

No effect of agmatine on the protective activity of clobazam and pregabalin against maximal electroshock-induced seizures in mice

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Abstract: The aim of this study was to assess the influence of agmatine (AGM – an endogenous neuromodulator / neurotransmitter in the brain) on the protective action of two antiepileptic drugs (clobazam [CLB] and pregabalin [PGB]) in the mouse maximal electroshock seizure (MES) model. Results indicate that AGM at a dose of 100 mg/kg, i.p., 45 min before the test had no significant effect on the anticonvulsant action of CLB or PGB in the MES test in mice. Moreover, the examined combination of AGM with CLB and PGB (at doses from the MES test) did not affect motor coordination in the chimney test and muscular strength in the grip-strength test in mice, indicating no acute adverse effects in animals. Based on the results from this study, it can be concluded that the lack of effect of AGM on the anti-convulsant action of CLB and PGB in the mouse MES model associated with no acute adverse effects in animals, make the combinations of AGM with CLB and PGB neutral from a preclinical point of view.

Key words: agmatine, clobazam, maximal electroshock seizure test, pregabalin

INTRODUCTION

Agmatine (AGM – 1-amino-4-guanidinobutane) is an endogenous polycationic amine synthesized by decarboxylation of L-arginine by the enzyme arginine decarboxylase and hydrolysed by agmatinase to putrescine and urea [1]. AGM has been detected in various mammalian organs, especially in the brain, where it exerts a wide range of biological activities, including neuroprotective, cognitive, anxiolytic, anticonvulsant, antinociceptive and antidepressant properties [2-9].

AGM has been proposed to act as a novel neurotransmitter/neuromodulator in the mammalian brain [1, 10]. Experimental evidence indicates that AGM enhances the anticonvulsant action of phenobarbital (PB) and valproate (VPA) in the mouse maximal electroshock-induced seizure (MES) model in mice [11]. In contrast, AGM had no impact on the antielectroshock activity of numerous conventional and second-generation antiepileptic drugs (AEDs), including carbamazepine (CBZ), lamotrigine (LTG), oxcarbazepine (OXC), phenytoin (PHT) and topiramate (TPM) in mice [11].

The aim of the study was to evaluate the effect of AGM on the protective activity of clobazam (CLB – a second generation AED) and pregabalin (PGB – a third generation AED) in the mouse MES model. The MES test in mice is thought to be an experimental model of tonic-clonic seizures and, to a certain

extent, of partial convulsions with or without secondary generalization in humans [12]. In this experimental test, the antiseizure potential of agents and compounds possessing the antiepileptic properties can be readily assessed, as well as determination of their effects on AEDs, fully effective in the suppression of tonic-clonic seizures in humans [12]. Therefore, it was appropriate to use the MES test in order to evaluate the effects of AGM on the protective action of CLB and PGB in the mouse MES model. Additionally, we investigated the combinations of AGM with CLB and PGB in relation to impairment of motor coordination and muscular strength by the use of the chimney and grip-strength tests.

MATERIALS AND METHODS

Animals and experimental conditions. Adult male Swiss mice (weighing 22-26 g) which were kept in colony cages with free access to food and tap water, under standardized housing conditions (natural light-dark cycle, temperature of 23±1°C, relative humidity of 55±5%), were used. After 7 days of adaptation to laboratory conditions, the animals were randomly assigned to experimental groups, each group comprised of 8 mice. Each mouse was used only once and all tests were performed between 08.00-15.00 hours. Procedures involving animals and their care were conducted in accordance with current European Community and Polish legislation on animal experimentation. Additionally, all efforts were made to minimize animal suffering and to use only the number of animals necessary to produce reliable scientific data. The experimental protocols and procedures were approved by

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the Second Local Ethics Committee at the University of Life Sciences (License No. 84/2009), and complied with the European Communities Council Directive of 24 November 1986 (86/609/EEC). The total number of animals used in this study was 160.

Drugs. The following drugs were used: AGM (sulfate salt – Sigma, St. Louis, MO, USA), CLB (Frisium[®], Sanofi-Aventis Deutschland GmbH, Frankfurt am Main, Germany), PGB (Lyrica[®], Pfizer Ltd., Sandwich, Kent, UK). CLB and PGB were suspended in a 1% solution of Tween 80 (Sigma, St. Louis, MO, USA) in distilled water, while AGM was directly dissolved in distilled water. All drugs were administered intraperitoneally (i.p.) as a single injection, in a volume of 5 ml/kg body wt. Fresh drug solutions were prepared on each day of experimentation and administered as follows: PGB was administered 120 min., AGM – 45 min. and CLB – 30 min. before electroconvulsions, motor coordination and grip-strength tests. The pretreatment times before testing of CLB and PGB were based upon information about their biological activity from the literature [13, 14] and own pilot studies. The times to the peak of maximum anticonvulsant effects for CLB and PGB were used as the reference times in all behavioural tests. The route of i.p. administration of AGM and the pretreatment time before testing of its antielectroshock effect were based upon information from the literature [4, 15].

Maximal electroshock seizure test. Electroconvulsions were produced by a current (50 Hz, 500 V, 0.2 s stimulus duration) delivered *via* ear-clip electrodes by a Rodent Shocker generator (constant-current stimulator Type 221, Hugo Sachs Elektronik, Freiburg, Germany). The criterion for the occurrence of seizure activity was the tonic hind limb extension (i.e., the hind limbs of animals outstretched 180° to the plane of the body axis). The protective activity of CLB and PGB was determined as their median effective doses (ED₅₀ values in mg/kg) against MES-induced seizures (fixed current intensity of 25 mA). CLB and PGB administered alone and in combination with AGM were tested for their ability to increase the number of animals not responding with tonus (i.e., protected from tonus) after stimulation. At least 3 groups of mice, each consisting of at least 8 animals and treated with a different dose of CLB or PGB alone or in combination with AGM, were challenged to collect data where close to 0%, 50%, and 100% of animals were protected from tonic seizures. After constructing a dose-effect curve (i.e., dose in mg/kg vs. percentage of mice protected), the protective median effective doses (ED₅₀ values of CLB and PGB were calculated according to a log-probit method [16]. Each ED₅₀ value represented a dose of CLB and PGB (in mg/kg) predicted to protect 50% of mice tested against MES-induced extension of the hind limbs. AGM was tested for its ability to affect the anticonvulsive potency of CLB and PGB. AGM was administered in a constant dose of 100 mg/kg that *per se* had no effect on seizure threshold in the maximal electroshock-induced seizure threshold test [11]. In this experimental protocol, an increase in the anticonvulsant potency of CLB and PGB in combination with AGM would be reflected by lower ED₅₀ values of CLB and PGB (i.e., lower dose of test drug was necessary to protect 50% of mice challenged). In the present study, CLB was administered at doses ranging between 10–20 mg/kg and PGB at doses ranging between 25–150 mg/kg. This experimental procedure has been described in detail in our earlier studies [17, 11].

Chimney test. The chimney test of [18] was used to quantify the adverse effect potential of CLB and PGB administered alone and in combination with AGM on motor performance in mice. In this test, the animals had to climb backwards up a plastic tube (3 cm inner diameter, 25 cm length), and impairment of motor performance was indicated by the inability of the mice to climb backward up the transparent tube within 60 s. The acute adverse effect potentials for CLB, PGB and their combination with AGM were determined at doses corresponding to the ED₅₀ values of CLB and PGB from the MES test when combined with AGM at the dose of 100 mg/kg. The impairment of motor coordination in mice was expressed in % of at least 8 determinations. This experimental procedure has also been described in detail in our earlier studies [17, 11].

Grip-strength test. The effects of combination of AGM with CLB and PGB at their ED₅₀ values from the MES test, on muscular strength (tone) in mice were quantified by the grip-strength test. The time before the commencement of the grip-strength test (after drug administration) was identical to that for the MES test. The grip-strength apparatus (BioSeb, Chaville, France) comprised a wire grid (8 × 8 cm) connected to an isometric force transducer (dynamometer). The mice were lifted by the tails so that their forepaws could grasp the grid. The mice were then gently pulled backward by the tail until the grid was released. The maximal force exerted by the mouse before losing grip was recorded. The mean of 3 measurements for each animal was calculated and subsequently, the mean maximal force of 8 animals per group was determined. The muscular strength in mice was expressed in N (newtons) as means ± S.E. of at least 8 determinations. This experimental procedure has been described in detail in our earlier study [11].

Statistics. ED₅₀ values with their 95% confidence limits were calculated and statistically analyzed by computer log-probit analysis according to Litchfield and Wilcoxon [16]. Qualitative variables from the chimney test were compared by use of the Fisher's exact probability test, whereas, the results from the grip-strength test were verified with one-way ANOVA. Differences among values were considered statistically significant if P<0.05. All statistical tests were performed using commercially available GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego, CA, USA).

RESULTS

Effects of AGM on the protective action of CLB and PGB in the mouse MES model.

CLB and PGB administered alone exhibited a clear-cut anticonvulsant activity in the MES test in mice and their ED₅₀ values are presented in Table 1. When AGM at a dose of 100 mg/kg was co-administered with CLB, it had no significant impact on the anticonvulsant effect of the latter drug against MES-induced seizures. The ED₅₀ value of CLB was reduced by 17% from 15.14 to 12.62 mg/kg (Table 1). Similarly, AGM at a dose of 100 mg/kg did not significantly affect the anticonvulsant activity of PGB against MES-induced seizures although the ED₅₀ value of PGB was reduced by 31% from 144.98 to 100.51 mg/kg (Table 1).

Table 1 Effect of agmatine (AGM) on the protective activity of clobazam (CLB) and pregabalin (PGB) against MES-induced seizures in mice.

Treatment (mg/kg)	ED ₅₀	n
CLB + vehicle	15.14 (12.63 – 18.14)	24
CLB + AGM (100)	12.62 (10.50 – 15.16)	24
PGB + vehicle	144.98 (112.71 – 186.47)	32
PGB + AGM (100)	100.51 (82.49 – 122.47)	32

Results are presented as median effective doses (ED₅₀ in mg/kg with 95% confidence limits in parentheses) of CLB and PGB that protected 50% of animals tested against MES-induced hindlimb extension. PGB was administered i.p. at 120 min., AGM at 45 min., and CLB at 30 min. prior to the MES test. Statistical analysis of data was performed with log-probit method. n – total number of animals used at those doses whose anticonvulsant effects ranged between 4–6 probits.

Effects of AGM in combination with CLB and PGB on motor performance and muscular strength of animals in the chimney and grip-strength tests. When AGM was administered in combination with CLB and PGB at doses corresponding to their ED₅₀ values from the MES test, motor performance as assessed by the chimney test was unaffected (Table 2). Furthermore, AGM concomitantly administered with CLB and PGB had no significant impact on skeletal muscular strength of the animals as assessed by the grip-strength test (Table 2).

Table 2 Effects of agmatine (AGM), clobazam (CLB), pregabalin (PGB) and their combinations on muscular strength and motor performance in mice.

Treatment (mg/kg)	Grip-strength (N)	Impairment of motor coordination (%)
Control (vehicle)	98.0 ± 5.67	0
AGM (100) + vehicle	94.8 ± 5.44	0
CLB (12.6) + vehicle	96.1 ± 5.62	0
CLB (12.6) + AGM (100)	94.6 ± 5.75	12.5
PGB (100.5) + vehicle	95.5 ± 5.33	0
PGB (100.5) + AGM (100)	94.0 ± 5.58	12.5

Results are presented as:

- 1) mean grip-strengths (in newtons ± S.E.) from the grip-strength test, assessing skeletal muscular strength in mice;
- 2) percentage of animals showing motor coordination impairment in the chimney test in mice.

Statistical analysis of data from the grip-strength test was performed with one-way ANOVA. The Fisher's exact probability test was used to analyze the results from the chimney test. The drugs were administered i.p. at times scheduled from the MES-induced seizure test, and at doses corresponding to the ED₅₀ values against MES-induced seizures in mice (for more details see legend to Table 1).

DISCUSSION

The results presented indicate that AGM administered i.p. at a dose of 100 mg/kg had no impact on the anticonvulsant action of CLB and PGB in the mouse MES model. This finding confirms our previous results documenting that AGM selectively enhanced the antiseizure activity of PB and VPA, having had no effect on the anticonvulsant efficacy of CBZ, PHT, LTG, TPM or OXC in the MES test in mice [11].

In the present study, the lack of significant enhancement of the antielectroshock action of CLB and PGB in mice by AGM, one can try to explain by considering the molecular mechanisms of action of the examined drugs. Experimental evidence indicates that AGM:

- 1) binds with high affinity to imidazoline I₁ receptors and α₂-adrenoceptors [19, 1, 10];
- 2) blocks NMDA, but not the AMPA or KA receptors [20];
- 3) blocks voltage-gated calcium channels [21].

AGM also affects synthesis of NO by activating the endothelial NOS [22], while inhibiting the inducible NOS [23], and neuronal NOS [24]. As for PGB, the drug binds with high affinity and specificity to the α_{2δ} subunit of P/Q-type voltage-gated calcium channels, which decreases Ca²⁺ influx at nerve terminals and reduces the release of excitatory neurotransmitters [25, 26, 13]. With respect to CLB, the drug like other 1,5-benzodiazepines, enhances GABA_A receptor-mediated inhibition in the brain [27]. Comparing molecular mechanisms of action of the studied AEDs and AGM one can ascertain that diverse mechanisms were not associated with the enhancement of the anticonvulsant action of CLB and PGB by AGM.

The evaluation of acute adverse-effect potentials for the combinations of AGM with PGB and CLB revealed that AGM did not affect the acute adverse effects produced by PGB and CLB in terms of the impairment of motor coordination or skeletal muscular strength. It is noteworthy that the estimation of acute side effects for the combinations of the AEDs with AGM in mice was performed at doses corresponding to their ED₅₀ values from the MES test. Similarly, the pretreatment times for the AEDs in combination with AGM were identical to those tested in the MES test in mice. It is important to note that our findings, showing no acute adverse effects in animals receiving AGM at a dose of 100 mg/kg, are consistent with our previous results [11], and with those documented earlier that AGM at doses up to 300 mg/kg did not produce any cardiovascular or locomotor effects in normal rats [3].

In conclusion, based on this study one can ascertain that modulation of AGM concentrations in the brain had no impact on the antiseizure action of PGB and CLB in the mouse MES model. However, more advanced studies should confirm the properties of AGM in combination with PGB and CLB in other experimental seizure models in animals.

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