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Activation of spinal kainate receptors after inflammation: behavioral hyperalgesia and subunit gene expression

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Abstract

We determined whether neural responses to inflammation and hyperalgesia involve activation of kainate receptors, a subgroup of glutamate receptors. Inflammation was introduced into the hind paw by intraplantar injection of complete Freund's adjuvant. The inflammation-induced thermal hyperalgesia was attenuated by intrathecal administration of a non-selective α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate receptor antagonist, 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo[f]quinoxaline-7-sulfonamide disodium (NBQX), as well as by selective kainate receptor antagonists, 6,7,8,9-tetrohydro-5-nitro-1*H*-benz[g]indole-2,3-dione 3-oxime (NS-102) and 3*S*,4a*R*,6*S*,8a*R*-6-(4-carboxyphenyl)methyl-1,2,3,4,4a,5,6,7,8,8a-deca-hydroisoquinoline-3-carboxylic acid (LY382884). Reverse transcription-polymerase chain reaction (RT-PCR) indicated that the GluR5 and GluR6, but not the GluR7, KA1 and KA2 subunits, exhibited increased mRNA expression at 2 h to 3 days following inflammation ($P<0.05$). Western blot showed an increase in GluR6 protein levels ($P<0.01$) with a time course consistent with the changes in its mRNA levels. cDNA sequence and *BbvI* endonuclease digestion of the GluR6 PCR product revealed that the upregulated GluR6 mRNAs were predominantly the unedited form (Q). These results suggest that a selective upregulation of kainate receptor subunit expression contributes to inflammatory hyperalgesia.

Keywords: Freund's adjuvant; LY382884; NS-102; NBQX; Q/R editing; (Rat); Kainate receptor; hyperalgesia

1. Introduction

Kainate receptors belong to the ionotropic glutamate receptor family that also includes α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and *N*-methyl-D-aspartate (NMDA) subtypes. While NMDA and AMPA receptors are key players in neural plasticity in the spinal cord, the role of kainate receptors in synaptic transmission and neural plasticity is not as well understood. Kainate receptor subunits are expressed in the spinal dorsal horn and dorsal root ganglion cells (Furuyama et al., 1993; Sato et al., 1993; Tölle et al., 1993). Recent studies indicate that kainate receptors are involved in nociceptive transmission and persistent pain states. High-intensity stimulation of primary afferents induces kainate receptor-mediated synaptic current in the dorsal horn of the spinal cord (Li et al., 1999). Systemic administration of kainic acid produces persistent hyperalgesia in rodents (Giovengo et al., 1999). Nocifensive responses to formalin and nerve injury-induced hyperalgesia are reduced by selective kainate receptor antagonists (Simmons et al., 1998; Sutton et al., 1999). The GluR5 subunit of the kainate receptor may play an enhanced role in spinal dorsal horn nociceptive processing after inflammation (Procter et al., 1998; Stanfa and Dickenson, 1999). However, there has been no direct evidence that there is a change in the genes coding for their receptors or their peptide products after inflammatory hyperalgesia.

The native kainate receptor is likely formed by a heteromeric combination of five subunits, GluR5–7 and KA1–2. Kainate-gated currents are observed in homomeric receptors of cloned GluR5–7 subunits (Egebjerg et al., 1991; Sommer et al., 1992; Schiffer et al., 1997). While homomeric KA1 or KA2 subunit clones do not appear to form functional channels, they may contribute to kainate channel activity when they form heteromeric kainate receptors with GluR5/6 subunits (Werner et al., 1991; Herb et al., 1992; Sakimura et

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al., 1992). A change in composition of the kainate receptor heteromer will result in alteration of its channel kinetics and gating. Similar to the GluR2 subunit of the AMPA receptor, an RNA editing of Q/R occurs in the second membrane domain of GluR5 and GluR6 (Belcher and Howe, 1997; Bleakman and Lodge, 1998, review). The edited form of GluR5/6 has lower Ca^{2+} permeability (Egebjerg and Heinemann, 1993; Burnashev et al., 1996). Since Q/R editing is incomplete for GluR5 and GluR6, both edited and unedited versions of GluR5 and GluR6 subunits coexist in adult brain (Bernard et al., 1999). The editing at the Q/R site of the GluR5/6 is subject to developmental modulation (Bernard et al., 1999). Prolonged epileptic activity may alter the editing process as well (Grigorenko et al., 1998).

We hypothesize that in addition to NMDA and AMPA subtypes of ionotropic glutamate receptors, kainate receptors are also involved in the response to tissue injury and the development of persistent pain. The present study examined the temporal profile of kainate receptor subunit mRNA expression in the spinal cord in a rat model of inflammatory hyperalgesia. The results show that hind paw inflammation induced a selective upregulation of GluR5 and GluR6 subunit mRNAs in the rat spinal cord. cDNA sequence and *BbvI* endonuclease digestion analyses of the GluR6 PCR product revealed that the upregulated GluR6 mRNAs were predominantly the unedited form (Q). The edited form (R) of GluR6 mRNA did not show significant change. Consistently, a selective kainate receptor antagonist attenuated behavioral hyperalgesia. Our results suggest that a selective upregulation of kainate receptor subunit mRNAs and changes in subunit composition may contribute to dorsal horn hyperexcitability that develops with inflammation and hyperalgesia.

2. Materials and methods

2.1. Animals

Adult male Sprague–Dawley rats weighing 150–250 g (Harlan, Indianapolis, IN) were used in all experiments. Rats were on a 12-h light/dark cycle and received food and water ad libitum. To produce inflammation and hyperalgesia, complete Freund's adjuvant (100 μg *Mycobacterium tuberculosis*, Sigma) suspended in an oil/saline (1:1) emulsion was injected subcutaneously into the plantar surface of one (behavioral studies) or two [reverse transcription-polymerase chain reaction (RT-PCR) and Western blot studies] hind paws. The complete Freund's adjuvant injection produced an intense tissue inflammation of the hind paw characterized by erythema, edema and hyperalgesia (Iadarola et al., 1988; Ren et al., 1992a). The inflamed animals tended to guard the inflamed paw but they ate and groomed normally and interacted with their cagemate. For RT-PCR and Western blot studies, naive and the complete Freund's adjuvant-treated rats (2 h to 14 days after treatment) were overdosed with pentobarbital sodium (100 mg/kg i.p.). The dorsal L4–5 spinal cord tissues were dissected. The Institutional Animal Care and Use Committee of the University of Maryland Dental School approved the experiments.

2.2. Behavioral hyperalgesia

To assess the thermal hyperalgesia, the rats were placed under a clear plastic chamber on an elevated glass surface and allowed to acclimate to their environment. A heat stimulus was applied from underneath the glass floor with a high intensity projector lamp bulb (Paw Thermal Stimulator System, University of California, San Diego, CA). The heat stimulus was directed onto the plantar surface of each hind paw and the paw withdrawal latency to the nearest 0.01 s was determined. A reduction in paw withdrawal latencies indicates the presence of thermal hyperalgesia (Hargreaves et al., 1988). Rats with inflamed hind paws also exhibited more integrative behaviors such as licking and guarding of the inflamed limb and exaggerated withdrawal duration that correlated with paw withdrawal latencies (Hargreaves et al., 1988).

2.3. Intrathecal (i.t.) procedure

The i.t. cannulation was performed under methohexital anesthesia (50 mg/kg i.p.). The atlanto-occipital membrane was exposed, and a 7.0–8.0-cm length of PE-10 tubing was inserted into the subarachnoid space through a slit made in the membrane. The cannula was advanced to the level of the lumbar spinal cord (Yaksh and Rudy, 1976). Upon recovery from anesthesia, animals with gross signs of motor impairment were excluded from the study. The location of the distal end of the i.t. catheter was verified at the end of the experiments. An AMPA/kainate receptor antagonist (RBI-Sigma), 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo[f]quinoxaline-7-sulfonamide disodium (NBQX, dissolved in saline; Sigma-RBI), a selective kainate receptor inhibitor, 6,7,8,9-tetrohydro-5-nitro-1*H*-benz[g]indole-2,3-dione 3-oxime (NS-102, dissolved in dimethyl sulfoxide, DMSO; Sigma-RBI) and a selective GluR5 kainate receptor antagonist, 3*S*,4a*R*,6*S*,8a*R*-6-(4-carboxyphenyl)methyl-1,2,3,4,4a,5,6,7,8,8a-deca-hydroisoquinoline-3-carboxylic acid (LY382884, dissolved in DMSO/saline; a gift from Eli Lilly) were used intrathecally. The drug vehicles were used as a control for i.t. drugs. Cumulative dosing was given in all experiments. Each experimental group consisted of six to eight animals. Analysis of variance (ANOVA) with post hoc tests (Fisher's Protected Least Significant Differences) was performed to compare data. $P < 0.05$ was considered statistically significant.

2.4. Rotarod test

Separate experiments were conducted to evaluate the effect of the receptor antagonists on motor performance.

Non-inflamed rats were placed on an accelerating Rotarod Treadmill (IITC Model 720A) and tested at two different speeds (15 and 45 rpm). The timer was set to zero when rats were in position and started simultaneously with the rotation of the rod. When the rat falls off the rod onto a platform, a switch is triggered and the timer stops. The time at which the rat was unable to stay on the rotarod cylinder was recorded and a 70-s cutoff was used. Prior to the formal test, a few 1-h training sessions were implemented to acclimate the animals to the rotarod apparatus. The rats were tested for three times at each speed at 10-min intervals.

2.5. Reverse transcription-polymerase chain reaction procedure

PCR primers targeted at GluR5–7 and KA1–2 subunits of kainate receptors were designed and synthesized (Sigma Genosys). Glyceraldehyde phosphate dehydrogenase (GAPDH) mRNA RT-PCR was used as an internal control since its expression is not regulated by manipulations within the design of these experiments (MacArthur et al., 1999; Zhou et al., 2001). The sequences of the PCR primers are: *GluR5*, Sense GCC CCT CTC ACC ATC ACA TAC/Antisense ACC TCG CAA TCA CAA ACA GTA CA [product = 208 base pair (bp)]; *GluR6*, Sense TTC CTG AAT CCT CTC TCC CCT/Antisense CAC CAA ATG CCT CCC ACT ATC (product = 260 bp); *GluR6-edited*, Sense TTC CTG AAT CCT CTC TCC CCT/Antisense CAG AAC CTT GCC GCA TGA GAG (product = 211 bp); *GluR6-unedited*, Sense TTC CTG AAT CCT CTC TCC CCT/Antisense ATG AGC TCA GAA CCT TGC TGC (product = 218 bp); *GluR7*, Sense TGG AAC CCT ACC GCT ACT CG/Antisense CCG CAA GCC ACT GGT TTT GTT (product = 356 bp); *KA1*, Sense AGC GTT ATG TCA TGC CCA GAC CAG/Antisense GGG GAG GAT CTG ACA CAT GGT (product = 316 bp); *KA2*, Sense TGA GGA GGG GAG GAA GAT GC/Antisense TGC AGC TCA AAG ATG TC (product = 228 bp); *GAPDH*, Sense TGA AGG TCG GTG TGA ACG GAT TTG GC/Antisense CAT GTA GGC CAT GAG GTC CAC CAC (product = 983 bp); *GAPDH*, Sense TGG GGT GAT GCT GGT GCT GA/Antisense CGC CTG CTT CAC CAC CTT CT (product = 537 bp).

To isolate total RNA, spinal tissues were homogenized in 1 ml of TRIzol Reagent (Life Sciences), incubated for 5 min at room temperature to permit the complete dissociation of nucleoprotein complex, and then 0.2 ml of chloroform per 1 ml of TRIzol Reagent was added. The samples were centrifuged at 12,000 × *g* for 15 min at 4 °C. The aqueous phase was transferred to a fresh tube, adding 0.5 ml of isopropyl alcohol per 1 ml of TRIzol Reagent, and centrifuged at 75,000 × *g* for 10 min at 4 °C. The supernatant was removed and the RNA pellet was washed once with 75% dethanol. The RNA pellet was dried and dissolved in RNAase-free water. $A_{260/280}$ ratio of the final RNA solution was no less than 1.6.

Deoxyribonuclease I (Amplification Grade, DNAase I, Gibco BRL) was used to digest single- and double-stranded genomic DNA (1 μg RNA/1 U DNAase I) before reverse transcription. The RNA sample was incubated with DNAase I for 15 min and then heated for 5 min at 65 °C to inactivate the deoxyribonuclease.

The first strand cDNA was synthesized with SuperScript RNase H^- Reverse Transcriptase using 3–6 μg of RNA. The PCR reaction mixture (100 μl) contained 1 × PCR buffer; synthesized cDNA (1–10 ng); 1.5 mM of $MgCl_2$; 0.2 μM of primers; 0.2 μM of GAPDH primers; 200 μM each of dCTP, dGTP, dATP and dTTP; 5 U Taq DNA polymerase (Gibco BRL). Template dilution analysis was conducted to determine that the amount of cDNA template to be used in the reaction was in the linear range. The optimal PCR cycles were also determined for each product. The temperature cycle (Robocycler Infinity, Stratagene) was: 94 °C/3–10 min (initial denaturing), 94 °C/1 min (denaturing), 41–59 °C/1 min (annealing), 72 °C/1 min (extension). A total of 25–30 cycles and a final 10-min extension at 72 °C were conducted. The PCR product samples were loaded in parallel with a 100-bp DNA ladder (Gibco BRL) on a 2% ethidium bromide-stained gel. The gel image was scanned for further densitometry analysis (Scion NIH Image 1.60). Our analysis of the PCR product should be considered semiquantitatively since the real copies of the cDNA were unknown and only relative abundance of the starting materials were compared. All specific PCR product bands were normalized to the respective GAPDH bands. The relative mRNA levels under different experimental conditions, as indicated by the PCR product, were then calculated as a percentage of the naive controls and compared (ANOVA). Data from three individual experiments were averaged. The positive PCR bands were purified (Wizard DNA Clean-Up kit, Promega) and sequenced (ABI 373 DNA Sequencer, Perkin Elmer), and the acquired sequences were verified by comparing to the targeted cDNA sequences. Mock RT-PCR reaction controls included the omitting of reverse transcriptase, primers or template. No specific PCR product was found in control reactions.

2.6. Western blot

The L4–5 spinal cord tissues were homogenized in solubilization buffer (50 mM Tris–HCl, pH 8.0; 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1% Triton-X100, 0.5% deoxycholic acid, 0.1% sodium dodecyl sulfate (SDS), 1 mM Na_3VO_4, 2.5 μg/ml aprotinin, 2 μg/ml leupetin, 2 μg/ml pepstatin A). The homogenate was centrifuged at 14,000 rpm for 10 min at 4 °C. The supernatant was removed. The protein concentration was determined using a detergent-compatible protein assay with a bovine serum albumin standard. Proteins (12 μg) were separated on a 7.5% SDS-PAGE gel and blotted to nitrocellulose membrane (Amersham) with a Trans–Blot Transfer Cell system (Bio-Rad). The blots were blocked with 5% milk in Tris buffered saline (TBS, 20 mM Tris, 150 mM NaCl pH 7.4) at room temperature for 30 min. After decanting the blocking buffer, the blot was incubated with anti-GluR6 (1:500–

1000, Santa Cruz) antibody overnight at 4 °C. The membrane was washed with TBS and incubated for 1 h with anti-goat immunoglobulin G (IgG) horseradish peroxidase (1:3000, Santa Cruz) in 5% milk/TBS. The membrane was then washed three times with TBS. The immunoreactivity was detected using Enhanced Chemiluminescence (ECL, Amersham). The loading and blotting of equal amount of proteins were verified with Coommassie blue staining. The ECL-exposed films were digitalized and densitometric quantification of immunoreactive bands was carried out using Scion National Institutes of Health (NIH) Image 1.60. ANOVA and unpaired two-tailed *t*-test were used to determine significant differences between sample groups. $P < 0.05$ was considered significant in all cases.

2.7. BbvI endonuclease analysis

To verify the presence of the specific restriction enzyme recognition sequence, GluR6 PCR products (0.01 ml) were digested with the endonuclease *BbvI* (2–4 U, New England

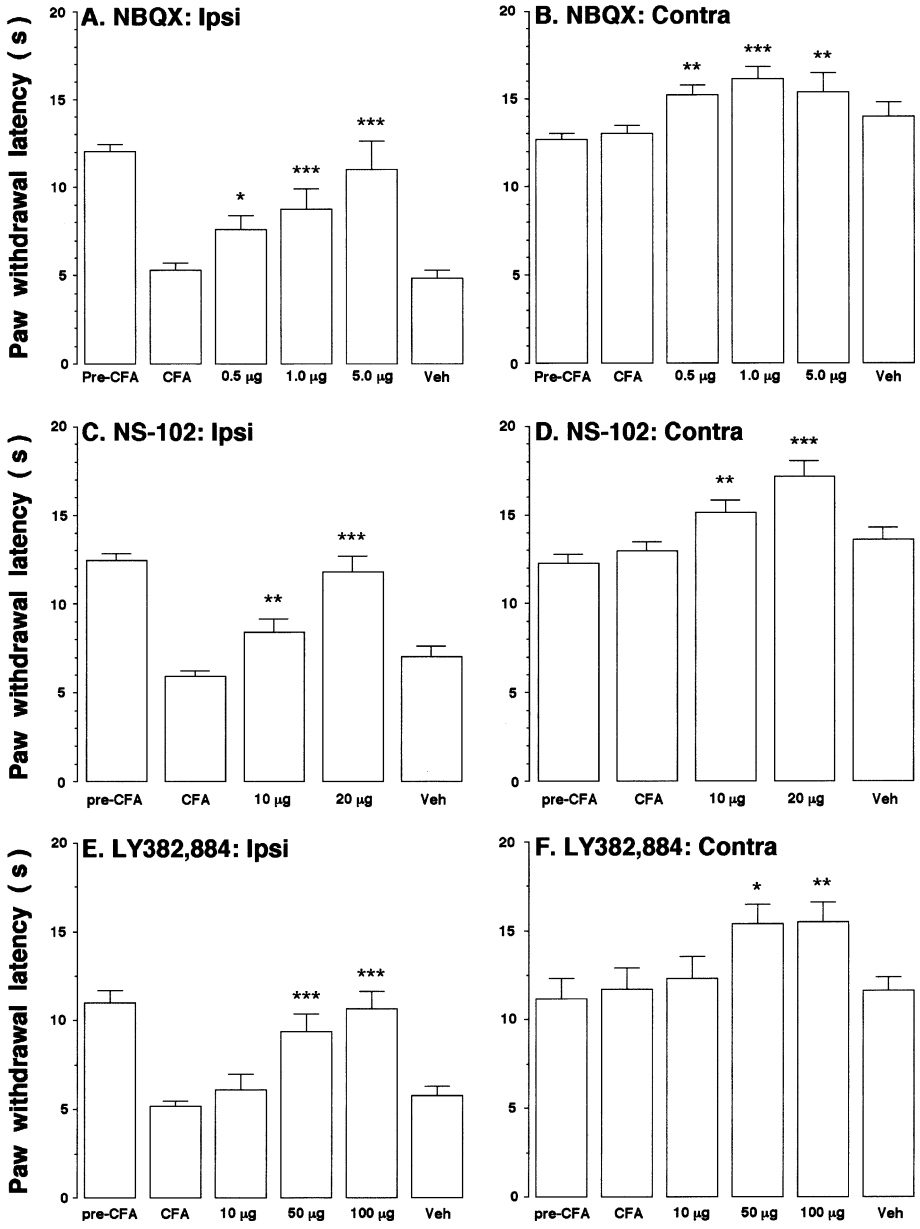

Fig. 1. Effects of intrathecal non-NMDA ionotropic glutamate receptor antagonists on paw withdrawal latencies of rats 24 h after complete Freund's adjuvant (CFA)-induced inflammation. There was a large reduction of paw withdrawal latencies in complete Freund's adjuvant-inflamed rats compared to pre-inflammation values (A, C and E), indicating the presence of thermal hyperalgesia. The receptor antagonists produced an increase in paw withdrawal latencies of both inflamed (Ipsi, ipsilateral) and non-inflamed (Contra, contralateral) paws, while drug vehicles (Veh) did not have a significant effect. Asterisks indicate significant differences from the complete Freund's adjuvant group, $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. $n = 6–8$ for each group.

Biolabs) at 37 °C for 1–2 h. The digested samples were fractionated on 2.5% agarose gels, stained with ethidium bromide and photographed.

3. Results

3.1. Behavioral hyperalgesia

After recovery from i.t. cannulation (3–7 days), the baseline paw withdrawal latency of the rat was determined and complete Freund's adjuvant was injected into one hind paw to produce inflammation and hyperalgesia. The inflammation of the hind paw resulted in a significant reduction in paw withdrawal latencies, indicating the development of hyperalgesia (Fig. 1A,C,E). The involvement of kainate receptors in the development of inflammatory hyperalgesia was evaluated pharmacologically in rats at 24 h after injection of complete Freund's adjuvant.

We first examined the effect of NBQX, a non-selective AMPA/kainate receptor antagonist (Sheardown et al., 1990; Smith et al., 1991; Randle et al., 1992; Mitsikostas et al., 1999), on inflammation-induced thermal hyperalgesia. As shown in Fig. 1A, NBQX, when injected intrathecally (0.5–5 μg), produced a significant and dose-dependent increase in paw withdrawal latency of the inflamed paw (ANOVA, $P<0.0001$). There was also a slight increase in paw withdrawal latency of the contralateral non-inflamed paw (ANOVA, $P<0.0001$) (Fig. 1B). We next studied the effect of NS-102, a non-NMDA ionotropic glutamate receptor antagonist which is selective against low-affinity kainate receptors (Johansen et al., 1993; Verdoorn et al., 1994; Wilding and Huettner, 1996; Mitsikostas et al., 1999). NS-

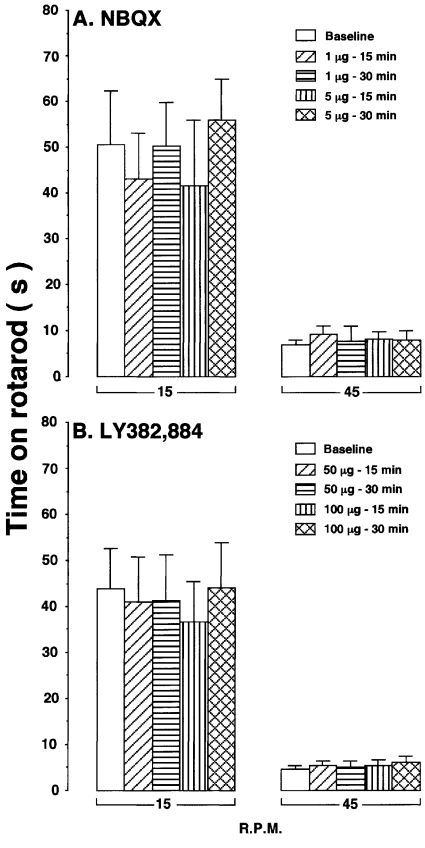

Fig. 2. Effects of receptor antagonists on motor performance of non-inflamed rats. Cumulative doses of NBQX (1 and 5 μg, $n=4$) and LY382884 (50 and 100 μg, $n=4$) were administered intrathecally. The rotarod performance was tested at two speeds (15 and 45 rpm) and at 15 and 30 min after injection of the drug. The two drugs did not produce a significant effect on the rotarod performance of the rats.

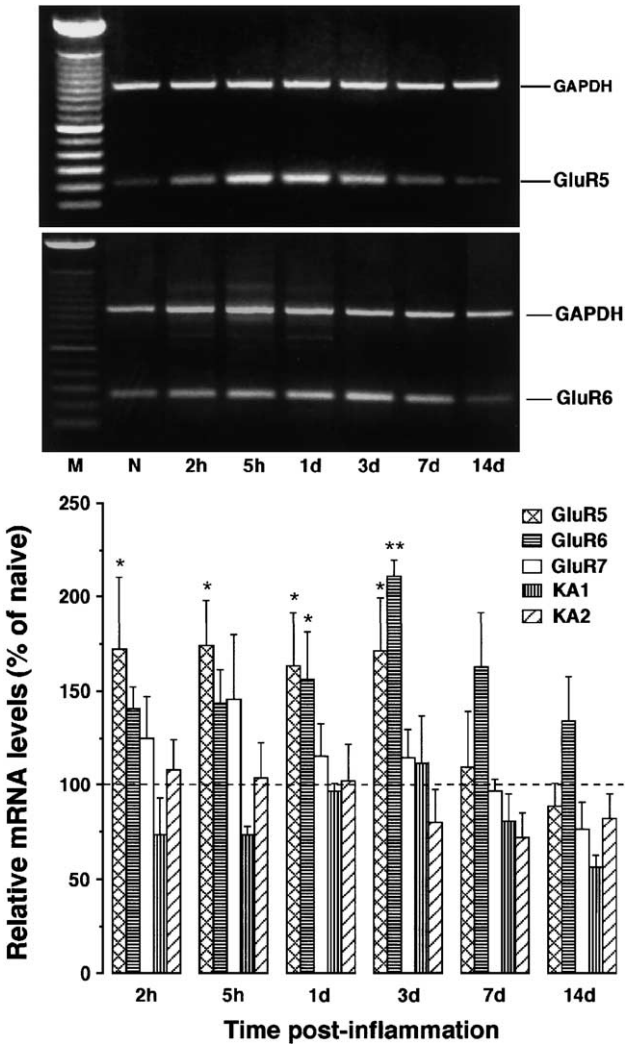

Fig. 3. Agarose gel electrophoresis (top and middle panels) and quantitative comparison (lower panel) of RT-PCR-amplified products of the kainate receptor subunit mRNAs from the rat spinal cord. PCR products were loaded in parallel with a 100-bp DNA ladder (M) on a 2% ethidium bromide-stained gel. All of the specific PCR products were normalized to GAPDH bands obtained in the same reaction. The agents used did not regulate GAPDH expression. The relative mRNA levels under different experimental conditions were illustrated as a percentage of the naive (N) controls (dashed line) for the purpose of comparison. Statistics was performed using raw data (ANOVA). Data from three to five independent experiments were averaged. Asterisks indicate significant differences from the naive control values, $P<0.05$.

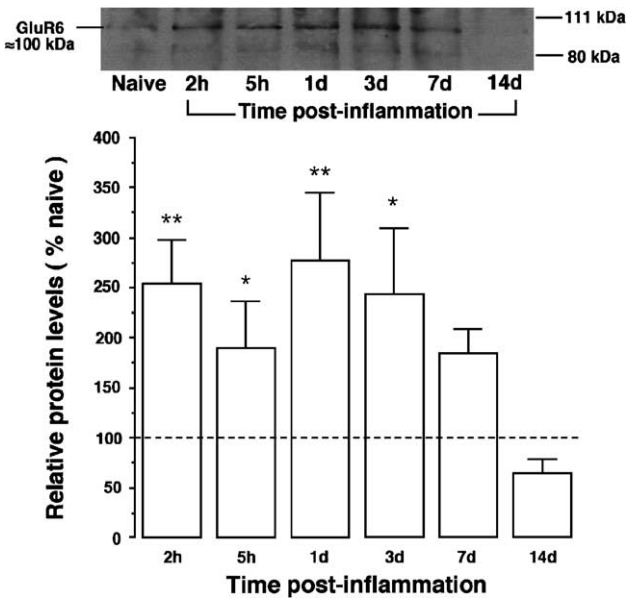

Fig. 4. Upregulation of GluR6 receptor proteins after inflammation. Upper panel: Western blot of L4–6 spinal cord tissues with antisera to the GluR6 subunit. Equal amount of protein from L4–5 spinal tissues was electrophoretically separated on a SDS-PAGE gel and incubated with GluR6 antibody. Very low levels of constitutive GluR6 subunits were observed in naive rats. Following inflammation, there was an increase in the protein product of the GluR6 subunit (2 h to 7 days), consistent with an increase in GluR6 transcription found in the RT-PCR experiments (Fig. 2). Lower panel: the relative protein levels under different experimental conditions were illustrated as a percentage of the naive controls for the purpose of comparison. Statistics was performed using raw data (ANOVA). Data from four independent experiments were averaged. Asterisks indicate significant differences from the naive control values, $P<0.05$.

102 increased the paw withdrawal latency to the pre-inflammation level at a 20-μg dose (ANOVA, $P<0.0001$) (Fig. 1C). NS-102 also produced a significant increase in contralateral paw withdrawal latency (Fig. 1D). We finally tested the effect of LY382884, a selective GluR5 kainate receptor antagonist (O'Neill et al., 1998; Simmons et al., 1998; Stanfa and Dickenson, 1999). Intrathecal LY382884 significantly elevated paw withdrawal latencies of both inflamed and non-inflamed paws at 50–100 μg (Fig. 1E,F). At higher doses, NBQX (10–50 μg), NS-102 (30 μg) and LY382884 (200 μg) often produced hind limb paralysis, thus prevented further testing.

Since the kainate receptor antagonists affected withdrawal latencies of both contralateral and ipsilateral paws in the inflamed rats and produced hind limb paralysis at high doses, the rotarod performance was tested to rule out the possibility that even a lower dose would have a non-specific effect on motor behavior. Cumulative doses of NBQX (1 and 5 μg, $n=4$) and LY382884 (50 and 100 μg, $n=4$) were administered i.t. The two drugs did not produce a significant effect on the rotarod performance of the rat (Fig. 2).

3.2. Kainate receptor gene expression

RT-PCR identified mRNA species for all five subunits of the kainate receptor in the spinal cord. Inflammation induced an upregulation of GluR5 and GluR6 subunit mRNAs (Fig. 3). The increase in GluR5 mRNA was present at 2 h to 3 days and returned to control level at 7 days after inflammation ($P<0.05$, ANOVA). The increase in GluR6 mRNA started at 2 h and peaked at 1–

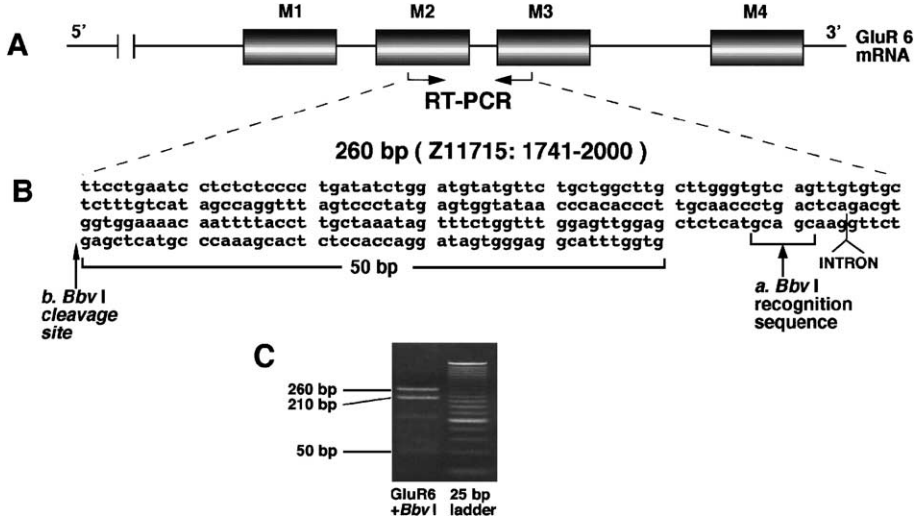

Fig. 5. DNA sequence and endonuclease *BbvI* digestion analysis of GluR6 RT-PCR product. (A) Diagram indicating the targeted segment of the GluR6 gene. The GluR6 PCR product (260 bp) spans GluR6 mRNA encoding the second (M2) and third (M3) membrane domains of the GluR6 subunit. (B) Sequence analysis indicates that the 260-bp segment contains a *BbvI* recognition sequence "gcagc" (arrow a) from unedited GluR6 mRNAs. The cleavage site (arrow b) for *BbvI* is eight nucleotides downstream of the recognition site. The *BbvI* cleavage of the GluR6 product will result in a 210- and 50-bp species. RNA editing ["a" to "g" switch in codon cag (Q) to cgg (R)] will suppress this restriction site for *BbvI* in the corresponding double-stranded cDNA. (C) The GluR6 PCR product was incubated in the presence of *BbvI* endonuclease. Note the appearance of the 210- and 50-bp segments after digestion, indicating the presence of the *BbvI* recognition sequence in the GluR6 product.

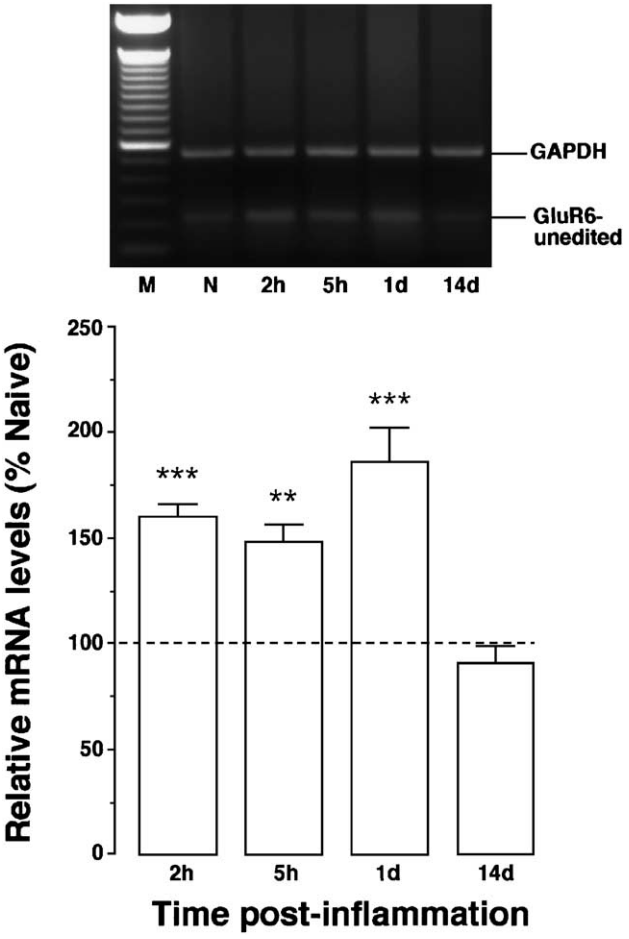

Fig. 6. Agarose gel electrophoresis (upper panel) and quantitative comparison (lower panel) of RT-PCR-amplified products of the GluR6 subunit mRNAs from the rat spinal cord tissues. The primers used in this experiment targeted the unedited form of GluR6 receptor mRNA (see Materials and methods). Similar to the PCR product (Fig. 3), using primers targeting a segment that includes both edited and unedited forms of GluR6 mRNAs, there was a significant increase in the unedited GluR6 mRNAs in the spinal cord after hind paw inflammation ($n=5$). Asterisks denote significant differences from the naive control (N), $**P<0.01$; $***P<0.001$.

3 days after inflammation ($P<0.05$, ANOVA). There were no statistically significant changes in the levels of GluR7 (ANOVA, $P=0.08$), KA1 (ANOVA, $P=0.20$) and KA2 (ANOVA, $P=0.06$) subunit mRNAs after inflammation, although there was a trend for an increase in GluR7 mRNA at 5 h after inflammation and a possible decrease in KA1 and KA2 mRNAs at some time points (Fig. 3). The upregulation of kainate receptor protein was verified by Western blot analysis. We chose to examine the protein levels of the GluR6 subunit that showed the largest increase in mRNA levels at 3 days and a trend toward increase at 7 days after inflammation. As shown in Fig. 4, there was an increase in GluR6 protein levels (ANOVA, $P<0.01$) with a time course consistent with the changes in its mRNA levels (Fig. 4).

3.3. GluR6 receptor Q/R editing

Through cDNA sequencing to verify the fidelity of RT-PCR products of five kainate receptor subunits, it was found that the 260-bp GluR6 cDNA from 24-h inflamed rats matched the unedited form of GluR6 mRNA, GluR6 (Q) gene sequence (Fig. 5A,B). The presence of the unedited GluR6 mRNA species was confirmed by *BbvI* digestion analysis. After incubation with *BbvI* that recognizes a "gcagc" sequence from the unedited GluR6 mRNA, the digestion products of 210 and 50 bp appeared in ethidium bromide-stained gels (Fig. 5C). The 50-bp band was weak, due to less incorporation of ethidium bromide into a small cDNA fragment.

To verify the selective upregulation of the unedited GluR6 mRNA, we next redesigned the primers to selectively target the cDNA that originated from the unedited form of GluR6 mRNA. The RT-PCR analysis indicated that the levels of the unedited form of GluR6 mRNA were upregulated after inflammation (Fig. 6). However, using the primers selectively targeting the edited form of GluR6

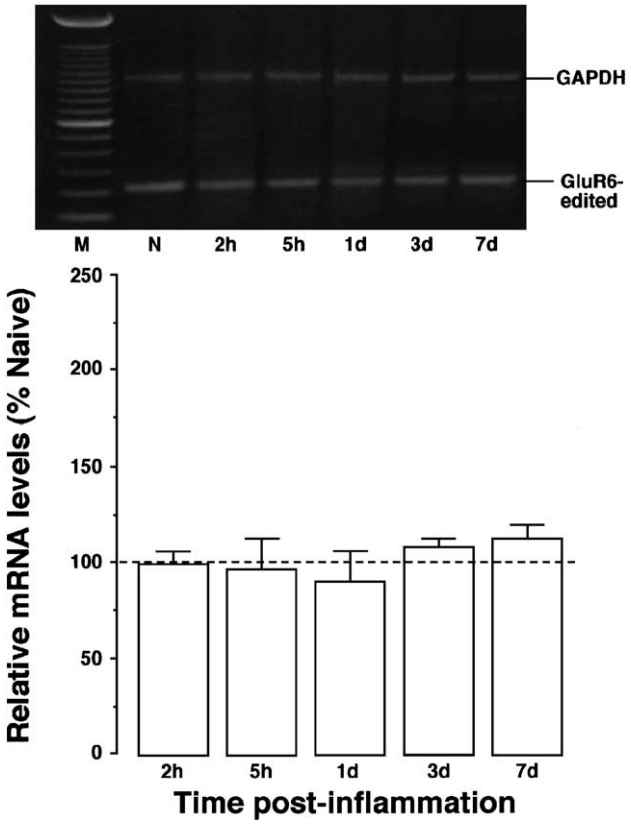

Fig. 7. Agarose gel electrophoresis (upper panel) and quantitative comparison (lower panel) of RT-PCR-amplified products of the GluR6 subunit mRNAs from the rat spinal cord tissues. The primers used in this experiment targeted the edited form of GluR6 receptor mRNA (see Materials and methods). In contrast to the PCR product using primers targeting a segment that includes both edited/unedited (Fig. 3) or only unedited (Fig. 6) forms of GluR6 mRNAs, there was no significant change in the edited GluR6 mRNAs in the spinal cord after hind paw inflammation ($n=4$).

mRNA, it was found that the levels of the edited form of GluR6 mRNAs were not affected by inflammation (Fig. 7). Interestingly, the upregulation of the unedited GluR6 mRNA, as demonstrated by selective primers targeting the unedited GluR6 mRNA (Fig. 6), reached statistical significance by 2–5 h, whereas the increase in GluR6 mRNAs did not reach statistical significance until 1 day after inflammation when non-selective primers were used (Fig. 3). These results suggest a selective upregulation of the unedited form of the GluR6 subunit after hind paw inflammation.

4. Discussion

The present study demonstrates activation of kainate receptors after persistent inflammation and hyperalgesia. We have shown that selective kainate receptor antagonists attenuated complete Freund's adjuvant-induced hyperalgesia and there was a selective upregulation of GluR5 and GluR6 subunit mRNA expression associated with the development of inflammation and hyperalgesia. The increase in mRNA expression was accompanied by an increase in protein translation product (GluR6). An interesting finding was that the increase in GluR6 mRNA appeared to be predominantly in the unedited form, which may allow an increase in calcium permeability of the kainate receptor channels. These convergent observations suggest that in addition to NMDA and AMPA subtypes of ionotropic glutamate receptors, kainate receptors also play a role in synaptic plasticity and hyperalgesia after tissue injury.

Our pharmacology analysis indicates that complete Freund's adjuvant-induced behavioral hyperalgesia was not only reversed by a non-selective AMPA/kainate receptor antagonist, but also by intrathecal administration of selective kainate receptor antagonists. Consistent with these results, a selective GluR5 receptor antagonist inhibited the C-fiber-evoked response and after-discharge of dorsal horn nociceptive neurons (Stanfa and Dickenson, 1999) and produced anti-nociception in the formalin model of pain (Simmons et al., 1998). These studies take advantage of the recent development of selective kainate receptor antagonists and clearly documented the involvement of kainate receptors in inflammatory hyperalgesia. It should be pointed out that the kainate receptor antagonist also produced an increase in paw withdrawal latency of the non-inflamed paw. This is in contrast with NMDA receptor antagonists that do not have an effect on the non-inflamed paw (Ren et al., 1992a,b). These results suggest that kainate receptors may produce analgesia rather than anti-hyperalgesia and are involved in nociceptive processing in the absence of inflammation. However, the role of kainate receptors in nociceptive processing appears to be enhanced after inflammation (Stanfa and Dickenson, 1999).

All five subunits of the kainate receptor mRNAs were identified in the spinal cord in the present study, although a previous study using in situ hybridization methodology did not detect GluR6 mRNA in the spinal cord (Tölle et al., 1993). The contamination by genome DNA is unlikely since we treated RNA samples with DNAase and the control reactions without reverse transcriptase did not give rise to positive PCR products. Apparently, the RT-PCR approach is more sensitive so that low levels of message can be detected. Using RT-PCR, Bernard et al. (1999) have also shown expression of GluR6 mRNAs in the spinal cord. These results suggest the presence of kainate receptors in intrinsic spinal cord neurons. The spinal kainate receptor subunit mRNAs are mainly located in the dorsal horn (Tölle et al., 1993). The mRNA that encodes the GluR5, but not the GluR6 subunit, has been identified in dorsal root ganglion cells (Bernard et al., 1999). All of the kainate receptor mRNA species have been found in the trigeminal ganglion (Sahara et al., 1997), indicating a possible pre-synaptic or peripheral site of action, or both. Convergent evidence suggests that kainate receptors are involved in both pre- and post-synaptic sensory transmission and modulation at the spinal level (Huettner, 1990; Li et al., 1999; Kerchner et al., 2001).

Among the five kainate receptor subunits, the present study found that only GluR5 and GluR6 mRNAs were significantly regulated by inflammation. These results imply that spinal GluR5/6 are "modulatory" subunits and responsible for adjustment of kainate receptor function in response to dramatic increases in synaptic input after injury. This view is consistent with in vitro studies that suggest GluR5/6 subunits are major contributors to kainate-gated current (Egebjerg et al., 1991; Sommer et al., 1992). Although high constitutive KA2 subunits are expressed in the spinal dorsal horn (Tölle et al., 1993), they may not form functional homomeric channels (Herb et al., 1992). In an in vitro expression system, the heteromeric GluR5/KA2 and GluR6/KA2 channels exhibit distinct channel kinetics (Herb et al., 1992). The in vivo composition of spinal kainate receptors is still unclear. Recent studies suggest that at least some kainate receptors in trigeminal ganglion have a GluR5/KA2 combination (Sahara et al., 1997). Thus, selective upregulation of the GluR5/6 subunits may result in an altered composition of the kainate receptors and changes in channel kinetics with increased functional activity. This may provide one mechanism to explain why GluR5 receptor antagonists are less effective (about fivefold) in reducing spinal nociceptive transmission in non-injured animals as compared to animals after inflammation (Stanfa and Dickenson, 1999).

Analysis of the PCR product cDNA sequence and the *BbvI* endonuclease digestion assay revealed that the upregulated GluR6 mRNAs were predominantly the unedited form (Q) and the edited form (R) of GluR6 mRNA, and did not show a significant change after inflammation. In fact, the unchanged GluR6 (R) mRNAs may have masked changes in GluR6 (Q) mRNAs at some points after inflammation. The upregulation of the unedited GluR6 mRNA reached statistical significance by 2–5 h, whereas the increase in pan-GluR6 mRNAs did not reach statistical significance until 1 day after inflammation. It is not known from this

study whether there was also a change in the editing status of GluR5 mRNAs since the specific segment responsible for the editing of GluR5 mRNAs was not targeted.

The editing status of the GluR6 subunit determines the ion flow of kainate receptors (Egebjerg and Heinemann, 1993). Heteromeric combinations of kainate receptors with GluR6 (Q) have higher conductance channels when compared to those with GluR6 (R) (Swanson et al., 1996). Similar to GluR2 subunits of the AMPA receptors, calcium permeability is reduced after Q/R editing of the GluR6 subunit (Burnashev et al., 1996). Unlike the GluR2 receptors, Q/R editing is incomplete for GluR6 receptors and subject to developmental regulation (Bernard et al., 1999). About 50% of GluR6 mRNAs are edited in the adult rat spinal cord (Bernard et al., 1999). Although the molecular mechanisms of glutamate receptor editing have been studied in great detail, its physiological or pathophysiological significance is just beginning to be appreciated. Recent studies have suggested a role of GluR6 Q/R editing in synaptic plasticity. Mice that are deficient in GluR6 Q/R editing, thus with increased GluR6 (Q), are more vulnerable to kainate-induced seizures (Vissel et al., 2001; also see Bernard et al., 1999). Interestingly, there appears to be an increase in GluR6 editing in surgically excised hippocampus of patients with refractory epilepsy (Grigorenko et al., 1998), possibly as a mechanism of neuroprotection. The present results provide an additional mechanism underlying the development of central sensitization and persistent pain. The findings suggest that the Q/R editing site of the GluR6 receptor is also a point of regulation after environmental challenge. An increase in the unedited form of GluR6 mRNAs suggests a reduced mRNA editing and an increase in GluR6 (Q) species. Since the Q/R editing on the GluR6 subunit reduces calcium flux through the kainate receptor channel, an increase in GluR6 (Q) will result in an increase in calcium flow into spinal neurons through the kainate receptor and increased channel conductance after inflammation. The increase in intracellular calcium will trigger kinase and receptor phosphorylation, increased signaling and neuronal hyperexcitability.

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