Capsaicin vanilloid receptor-1 mediates substance P release in experimental pancreatitis

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Capsaicin vanilloid receptor-1 (VR1) mediates substance P (SP) release in experimental pancreatitis. Am J Physiol Gastrointest Liver Physiol 281: G1322–G1328, 2001.—We examined whether the capsaicin vanilloid receptor-1 antagonist, at 4-h intervals. Neurokinin-1 receptor (NK1R) internalization in acinar cells, used as an index of endogenous SP release, was assessed by immunochemical quantification of NK1R endocytosis. The severity of pancreatitis was assessed by measurements of serum amylase, pancreatic myeloperoxidase (MPO) activity, and histological grading. Caerulein administration caused significant elevations in serum amylase and pancreatic MPO activity, produced histological evidence of pancreatitis, and caused a dramatic increase in NK1R endocytosis. Capsazepine treatment significantly reduced the level of NK1R endocytosis, and this was associated with similar reductions in pancreatic MPO activity and histological severity of pancreatitis. These results demonstrate that repeated caerulein stimulation causes experimental pancreatitis that is mediated in part by stimulation of VR1 on primary sensory neurons, resulting in endogenous SP release.

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activation induced by capsaicin, heat, and protons (29). Therefore, if SP release and subsequent NK1R activation in experimentally induced pancreatitis is mediated by VR1 activation, capsaepine should inhibit these events.

Endogenous SP release and stimulation of the NK1R can be assessed in vivo by evaluating cell-specific SP-induced NK1R endocytosis (14), because both ligand and receptor are rapidly internalized after binding of SP to the NK1R (4). With the use of an antiserum specific for the COOH-terminal 15 amino acids of the rat NK1R (30), functional NK1R activation by endogenous SP release can be assessed by immunocytochemical methods. Thus we quantified NK1R-immunoreactive endosomes in pancreatic acinar cells as an index of endogenous SP release in our model of secretagogue-induced experimental pancreatitis. This technique offers the advantage of measuring endogenous SP release at the cellular level, which is not reflected by measurements of total tissue concentration of peptide.

In this study, we tested the hypothesis that VR1-mediated SP release plays a role in experimental pancreatitis by evaluating the effects of capsazepine on SP release and the severity of pancreatic inflammation. We demonstrate that capsazepine treatment significantly inhibits SP release and diminishes pancreatic inflammation, suggesting that secretagogue-induced experimental pancreatitis in mice is mediated, in part, by stimulation of the VR1 in primary sensory neurons, resulting in endogenous SP release.

MATERIALS AND METHODS

Animal protocol and experimental design. Male C57/BL6 mice, 4 wk of age and weighing 12–15 g, were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were housed in climate-controlled rooms with a 12:12-h light-dark cycle. All animals were fed standard laboratory chow until a 14- to 15-h overnight fast before the experiment. Mice were permitted water ad libitum throughout the experiment. Mice were assigned to the following groups (n = 8): control (vehicle), caerulein only, and caerulein plus capsazepine. All procedures were approved by the institutional animal care and use committee.

Caeurulin-induced pancreatitis. The CCK analog caerulein was purchased from Bachem California (Torrance, CA), and the VR1 antagonist capsazepine was purchased from Sigma RBI (Natick, MA). Caerulein was dissolved in 0.1 M NaHCO3 and then diluted in isotonic saline. Capsazepine was dissolved in 100% DMSO and then diluted in absolute ethanol, Tween 80 (Sigma), and isotonic saline (10:10:10, vol/vol/vol). Both solutions were prepared the morning of the experiment and stored on ice.

Caerulein was administered as 12 hourly intraperitoneal injections at a supramaximal stimulating dose of 50 µg/kg per injection (3). Control mice received 12 hourly intraperitoneal injections of isotonic saline. Capsazepine (100 µmol/kg) was administered to the caerulein plus capsazepine group via subcutaneous injections at 4-h intervals commencing 1 h before the first caerulein injection. As an additional control, one group of caerulein-treated mice (n = 8) received subcutaneous capsazepine vehicle injections with a dosing schedule identical to capsazepine administration. One hour after the last caerulein or vehicle injection, animals were euthanized in a CO2 precharged chamber. Mixed arteriovenous blood was collected by decapitation for measurement of serum amylase concentration. The pancreas was then quickly removed and divided for histological grading, measurement of tissue myeloperoxidase (MPO) activity, and immunocytochemical analysis of SP release.

Serum amylase concentration. Mixed arteriovenous blood was centrifuged for 10 min at 1,500 g. The serum amylase concentration was measured using the procion yellow starch assay as previously described (10). Briefly, serum samples diluted with isotonic saline were added to glass culture tubes containing 800 µl of the 3% procion yellow starch solution in a buffer composed of 0.2 M NaH2PO4 and 0.2 M Na2HPO4 (pH 6.9). Blank tubes did not contain serum, and standard tubes contained a known amount of α-amylase. The blank, standard, and serum sample tubes were placed in a shaker bath and incubated at 37°C for 20 min. The reaction was stopped by the addition of 1.6 ml of 0.1 M HCl. The tubes were then centrifuged for 10 min, and the absorbance of the supernatant was read at 420 nm without disturbing the pellet. The standard curve was prepared using crude type VI-B α-amylase (Sigma).

MPO activity. Portions of the harvested pancreata were immediately frozen at −80°C. Tissue MPO activity was measured as previously described (5). Briefly, the tissue was homogenized in 0.5% hexadecyltrimethylammonium bromide (Sigma) in 50 mM KH2PO4 (pH 6). The homogenate was subjected to three freeze-thaw cycles and centrifuged at 4°C for 2 min. Absorbance was read at 460 nm at 0, 30, and 60 s after the addition of 2.9 ml of o-dianisidine dihydrochloride (Sigma) to 100 µl of the supernatant. The maximal change in absorbance per minute was used to calculate the units of MPO activity based on the oxidized o-dianisidine molar absorbency index of 1.13 × 104 M−1·cm−1.

Histological grading. Portions of the pancreata were fixed overnight at room temperature in a pH-neutral, phosphate-buffered, 10% formalin solution. The tissue was then embedded in paraffin, sectioned at 5 µm, stained with hematoxylin and eosin, and coded for examination by a pathologist blinded to the experimental design. The pathologist graded the severity of pancreatitis using modified scoring criteria as previously described (23). The results were expressed as a score of 0 to 3 for the histological parameters of edema and neutrophil infiltration and as a score of 0 to 7 for the parameter of tissue necrosis (Table 1). Immunochemistry analysis of SP release. SP release was assessed by analysis of NK1R endocytosis as described previously (14) with modifications. Briefly, portions of pancreata from control, caerulein-treated, and capsazepine-pre-treated caerulein-treated mice were fixed overnight in ice-cold freshly depolymerized paraformaldehyde (4% in PBS) at 4°C and then placed in ice-cold PBS-30% sucrose for 24 h. The tissue was then embedded in Tissue Tek OCT (Sakura, Torrance, CA), frozen, sectioned at 20 µm, mounted on Superfrost Plus glass slides (Fisher, Pittsburgh, PA), and dried with desiccant at room temperature for 4 h. After being

Table 1. Histological grading criteria for pancreatitis

<table>
<thead>
<tr>
<th>Histological Parameter</th>
<th>Possible Score</th>
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<tr>
<td>Edema</td>
<td>0 to 3 (None to diffuse expansion)</td>
</tr>
<tr>
<td>Neutrophil infiltration</td>
<td>0 to 3 (None to diffuse infiltration)</td>
</tr>
<tr>
<td>Necrosis</td>
<td>0 to 7 (None to substantial injury)</td>
</tr>
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</table>

Total histological score is combined scores of edema, neutrophil infiltration, and necrosis. Possible scores were given in increments of 0.5. Scoring criteria were modified from Spormann et al. (23).
washed, the sections were stained overnight at room temperature using a rabbit antiserum (no. 11886-5) specific for the COOH-terminal 15 amino acids of the rat NK1R (SPR393–407) at a dilution of 1:3,000 (30). The sections were then washed and incubated with cyanine 3-conjugated donkey anti-rabbit IgG secondary antibody (Jackson ImmunoResearch, West Grove, PA) at a dilution of 1:600 for 3 h at room temperature. The sections were washed and coverslipped using one drop of Aquamount (Lerner Laboratories, Pittsburgh, PA). Control sections were incubated with primary antiserum preabsorbed with 10 μmol/l SPR393–407 overnight at 4°C; specific NK1R immunostaining was abolished in these controls (data not shown).

Quantification of NK1R endocytosis. Immunostained sections were analyzed using a Zeiss LSM-410 inverted krypton-argon confocal laser scanning system coupled to a Zeiss Axiovert 100 microscope. Optical sections (0.5 μm) of 512 × 512 pixels were obtained and processed using Adobe Photoshop. Quantification of NK1R endocytosis was performed by analyzing 10 NK1R-immunoreactive acinar cells per mouse (n = 5) and determining the number of these cells containing >50 NK1R-immunoreactive endosomes. Cyttoplasmic endosomes were distinguished from plasma membrane-associated NK1R immunoreactivity by ensuring that the nucleus of the acinar cells was in the same optical section as the NK1R-immunoreactive endosomes.

Statistical analysis. Results are expressed as means ± SE. Statistical comparisons among groups were examined by one-way ANOVA with the Tukey post test, using GraphPad Prism version 2.00 (GraphPad Software, San Diego, CA). Statistical significance was set at P < 0.05.

RESULTS

Administration of supramaximal stimulating doses of caerulein (12 hourly injections of 50 μg·kg⁻¹·dose⁻¹) resulted in endogenous SP release as indicated by SP-induced endocytosis of the NK1R in pancreatic acinar cells (Fig. 1, top). Administration of the specific VR1 antagonist capsazepine to caerulein-treated mice significantly inhibited endogenous SP release. Endogenous SP release was quantified by immunocytochemical assessment of SP-induced NK1R endocytosis in pancreatic acinar cells. In control mice, only rare acinar cells (6%) contained more than 50 NK1R-immunoreactive endosomes. After caerulein administration, nearly all (94%) of the acinar

![Fig. 1. Top: confocal microscope images of neurokinin-1 receptor (NK1R)-immunoreactive pancreatic acinar cells. A: a control NK1R-immunoreactive acinar cell from a mouse treated with vehicle demonstrates a paucity of NK1R-immunoreactive endosomes. NK1R immunoreactivity is not evident on the plasma membrane because of the thinness of the optical section and its level through the nucleus; optical sections through the plasma membrane show plasma membrane-associated NK1R immunoreactivity (not shown). B: an NK1R-immunoreactive acinar cell from a mouse treated with caerulein alone indicates the release of substance P (SP) as demonstrated by SP-induced NK1R internalization into multiple cytoplasmic endosomes. C: an NK1R-immunoreactive acinar cell from a mouse treated with caerulein + capsazepine indicates capsazepine inhibition of caerulein-induced SP release. Bar = 10 μm. Bottom: quantification of caerulein-induced SP release and inhibition of caerulein-induced SP release by capsazepine. Quantification of endogenous SP release was performed by analyzing 10 NK1R-immunoreactive acinar cells per mouse (n = 5) and determining the number of these cells containing >50 NK1R-immunoreactive endosomes (%NK1R endocytosis). Caerulein significantly stimulated SP release, and capsazepine administration significantly inhibited caerulein-induced SP release. However, SP release was not reduced to control levels by capsazepine administration. *P < 0.001 vs. control; †P < 0.001 vs. caerulein.](http://ajpgi.physiology.org/)

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cells displayed >50 NK1R-immunoreactive endosomes per cell. Capsazepine treatment significantly reduced SP release as determined by the level of NK1R endocytosis ($P < 0.001$); only 32% of acinar cells contained >50 NK1R-immunoreactive endosomes per cell.

Repeated caerulein administration produced evidence of pancreatitis as determined by serum amylase levels, tissue MPO activity, and histological grading. Mice receiving caerulein exhibited a 10-fold increase in serum amylase concentration compared with control animals (Fig. 2). In addition, caerulein treatment caused a significant increase in the pancreatic activity of MPO (Fig. 3), an enzyme produced by neutrophils and used as a marker of inflammation associated with neutrophil infiltration. Histologically, caerulein treatment produced moderately severe pancreatitis characterized by pancreatic edema, neutrophil infiltration, and necrosis (Fig. 4). The histological parameters of edema, neutrophil infiltration, and necrosis were significantly elevated in mice receiving caerulein alone (Table 2).

Capsazepine administration was very effective in reducing MPO activity in caerulein-treated mice. As depicted in Fig. 3, animals receiving both caerulein and capsazepine exhibited an 84% reduction in MPO activity compared with caerulein treatment alone ($P < 0.001$). In contrast to the caerulein-treated group, the mice receiving both caerulein and capsazepine developed significantly less edema, neutrophil infiltration, and necrosis by histological criteria (Fig. 4). As demonstrated in Table 2, capsazepine treatment significantly reduced the scores of edema by 42% ($P < 0.001$), neutrophil infiltration by 40% ($P < 0.001$), and necrosis by 67% ($P < 0.05$). The total histological severity score was diminished by 47% in mice that received capsazepine ($P < 0.001$) (Fig. 5). Although the serum amylase concentration tended to be lower in the animals receiving capsazepine treatment, there was no statistically significant difference between the caerulein only and the caerulein plus capsazepine groups. Capsazepine vehicle had no effect on caerulein-induced pancreatitis (data not shown).

There was no mortality during the study period. The persistent elevation in serum amylase seen with significant reduction in tissue MPO activity and histological severity scores suggests that the initiating insult to the pancreas induced by caerulein was not affected by capsazepine.

**DISCUSSION**

Recent studies (3, 8) have suggested that SP plays a critical role in experimental pancreatitis. SP has been demonstrated to stimulate plasma extravasation in the pancreas, and this effect is blocked by administration of NK1R antagonists (8). Furthermore, genetic deletion of the NK1R in mice ameliorates secretagogue-induced pancreatitis, suggesting a significant role for SP stimulation of the NK1R in the pathogenesis of experimental pancreatitis (3). However, the mechanism of SP release in experimental pancreatitis has not yet been reported.

It has previously been shown (27) that stimulation of the capsaicin VR1 results in depolarization of the primary sensory neuron and subsequent activation of both afferent and efferent functions. SP is an undecapeptide neurotransmitter found in primary sensory neurons throughout the gastrointestinal tract and is released from nerve endings in both the spinal cord and gut after nerve stimulation and depolarization. We hypothesized that antagonism of the capsaicin VR1 would inhibit primary sensory neuronal activity (i.e., SP release) and thus reduce the severity of tissue inflammation.

In this study, we used an antiserum specific for the COOH-terminal 15 amino acids of the rat NK1R (30) to study the effects of repeated caerulein administration on endogenous SP release in the pancreas. Studies in vitro and in vivo (4, 15, 16) have demonstrated that after binding of SP, the NK1R is rapidly internalized by endocytosis and recycled to the plasma membrane after degradation of bound SP. By quantifying NK1R-immunoreactive endosomes in pancreatic acinar cells, we were able to assess the amount of endogenous SP...
stimulation after repeated caerulein administration and in caerulein-treated mice receiving the VR1 antagonist capsazepine.

The current study demonstrates that repeated caerulein administration in mice causes SP release and biochemical and histological evidence of acute pancreatitis. Furthermore, pharmacological antagonism of the capsaicin VR1 significantly inhibits SP release and reduces the severity of secretagogue-induced pancreatitis in mice. These findings suggest that primary sensory neurons play a critical role in the tissue inflammatory response to injury in pancreatitis. Thus we propose that repeated caerulein stimulation of the pancreas generates a signal that activates the capsaicin VR1 on primary sensory neurons, resulting in SP release and subsequent propagation of the inflammatory cascade, and that inhibition of primary sensory neurons through VR1 antagonism diminishes tissue inflammation in the pancreas via reduction in SP release.

Although supramaximal secretagogue stimulation causes mild pancreatitis in rats (12), it has been shown in mice to cause moderately severe pancreatitis characterized by tissue damage and elevated serum enzyme concentrations (9, 11). Capsazepine reduced the severity of tissue damage observed with caerulein administration by significantly diminishing pancreatic edema, neutrophil infiltration, and parenchymal injury and necrosis. Although serum amylase concentrations tended to be lower in the animals receiving capsazepine, these data were not significantly different. This finding suggests that caerulein-stimulated amylase release is an early event in the induction of pancreatitis and is not mediated by primary sensory neurons or correlated with the severity of experimental pancreatitis. Thus the initial insult to the pancreas (i.e., supramaximal caerulein stimulation) and consequent increased serum enzyme concentration are not blocked by capsazepine administration. However, capsazepine significantly diminished the severity of tissue damage and the extent of neutrophil infiltration, indicating that neural signaling participates in the entire complement of inflammatory changes, which is consistent with an important role for neurogenic inflammation.

In preliminary experiments, we have observed a dose dependence for effects of capsazepine in reducing the severity of pancreatitis. Low doses of capsazepine (30 μmol/kg sc once, 1 h before caerulein administration) had no effect on pancreatic MPO activity or histological score. However, more frequently administered doses of capsazepine (30 μmol/kg sc every 4 h) significantly diminished pancreatic MPO activity (data not shown). The highest doses reported here (100 μmol/kg sc every

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**Table 2. Effects of caerulein and capsazepine on pancreatic histology**

<table>
<thead>
<tr>
<th></th>
<th>Edema</th>
<th>Neutrophil Infiltration</th>
<th>Necrosis</th>
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<tbody>
<tr>
<td>Control</td>
<td>0.81 ± 0.19</td>
<td>0.13 ± 0.08</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>Caerulein alone</td>
<td>2.50 ± 0.13*</td>
<td>2.19 ± 0.13*</td>
<td>1.50 ± 0.33†</td>
</tr>
<tr>
<td>Caerulein + capsazepine</td>
<td>1.44 ± 0.15‡</td>
<td>1.31 ± 0.16‡</td>
<td>0.50 ± 0.33§</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE (n = 8). Histological parameters were scored by a pathologist blinded to the experimental design. *P < 0.001 vs. control; †P < 0.01 vs. control; ‡P < 0.001 vs. caerulein alone; §P < 0.05 vs. caerulein alone.

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**Fig. 5.** The effects of caerulein and capsazepine on total histological score of pancreatitis. Caerulein administration increased the total histological severity score of pancreatitis, and this effect was significantly inhibited by capsazepine. Results are expressed as means ± SE (n = 8). *P < 0.001 vs. control; †P < 0.001 vs. caerulein.
capsacin receptor by capsazepine (100 μmol/kg sc) has been reported previously (7, 18, 21) in rats and mice. These findings of dose-dependent inhibitory effects of capsazepine provide further support for the hypothesis that capsazepine acts via a specific receptor (e.g., VR1) and that stimulation of the VR1 mediates neurogenic inflammation.

The mechanism of VR1 activation in caerulein-induced experimental pancreatitis is currently unknown. The VR1, a nonselective cation channel expressed by primary sensory neurons, is directly activated by noxious heat (29). However, it is known that protons, even at moderately acidic conditions (pH ≤ 6.4), are capable of lowering the temperature threshold for activation of the VR1 at 37°C (29). Because pancreatic lysosomal and vacuole compartments have a high proton concentration and are increased in experimental pancreatitis (31), it is conceivable that the intra-acinar activation of trypsinogen (13, 25) damages these acidic compartments, subsequently lowering the tissue pH enough to activate the VR1 at 37°C. In addition to protons originating from pancreatic vacuoles and lysosomes, it is known that high proton concentrations are physiologically attainable in several injurious processes, including ischemia, infection, and inflammation (1, 24, 26).

Therefore, the inflammatory process associated with experimental pancreatitis may also serve as a source of protons, thereby lowering the temperature threshold enough to activate the VR1 and effector function of the primary sensory neuron. It is also possible that in addition to increasing proton concentration, a yet to be identified endogenous capsaicin-like molecule may bind and activate the VR1 in pancreatitis (32), as depicted in Fig. 6. Activation of the neuron leads to the release of various neuropeptides, particularly SP, thus mediating neurogenic inflammation through activation of target cell receptors, such as the NK1R.

The measurement of NK1R endocytosis in pancreatic acinar cells as an index of endogenous SP release is an extremely useful tool, as it allows the evaluation of neuropeptide secretion at a cellular level, which is not reflected in the quantification of total SP levels in tissue homogenates. The physiological significance of SP stimulation and subsequent NK1R endocytosis in acinar cells in experimental pancreatitis is unclear: is it involved in the inflammatory cascade of acute pancreatitis, or is it simply a secretagogue effect and thus irrelevant to pancreatic parenchymal damage? In vitro studies utilizing rat (17) and guinea pig (22) pancreatic acini have demonstrated that exposure to SP results in amylase release from acinar cells. Whether the interaction of SP with pancreatic acinar cells serves an additional proinflammatory role in experimental pancreatitis requires elucidation. However, a proinflammatory role for other nonacinar SP target cells (e.g., vascular endothelial cells) in pancreatitis has been suggested. Frossard et al. (9) demonstrated that endothelial cell surface expression of intracellular adhesion molecule 1 (ICAM-1) is increased in the pancreas in caerulein-induced pancreatitis and that ICAM-1 deficiency and neutrophil depletion reduce the severity of pancreatitis and pancreatitis-associated lung injury (9). These results, together with our biochemical and histological findings of a capsazepine-induced reduction in pancreatic neutrophil infiltration in caerulein-treated mice, suggest that the ability of capsazepine to reduce the severity of pancreatitis may occur by virtue of limiting the SP-induced extravasation of neutrophils.

In conclusion, the results of this study demonstrate that repeated caerulein stimulation in mice causes experimental acute pancreatitis that is mediated in part by stimulation of the capsaicin VR1 on primary sensory neurons, resulting in endogenous SP release. Endogenous SP release results in NK1R activation on target cells and subsequent propagation of the inflam-
matory cascade. Further study is required to determine the mechanism of VR1 activation in caerulein-induced pancreatitis.

REFERENCES