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Mechanosensitive ion channel Piezo2 is important for enterochromaffin cell response to mechanical forces

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Key points

- The gastrointestinal epithelial enterochromaffin (EC) cell synthesizes the vast majority of the body's serotonin. As a specialized mechanosensor, the EC cell releases this serotonin in response to mechanical forces. However, the molecular mechanism of EC cell mechanotransduction is unknown.
- In the present study, we show, for the first time, that the mechanosensitive ion channel Piezo2 is specifically expressed by the human and mouse EC cells.
- Activation of Piezo2 by mechanical forces results in a characteristic ionic current, the release of serotonin and stimulation of gastrointestinal secretion.
- Piezo2 inhibition by drugs or molecular knockdown decreases mechanosensitive currents, serotonin release and downstream physiological effects.
- The results of the present study suggest that the mechanosensitive ion channel Piezo2 is specifically expressed by the EC cells of the human and mouse small bowel and that it is important for EC cell mechanotransduction.

Abstract The enterochromaffin (EC) cell in the gastrointestinal (GI) epithelium is the source of nearly all systemic serotonin (5-hydroxytryptamine; 5-HT), which is an important neurotransmitter and endocrine, autocrine and paracrine hormone. The EC cell is a specialized mechanosensor, and it is well known that it releases 5-HT in response to mechanical forces. However, the EC cell mechanotransduction mechanism is unknown. The present study aimed to determine whether Piezo2 is involved in EC cell mechanosensation. Piezo2 mRNA was expressed in human jejunum and mouse mucosa from all segments of the small bowel. Piezo2 immunoreactivity localized specifically within EC cells of human and mouse small bowel epithelium. The EC cell model released 5-HT in response to stretch, and had Piezo2 mRNA and protein, as well as a mechanically-sensitive inward non-selective cation current characteristic of Piezo2. Both inward currents and 5-HT release were inhibited by Piezo2 small interfering RNA and antagonists (Gd^{3+} and D-GsMTx4). Jejunum mucosal pressure increased 5-HT release and short-circuit current via submucosal 5-HT3 and 5-HT4 receptors. Pressure-induced secretion was inhibited by the mechanosensitive ion channel antagonists gadolinium, ruthenium red and D-GsMTx4. We conclude that the EC cells in the human and mouse small bowel GI epithelium selectively express the mechanosensitive ion channel Piezo2, and also that activation of Piezo2 by force leads to

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inward currents, 5-HT release and an increase in mucosal secretion. Therefore, Piezo2 is critical to EC cell mechanosensitivity and downstream physiological effects.

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Abbreviations CFP, cyan fluorescent protein; DAPI, 4′,6-diamidino-2-phenylindole; EC, enterochromaffin; EP_{50}, half-point of the pressure–stimulus relationship; Gd^{3+}, gadolinium; GI, gastrointestinal; GR, GR 113808; G_t, tissue conductance; 5-HT, 5-hydroxytryptamine; IHC, immunohistochemsity; I_{sc}, short-circuit current; PB, phosphate buffer; PFA, paraformaldehyde; siRNA, small interfering RNA; TPH-1, tryptophan hydroxylase 1.

Introduction

In the 1930s, Vittorio Erspamer derived a biologically active amine, now known as serotonin (5-hydroxytryptamine; 5-HT), from the gut mucosa (Erspamer, 1957). He also showed that a unique chromium stained (hence enterochromaffin; EC) cell in the gut epithelium was solely responsible for production and presumably secretion of 5-HT into the peripheral circulation. 5-HT is now established as a neurotransmitter and an important autocrine, paracrine and endocrine molecule (Mawe & Hoffman, 2013). 5-HT controls gastrointestinal (GI) secretory (Brown, 1996; Hoffman *et al.* 2012), motor (Heredia *et al.* 2009; Hoffman *et al.* 2012; Heredia *et al.* 2013) and sensory (Zhu *et al.* 2001; Hoffman *et al.* 2012) functions. 5-HT is also taken up by platelets and becomes circulating 5-HT with important functions at a distance from the GI tract (Berger *et al.* 2009), such as platelet aggregation (Carneiro *et al.* 2008), vascular tone (Launay *et al.* 2002), skin sensation (Press *et al.* 2010), and even bone (Yadav *et al.* 2010) and metabolic health (Crane *et al.* 2015).

The EC cell is a part of a vast enteroendocrine sensory network that responds to chemical stimuli by hormone secretion. In 1959, Edith Bulbring's seminal work showed that small bowel mucosal pressure led to 5-HT release from the EC cell (Bulbring & Crema, 1959). The EC cell is now established as a primary mechanosensor of the gastrointestinal epithelium, meaning that the EC cell response to force defines the GI epithelium response to mechanical stimuli (Bulbring & Lin, 1958). Although we know that mechanical forces are strong stimuli for 5-HT release (Bertrand, 2004), the molecular mechanism of EC cell mechanotransduction remains unknown. Previous studies have implicated several molecules in EC cell mechanosensitivity, including G-protein coupled receptors (e.g. Gαq) (Kim *et al.* 2001) and purine receptors P2X and P2Y (Christofi, 2008; Chin *et al.* 2012; Linan-Rico *et al.* 2013). However, these molecules are probably downstream of the primary mechanosensor (Linan-Rico *et al.* 2013).

Mechanical force activates the EC cell within milliseconds (Bertrand, 2004). It is well known that fast cellular mechanosensation relies on mechanically gated ion channels, which serve as primary mechanosensors by converting mechanical energy into electrical signals (Arnadottir & Chalfie, 2010). Recent studies have shown that the mechanosensitive ion channel Piezo2 is the primary mechanosensor in several specialized mechanosensory cells, including proprioceptive (Woo *et al.* 2015) and light-touch dorsal root ganglion neurons (Ranade *et al.* 2014), as well as Merkel cells in the skin (Ikeda *et al.* 2014; Ranade *et al.* 2014; Woo *et al.* 2014). Merkel and EC cells share important functional and developmental properties. Both are specialized epithelial mechanosensors (Raybould *et al.* 2004; Nakatani *et al.* 2014), with a genetic lineage that depends on the transcription factor *Atoh1* (*Math1*) (Yang *et al.* 2001; Li *et al.* 2011; Roach *et al.* 2013; Wright *et al.* 2015), and secrete neurotransmitters in response to mechanical forces (Forsberg & Miller, 1983; Tachibana *et al.* 2005), and contact neurons (Haeberle *et al.* 2004; Bohorquez *et al.* 2015).

Piezo2 (encoded by *PIEZO2* or *FAM38B*) is a transmembrane protein that forms a cation selective mechanosensitive ion channel (Coste *et al.* 2010; Coste *et al.* 2012). In the Merkel cell, force activates Piezo2 mechanosensitive inward cation currents (Woo *et al.* 2014), leading to depolarization and activation of an L-type calcium channel (Ikeda *et al.* 2014), resulting in further increase of intracellular Ca^{2+}, presumably leading to exocytosis (Haeberle *et al.* 2004). Similarly, force applied to the EC cell leads to a Ca^{2+} influx via depolarization and subsequent activation of a voltage-gated L-type calcium channel (Raghupathi *et al.* 2013), which is important for 5-HT release.

Given the similarities between EC and Merkel cells, we investigated whether Piezo2 is involved in EC cell mechanosensation. In the present study, we found that Piezo2 is specifically expressed in human and mouse EC cells throughout the length of the small bowel and also that it is required for coupling of mechanical forces to 5-HT release and downstream GI physiological functions.

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Methods

Ethical approval

All experimental procedures were approved by the Institutional Review Board and Institutional Animal Care and Use Committee (A34414) of the Mayo Clinic.

Drugs

Fluoxetine and gadolinium (Gd^{3+}) were made as stock solution (10 mM in water). Ruthenium red was prepared fresh daily. All drugs were obtained from Sigma-Aldrich (St Louis, MO, USA). Working extracellular solutions were prepared from stock on the day of experiments. D-GsMTx4 was made as a working solution on the day of experiments by dissolving directly into the relevant extracellular solution.

Cell culture

QGP-1 cells. QGP-1 (passages 17–20) (a kind gift from Dr Valeria Giandomenico, Uppsala University, Uppsala, Sweden) were cultured in modified RPMI 1640 with 10% fetal bovine serum, 1% penicillin–streptomycin and 1% L-glutamine. Cells were grown to 50–60% confluence in either T25 flasks or laminin coated flexible membranes (Flexcell International, Burlington, NC, USA). When noted, Piezo2 was knocked down using 50 nM small interfering RNA (siRNA) (L-013925-02-0005; Dharmacon, Lafayette, CO, USA) for 48 h with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) transfection. Cells for electrophysiology were co-transfected with siGLO Green Transfection Indicator (D-001630-01-05; Dharmacon). On the day of experiments, cells for electrophysiology experiments were lifted with trypsin-EDTA (0.5%) and plated onto the test chamber.

Molecular biology

Real time RT-PCR. Tissue was collected from human jejunum and mouse mucosa of duodenum, jejunum and ileum. QGP-1 cells were trypsinized and collected. Collected tissues and cells were frozen in liquid nitrogen. Total RNA was isolated using RNA-bee (Tel-Test Inc., Friendswood, TX, USA) and cleaned using a RNeasy Kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions. Reverse transcription of the RNA was completed using a SuperScript VILO cDNA Synthesis Kit (Invitrogen) and a PCR reaction that included annealing the RNA for 10 min at 25°C, and also a 60 min cycle at 42°C and 5 min at 85°C to denature the enzyme. cDNA was diluted 10-fold and analysed for Piezo2 (primer from Origene, Rockville, MD, USA) by quantitative real-time PCR in accordance with the instructions of the manufacturer of the LightCycler 480 SYBR Green I Master (Roche, Basel, Switzerland). The mRNA expression of Piezo2 was normalized to species-dependent β-actin expression. Error bars indicate the SE.

Immunohistochemistry (IHC)

IHC protocols. Human jejunum tissues were obtained from patients undergoing Roux-en-Y gastric bypass surgery. Tissues were placed in ice-cold F12 medium (Invitrogen). A piece of tissue (2 × 2 cm) was dissected, pinned out in 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB) overnight, washed in 0.1 M PBS and then transferred into 30% sucrose in PBS overnight, and then frozen in OCT embedding compound (Sakura Finetek, Torrance, CA, USA) at −80°C until sectioning. Mouse tissues comprised flat sheets (1 × 0.5 cm) or tubes (length 0.5 cm) from duodenum, jejunum and ileum of TPH1-CFP mice. Tissues were fixed in 4% PFA-PB for 4 h separately, then washed in PBS and moved into 30% sucrose in PBS overnight before being frozen in OCT embedding compound (Sakura Finetek) at −80°C until sectioned. Tissues were cut into 12 μm thick sections, rinsed with PBS twice for 5 min and then blocked with 200 μl per slide of 1% BSA/PBS/0.3% Triton X/10% normal donkey serum in a humidity chamber. Primary antibodies (Piezo2 mouse antibody a kind gift from Dr Ardem Patapoutian) were added in 200 μl per slide of BSA/PBS/0.3% Triton/10% normal donkey serum and were incubated at 4°C overnight in a humidity chamber. Slides were then rinsed five times for 3 min in PBS. Secondary antibody was incubated for 1 h in the dark. Slides were mounted in slowfade gold with 4′,6-diamidino-2-phenylindole (DAPI) (Life Technologies, Grand Island, NY, USA) mounting buffer.

Cell quantification. 5-HT and/or Piezo2 positive cells were quantified in the epithelium only, and were defined by DAPI-staining as the luminal facing layer of the mucosa. Imaging was performed using Olympus BX51W1 (40×) and Olympus FV1000 confocal (20×, 0.95 NA and 60×, 1.2 NA, z-res 0.91 μm) microscopes (Olympus Corporation, Tokyo, Japan). We counted EC cells ($TPH1\text{-}CFP^{+}$) from three mice across all small bowel segments, each with three sections separated by z-distance >50 μm ($n = 3$ mice, $n = 97 \pm 23$ fields per mouse, $n = 217 \pm 30$ cells per mouse). Error bars indicate the SE.

Single cell electrophysiology

Solutions. The extracellular solution contained (in mM): 150 Na^{+}, 5 K^{+}, 2.5 Ca^{2+}, 1 Mg^{2+},160 Cl^{-}, 10 Hepes and 5.5 glucose (pH 7.35), 300 mmol kg^{-1}; the intracellular solution contained (in mM): 140 Cs^{+}, 150 Cl^{-}, 4 Mg^{2+}, 2 Ca^{2+}, 10 Hepes and 5 EGTA (pH 7.3), 300 mmol kg^{-1}.

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Data acquisition. Standard whole cell voltage clamp was used as described previously (Strege *et al.* 2003; Saito *et al.* 2009; Strege *et al.* 2012). Electrodes (Kimble KG12 glass) were pulled using a Sutter P97 puller (Sutter Instruments, Novato, CA, USA), coated with R6101 (Dow Corning, Auburn, MI, USA) and fire polished to 2–5 MΩ. Stimulation and data acquisition were performed using an an Axopatch 200B patch clamp amplifier, CyberAmp 320 signal conditioner, Digidata 1550A and pClamp, version 10.5 (Molecular Devices, Sunnyvale, CA, USA). Once the cells were voltage clamped, mechanical stimulation was applied via fire-polished glass microelectrodes (10–25% of cell size) driven by a piezo-transducer P-621.1CD attached to an E-625.CR controller (Physik Instrumente, Karlsruhe, Germany) (Coste *et al.* 2010; Maksimovic *et al.* 2014). Cells were subjected to a series of mechanical steps of 0.3 μm increments.

Data analysis. Whole cell patch clamp data were analysed in pClamp, version 10.5 (Molecular Devices). The peak currents within 10 ms of stimulus start were selected for analysis. pClamp (Molecular Devices) and Origin 2016 (OriginLab Co., Northampton, MA, USA) were used for electrophysiology data analysis. Current–voltage relationships were fit with a linear function, $V = A + B^{*}I$, where I is current, V is voltage, A is the y-intercept and B is the slope. Displacement-current curves were fit in using a Boltzmann function $I = A_2 + (A_1 - A_2)/(1 + exp[(x - x_0)/\mathrm{d}x])$, where I is current, A_1 is the y-intercept, A_2 is peak, x is displacement, x_0 is half-point displacement and dx is slope displacement. Error bars indicate the SE. $P < 0.05$ was considered statistically significant (ANOVA with Bonferroni post test when appropriate) using GraphPad Prism, version 6; GraphPad Software, San Diego, CA, USA).

Cell stretch on flexible substrates

QGP-1 cells or QGP-1 cells with Piezo2 siRNA on Flexcell plates were incubated in 3 ml of serum free media containing 2 μM fluoxetine and either vehicle or drug for 30 min. The substrate stretch protocol was 30 cycles min^{-1} at 25% strain with 1:2 ratio of relax:stretch using a validated custom instrument (Stroetz *et al.* 2001). At each time point (rest = 0 min and 1 min), samples were collected and analysed by a 5-HT enzyme-linked immunosorbent assay in accordance with the manufacturer's instructions (BA E-5900; Rocky Mountain Diagnostics, Colorado Springs, CO, USA).

Ussing chamber

Solutions. Krebs-Ringer (in mM): 120 NaCl, 5.9 KCl, 15 NaH_2CO_3, 1.6 NaH_2PO_4, 1.3 $CaCl_2$, 2.4 $MgCl_2$ (pH 7.4), gassed with a 95:5 mixture of O_2/CO_2. Glucose (10 mM) was added to the serosa side bath and mannitol (10 mM) was added to the mucosa bath to maintain osmotic balance.

Tissue preparation. Segments of jejunum (4 cm) were cut along the mesenteric border and the luminal contents were gently removed. Tissue was cut into 2 cm segments. During preparation, the tissues were bathed in ice-cold Krebs-Ringer solution.

Short-circuit current measurements. The full thickness preparations of mouse jejunum with a cross-sectional area of 0.3 cm^2 were mounted in 4 ml Ussing chambers (Physiologic Instruments, San Diego, CA, USA). The trans-epithelial potential difference was measured using paired Ag-AgCl electrodes via 3% agar with 3 M KCl bridges and clamped at 0 mV using another pair of Ag-AgCl electrodes. The mucosal and serosal surfaces of the tissue were bathed with 4 ml of Krebs-Ringer solution with mannitol and glucose, respectively, maintained at 37°C during the course of the experiments. Tissue was allowed to equilibrate to attain a stable basal short-circuit current (I_{sc}) and tissue conductance (G_t) for 30 min before conducting the experiment. Hydrostatic pressure was applied using a DPM-1 pneumatic transducer (Bio-Tek Instruments, Burlington, VT, USA) in a sealed mucosal chamber. From a resting applied pressure of 0 mmHg, pressure stimuli were applied until the peak response in I_{sc} current was achieved (1 min). Prior to these experiments, we determined the half-point of the pressure–stimulus relationship (EP_{50}) value using current responses (ΔI_{sc}) to graded pressure stimuli (data not shown). To test the effect of drugs on pressure-induced responses, three separate stimulations to the EP_{50} value (22 mmHg) were delivered to the mucosal side, with 15 min allowed between stimulations for recovery. After complete recovery to baseline following the third stimulus, drug or vehicle was added to the mucosal chamber and allowed to equilibrate for 20 min. In the presence of the drug, three separate stimuli were applied again. At baseline, recordings were made at 0 mmHg (atmospheric pressure). To ensure tissue viability, at the onset and conclusion of the experiment, ACh (100 μM) was applied to the serosal side to determine tissue viability and the response to stimulation (data not shown). Tissue was not used if there was no response to ACh either before or after the application of pressure. Data were recorded using Acquire and Analyse 2.3 (Physiologic Instruments).

Data analysis. Raw data were exported into text format and uploaded into Clampfit, version 10.5 (Molecular Devices). Pressure-induced peak I_{sc} was measured as the mean of three measurements $\Delta I_{sc} = I_{sc_peak} - I_{sc_baseline}$.

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Statistical analysis. All values represent the mean ± SE. All electrophysiology data and flex cell data were analysed using ANOVA with Bonferroni correction. A two-tailed paired *t* test was used in the Ussing chamber data analysis. $P < 0.05$ was considered statistically significant.

Results

Human and mouse jejunum epithelium expresses PIEZO2 mRNA

Piezo2 mRNA was expressed in human small bowel (jejunum; $n = 3$) and in mouse small bowel mucosa ($n = 3$) from duodenum, jejunum and ileum (Fig. 1).

Piezo2 is distributed specifically in the 5-HT positive EC cells in human jejunum epithelium

We then used IHC to determine the presence and distribution of Piezo2 protein in human jejunum epithelium (Fig. 2). Strikingly, Piezo2 was found only in a well defined population of epithelial cells morphologically similar to enteroendocrine cells. We immunolabelled both 5-HT (Fig. 2*A*) and Piezo2 (Fig. 2*B*) in human jejunum. We found that Piezo2 specifically labelled 91.2 ± 4.5% of 5-HT positive cells (Fig. 2*C*). Controls lacking Piezo2 or 5-HT primary antibodies or containing Piezo2 blocking peptide were appropriately negative (data not shown). Therefore, our data showed that Piezo2 was specifically localized within EC cells in human jejunum epithelium.

Piezo2 is distributed specifically in mouse small bowel EC cells

Next, we examined the murine small bowel. Tryptophan hydroxylase 1 (TPH-1) is the rate limiting enzyme found specifically within EC cells that converts tryptophan to 5-HT (Cote *et al.* 2003). We used a transgenic mouse model TPH1-CFP in which cyan fluorescent protein (CFP) expression was genetically encoded to express in EC cells (Li *et al.* 2014). The EC cell CFP fluorescence was easily observed ($n = 3$) (Fig. 3*A*) and Piezo2 immunofluorescence, using a different antibody from the one used above for human tissues (Ranade *et al.* 2014; Woo *et al.* 2014; Woo *et al.* 2015), revealed that Piezo2 positive cells were confined to the epithelium ($n = 3$) (Fig. 3*B*) and that they co-localized specifically with TPH1-CFP EC cells ($n = 3$) (Fig. 3*C*). Controls lacking Piezo2 primary antibody were appropriately negative (data not shown). Our data for the TPH1-CFP mouse model were consistent with those for the human small bowel, where Piezo2 was specifically expressed by EC cells.

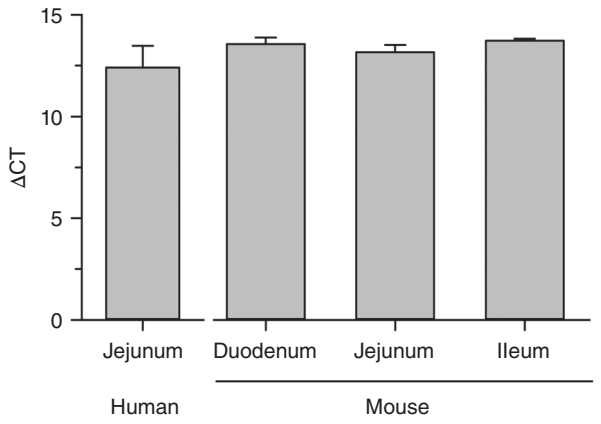


Figure 1. Piezo2 mRNA present in human jejunum and mouse small bowel mucosa
Piezo2 mRNA is found in human jejunum ($n = 3$) and mouse mucosa duodenum, jejunum and ileum ($n = 3$).

We then quantified Piezo2 distribution in the EC cells of duodenum, jejunum and ileum. Using the TPH1-CFP mouse model, we found that Piezo2 along the small intestine was exclusively localized in EC cells: duodenum (86.5 ± 14.3%, $n = 3$ animals, $n = 244$ cells), jejunum (91.3 ± 7.3%, $n = 3$ animals, $n = 264$ cells) and ileum (88.7 ± 8.1%, $n = 3$ animals, $n = 143$ cells) (Fig. 4). Overall, our mouse small bowel immunohistochemistry data showed that Piezo2 was specifically located within the EC cells.

Piezo2 mediates a mechanosensitive cation current in an EC cell model

Piezo2 encodes a mechanosensitive cation selective ion channel (Coste *et al.* 2010; Coste *et al.* 2012), and so we next examined whether Piezo2 is functional in EC cells. We used a validated EC cell model (QGP-1) that produces and releases 5-HT (Doihara *et al.* 2009; Kojima *et al.* 2014; Schulze *et al.* 2014) and expresses Piezo2 (data not shown). Whole cell voltage clamped cells had a variety of baseline inward currents and a fast pressure-induced inward current −8.3 ± 1.6 pA pF^{-1} ($n = 15$ out of 23 cells examined) (Fig. 5*A*) that inactivated with a mono-exponential time constant $\tau_i = 7.0 \pm 1.0$ ms at −60 mV (Fig. 5*A*), which was consistent with the published Piezo2 inactivation rates (6.2–7.3 ms) (Coste *et al.* 2010; Coste *et al.* 2012). The inactivation rate was also similar to that found for Piezo2 heterologously expressed in HEK-293 cells (data not shown). We observed an increase in current with an increasing stimulus, fit well by a two state Boltzmann function with a mid-point sensitivity of 3.30 ± 0.23 μm and slope of 0.67 ± 0.14 μm ($n = 8$) (Fig. 5*B*), consistent with Piezo2 (Schrenk-Siemens *et al.* 2015). Finally, mechanically induced currents had a linear current–voltage relationship with an *x*-intercept of −2.5±1.6 mV ($n = 3$) (Fig. 5*C*), suggestive of Piezo2 non-selective cation currents (Coste *et al.* 2010).

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We then tested whether Piezo2 antagonists and siRNA inhibited these mechanosensitive currents. Compared to no stretch controls (-8.3 ± 1.6 pA pF^{-1}, $n = 15$), the non-selective mechanosensitive ion channel blocker Gd^{3+} (30 μM) (Coste *et al.* 2010) inhibited the pressure-induced current (Fig. 6*A*) by 64% (-3.1 ± 0.8 pA pF^{-1} Gd^{3+}, $n = 7$, $P < 0.05$) (Fig. 6*B*) and the specific Piezo mechanosensitive ion channel antagonist D-GsMTx4 (5 μM) (Suchyna *et al.* 2000; Bae *et al.* 2011; Lee *et al.* 2014) inhibited Piezo2 currents in transfected HEK-293 cells (data not shown) and mechanosensitive inward currents in QGP-1 cells (Fig. 6*A*) by 89% (-1.0 ± 0.3 pA pF^{-1} GsMTx-4, $n = 6$, $P < 0.05$) (Fig. 6*B*). Importantly, Piezo2 siRNA knocked down Piezo2 mRNA by 70 $\pm$ 15% ($n = 3$, data not shown) and inhibited pressure-induced current (Fig. 6*A*) by 96% (-0.37 ± 0.38 pA/pF Piezo2 siRNA,

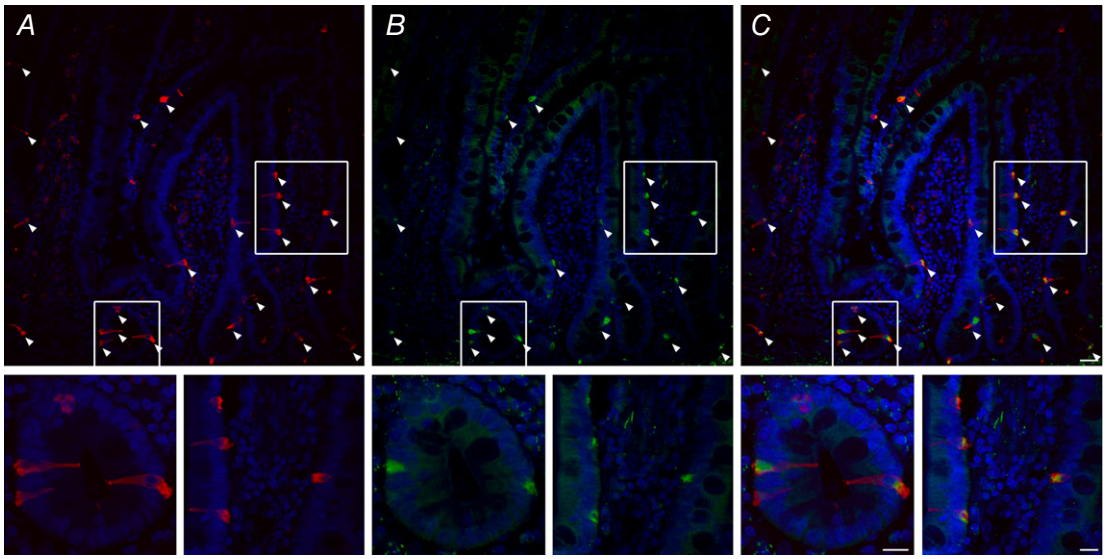


Figure 2. Piezo2 is a specific marker of the human small bowel EC cells
High resolution laser confocal microscopy of (*A*) 5-HT immunolabelling showing EC cells in red and DAPI labelling nuclei in blue, (*B*) Piezo2 immunolabelling in green and DAPI in blue and (*C*) overlap of 5-HT (red) and Piezo2 (green) and DAPI (blue). Arrowheads indicate EC cells and insets are enlarged sections from the areas enclosed by the white boxes. Scale bars = 30 μm (large field) and 15 μm (gland and villus insets).

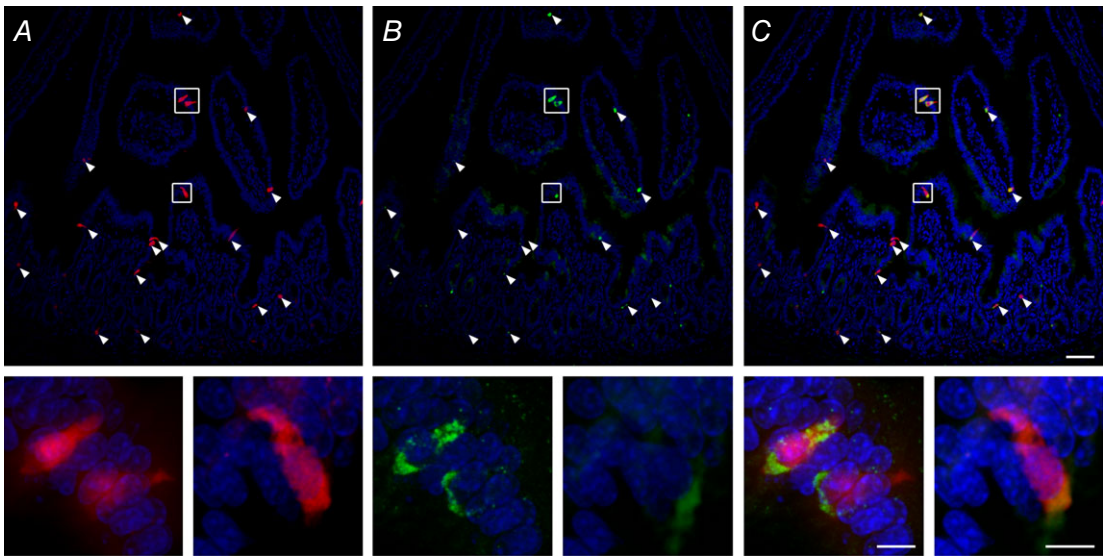


Figure 3. Piezo2 is selectively expressed in mouse small bowel 5-HT EC cells
High resolution laser confocal microscopy of jejunum from TPH1-CFP mouse showing (*A*) CFP positive (pseudo-coloured red) EC cells and DAPI, (*B*) Piezo2 immunolabelling (green) and DAPI and (*C*) co-localization of EC cell (red), Piezo2 (green) and DAPI. Arrowheads indicate EC cells and insets are enlarged sections from the areas enclosed by the white boxes. Scale bars = 50 μm (large field) and 10 μm (insets).

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$n = 6$, $P < 0.05$) compared to non-targeted (NT) siRNA (-6.5 ± 1.6 pA pF^{-1} NT, $n = 4$, $P > 0.05$) (Fig. 6*B*). NT siRNA was not different from the stretch controls ($P > 0.05$). Therefore, our data showed that an EC cell model had mechanosensitive currents with Piezo2 biophysical properties and inhibited by Piezo2 siRNA and pharmacological antagonists.

Piezo2 is necessary for mechanically induced release of 5-HT from EC cells

Next, to determine how mechanical activation of Piezo2 couples to 5-HT release, we stretched QGP-1 cells grown on laminin-coated flexible substrates and measured the released 5-HT. Compared to no stretch controls (Δ5-HT $= 0.10 \pm 0.10$ ng ml^{-1}, $n = 4$) (Fig. 7), depolarization by KCl and cyclic stretch (30 Hz for 1 min) significantly increased 5-HT release (Δ5-HT) by 0.91 ± 0.35 ng ml^{-1} ($n = 4$, $P < 0.05$) and by 0.62 ± 0.04 ng ml^{-1} ($n = 4$, $P < 0.05$), respectively (Fig. 7). Piezo2 pharmacologic inhibitors and Piezo2 siRNA inhibited stretch-induced 5-HT release compared to no stretch (Fig. 7): 30 μM Gd^{3+} (Δ5-HT $= 0.090 \pm 0.10$ ng ml^{-1}, $n = 4$, $P < 0.05$), 30 μM ruthenium red (Δ5-HT $= 0.18 \pm 0.11$ ng ml^{-1}, $P < 0.05$) and 5 μM D-GsMTx4 (Δ5-HT $= 0.012 \pm 0.046$ ng ml^{-1}, $n = 4$, $P < 0.05$). Importantly, Piezo2 siRNA inhibited pressure-induced 5-HT release (Δ5-HT $= 0.073 \pm 0.12$ ng ml^{-1}, $n = 7$, $P < 0.05$) compared to NT siRNA (Δ5-HT $= 0.50 \pm 0.16$ ng ml^{-1}, $n = 7$). NT siRNA did not significantly alter pressure-induced 5-HT release compared to stretch controls ($P > 0.05$). Therefore, our data show that Piezo2 is important for 5-HT release in response to force.

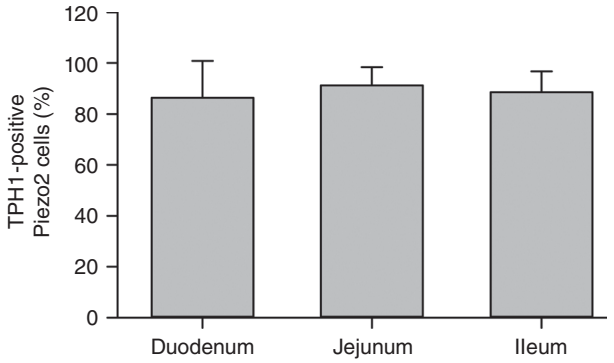


Figure 4. Piezo2 localizes specifically within EC cells in mouse small bowel
Quantification of Piezo2 positive cells that are TPH1 positive shows overlap in 86.5 ± 14.3% cells in duodenum ($n = 3$ animals, $n = 244$ cells), 91.3 ± 7.3% cells in jejunum ($n = 3$ animals, $n = 264$ cells) and 88.7 ± 8.1% cells in ileum ($n = 3$ animals, $n = 143$ cells).

Piezo2 is critical for the regulation of 5-HT mediated mucosal secretion in mouse small bowel epithelium

We wanted to understand how Piezo2 contributes to the physiological control of GI function. Previous studies showed that pressure applied to small bowel mucosa increases secretion, probably via 5-HT (Bulbring & Lin, 1958; Bulbring & Crema, 1959). To determine the involvement of Piezo2 in the mechanism of pressure-induced secretion from mouse jejunum

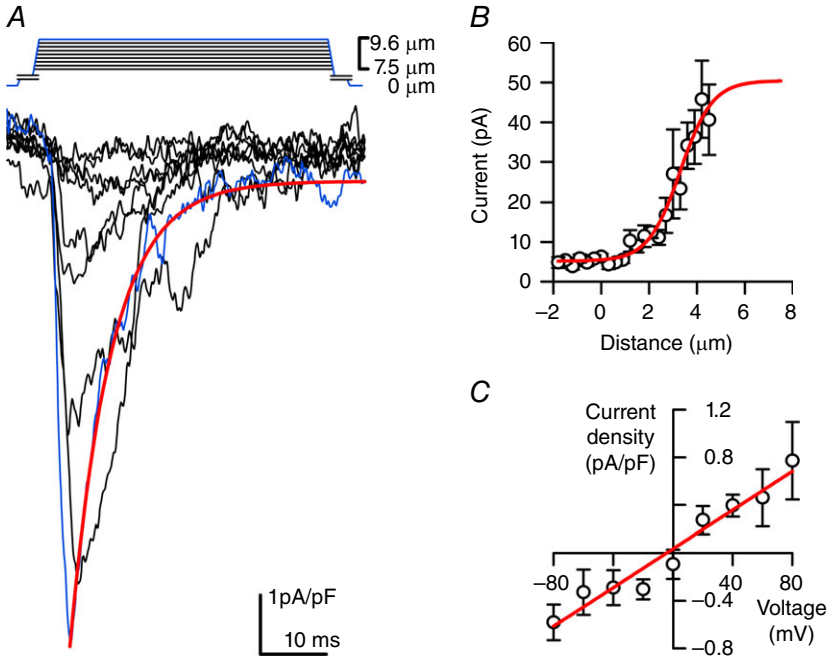


Figure 5. Membrane force leads to a fast non-rectifying inward current in QGP-1 EC cell model
A, stepwise (0.3 μm step^{-1}) increase in cell membrane deformation resulted in a typical set of rapidly activating and inactivating pressure induced inward currents with blue traces highlighting current in response to max deformation. Red line is a mono-exponential fit of the blue curve ($\tau_i = 5.8$ ms). *B*, peak current–deformation relationship ($n = 8$) fit by a two-state Boltzmann function (red line) with mid-point (x_0) 3.30 ± 0.23 μm and slope (dx) 0.67 ± 0.14 μm. *C*, peak current–voltage relationship ($n = 3$) fit by a linear function (red line) with x-intercept of -4.1 mV.

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mucosa, we modified our Ussing chamber to allow transient hydrostatic pressure application to mouse jejunum mucosa. Pressure stimulus-dependently increased I_{sc} with a pressure $EP_{50} = 22$ mmHg (data not shown), and so we used this pressure for the subsequent experiments. Transient mucosal pressure increased I_{sc} as shown in a typical trace (Fig. 8*A*) from 5.3 ± 16.0 μA to 59.0 ± 13.4 μA ($n = 12$, $P < 0.05$) (Fig. 8*B*) and simultaneously increased 5-HT levels at the mucosal side from 1.25 ± 0.07 ng ml^{-1} at rest to 1.43 ± 0.07 ng ml^{-1} ($n = 12$, $P < 0.05$) (Fig. 8*C*).

To determine whether 5-HT receptors and EC cell Piezo2 affect mechanically induced mucosal secretion, we designed a protocol in which three discrete control pressure steps were followed by three pressure steps in the presence of vehicle or drug (Fig. 9*A*). Addition of each drug at the testing concentrations without pressure did not alter the I_{sc} (data not shown). Because 5-HT stimulates mucosal secretion via 5-HT3 and 5-HT4 receptors (Vanner & Macnaughton, 2004), we blocked them with 1 μM ondansetron and 30 nM GR 113808 (GR), respectively. We tested these inhibitors at the mucosal and then basolateral sides of the tissue. When applied to the mucosal side, we found that these blockers (ondansetron + GR) did not affect pressure-induced short circuit increase (ΔI_{sc} from 39.6 ± 9.4 μA to 39.6 ± 9.5 μA, $n = 3$, $P > 0.05$) (Fig. 9*B*). By contrast, when ondansetron + GR were applied to the basolateral side, there was a 51% decrease in the short circuit response to pressure (ΔI_{sc} from 63.0 ± 11.2 to 30.8 ± 3.4 μA, $n = 4$, $P < 0.05$) (Fig. 9*B*), which is consistent with secretion block via the established submucosal 5-HT circuit (Vanner & Macnaughton, 2004).

We then investigated whether EC cell Piezo2 plays a role in pressure-induced increase in short circuit current. To specifically target the epithelium, we added drugs only to the mucosal side. Piezo2 blockers Gd^{3+} and ruthenium red (Coste *et al.* 2010; Coste *et al.* 2012) reduced ΔI_{sc} in response to pressure by 42% for 100 μM Gd^{3+} (ΔI_{sc} from 45.8 ± 5.9 μA to 26.4 ± 3.1 μA, $n = 5$, $P < 0.05$) (Fig. 9*B*) and by 46% for 100 μM ruthenium red (ΔI_{sc} from 49.6 ± 11.5 μA to 26.7 ± 10.3 μA, $n = 4$, $P < 0.05$) (Fig. 9*B*). We then used 5 μM D-GsMTx4, a specific blocker of mechanosensitive ion channels (Suchyna *et al.* 2000; Bae *et al.* 2011), and particularly Piezo2 (data not shown), and found that it inhibited the ΔI_{sc} response to pressure by 43% (ΔI_{sc} from 52.2 ± 15.1 μA to 29.6 ± 6.9 μA, $n = 4$, $P < 0.05$) (Fig. 9*B*). Therefore, our Ussing chamber results showed that mucosal pressure results in an EC cell Piezo2-dependent 5-HT mediated increase in mucosal secretion.

Discussion

Mechanical force applied to the GI mucosa leads to EC cell activation and the release of 5-HT(Bulbring & Crema, 1959), which plays important roles in GI and systemic physiology (Mawe & Hoffman, 2013). The EC cell is established as the specialized mechanosensor of the GI mucosa, whereas the identity of the EC cell primary mechanosensory molecule remained unknown. Interestingly, the skin Merkel cells are specialized mechanosensors (Ikeda & Gu, 2014; Maksimovic *et al.* 2014; Ranade *et al.* 2014; Woo *et al.* 2014) with important functional (Haeberle *et al.* 2004) and developmental similarities to EC cells (Yang *et al.* 2001; Wright *et al.* 2015). Mechanosensitive ion channel Piezo2 is fundamental for Merkel cell mechanosensitivity (Ikeda & Gu, 2014; Ranade *et al.* 2014; Woo *et al.* 2014).The similarities between Merkel and EC cell prompted us to examine whether Piezo2 was involved in EC cell mechanosensitivity.

We identified Piezo2 mRNA in human jejunum and all segments of mouse small bowel mucosa (Fig. 1) and, using immunolabelling with 2 different Piezo2 antibodies in human and mouse small bowel epithelium (Fig. 2 and 3), we found strong Piezo2 protein expression specifically within EC cells (Fig. 4).

We examined Piezo2 function using an established EC cell model (Doihara *et al.* 2009; Schulze *et al.* 2013; Kojima *et al.* 2014; Schulze *et al.* 2014) that has

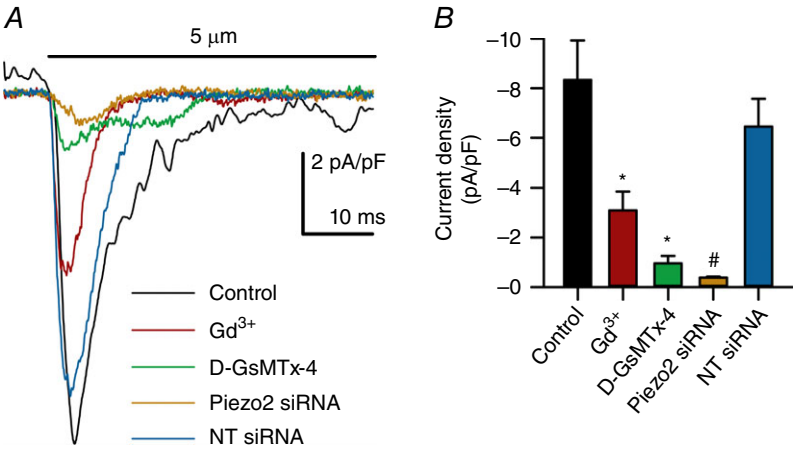


Figure 6. Piezo2 blockade inhibits mechanosensitive ion current in QGP-1 cells
A, superimposition of membrane force resulted in a fast inward current which was blocked by Gd^{3+}, GsMTx-4 and Piezo2 siRNA but not by NT siRNA. *B*, compared to the peak current in controls ($n = 15$), there were significant decreases in peak current when Piezo2 was inhibited by Gd^{3+} ($n = 7$, $^{*}P < 0.05$, ANOVA with Bonferroni correction) and D-GsMTx4 ($n = 6$, $^{*}P < 0.05$, ANOVA with Bonferroni correction) and knocked down by Piezo2 siRNA ($n = 6$, $^{\#}P < 0.05$ compared to NT siRNA, ANOVA) but not with NT siRNA ($n = 4$, $P > 0.05$ compared to controls, ANOVA with Bonferroni correction).

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Piezo2 and releases 5-HT (Doihara *et al.* 2009; Schulze *et al.* 2013; Schulze *et al.* 2014). Local force stimulated inward mechanosensitive currents with Piezo2 biophysical characteristics that were inhibited by Piezo2 siRNA and pharmacologic antagonists (Fig. 5 and 6). Therefore, mechanical activation of Piezo2 in EC cells results in an inward current that would depolarize the cell membrane, which is similar to other specialized mechanosensory cells, such as Merkel cells (Ikeda & Gu, 2014; Ranade *et al.* 2014; Woo *et al.* 2014), as well as proprioceptive (Woo *et al.* 2015) and touch sensitive (Ranade *et al.* 2014) neurons. In the EC cell, membrane depolarization leads to 5-HT release by exocytosis (Racke & Schworer, 1993; Lomax *et al.* 1999; Raghupathi *et al.* 2013). Indeed, we found that Piezo2 activation by stretch led to an increase in 5-HT release because Piezo2 siRNA and pharmacological antagonists diminished stretch-induced 5-HT release (Fig. 7). Therefore, our findings strongly suggest that Piezo2 activation by force is coupled to 5-HT release. Previous studies exploring EC cell mechanosensitivity suggest that other associated and downstream molecules are involved in EC cell mechanotransduction. For example, cholesterol-rich lipid rafts were found to be important (Kim *et al.* 2007) and recent studies suggest that Piezo2 sensitivity is indeed tuned by lipid rafts (Qi *et al.* 2015). It is also interesting that ATP release and purine receptors are often involved in cellular mechanotransduction and specifically EC cell mechanosensation (Chin *et al.* 2012; Linan-Rico *et al.* 2013). This is also consistent with our findings because, in bladder epithelium, ATP release was found downstream of Piezo1 activation (Miyamoto *et al.* 2014). Piezo1 is also found in the gut epithelial cells, although it is currently unknown whether Piezo1 contributes to the EC cell mechanosensitive response. The use of an EC cell model in the present study was advantageous because it allowed siRNA Piezo2 knockdown in pure cultures but was limited in terms of determining the detailed mechanism of EC cell mechanotransduction. Future work that aims to determine the coupling of Piezo2 to downstream signalling, including the source of intracellular Ca^{2+}, requires primary cultures and Piezo2 knockouts.

To examine the role of EC cell Piezo2 in mechanically stimulated GI epithelium secretion, we adapted Ussing

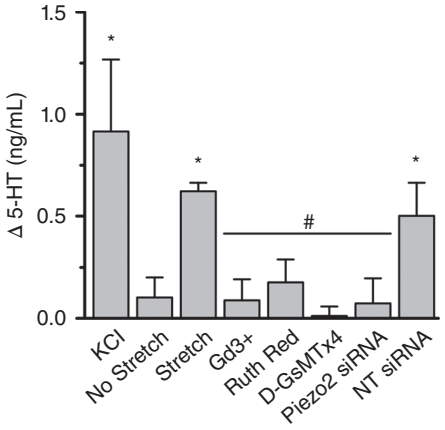


Figure 7. Piezo2 blockade inhibits stretch induced 5-HT release in QGP-1 cells
QGP-1 cells grown on laminin-coated flexible substrates released 5-HT in response to 50 mM KCl ($n = 4$, $^{*}P < 0.05$ compared to no stretch, ANOVA with Bonferroni correction) and cyclic tensile stretch ($n = 4$, $^{*}P < 0.05$ compared to no stretch, ANOVA with Bonferroni correction). Stretch-dependent 5-HT release was inhibited by Piezo2 blockers Gd^{3+}, ruthenium red and GsMTx-4 ($n = 4$ for each, all $^{\#}P < 0.05$ compared to stretch, ANOVA with Bonferroni correction). Piezo2 siRNA inhibited stretch-dependent 5-HT release, whereas NT siRNA did not ($n = 7$ for each, all $^{\#}P < 0.05$ compared to stretch, ANOVA with Bonferroni correction).

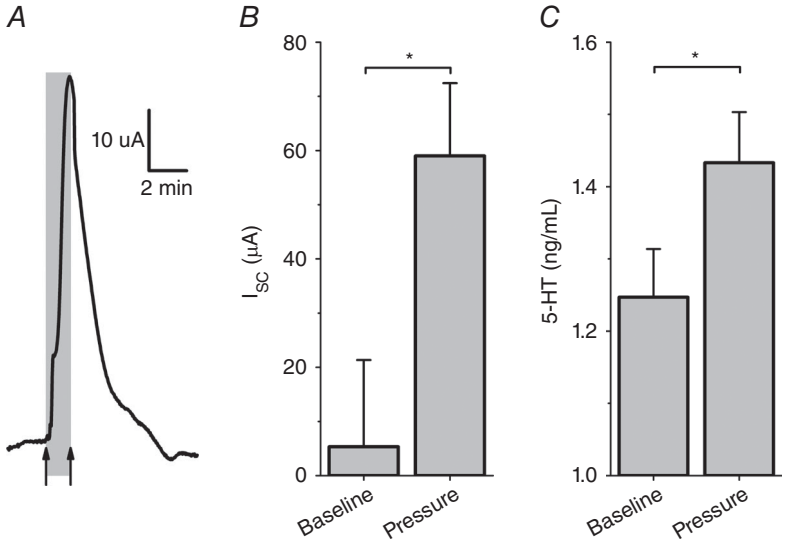


Figure 8. Mucosal pressure leads to an increase in secretion and release of 5-HT
A, typical trace of mouse jejunum in Ussing chamber with +22 mmHg of mucosal hydrostatic pressure resulted in a significant (*B*) Increase in I_{sc} ($n = 12$, $^{*}P < 0.05$) and (*C*) in the same tissues mucosal side 5-HT increase as measured by ELISA ($n = 12$, $^{*}P < 0.05$).

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chambers to pressure and voltage clamp mouse jejunum epithelium. Mucosal pressure acutely and reproducibly increased EC cell 5-HT release and I_{SC} (Fig. 8) via 5-HT acting on submucosal 5-HT3 and 5-HT4 receptors (Fig. 9), which are known to be important for 5-HT mediated secretomotor responses (Vanner & Macnaughton, 2004). EC cell Piezo2 was important for pressure-induced increase in secretion because Piezo2 pharmacological antagonists applied only to the mucosal side reduced the pressure-induced I_{SC} increase by ∼50% (Fig. 9). However, given the residual response, our data also suggest that another mechanism may be involved. This may include an additional EC cell mechanosensitive mechanism or mechanical activation of submucosa projecting neurons (Frieling *et al.* 1992; Vanner & Macnaughton, 2004), such as the intrinsic primary afferent neurons, which are well known for being mechanosensitive (Kunze *et al.* 2000).

Our results have important implications for future studies in GI mechanobiology. First, previous studies investigating how mechanically induced 5-HT release from EC cells affects GI motility and sensation have relied on the manipulation of 5-HT levels (Heredia *et al.* 2013) or its functional circuits (Spencer *et al.* 2011) and the results have been mixed, in part because no effective strategies exist to specifically inhibit EC cell mechanosensitivity. Our work presents Piezo2 as the first specific EC cell mechanotransducer, and so future studies can focus on Piezo2 to determine the specific downstream effects of EC cell activation by force. Second, EC cell 5-HT is an important hormone that is released in response to mechanical and chemical stimuli. Recent studies implicate EC cell 5-HT in a wide range of physiological processes outside the GI tract (Yadav *et al.* 2010; Crane *et al.* 2015). It would be interesting to determine the differences between the physiological effects as a result of EC cell mechano- vs. chemo-sensation.

In conclusion, in the present study conducted in the human and mouse small bowel GI epithelium, we show that Piezo2 is expressed specifically in EC cells, that mechanical forces result in ionic currents consistent with Piezo2 mechanosensitive ion channels and Piezo2-dependent 5-HT release, and that mucosal force results in a Piezo2-dependent increase in mucosal secretion via the 5-HT pathway. Our findings have significant implications for understanding how mechanical forces in the GI tract are coupled to downstream physiological effects.

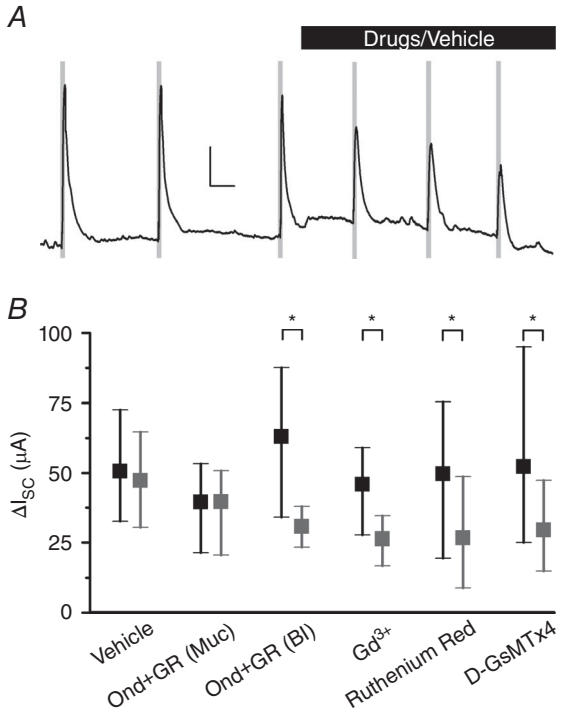


Figure 9. Pressure-induced increase in mucosal secretion is via 5-HT3/4 receptors and activation of Piezo2 mechanosensitive ion channels
A, typical Ussing experiment showing an increase in short-circuit current with pressure in the first three pressure applications (grey bars) and a block by mucosal side Gd^{3+} (black bar) for the subsequent three steps. Scale bars = 25 μA and 5 min. *B*, rise in ΔI_{sc} mean of three pressure steps without (black) and with the drug (grey) was unchanged for vehicle ($n = 4$) and ondansetron (1 μM) and GR 113808 (30 nM) on mucosal (Muc) side ($n = 3$, $P \geq 0.05$) but blocked by basolateral (bl) side ondansetron (1 μM) and GR 113808 (30 nM) ($n = 4$, $^*P < 0.05$), Gd^{3+} (100 μM, $n = 5$, $^*P < 0.05$), ruthenium red (100 μM, $n = 4$, $^*P < 0.05$) and D-GsMTx4 (5 μM, $n = 4$, $^*P < 0.05$).

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Additional information

Competing interests

The authors declare that they have no competing interests.

Author contributions

FW, DRL, SJG, PK, MG, RO, PAG, HJL, ABL, GF and AB were involved in the study conception or design and also conducted the study. FW, KK, CA and AB were involved in the acquisition, analysis or interpretation of data. FW, KK, CA, DRL, SJG, PK, MG, RO, PAG, HJL, ABL, GF and AB drafted the work or revised it critically for important intellectual content. All authors approved the final version of the manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and those who qualify for authorship are listed.

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