EFFECTS OF ZOPICLONE AND BENZODIAZEPINES ON SPINAL REFLEXES, ANEMIC DECEREBRATE RIGIDITY AND BENZODIAZEPINE BINDING

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Accepted July 15, 1983

Abstract—Whether or not zopiclone, a new sleep-inducing drug, exerted a similar effect to that of benzodiazepines was examined. The drug inhibited crossed extensor reflexes in chicks and augmented the dorsal root-dorsal root reflexes in rats without affecting the mono- and polysynaptic reflexes. The site of its action was considered to be located in the spinal cord since the effect on the dorsal root reflex was observed also in the spinal rats. Zopiclone selectively reduced the phasic responses of anemic decerebrate rigidity like benzodiazepines in rats. Despite the structural difference from the benzodiazepines, zopiclone dose-dependently inhibited $[^3H]$flunitrazepam binding in rat brain membranes, although the potency was weaker. These results suggested that zopiclone exerts CNS actions via benzodiazepine receptors.

It has been reported that zopiclone, [6-(5-chloro-2-pyridyl)-6,7-dihydro-7-oxo-5H-pyrrolo[3,4-b]pyrazin-5-yl]4-methyl-1-piperazine carboxylate, developed as a sleep-inducing drug (1), has similar pharmacological profiles to benzodiazepines, although this compound is unrelated to the benzodiazepines in chemical structure (2). Blanchard et al. (3) also reported that zopiclone was able to displace $[^3H]$diazepam and $[^3H]$flunitrazepam binding in rat brain membranes. These facts lead to an idea that zopiclone exerts its pharmacological activity through the benzodiazepine binding site in CNS (4, 5), despite the different chemical structure. Diazepam augments the dorsal root reflexes in the spinal cord (6), and in anemic decerebrate rats, diazepam inhibits the phasic responses of forelimbs (7). These effects, which are produced by an increase of GABAergic transmission (8-10), have not reported for other drugs except the benzodiazepines.

In the present study, it was examined whether zopiclone exerted a similar effect as the benzodiazepines on the motor systems, including spinal reflexes and rigidity. The displacing potency of zopiclone in $[^3H]$flunitrazepam binding was also examined in rat brain membranes.

Materials and Methods

Crossed extensor reflex in chicks: Chicks were anesthetized with $\alpha$-chloralose (50 mg/kg, i.p.). They were fixed on their backs and artificially respired through a cannula inserted into the trachea. The superficial fibular nerve of the left leg was exposed and stimulated (0.1 Hz, duration 1 msec, supramaximal, Nihon Kohden MSE-20) to induce the contralateral extensor reflex. Movement of the right leg following stimulation was recorded by means of an isotonic transducer (Nihon Kohden, TD-111s). Flunitrazepam and
nitrazepam were dissolved in a solvent consisting of 40% propylene glycol, 10% ethanol and 15% benzylalcohol in physiological saline. Zopiclone was dissolved in 0.1 N HCl and diluted in the aforesaid solvent. Each was injected into the jugular vein.

Anemic decerebrate rigidity in rats: The experiments were performed on male Wistar rats weighing 250–460 g (Nippon Rat Co.). The procedure for preparing the anemic decerebrate rigidity was the same as in the previous reports (7, 11). Briefly after anesthetizing rats with ether, the common carotid arteries were ligated bilaterally, and the occipital bone was exposed. The basilar artery was cauterized with a bipolar pincette electrode for high frequency coagulation (Mizuhoika Micro 1C) after making a trephined hole at the central part of the occipital bone. Recording of forelimb tension and mechanical stimulation of hind limbs were done according to the previous report (7). Records of the tonic and phasic responses were taken for 60 min after the administration of drugs, and the degree of drug effects was calculated by comparing the mean of the tension for each 5 min before the indicated time with the mean for 10 min before administration (control value). Drugs were suspended in 0.5% CMC-Na and administered intraduodenally.

Spinal reflexes in rats: Male Wistar rats (Nippon Rat Co.) weighing 350–450 g were anesthetized with urethane (1 g/kg, i.p.) and α-chloralose (25 mg/kg, i.p.). In some rats, the spinal cord was transected at the C1 level. Laminectomy was performed in the lumbo-sacral region. Ventral and dorsal roots below L4 were cut bilaterally, and dorsal and ventral roots of the segments L4 and L5 were isolated. A skin pouch was formed at the site of the dissection to cover the exposed tissues with liquid paraffin kept at 36–37°C. The dorsal root of L5 was placed on a bipolar silver wire electrode for stimulation (frequency of 0.2 Hz, pulse duration of 0.05 msec, supramaximal, Nihon Kohden MSE-3). The ipsilateral ventral root of L5 and the dorsal root of L4 were placed on bipolar silver wire electrodes for recording. Mono-synaptic (MSR) and polysynaptic (PSR) reflex potentials and dorsal root-dorsal root reflex potentials (DR-DRR) were evoked in the L5 ventral root and in the L4 dorsal root, respectively. MSR, PSR and DR-DRR were amplified with an a.c.-amplifier (Nihon Kohden, AVB-9), displayed on an oscilloscope (Nihon Kohden VC-9) and were averaged 8 times by an averaging computer (Nihon Kohden, ATAC-350). The analog output was recorded by an ink-writing recorder (San-ei Instrument, Rectigraph-8). Zopiclone was dissolved in 0.01 N HCl, diazepam injectable (Cercine®) was diluted in Cercine® solvent, and flurazepam was dissolved in Cercine® solvent. Each was injected into the femoral vein.

Assay of binding of [3H]nitrazepam to rat brain membranes: Crude synaptic membranes were prepared from the rat whole brain excluding cerebellum by the method of Zukin et al. (12). Briefly, brains were homogenized in 20 volumes of ice-cold 0.32 M sucrose, and the homogenate was centrifuged at 1,000 g for 10 min. The supernatant was centrifuged at 2,000 g for 20 min. The resultant pellet was suspended in 0.5% CMC-Na and administered intraduodenally.
were repeated twice, and the final pellet was suspended in the buffer and used for the binding assay.

Membranes were incubated with \(^{[3}\text{H}]\) flunitrazepam in the presence or absence of the test drugs for 30 min at 0–4°C, and the reaction was terminated by adding 4 ml of ice-cold 50 mM Tris-HCl (pH 7.4), passing the preparation through a Whatman GF/B filter and washing with 4 ml of the same buffer. In the displacement experiment, 1 nM \(^{[3}\text{H}]\) flunitrazepam was incubated with membranes in the presence or absence of various concentrations (10\(^{-9}\)–10\(^{-6}\) M) of drugs.

Specific \(^{[3}\text{H}]\) flunitrazepam binding was defined as the difference between the total binding and non-specific binding in the presence of 3×10\(^{-6}\) M diazepam. Protein was determined by the method of Lowry et al. (13).

Drugs used were zopiclone (Rhone-Poulanc), flurazepam (Sumitomo), nitrazepam (Rhone-Poulanc), diazepam (Rhone-Poulanc) and diazepam injectable (Cercine®, Takeda).

Results

Effects on crossed-extensor reflexes in chicks: Zopiclone, nitrazepam and flurazepam suppressed crossed-extensor reflexes immediately after the administration. As is evident in Fig. 1, recovery of the reflexes was fast in zopiclone, whereas that in the other two drugs was fairly slow. Figure 2 shows the dose-response relationship after 1–3 min (A) when the maximum suppression was shown and at 30 min (B).

Effects on anemic decerebrate rigidity: Intraduodenal administration of zopiclone (5 mg/kg) suppressed selectively the phasic responses without showing an appreciable effect on the tonic response (Fig. 3); the effect produced by 10 mg/kg was long-lasting. Nitrazepam (10 mg/kg, i.d.) gradually suppressed the tonic and more strongly the

Fig. 1. Effects of zopiclone and flurazepam on the crossed extensor reflex in chicks. Ordinate: relative amplitude of leg extension. Each point represents the mean±S.E.M. (n=4). \(\Delta\): solvent i.v. ; O: zopiclone 0.5 mg/kg i.v. ; •: flurazepam 1 mg/kg i.v. ; ⊠: nitrazepam 0.12 mg/kg i.v.

Fig. 2. Dose-response relationships of zopiclone (O), nitrazepam (□) and flurazepam (○) on the crossed extensor reflex in chicks. A: response at 1–3 min after injection when the depression is maximum. B: response at 30 min after injection.

Fig. 3. Effect of zopiclone (5 mg/kg, i.d.) on the tonic (●) and phasic (○) responses of rigid forelimbs in anemic decerebrate rats. Ordinate: relative tension of each response. Each point represents the mean±S.E.M. (n=6).
phasic responses (Fig. 4). Similar inhibitory patterns of the phasic and tonic responses were detected at the dose of 5 mg/kg (n=5). Flurazepam (10 mg/kg, i.d.) suppressed both phasic and tonic responses, and the degree of the inhibition increased gradually after the administration (Fig. 5). Similar inhibitory patterns of the responses were observed at the higher flurazepam dose of 50 mg/kg (n=5).

**Effects of zopiclone on spinal reflexes in rats:** The effects of zopiclone, flurazepam and diazepam on the spinal reflexes were assessed in either spinal or intact rats. MSR and PSR were not affected by zopiclone (0.1, 0.2 and 0.5 mg/kg, i.v.) (Fig. 6). However, DRR was markedly potentiated by zopiclone (0.2 and 0.5 mg/kg) in a dose-dependent manner. Flurazepam (0.5 mg/kg, i.v.) and diazepam (0.2 mg/kg, i.v.) (Figs. 6 and 7) augmented DR-DRR without affecting MSR and PSR.

**Inhibition of \[^{3}\text{H}]\text{flunitrazepam binding by zopiclone in brain membranes:** A Scatchard analysis of specific \[^{3}\text{H}]\text{flunitrazepam binding indicated the presence of only one class of binding sites with the affinity of 2.6 nM and maximal binding sites of 2.33 pmol/mg protein. } K_i \text{ values for each drug calculated from the IC50 value and } K_d \text{ were compared (Table I). The ability of zopiclone to inhibit } \[^{3}\text{H}]\text{flunitrazepam binding was about 3–20 times less potent than that of the benzodiazepines tested. The Hill coefficient of each drug was about 1.**

![Fig. 4. Effect of nitrazepam (10 mg/kg, i.d.) on the tonic (●) and phasic (□) responses of rigid forelimbs in anemic decerebrate rats.](image)

![Fig. 5. Effect of flurazepam (10 mg/kg, i.d.) on the tonic (●) and phasic (□) responses of rigid forelimbs in anemic decerebrate rats.](image)

![Fig. 6. Effects of zopiclone, flurazepam (FL) and diazepam (DZP) on spinal reflexes in intact (A) and C1 spinal rat (B). Ordinate: relative reflex amplitude 3 min after injection (i.v.) of drugs. Abscissa: doses of drugs. Each point represents the mean±S.E.M. (n=3). MSR: monosynaptic reflex, PSR: polysynaptic reflex, DRR: dorsal root reflex.](image)
Table 1. Inhibition of specific $[^3H]$flunitrazepam binding to rat brain membranes by benzodiazepines and zopiclone

| Drug    | $K_i$ (nM)       | Hill coefficient |
|---------|------------------|------------------|
| Diazepam | 6.47±0.87        | 0.94             |
| Clonazepam | 2.48±0.40        | 1.10             |
| Flurazepam | 15.1±3.10        | 1.04             |
| Zopiclone | 49.9±1.50        | 0.94             |

Specific $[^3H]$flunitrazepam (1.0 nM) binding to rat brain membranes was assayed in the presence of various concentrations ($10^{-9}$–$10^{-6}$ M) of drugs. $K_i$ values were calculated from the equation $K_i=1C50/(1+C/K_d)$, where $C$ is $[^3H]$flunitrazepam concentration and $K_d$ is 2.6 nM. Hill coefficients were calculated using $B_{max}=2.33$ pmol/mg protein and the data in the displacement experiments. Each $K_i$ value represents the mean of 4 experiments, with the S.E.M. indicated.

Discussion

It is generally accepted that enhancement of GABAergic pre- or postsynaptic inhibition is involved in the mechanisms of action of the benzodiazepines. Zopiclone dose-dependently inhibited $[^3H]$flunitrazepam binding in rat brain membranes, as was shown by Blanchard et al. (3), although the potency was weaker than the benzodiazepines tested. However, zopiclone was stronger than flurazepam in our pharmacological experiments (Figs. 1–3, 5 and 6). These may be due to the difference of pharmacokinetics or permeability to CNS of the drugs.

Zopiclone augmented the DR-DRR without affecting the mono- and polysynaptic reflexes (Figs. 6 and 7). The site of action was considered to be located in the spinal cord since the same effects on DRR were seen in both spinal and intact rats. Diazepam and flurazepam also augmented DRR. DRR enhancement, which is selectively produced by benzodiazepines, reflects the facilitation of GABAergic presynaptic inhibition (6, 14). Polc et al. (15) reported that zopiclone increased the dorsal root potential (DRR) which generates DRR in cats. Thus, it was suggested that zopiclone enhanced DRR by increasing the presynaptic inhibition like the benzodiazepines.

We have shown that diazepam selectively reduced the phasic tension of forelimbs without affecting the tonic tension in anemic decerebrate rats (7). The effect of diazepam on the phasic response was considered to be mediated by GABA (10). In the present
study, zopiclone selectively reduced the phasic responses like diazepam. However, the effect of nitrazepam was not so selective, and flurazepam reduced both responses equally. The cutaneous afferents in the hindlimbs is considered to activate the forelimb motoneuron directly or indirectly via the brain stem (16). Zopiclone may block these pathways at the spinal cord by enhancing GABAergic inhibitory transmission. However, the possibility that zopiclone may act on the brain stem is not excluded.

Acknowledgement: This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan.

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