Dual purinergic synaptic transmission in the human enteric nervous system


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Am J Physiol Gastrointest Liver Physiol 294: G554–G566, 2008. First published December 13, 2007; doi:10.1152/ajpgi.00500.2007.—Based on findings in rodents, we sought to test the hypothesis that purinergic modulation of synaptic transmission occurs in the human intestine. Time series analysis of intraneuronal free Ca\(^{2+}\) levels in submucosal plexus (SMP) from Roux-en-Y specimens was done using Zeiss LSM laser-scanning confocal fluo-4 AM Ca\(^{2+}\) imaging. A 3-s fiber tract stimulation (FTS) was used to elicit a synaptic Ca\(^{2+}\) response. Short-circuit current (I\(_sc\) = chloride secretion) was recorded in mucosa-SMP in flux chambers. A distension reflex or electrical field stimulation was used to study I\(_sc\) responses. Ca\(^{2+}\) imaging was done in 1,222 neurons responding to high-K\(^+\) depolarization from 61 surgical cases. FTS evoked synaptic Ca\(^{2+}\) responses in 62% of recorded neurons. FTS caused frequency-dependent Ca\(^{2+}\) responses (0.1–100 Hz). FTS Ca\(^{2+}\) responses were inhibited by \(\Omega\)-conotoxin (70%), hexamethonium (50%), TTX, high Mg\(^{2+}\)/low Ca\(^{2+}\) (≤100%), or capsaicin (25%). A P2γ1 receptor (P2γ1R) antagonist, MRS-2179 or PLC inhibitor U-73122, blocked FTS responses (75–90%). P2γ1R immunoreactivity occurred in 39% of vasoactive intestinal peptide-positive neurons. The selective adenosine A1 receptor (AdoA1R) agonist 2-chloro-N\(^6\)-(3-iodobenzyl)adenosine-5'-N-methylcarboxamid (2-Cl-IBMECA) caused concentration- and frequency-dependent inhibition of FTS Ca\(^{2+}\) responses (IC\(_{50}\) = 8.5 \times 10^{-8} M). The AdoA1R antagonist MRS-1220 augmented such Ca\(^{2+}\) responses; 2-Cl-IBMECA competed with MRS-1220. Knockdown of AdoA1R in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

In general, Ado modulates neural, immune, or sensory signals and may provide cytoprotection or neuroprotection in both the enteric nervous system (ENS) and central nervous system (14, 37, 48, 49). In the ENS, Ado suppresses synaptic transmission (10), efferent function of extrinsic capsaicin-sensitive sensory nerves (64), mucosal reflexes (19), neuroeffector transmission (11), and morphine withdrawal diarrhea (38).

Increasing the concentration of endogenous Ado (eAdo) is one mechanism by which several drugs may reduce gut inflammation (1, 2, 6, 28). An Ado kinase inhibitor or an AdoA1R agonist, \(\text{N}^{6}_-(3\text{-iodobenzyl})\text{adenosine-5'}\text{-N-methyluronamid (IB-MECA)}\), may be beneficial in murine models of colitis (42, 56), but the mechanisms involved remain poorly understood. The sites of action of these drugs and, in particular, whether AdoA1R is involved remain unclear. AdoA1R have a wide range of physiological and disease-related effects (18, 21, 34, 41) with promise for treatment of a variety of heart disease, uveitis, colorectal cancer, and inflammation (23, 29, 43, 51).

IB-MECA is in Phase I and II clinical trials for a chronic inflammatory disease, rheumatoid arthritis, and is apparently without toxicity (www.canfite.com/develop.html).

It is increasingly more evident that abnormalities in the ENS, or “little brain of the gut,” are involved in IBD or functional bowel diseases (irritable bowel syndrome) that underlie symptoms of constipation, diarrhea, dysmotility, malabsorption and transport, and painful sensations (59). Purinergic signaling pathways play an important role in sensory signaling in enterochromaffin cells (EC) and secretomotor reflexes in the intestinal tract; purinergic signaling operates at all levels of the EC-neural-secretomotor axis in gut reflexes (16, 18). Abnormalities in purinergic signaling, such as those that occur in experimental IBD (30), are expected to influence gut neural reflexes and contribute to symptomology in disease states.

Our recent study (30) assessed the protective effect of an AdoA1R agonist, IB-MECA, on gene dysregulation and injury in a rat chronic model of 2,4,6-trinitrobenzene sulfonic acid-induced colitis. Oral IB-MECA prevented abnormal gene expression in 92% of these genes, histopathology, gut injury, and weight loss. IB-MECA or Ado could suppress elevated free radicals in ex vivo inflamed gut. Oral IB-MECA blocked the colitis-induced upregulation of benzodiazepine, P2X\(_1\) receptor (P2X\(_1\)R), P2X\(_3\)R, P2X\(_7\)R, and P2Y\(_6\)R, A\(_{2a}\)R/A\(_{2b}\)R, but not P2X\(_1\)-P2X\(_7\) or metabotropic G-protein-coupled P2Y receptors P2Y1, P2Y4, P2Y6, P2Y11-P2Y14 with specific actions in gut neurophysiology (16, 18).

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not A1R or A2R genes, or downregulated P2X2R, P2Y1R, and P2Y2R (30).

Neural hypoxia elevates interstitial Ado in networks of enteric ganglia or CA1 hippocampal neurons that suppress transmitter release (26, 47) via AdoA1R and possibly AdoA3R, based on indirect pharmacological analysis. Such activation of low-affinity AdoA3R could protect the ENS by limiting excitability.

The physiological role of AdoA3Rs in the brain or ENS has been questioned (13, 21, 50), because the AdoA3R is a low-affinity receptor that requires high/micromolar concentrations of eAdo for activation: high levels of eAdo occur at sites of inflammation, infection, and metabolic stress (44, 48, 57, 58). However, in vitro studies demonstrated that ongoing release of eAdo differentially affects excitatory and inhibitory transmission to S or AH neurons in the gut. These effects at high affinity A1 or non-A1/putative A2 or A3 receptors (13) serve to complement the ability of Ado to shut down excitatory neural activity in gut microcircuits through its dual pre- and postsynaptic actions (9, 11, 19, 26).

The AdoA3R shows a species-specific distribution (40), pharmacology, function, and diversity of structure (40, 46). Differential expression of AdoA3R mRNAs occurs in the mammalian intestines (13, 27, 40), and AdoA3Rs, as well as AdoA2aR, AdoA2bR, and AdoA1Rs, are discretely expressed in human myenteric and submucosal neurons (13). Our laboratory’s initial studies also identified an inhibitory limb of the neural motor reflex that may be activated by putative AdoA3Rs in rat distal colon (3). In a guinea pig model of neurogenic diarrhea involving dimaprit/H2 activation of gut reflexes, neural AdoA3Rs are involved in inhibitory modulation of a stereotype cyclical pattern of coordinated motility and secretion (5). In addition, non-A1/A2 inhibitory receptors that may represent putative AdoA3Rs gate excitability of enteric AH neurons (14). Our findings in normal rodent gut, as well as reports by others (25), suggest a physiological role of low micromolar levels of eAdo at AdoA3Rs in intact neural circuits. A functional role for IB-MECA and AdoA3Rs is also indicated in synaptic plasticity that represents the likely substrates for learning and memory (21) that may operate in AH/intrinsic primary afferent neurons (IPANs) (18, 32, 65).

AdoA3Rs are, in general, inhibitory receptors in the ENS (F. L. Christof, unpublished observations; Ref. 16). In contrast, the P2Y1R is emerging as a significant target for nucleotides in excitatory modulation of EC cell secretion of 5-HT to remove anesthetic medications. It was necessary to first establish an optical recording technique suitable for monitoring synaptic transmission in the ENS. Fluo 4-acetyl methyl ester (AM) laser confocal Ca2+ imaging was used to record synaptic responses in postsynaptic neurons generated by focal electrical fiber tract stimulation (FTS) of internodal strands, connecting two ganglia in the SMP. Some of the data were presented in preliminary form in two abstracts at the American Gastroenterology Association Meetings (52, 69).

**MATERIALS AND METHODS**

**Collection of human jejunal specimens.** Specimens of human jejunum were collected from obese patients who underwent laparoscopic gastric bypass surgery. The study was approved by the biomedical science institutional review board of The Ohio State University. The human jejunum specimen was immediately given to the investigator by the surgeon within seconds after removal from the patient in the operating room and placed in 4°C oxygenated Krebs solution (in mM: 120 NaCl, 6.0 KCl, 1.2 MgCl2, 1.35 NaH2PO4, 14.4 NaHCO3, 2.5 CaCl2, 12.7 glucose). After approval of a pathologist, the specimen was used to carry out biological studies in gut neural layers. The time elapsed from tissue removal from the patient to the laboratory bench was 15 min maximum. The tissue was transferred to a large volume of Krebs (2 liters) at room temperature for 1 h initially, to remove anesthetic medications.

**Microdissection and laser scanning confocal microscope fluo-4 AM Ca2+ imaging in human SMP after electrical FTS.** The jejunal preparation was opened along the mesenterial border, and nonviable tissue that was damaged by surgical instruments or cautery was removed after careful microscopic inspection by an experienced physician. The whole thickness tissue was placed and perfused in oxygenated Krebs at room temperature, pinned and stretched to Sylgard, and carefully microdissected to remove mucosa, circular muscle, and myenteric plexus-longitudinal muscle layers. The dissection took ~1 h. The remaining SMP with intact network of ganglia in multiple layers was cut into ~1-cm SMP pieces for loading with Ca2+ indicator for separate experiments. We could routinely obtain 8–12 separate pieces of SMP from one jejunal specimen. Each piece of SMP was loaded with 30 μM fluo-4 AM (Molecular Probes, Eugene, OR) for 2 h and then incubated an additional 1 h in oxygenated Krebs solution at room temperature to cleave the AM from fluo-4 AM. The total time elapsed from the tissue removal from the patient to placement of the tissue preparation on the stage of the confocal microscope was ~5 h.

Time series analysis of intracellular free calcium at 2-s intervals was done in fluo-4 AM loaded submucosal neurons that were monitored by a Zeiss laser scanning confocal microscope (LSM) 410 laser-scanning confocal imaging system through a ×40 oil immersion objective (numerical aperture 1.3, working distance = 170 μm). LSM Ca2+ imaging was done in single submucosal ganglia of the SMP at 2-s intervals (12, 20). A schematic diagram of the preparation is illustrated in Fig. 1A.

Ca2+ imaging was carried out using an Ar-Kr laser to excite the cell at 488 nm, and fluorescence emissions were passed through a FTS10 dichroic mirror and collected through a photomultiplier tube equipped with a BP 505–550 filter, positioned in front of the pinhole and light path. Therefore, fluorescence emission is restricted to between 505 and 550 nm. Imaging was done using time series analysis LSM software. Scanned sections had a thickness of 17.5 μm. During the experiment, the tissue was perfused with oxygenated Krebs solution at 36 ± 0.5°C with a “solution in-line heater” (Warner Instruments, Hamden, CT) at a perfusion rate of 20 ml/min. An electrode, which was guided by a computerized Eppendorf Micromanipulator 5171, was placed on a fiber tract (or internodal strand connecting 2 ganglia) 0.3 mm away from the recorded ganglion (Fig.
agonists and antagonists (competitive and noncompetitive) on FTS-evoked \( \text{Ca}^{2+} \) transients were assessed on FTS before and 20–30 min after treatment. At the end of each experiment, the recorded ganglion was exposed to 75 mM high-potassium (K\(^+\)) solution. Neurons were included in the Ca\(^{2+}\) analysis when they responded to FTS before drug treatment and to high-K\(^+\) solution at the end of the experiments.

**Short-circuit current (chloride secretion) recordings.** Short-circuit current (I\(_{sc}\)) was recorded in mucosa-submucosa preparations in Ussing flux chambers under voltage clamp. For these experiments, longitudinal muscle with attached myenteric plexus and circular muscle layers were removed from the jejunum specimen. The remaining mucosa-submucosa tissue (M-SMP) was used as a flat sheet, which was pinned down on the inside of an Ussing chamber with a surface area of 1.767 cm\(^2\). Voltage clamp allowed us to record I\(_{sc}\), which is a measurement of chloride secretion. M-SMP were set up in flux chambers, according to published reports in rodent tissue (39).

**Distension reflexes, electrical field stimulation, and I\(_{sc}\) responses.** Distension, caused by the removal of 150 \( \mu \)l fluid for 30 s from the submucosal compartment, evoked a reflex I\(_{sc}\) response. Electrical field stimulation (EFS) with 10 or 20 Hz for 30 s (voltage 15 V, duration 0.5 ms) evoked an I\(_{sc}\) in the M-SMP. These frequencies were shown previously to evoke optimal EFS responses that can be used in pharmacological studies. Based on our frequency-response curves to FTS in the Ca\(^{2+}\) studies, a peak response occurred at 10–20 Hz (see below). Agonists and antagonists drugs were added to the submucosal side after an initial I\(_{sc}\) response was evoked by mechanical or electrical stimulation. Synaptic transmission was confirmed by blocking nerve conduction with tetrodotoxin in the end of each experiment.

**Colabeling for P2Y\(_1\) and vasoactive intestinal peptide in SMP.** Colabeling studies were done for P2Y\(_1\)R and vasoactive intestinal peptide (VIP) immunoreactivity (ir) in human submucous neurons with polyclonal antibodies. The submucosa was first incubated with P2Y\(_1\)R (Alamo, Jerusalem, Israel) and VIP primary antibodies (Peninsula, San Carlos, CA) overnight. FITC and Texas Red conjugated secondary antibodies were used to show P2Y\(_1\)R or VIP-positive neurons. Dual-labeling techniques were applied as previously described (13).

**Statistics.** Mean values ± SE were reported. Statistical significance was evaluated by paired or unpaired Student’s t-test, depending on the experimental design. The EC\(_{50}\)/IC\(_{50}\) values are obtained from sigmoid cumulative concentration-response curves fitted by a nonlinear curve-fitting program (GraphPad Prism 3). For multiple comparisons between different frequencies of FTS, ANOVA followed by post hoc (Dunnett’s and Newman-Keuls) tests was used. Some of the pharmacology (i.e., Cl-IBMECA concentration-response curve) was also tested with ANOVA. Statistical analysis was performed using StatView 54.51 (Abacus Concepts, Calabasas, CA). Differences are considered statistically significant for \( P < 0.05 \).

**RESULTS**

Tissue was obtained from postsurgical specimens in 61 patients undergoing Roux-en-Y gastric bypass surgery (age, 22–65 yr old; body mass index, 35–68 kg/m\(^2\)). Laser confocal Ca\(^{2+}\) imaging was done in 143 separate experiments in 143 ganglia in different tissues. The \( n \) values include all neurons that respond to FTS to the first electrical stimulus without treatment. Responses to agonists or antagonists included all neurons that had an initial FTS response, whether they had small or large effects. This included neurons with a very small response (i.e., even if it was 10%) vs. neurons that had a large response (i.e., 60%).

Each ganglion displayed three to four fiber tracts under the microscope. By stimulation of one fiber tract, a certain number of neurons within the visualized ganglion responded with a synaptic Ca\(^{2+}\) response. Our experimental protocol was restricted to stimulation of a single-fiber tract per recorded
ganglion. The ganglia in the human SMP (as is the case in rat SMP) are oriented in all directions; a fiber tract was randomly chosen for stimulation, with no preconception of orientation or direction. Seven hundred and sixty-three neurons (62.44%) out of 1,222 neurons that all responded to high-K⁺ depolarization gave a synaptic Ca²⁺ response to FTS stimulation. If FTS gave a response in two or more neurons in a ganglion, this was used for full analysis. FTS evoked responses in 2–20 neurons/ganglion.

High-K⁺ depolarization was used at the end of the experiment to confirm the number of neurons in the recorded ganglion that are “viable neurons”; this number was usually greater than that evoked by FTS. The total number of neurons in a ganglion in relation to the number of responsive neurons to high-K⁺ depolarization was not determined in our study. It is likely that only 60% of viable neurons respond to FTS, because we only stimulated one intermodal strand. In fact, when we switched the FTS electrode to a different fiber tract, some different neurons are often activated, suggesting that differential recruitment of neurons occurs by stimulating different fiber tracts. The question of recruitment of neurons, convergence and divergence of synaptic responses, deserves further consideration, but was not analyzed further here. To date, Dr. Jan Tack’s group has analyzed such responses in guinea pig myenteric neurons using Ca²⁺ imaging (62). EFS or distension experiments were done in 19 M-SMP from 8 postsurgical specimens.

**FTS and synaptic Ca²⁺ responses.** A frequency-dependent Ca²⁺ response curve was obtained in submucous neurons in response to FTS. Examples of time series images that depict neural Ca²⁺ responses at different frequencies are shown in Fig. 1B. The Ca²⁺ response increased in a frequency-dependent manner from 0.1 to 100 Hz, peaking at 10–25 Hz and gradually declining by 30–40% by 100 Hz (Fig. 1C).

Reproducibility of the Ca²⁺ response at different frequencies. FTS-Ca²⁺ responses were evoked repeatedly for 10 consecutive trials at 5-min intervals over a 45-min period at 5, 10, 25, and 35 Hz to assess reproducibility of the response (Fig. 2). At frequencies of 5, 10, and 35 Hz, ANOVA analysis revealed that the response declined significantly after repeated FTS trials. When stimuli of 25 Hz were applied to fiber tracts, no significant difference was seen during 10 repeated identical stimuli. This frequency was chosen for most pharmacological studies. At 35 Hz, repeated FTS caused a complete inhibition of the elicited Ca²⁺ response (Fig. 2D).

**P2Y₁R/Goαq/PLC signaling mechanism.** To assess the role of the PLC signaling pathway in the FTS Ca²⁺ response, we sought to investigate the effects of inhibition of PLC using U-73122 or a nucleotide P2Y₁R-selective antagonist to suppress FTS Ca²⁺ responses, because P2Y₁R agonists are known to be coupled to a Goαq/PLC/Ca²⁺ signaling in submucous neurons of the rat and guinea pig intestine and in human ECs (F. L. Christofi, unpublished observations; Refs. 12, 16, 17, 20, 68).

The selective P2Y₁R antagonist 2'-deoxy-NH₄-methyladenosine 3',5'-bisphosphate (MRS-2179; 20 μM) or the PLC inhibitor U-73122 (2 μM) could block 75–90% of the peak FTS Ca²⁺ response (Fig. 3, A–C). Examples of FTS Ca²⁺ responses in submucous neurons that were blocked by MRS-2179 are shown in Fig. 3A (time series of selected pseudocolor images displaying fluo-4 AM-Ca²⁺ fluorescence responses) and Fig. 3B (displaying the Ca²⁺ transients before and after MRS-2179).

![Fig. 2. Reproducibility of synaptic Ca²⁺ responses in submucous neurons.](image-url)

**Colocalization of P2Y₁R-ir in VIP-positive neurons.** Colabeling experiments revealed that 39% of VIP-ir neurons in the human SMP displayed strong ir for P2Y₁R (Fig. 3, D and E). An additional 30% of neurons were P2Y₁R⁺/VIP⁻, and 31% of
neurons were VIP+/P2Y$_1$R$^-$. An exhaustive immunochemical study of P2Y$_1$R distribution and chemical coding of submucous neurons was not attempted, as this was not the primary focus of our study.

**Functional viability of human submucous neurons.** Membrane depolarization and functional viability of human submucous neurons was assessed by K$^+$ depolarization and functional viability of human submucous neurons in intact M-SMP that served to identify them as neurons and their viability and ability to respond to stimulation. High K$^+$ causes membrane depolarization and functional viability of human submucous neurons in intact M-SMP that served to identify them as neurons and their viability and ability to respond to stimulation. High K$^+$ causes membrane depolarization and functional viability of human submucous neurons in intact M-SMP that served to identify them as neurons and their viability and ability to respond to stimulation. High K$^+$ causes membrane depolarization and functional viability of human submucous neurons in intact M-SMP that served to identify them as neurons and their viability and ability to respond to stimulation. High K$^+$ causes membrane depolarization and functional viability of human submucous neurons in intact M-SMP that served to identify them as neurons and their viability and ability to respond to stimulation. High K$^+$ causes membrane depolarization and functional viability of human submucous neurons in intact M-SMP that served to identify them as neurons and their viability and ability to respond to stimulation. High K$^+$ causes membrane depolarization and functional viability of human submucous neurons in intact M-SMP that served to identify them as neurons and their viability and ability to respond to stimulation. High K$^+$ causes membrane depolarization and functional viability of human submucous neurons in intact M-SMP that served to identify them as neurons and their viability and ability to respond to stimulation. High K$^+$ causes membrane depolarization and functional viability of human submucous neurons in intact M-SMP that served to identify them as neurons and their viability and ability to respond to stimulation. High K$^+$ causes membrane depolarization and functional viability of human submucous neurons in intact M-SMP that served to identify them as neurons and their viability and ability to respond to stimulation. High K$^+$ causes membrane depolarization and functional viability of human submucous neurons in intact M-SMP that served to identify them as neurons and their viability and ability to respond to stimulation. High K$^+$ causes membrane depolarization and functional viability of human submucous neurons in intact M-SMP that served to identify them as neurons and their viability and ability to respond to stimulation. High K$^+$ causes membrane depolarization and functional viability of human submucous neurons in intact M-SMP that served to identify them as neurons and their viability and ability to respond to stimulation. High K$^+$ causes membrane depolarization and functional viability of human submucous neurons in intact M-SMP that served to identify them as neurons and their viability and ability to respond to stimulation. High K$^+$ causes membrane depolarization and functional viability of human submucous neurons in intact M-SMP that served to identify them as neurons and their viability and ability to respond to stimulation. High K$^+$ causes membrane depolarization and functional viability of human submucous neurons in intact M-SMP that served to identify them as neurons and their viability and ability to respond to stimulation. High K$^+$ causes membrane depolarization and functional viability of human submucous neurons in intact M-SMP that served to identify them as neurons and their viability and ability to respond to stimulation. High K$^+$ causes membrane depolarization and functional viability of human submucous neurons in intact M-SMP that served to identify them as neurons and their viability and ability to respond to stimulation. High K$^+$ causes membrane depolarization and functional viability of human submucous neurons in intact M-SMP that served to identify them as neurons and their viability and ability to respond to stimulation. High K$^+$ causes membrane depolarization and functional viability of human submucous neurons in intact M-SMP that served to identify them as neurons and their viability and ability to respond to stimulation. High K$^+$ causes membrane depolarization and functional viability of human submucous neurons in intact M-SMP that served to identify them as neurons and their viability and ability to respond to stimulation. High K$^+$ causes membrane depolarization and functional viability of human submucous neurons in intact M-SMP that served to identify them as neurons and their viability and ability to respond to stimulation. 

**Synaptic Ca$^{2+}$ mechanisms.** The FTS-evoked Ca$^{2+}$ response in submucous neurons of the human jejunum was sensitive to blockade of nicotinic synaptic transmission by hexamethonium, blockade of Ca$^{2+}$-dependent synaptic transmission by high Mg$^{2+}$/low Ca$^{2+}$, or blockade of N-VOCC using Ω-conotoxin MVIIA (Fig. 4). In control experiments, repeated FTS Ca$^{2+}$ responses at 0, 5, 25, and 30 min caused only a very modest reduction in peak Ca$^{2+}$ response (<10%, Fig. 4A). Hexamethonium caused a 50% reduction in the FTS Ca$^{2+}$ response (Fig. 4B), and Ω-conotoxin blocked 70–80% of the response. High-Mg$^{2+}$/low-Ca$^{2+}$ perfusion caused an 80% suppression of the response that was reversible with washout (Fig. 4D).

**Blockade of the extrinsic and intrinsic pathways with capsaicin and TTX.** Capsaicin (10 μM) inhibited the FTS-evoked Ca$^{2+}$ response by ~25% in submucous neurons of the intact SMP (Fig. 5A). Blockade of nerve conduction with TTX could abolish FTS-evoked Ca$^{2+}$ responses (Fig. 5B). The distension-evoked I$\text{sc}$ response was reduced 29% by 10 μM capsaicin alone. Capsaicin, together with 1 μM TTX, reduced the I$\text{sc}$ response by 64% (Fig. 5C).

**MRS-1220 augments synaptic and distension-evoked responses in human intestine.** The human AdoA$_3$R-selective antagonist MRS-1220 alone at 1 or 10 μM concentrations could augment the FTS-Ca$^{2+}$ response (Fig. 4A).
selective AdoA3R antagonist MRS-1191 had no effect on synaptic Ca\(^{2+}\) responses; the synaptic Ca\(^{2+}\) response was not augmented by 1 μM MRS-1191 (38.2 ± 2.6 to 41.7 ± 4.1 pixels, \(P = 0.06\); 8 neurons) or 10 μM MRS-1191 (44.9 ± 3.1 to 49.7 ± 2.4 pixels, \(P = 0.081\); 43 neurons). MRS-1220 also enhanced the distension-evoked \(I_{sc}\) response by 31%, and enhancement was blocked and slightly further reduced by 1 μM TTX (Fig. 7B).

Inhibition of synaptic Ca\(^{2+}\) responses by activation of AdoA3Rs. The selective AdoA3R agonist 2-chloro-N\(^{6}\)-(3-iodobenzyl)adenosine-5’-N-methyl-carboxamide (2-Cl-IBMECA) (5 μM) could abolish the FTS-Ca\(^{2+}\) response at frequencies ranging from 0.5 to 25 Hz. At higher frequencies, 2-Cl-IBMECA was less effective, but significantly inhibited the FTS-Ca\(^{2+}\) response (Fig. 7A).

2-Cl-IBMECA caused a concentration-dependent inhibition of the 25-Hz FTS-evoked Ca\(^{2+}\) response. At a concentration of 5 μM 2-Cl-IBMECA, the Ca\(^{2+}\) response was nearly abolished. The apparent \(IC_{50}\) for 2-Cl-IBMECA inhibition of the FTS-evoked Ca\(^{2+}\) response is ~8.5 × 10\(^{-8}\) M (Fig. 7B).

The human AdoA3R selective antagonist MRS-1220 augmented the frequency-Ca\(^{2+}\) response at 1–35 Hz in human submucous neurons in response to FTS. The effect of MRS-1220 was greatest at 25 and 35 Hz. In contrast, in control experiments, vehicle instead of MRS-1220 did not augment the FTS-Ca\(^{2+}\) response (Fig. 7, C and D). In the presence of 5 μM 2-Cl-IBMECA, MRS-1220 could no longer augment the FTS-Ca\(^{2+}\) response (Fig. 7E).

It is possible that AdoA3Rs contribute to the inhibitory effect of the AdoA3R agonist 2-Cl-IBMECA, and, therefore, we carried out additional experiments in the presence of the irreversible AdoA3R antagonist 8-cyclopentyl-3-N-(3-[[3-(4-fluorosulphonyl)benzoyl]-oxy]-propyl)-1-N-propyl-xanthine (FSCPX) to knock down AdoA1R. FSCPX alone did not augment the FTS-Ca\(^{2+}\) response in submucous neurons (Fig. 7F). At a concentration of 1 μM FSCPX, which is sufficient to block all high-affinity AdoA1Rs (60), the AdoA3R agonist 2-Cl-IBMECA could still suppress the FTS-evoked Ca\(^{2+}\) response at frequencies ranging from 1 to 35 Hz (Fig. 7G).

EFS does not reveal an AdoA3R. EFS at 10 or 20 Hz was used to elicit a TTX-sensitive Cl\(^{-}\) secretory response in M-SMP. Knockdown of AdoA3Rs with FSCPX caused a modest but significant augmentation of the 20-Hz EFS-induced \(I_{sc}\) response in M-SMP; no effect was observed at 10 Hz. After knockdown, 2-Cl-IBMECA had no effect on the 10- or 20-Hz EFS-induced \(I_{sc}\) response in M-SMP (Fig. 8).

**DISCUSSION**

To our knowledge, this is the first study to provide proof that purinergic modulation of synaptic transmission occurs in the human SMP, which may be important in mucosal reflexes and distension reflexes (F. L. Christofi, unpublished observations; Ref. 16). Our study demonstrated that dual modulation of synaptic Ca\(^{2+}\) signaling occurs via activation of inhibitory AdoA3Rs or excitatory P2Y1Rs. The P2Y1/G\(\alpha/PLC/IP3/Ca\(^{2+}\) signaling is a major pathway in synaptic transmission of the huENS.

We developed a suitable approach to study synaptic transmission in the huENS. LSM Ca\(^{2+}\) imaging can be used to monitor synaptic Ca\(^{2+}\) responses evoked by electrical FTS of interganglionic connectives in the human SMP. Neurons in the intact ganglia are easily visualized by their fluo 4 fluorescence and are viable, responding with a robust Ca\(^{2+}\) transient to high-K\(^{+}\) depolarization and activation of VOCC or to FTS. Focal electrical stimulation of a neighboring interganglionic...
connective leads to a synaptic Ca\(^{2+}\) response in 64% of viable neurons in recorded ganglia; this implies that a majority of neurons within each ganglion receive significant synaptic inputs. The FTS response recorded in jejunum ganglia obtained from 61 different Roux-en-Y surgery cases involves neuronal conduction and synaptic transmission because it is sensitive to TTX, high Mg\(^{2+}\)/low Ca\(^{2+}\) solutions, \(\omega\)-conotoxin, and hexamethonium and displays inhibition to high-frequency stimulation. Previous work using calcium indicators revealed activity-dependent changes in intracellular calcium levels in response to electrical stimulation in both AH and S electrophysiological types of enteric neurons (4, 31, 35, 36, 55, 62). The FTS frequencies used in the present study are known to generate multiple action potentials associated with a transient elevation in Ca\(^{2+}\) levels. A high frequency or train stimulus (this study) would elicit a slow excitatory postsynaptic potential (EPSP) response with superimposed action potentials; the hexamethonium response observed would further imply that multiple fast nicotinic EPSPs contribute to the overall EPSP response. These fast EPSP responses are more easily recorded and distinguished by multsite optical recording techniques with a voltage-sensitive dye (53). Synaptic Ca\(^{2+}\) responses in the huENS are repeatable, reproducible, and quantifiable.

Synaptic transmission in human submucous ganglia. Our study provides proof for nerve conduction (TTX sensitivity), neurotransmission (high Mg\(^{2+}\)/low Ca\(^{2+}\), and \(\omega\)-conotoxin sensitivity), the types of neurotransmitters involved in neurotransmission (nucleotides, ACh), as well as the signaling pathways and receptors involved in synaptic transmission in the intact neural circuits of human submucous ganglia. High-Mg\(^{2+}\)/low-Ca\(^{2+}\) solutions remove Ca\(^{2+}\) involved in neuro-transmission in human submucous ganglia.

Fig. 5. Involvement of extrinsic and intrinsic neural pathways in synaptic and distension-evoked secretory responses. A: capsaicin caused a modest reduction in the FTS-evoked Ca\(^{2+}\) response \((n = 26, P < 0.001)\); B: sensitivity of the Ca\(^{2+}\) response to TTX \((n = 43, P < 0.0001)\). C: the distension-evoked short-circuit current \((I_{sc})\) response was reduced 29.3 \(\pm\) 7.1% by 10 \(\mu\)M capsaicin alone. 10 \(\mu\)M capsaicin plus 1 \(\mu\)M TTX reduced the \(I_{sc}\) response by 63.7 \(\pm\) 7.0% \([N = 5\) patients, 7 mucosa-SMP (M-SMP) tissues; \(P < 0.05\)].
transmission, or they would be acting to induce Ca\(^{2+}\)/H\(^{11001}\)-induced Ca\(^{2+}\)/H\(^{11001}\)-release from intracellular ryanodine-sensitive or IP\(_{3}\)-sensitive stores. N-type Ca\(^{2+}\)/H\(^{11001}\) channels that are sensitive to \(\omega\)-conotoxin are involved in the synaptic Ca\(^{2+}\)/H\(^{11001}\) response in human submucous neurons. At presynaptic sites, N-type Ca\(^{2+}\)/H\(^{11001}\) channels may play an important role in transmitter release (36, 63, 66). Therefore, the Ca\(^{2+}\)/H\(^{11001}\) response is synaptically driven, since it is sensitive to \(\omega\)-conotoxin. In guinea pig myenteric plexus, synaptic Ca\(^{2+}\)/H\(^{11001}\) transients were sensitive to the same manipulations of TTX, \(\omega\)-conotoxin, and high Mg\(^{2+}/\)low Ca\(^{2+}\) (4). N-type Ca\(^{2+}\)/H\(^{11001}\) channels could also be operating in the cell somas or enteric neurons. Action-potential-dependent Ca\(^{2+}\)/H\(^{11001}\) transients in the cell somas of enteric S neurons were shown to be blocked by \(\approx\)70% by \(\omega\)-conotoxin (55), and it has been suggested that \(\omega\)-conotoxin-resistant Ca\(^{2+}\)/H\(^{11001}\) responses may represent antidiromic activation of neurons, such as could occur with FTS.

**Species differences in synaptic Ca\(^{2+}\) signals between human and rat SMP.** In an earlier preliminary study, we characterized synaptic Ca\(^{2+}\) signaling pathways to electrical FTS in a rat preparation of SMP that is similar to human, at least anatomically, with several layers of submucous ganglia as in human (7). It is noteworthy that synaptic Ca\(^{2+}\) signaling mechanisms in the rat SMP differ significantly than those in the human SMP, stressing the importance of carrying out a study in human SMP, albeit a much more complex preparation. In rat SMP, unlike the human SMP, the synaptic Ca\(^{2+}\) response is mediated primarily by a cAMP-dependent mechanism and is blocked by a protein kinase A inhibitor. In human SMP (this study), the P2Y\(_{1}\)/Go\(_{q}\)/PLC/IP\(_{3}\)/Ca\(^{2+}\) signaling pathway predominates. In rat SMP, MRS-2179 could only block synaptic Ca\(^{2+}\) responses in 23% of neurons. Also different, nicotinic cholinergic transmission plays a minor role in synaptic Ca\(^{2+}\) responses in rat SMP. Such differences deserve further study in human SMP.

**Role of inhibitory AdoA\(_{3}\)R and excitatory P2Y\(_{1}\)Rs in synaptic transmission.** All previous work on purinergic regulation in the gut and gut reflexes was done in rodents (16, 18). The physiological role of AdoA\(_{3}\)Rs in the brain or ENS has been questioned (13, 21, 50) because the AdoA\(_{3}\)R is a low-affinity receptor that requires high/micromolar concentrations of eAdo for activation; high levels of eAdo occur at sites of inflammation, infection, and metabolic stress (22, 44, 48, 57, 58). Earlier studies showed that ongoing release of eAdo in micromolar
levels differentially affects excitatory and inhibitory transmission in the gut. These effects at high affinity A1 or non-A1/putative A2 or A3Rs (13) serve to complement the ability of Ado to shut down excitatory neural activity in gut microcircuits through its dual pre- and postsynaptic actions (9, 11, 19, 26).

In this study, activation of the AdoA3R inhibits synaptic transmission in the human SMP. Several lines of evidence support the hypothesis that AdoA3Rs are negatively coupled to synaptic transmission in the huENS. First of all, it is known that AdoA3R-ir is discretely localized in human submucous neurons (13). The IC50 concentration of the potent and selective AdoA3R agonist 2-Cl-IBMECA in suppressing the synaptic Ca2+ response was 8.5 × 10^{-8} M. To rule out the possibility that 2-Cl-IBMECA is acting at high-affinity AdoA1R (known to exist in submucous neurons) instead of AdoA3Rs, the irreversible AdoA1R antagonist FSCPX that forms a covalent bond with its receptor (60) was used to knock down AdoA1R. The ability of 2-Cl-IBMECA to suppress or abolish the synaptic Ca2+ response after knockdown and the antagonistic effect of the selective AdoA3R antagonist MRS-1220 strongly argue for an AdoA3R, not AdoA1R, in the 2-Cl-IBMECA effect. The potency of 2-Cl-IBMECA in human submucous neurons was 10-fold greater than that reported for guinea pig colonic enteric neurons (IC50 = 0.8 μM) in a model of secretory diarrhea, in which 2-Cl-IBMECA was able to suppress the dimaprit/H2-evoked cyclical coordinated pattern of motility and secretion (5). AdoA3R shows a species-specific distribution (40), pharmacology, and diversity of structure (41) that could explain the difference in potency of the AdoA3R agonist between human (this study) and guinea pig (5) submucous neurons. Other studies also identified an inhibitory limb of the neural motor reflex that may be activated by putative AdoA3Rs in rat distal colon, further arguing for a physiological role of AdoA3R in the ENS (3). In addition, non-A1/A2 inhibitory receptors that may represent putative AdoA3Rs gate excitability of enteric AH neurons (14). Our findings in normal rodent gut, as well as reports by others (25), suggest a physiological role of low micromolar levels of eAdo at AdoA3Rs in intact neural circuits. A functional role for IB-MECA and AdoA3Rs is also indicated in synaptic plasticity that represent the likely substrates for learning and memory (21) that may operate in AH/IPANs (18, 32, 65).

**Fig. 8.** Electrical field stimulation (EFS) reveals AdoA1R but not AdoA3Rs. A and B: EFS (10 or 20 Hz, 15, 0.5 ms, 30 s) increased the I_sc response in each of two M-SMP preparations from a Roux-en-Y specimen. Knockdown of AdoA3Rs with FSCPX (0.8 μM) augments the I_sc response, but the AdoA1R agonist 2-Cl-IBMECA (2.5 μM) does not inhibit the TTX-sensitive/neural-evoked I_sc response. C and D: pooled data reveal that EFS responses are TTX sensitive and, therefore, are mediated by activation of submucous neurons. FSCPX caused a significant augmentation of the I_sc response, but 2-Cl-IBMECA was without effect in the presence of FSCPX knockdown of AdoA1R (N = 4 patients; 6 M-SMP tissues at 10 Hz and 6 M-SMP tissues at 20 Hz EFS).
AdoA₃Rs are negatively coupled to nucleotide and cholinergic synaptic transmission. 2-Cl-IBMECA was most effective at lower frequencies of FTS, and, in the range of 0.5-25-Hz frequencies, activation of AdoA₃Rs could abolish synaptic transmission. At higher frequencies, it could only partially inhibit synaptic transmission, indicating an AdoA₃R-resistant component to synaptic transmission. We did not do an exhaustive analysis of the putative transmitters released at each of the frequencies that could be inhibited by AdoA₃Rs. However, at 25-Hz frequency, we can conclude that nucleotides (ATP or ADP) are a major contributor to the FTS synaptic Ca²⁺ response, since it could be blocked by a P2Y₁R antagonist. Nicotinic cholinergic transmission is involved in ~50% of responsive neurons that were sensitive to hexamethonium blockade of synaptic Ca²⁺ responses. Since 2-Cl-IBMECA could abolish the 25-Hz FTS response, it would follow that activation of AdoA₃Rs leads to suppression of purinergic and cholinergic transmission in the human SMP. At higher frequencies, 35-100 Hz, the identity of the transmitter(s) that is not sensitive to 2-Cl-IBMECA inhibition remains unknown. Also, we could not delineate whether effects of 2-Cl-IBMECA were at pre- and/or postsynaptic AdoA₃Rs. Both sites are possible, since AdoA₃R-ir has been identified on substance P varicose fibers and cell somas of SP or VIP neurons in rat and human intestine (F. L. Christofi, unpublished observations; Ref. 16). In rodent ENS, AdoA₃R-ir is expressed in both cell somas and processes of neurons, revealing their simple uniaxional morphology (Dogiel Type I) or their multipolar Dogiel Type II morphology (F. L. Christofi, unpublished observations; Ref. 16).

Our study provides strong pharmacological evidence for activation of AdoA₃R in intact neural circuits of the human SMP by eAdo; the AdoA₃R antagonist MRS-1220 alone that prefers the human AdoA₃R (but not a rodent selective antagonist MRS-1191) was effective in releasing an inhibitory influence of eAdo at AdoA₃R on synaptic transmission in human submucous ganglia. Collectively, this study in human submucous ganglia, our laboratory’s preliminary data in normal rodent gut (8), as well as reports by others (25), suggest a physiological role of low-micromolar levels of eAdo at AdoA₃Rs in intact neural circuits. A functional role for IBMECA and AdoA₃Rs is also suggested in synaptic plasticity that represents the likely substrates for learning and memory (29) that may operate in AH/IPANs (18, 32, 65). However, it is also very likely that these receptors are important in abnormal or disease states of the gut, given that low-affinity AdoA₃Rs can be more fully activated by high levels of Ado that can be released during disease and inflammation (22, 44, 48, 57, 58). AdoA₃Rs are subject to dysregulation in a profilin transgenic mouse with gut smooth muscle hypertrophy (F. L. Christofi and H. H. Hassani, unpublished observations), a rabbit Crohn’s/sleitis model (61), gut tissue placed in organotypic culture (F. L. Christofi, unpublished observations; Ref. 16), and 2,4,6-trinitrobenzene sulfonic acid colitis (30), and A₃K overexpression could be useful as a genetic therapy (13). Nerve terminals, neutrophils, and endothelial cells are known to release high levels of eAdo at sites of inflammation, infection, and metabolic stress that can activate the low-affinity AdoA₃R. Studies are underway to assess whether gut neural AdoA₃Rs behave differently in IBD (ulcerative colitis and Crohn’s disease).

Transgenic manipulation of A₃Rs provides a unique opportunity to study A₃Rs signaling and proof of physiological effect of A₃K agonists in gut reflexes (or protective effect in experimental colitis). Our current focus is on the use of transgenic A₃R knockout and profilin mouse models for the study of the physiological functions of the A₃R, including secretomotor function and synaptic transmission in intact neural circuits of the SMP, as well as the consequences of A₃R inhibition on animal models of inflammatory bowel disease (45).

The P2Y₁/Gqα/PLC/IP₃/Ca²⁺ signaling pathway in human submucous ganglia. AdoA₃Rs are, in general, inhibitory receptors in the ENS (F. L. Christofi, unpublished observations; Ref. 16). In contrast, the P2Y₁R is emerging as a significant target for nucleotides in excitatory modulation of EC cell secretion of 5-HT to trigger gut reflexes (8), excitatory purinergic signaling in mucosal stroking reflexes and synaptic transmission in submucous ganglia of the rodent intestine (12, 20), and slow synaptic transmission in SMP neurons (67). P2Y₁R are coupled to Gqα/PLC/IP₃/Ca²⁺ signaling in enteric neurons or EC cells (Refs. 8, 12, 16, 18, 20, 67).

In this study, we provide pharmacological evidence to support the hypothesis that P2Y₁/Gqα/PLC/IP₃/Ca²⁺ signaling is a predominant pathway involved in synaptic transmission in intact neural circuits of the human submucous nerve plexus. A P2Y₁-selective antagonist MRS-2179 prevents synaptic Ca²⁺ signaling in submucous ganglia, indicating that nucleotide release (ATP or ADP) during FTS activates postsynaptic excitatory P2Y₁R on the postsynaptic membrane of neurons to elevate intracellular free Ca²⁺ levels. The selective PLC inhibitor is effective in blocking ~80% of the synaptic Ca²⁺ response. Furthermore, recent evidence from detailed pharmacological and molecular signaling studies supports the hypothesis that modulation of 5-HT release from human ECs occurs via excitatory P2Y₁R coupled to the Gqα/PLC/IP₃/Ca²⁺ signaling pathway, leading to 5-HT release (68, 70). In parallel to this mechanism, during FTS in human submucous ganglia, calcium influx in neurons during synaptic activation and action potential generation involve α-conotoxin-sensitive N-type Ca²⁺ channels.

Our study provides pharmacological evidence for purinergic excitatory transmission via P2Y₁R. P2Y₁R-ir is expressed on 39% of submucous neurons with VIP, and another subpopulation of neurons expresses P2Y₁R but not VIP-ir (30% of neurons); the identity of these neurons remains unknown. In our laboratory’s previous study, it was shown that <10% of VIP neurons in the human SMP expressed AdoA₃R-ir, whereas most substance P immunoreactive neurons expressed AdoA₃R-ir (13). Therefore, it is likely that separate populations of submucous neurons exist with P2Y₁R and AdoA₃R, although colocalization studies would be necessary to provide direct proof. This was not technically feasible with the antisera available to us.

Mechanical or chemical activation of the mucosa can lead to an intestinal neural reflex and an increase in ion transport and fluid secretion. To date, only a few studies in rodents have investigated purinergic P2Y₁R regulation of secretomotor function (12, 17, 19, 20). Mucosal distortion by brush stroking or distension can elicit the reflex. P2Y₁-ir was identified in a majority of VIP, nitric oxide synthase, calretinin, neuropeptide Y, or somatostatin neurons, but not SP or calbindin submucous neurons (12). Mucosal touch/distension-evoked fluo 4/Ca²⁺
responses in submucous neurons were also inhibited by apyrase or blocked completely by MRS-2179; MRS-2179 only reduced I_A in stroking reflexes. It was concluded from those studies that several nucleotides may contribute to mechanically evoked secretomotor reflexes, including P2Y_Rs. It is likely that purinergic (P2Y_R) transmission in human gut is also involved in secretomotor reflexes, but this awaits proof.

Distension reflexes, EFS, and intrinsic/extrinsic nerves in the AdoA3R responses. In the M-SMP preparation, distension-evoked mucosal secretion (i.e., I_E/chloride secretion) involves both intrinsic (TTX-sensitive) and extrinsic (capsaicin-sensitive) nerve fibers. Distension-evoked secretion is augmented by the human selective AdoA3R antagonist MRS-1220, lending support to the concept that distension releases sufficient Ado or a related nucleotide precursor (i.e., AMP, ADP, ATP) that breaks down to Ado to activate a low-affinity AdoA3R. Extrinsic nerves with sensitivity to capsaicin are minor contributors to the synaptic Ca^{2+} response in submucous neurons; these are presumed to be nerve fibers that remain intact in the ganglia after acute microdissection of the M-SMP from human jejunum. The effect of MRS-1220 is blocked completely by TTX, indicating that the AdoA3R mechanism is restricted to intrinsic nerves in the human SMP.

Interestingly, when EFS stimulation was used to evoke a neural I_E/chloride secretory response, no AdoA3R could be revealed by using an AdoA3R-selective agonist 2-Cl-IB-MECA. EFS activates all neurons in the neural circuits of the SMP indiscriminately and in both the ortho- and antidromic directions, making it more difficult to reveal an inhibitory AdoA3R component at 10- or 20-Hz stimulation frequency. EFS did reveal an AdoA3R inhibitory component at 20 Hz with the AdoA3R antagonist FSCPX, suggestive of sufficient eAdo release to activate this high-affinity receptor. In contrast, FTS could not reveal an AdoA3R component. However, FTS stimulation of an internodal strand activates a more discrete synaptic pathway and can reveal a prominent AdoA3R component to synaptic transmission. In the intact mucosa-submucosa, AdoA3Rs are located on both ECs (sites of 5-HT release to trigger gut mucosal reflexes) and submucous neurons. In human EC cells, eAdo is sufficient to provide ongoing inhibition of basal and mechanically evoked 5-HT release via AdoA3Rs (8). Another possibility to explain the lack of AdoA3R functional responses in synaptic transmission is the potential downregulation or internalization of the receptor during the postsurgical period and processing of the tissue: from tissue removal in the operating room to Ca^{2+} imaging, it takes ~5 h, and, during this time, the AdoA3R could undergo changes in expression. Furthermore, it is know that the A1 becomes internalized after activation, and it takes up to 24 h for trafficking back to the membrane. In fact, we found that, in human EC cells, we could reveal an A1 inhibition response only after protection of the A1R from activation and internalization by eAdo by blocking it with an A1 antagonist overnight (8).

Conclusions. Collectively, data in human EC cells and the intact neural circuits of the human SMP (this study) support the unified hypothesis that inhibitory AdoA3Rs and excitatory P2Y_Rs modulate mucosal reflexes by acting at receptors in the EC-neural secretomotor reflex arc (8, 16, 18). In this study, 2-Cl-IB-MECA or eAdo can activate AdoA3Rs to suppress distension reflexes, as well as synaptic cholinergic and purinergic transmission in the human SMP. Nucleotides released during electrical stimulation of internodal strands release neurotransmitters that activate a major excitatory P2Y_R/Gq/PLC/ IP3/Ca^{2+} signaling pathway and N-type Ca^{2+} channels in the postsynaptic neurons; these mechanisms are important for communication between neurons in the “little brain” of the human gut.

REFERENCES


