Involvement of Spinal Substance P and Excitatory Amino Acids in Inflammatory Hyperalgesia in Rats

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ABSTRACT—To reveal possible involvement of NK-1 substance P receptors and *N*-methyl-D-aspartate (NMDA) and non-NMDA glutamate receptors in the production of inflammatory hyperalgesia, we examined the effects of intrathecal injections of antagonists at those receptors on the nociceptive threshold of inflammatory hyperalgesic rats in the paw-pressure test. Intrathecal injections of the NK-1 antagonist CP-96,345 (0.3-3 nmol/rat), the NMDA antagonist D-2-amino-5-phosphonovaleric acid (D-APV, 1-10 nmol/rat), and the non-NMDA antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 1-10 nmol/rat) dose-dependently suppressed adjuvant- and carrageenin-induced hyperalgesia, without effect on the nociceptive threshold of non-inflamed paws. Furthermore, to estimate whether inflammatory hyperalgesia is accompanied with an alteration of the responsiveness to substance P and excitatory amino acids, we examined the effects of injections of complete Freund's adjuvant (intradermal) and carrageenin (subcutaneous) on the aversive responses to intrathecal substance P and excitatory amino acid agonists. Both injections significantly potentiated the aversive behaviors elicited by intrathecal injections of excitatory amino acid agonists, NMDA (1 nmol/rat), α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA, 1 nmol/rat) and kainate (1 nmol/rat), but not those by substance P. The present results suggest that the enhancement of synaptic transmission mediated by substance P and excitatory amino acids in the spinal dorsal horn is at least partly involved in the production of inflammatory hyperalgesia, and that such a hyperalgesia is accompanied with the enhanced responsiveness to excitatory amino acids through NMDA and non-NMDA receptors, but not with changes in responsiveness to substance P.

Keywords: Substance P, Excitatory amino acid, Complete Freund's adjuvant, Carrageenin, Spinal cord

There are many findings suggesting that substance P (SP) and excitatory amino acids (EAAs), such as glutamate (Glu) and aspartate (Asp), in the primary afferent neurons are involved in transmission of information related to pain at the spinal dorsal horn. SP is contained in subpopulations of small and intermediate-sized dorsal root ganglion cells (1, 2), and SP-positive nerve terminals are highly concentrated in the superficial dorsal horn (3). An intrathecal injection of anti-SP antibody (4) or SP antagonist (5) produces antinociceptive effects, while an intrathecal injection of SP itself elicits nociceptive behavioral responses in rodents (5, 6). Furthermore, noxious skin stimulation (pinching) elicits the release of immunoreactive SP from the spinal dorsal horn (7, 8). Glu immunoreactivity exists with unmyelinated primary afferent terminals in the superficial dorsal horn (9), and Glu is co-localized with SP in small dorsal root ganglion neurons (10). Peripheral noxious stimulation increases the release of Glu and Asp from the dorsal spinal cord (11). Behaviorally, intrathecal injections of EAA agonists result in a dose-dependent aversive response in mice (5, 12), whereas EAA-receptor antagonists produce analgesia (5, 13). EAAs are thought to interact with SP in the sensitization of dorsal horn neurons (14) and are considered to underlie the development of certain models of hyperalgesia (15, 16).

Complete Freund's adjuvant or carrageenin injection into the rat hind paw plantar surface induces edema, hyperthermia and hyperalgesia; and these adjuvant- and carrageenin-induced hyperalgesia have been used as animal models of the hyperalgesia during inflammation (17, 18). Peripheral inflammation has been shown to in-

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crease the biosynthesis of SP in the dorsal root ganglia and spinal dorsal horn (19, 20), increase the release of SP from the dorsal horn (21), and increase Glu immunoreactivity in the dorsal horn (22). Therefore, the present experiments were carried out to examine whether SP and EAAs are involved in the adjuvant- and carrageenin-induced hyperalgesia and to determine whether such a hyperalgesia is concomitant with any alterations of the responsiveness to SP and EAAs in the spinal cord.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats weighing 230−280 g were used for all experiments. Rats were housed three in a cage and maintained under a 12 hr light-dark cycle (light cycle between 8:00 and 20:00) at a room temperature of 24±1℃ with free access to rat chow and water. They were allowed to acclimate for at least 24 hr prior to use in experiments. Each rat was used for only one experiment. The ethical guidelines for investigations of experimental pain in conscious animals (23) were followed.

Materials

The drugs injected intrathecally were substance P (Peptide Institute, Inc., Minoh), *N*-methyl-D-aspartic acid (NMDA) and kainic acid (Nacalai Tesque, Inc., Kyoto), α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA; Sigma Chemical Co., St. Louis, MO, USA). The antagonists used were D-2-amino-5-phosphonovaleric acid (D-APV; Cambridge Research Biochemicals, Ltd., Cambridge, UK), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; Tocris Neuramin, Buckhurst Hill, UK) and CP-96,345 ((2*S*,3*S*)-*cis*-2-(diphenylmethyl)-*N*-[(2-methoxyphenyl)-methyl]-1-azabicyclo[2.2.2]octan-3-amine (a kind gift from Central Research Division, Pfizer, Inc., Groton, CT, USA). CNQX was dissolved in 50% dimethyl sulfoxide (DMSO: special grade, Nacalai Tesque) in physiological saline and the other drugs were dissolved in physiological saline. *Mycobacterium butyricum* was purchased from Difco Laboratories (Detroit, MI, USA), and lambda carrageenin was from Sigma Chemical Co.

Intrathecal injection

Intrathecal injection was performed according to the method of Satoh et al. (24). Briefly, the skin of the back was incised along the spinous processes at the L_2-L_5 level under ether anesthesia, and then the wound was treated with the local anesthetic, 2% lidocaine jelly, and sutured. On the next day, after extracting the suture and treating the wound with 2% lidocaine jelly, 10 μl of drug was intrathecally injected into the freely moving rat through a lumbar puncture between L_3 and L_4 using a stainless steel needle (25-gauge). Ten microliters of physiological saline or 50% DMSO in saline alone were also injected intrathecally to the control group.

Antagonist study

Adjuvant arthritis was induced by intradermally inoculating 0.05 ml of paraffin oil suspension (12 mg/ml) of complete Freund's adjuvant (heat-killed *Mycobacterium butyricum*) into the right hind paw 10 days before intrathecal injection. Acute inflammation was produced by subcutaneous injection of lambda carrageenin (1.0 mg/0.1 ml saline) into the plantar region of the right hind paw 2 hr before intrathecal injection. The uninjected paw (left paw) served as the control paw.

The effects of antagonists on nociceptive responses were estimated using the paw-pressure test. For mechanical stimulation, pressure was applied to the hind paw by a wedge-shaped pusher at a loading rate of 48 g/sec using a pressure analgesimeter (Ugo Basile, Milan, Italy), and the pressure eliciting either paw withdrawal or a struggle response was determined.

Agonist study

Complete Freund's adjuvant (heat-killed *Mycobacterium butyricum*) suspended in paraffin oil (12 mg/ml) was intradermally inoculated into both hind paws 10 days before intrathecal injection. Lambda carrageenin dissolved into saline (10 mg/ml) was subcutaneously injected into the plantar region of both hind paws 2 hr before intrathecal injection. Uninjected rats served as controls.

The SP- and EAA agonists-elicited behaviors were observed using an acrylic resin cage (22×22×24 cm) as an observation cage, to which the rats were adapted for at least 15 min before the experiment. Immediately after the intrathecal injection, the rats were placed individually in the observation cage, and the duration of aversive behaviors (biting or licking the tail and hind paws) was measured for 5 min.

Statistical analyses

The results are expressed as means±S.E.M. Statistical analyses were performed by multiple comparison by Bonferroni's test following one-way analysis of variance (ANOVA) or Student's *t*-test; $P<0.05$ was considered significant.

RESULTS

Effects of intrathecal antagonists to SP and EAA on inflammatory hyperalgesia

In this series of experiments, adjuvant treatment decreased the nociceptive threshold of the treated paw

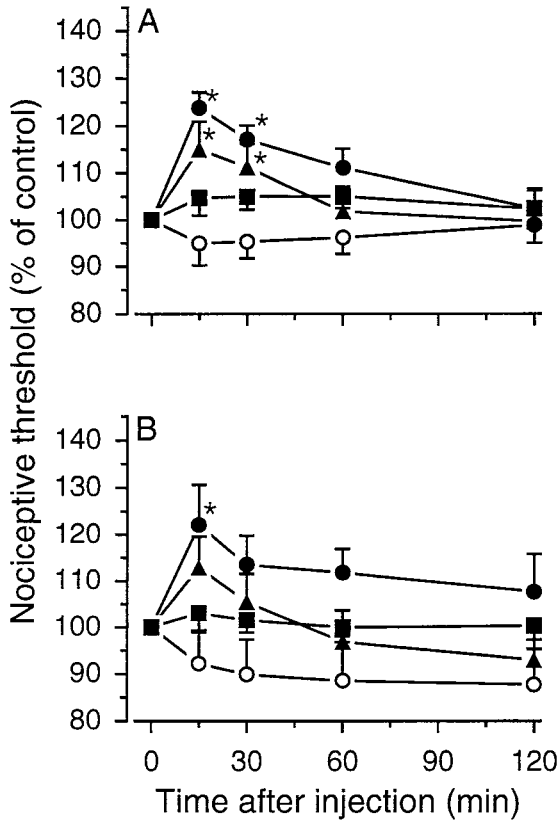

Fig. 1. Effects of intrathecal CP-96,345 on the nociceptive threshold of complete Freund's adjuvant (A) and carrageenin (B)-treated rats. CP-96,345 at doses of 0.3 (■), 1 (▲) and 3 (●) nmol or vehicle (○) were given intrathecally at time 0. The nociceptive threshold of each animal immediately before injection served as the control (100%). Values are each the mean and S.E.M. of 5–10 experiments. *$P<0.05$, when compared with the vehicle (multiple comparison by Bonferroni's test).

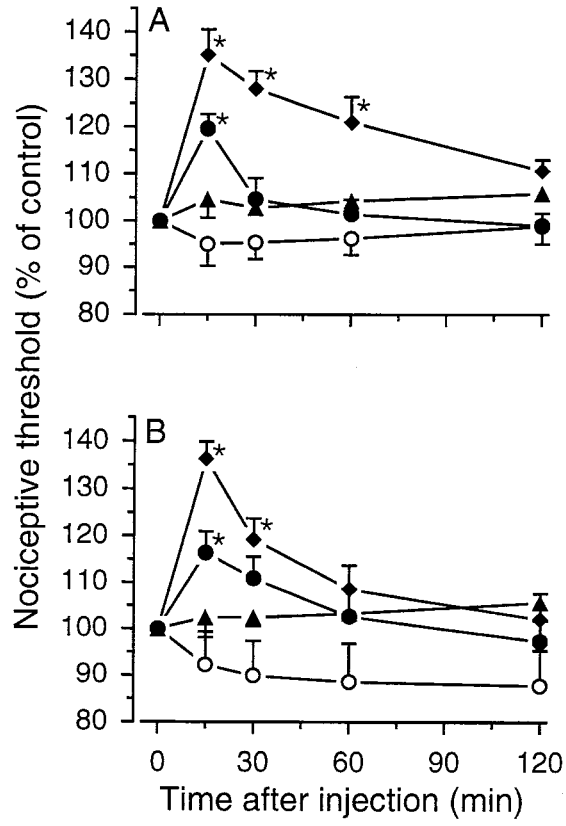

Fig. 2. Effects of intrathecal D-APV on the nociceptive threshold of complete Freund's adjuvant (A) and carrageenin (B)-treated rats. D-APV at doses of 1 (▲), 3 (●) and 10 (◆) nmol or vehicle (○) were given intrathecally at time 0. The nociceptive threshold of each animal immediately before injection served as the control (100%). Values are each the mean and S.E.M. of 5–10 experiments. *$P<0.05$, when compared with the vehicle (multiple comparison by Bonferroni's test).

from 315.5±3.0 g to 176.0±2.6 g (n=85) on day 10; carrageenin treatment decreased the threshold of the treated paw from 324.8±2.7 g to 179.3±2.4 g (n=85) at 2 hr. The nociceptive responses of the non-treated paw in both groups were not altered. The alteration in nociceptive threshold was not significantly different between adjuvant- and carrageenin-treated paws (Student's *t*-test). An intrathecal injection of either physiological saline or DMSO-containing saline did not alter the nociceptive thresholds of adjuvant-arthritic paw and carrageenin-inflamed paw. Intrathecal injections of antagonists examined did not alter gross behavior or motor function in any of the groups.

The intrathecal injection of CP-96,345 (0.3–3 nmol/rat) produced a dose-dependent elevation of the nociceptive threshold of both arthritic (Fig. 1A) and inflamed (Fig. 1B) paws, which peaked at 15 min. These peak nociceptive thresholds of adjuvant arthritic paw and carrageenin-inflamed paw after 3 nmol/rat CP-96,345 were 123.8±3.4% (n=10) and 122.1±8.6% (n=10) of the pre-injection level, respectively. The nociceptive thresholds of non-arthritic and non-inflamed paws were not significantly altered by CP-96,345 at a dose of 3 nmol/rat (n=10 each group).

An intrathecal injection of D-APV (1–10 nmol/rat) significantly increased the nociceptive threshold of both adjuvant arthritic (Fig. 2A) and carrageenin-inflamed (Fig. 2B) paws. These effects in a dose of 10 nmol/rat peaked at 15 min, when the nociceptive threshold were 135.0±5.5% (n=10) and 136.2±3.6% (n=10) of pre-injection level, respectively. The nociceptive thresholds of non-arthritic and non-inflamed paws were not significantly altered by D-APV at a dose of 10 nmol/rat (n=10 each group).

An intrathecal injection of CNQX (1–10 nmol/rat) produced a dose-related increase in the nociceptive thresholds of both adjuvant arthritic (Fig. 3A) and carrageenin-inflamed (Fig. 3B) paws, which peaked at 15

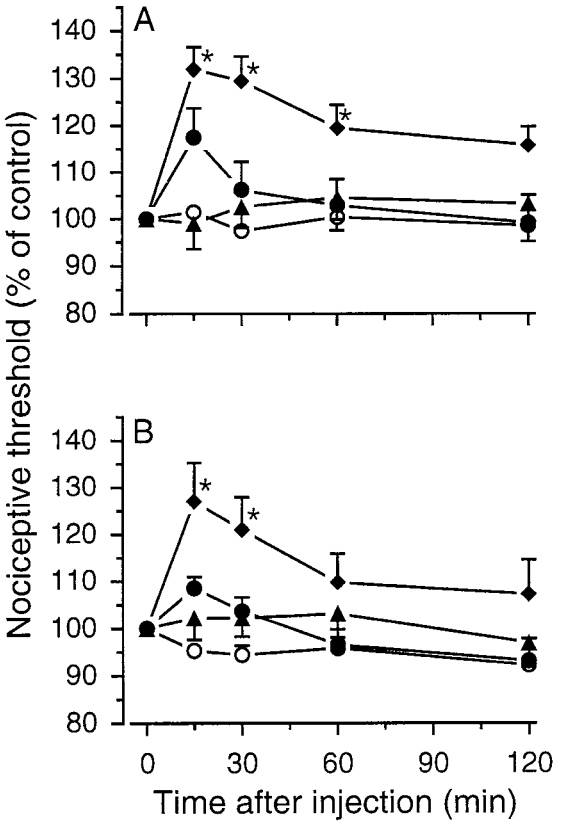

Fig. 3. Effects of intrathecal CNQX on the nociceptive threshold of complete Freund's adjuvant (A) and carrageenin (B)-treated rats. CNQX at doses of 1 (▲), 3 (●) and 10 (◆) nmol or vehicle (○) were given intrathecally at time 0. The nociceptive threshold of each animal immediately before injection served as the control (100%). Values are each the mean and S.E.M. of 5–10 experiments. *$P<0.05$, when compared with the vehicle (multiple comparison by Bonferroni's test).

min and subsided by 120 min. These peak nociceptive thresholds after 10 nmol/rat CNQX were 131.9±4.7% (n=10) and 126.9±8.3% (n=10) of pre-injection level, respectively. The nociceptive thresholds of non-arthritic and non-inflamed paws were not altered by CNQX at a dose of 10 nmol/rat (n=10 each group).

Effects of treatments with inflammatory agents on SP- and EAA-elicited behaviors

In rats with bilateral arthritic or inflamed paws, the nociceptive threshold of adjuvant-arthritic group was decreased from 335.4±2.2 g to 176.2±3.1 g (n=63) on day 10; the threshold of the carrageenin-inflamed group was decreased from 329.9±2.1 g to 175.2±1.7 g (n=90) at 2 hr.

Although there was a slight enhancement of the aversive response compared with the control group, the treatment with either adjuvant or carrageenin did not significantly affect the aversive behaviors produced by intrathecal SP (1 and 10 nmol/rat) (Fig. 4A).

The durations of aversive behaviors induced by intrathecal NMDA (1 nmol/rat) were significantly longer in both adjuvant- and carrageenin-treated groups than in the control one; the durations of NMDA-induced aversive behaviors for 5 min were 84.6±9.9 sec (n=7), 83.7±10.7 sec (n=10) and 45.6±3.1 sec (n=10), respectively (Fig. 4B). The aversive behaviors induced by intrathecal NMDA (10 nmol/rat) were not significantly changed by treatments with either adjuvant or carrageenin.

In the hyperalgesic rats, intrathecal AMPA (1 nmol/rat)-induced aversive behaviors were more pronounced than in control rats; the durations of AMPA-induced aversive behaviors for 5 min were 83.4±6.1 sec (n=7), 73.7±6.5 sec (n=10) and 44.9±5.3 sec (n=10) in the adjuvant- and carrageenin-treated and control rats, respectively (Fig. 4C). When a higher dose of AMPA (3 nmol/rat) was used as a test stimulus, the durations of AMPA-induced aversive behaviors were not significantly different between the control and either group of hyperalgesic rats.

The durations of aversive behaviors induced by intrathecal kainate (1 nmol/rat) were significantly longer in both adjuvant- and carrageenin-treated groups than in the control one; the durations of kainate-induced aversive behaviors for 5 min were 174.7±11.3 sec (n=7), 183.6±8.7 sec (n=10) and 115.6±13.2 sec (n=10) in the adjuvant- and carrageenin-treated and control rats, respectively (Fig. 4D). The aversive behaviors induced by intrathecal kainate (3 nmol/rat) were not significantly changed by treatments with either adjuvant or carrageenin.

DISCUSSION

Complete Freund's adjuvant and carrageenin-induced hyperalgesia which is due to inflammation of the treated region have been widely used as models to study mechanisms of nociception (17, 18, 25–27). It has been shown that peripheral inflammation increases the biosynthesis of SP in the dorsal root ganglia (19, 28, 29) and the release of SP from the spinal dorsal horn (21). Therefore, it is conceivable that inflammatory hyperalgesia is inhibited by NK-1 antagonists. Indeed, in the present study, intrathecal CP-96,345, a nonpeptide NK-1 antagonist, increased the nociceptive threshold in the paw-pressure test of the adjuvant- and carrageenin-treated paw in a dose-dependent manner without any effect on the threshold of untreated paw. Recently, it has been reported that the affinity of CP-96,345 to the NK-1 receptor is lower in rodents than in other mammalian species (such as humans, gerbils and hamsters) (30–32). However, competition

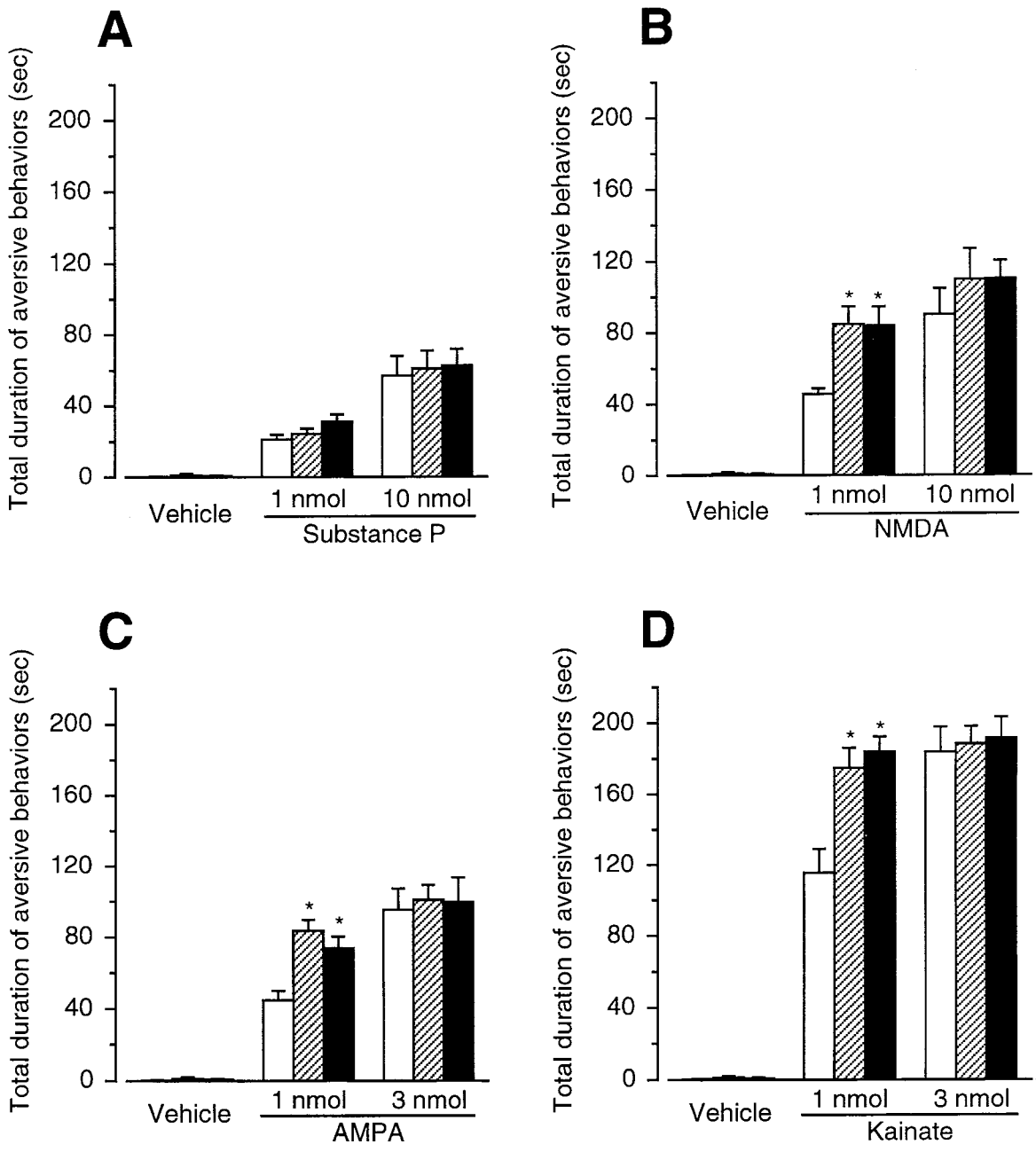

Fig. 4. Effects of treatments with complete Freund's adjuvant (hatched bars) and carrageenin (filled bars) on the aversive behaviors produced by intrathecal substance P (A), NMDA (B), AMPA (C) and kainate (D). Ordinate: duration of biting or licking behavior within 5 min after intrathecal injection (n=7–10). *P<0.05, when compared with the control group (open bars) (Student's *t*-test).

binding experiments have demonstrated that CP-96,345 bound with high affinity to the cloned rat NK-1 receptors and with low affinities to the cloned rat NK-2 and NK-3 receptors (33, 34). These data suggest that CP-96,345 is much more selective for NK-1 receptors than for NK-2 and NK-3 receptors even in rats. On the other hand, CP-96,345 was suggested to produce antinociceptive effect by interacting with calcium channels (35, 36). However, since our previous studies revealed that this antagonist at an intrathecal dose of 3 nmol/rat significantly suppressed aversive behaviors elicited by an intrathecal injection of SP but did not those of EAA agonists (5), the inhibitory effect of CP-96,345 at the dose used in this study is likely due to the blockade of NK-1 receptors rather than calcium channels. Therefore, the present results suggest that spinal NK-1 receptor activation is involved in inflamma-

tory hyperalgesia in rats. This is supported by the findings that an intrathecal injection of anti-SP antibody (37) or CP-96,345 (38) produced an improvement of inflammatory mechanical or thermal hyperalgesia, respectively.

EAAs are supposed to be involved in the nociceptive transmission in the spinal dorsal horn through NMDA receptors (39, 40). These are relevant to 'wind-up', a state of C-fiber input-dependent central sensitization (41, 42) which is considered to underlie hyperalgesia, and NMDA antagonists block this phenomenon (43). In the present study, both types of hyperalgesia induced by adjuvant and carrageenin were dose-dependently attenuated by D-APV, but intrathecal D-APV showed no effect on the nociceptive threshold of the untreated paw at the dose used in this study. These results suggest that NMDA receptors may play an important role for inflammatory hyperalgesia after intradermal adjuvant and subcutaneous carrageenin injection in the rat.

Glu may participate in nociceptive transmission also through non-NMDA receptors (44–46). In this study, intrathecal CNQX increased the nociceptive threshold of the inflammatory agent-injected paw in a dose-dependent manner and significantly suppressed the hyperalgesia induced by inflammatory agents at doses that did not alter the nociceptive threshold of the untreated paw. It should be considered that CNQX acts at the strychnine-insensitive glycine site of NMDA receptors (47). However, in our previous experiments, CNQX at the dose used in this study inhibited behavioral responses to intrathecal AMPA (3 nmol/rat) and kainate (3 nmol/rat), but not those to NMDA (10 nmol/rat) (5), suggesting that this antagonist at least at the dose used may not block NMDA receptors. Therefore, the present results support the idea that the activation of both NMDA and non-NMDA receptors is involved in the inflammatory hyperalgesia induced by adjuvant and carrageenin injection.

We found that intradermal injection of complete Freund's adjuvant and subcutaneous injection of carrageenin significantly potentiated the aversive responses to intrathecal EAA agonists, NMDA (1 nmol/rat), AMPA (1 nmol/rat) and kainate (1 nmol/rat). Thus, it appears that injections of the inflammatory agents enhance excitability of the spinal systems mediating the aversive behavior produced by intrathecal EAA agonists. Haley et al. (39) have reported that the prolonged chemical nociceptive stimulus produced by formalin induces a peripheral afferent drive that can activate central spinal NMDA receptors, and that the characteristics of the NMDA receptors may serve to amplify and/or prolong the neuronal activity produced by the afferent barrage in response to formalin. Kosher et al. (48) have shown that a part of the polymodal nociceptors located inside the inflamed area induced by carrageenin injection exhibits spontaneous activities. Possible explanations for the enhanced excitability observed in the present experiment are that C fibers sensitized by inflammatory agents maintain the activation of NMDA receptors by causing persistent release of glutamate or that membrane depolarization produced by non-NMDA activation enables excitation of the NMDA receptor–ion channel complex by removing the voltage-dependent NMDA receptor channel block. Thus, the potentiation of nociceptive behavior in inflamed animals may be mediated by central mechanisms underlying the plastic changes in synaptic transmission between primary afferents and dorsal horn neurons. It was confirmed that when NMDA (10 nmol/rat), AMPA (3 nmol/rat) and kainate (3 nmol/rat) were injected intrathecally in the doses that produced the maximum effect on aversive behavior of the control rats (5), aversive responses to these agonists at such higher doses were no longer augmented by inflammation.

SP co-exists with Glu in primary afferent terminals in the dorsal horn (10, 49). Micro-iontophoretic co-application of NMDA and SP enhanced the response to NMDA in primate spinothalamic tract neurons (14). We have found that CP-96,345, D-APV or CNQX alone inhibited intrathecal capsaicin-induced aversive behaviors and simultaneous applications of two of those antagonists in combination produced more marked inhibition than one alone (50). SP increased the release of Glu and Asp from the rat spinal cord (51). On the contrary, an activation of NMDA receptor caused release of SP in the rat spinal dorsal horn (52). SP augmented Glu-induced currents in isolated rat spinal dorsal horn neurons (53). These findings suggest that the pre- and post-synaptic interactions between SP and EAAs are relevant to spinal nociception and hyperalgesia. In the present experiments, the aversive response to intrathecal SP (1 and 10 nmol/rat) was slightly but not significantly potentiated by inflammation, indicating that NK-1 receptors are involved in the spinal processing of inflammatory hyperalgesia to a less extent than NMDA and non-NMDA receptors.

In conclusion, the present study indicates that spinal EAAs and SP systems play important roles in complete Freund's adjuvant- and carrageenin-induced hyperalgesia and that injections of inflammatory agents induce an enhanced excitability of the spinal systems mediating the aversive behaviors produced by intrathecal EAA agonists.

Acknowledgments

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