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Proconvulsant and 'Anxiogenic' Effects of n-Butyl β Carboline-3-Carboxylate, an Endogenous Benzodiazepine Binding Inhibitor From Brain

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NOVAS, M. L., C. WOLFMAN, J. H. MEDINA AND E. DE ROBERTIS. *Proconvulsant and 'anxiogenic' effects of n-butyl β carboline-3-carboxylate, an endogenous benzodiazepine binding inhibitor from brain.* PHARMACOL BIOCHEM BEHAV 30(2) 331–336, 1988.—The discovery of n-butyl β carboline-3-carboxylate (βCCB) as an endogenous substance of brain capable of interacting with the central benzodiazepine receptor [17], and the fact that this β carboline increases in the cerebral cortex of rats undergoing acute stress [12], led us to study the pharmacological properties of βCCB in mice. Using 3-mercaptopropionic acid in subconvulsant doses, it was found that this β carboline, although not being a convulsant, has a proconvulsant action, as indicated by the number of mice undergoing convulsions and the reduction in latency. This proconvulsant effect was observed both with IP or ICV injections and was blocked by the benzodiazepine receptor antagonist RO 15-1788. In an open-field test the injection of 0.3 mg/kg of diazepam increased the number of squares crossed, while βCCB had the opposite effect, reducing the squares crossed in a dose dependent manner between 1 and 30 mg/kg. This drug also increased the time of freezing and decreased the number of rearings. These changes were partially counteracted by the injection of 3.6 mg/kg of RO 15-1788. In the plus-maze test, 10 mg/kg chlordiazepoxide increased the number of entries and the time spent in the open arms, while the β carboline produced the opposite effect. The conclusion reached is that βCCB has both proconvulsant and anxiogenic actions, behaving as an inverse agonist for the central benzodiazepine receptor.

Benzodiazepine receptor Endogenous ligand Anxiogenic drugs Proconvulsant drug
N-Butyl β carboline-3-carboxylate

SINCE the discovery of specific binding sites for benzodiazepines [1,13], investigations to elucidate the mechanism of action of these drugs were greatly stimulated. An important line of research was the study of the possible existence of endogenous substances in brain which could interact physiologically with central benzodiazepine (BZD) receptors (see [6,7]). Most of these substances, which include purines, nicotinamide, peptides and proteins have low affinity for the BZD receptor. The best characterized peptide is that isolated by Guidotti *et al.* [5] called diazepam binding inhibitor (DBI), composed of 104 amino acids (Mr=11,000) and with a K_D in the μM range. A proteolytic DBI fragment of 18 amino acids called ODN retains some of the anxiogenic properties of the original peptide, but its affinity for the BZD receptor is even lower [4].

The ethyl β carboline-3-carboxylate (βCCE) isolated by Braestrup *et al.* [3] from urine and brain is the only compound with affinity in the nM range, similar to most used benzodiazepines. Unfortunately it was found that this substance was an artifact produced in the extraction procedure, which included prolonged heating in a highly acidic ethanol solution.

Using large amounts (18 kg) of bovine cerebral cortex and mild extraction with acetic acid-methanol, we were able to isolate and chemically identify n-butyl β carboline-3-carboxylate (βCCB), a substance capable of interacting specifically and with high affinity (K_D, 2–3 nM) with central BZD receptors [17]. The identification was made using mass spectrography, UV and fluorescence spectroscopy and HPLC. In this work several control experiments, which included avoiding use of any organic solvent during the extraction and purification steps were done to demonstrate that this β carboline is a genuine endogenous product, present in brain and not artificially formed during extraction. In an

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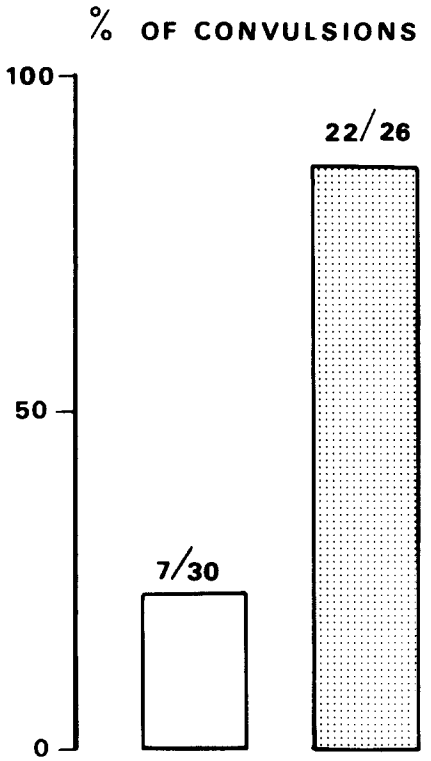

FIG. 1. Histograms showing the percentage change in the number of mice undergoing convulsions. Clear bar, mice injected only with the convulsant (35 mg/kg 3MPA IP); gray bar, mice injected with the convulsant plus 36 mg/kg βCCB IP (see description in the text).

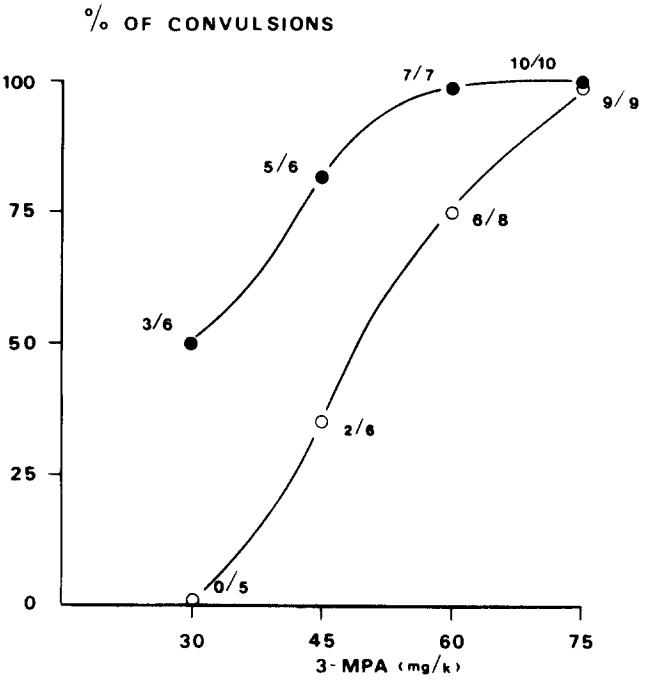

FIG. 2. Proconvulsant effect of βCCB after an intracerebroventricular injection of 40 μg per mouse. (○), mice injected only with the convulsant; (●), mice injected with the convulsant plus βCCB (see description in the text).

aqueous extraction, this endogenous βCCB increased 100% in the cerebral cortex of rats submitted to an acute swimming stress, a change that was counteracted by the prior injection of diazepam. This finding suggested that the level of this substance could be related to the state of 'anxiety' of the animal [12].

Different investigations about the action of substances interacting with the BZD receptor led to the concept of a three state model: drugs like diazepam, that are anxiolytic and anticonvulsants, are considered to be agonists; others, such as methyl β carboline-3-carboxylate (βCCM), with anxiogenic and convulsant effects, are inverse agonists, and a third group, such as RO 15-1788, are antagonists capable of blocking both agonist and inverse agonist actions (see [6]).

In this work we have studied some of the pharmacological effects of synthetic n-butyl β carboline-3-carboxylate in mice with the use of several tests to detect its action at the behavioral level. We reached the conclusion that this β carboline has proconvulsant and 'anxiogenic' effects, acting as an inverse agonist of the central BZD receptor.

METHOD

Proconvulsant Test

Adult mice of the A_2G strain and of either sex weighing between 22–26 g were used for the experiments of convulsion. As convulsant we used 3-mercaptopropionic acid (3MPA), which consistently produces convulsions in less than 6 min after an IP injection of 50 mg/kg or more. This drug is known to act on the GABA system, inhibiting glutamic acid decarboxylase [18]. Convulsions were positively registered when the mice had a complete episode involving clonic movements and tonic extension. The βCCB, suspended in saline with a few drops of Tween 80, was injected 5 min prior to the 3MPA, either IP or by intracerebroventricular (ICV) route. The latency, i.e., the time spent between the injection and the convulsions, was recorded in seconds.

Open-Field Test

We used an open-field test, which is considered as an indicator of the 'emotional state' in rats [9], to analyze the possible anxiogenic or anxiolytic effect of βCCB. We have also used the elevated plus-maze test of 'anxiety' that was applied to rats by Pellow *et al.* [15] and to mice by Lister [10]. This test is rapid, selective and equally capable of detecting anxiolytic and anxiogenic drug effects under identical conditions.

In the open-field experiments, two investigators recorded the visually exploratory activity of A_2G mice in a novel environment, using a mildly and uniformly illuminated green arena of 92×92 cm divided into 16 squares. The mice were brought to the low noise behavioral room, at least 1 hr before the experiment. After the injection of the βCCB or the vehicle, each mouse was put in a corner of the open field and observed for 15 min in three 5-min periods. During this time we recorded the number of squares crossed (SC), the time in seconds spent in total immobility (freezing), the number of groomings, rearings and boluses. Between tests the arena was carefully cleaned. The results obtained were compared with control mice injected with the vehicle and with mice injected IP with diazepam (0.3 mg/kg).

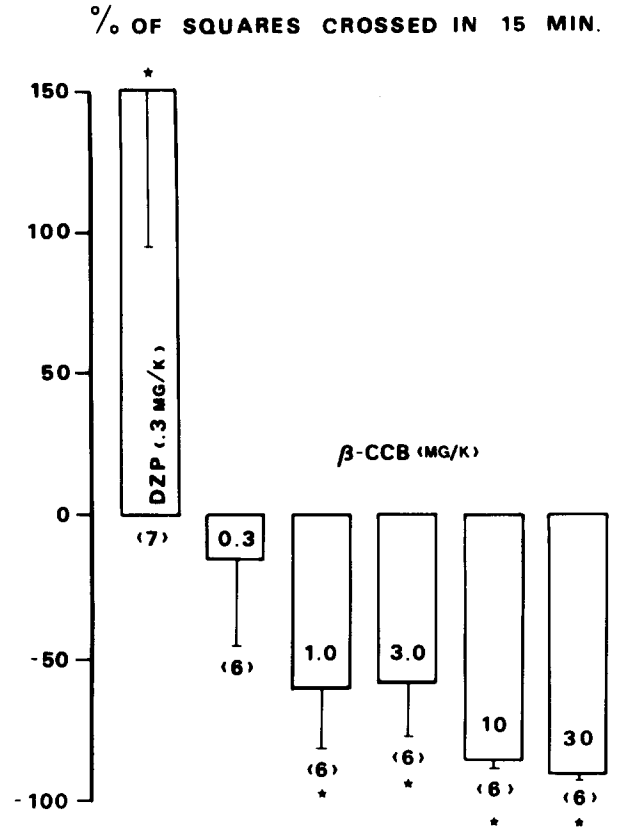

FIG. 3. Histograms showing the effect of diazepam (0.3 mg/kg IP) and of increasing doses of βCCB (0.3 to 30 mg/kg IP) on the number of squares crossed (SC) by A_2G mice in the open-field test during 15 min (see description of the experiment in the Method section). The base line (0%), equivalent to 116±10 SC, corresponds to mice injected only with the vehicle (*$p<0.002$, Student's t-test).

Elevated Plus-Maze Test

For the elevated plus-maze we used NIH mice of both sexes provided by the Malbran Institute of Argentina. The set-up consisted of two open arms of 25×5 cm, crossed with two closed arms of the same dimensions with walls 35 cm high. This maze is made of varnished wood to facilitate cleaning between each experiment. The maze was elevated 25 cm above the floor and was enclosed in a black cylinder of 0.80 cm diameter. Both the open and closed arms were uniformly illuminated with a rather dim light.

For each experiment, the mouse that stayed during an hour in the behavioral room in the original cage was weighed and injected IP with the vehicle, consisting of saline with a few drops of Tween 80, or with the drugs suspended by stirring and sonication. We studied the action of 3 and 10 mg/kg βCCB or of 10 mg/kg chlordiazepoxide, as a typical anxiolytic drug. After the injection, the mouse was kept isolated for five min and then was put in the open field for another five min. We confirmed the findings of Pellow and File [16] for rats, which, when were put in a novel environment before exposure to the plus-maze, had an increase in the overall activity in the last test. To start the experiment the mouse was placed in the center of the plus-maze and its behavior was observed by two investigators during five min (see the Results section).

Statistics

The statistical significance of the results obtained in the experiments of convulsion and the open-field test was calculated using the Student t-test. Analysis of variance (ANOVA) was applied to the results of the plus-maze, with drug treatment as a factor. Post hoc comparisons between individual treatment groups and control were performed using the Student t-test.

RESULTS

Proconvulsant Action of βCCB

The effect of βCCB on convulsions was manifested, both by the number of mice that suffered convulsions after a threshold-subthreshold dose of 3MPA (35 to 50 mg/kg), and by a change in latency. As shown in Fig. 1, these two parameters were affected by the injection of βCCB. Only 7 out of 30 mice showed convulsions in those injected with 35 mg/kg 3MPA IP. On the other hand, 22 out of 26 mice convulsed when they were injected IP with 36 mg/kg of βCCB, followed by 35 mg/kg of 3MPA IP. The effect was also very evident on the latency, which was reduced from 387±26 sec, in the 3MPA-injected mice, to 320±14 sec in those injected also with βCCB ($p<0.025$). Here it should be pointed out that βCCB has no convulsant effect per se even with doses of up to 100 mg/kg. In another series of experiments we observed that the proconvulsant effect of βCCB was completely blocked by 3.6 mg/kg IP of RO 15-1788 (n=8).

Figure 2 shows that the ICV injection of 40 μg per mice βCCB, which is not convulsive per se, produces a displacement of the dose-response curve for 3MP to the left. In these experiments the mice were injected IP with increasing doses of 3MPA between 30 and 75 mg/kg. The number of mice that convulsed at each dose of 3MPA with the injection of βCCB were compared with those receiving only the convulsant. For example, at 45 mg/kg 3MPA, 2 out of 6 mice convulsed, while 5 out of 6 did so when injected also with βCCB. The latency was also recorded and a certain decrease tendency was observed in the βCCB-injected mice. For example, at doses of 45 and 60 mg/kg 3MPA, the latency for convulsions was 444±48 sec for the control, against 333±28 sec for the βCCB-injected mice.

In some preliminary experiments it was shown that the proconvulsant effect of βCCB could be enhanced using the acetylcholinesterase inhibitor eserine (1 mg/kg) plus methylatropine (15 mg/kg IP), treatment that by itself did not produce convulsions. In this case, the proconvulsant effect of βCCB was already evident with one-third of the proconvulsant dose of βCCB (i.e., 10 mg/kg of βCCB instead of 30 mg/kg).

Effects of βCCB in the Open-Field Test

Figure 3 shows that diazepam (0.3 mg/kg IP), given 25 min earlier, increased significantly the number of squares crossed (292±65). In control mice, the number of SC was 116±10, a value that was taken as base line (0% in the figure). The injection of βCCB produced a significant reduction in SC, which is dose-dependent between 1 and 30 mg/kg ($p<0.002$).

In Fig. 4 the time spent in complete immobility (freezing) is recorded. There is a significant ($p<0.005$) increase in the time of immobility in the mice injected with 1 to 30 mg/kg βCCB, as compared to vehicle- or to diazepam-injected mice. At low doses of βCCB, both the changes in SC and

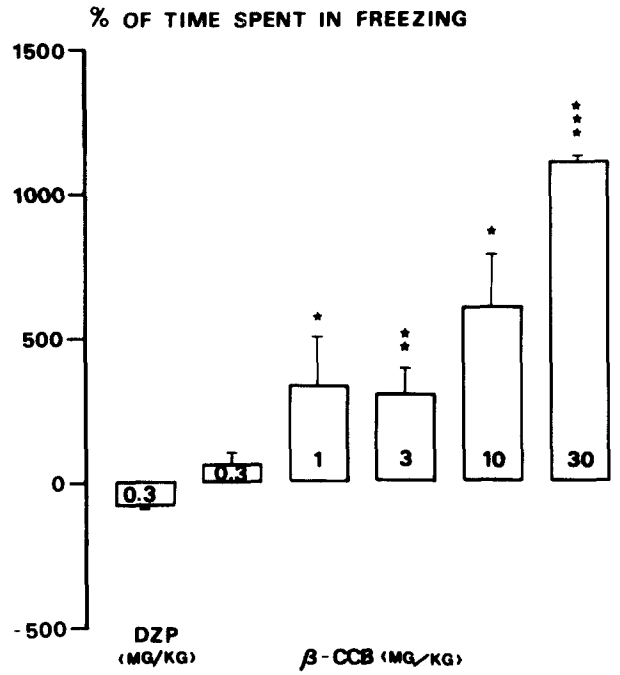

FIG. 4. Percent variation in the time (in sec) spent in immobility (freezing) by mice in the experiment described in Fig. 3. The base line (0%) corresponds to 41±31 sec. There is a significant increase in the time of freezing with doses of 1 to 30 mg/kg βCCB (*$p<0.0025$, **$p<0.025$, ***$p<0.001$, Student's t-test).

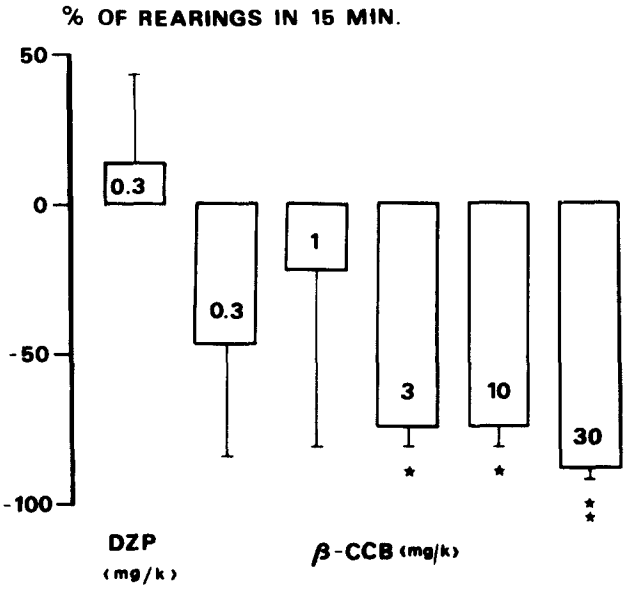

FIG. 5. Percent variation in the number of rearings by mice in the experiment described in Fig. 3. The base line (0%) corresponds to 13±2 rearings. There is a significant decrease in the number of rearings with doses of 3 to 30 mg/kg of βCCB (*$p<0.025$, **$p<0.005$, Student's t-test).

freezing do not depend on a decreased locomotion. When this was measured in an LKB-Animex apparatus, using 3 mg/kg βCCB IP, the locomotion was not impaired. Following the temporal course of the number of SC and of the time spent in freezing, both in control and treated mice, the frequency of SC tends to decrease, while the freezing increases during the test; both changes are considerably intensified by the βCCB.

As shown in Fig. 5, with 3 to 30 mg/kg βCCB, there is also a significant decrease in number of rearings ($p<0.002$) as compared to the vehicle- or the diazepam-injected mice. However, no changes in grooming or number of boluses between the groups were registered.

In experiments done in mice injected IP with 3.6 mg/kg RO 15-1788, we observed that the pharmacological effects of βCCB in the open field were partially counteracted by the benzodiazepine receptor antagonist. As shown in Table 1, the number of SC in the mice injected with 10 mg/kg βCCB, was reduced from 95.2±11 to 23.3±6 ($p<0.001$); while in those that received, in addition, a previous injection of 3.6 mg/kg of RO 15-1788, the number of SC increased to 79.1±7, approaching control values. The effect of βCCB on freezing was also partially reversed by RO 15-1788 (Table 1). These results suggest that the exploratory activity of mice, in a novel environment, decreases with the βCCB, a behavior opposite to that of a typical agonist for the central BZD receptor (i.e., diazepam) and that this effect is blocked by a benzodiazepine antagonist.

Effects of βCCB in the Plus-Maze Test

In the elevated plus-maze test we measured the total number of arm entries as an assessment of exploration and

TABLE 1

EFFECT OF βCCB AND RO 15-1788 ON NUMBER OF SQUARES CROSSED (SC) AND FREEZING (IN SEC) IN THE OPEN FIELD TEST

Treatment	n	SC	Freezing (sec)
Vehicle	15	95.2 ± 11	55.3 ± 28
βCCB	11	23.3 ± 7*	317.8 ± 56*
RO 15-1788 +βCCB	8	79.1 ± 7	203.0 ± 35†

(n) number of mice injected IP with vehicle or with 10 mg/kg βCCB or with 10 mg/kg βCCB plus 3.6 mg/kg RO 15-1788.

*$p<0.001$, βCCB versus vehicle; †$p<0.01$, RO + βCCB versus vehicle (Student t-test).

motor activity. We also recorded the number and percent of entries in the open and closed arms, as well as the time and percent of the time spent in both arms. It was considered an entry when the four feet of the mouse had entered either the open or closed arm, while the time spent at the cross was deducted from the total.

As shown in Table 2, the injection of 10 mg/kg of chlordiazepoxide increased the percent of entries in the open arms from 25.9±4.3 to 50.7±6.9 ($p<0.001$) and from 12.2±3.3 to 33±7.5 ($p<0.001$) the percent of time spent in the open arms. The percent of entries and time spent in closed arms was reduced by this benzodiazepine (data not shown). In the mice injected with 10 mg/kg βCCB there was a reduction in the percentage of time spent in the open arms, but the locomotion was also impaired. For this reason we took into consideration only those mice injected with 3 mg/kg βCCB, in which the locomotion was not significantly affected according to pilot experiments in the Animex. As shown in

TABLE 2

MEAN ± S.E.M. NUMBER OF ENTRIES OR TIME (IN SECONDS) SPENT IN THE OPEN OR THE CLOSED ARMS OF AN ELEVATED PLUS-MAZE, THE TOTAL NUMBER OF ARM ENTRIES, AND THE PERCENTAGE OF OPEN ARM ENTRIES OR OF TIME SPENT IN THE OPEN ARMS

	Number			Time		% Open	
Drug	Open	Closed	Total	Open	Closed	Number	Time
Vehicle (19)	4.2 ± 1	9.4 ± 0.6	13.0 ± 1.3	36.6 ± 10	170.3 ± 14.5	25.9 ± 4.3	12.2 ± 3.3
CZD (10)	9.2 ± 2.3	7.7 ± 1.1	16.4 ± 2.1	99.0 ± 22.7†	133.4 ± 19.7	50.7 ± 6.9‡	33.0 ± 7.5†
βCCB (14)	1.4 ± 0.4†	7.6 ± 0.8	9.0 ± 0.9†	16.3 ± 5	205.0 ± 16.1	13.3 ± 4*	5.4 ± 1.7

The number of mice, in parentheses, were injected IP with the vehicle or with 10 mg/kg chlorodiazepoxide (CZD) or 3 mg/kg βCCB, ten minutes before the test (see description in the text). *$p<0.05$, †$p<0.002$, ‡$p<0.001$, all in relation to control values (Student t-test, after ANOVA).

Table 2, in the elevated plus-maze, 3 mg/kg βCCB significantly reduced the open arm entries (from 4.2±1 to 1.4±0.4, $p<0.02$). There was also a reduction in the total number (from 13±1.3 to 9±0.9, $p<0.02$) and in the percent of entries (from 25.9±4.3 to 13.3±4, $p<0.05$) in the open arms. However, these parameters did not change for closed arms. The percentage of time spent in the open arms was reduced, however, it barely missed significance on the ANOVA.

DISCUSSION

The results presented here suggest that n-butyl β carboline-3-carboxylate, an endogenous substance present in brain which interacts with the central benzodiazepine receptor [17], has a proconvulsant action in mice, as well as a possible 'anxiogenic' effect. These actions were evidenced by the behavior in an exploratory open-field test (see [9]) and the elevated plus-maze test [15]. Both these properties are characteristic of an inverse agonist for the BZD receptor. This conclusion is supported by the blocking effect that the central BZD receptor antagonist RO 15-1788 has on the proconvulsant action of βCCB, as well as on the effect of this β carboline in the exploratory activity (Table 1). RO 15-1788 was effective at a dose of 3.6 mg/kg that has no action per se [16].

Opposite to other inverse agonists, such as methyl β carboline-3-carboxylate and methyl 6-7 dimethoxy 4-ethyl β carboline-3-carboxylate (DMCM), which can produce convulsions in mice, βCCB is not a convulsant, even at doses as high as 100 mg/kg. The proconvulsant effect is, however, manifested by the facilitation of seizures in mice injected with subconvulsant doses of 3MPA. The number of mice undergoing convulsions is increased by the injection of βCCB and the latency of the effect is significantly reduced (Fig. 1). These characteristics suggest a threshold reduction for the convulsant action of 3MPA. The action of this β carboline is even better manifested when the ICV route is used; in this case a net proconvulsant effect is produced with a dose of 40 μg of βCCB in the 3MPA-injected mouse (Fig. 2).

It is known that in rodents, esters of caboline can be rapidly hydrolyzed by blood cholinesterases [8]. We observed that when βCCB is injected 20 min before the 3MPA, the proconvulsant effect does not appear. This might be due to the hydrolysis of the butyl ester during this period.

When comparing the doses of βCCB needed to facilitate seizures with those producing an effect on the exploratory open-field test, it is evident that the last test is more sensitive. In fact, while 30 mg/kg IP are necessary to show a proconvulsant effect, doses of βCCB as low as 1–3 mg/kg suffice to reduce the number of squares crossed by the mice, to increase the time spent immobile, or to change the number of rearings (Figs. 1–3). These findings suggest that the occupancy of BZD receptors is probably smaller for the 'anxiogenic' than for the proconvulsant effect of βCCB. This problem is related to that of the in vivo binding of benzodiazepines and to the correlation between occupancy and efficacy of BZD receptor ligands as recently reported by Braestrup and Nielsen [2]. They observed that benzodiazepines needed higher receptor occupancies to show an anticonflict than an anticonvulsant effect. However, they point out that in humans the anxiolytic effects are obtained with lower doses of BZD than the antiepileptic effect.

The results obtained with the elevated plus-maze confirmed the findings of the open-field test. Mice injected with the vehicle had more entries and spent more time in the closed than in the open arms (Table 2). According to Montgomery [14] and Pellow *et al.* [15] exposure of rats to open elevated spaces produces significantly more anxiety-related behaviors than exposure to closed spaces. It has been hypothesized that the preference of rats and mice for the closed arms of the plus-maze reflects aversion caused by fear to height in open spaces. Here we confirm the previous findings of Pellow *et al.* [15], Pellow and File [16] in rats, and Lister [10] in mice, that anxiolytic drugs, such as chlordiazepoxide, increase the percent of entries and time spent in the open arms, suggesting a reduction in fear or 'anxiety.' On the other hand, a selective decrease in exploration of the open arms was observed by these authors with anxiogenic compounds such as picrotoxin, FG 7142, or caffeine. In our case βCCB, at the dose of 3 mg/kg, reduces the total number of entries, which depends exclusively on the reduction of entries in the open arms (Table 2). Analysis of covariance revealed that the reduction in open-arm entries is quite independent of the reduction in closed-arm entries. This behavior is that expected for a drug having 'anxiogenic' effects [15].

In conclusion, n-butyl β carboline-3-carboxylate, an en-

dogenous substance found in brain, although not a convulsant per se, has a net proconvulsant effect in mice. Both the results of the open-field and the elevated plus-maze tests suggest that βCCB is also 'anxiogenic.' These pharmacological properties characterize an inverse agonist of the BZD receptor.

ACKNOWLEDGEMENTS

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