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Review

Purinergic signalling in the enteric nervous system (An overview of current perspectives)

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ABSTRACT

Purinergic Signalling in the Enteric Nervous System involves the regulated release of ATP (or a structurally-related nucleotide) which activates an extensive suite of membrane-inserted receptors (P2X and P2Y subtypes) on a variety of cell types in the gastrointestinal tract. P2X receptors are gated ion-channels permeable to sodium, potassium and calcium. They depolarise cells, act as a pathway for calcium influx to activate calcium-dependent processes and initiate gene transcription, interact at a molecular level as a form of self-regulation with lipids within the cell wall (e.g. PIP_2) and cross-react with other membrane-inserted receptors to regulate their activity (e.g. nAChRs). P2Y receptors are metabotropic receptors that couple to G-proteins. They may release calcium ions from intracellular stores to activate calcium-dependent processes, but also may activate calcium-independent signalling pathways and influence gene transcription. Originally ATP was a candidate only for NANC neurotransmission, for inhibitory motoneurons supplying the *muscularis externa* of the gastrointestinal tract and bringing about the fast IJP. Purinergic signalling later included neuron–neuron signalling in the ENS, via the production of either fast or slow EPSPs. Later still, purinergic signalling included the neuro-epithelial synapse—for efferent signalling to epithelia cells participating in secretion and absorption, and afferent signalling for chemoreception and mechanoreception at the surface of the mucosa. Many aspects of purinergic signalling have since been addressed in a series of highly-focussed and authoritative reviews. In this overview however, the current focus is on key aspects of purinergic signalling where there remains uncertainty and ambiguity, with the view to stimulating further research in these areas.

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1. Introduction

Of the many fields of scientific endeavour addressed in this Special Issue, *Purinergic Signalling in the Enteric Nervous System* has arguably occupied the attention of researchers for the longest period of time. Accordingly, the historical development of this field is a long and complex story—taking as its beginning a proposal in 1970 that "*adenosine triphosphate or a related nucleotide*" was released by inhibitory nerves supplying the external layers of smooth muscle in the gastrointestinal tract (Burnstock et al., 1970). Thereafter, the term "*purinergic signalling*" entered the lexicon of neuro-transmission in 1971 (Burnstock, 1971).

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One might imaginatively extend the historical timeline to a beginning in 1963, when inhibitory junction potentials (IJPs) in response to electrical stimulation of intrinsic nerves were first observed in gastrointestinal smooth muscle (Burnstock et al., 1963). These IJPs, as well as their associated relaxation, were classed pharmacologically as non-adrenergic and non-cholinergic (NANC) (Burnstock et al., 1964, 1966). From their initial characterisation, it took a further 7 years before ATP was proposed as a neurotransmitter candidate for the fast IJP.

The fast hyperpolarizing action of intrinsic inhibitory nerves bore no resemblance (pharmacologically or temporally) to the slow hyperpolarizing action of extrinsic sympathetic (adrenergic) nerves to the smooth muscle of the gut (Gillespie, 1962a), nor any resemblance to the depolarising action of extrinsic parasympathetic (cholinergic) nerves to the same smooth muscles (Gillespie, 1962b). Furthermore, the smooth muscle IJP bore no resemblance to the cardiac pacemaker "IJP" (more correctly called the "inhibitory potential"), which is mediated by cholinergic vagal nerves and blocked by atropine (Del Castillo and Katz, 1955). Instead, NANC inhibition of the gut has become linked to purinergic signalling, as a new form of synaptic transmission in the mammalian and non-mammalian nervous systems (Burnstock et al., 1970, 1972). Yet, the characteristics of purinergic signalling in the muscularis externa of the gastrointestinal tract did not always sit comfortably with every example of NANC inhibition (Furness and Costa, 1973). In particular, purinergic signalling alone did not wholly account for NANC inhibition mediated by the vagal nerves to the stomach or by pelvic nerves to the colon and adjacent accessory muscles of defecation (anococcygeus and rectococcygeus muscles). Thus purinergic signalling is not the only form of NANC transmission in the gastrointestinal tract, but also includes inhibitory transmission by intrinsic motoneurons releasing nitric oxide (NO), vasoactive intestinal polypeptide (VIP), carbon monoxide (CO) and hydrogen sulphide (H_2S) (Farrugia and Szurszewski, 2014; Matsuda and Miller, 2010; Van Geldre and Lefebvre, 2004). It is too early to say how these inhibitory factors fully interact at a molecular level in neuro-effector tissues. Furthermore, the role of neuronal P2 receptors in releasing non-purinergic inhibitory transmitters requires further consideration.

2. ATP as an inhibitory transmitter

There was muted acclaim for ATP as the first non-classical transmitter candidate in the enteric nervous system. Initially, purinergic signalling was considered metabolically too costly to waste the universal energy currency of the cell on exocytosis. However, this objection and others were answered and a case firmly established for purinergic signalling in the gut (for evidential reviews, see: Burnstock, 2008, 2012; Burnstock et al., 2010). Today, the consensus of opinion is in favour of ATP (or a related nucleotide) acting primarily on P2Y1 receptors to mediate the fast IJP (for evidential reviews, see: King, 2012.; Goyal et al., 2013; Burnstock, 2014). P2Y1 transcripts are heavily expressed in the human gut (Janssens et al., 1996) and P2Y1-immunopositive material is present in the muscularis externa of rat gut (Van Crombruggen et al., 2007), as well as both the muscularis externa and myenteric plexus in the murine gut (Giaroni et al., 2002; Zhang et al., 2010). The fast IJP is blocked by P2Y1-selective antagonists, with an observed activity order of MRS2500 > MRS2279 > MRS2179 (Grasa et al., 2009). The fast IJP is absent in P2Y1$^{-/-}$ knockout mice, with concomitant loss of inhibitory activity by the selective P2Y1 agonist, MRS2365 (Gallego et al., 2012; Hwang et al., 2012). By contrast, the fast IJP is still present in nNOS$^{-/-}$ mice and therein inhibited by MRS2179 (Zhang et al., 2010).

Those early words "*adenosine triphosphate or a related nucleotide is the transmitter substance released by non-adrenergic inhibitory nerves*" were prophetic in many ways (Burnstock et al., 1970). The pharmacological characterisation of several cloned P2Y1 isoforms revealed that commercially-available ADP is just as efficacious as ATP (Filtz et al., 1994; Gao et al., 2006; Simon et al., 1995; Webb et al., 1993), whereas purified ADP was found to be a full agonist and purified ATP an antagonist at human P2Y1 (Léon et al., 1997). Ultimately, the pharmacological activity of nucleotides appears to depend more on whether they act as a full or partial agonist (rather than nucleotide purity) and on the receptor reserve in a particular cell (Palmer et al., 1998). Accordingly, ADP is believed to be a full agonist while ATP is a partial agonist at P2Y1 (Jacobson et al., 2015; Palmer et al., 1998).

As a further complication, the crystal structure of the human P2Y1 reveals two separate ligand-binding sites: one identified by the nucleotide antagonist, MRS2500, and another identified by the non-nucleotide antagonist, BPTU (Zhang et al., 2015). Both MRS2500 and BPTU inhibit the binding of radiolabelled [^{3}H]-2MeSADP, but it is too early to say how this occurs, or how ADP and ATP may interact with the nucleotide binding site. The BPTU site appears to occupy an allosteric binding site in a hydrophobic section of the P2Y1 molecule; it may be assumed that nucleotides cannot easily access this hydrophobic region. Additionally, a cross-comparison of crystal structures reveals fundamental differences between the ADP-activated human P2Y1 and P2Y12 (Zhang et al., 2014, 2015). For example, nucleotide and non-nucleotide antagonists bind in one of two ways to a region close to the agonist binding pocket at P2Y12 (Zhang et al., 2014), whereas two separate binding pockets exist for nucleotide and non-nucleotide antagonists at P2Y1 (Zhang et al., 2015). Accordingly, SAR studies for new and selective antagonists will have to acknowledge the existence of two subfamilies of P2Y receptors and eschew cross-comparison of the pharmacology of all the known ADP-activated P2Y subtypes.

3. ADP/ATP versus β-NAD$^+$/ADPr

There has been growing clamour for a related nucleotide (which is neither ADP nor ATP) as the mediator of the fast IJP. This related substance may be beta-nicotinamide adenine dinucleotide (β-NAD$^+$), or its CD38-generated bioactive metabolite, adenosine 5′-diphosphate ribose (ADPr) (Mutafova-Yambolieva et al., 2007; Durnin et al., 2012, 2013). Both β-NAD$^+$ and ADPr are agonists of recombinant P2Y1 isoforms, although weaker agonists compared to the potency of ADP/ATP (Gustafsson et al., 2011; Mutafova-Yambolieva et al., 2007). The inhibitory activity of β-NAD$^+$ is either unaffected, reduced or abolished in wild-type mice by the P2Y1 antagonist, MRS2500, and similarly unaffected, reduced or abolished in P2Y1$^{-/-}$ knockout mice (Gallego et al., 2012; Gil et al., 2013; Hwang et al., 2012). These troubling results with P2Y1 antagonists and in P2Y1$^{-/-}$ knockout mice cast doubt over a precise role for β-NAD$^+$/ADPr and its mode of action (Goyal, 2011; Goyal et al., 2013). Apart from P2Y1, β-NAD$^+$ may also activate A1 receptors in gut smooth muscle and in the presence of MRS2500 (Wang et al., 2015).

Based on the analysis of a series of pharmacological experiments, complementary lines of argument have been used as support for β-NAD$^+$/ADPr, and against ADP/ATP, as the principal transmitter for the fast IJP. Non-selective P2 receptor antagonists (PPADS and suramin), as well as a P2Y1-selective antagonist (MRS2179), block the fast IJP and hyperpolarisations to β-NAD$^+$, but not hyperpolarisations to ATP (Mutafova-Yambolieva et al., 2007; Hwang et al., 2012). A more potent P2Y1 antagonist, MRS2500, also blocked the fast IJP in wild-type mice (Gallego et al., 2012; Hwang et al., 2012), whereas MRS2500 failed to block hyperpolarisations to ADP and ATP in both wild-type and P2Y1$^{-/-}$ knockout mice (Hwang et al., 2012). The most sparing explanation for these observations is that β-NAD$^+$/ADPr may indeed activate P2Y1 receptors (as well as A1 receptors), but that ADP/ATP may additionally activate P2 receptors on intrinsic inhibitory motoneurons to release one or more non-purinergic inhibitory transmitters. This conclusion neither identifies β-NAD$^+$/ADPr, nor supplants ADP/ATP, as the principal candidate for purinergic signalling. An answer to this problem will certainly require further research. Also, research along these lines must include Up4A (uridine adenosine tetraphosphate), which has been proposed to be yet another candidate for purinergic signalling in the ENS (Durnin et al., 2014; Mutafova-Yambolieva and Durnin, 2014).