Characteristics of the Association of Brotizolam, a Thieno-Triazolo Diazepine Derivative, with the Benzodiazepine Receptor: A Selective and High Affinity Ligand of the Central Type I Benzodiazepine Receptor

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ABSTRACT—Characteristics of the association of brotizolam, a thieno-triazolo diazepine derivative, to central benzodiazepine receptors were examined. Brotizolam significantly displaced the [³H]flunitrazepam and [³H]β-carboline carboxylate ethylester bindings to crude synaptic membrane from the rat brain. This agent had the highest affinity for benzodiazepine receptors in the cerebellum, and it was found to be 2.1 times that in the spinal cord. Furthermore, a low concentration of brotizolam potentiated the GABA-stimulated ³⁶Cl⁻ influx into membrane vesicles. In contrast, the bindings of [³H]8-hydroxy-2-(di-n-propylamino)tetralin to 5-hydroxytryptamine₁A receptors and [³H]ketanserin to 5-hydroxytryptamine₂ receptors were not affected by brotizolam. The present results suggest that brotizolam may be a selective and high affinity ligand for the type I central benzodiazepine receptor. The anxiolytic and hypnotic actions of brotizolam seem to be not due to the association with 5-hydroxytryptamine receptor, but due to the activation of the GABAA receptor complex. Furthermore, the present results suggest that the lower affinity of brotizolam to benzodiazepine receptors in the spinal cord than those in the cerebellum may be related to the low muscle relaxation action of this drug.

Keywords: Brotizolam, Benzodiazepine receptor, Central type I benzodiazepine receptor, Spinal cord, Cerebellum

Classical benzodiazepine (BZP) derivatives such as diazepam and nitrazepam have various pharmacological actions such as anxiolytic, anticonvulsant, hypnotic and muscle relaxant properties (1, 2). Recently, various new types of anxiolytic drugs, both BZP derivatives and non-BZP derivatives, have been introduced (3, 4). These new drugs have more potent and selective pharmacological properties, especially possessing low or barely detectable muscle relaxation activity, as compared with that of classical BZP.

Brotizolam (8-bromo-6-(o-chlorophenyl)-1-methyl-4H-triazolo[3,4-c]thieno[2,3-e]-1,4-diazepine), one of the thieno-triazolo diazepine derivatives, has been clinically used as a hypnotic drug (5). The pharmacological significances of this drug are reported to be no alteration in the duration and frequency of REM sleep in addition to a low potency in muscle relaxation.

On the other hand, it has been well-established that the activation of central BZP receptor induces various central actions by affecting GABAergic neurons, since central BZP receptor couples with the GABAA receptor/Cl⁻ channel complex (6). Furthermore, recent pharmacological and molecular biological studies have indicated that both type I BZP receptor, a binding site having high affinity for CL218,872, and type II BZP receptor, which has lower affinity for CL218,872, are present in the mammalian central nervous system (7, 8).

In this study, we have examined the characteristics of the actions of brotizolam on the central GABAA receptor/BZP receptor/Cl⁻ channel complex using crude synaptic membrane and membrane vesicle preparations from the brain and spinal cord.
MATERIALS AND METHODS

Materials

[3H]Flunitrazepam (87.0 Ci/mmol), [3H]β-carboline carboxylate ethylester (β-CCE) (41.0 Ci/mmol), [3H]8-hydroxy-2-(di-n-propylamino)tetralin (8-OH DPAT) (157.1 Ci/mmol) and [3H]ketanserin (75.0 Ci/mmol) were obtained from New England Nuclear (Boston, MA, U.S.A.). Chlorine-36 ([36Cl]NaCl, 18.1 µCi/mg of NaCl) was purchased from Amersham (Des Plaines, IL, U.S.A.). Brotizolam was supplied by Nippon Boehringer Ingelheim (Kawanishi, Japan).

Binding assay of various 3H-ligands

For the measurement of receptor binding of various 3H-ligands, crude synaptic membranes from the cerebral cortex, cerebellum, spinal cord and/or whole brain of Wistar rats were prepared by the method of Zukin et al. (9).

The measurements of [3H]flunitrazepam and [3H]β-CCE bindings were carried out as previously described (10). [3H]8-OH DPAT and [3H]ketanserin binding assays were also employed as previously described (11, 12). In the assay of BZP receptor binding, crude synaptic membrane (100 µg protein) was incubated with 1 nM [3H]flunitrazepam for 60 min at 2°C or 1 nM [3H]β-CCE for 30 min at 2°C in 50 mM Tris-citrate buffer (pH 7.1), respectively. In the case of 5-hydroxytryptamine (5-HT) receptor binding, crude synaptic membrane (100 µg protein) was also incubated with 1 nM [3H]8-OH DPAT for 10 min at 37°C or 1.5 nM [3H]ketanserin for 15 min at 37°C in 50 mM Tris-HCl buffer (pH 7.4), respectively. After the incubation, the reaction mixture was filtered on a Whatman GF/B glass-fiber filter, which was pretreated in 0.05% polyethyleneimine, and then the filter was washed 3 times with 3 ml of cold buffer. The radioactivity trapped on the filter was measured by a liquid scintillation spectrometer.

RESULTS

The effect of brotizolam on [3H]flunitrazepam binding to BZP receptors was examined using crude synaptic membrane fractions obtained from the cerebral cortex, cerebellum and spinal cord. Among the various BZP receptor ligands examined, brotizolam exhibited the highest displacing capacity on [3H]flunitrazepam binding in the cerebral cortex, cerebellum and spinal cord (Fig. 1, A, B and C).

The inhibition by flunitrazepam of [3H]flunitrazepam binding showed had an IC50 value of 2.51 nM in the case of the cerebral cortex, and similar values were obtained in the cerebellum and spinal cord (Table 1). The IC50 value of nitrazepam in the cerebral cortex was found to be 15.85 nM, and a similar value was obtained in the case of the cerebellum and spinal cord (Table 1). The IC50 value of nitrazepam in the cerebral cortex was found to be 15.85 nM, and a similar value was obtained in the case of the cerebellum. In addition, it was found that the IC50 value of nitrazepam in the spinal cord was

| IC50 values (nM) in the displacement of [3H]flunitrazepam binding to the crude synaptic membranes from the cerebral cortex, cerebellum and spinal cord |
|----------------|----------------|----------------|
|                | Cerebral cortex | Cerebellum     | Spinal cord |
| Flunitrazepam  | 2.51           | 2.95           | 2.18        |
| Nitrazepam     | 15.85          | 14.45          | 8.91        |
| CL218,872      | 316.22         | 47.90          | 831.76      |
| Brotizolam     | 0.35           | 0.15           | 0.32        |
lower than those in the cerebral cortex and cerebellum. In contrast, CL218,872, a selective ligand for central type I BZP receptors, showed a higher affinity to BZP receptors in the cerebellum (IC\textsubscript{50}: 47.90 nM) than those in the cerebral cortex and spinal cord. In the case of brotizolam, its IC\textsubscript{50} value in the cerebellum was found to be 0.15 nM. This value was 2.13 times lower than that found in the spinal cord.

Effect of brotizolam on the binding of [\textsuperscript{3}H]f-CCE, which is known as an inverse agonist of the BZP receptor and radiolabelled ligand for the central type I BZP receptor (14), was also examined in the crude synaptic membrane fraction obtained from the rat whole brain. Brotizolam showed higher displacing capacity on [\textsuperscript{3}H]f-CCE binding as compared with those of \(\beta\)-CCE and CL218,872 (Fig. 2). Furthermore, the effect of brotizolam on GABA\textsubscript{A} receptor/BZP receptor/Cl\textsuperscript{-} channel was examined by measuring \(^{36}\text{Cl}^{-}\) influx into membrane vesicles from the rat whole brain (Fig. 3). Brotizolam (10\textsuperscript{-9} - 10\textsuperscript{-8} M) induced a significant enhancement of the GABA (30 \(\mu\)M)-stimulated \(^{36}\text{Cl}^{-}\) influx, although it had no effect on \(^{36}\text{Cl}^{-}\) influx in the absence of GABA. It is also noteworthy that the activating effect of brotizolam on \(^{36}\text{Cl}^{-}\) influx has been found to disappear if more than 10\textsuperscript{-7} M brotizolam is added to the assay system. The molecular mechanisms underlying this phenomenon is presently unknown.

On the other hand, both [\textsuperscript{3}H]8-OH DPAT binding to 5-HT\textsubscript{1A} receptor and [\textsuperscript{3}H]ketanserin binding to 5-HT\textsubscript{2} receptor were not affected by brotizolam, although these bindings were selectively inhibited by 5-HT and ketanserin (Fig. 4, A and B).

**Fig. 1.** Effect of various drugs on [\textsuperscript{3}H]flunitrazepam binding to crude synaptic membrane preparations from the cerebral cortex (A), cerebellum (B) and spinal cord (C). The control values for [\textsuperscript{3}H]flunitrazepam binding in the cerebral cortex (A), cerebellum (B) and spinal cord (C) were 629.9 ± 40.2, 412.9 ± 55.2 and 178.5 ± 2.8 fmol/mg protein, respectively. Each value represents the mean obtained from three separate experiments performed in triplicate. ○: Brotizolam, ●: Flunitrazepam, ▲: Nitrazepam, □: CL218872.

**Fig. 2.** Effect of various drugs on [\textsuperscript{3}H]\(\beta\)-CCE binding to crude synaptic membrane preparation from rat whole brain. The control value for [\textsuperscript{3}H]\(\beta\)-CCE binding was 101.5 ± 5.1 fmol/mg protein. Each value represents the mean obtained from two separate experiments performed in triplicate. ○: Brotizolam, ●: \(\beta\)-CCE, □: CL218872.
DISCUSSION

Brotizolam is known to exhibit various central actions similar to BZP derivatives (5). The present results indicate that brotizolam has a high affinity to both \[^{3}H\]flunitrazepam and \[^{3}H\]l-CCE binding sites. It was reported previously that brotizolam has a high displacing capacity on \[^{3}H\]flunitrazepam binding to normal human temporal cortex (15). This result has an essential agreement with that found in the present study. Central BZP receptors have been classified into both type I and type II receptors, although both receptor subtypes were found to be distributed differently in the mammalian central nervous system (7). Namely, the type I BZP receptor was found predominantly in the cerebellum, while the type II BZP receptor was mainly detected in the spinal cord. In contrast, both BZP type I and II receptors were almost equally distributed in the cerebral cortex (16, 17). Flunitrazepam, one of classical BZP derivatives, has been shown to have the same affinity to both BZP receptor subtypes. In fact, it has been reported that \[^{3}H\]flunitrazepam has the same binding capacity in the cerebellum and spinal cord (18).

In the case of brotizolam, the highest affinity to BZP receptor was found in the cerebellum which has predominantly type I BZP receptors (15, 16). The IC\(_{50}\) value in the cerebellum was 2.33 and 2.13 times lower than those found in the cerebral cortex and spinal cord. These results suggest that brotizolam may have a selective and high affinity to central type I BZP receptors. Similarly, it has been reported that lormetazepam has 2.9 times higher affinity to BZP receptors in the cerebellum than those in the spinal cord (19). It has been also reported that zolpidem and oxoquazepam bind to type I BZP receptors in the brain (3, 4).

The measurement of \(^{36}\text{Cl}^-\) influx in the presence of GABA has been considered to be a useful tool for examining the function of the GABA\(_A\) receptor complex (13). The addition of BZP derivatives such as flunitrazepam to this experimental system has been shown to potentiate the GABA-stimulated \(^{36}\text{Cl}^-\) influx (20). Brotizolam in a low concentration also showed a significant enhancement of GABA-stimulated \(^{36}\text{Cl}^-\) influx, although a higher concentration of brotizolam had no potentiating effects. Similarly, it was reported that flunitrazepam dose-dependently activated the GABA-stimulated \(^{36}\text{Cl}^-\) influx and exhibited a maximal activation at approximately at 1 \(\mu\)M (21). Similar bell-shaped activation curves have been also reported in the cases of triazolam and alprazolam, triazolobenzodiazepines (22). The molecular mechanisms underlying such a lack of an effect of a high concentration of BZP on the activation of GABA-stimulated \(^{36}\text{Cl}^-\) influx are unclear.
at present.

On the other hand, it has been reported that the muscle relaxant effect of brotizolam appears only at a high dose, and this dose is significantly higher than that which induces hypnotic and anxiolytic effects (23). These differential effects of brotizolam are clearly different from those of classical BZP derivatives such as nitrazepam (23). In fact, nitrazepam, which binds to both type I and type II BZP receptors, has significantly higher affinity to the spinal cord than brotizolam (Table 1).

In conclusion, the anxiolytic and hypnotic actions of brotizolam seem to be exclusively due to the activation of the cerebral GABA<sub>A</sub> receptor complex, since this agent has no affinity to 5-HT receptors which are also considered to be involved in the occurrence of anxiety. Brotizolam seems to bind BZP type I receptors in the central nervous system, especially in the brain. The binding of brotizolam to BZP receptors in the spinal cord seems weaker than those in other parts of the central nervous system, and this characteristic may be related to the weak muscle relaxation action of this drug (5).

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