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Purinergic Autocrine Regulation of Mechanosensitivity and Serotonin Release in a Human EC Model: ATP-gated $P2X_3$ Channels in EC are Downregulated in Ulcerative Colitis

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Abstract

Background—Alterations in 5-hydroxytryptamine (HT) signaling in inflamed gut may contribute to pathogenesis of inflammatory bowel diseases. Adenosine 5′-triphosphate (ATP) regulates mucosal-mechanosensory reflexes and ATP receptors are sensitive to mucosal inflammation. Yet, it remains unknown whether ATP can modulate 5-HT signaling in enterochromaffin cells (EC). We tested the novel purinergic hypothesis that ATP is a critical autocrine regulator of EC mechanosensitivity and whether EC expression of ATP-gated $P2X_3$-ion channels is altered in inflammatory bowel diseases.

Methods—Laser confocal (fluo-4) Ca^{2+} imaging was performed in 1947 BON cells. Chemical stimulation or mechanical stimulation (MS) was used to study 5-HT or ATP release in human BON or surgical mucosal specimens, and purine receptors by reverse transcription-polymerase chain reaction, Western Blot, or $P2X_3$-immunoreactivity in BON or 5-HT^+ human EC (hEC) in 11 control and 10 severely inflamed ulcerative colitis (UC) cases.

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Results—ATP or MS triggered Ca^{2+}-transients or 5-HT release in BON. ATP or adenosine diphosphate increased 5-HT release 5-fold. MS caused ATP release, detected after 5′ecto-ATPase inhibition by ARL67156. ARL67156 augmented and apyrase blocked Ca^{2+}/5-HT mechanosensitive responses. 2-Methyl-thio-adenosine diphosphate 5′-monophosphate-evoked ($P2Y_{1,12}$) or mechanically-evoked responses were blocked or augmented by a $P2Y_{1,12}$ antagonist, MRS2179, in different cells or inhibited by U73122. A $P2Y_{12}$ antagonist, 2MeSAMP, augmented responses. A $P2X_{1,3}$ agonist, α,β-MeATP, triggered Ca^{2+} responses, whereas a $P2X_{1,2/3,3}$ antagonist, 2′,3′-O-(2,4,6-trinitrophenyl)-ATP, blocked mechanical responses or cell-surface 5′ATP-^{TR} labeling. In hEC, α,β-MeATP stimulated 5-HT release. In UC, $P2X_3$-immunoreactivity decreased from 15% to 0.2% of 5-HT^+hECs. Human mucosa and BON expressed $P2X_1$, $P2X_3$, $P2X_4$, $P2X_5$, $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, and $P2Y_{12}$R-messenger RNA transcripts.

Conclusions—ATP is a critical determinant of mechanosensation and 5-HT release via autocrine activation of slow $P2Y_1$-phospholipase C/inositol-1,4,5-triphosphate-Ca^{2+} or inhibitory $P2Y_{12}$-purinergic pathways, and fast ATP-gated $P2X_3$-channels. UC downregulation of $P2X_3$-channels (or A_{2B}) is postulated to mediate abnormal 5-HT signaling.

Keywords

ATP; mechanosensitivity; 5-HT release; ulcerative colitis; ATP-gated $P2X_3$ channels

The enterochromaffin cell (EC) synthesizes and releases 5-hydroxytryptamine (HT) to initiate enteric neural reflexes and transmit information about visceral pain sensation.[1–8] Alterations in 5-HT release, content, or reuptake mechanisms may contribute to the pathogenesis of inflammatory bowel diseases (IBDs), diarrhea with bacterial toxin enterocolitis, and irritable bowel syndrome (IBS)[9–14]; associations also exist with diverticular disease, colorectal cancer, and celiac disease.[15] However, the basic mechanisms regulating 5-HT release in human enterochromaffin cells (hECs) remain poorly understood. Understanding how 5-HT release is regulated at cellular and molecular levels is a necessity for understanding the basis of these disorders.[16,17]

ECs have chemo- and mechanosensitive elements that detect changes in force or contents of the intestinal lumen.[2] The human carcinoid BON cells is an established hEC line shown to be a suitable model to study both chemo- and mechanosensation, in addition to receptor regulation, postreceptor signaling pathways, and physiological regulation of 5-HT release.[1,2,18–21] We found that a major component of mechanosensation is activation of the $G\alpha_q$/phospholipase C (PLC)/Ca^{2+} signaling pathway to increase 5-HT release. Because 5-HT release is a Ca^{2+}-dependent pathway, study of intracellular free Ca^{2+} levels in BON cells is a suitable target to study regulation of 5-HT release mechanisms at a single cell level.

Adenosine 5′-triphosphate (ATP) is a major player in mechanosensory reflexes, but its role in modulating 5-HT release was unknown.[18,22,23] We could show that mechanical stimulation of the mucosa in rodents releases ATP that is required for triggering secretomotor reflexes,[22,23] and we speculate that ATP, in part, acts on ECs to facilitate 5-HT release. In fact, in the studies in BON cells, we found that adenosine, a metabolite of ATP, is an important autoregulatory modulator of Ca^{2+}-dependent 5-HT release.[19]

The concept of purinergic signaling stems from studies that were designed to identify the nonadrenergic noncholinergic inhibitory neurotransmitter in the gut.[24] Purine receptors are classified as P1 for nucleosides (adenosine) and P2 for nucleotides (ATP). The P2 family can be subdivided into ion channel $P2X_{1–7}$ and metabotropic $P2Y_{1,2,4,6,11–14}$ receptor families.[25–27] ATP is a purinergic transmitter in the enteric nervous system, is involved in neuron-to-glial communication[28,29] and gliotransmission,[30] and is known to act at all levels of gut secretory and motility reflexes.[31,32] ATP is released by mechanical stimulation from organ structures that form tubes and sacs to participate in mechanosensory reflexes.[32] In the gastrointestinal (GI) tract, ATP release from epithelial cells by mechanical stimulation can initiate gut reflexes[22,23,33] or activate $P2X_3$ receptors on local afferent nerve endings to transmit pain sensation to the brain in animal models of IBS or IBD.[34]

We sought to test the novel purinergic hypothesis that ATP is a critical autocrine regulator of mechanosensitive 5-HT release. Purinergic pathways are very sensitive to inflammation leading to alterations in expression or function of purinergic targets (receptors or enzymes).[35–39] Gut inflammation causes discrete alterations in various purine messenger RNA (mRNA) gene transcripts in colitis models[36] or mucosal biopsies from Crohn's disease (CD) or ulcerative colitis (UC).[40] However, it is unknown if the expression of any receptor is altered in hEC from intact human gut in IBD. In preliminary experiments, we identified mRNA transcripts $P2X_1$–$P2X_6$ in hECs from intact gut mucosa. Transcripts for $P2X_{1–6}$ receptors are expressed in hEC. Because the ATP-gated $P2X_3$ ion channel is upregulated in enteric neurons in human IBD[41] and it can transmit pain sensation,[34,42] we tested whether $P2X_3$ expression is also altered in hEC of UC patients. Our strategy was to target a receptor that is linked to mechanosensitivity in hECs.

MATERIALS AND METHODS

Surgical Specimens for 5-HT Release

Surgical tissue from 8 patients were collected after patient consent and included 2 sigmoid colons from patients with polyposis, 4 jejunums from Roux-en-Y bypass procedures, 1 rectal colon, and 1 transverse colon from a colectomy. Tissue was placed in Krebs'/oxygenated buffer at 4°C and transported to the laboratory within 15 minutes. The 5-HT release was monitored in microdissected mucosa–submucosa tissues (~1 cm^2 tissues) floating in Krebs' solution oxygenated at 37°C. Tissues were equilibrated for 30 minutes before exposure to drugs for 30 minutes. Supernatants were collected, and a 5-HT enzyme-linked immunosorbent assay (ELISA) kit was used to determine the concentration of 5-HT release in supernatants (serotonin ELISA, BAE5900; Rocky Mountain Diagnostics, Inc., Colorado Springs, CO) using a Wallac-Victor^3 plate reader. Sensitivity of the assay is 0.005 ng/mL × *c* (correction for dilution).

Surgical Specimens for $P2X_3$-immunoreactivity in hEC

A trained clinical GI pathologist screened 70 specimens from different patients to identify and select 10 control diverticulitis (noninflamed portions) specimens and 11 UC specimens (inflamed) from the sigmoid colon. Three sections/specimen were analyzed and scored

according to Geboes et al.[43] Unpaired *t* test was used to calculate the difference in the grading score of inflammation (0–5.4 scale).

BON Cells in Culture

BON cells were a gift from C.M. Townsend Jr (University of Texas, Galveston, TX). Clone No. 7 was highly enriched with 5-HT. The cells were seeded on No. 0 cover slips (MatTek, Corp., Ashland, MA) at a density of 10^5 cells for touch experiments and at a density of 10^6 cells for shaking experiments. Cells were grown in Dulbecco-modified Eagle medium–nutrient mixture F-12 (1:1), supplemented with 10% fetal calf serum, 100 IU/mL penicillin, and 100 μg/mL streptomycin (Life Technologies, Grand Island, NY). Cells were grown in a humidified atmosphere of 95% air and 5% CO_2 at 37°C without reaching confluence for touch experiments and reaching confluence for shaking experiments.[2]

Mechanical Stimulation of a Single BON Cell

BON cells were loaded with 5 μM Fluo-4/AM in Dulbecco-modified Eagle medium–nutrient mixture F-12 (Life Technologies) for 20 minutes in a 95% air and 5% CO_2 incubator at 37°C for touch Ca^{2+} experiments using a modified-Zeiss LSCM 410/REN laser scanning confocal imaging system[19] described in Supplemental Methods, Supplemental Digital Content 1, http://links.lww.com/IBD/A230, and Figure 4.

Mechanical Stimulation of BON Cell Monolayers to Release 5-HT

Another mechanical stimulus was a mild rotational shaking at 80 rpm to measure 5-HT release from the population of cells, as we reported previously.[2,19] Briefly, the cells were exposed to mechanical stimulation on a shaker (Lab-Line, Melrose Park, IL), which rotated the culture plates containing the cells. The supernatants were collected as described previously (2) and frozen at -80C until the 5-HT assay was carried out.

ATP Quantification in Supernatants of BON Cell Monolayers After Rotational Shaking

To measure ATP release from BON cell monolayers, ATP release was obtained by using the luciferin/luciferase assay[44,45] and assay glow kits according to the manufacturer's instructions.

Statistical Analysis

Mean values ± standard error of the mean are reported. SPSS 17.0, GraphPad Prism 3.02, Stat-View 54.51, and laser scanning confocal microscope (LSM) Zeiss software were used for analysis. Dose-response curves were analyzed by analysis of variance (ANOVA) followed by Newman–Keuls multiple comparison test. For other parameters, simple unpaired/2-tailed *t* tests were used. Differences were considered statistically significant at $P < 0.05$.

Ethical Considerations

Patient consent was obtained for the procurement of surgical tissue that would otherwise be discarded following pathologic examination. This is outlined in our approved institutional

review board protocols 2004H0165 and 2012H0231 through the ethics committee at The Ohio State University Office of Responsible Research Practices.

RESULTS

Purinergic Ca^{2+} Responses in BON Cells

Ca^{2+} transients were analyzed in 1947 single BON cells—mechanical stimulation (touch) experiments were done in 1490 cells. Purinergic receptor agonists were tested in 457 cells. Additional experiments were done on 5-HT or ATP release. Our findings are summarized in Figures 1 to 11, Figures in Supplemental Digital Content 2 and 3, http://links.lww.com/IBD/A231 and http://links.lww.com/IBD/A232, respectively, Tables 1 and 2 and Table in Supplemental Digital Content 4, http://links.lww. com/IBD/A233.

ATP Responses

ATP (Tocris/R&D Systems, Minneapolis, MN) elicits a concentration-dependent increase in intracellular-free Ca^{2+} levels ($[Ca^{2+}]_i$) in BON cells with a 50% effective concentration (EC_{50}) of 10.8 ± 2.0 μM (Fig. 1). ATP can elicit several types of ATP Ca^{2+} transients or responses that can be distinguished by their kinetic profiles as shown in Figure 2: ATP can evoke a Ca^{2+} wave in about 30% to 50% of responsive cells (Fig. 2A) associated with Ca^{2+} oscillations. The frequency of oscillations ranges from 2 to 30 oscillations. Some Ca^{2+} transients have simple kinetic profiles (Fig. 2B, C). More complex multiphasic responses also occur in some cells (Fig. 2D, E) without oscillations in Ca^{2+} responses. Rarely, ATP evokes a slow rising Ca^{2+} response that persists for 200 to 300 seconds (Fig. 2F).

P2X and P2Y Responses and mRNA Transcripts in BON Cells and Human Intestinal Surgical Tissues

Laser confocal Ca^{2+} imaging reveals responses to ATP, the $P2Y_{1,12}$ agonist 2-methyl-thio-adenosine diphosphate 5′-monophosphate (2MeSADP; Tocris/R&D Systems), the $P2Y_{2,4,6}$ agonist uridine triphosphate (UTP; Sigma Aldrich, St. Louis, MO), and the $P2X_{1,3}$ agonist α,β-MeATP (Tocris/R&D Systems) in different proportions of BON cells (Fig. 3A). Various P2X and P2Y transcripts are revealed by reverse transcription-polymerase chain reaction (RT-PCR) as shown in Figure 3B–D. Primer sequences for P2X and P2Y receptor transcripts (Table, Supplemental Digital Content 4, http://links.lww.com/IBD/A233) and additional description of methodology are described in the Supplemental Methods, Supplemental Digital Content 1, http://links.lww.com/IBD/A230

Touch Evoked Ca^{2+} Response

Touch/stretch of a single BON cell causes a transient Ca^{2+} response that can be quantified and is reproducible (Fig. 4).[19] It is interesting that the second transient lasts longer on average than the first one—the mechanism for this “phenomenon” remains unknown. Eighty percent of BON cells are touch sensitive with a Ca^{2+} response. Touch causes a peak increase in $[Ca^{2+}]_i$ corresponding to a fluorescence pixel intensity of 56.3 ± 2.7 (n = 80). Low Ca^{2+} – Krebs’ solution does not affect touch Ca^{2+} responses (Table 1).

Effect of ARL67156 and Apyrase on Touch Ca^{2+} Responses

ARL67156 (Sigma-Aldrich) is an inhibitor of 5′-ectonucleotidases that is used to protect against breakdown of nucleotides such as ATP. It induced a concentration-dependent augmentation in the peak touch-Ca^{2+} response in 63% cells (Fig. 5A; n = 102, $P < 0.0001$). The EC_{50} for ARL67156 was 6.1×10^{-5} M. Apyrase (Sigma-Aldrich) reduced the touch-Ca^{2+} response by 79% ± 4.0 (n = 67) at concentrations between 0.5 and 5 U/mL (n = 256, ANOVA $P < 0.0001$). The EC_{50} for apyrase was 1 U/mL (Fig. 5B).

Depletion of 5-HT Content by ATP Stimulation

Exogenous ATP can cause depletion of 5-HT content in single BON cells as determined by quantitative analysis of 5-HT immunoreactivity (ir; Fig. 5C, $P < 0.0001$, n = 200).

Effect of Apyrase and ARL67156 on 5-HT Release Evoked by Mechanical Stimulation

In BON monolayers, apyrase (5 U/mL, 20 min) inhibited mechanically evoked 5-HT release (Fig. 5D, N = 4 dishes, $P < 0.05$). Incubation of BON cells with 10^{-5} M ATP or 10^{-5} M adenosine diphosphate (ADP; Sigma-Aldrich) caused a 4- to 5-fold increase in 5-HT release (Fig. 5E; N = 7, $P < 0.0001$).

ATP Release by Mechanical Stimulation Is Revealed by ARL67156

To test whether ATP was released by mechanical stimulation, BON cell monolayers were stimulated for 30 minutes by rotational shaking at 80 rpm. Mechanical stimulation increased ATP by 3.3-fold compared with baseline ATP (2.15 ± 0.4 pmol/well/30 min, N = 3; Fig. 5F). To evaluate if enzymes in BON cells are capable of degrading endogenous ATP release during mechanical stimulation, we determined the effect of ARL67156 on ATP release from BON cells in response to rotational shaking. As shown in Figure 5F, detectable levels of ATP release are revealed only if 1×10^{-5} M ARL67156 is used to protect against degradation of endogenous ATP release ($P < 0.005$). The activity of endogenous 5′-ectonucleotidases is confirmed by showing that exogenous ATP can be degraded by BON cells (Fig. 5G).

ADP is the preferred mediator for both $P2Y_1$ and $P2Y_{12}$ receptors, and MRS2179 (Tocris/R&D Systems) targets both of them. Therefore, we tested whether a $P2Y_{12}$ antagonist 2MeSAMP (Sigma-Aldrich) could influence mechanical stimulation. 2MeSAMP mimics only one component of the MRS2179 response by only augmenting touch-Ca^{2+} responses (Fig. 6D, E).

The $P2Y_1$ receptor is linked to the $G\alpha_q$/PLC/inositol-1,4,5-triphosphate (IP_3)-Ca^{2+} signaling pathway, and the PLC inhibitor U73122 could abolish the touch-Ca^{2+} response in 57% of touch-sensitive cells (0.1 μM–10 μM, n = 150), in contrast to the inactive analog U73343 (Tocris/R&D Systems) (Table 1). In the remaining 43% cells, U73122 (Tocris/R&D Systems) caused modest reduction in response, indicating a $G\alpha_q$/PLC/IP_3-insensitive mechanism. Additional interactions occur between MRS2179 and U73122 causing further reduction in basal Ca^{2+} levels (Fig. 6F). The threshold concentration was 1 μM for U73122. The 50% inhibitory concentration for U73122 was 2.3×10^{-6} M (Fig. 6G, n = 247).

Relationship Between 2MeSADP Sensitivity and Touch Sensitivity

In another experiment, "micropuff" application of 2Me-SADP (1 mM, 200 ms) increased $[Ca^{2+}]_i$ in ~54% of cells (79/156). 2MeSADP-sensitive cells (58/59 cells) responded with a Ca^{2+} transient to the touch stimulus. Therefore, a 1:1 relationship exists between 2MeSADP and touch sensitivity. Mechanically evoked or 2MeSADP-induced Ca^{2+} or 5-HT release responses are blocked by MRS2179 (Figure, Supplemental Digital Content 2, http://links.lww.com/IBD/A231).

Functional P2X Receptors in BON Cells

The $P2X_{1,3}$ agonist α,β-MeATP triggered various types of Ca^{2+} responses in BON cells (Fig. 7A, B). In 52% BON cells, touch-evoked Ca^{2+} responses were blocked by a P2X antagonist pyridoxal-phosphate-6-azophenyl-2′-4′-disulphonic acid (PPADS, 10 μM [R&D System, Minneapolis, MN], Fig. 7C) in a concentration-dependent manner. The apparent 50% inhibitory concentration for PPADS is 5×10^{-7} M (Fig. 7D). A selective $P2X_{1,3,2/3}$ receptor antagonist 2′,3′-O-(2,4,6-trinitrophenyl)-ATP (TNP-ATP; Sigma-Aldrich) prevented touch-Ca^{2+} responses in 27% BON cells. In another 18% cells, TNP-ATP augmented peak Ca^{2+} responses instead of blocking them, suggesting a different mechanism (Fig. 7E). Approximately 19% cells had $P2X_{1,3}$ agonist responses to α,β-MeATP (Table 1). A 100-millisecond micropuff of 5′ATP-Texas Red ($5'ATP\text{-}^{TR}$) caused cell membrane labeling and Ca^{2+} transients in BON cells (Fig. 8). The P2X antagonist PPADS could also block the $5'ATP\text{-}^{TR}$ response.

Dual Purinergic Modulation of 5-HT Release in hEC of Surgical Specimens

Data obtained in 8 surgical specimens from colon or jejunum illustrate dual modulation of 5-HT release by a $P2X_{1,3}$ agonist α, β-MeATP (Fig. 9A) and a metabolite of ATP, adenosine (Fig. 9B). Tetrodotoxin (Tocris/R&D Systems) was used to block nerve conduction. Fluoxetine (R&D Systems) increases 5-HT release (overflow) by blocking serotonin transporter-uptake of 5-HT. Western Blot (WB) identified a $P2X_3$ receptor in intact human mucosa (Fig. 9C), and immunofluorescent labeling identified $P2X_3$-ir in hECs (Fig. 10). Clear expression of $P2X_1$ or $P2X_2$ in mucosa was not observed in WB (not shown). $P2X_3$ expression by WB was done in specimens from the colon (N = 3). Whether regional differences exist in the receptor expressions was not evaluated.

Alterations in $P2X_3$ Receptor Expression in hECs From Surgical Cases of UC

Quantification of hEC expression of $P2X_3$ receptors was done in a pilot study of surgical specimens from 11 control cases (diverticulitis and noninflamed regions) and 10 inflamed cases (UC; Fig. 10A). Inflammation was confirmed by a blinded GI pathologist in UC (score = 5.4) compared with control (score = 0.65, $P < 0.0001$); 19 of 21 cases were sigmoid colon and 2 cases rectum. Control tissue is without inflammation or low level inflammation; in 5 of 11 cases, the score is 0.0 (no inflammation); in 3 cases, the score is 1.1 (low inflammation); and in 2 cases, the score is 1.3.

$P2X_3$-ir is colocalized in $5\text{-}HT^+$hECs in the human sigmoid colon (Fig. 10B). $P2X_3$-ir is expressed in 15% $5\text{-}HT^+$hECs, 0.43% epithelia, and 13% submucous ganglia. The number

of 5-HT$^+$ cells is reduced in UC by 50%. In UC, P2X$_3$-ir is reduced by 98% to <0.2% of 5-HT$^+$hECs in crypts (P = 0.025) and did not change in submucous neurons. P2X$_3$-ir is abundant in inflammatory cells infiltrating the lamina propria in UC. The A_{2B} receptor is expressed in 53% hEC, 46% ganglia, and 5.7% epithelia. In contrast to P2X$_3$, in UC, A_{2B} expression is reduced by 68% in hEC, 66% in epithelia, and 57% in neurons (Table 2; Fig. 10; Figure, Supplemental Digital Content 3, http://links.lww.com/IBD/A232).

DISCUSSION

The EC is a major player in initiating enteric and visceral nociceptive reflexes.[5–8] Alterations in 5-HT release or handling mechanisms may contribute to the pathogenesis of IBD, diarrhea, and IBS.[9–14] However, basic mechanisms regulating 5-HT release in hEC are poorly understood.[16,17] ATP is a key purinergic mediator in mechanosensory reflexes,[22,23] and purinergic pathways are sensitive to inflammation, but its role in modulating 5-HT release was unclear. Our study focused on BON cells and hECs. BON cells display an EC phenotype[2,5–7,18–21,46] relevant to hECs and are the only hEC model shown to be suitable for mechanosensitivity studies in single cells.[1,2,8,19] Our study proved that ATP release is a critical regulator of mechanosensitivity, Ca^{2+} signaling, and 5-HT release. Furthermore, in UC patients, ATP-gated P2X channels on hECs are very sensitive to inflammation.

Ca^{2+} responses to ATP in BON cells vary greatly in shape, size, and kinetics, and ATP can also trigger Ca^{2+} oscillations (or wave forms), suggesting multiple purinergic receptor mechanisms. Ca^{2+} waves are not involved in touch/stretch-induced responses, because mechanical stimulation could not trigger a Ca^{2+} wave in a single cell from ~1500 cells touched. Additional proof for multiple receptors is the finding that different proportions of cells respond to agonists (ATP, 2-MeSADP, UTP, and α,β-MeATP) or antagonists (MRS2179, 2MeSAMP, PPADS, and TNP-ATP) with selectivity for different receptors. Ten of 12 purine receptors are expressed in both BON and human mucosa—including P2X$_1$, P2X$_3$, P2X$_4$, P2X$_5$, P2Y$_1$, P2Y$_2$, P2Y$_4$, P2Y$_6$, P2Y$_{11}$, and P2Y$_{12}$. The cognate mediators for these receptors are ATP, ADP, UTP, uridine diphosphate, ADP, AMP, or β-NAD.[47]

ATP or ADP could stimulate a 5-fold increase in 5-HT release from BON cells and mechanical stimulation caused both ATP and 5-HT release. The ATP released is quickly inactivated by 5′ecto- ATPase and NTPDase enzymes.[48,49] ATP release could be revealed only after enzyme activity was inhibited by ARL67156, because these enzymes are very active in BON as shown in ATP degradation experiments. Exposure to purinergic antagonists (PPADS, TNP-ATP, MRS2179, and 2MeSAMP) or an enzyme that degrades nucleotides (apyrase) could block touch-Ca^{2+} responses or 5-HT release, whereas ARL67176 could augment responses. Eighty percent of cells are touch sensitive, and effects with apyrase or ARL67156 were evident in 77% and 63%, respectively, in touch-sensitive cells. We forward the concept that purinergic autoregulation via ATP is a primary mechanism in transducing of mechanical stimuli into intracellular Ca^{2+} signals leading to release of 5-HT to trigger mucosal reflexes. ATP release by mechanical stimulation from epithelial cells that make up 99% cells in the mucosa is also involved in mechanosensory reflexes,[31]as is ATP.[22] Therefore, it is likely that purines provide a local mechanism for fine-tuning autocrine modulation of 5-HT release that triggers gut reflexes.

Touch deforms the cell membrane of single cells and generates forces such as strain, tactile stimulation, stretch, and pressure. Turbulent shear stress, tactile forces, centrifugal forces, or changes in hydrostatic pressure are some of the forces generated by rotational shaking that lead to the release of 5-HT from the entire population of BON cells.[2] These forces are generated in the intestines during various patterns of motility. A recent study on fluorescence-activated cell sorting (FACS)-sorted hEC applied stretch–relaxation using a vacuum-operated Flexercell strain unit (FX-4000; Hillsborough, NC) and confirmed our earlier findings[19] that adenosine release acts via A_{2B} receptors to stimulate 5-HT release.[50] Our results show that the forces that comprise touch (stretch) and light shaking trigger a series of biological events leading to 5-HT release that is tightly regulated by ATP release.

Our study revealed 3 distinct purinergic signaling pathways in hEC that are linked to mechanosensitivity. First, $P2Y_1$ receptors are known to be linked to $G\alpha_q$/PLC/IP_3-Ca^{2+} pathway.[51] The $P2Y_1$ receptor is coupled to this Ca^{2+} pathway and a $P2Y_1$ antagonist (MRS2179) prevents or reduces responses in 65% cells, and all are sensitive to PLC inhibition (U73122). Furthermore, complex interactions occur between $P2Y_1$ and the PLC signaling pathway (Fig. 6F). ADP is the cognate agonist for $P2Y_1$ receptors, and a more potent, stable ADP analog 2MeSADP triggers Ca^{2+} responses and 5-HT release, and these responses are sensitive to blockade with MRS2179. All cells that responded to 2MeSADP were touch sensitive. It is concluded that a slow $P2Y_1$-$G\alpha_q$/PLC/IP_3-Ca^{2+} pathway is a major mechanism involved in autocrine regulation of mechanosensitivity in hEC.

However, ADP is also the cognate agonist for $P2Y_{12}$ (AC-coupled receptor), and data show that 2MeSADP (ADP analog) or touch-induced Ca^{2+} responses are dually modulated by MRS2179 in different cells. In a separate hEC population from those expressing a slow $P2Y_1$-$G\alpha_q$/PLC/IP_3-Ca^{2+} pathway, MRS2179 augments rather than block the Ca^{2+} response to 2MeSADP or mechanical stimulation. This suggests that MRS2179 is blocking ongoing inhibition mediated via $P2Y_{12}$ receptors resulting in augmentation of the response. This is likely the case, because a $P2Y_{12}$ antagonist 2MeSAMP could only augment touch-Ca^{2+} responses. Therefore, in a distinct population of hECs ADP activation of a $P2Y_{12}$/AC/cAMP signaling pathway provides ongoing inhibitory modulation of Ca^{2+} signals and hence 5-HT release.

A third purinergic mechanism regulating mechanosensitivity is activation of ATP-gated P2X ion channels in hECs. In human mucosa, blocking reuptake of 5-HT by serotonin transporter-inhibition elevates 5-HT release, and under these conditions, the $P2X_{1,3}$ agonist α,β-MeATP could then stimulate 5-HT release. In contrast, a metabolite of ATP, adenosine, inhibits 5-HT release. A $P2X_{1,3}$ agonist α,β-MeATP triggers Ca^{2+} responses, whereas a P2X (PPADS)[52] or $P2X_{1,2,3,2/3}$ antagonist (TNP-ATP)[53,54] blocks mechanical stimulation-Ca^{2+} responses or cell-surface P2X-labeling to ATP-^{TR}. of touch-Ca^{2+} responses with TNP-ATP suggests a different as yet unknown mechanism. However, reverse transcription-polymerase chain reaction identified $P2X_1$ and $P2X_3$ transcripts in BON and human mucosa, and $P2X_3$ protein in human mucosa; $P2X_2$ transcripts were absent in BON or human mucosa. Therefore, $P2X_2$ or $P2X_{2/3}$ heterodimers are unlikely to be involved. $P2X_3$ and $P2X_1$ channels are candidates for modulation of the mechanosensory signaling pathway in hEC leading to 5-HT release.

$P2X_3$ channels represent a therapeutic target for pain/analgesia.[34,42] ATP release from hEC (or epithelial cells) can activate ATP-gated $P2X_3$ channels on local afferent nerve endings to transmit pain sensation to the brain in IBD or IBS models.[32,34] The hEC is another target for $P2X_3$ modulation of 5-HT release that can act on intrinsic and extrinsic nerve endings of sensory neurons to modulate peristalsis and initiate nociception. In IBD and IBS models, $P2X_3$ upregulation is deemed pathologic in pain transmission. In human sigmoid colon, we found that $P2X_3$-ir is expressed in 15% of 5-HT^+hECs compared with a majority of hECs expressing A_{2B} receptors. In UC, discrete alterations occur in the expression of $P2X_3$ channels in hEC and other cell types. The most striking effect of mucosal inflammation in UC is on $P2X_3$ downregulation in hEC. Detectable $P2X_3$-ir is reduced from 15% to <0.2% of cells in 5-HT^+hEC, whereas expression did not change in neurons and is highly expressed in inflammatory cells. Downregulation of $P2X_3$ in UC may represent an adoptive response to attenuate reflexes and visceral pain. In IBD, there is also diminished P2X-purinergic vasoconstriction.[55] P2X expression is upregulated after injury to dorsal root ganglia,[56] in IBD and IBS models.[42] In CD, $P2X_3$ upregulation occurs in myenteric neurons,[41] which is expected to lead to abnormal motility. A new study is necessary to determine functional consequences of downregulation of $P2X_3$ in hEC from UC. A study evaluating $P2X_3$-expression in hEC in relation to visceral hyperalgesia in IBD (or IBS) patients could further evaluate the clinical importance of our findings. In our study, UC caused downregulation of A_{2B}-ir and was undetectable in 68% hECs in fixed intestinal sections, whereas in FACS-sorted hEC from CD patients, it was shown to cause upregulation of A_{2B}[50]; our study did not evaluate whether, in cells still expressing the receptor (e.g., 32%) in UC specimens, it was upregulated. A_{2B} receptors in biopsy were shown to be upregulated in UC and CD.[40] Differences between studies could also be because of the different diseases, severity, chronicity, or treatment of disease, EC handling during isolation, A_{2B} expression in cells other than hEC, or different ways to analyze expression.

CONCLUSIONS

Endogenous purines are critical determinants of 5-HT release evoked by mechanical stimulation, and our working model is depicted in Figure 11. Most cells are sensitive to mechanical forces and respond by releasing ~0.5% to 10% of intracellular pool of nucleotides. Mechanical stimulation activates ATP to diffuse from the cells into the extracellular compartment to bind to receptors on the surface of the cell and activate the mechanosensitive signaling pathway.[21] We forward the novel hypothesis that purines provide a mechanism for finetuning modulation of 5-HT release in triggering gut mechanical intrinsic and extrinsic reflexes. ATP could also act directly to activate the reflex.

In hEC, positive feedback modulation via a P2X channel or $P2Y_1$ would allow cells to translate small changes in mechanical stimulation into large changes in 5-HT release—P2X channels may provide speed and amplification whereas P2Y could provide slower/more sustained effects on reflexes. In a majority of hEC mechanically-evoked 5-HT release is tightly autoregulated by co-release of ATP via three distinct purinergic signaling mechanisms.

Mechanical stimulation releases ATP (or ADP) to activate a slow $P2Y_1$-PLC/IP_3-Ca^{2+}dependent 5-HT release. In distinct subsets of hEC, ongoing activation of a slow $P2Y_{12}$-AC/cAMP signaling pathway by ADP attenuates 5-HT release to dampen reflexes. ATP release in a minority of hEC in intact mucosa (<20%) can also activate a fast ATP-gated $P2X_3$ (or $P2X_1$) channel to stimulate 5-HT release. Overall, mechanical stimulation coreleases 5-HT, ATP, other purines, prostaglandins, PACAP, or other mediators[20] that influence or trigger reflexes or activate visceral sensory afferents.

Purinergic pathways are very sensitive to inflammation and severe downregulation of ATP-gated $P2X_3$ channels or A_{2B} receptors occurs in UC, and we postulate that this would alter 5-HT release and contribute to abnormal signaling, physiology, and visceral sensation—purinergic signaling pathways offer new potential therapeutic targets. Novel approaches to monitor 5-HT release by electrochemical detection will permit such studies in intact human mucosa or single hEC.[57]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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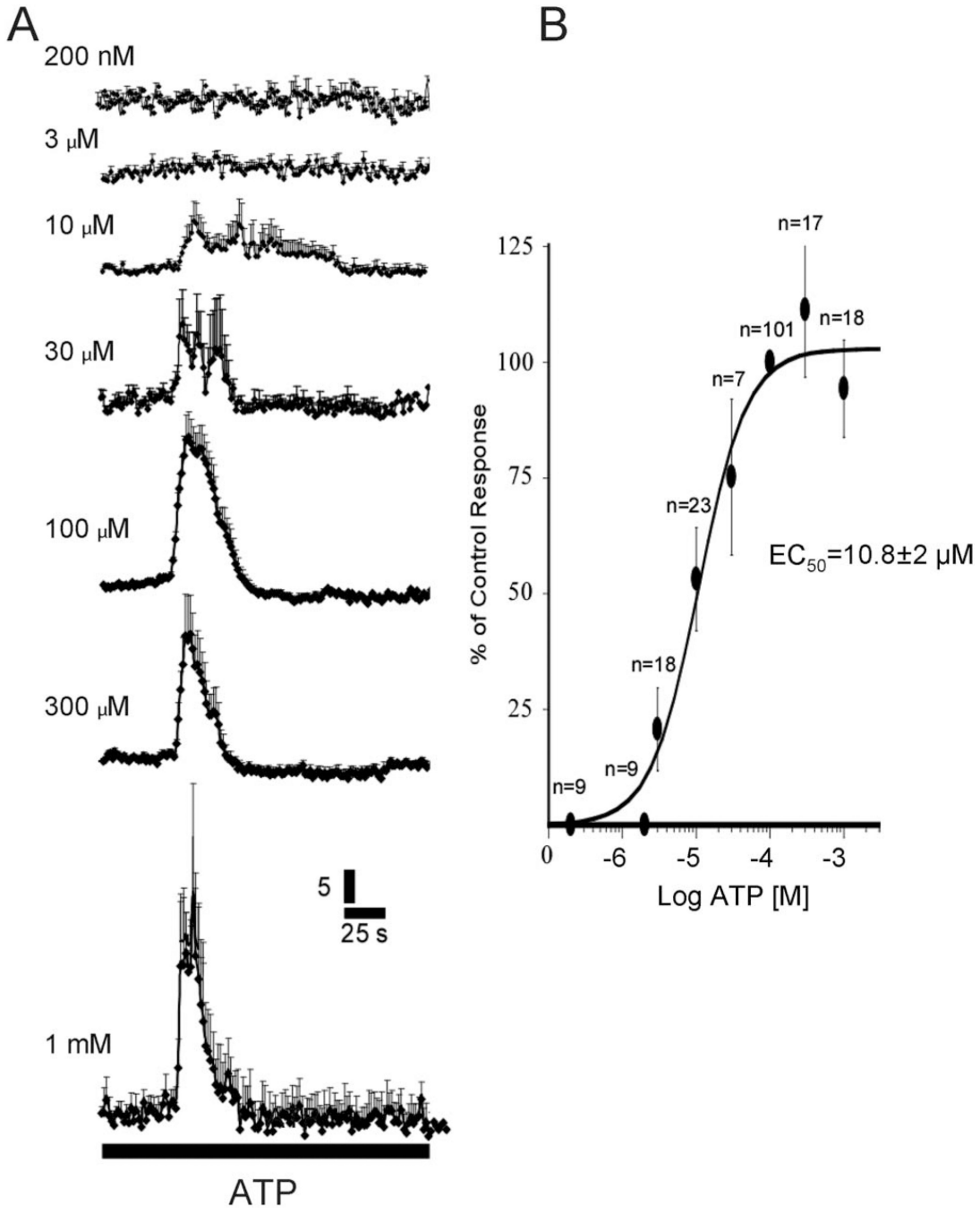

FIGURE 1.

ATP Ca^{2+} transients in BON cells. A, Ca^{2+} responses at different concentrations of ATP. B, ATP caused a concentration-dependent Ca^{2+} response with an EC_{50} at 10.8 ± 2 μM. n, number of cells; 100 μM ATP was used as the peak response for normalizing data. ANOVA, $P < 0.001$.

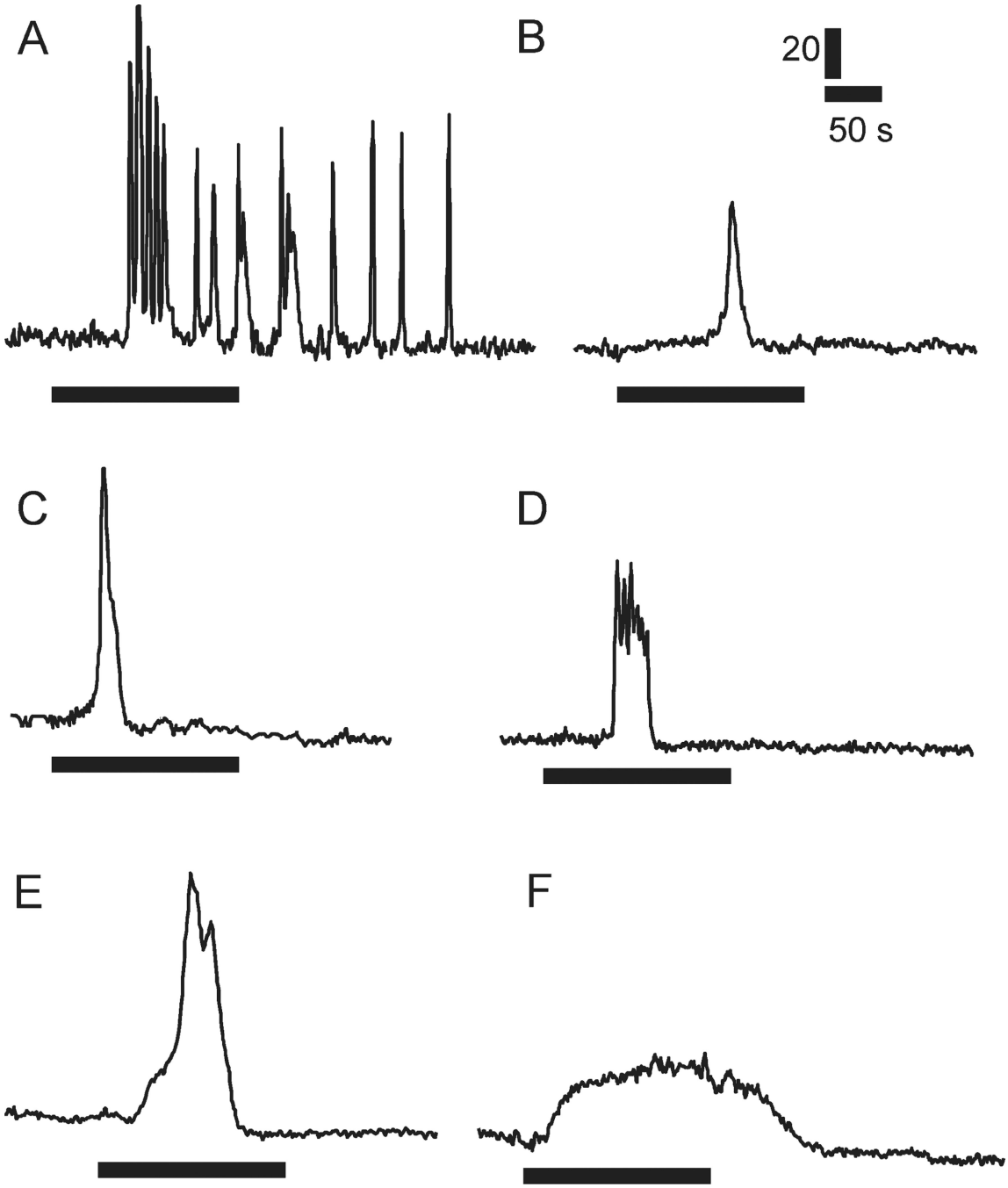

FIGURE 2.

ATP Ca^{2+} transients in BON cells with distinct kinetic-profiles: (A) Ca^{2+} waves or oscillations; (B,C) simple monophasic Ca^{2+} transients; (D,E). complex multiphasic responses with a burst of Ca^{2+}—spikes or multiple peaks; (F) rare Ca^{2+} response with very slow rising and falling phases. All responses were evoked with 100 μM ATP. Horizontal bar, duration of ATP perfusion. Vertical calibration, pixel intensity.

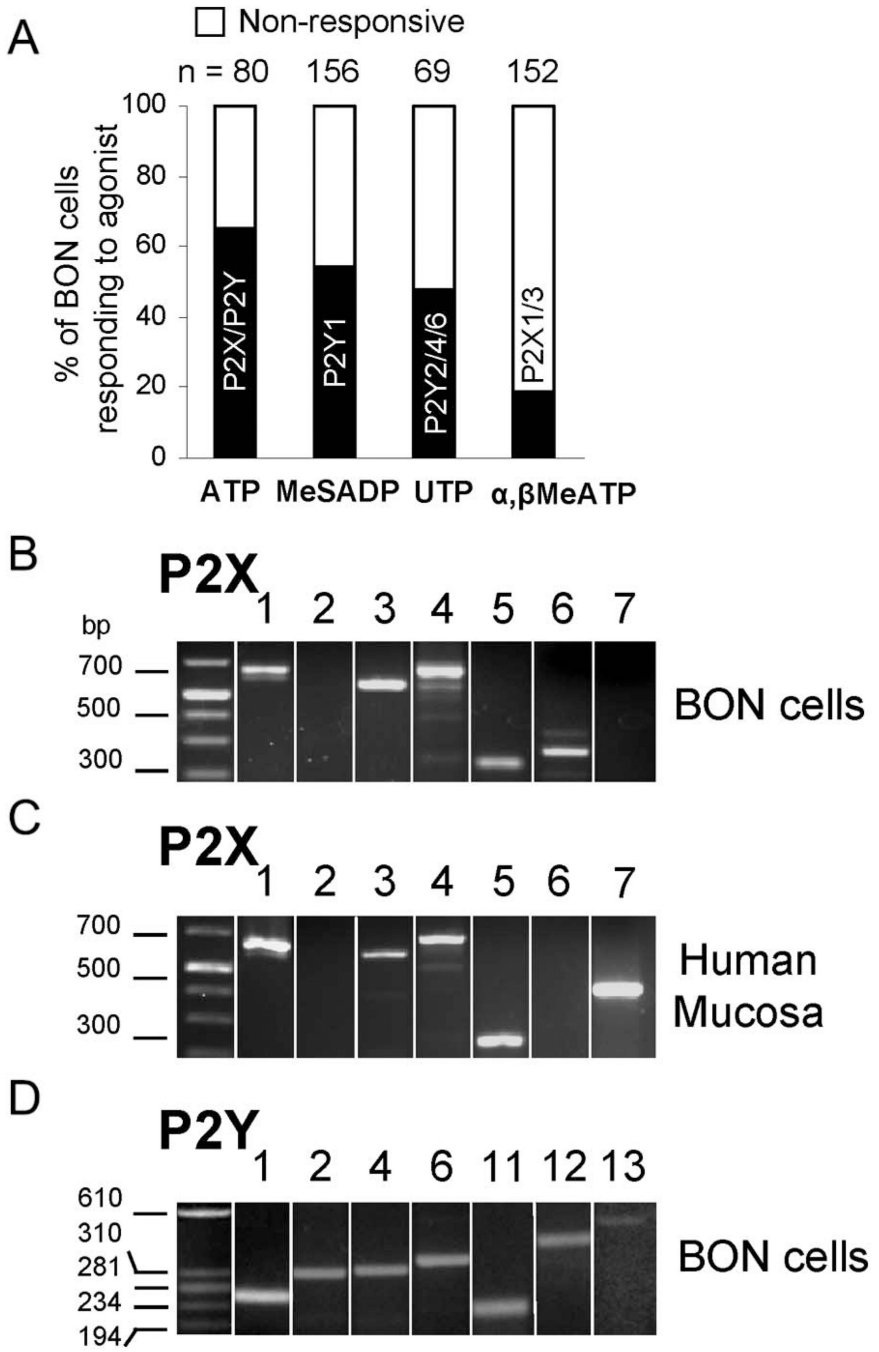

FIGURE 3.

Expression of different P2X and P2Y receptor subtypes in BON cells/EC. A, Calcium imaging reveals multiple functional receptors. ATP, a $P2Y_{1,12}$ agonist 2-MeSADP, a preferential $P2Y_{2,4,6}$ agonist UTP, or a $P2X_{1,3}$ agonist α,β-MeATP caused a Ca^{2+} response. B, mRNA transcripts for P2X receptors in BON cells. C, mRNA transcripts for P2X receptors in human mucosa. D, mRNA transcripts for P2Y receptors in BON cells.

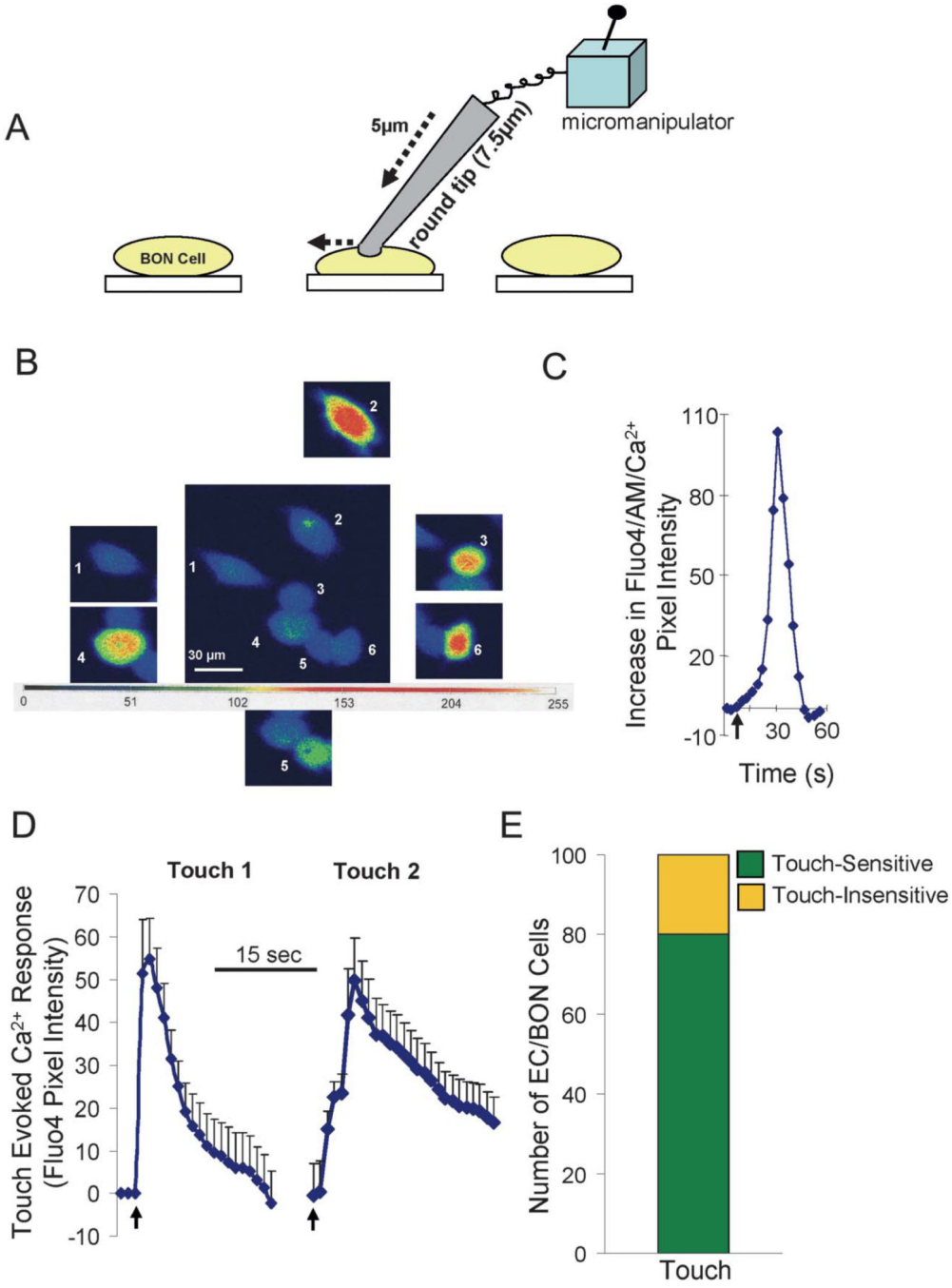

FIGURE 4.

Touch/stretch of single BON cells causes a Ca^{2+} response that can be quantified and is reproducible. A, Schematic of the touch/stretch technique—a piezo-micromanipulator is used to stretch the membrane of a single cell using a fire-polished glass tip (5–7.5 µm diameter). B, Touch of each BON cell individually elicited a Ca^{2+} response in cells 2 to 6 but not cell 1. Cells are pseudo-colored based on a gray scale of intensity ranging from 0 to 255. C, Kinetic Ca^{2+} response to touch in a single cell. D, The touch-evoked Ca^{2+} response indicated by arrows is reproducible if repeated after a 10-minute interval between trials

(touch 1 and touch 2; n = 80 cells individually touched). E, Eighty percent of BON cells are touch sensitive with a Ca^{2+} response.

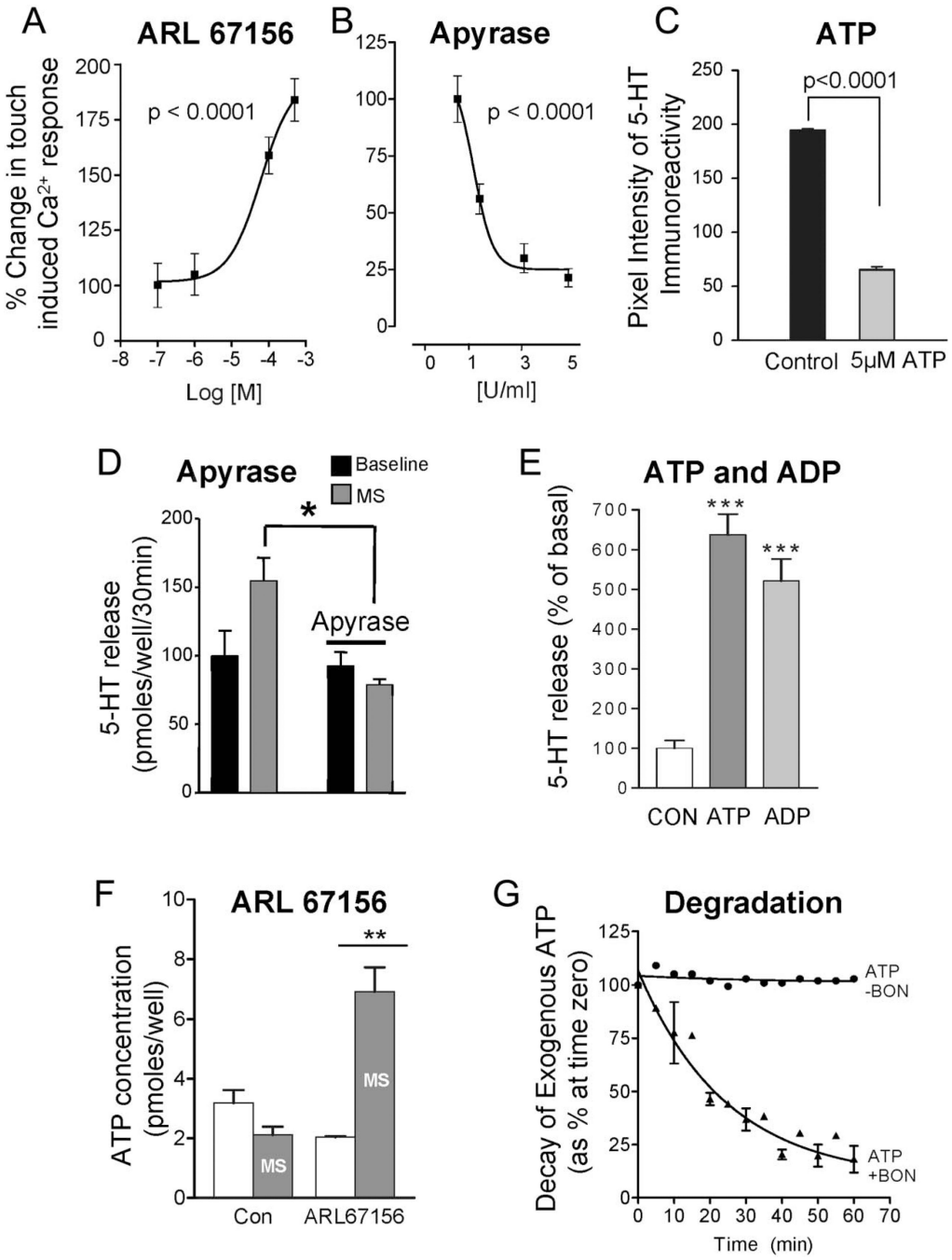

FIGURE 5.

Endogenous nucleotides are involved in the mechanically evoked Ca^{2+} response or 5-HT release in BON cells. A, The 5″-ectonucleotidase inhibitor ARL67156 augments touch-evoked Ca^{2+} responses. B, The ecto-ATPase inhibitor apyrase inhibits touch-Ca^{2+} responses. C, Exogenous ATP can deplete the 5-HT content in single BON cells as determined by quantitative analysis of 5-HT ir. 5-HT ir is quantified according to intensity levels using a Zeiss LSM/REN 410 imaging software and the same setting on the confocal (pinhole, contrast, brightness, power of laser, 40×/1.3 N.A objective). Cells were exposed to

ATP for 20 minutes at 378C in oxygenated Krebs' buffer. D, Rotational shaking evokes 5-HT release that is abolished by apyrase. E, Exogenous ATP or ADP causes a dramatic increase in 5-HT release from BON cells (N = 7, $P < 0.0001$); 10^6/well BON cells were seeded in a 12-well plate and incubated for 40 hours; cells were washed with OptiMEM solution (Life Technologies) and equilibrated for 30 minutes; 10^{-5} M ATP or 10^{-5} M ADP was incubated for 20 minutes, and supernatant was collected for 5-HT analysis by ELISA kit. Basal 5-HT = 1.12 ± 0.2 pmol/well/20 min. F, ATP release from BON cells in response to rotational shaking is only revealed if 10 μM ARL67156 is used to protect against degradation of endogenous ATP release; 10^6/well BON cells were incubated 40 hours, and cells were treated with 10^{-5} M ARL67156. Static incubation of cells was done at 37°C or mechanical stimulation was done by rotation shaking at 80 rpm and ATP detection in supernatants was done using an ATPlite kit using firefly luciferinluciferase; Rlu luminescence was measured in a Victor^3 (Perkin Elmer, Waltham, MA). *$P < 0.05$; **$P < 0.005$;*** $P < 0.0001$. G, Decay kinetics of 100 nM exogenous ATP by BON cells; the enzyme 5′-ectonucleotidase reduces the ATP-concentration over time by causing metabolic degradation.

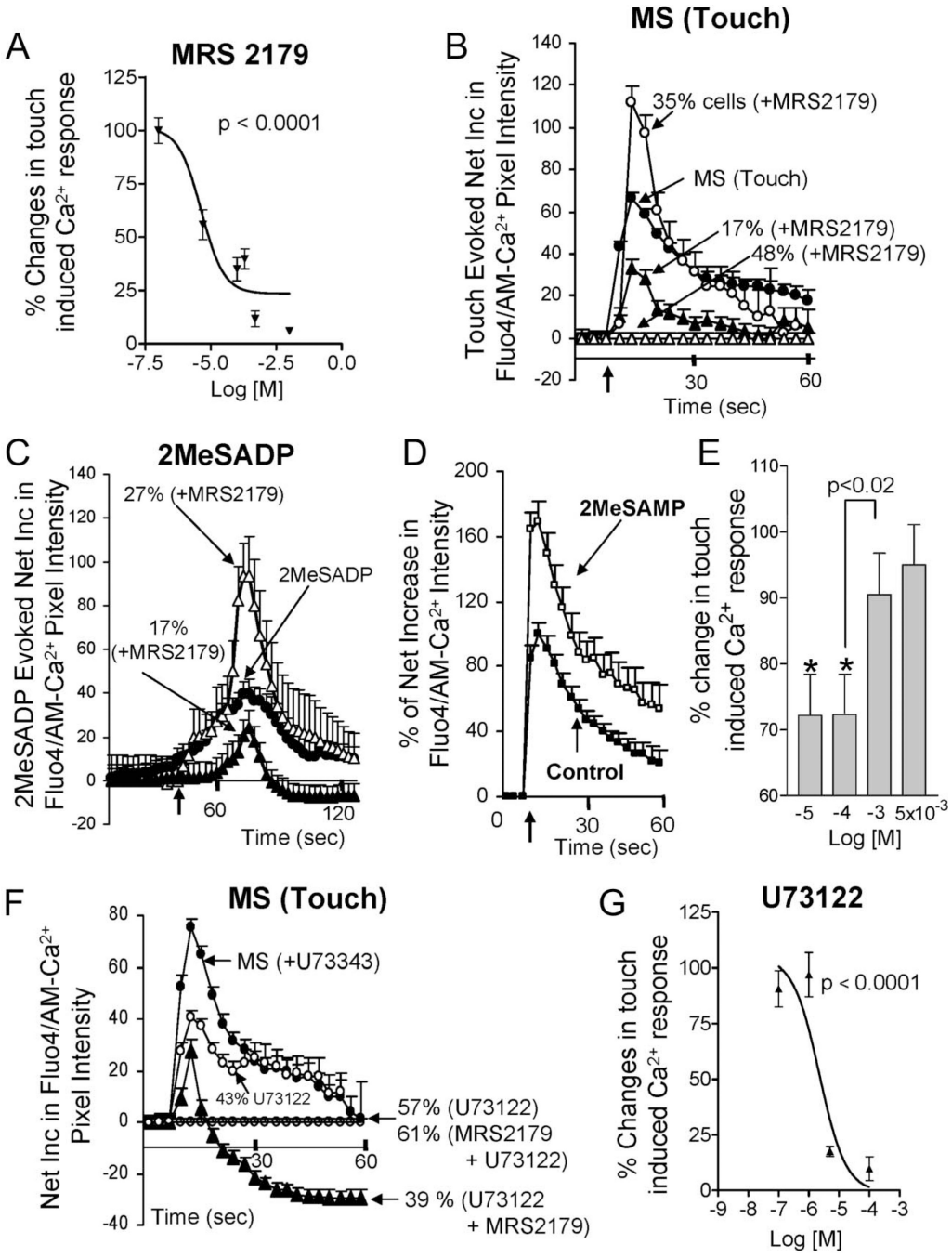

FIGURE 6.

$P2Y_1$ receptors, PLC signaling, and mechanosensitivity in BON cells. A, The prototypical $P2Y_1$ antagonist MRS2179 blocks the touch-evoked Ca^{2+} response in a concentration-dependent manner (n = 228). B, MRS2179 prevents or reduces touch-evoked Ca^{2+} responses in a majority of BON cells. In a different subset of cells (35%), MRS2179 augments the response. C, A $P2Y_1$ agonist 2MeSADP elicits a Ca^{2+} response in BON cells that is sensitive to MRS2179 (n = 152). Similar to touch responses, MRS2179 either blocks or augments the 2-MeSADP-induced Ca^{2+} response in different cells. D, The $P2Y_{12}$ antagonist

2MeSAMP augments the touch-Ca^{2+} response. E, Concentration-dependent response to 2MeSAMP. F, The PLC inhibitor U73122 abolishes the touch Ca^{2+} response in 57% of cells or reduces it in the remaining 43% of cells (n = 150). Interactions between MRS2179 and U73122 on touch-Ca^{2+} responses occur. U7312^{2+}MRS2179 can abolish touch Ca^{2+} responses and further reduce basal Ca^{2+} levels below the baseline. G, Concentration-dependent effect of the PLC inhibitor U73122 on touch-Ca^{2+} responses in sensitive cells (n = 247).

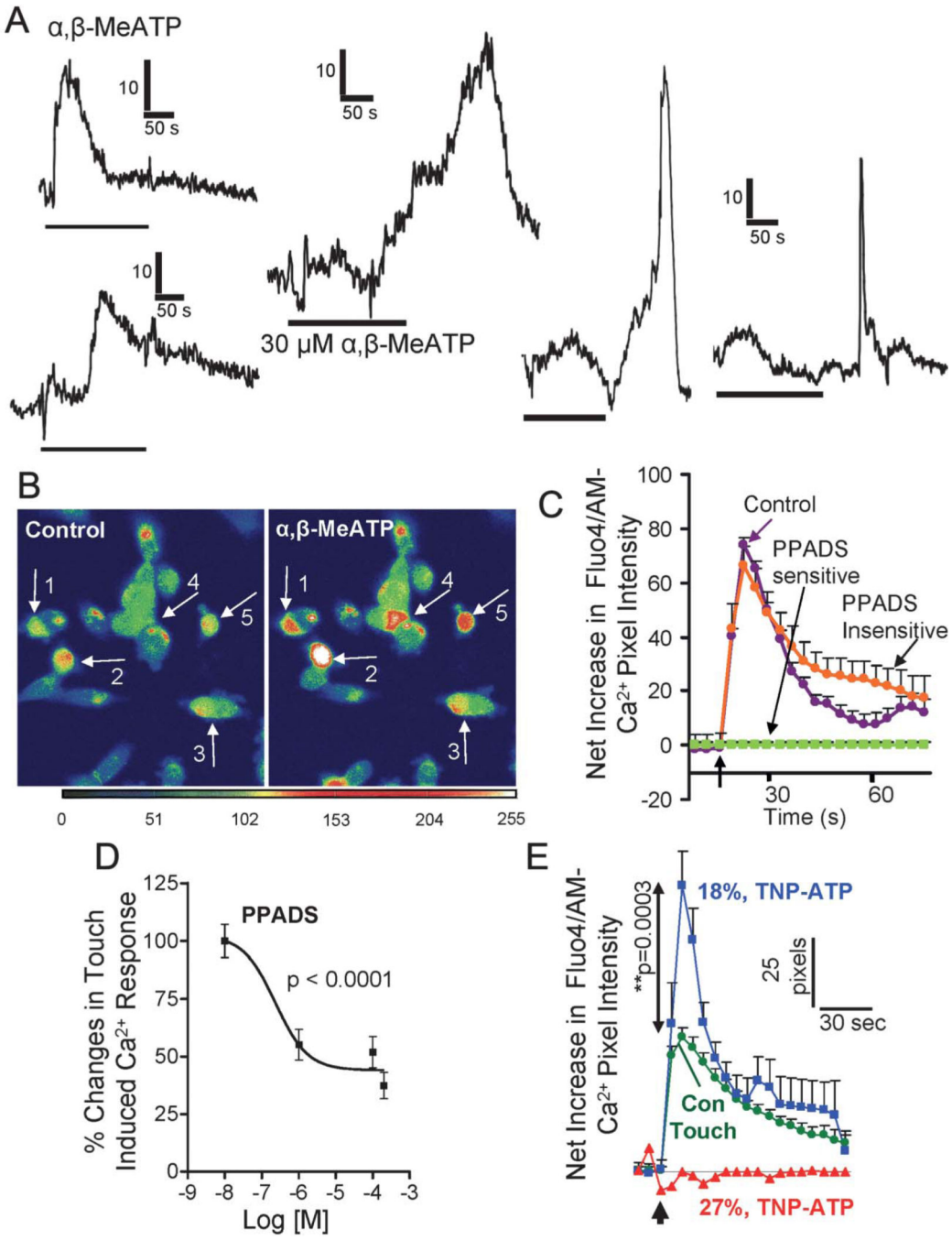

FIGURE 7.

P2X receptors are linked to mechanosensitivity in BON cells. A, Shapes of Ca^{2+} transients in response to perfusion of the $P2X_{1/3}$ agonist α,β-MeATP. B, Visualization of Ca^{2+} responses in BON cells exposed to α,β-MeATP; pseudocolor: images of Ca^{2+} responses obtained in 5 cells loaded with fluo-4/AM; image with α,β-MeATP taken after 20-second exposure to 1 mM puff of drug. C, In 52% BON cells, touch-evoked Ca^{2+} responses are blocked by a P2X antagonist PPADS (10 μM, PPADS-sensitive cells). In the remaining 48% cells, PPADS has no effect on mechanical stimulation. D, PPADS blocks touch-Ca^{2+}

responses in a concentration-dependent manner (n = 100). E, A selective $P2X_{1,3,2/3}$ receptor antagonist TNP-ATP blocks touch-Ca^{2+} responses in 27% cells. In an additional 18% cells, TNP-ATP augments the peak Ca^{2+} response.

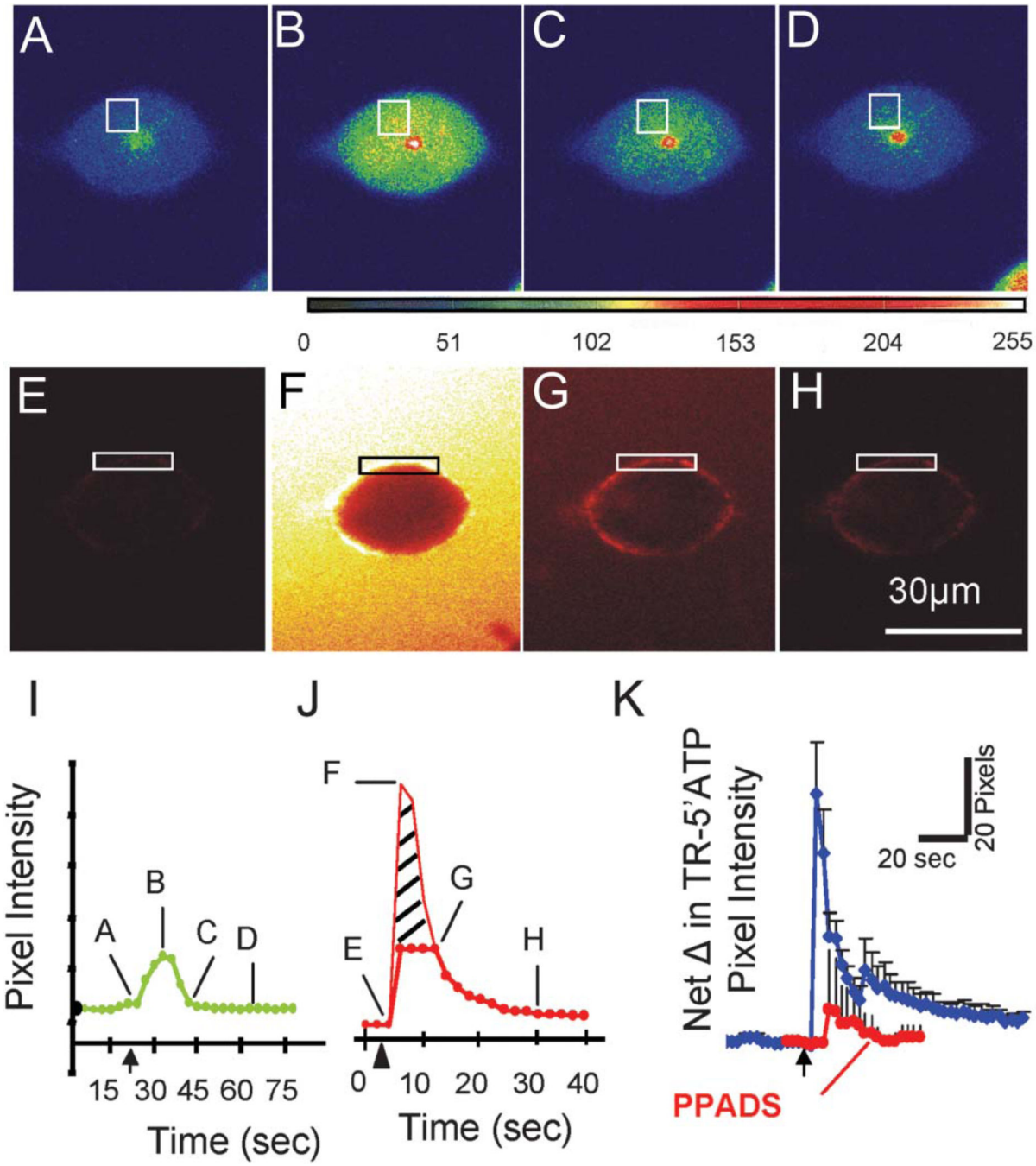

FIGURE 8.

Visualization of 5′ATP-^{TR} membrane binding in a live BON cell that is touch sensitive. A–D, The touch-sensitive Ca^{2+} response in a BON cell is revealed by laser confocal imaging; 10 × 10 pixels used in analysis. E–H, Fast puff application (100 ms) of 5′ATP-^{TR} causes cell membrane labeling of P2X sites. I, Touch-evoked Ca^{2+} transient of cell in A–D. J, 5′ATP-^{TR} fluorescence labeling of cell in E–H. F, entire cell is transiently visible. G, Specific membrane binding to P2X receptors at 12 seconds after the puff. K, Puff of 5′ATP-^{TR} membrane labeling is repeatable at 10-minutes intervals (not shown), and

exposure to a P2X antagonist 10 mM PPADS blocks 5′ATP membrane binding in response to a puff ($P < 0.0001$, n = 79).

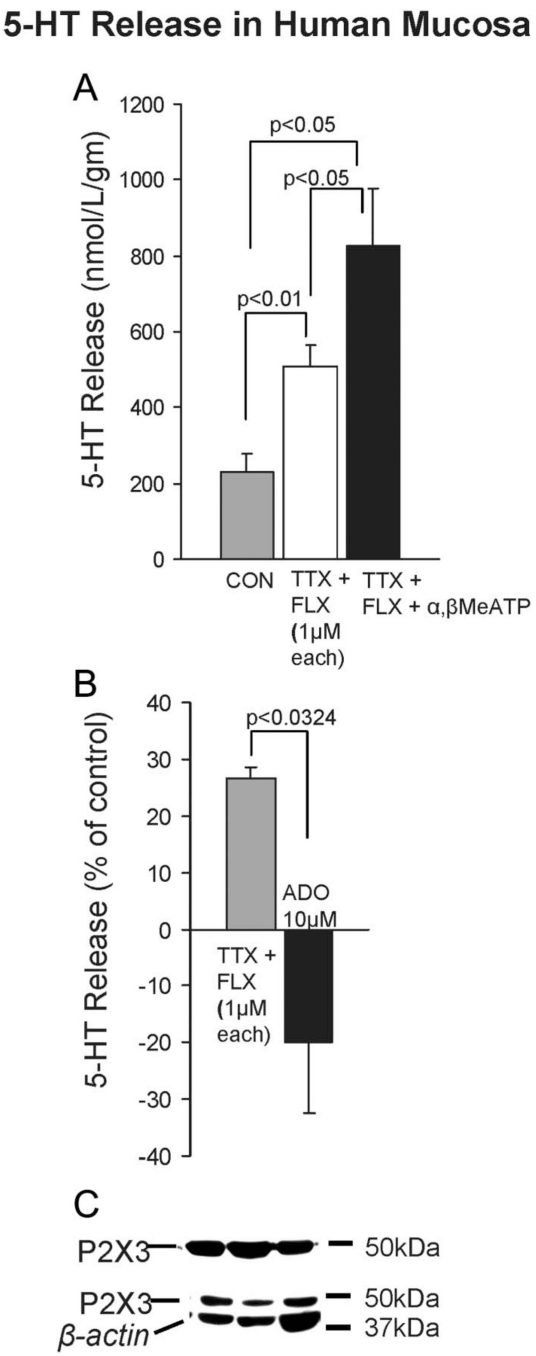

FIGURE 9.

Dual modulation of 5-HT release by α,β-MeATP and adenosine in human intestinal mucosa. A, $P2X_{1,3}$ receptor activation with 10 μM α,β-MeATP causes an increase in the 5-HT release from hECs collected in the supernatants. Inhibition of serotonin transporter-uptake of 5-HT by fluoxetine elevates 5-HT release (N = 6 each); experiments are done in tetrodotoxin to block nerve conduction. B, Adenosine (10 μM) can abolish basal 5-HT release occurring in the presence of fluoxetine and tetrodotoxin (N = 4). C, WB indicates expression of $P2X_3$ in human sigmoid colon; surgical specimens included 2 sigmoid colons,

4 jejunums, 1 rectal tissue, and 1 transverse colon, and data were pooled since release occurred in each case with α,β-MeATP; ELISA measured 5-HT release in supernatants during 30 minutes incubation at 37°C in oxygenated Krebs' buffer.

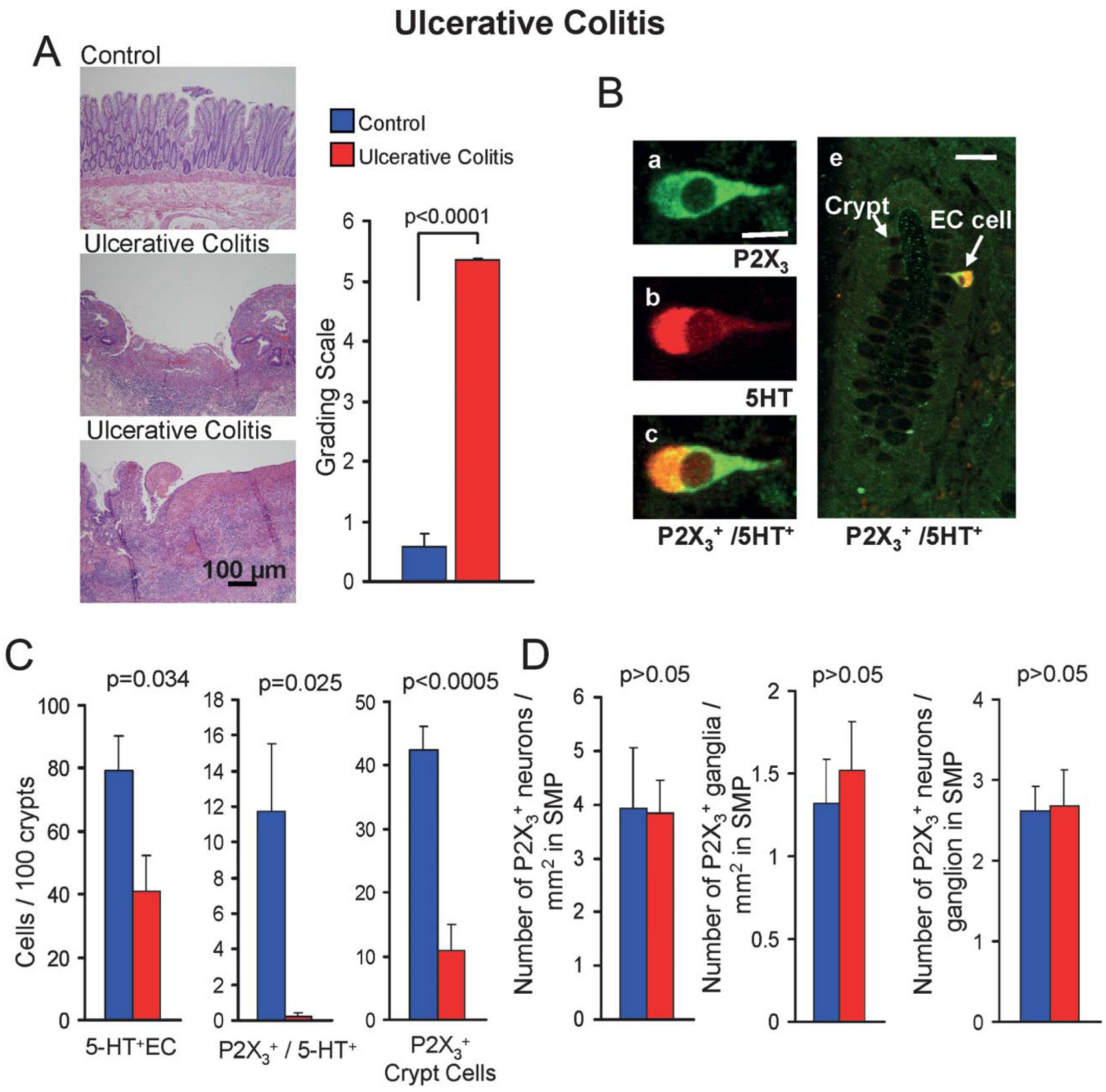

FIGURE 10.

Downregulation of $P2X_3$ receptor expression in hECs from human sigmoid colon in UC. A, Hematoxylin and eosin staining of tissue showing areas with severe crypt cell damage, inflammatory cell infiltration, and ulcers in UC. Control tissue appears fairly normal without inflammation. The clinical score for UC is significantly higher than control. Histopathologic assessment of inflammation in UC and controls was done according to Geboes et al.[43] Quantification of numbers of cells expressing receptors was done in areas with intact mucosa and crypt cells. B, $P2X_3$-ir is colocalized in 5-HT^+hECs in the human sigmoid colon. Top panel, single hEC in a crypt colabeled for $P2X_3$-ir and 5-HT-ir. Overlay images

for $P2X_3$-ir (green, fluorescein isothiocyanate-conjugated secondary antibody) and 5-HT-ir (red, TR-conjugated secondary antibody) to show colocalization. This approach was used to quantify the numbers of hECs expressing $P2X_3$-ir. D, In UC there is a reduction in the proportion of $5\text{-}HT^+$hECs in comparison with controls. There is also a dramatic reduction in hECs or crypt cells expressing $P2X_3$-ir. $P2X_3$ + hECs were reduced by <98% in UC. E, In submucous ganglia, $P2X_3$-ir expression is not altered.

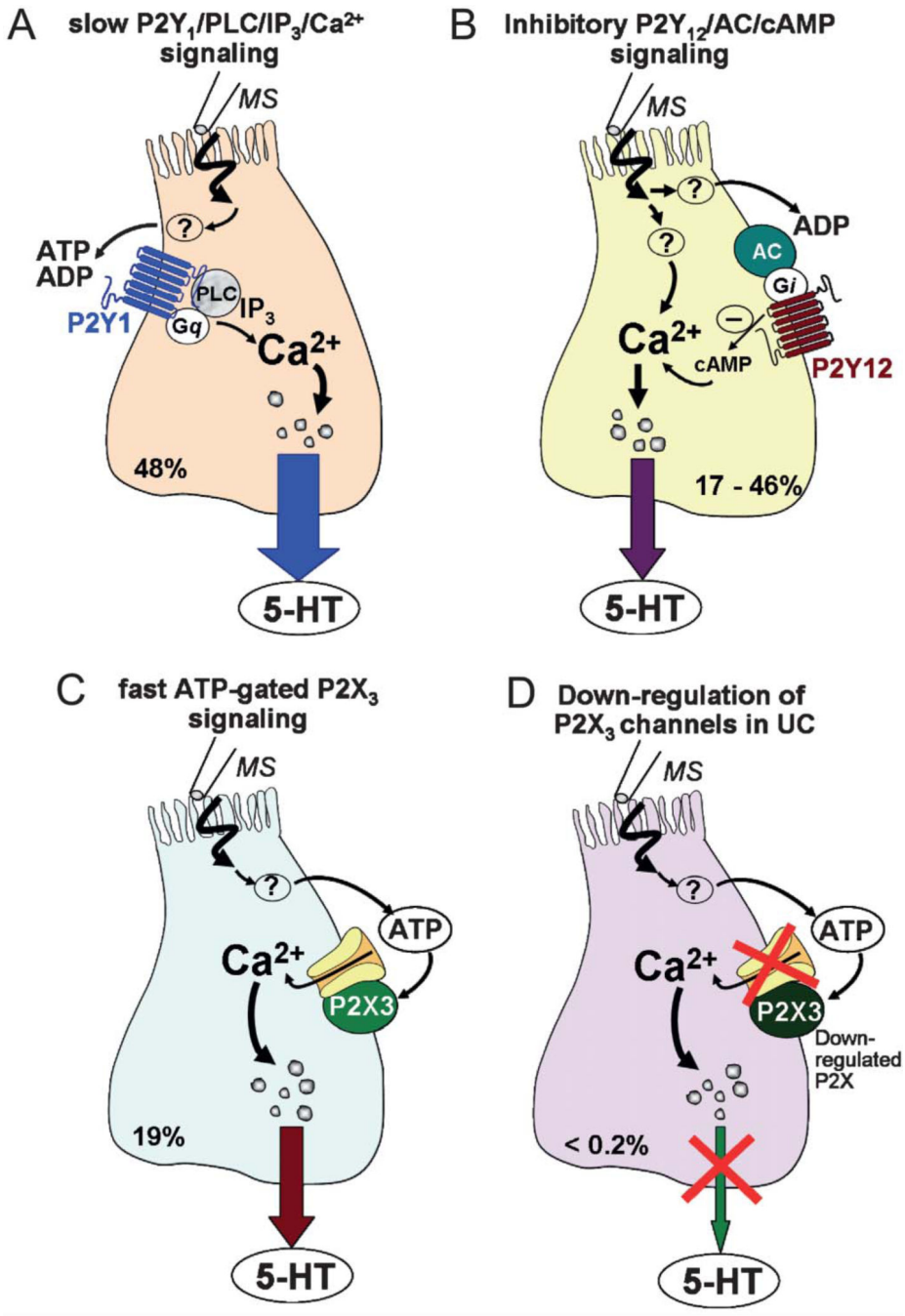

FIGURE 11.

Purinergic autoregulation model of mechanosensitivity in human ECs. In a majority of hEC, mechanically evoked 5-HT release is tightly autoregulated by corelease of ATP via 3 distinct purinergic signaling mechanisms. In the remainder of cells, either autoregulation does not occur or it involves nonpurinergic mechanisms. A, Mechanical stimulation releases ATP (or ADP) to activate a slow $P2Y_1$-PLC/IP_3-Ca^{2+}-dependent mechanism in 48% cells, leading to stimulation of 5-HT release. B, In a distinct subset of hEC, a slow $P2Y_{12}$AC/cAMP signaling pathway is activated by ATP (or ADP) release leading to attenuation of 5-

HT release. C, ATP release in 19% cells can alternatively activate a fast ATP-gated $P2X_3$ ion channel to stimulate 5-HT release. D, Purinergic pathways are very sensitive to inflammation, and severe downregulation of $P2X_3$ channels occurring in UC is expected to alter 5-HT release and contribute to abnormal signaling, physiology, and visceral sensation. In this model, purinergic autoregulation does not occur in all hEC. 5-HT release evoked by mechanical stimulation is not regulated by ATP in about 25% to 30% of hEC. Overall, mechanical stimulation coreleases 5-HT, ATP/other purines (ADP and adenosine), or other mediators (prostaglandins and PACAP[19]). 5-HT, ATP, and perhaps other mediators can trigger gut motor reflexes and visceral sensation, and any disruption in purinergic autoregulation and fine tune modulation of 5-HT release could have important consequences in IBD.

TABLE 1

Summary of the BON Cell Population Responses to Purinergic Drug/Manipulations for Touch or Purinergic Agonist-Evoked Ca^{2+} Transients

	Ca^{2+} Responses to Nucleotides/Analogs (Percentage of Cell Population*)		
Treatment	**Stimulate**	**No Effect**	**n**
ATP	65	35	80
UTP	48	52	69
α,β-MeATP	19	81	152
2MeSADP	54	46	156
Microejection (1 mM) or perfusion (10 and 100 μM)			

	Ca^{2+} Responses to Touch/Stretch (Percentage of Cell Population**)			
Antagonist/Blocker/Inhibitor	**Block/Abolish Response**	**Augment Response**	**No Effect**	**n**
Apyrase (5 U/mL)	77	0	23	67
ARL67156 (10 μM)	0	63	37	65
PPADS (10 μM)	52	0	48	100
MRS2179 (10 μM)	48	——	——	100
	17 (reduced)		35	
TNP-ATP (20 μM)	27	18	55	73
2MeSAMP	0	46	54	81
Piroxicam	0	73	27	41
Ca^{2+}-free medium (0.5 mM Ca^{2+}, 10 μM EGTA)	3.6	——	96.4	84
U73343	0	——	100	100
U73122	57	——	43	152

Data are expressed as *percentage of cell population responding to the drug for a single drug concentration or **touch/stretch.. Four different effects were analyzed including percentage of cells in which response was abolished, percentage of cells in which response was augmented, percentage with no effect, or percentage with partial inhibition of the response. Concentrations were chosen from concentration-response curves generated for touch-Ca^{2+} responses. The concentration of each drug used in most cases is >EC_{80} concentration. For MRS2179, an EC_{50} concentration is used. Antagonists were perfused for 10 to 20 minutes and agonists for 3 to 5 minutes.

TABLE 2

Discrete Alterations in $P2X_3$ and A_{2B} Purine Receptor Expression in hEC, Epithelia, and Lamina Propria From Sigmoid Colon of UC Surgical Cases Compared With Controls

				Δ in UC Compared With Noninflamed Diverticulitis Controls in Sigmoid Colon			
Purinergic Receptor/ Other	**Percentage of 5-HT$^+$hECs (1.2% Cells)**	**Percentage of Ganglia**	**Percentage of Epithelial Cells**	**hEC/5-HT$^+$ Cells (Δ in Expression)**	**Epithelial Cells**	**Lamina Propria/ Immune Cells (Δ in Number)**	**ENS (SMP) s100 +/HuC/D+ (Δ in +ve Ganglia)**
5-HT	NA	NA	NA	↓ 50% ($P < 0.0005$)	NA	NA	NA
$P2X_3$	15	13	0.43	↓ 98% ($P = 0.025$)	↓ 74% ($P < 0.005$)	↑ +++	Ø
A_{2B}	53	46	5.7*	↓ 68% ($P = 0.001$)	↓ 66% ($P = 0.011$)	↑ +++	↓ 57% ($P = 0.029$)

*Percentage of cells/A_{2B}+ crypt; percentage reduction is calculated on the basis of the numbers of fewer cells expressing the receptor in UC compared with control.

NA, not applicable; Ø, no change; Δ, change.