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A Synthetic Non-Benzodiazepine Ligand for Benzodiazepine Receptors: A Probe For Investigating Neuronal Substrates of Anxiety

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LIPPA, A. S., J. COUPET, E. N. GREENBLATT, C. A. KLEPNER AND B. BEER. *A synthetic non-benzodiazepine ligand for benzodiazepine receptors: A probe for investigating neuronal substrates of anxiety.* PHARMAC. BIOCHEM. BEHAV. 11(1) 99–106, 1979.—CL 218,872 is the first non-benzodiazepine to selectively displace brain specific ^{3}H-diazepam binding with a potency comparable to that of the benzodiazepines. Like the benzodiazepines, CL 218,872 increased punished responding in a conflict situation and protected against the convulsions induced by pentylenetetrazole. These three pharmacological properties are highly predictive of anxiolytic activity. Unlike the benzodiazepines, however, CL 218,872 was relatively inactive in tests designed to measure effects on neuronal systems which utilize GABA, glycine and serotonin as transmitters. Furthermore, CL 218,872 was relatively free of the ataxic and depressant side effects commonly associated with the benzodiazepines. Because of this high degree of selectivity, CL 218,872 may represent a new probe for investigating neuronal substrates of anxiety.

Triazolopyridazine Benzodiazepine GABA Glycine Serotonin Purines ^{3}H-Diazepam Benzodiazepine receptors

DESPITE their widespread use, the mechanism(s) by which the benzodiazepines produce their anxiolytic actions remains unknown. Considerable controversy has centered around the possibility that benzodiazepine actions on neurotransmitter systems utilizing serotonin (5HT) [37, 38, 40], gamma aminobutyric acid (GABA) [11, 12, 15, 38], or glycine [42,43] may somehow mediate the anxiolytic activity of these drugs. It has recently been demonstrated that the benzodiazepines affect 5HT, GABA and glycine systems within the same dose range that they affect animal procedures predictive of anxiolytic activity [17, 18, 21]. Benzodiazepines also produce various side effects unrelated to their anxiolytic actions (i.e., sedation, muscular incoordination, etc.), but within the same dose range that they produce their anxiolytic actions [21,29]. For this reason, it has not been possible to determine which neurotransmitter actions, if any, are responsible for the anxiolytic activity and which are responsible for producing side effects.

Recently, brain stereo-specific, high affinity binding sites for ^{3}H-diazepam and ^{3}H-flunitrazepam have been reported in several species including humans [5–8, 10, 24, 27, 28, 34, 35]. Significant correlations have been obtained between the in vivo and in vitro ability of benzodiazepines to inhibit ^{3}H-benzodiazepine binding and the clinical usefulness of these drugs in humans [6, 10, 27, 35]. Significant correlations were also reported between the ability of benzodiazepines to inhibit ^{3}H-diazepam binding and their ability to inhibit pentylenetetrazole (PTZ)-induced convulsions [27,35] and to increase punished responding in a conflict situation [19], procedures which are also highly correlated with the anxiolytic activity of benzodiazepines [21]. For these reasons, it has been suggested that these binding sites represent a common receptor mechanism through which benzodiazepines produce their anxiolytic actions [7, 8, 10, 20, 28, 35].

These benzodiazepine receptors also demonstrate a remarkable selectivity for benzodiazepines. Because of the inability of a large number of drugs, hormones and transmitters to affect ^{3}H-diazepam binding [8, 24, 27, 28, 35], it has been suggested that ^{3}H-diazepam binding sites represent receptors for some as yet unidentified endogenous ligand(s) [9, 19, 20, 27, 35]. This hypothesis is supported by the ability of brain extracts to alter ^{3}H-diazepam binding [2, 16, 25, 31].

In the present study, we report on the properties of CL 218,872(3-methyl-6-[3-(trifluoromethyl)phenyl]-1,2,4-triazolo [4,3-b]pyridazine), a representative of a new class of pharmacologically unique substances [1,22] (see Fig. 1 for chemical structure), with highly specific actions on benzodiazepine receptors. This degree of specificity makes CL 218,872 an

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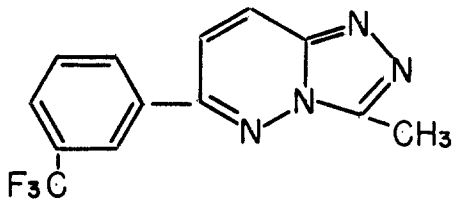

CL 218,872

3-methyl-6-[3-(trifluoromethyl)phenyl]-1,2,4-triazolo [4,3-b] pyridazine

FIG. 1. Chemical structure of CL 218,872.

excellent tool for assessing the relative importance of neuronal systems in mediating anxiolytic actions.

METHOD

Animals

Male Wistar rats (150–200 g) and Swiss Webster mice (20–25 g) were obtained from Royalhart Farms. All animals were housed 4–6 per cage with food (Purina Lab Chow) and water available ad lib. Subsequent deprivation conditions varied as function of testing procedure and are described below. For in vivo experiments, all drugs were suspended in 2% starch containing 5% PEG 400 and 1–2 drops of Tween 80 [21].

Receptor Binding Assays

A modification [4, 19, 20] of the binding assay described by Squires and Braestrup [35] and Mohler and Okada [27] was used to determine in vitro activity at the brain-specific benzodiazepine receptors. Food and water sated rats were sacrificed by decapitation and frontal cortex was dissected from the rest of the brain at the anterior border of the caudate nucleus. This tissue was weighed and gently homogenized (Potter-Elvehjem, teflon-glass homogenizer) in 20 volumes of ice cold 0.32 M sucrose solution. Homogenates were centrifuged twice at 1000 g for 10 min at 4°C. The pellets were discarded and supernatants recentrifuged at 30,000 g for 20 min at 4°C. The crude P_2 pellets thus formed were resuspended in twice the original homogenizing volume of cold 50 mM Tris·HCl buffer, pH 7.4. The binding assay consisted of 300 μl of the P_2-fraction suspension (approximately 350 μg tissue protein), 100 μl of test drug and 100 μl of ^{3}H-diazepam (39.8 Ci/mmole, New England Nuclear), which were added to 1.5 ml 50 mM Tris·HCl buffer, pH 7.4. The final concentration of ^{3}H-diazepam was 1.5 nM. Nonspecific binding controls and total binding controls received 100 μl of diazepam (3 μM final concentration) or 100 μl of deionized water, respectively, in place of test drug. After incubation at 0°C for 20 min, the reaction was terminated by rapid filtration under vacuum through Whatman GF/C filters. Following two 5 ml washes with iced Tris·HCl buffer, the filters were placed into scintillation vials and dried at 50–60°C for 30 min. After drying, 10 ml of (Beckman Ready-Solv HP) scintillation cocktail was added and radioactivity was determined in a Beckman Scintillation counter.

To assay specific binding of ^{3}H-spiroperidol (25.6 Ci/mmole, New England Nuclear) a P_2-fraction from striatal tissues was prepared as described above. This preparation was resuspended in cold 50 mM Tris·HCl buffer containing 0.5% ascorbic acid, 10 μM pargyline, 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$ and 1 mM $MgCl_2$ with an adjusted pH of 7.1. One hundred μl of this suspension (~300 μg of tissue protein) was incubated at 37°C for 10 min in the presence of 0.75 nM ^{3}H-spiroperidol in a final volume of 2.0 ml of buffer. After incubation, the tubes were placed into an ice bath and their contents were rapidly filtered under reduced pressure through Whatman GF/C filters. The filters were washed with two 5 ml rinses of ice-cold 50 mM Tris buffer, dried and the radioactivity determined by liquid scintillation spectrometry. Nonspecific binding of ^{3}H-spiroperidol was determined in the presence of 1 μM of unlabelled spiroperidol. Test substances, where indicated, were dissolved in diluted acid (0.01% acetic acid). At this concentration, this solvent has no effect on the binding of ^{3}H-spiroperidol.

Specific binding of ^{3}H-quinuclidinyl benzylate (^{3}H-QNB) (8.4 Ci/mmole, Amerstram/Searle) was assayed in rat striatal tissues by a slight modification of the method reported by Yamamura and Snyder [41]. Briefly, pooled striata were homogenized in sucrose as described above. The crude P_2 fraction was resuspended in 50 mM sodium-potassium phosphate buffer, pH 7.4. Fifty μl of this suspension (~150 μg of tissue protein) was incubated at 25°C for 1.0 hr with ^{3}H-QNB (2.5 nM) in a final volume of 2.0 ml of 0.05 M sodium potassium phosphate buffer, pH 7.4. Nonspecific binding of ^{3}H-QNB was determined in the presence of 100 μM of oxotremorine. Following incubation, the tubes were placed into an ice bath and 3.0 ml of ice-cold buffer were added to each sample. Ten minutes later, the contents were passed through a millipore filter (Type HAWP-02500, 0.45 μM) under moderate suction. The filters were rinsed twice with 5 ml of ice-cold buffer, and placed into counting vials. The filters were dried, 10 ml of scintillation cocktail was added and radioactivity was determined by liquid scintillation spectrometry.

Protein content of tissue samples in all binding assays described above was determined by the Lowry procedure [23] using bovine serum albumin as standard.

Rat Conflict Procedure

A modification [21] of an unconditioned passive avoidance procedure originally described by Vogel *et al.* [39] was used. Naive rats were water-deprived for 48 hr and food-deprived for 24 hr prior to testing. Sixty minutes after oral administration of graded doses of C L 218,872, diazepam or chlordiazepoxide,individual rats were placed in 4 1/4 in. wide ×6 1/2 in. deep, black Plexiglas chambers. A 10% dextrose solution was available from a single tap located in the rear of the chamber. A 0.3 mA constant current, 60 Hz, pulsed DC shocking circuit was established between the stainless steel grid floor and the tap. Each rat was allowed 20 sec of non-shocked drinking, after which time alternating cycles of 5-sec shock-off and 5-sec shock-on (where each lick on the tap was accompanied by shock) began and continued for a total of 5 min. The number of shocks received by

each rat was recorded and minimal effective doses (MED) were determined.

Ability to Protect Against Pentylenetetrazole (PTZ) Convulsions

Groups of 10 food (24 hr) deprived rats were treated orally with graded doses of CL 218,872, diazepam or chlordiazepoxide followed 60 min later by PTZ (23 mg/kg, intravenously into the caudal vein). This dose of PTZ was estimated to cause seizures in 99% of the rats. Animals were observed for at least 5 sec of uninterrupted clonic seizures, which occurred within several seconds of treatment. The number of animals protected from convulsions were recorded and median effective doses calculated [21].

Locomotor Activity

Groups of 8 food (24 hr) and water (48 hr) deprived rats were treated orally with graded doses of CL 218,872, diazepam or chlordiazepoxide. Sixty minutes after treatment, individual rats were placed into an activity meter (Animex®) and counts were recorded for 5 min. Percent change from parallel vehicle treated controls were calculated and the dose depressing locomotor activity by 50% (MD_{50}) was estimated by linear regression.

Ability to Remain on an Inclined Screen

The muscular incoordination produced by benzodiazepines was measured by determining the ability of rats tc remain on an inclined screen [21]. Groups of 8–10 rats were treated orally with graded doses of CL 218,872, diazepam or chlordiazepoxide and placed on a 60° inclined screen 60 min after treatment. Median effective doses for loss of the ability to remain on the screen were calculated [21].

Ability to Protect Against Convulsions Produced by Agents which Interfere with GABA or Glycine

Bicuculline and strychnine are believed to be receptor blockers for GABA and glycine, respectively [13, 14, 42], while isoniazid has been found to interfere with GABA synthesis [11,12]. Since benzodiazepines antagonize the convulsions produced by bicuculline, isoniazid and strychnine [11, 12, 17], we have utilized these procedures to estimate the possible GABA-ergic and glycine-ergic properties of CL 218,872.

Groups of 8 mice were treated orally with graded doses of CL 218,872 or diazepam. One hour later, all animals were treated with bicuculline solubilized in 0.1 N HCl in distilled water, at a dose of 0.6 mg/kg, intravenously, and observed for 5 min for tonic extensor seizures [17].

Groups of 8 mice were treated orally with graded doses of CL 218,872 or diazepam. Thirty minutes later all animals were treated with strychnine sulfate at a dose of 1.2 mg/kg, subcutaneously, in saline, and observed for 30 min for tonic extensor seizures. Median effective doses were calculated [17].

Groups of 5 rats were treated orally with graded doses of CL 218,872 or chlordiazepoxide. One hour later, isoniazid was administered subcutaneously at a dose of 450 mg/kg. This dose was estimated to cause seizures in all rats. Animals were observed for 60 min for convulsions.

Antagonism of l-Tryptophan-Induced Hyperthermia

The administration of *l*-tryptophan in combination with a monoamine oxidase inhibitor produces a measurable hyperthermia which appears to be 5HT mediated [22]. Since all anxiolytics tested to date inhibit this 5HT-mediated hyperthermia [22], we have utilized this procedure in an attempt to measure the anti-5HT properties of CL 218,872.

Groups of 6–8 rats were injected with tranylcypromine (20 mg/kg, IP) followed 30 min later by *l*-tryptophan (100 mg/kg, IP). Two hours after tranylcypromine, different groups of rats were injected intraperitoneally with either diazepam (1.25, 2.5, 5 or 10 mg/kg), chlordiazepoxide (5, 10 or 20 mg/kg), flurazepam (5, 10 or 20 mg/kg) or CL 218,872 (5, 10 or 20 mg/kg). Rectal temperatures were recorded 150–300 min after tranylcypromine injections.

RESULTS

In the ^{3}H-diazepam binding assay, diazepam, chlordiazepoxide and CL 218,872 each produced increasing amounts of inhibition in a dose related manner. The calculated IC_{50}'s were 6 nM, 116 nM and 248 nM for diazepam, CL 218,872 and chlordiazepoxide, respectively (Table 1). However, as can be seen in Fig. 2, the dose response curve for CL 218,872 was much flatter than the corresponding curves for diazepam and chlordiazepoxide. The Hill coefficient for CL 218,872 significantly deviated from unity, while the Hill coefficients for diazepam and chlordiazepoxide did not (Table 1). For this reason, an IC_{50} value may not accurately reflect the pharmacological potency of CL 218,872. In fact, it has recently been demonstrated that only 10–20% of benzodiazepine receptors must be occupied by diazepam in order to increase punished responding in a conflict situation [19,22] or to prevent PTZ-induced seizures [33]. Furthermore, IC_2 values of triazolopyridazines correlate best with the anti-PTZ properties of these drugs [1]. When IC_2 and IC_{20} values are calculated, the potency of CL 218,872 increases dramatically relative to the potency of diazepam (Table 1). By contrasts, CL 218,872 in concentrations as high as 1 μM did not significantly ($p>0.05$) alter ^{3}H-QNB or ^{3}H-spiroperidol binding (Table 2).

In the rat conflict procedure, CL 218,872 produced a dose-related increase in shocks, an effect similar to that produced by diazepam and chlordiazepoxide (see Fig. 3). The potency of CL 218,872 was comparable to that of diazepam and chlordiazepoxide with MED's being 0.8, 1.5 and 3.0 mg/kg for CL 218,872, diazepam and chlordiazepoxide, respectively. Likewise, CL 218,872 was also quite potent in its ability to inhibit PTZ-induced convulsions with ED_{50}'s in rats being 1.7, 1.6 and 2.4 mg/kg, for CL 218,872, diazepam and chlordiazepoxide, respectively (Table 3).

By contrast, CL 218,872 was very weak (both relative to the benzodiazepines and its own potency in inhibiting PTZ-induced convulsions) in its ability to inhibit the convulsions produced by bicuculline, isoniazid and strychnine. As can be seen in Table 4, chlordiazepoxide inhibited isoniazid-induced convulsions with an ED_{50} of 4 mg/kg. CL 218,872 was six times less potent than chlordiazepoxide with an ED_{50} of 25 mg/kg. CL 218,872 was approximately 15 times more potent in its ability to protect against PTZ than against isoniazid. Likewise, CL 218,872 was also weak in its ability to protect against bicuculline-induced convulsions (ED_{50} =31.2 mg/kg for CL 218,872 vs 1.6 mg/kg for diazepam) and even weaker in its ability to protect against strychnine-

TABLE 1

Drug	IC_{50} (nM)	IC_{20} (nM)	IC_2 (nM)	Hill Coefficient
Diazepam	6.0	1.5	0.12	0.99
Chlordiazepoxide	248.0	56.2	3.79	0.93
CL 218,872	116.2	9.1	0.09	0.54

TABLE 2

	^{3}H-QNB Binding* (p moles/mg protein)	^{3}H-Spiroperidol Bbinding 0051 (f moles/mg protein)
Control	1.90 ± 0.08	171.3 ± 2.3
CL 218,872 (1 μM)	1.76 ± 0.07	172.6 ± 3.2

*Data expressed as mean ± S.E.M.

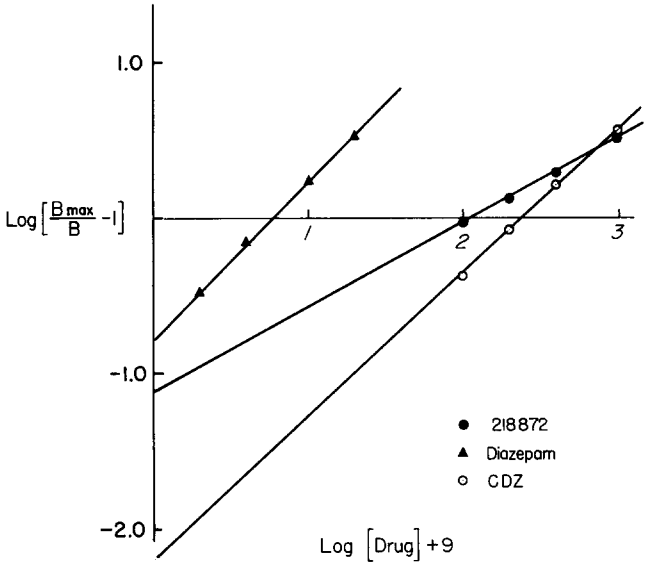

FIG. 2. Hill plots for diazepam, chlordiazepoxide and CL 218,872.

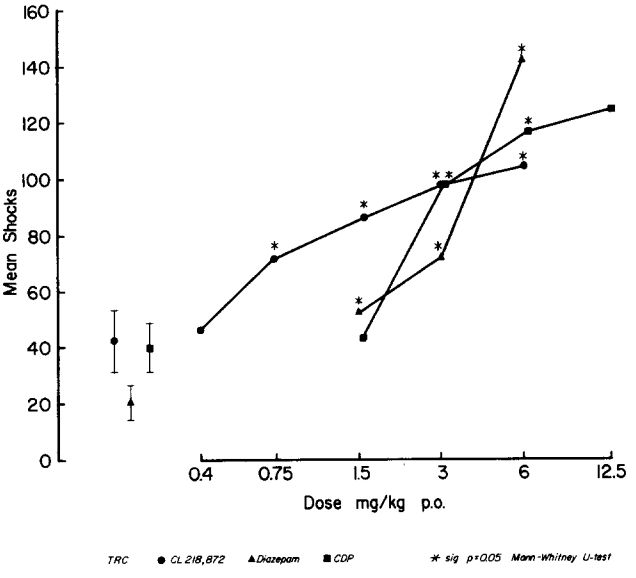

FIG. 3. Effects of diazepam, chlordiazepoxide and CL 218,872 on punished responding.

induced convulsions (ED_{50}=161.2 mg/kg for CL 218,872 vs 1.0 mg/kg for diazepam).

Diazepam, chlordiazepoxide and flurazepam produced dose-related reversals of the *l*-tryptophan and tranylcypromine-induced hyperthermia, with minimal effective doses being 2.5, 5.0 and 10.0 mg/kg, respectively (Fig. 4). CL 218,872 did not alter the hyperthermia with doses as high as 20 mg/kg (data not shown).

CL 218,872 depressed motor activity only very weakly relative to either the potency of the benzodiazepines (Table 5) or to its own potency in disinhibiting punished responding in the conflict test (Fig. 3). Similar potency differences between CL 218,872 and the benzodiazepines were observed in the inclined screen test (Table 5).

DISCUSSION

Several important facets of the mechanisms of benzodiazepine action may be discerned from these experiments. First, CL 218,872 displaced ^{3}H-diazepam from its binding sites on synaptosomal membrane fragments, with a potency intermediate between that of diazepam and chlordiazepoxide. The selectivity of this effect is reflected in the inability of CL 218,872 (1 μM) to affect ^{3}H-QNB and ^{3}H-spiroperidol binding. We believe this to be a highly significant finding, since it represents the first non-benzodiazepine

TABLE 3

TREATMENT	Median Effective Dose mg/kg, p.o. (A 95% Confidence Limit)
CL 218,872	1.7 (1.3–2.2)
Diazepam	1.6 (1.3–2.1)
Chlordiazepoxide	2.4 (1.8–3.2)

TABLE 4

Treatment	Loss of Ability to Remain on an Inclined Screen Median Effective Dose mg/kg, PO (95% Confidence Limit)	Dose Estimated to Reduce Locomotor Activity by 50% MD_{50}, mg/kg, PO
CL 218,872	396 (72–2165)	223
Diazepam	22 (12–38)	22
Chlordiazepoxide	34 (23–51)	52

TABLE 5

Treatment	Median Effective Dose (95% C.L.) for Protection against Seizures mg/kg, PO Bicuculline (mice)	Strychnine (mice)	Isoniazid (rats)
CL 218,872	31.2 (26–38)	161.2 (77–339)	25 16–39)
Diazepam	1.6 (1.1–2.3)	1.0 (0.8–1.4)	—
Chlordiazepoxide	—	—	4.0 (3.0–5.3)

which inhibits ^{3}H-diazepam binding with a potency comparable to that of the benzodiazepines.

Second, CL 218,872 protected against pentylenetetrazole-induced convulsions and increased punished responding in a conflict situation. Both of these properties are highly characteristic of anxiolytic drugs, and have been used as preclinical screens to predict anxiolytic activity [10]. Therefore, while CL 218,872 has yet to be tested in humans, the present results suggest the possibility that it may prove to be of some therapeutic value in the treatment of anxiety.

Third, CL 218,872 was very weak in tests believed to measure the muscular incoordination and sedation produced by the benzodiazepines [10]. Therefore, CL 218,872 produced inclined screen deficits only very weakly relative to either the potency of the benzodiazepines or its own potency in disinhibiting punished responding in the conflict test or protecting against pentylenetetrazole-induced convulsions. A similar lack of potency was observed in the ability of CL 218,872 to depress motor activity.

It is presently unclear why CL 218,872 is relatively devoid of side effects typically associated with benzodiazepines. One possibility may be that CL 218,872 selectively acts upon benzodiazepine receptors without affecting classical neurotransmitter systems. This is reflected in its relative inactivity in tests designed to detect actions on GABA, glycine and 5HT. Therefore, while the actions of benzodiazepines on GABA, glycine and 5HT may contribute to their anxiolytic properties, the present results minimize the necessity of

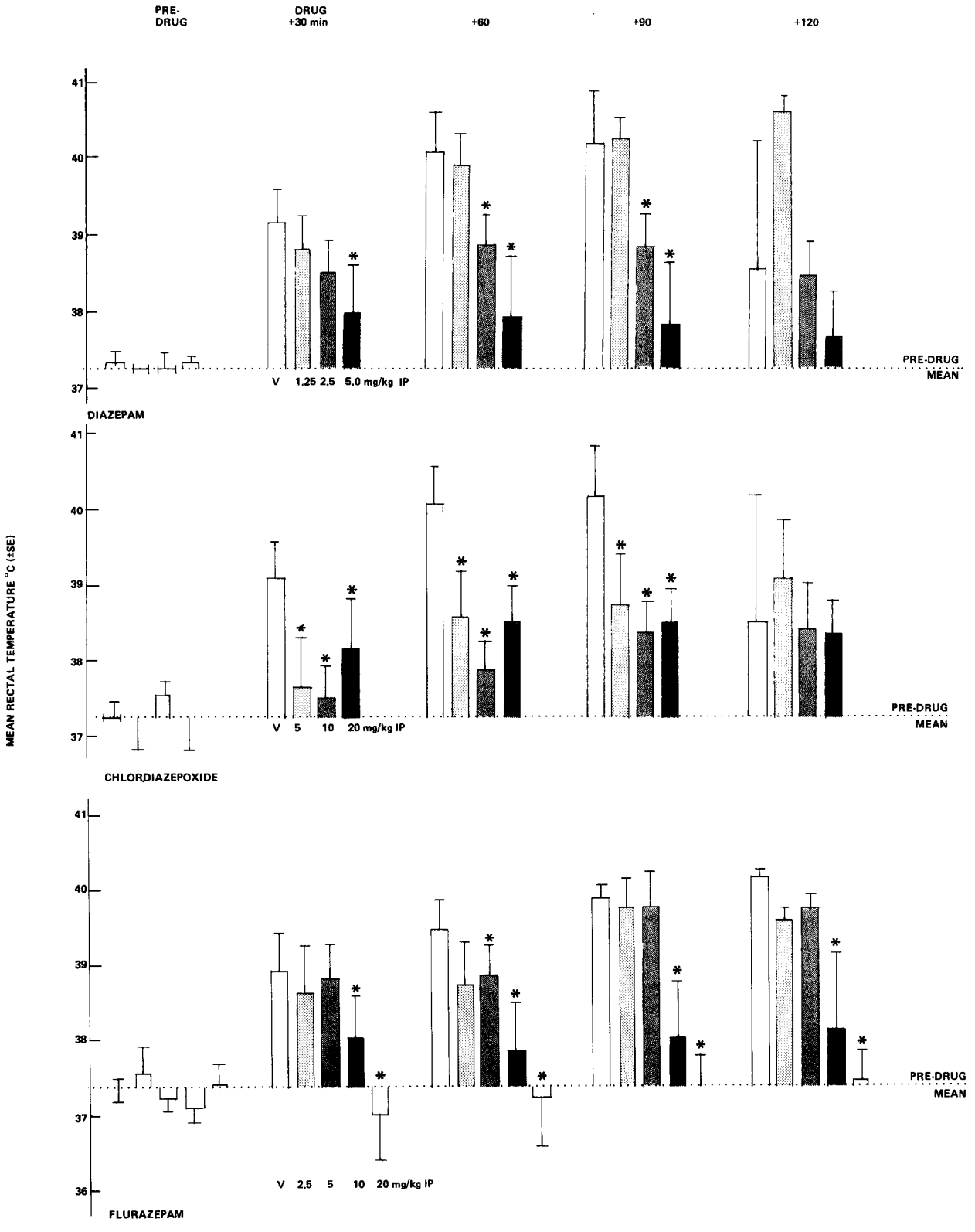

FIG. 4. Effects of diazepam, chlordiazepoxide and flurazepam on *l*-tryptophan and tranylcypramine induced hyperthermia.

these actions for producing anxiolytic activity and support their possible involvement in the production of some of the side effects typically associated with benzodiazepines.

The recent demonstration of multiple benzodiazepine receptors [30,36] also suggests the possibility that one class of receptors may be involved in the mediation of anxiolytic actions (Type I), while another class of receptors may be responsible for the production of side effects (Type II). According to this model, benzodiazepines have equal affinity for both types of receptors, and, thus, produce side effects along with their anxiolytic actions. CL 218,872, on the other hand, differentiates these multiple binding sites because of its higher affinity for Type I receptors [30,36]. Therefore, the relative lack of side effects produced by CL 218,872 may be due to its weaker actions on Type II receptors. Speculating further, the actions of benzodiazepines on Type II receptors may produce side effects by the intimate association of these receptors with other neurotransmitter systems (i.e., GABA, etc.).

Finally, reports of brain extracts containing endogenous, competitive inhibitors of benzodiazepine binding [2, 16, 25, 31] raise the possibility that these binding sites represent receptors for endogenous ligand(s), which may act as neurotransmitters. Quite recently, the purines, inosine and hypoxanthine, have been isolated from these brain extracts and suggested to be the endogenous ligands for the benzodiazepine receptors [2,31]. While these two substances are very weak in their ability to displace ^{3}H-diazepam (with Ki values of approximately 1 mM), intraventricular administration of inosine does protect somewhat against pentylenetetrazole (PTZ)-induced convulsions [32], a property also seen with benzodiazepines and highly correlated with their clinical efficacy [21]. As can be seen in Fig. 1, certain geometrical features of the CL 218,872 structure are surprisingly similar to the general structure of purines. Why then is CL 218,872 approximately 10^4 times more potent than inosine and hypoxanthine in its ability to act at the benzodiazepine receptor? One possibility may be that while the endogenous ligand is purine-like, it is neither inosine nor hypoxanthine, but these substances bear enough similarity to the ligand that they have still retained some, albeit, weak activity. Other purine-like substances such as cylic AMP can inhibit ^{3}H-diazepam binding [26] and have also been reported to display anti-conflict activity like benzodiazepines [3].

In summary, it is generally agreed that an understanding of the basic mechanisms by which the benzodiazepines produce their anxiolytic actions may provide valuable insights into those neuronal substrates responsible for the production of anxiety. While such an approach has yielded several hypotheses of benzodiazepine actions, interpretation of results from such studies has been complicated by the diverse pharmacological effects produced by the benzodiazepines. It is presently unclear as to which actions of the benzodiazepines are responsible for producing anxiolytic activity and which are responsible for producing side effects. CL 218,872, on the other hand, selectively acts upon so-called benzodiazepine receptors and displays anxiolytic properties in animals, while being relatively devoid of the side effects typically associated with benzodiazepines. For these reasons, it is our hope that CL 218,872 may represent a new probe for selectively investigating the neuronal substrates of anxiety.

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