Antihyperalgesic Effect of a Recombinant Herpes Virus Encoding Antisense for Calcitonin Gene–related Peptide

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Background: Calcitonin gene–related peptide (CGRP) is contained in and released by small-diameter, nociceptive primary afferent sensory neurons. Upon spinal release, one of the effects of CGRP seems to be to sensitize dorsal horn neurons to subsequent input from nociceptive afferents and, consequently, to induce a behavioral hyperalgesia. Therefore, attenuating evoked release of CGRP from central terminals of nociceptors should have an antihyperalgesic effect.

Methods: The authors applied a recombinant herpes vector, encoding an antisense sequence to the whole CGRP gene, to the dorsal surface of the hind paw of mice to knock down expression of the peptide selectively in primary afferents innervating this tissue.

Results: Herpes virus–based vector encoding an antisense sequence for the whole CGRP clearly reduced CGRP immunoreactivity in the infected spinal dorsal horn levels as well as in cultured dorsal root ganglia neurons. Selective knockdown of CGRP in primary afferents significantly attenuated the thermal, C-fiber hyperalgesia normally observed after topical application of capsaicin. The effect of viral vector–mediated knockdown of CGRP was comparable to the effect of intrathecal application of the CGRP antagonist CGRP8-37, but lasted for 14 weeks after one single application.

Conclusion: Viral vector–mediated knockdown of CGRP in primary afferent neurons provides a promising tool for treatment of chronic pain states as well as for studies investigating the pathophysiology underlying these conditions.

CGRP is a 37-amino acid peptide, a ubiquitously present marker of primary afferent fibers, where it is found in the majority of dorsal root ganglion (DRG) cell bodies at most spinal cord levels, in both myelinated and unmyelinated axons. Its specific expression is restricted exclusively in primary afferents by the use of the herpes simplex virus type 1, viruses, as vectors to latently infect and transduce DRGs via peripheral infection to alter the phenotypes of mature primary sensory neurons. The purpose of this study was to determine the effect of use herpes simplex vectors to knock down the expression of CGRP exclusively in primary afferents by the use of the herpes virus encoding an antisense sequence to the CGRP gene and to investigate the effects of such a knockdown on capsaicin-induced thermal hyperalgesia mediated by sensitization of C nociceptors.

Materials and Methods

Animals

Adult male Swiss Webster mice (22–25 g; Charles River Laboratories, Wilmington, MA) were housed in a 12-h
light: 12-h dark environment and provided food and water *ad libitum*. Effort was made to minimize discomfort and to reduce the number of animals used. All animal procedures were approved by University of Illinois at Chicago (Chicago, IL) and Stanford University (Stanford, CA) Institutional Animal Care and Use Committees.

**Virus Construct**

The herpes simplex virus vector was constructed as previously described, with modifications. In brief, a casette containing the human cytomegalovirus immediate–early promoter/enhancer, SV40 intron, a nearly full-length complimentary DNA for the rat CGRP precursor, and SV40 polyadenylation sequence was cloned from plasmid pCM-VrACGRP into pBluescript II SK(+) containing an inserted lox P site (fig. 1). This plasmid was reacted with Cre recombinase in the presence of ICP4 viral DNA containing a lox P site in the thymidine kinase (TK) sequence of the genome of an attenuated herpes simplex type 1 virus. Insertion into the TK sequence disables this gene and thus prevents replication in nondividing cells. A similar virus, KHZ, was constructed encoding for the bacterial reporter gene *lac-Z* and was used as a control. KACGRP = antisense CGRP viral vector; SV40 PA = simian virus 40 polyadenylation sequence; U₁ = unique long sequence, U₈ = unique short sequence.

**In Vitro Experiments**

**Cell Preparation and Assay.** In four separate experiments, two mice each were killed during deep isoflurane anesthesia, and the DRGs L3–L6 were removed, collected, and digested in collagenase (2 mg/ml, type 1A; Sigma, St. Louis, MO) and dispase (1 mg/ml; Sigma). After 40 min, the ganglia were triturated and returned to the water bath for an additional 10 min. Enzymatic digestion was halted by the addition of cold Dulbecco’s modified Eagle’s medium (DMEM). Cells were centrifuged at 400 rpm for 10 min. The cell pellet was resuspended in 5 ml DMEM and passed through a sterile 20-μm nylon mesh into a 50-ml conical tube. After centrifugation, cells were resuspended in 1–2 ml DMEM plus 10% fetal bovine serum. Cells were allowed to settle on poly-L-lysine–coated coverslips and placed in the incubator at 37°C. On the next day, 2 mM cytosine arabinoside was added to inhibit mitosis. Two days later, one half of the media was replaced with DMEM plus 10% fetal bovine serum. On the next day, cells were exposed to 5 mM capsaicin dissolved in ethanol and diluted in DMEM plus 10% fetal bovine serum and placed in the incubator for 10 min. The cell pellet was resuspended in 5 ml DMEM and passed through a sterile 20-μm nylon mesh into a 50-ml conical tube. After centrifugation, cells were resuspended in 1–2 ml DMEM plus 10% fetal bovine serum. Cells were allowed to settle on poly-L-lysine–coated coverslips and placed in the incubator at 37°C. On the next day, 2 mM cytosine arabinoside was added to inhibit mitosis. Two days later, one half of the media was replaced with DMEM plus 10% fetal bovine serum. On the next day, cells were exposed to 5 mM capsaicin dissolved in ethanol and diluted in DMEM plus 10% fetal bovine serum and placed in the incubator for 10 min. Cells were exposed to capsaicin to deplete intracellularly stored CGRP, thereby amplifying the signal for antisense-induced knockdown. Immediately after CGRP depletion, the media was changed such that either KACGRP or KHZ or vehicle was diluted in DMEM plus 10% fetal bovine serum to 1 × 10⁶ pfu/ml and added to the cells. After waiting 2 days to allow for replenishment of CGRP stores, cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (pH 7.4) on ice for 60 min.

**Fig. 1.** Photomicrographs of cultured mouse dorsal root ganglion (DRG) neurons. (A) Nomarski (1,000×) photo of DRG cells without calcitonin gene–related peptide (CGRP) primary antisera. (B) Strong CGRP immunoreactivity in a DRG neuron treated with capsaicin but not with virus (630×). (C) Strong CGRP immunoreactivity in DRG neurons treated with both capsaicin and control virus (KHZ) (630×). (D, bottom) Mild CGRP immunoreactivity in a DRG neuron treated with capsaicin and KACGRP (630×). (D, top) Diagram of recombinant herpes vector KACGRP. An antisense sequence to the gene for rat CGRP (A-CGRP) is inserted, under the control of a human cytomegaloviral immediate/early promoter/enhancer (hCMV IEP) sequence into the thymidine kinase (TK) sequence of the genome of an attenuated herpes simplex type 1 virus. Insertion into the TK sequence disables this gene and thus prevents replication in nondividing cells. A similar virus, KHZ, was constructed encoding for the bacterial reporter gene *lac-Z* and was used as a control. KACGRP = antisense CGRP viral vector; SV40 PA = simian virus 40 polyadenylation sequence; U₁ = unique long sequence, U₈ = unique short sequence.
Immunohistochemistry. To estimate infectivity of the viral vector used in this study, three wells of KHZ-infected cultures were stained for the presence of β-galactosidase, the protein product of the lacZ gene inserted by KHZ. β-Galactosidase was localized after blocking in 10% normal donkey serum and overnight incubation in rabbit anti-β-galactosidase (1:2,000; Chemicon, Temecula, CA) followed by a biotinylated donkey anti-rabbit secondary antibody (1:200) for 60 min at room temperature. Streptavidin-conjugated horse-radish peroxidase was used to amplify the signal. Visualization of β-galactosidase localization was performed by incubating the coverslips in 0.5 mg/ml 3,3′-diaminobenzidine in 0.1 M phosphate buffer, pH 7.4, for 20 min. Coverslips were washed extensively with 0.1 M phosphate buffer before dehydrating in a graded ethanol series and mounting in DPX (Sigma).

Cellular analysis was performed by determining the percentage of dexamethasone-positive intact neurons using a Leica DMRXA (Leica Microsystems GmbH, Wet- zlar, Germany).

In Vivo Experiments

Drug Administration. Intrathecal administration of drugs was accomplished via lumbar puncture (L5) with a 1-ml syringe (Beckton Dickinson, Franklin Lakes, NJ).18 Paw withdrawal latencies were recorded before intrathecal injection of drugs and at 0, 15, 30, 45, and 60 min after injection. Vehicle was comprised of preservative-free saline and India ink (10:1). Mice that did not show proper injection of drug, as indicated by the presence of India ink within the intrathecal space, were excluded from the study. CGRP (Sigma) was delivered intrathecally in 10 μl vehicle at doses ranging from 0.1 to 10 nmol. The CGRP antagonist (10 nmol CCGRP<sub>8–37</sub>; Sigma) was administered intrathecally immediately after topical application of capsaicin. There is typically a several-minute delay between topical application of capsaicin and activation of nociceptors22; therefore, the antagonist was administered at a time just before likely maximal CGRP release. Paw withdrawal latencies were recorded as described below.

Application of Virus. Mice were anesthetized with freshly prepared tribromoethanol (500 mg/kg). Calcium thioglycolate (Nair; Church & Dwight, Princeton, NJ) was applied liberally to the left hind paw. After 5 min, Nair was removed with a patch of sterile gauze. Another sterile patch of gauze was used to wash and dry the hind paw before the application of the virus. Five microliters (1 × 10<sup>6</sup> pfu/μl) of the herpes simplex type 1 vector, i.e., KACGRP or KHZ, respectively, was applied and spread over the hind paw with a pipette tip.18,20,21 The control virus was applied to the hind paws of control animals in an identical manner.

Behavioral testing was performed after topical application of capsaicin at 1.5, 4, 6, 8, 10, 14, and 17 weeks after application of KACGRP or KHZ, respectively, to determine the time course of antihyperalgesic effect after viral infection.

Behavioral Assay. Paw withdrawal responses to noxious radiant heat previously described for use in rats22–24 were adapted for use in mice.18,25 Mice were lightly anesthetized with pentobarbital (50 mg/kg intraperitoneal). Light anesthesia (i.e., animals are crawling around, lifting their heads, and sniffing) has been shown to be useful when using the A<sub>δ</sub>/C test for measuring thermal hyperalgesia.18,25 In this test, the dorsal as opposed to the plantar surface of the hind paw is being stimulated, which makes it necessary to optimize animals’ position. Light anesthesia does not affect nociceptive responses in this test18,22 and limits stress-induced analgesia as well as learning, both of which can be problems in behavioral nociceptive testing. Paw withdrawal latencies were recorded after thermal stimulation at two distinct heating rates, 0.9°C/s and 6.5°C/s, which selectively activate C and A<sub>δ</sub> thermoneceptors, respectively. We have previously demonstrated that withdrawal responses to these stimuli occur at average latencies of 13.4° ± 0.5° and 2.7° ± 0.1°C respectively, and surface skin temperatures of 47.2° ± 0.4° and 51.7° ± 0.5°C.25 Cutoff latencies of 20 and 6 s, which we have shown produce peak surface temperatures of 48.7° ± 0.2° and 62.9° ± 0.7°C, respectively, were used to prevent tissue damage. Sensitization of C fibers was achieved via the topical application of 20 μl capsaicin, 0.1%, dissolved in water:ethanol (50:50) applied to the viral vector-infected hind paw. Three baseline paw withdrawal latencies (PWLs) were measured before experimental manipulation (pre1, pre2, and pre3). Significant differences between groups (n = 8–10/group) and across time were determined by analysis of variance for repeated measures followed by a post hoc Bonferroni analysis.

Measurement of CGRP Levels by Radioimmunoassay. Dorsal root ganglia from either side were separately analyzed for CGRP immunoreactivity by radioimmunoassay. Each DRG section was weighted for later standardization by weight. The DRGs were homogenized in 10 volumes of artificial cerebrospinal fluid with 1 mg/ml complete peptidase inhibitor (Roche Bioscience, Palo Alto, CA) and centrifuged, and the supernatant was lyophilized before reconstitution for radioimmunoassay. CGRP levels were assayed using a radioimmunoassay kit (Peninsula Laboratories, San Carlos, CA) and analyzed using a Beckman gamma counter. CGRP levels were extrapolated from a standard curve. Significant differences between KACGRP-infected animals and controls were determined by the Student t test.
Institutes of Health, Bethesda, MD). Slides were coded (in gray pixel units) using Image J version 1.26 (National Institutes of Health, Bethesda, MD). The dorsal horns of both naive and antisense CGRP–infected mice were analyzed for area and mean optical density as previously reported, 21,22,24 topically application of capsaicin significantly decreased latency for C fiber–mediated responses. Table 1. Immunoreactivity in Cultured DRG Neurons

<table>
<thead>
<tr>
<th></th>
<th>No Primary Antibody</th>
<th>Vehicle</th>
<th>KHZ Virus</th>
<th>KACGRP Virus</th>
</tr>
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<tr>
<td>Total cells, n</td>
<td>125 ± 23</td>
<td>237 ± 44</td>
<td>136 ± 18</td>
<td>124 ± 27</td>
</tr>
<tr>
<td>% Dense staining</td>
<td>0</td>
<td>84.3 ± 11.2</td>
<td>83.1 ± 19.4</td>
<td>13.7* ± 9.2</td>
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Introduction of KACGRP virus but not control (KHZ or vehicle) decreased the percentage of small (< 20 μm) cultured dorsal root ganglion (DRG) neurons with dense staining for calcitonin gene–related peptide in capsaicin-treated cultures (data presented as mean ± SEM).

* Significant difference compared with KHZ virus (P < 0.05, t test).

KACGRP virus = calcitonin gene–related peptide antisense virus; KHZ virus = control virus.

infection with KHZ was not significantly different from uninfected cells.

In Vivo Experiments

Effects of Intrathecal CGRP on Paw Withdrawal Latencies. The effects of intrathecal injection of CGRP on PWL were examined for both Aδ and C fiber–selective heating rates. Figure 2 shows the percent change from baseline PWL for Aδ and C thermonociception after application of different doses of CGRP. PWLs for the low, i.e., C fiber–selective, heating rate were significantly shorter after administration of 1, 5, and 10 nmol CGRP, except at a dose of 5 nmol, and then only minimally (but significantly). The intrathecal administration of vehicle had no effect on PWL (data not shown).

Effects of Intrathecal CGRP<sub>8,37</sub> on Paw Withdrawal Latencies. As previously reported, 21,22,24 topically application of capsaicin significantly decreased latency for C fiber–mediated responses (fig. 3) but not Aδ-mediated paw withdrawal responses (data not shown). This effect lasted for at least 60 min and was unaffected by intrathecal application of vehicle. Conversely, intrathecal application of the CGRP antagonist CGRP<sub>8,37</sub> significantly

Results

In Vitro Experiments

The average transduction rate of lac-Z by KHZ as indicated by staining for the presence of β-galactosidase was 63% (data not shown). Knockdown of CGRP was assessed by comparing the number of neurons that demonstrated dense CGRP immunoreactivity in KACGRP-infected, KHZ-infected, and uninfected cultured DRG neurons after exposure to capsaicin. The number of densely labeled CGRP-immunoreactive cells was significantly lower in KACGRP when compared with KHZ-infected or uninfected neurons (table 1 and fig. 1). The percentage of cells with dense staining for CGRP after

Fig. 2. Dose-dependent effect of intrathecal calcitonin gene–related peptide (CGRP) on nociceptive responses. Filled triangles represent the percent decrease in latency of paw withdrawal responses to Aδ nociceptor activation. Filled circles represent the decrease in C fiber–mediated responses. * Significant decreases in response latency as compared with baseline, indicating hyperalgesia (P < 0.05, analysis of variance, n = 8/group).
attenuated capsaicin-evoked hyperalgesia for C fiber-mediated responses. Application of the antagonist in the absence of capsaicin hyperalgesia had no effect (data not shown).

CGRP Levels in DRGs after Viral Infection as Measured by Radioimmunoassay. Calcitonin gene-related peptide levels in DRGs L1–S1 6 weeks after unilateral KACGRP infection of the hind paw were significantly lower compared with contralateral control DRGs (fig. 4), indicating a sufficient knockdown of CGRP after viral infection.

CGRP Immunoreactivity in the Dorsal Horn of the Spinal Cord after Viral Infection. To investigate the effects of topical application of the KACGRP virus to one hind paw of rats on CGRP immunoreactivity in superficial layers (laminae I and II) of spinal dorsal horns, we collected spinal cords of animals 6 weeks after viral infection (fig. 5A). The reduction of CGRP immunoreactivity was only significant at the levels of L4–L5 (fig. 5B), which correspond to the levels that innervate the infected dorsal hind paw. 26 No significant decrease in CGRP immunoreactivity was seen at the C13–L1 level. Application of KHZ had no effect on CGRP immunoreactivity at either of the levels examined (data not shown).

Effects of KACGRP on Capsaicin-induced Hyper-sensitivity. After application of KACGRP or KHZ, baseline latencies for both AδH254 (data not shown) and C fibers remained unchanged (fig. 6). Topical application of capsaicin produced a profound C fiber-selective thermal hyperalgesia for at least 60 min in mice that were infected with the control virus KHZ. This hyperalgesia was not seen in mice that had been infected with KACGRP. The antihyperalgesic effect of KACGRP lasted for at least 60 min at the 10 weeks post–viral infection time point (fig. 6) and was significant from 1.5 to 14 weeks after infection, but was no longer observed at the 17 weeks time point (fig. 7). Four weeks after infection with KACGRP, there seemed to be an analgesic effect rather than an antihyperalgesic effect, i.e., PWLs longer than baseline PWLs.

Discussion

This study sought to investigate the effects of intrathecal administration of CGRP and CGRP8-37—a CGRP an-
The extent of CGRP knockdown after infection of cultured DRG neurons with KACGRP was approximately 80% compared with uninfected or KHZ infected neurons. These findings are comparable to those of Cheli et al.,27 who recently found a 74% decrease of the N-methyl-D-aspartate receptor NR1 subunit after infection of cultured neurons with their antisense herpes simplex–based vector. In vitro, CGRP levels in the lumbar DRGs as measured by radioimmunooassay were significantly lower compared with controls, indicating a satisfactory in vivo transfection rate.

Intrathecal application of CGRP led to a dose-dependent decrease in PWL to C fiber–selective noxious skin heating. The findings are consistent with the results of groups that found development of mechanical hyperalgesia after intrathecal application of CGRP.28,29 It should be mentioned that there is also one study that did not find nociceptive behavior after intrathecal administration of CGRP.30 However, doses used in this study were lower (10–100 pmol CGRP) as compared with the doses used in our study (0.1–10 nmol). Other laboratories reported reversal of capsaicin-induced hyperalgesia as assessed by paw pressure latency, paw immersion latency, and hot-plate latency by intraplantar injections of the CGRP antagonist CGRP₈₋₃⁷.31 and antinociceptive effects of an antiserum against CGRP on thermal and mechanical noxious stimuli, when administered intrathecally,17 indicating that several sites of action might exist for CGRP-induced hypersensitivity.

To our knowledge, there is no study investigating the differential behavioral effects of CGRP on C and Aδ thermonociceptors. The fact that the majority of neurons displaying CGRP immunoreactivity had a conduction velocity below 2.5 m/s32 indicates that at least the bulk of releasable CGRP is stored in C fibers. Furthermore, it has been shown that neonatal capsaicin treatment which destroys a large portion of C fibers significantly reduces CGRP levels in the dorsal horn of the spinal cord.33 These observations are consistent with our findings of decreased PWLs for C-fiber thermonociception, whereas PWLs for Aδ fiber–selective heating were unchanged after intrathecal CGRP injection.

Selective knockdown of CGRP in primary afferents using a viral vector–mediated antisense technique significantly attenuated the thermal, C-fiber hyperalgesia normally observed after topical application of capsaicin. This antihyperalgesic effect was comparable to the effect of intrathecal application of the CGRP antagonist CGRP₈₋₃⁷ but lasted for 14 weeks after one single application. There is a slight discrepancy in the time course of PWLs after application of capsaicin to the skin after viral infection (fig. 6) when comparing the previously published preliminary data20 and the results presented here, which can be explained by the larger number of animals per group used in this study.

Studies with aCGRP-deficient mice found no significantly different nociceptive behavior compared with wild-type mice as tested by the tail-flick and hot-plate test.34 However, they did show an attenuated response to chemical pain and inflammation.35 Another study with mice deficient in the aCGRP gene found normal responses to noxious radiant heat stimuli; however, in contrast to wild-type mice, animals did not develop signs of secondary hyperalgesia in an inflammatory pain model.36 These data may reflect some inherent differences.
between the knockout approach and the selective knockdown of peptides in adults using herpes simplex virus-directed transduction. Compensatory changes in the expression of other transmitters are more likely to occur in knockout mice and could explain discrepancies to studies with (vector-mediated) knockdown in adult animals. Such compensatory changes are known to occur in knockout animals. For example, with sns-null mutant mice, ie, mice lacking the Na1.8 channel, found a significant compensatory up-regulation of tetrodotoxin-sensitive sodium channels by 100%. In addition, CGRP, when administered into the periaqueuductal gray of rats, produced antinociceptive effects that were counteracted by application of a CGRP antagonist. These central anti- and peripheral pronociceptive effects of CGRP could compensate each other (in part) in CGRP-deficient mice. Therefore the method used in this study, i.e., the selective knockdown of CGRP solely in primary afferents of a specific anatomical region, in adults, has unique utility in determining the function of CGRP in primary afferent nociceptors.

Chronic administration of opioids results in a decrease of, or tolerance to, its pharmacologic effects, such as antinociception. CGRP has been shown to decrease the analgesia produced by μ or δ agonists. On the other hand, opioids have been shown to inhibit the release of CGRP. Simultaneous administration of morphine and the CGRP antagonist CGRP \( \text{CGRP}_8-37 \) prevented the development of tolerance to the antinociceptive effects of morphine in experimental pain models. These data indicate that treatment with a CGRP antagonist could be a useful adjuvant for treatment of pain, more specifically for the prevention or attenuation of tolerance to the antinociceptive effects of morphine. The long-lasting effect after single application and the transdermal bioavailability of the viral vector encoding for CGRP anti-sense used in this study make it a valuable tool for further studies.

Pohl and Braza used an antisense CGRP viral vector to reduce the enhanced synthesis of CGRP induced in DRG neurons by peripheral injection of complete Freund adjuvant. They found a 50% decrease in CGRP-like material in lumbar DRGs in rats that were infected with the vector encoding for antisense CGRP as compared with controls. In addition, the concomitant inflammatory paw edema was less in animals infected with the antisense CGRP virus. The effects of reduced CGRP up-regulation and reduced paw edema were only significant up to 12 days or investigated for 16 days after induction of inflammation, respectively. However, these authors used a different promoter (modified herpes simplex virus latency promoter) in their viral vector. Unfortunately, this study did not assess changes in behavior to noxious stimuli.

Monkeys treated with a similarly constructed enkephalin-encoding virus as used in this study demonstrated behavioral antihyperalgesic effects that lasted for at least 20 weeks in a thermal/capsaicin model. Therefore, we would expect that the antisense CGRP vector used in this study would also function in primates and thus may be applicable to human pain conditions. Using replication-deficient herpes vectors to alter the phenotype of primary afferent nociceptors is also a powerful tool to explore mechanisms of pain and hyperalgesia.

References

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