Transient receptor potential vanilloid 1, calcitonin gene-related peptide, and substance P mediate nociception in acute pancreatitis

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ALTHOUGH SEVERE PAIN is a common symptom of acute pancreatitis, pain in many tissues (reviewed in Ref. 6). During tissue injury and inflammation several factors may activate TRPV1. These factors include lipoxigenase products, heat, and hydrogen ions, which directly activate TRPV1. Furthermore, agonists of G protein-coupled receptors (e.g., proteases, bradykinin) and receptor-tyrosine kinases (e.g., nerve growth factor) can indirectly sensitize TRPV1 (2, 6) and thereby lower the threshold for activation.

Activation of TRPV1 releases SP and CGRP from the peripheral projections of primary spinal afferent neurons to induce neurogenic inflammation. SP interacts with the neurokinin 1 receptor (NK1R) to induce plasma extravasation and granulocyte infiltration from postcapillary venules. CGRP interacts with calcitonin-like receptor (CLR) and receptor activity modifying protein 1 (RAMP1) to cause arteriolar vasodilation. These processes contribute to acute pancreatitis (4, 9, 42). Thus antagonism or deletion of the NK1R inhibits cerulein-induced pancreatitis in mice (9). In contrast, deletion of the SP-degrading enzyme neutral endopeptidase exacerbates pancreatitis (23). CGRP also contributes to cerulein pancreatitis in rats (42). The TRPV1 agonist capsaicin induces pancreatitis in rats that is attenuated by an NK1R antagonist and is thus mediated by SP from sensory nerves (15). Ablation of sensory nerves by chronic treatment with capsaicin diminishes cerulein-induced pancreatitis in mice, providing further support for involvement of TRPV1 (29, 30).

Activation of TRPV1 on neurons also releases SP and CGRP from the central projections of sensory nerves in the dorsal horn of the spinal cord, where they contribute to somatic and visceral pain. TRPV1-deficient mice exhibit impaired thermal hypersensitivity in response to tissue inflammation (6). The intrathecal administration of antagonists to NK1R and CLR inhibits the frequency of abdominal contractions, an index of allodynia, associated with colorectal distension in rats (32). In the pancreas, indirect evidence suggests that TRPV1, SP, and CGRP contribute to pancreatic pain. Injection of capsaicin into the pancreatic duct induces expression of the protooncogene c-fos in spinal neurons of the dorsal horn (T10), indicative of SP release upon stimulation of pancreatic sensory nerves. Induction of necrotizing pancreatitis by treatment with L-arginine caused a 12-fold increase in the number of spinal neurons expressing the proto-oncogene c-fos in laminae I and II of L1, suggesting activation of nociceptive pathways. L-Arginine also caused a threefold increase in spontaneous abdominal contractions detected by electromyography, suggestive of referred pain. Systemic administration of the TRPV1 agonist capsazepine inhibited c-fos expression by 2.5-fold and abdominal contractions by 4-fold. Intrathecal, but not systemic, administration of antagonists of CGRP (CGRP8–37) and SP (SR140333) receptors attenuated c-fos expression in spinal neurons by twofold. Thus, necrotizing pancreatitis activates TRPV1 on pancreatic sensory nerves to release SP and CGRP in the dorsal horn, resulting in nociception. Antagonism of TRPV1, SP, and CGRP receptors may suppress pancreatitis pain.

hyperalgesia; inflammation; neurokinin receptors; visceral pain; nociception

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CGRP; 3) examine whether activation of TRPV1 in the pancreas induces neuropeptide release in the spinal cord; and 4) determine the contribution of TRPV1, SP, and CGRP to pancreatic pain using antagonists.

METHODS

Animals. Sprague-Dawley rats (male, 200–400 g; Charles River Laboratories, Hollister, CA) were kept in a temperature-controlled environment with a 12:12-h light-dark cycle, with standard laboratory food and water freely available. All procedures were approved by the UCSF Institutional Animal Care and Use Committee and were compliant with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Academy of Sciences, Bethesda, MD).

Materials. The source of antibodies and the dilutions used for staining tissues are shown in Table 1. Agonists and antagonists include the following: capsaicin (Sigma, St. Louis, MO), capsaazepine (Sigma), NK1R antagonist SR140333 (Sanofi, Montpellier, France), and CLR in spinal neurons.

Localization of TRPV1, SP, and CGRP in the pancreas. Rats were anesthetized with pentobarbital sodium (100 mg/kg ip) and transcardially perfused with 100 ml of 0.1 M PBS (pH 7.4) followed by 300 ml of 4% paraformaldehyde in PBS. The pancreas was removed, immersion-fixed (4 h), and cryoprotected in 30% sucrose in PBS overnight. Tissue was embedded in optimal cutting temperature compound (OCT; Miles, Elkhart, IN), and 15-μm frozen sections were prepared. Sections were incubated in blocking solution [3–10% normal goat serum (NGS) in PBS with 0.3% Triton-X 100] followed by the antibodies to TRPV1, SP, or CGRP, and fluorescent secondary antibodies (Table 1); with intervening washes. Sections were observed using a Zeiss Axiovert microscope (Thornwood, NY) and an MRC 1000 laser-scanning confocal microscope (Bio-Rad, Hercules, CA) by an investigator who was unaware of the experimental groups. Images were colored, and the brightness and contrast were adjusted using Adobe Photoshop 7.0 (Mountain View, CA).

Intraductal infusion of capsaicin and endocytosis of NK1R and CLR in spinal neurons. Capsaicin was injected into the pancreatic duct as described (14). Rats were anesthetized with pentobarbital sodium (20 mg/kg ip). The abdomen was opened with a midline incision. The antimesenteric side of the duodenum was punctured with a 25-gauge needle near the papilla of Vater. A catheter (PE-10 tubing) was inserted into the main hepatic duct, which was occluded with a clip to prevent misdirected leakage. Residual bile was allowed to drain by gravity for 5 min. The needle was removed, and the abdomen was closed. After 2.5 h, the rats were transcardially perfused, the spinal cord (T6 and T10) was removed, and 15-μm sections were collected. Sections were incubated with blocking solution (10% NGS in PBS with 0.3% Triton-X-100), then with antibodies to the NK1R or CLR, and then washed and incubated with fluorescent secondary antibodies (Table 1). Specimens were observed using confocal microscopy by an investigator unaware of the treatment groups (E. F. Grady). The receptor was considered internalized if greater than 20 endosomes were noted in the soma. Six spinal cord sections per treatment group were evaluated. Figure 2 contains representative images of spinal cord.

l-Arginine induced pancreatitis. Fasted (overnight), awake rats were injected twice with l-arginine (250 mg/100 g ip in 20% saline; Sigma) at time 0 and 1 h (10). Animals were kept in a quiet, undisturbed environment with free access to food and water until they were killed after 12–36 h. Pancreatitis was assessed by measuring amylase activity in the serum using the Infinity amylase assay (Thermo Electron, Arlington, TX) as described (9) and by histological examination of sections of pancreas stained with hematoxylin and eosin.

Pancreatic neutrophil sequestration was quantified by measuring MPO activity in the pancreas. The pancreas was removed after perfusion and stored at 70°C. The tissue was then thawed and homogenized in 20 mM phosphate buffer (pH 7.4), centrifuged (10,000 g for 10 min at 4°C), and the resulting pellet was resuspended in 50 mM phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide (Sigma). The suspension was subjected to four cycles of freezing and thawing and further disrupted by sonication (60 s), followed by centrifugation (10,000 g for 5 min at 4°C). The supernatant (100 μl) was tested for MPO activity with 100 μl of o-dianisidine dihydrochloride in sodium phosphate buffer (50 mM, pH 6.0) mixed with 10 μl hydrogen peroxide. MPO is expressed as milliunits per milligram protein.

Two strategies were used to exclude the possibility that l-arginine-induced pain was due to nonspecific peritoneal irritation resulting from the intraperitoneal route of drug administration. First, l-arginine (250 mg/100 g in 20% saline at t = 0 and t = 1 h) was administered to a separate group of animals subcutaneously in the dorsum of the rat. A second group of animals was injected with intraperitoneal glycine, an amino acid which does not cause pancreatitis (Sigma, 250 mg/kg in 20% saline at t = 0 and t = 1 h).

Detection of immunoreactive Fos in the spinal cord by immunohistochemistry. Rats were anesthetized with pentobarbital sodium (100 mg/kg ip) and transcardially perfused with 100 ml of 0.1 M PBS followed by 300 ml of 3.7% paraformaldehyde in PBS. The head of the pancreas was removed after perfusion and stored in formalin. Sections of thoracic and lumbar spinal cord (T3, T9, T11, and L1) were immersion-fixed (4 h), and cryoprotected in 30% sucrose in PBS. The sections were then embedded in OCT (Thermo Electron, Arlington, TX) as described (9) and by histological examination of sections of pancreas stained with hematoxylin and eosin.

Table 1. Antibody sources and incubation conditions

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guinea pig anti-rat TRPV1 (COOH terminus)</td>
<td>Chemicon, Temecula, CA</td>
<td>1:1,000, 24 h, 4°C</td>
</tr>
<tr>
<td>Rabbit anti-rat SP</td>
<td>Sigma, St. Louis, MO</td>
<td>1:500, 24 h, 4°C</td>
</tr>
<tr>
<td>Mouse monoclonal anti-rat CGRP (code 4901)</td>
<td>CURE Digestive Diseases Research Center, UCLA</td>
<td>1:500, 24 h, 4°C</td>
</tr>
<tr>
<td>Rabbit anti-nK1R (code 94168)</td>
<td>CURE Digestive Diseases Research Center, UCLA</td>
<td>1:2,000, 24 h, 4°C</td>
</tr>
<tr>
<td>Goat anti-rabbit IgG Rhodamine Red</td>
<td>Jackson Immunoresearch Laboratories, West Grove, PA</td>
<td>1:200, 24 h, 4°C</td>
</tr>
<tr>
<td>Goat anti-mouse IgG Rhodamine Red</td>
<td>Jackson Immunoresearch Laboratories</td>
<td>1:200, 2 h, room temperature</td>
</tr>
<tr>
<td>Goat anti-rabbit IgG Alexa Fluor 488</td>
<td>Molecular Probes, Eugene, OR</td>
<td>1:1,000, 2 h, room temperature</td>
</tr>
<tr>
<td>Goat anti-guinea pig IgG FITC</td>
<td>Molecular Probes</td>
<td>1:500, 2 h, room temperature</td>
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<tr>
<td>Rabbit anti-c-Fos</td>
<td>Molecular Probes</td>
<td>1:20,000, 24 h, 4°C</td>
</tr>
<tr>
<td>Biotinylated goat anti-rabbit IgG</td>
<td>Vector Labs, Burlingame, CA</td>
<td>1:200, 2 h, room temperature</td>
</tr>
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TRPV1, transient receptor potential vanilloid 1; SP, substance P; NK1R, neurokinin 1 receptor; CLR, calcitonin-like receptor.
blocking solution (3% NGS in PBS, 1 h, room temperature) and then Fos antibody (Table 1). Sections were washed and incubated with biotinylated goat anti-rabbit antibody, followed by an avidin-biotin-peroxidase complex (Vector Labs, Burlingame, CA). For the staining reaction, 1% hydrogen peroxide (Sigma) was used as a hydrogen ion donor and 0.05% diaminobenzidine (Sigma) was used as the chromogen. Slides were examined by an investigator unaware of the experimental group using microscopy at ×20 magnification. The number of Fos-stained nuclei was counted in six sections per animal from each spinal cord level. Fos staining was subdivided into three groups (laminae I and II, laminae III and IV, and laminae V–X), and each group was averaged per section.

Electromyographic recordings. Rats were anesthetized with ketamine (75 mg/kg im) and xylazine (5 mg/kg im). Six NiCr wire electrodes (80-mm diameter) were implanted bilaterally in the upper half of the external oblique muscle of the abdominal wall, and the wires were exteriorized on the back of the neck and secured to the skin (28). Animals were allowed to recover for 3 days. Rats were then acclimatized to plastic restrainers twice a day for 2 days. The electrical activity of the abdominal muscles were amplified (GrassLink 15 Amplifier; Grass Telefactor, West Warwick, RI) and recorded (Grass Polyview System; Grass Telefactor) for 20-min periods. Two sets of baseline recordings were obtained from each animal prior to induction of pancreatitis. Pancreatitis was induced with L-arginine as described. Recordings were then obtained at 12, 24, and 36 h after induction of pancreatitis. Data are expressed as the percent increase in the total number of abdominal contractions per 20 min.

Administration of antagonists. For systemic antagonism of TRPV1, rats were pretreated with capsazepine (25 mg/kg sc) or vehicle (10% ethanol and 10% Tween-80 is) 1 h prior to induction of pancreatitis and then re-treated with capsazepine (25 mg/kg) every 12 h until they were killed. The dose of capsazepine was selected based on efficacy in inhibiting carrageenan-induced Fos expression in the hindpaw of the rat (21). For central antagonism of CLR and NK1R, CGRP8–37 (50 μg), SR140333 (30 μg), or vehicle (saline) was administered by intrathecal injection at 22 h after the induction of pancreatitis. Experiments using antagonist- and vehicle-treated animals were conducted simultaneously. Rats were briefly anesthetized with halothane. As a guide, a blunt, grooved 25-gauge needle was inserted into the intrathecal space of the lumbar region of the spinal cord in a sterile fashion. A catheter was placed into the intrathecal space through the groove (Harvard Apparatus, Harvard, MA). Drugs were injected in a volume of 50 μl and flushed in with 10 μl saline, and the needle and catheter were removed. Animals were then awakened from anesthesia, monitored for disability, and killed 2 h later. At the time of spinal cord dissection, the tract of the needle was inspected to ensure that the drug was delivered into the intrathecal space. Accurate placement of the intrathecal catheter was assessed by the ease of placement and the ability of the catheter itself to advance (thread) without resistance as has been previously described (16). For systemic antagonism of CLR and NK1R, rats were pretreated subcutaneously with CGRP8–37 (100 μg), SR140333 (60 μg), or vehicle 1 h prior to induction of pancreatitis and then retreated with antagonists every 12 h until they were killed. Doses of CGRP8–37 and SR140333 were selected based on efficacy in colorectal distension models of visceral pain (18, 32).

Statistical analysis. Data are means ± SE. Differences between experimental and control groups were assessed using one-way analysis of variance, with P < 0.05 considered significant.

RESULTS

Pancreatic sensory nerves coexpressed TRPV1, SP, and CGRP. We determined whether TRPV1 is expressed in nerve fibers within the pancreas using immunofluorescence. Immunoreactive TRPV1 was detected in numerous fibers in close proximity to pancreatic acini (Fig. 1). These nerve fibers also coexpressed CGRP (Fig. 1A) and SP (Fig. 1B). Thus TRPV1 is expressed in the peripheral projections of primary spinal afferent neurons, where it may be exposed to inflammatory mediators and thereby mediate hypersensitivity.

Fig. 1. Representative images (n = 3 rats) showing localization of transient receptor potential vanilloid 1 (TRPV1), CGRP, and substance P (SP) in the body of the pancreas. TRPV1 was detected in nerve bundles and fibers between acinar cells. These nerves coexpressed CGRP (A) and SP (B) (arrows). Scale bar = 20 μm.
Intraductal administration of capsaicin induced endocytosis of the NK1R but not CLR in spinal neurons. SP induces endocytosis of the NK1R in many cell types. Sites of SP release in the spinal cord can be detected using confocal microscopy to identify the disappearance of NK1R from the cell surface and appearance of the receptor in endosomes (25). We examined whether activation of TRPV1 in the pancreas by intraductal capsaicin induced endocytosis of the NK1R in the dorsal horn, which would be indicative of SP release from the central projections of pancreatic sensory nerves. In control experiments in which vehicle was injected into the pancreatic duct, immunoreactive NK1R was detected at the plasma membrane of the soma and dendrites of spinal neurons in laminae I–III of the dorsal horn of the spinal cord (T10, Fig. 2A). At 2.5 h after injection of the TRPV1 agonist capsaicin into the pancreatic duct, NK1R was detected in numerous endosomes in the dendrites and soma of neurons in lamina I and to a lesser extent in lamina III (Fig. 2A). Thus activation of pancreatic sensory nerves by intrapancreatic capsaicin induced the release of SP from the central projections of primary spinal afferent neurons in the dorsal horn, where SP activated the NK1R to induce endocytosis.

In transfected cell lines that overexpress CLR and RAMP1, CGRP induces endocytosis of CLR (7). To determine whether activation of TRPV1 in the pancreas induces the release of CGRP within the dorsal horn and consequent endocytosis of CLR, we localized CLR in the spinal cord after intraductal injection of capsaicin. In vehicle-treated animals, immunoreactive CLR exhibited an intense punctate distribution in laminae I and II of the dorsal horn, suggestive of localization in nerve endings or fibers (Fig. 2B). CLR was rarely detected in the soma of spinal neurons. Notably, there was no detectable receptor at the plasma membrane of the soma or fibers. This punctate localization of CLR was markedly different from that of the NK1R, which was prominently detected at the plasma membrane of the soma and dendrites of spinal neurons in the vehicle-treated animals. The localization of CLR was unaffected by intraductal administration of capsaicin (Fig. 2B).

Fig. 2. Representative images (n = 3 rats) showing localization of neurokinin 1 receptor (NK1R) and calcitonin-like receptor (CLR) with CGRP in the dorsal horn of T10 spinal cord. A: capsaicin or vehicle was injected into the pancreatic duct, and 2.5 h later tissue was collected for analysis. In vehicle-treated rats, NK1R was detected at the plasma membranes of the somata and dendrites of spinal neurons in laminae I–III (arrowheads). Intraductal capsaicin induced endocytosis of the NK1R in neurons in lamina I and to a lesser extent in lamina III (arrows). B: in vehicle-treated rats, CLR was present in punctate structures in nerve fibers of laminae I–III (arrows). This distribution was unaffected by intraductal capsaicin. Scale bar = 10 μm.
Nerve terminals or fibers that stained for CLR were in close proximity to nerve fibers containing immunoreactive CGRP, which was also detected in laminae I and II of the spinal cord (Fig. 2, A and B). Thus CLR is appropriately localized to interact with CGRP in the dorsal horn. However, although we found that CLR was highly expressed in the spinal cord in regions that are innervated by nerve fibers containing CGRP, activation of sensory nerves did not alter the subcellular distribution of this receptor in these experiments.

\textbf{L-Arginine induced necrotizing pancreatitis.} L-Arginine induced a severe form of pancreatitis that was evident on gross inspection by tissue necrosis and hemorrhage involving the pancreas and peripancreatic and mesenteric fat, which was similar to findings in humans with severe pancreatitis. Serum amylase was elevated 12 h after L-arginine administration (data not shown), and amylase levels peaked at 24 h (L-arginine, 12,465 ± 882 U/ml; control, 8,088 ± 220 U/ml; n = 6, \( P < 0.05 \); Fig. 3A). Histological examination of the pancreas 24 h after L-arginine treatment revealed neutrophil and monocyte infiltration, interlobular edema, and acinar cell necrosis, whereas the pancreata of animals treated with vehicle were normal (Fig. 3B).

\textbf{L-Arginine induced expression of Fos in spinal neurons and caused increased frequency of abdominal contractions.} Expression of the proto-oncogene c-fos in spinal neurons is well established as an indirect marker of activated neurons in spinal nociceptive pathways. To determine whether pancreatitis induced activation of nociceptive neurons in the spinal cord, we quantified expression of Fos in spinal neurons. L-Arginine stimulated a marked increase in the number of Fos-stained nuclei at T9, T11, and L1 in laminae I and II at 24 h. The most prominent increase was seen at L1, where L-arginine caused a 12-fold increase in the number of Fos stained nuclei (L-arginine, 73 ± 6; vehicle, 5 ± 2; \( n = 6 - 9, P < 0.05 \); Fig. 4A). Most Fos stained nuclei were detected in laminae I and II of the dorsal horn of the spinal cord, the region where the majority of the sensory fibers synapse.

To confirm that the observed increase in Fos immunoreactivity seen at 24 h was due to acute pancreatitis inflammation and not simply peritoneal irritation from administration of L-arginine, we conducted two additional control experiments. In the first control experiment, glycine was administered intraperitoneally as a control for nonspecific pancreatic inflammation associated with amino acid administration. Spinal cord Fos-stained nuclei per section at 24 h in the glycine group was equivalent to that seen in saline-treated animals (glycine, 9 ± 4; vehicle, 11 ± 2; \( n = 4, P > 0.05 \)). In the second control experiment, a similar dose of L-arginine was administered subcutaneously and the animals were killed at 24 h. Spinal cord Fos staining was blunted compared with intraperitoneal administration. Again, the most prominent increase was seen at L1, where L-arginine caused a twofold increase in the number of Fos-stained nuclei (L-arginine, 17 ± 3; vehicle 9 ± 2; \( n = 4, P < 0.05 \)). Interestingly, in the animals treated with L-arginine subcutaneously, there was evidence of mild pancreatitis as noted by mild elevation in serum amylase. Hence, subcutaneous administration of L-arginine is able to induce pancreatitis, albeit, to a lesser degree, which is likely explained by the efficiency of the intraperitoneal vs. subcutaneous absorption of large volumes in the rat. This is further confirmed by the glycine control experiment where the lack of Fos immunoreactivity at 24 h was associated with normal serum amylase.

There is an increased frequency of abdominal contractions in animals with visceral pain induced by colorectal distension with a balloon catheter, and measurement of these contractions is an established behavioral correlate of visceral pain (28). To assess whether the noted increased Fos staining observed in L-arginine pancreatitis represented activation of nociceptive neuronal activation, we measured the frequency of spontaneous abdominal contractions in animals with pancreatitis using chronically implanted electrodes. The baseline frequency of spontaneous abdominal contractions ranged from 20–39 per 20-min interval. Treatment with L-arginine increased the frequency of contractions within 12 h, and effects were maximal at 24 h (Fig. 4B). At 24 h the frequency was 290 ± 48% over baseline in L-arginine-treated animals and 102 ± 19% in vehicle-treated animals (\( P < 0.05, n = 6 \)). Thus pancreatitis induces abdominal musculature contraction, which is indicative of pain-related behavior.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig3.png}
\caption{L-Arginine (L-Arg)-induced pancreatitis. A: serum amylase activity in rats 24 h after treatment with vehicle or L-Arg (\( n = 6 \) rats, \( P < 0.05 \)). B: representative hematoxylin and eosin-stained sections of pancreas 24 h after vehicle or L-Arg. Note the infiltration of neutrophils, vacuolization, and acinar necrosis in pancreatitis. Scale bar = 100 \( \mu \)m; \( n = 6 \) rats.}
\end{figure}
Antagonism of TRPV1 diminished Fos expression and abdominal contractions. We treated animals with the TRPV1 antagonist capsazepine to assess the role of TRPV1 in pancreatic pain. Systemic administration of capsazepine inhibited L-arginine-induced activation of nociceptive neurons at T9 and T11 as evaluated by counting nuclei in the dorsal horn that expressed Fos (Fig. 5A). The greatest effect of capsazepine on Fos staining was noted at T11 (capsazepine, 23.4 ± 3.1; vehicle, 10.4 ± 1.7; n = 6, P < 0.05). There was minimal effect of capsazepine on the number of Fos-stained nuclei at T3, which is anticipated since T9, T11, and L1 are the regions that receive the predominant innervation by pancreatic afferent fibers. Furthermore, capsazepine had no effect on the number of Fos-stained nuclei in animals treated with the L-arginine vehicle. Of note, the number of Fos-stained nuclei in the vehicle-treated animals (Fig. 5A) was lower than observed in the L-arginine animals (Fig. 5A), which may reflect an effect of the capsazepine vehicle (DMSO, alcohol, and saline) on either activation of nociceptors or Fos expression. Furthermore, the repeated subcutaneous injections associated with capsazepine or carrier delivery may increase the threshold for nociceptive neuron activation.

In addition to inhibiting expression of Fos, capsazepine also strongly decreased the frequency of abdominal contractions induced by L-arginine pancreatitis (vehicle, 667 ± 43% basal; capsazepine, 145 ± 18% basal; P < 0.05, Fig. 5B). Thus
Antagonism of TRPV1 inhibits activation of nociceptive neurons in the spinal cord and prevents a behavioral index of pain in pancreatitis. To determine whether the antinociceptive effects of capsaizepine were secondary to an anti-inflammatory action, which would also suppress pain, we determined the effects of capsaizepine on severity of pancreatitis. At 24 h after treatment with L-arginine, the levels of serum amylase were not significantly different in animals receiving capsaizepine or vehicle (13,457 ± 680 U/ml or vehicle 13,217 ± 223 U/ml; n = 6–9 rats, *P < 0.05 vs. vehicle. B: abdominal contractions measured 12, 24, and 36 h after treatment with L-arginine plus systemic administration of capsaizepine or vehicle. Note that capsazepine inhibited abdominal contractions at all time points; n = 6–9 rats, *P < 0.05 vs. vehicle.

Fig. 5. Effects of TRPV1 antagonism on L-Arg pancreatitis. A: Fos expression in the nucleus of spinal neurons in laminae I–III of the dorsal horn of T3, T9, T11, and L1 at 24 h after treatment with L-Arg plus systemic administration of capsaizepine or vehicle. Note that capsazepine inhibited Fos expression in T9, T11, and L1; n = 6–9 rats, *P < 0.05 vs. vehicle. B: abdominal contractions measured 12, 24, and 36 h after treatment with L-arginine plus systemic administration of capsaizepine or vehicle. Note that capsazepine inhibited abdominal contractions at all time points; n = 6–9 rats, *P < 0.05 vs. vehicle.

Antagonism of CGRP and SP receptors in the spinal cord inhibited Fos expression in spinal neurons. The release of CGRP and SP from the central projections of pancreatic afferent nerves in the dorsal horn of the spinal cord may activate nociceptive neurons and induce Fos expression. To investigate this possibility, we administered antagonists of CGRP and SP receptors by intrathecal injection, thereby avoiding any anti-inflammatory effects in peripheral tissues. Antag-

Fig. 6. Effects of capsazepine on L-Arg-induced pancreatitis. A: serum amylase activity measured at 24 h was the same in rats treated with L-Arg plus capsaizepine or vehicle; n = 6–9 rats, P > 0.05. B: representative images (n = 6 rats) showing hematoxylin and eosin stained sections of pancreas in rats treated with L-Arg plus capsaizepine or vehicle. Note that the pancreas of capsaizepine-treated rats showed infiltration of neutrophils, vacuolization, and acinar necrosis. Scale bar = 100 μm. C: pancreatic MPO activity measured at 24 h was the same in rats treated with L-Arg plus capsaizepine or vehicle; n = 5 rats, P > 0.05.
CGRP receptor antagonist CGRP8–37 markedly inhibited expression of Fos in the dorsal horn was assessed 2 h later. Antagonists inhibited Fos expression in the spinal cord (T11: vehicle, 19 ± 1.0; SR140333, 11 ± 0.8; n = 6, P < 0.05; Fig. 7B). Overall, the number of Fos-stained nuclei in the spinal cord was lower than that seen in l-arginine-treated animals (Fig. 4), which probably reflects the antinoceptive effects of the anesthesia required for intrathecal injections. However, the intrathecally administered antagonists had no effect on serum amylase or pancreatic histology (data not shown) and thus did not influence pancreatitis. Peripheral administration of CGRP8–37 and SR140333 and had no effect on Fos staining in the spinal cord (T11: vehicle, 43 ± 8.6; CGRP8–37, 38 ± 4; T9: vehicle, 21 ± 3; SR140333: 28 ± 4; n = 4, P > 0.05).

**DISCUSSION**

We and others have previously shown that TRPV1 activation in the pancreas causes local release of SP and CGRP and induces neurogenic inflammation (9, 23, 24, 29). TRPV1 is also known to mediate pain signaling in somatic models of pain (6). We therefore hypothesized that activation of TRPV1 in the inflamed pancreas promotes release of SP and CGRP in the spinal cord to induce pain. We found that sensory nerves innervating the pancreas coexpressed TRPV1, SP, and CGRP and that activation of TRPV1 in the pancreas stimulated release of SP within the spinal cord as evidenced by NK1R endocytosis within spinal neurons. Pancreatitis resulted in activation of spinal neurons, as evidenced by increased Fos expression, and promoted contraction of abdominal muscles, a behavioral correlate of pain. Systemic antagonism of TRPV1 and central antagonism of SP and CGRP receptors inhibited nociception in pancreatitis.

**Assessment of pancreatitis pain.** We selected the l-arginine model for the study of inflammatory pain in the pancreas. This model is particularly well-suited for pain studies as the animals develop a more severe pancreatitis than is observed following secretagogue administration, and yet it is a nonsurgical model, thereby eliminating the confounding effects of surgical pain. The mechanism of l-arginine-induced pancreatitis is only partially understood. l-Arginine is directly toxic to acinar cells and also promotes release of nitric oxide and oxygen free radicals that enhance the cellular damage (10).

We used two approaches to measure inflammatory pancreatic pain. We indirectly assessed activation of spinal neurons in nociceptive pathways by examining Fos expression, and we measured the frequency of abdominal contractions as a behavioral correlate of nociception. Although expression of Fos is not specific for nociceptive inputs, it is widely accepted as an indirect marker of activation of Fos-expressing nociceptive neurons in the dorsal horn (5) and has been used by our group and others to study activation of nociceptive nerves in the pancreas (13, 14, 20). Thus following administration of the secretagogue cerulein, which causes a mild interstitial pancreatitis, Fos expression is increased in the superficial laminae (I and II) of the spinal cord at levels T9 and T10 in the rat (20). This pattern of Fos activation is similar to that produced by injection of capsaicin or trypsin directly into the pancreas (T8–10) (13, 14) and is consistent with retrograde tracing studies indicating that pancreas afferent fibers synapse at levels T6–L1 of the spinal cord (33, 44). In the present study, prominent Fos immunoreactivity was noted at T9 and T11; however, the highest numbers of Fos-positive nuclei were noted at L1. The cause of this peak at L1 is unclear. It is not a nonspecific effect of intraperitoneal injection, since glycine injection into the peritoneum had no effect, and the subcutaneous route of administration of l-arginine produced a similar distribution of Fos-positive nuclei in the spinal cord. l-Arginine does not cause inflammation of other abdominal organs, such as the liver or bowel (10). Increased Fos staining at L1 could reflect activation of additional nociceptors in this more severe model of pancreatitis because l-arginine induces tissue necrosis not only the gland itself but also in peripancreatic and mesenteric tissues.

Electromyographic recording of abdominal contractions is a technique that is widely used to assess visceral pain caused by
colorectal distension (17, 18). Visceral pain is referred to the abdominal wall and causes contractions of the rectus muscle in rodents. We detected a marked increase in abdominal contractions in animals with L-arginine pancreatitis, which correlated with the severity of inflammation and with expression of Fos. Our results are in agreement with studies using von Frey hairs applied to the abdominal wall as an index of inflammatory pancreatic pain (12, 43). The electromyography results together with the Fos staining support the finding that the L-arginine model of pancreatitis is painful.

Contribution of TRPV1 to pancreatitis pain. We detected TRPV1 immunoreactivity in sensory nerves within the pancreas that also contained SP and CGRP. Within the pancreas, SP and CGRP are present in the projections of primary spinal afferent neurons derived from T6–L2 (33, 35). Thus TRPV1 colocalizes with SP and CGRP in the peripheral projections of primary spinal afferent neurons in the pancreas. Our findings support the importance of TRPV1-mediated neurogenic mechanisms of pancreatic inflammation (15).

Systemic administration of the TRPV1 antagonist capsazepine attenuated Fos expression and prevented abdominal contractions induced by L-arginine pancreatitis. However, serum amylase levels were unaffected by capsazepine, suggesting that antagonism of TRPV1 did not affect acinar cell injury. Moreover, histological examination revealed substantial inflammation of the pancreas in animals receiving capsazepine. Furthermore, pancreatic MPO activity was not significantly decreased in animals treated with capsazepine as opposed to carrier in L-arginine-induced pancreatitis. We and others have reported that capsazepine inhibits cerulein-induced pancreatitis in mice and rats (15, 29). NK1R antagonists also suppress cerulein pancreatitis (4, 9). Differences in sensitivity to capsazepine may reflect disparity in the mechanisms and severity of the inflammation. Thus L-arginine causes a more severe inflammation due to direct toxicity of acini, whereas cerulein causes a mild interstitial pancreatitis due to hyperstimulation of acinar cells. Alternatively, pancreatic nociception may be more sensitive to TRPV1 antagonism than pancreatic neurogenic inflammation. Interestingly, intradermal administration of capsazepine attenuates both carrageenan-induced and formalin-induced inflammatory pain in rats and mice without affecting inflammation, which supports our results (21, 34). Others have reported only modest antinociceptive actions of capsazepine (41), possibly due to limited bioavailability (38). We pretreated animals with capsazepine and then redosed throughout the experiment, which may have overcome this problem. Since capsazepine can have nonspecific effects (38), we cannot exclude the possibility that capsazepine attenuated pancreatitis pain by mechanisms other than TRPV1 antagonism.

The mechanism by which TRPV1 is activated in pancreatitis remains to be determined, although there are many possibilities. Protons, lipids, and elevated temperatures could directly activate TRPV1 (6). In addition, inflammatory agents such as proteases, bradykinin, ATP, and prostaglandin E₂ can indirectly activate TRPV1 to cause hyperalgesia (2, 6). Of these, proteases may be especially important in the inflamed pancreas. Primary spinal afferent neurons, including those innervating the pancreas, express protease-activated receptor-2 (PAR-2), a receptor for trypsin and mast cell tryptase (13, 14, 37, 40). During pancreatitis, there is premature activation of trypsinogen (11) and infiltration of mast cells containing trypsin (12). Activation of PAR-2 on sensory nerves induces the release of neuropeptides in peripheral tissues to induce neurogenic inflammation (37). PAR-2 agonists also sensitize TRPV1, by protein kinase C-ε-mediated phosphorylation of the channel, and thereby induce hyperalgesia (2). Indeed, administration of PAR-2 agonists into the pancreatic duct enhances Fos expression in spinal neurons, providing evidence for a role of PAR-2 in pancreatic pain (13, 14). Our work implicates TRPV1 in pain signaling from the pancreas, but it does not elucidate the mechanism of activation or sensitization of this channel. Further experiments are necessary to identify the mediator(s) of TRPV1 sensitization in pancreatitis.

Contributions of SP and CGRP to pancreatitis pain. Our laboratory was the first to demonstrate endocytosis of a GPCR (the NK1R) in vivo, and this approach has been used by many other groups to assess neuropeptide (SP) release (25, 26). The use of this approach to study peptide release requires that the receptor be present on the cell surface in the unstimulated state. Activation of TRPV1 on pancreatic sensory nerves by intraductal capsaicin resulted in endocytosis of the NK1R in the dorsal horn of the spinal cord at T10. Our results suggest that activation of TRPV1 in the pancreas promotes SP release from the central projections of pancreatic sensory nerves to activate the NK1R on nociceptive spinal neurons.

CLR was prominently detected in punctate structures, in nerve terminals or fibers, in the dorsal horn in close association with CGRP in fibers projecting from the DRG. CLR was never detected at the plasma membrane of neurons in the dorsal horn of the spinal cord, even under unstimulated conditions. Indeed, most of the immunoreactivity had a punctate distribution in nerve fibers, and it was very rarely detected in the cell bodies. This pattern of staining was unaffected by induction of pancreatitis. This punctate staining could represent either an internalized receptor or newly synthesized or stored receptor. Thus CLR is expressed in regions of the cord that receive innervation by pancreatic sensory nerves and could mediate the effects of released CGRP; however, additional studies are needed to investigate this possibility. In heterologous expression systems, CLR and RAMP1 are present at the plasma membrane and CGRP induces endocytosis of CLR (7).

We found that antagonism of CGRP and SP receptors in the spinal cord, but not the periphery, inhibited Fos expression during pancreatitis, suggesting that CLR and NK1R in the dorsal horn of the spinal cord play an important role in pain transmission. Our results are in accordance with observations that antagonism or deletion of the NK1R suppresses visceral pain caused by colorectal distension (17). In support of a role for the SP and the NK1R in pancreatitis pain, NK1R mRNA in patients with chronic pancreatitis correlates with the intensity of the pain (36). We did not observe that systemic antagonism of the NK1R affected Fos expression in L-arginine pancreatitis. In contrast to our results, peripheral, but not central, antagonism of the NK1R using CP-99,994, a tachykinin NK-1 receptor antagonist, inhibited von Frey filament hypersensitivity in dibutyltin dichloride-induced pancreatitis in rats (39). However, since CP-99,994 can cross the blood-brain barrier, the mechanism of activation or sensitization of this channel remains to be determined, although there are many possibilities.

We also observed that central antagonism of CLR suppressed Fos expression. CGRP release in the dorsal horn...
promotes pain signaling and may even work in synergy with SP to augment the transmission of painful stimuli. Thus intra- thecal CGRP antibody and CGRP (1-37) suppress mechanical and thermal hyperalgesia and allodynia, which are induced by paw inflammation or spinal cord hemisection in rats (3, 19). CGRP potentiates the release of SP from primary spinal afferent neurons (31), presumably by activating neuronal receptors. Although CGRP inhibits the degradation of SP by neutral endopeptidase (22), potentiated SP release occurs in the presence of peptide inhibitor conditions (31), suggesting a direct stimulation of SP release.

In conclusion, necrotizing pancreatitis may activate TRPV1 on pancreatic sensory nerves to induce release of CGRP and SP in the dorsal horn, where they mediate nociception. Antagonism of TRPV1, SP, and CGRP receptors may offer new therapies for pancreatitis pain and hyperalgesia.

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