Cathepsin S Causes Inflammatory Pain via Biased Agonism of PAR2 and TRPV4*

Peishen Zhao†1, TinaMarie Lieu†1, Nicholas Barlow†1, Matthew Metcalf‡, Nicholas A. Veldhuis§, Dane D. Jensen‡, Martina Kocan†, Silvia Sostegni, Silke Haertes†, Vera Baraznenok¶, Ian Henderson†, Erik Lindström‡, Raquel Guerrero-Alba, Eduardo E. Valdez-Morales, Wolfgang Liedtke**, Peter McIntyre†‡, Stephen J. Vanner§, Christoph Korbmacher¶, and Nigel W. Bunnett†‡§¶¶2

From the †Monash Institute of Pharmaceutical Sciences, Parkville 3052, Australia, §Institut für Zelluläre und Molekulare Physiologie, Friedrich-Alexander-Universität Erlangen-Nürnberg, 91054 Erlangen, Germany, **Medivir AB, 141 Huddinge, Sweden, ‡Gastrointestinalal Disease Research Unit, Division of Gastroenterology, Queen’s University, Kingston, Ontario N6L 3N6, Canada, ¶¶ARC Centre of Excellence in Convergent Bio-Nano Science and Technology, Monash University, Parkville 3052, Australia

Background: Proteases trigger inflammation and pain by cleaving protease-activated receptors (PARs) at defined sites. Serine proteases such as trypsin and mast cell tryptase cleave PAR2 at R36=S37 and reveal a tethered ligand that excites nociceptors, causing neurogenic inflammation and pain. Whether proteases that cleave PAR2 at distinct sites are biased agonists that also induce inflammation and pain is unexplored. Cathepsin S (Cat-S) is a lysosomal cysteine protease of antigen-presenting cells that is secreted during antigen presentation and adaptive immunity (1). Inflammatory mediators also stimulate secretion of Cat-S from macrophages and microglial cells (2, 3), and increased Cat-S activity has been detected in inflamed tissues, including synovial fluid from patients with rheumatoid arthritis (4) and colonic secretions from mice with colitis (5). Since Cat-S retains activity at neutral pH (3), the secreted protease may contribute to disease processes, including pain and inflammation. Indeed, nerve injury leads to up-regulation of Cat-S in macrophages of dorsal root ganglia (5) and in microglial cells of the spinal cord (7). The peripheral or central administration of Cat-S causes allosdynia and hyperalgesia, and Cat-S inhibition or deletion attenuates neuropathic and inflammatory pain in rodents (5–7). In the spinal cord, Cat-S induces pain by an indirect mechanism: Cat-S released from microglial cells liberates membrane-tethered fractalkine from neurons, which activates CX3CR1 on microglial cells to trigger inflammatory signals that contribute to central sensitization of pain (1, 7, 8). The mechanisms by which Cat-S in the periphery causes pain and inflammation are unknown, and whether Cat-S can directly regulate neurons via specific receptors has not been studied.

Results: Cathepsin S (Cat-S) cleaved PAR2 at a unique site E56=T57, leading to Gαs-mediated cAMP accumulation and TRPV4-dependent inflammation and pain. Cat-S is a biased agonist of PAR2- and TRPV4-dependent inflammation and pain. Our results identify Cat-S as a biased agonist of PAR2 that causes PAR2- and TRPV4-dependent inflammation and pain. They expand the role of PAR2 as a mediator of protease-driven inflammatory pain.

Significance: PARs integrate responses to diverse proteases.

Cathepsin S (Cat-S)3 in lysosomes of macrophages, microglial cells, B-lymphocytes, and dendritic cells contributes to antigen presentation and adaptive immunity (1). Inflammatory mediators also stimulate secretion of Cat-S from macrophages and microglial cells (2, 3), and increased Cat-S activity has been detected in inflamed tissues, including synovial fluid from patients with rheumatoid arthritis (4) and colonic secretions from mice with colitis (5). Since Cat-S retains activity at neutral pH (3), the secreted protease may contribute to disease processes, including pain and inflammation. Indeed, nerve injury leads to up-regulation of Cat-S in macrophages of dorsal root ganglia (5) and in microglial cells of the spinal cord (7). The peripheral or central administration of Cat-S causes allosdynia and hyperalgesia, and Cat-S inhibition or deletion attenuates neuropathic and inflammatory pain in rodents (5–7). In the spinal cord, Cat-S induces pain by an indirect mechanism: Cat-S released from microglial cells liberates membrane-tethered fractalkine from neurons, which activates CX3CR1 on microglial cells to trigger inflammatory signals that contribute to central sensitization of pain (1, 7, 8). The mechanisms by which Cat-S in the periphery causes pain and inflammation are unknown, and whether Cat-S can directly regulate neurons via specific receptors has not been studied.

* This work was supported by NHMRC 63030, 1049682, 1031886, ARC Centre of Excellence in Convergent Bio-Nano Science and Technology and Monash University (to N. W. B.) and by a PhD fellowship from the Bayerische Forschungsstiftung (to S. S.).
† These authors contributed equally to this manuscript.
‡† The abbreviations used are: Cat-S, cathepsin S; PAR2, protease-activated receptor 2; DRG, dorsal root ganglia; GPCR, G protein-coupled receptor; TRP, transient receptor potential; ERK, extracellular signal-regulated kinase; MMP-1, matrix metalloprotease-1; APC, activated protein C; AP, activating peptide; BRET, bioluminescence resonance energy transfer.
We investigated whether Cat-S causes pain by cleaving protease-activated receptor-2 (PAR2), which is expressed by keratinocytes (9) and nociceptive neurons (10). PAR2 is a member of a family of four G-protein coupled receptors (GPCRs) with a unique mechanism of activation: proteases cleave within the extracellular N-terminal domains of PARs to reveal tethered ligands that bind to and activate the cleaved receptors (11). Trypsin cleaves human PAR2 at $\text{R}^{36} \downarrow \text{S}^{37}$ to expose the tethered ligand $\text{SLIGKV}$, and synthetic peptides that mimic this domain can directly activate the receptor (12, 13). Any protease that cleaves at this canonical site would be expected to trigger the same signaling events and patho-physiological outcome. Serine proteases that activate PAR2 include trypsin I/II (12, 13), trypsin IV (14, 15), trypstatase (16, 17), coagulation factors VIIa and Xa (18), acrosin (19), granzyme A (20), membrane-type serine protease 1 or matriptase (21), TMPRSS2 (22), and kalikrein 2, 4, 5, 6, and 14 (23–26). During injury and inflammation, these proteases can activate PAR2 on nociceptive neurons to stimulate $\text{Ca}^{2+}$-dependent release of neuropeptides that cause neurogenic inflammation (10). PAR2 can also sensitize transient receptor potential (TRP) ion channels, including TRP vanilloid 1 (TRPV1) (27), TRPV4 (28–30), and TRP ankyrin A1 (TRPA1) (31), leading to the release of neuropeptides in the dorsal horn of the spinal cord that induce pain transmission (32). Besides proteases that cleave the receptor at the canonical site, certain proteases cleave PAR2 at distinct sites to destroy or remove the tethered ligand domain. These cleavage events disarm the receptor by rendering it unable to respond to activating proteases. For example, elastase cleaves PAR2 at $\text{S}^{68} \downarrow \text{V}^{69}$, which removes the tethered ligand and thereby prevents trypsin-stimulated PAR2 signaling (33, 34). However, the patho-physiological relevance of this PAR2 disarming mechanism is uncertain.

We report that Cat-S, like elastase, cleaves PAR2 distal to the canonical trypsin site. Cleavage exposes a unique tethered ligand domain that induces distinct signaling events that sensitize TRPV4 and cause hyperexcitability of nociceptive neurons, which induce neurogenic inflammation and pain. This mechanism of biased agonism of GPCRs can explain how different endogenous ligands or drugs that interact with the same GPCR can activate divergent signaling pathways with unique outcomes (35). Proteases that cleave PARs at different sites may also act as biased agonists. Elastase cleavage of PAR2 at $\text{S}^{68} \downarrow \text{V}^{69}$ induces PAR2-dependent activation of extracellular signal regulated kinases 1/2 (ERK1/2) by a Rho-kinase dependent pathway (34) that is distinct from trypsin-induced MAPK activation that is mediated by $\beta$-arrestins (36). Potential biased agonists of PAR2 include elastase (37), matrix metalloprotease-1 (MMP-1) (38–40), and activated protein C (APC) (41, 42). However, although biased agonism is emerging as potential mechanism of PAR activation, the patho-physiological relevance of biased agonism is not fully understood, and nothing is known about the contribution of biased agonism for protease-induced inflammation and pain.

**EXPERIMENTAL PROCEDURES**

**Animals**—The Animal Ethics Committee of Monash University and Queen’s University approved procedures using mice. C57BL/6 mice, $\text{par2}^{-/-}$ and $\text{par2}^{+/+}$ littermates (43) and $\text{trpv4}^{+/+}$ and $\text{trpv4}^{-/-}$ littermates (44) (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 (45) and with approval of the animal welfare officer for the University of Erlangen-Nürnberg.

**Materials**—2-Fuoryl-LIGRLO-NH$_2$ and peptides corresponding to sequences of human PAR2 were from American Peptide Company, Inc. The Cat-S inhibitor MV026031 was from Medivir AB. K$_V$ values are human Cat-S 47 nM, mouse Cat-S 22 nM, human Cat-K 410 nM, mouse Cat-K 4,200 nM, human Cat-B and Cat-H >200,000 nM, human Cat-L 7,800 nM, and human Cat-V 2,600 nM. The PAR2 antagonist GB88 was a gift from the Ferring Research Institute. Anti-HA antibody was from Roche Applied Science. Monoclonal mouse antibody against FLAG sequence (DYKDDDDK) was generated by CSIRO. Goat anti-rat and anti-mouse IgG conjugated to Alexa Fluor 488 or 597 were from Invitrogen. Alpha Screen ERK1/2 activity kit was from PerkinElmer Life Sciences. Fluorogenic substrate for Cat-S (Acetyl-KQKLR-AMC) was from Bachem AG. Unless otherwise indicated, other reagents were from Sigma-Aldrich.

**Recombinant Human Cat-S**—Human pro-Cat-S with a hexahistidine tag was expressed in SF9 cells using a recombinant baculovirus. Medium was collected 3 days after infection, cleared by centrifugation (8000 × g, 1 h, 4 °C) and filtration (0.22 μm), and pro-Cat-S was purified by affinity chromatography over a 5-ml HiTrap Chelating HP column (GE Healthcare), charged with 0.2 mL cobalt sulfate. Pro-Cat-S was eluted in 0–0.15 M gradient of imidazole in buffer S (PBS, pH 7.4–7.5, supplemented with 0.36 M NaCl, 10% glycerol, and 0.2 mM PMSF). Pro-Cat-S was dialyzed overnight against buffer S, containing 2 mM EDTA. Pro-Cat-S was activated by incubation in activation buffer (NaOAc 0.1 M, NaCl 0.1 M, EDTA 5 mM, DTT 1 mM, pH 4.5) at 37 °C for 15 to 30 min (determined by measuring the time to peak activity). Cat-S was then buffer-exchanged into PBS (pH 7.4) using an Econo-Pac 10DG column (Bio-Rad). The active site concentration of Cat-S was determined by titration with E-64 (3-carboxy-trans-2,3-epoxypropyl-leucylamido(4-guanidino)butane) in a buffer of 0.1 M Na phosphate, 0.1 M NaCl, 0.1% PEG-4000, 1 mM DTT, pH 6.5 using 100 μM boc-Val-Leu-Lys-AMC (Bachem) as substrate. Fluorescence was measured at 390 nm excitation and 460 nm emission.

**Generation of cDNA Constructs, Transfections, and Cell Culture**—Human PAR2 cDNA with N-terminal Flag and C-terminal HA11 epitopes has been described (46). The Cat-S cleavage site of human PAR2 was mutated using QuickChange II Site-directed Mutagenesis Kit (Agilent Technologies). The primers were: g184a_t185g_g187c_a188c_c191a-sense: 5′-gca cat ccc acg tca ctg gaa aag gag tta caa gtc caa aag tct ttt ctg tgg atg-3′; antisense: 5′-aaa act cat cca cag aaa aga ctt ttg gac ttg-3′; primer 2: 5′-gca cat ccc acg tca ctg gaa aag gag tta caa gtc caa aag tct ttt ctg tgg atg-3′; antisense: 5′-aaa act cat cca cag aaa aga ctt ttg gac ttg-3′.

Human embryonic kidney (HEK) 293 and KNRK (rat sarcoma virus transformed kidney epithelial) 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 has been described (12, 36, 46, 47).
46, 47). HEK293 and KNRK cells were transiently transfected with PAR2 constructs as described (29, 36). Cells were co-transfected with PAR2 constructs and GFP to identify transiently transfected cells for measurements of [Ca^{2+}], in individual cells. HEK-Flp-In TREX-TRPV4 cells were generated and maintained as reported, and cells were incubated with tetracycline (100 ng/ml) for 16 h before study to induce TRPV4 expression (48). Cells were plated in poly-D-lysine-coated 96-well plates (BRET, Ca^{2+}, cAMP, ERK1/2 assays) or glass coverslips (microscopy, single cell Ca^{2+} assays) for 16–48 h before assays.

**Cat-S Degradation of N-terminal PAR2 Peptides—Peptides corresponding to N-terminal fragments of human PAR2 (320–370 μM) were incubated with Cat-S (10 nM) in Hank’s Balanced Salt Solution (HBSS) pH 7.4 for 0, 5, 30, or 60 min at 37 °C. Reactions were quenched with equal volume of 50% acetonitrile and 0.1% trifluoroacetic acid in H2O. Degradation was assessed using an Agilent 1260 Infinity HPLC System with Poroshell 120, SB-C18, 2.1 × 30 mm, 2.7 μm column, 5–95% acetonitrile in water over 9 min, 0.1% TFA throughout. The reaction products were identified by mass spectrometry using a Shimadzu LCMS 2020, single quadrupole in electrospray in positive ionization mode with a mass range of 200–2000 m/z.

**Cat-S Cleavage of Cell Surface PAR2—HEK293 cells stably expressing human PAR2 with N-terminal extracellular Flag and C-terminal intracellular HA11 epitopes were equilibrated in HBSS for 30 min, incubated with trypsin (100 nM), 2-furolyl-LIGRLO-NH2 (10 μM), Cat-S (100 nM), Cat-S-AP (50 μM), or vehicle (control) for 30 min, and fixed with 4% paraformaldehyde (4 °C, 20 min). Cells were incubated in PBS containing 1% horse serum with mouse anti-Flag (2.5 μg/ml) and rat anti-HA (1:1000) antibodies (4 °C, overnight), washed and then incubated with Alexa-488 goat anti-mouse IgG and Alexa-597 goat anti-rat IgG secondary antibodies (1:1000, 1 h, room temperature). Images were obtained with Leica TCS SP8 Laser-scanning Confocal Microscope using a HCX PL APO 63 oil immersion objective.

**BRET Analysis of PAR2 Association with Heterotrimeric G Proteins and β-Arrestins—PAR2, G protein, and β-arrestin BRET was analyzed as described (48–50). For PAR2 and G protein BRET, HEK-FT cells were transiently transfected with PAR2-RLuc8 (0.18 μg), Gα2-Venus (0.4 μg), Gβ1 (0.266 μg), and either Goq or Goss (0.266 μg) and using GeneJuice (Novagen). Go was omitted from controls. For PAR2 and β-arrestin BRET, HEK293 cells were transiently transfected with PAR2-RLuc8 (1 μg) and β-arrestin1-YFP or β-arrestin2-YFP (4 μg). At 24 h after transfection, cells were seeded in 96-well plates. After 48 h, cells were equilibrated in HBSS for 30 min at 37 °C, and then incubated with the luciferase substrate coelenterazine H (Promega; 5 μM final) for 3 min. RLuc8 luminescence (480 nm) and Venus/YFP fluorescence (530 nm) were measured for a 2 min baseline and at various times after incubation with trypsin, Cat-S or Cat-S AP using LUMistar Omega (BMG Labtech).

**Signaling Assays in Cell Lines—For measurement of [Ca^{2+}], 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 probrucinid and 0.5% BSA for 1 h at 37 °C. Measurement of [Ca^{2+}], in cell populations, fluorescence was measured at 340 nm and 380 nm excitation and 530 nm emission using a FlexStation Microplate Reader (Molecular Devices). After a baseline reading for 60 s, cells were exposed to graded concentrations of trypsin or Cat-S, followed by ionomycin (10 μM) as a positive control. To examine Cat-S disarming of PAR2, HEK293 cells were pre-incubated with vehicle (control) or Cat-S (100 nM) for 30 min, washed three times with assay buffer, and then challenged with trypsin (100 nM). For measurement of [Ca^{2+}], in individual cells, cells were mounted in an open chamber and were observed using a Leica DMi6000B microscope with a HC PL APO 20× NA0.75 objective. Fluorescence was measured at 340 nm and 380 nm excitation with 530 nm emission using an Andor iXon 887 camera (Andor) and MetaFluor v7.8.0 software (Molecular Devices). Results are normalized to the ionomycin response or are expressed as change from basal in 340/380 nm ratio. cAMP accumulation was measured using the CAMYEL BRET sensor (48). HEK-PAR2, KNRK-PAR2, or KNRK-VC cells were transfected with 4 μg of cDNA encoding CAMYEL sensor (YFP-Epac-RLuc). In some experiments, KNRK cells were co-transfected with CAMYEL sensor plus 2 μg of wild-type PAR2, a mutant of the Cat-S cleavage site (KNRK-PAR2ΔV55S/E56P/T57K), or empty vector control pcDNA3.1. After 24 h, cells were seeded in 96-well plates and incubated overnight. Medium was replaced with HBSS 30 min before assays. Cells were loaded with coelenterazine H and BRET was measured as described above. After a 2-min basal period, cells were challenged with graded concentrations of trypsin, Cat-S, or Cat-S AP. Forskolin (10 μM) was used as a positive control. For assays of ERK activity, KNRK-PAR2 cells were incubated in serum-free medium overnight. Cells were challenged at 37 °C with trypsin (100 nM) or Cat-S (100 nM) for 0–60 min for time course measurements, or with graded concentrations of trypsin or Cat-S for 5 min to generate concentration response curves. ERK1/2 activity was measured using AlphaScreen SureFire phosphor-ERK assay (PerkinElmer Life Sciences). FBS (10%) was used as a positive control.

**Signaling Assays in Neurons—DRG (C1-L5) from C57BL/6 wild-type, par2<sup>−/−</sup> or trpv4<sup>−/−</sup> mice were dispersed by incubation in collagenase (2 mg/ml, InVitrogen) and dispase (2 mg/ml, Roche) for 30 min at 37 °C, triturated with a fire-polished Pasteur pipette, and incubated for an additional 20 min at 37 °C, triturated again, and incubated for an additional 10 min at 37 °C. Neurons were plated onto coverslips coated with laminin (0.004 mg/ml) and poly-d-lysine (0.1 mg/ml) in 12-well plates. Neurons were cultured in L-15 Lebovitz medium containing 10% fetal calf serum, with penicillin and streptomycin and maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO2 until retrieval (16 h) for signaling assays. For measurement of cAMP accumulation, neurons were preincubated with 3-isobutyl-1-methylxanthine (1 mM) for 45 min before assays. For assays of ERK activity, neurons were incubated in serum-free medium overnight. Neurons were challenged with trypsin (100 nM), Cat-S (100 nM), forskolin (10 μM, positive cAMP control), or phorbol 12,13-dibutyrate (200 nM, positive ERK1/2 control) for 30 min (cAMP assays) or 20 min (ERK1/2 assays) at 37 °C. cAMP accumulation was measured using AlphaScreen cAMP assay and ERK1/2 activity was measured using Cathepsin S Biased Agonism of PAR2

SEPTEMBER 26, 2014 • VOLUME 289 • NUMBER 39
AlphaScreen SureFire phosphor-ERK assay (PerkinElmer Life Sciences). For measurement of \([\text{Ca}^{2+}]_i\), neurons were loaded with Fura-2/AM (2 \mu M) and fluorescence was measured in individual neurons as described for cell lines. Neurons were challenged sequentially with either trypsin (100 nM) or Cat-S (100 nM), capsaicin (1 \mu M), 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 \mu M), adenylyl cyclase (SQ22536, 20 \mu M) or a TRPV4 antagonist (10 \mu M) (60-min preincubation). Images were analyzed using a custom journal in MetaMorph v7.8.2 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 \mu m diameter) were selected.

**Cat-S Disarming of PAR\(_2\) and Sensitization of TRPV4 in Xenopus Laevis Oocytes**—Linearized plasmids were used as templates for cRNA synthesis (mMessage mMachine; Ambion) using T7 as promoter. Defolliculated stage V–VI oocytes were injected with cRNA encoding human PAR\(_2\) alone (10 ng), human TRPV4 alone (0.5 ng) or both PAR2 (10 ng) plus TRPV4 (0.5 ng). The cRNAs were dissolved in RNase-free water and the total volume injected was 46 nl. Injected oocytes were stored at 19 °C in ND96 solution (in mM: NaCl 96, KCl 2, MgCl\(_2\) 1, HEPES 5, pH 7.4 with NaOH) at room temperature. To examine disarming of PAR\(_2\), oocytes expressing PAR\(_2\) alone were pre-incubated with Cat-S (1 \mu M), Cat-S AP (50 \mu M), trypsin (8 \mu M), or vehicle (control) for 5 min, and whole-cell currents were measured after challenge with trypsin (8 \mu M). To examine PAR\(_2\)-dependent sensitization of TRPV4, oocytes expressing TRPV4 alone or TRPV4 plus PAR\(_2\) were pre-incubated with trypsin (8 \mu M), Cat-S (1 \mu M) or Cat-S AP (50 \mu M) for 5 min, and whole-cell currents were measured after challenge with GSK1016790A (50 \mu M) and HC067047 (100 \mu M). All recordings were obtained at a holding potential of −60 mV. Downward deflections in the current traces correspond to inward currents (i.e. movement of positive charge into the cell). Recordings were obtained using an OC-725C amplifier (Warner Instruments Corp.) and were analyzed using PULSE 8.67 software (HEKA).

**Cat-S Sensitization of TRPV4 in HEK Cells**—[Ca\(^{2+}\)_i] was measured in individual HEK-TRPV4 cells as described above. Cells were pre-incubated with Cat-S (100 nM), trypsin (100 nM), or vehicle for 5 min, and then challenged with GSK1016790A (100 pM). The maximal increase in [Ca\(^{2+}\)_i], above basal within 15 min of challenge with GSK1016990A was determined.

**Cat-S hyperexcitability of Nociceptive Neurons**—DRG (T9–T13) from C57BL/6 mice were dispersed by incubation in collagenase (1 mg/ml, Worthington) and dispase (4 mg/ml, Roche) for 10 min at 37 °C, triturated with a fire-polished Pasteur pipette, and incubated for an additional 5 min at 37 °C. Neurons were plated onto coverslips coated with laminin (0.017 mg/ml) and poly-d-lysine (2 mg/ml) in 24-well plates. Neurons were cultured in F12 medium containing 10% fetal calf serum, with penicillin and streptomycin and maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO\(_2\) until retrieval (16 h) for electrophysiological studies. Cells were pre-incubated with Cat-S (500 nM) for 1 h. The PKA inhibitors PKI (10 \mu M) or H-89 (10 \mu M), the adenyl cyclase inhibitor SQ22536 (20 \mu M) or the PKC inhibitor GF-109203X (10 \mu M) were applied 30 min before Cat-S. Perforated patch-clamp recordings were made using amphotericin B (240 g/ml, Sigma Aldrich) from small-diameter neurons (<30 pF capacitance) in current clamp mode at room temperature. Changes in excitability were quantified by measuring rheobase and numbers of action potentials discharged at twice rheobase. Recordings were made using Multi-clamp 700B or Axopatch 200B amplifiers, digitized by Digidata 1440A or 1322A and stored and processed using pClamp 10.1 software (Molecular Devices). The recording chamber was continuously perfused with external solution at 2 ml/min. Solutions has the following composition (mm): pipette solution: K-glucanote 110, KCl 30, HEPES 10, MgCl\(_2\) 1, CaCl\(_2\) 2; pH 7.5 with 1 M KOH; external solution - NaCl 140, KCl 5 HEPES 10, glucose 10, MgCl\(_2\) 1, CaCl\(_2\) 2; pH to 7.3 to 7.4 with 3 M NaOH.

**Mechanical Hyperalgesia and Edema in Mice**—For behavioral assessments, mice were placed in individual cylinders on a mesh stand. Mice were acclimatized to the experimental room, restraint apparatus, and investigator for 2-h periods on 2 successive days before experiments, and the investigator was blinded to the experimental treatments. To assess mechanical pain, paw withdrawal in response to stimulation of the plantar surface of the hind paw with graded von Frey filaments (0.078, 0.196, 0.392, 0.686, 1.569, 3.922, 5.882, 9.804, 13.725, and 19.608 mN) was determined using the “up-and-down” paradigm (51). In this analysis, 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 (52). On the day before the study, von Frey scores were measured in triplicate to establish a baseline for each animal. To examine the effects of Cat-S, mice were sedated with 5% isoflurane and Cat-S (1.4–14 \mu M, 10 \mu l) or vehicle (0.9% NaCl, 10 \mu l) was injected subcutaneously into the plantar surface of one hind paw. To evaluate the contribution of cAMP to Cat-S evoked pain, the adenyl cyclase inhibitor SQ22536 (1 \mu g in 2.5 \mu M in 10 \mu l) or vehicle (0.9% NaCl) was injected into the paw 30 min before Cat-S (2.5 \mu M, 10 \mu l). Injection of SQ22536 or vehicle was preceded by an injection of 2.5 \mu l distilled water to cause a transient hyposmotic permeabilization. To investigate the contribution of Cat-S and PAR\(_2\) in formalin-induced pain and inflammation, mice were pre-treated with Cat-S inhibitor MV026031 (50 mg/kg), PAR\(_2\) antagonist GB88 (10 mg/kg) or vehicle by gavage. Formalin (4%, 10 \mu l) or NaCl (0.9%, 10 \mu l) was injected into the plantar surface of the hindpaw 2 h later. Mechanical hyperalgesia and edema were measured between 30–240 min after intraplantar injections. Afterward, the paws were collected for Cat-S activity assays.
Cat-S Activity Assays—Paws were removed 4 h after formalin or saline injection. The skin of the pad was excised, homogenized, and sonicated in HBSS, and centrifuged (20,000 × g, 15 min, 4 °C). Supernatants (10 μg protein) were incubated with 50 μM Cat-S substrate Acetyl-KQKLR-AMC in the presence or absence of Cat-S inhibitor MV026031 (1 μM) for 30 min at 37 °C. Fluorescence was measured at 354 nm excitation and 442 nm emission. Activity that was susceptible to inhibition by MV026031 was attributed to Cat-S and results are expressed as fold-change over saline-treated tissues.

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 an ANOVA and a Bonferroni’s or Dunnett’s post-hoc test. A p value <0.05 was considered to be significant.

RESULTS

Cat-S Cleaves within the Extracellular N Terminus of Human PAR2 at E56 ▼ T57—To determine whether Cat-S can cleave PAR2, we incubated recombinant human Cat-S (10 nM) with three 30 residue peptides (320–370 μM) corresponding to most of the amino terminus of PAR2 residues 21–90 and examined degradation by high pressure liquid chromatography (HPLC) and mass spectrometry (Fig. 1A). Cat-S rapidly degraded the PAR2 31–60 fragment, with detectable degradation after 5 min and ~50% degradation after 60 min (Fig. 1B). Two products were identified, corresponding to PAR2 31–56 and PAR2 57–60. These findings indicate that Cat-S hydrolyzes PAR2 31–60 at E56 ▼ T57, 20 residues downstream of the canonical trypsin cleavage site R56 ▼ S57. Cat-S did not degrade PAR2 21–50 after 60 min (Fig. 1C), and there was minimal degradation of PAR2 61–90 after 60 min (Fig. 1D). Thus, Cat-S cleaves at a major single site within the extracellular N terminus of human PAR2; E56 ▼ T57.

Cat-S Cleaves PAR2 Expressed in HEK Cells but Does Not Stimulate Receptor Endocytosis—Although Cat-S can hydrolyze a synthetic fragment of PAR2, steric restrictions or the presence of other components in the plasma membrane could affect cleavage of the intact receptor in cells. To assess whether Cat-S can cleave the PAR2 at the plasma membrane, we expressed in HEK293 cells human PAR2 with an extracellular N-terminal Flag epitope and an intracellular C-terminal HA11 epitope (Fig. 2A). We exposed the cells to vehicle, proteases or synthetic PAR2 agonists, and localized Flag and HA11 epitopes by immunofluorescence and confocal microscopy. In cells treated with vehicle, Flag and HA11 remained at the plasma membrane (Fig. 2B). After incubation with trypsin (100 nM, 30 min), Flag was removed from the cell surface and HA11 was detected in endosomes (Fig. 2C). This result is consistent with trypsin cleavage of PAR2, which would remove the Flag epitope, expose the canonical tethered ligand, and activate and internalize the cleaved receptor, as we have previously described (46, 47). After incubation with 2-furoly-LIGRLO-NH2 (10 μM, 30 min), a synthetic analog of the trypsin-revealed tethered ligand and a potent PAR2 agonist (53), both Flag and HA11 were colocalized in endosomes, consistent with receptor activation and endocytosis without cleavage (Fig. 2C). After incubation with Cat-S (100 nM, 30 min), Flag was removed yet HA11 remained at the cell surface (Fig. 2D). Cat-S cleavage of PAR2 at E56 ▼ T57 would expose a potential tethered ligand domain beginning T57TVFSV. Incubation of cells with the decapeptide TVFSVDEFSVA (50 μM, 30 min), which corresponds to the putative tethered ligand and is hereafter referred to as Cat-S activating peptide (AP), did not affect the subcellular localization of Flag or HA11, which remained at the plasma membrane (Fig. 2D). Thus, trypsin cleaves and activates PAR2, which results in receptor endocytosis. Although Cat-S cleaves PAR2, it does not trigger receptor endocytosis. Whereas the trypsin-revealed AP stimulates PAR2 endocytosis, Cat-S AP is unable to internalize this receptor.

Cat-S Removes the Canonical Trypsin Cleavage Site and the Tryptsin-exposed Tethered Ligand and Prevents Trypsin-induced Activation of PAR2—Cat-S cleavage of PAR2 at E56 ▼ T57 would be expected to remove the trypsin-exposed tethered ligand domain (T57SLIGKV42), which is upstream of the Cat-S site, and thereby prevent trypsin-induced activation of this receptor, as we have previously reported for elastase (33, 46). To examine this possibility, we pre-incubated HEK293 cells, which express endogenous PAR2, with vehicle (control) or Cat-S (100 nM) for 30 min. Cells were washed and trypsin (100 nM)-evoked increases in [Ca2+]i were measured to assess PAR2 activation (Fig. 3A). In cells treated with vehicle, trypsin caused a rapid and transient increase in [Ca2+]i (Fig. 3B). Pre-incubation with Cat-S caused a >2-fold reduction in the response to trypsin (Fig. 3, B and C). Since HEK cells also express PAR1, which can also be activated by trypsin (15), we confirmed the specific attenuation of PAR2 activation in Xenopus laevis oocytes. We expressed PAR2 in oocytes and examined trypsin-evoked PAR2 activation by measurement of whole-cell currents using the two-electrode voltage-clamp technique. In oocytes pre-incubated with vehicle, trypsin (8 nM) stimulated a transient inward current, consistent with the activation of Ca2+-sensitive Cl− channels (Fig. 3, D–F). There was no or minimal response to trypsin in non-injected oocytes, which indicates the requirement of PAR2 expression for the trypsin response (not shown). Pre-incubation with trypsin (8 nM, 5 min), abolished the response to subsequent trypsin challenge (Fig. 3, E and F), consistent with PAR2 cleavage, exposure of the tethered ligand, and desensitization (54). Pre-incubation with Cat-S (1 μM, 5 min) suppressed trypsin-evoked currents by >3-fold (Fig. 3, E and F). Our results suggest that Cat-S removes the canonical trypsin cleavage site and trypsin-exposed tethered ligand and thereby disarms PAR2. Notably, pre-incubation of oocytes with Cat-S AP (50 μM, 5 min) also inhibited trypsin-evoked currents by >2-fold (Fig. 3, E and F). A possible explanation for this effect is that Cat-S AP can activate PAR2 and thereby desensitize responses to trypsin PAR2, as is the case for the trypsin-exposed AP, which desensitizes PAR2 (54).

Cat-S Stimulates PAR2 Coupling to Gα but Not Goq—After interaction with agonists, GPCRs adopt conformational changes that facilitate coupling to heterotrimeric G-proteins, which initiate intracellular signaling events. Trypsin cleavage of PAR2 leads to mobilization of intracellular Ca2+, which is consistent with PAR2 coupling to Goq, activation of phospholipase Cβ and formation of inositol trisphosphate. To determine whether Cat-S also induces coupling of PAR2 to heterotrimeric
G proteins, we used bioluminescence resonance energy transfer (BRET) to examine changes in conformation/proximity between PAR₂ and G₁₁₂₅ in response to different ligands (Fig. 4A). We expressed in HEK293 cells PAR₂ with a C-terminal RLuc8 together with G₁₁₂₅₂-Venus, G₁₁₂₅₁ and various Gα subunits. By expressing either Gαq or Gαs, we were able to assess PAR₂ coupling to different Gα subunits, as described for other GPCRs (49). Thus, a change in the agonist-induced BRET signals (increase or decrease) in cells overexpressing Gα subunits would suggest a conformational change in the receptor that favors coupling to the Gα subunits in question. Cells not transfected with Gα were used as a control. In control cells, trypsin (100 nM), Cat-S (100 nM), or Cat-S AP (10 μM) did not affect the BRET signal between PAR₂-RLuc8 and G₁₁₂₅₂-Venus (Fig. 4B). In cells overexpressing Gαq, trypsin caused a rapid increase in BRET that was maximal at 6 min, sustained for 8 min, and declined to baseline after 10 min (Fig. 4C). This finding suggests that trypsin induces a conformational change in PAR₂ that facilitates coupling to Gαq, which would lead to the expected mobilization of intracellular Ca²⁺ stores. In contrast, Cat-S did

**FIGURE 1.** Cat-S degradation of N-terminal fragments of human PAR₂. A, N terminus of human PAR₂ showing the sequences of the synthetic peptides that were used for degradation studies. The residues in red denote the canonical tethered ligand and a corresponding AP that is revealed after trypsin cleavage. The residues in blue denote the presumed tethered ligand and a corresponding AP that would be revealed after Cat-S cleavage. Gray shading represents membrane. B, Cat-S degradation of PAR₂ 31–60. HPLC traces (left) show elution of the substrate and products (P) after incubation with Cat-S for 0 min or 60 min. Time course (right) shows the kinetics of substrate degradation and product formation. Analysis of the products is consistent with Cat-S cleaving human PAR₂ at E₁₅₆-T₁₅₇. C, time course of levels of PAR₂ 21–50 after incubation with Cat-S, showing no detectable degradation. D, time course of levels of PAR₂ 61–90 after incubation with Cat-S, showing minimal degradation and the appearance of 4 minor products that were not identified. n = 3 experiments.
not induce BRET in cells overexpressing Gaq. In cells overexpressing Gαs, both Cat-S and trypsin caused a change in BRET that was maximal after 5 min, but in opposite directions, and BRET returned to baseline after 10 min (Fig. 4D). These results suggest that Cat-S- and trypsin-activated PAR2 couples to Gαs, which would be expected to activate adenylyl cyclase and generate cAMP. The opposite direction of BRET signal suggests that Cat-S and trypsin induce distinct conformational changes in PAR2. Cat-S AP also caused a large change in BRET in Gαs-transfected cells, but here the BRET signal rapidly declined (Fig. 4E). Considered together, our results suggest that Cat-S couples to Gaq and mobilization of intracellular Ca^{2+}, to Gαs and gener-

**FIGURE 2.** Cat-S removal of extracellular epitope of human PAR2. A, human PAR2 with N-terminal Flag and C-terminal HA11 epitopes that was expressed in HEK cells. B–D, confocal photomicrographs showing immunoreactive Flag (green) and HA11 (red) epitopes after incubation with vehicle (B), trypsin (100 nM) or 2-furoyl-LIGRLO-NH₂ (10 μM) (C), or Cat-S (100 nM) or TVFSVDEFSA (50 μM) (D). Arrowheads show plasma membrane and arrows show endosomes. Scale bar, 10 μm. Representative images from three experiments.

**FIGURE 3.** Cat-S removal of the canonical tethered ligand and inhibition of trypsin-evoked activation of PAR2. A, HEK293 cells were pre-incubated with vehicle (control) or Cat-S (100 nM) for 30 min, washed and then challenged with trypsin (arrow, 100 nM). Changes in [Ca^{2+}] were measured using Fura-2/AM. B, trypsin responses in cells pre-incubated with vehicle or Cat-S (n = 3). C, maximal trypsin responses in cells pre-incubated with vehicle or Cat-S (n = 3). *, p < 0.05 compared with vehicle. D, *Xenopus laevis* oocytes expressing PAR2 were pre-incubated with vehicle (control), trypsin (8 nM), Cat-S (1 μM), or Cat-S AP (50 μM) for 5 min. After pre-incubation, trypsin (8 nM)-evoked whole-cell currents (black bar) were measured. E, representative whole-cell current traces. F, mean Δ_{trypsin} values of pooled experiments. n indicates number of individual oocytes studied. N indicates the number of batches of oocytes. ***, p < 0.001 compared with vehicle.
ation of cAMP, and to β-arrestins which mediate receptor endocytosis and activation of extracellular signal regulated kinases 1/2 (ERK1/2) from endosomal signalosomes (11). To examine PAR2-dependent signals, we compared responses of KNRK cells expressing human PAR2 (KNRK-PAR2) or empty vector control (KNRK-VC). We also examined signaling in HEK293 cells overexpressing PAR2, and used the Cat-S inhibitor MV026031 and the PAR2 antagonist GB88 (55) to ascertain the respective requirements of enzymatic activity and PAR2 activation for responses.

As we have previously shown, trypsin stimulated a rapid, transient and concentration-dependent increase in \([\text{Ca}^{2+}]_i\) in KNRK-PAR2 cells but not in KNRK-VC cells (12) (Fig. 5). Cat-S did not affect \([\text{Ca}^{2+}]_i\) in KNRK-PAR2 cells at any concentration or time point studied (Fig. 5).

We first studied cAMP signaling in HEK-PAR2 cells, which were readily transfected with PAR2 and the CAMYEL YFP-Epac-RFP cAMP sensor. Trypsin stimulated a rapid, transient and concentration-dependent increase in cAMP formation in HEK-PAR2 cells (Fig. 6, A and B). Cat-S also caused a time and concentration-dependent increase in cAMP formation in HEK-PAR2 cells, with an EC_{50} of 128 ± 4.0 nM, similar to that of trypsin (Fig. 6, A and B). The effect of Cat-S (100 nM) was abolished when the protease was pre-incubated with the selective Cat-S inhibitor MV026031 (1 μM, 30 min before assay), and is thus dependent on enzymatic activity (Fig. 6C). Pre-incubation of HEK-PAR2 cells with the PAR2 antagonist GB88 (10 μM, 60 min) (55) abolished the cAMP response to Cat-S (Fig. 6C). Cat-S (100 nM) also stimulated cAMP formation in KNRK-PAR2 cells, and had a small stimulatory effect in KNRK-VC cells, presumably by a PAR2-independent process since KNRK-VC cells do not express appreciable levels of PAR2 (Fig. 6D). Cat-S AP caused a concentration-dependent stimulation of cAMP formation in KNRK-PAR2 cells but not in KNRK-VC cells (EC_{50} 6.85 ± 0.33 μM, Fig. 6E). A control peptide in which the first 2 residues were substituted (PGFSVDEFSA) had no effect on cAMP formation in KNRK-PAR2 or KNRK-VC cells (Fig. 6F).

Trypsin-activated PAR2 interacts with β-arrestins, which mediate endocytosis of PAR2 and assemble signalosomes that are required for ERK1/2 activation (36, 47). To determine whether Cat-S induces interaction between PAR2 and β-arrestins, we coexpressed PAR2-RLuc8 and β-arrestin1-YFP or β-arrestin2-YFP in HEK293 cells, and examined protease-in-
duced BRET signals (50). As we have previously reported, trypsin stimulated a time- and concentration-dependent BRET between PAR2-RLuc8 and H9252-arrestin1-YFP or H9252-arrestin2-YFP (48) (Fig. 7, A–D). Cat-S did not induce a detectable BRET signal at any time or concentration tested (Fig. 7, A–D). The inability of Cat-S to recruit H9252-arrestins is in accordance with the finding that Cat-S did not stimulate endocytosis of PAR2 (Fig. 2A), and agrees with our report that H9252-arrestins mediate endocytosis of trypsin-activated PAR2 (47). Challenge of KNRK-PAR2 cells with trypsin stimulated a rapid and concentration-dependent activation of ERK1/2, whereas Cat-S did not stimulate ERK1/2 activation (Fig. 7, E and F). These findings are consistent with the report that β-arrestins mediate PAR2-mediated ERK1/2 activation in KNRK-PAR2 cells (36).

Our results suggest that Cat-S is a biased agonist of PAR2. In contrast to trypsin, which mobilizes intracellular Ca2+, generates cAMP and recruits β-arrestins that mediate PAR2 endocytosis and ERK1/2 activation, Cat-S stimulates only PAR2-dependent formation of cAMP. The observation that Cat-S also stimulates PAR2-dependent cAMP formation suggests that the stimulatory action of Cat-S, like that of trypsin, involves proteolytic exposure of a tethered ligand.

Cat-S-evoked Activation of PAR2 Requires Receptor Cleavage—To verify the requirement of PAR2 cleavage for Cat-S signaling, we mutated the putative cleavage site. We first determined whether Cat-S cleaved a decapeptide in which the P2, P1, and P1′ positions were substituted: V55S, E56P, and T57K (52GVTSPKVFSVD62). As expected, Cat-S was unable to cleave this peptide at concentration up to 880 μM (Fig. 8A). Thus, when Cat-S (10 nM) was incubated with G52VTSPKVFSVD62 for 60 min, 0.6 ± 1.4% of the fragment was degraded (Fig. 8A). Thus, when Cat-S (10 nM) was incubated with G52VTSPKVFSVD62 for 60 min, 0.6 ± 1.4% of the fragment was degraded (Fig. 8A).
alent residues in human PAR2 and transiently expressed the mutant (PAR2/H9004V55S/E56P/T57K) or wild-type PAR2 (both with C-terminal HA11 epitopes) in HEK293 or KNRK cells. PAR2/H9004V55S/E56P/T57K, like PAR2 wild-type, was normally localized at the plasma membrane of HEK cells (Fig. 8B). Trypsin similarly increased $[Ca^{2+}]_i$ in KNRK-PAR2/H9004V55S/E56P/T57K and in KNRK-PAR2 cells (Fig. 8C). However, whereas Cat-S stimulated a concentration-dependent formation of cAMP in KNRK-PAR2 cells, Cat-S did not stimulate cAMP formation in KNRK-PAR2/H55S/E56P/T57K cells (Fig. 8D). Our results suggest that Cat-S cleaves PAR2, which activates adenylyl cyclase and generates cAMP.

Cat-S Induces PAR2-dependent Sensitization of TRPV4—Trypsin-cleaved PAR2 can activate and sensitize TRPV4 by mechanisms that involve channel phosphorylation by protein kinase C (PKC) and tyrosine kinases, and the generation of arachidonic acid metabolites that directly activate TRPV4, which result in inflammation and hyperalgesia (28–30). To determine whether Cat-S-activated PAR2 can sensitize TRPV4, we expressed in *Xenopus laevis* oocytes human TRPV4 alone or TRPV4 plus human PAR2. We exposed oocytes to vehicle (control), trypsin (8 nM), Cat-S (1 μM) or Cat-S AP (50 μM) for 5 min, and then activated TRPV4 with the selective agonist GSK1016790A (50 nM) followed by the TRPV4 antagonist HC067047 (100 nM). We compared the magnitude of the response to the TRPV4 agonist in oocytes expressing TRPV4 alone to that in oocytes expressing PAR2 plus TRPV4. In vehicle-treated oocytes expressing TRPV4 alone (control), trypsin (8 nM), Cat-S (1 μM) or Cat-S AP (50 μM) for 5 min, and then activated TRPV4 with the selective agonist GSK1016790A (50 nM) followed by the TRPV4 antagonist HC067047 (100 nM). We compared the magnitude of the response to the TRPV4 agonist in oocytes expressing TRPV4 alone to that in oocytes expressing PAR2 plus TRPV4. In vehicle-treated oocytes expressing TRPV4 alone, GSK1016790A caused a small inward current that was reversed by HC067047 and is thus attributable to TRPV4 activation (Fig. 9A). Pretreat-
ment of TRPV4-expressing oocytes with trypsin, Cat-S or Cat-S AP did not alter the TRPV4 response (Fig. 9, B–D). In vehicle-treated oocytes expressing TRPV4 plus PAR2, the response to GSK1016790A was the same as in oocytes expressing TRPV4 alone (Fig. 9A). Pre-incubation of oocytes expressing TRPV4 plus PAR2 with trypsin, Cat-S or Cat-S AP for 5 min amplified the response to GSK1016790A, indicating TRPV4 sensitization (Fig. 9, B–D). Compared with the response in oocytes expressing TRPV4, the responses of oocytes expressing TRPV4 plus PAR2 was amplified by ~8-fold after trypsin and ~5-fold after Cat-S and Cat-S AP (Fig. 9E). Thus, Cat-S induces PAR2-dependent sensitization of TRPV4.

We similarly examined whether Cat-S can sensitize TRPV4 stably expressed in HEK293 cells. HEK-TRPV4 cells were incubated with vehicle (control), Cat-S (100 nM) or trypsin (100 nM) for 5 min and were then challenged with GSK1016790A (100 pm). In vehicle-treated cells, GSK1016790A caused a gradual and sustained increase in [Ca^{2+}]_i, consistent with TRPV4 activation (Fig. 9F). Trypsin, but not Cat-S, increased [Ca^{2+}]_i, and preincubation with both proteases resulted in a ~3-fold

FIGURE 8. Requirement of PAR2 cleavage for activation by Cat-S. A, Cat-S degradation of PAR2, HPLC traces (left) show elution of the substrate after incubation with Cat-S for 0 min or 60 min. Time course (right) shows the kinetics of substrate degradation and product formation. In red are the residues replaced from the wild-type sequence. B, localization of wild-type PAR2 and PAR2 ΔV^{55S/E^{55P}T^{57K}} expressed in HEK293 cells by immunofluorescence, using antibody to intracellular C-terminal HA11 epitope, and confocal microscopy. Scale bar 10 μm. C, trypsin (100 nM)-evoked Ca^{2+} signals in individual KNRK cells transiently expressing wild-type PAR2 or PAR2 ΔV^{55S/E^{55P}T^{57K}}. Cells were co-transfected with GFP for identification. D, Cat-S-evoked cAMP formation in KNRK cells transiently expressing wild-type PAR2 and PAR2 ΔV^{55S/E^{55P}T^{57K}}. Triplet measurements of n = 3–4 experiments.
increase in the maximal response to GSK1016790A (Fig. 9, F and G). Thus, Cat-S sensitizes TRPV4 in HEK293 cells as well as in oocytes.

**Cat-S Is a Biased Agonist of PAR₂ in DRG Neurons—** PAR₂ is expressed by nociceptive neurons, where activation induces neurogenic inflammation (10) and pain (32). To determine whether Cat-S is a biased agonist of PAR₂ in nociceptive neurons, we challenged mouse DRG neurons with trypsin or Cat-S (100 nM) and measured cAMP accumulation, ERK1/2 activation and [Ca²⁺]ᵢ. Trypsin and Cat-S stimulated cAMP accumulation to a similar extent that was maximal after 30 min (Fig. 10 A). Trypsin but not Cat-S stimulated ERK1/2 activation, which was maximal after 20 min (Fig. 10 B). Trypsin stimulated an increase in [Ca²⁺]ᵢ in 24.7 ± 7.1% and Cat-S stimulated an increase in [Ca²⁺]ᵢ in 40.4 ± 10.7% of small diameter neurons from wild-type mice, similar to the proportion of neurons that responded to capsaicin (41.0 ± 4.8%) (Fig. 10 C, D, F). In the absence of extracellular Ca²⁺ ions, the response to trypsin was diminished but not abolished (Fig. 10, C and E), whereas the response to Cat-S was undetectable (Fig. 10, D, F). Significantly fewer neurons from par2⁻/⁻ or trpv4⁻/⁻ mice responded to Cat-S with a detectable increase in [Ca²⁺]ᵢ (wild-type 40.4 ± 10.7%, par2⁻/⁻ 14.3 ± 0.5%, trpv4⁻/⁻ 4.7 ± 2.5% of small diameter neurons, p < 0.05 to wild-type) (Fig. 10H). The TRPV4 antagonist HC067047 inhibited Cat-S responses of neurons from wild-type mice (Fig. 10 H). The PKA inhibitor PKI and the adenylyl cyclase inhibitor SQ22536 both suppressed Cat-S responses of neurons from wild-type mice (Fig. 10 I). These results suggest that the Cat-S-evoked increase in [Ca²⁺]ᵢ depends on activation of PAR₂ and entails TRPV4-dependent influx of Ca²⁺ ions, rather than mobilization of intracellular Ca²⁺ stores. They are consistent with activation of TRPV4 in neurons via biased signaling of the PAR₂/adenyl cyclase/cAMP/PKA pathway. The residual Ca²⁺ responses in neurons from par2⁻/⁻ and trpv4⁻/⁻ mice occur by unknown mechanisms, possibly involving other PARs and TRP channels. As in HEK and KNRK cell lines, Cat-S preferentially signals via cAMP in DRG neurons.

**Cat-S Induces a PKA-induced Hyperexcitability of Nociceptive Neurons—** Cat-S induces hyperexcitability of nociceptive neurons from wild-type but not par2⁻/⁻ mice by unknown mechanisms (5). To determine whether PAR₂ biased signaling...
accounts for this effect, we examined the actions of inhibitors of PKA (PKI, H-89), adenylyl cyclase (SQ22536), and PKC (GF-109203X) on Cat-S-evoked neuronal hyperexcitability. We incubated small diameter mouse DRG neurons with Cat-S (100 nM) or vehicle (control) for 60 min, and then measured the rheobase (minimum current to generate an action potential) and the action potential discharge at twice rheobase by patch-clamp recording to assess neuronal hyperexcitability. Cat-S decreased the rheobase (vehicle, 69.8 ± 2.8 pA, n = 57 neurons; Cat-S, 39.6 ± 2.8 pA, n = 55 neurons; p < 0.0001) and increased the frequency of action potential discharge (vehicle, 1.21 ± 0.06; Cat-S, 1.98 ± 0.13; p < 0.0001), indicating hyperexcitability (Fig. 11). Inhibition of PKA with PKI or H-89 abolished the effects of Cat-S on rheobase and action potential firing (Fig. 11, A–C). Inhibition of adenylyl cyclase with SQ22536 inhibited the effects of Cat-S on rheobase (Fig. 11D). In contrast, inhibition of PKC with GF-109203X had no effect on Cat-S-evoked changes in rheobase or action potential discharge (Fig. 11E). None of the inhibitors affected the rheobase of action potential firing of vehicle-treated neurons. Thus, three distinct inhibitors (PKI, H-89, SQ22526) of the adenylyl cyclase/cAMP/PKA pathway blocked the effects of Cat-S on nociceptor hyperexcitability. These results suggest that Cat-S evokes PAR2-dependent hyperexcitability of nociceptive neurons via biased activation of adenylyl cyclase/cAMP/PKA but not PKC signaling pathways.

Cat-S Induces PAR2- and TRPV4-dependent Inflammation and Pain—Intraplantar injection of trypsin or trypsin-revealed AP induces neurogenic inflammation and hyperalgesia by PAR2- and TRP-dependent mechanisms (10, 27, 28, 32). TRPV4 agonists also cause neurogenic inflammation and mechanical hyperalgesia (28, 52). To examine whether Cat-S causes inflammation and pain by similar mechanisms, we made intraplantar injections of Cat-S (1.4–14 nM, 10 μl) to wild-type, par2+/−, trpv4+/−, and wild-type mice treated with the TRPV4 antagonist HC067047. I, proportion of small diameter neurons responding to Cat-S in neurons from wild-type (WT) mice, par2+/− or trpv4+/− mice, or wild-type mice treated with the TRPV4 antagonist HC067047. I, proportion of small diameter neurons responding to Cat-S in neurons from wild-type mice treated with vehicle (Veh.) the PKA inhibitor PKI or the adenylyl cyclase inhibitor SQ22536. A–C, H, I, n = 4–6 mice. C–F, records from 15–20 individual neurons. p < 0.05 compared with wild-type or vehicle.

FIGURE 10. Cat-S biased signaling in DRG neurons. The effects of trypsin (Tryp., 100 nM) and Cat-S (100 nM) on cAMP accumulation (A), ERK1/2 activation (B) and [Ca²⁺]i (C–I) were measured in DRG neurons. C–F, effects of trypsin and Cat-S on [Ca²⁺]i. E and F, assays in Ca²⁺-free extracellular fluid. G, proportion of small diameter neurons responding to trypsin, Cat-S or capsaicin (Cap.). H, proportion of small diameter neurons responding to Cat-S in neurons from wild-type (WT) mice, par2+/− or trpv4+/− mice, or wild-type mice treated with the TRPV4 antagonist HC067047. I, proportion of small diameter neurons responding to Cat-S in neurons from wild-type mice treated with vehicle (Veh.) the PKA inhibitor PKI or the adenylyl cyclase inhibitor SQ22536. A–C, H, I, n = 4–6 mice. C–F, records from 15–20 individual neurons. p < 0.05 compared with wild-type or vehicle.
maximal after 1 h and sustained for at least 4 h, indicative of mechanical hyperalgesia (Fig. 12, A and B). Cat-S (14 μM, 10 μl) also caused edema that was maximal at 1 h and maintained for 4 h, indicative of edema (Fig. 12C). PAR2 deletion inhibited Cat-S-induced mechanical hyperalgesia and edema at all time points, whereas TRPV4 deletion attenuated paw edema after 2 h but did not prevent the hyperalgesia (Fig. 12, B and C). Thus, Cat-S evokes pain and inflammation by a PAR2-dependent process, and TRPV4 contributes to the sustained Cat-S-induced inflammation.
To determine whether Cat-S causes mechanical hyperalgesia via biased agonism of PAR₂, we examined the effects of inhibiting the adenylyl cyclase/PKA pathway on Cat-S-evoked pain. Wild-type mice received an intraplantar injection of the adenylyl cyclase inhibitor SQ22536 or vehicle, followed by Cat-S (2.5 μM). In vehicle-treated mice, Cat-S caused a sustained mechanical hyperalgesia (Fig. 11D). SQ22536 inhibited Cat-S-evoked hyperalgesia at 1 and 2 h (Fig. 12D). These results suggest that Cat-S induces mechanical hyperalgesia via the adenylyl cyclase pathway, and are consistent with the capacity of Cat-S to cause hyperexcitability of nociceptive neurons by an adenylyl cyclase- and PKA-dependent process (Fig. 11). It was not possible to examine directly the contribution of PKA to Cat-S-evoked pain, since the PKA inhibitor WIPTIDE, which is commonly used to study the role of PKA in pain in vivo, is a peptide and would be likely degraded by Cat-S.

To determine whether endogenous Cat-S contributes to pain and inflammation, we made intraplantar injections to mice of formalin, which causes PAR₂-dependent hyperalgesia (32). Intraplantar formalin (4%, 10 μl) induced a 12-fold increase in Cat-S activity in the paw tissue, as determined using the Cat-S substrate acetyl-KQKLR-AMC (Fig. 12E). Pre-treatment of mice with the Cat-S inhibitor MV026031 (50 mg/kg p.o.) 2 h before formalin injection suppressed formalin-induced activation of Cat-S, which confirms the effectiveness of this inhibitor at the dose given in vivo. Formalin induced the expected rapid
and sustained mechanical hyperalgesia and edema (Fig. 12, F and G). MV026031 inhibited formalin-induced mechanical hyperalgesia at all times and suppressed edema at 1 h (Fig. 12, F and G). Pre-treatment with the PAR2 antagonist GB88 (10 mg/kg p.o.) inhibited mechanical hyperalgesia after 1 h and also suppressed edema at 1 h (Fig. 12, F and G). These results reveal a role for endogenous Cat-S and PAR2 in formalin-induced inflammation and pain.

**DISCUSSION**

We report a new mechanism by which Cat-S activates PAR2 and TRPV4 to cause inflammation and pain. Our major finding is that Cat-S is a biased agonist of PAR2. By cleaving PAR2 at a unique site (E56\(\rightarrow\)T57), which is distal to the canonical tethered ligand that is exposed by trypsin cleavage, Cat-S reveals a distinct tethered ligand domain. Cat-S cleavage stabilizes conformations of PAR2, that signal by mechanisms that are distinctly different from those activated by trypsin. After cleavage by Cat-S, PAR2 couples to G\(\alpha\)s, leading to the formation of cAMP. In contrast to trypsin-activated PAR2, Cat-S cleavage fails to mobilize intracellular Ca\(^{2+}\), activate ERK1/2, recruit \(\beta\)-arrestins or cause receptor endocytosis. Cat-S-cleaved PAR2 sensitizes TRPV4 and causes hyperexcitability of nociceptive neurons by an adenylyl cyclase- and PKA-mediated pathway. The intraplantar administration of Cat-S causes sustained mechanical hyperalgesia and inflammatory edema in mice by PAR2\(\rightarrow\), TRPV4\(\rightarrow\), and adenylyl cyclase-dependent mechanisms. Intraplantar formalin, which induces sustained inflammation and pain, activates Cat-S, and a Cat-S inhibitor and PAR2 antagonist both suppress formalin-induced inflammation and pain. Given that Cat-S is activated and secreted in inflammatory diseases, our findings indicate that antagonists of Cat-S, PAR2 and TRPV4 may be useful treatments for inflammation and pain.

**Cat-S Is a Biased Agonist of PAR2.**—We have identified a new mechanism by which Cat-S activates PAR2. The established mechanism by which serine proteases such as trypsin, tryptase and kallikreins activate PAR2 involves hydrolysis of the R\(^{36}\)S\(^{37}\) bond and exposure of the tethered ligand domain \(SLIGKV\), which binds to and activates the cleaved receptor. By incubating synthetic fragments of the extracellular N terminus of PAR2 with Cat-S, we identified that Cat-S cleaves within the N terminus of PAR2 at a single major site: E56\(\rightarrow\)T57. In experiments in HEK293 cells expressing PAR2 with N-terminal Flag and C-terminal HA11 epitopes, we observed that Cat-S removed the extracellular Flag epitope, which indicates that Cat-S can cleave intact PAR2 at the cell surface as well as receptor fragments. To ascertain the importance of cleavage at the E56\(\rightarrow\)T57 for Cat-S activation of PAR2, we studied the capacity of Cat-S to cleave and activate a mutant receptor in which the P2, P1 and P1\' positions were replaced: V\(^{56}\)S\(\rightarrow\)S, E56\(\rightarrow\)P, and T57\(\rightarrow\)K. Cat-S did not cleave a fragment with these substitutions (\(^{52}GVTSPKVFSVD\)) and Cat-S was unable to stimulate formation of cAMP in KNRK cells expressing PAR2\(\rightarrow\)V\(^{56}\)S/ E56\(\rightarrow\)P/T57\(\rightarrow\)K. PAR2\(\rightarrow\)Δ\(^{56}\)S/E56\(\rightarrow\)P/T57\(\rightarrow\)K was normally localized at the plasma membrane and trypsin stimulated Ca\(^{2+}\) signaling in KNRK-PAR2\(\rightarrow\)Δ\(^{56}\)S/E56\(\rightarrow\)P/T57\(\rightarrow\)K cells, which indicate that membrane trafficking and trypsin activation are unaffected by these mutations. Identification of E56\(\rightarrow\)T57 as the major site at which Cat-S cleaves within the N terminus of PAR2 is consistent with the known requirements for Cat-S substrate recognition (56, 57). Aliphatic residues at the P2 position, including valine and leucine, direct Cat-S selectivity. The P1 position can tolerate several other residues including glycine, lysine, glutamic acid, glutamine, tyrosine, and alanine, while other positions contribute little to Cat-S recognition. We identified a single site of Cat-S cleavage, despite numerous aliphatic residues within the PAR2 N terminus, including eight valine residues. Thus, the selectivity with which Cat-S cleaves PAR2 probably relies on a series of mutual interactions from numerous sites, which requires future study. In addition to the E56\(\rightarrow\)T57 site, Cat-S can also cleave PAR2 more proximally at G\(^{41}\)\(\rightarrow\)K\(^{52}\) (58). However, this study did not assess whether Cat-S cleaves PAR2 at the site that we have identified.

The Cat-S cleavage site (E56\(\rightarrow\)T57) is distal to the trypsin cleavage site (R\(^{36}\)S\(^{37}\)). We observed that pre-incubation with Cat-S attenuated trypsin-evoked Ca\(^{2+}\) signaling in HEK293 cells and trypsin-induced activation of calcium-activated chloride channels in oocytes. These findings are consistent with the proposal that Cat-S, by removing the trypsin cleavage site, disarm PAR2 for trypsin activation. Similarly, neutrophil elastase cleaves PAR2 at S\(^{58}\)\(\rightarrow\)V\(^{59}\), which also prevents trypsin-stimulated PAR2 signaling (33, 34). The path-physiological relevance of these mechanisms of PAR2 disarming is uncertain.

We found that Cat-S-activated PAR2 signals by mechanisms that are distinctly different from those arising from trypsin-activated PAR2. We examined PAR2 coupling to heterotrimeric G proteins using BRET. Proteases stimulated distinct BRET signals between PAR2\(\rightarrow\)RLuc8 and Gly2-Venus. Cat-S induced a sustained increase in BRET only in the presence of overexpressed G\(\alpha\)s, whereas trypsin induced a sustained increase in BRET in the presence of Goq and a transient decrease in BRET in the presence of G\(\alpha\)s. Similar differences in coupling between PAR2 and different G proteins have been observed in Cos-7 cells, where trypsin induces a sustained signal between PAR2 and G\(\alpha\)12, but a rapid and transient signal between PAR2 and G\(\alpha\)1 (59). The rapid decline in BRET signal may due to receptor desensitization or dissociation of Goq by dimer from the complex. The reason for the differences in trypsin- and Cat-S-induced BRET in cells expressing G\(\alpha\)s is unclear. One possible explanation is that Cat-S and trypsin cleave PAR2 at distinct sites, and that Cat-S cleavage results in a higher affinity or more stable association between the tethered ligand and the receptor. Alternatively, the trypsin- and Cat-S cleaved receptor may adopt distinct conformations that interact differently with G proteins. Although the structural determinants for PAR2 coupling to Goq and G\(\alpha\)s remain to be identified, our results suggest that proximal regions of PAR2 that are revealed by trypsin cleavage mediate Goq coupling, whereas distant regions exposed by Cat-S or trypsin cleavage mediate Goq coupling.

The observations that Cat-S-activated PAR2 couples to G\(\alpha\)s alone, whereas trypsin-activated PAR2 couples to both G\(\alpha\)s and Goq, are consistent with the capacity of Cat-S to generate cAMP but not to mobilize intracellular Ca\(^{2+}\), whereas trypsin induces both signals. Trypsin, but not Cat-S, also activated ERK1/2, recruited \(\beta\)-arrestins and caused PAR2 endocytosis. The inability of Cat-S-cleaved PAR2 to activate ERK1/2 and to...
internalize agrees with the lack of interactions with β-arrestins, which mediate PAR2 ERK1/2 signaling (36) and endocytosis (47). The Cat-S inhibitor MV026031 prevented Cat-S induced formation of cAMP in HEK-PAR2 cells, indicating a requirement for enzymatic activity. Cat-S-induced cAMP formation is attributable to cleavage and activation of PAR2 since the PAR2 antagonist GB88 (55) prevents Cat-S stimulation of cAMP generation in HEK-PAR2 cells, and because Cat-S stimulated cAMP formation in KNRK cells expressing wild-type PAR2, but not the cleavage-resistant mutant PAR2ΔV55S/E56P/T57K.

A synthetic peptide corresponding to the first 10 residues distal to the Cat-S cleavage site (Samientos, Cat-S AP) induced Gαs-dependent BRET signals between PAR2-RLuc8 and Gγ2-Venus, and stimulated cAMP formation in KNRK-PAR2 cells but not in KNRK-VC cells. Whereas Cat-S and trypsin increased Gαs-dependent BRET signals, Cat-S AP caused a large and sustained decrease in BRET. The reason for this difference is unknown, but the results suggest that the receptor adopts distinctly different conformations after cleavage by Cat-S or by trypsin (Cat-S AP). Our results indicate that Cat-S, like trypsin, reveals a tethered ligand that binds to and activates the cleaved receptor. After trypsin cleavage, the tethered ligand interacts with domains in the second extracellular loop of PAR2 (60). Further studies are required to ascertain whether the Cat-S-revealed tethered ligand similarly activates PAR2 and to identify key residues that are required for such interactions. However, PAR2 cleavage may also be sufficient to induce conformational changes that result in receptor activation without tethered ligand binding. In case of elastase, synthetic peptides that mimic a potential tethered ligand are unable to activate PAR2, possibly because the elastase cleavage site (S58↑V69) is close to the first transmembrane domain (34). In contrast, elastase can activate PAR2 by a tethered ligand mechanism (37). A recent report suggests that Cat-S can cleave PAR2 at G41↑K42 to reveal a tethered ligand (42)KVDGTS, which, like the trypsin-exposed AP SLIGRL, stimulates Ca2+ signaling in HeLa cells, albeit with reduced potency (58). However, we found no evidence that Cat-S induced mobilization of intracellular Ca2+ in KNRK or HEK cells expressing PAR2, but instead observed that Cat-S stimulates a TRPV4-dependent influx of extracellular Ca2+ ions in nociceptive neurons.

Biased agonism, by which different endogenous ligands or drugs induce distinct conformations of the same GPCR leading to diverse signaling patterns and outcomes, is an emerging theme in the GPCR field (35). However, in contrast to most instances, where biased agonists activate the same set of signaling pathways but with differential potencies, Cat-S activated PAR2 by a single mechanism, Gαs-dependent formation of cAMP, and was completely inactive in all other pathways at any tested concentration. Neutrophil elastase is another biased agonist of PAR2, since elastase stimulates PAR2-dependent activation of ERK1/2 by a Rho-kinase dependent but β-arrestin-independent pathway (34). The observation that Cat-S- and elastase-activated PAR2 neither interacts with β-arrestins nor internalizes has implications for PAR2 desensitization and down-regulation, which involve β-arrestin-mediated uncoupling of PAR2 from heterotrimeric G-proteins and PAR2 endocytosis and lysosomal degradation (36, 47, 54). We found that Cat-S-activated PAR2 remained at the cell surface, and that Cat-S induced sustained Gαs-dependent PAR2-RLuc8 and Gγ2-Venus BRET. These findings suggest that Cat-S could induce sustained signals from PAR2 at the plasma membrane. Further studies are necessary to elucidate the importance of such signals and to investigate the fate of PAR2 after cleavage by proteases that fail to recruit β-arrestins and cause receptor endocytosis.

**Patho-physiological Importance of Cat-S PAR2 Signaling**—Our results show that Cat-S-activated PAR2 couples to signaling pathways that sensitize and activate TRPV4 and cause inflammation and pain. We have thus identified a patho-physiologically relevant outcome of Cat-S biased agonism of PAR2.

We observed that pre-incubation with Cat-S and Cat-S AP strongly (5-fold) amplified TRPV4 currents in oocytes co-expressing PAR2 and TRPV4, but did not affect TRPV4 currents in oocytes expressing TRPV4 alone. Cat-S similarly amplified TRPV4 Ca2+ signals in HEK-TRPV4 cells. Thus, Cat-S-activated PAR2 can sensitize TRPV4. Our results suggest that Cat-S is a biased agonist of PAR2 in nociceptive neurons that control pain and neurogenic inflammation. Both Cat-S and trypsin stimulated cAMP formation in DRG cultures, whereas trypsin alone stimulated ERK1/2 activation. These results are consistent with observations in KNRK and HEK cells. Although both Cat-S and trypsin increased [Ca2+]i in DRG neurons, the response to Cat-S was prevented by removal of extracellular Ca2+, whereas the response to trypsin was only slightly reduced. Thus, Cat-S-activated PAR2 couples to mechanisms that induce Ca2+ influx in neurons but not mobilization of intracellular Ca2+ stores. PAR2-deletion or deletion or antagonism of TRPV4 markedly reduced Cat-S-evoked Ca2+ signals in neurons, which indicates that Cat-S causes a PAR2-dependent activation of TRPV4, leading to Ca2+ influx. The adenylyl cyclase inhibitor SQ22536 and the PKA inhibitor PKI both suppressed Cat-S Ca2+ signals, suggesting a major role for the PAR2 biased adenylyl cyclase/cAMP/PKA signaling pathway in TRPV4 activation. PKA and PKC play a major role in sensitizing TRPV4 through phosphorylation of serine and threonine residues, including S524 in the case of PKA, and assembly of a complex with the scaffolding protein AKAP79 (61). After activation by trypsin or trypsin AP, PAR2 sensitzes and activates TRPV4 by several mechanisms, which include PKC- and tyrosine kinase-dependent processes, as well as formation of arachidonic acid metabolites that are TRPV4 agonists (28, 29). Thus, multiple proteases that can activate PAR2 by distinct mechanisms are capable of sensitizing TRP channels by divergent signaling processes.

We have previously shown that Cat-S causes hyperexcitability of nociceptive neurons in wild-type but not par2−/− mice, but by unknown signaling pathways (5). In the present study we found that Cat-S also reduced the rheobase and increased action potential discharge, confirming hyperexcitability. Pharmacological inhibitors of adenylyl cyclase (SQ22536) and two different PKA inhibitors (PKI, H-89) suppressed this sensitization, which further implicates PAR2 biased signaling by the Gαs, adenylyl cyclase, cAMP and PKA pathway in this process.

Intraplantar injection of Cat-S to mice caused sustained inflammatory edema and mechanical hyperalgesia. Deletion of
par₂ strongly inhibited inflammation and pain at all time points, whereas deletion of trpv4 inhibited only the later stages of inflammation, consistent with the requirement of TRPV4 for sustained inflammatory signaling of PAR₂ (29). The residual inflammation and pain observed in par₂−/− mice may be mediated by Cat-S activation of other PARs, which may also explain the small PAR₂-independent Ca²⁺ signals to Cat-S in DRG neurons from par₂−/− mice. Other proteases (trypsin 4) can also cause inflammation by activating both PAR₁ and PAR₂ (15). Cat-S induced inflammation and pain could also be caused by sensitization and activation of other TRP channels, since PAR₂ can sensitize TRPV1 and TRPA1 and induce neurogenic inflammation and pain (27, 31). The finding that Cat-S causes PAR₂-dependent inflammation and pain is consistent with our previous report that Cat-S causes visceral pain, which required expression of PAR₂ (5). Inhibition of adenylyl cyclase strongly inhibited Cat-S-evoked mechanical hyperalgesia, which implicates PAR, biased signaling. Antagonism of Cat-S and PAR₂ suppressed formalin-induced pain and inflammation, which suggests that endogenous Cat-S can activate PAR₂ to cause algesic and inflammatory signals. The inplantar injection of formalin resulted in Cat-S activation, possibly due to the infiltration of macrophages that are a major source of Cat-S. The Cat-S inhibitor MV026031 suppressed Cat-S activity in tissues and blocked formalin-induced mechanical hyperalgesia and inflammation, confirming the importance of endogenous Cat-S. In agreement with the report that PAR₂ deletion attenuates formalin-induced pain (32), we observed that the PAR₂ antagonist GB88 inhibited the algesic and inflammatory actions of formalin. GB88 also blocks the inflammatory effects of PAR₂ agonists and of carrageenan (62).

Our finding that Cat-S is a biased agonist of PAR₂- and TRPV4-dependent inflammation and pain has implications for the mechanism and treatment of disease. Cat-S is activated in DRG macrophages (6) and spinal microglial cells (7) after nerve injury. Cat-S from microglial cells liberates fractalkine from spinal neurons, which activates CX3CR1 on microglial cells to trigger inflammatory signals that contribute to central sensitization of pain (1, 7, 8). Whether Cat-S can activate PAR₂ on the central projections of primary sensory neurons within the dorsal horn to cause pain remains to be investigated. Cat-S is also activated in inflammatory diseases including rheumatoid arthritis (4) and colitis (5). Given the established contributions of PAR₂ to TRPV4 to arthritis (63) and colitis (30, 64, 65), antagonists of Cat-S, PAR₂, and TRPV4 may be valuable treatments for these and other inflammatory diseases.

Acknowledgments—We thank Dr. Bimbil Graham for expert advice, and Tao Yu and Cameron Nowell for technical assistance.

REFERENCES

1. Clark, A. K., and Malcangio, M. (2012) Microglial signalling mechanisms: Cathepsin S and Fractalkine. Exp. Neurol. 234, 283–292
2. Clark, A. K., Wodarski, R., Guida, F., Sasso, O., and Malcangio, M. (2010) Cathepsin S release from primary cultured microglia is regulated by the P2X7 receptor. Glia 58, 1710–1726
3. Liuzzo, J. P., Petanceska, S. S., Moscatelli, D., and Devi, L. A. (1999) Inflammatory mediators regulate cathepsin S in macrophages and microglia: A role in attenuating heparan sulfate interactions. Mol. Med. 5, 320–333
4. Pozgan, U., Caglic, D., Rozman, B., Nagase, H., Turk, V., and Turk, B. (2010) Expression and activity profiling of selected cysteine cathepsins and matrix metalloproteinases in synovial fluids from patients with rheumatoid arthritis and osteoarthritis. Biol. Chem. 391, 571–579
5. Catarruzzo, F., Lyo, Y., Jones, E., Pham, D., Hawkins, J., Kirkwood, K., Valdez-Morales, E., Ibeakanma, C., Vanner, S. J., Bogoy, M., and Bunnell, N. W. (2011) Cathepsin S is activated during colitis and causes visceral hyperalgesia by a PAR2-dependent mechanism in mice. Gastroenterology 141, 1864–1874 e1861–e1863
6. Buxton, F., Clark, A. K., Gennari, F., Gentry, C., Patel, S., Wotherspoon, G., Buxton, F., Song, C., Ullah, J., Winter, J., Fox, A., Bevan, S., and Malcangio, M. (2007) Role of the cysteine protease cathepsin S in neoplastic hyperalgesia. Pain 130, 225–234
7. Clark, A. K., Yip, P. K., Grist, J., Gentry, C., Staniland, A. A., Marchand, F., Dehvari, M., Wotherspoon, G., Winter, J., Ullah, J., Bevan, S., and Malcangio, M. (2007) Inhibition of spinal microglial cathepsin S for the reversal of neuropathic pain. Proc. Natl. Acad. Sci. U.S.A. 104, 10655–10660
8. Clark, A. K., Yip, P. K., and Malcangio, M. (2009) The liberation of fractalkine in the dorsal horn requires microglial cathepsin S. J. Neurosci. 29, 6945–6954
9. Steinhoff, M., Corvera, C. U., Thoma, M. S., Kong, W., McAlpine, B. E., Caughey, G. H., Ansel, J. C., and Bunnell, N. W. (1999) Proteinase-activated receptor-2 in human skin: tissue distribution and activation of keratinocytes by mast cell tryptase. Exp. Derm. 8, 282–294
10. Steinhoff, M., Vergnolle, N., Young, S. H., Tognotto, M., Amadesi, S., Ennes, H. S., Trevisani, M., Hollenberg, M. D., Wallace, J. L., Caughey, G. H., Mitchell, S. E., Williams, L. M., Geppetti, P., Mayer, E. A., and Bunnell, N. W. (2000) Agonists of proteinase-activated receptor 2 induce inflammation by a neurogenic mechanism. Nature Med. 6, 151–158
11. Ossovskaya, V. S., and Bunnell, N. W. (2004) Protease-activated receptors: contribution to physiology and disease. Physiol. Rev. 84, 579–621
12. Bohm, S. K., Kong, W., Bromme, D., Smeekens, S. P., Anderson, D. C., Connolly, A., Kahn, M., Nelenk, N. A., Coughlin, S. R., Payan, D. G., and Bunnell, N. W. (1996) Molecular cloning, expression and potential functions of the human proteinase-activated receptor-2. Biochem. J. 314, 1009–1016
13. Nystedt, S., Emilsson, K., Wahlestedt, C., and Sundelin, J. (1994) Molecular cloning of a potential proteinase activated receptor. Proc. Natl. Acad. Sci. U.S.A. 91, 9208–9212
14. Cottrell, G. S., Amadesi, S., Grady, E. F., and Bunnell, N. W. (2004) Trypsin IV, a novel agonist of protease-activated receptors 2 and 4. J. Biol. Chem. 279, 13532–13539
15. Knecht, W., Cottrell, G. S., Amadesi, S., Mohlin, J., Kärägård, A., Gedda, K., Peterson, A., Chapman, K., Hollenberg, M. D., Vergnolle, N., and Bunnell, N. W. (2007) Trypsin IV or mesotrypsin and p23 cleave protease-activated receptors 1 and 2 to induce inflammation and hyperalgesia. J. Biol. Chem. 282, 26089–26100
16. Corvera, C. U., Déry, O., McConalogue, K., Böhm, S. K., Khttin, L. M., Caughey, G. H., Payan, D. G., and Bunnell, N. W. (1997) Mast cell tryptase regulates rat colonic myocytes through proteinase-activated receptor 2. J. Clin. Invest. 100, 1383–1393
17. Molino, M., Barnathan, E. S., Numerof, R., Clark, J., Dreyer, M., Cumashi, A., Hoxie, J. A., Schechter, N., Woolkalis, M., and Brass, L. F. (1997) Interactions of mast cell tryptase with thrombin receptors and PAR-2. J. Biol. Chem. 272, 4043–4049
18. Camerer, E., Huang, W., and Coughlin, S. R. (2000) Tissue factor and factor X-dependent activation of protease-activated receptor 2 by factor VIIa. Proc. Natl. Acad. Sci. U.S.A. 97, 5255–5260
19. Smith, R., Jenkins, A., Lourbakos, A., Thompson, P., Ramakrishnan, V., Toomilinson, J., Deshpande, U., Johnson, D. A., Jones, R., Mackie, E. J., and Pike, R. N. (2000) Evidence for the activation of PAR-2 by the sperm protease, acrosin: expression of the receptor on oocytes. FEBS letters 484, 285–290
20. Hansen, K. K., Sherman, P. M., Cellars, L., Andrade-Gordon, P., Pan, Z., Baruch, A., Wallace, J. L., Hollenberg, M. D., and Vergnolle, N. (2005) A major role for proteolytic activity and proteinase-activated receptor-2 in
the pathogenesis of infectious colitis. Proc. Natl. Acad. Sci. U.S.A. 102, 8363–8368.

21. Takeuchi, T., Harris, J. L., Huang, W., Yan, K. W., Coughlin, S. R., and Craik, C. S. (2000) Cellular localization of membrane-type serine protease 1 and identification of protease-activated receptor-2 and single-chain urokinase-type plasminogen activator as substrates. J. Biol. Chem. 275, 26333–26342.

22. Wilson, S., Greer, B., Hooper, J., Zijlstra, A., Walker, B., Quigley, J., and Hawthorne, S. (2005) The membrane-anchored serine protease, TM-PRSS2, activates PAR-2 in prostate cancer cells. Biochem. J. 386, 967–972.

23. Mize, G. J., Wang, W., and Takayama, T. K. (2008) Prostate-specific kalireniks-2 and -4 enhance the proliferation of DU-145 prostate cancer cells through protease-activated receptors-1 and -2. Mol. Cancer Res.: MCR 6, 1043–1051.

24. Okkonenpalou, K., Hansen, K. K., Saijieddine, M., Tea, I., Blaber, M., Blaber, S. I., Scarsbrick, I., Andrade-Gordon, P., Cottrell, G. S., Bunnell, N. W., Diamantis, E. P., and Hollenberg, M. D. (2006) Proteinase-activated receptors, targets for kalirenik signalling. J. Biol. Chem. 281, 32095–32112.

25. Ramsay, A. J., Dong, Y., Hunt, M. L., Linn, M., Samaratunga, H., Clemens, J. A., and Hooper, J. D. (2008) Kalirenik-related peptide 4 (KLK4) initiates intracellular signaling via protease-activated receptors (PARs). KLK4 and PAR-2 are co-expressed during prostate cancer progression. J. Biol. Chem. 283, 12293–12304.

26. Ramsay, A. J., Reid, J. C., Adams, M. N., Samaratunga, H., Dong, Y., Clemens, J. A., and Hooper, J. D. (2008) Prostatic trypsin-like kalirenik-related peptides (KLKs) and other prostate-expressed trypsin proteinases as regulators of signalling via protease-activated receptors (PARs). Biol. Chem. 389, 653–668.

27. Amadesi, S., Nie, J., Vergnolle, N., Cottrell, G. S., Grady, E. F., Trevisani, M., Manni, C., Geppetti, P., McRoberts, J. A., Ennes, H., Davis, J. B., Mayer, E. A., and Bunnell, N. W. (2004) Protease-activated receptor 2 sensitizes the capsacin receptor transient receptor potential vanilloid receptor 1 to hyperalgesia. J. Neurosci. 24, 4300–4312.

28. Grant, A. D., Cottrell, G. S., Amadesi, S., Trevisani, M., Nicoletti, P., Materazzi, S., Altier, C., Cenac, N., Zamponi, G. W., Bautista-Cruz, F., Lopez, C. B., Joseph, E. K., Levine, J. D., Lidtke, W., Vanner, S., Vergnolle, N., Geppetti, P., and Bunnell, N. W. (2007) Protease-activated receptor 2 sensitizes the transient receptor potential vanilloid 4 ion channel to cause mechanical hyperalgesia in mice. J. Physiol. 578, 715–733.

29. Poole, D. P., Amadesi, S., Veldhuis, N. A., Abogadie, F. C., Lieu, T., Darby, W., Lidtke, W., Lew, M. J., McIntyre, P., and Bunnell, N. W. (2013) Protease-activated receptor 2 (PAR2) protein and transient receptor potential vanilloid 4 (TRPV4) protein coupling is required for sustained inflammatory signaling. J. Biol. Chem. 288, 5790–5802.

30. Sipe, W. E., Brierley, S. M., Martin, C. M., Phillips, B. D., Cruz, F. B., Grady, E. F., Lidtke, W., Cohen, D. M., Vanner, S., Blackshaw, L. A., and Bunnell, N. W. (2008) Transient receptor potential vanilloid 4 (TRPV4) protein coupling is required for sustained inflammatory signaling. Am. J. Physiol. Lung Cell Mol. Biol. 302, 411–419.

31. Dey, O., Thoma, M. S., Wong, H., Grady, E. F., and Bunnell, N. W. (1999) Trafficking of proteinase-activated receptor-2 and β-arrestin-1 tagged with green fluorescent protein. β-Arrestin-dependent endocytosis of a proteinase receptor. J. Biol. Chem. 274, 18524–18535.

32. Jensen, D. D., Godfrey, C. B., Niklas, C., Canals, M., Kocan, M., Poole, D. P., Murphy, J. E., Bunell, N. W., and Korbmacher, C. (2012) Proteolytic activation of the epithelial sodium channel (ENaC) by the cytosine proteinase cathepsin-S. Pflugers Arch. 464, 355–365.

33. Liedtke, D., Leduc, D., Cottrell, G. S., D’Alayer, J., Hansen, K. K., Bunnell, N. W., Hollenberg, M. D., Pidard, D., and Chignard, M. (2005) Pseudomonas aeruginosa elastase enables proteinase-activated receptor 2 in respiratory epithelial cells. Am. J. Resp. Cell Mol. Biol. 32, 411–419.

34. Gilad, C., Rebois, R. V., Hogue, M., Trieu, P., Breit, A., Hebert, T. E., and Bouvier, M. (2005) Real-time monitoring of receptor and G-protein interactions in living cells. Nature Methods 2, 177–184.

35. Galés, C., Damerell, B., Sebeer, R. M., Feldman, B. J., and Pfleger, K. D. (2011) Enhanced BRET Technology for the Monitoring of Agonist-Induced and Agonist-Independent Interactions between GPCRs and β-Arrestins. Frontiers in Endocrinology 1, 12.

36. Chaplan, S. R., Bach, F. W., Pogrel, J. W., Chung, J. M., and Yaksh, T. L. (1994) Quantitative assessment of tactile allodynia in the rat paw. J. Neurosci. Methods 53, 55–63.

37. Mihara, K., Ramachandran, R., Zamponi, G. W., Materazzi, S., Nussini, R., Lidtke, W., Cattaruzza, F., Grady, E. F., Geppetti, P., and Bunnell, N. W. (2010) A role for transient receptor potential vanilloid 4 in nociception and tumorigenesis of breast cancer cells. Cell 120, 303–313.

38. Trivedi, V., Boire, A., Letcher, B., Bouvier, M., and Hollenberg, M. D. (2013) Neutrophil Elastase and Proteinase-3 Trigger G Protein-Biased Signaling through Proteinase-Activated Receptor-1 (PAR1). J. Biol. Chem. 288, 32979–32990.

39. Boire, A., Covic, L., Agarwal, A., Jannes, S., Sherifi, S., and Kuliopulos, A. (2005) PAR1 is a matrix metalloprotease-1 receptor that promotes invasion and tumorigenesis of breast cancer cells. Cell 120, 303–313.

40. Austin, K. M., Covic, L., and Kuliopulos, A. (2013) Matrix metalloproteases and PAR1 activation. Blood 121, 431–439.

41. Mosnier, L. O., Sinha, R. K., Burnier, L., Bouwens, E. A., and Griffin, J. H. (2012) Biased agonism of proteinase-activated receptor 1 by activated protein C caused by noncanonical cleavage at Arg46. Blood 120, 5237–5246.

42. Schuepbach, R. A., Madon, J., Ender, M., Galli, P., and Riewald, M. (2012) Protease-activated receptor-1 cleaved at R46 mediates cytoprotective effects. J. Thromb. Haemost. 10, 1675–1684.

43. Lindner, J. R., Kahn, M. L., Coughlin, S. R., Sambrano, G. R., Schable, E., Bernstein, D., Doy, F., Hafezi-Moghadam, A., and Ley, K. (2000) Delayed onset of inflammation in proteinase-activated receptor-2-deficient mice. J. Immunol. 165, 6504–6510.

44. Dulon, S., Leduc, D., Cottrell, G. S., D’Alayer, J., Hansen, K. K., Bunnell, N. W., Hollenberg, M. D., Pidard, D., and Chignard, M. (2005) Pseudomonas aeruginosa elastase enables proteinase-activated receptor 2 in respiratory epithelial cells. Am. J. Resp. Cell Mol. Biol. 32, 411–419.

45. Geppetti, P., and Bunell, N. W. (2010) A role for transient receptor potential vanilloid 4 in tonicity-induced neurogenic inflammation. J. Immunol. 184, 1215–1222.

46. Herkens, S., Krappitz, M., Bertog, M., Krappitz, A., Baraznenok, V., Henke, S. I., Scarisbrick, I., Andrade-Gordon, P., Cottrell, G. S., and Bunnett, N. W. (2000) Neutrophil elastase acts as a biased agonist for proteinase-activated receptor-2. J. Biol. Chem. 275, 22942–22960.

47. McGuire, J. J., Saifeddine, M., Triggle, C. R., Sun, K., and Hollenberg, M. D. (2004) 2-furyl-LIGRLO-amide: a potent and selective proteinase-activated receptor 2 agonist. J. Pharmacol Experimental Therapeutics 309, 1124–1131.
Cathepsin S Biased Agonism of PAR$_2$

54. Böhm, S. K., Khitin, L. M., Grady, E. F., Aponte, G., Payan, D. G., and Bunnett, N. W. (1996) Mechanisms of desensitization and resensitization of proteinase-activated receptor-2. *J. Biol. Chem.* **271**, 22003–22016

55. Suen, J. Y., Barry, G. D., Lohman, R. J., Halili, M. A., Cotterell, A. J., Le, G. T., and Fairlie, D. P. (2012) Modulating human proteinase activated receptor 2 with a novel antagonist (GB88) and agonist (GB110). *Br. J. Pharmacol.* **165**, 1413–1423

56. Biniossek, M. L., Nágler, D. K., Becker-Pauly, C., and Schilling, O. (2011) Proteomic identification of protease cleavage sites characterizes prime and non-prime specificity of cysteine cathepsins B, L, and S. *J. Proteome Res.* **10**, 5363–5373

57. Choe, Y., Leonetti, F., Greenbaum, D. C., Lecaille, F., Bogyo, M., Brömme, D., Ellman, J. A., and Craik, C. S. (2006) Substrate profiling of cysteine proteases using a combinatorial peptide library identifies functionally unique specificities. *J. Biol. Chem.* **281**, 12824–12832

58. Elmariah, S. B., Reddy, V. B., and Lerner, E. A. (2014) Cathepsin S Signals via PAR2 and Generates a Novel Tethered Ligand Receptor Agonist. *PloS one* **9**, e99702

59. Ayoub, M. A., and Pin, J. P. (2013) Interaction of Protease-Activated Receptor 2 with G Proteins and β-Arrestin 1 Studied by Bioluminescence Resonance Energy Transfer. *Frontiers in Endocrinology* **4**, 196

60. Lerner, D. J., Chen, M., Tram, T., and Coughlin, S. R. (1996) Agonist recognition by proteinase-activated receptor 2 and thrombin receptor. Importance of extracellular loop interactions for receptor function. *J. Biol. Chem.* **271**, 13943–13947

61. Fan, H. C., Zhang, X., and McNaughton, P. A. (2009) Activation of the TRPV4 ion channel is enhanced by phosphorylation. *J. Biol. Chem.* **284**, 27884–27891

62. Lohman, R. J., Cotterell, A. J., Barry, G. D., Liu, L., Suen, J. Y., Vesey, D. A., and Fairlie, D. P. (2012) An antagonist of human protease activated receptor-2 attenuates PAR2 signaling, macrophage activation, mast cell degranulation, and collagen-induced arthritis in rats. *FASEB J.* **26**, 2877–2887

63. Denadai-Souza, A., Martin, L., de Paula, M. A., de Avellar, M. C., Muscará, M. N., Vergnolle, N., and Cenac, N. (2012) Role of transient receptor potential vanilloid 4 in rat joint inflammation. *Arthritis Rheumatism* **64**, 1848–1858

64. Cenac, N., Andrews, C. N., Holzhausen, M., Chapman, K., Cottrell, G., Andrade-Gordon, P., Steinhoff, M., Barbara, G., Beck, P., Bunnett, N. W., Sharkey, K. A., Ferraz, J. G., Shaffer, E., and Vergnolle, N. (2007) Role for protease activity in visceral pain in irritable bowel syndrome. *J. Clin. Invest.* **117**, 636–647

65. Fichna, J., Mokrowiecka, A., Cygankiewicz, A. I., Zakrzewski, P. K., Malecka-Panas, E., Janecka, A., Krajewska, W. M., and Storr, M. A. (2012) Transient receptor potential vanilloid 4 blockade protects against experimental colitis in mice: a new strategy for inflammatory bowel diseases treatment? *Neurogastro Motility* **24**, e557–e560