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Synergistic Role of TRPV1 and TRPA1 in Pancreatic Pain and Inflammation

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Abstract

Background & Aims—The transient receptor potential (TRP) channels TRPV1 and TRPA1 have each been associated with regulation of efferent properties of primary afferent neurons that initiate neurogenic inflammation and are required for the development of inflammatory hyperalgesia. To evaluate the role of these channels in producing pain during pancreatic inflammation, we studied pancreatic nodose (NG) and dorsal root (DRG) ganglion sensory neurons (identified by content of retrograde tracer) and behavioral outcomes in a mouse model of acute pancreatitis.

Methods—Pancreatic inflammation was induced by 8 hourly injections of caerulein (50 µg/kg). The extent of inflammation, pancreatic neuron TRP channel expression and function and excitability, and pain-related behaviors were evaluated over the course of the following week.

Results—Histology and myeloperoxidase activity confirmed pancreatic inflammation that was associated with increased excitability and mRNA expression of the TRP channels in NG and DRG pancreatic neurons. Calcium imaging of pancreatic NG and DRG neurons from mice given caerulein revealed increased responses to TRP agonists. TRPV1 and TRPA1 antagonists attenuated caerulein-induced pain behaviors and pancreatic inflammation; they had a synergistic effect.

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Conclusions—Pancreatic inflammation significantly increased the expression and functional properties of TRPV1 and TRPA1, as well as the excitability of pancreatic sensory neurons in vagal and spinal pathways. TRP channel antagonists acted synergistically to reverse pancreatic inflammation and associated pain behaviors; reagents that target interactions between these channels might be developed to reduce pain in patients with acute pancreatitis.

Keywords

nervous system; analgesia; pain relief; pancreas

INTRODUCTION

The symptom that most often brings patients with acute pancreatitis to the clinic is abdominal pain. Acute pancreatitis (AC) is a mild, self-limited condition, typically present with elevated serum levels of pancreatic enzymes¹. The incidence of acute pancreatitis has been increasing over recent years² and the mechanisms leading to acute pancreatitis are incompletely understood. Recent studies, have illustrated a role for sensitization of pancreatic sensory neurons and neurogenic inflammation in the development of painful symptoms³. During acute pancreatitis, pancreatic sensory (afferent) nerve endings are exposed to a rich milieu of inflammatory mediators which, by mechanisms that remain to be determined, sensitize them^{3–4}.

Primary afferent neurons have efferent functions that mediate neurogenic inflammation in the presence of tissue injury or inflammation⁵. These efferent functions include the release of neurogenic mediators, such as substance P (SP) and calcitonin gene-related peptide (CGRP). These mediators are released at peripheral afferent terminals and bind to endothelial, epithelial and acinar cells leading to vasodilation, edema, neutrophil infiltration and mast cell degranulation, hallmark characteristics of pancreatitis^{3–6}. Recent data suggest that activation of primary sensory neurons and the release of these inflammatory mediators are important in determining the severity of pancreatitis⁷. Blocking this neurogenic inflammation in animal models of pancreatitis significantly improves disease outcome and reduces pain behaviors⁸. Likewise, exacerbating neurogenic inflammation by upregulating endogenous transient receptor potential (TRP) channels in sensory neurons^{9, 10} can worsen disease outcomes. Enhanced TRPV1 function on pancreatic afferent terminals leads to increased release of proinflammatory neuropeptides in the pancreas³. This TRPV1-mediated release of proinflammatory neuropeptides has a ‘feed-forward’ effect, whereby the release of inflammatory mediators sensitizes TRPV1, leading to increased activity and further release of neuropeptides. A related TRP channel, TRPA1, is co-expressed with TRPV1 in many dorsal root (DRG) and nodose (NG) ganglion neurons and also has been implicated in pancreatic pathophysiology^{11,12} and inflammatory pain signaling^{13–15}.

We hypothesized that the expression and function of TRPV1 and TRPA1 are both significantly upregulated in acute pancreatitis and, further, that they act together to produce inflammatory pain. To determine the relative contribution of both sources of pancreatic sensory innervation, we studied spinal and vagal pancreatic sensory neurons in DRG and NG, respectively. Our results indicate that both TRPV1 and TRPA1 contribute to pancreatic inflammation and pain, as well as provide the first evidence of a synergistic interaction between TRPV1 and TRPA1 that is essential to the development of pancreatitis.

MATERIAL AND METHODS

Animals

Experiments were performed on 8-week old male C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME) housed in the AAALAC accredited Division of Laboratory Animal Resources at the University of Pittsburgh. Mice had access to water and food ad libitum. All protocols were approved by the Institutional Animal Care and Use Committee.

Surgical Procedures and Cell Labeling

All surgical procedures were performed under aseptic conditions in a designated surgery area. Anesthesia was maintained with 2% isoflurane. For back-labeling of pancreatic DRG and NG neurons, a laparotomy was performed to expose the pancreatic head into which three injections of Alexa Fluor 488-conjugated cholera toxin B (CTB; 2 mg/ml in sterile saline; Molecular Probes; Eugene, OR; total volume injected ~5 μ l) were made¹⁶. CTB was employed because of previous studies indicating its efficacy for labeling pancreatic afferents¹⁶ and because it appears to have no effect on afferent membrane properties including resting membrane potential, rheobase, or action potential characteristics, when compared to DiI-back-labeled afferents (data not shown). The abdominal muscles and overlying skin were sutured separately and mice were allowed to recover for at least 4 days.

Acute Pancreatitis

Acute pancreatitis was induced by repeated injections of the cholecystokinin analog caerulein (Sigma-Aldrich, St Louis, MO) dissolved in 0.01 M phosphate-buffered saline (PBS). Mice received intraperitoneal (i.p.) injections of either caerulein (50 μ g/kg in 100 μ l) or vehicle (PBS) hourly for 8 hrs. Mice were sacrificed 1, 3 or 7 days after the second hourly injection and pancreata, T9-12 DRG, and NG were harvested. Pancreata were processed immediately for myeloperoxidase activity or histology to assess the extent of inflammation. DRG and NG were acutely dissociated, cultured (see below) and employed for single cell PCR (expression of TRPV1 and TRPA1), Ca²⁺ imaging or whole cell patch clamp.

Myeloperoxidase (MPO) Assay and histology

Briefly, mice were overdosed with inhaled isoflurane and pancreata were dissected, weighed, added to a beaker containing 1.0 mL 0.5% hexadecyltrimethylammonium bromide (HTAB; Sigma) and finely minced using spring scissors. 2mL HTAB was added and the sample was sonicated for 10 sec prior to homogenization for 30 sec. The samples underwent three freeze thaw cycles, were centrifuged twice, reacted with O-dianisidine dihydrochloride (Sigma) and read on a plate reader at 460nm. Histological assessments were made on paraffin-embedded cross-sections of pancreata stained with hematoxylin and eosin (H&E) in a blinded fashion.

Cell Culture, Calcium Imaging and Whole Cell Recordings

1 or 3 days following vehicle or caerulein treatment, mice were overdosed with isoflurane, perfused with ice-cold Ca²⁺/Mg²⁺-free Hank's Balanced Salt Solution (HBSS, Invitrogen). T9-12 DRG and NG were rapidly dissected and prepared for culture as described previously¹⁷. Dissociated cells were resuspended in F12 media (Invitrogen) containing 10% fetal calf serum and antibiotics (penicillin/streptomycin, 50 U/ml) and plated onto laminin (0.1mg/ml) and poly-D-lysine (5mg/ml) coated glass coverslips. No additional growth factors were added to the culture medium. Cells were incubated overnight at 37°C and only CTB-labeled pancreatic T9-12 DRG and NG neurons were either imaged or whole cell recordings were performed the following day as previously described^{16, 18} (see supplemental methods). No unlabeled cells were investigated for the purposes of comparison to non-pancreatic afferents

because it was possible that unlabeled cells were in fact pancreatic afferents that were not back-labeled by our limited dye injections.

Single-Cell RT-PCR and qRT-PCR

Individual CTB-labeled pancreatic neurons were collected with large-bore (~50 μ m) glass pipettes and expelled into microcentrifuge tubes containing reverse-transcriptase (RT) mix (Invitrogen). For each experiment, negative controls consisted of omitting reverse transcriptase or using a cell-free bath aspirate as template. The first-strand cDNA from a pancreatic sensory neuron was used as template in a PCR reaction containing 1 $\times$ GoTaq reaction buffer (Promega Madison, WI), 20 mM outer primers, 0.2 mM dNTPs, and 0.2 mL GoTaq DNA polymerase (Promega); primer sequences are listed in supplemental Table 1. Each initial PCR product served as template in a subsequent PCR reaction using a nested primer pair. Each initial PCR product served as template in a subsequent PCR reaction using a nested primer pair, the products of which were electrophoresed on 2% agarose–ethidium bromide gels and photographed. Only neurons producing detectable amplification of a housekeeping gene (GAPDH) were analyzed further. For qRT-PCR the first-strand cDNA of cells expressing the target genes was pre-amplified (26 cycles) using the PCR condition as described above. The products were used as template for real-time PCR using AbsoluteTM QPCR SYBR[®] Green ROX mix (ABgene, Rochester, NY) in an Applied Biosystems (Foster City, CA) 5700 real-time thermal cycler. Threshold cycle (Ct) values were recorded as a measure of initial template concentration and relative fold changes in RNA levels were calculated by the $\Delta\Delta$ Ct method using GAPDH as a reference standard¹⁹

TRP Receptor Antagonists

Mice were treated with caerulein (n=9) according to the hourly protocol described above and either the TRPA1 antagonist HC-030031 (Hydra Bioscience, Cambridge, MA; 100 and 300 mg/kg b.w.), the TRPV1 antagonist AMG 9810 (100 and 300 mg/kg; TOCRIS, Chicago, IL) or vehicle (PBS) was administered twice i.p. 1 hr before the first caerulein injection and again 4 hr later. The interaction between TRPA1 and TRPV1 was studied by administering both antagonists together at doses of 50 or 100 mg/kg each following the same dosing schedule as above.

Behavioral Testing

To assess pain-related behaviors, mice were placed in Plexiglas boxes and exploratory behaviors were monitored photoelectrically for 15 min periods at 9, 10, 12, 14 and 24 hr post-treatment. Photoelectric beams were spaced 1.5 cm apart, providing 0.75 cm spatial resolution. TruScan software (Coulbourn Instruments; Whitehall, PA) analyzed time spent in different parts of the arena, path information, distance travelled and total movements simultaneously in the X-Y plane. The software also analyzed the amount of time each mouse spent in the “vertical plane” or standing position that required stretch of the abdominal muscles, a position that is assumed to be uncomfortable in the presence of abdominal hypersensitivity. Modulation of exploratory behavior was evaluated after combined TRPV1 and TRPA1 antagonist administration (100mg/kg each at a 4-hr interval as above) and, in different mice, also after morphine (2.5 mg/kg, subcutaneous) administered 30 min prior to the 24 hr post-treatment time point.

Data Presentation and Analysis

Data are presented as mean $\pm$ SEM and were analyzed using SigmaStat (Version 3.1, Systat Software). Statistical analyses for differences in changes over time in multiple groups were performed using two-way ANOVA followed by the Holm-Sidak *post hoc* test. Differences between groups were tested using Student's *t* test (when comparing two groups) or one-way

ANOVA (when comparing more than two groups) followed by *post hoc* Bonferroni-protected pairwise comparisons. Statistical significance was set at $p \leq 0.05$.

RESULTS

Pancreatic Inflammation

The severity of pancreatitis was assessed by microscopic examination of H&E-stained sections. No significant morphological abnormalities were observed in vehicle-treated mice whereas pancreata from caerulein-treated mice exhibited inflammatory cell infiltrates (neutrophils) and edema. These morphological changes were greatest 1 day after caerulein treatment and less severe 3 days after caerulein treatment (Figure 1A–C). No significant pancreatic pathology was observed 7 days post treatment or in liver or lungs on any day after treatment (Figure 1D).

Inflammation was quantified by MPO assay²⁰ and histological scoring (Supplemental Table 2). MPO activity was significantly increased in pancreata from caerulein-treated mice on day 1 and 3 (Figure 1E; control = 0.88 ± 0.10 U/mg; day 1 = 3.33 ± 0.54 U/mg; day 3 = 6.53 ± 0.78 U/mg). MPO activity 7 days after treatment (1.22 ± 0.59 U/mg) was not significantly different from vehicle-treated mice (Figure 1E). Pancreatic weight was also significantly increased after caerulein treatment at both 1 and 3 days after treatment (data not shown).

Pancreatitis Increases Neuronal Excitability

Persistent or ongoing pain in acute pancreatitis is thought to be initiated and maintained by activation and sensitization of pancreatic afferents⁴. Significant decreases in resting membrane potential and rheobase were observed, as well as a greater magnitude of afterhyperpolarization (AHP) of pancreatic DRG and NG neurons 1 day following caerulein treatment compared to vehicle control (Table 1) indicating increased neuronal excitability consistent with inflammatory-induced sensitization. NG neurons discharged more action potentials than their DRG counterparts upon depolarizing current injections (3X rheobase) in both vehicle and caerulein treated groups, revealing greater excitability of the pancreatic nodose afferents.

Pancreatitis Increases Neuronal TRP Expression

The majority of pancreatic DRG neurons from vehicle-treated mice expressed mRNA for TRPV1 and TRPA1, whereas significantly fewer NG neurons expressed these transcripts (Table 2). All of the TRPA1-expressing DRG and NG pancreatic neurons also expressed TRPV1. The proportions of pancreatic DRG and NG neurons expressing either TRPV1 or TRPA1 transcripts were increased significantly 1 day after caerulein treatment (Table 2). Quantitatively (single cell qRT-PCR), the caerulein treatment protocol also significantly increased the relative content of TRP channel mRNA in pancreatic DRG and NG neurons (relative to vehicle treatment) both 1 and 3 days after treatment (with the exception of TRPV1 in NG neurons 3 days post-treatment; Figure 2A, B).

Pancreatitis Enhances TRP-produced Ca^{2+} Transients

Ca^{2+} transients produced by the TRPV1 agonist capsaicin (CAP) and TRPA1 agonist mustard oil (MO) were measured in dissociated pancreatic DRG and NG neurons 1 and 3 days after caerulein or vehicle treatment. Similar to the increase in TRPV1 mRNA expression following caerulein treatment, the percentage of CAP-responsive DRG neurons was significantly increased for all concentrations tested on both days 1 and 3 after caerulein relative to vehicle treatment (Figure 3A). The percentage of CAP-responsive NG neurons was significantly increased only at the greatest concentration of CAP (1 μM) 1 day after caerulein treatment (Figure 3A). The peak Ca^{2+} signal was also significantly increased in

pancreatic DRG neurons at all concentrations of CAP tested and at both times after caerulein treatment, but only at 1 day after caerulein treatment in NG neurons (Supplemental Figure 1 and Supplemental Table 3). Greater functional changes were observed for TRPA1, as the percentage of MO-responsive pancreatic DRG neurons was significantly increased for all concentrations tested and at both times after caerulein treatment (Figure 3B). The percentage of MO-responsive NG neurons was also significantly increased for both concentrations tested, but only at 1 day after caerulein treatment. The peak Ca^{2+} transient elicited by MO was also significantly increased in both DRG and NG neurons for all concentrations and times after caerulein (Supplemental Figure 1 and Supplemental Table 3).

TRPV1 and TRPA1 Antagonists Reduce Inflammation and Pain Behaviors

To examine whether the observed changes in TRP channel expression and function contributed to inflammation and pain in this model of acute pancreatitis, TRPV1 and TRPA1 antagonists were administered once at the beginning of caerulein (or vehicle) treatment and again after the 4th caerulein (or vehicle) injection. Administration of 100 mg/kg of either the TRPV1 (Figure 4B) or TRPA1 (data not shown) antagonist prevented the morphological changes produced by caerulein (Figure 4A). Combined treatment with 100mg/kg of both antagonists (Figure 4C) was more effective than 100mg/kg or 300mg/kg of either antagonist given alone (data not shown). The morphological observations are supported by results from the MPO assay. Notably, neither antagonist affected MPO activity in control mice, but both antagonists dose-dependently reduced pancreatic MPO activity 1 day after caerulein treatment (Figure 4D). Co-administration of 100 mg/kg of each antagonist reduced MPO activity to an extent equivalent to or greater than 300 mg/kg of either antagonist given alone, suggesting a greater than additive effect.

Because of the apparent synergy between the two antagonists we wanted to confirm their specificity on cultured pancreatic afferents. Using calcium imaging we found that pretreating cultures with either antagonist did block the action (size of response or the number of cells responding) of the cognate agonist but did not block the response of the non-cognate agonist (e.g., the TRPV1 antagonist blocked the CAP response, but had no effect on MO response; data not shown). These results indicate that the antagonists are selective and that blocking one TRP channel does not prevent the second channel from responding to its specific ligand. These results also suggest that the observed synergistic effects are due to downstream signaling events that occur downstream of ligand binding.

We hypothesized that pancreatitis-induced discomfort and pain would be reflected in significantly decreased exploratory behavior. Figure 5 shows that the total distance traveled and the time spent moving in the horizontal plane, as well as rearing events in the vertical plane (Figure 5C), were all significantly reduced in caerulein-treated mice. Confirming the pain-related feature of these behaviors, morphine (given 30 min prior to behavioral testing at 24 hr after caerulein) significantly reversed all three behavioral measures (Figure 5A–C). In vehicle-treated mice, this dose of morphine had no effect. Combined treatment with TRPV1 and TRPA1 antagonists significantly attenuated decreases in all exploratory behaviors in caerulein-treated mice (Figure 5A–C).

DISCUSSION

Previous studies have shown that TRPV1 is upregulated in pancreatic DRG neurons during chronic pancreatitis⁸ and that antagonism of TRPV1 can attenuate caerulein-induced pancreatitis in both mice and rats^{21, 22}. Knockout mice lacking either TRPV1 or TRPA1 have been shown to have reduced inflammatory responses^{23, 14, 24}; however, caerulein-induced pancreatitis has been reported to be unaffected by genetic deletion of TRPV1²². The reported failure of TRPV1 deletion to prevent pancreatitis may reflect the presence of and

compensation by TRPA1 in the same pancreatic neuronal population considering that the present studies and others have documented significant co-expression of TRPV1 and TRPA1 in trigeminal and DRG neurons^{25–26}. This hypothesis is supported by the observation from the studies showing that ablation of TRPV1-expressing neurons with neonatal capsaicin treatment was sufficient to block caerulein-induced pancreatitis²².

Using calcium imaging to assess TRPV1 and TRPA1 receptor function, we found that the majority of DRG and many NG pancreatic neurons responded to both CAP and MO, consistent with the prevalence of TRPV1 and TRPA1 mRNA in these neuronal populations. DRG and NG pancreatic neurons significantly differed in the proportions of neurons that expressed one or both of these TRP channels (DRG, ~70%; NG, ~40%), and these differences were also observed after inducing pancreatitis. Neurons from both ganglia exhibited significant increases in both the magnitude of response and percentage of cells responding to CAP and MO after induction of pancreatitis, revealing an inflammatory-induced sensitization to these agonists. We also established, in related whole cell patch clamp experiments, that both DRG and NG pancreatic neurons exhibited significant increases in excitability (decreases in both rheobase and resting membrane potential). Significantly, changes in TRP receptor functionality and neuron excitability developed rapidly over the same time course and were readily apparent 1 day after initiating pancreatic inflammation. Thus, changes in TRPV1 and TRPA1 expression and function are only part of more widespread changes in excitability that occur in pancreatitis. These results complement previous studies in rat showing long-lasting changes in TRPV1 expression and function after injection of TNBS into the pancreatic duct^{8, 27} and indicate that pancreatic sensory neurons plasticity is both sufficiently rapid and potentially long-lasting to contribute to the pathophysiology of pancreatic disease.

It has long been advanced that spinal splanchnic pathways (arising from DRG neurons) encode and process visceral nociceptive information, whereas vagal afferents contribute to nausea and the affective aspects of visceral nociception such as fear and depression²⁸. However, recent studies of vagal gastric and esophageal afferents have shown that they are capable of encoding noxious stimuli and 30–50% express TRPV1^{29–32}. Indeed, pancreatic NG neurons exhibited a ~20% decrease in resting membrane potential and a ~70% decrease in rheobase 1 day following caerulein treatment. These changes were much greater than observed in pancreatic DRG neurons, suggesting greater sensitization of vagal afferents in response to inflammatory changes in the pancreas. Regardless of the exact role played by vagal and spinal afferents in the perception of pain, these results suggest that changes in both afferent populations contribute to altered visceral sensation, perhaps performing complementary roles as has been described in the bronchopulmonary innervation³³.

Although visceral pain, and particularly pancreatic pain, is difficult to evaluate directly, we hypothesized that mice with pancreatitis pain would spend less time pursuing exploratory behaviors (open field measurements of vertical and horizontal movements). We were particularly interested in movements in the vertical plane that require mice to stretch their abdominal muscles, considering that patients suffering from acute pancreatitis report the greatest pain relief when in a hunched position³⁴. Overall, mice with caerulein-induced pancreatitis showed a significant reduction in exploratory behavior in both the horizontal and vertical planes compared to vehicle-treated mice. The reduced exploratory behavior 1 day after caerulein treatment coincided with the greatest observed pancreatic inflammation following caerulein treatment as evidenced by histological examination and MPO activity. To confirm that the decreased behaviors were pain-related, low dose morphine was shown to reverse the decreases in behavior of caerulein-treated mice, but had no effect on the behavior of vehicle-treated mice. Importantly, morphine was efficacious as a single treatment given at a time when both pancreatitis and behavioral deficits were well established, thus validating

the utility of this behavioral methodology for quantifying ongoing pancreatic pain. As a final test of the original hypothesis, TRPV1 or TRPA1 receptor antagonists were found to significantly attenuate both pancreatic inflammation and pancreatitis-induced decreases in exploratory behaviors. When given in combination, the antagonists performed better than either one given alone, and at a lower concentration, suggesting a synergistic relationship between the two channels that has been previously illustrated only in *in vitro* systems ²⁵.

In summary, these results highlight a role for TRP channel interactions that contribute to the development of experimental pancreatitis. Caerulein induced inflammatory changes in the pancreas, increased TRP channel expression in pancreatic DRG and NG neurons, increased excitability of pancreatic neurons and produced behaviors consistent with discomfort and pain. The changes in excitability and functionality of TRPV1 and TRPA1 receptors in both spinal and vagal neurons demonstrate that consequences of pancreatic inflammation are not restricted to one pathway of innervation and may provide a mechanism by which neurogenic inflammation could contribute to exacerbation and/or maintenance of inflammatory processes. The experiments employing TRPV1 and TRPA1 receptor antagonists demonstrate that blocking these channels is sufficient to prevent pancreatic inflammation and pain behaviors, suggesting that 1) current flow through these channels may act as a common pathway for release of neurogenic peptides and 2) sensitization of this population of afferents is required for pancreatitis-induced pain. The synergistic relationship between TRPV1 and TRPA1 in this model of acute pancreatitis suggests that potential therapeutics engineered to target both channels simultaneously could be used at a lower dosage than required to block either receptor alone, thereby potentially avoiding the unwanted side effects of previously tested TRP antagonists, such as hypothermia.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Artn	Artemin
Caer	Caerulein
Cap	Capsaicin
CTB	Cholera toxin β
DRG	Dorsal Root Ganglion
MO	Mustard Oil
MPO	Myeloperoxidase
NG	Nodose Ganglion
NGF	Nerve Growth Factor
TRPA1	Transient Receptor Potential Ankyrin 1
TRPV1	Transient Receptor Potential Vanilloid 1

Veh	Vehicle
VP	Vertical Plane

References

1. Singer, MV.; Gyr, K.; Sarles, H. Revised classification of pancreatitis. *Gastroenterology*; Report of the Second International Symposium on the Classification of Pancreatitis in Marseille; France. March 28–30, 1984; 1985. p. 683–5.
2. Imrie CW. Classification of acute pancreatitis and the role of prognostic factors in assessing severity of disease. *Schweiz Med Wochenschr.* 1997; 127:798–804. [PubMed: 9175217]
3. Liddle RA, Nathan JD. Neurogenic inflammation and pancreatitis. *Pancreatology.* 2004; 4:551–9. discussion 559–60. [PubMed: 15550764]
4. Liddle RA. The role of Transient Receptor Potential Vanilloid 1 (TRPV1) channels in pancreatitis. *Biochim Biophys Acta.* 2007; 1772:869–78. [PubMed: 17428642]
5. Richardson JD, Vasko MR. Cellular mechanisms of neurogenic inflammation. *J Pharmacol Exp Ther.* 2002; 302:839–45. [PubMed: 12183638]
6. Forrest SL, Keast JR. Expression of receptors for glial cell line-derived neurotrophic factor family ligands in sacral spinal cord reveals separate targets of pelvic afferent fibers. *J Comp Neurol.* 2008; 506:989–1002. [PubMed: 18085594]
7. Nathan JD, Peng RY, Wang Y, McVey DC, Vigna SR, Liddle RA. Primary sensory neurons: a common final pathway for inflammation in experimental pancreatitis in rats. *Am J Physiol Gastrointest Liver Physiol.* 2002; 283:G938–46. [PubMed: 12223354]
8. Xu GY, Winston JH, Shenoy M, Yin H, Pendyala S, Pasricha PJ. Transient receptor potential vanilloid 1 mediates hyperalgesia and is up-regulated in rats with chronic pancreatitis. *Gastroenterology.* 2007; 133:1282–92. [PubMed: 17698068]
9. Nilius B, Mahieu F, Karashima Y, Voets T. Regulation of TRP channels: a voltage-lipid connection. *Biochem Soc Trans.* 2007; 35:105–8. [PubMed: 17233613]
10. Nilius B, Owsianik G, Voets T, Peters JA. Transient receptor potential cation channels in disease. *Physiol Rev.* 2007; 87:165–217. [PubMed: 17237345]
11. Ceppa EPCF, Amadesi S, Vaksman N, Grady E, Liedtke W, Bunnett NW, Kirkwood KS. The Transient Receptor (TRP) Ion Channels TRPA1 and TRPV4 Mediate Inflammatory Pain in the Pancreas. *Gastroenterology.* 2008; 142:A-71–A-722008.
12. Schwartz ES, Christianson JA, Bielefeldt K, Davis BM, Gebhart GF. Characterization of TRPV1 and TRPA1 in Pancreatic Nodose and Dorsal Root Ganglion Neurons. *Dig Des Week.* 2009 Abstr 911.
13. McMahon SB, Wood JN. Increasingly irritable and close to tears: TRPA1 in inflammatory pain. *Cell.* 2006; 124:1123–5. [PubMed: 16564004]
14. Bautista DM, Jordt SE, Nikai T, Tsuruda PR, Read AJ, Poblete J, Yamoah EN, Basbaum AI, Julius D. TRPA1 mediates the inflammatory actions of environmental irritants and proalgesic agents. *Cell.* 2006; 124:1269–82. [PubMed: 16564016]
15. Schmutzler BS, Roy S, Hingtgen CM. Glial cell line-derived neurotrophic factor family ligands enhance capsaicin-stimulated release of calcitonin gene-related peptide from sensory neurons. *Neuroscience.* 2009; 161:148–56. [PubMed: 19285119]
16. Fasanella KE, Christianson JA, Chanthaphavong RS, Davis BM. Distribution and neurochemical identification of pancreatic afferents in the mouse. *J Comp Neurol.* 2008; 509:42–52. [PubMed: 18418900]
17. Malin SA, Davis BM, Molliver DC. Production of dissociated sensory neuron cultures and considerations for their use in studying neuronal function and plasticity. *Nat Protoc.* 2007; 2:152–60. [PubMed: 17401349]
18. Chen X, Molliver DC, Gebhart GF. The P2Y2 receptor sensitizes mouse bladder sensory neurons and facilitates purinergic currents. *J Neurosci.* 30:2365–72. [PubMed: 20147562]

19. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*. 2001; 25:402–8. [PubMed: 11846609]
20. Klebanoff SJ. Myeloperoxidase: friend and foe. *J Leukoc Biol*. 2005; 77:598–625. [PubMed: 15689384]
21. Hutter MM, Wick EC, Day AL, Maa J, Zerega EC, Richmond AC, Jordan TH, Grady EF, Mulvihill SJ, Bunnett NW, Kirkwood KS. Transient receptor potential vanilloid (TRPV-1) promotes neurogenic inflammation in the pancreas via activation of the neurokinin-1 receptor (NK-1R). *Pancreas*. 2005; 30:260–5. [PubMed: 15782105]
22. Romac JM, McCall SJ, Humphrey JE, Heo J, Liddle RA. Pharmacologic disruption of TRPV1-expressing primary sensory neurons but not genetic deletion of TRPV1 protects mice against pancreatitis. *Pancreas*. 2008; 36:394–401. [PubMed: 18437086]
23. Story GM, Gereau RWt. Numbing the senses: role of TRPA1 in mechanical and cold sensation. *Neuron*. 2006; 50:177–80. [PubMed: 16630827]
24. Caterina MJ, Leffler A, Malmberg AB, Martin WJ, Trafton J, Petersen-Zeitze KR, Koltzenburg M, Basbaum AI, Julius D. Impaired nociception and pain sensation in mice lacking the capsaicin receptor. *Science*. 2000; 288:306–13. [PubMed: 10764638]
25. Salas MM, Hargreaves KM, Akopian AN. TRPA1-mediated responses in trigeminal sensory neurons: interaction between TRPA1 and TRPV1. *Eur J Neurosci*. 2009; 29:1568–78. [PubMed: 19419422]
26. Obata K, Katsura H, Mizushima T, Yamanaka H, Kobayashi K, Dai Y, Fukuoka T, Tokunaga A, Tominaga M, Noguchi K. TRPA1 induced in sensory neurons contributes to cold hyperalgesia after inflammation and nerve injury. *J Clin Invest*. 2005; 115:2393–401. [PubMed: 16110328]
27. Xu GY, Winston JH, Shenoy M, Yin H, Pasricha PJ. Enhanced excitability and suppression of A-type K⁺ current of pancreas-specific afferent neurons in a rat model of chronic pancreatitis. *Am J Physiol Gastrointest Liver Physiol*. 2006; 291:G424–31. [PubMed: 16645160]
28. Grundy D. Neuroanatomy of visceral nociception: vagal and splanchnic afferent. *Gut*. 2002; 51 (Suppl 1):i2–5. [PubMed: 12077054]
29. Bielefeldt K, Lamb K, Gebhart GF. Convergence of sensory pathways in the development of somatic and visceral hypersensitivity. *Am J Physiol Gastrointest Liver Physiol*. 2006; 291:G658–65. [PubMed: 16500917]
30. Blackshaw LA, Page AJ, Partosoedarso ER. Acute effects of capsaicin on gastrointestinal vagal afferents. *Neuroscience*. 2000; 96:407–16. [PubMed: 10683581]
31. Zhang L, Jones S, Brody K, Costa M, Brookes SJ. Thermosensitive transient receptor potential channels in vagal afferent neurons of the mouse. *Am J Physiol Gastrointest Liver Physiol*. 2004; 286:G983–91. [PubMed: 14726308]
32. Zhong F, Christianson JA, Davis BM, Bielefeldt K. Dichotomizing axons in spinal and vagal afferents of the mouse stomach. *Dig Dis Sci*. 2008; 53:194–203. [PubMed: 17510799]
33. Taylor-Clark TE, McAlexander MA, Nassenstein C, Sheardown SA, Wilson S, Thornton J, Carr MJ, Udem BJ. Relative contributions of TRPA1 and TRPV1 channels in the activation of vagal bronchopulmonary C-fibres by the endogenous autacoid 4-oxononanal. *J Physiol*. 2008; 586:3447–59. [PubMed: 18499726]
34. Ceyhan GO, Michalski CW, Demir IE, Muller MW, Friess H. Pancreatic pain. *Best Pract Res Clin Gastroenterol*. 2008; 22:31–44. [PubMed: 18206811]

Figure 1. Caerulein produces pancreatitis

Pancreatic histology, examined 1 day after repeated injection of vehicle (PBS, **A**) or 1 (**B**) or 3 (**C**) and 7 (**D**) days after repeated injection of caerulein (N=6/group), revealed significant edema (filled arrowhead) and neutrophil infiltration (unfilled arrowheads) as early as 1 day after caerulein treatment with recovery by 3 days. No evidence of inflammation was observed in vehicle-treated pancreas. (**E**) MPO activity (units/mg tissue) was also significantly increased 1 and 3 days following caerulein (caer; N=8 each day), but was not significant from vehicle (veh, N=6) treatment by day 7 ($F_{3,24} = 18.90$ one-way ANOVA, $p < 0.001$). *, $p < 0.05$ relative to vehicle treatment (Bonferroni posthoc test).

Figure 2. Pancreatic inflammation increases TRP channel mRNA expression

The expression levels of TRPV1 and TRPA1 mRNA in pancreatic DRG (A) and NG (B) neurons were also elevated 1 and 3 days after caerulein treatment. N=6 for vehicle and N= 9 for Caer-1d and 3d treated mice and the number of cells (n) used for mRNA quantification is given in each bar. * $p < 0.05$, two-way ANOVA with a Holm-Sidak posthoc test to compare vehicle- vs caerulein-treated groups.

Figure 3. Pancreatitis increases the percentage of pancreatic neurons responding to capsaicin (TRPV1) and mustard oil (TRPA1)

Ca^{2+} imaging was performed on pancreatic DRG (left panels) and NG (right panels) neurons from vehicle- and caerulein (caer)-treated mice in response to capsaicin (CAP) (**A**) or mustard oil (MO) (**B**). In vehicle treated mice (open circles), the percentage of neurons expressing functional TRPV1 was significantly higher in pancreatic DRG neurons, than in NG neurons. In contrast, the functional expression of TRPA1 was similar between the two ganglia types. Caerulein treatment significantly increased the percentage of pancreatic DRG neurons that express functional TRPV1 or TRPA1. Caerulein treatment only produced a transient (1 day post-treatment) significantly increase in the percentage of pancreatic NG neurons expressing TRPV1 or TRPA1. All data analyzed by two way ANOVAs and in all cases $F > 10.2$, $p < 0.01$; * $p < 0.05$ with a Holm-Sidak posthoc test to compare groups (N =at least 8 animals and $n=38$ cells /group).

Figure 4. Pharmacologic antagonism of TRP channels reduces pancreatic inflammation
Pancreatic histology, examined 1 day after repeated injections of caerulein (A) or TRP channel antagonists: 100 mg TRPV1 (B), 100 mg TRPV1 (AMG9810) + 100 mg TRPA1 (HC-030031) (C) (N=6/group). TRPV1 (AMG9810) or TRPA1 (HC-030031) receptor antagonists had no effect on MPO activity (D) (units/mg tissue) in vehicle (veh)-treated mice (N=6), but dose-dependently attenuated pancreatic inflammation in caerulein (caer)-treated mice (doses in mg/kg given in bars; N=5–8/treatment group; $F_{9,50}=8.748$ one-way ANOVA with a Bonferoni posthoc test; * (vs vehicle); † (vs caer) $p<0.01$. Combined treatment with 100mg/kg of both antagonists was comparable to or more effective than 300mg/kg of either antagonist given alone (** $p<0.05$).

Figure 5. Pharmacological antagonism of TRP channels blocks pancreatitis pain

Activity monitoring revealed that the total distance traveled (A), time spent moving in the horizontal plane (B), and rearing events in the vertical plane (VP) (C) were all significantly reduced in caerulein (caer)- vs vehicle-treated mice (N=6/group; All data analyzed by two way ANOVAs and in all cases $F > 10.1$, $p < 0.01$). Morphine (2.5 mg/Kg, subcutaneous) given 30 min prior to testing significantly reversed all three behavioral parameters in caerulein-treated mice (all data analyzed by two way ANOVAs and in all cases $F > 7.2$, $p < 0.01$). Combined treatment with TRPV1 and TRPA1 antagonists (each at 100 mg/kg) significantly prevented caerulein-produced behaviors at all times tested. All data

analyzed by two way ANOVAs and in all cases $F > 10.2$, $p < 0.01$. * $p < 0.01$ (vs caerulein); ** $p < 0.001$ (vs vehicle); Holm-Sidak posthoc test.

Table 1

Passive and Active Electrical Properties of Pancreatic DRG and NG Neurons 1day Post-Vehicle or Post-Cerulein Treatment

	Vehicle-treated		Cerulein-treated	
	DRG (n=22)	NG(n=13)	DRG (n=21)	NG (n=9)
RMP (mV)	-61.5±1.5	-62.9±1.9	-54.5±1.9**	-51.9±1.3**
Input Resistance (MΩ)	359.3±49.8	325.8±47.0	319.8±25.7	271.8±50.0
Rheobase (pA/pF)	2.64±0.33	2.14±0.46	1.60±0.36*	0.95±0.15*
AP Threshold (mV)	-20.7±1.7	-22.5±1.6	-18.4±1.6	-22.7±0.9
AP Amplitude (mV)	94.0±2.3	95.1±1.8	90.6±2.5	86.5±3.4*
AP Overshoot (mV)	32.5±1.5	32.3±1.5	36.1±1.6	34.6±2.3
AP Duration (ms)	4.6±0.63	4.8±0.6	4.9±0.5	4.8±0.7
AHP (mV)	-10.7±1.3	-10.1±1.6	-16.9±1.7**	-20.6±1.3**

Data are mean ± SEM.

* $P < 0.05$ vs. vehicle-treated

** $p < 0.001$ vs. vehicle-treated

¹ RMP, resting membrane potential

² AP, action potential

³ AHP, after-hyperpolarization.

Changes in Gene Expression of TRP Transcripts Before and After Inflammation in CTB-labeled pancreatic afferents

CTB-Alexa488-labeled pancreatic neurons were collected after plating and processed for single cell PCR analysis. N=6 for vehicle treated and N=9 for caer-1 d and 3 d treated mice. Numbers (n) in parenthesis indicate the number of total cells analyzed in corresponding group. Each group represents Veh: NG (71) DRG (69); Caer-1d: NG(67) DRG (72); Caer-3d: NG(67) DRG (64). Error (SEM) represents animal-to-animal variations in percentage.

Table 2

	TRPV1			TRPA1		
	Veh	Caer 1d	Caer 3d	Veh	Caer id	Caer 3d
DRG	72±3.2%	84±4.1% *	80±2.7%	68±3.8%	79±4.1% *	78±4.3% *
NG	41±2.8%	49±3.7% *	45±1.9%	36±1.7%	47±3.9% *	44±2.3%

* p <0.05 vs Veh by chi-square test.