Blockade of N- and P/Q-Type Calcium Channels Reduces the Secondary Heat Hyperalgesia Induced by Acute Inflammation

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Accepted for publication June 1, 1998
This paper is available online at http://www.jpet.org

ABSTRACT
High voltage calcium channels are implicated in nociceptive transmission after nerve injury, capsaicin or formalin injection. The purpose of this study was to investigate the role of calcium channels in secondary heat hyperalgesia associated with acute joint inflammation. After induction of acute inflammation (knee joint injection of kaolin and carrageenan), decreased paw withdrawal latency (PWL) to radiant heat (i.e., secondary hyperalgesia), increased guarding of the limb and increased joint circumference occurs. Spinal administration (through a microdialysis fiber placed in dorsal horn) of an N-type calcium channel blocker (MVIIA, SNX 111, ziconotide, 0.001–0.1 mM), before induction of inflammation, prevents the decrease in PWL. Treatment with SNX 111 4 hr after induction reverses heat hyperalgesia. A small reduction in spontaneous pain-related behaviors (guarding of the limb) occurs after pre- or post-treatment with SNX 111. Spinal blockade of P/Q-type calcium channels (with ω-agatoxin IVA) had no effect on the decrease in PWL to radiant heat when administered after induction of inflammation. However, pre-treatment with ω-agatoxin IVA prevents secondary heat hyperalgesia. ω-Agatoxin IVA has no effect on spontaneous pain-related behaviors whether administered before or after induction of inflammation. In contrast, pre or post-treatment with nifedipine (L-type calcium channel blocker, 0.01–1.0 mM), had no effect on heat hyperalgesia or spontaneous pain-related behaviors induced by acute inflammation. There were no differences in joint circumference between groups with any treatment. Thus, N-type calcium channels contribute to both the development and maintenance of secondary heat hyperalgesia while P-type calcium channels are only involved during development of hyperalgesia.

Received for publication April 1, 1998.

ABBREVIATIONS: PWL, paw withdrawal latency; ANOVA, analysis of variance; ACSF, artificial cerebrospinal fluid; NMDA, N-methyl-D-aspartate.
sive behaviors, specifically spontaneous pain behaviors and mechanical hyperalgesia and allodynia (Malmberg and Yaksh, 1994; Chaplan et al., 1994; Bowersox et al., 1996; Sluka, 1997). For example, Malmberg and Yaksh (1994) demonstrated that intrathecal administration of a P/Q-type calcium channel blocker reduced the nocifensive behaviors induced by formalin if delivered before injection of formalin but not after injection. From this data it could be concluded that spinal P/Q-type calcium channels were important in the initiation but not in the maintenance of the behavioral responses in the formalin test. In the same study, blockade of N-type calcium channels reduced nocifensive pain behaviors if delivered either before or after formalin injection while L-type calcium blockers had minimal effects (Malmberg and Yaksh, 1994). In a more chronic pain model, neuropathic pain, blockade of N-type channels reduced mechanical allodynia while blockade of L- and P/Q-type channels had no effect (Chaplan et al., 1994). Thus, different pain models, different times of activation of calcium channels and different test stimuli may be important factors in the role of calcium channels in the development and maintenance of nocifensive behaviors induced by tissue injury.

Intraarticular injection of kaolin and carrageenan results in an acute inflammation that is associated with nocifensive behaviors including secondary heat hyperalgesia and guarding of the inflamed hindlimb (Sluka and Westlund, 1993). Recordings from peripheral nerves innervating the knee joint show an increased activity in type II, III and IV primary afferent fibers (Schaible and Schmidt, 1985, 1988). This increased activity in primary afferent fibers is transmitted centrally to the spinal cord and results in sensitization of dorsal horn neurons. Extracellular recordings of dorsal horn neurons demonstrate an increase in receptive field size, increase in background activity and an increase in responsiveness to innocuous mechanical stimuli (Schaible et al., 1987; Dougherty et al., 1992). The secondary hyperalgesia is thought to reflect changes in central neurons (Willis and Coggeshall, 1991). Hypercalcitability of dorsal horn neurons is reduced by calcium channel blockers (Neugebauer et al., 1996; Nebe et al., 1997).

This study was designed to address the spinal roles of the N-, P/Q- and L-type calcium channels in the nocifensive behaviors associated with acute joint inflammation. Knee joint inflammation induced by intraarticular injection of kaolin and carrageenan into the knee joint was used as a model of acute arthritis. The calcium channel antagonists were delivered either before or after induction of inflammation to assess the time-dependent nature of the calcium channels. The hypotheses tested were that 1) spinal blockade of N-, P/Q- and L-type calcium channels would prevent the heat hyperalgesia (if delivered before knee joint injection) or 2) spinal blockade of N- and L-type but not P/Q-type calcium channels would reduce the hyperalgesia (if delivered after development of hyperalgesia).

Methods

Placement of microdialysis fiber. All experiments were approved by the Animal Care and Use Committee at our institution. A microdialysis fiber was implanted into the spinal dorsal horn of the rat (male Sprague-Dawley, n = 64) according to the protocol of Skilling et al. (1998), as described previously (Sluka and Westlund, 1992). Briefly, rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and a microdialysis fiber (200 μm o.d., 45,000 MW cut-off, Hospal AN69) was passed transversely through the deep dorsal horn of the spinal cord (L5 spinal segment) and stabilized with dental cement applied to the bone. The microdialysis fiber was permeable only where it was positioned in the grey matter of the spinal cord (2 mm gap). ACSF was infused through the microdialysis fiber at a rate of 5 μl.min⁻¹ for delivery of drugs. This method is used for local delivery of drugs to the lumbar enlargement, one spinal segment (Sluka and Westlund, 1993). All animals had normal gross motor functioning as evidenced by normal gait, stepping reflex, righting reflex and rotorod test the day after insertion of microdialysis fibers.

Knee joint injection. Knee joint inflammation was induced while the rat was briefly anesthetized with halothane (2–4%) delivered via a vaporizer. One knee joint of each of the animals was injected with a mixture of 3% kaolin and 3% carrageenan (0.1 ml; pH 7.4) in sterile saline.

Behavioral testing and assessment of inflammation. As a measure of heat hyperalgesia, animals were tested for PWL to radiant heat according to the protocol first described by Hargreaves et al. (1988). Briefly, animals were placed in clear plastic cages on an elevated glass plate. Radiant heat was applied to the plantar surface of the hindpaw until the rat lifted its paw. The time at which this occurred was considered the PWL. Both paws were tested independently for five trials per side with a 5-min waiting period between trials. The right and left paws were tested consecutively, alternating which paw was tested first between each of the five trials. Each testing period lasted 30 min. Since inflammation is confined to the knee joint (site of injury) and we are testing the paw to radiant heat stimuli (outside the site of injury) this test is a measure of secondary heat hyperalgesia. Secondary hyperalgesia is thought to reflect changes in central neurons (see Willis and Coggeshall, 1991).

To quantify the abnormal posture of the hindpaw induced by the arthritis, the animals were rated on a subjective spontaneous pain-related behavior scale (0–5) based on position of the hindpaw and weight bearing status as previously published (Sluka and Westlund, 1993). The following scale was used: 0 was normal; 1 was curling toes; 2 was eversion of the foot; 3 was partial weight bearing; 4 was non-weight bearing and guarding; and 5 avoidance of any contact with the limb. The spontaneous pain-related behaviors are a measure of limb guarding that commonly occurs after injury.

Knee joint circumference was measured with a flexible tape measure around the center of the knee joint while the joint was held in extension. The knee joints were measured before (baseline), and at varying time points after injection of the knee joint with kaolin and carrageenan. Joint circumference is a measure of joint swelling commonly used clinically to assess the degree of inflammation.

Experimental groups. Male Sprague-Dawley rats (250–350 g) were treated with the N-type calcium channel blocker, SNX 111 (ziconotide, MVIIA, 0.001–0.1 mM; n = 15); the P/Q-type calcium channel blocker, ω-agatoxin IVA (0.01–1 μM, n = 16) or the L-type calcium channel blocker, nifedipine (0.01–1 mM; n = 11, 1 mM nifedipine was dissolved in 30% DMSO) regimens: 1) pretreatment through a microdialysis fiber placed in the dorsal horn with the highest effective dose for 1 hr immediately before induction of arthritis (SNX111, 0.1 mM; ω-agatoxin IVA, 1 μM; nifedipine, 1 mM); 2) post-treatment through a microdialysis fiber in the dorsal horn 4 hr after induction of arthritis with continuous infusion of cumulative concentrations.

Doses for the calcium channel blockers were based on those used in a previous study with the same method of administration that showed a reduction of mechanical hyperalgesia (Sluka, 1997). The dose of nifedipine was limited to 1 mM because of its solubility. At 1 mM 30% DMSO was required for nifedipine to go into solution.

Control groups of arthritic rats included: 1) pretreatment (n = 6) and post-treatment (n = 6) with ACSF in 30% DMSO through a
microdialysis fiber (pH 7.2–7.4); 2) Pretreatment through a microdialysis fiber with the inactive conopeptide, SNX 157 (n = 6).

In experimental and control groups, a microdialysis fiber was implanted in the dorsal horn 1 day before the experiment for delivery of receptor antagonists locally in the spinal cord. On day 2, the animals were housed in small Lucite cubes, which limited their movement but provided food and water. Baseline testing (see below) of the PWL was done for comparison with latencies after induction of knee joint inflammation.

In animals pretreated with receptor antagonists through a microdialysis fiber the drug was delivered for 1 hr after baseline behavioral testing. After drug administration, PWL to radiant heat was again tested. The knee joint was then injected with a mixture of 3% kaolin and 3% carrageenan. The animals were allowed to awaken and to move about their cages. The PWL test was then performed at 4 hr, 6 hr and 8 hr after induction of arthritis. In animals treated after the development of heat hyperalgesia, the knee joint was injected after baseline behavioral testing. Receptor antagonists or their inactive stereoisomers were administered after PWL testing at 4 hr since hyperalgesia is fully developed (Sluka and Westlund, 1993). Successively increasing concentrations were administered for a total of 1.25 hr per dose. Behavioral testing was repeated during the last 30 min after each dose was given.

Statistical analysis. A repeated measures analysis of variance (ANOVA) was used to compare the PWL and joint circumference before, after induction of arthritis, and after administration of receptor antagonists for both the ipsilateral and the contralateral paws. If significance was obtained (P < .05) differences between groups were compared by t tests. Since pain-related behavior ratings did not have a normal distribution, a Friedman’s ANOVA was performed. Post-hoc testing with a sign test compared differences between groups. All data are expressed as the mean ± S.E.M.

Results

Pretreatment with calcium channel blockers resulted in a significant effect for time (F_{4,100} = 56.26, P = .0001) and time × group (F_{4,100} = 5.77, P = .0001) for changes in PWL. Post-treatment with calcium channel blockers also resulted in a significant effect for time (F_{4,88} = 48.0, P = .0001) and time × group (F_{12,88} = 22.95, P = .05) for changes in PWL. Furthermore, there was an overall effect for group for changes in spontaneous pain-related behaviors after pretreatment with calcium channel blockers (χ^2 = 7.1, P = .03) but not post-treatment (χ^2 = 3.0, P = .39). No difference between groups was observed for joint circumference with either pretreatment or post-treatment. Thus, there were significant differences between groups for changes in PWL and pain-related behaviors, but not joint circumference. Specific details are described below.

Control arthritic animals. After induction of acute inflammation there was a significant decrease in the latency of withdrawal from radiant heat applied to the paw (i.e., heat hyperalgesia), increased pain-related behavior ratings, an increased joint circumference. The PWL decreased from 10.87 ± 0.45 sec to 7.57 ± 0.32 sec in control arthritic animals treated with ACSF (fig. 1). The pain-related behavior ratings before induction of inflammation were zero and increased by 4 hr to ∼4 in control arthritic animals (see fig. 2 for specific numbers). This increase in pain-related behaviors remained increased through 8 hr. The ipsilateral joint circumference increased by 15 to 20 mm in control arthritic animals by 4 hr from baseline of 60 to 70 mm, and it remained increased 8 hr after induction of inflammation. There were no changes on the contralateral side for PWL or joint circumference.

Microdialysis administration of calcium channel blockers

Blockade of N-type calcium channels with SNX 111 (ziconotide). Spinal treatment with SNX 111 before induction of arthritis significantly prevented the decrease in PWL 4, 6 and 8 hr after inflammation compared with animals treated with the inactive conopeptide SNX 157 (fig. 1). Similarly, treatment with SNX 111 after induction of arthritis reversed the decrease in PWL. A significant increase in PWL was observed compared with animals treated with ACSF as a control for the 0.1 mM dose (fig. 1).

Spontaneous pain-related behaviors from animals pretreated with SNX 111 were significantly less than those from animals pretreated with SNX 157 8 hr after induction of inflammation (fig. 2). The effects of pretreatment with SNX 111 on spontaneous pain-related behaviors were minimal, resulting behaviorally in an increase in weight bearing on the limb. Post-treatment with SNX 111 decreased the pain-related behaviors, but not post-treatment (χ^2 = 5.77, P = .0001) and time × group (F_{12,88} = 22.95, P = .05) for changes in PWL.
iors from animals treated with ACSF and those treated with controls treated with ACSF. There was also no significant decrease in PWL induced by acute inflammation compared to animals pretreated (top) or post-treated (bottom) with calcium channel antagonists or ACSF. Pretreatment with SNX 111 resulted in a minimal but significant effect on pain-related behavior ratings 8 hr after induction of inflammation compared with ACSF control animals. Post-treatment with 0.1 mM (D3) SNX 111 significantly reversed the pain-related behavior ratings compared with ACSF control animals. The doses (D1, D2 and D3) are the same as for figure 1. *Significantly different from control animals, P < .05.

related behaviors with the highest dose (0.1 mM), showing a significant difference from animals treated with ACSF.

Blockade of P/Q-type calcium channel with ω-agatoxin IVA. Spinal treatment with ω-agatoxin IVA before the induction of arthritis significantly prevented the decrease in PWL 4 and 6 hr after inflammation, but PWL decreased significantly by 8 hr, compared with control animals treated with ACSF (fig. 1). In contrast, post-treatment with increasing doses of ω-agatoxin IVA had no significant effect on the decrease in PWL induced by acute inflammation compared with controls treated with ACSF. There was also no significant difference between the spontaneous pain-related behaviors from animals treated with ACSF and those treated with ω-agatoxin IVA. Thus, pretreatment with ω-agatoxin IVA prevents the decrease in PWL, but not the spontaneous pain-related behaviors.

Blockade of L-type calcium channels with nifedipine. Spinal treatment with nifedipine either before or after induction of inflammation had no effect on the decrease in PWL or the pain-related behaviors (figs. 1 and 2).

Discussion

The current study demonstrated a secondary heat hyperalgesia and increased spontaneous pain-related behaviors after intraarticular injection of kaolin and carrageenan in rats. A spinal role for the N- and P/Q-type calcium channels for the development of secondary heat hyperalgesia induced by joint inflammation was demonstrated. The doses used in these studies were based on those used in a previous study with the same method of delivery by microdialysis (Sluka, 1997). The maximal doses used in the current study all prevented mechanical allodynia observed after capsaicin injection in a previous study (Sluka, 1997). In the current study, the lack of effect by calcium channel blockers on baseline responses to heat before injection of capsaicin or on the contralateral side indicated that the doses used did not reduce or block normal neuronal responses in the unsensitized animal.

Several investigators have shown a role for calcium channels in spontaneous pain behaviors and mechanical allodynia. However, this is the first study to demonstrate a role for calcium channels in secondary heat hyperalgesia. The same pattern of inhibition of nocifensive behaviors was observed by Malmberg and Yaksh (1994) in the formalin test when the antagonists were delivered spinally. They observed that blockade of 1) N-type calcium channels reduced the number of flinches if delivered either before or after injection of formalin; 2) P/Q-type calcium channels prevented the number of flinches if given before but not after injection of formalin; and 3) L-type calcium channels produced a minimal effect. Recordings from dorsal horn neurons after formalin injection also demonstrated the same pattern of inhibition (Diaz and Dickenson, 1997). Codere and Melzack (1992) reduced nocifensive behaviors associated with formalin with an L-type calcium channel antagonist. However, only a minimal reduction in nocifensive behaviors was observed as compared to a NMDA glutamate receptor antagonist (MK801). The secondary mechanical allodynia observed after capsaicin injection was prevented by spinal administration of antagonists at N-, P/Q- and L-type calcium channels before injection of capsaicin (Sluka, 1997). However, the threshold to mechanical stimuli remained at baseline after pretreatment with a P/Q-type calcium channel blocker (completely prevented) and was significantly elevated (but not completely prevented) with N-type and L-type calcium channel blockers.

In a more chronic pain model, mechanical alldynia associated with neuropathic pain was reduced by N-type but not P/Q- or L-type calcium channel blockers (Chaplan et al., 1994; Bowersox et al., 1996).

Using the kaolin and carrageenan model of knee joint inflammation, Neugebauer et al. (1996) demonstrated that blockade of N-type or L-type calcium channels reduced the mechanical hypersensitivity of dorsal horn neurons. However, there was also a reduction in the responses to mechanical input from the normal non-inflamed knee joint. This is in contrast to the current study and a previous report (Sluka, 1997) which demonstrated that at doses administered spinally there was no effect on baseline responses to heat or mechanical stimuli but a reduction in secondary hyperalgesia. This suggests a qualitatively higher dose of antagonist was used in the studies by Neugebauer et al. (1996) compared with the current study. Similar to the current study, Nebe et al. (1997) showed that blockade of P-type calcium channels reduced responses to mechanical stimulation from the inflamed but not the normal knee joint. Neither of these studies tested the responses of dorsal horn neurons to heat stimuli before or after induction of inflammation. The majority of the data support a role for N-type calcium channels in nociception associated with tissue injury induced by a variety of different stimuli. However, the role for P/Q-type and L-type calcium channels in nociceptive transmission after tissue injury is more variable between studies. Most of the data...
support that spinal P/Q-type calcium channels are involved early in the development of hyperalgesia while L-type calcium channels are only minimally involved in nociceptive behaviors. Thus, different pain models (formalin, carrageenan, capsaicin, neuropathy) the time of activation of calcium channels (early or late), different assessments (spontaneous behaviors, mechanical or heat) may be important factors in deciphering the role of the calcium channels in the development and maintenance of nociceptive behaviors induced by tissue injury.

N-type calcium channels in the dorsal horn. From the current study, it is concluded that spinal N-type calcium channels are involved in both the induction and maintenance of secondary heat hyperalgesia induced by acute joint inflammation. It is not possible to conclude if the channels are located presynaptically on primary afferent terminals or on dorsal horn neurons. However N-type calcium channels are found predominately in neuronal tissue and have been shown to be involved presynaptically in regulating neurotransmitter release (Miller, 1987; Hirning et al., 1988). Anatomical localization of N-type calcium channels has been demonstrated in the dorsal horn by autoradiography (Kerr et al., 1988; Takemura et al., 1989). The greatest concentration of these calcium channels is in the superficial dorsal horn where primary afferent fibers terminate. In support of a presynaptic role for N-type calcium channels on primary afferent fibers, release of neuropeptides contained in primary afferent fibers in spinal dorsal horn tissue is blocked by the N-type calcium channel blocker, omega-conopeptide GVIA (Maggi et al., 1990; Santicioli et al., 1992). Thus, the blockade of N-type calcium channels is presumed to be presynaptic on primary afferent terminals in the dorsal horn and would reduce release of neurotransmitters from the central terminals of primary afferents. Blockade of release from primary afferent fibers would reduce activity in dorsal horn neurons and thus further reduce the release of neurotransmitters. Overall this would present as a decrease in central sensitization and thus secondary hyperalgesia.

P-type calcium channels in the dorsal horn. The current study demonstrated that pretreatment but not post-treatment with omega-agatoxin IVA reduced the secondary heat hyperalgesia associated with joint inflammation. Anatomical localization of the P-type channel has been shown in cerebellum, cortex, ventral periaqueductal gray, substantia nigra, hippocampus and some brainstem nuclei (Hillman et al., 1991). However, anatomical localization of P-type calcium channels in the spinal cord and dorsal root ganglion has not been demonstrated. P-type calcium channels have been demonstrated on spinal and dorsal root ganglia neurons using electrophysiological techniques (Mintz et al., 1992a). Blockade of P-type channels may reduce calcium entry postsynaptically or neurotransmitter release presynaptically. In addition, P-type calcium channels show little inactivation and thus may contribute to continued calcium influx either through presynaptic or postsynaptic membranes upon activation (Llinas et al., 1989a, 1989b; Mintz et al., 1992a, 1992b; Spedding and Paoletti, 1992). Continued calcium influx would then result in increased release of neurotransmitters and increased intracellular calcium which would activate second messenger systems resulting in long term changes.

Role of L-type. The lack of effect with the L-type calcium channel blocker on secondary hyperalgesia supports the view that calcium influx through L-type calcium channels are not involved in central sensitization or secondary hyperalgesia associated with joint inflammation. L-type calcium channels are located on dorsal horn neurons, in particular cell bodies throughout the spinal cord with an equal distribution of cells across the dorsal and ventral horns of the spinal cord (Ahlijanian et al., 1990). From the work of Neugebauer et al. (1996), it appears that L-type calcium channels are involved in sensitization of central neurons to mechanical stimuli induced by joint inflammation. However, the dose administered also reduced the responses of unsensitized neurons. The dose in the study by Neugebauer et al. (1996) is most likely higher than that used in the current study (nifedipine had no effect on baseline PWL to heat). Similarly, mechanical allodynia induced by capsaicin injection is prevented by blockade of L-type calcium channels (Sluka, 1997). L-type calcium channels are not thought to be involved in release of neurotransmitters (see Spedding and Paoletti, 1992) but rather to be located postsynaptically. These channels would therefore be involved in neuron depolarization and influx of calcium intracellularly. Influx of calcium intracellularly might then result in activation of signal transduction cascades.

In summary, blockade of N-type calcium channels were most effective in reducing secondary heat hyperalgesia and spontaneous pain-related behaviors associated with acute joint inflammation. SNX 111, delivered spinally before inflammation or after the development of hyperalgesia, reduced the nociceptive behaviors. Blockade of P-type calcium channels was only effective if delivered before the induction of inflammation while blockade of L-type calcium channels had no effect. Thus, spinal N-type calcium channels contribute to both the development and maintenance of secondary heat hyperalgesia while spinal P-type calcium channels are only involved during the development of secondary hyperalgesia.

Acknowledgements
I thank Drs. G. F. Gebhart, T. J. Brennan and C. Cleland for critically reading the manuscript and Chis Fisher for his excellent technical assistance. I also thank Neurex Corporation for supplying the ziconotide (SNX 111) and SNX 157.

References

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