Published in final edited form as:

J Pain. 2008 December ; 9(12): 1096–1105. doi:10.1016/j.jpain.2008.06.005.

Spinal Dynorphin and Bradykinin Receptors Maintain Inflammatory Hyperalgesia

Miaw-Chyi Luo^{*}, Qingmin Chen^{*}, Michael H. Ossipov, David R. Rankin, Frank Porreca, and Josephine Lai

Department of Pharmacology, University of Arizona Health Sciences Center, Tucson, Arizona 85724, U.S.A

Abstract

An upregulation of the endogenous opioid, dynorphin A, in the spinal cord is seen in multiple experimental models of chronic pain. Recent findings implicate a direct excitatory action of dynorphin A at bradykinin receptors to promote hyperalgesia in nerve injured rats, and its upregulation may promote, rather than counteract, enhanced nociceptive input due to injury. Here we examined a model of inflammatory pain by unilateral injection of Complete Freund's Adjuvant (CFA) into the rat hind paw. Rats exhibited tactile hypersensitivity and thermal hyperalgesia in the inflamed paw by 6 hr after CFA injection, while a significant elevation of prodynorphin transcripts in the lumbar spinal cord was seen at day 3 but not at 6 hr. Thermal hyperalgesia at day 3, but not at 6 hr, after CFA injection was blocked by intrathecal administration of anti-dynorphin antiserum or by bradykinin receptor antagonists. The antihyperalgesic effect of the latter was not due to *de novo* production of bradykinin or upregulation of spinal bradykinin receptors. These data suggest that elevated spinal dynorphin upon peripheral inflammation mediates chronic inflammatory hyperalgesia. The antihyperalgesic effect of bradykinin receptor antagonists requires the presence of upregulated spinal dynorphin but not of *de novo* production of bradykinin, supporting our hypothesis that pathological levels of dynorphin may activate spinal bradykinin receptors to mediate inflammatory hyperalgesia.

Perspective—This study shows that chronic peripheral inflammation induces a significant upregulation of the endogenous opioid peptide dynorphin. Elevated levels of spinal dynorphin and activation of spinal bradykinin receptors are essential to maintain inflammatory hyperalgesia. The results suggest that blockade of spinal bradykinin receptors may have therapeutic potential in chronic inflammatory pain.

Keywords

spinal cord; G protein coupled receptor; nociceptors; Complete Freund's Adjuvant; pain; inflammation

Corresponding Author: Josephine Lai, Ph.D., Department of Pharmacology, The University of Arizona Health Sciences Center, 1501 N. Campbell Ave., Tucson, AZ 85724, U.S.A., Tel: (520) 626-2147, FAX: (520) 626-4182, E-mail: lai@u.arizona.edu.

^{*}M.C.L. and Q.C. contributed equally to this study

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Introduction

The endogenous opioid peptides consist of the enkephalins, the endorphins and the dynorphins³¹ which are proteolytic products of proenkephalin, proopiomelanocortin, and prodynorphin, respectively. Dynorphin A(1–17) [YGGFLRRIRPKLKWDNQ] is one of the major proteolytic fragments of prodynorphin⁶ and is widely distributed in the central nervous system⁶. Unlike the enkephalins or endorphins, however, administration of dynorphin A either systemically or intrathecally produces little or no antinociception^{9,14,45}. This unexpected property of an endogenous opioid was thought to be due to the rapid breakdown of dynorphin A upon release⁵, but subsequent analyses showed that dynorphin A and its proteolytic products had other, non-opioid effects that may confound its actions at opioid receptors, including motor deficits^{9,35}, neurotoxicity²⁶, release of excitatory neurotransmitters^{12,22,33} and inflammatory mediators²², and prolonged hyperalgesia^{39,40}. Many experimental models of pathological pain, including inflammatory pain^{17,28}, neuropathic pain^{21,27}, sustained opioid induced pain⁴⁰, bone cancer pain²⁹, spinal cord trauma^{1,10,37}, arthritis⁴⁸, and chronic pancreatitis⁴² show a significant regional elevation of dynorphin A in the spinal cord. Elevated levels of dynorphin appear critical for the expression of some aspects of these chronic pain conditions, even though dynorphin also activates opioid receptors concurrently to mitigate, to some degree, its pronociceptive effects in neuropathic pain states⁵¹. The hypothesis that upregulation of spinal dynorphin produces net pronociceptive effects and is important in the maintenance of chronic pain is supported by the following findings: spinal administration of an anti-dynorphin A antiserum reduces neurological impairment after nerve injury¹¹, blocks the increased sensitivity to noxious thermal and innocuous mechanical stimuli and attenuates trauma induced changes in the spinal cord⁴⁹, but does not alter normal sensory thresholds in non-injured rats^{27,44}. Transgenic mice that carry a null mutation in the prodynorphin gene do not exhibit persistent pain states after peripheral nerve injury when compared with their wild type littermates⁴⁷.

We reported recently that dynorphin A and its non-opioid fragment, dynorphin A_{2–13}, bind to B1 and B2 bradykinin receptors²⁴. Dynorphin A_{2–13}, which has very low affinity (>10 μM) for opioid receptors, activates the B2 receptors in a dorsal root ganglion X neuroblastoma cell line, F-11, to cause a transient influx of calcium via L-type and P/Q type voltage-gated calcium channels. Similar response to dynorphin was seen in primary cultures of dorsal root ganglia. As high concentration of dynorphin promotes its activation of bradykinin receptors, regional upregulation of spinal dynorphin associated with many pathological pain states may be pronociceptive by activating bradykinin receptors in the spinal cord. Evidence from rats with peripheral nerve injury showed that bradykinin receptor antagonists were effective in reversing nerve injury induced tactile and thermal hypersensitivity only at times when spinal dynorphin was upregulated. Here we examined an experimental model of inflammatory hyperalgesia in response to unilateral intraplantar injection of Complete Freund's Adjuvant (CFA) into the rat hind paw. The treatment evokes a rapid onset of sensory hyper-responsiveness in the injected paw and significant upregulation of spinal dynorphin. We determined whether upregulation of spinal dynorphin upon a peripheral inflammatory insult acts to promote pain, and if so, whether activation of spinal bradykinin receptors is necessary for maintaining the inflammatory hyperalgesia associated with elevated spinal dynorphin.

Materials and Methods

Animal surgery and drug administration

Male Sprague-Dawley rats (225–300g) were used. All procedures were approved by the Institutional Animal Care and Use Committee and followed guidelines of the International Association for the Study of Pain and the National Institutes of Health. For experiments with intrathecal injection, rats were implanted with intrathecal (*i.th.*) catheters (polyethylene-10

tubing in length of 7.5 cm) as described previously⁵² and allowed 5 days to recover from surgery prior to further experiment. Animals that showed signs of motor dysfunction or behavioral abnormality were euthanized. To induce inflammation, rats received an intraplantar injection of 100 µl CFA (Sigma, St. Louis, MO) into the plantar surface of the left hind paw under brief isoflurane anesthesia. Baseline tactile response threshold and thermal response latency were determined prior to CFA injection and 72 hr post-CFA injection before drug administration. Bradykinin (0.15, 2 and 10 µg in 5 µl saline, Bachem Inc., Torrance, CA), control serum (5 µl, Calbiochem, San Diego, CA), anti-dynorphin antiserum (200 µg in 5 µl saline, Peninsula Laboratories Inc., San Carlos, CA), vehicle (saline, 5 µl), [Des-Arg⁹, Leu⁸]-bradykinin (DALBK, 50 nmol in 5 µl saline, Bachem Inc., Torrance, CA), or D-Arg-[Hyp³, Thr⁵, D-Tic⁷, Oic⁸]-bradykinin (HOE 140, 10 pmol in 5 µl saline, American Peptide Company, Inc., Sunnyvale, CA) was delivered to the lumbar region of the spinal cord via intrathecal catheters followed by a 9 µl saline flush. Nociceptive testing was then carried out on the left hind paw as described below 30 minutes after serum administration and 45 minutes after DALBK or HOE 140 injection.

Nociceptive testing – tactile hypersensitivity

Rats were placed in a suspended plastic chamber with a wire mesh platform and allowed to habituate for 15 minutes. Tactile hypersensitivity was determined by measuring paw withdrawal threshold in response to probing the plantar surface of the left hind paw with a series of eight calibrated von Frey filaments (0.40, 0.70, 1.20, 2.00, 3.63, 5.50, 8.50, and 15.0 g). Paw withdrawal threshold was determined by sequentially increasing and decreasing stimulus intensity (“up and down” method) and analyzed using a Dixon non-parametric test⁴.

Nociceptive testing – thermal hypersensitivity

Nociceptive responses to noxious heat were evaluated against noxious radiant heat and on the 52°C hot-plate. Animals were placed in clear plastic chambers on a glass surface and were habituated for 15 min prior to testing. Thermal sensitivity was determined by the withdrawal latency to an infrared heat source directed onto the plantar surface of the left hind paw, and was indicated by a motion detector that halted the source and a timer¹⁶. A maximum cut-off of 33 sec was employed to prevent tissue damage. The hot-plate test was performed by placing the animal in a glass cylinder on a temperature-controlled heated plate maintained at 52°C and determining the latency to hind paw licking. A cutoff time was set at 40 sec to prevent injury. The latencies were measured prior to surgery, and before and after the administration of drug or vehicle.

Experimental Design

Experiment 1: Rats were divided into 2 groups of 9. Baseline thermal latency (TL) and paw withdrawal threshold to von Frey filaments (PWT) were determined for all subjects. One group received unilateral intraplantar injection of saline and one group received unilateral intraplantar injection of CFA. TL and PWT were determined at time 6 hr, 24 hr, 48 hr and 72 hr. Three rats from each group were taken after the 6 hr testing and up to 6 were taken after the 72 hr testing for isolation of the ipsilateral dorsal horn of the spinal cord and L5 DRG. The tissues were used for qPCR analysis of prodynorphin, kininogen, and bradykinin B1 and B2 receptors. Additional rats, either untreated (naïve), or treated with saline or CFA were source of additional tissues for radioligand binding.

Experiment 2: Rats were implanted with *i.th.* catheters. Subjects were divided into 4 groups of 6. Baseline TL and PWT were determined for all subjects. All groups received unilateral intraplantar CFA. Two groups received either a control serum or an anti-dynorphin A antiserum intrathecally at 6 hr after CFA treatment. TL and PWT were determined before and 30 min

after *i.th.* administration. Two groups received the same treatment and testing at 72 hr after CFA treatment.

Experiment 3: Rats were implanted with *i.th.* catheters. Subjects were divided into 6 groups of 6. Baseline TL and PWT were determined for all subjects. All groups received unilateral intraplantar CFA. Three groups received either saline, HOE 140 or DALBK intrathecally at 6 hr after CFA treatment. TL and PWT were determined before and 45 min after *i.th.* administration. Three groups received the same treatment and testing at 72 hr after CFA treatment.

Experiment 4: Rats were implanted with *i.th.* catheters. The dose-effect of *i.th.* bradykinin on the sensory threshold of the hind paw to radiant heat or hot plate, or in response to von Frey filament probing, was measured over a period of 120 min after injection. For each behavioral test, subjects were divided into 3 groups of 6, and each group received 0.15, 2, or 10 μ g of bradykinin. Baseline sensory threshold was determined before injection and at designated intervals after injection.

Quantitative RT-PCR

Total RNA was isolated from the ipsilateral lumbar dorsal horn of the spinal cord and ipsilateral L5 dorsal root ganglion (DRG) using AurumTM total RNA mini kit (Bio-Rad, Hercules, CA). Quantitative RT-PCR was performed using the iCycler iQ Multicolor Real-Time PCR Detection System with iScript cDNA Synthesis Kit and iQ SYBR Green Supermix (Bio-Rad, Hercules, CA). Samples were run in triplicate using an annealing temperature of 60 °C. Primers for the amplification were: prodynorphin [forward: 5'-GCAAATACCCCAAGAGGAG-3' (nuc. 512 ~ 530); reverse: 5'-CGCAGAAAACCACCATAGC-3' (nuc. 659 ~ 677)]; kininogen [forward: 5'-CATGAGACCTTGGGAGAAC-3' (nuc. 1068 ~ 1086); reverse: 5'-GCACCCTGGCAATGTAGG-3' (nuc. 1214 ~ 1231)]; bradykinin B1 receptor [forward: 5'-GCATCTTCCTGGTGGTGG-3' (nuc. 401 ~ 418); reverse: 5'-CAGAGCGTAGAAGGAATGTG-3' (nuc. 543 ~ 562)]; bradykinin B2 receptor [forward: 5'-CTTTGTCCTCAGCGTGTTC-3' (nuc. 242 ~ 260); reverse: 5'-CAGCACCTCTCCGAACAG-3' (nuc. 385 ~ 402)]; GAPDH [forward: 5'-ATCATCCCTGCATCCACTG-3' (nuc. 610 ~ 628); reverse: 5'-GCCTGCTTCACCACCTTC-3' (nuc. 771 ~ 788)]. PCR efficiency for these gene targets was between 97 % and 100 %. The expression level of each target gene in the lumbar spinal cord was normalized to the expression of GAPDH. The differences of target gene expression between treatments were analyzed using the Comparative C_T Method (ABI Prism 7700 Sequence Detection System User Bulletin #2, p11–15, 2001). The threshold cycle (C_T) is defined as the cycle at which the amount of amplified PCR product from the target cDNA reaches a fixed threshold. In each treatment, $\Delta C_T = C_T$ for gene target – C_T for the endogenous reference, GAPDH. $-\Delta \Delta C_T = \Delta C_{T, \text{treatment}} - \Delta C_{T, \text{control}}$. The equation, $2^{-\Delta \Delta C_T}$, denotes the level of target transcripts in the treated group relative to that of the control group. The expression level of each target gene is converted to the copy number of the target relative to 500,000 copies of GAPDH where the amount has been generated after 35 cycles of PCR amplification. Data are mean $\pm$ S.E.M. of three independent tissue samples done in triplicates in parallel. Unpaired t-test was used for between group comparisons.

Receptor binding analysis

Lumbar spinal cords were rapidly dissected to isolate the dorsal horn ipsilateral to the injected paw. Tissues from four or five rats within each treatment group were pooled for membrane preparation. The tissues were homogenized in ice-cold 50mM Tris buffer, pH 7.4, supplemented with 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and pelleted by low speed centrifugation at 2,460 X g for 15 min at 4 °C. The supernatant was recovered and centrifuged

again at 2,460 X g for 15 min at 4 °C. Particulate matter in the supernatant recovered from the second centrifugation was pelleted at 28,000 X g for 45 min at 4 °C. The pellets were resuspended in binding buffer (50 mM Tris, pH 7.4, supplemented with 0.5% bovine serum albumin and protease inhibitor cocktail (10 µM captopril, 50 µg/ml bacitracin, 0.1 mM PMSF)). Total and non-specific binding of [³H]kallidin (76.1 Ci / mmol, PerkinElmer) were determined at a saturating concentration of 3.1 ± 0.36 nM based on published data^{24,53}; non-specific binding of [³H]kallidin was defined as that bound in the presence of excess cold kallidin (10 µM, American Peptide Company). All samples were assayed in duplicates in a final reaction volume of 1.0 ml, and incubated at 25 °C for 90 min in a shaking water bath. Reactions were terminated by rapid filtration through Whatman GF/B filters presoaked in polyethyleneimine and washed 4 times each with 4 ml of cold saline. Radioactivity was determined by liquid scintillation counting. Data are mean $\pm$ S.E.M. of three independent experiments.

Statistical analysis

Significant differences among paw withdrawal thresholds and among paw withdrawal latencies over time between saline and CFA treated rats were determined by two-way ANOVA followed by Bonferroni post test. Unpaired t-test was used to compare differences in paw withdrawal latencies between control and treatment groups, and non-parametric Mann-Whitney U test was used to compare paw withdrawal threshold measurements. All other data were compared by unpaired t-test unless otherwise stated. Significance is set at 95% confidence limit ($P < 0.05$).

Results

Intraplantar CFA induces tactile hypersensitivity and thermal hyperalgesia

Unilateral intraplantar administration of CFA into the left hind paw of rats induced significant hypersensitivities to touch (tactile hypersensitivity) and to noxious heat (thermal hyperalgesia) by 6 hr after injection and persisted throughout the 3 days of testing when compared with saline control group (Fig. 1). The average baseline paw withdrawal latency to noxious radiant heat before injections was 22 ± 1.45 s ($n = 18$) (Fig. 1A). Thermal hyperalgesia was indicated by a significant reduction in the paw withdrawal latency post-CFA when compared with the respective saline controls ($F_{(1,80)} = 435$; $P = 4.5e-34$, two-way ANOVA)(Fig. 1A). The baseline paw withdrawal threshold to probing with von Frey filaments before injections was 15 ± 0.00 g ($n = 18$) (Fig. 1B). Tactile hypersensitivities were indicated by a significant reduction in the paw withdrawal threshold post-CFA when compared with the respective saline control group ($F_{(1,80)} = 161$; $P = 0.69e-20$, two-way ANOVA) (Fig. 1B). Bonferroni post test showed that sensory thresholds of the CFA treated animals were significantly different from the saline controls at all the time points.

Upregulation of spinal dynorphin is seen 72 hr, but not 6 hr, after intraplantar CFA

Spinal prodynorphin (PDYN) transcripts in the lumbar dorsal horn of the spinal cord was significantly upregulated 72 hr after intraplantar CFA injection ($P = 0.0005$, *c.f.* saline injected control), but not at an earlier time point of 6 hr post-CFA ($P > 0.05$) (Fig. 2). Very low level of prodynorphin transcripts were detected in extracts from the ipsilateral L5 DRG from both saline-treated and CFA-treated rats at both time points, but there was no significant difference between the control and CFA-treated group either at 6 hr or 72 hr (Fig. 2).

Intrathecal anti-dynorphin antiserum reverses CFA-induced thermal hyperalgesia at 72 hr, but not at 6 hr after intraplantar CFA administration

The effect of *i.th.* anti-dynorphin antiserum on the CFA-induced inflammatory hyperalgesia was examined 6 hr and 72 hr after injection of CFA (Fig. 3A). Baseline paw withdrawal latency was 21 ± 0.56 s ($n = 24$) before intraplantar CFA (Baseline). After CFA injection, paw

withdrawal latency was 7.4 ± 0.81 s after 6 hr (CFA baseline, $P < 0.0001$ *c.f.* Baseline), or 11 ± 0.60 s after 72 hr (CFA baseline, $P < 0.0001$ *c.f.* Baseline). Control serum injection at 6 or 72 hr did not alter the thermal hyperalgesia induced by CFA ($P > 0.05$). Intrathecal administration of anti-dynorphin antiserum 6 hr after CFA also had no effect on the paw withdrawal latency when compared with rats receiving control serum 6 hr after CFA ($P > 0.05$). In contrast, *i.th.* administration of anti-dynorphin antiserum 72 hr after CFA significantly attenuated thermal hyperalgesia in these rats. The mean paw withdrawal latency was 17 ± 0.94 s, which differed significantly from that receiving control serum at 72 hr after CFA ($P < 0.01$). The *i.th.* anti-dynorphin antiserum treatment did not fully block thermal hyperalgesia at this time point because the mean paw withdrawal latency was statistically different from the baseline paw withdrawal latency (i.e. Baseline) prior to CFA treatment ($P < 0.01$).

Before intraplantar CFA injection, the baseline paw withdrawal threshold to von Frey probing was 15 ± 0.0 g ($n = 24$). After CFA injections, tactile hypersensitivity was seen as a reduction of paw withdrawal thresholds to 3.1 ± 0.33 g ($P < 0.0001$) at 6 hr (CFA baseline) and 2.3 ± 0.30 g ($P < 0.0001$) at 72 hr (CFA baseline), respectively (Fig. 3B). Neither control serum nor anti-dynorphin antiserum, given intrathecally, altered the tactile hypersensitivity seen at 6 hr or 72 hr post-CFA treatment.

Intrathecal bradykinin receptor antagonists reverse CFA-induced thermal hyperalgesia 72 hr, but not 6 hr, after intraplantar CFA administration

In this experiment, the baseline paw withdrawal latency prior to CFA was 21 ± 0.37 s (Baseline, $n = 36$), and was significantly reduced at both 6 hr and 72 hr after CFA treatment ($P < 0.0001$ *c.f.* Baseline) (Fig. 4A). Saline injection at either 6 hr or 72 hr after CFA into CFA pretreated rats did not alter the paw withdrawal latency ($P > 0.05$ *c.f.* CFA baseline). Intrathecal administration of the bradykinin B1 receptor antagonist, DALBK (50 nmol), or the B2 receptor antagonist, HOE140 (10 pmol), significantly reversed CFA-induced thermal hyperalgesia when given at the 72 hr time point compared with saline treatment 72 hr after CFA. The paw withdrawal latency after DALBK was 16 ± 2.15 s, ($P = 0.02$ *c.f.* CFA-saline), and that after HOE 140 was 15 ± 1.27 s ($P < 0.01$ *c.f.* CFA saline). The same treatments with DALBK or HOE 140 at 6 hr after CFA had no effect on the thermal hyperalgesia seen after CFA.

Baseline paw withdrawal threshold to von Frey probing was 15 ± 0.24 g ($n = 36$, Baseline). Tactile hypersensitivity was seen 6 hr and 72 hr ($P < 0.0001$ *c.f.* Baseline) post-CFA (Fig. 4B). Neither DALBK nor HOE 140 given at 6 hr altered the tactile hypersensitivity due to CFA. At 72 hr, both *i.th.* DALBK and HOE 140 had a statistically significant, but very minor effect on the paw withdrawal threshold when compared with the saline control ($P < 0.05$ for DALBK; $P < 0.01$ for HOE140). Saline treatment did not alter CFA-induced tactile hypersensitivity at either 6 or 72 hr after CFA ($P > 0.05$ *c.f.* CFA baseline).

Kininogen and bradykinin receptor expression in the ipsilateral lumbar spinal cord and DRG

There is no evidence for *de novo* production of kininogen, the precursor for bradykinin, in either DRG or spinal cord as quantitative RT-PCR failed to detect any transcripts for kininogen using tissues from saline treated or CFA treated rats at 6 hr or 72 hr post CFA. Data are based on 6 rats per treatment group for each time point; the spinal cords were extracted separately and the RT-PCR reaction done on each extract in triplicate. The negative outcome is not due to a low efficiency of the kininogen primers, which have been previously tested on skin extracts²⁴. In the same extracts, transcripts for prodynorphin (Fig. 2) and bradykinin receptors (Fig. 5) were readily detectable.

Quantitative RT-PCR was used to evaluate bradykinin B1 and B2 receptor mRNA in the ipsilateral dorsal horn of the spinal cord (Fig. 5A) and ipsilateral L5 DRG (Fig. 5B). In naïve

rats, the expression of B1 receptors was negligible in both spinal cord and DRG from naïve rats (< 10 copies / 500,000 copies of GAPDH generated after 35 PCR cycles), while B2 receptor transcripts were predominantly found in the DRG of naïve rats. Intraplantar injection of either saline or CFA induced a significant increase in the level of transcripts for both B1 and B2 receptor in the spinal cord (Fig. 5A) as well as in the DRG (Fig. 5B) when compared with that in naïve rats. The increase seen was comparable between intraplantar saline and intraplantar CFA in both tissues at 6 hr and at 72 hr.

The apparent upregulation in transcript levels for B1 and B2 receptors did not produce a concurrent increase in bradykinin receptors in the ipsilateral dorsal horn of the lumbar spinal cord based on the specific binding of the non-selective bradykinin receptor agonist, [³H] kallidin at a saturating concentration of 3.1 ± 0.36 nM (Fig. 5C). Neither saline nor CFA treatment produced a significant change in the specific binding of [³H]kallidin at 6 or 72 hr when compared with tissues from naïve rats (ANOVA, $P > 0.05$). There was no significant difference between the saline- and CFA-pretreated group at 72 hr ($P > 0.05$). Unpaired t-test indicates a small but significant difference between the saline- and CFA-pretreated group at 6 hr ($P = 0.046$).

Intrathecal administration of bradykinin

In attempt to activate spinal bradykinin receptors directly, bradykinin (0.15, 2 and 10 µg) or saline was injected intrathecally. Intrathecal bradykinin elicited immediate, but transient bouts of hyperactivity and escape behaviors that lasted less than 30 sec, and normal behavior returned within one min. Spinal bradykinin did not alter behavioral responses to tactile stimuli or to noxious thermal stimuli (Figure 6). Paw withdrawal thresholds to probing with von Frey filaments ranged from a high of 15 ± 0 g to a low of 13.0 ± 1.3 g over the entire testing period (Figure 6A). There were no significant differences in responses among the dosing groups. The paw withdrawal latencies to noxious radiant heat ranged between 19.8 ± 0.8 sec and 24.9 ± 0.6 sec over the testing period and there were no significant differences in responses among the 3 dosing groups (Figure 6B). Likewise, hot-plate latencies were not different across time for all treatment groups receiving *i.th.* bradykinin (Figure 6C). Intrathecal injection with saline had no significant behavioral effects at any time point (data not shown).

Discussion

Significant advances have been made in elucidating the cellular and molecular processes that mediate neuronal hyperexcitability in the periphery and in the spinal cord upon an inflammatory insult^{15,18}. Intraplantar injection of CFA is a well accepted rodent model of chronic inflammation which induces persistent hypersensitivity to both touch and noxious heat in the inflamed paw. This model was used in the present study to evaluate the role of spinal dynorphin in inflammatory pain.

An upregulation of spinal dynorphin and its precursor, prodynorphin, following intraplantar CFA is well-documented³². The induction of prodynorphin transcripts is evident by as early as 8 to 12 hr after CFA administration¹⁷; by 24 hr, prodynorphin immunoreactive neurons are evident in both superficial laminae and lamina V of the dorsal horn¹⁹. The upregulation of spinal dynorphin transcripts seen in the present study is in good agreement with that previously reported. Our data also show that the anti-dynorphin antiserum is anti-hyperalgesic only at a later time (72 hr) when spinal dynorphin is upregulated, but has no effect at an early time (6 hr) when rats exhibit hyperalgesia but no sign of upregulation of prodynorphin transcripts (Fig. 2) or of dynorphin peptides¹⁷. Thus, the rapid onset of inflammatory hyperalgesia is independent of the basal level of spinal dynorphin, but the persistent hyperalgesic state is likely maintained by elevated spinal dynorphin.

In several experimental models of abnormal pain, including spinal nerve ligation^{3,30,43}, chronic constriction of the sciatic nerve⁷, sustained morphine exposure^{41,50}, or visceral inflammation⁴², the bulbospinal projection neurons from the rostral ventromedial medulla have been found to be critical for maintaining pathological pain states and regulating the expression of spinal dynorphin. Modifications of this descending pain modulatory pathway also occur upon peripheral inflammation as a result of the enhanced nociceptive input to supraspinal sites³⁸, and may thus similarly contribute to the upregulation of spinal dynorphin. Blockade of the lamina I spinothalamic neurons by lesioning the NK-1 receptor containing neurons blocks the pain facilitatory influence of the descending input³⁶, and blockade of the NK-1 receptors prevents the upregulation of spinal dynorphin by intraplantar CFA¹⁹. Thus, a robust activation of the spinothalamic neurons, including the substance P responsive neurons, may induce a rapid activation of the descending pain modulatory pathway that in turn mediates an over-expression of spinal dynorphin.

The antihyperalgesic effect of bradykinin receptor antagonists, like the anti-dynorphin antiserum, occurred at 72 hr but not at 6 hr after CFA (Fig. 4). We found no evidence for *de novo* synthesis of kininogen in the spinal cord either after intraplantar CFA in the present study, or after spinal nerve ligation described previously²⁴. For bradykinin to activate spinal bradykinin receptors, it must be produced from local kininogen through proteolytic cleavage by tissue kallikrein either in storage or upon release within the spinal cord. A recent study reported the presence of extremely low bradykinin-like immunoreactivity (< 0.4 pg / mg) in the spinal cord after intraplantar capsaicin⁴⁶, and suggested that spinal bradykinin receptors are activated by endogenous bradykinin. Whether kininogen is localized in presynaptic terminals or bradykinin is a neurotransmitter remains controversial. As our quantitative RT-PCR analysis could not detect any kininogen transcripts, our evidence does not support the postulation that bradykinin receptor antagonists reverse CFA induced thermal hyperalgesia by blocking the action of endogenous bradykinin in the spinal cord.

In the absence of bradykinin in the spinal cord, our previous studies have suggested a direct agonist action of dynorphin at bradykinin receptors²⁴. The pronociceptive action of *i.th.* dynorphin requires B2 bradykinin receptors as transgenic mice with a null mutation of the B2 receptor did not exhibit hyperalgesia or tactile hypersensitivity after *i.th.* dynorphin²⁴. Elevated levels of spinal dynorphin may activate spinal bradykinin receptors was first proposed in a model of neuropathic pain where bradykinin receptor antagonists were found to be antihyperalgesic only at the time when spinal dynorphin was upregulated²⁴. A similar relationship between the antihyperalgesic effect of anti-dynorphin antiserum and that of bradykinin antagonists is seen here for inflammatory hyperalgesia.

The antihyperalgesic effect of *i.th.* anti-dynorphin antiserum or bradykinin receptor antagonists at 72 hr but not at 6 hr after CFA is not due to changes in the level of spinal bradykinin receptors 72 hr after CFA compared with that at 6 hr after CFA. Based on the high affinity binding of the agonist [³H]kallidin (which labels both B1 and B2 receptors), none of the treatment groups (saline or CFA) differs significantly from that seen in the spinal cord of naïve rats (Fig. 5C). This is despite the upregulation of transcripts for both receptor types evident at both 6 hr and 72 hr after saline or CFA treatment. Thus, the transcription of bradykinin receptors in the sensory afferent pathway is easily perturbed by seemingly minor insult to the skin such as injections of saline but does not correlate with hyperalgesia. The data may suggest that transcriptional upregulation does not result in translational upregulation of bradykinin receptors defined by the high affinity binding of kallidin. Alternatively, it is possible that transcriptional upregulation yields more bradykinin receptors, but under the condition of peripheral inflammation, the newly synthesized bradykinin receptors in the DRG are transported predominantly to the peripheral terminals. Differential trafficking of proteins to the periphery has been demonstrated for the vanilloid receptor TRPV1 under inflammatory

conditions²⁰. The relationship between transcriptional upregulation and the anatomical disposition of bradykinin receptors upon inflammation requires further analysis.

The lack of antihyperalgesic effect of *i.th.* anti-dynorphin antiserum or bradykinin receptor antagonists at 6 hr after CFA is not likely due to a lack of functional competence of the spinal bradykinin receptors for the following reasons: first, the tissues from these rats bind [³H] kallidin to the same extent as those from other treatment groups. Uncoupled receptors tend to bind agonists with low affinity, reflected by significantly lower specific binding of an agonist. Second, when dynorphin A(2–13) is given intrathecally to naïve rats at doses that do not cause motor dysfunction, the peptide induces reversible tactile and thermal hypersensitivity that are blocked by the B2 receptor antagonist HOE 140, suggesting that under physiological conditions, spinal B2 receptors are activated by intrathecal dynorphin directly or indirectly²⁴. This finding is consistent with a number of earlier studies. One showed that bradykinin enhanced dorsal root stimulation-evoked release of CGRP-like immunoreactivity from rat spinal cord tissues *in vitro*, suggesting that there are active B2 receptors in primary afferent terminals². Another found that in a rat spinal cord preparation with attached tail, bradykinin stimulates ventral root depolarization both by superfusion of the tail and by superfusion of the spinal cord, suggesting that functional bradykinin receptors are present both peripherally and centrally⁸.

A logical approach to demonstrate possible nociceptive function of spinal bradykinin receptors *in vivo* is by the *i.th.* administration of bradykinin. The reported outcome of this approach varies widely, ranging from transient antinociceptive effect in animals within one minute and the effect lasted less than 15 minutes^{25,34}, to long lasting hyperalgesia (up to 4 hr) that peaked at 90 min^{23,46}. We could not detect any hyperalgesic effect induced by intrathecal bradykinin at the dose range as previously reported⁴⁶ and measured with three behavioral tests (Fig. 6). Because our data clearly show the presence of bradykinin receptors in the spinal cord (Fig. 5), and that they mediate the acute effects of *i.th.* dynorphin A(2–13){Lai, 2006 #119}, we conclude that the lack of effect of the bradykinin treatment is most likely due to the known short half-life of the peptide.

As we rule out the role of altered expression and/or functional competence of spinal bradykinin receptors as the cause for the distinct antihyperalgesic effect of bradykinin antagonists at 72 hr after CFA, we postulate that a delayed onset of activation of the spinal bradykinin receptors is due to a delay in the production of agonist(s) at these receptors. The concurrent effect of the anti-dynorphin antiserum and bradykinin antagonists is consistent with the hypothesis that spinal dynorphin ultimately activate spinal bradykinin receptors to promote inflammatory pain.

The effect of anti-dynorphin antiserum or bradykinin antagonists on tactile hypersensitivity is insignificant or very small (Figs. 3, 4). The differential effect of bradykinin receptor antagonists on touch and noxious heat seen here is in good agreement with previous studies¹³, and suggests that tactile hypersensitivity arising from peripheral inflammation is propagated through a distinct pathway, and/or nerves which do not express bradykinin receptors. The antagonists' lack of effect on CFA induced tactile hypersensitivity is unlikely to be due to insufficient dosage²⁴. The similarity between the effects of the dynorphin antiserum and that of bradykinin receptor antagonists on inflammatory pain suggests that spinal dynorphin and bradykinin receptors activate similar nociceptive pathways. In conclusion, the antihyperalgesic effect of bradykinin receptor antagonists requires the presence of upregulated spinal dynorphin but not of *de novo* production of bradykinin, supporting our hypothesis that pathological levels of dynorphin may activate spinal bradykinin receptors to mediate inflammatory hyperalgesia.

Acknowledgements

This study was supported by grants from the National Institute on Drug Abuse DA11823 and DA06284.

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Figure 1.

Thermal (A) and tactile (B) hypersensitivities are produced after an intraplantar injection of complete Freund's adjuvant (CFA) into the rat's left hind paw. Paw withdrawal latency (A) or paw withdrawal thresholds (B) were measured prior to CFA injection as well as 6 hr, 24 hr, 48 hr and 72 hr post CFA administration. Data are mean $\pm$ S.E.M. from nine rats per group.

*** $P < 0.001$, Bonferroni post test.

Figure 2.

Quantitative RT-PCR of prodynorphin transcripts in lumbar spinal cord and DRG from saline or CFA treated rats. Ipsilateral lumbar dorsal horn of the spinal cord (iLSC) and ipsilateral lumbar 5 DRG (iL5DRG) were harvested at 6 hr and 72 hr post saline or post CFA treatment. Data are mean $\pm$ S.E.M. from six rats per group. *** $P < 0.001$, unpaired t-test between saline and CFA treatment at each time point.

Figure 3.

Effect of *i.th.* anti-dynorphin antiserum (200 μ g) on the thermal (A) and tactile (B) hypersensitivities exhibited by the rats' left hind paws after intraplantar CFA. In (A) and (B), the baseline latencies or thresholds were obtained prior to CFA administration, and the CFA baselines were obtained at 6 hr or 72 hr post CFA injection prior to intrathecal anti-dynorphin antiserum, or control serum administration. Both baselines represent pooled data from all treatment groups. Data are mean $\pm$ S.E.M. from six rats per group. (A) $**P = 0.002$, Baseline vs. CFA + Dyn antiserum; $**P = 0.0016$, CFA + control serum vs. CFA + Dyn antiserum, unpaired t-test.

Figure 4.

Effect of *i.th.* saline, DALBK or HOE 140 on the thermal (A) and tactile (B) hypersensitivities exhibited by the rats' left hind paws after intraplantar CFA. In (A) and (B), the baselines were obtained prior to CFA administration, and the CFA baselines were obtained at 6 hr or 72 hr post CFA injection prior to *i.th.* drug administration. Both baselines represent pooled data from all treatment groups. Data are mean $\pm$ S.E.M. from six rats per group. Significance test is in comparison between treatment and saline control.

Figure 5.

Quantitative RT-PCR of bradykinin B1 and B2 receptor transcripts in lumbar spinal cord (A) and DRG (B) from naïve, saline-treated or CFA-treated rats. (A) Ipsilateral lumbar dorsal horn of the spinal cord and (B) ipsilateral L5 DRG were harvested from naïve rats 6 hr and 72 hr after intraplantar saline or CFA treatment. Data are mean $\pm$ S.E.M. from six rats per group. (C) Specific binding of [³H]kallidin (high affinity B1 and B2 receptor agonist) in crude membranes prepared from the ipsilateral dorsal horn of lumbar spinal cord from naïve, saline-treated, or CFA-treated rats (n=5 per group). Data are mean $\pm$ S.E.M. from three independent experiments.

Figure 6.

The behavioral responses to intrathecal administration of bradykinin are shown as paw withdrawal threshold to innocuous light touch with von Frey filaments (A), response latencies to noxious radiant heat applied to the hindpaw (B) or to the 52°C hot-plate (C) in male Sprague Dawley rats. Intrathecal administration of bradykinin did not produce any changes in behavioral responses to tactile or thermal stimuli over the dose range administered.