Pharmacology Biochemistry & Behavior, Vol. 39, pp. 379–382. © Pergamon Press plc, 1991. Printed in the U.S.A. 0091-3057/91 $3.00 + .00

Role of cGMP in the Mechanism of Anxiolytic Activity of U-78875

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Received 16 July 1990

SETHY, V. H. AND T. T. OIEN. *Role of cGMP in the mechanism of anxiolytic activity of U-78875.* PHARMACOL BIOCHEM BEHAV **39**(2) 379–382, 1991.—The inhibition constant (K_i) of U-78875 was investigated without and with muscimol in the incubation medium using in vitro (^{3}H)-flunitrazepam [(^{3}H)-FNZ] binding to cortical membrane preparation. Also, the effect of U-78875 on cerebellar cyclic 3′,5′-guanosine monophosphate (cGMP) was studied in control and stressed (electric footshock) mice. The K_i of U-78875 was 1.56 nM for inhibition of (^{3}H)-FNZ binding. The presence of muscimol (10^{-5} M) had no significant effect on the K_i of U-78875. U-78875 and diazepam significantly decreased cerebellar cGMP, and this effect was antagonized by flumazenil. Both U-78875 and diazepam dose-dependently antagonized electric footshock-induced increases in cGMP, and U-78875 was two orders of magnitude more potent in stressed animals as compared to control animals. These biochemical investigations indicate that U-78875 is an agonist of benzodiazepine receptors, and cGMP may mediate its anxiolytic activity.

U-78875 (^{3}H)-FNZ binding cGMP Electric footshock

U-78875 (3-[5-cyclopropyl-1,2,4-oxadiazol-3-yl]-5-[1-methylethyl]-imidazo-[1,5-a]quinoxalin-4[5H]one) is a nonbenzodiazepine anxiolytic agent. In behavioral tests designed to determine anxiolytic activity, this compound increased lever-press responding in Geller's conflict test, punished water-licking in Vogel's behavioral paradigm, social interaction in the face to face test, and time away from the walls of a cage in the center test. U-78875 had anxiolytic activity similar to diazepam in these behavioral tests (19,27). U-78875 was more potent than diazepam in blocking isolation-induced aggression and stress-induced elevations in dopamine metabolism (28). In addition to the anxiolytic activity, U-78875 was also found to antagonize some of the pharmacological effects of benzodiazepines (BZ). Diazepam-induced reductions in motor activity, amnesia, impairment in conditioned avoidance response, and rotorod performance were blocked by treatment with U-78875 (27). U-78875 was as potent as flumazenil in antagonizing triazolam-induced muscle relaxation (28). Alprazolam-induced suppression of 2-deoxyglucose metabolism in various areas of the brain was also antagonized by U-78875 (17). U-78875 had a high affinity for benzodiazepine receptors of the cerebral cortex, as determined by in vitro (^{3}H)-FNZ binding. However, this compound was very weak in enhancing GABA-mediated $^{36}Cl^-$ uptake into rat cerebrocortical synaptoneurosomes (27). The behavioral and biochemical investigations indicate that U-78875 has both agonist and antagonist properties for pharmacological effects mediated by BZ receptors (BZR).

The purpose of the present study was to further investigate the neurochemical profile of U-78875, and to compare its spectrum of activity with diazepam. First, we investigated the binding characteristics of U-78875 in the presence of muscimol because GABA-ergic drugs enhance the affinity of BZ anxiolytics for BZR (10,25). Secondly, we investigated the effect of U-78875 on cerebellar cGMP levels because anxiolytics decrease cerebellar cGMP (1, 9, 14), and a BZ antagonist, flumazenil (up to 30 mg/kg), does not alter this parameter (3,14). Finally, the attenuating effect of U-78875 on electric footshock-induced elevations in cerebellar cGMP was investigated to determine the potency and efficacy of this drug in a biochemical model of stress. The stress-induced increase in cerebellar cGMP is blocked by anxiolytics, and is a very sensitive parameter for biological evaluation of anxiolytics (7).

METHOD

Animals

Male Sprague-Dawley rats (125–180 g) and male CF-1 mice (18–20 g) bred at The Upjohn Company were used in this study. Animals were kept under constant diurnal (12 h:12 h) lighting and temperature conditions for 5–7 days prior to their use. Rats were sacrificed between 8 and 10 a.m. for binding studies, and mice were sacrificed between 1 and 3 p.m. for determination of cerebellar cGMP.

(^{3}H)-FNZ Binding

In vitro (^{3}H)-FNZ binding to crude rat cerebral cortical membrane preparations for the determination of inhibition constants (K_i) of U-78875, diazepam, flumazenil, and 3-carbomethoxy-β-carboline (β-CCM) without and with muscimol was carried out by the method previously described (20,22). Rats were decapitated, the brain was quickly removed from the skull, and the bilateral cerebral cortex was dissected out. The tissue was weighed and then homogenized in 25.0 ml of ice-cold (0°C) 50 mM Tris-citrate buffer, pH 7.1, using a Brinkman polytron homogenizer for 30 s at setting #6. The homogenate was centrifuged at

48,000 × g for 10 min, and the pellet was washed 6 times by resuspension and recentrifugation as described before. The final pellet was stored at −70°C for a minimum of 18 h. On the day of the assay, the pellet was thawed in 25 ml of Tris-citrate buffer and was washed once again. The final pellet was suspended in 50 volumes (w/v) of the same buffer.

The binding of (^{3}H)-FNZ was determined by incubating 1.0 ml aliquots of membrane suspension with 0.1 ml of (^{3}H)-FNZ (final concentration of ligand was 0.98 to 1.0 nM, specific activity 81.8 Ci/mmol, New England Nuclear, Boston, MA), 0.1 ml of distilled water or drug as indicated, and 0.8 ml of Tris-HCl buffer, pH 7.4, to give a final volume of 2.0 ml. Each drug was investigated at five to seven concentrations. Muscimol (0.1 ml), at a concentration of 10^{-5} M, was used for studies involving the presence of a GABA agonist. The volume of buffer was adjusted accordingly. The mixture was incubated at 0°C for 60 min. The binding reaction was terminated by filtering the mixture under vacuum through a Whatman GF/B filter. The incubation tube was rinsed with ice-cold buffer, and this rinse also was filtered through the same filter. The filter was then washed three times using 5.0 ml aliquots of buffer each time. Finally, the filter paper was placed in a scintillation vial to which 15.0 ml of Amersham Searle ACS® cocktail was added. The vials were shaken for 30 min on a mechanical shaker (Eberbach), and then the radioactivity was counted by liquid scintillation spectrometry.

Specific binding was defined as the total binding minus binding in the presence of 10 μM flurazepam. Specific binding represented over 95% of total binding. The IC_{50} was obtained by logit-log plot of the data. K_i's were calculated by the following equation: $K_i = IC_{50}/(1 + c/K_d)$, where c = concentration of ligand (0.98 to 1.0 nM) and K_d = dissociation constant (0.94 nM).

Cerebellar cGMP

U-78875, diazepam, and flumazenil were dissolved in 20% N,N-dimethylacetamide. Both U-78875 and diazepam were administered orally, whereas flumazenil was injected intraperitoneally. Control mice received an equal volume of the vehicle (1 ml/100 g). Thirty min after treatment with U-78875, diazepam, or flumazenil, or combined treatment with U-78875 or diazepam plus flumazenil, mice were sacrificed by a beam of microwave radiation focused on the skull for 0.6 s (Metabostat™, model 4094, developed by Gerling-Moore). The procedure for estimation of cGMP was the same as that described by Burkard et al. (3). The cerebellum was quickly removed from the skull and homogenized in approximately 10 volumes of 1% perchloric acid, using a Brinkman polytron PCU-110 homogenizer for 15 s at setting #6. The samples were kept on ice for 30 min, boiled for 2 min, and then centrifuged at 17000 × g for 20 min. The content of cGMP in the supernatant was measured by a radioimmunoassay. The results are expressed as percent of control. Statistical analysis was done using one-way analysis of variance and subsequently by a paired *t*-test.

Electric Footshock

U-78875 or diazepam was administered orally 30 min prior to subjecting the mice to electric footshock. The animals were subjected to inescapable electric footshock (0.5 mA for 5 s) stress in a behavioral box with a stainless steel grid floor. Mice were immediately sacrificed as described before for estimation of cGMP.

RESULTS

The K_i's of U-78875, diazepam, flumazenil, and β-CCM for inhibition of (^{3}H)-FNZ binding without and with muscimol are described in Table 1. Muscimol did not alter the K_i's of U-78875 or flumazenil, and the K_i ratios (with/without) were 0.97 and 1.07, respectively. The K_i of diazepam was significantly ($p<0.01$) decreased by muscimol and had a K_i ratio of 0.69. The K_i of β-CCM was significantly ($p<0.01$) increased by muscimol, and the K_i ratio was 1.53 (Table 1).

TABLE 1

EFFECT OF MUSCIMOL (10^{-5} M) ON K_i OF BENZOERGIC DRUGS IN (^{3}H)-FNZ BINDING ASSAYS

Drug	K_i (nM)* Without Muscimol	With Muscimol	K_i Ratio With/Without
U-78875	1.56 ± 0.07	1.51 ± 0.06	0.97
Diazepam	7.04 ± 0.06	4.85 ± 0.11	0.69
Flumazenil	0.91 ± 0.05	0.98 ± 0.04	1.07
β-CCM	0.73 ± 0.06	1.12 ± 0.02	1.53

*Mean ± SE of 6 observations.

Cerebellar cGMP concentration in control mice ranged between 217 and 260 pmol/g of tissue (N = 10). U-78875 (30 and 60 mg/kg orally) and diazepam (30 mg/kg) significantly ($p<0.01$) decreased cerebellar cGMP levels. Treatment with flumazenil significantly ($p<0.01$) blocked U-78875- or diazepam-induced reductions in cGMP levels. Flumazenil (10 mg/kg) had no significant effect on cGMP levels (Fig. 1).

Electric footshock increased cerebellar cGMP levels to 181% of control. Pretreatment with U-78875 and diazepam significantly ($p<0.01$) blocked the footshock-induced increase in cGMP levels. The attenuating effects of U-78875 or diazepam on footshock-induced increases in cGMP were dose dependent. U-78875 (1 and 10 mg/kg) and diazepam (0.1 mg/kg) had no effect on cGMP levels in control mice (Fig. 2).

DISCUSSION

The BZ are psychoactive drugs with wide therapeutic applications as anxiolytics, anticonvulsants, and muscle relaxants (29). The site of action of this class of drugs at the BZR has

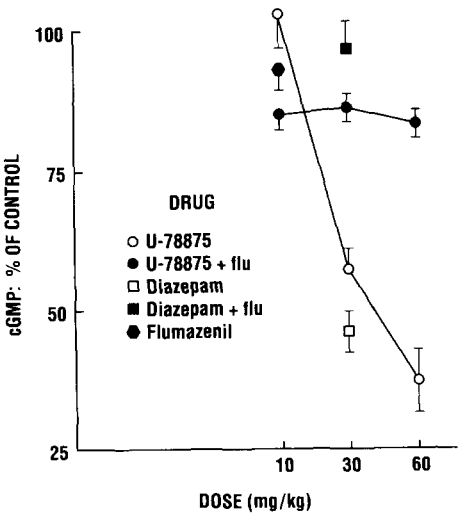

FIG. 1. Effect of U-78875, diazepam, and flumazenil on cerebellar cGMP in mice. Each observation is the mean ± SE of 4–8 animals.

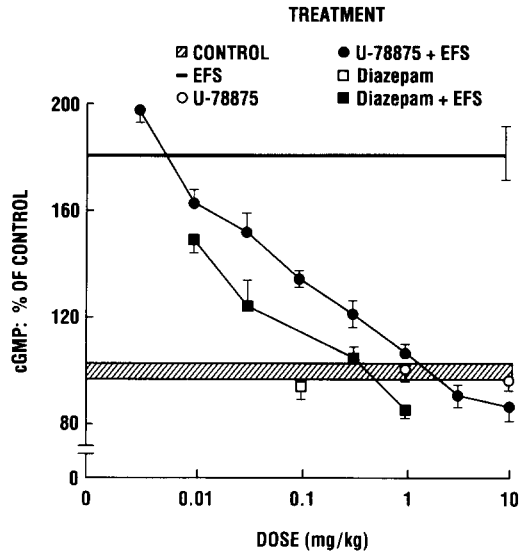

FIG. 2. Effect of U-78875 or diazepam on electric footshock (EFS)-induced increases in cerebellar cGMP in mice. Each observation is the mean ± SE of 4–9 animals.

been extensively investigated in vitro, ex vivo, and in vivo (15, 21, 24, 26). The binding of BZR agonists is increased by GABA-ergic drugs (10,25). On the other hand, BZ increase the affinity of GABA agonists, and thus facilitate transmission of GABA-mediated effects (5). Stimulation of the BZ-GABA receptor complex by BZ also increases Cl^- flux (2,4) and decreases the concentration of cerebellar cGMP (1, 9, 13, 18).

U-78875 and diazepam bind to BZR, and both drugs significantly decrease cerebellar cGMP levels. Flumazenil, a BZ antagonist, blocked U-78875- and diazepam-induced reductions in cGMP. These observations indicate that U-78875 has an agonist effect on the BZ-GABA receptor complex which may be responsible for the reduction in cerebellar cGMP levels.

Acute stress by electroconvulsive shock, forced swimming in ice-cold water, or immobilization increases cerebellar cGMP levels (6–8). Likewise, electric footshock was found to increase cerebellar cGMP levels in the present study. Pretreatment with U-78875 or diazepam blocked the electric footshock-induced elevation in cGMP. The doses for both U-78875 and diazepam were one to two orders of magnitude lower for blocking the electric footshock-induced elevation in cGMP as compared to those needed to lower cGMP levels in control animals. These observations indicate that doses of U-78875 which do not alter cGMP levels in normal mice do normalize a perturbation caused in these animals by electric footshock. Thus blockade of stress-induced elevations in cerebellar cGMP levels seems to be a sensitive biochemical correlate for determination of the anxiolytic profile of a compound in mice.

Muscimol had no effect on binding affinity of U-78875. Similarly, the binding affinity of flumazenil was not altered by GABA-ergic agonists, which is consistent with previously reported observations (12, 20, 23). Like flumazenil (11,16), U-78875 had no effect on ^{36}S-TBPS (a marker for Cl^- channels) binding, and it was very weak in potentiating GABA-mediated $^{36}Cl^-$ flux. Furthermore, U-78875 antagonized the potentiating effect of diazepam on GABA-mediated $^{36}Cl^-$ flux and TBPS binding (27). The BZR antagonist property of U-78875 seems to be responsible for blocking some of the pharmacological effects of BZ as described before (17, 27, 28).

The neurochemical and behavioral studies with U-78875 indicate that this compound is an agonist for reducing cerebellar cGMP levels, for increasing lever-press in Geller's conflict test, and water-licks in Vogel's procedure, and also in blocking isolation-induced aggression. It is an antagonist of BZ-induced muscle relaxation, with decrease in motor activity and amnesia. This pharmacological profile of U-78875 is consistent with the concept that this compound is a partial agonist of BZR.

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