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European Journal of Pharmacology 366 (1999) 137–142

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N^G-Nitro-L-arginine impairs the anticonvulsive action of ethosuximide against pentylenetetrazol

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Received 16 July 1998; revised 9 December 1998; accepted 10 December 1998

Abstract

N^G-nitro-L-arginine (NNA; an inhibitor of nitric oxide synthase) in a dose of 40 mg/kg impaired the protective activity of ethosuximide against the clonic phase of pentylenetetrazol-induced seizures in mice. The ED_{50} value of ethosuximide was significantly increased from 108 to 158 mg/kg. NNA (40 mg/kg) was ineffective against the protective effects of diazepam, phenobarbital and valproate against pentylenetetrazol-induced seizures. NNA (40 mg/kg) did not influence the plasma levels of the antiepileptic drugs studied, so a pharmacokinetic interaction is not probable. L-Arginine (500 mg/kg) prevented the NNA-induced reduction of the anticonvulsive activity of ethosuximide. It can be concluded that nitric oxide participates in the expression of the anticonvulsive action of ethosuximide, but not that of diazepam, phenobarbital and valproate, against pentylenetetrazol-induced seizures.

Keywords: Nitric oxide (NO); L-Arginine; N^G-Nitro-L-arginine; Antiepileptic drug; Pentylenetetrazol; Seizure

1. Introduction

Nitric oxide (NO) may behave as a second messenger or a neurotransmitter, influencing various physiological functions (Moncada et al., 1991). For instance, NO seems to affect synaptic plasticity, long-term potentiation (Böhme et al., 1991; O'Dell et al., 1991; Schuman and Madison, 1991), long-term depression (Shibuki and Okada, 1991) and desensitization of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors (Ito and Karachot, 1991). Also, its involvement in the induction of the release of several neurotransmitters has been suggested (Montague et al., 1994). Apart from playing a role in the physiological events, NO has been implicated in the pathophysiology of some neurodegenerative diseases (e.g., Parkinson's or Huntington's disease) and neuronal damage induced by brain ischemia (Hoffman, 1991; Moncada et al., 1991). Besides serving central functions, NO may also modulate the macro- and microvessels of the blood–brain barrier, influence pyloric activity, or produce apoptosis (Shukla et al., 1995; Willis et al., 1996; Brüne et al., 1998).

Less than a decade ago, Meldrum and Garthwaite (1990) considered a possible role of NO in the pathophysiology of epilepsy. Although inhibitors of NO synthase did affect seizure susceptibility to some convulsive stimuli in experimental animals, the obtained data were contradictory. These agents were documented to have anticonvulsive and proconvulsive activity, depending upon the seizure model used or routes of administration (Buisson et al., 1993; Penix et al., 1994; Przegaliński et al., 1994, 1996; Rundfeldt et al., 1995; Van Leeuwen et al., 1995; Smith et al., 1996; Tutka et al., 1996; Urbańska et al., 1996; Alexander et al., 1998). In this context, we studied the influence of N^G-nitro-L-arginine (NNA; an inhibitor of NO synthase) on the anticonvulsive action of diazepam, ethosuximide, phenobarbital and valproate against pentylenetetrazol-induced seizures in mice. A modulatory effect of NNA on the activity of antiepileptic drugs would provide information about the involvement of NO-mediated events in the mechanism of action of conventional antiepileptic drugs.

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0014-2999/99/$ - see front matter
PII: S0014-2999(98)00927-3

2. Materials and methods

2.1. Animals and experimental conditions

The experiments were carried out with male Swiss mice (purchased from a licensed dealer: T. Górzkowska, Warsaw, Poland) weighing 20–25 g, between 10.00 and 13.00 h. The animals were housed in colony cages with free access to food (chow pellets) and tap water. The temperature during the experimental procedures was 21 ± 1°C and the mice were on a natural light–dark cycle. The experimental groups, consisting of 8–10 animals, were chosen by means of a randomized schedule.

2.2. Drugs

Phenobarbital (Polfa, Cracow, Poland), valproate magnesium (Polfa, Rzeszów, Poland), ethosuximide and pentylenetetrazol (both drugs from Sigma, St. Louis, MO, USA) were brought into solution with sterile saline and injected intraperitoneally, except for pentylenetetrazol, which was given subcutaneously. Diazepam (Polfa, Warsaw, Poland), NNA and L-arginine (both substances from RBI, Natick, MA, USA) were suspended in a 1% solution of Tween 80 (Sigma, St. Louis, MO, USA) and administered intraperitoneally. The injection volume for antiepileptic drugs and pentylenetetrazol was 5 ml/kg whilst that for NNA and L-arginine was 10 ml/kg body weight. The treatment times prior to pentylenetetrazol injection were diazepam, pheno-barbital and L-arginine 60 min, and ethosuximide, valproate and NNA 30 min.

2.3. Pentylenetetrazol-induced seizures

Pentylenetetrazol (Sigma) was administered subcutaneously in a dose of 96 mg/kg, which was equal to its CD_{97} (convulsive dose 97%) for the induction of clonic seizures. In order to define the influence of L-arginine (500 mg/kg) upon the convulsive threshold for the clonic phase of pentylenetetrazol-induced convulsions, the CD_{50} (convulsive dose 50%) of pentylenetetrazol was evaluated. Clonic seizure was defined as clonus of the whole body with loss of the righting reflex for at least 3 s. Pentylenetetrazol-injected animals were put individually into transparent Plexiglass cages (25 × 15 × 10 cm) and observed for the occurrence of clonic seizures for 30 min. Mortality was assessed 60 min after the injection of pentylenetetrazol.

2.4. Chimney test

The effect of ethosuximide alone or combined with NNA on motor impairment was quantified with the chimney test of Boissier et al. (1960). In this test, animals had to climb backwards up a plastic tube (3 cm inner diameter, 25 cm length). Motor impairment was indicated by the inability of mice to climb backwards up the tube within 60 s, and results are given as the percentage of animals which failed to perform the test.

2.5. Estimation of plasma levels of antiepileptic drugs

The animals were administered vehicle + antiepileptic or NNA (40 mg/kg) + antiepileptic drug. In the case of ethosuximide, the effect of L-arginine (500 mg/kg) + NNA (40 mg/kg) on the plasma levels of this antiepileptic drug was also studied. Mice were decapitated at time of pentylenetetrazol injection and samples of blood of approximately 1 ml were collected into Eppendorf tubes. They were subsequently centrifuged at 10,500 g (Abbott centrifuge, Irving, TX, USA) for 3 min. Plasma samples of 70 μl were put into Abbott system cartridges. The plasma levels of antiepileptic drugs were estimated by immunofluorescence, using an Abbott TDx analyzer (Abbott), and expressed in μg/ml as means ± S.D. of at least eight determinations.

2.6. Statistics

In order to calculate CD_{97} or CD_{50} values for pentylenetetrazol, at least four groups of mice were injected with various doses of the convulsant to obtain 10–30, 30–50, 50–70 and 70–90% of mice with clonic convulsions. Then, the dose–response curves were constructed. Similarly, the dose–response curves for the protection provided by antiepileptic drugs against the clonic phase of pentylenetetrazol (at its CD_{97})-induced seizures were plotted. CD_{97}s or CD_{50}s for pentylenetetrazol or ED_{50} values for antiepileptic drugs and statistical analysis of the data, obtained in the convulsive test, were estimated by computer probit analysis, according to Litchfield and Wilcoxon (1949). CD_{50}s and ED_{50}s are accompanied by 95% confidence limits (in parentheses). The results from the chimney test and mortality were compared statistically by using Fisher's exact probability test. Plasma levels of antiepileptic drugs were compared by using Student's unpaired *t*-test.

3. Results

3.1. Influence of NNA and L-arginine on pentylenetetrazol-induced seizures

The CD_{97} for the induction of clonic seizures by pentylenetetrazol was 96 mg/kg. The incidence of clonic convulsions in mice injected with pentylenetetrazol at doses of 50, 60 and 80 mg/kg was 25, 50 and 88%, respectively (the mortality rate was 0, 0, 25 and 50%, respectively). Out of eight mice injected with pentylenetetrazol at the dose of 96 mg/kg, 4 of them did not survive the 60-min observation period. NNA (up to 40 mg/kg) was without effect upon the control CD_{50} of pentylenetetrazol for the

induction of the clonic phase (Tutka et al., 1996). The NO donor, L-arginine (500 mg/kg), did not affect the convulsive threshold for the clonic phase of pentylenetetrazol-induced seizures. The control CD_{50} of pentylenetetrazol of 57.6 (50.6–65.4) mg/kg was insignificantly elevated to 61.9 (50.6–75.7) mg/kg. Pentylenetetrazol-induced lethality was not statistically different in NNA (40 mg/kg)- or L-arginine (500 mg/kg)-injected groups vs. the control group.

3.2. Effect of NNA on the anticonvulsive activity of antiepileptic drugs

As shown in Table 1, NNA (40 mg/kg) impaired the anticonvulsive activity of ethosuximide against the clonic phase of pentylenetetrazol-induced convulsions, increasing its ED_{50} value from 108 to 158 mg/kg. At the lower doses of 1 and 10 mg/kg, NNA did not significantly modify the protective activity of ethosuximide. No mortality was observed in mice injected with ethosuximide at doses of 100 mg/kg and higher (125 and 150 mg/kg) whilst two out of eight mice died when ethosuximide (100 mg/kg) was combined with NNA (40 mg/kg), and this was not statistically significant. Co-administration of NNA (40 mg/kg) with ethosuximide (125, 150 and 200 mg/kg) resulted in 12.5, 0 and 0% lethality over the 60-min observation period (results not shown in Table 1). NNA (40 mg/kg) did not affect the anticonvulsive action of the remaining antiepileptic drugs (diazepam, phenobarbital and valproate) against pentylenetetrazol (Table 1). However, the NO donor L-arginine (500 mg/kg) partially attenuated the effect of NNA (40 mg/kg) on the anticonvulsive activity of ethosuximide. The re-evaluated control ED_{50} value of ethosuximide against the clonic phase of pentylenetetrazol-induced convulsions was 121 (98–151) mg/kg and was elevated to 164 (134–200) mg/kg by NNA (40 mg/kg; $P < 0.05$). Co-administration of NNA (40 mg/kg) and L-arginine (500 mg/kg) with ethosuximide resulted in an ED_{50} of 145 (123–170) mg/kg, which was not significantly different from the control ED_{50} value of ethosuximide (results not shown in Table 1).

Table 1
Influence of N^G-nitro-L-arginine (NNA) on the anticonvulsive activity of conventional antiepileptic drugs against pentylenetetrazol-induced clonic convulsions in mice

Antiepileptic drug	NNA (mg/kg)		
	0	10	40
Ethosuximide	108 (88–134)	125 (105–149)	158[a] (132–190)
Diazepam	0.40 (0.31–0.52)	NT	0.42 (0.32–0.56)
Phenobarbital	14.4 (10.8–19.3)	NT	13.0 (10.0–16.8)
Valproate	124 (98–157)	NT	122 (107–157)

Table data are ED_{50} values (in mg/kg) with 95% confidence limits in parentheses.
Antiepileptic drugs were administered i.p.
Ethosuximide, valproate and NNA were given 30 min, whilst diazepam and phenobarbital were given 60 min prior to maximal electroshock.
The calculation of ED_{50} values and their statistical evaluation were based upon the method of Litchfield and Wilcoxon (1949), but modified in that dose–effect curves were calculated on a computer.
[a] $P < 0.01$ vs. respective control group.
NT—not tested.

Table 2
Influence of NNA upon the total plasma levels of antiepileptic drugs in mice

Treatment (mg/kg)	NNA (mg/kg)	
	0	40
Ethosuximide (158)	138 ± 10.8	153 ± 27.6
Diazepam (0.42)	0.24 ± 0.028	0.24 ± 0.027
Phenobarbital (13)	13.6 ± 1.07	14.1 ± 0.70
Valproate (122)	147 ± 10.1	144 ± 13.0

Table values are the means (in μg/ml) of eight determinations ± S.D.
Student's unpaired *t*-test was used for statistical comparisons.
For treatment times refer to Table 1.

3.3. Motor performance of mice pretreated with NNA and ethosuximide alone or in combination

NNA (40 mg/kg) and ethosuximide (108 or 158 mg/kg) given alone did not influence the motor performance of mice in the chimney test (7, 10 and 9 mice, respectively, out of 10 per experimental group performed the test correctly). In the control group, no animal showed motor impairment. The co-administration of NNA (40 mg kg) with ethosuximide resulted, however, in motor impairment (5 mice out of 10 did not perform the test; $P < 0.01$).

3.4. Influence of NNA on the plasma levels of antiepileptic drugs

The data in Table 2 demonstrate that in no case did NNA (40 mg/kg) affect the total plasma levels of the antiepileptic drugs. Also, when NNA (40 mg/kg) and L-arginine (500 mg/kg) were co-administered with ethosuximide (145 mg/kg), the plasma level of ethosuximide (153 ± 19.2 μg/ml) did not differ significantly from that in the control group (163 ± 15.5 μg/ml; result not shown in Table 2).

4. Discussion

The present study revealed that NNA (40 mg/kg) significantly decreased the protective activity of ethosuximide against the clonic phase of pentylenetetrazol-induced convulsions. The anticonvulsive effects of diazepam, phenobarbital and valproate were not affected by this inhibitor of NO synthesis, which may indicate that NO does not play a role in their mechanism of action. NNA did not affect the plasma level of ethosuximide, so a pharmacokinetic interaction does not seem to be involved

in the NNA-induced impairment of the anticonvulsive activity of this antiepileptic drug. It is noteworthy that ethosuximide is only minimally bound to plasma proteins (Chang, 1989), so the total plasma level in this case is a good indicator of the levels of bioactive ethosuximide. The inhibitor of NO synthesis did not influence the plasma levels of the remaining antiepileptic drugs studied, either. This appears to exclude the possibility of pharmacokinetic (in terms of the total plasma levels at least) and pharmacodynamic interactions between NNA and these antiepileptics.

Previous studies indicated that another unspecific inhibitor of NO synthase, N^G-nitro-L-arginine methyl ester (40 mg/kg), reduced the anticonvulsive action of phenobarbital and valproate against maximal electroshock-induced convulsions in mice. The protective activity of carbamazepine and diphenylhydantoin was not affected (Borowicz et al., 1998). However, 7-nitroindazole (a selective neuronal NO synthase inhibitor) influenced only the protection offered by phenobarbital against maximal electroshock and this protection was actually increased (Borowicz et al., 1997). Attempts to reverse the impairing effect of N^G-nitro-L-arginine methyl ester or the potentiating effect of 7-nitroindazole with the NO precursor, L-arginine (500 mg/kg), were unsuccessful (Borowicz et al., 1997, 1998). This may indicate that NO synthesis inhibitors interact in a non-specific manner with antiepileptic drugs in the maximal electroshock test. A similar hypothesis was also suggested by Baran et al. (1997), who studied the interaction between N^G-nitro-L-arginine methyl ester, 7-nitroindazole or molsidomine (an NO donor) and conventional antiepileptic drugs against maximal electroshock in mice. 7-Nitroindazole was additionally shown to enhance the anticonvulsive activity of flurazepam against electroconvulsions in mice (Deutsch et al., 1995). In the present study, however, L-arginine prevented the NNA-induced reduction in the anticonvulsive action of ethosuximide. Again, this could not be attributed to a pharmacokinetic mechanism since the plasma level of ethosuximide was not affected by NNA + L-arginine. Thus, mediation of the anticonvulsive effect of ethosuximide may be to some extent dependent on NO. Since NO synthase inhibitors have been reported to exert opposite, apparently dose-dependent effects in the same seizure model (Rundfeldt et al., 1995) or contrasting effects against seizures induced by the excitatory amino acids, glutamate or kainate (Tutka et al., 1996), any general conclusion about the role of NO in seizure activity or the mechanisms of action of antiepileptic drugs cannot be drawn at the moment. The finding that the effect of NNA on ethosuximide could be partially reversed by L-arginine does not necessarily mean that the action of ethosuximide was dependent upon neuronal NO synthase activity. It may also result from the inhibition of the peripheral actions of NO since arginine analogues, such as NNA, actually inhibit the activity of both endothelial and neuronal NO synthase (Dwyer et al., 1991). However, the possibility that NNA interferes with the penetration of pentylenetetrazol into the brain seems unlikely. First, NNA (up to 40 mg/kg) under our experimental conditions did not affect the susceptibility of mice to pentylenetetrazol (Tutka et al., 1996). Second, the anticonvulsive activity of the other studied antiepileptic drugs against pentylenetetrazol-induced seizures remained unchanged in the presence of NNA. Phenobarbital induced a three-fold increase in neuronal NO synthase mRNA expression followed by a more modest induction of enzyme activity in the rat cerebellum (Thompson et al., 1997). Considering that NNA was ineffective against the anticonvulsive activity of this antiepileptic drug, phenobarbital-induced alterations in the activity of the NO system seem of minor importance in the mechanism of its protection against pentylenetetrazol-induced convulsions.

The possibility exists that the interaction between NNA and ethosuximide may involve an increase in the intraneuronal Ca^{2+} concentration since this NO synthase inhibitor actually augments the NMDA- and kainate-induced increase in intracellular Ca^{2+} concentration (Tanaka et al., 1993). Ca^{2+} ions play an essential role in the induction and maintenance of seizure activity (Speckmann et al., 1993), and it has been well documented that various convulsive procedures lead to a substantial increase in glutamate-mediated events (Urbańska et al., 1998). It is noteworthy that the Ca^{2+} inhibitor, diltiazem, distinctly potentiated the anticonvulsive action of ethosuximide against pentylenetetrazol-induced convulsions in mice and a pharmacokinetic factor was excluded (Czuczwar et al., 1990). This may indicate that the expression of the protective activity of ethosuximide may depend upon the intracellular Ca^{2+} concentration. In contrast, diltiazem did not affect the anticonvulsive efficacy of either valproate or phenobarbital. Although another Ca^{2+} inhibitor, nifedipine, increased the protective action of ethosuximide, valproate or phenobarbital against pentylenetetrazol, the most spectacular interaction was observed in the case of ethosuximide. The anti-pentylenetetrazol activity of diazepam was not influenced by Ca^{2+} inhibitors (Czuczwar et al., 1990).

The combined treatment of NNA and ethosuximide not only reduced the protective potency of this antiepileptic drug against pentylenetetrazol-induced seizures but also resulted in motor impairment which was not seen when ethosuximide was administered alone. Motor impairment was also observed when another inhibitor of NO synthesis, L-N^G-nitroarginine methyl ester, was combined with phenobarbital or valproate (Borowicz et al., 1998). These results are in agreement with the hypothesis that constitutive NO synthase is essential for normal movement (Starr and Starr, 1995). Moreover, NNA (5–80 mg/kg) was shown to produce dose dependently catalepsy in mice (Marras et al., 1995), which could account for the impaired motor performance seen when it was given in combination with ethosuximide.

According to Löscher and Schmidt (1988), pentylenetetrazol-induced convulsions may serve as a model of human myoclonic seizures and, with some limitations, as a model of human absence seizures. In as far as the results of experimental studies can be extrapolated to the clinical situation, the obtained results seem to support the notion that the anticonvulsive and adverse potential of ethosuximide may be dependent upon endogenous NO in epileptic patients. This leads to the conclusion that derangements in the activity of the NO system could result in modification of the anticonvulsive potency of this antiepileptic drug. The protective activity of diazepam, phenobarbital, and valproate against pentylenetetrazol seems to be unrelated to the inhibition of NO synthase.

Acknowledgements

This study was supported by a KBN grant no. 4 P05F 042 10. Generous gifts of valproate magnesium from Polfa (Rzeszów, Poland) and phenobarbital sodium from Polfa (Cracow, Poland) are greatly acknowledged.

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