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The Neurokinin-1 Receptor Antagonist LY306,740 Blocks Nociception-Induced Increases in Dorsal Horn Neurokinin-1 Receptor Gene Expression

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SUMMARY

Dilute formalin injected into the rat hindpaw as a nociceptive stimulus increases neurokinin-1 receptor (NK-1R) mRNA levels in the dorsal horn of the spinal cord. Increased NK-1R mRNA levels could result from increased mRNA stability or an increased rate of NK-1R mRNA transcription. In this study, RNA samples prepared from the spinal cords of rats receiving an injection of formalin into the right hindpaw were assayed for NK-1R mRNA with the use of solution hybridization-nuclease protection assays. The mature and incompletely spliced NK-1R-encoding RNAs protected by endogenous rat RNAs were characterized by the use of NK-1R plasmid constructs containing intron sequences. NK-1R pre-mRNA species were enriched in the nuclear fractions of spinal cord samples, and in the steady state, total molar amounts of NK-1R pre-mRNAs were 2-fold greater than those of mature NK-1R mRNA. In formalin-treated animals, the temporal pattern of peak pre-mRNA levels compared with mature mRNA indicated that chemogenic nociception either activates transcription of the NK-1R gene or decreases the rate of pre-mRNA splicing. In the ipsilateral lumbar dorsal horn, NK-1R mRNA levels were significantly increased at 2 and 6 hr after formalin injection. Levels of the NK-1R pre-mRNA containing both introns A and B were significantly increased 1 hr after formalin treatment, and levels of NK-1R pre-mRNAs containing intron A were significantly elevated at 2 hr after formalin treatment. Pretreatment of rats with the selective NK-1R antagonist LY306,740 was used to determine the role of NK-1R activation in the regulation of nociception-induced NK-1R mRNA levels. Rats were pretreated with either LY306,740 or LY307,679 (an inactive enantiomer) before injection of formalin into the right hindpaw. Pretreatment with LY306,740 (but not LY307,679) completely blocked the formalin-induced increase in dorsal horn NK-1R mRNA levels and significantly reduced formalin-induced behavioral activity. Thus, activation of the NK-1R during nociception increases dorsal horn NK-1R mRNA levels through activation of transcriptional or splicing mechanisms. The stimulation of NK-1R gene expression by activation of the NK-1R provides a homologous mechanism for altering the sensitivity of dorsal horn cells to substance P, potentially via the actions of second messengers, which presumably results in the maintenance of proper sensory information processing during long term nociception.

NK-1R (1, 2) is a G protein-coupled receptor whose activation induces phospholipase C-mediated phosphoinositol hydrolysis (3), mobilization of internal Ca^{2+} stores (4), and activation of protein kinase C (5). Other reported response properties of the NK-1R include the release of arachidonic acid for the generation of prostanoid messengers (6) and, in a limited number of experimental situations, the generation of cAMP (for a discussion, see Ref. 7). NK-1R and its preferred natural tachykinin neuropeptide ligand SP are widely associated with the mediation of nociception. NK-1Rs are located in laminae I and II of the spinal cord dorsal horn (for a review, see Ref. 8), which is also the site of SP-containing primary afferent synaptic terminals. The intraspinal release of SP can be evoked by noxious peripheral stimuli. Results of anatomic (9), electrophysiological (10), and behavioral (11) studies support this association. The injection of dilute formalin into one hindpaw as a unilateral inflammatory stimulus (12 and references therein) alters the levels of tachykinin peptides and their mRNAs and of neurokinin receptor mRNAs in spinal systems (13–15 and references therein). The increases in steady state levels of NK-1R mRNA occur 2–6 hr after the onset of intense nociception (14). The cellular and molecular mechanisms underlying this nociception-induced up-regulation of NK-1R mRNA expression are unknown. Although agents that inhibit SP release from primary afferent neurons also block the up-regulation of NK-1R gene expression induced by inflammatory nociception (15),

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ABBREVIATIONS: NK-1R, neurokinin-1 receptor; SP, substance P; PPT, preprotachykinin; DRG, dorsal root ganglia.

the role of NK-1R activation in regulating its own mRNA expression levels is not known. Newly developed nonpeptide NK-1R antagonists have greatly aided the study of the role of NK-1R activation in sensory processes; recent reports suggest that the NK-1R may specifically mediate long term or inflammatory nociception (16–18).

The *NK-1R* gene has been structurally characterized in several species and consists of a total of five exons, each encoding portions of the NK-1R protein-coding region (for a review, see Ref. 7). The positions of the introns relative to the protein-coding regions of the three members of the tachykinin receptor family are identical, but their sizes dramatically differ. For NK-1R, introns A and B are >15 and >23.3 kb, respectively, in both rats and humans, whereas introns C and D are 1140 and 1800 bases. In the rat, significant amounts of specifically hybridizing RNAs consistent with intron-containing NK-1R transcripts are present in diverse tissues (e.g., neuronal tissues and urinary bladder; 19). Therefore, it has been proposed that the large introns of the rat NK-1R pre-mRNAs may be spliced more slowly than the smaller introns (19). The splicing of exons and removal of intervening sequences from nascent RNA transcripts are important post-transcriptional events in the formation of mature, translatable mRNAs. The analysis of incompletely spliced NK-1R transcripts using solution hybridization-nuclease protection assays could be useful in assessing changes in gene expression that determine steady state NK-1R mRNA levels.

We examined some cellular and molecular mechanisms responsible for the nociception-induced alteration of NK-1R mRNA levels in the dorsal horn cell during long term neuronal activation. Rapid internalization and perhaps degradation of cell surface receptor proteins may be responsible for the rapid desensitization of NK-1R (20, 21). Rapid turnover of NK-1R mRNA and NK-1R protein may be regulated by activation of NK-1R and concomitant cytoplasmic and nuclear mechanisms. This may provide a means for resetting target cell sensitivity to SP and modifying the sensitivity and biological function of dorsal horn cells expressing NK-1R. As such, regulation of NK-1R expression may be critical for maintenance of the proper processing of sensory information during long term nociception. This study provides evidence that during neuronal activation of the dorsal horn of the rat spinal cord, increased NK-1R gene expression is regulated by activation of NK-1R, followed by subsequent activation of mRNA regulatory mechanisms.

Experimental Procedures

Animals and Materials. A total of 80 male Sprague-Dawley rats (250–300 g; Harlan Sprague-Dawley, Indianapolis, IN) were used for the three groups of experiments described. All animal procedures were performed in accordance with National Institutes of Health guidelines and were reviewed and approved by the Washington University Institutional Animal Care and Use Committee.

Formalin, ampicillin, *Escherichia coli* tRNA, nuclease S_1, ribonucleases A and T_1, rNTPs, and placental ribonuclease inhibitor were purchased from Sigma Chemical (St. Louis, MO). Restriction endonucleases and RQ1 DNase were purchased from Promega (Madison, WI). T4 DNA ligase, T3 and T7 RNA polymerases, and proteinase K were purchased from Boehringer-Mannheim (Indianapolis, IN). pBluescript II SK^- was purchased from Stratagene (La Jolla, CA). [α-^{32}P]UTP (3000 Ci/mmol) was purchased from Amersham (Arlington Heights, IL). Sodium pentobarbital was obtained from Vet Labs (Lenexa, KS). The selective nonpeptide NK-1R antagonist LY306,740 and its inactive isomer LY307,679 were generously provided by Eli Lilly and Co. (Indianapolis, IN). These compounds are from a diacetamidopropane series of antagonists recently described (22). Other chemicals were of the highest purity commercially available.

Analysis of NK-1R pre-mRNAs in RNA prepared from subcellular fractions of rat tissues. Spinal cord and urinary bladder tissues were removed from two groups of untreated rats (20 and 10 each, respectively) for isolation of RNA from cytoplasmic and nuclear subcellular fractions. Spinal cord tissues from each group of animals were rapidly removed using hydraulic pressure as described below. The spinal cords were divided into thoracic (T1–T12) and lumbar enlargement (L1–L6) regions, and the resulting dorsal horn samples within each region were pooled. Urinary bladder tissues were also dissected free of adipose and connective tissue from the animals after decapitation, and the tissue samples were pooled for the isolation of nuclear and cytoplasmic RNAs. RNA samples were then assayed for NK-1R mRNAs using solution hybridization-nuclease protection assays (see below).

Effects of formalin-induced nociception on spinal cord dorsal horn NK-1R mRNA and pre-mRNA content. Fifteen rats received a subcutaneous injection of 100 μl of 5% formalin into the plantar aspect of the right hindpaw. Formalin-treated animals were visually monitored during the 30 min immediately after hindpaw treatment to confirm the appearance of hindpaw inflammation and the appropriate early- and late-phase pain-related behaviors involving the treated paw (e.g., biting, licking, and lifting; 12). Spinal cord tissues were removed from these subjects either 1, 2, or 6 hr after hindpaw treatment, resulting in three formalin-treated experimental groups (five animals each). Five additional naive animals served as treatment controls (five animals). At the various time points (1, 2, or 6 hr) after hindpaw treatment, rats were decapitated, and spinal cord tissues were rapidly removed using hydraulic pressure (a forceful injection of ice-cold isotonic saline) applied to the caudal end of the vertebral canal with a 30-ml syringe and 18-gauge needle. This method is a modification of that described by deSousa and Horrocks (23). After removal, spinal cord tissues were quickly rinsed in cold isotonic saline and dissected on ice. The lumbar enlargement (L1–L6) regions were dissected and divided into longitudinal quarters along the saggital midline and just above the central canal. The left and right lumbar dorsal horn tissues from formalin-treated and naive experimental animals were assayed individually. The resulting total RNA samples were assayed for NK-1R and β-actin mRNAs using solution hybridization-nuclease protection assays (see below).

Effects of NK-1R antagonist pretreatment on formalin-induced NK-1R mRNA expression. Three groups of 10 rats each were administered an intraperitoneal injection of the nonpeptide NK-1R antagonist LY306,740 (30 mg/kg; 22), an inactive enantiomer LY307,679 (30 mg/kg), or isotonic saline (1 ml/kg). These doses were chosen to provide adequate NK-1R blockade, producing analgesia in the formalin test for ≥4 hr.[2] Thirty minutes later, the rats in each drug treatment group received a subcutaneous injection of either 100 μl of 5% formalin into the plantar aspect of the right hindpaw or a sham injection. These treatments resulted in the creation of six experimental groups: saline-sham, saline-formalin, LY306,740-sham, LY306,740-formalin, LY307,679-sham, and LY307,679-formalin (i.e., drug pretreatment-hindpaw treatment; five animals each). Formalin-induced pain-related behaviors were quantified during a 10-min period beginning 30 min after hindpaw treatment. Rats were placed into 256-in^2 observation chambers, and the number of hindpaw flinches/min were counted by an observer who was blinded to drug and hindpaw treatments as described previously (15). Various tissues were removed from the rats 4 hr after hindpaw treatment. Before tissue removal during surgery, rats were anesthetized imme-

[2] S. Iyengar, personal communication.

diately with the use of sodium pentobarbital (~40 mg/kg intraperitoneally). A laminectomy was performed to expose the lumbar spinal cord and six lumbar DRG (three from each side, L4–L6). The spinal cord lumbar enlargements were dissected into quarters as described above, and the three DRG from each side of each rat were combined into one tissue sample. Animals were then killed with an overdose of sodium pentobarbital (~150 mg/kg intraperitoneally). Total RNA samples from DRG were assayed for SP-encoding PPT and β-actin mRNAs, and lumbar dorsal horn samples were assayed for NK-1R and β-actin mRNAs using solution hybridization-nuclease protection assays (see below).

Isolation of total, nuclear, and cytoplasmic RNAs. Nuclear and cytoplasmic RNA were isolated from spinal cord and urinary bladder crude subcellular fractions. Homogenates were prepared from pooled samples of ~1-2 g of spinal cord dorsal horn tissue and ~0.5 g of urinary bladder tissue. Subcellular fractions were prepared through homogenization of 50 mg of wet weight of tissue/ml of ice-cold potassium phosphate buffer (100 mM) containing 0.32 M sucrose and 1 mM $MgCl_2$, pH 7.2, using 10 strokes in a Potter-Elvehjem glass/Teflon homogenizing vessel powered by a motor (Arthur H. Thomas Co., Philadelphia, PA) at 160 rpm. The homogenate was centrifuged at 1000 × *g* for 10 min, and the resulting cytosol-containing supernatant was decanted from the crude nuclear pellet. This method was adapted from the method described by Krause and Karavolas (24).

Total cellular RNAs were extracted from individual spinal cord and DRG tissue samples and from nuclear and cytoplasmic subcellular fractions using a previously described rapid guanidinium isothiocyanate-phenol-chloroform extraction method (14, 15). This method is a modification of the method described by Chomczynski and Sacchi (25).

Plasmids, cRNA transcription reactions, and quantification of cRNA standards. We used nine different plasmid constructs for analysis of the RNA. For the analysis of NK-1R-encoding RNAs, an NK-1R 5′ coding region plasmid [pSPR (+577 → +1218)] and a full coding region plasmid [pSPR (+577 → +1800)] were used. For the analysis of β-actin mRNA, a β-actin coding region plasmid (pBS-rβA_{210}) was used. For the analysis of SP-encoding PPT mRNAs, the pG1-β-PPT and pG2-31-1 (γ-PPT) plasmids were used. The use of these five plasmids in solution hybridization-nuclease protection assays has been described previously (14, 15, 19, 26).

Four additional plasmids containing both exon and intron portions of the rat NK-1R cDNA were used to characterize the potential RNA species protected by annealing of the NK-1R 5′ coding region probe to incompletely spliced forms of NK-1R mRNA. These included two previously described rat genomic subclones. The first was pBS-SPRλ5-1 BB3000, which contains a 3-kb insert corresponding to a *Bam*HI restriction fragment of a genomic clone containing the first (965 bp) exon of the rat *NK-1R* gene, with 5′ flanking, exon 1, and sequences corresponding to the 5′ portion of the first intron. The second was pBS-SPRλ6-1 EE1650, which contains a 1650-bp *Eco*RI fragment containing the second exon (195 bp) of the rat NK-1R, with flanking sequences corresponding to portions of the first and second introns (19). Two additional plasmids containing both exon and intron portions of NK-1R cDNA were constructed using the pBS-SPRλ6-1 EE1650 and pSPR (+577 → +1800) plasmids. The first plasmid was generated by initially ligating a 465-bp *Xho*I/*Sin*I coding region fragment of pSPR (+577 → +1800), which contained 389 bp of exon 1 and a 76-bp portion of exon 2, to a 1495-bp *Sin*I/*Eco*RI fragment of pBS-SPRλ6-1 EE1650, which contained the 120-bp 3′ portion of exon 2 and a 1375-bp portion of the second intron. The resultant *Eco*RI/*Xho*I fragment was ligated into appropriately digested pBluescript II SK⁻, resulting in a 4.89-kb plasmid called pBS-rSPR (exons 1–2 + intron B). The second plasmid was generated by ligating a 155-bp *Eco*RI/*Sin*I fragment of pBS-SPRλ6-1 EE1650, which contained 79 bp of the first intron and a 76-bp portion of exon 2 to a 759-bp *Sin*I/*Xba*I fragment of pSPR (+577 → +1800), which contained the remainder of the coding region. This *Eco*RI/*Xba*I fragment was ligated into appropriately digested pBluescript II SK⁻, resulting in a 3.85-kb plasmid called pBS-rSPR (intron A + exons 2–5). *E. coli* DH-5α cells were electroporated with the ligation reaction products. Ampicillin-resistant colonies were isolated, and plasmids were purified and characterized using restriction mapping.

Antisense ^{32}P-labeled cRNA probes were synthesized with the use of [α-^{32}P]UTP. The 5′ coding region NK-1R plasmid was linearized with *Xho*I, and probe was generated using T3 RNA polymerase; the pBS-rβA_{210} plasmid was linearized with *Xba*I, and the pG1-β-PPT plasmid was linearized with *Hin*dIII; and cRNA probes were generated using T7 RNA polymerase as described previously (14, 15, 19). Unlabeled message-sense cRNAs were also generated for use as quantification standards in nuclease protection assays. The PPT, NK-1R full coding region, and β-actin plasmids were linearized and sense RNAs was generated as described previously (14, 15). All transcription reactions were carried out using procedures suggested by Promega. Template DNA was subsequently digested using RQ1 DNase. Message-sense cRNAs were quantified using spectrophotometric analysis and confirmed by electrophoresis and subsequent visualization of similar amounts of each cRNA on denaturing polyacrylamide gels stained with bromphenol blue, as described previously (14).

Linearized plasmids were also used in nuclease protection assays to characterize the sizes of RNA species protected by annealing of NK-1R 5′ coding region probe to NK-1R-encoding cDNA sequences containing various combinations of exons and introns. For this purpose, pBS-SPRλ5-1 BB3000 (exon 1 only) was linearized with *Xba*I, pBS-SPRλ6-1 EE1650 (exon 2 only) was digested with *Eco*RI, pBS-rSPR (exons 1–2 + intron B) was linearized with *Bam*HI, pBS-rSPR (intron A + exons 2–5) was linearized with *Hin*dIII, and the pSPR (+577 → + 1800) full coding region (exons 1–5) plasmid was linearized with *Xba*I.

Solution hybridization-nuclease protection assays. RNA samples were assayed for PPT, NK-1R, or β-actin mRNAs using solution hybridization-nuclease protection assays. The use of protection assays to quantify these mRNAs has been described previously (14, 15, 19, 26). Briefly, 2 × 10^5 dpm of the specific ^{32}P-labeled antisense cRNA probe was coprecipitated with 10 μg of total cellular RNA, 20–50 μg of nuclear or cytosolic RNA, 5–200 pg of cRNA quantification standards, 100 pg of linearized NK-1R plasmids, or *E. coli* tRNA for negative controls (each sample of <25 μg of RNA was made up to 25 μg with *E. coli* tRNA). The RNA-[^{32}P]RNA coprecipitates were each resuspended in 10 μl of hybridization buffer [40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid), pH 6.4, 400 mM NaCl, 1 mM EDTA, 80% (v/v) deionized formamide], and the mRNAs were allowed to anneal with cRNA probes for 16–20 hr at 45°. Annealed portions of the RNAs were protected from nuclease digestion by nucleases A (40 μg/ml) and T_1 (2 μg/ml), which was performed for 30 min at 37° and followed by a 15-min digestion at 37° with proteinase K (100 μg/sample). Nuclease S_1 (1000 units/ml; Sigma) digestion was used for PPT assays and was terminated by the addition of 75 μl of stop buffer (2.5 M NH_4OAc, 50 mM EDTA). β-Actin mRNA was assayed in a similar solution hybridization-nuclease protection assay using only 2 μg of total RNA, 2 × 10^5 dpm of ^{32}P-labeled antisense cRNA probe, and digestion with nucleases A and T_1. In all cases, the digestion reaction products were precipitated with an equal volume of 2-propanol before resuspension and electrophoresis at 25 V/cm on 6% acrylamide gels containing 7 M urea. Gels were fixed, dried, and exposed to phosphor plates (Molecular Dynamics, Sunnyvale, CA) for 16–48 hr. Densitometric images were generated and analyzed using a Molecular Dynamics PhosphorImager SF.

Densitometric signals for the total RNA samples were compared with those for the message-sense cRNA quantification standards to calculate the relative amount of specific mRNA in each total RNA sample using linear regression analysis. For the shortened RNA fragments corresponding to incompletely spliced pre-mRNAs, the signal intensity was compared with that of the band corresponding to the full-length cRNA quantification standard (642 bases) signal. To

determine the relative molar amounts of individual pre-mRNA transcripts corresponding to each signal, the quantification of each band included an adjustment based on the relative specific activity of each protected RNA species (2). [a-^{32}P]UTP was used to prepare the ^{32}P-labeled antisense 5′ NK-1R probe. Consequently, the radioactive base content of each of the protected species was as follows: full-length mRNA, 132 units/642 bases; NK-1R pre-mRNA containing only intron B, 122 units/584 bases; NK-1R pre-mRNA containing intron A, 79 units/389 bases; and NK-1R pre-mRNA containing both introns A and B, 43 units/195 bases. The resulting differences in specific radioactivities of each species were used to calculate the relative number of transcripts in each band. For example, the 389-base band had a specific activity of 79/132, or 60% that of the 642-base mRNA band; thus, for equal radioactive signal intensities, the 389-base band contained a specific activity of 132/79, or 167% of the number of transcripts contained in the 642-base mRNA signal. Quantification of PPT mRNAs was obtained by analysis of the γ-PPT 295-base band protected from β-PPT cRNA (14, 15).

Statistical analysis. Data values are reported as mmol of specific RNA/mol of β-actin mRNA (mean ± standard error). Data were analyzed using analysis of variance or analysis of covariance, with Dunnett's test or Fisher's PLSD used for post hoc comparisons. Significance was considered to be $p \leq 0.05$. All comparisons were made with the naive or sham-treated control groups of the corresponding side.

Results

Characterization of the RNA species protected from the 5′NK-1R probe by NK-1R plasmids containing intron sequences. The use of the 5′ NK-1R coding region probe in a solution hybridization-nuclease protection assay using various plasmid constructs and synthetic RNAs is illustrated (Fig. 1), characterizing the RNA species potentially protected by individual NK-1R pre-mRNA species. Fig. 1A shows the 740-base antisense 5′ NK-1R coding region probe, 642 bases of which span the first two and a portion of the third exon regions of NK-1R mRNA sequence; the full-length NK-1R sense cRNA; and the five plasmid constructs used to confirm the size of RNA species protected by various intron/exon combinations of NK-1R sequence. Fig. 1B shows a densitometric image of a gel, demonstrating the use of these plasmid constructs in a solution hybridization-nuclease protection assay. The results of this assay demonstrated that the 5′ NK-1R coding region probe was able to anneal to and protect from nuclease digestion RNAs of smaller sizes than the 642-base band protected by the mature NK-1R mRNA. Some residual 740-base undigested antisense probe is evident in each of the gel lanes shown in Fig. 1B. Similar 642-base bands were produced by annealing the 5′ NK-1R coding region probe with a full-length NK-1R sense mRNA or a plasmid bearing the full-length NK-1R cDNA sequence [pSPR (+577 → +1800)]. Additional shorter bands were protected by plasmids containing intron sequences that were not complementary to the entire sequence of the antisense RNA probe. The plasmid containing exons 1 and 2 followed by the intron B of NK-1R cDNA [pBS-rSPR (exons 1–2 + intron B)] protected a 584-base band; the plasmid containing only exon 1 flanked by portions of the 5′ untranslated region, and intron A (pBS-SPRλ5-1 BB3000) protected a 389-base band; the plasmid containing a portion of intron A followed by exons 2–5 [pBS-rSPR (intron A + exons 2–5)] protected a 253-base band; and the plasmid containing exon 2 flanked by portions of intron A and B (pBS-SPRλ6-1 EE1650) protected a 195-base band. The 195-base band was broad in appearance, indicating heterogeneity of the RNA species protected by this region of the 5′ NK-1R coding region probe. Five additional bases in the vector sequence can anneal to intron sequences adjacent to the second exon (3 bases at the 5′ side,

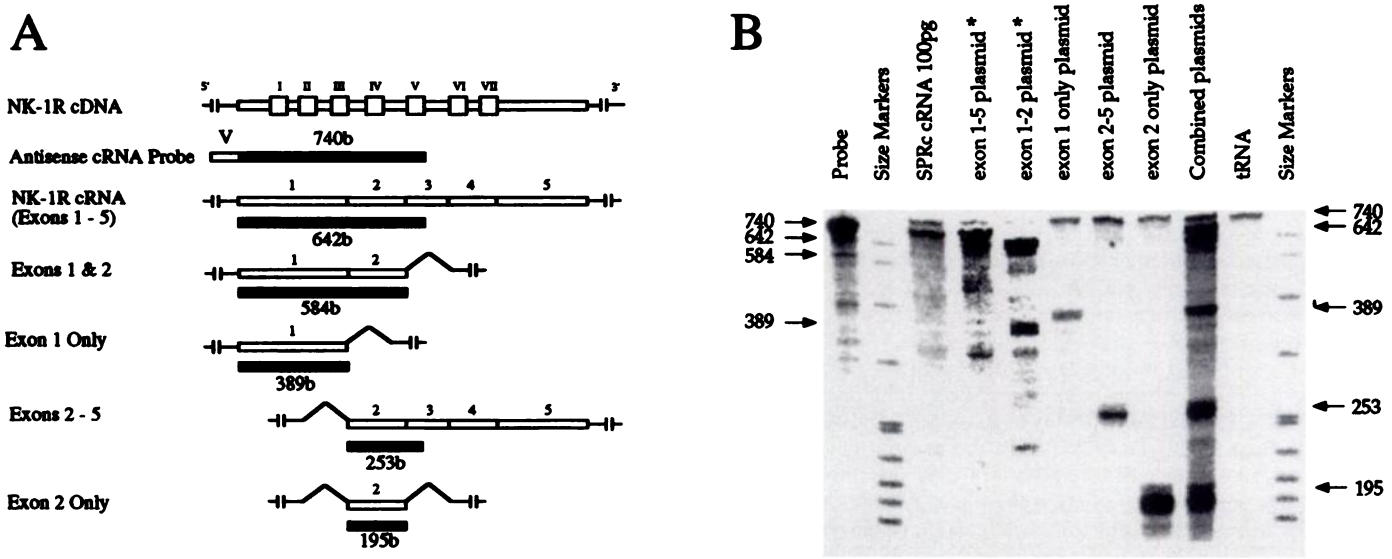

Fig. 1. Characterization of NK-1R RNA species protected using a solution hybridization-nuclease protection assay with a 5′ coding region NK-1R probe and plasmid constructs of NK-1R pre-mRNAs. A, Sizes of NK-1R cDNA, the 5′ coding region antisense cRNA probe, plasmid constructs containing various intron/exon combinations of NK-1R, and their respective protected RNA species. *Roman numerals*, putative transmembrane domains of the deduced protein; *arabic numerals*, specific exon regions of NK-1R mRNA. B, Results of solution hybridization-nuclease protection assay for NK-1R with the plasmid constructs shown in A. *Arrows*, sizes of the undigested NK-1R probe (740 bases) and RNA species protected by NK-1R mRNA (642 bases), 100 pg of message-sense NK-1R cRNA quantification standard or coding region (exons 1–5) plasmid (642 bases), plasmid containing exons 1 and 2 followed by intron B (584 bases), plasmid containing exon 1 followed by intron A (389 bases), plasmid containing intron A followed by exons 2–5 (253 bases), and plasmid containing exon 2 flanked by introns A and B (195 bases). *, Different gray-scale image of the gel, allowing proper visualization of the protected bands; however, the entire image results from a single phosphor plate exposure to the same gel.

2 bases at the 3′ side). In addition, there is an A-T-rich region of sequence at the 3′ end of the second exon (8 of 10 bases are A or T from +1149 to +1158 of NK-1R cDNA) that could partially anneal to the probe. Thus, RNAs ~190-200 bases long result from this hybridization, producing a broadened band on the acrylamide gel. Although the species protected by this portion of the 5′ NK-1R coding region probe are heterogeneous in size, the species composing this band are, for convenience, referred to in the text as the 195-base signal.

Characterization of the RNA species protected from the 5′NK-1R probe by total RNA from rat tissues. The use of the 5′ NK-1R coding region probe in solution hybridization-nuclease protection assays is shown for the analysis of partially spliced NK-1R pre-mRNA species in RNA samples isolated from rat tissues (Fig. 2). Fig. 2A gives a schematic description of NK-1R cDNA, mRNA, and pre-mRNAs that contain sequences from either intron A or intron B (or both), and the portions of those RNAs complementary to the 5′ NK-1R coding region antisense probe. Fig. 2B, a densitometric image of a representative solution hybridization-nuclease protection assay with experimental samples of lumbar spinal cord total RNA from formalin-treated rats, shows that the RNA species protected from total RNA samples derived from rat tissues correspond precisely in size with the species predicted using hybridization with NK-1R plasmid constructs described in Fig. 1. In addition to the residual undigested NK-1R probe (740 bases) and RNA species protected by NK-1R mRNA or 100 pg of message-sense NK-1R cRNA quantification standard (642 bases), bands corresponding to NK-1R pre-mRNAs containing intron A (389 bases) or NK-1R pre-mRNAs containing both introns A and B (195 bases) are readily detected in spinal cord samples. The 389-base band corresponds to NK-1R pre-mRNAs containing intron A alone or containing intron A with intron B; for clarity, this group of NK-1R pre-mRNAs is referred to as "intron A-containing NK-1R pre-mRNAs." The 584-base band corresponding to an NK-1R pre-mRNA containing only intron B was at the limit of detectability in spinal cord RNA samples in the exposure shown. The phophor image also shows the bands protected by a sample of rat urinary bladder RNA, with a much more robust signal in the 389-base band, confirming the initial evidence for the presence of partially spliced NK-1R pre-mRNA species described by Hershey *et al.* (19). The 642-, 584-, 389-, and 195-base bands were used to quantify the levels of the intron-containing NK-1R pre-mRNA species shown in Fig. 2A. Levels of NK-1R mRNA (642 bases) in the lumbar spinal cord dorsal horns of naive control rats corresponded to 12 ± 2 pg/μg of total RNA (left side) and 13 ± 2

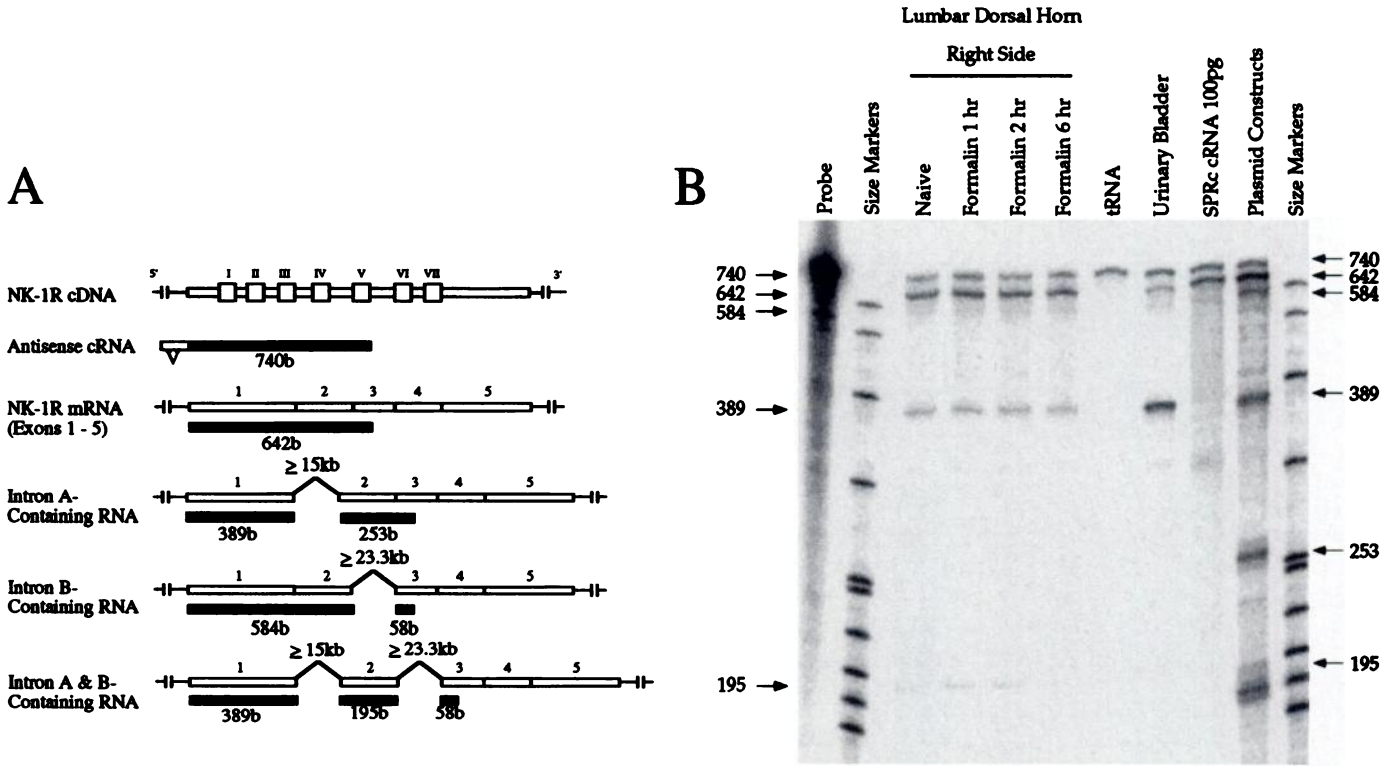

Fig. 2. A 5′ coding region NK-1R solution hybridization-nuclease protection assay for analysis of NK-1R pre-mRNA species in RNA samples isolated from rat tissues. A, Sizes of NK-1R cDNA, the 5′ coding region antisense cRNA probe; potential partially spliced NK-1R mRNA precursors containing intron A, intron B, or both introns A and B; and their respective protected RNA species. *Roman numerals*, putative transmembrane domains of the deduced protein; *arabic numerals*, specific exon regions of NK-1R mRNA and minimum sizes of the first two intron sequences. B, Results of representative solution hybridization-nuclease protection assay for quantification of NK-1R mRNA and mRNA precursors in formalin-treated and naive experimental animals. Each spinal cord sample contained 10 μg of total RNA. *Arrows*, sizes of the undigested NK-1R probes (740 bases) and RNA species protected by NK-1R mRNA or 100 pg of message-sense NK-1R cRNA quantification standard (642 bases), an NK-1R pre-mRNA containing only intron 2 (584 bases), NK-1R pre-mRNA containing intron A (either alone or with intron B; 389 bases), an NK-1R pre-mRNA containing only intron A (253 bases), and an NK-1R pre-mRNA containing introns A and B (195 bases). The plasmid constructs *lane* contains ~100 pg each of the constructs described in legend to Fig. 1. Note that the RNA species protected from rat tissue samples correspond in size to those protected using intron-containing plasmid constructs in Fig. 1. *Lanes*, RNA samples from only one individual experimental subject; thus, treatment effects may not be fully appreciable by viewing only one assay.

pg/μg total RNA (right side). These amounts are similar to those described in our previous studies using solution hybridization-nuclease protection assays with a 3′ NK-1R coding region probe (14, 15).

Analysis of NK-1R pre-mRNAs in RNA prepared from subcellular fractions of rat tissues. If the protected RNA species described above correspond to partially spliced NK-1R pre-mRNA species, they should be enriched in nuclear subcellular fractions. Fig. 3, the densitometric image of a representative 5′ NK-1R coding region probe solution hybridization-nuclease protection assay of RNAs derived from nuclear (50 μg) and cytosolic (20 μg) subcellular fractions of pooled rat spinal cord dorsal horn tissues, shows that although the RNA species protected by the 5′ NK-1R antisense probe in these samples are similar in size to those described in the legends to Figs. 1 and 2, the major RNA species protected by NK-1R pre-mRNA species (389 and 195 bases) are predominantly expressed in the nuclear fractions of both thoracic and lumbar spinal cord samples. Quantification of the amounts of NK-1R mRNA and pre-mRNAs in spinal cord and urinary bladder RNA samples revealed somewhat different expression patterns among the tissues. Of the total number of transcripts of each pre-mRNA species in the spinal cord samples (nucleus plus cytosol), the nuclear portion accounted for 83% of the total number of transcripts in the 389-base signal and 81% of the total number of transcripts in the 195-base signal. In contrast, in the 389- and 195-base pre-mRNA bands in urinary bladder RNA samples, the number of transcripts found in the nuclear fractions represented >99% of the total number of transcripts of those species (nucleus plus cytosol; data not shown). In spinal cord samples, the number of transcripts in the 389-base signal was 160%, and the number of transcripts in the 195-base signal

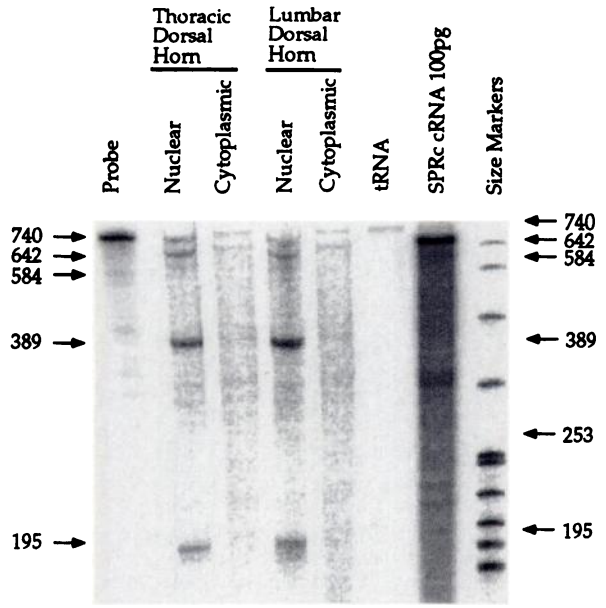

Fig. 3. Results of representative solution hybridization-nuclease protection assay of NK-1R mRNA and pre-mRNAs in nuclear and cytosolic subcellular fractions of rat spinal cord dorsal horn tissues. Each nuclear fraction sample assayed contained 50 μg of RNA, and each cytosolic sample contained 20 μg of RNA. *Arrows*, sizes of RNA species described in legends to Figs. 1 and 2. Note that the RNA species protected by incompletely spliced mRNA precursors are primarily expressed in the nuclear RNA fractions.

was 50% of the total number (nucleus plus cytosol) of mature NK-1R mRNA transcripts (642 bases). The pattern of relative molar ratios of NK-1R pre-mRNA and mRNA species evident in Fig. 4 are generally similar to this result. However, in urinary bladder, the number of transcripts in the 389- and 195-base signals represented 960% and 336%, respectively, of the total number of mature NK-1R mRNA transcripts (Fig. 2).

Effects of formalin-induced nociception on spinal cord dorsal horn NK-1R mRNA and pre-mRNA content. The amounts of NK-1R-encoding mRNA and pre-mRNAs in the lumbar spinal cord dorsal horns of rats receiving a formalin injection into one hindpaw are shown in Fig. 4. The levels of NK-1R-encoding RNAs were normalized in this study by the measurement of β-actin mRNA in 2-μg samples of total RNA from the same tissues using solution hybridization-nuclease protection assays (data not shown). The expression of β-actin mRNA was not significantly altered by formalin treatment at any time point, and the mean content of 267 ± 12 pg/μg of total RNA in lumbar spinal cord dorsal horn tissues of naive controls seen in this study is similar to the values reported previously (14, 15). Fig. 4A shows that in the spinal cord lumbar dorsal horn ipsilateral to the formalin-injected hindpaw (right side), levels of NK-1R-encoding mRNA and pre-mRNAs were altered at various time points after hindpaw treatment. The levels of NK-1R mRNA (642-base signal in Fig. 4B) were significantly increased above naive control values (by ~2-fold) at 2 and 6 hr after injection of formalin into the hindpaw, confirming the effects of formalin-induced inflammation described previously using a 3′ NK-1R coding region probe (14, 15). The time course of alterations in the levels of NK-1R pre-mRNAs, however, was different from that of the mature NK-1R mRNA transcript. The levels of NK-1R pre-mRNAs containing intron A (389-base signal in Fig. 4B) trended toward an increase at 1 hr, were significantly increased at 2 hr, and decreased to control levels by 6 hr after formalin. The levels of NK-1R pre-mRNA containing both introns A and B (195-base signal in Fig. 4B) were significantly increased at 1 hr, but this increase was reduced to control values within 2–6 hr after formalin treatment. Levels of NK-1R pre-mRNA containing only intron B (584-base signal in Fig. 4B) were very low (at or near the detection limit of the protection assay) and were not significantly altered at any time point after formalin treatment.

Our previous studies have demonstrated a trend toward an increase in the mature NK-1R mRNA transcript in the contralateral spinal cord as a consequence of a unilateral inflammatory stimulus (14, 15). Fig. 4B shows that alterations in the amount of NK-1R mRNA and pre-mRNAs in the contralateral (left) dorsal horn were similar to, but generally less than, those in the ipsilateral lumbar dorsal horn. The only significant change in expression levels of any NK-1R-encoding RNA species in the contralateral dorsal horn was the intron A-containing NK-1R pre-mRNAs (389-base signal in Fig. 4B) 2 hr after formalin, which was significantly higher than naive controls of the same side.

Effects of LY306,740 pretreatment on formalin-induced NK-1R mRNA expression. Fig. 5 shows a pain-related behavior, hind limb flinches, resulting from hindpaw treatments in rats pretreated with the selective nonpeptide NK-1R antagonist LY306,740. During the peak 10 min of the late phase of the formalin test (30–40 min after hindpaw

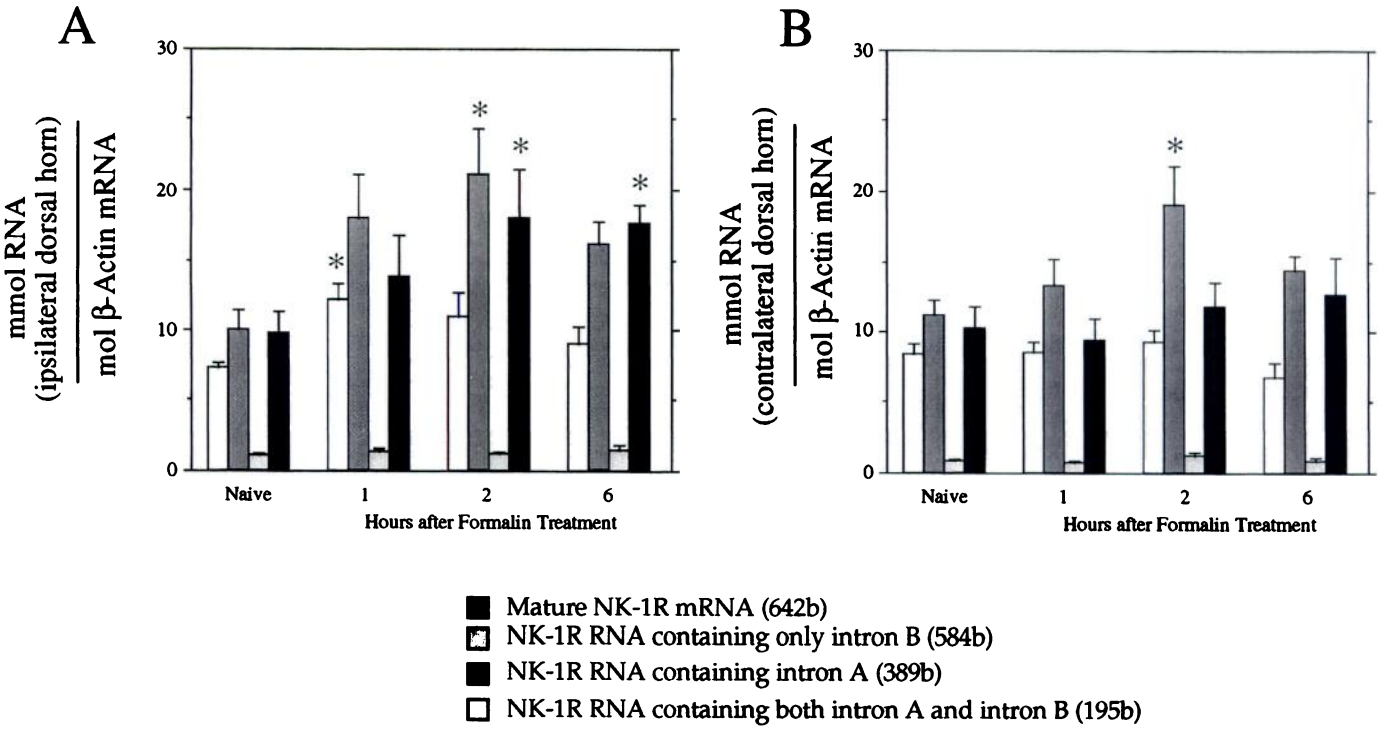

Fig. 4. Levels of NK-1R-encoding mRNA and pre-mRNAs in the lumbar spinal cord dorsal horns of rats at various times after a formalin injection into the right hindpaw. A, Ipsilateral (right) dorsal horn. Note that levels of NK-1R mRNA [642 bases (*642b*)] were significantly increased above naive levels at 2 and 6 hr. The levels of the intron A-containing NK-1R pre-mRNAs [389 bases (*389b*)] were significantly increased at 2 hr, and the level of NK-1R pre-mRNA containing both introns A and B [195 bases (*195b*)] was significantly increased at 1 hr. The levels of NK-1R pre-mRNA containing only intron B [584 bases (*584b*)] were very low and not significantly altered after formalin treatment. B, Contralateral (left) dorsal horn. Note that changes in the contralateral lumbar dorsal horn were similar but smaller in magnitude than those in the ipsilateral lumbar dorsal horn. Only the level of NK-1R pre-mRNAs containing intron A (389 bases) was significantly higher than naive controls 2 hr after formalin treatment. *, $p < 0.05$ compared with naive controls of the same side.

treatment), controls showed no flinches of the right hindlimb (Fig. 5, *Saline-Sham*). Injection of formalin into the hindpaw of saline-pretreated rats produced a significant amount of flinching behaviors during the late phase of the formalin test (Fig. 5, *Saline-Formalin* versus *Saline-Sham*). Pretreatment with LY307,679 had no appreciable effects on the development of late-phase formalin-induced hindlimb flinches (Fig. 5; *LY307,679-Formalin* versus *Saline-Formalin*). However, pretreatment with LY306,740 significantly reduced the number of late-phase hindlimb flinches induced with formalin by ~50% (Fig. 5, *LY306,740-Formalin* versus *Saline-Formalin*).

Fig. 6 shows the expression levels of SP-encoding PPT mRNA in the DRG of rats pretreated with LY306,740. Average γ-PPT mRNA levels in this study corresponded to an expression level of ~4 pg/μg of DRG total RNA, similar to previous studies (14, 15). After 4 hr, γ-PPT mRNA levels were significantly increased in the lumbar DRG ipsilateral to the hindpaw injected with formalin (Fig. 6; *Saline-Formalin* versus *Saline-Sham*). Pretreatment with NK-1R antagonist LY306,740 or its inactive isomer LY307,679 did not alter formalin-induced γ-PPT mRNA expression levels (Fig. 6; *LY306,740-Formalin* or *LY307,679-Formalin* versus *Saline-Formalin*). There was no significant difference in γ-PPT mRNA expression levels between treatment groups in the contralateral (left) DRG. Fig. 7 shows the expression levels of NK-1R mRNA in the lumbar dorsal horns of rats pretreated with LY306,740 or LY307,679. Amounts of NK-1R mRNAs in the ipsilateral lumbar dorsal horn were increased 4 hr after injection of formalin into the right hindpaw (Fig. 7, *Saline-Formalin* versus *Saline-Sham*). Pretreatment with NK-1R antagonist LY306,740 blocked the formalin-induced expression of NK-1R mRNAs to levels not significantly different from those of controls (Fig. 7; *LY306,740-Formalin* versus *Saline-Sham* or *LY306,740-Sham*). Pretreatment with the inactive NK-1R antagonist isomer LY307,679 did not alter the pattern of increased NK-1R mRNA expression after formalin injection into the hindpaw; NK-1R mRNA levels were increased after formalin as in saline-pretreated rats (Fig. 7; *LY307,679-Sham* and *LY307,679-Formalin* versus *Saline-Sham* and *Saline-Formalin*). Antagonist treatment alone (without the nociceptive formalin stimulus) did not change steady state NK-1R mRNA expression levels (Fig. 7; *LY306,740-Sham* versus *Saline-Sham*). The levels of NK-1R pre-mRNAs containing intron A (389 bases) were significantly increased (by ~2-fold) compared with saline-sham controls in the same groups with significantly increased mature NK-1R mRNA levels (*Saline-Formalin* and *LY307,679-Formalin*; data not shown). There were no significant differences in NK-1R mRNA levels between treatment groups in the contralateral dorsal horns.

Discussion

This study was undertaken to assess some of the cellular and molecular mechanisms responsible for the nociception-induced up-regulation of NK-1R mRNA in the lumbar dorsal horn of the rat spinal cord after inflammation of one hindpaw via an injection of formalin. This model system provides a

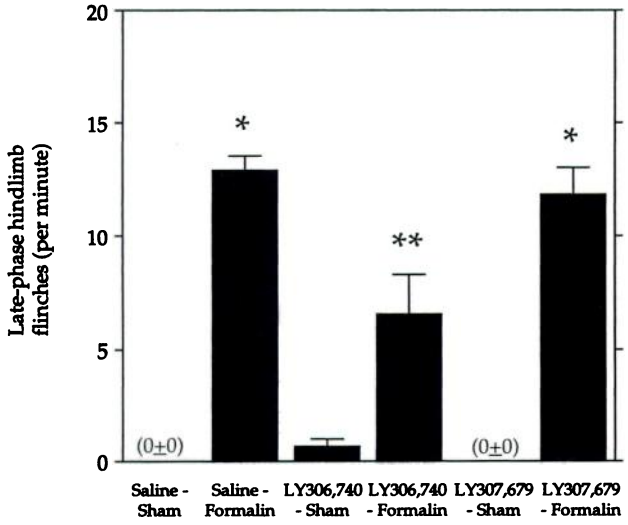

Fig. 5. Hindlimb flinches during the late phase of the formalin test in rats pretreated with LY306,740, LY307,679, or saline. Formalin produced a significant amount of flinching behaviors during the late phase of the formalin test in saline- or LY307,679-pretreated rats; pretreatment with NK-1R antagonist LY306,740 significantly reduced the number of hindlimb flinches induced by formalin. All values are given as number of hindlimb flinches/min (mean ± standard error; five animals). *, $p < 0.01$ versus all sham-treated control groups. **, $p < 0.01$ versus sham-treated control groups, saline-formalin, and LY307,679-formalin groups (analysis of variance and Fisher's PLSD).

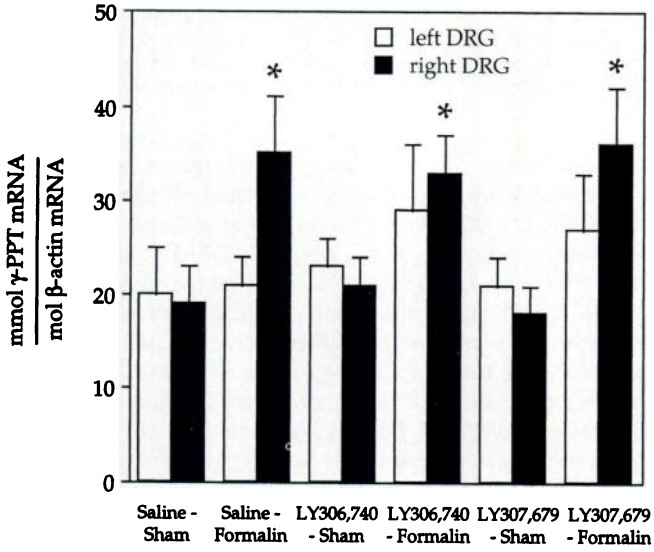

Fig. 6. SP-encoding γ-PPT mRNA expression in the L4–L6 DRG 4 hr after a unilateral injection of formalin in rats pretreated with LY306,740, LY307,679, or saline. In all pretreatment groups, γ-PPT mRNA levels were significantly increased in the lumbar DRG ipsilateral to the hindpaw injected with formalin. All values are given as nmol of γ-PPT mRNA/mol of β-actin mRNA (mean ± standard error; five animals, except the right side LY306,740-formalin group, for which there were four animals). *, $p < 0.05$ versus saline/sham-treated controls (analysis of covariance with Fisher's PLSD).

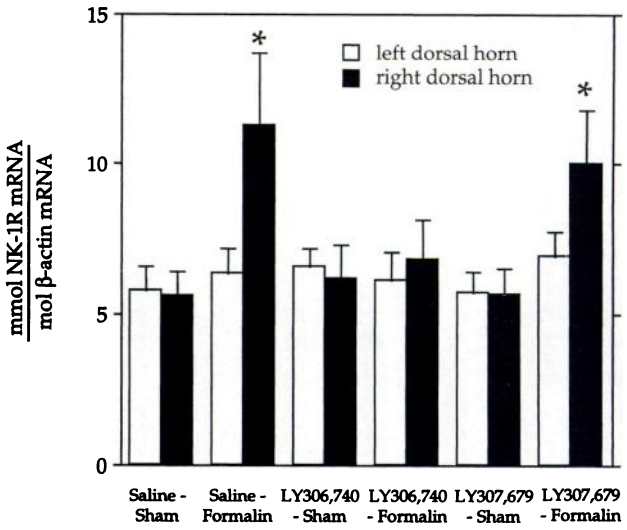

Fig. 7. NK-1R mRNA expression in the lumbar dorsal horn 4 hr after a unilateral injection of formalin in rats pretreated with LY306,740, LY307,679, or saline. NK-1R mRNA levels were increased after formalin in saline- or LY307,679-pretreated rats. Pretreatment with NK-1R antagonist LY306,740 blocked the formalin-induced expression of NK-1R mRNAs to levels not significantly different from those of saline/sham controls. LY307,679 treatment alone (without the nociceptive formalin stimulus) did not change NK-1R mRNA expression levels. All values are given as nmol of NK-1R mRNA/mol of β-actin mRNA (mean ± standard error, five animals). *, $p < 0.05$ versus saline/sham-treated controls (analysis of covariance and Fisher's PLSD).

means by which the physiological changes in receptor expression induced by intense, long term neuronal activation can be studied in a discrete, well-studied, *in situ* neuronal network. The results of this study indicate that activation of transcriptional or post-transcriptional mechanisms or possibly blockade of splicing contributes to the nociception-induced increase in NK-1R mRNA levels in the spinal cord. Importantly, blockade of NK-1R during formalin-induced nociception blocks the development of this increase in NK-1R mRNA levels.

RNA regulatory mechanisms in the dorsal horn of the spinal cord during inflammatory nociception were assessed through quantification of slowly spliced NK-1R pre-mRNAs during the development of increased mature NK-1R mRNA levels. Hershey *et al.* (19) used the 5′ NK-1R coding region antisense RNA probe [pSPR (+577 → +1218)] to characterize steady state NK-1R mRNA levels in various tissues. They showed that in addition to the mature NK-1R mRNA, significant amounts of shorter, specifically hybridizing RNAs were evident in many RNA preparations, including those from neuronal tissues and particularly those in urinary bladder (19). It was proposed that these RNA signals corresponded in length to RNA species that would be produced by annealment of the probe to intron-containing NK-1R transcripts. Because of the large sizes of introns A and B of the rat *NK-1R* gene sequence (>15.3 and >23.3 kb, respectively), it was suggested that they may be spliced very slowly compared with the smaller introns C and D (1140 and 1800 bases, respectively; 19). Tsuchida *et al.* (27) reported only one hybridizing band with a rat NK-1R probe spanning exons 1–3 in their study in which they quantified NK-1R mRNA level expression in rat tissue poly(A)$^+$ RNA fractions, but poly(A)$^+$ RNA isolation primarily selects mature RNA transcripts. In addition, the anticipated size or sizes of the partially protected pre-mRNA species would not have been visible in the portion of the autoradiogram shown. Furthermore, there is evidence in IM-9 cells expressing the human NK-1R and in human

synovial cells and glial cells (7 and references therein)[3] of shortened protected species corresponding to NK-1R pre-mRNAs. In this study, we further characterized the 5′ NK-1R coding region probe for the analysis of partially spliced NK-1R pre-mRNA species and used this to assess the dynamics of transcriptional and/or splicing processes by measuring the relative amounts of accumulated pre-mRNA species. The extremely low abundance of NK-1R mRNA in rat neuronal tissues (<0.001% of total RNA in this study; also, Refs. 14, 15, and 19) prevented the successful use of more-traditional transcriptional "run-on" assays. The extensive use of plasmid constructs containing portions of the first two intervening intron sequences of *NK-1R* gene in solution hybridization-nuclease protection assays (see Figs. 1–3) verified that the RNA species protected by RNA samples from rat tissues correspond precisely in size with those that would be protected by incompletely processed pre-mRNAs. Importantly, NK-1R pre-mRNA species are shown to be concentrated within the nucleus (Fig. 3).

Either transcriptional or post-transcriptional mechanisms could be responsible for the nociception-induced changes in spinal cord NK-1R mRNA content. The rate of transcription *in vivo* is not constant along the entire length of the DNA template, resulting in the accumulation of nascent RNA of discrete sizes (28). A pool of RNAs associated with polymerase complexes "paused" along the DNA template would be indistinguishable from capped and polyadenylated but incompletely spliced pre-mRNAs on nuclease protection. Although RNA species still associated with paused RNA polymerase may contribute to NK-1R pre-mRNAs detected by nuclease protection assays, the specific identification of those RNA species is beyond the scope of this study. Although processing of transcripts may occur during transcription (29), capping and polyadenylation are kinetically faster processes than splicing (30). Thus, intron removal is generally regarded as a post-transcriptional event. As previously suggested, the splicing of large intron sequences may be rate limiting in the formation of mRNA (19). The pattern of intron removal is, in some cases, tissue or species specific, but it is generally understood that the rate of splicing and the order of intron removal are kinetically determined and may not be subject to regulation associated with the activational state of the cell (30). However, recent evidence shows that post-transcriptional processing events may be subject to regulation in some systems. For example, the repression of the RNA splicing pathway of T cell receptor-β via the inhibition of exon splicing events may involve a labile repressor protein (31). Similarly, Ha-*ras* repression of fibronectin mRNA production is a nuclear post-transcriptional event resulting in an accumulation of nuclear fibronectin pre-mRNA, with no alteration in cytoplasmic fibronectin mRNA stability (32). Also, the induction of interleukin-1β gene transcription by retinoic acid involves regulated pre-mRNA processing (33).

Our results provide a novel description of the relative amounts of NK-1R pre-mRNA transcripts compared with mature NK-1R mRNAs in an activated, *in situ* neuronal system. The molar amounts of NK-1R pre-mRNA species in the spinal cord dorsal horn are very large. Indeed, intron-containing NK-1R pre-mRNA transcripts amount to approximately twice the number of NK-1R mRNAs in spinal cord samples; in urinary bladder, pre-mRNAs may outnumber mature mRNA by more than 10-fold (see Results). In rat neuronal AR42J cells, NK-1R message half-life is ~30 min (34); it is ~2 hr in U373 cells bearing the human NK-1R.[4] A similar half-life of NK-1R mRNA *in vivo* of ~1-2 hr would be quite short in comparison to most other mRNAs, which typically have half-lives of >24 hr (35). mRNAs are generally considered to be stable in cytoplasm; the primary determinant of mRNA half-life is the strength of destabilizing elements. There are multiple attributes of a specific mRNA that determine its rate of degradation in the cytoplasm. Secondary structure in the 5′- or 3′-untranslated regions, the presence of premature stop codons, open reading frame sequences, or A-U-rich regions in the 3′-untranslated region; and the degree of polyadenylation contribute to the regulation of mRNA stability (35). The 3′-untranslated region of NK-1R mRNA contains pentanucleotide sequence repeats (AUUUA) that may affect mRNA stability and allow rapid turnover of the mature transcript (19, 34).[5] The rapid desensitization of NK-1R after the application of ligand may be indicative of internalization and rapid degradation of cell surface receptor proteins (20, 21). Thus, it seems reasonable to hypothesize that dorsal horn neuronal NK-1R mRNA turnover is rapid and regulated by the activational state of the cell.

Both mature NK-1R mRNA and intron-containing pre-mRNA levels are increased after formalin-induced nociception. Increased levels of NK-1R mRNA 2 and 6 hr after formalin (Fig. 4A; *filled bars*) confirm the findings of our previous studies (14, 15) and are consistent with the nociception-evoked increase in NK-1R mRNA expression detected using *in situ* hybridization (36). Specific NK-1R pre-mRNA species, however, increased over a more rapid time course than the mature NK-1R transcript. The time course of peak levels of each of the pre-mRNAs (the pre-mRNA species containing both introns A and B peaked at 1 hr; the intron A-containing pre-mRNA was highest at 2 hr; and then the mature mRNA, containing no introns, increased later, at 2 and 6 hr after formalin; Fig. 4A) suggests that the *de novo* synthesis of NK-1R-encoding transcripts is increased in the spinal cord dorsal horn after inflammation of the ipsilateral hindpaw. The consistent pattern of mRNA splicing intermediates, with nearly undetectable amounts of pre-mRNA containing only intron B, suggests a splicing pathway in which the small introns C and D are removed very rapidly, intron B is removed before intron A, and significant amounts of the pre-mRNAs containing intron A accumulate at apparently rate-limiting splicing steps. The order of removal of specific introns does not seem to change according to the state of dorsal horn activation.

Alterations in the levels of NK-1R mRNA and pre-mRNAs in the contralateral (left) dorsal horn were similar to, but generally less than, those in the ipsilateral lumbar dorsal horn (Fig. 4B). Only the intron A-containing NK-1R pre-mRNAs were significantly increased in the contralateral dorsal horn 2 hr after formalin. This pattern of activation of both sides of the cord has also been observed in previous studies (14, 15). These contralateral effects and their physiological basis have been discussed in detail previously (15). Because

[3] D. DiMaggio and J. E. Krause, unpublished observations.

[4] J. Takeda and J. E. Krause, unpublished observations.

[5] J. Takeda and J. E. Krause, unpublished observations.

a unilateral nociceptive stimulus is evidently capable of altering the physiological state of the contralateral dorsal horn, all comparisons in this study were made with the same side of naive subjects rather than between the ipsilateral and contralateral sides of the cord within individual formalin-treated subjects.

The specific role of NK-1R activation in the regulation of activity-induced NK-1R expression has not been investigated. We previously suggested a homologous mechanism for the regulation of *NK-1R* gene transcription (14, 15, 19). Changes in the level of receptor-encoding mRNA during activation of surface receptors have been described in only a limited number of G protein-coupled receptor systems. For example, increased cAMP levels resulting from activation of the β_2-adrenergic receptor increase transcription of the β_2-adrenergic receptor gene, perhaps by interacting with the cAMP response element present in the 5′ flanking region of the β_2-adrenergic receptor gene (37). Similar homologous mechanisms for the regulation of receptor mRNA levels have been described for the thyrotropin-releasing hormone receptor (38), muscarinic receptor subtypes (39), and the luteinizing hormone/chorionic gonadotropin receptor (40). This study shows that activation of NK-1R plays an important role in regulating NK-1R mRNA levels after formalin-induced nociception. In saline-pretreated rats, formalin injection significantly increased nociceptive behavioral activity, levels of SP-encoding PPT mRNA in the ipsilateral lumbar DRG, and levels of NK-1R mRNA in the ipsilateral lumbar dorsal horn (Figs. 5–7). However, pretreatment of rats with LY306,740 (a selective nonpeptide NK-1R antagonist) significantly reduced the number of hindlimb flinches 30–40 min after formalin (Fig. 5) and, importantly, blocked the formalin-induced increase in dorsal horn NK-1R mRNA levels (Fig. 7). Pretreatment of rats with LY307,679 (an inactive isomer of NK-1R antagonist) resulted in a pattern of formalin-induced behaviors and PPT/NK-1R mRNA expression similar to that of saline-injected controls (Figs. 5–7), indicating that this compound did not have nonspecific effects on these parameters. Nociception-evoked SP-encoding PPT mRNA levels in the DRG of antagonist LY306,740-pretreated rats were similar to those of saline-pretreated rats (Fig. 6). This indicates that the major effect of NK-1R antagonist was not inhibition of increased PPT mRNA production or alteration of SP turnover in primary afferent neurons. The blockade of nociception-induced NK-1R mRNA levels by LY306,740 demonstrates that activation of NK-1R regulates the expression of NK-1R mRNA after formalin-induced nociception, providing a homologous mechanism for altering the sensitivity of dorsal horn cells to the SP released from primary afferent neurons. Levels of the intron A-containing NK-1R pre-mRNA were similar in saline-sham and LY306,740-formalin groups (data not shown), suggesting that NK-1R antagonist pretreatment blocks transcriptional activation induced by NK-1R activation rather than blocking the splicing of NK-1R pre-mRNAs. The possible mediators responsible for changes in NK-1R mRNA levels include diacylglycerol, Ca^{2+} or Ca^{2+}-activated systems via inositol-1,4,5-phosphate, and arachidonic acid or its metabolic products (3–6).

Direct responsiveness to the activity of SP released by peripheral nociceptive stimuli and the subsequent rapid turnover of NK-1R mRNA and protein may provide a mechanism to quickly reset the cellular sensitivity of dorsal horn cells to SP. Altered receptor expression or function resulting from cellular activation could be a means by which the dorsal horn cell maintains proper biological function during long term neuronal activation (e.g., that induced by chronic inflammatory nociception). Changes in the expression of NK-1R at the dorsal horn cell surface may be important in balancing the influence of other activated transmitter/receptor systems or cell types and may be critical for the continued accurate processing of sensory information during long term nociception.

Acknowledgments

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