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Benzodiazepine/$GABA_A$ receptors are involved in magnesium-induced anxiolytic-like behavior in mice

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Abstract:
Behavioral studies have suggested an involvement of the glutamate pathway in the mechanism of action of anxiolytic drugs, including the NMDA receptor complex. It was shown that magnesium, an NMDA receptor inhibitor, exhibited anxiolytic-like activity in the elevated plus-maze test in mice. The purpose of the present study was to examine interaction between magnesium and benzodiazepine/$GABA_A$ receptors in producing anxiolytic-like activity. We examined behavior of mice treated with magnesium and benzodiazepine/$GABA_A$ receptor ligands, in the elevated plus maze. The anxiolytic-like effect of magnesium (20 mg/kg) was antagonized by flumazenil (10 mg/kg) (benzodiazepine receptor antagonist) while combined treatment with the non-effective doses of magnesium (10 mg/kg) and benzodiazepines (diazepam (0.5 mg/kg) or chlordiazepoxide (2 mg/kg)) produced synergistic interaction (increased time in open arms and number of open arm entries) in this test. The obtained data indicate that benzodiazepine receptors are involved in the anxiolytic-like effects of magnesium.

Key words:
magnesium, flumazenil, chlordiazepoxide, diazepam, anxiety, elevated plus-maze, mice

Introduction

Benzodiazepines were the first drugs for the treatment of anxiety disorders [2] despite a high incidence of unwanted side effects (tolerance, sedation and risk of abuse) [26].

Chronic administration of benzodiazepines produced physiological dependence and severe withdrawal syndrome could occur [8]. Moreover, these treatments are effective in only 70% of patients and full remission is observed only in 40% of patients. Several studies have demonstrated that antagonists of the NMDA-glutamate receptor exhibited an anxiolytic-like effect in animal tests of anxiety [1, 4, 6, 12, 17, 22] and that combined administration of an NMDA antagonist (MK-801) with diazepam had additive effects [22].

Magnesium (Mg) is a very potent inhibitor of the NMDA receptor complex. This ion blocks the activation of NMDA receptor ion channel in a voltage-dependent manner [24]. Our previous studies have indicated that Mg produced antidepressant and anxiolytic-like activity without development of tolerance to these effects [13]. Moreover, we have also shown that Mg administered at a dose of 10 mg/kg, ineffective *per se*, normalized the increased immobility time in restraint stress-exposed mice [15]. Our subsequent

studies proved that the antidepressant activity of Mg (like organic NMDA antagonists) involves the NMDA/glutamate pathway [14, 16]. In addition Mg depletion leads to an increase in depression- and anxiety-related behavior as revealed by using various behavioral paradigms [9, 23]. Clinical observations demonstrated low levels of Mg in patients with anxiety and depression [3, 5, 20, 21]. It was shown that anxiety increased the need for Mg [20, 21]. Therefore, it is important to investigate the involvement of Mg in the mechanism of anxiolytic drug action.

In this study, we investigated the interaction between Mg and benzodiazepine/$GABA_A$ receptor ligands in the elevated plus maze test in mice.

Materials and Methods

Animals

All procedures were approved by the Ethical Committee of the Medical University, Lublin. The experiments were carried out on male albino Swiss mice (25–30 g). The animals were kept on a natural day-night cycle with free access to food and water. Each experimental group consisted of 9–12 animals.

Drug administration

Magnesium hydroaspartate – Mg (Farmapol, Poznań, Poland) alone or in combination with other drugs was administered intraperitoneally (*ip*) 0.5 h before the test. Chlordiazepoxide (Polfa, Poland), diazepam (Polfa, Poland), flumazenil (Hoffman-La Roche – Basel, Switzerland) were suspended in a 1% aqueous solution of Tween 80 just prior to use and administered 1 h before the test. Control animals received an *ip* injection of 1% aqueous solution of Tween 80 (vehicle). All vehicle and drug suspensions were administered in a volume of 10 ml/kg.

Elevated plus-maze test

The studies were carried out on mice according to the method of Lister [7]. The plus-maze apparatus was made of Plexiglas and consisted of two open (30 × 5 cm) and two closed (30 × 5 × 15 cm) arms. The arms extended from a central platform of 5 × 5 cm. The apparatus was mounted on a Plexiglas base raising it 38.5 cm above the floor and illuminated by red light. The test consisted in placing a mouse in the center of the apparatus (facing a closed arm) and allowing it to freely explore. The number of entries into the open arms and the time spent in these arms were scored for a 5-min test period. An entry was defined as placing all four paws within the boundaries of the arm. The following measures were obtained from the test: the total number of arm entries; the percentage of arm entries into the open arms; the time spent in the open arms expressed as a percentage of the time spent in both the open and closed arms. Anxiolytic activity was indicated by increases in time spent in open arms or in number of open arm entries. Total number of entries into either type of arm was used as a measure of overall motor activity.

Statistical analysis

The obtained data were evaluated by the analysis of variance (ANOVA) followed by Bonferroni *post-hoc* test. All results are presented as the means ± SEM; $p < 0.05$ was considered as statistically significant.

Results

Effect of flumazenil on the anxiolytic-like activity of magnesium

Mg given at a dose of 20 mg/kg induced an anxiolytic-like effect significantly increasing the percentage of the time spent in the open arms, and the percentage of the open arm entries (Fig. 1). The increase in the percentage of the time spent in the open arms induced by Mg (20 mg/kg) was significantly reversed by flumazenil (10 mg/kg) [Fig. 1A; $F(3, 37) = 5.848$, $p = 0.0023$]. The increase in the percentage of the open arm entries induced by Mg (20 mg/kg) was partially reversed by flumazenil (10 mg/kg) [Fig. 1B; $F(3, 37) = 2.825$, $p = 0.0518$]. Flumazenil given alone had no effect on either the time spent or the entries into the open arms in the plus-maze test (Fig. 1A, B).

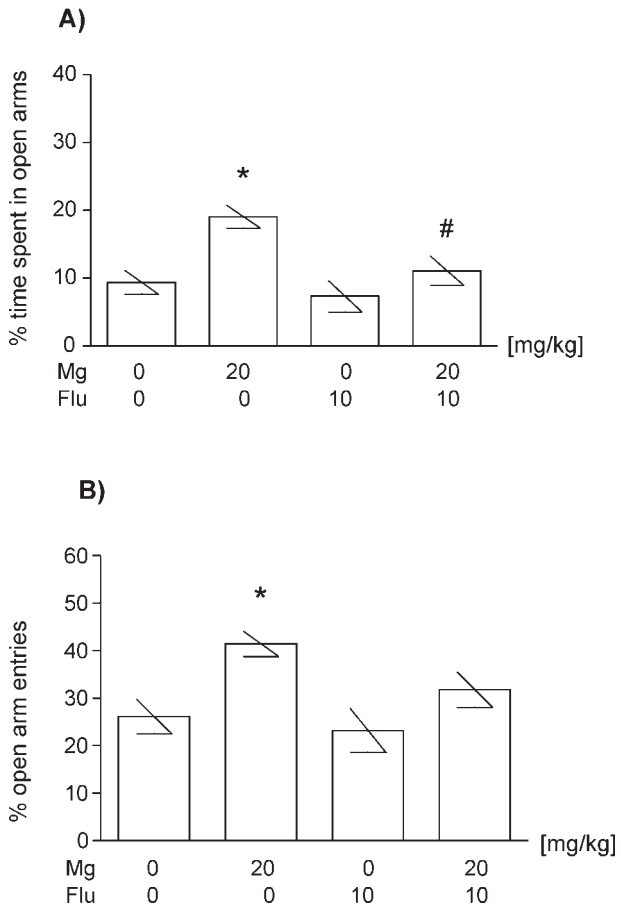

Fig. 1. Effect of flumazenil (Flu) on the action of magnesium (Mg) in the elevated plus-maze procedure in mice (percentage of time spent in open arms – **A**, and percentage of open arm entries – **B**). Flu was administered 60 min before the test, Mg was administered 30 min after the Flu injection. The values represent the means ± SEM (n = 9–12 mice per group). The absolute values in vehicle-treated mice were as follows: time 28.2 ± 5.4 and number of open arm entries 3.8 ± 0.4. * $p < 0.01$ *vs.* control (vehicle-treated groups), # $p < 0.05$ *vs.* Mg-treated group (Bonferroni's test)

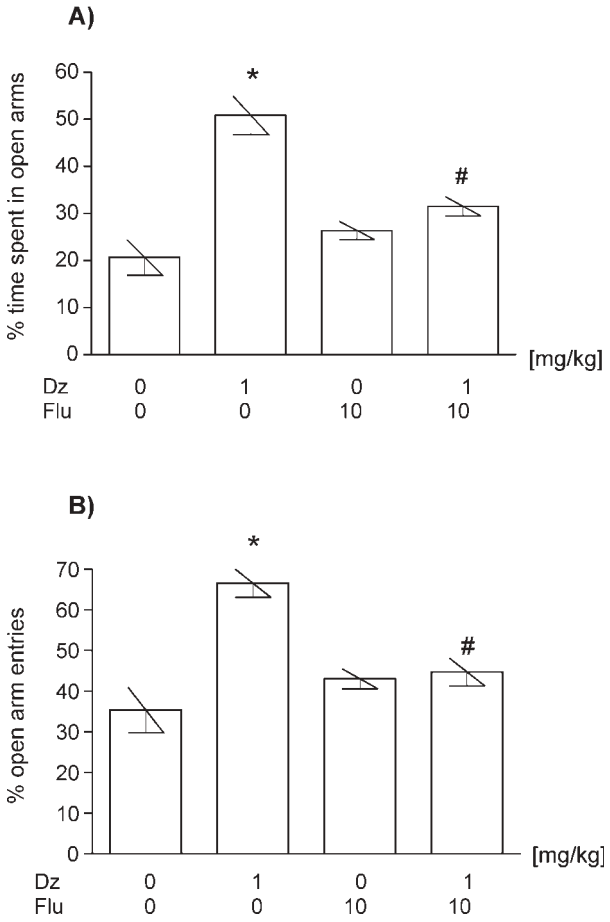

Fig. 2. Effect of flumazenil (Flu) on the action of diazepam (Dz) in the elevated plus-maze procedure in mice (percentage of time spent in open arms – **A**, and percentage of open arm entries – **B**). Flu and Dz were administered 60 min before the test. The values represent the means ± SEM (n = 9–10 mice per group). The absolute values in vehicle-treated mice were as follows: time 31.6 ± 5.7 and number of open arm entries 3.4 ± 0.6. * $p < 0.001$ *vs.* control (vehicle-treated groups), # $p < 0.001$ *vs.* Dz-treated group (Bonferroni's test)

Effect of flumazenil on the anxiolytic-like activity of diazepam

In order to verify the ability of flumazenil to antagonize benzodiazepine action in our behavioral paradigm, we examined the effect of combined diazepam and flumazenil administration. Diazepam given at a dose of 1 mg/kg induced an anxiolytic-like effect significantly increasing the percentage of the time spent in the open arms and the percentage of the open arm entries (Fig. 2). The increase in the percentage of the time spent in the open arms induced by diazepam (1 mg/kg) was significantly reversed by flumazenil (10 mg/kg) [Fig. 2A; $F(3, 34) = 18.31$, $p < 0.0001$]. The increase in the percentage of the open arm entries induced by diazepam was reversed by flumazenil (10 mg/kg) [Fig. 2B; $F(3, 34) = 12.49$, $p < 0.0001$]. Flumazenil given alone had no effect on either the time spent or the entries into the open arms in the plus-maze test (Fig. 2A, B).

Anxiolytic-like effect of joint administration of diazepam and magnesium

Mg administered at the dose of 10 mg/kg and diazepam administered at the dose of 0.5 mg/kg did not change the percentage of the time spent and entries into the open arms (Fig. 3). The joint administration

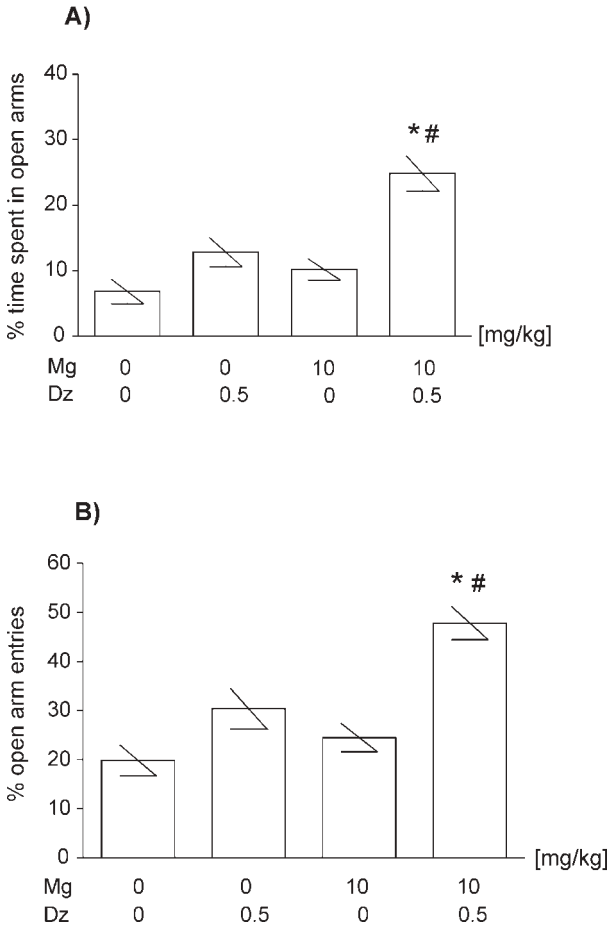

Fig. 3. Effect of joint administration of diazepam (Dz) and magnesium (Mg) in the elevated plus-maze procedure in mice (percentage of time spent in open arms – **A**, and percentage of open arm entries – **B**). Dz was administered 60 min before the test, Mg was administered 30 min after the Dz injection. The values represent the means ± SEM (n = 10–12 mice per group). The absolute values in vehicle-treated mice were as follows: time 20.7 ± 5.8 and number of open arm entries 2.8 ± 0.5. * $p < 0.001$ *vs.* control (vehicle-treated groups), # $p < 0.001$ *vs.* Mg or 0.001 *vs.* Dz in part A or 0.001 *vs.* Mg or 0.01 *vs.* Dz-treated groups in part B (Bonferroni's test)

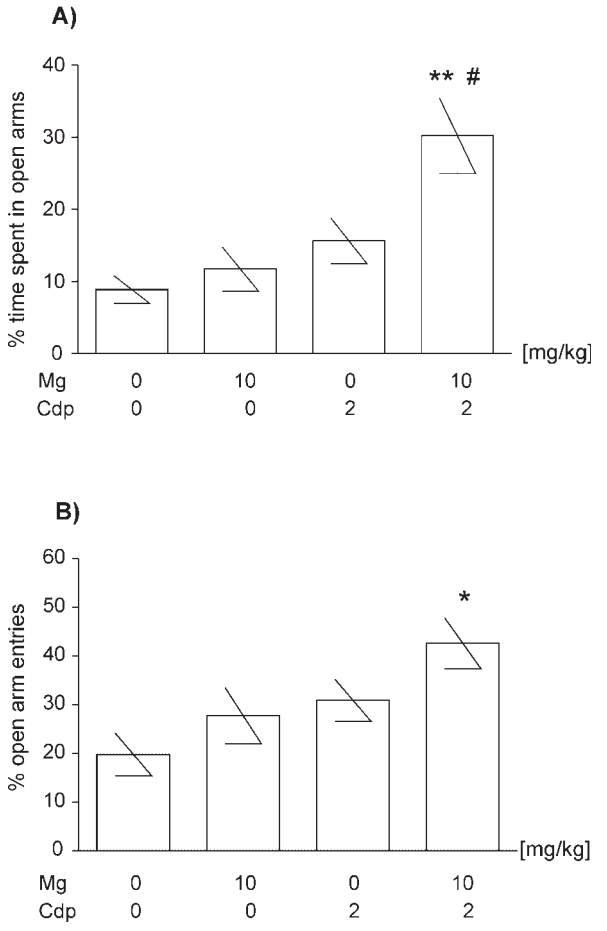

Fig. 4. Effect of joint administration of chlordiazepoxide (Cdp) and magnesium (Mg) in the elevated plus-maze procedure in mice (percentage of time spent in open arms – **A**, and percentage of open arm entries – **B**). Cdp was administered 60 min before the test, Mg was administered 30 min after the Cdp injection. The values represent the means ± SEM (n = 9–10 mice per group). The absolute values in vehicle-treated mice were as follows: time 26.7 ± 5.8 and number of open arm entries 4.2 ± 1.3. * $p < 0.01$, ** $p < 0.001$ *vs.* control (vehicle-treated groups), # $p < 0.05$ *vs.* Cdp or 0.01 *vs.* Mg-treated group (Bonferroni's test)

of Mg and diazepam significantly increased the percentage of time spent in the open arms [Fig. 3A; $F(3, 41) = 13.52$, $p < 0.0001$]. The joint administration of Mg and diazepam significantly increased number of open arm entries [Fig. 3B; $F(3, 41) = 13.10$, $p < 0.0001$].

Anxiolytic-like effect of joint administration of chlordiazepoxide and magnesium

Mg administered at the dose of 10 mg/kg and chlordiazepoxide administered at the dose of 2 mg/kg did not change the percentage of the time spent and entries into the open arms (Fig. 4). The joint administration of Mg and chlordiazepoxide significantly increased the percentage of time spent in the open arms [Fig. 4A; $F(3, 35 = 7.093$, $p = 0.0008$]. The joint administration of Mg and chlordiazepoxide significantly increased the number of open arm entries [Fig. 3B; $F(3, 35) = 3.756$, $p = 0.0194$].

Effect of magnesium and benzodiazepine ligands on the total arm entries

Mg and flumazenil did not alter the number of total arm entries (Tab. 1). However, diazepam (0.5 and

1 mg/kg) alone, and combined treatment with magnesium (10 mg/kg) and diazepam (0.5 mg/kg) or chlordiazepoxide (2 mg/kg) significantly increased the total arm entries (Tab. 1).

Tab. 1. The number of total arm entries for all experimental groups

Treatment and dose	Number of total arm entries
Vehicle	15.5 ± 1.03
Magnesium 20 mg/kg	18.58 ± 1.68
Flumazenil 10 mg/kg	16.8 ± 1.76
Magnesium and flumazenil	17.67 ± 1.42
	F(3, 37) = 0.7570, p = 0.5254
Vehicle	19.33 ± 1.09
Diazepam 1 mg/kg	33.4 ± 3.75**
Flumazenil 10 mg/kg	19.90 ± 1.47
Diazepam and flumazenil	28.56 ± 2.32
	F(3, 34) = 7.966, p = 0.0004
Vehicle	14.83 ± 0.80
Diazepam 0.5 mg/kg	20.55 ± 1.34*
Magnesium 10 mg/kg	14.50 ± 1.00
Magnesium 10 mg/kg and diazepam 0.5 mg/kg	22.25 ± 1.72***
	F(3, 41) = 9.443, p < 0.0001
Vehicle	19.0 ± 1.88
Magnesium 10 mg/kg	20.56 ± 3.16
Chlordiazepoxide 2 mg/kg	27.8 ± 1.97
Magnesium 10 mg/kg + chlordiazepoxide 2 mg/kg	28.3 ± 2.42*
	F(3, 35) = 4.165, p = 0.0127

Data represent the means ± SEM, n = 9–12. Chlordiazepoxide, diazepam and flumazenil were administered 60 min before the test, magnesium was administered 30 min before the test. * p < 0.05; ** p < 0.01; *** p < 0.001 *vs.* control-vehicle-treated group (Bonferroni's test)

Discussion

Preclinical evidence suggested that NMDA receptor antagonists might be useful for treatment of anxiety disorders [28, 29], however, serious untoward effects precluded their clinical use. A number of behavioral data have suggested the involvement of glutamate-mediated neurotransmission in anxiolytic-like behavior.

In animals, NMDA receptor antagonists exhibit anxiolytic-like activity. The antidepressant-like activity was demonstrated for a non-competitive NMDA antagonist, dizocilpine (MK-801) [1, 4, 22], competitive NMDA antagonist, 2-amino-7-phosphonoheptanoic acid (AP7) [12], DL-(E)-2-amino-4-methyl-5-phosphono-3-pentenoic acid (CGP 37849) [17], and partial NMDA agonist 1-aminocyclopropanecarboxylic acid (ACPC) [4, 17] and antagonist L-701,324 [6] of glycine$_B$ site of the NMDA receptor.

The anxiolytic-like activity of MK-801 was potentiated by diazepam and reversed by the anxiogenic β-carboline agent (FG-7142) and the benzodiazepine receptor antagonist (Ro-15-1788) [22] and the effect of CGP 37849 has been antagonized by flumazenil [16] in the rat elevated plus maze (EPM) test. Thus, the benzodiazepine receptors are involved in the anxiolytic-like activity of NMDA antagonists and this is due to a possible interaction between NMDA and GABA/benzodiazepine system [17].

Similarly to organic antagonists of NMDA receptor complex, Mg also exhibits anxiolytic-like activity [13]. Our previous studies showed that Mg significantly increased time spent in open arms and number of open arm entries in the EPM procedure [13]. Validation of the EPM procedure has shown that it is sensitive to drugs that produce anxiolytic or anxiogenic effects in humans [10], including drugs that have non-benzodiazepine sites of action [11].

The present study has demonstrated the effect of Mg on the action of benzodiazepines, the most common anxiolytic drugs [2]. We examined the influence of Mg on the anxiolytic-like effects of diazepam and chlordiazepoxide. Both these drugs are positive modulators of GABA$_A$ receptors and facilitate GABA neurotransmission [30]. Mg at a dose ineffective *per se* given jointly with benzodiazepines (also at ineffective doses) produced synergistic effect. This apparent synergism was manifested as a significantly increased time spent in open arms. Moreover, also the number of open arm entries was increased, which was reflected by enhancement of the total arm entries. Because both tested drugs specifically modify GABA-ergic neurotransmission, thus, anxiolytic-like effects of Mg seem to be mediated, at least in part, through an interaction with benzodiazepine/GABA$_A$ system. Moreover, the direct evidence for the involvement of benzodiazepine/GABA$_A$ system in the anxiolytic ac-

tivity of Mg came from flumazenil experiments. The ability of flumazenil, a specific antagonist of the benzodiazepine/$GABA_A$ receptors, to antagonize the anxiolytic-like action of Mg clearly demonstrates a crucial role of this receptor complex in the above-mentioned behavior.

The main mechanisms of anxiolytic action of Mg may be mediated *via* a voltage-gated antagonistic properties at the NMDA receptor [31], and simultaneous activation of the $GABA_A$-gated chloride channels ([19], present results). In fact, Mg-induced anxiolytic-like effect in EPM was enhanced by NMDA receptor antagonists and antagonized by D-serine (a glycine site agonist) (our unpublished data). Lowering of Mg level leads to central hyperexcitability due to disinhibition of the NMDA receptor channels and by simultaneous activation of the $GABA_A$-gated chloride channels [19]. Moreover, neuroanatomical, electrophysiological and neurochemical evidences indicate the functional relationships between NMDA and benzodiazepine/$GABA_A$ systems, which may also take place in Mg antianxiety mechanism(s) (e.g. [18, 25, 27]).

In summary, this is the first evidence that anxiolytic-like activity of Mg in EPM test involves GABAergic neurotransmission. This study indicates that benzodiazepine receptors are involved in the anxiolytic-like effects of Mg.

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