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Anxiogenic Effects of Methyl-β-Carboline-3-Carboxylate in a Light/Dark Choice Situation

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BELZUNG, C., R. MISSLIN, E. VOGEL, R. H. DODD AND G. CHAPOUTHIER. *Anxiogenic effects of methyl-β-carboline-3-carboxylate in a light/dark choice situation.* PHARMACOL BIOCHEM BEHAV **28**(1) 29–33, 1987.—Doses of benzodiazepine, clorazepate, and also of the inverse agonist of the benzodiazepine receptor, β-CCM, which failed to present sedative or postictal depressive effects, were at first determined in a free exploratory situation. Then, the effects of clorazepate dosed at 1.0, 2.0 and 4.0 mg/kg and β-CCM dosed at 1.0 and 2.5 mg/kg were studied in the light/dark box choice procedure. Clorazepate tended to produce an increase of the time spent by mice in the lit box as well as of the number of transitions between the two boxes, whereas the dose of 1.0 mg/kg of β-CCM had opposite effects. The benzodiazepine antagonist RO 15-1788 completely counteracted the anxiolytic effects of clorazepate dosed at 2.0 mg/kg and the anxiogenic effects of β-CCM.

β-CCM RO 15-1788 Clorazepate Light/dark test Anxiolytic and anxiogenic drug effects Mice Locomotor activity

THE classical agonists of the benzodiazepine receptor (BZR), benzodiazepines, are known to have anxiolytic properties. Recent evidence has shown that several β-carbolines, known to be inverse agonists of the BZR, have opposite properties and induce anxiety in animals. In rhesus monkeys, Ninan *et al.* [15] found that ethyl-β-carboline-3-carboxylate (β-CCE) produces a wide range of behaviors that have been proposed to represent "anxious" responses in other primate models of anxiety. In rodents, File *et al.* [7] observed that β-CCE significantly reduced active social interaction. Prado de Carvalho *et al.* [20] showed that methyl-β-carboline-3-carboxylate (β-CCM) caused a significant reduction in pressing rates in mice trained in a classical conflict test with food reinforcement. These effects are readily described as "proconflict" since they are opposite to the anticonflict effects of anxiolytic drugs [3]. Such proconflict effects of several β-carbolines were also demonstrated by Corda *et al.* [2] and Petersen and Jensen [18] in rats using a conflict situation with water reinforcement. All these effects (see for review Pellow and File [16] have been interpreted as anxiogenic and most of them can be suppressed by co-administration of the classical antagonist RO 15-1788. However, Crawley *et al.* [6], using a simple animal model for behavioral actions of tranquilizers, found no anxiogenic activity of β-CCM in this model system.

The purpose of the present study was to investigate the behavioral effects of β-CCM and to compare them to the effects of a benzodiazepine, clorazepate, in a test especially adapted to mice that is based on the natural tendency of this species to prefer dark environments. Indeed, it has been known for more than 5 years that in a two-chambered system where mice can freely move between a brightly lit cage and a dark corner, they display a preference for the dark chamber and, after treatment with benzodiazepines, show more crossing between the two chambers [1, 4, 5]. It is believed that benzodiazepines produce a dose-dependent facilitation of exploratory behavior, presumably by suppressing novelty-induced behavioral inhibition. However, several studies have shown that in experimental situations in which animals are forced into a novel environment, they actually attempt to escape rather than explore [13, 14, 22, 24, 25]. One of us (R.M.) has modified the test described by Crawley *et al.* [1, 4, 5] so that the apparatus consists of two boxes of the same size, one lit and the other dark, which communicate with one another. Furthermore, in addition to the number of transitions between the two compartments, the time spent by mice in the brightly-lit compartment was also recorded. Indeed, in a preliminary investigation, anxiolytic drugs significantly increased the time spent by mice in the lit box (unpublished data). This behavioral model is presented here for the first time.

Experiment 1 was undertaken to investigate the effects of several doses of clorazepate and β-CCM on locomotion in order to determine at which doses these drugs begin to re-

duce animals' locomotor activity and thus prevent normal expression of behavior. Indeed, it is well known that most anti-anxiety agents such as benzodiazepines have sedative properties when administered at high doses. In addition, β-CCM is a potent convulsant which produces convulsions in a dose-dependent manner, for example in mice [21]. Therefore, convulsive episodes are often followed by a variable period of post-ictal depression during which animals are unable to normally exhibit behavioral sequences.

Experiment 2 was aimed at confirming that the modified lit/dark procedure is a valid screening test for anxiolytic drugs, such as the benzodiazepine clorazepate. We expected a significant increase in the time spent in the lit box as well as in the number of transitions by mice treated with this drug, and an antagonistic action of RO 15-1788. Furthermore, since intrinsic actions of benzodiazepine antagonists have been demonstrated [7, 8, 10, 23], we also observed the effects of a normally antagonistic dose of RO 15-1788 given alone. Experiment 3 examined the effects of β-CCM as well as the effects of a combination of β-CCM and RO 15-1788 on the behavior in mice confronted with the lit/dark choice procedure.

GENERAL METHOD

Animals

Male Swiss albino mice from Centre d'Elevage R. Janvier, 13 weeks of age at time of testing, were used. Prior to experimental testing, they were housed five to a standard cage containing a constant supply of food pellets and water, and kept on a 12/12 hr light-dark cycle with lights on at 1 a.m. in order to observe animals in their high activity period, that is, when lights are off.

EXPERIMENT 1

Apparatus

The prototype of the apparatus has been devised by Hughes [11] and adapted for mice by Misslin and Ropartz [12]. It consisted of a polyvinyl chloride box (30×20×20 cm), subdivided into six equal, square exploratory units and covered with Plexiglas. It could be divided in half by means of three temporary partitions. The apparatus was kept on a stand in the room which housed the mice. During observation, the experimenters stood next to the boxes always at the same places.

Procedure

Each subject was placed in one-half of the apparatus with the temporary partitions in place, in order for familiarization to occur. Approximately 24 hr after being placed in the apparatus, the subject was exposed to the familiar and novel environments by the removal of the temporary partitions. The subject was then observed, in red light, for ten minutes. The number of units entered by the subjects was recorded as locomotor activity.

Drugs were administered intraperitoneally, 20 minutes before testing, in concentrations giving an injection volume of 10 ml/kg of mouse. Mice were randomly allocated to the following groups: (a) vehicle control (physiological saline; n=20), dipotassium clorazepate (1.0, 2.0, 4.0 and 8.0 mg/kg in saline; respectively n=10, 10, 10 and 20); (b) vehicle control (saline with a drop of 0.1 HCl; n=10), β-CCM (1.0, 2.5 and 5.0 mg/kg; n=10).

TABLE 1

EFFECTS OF CLORAZEPATE AND β-CCM ON THE LOCOMOTOR ACTIVITY

Drugs (mg/kg)	Locomotion	N
Clorazepate		
0.0	118.95 ± 6.87	20
1.0	128.8 ± 15.73	10
2.0	106.0 ± 7.58	10
4.0	123.2 ± 12.96	10
8.0	74.1 ± 12.96*	20
β-CCM		
0.0	112.6 ± 7.73	10
1.0	113.6 ± 8.02	10
2.5	99.1 ± 7.52	10
5.0	67.8 ± 7.58†	10

Male Swiss mice were given (IP) 1.0–8.0 mg/kg of clorazepate or saline and 1.0–5.0 mg/kg of methyl-β-carboline-3-carboxylate (β-CCM) or saline 20 min before testing in an exploratory model in the mouse. Data are expressed as mean ± S.E.M. Statistical significance represents Newman-Keuls significance of individual group means following analysis of variance. Clorazepate dosed at 8.0 mg/kg and β-CCM dosed at 5.0 mg/kg significantly decreased locomotor activity.

*$p<0.05$ vs. control mice (vehicle).

†$p<0.01$.

EXPERIMENT 2

Apparatus

The apparatus consisted of two polyvinylchloride boxes (20×20×14 cm) covered with Plexiglas. One of these boxes was darkened with cardboard. A light from a 100 W desk lamp above the other box provided the only room illumination. An opaque plastic tunnel (5×7×10 cm) separated the dark box from the lit one. During observation the experimenters always sat at the same place, next to the apparatus.

Procedure

The subjects were individually tested in five minute sessions in the apparatus described above. Testing was performed between 2 and 4 p.m. Mice were naive to the apparatus and had no previous drug treatment. All mice were placed in the lit box to initiate the test session. The amount of time spent in the lit box and the number of transitions across the tunnel were recorded, minute by minute, during 5 minutes after the first entry in the dark box. A mouse whose four paws were in the new box was considered as having changed boxes.

Drugs were administered intraperitoneally, 20 min before testing, in concentrations giving an injection volume of 10 ml/kg of mouse. Only doses without sedative properties were chosen. Mice were randomly allocated to the following groups: (a) vehicle control (physiological saline with a drop of Tween 60; n=33), dipotassium clorazepate [1.0, 2.0 and 4.0 mg/kg plus saline/Tween 80; respectively n=25, 47 and 25)], a combination of clorazepate (2.0 mg/kg) and RO 15-1788 (3.0 mg/kg) in saline/Tween 60; n=25; (b) vehicle (saline with a drop of Tween 80; n=30), RO 15-1788 (3.0 mg/kg; n=30) in vehicle.

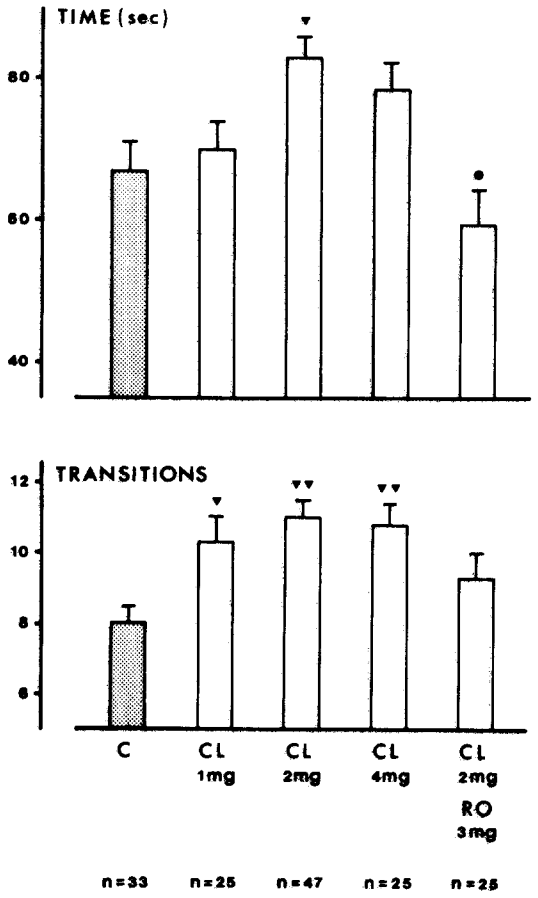

FIG. 1. Clorazepate (CL) 1.0–4.0 mg/kg (IP) administered 20 min before behavioral testing tended to increase the time spent by mice in the lit box and the number of transitions between the light and dark boxes. RO 15-1788 (RO) 3 mg/kg antagonized the 2.0 mg/kg clorazepate-induced increase in time spent by mice in the lit box as well as light/dark transitions. ▼$p<0.05$; ▼▼$p<0.01$: clorazepate mice versus controls (C). ●$p<0.05$: clorazepate (2.0 mg/kg) combined with RO 15-1788 mice versus 2.0 mg/kg clorazepate mice.

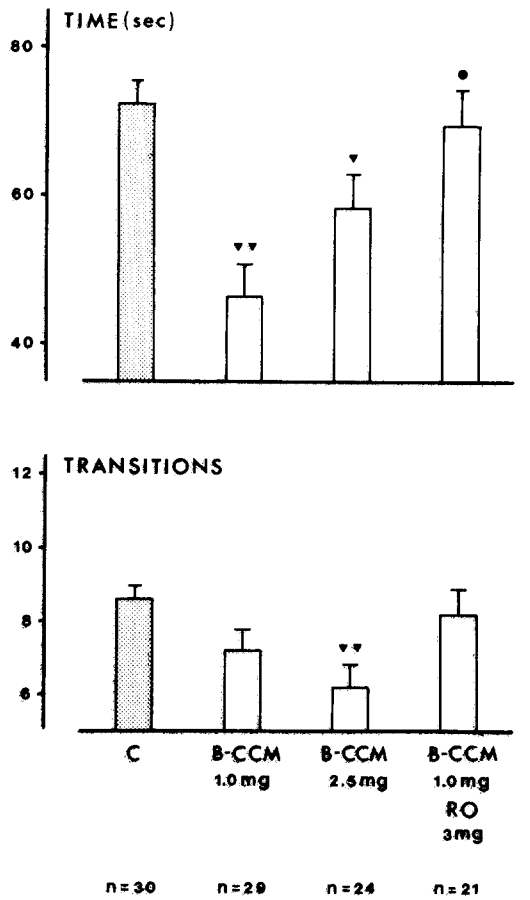

FIG. 2. Methyl-β-carboline-3-carboxylate (β-CCM) 1.0 and 2.5 mg/kg (IP) was administered 20 min before testing. Dose at 1.0 mg/kg decreased the time spent by mice in the lit box. Dose at 2.5 mg/kg decreased the time spent in the lit box as well as the number of transitions between the two boxes. RO 15-1788 (RO) 3 mg/kg antagonized the effects of 1.0 mg/kg of β-CCM. ▼$p<0.05$; ▼▼$p<0.01$: β-CCM mice versus controls (C). ●$p<0.05$ β-CCM (1.0 mg/kg) combined with RO 15-1788 versus 1.0 mg/kg β-CCM mice.

EXPERIMENT 3

The testing procedure was strictly the same as that described in Experiment 2. Mice were randomly allocated to the following groups: vehicle control (saline with a drop of 0.1 HCl and a drop of Tween 80; n=30), β-CCM (1.0 and 2.5 mg/kg) dissolved in 0.1 HCl and diluted to volume with saline plus a drop of Tween 80 (n=29 and 24), β-CCM (1.0 mg/kg) plus RO 15-1788 (3.0 mg/kg; n=21).

Statistical Analysis

Statistical significance of differences between control and treated groups was ascertained by a combined analysis of variance and an unpaired two-tailed range *t*-test using the Newman-Keuls method as described by Wiener [26]. An ANOVA was carried out and the pooled variance was used for multiple *t* comparisons (drug groups vs. controls).

RESULTS

Experiment 1

As can be seen in Table 1, clorazepate at a dose of 8.0 mg/kg and β-CCM at a dose of 5.0 mg/kg produced a significant decrease in locomotor activity. We also noted that mice treated with β-CCM dosed at 2.5 and 5.0 mg/kg exhibited 2 minutes after administration of the drug a typical preconvulsant behavioral profile that consisted of motor suppression, head rigidity and increase of respiratory rate, but only one animal, treated with the dose of 5.0 mg/kg, showed tonico-clonic seizures. These findings led us to only use the doses without sedative or preconvulsant properties for the following experiments.

EXPERIMENT 2

Figure 1 shows that clorazepate at 2.0 mg/kg produced a significant increase in the time spent by mice in the lit box. Furthermore, this durg at doses of 1.0, 2.0 and 4.0 mg/kg also significantly increased the number of transitions across the tunnel. Mice treated with the combination of clorazepate and RO 15-1788 did not differ from controls in respect to these parameters, while they spent significantly less time in the lit box when compared to the clorazepate 2.0 mg/kg mice. These results suggest that RO 15-1788 completely coun-

TABLE 2
EFFECTS OF β-CCM ON THE TIME INDEX

Drug (mg/kg)	Time Index	N
β-CCM		
0.0	8.62 ± 0.42	30
1.0	6.5 ± 0.55*	29
2.5	9.96 ± 0.42	24
RO 15-1788		
1.0		
+3.0	9.19 ± 0.73	21

Methyl-β-carboline-3-carboxylate (β-CCM) 1.0 and 2.5 mg/kg (IP) was administered 20 min before testing. β-CCM dosed at 1.0 mg/kg reduced the index of time spent by mice in the lit box to the number of transitions between the light and dark boxes (time index). This effect was antagonized by RO 15-1788 (3.0 mg/kg).
*$p<0.05$ vs. controls (vehicle).

teracted the effects of clorazepate. Furthermore, there were no significant differences between control mice and RO 15-1788 treated animals. Mean and S.E.M. were respectively as follows: 23.6±1.7 and 27.0±1.6 (N=30) for the time spent in the lit box and 9.1±0.5 and 8.6±0.6 for the number of transitions.

EXPERIMENT 3

As can be seen from Fig. 2, β-CCM at a dose of 1.0 mg/kg decreased the time spent by mice in the lit box as well as the number of transitions. However, there were significant differences between treated mice and controls only in respect to the first parameter. This effect was completely antagonized by RO 15-1788. Furthermore, β-CCM at a dose of 2.5 mg/kg significantly reduced the time spent by mice in the lit box as well as the number of transitions. We also observed that the latter mice moved more slowly than controls as if they were under sedation. It appeared that these results were somewhat surprising. The time spent by mice in the lit box was reduced by β-CCM dosed at 2.5 mg/kg, but even more so by the same drug dosed at 1.0 mg/kg, while the number of transitions was also reduced by 2.5 mg/kg of β-CCM, but this time less than when a 1.0 mg/kg was used. Thus, we calculated the index of time spent in the lit box to the number of transitions (time index). As can be seen from Table 2, 1.0 mg/kg of β-CCM significantly reduced the time index, while 2.5 mg/kg failed to significantly affect it.

GENERAL DISCUSSION

It is well known that mice, when confronted with a dark and a lit cage, naturally tend to prefer the dark one. Using the lit/dark choice procedure Crawley *et al.* [1, 4, 5] found that anxiolytic drugs as well as pentobarbital induced an increase in mouse transitions between the brightly-lit compartment and the dark one. In a preliminary investigation, one of us recently observed that some anxiolytic compounds significantly increased the time spent by mice in the lit box, probably by reducing aversive properties of lit places (unpublished data). These findings led us to suppose that this test procedure could be considered as a conflict paradigm that is at once based on the innate tendency of mice to seek refuge in a dark box, and their propensity to escape novel places by moving around.

Experiment 1 allowed us to determine the doses of clorazepate and β-CCM which induced a significant decrease of locomotion in mice freely confronted with familiar and novel places. The findings obtained show that the effective dose for clorazepate was 8.0 mg/kg while β-CCM significantly depressed the locomotor activity in mice treated with 5.0 mg/kg. At the latter dose, soon after injection, only one mouse fell on its flanks and showed a convulsive episode, but most mice treated with 2.5 and 5.0 mg/kg of β-CCM exhibited a preconvulsive pattern of behaviors such as locomotion suppression, increase of respiratory rate and head rigidity. Therefore, it can be suggested that the decrease of locomotion that we observed in mice treated with 5.0 mg/kg of β-CCM seems to be due in fact to postictal depression. At 7.5 and 10.0 mg/kg of β-CCM, tonico-clonic seizures appeared in 30% and 60% of mice (unpublished data). These results are comparable to those obtained by Prado de Carvalho *et al.* [21] and confirm the potent convulsant properties of this drug.

The findings obtained in Experiment 2 confirm the predictive value of the lit/dark two box test, as clorazepate dosed at 1.0–4.0 mg/kg tend to increase the time spent in the lit box as well as the number of transitions. These results are in agreement with the finding that anti-anxiety agents induce an increase of the number of transitions in the lit/dark choice procedure [1, 4, 5]. Graeff and Rawlins [9] suggested that in the conflict tests anxiolytics depress the behavioral inhibition system as well as the brain aversive system. The present results also confirm the benzodiazepine antagonistic properties of RO 15-1788, since this drug completely abolished the anticonflict effects of clorazepate dosed at 2.0 mg/kg. This antagonistic action did not seem to be due to the intrinsic effects of RO 15-1788, since this drug, given alone, had no effect on the behavior of mice tested in our apparatus.

The results we obtained in Experiment 3 allowed us to demonstrate that β-CCM dosed at 1.0 mg/kg tends to reduce the time spent by mice in the lit box as well as the number of their transitions, although the latter parameter did not reach a significant level. These data are strictly similar to those we obtained in a preliminary study (unpublished) and can be opposed to those of clorazepate, confirming the anxiogenic properties of β-CCM as has been reported by several authors [2, 7, 15, 17, 18, 20]. For example, it can be emphasized that Prado de Carvalho *et al.* [20] observed anxiogenic activity of β-CCM in their model in mice at the same dose as we did. Furthermore, our results show that RO 15-1788 completely antagonized the effects of β-CCM. This is in agreement with the finding that RO 15-1788 potently blocked the convulsant effect of β-CCM [19] and that both of these compounds are highly affinity ligands of the benzodiazepine binding site.

The effects of 2.5 mg/kg β-CCM may seem surprising insofar as the drug induced a significant decrease in the number of transitions without significantly affecting the time spent by mice in the lit box. However, as has been noted in Experiment 1, most of these animals exhibited a short preconvulsive episode soon after injection. Therefore, it is likely that the decrease of the number of transitions could be due to postictal depression. This interpretation is supported by the reduction of locomotor activity following high doses of β-CCM, in spite of the fact that this effect became significant only at the dose of 5.0 mg/kg. Similar results demonstrating a decrement in lit/dark transitions as well as in general activity following high doses of β-CCM (from 5.0 to 50

mg/kg) were also described by Crawley *et al.* [6]. These latter authors suggested as we are doing here that this decrement is very likely related to subclinical seizures rather than to a specific anxiogenic action of β-CCM. This is in agreement with the effects of β-CCM on the time index that we observed in the present study, since 1.0 mg/kg reduced this parameter, while 2.5 mg/kg did not affect it. Therefore, we may suggest that the dose of 1.0 mg/kg actually has anxiogenic properties in so far as these mice, at every entry, spent less time in the lit box than controls. On the contrary, the 2.5 mg/kg mice showed less entries into the lit box than controls, but spent there the same length of time at each entry.

In conclusion, our results support earlier data that showed anxiogenic properties of β-CCM in mice. Furthermore, the present data validate the usefulness of the behavioral model used here which may provide a simple and selective test in mice for anxiolytic drugs as well as anxiogenic compounds. Since benzodiazepines and inverse agonists modify food intake, pain threshold or motivation, the lit/dark box test which does not use these factors appears to be well designed to detect pro- and anti-conflict effects of drugs.

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