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Effects of Acute and Chronic Barbiturate Administration on Synaptosomal Calcium Accumulation

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SUMMARY

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Synaptosomes were isolated from four treatment groups of DBA/2J mice: Group I-Control; Group II-7 day tolerant (received dietary phenobarbital, 2.5 mg/g Purina Lab Chow for 7 days); Group III-20 hr withdrawn; and Group IV-7 day recovered (received dietary phenobarbital for 7 days followed by normal diet for 7 days). Pentobarbital (0.45 mM) added *in vitro* to synaptosomes from control mice significantly depressed (21.4%) potassium depolarized $^{45}Ca^{++}$ accumulation but did not significantly alter nondepolarized $^{45}Ca^{++}$ uptake or subsequent $^{45}Ca^{++}$ efflux. Chronic administration of phenobarbital to mice in the tolerant and withdrawn conditions resulted in behavioral tolerance and subsequent withdrawal symptomatology, but neither KCl-induced $^{45}Ca^{++}$ accumulation nor $^{45}Ca^{++}$ efflux was significantly altered. *In vitro* addition of pentobarbital (0.45 mM) to synaptosomes from tolerant mice did not significantly depress KCl-induced $^{45}Ca^{++}$ accumulation (8.1%) but did significantly depress KCl-induced $^{45}Ca^{++}$ accumulation by synaptosomes from withdrawn mice (15.2%) and recovered mice (16.8%). These data suggest that barbiturates may depress calcium-mediated stimulus-secretion coupling events contributing to central nervous system depression. Further, functional tolerance after chronic barbiturate treatment may result from a membrane adaptation facilitating calcium-mediated secretory functions.

INTRODUCTION

Recent evidence by Blaustein and Ector (1) has shown that pentobarbital[2] inhibits depolarization-induced $^{45}Ca^{++}$ influx across synaptosomal membranes. Barbiturates also depress potassium-facilitated calcium uptake by rat sympathetic ganglia (2). It was suggested that central nervous system depression produced by barbiturates may be mediated by membrane events that disrupt "stimulus-secretion coupling" (1), although postsynaptic inhibition of calcium permeability by barbiturates was also demonstrated in rat sympathetic ganglia (2).

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[2] The abbreviations used are GABA, γ-aminobutyric acid; PB, phenobarbital; PT, pentobarbital.

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Sohn and Ferendelli (3) also demonstrated a significant inhibition of $^{45}Ca^{++}$ influx into rabbit cortex synaptosomes by *in vitro* addition of PB. The hypothesis that barbiturates act presynaptically is further supported by Haycock *et al.* (4) who showed that potassium-facilitated, calcium-dependent norepinephrine and GABA secretion from synaptosomes is inhibited by PT. Inhibition of this transmitter release by PT was attributed to a selective inhibition of depolarization-induced calcium influx.

The present study was performed to examine the presynaptic effects of barbiturates and to determine if tolerance develops to the observed depression of depolarization-induced synaptosomal calcium accumulation. A relationship between alterations in transmembrane calcium movements and the production of barbiturate tolerance and dependence is suggested by this investigation.

METHODS

Male DBA/2J mice, 25–30 g (Jackson Laboratories, Bar Harbor, Maine) were housed individually for at least five days prior to experimentation and kept on a 12/12 hour light-dark cycle with food and water available *ad libitum*. Mice were then randomly divided into four experimental groups: control, tolerant, withdrawn, and recovered. Control mice were maintained on a Purina Lab Chow diet *ad libitum*. Barbiturate dependence was induced by utilizing a procedure with PB as a dietary adjunct to a milled lab chow diet. This procedure has been shown to reliably produce physical dependence in mice (5). Animals comprising the tolerant group were maintained on this diet for seven days prior to sacrifice. Animals used in the withdrawal phase were prepared by substituting diet without PB on the morning of the seventh day. These animals were observed at four hour intervals beginning six hours after diet substitution and sacrificed at peak withdrawal severity (defined by the time of greatest tonic-clonic convulsion incidence). The recovery group consisted of animals successfully completing both dependence and withdrawal, followed by subsequent maintenance on standard Purina Lab Chow for seven days post-withdrawal.

Preparation of synaptosomes. To provide a synaptosomal preparation, DBA/2J mice from each of the treatment groups (control, tolerant, withdrawn, recovered) were decapitated, and whole brains dissected free and weighed. The whole brains were placed in a glass-on-glass conical homogenizer (Duall tissue grinder, Kontes Glass Co.) and gently homogenized with a Sorvall Omni-mixer at the lowest setting. From this homogenate, the synaptosomal fraction was isolated by a modified method of Gray and Whittaker (6). The final pellet, sedimented at 100,000 g_{max}, was rinsed with incubation media (Table 1) to remove the sucrose and was resuspended in 8.0 ml of the same media for $^{45}Ca^{++}$ accumulation and efflux studies.

Calcium accumulation and efflux. The accumulation and efflux of $^{45}Ca^{++}$ associated with the synaptosomal preparations was studied by modifications of methods of Blaustein and Ector (7). The composition of the solutions utilized for $^{45}Ca^{++}$ accumulation and efflux experiments is given in Table 1. For depolarized loading, 600–1200 μg of protein in a 0.5 ml aliquot was added to 0.5 ml of incubation media (with or without Na PT, 0.45 mM final concentration) and allowed to equilibrate for 12 min at 30° in a Dubnoff metabolic shaker. After 12 min, $^{45}Ca^{++}$ loading was initiated by the addition of 0.5 ml of depolarization media (Table 1) containing $^{45}Ca^{++}$ (1.2 mM final concentration) and 213 mM KCl (71 mM final concentration). After 1 min of incubation at 30°, 0.5 ml of the 1.5 ml volume was drawn off and pipetted into a 25 ml Erlenmeyer flask containing 10.0 ml of efflux media (Table 1), also at 30°. The re-

TABLE 1

Composition of solutions utilized for investigation of calcium influx and efflux[a,b]

Solution	NaCl	KCl	$CaCl_2$
1. Incubation	132 mM	5 mM	0
2. Efflux	132 mM	5 mM	1.2 mM
3. Depolarizing	0	213 mM	1.2 mM
4. Nondepolarizing	0	5 mM	1.2 mM

[a] In addition to the constituents listed, all solutions also contained $MgCl_2$, 1.3 mM; NaH_2PO_4, 1.2 mM; dextrose, 10.0 mM; Tris base, 20.0 mM.

[b] The solutions were buffered to pH 7.65 at 23° by titration with 1.0 M maleic acid.

maining 1.0 ml was removed with a Pasteur pipette and filtered on a prewashed 0.45 μm Gelman cellulose acetate filter (25 mm diameter) with a Millipore microfiltration apparatus. Each filter was washed with 15.0 ml of incubation media, removed, and placed in a scintillation vial. The efflux solution was allowed to incubate for one minute and filtered and washed in the same manner. Nondepolarized samples were treated in the same fashion, except the $^{45}Ca^{++}$ was added in a nondepolarizing media (Table 1) containing 5 mM KCl. After drying, 15.0 ml of scintillation cocktail (Type 3A40, Research Products International) was added to the filters and counting was done on a Packard Tricarb liquid scintillation counter. All samples were corrected for filter bound $^{45}Ca^{++}$. Calcium efflux was computed as the difference between the $^{45}Ca^{++}$ remaining in the synaptosomes (i.e., on the filters) after the 1 min efflux period and the $^{45}Ca^{++}$ content after loading (see legends for Tables 2–5).

Protein assay. Synaptosomal protein concentration was assayed by the method of Lowry *et al.* (8).

Phenobarbital assay. Phenobarital was assayed by gas chromatography as the butyl derivative following a double extraction of the brain homogenate after the method of Greeley (9). The average extraction recovery was 69% for PB and 94% for PT.

RESULTS

Behavioral assessment of barbiturate tolerance and withdrawal. To demonstrate that animals exposed to the PB adulterated diet for seven days were functionally tolerant, changes in the animal's responses to the same brain level of drug were measured. This data is depicted in Figure 1.

Brain levels were plotted against grid test scores (a measure of neuromuscular impairment (5)) for the indicated days just prior to sacrifice. The progressively reduced grid test scores ($p < 0.001$, t-test, day 6 vs day 1) at equivalent brain drug levels indicated the development of functional (pharmacodynamic) tolerance, consistent with previous work indicating that functional tolerance develops progressively (5). Animals subsequently utilized for biochemical studies to investigate the phenomenon of functional tolerance were sacrificed on the morning of the seventh day of chronic PB exposure and were assumed to be functionally tolerant at this time.

Withdrawal was assessed by noting behavioral symptoms that included gross tremor, muscular rigidity, straub tail, hyperactivity, and convulsive activity. The time course for the development of withdrawal symptoms in DBA/2J mice ($n = 25$) is presented in Figure 2.

The full spectrum of withdrawal behaviors and scoring procedures are described elsewhere (5). As seen in Figure 2, the peak of withdrawal severity occurs at approximately 20 hr, an interval that corresponds to the time of maximum probability of occurrence of tonic-clonic convulsions. For subsequent $^{45}Ca^{++}$ accumulation and efflux studies, all animals used in the withdrawal portion of this series of experiments were sacrificed at this point. To ensure comparability of intensity of withdrawal, the animals were stressed via twirling briefly by the tail and observed for "convulsion on handling" (10) and other symptoms. Two mice showing similar withdrawal signs were sacrificed immediately and their brains were pooled for synaptosomal preparation. Mice demonstrating lethal tonic extensor seizures at this time were not utilized. Gross withdrawal signs were rare after 48 hr post-withdrawal (see Fig. 2), and animals assessed at seven days post-withdrawal showed no appreciable behavioral differences from controls.

Time parameters for calcium accumulation. To select the point of maximum $^{45}Ca^{++}$ loading (net accumulation) of control synaptosomes following potassium-induced depolarization, incubation times of 15, 30, 60 and 120 sec were investigated and displayed in Figure 3. A true 0 sec value must be extrapolated, as accumulation begins immediately upon $^{45}Ca^{++}$ introduction. The data for depolarized synaptosomes indicated that potassium-induced accumulation attained a maximum value at 60 sec; this time period was used for subsequent uptake studies with $^{45}Ca^{++}$. The data for synaptosomes incubated without excess potassium indicated that the accumulation of

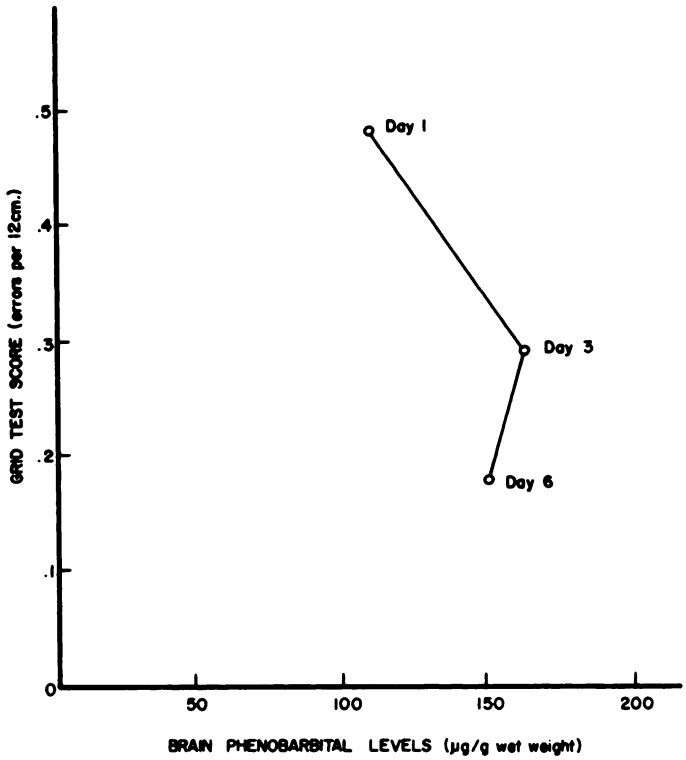

FIG. 1. *Grid test scores as a function of whole brain phenobarbital concentration*

Each point represents the mean of six observations. Each mouse was tested only once immediately prior to sacrifice. The grid tests were conducted as described by Belknap *et al.* (5). The means ± standard deviations for brain phenobarbital levels shown in this figure are 112 ± 21, 116 ± 31 and 154 ± 37 µg/g wet weight for days 1, 3, and 6 respectively.

$^{45}Ca^{++}$ by nondepolarized synaptosomes was not only of a lesser magnitude, but increased monotonically with longer incubation times. An analysis of variance among points of the two curves yielded a significant depolarized/nondepolarized by time interaction ($p < 0.03$), indicating a significant difference in uptake values evident at 60 sec ($p < 0.01$). To further illustrate the differences between depolarized and nondepolarized preparations, the efflux of $^{45}Ca^{++}$ from synaptosomes following $^{45}Ca^{++}$ incubation (60 sec) was monitored and expressed as percent loss from "loaded" synaptosomes. The overall mean for percent loss across groups for depolarized fractions was 85.3%, while the mean percent loss for nondepolarized fractions was 72.7% ($p < 0.001$). These data indicate that the $^{45}Ca^{++}$ that was loaded as a result of depolarization-induced uptake was subsequently extruded more completely from synaptosomes than the $^{45}Ca^{++}$ that was accumulated following incubation without excess potassium.

Acute in vitro inhibition of synaptosomal $^{45}Ca^{++}$ accumulation by PT. Pentobarbital (0.45 mM, final concentration) was added to synaptosomes isolated from control mice to quantitate the *in vitro* inhibition of $^{45}Ca^{++}$ accumulation and/or efflux. Table 2 shows that PT administration significantly inhibits KCl-induced synaptosomal $^{45}Ca^{++}$ accumulation by 21.4%. Net KCl-induced influx (Δ_4) of $^{45}Ca^{++}$ across synaptosomal membranes was also significantly inhibited by PT. This level of PT was chosen because it approaches plasma levels which produce general anesthesia and is known to depress $^{45}Ca^{++}$ influx in synaptosomes isolated from rats by approximately 30–50% (1, 3). *In vitro* PT addition

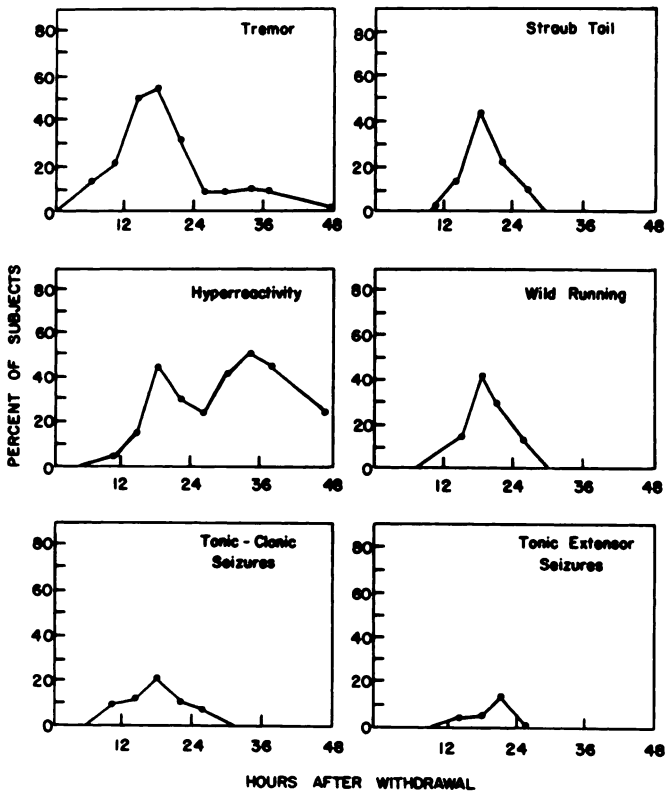

FIG. 2. *Time-course for withdrawal symptomatology*

Each point represents the mean of the observations from 25 mice. All mice were maintained on a phenobarbital-adulterated diet for six days (as described under METHODS) prior to withdrawal.

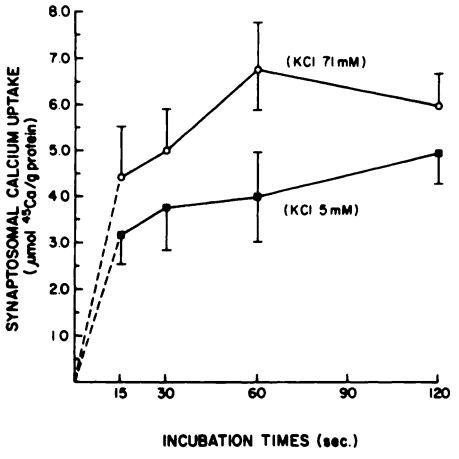

FIG. 3. *Calcium accumulation as a function of different incubation times for depolarized and nondepolarized synaptosomes*

Data represent depolarized (○) and nondepolarized (■) synaptosomes. The points at the 60 sec time period are significantly different ($p < .01$). Each point represents the mean ± S.E.M. of four experiments.

did not depress $^{45}Ca^{++}$ efflux from either synaptosomes that had been previously depolarized by KCl or from nondepolarized synaptosomes. In addition, Table 2 shows that PT did not significantly inhibit synaptosomal $^{45}Ca^{++}$ accumulation by nondepolarized synaptosomes.

Synaptosomal $^{45}Ca^{++}$ accumulation after tolerance development and the effects of in vitro addition of PT. In these experiments synaptosomes were isolated from 7 day tolerant mice to demonstrate, first, the effects of chronic barbiturate administration (*in vivo*) on synaptosomal $^{45}Ca^{++}$ accumulation and efflux, and second, the effects of *in vitro* PT addition (0.45 mM) on synaptosomal $^{45}Ca^{++}$ accumulation and efflux. The second phase of this study was carried out to examine the effects of addition of barbiturate, *in vitro*, to synaptosomes isolated from tolerant mice. Table 3 shows that $^{45}Ca^{++}$ depolarized uptake of no-drug adjunct (no *in vitro* PT addition) is

TABLE 2

Effects of acute in vitro barbiturate administration on synaptosomal $^{45}Ca^{++}$ accumulation[a]

Synaptosomes were isolated from control mice and $^{45}Ca^{++}$ accumulation and efflux were determined as described under METHODS. *Total Accumulation* refers to the $^{45}Ca^{++}$ accumulated during a one min loading period. *Total Efflux* refers to the quantity of $^{45}Ca^{++}$ extruded from synaptosome during a one min efflux period. Δ_k *Influx* refers to the net KCl-facilitated $^{45}Ca^{++}$ influx (depolarized total accumulation minus nondepolarized total accumulation).

	Depolarized (71 mM KCl)			Nondepolarized (5 mM KCl)			Δ_k Influx
	Total Accumulation	Total Efflux	% Loss (Efflux)[c]	Total Accumulation	Total Efflux	% Loss (Efflux)[c]	
	(μmol $^{45}Ca^{++}$/min/g protein)			*(μmol $^{45}Ca^{++}$/min/g protein)*			
No drug adjunct	7.01 ± 0.80	5.91 ± 0.71	84.1 ± 1.5	6.13 ± 0.71	4.40 ± 0.44	72.4 ± 1.7	0.88 ± 0.21
Pentobarbital adjunct (*in vitro*) (0.45 mM)	5.52 ± 0.64[b]	4.78 ± 0.57	86.3 ± 1.2	5.19 ± 0.63	3.72 ± 0.46	73.3 ± 1.3	0.33 ± 0.08[b]
% Depression	21.4%		0%	15.3%		0%	

[a] Data are expressed as means ± S.E.M. of 6 experiments.

[b] Significantly different from no drug adjunct values ($p < 0.01$) using a *t*-test for matched samples.

[c] Calculations were performed using the equation: % Loss (Efflux) = $\frac{\text{Total Efflux}}{\text{Total Accumulation}} \times 100$.

TABLE 3

Effects of chronic phenobarbital treatment in vivo (7 days) on synaptosomal $^{45}Ca^{++}$ accumulation[a]

Synaptosomes were isolated from tolerant mice and $^{45}Ca^{++}$ accumulation and efflux were determined as described under METHODS. *Total Accumulation, Total Efflux,* and Δ_k *Influx* values were obtained as outlined in Table 2.

	Depolarized (71 mM KCl)			Nondepolarized (5 mM KCl)			Δ_k influx
	Total accumulation	Total efflux	% Loss (efflux)[c]	Total accumulation	Total efflux	% Loss (efflux)[c]	
	(μmol $^{45}Ca^{++}$/min/g protein)			*(μmol $^{45}Ca^{++}$/min/g protein)*			
No drug adjunct	6.02 ± 0.51	5.10 ± 0.40	85.1 ± 1.7	5.61 ± 0.38	4.29 ± 0.24	75.0 ± 1.2	0.41 ± 0.24
Pentobarbital[b] adjunct (*in vitro*) (0.45 mM)	5.53 ± 0.42	4.83 ± 0.34	88.1 ± 1.5	5.35 ± 0.35	3.96 ± 0.16	73.4 ± 2.5	0.18 ± 0.24
% Depression	8.1%		0%	4.6%		0%	

[a] Data are expressed as means ± S.E.M. of 6 experiments.

[b] Pentobarbital was added *in vitro* in a final concentration of 0.45 mM.

[c] Calculations were performed using the equation: % Loss (Efflux) = $\frac{\text{Total Efflux}}{\text{Total Accumulation}} \times 100$.

6.02 ± 0.51 μmol $^{45}Ca^{++}$ per minute per g protein as compared to 7.01 ± 0.80 μmol $^{45}Ca^{++}$ per minute per g protein in no-drug adjunct synaptosomes isolated from control mice (Table 2). Although this shows an apparent reduction in $^{45}Ca^{++}$ accumulation by synaptosomes isolated from tolerant mice, this difference was not significant. A similar trend was also present for net $^{45}Ca^{++}$ influx (Δ_k) but the differences were not significant. Likewise, when PT (0.45 mM final concentration) was added to synaptosomes isolated from tolerant animals (PT adjunct), no significant depression of $^{45}Ca^{++}$ accumulation was observed. These observations suggest a development of tolerance at the membrane level in that a significant (21.4%) depression of $^{45}Ca^{++}$ accumulation in control synaptosomes subsequent to PT addition was reduced to a nonsignificant depression (8.1%) when PT was added to synaptosomes isolated from tolerant mice.

Data in Table 3 agree with data obtained from control animals in that efflux of $^{45}Ca^{++}$ from depolarized and nondepolarized synaptosomes was not depressed by barbiturate administration (either *in vitro* or *in vivo*).

Synaptosomal $^{45}Ca^{++}$ accumulation during withdrawal and the effects of in vitro addition of PT. The effects of withdrawal on synaptosomal $^{45}Ca^{++}$ accumulation and efflux and the effects of addition of PT, *in vitro*, on $^{45}Ca^{++}$ accumulation and efflux are shown in Table 4. The depolarized uptake of $^{45}Ca^{++}$ into synaptosomes derived from withdrawn mice (no-drug adjunct) appeared to be quantitatively similar to the uptake in synaptosomes from tolerant mice (6.22 and 6.02 μmol $^{45}Ca^{++}$ per minute per g protein, respectively) and was not significantly different from synaptosomal $^{45}Ca^{++}$ accumulation in control synaptosomes (Table 2). However, it is interesting that the 15.0% depression by PT addition, *in vitro*, in synaptosomes from withdrawn mice is significantly different from no-drug adjunct ($p < 0.01$). This depression is also significantly different from the 8.1% depression seen in tolerant synaptosomes (Table 3). This suggests that synaptosomal membranes may readapt during withdrawal to approach control sensitivity to barbiturate exposure.

Synaptosomal $^{45}Ca^{++}$ accumulation after recovery and the effects of in vitro addition of PT. Table 5 shows that no-drug adjunct synaptosomal $^{45}Ca^{++}$ accumulation is quite similar to uptake values obtained for control, no-drug adjunct accumulation (Table 2). In addition, as seen with synaptosomes from control and withdrawn mice, addition of a PT adjunct (0.45 mM) to these synaptosomes resulted in a significant ($p < 0.01$) 16.8% depression in $^{45}Ca^{++}$ depolarized accumulation. Likewise, examination of nondepolarized data showed a significant ($p < 0.01$) barbiturate adjunct depression. Interestingly, however, there was no apparent inhibition of net $^{45}Ca^{++}$ influx (Δ_k) by *in vitro* PT addition (Table 5). *In vitro* addition of PT to control synaptosomes did significantly depress Δ_k values (Table 2).

DISCUSSION

Barbiturates are known to produce functional tolerance and physical dependence after repeated administration. Therefore, from a mechanistic standpoint, disruptions in membrane function induced by barbiturates would be expected to correlate with the central depressant actions. Also, compensatory changes in membrane function should occur which temporally parallel the development of behavioral tolerance and

TABLE 4

Effects of withdrawal (20 hr) following chronic phenobarbital treatment on synaptosomal $^{45}Ca^{++}$ accumulation[a]

Synaptosomes were isolated from withdrawn mice and $^{45}Ca^{++}$ accumulation and efflux were determined as described under METHODS. Total *Accumulation*, *Total Efflux*, and Δ_k *Influx* values were obtained as outlined in Table 2.

	Depolarized (71 mM KCl)			Nondepolarized (5 mM KCL)			Δ_k Influx
	Total accumulation	Total efflux	% Loss (efflux)[c]	Total accumulation	Total efflux	% Loss (efflux)[c]	
	(μmol $^{45}Ca^{++}$/min/g protein)			*(μmol $^{45}Ca^{++}$/min/g protein)*			
No drug adjunct	6.22 ± 0.49	5.13 ± 0.55	81.1 ± 3.4	5.58 ± 0.49	3.99 ± 0.40	71.1 ± 2.5	0.64 ± 0.26
Pentobarbital[b] adjunct (*in vitro*) (0.45 mM)	5.29 ± 0.49[d]	4.61 ± 0.56	85.5 ± 3.7	5.07 ± 0.55	3.66 ± 0.42	71.6 ± 3.1	0.22 ± 0.27
% Depression	15.0%		0%	9.1%		0%	

[a] Data are expressed as means ± S.E.M. of 6 experiments.

[b] Pentobarbital was added *in vitro* in a final concentration of 0.45 mM.

[c] Calculations were performed using the equation: $\text{Loss (Efflux)} = \frac{\text{Total Efflux}}{\text{Total Accumulation}} \times 100$.

[d] Significantly different from no drug adjunct ($p < .01$), using a *t*-test for matched samples.

TABLE 5

Effects of acute, in vitro barbiturate administration in animals "recovered" from barbiturate dependence and withdrawal (7 days post-withdrawal) on synaptosomal $^{45}Ca^{++}$ accumulation[a]

Synaptosomes were isolated from recovered mice and $^{45}Ca^{++}$ accumulation and efflux were determined as described under METHODS. *Total Accumulation, Total Efflux,* and Δ_k *Influx* values were obtained as outlined in Table 2.

	Depolarized (71 mM KCl)			Nondepolarized (5 mM KCl)			Δ_k influx
	Total accumulation	Total efflux	% Loss (efflux)[c]	Total accumulation	Total efflux	% Loss (efflux)[c]	
	(μmol $^{45}Ca^{++}$/min/g protein)			*(μmol $^{45}Ca^{++}$/min/g protein)*			
No drug adjunct	7.05 ± 0.24	5.94 ± 0.24	84.4 ± 1.5	6.38 ± 0.19	4.70 ± 0.21	73.6 ± 1.7	0.67 ± 0.21
Pentobarbital adjunct (*in vitro*) (0.45 mM)	5.87 ± 0.22[b]	5.13 ± 0.19	87.5 ± 0.6	5.08 ± 0.08[b]	3.64 ± 0.10	70.9 ± 1.5	0.79 ± 0.15
% Depression	16.8%		0%	20.4%		0%	

[a] Data are expressed as means ± S.E.M. of 6 experiments.

[b] Significantly different from no drug adjunct ($p < 0.01$) using a *t*-test for matched samples.

[c] Calculations were performed using the equation: $\%\ \text{Loss (Efflux)} = \frac{\text{Total Efflux}}{\text{Total Accumulation}} \times 100.$

physical dependence. Several recent investigations have implicated changes in transmembrane calcium uptake and/or binding in the central nervous system depression associated with acute barbiturate action. Blaustein and Ector (1) have shown that the addition of PT *in vitro* inhibits potassium-, veratridine-, and gramicidin-induced $^{45}Ca^{++}$ uptake into rat brain synaptosomes. An extension of this finding was provided by Haycock *et al.* (4), who showed that PT depressed calcium-dependent release of [^{3}H]norepinephrine and [^{14}C]GABA from mouse forebrain synaptosomes subsequent to depolarization with potassium or veratridine. These data suggest that barbiturates may interfere with transmitter release and/or synaptic transmission by reducing the depolarization-triggered calcium entry into presynaptic terminals. The interference of calcium transport and/or binding by barbiturates is also supported by recent work by Matthews and Nordmann (11) which showed that amylobarbital decreased the surface charge of synaptic vesicles. This effect would, by reducing available binding sites for calcium, alter membrane stability (12) and ultimately affect neurotransmitter exocytosis. In a more global sense, then, disruptions of calcium concentration, binding or flux within the central nervous system should profoundly affect neuronal function and may provide, at least in part, a mechanism for the observed behavioral effects. These presynaptic actions of barbiturates should, naturally, be evaluated within the context of literature that also suggests postsynaptic actions (13–16). However, it should be pointed out that general depressant actions affecting membrane function might be expected to appear *a priori* in both pre- and postsynaptic membranes. Moreover, chronic studies exploring tolerance to these postsynaptic effects have not been done. Thus, a complete characterization of barbiturate action at such synaptic sites is required in order to fully explain the observed behavioral correlates, both acutely and chronically.

Although inhibition of calcium uptake and/or binding may be involved in central nervous system depression, this barbiturate-mediated effect does not necessarily provide the sole basis for adaptational changes at the membrane level that can be expected to occur in a correlative manner with the accrual of tolerance and physical dependence. The data from our experiments with acute, *in vitro* PT administration agree with that obtained by Blaustein and Ector (1) and Haycock *et al.* (4) since addition of PT *in vitro* to a synaptosomal preparation isolated from control (naive) mice significantly inhibited potassium-in-

duced calcium uptake. However, our data show that after chronic administration of PB, the inhibitory effect of PT *in vitro* was significantly attenuated. This effect on calcium accumulation occurred concomitantly with the development of behavioral indices of functional tolerance and physical dependence, suggesting that adaptational changes in membrane calcium produced by chronic barbiturate administration may be involved in tolerance and dependence development. The addition of PT to synaptosomes from tolerant mice was necessary to demonstrate "tolerance" at the membrane level after chronic PB treatment. That is, there were no significant differences in $^{45}Ca^{++}$ accumulation in the no-drug adjunct groups among the four different animal treatment groups (control, tolerant, withdrawn, and recovered). However, when PT was added *in vitro* to the synaptosomal preparation from tolerant animals, there was no longer a significant depression of $^{45}Ca^{++}$ accumulation as compared to the no-drug adjunct. Conversely, $^{45}Ca^{++}$ accumulation in synaptosomes isolated from mice in the withdrawn group was again depressed significantly by PT addition, *in vitro,* as was $^{45}Ca^{++}$ uptake in synaptosomes from the recovery group. These data suggest that tolerance develops to the inhibitory effects of barbiturates on synaptosomal $^{45}Ca^{++}$ accumulation. Further, readaptation appears to occur rapidly during withdrawal and is essentially complete after a recovery period of seven days. The fact that PT had to be added *in vitro* to the synaptosomal preparations to demonstrate membrane changes is not surprising. Chin and Goldstein (17) have found that 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 alcohol exhibited different fluidity parameters than controls. However, when the same preparations were tested in the presence of ethanol, synaptosomal membranes from ethanol-treated mice demonstrated significantly smaller fluidity changes to ethanol *in vitro* than membranes from the control animals. These results are further supported by Curran and Seeman (18) who demonstrated that a significant difference between control and ethanol-tolerant groups in miniature end plate potential frequency in rat phrenic nerve terminals could only be revealed by readdition of ethanol. The conclusions of these studies are all consistent with the existence of homeostatic mechanisms in which the chronic presence of a depressant drug (such as ethanol or barbiturates) provides the stimulus for an adaptive response in the neuronal membrane itself. Tolerance, then, may occur as a result of membrane changes which cause an adaptation to this inhibitory action.

A final point concerning the generalization of this phenomenon can be made by the observation in this investigation that cellular tolerance to the inhibitory effects of *in vitro* PT was apparent following chronic *in vivo* treatment with PB. Sohn and Ferendelli (3) have shown that PB will also depress potassium-induced calcium influx, although Blaustein and Ector (1) failed to find a significant effect. Thus, if barbiturates share a commonality of action in terms of their mechanisms of dependence production, the suggestion of cross tolerance between the two barbiturates observed in this study is not unexpected.

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