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# Tolerance to barbiturate and chlorpromazine-induced central nervous system sedation—Involvement of calcium-mediated stimulus-secretion coupling

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It has been well documented that calcium ions are essential for neurotransmitter secretion in the central nervous system [1–3]. Subsequent to depolarization of the nerve end, calcium ions flow down a concentration gradient and couple cytoplasmic events which result in neurotransmitter release. This process is known as "stimulus–secretion coupling". "Stimulus–secretion coupling" has become of interest recently as a potential site of action for drugs which produce sedation. For example, recent work has shown that barbiturates depress depolarization-induced calcium influx across synaptosomal membranes, and subsequent transmitter release, in concentrations which are consistent with *in vivo* sedative doses [4, 5]. To implicate further the involvement of "stimulus–secretion coupling" in the production of central nervous system sedation, recent work in our laboratories has shown that chronic *in vivo* barbiturate administration results in the development of tolerance to depolarization-induced synaptosomal calcium accumulation in parallel with the development of behavioral signs of tolerance [6]. A similar correlation between behavioral tolerance and tolerance to synaptosomal membrane calcium movement also has been reported recently for morphine [7, 8].

In the present report, we have used chlorpromazine to characterize further the involvement of "stimulus–secretion coupling" in the production of sedation. Chlorpromazine was chosen for this study because it is a drug known to produce marked sedation followed by tolerance [9], but on the other hand it produces an antipsychotic effect to which tolerance does not develop or develops quite slowly. Our results show, as we have found with barbiturates, that behavioral indices of sedation and tolerance with chlorpromazine agree quite well with depression of depolarization-induced synaptosomal calcium influx and the development of tolerance at the membrane level to this effect.

Ten- to twelve-week-old male DBA/2J mice, 20–25 g, were housed individually for at least 5 days prior to experimentation and kept on a 12/12 hr light–dark cycle with food and water *ad lib.* Mice were then divided randomly into three experimental groups: control, acute and tolerant. Control mice were maintained on a milled Purina Lab Chow diet *ad lib.* Acute animals received a single dose of chlorpromazine hydrochloride, 15 mg/kg, i.p., and then were killed 30 min later during the peak sedative effect (after loss of righting reflex). Animals in the tolerant group received a food cup containing a milled diet (Purina Lab Chow) thoroughly mixed with 1.0 mg chlorpromazine/g diet as their sole food source. This procedure for dietary drug administration is described more completely elsewhere [10]. Animals in the tolerant group were maintained on the chlorpromazine diet for 7 days prior to being killed. During the 7 days, the animals showed no appreciable loss in weight (average weight day 0 = 23.86 ± 2.65 g; average weight day 6 = 22.28 ± 2.10), and food and chlorpromazine consumption increased markedly (Fig. 1).

Grid test scores (a measure of neuromuscular impairment) were used to determine the degree of intoxication and tolerance development for animals in the tolerant group. This procedure is described in detail elsewhere [10], but briefly involves a determination of the number of times that a mouse's foot slips through a wire grid floor (number of errors) over a 12 cm distance. A reduction in grid test scores over a period of time represents a reduction in intoxication, which can be used as an index of fuctional tolerance. It has been established that tolerance to chlorpromazine-induced sedation is a functional or tissue tolerance and not metabolic, although it has been suggested that chlorpromazine may induce its own metabolism [9]. Figure 1 shows that there was a significant reduction in grid test scores over the 7-day diet period, while at the same time there was a dramatic increase in food and chlorpromazine consumption. Behaviorally, the mice showed marked signs of intoxication after day 1 of the chlorpromazine diet, while the mice on day 6 appeared much more alert. These data provide evidence for substantial tolerance development by day 6.

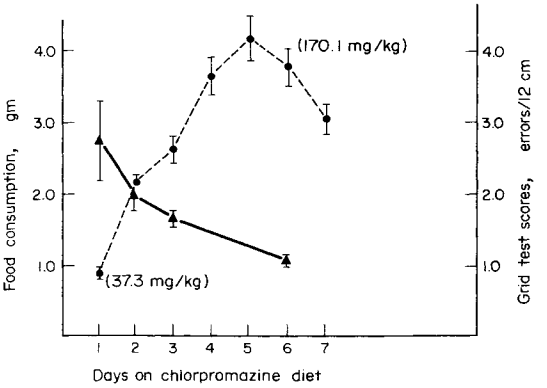


Fig. 1. Grid test scores and chlorpromazine diet consumption over a 7-day period. Each point represents the mean ± S.E.M. of five observations. Each mouse was tested only once to avoid learning effects. The grid tests were performed as described by Belknap *et al.* [10]. The numbers in parentheses represent the dietary consumption of chlorpromazine on day 1 (37.3 mg/kg) and day 6 (170.1 mg/kg). Statistical analysis (Student's *t*-test) showed that grid test scores on day 1 vs day 6 were significantly different ($P < 0.05$) and that chlorpromazine consumption on day 6 was significantly greater than that for day 1 ($P < 0.001$).

To characterize the behavioral similarities between chlorpromazine- and phenobarbital-induced sedation, cross-tolerance studies were performed. The results of this work are shown in Fig. 2. Male C57 (panels A and B) and DBA (panels C and D) mice (20–25 g) were divided into three groups: a non-treated, pair-fed control group (control), a chlorpromazine (CPZ) group which was maintained on a 1 mg/kg of chlorpromazine hydrochloride diet for 7 days as described above and a phenobarbital (PB) group which was fed a milled phenobarbital diet (3.0 mg/g diet) for 7 days [10] and then placed on a control diet for 2 days prior to use. Panels A and C of Fig. 2 show that an intraperitoneal injection of a chlorpromazine challenge dose, 15 mg/kg, produced a loss of righting reflex which was maintained for over 12 hr in control C57 mice and for about 2–3 hr in control DBA mice. However, when this challenge dose of chlorpromazine was given to C57 mice from the CPZ and PB groups (Fig. 2A), 50 per cent of the animals regained their righting reflex after approximately 8 and 5 hr respectively. Fifty per cent of the DBA mice (Fig. 2C) from the CPZ group regained their righting reflex in less than 30 min and over 75 per cent of the animals in the PB group did not lose their righting reflex during the measurement period. Similarly, panels B and D of Fig. 2 show that a challenge dose of phenobarbital, 125 mg/kg, produced a loss of the righting reflex in control mice

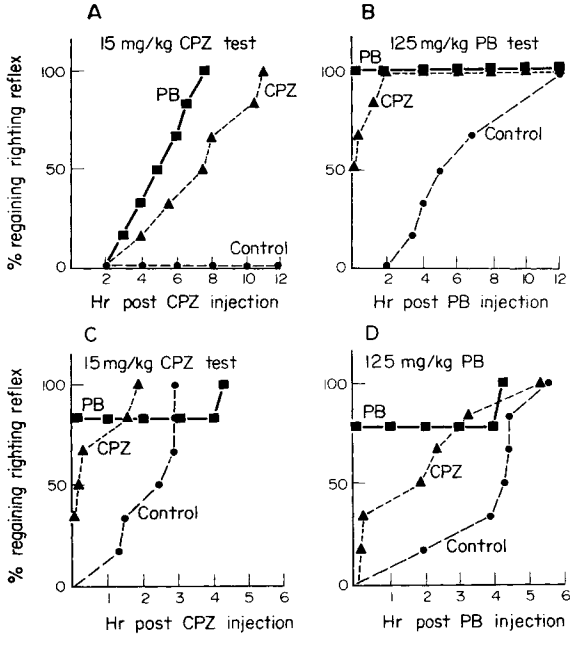


Fig. 2. Cross-tolerance between phenobarbital- and chlorpromazine-induced sedation. Panels A and B represent data from male C57 mice (20–25 g), while panels C and D represent data from male DBA mice (20–25 g). Control mice were maintained on a Purina Lab Chow diet *ad lib.* Chlorpromazine-treated (CPZ) animals were maintained for 7 days on a milled Purina Lab Chow diet adulterated with 1 mg/g of chlorpromazine hydrochloride as their only food source. Phenobarbital-treated (PB) mice were given 3 mg phenobarbital/g of milled Purina Lab Chow diet as their only food source [10]. Panels A and C show the effects of a 15 mg/kg, i.p., challenge dose of chlorpromazine on the per cent of mice from each of the above animal groups (control, CPZ, and PB) regaining their righting reflex over a 12-hr measurement period. Panels B and D show the effect of a 125 mg/kg, i.p., phenobarbital challenge dose on the per cent of mice able to regain the righting reflex over 12 hr. Each animal group consisted of six mice.

* PPO = 2,5-diphenyloxazole; POPOP = 1,4-bis-[2-(4-methyl-5-phenyloxazolyl)] benzene.

whereby 50 per cent of the mice regained their righting reflex within 4–5 hr (C57 and DBA) and all animals regained the righting reflex within 12 and 6 hr for C57 and DBA mice respectively. However, when the challenge dose of phenobarbital was administered to mice from the CPZ and PB groups, a marked tolerance was again observed. None of the C57 mice (Fig. 2B) in the PB group lost their righting reflex, and the maximum sleeping time for any of the mice in the CPZ group was about 2 hr. Fifty per cent of the animals in the CPZ group did not lose their righting reflex. Similar results were obtained with DBA mice (Fig. 2D). These data suggest that there is a marked cross-tolerance between the sedative effects produced by chlorpromazine and phenobarbital. This is particularly interesting in view of work which has shown that chlorpromazine actually worsens withdrawal symptomatology subsequent to withdrawal of chronic barbiturate [11] and ethanol [12] administration.

At the appropriate times, mice from each of the three chlorpromazine treatment groups (control, acute chlorpromazine and chronic dietary chlorpromazine) were killed and synaptosomes were isolated by the method of Cotman [13]. The presence of synaptosomes was verified by electron microscopy. 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 [13]. The final synaptosomal pellet was resuspended in incubation medium to give a protein concentration of approximately 0.5 to 0.9 mg/ml.

The accumulation of $^{45}Ca^{2+}$ by synaptosomes was studied as described by Blaustein and Ector [4]. A 0.5-ml aliquot of the synaptosomal preparation from each treatment group (control, acute or chronic) was added to 0.5 ml of incubation medium (NaCl, 136 mM; KCl, 5 mM; $CaCl_2 \cdot 2H_2O$, 1.2 mM; $MgCl_2$, 1.3 mM; glucose 10.0 mM; Tris base, 20.0 mM; pH adjusted to 7.65 with 1 M maleic acid) and allowed to equilibrate for 12 min at 30° in a Dubnoff metabolic shaker. Chlorpromazine, 50 $\mu$M or 5 $\mu$M, was added to some of the incubating samples (for 12 min) to examine the inhibitory effects of an *in vitro* chlorpromazine challenge. For depolarized samples, $^{45}Ca^{2+}$ loading was initiated by the addition of 0.5 ml of depolarization medium (KCl, 213 mM; $CaCl_2 \cdot 2H_2O$, 1.2 mM; $MgCl_2$, 1.3 mM; glucose, 10 mM; Tris base, 20 mM; pH adjusted to 7.65 with 1 M maleic acid) containing $^{45}Ca^{2+}$ (specific activity = 2 $\mu$Ci $^{45}Ca^{2+}$/$\mu$mole of $^{40}Ca^{2+}$). This resulted in a 1.5-ml incubation volume containing 71 mM KCl to depolarize the synaptosomal membranes [4]. After a 2-min incubation period, $^{45}Ca^{2+}$ loading was stopped by adding 5.0 ml of ice-cold EGTA stopping solution (NaCl, 132 mM; KCl, 5 mM; EGTA, 3 mM; $MgCl_2$, 1.3 mM; glucose, 10 mM; Tris base, 20 mM; pH adjusted to 7.65 with 1 M maleic acid). Each sample was filtered immediately on a presoaked (250 mM KCl) 0.45 $\mu$m Millipore cellulose acetate filter using a Millipore microfiltration apparatus. 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 of $^{40}Ca^{2+}$) was added. Each filter was washed with 10.0 ml of 0.32 M sucrose and placed in a scintillation vial with a mixture containing 0.6% PPO * and 0.01% POPOP in 1:1 toluene and 2-ethoxyethanol. Samples were counted using a Beckman LS-8000 liquid scintillation counter. Counting efficiency was determined using the H number method and was found to be approximately 63 per cent. Synaptosomal protein concentration was assayed by the method of Lowry *et al.* [14].

Figure 3 shows $^{45}Ca^{2+}$ accumulation by control synaptosomes in depolarizing (71 mM) and nondepolarizing (5 mM) media as a function of time. Accumulation of $^{45}Ca^{2+}$ in the nondepolarizing medium was a slow monotonic process over a 2-min time period. On the other hand, uptake of $^{45}Ca^{2+}$ by synaptosomes placed in a depolarizing medium was increased by approximately 2- to 3-fold over nondepolarized values at

Table 1. Effect of acute and chronic chlorpromazine administration on synaptosomal $^{45}Ca^{2+}$ influx*

| | ($\mu$moles $^{45}Ca^{2+}$/g protein) | | |
|---|---|---|---|
| | Non-treated controls (N = 4) | Acute chlorpromazine, 15 mg/kg, i.p. (N = 5) | Chronic chlorpromazine 1 mg/g diet/7 days (N = 4) |
| No drug, depolarized | 6.33 ± 0.33[a] | 8.34 ± 0.79[c] | 6.18 ± 0.85 |
| Depolarized plus chlorpromazine, 50 $\mu$M | 3.10 ± 0.28[b] | 3.72 ± 0.58[d] | 3.88 ± 0.48 |
| Depolarized plus chlorpromazine, 5 $\mu$M | 5.10 ± 0.87 | 6.53 ± 0.88 | 5.54 ± 0.64 |
| No drug, nondepolarized | 2.52 ± 0.39 | 3.32 ± 0.65 | 2.36 ± 0.30 |
| Nondepolarized plus chlorpromazine, 50 $\mu$M | 2.40 ± 0.32 | 2.70 ± 0.40 | 2.18 ± 0.37 |
| Nondepolarized plus chlorpromazine, 5 $\mu$M | 2.69 ± 0.45 | 3.71 ± 0.20[e] | 2.11 ± 0.46[f] |
| $\Delta_k$ No drug | 3.81 ± 0.33[g] | 5.02 ± 0.46[i] | 3.79 ± 0.79[l] |
| $\Delta_k$ Chlorpromazine, 50 $\mu$M | 0.70 ± 0.22[h] | 1.02 ± 0.22[j] | 1.70 ± 0.22[m] |
| $\Delta_k$ Chlorpromazine, 5 $\mu$M | 2.49 ± 0.66 | 3.53 ± 0.59[k] | 3.44 ± 0.35 |

* Depolarized data represent synaptosomal $^{45}Ca^{2+}$ accumulation in the presence of 71 mM KCl. Non-depolarized data represent synaptosomal $^{45}Ca^{2+}$ accumulation in the presence of 5 mM KCl. The $\Delta_k$ values were calculated as the difference between the appropriate potassium-depolarized and nondepolarized data (obtained from paired samples of synaptosomes) and represent $^{45}Ca^{2+}$ influx across the synaptosomal membrane. The data are expressed as the means ± S.E.M. Chlorpromazine, 50 $\mu$M or 5 $\mu$M, was added *in vitro* to samples from each of the three treatment groups [nontreated controls, acute chlorpromazine (15 mg/kg, i.p.) or chronic chlorpromazine (1 mg/g diet/7 days] to determine the membrane effects of an *in vitro* challenge dose of chlorpromazine. Statistical analysis was performed by Analysis of Variance using Neuman–Keuls as the *post hoc* test. Significant comparisons reaching at least the $P < 0.05$ level are as follows: a vs b, c vs d, e vs f, g vs h, i vs j, i vs k, h vs m, and l vs m.

both 1- and 2-min time periods. The difference (at either the 1-min or the 2-min time period) between the nondepolarized and depolarization-induced accumulation is a measure of the net potassium facilitated influx ($\Delta_k$) across the synaptosomal plasma membrane [4]. The $\Delta_k$ values, therefore, represent the concentration of $Ca^{2+}$ intimately involved in stimulus–secretion coupling events subsequent to membrane depolarization.

Table 1 shows that neither acute nor chronic *in vivo* administration of chlorpromazine alters the total accumulation or net influx ($\Delta_k$) of $^{45}Ca^{2+}$ subsequent to depolarization. However, an *in vitro* chlorpromazine challenge added to synaptosomes isolated from the three treatment groups (control, acute, and tolerant) provided interesting results. Total accumulation of $^{45}Ca^{2+}$ in the absence of depolarization was not altered by chlorpromazine, regardless of whether the chlorpromazine was administered *in vivo* or *in vitro*. This is consistent with our recent finding that chlorpromazine does not affect binding of $^{45}Ca^{2+}$ by microsomal membranes from bovine adrenal medulla [15]. An analysis of chlorpromazine-induced changes in net $^{45}Ca^{2+}$ influx across synaptosomal membranes subsequent to KCl-induced depolarization shows that neither acute nor chronic *in vivo* chlorpromazine administration altered $\Delta_k$ values. However, when 50 $\mu$M chlorpromazine was added *in vitro*, statistical analysis showed that there was a significant depression of $^{45}Ca^{2+}$ influx in synaptosomes from all three groups of mice (control, acute and tolerant), but there was a marked difference in the magnitude of inhibition produced by chlorpromazine in tolerant vs control mice. Although "no drug" $\Delta_k$ values are similar for control and tolerant synaptosomes, the addition of 50 $\mu$M chlorpromazine resulted in an approximate 2.5-fold increase in $^{45}Ca^{2+}$ influx in tolerant as compared to control synaptosomes (1.70 ± 0.22 and 0.70 ± 0.22 $\mu$moles $^{45}Ca^{2+}$/g of protein for tolerant and control synaptosomes respectively). This suggests the development of membrane tolerance to the inhibitory effects of chlorpromazine. The same general trend was seen with 5 $\mu$M chlorpromazine *in vitro*, but these values were not statistically significant. These results suggest that, in order to demonstrate membrane tolerance, synaptosomal membranes from tolerant animals must be challenged with the drug *in vitro* and the resulting $^{45}Ca^{2+}$ influx compared to membrane preparations from control and tolerant animals without *in vitro* drug administration. The fact that chlorpromazine had to be added *in vitro* to our synaptosomal preparation to demonstrate membrane changes is not surprising. Chin and Goldstein [16] have likewise found that, in order to demonstrate tolerance to the action of ethanol in increasing membrane fluidity, ethanol must be added *in vitro* to membrane preparations from control and tolerant mice for comparison of results. These investigators were not able to show that ethanol-adapted (tolerant) membranes tested in the absence of ethanol were ordered differently than those from controls. However, when the same preparations were tested in

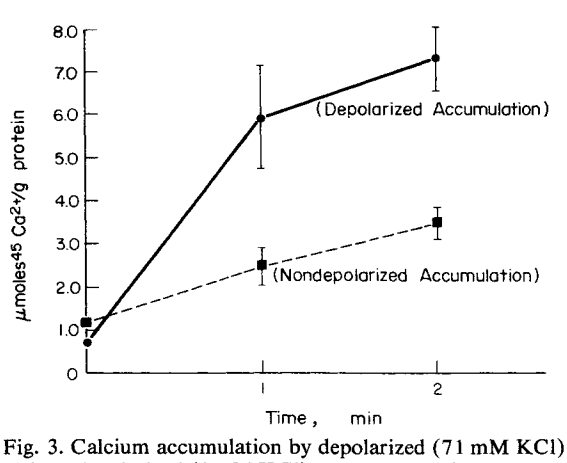


Fig. 3. Calcium accumulation by depolarized (71 mM KCl) and nondepolarized (5 mM KCl) synaptosomes from control mice as a function of different incubation times. The points at both 1 and 2 min are significantly different ($P < 0.05$; Student's *t*-test). Each point represents the mean ± S.E.M. of three experiments.

the presence of ethanol, synaptosomal membranes from ethanol-treated mice were less responsive to ethanol *in vitro* than membranes from the control animals. Recent work by Curran and Seeman [17] also supports this finding. These investigators showed that a significant difference between control and ethanol-tolerant groups in miniature end plate potential frequency in phrenic nerve terminals could be brought about only by readdition of ethanol. Recent work in our laboratories has provided a similar finding in that membrane tolerance to chronic barbiturate administration was only observable when synaptosomes were challenged with *in vitro* pentobarbital [6].

These studies point to a cellular mechanism involving calcium-mediated stimulus–secretion coupling events for the production of sedation. Previous work in our laboratories [6] and work done recently by other [4, 5] have shown that barbiturates inhibit synaptosomal calcium influx. We have shown that tolerance develops to this inhibitory effect on $^{45}Ca^{2+}$ influx at the same time as the appearance of behavioral signs of tolerance. The data we have obtained with chlorpromazine are particularly interesting since this drug produces sedation to which tolerance develops, but chronic administration does not result in the production of physical dependence, at least not to the extent of the dependence which is produced by ethanol or barbiturates. Our work shows that, as occurs with barbiturates, tolerance at the membrane level (to the inhibitory effects of chlorpromazine on synaptosomal $^{45}Ca^{2+}$ influx) occurs during the same time frame as the development of behavioral signs of tolerance.

Also of interest is our finding that phenobarbital and chlorpromazine-induced sedation are cross-tolerant. DBA mice rendered tolerant to phenobarbital sedation were also tolerant to chlorpromazine-induced sedation and vice versa. On the other hand, previous work has shown that if chlorpromazine is administered to animals physically dependent upon barbiturates [11] or ethanol [12], withdrawal symptomatology is made worse. This information has significant implications in that it suggests that cellular mechanisms involved in tolerance to sedation may be different from mechanisms involved in physical dependence production. It appears plausible to suggest that "stimulus–secretion coupling" events may be involved in drug-induced sedation and in the development of tolerance to sedation but that other factors, which as yet remain undefined, may be more intimately involved in physical dependence production.

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# Incorporation of ψ-isocytidine, a new antitumour C-nucleoside, into mammalian RNA and DNA

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ψ-Isocytidine [5-(β-D-ribofuranosyl)-isocytosine], synthesized by Chu *et al.* [1], is effective against several transplantable murine leukemias including the leukemic cell line P815/ara-C that is resistant to treatment by ara-C (1-β-D-arabinofuranosylcytosine) [2]. This is probably due to the fact that, whereas ara-C is phosphorylated by deoxycytidine kinase, the activity of which is reduced markedly in P815/ara-C [3], ψ-isocytidine is apparently phosphorylated by cytidine kinase [4]. ψ-isocytidine differs from other cytosine nucleosides, such as ara-C, in that it is a C-nucleoside in which the ribose moiety is attached to the base via a carbon–carbon linkage (Fig. 1). This structural feature renders ψ-isocytidine significantly more resistant than cytidine or ara-C to deamination by pyrimidine nucleoside deaminase [2]. The mechanism of action of ψ-isocytidine is different from that of ara-C. Whereas the triphosphate nucleotide form of ara-C inhibits DNA polymerase and thereby DNA synthesis [5], DNA synthesis in mouse small intestine appears not to be directly inhibited by ψ-isocytidine (unpublished observations). Another analog of cytidine which is also isosteric with ψ-isocytidine, 5-azacytidine (Fig. 1), in the monophosphate form inhibits orotidylate decarboxylase [6]. Preliminary studies indicate that, unlike that found using 5-azacytidine, orotidylate decarboxylase activity in homogenates of livers from rats treated with ψ-isocytidine is not inhibited. Since both ara-C [7] and 5-azacytidine [8] are incorporated into nucleic acids, it was of interest, as a possible explanation for the biological activity of ψ-isocytidine, to determine whether this substance could also be utilized as a precursor for RNA and DNA syntheses.