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Relationship of the Effects of the Benzodiazepine Derivative Clorazepate on Corticosterone Secretion With its Behavioural Actions. Antagonism by Ro 15-1788

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MORMEDE, P., R. DANTZER AND A. PERIO. *Relationship of the effects of the benzodiazepine derivative clorazepate on corticosterone secretion with its behavioural actions. Antagonism by Ro 15-1788.* PHARMACOL BIOCHEM BEHAV **21**(6) 839–843, 1984.—The effectiveness of Ro 15-1788, a benzodiazepine receptor antagonist, in modifying the effects of the benzodiazepine derivative clorazepate on schedule-controlled behaviour and pituitary-adrenal activity of rats was investigated. At low doses (5 mg/kg) clorazepate increased punished responding and slightly decreased basal plasma corticosterone levels. At high doses (40 mg/kg), clorazepate suppressed fixed ratio responding, raised basal plasma levels of corticosterone and reduced the stress response produced by exposure to a novel environment. Pretreatment with Ro 15-1788 blocked the behavioural and neuroendocrine effects of the high dose of clorazepate. These results suggest that both types of effects of clorazepate share a common mechanism which involves brain benzodiazepine receptors.

Benzodiazepines Clorazepate Benzodiazepine antagonist Ro 15-1788 Punished responding FR responding Plasma corticosterone Anxiety Stress Novelty Rat

THE effects of anti-anxiety drugs on pituitary-adrenal activity have been documented in many reports but the results are still somewhat conflicting. Response to stress, measured by increases in plasma corticosteroids, has been shown to be attenuated by various benzodiazepines when injected in acute studies. This effect usually requires relatively high doses (e.g., 5–10 mg/kg diazepam in rats; [2, 15–17, 25]) although it has also been occasionally observed with very low doses (e.g., 0.1–0.5 mg/kg diazepam; [19]). Negative results have also been reported [1,8]. Baseline pituitary-adrenal activity may also be altered since a few studies suggest an increase in basal plasma corticosteroids under the influence of high doses of benzodiazepines [1, 15, 25].

An important question concerns the relationship between the effects of benzodiazepines on pituitary-adrenal activity and their anxiolytic and sedative properties. Reduction of the stress response has been suggested to reflect the anxiolytic activity of benzodiazepines while the enhanced basal plasma corticosteroid values have been attributed to non-specific stress effects arising from sedation and ataxia [17,23]. However, few attempts have been made to compare in the same study both behavioural and neuroendocrine effects of benzodiazepines.

The present experiment was undertaken to study the effects of a benzodiazepine derivative, clorazepate, on plasma corticosterone levels in rats, using doses selected on the basis of their behavioural effects in a conflict punishment procedure. In addition, we determined whether these effects were counteracted by the blockade of benzodiazepine receptors with Ro 15-1788 [14].

METHOD

Animals

Male Sprague-Dawley rats (IFFA-CREDO. Lyon) were used. For behavioural experiments they were housed one per cage and were kept at 80% of their free running body weight. For endocrine experiments, they were housed five or three per cage and had free access to food. Cages were in polypropylene with a sawdust litter. Water was available ad lib. The animal room was air conditioned (21°C, 55% relative humidity) and light controlled (light on from 7.00 to 19.00).

Apparatus

Behavioural experiments were run in standard Skinner boxes (Campden) fitted with one lever and a recessed food

tray. The cage contained a floor grid through which scrambled electric shocks (Shock Generator Campden, ref. 4104) could be delivered. Control of the operant schedule and recording of the results were done automatically by a minicomputer (PDP 8/F).

Drug

Clorazepate, dipotassium salt, was kindly supplied by Clin-Midy Laboratories (Montpellier, France). Doses are given as the salt. The drug was initially dissolved in saline, but precipitation could occasionally be observed with high concentrations in these conditions. It was therefore subsequently dissolved in distilled water and control animals received a 0.02 N NaOH solution in distilled water of the same pH (12.0) as the clorazepate solution.

PROCEDURES

All experiments took place in the morning between 9.00 and 13.00. Five experiments were carried out.

Dose Response Studies

Punished Behaviour. Six rats (300–320 g) trained for several months in an approach-avoidance conflict test modified from Davidson and Cook [7] were used to study the effects of clorazepate on punished behaviour. Lever presses in the Skinner box were reinforced according to a multiple schedule consisting of 10-min periods of variable interval (VI30) for food (45-mg Noyes pellet) alternating with 2-min periods of fixed ratio (FR3) for both food and electric shock (0.15–0.50 mA, 0.5-sec scrambled shock delivered through the grid floor). The FR3 component was signaled by a light located above the response lever. The session terminated 6 min after the beginning of the third VI period so that its total duration was 30 min. Clorazepate dissolved in distilled water was administered intraperitoneally at different dosages (1 to 32 mg/kg) to each rat used as its own control, 30 min before the beginning of the session. The order of treatment was randomized for each rat and a minimum of two drug-free sessions preceded each drug session. The total number of responses during the non-punished and the punished segments of the conflict session were recorded.

Plasma corticosterone levels and response to stress. Fifty rats, weighing 160 g (coefficient of variation=3%) and housed five per cage were used. Clorazepate was dissolved in saline and injected intraperitoneally in volume of 1 ml per animal. Controls received saline. The five treatments (four doses of clorazepate and saline) were randomized within each cage. Ninety minutes after the injection, half the animals were killed by decapitation. The other animals were placed individually in an opaque plastic bucket with a sawdust litter and killed by decapitation 30 min later, i.e., 2 hours after the injection.

Time Course Study of the Effects of Clorazepate on Plasma Corticosteroid Levels

Seventy-two rats, weighing 240 g (coefficient of variation=2.6%) and housed three per cage, were used. Clorazepate was dissolved in distilled water and was injected intraperitoneally at a dose of 5 or 40 mg/kg, in a volume of 0.5 ml per animal. Animals were killed by decapitation 15, 30, 60 or 120 min after the injection. Treatments were randomized between cages and each experimental group included 6 animals, in two cages.

Ro 15-1788 Antagonism of the Effects of Clorazepate

Behavioural study. Five rats (300–320 g) trained for several months in a FR10 schedule (one reinforcement for every 10 responses) were used. Ro 15-1788 at a dose of 25 mg/kg was suspended in a solution of 1% carboxymethylcellulose (CMC) and injected intraperitoneally 15 min before the injection of 40 mg/kg clorazepate dissolved in distilled water. The test session began 15 min later. Each animal was used as its own control and received in a random order either the combined treatment, the vehicles or CMC + clorazepate. Each drug session was separated from the preceding one with a minimum interval of 3 days. The total number of reinforcements obtained during a 15 min session was recorded.

Neuroendocrine effects. Forty-eight rats, weighing 188 g (coefficient of variation=2.9%) and housed three per cage, were used. Ro 15-1788 at a dose of 25 mg/kg was suspended in a solution of 1% CMC and injected intraperitoneally 15 min before the injection of 40 mg/kg clorazepate which was dissolved in distilled water. CMC and NaOH 0.02 N were used as control injections. Half the animals were put back in their home cage after the last injection while the others were placed individually in a plastic bucket with a sawdust litter. All animals were killed by decapitation 30 min after the last injection. Treatments were randomized between cages and each experimental group included 6 animals in 2 cages.

Corticosterone Determination

Trunk blood samples were collected on EDTA (50 μl of a 10% solution). After centrifugation, plasma was removed and stored at −20°C. Plasma corticosterone was measured by a competitive protein binding assay [22] with transcortin as 1% pregnant woman serum (0.4 to 12.8 ng/tube) or 0.5% male rhesus monkey serum (0.04 to 1.28 ng/tube) according to corticosterone levels in the sample. Tritiated corticosterone was used as the tracer and dextran-coated charcoal as absorbant of free radioactivity.

Statistical Analysis

In the approach-avoidance conflict test, non-punished responses on the drug session were expressed as percentage of non-punished responses on the last free-drug session. Punished responses were subjected to a logarithmic transformation in order to homogenize variances. Results were analyzed by analysis of variance with block effects.

Because the data exhibited a log-normal distribution, the plasma corticosterone levels were transformed into logarithmic values. Results are expressed as mean ± standard error of the mean (SEM). Two-way or 3-way analyses of variance were carried out, followed, when necessary, by a Newman-Keuls test for post hoc two by two comparisons of group means.

RESULTS

Dose-Response Studies

Punished behaviour. Figure 1 shows the effects of clorazepate on the approach-avoidance conflict test. Clorazepate increased the number of punished responses at doses as low as 2 mg/kg and a similar effect was also produced by all higher doses that were studied, $F(6,30)=3.81$ $p<0.01$. Nonpunished responses were depressed after 16 and 32 mg/kg clorazepate, $F(6,30)=25.1$ $p<0.001$.

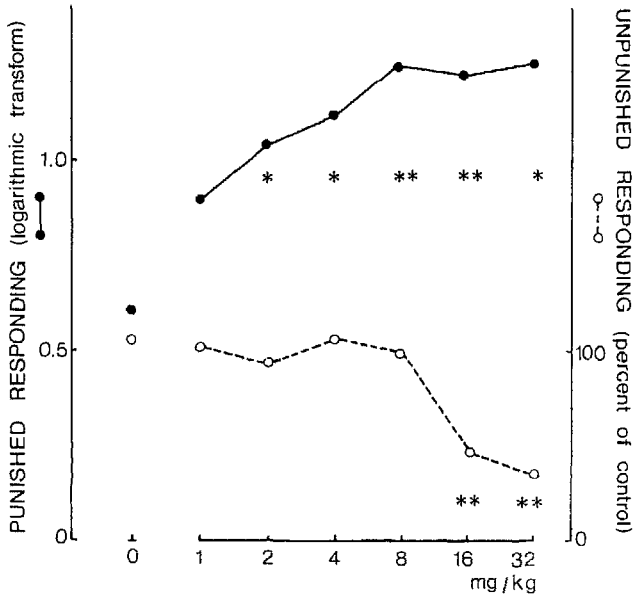

FIG. 1. Effects of clorazepate on punished behaviour and nonpunished behaviour in an approach-avoidance conflict. Solid line indicates effects on punished responses (after logarithmic transformation), broken line indicates effects on nonpunished responses (expressed as percent of response rate on the day preceding drug treatment: 1068±80 unpunished and 3.17±0.31 punished responses). Each point is the mean of six animals. * $p<0.05$, ** $p<0.01$ With respect to placebo-treated animals (dose 0).

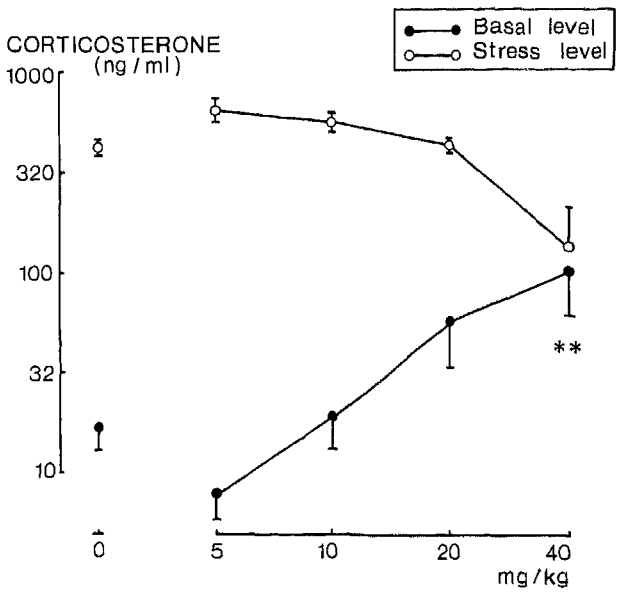

FIG. 2. Dose-response relationships of clorazepate on plasma corticosterone levels in rats left in their home cage (full circles) and in rats exposed for 30 min to a new environment (open circles). Each point represents the mean (±s.e.m.) of 5 rats (** $p<0.01$ with respect to control injection).

Plasma corticosterone levels and response to stress. The effects of clorazepate on basal plasma corticosterone levels and on response to stress are shown in Fig. 2. Two-way analysis of variance revealed that the dose factor was not significant, $F(1,40)=1.45$, unlike the significant stress factor, $F(1,40)=140.4$ $p<0.001$, and the significant interaction of stress with dose, $F(4,40)=10.3$, $p<0.01$. Plasma corticosterone levels were significantly increased by stress in all groups except after 40 mg/kg clorazepate. This dose significantly increased the basal levels of plasma corticosterone and decreased the response to stress. A slight decrease in basal plasma corticosterone levels was observed after 5 mg/kg clorazepate, but this was not significant.

Time Course Study

Figure 3 shows the time-response curve for clorazepate on basal plasma corticosterone levels. A two-way analysis of variance revealed that the dose factor, $F(2,60)=23.1$, $p<0.001$, the time factor, $F(3,60)=6.11$, $p<0.001$, and the interaction of dose with time, $F(6,60)=4.89$, $p<0.001$, were all significant. Post hoc comparisons of group means showed that in comparison with placebo-treated animals, 5 mg/kg clorazepate induced a significant drop in plasma corticosterone 2 hours after the injection and that 40 mg/kg clorazepate induced a significant increase which was maximum 30 min after the injection and still apparent at 60 min.

Ro 15-1788 Antagonism

Behavioural data. Rats trained under the FR10 schedule of food reinforcement and treated with 40 mg/kg clorazepate 15 min before the FR10 session totally ceased responding for the duration of the session. Rats treated with placebo ob-

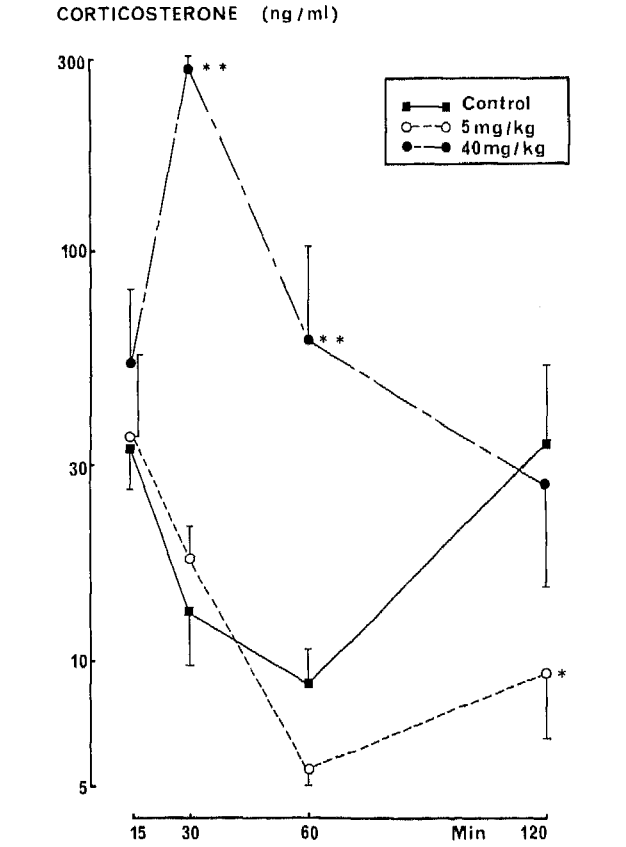

FIG. 3. Corticosterone levels at different times after clorazepate treatment. Rats injected at time 0 with clorazepate were put back into their home cage and killed at different intervals. Each point is the mean (±s.e.m.) of 6 rats (* $p<0.05$, ** $p<0.01$ with respect to controls).

tained 88±6.8 (mean±SEM) reinforcements per session. Pretreatment with 25 mg/kg Ro 15-1788 reversed the effects of clorazepate (mean number of reinforcements: 65±13.7, $p>0.10$ with comparison to placebo-treated animals).

Neuroendocrine effects. Table 1 shows the effects of a combined treatment with clorazepate and Ro 15-1788 on basal plasma corticosterone levels and response to stress. A 3-way analysis of variance revealed that the main effect of stress, $F(1,40)=52.2$, $p<0.001$, was significant, together with the interaction stress × clorazepate, $F(1,40)=25.9$, $p<0.001$, stress × Ro 15-1788, $F(1,40)=6.61$, $p<0.05$, and stress × clorazepate × Ro 15-1788, $F(1,40)=11.26$, $p<0.01$. Post hoc comparisons of group means showed that 40 mg/kg clorazepate induced an increase in basal plasma corticosterone levels which was blocked by prior administration of Ro 15-1788. In animals placed in the novel environment, 40 mg/kg clorazepate induced a drop of plasma corticosterone levels, and this effect was blocked by prior administration of Ro 15-1788. This last compound had no effect of its own on either basal plasma corticosterone levels or on response to stress.

TABLE 1

ANTAGONISM BY Ro 15-1788 OF THE EFFECTS OF CLORAZEPATE ON PITUITARY-ADRENAL ACTIVITY

Basal levels	Ro 0	CZ 0	12.3 (9.36–16.2)	
		CZ 40	158.0 (125–201)	*
	Ro 25	CZ 0	16.5 (11.5–23.7)	
		CZ 40	32.8 (22.1–48.8)	
Stress levels	Ro 0	CZ 0	255.0 (228–286)	†
		CZ 40	65.7 (35.0–123)	*
	Ro 25	CZ 0	238.0 (152–373)	†
		CZ 40	211.9 (181–247)	†

For each group is given the mean value of plasma corticosterone levels, in ng/ml, and the range of variation (6 rats per group). *$p<0.01$ with comparison to placebo-treated animals (Ro 0, CZ 40); †$p<0.01$ with comparison to basal levels.

DISCUSSION

The ability of agents with clinical antianxiety activity to restore responding suppressed by punishment has been documented by many investigators [5] and the approach-avoidance conflict test appears to be one of the most sensitive test for detecting this activity. This technique allows also to determine the extent to which the drug is selectively affecting punished, as opposed to non-punished, behaviour [4]. In the present case, clorazepate produced significant anticonflict activity over a wide range of doses, while unpunished behaviour was only affected at high doses. This decrease in unpunished responding is generally interpreted as indicating the onset of general depressant effects while the increase in punished responding is interpreted as specifically reflecting the antianxiety activity. From the results of the approach-avoidance conflict test, it is therefore possible to select doses of a benzodiazepine compound which has predominant anxiolytic activity or predominant sedative effects.

The present experiments show that clorazepate has three main effects on pituitary-adrenal activity. At sedative doses (40 mg/kg), it raises basal plasma corticosterone levels and inhibits the stress response to a novel environment. High acute dosages of benzodiazepines have been shown to antagonize the activation of the pituitary-adrenal axis caused by stressful situations, e.g., novel environments and/or sound stimulation [1, 9, 10, 17, 25] electric footshocks [2,16], forced swimming [19] and physical restraint [9,15]. When basal levels were measured, they were shown to be increased by similar doses of the drugs [1, 15, 18, 25] suggesting that the same mechanism is responsible for both the increase of basal levels and the reduction of post stress values. At anxiolytic doses (5 mg/kg), clorazepate slightly decreases basal plasma corticosterone levels. This effect appears later than the classical action observed with sedative doses, but has no counterpart on the stress response.

One major question is the relationship between the anxiolytic properties of benzodiazepines and their action on the hypophysio-adrenocortical system. The present results show that the classical endocrine effects of benzodiazepines are obtained with high, sedative, doses of clorazepate. The same result was obtained with diazepam (unpublished results) studied in the same experimental paradigms: punished responding was significantly increased at 2 mg/kg (anxiolytic dose), unpunished responding was decreased by more than 50% at 8 mg/kg (sedative dose) and 10–20 mg/kg were necessary to induce endocrine effects (increase of basal levels and reduction of stress response). It is therefore obvious that the action of benzodiazepine on the hypophysio-adrenocortical system is not related to their primary anxiolytic properties, which may be demonstrated with low doses of the drugs, but rather to their sedative effect, which becomes predominant when doses are increased. This conclusion is consistent with the most of the experimental evidence obtained with acute benzodiazepine administration. One report is at variance with these results. Le Fur and colleagues [19] described a decrease of stress corticosteroid levels after low doses of benzodiazepines such as 0.5 mg/kg of diazepam. Basal corticosteroid levels under treatment were not reported. However, the short delay between stress induction and blood sampling (5 min) suggest that post stress values could have been influenced by a possible reduction of basal levels by the low doses of drugs used. Furthermore, Fekete and colleagues [8] could not replicate these results, so that they need confirmation.

The relationship between the effects of benzodiazepines on pituitary-adrenal activity and their anxiolytic and sedative effects can also be investigated by recourse to chronic drug studies. The classical view is that there is tolerance to the sedative effects of benzodiazepines but not their anxiolytic activity during the course of repeated treatments (e.g., [4]). Therefore, a tolerance to the drug-induced increases in basal plasma corticosterone levels but not to the drug-induced decreases in response to stress would indicate that the former effect is related to the sedative properties of benzodiazepines while the latter would be accounted for by their anxiolytic activity. However, the literature on the neuroendocrine effects of chronic benzodiazepine treatment is still more confusing than in the case of acute studies. This effect has been described to be either enhanced by diazepam [18] or chlordiazepoxide [10] or reduced by the same drugs [1,25] during chronic treatment. We were unable to show any change in the effect of clorazepate during the course of a 5-day treatment [21]. Differences in experimental design, doses, length of treatment could explain these divergent results, as shown by File [9]. This point remains open to further investigation.

The mechanisms of the neuroendocrine effects of benzodiazepines are not peripheral since the in vitro and in vivo

reactivity of the adrenal cortex to ACTH is not altered in benzodiazepine-treated rats [15,17]. There is the possibility that the effects of benzodiazepines on the pituitary-adrenal axis are mediated by the central neural pathways which modulate the synthesis and release of ACTH [12]. Benzodiazepines have been shown to exert their effects at specific brain benzodiazepine receptors [20,24]. The understanding of the molecular mechanisms of action of benzodiazepines has been greatly improved recently by the synthesis of specific antagonists at the receptor level. Ro 15-1788, an imidazobenzodiazepine derivative, antagonizes many pharmacological effects of benzodiazepines mediated by the high affinity binding sites in brain membranes [14]. This antagonist blocks both the anticonflict and the sedative properties of benzodiazepine agonists [3,26]. Although this compound has been found to have partial agonist properties [6, 11, 13], we did not find in preliminary experiments any effect of Ro 15-1788 per se on pituitary-adrenal activity in doses up to 100 mg/kg. The demonstration in the present experiment of a blockade by Ro 15-1788 of the effects of clorazepate on plasma corticosterone in rats enlarges the range of benzodiazepine effects antagonized by this compound. Both behavioural and endocrine effects of clorazepate were shown here to be antagonized by Ro 15-1788, which suggests that they share a common receptor mechanism, the level of which is not yet known.

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