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CHLORPROMAZINE INDUCES MEMBRANE POTENTIAL DEPOLARIZATION AND ATP LOSS, AND INHIBITS MICROSOMAL ATPase IN SOYBEAN (*GLYCINE MAX* L.) ROOTS

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In intact soybean roots, chlorpromazine causes a depolarization of the membrane potential at low concentrations (as low as 30 μM, half-maximally at about 150 μM), and induces a marked decrease in ATP levels at higher concentrations (half-maximal at about 0.5 mM) over longer periods of time. In root microsomal suspensions, chlorpromazine inhibits an apparently specific ATPase activity component (half-maximally at about 0.3 mM). Chlorpromazine inhibits *N,N'*-dicyclohexylcarbodiimide-, diethylstilbesterol- and azide-inhibited ATPase activities. On linear sucrose gradients, chlorpromazine inhibition of ATPase activity occurs in two peaks, at 1.12 g/ml and 1.14–1.17 g/ml, which may represent a tonoplast and plasma membrane ATPase, respectively. Neither peak corresponds to the F_1 ATPase. It is unclear whether ATPase inhibition or ATP loss is the cause of the membrane potential depolarization. Clearly chlorpromazine has multiple effects which are probably unrelated to its calmodulin-inhibition activity.

Introduction

The phenothiazine type tranquilizer, chlorpromazine, is often used as a calmodulin inhibitor, even though it affects numerous other systems at a concentration similar to that required to inhibit calmodulin-mediated systems [1–3]. Some effects which apparently do not involve calmodulin include inhibition of soluble F_1 ATPase [4], an ecto ATPase of leucocytes [5] and a glutamate dehydrogenase [6]. At higher concentrations, chloropromazine inhibits all components of the mitochondrial electron-transport system [7]. It is also an activator of a membrane-associated cryptic enzyme system which activates a galactosyltransferase important in the osmotic response volume changes of a wall-less golden brown alga [8]. In none of these instances is the mechanism of action clearly known, although binding to the enzyme [6], and its detergent-like activity [8] have been implicated.

Using soybean (*Glycine max* L.) roots, we examined the effect of chlorpromazine on the membrane potential, and found that it induces a depolarization. This was unexpected, since Lado et al. [9] reported that chlorpromazine (and other phenothiazines) and fusicoccin synergistically induce proton efflux from maize roots, and trifluoperazine and fusicoccin synergistically induce a membrane hyperpolarization. To determine the site of chlorpromazine action, we examined its effect on intact root ATP levels and its inhibition of microsomal ATPase activity, on the assumption that it might inhibit the electrogenic ATPase pump assumed to be located at the plasma membrane. We find that chlorpromazine induces ATP loss and inhibits apparently specific ATPase compo-

Abbreviations: APW 6, artificial pond water, pH 6; DCCD, *N,N'*-dicyclohexylcarbodiimide; Mes, 2-(*N*-morpholino)-ethanesulfonic acid; Bistris propane, 1,3-bis(tris(hydroxymethyl)methylamino)propane.

nents, both at concentrations near those required to depolarize the membrane potential. None of these effects necessarily involve calmodulin.

Materials and Methods

Materials. ATP was from Boehringer Mannheim (Indianapolis, IN) and was prepared in a salt-free form by passing it through a cation-exchange resin (Dowex-50W, 8% crosslinked) prewashed with distilled deionized water and 1 N HCl, and pH adjusted to 6.5 with solid Bistris propane (Sigma). Firefly extract was prepared for measurements of ATP as described [10]. *p*-Nitrophenyl phosphate and firefly extract were from Sigma Chemical Co.

Membrane preparation. Soybean seeds (Wayne cultivar) were rinsed in tap water, then with distilled water, and sown on distilled water-saturated vermiculite in trays. The trays were covered with aluminium foil and placed in a incubator at 27°C for 4 days. The tip (1.5 cm) of the main root and the lateral roots were cut into a 0.1 mM $CaCl_2$ aerated solution, blotted and weighed, then homogenized with mortar and pestle on ice in a volume of grinding medium five times the weight of the root tips. The grinding medium consisted of 0.25 M sucrose, 10 mM Na_2EDTA, 5 mM $MgSO_4$, 25 mM Mes, 2.5 mM dithiothreitol, 1% w/v bovine serum albumin; pH adjusted to 7.00 with KOH or Bistris propane. The homogenate was filtered through four layers of cheesecloth and this filtrate centrifuged twice at $13\,000 \times g$ in a Beckman SW 27 rotor for 10 min, the pellets being discarded each time. The final supernatant was centrifuged a $83\,000 \times g$ for 30 min and the resulting pellet resuspended with a brush in a suspending medium (0.25 mM sucrose/2.5 mM dithiothreitol/Mes; pH adjusted to 7.00 with solid Bistris propane). This microsomal suspension was used as is or layered on either a step gradient or a linear gradient prepared as follows: 5 ml each of 25, 30, 35 and 40% w/w sucrose solutions containing 2.5 mM Na_2EDTA and 1 mM dithiothreitol were layered under the microsomal suspension (10 ml); this was centrifuged at $83\,000 \times g$ for 2 h, and the interfaces removed with a bent-tip pasteur pipette. Continuous linear gradients, 20–45% w/w sucrose (32 ml total), were prepared and 5 ml of microsomal suspension layered on; this was centrifuged at $83\,000 \times g$ for 2 h, then fractionated into fractions of about 2 ml.

Measurement of ATP levels. 4-day-old seedlings were washed in distilled water and three seedlings incubated in APW6 + 0.4 mM KCl [11] (8 ml total) with various concentrations of chlorpromazine. The roots were blotted, excised, weighed, and ground in a mortar and pestle on ice in 2 ml of grinding mix (5% trichloroacetic acid containing 1 g/l Na_2EDTA). The homogenate was diluted with 2 ml of the grinding mix, filtered through a Whatman No. 2 filter paper, and the mortar and filter washed with 2 ml of the grinding mix. The filtrate was stored at −20°C or assayed the same day for ATP. The ATP levels were assayed according to the method described in Ref. 10.

Membrane potential measurements. 3-day-old seedlings were grown as described, placed in a plexiglas holder and continuously bathed with APW 6 + 0.4 mM KCl at a flow rate of about 10 ml/min. The microelectrode preparation and circuitry are as described in Ref. 12. The microelectrode was inserted into cells of the outer cortical layer above the zone of root elongation. Chlorpromazine in APW 6 + 0.4 mM KCl was used at the same flow rate as APW 6 + 0.4 mM KCl alone.

Assays. Cytochrome *c* oxidase and antimycin A-insensitive NADPH-cytochrome *c* reductase were assayed according to the method described in Ref. 13 with modifications as given in Ref. 14. ATPase was assayed at 30°C using 5.0 mM $MgSO_4$ and Bistris propane/ATP (pH 6.5), and 25 mM Mes/Bistris propane (pH 6.5). The reaction was initiated by the addition of 0.1 or 0.05 ml of sample (final volume, 0.5 ml), and stopped by the addition of 1 ml of Ames reagent [15] containing 2.1% sodium lauryl sulfate (required to avoid complexing of chlorpromazine with molybdate). The final pH was 6.5. Phosphatase was measured using *p*-nitrophenyl phosphate as the substrate. Protein was measured using the Biorad assay (Biorad Laboratories, CA) [16].

Results

Chlorpromazine-induced depolarization. Partial depolarization of the membrane potential occurred

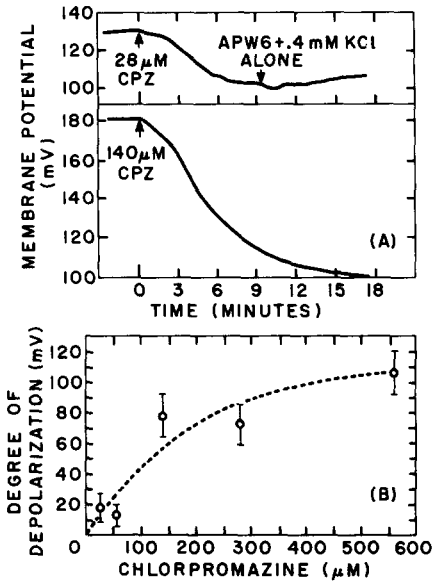

Fig. 1. Chlorpromazine-induced membrane potential depolarization. A, Examples of membrane potential depolarizations induced by 28 and 140 μM chlorpromazine (CPZ). The membrane potential is negative. B, Concentration dependence of the extent of depolarization, calculated as steady-state membrane potential minus the potential in the presence of chlorpromazine. Data are shown as mean ± S.D. Three to five measurements were made per treatment.

at concentrations of chlorpromazine as low as 28 μM, the degree of depolarization increasing at least up to 560 μM (Fig. 1B). The onset of depolarization was rapid, and came down to a new steady-state level after about 12 min (Fig. 1A). At low concentrations, the rate of depolarization was usually slower. The depolarization was usually not reversible.

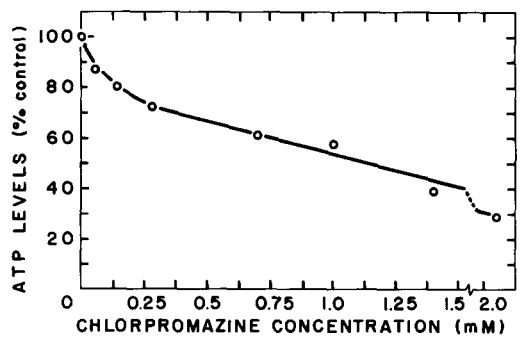

Fig. 2. Chlorpromazine-induced ATP loss in intact roots. Three seedlings per replicate (three replicates per treatment) were incubated for 30 min at the concentrations shown. Two experiments were done for chlorpromazine treatments 0.14, 0.28 and 0.7 mM.

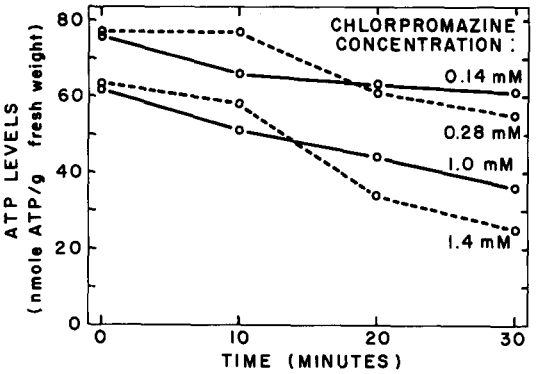

Fig. 3. Time course of chlorpromazine-induced ATP loss. Three seedlings per replicate (three replicates per treatment) were incubated at the chlorpromazine concentrations shown. Two experiments were done for 0.14 and 0.28 mM chlorpromazine. Each point is the mean.

ATP levels. The chlorpromazine concentration required to decrease ATP levels by 50% was about 0.5 mM when a 30 min incubation period was used (Fig. 2). Furthermore, the time course of ATP loss was quite slow, half-maximal loss occurring after about 15 min at various chlorpromazine concentrations (Fig. 3).

ATPase and phosphatase. Microsomal ATPase activity was inhibited 50% at a chlorpromazine concentration of about 300 μM; chlorpromazine-

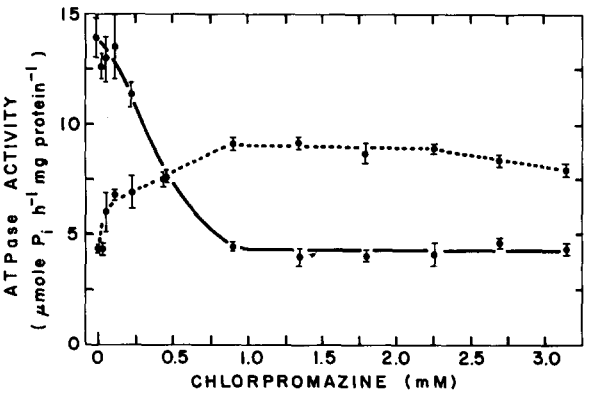

Fig. 4. Concentration dependence of chlorpromazine-induced inhibition and stimulation of ATPase and phosphatase. 50-μl aliquots of 0.086 mg/ml sample were added to the reaction mix and incubated for 30 min at 30°C. ATPase activity, ———; phosphatase activity, - - - - - -. Data are shown as mean ± S.D.

TABLE I

STEP GRADIENT LOCALIZATION OF CHLORPROMAZINE AND AZIDE EFFECTS ON ATPase AND PHOSPHATASE ACTIVITY

Data are shown as mean ± S.D. for aliquots from the interfaces of a sucrose step gradient on which a microsomal suspension had been centrifuged to equilibrium. Numbers in parentheses are the % of control activity for each interface. Reaction mixtures were incubated for 30 min at 30°C. Data for the microsomal suspension prior to step gradient centrifugation are also shown. Inhibitor concentrations were: chlorpromazine (1.12 mM) and azide (6 mM). Values are μmol P_i/h per mg protein.

Interface	Control	Substrate and treatment			
		ATP		*para*-Nitrophenyl phosphate	
		Chlorpromazine	Azide	Control	Chlorpromazine
Overlay					
25%	1.14 ± 0.14	1.20 ± 0.06 (105)	1.13 ± 0.15 (99)	2.11 ± 0.08	2.32 ± 0.03 (110)
25–30%	4.67 ± 0.39	3.82 ± 0.23 (92)	5.35 ± 0.36 (115)	5.02 ± 0.06	6.03 ± 0.19 (120)
30–35%	7.69 ± 0.42	5.89 ± 0.45 (77)	8.14 ± 0.10 (106)	7.62 ± 0.22	7.82 ± 0.17 (102)
35–40%	9.01 ± 1.08	6.86 ± 0.12 (76)	9.23 ± 0.12 (102)	9.40 ± 0.57	9.83 ± 0.16 (105)
Pellet	5.16 ± 1.42	1.87 ± 1.51 (26)	4.00 ± 1.07 (78)	4.53 ± 0.44	5.24 ± 0.18 (116)
Microsomal suspension	8.69 ± 0.36	4.61 ± 0.24 (53)	8.41 ± 0.29 (97)	5.23 ± 0.04	7.33 ± 0.05 (140)

induced stimulation of phosphatase activity had a very similar concentration dependence (Fig. 4). On step gradients, chlorpromazine-inhibited ATPase activity and stimulated phosphatase activity did not migrate entirely to the same density region (Table I). Azide at a concentration of 6 mM was used to determine if chlorpromazine-inhibited ATPase activity was in fact F_1 ATPase [17]. Azide did not significantly inhibit ATPase activity (Table I). The time course of chlorpromazine inhibition was also examined. The chlorpromazine-induced decrease in ATPase activity occurred within 2 min after 1.1 mM chlorpromazine was added (data not shown).

Inhibitor interactions. DCCD (0.1 mM), diethylstilbesterol (0.1 mM), and chlorpromazine inhibition were not additive; based on absolute inhibited activities, chlorpromazine inhibits DCCD-, diethylstilbesterol- and azide-inhibited activities (Table II). The concentration dependence of DCCD and diethylstilbesterol inhibition was examined in the presence of various chlorpromazine concentrations

TABLE II

INHIBITOR-SENSITIVE ATPase ACTIVITY IN A MICROSOMAL SUSPENSION

All values, except for the control, are the control value minus residual ATPase activity in the presence of inhibitor or combination of inhibitors. They thus represent the absolute activity which is inhibited. The data are shown as nmol P_i per h, and are mean ± S.D. Values in parentheses are % inhibition. Specific activity of the control was 10.9 μmol P_i per h per mg protein. Inhibitor concentrations were: DCCD (0.1 mM), diethylstilbesterol (0.1 mM), KCl (50 mM), vanadate (0.02 mM), chlorpromazine (1.12 mM) and azide (6 mM). As can be seen, in this experiment KCL did not stimulate ATPase activity, although stimulation was seen in other experiments.

	Inhibitors					
	Control	KCl	DCCD	Diethylstilbesterol	Vanadate	Chlorpromazine
Control	283.8 ± 2.2 (0)					
KCl	1.4 ± 11.1 (0)					
DCCD	105.9 ± 5.0 (37)	111.1 ± 8.8 (39)				
Diethylstilbesterol	86.8 ± 12.1 (31)	95.1 ± 19.3 (34)	134.6 ± 3.0 (47)			
Vanadate	185.2 ± 4.2 (65)	193.5 ± 6.1 (68)	225.8 ± 3.6 (80)	210.9 ± 3.6 (74)		
Chlorpromazine	160 ± 4.4 (56)	162.8 ± 3.7 (57)	170 ± 4.7 (60)	168.9 ± 2.2 (60	237.1 ± 5.5 (84)	
Azide	20.5 ± 3.3 (7)	34.6 ± 12.5 (12)	65.3 ± 8.1 (23)	131.3 ± 5.5 (46)	189.9 ± 2.5 (67)	168.6 ± 5.0 (59)

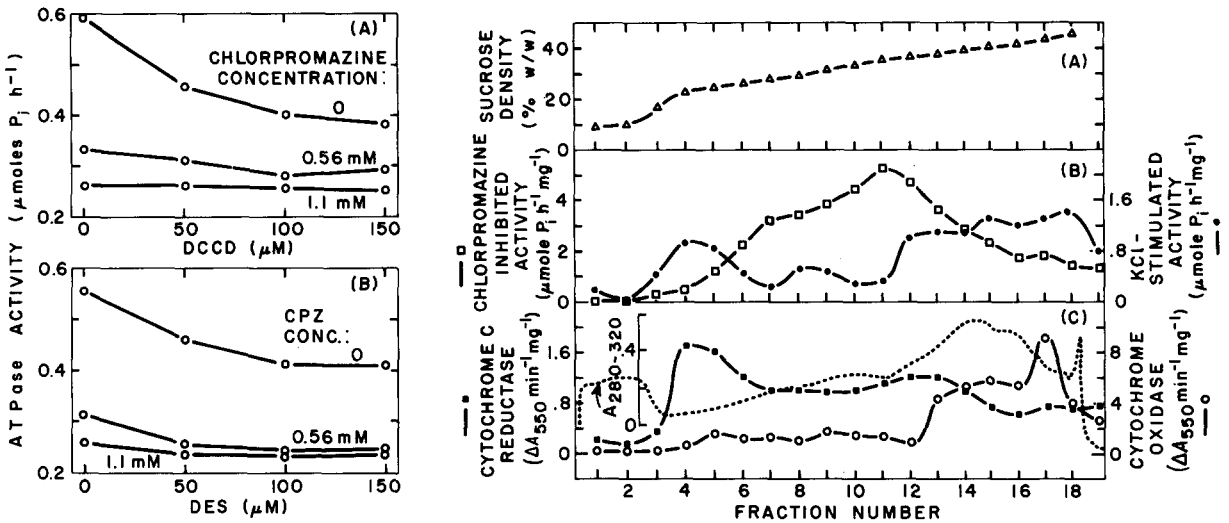

Fig. 5. DCCD- and diethylstilbesterol-induced ATPase inhibition at various chlorpromazine (CPZ) concentrations. ATPase activity in the presence of DCCD (A) and diethylstilbesterol (DES) (B) was measured at the chlorpromazine concentrations shown. Reaction mixtures were incubated for 20 min at 30°C. Data are shown as mean ± S.D.

Fig. 6. Linear sucrose gradient localizations of chlorpromazine-inhibited ATPase activity. Chlorpromazine-inhibited (□) and KCl-stimulated (●) activities were calculated by subtracting the activity in the presence of chlorpromazine (1.12 mM) or KCl (50 mM) from the control activity. Reactions mixtures were incubated for 1 h at 30°C.

to show the interaction of DCCD and diethylstilbesterol with chlorpromazine explicitly (Fig. 5). Vanadate and chlorpromazine activities are partially additive; some component of the ATPase activity may be inhibited by only one of these inhibitors (Table II). However, vanadate inhibition

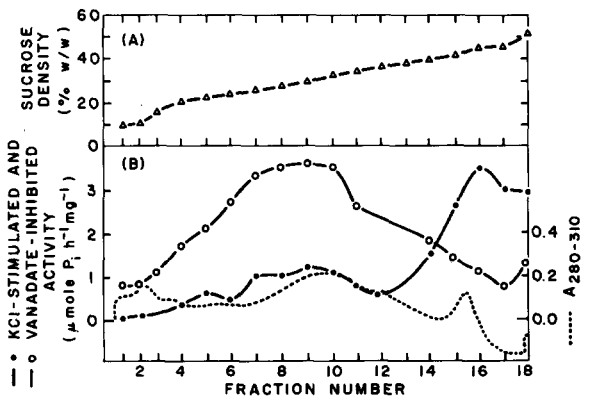

Fig. 7. Linear sucrose gradient localization of vanadate-sensitive ATPase activity. Vanadate-inhibited (○) and KCl-stimulated (●) activities were calculated as in Fig. 6. Vanadate was at 20 μM and KCl at 50 mM. Reaction mixtures were incubated for 45 min at 30°C.

apparently does not saturate [18], so the increased inhibition seen in the presence of both chlorpromazine and vanadate may not be caused by inhibition of different ATPase activities.

Linear gradient characterization. On linear gradients, chlorpromazine inhibition is seen as a broad peak (at about 1.16 g/ml) with a shoulder (at 1.2 g/ml), neither of which corresponds to the cytochrome *c* oxidase peak marking mitochondrial activity (Fig. 6). On these gradients, azide inhibition of ATPase activity is insignificant (data not shown). Vanadate inhibition forms a very broad peak throughout the gradient, with greatest inhibition at about 1.13 g/ml (Fig. 7). Chlorpromazine and vanadate inhibition have the same general localization on the linear gradients, overlapping to some extent.

Discussion

The three effects of chlorpromazine seen in this study probably correspond to two different mechanisms of action. The membrane potential is sensitive to low concentrations of chlorpromazine. We considered the most likely cause of the depolariza-

tion to be inhibition of mitochondrial activity or plasma membrane electrogenic ATPase pump activity. The depolarization was similar to that caused by carbonyl cyanide *m*-chlorophenylhydrazone in soybean roots (Chen, T.H. and Spanswick, R.M., unpublished data), but markedly different from that caused by KCN in oat coleoptiles (cf. Ref. 19), where there is an initial strong depolarization which repolarizes to a new steady-state membrane potential. The membrane potential could be due to the ATP loss caused by the action of chlorpromazine as an uncoupler or inhibitor of the F_1 ATPase [20,21]. The time course of ATP loss is slower than the induction of membrane depolarization. However, the ATP level in the cell in which the microelectrode is inserted may be affected before bulk ATP levels fall. In oat coleoptiles, even small KCN-induced decreases in ATP levels caused some membrane depolarization [19]. Although the onset of depolarization was rapid at low concentrations of chlorpromazine, the rate of depolarization was usually relatively slow; thus, ATP loss is a possible cause of depolarization.

Another possible cause of the depolarization is direct chlorpromazine-induced inhibition of the plasma membrane electrogenic pump [22]. Microsomal ATPase activity is inhibited by chlorpromazine with a concentration dependence similar to that for both membrane depolarization and ATP loss. The time course of chlorpromazine inhibition was fast, but it is not possible to extrapolate from time course results in vitro to depolarization induced in vivo. We examined the characteristics of the chlorpromazine-induced ATPase inhibition to determine whether it was inhibiting the plasma membrane ATPase.

Chlorpromazine inhibition of ATPase activity appears to be relatively specific, based on its interaction with other inhibitors and its localization on linear sucrose gradients. DCCD and diethylstilbesterol cause no additional inhibition in the presence of chlorpromazine. This suggests that chlorpromazine inhibits ATPase activity sensitive to DCCD and diethylstilbesterol, known to inhibit both the KCl-stimulated plasma membrane ATPase [18] and the chloride-stimulated tonoplast ATPase [26]. Vanadate inhibition is probably not saturated, so it is not clear if chlorpromazine is inhibiting vanadate-sensitive activity.

The main peak of chlorpromazine is found at a sucrose density which corresponds approximately to that of the plasma membrane ATPase activity based on the criterion of KCl stimulation [13] for soybeans [23–25]. In our preparations, KCl stimulation was always low, presumably due to the low reaction incubation temperature used in these experiments (the low temperature was used since it represents a more likely in vivo temperature). The KCl stimulation, when it was significant, was found in three regions of the sucrose gradients. One of these, at about 1.1 g/ml is probably due to the chloride-stimulated tonoplast ATPase [14]. The other two were at 1.13 and 1.2 g/ml, and probably correspond to the plasma membrane and mitochondrial ATPase, respectively. Chlorpromazine and vanadate inhibition peak at about 1.14–1.17 g/ml and 1.11–1.15 g/ml respectively; both peaks are very broad. The difference in chlorpromazine- and vanadate-inhibited ATPase localization, considered in conjunction with inhibitor interaction results, suggests that these two inhibitors act on ATPase components which are to some extent different. Overall, the results suggest that chlorpromazine inhibits a specific class of ATPases, one of which may be the plasma membrane ATPase.

Initially, it was thought that chlorpromazine was binding to a particular class of ATPases, causing a change in conformation which led to a change in substrate specificity, and thus a stimulation of phosphatase activity. The inhibitory and stimulatory action of chlorpromazine can be at least partially separated on step gradients, but we cannot discount the possibility that the phosphatase represents a dislodged form of the membrane-bound ATPase, because of the similarity in concentration dependence of the two activities.

There is no indication that the chlorpromazine effects reported in this paper involve calmodulin-mediated systems, because of the high concentration required to saturate the inhibitory effects. This suggests that great care must be taken in intact systems when assigning any phenothiazine-induced effect to calmodulin-mediated systems.

We are unable to determine the cause of plasma membrane depolarization induced by chlorpromazine. Either ATP loss or inhibition of the plasma membrane electrogenic ATPase could

account for it but, in our system, the concentration dependence and time course of all three effects is too similar to be able to choose on this basis alone; either mechanism, or a combination of both, is possible.

Note added in proof (Received April 7th, 1983)

A recent paper by Bonetti et al. [27] reports that chlorpromazine inhibits acid secretion and induces membrane potential depolarization in beet roots, and in other tissues, when treated with optimal levels of fusicoccin. They further report that chlorpromazine does not cause a significant change in ATP levels at low concentrations. We have examined the effect on ATP levels in soybean roots in more detail and determined that at concentrations up to 140 μM the ATP loss is not significant. Furthermore, we have compared chlorpromazine's effect with that of the uncoupler, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), and found that, for the same degree of membrane depolarization, CCCP causes twice the ATP loss that chlorpromazine does. This suggests that ATP loss is not the cause of chlorpromazine-induced depolarization.

Acknowledgements

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