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Chlordiazepoxide and Theophylline Alter Calcium Levels in Subcellular Fractions of Rat Brain Cortex (40515)

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Benzodiazepines modify several neuronal systems including those mediated by norepinephrine, serotonin, γ amino butyric acid and glycine (1, 2). We postulated that benzodiazepines might alter calcium-mediated secretion mechanisms common to many neurosecretory processes and thereby produce changes in different nerve types. Since chlorpromazine and methylxanthines are known to alter calcium metabolism in isolated tissue preparations (3, 4), we compared the effects of chlordiazepoxide with those of chlorpromazine and theophylline on calcium levels in subcellular fractions of rat brain cortex.

Methods. The 196 rats (male, Sprague-Dawley derived, 280–320 g) used in these studies were given food and water *ad lib.* prior to drug administration. All drug solutions were injected ip 45 min prior to decapitation of the animals. After sacrifice, brain cortex (2/sample) was rapidly removed, weighed and homogenized in 15 vol of ice cold 0.32 *M* sucrose (with 0.005 *M* Tris buffer, pH 7.4) using a Potter Elvehjem glass homogenizer with a teflon pestle. All subsequent steps for isolation of subcellular particles were done at 0–4°. The homogenate was spun at 1000 g and the pellet was resuspended in sucrose and spun again at the same speed. The combined supernatants were spun at 17,000 *g* for 20 min. The 17,000 g supernatant was spun at 105,000 g for 1 hr. to obtain the microsomal fraction. The pellet from the 17,000 *g* centrifugation was resuspended in 1.5 ml of 0.32 *M* sucrose and layered on a gradient made up of 5 ml of 1.2 *M* sucrose and 5 ml of 0.8 *M* sucrose. The gradient was spun at 58,000 *g* for 2 hr. The upper layer containing myelin was discarded but the pellet, primarily made up of mitochondria, was retained. The middle layer composed of synaptosomes was further fractionated as follows. The synaptosome fraction was diluted with distilled water (2 ml/g original tissue) and gently drawn up into and released from a pipet about 10 times. This suspension was layered on a sucrose gradient (0.4, 0.6, 0.8, 1.0 and 1.2 *M*, 2 ml each) and centrifuged for 2 hr at 58,000*g*. The layers were separated using a pasteur pipet and correspond to the fractions of Whittaker *et al.* (5). The lighter layer of "synaptosome ghosts" appears to be rich also in synaptic vesicles (see Fig. 1 and 3).

Two mitochondrial fractions were obtained from each sample, one from the broken synaptosomes and one resulting from disruption of cells during the original homogenization of the tissue.

Aliquots of each of the fractions were wet digested using HNO_3 and H_2O_2. Samples were taken to dryness and the residue dissolved in 0.1 ml concentrated HNO_3 and 0.9 ml of doubled distilled water. Aliquots of these digests were analyzed for calcium in the Perkin-Elmer model 290B atomic absorption spectrometer. Lanthanum 1% was added to each sample to eliminate any interference by phosphate. Protein in each fraction was determined by the method of Lowry *et al.* (6) using bovine serum albumin as the standard.

Drugs used were chlordiazepoxide HCl (Hoffman-LaRoche), theophylline sodium acetate (K & K Labs) and chlorpromazine HCl (Smith-Kline-French). Controls received saline or an equivalent amount of sodium acetate. Three treated and three control samples were done in each experiment. Comparisons were made only between treatment and controls done simultaneously. The theophylline experiments were done in spring and the others in mid and late summer.

Results. Effects of chlordiazepoxide and chlorpromazine on calcium in rat brain cortex. Forty-five minutes after administration of chlordiazepoxide in a dose which produced mild ataxia, calcium is increased in vesicle-rich-synaptosomal subfractions but mito-

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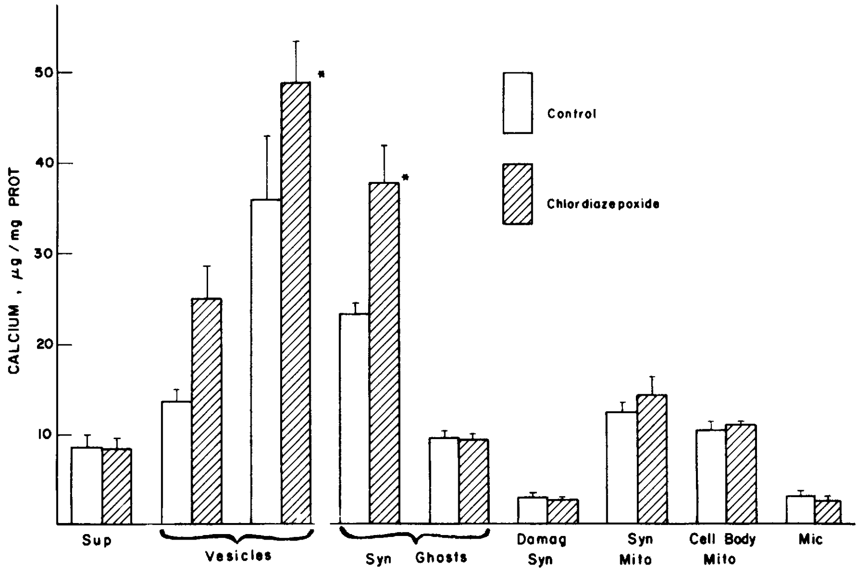

FIG. 1. Effect of Chlordiazepoxide on Subcellular Calcium Distribution in Rat Brain Cortex. Animals were given 20 mg/kg chlordiazepoxide HCl ip and 45 min later were decapitated, brain cortex removed and homogenized. Subcellular fractions were separated by differential and sucrose gradient density centrifugation. It is likely that the lighter of the 2 "synaptosomal ghost" fractions is also rich in vesicles. Means ± SE are given for $n = 6$ except for mic for which $n = 8$. Fractions are labeled according to Whittaker *et al.* (5): Sup = synaptosomal supernatant, syn ghosts = synaptosomal ghosts, damag Syn = damaged synaptosomes, syn Mito = synaptosomal mitochondria, cell body mito = cell body mitochondria, mic = microsomes. (Note that cell body and synpatosomal mitochondria are obtained in separate fractions by this procedure). * Significant at the 5% level by ANOVA and Newman-Keuls comparison of means.

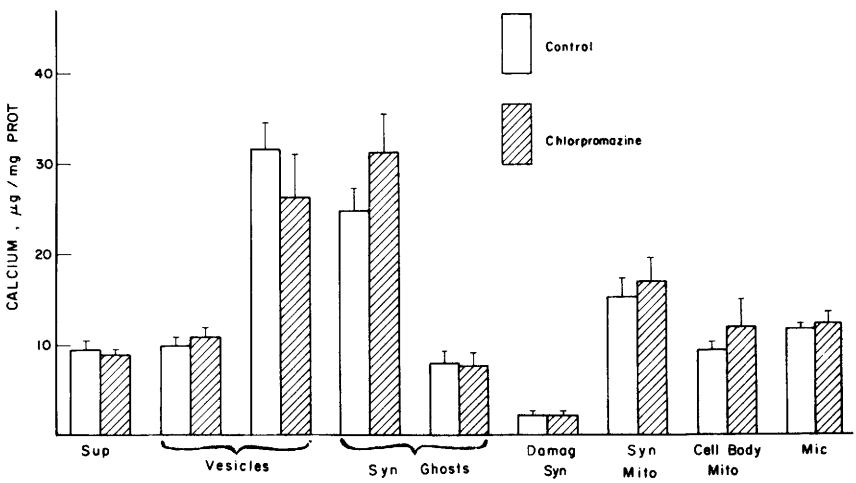

FIG. 2. Effect of Chlorpromazine on Subcellular Calcium Distribution in Rat Brain Cortex. Chlorpromazine HCl 50 mg/kg was given ip 45 min before decapitation of the animals. Subcellular fractions were separated and are designated as in the legend of Fig. 1. Means and SE are given for $n = 6$ except for mic for which $n = 8$. No significant differences were seen due to chlorpromazine treatment.

chondrial, microsomal and other fractions were unaffected (Fig. 1). By contrast, a dose of chlorpromazine which produced a marked sedation of the animals, caused no changes in calcium in subcellular fractions of rat brain cortex (Fig. 2).

Effect of theophylline on calcium content of rat brain cortex. A dose of 20 mg/kg ip of

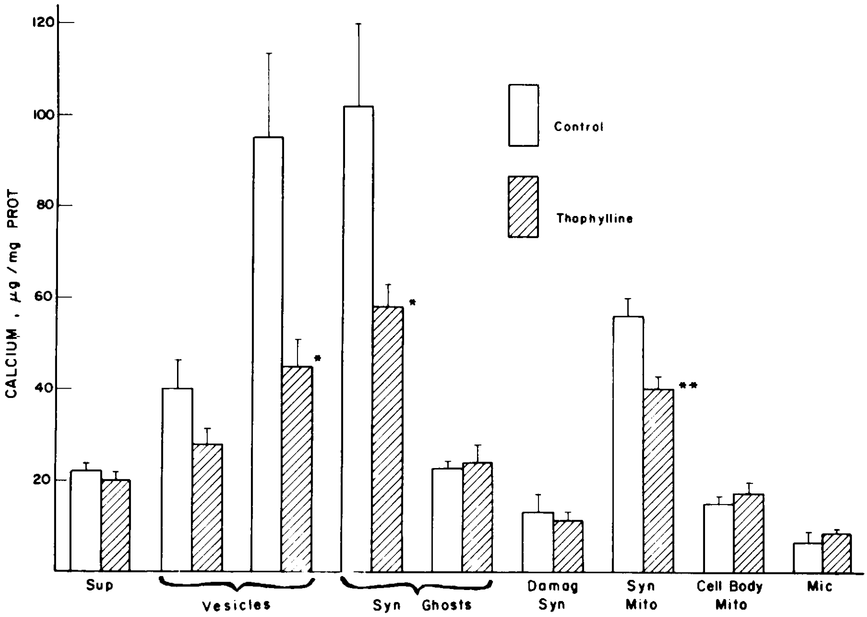

FIG. 3. Effect of Theophylline on Subcellular Calcium Distribution in Rat Brain Cortex. Theophylline sodium acetate, 156 mg/kg was given ip 45 min prior to decapitation of the animals. Controls received either saline or an equivalent amount of sodium acetate and similar results were obtained whether or not sodium acetate was used in controls. Subcellular fractions were separated and designated as in the legend of Fig. 1. Means and SE are given for $n = 9$ except for mic for which $n = 12$. It is likely that the lighter of the 2 "synaptosomal ghost" fractions is also rich in vesicles. * Significantly different at the 5% level by ANOVA and Newman Keuls comparison of means. ** Significantly different at the 5% level by students t test.

theophylline sodium acetate produced a mild hyper-reactivity of the rats but did not alter calcium contents of subcellular fractions of brain cortex ($n = 12$ except for the microsomes where $n = 18$). These results are in agreement with a previous report that 25 mg/kg of caffeine had no effect on calcium or magnesium ions in any rat brain part (7).

A higher dose of theophylline sodium acetate (156 mg/kg ip) which produced a marked hyper-reactivity and increased motor activity, decreased calcium content in the same subfractions of rat cortical synaptosomes that were affected in the opposite manner by chlordiazepoxide (Fig. 3). In addition, this dose of theophylline decreased the calcium content of synaptosomal but not of cell body mitochondria.

In one experiment involving three theophylline treated (156 mg/kg, ip, of the sodium acetate salt) and 3 saline treated control rat cortical samples, the animals were pretreated with $^{45}CaCl_2$ (0.167 μCi/g body wt ip) 8 hr prior to drug treatment. Aliquots of wet digested subcellular fractions were counted by liquid scintillation. A marked decrease in ^{45}Ca (to 30% of control) in the vesicle fractions was noted in the theophylline-treated samples. Calcium-45 in synaptosomal mitochondria was also decreased but cell body mitochondria were not different from control. These results correspond to the data of Fig. 3 which shows decreases in ^{40}Ca in certain brain fractions, following theophylline administration.

Results were analyzed using analysis of variance (ANOVA) and Newman-Keuls range test. This test is essentially a multiple t test among a pool of means. Sometimes a difference in two means of intermediate size is not fairly evaluated and this appeared to be the case with cell body mitochondria in rats treated with theophylline in Fig. 3. Therefore a student's t test was applied to these data of Fig. 3.

Discussion. Essman (8) showed previously that chlordiazepoxide (2–10 mg/kg) increased sodium and decreased magnesium levels in guinea pig cortical synaptosomes. Consistent changes in myelin and mitochondrial fractions of this tissue were not evident after chlordiazepoxide administration. These observations suggest that changes in electrolytes produced by benzodiazepines, are limited to synaptosomes at least in cortical neurons. Our results similarly show that changes in calcium in rat brain cortex caused by chlordiazepoxide are limited to synaptosomal fractions. These changes in calcium are not produced by doses of chlorpromazine which have a marked sedative effect. The benzodiazepines appear to have specific effects on ion metabolism in brain cortical synaptosomes.

Chlorpromazine (0.01–0.1 m*M*) inhibits calcium uptake in isolated sarcoplasmic reticulum and in isolated mitochondria of frog skeletal muscle (4). The highest concentration (0.1 m*M*) releases calcium from mitochondria in this tissue. Calcium uptake by partially purified rat brain mitochondria is markedly inhibited by 0.1 m*M* chlorpromazine (9). Chlorpromazine (1 m*M*) also releases calcium from mitochondria in isolated bovine adrenal medulla, but not from endoplasmic reticulum of this tissue (3). Our *in vivo* results show that chlorpromazine produces no changes in calcium levels in rat cortical subcellular fractions. Either brain tissue calcium metabolism is resistant to the action of this drug or the effects of high concentrations of chlorpromazine in isolated tissues do not reflect the true action of this agent on calcium metabolism in intact animals.

Many studies show changes in calcium in isolated tissues resulting from methylxanthine treatment. For example, caffeine, 2–10 m*M*, inhibits calcium uptake by both sarcoplasmic reticulum and mitochondria isolated from frog skeletal muscle (4). The results of the present study are the first to show decreases in brain calcium levels after methylxanthine administration to whole animals. It has been noted however, that theophylline (1–2 mg/kg/min, iv) in sheep causes hypocalcemia (10). Also, rats pretreated with cortisone (10 mg/kg/day for 4 days) show a hypocalcemia in response to theophylline (30–180 mg/kg) although in control animals no change in blood calcium is seen after theophylline administration (11). The hypocalcemia occurred even after removal of the kidneys, but the effect was lost in thyroparathyroidectomized rats. The hypocalcemic effect of theophylline is probably mediated through thyrocalcitonin (11). It seems unlikely however, that relatively small changes in blood calcium caused by theophylline account for the large changes in calcium in brain cortical synaptosomal subfractions noted in the present study.

Cell body mitochondria were unaffected by theophylline but synaptosomal mitochondria lost calcium following theophylline treatment. This reflects the different functional nature of mitochondria from these two sources which has been noted previously (see 12).

Snyder *et al.* (1) state that it is possible that no single neural action will explain the anxiolytic, hypnotic, anticonvulsant and muscle relaxant effects of the benzodiazepines. However, altered calcium metabolism as produced by chlordiazepoxide in brain tissue in the present study may account for the effects of benzodiazepines on different nerve pathways requiring calcium for neurotransmitter secretion.

Summary. Forty-five minutes after ip injection of chlordiazepoxide, vesicles from rat brain cortical synaptosomes contained significantly less calcium per unit protein than controls. By contrast, a relatively high dose of chlorpromazine failed to change calcium content in rat brain cortical subfractions. A high dose of theophylline increased calcium in both vesicles and mitochondria isolated from rat brain cortical synaptosomes. It is suggested that chlordiazepoxide and high doses of theophylline may influence calcium mediation of transmitter secretion in brain cortex by effects on calcium transfer mechanisms.

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