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Comparison of the *in vivo* effects of convulsant and optically active hypnotic barbiturates with their effects on the *in vitro* K^+-stimulated release of [^{3}H]acetylcholine*

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Barbiturates inhibit the release of acetylcholine (ACh) from the exposed cerebral cortex *in vivo* [1–3] and from the electrically stimulated, perfused superior cervical ganglion [4]. To examine inhibition of neurotransmitter release in the CNS *in vitro*, K^+-stimulated brain slice preparations have been used. Early studies showed that amobarbital and thiopental inhibit K^+-stimulated ACh and [^{14}C]ACh release from cerebral cortex slices [5, 6]. Later investigations indicated that this inhibition of K^+-stimulated ACh release occurs in other brain regions [7], is dose dependent and is a common characteristic of several members of the barbiturate drug class [8]. Further work using brain slices *in vitro* excluded the possibility that the effect on K^+-stimulated ACh release was a result of ACh synthesis inhibition by the barbiturates since there was no difference, as determined by a comparison of dose–response curves, between the phenobarbital inhibition of Ca^{2+}-dependent K^+-stimulated release of endogenous ACh and preformed radioactive ACh [9].

To test more rigorously the inhibition of K^+-stimulated ACh release in brain slices as a possible model system for the study of the mechanism of barbiturate depression of the CNS, we compared the *in vivo* effects of the stereoisomers of pentobarbital [*R*(+)-PB, *S*(−)-PB] and secobarbital [*R*(+)-SB, *S*(−)-SB] and the effects of the convulsant barbiturate 5-(2-cycohexylideneethyl)-5-ethyl barbituric acid (CHEB) [10] with their effects *in vitro* on K^+-stimulated [^{3}H]ACh release.

Materials and methods

PB and SB in their free acid forms were purchased from the Sigma Chemical Co., St. Louis, MO. The stereoisomers of these drugs were supplied through the offices of Dr. Robert E. Willette of NIDA. CHEB was provided by Dr. Hall Downes of the University of Oregon Health Sciences Center, Portland, OR.

Brain levels of barbiturates were determined in tissue from male ICR Swiss mice (25–35 g, Harlan Industries, Inc., Indianapolis, IN) that had been decapitated at loss of righting reflex after an intraperitoneal (i.p.) injection of racemic PB and SB or their stereoisomers and at convulsion after i.p. CHEB. The brains were removed, rinsed with saline, weighed, and stored in glass vials at −70° until time for assay. The barbiturates were extracted and measured spectrophotometrically by the method of Brodie *et al.* [11].

In those experiments in which inhibition of K^+-stimulated [^{3}H]ACh release was studied, the mice were decapitated and the brains were removed and dissected on ice. The forebrain region was obtained by making a vertical cut just caudal to the inferior colliculi. The tissue was cut into 0.4 mm slices by chopping in two directions at a 90° angle with a Brinkmann–McIlwain tissue chopper. The slices were suspended in 15 ml of low K^+ medium with 1 μCi/ml (0.1 μM) [^{3}H]choline (Amersham/Searle, Arlington Heights, IL, 10.1 Ci/mmole) and incubated for 10 min at 37° with gentle shaking. After this loading phase the slices were centrifuged at 4° and 19,000 *g* for 5 min. The resulting pellet was resuspended in 15 ml of low K^+ medium and 1-ml samples were placed in polyethylene tubes on ice. Following centrifugation as before, the pellets were resuspended in one of several different incubation media and incubated for 10 min at 37° with vigorous shaking. After this release phase the slices were again centrifuged as before. An 800-μl sample of the supernatant fluid was removed to assay for [^{3}H]ACh released into the medium. The remaining supernatant fluid was discarded and the pellet was homogenized in 1 ml of low K^+ medium. A 700-μl sample of the homogenate was taken to assay for [^{3}H]ACh remaining in the tissue. [^{3}H]ACh was measured in supernatant fluid and homogenate samples after extraction and purification as descibed previously [9, 12].

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The low K^+ medium contained (mM): 120 NaCl; 5 KCl; 15 Na^+ phosphate buffer (pH 7.4); 1.2 $MgCl_2$; 0.75 $CaCl_2$; and 10 glucose. Acetylcholinesterase was inhibited by the addition of 5×10^{-5} M paraoxone (diethyl-*p*-nitrophenyl-phosphate, Sigma Chemical Co.). The KCl concentration was increased to 50 mM to make the high K^+ medium that was used to stimulate the release of [^{3}H]ACh. The drugs were dissolved in either low K^+ or high K^+ medium with the addition of NaOH. After dilution to the appropriate concentration the pH of the solutions never varied more than 0.1 unit from 7.4.

The amount of [^{3}H]ACh released was the [^{3}H]ACh in the medium divided by the total amount of [^{3}H]ACh in the tissue before the release phase began. In a typical experiment, there were approximately 350,000 dpm [^{3}H]ACh in the tissue after the loading phase. Under control conditions low K^+ medium released 0.177 ± 0.007 (mean ± S.E.M.; N = 21) and high K^+ medium released 0.656 ± 0.001 (mean ± S.E.M.; N = 20) of the total [^{3}H]ACh available. In order to calculate the percent inhibitions of K^+-stimulated [^{3}H]ACh release by CHEB and the stereoisomers of PB and SB, the amounts of [^{3}H]ACh released under the appropriate conditions were used in the following formula:

$$\frac{(\text{high } K^+_{\text{control}} - \text{low } K^+_{\text{control}}) - (\text{high } K^+_{\text{drug}} - \text{low } K^+_{\text{drug}})}{(\text{high } K^+_{\text{control}} - \text{low } K^+_{\text{control}})} \times 100$$

The percentages of inhibition were converted to probits so that the concentration of drug required to cause 50 per cent inhibition of K^+-stimulated [^{3}H]ACh release (IC_{50}) and the slopes of the log dose–response curves could be determined along with their 95 per cent confidence intervals [13].

Results and discussion

Intraperitoneal doses of the stereoisomers of PB and SB that caused a loss of righting reflex in all mice were determined in preliminary experiments and then used to establish the CNS stereospecificity for the hypnotic action of these drugs. The actual dose of drug injected was not critical. When lower doses of the PB and SB stereoisomers were injected, a smaller percentage of mice lost the righting reflex. Those animals losing the righting reflex had brain levels not significantly different from brain levels at this end point in mice injected with higher doses. The brain levels of the *S*(−)-stereoisomers of both PB and SB were significantly less than the *R*(+)-stereoisomers at loss of righting reflex (Table 1). Differences in hypnotic potency between barbiturate stereoisomers have been noted by previous investigators who compared injected doses [14–19]. The use of brain levels in our study at an observable endpoint (loss of righting reflex) eliminated the possibility that these differences in potency were caused by selective stereoisomer metabolism, plasma protein binding, or penetration across the blood–brain barrier. Brain levels of CHEB, after a 30 mg/kg dose, were measured at convulsion and found to be 46 nmoles/g brain (mean ± S.E.M., N = 6).

Table 1. Brain levels of pentobarbital and secobarbital racemates and stereoisomers at loss of righting reflex after i.p. administration in mice*

	S(−)	*RS*(±) (nmoles/g brain)	*R*(+)
Pentobarbital	123 ± 1†	153 ± 10†	231 ± 16
Secobarbital	89 ± 4†	122 ± 5†	173 ± 7

* Brain levels were determined spectrophotometrically by the method of Brodie *et al.* [11]. The doses administered were 35 mg/kg of *S*(−), 50 mg/kg of *RS*(±) and 75 mg/kg of *R*(+). The results are means ± S.E.M.; N = 6.

† Represents a value significantly different than *R*(+) at $P < 0.05$ by a one-way analysis of variance followed by the Student–Newman–Keuls test to determine the significant differences among the group means.

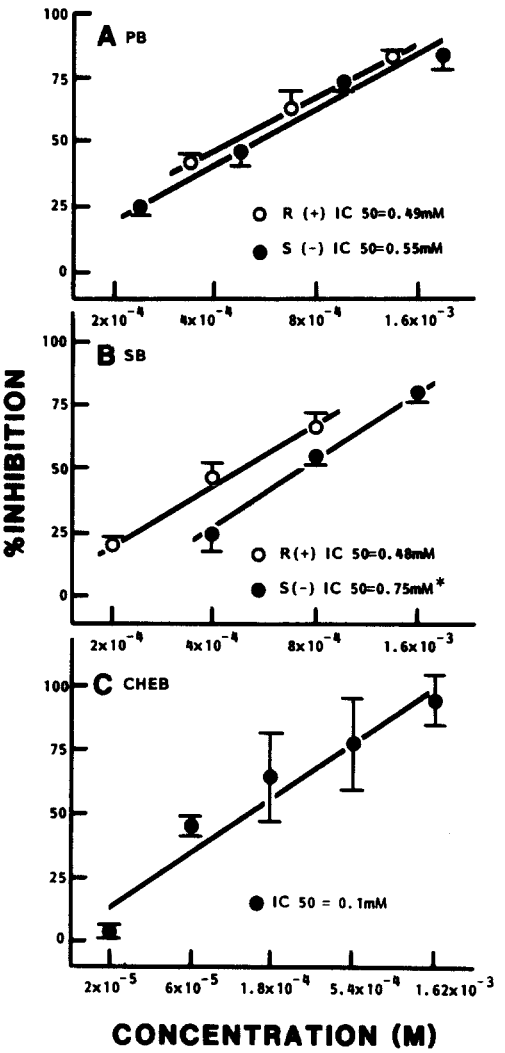

Fig. 1. Inhibition of K^+-stimulated [^{3}H]ACh release from mouse forebrain slices. (A) Inhibition by pentobarbital (PB) stereoisomers. (B) Inhibition by secobarbital (SB) stereoisomers. (C) Inhibition by 5-(2-cyclohexylidene-ëthyl-5-ethyl barbituric acid (CHEB). The slices were incubated with [^{3}H]choline at 1 μCi/ml (0.1 μM) for 10 min. Following centrifugation and wash, the slices were reincubated at different concentrations of the PB and SB stereoisomers or of CHEB for 10 min. [^{3}H]ACh was assayed in the medium and tissue as described previously [9, 12]. The concentration of drug required to inhibit 50 per cent of K^+-stimulated [^{3}H]ACh release (IC_{50}) and its 95 per cent confidence interval were determined from the per cent inhibitions as described in Materials and Methods. The 95 per cent confidence intervals are, respectively: (0.41 to 0.58 mM) and (0.46 to 0.65 mM) for *R*(+) and *S*(−)-PB; (0.39 to 0.58 mM) and (0.61 to 0.97 mM) for *R*(+) and *S*(−)-SB; and (0.05 to 0.25 mM) for CHEB. Each point is a mean ± S.E.M.; N = 3–6 determinations. The asterisk (*) represents a value significantly different from *R*(+) at P = 0.05.

IC_{50} Values for the inhibition of K^+-stimulated [^{3}H]ACh release were then determined *in vitro* for the PB and SB stereoisomers. The difference in IC_{50} values for the PB stereoisomers was not significant, although *R*(+)-PB tended to be more potent than *S*(−)-PB (Fig. 1A). The difference in IC_{50} values between the stereoisomers of SB did reach significance, with *R*(+)-SB more potent than *S*(−)-SB (Fig. 1B). It should be noted that this stereospecificity found *in vitro* was the opposite of that found *in vivo* for the hypnotic action of these drugs. CHEB was tested in the *in vitro* brain slice system and was also found to inhibit the K^+-stimulated release of [^{3}H]ACh (Fig. 1C). The dose–response curves for the PB and SB stereoisomers and of CHEB were parallel since the 95 per cent confidence intervals for their slopes all overlapped.

The inhibition of K^+-stimulated [^{3}H]ACh release by CHEB represents an interesting finding when compared with the effects of barbiturates on the K^+-stimulated uptake of $^{45}Ca^{2+}$ into brain synaptosomes. Blaustein and Ector [20] showed that the hypnotic barbiturates pentobarbital and thiopental inhibit the K^+-stimulated uptake of $^{45}Ca^{2+}$ into rat brain synaptosomes. They also observed that (+)-5-(1,3 dimethylbutyl)-5-ethyl barbituric acid (DMBB), a convulsant barbiturate, significantly inhibits this same process. The results of the present study are consistent with the hypothesis of these investigators that barbiturates inhibit the depolarization-induced uptake of Ca^{2+} into presynaptic nerve terminals, but the observation that convulsant barbiturates act as hypnotic barbiturates in both *in vitro* systems raises the question of the usefulness of these *in vitro* systems as valid models for barbiturate hypnotic action. A further test of the inhibition of the K^+-stimulated $^{45}Ca^{2+}$ uptake model of barbiturate action would be to determine whether or not the stereospecificity of this *in vitro* action is the same as it is *in vivo* for PB and SB.

In summary, the K^+-stimulated [^{3}H]ACh release brain slice system does not appear to be an appropriate *in vitro* model in which to study the hypnotic action of barbiturates. The K^+-stimulated release of [^{3}H]ACh *in vitro* was inhibited to a greater extent by the stereoisomers of PB and SB which were less active in causing loss of righting reflex *in vivo*, and was also inhibited by the convulsant barbiturate CHEB.

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