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Effects of drugs acting on the GABA-benzodiazepine receptor complex on flurothyl-induced seizures in Mongolian gerbils

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Abstract

In the present study, the mechanism behind flurothyl-induced seizures was examined using drugs acting on the GABA-benzodiazepine receptor complex in Mongolian gerbils. In addition, amino acid concentrations in the brain were also investigated. In behavioral experiments, the incidence of tonic extensor was 83.3% in both the control and picrotoxin (0.5 mg/kg)-treated groups, 0% in the valproate (200 mg/kg)-treated group, and 50% in the picrotoxin plus valproate-treated group. However, picrotoxin did not antagonize the effect of valproate on clonic seizure latency at all. Flumazenil, a benzodiazepine receptor antagonist, was found to have an inhibitory effect on the anticonvulsant action of diazepam (0.5 mg/kg). The incidence of tonic extensor was 83.3% in flumazenil (10 mg/kg)-treated group, 0% in the diazepam (0.5 mg/kg)-treated group, and 83% in the flumazenil plus diazepam-treated group as well as the control group. Flumazenil also completely reversed the effect of diazepam on clonic seizure latency. In biochemical experiments, the concentration of the inhibitory amino acid, GABA, was significantly increased in the hippocampus ($P<0.05$) and cerebellum ($P<0.01$) in diazepam-treated animals. The increase of GABA in the hippocampus and cerebellum was antagonized by the administration of flumazenil. These results suggested that the anticonvulsant action of diazepam may be linked to increase in hippocampus and cerebellum GABA concentrations. The findings suggest that the mechanism of flurothyl-induced seizures, in part, is related to the highly sensitive benzodiazepine site of the GABA-benzodiazepine receptor complex.

Keywords: Flurothyl; GABA-benzodiazepine receptor; Amino acid; (Mongolian gerbil)

1. Introduction

The prevalence of epilepsy is 0.7–1% (Engel et al., 1989), making it the most common serious neurological disorder in the world (Bradford, 1995). Despite advances in antiepileptic therapy, at least 25% of patients with epilepsy are still not free of seizures (Lindekens et al., 2000).

Applegate et al. (1997) produced an animal model of seizure based on the convulsant action of flurothyl in C57BL/6 mice. The model can exhibit various phenotypes, acute clonic and tonic seizures, a kindled state and the propagation of seizures, using different methods of exposure. Therefore, elucidation of the mechanism of this model would be useful for screening antiepileptic drugs in the various states of seizure. An imbalance between excitatory and inhibitory amino acids produced by a decrease in γ-aminobutyric acid (GABA)-ergic transmission and/or an increase in glutamatergic transmission has been associated with the epileptic pathology, both in animal models and in humans (Sugaya and Onozuka, 1978; Bradford, 1995; Pena and Tapia, 2000). In general, agents that stimulate the GABA-benzodiazepine receptor have an anticonvulsant effect (Loscher and Schmidt, 1988; Costa and Guidotti, 1996; Hoogerkamp et al., 1996), and such action is reversed by flumazenil, an antagonist of this receptor (Przegalinski et al., 2000). Valproate and diazepam are well-established therapeutic agents producing a prompt anticonvulsant effect when administered during an epileptic seizure (Costa and Guidotti, 1996; Hoogerkamp et al., 1996; Johannessen, 2000).

The most widely used animal models of seizure are the traditional maximal electroshock model and the pentylenetetrazol (PTZ)-induced model. The former is considered a predictor of therapeutic efficacy against generalized tonic–clonic seizures, while the latter represents a valid model of

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generalized clonic seizures. In fact, Li et al. (2004) showed that PTZ-induced convulsions were augmented by excitatory amino acids, glutamate and aspartate, and markedly inhibited by inhibitory amino acids, GABA and taurine, in rats. Rowley et al. (1997) reported that glutamate and GABA levels in the rat hippocampus were changed by maximal electroshock-induced seizures; that is, glutamate concentrations were elevated above basal levels while GABA concentrations were decreased in a sustained fashion.

While flurothyl can produce several types of seizure as well, there are few reports about the mechanism(s) of this model. The anticonvulsant action of diazepam against PTZ-induced clonic seizures is mediated by augmentation of the GABA concentration via the GABA-benzodiazepine receptor because it is reversed by flumazenil (Nutt et al., 1982). The efficacy of diazepam against maximal electroshock-induced convulsions at toxic doses suggests that its actions are mediated via binding to voltage-sensitive sodium channels (McLean and Macdonald, 1988). Valproate, on the other hand, one of the major antiepileptic drugs, is used to treat both generalized and partial seizures (Loscher, 1999). This wide spectrum of anticonvulsant actions is reflected by multiple mechanisms; inhibition of voltage-dependent sodium channels, blockade of T-type calcium channels, and enhancement of GABAergic transmission (Loscher, 1999). The tonic seizures induced by 4-aminopyridine are suppressed by drugs which inhibit voltage-dependent sodium channels (Yamaguchi and Rogawski, 1992). The mechanism of the anticonvulsant action of valproate against PTZ-induced seizures is only partially understood, but the seizures are based on an inhibition of chloride conductance caused by binding the picrotoxin sites of GABA-benzodiazepine receptors, and drugs which enhance GABA transmission exhibit anticonvulsant action in this model (De Deyn and Macdonald, 1989). Therefore, it is thought that valproate acts by augmenting GABA transmission. As described above, these two drugs exert their pharmacological actions by allosterically modulating the GABA-benzodiazepine receptor complex to produce a facilitatory effect on the GABA-mediated inhibitory neurotransmission in the central nervous system. By contrast, drugs antagonism to the GABA-benzodiazepine receptor, for instance, picrotoxin and flumazenil, produce a proconvulsant effect.

In our laboratory, we examined the effects of novel antiepileptic drugs and of MK-801, which is a non-competitive NMDA receptor antagonist, on FE-induced seizures (Hashimoto et al., 2003a,b). Furthermore, Araki et al. (2002) reported a difference between mice and Mongolian gerbils in the timing of tonic convulsions; i.e. several minutes after clonic seizures in the mice, and immediately after or within 1 min of the clonic seizures in the gerbils. Based on these previous studies, GABA-benzodiazepine receptor is the most conceivable target in flurothyl-induced seizures, and the characteristics of Mongolian gerbils make them suitable for elucidating the progression from clonic seizure to tonic extensor and mechanism of seizures. However, precisely how flurothyl induces seizures is unclear. Moreover, it is unclear which amino acids in the brain are related to the expression of seizures. Therefore, in this study, the mechanism of flurothyl-induced seizures was investigated using GABA-benzodiazepine receptor antagonists, picrotoxin for valproate and flumazenil for diazepam, in Mongolian gerbils. Additionally, the relationship between the anticonvulsant action of the drugs and changes in amino acid concentrations in the brain were examined.

2. Materials and methods

2.1. Animals

Male Mongolian gerbils ($n=114$), 7 weeks old and weighting 50–58 g, were obtained from Seac Yoshitomi (Fukuoka, Japan). The animals were given at least 1 week to acclimatize before the experiments begun. The animals were housed in a temperature-controlled environment under a 12:12 h light/dark cycle (lights on at 7:00 a.m.). Food and water were provided ad libitum. All experiments were conducted according to the guidelines for Animal Experimentation at Okayama University Medical School.

2.2. Experimental procedures and methods of measuring amino acid content in the brain using high performance liquid chromatography with electrochemical detection (HPLC–ECD)

The method used to generate flurothyl-induced seizures has been described elsewhere (Araki et al., 2002). Briefly, Mongolian gerbils were placed individually in 2-l closed Plexiglas chambers (Bell jar). Seizures were elicited using 10% flurothyl (2,2,2-trifluoroethyl ether; Aldrich Chem. Co., Milwaukee, WI) in 95% ethanol. Flurothyl was administered by infusion (0.2 ml/min) using a 10-ml syringe driven by an infusion pump (kd Scientific Model 100, Neuroscience, Inc.). Sustained loss of posture control (>2 s) was defined as a clonic seizure. The latency from the start of the infusion to the onset of clonic seizures was measured. Flurothyl continued to be infused while the animals exhibited clonic seizures. As shown by our previous study (Araki et al., 2002), Mongolian gerbils with no drug treatment (control) exhibited tonic extensor immediately after clonic seizure. Most Mongolian gerbils exhibited tonic extensor within several seconds and a maximum response at 1 min (about 5–6% of Mongolian gerbils). The gerbils in which tonic extensor did not occur exhibited symmetrical hindlimb treading and loss of posture similar to animals with PTZ-induced seizures within several seconds after clonic seizure. Thus, it is the point that we could reliably determine whether tonic extensor occurred or not. Therefore, the infusion of flurothyl was stopped when the animals exhibited clonic seizure and we recorded the clonic seizure latency and the incidence of tonic extensor. After observation of the seizure phenotype, the gerbils were killed by decapitation, and the brains removed from their skulls for the measurement of amino acids (glutamate, glycine and GABA). Amino acids in the brain were measured using HPLC–ECD, according to the method of Ueda et al. (2001). The brain was divided into four parts, the frontal cortex, hippocampus, striatum and cerebellum, and weighed. Each part was homogenized with methanol, including

Table 1
Effect of picrotoxin on flurothyl-induced seizures in Mongolian gerbils

	Control ($n=6$)	0.5 mg/kg ($n=6$)	1.0 mg/kg ($n=6$)
Latency (s)	394±16	386±12	342±10 [a]

All values are presented as mean±S.E.M.
[a] $P<0.05$ vs. control, ANOVA followed by Dunnett's test.

10 mM homoserine as an internal standard. The homogenates were centrifuged at 15,000×*g* for 15 min at 4 °C. The supernatants were filtered and then stored in a deep freezer at −80 °C so as to not degenerate before being measured. On the day of measurement, homogenized samples were subjected to pre-column derivation of amino acids with *o*-phthaldialdehyde/2-mercaptoethanol prior to analysis. The HPLC–ECD system consisted of a pump (EP-300), electrochemical detector (ECD-300) and sample injector (SI-300). The electrochemical detector utilized a glassy carbon working electrode (+0.60 V). For HPLC, a stainless steel ODS column (EICOMPAK SC-5ODS 2.1×150 mm^2) was used to maintain a constant temperature of 30 °C in the column oven (ATC-300). The mobile phase was prepared by mixing 29% methanol (HPLC grade, Kanto Chemical Co., Tokyo) with 0.1 M phosphate buffer (pH6.0). Its rate of flow was 0.23 ml/min.

2.3. Drugs and administration

Dosages and drugs were as follows: diazepam (Cercine, Takeda Pharmaceutical, Osaka) at 0.5 mg/kg; valproic acid–Na (Biochemicals Inc., Tokyo) at 200 mg/kg; flumazenil (Sigma) at 10 mg/kg; picrotoxin (Sigma) at 0.5 and 1.0 mg/kg. Diazepam was diluted with saline. Valproic acid–Na was dissolved in distilled water, and flumazenil and picrotoxin were dissolved in saline. All drugs were administered intraperitoneally (i.p.) at a volume of 0.1 ml/100 g body weight. Dosages were chosen to match those that previously demonstrated anticonvulsant action (Araki et al., 2002). All drugs were administered 30 min before the beginning of flurothyl infusion. In antagonistic experiments, diazepam was injected i.p. 2 min prior to flumazenil or vehicle, and valproic acid–Na was injected i.p. 5 min prior to picrotoxin or vehicle. All control groups were administered the appropriate vehicle; saline for diazepam, flumazenil, and picrotoxin, and distilled water for valproate, instead of the drugs. However, the control groups were exposed to flurothyl the same as the drug treatment groups. The gerbils that showed spontaneous convulsions before the experiments, or immediately after drug injections, were excluded from the study. In the present study, none of the animals showed spontaneous convulsions.

2.4. Statistical analysis

The latency of the clonic seizures induced by flurothyl and the concentrations of amino acids in the brain were evaluated using an analysis of variance (ANOVA) followed by Dunnett's test for multiple comparisons. The incidence of hindlimb tonic extensor was analyzed with the χ^2 test. Probability values less than 0.05 were considered to show a significant difference.

3. Results

3.1. Effect of picrotoxin on flurothyl-induced seizures in Mongolian gerbils

Table 1 shows the effect of picrotoxin, a $GABA_A$ receptor antagonist, on flurothyl-induced seizures in Mongolian gerbils. The latency of clonic seizures decreased in a dose-dependent manner. At 1.0 mg/kg of picrotoxin, the latency was significantly reduced ($P<0.05$).

3.2. Effect of 95% ethanol on the expression of seizures in Mongolian gerbils treated with drugs acting on the GABA-benzodiazepine receptor complex

To know the effect of 95% ethanol on seizure expression, we set up the without flurothyl groups. Mongolian gerbils were treated with picrotoxin ($n=3$), valproate ($n=3$), flumazenil ($n=3$), diazepam ($n=3$), picrotoxin+valproate ($n=3$), or flumazenil+diazepam ($n=3$), and were individually placed in Bell jars. The cutoff time was 30 min from the infusion of 95% ethanol. None of the animal exhibited any seizures.

3.3. Inhibitory effect of picrotoxin on the anticonvulsant effect of valproate on flurothyl-induced seizures in Mongolian gerbils

Fig. 1 shows the antagonistic action of picrotoxin on the anticonvulsant effect of valproate. A picrotoxin dose of 0.5 mg/kg was calculated using the above data to be the maximum no efficacy dose. The control group and the group treated with picrotoxin at 0.5 mg/kg had an 83.3% incidence of tonic extensor (Fig. 1A). The rest of the animals showed only clonic seizures. The valproate group, by contrast, had a 0% incidence of tonic extensor ($P<0.01$) induced by flurothyl, although all

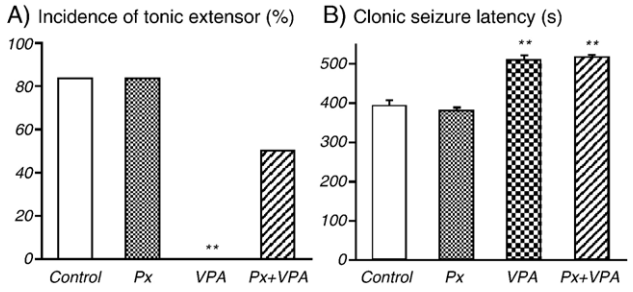

Fig. 1. Inhibitory effect of picrotoxin on the anticonvulsant action of valproate in Mongolian gerbils with flurothyl-induced seizures. Picrotoxin (0.5 mg/kg) or valproate (200 mg/kg) was intraperitoneally administered 30 min before exposure to flurothyl. When the animal was co-administered these two drugs, valproate was given 5 min prior to picrotoxin. After drug administration(s), the animal was returned to his cage. Mongolian gerbils were individually placed in airtight Bell jars when exposed to flurothyl. Clonic seizure latency was measured from the start of flurothyl infusion to expression of clonic seizure. The incidence of tonic extensor after clonic seizure was recorded. In the control group, the animals were administered the appropriate vehicle, according to the same experimental procedure used for the drug treatment group. (A) Incidence of tonic extensor (%). $^{**}P<0.01$ vs. control χ^2 test ($n=6$). (B) Clonic seizure latency (s). Each column represents the mean±S.E.M. $^{**}P<0.01$ vs. control, ANOVA followed by Dunnett's test ($n=6$). VPA: valproate; Px: picrotoxin.

animals exhibited clonic seizures. Although the gerbils were co-administered valproate and picrotoxin, the incidence of tonic extensor was 50%; there was no significant difference between the control group and the picrotoxin + valproate-treated group (Fig. 1A). The latency of clonic seizures induced by flurothyl in both the controls and the animals treated with 0.5 mg/kg of picrotoxin was about 400 s. The prolonged latency (509 s, $P<0.01$) due to 200 mg/kg of valproate was not antagonized by picrotoxin at all ($P<0.01$), as shown in Fig. 1B.

3.4. Inhibitory effect of flumazenil on the anticonvulsant effect of diazepam on flurothyl-induced seizures in Mongolian gerbils

Fig. 2 shows the antagonistic effect of flumazenil on the anticonvulsant action of diazepam. In both the control and flumazenil-treated groups, the incidence of tonic extensor was 83.3% (Fig. 2A). In the diazepam group, by contrast, the incidence of flurothyl-induced tonic extensor was 0% ($P<0.01$). When the gerbils were co-administered diazepam and flumazenil, the incidence was again 83.3% (Fig. 2A). The latency of clonic seizures induced by flurothyl in both the controls and the gerbils treated with 10 mg/kg of flumazenil was about 430 s. The prolonged latency due to 0.5 mg/kg of diazepam (about 544 s, $P<0.01$) was antagonized by flumazenil, as shown in Fig. 2B. The efficacy of diazepam was completely reversed by flumazenil (Fig. 2B).

3.5. Effect of various drugs on brain amino acid concentrations in Mongolian gerbils with flurothyl-induced seizures

Table 2 summarizes the effects of drugs acting on the GABA-benzodiazepine receptor complex on amino acid concentrations in the brain of Mongolian gerbils with flurothyl-induced seizures. Concerning glutamate and glycine, there was no significant difference in any of the four regions, the cortex, hippocampus, striatum or cerebellum. The concentration of GABA was significantly increased in the hippocampus ($P<0.05$) and in the cerebellum ($P<0.01$) in the diazepam-treated group. However, there were no significant differences in the cortex and striatum in the diazepam-treated group. In addition, there were no significant changes due to the other drugs in any of the four regions.

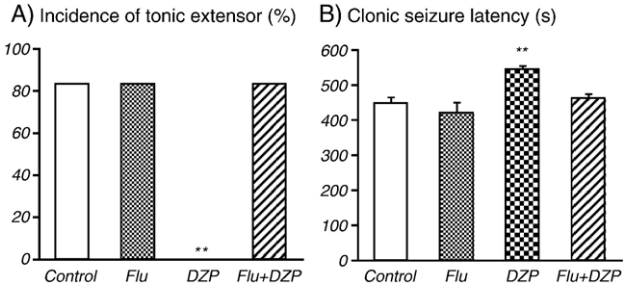

Fig. 2. Inhibitory effect of flumazenil on the anticonvulsant action of diazepam in Mongolian gerbils with flurothyl-induced seizures. Flumazenil (10 mg/kg) or diazepam (0.5 mg/kg) was intraperitoneally administered 30 min before exposure to flurothyl. When the animal was co-administered these two drugs, diazepam was given 2 min prior to flumazenil. After drug administration(s), the animal was returned to his cage. Mongolian gerbils were individually placed in airtight Bell jars when exposed to flurothyl. Clonic seizure latency was measured from the start of flurothyl infusion to expression of clonic seizure. The incidence of tonic extensor after clonic seizure was recorded. In the control group, the animals were administered the appropriate vehicle, according to the same experimental procedure used for the drug treatment group. (A) Incidence of tonic extensor (%). $^{**}P<0.01$ vs. control χ^2 test ($n=6$). (B) Clonic seizure latency (s). Each column represents the mean ± S.E.M. $^{**}P<0.01$ vs. control, ANOVA followed by Dunnett's test ($n=6$). DZP: diazepam; Flu: flumazenil.

Table 2
Effects of GABA-benzodiazepine modulating drugs on brain amino acid concentrations (nmol/mg) in Mongolian gerbils with flurothyl-induced seizures

	n	Cortex	Hippocampus	Striatum	Cerebellum
Glutamate					
Control	6	31.2±0.9	30.0±1.1	28.6±1.3	30.3±1.1
DZP	6	32.6±0.8	30.5±1.5	31.3±1.2	29.8±1.0
DZP+Flu	6	29.5±1.0	29.2±1.0	27.5±1.0	27.7±1.0
VPA	6	33.5±0.5	32.3±1.1	30.6±0.7	31.9±0.8
VPA+Px	6	30.7±0.6	31.0±0.7	28.6±0.9	29.5±0.6
Glycine					
Control	6	7.6±0.9	7.3±0.5	8.2±0.7	6.5±0.4
DZP	6	6.9±0.4	6.5±0.3	6.3±1.4	5.6±0.4
DZP+Flu	6	8.6±0.5	8.0±0.7	8.4±0.5	6.5±0.3
VPA	6	7.1±0.3	6.3±0.2	6.9±0.5	6.0±0.3
VPA+Px	6	7.2±0.2	6.9±0.3	6.8±0.3	6.7±0.5
GABA					
Control	6	9.3±1.1	9.4±0.6	10.1±0.8	8.6±0.6
DZP	6	11.6±0.7	11.4±0.5[a]	13.7±1.7	11.3±0.7[b]
DZP+Flu	6	11.4±0.6	10.4±0.3	11.6±1.0	10.1±0.4
VPA	6	10.8±0.5	11.0±0.3	13.1±0.6	10.0±0.4
VPA+Px	6	11.0±0.8	10.4±0.7	13.1±0.7	10.1±0.7

DZP: diazepam; Flu: flumazenil; VPA: valproate; Px: picrotoxin.
All values are presented as mean ± S.E.M.
[a] $P<0.05$ vs. control, ANOVA followed by Dunnett's test.
[b] $P<0.01$ vs. control, ANOVA followed by Dunnett's test.

4. Discussion

In this study, the effects of drugs targeting the GABA-benzodiazepine receptor complex on flurothyl-induced seizures in Mongolian gerbils were investigated. Flurothyl, a volatile convulsant, which has been established as effective for measuring susceptibility to seizures, reliably elicits convulsions from animals when delivered by inhalation (Alder et al., 1967). Other chemoconvulsants (for instance PTZ and kainic acid) are administered systematically (i.p. or i.v.) and they are not able to be controlled the severity of seizures until their inactivation. In the case of flurothyl, however, once sustained seizure activity begins, the intensity can be controlled by opening the chamber because flurothyl does not depend on metabolism for its action, hence its is rapidly eliminated. Flurothyl can produce two different phenotypes of seizures, i.e. forebrain and brainstem ones. The former occurs in animals exposed to flurothyl at lower thresholds with clonus of the face and forelimb, while the latter occurs at higher thresholds with tonus of the forelimb and hindlimb after forebrain seizure. Applegate et al. (1997) reported a model of epileptogenesis, which is based on the convulsant action of flurothyl: C57BL/6 mice were exposed to

flurothyl until they developed generalized seizures (forebrain seizure), once daily for 8 days. After this period, the mice were left undisturbed for 28 days and then re-exposed to flurothyl, to trigger brainstem seizures. Samoriski and Applegate (1997) reported that the number of generalized seizures and the duration of the stimulation-free interval are important factors for long-lasting reductions of seizure thresholds and for the expression of brainstem seizure. This model can be used to observe the behavioral change in kindling development during the stimulation-free period and also to observe tonic extensor, which is not possible with electrical kindling model. Thus, flurothyl is very useful because it can produce several phenotypes; acute clonic and tonic seizures, kindled state and seizure propagation. Our previous studies implied that drugs acting on GABA-benzodiazepine were somehow related to the mechanism of flurothyl-induced seizures. Therefore, to elucidate the mechanism involved, appropriate antagonists, picrotoxin for valproate and flumazenil for diazepam, were used in this study.

In this study, picrotoxin, a non-competitive antagonist of the $GABA_A$ receptor, slightly antagonized the anticonvulsant effect of valproate on the incidence of flurothyl-induced tonic extensor. Valproate possesses a wide spectrum of anticonvulsant effects and is clinically useful at the epileptic stage. It was reported that the anticonvulsant potency of valproate strongly depends on the species, the administration route, seizure induction, and the time interval between drug administration and seizure induction (Loscher, 1993). In this study of flurothyl-induced seizures in Mongolian gerbils, valproate exhibited marked anticonvulsant action, but the action of valproate was not antagonized by picrotoxin completely. Krasowski (2000) showed, using the whole-cell patch-clamp technique, that picrotoxin and flurothyl antagonize GABA responses at distinct sites or with different mechanisms of action. Furthermore, Gurley et al. (1995) suggested that flurothyl occupied the $GABA_A$ receptor more strongly than did picrotoxin, and the antagonistic action of picrotoxin was masked. Therefore, it is conceivable that the mechanism behind the seizures differs between flurothyl and picrotoxin. Pribilla et al. (1992) reported that although the only receptor targeted by flurothyl is the $GABA_A$ receptor, picrotoxin has a potent antagonistic action not only on the $GABA_A$ receptor but also on a homomeric glycine receptor. Consequently, picrotoxin did not reverse the anticonvulsant action of valproate in flurothyl-induced seizures in this study.

It was reported that flumazenil, a central benzodiazepine receptor antagonist, at a dose of 10 mg/kg, antagonizes various pharmacological effects of benzodiazepines (File et al., 1985; Loscher and Honack, 1994; Khan et al., 2000). In the preset study, flumazenil completely reversed the anticonvulsant effect of diazepam on the incidence of flurothyl-induced tonic extensor. It was established that flumazenil can suppress the anticonvulsant action of diazepam induced by various stimuli (Manocha et al., 2003). Flumazenil alone did not have any effect on the flurothyl-induced seizures, but it counteracted the anticonvulsant action of diazepam. There are two mechanisms behind the anticonvulsant action of diazepam as mentioned in the Introduction. In this study, we elucidated that flumazenil can reverse the action of diazepam in the flurothyl model. Therefore, it is speculated that, at least in part, flurothyl-induced seizures involve in the benzodiazepine receptor.

In this study, the effect of flurothyl on amino acid concentrations in the brain in Mongolian gerbils was also investigated. One of our new findings is the relationship between the anticonvulsant action of diazepam and amino acid levels. Glutamate and GABA concentrations during seizures are elevated in humans (During and Spencer, 1993). It generally seemed that glutamatergic transmission was enhanced and the extracellular glutamate concentration was increased (Bradford, 1995). However, in animal models, the results were different. Some reports indicated that the occurrence of seizures correlated with the increase in the extracellular concentrations of excitatory amino acids (Walker et al., 1995), whereas others showed neither changes (Lehman et al., 1985; Millan et al., 1991) nor a decrease (Sierra-Paredes et al., 2000). These discrepancies would be due to differences between species and the convulsion-inducing methods.

The concentrations of glutamate and glycine in the brain were not changed by the administration of various drugs in Mongolian gerbils with flurothyl-induced seizures. Ahmad et al. (2005) reported that the extracellular glutamate concentration in the brain was not significantly altered by valproate treatment. Li et al. (2004) also showed that glutamate and glycine concentrations in the rat hippocampus were not significantly changed 30 min after the administration of valproate. Extracellular concentrations of excitatory and inhibitory amino acids have been investigated in different types of epileptic seizures in humans and several animal models as indirect evidence of neurochemical modifications in the synaptic cleft.

In contrast to glutamate and glycine, GABA concentrations in the hippocampus and cerebellum were significantly increased by the treatment with diazepam. Hiramatsu et al. (1988) indicated that the brain GABA concentration was not changed by diazepam. However, Saad (1972) and Polc et al. (1974) showed that diazepam increased GABA concentrations in rodents, and Loscher and Schmidt (1987) reported similar findings in humans. They concluded that although the mechanism by which diazepam increases endogenous GABA levels is not clear, a reduction of GABA turnover in response to postsynaptic GABA potentiation is involved. Moreover, Battistin et al. (1984) showed that diazepam inhibited GABA transaminase activity, suggesting that it increased the GABA concentration symmetrically. In fact, there are indications that GABA content is abnormally low in some regions of the brain in seizure-sensitive gerbils (Loskota, 1974), and the GABA-benzodiazepine receptor function of this species is impaired (Olsen et al., 1984). It has been well established that benzodiazepines allosterically modulate GABA receptors function. Macdonald and Kapur (1999) proposed the use of benzodiazepines in the treatment of status epilepticus due to their ability to enhance $GABA_A$ receptor-mediated inhibitions. Eghbali et al. (1997) reported that hippocampal $GABA_A$ channel conductance was increased by diazepam. Therefore, the GABA mechanism is important for the occurrence of

seizures, and for the inhibitory effect of diazepam on seizures. However, in this study, the increases in the GABA concentration induced by diazepam were not significantly reversed by flumazenil despite the fact that flumazenil antagonized the seizures. To our knowledge, there is no report that flumazenil directly affects the concentration of GABA. Little (1984) reported that the effect of flumazenil on the response to GABA was dependent on concentration; at high concentrations, flumazenil significantly decreased the response to GABA and at low concentrations, increased it. In this study, we used Mongolian gerbils as an experimental animal, in which it is known that GABAergic system, including the GABA-benzodiazepine receptor, is impaired. Also, the dose of flumazenil used, 10 mg/kg, may be high for this species. Consequently, though the level of GABA increased when the gerbils were co-administered diazepam and flumazenil, flumazenil might have reversed the anticonvulsant action of diazepam.

It generally seems that the impairment of GABAergic inhibitory neurotransmission can lead to convulsions, whereas the potentiation of the GABAergic system results in anticonvulsant actions (Bradford, 1995; Loscher, 1998). In the present study, valproate protected against flurothyl-induced tonic extensor, but the concentration of GABA in the brain was not increased. Lindekens et al. (2000) made a similar finding, that valproate had an anticonvulsant effect on pilocarpine-induced seizures, but did not significantly alter extracellular GABA release. However, increases in brain GABA concentrations in rodents are only seen after the administration of relatively high doses of valproate; whereas lower doses, which still exert an anticonvulsant action, do not change the GABA concentration (Morre et al., 1984; Biggs et al., 1992). Taken together, it might be suggested that the anticonvulsant action of valproate is not always correlated in the increase in the concentrations of GABA in the brain, and that the effect of diazepam on the GABA concentration is related to its anticonvulsant action.

In conclusion, both diazepam and valproate had strong anticonvulsant effects on flurothyl-induced seizures. Although picrotoxin did not completely antagonize the anticonvulsant action of valproate, flumazenil was able to reverse the action of diazepam completely. In a biochemical study, diazepam increased GABA concentrations in the hippocampus and cerebellum. These findings in the flurothyl model showed that the relationship between the anticonvulsant action of diazepam and increase in the concentration of GABA in these regions may be produced by GABA-shift; i.e., an increase in sensitivity of the benzodiazepine receptor through the increase in GABA concentrations. These results suggest that the mechanism of flurothyl-induced seizures, in part, is related to the highly sensitive benzodiazepine site of the GABA-benzodiazepine receptor chloride ionophore complex.

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