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Selective Antinociceptive Effect of Excitatory Amino Acid Antagonists in Intact and Acute Spinal Rats[1]

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ADVOKAT, C. AND D. RUTHERFORD. *Selective antinociceptive effect of excitatory amino acid antagonists in intact and spinal rats.* PHARMACOL BIOCHEM BEHAV **51**(4) 855–860, 1995.—Results of neurophysiologic and behavioral studies suggest that excitatory amino acid (EAA) antagonists may provide a new class of analgesic agents, which might be selective for neuropathic pain states that are resistant to opiate treatment. Most of these paradigms involve animal models of peripheral injury. The present study evaluated the antinociceptive effect of spinally [intrathecally (IT)] administered EAA antagonists after central injury, produced by spinal transection. Intrathecal injection of the AMPA/kainate receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione produced dose-dependent antinociception on the thermal tail withdrawal [tail-flick (TF)] reflex test in Intact rats, which was significantly potentiated after spinal transection. In contrast, IT injection of the NMDA antagonist, 2-amino-5-phosphonopentanoic acid (AP5) did not affect the TF in intact rats, but significantly blocked this response in spinal rats. However, some of the spinal rats did not recover the reflex, suggesting a possible toxic action of AP5.

Excitatory amino acid antagonists CNQX AP5 Antinociception Spinal rat Intrathecal Tail-flick

ALTHOUGH it is fairly well established that the excitatory amino acids (EAAs), primarily glutamate and aspartate, are involved in spinal pain processing, the specific role of the various receptor subtypes in nociceptive transmission is not well defined. These receptors have been categorized as either ionotropic NMDA (activated by *N*-methyl-D-aspartate) or non-NMDA subtypes, which include the AMPA/kainate (activated by alpha-amino-3-hydroxy-5-methyl-4-isoxazole-proprionic acid and kainate) class, and metabotropic receptors (9,19).

Whereas AMPA receptors exhibit conventional, ligand-gated conductances, the NMDA receptor has some unique properties. Under normal physiologic conditions, the NMDA ion channel is blocked by Mg^{++} and the receptor complex is not activated. Only after this blockade is removed by excessive, intense, or repetitive depolarizing stimulation are receptor channels opened and conductances generated (10–12). When this occurs, the initial neural response to the first few stimuli suddenly increases dramatically (becomes sensitized), although subsequent input remains constant. This phenomenon, exhibited by certain dorsal horn neurons in the spinal cord, is termed "wind-up" and is believed to be responsible for the hyperalgesic reactions (increased response to noxious stimulation) that characterize a variety of pain states that are clinically referrred to as neuropathic pain. As a result, the possibility that antagonists of EAA receptors may provide a new class of analgesic agents has generated much interest (9–12).

There is substantial neurophysiologic support for this proposal. Monosynaptic, fast responses of dorsal horn cells, evoked by electrical stimulation of myelinated primary afferents (i.e., below C-fiber threshold) are selectively blocked by the AMPA/kainate antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (20,22) but not NMDA antagonists (28, 44). Conversely, intrathecal (IT) application of the selective, competitive NMDA antagonist APV, (+)-2-amino-5-phos-

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phonopentanoic acid (AP5), blocked the sensitized response of dorsal horn neurons without affecting evoked responses under a variety of conditions, including electrical stimulation of the receptive field (14,35), injection of formalin into the hindpaw (18), tail ischemia (41) or prior administration of EAA agonists (1,40). Several studies examining both CNQX and AP5 (or the related compound AP7) on spinal neurons have demonstrated a consistent antagonism of CNQX against all types of innocuous and noxious inputs, whereas NMDA antagonism was limited to noxious input and the resulting sensitization (3,15,31,47). On the basis of these results, it might be predicted that CNQX should reduce behavioral reactions to a variety of stimuli, whereas AP5 should have a selective action against intense or chronic noxious stimulation (11,13).

Behavioral paradigms provide support for some of these predictions. NMDA antagonists effectively block hyperalgesic reactions, produced by formalin (7,8,29,30), carregeenan (37), ischemia (39), heat (6), and NMDA itself (2,26,27), as well as peripheral nerve damage (24,38,46). In most of these studies the response of the untreated limb was unaffected [(2,26, 30,37,46); also see (45)]. However, some investigators report an antinociceptive effect of NMDA antagonists, particularly AP5, against acute, transient nociceptive stimulation, such as the tail and hindpaw withdrawal reflexes (5,29,34–36). Although these effects are often observed at higher doses, which produce motor impairments, the analgesic effects have been dissociated from the motor deficits (5,35,36). Such results are inconsistent with the proposed selective effect of NMDA antagonists against chronic pain.

There are additional discrepancies between the behavioral and neural data with regard to the non-NMDA antagonist CNQX (and the related compounds DNQX and NBQX). In some studies, these drugs have been used to block the hyperalgesic effect of AMPA on reflex withdrawal tests (23,25,27). However, when tested alone in rats (25,27), the antagonists did not produce a hypoalgesic reaction. This would seem to be inconsistent with the proposed role of AMPA receptors in acute nociceptive transmission. As suggested by Meller et al. (25), this could be due to the low doses used (1–27 nM) or to a species difference, because comparable doses produced antinociception on the hot plate and tail-flick in mice (30).

A similar argument could be applied to the effect of AMPA antagonists on tonic nociceptive behaviors. Coderre and Melzack (7) reported that IT AMPA did not facilitate, and 10–50 μg CNQX did not reduce, the response to formalin or to thermally induced, secondary hyperalgesia (6). In contrast, other studies have shown that CNQX is effective against formalin [when given systemically (21)], carregeenan [161 μg given IT (37)], and chronic peripheral neuropathy [multiple IT injections of 20 μg (24)]. It has also recently been reported that microdialysis infusion of 2 mM of CNQX not only prevented the spinal release of EAAs in an experimental paradigm of arthritis (42) but also reduced behavioral pain reactions and peripheral inflammation (43). Although these results support the role of AMPA receptor activation in a variety of pain syndromes, it is perhaps surprising that relatively high doses were required considering the implication of the neurophysiologic evidence that CNQX would be effective against even innocuous input.

Finally, in the majority of these studies, EAA antagonists have only been assessed in peripheral models of tonic or chronic injury. Yet, it has been shown that spinal cord trauma produces a profound increase in the concentration of extracellular EAAs (32) and that NMDA antagonists are preferentially effective against hyperalgesia after spinal cord transection (17,33,34). As yet, no AMPA antagonist has been examined in the spinal preparation. The present study therefore evaluated the effect of IT CNQX and AP5 on the thermally elicited tail withdrawal reflex, in both intact and acutely spinalized rats.

METHOD

Subjects

A total of 61 male albino Holtzman rats (Harlan Sprague-Dawley Laboratories, Madison, WI), weighing 300–500 g, were used as subjects. All rats were individually housed in suspended wire mesh cages in a colony room maintained on a 12L : 12D cycle, with access to food and water throughout the experiments.

Surgical Procedures

Intrathecal catheterization. Animals were anesthetized with a mixture of isoflurane (AErrane; Anaquest, Madison, WI) and oxygen and placed in a stereotaxic frame. An incision was made behind the ears and the neck muscles were scraped to expose the back of the skull. An incision of the atlanto-occipital membrane allowed insertion of an 8-cm-long catheter of PE-10 polyethylene tubing filled with sterile saline into the spinal subarachnoid space. Prior to insertion, a loose knot was tied in the catheter and coated with dental cement so that it could be held in place against the skull with adhesive (Superglue; Bel-Art Products, Pequannock, NJ). The incision was closed and the exposed tip of the catheter was heat-sealed. Within 30 min all rats had recovered from the anesthesia. Any rat showing a postoperative neurologic deficit (i.e., a crippled limb) was euthanized and eliminated from the study. Even in these situations (~10%) there was no overt evidence of pain. Intact rats were tested 5 days after surgery.

Spinal transection. In addition to the catheter implantation, several groups of rats (n = 28) also sustained a spinal transection, as described previously (17). The skin incision was extended and after retraction of the paraspinal muscles a laminectomy was performed between thoracic vertebrae 6 and 9. A 1–2-mm portion of the spinal cord was crushed and severed, leaving the catheter intact. The space left in the spinal cord was replaced with gelfoam to reduce bleeding, after which the incision was closed in layers and the cages placed under a heat lamp to maintain body temperature. On the morning after surgery the hindquarters of each rat were washed with soap and warm water and their urine expressed manually by the application of pressure to their bladders. Food was placed in the cages of the spinal rats, and they were able to eat and drink after recovering from the anesthesia. Spinal rats did not exhibit overt reactions of distress (no squealing or struggling) when handled during voiding or testing. Nevertheless, all experiments with spinal rats were completed as soon as possible, within approximately 24 h after surgery.

Tail Flick Assessment

Noxious stimulation was provided by a beam of high-intensity light (IITC Life Sciences, Woodland Hills, CA) focused on the tail. The response time was measured automatically and was defined as the interval between the onset of the thermal stimulus and the abrupt flick of the tail. Each determination consisted of three to five trials; the mean score was taken as the response latency. Animals not responding

within 14 s were removed from the apparatus to prevent tissue damage and assigned a score of 14 s.

Drug Administration and Procedure

For IT injections, the tip of the heat-sealed catheter was cut, a 30-ga needle was inserted into the catheter, and 10 μl of the drug solution was infused followed by a 10-μl wash of the saline vehicle. Injections were performed manually with a 50-μl Hamilton syringe (Hamilton Co., Reno, NV) over a 2-3-min period, after which the catheters were again heat-sealed.

The AMPA antagonist, CNQX (Research Biochemicals International, Natick, MA) was dissolved in a solution of 50-60% dimethylsulfoxide (DMSO)/50-40% saline. Individual groups of intact and spinal rats were injected with either this vehicle or a dose of 10 μg (43 nM) or 20 μg (86 nM) CNQX, dissolved in the vehicle. AP5 was administered to intact and spinal rats in a saline vehicle at a dose of either 5 (25.4 nM), 10 (51 nM), or 30 μg (152 nM; given to intact rats only). On the day of the experiment all rats were pretested on the tail-flick (TF) for baseline assessment, injected with the respective drug, and retested 15, 30, and 60 min later. Any rat with a score of 14 s at 60 min was tested again for recovery to determine whether loss of the reflex was permanent. In the event that recovery did not occur, autopsies were performed the next day to determine whether the catheter was inadvertently placed within the spinal cord. Each animal was used only once and contributed a single pre- and postdrug data set.

All surgical and test procedures were reviewed and approved by the Institutional Animal Care and Use Committee of Louisiana State University.

Statistical Analyses

The effects of the drugs were analyzed by Student's *t*-test and one-way or two-way analyses of variance (ANOVAs) performed with the aid of a computer program (Sigma-Stat; Jandel, San Rafael, CA), followed by post hoc Newman-Keuls and Dunnett's tests. Analyses were performed on the area under the curve (AUC). This value was obtained with the aid of a computer program (PHARM/PCS). For each animal the AUC was determined by entering each *xy* data pair, in which

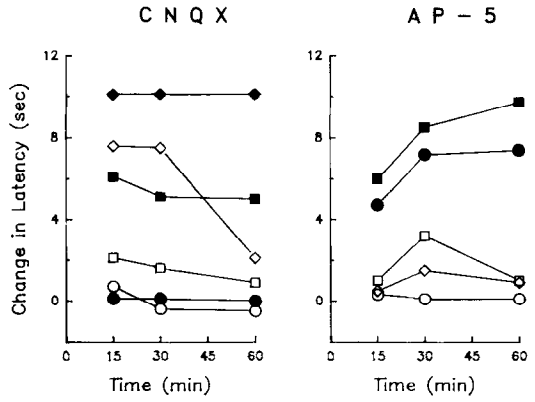

FIG. 1. Mean change in TF latency of separate groups (n = 5-7) of intact (open symbols) and spinal (filled symbols) rats after IT injection of either CNQX or AP5. For CNQX, the doses were saline (circles), 10 μg (squares) or 20 μg (diamonds). For AP5 the doses were 5 μg (circles), 10 μg (squares) or 30 μg (diamonds, intact only). For clarity, SE values were not included.

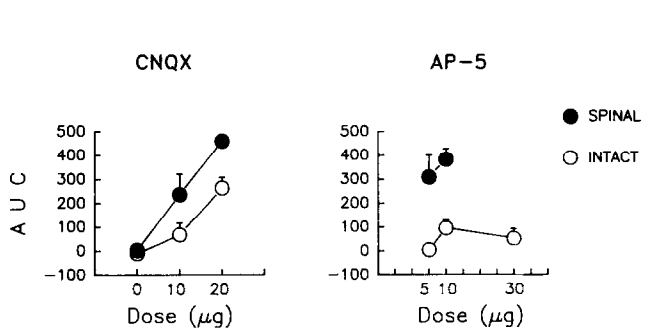

FIG. 2. Dose-response effect of IT administered CNQX and AP5 on the thermally elicited TF reflex of intact (○) and spinal (●) rats. Each symbol represents the mean ± SEM AUC (calculated from the data in Fig. 1) for each of the indicated doses, obtained from separate groups (n = 5-7), which were assessed before and 15, 30, and 60 min after the respective injections.

x = the difference in TF latency between the pretest score and the latency obtained at each time point, and y = 15, 30, and 60 min. The computer program calculated the total area (i.e., the integral) based on an approximation using the trapezoidal rule. This procedure provided a single value that incorporated the latencies over all time points. Statistical tests were performed on the AUC values of each group; results were considered to be significant at $p < 0.05$.

RESULTS

Figure 1 shows the mean (± SEM) change in TF latency of each group as a function of postinjection time. The left side of the figure summarizes the effect of the three doses of CNQX in intact and spinal rats; the right side presents the corresponding data for AP5. These values were used to obtain the AUC score for each rat, at each drug dose. Figure 2 summarizes the mean (± SEM) AUC scores as a function of dose for intact (○) and spinal rats (●). The left side of the figure shows the results of CNQX. A one-way ANOVA performed on the AUC scores of the intact rats showed that there was an overall effect of dose [n = 18; $F(2, 17) = 14.1$, $p < 0.001$]. Post hoc tests indicated that the effect of 20 μg was significantly greater than that of either 10 μg or the vehicle ($p < 0.05$). A one-way ANOVA performed on the scores of the spinal rats also showed an effect of dose [n = 18; $F(2, 17) = 13.5$, $p < 0.001$]. Post hoc tests indicated that the effect of 20 μg of CNQX was significantly greater than that of the vehicle or 10 μg and that the effect of 10 μg was significantly greater than the vehicle ($p < 0.05$).

A two-way ANOVA performed on the AUC scores of intact and spinal rats indicated that there was a significant effect of the transection [n = 23; $F(1, 22) = 8.2$; $p = 0.01$] and of dose [$F(1, 22) = 10.9$; $p = 0.004$], with no interaction. Post hoc comparisons did not support a significant difference between intact and spinal rats at either of the two doses separately. However, there was no overlap of AUC values between these two conditions at the 20-μg dose. A nonparametric, Mann-Whitney U-test showed a significant difference between these two groups [$U(5, 5) = 0$; $p < 0.008$].

The right side of Fig. 1 summarizes the results of the competitive NMDA antagonist AP5. A one-way ANOVA showed no difference among the AUC values of the intact rats [n =

15; $F(2, 14) = 2.1$, NS]. A t-test on the data of the two spinal groups showed no difference between the two doses of 5 and 10 μg [$t(8)$-0.7, NS].

A two-way ANOVA on the scores of intact and spinal rats that received the two common doses showed a significant effect of transection [$n = 20$; $F(1, 19) = 29.4$; $p < 0.001$] but not of dose [$F(1, 19) = 2.2$, NS], with no interaction. Post hoc comparisons showed that the scores of spinal rats were significantly greater than those of intact rats at each dose ($p < 0.05$ in each case).

In this study, there was no saline control included with the drug treatments. This minimized the number of animals subjected to surgical preparation. Therefore, to further evaluate the effect of AP5 in intact rats, a repeated measures one-way ANOVA was performed on the TF latencies across all time points (pretest, 15, 30, and 60 min). The results showed no significant effect of either 5 or 10 μg at any time point.

However, an additional effect was noted after IT injection of AP5 in spinal rats. The response of three of five rats that received 5 μg was still 14 s at 60 min postinjection. One of these animals did not recover the reflex by the next day. The response of five of the six rats that received 10 g was also 14 s at the final 60-min test and four did not recover the reflex by the next day. In each case, the autopsies showed that the catheter was correctly placed outside of the spinal cord. In contrast, every rat injected with CNQX that reached the 14 s cutoff recovered the TF reflex during the day of the test.

DISCUSSION

The development of selective agents for the non-NMDA and NMDA types of EAA receptors made it possible to examine their respective roles in spinal pain processing. Neurophysiologic evidence indicated that non-NMDA, particularly AMPA receptors, mediated fast excitatory transmission involving both innocuous and acute nociceptive input, whereas NMDA receptors were implicated specifically in nociceptive reactions, particularly those induced by intense prolonged stimulation, sufficient to produce the hyperalgesic state underlying neuropathic pain. This dichotomy suggested that antagonism of AMPA receptors might produce a nonspecific blockade of sensory input, which would have limited therapeutic benefit (11). Antagonism of NMDA receptors, however, appeared to provide a novel mechanism for the development of analgesic drugs.

The application of these agents in behavioral paradigms has not entirely supported this distinction. In particular, the AMPA antagonist CNQX has been shown to reduce a variety of hyperalgesic behaviors, without affecting the normal response to noxious stimulation. Results of AP5 in these same procedures do provide support for the efficacy of NMDA antagonism in chronic nociceptive conditions. Although AP5 has also been shown to have analgesic effects in some acute reflex assays, these are often transient (29,35) or require doses that are near threshold for motor impairment (5).

The present study assessed the effect of the IT administration of CNQX and AP5 on the acute, thermally elicited TF withdrawal reflex. In most previous investigations this response (or that of the hindlimb) was made hyperalgesic by a variety of peripheral treatments such as chemical irritants, ischemia, spinal injection of nociceptive agents, or nerve damage. In the present study the hyperalgesic reaction was induced by central injury resulting from spinal transection. Spinalization produces several characteristics of a hyperalgesic state, including an increase in the release of EAAs (32), an increase in the excitation of dorsal horn cells (16), behavioral facilitation (decrease in latency) to constant nociceptive input (17), and a selective antinociceptive effect of the noncompetitive NMDA antagonist, ketamine (17,33). Spinalization also potentiates the reflex scratching response elicited by IT L-glutamic acid (4).

Because motor function cannot be easily evaluated in the spinally transected preparation, it is conceivable that all of the effects listed might be due to changes in motor output rather than, or in addition to, sensory transmission (44). Without any accompanying indices of motor function it is difficult to differentiate between these alternatives. It is known that at the doses used in these nociceptive paradigms, the NMDA antagonist ketamine does have analgesic properties (17). It has also been reported that the excitation of dorsal horn convergent neurons produced by IT NMDA is reduced by the application of diffuse noxious inhibitory controls [i.e., noxious pinch of areas outside the neuron's receptive field (41)]. This suggests some degree of specificity for sensory processing.

However, the possibility that spinal application of these drugs may influence motor output warrants investigation, particularly considering the apparently toxic effect produced by AP5 on the spinal reflex. It is most likely that this neurologic deficit was produced by the drug because: a) autopsies indicated that the catheter was placed correctly, b) more rats were affected by the higher than the lower dose, c) only AP5 produced this reaction, and d) only spinal rats were affected.

Nevertheless, the results of this study are generally consistent with those obtained in paradigms that involved peripheral injury. IT administration of CNQX significantly increased TF latencies in intact rats at the higher, 20-μg dose, and the antinociceptive effect was potentiated after spinal transection. As predicted, IT AP5 did not modify the reflex in intact rats but produced a substantial antinociceptive effect after spinalization. Further investigation is necessary to determine whether these differential actions represent a specific action against centrally induced hyperalgesia and/or an additional effect on hyperreflexia.

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