

ROLE OF NITRIC OXIDE IN ANTICONVULSANT EFFECTS OF BENZODIAZEPINES IN MICE

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S. TALAREK, S. FIDECKA. Pol. J. Pharmacol., 2003, 55, 181–191.

The influence of nitric oxide (NO) on anticonvulsant activity of diazepam and clonazepam was examined in the pentetrazole- and electroshock-induced seizure models in mice. Protective efficacy of the threshold dose of diazepam against pentetrazole-induced clonic and tonic seizures, and death was significantly increased by N^G-nitro-L-arginine methyl ester hydrochloride (L-NAME) while 7-nitroindazole (7-NI) was slightly less effective. The above intensifying effect of L-NAME on antiepileptic activity of diazepam was reversed by L-arginine, a substrate for NO formation, but not by D-arginine. Methylene blue, the guanylate cyclase inhibitor, increased the protective efficacy of diazepam and clonazepam in the pentetrazole-induced seizures. 7-NI was able to potentiate the protective efficacy of diazepam and clonazepam in electroshock-induced tonic hindlimb extension. These findings suggest that the cGMP/NO system may participate in antiepileptic effects of benzodiazepines.

Key words: *nitric oxide, benzodiazepines, seizures, mice*

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INTRODUCTION

Benzodiazepines are in clinical use as sedative, antianxiety, hypnotic and anticonvulsant drugs. They bind to γ -aminobutyric acid-A (GABA_A) receptors and act to enhance the action of the inhibitory neurotransmitter GABA at these receptors [19].

Nitric oxide (NO), a small diffusible messenger molecule synthesized from the amino acid L-arginine by the enzyme NO synthase (NOS), appears to play a crucial role in a number of physiological and pathophysiological processes [28]. NO acts as an endogenous activator of guanyl cyclase and thereby increases the level of an intracellular second messenger, cGMP [43]. It has been found that NO produced in response to N-methyl-D-aspartate (NMDA) receptor stimulation may be involved in the modulation of seizure activity [5] but the precise role of NO in the expression of seizures is unclear. Although many studies have revealed that NOS inhibitors, which block the formation of NO, can potentiate behavioral or electrographic effects of convulsant drugs suggesting that NO acts as an endogenous anticonvulsant [5, 33, 39], but other studies indicate that NO is proconvulsant [1, 31]. These paradoxical differences may be explained by different experimental conditions (species and age of animals, model of seizures, the dose or type of NO pathway modulators used, the brain structures) [10, 32, 48]. Practically, all the NOS inhibitors used in the experiments affect not only neuronal but also endothelial NOS which leads to an increase in blood pressure [28], that in turn may affect the excitability of central neurons [15]. Furthermore, the specificity of these NOS inhibitors is limited by their muscarinic receptor blocking properties [6]. Penix et al. [32] suggested that whereas neuronal NO possesses proconvulsive activity, vascular (peripheral) NO suppresses seizures. Consistent with this view some authors suppressing the production of NO selectively within the brain have revealed anticonvulsant effects [11, 30]. The innovative study of Mülsch et al. [30] may be particularly relevant in the light of this, as the administration of either 7-NI, a brain-selective inhibitor of NOS, or diazepam, reduced both severity of seizures and the concomitant accumulation of central NO following the administration of kainate. Other authors in their studies in which the production of NO was suppressed non-selectively (both centrally

and peripherally) revealed proconvulsant effects [5, 32, 39].

Literature data point to the relationship between L-arginine:NO:cGMP pathway and GABA-mediated transmission in the central nervous system. A number of *in vivo* and *in vitro* studies suggest that NO plays a modulatory role in either release or uptake of neurotransmitters including glutamate [18] and GABA [18, 22, 41]. Histochemical mapping of NOS revealed that NOS-positive neurons are co-localized with GABA [50]. It has also been postulated that NO can modulate the activity of GABA_A receptors [52] or act directly on GABA_A receptors [21].

The present research was aimed to find out whether L-arginine : NO : cGMP pathway plays any role in antiepileptic activity of diazepam and clonazepam. In order to examine the possible involvement of NO in the anticonvulsant effects of benzodiazepines we used L-NAME and 7-NI (NOS inhibitors), L-arginine (a substrate for NO formation) and methylene blue (guanyl cyclase inhibitor). The threshold doses of benzodiazepines were chosen during pilot experiments. Benzodiazepines given at higher doses had significant protective efficacy. The aim of our study was to test if concomitant administration of compounds, affecting the level of NO, and benzodiazepines results in synergy of their anticonvulsant effects. Therefore, the doses which produced only slight antiepileptic effects were chosen.

Some of results presented in the paper have been published previously in abstract form [44].

MATERIALS and METHODS

Animals

The experiments were carried out on male Albino Swiss mice weighing 18–25 g. The animals were kept 8–10 to a cage at room temperature of $20 \pm 1^\circ\text{C}$ under natural day-night cycle with free access to food and water. All experiments were performed between 9:00 a.m. and 3:00 p.m.

The experiments were performed in accordance with the ethical requirements.

Drugs

The following drugs were used: diazepam (Relanium, Polfa, Poland), clonazepam (Rivotril, Hoffmann-La Roche, Germany), 7-nitroindazole (7-NI, RBI, USA) N^G-nitro-L-arginine methyl ester hy-

drochloride (L-NAME), methylene blue, L-arginine hydrochloride, D-arginine hydrochloride (all from Sigma, USA), pentetrazole (Aldrich, Germany). Diazepam and clonazepam were diluted to adequate concentration, and L-NAME, 7-NI, L-arginine, D-arginine, methylene blue and pentetrazole were dissolved in 0.9% NaCl solution. All substances were administered intraperitoneally (*ip*), except for methylene blue which was given intravenously (*iv*) and pentetrazole which was given subcutaneously (*sc*). The injection volume was 10 ml/kg, except for methylene blue which was given in volume of 5 ml/kg. The control animals were injected with an appropriate volume of the solvent at the respective time before the test.

Pentetrazole-induced convulsions

Pentetrazole was given at the dose of 110 mg/kg *sc*. The animals were observed for 60 min after pentetrazole administration and the number of mice developing clonic seizures and tonic convulsions (also the number of episodes), and animals which died was recorded [51]. Diazepam (0.05 mg/kg) and clonazepam (0.0125, 0.005, 0.025 mg/kg) were given *ip* 30 min before pentetrazole, L-NAME (1, 10, 100, 200 mg/kg), 7-NI (100 mg/kg), L-arginine (125 mg/kg) and D-arginine (125 mg/kg) were given 35 min before pentetrazole, while methylene blue (5 mg/kg) was given 5 min prior to the seizure-inducing drug. The absolute mean values of a number of clonic and tonic episodes in control pentetrazole-treated group ranged from 17 to 35 and from 8 to 10 (\pm SEM), respectively. These values and mortality rate after the treatment with pentetrazole alone were assumed to be 100%.

Electroconvulsions

The procedure was carried out as described by Swinyard and Castellion [44]. Convulsions were evoked with alternating current (50 Hz, 50 mA, 0.2 s) passing through ear clip electrodes and generated by a stimulator GE-01 (COMT, Białystok, Poland). Duration (in seconds) of tonic hindlimb extension phase was recorded. Mice were given diazepam (2.5 mg/kg) and clonazepam (0.25 mg/kg) 60 min before electroshock and 7-NI (10, 50 and 100 mg/kg) was administered 30 min before the seizure induction.

Statistical analysis

The data obtained were evaluated statistically and presented in two ways:

1) using χ^2 test with Yates correction (the number of mice with seizures in pentetrazole-induced convulsions, data in the table),

2) Student's *t*-test (electrogenic convulsions and the number of seizure episodes in pentetrazole-induced convulsions, data in the figures).

The results are expressed as means \pm SEM of groups consisting of 10 mice. A probability (*p*) value of 0.05 or more is reported as statistically not significant (NS).

RESULTS

The influence of L-NAME on anticonvulsant activity of diazepam in pentetrazole-induced seizures in mice (Fig. 1 and Tab. 1)

Pentetrazole (110 mg/kg) produced clonic seizures in all control mice and tonic seizures in 9 mice and death of 8 animals. The administration of the threshold dose of diazepam (0.05 mg/kg) resulted in slight and not significant effect on pentetrazole-induced seizures. The effect of the threshold dose of diazepam (counted as the number of seizure episodes, Fig. 1) on the clonic convulsions was potentiated by the administration of 200 mg/kg and on the tonic seizure by 10, 100 and 200 mg/kg of the drug. The results are additionally presented as the number of animals which developed seizures and died (Tab. 1). Administration of L-NAME did not affect the number of animals which developed clonic seizures but the differences for tonic seizures were significant even in case of administration of 1 mg/kg of the drug. L-NAME (1, 10, 100 and 200 mg/kg) potentiated the effect of threshold dose of diazepam against mortality in the dose-dependent way (Fig. 1 and Tab. 1). Administration of L-arginine (125 mg/kg), but not D-arginine (125 mg/kg) resulted in reversal of the effects of L-NAME. It was expressed as significant increase in a number of seizure episodes and mortality rate (Fig. 1). Similar effect of L-arginine but without influence on clonic convulsions was observed when the results were expressed as the number of mice which developed seizures (Tab. 1).

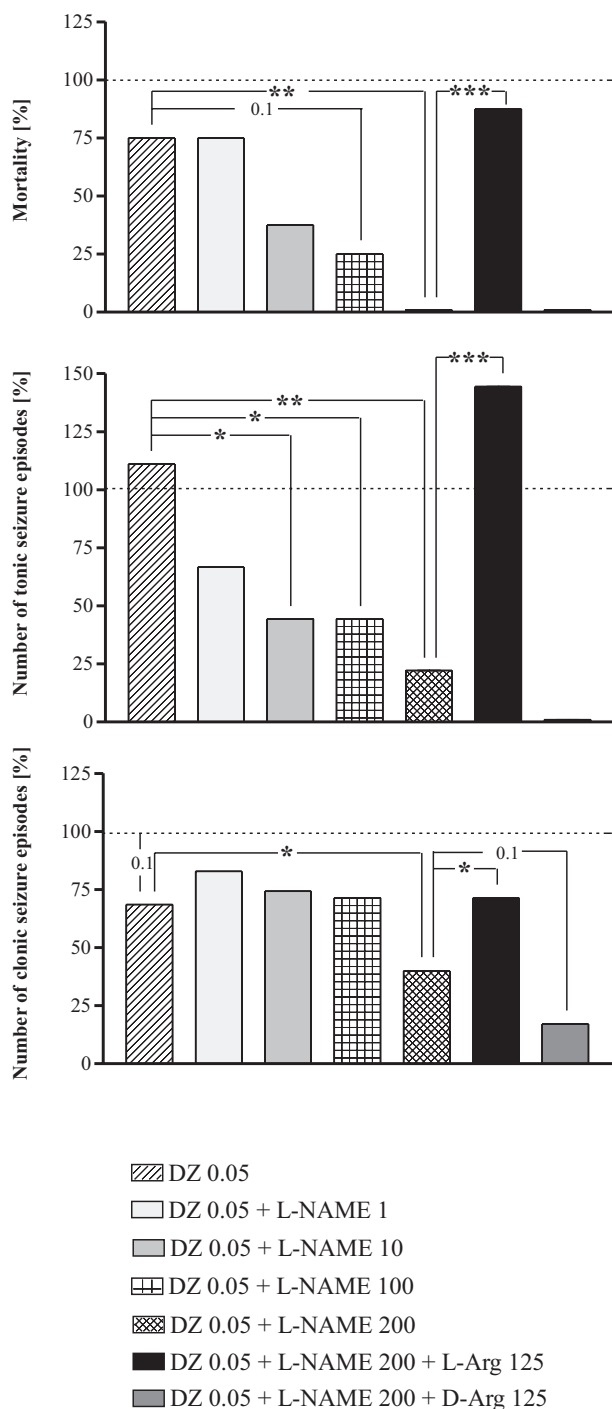


Fig. 1. The influence of L-NAME (1–200 mg/kg) on anticonvulsant activity of diazepam (DZ, 0.05 mg/kg) in pentetrazole-induced seizures in mice. The results are expressed as means \pm SEM of groups consisting of 10 mice. The mean value of the number of clonic and tonic episodes, and mortality rate in mice treated with saline + pentetrazole was assumed to be 100%. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (Student's *t*-test)

The influence of L-NAME on anticonvulsant activity of clonazepam in pentetrazole-induced seizures in mice (Fig. 2 and Tab. 1)

Administration of the threshold dose of clonazepam (0.0125 mg/kg) significantly reduced the number of clonic seizure episodes (Fig. 2) but did not influence the number of convulsing animals (Tab. 1). Tonic seizures and mortality in pentetra-

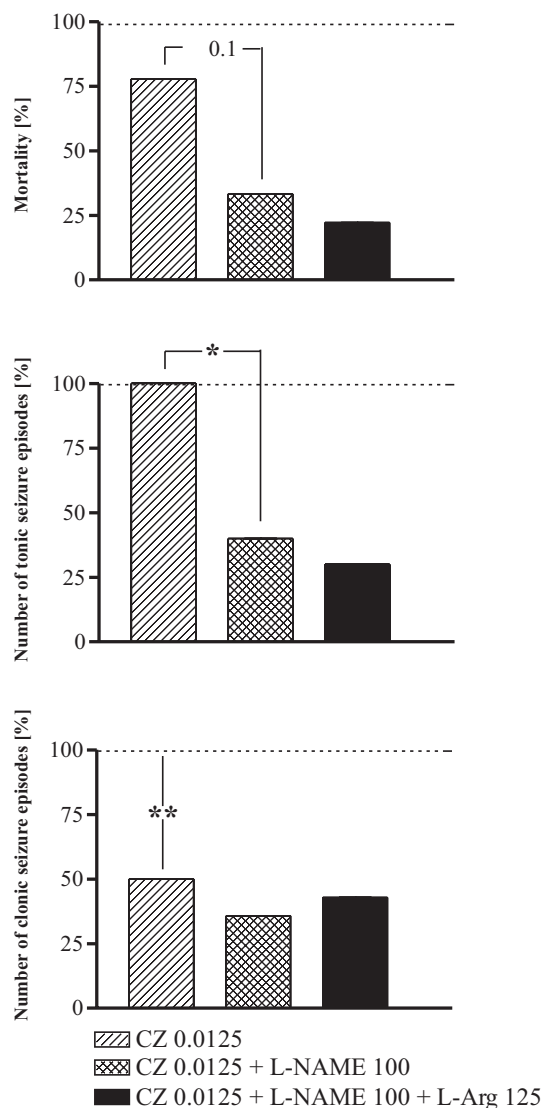


Fig. 2. The influence of L-NAME (100 mg/kg) on anticonvulsant activity of clonazepam (CZ, 0.0125 mg/kg) in pentetrazole-induced seizures in mice. The results are expressed as means \pm SEM of groups consisting of 10 mice. The mean value of the number of clonic and tonic episodes, and mortality rate in mice treated with saline + pentetrazole was assumed to be 100%. * $p < 0.05$, ** $p < 0.01$ (Student's *t*-test)

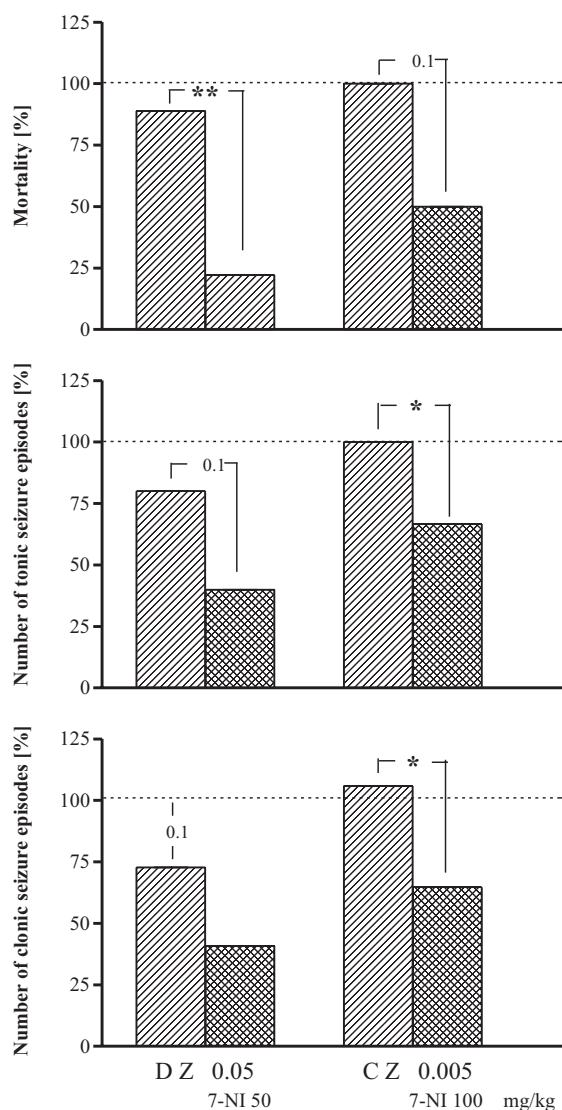


Fig. 3. The influence of 7-nitroindazole (7-NI) on anticonvulsant activity of diazepam (DZ) and clonazepam (CZ) in pentetrazole-induced seizures in mice. The results are expressed as means \pm SEM of groups consisting of 10 mice. The mean value of the number of clonic and tonic episodes, and mortality rate in mice treated with saline + pentetrazole was assumed to be 100%. * $p < 0.05$, ** $p < 0.01$ (Student's *t*-test)

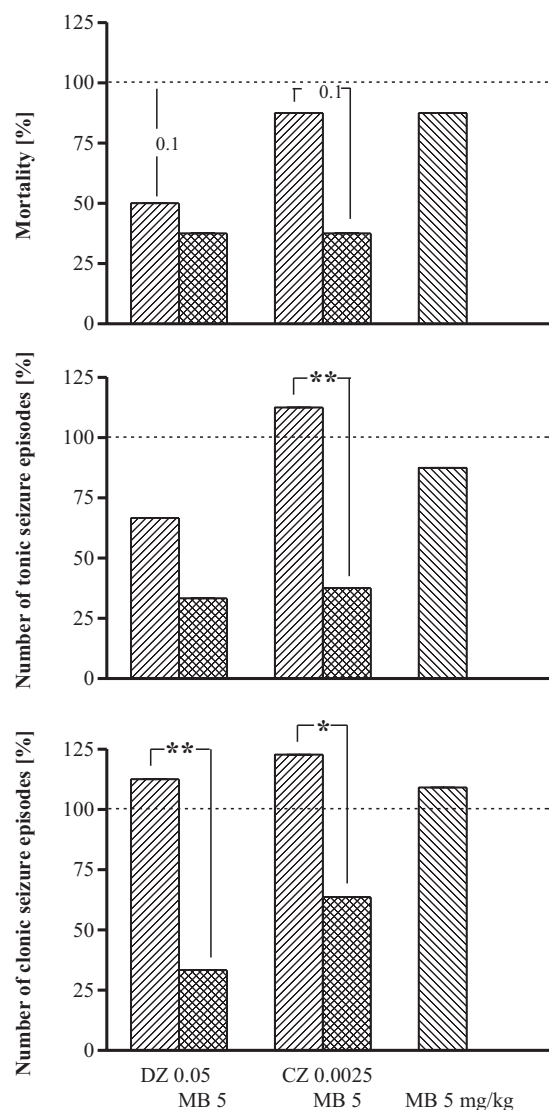


Fig. 4. The influence of methylene blue (MB) on anticonvulsant activity of diazepam (DZ) and clonazepam (CZ) in pentetrazole-induced seizures in mice. The results are expressed as means \pm SEM of groups consisting of 10 mice. The mean value of the number of clonic and tonic episodes, and mortality rate in mice treated with saline + pentetrazole was assumed to be 100%. * $p < 0.05$, ** $p < 0.01$ (Student's *t*-test)

zole-treated mice were not changed by clonazepam (0.0125 mg/kg). L-NAME (100 mg/kg) coadministered with clonazepam did not change the number of clonic seizure episodes (Fig. 2) as well as the number of mice which developed clonic seizures (Tab. 1). Administration of L-NAME with clonazepam resulted in significant decrease in the number of tonic seizure episodes (Fig. 2) and the number of mice which developed tonic seizures (Tab. 1). L-NAME also markedly diminished mortality of animals (Tab. 1). The effect of coadministration of

L-NAME with clonazepam on both clonic and tonic convulsions, and mortality rate was not changed by 125 mg/kg of L-arginine (Tab. 1).

The influence of 7-NI on anticonvulsant activity of diazepam and clonazepam in pentetrazole-induced seizures in mice (Fig. 3 and Tab. 1)

Pentetrazole-induced seizures and mortality of mice were not changed by threshold dose of both

Table 1. The influence N^G-nitro-L-arginine methyl ester (L-NAME), 7-nitroindazole (7-NI) and methylene-blue (MB) on protective efficacy of diazepam (DZ) and clonazepam (CZ) against pentetrazole-induced seizures expressed as the number of mice with clonic and tonic seizure episodes and mortality rate. Groups consisted of 10 mice. * p < 0.05, ** p < 0.01, *** p < 0.001 vs pentetrazole + benzodiazepine-treated mice; • p < 0.05 vs pentetrazole-treated mice; ^Δ p < 0.01 vs pentetrazole + DZ + L-NAME-treated mice (test χ^2 with Yates correction)

Substances (mg/kg)	Number of animals with clonic seizures	Number of animals with tonic seizures	Mortality
Pentetrazole 110	10	9	8
Diazepam (DZ) 0.05	10	8	6
DZ 0.05 + L-NAME 1	10	6*	6
DZ 0.05 + L-NAME 10	10	4**	3**
DZ 0.05 + L-NAME 100	9	4**	2**
DZ 0.05 + L-NAME 200	8	2***	0***
DZ 0.05 + L-NAME 200 + L-arg 125	10	10 ^{ΔΔ}	7 ^{ΔΔ}
DZ 0.05 + L-NAME 200 + D-arg 125	5	0	0
Pentetrazole 110	10	10	9
DZ 0.05	10	8	8
DZ 0.05 + 7-NI 50	4*	4*	2***
DZ 0.05 + 7-NI 50 + L-arg 125	4	0	0
Pentetrazole 110	10	9	8
DZ 0.05	10	6•	4•
DZ 0.05 + MB 5	7	3	3
Pentetrazole 110	10	10	9
Clonazepam (CZ) 0,0125	10	9	7
CZ 0.0125 + L-NAME 100	7	4**	3*
CZ 0.0125 + L-NAME 100 + L-arg 125	8	3	2
Pentetrazole 110	10	8	8
CZ 0.005	10	9	8
CZ 0.005 + 7-NI 50	8	7	5
CZ 0.005 + 7-NI 100	7	6	4*
CZ 0.005 + 7-NI 100 + L-arg 125	6	5	5
Pentetrazole 110	10	8	8
CZ 0.005	9	4•	4•
CZ 0.0025	10	9	7
CZ 0.0025 + MB 5	8	3***	3*

diazepam (0.05 mg/kg) and clonazepam (0.005 mg/kg). 7-NI (50 mg/kg) administered with diazepam resulted in a significant decrease in the number of animals which developed clonic and tonic convulsions and in mortality (Tab. 1). The number of clonic and tonic seizure episodes was markedly (about 50%) but not significantly decreased by 7-NI (Fig. 3). L-arginine (125 mg/kg) did not change the effect of coadministration of 7-NI with

diazepam on the seizure activity and mortality of mice (Tab. 1). Coadministration of 7-NI (100 mg/kg) with clonazepam (0.005 mg/kg) resulted in potentiation of the effect of threshold dose of clonazepam on the clonic and tonic seizures and mortality (counted as the number of episodes, Fig. 3). The effect of administration of 7-NI with clonazepam on mortality was not changed by L-arginine (Tab. 1).

The influence of methylene blue on anti-convulsant activity of diazepam and clonazepam in pentetrazole-induced seizures in mice (Fig. 4 and Tab. 1)

Methylene blue (5 mg/kg) administered with diazepam (0.05 mg/kg) significantly reduced only the number of clonic seizure episodes (Fig. 4) but did not change the number of animals with clonic and tonic convulsions, and mortality rate (Tab. 1). Coadministration of methylene blue (5 mg/kg) with clonazepam (0.0025 mg/kg) significantly reduced the number of clonic and tonic seizure episodes and not significantly the mortality of mice (Fig. 4). The number of responding animals was significantly decreased by methylene blue coadministered with clonazepam in the case of mice with tonic seizures, and mortality (Tab. 1). Methylene blue administered alone did not affect the values observed after pentetrazole treatment (Fig. 4).

The influence of 7-NI on protective efficacy of diazepam and clonazepam against electroshock-induced tonic hindlimb extension in mice (Fig. 5)

7-NI administered alone at doses of 50 and 100 mg/kg significantly and dose-dependently reduced the duration of tonic hindlimb extension. Anticonvulsive activity of diazepam (2.5 mg/kg) was significantly potentiated by 7-NI (50, 100 mg/kg). 7-NI (100 mg/kg) was also able to intensify the anticonvulsant effect of clonazepam (0.25 mg/kg). Protective efficacy of both diazepam and clonazepam was not changed by 7-NI administered at the dose of 10 mg/kg.

DISCUSSION

It is now well established that the principal molecular target of the antiepileptic benzodiazepines is postsynaptic macromolecular GABA_A receptor complex. Activation of GABA_A receptors by the endogenous ligand, GABA, in mature neurons results in membrane hyperpolarization secondary to inward chloride flux. Binding of benzodiazepines to a specific recognition site on the GABA_A receptor results in augmentation of GABA-activated currents. This enhancement occurs through an increased frequency of channel openings without substantial changes in the mean open time or con-

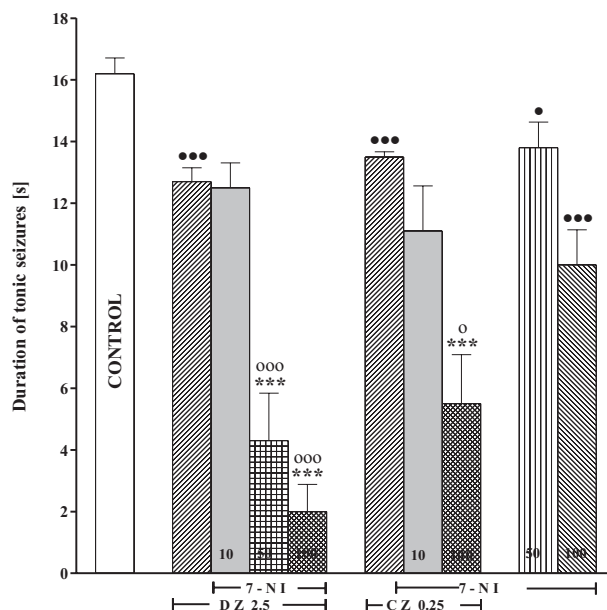


Fig. 5. The influence of 7-nitroindazole (7-NI) on protective efficacy of diazepam (DZ) and clonazepam (CZ) against electroshock-induced tonic hindlimb extension in mice. ● $p < 0.05$ vs control, ●● $p < 0.001$; *** $p < 0.001$ vs benzodiazepine; ○ $p < 0.05$, ○○○ $p < 0.001$ vs 7-NI (Student's *t*-test)

ductance of the channel [26]. Benzodiazepines act also on targets other than the GABA_A receptor. Through unknown mechanisms, benzodiazepines may produce elevations of GABA levels in the cerebrospinal fluid [23]. At high concentrations, especially those that might be achieved in the treatment of status epilepticus, benzodiazepines inhibit currents through voltage-gated sodium channels [24] and, to a lesser extent, calcium channels [42].

Recent evidences point to the interactions between NO and GABA. It is known that high concentration of NO potentiates the release of GABA [17, 18, 22], and postsynaptically potentiates GABA-mediated increases in chloride conductance at GABA_A receptors [16]. In contrast, low concentrations of NO can exert inhibitory effects on GABAergic transmission [16, 17] indicating that the effects of NO can be biphasic.

Reports that NO is able to modulate some effects of benzodiazepines also suggest interactions between NO and benzodiazepines/GABA_A systems. NO has been implicated in benzodiazepine-induced antinociception [46], anxiolytic activity [35] and also tolerance and dependence [20].

The role of NO in seizure has been widely investigated, using various NOS inhibitors and donors. Some studies suggested that NO acts as a procon-

vulsant, but others have suggested that it acts as an anticonvulsant, depending on the stimulants and NO-related chemicals present [see Introduction].

The pentetrazole model has proven to be a good predictor of clinical efficacy against generalized spike-wave epilepsies of the absence type [37]. It is generally known that benzodiazepines prominently prevent pentetrazole-induced seizures [44]. To examine possible involvement of NO in the anticonvulsant effects of benzodiazepines, the threshold doses of the drug were used. It is known that L-NAME is a non-selective antagonist that inhibits the activity of both endothelial and neuronal NOS and causes pronounced increases in arterial blood pressure [36]. In the present studies, we showed that L-NAME, given at the dose of 200 mg/kg, potentiated the effect of threshold dose of diazepam against pentetrazole-induced clonic and tonic seizures, and mortality in mice. The tonic phase of seizures was diminished also by the lower doses (10, 100 mg/kg) of L-NAME coadministered with diazepam. The potentiating effect of L-NAME coadministered with diazepam was reversed by L-arginine (NO precursor) but not D-arginine. In our previous study, we observed the lack of anticonvulsant effect of both L- and D-arginine administered solely in pentetrazole-induced seizures (unpublished data). We also found in the present experiments that the coadministration of L-NAME with clonazepam resulted in potentiation of the effect of the threshold dose of clonazepam on pentetrazole-induced tonic seizures. Literature data demonstrated that compounds, changing the level of endogenous NO might differentially interact with anti-epileptic drugs. Tutka et al. [49] demonstrated that molsidomine, a donor of nitric oxide, enhanced the protective activity of valproate against the clonic phase of pentetrazole-induced seizures and did not alter the anticonvulsive efficacy of clonazepam, ethosuximide, and phenobarbital. Studies of Czuczwar et al. [8] have shown that N^G-nitro-L-arginine, an unspecific NOS inhibitor, impaired the protective activity of ethosuximide against the clonic phase of pentetrazole-induced seizures and was ineffective with respect to the protective action of phenobarbital, valproate and diazepam.

It is necessary to emphasize that in our previous studies (unpublished data) L-NAME given alone at doses of 100 and 200 mg/kg did not influence the pentetrazole-induced seizures. Anticonvulsive effects of L-NAME have been observed after its *ip*

administration at the dose of 500 mg/kg. However, Salter et al. [40] in biochemical studies demonstrated that L-NAME even at the dose of 10 mg/kg *ip* inhibited cerebellar activity by 50% when measured up to 0.5 h after administration. Literature data show contradictory results on seizure phenomena following administration of NOS inhibitors. Przegaliński et al. [34], similarly to our results, reported a lack of effect of lower doses of L-NAME on pentetrazole-induced seizures. Osonoe et al. [31] demonstrated that NOS inhibitors attenuated the tonic, but not the clonic seizures in rats produced by pentetrazole. Proconvulsant effects of NOS inhibition are suggested to occur in the pilocarpine model and in the limbic components in pentetrazole-induced seizures, while an anticonvulsant role is suggested for the tonic seizures induced by higher doses of pentetrazole [10]. The discrepancy may be due to differences in the selectivity and doses of the inhibitors used.

In order to avoid the cardiovascular effects of arginine-derived NOS inhibitors we used 7-NI, an inhibitor of neuronal NOS, which does not affect endothelial NOS (*in vivo*) and blood pressure [29]. We have shown that administration of 7-NI resulted in potentiation of the protective efficacy of diazepam and clonazepam against pentetrazole-induced seizures and this effect was not affected by L-arginine. Similarly, Borowicz et al. [3] have shown that 7-NI was able to potentiate the protective efficacy of clonazepam against pentetrazole-induced seizures, and this effect was also not reversed by L-arginine. It is difficult to explain these different effects of L-arginine on various NOS inhibitors. Some pharmacokinetic interactions could not be excluded.

Additionally, we investigated the effects of 7-NI on the antiseizure activity of benzodiazepines in the maximal electroshock test in mice. The electroshock assay in mice is used primarily for screening the compounds which are effective in grand mal epilepsy. Tonic hindlimb extension is evoked by electric stimuli which is suppressed not only by antiepileptic drugs but also by other centrally active drugs [51]. Our results indicate that 7-NI, a selective neuronal NOS inhibitor, given at doses of 50 and 100 mg/kg, shows anticonvulsant effect in this test. However, other authors reported a lack of influence of as well lower doses (10 and 30 mg/kg) [34] as the same dose (100 mg/kg) [12] of 7-NI as used by us in electroshock-induced convulsions. This contrast to the results presented here is diffi-

cult to elucidate but some differences in procedure of seizures model, delivery and weight of animals [12], and use of lower doses of 7-NI [34] may partially explain it.

In our studies, 7-NI significantly and in the dose-dependent way increased the protective efficacy of diazepam and clonazepam against maximal electroshock in mice. A similar results were also obtained by Deutsch et al. [12], who indicated potentiating influence of 7-NI (100 mg/kg) on the protective efficacy of flurazepam against electrically precipitated seizures in mice. In another study, 7-NI enhanced the protective activity of phenobarbital against electroshock-induced convulsions but did not impair that of carbamazepine, diphenylhydantoin and valproate [2]. Our earlier studies showed that L-NAME, a non-selective NOS inhibitor, did not affect the antiepileptic activity of diazepam in electroconvulsions [45]. Borowicz et al. [4] have demonstrated that L-NAME diminished the protective activity of phenobarbital and valproate, but not that of diphenylhydantoin and carbamazepine against maximal electroshock in mice. Thus, NOS inhibitors may differentially interact with antiepileptic drugs.

Several studies have reported that NO released upon NMDA receptor stimulation, may subsequently modulate NMDA receptor function through the redox binding site [47]. Moreover, it is widely accepted that the NMDA receptor complex includes a special modulatory redox site with a number of SH groups. NO is responsible for oxidizing them which is followed by a closure of NMDA-related ion channels and a decrease in an intracellular influx of Ca^{2+} [21]. There have been reports that NMDA receptors regulate the release of GABA [27] and that activation of NMDA-type glutamate receptors causes a reduction in the effect of GABA [38]. It was also shown that NMDA receptor antagonists reduce seizures induced by GABA_A receptor antagonists as pentetrazole, picrotoxin, bicuculline and also by electroshock [13]. Moreover, there is evidence that most NMDA receptor antagonists, apart from their own anticonvulsive properties, potentiated the protective efficacy of conventional antiepileptic drugs against maximal electroshock-induced seizures in mice [7]. Deutsch et al. [12] have shown that MK-801, a non-competitive NMDA receptor antagonist, possessing protective activity against electroconvulsions *per se*, was able to enhance the anticonvulsive activity of

flurazepam. Therefore, it is possible that lowering of the level of NO by NOS inhibitors would lead to a potentiated anticonvulsive activity of some anti-epileptic drugs. However, the results of studies on an involvement of NO in NMDA-induced seizures are contradictory. De Sarro et al. [11] have shown the protective effect of NOS inhibitor N^G-monomethyl-L-arginine against seizures induced by NMDA in rats. On the other hand, Eblen et al. [14] have reported the lack of significant protection by 7-NI and L-NAME against NMDA-induced seizures in mice.

The major action of NO is to activate soluble guanyl cyclase and to raise cGMP levels in target cell [9]. Methylene blue, a simple inhibitor of guanyl cyclase [25], has been widely applied in experiments to determine the contribution of the cGMP pathway in the effects of NOergic system. The results of the present study demonstrate that methylene blue (5 mg/kg *iv*) much stronger intensified the effects of threshold dose of clonazepam than that of diazepam in pentetrazole-induced seizures in mice. Methylene blue given alone at the same dose did not affect the convulsions induced by pentetrazole. The potentiation effect of methylene blue on anticonvulsant effect of flurazepam was demonstrated by Deutsch et al. [12] but in electroshock test in mice. However, the comparison of the effects of methylene blue in such different models as pentetrazole-induced seizures and maximal electroshock-induced seizures is controversial but the results seem to suggest involvement of NO in anticonvulsant action of benzodiazepines.

In summary, the anticonvulsant effects of diazepam and clonazepam was intensified by both, a non-selective NOS inhibitor, L-NAME and selective NOS inhibitor, 7-NI in pentetrazole-induced seizures in mice. The above effect of L-NAME was reversed by L-arginine, endogenous donor of NO, but not by D-arginine. The participation of NO : cGMP pathway in the anticonvulsant effects of benzodiazepines seems to be supported by intensification of these effects by methylene blue, the guanylate cyclase inhibitor. Additionally, 7-NI was able to potentiate the protective efficacy of diazepam and clonazepam in electroshock-induced tonic hind-limb extension in mice.

Finally, the results of the present study seem to indicate that NO is involved, at least partly, in the anticonvulsant effects of benzodiazepines.

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Received: October 21, 2002; in revised form: March 10, 2003.