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β-Carbolines enhance shock-induced suppression of drinking in rats

(benzodiazepines/phenylpyrazoloquinolines/punished drinking behavior/proconflict action/anxiety)

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ABSTRACT **By using Vogel's method to test the anxiolytic action of benzodiazepines and reducing the intensity of the current delivered to the drinking tube, it is possible to distinguish the pharmacological activity of three types of ligands for the benzodiazepine recognition site. An anticonflict action typical of anxiolytic benzodiazepines, a proconflict action typical of many β-carbolines, including FG 7142 (β-carboline-3-carboxylic acid ethyl ester methyl amide), and an antagonistic action of the proconflict and anticonflict actions typical of RO 15-1788 (ethyl-8-fluoro-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazol[1,5-*a*]-[1,4]-benzodiazepine-3-carboxylate) and CGS 8216 (2-phenylpyrazolo[4,3-*c*]quinolin-3-(5*H*)-one). Pentylenetetrazole, which causes convulsions by interacting with a subunit of the γ-aminobutyric acid receptor that is different from the benzodiazepine recognition site, also induces a proconflict action that is antagonized by anxiolytic benzodiazepines but not by RO 15-1788.**

A number of ligands that bind with high affinity to the benzodiazepine recognition site differ from the anxiolytic benzodiazepines pharmacologically (1–10). When a ligand for the benzodiazepine recognition site differs from anxiolytic benzodiazepines pharmacologically, it also differs in the way it interacts with γ-aminobutyric acid (GABA) receptors biochemically (7, 11). Anxiolytic benzodiazepines relieve convulsions due to an impairment of GABAergic transmission (12), and increase the B_{max} (maximal binding) of the high-affinity GABA recognition site; in contrast, the affinity of benzodiazepine recognition sites is increased by GABA (13, 14). A second group of benzodiazepine recognition site ligands, such as ethyl-8-fluoro-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazol[1,5-*a*]-[1,4]-benzodiazepine-3-carboxylate (RO 15-1788), 2-phenylpyrazolo[4,3-*c*]quinolin-3-(5*H*)-one (CGS 8216), and β-PrCC (β-carboline-3-carboxylic acid propyl ester) fail to relieve anxiety, bind to the benzodiazepine recognition site in a GABA-independent manner, and fail to modulate the B_{max} of GABA binding (6, 7, 11, 14). A third group of benzodiazepine recognition site ligands, such as the derivatives of β-carboline-3-carboxylic acid ethyl ester (β-CCE), elicit or facilitate convulsions, have their affinity for the benzodiazepine recognition site reduced by GABA (7), and block the increase of the B_{max} of GABA binding induced by anxiolytic benzodiazepines (14). One wonders whether these differences can help to predict the action of various benzodiazepine recognition site ligands on anxiety-regulating mechanisms in humans. The ability of drugs to increase the number of punished responses in rats operating in a conflict situation appears to be related to an anxiolytic action in humans (15, 16). Those β-carbolines that trigger panic–anxiety in humans (7) and attenuate the punishment-lessening effect of benzodiazepines in rats (3) perhaps may even increase fear of punishment in experimental animals. The present report shows that β-carboline-3-carboxylic acid methyl ester (β-CCM), 6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylic acid methyl ester (DMCM), β-carboline-3-carboxylic acid ethyl ester methyl amide (FG 7142), and β-CCE facilitate the suppression induced by punishment, studied in a behavioral model test for anxiolytic drugs in rats. This effect of β-carbolines is blocked by ligands of benzodiazepine recognition sites that are devoid of intrinsic activity such as RO 15-1788 and CGS 8216 and by ligands with anxiolytic activity.

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MATERIAL AND METHODS

Animals. Male Sprague–Dawley rats weighing 120–150 g were housed in our facilities at 24°C with light–dark cycles of 12 hr (light on: 0700–1900) and they were fed Purina Lab Chow and water ad lib for at least 3 days before preparation for experiments. Groups of 16–20 rats to be prepared for the operant behavioral tests were deprived of water for 72 hr prior to the conflict session.

Conflict–Punishment Procedure. *Apparatus.* The rat to be tested was placed in a clear Plexiglas box 28 × 20 × 20 cm with a stainless steel grid floor. This chamber was enclosed in a sound-attenuated ventilated box. Water was provided through an electrically shielded stainless steel drinking tube extending 2 cm into the box, 3 cm above the floor. The unshielded tip of the drinking tube and the grid floor were connected to a constant current shock generator and to a drinkometer. The drinkometer output and the shock generator were connected to timer controlled relays delivering one shock lasting for 1 sec for every 3 sec of cumulative drinkometer output. This 3-sec cumulative drinking period was termed "licking period." To study the anticonflict drug actions the intensity of the current delivered to the drinking tube was 0.8 mA; to study proconflict drug actions the shock duration was kept constant but its intensity was lowered to 0.35 mA. When unpunished drinking was measured the shock was omitted. A counter recorded the total number of drinking periods. All behavioral equipment was purchased from Lafayette Instrument, Lafayette, IN.

Procedure. All experiments were carried out between 1100 and 1800. One hour before the test, each rat was placed in the

Abbreviations: GABA, γ-aminobutyric acid; RO 15-1788, ethyl-8-fluoro-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazol[1,5-*a*]-[1,4]-benzodiazepine-3-carboxylate; CGS 8216, 2-phenylpyrazolo[4,3-*c*]quinolin-3-(5*H*)-one; β-CCE, β-carboline-3-carboxylic acid ethyl ester; β-CCM, β-carboline-3-carboxylic acid methyl ester; DMCM, 6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylic acid methyl ester; FG 7142, β-carboline-3-carboxylic acid ethyl ester methyl amide; PTZ, pentylenetetrazole; EEG, electroencephalogram; i.v., intravenous(ly).

chamber and the time needed to find the drinking tube was measured; the rat was removed from the chamber immediately after. Those animals taking more than 3 min to find the tube were discarded. Each rat was allowed to habituate to the chamber without the drinking tube for 15 min immediately before the test to avoid exploration-induced delay of drinking. The drinking tube was inserted into the cage and the animals were allowed to complete a 3-sec licking period before the first shock was delivered. A 3-min test period started at the end of the first shock.

Measurement of Threshold to Painful Stimuli. Threshold for painful stimuli was measured with the tail flick test of D'Amour and Smith (17). The tail flick latency was automatically determined by a timer to within $^1/_4$ sec.

Electroencephalogram (EEG) Recording. Male Sprague–Dawley rats (250 g) were implanted with $^1/_8$-inch stainless steel screw electrodes resting on the dura, and 0.005-inch stainless steel wire electrodes for recording the EEG and nuchal electromyogram (EMG), respectively (1 inch = 25.4 mm). Electrode placements were for bipolar bifrontal (channel on tracings) and frontooccipital recordings. After a 1-week recovery period, EEG and EMG were recorded starting at 0900 on a Grass model 78 polygraph calibrated to 50 μV/10 mm and a paper speed of 10 mm/sec. State of consciousness was conventionally determined (18), and samples of tracings were converted into amplitude vs. frequency plots by an automatic fast Fourier transformation apparatus (Bruel and Kjaer, Naerum, Denmark).

Drugs. The following drugs were used: β-CCE, β-CCM, DMCM, and FG 7142 (gifts from C. Braestrup, Ferrosan, Copenhagen, Denmark); diazepam and RO 15-1788 (H. Mohler, Hoffmann–La Roche, Basel, Switzerland); CGS 8216 (B. Petrack, CIBA–Geigy, Ardsley, NY); pentylenetetrazole (PTZ) and strychnine (Sigma). FG 7142, β-CCM, β-CCE, DMCM, diazepam, RO 15-1788, and CGS 8216 were dissolved in 200 μl of dimethyl sulfoxide and 1 drop of Tween 80, brought up to volume with saline, and injected intravenously (i.v.). PTZ and strychnine were dissolved in saline and administered intraperitoneally or subcutaneously, respectively. The injection volume for all the drugs was 3 ml/kg of body weight. Depending on the specific time latency for the onset of the drug actions, rats were placed in the test cage either before or immediately after drug injection.

Statistics. Significant differences between control and treated groups were determined as $P < 0.05$ by using the Dunnett multiple comparison test (19).

RESULTS

When water-deprived animals were placed in the experimental chamber, in the absence of shock, they licked without interruption for the test period (3 min), totaling approximately 25 licking periods (Fig. 1*A*). When a 0.8-mA current was coupled every 3 sec to the drinking tube it reduced the number of licking periods significantly (Fig. 1). This conflict situation is similar to the one described by Vogel *et al.* (20). Diazepam elicited an anticonflict effect as shown by the reinstatement of licking activity (Fig. 1*B*). However, no anticonflict effect was detected when β-carbolines or other nonanxiolytic ligands of benzodiazepine recognition sites were studied (Fig. 1*B*). We then wondered whether some of these ligands induce "proconflict effects" by enhancing the suppressing action of punishment on drinking. To test this possibility we lowered the shock intensity to 0.35 mA because we knew that this current intensity (see Fig. 1*A*) would minimally decrease spontaneous water drinking and hence allow drug-induced facilitation of drinking suppression to be more easily observed. FG 7142, β-CCM, DMCM, and β-CCE induced a dose-dependent decrease in the number of shocks received by the animals during the 3-min testing (Fig. 2).

The suppression of punished drinking by different β-carbolines occurs at dose levels that fail to change unpunished drinking (Fig. 2). Of the β-carbolines tested, the most potent was β-CCM (IC_{30}, 0.1 mg/kg) and the least potent was FG 7142 (IC_{30}, 1.8 mg/kg). The separation of the changes on punished and unpunished licking elicited by β-CCE was less

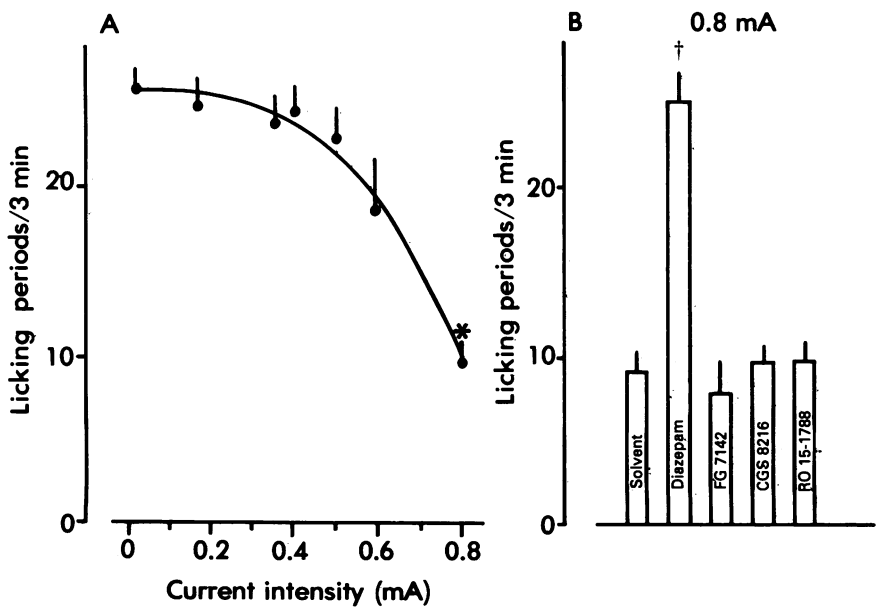

FIG. 1. (*A*) Relationship between current intensity and number of licking periods in 3 min. Each point represents the mean + SEM of three to six rats per group. (*B*) Number of licking periods in 3 min with current intensity set at 0.8 mA in rats receiving i.v. various ligands of the benzodiazepine recognition site. Diazepam (0.5 mg/kg), FG 7142 (2.5 mg/kg), CGS 8216 (1 mg/kg), and RO 15-1788 (2 mg/kg) were injected 10 min before the test. Each value represents the mean + SEM of five rats per group.
*$P < 0.05$ compared to the rats performing in an unpunished situation.
†$P < 0.05$ compared to the vehicle-treated group.

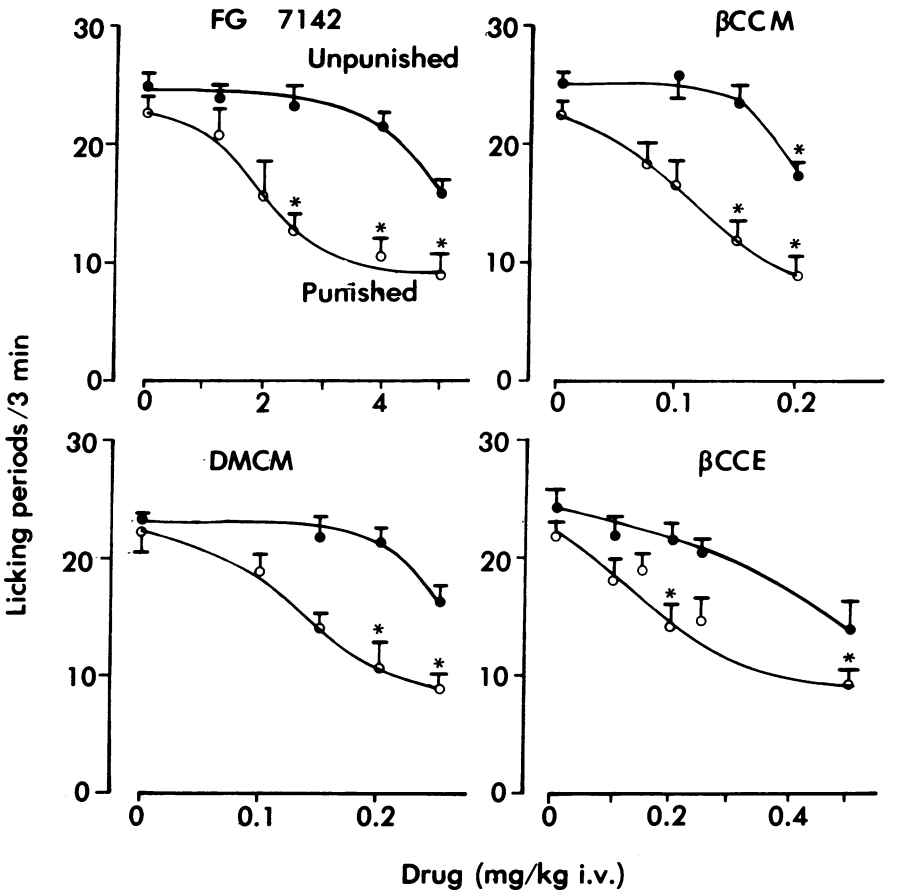

FIG. 2. Number of licking periods in 3 min under punished (○) and unpunished (●) conditions in rats receiving one of four β-carbolines i.v. FG 7142, β-CCM, DMCM, and β-CCE were injected 15, 10, 10, and 5 min before the test, respectively. Each point represents the mean ± SEM of 5–11 rats per group.
*$P < 0.05$ compared to the respective vehicle-treated group.

pronounced and more variable than that observed with the other β-carbolines. At the IC_{30} dose none of the drugs produced readily observable alterations in gross behavior or alterations in the pain threshold. Doses of β-carbolines 2–3 times higher than those required to lower the current threshold that suppresses drinking decreased unpunished drinking and caused

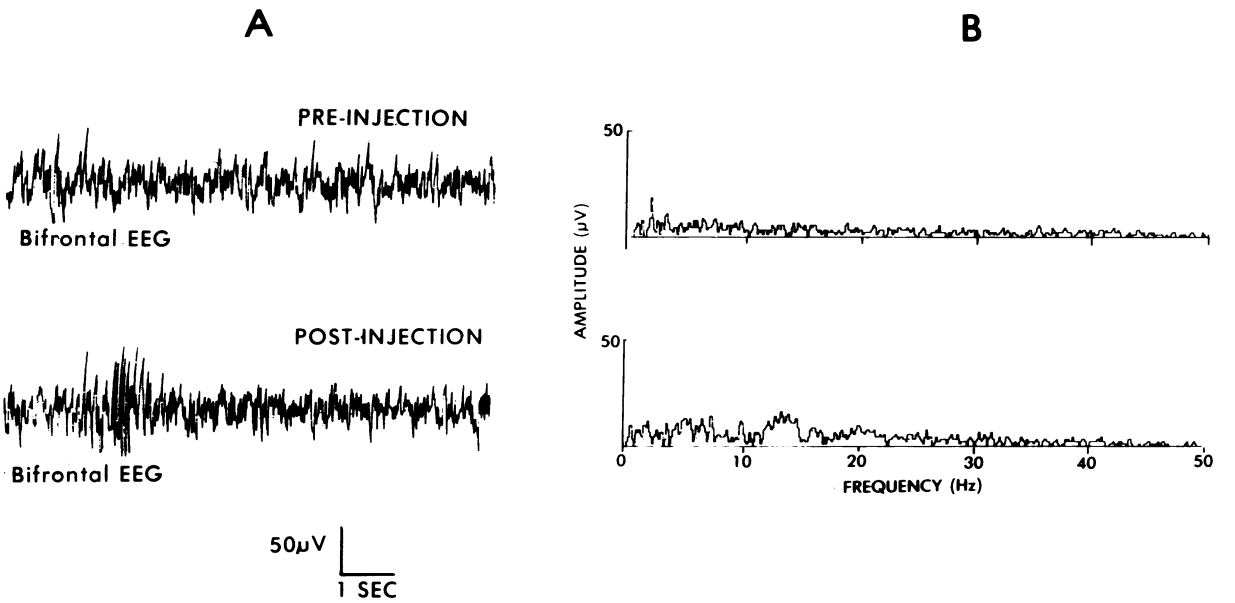

FIG. 3. EEG effect of β-CCM (0.15 mg/kg, i.v.). (*A*) Bifrontal EEG from control (preinjection) and β-CCM (10 min postinjection) treated animals. (*B*) The identical EEG signals transformed by fast Fourier analysis into an amplitude vs. frequency plot. The data are from representative animals. FG 7142 (10 mg/kg, i.v.) injection produced records similar to control.

Table 1. Antagonism by RO 15-1788, CGS 8216, and diazepam of the proconflict effect of β-carbolines

Antagonist	Licking periods in 3 min			
	Saline	FG 7142	β-CCM	DMCM
Saline	22 ± 2.0	10 ± 0.9*	9 ± 2.1*	11 ± 1.5*
RO 15-1788	21 ± 1.9	21 ± 2.3	21 ± 1.5	24 ± 1.4
CGS 8216	18 ± 1.4	17 ± 2.7	16 ± 4.4	15 ± 2.5
Diazepam	26 ± 2.3	21 ± 2.4	27 ± 1.7	21 ± 2.0

RO 15-1788 (2 mg/kg), CGS 8216 (1 mg/kg), diazepam (0.5 mg/kg), FG 7142 (4 mg/kg), β-CCM (0.15 mg/kg), and DMCM (0.2 mg/kg) were injected i.v. 10 min before the test. Each value represents the mean ± SEM of 6–10 animals per group.
*$P < 0.05$ compared to saline-treated group.

sedation. EEG tracings taken at various times after drug injections indicated that FG 7142 produced no evidence of EEG or motor sequence activity even in doses up to 10 mg/kg i.v. β-CCM in doses of 0.15 mg/kg produced occasional brief sharp wave activity in the EEG but not motor convulsant effects (Fig. 3). Substantially higher doses (0.6 mg/kg) were needed to elicit major sharp wave activity and motor convulsions.

The proconflict effects of β-carbolines appear to be specific because diazepam (0.5 mg/kg, i.v.), RO 15-1788 (2 mg/kg, i.v.), and CGS 8216 (1 mg/kg, i.v.) failed to alter the direction of pattern of punished licking under conditions of low current intensity (Table 1). However, these drugs antagonized the suppression of punished drinking elicited by FG 7142, DMCM, or β-CCM.

In order to study the specificity of the response induced by β-carbolines, we have tested PTZ because this drug is known to affect operant conflict behavior in the rat (21) and to cause convulsions by reducing GABAergic transmission (22) and strychnine because this drug reduces glycinergic transmission (23). As shown in Fig. 4, strychnine in subconvulsive doses failed to alter duration and pattern of punished drinking. In contrast, PTZ significantly decreased the current threshold to suppress drinking. The rats receiving PTZ doses that fail to change unpunished drinking took a smaller number of shocks when compared to untreated rats. This effect, however, was not reversed by doses of RO 15-1788 that change the proconflict affects of β-carbolines (Table 1).

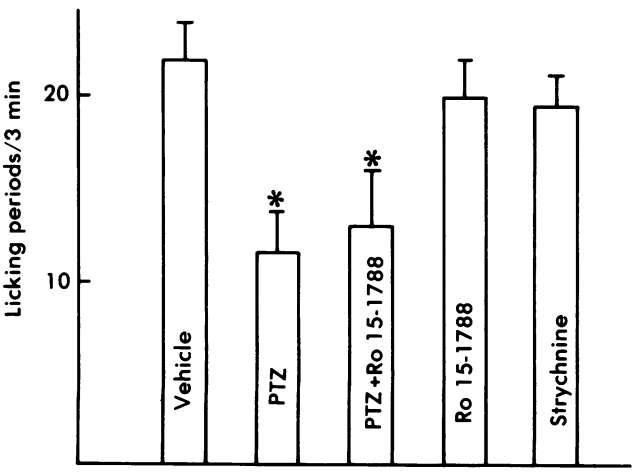

FIG. 4. Number of licking periods in 3 min with electric shock at 0.35 mA in rats receiving PTZ or strychnine. PTZ (15 mg/kg, intraperitoneally) was injected 15 min before the test. Strychnine (0.6 mg/kg, subcutaneously) was injected 20 min before the test. RO 15-1788 (2 mg/kg, i.v.) was injected 5 min after the PTZ. Each value represents the mean + SEM of 8–12 rats per group.
*$P < 0.05$ compared to vehicle-treated group.

DISCUSSION

It has been reported that the β-carbolines RO 15-1788 and CGS 8216 prevent the anticonflict action of benzodiazepines (3, 5, 6, 9, 10). Our experiments indicate that RO 15-1788 and CGS 8216 are almost devoid of intrinsic activity because these two ligands fail to elicit a proconflict or anticonflict action in the doses studied here.

In contrast, the present experiments show that β-CCM, DMCM, FG 7142, and PTZ elicit a proconflict action in rats. The action of β-carbolines is antagonized by RO 15-1788 and CGS 8216; however, both drugs fail to modify the proconflict activity of PTZ. These findings suggest that, on the basis of behavioral action in rats, the benzodiazepine recognition site ligands can be divided into three groups: (*i*) ligands such as diazepam that possess anticonflict action and inhibit the proconflict action of β-carbolines; (*ii*) ligands such as β-CCM that possess proconflict action and inhibit the anticonflict action of diazepam; (*iii*) ligands such as RO 15-1788 and CGS 8216 that at low doses possess neither proconflict nor anticonflict action, but block the anticonflict and proconflict actions of benzodiazepines and β-carbolines, respectively. Thus, it appears that specific ligands are encoded with specific information for the benzodiazepine recognition site. If the ligands lack this specific information, then they function as simple antagonists like RO 15-1788 and CGS 8216. This view is upheld by the observation that the relative potency of the β-carbolines tested in the present experiments correlates positively with their relative potency in displacing [^{3}H]flunitrazepam and [^{3}H]diazepam from benzodiazepine binding sites *in vitro* and *in vivo* (7). The only exception is β-CCE, which appears to be more potent in the *in vitro* binding studies than in the behavioral experiments. This exception is probably due to its extremely short half-life in plasma (24). Moreover, the proconflict action of β-carbolines is due neither to a decrease in pain threshold nor to EEG sharp wave activity, insofar as proconflict actions occurred with FG 7142 and doses of β-CCM lower than those that induced sharp wave activity.

PTZ, which has been widely used to antagonize the anxiolytic action of benzodiazepines (15), has a pharmacological profile very similar to that of β-carbolines. Although PTZ is chemically unrelated to the β-carbolines and has an extremely low affinity for benzodiazepine recognition sites (7), it causes convulsions that are antagonized by benzodiazepines and produces anxiety in humans (21, 25). In the present experiments PTZ causes a proconflict action in doses that are $^1/_3$ to $^1/_4$ of those required to induce convulsions. However, this proconflict action cannot be antagonized by RO 15-1788 in doses that prevent comparable proconflict effects of β-carbolines, but it is inhibited when the benzodiazepine recognition sites are occupied by anxiolytic benzodiazepine. Electrophysiological studies indicate that PTZ is a selective antagonist of GABA-mediated postsynaptic inhibition (22), conceivably through an inhibition of the opening of the GABA-regulated Cl^- channels. On the other hand, strychnine, which inhibits glycine-modulated Cl^- conductance (23), has no proconflict effect at doses close to those producing generalized convulsions. This finding indicates that the proconflict actions elicited by PTZ and β-carbolines have similar pharmacological profiles, and electrophysiological studies indicate that both drugs down-regulate GABA-mediated postsynaptic responses (22, 26); hence the behavioral actions of β-carbolines may be mediated by a down-regulation of GABA receptor function. This conclusion agrees with previous findings that β-carbolines block the benzodiazepine-induced enhancement of specific GABA binding to synaptic membrane preparations, whereas GABA reduces

the binding of β-carbolines to the benzodiazepine binding sites (7, 14).

It has been reported that in the behavioral models of conflict punishment proposed by Geller and Seifter (27) or Vogel *et al.* (20) the anticonflict effect of benzodiazepines is potentiated or mimicked by stimulation of GABA receptors with muscimol (28). Conversely, blockade of GABA receptor function with bicuculline, picrotoxin, or PTZ abolished the anticonflict action of diazepam (16, 29, 30). These results strongly suggest that GABA receptors are operative in the regulation of behavioral inhibition induced by punishment and they are consistent with the view that a facilitation of GABAergic transmission mediates the anxiolytic action of benzodiazepines (31). This facilitation occurs at the postsynaptic GABA receptor complex, which functions as a supramolecular unit composed of a number of subunits, including GABA recognition sites, Cl^- channel, regulatory sites of this channel, benzodiazepine recognition sites, and the coupler protein GABA-modulin (32). Presumably a spectrum of behavioral states ranging from diazepam-induced anticonflict action to the proconflict action elicited by β-carbolines and PTZ reflects the interaction of the various subunits operative in the supramolecular organization of GABA receptors linked to a Cl^- ionophore. The importance of the present observations is that proconflict and anticonflict tests can be adopted to further investigate the relationship between drug-induced shifts in conflict behavior with functional shifts in the GABA/benzodiazepine/Cl^- ionophore complex.

Because PTZ exacerbated anxiety in patients (21) and FG 7142 elicits severe anxiety in human volunteers (7), one can hypothesize that the supramolecular organization of GABA receptors plays a pivotal role in the regulation of the level of anxiety in physiological and pathological conditions. Hence, studies on the endogenous modulators of GABA receptor function such as the endogenous ligand of benzodiazepine recognition sites may elucidate some molecular aspects of the neurobiology of anxiety and may open new avenues for the therapeutic modulation of abnormal levels of anxiety.

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