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DIFFERENTIAL EFFECTS OF CHLORPROMAZINE AND CHLORPROMAZINE FREE RADICAL ON CALCIUM TRANSPORT IN SARCOPLASMIC RETICULUM VESICLES

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Abstract—Chlorpromazine activates, and chlorpromazine free radical inhibits, the calcium transport system of fragmented sarcoplasmic reticulum. The associated calcium-dependent ATPase is affected in a similar manner. However, the calcium independent (basal) ATPase is not reduced in activity by the free radical. It is suggested that unmodified chlorpromazine rather than its free radical form is the pharmacologically active moiety.

The exact locus of action of chlorpromazine, and phenothiazines generally, is not known. Tranquillizers, in rendering target cells less responsive to stimuli, could operate through a variety of mechanisms and chlorpromazine is known to have a multiplicity of actions on a wide selection of tissues. It effects oxidative phosphorylation [1], flavin enzymes [2], enzymes of glycolysis [3], membrane bound calcium [4] and the permeability of membranes to a large number of substances [5]. In a review of the diversity of effects of phenothiazines Guth and Spirtes [5] concluded that "the alteration of membrane permeability may constitute the major mode of action of the phenothiazine tranquillizers". Seeman [6] has reviewed the evidence that phenothiazines exert their action by dissolving in the cell membrane, thereby obeying the Meyer–Overton rule of anaesthesia that "narcosis commences when any chemically indifferent substance has attained a certain molar concentration in the lipids of the cell" [7, 8]. The degree of stabilization conferred on the cell membrane can be conveniently followed by determining the resistance to haemolysis of red cells under lytic conditions [9]. Chlorpromazine has been shown to increase the surface area of the cell membrane, thereby reducing loss of cell contents during swelling [10].

Another system sensitive to small changes in cell permeability is fragmented sarcoplasmic reticulum of skeletal muscle [11]. The vesicles contain a membrane-bound ATP-dependent calcium transport system which requires that the membrane be largely intact for significant calcium accumulation to occur. With aging or the use of membrane disrupting agents the capability of transporting calcium decreases due to the vesicles becoming increasingly leaky and the ATPase activity increases as the system becomes uncoupled [12]. It is thus a useful model for studying pharmacological agents thought to interact with cell membranes *in vivo*.

There have been reports of inhibiting effects of phenothiazines [13, 14] and barbiturates [15] on the calcium transport system which would not be anticipated if they were acting as stabilizing agents. More recently, by removing activating potassium ions, we have shown [16] that chlorpromazine and other phenothiazines as well as various barbiturates considerably stimulated calcium transport and hypothesized that the increased uptake was due to improvement in the integrity of the membranes of the isolated vesicles.

A major metabolite of chlorpromazine is the pharmacologically inactive sulphoxide which is formed through the chlorpromazine free radical intermediate [17] (Fig. 1). Akera and Brody [18, 19] have demonstrated that chlorpromazine free radical is a potent inhibitor of membrane-bound Na + K - ATPase of rat brain and have suggested that this may be its active form. We have, therefore, studied the effect of chlorpromazine free radical on the sarcoplasmic reticulum calcium pump.

MATERIALS AND METHODS

Sarcoplasmic reticulum vesicles. Rabbit skeletal muscle was homogenized at 4° in three volumes of 1 M sucrose, and after dilution to about 0·65 M sucrose, 450 ml of the 10,000 *g* "post mitochondrial" supernatant was placed on 100 ml of 1 M sucrose in an MSE BXIV zonal rotor [20]. After centrifugation at 25,000 rev/min for 60 min in an MSE superspeed 50 ultracen-

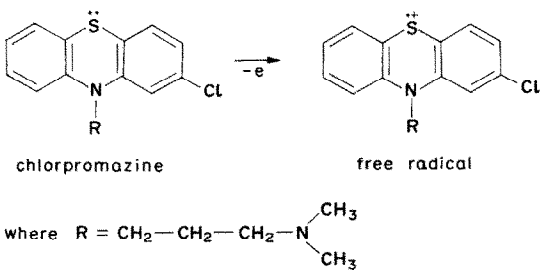

Fig. 1. Formation of chlorpromazine free radical.

trifuge, the fragmented sarcoplasmic reticulum was collected in four 25-ml fractions. The second-lightest fraction, which has the highest calcium transport activity [20] was used for this study.

Chlorpromazine free radical. This was prepared either by condensation of chlorpromazine sulphoxide and chlorpromazine [18] or by the *in situ* oxidation of chlorpromazine by hydrogen peroxide and peroxidase [21]. While the actual concentration of chlorpromazine free radical can be determined in strong acid medium [22] the steady-state concentration could not be determined experimentally at neutral pH values due to the very rapid decay rate [22]. For this reason it is the initial chlorpromazine concentration in the various media that is referred to in the figures.

Chemicals. Chlorpromazine and chlorpromazine sulphoxide were kindly supplied by May & Baker Ltd. All chlorpromazine solutions were stored in the dark

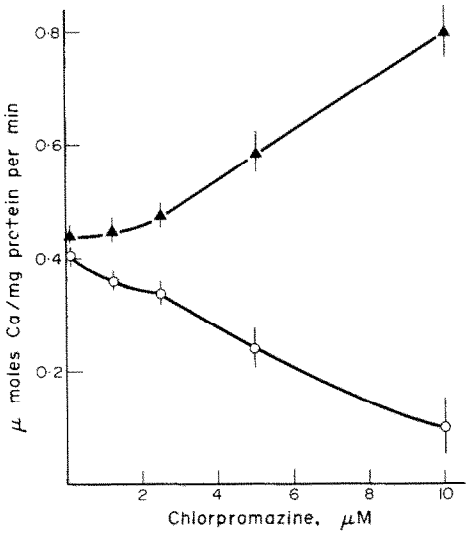

Fig. 2. Effects of chlorpromazine and chlorpromazine free radical on the rate of calcium accumulation of muscle microsomes. Microsomes (40 μg protein) were preincubated with chlorpromazine (▲) and chlorpromazine, 40 μM hydrogen peroxide and 4 μg peroxidase (○) in a volume of 0·5 ml at 37° for 5 min. Then 0·5 ml of calcium uptake medium was added and the reaction terminated after 1 min. Mean of five experiments. Vertical line indicates S.E.M.

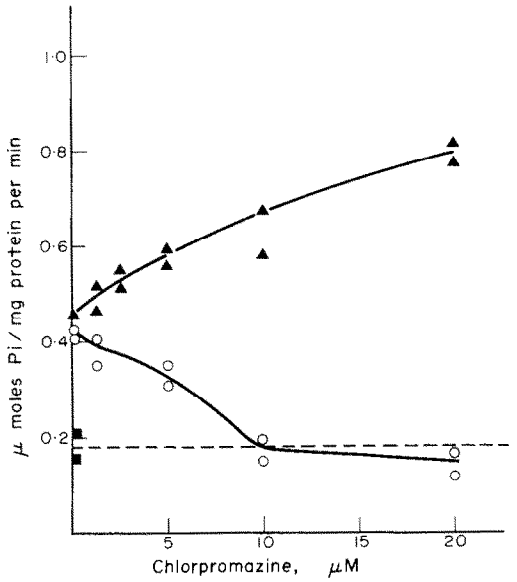

Fig. 3. Effects of chlorpromazine and chlorpromazine free radical on the calcium-dependent ATPase of muscle microsomes. Conditions as in Fig. 1. Chlorpromazine (▲) and chlorpromazine, hydrogen peroxide and peroxidase (○). Results of two experiments. The dashed line represents the mean activity of the calcium-independent (basal) ATPase in the presence of 1 mM EGTA (■).

to reduce photodecomposition. Disodium ATP(Boehringer Gmbh, Mannheim) was converted to the imidazole salt by the procedure of Schwartz *et al.* [23]. All other chemicals were of analytical grade.

Calcium uptake. This was measured [16] after filtration of the medium through Millipore filter, pore size 0·45 μm, and counting ^{45}Ca in a Nuclear Chicago liquid scintillation counter 720 series.

ATPase activity. This was measured as described previously [16].

RESULTS

Figure 2 shows that pre-incubation of microsomes with varying concentration of chlorpromazine, hydrogen peroxide and peroxidase for 5 min causes a progressive decrease in calcium uptake with increasing free radical concentration whereas unmodified chlorpromazine has a marked stimulatory effect. The effects on the calcium-dependent ATPase follow a similar pattern (Fig. 3). It is to be noted that in the absence of chlorpromazine, hydrogen peroxide and peroxidase have only a minimal effect on calcium uptake and ATPase activity. As the transport system remains tightly coupled [16] chlorpromazine also increases the calcium-dependent ATPase. Only that fraction of the total ATPase activity associated with the calcium pump is inhibited by the free radical; the remaining activity is due to the calcium-independent (basal) ATPase [24]. The dashed line in Fig. 3 shows the

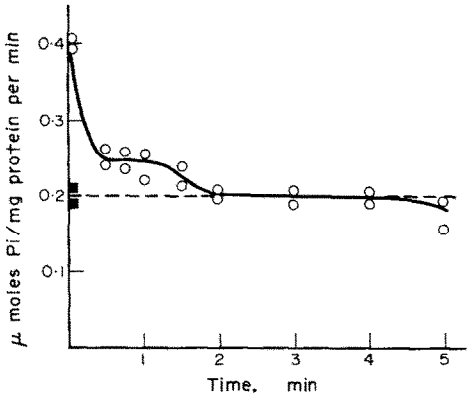

Fig. 4. Effect of time of pre-incubation in 10 μM chlorpromazine, hydrogen peroxide and peroxidase on the calcium-dependent ATPase of muscle microsomes. Results of two experiments. The dashed line represents the mean activity of the calcium independent (basal) ATPase in the presence of 1 mM EGTA. (■)

ATPase activity in the presence of the selective calcium chelating agent ethylene glycol *bis* (amino-ethyl ether) *N,N'*tetra-acetic acid (EGTA). While preincubation was usually continued for 5 min, full inhibition of the calcium pump associated ATPase had occurred within 2 min in 10 μM chlorpromazine free radical (Fig. 4).

When the pure stable chlorpromazine free radical [18] is added to the incubation medium there is a smaller reduction of calcium uptake, due perhaps to the less efficient interaction with the vesicular membrane (Fig. 5). Chlorpromazine sulphoxide is without effect on the system.

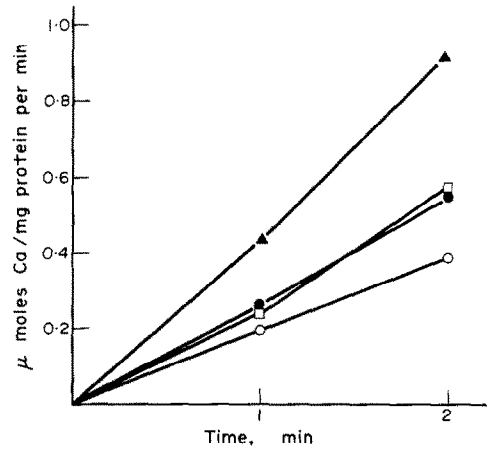

Fig. 5. Effects of chlorpromazine, chlorpromazine free radical and chlorpromazine sulphoxide on the calcium uptake of muscle microsomes. Solutions of the drugs were prepared from the solid state immediately before addition to uptake medium at a final concentration of 5 μM. Control (●), chlorpromazine (▲) chlorpromazine sulphoxide (□) and chlorpromazine free radical (○).

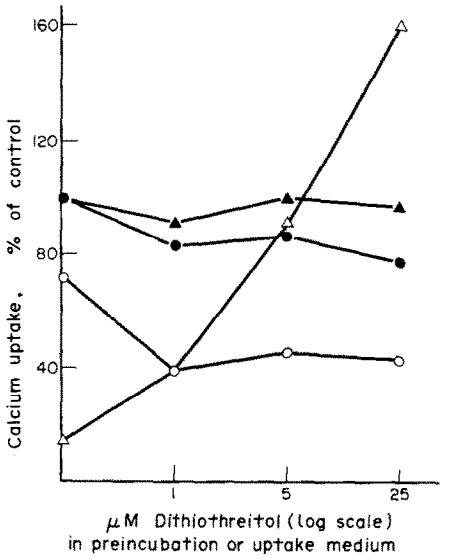

Fig. 6. The effects of chlorpromazine and chlorpromazine free radical on the calcium uptake of muscle microsomes in the presence of dithiothreitol (DTT). Conditions as in Fig. 2. DTT was added to the uptake medium without (●) and with (○) 10 μM chlorpromazine hydrogen peroxide and peroxidase in the preincubation medium. DTT was added to the preincubation medium without (▲) and with (△) 10 μM chlorpromazine, hydrogen peroxide and peroxidase in the pre-incubation medium.

Akera and Brody [18] found that the inhibition of Na + K-ATPase by chlorpromazine free radical was reversed by the addition of dithiothreitol (DTT) to the medium. We observed little effect of DTT when incorporated in the uptake medium after pre-incubation to generate the free radical. However, when 25 μM DTT was present in the preincubation medium with hydrogen peroxide, peroxidase and chlorpromazine, not only was inhibition prevented but definite activation occurred (Fig. 6). This is being investigated further.

DISCUSSION

It is thus clearly shown that chlorpromazine free radical has effects opposite to those of chlorpromazine in this *in vitro* system, decreasing accumulation of calcium and inhibiting the associated ATPase activity. While the free radical is formed in the detoxication of chlorpromazine to the sulphoxide [17] and Gubitz *et al.* [21] subsequently showed that it was the free radical form of each phenothiazine that was inhibiting brain Na + K-ATPase, there is little relationship between the ease of formation of such free radicals and pharmacological activity. Thus promazine, with mild tranquillizing properties, readily forms inhibitory free radicals whereas trifluoperazine and triflupromazine, acknowledged tranquillizers, do not [21]. Also, Seeman [9] did not find any correlation between the half wave potential for the one-electron oxidation step (to

form the free radical of the phenothiazine) and the daily clinical dose required to control acute schizophrenia.

The role of calcium in stabilizing membranes has been known since the work of Locke in 1894 [25]. Calcium removal results in depolarization and spontaneous electrical discharges [26]. It would be expected, therefore, that agents increasing the binding of calcium to membranes or decreasing the amount released on receipt of a stimulus would exert a stabilizing effect. That the effect of chlorpromazine in increasing calcium transport in this model system can be extrapolated to whole tissue may be inferred from the finding of Balzer and Hellenbrecht [27] who observed a lack of response of sartorius muscle to 5 μg/ml acetylcholine when 30 μM chlorpromazine was added to the medium.

The generation of free radicals from phenothiazines by ultraviolet irradiation may have some physiological significance however. Chlorpromazine, and phenothiazines generally are concentrated in the eye [28] and it has been suggested [29] that light-induced free radical formation may be a factor in the causation of opacities of cornea and lens by reaction with SH groups of susceptible proteins.

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