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Effects of anticonvulsants *in vivo* on high affinity choline uptake *in vitro* in mouse hippocampal synaptosomes

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1 The effects of several anticonvulsant drugs on sodium-dependent high affinity choline uptake (HACU) in mouse hippocampal synaptosomes was investigated. HACU was measured *in vitro* after *in vivo* administration of the drug to mice.

2 HACU was inhibited by drugs which have in common the ability to facilitate γ-aminobutyric acid (GABA) transmission, pentobarbitone, phenobarbitone, barbitone, diazepam, chloridiazepoxide, and valproic acid.

3 Dose-response relationships were determined for these drugs and the drugs' potencies at inhibiting HACU correlated well with their anticonvulsant potencies.

4 Clonazepam, ethosuximide, carbamazepine, and barbituric acid had no effect on HACU in the doses used while phenytoin and trimethadione stimulated HACU.

5 These results suggest that certain anticonvulsants may elicit a part of their anticonvulsant activity by modulating cholinergic neurones. This effect may be mediated through a GABA mechanism.

Introduction

The central cholinergic system may play a role in the expression of convulsions and the anticonvulsant effects of drugs (Maynert *et al.*, 1975). Decreased brain acetylcholine (ACh) levels have long been known to accompany convulsions (Richter & Crossland, 1949; Crossland, 1953; Beani *et al.*, 1969). The decrease in ACh levels has since been shown to be related to an increase in ACh release (Celesia & Jasper, 1966; Szerb *et al.*, 1970). By itself, ACh applied locally in the hippocampus can reduce the number of stimuli to the fornix necessary to obtain hippocampal seizure discharge (Nasello & Marichich, 1973). Also, centrally applied prostigmine, an acetylcholinesterase inhibitor, can elicit epileptiform discharge which is blocked by atropine (Celesia & Jasper, 1966). Conversely, cholinergic neurones are inhibited by several anticonvulsant drugs. For example, inhibition of ACh release *in vivo* occurs with diazepam (Bianchi *et al.*, 1975) and pentobarbitone (Beani *et al.*, 1968; 1969; Pepeu & Bartolini, 1968). Also pentobarbitone reduces ACh turnover in the cortex *in vivo* (Trabuchi *et al.*, 1975).

While pentobarbitone and phenobarbitone inhibit K^+ stimulated ACh release from hippocampal slices *in vitro* (Waller & Richter, 1980; Richter & Werling, 1979; Richter & Jackson, 1980), this *in vitro* effect of the drugs may not be related to their *in vivo* actions (Holtman & Richter, 1981; see review by Richter & Holtman, 1982). Another more appropriate test of these drug effects is to administer the drug *in vivo* and then subsequently measure the high affinity choline uptake (HACU) *in vitro*, as an index of cholinergic nerve activity as it was *in vivo* at the time of death. This approach has been employed in examining the inhibition of HACU by pentobarbitone (Simon *et al.*, 1976; Richter *et al.*, 1982) and the stimulation of HACU by some convulsant drugs (Richter *et al.*, 1982).

The sodium-dependent HACU associated with cholinergic nerve terminals (Haga & Noda, 1973; Kuhar, 1973 Yamamura & Snyder, 1973) has been proposed to be a rate limiting and regulatory step in the synthesis of acetylcholine (Simon *et al.*, 1976). However it has also been argued that HACU is not directly coupled to acethylcholine synthesis (Kessler & Marchbanks, 1979) or release (Murrin *et al.*, 1977). By increasing the activity of cholinergic neurones *in vivo* by electrical stimulation (Simon *et*

al., 1976) one can elevate HACU. Similarly, intraseptal administration of bicuculline reverses the i.p. pentobarbitone-induced decrease in ACh turnover in the hippocampus (Brunello & Cheney, 1981). Pentobarbitone injected directly into the hippocampus *in vivo* has no effect on *in vitro* HACU in hippocampal synaptosomes (Richter & Gormley, 1982). Also, there is no inhibition of HACU in hippocampal synaptosomes incubated with pentobarbitone (Simon *et al.*, 1976). Thus, it appears that the effect of pentobarbitone on HACU is indirect. In the present study we have examined the effects of several anticonvulsants *in vivo* on the high affinity uptake of choline into hippocampal synaptosomes *in vitro* to determine whether or not the inhibition initially observed with pentobarbitone is unique to this class of anticonvulsants.

Methods

In these experiments mice were injected intraperitoneally with the drugs. Chlordiazepoxide, diazepam, clonazepam and trimethadione were dissolved in a 30% solution of polyethylene glycol in water. Barbituric acid was dissolved in water and the pH was adjusted to 7–8 with 1 N NaOH. All other drugs were dissolved in saline. All drug concentrations were adjusted so the final volume for injection was 0.25 ml $20\,g^{-1}$. Control animals were treated with the appropriate vehicle, either saline or polyethylene glycol. By itself, polyethylene glycol had no effect on HACU compared with saline controls. The animals were decapitated at the time after injection of the peak anticonvulsant effect, as described by Raines *et al.*, (1979) for barbitone, or Krall *et al.*, (1978) for all other drugs (see Tables 1 and 2 for times). The hippocampus was removed and HACU was measured in a synaptosomal preparation of this tissue by the method of Simon *et al.* (1976). Briefly, the whole hippocampus was weighed and homogenized in 20 volumes of 0.32 M sucrose. Synaptosomes were prepared by centrifugation and resuspended in 30 volumes of 0.32 M sucrose. Choline uptake was measured in 50 μl (approximately 50 μg of protein) in a final volume of 0.5 ml by incubation at 30 °C for 4 min. [^{3}H]-choline (80 Ci $mmol^{-1}$, 1 μCi μl^{-1}, New England Nuclear) was diluted 1:50 with 25 μM choline chloride (Sigma Chemical Co.) and this [^{3}H]-choline solution was used in the incubation medium at a final concentration of 0.5 μM, 0.25 μCi 0.5 ml^{-1}). The sodium-dependent portion of choline uptake was determined by subtracting the uptake of [^{3}H]-choline in the absence of sodium from the total uptake in the presence of sodium. HACU refers to this sodium-dependent portion of the uptake, HACU was linear with time and protein concentration in the ranges used. Protein was determined by the method of Lowry *et al.* (1951). Choline levels were measured by a radioenzymatic procedure (Goldberg & McCaman, 1973).

The dose-response effects of the drugs were analysed with a computerized nonlinear least-square curve fitting technique using a multivariate secant method of false position (DUD method; Ralston & Jennrich, 1979). Because the data for only half the drugs conformed to the conventional rectangular hyperbola (by the method of Goldstein, 1964) data for all drugs were fitted to a modified hyperbolic (logistic) equation proposed by Parker & Waud (1971) for allosteric receptors:

$$\%\ \text{Inh of HACU} = \frac{(\text{maximum-inhibition}) \times (\text{dose})^p}{(\text{dose})^p + (\text{ED}_{50})^p}$$

where dose and ED_{50} are in mg kg^{-1}. ED_{50} is the dose of the drug which gives 50% of the drug's maximal inhibition and p is the slope factor. Further analyses involved correlation of the ED_{50} for inhibition of HACU with the anticonvulsant potency described in the literature (Krall *et al.*, 1978; Raines *et al.*, 1979) by a least-squares linear regression. Analyses of the stimulation of HACU was by a one way analysis of variance followed by a Dunnett's t test for comparison with the control.

The mice were tested for loss or righting reflex (LRR) immediately before decapitation. The animal was considered to have lost its righting reflex if it could not right itself after being placed on its back in three tests over one minute. It should be noted that while none of the mice met this test at the time of decapitation after even high doses of diazepam, chloridiazepoxide and clonazepam, they were not always able to right themelves at earlier times after injection.

Sodium phenobarbitone and sodium barbitone were purchased from the J.T. Baker Chemical Co., Phillipsburg, N.J. Sodium pentobarbitone and phenytoin were purchased from Sigma Chemical Co., St. Louis, MO. Chloridiazepoxide and diazepam were purchased from Hoffmann-LaRoche, Nutley, N.J. Valproic acid was purchased from Saber Laboratories, Morton Grove, IL. Trimethadione was kindly given to us by Dr E.L. Worech of Abbot Laboratories, North Chicago, IL. Clonazepam was a gift from Dr W.E. Scott of Roche Laboratories, Nutley N.J. Ethosuximide was a gift from Dr M.L. Black or Warner-Lambert Co., Ann Arbor, MI. Carbamazepine was kindly provided for us by James Pensalue of CIBA-GEIGY, Summit, N.J. Male ICR-Swiss mice, 25 to 35 g, were obtained from Harlan Industries, Indianapolis, Indiana.

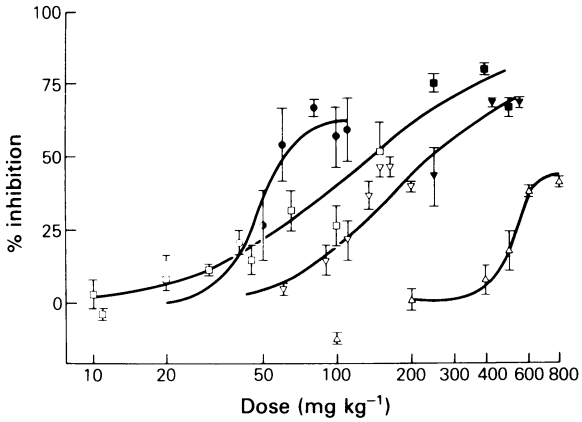

Figure 1 Effects of barbiturates and valproic acid on high affinity choline uptake (HACU) in mouse hippocampal synaptosomes. Computer fitted curves are given for pentobarbitone (○, ●), phenobarbitone (□, ■), barbitone (▽, ▼) and valproic acid (△). Each point represents the actual data (means, with vertical lines giving s.e.mean, $n = 3$ or more animals). Open symbols indicate that the animals were awake at the time of death. Closed symbols indicate that the animals had lost their righting reflex (LRR) at time of death.

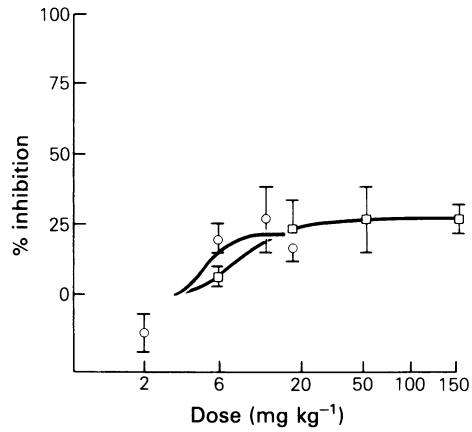

Figure 2 Effect of two benzodiazepines on high affinity choline uptake (HACU) in mouse hippocampal synaptosomes. Computer fitted curves are given for diazepam (○) and chlordiazepoxide (□). Each point represents the actual data for the mean of four animals. Vertical lines show the s.e. mean.

Results

The barbiturate derivatives (phenobarbitone, barbitone and pentobarbitone) all inhibited HACU (Figure 1). Phenobarbitone and barbitone did not cause a loss of righting reflex at doses up to and including the ED_{50} for inhibition of HACU while the ED_{50} for pentobarbitone against HACU approaches the dose at which some of the animals (2 or 5) lost their righting reflex. Since the doses required for the hypnotic and anticonvulsant effects of phenobarbitone and barbitone are more widely separated than the doses of pentobarbitone, our results suggest that the inhibition of HACU may be more closely associated with the anticonvulsant effect of these drugs

Table 1 Computer derived estimates for dose-response curve parameters for the inhibition of high affinity choline uptake (HACU) in mouse hippocampal synaptosomes

Drug	*Time* (min)	*ED_{50}* (mg kg^{-1})	*Maximum % inhibition*	*Slope factor*
PB	(15)	48.2 (40.0–56.3)	62.7 (48.2–77.2)	5.75 (−0.72–12.2)
PheB	(60)	113.2 (60.6–166.2)	85.7 (66.4–105.1)	1.48 (0.94–2.02)
Barb	(60)	158.0 (98.5–218.2)	71.7 (49.1–94.2)	2.15 (0.74–3.56)
VPA	(15)	507.0 (441.5–572.6)	44.4 (30.2–58.6)	9.00 (0.11–17.9)
CDZ	(15)	9.4 (−4.1–22.8)	27.0 (13.3–40.7)	2.57 (−4.04–9.11)
DZP	(15)	4.93 (−11.2–11.2)	21.2 (9.7–31.2)	14.0 (−5.50–55.7)

Estimates of the ED_{50} for inhibition of HACU, maximum % inhibition of HACU, and the slope factor (p) are given as the mean with the 95% confidence intervals in parentheses for the anticonvulsants shown in Figures 1 and 2. The drugs were given intraperitoneally and the animals were kelled at peak anticonvulsant activity as described by Raines *et al.* (1979) for barbitone (Barb) and by Krall *et al.* (1978) for chloridiazepoxide (CDZ), diazepam (DZP), pentobarbitone (Pb), phenobarbitone (PheB) and valproic acid (VPA).

than with the hypnotic effect. To rule out a non-specific effect of this class of drugs, barbituric acid, which lacks sedative/hypnotic and anticonvulsant actions, was tested and found to be ineffective on HACU at doses from 50 mg kg^{-1} up to 400 mg kg^{-1} at which point solubility limited our ability to increase the dose. The potency of the barbiturates to inhibit HACU (Table 1) follows the same order (pentobarbitone < phenobarbitone < barbitone) as the anticonvulsant effects (Raines *et al.*, 1979). The

Table 2 Effects of several drugs which did not inhibit high affinity choline uptake (HACU)

Drug	*Time* (min)	*Dose* (mg kg^{-1})	*Control HACU* (pmol mg^{-1} P4 minZW^1)	*HACU (% control)*
PHT	120	0	20.1 ± 1.0 (8)	
		10		98.2 ± 4.6 (3)
		20		94.8 ± 5.2 (4)
		40		99.0 ± 7.3 (4)
		50		119.6 ± 5.6 (3)
		80		120.2 ± 10.9 (4)
		100		123.1 ± 9.1 (3)
		200		148.4 ± 7.0* (3)
		400		110.5 ± 9.4 (3)
ESI	30	0	19.7 ± 1.8 (6)	
		100		120.1 ± 8.0 (3)
		150		115.4 ± 11.2 (3)
		200		124.6 ± 11.0 (3)
		400		125.9 ± 9.8 (3)
CBZ	15	0	18.7 ± 1.2 (8)	
		5		112.3 ± 15.4 (4)
		10		102.9 ± 4.4 (4)
		20		105.7 ± 12.1 (4)
		40		105.4 ± 9.9 (4)
TMD	60	0	17.5 ± 0.7 (6)	
		100		142.9 ± 8.3* (3)
		300		124.1 ± 12.5 (3)
		600		110.3 ± 11.8 (3)
		900		91.0 ± 4.8 (3)
BA	60	0	23.0 ± 1.1 (6)	
		50		97.9 ± 8.2 (3)
		100		113.9 ± 6.5 (3)
		200		90.3 ± 6.6 (3)
		400		92.1 ± 2.8 (3)
CLZ	30	0	18.1 ± 1.0(14)	
		.01		76.7 ± 12.5 (4)
		.1		97.2 ± 8.4 (4)
		1		85.3 ± 20.1 (4)
		10		100.3 ± 10.1 (4)
		20		111.6 ± 9.8 (3)
		40		96.9 ± 5.9 (3)
		80		101.3 ± 13.6 (3)
		160		101.4 ± 19.4 (3)

The HACU for the vehicle control is given as the mean ± s.e. mean of n (number in parentheses) animals. The effect of the drugs on HACU is given as % of the control value ± s.e. mean. The drugs were given intraperitoneally and the animals were killed at the time of peak anticonvulsant activity, as described by Krall *et al.* (1978), for phenytoin (PHT), ethosuximide (ESI), carbamazepine (CBZ) trimethadione (TMD) and clonazepam (CLZ). Barbituric acid (BA) treated animals were killed at the same time post injection as phenobarbitone and barbitone. $P < 0.05$ (analysis of variance and Dunnett's t test).

dose-response curves for phenobarbitone and barbitone are parallel to one another (Figure 1). While the curves for pentobarbitone and valproic acid appear to be steeper, the confidence limits for the slope factor for pentobarbitone and valproic acid overlap those of phenobarbitone and barbitone, so this trend is not statistically significant (Table 1).

The benzodiazepines diazepam and chloriazepoxide inhibited HACU but the maximal effect was lower than that found with the barbiturates (Figure 2 and Table 1). Within the benzodiazepine class the order of potency to inhibit HACU (diazepam > chlordiazepoxide) (Table 1) follows that of their anticonvulsant potency (Krall *et al.*, 1978). Clonazepam had no significant effect on HACU over a wide range of doses (Table 2). According to Krall *et al.* (1978) clonazepam is ten thousand times more potent as an anticonvulsant against metrazole than against maximal electroshock. This indicates that inhibition of HACU is better at predicting drug action in the maximal electroshock model than in the metrazole model.

Although differences in efficacy complicate comparisons in potency it is apparent that diazepam and chloridiazepoxide are more potent than the barbiturates or valproid acid. The overall relationship of potencies for inhibiting HACU is diazepam > chlordiazepoxide > pentobarbitone > phenobarbitone > valproic acid and this follows the same order as their anticonvulsant potencies (Krall *et al.*, 1978; Raines *et al.*, 1979). For all these anticonvulsants their potency as inhibitors of HACU correlates well with their anticonvulsant potency against metrazole with a slope of 3.25 ± 0.72 (Figure 3a) ($r = 0.987$) or maximal electroshock with a slope of 1.61 ± 1.00 (Figure 3b) ($r = 0.913$).

Of the other anticonvulsant drugs tested none had any inhibitory effect on HACU (Table 2). In fact phenytoin and ethosuximide had a tendency to stimulate HACU. Phenytoin at high doses caused convulsions (see Gruber *et al.*, 1940). The greatest stimulation of HACU by phenytoin occured at the lowest dose that caused convulsions (200 mg kg^{-1}). The loss of the stimulatory effect on HACU at the highest dose (400 mg kg^{-1}) may have been caused by anoxia which probably accompanied the severe convulsions at this dose.

Choline levels were measured in the incubation media and synaptosomal pellet of samples incubated similarly to those used to measure choline uptake. Choline levels in samples from phenobarbitone, valproic acid, diazepam, phenytoin or control treated animals were not significantly different. Thus, it is unlikely that the increases or decreases in HACU described here are the result of changes in endogenous choline levels having an effect on the specific activity of [^{3}H]-choline.

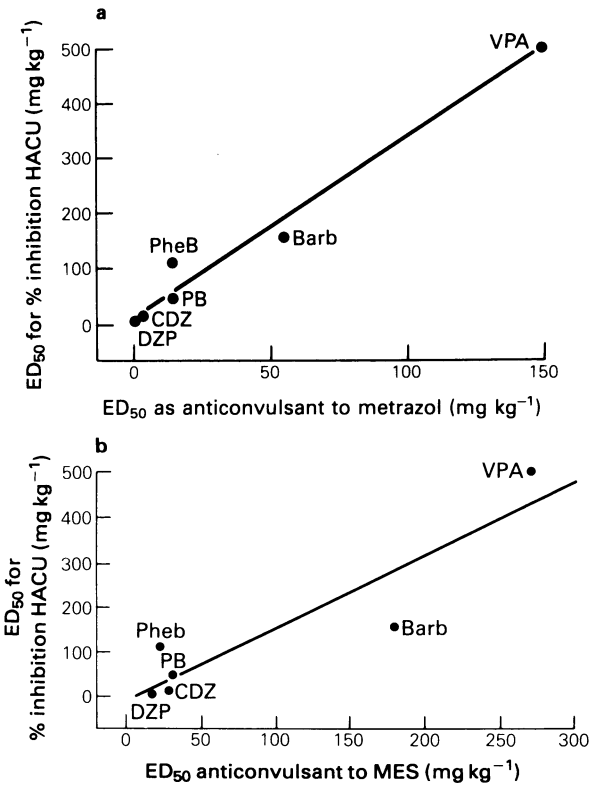

Figure 3 Correlation of anticonvulsant potency with ED_{50} for inhibition of high affinity choline uptake (HACU). The anticonvulsant potency (as ED_{50} in mg kg^{-1}) for barbitone (Barb) was from Raines *et al.* (1979) and for diazepam (DZP), chlordiazepoxide (CDZ), pentobarbitone (Pb), phenobarbitone (PheB), barbitone (Barb) and valproic acid (VPA) were from Krall *et al.* (1978). Inhibition of HACU as the ED_{50} in mg kg^{-1} are the data given in Table 1. (a) Least-squares linear regression of the metrazole anticonvulsant potency versus the ED_{50} for inhibiting HACU. The linear regression line has a slope of 3.25 and $r = 0.987$. (b) Least-squares linear regression of the maximal electroshock (MES) anticonvulsant potency versus the ED_{50} for inhibiting HACU. The linear regression line has a slope of 1.61 and $r = 0.913$.

Discussion

In vitro HACU is an indicator of cholinergic activity *in vivo* (Simon *et al.*, 1976, Richter *et al.*, 1982). The barbiturates (phenobarbitone barbitone and pentobarbitone), two of the benzodiazepines (diazepam and chlordiazepoxide) and valproic acid all inhibit sodium-dependent HACU uptake in hippocampal synaptosomes. Czuczwar *et al.* (1982) have suggested that phenobarbitone and diazepam need an intact

hippocampus for the full development of their anticonvulsant activity. Phenytoin and carbamazepine, which did not inhibit HACU in our study, were shown by Czuczwar *et al.* (1982) to exert anticonvulsant effects despite kainic acid lesions of the hippocampus. The inhibitory effects of the barbiturates, benzodiazepines and valproic acid on the cholinergic neurones in the hippocampus may therefore be integrally related to their anticonvulsant actions.

Willow & Catterall (1982) presented a classification of anticonvulsants divided into three groups. The first class, effective against partial seizures and tonic-clonic (grand mal) seizures, is composed of phenytoin and carbamazepine. Both drugs failed to inhibit HACU in our studies. In fact phenytoin stimulated HACU at a dose which caused convulsions. The second group, containing phenobarbitone, the benzodiazepines and valproic acid, are broad spectrum anticonvulsants and all were able to inhibit HACU. The drugs in this second group share a principal mechanism of action to enhance inhibitory transmission using the neurotransmitter γ-aminobutyric acid (GABA; Olsen, 1982) and it is possible that the inhibition of HACU occurs indirectly via this mechanism (Richter & Gormley 1982). The lack of an effect of clonazepam on HACU may be the result of the generally low efficacy of the benzodiazepines on the GABA-receptor complex; in the case of clonazepam the efficacy may be so low as to be indistinguishable from the vehicle control. Valproic acid also inhibited HACU (Figure 1) and its dose-response curve appears to be parallel to that of pentobarbitone. Although valproic acid has other effects, it has been suggested that its anticonvulsant effect is related to its ability to raise brain GABA levels by inhibiting the neurotransmitter's degradation by GABA-transaminase (EC 2.6.1.19; Metcalf, 1979). Thus valproic acid fits in with the second group of Willow & Catterall (1982). However, its efficacy as an anticonvulsant distinguishes it from the other drugs in this group. The third group, containing ethosuximide and trimethadione, are effective against petit mal seizures and have no known effect on GABA receptors. Neither of these two drugs inhibited HACU, and trimethadione actually stimulated HACU at a dose of 100 mg kg^{-1}.

The difference between the efficacy and potency of the benzodiazepines, diazepam and chlordiazepoxide, and that of the barbiturates correlates with their effects on the GABA-receptor complex (Simmonds, 1983). In enhancing GABA binding to $GABA_A$-type receptors the benzodiazepines are potent but with only a modest effect while the barbiturates are moderately potent with a marked effect; we found similar differences between the barbiturates and active benzodiazepines on HACU. Even though the slope factors (p) were not different among any of the drugs, the model of Parker & Waud (1971) gave tighter confidence intervals in all cases for the ED_{50} and maximum % inhibition than did the standard model. Thus, the more complex model is apropriately used and in the case where the slope factor (p) = 1, this model is equivalent to the standard hyperbola for drug-response relations. In all cases the fit of the data to the Parker & Waud model was as good or better than the standard model.

The correlation between anticonvulsant potencies and inhibition of HACU for some of these drugs is suggestive but cannot imply cause and effect. Further experiments are progressing in our laboratory to test more directly the involvement of cholinergic projections in convulsions. Also, the role of GABA in the inhibition of HACU needs to be tested. Further investigation of the effects of GABA-mimetics on HACU in the hippocampus is currently being pursued.

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