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Neuropharmacology of purinergic receptors in human submucous plexus: Involvement of $P2X_1$, $P2X_2$, $P2X_3$ channels, P2Y and A_3 metabotropic receptors in neurotransmission

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ABSTRACT

Rationale: The role of purinergic signaling in the human ENS is not well understood. We sought to further characterize the neuropharmacology of purinergic receptors in human ENS and test the hypothesis that endogenous purines are critical regulators of neurotransmission.
Experimental approach: LSCM-Fluo-4-(Ca^{2+})-imaging of postsynaptic Ca^{2+} transients (PSCaTs) was used as a reporter of neural activity. Synaptic transmission was evoked by fiber tract electrical stimulation in human SMP surgical preparations. Pharmacological analysis of purinergic signaling was done in 1520 neurons from 241 separate ganglia, 104 patients; immunochemical labeling for P2XRs of neurons in ganglia from 19 patients. Real-time MSORT (Di-8-ANEPPS) imaging was used to test effects of adenosine on fast excitatory synaptic potentials (fEPSPs).
Results: Synaptic transmission is sensitive to pharmacological manipulations that alter accumulation of extracellular purines. Apyrase blocks PSCaTs in a majority of neurons. An ecto-NTPDase-inhibitor 6-N,N-diethyl-D-β,γ-dibromomethyleneATP or adenosine deaminase augments PSCaTs. Blockade of reuptake/deamination of eADO inhibits PSCaTs. Adenosine inhibits fEPSPs and PSCaTs (IC_{50} = 25 μM), sensitive to MRS1220-antagonism (A_3AR). A P2Y agonist ADPβS inhibits PSCaTs (IC_{50} = 111 nM) in neurons without stimulatory ADPβS responses (EC_{50} = 960 nM). ATP or a $P2X_{1,2,2/3}$ (α,β-MeATP) agonist evokes fast, slow, biphasic Ca^{2+} transients or Ca^{2+} oscillations (EC_{50} = 400 μM). PSCaTs are sensitive to $P2X_1$ antagonist NF279. Low (20 nM) or high (5 μM) concentrations of P2X antagonist TNP-ATP block PSCaTs in different neurons; proportions of neurons with P2XR-ir follow the order $P2X_2 > P2X_1 >> P2X_3$; $P2X_1 + P2X_2$ and $P2X_3 + P2X_2$ are co-localized. RT-PCR identified mRNA-transcripts for $P2X_{1\text{-}7}$,$P2Y_{1,2,12-14}R$. Responsive neurons were also identified by HuC/D-ir.
Conclusions: Purines are critical regulators of neurotransmission in the human enteric nervous system. Purinergic signaling involves $P2X_1$, $P2X_2$, $P2X_3$ channels, $P2X_1 + P2X_2$ co-localization and inhibitory P2Y or A_3 receptors. These are potential novel therapeutic targets for neurogastroenterology.
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Abbreviations: ARL67156, 6-N,N-diethyl-D-β,γ-dibromomethyleneATP; ATP, adenosine 5′-triphosphate; Ca^{2+}, calcium; $[Ca^{2+}]_i$, intracellular free calcium concentration; cDNA, cyclic deoxyribonucleic acid, eADO; endogenous adenosine, EHNA; erythro-9-Amino-β-hexyl-α-methyl-9H-purine-9-ethanol hydrochloride, ENS; enteric nervous system, fEPSPs; fast excitatory synaptic potentials, FITC; Fluorescein isothiocyanate, FTS; fiber tract stimulation, h; hour, $[Ca^{2+}]_i$; intracellular free Ca^{2+} levels, IBD; inflammatory bowel diseases, IBS; irritable bowel syndrome, IP_3; inositol-1,4,5-triphosphate, LSCM; laser scanning confocal microscope, mRNA; messenger ribonucleic acid, MSORT; multi-site optical recording technique, nAChR; nicotinic ACh receptor, NBTI; N^6-nitrobenzyl-thioinosine, PSCaT; postsynaptic calcium transient, PBS; phosphate buffer solution, R(s); receptor(s), RT-PCR; reverse transcription-polymerase chain reaction, SB218795, (*R*)-[[2-Phenyl-4-quinolinyl)carbonyl]amino]-methyl ester benzeneacetic acid; sec, second; SEM, standard error of the mean; TNP-ATP, 2′3′-o-(2,4,6-trinitrophenyl)-ATP; TK, tachykinin.

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1. Introduction

The concept of purinergic signaling stems from studies that were designed to identify the non-adrenergic, non-cholinergic (NANC) inhibitory neurotransmitter in the gut (Burnstock et al., 1972). Purine receptors are broadly classified as P1 for nucleosides (adenosine) and P2 for nucleotides (ATP). They can be subdivided into adenosine (A_1,A_{2A},A_{2B},A_3), $P2X_{1-7}$ and $P2Y_{1,2,4,6,11\text{-}14}$. Most, if not all of these receptors, are expressed in the gastrointestinal tract where they are known to be involved in the physiological regulation of gut reflexes in animal models (Burnstock, 2008, 2012; Christofi, 2008). For example, purine release from enterochromaffin cells (EC) modulates serotonin release that triggers gut neural reflexes (Liñán-Rico et al., in press). ATP is also released from epithelial cells to influence gut reflexes (Burnstock, 2008, 2009). In animal models, purines (ATP, ADP, AMP, β-NAD^+, ADP ribose) are potential transmitter(s) in the enteric nervous system (ENS) and gut reflexes, and are known to act at all levels of gut motor reflexes (Burnstock, 2008, 2012; Christofi et al., 2004; Cooke et al., 2004; Christofi, 2008; Gomes et al., 2009; Gulbransen and Sharkey, 2009; Gulbransen et al., 2012). Emerging evidence supports the concept that purine nucleotides other than ATP such as β-NAD^+ or its metabolite ADP ribose are involved in $P2Y_1$-mediated inhibitory neuromuscular transmission in rodents, primates and humans (Durnin et al., 2012; Gallego et al., 2011; Hwang et al., 2011, 2012). Adenosine, a metabolite of ATP, acts as an inhibitory neuromodulator in the ENS (via A_1 AR or A_3AR; Bozarov et al., 2009; Christofi, 2001, 2008). In contrast to neuromuscular transmission, our knowledge of purinergic signaling between neurons in the human ENS for adenosine or nucleotides is rather limited.

It is difficult to translate data on purinergic signaling mechanisms and neural receptors identified in animal models to human ENS, since species differences are known to exist in neurochemistry, neurophysiology, and receptor pharmacology of enteric neurons (Breunig et al., 2007; Burnstock, 2012; Furness et al., 1995; Schemann et al., 2002; Schemann and Neunlist, 2004; Timmermans et al., 2001). Our earlier studies identified species differences in purinergic receptors. The difference was that A_3, but not A_1, adenosine receptors can be identified in human ENS, whereas both functional receptors are present in guinea pig (Bozarov et al., 2009; Christofi and Wood, 1993; Wunderlich et al., 2008). Marked species differences in the distribution of $P2X_2$ and $P2X_3$ receptors exist in mouse, guinea-pig, rat and human (Van Nassauw et al., 2002; Yiangou et al., 2001; Xiang and Burnstock, 2006; Poole et al., 2002; Ren et al., 2003). In addition, mouse and guinea pig P2X receptors are known to have different pharmacological properties (Burnstock, 2012).

Neuroimaging techniques for monitoring intracellular free Ca^{2+} levels ($[Ca^{2+}]_i$) or multi-site optical recording (MSORT) of membrane potential (V_m) have made it possible to perform in vitro neuropharmacology studies in human submucous plexus (a component of the ENS) from surgical tissue or biopsy, and identified neuronal receptors for histamine (H1–H4), serotonin (5-HT_3), proteases (PAR-2), purine nucleotides (e.g. $P2Y_1$) and noradrenaline in surgical tissues (Cirillo et al., 2013; Michel et al., 2011; Neunlist et al., 1999; Schemann et al., 2010; Wunderlich et al., 2008;). In contrast to animals, little information exists on receptor subtypes or purinergic signaling mechanisms of activation/inactivation in the human ENS (other than a single study by our group; Wunderlich et al., 2008). A better understanding of the neuropharmacology of purinergic receptors and signaling pathways is essential, as they are potential therapeutic targets for Inflammatory Bowel Diseases, Irritable Bowel Syndrome and diarrheal disorders (Antonioli et al., 2008; Bozarov et al., 2009; Burnstock, 2008; Cockayne et al., 2000; Eltzschig et al., 2012; Gulbransen et al., 2012; Guzman et al., 2006; Liñán-Rico et al., in press; Ren et al., 2011).

We further tested the general hypothesis that endogenous purines are key regulators of neurotransmission in the human submucous plexus, acting at ATP-gated P2X ion-channels, metabotropic P2Y or P1 receptors, as they are in animal models. Experiments were done using in vitro micro-dissected submucous plexus (SMP) from surgical cases. Ca^{2+} neuroimaging was used to study purinergic synaptic transmission by pharmacological manipulation with selective drugs (agonists or antagonists) for P2X-ion channels, metabotropic P2Y and A_3 receptors, as well as enzymes or inhibitors that either increase or decrease extracellular levels of endogenous purines. Responsive neurons were identified by their immunoreactivity for HuC/D, response to high K^+ depolarization, size/shape and location; protein and mRNA expression was used to identify receptors. Novel findings support the hypothesis that endogenous purines are critical regulators of neurotransmission in human ENS acting at $P2X_1$, $P2X_2$, $P2X_3$ channels, as well as inhibitory P2Y or A_3 receptors. Purine nucleotides and adenosine, together with tachykinin(s) and ACh are key regulators of neurotransmission or co-activation in human SMP.

2. Materials and methods

2.1. Chemicals

Drugs were purchased from either Sigma-Chemical Company (ARL67156, ATP, adenosine, EHNA, TNP-ATP, suramin, ADPβS, PPADS, apyrase, hexamethonium, adenosine deaminase, NF279) or Tocris (α,β-Me-ATP, MRS1220, MRS2179, SB218795) and 1–10 mM stocks were dissolved in distilled water or DMSO (final concentration ≤0.1%) and diluted into Krebs buffer. Drugs >0.2 mM concentrations were adjusted for pH if needed.

2.2. Institutional IRB approval of tissue procurement and LSCM Fluo-4 AM Ca^{2+} imaging in human SMP

In vitro Ca^{2+} imaging was conducted on freshly procured surgical tissue from minimal invasive Roux-en-Y gastric-bypass after patient consent as outlined in IRB protocols 2004H0165 and 2012H0231 approved at The Ohio State University. The surgical specimen was placed directly into 4 °C oxygenated Krebs solution and the time elapsed from tissue removal from the patient to the laboratory bench was kept to ~15 min. For SMP dissections in the lab, whole-thick tissue was placed in oxygenated Krebs buffer at room temperature and micro-dissected to remove mucosa, after initial blunt dissection of muscular layers with attached myenteric plexus (to remove longitudinal muscle – myenteric plexus and attached circular muscle). The SMP with intact networks of ganglia was cut into ~1 cm^2 pieces for loading with Ca^{2+} indicator to run several experiments (Wunderlich et al., 2008).

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 at room temperature to cleave the AM. The time that elapsed from tissue procurement to neuroimaging was ~5 h. Ca^{2+} imaging was done using a modified-custom fitted Zeiss LSCM 410/REN laser scanning confocal imaging system. A solution in-line heater was used to maintain perfusion temperature at 36.5 °C ± 0.5 °C at a perfusion-rate of 9 ml/min. Time-series analysis of $[Ca^{2+}]_i$ at 1-sec intervals was done in Fluo-4/AM loaded submucous neurons imaged through a 40× oil immersion apofluor objective (numerical aperture 1.3, working distance = 170 μm). Ca^{2+} imaging was carried out using an Ar–Kr laser to excite the cells at 488 nm, and fluorescence emissions were passed through a FT510 dichroic mirror and collected through a photomultiplier tube equipped with a BP 505–550 filter, positioned in front of pinhole and light path. Scanned optical sections had a thickness of 17.5 μm. Fig. 1 describes the recording technique for fiber tract stimulation (FTS) to evoke a synaptic response observed as a post-synaptic Ca^{2+} transient (PSCaT). After an equilibration period, a 3-s FTS at 0.1–100 Hz could be applied by a 25-μm Teflon coated platinum electrode (at 50 V, 400 μs). The effects of superfusion of agonists, antagonists, enzymes or enzyme inhibitors on basal or FTS-evoked PSCaTs were assessed before and 10–30 min after treatment. TTX, high Mg^{2+}/low Ca^{2+} Krebs or hexamethonium were used to confirm synaptic transmission. At the end of each experiment, the recorded ganglion was exposed to modified Krebs buffer containing 75 mM high-potassium (K^+). Neurons were included in the Ca^{2+} analysis if they could respond

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