TRPA1 in mast cell activation-induced long-lasting mechanical hypersensitivity of vagal afferent C-fibers in guinea pig esophagus

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Yu S, Gao G, Peterson BZ, Ouyang A. TRPA1 in mast cell activation-induced long-lasting mechanical hypersensitivity of vagal afferent C-fibers in guinea pig esophagus. Am J Physiol Gastrointest Liver Physiol 297: G34–G42, 2009. First published May 7, 2009; doi:10.1152/ajpgi.00068.2009.—Sensitization of esophageal sensory afferents by inflammatory mediators plays an important role in esophageal nociception. We have shown esophageal mast cell activation induces long-lasting mechanical hypersensitivity in vagal nodose C-fibers. However, the roles of mast cell mediators and downstream ion channels in this process are unclear. Mast cell tryptase via protease-activated receptor 2 (PAR2)-mediated pathways sensitizes sensory nerves and induces hyperalgesia. Transient receptor potential A1 (TRPA1) plays an important role in mechanosensory transduction and nociception. Here we tested the hypothesis that mast cell activation via a PAR2-dependent mechanism sensitizes TRPA1 to induce mechanical hypersensitivity in esophageal vagal C-fibers. The expression profiles of PAR2 and TRPA1 in vagal nodose ganglia were determined by immunostaining, Western blot, and RT-PCR.Extrasellar recordings from esophageal nodose neurons were performed in ex vivo guinea pig esophageal-vagal preparations. Action potentials evoked by esophageal distension and chemical perfusion were compared. Both PAR2 and TRPA1 expressions were identified in vagal nodose neurons by immunostaining, Western blot, and RT-PCR. Ninety-one percent of TRPA1-positive neurons were of small and medium diameters, and 80% coexpressed PAR2. Esophageal mast cell activation significantly enhanced the response of nodose C-fibers to esophageal distension (mechanical hypersensitivity). This was mimicked by PAR2-activating peptide, which sustained for 90 min after wash, but not by PAR2 reverse peptide. TRPA1 inhibitor HC-030031 pretreatment significantly inhibited mechanical hypersensitivity induced by either mast cell activation or PAR2 agonist. Collectively, our data provide new evidence that sensitizing TRPA1 via a PAR2-dependent mechanism plays an important role in mast cell activation-induced mechanical hypersensitivity of vagal nodose C-fibers in guinea pig esophagus.

visceral afferent; sensitization; nociception; protease-activated receptor 2; transient receptor potential A1

ABNORMAL ESOPHAGEAL SENSATIONS are generated from sensory afferent nerve endings in the wall of the esophagus and transmitted to the central nervous system by both spinal (27, 31) and vagal (7, 25, 30, 38) pathways. Esophageal sensory afferent nerves usually are polymodal with their cell bodies situated in either dorsal root ganglion (DRG) or nodose/jugular ganglia (21). A subtype of these afferent nerves, nociceptive afferent, can innervate noxious mechanical, chemical, and thermal stimuli to induce nociception (31, 36, 37). Under certain conditions, such as tissue damage or inflammation, these processes could be enhanced by peripheral and/or central sensitization and result in esophageal hyperalgesia (14, 29). The mechanism of peripheral sensitization of esophageal afferents is currently unclear.

Esophageal mast cells are mainly distributed along the lamina propria of the mucosal layer and may release a variety of mediators, primarily histamine and tryptase, upon activation. These mediators not only directly mediate allergic or inflammation responses, but also sensitize sensory afferent nerve terminals in their proximity to sense the potential damage. Our previous study (34) showed that esophageal mast cell activation induces a long-lasting increase in the mechanoeexcitability of esophageal vagal nodose C-fibers. This is associated with a significant increase in histamine release and a significant decrease in tryptase-positive mast cells in the esophagus. The histamine H1-receptor antagonist inhibits the increased mechanoeexcitability of the esophageal nodose C-fibers only when given before, but not after, mast cell activation. In addition, exogenous histamine initiates but does not maintain the potentiation effect of mast cell activation on esophageal nodose C-fibers. This indicates that, although histamine is essential, additional mediators are critical in this potentiation process. It is likely that other mast cell mediators, such as tryptase via a protease-activated receptor 2 (PAR2)-dependent mechanism (24), play a role in this sensitization process.

PAR2 is a G protein-coupled receptor and has been identified in sensory nerves in the gastrointestinal tract. PAR2 agonists, such as mast cell tryptase, activate submucosal (28), myenteric neurons (13) and jejunal and colonic afferent nerves (17, 32). Although PAR2 expression has been identified in DRG (39) and enteric neurons (9, 28), there is no study examining PAR2 expression in vagal nodose ganglion neurons. Moreover, few studies have provided direct evidence that mast cell tryptase released after mast cell degranulation acts on PAR2 to induce mechanical hypersensitivity in primary sensory afferent nerves in the gastrointestinal tract (17, 32).

Transient receptor potential A1 (TRPA1) is a new member of the TRP channel family. It is expressed in sensory nerves and mediates cold, mechanical, and chemical nociception (5, 8, 16, 33). Two strains of TRPA1 knockout mice both display impaired responses to noxious mechanical stimulation (6, 18). This suggests a specific role for TRPA1 in mechanonociception. After inflammation or nerve injury, nerve growth factor induced an increase of TRPA1 in DRG neurons contributing to cold hyperalgesia (23). PAR2 can sensitize TRPA1 in DRG to induce inflammatory pain (11). However, direct evidence of the involvement of TRPA1 in mast cell activation-induced mechanical hypersensitivity in sensory afferents, such as esophageal nodose C-fibers, is still lacking.
In our previous study, we have shown that TRPA1 played a pivotal role in bradykinin-induced mechanical hypersensitivity in esophageal nodose and jugular C-fibers but not nodose Aβ fibers (35). In the present study, we addressed the hypothesis that mast cell activation via a PAR2-dependent mechanism sensitizes TRPA1 to induce mechanical hypersensitivity in esophageal vagal nodose C-fibers. Our data demonstrated that both PAR2 and TRPA1 were expressed and coexpressed in small- and medium-size nodose neurons. Mast cell tryptase via a PAR2-mediated mechanism sensitized TRPA1 and played an important role in mast cell activation-induced long-lasting mechanical hypersensitivity of vagal nodose C-fibers in guinea pig esophagus.

MATERIALS AND METHODS

Male Hartley guinea pigs (Hilltop Laboratory Animals, Scottsdale, PA) weighing 100–300 g were used. All experiments were approved by the Pennsylvania State University Animal Care and Use Committee.

Sensitization and allergen challenge to induce mast cell activation. Guinea pigs were sensitized with three intraperitoneal injections of ovalbumin (OVA, 10 mg/kg in 0.9% saline) every 48 h. After the last injection (3 wk), guinea pigs were killed by CO2 inhalation and exsanguinations, and esophageal-vagal preparations were carefully dissected as previously described (37). Antigen challenge was performed by using OVA (10 μg/ml) diluted in Krebs bicarbonate solution [KBS, composed of (in mM): 118 NaCl, 5.4 KCl, 1.0 NaH2PO4, 1.2 MgSO4, 1.9 CaCl2, 25.0 NaHCO3, and 11.1 dextrose, and gassed with 95% O2-5% CO2]. The dissected esophageal-vagal preparation from sensitized animals was immersed in the KBS-containing antigen (OVA, 10 μg/ml) for 30 min to induce antigen-mediated mast cell degranulation.

Immunofluorescence staining. Eight nodose ganglia (from 8 guinea pigs) were first dissected and fixed in 4% formaldehyde in PBS overnight at 4°C. The tissues were then rinsed in PBS, cryoprotected with 18% sucrose in PBS for 18–24 h. Afterward, the tissues were covered with optimum-cutting temperature mounting medium and frozen on dry ice. The tissues were cut in serial sections of 12 μm thickness on a cryostat, collected on silane-coated slides, and air-dried for 30 min.

Sections were incubated with blocking solution containing 1% BSA, 10% normal goat serum, and 0.1% Tween 20 in PBS for 60 min. The tissues were then rinsed with PBS, cryoprotected with 18% sucrose in PBS for 18–24 h. Afterward, the tissues were covered with optimum-cutting temperature mounting medium and frozen on dry ice. The tissues were cut in serial sections of 12 μm thickness on a cryostat, collected on silane-coated slides, and air-dried for 30 min.

Sections were incubated with blocking solution containing 1% BSA, 10% normal goat serum, and 0.1% Tween 20 in PBS for 60 min. The tissues were then rinsed with PBS, cryoprotected with 18% sucrose in PBS for 18–24 h. Afterward, the tissues were covered with optimum-cutting temperature mounting medium and frozen on dry ice. The tissues were cut in serial sections of 12 μm thickness on a cryostat, collected on silane-coated slides, and air-dried for 30 min.

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In a separate study, 1,1’-dioctadecyl-3,3’,3’-tetramethylindocarbocyanine perchlorate (DiI) (Molecular Probes) retrograde labeling of nodose neurons from the esophagus was performed in two guinea pigs according to the previously described method (37). Four nodose ganglia (2 from each animal) were collected 10 days after DiI injection with 1 μl DiI solution (1% diluted in 50% dimethyl sulfoxide in saline) in the wall of the esophagus at 50 mm above the gastric-esophageal junction and processed for immunofluorescence staining with either PAR2 or TRPA1 as described above. The secondary antibodies included goat anti-mouse antibody labeled with Alexa 488 for PAR2 and goat anti-rabbit labeled with Alexa 488 for TRPA1. The slides were analyzed and counted using a conventional epifluorescence microscope (Olympus DX60) with the filters set to allow visualization of Alexa 488 (for PAR2 and TRPA1) and Alexa 594 (for DiI). The numbers of DiI-labeled neurons and DiI-labeled PAR2-, TRPA1-positive neurons were counted by identifying from pixel intensity above background fluorescence from four sections of each ganglion (n = 4). The percentages of PAR2- or TRPA1-positive nodose neurons among DiI-labeled esophageal neurons were then calculated.

Western blot. Vagal nodose ganglia and jugular ganglia, DRG, and liver were freshly obtained from guinea pigs (n = 4). The tissues were first homogenized in lysis buffer (20 mM Tris, pH 7.5, 1% TX, 10% glycerol, 137 mM NaCl, 2 mM EDTA, 25 mM glycerophosphate, 1 mM Na2VO4, and EDTA-free complete protease inhibitor cocktail from Roche). Next, the lysates were centrifuged at 5,000 g at 4°C for 30 min, and supernatants were transferred to new tubes, followed by protein concentration measurement by BCA assay. Lysates (20 μg) were separated by 4–12% NuPage, transferred to a polyvinylidene difluoride membrane, and blotted with primary (overnight), then secondary (1-h) antibodies. The primary antibodies included a mouse monoclonal anti-PAR2 antibody (1:200; Santa Cruz), a rabbit polyclonal anti-TRPA1 antibody (1:200; Novus Biologicals), and a mouse monoclonal anti-β-tubulin antibody (Sigma). The secondary antibodies included anti-mouse Ig, horseradish peroxidase-linked F(a)b’2 fragment (from sheep) for PAR2 (1:3,000, catalog no. NA9310) and anti-rabbit Ig, horseradish peroxidase-linked F(a)b’2 fragment (from donkey) for TRPA1 (1:3,000, catalog no. NA9340) (GE Life Sciences, Piscataway, NJ).

RT-PCR. Vagal nodose and jugular ganglia, DRG, and liver were freshly obtained from guinea pigs (n = 4). The total RNA was extracted using TRIzol reagent (Invitrogen) essentially according to the manufacturer’s instructions and digested with DNase RQI (Promega) to remove any contaminating genomic DNA. RNA concentrations were measured using the NanoDrop ND-1000 Fluorospectrometer. The first-strand cDNA was then produced from 1.0 μg of the total RNA using Omniscript Reverse Transcrip tapeddase (Qiagen) and Superscript First-Strand Synthesis System for RT-PCR (Invitrogen) with random hexamer primers according to the manufacturer’s instructions. The concentration and quality of the resulting cDNA was quantified and analyzed using the NanoDrop ND-1000 Fluorospectrometer. PCR amplification was performed using PfU Ultra High-fidelity DNA polymerase (Stratagene) with the same amount of sample cDNAs. One pair of primers specific for guinea pig PAR2 (9) (sense, 5’-CAT GTT CAG CTAC TTC TCC TTC TT-3’; antisense, 5’-GGT TT AAT AAC ACT GGT GGA GCT TGA-3’) was used to amplify a 472-bp fragment, and two pairs of primers specific for guinea pig TRPA1 (1st pair of primers: sense 1, 5’-CAT TTT GCT GCM ACC CAG GGA GCC ACT G-3’; and antisense 1, 5’-ATA AGT GGA GAG CGT CCT TCA GAA TC-3’; 2nd pair of primers: sense 2, 5’-AAG ATG CAT AGT GAT GGA GAA C-3’; and antisense 2, 5’-TCT GTG TCH GAG ATG ATC TCC ATC-3’) were used to amplify a 212-bp fragment and a 319-bp fragment. As a control, a 332-bp fragment of guinea pig glyceraldehyde-3-phosphate dehydrogenase cDNA was amplified using a pair of primers (sense, 5’-CATCAC CAT CTT CCA GGA GGC A-3’; and antisense, 5’- GTC TTC TGG GTG GCA GTA TG-3’) (26).
Electrophysiology. The esophageal-vagal preparations were set up, and extracellular recordings of action potential discharges from nodose neuron C-fibers were performed as previously described (37, 34). The esophagus and trachea were dissected with intact bilateral extrinsic vagal innervation (including jugular and nodose ganglia). The tissue was pinned in a small Sylgard-lined Perspex chamber filled with KBS (at 35°C, and gassed with 95% O₂-5% CO₂). The chamber had two compartments: the esophagus with attached trachea (to support the recurrent laryngeal nerves) and the vagus were pinned in the tissue compartment, and the rostral aspect of the vagus nerves including the nodose and jugular ganglia were pinned in the recording compartment. The two compartments were separated by a silicone grease plug and were separately superfused with KBS (pH 7.4; 35°C; 4–6 ml/min). Polyethylene tubing was inserted 3–5 mm in the cranial and caudal esophagus and secured for perfusion.

The pressure in the fluid (KBS)-filled esophagus was measured with a differential pressure transducer connected in series to the esophagus and recorded simultaneously with neural activity by the chart recorder (TA240S; Gould, Valley View, OH). Isobaric esophageal distension for 20 s with an intraluminal pressure of 10–100 mmHg separated by at least 60 s was used to determine the distension pressure-nerve activity relationship of an esophageal afferent fiber. Extracellular recordings were performed using an aluminosilicate glass microelectrode (pulled with a Flaming-Brown micropipette puller; Sutter Instruments, Novato, CA) filled with 3 M sodium chloride (electrode resistance 2 MΩ). The microelectrode was placed in a nodose ganglion with an electrode holder connected directly to the headstage (A-M Systems, Everett, WA). A return electrode of silver-silver chloride wire and earthed silver-silver chloride pellet was placed in the perfusion fluid of the recording compartment. The recorded signal was amplified (Microelectrode AC amplifier 1800; A-M Systems) and filtered (low cut-off, 0.3 kHz; high cut-off, 1 kHz), and the resultant activity was displayed on an oscilloscope (TDS 340; Tektronix, Beaverton, OR) and chart recorder. The data were stored and analyzed on a Macintosh computer using the software TheNerveOff (sampling frequency 33 kHz; PHOCIS, Baltimore, MD) and further processed using spreadsheet software (Microsoft Excel 2004 for Mac). The nerve fiber was considered a C-fiber if it conducted action potentials at <1 m/s. Conduction velocity was calculated by dividing the length of the approximated nerve pathway (from the recorded nodose/jugular neurons to the mechanosensitive receptive field in the esophagus) by conduction time.

The chemicals diluted in KBS solution were delivered to the esophagus in the external perfusate for 10–20 min. The nerve activity (action potential discharge) was monitored continuously and analyzed in 1-s bins (yielding the number of action potentials in each second, Hz). The peak of frequency of action potentials in response to each stimulation was analyzed and compared. The compounds used in the experiment included: PAR2-activating peptide (PAR2-AP: 2-Furoy-LIGRL-Amide) and PAR2 reverse peptide (PAR2-RP: 2-Furoy-OLRGIL-Amide) (Peptide International); allyl isothiocyanate (AITC), leupeptin, and OVA (Sigma-Aldrich); and HC-030031 (Chembridge). The stock solutions, 2-Furoy-LIGRL-Amide (10 mM in water), 2-Furoy-OLRGIL-Amide (10 mM in water), HC-030031 (10 mM in dimethyl sulfoxide), and leupeptin (10 mM, in water) were stored at −20°C and diluted in KBS to final concentration on the day of use. AITC (95%) was diluted with KBS in the final concentration (1 mM) on the day of use.

Experimental protocol. In electrophysiological studies, only the results from AITC-responsive nodose C-fibers, which were confirmed by the end of each recording, were selected for analysis. We performed one study per day and recorded afferent nerve activities from one nodose C-fiber per animal. First, the effect of mast cell activation-induced long-lasting mechanical hypersensitivity was confirmed, and the effect of a trypsin inhibitor on this potentiation was tested; second, whether PAR2 agonist (PAR2-AP) mimicked mast cell activation-induced mechanical hypersensitivity was determined; third, whether TRPA1 inhibitor HC-030031 pretreatment prevented mechanical hypersensitivity induced by either mast cell activation or PAR2-AP was investigated.

Data analysis. The distension-evoked nerve response was quantified as the peak frequency of the action potential discharge within a 20-s distention period. Chemical perfusion-evoked response was quantified as the peak frequency of action potential discharge within 10–30 min from the start of the response after the spontaneous activity (if present) was subtracted. The peak frequencies (Hz) of the action potential discharges were presented as means ± SE and compared by paired t-test. For all experiments, the significance was defined as p < 0.05.

RESULTS

Expression of PAR2 and TRPA1 in nodose ganglia neurons. To determine the roles of PAR2 and TRPA1 in mast cell activation-induced mechanical hypersensitivity in esophageal nodose C-fibers, we examined their expression and colocalization in nodose neurons by immunostaining. Both PAR2 and TRPA1 expression were identified in nodose neurons (Fig. 1, A and B). In the total 620 counted nodose neurons (from 8 guinea pigs), ~91% (563/620) TRPA1-positive neurons were small- and medium-size neurons (diameters: 10–30 μm), and 80% (498/620) TRPA1-positive neurons were also positively labeled with PAR2 (Fig. 1, C and D). In a separate study, we examined the percentages of PAR2- or TRPA1-positive nodose neurons in DiI retrograde-labeled esophageal nodose neurons. In a total of 78 DiI-labeled esophageal nodose neurons (from 4 ganglia × 4 sections) that were counted, most of them also displayed PAR2-positive (97 ± 2%, image not shown) and TRPA1-positive (92 ± 1%) immunoreactivities (Fig. 1, E–H).

Next, we detected PAR2 and TRPA1 mRNA in nodose ganglia by RT-PCR. One pair of primers specific for guinea pig PAR2 (9) was applied. We identified the PCR products with 472 bp from RNA extracted from nodose (and jugular) ganglia, as well as from positive (DRG) and negative (liver) controls (39, 3) (Fig. 2A). Currently, there are no reports of TRPA1 expression in guinea pig nodose neurons. We designed two pairs of primers according to the published sequences from human (accession no.: NM 007332), monkey (accession no.: XM 001083172), mouse (accession no.: NM 177781), and rat (accession no.: NM 207608). Two PCR products with 212 and 312 bp were identified from RNA extracted from nodose (and jugular) ganglia and were also detected in positive (DRG) but not in negative (liver) controls (33) (Fig. 3A).

Western blot analysis with an anti-PAR2 antibody showed the presence of PAR2 in nodose ganglia. Probing with PAR2 antibody revealed strong immunoreactive bands in lysates from nodose (and jugular) ganglia. The same band was also found in the DRG, which is known to express high levels of PAR2, but not in the liver (3, 39) (Fig. 2B). We also determined the expression of TRPA1 in nodose ganglia by Western blot. Probing with anti-TRPA1 antibody identified strong immunoreactive bands in lysates from nodose (and jugular) ganglia as well as from the positive control (DRG), but not the negative control (liver) (33) (Fig. 3B).

In this part of the study, the expression profiles of PAR2 and TRPA1 were identified consistently by immunostaining, Western blot, and RT-PCR. These findings provide the first evidence for the expression and colocalization of PAR2 and TRPA1 in guinea pig nodose ganglia (neurons). The high
coexpression rate (80%) may indicate a frequent interaction of PAR2 and TRPA1.

Mast cell activation-induced mechanical hypersensitivity in esophageal nodose C-fibers. To confirm mast cell activation-induced mechanical hypersensitivity in esophageal nodose C-fibers in the present study, we performed extracellular recordings in esophageal-vagal preparations from OVA-sensitized guinea pigs and those from naive animals. Esophageal distension-evoked action potentials were compared before and after OVA-induced mast cell activation. Consistent with our previous study (34), we found that in vitro OVA challenge (OVA, 10\(\mu\)g/ml, 30 min) in esophageal-vagal preparations from OVA-sensitized guinea pigs led to two- to threefold increases of excitabilities of esophageal nodose C-fibers in response to esophageal distension. The peaks of frequency of action potential discharges evoked by esophageal distensions increased significantly from 2.8 ± 0.3, 5.8 ± 0.6, and 9.8 ± 0.9 Hz to 7.5 ± 0.6, 13.8 ± 1.3, and 18.3 ± 1.4 Hz at distension pressures of 10, 30, and 60 mmHg for 20 s, respectively (Fig. 4A and Table 1). Similar to our previous study (34), this mechanical hypersensitivity lasted 90 min after washing OVA out (data not shown). As controls, OVA challenge in esophageal-vagal preparations from naive animals did not induce mechanical hypersensitivity in esophageal nodose

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**Fig. 1.** Immunofluorescence staining of transient receptor potential A1 (TRPA1; A) and protease-activated receptor 2 (PAR2; B), and their coexpression in nodose ganglia neurons (C). D: ~91% (563/620) TRPA1-positive nodose neurons have small and medium (10–30 \(\mu\)m) diameters, and 80% (498/620) of them also positively labeled with PAR2 (n = 620, 20 neurons × 4 sections × 8 animals, excluding 1 section falling off). In a total of 78, 1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine perchlorate (DiI)-labeled esophageal nodose neurons (from 4 ganglia × 4 sections) that were counted, most displayed PAR2-positive (97 ± 2%, image not shown) and TRPA1-positive (92 ± 1%) immunoreactivities (E–H).

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**Fig. 2.** Detecting the expression of PAR2 mRNA by RT-PCR (A) and PAR2 protein by Western blot (B) in guinea pig nodose (and jugular) ganglia compared with positive [dorsal root ganglion (DRG)] and negative (liver) controls. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Ab, antibody.
C-fibers. In these animals, no significant changes were found in action potential discharges evoked by 20-s esophageal distensions before and after OVA challenge (2.8 ± 0.4, 6 ± 1.1, and 8.6 ± 1.2 Hz vs. 2.6 ± 0.2, 5.4 ± 0.4, and 9.4 ± 1.2 Hz at distension pressures of 10, 30, and 60 mmHg, respectively, \( P > 0.05, n = 5 \)) (Fig. 4B). Only nodose C-fibers that were confirmed to respond to TRPA1 agonist AITC (1 mM) at the end of each study were included in the analysis.

The data from our previous study have shown that mast cell activation induced by in vitro OVA challenge leads to a significant decrease in the number of tryptase-positive mast cells in the esophagus (34). However, functional evidence of whether mast cell tryptase plays a role in sensitizing esophageal vagal sensory afferent is still lacking. In the present study, a serine protease inhibitors leupeptin was applied to determine whether mast cell tryptase participated in mast cell activation-induced mechanical hypersensitivity in esophageal nodose C-fibers. Pretreatment with leupeptin (30 min, 20 \( \mu \)M), a known inhibitor of guinea pig mast cell tryptase (28), significantly inhibited mast cell activation-induced mechanical hypersensitivity of nodose C-fibers. After 30 min perfusion and continuously in the presence of leupeptin, OVA challenge did not significantly change the action potential discharges evoked by 20-s esophageal distensions (control vs. OVA + leupeptin: 2.3 ± 0.3, 6 ± 1.1, and 10.8 ± 0.9 Hz vs. 2.5 ± 0.3, 6.8 ± 0.8, and 11.8 ± 0.9 Hz at distension pressures of 10, 30, and 60 mmHg, respectively, \( P > 0.05, n = 4 \)) (Fig. 4C). This suggested that mast cell tryptase played a crucial role in mechanical hypersensitivity of nodose C-fibers induced by mast cell activation, and this effect was likely mediated by a PAR2-dependent mechanism.

PAR2 agonist, PAR2-AP, mimicked the effect of mast cell activation-induced mechanical hypersensitivity in esophageal nodose C-fibers. To determine the role of PAR2 in mast cell activation-induced mechanical hypersensitivity in nodose C-fibers, we tested the hypothesis that PAR2 agonist (using PAR2-AP) mimicked the mechanical hypersensitivity induced by mast cell activation. We chose 2-Furoyl-LIGRLO-Amide, a potent and selective PAR2-AP (18), and its negative control peptide (2-Furoyl-OLRGIL-Amide) for our study. Perfusion with 2-Furoyl-LIGRLO-Amide (10 \( \mu \)M, 30 min) evoked action potential discharges that were significantly increased over the baseline spontaneous activity (action potentials subtracting baseline spontaneous activity: 3.3 ± 1.1 Hz, \( n = 6 \)) in esophageal distensions (Fig. 4A, B, C)
Mechanical hypersensitivity

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Values are means ± SE. MC, mast cell; PAR2-AP, protease-activated receptor 2-activating peptide. *P < 0.05 (n = 4 experiments), †P < 0.05 (n = 6), ‡P > 0.05 (n = 6), and §P > 0.05 (n = 6) vs. control.

This was associated with a significant increase in their responses to esophageal distension. The peaks of frequency of action potential discharges (subtracting baseline spontaneous activity and PAR2-induced activity) evoked by 20-s esophageal distensions significantly enhanced from 3.2 ± 0.7, 6.3 ± 1.2, and 11.2 ± 2.3 Hz to 8.8 ± 1.6, 16.5 ± 2.6, and 22 ± 2.9 Hz at distension pressures of 10, 30, and 60 mmHg for 20 s, respectively (P < 0.05, n = 6) (Fig. 5A). Moreover, the induced mechanical hypersensitivity was examined every 30 min for 90 min after washing PAR2-AP out. A sustained increase in action potential discharges in response to esophageal distensions was observed for 90 min as determined by the significant fold increases in action potential discharges evoked by esophageal distension at 60 mmHg after washing PAR2-AP out for 30, 60, and 90 min (compared with that before PAR2-AP, P < 0.05, n = 6) (Fig. 5, C and D and Table 1). In control experiments, perfusion with PAR2-RP (2-Furoyl-OLRGIL-Amide, 10 μM, 30 min) neither evoked any action potential discharges nor increased the responses to esophageal distensions in esophageal nodose C-fibers. The action potentials evoked by esophageal distensions did not significantly change before and after perfusion with 2-Furoyl-OLRGIL-Amide (1.8 ± 0.3, 5.8 ± 1.1, and 10.5 ± 1 Hz vs. 2 ± 0.4, 5.5 ± 0.9, and 11 ± 1 Hz at distension pressures of 10, 30, and 60 mmHg, respectively, P > 0.05, n = 4) (Fig. 5B). These fibers were then confirmed not only to be activated by 2-Furoyl-LIGRLO-Amide (PAR2-AP) but also to display the increased responses to esophageal distensions (action potentials increased to: 6.5 ± 1.2, 14.3 ± 2.3, and 19.5 ± 1 Hz at distension pressures of 10, 30, and 60 mmHg, respectively, vs. baseline and PAR2-RP: P < 0.05, n = 4) (data not shown in Fig. 5). Only nodose C-fibers that were confirmed to respond to TRPA1 agonist AITC (1 mM) at the end of each study were included in the analysis.

Currently, there is no PAR2 antagonist available to further investigate the role of PAR2 in mast cell activation-induced mechanical hypersensitivity. Our data that PAR2 agonist mimicked the effect of mast cell activation-induced long-lasting mechanical hypersensitivity supported a role of PAR2 in mast cell activation-induced potentiation effect in esophageal vagal nodose C-fibers.

TRPA1 inhibitor, HC-030031, prevented mechanical hypersensitivity induced by either mast cell activation or PAR-AP in esophageal nodose C-fibers. To clarify the involvement of downstream TRPA1 in this potentiation process, we tested the hypothesis that pretreatment with TRPA1 antagonist prevented mechanical hypersensitivity induced by either mast cell acti-
vation or PAR2-AP in esophageal vagal nodose C-fibers. We chose a TRPA1 inhibitor (HC-030031) for our study because it has been shown to have a specific inhibitory effect on TRPA1 agonist in guinea pig jugular ganglia neurons (4). First, we determined whether perfusion with HC-030031 (10 μM) itself for 30 min changed the mechanoexcitability in esophageal nodose C-fibers from OVA-sensitized guinea pigs. Before and after perfusion with HC-030031 (10 μM) for 30 min, action potentials evoked by esophageal distension did not change significantly (2.2 ± 0.3, 5.2 ± 0.6, and 9.7 ± 0.8 Hz vs. 2.2 ± 0.3, 5.7 ± 0.8, and 10.2 ± 1.1 Hz at distension pressures of 10, 30, and 60 mmHg, respectively, P > 0.05, n = 6) (Fig. 6A).

Next, we studied the inhibitory effect of HC-030031 on mast cell activation-induced mechanical hypersensitivity. Following 30 min perfusion with and in the presence of HC-030031 (10 μM), mast cell activation induced by OVA challenge (OVA, 10 μg/ml, 30 min) failed to induce mechanical hypersensitivity in esophageal nodose C-fibers from OVA-sensitized guinea pigs (action potentials: 2.2 ± 0.3, 5.7 ± 0.8, and 10.2 ± 1.1 Hz vs. 2.7 ± 0.6, 6.2 ± 0.6, and 10.3 ± 1.4 Hz before and after mast cell activation at distension pressures of 10, 30, and 60 mmHg, respectively, P > 0.05, n = 6) (Fig. 6A and Table 1). Only nodose C-fibers that were confirmed to respond to TRPA1 agonist AITC (1 mM) at the end of each study were included in the analysis. These data provided new evidence that pretreatment with TRPA1 inhibitor HC-030031 significantly inhibited mechanical hypersensitivity induced by either mast cell activation or PAR2-AP in esophageal vagal nodose C-fibers.

**DISCUSSION**

Mast cell activation releases a variety of mediators, including preformed mediators (histamine and tryptase), newly synthesized mediators (platelet-activating factor, prostaglandin D2, and leukotriene C4), and cytokines (tumor necrosis factor) (1). Among them, tryptase may be one of the most important mediators that regulate the excitability of sensory afferents. Mast cell tryptase has been shown to increase the excitability of sensory afferents. This effect is usually mediated via PAR2 on sensory nerves (17, 32). PAR2 expression has been reported on DRG, submucosal, and myenteric neurons but not in nodose neurons. Here, we identified, for the first time, the expression of PAR2 in guinea pig vagal nodose neurons by immunostaining, Western blot, and RT-PCR. We observed that most PAR2-positive immunoreactivities were on small and medium neurons, which also coexpressed TRPA1. This is in agreement with the previous report that PAR2 was expressed mostly in small and unmyelinated rat DRG neurons (39, 22). Both PAR2 and TRPA1 were further identified to be highly expressed in DiI-labeled esophageal nodose neurons. TRPA1 expression has recently been reported in small and medium nodose neurons from rat (12) and mouse (20). Our data add new evidence by identifying TRPA1 expression in guinea pig nodose neurons using immunostaining, Western blot, and RT-PCR. About 91% of TRPA1-positive immunoreactivities were found in small and medium nodose neurons, and 80% of them colocalized with PAR2-positive immunoreactivities. These data strongly suggest a functional interaction between PAR2 and TRPA1 on nodose afferent unmyelinated C-fibers, the axons of small- and medium-size neurons.

Vagal afferents project to the guinea pig esophagus with four subtypes of nerve fibers, including nodose C and Aδ fibers, and jugular C and Aδ fibers (33). We chose nodose C-fibers in the present study to determine the functional involvement of PAR2 and TRPA1 in mast cell activation-induced mechanical hypersensitivity. This was based on the above expression data and
our previous studies. First, mechanical hypersensitivity induced by mast cell activation occurs in nodose C but not Aδ fibers (34). Second, TRPA1 agonist AITC activates both nodose and jugular C-fibers but not nodose Aδ fibers (35). Third, TRPA1 mediated bradykinin-induced mechanical hypersensitivity in nodose and jugular C-fibers but not in nodose Aδ fibers (35).

Our previous study showed that mast cell activation induced a long-lasting mechanical hypersensitivity of nodose C-fibers in the guinea pig esophagus (34). This was reconfirmed in the present study where mast cell activation induced by in vitro OVA challenge significantly enhanced the mechanoexcitability of esophageal nodose C-fibers in esophageal-vagal preparation only from OVA-sensitized guinea pigs but not from naive animals. This mechanical hypersensitivity lasted for 90 min after washing antigen out. This is similar to the previous observation on submucosal neurons that mast cell tryptase and PAR2-AP induced a long-lasting hyperexcitability (28). In our previous study, we have shown that mast cell activation led to a significant decrease in the number of tryptase-positive mast cells in the esophagus (34). This indicated that a significant amount of tryptase was released after mast cell activation. In the present study, we further demonstrated that pretreatment with a serine protease inhibitor (leupeptin) prevented mechanical hypersensitivity induced by mast cell activation in esophageal vagal C-fibers. This again suggested that mast cell tryptase could cleave PAR2 at a specific site within the extracellular amino terminus of the receptor. This exposes a new amino terminus that serves as a tethered ligand domain, which binds to the conserved regions in the second extracellular loop of the cleaved receptor to activate the receptor itself (24).

To determine the role of PAR2 in this process, the most definitive approach would be to demonstrate that PAR2 antagonist blocks the mast cell activation-induced effect. Currently, to the best of our knowledge, there is no specific PAR2 antagonist available. Thus, we applied PAR2 agonist, 2-Furoyl-LIGRLO-Amide, to determine whether it mimics mast cell activation-induced mechanical hypersensitivity of nodose C-fibers in the guinea pig esophagus. Our data strongly support a role of PAR2 in this potentiation process. First, perfusion with PAR2 agonist 2-Furoyl-LIGRLO-Amide resulted in a significant enhancement in mechanoexcitability of esophageal nodose C-fibers. Second, this increased mechanoexcitability was sustained for 90 min after washing PAR2 agonist out. These two features are consistent with our previous study (34) and parallel to current results showing that mast cell activation induced a long-lasting mechanical hypersensitivity in esophageal nodose C-fibers. In addition, we observed a modest activation response during PAR2-AP perfusion that is different from the observations that mast cell degranulation alone did not evoke a significant activation response in esophageal nodose C-fibers in our previous (34) and current studies. We suspect that two factors may contribute to this difference. First, 2-Furoyl-LIGRLO-Amide is a new and potent PAR2 agonist (19) that may induce a stronger activation response than that induced by mast cell tryptase. Second, perfusion with exogenous PAR2 agonist may be equivalent to a higher concentration of endogenous tryptase that would reach the sensory afferent nerve endings after mast cell activation.

PAR2 activation has been proven to sensitize several downstream TRP channels via its G protein-coupled receptor on sensory afferent to induce thermal, mechanical, and chemical hyperalgesia. The roles of TRPV1 (2, 3, 10) and TRPV4 (15, 32) in PAR2-mediated thermal- and mechanohyperalgesia are well defined. The role of TRPA1 in this process is less understood. TRPA1 is of great importance in mechanosensory transduction (6, 18). TRPA1 could be sensitized by PAR2 activation in disassociated DRG neurons and contributed to somatic pain in an animal behavioral model (11). Thus we speculated that TRPA1 might contribute to mechanical hypersensitivity of visceral afferents induced by PAR2 activation as we have defined above. The results from the present study support the conclusion that TRPA1 participates in PAR2 activation-induced mechanical hypersensitivity of esophageal nodose C-fibers. Pretreatment of the tissue with TRPA1 inhibitor HC-030031 completely prevented mechanical hypersensitivity induced by mast cell activation in esophageal nodose C-fibers. Moreover, pretreatment with TRPA1 inhibitor HC-030031 significantly inhibited PAR2 agonist-induced mechanical hypersensitivity in esophageal nodose C-fibers. Sensory afferent neurons are usually polymodal and could concurrently express a variety of ion channels. It should be noted that our study did not exclude the possible involvements of TRPV1 and TRPV4 in PAR2 activation-induced mechanical hypersensitivity due to the technique limit and the fact that both TRPA1 and TRPV1 agonists could sensitize mechanoexcitability of esophageal nodose C-fibers (35, 37).

In summary, our expression and functional data provide new evidence that sensitizing TRPA1 via a PAR2-dependent mechanism plays an important role in mast cell activation-induced long-lasting mechanical hypersensitivity of nodose C-fiber in the guinea pig esophagus. Mast cell is an important proinflammatory cell in the gastrointestinal tract. It not only participates in host-defense immune responses but also regulates the functions of peripheral nerves and smooth muscles in the gastrointestinal tract. Increased numbers of esophageal mast cells have been reported in certain esophageal disorders. Upon activation, mast cells release mediators such as tryptase which may act on PAR2 to sensitize sensory afferents in the proximity. Our data reveal a new mechanism of esophageal peripheral sensitization. Whether this peripheral sensitization effect would contribute to the development of abnormal esophageal sensations such as esophageal-related noncardiac chest pain and heartburn in the clinic needs to be clarified further.

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GRANTS

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