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Short communication

Glutamate participates in the peripheral modulation of thermal hyperalgesia in rats

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Abstract

While the effects of excitatory amino acids have been well characterized in the central nervous system, relatively little is known about their possible modulation of elements responsible for hyperalgesia within peripheral tissue. The presented experiments demonstrate that the intraplantar (i.pl.) injection of L-glutamate (30 nmol) evokes a thermal hyperalgesic response in the paw withdrawal latencies of normal rats which is stereospecific. In addition, the i.pl. injection of either the non-competitive *N*-methyl-D-aspartate (NMDA) receptor antagonist MK-801 (10 nmol) or the competitive α-amino-3-hydroxy-4-methyl-5-isoxazolepropionic acid (AMPA)/kainate receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (100 nmol) into hindpaws inflamed with carrageenan significantly reduced the thermal hyperalgesic response in rats. Collectively, these results suggest that excitatory amino acids activate a peripheral target which facilitates a hyperalgesic behavioral response to thermal stimulation via a receptor mediated process.

Keywords: Glutamate; MK-801; CNQX (6-cyano-7-nitroquinoxaline-2,3-dione); Inflammation; Pain; Analgesia

1. Introduction

With the discovery that excitatory amino acids such as glutamate function as specific receptor-dependent neurotransmitters within the central nervous system, much attention has been given to the characterization of the central systems which they modulate. Several lines of experimental evidence are consistent with the hypothesis that excitatory amino acids participate in central processes like learning and memory as well as the manifestations of certain neurodegenerative diseases and neuronal death.

Glutamate has been implicated as an excitatory neurotransmitter at the level of the spinal cord (Aanonsen and Wilcox, 1986, 1987; Wilcox, 1993). Glutamate immunoreactivity has been localized in the axons of myelinated and unmyelinated primary afferent neurons (Westlund et al., 1989) and glutamate immunoreactivity has also been colocalized with substance P in the dorsal root ganglion (Battaglia and Rustioni, 1988). In addition, in vitro experiments have demonstrated that glutamate release is evoked from dorsal root ganglia cultures with capsaicin, a stimulant selective for a distinct subpopulation of primary afferent neurons consisting primarily of unmyelinated nociceptors (Jeftinija et al., 1991). The results of in vivo experiments also suggest that glutamate may function as a neurotransmitter at the level of the spinal dorsal horn. Specifically, a characteristic abdominal biting and scratching behavior is elicited in rats immediately following the intrathecal injection of glutamate in the lumbar region. Additionally, a decreased threshold to thermal stimulation is produced, as measured by the tail-flick and hot plate assays (Aanonsen and Wilcox, 1986, 1987). These hyperalgesic behaviors in response to intrathecally administered glutamate can be inhibited by the intrathecal administration of various antag-

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onists acting at the *N*-methyl-D-aspartate (NMDA) receptor, a glutamate receptor subtype.

Comparatively few studies however have evaluated peripheral actions of excitatory amino acids in the development of hyperalgesia. The localization of glutamate in the axons of primary afferent neurons and dorsal root ganglia suggests that excitatory amino acids may be transported and released in peripheral tissue as well as the dorsal horn, and may modulate the activity of peripheral nociceptors. We have demonstrated previously that administration of 50 mM potassium or capsaicin evokes the release of glutamate and aspartate from bovine dental pulp as measured by high-performance liquid chromatography (Jackson et al., 1993). We have also demonstrated using in vitro superfusion, that the addition of glutamate, kainate, or α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) to the superfusion buffer evokes a dose-dependent increase in the release of immunoreactive calcitonin gene-related peptide from bovine dental pulp relative to spontaneous baseline release. These results suggest that excitatory amino acids may be released from primary afferent neurons and that excitatory amino acids may activate a population of small diameter peptide-containing neurons. The aims of the present study were to evaluate whether the administration of glutamate in peripheral tissue evokes thermal hyperalgesia in a rat behavioral assay, and whether glutamate receptor subtypes in peripheral tissue contribute to the thermal hyperalgesia observed in inflamed tissue.

2. Materials and methods

Thermal hyperalgesia was evaluated by the paw withdrawal test (Hargreaves et al., 1988). Male Sprague-Dawly rats (200–225 g) were maintained in the animal facility for at least one week before the experiments with food and water ad libitum and lighting from 06:00–18:00 h. On the day of the experiment animals were placed in clear plastic chambers on the glass floor of the testing apparatus (7370 Plantar Test, Ugo Basile, Italy) and allowed to acclimate to their surroundings for 5 min. Following acclimation, a radiant heat source was aimed at the plantar surface of one of the hindpaws through the glass floor. A photoelectric cell automatically turns the heat source off when the reflected light beam is interrupted (i.e. when the animal withdraws the paw) and records the paw withdrawal latency. The person performing the hindpaw latency tests was blinded to treatment allocations. This protocol was approved by the University of Minnesota's Animal Care and Use Committee.

After baseline paw withdrawal latencies were recorded, one hindpaw of the rat was randomly injected (i.pl.) with either glutamate (30 nmol) or the phosphate buffered saline vehicle control (50 μl) and paw withdrawal latency values were evaluated 5 min later. Changes in hindpaw temperature were also evaluated. The hindpaw of rat was gently placed on a contact thermocouple (YSI No. 408, Yellow Springs Instruments, OH, USA) until the digital temperature reading stabilized (15 s maximum) at the baseline and 5 min time points.

In another series of experiments, unilateral hindpaw inflammation was induced with carrageenan (2 mg/paw). The characteristic inflammatory response of carrageenan was allowed to develop for 2.5 h. At this time point either an i.pl. injection of the non-competitive NMDA receptor antagonist MK-801 (10 nmol), the competitive AMPA/kainate receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (10, 100 nmol), or the vehicle control (50 μl) was administered directly into the inflamed paw. Since MK-801 can mediate antinociceptive effects through a centrally mediated mechanism, this compound was also administered into the subcutaneous tissue of the neck in a double-dummy random design to evaluate possible systemic effects at this dose. Five minutes after these injections paw withdrawal latency values were recorded again.

L-Glutamate (Fluka Biochemika, Switzerland), D-glutamate, lidocaine hydrochloride (Sigma Chemical Co., St. Louis, MO, USA), and CNQX (Tocris Cookson, Bristol, UK) were dissolved in phosphate buffered saline. The (+)-MK-801 (a gift from Merck Sharp & Dohme Research Lab, Rahway, NJ, USA), and (−)-MK-801 (Research Biochemicals International, Natick, MA, USA) stock solutions were dissolved in ethanol and subsequently diluted in phosphate buffered saline. The pH of all injected solutions was adjusted to 7.4. Carrageenan (Sigma Chemical Co., St. Louis, MO,

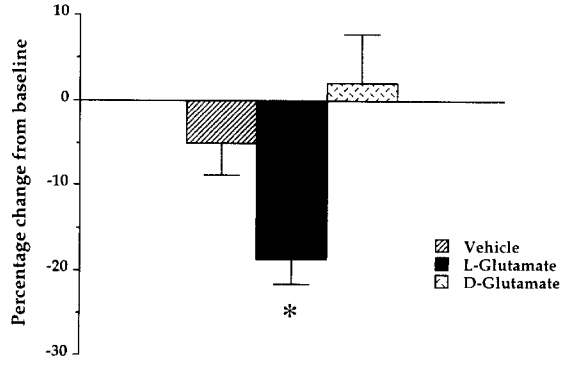

Fig. 1. Intraplantar injection of glutamate modulates thermal nociception in normal tissue. L-Glutamate (30 nmol) or PBS were injected into the rat hindpaw. Measurement of paw withdrawal latencies to radiant heat was then repeated 5 min later. Data in this figure display the percentage change in paw withdrawal latency from baseline 5 min after glutamate injection. Average baseline paw withdrawal latency across all groups = 10.3 ± 0.2 s. Error bars = S.E.M. (n = 17–25/group), $^{*}P < 0.05$ (compared to vehicle).

USA) was solubilized in 0.9% saline and 100 μl intraplantar (i.pl.) injections were made into the hindpaw.

All data are presented as the mean ± standard error of the mean. A one- or two-way analysis of variance was used to determine statistical differences between the groups according to the experimental design. Duncan's multiple range test was used for post-hoc comparisons between groups.

3. Results

The effects of intraplantar administration of glutamate on thermal hyperalgesia are presented in Fig. 1. The injection of 30 nmol of glutamate reduced the threshold of thermal nociception in the rat hindpaw withdrawal assay within 5 min (average baseline paw withdrawal latency across all groups = 10.3 ± 0.2 s). This dose of glutamate reduced the paw withdrawal latency by 18.6 ± 3.0% relative to baseline levels when compared to the 5.0 ± 3.7% change from baseline observed in the vehicle-treated animals ($F(2,60) = 6.33$; $P < 0.05$). To evaluate whether this effect was receptor mediated, the metabolically inactive stereoisomer D-glutamate was also evaluated. In the same experimental design and conditions, the D-isomer tended to increase the paw withdrawal latency at 5 min (2.1 ± 5.7%); however, this effect was not statistically different from that observed with the vehicle control.

Despite the stereospecificity of this behavioral response, it was possible that the observed changes in latencies could have been produced by factors unrelated to pain or hyperalgesia. One possible factor may have been an increase in cutaneous temperature which might facilitate responses without evoking hyperalgesia. To determine whether the observed changes in paw withdrawal latency may have been influenced by changes in cutaneous temperature, paw temperature was directly evaluated. The i.pl. administration of L-glutamate (29.9 ± 0.3°C) or phosphate buffered saline vehicle (29.8 ± 0.3°C) into the hindpaw did not change the local temperature of the hindpaw at 5 min relative to the baseline temperature (pooled average = 29.5 ± 0.2°C), ($F(1,14) = 1.11$; P = N.S.).

We next evaluated the hypothesis that endogenous excitatory amino acids are released in inflamed tissue and contribute to the development of thermal hyperalgesia during inflammation. The participation of peripheral NMDA receptors in contributing to thermal hyperalgesia during inflammation was evaluated with the non-competitive receptor antagonist (+)-MK-801. An intraplantar injection of either (+)-MK-801 (10 nmol) or vehicle control was performed in the inflamed hindpaw 2.5 h after the carrageenan injection and paw withdrawal latency was measured 5 min later. These results are presented in Fig. 2A. As depicted by the

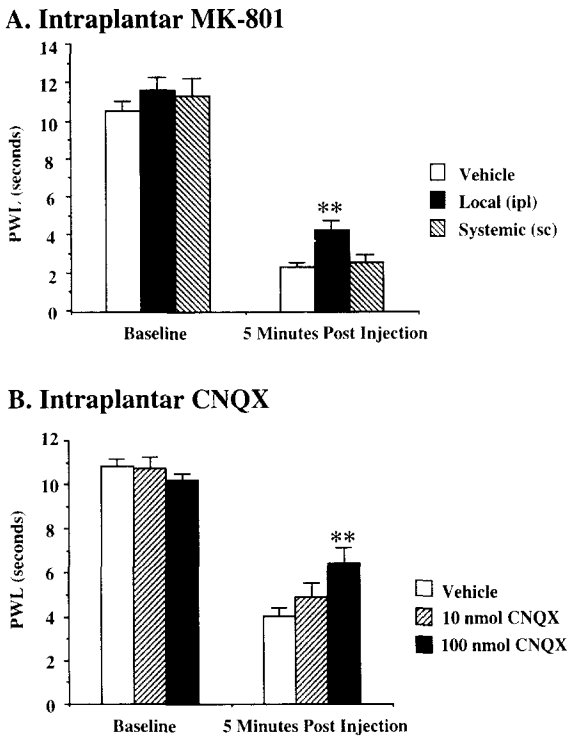

Fig. 2. Intraplantar injections of MK-801 and CNQX reduce thermal hyperalgesia in inflamed tissue. Inflammation was induced by the i.pl. injection of carrageenan (2 mg) into the hindpaw of male Sprague-Dawley rats. Inflammation was allowed to develop for 2.5 h before the injection of either MK-801, CNQX, or vehicle into the inflamed paw. Panel A displays paw withdrawal latency results 5 min after the injection of MK-801 (10 nmol). To evaluate for potential systemic effects at this dose, one group of animals received an equivalent s.c. dose of MK-801. (Baseline paw withdrawal latencies across all groups = 11.2 ± 0.4 s.) Panel B displays the paw withdrawal latency results 5 min after the i.pl. injection of CNQX (10 and 100 nmol). (Baseline paw withdrawal latencies across all groups = 10.6 ± 0.2 s.) Error bars = S.E.M., n = 9–15/group; $^{**}P < 0.01$ (compared to vehicle).

shaded bars in this figure, the administration of (+)-MK-801 significantly increased paw withdrawal latency values as compared to vehicle injection (unfilled bars) at 5 min (4.3 ± 0.5 s vs. 2.4 ± 0.2 s; $F(2,36) = 8.07$; $P < 0.01$). This effect appears to be receptor specific since the i.pl. administration of the stereoisomer (−)-MK-801 (30 nmol) in another series of experiments resulted in no change in the paw withdrawal latency relative to vehicle at 5 min (3.6 ± 0.4 s vs. 3.4 ± 0.3 s; $F(2,70) = 3.65$; P = N.S.).

To evaluate whether the observed effects of (+)-MK-801 were attributable to peripheral NMDA receptors in the inflamed tissue and not a centrally mediated effect, (+)-MK-801 (10 nmol) or vehicle control were injected 2.5 h after carrageenan injections into either the hindpaw or the subcutaneous tissue of the neck using a double-dummy experimental design. Measurement of paw withdrawal latency was made 5 min after these injections. The striped bars in Fig. 2A demonstrates that the systemic injection of this dose of (+)-

MK-801 did not alter the paw withdrawal latency relative to the vehicle control (subcutaneous (+)-MK-801 = 2.6 ± 0.4 s; P = N.S. relative to vehicle), signifying a peripherally mediated effect in this behavioral assay.

CNQX, a competitive antagonist of AMPA/kainate receptors, was also evaluated in this paradigm for peripheral antinociceptive effects. Similar to the (+)-MK-801 experiments described above, 2.5 h after inducing inflammation in the hind paw with carrageenan, either vehicle or CNQX (10 or 100 nmol) was injected into the inflamed paw and latencies were measured 5 min later. Fig. 2B shows the attenuation of the paw withdrawal latency by CNQX. As depicted by the shaded bars, the intraplantar injection of CNQX (100 nmol) significantly increased the paw withdrawal latency as compared to the vehicle treated hindpaw (6.4 ± 0.7 s vs. 4.0 ± 0.4 s; $F(2,31) = 4.43$; $P < 0.01$).

The local effects of MK-801 and CNQX were also evaluated in normal tissue to determine if the antinociceptive behavioral effects were specific for inflamed tissue or could be attributed to other effects such as local anesthesia. While the i.pl. injection of lidocaine hydrochloride (1.0 mg/paw) produced a profound antinociceptive effect at 5 min post-injection relative to phosphate buffered saline as measured by paw withdrawal latency (19.0 ± 1.7 s vs. 8.8 ± 0.4 s; $F(3,41) = 22.3$; $P < 0.01$), (+)-MK-801 (10 nmol) had no effect in normal tissue (8.5 ± 0.9 s; P = N.S.). Interestingly, i.pl. CNQX (100 nmol) reduced paw withdrawal latency (6.3 ± 0.6 s; $P < 0.05$) in normal tissue.

4. Discussion

The present study used two independent experimental approaches to evaluate whether excitatory amino acids have a peripheral site of action for modulating thermal nociception. The results indicate that glutamate evokes a stereospecific thermal hyperalgesic response following local intraplantar injection in rats. Further, local intraplantar administration of NMDA or AMPA/kainate receptor antagonists attenuates carrageenan-induced thermal hyperalgesia. Collectively, these results support the hypothesis that excitatory amino acids act in peripheral tissue to activate primary afferent neurons involved in nociceptive signalling.

The presence of glutamate binding sites in peripheral tissue has been evaluated by our group (Jackson et al., 1993). Using tritiated glutamate in a radioreceptor binding assay, it was determined that there are specific binding sites for glutamate within peripheral tissue such as dental pulp. The relative glutamate receptor subtype population within this tissue could be quantitatively distinguished and was characterized as NMDA > kainate > AMPA on the basis of total binding.

Peripheral activation of primary afferent neurons by glutamate has been suggested by an in vitro neonatal rat spinal cord-tail preparation which measures reflexive ventral root potentials (Ault and Hildebrand, 1993a). This effect was stereospecific (the D-isomer was inactive) and none of the other amino acids tested evoked a response, including aspartate. This response is presumed to be a nociceptive reflex since the algogens capsaicin and bradykinin also evoke ventral root potentials in this model. Using the same preparation, it has also been demonstrated that the excitatory amino acid analogues kainate and domoate produce ventral root potentials that can be antagonized by the AMPA/kainate receptor antagonist DNQX (Ault and Hildebrand, 1993b). Caution should be observed in the interpretation and generalization of these effects in peripheral tissue however since the work of Ault et al. was performed in neonatal animals, a model which may differ from the peripheral environment of adult animals.

Our present results indicate that glutamate is capable of decreasing the thermal nociceptive threshold in normal tissue of adult rats. This peripheral response is probably receptor mediated since the stereoisomer D-glutamate is inactive in this assay. Since there were no changes in the temperature of the hindpaws after the i.pl. injections of glutamate relative to baseline, it is suggested that the observed response can be attributed to hyperalgesia and not to local changes in the tissue, like altered blood flow or cutaneous temperature.

Additionally, our results suggest that activation of peripheral NMDA and AMPA/kainate receptors contributes to the development of the thermal hyperalgesia during carrageenan-induced inflammation, since the non-competitive NMDA receptor antagonist MK-801 as well as the competitive AMPA/kainate receptor antagonist CNQX both significantly reduce thermal hyperalgesia in rats. Our results suggest that the local antinociceptive effect of MK-801 is receptor mediated, since the stereoisomer (−)-MK-801 is inactive in this behavioral assay. While it is recognized that (+)-MK-801 can attenuate thermal hyperalgesia through mechanisms mediated within the central nervous system, our results indicate an additional, peripheral site of action since the systemic administration of (+)-MK-801 at this low dose was without effect. The antinociceptive effects observed with the local administration of the glutamate receptor antagonists (+)-MK-801 and CNQX appear to be limited to inflamed tissue because they displayed no antinociceptive effects in paw withdrawal latencies when injected into normal tissue at the same doses.

Collectively, these results suggest that excitatory amino acids can modulate thermal hyperalgesia via peripheral activation of small diameter afferents in addition to the well characterized central mechanisms.

These findings are of physiologic and pharmacologic significance because activation of excitatory amino acid receptors in peripheral tissue may partially contribute to inflammatory mediated pain states. Accordingly, these peripheral receptors may represent an additional target for analgesic therapies in the future. The source of endogenous glutamate released into inflamed tissue and the cellular location of glutamate receptors require additional research.

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