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PHENOTHIAZINE ANTAGONISM OF CALMODULIN: A STRUCTURALLY-NONSPECIFIC INTERACTION

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SUMMARY: Phenothiazine antagonism of calmodulin-stimulated (Mg^{2+} + Ca^{2+})-ATPase activity in erythrocyte ghosts was examined to determine the structural specificity of the interaction. Four analogs of chlorpromazine, differing in the position of the chlorine substitution of the aromatic ring, were about equally potent in antagonizing calmodulin activation, while only the 2-chloro analog (chlorpromazine) has tranquilizer activity and antagonizes dopamine-sensitive adenylate cyclase. As all four analogs have similar hydrophobicity and surface activity, the results indicate that the antagonism of calmodulin by phenothiazines is unrelated to their pharmacological specificity and occurs at a structurally-nonspecific hydrophobic site on the protein.

INTRODUCTION

Calmodulin, an intracellular Ca^{2+} receptor widely distributed in many cell types (1), is thought to play an important role in synaptic transmission in the central nervous system (2). Levin and Weiss (3) showed that antipsychotic agents, such as trifluperazine, thioridazine, benperidol and pimozide, are potent, competitive antagonists of the calmodulin activation of cyclic AMP phosphodiesterase of brain, whereas antianxiety agents and antidepressants are considerably less potent antagonists. They showed further that chlorpromazine sulfoxide and promethazine, which are ineffective as antipsychotic tranquilizing agents, are also ineffective in antagonizing calmodulin activation, implying that calmodulin contained specific binding sites for tranquilizers. However, these studies were complicated by the fact that all of the less potent antagonists tested are also less hydrophobic, raising the possibility that the antipsychotic agents bound to a structurally nonspecific hydrophobic site on calmodulin. This possibility was suggested by an earlier study showing that the antipsychotic agents were also the most potent inhibitors of membrane-

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bound Na^+, K^+-ATPase in brain (4) and by a recent report that calmodulin contains a hydrophobic domain which is exposed by Ca^{2+} (5).

The presence of a specific binding site on calmodulin for antipsychotic tranquilizing agents is inconsistent with a number of other findings. Haloperidol (a butyrophenone) binds to calmodulin with a lower affinity (6), and is less potent in antagonizing calmodulin activation of phosphodiesterase (7), than chlorpromazine, yet haloperidol is about 50 fold more potent than chlorpromazine clinically as an antipsychotic agent (see 8). In addition, although Levin and Weiss (6) showed about a five fold difference in binding of (+)- and (-)-stereoisomers of butaclamol (a benzocycloheptapyridoisoquinoline tranquilizer) to calmodulin, it was reported that the isomers were equieffective in antagonizing calmodulin activation of phosphodiesterase (7), while only the (+)-stereoisomer is an effective tranquilizer (9). A similar lack of stereospecific antagonism of calmodulin was seen with the thioxanthene class of tranquilizers, cis- and trans-chlorprothixene and flupenthixol (7). Despite the apparent contradictions between clinical potency and calmodulin antagonism within the structurally diverse group of antipsychotic agents discussed above, it was possible that calmodulin contained a specific phenothiazine tranquilizer binding site, as trifluperazine, one of the most potent phenothiazine tranquilizers *in vivo* and *in vitro* (8), is also one of the most potent calmodulin antagonists known at present. To examine this possibility, a series of phenothiazine analogs which differ in their tranquilizer (10) and anti-dopamine (11) activity, but which have similar hydrophobicity, membrane actions and surface activity (10), have been used in this study. It is shown that calmodulin fails to discriminate between these compounds, suggesting that it lacks a structurally specific phenothiazine binding site of similar specificity to the site of tranquilizer action in the central nervous system.

MATERIALS AND METHODS

Erythrocyte ghosts were prepared by hemolyzing 4-day old washed human erythrocytes, obtained from the Red Cross and stored in acid-citrate-dextrose, in 20 imOsm sodium phosphate buffer, pH 7.4 by the procedure of Dodge *et al.*

(12), as previously described (13). The ghosts were treated further with 0.1 mM EDTA-1.0 mM Tris, pH 8.0 to remove endogenous activators, as previously described (13). (Mg^{2+} + Ca^{2+})-ATPase was assayed with [γ-^{32}P]ATP in a medium containing (in mM) 66 NaCl, 55 Tris-maleate, 0.1 ouabain, 6.5 $MgCl_2$, 0.1 EGTA and 87 µM free Ca^{2+} (calculated as in 13) in a final volume of 0.6 ml at pH 7.2, unless otherwise stated. Membrane suspension (50 µl, approx. 0.2 mg protein), 0.5 µg calmodulin (when required) and phenothiazines were incubated for 10 min prior to starting the reaction by the addition of 2 mM ATP. The mixture was incubated for 60 min at 37°C and the reaction terminated with trichloracetic acid and [$^{32}P_i$] estimated as previously described (13). Phenothiazine solutions were freshly prepared and assayed in the dark, as preliminary studies showed a pronounced increase in inhibition of both basal and calmodulin-stimulated ATPase activity after preincubation of membranes with chlorpromazine for 60 min in direct daylight. All (Mg^{2+} + Ca^{2+})-ATPase activities were corrected for Mg^{2+}-ATPase activity in the absence of added $CaCl_2$ and the activity expressed as µmoles P_i/hr per ml of original packed cells.

Chemicals. Calmodulin was a highly purified preparation from bovine brain kindly supplied by Dr. J. Wang, University of Manitoba. [γ-^{32}P]ATP (20-40 Ci/mmol) was from Amersham. Charcoal (Norit A) was from Fisher Chemical Co. and EGTA was from Sigma. Inorganic salts were Analytical-Reagent grade. Chlorpromazine analogs were kindly supplied by Dr. A.R. Green, Smith, Kline and French (Australia).

RESULTS

(Mg^{2+} + Ca^{2+})-ATPase activity in EDTA-treated ghosts was shown in preliminary studies to be maximally activated by 0.5 µg of calmodulin per assay tube (0.6 ml). The effect of increasing concentrations of chlorpromazine hydrochloride (2-chloro-10-dimethylamino-propylphenothiazine hydrochloride) and the 1-, 3- and 4-chloro analogs was investigated in the presence and absence of calmodulin (Fig. 1). None of the chlorpromazine analogs significantly inhibited the non-activated (Mg^{2+} + Ca^{2+})-ATPase activity. All of the chlorpromazine analogs progressively inhibited the calmodulin stimulated (Mg^{2+} + Ca^{2+})-ATPase activity toward the basal level with a similar potency. The mean concentrations necessary to inhibit calmodulin activation 50% (I_{50}) were 300 µM, 135 µM, 125 µM and 120 µM for the 1-,2-,3- and 4-chloro analogs, respectively. None of the chlorpromazine analogs inhibited the Mg^{2+}-ATPase activity in the absence of Ca^{2+} (results not shown). The potency of chlorpromazine (2-chloro) and the 4-chloro analog were not significantly affected by increasing the free Ca^{2+} concentration in the medium from 2.6 µM to 173 µM (Fig. 2).

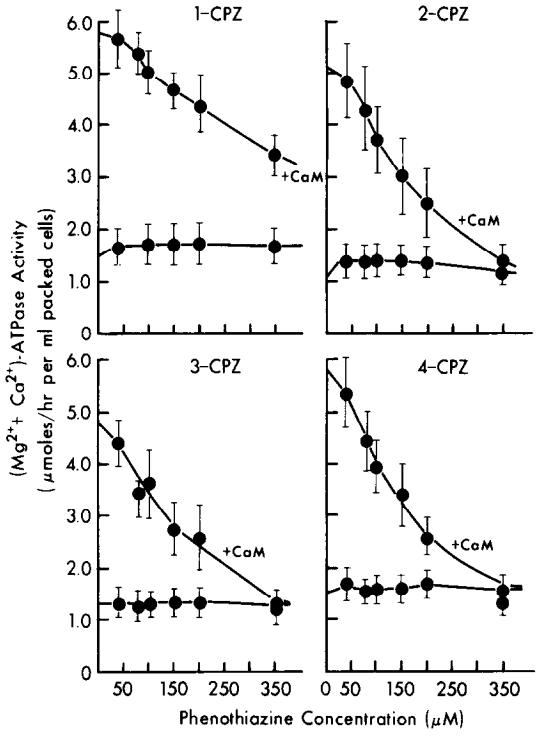

Fig. 1. Antagonism of calmodulin-stimulated (Mg^{2+} + Ca^{2+})-ATPase activity by chlorpromazine analogs. The concentration of phenothiazines was increased in the absence (bottom-curve) and in the presence of 0.5 μg of calmodulin (top curve). The chlorpromazine analogs were substituted with chlorine in position 1-, 2- (chlorpromazine), 3- and 4-. The results represent the mean ± standard error of the mean of 3 experiments.

DISCUSSION

Antipsychotic tranquilizers interact stereospecifically with dopamine binding sites in the caudate nucleus of the brain (9), and their specificity of binding correlates well with their clinical potency (14). These agents also antagonize dopamine-stimulation of adenylate cyclase in the caudate nucleus, but in this system there is not a complete correlation with antipsychotic activity when a structurally diverse group of antipsychotic agents is considered (11). Nevertheless, the dopamine binding site coupled to adenylate cyclase discrimates between various chlorpromazine analogs differing in the position of the 2-chloro substitution on the phenothiazine ring (11). While all the analogs tested inhibit membrane Na^+, K^+-ATPase activity, only the 2-chloro

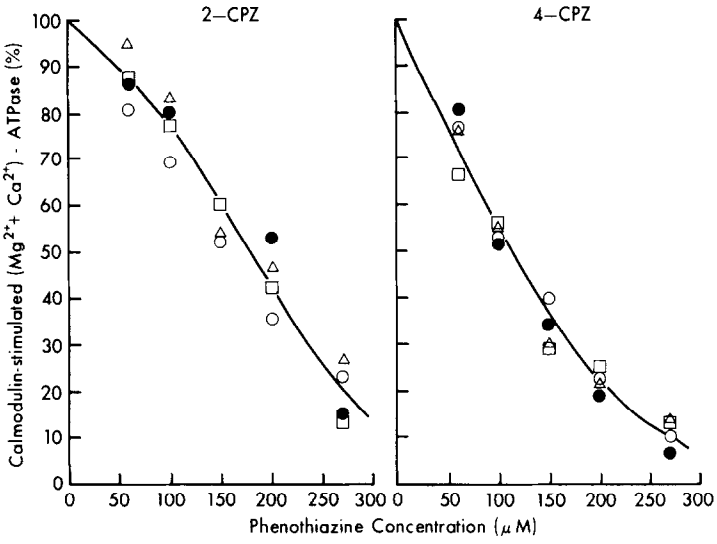

Fig. 2. Effect of Ca^{2+} on the antagonism of the calmodulin stimulation of (Mg^{2+} + Ca^{2+})-ATPase activity by chlorpromazine (left panel) and the 4-chloro analog of chlorpromazine (right panel). Calmodulin stimulation of (Mg^{2+} + Ca^{2+})-ATPase activity in EDTA-treated erythrocyte ghosts was normalized to 100% at each Ca^{2+} concentration. The free Ca^{2+} concentration was 2.6 μM (○), 14.8 μM (△), 57.7 μM (□) and 172.8 μM (●). Phenothiazines did not significantly inhibit basal (Mg^{2+} + Ca^{2+})-ATPase activity in the absence of calmodulin at any of the Ca^{2+} concentrations examined.

analog (chlorpromazine) shows tranquilizer activity (10) and antagonizes dopamine-sensitive adenylate cyclase (11). The Na^+, K^+-ATPase inhibition (I_{50} = 50-150 μM) was probably due to a general membrane interaction, as all four analogs have a similar hydrophobicity and surface activity, and penetrate the central nervous system equally (10). While in the present study none of the chlorpromazine analogs inhibited the basal (Mg^{2+} + Ca^{2+})-ATPase activity in EDTA-treated erythrocyte membranes at the doses used, all four analogs antagonized calmodulin activation with similar potency. The antagonism of calmodulin by the four analogs tested could only be demonstrated in the presence of Ca^{2+} and calmodulin. The mechanism of antagonism of calmodulin-activation of (Mg^{2+} + Ca^{2+})-ATPase was similar for both tranquilizer and non-tranquilizer chlorpromazine analogs, as the inhibition was independent of Ca^{2+} from 2.6 μM to 173 μM in both cases. Levin and Weiss (3) also

reported that phenothiazine antagonism of calmodulin activation of phosphodiesterase is independent of Ca^{2+}.

Previous attempts to examine the relationship between inhibition of calmodulin and clinical efficacy of antipsychotic tranquilizers utilized a structurally diversed group of agents (3, 7). Since the mechanism of antipsychotic activity is not completely understood and may be different for various classes of drugs, it was possible that calmodulin was specific for the phenothiazine antipsychotic agents. It was suggested that the relative binding of phenothiazine derivatives paralleled their clinical antipsychotic activity (6), but in that study the inactive phenothiazines used were also less hydrophobic, and are expected to be less potent if a hydrophobic interaction determined the partitioning and free energy of binding. This difficulty was overcome in the present study, which shows conclusively that antagonism of calmodulin by phenothiazines parallels the hydrophobicity of the compounds rather than their clinical efficacy as tranquilizers. This study suggests that antagonism of Ca^{2+}-dependent events by phenothiazines is not a sufficient criterion for establishing a physiological role of calmodulin and results obtained with phenothiazines and other hydrophobic compounds should be viewed with caution.

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