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# $Ca^{2+}$ channel blockade and the antielectroshock activity of NMDA receptor antagonists, CGP 40116 and CGP 43487, in mice

Maciej Gasior [1], Kinga Borowicz, Radosław Starownik, Zdzisław Kleinrok, Stanisław J. Czuczwar *

*Department of Pharmacology and Toxicology, Medical School, Jaczewskiego 8, 20-090 Lublin, Poland*

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**Abstract**

Nicardipine, nifedipine and flunarizine showed anticonvulsive activity (reflected by significant elevations of the seizure threshold for tonic hindlimb extension) in doses of 20, 20 and 15 mg/kg, respectively. In combination studies, CGP 40116 [D-(*E*)-2-amino-4-methyl-5-phosphono-3-pentenoic acid] or its methyl ester derivative (CGP 43487) was administered in a constant dose of 0.25 and 3.5 mg/kg, respectively. At these doses both competitive NMDA receptor antagonists were able to elevate significantly the convulsive threshold. Nicardipine, nifedipine, and flunarizine were administered at maximal doses (or lower) not affecting the convulsive threshold (15, 15 and 10 mg/kg, respectively). The protective activity of CGP 40116 and CGP 43487 was dose dependently potentiated by all three $Ca^{2+}$ channel inhibitors. The combined treatment caused motor impairments (evaluated in the chimney test) and long-term memory deficits (measured in the passive avoidance task) similar to these produced by CGP 40116 or CGP 43487 alone. Our results indicate that nicardipine, nifedipine and flunarizine significantly potentiate the protective activity, but not the adverse effects, of CGP 40116 and CGP 43487 in mice.

*Keywords:* NMDA receptor antagonist; CGP 40116; CGP 43487; $Ca^{2+}$ channel inhibitor; Flunarizine; Nicardipine; Nifedipine; Seizure

## 1. Introduction

Strong evidence has been provided that excessive calcium ($Ca^{2+}$) influx into a neuron plays a crucial role in epileptogenesis (Hotson and Prince, 1981; Purmain et al., 1983; Speckmann et al., 1993; Vreugdenhill and Wadman, 1992). $Ca^{2+}$ ions may enter a cell via the NMDA receptor ion complex or/and through voltage-dependent $Ca^{2+}$ channels (Jahr and Stevens, 1987; MacDermott et al., 1986; Sher et al., 1991). Activation of either NMDA receptors or L-type voltage-dependent $Ca^{2+}$ channels leads to seizure activity in experimental animals (Bolger et al., 1985; Czuczwar et al., 1985; Gasior et al., 1995b; Shelton et al., 1987; Leander et al., 1988; Palmer et al., 1993). NMDA receptor antagonists exert potent anticonvulsant effects in a variety of experimental models of epilepsy (for review see Löscher and Schmidt, 1994; Rogawski, 1992). $Ca^{2+}$ channel inhibitors have also been effective against e.g.: electroconvulsions, pentetrazol-, sound-, *N*-methyl-D,L-aspartic acid-, BAY k-8644 [methyl-1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoro-methylphenyl)-pyridine-5-carboxylate; a $Ca^{2+}$ channel agonist of the dihydropyridine class]-, and bicuculline-induced seizures in mice (Shelton et al., 1987; De Sarro et al., 1988; Dolin et al., 1988; Czuczwar et al., 1990a,b; Palmer et al., 1993). Moreover, $Ca^{2+}$ channel inhibitors enhance the protective activity of conventional antiepileptic drugs against maximal electroshock- and pentetrazol-produced seizures in mice (Czuczwar et al., 1990a,b).

More recently, Czuczwar et al. (1994) reported that BAY k-8644 impaired the anticonvulsant efficacy of two competitive NMDA receptor antagonists, CGP 37849 [D,L-(*E*)-2-amino-4-methyl-5-phosphono-3-pentenoic acid] and D-CPP-ene [3-(2-carboxypiperazine-4-yl)-1-propenyl-1-phosphonic acid], against electroconvulsions in mice. Further, the protective activity of a non-competitive NMDA receptor antagonist, MK-801 (dizocilpine), and of the non-NMDA receptor antagonists, NBQX [2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(*F*)quinoxaline] and GYKI

* Corresponding author. Tel./fax: (+48-81)-77-89-24; e-mail: kornas@ursus.ar.lublin.pl

[1] Present address: NIH/NIDA, Addiction Research Center, P.O. Box 5180, Baltimore, MD 21224, USA; e-mail: mgasior@irp.nida.nih.gov


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52466 [1-(4-aminophenyl)-4-methyl-7,8-methylene-dioxy-5*H*-2,3-benzodiazepine], was not affected by BAY k-8644 (Czuczwar et al., 1994).

This prompted us to study the influence of $Ca^{2+}$ channel inhibitors, nicardipine, nifedipine and flunarizine, on the antielectroshock activity of two competitive NMDA receptor antagonists, CGP 40116 [D-(*E*)-2-amino-4-methyl-5-phosphono-3-pentenoic acid] and its methyl ester derivative, CGP 43487, in mice. According to Porter (1990), electroconvulsions are useful for detecting drugs effective against partial or generalized tonic-clonic seizures in humans. It is important to note that NMDA receptor antagonists produce numerous adverse effects, for instance: locomotor depression or stimulation and learning or memory impairment (for recent reviews see Schmidt, 1994; Witkin, 1995). We studied whether the coadministration of $Ca^{2+}$ channel inhibitors and NMDA receptor antagonists affects the performance of mice in the chimney test (motor impairment screen test according to Boissier et al., 1960) and in a passive avoidance task (long-term memory impairment, Venault et al., 1986).

## 2. Materials and methods

### 2.1. General

Experiments were performed with male Swiss mice weighing 24–28 g. After 10 days of adaptation to laboratory conditions, experimental groups (8–12 animals) were formed randomly. Mice were kept in colony cages with free access to tap water and food (chow pellets) under standard laboratory conditions with a natural light/dark cycle. All experiments were performed between 9:00 a.m. and 2:00 p.m.

### 2.2. Electroconvulsions

Seizures were produced by means of an alternating current generator (Hugo Sachs Rodent Shocker, Type-221, Freiburg, Germany). The stimulus, of constant 0.2 s duration, was delivered via ear-clip electrodes. To evaluate the convulsive threshold ($CS_{50}$), at least four groups of mice were challenged with electric shocks of increasing intensity, until they produced tonic extension of the hind limbs (the endpoint in this test). Subsequently, an intensity-response curve was calculated on the basis of the percentage of the animals showing the endpoint. The $CS_{50}$ value represents the current strength (in mA) necessary to produce tonic hindlimb extension in 50% of mice.

### 2.3. Drugs

CGP 40116 [D-(*E*)-2-amino-4-methyl-5-phosphono-3-pentenoic acid] and CGP 43487 [D-(*E*)-2-amino-4-methyl-5-phosphono-3-pentenoic methyl ester], both generously supplied by Ciba-Geigy (Basel, Switzerland), were used as competitive NMDA receptor antagonists. Three $Ca^{2+}$ channel inhibitors were used throughout the study: nicardipine, nifedipine (both obtained from Sigma, St. Louis, MO, USA) and flunarizine (Polfa, Starogard, Poland). Sterile saline was used to bring CGP 40116 and CGP 43487 into solution. All $Ca^{2+}$ channel inhibitors were suspended in a 1% solution of Tween 81 (Loba Chemie, Vienna, Austria). Solutions of nicardipine and nifedipine were handled under dark conditions. The NMDA and $Ca^{2+}$ channel inhibitors were administered intraperitoneally (i.p.) 90 and 60 min before electroconvulsions, respectively. The injection volume was always 10 ml/kg body weight.

### 2.4. Chimney test

The chimney test of Boissier et al. (1960) was used to evaluate the influence of CGP 40116 or CGP 43487 alone or in combination with the $Ca^{2+}$ channel inhibitors on motor performance. Motor impairment in this test was indicated by the inability of the animals to climb backwards up the tube (3 cm inner diameter, 25 cm length) within 60 s. The animals were pretrained 24 h before treatment and those unable to perform the test were rejected from the experimental groups. On the following day, mice were treated with the compounds either alone or in combination. Results were calculated as a percentage of animals failing to perform the test.

### 2.5. Passive avoidance acquisition and retention testing

According to Venault et al. (1986), the step-through passive avoidance task may be used as a measure of long-term memory. We used this test to compare the influence of CGP 40116, CGP 43487, nicardipine, nifedipine, flunarizine alone or in combination (an NMDA receptor antagonist + a $Ca^{2+}$ channel inhibitor) on passive avoidance acquisition in mice. Procedural details have been published elsewhere (Borowicz et al., 1995). Shortly, mice avoiding the dark compartment for over 60 s (on the day after their entry of this compartment had been punished by an electric footshock of 0.8 mA for 2 s) showed no long-term memory impairment and were regarded as remembering the task. Retention was expressed as the percentage of mice with no memory impairment.

### 2.6. Statistics

$CS_{50}$ values (with 95% confidence limits) and their statistical analysis were calculated by computer probit analysis, according to Litchfield and Wilcoxon (1949). Additionally, the current strength-response curves evaluated in the drug-free group and groups treated with NMDA

receptor antagonists alone or combined with $Ca^{2+}$ channel inhibitors are presented in Fig. 2. The results obtained in the chimney and passive avoidance tests were compared statistically by using Fisher's exact probability test. A $P$ value of less than 0.05 was accepted as statistically significant.

## 3. Results

### 3.1. Effects of nicardipine, nifedipine and flunarizine upon the electroconvulsive threshold

Nicardipine (20 mg/kg) and nifedipine (20 mg/kg) significantly raised the convulsive threshold, increasing the $CS_{50}$ value from 6.2 (5.9–6.5; vehicle-treated group) to 7.6 (7.1–8.1) and 7.4 (6.9–7.9) mA, respectively. Similarly, flunarizine in doses of 15 and 20 mg/kg was reported to elevate the $CS_{50}$ value (Gasior et al., 1995b). Nicardipine, nifedipine and flunarizine did not affect the convulsive threshold at doses up to 15, 15 and 10 mg/kg, respectively (see inset in Fig. 1). Based on these results, maximal ineffective doses (or lower ones) of $Ca^{2+}$ channel inhibitors were selected for the following experiments with the NMDA receptor antagonists.

### 3.2. Influence of nicardipine, nifedipine and flunarizine on the convulsive threshold elevated by CGP 40116

CGP 40116 (0.25 and 0.5 mg/kg) significantly elevated the convulsive threshold. Specifically, the respective $CS_{50}$ values were 6.4 (6.0–6.7) and 7.5 (6.7–8.5) mA versus the control value of 5.6 (5.1–6.2) mA. CGP 40116, administered in the dose of 0.125 mg/kg, did not influence the convulsive threshold.

Administration of either nicardipine (15 mg/kg), nifedipine (3.75–15 mg/kg) or flunarizine (10 mg/kg) in combination with CGP 40116 (0.25 mg/kg) led to a considerable potentiation of the protective efficacy of this competitive NMDA receptor antagonist against electroconvulsions (Fig. 1a, significant elevation of the respective $CS_{50}$ values; Fig. 2A, shift of the current intensity-response curves to the right).

### 3.3. Effects of $Ca^{2+}$ channel inhibitors on the convulsive threshold elevated by CGP 43487

After administration of CGP 43487 (3.5 mg/kg) a significant elevation of the $CS_{50}$ value from 6.7 (6.3–7.1) to 7.7 (7.1–8.3) mA was found. No effect upon the

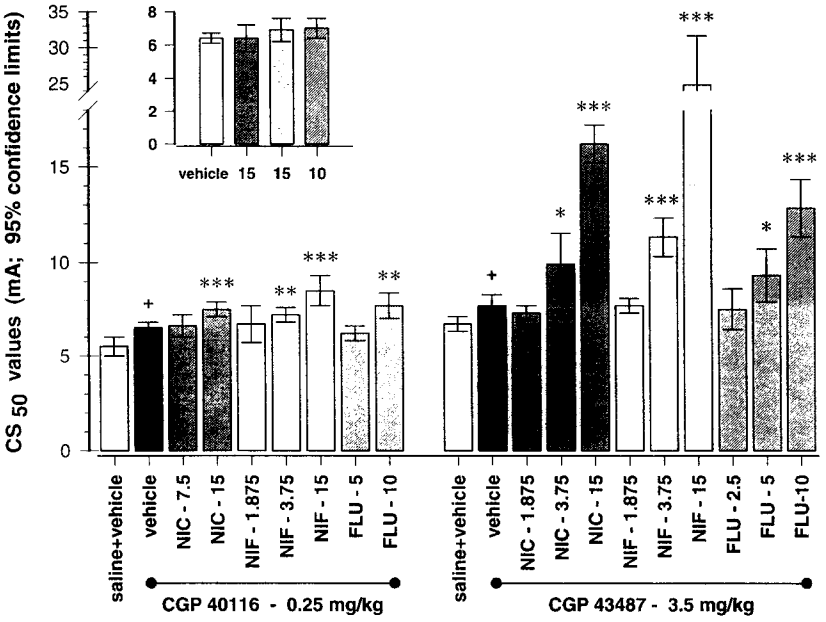


Fig. 1. Bars represent the convulsive threshold, expressed as the $CS_{50}$ value in mA. Error bars show 95% confidence limits of the $CS_{50}$ values. The $CS_{50}$ values evaluated in (saline + vehicle)-treated group are illustrated by the open bars. The solid bars refer to the convulsive threshold evaluated in mice pretreated with CGP 40116 (0.25 mg/kg) or CGP 43487 (3.5 mg/kg) alone 90 min prior to the electroconvulsive test. The shaded bars represent the $CS_{50}$ values assessed in groups co-administered one of the NMDA receptor antagonists and one of the $Ca^{2+}$ channel inhibitors (60 min before the test). All compounds were injected i.p. in a single dose. The doses of nicardipine, nifedipine and flunarizine are shown below each bar in mg/kg. The inset is a graph of the $CS_{50}$ values evaluated in the vehicle group (open bar) and in the groups pretreated with nicardipine (15 mg/kg), nifedipine (15 mg/kg) and flunarizine (10 mg/kg) alone, respectively. The calculation of the $CS_{50}$ values and statistical analysis were performed according to Litchfield and Wilcoxon (1949). $^{+}$ $P < 0.05$ vs. (saline + vehicle)-treated group; $^{*}$ $P < 0.05$; $^{**}$ $P < 0.01$; $^{***}$ $P < 0.001$ vs. the group treated with either CGP 40116 or CGP 43487 alone, respectively. Abbreviations: NIC, nicardipine; NIF, nifedipine; FLU, flunarizine.

convulsive threshold was noted when the competitive NMDA receptor antagonist was given at 3.0 mg/kg.

All three $Ca^{2+}$ channel inhibitors, nicardipine (3.75–15 mg/kg), nifedipine (3.75–15 mg/kg) and flunarizine (5–10 mg/kg), in a dose-dependent manner, markedly potentiated the protective efficacy of CGP 43487 (3.5 mg/kg) against electroconvulsions (Fig. 1). The rightward shift of the current strength-response curves is shown on Fig. 2B.

### 3.4. Chimney test

Administration of CGP 40116 or CGP 43487, at their effective doses against electroconvulsions (0.25 and 3.5 mg/kg, respectively), caused a significant motor impairment only in the case of CGP 43487 (in 33.3% of mice). Nicardipine (15 mg/kg), nifedipine (15 mg/kg) and flunarizine (10 mg/kg) did not affect the motor performance of mice (Table 1). Co-administration of the $Ca^{2+}$ channel inhibitors in the doses listed above and either CGP 40116 (0.25 mg/kg) or CGP 43487 (3.5 mg/kg) did not worsen the performance of mice when compared to that induced by NMDA receptor antagonists alone (Table 1). Generally, however, the combined treatment produced a significant motor impairment in comparison with the vehicle + saline group (Table 1).

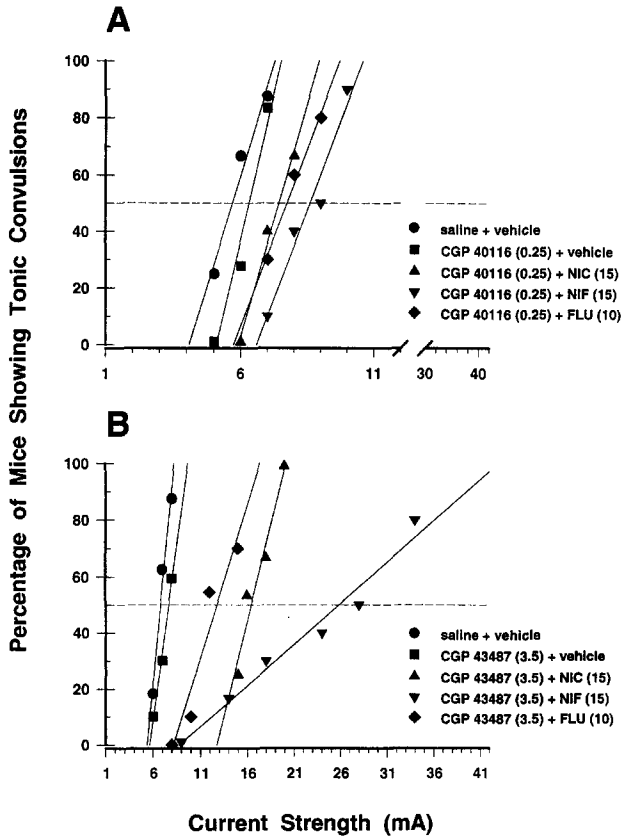


Fig. 2. The current strength (mA)-response (%) plot with computed linear regressions (solid lines) evaluated in (saline + vehicle)-treated mice (●) or mice pretreated with either CGP 40116 (■, upper panel), CGP 43487 (■, lower panel) alone or in combination with nicardipine (▲), nifedipine (▼) and flunarizine (◆). CGP 40116 (A) and CGP 43487 (B) were administered i.p. in the doses of 0.25 and 3.5 mg/kg, respectively. Nicardipine, nifedipine and flunarizine were injected in the doses of 15, 15 and 10 mg/kg, respectively. The points of interception at the dashed lines reflect the current strength (in mA) required to produce tonic hindlimb extension in 50% of mice tested. See also legend of Fig. 1 for treatment times and abbreviations.

Table 1
Effect of CGP 40116, CGP 43487, nicardipine, nifedipine and flunarizine alone or in combination on motor performance and on retention of a passive avoidance task by mice

| Treatment (mg/kg) | Motor impairment (%) | Retention (%) |
|---|---|---|
| Saline + vehicle | 0 | 100 |
| NIC (15) + saline | 25 | 50 [a] |
| NIC (15) + saline | 25 | 41.6 [a] |
| FLU (10) + saline | 8.3 | 75 |
| CGP 40116 (0.25) + vehicle | 25 | 58.3 [a] |
| CGP 40116 (0.25) + NIC (15) | 33.3 [a] | 58.3 [a] |
| CGP 40116 (0.25) + NIF (15) | 50 [a] | 33.3 [a] |
| CGP 40116 (0.25) + FLU (10) | 33.3 [a] | 58.3 [a] |
| CGP 43487 (3.5) + vehicle | 33.3 [a] | 33.3 [a] |
| CGP 43487 (3.5) + NIC (15) | 41.6 [a] | 16.6 [a] |
| CGP 43487 (3.5) + NIF (15) | 66.6 [a] | 8.3 [a] |
| CGP 43487 (3.5) + FLU (10) | 25 | 25 [a] |

The results are expressed as a percentage of animals showing motor impairment in the Chimney test and no long-term memory deficit in the passive avoidance task, respectively (see Materials and methods). [a] $P$ at least $< 0.05$ vs. saline + vehicle-treated group, Fisher's exact probability test. Each group consisted of 12 animals. Abbreviations: NIC: nicardipine; NIF: nifedipine; FLU: flunarizine. See also legends of Fig. 1 and Fig. 2.

### 3.5. Dark avoidance task

CGP 40116 (0.25 mg/kg) and CGP 43487 (3.5 mg/kg) worsened long-term memory, since 58.3% and 33.3% of mice tested, respectively, correctly performed the task. A similar trend was observed in the case of nicardipine (a 50% impairment of retention) and nifedipine (a retention of 41.6%) administered alone, each in the dose of 15 mg/kg. Co-administration of nicardipine (15 mg/kg), nifedipine (15 mg/kg) or flunarizine (10 mg/kg) with CGP 40116 (0.25 mg/kg) or CGP 43487 (3.5 mg/kg) resulted in a similar impairment of long-term memory to that caused by the NMDA receptor antagonists alone (Table 1). However, there was a deficit of long-term memory upon the combined treatment of NMDA receptor antagonists and $Ca^{2+}$ channel inhibitors compared to that of the vehicle + saline group (Table 1).

## 4. Discussion

The most important finding of this study is that the anticonvulsant efficacy of two competitive NMDA receptor antagonists, CGP 40116 and CGP 43487, was markedly enhanced by nicardipine, nifedipine and flunarizine against

electroconvulsions in mice. Each $Ca^{2+}$ channel inhibitor (at subprotective doses) co-administered with CGP 40116 or CGP 43487 caused a rightward shift of the current strength-response curve which resulted in significant increases in the respective $CS_{50}$ values. The most marked quantitative effect was observed when CGP 43487 (3.5 mg/kg) was combined with nicardipine (15 mg/kg), nifedipine (15 mg/kg), and flunarizine (10 mg/kg) – the respective increases in the convulsive threshold were 216%, 330% and 166%. Further, the motor performance of mice after the combined treatment was similar to that after administration of the NMDA receptor antagonists alone. In the passive-avoidance test, all compounds (except flunarizine) produced a significant long-term memory deficit compared with the drug-free group. Co-administration of $Ca^{2+}$ channel inhibitors did not worsen long-term memory when compared to the retention observed after NMDA receptor antagonists alone. The combined treatment, however, in most cases resulted in motor and memory deficits in comparison with the untreated controls. It is noteworthy that agents affecting different neurotransmitter systems (atropine, γ-hydroxybutyric acid, γ-butyrolactone, and baclofen) were also found to potentiate the protective activity of a competitive NMDA receptor antagonist, 2-amino-5-phosphonopentanoic acid. Muscimol, however, was completely ineffective in this respect (Czuczwar et al., 1983). Also, simultaneous blockade of more than one subtype of glutamate receptor (e.g. NMDA and non-NMDA) resulted in a more pronounced anticonvulsive effect when compared to the effect of blockade of an individual receptor subtype (Czuczwar et al., 1995).

As mentioned in the Introduction, it is very likely that enhanced $Ca^{2+}$ currents, due to enhanced excitatory neurotransmitter release, may trigger seizure activity. Therefore, a reduction in neuronal $Ca^{2+}$ influx produces an antiepileptic effect. This concept is supported by experimental findings showing that the anticonvulsive actions of phenobarbital, carbamazepine, valproate, ethosuximide and diphenylhydantoin are, at least partially, due to their inhibition of $Ca^{2+}$ influx (see discussions in Rogawski and Porter, 1990; Gasior et al., 1995b). This mode of action is also believed to account for the anticonvulsive activity of voltage-operated $Ca^{2+}$ channel inhibitors (Faingold, 1992; Speckmann et al., 1993). The exact nature of the interaction between $Ca^{2+}$ channel inhibitors and NMDA receptor antagonists is not clear. We cannot totally exclude a pharmacokinetic interaction, because the plasma levels of the compounds studied were not measured. In our opinion, the pharmacokinetic mechanisms do not seem probable because the adverse effects of NMDA receptor antagonists were not significantly affected by the combined treatment. Studies performed with $Ca^{2+}$ channel inhibitors and currently available antiepileptic drugs (Czuczwar et al., 1990a,b, 1992) indicate that, generally, a pharmacokinetic interaction is not responsible for the $Ca^{2+}$ channel inhibitor-induced potentiation of the protective activity of conventional antiepileptics. Only nifedipine increased the total plasma levels of phenobarbital (by 14%) or valproate (by 43%) and nicardipine increased the plasma levels of carbamazepine by 87% (Czuczwar et al., 1990b, 1992). Nifedipine did not affect the total plasma levels of carbamazepine, diphenylhydantoin, ethosuximide, phenobarbital, or valproate (Czuczwar et al., 1990a,b) and flunarizine did not affect those of carbamazepine, diphenylhydantoin, or valproate (Czuczwar et al., 1992).

It has been shown that the $Ca^{2+}$ channel agonist, BAY k-8644, exclusively impairs the anticonvulsant action of competitive NMDA receptor antagonists (CGP 37849 and D-CPP-ene) and is completely ineffective in the case of non-competitive NMDA (MK-801) and non-NMDA (NBQX and GYKI 52466) receptor antagonists against electroconvulsions in mice (Czuczwar et al., 1994). These findings could suggest that the potentiation of the anticonvulsant efficacy of CGP 40116 and CGP 43487 was due to the centrally mediated actions of $Ca^{2+}$ channel inhibitors. Similarly, the anticonvulsive efficacy of conventional antiepileptics was enhanced by centrally active $Ca^{2+}$ channel inhibitors, but not by verapamil (Czuczwar et al., 1990a,b) whose penetration into the brain is limited (Hamann et al., 1983). Epileptiform activity is a sequence of events which may be triggered by excitatory amino acids (Hayashi, 1954; Bradford and Peterson, 1987; Meldrum, 1991). First, L-glutamate activates the NMDA receptor/channel complex, which results in $Ca^{2+}$ ion entry through receptor-operated channels following initial depolarization (Mayer and Miller, 1990). Further, the NMDA-activated depolarization spreads to the voltage-operated $Ca^{2+}$ channels and, finally, leads to their activation and a subsequent large $Ca^{2+}$ influx (Courtney et al., 1990; Mayer and Miller, 1990). Therefore, NMDA receptor antagonists may attenuate $Ca^{2+}$ influx either directly through NMDA receptors or indirectly via voltage-operated $Ca^{2+}$ channels. By analogy, similar 'cross-talk' may be expected in the case of $Ca^{2+}$ channel inhibitors. This interpretation might explain the anticonvulsive activity of $Ca^{2+}$ channel inhibitors per se and in combination with CGP 40116 or CGP 43487. There is also evidence that some $Ca^{2+}$ channel inhibitors directly bind to the NMDA receptor complex and produce a specific block of its function (Hashim et al., 1988; Skeen et al., 1993). However, with only few exceptions, there is little experimental support for this hypothesis. Moreover, the presynaptic prevention of excitatory amino acid release by $Ca^{2+}$ channel inhibitors cannot be completely ruled out (De Sarro et al., 1988; Janis and Triggle, 1991). The fact that nicardipine, nifedipine and flunarizine enhanced the anticonvulsant action of CGP 40116 and CGP 43487 in a similar fashion may point out that it is $Ca^{2+}$ channel blockade which is responsible for this effect. Flunarizine, apart from this mechanism of action, may also block sodium conductances (Ashton and Wauquier, 1986) and this effect could be also involved.

Unfortunately, the potential antiepileptic utility of

NMDA receptor antagonists is limited by their side-effects (Schmidt, 1994; Witkin, 1995). In fact, the marked potentiation of the protective action of CGP 40116 or CGP 43487 by $Ca^{2+}$ inhibitors was accompanied by behavioral impairment. However, the novel strategy ($Ca^{2+}$ channel inhibitors + NMDA receptor antagonists) for the treatment of epilepsy should not be immediately discarded since more specific and less toxic compounds may be soon available. Whether such a polypharmacological approach might be useful in the treatment of other neurological disorders is a question for further studies.

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