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# EFFECTS OF CHLORDIAZEPOXIDE ON DEPOLARIZATION-INDUCED CALCIUM INFLUX INTO SYNAPTOSOMES

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**Abstract**—Female, Swiss–Webster mice (20–28 g) were randomly divided into three experimental groups: control, acute (received a single i.p. dose of chlordiazepoxide, 20 mg/kg) and tolerant [received dietary chlordiazepoxide (2.5 mg/g milled Purina Lab Chow), for 8 days]. Synaptosomes were isolated from whole brains of mice from each group, and the inhibitory effects of chlordiazepoxide on KCl-induced $^{45}Ca^{2+}$ influx were examined. Both *in vitro* (0.15 mM), and acute *in vivo* (20 mg/kg, i.p.) chlordiazepoxide significantly inhibited KCl-induced $^{45}Ca^{2+}$ influx ($\Delta_K$) into synaptosomes isolated from control and acute mice, respectively. Chronic dietary chlordiazepoxide administration resulted in the development of tolerance to the inhibitory effects of chlordiazepoxide during the same time period that behavioral tolerance developed. The results suggest that chlordiazepoxide-induced sedation may be mediated, at least partially, by inhibition of calcium influx into nerve ends. In addition, tolerance to chlordiazepoxide sedation may be mediated by an adaptation of the nerve end such that the degree of inhibition of calcium influx is reduced after chronic chlordiazepoxide administration.

Although the cellular mechanisms underlying the sedative, antianxiety, and/or anticonvulsant actions of benzodiazepines are unknown, recent work has clearly established that some or possibly all of these effects are related to changes in γ-aminobutyric acid (GABA). Low concentrations of benzodiazepines ($10^{-6}$M) facilitate KCl-induced pre-synaptic GABA release from rat frontal cortex prisms, whereas higher concentrations ($10^{-4}$M) inhibit GABA release [1]. It has been suggested that benzodiazepines may facilitate GABA-mediated activity post-synaptically by competing with an endogenous inhibitory protein that modulates GABA receptor activity [2]. Benzodiazepines specifically modulate GABA-mediated post-synaptic inhibition in cultured mammalian neurons [3]. Low doses of diazepam and chlordiazepoxide facilitate, and high doses antagonize GABA-mediated post-synaptic inhibition. Geller *et al.* [4] recently showed that diazepam enhanced GABA-mediated inhibition in rat cerebellum *in situ* at doses that did not change spontaneous firing rates. They suggested that the actions of diazepam may be mediated, at least in part, by a specific increase in GABA-mediated inhibition in the central nervous system.

Although the evidence in the literature clearly shows a relationship between GABA and benzodiazepines, it has not been established conclusively whether the GABA-mediated actions contribute to sedation, relief of anxiety, or suppression of seizure activity or to all of these. Recent evidence has shown that a functional loss of GABAergic inhibitory synapses in sensorimotor cortex leads to epileptic activity of cortical pyramidal neurons [5].

The purpose of the present investigation was to examine chlordiazepoxide-mediated changes of calcium influx into synaptosomes to determine if a relationship exists between sedation produced by chlordiazepoxide and alterations in calcium-mediated 'stimulus–secretion coupling' events. Recent work in our laboratories has shown that barbiturates [6–8] and chlorpromazine [9] markedly inhibit depolarization-induced synaptosomal $^{45}Ca^{2+}$ influx at concentrations that are consistent with those required to produce sedation. In addition, chronic administration of both barbiturates and chlorpromazine resulted in the development of tolerance to this inhibition of depolarization-induced synaptosomal $^{45}Ca^{2+}$ influx at the same time that behavioral tolerance developed. We report here that chlordiazepoxide appears to have a similar inhibitory effect on $^{45}Ca^{2+}$ influx across the nerve end, which may contribute to its sedative effects.

## MATERIALS AND METHODS

Female, Swiss–Webster mice (20–28 g) were kept on a 12/12 hr light–dark cycle with food and water *ad lib.* for at least 5 days. The mice were then divided into three experimental groups: control, acute and tolerant. Control mice were maintained on a Purina Lab Chow diet *ad lib.* Acute mice received a single dose of chlordiazepoxide (20 mg/kg, i.p.) and were killed 30 min later. Animals in the tolerant group received a food cup containing a milled diet (Purina Lab Chow) thoroughly mixed with 2.5 mg chlordiazepoxide/g diet as their sole food source, using the method of Belknap *et al.* [10]. Mice in the tolerant group were maintained on the chlordiazepoxide diet for 8 days and were then killed.

Tolerance was assessed by administering an i.p. dose of chlordiazepoxide hydrochloride (20 mg/kg) to mice that had received the chlordiazepoxide diet (2.5 mg chlordiazepoxide/g milled Purina Lab Chow)

for 8 days or to control mice. Fifteen minutes after injection and at the same time in the afternoon, spontaneous activity was measured in a photocell activity monitor (43 × 31 × 12 cm) divided into four equal compartments with the openings between the compartments being at alternate ends. Activity scores were expressed as the number of beam crossings in a 5-min test period.

At the appropriate times, mice from each of the three treatment groups were killed and synaptosomes were isolated by the method of Cotman [11]. Briefly, whole brains were homogenized using a Thomas size C homogenizing tube with a Teflon pestle at the lowest possible speed setting on a Sorvall Omni-Mixer using eight up and down strokes. Centrifugation procedures were identical to those described by Cotman [11]. The final synaptosomal pellet was resuspended in incubation medium to give a protein concentration of approximately 0.7–1.3 mg/ml.

The accumulation of $^{45}Ca^{2+}$ by synaptosomes was studied as described by Leslie *et al.* [9]. A 0.5-ml aliquot of the synaptosomal preparation from each treatment group was added to 0.5 ml of incubation medium (136 mM NaCl, 5 mM KCl, 1.2 mM $CaCl_2 \cdot 2H_2O$, 1.3 mM $MgCl_2$, 10 mM glucose, 20 mM Tris base, with the pH adjusted to 7.65 with 1 M maleic acid) and allowed to equilibrate for 12 min at 30° in a Dubnoff metabolic shaker. Chlordiazepoxide–HCl (0.15 mM, final concentration) was added to some of the incubating samples (for 12 min) to examine the inhibitory effects of an *in vitro* chlordiazepoxide challenge. For depolarized samples, $^{45}Ca^{2+}$ loading was initiated by the addition of 0.5 ml of depolarization medium (213 mM KCl, 1.2 mM $CaCl_2 \cdot 2H_2O$, 1.3 mM $MgCl_2$, 10 mM glucose, and 20 mM Tris base, with the pH adjusted to 7.65 with 1 M maleic acid) containing $^{45}Ca^{2+}$ (sp. act. = 2 $\mu$Ci $^{45}Ca^{2+}/\mu$mole $^{40}Ca^{2+}$). This resulted in a 1.5 ml incubation volume containing 74 mM KCl to depolarize the synaptosomal membranes [12]. After a 2-min incubation period, $^{45}Ca^{2+}$ loading was stopped by adding 5.0 ml of ice-cold EGTA stopping solution (136 mM NaCl, 5 mM KCl, 1.3 mM $MgCl_2$, 3 mM EGTA [ethyleneglycol-bis-(beta-aminoethyl ether)*N,N'*-tetraacetic acid], 10 mM glucose, and 20 mM Tris base with the pH adjusted to 7.65 with 1 M maleic acid). The 2-min $^{45}Ca^{2+}$ loading period was chosen because our previous work had shown that peak influx occurs at this time [6, 9]. Each sample was immediately filtered on a presoaked (250 mM KCl) 0.45 $\mu$m Millipore cellulose acetate filter using a Millipore microfiltration manifold. Each filter was washed with 10.0 ml of 0.32 M sucrose and placed in a scintillation vial with 15 ml of Beckman Ready-Solv™. Samples were counted using a Beckman LS-8000 liquid scintillation counter. Counting efficiency was determined using an H number and was found to be approximately 72 per cent. Non-depolarized samples were handled in the same manner except that after the 12-min incubation period 0.5 ml of incubation medium (5 mM KCl) containing $^{45}Ca^{2+}$ (2 $\mu$Ci $^{45}Ca^{2+}/\mu$mole $^{40}Ca^{2+}$) was added. Synaptosomal protein concentration was assayed by the method of Lowry *et al.* [13]. Net influx of calcium across the synaptosomal membrane was calculated by subtracting the non-depolarized (5 mM KCl) values from the depolarized (74 mM KCl) values. This difference is expressed as $\Delta_k$ (potassium-induced change) and represents the amount of calcium that traverses the plasma membrane.

## RESULTS

Table 1 shows the effects of *in vitro* chlordiazepoxide on depolarization-induced (74 mM KCl) $^{45}Ca^{2+}$ accumulation, depolarization-independent (5 mM KCl) $^{45}Ca^{2+}$ accumulation, and net $^{45}Ca^{2+}$ influx ($\Delta_k$) into synaptosomes isolated from control mice. Chlordiazepoxide (0.15 mM) significantly

Table 1. Effects of *in vitro* chlordiazepoxide on $^{45}Ca^{2+}$ accumulation by synaptosomes isolated from control mice*

| | ($\mu$moles $^{45}Ca^{2+}$ †/g protein) | Depression (%) |
|---|---|---|
| Depolarized accumulation | 6.58 ± 0.41 | |
| Depolarized accumulation plus 0.15 mM chlordiazepoxide | 5.17 ± 0.21‡ | 21.4 |
| Non-depolarized accumulation | 2.85 ± 0.09 | |
| Non-depolarized accumulation plus 0.15 mM chlordiazepoxide | 3.14 ± 0.13 | 0 |
| $\Delta_k$ | 3.73 ± 0.12 | |
| $\Delta_k$ 0.15 mM chlordiazepoxide | 2.03 ± 0.06§ | 45.6 |

* Synaptosomes were isolated from naïve Swiss–Webster mice; drug-treated synaptosomes were challenged *in vitro* with 0.15 mM (final concentration) chlordiazepoxide. For depolarized accumulation of $^{45}Ca^{2+}$, synaptosomes were incubated for 2 min with 74 mM KCl. Non-depolarized synaptosomes were incubated with incubation medium (5 mM KCl) containing $^{45}Ca^{2+}$ for 2 min. $\Delta_k$ values (net $^{45}Ca^{2+}$ influx) were calculated by subtracting non-depolarized accumulation values from depolarized accumulation values for each of the respective control or chlordiazepoxide-treated samples. Statistical significance was determined by analysis of variance using Neuman-Keuls as the post-hoc test.

† Mean ± S.E.; N = 6.

‡ Indicates significant ($P < 0.05$) inhibition, compared to depolarized accumulation.

§ Indicates significant ($P < 0.05$) inhibition, compared to $\Delta_k$ values.

Table 2. Effects of *in vitro* chlordiazepoxide on net $^{45}Ca^{2+}$ influx ($\Delta_k$) into synaptosomes isolated from control, acute and tolerant mice groups*

| | Net $^{45}Ca^{2+}$ influx ($\Delta_k$) ($\mu$moles $^{45}Ca^{2+}$/g protein) | Depression (%) |
|---|---|---|
| Control (N = 6) | 3.72 ± 0.12[a] | |
| Control plus 0.15 mM chlordiazepoxide (N = 6) | 2.03 ± 0.06[b] | 45.6 |
| Acute (N = 6) | 2.72 ± 0.29[d] | |
| Acute plus 0.15 mM chlordiazepoxide (N = 6) | 1.65 ± 0.24[e] | 39.5 |
| Tolerant (N = 8) | 3.43 ± 0.16[g] | |
| Tolerant plus 0.15 mM chlordiazepoxide (N = 8) | 2.63 ± 0.09[h] | 23.3 |

* Synaptosomes were isolated from Swiss–Webster mice from control, acute and chronic groups; drug-treated synaptosomes were challenged *in vitro* with 0.15 mM (final concentration) chlordiazepoxide. Data represent net $^{45}Ca^{2+}$ influx into synaptosomes, which was calculated by subtracting non-depolarized $^{45}Ca^{2+}$ accumulation values from depolarization-induced (74 mM KCl) $^{45}Ca^{2+}$ accumulation. Statistical significance was determined by analysis of variance using Neuman-Keuls as the post-hoc test. Values significantly different from one another ($P < 0.05$) include: *a* vs *b*, *a* vs *d*, *a* vs *e*, *a* vs *h*, *d* vs *g*, *g* vs *h*, *h* vs *b*, and *h* vs *e*; values are means ± S.E.M.

inhibited ($P < 0.05$) the depolarized accumulation of $^{45}Ca^{2+}$ (21.4 per cent), which represents the total amount of $^{45}Ca^{2+}$ accumulated by control synaptosomes in the presence of 74 mM KCl. Non-depolarized accumulation, which represents the total amount of $^{45}Ca^{2+}$ accumulated in the absence of depolarization by KCl, was not altered significantly by 0.15 mM chlordiazepoxide. This suggests that *in vitro* chlordiazepoxide inhibits depolarization-induced $^{45}Ca^{2+}$ uptake by synaptosomes but not $^{45}Ca^{2+}$ accumulation by resting synaptosomes. Consequently, the subtraction of the respective non-depolarized $^{45}Ca^{2+}$ accumulation from the depolarized accumulation of $^{45}Ca^{2+}$ revealed a 45.6 per cent inhibition of net $^{45}Ca^{2+}$ influx ($\Delta_k$) by chlordiazepoxide (Table 1).

Table 2 shows the inhibitory effects of *in vitro* chlordiazepoxide on net $^{45}Ca^{2+}$ influx ($\Delta_k$) into synaptosomes isolated from control, acute and tolerant mice groups. *In vitro* chlordiazepoxide (0.15 mM) inhibited by 45.6 per cent the net influx of $^{45}Ca^{2+}$ into synaptosomes isolated from control mice. Addition of chlordiazepoxide (0.15 mM) to synaptosomes isolated from mice of the acute group resulted in a 39.5 per cent depression of $^{45}Ca^{2+}$ influx.

Table 2 also shows the effects of *in vitro* chlordiazepoxide on $^{45}Ca^{2+}$ influx into synaptosomes isolated from tolerant mice; addition of 0.15 mM chlordiazepoxide to these synaptosomes resulted in a 23.3 per cent inhibition of $^{45}Ca^{2+}$ influx. Chronic chlordiazepoxide treatment apparently resulted in an adaptive response to *in vitro* chlordiazepoxide inhibition of $^{45}Ca^{2+}$ influx into isolated synaptosomes. This is supported by the observation that net $^{45}Ca^{2+}$ influx into synaptosomes isolated from tolerant mice, in the absence of an *in vitro* chlordiazepoxide challenge, was the same as that observed for control synaptosomes (3.43 ± 0.16 and 3.72 ± 0.12 $\mu$moles $^{45}Ca^{2+}$/g protein, respectively), whereas acute *in vivo* chlordiazepoxide administration (20 mg/kg, i.p.) resulted in a significant reduction in $^{45}Ca^{2+}$ influx (2.72 ± 0.29 $\mu$moles $^{45}Ca^{2+}$/g protein; 26.9 per cent reduction) as compared to control.

Figure 1 summarizes the *in vivo* effects of chlordiazepoxide on $^{45}Ca^{2+}$ accumulation by synapto-

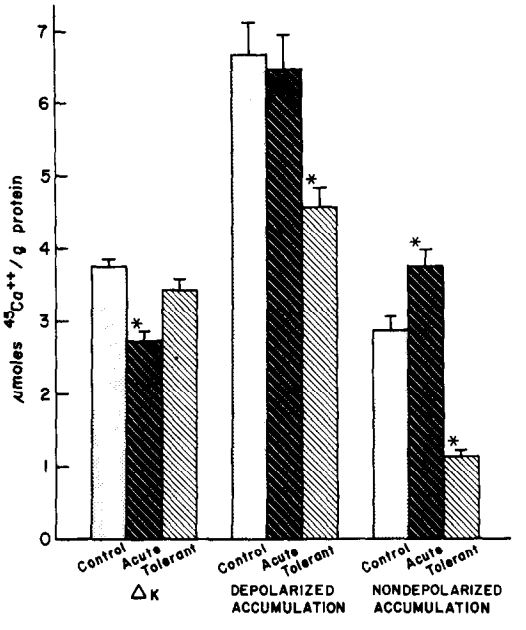


Fig. 1. Effects of *in vivo* chlordiazepoxide on $^{45}Ca^{2+}$ accumulation by synaptosomes isolated from control, acute and tolerant mice groups. Each bar represents the mean ± S.E. of six to eight experiments. Control mice were maintained on a Purina Lab Chow diet *ad lib.* prior to being killed. Acute mice were given 20 mg/kg chlordiazepoxide, i.p. and were killed 30 min later. Tolerant mice were maintained on a chlordiazepoxide-adulterated, milled Purina Lab Chow diet (2.5 mg chlordiazepoxide–HCl/g diet) for 8 days prior to being killed. Data were statistically analyzed by analysis of variance using Neuman-Keuls as the post-hoc test. Asterisks indicate a significant difference ($P < 0.05$) between acute or chronic values and their respective control values.

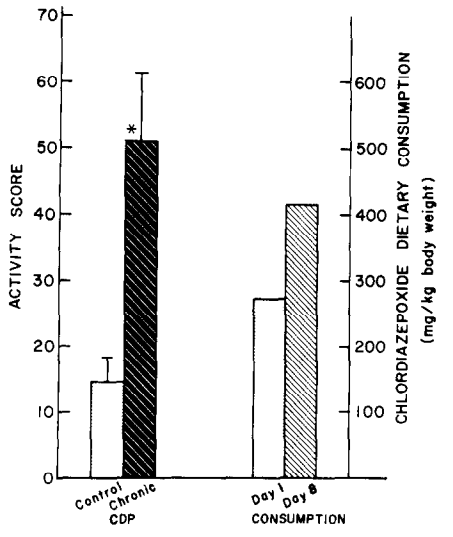


Fig. 2. Behavioral assessment of chlordiazepoxide (CDP) tolerance development using a photocell activity monitor and dietary consumption. Each bar on the left side of the figure represents the mean ± S.E. value for activity scores obtained from ten mice. Each bar on the right side of the figure represents mean dietary chlordiazepoxide consumption data obtained from ten mice. Tolerance was assessed by administering an intraperitoneal dose of chlordiazepoxide–HCl (20 mg/kg) to mice that had received the chlordiazepoxide diet (2.5 mg CDP–HCl/g milled Purina Lab Chow) for 8 days (chronic) or to mice that had received no prior treatment (control). Fifteen minutes after chlordiazepoxide injection, activity was measured in a photocell activity monitor (43 × 31 × 12 cm) divided into four equal compartments with openings between them at alternate ends. The activity scores represent the number of beam crossings in a 5-min test period.

somes that were isolated from control, acute and tolerant mice groups. As suggested from the results in Table 2, Fig. 1 shows that acute i.p. administration of chlordiazepoxide (20 mg/kg) resulted in significant reduction in $^{45}Ca^{2+}$ influx ($\Delta_k$) compared to control values. However, after chronic administration of chlordiazepoxide (2.5 mg/g Purina Lab Chow diet for 8 days), $^{45}Ca^{2+}$ influx into synaptosomes isolated from tolerant mice was the same as that observed in control synaptosomes. Figure 1 also shows that depolarized accumulation and non-depolarized accumulation of $^{45}Ca^{2+}$ by synaptosomes from tolerant mice were significantly reduced from control values. This suggests that, although net influx of $^{45}Ca^{2+}$ into synaptosomes isolated from tolerant mice was no different from that observed from control mice, other mechanisms involving calcium, such as membrane binding, may have been significantly reduced.

Figure 2 shows the behavioral assessment of chlordiazepoxide tolerance development in mice maintained on the chlordiazepoxide diet for 8 days. To assess tolerance development, both control mice and mice that were maintained on the chlordiazepoxide diet for 8 days were given an i.p. injection of chlordiazepoxide (20 mg/kg); spontaneous activity was measured 15 min later. Figure 2 shows that spontaneous activity of chronically treated chlordiazepoxide mice was markedly increased over that of control mice, suggesting behavioral tolerance development subsequent to chronic exposure to chlordiazepoxide. Figure 2 shows that consumption of dietary chlordiazepoxide was markedly increased (39 per cent) on day 8 vs day 1. This further suggests the development of tolerance to the drug since activity scores were higher in chronic mice, compared to control mice.

## DISCUSSION

Although recent evidence suggests that benzodiazepines act specifically to augment GABA-mediated inhibitory activity in the central nervous system [14], the results of the present investigation demonstrate that chlordiazepoxide, in sedative-hypnotic concentrations (20 mg/kg i.p. and 0.15 mM *in vitro*) [15, 16] markedly inhibits depolarization-induced $^{45}Ca^{2+}$ influx into synaptosomes isolated from control animals. Because calcium is so intimately coupled with exocytotic release of neurotransmitters [17], a process known as 'stimulus–secretion coupling', these results suggest that chlordiazepoxide may acutely inhibit calcium-mediated 'stimulus–secretion coupling' events to cause sedation. Chronic administration of dietary chlordiazepoxide resulted in the development of tolerance to this inhibitory effect on $^{45}Ca^{2+}$ influx during the same time period in which behavioral tolerance developed. This adaptation after chronic chlordiazepoxide exposure may be involved in functional tolerance development. The effects of chlordiazepoxide on synaptosomal calcium uptake are strikingly similar to the effects of barbiturates [6, 7] and chlorpromazine [9], both of which are potent central nervous system depressants. As with pentobarbital and chlorpromazine, acute, *in vitro*, chlordiazepoxide addition markedly inhibited depolarization-induced $^{45}Ca^{2+}$ uptake but did not alter non-depolarized $^{45}Ca^{2+}$ accumulation. These results suggest that all three drugs depress depolarization-induced, passive calcium movement but do not alter calcium movements or binding under resting conditions.

Chlordiazepoxide also appears to be similar to barbiturates and chlorpromazine in its actions on synaptosomal $^{45}Ca^{2+}$ influx, in that chronic dietary administration resulted in a reduced sensitivity to the depressant effect of the drug. The same type of reduced sensitivity to the inhibitory effects of barbiturates and chlorpromazine is observed after chronic administration of each of these drugs [6, 7, 9].

The results of this investigation suggest that, although chlordiazepoxide may have specific effects on GABA-mediated inhibitory functions which are not shared by barbiturates and other sedative drugs [14], it shares a common action with barbiturates and chlorpromazine of inhibiting calcium influx across the nerve end which might possibly contribute to sedation [6, 7, 9, 12].

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