

Calmodulin-Binding Drugs Affect Responses to Cytokinin, Auxin, and Gibberellic Acid¹

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ABSTRACT

Trifluoperazine, a phenothiazine tranquilizer, and tetracaine, a local anesthetic, have been found to inhibit a variety of plant hormone responses at concentrations compatible with their known inhibition of Ca²⁺-calmodulin-dependent enzyme activities. Among these responses are cytokinin-dependent betacyanin synthesis and increase in fresh weight in *Amaranthus tricolor* cotyledons, auxin-dependent increase in length of wheat coleoptile segments and gibberellic acid-dependent induction of α -amylase synthesis in barley aleurone layers. The reversibility of some of these inhibitory effects has been demonstrated, indicating that, up to a point, a generalized membrane destruction can be ruled out. The evidence, taken in conjunction with numerous examples from the literature showing calcium involvement in the action of all of the plant hormones, support a unifying theory of hormone action.

The importance of calcium in plant membranes has long been recognized, initially in a structural role related to membrane integrity (17) and in such processes as the mechanism of uptake of monovalent ions (see references in 9). More recently, membrane storage of calcium and membrane control of calcium transport have emerged as key processes necessary to trigger initial events in many biological responses which may depend on changes in cytoplasmic calcium concentration (1, 2, 5).

Epel (14) proposed that many cellular or developmental processes may be triggered by some event such as the early changes in cation permeability, particularly the increased Ca²⁺ concentration in the cytoplasm, seen in the fertilization of the sea urchin egg. One example given was the photoinduction of developmental programs in plants. Trewavas (27-29) has taken this fertilization system as a model to propose a unifying theory for developmental control in plants, depending on a membrane being the controlling system, with alterations in cytoplasmic ion balance leading to subsequent changes.

Changes in ion transport were proposed as key events in cytokinin-dependent cell enlargement (15, 16, 21, 22, 25). Comparison with effects of the phytotoxin fusicoccin led Marrè (23) to suggest that the growth effect of cytokinins (as well as effects of IAA on cell enlargement, ABA-induced inhibition of stomatal opening and of seed germination, and some aspects of GA₃-induced seed germination) depends on the activation of the fusicoccin-sensitive H⁺/K⁺-exchange mechanism of ion transport, but that the activation may be an indirect one through a complex series of events possibly involving the synthesis of some short lived protein(s). In *Amaranthus tricolor* cotyledons, fusicoccin was found to be syn-

ergistic with BA in induction of betacyanin synthesis, thus leading to a modification of Marrè's proposal, with expansion to a general theory of cytokinin action (8, 10). Recent work has followed the line that calcium interactions with cytokinins may mean that the calcium-dependent regulator protein calmodulin is involved. It was found that drugs which bind to calmodulin and inhibit its action in other systems do indeed inhibit betacyanin accumulation, whether induced by cytokinin, fusicoccin, or red light, and also another cytokinin-dependent response, cell division in soybean callus tissues (13).

This general theory of cytokinin action has now been integrated with the idea of a unifying theory of plant hormone action as referred to above (14, 23, 27, 28) and these drugs have been used to test this unifying theory in another cytokinin-dependent response, and in classical responses to auxin and to GA₃. The drugs that have been used in this study are trifluoperazine and tetracaine, representatives of the two classes of calmodulin-binding compounds, neuroleptic drugs (31) and local anesthetics (26, 30), respectively. The hormone responses chosen were cell expansion growth in excised *Amaranthus* cotyledons (cytokinin-dependent), cell expansion growth in wheat coleoptiles (auxin-dependent), and α -amylase induction in barley aleurone layers (gibberellic acid-dependent).

MATERIALS AND METHODS

Chemicals. BA, IAA, GA₃, and TC² HCl were from Sigma. TFP HCl was a gift from Smith Kline and French Laboratories; it was made up as a 10 mM stock aqueous solution, adjusted to pH 6.1 with 1 N NaOH.

Cell Expansion Growth and Betacyanin Accumulation in *Amaranthus* Cotyledons. *Amaranthus tricolor* seeds (1 g) were germinated in the dark at 25 ± 1°C for 96 h under standard conditions (11). The roots were removed and the remaining cotyledons plus hypocotyls (half-seedlings) were placed in 20 ml distilled H₂O in a 15-cm Petri dish and were transferred to 40 ± 1°C in the dark for 1.5 h and a further 1.5 h at 25°C with the lid off. This treatment had been found to potentiate subsequent betacyanin induction by BA (11 and references therein). After aging, the cotyledons were pinched off from the hypocotyl and placed on filter papers covered by incubation medium. Each treatment comprised 30 cotyledons/9-cm Petri dish with 5 ml buffer (10 mM K₂HPO₄-NaH₂PO₄, pH 6.8), ±5 μM BA, with and without drug additions. All treatments were in triplicate. After 24-h incubation at 25°C in the dark, increases in fresh weight and levels of betacyanin accumulation (12) were measured.

Cell Expansion Growth in Wheat Coleoptiles. Dry wheat seeds (*Triticum aestivum* L. var Olympic) were sown on saturated but thoroughly drained vermiculite and grown for 72 h in the dark at

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²Abbreviations: IC₅₀, concentration giving 50% inhibition; TC, tetracaine; TFP, trifluoperazine.

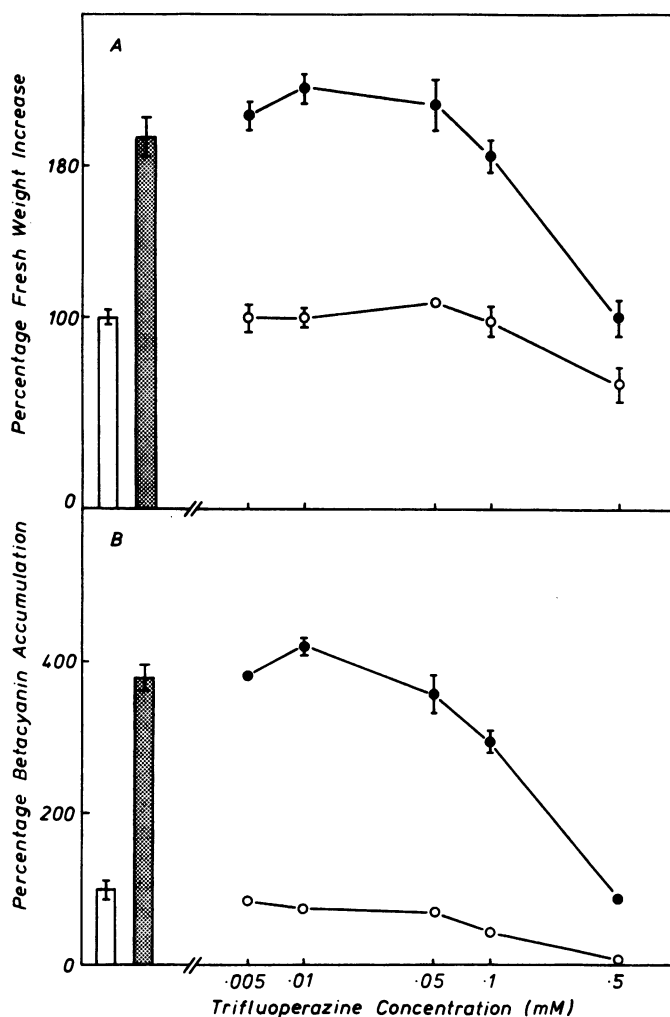


FIG. 1. Effect of TFP on control and cytokinin-stimulated increase in fresh weight (A) and betacyanin level (B). Histograms represent the control (unshaded) and cytokinin-stimulated (shaded) levels in the absence of TFP. *Amaranthus* cotyledons were incubated for 24 h in phosphate buffer, pH 6.8 (○), or in buffer plus 5 μ M BA (●). In A, the results of three experiments, triplicate treatments in each, have been normalized. In the control, in the absence of BA and TFP, the increase in fresh weight was 12.06 ± 0.57 mg/30 cotyledons. Results are reported as a percentage of this control, means \pm SE. In B, the results of two experiments, triplicate treatments in each, have been normalized. In the control, the betacyanin content was 0.145 ± 0.015 nmol/cotyledon, means \pm SE. Where no bar is seen, the SE fell within the symbol.

$23 \pm 2^\circ\text{C}$. Coleoptiles were selected for size (20 ± 2 mm) and a 5-mm section was obtained with a two-bladed cutter 3 mm below the tip. The required number of sections for a given experiment were first floated on distilled H_2O . Twenty sections were then collected at random (surface water removed by blotting) and placed in 5 ml of test solution contained in 20-ml capped vials. The vials were continuously shaken (60 oscillations/min) during the experiment. Growth measurements were obtained at intervals by removing the sections from the vials, lining on a wet glass plate, and measuring under a dissecting microscope equipped with a vernier eyepiece giving an accuracy of 0.01 mm at the chosen magnification. The sections were placed back into the appropriate vial immediately after measurement. When sections had to be transferred to different solutions during an experiment, they were thoroughly washed in aliquots of the next solution, blotted, and placed in a fresh lot of that solution.

α -Amylase Induction in Barley Aleurone Layers. Barley (*Hor-*

deum vulgare L. var Clipper) seeds were de-embryonated and imbibed, and aleurone layers were prepared and incubated according to Chrispeels and Varner (4) except that 10 mM CaCl_2 was used instead of 100 mM. The aleurone layers were incubated for 24 h at $25^\circ\text{C} \pm 1 \mu\text{M GA}_3$ and with and without the calmodulin inhibitors. α -Amylase in the layers was assayed (3) with the modification of Locy and Kende (20) whereby Triton X-100 (0.1%, v/v) was included in the NaCl used for homogenization, in order to release latent α -amylase. One unit of enzyme activity is defined as the amount which brings about an $A_{620\text{nm}}$ change of 1.0 in 5 min.

Reversibility of the inhibition caused by TFP was tested by replacing all solutions after 5-h incubation by medium with and without inhibitor, $\pm\text{GA}_3$, as detailed in Table III.

RESULTS

The cytokinin-dependent increase in fresh weight in *Amaranthus* cotyledons is shown in Figure 1, together with the effect of varying concentrations of TFP. The presence of BA during the 24-h incubation at 25°C caused a 100% fresh weight increase. TFP at low concentrations (10 μM) caused a small increase in this response, with inhibition markedly increasing above this concentration until at 0.5 mM the BA-independent response is almost completely prevented. The betacyanin accumulation in these cotyledons is shown also in Figure 1 with a concentration curve response to TFP very similar to that shown for half-seedlings (12). The latter experiment differed in the BA concentration (0.5 μM , i.e. half-maximal) and in the addition of 10 mM tyrosine (an external source of betacyanin precursor). BA-dependent fresh weight increase is more sensitive to TFP than is betacyanin accumulation ($\text{IC}_{50} = 50$ and 150 μM , respectively). Parallel experiments measuring acid secretion showed effects of TFP similar to those shown on fresh weight increase (data not shown).

Auxin-dependent increase in length of segments of wheat coleoptiles, in the presence and absence of sucrose, and the effect of three concentrations each of TFP and TC is shown in Figure 2. The calculated IC_{50} values are given in Table I. A greater auxin response is seen in the presence of sucrose and since the effect of the inhibitors was similar in both presence and absence of sucrose, the sugar was included in future experiments. The slight stimulation caused by low concentrations of TFP (10 μM) is again seen, as with cytokinin-dependent betacyanin synthesis (9, 10 and Fig. 1) and fresh weight increase (Fig. 1).

A time course of the increase in length after addition of auxin with and without the inhibitors is shown in Figure 3. The effect of auxin is seen at 3 h with inhibition by both TFP and TC becoming apparent by 6 h. In Figure 4, the effect of replacing the solutions with inhibitor with fresh sucrose + IAA (–inhibitor) at 30 min, 3 h, and 6 h is shown. It can be seen that this procedure resulted in recovery of the growth potential at times up to 6 h. The stimulation seen with the 30-min replacement of TC may be due to a low concentration being built up in the cells during this short time, giving rise to the stimulation indicated above with low concentration.

On the other hand, treatment of the segments for 10 h before replacing the medium with fresh IAA + sucrose (–inhibitor) resulted in permanent inhibition of growth (Table II). A dramatic difference is seen if the 10-h drug treatment is made at 10°C instead of 25°C , and the segments transferred to 25°C for the succeeding 20 h. In this case, there was little difference in the inhibitor-treated (0–10 h) sets from the sucrose + IAA control. Further work is needed to distinguish between the possibilities of either a slow entry of inhibitors at low temperature or an unaffected entry but no permanent effect under conditions of suppressed growth by low temperature.

GA_3 -dependent induction of α -amylase in barley aleurone layers and the effect of TFP and TC on this activity is shown in

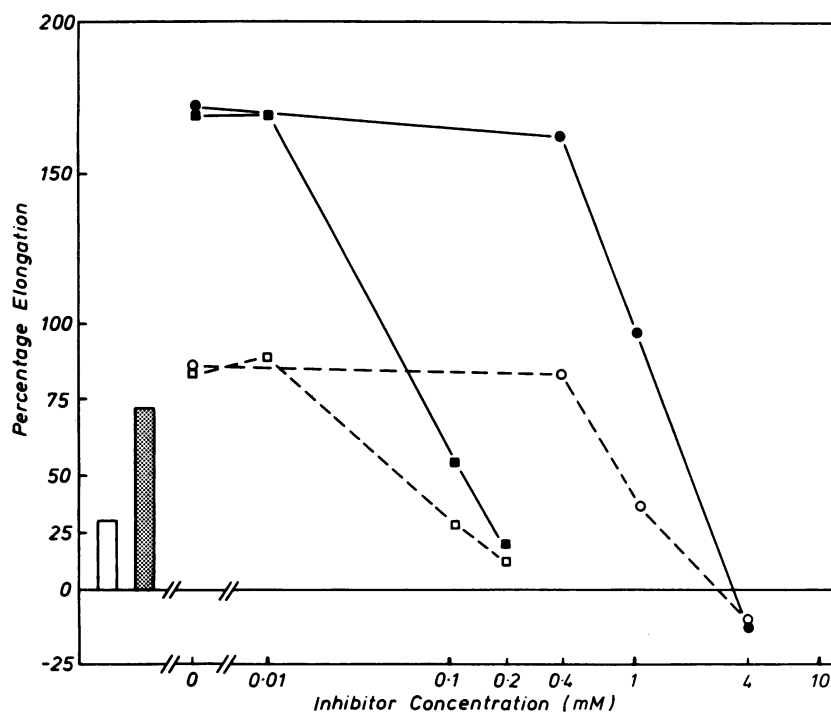


FIG. 2. Effect of three concentrations of TC (○—○, minus 2% sucrose; ●—●, plus 2% sucrose) or TFP (□—□, minus 2% sucrose; ■—■, plus 2% sucrose) on the elongation of wheat coleoptile sections tested in the presence of 0.01 mM IAA and measured after 21-h growth. Histograms: unshaded, water control; shaded, 2% sucrose control.

Table I. Comparison of the IC_{50} Values for Trifluoperazine and Tetracaine in a Number of Plant Hormone- and Red Light-Stimulated Biological Systems

Biological System	TFP	TC
	μM	
Red light-dependent betacyanin synthesis ^a	70	300
Cytokinin-dependent soybean callus growth ^a	70	500
Cytokinin-dependent betacyanin synthesis in <i>Amaranthus</i> half-seedlings ^a	150	600
Cytokinin-dependent cotyledon growth	50	ND ^b
Cytokinin-dependent betacyanin synthesis in <i>Amaranthus</i> cotyledons	150	ND
Auxin-dependent coleoptile growth (in absence of sucrose)	50	800
Auxin-dependent coleoptile growth (in presence of sucrose)	25	600
GA ₃ -dependent α -amylase induction (Triton X-100 extracted)	400	1000

^a Data from Elliott (12, 13).

^b ND, not determined.

Figure 5. There was a marked increase in activity recoverable after extraction with Triton X-100. However, both easily extracted (results not shown) and latent α -amylase synthesis were inhibited by similar concentrations of TFP and TC. IC_{50} values are shown in Table I, 0.4 mM for TFP and 1 mM for TC. Small but significant ($p < 0.1$) stimulations are seen with 10 μM TFP and 50 μM TC.

Table III gives the results of a reversal experiment showing that no permanent inhibition of α -amylase induction is caused by TFP if it is removed after a 5-h treatment.

DISCUSSION

The results described in this paper show remarkably similar concentration response curves to TFP and TC by the hormone-stimulated system used. IC_{50} values obtained are compared in

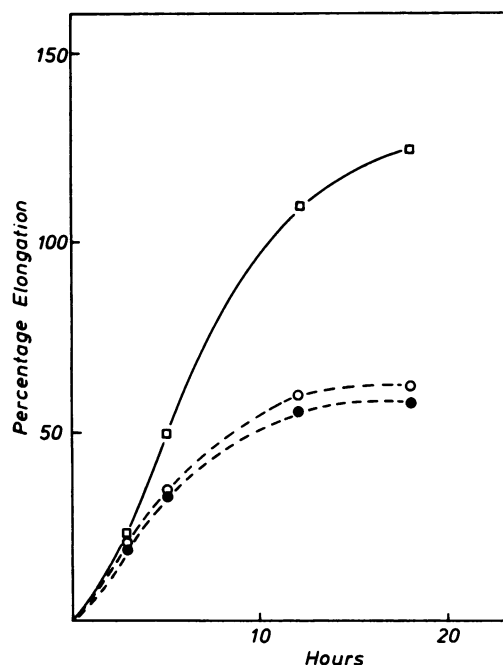


FIG. 3. Time course of wheat coleoptile section growth in the presence of TC (1 mM) or TFP (0.06 mM). Standard errors are not indicated but there was no significant difference between the two inhibitor treatments. (□—□), 2% sucrose + 0.01 mM IAA. (○—○), 2% sucrose + 0.01 mM IAA + TC; (●—●), 2% sucrose + 0.01 mM IAA + TFP.

Table I with those already described for two other cytokinin-dependent systems and for the red light-stimulated betacyanin synthesis in *Amaranthus* seedlings (12, 13). The close similarities between the IC_{50} values is consistent with a uniform theory of developmental control; the inhibition by these two compounds is suggestive of an involvement of calmodulin in this control, especially in view of the many indications of calcium interactions in

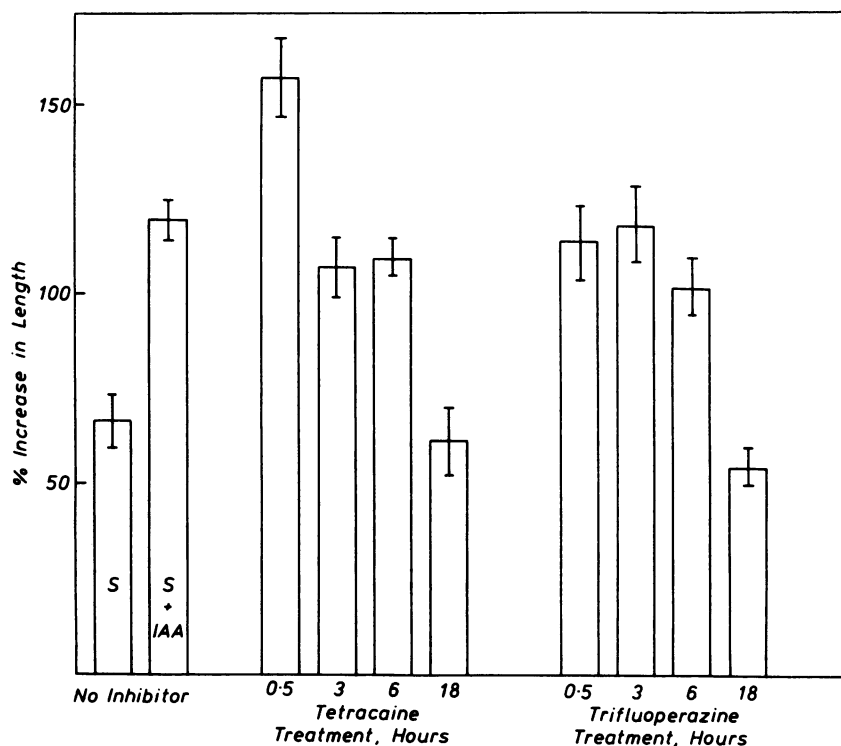


FIG. 4. Effect of duration of exposure to TC (1 mM) or TFP (0.06 mM) on the final length of wheat coleoptile sections after 18-h growth. The sections were exposed to the inhibitors at the beginning of the experiment, and for the periods indicated, before being transferred to inhibitor-free solutions. S, 2% sucrose; IAA, 0.01 mM IAA. The effect of both inhibitors was tested in the presence of these concentrations of sucrose + IAA throughout the experiment.

Table II. Effect of Loading Wheat Coleoptile Sections with Tetracaine or Trifluoperazine during Exposure to Different Temperatures, on the Subsequent Growth of the Sections

Sections incubated in all solutions contained 2% sucrose and 0.01 mM IAA. Inhibitor concentrations: TC = 1 mM; TFP = 0.06 mM.

Solutions		Temperature Treatments ^a			
		0-10 h (25°C), 10-20 h (25°C)		0-10 h (10°C), 10-30 h (25°C)	
First 10 h	Remaining 10 or 20 h	Total growth at		Total growth at	
		10 h	20 h	10 h	30 h
		%			
No inhibitor	No inhibitor	94 ± 5	174 ± 8	30 ± 1	170 ± 29
TC	TC	68 ± 5	58 ± 8	24 ± 2	49 ± 10
TFP	TFP	64 ± 7	63 ± 8	28 ± 3	88 ± 7
TC	No inhibitor	64 ± 5	73 ± 11	27 ± 2	154 ± 11
TFP	No inhibitor	61 ± 4	61 ± 8	30 ± 3	151 ± 10
2% Sucrose alone throughout		31 ± 2	93 ± 6	10 ± 1	95 ± 9

^a Per cent growth calculated on the basis of the initial length of the sections.

plant hormone and phytochrome action (27). The inhibitory effect of chlorpromazine on geotropism in *Avena* coleoptiles (3) is another indication of possible calmodulin-mediated regulation of plant development. The recent demonstration of a calmodulin-mediated activation of calcium uptake by microsomes from zucchini hypocotyl hooks, and its abolition by fluphenazine (6) provides a mechanism whereby regulation of cytoplasmic calcium may control developmental processes. Caution does need to be exercised, however, for the reasons outlined in other places (13 and references therein) to accommodate the possibility that the drugs may be inhibiting a calcium-phospholipid-dependent event

or indeed some membrane process quite unrelated to calcium regulation. It may be noted that in three of the biological responses shown here (Figs. 1 and 5) the drugs inhibit not only the hormone-induced response but also the control level of activity. One interpretation of this is that the control level is due to endogenous hormone content. It is also consistent with the interpretation that the calmodulin-mediated step is some fundamental growth process which, although related to hormone action, may not occur at the same time or at the same rate.

A recent report (19) describes the stimulatory affects of low concentrations of some calmodulin-Ca²⁺ complex inhibitors on

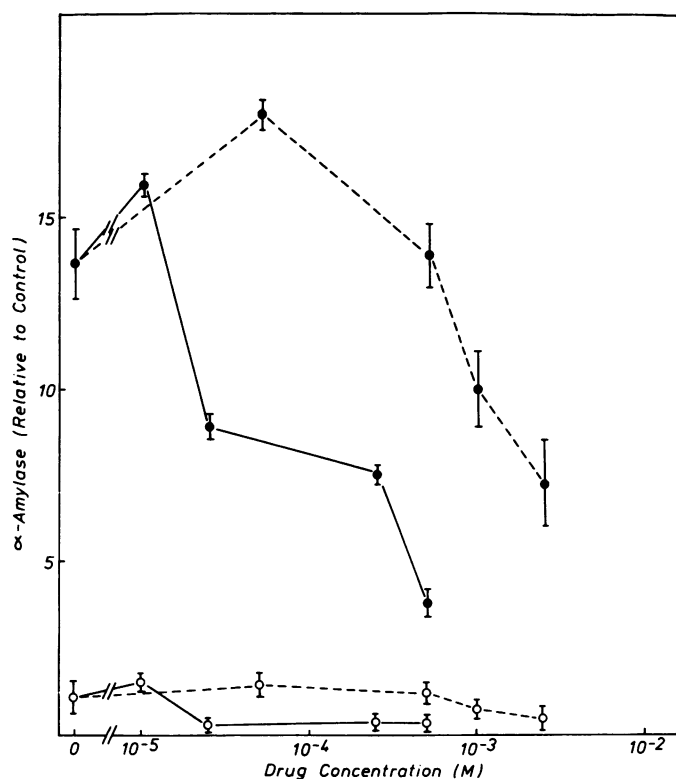


FIG. 5. Effect of TFP and TC on endogenous (control) and GA_3 -stimulated α -amylase production in barley aleurone layers. The results of three experiments, duplicate treatments in each, have been normalized. In the control, in the absence of GA_3 and drug additions, the α -amylase content was 0.231 ± 0.113 enzyme unit/aleurone layer. One enzyme unit was defined as the amount which brings about an A change of 1.0 at 620 nm in 5 min in the assay described by Chrispeels and Varner (4). Results are reported relative to the control, mean \pm SE. (O) control; (●) plus $1 \mu M$ GA_3 . (—) TFP; (---) TC.

Table III. Reversibility of Trifluoperazine Inhibition of α -Amylase Induction

Conditions during 24-h Induction ^a		α -Amylase Activity	
Inhibitor present during first 5 h	Conditions during remaining induction period (19 h)	- GA_3	+ GA_3
		units/aleurone layer	
No inhibitor	No inhibitor	0.313 ± 0.004	3.083 ± 0.032
TFP	TFP	0.480 ± 0.004	2.415 ± 0.021
TFP	No inhibitor	0.525 ± 0.092	3.065 ± 0.053

^a Aleurone layers (10) were incubated in standard medium (2 ml) containing 1 mM acetate buffer, pH 4.8, 10 mM $CaCl_2$, 15 μg chloramphenicol $\pm 1 \mu M$ GA_3 ± 0.25 mM TFP for 5 h as in the first column. Fresh solutions were then supplied as in the second column for the remainder of the incubation period (19 h). Aleurone layers were thus extracted at 24 h and α -amylase was estimated as in "Materials and Methods." Values are means of a single representative experiment \pm SE ($n = 3$).

fusicoccin-stimulated growth, acid secretion, and hyperpolarization of the potential difference. These experiments were over shorter time periods than those reported here, but the tendency for stimulation at low concentrations followed by inhibition at high concentrations has been noted in a number of systems (12, 13, and this paper, Figs. 1, 2, 4, and 5; Table II).

A recent study of ATP-dependent calcium transport in membrane preparations from soybean hypocotyls showed that IAA

pretreatment increases Ca^{2+} transport activity of preparations from the elongating zone, while with preparations from meristematic and maturing zones, pretreatment with zeatin promotes (18). In a purified mitochondrial fraction from etiolated oat shoots, phytochrome induces photoreversible calcium fluxes (24). Calmodulin-stimulated calcium uptake (6) is no longer observed in vesicles from corn coleoptiles after exposure to far-red light (7). All these results are clearly compatible with those in the present paper which show that an underlying control point for different hormones and phytochrome is one which is affected in the same way by Ca^{2+} -calmodulin inhibitors.

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