Pharmacology Biochemistry & Behavior, Vol. 19, pp. 951–955, 1983. © Ankho International Inc. Printed in the U.S.A.

Strain Differences in Susceptibility to the Convulsant Actions of 3-Carbomethoxy-β-Carboline[1]

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Received 17 May 1983

SCHWERI, M. M., S. M. PAUL AND P. SKOLNICK. *Strain differences in susceptibility to the convulsant actions of 3-carbomethoxy-β-carboline.* PHARMACOL BIOCHEM BEHAV **19**(6), 951–955, 1983.—NIH mice were found to be approximately three-fold more sensitive than NIH General Purpose mice to the convulsant actions of 3-carbomethoxy-β-carboline. The convulsant action of 3-carbomethoxy-β-carboline has been previously demonstrated to be mediated via an interaction with C.N.S. benzodiazepine receptors. The characteristics of the benzodiazepine receptor from the two strains appeared to be identical with respect to both binding affinity and capacity for [^{3}H]3-carbomethoxy-β-carboline and [^{3}H]diazepam, as well as the relative decreases in apparent receptor affinity for [^{3}H] 3-carbomethoxy-β-carboline in the presence of 10 μM γ-aminobutyric acid. Although the rate of degradation of 3-carbomethoxy-β-carboline in plasma was similar in the two strains, a marked difference in brain levels of the drug (or an active metabolite) was observed after *in vivo* administration. These results suggest that pharmacokinetic, rather than pharmacodynamic factors are primarily responsible for the observed strain differences in sensitivity to 3-carbomethoxy-β-carboline.

3-Carbomethoxy-β-carboline Benzodiazepine antagonists NIH mice NIH General Purpose mice Strain differences

THE ester derivatives of β-carboline-3-carboxylic acid bind to benzodiazepine receptors with high affinities [2,15] and antagonize many of the pharmacological actions of the benzodiazepines [5, 10, 17]. More recent studies have demonstrated that several β-carbolines not only antagonize the pharmacologic actions of the benzodiazepines, but under appropriate conditions display intrinsic pharmacologic actions that are opposite to those of the benzodiazepines [6, 7, 9]. For example, 3-carbomethoxy-β-carboline (β-CCM) and 6,7-dimethoxy-4-ethyl-3-carbomethoxy-β-carboline (DMCM) are convulsants [3,12], 3-carboethoxy-β-carboline elicits a behavioral syndrome reminiscent of human anxiety in primates [9], and 3-hydroxymethyl-β-carboline increases sleep latency and decreases total sleep time in rats [7]. The molecular mechanisms by which such "active antagonists" elicit their intrinsic pharmacologic effects are not known. However, pharmacologic evidence [3, 9, 12] suggests these actions are mediated through benzodiazepine receptors. Studies using [^{3}H]-β-carbolines have shown that under the appropriate conditions, the binding of these compounds to brain benzodiazepine receptors is inhibited by γ-aminobutyric acid (GABA) and the GABAmimetic, muscimol [1,3], and the extent to which GABA or muscimol inhibits the binding of these [^{3}H]-β-carbolines appears to be correlated with the relative potency of these compounds as convulsants [3]. During the course of studies in our laboratories demonstrating that certain benzodiazepine antagonists could block β-CCM induced convulsions in mice [12], we observed that the NIH General Purpose and NIH mouse strains displayed a markedly different susceptibility to the convulsant actions of β-CCM. Since an approximately threefold difference in susceptibility to β-CCM was observed (i.e., the percentage of mice having convulsions under optimum conditions), it appeared these strains might be appropriate models for investigating the molecular mechanisms by which β-carbolines such as β-CCM elicit seizures. We now report that pharmacokinetic factors, reflected as elevated brain concentrations of β-CCM (or an active metabolite) may be primarily responsible for the differences in susceptibility to seizures. Pharmacodynamic factors, including benzodiazepine receptor number and affinity, as well as the coupling of GABA and benzodiazepine receptors apparently do not contribute to this difference in susceptibility.

METHOD

Animals

Male NIH General Purpose (GP) and NIH mice (4–6 weeks old unless otherwise stated) were obtained from colonies maintained by the Veterinary Resources Branch, Na-

[1]Abbreviations used: β-CCM, 3-carbomethoxy-β-carboline; DMCM, 6,7-dimethoxy-4-ethyl-3-carbomethoxy-β-carboline; GABA, γ-aminobutyric acid; GP, NIH General Purpose; IP, intraperitoneal; PTZ, pentylenetetrozol.

tional Institutes of Health, Bethesda, MD. Mice were housed in our facilities for at least 48 hours prior to use.

Convulsant Activity of β-CCM and Pentylenetrazole (PTZ)

β-CCM was dissolved in 0.55 N HCL and diluted sevenfold with phosphate-buffered saline (pH 7.2). PTZ was dissolved in phosphate-buffered saline. Drugs were injected intraperitoneally (IP) in a volume of 0.1 ml. Mice were observed for fifteen minutes after injection for the presence or absence of tonic and clonic convulsions.

[^{3}H]-Diazepam and [^{3}H] β-CCM Binding to Brain Tissue

Mice were killed by cervical dislocation and forebrains (defined by an oblique cut on the dorsal surface posterior to the superior colliculus and ending at the mammillary bodies on the ventral surface) were removed. [^{3}H] Diazepam and [^{3}H] β-CCM binding was determined in whole homogenates prepared by disrupting the tissue in 20 volumes of 50 mM Tris-HCl buffer (pH 7.4) using a Brinkmann Polytron (Brinkmann Instruments, Westbury, NY), setting 7 (15 sec). Incubations were performed in a total volume of 1 ml consisting of: 0.2 ml tissue homogenate, 0.7 ml buffer, 0.075 ml radioligand, and 0.025 ml of water or diazepam (final concentration, 3 μM). The samples were incubated at 0–4° for 30–45 min with shaking, and the reaction terminated by rapid filtration through Whatman GF/B filters with 2 five ml washes of ice-cold buffer. In other experiments examining the effects of GABA on the binding of these radioligands to extensively washed membranes, the procedure described by Braestrup and Nielsen [1] was employed with the following modifications: (a) the tissue pellet was resuspended in 250 rather than 500 volumes of Tris citrate buffer (pH 7.1), and (b) the samples were incubated for 45 min.

Metabolism of β-CCM in Mouse Plasma

The degradation of β-CCM was determined in mouse plasma using a radioreceptor assay [14]. In brief, 10 μl of β-CCM (3.1 mM) was added to rat plasma (300 μl) to yield a final concentration of 100 μM. Plasma was maintained at 37°C for 1 min prior to addition of β-CCM. Three minutes later, 930 μl of ethanol was added to stop the reaction. The ability of an aliquot of this extract to inhibit [^{3}H]diazepam binding in rat cortical membranes was determined and compared with a standard curve constructed by adding known amounts of β-CCM to plasma maintained at 0–4°, and the concentration of "β-CCM equivalents" was calculated by probit analysis. Plasma containing no added β-CCM did not inhibit [^{3}H] diazepam binding. Since the identity of the compound(s) which displace [^{3}H] diazepam from brain membranes was not identified, the term "β-CCM equivalents" is used to denote this activity. However, recent studies from our laboratory [16] suggest that β-carboline esters such as β-CCE are metabolized to β-carboline-3-carboxylic acid, which binds to benzodiazepine receptors with relatively low affinity (K_i~20 μM) [4] and would not significantly contribute to the displacement of [^{3}H] diazepam under these conditions.

Concentration of β-CCM in Mouse Forebrain

Mice were injected with β-CCM (30 mg/kg). Ten minutes later, the mice were sacrificed by cervical dislocation and the forebrains removed. Tissue was homogenized in 15 ml of 50 mM Tris-HCl buffer (pH 7.4), placed on ice for 15 minutes, then centrifuged at 20,000 × g for 20 min (4°). The supernatant was decanted and saved, and the homogenization and centriguation repeated twice more. The resulting supernatants were combined, and the "β-CCM equivalents" determined by comparing the inhibition of [^{3}H] β-CCM binding in an unwashed homogenate from untreated mouse forebrain caused by an aliquot of the combined supernatants to that caused by known amounts of β-CCM added to these homogenates. Each assay sample contained: 0.2 ml of the combined supernatants, 0.2 ml tissue (containing about 10 mg forebrain), 0.9 ml buffer, 0.1 ml GABA (100 μM, final concentration), 0.0375 ml water, and 0.0625 ml [^{3}H] β-CCM (0.7 nM, final concentration). Each standard (as well as samples measuring total and nonspecific binding) contained 0.2 ml of the combined supernatants from the brains of untreated mice to correct for inhibition of [^{3}H] β-CCM binding not due to β-CCM (the specific binding of [^{3}H] β-CCM was inhibited approximately 10% by endogenous factors). Specific binding of [^{3}H] β-CCM and [^{3}H] diazepam was defined as the difference in binding observed in the presence and absence of 3 μM diazepam.

Materials

β-CCM was synthesized by Dr. M. Cain as previously described [4]. Diazepam was a gift of Hoffmann-LaRoche, Nutley, NJ. PTZ was purchased from K and K Laboratories, Plainview, NY. [^{3}H] Diazepam (Sp. Act. 76.8 Ci/mmol) and [^{3}H] β-CCM (Sp. Act. 83.4 Ci/mmol) were purchased from New England Nuclear, Boston, MA. Other chemicals and reagents were purchased from standard commercial sources.

RESULTS

Strain Differences in Sensitivity to the Convulsant Actions of β-CCM

Administration of β-CCM (30 mg/kg) to 4–6 week old male NIH mice elicited tonic and clonic convulsions in 81% of the animals. In contrast, only 28% of age-matched GP mice had convulsions (Table 1). However, with increasing age this difference in seizure susceptibility diminished so that by 8–10 weeks, 76% of GP mice exhibited convulsions following this dose of β-CCM. This value was not significantly different from the percentage (91%) of age-matched NIH mice which convulsed following β-CCM.

A parabolic dose response curve for the convulsant actions of β-CCM has previously been reported in NIH mice [3,12]. Since a parabolic dose response curve predicts that higher doses of β-CCM would elicit a lower percentage of mice convulsing, it was necessary to determine if the altered sensitivity of the GP strain to β-CCM would be observed over a wide dose range. Table 2 demonstrates that β-CCM is both less efficacious and less potent in the GP strain of mice.

The reduced sensitivity of the GP strain to the convulsant actions of β-CCM does not appear to be generalized lack of response to all chemical convulsants, since no differences in the susceptibility to PTZ-induced seizures were observed in the two strains (data not shown). When dose-response curves for the two strains using PTZ were compared, both groups of mice displayed very steep dose-response curves, with ED_{100} values of approximately 50 mg/kg (data not shown).

Characterization of [^{3}H] β-CCM and [^{3}H] Diazepam Binding in the NIH and GP Mouse Brain

The binding of benzodiazepine receptor ligands to NIH

TABLE 1

EFFECTS OF β-CCM IN NIH AND GP MICE

Age (weeks)	% Convulsions NIH	GP
4–6	81 ± 6 (8)	28 ± 4 (8)*
8–10	91 ± 4 (6)	76 ± 5 (7)

Mice were observed (15 min) for convulsions following IP injection of 30 mg/kg β-CCM. Values represent the Mean ± S.E.M. of the percent convulsing. Numbers in parentheses indicate the number of trials; each trial consisted of 5–10 mice per group.

*Significantly different from 4–6 week NIH mice. Student's t-test, $p<0.001$.

Significantly different from 4–6 week GP mice. Student's t-test, $p<0.001$.

TABLE 2

RESPONSE OF NIH AND GP STRAINS TO β-CCM

Dose β-CCM (mg/kg)	% Convulsions NIH	GP
5	10	0
10	70	50
20	70	40
30	80	40
50	20	0
60	50	20

At each dose, 10 mice from each strain were injected (IP) with β-CCM and observed (15 min) for convulsions. Mice were 4–5 weeks old. The percentage of NIH mice having convulsions is significantly greater than the percentage of GP mice having convulsions (paired t-test, $p<0.005$) when compared at doses of 5–60 mg/kg β-CCM.

TABLE 3

[^{3}H] β-CCM AND [^{3}H] DIAZEPAM BINDING TO GP AND NIH FOREBRAIN PREPARATIONS

Tissue Preparation	Ligand	[Ligand] (nM)	10 μM GABA	Mouse Strain NIH*	GP*
1) Unwashed homogenates	[^{3}H] β-CCM	4.5	−	321.4 ± 8.8 (4)	317.6 ± 15.5 (5)
2) Unwashed homogenates	[^{3}H] β-CCM	0.56	−	82.9 ± 5.6 (5)	82.9 ± 5.2 (5)
3) Unwashed homogenates	[^{3}H] Diazepam	20.1	−	1283.6 ± 23.4 (4)	1223.4 ± 54.6 (5)
4) Unwashed homogenates	[^{3}H] Diazepam	0.524	−	95.9 ± 2.3 (4)	99.0 ± 3.8 (5)
5) Washed membranes (five times)	[^{3}H] β-CCM	0.302	−	95.9 ± 3.1 (5)	97.5 ± 2.6 (5)
6) Washed membranes	[^{3}H] β-CCM	0.302	+	88.2 ± 4.3 (5)†	90.5 ± 2.4 (5)‡

*Specifically bound ligand, fmols/mg protein. Values represent the mean ± S.E.M. Numbers in parentheses denote number of samples. Each sample was obtained from a separate mouse; individual samples were assayed in triplicate. Each assay contained about 1 mg protein. Procedure described in the Method section.

†Significantly different from binding in five times washed membranes from NIH mice containing no GABA. Paired t-test, $p<0.005$.

‡Significantly different from binding in five times washed membranes from GP mice containing no GABA. Paired t-test, $p<0.001$.

and GP mouse brain was examined in unwashed homogenates using either [^{3}H] diazepam or [^{3}H] β-CCM as a radioligand, and in extensively washed membranes using [^{3}H] β-CCM in the presence of absence of 10 μM GABA. Saturating and subsaturating concentrations of both radioligands were used in forebrain homogenates to examine possible affinity and/or receptor number differences which could account for the strain difference in sensitivity to β-CCM. However, no significant differences between strains were observed in the binding of either radioligand to unwashed homogenates at any of the ligand concentrations used (Table 3). In extensively washed membranes, 10 μM GABA elicited a small but statistically significant inhibition of [^{3}H] β-CCM binding in both strains. However, the reduction in [^{3}H] β-CCM binding was not different between the two strains (Table 3).

Metabolism of β-CCM in Mouse Plasma

Rat plasma has previously been shown to rapidly metabolize the methyl and ethyl esters of β-carboline-3-carboxylic acid [7,16]. The rate of degradation of β-CCM was examined in plasma (*in vitro*) in both NIH and GP strains to determine if differences in the rate of degradation could play a significant role in the strain differences in response to β-CCM observed *in vivo*. Mice were administered β-CCM (30 mg/kg) and observed 15 min for convulsions. After a two day washout period, the degradation of β-CCM in plasma from convulsing mice was compared with the plasma from non-convulsing GP mice. Table 4 demonstrates that under these *in vitro* conditions, no significant differences are observed in the rate of degradation of β-CCM between the two strains.

Brain Concentrations of β-CCM in NIH and GP Mice

Brain concentrations of β-CCM equivalents were measured in mice 10 min after injection with 30 mg/kg of β-CCM (Table 5). The presence of absence of convulsions was noted in order to correlate the incidence of convulsions with brain

TABLE 4

METABOLISM OF β-CCM IN PLASMA

Experiment	% of Initial β-CCM Equivalents: NIH mice with convulsions	GP mice without convulsions
1	49.6 ± 3.7 (4)	48.5 ± 3.4 (3)
2	39.2 ± 1.2 (5)	40.9 ± 0.7 (3)

Values represent the mean ± S.E.M. Numbers in parentheses denote number of samples in each group. Each sample consisted of combined plasma from two mice. Plasma was prepared from trunk blood collected from mice whose convulsive potential had been established two days earlier. The plasma was incubated at 37°C in the presence of 100 μM β-CCM. β-CCM equivalents remaining in the plasma after 3 min were determined as described in the Method section and compared with the β-CCM equivalents initially present.

TABLE 5

FOREBRAIN CONCENTRATION OF β-CCM EQUIVALENTS

Strain	[β-CCM equivalents] μM: Convulsions	No Convulsions
GP	3.6 ± 0.8 (3)*	1.4 ± 0.4 (3)
NIH	3.4 ± 0.3 (3)†	1.2 ± 0.6 (2)

Average ±S.E.M. of the means obtained from separate experiments. In each experiment, 4–5 mice from each strain were used. Number in parentheses denotes number of experiments from which data was obtained. Mice were injected IP with 30 mg/kg β-CCM. After 10 min they were sacrificed and the concentration of β-CCM in their forebrains determined as described in the Method section.

*Significantly different from "GP, No Convulsions" group by paired t-test, $p<0.05$.

†Significantly different from "GP, No Convulsions" group by paired t-test, $p<0.005$.

concentrations of β-CCM. The concentration of β-CCM in the forebrains of convulsing NIH mice was 3.4±0.3 μM, while the concentration in the forebrains of non-convulsing GP mice was 1.4±0.4 μM. Moreover, the brain concentrations of β-CCM in the forebrains of GP mice which did convulse was not significantly different (3.6±0.8 μM) from those of NIH mice with convulsions, yet were significantly higher than in GP mice which did not convulse. Brain levels of β-CCM in non-convulsing NIH mice were not significantly different from GP mice which did not convulse.

DISCUSSION

These data demonstrate that two genetically related strains of mice display large differences in their sensitivity to the convulsant actions of β-CCM. The GP mouse (the strain from which the NIH mouse was derived) is far less sensitive to the convulsant actions of β-CCM than the NIH mouse. As reported previously for the NIH strain [12] a parabolic dose-response curve is also generated when β-CCM is given to GP mice; however, the maximum response is significantly less than is obtained with the NIH mice (Table 2).

The primary determinant of suceptibility to the convulsant actions of β-CCM appears to be a difference in brain levels of this compound or an active metabolite. NIH mice (which convulsed with a frequency 2.9-fold greater than the GP mice following optimum doses of β-CCM, Table 1) and 2.4-fold higher brain concentrations of β-CCM (or an active metabolite) than GP mice which did not convulse (Table 5). The importance of brain levels of β-CCM is further supported by the observation that forebrain concentrations of active drug forms in the GP mice which did have convulsions were not significantly different from those found in the convulsing NIH mice.

Since forebrain concentrations of β-CCM in both strains are well above the reported K_d values of this compound (~1 nM, [1] it might be argued that all the animals should exhibit convulsions if this is indeed a receptor-mediated event. The convulsant actions of β-CCM have been shown to be potently antagonized not only by diazepam, but by the specific benzodiazepine antagonists Ro 15-1788 and CGS 8216 [12] strongly implicating a benzodiazepine receptor in the convulsant actions of β-CCM. Receptor occupation, rather than whole brain levels of a compound, may be the critical factor for the pharmacological action of both benzodiazepines [11] and β-carbolines [3]. Furthermore, at 37°, the K_d of both benzodiazepine agonists and antagonists is elevated by as much as one order of magnitude [8]. Since the brain concentrations reported are essentially an average of the concentrations found in discrete compartments, they may only be proportional to the actual concentration of β-CCM or an active metabolite near the benzodiazepine receptor. The absolute values, therefore, may not represent a true concentration, but rather reflect a relative concentration at the receptor. The data presented here (Table 5) suggest that animals which convulse have significantly higher levels of β-CCM at the benzodiazepine receptor than those that do not.

The difference in sensitivity to the convulsant actions of β-CCM is present only within a narrow time frame such that, at 8–10 weeks of age, both the NIH and GP mice are equally sensitive to the convulsant actions of β-CCM (Table 1). This age dependance may be due to differences in the rate of development of enzymes which degrade β-CCM or of systems which control the distribution of the drug in the CNS.

The metabolism of β-CCM by plasma is one pharmacokinetic factor which apparently does not contribute to the difference in brain levels between the two strains (Table 4). Recent work from this laboratory [14,16] suggests that the rates of metabolism of the β-carboline-3-carboxylic acid esters by plasma *in vitro* may provide a useful index of the rates of their metabolism *in vivo*, even though the liver likely plays an important role in the degradation of these compounds in the intact animal. These findings demonstrated strong correlations between many of the pharmacologic differences among β-carboline-3-carboxylic acid esters and their rate of degradation by plasma [16], and accounted for the differences in species responses to these compounds as well [13]. Therefore, the rate of plasma metabolism of β-CCM was compared in the two strains. Although the rate of degradation of β-CCM was not different between strains, the $t_{1/2}$ obtained in these studies (~3 min) was comparable to values previously obtained in Sprague Dawley rats [16].

The pharmacokinetic nature of this strain difference is supported by the observation that PTZ is equally effective in eliciting convulsions in both strains. The observed difference in response to β-CCM, therefore, is not due to a generalized lowering of the seizure threshold in NIH mice.

No evidence for a pharmacodynamic difference between the strains at the level of the benzodiazepine receptor was observed. Several lines of evidence [3,12] suggest that β-CCM convulsions are mediated via benzodiazepine receptors. Comparison of the binding of both [^{3}H] β-CCM and [^{3}H] diazepam to brain benzodiazepine receptors at saturating and subsaturating conditions, however, revealed no significant difference in ligand-receptor interactions between the two strains (Table 3). In recent reports [12], GABA has been demonstrated to selectively elicit a slight but significant reduction in the binding of convulsant β-carbolines (e.g., β-CCM and the closely related 6, 7-dimethoxy-4-ethyl derivative, DMCM) to benzodiazepine receptors *in vitro*. Although 10 μM GABA did elicit a slight but statistically significant decrease in the binding of [^{3}H] β-CCM to forebrain membranes (Table 3), these reductions were identical in both strains.

Our data demonstrate significant strain differences in response to the convulsant actions of β-CCM in mice. Furthermore, the differences in sensitivity appear to be related to pharmacokinetic, rather than pharmacodynamic factors, reflected in significant differences in the amounts of β-CCM (or an active metabolite) in brain at the time of seizures. Whether these differences in sensitivity relate to differences in metabolism or transport of β-CCM into the CNS of these mice is currently under investigation.

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