Effects of a Benzodiazepine Antagonist, Ro 15-1788, on Hippocampal Field Potentials in Freely Moving Rats

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SUZUKI, H. Effects of a Benzodiazepine Antagonist, Ro 15-1788, on Hippocampal Field Potentials in Freely Moving Rats. Tohoku J. Exp. Med., 1991, 165 (4), 261-270 — Effects of a benzodiazepine receptor agonist (diazepam) and an antagonist (Ro 15-1788, flumazenil) administered separately or in combination on field potentials recorded from the hippocampal dentate area were examined in unanesthetized, unrestrained rats. Population excitatory postsynaptic potentials (EPSPs) evoked by stimulation of the perforant path were depressed significantly by diazepam (4 mg/kg, i.p.). However, diazepam did not affect the firing (spike) threshold of dentate granule cells. The injection of Ro 15-1788 (4 mg/kg, i.p.) alone affected neither excitatory synaptic transmission nor population spike threshold. Strength of γ-amino butyric acid-mediated recurrent inhibition as measured by the paired-pulse technique was potentiated by diazepam but unaffected by Ro 15-1788. However, the diazepam-enhanced inhibition was reversed by a subsequent administration of Ro 15-1788. Previous studies indicate that Ro 15-1788 acts not only as a selective benzodiazepine antagonist but also as a partial agonist-antagonist or an inverse agonist depending probably on doses. The present study demonstrated that Ro 15-1788 acted as a pure antagonist at low doses. These data suggest that the clinical use of Ro 15-1788 at high doses against comas induced by unidentified drugs could worsen the conditions and that low doses are recommendable for initial treatments because of its pure antagonist action.

benzodiazepine receptor; diazepam; Ro 15-1788; dentate field potential; paired-pulse depression.

Ro 15-1788 (ethyl-8-fluoro-5, 6-dihydro-5-methyl-6-oxo-4H-imadazo-[1, 5]-[1, 4]benzodiazepine-3-carboxylate; flumazenil) antagonizes benzodiazepine actions and has been used clinically against benzodiazepine overdosage (Scollo-Lavizzari 1983; Knudsen et al. 1988). Despite its benzodiazepine antagonizing effects, Ro 15-1788 was recently reported to act as a weak benzodiazepine agonist when administered alone (David et al. 1982; Skerritt and Macdonald 1983; Robertson et al. 1984; Kemp et al. 1987; Lavie et al. 1987; Albertson and Joy 1989). Using acute urethane-anesthetized rats, Albertson and Joy (1989) demonstrated that Ro 15-1788 reversed a diazepam-enhanced recurrent inhibition mediated by β-amino butyric acid (GABA) in the hippocampal dentate gyrus and that a

Received September 9, 1991; revision accepted for publication October 23, 1991.
pretreatment with Ro 15-1788 diminished the inhibition-enhancing effect of diazepam. Interestingly, Ro 15-1788 (40 mg/kg) was also shown to have a mild inhibition-enhancing effect when injected alone.

In order to preclude the potential confounding effects of anesthetic agents such as urethane, it is clearly important to examine central effects of Ro 15-1788 in unanesthetized animals. Therefore, the present study was designed to investigate the central actions of Ro 15-1788 (4 mg/kg) and diazepam (4 mg/kg), and their time courses in freely moving rats. A standard field potential technique was employed to examine the following four questions in detail. (1) How do diazepam and Ro 15-1788 affect excitatory synaptic transmission in the dentate gyrus? (2) How do these drugs affect the excitability (action potential threshold) of dentate granule cells? (3) How do these drugs affect the so-called paired-pulse depression known to reflect the strength of GABA-mediated recurrent inhibition? (4) Does Ro 15-1788 antagonize the diazepam-induced enhancement of paired-pulse depression?

MATERIALS AND METHODS

Adult male Wistar rats (n = 24) weighing 320-460 g at the time of surgery were used. The animals were randomly divided into three groups: the diazepam alone group (n = 8), the Ro 15-1788 alone group (n = 8), and the diazepam-Ro 15-1788 serial injection group (n = 8).

Under pentobarbital anesthesia (45 mg/kg, i.p.), the animals were implanted with polyurethane-coated, stainless steel stimulating (100 μm in diameter) and recording (50 μm in diameter) electrodes into the angular bundle (perforant path) and dentate hilus, respectively. Stereotaxic coordinates were 7.8 mm posterior to bregma and 4.5 mm from midline for the former, and 3.8 mm from bregma and 2.5 from midline for the latter. The final depths of these electrodes were adjusted under electrophysiological guidance to maximize evoked field potentials. Small stainless steel screws were fixed to the nasal, frontal and interparietal bones to serve as electrodes for ground, recording reference and stimulation reference, respectively. Wires from all electrodes were led to a 9-pin connector (Science Technology Center, Carleton University, Ottawa, Canada), which was then fixed to the skull with dental acrylic.

Following a 3-week postsurgical recovery period, field potentials were recorded from the unrestrained animal placed in an observation box (22 × 30 × 40 cm). Single-pulse stimulation of the perforant path, a major afferent pathway to the dentate gyrus, evoked in the dentate hilus a negative population spike superimposed on a slow positive population excitatory postsynaptic potential (EPSP). The former potential is known to reflect a synchronous firing of dentate granule cells. Perforant path stimuli were delivered at 15 sec intervals. Stimulus intensity was varied by changing pulse width in 16 steps from 10-250 μsec, while pulse height (current) was fixed.
As in the previous studies (Maru and Goddard 1987a, b; Suzuki et al. 1988), an initial rising slope (in mV/msec) of the population EPSP was measured as an index of EPSP strength, while the population spike amplitude (in mV) was derived from the vertical length between the line connecting the two positive peaks on the population EPSP and the negative peak of the population spike (Fig. 1). Stimulus-EPSP curves were obtained by plotting EPSP slopes against stimulus intensities (i.e., pulse widths). The slope of the stimulus-EPSP curve was used as an index of excitatory synaptic transmission efficacy. EPSP-spike (E-S) curves were generated by plotting population spike amplitudes against EPSP slopes. The x-intercept of the E-S curve was used as an estimate of the firing threshold of a granule cell population. Furthermore, the amplitude of each population EPSP at population spike onset was used as another estimate of the population spike threshold.

When two identical pulses at 20–30 msec intervals were applied to the perforant path, a population spike to the second pulse (PS2) was usually smaller than that to the first pulse (PS1). This is called "paired-pulse depression" and has been considered to reflect the operation of recurrent inhibitory circuits mediated by GABA (Adamec et al. 1981; Tuff et al. 1983; Suzuki et al. 1988). PS2/PS1 ratios were obtained for a fixed range of PS1 values. An estimate of the relative strength of paired-pulse depression was derived from (1-PS2/PS1)×100.

In all groups of animals, control recordings were made for 20 min before and

![Fig. 1. A typical field potential in the dentate hilus evoked by perforant path stimulation. The potential consists of two components, a negative population spike superimposed on a slow positive population EPSP. The former represents a sink current due to a synchronous discharge of granule cells, while the latter reflects a source current resulting from EPSPs on granule cell dendrites. EPSP slope: measured in mV/msec at an initial rising phase of the population EPSP. PS: population spike amplitude, corresponding to the vertical length between the tangent line connecting the two positive waves on the population EPSP and the negative peak of the population spike. T: population spike threshold defined as population EPSP amplitude at population spike onset.](image)
for 30 min after an intraperitoneal injection of 0.5 ml saline. Subsequently, the three groups were treated as follows. The diazepam alone group received a dose (4 mg/kg, i.p.) of diazepam (Sercine®; Takeda, Osaka) and 20 min later additional 0.5 ml saline. The effects on field potentials and behavior were examined for 3 hr. For the Ro 15-1788 alone group, 20 min recordings were made after an additional injection of 0.5 ml saline. Then, a dose (4 mg/kg, i.p.) of Ro 15-1788 (a gift of Hoffmann-La Roche) dissolved in saline at a concentration of 0.5 mg/ml was administered, and field potentials and behavior were observed for 3 hr. For the diazepam-Ro 15-1788 serial injection group, diazepam (4 mg/kg, i.p.) was injected first, and 20 min later Ro 15-1788 (4 mg/kg, i.p.) was given. Combined effects of these two drugs were examined for 3 hr.

Field potential data were analyzed statistically by using a paired-t test and one- or two-way analyses of variance (ANOVA). The level of significance was p <0.05. Since effects of diazepam and Ro 15-1788 were presumed to appear at 5-20 min after i.p. injection, statistical comparison by the paired-t test was made between the saline control value just before injection and the value obtained at 10-20 min after injection.

RESULTS

Within several min after diazepam (4 mg/kg, i.p.), the animal became inactive and ataxic in its extremities. During this period population EPSPs were reduced slightly (Fig. 2A, B). The slope of the stimulus-EPSP curve at 10-20 min after diazepam (5.16±1.40) was significantly smaller than that during the saline control period (5.96±1.25) (paired t=5.67, df=7, p <0.01). However, the slope of the stimulus-EPSP curve at 10-20 min after Ro 15-1788 (4 mg/kg, i.p.) (7.76±1.64) was not significantly different from that during the saline control period (7.82±1.52) (paired t=0.46, df=7, p >0.05) (Fig. 2C).

As indicated above, the x-intercept of the E-S curve has been used as an estimate of population spike threshold or an index of granule cell excitability. X-intercept values were not altered by diazepam (saline control, 3.10±0.83 mV/msec; 10-20 min after injection, 3.06±1.26 mV/msec; paired t=0.15, df=7, p > 0.05) or Ro 15-1788 (saline control, 3.55±1.38 mV/msec; 10-20 min after injection, 3.52±1.08 mV/msec; paired t=0.12, df=7, p >0.05). Furthermore, the amplitude of individual population EPSPs at population spike onset was not affected by diazepam (saline control, 6.98±1.13 mV; 10-20 min after injection, 6.51±1.40 mV; paired t=1.98 df=7, p >0.05) or Ro 15-1788 (saline control, 7.45±1.72 mV; 10-20 min after injection, 7.02±1.35 mV; paired t=1.67, df=7, p >0.05).

The strength of paired-pulse depression was greatly enhanced immediately after diazepam and remained elevated for at least 3 hr (one-way ANOVA, F[15, 105] =23.9, p <0.01) (Fig. 3). The relative strength at 10-20 min after diazepam (82.79±15.7%) was significantly greater than the saline control value.
Fig. 2. Effects of diazepam on excitatory synaptic transmission in the dentate gyrus. A, stimulus-EPSP scatter diagrams showing EPSP slopes plotted against stimulus intensities (pulse widths). Open circles and a dotted line are EPSP slopes and their regression line, respectively, at control recordings, while filled circles and a solid line are those obtained 10-20 min after diazepam (4 mg/kg). B, slopes of stimulus-EPSP regression lines plotted against time for the diazepam group. Values are means (n=8) with standard deviations (vertical bars). Open inverse triangles, preinjection control recordings; open circles, saline control recordings; filled circles, recordings after diazepam. The value at 10-20 min after diazepam was significantly different from the last saline control value. C, slopes of stimulus-EPSP regression lines for the Ro 15-1788 group. Filled squares, recordings after Ro 15-1788; other symbols are the same as in B. The value at 10-20 min after Ro 15-1788 is not statistically different from the last saline control value.
A significant increase in paired-pulse depression was also observed in the Ro 15-1788 alone group (one-way ANOVA, $F_{[15, 105]} = 4.03, p < 0.01$). However, as shown in Fig. 4A, this tendency for an increasing depression was apparent even one hr before the Ro 15-1788 injection, and therefore might not be due to a specific

(26.44±33.03%) (paired $t=6.72$, df=7, $p<0.01$).

Fig. 3. Enhancement of paired-pulse depression by diazepam and its reversal by Ro 15-1788. A, representative field potentials evoked by paired pulses. Each pair of responses was evoked by two identical pulses at 25 msec intervals. Stimulus intensities were adjusted to elicit similar 1st population spike amplitudes. The middle pair was obtained at 15 min after diazepam, while the bottom pair was recorded at 15 min after Ro 15-1788 (i.e., 35 min after diazepam). B, time course of diazepam-induced enhancement and its reversal by Ro 15-1788. Relative strength values of paired pulse depression were plotted against time. Solid line, the diazepam alone group; dotted line, the diazepam—Ro 15-1788 group. Symbols are the same as in Fig. 2B.
effect of the drug. In order to examine this possibility, two animals were chosen randomly from this group and given, two weeks later, an additional test with 3 injections of saline alone. The possibility was supported in that a similar tendency of increasing depression was observed without Ro 15-1788 injection (Fig. 4B). Furthermore, the relative strength of paired-pulse depression measured at 10–20 min after Ro 15-1788 was not significantly different from the saline control value (paired t = 0.47, df = 7, p > 0.05). In conclusion, these data indicated that Ro 15-1788 did not have a specific effect on paired-pulse depression.

When Ro 15-1788 was administered 20 min after diazepam, the diazepam-enhanced paired-pulse inhibition was reduced for approximately 90 min (Fig. 3B).
The time course of these drug effects was significantly different from that of the diazepam-alone group (two-way ANOVA, group × time interaction, $F[15, 210] = 4.57$, $p < 0.01$). The antagonist effect of Ro 15–1788 against diazepam disappeared in 2 hr and thereafter the diazepam-enhanced paired-pulse inhibition reappeared.

**DISCUSSION**

With respect to benzodiazepine receptors, Ro 15–1788 has been reported to act not only as a selective antagonist (Hunkeler et al. 1981; Darragh et al. 1983) but also as a partial agonist-antagonist (David et al. 1982; Skerritt and Macdonald 1983; Robertson et al. 1984; Kemp et al. 1987; Lavie et al. 1987; Albertson and Joy 1989) or an inverse agonist (King et al. 1985). Using the paired-pulse stimulation method to estimate the strength of GABA-mediated recurrent inhibition, Albertson and Joy (1989) showed that high doses of Ro 15–1788 (20 or 40 mg/kg, i.p.) reversed the diazepam-enhanced recurrent inhibition and thus acted as a benzodiazepine antagonist. However, the same high doses, when given alone, enhanced the recurrent inhibition slightly and thus had a weak agonist action. These complex effects have been interpreted to indicate that, in addition to a weak GABA-potentiating action, Ro 15–1788 reduces a diazepam-enhanced GABAergic inhibition by means of its high affinity binding to benzodiazepine receptors. The present study demonstrated that Ro 15–1788 (4 mg/kg, i.p.) could antagonize the diazepam-enhanced GABAergic inhibition at such a low dose having no intrinsic action on GABAergic synapses. In other words, the low dose of Ro 15–1788 was shown to act as a pure antagonist rather than a partial agonist-antagonist against the diazepam-enhanced GABAergic inhibition.

These findings may have clinical implications for the use of Ro 15–1788 against comas induced by unidentified drugs. When high doses of Ro 15–1788 are used to treat comas induced by non-benzodiazepine drugs, the conditions may worsen due to the agent's GABA potentiating action. Therefore, comas induced by unidentified drugs may be treated initially with low doses of Ro 15–1788 having a pure antagonist action. Subsequently, higher doses may be used when the involvement of benzodiazepine drugs is suspected. The present study cannot indicate the appropriate dose of Ro 15–1788 for clinical use. However, available human studies (Fragen 1988; Knudsen et al. 1988) suggest an initial dose of 0.2 mg i.v. with supplemental doses at 0.1–0.2 mg/min up to a total of 0.5–1.0 mg. If this procedure fails to improve comas induced by unidentified drugs, benzodiazepine drugs may not be involved in the conditions.

The present study indicated that diazepam (4 mg/kg) reduced the amplitude of population EPSPs in the dentate gyrus while leaving the population spike threshold (i.e., granule cell excitability) unaffected. However, Albertson and Joy (1989), who examined the diazepam actions on dentate field potentials in urethane-anesthetized rats, reported that the same dose of the drug (4 mg/kg) had
no significant effect on the threshold or amplitude of population EPSPs. Furthermore, using an unanesthetized rat preparation similar to ours, Adamec et al. (1981) found no significant effect of diazepam (1 or 2 mg/kg) on either population EPSPs or population spikes. Interestingly, our preliminary data (unpublished) indicated that diazepam at a higher dose (6 mg/kg) strongly depressed both population EPSPs and population spikes but that this depression was eliminated simply by arousing the animal with the use of strong stimuli such as tail-pinching. These data suggest that the diazepam-induced depression of population EPSPs and spikes was not caused directly by the drug's action on perforant path-dentate synaptic transmission, but indirectly through the reduction in arousal. Therefore, the diazepam-induced depression of population EPSPs found in the present study might have also been due to a drug-induced reduction in arousal. Conceivably, diazepam did not affect population EPSPs under urethane anesthesia (Albertson and Joy 1989), since they had already been reduced and stabilized by urethane.

The present study demonstrated that Ro 15-1788 (4 mg/kg) had no significant effect on population EPSPs or population spikes in the dentate gyrus of unanesthetized rats. In contrast, using urethane-anesthetized rats, Albertson and Joy (1989) showed that Ro 15-1788, while having no effect on population EPSPs, produced an increase in population spike threshold and a decrease in spike amplitude. Urethane has been shown to depress population spikes and to modify GABAergic inhibition (Maggi and Meli 1986; Kamondi et al. 1988; Moody et al. 1988). The reason for the inconsistent effects of Ro 15-1788 on population spikes is unknown but may be due to the differential doses used, or the presence or absence of anesthesia, or both. Further experiments, particularly those employing higher doses in unanesthetized animals, are needed to clarify this point.

References


