). Despite these caveats, calcium/calmodulin antagonists have been used in plant systems to gain initial evidence for calcium/calmodulin-dependent processes. Most notable are the reports by Elliott et al. (14), Saunders and Hepler (28), and Raghothama et al. (27) concerning the inhibition of growth regulatory-stimulated growth and development. These investigators examined antagonist specificity by comparing struc-

tural analogs having different affinities for calcium-binding proteins and demonstrating a comparable efficacy in vivo or demonstrating that other physiological parameters were not indiscriminately impaired.

The results presented here suggest that a calcium-binding protein may play a role in the signal transduction chain for elicitor-induced sesquiterpene metabolism. First, specificity of the antagonists was demonstrated by the selective suppression of elicitor-inducible sesquiterpene cyclase by W7 but not W5. This is consistent with the known binding affinities of these compounds for calcium/calmodulin complexes (18). The demonstration that two different classes of antagonists, the phenothiazines and naphthalenesulfonamides, had similar effects supports the suggestion of a common but specific mode of action. Second, although PAL is induced with a similar time course and is transcriptionally regulated like sesquiterpene cyclase, the antagonists inhibited the induction of cyclase at all levels examined but not the induction of PAL. This type of result is not consistent with a general or nonspecific effect of the antagonists. Third, the antagonists are effective inhibitors of the inducible cyclase activity only if they are added before or simultaneously with elicitor. This result is consistent with the antagonists' interference with a signal transduction chain resulting in cyclase gene expression. Finally, the antagonists did not interfere with other general physiological parameters such as [^{35}S]methionine uptake, protein synthesis, and mRNA translational activities. The combined results argue that the antagonists are not altering plant metabolism in some nonspecific way but are selectively suppressing the mechanism(s) responsible for the induction of sesquiterpene cyclase enzyme activity.

Whether the antagonists are capable of suppressing the induction of other sesquiterpene biosynthetic enzymes such as 3-hydroxy-3-methylglutaryl CoA reductase (9, 36) is currently unknown. However, we suspect that this is the case. Such an interpretation could also account, to some extent, for the greater suppression of capsidiol accumulation by the antagonist treatments when the suppression of inducible sesquiterpene cyclase was more modest. It is also important to note that the antagonists at high concentrations stimulated the elicitor-inducible PAL activity. Although the significance of this finding is also unknown, it may represent a chemical stress-induced response.

A number of other reports support our observations and provide provocative implications for a calcium-based signal transduction mechanism controlling elicitor-induced phytoalexin biosynthesis in tobacco. Earlier work implicated calcium as a potential second messenger in elicitor-induced phytoalexin biosynthesis in potato (40), carrot (22), and soybean (33) but perhaps not in pea (20). More recently, Knight et al. (21) used transgenic plants expressing aequorin to document transient increases in the intracellular calcium levels in tobacco seedlings stimulated with elicitor. The increased calcium occurred very rapidly and returned to normal levels within minutes of the initial stimulus.

The transient nature of the cytoplasmic calcium levels is suggestive of a signal transduction chain and implies that components of the chain would be sensitive to inhibitors or antagonists at discrete times, as observed in the current work. Exactly how the calcium/calmodulin antagonists interfere within this chain is not understood. However, recent characterization of calcium-dependent kinases that are also sensitive to these antagonists provides an intriguing possibility (21, 24). Perhaps, elicitor recognition results in a transient elevation of cytoplasmic calcium, which activates a calcium-dependent kinase. This could be a type II kinase that requires both calcium and calmodulin for optimal activity (31) or one related to the calcium-dependent, calmodulin-independent kinases recently described by Harper et al. (17) and Li et al. (24). The calcium-stimulated kinase would then be responsible for phosphorylating additional proteins, one of which is directed toward the activation of sesquiterpene cyclase gene expression. Evidence of transient changes in the phosphorylation patterns of proteins in elicitor-treated cells (16) and in extracts incubated with oligosaccharides (15) and activation of gene expression by phosphorylation of *trans*-acting factors (19) provides additional support for such speculation. A possible test of this hypothesis would be to determine whether cyclase gene expression was constitutive in cells engineered with a constitutive calcium-independent protein kinase, much like that described by Kapiloff et al. (19).

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