Transient receptor potential ion channels V4 and A1 contribute to pancreatitis pain in mice

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Ceppa E, Cattaruzza F, Lyo V, Amadesi S, Pelayo J, Poole DP, Vaksman N, Liedtke W, Cohen DM, Grady EF, Bunnett NW, Kirkwood KS. Transient receptor potential ion channels V4 and A1 contribute to pancreatitis pain in mice. Am J Physiol Gastrointest Liver Physiol 299: G556–G571, 2010. First published June 10, 2010; doi:10.1152/ajpgi.00433.2009.—The mechanisms of pancreatic pain, a cardinal symptom of pancreatitis, are unknown. Proinflammatory agents that activate transient receptor potential (TRP) channels in nociceptive neurons can cause neurogenic inflammation and pain. We report a major role for TRPV4, which detects osmotic pressure and arachidonic acid metabolites, and TRPA1, which responds to 4-hydroxynonenal and cyclopentenone prostaglandins, in pancreatic inflammation and pain in mice. Immunoreactive TRPV4 and TRPA1 were detected in pancreatic nerve fibers and in dorsal root ganglia neurons innervating the pancreas, which were identified by retrograde tracing. Agonists of TRPV4 and TRPA1 increased intracellular Ca2+ concentration ([Ca2+]i) in these neurons in culture, and neurons also responded to the TRPV1 agonist capsaicin and are thus nociceptors. Intraductal injection of TRPV4 and TRPA1 agonists increased c-Fos expression in spinal neurons, indicative of nociceptor activation, and intraductal TRPA1 agonists also caused pancreatic inflammation. The effects of TRPV4 and TRPA1 agonists on [Ca2+]i, pain and inflammation were markedly diminished or abolished in trpv4 and trpa1 knockout mice. The secretogogue cerulein induced pancreaticitis, c-Fos expression in spinal neurons, and pain behavior in wild-type mice. Deletion of trpv4 or trpa1 suppressed c-Fos expression and pain behavior, and deletion of trpa1 attenuated pancreatitis. Thus TRPV4 and TRPA1 contribute to pancreatic pain, and TRPA1 also mediates pancreatic inflammation. Our results provide new information about the contributions of TRPV4 and TRPA1 to inflammatory pain and suggest that channel antagonists are an effective therapy for pancreatitis, when multiple proinflammatory agents are generated that can activate and sensitize these channels.

Severe pain is the predominant symptom of pancreatitis (4). However, the cause of pancreatitis pain is unknown and it is difficult to manage with current therapies. The mechanisms of inflammatory pain have been extensively examined in tissues other than the pancreas (6). Proinflammatory and noxious agents activate a subpopulation of primary spinal afferent neurons (C and Aδ fibers) that express substance P (SP) and calcitonin gene-related peptide (CGRP). These agents include agonists of G protein-coupled (e.g., serine proteases, bradykinin, prostaglandins) and tyrosine kinase receptors (e.g., nerve growth factor), and activators of ligand-gated ion channels. Activated neurons release SP and CGRP from their peripheral projections in inflamed tissues, and centrally in the dorsal horn of the spinal cord. In the periphery, SP and CGRP cause neurogenic inflammation, which is characterized by extravasation of plasma proteins, granulocyte infiltration, and hyperemia. In the spinal cord, SP and CGRP activate nociceptive neurons. Proinflammatory agents can also sensitize ion channels by enhancing their activity and by inducing their expression and cell-surface translocation. This sensitization amplifies neuronal responsiveness to cause allodynia, hyperalgesia, and exacerbated neurogenic inflammation.

Members of the transient receptor potential (TRP) family of ion channels that are expressed in nociceptive neurons, notably TRP vanilloid 1 (TRPV1), TRPV4, and TRP ankyrin 1 (TRPA1), make important contributions to inflammation and pain (34). The capsaicin receptor, TRPV1, responds to heat (>42°C) (11) and eicosanoids (23), and proinflammatory agents sensitize TRPV1 to induce hyperalgesia to thermal stimuli (10). We previously showed that TRPV1 mediates pancreatic inflammation and pain via release of SP and CGRP (18, 43). TRPV4 responds to altered tonicity (29, 30) and warmth (>27°C) (18) and mediates mechanical pain (44). Hypoosmotic stimuli cause cell swelling and activate phospholipase A2, resulting in generation of arachidonic acid (35). 5,6-Epoxyeicosatrienoic acid (EET), a cytochrome P-450 product of arachidonic acid, is an endogenous agonist of TRPV4, whereas 4x-phorbol 12,13-didecanoate (4xPDD) is a synthetic TRPV4 agonist (50, 51). Proinflammatory agents also sensitize TRPV4 to cause hyperalgesia to mechanical stimuli (1, 17, 41). TRPA1 responds to the pungent ingredients of spices, including mustard oil (MO), garlic, and cinnamon (25); environmental irritants, such as acrolein (7), formaldehyde (33), and cigarette smoke (5); and irritating inhaled anesthetics (32). Endogenous agonists of TRPA1 include 4-hydroxy-2-nonenal (HNE), an endogenous agonist of TRPV4, whereas 4x-phorbol 12,13-didecanoate (4xPDD) is a synthetic TRPV4 agonist (50, 51). Proinflammatory agents also sensitize TRPV4 to cause hyperalgesia to mechanical stimuli (1, 17, 41). TRPA1 responds to the pungent ingredients of spices, including mustard oil (MO), garlic, and cinnamon (25); environmental irritants, such as acrolein (7), formaldehyde (33), and cigarette smoke (5); and irritating inhaled anesthetics (32). Endogenous agonists of TRPA1 include 4-hydroxy-2-nonenal (HNE), an endogenous α,β-unsaturated aldehyde generated from membrane lipids in response to oxidative stress during inflammation and tissue injury (47), and cyclopentenone prostaglandins, including PGA2, PGI2, and PGD2, which are metabolites of PGE2, PGE1, and PGD2, respectively (31, 46). Proinflammatory agents also sensitize TRPA1, resulting in hyperalgesia (12, 15, 49). Agonists of both TRPV4 and TRPA1 also induce the release of neuropeptides that cause neurogenic inflammation (5, 47, 48). Although inflammatory mediators can activate and sensitize TRPV4 and TRPA1 to induce pain and neurogenic inflammation, little is known about the contributions of these channels to...
inflammatory pain of diseased organs. Nothing is known about the roles of TRPV4 and TRPA1 in pancreatitis and pancreatic pain. We evaluated the contribution of TRPV4 and TRPA1 to inflammation and pain in acute pancreatitis, a condition in which multiple inflammatory mediators are generated that could activate and sensitize these channels. Elevated ductal pressures and altered extracellular tonicity of the inflamed pancreas (8), and arachidonic acid metabolites such as EET that are abundantly generated during pancreatitis (52, 56), may activate TRPV4 on pancreatic sensory nerves to induce neurogenic inflammation and pain. Pancreatitis results in generation of HNE (2, 24) and prostaglandins (13, 57) that could activate TRPA1 to cause inflammatory pain. Moreover, trypsins that are prematurely activated within the inflamed pancreas can activate protease-activated receptor 2 (PAR2) on pancreatic sensory nerves (19, 21) and thereby sensitize TRPV4 (17) and TRPA1 (15) to cause hyperalgesia and exacerbated neurogenic inflammation.

To evaluate the contributions of TRPV4 and TRPA1 to pancreatitis and pancreatic pain we 1) localized TRPV4 and TRPA1 in pancreatic nociceptive neurons; 2) examined whether TRPV4 and TRPA1 agonists directly activate these neurons; 3) determined whether endogenous TRPV4 and TRPA1 agonists injected into the pancreatic duct cause pancreatic inflammation and activate spinal nociceptive neurons; and 4) evaluated whether deletion of trpv4 and trpa1 genes ameliorates experimental pancreatitis and pancreatic pain. Our results show that both TRPV4 and TRPA1 mediate pancreatitis pain and that TRPA1 also mediates pancreatic inflammation. To our knowledge, this is the first evidence that TRPV4 and TRPA1 contribute to pancreatitis, and our results suggest that antagonists of these channels may represent novel therapies for pancreatitis and pancreatic pain.

MATERIALS AND METHODS

Animals. The University of California, San Francisco (UCSF) Institutional Animal Care and Use Committee approved all procedures with animals. The trpv4 and trpa1 wild-type (wt) and knockout (ko) mice in a C57Bl6 background have been described (7, 12, 30, 41). Age-matched littermates (male and female, 20–25 g) were used. Mice were maintained in a temperature-controlled environment with a 12-h light-dark cycle and free access to food and water. Mice were killed with pentobarbital sodium (200 mg/kg ip) after the experiments.

Materials. HNE was from Alexis Biochemical (San Diego, CA) and 15-deoxy-Δ12,14-prostaglandin J2 (15dPGJ2) was from Cayman Chemical (Ann Arbor, MI). 4PDD and human myeloperoxidase (MPO) were from Calbiochem (La Jolla, CA). 1,1'-Dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate (DiI), Alexa Fluor 594-conjugated cholera toxin B (Alexa-CTB), Prolong Gold, and fura-2-AM were from Invitrogen (Carlsbad, CA). Unless specified differently, other reagents were from Sigma Chemical (St. Louis, MO).

Retrograde tracing of pancreatic sensory nerves. Retrograde tracers were injected into the pancreas to identify pancreatic sensory neurons within dorsal root ganglia (DRG) innervating the pancreas, as previously described (39, 40, 43). Mice were anesthetized with pentobarbital sodium (50 mg/kg ip). The pancreas was exposed through a midline laparotomy. The retrograde tracer DiI (17 mg/ml DiI in...
activity (LI) is retained in tissue from detection of TRPV4 (55). We observed that TRPA1-like immunoreacted. The TRPV4 antibody detects TRPV4 in TRPV4-transfected antibodies used in the present study have been extensively characterized/H9262 M) (1:200, overnight or 3 days, 4°C). The TRPV4 and TRPA1 sorption of the diluted antibodies with immunizing peptide (10 25 formic acid (2–4 h, room temperature), washed, and cryoprotected by incubation in 30% sucrose in PBS (overnight, 4°C). Tissues were embedded in Optimal Cutting Temperature Embedding medium (Sakura Finetek, Torrance, CA), and frozen sections (12 14 mm) were prepared. Sections were permeabilized in blocking buffer (0.1% Triton X-100 or 0.1 0.5% saponin, 10% NGS, 100 mM PBS, pH 7.4) for 4 h at room temperature. DiI signals were similar in sections permeabilized with Triton X-100 or saponin, but TRP staining was diminished in saponintreated tissues, suggesting inadequate permeabilization (not shown). Sections were incubated with primary antibodies overnight at 4°C: rabbit anti-TRPV4 [1:200 400, (17, 41, 55)] or rabbit-anti-TRPA1 [1:400, no. 110-407-63, Novus Biologicals, Littleton, CO (12)]. Slides were washed and incubated with FITC-conjugated goat anti-rabbit IgG (1:200, Jackson ImmunoResearch, West Grove, PA) for 1 h at room temperature. Slides were washed and mounted with Prolong. TRPV4 and TRPA1 antibody specificity was determined by preadsorption of the diluted antibodies with immunizing peptide (10–25 mM) (1:200, overnight or 3 days, 4°C). The TRPV4 and TRPA1 antibodies used in the present study have been extensively characterized. The TRPV4 antibody detects TRPV4 in TRPV4-transfected HEK cells, determined by Western blotting and immunofluorescence, and there is no signal in untransfected cells, suggesting specific detection of TRPV4 (55). We observed that TRPA1-like immunoreactivity (LI) is retained in tissue from trpa1-ko mice (N. W. Bunnett, unpublished observations), consistent with the expression of a truncated TRPA1 channel and conservation of the target sequences of the TRPA1 antibody used in the present study (7, 42).

Confocal microscopy. Images (512 512 or 1,024 1,024 pixels) were acquired with a Zeiss LSM510 Meta System, using a Zeiss Axiovert microscope with a Zeiss LSM510 software (version 4.2). Images (512 or 1,024 pixels) were acquired with a Zeiss LSM510 Meta System, using a Zeiss Axiovert microscope with a Zeiss LSM510 software (version 4.2). Images were collected at 0.43- or 0.37-μm intervals by using a pinhole of one airy unit for each channel. Images for peptide preadsorption controls were collected and processed identically to the positively stained images. Images were colored and merged by use of the Zeiss LSM510 software (version 4.2).

Neuronal counting in DRG sections. Neurons were counted by two investigators in 12–20 sections of DRG (T8–T10) from each of three wt mice. The total number of neurons and the number of neurons expressing detectable TRPV4-LI, TRPA1-LI, DiI, or Alexa-CTB signals were determined. Positively stained neurons were identified using the threshold function of the Zeiss LSM510 software (version 4.2). Neurons were considered positive for TRPV4-LI, DiI or Alexa-CTB if the fluorescent signal exceeded the average background fluorescence signal of control tissues, which was used to determine the minimum threshold.

Isolation and culture of mouse DRG neurons. DRG (T8–T10) were minced in ice-cold Hank’s balanced salt solution (HBSS), and digested by incubation in DMEM containing 1 mg/ml collagenase type IA and 0.8 mg/ml DNase type IV for 60 min at 37°C, and then in DMEM containing 0.25% trypsin for 10 min at 37°C. After digestion, soybean trypsin inhibitor was added to neutralize trypsin. Neurons were pelleted; suspended in DMEM containing 5% fetal bovine serum, 5% horse serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 2 mM glutamine; dissociated by gentle trituration; and plated on glass coverslips coated with poly-L-lysine (0.1 mg/ml) and laminin (5 μg/ml). After 24 h, the antimitotic agent cytosine-β-d-arabino-furanoside (1 μM) was added to the culture medium. Neurons were cultured for 2–4 days before use.

Measurement of [Ca2+]i in DRG neurons. Expression of functional TRP channels was assessed by ratiometric imaging of intracellular Ca2+ concentration ([Ca2+]i) in cultured DRG neurons. Neurons were incubated in HBSS containing Ca2+ and Mg2+, 20 mM HEPES, pH 7.4, 0.1% BSA, and fura 2-AM (5 μM) for 45 min at 37°C, washed, and mounted in an open chamber at 37°C. Fluorescence of individual neurons was measured at 340- and 380-nm excitation and 510-nm emission by using a Zeiss Axiovert microscope, an ICCD video camera (Stanford Photonics, Stanford, CA), and a video microscopy acquisition program (Axon Instruments, Union City, CA). Agonists were added directly to the chamber (50 μl/injection into 350 μl volume). Neurons were challenged with 4αPDD (10 μM), MO (100 μM), 15PGD2 (100 μM), or HNE (300 μM). Neurons were then challenged with capsaicin (1 μM) and KCl (50 mM) to identify nociceptive neurons. Results are expressed as the 340/380 nm emission ratio, which is proportional to the [Ca2+]i, of the responding neurons. For studies of trpv4-wt and trpa1-wt mice, only DiI or Alexa-CTB-labeled neurons were analyzed. For studies of trpv4-ko and trpa1-ko mice, all neurons were analyzed to ensure that diminished responses were not due to the lack of channel in the chosen cells but instead due to selective gene deletion. Results are from neurons of three to four mice.

Intraductal injections. TRPV4 and TRPA1 agonists were administered into the pancreatic duct to determine whether they induced pancreatic inflammation and activated spinal nociceptive neurons. Mice were anesthetized with pentobarbital sodium (50 mg/kg ip). The duodenum was exposed through the midline, and the common bile duct was temporarily occluded with a hemoclip on the hepatic end. A catheter (PE-10) was inserted through the duodenum and ampulla of Vater into the pancreatic duct, and the following agonists were infused into the pancreatic duct (25 μl over 1 min): 4αPDD (0.025 mg/ml in 5% horse serum, 100 U/ml penicillin, and 0.1 mg/ml streptomycin); and 2 mM glutamine. After 24 h, the antimitotic agent cytosine-β-d-arabino-furanoside (1 μM) was added to the culture medium. Neurons were cultured for 2–4 days before use.

TRPV4 and TRPA1 contribute to pancreatitis pain. The results of this study suggest that TRPV4 and TRPA1 contribute to the pathogenesis of pancreatitis pain. The pancreas is a complex organ that plays a crucial role in digestion and metabolism. Pancreatitis, an inflammation of the pancreas, can lead to significant pain and complications. The nervous system plays a critical role in the perception and modulation of pain, and the role of TRPV4 and TRPA1 in pancreatitis pain has been a topic of interest.

TRPV4 (transient receptor potential vanilloid 4) is a cation channel that is activated by a wide range of noxious stimuli, including high temperatures, alcohols, and endogenous chemical messengers. It is expressed in sensory neurons and is involved in the transmission of pain signals. TRPA1 (transient receptor potential ankyrin 1) is another cation channel that is activated by a variety of stimuli, including cold, osmotic stress, and inflammatory mediators. It is expressed in nociceptive neurons and is also involved in pain transmission.

The authors of this study have observed that TRPV4 and TRPA1 expression is retained in tissue from patients with pancreatitis, suggesting a role for these channels in the pain associated with this condition. They have used immunohistochemistry and immunofluorescence techniques to localize these channels in the pancreas and to assess their expression in neuronal tissue.

To further investigate the role of TRPV4 and TRPA1 in pancreatitis pain, the authors performed in vivo experiments. They infused the agonists capsaicin and 4αPDD into the pancreatic duct of mice, and monitored the response of spinal nociceptive neurons. The results showed that infusion of these agonists into the pancreatic duct induced a significant increase in the activity of nociceptive neurons, as measured by an increase in [Ca2+]i. This suggests that TRPV4 and TRPA1 contribute to the activation of nociceptive neurons in response to pancreatitis.

The study also employed genetic approaches to determine the contribution of TRPV4 and TRPA1 to pancreatitis pain. The authors used mice genetically deficient in TRPV4 and TRPA1 to determine whether the observed effects were specific to these channels. The results showed that the effects were specific to TRPV4 and TRPA1, as the deletion of these channels resulted in a significant decrease in the pain response to pancreatitis.

In conclusion, the results of this study provide evidence for the involvement of TRPV4 and TRPA1 in the pathogenesis of pancreatitis pain. These channels may represent potential targets for the development of new therapies for the treatment of pancreatitis pain.
2.5% DMSO), hypotonic NaCl (150 mosM), isotonic NaCl (300 mosM), MO (10 mg/ml in 1% ethanol, 0.5% Tween 20, 0.9% NaCl), HNE (100 μM in 0.1% DMSO), 15dPGJ2 (600 μM in 6% DMSO), or vehicle (controls). The hemoclip and catheter were removed, the abdomen was closed, and animals were killed 2.5 h later. Tissues were collected for assessment of pancreatitis and activation of spinal nociceptive neurons. The injection volume (25 μl) was selected to ensure adequate delivery of test agents to pancreatic tissues and was

![Image](http://ajpgi.physiology.org/)

**Fig. 3.** TRP channel-mediated calcium signaling in DRG neurons innervating the pancreas. DiI or Alexa Fluor 594-conjugated cholera toxin B (Alexa-CTB) was injected into the pancreas. After 10–15 days (DiI) or 4 days (Alexa-CTB), DRG (T8–T10) were isolated and cultured. Intracellular Ca²⁺ concentration ([Ca²⁺]ᵢ) was measured in individual neurons challenged with 4α-phorbol 12,13-didecanoate (4αPDD; A), mustard oil (MO; B), 15-deoxy-Δ12,14-prostaglandin J₂ (15dPGJ₂; C), or 4-hydroxy-2-nonenol (HNE; D). **Left:** changes in 340/380 ratio, which is proportional to [Ca²⁺]ᵢ, in individual DiI-labeled neurons from trpv4- (trpv4-wt; A) and trpv1-wild-type mice (trpa1-wt; B–D). Traces are from individual neurons indicated by arrows (middle) (A, C, and D are fluorescence images; B is merged fluorescence and phase image). **Right:** maximal increases over basal values in 340/380 ratio (Δ340/380) in neurons from trpv4-wt and trpv4-ko mice (A) and trpa1-wt and trpa1-ko mice (B–D). *P < 0.05.
based on published studies where injection volumes range from 50–100 μl in mice (20, 28) to 500 μl in rats (54). To verify that this volume of solution injected into the pancreatic duct effectively delivered test agents to the pancreas, and to ensure that they did not extravasate into surrounding tissues, 25 μl of dye [5:1 Procion Yellow (Polysciences, Warrington, PA) and Evans blue (Sigma) in saline] was injected into the pancreatic duct over 1 min. The hemoclip and catheter were then removed, and the pancreas was observed immediately or 30 min after injection. This analysis indicated that the dye penetrated the pancreas and did not leak into other tissues (not shown). This volume did not induce observable edema or damage to the pancreas.

Cerulein-induced pancreatitis. Mice received hourly subcutaneous injections (10 μl) of cerulein (50 μg/kg) or vehicle (0.9% NaCl) for 12 h. Tissues were collected for assessment of pancreatitis and activation of spinal nociceptive neurons.

Assessment of pancreatitis. Pancreatitis was assessed by measurement of serum amylase activity, pancreatic MPO activity, and pancreatic histology severity score (HSS). Activation of spinal nociceptive neurons was assessed by detection of c-Fos, as described (22, 53). For amylase assays, blood was collected by puncture of the left ventricle and centrifuged (10,000 × g, 5 min), and serum was obtained for measurement of amylase activity by use of Infinity Amylase Liquid Stable Reagent (Thermo Electron, Louisville, CO). For pancreatic MPO assays and HSS, mice were transcardially perfused with 50 ml of 100 mM PBS, pH 7.4, and the pancreas was removed for MPO assays and histochemical analysis. Perfusion was continued with 100 ml of 3.7% formaldehyde in PBS, and the spinal cord was removed for detection of c-Fos by immunohistochemistry. The body of the pancreas was snap frozen and stored at −80°C for measurement of MPO (16). Tissues were homogenized in 1 ml 0.5% hexadecyltrimethyl ammonium bromide in phosphate buffer (pH 6.0) and subjected to three freeze-thaw cycles. The homogenates were centrifuged (15,000 g, 20 min, 4°C) and the supernatant (5 μl) was mixed with 3,3′,5,5′-tetramethylbenzidine liquid substrate solution (200 μl). The reaction continued for 5 min at room temperature and the absorbance at 650 nm was used to calculate pancreas MPO activity, based on a standard curve against human MPO. Data are expressed as mU MPO/mg protein. For histological analysis, the tail of the pancreas was placed in 10% formalin (overnight, 4°C), embedded in paraffin, and processed for hematoxylin and eosin staining. Sections were evaluated by an investigator unaware of the experimental groups, using a three-point scale for each of the following criteria: 1) macrolobular (interlobar) edema, 2) microlobular (intralobar) edema, 3) polymorphonuclear leukocyte infiltration, 4) presence of polymorphonuclear leukocytes in peripancreatic fat, 5) presence of vacuoles, and 6) necrosis (22). The scores were tabulated and the mean value defined the HSS.

Assessment of activation of spinal nociceptive neurons. To assess activation of nociceptive neurons in the spinal cord, c-Fos-LI was localized by immunohistochemistry (53). Segments of spinal cord (T6–T12) were placed in 3.7% formaldehyde (1 h, room temperature) and cryoprotected by incubation in 30% sucrose in PBS (overnight, 4°C). Sections (40–50 μm) were cut in the transverse plane using a sliding microtome and placed in 100 mM PBS, pH 7.4. Samples were incubated in PBS containing 3% normal goat serum (1 h, room temperature) and incubated with c-Fos antibody (1:20,000, 16 h, room temperature; Calbiochem, La Jolla, CA). Sections were washed and incubated with biotinylated goat anti-rabbit antibody (1:200, 1 h, room temperature; Vector Laboratories, Burlingame, CA), followed by an avidin-biotin-peroxidase complex (Vector), and 1% hydrogen peroxide. Slides were examined by investigators unaware of the experimental groups. Fos-stained nuclei in laminae I and II of the spinal cord were counted in six sections per spinal cord segment in each mouse with use of a ×20 objective, and mean data were determined for each mouse.

Pain behavior. Pain-related behavioral was evaluated as described for evaluation of visceral pain (27, 38). After the last subcutaneous administration of cerulein, mice were observed in a blinded manner for 30 min by two investigators. The number of abdominal retractions (including hunching), abdominal squashes,
abdominal stretches, and abdominal licking events were recorded and summed.

Statistical analysis. Results are expressed as means ± SE from \( n \geq 6 \) mice per group (unless otherwise stated). Results were compared by Student’s \( t \)-test (2 comparisons) or ANOVA and Student-Newman-Keuls test (2 comparisons). HSSs (nonparametric data) were statistically assessed by Wilcoxon’s signed-rank test.

RESULTS

Pancreatic nociceptive neurons express TRPV4 and TRPA1. Peptidergic sensory neurons innervating the pancreas, including those containing SP and CGRP, have been previously identified in DRG by injecting retrogradely transported tracers into the pancreas (39, 40, 43). We used a similar approach to determine whether sensory neurons innervating the pancreas express TRPV4 and TRPA1. The retrograde tracers DiI or Alexa-CTB were injected into the pancreas, and TRPV4- and TRPA1-LI were then localized in DRG (T8–T10) by immunofluorescence and confocal microscopy. DiI (Fig. 1) and Alexa-CTB (not shown, but see Fig. 3B) were similarly detected in the soma of a proportion of DRG neurons at 10–15 days (DiI) or 4 days (Alexa-CTB) after tracer injection into the pancreas. Only DiI-injected neurons were used for quantification. TRPV4-LI and TRPA1-LI were detected in the soma of a proportion of DRG neurons (Fig. 1, A and B). The diameter of neurons expressing TRPV4-LI or TRPA1-LI was \( \sim 15–20 \mu m \), which is consistent with expression by small diameter nociceptive neurons. Some DiI-positive neurons innervating the pancreas also expressed TRPV4-LI or TRPA1-LI (Fig. 1, A and B, arrows). However, TRPV4-LI and TRPA1-LI were also detected in neurons that did not contain DiI (Fig. 1, A and B, arrowheads), and some DiI-containing neurons did not express detectable TRPV4-LI or TRPA1-LI. For localization of TRPV4-LI and DiI, of 2,089 counted neurons (\( n = 3 \) mice), 24.1 ± 1.9\% (512/2,089) expressed TRPV4-LI, and 7.6 ± 1.3 (157/2,089) contained both TRPV4-LI and DiI (Fig. 1B). In the case of TRPA1-LI and DiI, of 1,041 counted neurons (\( n = 3 \) mice), 20.3 ± 1.5\% (213/1,041) expressed TRPA1-LI, and 10.1 ± 2.8 (109/1,041) contained both TRPA1-LI and DiI (Fig. 1B). Of all neurons that were labeled with DiI, 37.5 ± 4.8\% (157/417) expressed TRPV4-LI and 44.3 ± 8.7\% (109/234) expressed TRPA1-LI. Thus TRPV4-LI was detected in \( \sim 38\% \) and TRPA1-LI was detected in \( \sim 44\% \) of pancreatic sensory neurons.

To determine whether TRPV4 and TRPA1 were present in nerves fibers within the pancreas, we also localized TRV4-LI and TRPA1-LI in pancreatic sections. Although we could not detect TRPV4-LI and TRPA1-LI in individual fibers, both TRPV4-LI (Fig. 2A) and TRPA1-LI (Fig. 2B) were detected in bundles of nerve fibers between acinar cells. This pattern of staining resembles that which we have previously reported for TRPV1-LI within the pancreas (53).

![Figure 5](http://www.ajpgi.org) Effects of TRPV4 agonists on c-Fos expression in spinal neurons. 4aPDD or vehicle control (A and C) or hypotonic or isotonic NaCl (B) were injected into the pancreatic duct of trpv4-wt and trpv4-ko mice. After 2.5 h, c-Fos-LI was localized in the dorsal horn of the spinal cord (T6–T12) and the number of c-Fos-LI-positive nuclei per field was determined. 4aPDD and hypotonic NaCl increased c-Fos-LI in the nucleus of neurons in superficial laminae (arrows) of T6–T12 of trpv4-wt but not trpv4-ko mice. There was no increase in c-Fos-LI in T6 or T12. \*,\#P < 0.05. Scale bar = 100 \( \mu m \).
Preadsorption of the TRP antibodies with the peptides used for immunization abolished staining of the soma of DRG neurons (Fig. 1C) and of nerve fibers within the pancreas (Fig. 2, A and B, right).

Together, these results show that TRPV4-LI and TRPA1-LI are expressed in DRG neurons innervating the pancreas and are also present in nerve fibers within the pancreas. Thus these channels are suitably localized to detect painful stimuli from the inflamed pancreas.

Agonists of TRPV4 and TRPA1 signal to pancreatic nociceptive neurons. To confirm the expression of functional TRPV4 and TRPA1 in pancreatic sensory neurons, we examined the effects of agonists of these channels on [Ca\(^{2+}\)]\(_i\) of isolated DRG neurons. DRG neurons innervating the pancreas were identified by pancreatic injection of the retrograde tracers DiI or Alexa-CTB. The TRPV4 agonist 4\(\alpha\)PDD (50) (10 μM) induced a prompt and sustained increase in [Ca\(^{2+}\)]\(_i\) (340/380 nm ratio, 1.49 ± 0.38 over basal) in 18% of DiI-labeled neurons (11 of 61 DiI-positive cells, 4 mice) from trpv4-wt mice (Fig. 3A). 4\(\alpha\)PDD had no detectable effect on [Ca\(^{2+}\)]\(_i\) in DRG neurons from trpv4-ko mice (0 of 69 cells, 4 mice). The TRPA1 agonist MO (25) (100 μM) rapidly increased [Ca\(^{2+}\)]\(_i\) (340/380 nm ratio, 0.61 ± 0.08 over basal) in 56% of Alexa-CTB-labeled neurons (71 of 128 DiI-positive cells, 4 mice) from trpa1-wt mice (Fig. 3B). MO had no detectable effect on [Ca\(^{2+}\)]\(_i\) in DRG neurons from trpa1-ko mice (0 of 245 cells, 3 mice). The TRPA1 agonist 15dPGJ\(_2\) (31, 46) (100 μM) gradually increased [Ca\(^{2+}\)]\(_i\) with a maximum effect at ±1 min (340/380 ratio, 1.21 ± 0.24 over basal) in 90% of the DiI-positive neurons (43 of 48 cells, 3 mice) from trpa1-wt mice (Fig. 3C). Although 15dPGJ\(_2\) also increased [Ca\(^{2+}\)]\(_i\) in DRG neurons from trpa1-ko mice, the magnitude of the effect was reduced by 54% compared with neurons from trpa1-wt mice, and responses were observed only in 43% of the neurons (23 of 53 cells, 3 mice). In DiI-labeled neurons from trpa1-wt mice, the TRPA1 agonist HNE (47) (300 μM) caused a biphasic increase in [Ca\(^{2+}\)]\(_i\), with a rapid first phase within 1 min (340/380 ratio, 1.47 ± 0.33 over basal) in 21% of DiI-labeled
neurons (19 out of 92 cells, 3 mice), and delayed second phase after ≥1.5 min (340/380 ratio, 2.29 ± 0.15 over basal) in 50% of the analyzed neurons (46 of 92 cells, 3 mice) (Fig. 3D). Although HNE also increased [Ca^{2+}]_i in neurons from trpal-ko mice, the rapid and delayed responses were reduced by 48 and 45%, respectively, compared with neurons from trpal-wt mice, and were observed only in 7 and 36% of the analyzed neurons (5 of 72 cells, and 26 of 72 cells respectively, 3 mice). Dil- or CTB-labeled neurons from trpv4-wt and trpal-wt mice that responded to their respective agonists also responded to capsaicin (1 μM) and KCl (50 mM) (not shown) and can thus be considered to be nociceptive neurons. Thus pancreatic sensory neurons respond to the TRPV4 agonist 4αPDD and the TRPA1 agonists MO, 15dPGJ_2, and HNE. The response to 4αPDD requires expression of trpv4, and the response to MO requires expression of trpal. Although responses to 15dPGJ_2 and HNE depend in large part on expression of trpal, these agonists can also increase [Ca^{2+}]_i in pancreatic DRG neurons in mice by a TRPA1-independent mechanism, which remains to be defined. These data support the presence of TRPV4 and TRPA1 in pancreatic sensory neurons that transmit pain.

Activation of TRPV4 in the pancreas does not cause pancreatic inflammation but activates nociceptive spinal neurons. Since TRPV4 is expressed by nociceptive neurons within the pancreas, we examined whether stimulation of TRPV4 within the pancreas could cause neurogenic inflammation and activate nociceptive pathways. We injected the TRPV4 agonists 4αPDD (0.025 mg/ml) (50) or hypotonic solution (150 mOsm NaCl) (29), or control agents (vehicle or 300 mOsm NaCl) into the

Fig. 7. Effects of TRPA1 agonists on pancreatic histology. MO, HNE, or vehicle control were injected into the pancreatic duct of trpal-wt (top) and trpal-ko (bottom) mice. After 2.5 h, tissue was collected for histology. In trpal-wt mice, MO and HNE induced edema, cellular infiltration, formation of vacuoles, and necrosis, which were diminished in trpal-ko mice. Scale bar = 50 μm.
pancreatic duct of mice. After 2.5 h, we measured serum amylase and pancreatic MPO activity and assessed pancreatic histology as indexes of pancreatitis. Intraductal injection of 4αPDD or hypotonic NaCl did not affect serum amylase or pancreatic MPO compared with controls, in either trpv4-wt or trpv4-ko mice (Fig. 4, A and B). Intraductal injection of 4αPDD did not increase HSS compared with vehicle. However, hypotonic NaCl increased HSS that was not due to TRPV4 activation, as it was not diminished in trpv4-ko mice. Differences in pancreatic MPO activity (~3–5 U/mg protein) and HSS (~2–3 U) between the control groups are probably due to the different vehicles that were administered to control mice (2.5% DMSO for 4αPDD group; 300 mOsm NaCl for hypotonic NaCl group).

To assess activation of nociceptive neurons, we localized c-Fos-LI in neurons in laminae I/II of the dorsal horn of the spinal cord (T6–T12). Both 4αPDD and hypotonic NaCl caused a 1.5- to 2-fold increase in the number of c-Fos-positive neurons of T8–T10 in trpv4-wt mice but not in trpv4-ko mice (Fig. 5, A–C). There were no effects on c-Fos-LI levels in spinal neurons of T6 or T12 of trpv4-wt mice, indicating that effects are confined to regions of the spinal cord receiving input from pancreatic sensory neurons. Small differences in the number of c-Fos-positive neurons between control groups probably relates to the different vehicles that were administered to control mice.

Thus activation of TRPV4 in the pancreas does not cause detectable pancreatic inflammation but does activate nociceptive neurons in the dorsal horn of the spinal cord. Activation of TRPA1 in the pancreas causes pancreatic inflammation and activates nociceptive spinal neurons. We similarly examined whether activation of TRPA1 in the pancreas causes inflammation and activation of nociceptive neurons. Intraductal injections of the TRPA1 agonists MO (10 mg/ml) (25), 15dPGJ2 (600 μM) (31, 46), or HNE (100 μM) (47) caused a small but significant increase in serum amylase and HSS, and a marked increase in MPO in trpa1-wt mice, consistent with acute pancreatic inflammation (Fig. 6, A–C). Activation of TRPA1 by MO and HNE in trpa1-wt mice caused a marked increase in necrosis, with a loss of nuclei and zymogen granules and a presence of vacuoles (Fig. 7). These pathological changes were more pronounced with MO than with HNE. The proinflammatory effects were not observed or were diminished in trpa1-ko mice and thus depend on activation of this channel. A striking finding was that the inflammatory cell infiltrate in the parenchyma of the pancreas and in adjacent fat was diminished in trpa1-ko mice, which supports the MPO results. Edema was less indicative of specific activation of TRPA1, since it was present in both trpa1-wt and trpa1-ko mice, and may be due in part to the intraductal injection. Differences in serum amylase activity (~8–20 U/ml)}
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and pancreatic MPO activity (~1–3 U/mg protein) between the control groups are probably due to the different vehicles that were administered to control mice (1% ethanol, 0.5% Tween 20, 0.9% NaCl for MO group; 0.1% DMSO for HNE group; 6% DMSO for 15dPGJ2 group).

Intraductal injections of MO, 15dPGJ2, or HNE also caused a two- to threefold increase in the number of neurons expressing c-Fos-LI in T8–T10 in trpa1-wt mice but not in trpa1-ko mice (Fig. 8, A–C). These effects were not observed at levels T6 or T12 in trpa1-wt mice. Minor differences in the number of c-Fos-positive neurons between control groups probably relate to the different vehicles that were administered to control mice.

Thus activation of TRPA1 within the pancreas causes inflammation and activates nociceptive neurons in the dorsal horn of the spinal cord.

TRPV4 contributes to pain and TRPA1 contributes to inflammation and pain of cerulein-induced pancreatitis. Since activation of TRPV4 and TRPA1 in the pancreas resulted in pancreatic inflammation (TRPA1) and activation of nociceptive neurons (TRPV4 and TRPA1), we evaluated the contribu-

Fig. 9. Cerulein-induced pancreatitis. The trpv4-wt or trpv4-ko mice (A and C) and trpa1-wt or trpa1-ko mice (B and C) were treated with cerulein (CE) or vehicle control. After 12 h, serum amylase and pancreatic MPO and HSS were measured. In trpv4-wt and trpa1-wt mice, cerulein increased serum amylase, pancreatic MPO, and HSS, which was characterized by edema, cellular infiltration, formation of vacuoles, and necrosis. Inflammation was similar in trpv4-ko mice but was diminished in trpa1-ko mice. *, #P < 0.05. Scale bar = 50 μm.
tion of these channels to pancreatitis and pancreatic pain. To induce pancreatitis, we treated mice with the acinar cell secretagogue cerulein, which induces robust pancreatic inflammation (36). Cerulein treatment of trpv4-wt or trpa1-wt mice for 12 h resulted in elevated serum amylase, a two- to fourfold increase in MPO and a five- to sevenfold increase in HSS, which included edema, granulocyte infiltration, and acinar cell necrosis (Fig. 9, A–C). Cerulein caused a marked infiltration of inflammatory cells, consisting of neutrophils and monocytes, that was accompanied by necrosis and loss of nuclei. Cerulein also caused pancreatic edema, although a slight edema was evident in vehicle-treated animals, perhaps related to transcardial perfusion with fixative. Indexes of pancreatitis were unaffected by deletion of trpv4 but were significantly reduced in trpa1-ko mice, consistent with the proinflammatory effects of agonists of TRPA1 but not TRPV4. The HSS of control mice (<1 U, Fig. 9) was markedly less than in control mice that received intraductal injections of vehicles (2–3 U, Figs. 4 and 6), suggesting that ductal injection per se affected the HSS.

Cerulein treatment of trpv4-wt and trpa1-wt mice caused a two- to fourfold increase in the number of c-Fos-LI-positive neurons in the dorsal horn, which was most apparent at T8–T10, but also observed at T6 and T12, indicative of activation of pain pathways originating from the inflamed pancreas and surrounding tissues (Fig. 10, A and B). Cerulein also caused a marked increase in pain-related behaviors (abdominal retraction, squashes, stretches, licking) in trpv4-wt and trpa1-wt mice (Fig. 11, A and B). Expression of c-Fos-LI was markedly reduced in trpv4-ko mice and absent from trpa1-ko mice, and deletion of either channel reduced behavioral correlates of pain by >50%. These results are consistent with the effects of intraductal injection of agonists of TRPV4 and TRPA1 on c-Fos-LI expression. Fewer c-Fos-positive neurons were identified in control mice treated with cerulein vehicle (Fig. 10) compared with control mice that received intraductal injections of vehicle (Figs. 5 and 8), suggesting that ductal injection per se affected c-Fos expression.

Together, these results suggest that TRPV4 contributes only to pain, and TRPA1 contributes to both inflammation and pain of cerulein-induced pancreatitis in mice.

**DISCUSSION**

Members of the TRP family of ion channels permit nociceptive neurons to detect diverse noxious and proinflammatory stimuli and to regulate pain transmission and neurogenic inflammation (34). However, nothing is known about the contributions of TRPV4 and TRPA1 to pancreatitis pain. Our results show that TRPV4 and TRPA1 contribute to inflammation and pain in acute pancreatitis. We observed that TRPV4 and
TRPV4 and TRPA1 contribute to pancreatitis pain.

TRPV4 and TRPA1 are expressed in sensory neurons innervating the pancreas. Agonists of TRPV4 and TRPA1, including those that are generated in pancreatitis, directly activate pancreatic neurons, providing functional evidence of TRP channel expression. Administration of TRPV4 and TRPA1 agonists into the pancreatic duct induced expression of c-Fos in spinal neurons of the dorsal horn that receive input from pancreatic sensory nerves, indicating activation of spinal nociceptive neurons. Agonists of TRPV4, but not TRPA1, also caused inflammation of the pancreas. Consistent with these findings, deletion of trpv4 and trpa1 markedly reduced pancreatitis-induced activation of spinal neurons and pain-related behavior, and deletion of trpa1 but not trpv4 attenuated pancreatic inflammation. Our results indicate that TRPV4 and TRPA1 contribute to pancreatitis pain and TRPA1 alone contributes to pancreatic inflammation.

Sensory nerves innervating the pancreas express TRPV4 and TRPA1. We detected TRPV4-LI and TRPA1-LI in the soma of 38–44% of pancreatic neurons, respectively, which were identified by retrograde tracing. We confirmed expression of functional TRPV4 and TRPA1 in pancreatic sensory nerves by examining the ability of agonists to regulate [Ca²⁺]i in retrogradely labeled neurons. Pancreatic DRG neurons from trpv4-wt mice responded to the TRPV4 agonist 4αPDD (50), whereas neurons from trpv4-ko mice were completely unresponsive. These results suggest that 4αPDD is a selective agonist of TRPV4 in pancreatic nociceptive neurons of mice and are consistent with the finding that 4αPDD enhances mechanical pain in trpv4-wt and not trpv4-ko mice (17). Pancreatic DRG neurons from trpa1-wt mice responded to the TRPA1 agonists MO (25), 15dPGJ2 (31, 46), and HNE (47). Neurons from trpa1-ko mice were unresponsive to MO, indicating that the effects of MO are completely dependent on expression and activation of TRPA1 and suggesting that MO is a TRPA1-selective agonist in DRG neurons. Although neurons from trpa1-ko mice retained some responsiveness to 15dPGJ2 and HNE, both the magnitude of the responses and the proportion of responsive neurons were markedly diminished, suggesting that these agonists signal to pancreatic nociceptive neurons in large part by activating TRPA1. We and other groups have reported that 15dPGJ2 and HNE signal to nociceptive neurons exclusively by activating TRPA1, since neurons from trpa1-ko mice were completely unresponsive (31, 46, 47). However, these studies did not examine TRP signaling in defined populations of DRG neurons innervating a particular organ. The mechanism by which 15dPGJ2 and HNE signal to DRG neurons cultured from trpa1-ko mice remains to be determined. Neurons that responded to agonists of TRPV4 and TRPA1 responded to capsaicin and thus express TRPV1 and are nociceptive neurons. Pancreatic sensory nerves that express TRPV1 also express SP and CGRP (53), which are thus likely to be coexpressed with TRPV4 and TRPA1.

The localization of TRPV4-LI and TRPA1-LI to pancreatic neurons supports our functional analysis of neuronal responsiveness to selective agonists. However, we observed differences in the proportion of pancreatic neurons expressing TRP channels that were detected by immunofluorescence, and the proportion on neurons that responded to TRP channel agonists with elevated [Ca²⁺]i. Whereas 38% of DiI-labeled pancreatic neurons expressed TRPV4-LI, only 18% of cultured neurons responded to the TRPV4-selective agonist 4αPDD. Similarly, 44% of pancreatic neurons expressed TRPA1-LI, and the proportion of neurons that responded to the TRPA1-selective agonist MO was 56%. These discrepancies may be attributable to differences in the sensitivity of the detection methods, or the concentration of agonists used to stimulate cultured neurons (we did not examine a full range of agonist concentrations). An alternative explanation could be that short-term culture induced alterations in the expression of functional TRPV4 and TRPA1 in DRG neurons.

Although we used two different retrograde tracers to identify DRG neurons innervating the pancreas for assessment of TRPV4 and TRPA1 expression and function, we cannot exclude the possibility that these tracers also labeled nonpancreatic neurons. We ensured that tracers did not leak from the pancreas during the injection procedure by injecting small volumes of tracers into the organ and by careful observation. However, the pancreas is friable and lacks a serosal covering, and it is possible that tracers subsequently leaked to surrounding organs where they may be taken up and transported to DRG. However, the general agreement of the immunohistochemical studies of DRG, the functional studies of isolated neurons, and the effects of intraductal injection of selective TRP agonists support our conclusion that TRPV4 and TRPA1 are expressed by sensory neurons innervating the pancreas.

Activation of TRPV4 and TRPA1 in the pancreas causes inflammation and pain. Administration of the TRPV4 agonists 4αPDD (50) or hypotonic NaCl (29) into the pancreatic duct failed to elicit detectable pancreatic inflammation in either trpv4-wt or trpv4-ko mice, as assessed by determination of serum amylase, pancreatic MPO, and HSS. However, in trpv4-wt mice, both agonists increased c-Fos-LI in neurons of superficial laminae in regions of the spinal cord that receive input from pancreatic sensory neurons. This response was completely absent from trpv4-ko mice, a result that is consistent with the lack of response of cultured neurons from trpv4-ko mice to a TRPV4 agonist. Thus TRPV4 activation
within the pancreas activates nociceptive spinal neurons. In contrast, intraductal administration of the TRPA1 agonists MO (25), HNE (47), or 15dPGJ2 (31, 46) to trpa1-wt mice increased serum amylase and pancreatic MPO and HSS, indicative of pancreatic inflammation, and also increased c-Fos-LI in spinal neurons. These responses were absent in trpa1-ko mice, indicating that MO, HNE, and 15dPGJ2 cause pancreatic inflammation and activate spinal nociceptive neurons by specifically activating TRPA1. The in vitro experiments in which MO evoked a calcium signal in cultured neurons from TRPA1-wt but not TRPA1-ko mice support these in vivo results and indicate that the effects of MO are completely dependent on TRPA1. Although deletion of TRPA1 abolished the effects of the TRPA1 agonists HNE and 15dPGJ2 on inflammation and pain, TRPA1 deletion markedly diminished but did not abolish responses of cultured neurons to these agonists. Further experiments are required to define the mechanisms of the residual effects of HNE and 15dPGJ2 on neurons from trpa1-ko mice.

Contribution of TRPV4 and TRPA1 to pancreatitis and pancreatitis pain. Although visceral pain, and particularly pancreatic pain, is difficult to evaluate, we used two end points to assess pain in mice with cerulein-induced pancreatitis: c-Fos-LI in spinal neurons and observation of pain-related behavior (abdominal retractions, squashes, stretches, and licking). These validated end points have been used by us and others to evaluate pancreatic pain (53, 54). In trpv4-wt and trpa1-wt mice, we observed that the secretagogue cerulein caused pancreatitis, as determined by increased serum amylase, pancreatic MPO and HSS, and pain as determined by elevated c-Fos-LI in spinal neurons and appearance of pain-related behaviors. Of note, c-Fos-LI was elevated in spinal neurons that receive input from the pancreas and also other abdominal organs, which may reflect a widespread inflammatory response that we have previously observed in animals with pancreatitis (53). Deletion of either trpv4 or trpa1 markedly attenuated expression of c-Fos-LI in spinal neurons and pain-related behavior, whereas deletion of TRPA1 also reduced pancreatitis. Our results are consistent with the effects of intraductal TRP agonists, where both TRPV4 and TRPA1 agonists activated spinal neurons, and only TRPA1 agonists caused pancreatic inflammation. These results suggest a role for TRPV4 in pancreatitis pain, and involvement of TRPA1 in both pain and inflammation.

Our results add to our understanding of the contribution of TRP channels to pancreatitis and visceral pain. TRPV1 also contributes to pancreatic inflammation and pain. Pancreatic nociceptive neurons coexpress TRPV1, SP, and CGRP (53, 54), and the TRPV1 agonist capsaicin stimulates release of SP and CGRP within the pancreas, causing...
neurogenic inflammation, and in the dorsal horn, to activate spinal neurons (22, 53). TRPV1 is upregulated in pancreatic neurons during chronic pancreatitis, induced by intraductal administration of trinitrobenzene sulfonic acid to rats (54). Antagonism of TRPV1 attenuates pancreatitis induced by cerulein in rats and mice (22, 36) and also suppresses inflammatory pancreatic pain induced by l-arginine and trinitrobenzene sulfonic acid in rats (53, 54). Notably, whereas trpv1 deletion does not attenuate cerulein-induced pancreatitis, possibly because of compensation by other channels such as TRPV4 and TRPA1, ablation of primary sensory nerves with the TRPV1 agonist resiniferatoxin is protective (36). These results confirm the importance of primary sensory nerves in pancreatic inflammation. It will be of interest to determine whether antagonism of TRPV4 and TRPA1 also ameliorates pancreatitis and pancreatic pain induced by diverse inflammatory agents, especially those that cause chronic pancreatitis, where pain is a severe problem and is particularly difficult to manage. TRPV4 also contributes to mechanosensation in the intestine (9), and TRPV4 (41) and TRPA1 (12) contribute to mechanical hyperalgesia in the colon induced by PAR2 agonists and inflammatory agents, suggesting its widespread involvement of these channels in visceral mechanosensation and inflammatory pain.

The observation that intraductal injection of TRPV4 agonists activates nociceptive neurons (i.e., stimulate c-Fos expression in spinal neurons) but does not induce pancreatic inflammation is consistent with the finding that trpv4 deletion attenuates pancreatitis pain without affecting inflammation. However, the lack of effects of TRPV4 agonists on pancreatic inflammation is at odds with our report that TRPV4 agonists stimulate neuropeptide release from sensory nerves and induce neurogenic inflammation in other organs (48) and is surprising because activation of other TRP channels (e.g., TRPV1) (53) in the pancreas stimulates neuropeptide secretion to cause neurogenic inflammation. Although we cannot explain this contradiction, the inability of TRPV4 agonists to cause neurogenic inflammation in the pancreas may relate to the doses of agonists that were used or the rapid degradation of released neuropeptides by pancreatic proteases, especially in the inflamed states when these proteases are prematurely activated. It is also possible that TRPV4 agonists release neuropeptides such as CGRP that can protect against pancreatic inflammation (37).

We propose that pancreatitis results in the generation of proinflammatory agents within the pancreas that either directly activate or sensitize TRPV4 and TRPA1 on sensory nerve fibers, causing neurogenic inflammation and pain (Fig. 12). Factors that may directly activate TRPV4 include elevated ductal pressures and altered toxicity of the inflamed pancreas (8) and generation of arachidonic acid metabolites such as EET, which is abundant in the inflamed pancreas (52, 56). Factors that may directly activate TRPA1 include HNE (2, 24) and cyclopentenone prostaglandins such as PGA2, PGA1, and PGJ2 (13, 57) that are generated in pancreatitis. Prominent among the agents that can sensitize TRP channels are proteases, and the premature generation of trypsins in the pancreas is a consistent feature of pancreatitis. Trypsins, including trypsin IV or mesotrypsin, which may be preferentially formed in pancreatitis (45), cleave PAR2 on primary spinal afferent neurons to induce neurogenic inflammation and pain (26). Activated PAR2 sensitizes TRPV4 (17), TRPA1 (15), and TRPV1 (3, 14). The mechanisms of sensitization include channel phosphorylation by second messenger kinases, and release of inhibition by phosphatidylinositol-4,5-bisphosphate. Other inflammatory agents such as nerve growth factor, bradykinin, and prostaglandins are also likely to sensitize primary spinal afferent neurons in the inflamed pancreas to exacerbate neurogenic inflammation and pain. Although our results suggest that proinflammatory agents activate or sensitize TRPV4 and TRPA1 expressed by pancreatic sensory nerve fibers, we cannot exclude the possibility that they also activate TRP channels expressed by other cell types, such as duct epithelial cells and acinar cells. Although we did not observe prominent expression of TRPV4-LI or TRPA1-LI by pancreatic acinar or duct cells, further experiments are required to determine whether nonneuronal cells in the pancreas express TRP channels and to evaluate whether activation of channels in these cells causes inflammation.

The observation that several TRP channels (TRPV1, TRPV4, and TRPA1) contribute to both inflammation and pain in the pancreas raises the possibility of redundancy or functional interactions between these channels. Additional work is required to examine the possible mechanisms by which TRPV4 and TRPA1 may interact to regulate neurogenic inflammation and pain in the pancreas.

In summary, our results show a major role for TRPV4 and TRPA1 in inflammatory pain during acute pancreatitis. Since deletion of these channels attenuates activation of nociceptive neurons and pain-related behavior during pancreatitis, we propose that antagonists of TRPV4 and TRPA1 may represent novel therapies for pancreatic pain.

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DISCLOSURES

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