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Benzodiazepine System is Involved in Hyperalgesia in Rats Induced by the Exposure to Extremely Low Frequency Magnetic Fields

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Many reports demonstrate that extremely low frequency magnetic fields (ELF MFs, 60 Hz) may be involved in hyperalgesia. In a previous investigation, we suggested that MFs may produce hyperalgesia and such a response may be regulated by the benzodiazepine system. In order to further confirm this effect of MFs, we used diazepam and/or flumazenil with MFs exposure. When testing the pain threshold of rats using hot plate tests, MFs or diazepam (0.5 μg, i.c.v.; a benzodiazepine receptor agonist) induced hyperalgesic effects with the reduction of latency. These effects were blocked by a pretreatment of flumazenil (1.5 mg/kg, i.p.; a benzodiazepine receptor antagonist). When the rats were exposed simultaneously to MFs and diazepam, the latency tended to decrease without statistical significance. The induction of hyperalgesia by co-exposure to MFs and diazepam was also blocked by flumazenil. However, the pretreatment of GABA receptor antagonists such as bicuculline (0.1 μg, i.c.v.; a $GABA_A$ antagonist) or phaclofen (10 μg, i.c.v.; a $GABA_B$ antagonist) did not antagonize the hyperalgesic effect of MFs. These results suggest that the benzodiazepine system may be involved in MFs-induced hyperalgesia.

Key words: Magnetic fields, Hyperalgesia, Diazepam, Flumazenil, GABA

INTRODUCTION

It has been reported that extremely low frequency (ELF, <300 Hz) magnetic fields (MFs) produce a variety of behavioral and physiological functions in animals (Adey, 1981; Gould, 1984; Frey, 1993). Some investigations have also reported on animal "hypersensitivity" to electric or MFs (Hillert *et al.*, 1997). These studies examined whether pains, depression, lethargy, sleeping disorders, and even epileptic seizures are associated with electromagnetic field exposure. Kavaliers and Ossenkopp (1986) suggested that exposure to magnetic stimuli might alter morphine-induced responses in mice. Exposure of mice to earth strength, 0~1.5 gauss (G), 60 Hz MFs results in a reversible, dose-dependent inhibition of nocturnal peak in the day-night rhythmicity of morphine-induced analgesia (Kavaliers *et al.*, 1984). Furthermore, several types of MFs alter the effects of morphine-induced analgesia in mice (Ossenkopp and Ossenkopp, 1983; Ossenkopp *et al.*, 1985). In addition to the action on exogenous opioid-mediated analgesia, MFs stimuli reduce the endogenous opioid-mediated analgesia in various species, including humans (Kavaliers and Ossenkopp, 1986; Papi *et al.*, 1995; Kavaliers *et al.*, 1998; Jeong *et al.*, 2000).

Several studies have pointed out a significant interaction between an opiate and benzodiazepine in the brain. The involvement of opioid and benzodiazepine systems has also been reported by Mantegazza *et al.* (1982). They showed that an intracerebroventricular (i.c.v.) administration of diazepam or midazolam decreases the antinociceptive effect of morphine in rats as measured by the tail flick and hot plate methods (Mantegazza *et al.*, 1982; Rosland *et al.*, 1990; Luger *et al.*, 1994). Midazolam also causes hyperalgesia when administered intracerebroventricularly. Furthermore, flumazenil was shown to block the hyperalgesia induced by midazolam in a tail-flick test (Niv *et al.*, 1988; Tatsuo *et al.*, 1999). In previous work, we found that exposure of 20 G MFs alone induces a significant reduction of latency in normal mice (Jeong *et*

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al., 2000). From these investigations, we hypothesized that MFs-induced hyperalgesia occurs *via* the benzodiazepine receptor. The objective of the present work is to elucidate the interaction between MFs and the benzodiazepine-GABA complex system.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (Hanlim, Korea) weighing 250-350 g each were used in all experiments. These animals were maintained in a temperature-controlled room (25 ± 2 °C) and kept on a 12:12 light dark cycle (lights on at 08:00 h). Food and water were available *ad libitum* during all experiments. The animals received i.c.v. catheters and were housed individually.

Hot plate test

Pain thresholds were evaluated with the hot plate test. The hot plate test was conducted by previously reported methods (Jeong *et al.*, 2000). The rats were placed individually on the hot plate (50.2 ± 0.2 °C) and latency was recorded. Latency is defined as the time elapsed to obtain one of the following responses: licking the feet, jumping or rapidly stamping the feet, whichever occurred first. To prevent tissue damage the cut-off time was 50 sec. Hyperalgesia is defined as a statistically significant difference in the latency between control and treated rats. The rats were placed on the hot plate after i.c.v. administration.

Intracerebroventricular catheters

Each rat was placed in a stereotaxic frame following pentobarbital (50 mg/kg, intraperitoneal) anesthesia. The skull was exposed and then a hole was bored at coordinates overlaying the left lateral ventricle: 1 mm posterior to the bregma and 1 mm left to the midline, according to the atlas of Paxinos and Watson (1986). A guide cannula was inserted 4 mm into the lateral ventricle. The cannula was fixed to the skull with dental acrylic and secured by stainless steel screws. Those animals with normal motor function and behavior were used 4-7 days after this procedure for the experiment. Intracerebroventricular (i.c.v.) administration was performed using a Hamilton syringe. After the experiments, the rats were sacrificed 5 min after a methylene blue injection followed by rapid removal of the brain in order to confirm accurate injection of the drug with the dye deposition around the ventricular system (Tatsuo *et al.*, 1999).

Magnetic fields, drugs and procedures

MFs were generated with Helmholtz coils set parallel to each other in a wood frame. MFs consisted of 60 Hz time-varying fields. The generated MFs were within a range of 0-25 G intensity with the change of input voltage.

Diazepam was purchased from Samjin Pharm. Co. (Seoul. Korea). Diazepam was dissolved in 0.7% ethanol. Diazepam was administered intracerebroventricularly. Roche Korea (Seoul, Korea) kindly presented flumazenil to us. Flumazenil was administered intraperitonealy (i.p.). Bicuculline methobromide and phaclofen were bought from Sigma (St. Louis, MO, USA). Bicuculline was dissolved in saline. Phaclofen was dissolved in 0.1 N NaOH. All drugs were administered intracerebroventricularly except flumazenil.

Seven to ten rats were used in each group. All hot plate experiments were carried out from 16:00 to 17:00 in order to avoid an interruption of the circadian rhythm. MFs exposure started at fixed times considering exposure duration and lasted till just before the hot plate test. All drugs administered i.c.v. were given 5 min before the hot plate test. Flumazenil was injected i.p.15 min before the hot plate test to allow enough time for systemic absorption in the rats.

Statistical analysis

Data are presented as the mean ± SEM. Differences between groups were tested using Students *t*-test. Differences between multiple groups were tested using analysis of variance (ANOVA) for repeated measures and checked for significance using Scheffe's *F*-test. Differences were considered statistically significant if a value of $P < 0.05$ was obtained.

RESULTS

Table I shows the results of the rats' responses to thermal stimuli in the hot plate test. Exposure to 10 and 20 G of MFs for 6 h induced a significant reduction of latency (pain threshold; $P<0.05$) but the rats did not respond at

Table I. Reduction of pain threshold dependent on the intensity and duration of magnetic field exposure

	Exposure time			
	0 h	1 h	3 h	6 h
control	27.2 ± 3.5	30.1 ± 2.0	27.5 ± 2.5	29.7 ± 2.2
5 G	30.2 ± 1.9	29.1 ± 1.8	29.8 ± 2.7	27.1 ± 1.2
10 G	29.5 ± 2.7	28.9 ± 1.6	26.9 ± 2.8	24.9 ±0.8 *
20 G	30.1 ± 2.3	28.7 ± 1.9	27.1 ± 2.4	22.8 ± 2.1 *

We exposed rats to MFs and conducted the hot plate test at 0, 1, 3 and 6 h after exposure. A significant hyperalgesic effect occurred after exposure of 20 G (6 h). The results are presented as the mean ± SEM for latency. *designates significant differences ($P<0.05$) compared to the sham control in corresponding time and baseline, respectively.

the lower intensity of 5 G of MFs. With this result, we fixed the intensity and the duration of MFs at 20 G and 6 h, respectively, to induce hyperalgesia by MFs in the following experiments.

The effect of MFs-exposure or an injection of diazepam (i.c.v.) on the latency is shown in Fig. 1. A 0.5 μg administration of diazepam induced a hyperalgesic effect as significantly as did MFs ($P<0.05$). The time-response curve also shows high similarity between both groups. The hyperalgesic effects of MFs and diazepam were completely inhibited by the treatment of flumazenil (1.5 mg/kg, i.p.; Fig. 2 A and B).

We examined the effect of simultaneous exposure of rats to MFs and diazepam as shown in Fig. 3. The administration of diazepam (0.5 μg, i.c.v.) after exposure to MFs significantly reduced the latency compared to the control. The reduction of the latency was increased by long MFs exposures (40, 50, and 60 min) more than that by MFs or diazepam alone, but there was no significant difference between the groups.

When we treated the rats with flumazenil (1.5 mg/kg, i.p.) in simultaneous exposure to MFs and diazepam, its inhibitory effect was apparent; however, it did not completely reverse hyperalgesia as much as was reversed in MFs or diazepam alone (Fig. 4).)

The effect of bicuculline or phaclofen on the hyperalgesia induced by MFs is shown in Fig. 5A and B, respectively. Bicuculline (0.1 μg, i.c.v.), a $GABA_A$ receptor antagonist, did not blunt the hyperalgesic effect of MFs. Phaclofen, a $GABA_B$ receptor antagonist, also did not inhibit the hyperalgesia induced by MFs. At these doses, bicuculline or phaclofen did not produce any significant effect.

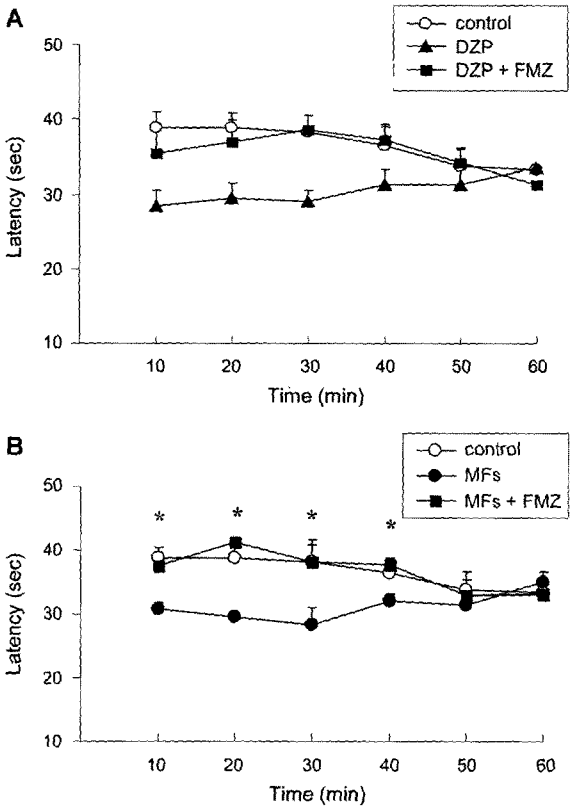

Fig. 2. Antagonism of diazepam or MFs-induced hyperalgesia by flumazenil. **A:** Rats received flumazenil (1.5 mg/kg, i.p.) 10 min before diazepam (0.5 μg, i.c.v.). The hot plate test was performed 5 min after the administration of diazepam. **B:** Rats were injected with flumazenil i.p. 15 min before MFs exposure finished (20 G, 6 h). The hot plate test was performed as soon as MFs exposure ended. Results are expressed as the means ± SEM of the latency. In both experiments, flumazenil completely inhibited the hyperalgesia induced by diazepam or MFs ($P<0.05$; ANOVA). DZP: diazepam; FMZ: flumazenil; MFs: Magnetic fields.

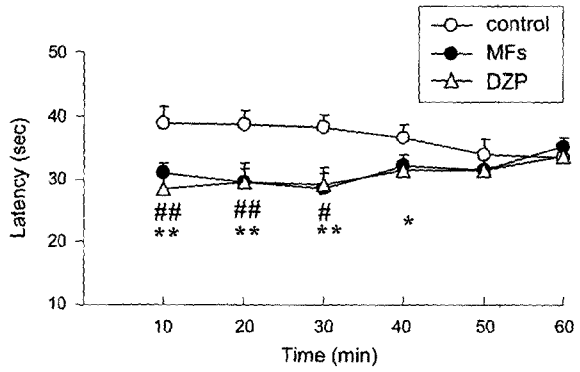

Fig. 1. Hyperalgesia induced by MFs or diazepam. The rats were exposed to 20 G of MFs or a sham treatment for 6 h. Diazepam, 0.5 μg, was administered i.c.v. 30 min before the hot plate test. Results are expressed as the means ± SEM of the latency. Both MFs and diazepam induced significant differences (MFs: $P<0.05$, diazepam: $P<0.05$; ANOVA) compared to the control. DZP: diazepam; MFs: Magnetic fields.

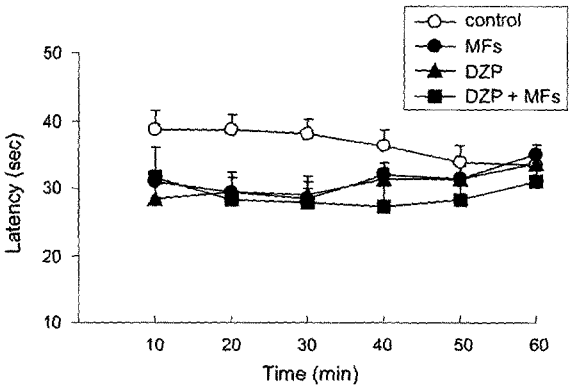

Fig. 3. Interaction between diazepam and MFs. Rats were administered with diazepam (0.5 μg, i.c.v.) before the end of the 6 h 20 G MFs exposure. The hot plate test was performed after finishing the MFs exposure. Results are expressed as the means ± SEM of the latency. The co-exposure of rats to MFs and diazepam induced a lower latency ($P<0.05$; ANOVA), but there were no significant differences compared to MFs or diazepam alone. DZP: diazepam; MFs: Magnetic fields

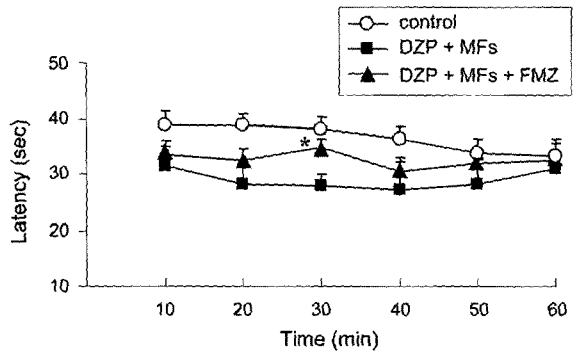

Fig. 4. Inhibition of diazepam and MFs-induced hyperalgesia by flumazenil. Flumazenil (1.5 mg/kg, i.p.) and diazepam (0.5 μg, i.c.v.) were injected 15 and 5 min, respectively, before the 6 h 20 G MFs exposure ended. The hot plate test was performed after finishing the MFs exposure. Results are expressed as the means ± SEM of the latency. The hyperalgesia induced by co-exposure to MFs and diazepam was reversed by flumazenil in single check point ($P<0.05$; ANOVA). DZP: diazepam; FMZ: flumazenil; MFs: Magnetic fields

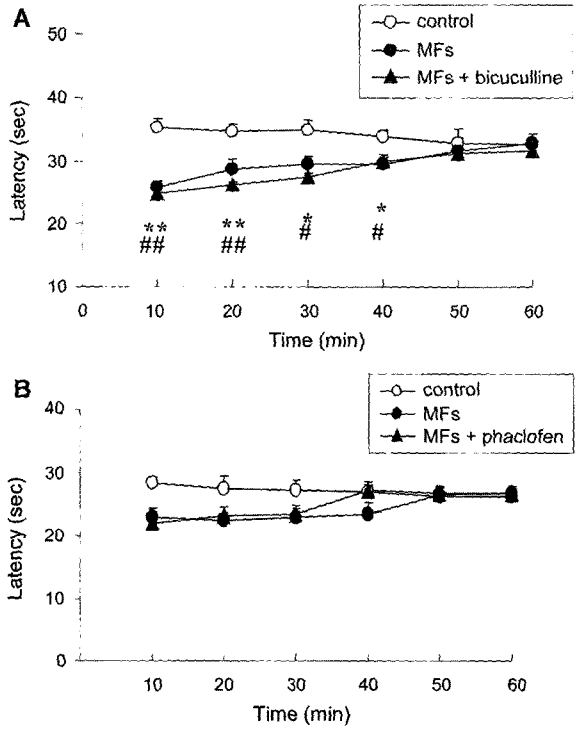

Fig. 5. No influence by $GABA_A$ receptor antagonists on MFs-induced hyperalgesia. **A:** Rats were given i.c.v. 0.1 μg of bicuculline 5 min before the 6 h 20 G MFs exposure ended. **B:** Rats were given i.c.v. 10 μg of phaclofen 5 min before the end of the 6 hrs 20 G MFs exposure. The hot plate test was performed after finishing the MFs exposure. Results are expressed as the means ± SEM of the latency. The GABA antagonists did not inhibit the MFs-induced hyperalgesia. MFs: Magnetic fields

DISCUSSION

We have previously reported that exposure to 20 G of MFs for 6 h induces a significant reduction of latency in normal mice (Jeong *et al.*, 2000). The results of the present study also show that exposure to 10 and 20 G of MFs for 6 h induced a significant hyperalgesic effect in rats. MFs-induced hyperalgesia significantly persisted for 30 min. These repeated results confirm a biological effect of MFs. To further clarify the mechanism of this MFs effect, we fixed MFs at 20 G for 6 h because the hyperalgesic effect of 10 G of MFs was not apparent in our previous experiments (Jeong *et al.*, 2000).

MFs-induced hyperalgesia was similar to the hyperalgesic effect induced by diazepam. Tatsuo (1999) reported that midazolam causes hyperalgesia when administered intracerebroventricularly. Additionally, flumazenil was shown to block the hyperalgesia induced by midazolam in a tail-flick test (Niv *et al.*, 1988; Tatsuo *et al.*, 1999). This result is consistent with our finding that flumazenil, a benzodiazepine receptor antagonist, inhibited the hyperalgesia induced by diazepam at the tested dose, at which the flumazenil dose did not produce any significant effect (Data not shown). In addition, this flumazenil dose completely reversed the reduced latency by MFs exposure. These results indicate that MFs and diazepam-induced hyperalgesia may have a similar pathway such as the activation of a benzodiazepine receptor. However, considering MFs are not chemical compounds like diazepam, we are not able to directly compare MFs to diazepam.

Diazepam has been known to act in the central nervous system by binding to a benzodiazepine receptor that opens the chloride channel. The MFs used in the present study are invisible and extremely low frequency radiation. They also have no ionizing effect on molecules. Therefore, the biological effect of MFs precludes the interaction between molecules like diazepam and a benzodiazepine receptor. One hypothesis is that MFs may cause a conformational change in the benzodiazepine receptor, resulting in an increase in the influx of chloride ions. Time varying MFs, if the exposure is strong and long enough, interfere with the electrostatic stability of the cell environment and animals. In the current case, all biological systems did not respond to MFs. However, this finding may not exclude that a highly sensitive molecular system and biological environment that respond to MFs are present. For instance, nerve cells acting by ion flow may be highly sensitive to MFs. It is interesting to note that the benzodiazepine receptor antagonist completely blocked MFs influence on pain threshold even if it is not a molecule.

As we mentioned earlier, the effects of benzodiazepines are mediated by the interaction of GABA with $GABA_A$

receptors (Costa and Guidotti, 1979; Tallman and Gallaher, 1985; Matsumoto, 1989). Furthermore, GABA receptors are divided into $GABA_A$ and $GABA_B$ receptors (Enna and Karbon, 1986; Bormann, 1988; Bowery, 1989). The present results have shown that MFs-induced hyperalgesia was not changed by bicuculline (a $GABA_A$ receptor antagonist) or phaclofen (a $GABA_B$ receptor antagonist). At the tested dose, bicuculline or phaclofen did not produce any significant effect (Data not shown). In this experiment, we could not obtain a similar result with flumazenil. Since GABA and benzodiazepine binding sites are components in the GABA-benzodiazepine complex, we expected that the treatment with the GABA antagonist would give us a similar result as that found with flumazenil. However, this did not occur. From these results, we suggest that the influence of MFs on hyperalgesia may have specificity in the benzodiazepine system. Further study will be done on what mechanism is associated with this specific effect of MFs on the benzodiazepine system.

In summary, the exposure of rats to MFs or diazepam produced hyperalgesia with a reduction of latency in a hot plate test. The hyperalgesia induced by MFs was completely inhibited by flumazenil, a benzodiazepine receptor antagonist, but not by bicuculline or phaclofen (a $GABA_B$ receptor antagonist). These results suggest that the benzodiazepine system of the benzodiazepine-GABA complex may be involved in MFs-induced hyperalgesia.

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