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Differential Effects of Calmodulin Antagonists on Indol-3-ylacetic Acid- and Gibberellic Acid-Induced Biphasic Growth Responses

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Abstract. The effects of chlorpromazine and calmidazolium on rapid IAA- and GA_3-induced changes in growth rate of etiolated pea stems were measured. During the initial period of up to 160 min after hormone application, the responses to both IAA and GA_3 were seen to be biphasic, showing two acceleration peaks. Chlorpromazine or calmidazolium applied simultaneously with the hormones blocked the first IAA-induced acceleration peak, but did not affect the second one. In contrast, the first GA_3-induced peak was not prevented by chlorpromazine or calmidazolium, while the second one was completely abolished. The results support the concept that the active calmodulin-calcium complex may be an essential component of hormone-controlled stem elongation. They also point to differential mechanisms of IAA and GA_3 actions.

Various physiological processes in plants that are regulated by external signals involve a second messenger system in which Ca^{2+} ions and their receptor protein calmodulin play an essential role (Hepler and Wayne 1985, Poovaiah and Reddy 1987). The involvement of Ca^{2+} ions in response to IAA (Cleland and Rayle 1977, Hasenstein and Evans 1986) and gibberellin (Moll and Jones 1981b) was shown before. Recent data also indicate that calmodulin antagonists prevent many hormone effects, including stem elongation (Elliott *et al.* 1983, Raghothama *et al.* 1985). In addition to long-term effects, application of hormones elicits rapid changes in stem growth rate, and a biphasic growth response has been demonstrated for auxins (Penny *et al.* 1972, Vanderhoef and Stahl 1975) and for gibberellins (Moll and Jones 1981b). The possible involvement of calcium-calmodulin complex in rapid responses has apparently not been studied. The present paper reports on the effects of two calmodulin antagonists, chlorpromazine and calmidazolium, on etiolated pea stem elongation. The inhibitors were administered under the conditions when IAA and GA_3 induce rapid changes in elongation rate and the results point to the possible differential involvement of calmodulin in these processes.

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MATERIAL AND METHODS

Pea seeds (*Pisum sativum* L. cv. Meteor) were obtained from C. Sharpe & Co., Sleaford, England. The seedlings were grown in complete darkness for 8 d and plants with 1.5–2.5 cm long third internodes were selected for experiments. The seedlings were decapitated below the hook region and their growth rate was measured using an angular position sensing transducer (Brush Instrument Division of Clevite Corp., Cleveland, USA) essentially as described by Naumović and Nešković (1979). Since decapitation provoked spontaneous oscillations in growth rate, measurements and application of various substances were begun after 120 min, at which time a constant growth rate was established. Growth was continuously recorded and the rate calculated from the tracings in 10 min intervals. At least ten individual plants were used per treatment and the results were normalized, by setting initial growth rate at 100 %. All manipulations were performed under a weak green safe light.

The decapitated epicotyl was inserted into a glass tube and various substances were applied in 10 µl microdrops onto its surface. A microdrop of buffer added to the control plants had no effect on growth rate. All substances were dissolved in a buffer solution consisting of 2.5 mM HEPES, 2.5 mM MES and 1 mM succinic acid, adjusted to pH 5.0 with Tris base (Moll and Jones 1981a). Test solutions included: gibberellic acid (Sigma), indol-3-ylacetic acid (BDH), and the calmodulin antagonists chlorpromazine hydrochloride (Galenika) and calmidazolium (Sigma). Calmidazolium was dissolved in buffer containing 0.25 % dimethylsulfoxid, which alone had no effect on growth.

RESULTS

The effect of CPZ and calmidazolium on pea stem growth rate was tested in preliminary experiments. Both substances decreased the initial growth rate within the first 10 min, and subsequently a new but constant growth rate was established (see the corresponding curves in Fig. 2 C, D). A concentration decreasing the growth rate to about 50 % of the initial was chosen for further work. This was 10 mM for CPZ and 10 µM for calmidazolium. Since CPZ had to be used in high concentration, experiments to study the reversal of growth inhibition were performed. It was shown that washing the plants with water 40 min after CPZ application restored the high initial growth rate (Fig. 1). It was assumed therefore that the CPZ effect was not due to unspecific toxic action of the drug.

Abbreviations used: HEPES = N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; MES = 2-(N-morpholino)ethanesulfonic acid; Tris = tris-(hydroxymethyl)-aminomethane base; GA_3 = gibberellic acid; IAA = Indol-3ylacetic acid; CPZ = chlorpromazine hydrochloride; calmidazolium = 1-[bis(4-chlorophenyl)-methyl]-3-[2-(2,4-dichlorophenyl)-2-[(2,4-dichlorophenyl)methoxyl]-ethyl]--1H-imidazolium chloride, compound R 24571.

In measurements of elongation rate which were extended to 160 min, we have observed that the responses to both hormones were biphasic (Fig. 2A, B). In response to IAA the peaks occurred after 20 and 140 min and were well separated by a slowing of growth with a minimum at about 60 min. The acceleration peaks for GA_3 response were much closer to each other and occurred at 30 and 60 min, but were clearly discernible in all plants. When added simultaneously with IAA, both CPZ and calmidazolium inhibited the first stimulation peak, but did not affect the second one (Fig. 2C). In contrast, the first GA_3-induced stimulation peak was not prevented by simultaneous CPZ and calmidazoloum application, while the second peak was completely abolished (Fig. 2D).

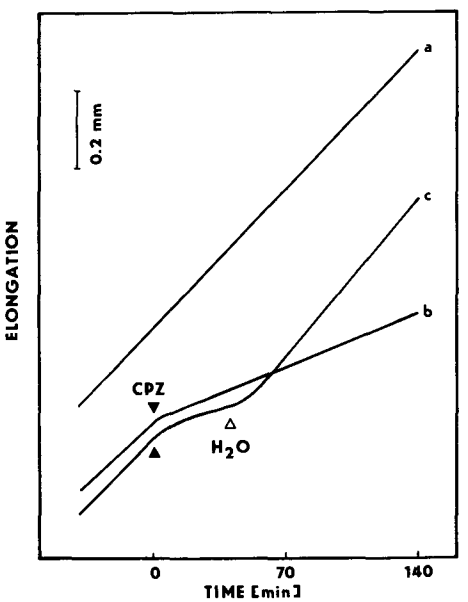

Fig. 1. Reversal of growth rate decrease caused by chlorpromazine. Traces showing the elongation of three representative plants are presented: (a) trace of a control plant; (b) a plant treated with CPZ at zero time (black arrow); (c) CPZ applied at zero time (black arrow) and amply washed with water 40 min later (white arrow).

DISCUSSION

Evidence for the involvement of calcium ions and calmodulin in hormone-induced elongation comes mainly from experiments in which the calcium-calmodulin action was blocked by CPZ or some other antagonists, the response being measured after a longer incubation time (Elliott *et al.* 1983, Raghothama *et al.* 1985). We have demonstrated that two calmodulin antagonists also interfere with rapid responses to IAA and GA_3, which suggests the involvement of calmodulin in the early steps of

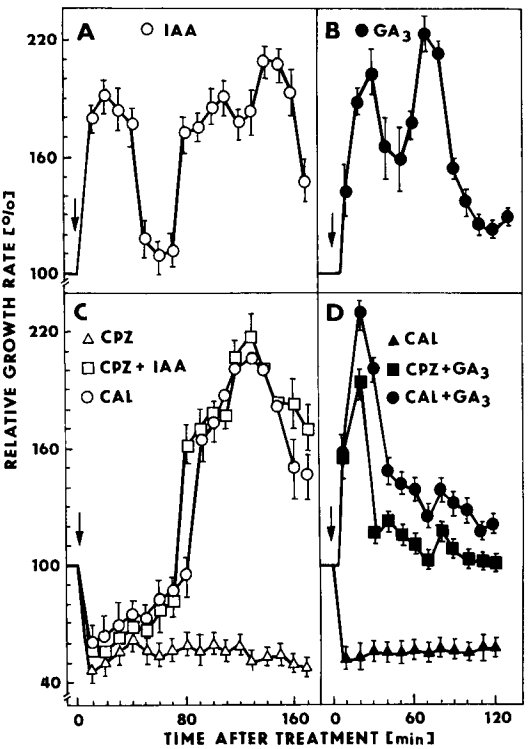

Fig. 2. Effect of chlorpromazine (CPZ) and calmidazolium (Cal) on growth rate induced by IAA and CA_3. A. IAA (10 μg) added at zero time (arrow). B. GA_3 (10 μg) added at zero time (arrow). C. CPZ (10 mM), or calmidazolium (10 μM) added with IAA at zero time (arrow). D. CPZ (10 mM), or calmidazolium (10 μM) added with GA_3 at zero time (arrow). Note that the controls for CPZ and calmidazolium are presented once, in C and D respectively. All values represent the average growth rate of 20 plants, (% of initialrate), vertical bars represents SE.

stimulus transmission. It is well known that the antagonists have unspecific effects which may obscure their interaction with calmodulin and the data, therefore, should be interpreted with caution (Roberts *et al.* 1986). The experiment in Fig. 1 shows that the CPZ effect could be reversed by washing out the drug. An indirect confirmation that the observed effects result from the direct drug action on calmodulin activity may be inferred from the following points: (i) We have applied two calmodulin antagonists that differ structurally from each other and do not affect identical cellular targets (Gietzen *et al.* 1981); nevertheless they both prevent the IAA- and GA_3-induced increase in growth rate in a similar manner. (ii) Both antagonists inhibit growth from 10 to 50 min after IAA application and from 50 to 100 min after GA_3 application, which suggests that they do not affect growth independently of hormones, but inhibit specific hormone effects.

It has been demonstrated that the first IAA peak is similar to acid-induced growth (Vanderhoef and Stahl 1975) and may be partly due to IAA-induced proton efflux.

In our experiments as well, this peak can be suppressed by higher (> 7.0) pH buffers (data not shown). Auxin-induced H^+-efflux seems to be preceded by the acidification of the cytoplasm (Göring and Bleiss 1982, Felle *et al.* 1986). It is suggested that this acidification involves transient intracellular release and sequestration of Ca^{2+} ions (Brummel and Hall 1987). The results showing that CPZ and calmidazolium inhibit the first IAA effect (Fig. 2C) indicate the possible involvement of calmodulin in these processes.

Indol-3-ylacetic acid also induces rapid changes in mRNA, protein and cell wall synthesis (for review see Evans 1984). Some of these events may be responsible for the second phase of IAA-induced growth (Vanderhoef *et al.* 1976) and it seems that they are not calmodulin-dependent (Fig. 2C). This further implies that the first and second responses do not result from a single primary reaction. The recently proposed model for auxin action (Brummel and Hall 1987) envisages two separate receptors for proton extrusion and gene activation.

There is generally little information concerning the molecular aspects of gibberellin effect on stem elongation. The finding that the first phase of GA_3-induced growth acceleration is not prevented by CPZ and calmidazolium (Fig. 2D) indicates that GA_3 acts by a mechanism different from that of auxin. It was already stated that GA treatment did not bring about proton extrusion and wall acidification in lettuce hypocotyls (Stuart and Jones 1978) and bean leaves (Brock and Cleland 1989). Moreover, in contrast to auxin response, the initial gibberellin effect can be prevented by protein synthesis inhibitor cycloheximide (Rose 1974). Chory *et al.* (1987) found that GA_3 application induced changes in mRNA and polypeptide synthesis in maize and dwarf pea stems within 30 min. It remains to be seen if these polypeptides are required for growth responses. According to our results, the events occurring about 50 min after GA_3 application seem to be mediated by calmodulin. A further search for such calmodulin-dependent growth-limiting proteins is warranted.

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