Protease-activated receptor-2 in endosomes signals persistent pain of irritable bowel syndrome

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Once activated at the surface of cells, G protein-coupled receptors (GPCRs) redistribute to endosomes, where they can continue to signal. Whether GPCRs in endosomes generate signals that contribute to human disease is unknown. We evaluated endosomal signaling of protease-activated receptor-2 (PAR2), which has been proposed to mediate pain in patients with irritable bowel syndrome (IBS). Trypsin, elastase, and cathepsin S, which are activated in the colonic mucosa of patients with IBS and in experimental animals with colitis, caused persistent PAR2-dependent hyperexcitability of nociceptors, sensitization of colonic afferent neurons to mechanical stimuli, and somatic mechanical allodynia. Inhibitors of clathrin- and dynamin-dependent endocytosis and of mitogen-activated protein kinase kinase-1 prevented trypsin-induced hyperexcitability, sensitization, and allodynia. However, they did not affect elastase- or cathepsin S-induced hyperexcitability, sensitization, or allodynia. Trypsin stimulated endocytosis of PAR2, which signaled from endosomes to activate extracellular signal-regulated kinase. Elastase and cathepsin S did not stimulate endocytosis of PAR2, which signaled from the plasma membrane to activate adenyl cyclase. Biopsies of colonic mucosa from IBS patients released proteases that induced persistent PAR2-dependent hyperexcitability of nociceptors, and PAR2 association with β-arrestins, which mediate endocytosis. Conjugation to cholestanol promoted delivery and retention of antagonists in endosomes containing PAR2. A cholestanol-conjugated PAR2 antagonist prevented persistent trypsin- and IBS protease-induced hyperexcitability of nociceptors. The results reveal that PAR2 signaling from endosomes underlies the persistent hyperexcitability of nociceptors that mediates chronic pain of IBS. Endosomally targeted PAR2 antagonists are potential therapies for IBS pain. GPCRs in endosomes transmit signals that contribute to human diseases.

There is a growing realization that G protein-coupled receptors (GPCRs), which were formerly considered to function principally at the surface of cells, can continue to signal from endosomes by mechanisms that involve β-arrestins (βARRs) and G proteins (1). Although GPCR signaling begins at the plasma membrane, activated receptors associate with βARRs, which mediate receptor desensitization and endocytosis (2). These processes efficiently terminate GPCR signaling at the plasma membrane. The detection of GPCR signaling complexes in endosomes, and the finding that disruption of endocytosis can suppress signaling, both suggest that GPCRs signal from endosomes (3–12). GPCRs in endosomes can generate persistent signals in subcellular compartments that control gene transcription and neuronal excitation (8, 11, 12). Although endosomal signaling of GPCRs can regulate important physiological processes, including pain transmission (8, 12), the contribution of endosomal signaling to human disease is far from clear.

Protease-activated receptor-2 (PAR2) mediates the inflammatory and pronociceptive actions of proteases (13). Given the irreversible mechanism of proteolytic activation, PAR2 may be capable of persistent signaling at the plasma membrane and in endosomes | receptors | pain | proteases

Significance

Activated G protein-coupled receptors (GPCRs) internalize and can continue to signal from endosomes. The contribution of endosomal signaling to human disease is unknown. Proteases that are generated in the colon of patients with irritable bowel syndrome (IBS) can cleave protease-activated receptor-2 (PAR2) on nociceptors to cause pain. We evaluated whether PAR2 generates signals in endosomes of nociceptors that mediate persistent hyperexcitability and pain. Biopsies of colonic mucosa from IBS patients released proteases that induced PAR2 endocytosis, endosomal signaling, and persistent hyperexcitability of nociceptors. When conjugated to the transmembrane lipid cholestanol, PAR2 antagonists accumulated in endosomes and suppressed persistent hyperexcitability. The results reveal the therapeutic potential of endosomally targeted PAR2 antagonists for IBS pain, and expand the contribution of endosomal GPCR signaling to encompass processes that are relevant to disease.


Conflict of interest statement: P.M. and G.A.H. work for Takeda Pharmaceuticals, Inc. and N.W.B. wrote the paper. N.W.B. and G.A.H. are founding scientists of Endosome Therapeutics, Inc.

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endosomes. Trypsin and mast cell tryptase activate PAR₂ by canonical mechanisms that induce receptor association with βARRs, endocytosis, and endosomal signaling (5, 14). Neutrophil elastase (NE) and macrophage cathepsin S (CS) cleave PAR₂ at different sites and activate PAR₂ by biased mechanisms that do not induce receptor interactions with βARRs or endocytosis (15, 16). The contribution of PAR₂ signaling at the plasma membrane and in endosomes to the disease-relevant actions of proteases is unknown.

Proteases and PAR₂ have been implicated in the hypersensitivity of sensory nerves in the colon that may account for chronic pain in patients with irritable bowel syndrome (IBS) (17). Biopsies of colonic mucosa from IBS patients secrete proteases, including tryptase and trypsin-3, which induce PAR₂-dependent hyperexcitability of nociceptors and colonic nociception in mice (18–21). PAR₂ agonists induce a remarkably long-lasting hyperexcitability of neurons by unknown mechanisms (22, 23). Whether PAR₂ at the plasma membrane or in endosomes is a target for the treatment of IBS pain remains to be determined.

We examined the hypothesis that two components of PAR₂ signaling contribute to the persistent hyperexcitability of nociceptors in IBS: the irreversible mechanism of proteolytic activation and the capacity of PAR₂ to generate sustained signals from endosomes.

**Results**

**PAR₂-Mediated Nociception.** Proteases may induce pain by activating PAR₂ on nociceptors or other cell types. To determine the contribution of PAR₂ on nociceptors, we bred mice expressing Par₂ flanked by LoxP sites (Par₂<sup>fluor</sup>) with mice expressing Cre recombinase targeted to nociceptors using the Na<sub>1.8</sub> promoter (Scn10a). Par₂<sup>-Na<sub>1.8</sub></sup> mice lacked immunoreactive PAR₂ in Na<sub>1.8</sub> neurons of the dorsal root ganglia (DRG) (Fig. 14). Whereas 31% (20 of 65) of small-diameter (<25 μm) DRG neurons from WT mice responded to trypsin (100 nM) with increased [Ca<sup>2+</sup>]<sub>i</sub>, only 6% (3 of 51) of neurons from Par₂<sup>-Na<sub>1.8</sub></sup> mice responded (Fig. 1B and SI Appendix, Fig. S1 A and B). We assessed nociception by measuring withdrawal responses to stimulation of the plantar surface of the hindpaw with von Frey filaments (VFF). In WT mice, intraplantar injection (10 μL) of trypsin (80 nM), NE (3.9 μM), or CS (5 μM) induced mechanical allodynia within 30 min, which was maintained for 180 min (Fig. 1 C–E). In Par₂<sup>-Na<sub>1.8</sub></sup> mice, the initial responses were maintained, but responses after 120 min were diminished. At 180 min, when mechanical allodynia in WT mice was fully maintained, responses in Par₂<sup>-Na<sub>1.8</sub></sup> mice had returned to baseline (trypsin, NE) or were significantly attenuated (CS). In WT mice, intraplantar trypsin increased paw thickness—measured using calipers—which peaked at 1 h and was maintained for 4 h, and stimulated an influx of neutrophils after 4 h, consistent with inflammation (SI Appendix, Fig. S1 C and D). Tryptsin-induced inflammation was markedly diminished in Par₂<sup>-Na<sub>1.8</sub></sup> mice.

To assess the contribution of endocytosis to protease-induced nociception, Dynogoa4a (Dy4, dynamin inhibitor) (24), PitStop2 (PS2, clathrin inhibitor) (25), inactive (inact) analogs (50 μM), or vehicle (0.2% DMSO, 0.9% NaCl) (10 μL) was administered by
intraplantar injection to mice. After 30 min, trypsin (10 nM), NE (1.2 μM), or CS (2.5 μM) (10 μL) was injected into the same paw. In controls (vehicle or inactive analogs), trypsin, NE, and CS induced mechanical allodynia that was fully maintained for 4 h (Fig. 1 F–K). Dy4 and PS2 inhibited trypsin-induced allodynia at 1 and 2 h (Fig. 1 F and J), whereas NE- (Fig. 1 G and J) and CS- (Fig. 1 H and K) induced allodynia was unchanged. Endocytic inhibitors or proteases did not influence withdrawal responses of the noninjected contralateral paw (SI Appendix, Fig. S2 A and B). Trypsin, NE, and CS increased paw thickness, consistent with edema (SI Appendix, Fig. S2 C–H). Dynamin and clathrin inhibitors did not affect edema.

The results suggest that proteases induce persistent nociception and neurogenic inflammation in large part by activating PAR2 on Nav1.8+ neurons. PAR2 endocytosis is necessary for the nociceptive actions of trypsin, but not NE or CS.

**PAR2-Mediated Hyperexcitability of Nociceptors.** To evaluate the contribution of endocytosis to protease-induced hyperexcitability of nociceptors, the rheobase (the minimal input current required to fire one action potential) of small diameter neurons of mouse DRG was measured by patch-clamp recording. Neurons were preincubated with trypsin (50 nM, 10 min), NE (390 nM, 30 min), CS (500 nM, 60 min) (conditions selected to cause robust hyperexcitability), or vehicle, and washed. Rheobase was measured 0 or 30 min after washing. The mean rheobase of protease- or vehicle-treated neurons was calculated. Trypsin, NE, and CS decreased rheobase at 0 and 30 min, indicating an initial hyperexcitability that is maintained for at least 30 min (Fig. 2). Dy4 (30 μM) or PS2 (15 μM) did not affect the capacity of trypsin, NE, or CS to cause initial hyperexcitability (0 min). Dy4 and PS2 abolished the persistent effects of trypsin (Fig. 2 A–C), but not of NE (Fig. 2 D and E) or CS (Fig. 2 F and G) (30 min). Dy4, PS2, or vehicle (0.3% DMSO) did not affect the excitability of DRG neurons (SI Appendix, Fig. S3).

I-343 is an imidazopyridazine derivative that has been described as a potent PAR2 antagonist in the patent literature (26) (SI Appendix, Fig. S4A). I-343 belongs to the same family of PAR2 antagonists as I-191, a full antagonist of PAR2 that inhibits multiple components of PAR2 signaling, including those that may mediate protease-induced pain (27). We investigated whether I-343 inhibits PAR2 signaling in HT-29 and HEK293 cells, which express endogenous PAR2, and in KNRK cells transfected with human (h) PAR2. Accumulation of inositol phosphate-1 (IP1) was measured in response to trypsin or the PAR2-selective agonist 2-Furoyl-LIGRLO-NH2 (2F), an analog of the trypsin-exposed tethered ligand. I-343 inhibited 2F (300 nM)-induced IP1 in HT-29 cells (pIC50 8.93 ± 0.11, IC50 1.1 nM) and 2F (100 nM)-induced IP1 in KNRK-hPAR2 cells (pIC50 6.18 ± 0.11, IC50 666 nM) (SI Appendix, Fig. S4 B–D). I-343 inhibited trypsin (30 nM)-induced IP1 in HEK293 cells (pIC50 9.36 ± 0.20, IC50 0.4 nM) and in KNRK-hPAR2 cells (pIC50 5.13 ± 0.14, IC50 7507 nM). I-343 did not affect ATP (10 μM)-stimulated IP1 in KNRK cells (SI Appendix, Fig. S4E).

I-343 (10 μM) prevented the decrease in rheobase 30 min after trypsin and CS, but not NE (Fig. 3 A–C). However, I-343 prevented the decrease in rheobase 0 min after NE (Fig. 3D). I-343 (100 nM, 300 nM) also prevented the decrease in rheobase 0 min after trypsin (SI Appendix, Fig. S5A). When neurons were incubated with thrombin (50 nM, 20 min) and washed, there was an immediate decrease in rheobase that was prevented by preincubation with the PAR2 antagonist SCH97979 (1 μM, 10 min) (28); SCH97979 alone had no effect (SI Appendix, Fig. S5B) and SCH97979 did not affect the response to trypsin (SI Appendix, Fig. S5C). Thus, PAR2 mediates the immediate and persistent actions of trypsin, the persistent actions of CS, and the initial effects of NE; NE causes persistent hyperexcitability by a different mechanism. PAR2 does not mediate the initial actions of trypsin.

Another PAR2 antagonist, GB88, also prevents trypsin, NE, and CS activation of nociceptors (29). Trypsin-activated PAR2 signals from endosomes by βARR- and Raf-1–dependent processes, which activate ERK (5). PD98059 (50 μM), which inhibits activation of mitogen-activated protein kinase kinase-1 (MEK1) (30), did not affect initial trypsin-induced hyperexcitability, but prevented persistent trypsin-induced hyperexcitability (Fig. 3E). In contrast, GFI09203X (Bis-1, 10 μM), which inhibits PKCα and other kinases (30), prevented the initial but not the persistent effects of trypsin (Fig. 3F).

The results suggest that trypsin induces initial hyperexcitability of nociceptors by PAR2/PKC signaling from the plasma membrane, and persistent hyperexcitability by PAR2/ERK signaling from endosomes. Adenylyl cyclase and PKA mediate NE- and CS-induced hyperexcitability of nociceptors (15, 16), which was not further studied.
PARβ Endocytosis and Compartmentalized Signaling in Nociceptors.

To assess endocytosis of PARβ in nociceptors, we transfected mouse (m) PARβ-GFP into mouse DRG neurons. In vehicle-treated neurons, mPARβ-GFP was detected at the plasma membrane and in intracellular compartments that may correspond to the prominent stores of PARβ in the Golgi apparatus (Fig. 4A) (31). Trypsin, but not NE or CS (100 nM, 30 min), induced removal of mPARβ-GFP from the plasma membrane and accumulation in endosomes (Fig. 4 A and B). Dy4, but not Dy4 inact, inhibited trypsin-induced endocytosis of mPARβ-GFP (Fig. 4C).

To determine whether PARβ recruits βARRs, which mediate endocytosis of PARβ (14), we expressed bioluminescence resonance energy transfer (BRET) sensors for PARβ–RLuc8 (donor) and βARR2-YFP (acceptor) in mouse DRG neurons. Trypsin, but not NE or CS, stimulated PARβ–RLuc8/βARR2-YFP BRET over 25 min (Fig. 4D).

To determine whether trypsin causes PARβ-dependent activation of PKC and ERK, which respectively mediate the initial and persistent phases of trypsin-induced hyperexcitability of nociceptors, we expressed genetically encoded FRET biosensors in neurons. The biosensors are targeted to subcellular compartments and are reversibly modified by kinases and phosphatases. They are suitable for analysis of signaling in subcellular compartments with high spatial and temporal resolution (8, 12). Biosensors for plasma membrane PKC (pmCKAR), cytosolic PKC (CytoCKAR), cytosolic ERK (CytoERK), and nuclear ERK (NucERK) were expressed in DRG neurons from rat, because pilot studies revealed more robust and consistent PARβ responses than in mouse neurons. Trypsin (100 nM) activated PKC at the plasma membrane but not in the cytosol (Fig. 4 E–G), and activated ERK in the cytosol and nucleus (Fig. 4 H–J). The PARβ antagonist I-343 (10 μM) inhibited trypsin-induced activation of PKC and ERK, whereas the PARβ antagonist SCH530348 (100 nM) had no effect (Fig. 4 F and I). At the end of experiments, neurons were challenged with the positive controls phorbol 12,13-dibutyrate (PDBu) for EKAR biosensors or PDBu plus phosphatase inhibitor mixture-2 for CKAR biosensors, to ensure that the response of the biosensor was not saturated.

The results suggest that trypsin, but not NE or CS, stimulates βARR recruitment and dynamin-dependent endocytosis of PARβ in nociceptors. Trypsin causes PARβ-dependent activation of PKC at the plasma membrane and ERK in the cytosol and nucleus.

Mechanisms of PARβ Endocytosis and Endosomal Signaling. We examined the mechanism of PARβ endocytosis and endosomal signaling in HEK293 cells. To quantify the removal of PARβ from the plasma membrane and its accumulation in early endosomes, we measured bystander BRET between PARβ and proteins that are resident at the plasma membrane (Ras-like protein expressed in many tissues, or RIT) and early endosomes (Ras-related protein Rab5a) (8, 12). This application of BRET takes advantage of nonspecific protein–protein interactions to track movement of membrane proteins through different compartments (32). Trypsin induced a decrease in PARβ–RLuc8/RIT-Venus BRET (EC50 2.9 nM), and an increase in PARβ–RLuc8/Rab5a-Venus BRET (EC50 2.7 nM) (Fig. 5 A and B and SI Appendix, Fig. S6 A–D). Neither NE nor CS (100 nM) affected PARβ–RLuc8/RIT-Venus or Rab5a-Venus BRET (Fig. 5 A and B). PS2, but not PS2 inact, suppressed the trypsin-induced decrease in PARβ–RLuc8/RIT-Venus BRET and increase in PARβ–RLuc8/Rab5a-Venus BRET (Fig. 5 C and D and SI Appendix, Fig. S6 E and F). Dominant-negative dynaminK44E (DynK44E), which is deficient in GTP binding (33), increased the trypsin-induced decrease in PARβ–RLuc8/Rab5a-Venus BRET, but did not affect PARβ–RLuc8/RIT-Venus BRET (Fig. 5 C and D and SI Appendix, Fig. S6 G and H). WT dynamin (DynWT) had minimal effects. Because GTP binding is required for scission of budding vesicles from the plasma membrane, DynK44E presumably traps PARβ in membrane vesicles, which would impede interaction with Rab5a but not RIT. Thus, trypsin, but not CS or NE, induces clathrin- and dynamin-dependent endocytosis of PARβ.

We investigated the contribution of endocytosis to trypsin-induced signaling in HEK293 cells expressing Flag-PARβ–HA11 and FRET biosensors for cytosolic and nuclear ERK (CytoERK, NucERK), plasma membrane and cytosolic PKC (pmCKAR, CytoCKAR), and plasma membrane and cytosolic cAMP (pmEpac, CytoEpac). Trypsin (10 nM), but not NE or CS (100 nM), stimulated a rapid and persistent activation of ERK in the cytosol and nucleus (EC50, 5 nM) (Fig. 5 E and F and SI Appendix, Fig. S7 A–F). I-343 (10 μM) but not the PARβ antagonist SCH530348 (100 nM) inhibited trypsin activation of cytosolic and nuclear ERK (Fig. 5G). PS2 and DynK44E inhibited trypsin-stimulated activation of cytosolic and nuclear ERK compared with PS2 inact and DynWT controls (Fig. 5 H and I and SI Appendix, Fig. S7 G–J). AG1476 (1 μM), an inhibitor of EGFR receptor tyrosine kinase (34), UBO-QIC (100 nM), which inhibits Gq, and certain Gq signals (35), and G66983 (1 μM), which inhibits all isoforms of PKC (36), suppressed trypsin-stimulated activation of cytosolic ERK (Fig. 5I and SI Appendix, Fig. S7K). UBO-QIC and G66983 also inhibited activation of nuclear ERK (Fig. 5K and SI Appendix, Fig. S7L). The results suggest that PARβ signals from endosomes by Gq, q-dependent mechanisms to activate ERK in the cytosol and nucleus.

To determine whether trypsin induces translocation of βARR and Gq, to endosomes, we measured BRET between βARR1–RLuc8 or Gq,–RLuc8 and Rab5a-Venus in HEK293 cells. Trypsin (100 nM)
ERK + B and PAR in endosomes is a signaling, which sustains the hyperexcitability of nociceptors exposed to signalosome in early endosomes.

Stimulation of cells with the positive controls PDBu (EKar), PDBu + phosphatase inhibitor mixture-2 (CKAR), or forskolin + 3-isobutyl-1-methylxanthine (Epac) revealed that responses to proteases did not saturate the FRET biosensors (Fig. 5 E and F and SI Appendix, Fig. S9 A–D).

**IBS-Induced Hyperexcitability of Nociceptors.** We investigated whether proteases from mucosal biopsies of IBS patients cause a persistent hyperexcitability of nociceptors by a mechanism that entails endosomal signaling of PAR2. Biopsies of colonic mucosa from patients with diarrhea-predominant IBS (IBS-D) or healthy control (HC) subjects were placed in culture medium (24 h, 37 °C). Mouse DRG neurons were then exposed to biopsy supernatants (30 min, 37 °C) and washed. Rheobase was measured 30 min after washing to assess persistent hyperexcitability. Supernatants of biopsies from IBS-D patients caused a persistent decrease in rheobase, consistent with hyperexcitability, compared with supernatants from HC subjects (rheobase at 30 min: HC, 78.33 ± 4.41 pA; 12 neurons, supernatant from four HC: IBS-D, 54.55 ± 4.74 pA, 11 neurons, supernatant from four IBS-D: P < 0.05; ANOVA, Tukey’s multiple comparisons test) (Fig. 6 A and B). I-343 (PAR2 antagonist, 10 μM), Dy4 (dynamin inhibitor, 30 μM), and PD98059 (MEK1 inhibitor, 50 μM) abolished IBS-D–induced hyperexcitability of nociceptors (Fig. 6 A–D). Dy4 caused a nonsignificant decrease in rheobase of neurons exposed to HC supernatant, but I-343 and PD98059 had no effect.

To examine whether proteases in IBS-D supernatants can stimulate endocytosis of PAR2, BRET was used to assess the proximity between PAR2–RLuc8 and Rab5a–Venus expressed in HEK293 cells. IBS-D supernatant increased PAR2–RLuc8/Rab5a–Venus BRET after 60 min compared with HC supernatant (Fig. 6F). Trypsin (10 nM, positive control) also increased PAR2–RLuc8/Rab5a–Venus BRET.

These results suggest that proteases that are released from biopsies of colonic mucosa from patients with IBS-D cause long-lasting hyperexcitability of nociceptors by a mechanism that requires dynamin-dependent endocytosis of PAR2 and PAR2 ERK signaling from endosomes.

**Antagonist Delivery to PAR2 in Endosomes.** If endosomal signaling of PAR2 sustains the hyperexcitability of nociceptors exposed to supernatants from IBS-D patients, do PAR2 antagonists that are targeted to endosomal receptors effectively reverse this process? Conjugation to the transmembrane lipid cholesterol facilitates endosomal delivery of antagonists of the neurokinin 1 receptor (NK1R) and calcitonin receptor-like receptor (CLR), which provide more efficacious and long-lasting antinociception than conventional antagonists that do not target receptors in endosomes (8, 12). To evaluate whether PAR2 in endosomes is a therapeutic target, tripartite probes were synthesized comprising: cholesterol to anchor probes to membranes or ethyl ester that does not incorporate into membranes; a polyethylene glycol (PEG) 12 linker to facilitate presentation in an aqueous environment; and a cargo of cyanine 5 (Cy5) for localization or PAR2 targeted to endosomal receptors effectively reverse this process?

**Fig. 4.** PAR2 endocytosis, βARR2 recruitment, and compartmentalized signaling in nociceptors. (A–C) PAR2 endocytosis. (A) Representative image of three experiments) of effects of trypsin (Tryp) on the distribution of mPAR2–GFP in mouse DRG neurons. Arrowheads (A, Left) show PAR2–GFP at the plasma membrane. Arrows (A, Right) show PAR2–GFP in endosomes. (B and C) Cytosol/plasma membrane ratio of mPAR2–GFP in mouse DRG neurons after 30-min incubation with trypsin, NE, or CS (B), or after preincubated with Dy4 or Dy4 inact and then trypsin (C). (D) PAR2–RLuc8/RAB2–YFP BRET in mouse DRG neurons exposed to trypsin, NE or CS. AUC area under curve (25 min) *P < 0.05 to vehicle. n, experimental replicates, triplicate observations. (E–F) Compartmentalized signaling. Effects of trypsin on PKC activity at the plasma membrane (E and F) and in the cytosol (G), and on ERK activity in the cytosol (H and I), and nucleus (J) of rat DRG neurons. Numbers in bars denote neuron numbers. *P < 0.05, **P < 0.01 to vehicle.
Cy5-Chol. Video-imaging revealed frequent association of endosomes containing PAR2-GFP and Cy5-Chol (Movie S1). I-343–PEG-cholesterol (MIPS15479) (SI Appendix, Fig. S10A) antagonized 2F-stimulated IP3 accumulation in HT-29 cells (pIC50 6.18 ± 0.07; IC50 670 nM), albeit with reduced potency compared with the parent compound I-343 (pIC50 8.96 ± 0.10; IC50 1.1 nM) (SI Appendix, Fig. S10C).

Antagonism of Endosomal PAR2 and Hyperexcitability of Nociceptors.

To evaluate the capacity of an endosomally targeted PAR2 antagonist to inhibit protease-induced hyperexcitability of nociceptors, mouse DRG neurons were preincubated with MIPS15479 (30 μM) or vehicle (60 min, 37 °C), washed, and recovered in antagonist-free medium for 180 min to allow accumulation of antagonists in endosomes (Fig. S4). Transient incubation with trypsin decreased rheobase of vehicle-treated neurons at 0 and 30 min (Fig. S9). MIPS15479 did not affect the initial excitability at 0 min, but prevented the persistent response at 30 min. MIPS15479 had no effect on baseline rheobase at either time point. Similarly, transient incubation with IBS-D supernatant decreased rheobase at 30 min compared with HC supernatant (Fig. 8C). MIPS15479 completely prevented the persistent actions of IBS-D supernatant on nocicept excitability (rheobase at 30 min: vehicle IBS-D, 40.3 ± 3.9 pA; 12 neurons, supernatant from four patients; MIPS15479 IBS-D, 64.7 ± 3.84 pA; 17 neurons, supernatant from four patients; P < 0.05) (Fig. 8C). MIPS15479 did not affect excitability of neurons treated with HC supernatant. These results support the hypothesis that PAR2 in endosomes generates signals that underlie the persistent hyperexcitability of nociceptors, and is a potential therapeutic target for IBS pain.

PAR2 Endosomal Signaling Mediates Trypsin-Induced Sensitization of Colonic Afferent Neurons and Colonic Nociception.

The sensitization of colonic afferent neurons to mechanical stimuli is a leading hypothesis for IBS pain (17). To examine whether proteases cleave PAR2 on the peripheral terminals of colonic nociceptors to induce mechanical hypersensitivity, we made single-unit recordings from afferent neurons innervating the mouse colon. Receptive fields were identified by mechanical stimulation of the mucosal surface with VFF; proteases were applied to the mucosal receptive fields, and mechanical responses were reevaluated to assess sensitization. Under basal conditions, repeated mechanical stimulation (2-g filament) induced reproducible firing (Fig. 9A). Exposure to trypsin (10 nM, 10 min) amplified the frequency of firing to mechanical stimulation by 35.8 ± 5.9%, to NE (100 nM, 10 min) by 41.0 ± 11.8%, and to CS (100 nM, 10 min) by 52.0 ± 13.2% (Fig. 9B–E).

Colitis in mice induces hypersensitivity of colonic afferent neurons that persists even after inflammation is resolved (17). This chronic hypersensitivity resembles postinfectious/inflammatory IBS. To determine whether proteases can further amplify chronic hypersensitivity, mice were treated with trinitrobenzene sulphonic acid (TNBS enema) to induce colitis. At 28 d post-TNBS, when inflammation was resolved, mechanical stimulation

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of the colon induced a larger firing rate than in healthy control mice, consistent with chronic hyperexcitability (SI Appendix, Fig. S11 A–D). Compared with basal responses, trypsin further amplified responses by 16.4 ± 7.9%, NE by 30.6 ± 9.0%, and CS by 29.6 ± 9.2%. Thus, proteases can still amplify the excitability of colonic nociceptors even when they are already sensitized as a result of prior inflammation.

To determine the contribution of endosomal PAR2 signaling to trypsin-induced sensitization of colonic afferent neurons in normal healthy mice, I-343 (10 μM), PS2, or PS2 inact (50 μM) was applied to the receptive fields before exposure to trypsin. I-343 and PS2 did not affect basal mechanical sensitivity, but abolished trypsin-induced sensitization of mechanical responses (Fig. 9 F and G). PS2 inact did not affect basal responses or trypsin-induced sensitization (Fig. 9H).

Noxious colorectal distension (CRD) triggers the visceromotor response (VMR), a nociceptive brainstem reflex consisting of contraction of abdominal muscles, which can be monitored by electromyography. This approach allows assessment of visceral sensitivity in awake mice (38). To examine protease-induced hyperexcitability, a protease mixture (10 nM trypsin + 100 nM NE + 100 nM CS) or vehicle (saline) (100 μL) was instilled into the colon (enema) of healthy mice. After 15 min, the VMR was measured in response to graded CRD (20–80 mm Hg) with a barostat balloon. In vehicle-treated mice, CRD induced a graded VMR (Fig. 9F). The protease mixture amplified VMR at all pressures from 40 to 80 mm Hg. Administration of I-343 (30 mg/kg) into the colon (100 μL enema) 30 min before the protease mixture, abolished the response (Fig. 9F). Because alterations in the compliance of the colon can alter VMR to CRD, the pressure/volume relationship was measured at all distending pressures. Compliance of the colon was unaffected by the protease mixture or I-343 (SI Appendix, Fig. S11 E and F).

The results support the hypothesis that PAR2 endocytosis is required for trypsin-induced sensitization of colonic afferent neurons and colonic nociception.

Discussion

We propose that two components of PAR2 signaling contribute to persistent hyperexcitability on nociceptors: irreversible proteolytic activation and sustained signaling from endosomes or the plasma membrane, depending on the mechanism of activation (Fig. 10 and Movie S2). In the case of trypsin and proteases from the mucosa of patients with IBS-D, PAR2 endocytosis and endosomal ERK signaling mediate persistent hyperexcitability. In the case of NE and CS, which do not cause PAR2 endocytosis, plasma membrane signaling underlies persistent hyperexcitability. The observation that an endosomally targeted PAR2 antagonist blocks the persistent actions of trypsin and IBS proteases identifies PAR2 in endosomes as a therapeutic target. The combined results reveal that endosomal GPCRs signaling can contribute to processes that are relevant to human disease, and support the proposal that GPCRs in endosomes are a target for therapy (8, 12).

Mechanisms of Persistent Hyperexcitability of Nociceptors and Nociception.

The contribution of plasma membrane and endosomal signaling of PAR2 to protease-induced hyperexcitability of nociceptors depends
on the protease and the timing of the response. Trypsin, but not NE or CS, caused dynamin- and clathrin-dependent endocytosis of PAR2 in nociceptors. Because inhibitors of endocytosis did not affect the initial effects of trypsin, NE, or CS on excitability, hyperexcitability initially involves PAR2 signaling at the plasma membrane. Clathrin and dynamin inhibitors prevented persistent trypsin-induced hyperexcitability (at a time when activated PAR2 was in endosomes), and also suppressed trypsin-induced sensitization of colonic afferent nociceptors and somatic mechanical allostheny. Thus, PAR2 endocytosis and continued endosomal signaling are necessary for persistent trypsin-induced hyperexcitability of nociceptors and nociception. Inhibitors of endocytosis did not affect NE- or CS-induced neuronal hyperexcitability or mechanical allodynia, which is consistent with the inability of these proteases to induce PAR2 endocytosis.

PAR2/PKC signaling at the plasma membrane mediates initial trypsin-induced hyperexcitability of nociceptors, because trypsin induced PAR2-dependent PKC activation at the plasma membrane, and a PKC inhibitor blocked the initial hyperexcitability. Several observations indicate that PAR2/ERK signaling from endosomes mediates persistent trypsin-induced hyperexcitability: trypsin stimulated PAR2-dependent ERK activity in the cytosol and nucleus, clathrin and dynamin inhibitors suppressed ERK activation, and a MEK1 inhibitor prevented the persistent actions of trypsin. The finding that inhibitors of Gia, PKC, and the EGF receptor block trypsin activation of ERK, suggest that several pathways mediate PAR2 signaling in endosomes. A PAR2/βAR/ Ral1/MEK signaling complex in endosomes mediates activation of ERK in the cytosol (5), and Gqα and Goq transduce signals from other GPCRs in endosomes (7, 8, 12). Gqα was detected in early endosomes of HEK293 cells under basal conditions and after exposure to trypsin by immunofluorescence and superresolution microscopy. In agreement with previous studies (5, 14), trypsin induced translocation of PAR2 and βARR to early endosomes, detected by BRET and microscopy. Together, these findings support involvement of Gia and βARR in endosomal signaling of PAR2. NE and CS induce PAR2-dependent stimulation of adenyl cyclase and protein kinase A-mediated activation of transient receptor potential vanilloid 4 in nociceptors, which likely underlie hyperexcitability (15, 16). The inability to recruit βARRs and internalize may contribute to the sustained signaling of NE- and CS-activated PAR2 at the plasma membrane.

The finding that a cholesterol-conjugated PAR2 antagonist inhibited the ability of trypsin and IBS-D proteases to cause persistent hyperexcitability of nociceptors reinforces the importance of endosomal PAR2 signaling. After transient incubation and recovery, cholesterol-conjugated tripartite probes were removed from the plasma membrane of nociceptors and accumulated in endosomes containing PAR2, which demonstrates effective targeting. The observation that I-343–PEG-cholesterol and inhibitors of clathrin and dynamin inhibited the persistent, but not initial, actions of trypsin and IBS-proteases on nociceceptor excitability suggests selective targeting of PAR2 in endosomes represents a viable therapeutic strategy. Because I-343–PEG-cholesterol abolished persistent hyperexcitability despite reduced potency, endosomal targeting, retention, and local concentration are probably important determinants of efficacy.

There are several limitations of this investigation. Further studies are required to characterize the composition of PAR2 signaling complexes in different subcellular domains, and to define the mechanisms by which PAR2/PKC signaling at the plasma membrane and PAR2/ERK signaling in endosomes amplify the sensitivity of nociceptors. The potency, endosomal targeting, retention, and local concentration of I-343 are probably important determinants of efficacy. There are several limitations of this investigation. Further studies are required to characterize the composition of PAR2 signaling complexes in different subcellular domains, and to define the mechanisms by which PAR2/PKC signaling at the plasma membrane and PAR2/ERK signaling in endosomes amplify the sensitivity of nociceptors. The potency, endosomal targeting, retention, and local concentration of I-343 are probably important determinants of efficacy.

Fig. 8. Antagonism of endosomal PAR2 and hyperexcitability of nociceptors. (A and B) Trypsin-induced hyperexcitability of mouse DRG neurons. Neurons were preincubated with MIP515479 or vehicle (control, con) for 60 min, washed, and recovered for 170 or 140 min. Neurons were then exposed to trypsin (10 min). Rheobase was measured 0 or 30 min after trypsin and 180 min post-MIP515479. (C) IBS-induced hyperexcitability of mouse DRG neurons. Neurons were preincubated with MIP515479 or vehicle (control, con) for 60 min, washed, and recovered for 60 min. Neurons were then exposed to HC or IBS-D supernatant for 30 min, washed, and rheobase was measured 30 min later (T 30 min), 120 min post-MIP515479. *P < 0.05, **P < 0.01. Numbers in bars denote neuron numbers.

PAR2 Endosomal Signaling and IBS Pain. PAR2 endosomal signaling may underlie persistent hyperexcitability of nociceptors in patients with IBS-D, because an endosomally targeted PAR2 antagonist and inhibitors of dynamin and MEK1 prevented the persistent effects of IBS-D proteases on the hyperexcitability of nociceptors. Although Dy4 may have off-target effects (42), we obtained similar results by overexpressing dominant-negative dynamin or inhibiting clathrin. Dy4 and PS2 did not affect basal excitability of nociceptors or affect hyperexcitability or allodynia to NE or CS, which do not cause PAR2 endocytosis. These findings argue against nonselective effects of inhibitors on the function of nociceptors. The finding that an endosomally targeted PAR2 antagonist (I-343–PEG-cholesterol) replicated the inhibitory actions of Dy4 and PS2 on trypsin-induced hyperexcitability, supports the involvement of endosomal PAR2 signaling.
Hypersensitivity to colorectal distension is a hallmark of IBS (17). Our results show that proteases that activate PAR2 by canonical (trypsin) and biased (NE, CS) mechanisms sensitize colonic nociceptors to mechanical stimuli in basal and postinflammatory states. Trypsin-evoked sensitization requires PAR2 endocytosis and endosomal signaling. The administration of a mixture of proteases (trypsin, NE, CS) into the colonic lumen of mice amplified VMR to CRD, which is consistent with mechanical hyperalgesia in the colon. I-343 abolished these effects, which are thus dependent on PAR2. We did not determine whether inhibitors of endocytosis or endosomally targeted PAR2 antagonists suppress trypsin-evoked colonic nociception due to the impracticality of systemic administration of broadly acting and lipophilic drugs. Such studies will require pharmacokinetic studies of endocytic inhibitors and I-343–PEG-cholestanol to determine whether these drugs are capable of targeting pain-sensing neurons in the colonic wall. However, when administered by local injection into the paw, inhibitors of dynamin and clathrin prevented the ability of trypsin to cause mechanical allodynia. These results support a role for endosomal signaling in mechanical nociception.

Our results show that PAR2 expressed by NaV1.8+ neurons mediates the long-lasting pronociceptive actions of trypsin, NE,
and CS, because the sustained actions of these proteases were absent or diminished in Parβ-Parγ-1.8 mice. Because global deletion of Par2 attenuates the algesic actions of these proteases (15, 16, 43), the initial effects may involve activation of Par2 on other cell types involved in nociception, including keratinocytes and colonocytes that highly express Par2 (13). The findings that deletion of Par2 in NaV1.8 neurons or treatment with a Par2 antagonist significantly impairs nociceptive signalling in DRG cultures support the suggestion that proteases can directly activate nociceptors by cleaving Par2. However, we cannot exclude the possibility that proteases activate Par2 on nonneuronal cells that control nociceptor function, or that proteases may cause pain by activating other receptors, such as Par1, Par2, or NaV1.8 neurons markedly inhibited tryptase-induced edema and neutrophil infiltration, which supports the proposal that tryptase causes inflammation by a neurorigenic mechanism (44).

Our results have implications for the treatment of IBS pain. GPCRs are the target of over one-third of therapeutic drugs, most of which are designed to target cell surface receptors. The realization that GPCRs can continue to signal from endosomes to control important pathophysiological processes, has led to the proposal that receptors in endosomes are a target for therapy (1, 8, 12). The capacity of endosomally targeted Par2 antagonists to abolish IBSP-dependent persistent hyperexcitability of nociceptors highlights the importance of endosomal signalling of GPCRs for human disease, and reveals endosomal PAR2 as a therapeutic target for IBS pain.

Materials and Methods

Human Subjects. The Queen’s University Human Ethics Committee approved human studies. All subjects gave informed consent.

Animal Subjects. Institutional Animal Care and Use Committees of Queen’s University, Monash University, Flinders University, New York University, and the South Australian Health and Medical Research Institute approved studies in mice and rats.

Nociception, Inflammation, Nociceptor Hypermectitability. The analysis of somatic nociception and inflammation (8, 12), nociceptor hyperexcitability (21), sensitization of colonic afferent neurons, and colonic nociception (38) have been described.

BRET, FRET Assays. Endocytosis was studied by BRET and compartmentalized signaling was analyzed by FRET, as described previously (8, 12).

Results. Statistics. Results are mean ± SEM. Differences were assessed using Student’s t test (two comparisons) or one- or two-way ANOVA (multiple comparisons).

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