CALCIUM EFFLUX AND RESPIRATORY INHIBITION IN BRAIN MITOCHONDRIA: EFFECTS OF CHLORPROMAZINE METABOLITES

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Summary: 7-Hydroxychlorpromazine was found to be similar to chlorpromazine in its ability to inhibit brain mitochondrial calcium accumulation and respiration. 7,8-Dihydroxychlorpromazine, however, was found to produce a marked efflux of calcium accumulated by these mitochondrial preparations in the presence of ATP. In addition, this metabolite or one of its orthoquinone forms was observed to gradually inhibit state 4 respiration and prevent the stimulation of oxygen uptake normally observed upon the addition of phosphate and phosphate acceptor. The possible importance of sulfhydryl groups in these inhibitory actions is discussed.

Chlorpromazine (CPZ) has previously been shown to inhibit respiration or ATP supported calcium accumulation by partially purified preparations of rat brain mitochondria, while chlorpromazine sulfoxide, one of the major metabolites of this drug, was found to be comparatively inactive (1). CPZ is known to undergo 7-hydroxylation (2); the resulting 7-hydroxychlorpromazine (7-OH-CPZ) possesses pharmacological activity similar to that of chlorpromazine (3) and has been shown to appear in the central nervous system of experimental animals (4, 21). When incubated with liver microsomes, monohydroxylated chlorpromazine derivatives are further hydroxylated to form orthodihydroxy metabolites which then undergo mono-O-methylation (5, 6). 7,8-Dihydroxychlorpromazine (7,8-diOH-CPZ) and its methoxylated analogs have recently been found in biological samples taken from chronic schizophrenic patients solely on CPZ therapy (7). In the current investigation the effects of 7-OH-CPZ and 7,8-diOH-CPZ on brain mitochondrial calcium transport and respiration were studied.

Experimental

The hydroxylated chlorpromazine derivatives used in this study were obtained from the Psychopharmacology Research Branch, N. I. M. H. Chlor-

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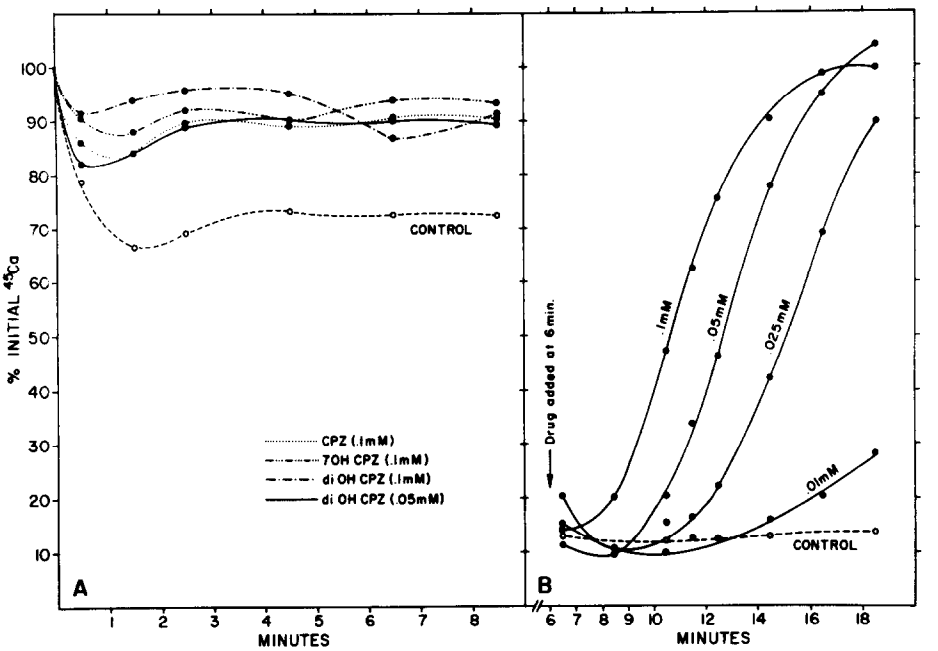

Figure 1: The percentage of the initial concentration of calcium has been plotted as a function of time of incubation. Composition of reaction mixture 1A: 38.0 mM HEPES (pH 7.4), 1.9 mM phosphate, 5.7 mM potassium glutamate, 4.75 mM Mg Cl_2, .05 mM $Ca^{45}Cl_2$, 6 mM mannitol, 4 μM histidine, .04 mg bovine serum albumin per ml, KCl to 250 mosmolar, .312 mg mitochondrial protein per ml. 1B: 36 mM HEPES (pH 7.4), 4.5 mM $MgCl_2$, 4.5 mM ATP, .1 mM $Ca^{45}Cl_2$, 6 mM mannitol, 4 μM histidine, .04 mg BSA per ml, KCl to 250 mosmolar, .326 mg mitochondrial protein per ml. T = 24°C.

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promazine hydrochloride was a gift from Dr. Harry Green of Smith, Kline and French Laboratories. Fresh solutions of CPZ and its metabolites were prepared for each experiment. Calcium-45 chloride ($^{45}CaCl_2$) obtained from New England Nuclear Corp. was contributed by Dr. Emil Bozler. Dithiothreitol (DTT), HEPES buffer and DTNB (5,5'-dithio-bis-(2nitrobenzoic acid)) were purchased from Calbiochem.

Mitochondria were prepared from rat brain as previously described (8) except that the mitochondrial pellet was collected at 5000 x g. In respiration-supported calcium uptake studies, mitochondria were incubated in appropriate reaction media containing 0.05 mM $^{45}CaCl_2$ (.22μCi per 1.0 μmole); 0.1 mM $^{45}CaCl_2$ (.45μCi per μmole) was chosen for ATP-supported uptake. At frequent time intervals samples were filtered through 0.45

micron Millipore filters. Aliquots of the filtrates were dried on ringed aluminum planchets and counted in a gas flow counter (Nuclear Chicago) in order to calculate ^{45}Ca uptake.

The effects of CPZ and its hydroxylated metabolites on calcium transport are illustrated in Fig. 1. The hydroxylated metabolites appear to be about as effective as CPZ in blocking calcium accumulation in the presence of glutamate plus inorganic phosphate (Fig. 1A). With ATP-supported calcium uptake, CPZ and 7-OH-CPZ exert about the same degree of inhibition (not illustrated). In contrast, with 7,8-diOH-CPZ present, calcium uptake proceeded for the first two or three minutes, but this was followed by a marked efflux of calcium from the mitochondria.

When mitochondria were calcium loaded by incubation with 0.1 mM $^{45}CaCl_2$ for 6 minutes, the addition of 0.1 mM 7,8-diOH-CPZ (Fig. 1B) promoted a dramatic and complete efflux of accumulated calcium. At lower drug concentrations onset of efflux was delayed. In experiments of longer duration a release of essentially all accumulated calcium was found at 7,8-diOH-CPZ concentrations as low as 0.01mM. 7,8-diOH-CPZ assumes a red color upon dissolving and becomes darker with time, a change which probably reflects the formation of an orthoquinone. Efflux from calcium loaded mitochondria was delayed if DTT was added before the dihydroxy metabolite. Prior addition of DTT (.6mM) prevented the color from appearing initially; with time, however, the red color did return. The calcium efflux which was observed in the presence of DTT and 7,8-diOH-CPZ was well under way before the color in the incubation mixture became visible.

Chlorpromazine and 7-OH-CPZ had similar effects on mitochondrial respiration. With glutamate or succinate as substrate, state 4 respiration was stimulated with either of these phenothiazines (0.1mM) while state 3 respiration was slowed and the state 4-3 transition became less sharply defined. Effects of 7,8-diOH-CPZ on respiration with glutamate or succinate as substrate are recorded in Figure 2. This metabolite appears to stimulate state 4 respiration initially. Unlike the other phenothiazines the dihydroxy metabolite gradually caused state 4 respiration to decrease. This inhibitory effect appeared after about 1.5 minutes of exposure to the drug with glutamate and after about 5 minutes with succinate as substrate. Once this inhibitory effect had appeared addition of ADP plus Pi caused no

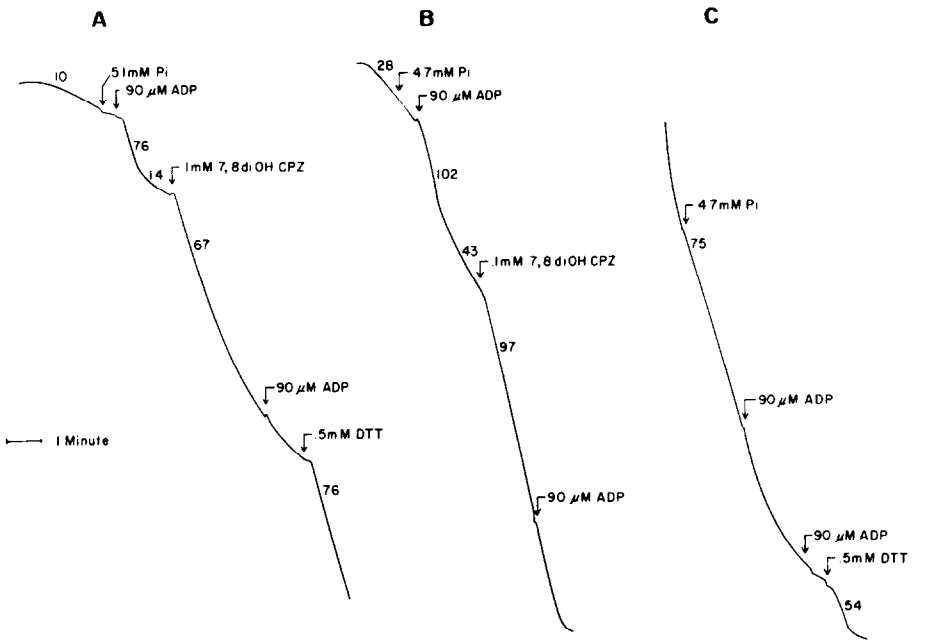

Figure 2: Effects of 7,8-diOH-CPZ on oxygen uptake. Composition of reaction mixture: 32 mM HEPES (pH 7.4), 82 mM KCl, 5 mM glutamate (trace A) or 5 mM succinate (trace B and C), 30 mM mannitol, .02 mM EDTA, .2 mg BSA per ml, T = 21°C. Mitochondrial protein: 1.49 mg per ml. In trace C mitochondria were preincubated for 2 minutes with .1 mM 7,8-diOH-CPZ before oxygen uptake was recorded. Figures next to tracings refer to nano atoms oxygen per minute per mg.

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further change in the rate of oxygen uptake, i.e. transition to state 3 respiration was prevented. No change from an inhibited state 4 rate was observed when 2,4-dinitrophenol (DNP) was added; DTT however, was effective in releasing the inhibition. After the addition of DTT a rapid rate of oxygen uptake similar in magnitude to the initial (stimulated) state 4 rate was observed. As before, 7,8-diOH-CPZ imparted a red color to the mixture. As the red color deepened respiration became inhibited. The addition of small amounts of DTT prevented the color initially; when the deep red color returned the inhibition of respiration also returned.

After glutamate-supported state 4 respiration had been blocked by 7,8-diOH-CPZ, succinate-addition produced an increase in the rate of oxygen uptake even when the drug had been present for more than 4 minutes. When ADP plus Pi were added after succinate no further increase in the rate

of oxygen uptake was observed, suggesting state 3 was still blocked. Addition of DTNB (0.1mM) just prior to succinate prevented the increased oxygen uptake. It was also found that prolonged incubation with DTNB caused a gradual decrease in state 4 respiration, an effect similar to that observed with 7,8-diOH-CPZ.

Discussion

Effects of 7-OH-CPZ on brain mitochondrial preparations appear similar to those of CPZ in its actions on calcium uptake and on respiration. The effects of 7,8-diOH-CPZ are more dramatic. Few agents other than dinitrophenol have been shown to cause such a marked efflux of accumulated calcium from mitochondria. Chlorpromazine produces an efflux of calcium from mitochondria (1) but higher concentrations (0.1 mM or greater) are necessary to demonstrate this effect. With ATP present the initial rate of calcium accumulation by the mitochondria is not altered by the dihydroxy metabolite, but the ability to retain accumulated calcium is lost. It is not yet clear whether this effect is produced by the 7,8-dihydroxy compound, or its semiquinone free radical,or its corresponding orthoquinone. Non-enzymatic oxidation of the dihydroxy metabolite to the semiquinone-quinone has recently been demonstrated (9); both the 7-OH-CPZ and the 7,8-diOH-CPZ are easily oxidized to their semiquinone free radical form in 50 percent HCl or H_2SO_4 solutions (9).

It seems likely that sulfhydryl groups are involved in these calcium movements since it has been shown that DTNB, an agent known to react with sulfhydryl groups, inhibits ATP-supported calcium accumulation (1). In experiments with 7,8-diOH-CPZ the delay in calcium efflux caused by prior addition of DTT suggests a sulfhydryl involvement. This observation does not clearly establish the direct involvement of thiol groups in this process, however, since DTT can both reduce mitochondrial thiol groups and delay the appearance of the quinone (red) form of the drug metabolite.

Similarities between the inhibition of state 4 respiration seen with prolonged incubation in the presence of DTNB and that observed with 7,8-diOH-CPZ or its quinone form suggest that sulfhydryl groups may be important in this inhibitory action also. Somewhat analogous effects on respiration have been observed with napthoquinone compounds. Young (10) has shown that menadione inhibits DNP-stimulated oxygen uptake although only slight changes

in state 4 respiration occur. He has postulated that mitochondrial sulfhydryl groups are affected. In contrast, 2-heptyl-4-hydroxyquinoline-N-oxide produces a rapid inhibition of state 4 respiration with succinate, an effect which is released by DNP (11). It has been shown that free sulfhydryl groups are present during state 4 respiration and that these groups increase in number during state 3 respiration (12). It is known that agents which react with sulfhydryl groups also inhibit oxidative phosphorylation (13-17). Results observed here are similar to those observed in the presence of tellurite, another agent known to interact with thiol groups (18). Tellurite inhibits the oxidation of NADH linked substrates, an effect which is relieved upon the addition of succinate. Unlike tellurite, however, 7,8-diOH-CPZ is effective at a lower concentration and inhibits succinate oxidation as well.

These effects by CPZ metabolites in brain mitochondria may play an important role in the in vivo actions or toxic effects of CPZ. There is considerable evidence which suggests that oxidative metabolism is essential for numerous neural functions. Likewise, it is important to maintain low intracellular calcium levels to preserve normal nerve function. Godfraind et al (19) have recently shown that DNP, which promotes Ca efflux from isolated mitochondria, produced membrane hyperpolarization, a fall in membrane resistance and a fall in electrical excitability in some populations of cat cortical neurons. Tasaki (20) has shown somewhat similar results in squid axions perfused with increased levels of intracellular calcium. Thus drug metabolites which can potentially alter calcium uptake and oxidative processes in neuronal mitochondria may have the capacity to alter in vivo neuronal processes.

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