Neutrophil Elastase Activates Protease-activated Receptor-2 (PAR2) and Transient Receptor Potential Vanilloid 4 (TRPV4) to Cause Inflammation and Pain*

Received for publication, February 2, 2015, and in revised form, April 8, 2015. Published, JBC Papers in Press, April 15, 2015, DOI 10.1074/jbc.M115.642736

Peishen Zhao1, TinaMarie Lieu1, Nicholas Barlow3, Silvia Sostegni3, Silke Haerteis2, Christoph Korbmacher2, Wolfgang Liedtke3, Nestor N. Jimenez-Vargas4, Stephen J. Vanner4, and Nigel W. Bunnett*1

From the 1Monash Institute of Pharmaceutical Sciences and the **ARC Centre of Excellence in Convergent Bio-Nano Science and Technology, Monash University, Parkville 3052, Australia, the 3Institut für Zelluläre und Molekulare Physiologie, Friedrich-Alexander-Universität Erlangen-Nürnberg, Erlangen 91054, Germany, the 2Department of Neurology, School of Medicine, Duke University, Durham, North Carolina 27710, the 4Institut für Zelluläre und Molekulare Physiologie, Friedrich-Alexander-Universität Erlangen-Nürnberg, Erlangen 91054, Germany, and the 4Institut für Zelluläre und Molekulare Physiologie, Friedrich-Alexander-Universität Erlangen-Nürnberg, Erlangen 91054, Germany.

Proteases that cleave protease-activated receptor-2 (PAR2) at Arg36† Ser37 reveal a tethered ligand that binds to the cleaved receptor. PAR2 activates transient receptor potential (TRP) channels of nociceptive neurons to induce neurogenic inflammation and pain. Although proteases that cleave PAR2 at non-canonical sites can trigger distinct signaling cascades, the functional importance of the PAR2-biased agonism is uncertain. We investigated whether neutrophil elastase, a biased agonist of PAR2, causes inflammation and pain by activating PAR2 and TRPV4. Elastase cleaved human PAR2 at Ala66↓ Ser67 and Ser67↓ Val68. Elastase stimulated PAR2-dependent cAMP accumulation and ERK1/2 activation, but not Ca2+ mobilization, in KNRK cells. Elastase induced PAR2 coupling to Gαq but not Gαi in HEK293 cells. Although elastase did not promote recruitment of G protein-coupled receptor kinase-2 (GRK2) or β-arrestin to PAR2, consistent with its inability to promote receptor endocytosis, elastase did stimulate GRK6 recruitment. Elastase caused PAR2-dependent sensitization of TRPV4 currents in Xenopus laevis oocytes by adenylyl cyclase- and protein kinase A (PKA)-dependent mechanisms. Elastase stimulated PAR2-dependent cAMP formation and ERK1/2 phosphorylation, and a PAR2- and TRPV4-mediated influx of extracellular Ca2+ in mouse nociceptors. Adenylyl cyclase and PKA-mediated elastase-induced activation of TRPV4 and hyperexcitability of nociceptors. Intraplantar injection of elastase to mice caused edema and mechanical hyperalgesia by PAR2- and TRPV4-mediated mechanisms. Thus, the elastase-biased agonism of PAR2 causes Gαq-dependent activation of adenylyl cyclase and PKA, which activates TRPV4 and sensitizes nociceptors to cause inflammation and pain. Our results identify a novel mechanism of elastase-induced activation of TRPV4 and expand the role of PAR2 as a mediator of protease-driven inflammation and pain.

Protease-activated receptors (PARs)2 are a unique family of four G protein-coupled receptors that are activated by extracellular and membrane-tethered proteases (1). Proteases such as thrombin and trypsin cleave at specific sites within the extracellular N terminus of PAR1, PAR2, and PAR4, which reveals tethered ligand domains that bind to and activate the cleaved receptors. When activated by this canonical mechanism, PAR1, PAR2, and PAR4 predominantly couple to Gαq and β-arrestins, leading to downstream signaling events that mediate hemostasis, inflammation, pain, and repair mechanisms (2, 3).

PAR2 is an important mediator of protease-driven inflammation and pain. PAR2 is expressed by epithelial, endothelial, and smooth muscle cells in multiple tissues, as well as by cells of the immune system and the central and peripheral nervous systems (4). Proteases that activate PAR2 on nociceptive neurons can stimulate the release of substance P and calcitonin gene-related peptide, which cause neurogenic inflammation in peripheral tissues and mediate pain transmission in the dorsal horn of the spinal cord (5, 6). PAR2 sensitizes transient receptor potential

* This work was supported by NHMRC Grants 63303, 1049682, and 1031886, grants from the ARC Centre of Excellence in Convergent Bio-Nano Science and Technology and Monash University (to N.W.B.), a Ph.D. fellowship from the Bayerische Forschungsstiftung (to S.S.), and the Else Kröner-Fresenius-Stiftung (to S.H.).

1 To whom correspondence should be addressed: Monash Institute of Pharmaceutical Sciences, 381 Royal Parade, Parkville, Victoria 3052, Australia. Tel.: 61-3-9903-9136; Cell: 61-407-392-619; Fax: 61-3-9903-9581; E-mail: Nigel.Bunnett@Monash.edu.

2 The abbreviations used are: PAR2, protease-activated receptor 2; TRPV, transient receptor potential; GRK, G protein-coupled receptor kinases; PKA/C, protein kinase A/C; DRG, dorsal root ganglia; BRET, bioluminescence resonance energy transfer; VC, vector control.

Background: Proteases cleave protease-activated receptor-2 (PAR2), which activates transient receptor potential (TRP) ion channels to cause inflammation and pain.

Results: Neutrophil elastase cleaves PAR2, resulting in Gαq-mediated cAMP formation, transient receptor potential vanilloid 4 (TRPV4) activation, and sensitization of nociceptive neurons, inflammation, and pain.

Conclusion: Elastase causes PAR2- and TRPV4-mediated inflammation and pain.

Significance: PARs and TRP channels mediate responses to diverse proteases.
Elastase Activation of PAR2 and TRPV4

(TRP) ion channels of nociceptors, including TRP vanilloid 1 (TRPV1) (7), TRPV4 (8–10), and TRP ankyrin A1 (TRPA1) (11), which amplifies the proinflammatory and hyperalgesic actions of proteases.

Pancreatic trypsin I and II, the most widely studied and first identified agonists of PAR2, cleave the receptor at Arg36→Ser37, to reveal the tethered ligand SLIGKV (in human) (4, 12). Trypsin-activated PAR2 couples to Gαq, leading to mobilization of intracellular Ca²⁺ and activation of second messenger kinases such as protein kinase C (PKC) and D (PKD) (8, 13, 14). Trypsin-activated PAR2 also recruits G protein receptor kinase 2 (GRK2) and β-arrestins, which uncouple PAR2 from G proteins and mediate clathrin- and dynamin-dependent receptor endocytosis (15, 16). By recruiting PAR2 and Src to endosomal signalosomes, β-arrestins can mediate the activation of extracellular signal-regulated kinases 1 and 2 (ERK1/2) within the cytosol (15).

Although pancreatic trypsins effectively activate PAR2, the identity of the proteases that activate PAR2 under pathophysiological conditions is uncertain. In particular, given the widespread distribution of PAR2 and the restricted expression of trypsin I/II to the pancreas, where activity is tightly controlled by endogenous inhibitors, the proteases that activate PAR2 in tissues other than the pancreas and intestine remain to be identified. Other proteases that can cleave PAR2 at Arg36→Ser37 include trypsin IV (mesotrypsin) (17, 18), tryptase (19, 20), cathepsin S, which cleaves at Gly41→Lys42 (30), cathepsin G, which cleaves at Phe64→Thr65 (31), neutrophil elastase, which cleaves at Thr57 (31), and proteinase 3, which cleaves at Val61→Asp62 (32). However, the precise molecular mechanisms and the physiological consequences of biased protease signaling are poorly defined.

We investigated the mechanisms and pathophysiological outcomes of neutrophil elastase-induced biased agonism of PAR2. Elastase is one of the major proteases released from infiltrating neutrophils in inflamed tissues. Given its high circulating concentration (up to 1 μM) and long half-life (6–8 h) during inflammation, elastase has been proposed as a target for anti-inflammatory therapy (33). Elastase is a biased agonist of both PAR1 and PAR2, but by distinctly different mechanisms. Elastase cleaves PAR2 at Leu15→Arg16, distal to the thrombin cleavage site, which reveals a tethered ligand domain (RNPND-KYEFP-NH₂) that activates Gαq-mediated ERK signaling (34). Elastase cleaves PAR2 at Ser67→Val68, distal to the trypsin cleavage site, which activates PAR2 by a mechanism that does not involve exposure of a tethered ligand domain (32). However, the functional importance of the elastase-biased agonism of PAR2 is uncertain.

Given the important proinflammatory and pro-nociceptive actions of elastase, PAR2, and trypsin channels, we investigated whether the elastase-biased agonism of PAR2 activates TRPV4 and causes inflammation and pain. Our results reveal that elastase-activated PAR2 robustly couples to Gαq, leading to a PKA-dependent activation of TRPV4 and hypersensitivity of nociceptive neurons, and PAR2- and TRPV4-mediated inflammatory edema and mechanical hyperalgesia.

**Experimental Procedures**

**Animals**—Institutional animal ethics committees approved all experiments. C57BL/6 mice, PAR2−/− and PAR2+/+ littermates (35) and TRPV4+/+/ and TRPV4−/− littermates (36) (8–12 weeks, male) were studied. Mice were maintained under temperature (22 ± 4 °C) and light- (12-h light/dark cycle) controlled conditions with free access to food and water. Oocytes were collected from Xenopus laevis as described (37).

**Materials**—Human sputum elastase was from SERVA Electrophoresis GmbH (10 units/mg) for oocyte experiments, and from Elastin Products Company Ltd. (864 units/mg) for other experiments. AlphaScreen SureFire phosphor-ERK and cAMP activity assays were from PerkinElmer Life Sciences Inc. Coelenterazine H was from Nanolight Technology, Prolume Ltd. Adenyl cyclase inhibitor SQ22536 and PKC inhibitor GF109203X were from Cayman Chemicals. Unless otherwise indicated, other reagents were from Sigma.

**Generation of cDNA Constructs, and Cell Culture**—Generation of human PAR2 and human TRPV4 constructs for expression in X. laevis oocytes have been described (38). PAR2 constructs for expression in mammalian cells have been described (9, 15). Human embryonic kidney (HEK) 293 cells and sarcoma virus-transformed rat kidney epithelial (KNRK) cells were maintained in DMEM with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin. Generation and maintenance of HEK293 and KNRK cells stably expressing human PAR2 constructs have been described (12, 15, 39, 40).

**Elastase Cleavage of N-terminal PAR2 Fragments**—Peptides corresponding to N-terminal fragments of human PAR2 (100 μM) were incubated with elastase (10 units/ml (390 nm)) in Hanks’ balanced salt solution, pH 7.4, for 1, 15, or 60 min at 37 °C. Reactions were quenched with an equal volume of 0.1% trifluoroacetic acid over 10 min. Products were identified by mass spectrometry using a Shimadzu LCMS 2000.

**Signaling Assays in Cell Lines**—For measurement of [Ca²⁺], KNRK-PAR2, or KNRK-empty vector control (VC) cells were seeded into 96-well poly-D-lysine-coated plates at a density of 30,000 cells/well 16–24 h before the assay. Cells were loaded with Fura-2/AM (1 μM) in assay buffer (150 mM NaCl, 2.6 mM KCl, 0.1 mM CaCl₂, 1.18 mM MgCl₂, 10 mM D-glucose, 10 mM HEPES, pH 7.4) containing 4 mM probenecid and 0.5% BSA for 1 h at 37 °C. Fluorescence was measured at 340 and 380 nm excitation and 530 nm emission using a FlexStation Microplate reader.
Elastase Activation of PAR2 and TRPV4

Reader (Molecular Devices). After a baseline reading for 60 s, cells were exposed to trypsin (1 × 10^{-4}–30 units/ml (1 × 10^{-12}–10^{-11} M)), elastase (1 × 10^{-4}–30 units/ml (1.2 × 10^{-11}–1.2 × 10^{-10} M)), or ATP (10 μM, positive control). Peak responses to each agonist were measured. For cAMP accumulation assays, CAMYEL BRET sensors were used (41). KNKPAR2 or KNKR-VC cells were plated into 10-cm dishes the day before transfection, and transfected with 4 μg of cDNA encoding CAMYEL sensor (YFP-Epac-RLuc). After 24 h, cells were seeded in poly-D-lysine-coated 96-well plates and cultured overnight. Medium was replaced with 0.5 ng of TRPV4 cRNA alone, or both TRPV4 and PAR2 cRNA, or GRK6-Venus, or PAR2 or KNRK-Venus (4 μg) using PEI in 10-cm dishes. After 24 h, cells were seeded into poly-D-lysine-coated 96-well plates and the ligand-induced BRET signal was measured as described above (41–43).

BRET Analysis of PAR2 Association with G Proteins—HEK293 cells were transiently transfected with PAR2-RLuc8 (1.8 μg), Gγ2-Venus (0.4 μg), Gβ1 (0.266 μg) and either Gαq or Gαs (0.266 μg) using PEI in 6-well dishes. After 24 h, cells were seeded into poly-D-lysine-coated 96-well plates and the ligand-induced BRET signal was measured as described above (41–43).

BRET Analysis of PAR2 Association with G蛋白质, β-Arrestins, and Plasma Membrane Proteins—HEK293 cells were transiently transfected with PAR2-RLuc8 (1 μg) and ORK2-Venus, ORK6-Venus, β-arrestin1-YFP, β-arrestin2-YFP, RIT-Venus, or KRas-Venus (4 μg) using PEI in 10-cm dishes. After 24 h, cells were seeded in 96-well plates. After 48 h, cells were equilibrated in Hanks’ balanced salt solution for 30 min at 37 °C, and the ligand-induced BRET signal was measured as described above.

Two-electrode Voltage Clamp Studies of X. laevis Oocytes—Oocytes were obtained from adult X. laevis as described (38). Defolliculated stage V–VI oocytes were injected (Nanoject II automatic injector, Drummond) with 0.5 ng of TRPV4 cRNA alone, 10 ng of PAR2 cRNA alone, or both TRPV4 and PAR2 cRNA. Oocytes were studied 2 days after injection using the two-electrode voltage-clamp technique as described (37, 38, 44). A Ca^{2+}-free solution was used to prevent activation of endogenous Ca^{2+}-activated chloride channels by TRPV4-mediated Ca^{2+} influx and to delay a Ca^{2+}-induced decay of TRPV4 current (31, 45). Oocytes were voltage-clamped at −60 mV. Oocytes were incubated with trypsin (2.48 units/ml (8 nM)), elastase (1 units/ml (3 μM)), or vehicle, and were then challenged with the TRPV4 agonist GSK1016790A (50 nM) and the TRPV4 antagonist HCO67047 (100 nM).

Signaling Assays in Neurons—Dorsal root ganglia (DRG) (C1-L5) from C57BL/6 wild-type, PAR2^{-/-} or Trpv4^{-/-} mice were isolated as described (31). Neurons were incubated in serum-free medium overnight before ERK1/2 and cAMP assays. For cAMP accumulation assays, neurons were preincubated with 3-isobutyl-1-methylxanthine (1 μM) for 45 min before exposure to agonists. Neurons were challenged with trypsin (10 units/ml (34.7 nM)), elastase (1 units/ml (39 nM) or 10 units/ml (390 nM)), forskolin (10 μM, cAMP positive control), or phorbol 12,13-dibutyrate (200 nM, ERK1/2 positive control) for 45 (cAMP assays) or 30 min (ERK1/2 assays) at 37 °C. cAMP accumulation was measured using AlphaScreen cAMP assay and ERK1/2 activity was measured using AlphaScreen SureFire phosphor-ERK assay (PerkinElmer Life Sciences). For measurement of [Ca^{2+}]_i, neurons were loaded with Fura-2/AM (2 μM) for 1.5 h at 37 °C, and the cells were observed using a Leica DMi600B microscope with a PL APO ×20 NA0.75 objective. Fluorescence was measured at 340 and 380 nm excitation with 530 nm emission using an Andor iXon 887 camera (Andor) and Meta-Fluor version 7.8.0 software (Molecular Devices). Neurons were challenged sequentially with either trypsin (10 units/ml (34.7 nM)) or elastase (10 units/ml (390 nM)), capsaicin (1 μM), and KCl (50 mM). In some experiments, neurons were assayed in Ca^{2+}-free buffer containing 2 mM EDTA. Neurons were also treated with inhibitors of PKA (PKI, 10 μM), adenylyl cyclase (SQ22536, 20 μM), PKC (GF109203X, 1 μM), or Rho kinase (Y-27632, 10 μM) (60 min preincubation and present through the experiments). Images were analyzed using a custom journal in MetaMorph version 7.8.2 software (Molecular Devices). A maximum intensity image was generated and projected through time to generate an image of all cells. Cells were segmented and binarized from this image using the Multi-Wavelength Cell Scoring module on the basis of size and fluorescence intensity. Neurons of interest (<25 μm diameter) were selected.

Hyperexcitability of Nociceptive Neurons—DRG (T9-T13) from C57BL/6 mice were dispersed and cultured overnight (31). Cells were preincubated with elastase (10 units/ml (390 nM)) for 1 h. The PKA inhibitors PKI (10 μM), Rho kinase inhibitor (Y-27632, 10 μM), or the PKC inhibitor GF109203X (1 μM) were applied 30 min before elastase. Perforated patch clamp recordings were made from small-diameter neurons (<30 pF capacitance) in current clamp mode at room temperature as described (31). Changes in excitability were quantified by measuring rheobase and numbers of action potentials discharged at twice rheobase.

Mechanical Hyperalgesia and Edema in Mice—For behavioral assessments, C57BL/6 wild-type, PAR2^{-/-} or Trpv4^{-/-} mice were acclimatized to the experimental room, restrained apparatus, and investigator for 2-h periods on 2 successive days before experiments, and the investigator was blinded to the experimental treatments. von Frey filaments were used to determine mechanical pain response as described (31). An increase in the filament stiffness required to induce paw withdrawal indicates mechanical analgesia, whereas a decrease in the filament stiffness required to induce withdrawal indicates mechanical hyperalgesia. To assess inflammatory edema of the paw, hind paw thickness was measured using digital calipers before and after treatments (46). Baseline von Frey scores were taken the day before the experiments. To examine the effects of elastase, mice were sedated with 5% isoflurane and elastase (100 units/ml (3.9 μM), 10 μl) or vehicle (0.9% NaCl, 10 μl) was injected subcutaneously into the plantar surface of one hind paw. Mechanical hyperalgesia and edema were measured between 30 and 240 min after intraplantar injections.

Statistical Analyses—Results are expressed as mean ± S.E. Differences between two groups were examined using unpaired t-tests. Differences between multiple groups were examined using ANOVA.
Elastase Activation of PAR₂ and TRPV4

Results

Elastase Cleaves PAR₂ at Ala⁶⁶↓Ser⁶⁷ and Ser⁶⁷↓Val⁶⁸—To identify the sites at which elastase cleaves human PAR₂, we incubated elastase (10 units/ml (390 nM)) with two synthetic peptides spanning the N terminus of PAR₂ including trypsin cleavage site (Arg³⁶↓Ser³⁷), cathepsin S cleavage site (Glu⁵⁶↓Thr⁵⁷), and the remaining extracellular domain up to the first transmembrane domain (residues 31–75) (Fig. 1A). Elastase rapidly cleaved PAR₂ peptide 2 (residues 61–75) but not PAR₂ peptide 1 (residues 31–60) (Fig. 1, B–D). We observed ~50% degradation of peptide 2 at 15 min and almost complete degradation after 60 min (Fig. 1D). The products that were identified by mass spectrometry are consistent with elastase cleaving human PAR₂ at Ala⁶⁶↓Ser⁶⁷ and Ser⁶⁷↓Val⁶⁸ (Fig. 1B). This finding is consistent with a previous report in which a lower concentration of elastase was used to cleave PAR₂ fragments (32).
Elastase Activation of PAR2 and TRPV4

Elastase Stimulates PAR2-dependent cAMP Accumulation but Not Ca2+ Mobilization—Elastase cleavage of PAR2 at Ser67/Val68 stimulates Gα12/13- and Rho kinase-dependent phosphorylation of ERK1/2 (32). Whether elastase activates other signaling pathways is uncertain. Cathepsin S, a lysosomal protease that is also a biased agonist of PAR2, stimulates cAMP-mediated cAMP accumulation (31). To investigate whether elastase also stimulates PAR2-dependent cAMP activation, we compared responses of KNRK cells expressing empty vector control (KNRK-VC) or human PAR2 (KNRK-hPAR2). As previously reported, trypsin stimulated a concentration-dependent increase in [Ca2+]i in KNRK-PAR2 cells but not KNRK-VC cells (12, 31) (Fig. 2, A and B). Conversely, elastase had no effect on [Ca2+]i at any concentration in both cell lines (Fig. 2, A and B).

We studied cAMP activation in response to trypsin or elastase in KNRK-VC and KNRK-PAR2 cells transiently transfected with a CAMYEL YFP-Epac-RFP cAMP sensor (41). Both trypsin and elastase stimulated a concentration-dependent increase in cAMP formation in KNRK-PAR2 cells but not KNRK-VC cells with similar potency (EC50: trypsin, 3.90 ± 0.22 units/ml (12.33 ± 0.22 nm); elastase, 2.13 ± 0.21 units/ml (80.90 ± 0.21 nm)) (Fig. 2, C and D). Thus, elastase stimulates PAR2-dependent cAMP accumulation but not Ca2+ mobilization in KNRK cells.

Elastase Induces PAR2 Coupling to Gαq but Not Gαi—cAMP formation may due to direct activation of Gαq, which activates adenylyl cyclase, or a result of indirect activation of other signaling pathways, such as the Gq pathway via activation of Ca2+-sensitive adenyllyl cyclases (47). As an indicator of direct activation of G proteins, we used BRET to examine the change in proximity between PAR2 and heterotrimeric G proteins in HEK293 cells (31). We transiently expressed PAR2-RLuc8 with Gβγ, Gly2-Venus and different Gα proteins, including Gαq and Gαo. Changes in BRET ratios between PAR2-RLuc8 and Gly2-Venus were monitored in cells treated with trypsin or elastase. Trypsin (10 units/ml (34.7 nM)) stimulated and increased the BRET signal in cells expressing either Gαq or Gαo (Fig. 3, A and B). In contrast, elastase (10 units/ml (390 nM)) only induced a change in BRET in cells expressing Gαq but not Gαo (Fig. 3, A and B). These findings are consistent with our signaling data showing that trypsin-activated PAR2 mobilizes Ca2+ and generates cAMP, whereas elastase-activated PAR2 generates cAMP but does not mobilize Ca2+. They suggest that elastase activates PAR2-dependent Gαq signaling that leads to cAMP formation. Notably, in Gαo-expressing cells, whereas trypsin increased BRET, elastase decreased BRET. This observation suggests that trypsin-activated PAR2 adopts a different conformation from elastase-activated PAR2 relative to G proteins. The Gγ2-Venus fluorescence was similar in all experiments, suggesting comparable levels of expression (Fig. 3C).

Elastase Recruits GRK6 but Not β-Arrestin to PAR2—Trypsin-activated PAR2 associates with β-arrestins, which mediate PAR2 desensitization and endocytosis (12, 15, 39). β-Arrestins also recruit Src and PAR2 to endosomes, where it assembles a signalosome that activates ERK1/2 in the cytosol (15). GRKs can phosphorylate activated GPCRs and thereby promote receptor interaction with β-arrestins (48). We examined whether elastase-biased agonism of PAR2 leads to differential Gαq and β-arrestin recruitment. We expressed in HEK293 cells PAR2-RLuc8, GRK2-Venus, GRK6-Venus, β-arrestin1-YFP, or β-arrestin2-YFP, and examined BRET after stimulation of cells with trypsin or elastase. Trypsin (10 units/ml (34.7 nM)) but not elastase (10 units/ml (390 nM)) increased BRET between PAR2-RLuc8, GRK2-Venus, β-arrestin1-YFP, and β-arrestin2-YFP (Fig. 4, A, C, and D). These results support the hypothesis that GRK2 phosphorylates trypsin-activated PAR2, which induces β-arrestin recruitment, receptor desensitization, and internalization. Although elastase failed to recruit GRK2 and β-arrestins to PAR2, both elastase and trypsin caused a decrease in BRET between PAR2-RLuc8 and GRK6-Venus (Fig. 4B). Although further studies are required to determine the importance of the potential association of PAR2 with GRK6, it is possible that GRK6 may play an important regulatory role for elastase-activated PAR2.

Previous studies using immunofluorescence and confocal microscopy suggest that whereas trypsin-activated PAR2 undergoes endocytosis, elastase-activated PAR2 remains at the plasma membrane (32, 49). To quantitatively assess PAR2 trafficking at the plasma membrane, we measured the BRET signal between PAR2-RLuc8 and two proteins that reside at the plasma membrane: KRas-Venus, which is present in cholesterol-independent microdomains (50), and RIT-Venus, which is uniformly distributed. Trypsin (10 units/ml (34.7 nM)) but not elastase (10 units/ml (390 nM)) induced a rapid decrease in BRET between PAR2-RLuc8 and both RIT-Venus and KRas-Venus (Fig. 4, D and E). These results are consistent with the
Elastase Activation of PAR<sub>2</sub> and TRPV4

FIGURE 3. PAR<sub>2</sub> and G protein associations. A and B, PAR<sub>2</sub>-RLuc8 and Gγ-Venus were co-expressed in HEK293 cells with Gβ<sub>2</sub> and various G<sub>α</sub> subunits. The effects of trypsin or elastase (arrow) on BRET ratios between PAR<sub>2</sub>-RLuc8 and Gγ-Venus in cells overexpressing G<sub>αq</sub> (A) or G<sub>αs</sub> (B) were measured. C, Gγ-Venus fluorescence levels were measured. Duplicate measurements of n = 4 experiments are shown.

FIGURE 4. PAR<sub>2</sub> association with GRKs, β-arrestins, and plasma membrane proteins. PAR<sub>2</sub>-RLuc8 was co-expressed in HEK293 cells with GRK2-Venus (A), GRK5-Venus (B), β-arrestin1-YFP (C), β-arrestin2-YFP (D), RIT-Venus (E), or Kras-Venus (F). The effects of trypsin or elastase (arrow) on BRET ratios were measured. Triplicate measurements of n = 3–4 experiments are shown.

observation that trypsin- but not elastase-activated PAR<sub>2</sub> couples to β-arrestins and internalizes.

Elastase Evokes PAR<sub>2</sub>-dependent Sensitization of TRPV4 Currents—After activation by canonical agonists such as trypsin, or biased agonists such as cathepsin S, PAR<sub>2</sub> activates and sensitizes TRPV4, which contributes to neurogenic inflammation and mechanical hyperalgesia (8, 31). To evaluate whether elastase-activated PAR<sub>2</sub> sensitizes TRPV4, we expressed TRPV4 alone or both PAR<sub>2</sub> and TRPV4 together in X. laevis oocytes, and measured whole cell currents using the two-electrode voltage-clamp technique. Preincubation of oocytes expressing PAR<sub>2</sub> and TRPV4 with elastase (1 units/ml (3 μM)) for 5 min resulted in a 6-fold increase in the response to the TRPV4-selective agonist GSK1016790A (50 nM) compared with vehicle (Fig. 5, A and B, second and fourth columns). In contrast, elastase did not affect the response to GSK1016790A in oocytes expressing TRPV4 but not PAR<sub>2</sub> (Fig. 5B, first and third columns). These results suggest that elastase induces a PAR<sub>2</sub>-dependent sensitization of TRPV4 in X. laevis oocytes, as we have previously described (38). To evaluate the mechanism of this sensitization, we preincubated oocytes with a PKC inhibitor GF109203X (1 μM), adenylyl cyclase inhibitor SQ22536 (20 μM), or PKA inhibitor PKI (10 μM). SQ22536 and PKI prevented elastase-induced and PAR<sub>2</sub>-mediated sensitization of TRPV4, whereas GF109203X partially inhibited sensitization (Fig. 5, A and B, fifth to seventh columns). All three inhibitors had no effect on GSK1016790A-stimulated TRPV4 currents in oocytes expressing TRPV4 alone (data not shown). In control experiments, elastase activation of the epithelial sodium channel was preserved in the presence of protein kinase inhibitors (data not shown). Thus, the inhibitory effect of the kinase inhibitors on the PAR<sub>2</sub>-mediated sensitization of TRPV4 by elastase is not due to an inhibition of enzymatic activity. These results suggest that adenylyl cyclase and PKA are critically important for elastase-stimulated and PAR<sub>2</sub>-dependent sensitization of TRPV4, and are consistent with the capacity of elastase-activated PAR<sub>2</sub> to couple to G<sub>αq</sub> and cAMP accumulation.

Because elastase cleaves PAR<sub>2</sub> distal from the trypsin site, elastase can remove the trypsin-exposed tethered ligand and disarm the receptor to subsequent activation by trypsin (32, 49). To determine whether elastase also disarms PAR<sub>2</sub> in oocytes, we expressed PAR<sub>2</sub> and examined trypsin-evoked PAR<sub>2</sub> activation of whole cell currents. In oocytes preincubated with vehicle, trypsin (2.48 units/ml (8 nM)) stimulated a transient inward current, consistent with the activation of Ca<sup>2+</sup>-sensitive Cl<sup>-</sup> channels (Fig. 5C). Non-injected oocytes showed no or minimal response to trypsin (38). Preincubation with elastase (1 units/ml (3 μM), 5 min) inhibited trypsin-evoked currents by...
Elastase Activation of PAR2 and TRPV4

In DRG from wild-type mice, trypsin (10 units/ml) and elastase (1 or 10 units/ml) stimulated ERK1/2 activation (Fig. 6A). Elastase robustly stimulated ERK1/2 activation to a similar degree at both 1 (39 nM) and 10 units/ml (390 nM). Elastase (10 units/ml) did not stimulate ERK1/2 activation in DRG from Par2−− mice, but instead inhibited ERK1/2 activation by mechanisms that remain to be elucidated (Fig. 6B). Similarly, trypsin and elastase (both 10 units/ml) stimulated cAMP accumulation in DRG from wild-type mice (Fig. 6C), and the stimulatory effect of elastase was not observed in DRG from Par2−− mice (Fig. 6D). These results suggest that elastase stimulates ERK1/2 activation and cAMP accumulation in DRG by a PAR2-dependent process, consistent with our current observations and with published studies showing similar stimulator actions of elastase in KNRK-PAR2 but not KNRK-VC cells (32).

To investigate whether elastase cleavage of PAR2 leads to activation of TRP channels, we measured [Ca2+] in small to medium diameter neurons. Elastase (10 units/ml) stimulated a rapid and sustained increase in [Ca2+] in neurons from wild-type mice (Fig. 7A). Responses to elastase were detected in ~50% of these neurons, similar to the proportion of neurons responding to trypsin (Fig. 7B). The magnitude of response to elastase was markedly diminished in neurons from Par2−− mice (Fig. 7C) and TRPV4−− mice (Fig. 7D). Fewer neurons from Par2−− mice and TRPV4−− mice responded to elastase (Fig. 7E). Removal of extracellular Ca2+ ions strongly inhibited the proportion of elastase-responsive neurons in wild-type mice (Fig. 7F). Our results suggest that elastase activates PAR2

FIGURE 6. Elastase-evoked ERK1/2 activation and cAMP accumulation in DRG. The effects of trypsin and elastase on ERK1/2 activation (A and B) and cAMP accumulation (C and D) in DRG from wild-type and Par2−− mice. *, p < 0.05; **, p < 0.01 unpaired t test (B and D) one-way analysis of variance (A and C) compared with control groups.

Elastase Activation of Endogenous PAR2 and TRPV4 in DRG Neurons—Proteases can activate PAR2 on nociceptive neurons to stimulate the release of neuropeptides in peripheral tissues, resulting in neurogenic inflammation, and in the dorsal horn of the spinal cord, leading to pain transmission (5, 6). PAR2 activates TRP channels, which amplify the proinflammatory and pro-nociceptive actions of proteases (8, 11, 13). We have previously reported that proteases that activate PAR2 by canonical mechanisms, such as trypsins and tryptase (6, 7), and biased mechanisms, including cathepsin S (31), can signal to nociceptors by activating PAR2 and TRP channels. To determine whether elastase similarly signals to nociceptors, we assessed cAMP accumulation, ERK1/2 activation, and [Ca2+] in mouse DRG neurons in short term culture.

~80% (Fig. 5C). Thus, elastase disarms PAR2 by removing the tethered ligand.

FIGURE 5. Elastase-induced sensitization of TRPV4 and disarming of PAR2. A and B, X. laevis oocytes co-expressing TRPV4 and PAR2, or expressing TRPV4 alone were preincubated with vehicle or elastase for 5 min. Elastase-treated oocytes were treated with vehicle or inhibitors of adenylyl cyclase (SQ22536), PKA (PKI), or PKC (GF109203X). Oocytes were treated with the TRPV4 agonist GSK1016790A (GSK) and the TRPV4 antagonist HC067047 (HC). The effects of elastase on TRPV4 currents were measured. A, representative traces from oocytes co-expressing TRPV4 alone or TRPV4 and PAR2. B, mean ΔGSK1016790A values of pooled data from oocytes expressing TRPV4 alone or TRPV4 and PAR2. C, oocytes expressing PAR2 were preincubated with vehicle (veh) or elastase for 5 min, washed (W), and trypsin-evoked whole cell currents were measured. Columns represent mean Δ current values. n indicates number of batches of oocytes measured. N indicates the number of individual oocytes measured. N indicates the number of batches of oocytes. ***, p < 0.001, unpaired t test; §§§, p < 0.0001, unpaired t test.

In DRG from wild-type mice, trypsin (10 units/ml) and elastase (1 or 10 units/ml) stimulated ERK1/2 activation (Fig. 6A). Elastase robustly stimulated ERK1/2 activation to a similar degree at both 1 (39 nM) and 10 units/ml (390 nM). Elastase (10 units/ml) did not stimulate ERK1/2 activation in DRG from Par2−− mice, but instead inhibited ERK1/2 activation by mechanisms that remain to be elucidated (Fig. 6B). Similarly, trypsin and elastase (both 10 units/ml) stimulated cAMP accumulation in DRG from wild-type mice (Fig. 6C), and the stimulatory effect of elastase was not observed in DRG from Par2−− mice (Fig. 6D). These results suggest that elastase stimulates ERK1/2 activation and cAMP accumulation in DRG by a PAR2-dependent process, consistent with our current observations and with published studies showing similar stimulator actions of elastase in KNRK-PAR2 but not KNRK-VC cells (32).
FIGURE 7. Elastase-evoked Ca\(^{2+}\) influx in DRG neurons. [Ca\(^{2+}\)]\(_i\) was measured in individual small to medium diameter DRG neurons. A, C, and D, representative traces from 15 to 20 individual neurons from wild-type (A), PAR\(_2^{-/-}\) (C), or Trpv4\(^{-/-}\) (D) mice challenged with elastase (arrow). B and E–H, proportion of neurons responding to elastase or trypsin from wild-type mice (B, F–H) or PAR\(_2^{-/-}\) or Trpv4\(^{-/-}\) mice (E). F, proportion of elastase-responsive neurons in 2 mM Ca\(^{2+}\)-free buffer. G, proportion of elastase-responsive neurons treated with vehicle, PKA inhibitor PKI, adenylyl cyclase inhibitor SQ22536, PKC inhibitor GF109203X or Rho kinase inhibitor Y27632. H, proportion of neurons responding to elastase with or without preincubation with elastase inhibitor elafin. *, p < 0.05; **, p < 0.01, unpaired t test (F and H), and one-way analysis of variance (E and G) compared with vehicle or wild-type groups.
on nociceptors, which in turn activates TRPV4, allowing the influx of extracellular Ca\(^{2+}\) ions. The residual responses in mice lacking PAR\(_2\) may be attributable to activation of other PARs, including PAR\(_1\), which is also expressed by nociceptors (51) and which responds to elastase (34), or due to elastase activation of ion channels, such as the epithelial sodium channel (52, 53). Residual responses in TRPV4-deficient mice may be due to PAR\(_2\)-dependent activation of other TRPs, such as TRPV1 or TRPA1 (7, 11).

To characterize the signaling mechanism underlying elastase-stimulated and PAR\(_2\)-dependent activation of TRPV4, we pretreated neurons with inhibitors of adenylyl cyclase, PKA, PKC, or Rho kinase. The adenylyl cyclase inhibitor SQ22536 (20 \(\mu\)M), the PKA inhibitor PKI (10 \(\mu\)M), and the Rho kinase inhibitor Y27632 (10 \(\mu\)M) all reduced the percentage of neurons responsive to elastase, whereas the PKC inhibitor GF109203X (1 \(\mu\)M) had no effect (Fig. 7G). These results suggest that elastase-cleaved PAR\(_2\) activates TRPV4 in nociceptors by G\(_{\alphaq}\), adenylyl cyclase-, and PKA-dependent mechanisms, as well as by a Rho kinase-dependent process. They are consistent with the role of adenylyl cyclase and PKA in mediating elastase-stimulated and PAR\(_2\)-dependent sensitization of TRPV4 in X. laevis oocytes. Our findings also agree with the reports that elastase-activated PAR\(_2\) stimulates Rho kinase (32), and that Rho kinase contributes to PAR\(_2\)-mediated TRPV4 sensitization in oocytes (38). Preincubation of elastase with its specific inhibitor elastatin (10 \(\mu\)M, 30 min) abolished elastase stimulation of [Ca\(^{2+}\)]\(_i\) in nociceptors, indicating a requirement for proteolytic activity (Fig. 7H).

Elastase Evoke PKA-dependent, and PKC- and Rho Kinase-independent Hyperexcitability of Nociceptive Neurons—Hyperexcitability of nociceptors can lead to exacerbated pain transmission. We have previously shown that canonical agonists, such as trypsin and tryptase (54, 55) and biased agonists, such as cathepsin S (31), cause hyperexcitability of DRG neurons from mice. To directly determine whether elastase induces hyperexcitability, we made patch clamp recordings from small diameter DRG neurons in short term culture. We assessed excitability by measuring the rheobase (minimum current required to fire a single action potential) and the action potential discharge frequency at twice rheobase. Preincubation with elastase (10 units/ml (390 nM), 1 h) resulted in a >50% reduction in rheobase, which is indicative of hyperexcitability (Fig. 8A, B and C). Elastase-evoked hyperexcitability was abolished by the PKA inhibitor PKI (10 \(\mu\)M), but unaffected by the PKC inhibitor GF109203X (1 \(\mu\)M) or the Rho kinase inhibitor Y-27632 (10 \(\mu\)M) (Fig. 8A, B and C). Elastase did not affect the frequency of action potential discharge at the current to twice rheobase (data not shown). Our results suggest that elastase causes hyperexcitability of nociceptive neurons by a PKA-dependent process, consistent with elastase stimulation of PAR\(_2\)-dependent cAMP accumulation in DRG.

Elastase Evoke Inflammation and Mechanical Hyperalgesia by Activating PAR\(_2\) and TRPV4—Both canonical agonists, such as trypsin and tryptase, and biased agonists, such as cathepsin S, can activate PAR\(_2\) and TRP channels at the periphery terminals of primary nociceptive neurons, which causes neurogenic inflammation and hyperalgesia (5–8, 31). We investigated whether elastase, as a biased agonist for PAR\(_2\), induces inflammation and pain by activating PAR\(_2\) and TRPV4. We administered elastase (100 units/ml (3.9 \(\mu\)M), 10 \(\mu\)l) to mice by intraplantar injection, and measured mechanical pain using calibrated von Frey filaments applied to the plantar surface of the paw, and assessed inflammatory edema by measuring paw thickness with calipers. In wild-type mice, elastase induced marked mechanical hyperalgesia that was sustained for at least 4 h (Fig. 9A). Elastase also induced a >3-fold increase in paw thickness that declined after 1 h but was sustained for at least 4 h (Fig. 9B). Deletion of Par2 attenuated elastase-evoked mechanical hyperalgesia and inflammatory edema. Deletion of Trpv4 attenuated elastase-induced inflammation to a similar degree as deletion of Par2. However, Trpv4 deletion caused a greater reduction in elastase-induced hyperalgesia than Par2 deletion. Thus, both PAR\(_2\) and TRPV4 contribute to elastase-evoked inflammation and pain. The residual pain and inflammation observed in Par\(_2^{-/-}\) and Trpv4\(^{-/-}\) mice may be attributable to elastase activation of other PARs or ion channels.

Discussion

We report that neutrophil elastase is a biased agonist of PAR\(_2\) that stimulates G\(_{\alphaq}\)-mediated cAMP accumulation and ERK1/2 activation in cell lines expressing human PAR\(_2\), and in DRG neurons from mice. In X. laevis oocytes and mouse nociceptive neurons, elastase activation of PAR\(_2\) sensitizes and activates TRPV4 by adenylyl cyclase- and PKA-dependent mechanisms. Elastase causes PKA-dependent hyperexcitability of nociceptors, and evokes PAR\(_2\)- and TRPV4-mediated mechanical hyperalgesia and inflammation.
Elastase Activation of PAR2 and TRPV4

**A**

- Elastase, wild-type (n=12)
- Elastase, PAR2+/− (n=8)
- Vehicle, wild-type (n=3)
- Elastase, Trpv4+/− (n=8)

**B**

- Elastase, wild-type (n=12)
- Elastase, PAR2+/− (n=8)
- Vehicle, wild-type (n=3)
- Elastase, Trpv4+/− (n=8)

**FIGURE 9.** Elastase-evoked and PAR2- and TRPV4-dependent inflammation and pain. Elastase or vehicle was administered by intraplantar injection to wild-type, PAR2+/−, and Trpv4+/− mice. A, mechanical hyperalgesia was measured using von Frey filaments. B, paw edema was assessed by measurement of paw thickness with calipers. **p < 0.05; **** p < 0.0001** two-way analysis of variance compared with vehicle. n = 3–12 mice.

**Elastase Is a Biased Agonist of PAR2 that Stimulates a Distinct Signaling Profile** — We observed that elastase-activated PAR2 generates a signaling profile that is distinctly different from that evoked by trypsin. By using BRET to examine the proximity of PAR2 to G\(_{\text{q}}\) in HEK293 cells, we found that elastase stimulated G\(_{\text{q}}\) but not G\(_{\text{q}}\)-dependent BRET, whereas trypsin stimulated a G\(_{\text{q}}\)- and G\(_{\text{q}}\)-dependent BRET. These results suggest that whereas elastase-activated PAR2 couples to G\(_{\text{q}}\) alone, trypsin-activated PAR2 couples to G\(_{\text{q}}\) and G\(_{\text{q}}\). Our BRET results are consistent with the observations that elastase stimulates accumulation of cAMP but not mobilization of intracellular Ca\(^{2+}\) in KNRK-PAR2 cells, whereas trypsin stimulates both cAMP accumulation and Ca\(^{2+}\) mobilization. These responses are PAR2-dependent, because elastase and trypsin did not affect cAMP or Ca\(^{2+}\) levels in KNRK-VC cells. Our results confirm the inability of elastase to mobilize intracellular Ca\(^{2+}\) in KNRK-PAR2 cells (32). Elastase also stimulates a PAR2-dependent activation of ERK1/2 in KNRK-PAR2 cells that is blocked by a Rho kinase inhibitor, suggesting a G\(_{\text{q}12/13}\)-mediated mechanism (32). Considered together, our findings are consistent with the proposal that elastase is a biased agonist of PAR2 that stimulates PAR2-coupling to G\(_{\text{q}}\), accumulation of cAMP, and activation of ERK1/2, but which is unable to evoke G\(_{\text{q}}\)-mediated Ca\(^{2+}\) signaling. In this regard, elastase resembles cathepsin S, another PAR2 biased agonist that selectively stimulates coupling to G\(_{\text{q}}\), cAMP accumulation, but not Ca\(^{2+}\) mobilization (31).

The structural basis that underlies the different signaling properties of trypsin-, elastase-, and cathepsin S-cleaved PAR2 remains to be determined. However, because these proteases cleave PAR2 at distinct sites, and promote activation by different mechanisms, differences in receptor conformation are likely to underlie these divergent signaling mechanisms. Whereas trypsin cleaves human PAR2 at Arg\(^{16}\) \(\downarrow\) Ser\(^{37}\) to reveal the tethered ligand SLIGKV (4), and cathepsin S cleaves at Glu\(^{56}\) \(\downarrow\) Thr\(^{57}\) to expose the tethered ligand TVFSVDEFSA, elastase acts distal to both the trypsin and cathepsin S sites at Ser\(^{67}\) \(\downarrow\) Val\(^{68}\) (32). Given the close proximity of the elastase site to the first transmembrane domain, it is not surprising that elastase activates PAR2 by a mechanism that does not involve exposure of a tethered ligand (32). The observation in G\(_{\text{q}}\)-expressing cells that elastase and trypsin induced opposite changes in BRET between PAR2-RLuc8 and G\(_{\text{q}}\)-Y Venus suggests that elastase-cleaved PAR2 adopts a different conformation from trypsin-cleaved PAR2 relative to G\(_{\text{q}}\). BRET analysis also suggest that cathepsin S-cleaved PAR2 adopts a different conformation from the trypsin-cleaved receptor (31). Whether these differences underlie the biased agonism of elastase and cathepsin S remains to be determined.

Elastase cleavage of PAR2 was first reported *in vitro* in *Escherichia coli* expressing the N-terminal domain of the receptor (56). Because elastase cleaves PAR2 distal to the trypsin cleavage site, elastase pretreatment can prevent subsequent activation by trypsin, and thereby “disarm” the receptor (49, 56). We found that pretreatment with elastase prevented trypsin signals in oocytes, which confirms this mechanism. Cathepsin S can similarly disarm PAR2 in both mammalian cells and oocytes (31).

**Regulation and Trafficking of Elastase-activated PAR2** — After activation by trypsin, PAR2 is phosphorylated by second messenger kinases and GRKs, and associates with β-arrestins, which mediate receptor desensitization and endocytosis (15, 39). We observed that trypsin stimulated a large and sustained increase in BRET between PAR2 and GRK2 and β-arrestins, consistent with GRK2 and β-arrestin recruitment. This recruitment coincided with a decreased BRET between PAR2 and the resident plasma membrane proteins RIT and K-Ras, suggesting trypsin-induced endocytosis of PAR2. Conversely, we observed that elastase-activated PAR2 was unable to recruit GRK2, interact with β-arrestins, or undergo endocytosis. The lack of GRK2 recruitment by elastase-activated PAR2 is consistent with the lack of β-arrestin recruitment, which is known to rely on GRK-mediated phosphorylation of agonist-occupied receptors (57). Our findings support a previous report that elastase-activated PAR2 is unable to recruit β-arrestins (32). Cathepsin S-activated PAR2 also fails to recruit β-arrestins and does not internalize (31). Further studies are required to define the mechanisms that regulate signaling of elastase- and cathepsin S-activated PAR2.

The inability of elastase to promote the recruitment of GRK2 and β-arrestins to PAR2 and to stimulate receptor endocytosis
Elastase Activation of PAR$_2$ and TRPV4

is likely to affect signaling of elastase-activated PAR$_2$. Trypsin-activated PAR$_2$ undergoes β-arrestin-dependent endocytosis in KNRK-PAR$_2$ cells. β-Arrestins assemble an endosomal signalingosome comprising PAR$_2$, Raf, and MEKK that is necessary for activation and cytosolic retention of ERK1/2 (15). A mutant PAR$_2$ that is unable to associate with β-arrestins and internalize activates nuclear ERK1/2 by a mechanism that involves trans-activation of the epidermal growth factor receptor. Because elastase-cleaved PAR$_2$ neither recruits β-arrestins nor internalizes, it may only activate nuclear ERK1/2 but not cytosolic ERK1/2. Further experiments will be required to investigate the mechanisms by which elastase-activated PAR$_2$ stimulates ERK1/2, and to determine the importance of PAR$_2$ trafficking for protease-evoked inflammation and pain.

Because elastase was unable to stimulate GRK2 translocation to PAR$_2$ at the plasma membrane, we investigated the possibility that elastase alters the potential association of PAR$_2$ with plasma membrane-localized GRKs. Of 7 known GRKs, GRK4, GRK5, and GRK6 are primarily localized to plasma membrane (58, 59), and GRK6 is expressed in spinal cord and enteric neurons that also express PAR$_2$ (60 – 62). Deletion of GRK6 leads to an increase in capsaicin-induced post-colitis hyperalgesia, suggesting that GRK6, like PAR$_2$, contributes to post-inflammatory pain (62). Here we show that both trypsin and elastase lead to a decrease in BRET between PAR$_2$ and GRK6. These data suggest that GRK6 contributes to PAR$_2$ regulation or signaling in response to proteases that activate the receptor by canonical and biased mechanisms. Because GRK6 is primarily located at the plasma membrane, a decrease in the BRET signal may reflect PAR$_2$ moving away from the membrane. However, this possibility is unlikely because elastase does not cause PAR$_2$ internalization, demonstrated by both immunofluorescence (32) and our BRET analysis of the association of PAR$_2$ with plasma membrane-resident proteins.

**Elastase Activates Nociceptive DRG Neurons, and Leads to PAR$_2$- and TRPV4-dependent Inflammation and Pain**—Our results show that elastase stimulates cAMP accumulation and ERK1/2 activation in DRG from wild-type but not Par$_2^{-/-}$ mice, which is consistent with our findings in KNRK-PAR$_2$ and KNRK-VC cells. However, in marked contrast to observations in KNRK-PAR$_2$ cells, in which elastase failed to increase [Ca$^{2+}$], elastase stimulated a robust increase in [Ca$^{2+}$], in DRG neurons from wild-type mice. Elastase-evoked Ca$^{2+}$ signals in neurons from wild-type mice were strongly inhibited by removal of extracellular Ca$^{2+}$ ions, and also suppressed by deletion of Par2 or Trpv4. These results are consistent with the proposal that elastase-activated PAR$_2$ triggers the activation of TRPV4, which allows influx of extracellular Ca$^{2+}$ ions. Residual responses to elastase in Par$_2^{-/-}$ and TRPV4$^{-/-}$ mice may be due to activation of other PARs and TRP channels.

We used pharmacological inhibitors to characterize the mechanism by which elastase-activated PAR$_2$ may stimulate TRPV4 in DRG neurons. Adenylyl cyclase, PKA, and PKC are the major effectors of Go$_q$ and Go$_q$ signaling. To investigate their involvement in elastase-mediated Ca$^{2+}$ signaling, we treated neurons with inhibitors of adenylyl cyclase, PKA, and PKC. Both adenylyl cyclase and PKA inhibitors attenuated elastase-evoked Ca$^{2+}$ influx. However, inhibition of PKC did not affect Ca$^{2+}$ influx. Considered together, these results suggest that in DRG neurons elastase-activated PAR$_2$ causes an adenyly cyclase- and PKA-dependent activation of TRPV4. They are consistent with the known involvement of PKA in regulating TRPV4 (63), and our observation that cathepsin S also causes a PKA-mediated activation of TRPV4 in DRG neurons (31). PAR$_2$ coupling to TRPV4 has been observed in multiple systems with a variety of PAR$_2$ agonists. However, the mechanism of coupling is agonist-dependent. Trypsin-activated PAR$_2$ stimulates TRPV4 in sensory neurons by PKC- and tyrosine kinase-dependent processes, and by the generation of arachidonic acid metabolites (8, 9). In contrast, diesel exhaust particles can activates the PAR$_2$-TRPV4 axis in airway epithelial cells by a G$_{q/o}$ and phosphatidylinositide 3-kinase-dependent mechanism (64).

Elastase also increased the excitability of DRG nociceptors, as revealed by a decrease in the input current required to fire action potentials. Although further studies are required to identify the mechanism of elastase-evoked neuronal sensitization, this sensitization was also suppressed by an inhibitor of PKA but unaffected by a PKC inhibitor, in line with our previous observations of cathepsin S-evoked neuronal hyperexcitability (31).

In X. laevis oocytes co-expressing PAR$_2$ and TRPV4, we observed that pretreatment with elastase caused a 6-fold increase in GSK1016790A-stimulated current, indicative of TRPV4 sensitization. This effect was not observed in oocytes expressing TRPV4 alone, and is thus PAR$_2$-dependent. Inhibitors of adenyly cyclase and PKA abolished elastase-evoked sensitization of TRPV4, which is consistent with observations in neurons. However, in oocytes and PKC the inhibitor also partially inhibited sensitization, whereas a PKC inhibitor had no effect on elastase-induced sensitization of TRPV4 in DRG neurons. Species differences may account for this discrepancy.

We found that intraplantar injection of elastase leads to sustained mechanical hyperalgesia and inflammatory edema. Deletion of Par$_2$ partly blocked elastase-mediated inflammation and pain, whereas deletion of Trpv4 strongly inhibited both pain and inflammation. The residual effects of elastase in Par$_2^{-/-}$ and Trpv4$^{-/-}$ mice may be attributable to activation of other PARs and TRP channels that are expressed by nociceptors.

Infiltration of neutrophils is an essential component of the host defense mechanism against invading pathogens during acute inflammation. Proteases such as elastase, proteinase 3, and cathepsin G, which are released from neutrophils during inflammation, mediate killing of invading microorganisms. Our results suggest that proteases such as elastase can also cleave PAR$_2$ and TRPV4 on sensory nerves to induce the acute neurogenic inflammation and pain, which may also serve as an acute protective mechanism.

**Acknowledgment**—We thank Cameron Nowell for technical assistance.

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