Clathrin and GRK2/3 inhibitors block δ-opioid receptor internalization in myenteric neurons and inhibit neuromuscular transmission in the mouse colon

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Introduction

G protein-coupled receptors (GPCRs) enable cells to detect and respond appropriately to changes in their extracellular environment. GPCRs represent a significant therapeutic target, with 30% of currently approved drugs targeting this family of receptors (18). Traditionally, GPCRs have been described as proteins that transduce signals from the cell surface. These signals are effectively terminated by two major mechanisms: receptor desensitization and endocytosis. The demonstration that activated GPCRs can continue to signal from within endosomes, resulting in cellular responses that are spatially and temporally distinct from those derived from the cell surface, represents a major conceptual advancement in the field of GPCR biology. In this model, internalized activated GPCRs form endosomal signaling complexes, known as “signalosomes,” that elicit signals distinct from those initiated at the plasma membrane. This “positional dynamism” defines both the nature and duration of the ensuing cellular signaling events downstream of receptor activation (33, 46). Intracellular GPCRs can drive unique signaling events and may play a potential role in pathophysiology or represent a distinct therapeutic target. For example, “location bias” has been described for endogenous opioid peptides and their synthetic derivatives, which can activate the μ-opioid and δ-opioid receptors (MOR and DOR, respectively) at the cell surface to promote internalization of

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NEW & NOTEWORTHY Internalization of activated G protein-coupled receptors is a major determinant of the type and duration of subsequent downstream signaling events. Inhibitors of endocytosis effectively block opioid receptor internalization in enteric neurons. The clathrin-dependent endocytosis inhibitor Pitstop2 blocks effects of opioids on neurogenic contractions of the colon in an internalization-independent manner. These inhibitors also significantly impact cholinergic neuromuscular transmission. We conclude that these tools are unsuitable for examination of the contribution of neuronal G protein-coupled receptor endocytosis to gastrointestinal motility.
activated receptors into endosomes. In contrast, clinically important opioid drugs, such as morphine, can cross the plasma membrane to directly activate MOR in the Golgi network. What remains to be defined are the implications that potential activation of three spatially distinct signaling pathways downstream of the same GPCR could have on neuronal functions, such as the differential control of neuronal excitability (44). Similar differences between the relative accessibility of endogenous ligands and drugs to preformed receptors at the Golgi apparatus have been shown for the \( \beta_1 \)-adrenergic receptor (19). Location bias has also been described for metabotropic glutamate receptor 5 (mGlur5), which is localized to both the cell surface and intracellular membranes in dorsal horn neurons of the spinal cord. Neuropathic pain induced by nerve injury was associated with increased mGlur5 at the nuclear membrane of these neurons. Inhibition of nuclear mGlur5, but not cell surface receptor, was effective at attenuating pain behavior and related cellular signaling (48). These studies demonstrate that pharmacological targeting of GPCR signaling at specific subcellular locales may prove to be a novel and effective therapeutic approach.

Recent studies have defined the importance of endosomal signaling to physiological and pathophysiological processes, including acute pain. This has significant implications for therapy, and the potential for selectively targeting endosomal GPCR (eGPCR) signaling for the control of pain has recently been highlighted. In these studies, small molecule inhibitors of endocytosis and delivery of lipidated antagonists to endosomes was effective in suppressing acute mechanical algies in mice (20, 21, 53), with the conclusion that endosomal signaling by pronociceptive GPCRs drives prolonged pain behavior. Whether eGPCR signaling similarly contributes to the enteric neuronal hyperexcitability and associated gastrointestinal (GI) dysfunction that occurs following inflammation or infection of the intestine (28) is presently unknown.

Classically, the primary function of endocytosis is to effectively terminate downstream signaling through sequestration of the GPCR away from the cell surface. Activated GPCRs are phosphorylated by G protein-coupled receptor kinases (GRKs) to promote \( \beta \)-arrestin (\( \beta \)Arr) recruitment and subsequent internalization of receptors through an adaptor protein (AP2-), clathrin- and dynamin-dependent mechanism. It is now appreciated that internalization of GPCRs is required for the full signaling repertoire and that some specific signals depend on receptors forming additional signaling platforms in intracellular compartments. Key proteins essential for effective endocytosis can be targetted genetically or through the use of small molecule inhibitors. The processes that these proteins control include GRK-dependent phosphorylation, \( \beta \)Arr interaction with clathrin adaptor protein AP2, dynamin GTPase activity, and clathrin-coated pit formation. The importance of endocytosis for the control of enteric neuron function is unknown beyond its role in receptor desensitization and resensitization. Endocytic inhibitors have been used to examine mechanisms of agonist-evoked GPCR endocytosis and GPCR-mediated signaling in enteric neurons (10, 34, 36, 56). However, at present there has been very limited examination of the role that internalized GPCRs may play in gut function, or of eGPCR signaling in enteric neurons in general. Agonist-evoked internalization of the neurokinin 1 receptor (NK1R) in myenteric neurons leads to endosomal signaling through mitogen-activated protein kinase (MAPK), which is sustained if endosomal retention of the receptor is favored (6, 34). Although these studies demonstrated that the endosomal endopeptidase endothelin-converting enzyme 1 (ECE-1) controls the duration of this signaling and subsequent NK1R recycling and resensitization at the cell surface, the role of receptor endocytosis in physiologically important neurogenic processes in the GI tract, such as the coordination of motility, remains undetermined.

In the present study, we examined the utility of small molecule inhibitors of clathrin and GRK2/3 to determine the importance of endocytosis for the actions of GPCRs on intestinal motility, the primary function of myenteric neurons. We focused on MOR and DOR as representative GPCRs due to existing knowledge about the trafficking and function of these receptors in the enteric nervous system (ENS) (8, 22, 37, 52). MOR and DOR are established and emerging targets for the treatment of moderate to severe pain, respectively, and clinically important opioids, such as morphine, can promote severe, intractable constipation through MOR activation on enteric neurons (52). MOR and DOR share a common mechanism of action in the ENS. Both are G\(_1\)-coupled receptors and inhibit electrically stimulated contractions, and their respective agonists evoke contractions when directly applied to intestinal tissue (8, 12, 13, 41). MOR and DOR expression in the colon is almost exclusively restricted to enteric neurons (22, 35, 37), and the effects of both MOR and DOR agonists on colonic motility are mediated through a neurogenic mechanism. Finally, agonists that robustly or weakly promote MOR or DOR endocytosis in myenteric neurons have been identified (4, 22, 37, 43). Thus, examination of opioid receptors provides an ideal opportunity to determine the functional importance of GPCR endocytosis in the control of neuromuscular transmission in the GI tract. As a first and fundamental step toward defining the importance of GPCR internalization in enteric neurotransmission, the suitability of established endocytosis inhibitors for use in standard assays of GI motility must be determined. This study examined the effectiveness of small molecule inhibitors of clathrin and GRK2/3 to inhibit agonist-evoked DOR internalization and their potential impact on the neurogenic control of intestinal smooth muscle contractions. We demonstrate that, although these compounds can inhibit GPCR endocytosis in enteric neurons, significant effects on neuromuscular transmission and direct actions on smooth muscle can confound interpretation of the functional role that GPCR endocytosis plays in GI motility.

**MATERIALS AND METHODS**

**Animals**

C57BL/6J mice and knockin mice expressing DOR tagged with COOH-terminal enhanced green fluorescent protein (DOR eGFP) (42) (6–8 wk, male) were purpose bred by the Monash Animal Research Platform. Wnt1-GCaMP3 mice (4–6 wk, male and female) were generated as described (17). Mice were housed under 12:12-h light-dark cycle, temperature-controlled conditions (24°C), and with free access to food and water. All procedures involving mice were approved by the Monash Institute of Pharmaceutical Sciences and The University of Melbourne animal ethics committees.
Reagents

Reagents used were from the following suppliers: Pitstop2 (PS2) and Pitstop2 inactive control (PS2i; Abcam); Takeda compound 101 (Compd101) and SN80 (Tocris); morphine hydrochloride (MacFarlan Smith); tetrodotoxin citrate (TTX; Alomone); nicardipine hydrochloride, carbamoyl choline (carbachol), and ATP (Sigma-Aldrich). TTX, nicardipine, and carbachol were diluted in water. All other compounds were diluted in DMSO. Final bath concentrations of DMSO did not exceed 0.15%.

Endocytosis Assays

The effectiveness of endocytosis inhibitors was determined using SN80-induced DOREGFP endocytosis as a standard assay (8, 37). Briefly, DOREGFP knockin mice were euthanized by cervical dislocation, and the distal colon was harvested and placed in modified Krebs buffer (containing 10 μM nicardipine and 1 μM TTX). Whole mount preparations were recovered (Krebs + TTX + nicardipine, 37°C, 1 h, bubbled with 95% O2-5% CO2). Preparations were incubated with inhibitors or vehicle (37°C, 20 min) and then washed with ice-cold Krebs (3 × 5-min washes). These were then incubated with SN80 (1 μM) with or without endocytosis inhibitors (4°C, 1 h), washed (3 × 5-min washes, ice-cold Krebs), and then recovered (agonist-free Krebs, 37°C, 30 min). Tissues were fixed (4% paraformaldehyde, overnight, 4°C). Fixative was cleared (3 × 10-min washes, PBS), and circular muscle-myenteric plexus whole mounts were prepared. These were blocked (5% normal horse serum, 0.1% Triton X-100 in PBS containing 0.1% sodium azide, 1 h, room temperature) and then labeled by indirect immunofluorescence for eGFP (rabbit anti-GFP, 1:1,000, Thermo Fisher, no. AA11122), neuronal NO synthase (nNOS; goat anti-nNOS, 1:1,000, Genetex, no. 89962), and the pan-neuronal marker Hu [human anti-Hu, 1:25,000 (26), 48 h at 4°C, diluted in blocking buffer]. Primary antibody binding was detected using donkey secondaries conjugated to Alexa 488 (anti-rabbit, Thermo Fisher, 1:500), Alexa 568 (anti-goat, Thermo Fisher, 1:500), and Alexa 647 (anti-human, Jackson ImmunoResearch, 1:500, 1 h at room temperature, diluted in PBS). Tissues were washed (3 × 10 min, PBS) and then mounted using ProLong Diamond antifade mountant (Thermo Fisher).

Microscopy and Image Analysis

Images were captured using an SP8 TCS confocal system. Five images including myenteric ganglia were captured per preparation (×40 objective, numerical aperture 1.3, >2.0 zoom, 16-bit depth, 1,024 × 1,024 pixel resolution). The subcellular distribution of DOREGFP within the neuronal soma was determined using nNOS and Hu immunoreactivities to define cellular morphology. Images were converted to binary (i.e., positive or negative pixels), using the nucleus to define the threshold for positive staining. At least 35 neurons from 3–5 preparations were analyzed per treatment group. The subcellular distribution of DOREGFP was expressed as the relative percentage of total cellular DOREGFP that was associated with the plasma membrane. Single optical sections containing the neuronal soma and nucleus were included in analysis. A threshold based on the absence of nuclear DOREGFP was set for each image. Images were converted to binary with pixels set to either 0 (negative) or 255 (positive). Individual neurons were outlined based on Hu and nNOS immunoreactivities, and total positive pixels were determined. A second region of interest was drawn immediately beneath the plasma membrane, allowing measurement of intracellular pixels. Finally, nuclear labeling was quantified and subtracted from total and intracellular values. The relative percentages of membrane [= total – (intracellular + nuclear positive pixels)] and cytosolic (= intracellular – nuclear positive pixels) labeling were then determined.

Calcium Imaging of Whole Mount Preparations

The effects of PS2 and PS2i on neuronal function were assessed by calcium imaging of circular muscle-myenteric plexus whole mount preparations of the colon from Wnt1-GCaMP3 mice, which express the genetically encoded calcium biosensor GCaMP3 only in neural crest-derived cells [i.e., enteric neurons and glia (17)]. Tissues were imaged in calcium assay buffer (10 mM HEPES, 0.5% BSA, 10 mM d-glucose, 2.2 mM CaCl₂·H₂O, MgCl₂·6H₂O, 2.6 mM KCl, 150 mM NaCl) using a Leica SP8-MP multiphoton system (HC PLAN APO 0.95 NA/25x water immersion objective). Images were captured at 16-bit depth, pinhole of 8–10 airy units, 1,024 × 1,024 pixel resolution at a frame rate of one image every 0.86 s). Preparations were incubated with PS2 or PS2i (both 15 μM) for 20 min before and during imaging. Tissues were challenged with ATP (100 μM), and the responses were recorded over a 2-min period. Whole mounts were then fixed and stained for protein gene product (PGP)9.5 (rabbit clone EPR4118; Abcam, ab108986) and glial fibrillary acidic protein (GFAP; chicken, 1:1,000, ab4674, Abcam) immunoreactivities to identify all neurons, nerve fibers, and enteric glial cells, respectively. Nuclei were labeled with DAPI. Immunofluorescence was imaged (×20 objective, 1.3 numerical aperture). The calcium imaging and immunolabeled images were overlaid using key landmarks with the bUnwarp plugin in ImageJ (v.1.52i) (2). Myenteric neurons were identified by PGP9.5 labeling, and enteric glial cells were identified based on GFAP immunoreactivity and DAPI staining. The calcium responses were extracted using ImageJ and normalized to baseline (F₀/F₀). Positive responses were defined based on an elevation above baseline of greater than 10%.

Tissue Contracture Assays

Muscle strips were prepared from the distal colon as described (8). Briefly, tissues were placed in 10-ml water-jacketed organ baths containing Krebs solution (in mMol/l): 118 NaCl, 4.70 KCl, 1 NaH₂PO₄-2H₂O, 25 NaHCO₃, 1.2 MgCl₂-6H₂O, 11 t-glucose, and 2.5 CaCl₂-2H₂O: maintained at 37°C and bubbled with 95% O₂-5% CO₂. Isometric contractions of the circular muscle were measured by a Grass FT03 force displacement transducer (Grass Instruments, Quincy, MA). Data were acquired with a PowerLab 4/SP system and were viewed using LabChart software (v.5; AD Instruments, Castle Hill, NSW, Australia). Strips were placed under a resting tension of 0.5–1 g and were equilibrated for 30 min before use. Drugs were applied at a volume of 10–15 μl. After the completion of each experiment, tissues were contracted with carbachol (10 μM) to evaluate viability. Tissues that were unresponsive to carbachol were omitted from analysis.

Electrically stimulated contractions. Neurogenic contractions were evoked by transmural electrical field stimulation (EFS; 0.5 ms duration, 3 pulses/s, 60 V), which was applied through platinum electrodes incorporated into the tissue holders (8). Once reproducible baseline contractile responses to EFS were maintained (minimum of 3 sets, 5-min intervals), tissues were washed and preincubated (10 min) with either vehicle (0.1% DMSO), PS2 (15 μM), or Compd101 (10 μM). EFS (3 sets, 5-min intervals) were subsequently applied to the muscle strips. The amplitudes of EFS-evoked contractions were compared with baseline responses (i.e., in the absence of inhibitors). Data were expressed as percent baseline EFS-evoked contractions.

Drug-evoked contractions. After the equilibration period, EFS was applied (2 sets, 2-min intervals) to determine tissue viability. Muscle strips were washed and preincubated (10 min) with either vehicle or inhibitors as described above. TTX was added to the bath to determine the effects on inhibitory neurotransmission. To measure the direct effects of the inhibitors on smooth muscle contractility, the nonselective muscarinic agonist carbachol, which evokes contractions mainly through actions on smooth muscle (1), was added cumulatively to the bath (1 nM–10 μM). Data were
fitted to three-parameter nonlinear regression curves using Prism (v.8.01; GraphPad Software) and EC50 and Emax values were determined. In another set of experiments, tissues were exposed to either SNC80 (1 nM–10 μM, cumulative additions every 2 min) or morphine (1 μM). For each experiment, the maximum contraction amplitude was measured and corrected to baseline activity (2 min before addition of endocytic inhibitors). Data were expressed as grams of tension (g) generated.

**Statistical Analysis**

Data were expressed as means ± SE, and graphs were constructed in Prism v.8.0.1. All groups for image analysis were compared by one-way ANOVA. For contraction assays, specific statistical analyses used for each experiment are stated in the respective figure legends. P < 0.05 was defined as significantly different to the null hypothesis of no difference between means at the 95% confidence level.

**RESULTS**

PS2 is a tool compound that is routinely used to examine the importance of clathrin and the process of endocytosis for cell surface protein function. PS2 complexes with the terminal domain of clathrin, preventing interaction with endocytic ligands containing clathrin box motifs (49). Recently, PS2 has enabled determination of the relative contribution of intracellular signaling for physiological and pathophysiological effects of GPCR activation (16, 20). Cmpd101 is a GRK2/3 inhibitor that has been used to inhibit MOR internalization and desensitization in model cell lines and neurons (25, 29). In the present report, we determined the potential utility of these small molecule inhibitors for the study of the neurogenic actions of GPCRs on GI functions.

**DOR Endocytosis Is Significantly Inhibited by Clathrin and GRK2/3 Inhibition**

To assess the effectiveness of PS2 and Cmpd101 in inhibiting native GPCR endocytosis in myenteric neurons, we examined SNC80-evoked redistribution of DOR, using colon tissue from knockin mice expressing DOReGFP. Under basal conditions DOReGFP was localized to the cell surface of a subset of myenteric neurons (% cell surface-associated: means ± SE 78.6% ± 1.1%, 95% confidence interval: 76.5–80.8; n = 70 neurons; Fig. 1, inset 1). Treatment with PS2 (15 μM) or Cmpd101 (10 μM) alone did not significantly alter the subcellular distribution of DOReGFP under basal conditions (PS2: 80.7% ± 1.3, 78.1–83.2; n = 35; Cmpd101: 82.2% ± 0.7, 80.7–83.7, n = 62). Exposure to the DOR agonist SNC80 (1 μM, 30 min, EC80 concentration) promoted significant redistribution of DOReGFP from the cell surface to endosome-like structures (49.5% ± 1.02, 47.5–51.6; n = 106 neurons, P < 0.001). PS2 pretreatment significantly diminished DOReGFP internalization to SNC80 (79.8 ± 0.8%, 78.2–81.4, n = 127, P < 0.001). The inactive control for PS2, PS2i (15 μM) did not significantly affect SNC80-induced DOReGFP endocytosis (44.7 ± 1.0%, 42.7–46.8, n = 66, P = 0.12). Inhibition of GRK2/3 with Cmpd101 also significantly attenuated SNC80-evoked DOReGFP endocytosis (69.5 ± 1.7%, 66.3–72.8, n = 101, P < 0.001).

**Inhibitory Neurotransmission Is Unaffected by Endocytic Inhibitors**

The resting tone of the colon is maintained by nitricergic inhibitory motor neurons. Disinhibition of this input results in a sustained contraction of the external smooth muscle layers (47). The basal tone of the colon was unaffected by the addition of either PS2 or Cmpd101, indicating that the inhibitory input remained intact (Fig. 2, A–C). Removal of this inhibition by the neurotoxin TTX resulted in a tonic contraction (0.12 ± 0.04 g, n = 6; Fig. 2 A and D), which was unaffected by PS2 (0.24 ± 0.09 g, n = 6; Fig. 2, B and D) and Cmpd101 (0.23 ± 0.13 g, n = 6; Fig. 2, C and D). This confirms that these compounds have no detectable influence on inhibitory neuromuscular transmission.

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**Fig. 1.** Pitstop2 (PS2) and Takeda compound 101 (Cmpd101) are effective inhibitors of δ-opioid receptor (DOR) endocytosis in myenteric neurons. A: under basal conditions, DOR tagged with COOH-terminal enhanced green fluorescent protein (DOReGFP) was localized to the cell surface of myenteric neurons (inset 1, arrows). SNC80 (1 μM, 30 min) stimulated internalization of DOReGFP from the cell surface to endosomes (inset 2, arrowheads), which was effectively blocked by PS2 (inset 3, arrows) and by Cmpd101 (inset 4, arrows). Inhibitors alone had no effect on the cellular distribution of DOReGFP. Scale, 20 and 10 μm (inset images). B: quantitative analysis of percent total DOReGFP at the cell surface under each treatment condition (mean ± 95% confidence interval). n = 35–127 neurons per group, with individual responses represented by triangles; n = 3–5 mice. ****P < 0.0001, by one-way ANOVA with Tukey’s post hoc test. Comparisons shown for inhibitors relative to SNC80 treatment.
Transmural EFS produces a neurogenic contraction that is mediated primarily by the release of acetylcholine (ACh) and activation of muscarinic receptors on GI smooth muscle cells. Inhibition of electrically evoked intestinal contractions is commonly used to characterize opioid receptor agonists. We examined the effect of PS2 and Cmpd101 on the amplitude of EFS contractions to assess their suitability for use in this assay. Both PS2 (39.7 ± 3.6% of baseline EFS, n = 8) and Cmpd101 (46.9 ± 8.7%, n = 6) significantly suppressed EFS-evoked contractions compared with vehicle (88.7 ± 8.8%, n = 6, P < 0.001 and P < 0.01, respectively; Fig. 3, A and C–E). PS2i, the inactive control for PS2, did not affect EFS-evoked contractions (91.9 ± 8.5%, n = 6; Fig. 3, B and E). In contrast to Cmpd101, PS2 produced a graded reduction in contraction amplitude with each successive electrical stimulus (1st stimulation: 64.1 ± 4.8%, 2nd: 37.1 ± 4.3%, P < 0.001, and 3rd: 26.0 ± 4.9%, P < 0.001; Fig. 3E). This is consistent with depletion of existing stores of ACh and a reduced capacity to effectively release vesicular stores. These observations indicate that both PS2 and Cmpd101 are unsuitable for examination of GPCR function in assays involving assessment of electrically evoked contractions.

The direct actions of endocytic inhibitors on smooth muscle were measured using carbachol, which produced robust concentration-dependent contractions (pEC50 = 5.96 ± 0.37, Emax = 1.77 ± 0.35 g, n = 5; Fig. 4, A and D). These responses were unaffected by PS2 (pEC50 = 5.67 ± 0.18, Emax = 1.52 ± 0.16 g, n = 6; Fig. 4, B and D) but were significantly decreased by Cmpd101 (pEC50 = 5.37 ± 0.35, Emax = 0.76 ± 0.20 g, n = 5, P = 0.02; Fig. 4, C and D). Together, these findings suggest that PS2 and Cmpd101 exert anticholinergic effects through distinct mechanisms; whereas PS2 reduces cholinergic contractions via a neurogenic mechanism, Cmpd101 exerts its inhibitory effects, in part, through actions on muscarinic signaling in smooth muscle cells.

**PS2 Does Not Affect Activation of Myenteric Neurons**

The inhibition of EFS-evoked contractions by PS2 could be due to a general inhibitory or deleterious effect on myenteric neuron function or through decreased synaptic vesicle formation and vesicular release of excitatory neurotransmitters. To determine the mechanism of action, the potential inhibitory effects of PS2 on myenteric neurons were examined using Ca2+ imaging of myenteric whole mount preparations. Preparations were challenged with ATP, which activates both GPCRs (P2Y) and ion channels (P2X) (3). The effects of PS2 and PS2i on ATP-evoked (100 μM) increases in intracellular Ca2+ levels in neurons and glia were examined. PS2 or PS2i did not significantly alter the peak amplitude of ATP responses by neurons (n = 333) and glia (n = 517) relative to vehicle controls (Fig. 5, A–C; n = 3–4 mice per group). The PGP9.5-positive nerve fibers associated with the circular muscle layer also responded to ATP in the presence of PS2 (Fig. 5D). These data demonstrated that PS2-dependent inhibition of neurogenic contractions is unlikely to be through adverse effects on neuronal activation or neuronal viability.

**PS2 Reduces GPCR-Mediated Responses Via an Internalization-Independent Mechanism**

PS2 has been used to investigate GPCR internalization and signaling in recombinant cell systems and in natively expressing cells and tissues (16, 20, 36). We have demonstrated that PS2 alone does not influence contractions to TTX. Like TTX, opioid receptor agonists produce a neurogenic smooth muscle contraction that is mediated via the disinhibition of the resting tone (8, 31). Thus, we hypothe-
sized that contractile responses to both internalizing and noninternalizing opioid receptor agonists would be similarly unaffected by PS2. To test this prediction, the functional effects of PS2 on GPCR-mediated responses of the colon were examined using SNC80 and morphine, which were chosen due to their markedly different abilities to promote opioid receptor endocytosis. SNC80 strongly internalizes DOR (8, 37), whereas acute exposure to morphine activates MOR signaling in myenteric neurons without promoting receptor endocytosis (10, 32, 43). SNC80 elicited sustained contractions in a concentration-dependent manner. These responses were biphasic with a maximum response at 100 nM (0.09 ± 0.03 g, n = 11). PS2 effectively abolished these responses (Fig. 6B). Morphine (1 µM) also evoked a rapid and sustained contraction (0.14 ± 0.04 g, n = 6), which was significantly reduced by PS2 (0.03 ± 0.04 g, n = 6, P < 0.05; Fig. 6, A and B). The effects of PS2 on opioid-dependent suppression of neurogenic contractions and of Cmpd101 on opioid-evoked contractions were not examined due to the effects of these inhibitors on EFS- and carbachol-stimulated contractions, respectively. These data indicate that the PS2-dependent inhibition of GPCR-mediated colonic contractions is via an internalization-independent mechanism.

Fig. 3. Endocytic inhibitors reduce electrically stimulated contractions of the colon. A: amplitude of electrical field stimulation (EFS)-evoked contractions was unaffected by addition of vehicle. Solid circles represent where EFS was applied. Exposure to Pitstop2 inactive control (PS2i; 15 µM) had no effect on contractions (B), whereas Pitstop2 (PS2; 15 µM) inhibited subsequent EFS-evoked contractions in a graded manner (C). D: G protein-coupled receptor kinase (GRK)2/3 inhibition by Takeda compound 101 (Cmpd101; 10 µM) suppressed EFS-evoked contractions. E: quantitative analysis of contractions to sequential electrical stimulations following exposure to the different treatments. Data are expressed as means ± SE; n = 6–8 mice per group, with individual responses represented by triangles. Individual EFS amplitudes were compared using repeated-measures two-way ANOVA followed by Tukey’s post hoc test. **P < 0.01 and ***P < 0.001.
DISCUSSION

Use of Endocytic Inhibitors for Physiological Studies of Gastrointestinal Function

Our data show that the inhibitor of clathrin-mediated endocytosis PS2 effectively prevents agonist-evoked GPCR endocytosis in enteric neurons. However, PS2 blocked contractions evoked by agonists that either strongly or weakly internalize opioid receptors (SNC80 and morphine, respectively), indicating that this effect was independent of receptor endocytosis. Moreover, our functional studies support a model in which the effects of PS2 are mediated through actions on neurotransmitter release from the ENS rather than through block of target GPCR endocytosis or inhibition of smooth muscle activity (Fig. 7). PS2 has been used to define the importance of endosomal signaling in model cells and neurons (16, 20, 21, 53) and has also been administered intrathecally to block nociceptive signaling from second-order neurons of the spinal cord (20). Although direct examination of compartmentalized signaling in enteric neurons can be similarly determined using PS2, the present study demonstrates that currently available tools cannot be used to determine the functional importance of GPCR endocytosis and endosomal signaling to GI motility. Approaches such as endosomally targeted antagonists (20, 21, 53) or evaluation of receptor internalizing vs noninternalizing agonists will be better suited for this type of study.

Cmpd101 is an inhibitor of GRK2 and -3 (45), which are key enzymes required for phosphorylation of activated GPCRs and for ensuing βArr recruitment, receptor endocytosis, and desensitization. Cmpd101 blocks MOR desensitization and endocytosis in model cell lines and locus coeruleus neurons (25, 27, 53), or evaluation of receptor internalizing vs noninternalizing agonists will be better suited for this type of study.

Cmpd101 is an inhibitor of GRK2 and -3 (45), which are key enzymes required for phosphorylation of activated GPCRs and for ensuing βArr recruitment, receptor endocytosis, and desensitization. Cmpd101 blocks MOR desensitization and endocytosis in model cell lines and locus coeruleus neurons (25, 27, 29), consistent with the GRK2/3-mediated multiosite phosphorylation of this receptor (9, 27). In contrast, the COOH terminus of DOR is not highly phosphorylated (14) and the requirement for GRK2 activity for DOR trafficking appears to be dependent on the cellular background and potentially on the neuronal subtype examined (5, 55). While we did not detect a significant difference in SNC80-evoked internalization profiles across nNOS positive and negative neurons upon treatment with SNC80 (J. DiCello and D. Poole, unpublished data), we show that Cmpd101 was an effective inhibitor of SNC80-evoked DOR-eGFP endocytosis in myenteric neurons. However, Cmpd101 suppressed cholinergic contractions, rendering it unsuitable for use in standard assays of GI function.

PS2 Suppresses Neuromuscular Transmission

Although PS2 attenuates excitatory neuromuscular transmission, it is possible that this is mediated through altered synaptic vesicle formation, rather than through reduced transmitter release or block of neuronal depolarization. Inhibitory neurotransmission in the ENS is primarily mediated through the gaseous transmitter NO. TTX contracts GI smooth muscle through suppression of nitrergic signaling, which effectively removes an underlying inhibitory influence to reveal myogenic activity. Our data suggest that release of transmitter stores, such as those for ACh, is a clathrin-dependent process, whereas NO generation and release are mediated through a clathrin-independent mechanism. This is consistent with the effect of clathrin depletion on the quantal size and relative amount of readily releasable ACh-containing vesicles (24), the loading of ACh into clathrin-sculpted synaptic vesicles (39), and the clathrin-dependent uptake of choline into endosomes (39, 40). In contrast, NO release is through diffusion across membranes and is therefore unlikely to be impacted by clathrin inhibition (23). This model is supported by key findings of the present study: 1) PS2 diminished EFS-evoked cholinergic contractions in a graded manner, which is consistent with depletion of
existing stores and a reduced capacity to effectively replace vesicular stores; 2) nitrergic transmission and TTX-evoked contractions were unaffected by PS2; 3) direct activation of GI smooth muscle by carbachol was unaffected by PS2; and 4) myenteric neurons responded normally to exogenous application of ATP in the presence of PS2. Mechanistically, these data support a clathrin-dependent effect on vesicle formation rather than a direct suppression of neuromuscular transmission or effect on GI smooth muscle.

**PS2 Influences Opioid Receptor Signaling Independently of Endocytosis**

The effects of DOR agonists on neurogenic contractions of the colon are correlated with the receptor-internalizing properties of the agonist. SNC80, which strongly internalizes DOR, evokes robust contractions, effectively inhibits electrically stimulated contractions, and rapidly desensitizes DOR (8). In contrast, the related compound AR-M 1000390 (ARM390),
which only weakly internalizes DOReGFP at higher concentrations, exhibits a reduced capability to evoke contractions, inhibits EFS-contractions, and desensitizes DOR (7). The distinct antinociceptive capacities of these two ligands and the subsequent development of analgesic tolerance have been attributed to their respective internalizing properties (38). However, ARM390 is a partial agonist in the ENS, and reduced effector coupling capacity is the most likely explanation for the agonist-dependent differences observed in the colon (8). The block of morphine-evoked contractions by PS2 supports this hypothesis, as morphine does not promote MOR endocytosis in myenteric neurons (30, 32, 43). The equivalent block of contractions to both SNC80 and morphine indicates that PS2 is likely to have effects on downstream signaling from opioid receptors that are endocytosis independent and not directly related to effects of PS2 on neurotransmission. This does not exclude effects of PS2 on lateral movement and clustering of MOR within the plasma membrane, which can also significantly influence downstream signaling (16).

Cmpd101 Suppresses Cholinergic Smooth Muscle Contractions

Cmpd101 inhibited electrically evoked neurotransmitter release, or muscarinic receptor signaling in smooth muscle effector cells. Direct examination of contractions to carbachol demonstrated a significant reduction in efficacy, but not potency, in the presence of Cmpd101. Thus, Cmpd101 has a direct effect on GPCR-mediated smooth muscle activity. The specific effects of Cmpd101 on signaling downstream of GPCRs in GI smooth muscle and enteric neurons is presently undefined. However, our proposed mechanism of action is consistent with a previous report that Cmpd101 inhibits GPCR- and electrically evoked prostatic smooth muscle contractions (54).

Nonspecific Actions of Endocytic Inhibitors

The nonselective and endocytosis-independent actions of commonly used inhibitors have been reported. In particular, the mode of action and selectivity of PS2 has been challenged, based on studies using cells expressing clathrin heavy chain with NH2-terminal domain interaction site mutations (51), demonstration of inhibitory effects on non-clathrin-mediated endocytosis (11, 15), and the negative effects of PS2 on GPCR expression at the cell surface (15). However, these compounds are still routinely used to define the importance of cell surface and intracellular GPCR signaling. These inhibitors are extremely useful tools for the study of receptor signaling in smooth muscle.

Fig. 7. Proposed mechanisms of action of endocytic inhibitors on neuromuscular transmission in the enteric nervous system. A: Pitstop2 (PS2) suppresses synaptic transmission through effects on vesicle formation and release, reuptake of neurotransmitters, and negative modulation of G protein-coupled receptor (GPCR) signaling. PS2 influences vesicle size and number and suppresses excitatory neurotransmitter release at synapses and neurotransmitter uptake into presynaptic terminals. PS2 also inhibits downstream signaling from postsynaptic GPCRs. B: PS2 blocks release of transmitter from nerve fibers associated with the circular muscle layer. C: Takeda compound 101 (Cmpd101) does not block neurotransmitter release but inhibits cholinergic contractions through direct influence on signaling by GPCRs on smooth muscle cells.
isolated cells (16, 20). In these systems it is possible to use additional genetic approaches to confirm that effects are specific and reflect actions on the endocytic machinery (e.g., expression of dominant negative dynamin, small interfering RNA knockdown of dynamin, use of knockout cell lines). The use of equivalent validation approaches in physiological systems, such as the ENS, is challenging or impossible. The potential for off-target actions must therefore be considered when interpreting any data derived using these inhibitors. This is clearly illustrated by the findings outlined in the present study.

In conclusion, commonly used and commercially available endocytosis inhibitors are effective tools for the study of GPCR signaling in isolated neurons (16). However, the present study demonstrates that they are likely to prove unsuitable for studies of the neurogenic control of complex physiological processes in the GI tract. Clathrin inhibition is associated with altered neurotransmitter release and uptake in the central nervous system (39, 50), and we provide evidence that PS2 negatively impacts neuromuscular transmission in the gut. This presents a potentially significant challenge to their use for studying GI function, as many processes are mediated through agonist actions at the level of the ENS. Moreover, our data indicate that there are inhibitory effects of PS2 on GPCR signaling that are likely to be endocytosis independent. Thus, caution should be taken when interpreting physiological studies derived using these inhibitors.

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AUTHOR CONTRIBUTIONS
J.J.D., P.R., S.E.C., and D.P.P. conceived and designed research; J.J.D., P.R., S.E.C., and D.P.P. performed experiments; J.J.D., P.R., E.M.E., A.S., and D.P.P. analyzed data; J.J.D., P.R., A.B.G., N.A.V., M.C., S.E.C., and D.P.P. interpreted results of experiments; J.J.D., P.R., and D.P.P. prepared figures; J.J.D. and D.P.P. drafted manuscript; J.J.D., A.B.G., M.C., S.E.C., and D.P.P. edited and revised manuscript; J.J.D., P.R., S.E.C., and D.P.P. approved final version of manuscript.

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