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RAPID COMMUNICATION

Interference With Nitric Oxide Production and Action Potentiates the Antiseizure Efficacy of Flurazepam

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DEUTSCH, S. I., R. B. ROSSE, C. MORN, L. KOETZNER AND J. MASTROPAOLO. *Interference with nitric oxide production and action potentiates the antiseizure efficacy of flurazepam.* PHARMACOL BIOCHEM BEHAV 51(1) 133–137, 1995. – The effect of inhibiting "downstream" consequences of NMDA receptor stimulation with 7-nitroindazole, an inhibitor of the neuronal form of nitric oxide synthase (NOS), and methylene blue, an inhibitor of the nitric oxide (NO)-sensitive soluble guanylyl cyclase, on electrically precipitated tonic hindlimb extension in mice was studied. Moreover, the abilities of these compounds to potentiate the antiseizure efficacy of flurazepam were also examined. When administered alone, 7-nitroindazole (10.0–100 mg/kg) and methylene blue (1.0–100 mg/kg) did not share the ability of MK-801 (0.1 to 1.0 mg/kg) to antagonize electrically precipitated tonic hindlimb extension. However, doses of MK-801 (0.18 mg/kg), 7-nitroindazole (100 mg/kg), and methylene blue (10.0 and 100 mg/kg) that were devoid of apparent antiseizure efficacy by themselves potentiated the ability of flurazepam to antagonize electrically precipitated seizures. NMDA receptor antagonists cause neuronal toxicity, interfere with acquisition of spatial memory and induction of long-term potentiation in the hippocampal CA1 region, and precipitate psychoses in susceptible individuals. Thus, the development of both open-channel blockers of the NMDA receptor complex that can be administered in lower doses, and inhibitors of the "downstream" consequences of NMDA receptor-gated transient elevations of intraneuronal calcium ions as potential adjunctive antiseizure medications should be considered. Moreover, administration of these compounds with benzodiazepines may attenuate some of the neurotoxicity that may result from NMDA receptor antagonism.

NMDA receptor MK-801 7-Nitroindazole Methylene blue Nitric oxide Calcium ions Seizures

PHARMACOLOGICAL strategies for interfering with excitatory neural transmission and the consequences of calcium ion entry are being developed as potential interventions for the treatment of seizure disorders and neuroprotection (7,10). Glutamate-stimulated calcium ion conduction through the NMDA receptor-associated ionophore leads to the production of nitric oxide (NO) as one of its "downstream" consequences (3,11). Many effects attributable to NMDA receptor stimulation may be mediated by generation of NO, a gaseous intercellular messenger, and its subsequent stimulation of the soluble heme-containing form of guanylyl cyclase to produce cyclic GMP (3,11). MK-801 has been shown to antagonize the electrical precipitation of tonic hindlimb extension in mice (16), and has been explored for its potential development as a medi-

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cation with anticonvulsant and neuroprotective properties (7,10). The present study was undertaken to examine whether compounds that only interfere with downstream consequences of glutamate-stimulated calcium ion entry share this anticonvulsant property of MK-801. Moreover, in view of the delicate interaction between NMDA and $GABA_A$ receptor-mediated events, we wondered whether these downstream blockers would potentiate the antiseizure efficacy of flurazepam in the IECS procedure.

In order to examine the anticonvulsant efficacy of interfering with some of the consequences of NMDA receptor stimulation (i.e., calcium ion entry, NO generation, and cyclic GMP production), the abilities of MK-801 {(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine maleate; dizocilpine}, 7-nitroindazole, and methylene blue to antagonize electrically precipitated tonic hindlimb extension in mice and their abilities to potentiate the antiseizure efficacy of flurazepam were studied. MK-801 is an uncompetitive allosteric antagonist of the NMDA receptor complex that binds to a hydrophobic channel domain interfering with calcium ion conductance (6). 7-Nitroindazole is a selective inhibitor of the neuronal isoenzyme of nitric oxide synthase (NOS), the enzyme responsible for the production of nitric oxide from arginine in neurons (12). 7-Nitroindazole has been shown to cause a dose-related inhibition of the neuronal form of NOS (1). Moreover, 7-nitroindazole was shown to be centrally effective when administered peripherally to rats. Methylene blue interferes with the ability of NO to stimulate cyclicGMP production by binding to the heme moiety of the soluble guanylyl cyclase (8); methylene blue may also interfere with other steps along the NO "cascade" (9). In a dose-dependent manner, methylene blue interfered with the ability of acetylcholine to cause endothelium-dependent relaxation of rabbit aortic rings (8). Presumably, this relaxation is mediated by the acetylcholine-dependent elevation of cyclic GMP levels, which can be blocked by methylene blue. The central effects of peripherally administered methylene blue have a long history of study dating back to Bodoni in 1899 (2). Because of its possession of a phenothiazine nucleus and theoretical benefits thought to be associated with its ability to participate in electron transfer reactions, methylene blue has been tried empirically in the treatment of manic-depressive illness with some therapeutic success (15). These empirical trials in patients support a centrally active effect of methylene blue.

METHOD

Animals

An outbred strain of experimentally naive, male NIH Swiss mice weighing approximately 30 g and housed in hanging wire cages (five mice/cage) were used throughout the experiments. Animals were maintained in a temperature-controlled vivarium on a 12 h light-dark cycle with free access to food and water. Animals were transported to the laboratory on the day of the experiment.

Drugs

MK-801 (Research Biochemicals International, Natick, MA) and flurazepam HCl (Hoffmann-La Roche, Nutley, NJ) were dissolved in 0.9% saline. 7-Nitroindazole (Biomol, Plymouth Meeting, PA) was suspended by ultrasonic agitation in sesame oil (Sigma Chemical Co., St. Louis, MO). Methylene blue was obtained from American Regent Laboratories (Shirley, NY) as a 1% solution; the stock solution was diluted with normal saline. All drugs were injected intraperitoneally in a volume of 0.01 ml/g of body weight. Flurazepam was always injected 20 min prior to the IECS procedure. MK-801, 7-nitroindazole, and methylene blue were injected 10, 40, and 10 min prior to flurazepam, and 30, 60, and 30 min prior to the incremental electroconvulsive shock (IECS) procedure, respectively.

Incremental Electroconvulsive Shock (IECS) Procedure

In the IECS procedure, a Hittman electroconvulsive shock generator (Medcraft model B24-II) was utilized to administer 0.3 s of voltage via earclip electrodes. To determine the threshold voltage for the precipitation of tonic hindlimb extension, the procedure began with 70 V and was increased in 10-V increments every 2 s until maximal tonic hindlimb extension occurred or 170 V was reached. A voltage of 180 was recorded for animals that did not show tonic hindlimb extension.

Analysis

In all experiments, groups of at least 12 mice were tested in each of the experimental conditions. Data from the experiments were analyzed with either a one-way or two-way analysis of variance (ANOVA) and subsequent post hoc tests when appropriate. All reports of statistical significance were based upon a value of $p < 0.05$.

RESULTS

As illustrated in Fig. 1, consistent with our previous results, a one-way ANOVA revealed that animals treated with MK-801 showed a statistically significant, $F(1, 5) = 17.73$, dose-related increase in the threshold voltage required for the electrical precipitation of tonic hindlimb extension (16). One-way ANOVAs showed that 7-nitroindazole, $F(1, 5) = 0.61$, and methylene blue, $F(1, 3) = 2.27$, were devoid of any apparent antiseizure efficacy when administered alone in the IECS procedure (Figs. 2 and 3, respectively).

With the combination of MK-801 (0.18 mg/kg) and flurazepam, the two-way ANOVA revealed a significant main effect

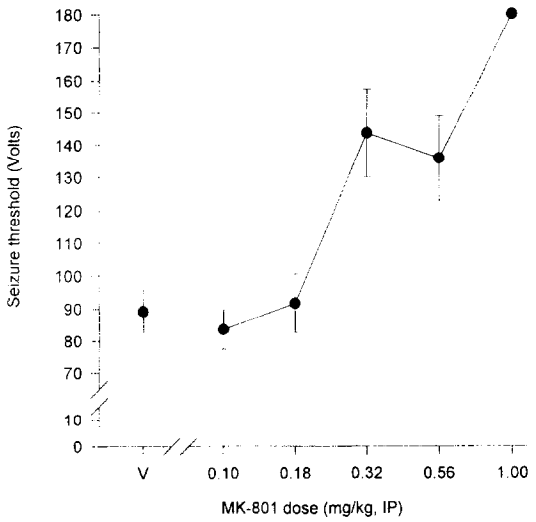

FIG. 1. The mean seizure voltage (±SEM) of groups of mice (n = 12–14/group) injected with saline (the point above V) or various doses of MK-801.

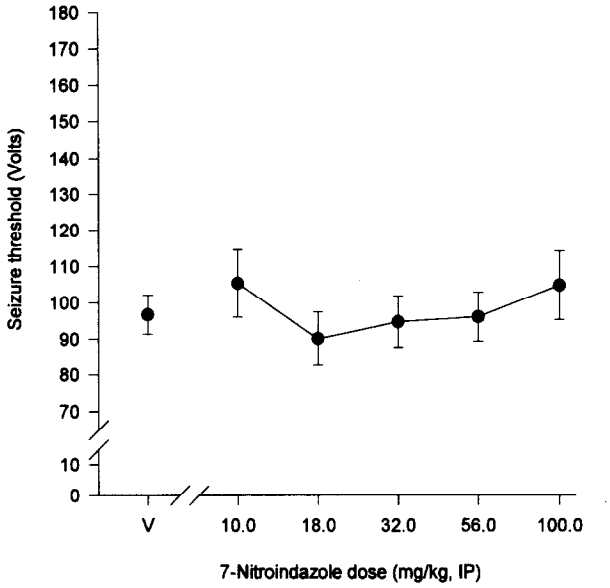

FIG. 2. The mean seizure voltage (±SEM) of groups of mice (n = 12–14/group) injected with sesame oil (the point above V) or various doses of 7-nitroindazole.

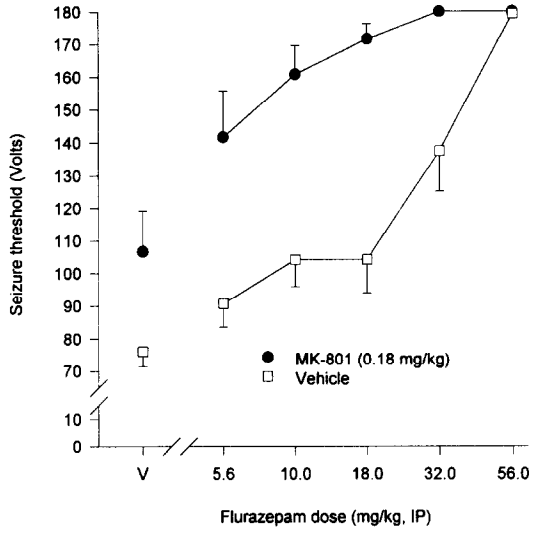

FIG. 4. The mean seizure voltage (±SEM) of groups of mice (n = 12–14/group) injected with saline (open squares) or 0.18 mg/kg of MK-801 (closed circles) prior to an injection of saline (points above V) or various doses of flurazepam.

for flurazepam, $F(2, 5) = 28.35$, a significant main effect for MK-801, $F(1, 1) = 75.85$, and a significant interaction, $F(12, 5) = 4.07$. This indicates that an ineffective dose of MK-801 (Scheffe test for MK-801 + flurazepam vehicle vs. MK-801 vehicle + flurazepam vehicle, $p = 0.81$) significantly potentiated the ability of flurazepam to antagonize electrically precipitated tonic hindlimb extension (Fig. 4). As illustrated in Fig. 5, although 7-nitroindazole was devoid of any apparent antiseizure efficacy when administered alone (Fig. 2), a two-way ANOVA revealed a main effect for flurazepam, $F(2, 5) = 31.45$, and a main effect for 7-nitroindazole, $F(1, 1) = 22.02$, indicating that 100 mg/kg of 7-nitroindazole significantly potentiated this action of flurazepam. Finally, three doses of methylene blue were tested for their ability to potentiate flurazepam (Figs. 6, 7, and 8). As can be seen, although 1.0 mg/kg of methylene blue had no effect [two-way

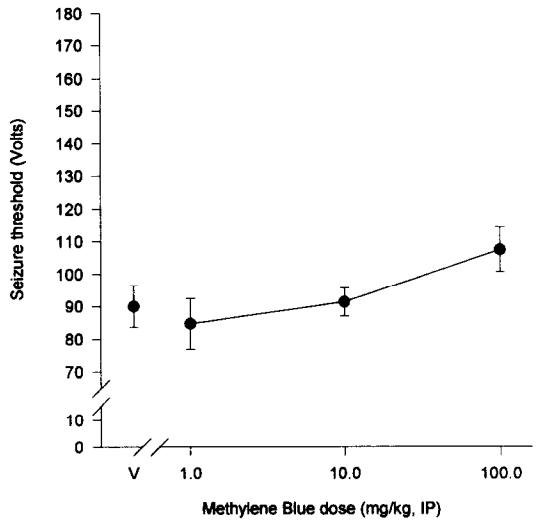

FIG. 3. The mean seizure voltage (±SEM) of groups of mice (n = 12–14/group) injected with saline (the point above V) or various doses of methylene blue.

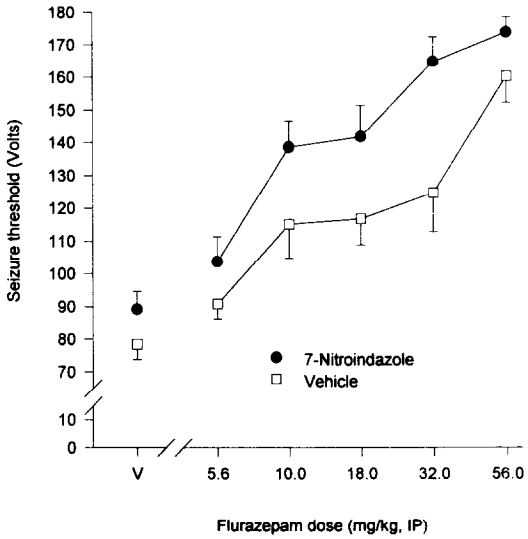

FIG. 5. The mean seizure voltage (±SEM) of groups of mice (n = 12–14/group) injected with sesame oil (open squares) or 100 mg/kg of 7-nitroindazole (closed circles) prior to an injection of saline (points above V) or various doses of flurazepam.

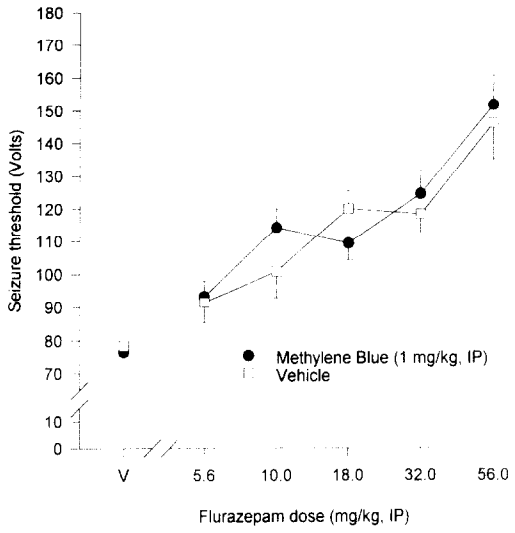

FIG. 6. The mean seizure voltage (±SEM) of groups of mice (n = 12–14/group) injected with saline (open squares) or 1 mg/kg of methylene blue (closed circles) prior to an injection of saline (points above V) or various doses of flurazepam.

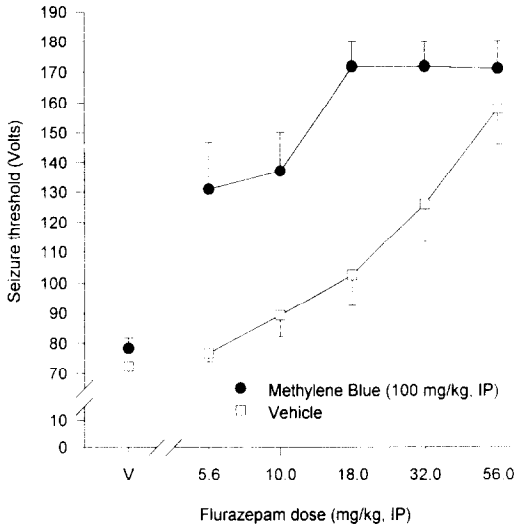

FIG. 8. The mean seizure voltage (±SEM) of groups of mice (n = 12–14/group) injected with saline (open squares) or 100 mg/kg of methylene blue (closed circles) prior to an injection of saline (points above V) or various doses of flurazepam.

ANOVA, $F(1, 1) = 0.43$], both 10.0 and 100 mg/kg significantly potentiated the anticonvulsant effects of flurazepam [$F(1, 1) = 4.23$ and 49.48, respectively].

DISCUSSION

Interference with receptor-gated calcium ion conductance through the NMDA receptor-associated ionophore has been shown to possess anticonvulsant and neuroprotective effects. Unfortunately, competitive NMDA antagonists and uncompetitive open-channel blockers that bind to the hydrophobic channel domain of the NMDA receptor complex are associated with intracytoplasmic vacuole formation in neurons of the cingulate and retrosplenial cerebral cortices (17,18). Furthermore, inhibition of NMDA-mediated calcium ion conduction can disrupt acquisition of spatial memory and induction of long-term potentiation in the hippocampal CA1 region (14), as well as precipitate schizophreniform psychoses in susceptible individuals (4). Thus, there may be severe limitations to the development of NMDA antagonists as anticonvulsant and neuroprotective medications. Interestingly, MK-801-induced vacuole formation in rat cingulate neurons can be significantly attenuated by diazepam (18). The latter data encourage strategies for developing the anticonvulsant and neuroprotective properties of NMDA open-channel blockers, while avoiding their neurotoxic side effects with GABAergic interventions.

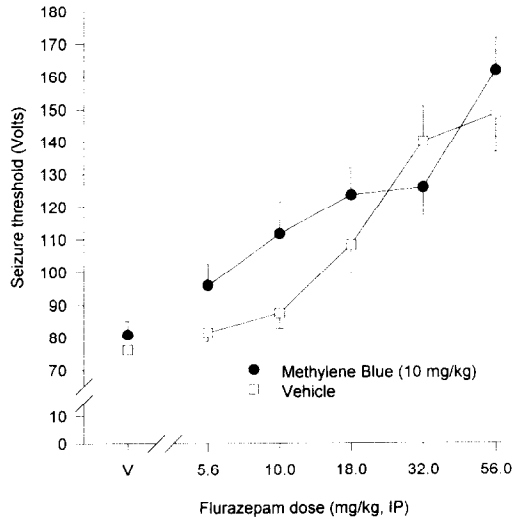

FIG. 7. The mean seizure voltage (±SEM) of groups of mice (n = 12–14/group) injected with saline (open squares) or 10 mg/kg of methylene blue (closed circles) prior to an injection of saline (points above V) or various doses of flurazepam.

The current study replicated previous results showing that MK-801 possesses a dose-related ability to raise the threshold voltage for electrically precipitated tonic hindlimb extension in mice (16). Moreover, a dose of MK-801 that was devoid of this pharmacological action when tested alone potentiated the antiseizure efficacy of flurazepam in this paradigm. Conceivably, there is a role for the development of open-channel blockers as adjuvant antiseizure medications; moreover, in this instance, flurazepam may attenuate pathologic intraneuronal vacuolization associated with MK-801 administration.

The current study also showed that interference with at least some of the downstream consequences of transiently elevated intraneuronal calcium ion concentrations (i.e., NO and cyclic GMP production) potentiated the ability of flurazepam to antagonize electrically precipitated seizures. These data suggest that neuronal NOS inhibitors and inhibitors of NO-

sensitive soluble guanylyl cyclase activity deserve serious consideration for development as adjuvant medications for the treatment of seizures. There are data consistent with a positive association between elevated intraneuronal cyclic GMP levels and seizures (5,13,19). Interestingly, although 7-nitroindazole and methylene blue were shown to potentiate the antiseizure efficacy of flurazepam, they were devoid of any obvious intrinsic antiseizure activity by themselves in the dose ranges examined in this investigation. Thus, the precise mechanism(s) by which inhibition of NO and cyclic GMP production results in the potentiation of the action of flurazepam in the IECS procedure remains unclear.

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