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F 2692: Flumazenil-Reversible Anxiolytic Effects but Inactive on [^{3}H]-Ro 15-4513 Binding

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FILE, S. E. AND N. ANDREWS. *F 2692: Flumazenil-reversible anxiolytic effects but inactive on [^{3}H]-Ro 15-4513 binding.* PHARMACOL BIOCHEM BEHAV **48**(1) 223-227, 1994.—F 2692, a pyridazine derivative, has little affinity for benzodiazepine receptors, yet in two animal tests its anxiolytic effects have been reported to be reversed by benzodiazepine antagonists. In the rat social interaction test, after 5 days of IP treatment, F 2692 (3, 10, or 30 mg/kg) produced greater increases in social interaction than diazepam (0.3, 1, or 3 mg/kg). A comparison of acute and 5 day administration of F 2692 showed rapidly developing tolerance at all doses. The acute anxiolytic effects of F 2692 (1 mg/kg) were reversed by the benzodiazepine antagonists flumazenil (4 mg/kg) and ZK 93426 (4 mg/kg). We, therefore, examined whether F 2692 was active at a benzodiazepine binding site (the diazepam-insensitive portion of [^{3}H]-Ro 15-4513) to which flumazenil but not flunitrazepam binds. However, F 2692 (10^{-9} to 10^{-4} M) was without effect on this binding. Thus, F 2692 has anxiolytic actions in the social interaction test, that are greater than those of diazepam, and which can be reversed by benzodiazepine antagonists. However, the site of action of the compound remains unknown.

Anxiety Benzodiazepines 5-HT Rat social interaction Ro 15-4513 binding

A POTENTIAL anxiolytic compound, F 2692 (1-(3′-trifluoro-methylphenyl) 1,4-dihydro 3-amino 4-oxo 6-methyl pyridazine) has recently been described, which has no affinity for $GABA_A$, α_2, $5\text{-}HT_{1A}$, or $5\text{-}HT_2$ receptors and only very weak affinity for benzodiazepine receptors in vitro or in vivo (1). In the elevated plus-maze test in the rat and the black/white crossing test in the mouse, F 2692 had anxiolytic actions and, surprisingly, these were antagonised by the benzodiazepine receptor antagonists, flumazenil and ZK 93426 (1).

A possible mechanism for its anxiolytic actions is suggested by the report that F 2692 inhibits the enhanced 5-HT release in the ventral hippocampus that was found in rats exposed to the elevated plus maze (8). There is evidence from factor analysis that different animal tests of anxiety are measuring different underlying factors (5) and exposure to the plus maze and social interaction tests has different effects on synaptic availability of 5-HT and GABA (8,6). The purpose of the present experiments was, therefore, to extend the investigation of the potential anxiolytic effects of F 2692 to the social interaction test. Experiment 1 compared the effects of 5 days of treatment with diazepam or F 2692, and Experiment 2 compared the effects of acute and 5 days treatment with F 2692. Experiment 3 examined whether the anxiolytic effects of acute administration of F 2692 could be reversed by the benzodiazepine receptor antagonists flumazenil and ZK 93426.

Finally, we explored a possible site of action of F 2692. Assié et al. (1) showed that F 2692 only weakly inhibits [^{3}H]-flunitrazepam binding to cortical membranes, but it is possible that F 2692 binds to a benzodiazepine binding site to which flunitrazepam does not bind. Such a binding site (termed diazepam insensitive) has been identified by using [^{3}H]-Ro 15-4513 as the radioligand (7). The binding of this ligand has both a diazepam sensitive and diazepam insensitive component. However, both flumazenil and ZK 93426 antagonised the diazepam insensitive binding. We, therefore, investigated the effects of F 2692 on this binding.

METHOD

Animals

Male hooded Lister rats (Olac, Bicester), weighing approximately 200 g at the start of each experiment, were housed in

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groups of five until 5 days before social interaction testing, when they were singly housed. Food and water were available at all times, room temperature was maintained at 22°C, and lights were on from 0700–1700 h.

Apparatus

The social interaction test arena was a wooden box 60 × 60 cm, with 35 cm high walls and was lit by high (350 lux) or low (<35 lux) light. A camera was mounted vertically above the arena and the rats were observed from a video monitor in the adjacent room. Infrared photocells were mounted in the walls, 4.5 cm and 12.5 cm from the floor, and the interruption of these beams provided automated measures of locomotor activity and rearing, respectively. The output from the photocells and the scores of the observer were entered into a computer.

Drugs

For the behavioural studies, F 2692 (Pierre Fabre Medicament), ZK 93426 (Schering), diazepam, and flumazenil (Roche Products Ltd.) were prepared in a vehicle of distilled water and Tween 20 and sonicated in an ultrasonic water bath for 30 min prior to injection. All drugs were injected IP in a volume of 2 ml/kg. For radioligand binding [7,9-^{3}H]-Ro 15-4513 (28.8 Ci/mmol) was obtained from NEN, UK. Diazepam, flumazenil and F 2692 were dissolved in ethanol and diluted to the relevant concentrations immediately before incubation. TRIS base (Sigma) was dissolved in distilled water at a concentration of 50 mM and the pH adjusted to 7.4 at 0–4°C with concentrated HCl.

Procedure

Behavioural studies.

Experiment 1. Five days prior to social interaction testing, animals were randomly assigned to either control (distilled water/Tween 20; n = 16 pairs), F 2692 (3, 10, or 30 mg/kg; n = 16 pairs per dose), or diazepam (0.3, 1, or 3 mg/kg; n = 14 pairs per dose) groups. Each rat received an IP injection of vehicle or drug, as appropriate, once per day for 5 days. On the day prior to testing, animals were allocated to test partners on the basis of body weight such that each pair did not differ by more than 5 g. Both members of a pair always received the same drug treatment; each rat was injected 30 min prior to testing. Both high and low lighting conditions were employed, and all animals were unfamiliar with the test arena [for more details of social interaction testing, see File (3)]. Eight pairs of each treatment group were tested in the high light condition and eight pairs of the control and F 2692 groups and six pairs of the diazepam groups were tested in the low light condition.

Pairs of rats were tested for a period of 7.5 min, in an order randomised for drug treatment between 0800 and 1200 h. The time spent in active social interaction was scored by an observer blind to drug treatment. Behaviours classed as active social interaction were sniffing, following, allogrooming, crawling under or over the partner, boxing, kicking, and biting.

Experiment 2. Animals were assigned to pretreatment groups receiving five daily injections of either vehicle (distilled water/Tween 20) or F 2692 (0.3, 1, or 3 mg/kg). On the test day, the vehicle-pretreated animals received an IP injection of either vehicle (n = 8 pairs per group) or F 2692 (0.3 mg/kg, n = 8 pairs/group; 1 or 3 mg/kg, n = 4 pairs/group). Those pretreated for 5 days with F 2692 received an IP injection of the same dose of F 2692 on the test day (n = 8 pairs/group).

Testing took place 30 min after drug injection in the high light, unfamiliar test condition, and was as described for Experiment 1, except that the animals were tested for 4.5 min.

Experiment 3. Rats were randomly allocated to the following acute treatment groups (n = 8 pairs/group): control (two injections of distilled water/Tween 20); control + flumazenil (4 mg/kg); control + ZK 93426 (4 mg/kg); F 2692 (1 mg/kg) + vehicle; F 2692 (1 mg/kg) + flumazenil (4 mg/kg); F 2692 (1 mg/kg) + ZK 93426 (4 mg/kg). The first injections of vehicle, F 2692, or ZK 93426 were administered 30 min prior to testing, the second injection of vehicle or flumazenil was administered 20 min before testing. All injections were IP, and rats were tested in an order randomised for drug treatment. Testing was as described for Experiment 2.

[^{3}H]-Ro 15-4513 binding to cerebellar membranes.

Preparation of membranes. Rats were stunned and killed by cervical dislocation and the cerebellum dissected. The tissue was homogenised in distilled water using a Polytron homogen-

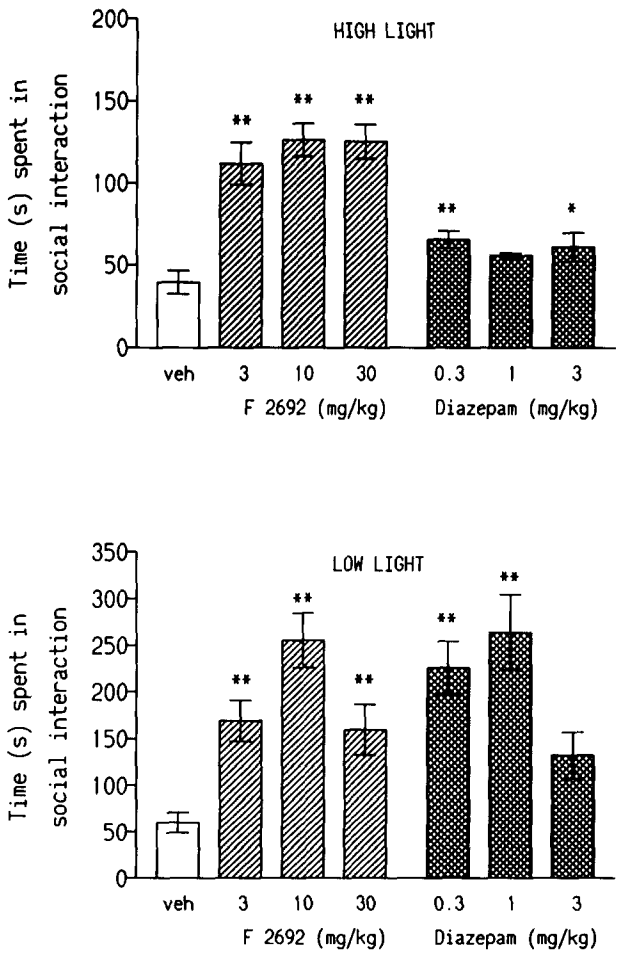

FIG. 1. Mean (±SEM) time (s) spent in social interaction by pairs of rats treated for 5 days and tested 30 min after one further injection of either vehicle, F 2692 (3, 10, or 30 mg/kg) or diazepam (0.3, 1, or 3 mg/kg). Animals were tested in either high (upper panel) or low (lower panel) light conditions. *P < 0.05, **P < 0.01 post hoc Duncans tests after ANOVA compared with vehicle group.

iser (setting 6.5) for 15 s at a concentration of 6.6 mg/ml. The homogenate was then centrifuged at 32000 × *g* for 20 min at 0–4°C. The resulting pellet was resuspended in Tris buffer and spun a further three times, the final pellet being frozen overnight at −20°C. The following day the pellet was thawed, resuspended in Tris buffer, centrifuged, and the pellet resuspended at a final tissue concentration of 3.3 mg/ml in Tris buffer.

Assay

Total binding was defined by incubating cerebellar tissue with [^{3}H]-Ro 15-4513 (2 nM) in a final assay volume of 0.5 ml. Nonspecific binding was defined by the addition of 100 μM flumazenil. The diazepam insensitive binding was demonstrated by subtracting the nonspecific binding defined by 100 μM flumazenil from the binding found following addition of 100 μM DZ (a concentration at which maximal inhibition of the diazepam sensitive is achieved; see Fig. 4). The effect of F 2692 on the diazepam-insensitive binding site was investigated by incubating 100 μM DZ with increasing concentrations of F 2692 (10^{-9}–10^{-4} M). Incubation (in triplicate) was for 90 min on ice and was stopped by rapid filtration through Whatman GF/B filter papers using a Millipore manifold system. Filters were placed in vials with 3 ml of emulsifier safe (Packard) and left overnight before liquid scintillation counting using an LKB Rackbeta 1214 liquid scintillation counter.

Statistics

The data from Experiment 1 were analysed by single factor analyses of variance (ANOVA) with post hoc Duncan's tests for comparisons between individual groups. The data from Experiments 2 and 3 were analysed by two-factor ANOVAs, followed by Duncan's tests. In Experiment 2, the two factors were the chronic and acute treatments (a significant acute × chronic treatment interaction would show a significant modification of the acute effect). In Experiment 3, the two factors were F 2692 treatment and either flumazenil or ZK 93426 treatment (a significant F 2692 × antagonist interaction would show that the acute anxiolytic effect of F 2692 was modified by antagonist administration).

TABLE 1

MEAN (±SEM) LOCOMOTOR AND REARING SCORES OF PAIRS OF RATS DURING SOCIAL INTERACTION IN HIGH LIGHT, UNFAMILIAR TEST CONDITIONS

Drug Group Control	Locomotor Activity	Rears
Acute F 2692		
(mg/kg IP)	64.8 ± 8.7	24.8 ± 2.7
0.3	62.4 ± 5.8	32.4 ± 4.2
1	66.8 ± 8.6	31.8 ± 2.8
3	59.8 ± 11.4	30.8 ± 6.0
Subchronic F 2692		
(mg/kg IP)		
0.3	85.1 ± 12.6	29.3 ± 4.0
1	82.0 ± 11.4	29.1 ± 3.8
3	73.3 ± 7.3	25.6 ± 1.3

Rats were tested 30 min after IP injection of either vehicle (control), acute F 2692 (0.3, 1 or 3 mg/kg) or subchronic F 2692 (0.3, 1, or 3 mg/kg).

RESULTS

Experiment 1

The rats treated with F 2692 (3, 10, or 30 mg/kg for 5 days) showed increased social interaction in both the high, $F(3, 28) = 16$, $p < 0.0001$, and low, $F(3, 28) = 11.6$, $p < 0.0001$, light test conditions. Post hoc comparisons showed that F 2692 increased social interaction at all doses tested, see Fig. 1. The increase in social interaction was specific, as none of the doses increased locomotor activity or rearing; in the low light testing condition the 30 mg/kg dose lowered levels of rearing ($p < 0.05$).

Diazepam (0.3, 1, or 3 mg/kg for 5 days) also increased social interaction in both lighting conditions [high light: $F(3, 28) = 3.3$, $p < 0.05$; low light, $F(3, 22) = 11.9$, $p < 0.001$].

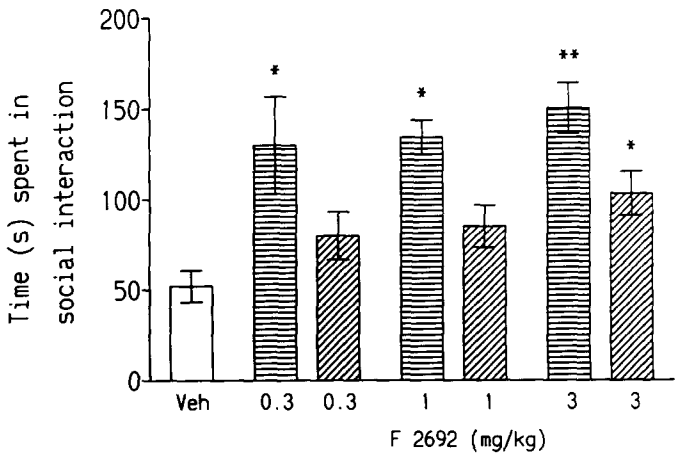

FIG. 2. Mean (±SEM) time (s) spent in social interaction by pairs of rats tested in high light after five daily injections and an acute injection of vehicle (veh), five daily injections of vehicle and an acute injection of F 2692 (0.3, 1, or 3 mg/kg; horizontal shading), or five daily injections and a test injection of F 2692 (0.3, 1, or 3 mg/kg; hatched bars). *$P < 0.05$ compared with vehicle group.

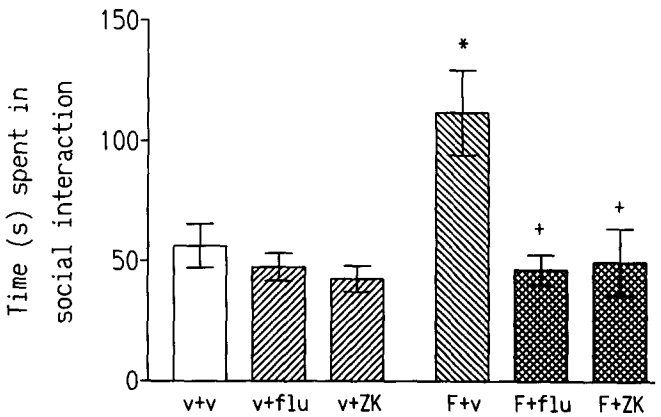

FIG. 3. Mean (±SEM) time (s) spent in social interaction by pairs of rats tested in high light after injection with vehicle (v) or F 2692 (1 mg/kg, F) plus vehicle (v), flumazenil (4 mg/kg, flu), or ZK 93426 (4 mg/kg, ZK). *$p < 0.05$ compared with v+v group; $^+p < 0.05$ compared with F+v group.

In contrast to F 2692, post hoc comparisons showed that not all doses of diazepam significantly increased social interaction in both lighting conditions (see Fig. 1). While locomotor activity was not altered by any dose of diazepam in either lighting level, the highest dose decreased rearing in both high ($p < 0.05$) and low ($p < 0.05$) lighting conditions.

Experiment 2

F 2692 was less effective following 5 days of treatment than after acute administration, $F(1, 34) = 1.5$, $p < 0.01$. After acute administration, all doses of F 2692 increased social interaction, whereas after 5 days of treatment only the 3 mg/kg dose reached significance (see Fig. 2). There were no significant effects on locomotor activity or rearing activity, see Table 1.

Experiment 3

There was a significant increase in social interaction following acute administration of F 2692 [F 2692 factor, $F(1, 28) = 6.4, p < 0.05$] and a significant decrease in social interaction following administration of ZK 93426 [ZK 93426 factor, $F(1, 28) = 9.4$. $p < 0.01$]; (see Fig. 3) the F 2692 × ZK 93426 interaction just failed to reach significance ($p = 0.06$) There were no effects on locomotor activity or rearing by any of the drug combinations when compared with control.

There was a significant F 2692 × flumazenil interaction, $F(1, 28) = 6.9$, $p < 0.05$, which showed that the anxiolytic effects of F 2692 were significantly altered by the presence of flumazenil (see Fig. 3). There were no effects on locomotor activity or rearing by any of the drug combinations when compared with control.

[^{3}H]-Ro 15-4513 Binding

The results demonstrate a diazepam sensitive component (approximately 65% of total specific binding) and the IC_{50} for diazepam was 29.1 ± 0.7 nM. Flumazenil (100 μM) antagonised all but 10% of the total [^{3}H]-RO 15-4513 binding. Thus, the difference between the binding displaced maximally by diazepam and the binding displaced by flumazenil produced a diazepam insensitive component of approximately 25%. F 2692 was found to have a negligible effect on the diazepam insensitive portion of the [^{3}H]-RO 15-4513 binding, see Fig. 4.

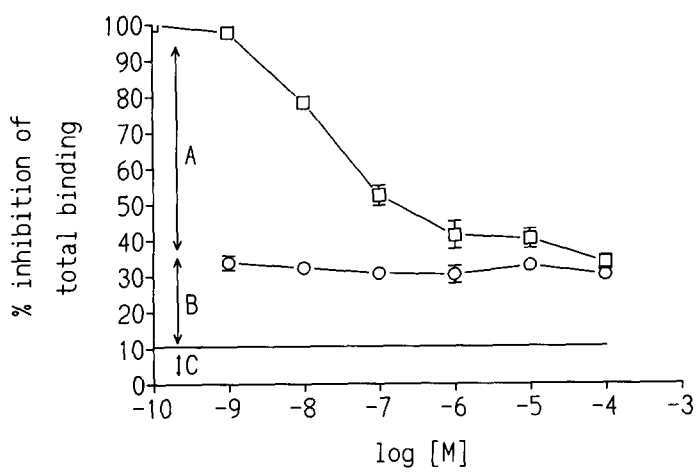

FIG. 4. Displacement of [^{3}H]-RO 15-4513 (2 nM) binding to rat cerebellar membranes by diazepam. Membranes were prepared as decribed in the Method section. Nonspecific binding was defined with 100 μM flumazenil. Effect of F 2692 on diazepam insensitive binding was studied by adding varying concentrations of F 2692 in the presence of 100 μM diazepam. The results are the mean of four separate experiments performed in triplicate.

DISCUSSION

Our results show that F 2692 has strong anxiolytic actions in the rat social interaction test at doses lower than those previously reported effective in the elevated plus maze (1). After 5 days of treatment there was development of tolerance to the effects of all the doses that we tested (0.3, 1, and 3 mg/kg). This is a much more rapidly developing tolerance than has generally been found for the anxiolytic properties of diazepam (4) and is further evidence that F 2692 is not acting at the same receptor as diazepam. However, our tolerance results with F 2692 contrast with the previous report of no tolerance after 21 days of treatment with a higher dose range (3–30 mg/kg) to the effects in the plus maze (2). It is unlikely that the difference between the two studies lies simply with the doses investigated, because we found equal loss of efficacy with all doses, and Chopin et al. (2) found no tolerance with any dose. The development of tolerance to anxiolytic effects in one animal test, but not another, further underlines suggestions that different animal tests may be measuring distinct underlying factors and are sensitive to different neuropharmacological manipulations. However, in agreement with results from both the plus maze and the mouse black/white crossing test, we found the effects of F 2692 were reversed by the benzodiazepine antagonists flumazenil and ZK 93426.

It has been found that F 2692 can reduce the increased 5-HT release measured in the ventral hippocampus following exposure to the elevated plus maze (8). Indeed, the authors proposed that the ability to decrease 5-HT release could be the mechanism by which F 2692 exerted its anxiolytic effects. Unfortunately, this does not provide an explanation of the reversal by flumazenil of the anxiolytic effects of F 2692, because flumazenil has also been shown to decrease 5-HT release in the ventral hippocampus of animals exposed to the elevated plus maze (8). The results of our binding study also exclude the possibility that F 2692 acts on the diazepam-insensitive component of the [^{3}H]-RO 15-4513 binding site. The reversibility of the effects of F 2692 by flumazenil raises the possibility that it has actions at a nonbenzodiazepine site, in which case antagonism of a drug effect by flumazenil can no longer be regarded as a sufficient condition for establishing a benzodiazepine site of action.

In conclusion, F 2692 has anxiolytic effects in the rat social interaction test, to which tolerance developed after 5 days of administration. Furthermore, the acute anxiolytic effect of F 2692 was completely reversed by administration of the benzodiazepine antagonist flumazenil, even though F 2692 possesses only micromolar affinity for the $GABA_A$ receptor and flunitrazepam binding sites, and does not act at the diazepam-insensitive [^{3}H]-RO 15-4513 binding site.

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