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Involvement of Spinal NMDA Receptors in Capsaicin-Induced Nociception

TSUKASA SAKURADA,* KENJI WAKO,† AKINORI SUGIYAMA,† CHIKAI SAKURADA,* KOICHI TAN-NO† AND KENSUKE KISARA†

*Department of Biochemistry, Daiichi College of Pharmaceutical Sciences, 22-1 Tamagawa-cho, Minami-ku, Fukuoka 815, Japan, and
†Department of Pharmacology, Tohoku College of Pharmacy, 4-4-1 Komatsushima, Aoba-ku, Sendai 981, Japan

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SAKURADA T., K. WAKO, A. SUGIYAMA, C. SAKURADA, K. TAN-NO AND K. KISARA. *Involvement of spinal NMDA receptors in capsaicin-induced nociception.* PHARMACOL BIOCHEM BEHAV **59**(2) 339–345, 1998.—Intraplantar injection of capsaicin into the mouse hindpaw produced an acute nociceptive response. The involvement of *N*-methyl-D-aspartate (NMDA) receptors was examined by intrathecal administration of various excitatory amino acid (EAA) receptor antagonists. The selective and competitive NMDA receptor antagonists, D(−)-2-amino-5-phosphono-valeric acid (APV) and (±)-3-(2-carboxypiperazin-4-yl) propyl-1-phosphoric acid (CPP), were most potent in inhibiting the nociceptive response induced by capsaicin (ED_{50}, 0.23 nmol and 0.12 nmol). The noncompetitive NMDA receptor antagonist dizocilpine (MK-801) and the non-NMDA antagonist, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) had similar effects on the capsaicin-induced nociception (ED_{50}, 2.90 and 7.98 nmol), while ketamine and 7-chlorokynurenic acid were without effect. Ifenprodil, an antagonist at the receptor-coupled polyamine site, showed a significant reduction of the nociceptive response (ED_{50}, 13.8 nmol). The inhibitory effects of APV, CPP, MK-801, and ifenprodil were reversed by co-administration of NMDA. Coadministration of α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) or kainate resulted in a marked reduction of CNQX-induced antinociception. The present results suggest that the NMDA receptor plays a key role in spinal nociceptive processing as measured by the capsaicin test in mice. This nociceptive test may be useful for evaluating competitive NMDA antagonists. © 1998 Elsevier Science Inc.

NMDA receptors NMDA receptor antagonists Antinociception Capsaicin Intrathecal injection Mouse spinal cord

THERE is evidence that primary afferent fibers utilize the excitatory amino acids (EAA), glutamate (Glu) and aspartate (Asp) as their neurotransmitters. Receptors for EAAs have been classified into at least three different types according to their specific affinity for selective agonists; *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA), and kainic acid. EAAs appear to participate in spinal nociceptive mechanisms. Application of EAA receptor agonists elicits hyperalgesia (2) and selective EAA receptor antagonists have been shown to produce antinociception (7, 32). Electrophysiological studies have shown that receptors of EAAs may be involved in the excitation of a proportion of both superficial and deep dorsal horn neurons (3,16,19). The presence of Glu in small-diameter dorsal root ganglion neurons (5), and in terminals in the superficial spinal cord dorsal horn (14,31) was demonstrated immunohistochemically. Glu and substance P have been also found to coexist in the terminals of primary sensory afferents (5). Behavioral studies have indicated that intrathecal (IT) administration of NMDA or non-NMDA receptor agonists produces biting and scratching behavior resembling that of substance P (1,35). The NMDA receptor antagonist has been shown to be effective in relieving hyperalgesia in a model of peripheral neuropathy (29,44) and in abolishing tactile allodynia induced by IT injection of a glycine antagonist, strychnine (45). Several authors have shown release of both substance P and neurokinin A by injection of capsaicin (17,18,23).

We previously reported the capsaicin test as a behavioral model for evaluating tachykinin antagonists in the mouse spinal cord. Subcutaneous injection of dilute capsaicin into a

Requests for reprints should be addressed to Tsukasa Sakurada, Department of Biochemistry, Daiichi College of Pharmaceutical Sciences, 22-1 Tamagawa-cho, Minami-ku, Fukuoka 815, Japan.

mouse's hindpaw produces a short-lasting nociceptive response (36). It has been demonstrated that IT administration of CP-96,345, an NK_1 receptor antagonist, inhibits the capsaicin-induced paw licking response (37). Glu and Asp are released within the spinal cord following formalin injection into the hindlimb and after IT administration of substance P (39, 40). In addition, capsaicin elicits a significant release of Glu from the dorsal spinal cord in vivo (41) and in vitro (26,42).

To determine whether EAA receptor antagonists have different roles in capsaicin-induced nociception, the antinociceptive effect of antagonists with different selectivities of action at EAA receptor subtypes was investigated when administered IT into mice.

The experiments were performed with the approval of the Committee of Animal Experiments in Tohoku College of Pharmacy.

METHOD

The study included 410 male ddY mice (Shizuoka Laboratory Center, Japan), body weight 22–25 g at the outset. Animals were housed in a temperature (22 ± 24°C)- and humidity (60–70%)-controlled room illuminated on a 12 L:12 D cycle. Food (Clea Japan, Inc., Osaka, Japan) and water were provided ad lib throughout the course of the study.

Intrathecal Injection

The procedure of IT administration of drugs was based on the technique described by Hylden and Wilcox (25). Briefly, the intervertebral (between L5 and L6) space was punctured directly using a 28-gauge needle attached to a 50-μl Hamilton microsyringe. The mice were not anesthetized during these procedures.

Capsaicin Test

The capsaicin test was performed as described in detail previously (36). The mice were placed in individual cages (22.0 × 15.0 × 12.5 cm), which served as observation chambers after intraplantar injection of capsaicin. To reduce variability they were habituated to this environment for at least 1 h prior to injection of capsaicin. A volume of 20 μl capsaicin (1600 ng) solution was injected subcutaneously into the dorsal surface of the right hindpaw. The amount of time spent licking the injected paw in the 5 min after the capsaicin injection was measured and expressed as the cumulative licking time. The measurement of the behavioral responses was performed blind, i.e., the observer had no information as to group designation.

Drugs

The following drugs were used: D(−)-2-amino-5-phosphonovaleric acid (APV), (±)-3(2-carboxypiperazin-4-yl)-propyl-1-phosphoric acid (CPP), (5R,10S)-(±)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclo-hepten-5,10-imine hydrogen maleate (MK-801), ketamine hydrochloride, 7-chlorokynurenic acid (7-Cl-Kyn), and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (Research Biochemical Incorporated, Natick, MA), ifenprodil and AMPA (Sigma, St. Louis, MO), NMDA, kainate (Nacalai Tesque, Kyoto, Japan), and capsaicin (Merck, West Point, PA). Capsaicin was dissolved in 60% dimethylsulfoxide (DMSO) for preparing concentrated stock solutions and working solutions were then diluted in saline, in a step-wise fashion. Final concentration of capsaicin used in the present study was 1600 ng/7.5% DMSO 20 μl. 7-Cl-Kyn, ifenprodil, and CNQX were dissolved in 1:1 mixture of DMSO in artificial cerebrospinal fluid (CSF), and the other drugs were dissolved in artificial CSF. All EAA receptor antagonists were given IT 5 min prior to testing as an IT injection except in the time course experiment. All IT antagonists were given in a volume of 5 μl. NMDA, AMPA, or kainate in combination with each antagonist was also coadministered IT in a total volume of 5 μl.

Calculations of ID_{50} and Data Analysis

Results are presented as the mean ± SEM. ID_{50} values with 95% confidence limits were determined for reduction in capsaicin-induced nociceptive response by the method of Litchfield and Wilcoxon (27). Statistical evaluations were performed using multiple analysis of variance (ANOVA). Significant differences between groups were determined by Dunnett's test when various EAA receptor antagonists were given in a single injection. The results obtained by coadministration were evaluated by Tukey's test. Statistical significance was set at $p < 0.05$.

RESULTS

Antagonists Given IT in the Mouse Capsaicin Model

The subcutaneous injection of capsaicin (1600 ng) into the dorsal surface of the hindpaw produced a licking response toward the injected paw, as previously reported (36). This characteristic behavior appeared immediately, peaked at 0–5 min, and then decayed. The antinociceptive effects of the EAA receptor antagonists were evaluated 5 min, 15 min, and 30 min after IT injection in the capsaicin test. From the time-course examination of the antinociceptive effect of EAA antagonists used (Table 1), it is clear that the maximal effect occurred at 5 min after IT injection. Therefore, in further experiments, each antagonist was injected 5 min before intraplantar injection of capsaicin.

The competitive receptor antagonists, APV, and CPP, reduced capsaicin-induced licking in a dose-dependent manner (Fig. 1A, and B). The ID_{50} values for APV and CPP were 0.23

TABLE 1

THE TIME COURSE OF THE ANTINOCICEPTIVE EFFECT OF INTRATHECALLY ADMINISTERED EXCITATORY AMINO ACID ANTAGONISTS IN THE CAPSAICIN TEST

		Time After Injection		
Dose (nmol)		5 min	15 min	30 min
CSF control		63.6 ± 2.0	69.9 ± 4.1	72.4 ± 6.3
APV	1.0	25.8 ± 2.0*	29.9 ± 4.3*	41.6 ± 4.0*
CPP	0.5	18.8 ± 1.9*	28.9 ± 4.8*	33.6 ± 5.0*
MK-801	5.0	21.7 ± 4.3*	35.1 ± 4.9*	47.3 ± 2.5*
Vehicle control		58.7 ± 2.3	65.2 ± 2.7	69.7 ± 3.9
CNQX	20.0	20.0 ± 1.3‡	37.1 ± 4.0‡	49.4 ± 2.8†
Ifenprodil	20.0	19.7 ± 3.4‡	35.2 ± 4.3‡	38.4 ± 3.8‡

Each antagonist was injected intrathecally 5 min, 15 min, and 30 min before intraplantar injection of capsaicin. The duration of licking induced by capsaicin was determined using the 5-min period starting immediately after capsaicin injection. The CSF- or vehicle-control value was compared with the effect of excitatory amino acid antagonists at the designated times. The data are given as the mean ± SEM for groups of 10 mice. *$p < 0.01$ when compared to CSF-controls. †$p < 0.05$, ‡$p < 0.01$ when compared to vehicle controls.

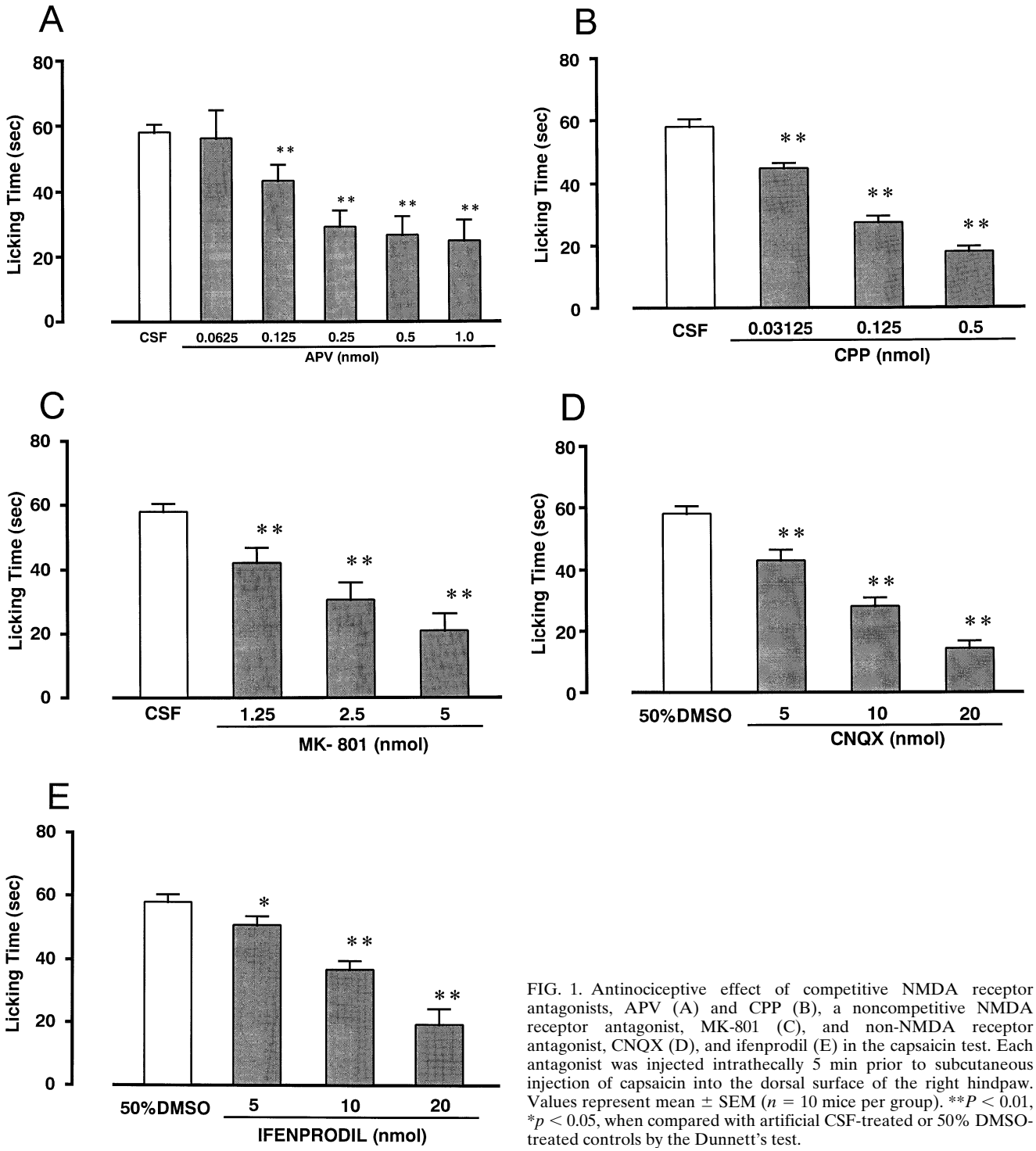

FIG. 1. Antinociceptive effect of competitive NMDA receptor antagonists, APV (A) and CPP (B), a noncompetitive NMDA receptor antagonist, MK-801 (C), and non-NMDA receptor antagonist, CNQX (D), and ifenprodil (E) in the capsaicin test. Each antagonist was injected intrathecally 5 min prior to subcutaneous injection of capsaicin into the dorsal surface of the right hindpaw. Values represent mean ± SEM ($n = 10$ mice per group). $^{**}P < 0.01$, $^{*}p < 0.05$, when compared with artificial CSF-treated or 50% DMSO-treated controls by the Dunnett's test.

nmol and 0.12 nmol, respectively (Table 2). MK-801 also dose dependently reduced the capsaicin-induced nociceptive response with an ID_{50} of 2.90 nmol (Fig. 1C, Table 1). Ketamine (40 nmol) and 7-Cl-Kyn (20 nmol) had no substantial effect on the capsaicin-induced nociception (Table 2). Capsaicin-induced nociception was significantly reduced by the AMPA receptor antagonist CNQX, and ifenprodil, an antagonist at the receptor coupled polyamine site, was antinociceptive in a dose-dependent manner (Fig. 1D, and E). The ID_{50} values for CNQX and ifenprodil were 7.98 and 13.8 nmol, respectively (Table 2). Drugs in doses used in the present experiment gave no hind-leg muscle flaccidity, except for 7-Cl-Kyn (20 nmol), which caused a slightly impaired walking. 7-Cl-Kyn at a dose of 40 nmol produced a marked muscle flaccidity that lasted for 60 min.

TABLE 2

ANTINOCICEPTIVE EFFECT OF INTRATHECALLY ADMINISTERED EXCITATORY AMINO ACID ANTAGONISTS IN THE CAPSAICIN TEST

Drugs	ID_{50}
APV	0.23 (0.18–0.29)
CPP	0.12 (0.04–0.35)
MK-801	2.90 (1.42–5.92)
Ketamine	>40.0
7-Cl-Kyn	>20.0
CNQX	7.98 (5.83–16.4)
Ifenprodil	13.8 (9.07–21.0)

Values in parentheses are 95% confidence limits. Each antagonist was injected intrathecally 5 min prior to intraplantar injection of capsaicin. A minimum of three doses with 10 animals per dose was used to construct dose-response curves.

Coadministration Studies with the Capsaicin Model

Experiments with combinations of EAA agonists and antagonists were performed to identify the effects of EAA receptor antagonists by combination with a selective receptor agonist, NMDA, AMPA, or kainate. In these experiments, a single 5 μl injection containing a mixture of the two agents was given. The antinociceptive effects of APV, CPP, MK-801, and ifenprodil were reversed almost completely by coadministration of NMDA (0.2 nmol) (Fig. 2A, B, C, and E). CNQX-induced antinociception was reversed readily by coadministration of AMPA (0.01 nmol) or kainate (0.005 nmol) (Fig. 2D). A significant antagonistic effect on CNQX-induced antinociception was also seen by coadministration of NMDA. Single administrations of NMDA (0.2 nmol), AMPA (0.01 nmol), or kainate (0.005 nmol) used in coadministration studies gave no significant effect of capsaicin-induced nociception.

DISCUSSION

In a recent report, we showed that the capsaicin test involving peripheral nociception, which produces behavior similar to that elicited by formalin, may be a more suitable method for evaluating the antinociceptive effect of tachykinin antagonists in the mouse spinal cord than the formalin test (36). The difference between the capsaicin- and formalin-induced behavioral response is that the nociceptive response induced by capsaicin evokes only an early phase nociception without a late phase response. The results resemble those reported in rats following intraplantar injection of capsaicin (20); capsaicin evoked nocifensive behavior in rats characterized by lifting and guarding the injected paw, which was of relatively short duration. It is, therefore, apparent that subcutaneous injection of capsaicin into the dorsum of the hindpaw of mice and rats evokes only a single phase of nociceptive behavior.

The present study examined the antinociceptive effects of various EAA antagonists on capsaicin-induced nociception in mice. Several studies have provided evidence that the NMDA receptor mediates nociceptive response to prolonged C-fiber stimulation (13,16,21,43), but not acute nociceptive behavioural responses such as tail-flick or hot-plate latencies (2,7). The primary finding in the present study is that competitive NMDA receptor antagonists, APV and CPP, were the most potent of all antagonists tested in reducing the nociceptive response in the capsaicin test. These results are in marked contrast to a number of previous reports that the early acute response in the formalin test is not affected by NMDA antagonists (12,21,44). Acute nociception induced by intraplantar capsaicin was markedly but not completely inhibited by APV at a dose that almost abolished the behavioural response elicited by IT NMDA (35). This implies that the capsaicin-induced nociceptive response may not only be mediated by the release of Glu and Asp but also other neurotransmitters or neuromodulators, possibly the release of substance P. Recently, it has been reported that Glu and Asp are both released into the extracellular space of the dorsal horn of the lumbar spinal cord following capsaicin injection into the hindlimb (41). In addition, capsaicin evoked the release of Glu from rat primary afferent neurons in vitro (26) and from rat spinal dorsal horn slices (42). Antinociception induced by the selective NMDA antagonist APV was reversed drastically by coadministration of NMDA in the capsaicin test, in contrast to coadministered AMPA and kainate, suggesting that the antinociceptive effect of APV is mediated by the NMDA receptors in the spinal cord. Similarly, the antinociceptive response of CPP was antagonized markedly by coadministration of NMDA in the capsaicin test. However, CPP-induced antinociception was reversed slightly but significantly by coadministration of AMPA or kainate. This partial antagonism by AMPA may be explained by the previous report that CPP at a relatively high concentration inhibited [^{3}H]AMPA binding to rat cortical membranes (22).

IT treatment with MK-801, a noncompetitive NMDA receptor antagonist, has been reported to reduce thermal hyperalgesia in rats following chronic constrictive nerve injury (29,38). However, MK-801 did not produce a significant reduction in nociceptive responses during the early phase of the formalin test in mice and rats (12,32). Electrophysiological data also showed that intravenous MK-801 produced a marked inhibition of dorsal horn activity in the late phase of the formalin-induced response, but only small nonsignificant effects on activity during the early phase (21). In the present study, IT MK-801 produced a dose-dependent antinociceptive effect, which was much less potent than the competitive antagonists, APV and CPP. Taken together, it is suggested that the nociceptive mechanisms of capsaicin is different from those of formalin-induced early phase nociceptive responses in the spinal cord level. The antinociceptive effect of MK-801 was antagonized by coadministration of NMDA, but not by AMPA or kainate. The coadministration study suggests that antinociception induced by IT MK-801 may be produced through the channel site linked with the NMDA receptors. Ketamine has been reported to eliminate the hyperalgesic state induced by a single unilateral injection of carrageenan and peripheral nerve injury (10,28,34). However, IT-injected ketamine was not found to affect acute nociceptive behavioral response as assayed by the tail-flick and hot-plate tests (32), which would support our data that ketamine did not produce antinociceptive effects in the capsaicin test. Very recent behavioral evidence has also shown that ketamine was without affecting formalin hyperalgesia seen in the late phase (10 to 40 min) of formalin-induced nociceptive response (10).

Recently, polyamines, such as spermidine and spermine, have been found to increase the specific binding of noncompetitive antagonists with the ion channel of the NMDA receptor complex (33). The site of action could be different from the Glu recognition site and the glycine modulatory site (33).

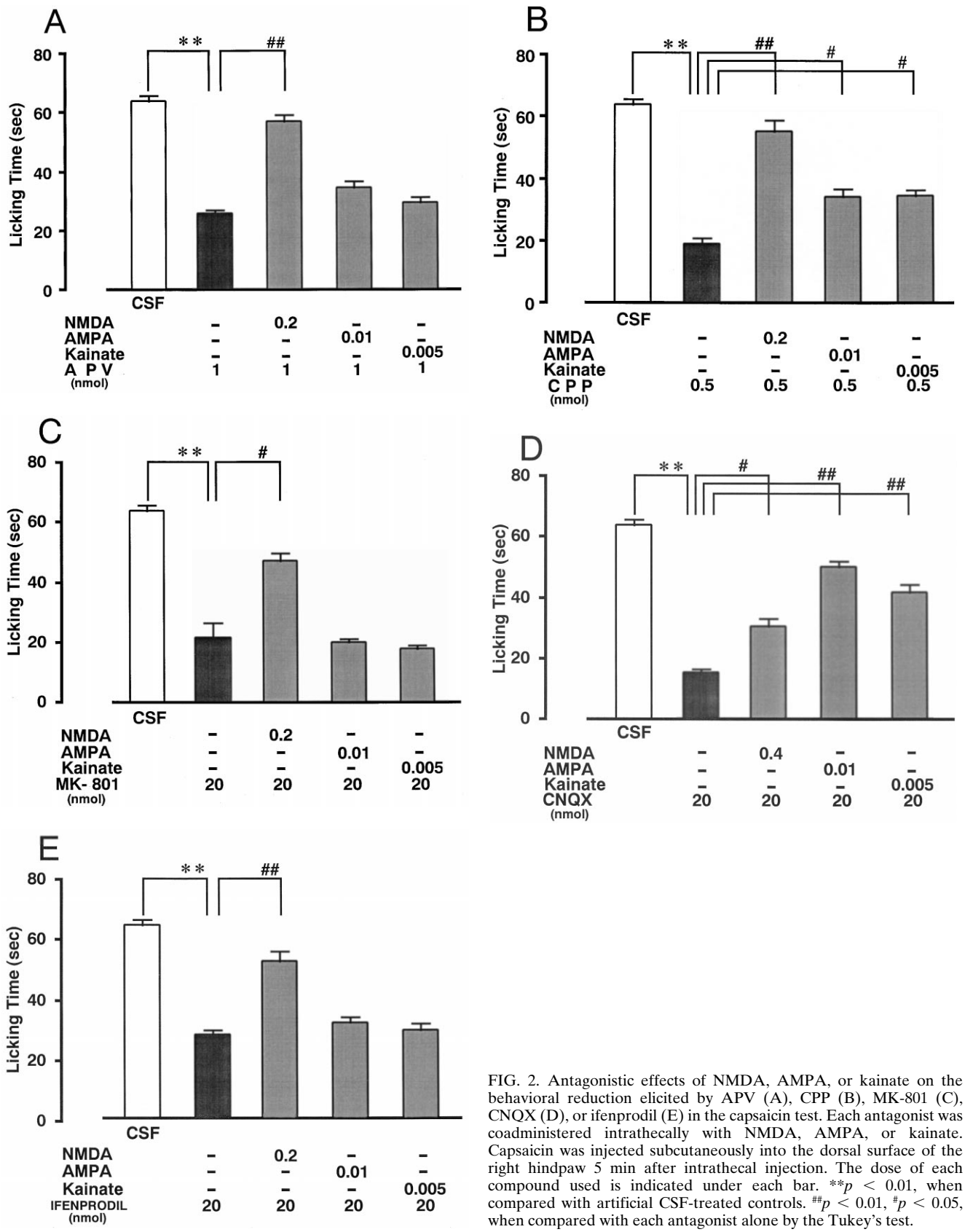

FIG. 2. Antagonistic effects of NMDA, AMPA, or kainate on the behavioral reduction elicited by APV (A), CPP (B), MK-801 (C), CNQX (D), or ifenprodil (E) in the capsaicin test. Each antagonist was coadministered intrathecally with NMDA, AMPA, or kainate. Capsaicin was injected subcutaneously into the dorsal surface of the right hindpaw 5 min after intrathecal injection. The dose of each compound used is indicated under each bar. $^{**}p < 0.01$, when compared with artificial CSF-treated controls. $^{\#\#}p < 0.01$, $^{\#}p < 0.05$, when compared with each antagonist alone by the Tukey's test.

There is evidence that ifenprodil is a noncompetitive NMDA antagonist that acts selectively at the polyamine recognition site on the NMDA receptor (8,9). In the present study, IT-administered ifenprodil inhibited the nociceptive response to capsaicin in a dose-dependent manner, an inhibition that was antagonized by coadministration of NMDA. This result suggests that antinociception induced by IT ifenprodil may be due to an inhibition of polyamine stimulation of binding of NMDA receptor ligands to the complex. IT injection of 7-Cl-Kyn, a glycine site antagonist, did not affect the nociceptive response to capsaicin. The antinociceptive effects of the two glycine site antagonists 7-Cl-Kybn and HA-966, injected IT, are much less than the competitive NMDA antagonists, APV and CPP, in the hot-plate, tail-flick, and early phase-formalin tests (32). Recent studies have demonstrated that the second phase of the formalin response is reduced by subcutaneous injection of HA-966, a partial glycine receptor agonist (11,15, 30). Therefore, it seems unlikely that the glycine site of the NMDA receptor complex could contribute to spinal sensitization following subcutaneous capsaicin injection, as well as acute nociceptive responses. CNQX was originally considered to be a potent and competitive antagonist at non-NMDA EAA receptors (22), particularly at AMPA binding sites, rather than kainate binding sites. However, CNQX also acts at the strychnine-insensitive glycine site of the NMDA receptor (6). Antinociceptive effects were reported earlier for IT-injected CNQX in the several nociceptive assays in mice (32), but not in the rat formalin test (12). However, the AMPA receptor selective antagonist NBQX has recently been shown to have a positive effect on acute phase of formalin response (24). Much higher doses of CNQX were produced antinociceptive effects on the capsaicin-induced nociceptive response. There is an interesting report to note that low concentrations of selective AMPA/kainate receptor antagonists, CNQX and DNQX, can be selective for acute nociceptive transmission in the rat isolated spinal cord-tail preparation, while these antagonists at higher concentrations did not have selectivity for capsaicin-induced nociceptive responses (4). In addition, blockade of the NMDA-induced response by CNQX has also been reported to be noncompetitive in dissociated dorsal horn neurons of the rat, although the quisqualate and kainate responses were competitively blocked by CNQX (46). CNQX produces an unsurmountable antagonism of responses to NMDA in the hemisected rat spinal cord, and D-serine and glycine can selectively reduce this antagonism (6). In the present study, antinociception induced by CNQX at a high dose (20.0 nmol) was reversed significantly by coadministration of AMPA and kainate. This is consistent with the radioligand binding data that CNQX is most effective at [^{3}H]AMPA binding sites, effective at [^{3}H]kainate binding sites, and very weak at [^{3}H]CPP binding sites (22). On the other hand, NMDA enhances formalin-nociceptive responses by coadministration of AMPA, which had no effect alone (12). Thus, it is evident that there may be an important interactive effect of agonists and antagonists acting at NMDA and AMPA/kainate receptors.

In conclusion, this study suggests that NMDA receptors have a more pronounced role than AMPA/kainate receptors in the spinal processing of capsaicin-induced nociception. Antinociception elicited by APV, CPP, MK-801, and ifenprodil was blocked by coadministration of NMDA, suggesting that the antinociceptive response of these NMDA antagonists is mediated by the NMDA receptor in the mouse spinal cord. It remains unproven what accounts for the wide range of efficacies among NMDA receptor antagonists.

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