



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 adenylyl 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.

endosomes | receptors | pain | proteases

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 (β ARRs) and G proteins (1). Although GPCR signaling begins at the plasma membrane, activated receptors associate with β ARRs, 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 com-

partments 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 proinflammatory 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

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.

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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^{lox/lox}*) with mice expressing Cre recombinase targeted to nociceptors using the *Nav1.8* promoter (*Scn10a*). *Par₂-Nav1.8* mice lacked immunoreactive PAR₂ in Nav1.8⁺ neurons of the dorsal root ganglia (DRG) (Fig. 1A). Whereas 31% (20 of 65) of small-diameter (<25 μm) DRG neurons from WT mice responded to trypsin (100 nM) with increased [Ca²⁺]_i, only 6% (3 of 51) of neurons from *Par₂-Nav1.8* 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. 1C–E). In *Par₂-Nav1.8* 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-Nav1.8* 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*). Trypsin-induced inflammation was markedly diminished in *Par₂-Nav1.8* mice.

To assess the contribution of endocytosis to protease-induced nociception, Dyngo4a (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

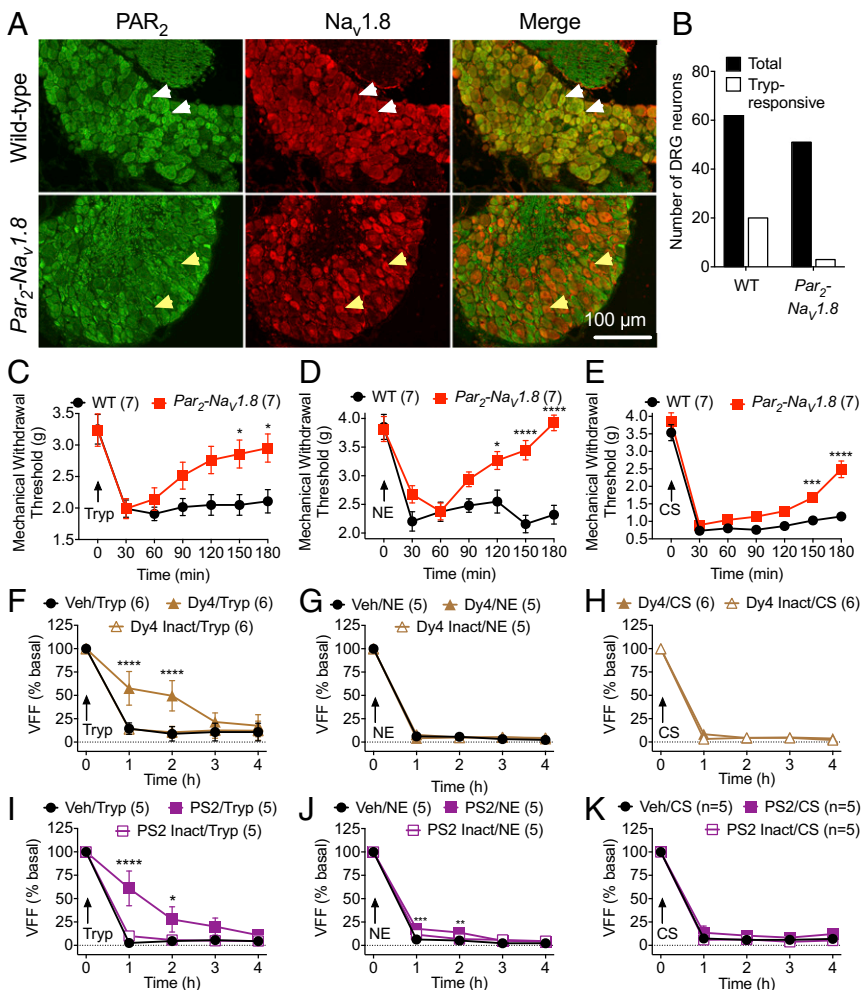


Fig. 1. Protease-induced mechanical nociception. (A) Localization of PAR₂ and Nav1.8 immunoreactivity in DRG from WT or *Par₂-Nav1.8* mice. White arrowheads: neurons coexpressing PAR₂ and Nav1.8 in WT mice. Yellow arrowheads: neurons expressing Nav1.8 but not PAR₂ in *Par₂-Nav1.8* mice. (B) Total number and number of trypsin (100 nM)-responsive DRG neurons (<25 μm) from WT and *Par₂-Nav1.8* mice. (C–E) VFF withdrawal responses in WT and *Par₂-Nav1.8* mice after intraplantar injection of trypsin (C, Tryp), NE (D), or CS (E). (F–K) VFF withdrawal responses in WT mice after intraplantar injection of Dy4 or Dy4 inact (F–H, dynamin inhibitor), PS2 or PS2 inact (I–K, clathrin inhibitor), or vehicle (Veh), followed 30 min later by intraplantar trypsin (F and I), NE (G and J), or CS (H and K). **P* < 0.05, ****P* < 0.01, *****P* < 0.001, ******P* < 0.0001. Numbers in parentheses denote mouse number.

intraplantar injection to mice. After 30 min, trypsin (10 nM), NE (1.2 μ M), or CS (2.5 μ M) (10 μ L) 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 *I*), 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 Na_v-1.8⁺ 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 μ M) or PS2 (15 μ M) 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 basal 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 \pm 0.11, IC₅₀ 1.1 nM) and 2F (100 nM)-induced IP₁ in KNRK-hPAR₂ cells (pIC₅₀ 6.18 \pm 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 \pm 0.20, IC₅₀ 0.4 nM) and in KNRK-hPAR₂ cells (pIC₅₀ 5.13 \pm 0.14, IC₅₀ 7507 nM). I-343 did not affect ATP (10 μ M)-stimulated IP₁ in KNRK cells (*SI Appendix*, Fig. *S4E*).

I-343 (10 μ M) 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. 3*D*). 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 PAR₁ antagonist SCH79797 (1 μ M, 10 min) (28); SCH79797 alone had no effect (*SI Appendix*, Fig. *S5B*) and SCH79797 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. PAR₁ does not mediate the initial actions of trypsin.

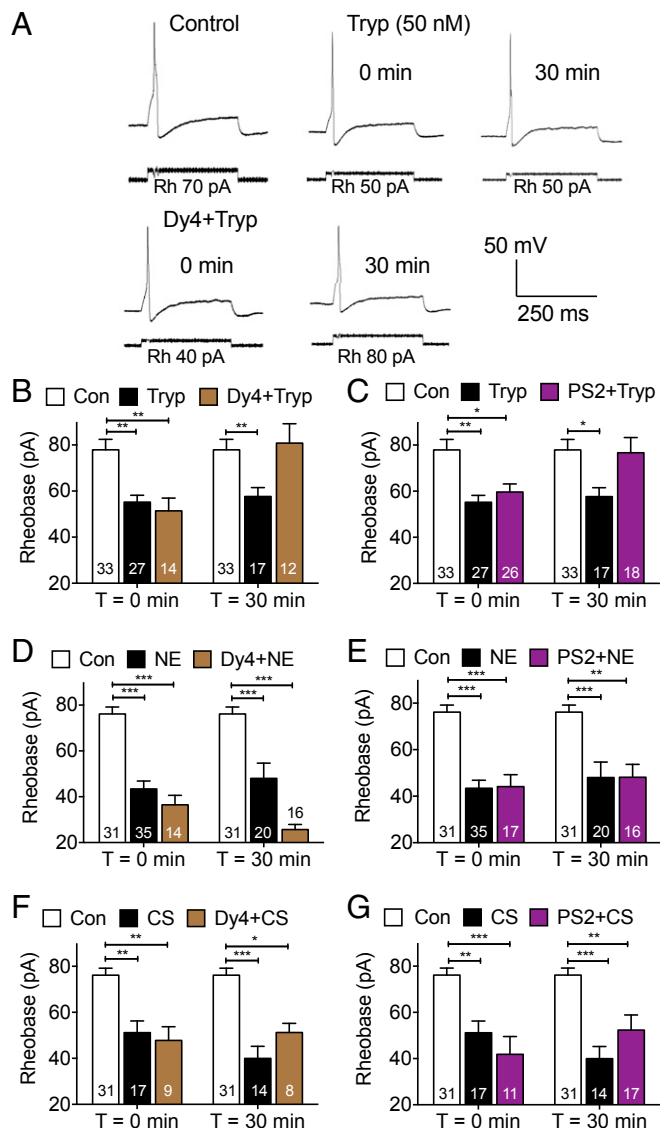


Fig. 2. Protease-induced hyperexcitability of nociceptors. Rheobase of mouse DRG neurons preincubated with Dy4 (*A*, *B*, *D*, and *F*, dynamin inhibitor), PS2 (*C*, *E*, and *G*, clathrin inhibitor), or vehicle control (Con). Neurons were challenged with trypsin (*A–C*), NE (*D* and *E*), or CS (*F* and *G*), washed, and rheobase was measured 0 or 30 min later. (*A*) Representative traces. Rh, rheobase. (*B–G*) Mean responses. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Numbers in bars denote neuron numbers.

Another PAR₂ antagonist, GB88, also prevents trypsin, NE, and CS activation of nociceptors (29). Trypsin-activated PAR₂ signals from endosomes by β ARR- and Raf-1-dependent processes, which activate ERK (5). PD98059 (50 μ M), 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. 3*E*). In contrast, GF109203X (Bis-1, 10 μ M), which inhibits PKC α and other kinases (30), prevented the initial but not the persistent effects of trypsin (Fig. 3*F*).

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.

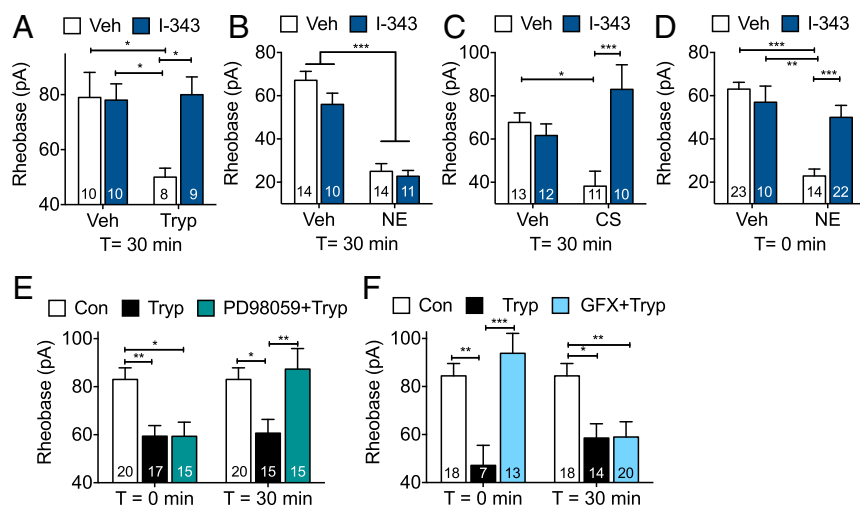


Fig. 3. Mechanisms of protease-induced hyperexcitability of nociceptors. Rheobase of mouse DRG neurons preincubated with I-343 (A–D, PAR₂ antagonist), PD98059 (E, MEK1 inhibitor), or GF109203X (F, GFX, PKC inhibitor). Neurons were challenged with trypsin (A, E, and F, Tryp), NE (B and D), or CS (C), washed, and rheobase was measured 0 or 30 min later. **P* < 0.05, ***P* < 0.01, ****P* < 0.0001. Numbers in bars denote neuron numbers.

PAR₂ Endocytosis and Compartmentalized Signaling in Nociceptors.

To assess endocytosis of PAR₂ in nociceptors, we transfected mouse (m) PAR₂-GFP into mouse DRG neurons. In vehicle-treated neurons, mPAR₂-GFP was detected at the plasma membrane and in intracellular compartments that may correspond to the prominent stores of PAR₂ in the Golgi apparatus (Fig. 4A) (31). Trypsin, but not NE or CS (100 nM, 30 min), induced removal of mPAR₂-GFP from the plasma membrane and accumulation in endosomes (Fig. 4A and B). Dy4, but not Dy4 inact, inhibited trypsin-induced endocytosis of mPAR₂-GFP (Fig. 4C). To determine whether PAR₂ recruits βARRs, which mediate endocytosis of PAR₂ (14), we expressed bioluminescence resonance energy transfer (BRET) sensors for PAR₂-RLuc8 (donor) and βARR2-YFP (acceptor) in mouse DRG neurons. Trypsin, but not NE or CS, stimulated PAR₂-RLuc8/βARR2-YFP BRET over 25 min (Fig. 4D).

To determine whether trypsin causes PAR₂-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 (CytoEKAR), and nuclear ERK (NucEKAR) were expressed in DRG neurons from rat, because pilot studies revealed more robust and consistent PAR₂ responses than in mouse neurons. Trypsin (100 nM) activated PKC at the plasma membrane but not in the cytosol (Fig. 4E–G), and activated ERK in the cytosol and nucleus (Fig. 4H–J). The PAR₂ antagonist I-343 (10 μM) inhibited trypsin-induced activation of PKC and ERK, whereas the PAR₁ antagonist SCH530348 (100 nM) had no effect (Fig. 4F 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 βARR2 recruitment and dynamin-dependent endocytosis of PAR₂ in nociceptors. Trypsin causes PAR₂-dependent activation of PKC at the plasma membrane and ERK in the cytosol and nucleus.

Mechanisms of PAR₂ Endocytosis and Endosomal Signaling. We examined the mechanism of PAR₂ endocytosis and endosomal signaling in HEK293 cells. To quantify the removal of PAR₂ from the plasma membrane and its accumulation in early endosomes, we measured bystander BRET between PAR₂ 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 PAR₂-RLuc8/RIT-Venus BRET (EC₅₀ 2.9 nM), and an increase in PAR₂-RLuc8/Rab5a-Venus BRET (EC₅₀ 2.7 nM) (Fig. 5A and B and *SI Appendix, Fig. S6A–D*). Neither NE nor CS (100 nM) affected PAR₂-RLuc8/RIT-Venus or Rab5a-Venus BRET (Fig. 5A and B). PS2, but not PS2 inact, suppressed the trypsin-induced decrease in PAR₂-RLuc8/RIT-Venus BRET and increase in PAR₂-RLuc8/Rab5a-Venus BRET (Fig. 5C and D and *SI Appendix, Fig. S6E and F*). Dominant-negative dynaminK44E (DynK44E), which is deficient in GTP binding (33), inhibited the increase in PAR₂-RLuc8/Rab5a-Venus BRET, but did not affect PAR₂-RLuc8/RIT-Venus BRET (Fig. 5C and D and *SI Appendix, Fig. S6G 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 PAR₂ 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 PAR₂.

We investigated the contribution of endocytosis to trypsin-induced signaling in HEK293 cells expressing Flag-PAR₂-HA11 and FRET biosensors for cytosolic and nuclear ERK (CytoEKAR, NucEKAR), 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. 5E and F and *SI Appendix, Fig. S7A–F*). I-343 (10 μM) but not the PAR₁ 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. 5H and I and *SI Appendix, Fig. S7G–J*). AG1478 (1 μM), an inhibitor of EGF receptor tyrosine kinase (34), UBO-QIC (100 nM), which inhibits Gα_q and certain Gβγ signals (35), and Gö6983 (1 μM), which inhibits all isoforms of PKC (36), suppressed trypsin-stimulated activation of cytosolic ERK (Fig. 5J and *SI Appendix, Fig. S7K*). UBO-QIC and Gö6983 also inhibited activation of nuclear ERK (Fig. 5K and *SI Appendix, Fig. S7L*). The results suggest that PAR₂ signals from endosomes by Gα_q-dependent mechanisms to activate ERK in the cytosol and nucleus.

To determine whether trypsin induces translocation of βARR and Gα_q to endosomes, we measured BRET between βARR1-RLuc8 or Gα_q-RLuc8 and Rab5a-Venus in HEK293 cells. Trypsin (100 nM)

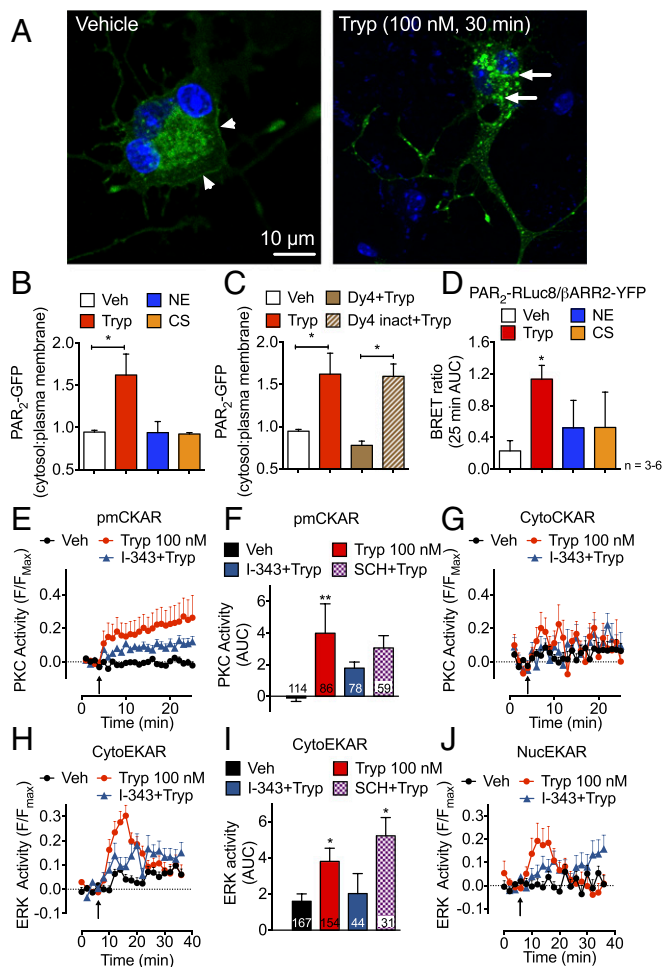


Fig. 4. PAR₂ endocytosis, β ARR2 recruitment, and compartmentalized signaling in nociceptors. (A–C) PAR₂ endocytosis. (A) Representative images (of three experiments) of effects of trypsin (Tryp) on the distribution of mPAR₂-GFP in mouse DRG neurons. Arrowheads (A, Left) show PAR₂-GFP at the plasma membrane. 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/ β ARR2-YFP BRET in mouse DRG neurons exposed to trypsin, NE or CS. Area under curve (25 min) **P* < 0.05 to vehicle. *n*, experimental replicates, triplicate observations. (E–J) 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.

stimulated an increase in β ARR1-RLuc8/Rab5a-Venus BRET and in α_q -RLuc8/Rab5a-Venus BRET (SI Appendix, Fig. S8 A and B). We used immunofluorescence and structured illumination microscopy to localize PAR₂-HA, α_q and early endosomal antigen-1 (EEA1) in HEK-293 cells. In unstimulated cells, PAR₂ was confined to the plasma membrane, although α_q was detected in early endosomes (SI Appendix, Fig. S8C). Trypsin (10 nM, 30 min) induced translocation of PAR₂ to early endosomes containing α_q . The results support the hypothesis that trypsin causes assembly of a PAR₂/ β ARR/ α_q signalosome in early endosomes.

Trypsin (10 nM) caused a rapid and sustained activation of PKC and generation of cAMP at the plasma membrane and in the cytosol of HEK293 cells (SI Appendix, Fig. S9 A–H). DynK44E strongly inhibited these signals, but DynWT had no effect. I-343, but not SCH530348, inhibited trypsin stimulation of PKC and cAMP, which thus depend on PAR₂ (SI Appendix, Fig. S9 G and H). These results suggest that endocytosis is necessary

for multiple components of PAR₂ signaling. cAMP signaling at the plasma membrane is usually desensitized by β ARR delivery of phosphodiesterases, which degrade cAMP (37). The sustained plasma membrane cAMP response to trypsin support the existence of mechanisms that allow persistent PAR₂ signaling, which warrant further investigation. 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 \pm 4.41 pA, 12 neurons, supernatant from four HC; IBS-D, 54.55 \pm 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 μ M), Dy4 (dynamin inhibitor, 30 μ M), and PD98059 (MEK1 inhibitor, 50 μ M) 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. 6E). 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 antagonist I-343 (SI Appendix, Fig. S10 A and B). To determine whether tripartite probes accumulate in endosomes containing PAR₂, mouse DRG neurons expressing mPAR₂-GFP were incubated with Cy5-PEG-cholesterol (Cy5-Chol) or Cy5-PEG-Ethyl ester (Cy5-Ethyl ester) (200 nM, 60 min, 37 °C). Neurons were washed and imaged (37 °C). Cy5-Ethyl ester was not taken up by neurons, whereas Cy5-Chol inserted into the plasma membrane and then accumulated in endosomes of the soma and neurites over 3 h (Fig. 7). Trypsin induced endocytosis of PAR₂-GFP into endosomes in close proximity to vesicles containing

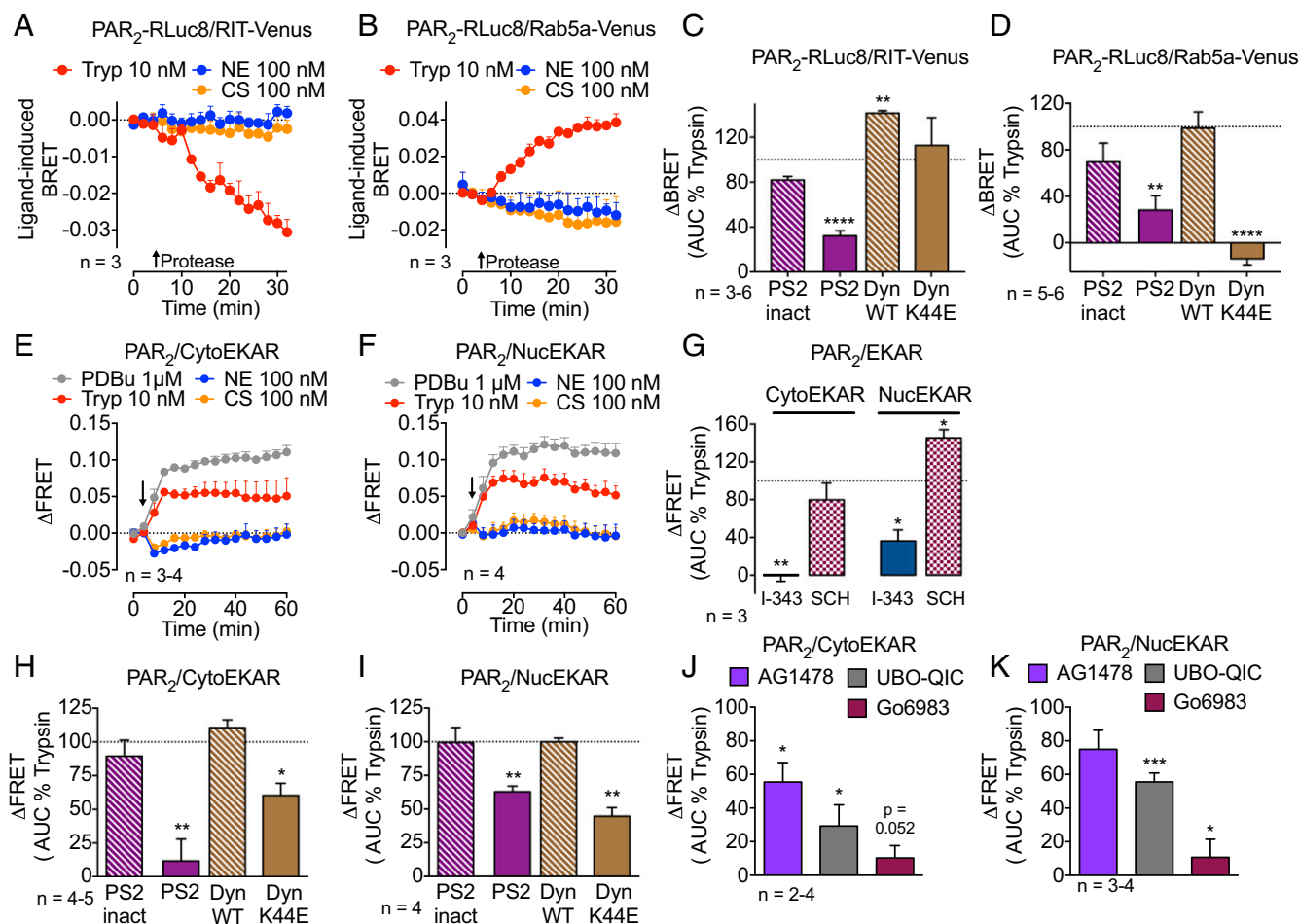


Fig. 5. PAR₂ endocytosis and compartmentalized ERK signaling in HEK293 cells. (A–D) BRET assays of endocytosis. PAR₂-RLuc8/RIT-Venus BRET (A and C) and PAR₂-RLuc8/Rab5a-Venus BRET (B and D). (E–K) FRET assays of cytosolic (E, G, H, and J) and nuclear (F, G, I, and K) ERK activity. AUC, area under curve. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001 compared with trypsin alone. *n*, experimental replicates in triplicate.

Cy5-Chol. Video-imaging revealed frequent association of endosomes containing PAR₂-GFP and Cy5-Chol (Movie S1). I-343-PEG-cholestanol (MIPS15479) (SI Appendix, Fig. S10A) antagonized 2F-stimulated IP₁ 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 antagonist in endosomes (Fig. 8A). Transient incubation with trypsin decreased rheobase of vehicle-treated neurons at 0 and 30 min (Fig. 8B). 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 nociceptor excitability (rheobase at 30 min: vehicle IBS-D, 40 ± 3.89 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

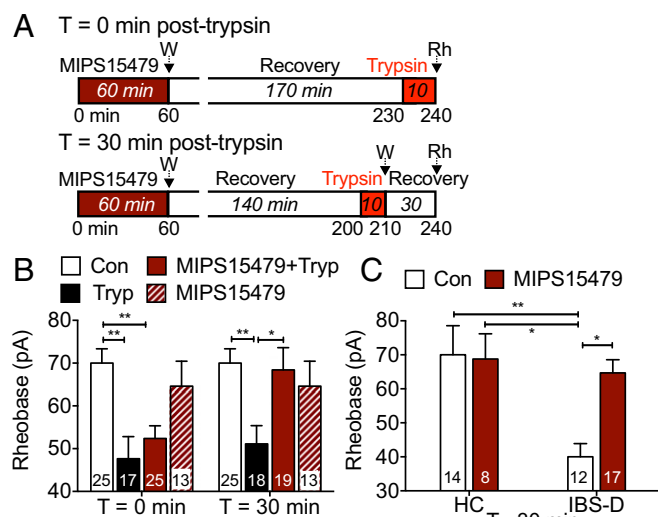


Fig. 8. Antagonism of endosomal PAR₂ and hyperexcitability of nociceptors. (A and B) Trypsin-induced hyperexcitability of mouse DRG neurons. Neurons were preincubated with MIPS15479 or vehicle (control, con) for 60 min, washed, and recovered for 170 or 140 min. Neurons were then exposed to trypsin (10 min). Rheobase was measured 0 or 30 min after trypsin and 180 min post-MIPS15479. (C) IBS-induced hyperexcitability of mouse DRG neurons. Neurons were preincubated with MIPS15479 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-MIPS15479. **P* < 0.05, ***P* < 0.01. Numbers in bars denote neuron numbers.

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 allodynia. 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 G α_q , PKC, and the EGF receptor block trypsin activation of ERK, suggest that several pathways mediate PAR₂ signaling in endosomes. A PAR₂/ β ARR/Raf-1/MEK signaling complex in endosomes mediates activation of ERK in the cytosol (5), and G α_q and G α_x transduce signals from other GPCRs in endosomes (7, 8, 12). G α_q 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 β ARR to early endosomes, detected by BRET and microscopy. Together, these findings support involvement of G α_q and β ARR in endosomal signaling of PAR₂. NE and CS induce PAR₂-dependent stimulation of adenylyl

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 β ARRs and internalize may contribute to the sustained signaling of NE- and CS-activated PAR₂ at the plasma membrane.

The finding that a cholestanol-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, cholestanol-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-cholestanol and inhibitors of clathrin and dynamin inhibited the persistent, but not initial, actions of trypsin and IBS-proteases on nociceptor excitability suggests selective targeting of PAR₂ in endosomes represents a viable therapeutic strategy. Because I-343-PEG-cholestanol 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. Involvement of PKC and ERK are consistent with reports that painful stimulants activate PKC ϵ and ERK1/2 in primary sensory neurons, and that inhibitors of PKC ϵ and MEK attenuate nociception (39, 40). PKC and ERK can activate channels that control the sensitivity of nociceptors (41). GPCRs in endosomes can also regulate transcription, which may contribute to chronic hyperexcitability of nociceptors (8, 11). Additional studies are required to define the specificity of I-343 and I-343-PEG-cholestanol (MIPS15479) for PAR₂ over other GPCRs. Although I-343 shows remarkable potency for PAR₂, high concentrations of I-343 can inhibit thrombin signaling, suggesting antagonism of PAR₁ (26). However, I-343, at concentrations that do not affect thrombin signaling, prevented trypsin-induced hyperexcitability of nociceptors, whereas a PAR₁-selective antagonist had no effect. Thus, PAR₂, not PAR₁, mediates these actions of trypsin.

A limitation to the use of inhibitors of endocytosis is that clathrin and dynamin control the trafficking of many receptors and channels that control neuronal activity and nociception. 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-cholestanol) replicated the inhibitory actions of Dy4 and PS2 on trypsin-induced hyperexcitability, supports the involvement of endosomal PAR₂ signaling.

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 we did not identify the proteases that are released from colonic biopsies that mediate these effects, trypsin and trypsin-3 are likely candidates; both proteases are activated in the colonic mucosa of IBS patients, and trypsin and trypsin inhibition or PAR₂ deletion prevent IBS-dependent hyperexcitability of nociceptors (18–21). The finding that a dynamin inhibitor and an endosomally targeted PAR₂ antagonist prevented hypersensitivity supports involvement of proteases that activate PAR₂ by canonical mechanisms and induce receptor endocytosis, such as trypsin and trypsin-3 (13).

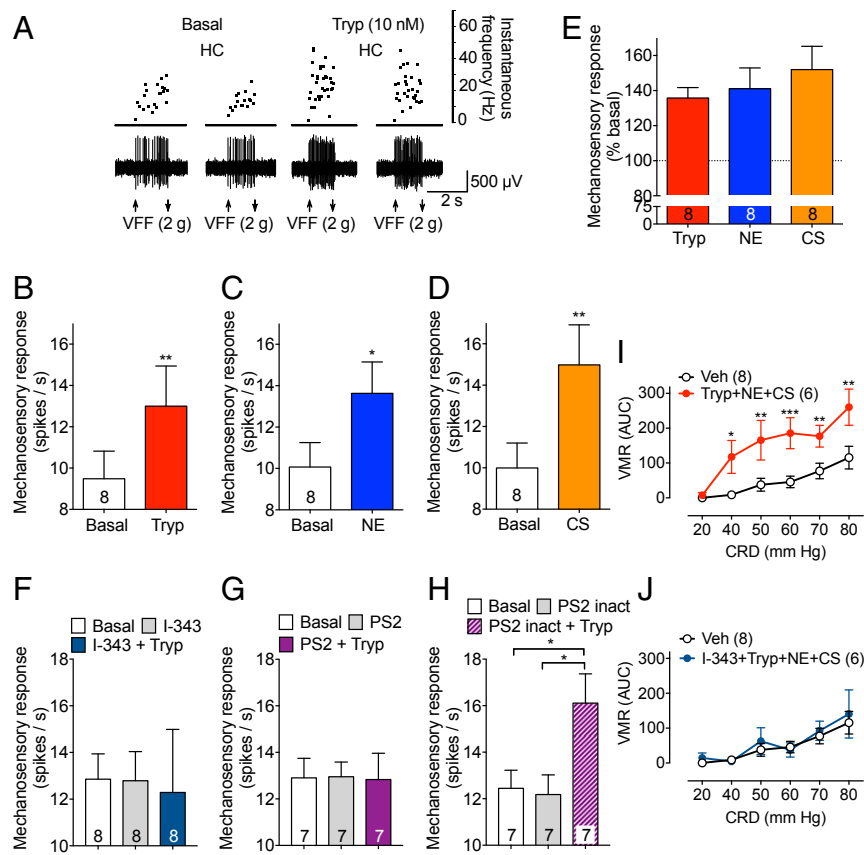


Fig. 9. Sensitization of colonic afferents and colonic nociception. (A–H) Mechanosensory responses in healthy control mice to stimulation of the colonic mucosa with a 2 g VFF under basal conditions and after exposure of receptive fields to trypsin (A, B, and E–H, Tryp), NE (C and E), or CS (D and E). (A) Representative results. (B–D and F–H) Mean responses. (E) Responses as percent basal. Numbers in bars denote afferent numbers. (I and J) VMR to CRD in awake normal mice. Numbers in parentheses denote mouse number. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

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 Na_v1.8⁺ neurons mediates the long-lasting pronociceptive actions of trypsin, NE,

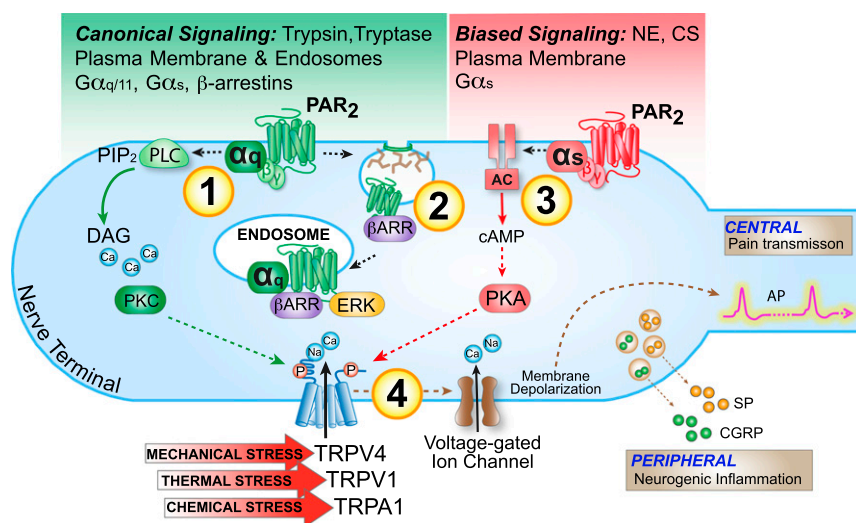


Fig. 10. Mechanisms of protease- and PAR₂-induced hyperexcitability of nociceptors. After activation by canonical mechanisms, PAR₂ signals at the plasma membrane to activate PLC, which mediates initial hyperexcitability (1). PAR₂ then undergoes clathrin-, dynamin-, and βARR-dependent endocytosis (2). PAR₂ continues to signal from endosomes by βARR- and Gα_q-mediated mechanisms to activate ERK, which mediates persistent hyperexcitability. After activation by biased mechanisms, PAR₂ signals from the plasma membrane to activate adenylyl cyclase (AC) and PKA, which mediate the initial and persistent hyperexcitability (3). Kinases may regulate the activity of TRP channels and voltage-gated ion channels, to control nociceptor hyperexcitability (4).

and CS, because the sustained actions of these proteases were absent or diminished in *Par₂-Nav1.8* mice. Because global deletion of PAR₂ attenuates the algescic 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 suppressed trypsin signaling 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 in Nav1.8⁺ neurons markedly inhibited trypsin-induced edema and neutrophil infiltration, which supports the proposal that trypsin causes inflammation by a neurogenic 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 IBS-dependent persistent hyperexcitability of nociceptors highlights the importance of endosomal signaling of GPCRs for human disease, and reveals endosomal PAR₂ as a therapeutic target for IBS pain.

- Murphy JE, Padilla BE, Hasdemir B, Cottrell GS, Bunnett NW (2009) Endosomes: A legitimate platform for the signaling train. *Proc Natl Acad Sci USA* 106:17615–17622.
- DeWire SM, Ahn S, Lefkowitz RJ, Shenoy SK (2007) Beta-arrestins and cell signaling. *Annu Rev Physiol* 69:483–510.
- Cheloha RW, Gellman SH, Vilaradga J-P, Gardella TJ (2015) PTH receptor-1 signalling-mechanistic insights and therapeutic prospects. *Nat Rev Endocrinol* 11:712–724.
- DeFoa KA, et al. (2000) The proliferative and antiapoptotic effects of substance P are facilitated by formation of a beta-arrestin-dependent scaffolding complex. *Proc Natl Acad Sci USA* 97:11086–11091.
- DeFoa KA, et al. (2000) Beta-arrestin-dependent endocytosis of proteinase-activated receptor 2 is required for intracellular targeting of activated ERK1/2. *J Cell Biol* 148:1267–1281.
- Eichel K, Jullié D, von Zastrow M (2016) β -Arrestin drives MAP kinase signalling from clathrin-coated structures after GPCR dissociation. *Nat Cell Biol* 18:303–310.
- Irannejad R, et al. (2013) Conformational biosensors reveal GPCR signalling from endosomes. *Nature* 495:534–538.
- Jensen DD, et al. (2017) Neurokinin 1 receptor signaling in endosomes mediates sustained nociception and is a viable therapeutic target for prolonged pain relief. *Sci Transl Med* 9:eal3447.
- May V, Parsons RL (2017) G protein-coupled receptor endosomal signaling and regulation of neuronal excitability and stress responses: Signaling options and lessons from the PAC1 receptor. *J Cell Physiol* 232:698–706.
- Thomsen ARB, et al. (2016) GPCR-G protein- β -arrestin super-complex mediates sustained G protein signaling. *Cell* 166:907–919.
- Tsvetanova NG, von Zastrow M (2014) Spatial encoding of cyclic AMP signaling specificity by GPCR endocytosis. *Nat Chem Biol* 10:1061–1065.
- Yarwood RE, et al. (2017) Endosomal signaling of the receptor for calcitonin gene-related peptide mediates pain transmission. *Proc Natl Acad Sci USA* 114:12309–12314.
- Ossovskaya VS, Bunnett NW (2004) Protease-activated receptors: Contribution to physiology and disease. *Physiol Rev* 84:579–621.
- Déry O, Thoma MS, Wong H, Grady EF, Bunnett NW (1999) Trafficking of proteinase-activated receptor-2 and beta-arrestin-1 tagged with green fluorescent protein. beta-arrestin-dependent endocytosis of a proteinase receptor. *J Biol Chem* 274:18524–18535.
- Zhao P, et al. (2014) Cathepsin S causes inflammatory pain via biased agonism of PAR2 and TRPV4. *J Biol Chem* 289:27215–27234.
- Zhao P, et al. (2015) Neutrophil elastase activates PAR2 and TRPV4 to cause inflammation and pain. *J Biol Chem* 290:13875–13887.
- Azpiroz F, et al. (2007) Mechanisms of hypersensitivity in IBS and functional disorders. *Neurogastroenterol Motil* 19(Suppl 1):62–88.
- Barbara G, et al. (2007) Mast cell-dependent excitation of visceral-nociceptive sensory neurons in irritable bowel syndrome. *Gastroenterology* 132:26–37.
- Cenac N, et al. (2007) Role for protease activity in visceral pain in irritable bowel syndrome. *J Clin Invest* 117:636–647.
- Rolland-Fourcade C, et al. (2017) Epithelial expression and function of trypsin-3 in irritable bowel syndrome. *Gut* 66:1767–1778.
- Valdez-Morales EE, et al. (2013) Sensitization of peripheral sensory nerves by mediators from colonic biopsies of diarrhea-predominant irritable bowel syndrome patients: A role for PAR2. *Am J Gastroenterol* 108:1634–1643.
- Kayssi A, Amadesi S, Bautista F, Bunnett NW, Vanner S (2007) Mechanisms of protease-activated receptor 2-evoked hyperexcitability of nociceptive neurons innervating the mouse colon. *J Physiol* 580:977–991.

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 Hyperexcitability. 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).

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

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- Reed DE, et al. (2003) Mast cell tryptase and proteinase-activated receptor 2 induce hyperexcitability of guinea-pig submucosal neurons. *J Physiol* 547:531–542.
- Robertson MJ, Deane FM, Robinson PJ, McCluskey A (2014) Synthesis of Dynole 34-2, Dynole 2-24 and Dyngo 4a for investigating dynamin GTPase. *Nat Protoc* 9:851–870.
- Robertson MJ, et al. (2014) Synthesis of the Pstptso family of clathrin inhibitors. *Nat Protoc* 9:1592–1606.
- Farmer LJ (2013) Imidazopyridazines useful as inhibitors of the PAR-2 signaling pathway. US Patent Appl WO 2015/048245.
- Jiang Y, et al. (2018) A potent antagonist of protease-activated receptor 2 that inhibits multiple signaling functions in human Cancer cells. *J Pharmacol Exp Ther* 364:246–257.
- Ahn HS, et al. (2000) Inhibition of cellular action of thrombin by N3-cyclopropyl-7-[[4-(1-methylethyl)phenyl]methyl]-7H-pyrrolo[3, 2-f]quinazoline-1,3-diamine (SCH 79797), a nonpeptide thrombin receptor antagonist. *Biochem Pharmacol* 60:1425–1434.
- Lieu T, et al. (2016) Antagonism of the proinflammatory and pronociceptive actions of canonical and biased agonists of protease-activated receptor-2. *Br J Pharmacol* 173:2752–2765.
- Davies SP, Reddy H, Caivano M, Cohen P (2000) Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem J* 351:95–105.
- Jensen DD, et al. (2016) Protein kinase D and G β subunits mediate agonist-evoked translocation of protease-activated receptor-2 from the Golgi apparatus to the plasma membrane. *J Biol Chem* 291:11285–11299.
- Lan TH, Liu Q, Li C, Wu G, Lambert NA (2012) Sensitive and high resolution localization and tracking of membrane proteins in live cells with BRET. *Traffic* 13:1450–1456.
- Herskovits JS, Burgess CC, Obar RA, Vallee RB (1993) Effects of mutant rat dynamin on endocytosis. *J Cell Biol* 122:565–578.
- Levitzki A, Gazit A (1995) Tyrosine kinase inhibition: An approach to drug development. *Science* 267:1782–1788.
- Gao ZG, Jacobson KA (2016) On the selectivity of the G α_q inhibitor UBO-QIC: A comparison with the G α_i inhibitor pertussis toxin. *Biochem Pharmacol* 107:59–66.
- Gschwendt M, et al. (1996) Inhibition of protein kinase C mu by various inhibitors. Differentiation from protein kinase c isoenzymes. *FEBS Lett* 392:77–80.
- Perry SJ, et al. (2002) Targeting of cyclic AMP degradation to beta 2-adrenergic receptors by beta-arrestins. *Science* 298:834–836.
- Castro J, et al. (2017) Cyclic analogues of alpha-conotoxin Vc1.1 inhibit colonic nociceptors and provide analgesia in a mouse model of chronic abdominal pain. *Br J Pharmacol* 175:2384–2398.
- Aley KO, et al. (2001) Nociceptor sensitization by extracellular signal-regulated kinases. *J Neurosci* 21:6933–6939.
- Khasar SG, et al. (1999) A novel nociceptor signaling pathway revealed in protein kinase C epsilon mutant mice. *Neuron* 24:253–260.
- Cheng JK, Ji RR (2008) Intracellular signaling in primary sensory neurons and persistent pain. *Neurochem Res* 33:1970–1978.
- Park RJ, et al. (2013) Dynamin triple knockout cells reveal off target effects of commonly used dynamin inhibitors. *J Cell Sci* 126:5305–5312.
- Vergnolle N, et al. (2001) Proteinase-activated receptor-2 and hyperalgesia: A novel pain pathway. *Nat Med* 7:821–826.
- Steinhoff M, et al. (2000) Agonists of proteinase-activated receptor 2 induce inflammation by a neurogenic mechanism. *Nat Med* 6:151–158.