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Effect of Calmodulin Antagonists on Auxin-Induced Elongation[1]

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ABSTRACT

Coleoptile segments of oat (*Avena sativa* var Cayuse) and corn (*Zea mays* L. var Patriot) were incubated in different concentrations of calmodulin antagonists in the presence and absence of α-naphthaleneacetic acid. The calmodulin antgonists (chlorpromazine (CP), trifluoperazine, and fluphenazine) inhibited the auxin-induced elongation at 5 to 50 micromolar concentrations. Chlorpromazine sulfoxide, an analog of chlorpromazine, did not have significant effect on the elongation of oat and corn coleoptiles. A specific inhibitor of calmodulin *N*-(6-aminohexyl)5-chloro-1-naphthalenesulfonamide hydrochloride (W-7, a naphthalenesulfonamide derivative) inhibited coleoptile elongation, while its inactive analog *N*-(6-aminohexyl)-1-naphthalenesulfonamide hydrochloride (W-5) was ineffective at similar concentrations. During a 4-hour incubation period, coleoptile segments accumulated significant quantities of ^{3}H-CP. About 85 to 90% of auxin-induced growth was recovered after 4 hours of preincubation with CP or 12 hours with W-7 and transferring coleoptiles to buffer containing NAA. Leakage of amino acids from coleoptiles increased with increasing concentration of CP, showing a rapid and significant increase above 20 micromolar CP. The amount of amino acids released in the presence of W-7 and W-5 was significantly lower than the amount released in the presence of CP. Both W-5 and W-7 increased amino acid release but only W-7 inhibited auxin-induced growth. Calmodulin activity measured by phosphodiesterase activation did not differ significantly between auxin-treated and control coleoptile segments. These results suggest the possible involvement of calmodulin in auxin-induced coleoptile elongation.

The regulation of germination, growth, and senescence by plant hormones is influenced by the presence of calcium (14, 17, 18). Auxin (IAA) at growth-promoting concentrations resulted in 100% increase in ATP-dependent calcium transport across membranes (13). In addition, auxin treatment resulted in the release of calcium ions from membrane vesicles (4), and stimulated ATPase activity under *in vitro* conditions (23). Auxin-calcium interaction in cellular processes could be regulated through calmodulin, an intracellular calcium-binding protein which is known to have many regulatory roles in the cellular processes of animals and plants (1, 7, 8, 16, 20, 25). Enzymes such as NAD kinase, Ca-ATPase, quinate:NAD$^+$ oxidoreductase, isofloridoside phosphate synthase, and protein kinases are known to be activated by calmodulin in plants (1, 9, 12, 25, 26). Therefore, it appeared that calmodulin could be involved in auxin-induced growth processes in plants.

The role of calmodulin in auxin-induced elongation was studied by using potent antagonists of calmodulin such as CP,[3] FPZ, TFP, and naphthalenesulfonamide derivatives.

MATERIALS AND METHODS

Chemicals and Reagents. Calmodulin antagonists FPZ and TFP along with CPS were a gift from Smith Kline and French Co., Philadelphia. Activator-deficient cAMP phosphodiesterase (from bovine heart) and its activator (calmodulin from bovine brain), cAMP and CP were obtained from Sigma. ^{3}H-CP was purchased from New England Nuclear, Boston, MA. The naphthalenesulfonamide derivatives (W-5 and W-7) were kindly supplied by Dr. Hiroyoshi Hidaka of Mie University, Edobashi, Japan and later purchased from Seikagaku America, Inc., St. Petersburg, FL. Both W-7 and W-5 were dissolved in distilled water, pH adjusted to 6.75 with NaOH. The solutions were filtered through Whatman No. 1 filter paper before use.

Plant Material. Oat (*Avena sativa* var Cayuse) and corn (*Zea mays* L. var Patriot) seeds were sown in plastic trays filled with vermiculite and kept in dark for about 5 d at 24°C. The dark grown coleoptiles were harvested under a dim green light and 8-mm segments, excluding 3-mm tip, were excised using a plexiglass coleoptile cutter specially designed for this purpose. The leaf blades were not removed from coleoptile segments except during ^{3}H-CP uptake studies. Coleoptile segments were transferred to a beaker containing distilled water and kept floating for about 1 h prior to the treatment.

Sets of 10 presoaked coleoptiles were transferred to Petri dishes (5 cm diameter) containing 10 ml of incubating medium consisting of 10 mM KH_2PO_4 (pH 6.3), 1.5% w/v sucrose, 10 mM sodium citrate, and 0.1% v/v DMSO. Various test solutions and 10 μM NAA were added as required. NAA was prepared in 0.1% v/v DMSO using the incubating medium. All dilutions of the calmodulin antagonists and detergents were made using the same buffer. Coleoptiles were incubated in dark for 18 h at 24°C and the length of coleoptiles was measured using a millimeter graph paper. In reversal and short-term experiments the length of coleoptiles was measured by shadowgraph technique using an overhead projector. The treatments involving W-5 and W-7 were carried out in 3.5-cm diameter Petri dishes using 2 ml of buffer. Short-term growth studies were performed by incubating coleoptile segments for 2, 4, and 6 h in the buffer consisting of 10 μM NAA with or without 15 μM CP.

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[3] Abbreviations: CP, chlorpromazine; NAA, naphthaleneacetic acid; CPS, chlorpromazine sulfoxide; TFP, trifluoperazine; FPZ, fluphenazine; PDE, phosphodiesterase; W-7, *N*-(6-aminohexyl)5-chloro-1-naphthalenesulfonamide hydrochloride; W-5, *N*-(6-aminohexyl)-1-naphthalenesulfonamide hydrochloride; EGTA, ethyleneglycol-bis-(β-aminoethyl ether) *N,N,N',N'*-tetra acetic acid.

Uptake of ^{3}H-Chlorpromazine by Elongating Coleoptile Segments. Three sets of 10 coleoptile segments free of leaf blades were transferred to Petri dishes containing 5 ml of buffer consisting of 15 μM CP, 2 × 10^6 dpm ^{3}H-CP (22 Ci/mmol) in the presence or absence of 10 μM NAA. Incubation was carried out in dark and the coleoptiles were removed from buffer after 1, 2, 4, and 18 h of incubation. The coleoptiles were blotted free of solution and washed in a large volume of distilled water for 30 s to remove the label bound to the surface of coleoptiles. The washed segments were kept frozen at −20°C for 2 h. The frozen coleoptiles were transferred to scintillation vials containing 400 μl of distilled water and mascerated with glass rod. A tissue digesting cocktail (Scinti-Gest, Fisher Scientific Company, Pittsburgh) was added at a rate of 2 ml/10 coleoptiles and the digestion was carried out at 50°C for 24 h in a hot water bath. Radioactivity in the digested sample was counted in a Packard-Tricarb liquid scintillation spectrometer. Uptake of CP was expressed as nmol/g fresh weight of coleoptile segments.

Estimation of Free Amino Acids. The quantity of free amino acids released into the media during incubation was used as a measure of membrane integrity (24). One ml of incubation medium was used for measuring free amino acids with ninhydrin (30).

Reversal of Growth Inhibition Caused by Antagonists. Three sets of 10 coleoptile segments were preincubated in dark for 4 h in buffer containing 15 μM CP. Similarly, the incubation of coleoptiles in buffer containing 60 μM of W-7 was carried out for 12 h. The preincubated coleoptiles were washed in a large volume of water with five changes. The washed coleoptiles were transferred to buffer containing 10 μM NAA and the incubation was continued in dark. The length of coleoptiles was measured after 18 h of incubation by shadowgraph technique using an overhead projector. The coleoptiles preincubated in buffer and then transferred to 10 μM NAA served as controls for the above treatments.

Estimation of Calmodulin Activity. Calmodulin activity in the tissue extract was assayed by measuring its ability to stimulate the activator-deficient PDE, which converts cAMP to 5′-AMP (28). The 5′-AMP released during the reaction was measured by HPLC (Waters Associates model 6000A). For PDE assay, the tissue was extracted in 40 mM Hepes buffer (pH 7), 0.5 M NaCl, 5 mM $CaCl_2$, and 28 mM L-ascorbic acid (10 ml/g fresh weight) (6). The homogenate was passed through cheesecloth and centrifuged at 100,000*g* for 60 min in a Beckman L-5-50B Ultracentrifuge. The supernatant was heated to 90°C for 5 min in a hot water bath and the heat-denatured proteins were removed by spinning at 12,500*g* for 5 min. The supernatant obtained was used for assaying calmodulin activity.

The PDE assay mixture (0.5 ml) was composed of 40 mM Tris-HCl (pH 8.0), 1 mM $CaCl_2$, 0.4 mM $MnCl_2$, and 2 mM cAMP (29). A standard curve was obtained with increasing concentrations of bovine heart calmodulin with constant amount of the enzyme (PDE). The reaction was started by adding PDE to a final concentration of 0.02 units/ml. The reaction mixture was incubated at 30°C for 20 min and the reaction was stopped by heating in a boiling water bath for 5 min. 5′-AMP produced during the reaction was quantitated by the modified procedure of Watterson *et al.* (29), using a C_{18}-μBondapak column (3.0 mm × 30 cm). The mobile phase consisted of 10 mM KH_2PO_4 (pH 2.5) delivered at the rate of 1.5 ml/min by a Waters 6000 model solvent delivery system. The absorbance was monitored using a variable wavelength detector (Waters model 450) set at 260 nm.

Calmodulin levels were expressed on the basis of units/coleoptile and units/mg protein. One unit of calmodulin is defined as that amount which stimulates 0.01 unit of PDE to 50% of its maximum activity at pH 7.5 and 30°C. One unit of PDE is defined as that amount which hydrolyzes 0.5 μmol of cAMP to 5′-AMP/min at pH 7.5 and 30°C.

RESULTS

Effect of Calmodulin Antagonists. The addition of increasing concentrations of calmodulin antagonists to the incubation me-

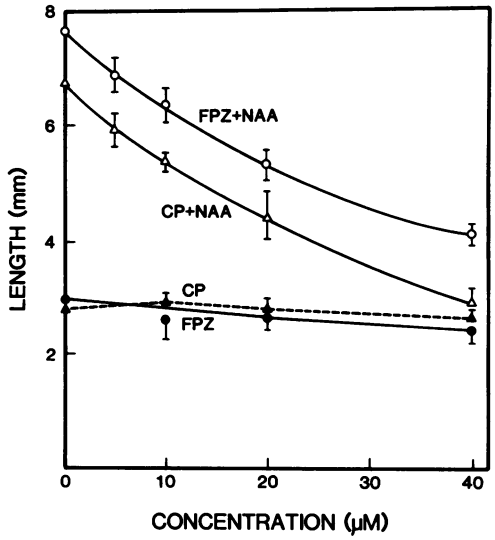

FIG. 1. Effect of calmodulin antagonists on NAA induced oat coleoptile elongation. Eight-mm long coleoptile segments were incubated in the dark for 18 h at room temperature (24°C). The incubating medium consisted of 10 mM KH_2PO_4, 1.5% w/v sucrose, 10 mM sodium citrate, and 0.1% v/v DMSO (pH 6.3). All the treatments except control had 10 μM NAA. The calmodulin antagonists were added to the media at the beginning of incubation. The values represent the average elongation of 20 coleoptile segments floated in two Petri dishes (5 cm diameter), each containing 10 ml of test solution. All the experiments were repeated at least three times. The vertical bars represent SE.

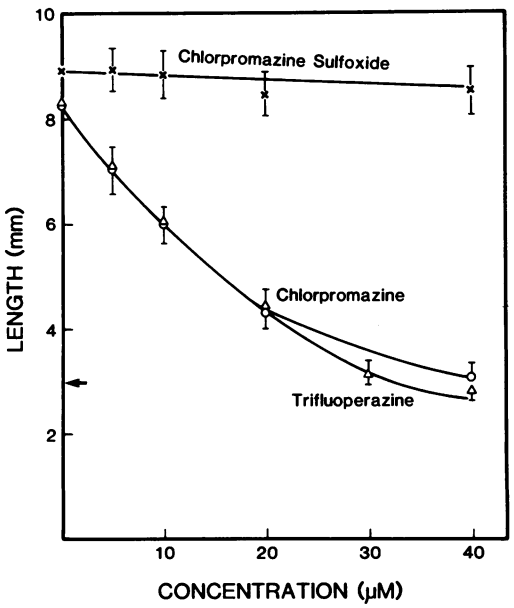

FIG. 2. Effect of calmodulin antagonists and chlorpromazine sulfoxide, an inactive analog of chlorpromazine, on NAA-induced oat coleoptile elongation. The elongation of coleoptiles was measured as described in Figure 1. ←, Coleoptile elongation without NAA. The vertical bars represent SE.

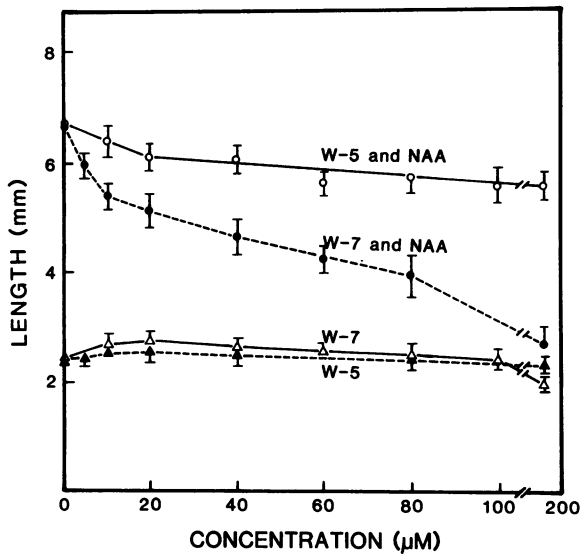

FIG. 3. Effect of naphthalenesulfonamides, the calmodulin inhibitor W-7 and its analog W-5 on oat coleoptile elongation induced by auxin. Sets of 10 oat coleoptile segments were incubated in 2 ml of test solution for 18 h in the dark. The values are the mean of 20 observations. The experiment was repeated three times resulting in similar observations. The vertical bars represent SE.

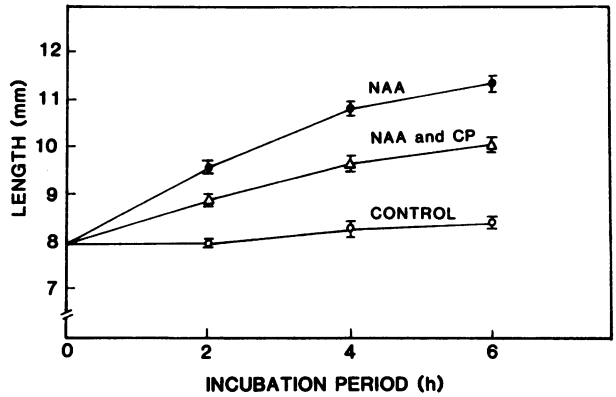

FIG. 4. Effect of 15 μM CP on the short-term growth of oat coleoptile segments in the presence or absence of 10 μM NAA. Three sets of 10 coleoptile segments were floated in test solutions for 2, 4, and 6 h in dark. The vertical bars represent SE.

dia progressively decreased auxin-induced elongation in both oat (Fig. 1) and corn (data not shown) coleoptiles. Oat coleoptiles floated in 10 μM NAA solution showed more than 100% increase in length from that of the control. NAA-induced elongation was inhibited by 50% (Ic_{50}) in the presence of 10 to 15 μM CP (Fig. 1) and TFP (Fig. 2). The Ic_{50} value for FPZ varied between 20 to 30 μM. Corn coleoptiles also showed similar trend of inhibition in the presence of calmodulin antagonists even though their response to NAA treatment was lower than that of oat coleoptiles (data not shown). CP and TFP were more effective in inhibiting the NAA-induced elongation than FPZ. Among the naphthalenesulfonamide derivatives, W-7 inhibited auxin-induced elongation (Ic_{50} 40–60 μM) without any effect on the basal growth (*i.e.* growth without added NAA), whereas W-5 was almost inactive (Fig. 3).

In all our experiments, CP and FPZ did not inhibit the basal elongation of coleoptiles up to 40 μM. Short-term growth studies were carried out to understand the effect of CP during initial periods of coleoptile elongation. The results presented in Figure 4 indicated significant differences among the treatments. The effect of CP on NAA-induced coleoptile elongation became clear at the end of 2 h of incubation. A similar trend continued over a period up to 6 h. There was nearly 45% inhibition of auxin-induced growth at the end of 4 h of incubation of coleoptile segments in the presence of 15 μM CP.

Chlorpromazine sulfoxide, which is an inactive analog of chlorpromazine, was used to distinguish the calmodulin antagonism of CP from its phenothiozine drug action in affecting elongation (Fig. 2). At all the concentrations tested (5–500 μM), CPS had little or no effect on auxin-induced elongation of oat coleoptiles. In contrast, CP completely inhibited auxin-induced elongation at 40 μM (Figs. 1 and 2).

Uptake of [^{3}H]Chlorpromazine by Elongating Coleoptiles. Studies on the uptake of chlorpromazine by coleoptile segments were performed in the presence of [^{3}H]CP (Table I). The uptake of CP increased with time and nearly doubled between 2 h (115 nmol/g fresh weight) and 4 h (193 nmol/g fresh weight) of incubation. The coleoptile segments accumulated 339 nmol/g fresh weight at the end of 18 h of incubation.

Leakage of Amino Acids. There was a drastic increase in the amount of amino acids released to the incubating medium in the presence of CP at and above 40 μM (Fig. 5). There was little or no difference in the amount of amino acids released in the presence of CP from 10 to 20 μM. CPS had no effect on the release of amino acids from tissue up to 500 μM concentration. Both W-7 and W-5 enhanced amino acid leakage (Fig. 6). The total quantity of amino acids released in the presence of W-7

Table I. *Uptake of [^{3}H]Chlorpromazine by Oat Coleoptile Segments*

Uptake studies were performed in the presence of 2×10^6 dpm of 15 μM [^{3}H]CP. After incubation, the segments were washed in a large volume of distilled water and digested in a tissue solubilizer. The radioactivity in digested sample was counted in a liquid scintillation spectrometer.

Time	CP Uptake
h	*nmol/g fresh wt*
1	97.7 (±2.52)[a]
2	115.8 (±5.10)
4	193.0 (±14.56)
18	339.4 (±5.15)

[a] Numbers in parentheses, SD.

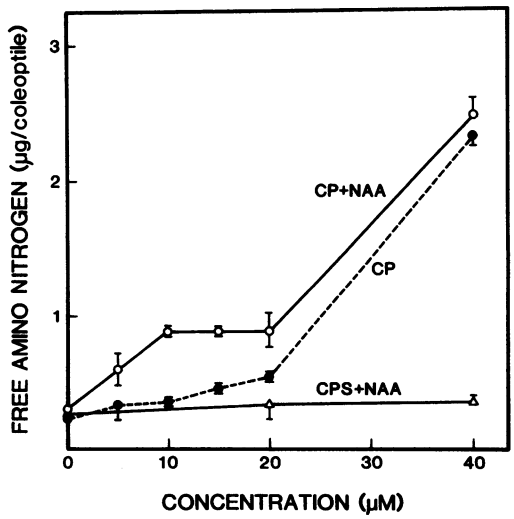

FIG. 5. Effect of calmodulin antagonist CP and its analog CPS on leakage of amino acids into the incubation media in the presence and absence of NAA. The coleoptiles were incubated in the test solutions for 18 h in the dark. One ml of the test solution was used for free amino acid analysis. The vertical bars represent SE.

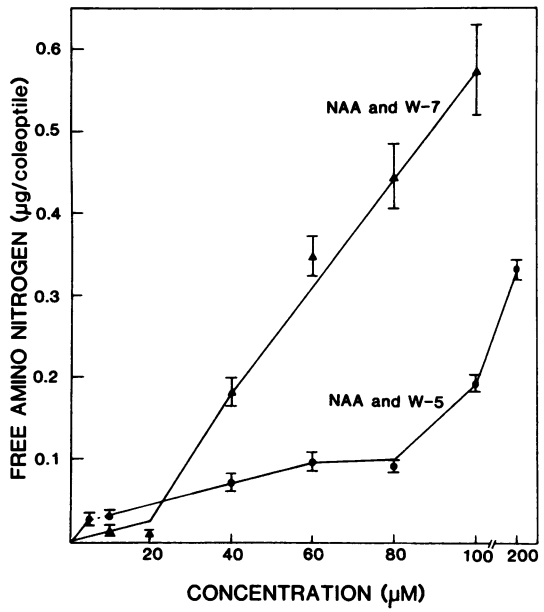

FIG. 6. Effect of W-7 and W-5 on the leakage of amino acids from elongating coleoptiles. Two sets of 10 coleoptiles were floated in 5 ml of buffer containing 10 μM NAA and different concentrations of calmodulin antagonists. The amino acid released during 18-h incubation period was measured by the method described by Yemm and Cocking (30). All the values were subtracted from the value of amino acid released in the presence of NAA alone. The vertical bars represent SE.

Table II. *Reversal of Growth Inhibition Caused by Chlorpromazine and W-7*

Three sets of 10 oat coleoptile segments were preincubated in the dark in the presence and absence of 15 μM CP and 60 μM W-7 for 4 and 12 h, respectively. The coleoptile segments were washed in a large volume of distilled water with several changes of water before incubating in buffer consisting of 10 μM NAA for 18 h. The elongation of coleoptile segments preincubated in buffer and then transferred to 10 μM NAA at 4 and 12 h was considered as 100% elongation.

Additions	Elongation/Preincubation Time	
	4 h	12 h
	mm	*mm*
	5.32 (0.42)[a]	5.88 (0.27)
15 μM CP	4.51 (0.21)	
60 μM W-7		5.39 (0.32)

[a] Numbers in parentheses, SE.

was significantly less than that in the presence of CP. There was no significant difference in the amino acids released up to 20 μM of W-7. The differences became significant at and above 40 μM of W-7. The amount of amino acids released in the presence of 200 μM W-5 was 0.334 μg/coleoptile compared to 0.575 μg/coleoptile in the presence of 100 μM of W-7.

Reversal of Growth Inhibition Caused by Antagonists. Experiments to study the reversal of growth inhibition caused by CP and W-7 treatments for 4 and 12 h, respectively, were performed by incubating the coleoptile segments in 10 μM NAA for 18 h (Table II). After preincubation in W-7 for 12 h, the coleoptiles recovered nearly 90% of NAA-induced growth obtained in the absence of W-7. Similarly, there was 85% recovery in NAA-induced growth following pretreatment with CP for 4 h.

Calmodulin Activity. Calmodulin activity was measured by the activation of PDE (Table III). The activity varied from 2.17 units (NAA + CP) to 2.35 units (control)/coleoptile and there was no signficant difference in calmodulin activity among the treatments.

DISCUSSION

The results showed that all the three calmodulin antagonists with the phenothiazine structure (CP, TFP and FPZ) inhibited auxin-induced coleoptile elongation with increasing concentrations. The range of concentration inhibiting coleoptile elongation is comparable to the inhibition of other calcium-calmodulin mediated responses (9). Both CP and TFP had similar Ic_{50} values (10–15 μM) in inhibiting coleoptile elongation, whereas FPZ exhibited a higher value (20–35 μM). Previous reports indicated similar Ic_{50} values for the inhibition of calmodulin mediated Ca-ATPase activity (9), cell proliferation (21), and hormone-induced growth (10, 19).

Phenothiazine drugs have been attributed to possess properties other than calmodulin antagonism and the inactive structural analog of CP, CPS was used to distinguish the phenothiazine drug action from calmodulin antagonism (5). The inability of CPS to inhibit coleoptile elongation indicated that the phenothiazine structure of these antagonists is not the major feature which caused the inhibition of auxin-induced elongation. The inactivity of CPS could be due to large differences in membrane affinity between CP and CPS. This work is in conformity with previous reports (5, 12, 15, 27). CP at 40 μM did not show significant effect on the basal growth of coleoptiles, whereas auxin-induced growth was totally inhibited at this concentration. Roux and Slocum (20) and Biro *et al.* (2) observed inhibition of basal growth of coleoptiles in the presence of >10 μM CP. This difference could be due to variation in techniques and preparation of plant material. Our observations on the effect of low concentration of CP (less than 10^{-6} M) indicated slight increase in coleoptile elongation (data not presented). Similar observations were reported by Biro *et al.* (2), but its significance is not clear. More work is needed to distinguish between the calmodulin inhibition and nonspecific effects of phenothiozine drugs. In short-term growth studies, CP at 15 μM was effective in inhibiting the coleoptile elongation at the end of 2 h of incubation in the presence of 10 μM NAA. Nearly 45% inhibition in NAA-induced growth was observed at the end of 4 h of incubation.

Naphthalene sulfonamide derivatives are another group of active calmodulin antagonists (11). W-7, which is a specific antagonist of calmodulin, inhibited auxin-induced coleoptile elongation, whereas its structural analog W-5 (same as W-7 but lacking a Cl), which is 10 times less active as a calmodulin antagonist with the same membrane affinity, did not significantly inhibit elongation. This further supported the involvement of calmodulin in auxin-induced elongation.

The release of free amino acids from the tissue has been used as a parameter for measuring cellular integrity (24). The amount of amino acids released in the presence of 5 μM of CP (0.54 μg/coleoptile) nearly doubled at 10 μM CP (0.93 μg/coleoptile) and remained almost constant up to 20 μM of CP. There were two distinct phases in the release of amino acids, a short increase at lower concentrations and a drastic increase above 20 μM of CP. The Ic_{50} value of calmodulin antagonism of CP in our experiments fell in the concentration range of 10 to 15 μM, where the possibility of detergent action seems to be less. The structural analog of CP, CPS did not cause any drastic increase in amino acids released at the concentrations tested (20–100 μM). This release of amino acids at higher concentrations of CP may be due to irreversible membrane damage. The release of amino acids in the presence of W-5 and W-7 revealed interesting information. The amount of amino acids released in the presence of W-7 was less than one-tenth of that released by CP at similar concentrations. Both W-5 and W-7 enhanced the release of

Table III. *Calmodulin Activity in Oat Coleoptiles as Determined by PDE Activation*

The procedure for measuring calmodulin activity by PDE activation is outlined in the text. The coleoptile segments were incubated in the test solutions for 18 h in dark before extraction. The coleoptiles harvested 60 min before the assay served as 0 time control. Protein in the soluble fraction was determined by dye binding assay (3).

Treatment	PDE Activation		Protein
	units of calmodulin/coleoptile	*units of calmodulin/mg protein*	*mg/g fr wt*
0 Time control	2.35 ± 0.10	45.65	3.66
Control	2.27 ± 0.27	51.74	3.57
NAA (10 μM)	2.24 ± 0.26	53.40	3.15
NAA + CP (40 μM)	2.17 ± 0.19	61.69	2.72

amino acids. Coleoptiles incubated in 200 μM of W-5 released 0.348 μg of amino acid/coleoptile which was comparable to those treated with 60 μM of W-7 (Fig. 6). At this concentration (200 μM) W-5 did not inhibit auxin-induced elongation, whereas 60 μM of W-7 inhibited coleoptile elongation by more than 50%. W-7 is known to be a more specific calmodulin antagonist than W-5 (11) and this could be used effectively in studying the processes involving calmodulin.

The uptake of CP was studied using [^{3}H]CP. The uptake increased with time and it nearly doubled from 2 to 4 h of incubation. The high uptake of CP (97 nmol/g fresh weight) at the end of 1 h could be due to high nonspecific binding of chlorpromazine to the external surface of tissue. Similar type of uptake was observed by Biro *et al.* (2). Scharff and Foder (22) observed in their experiments that 70% of trifluoperazine was bound to membranes. They indicated that most of TFP was nonspecifically bound. The short-term reversal studies in the presence or absence of 10 μM NAA indicated that 85% of NAA-induced growth could be recovered after 4 h of pretreatment with 15 μM of CP. This observation also supports the possibility that most of CP taken up by the tissue could be due to nonspecific accumulation of the chemical. Caution should be exercised in interpreting these results.

The reversal of inhibition of NAA-induced growth caused by antagonists was studied by preincubating the coleoptile segments in CP (15 μM) and W-7 (60 μM) for 4 and 12 h, respectively, before transferring them to 10 μM NAA. Nearly 91% of NAA-induced growth was obtained after W-7 pretreatment for 12 h. Similarly, 85% growth could be obtained in the presence of NAA after 4 h of CP treatment. The inhibitory effects of W-7 were not significant at 6 h of treatment but significant differences were recorded when measured at 12 h of treatment. These results along with uptake studies indicated that most of CP in tissue is held in a nonspecific manner. Biro *et al.* (2) have shown that the coleoptiles removed from CP medium recovered the ability to respond to the gravitational stimulus. It seems that CP effect could be reversed by removing coleoptiles from medium containing CP during early periods of incubation.

Our results suggest the possible involvement of calmodulin in auxin-mediated coleoptile elongation. The involvement of calmodulin in cellular processes may be due to changes in the actual content of calmodulin (21) and/or by the modulation of its activity due to altered calcium levels in cytoplasm or by the levels of endogenous inhibitors (16). The results from PDE assay did not reveal any significant changes in the levels of calmodulin in response to auxin treatment (Table III). These results suggest that the calmodulin antagonists affect calmodulin activity and thereby decrease the auxin-induced coleoptile elongation.

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