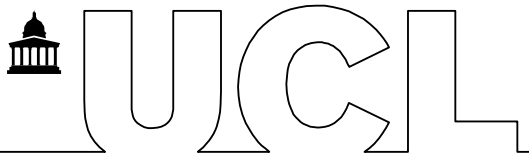

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Purinergic signalling in the enteric nervous system (An overview of current perspectives)

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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 epithelial 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.

Keywords: ATP, nonadrenergic noncholinergic (NANC), ENS, Neurotransmission, ATP release, P2Y receptor, P2X receptor

Contents

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). 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 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, non-cholinergic non-adrenergic (NANC) inhibition of the gut has become linked to purinergic signalling, as 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 (Leon 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 receptors (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 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., 2011). 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).

4. ATP as a fast excitatory transmitter in the ENS

Evidence has accrued for purinergic transmission in the ENS. Numerous P2X and P2Y receptor subtypes occur on enteric motoneurons (Van Crombruggen et al., 2007) and locally-applied ATP may evoke fast and slow depolarisations in identified inhibitory motoneurons, presumptive evidence for the involvement of ion-channels (P2X) and metabotropic receptors (P2Y), respectively (Thornton et al., 2013).

ATP opened P2X ion-channels in cultured myenteric neurons (in 90% tested) and evoked PPADS-sensitive fast EPSCs in myenteric neurons (Barajas-Lopez et al., 1996; Zhou and Galligan, 1996). In intact ganglia, electrical stimulation of nerve tracts elicited suramin-sensitive fast EPSPs in myenteric S/type I neurons (Galligan and Bertrand, 1994). Subsequently, suramin-sensitive fast EPSPs were identified in anally-projecting myenteric neurons in descending inhibitory pathways (Johnson et al., 1999). Mucosally-applied ATP caused a PPADS-sensitive activation of myenteric AH/Type II neurons (in 74% tested), most of which were also activated by mucosally-applied 5-HT (Bertrand and Bornstein, 2002).

About 25% of enteric neurons are strongly immunopositive for the P2X2 subunit, which is present in inhibitory motoneurons (NOS-positive) and intrinsic primary afferent neurons (Calbindin-positive IPANs), as well as secretomotor neurons to the mucosa (VIP-positive) (Castelucci et al., 2002). The P2X3 subunit was found to a lesser extent in inhibitory motoneurons (NOS-positive), but also in neurons which were phenotypically different from P2X2-containing cells (e.g. Calretinin-positive interneurons) (Poole et al., 2002). The P2X5 subunit is present in enteric neurons, with co-localisation of P2X5-IR with NOS-IR, Calbindin-IR and VIP-IR (Ruan and Burnstock, 2005). Lately, it was shown that cultured myenteric neurons were strongly immunopositive for the P2X4 subunit (Nieto-Piscador et al., 2013), although P2X4-IR is not present in intact ganglia and found only in tissue-resident macrophages (Yu et al., 2010). On the other hand, AH/type II enteric neurons in intact ganglia were strongly immunopositive for the P2X6 subunit, which was colocalised with Calbindin-IR and Calretinin-IR (Yu et al., 2010).

Based on their operational and pharmacological profiles, attempts have been made to identify the P2X receptor subtypes in enteric neurons. Early work suggested that the P2X2-like receptor was highly prominent in enteric neurons (Zhou and Galligan, 1996), whereas a comparable study around the same time suggested that P2X4-like receptor was present instead (Barajas-Lopez et al., 1996). At that time, there was a limited range of pharmacological tools to identify P2X receptor subtypes and, accordingly, there remains doubt over the major P2X receptor subtypes present in the ENS based on pharmacological profiling. Progress has been made with highly-selective antagonists for a few key P2X subtypes (P2X1, P2X3, P2X2/3 and P2X7) (for evidential reviews, see: Felix et al., 2012; Ochoa-Cortes et al., 2014).

The activation and inactivation characteristics of P2X mediated responses in enteric neurons have been used to infer the presence of particular P2X receptor subtypes. This approach is open to misinterpretation, because cell membrane lipids such as PIP2 may dock with P2X receptors and considerably modify channel properties (Fujiwara and Kubo, 2006; Zhao, et al., 2007; Bernier et al., 2008a,b; Mo et al., 2010; Ase et al., 2010; Hansen, 2015]. In this respect, PIP2 levels themselves are affected by GPCR activation and, accordingly, by the recent synaptic history of the host neuron. Additionally, the efficacy of known allosteric

modulators of P2X receptors may change according to P2X receptor density (Clyne et al., 2003) and with the impact of PIP2 on P2X ion-channel function (Mo et al., 2009).

P2X receptors may cross react with other ligand-gated ion-channels in enteric neurons: nACHRs (Decker and Galligan, 2009), GABAA (Karanjia et al., 2006) and 5-HT3 (Barajas-Lopez et al., 2002). P2X receptor activation may blunt the response of non-purinergic receptors and, conversely, prior activation of non-purinergic receptors may alter the amplitude and time-course of P2X mediated responses. Again, the recent synaptic history may have a bearing on the availability and operational profile of enteric P2X receptors.

Currently, it may be said with a degree of confidence that phenotypically-distinct enteric neurons contain different suites of P2X receptors. Most likely, enteric neurons contain a combination of homomeric and heteromeric P2X receptors. Homomeric P2X2 and P2X3 receptors have been functionally characterised in heterologous expression systems and P2X2- and P2X3-like receptors are present in the ENS (Galligan and North, 2004). Heteromeric P2X2/3, P2X2/5 and P2X2/6 receptors have been functionally characterised in heterologous expression systems and in some native tissues, and also may be present in the ENS (Compan et al., 2012; Hausmann et al., 2012). Purinergic signalling still occurs in the ENS, without serious impairment of gastrointestinal motility, after gene deletion for P2X2 and P2X3 subunit proteins (Bian et al., 2003; Ren et al., 2003; Devries et al., 2010).

5. ATP as a slow excitatory transmitter in the ENS

ATP caused a slow depolarisation and nerve tract stimulation caused a slow EPSP and the discharge of action potentials in S/Type I myenteric neurons, ostensibly through the inactivation of a calcium-dependent potassium conductance (Katayama and Morita, 1989). Similar observations were made in phenotypically-identified secretomotor neurons in the guinea-pig submucous plexus, where both ATP depolarisations and slow EPSPs evoked action potentials; this slow excitation was inhibited by the P2Y1-selective antagonist, MRS2179 (Hu et al., 2003; Monro et al., 2004). Subsequently, a P2Y receptor was cloned from the guinea-pig submucous plexus and both functionally and structurally characterised as guinea-pig P2Y1 (Gao et al., 2003). Electrical stimulation of nerve endings in the mucosal layer evoked slow EPSPs which were inhibited by MRS2179 in guinea-pig myenteric neurons (Gwynne and Bornstein, 2009). Such P2Y1-containing myenteric neurons are proposed to be on descending inhibitory pathways and to excite inhibitory motoneurons, thereby evoking descending inhibition in response to mucosal stimulation (Thornton et al., 2013).

P2Y1 receptor mRNA is present, and P2Y1-immunopositive material co-localises with NOS-IR, in the myenteric plexus in the murine gut (Giaroni et al., 2002; Zhang et al., 2010). Additionally, P2Y2-immunopositive material was found in guinea-pig myenteric neurons throughout the ENS (in 40-60% of counted cells) (Xiang and Burnstock, 2005). Since P2Y1 and P2Y2 are activated by ATP, caution must be taken in inferring the P2Y receptor mediating a membrane response - on the basis of ATP agonism and where P2Y1 antagonists show incomplete inhibition of agonist responses.

P2Y6-immunopositive material was found in guinea-pig myenteric neurons throughout the ENS (in about one-third of counted cells) (Xiang and Burnstock, 2006). Of the P2Y6-positive cells, a subset also contained NOS-IR (30-35% of counted cells). Additionally, P2Y12 immunopositive material was found together with Calbindin-IR in AH/Type II neurons in the guinea-pig myenteric neurons (Xiang et al., 2006). Since P2Y6 and P2Y12 are activated by ADP, caution must be taken in inferring the P2Y receptor mediating a membrane response - on the basis of ADP agonism and where either P2Y6 or P2Y12 antagonists show incomplete inhibition of agonist responses.

P2Y receptors involved in synaptic transmission in the ENS may be distinguished by pharmacology. Thus P2Y1 receptors are blocked by MRS2179 (although MRS2279 and MRS2500 are more potent); on the other hand, P2Y2, P2Y6 and P2Y12 receptors are unaffected by either MRS2179 or MRS2279 (von Kügelgen, 2006; Felix et al., 2012). As a precautionary note, MRS2179 also blocks P2X1, although the antagonist is 10-fold less potent at P2X1 than at P2Y1 (Brown et al., 2000). As a further cautionary note, P2X1-IR was observed in 10% of human submucous neurons and, here, □□□-meATP evoked calcium responses which were inhibited by very low concentrations of the P2X1-selective antagonist, NF279 (100 nM) (Liñán-Rico et al., 2015). P2Y6 receptors are blocked by MRS2578, a compound which does not block P2Y2, P2Y4 and P2Y11 receptors but is weakly active against P2Y1 (Mamedova et al., 2004). P2Y12 receptors are blocked by a number of antagonists (including the highly potent PSB 0739) (Baqi et al., 2009; Hoffman et al., 2009), although little is known about the actions of P2Y12 antagonists in the ENS (Ochoa-Cortes et al., 2014).

6. ATP release by inhibitory motoneurons

The originally-defined pharmacological profile for P2Y receptors gave an agonist potency order of 2-MeSATP>>ATP>α,β-meATP for NANC relaxations in guinea-pig taenia coli (Burnstock and Kennedy, 1985). 2-MeSATP and ATP are agonists at recombinant P2Y1 receptors but, perplexingly, α,β-meATP is ineffective (Webb et al., 1993; Burnstock et al., 1994; Filtz et al., 1994; Simon et al., 1995; Tokuyama et al., 1995). However, α,β-meATP is a potent stimulant at some P2X ion-channels found in the ENS (Galligan and North, 2004; Burnstock, 2014). Thus, the ability of α,β-meATP to evoke NANC relaxations may be secondary to the process of release of ATP (or a related nucleotide).

A number of receptor-gated ion-channels can depolarise myenteric neurons to evoke fast IJPs and NANC relaxations. This was shown first for nicotine and DMPP (at nACHRs) (Gillespie and MacKenna, 1960), thereafter for GABA and muscimol (at GABAA) (for example, see: Boeckxstaen et al., 1991; Bayer et al., 2002) and subsequently, for 5-HT and 2-methyl-5-HT (at 5-HT3Rs) (Zhou and Galligan, 1999; Neal and Bornstein, 2007; Dickson et al., 2010). In each of these cases, the actions of neuronal stimulants are blocked either by tetrodotoxin (TTX; an inhibitor of voltage-gated sodium ion channels (NaV)) or by selective antagonists of the above receptor subtypes, to inhibit the output of purines (Durnin et al., 2013). ATP and α,β-meATP-gated ion-channels can join this list of neuronal stimulants, but they may act in a different way and at different locations (Boeckstaen et al., 1991; De Man et al., 2003; Van Crombruggen et al., 2007; King and Townsend-Nicholson, 2008).

ATP-mediated NANC relaxations in the muscularis externa were blocked by TTX when the stimulatory concentration of ATP was low (≤100 μM) (Boeckxstaen et al., 1991), but recovered in the presence of TTX when higher stimulatory concentrations of ATP were used (≥100 μM) (Van Crombruggen et al., 2007). The inhibitory activity of α,β-meATP was mostly spared by TTX and yet blocked by apamin (500 nM), an inhibitor of the SK potassium channels that underpin the fast IJP (Van Crombruggen et al., 2007). Thus, the possibility exists that some nucleotides release inhibitory transmitters without the need for TTX-sensitive NaV channels or for action potential conduction. There is supporting evidence from this proposal.

The P2X receptor family is part of a superfamily of ion-channels classified as ligand-gated calcium channels (Pankratov and Lalo, 2014). The degree of calcium permeability relative to sodium permeability (PCa/PNa) is 1.2-4.8, for a range of native and recombinant P2X receptors, and indicates that P2X ion-channels are calcium preferring (Pankratov and Lalo, 2014). Accordingly, the fractional calcium current for activated P2X1-5,7 receptors is high (2.7-12.4%), regardless of membrane potential and despite low levels of extracellular Ca^{2+}

relative to extracellular Na^+ ions (Egan and Khakh, 2004). Calcium entry through ATP- and α,β-meATP-activated P2X receptors stimulates glutamate release from the nerve terminals of sensory neurons in the CNS, as judged by the elevated mEPSCs frequency in second-order neurons (Khakh and Henderson, 1998; Kato and Shigetomi, 2001; Nakatsuka and Gu, 2001; Khakh et al., 2003; Shigetomi and Kato, 2004]. Here, mEPSC frequency was unaffected by either TTX or inhibitors of voltage-dependent calcium channels (VDCCs), but inhibited completely by P2 receptors antagonists (such as PPADS, suramin and TNP-ATP) and by postsynaptically-acting glutamate receptor antagonists (such as CNQX).

Returning to the ENS, NANC relaxations evoked by α,β-meATP in the guinea-pig taenia coli were inhibited by the P2X3 and P2X2/3 antagonist, A317491, and by the P2Y1 antagonist, MRS2179 (King and Townsend-Nicholson, 2008). On the other hand α,β-meATP responses were unaffected by the NaV inhibitor, TTX, or the VDCC inhibitor, ω-conotoxin (ωCTX). Similar findings were reported for mouse jejunum, where α,β-meATP responses in circular muscle were inhibited by two P2X antagonists, NF279 and Evans Blue, and by the P2Y1 antagonist, MRS2179 (De Man et al., 2003). Here again, α,β-meATP responses were unaffected by TTX (De Man et al. 2003). Lastly, in wild-type and $nNOS^{-/-}$ mice, the fast IJP in circular muscle was blocked P2Y1 antagonist, MRS2179, and only partially inhibited by prolonged application of α,β-meATP (Zhang et al., 2010). Here, α,β-meATP may have desensitised neuronal P2X3 and P2X2/3 receptors. Taken together, these observations indicate that prejunctional P2X receptors act as neuronal calcium channels, to facilitate in a TTX-insensitive manner the release of ATP (and perhaps other NANC transmitters); thereafter, released ATP (or a related nucleotide) may act on postjunctional P2Y1 receptors. In a similar vein, a significant proportion of acetylcholine release from enteric motoneurons is resistant to TTX, dependent on the levels of extracellular calcium and enhanced by the depolarising action of excess extracellular potassium (Paton et al., 1971).

7. ATP release in the ENS

In enriched fractions of neuronal varicosities prepared from the myenteric plexus, stimulated ATP release may occur by two mechanisms: (1) veratridine-mediated release which is blocked by TTX; (2) potassium-mediated release which is dependent on extracellular Ca^{2+} influx and is not blocked by TTX (White and Leslie, 1982). For intact enteric plexuses (*in situ*), the process of ATP release is more complex than for isolated neuronal varicosities. Either field electrical stimulation or neuronal stimulants cause the release of both ATP and β-NAD^+, yet only the release of the latter is significantly inhibited by the NaV inhibitor, TTX, or the VDCC inhibitor, ω-CTX (Mutafova-Yambolieva et al., 2007; Durnin et al., 2013). In similar experiments, field electrical stimulation enhanced the release of Up4A, but neuronal stimulants failed to do so (Durnin et al., 2014). Thus, the mechanism of release (and, perhaps, even the site of release) may vary for three purine candidates proposed to mediate NANC inhibition. One proposal is that ATP may be released from cell bodies of myenteric motoneurons and β-NAD^+ may be released from myenteric nerve terminals (Durnin et al., 2013), whereas the site of Up4A release is undecided (Durnin et al., 2014). Another proposal is that only some purines may be utilised in some anatomical regions of the ENS, with β-NAD^+ activity more prominent in colonic tissue and less so, or even not at all, in other areas (e.g. stomach and caecum) (Chaudhury et al., 2012; Goyal et al., 2013; Mutafova-Yambolieva and Durnin, 2014).

The SLC17A9 protein has been identified as a vesicle nucleotide transporter (VNUT), and is expressed in various organs and particularly concentrated in human, bovine and mouse brain and adrenal glands (Sawada et al., 2008). Reduced expression of SLC17A9/VNUT significantly reduces the release of ATP from rat PC12 cells (a noted model of ATP exocytosis), indicating a prominent role for this solute carrier in regulated exocytosis (Sawada et al., 2008). A role for SLC17A9/VNUT in loading storage vesicles with ATP and

other nucleotides has been increasingly recognized and acknowledged (Lazarowski, 2012). Thus, SLC17A9 immunopositive material is present in nNOS-positive enteric nerve varicosities (Chaudhury et al., 2012) and, accordingly, purinergic and nitrergic transmission may occur at the same sites of release. As yet, there is insubstantial evidence for SLC17A9/VNUT loading for β-NAD^+ (Goyal et al., 2013), although ATP and β-NAD^+ are co-released from rat PC12 cells when stimulated by excess extracellular potassium or by nicotine (Yamboliev et al., 2009). Here, the co-release of ATP and β-NAD^+ occurs through distinct mechanisms, with the release of β-NAD^+ but not ATP inhibited by botulinum toxin. Accordingly, at this point in time, there is no consensus on how ATP and β-NAD^+ may be stored and released in the ENS.

8. ATP and the neuro-epithelial synapse

A case has been made for ATP release as the primary stimulus to mechanoreception in mucosal epithelial cells (Cooke, et al., 2003; Liñán-Rico et al., 2013). Here, ATP may act in an autocrine function to stimulate enterochromaffin (EC) cells, to release 5-HT which in turn activates 5-HT3 receptors on mucosal nerve endings. The process of 5-HT release may involve P2Y1 receptors or other receptors subtype coupled to Gαq in EC cells (Cooke et al., 2003; Kim et al., 2007) and also is regulated by P2Y12 and P2X3 receptors in EC cells (Liñán-Rico et al., 2013). Locally released ATP may activate P2X receptors on the mucosal nerve endings (Bertrand and Bornstein, 2002; Bertrand, 2003). However 5-HT appears to be the principal stimulus for mucosal sensory nerves, whereas P2X receptor activation may only be important in post-infectious mechano-hypersensitivity (Rong et al., 2009). The reasons for this switch are as yet unclear.

More is known about the process of chemoreception and, accordingly, significant progress has been made in understanding the related process of gustation, where ATP has a more prominent role than 5-HT (for evidential reviews, see: Roper, 2006, 2013). Abundant P2X3- and P2X2-immunopositive material was found in nerve-ending plexuses of the chorda tympani and glossopharyngeal nerves beneath the epithelial lining of the tongue (Bo et al., 1999; Finger et al, 2005). Taste reception is lost to nearly all sapid tastants in double knockout $P2X2^{-/-}$ and $P2X3^{-/-}$ mice (Finger et al., 2005) and, in a similar vein, taste reception is lost to sapid tastants in the presence of a novel P2X3 and P2X2/3 receptor antagonist, AF353 (Vandenbeuch et al., 2015). There was no appreciable reduction in ATP release from taste-bud epithelial cells in the presence of AF-353 (100 μM) and behavioural studies in mice showed that a preference for sweeteners was inhibited in a concentration-dependent fashion to intraperitoneal applied AF353 (Vandenbeuch et al., 2015).

The process of ATP release is complex in taste-buds, as is its modulation by other neurotransmitter substances (Kinnamon and Finger, 2013; Roper 2013). Locally-released ATP, acetylcholine, 5-HT, GABA and perhaps noradrenaline appear to subserve autocrine and paracrine roles in taste-buds, to regulate the further release of ATP and 5-HT as excitatory substances at sensory nerve endings. Here, an important autocrine role is played by P2Y1 and P2X2 in augmenting further release of ATP by Type II taste-bud cells. Accordingly, the P2Y1 antagonist MRS2179 limits ATP release in wild-type mice (Huang et al., 2009) and $P2X2^{-/-}$ knockout similarly limits ATP release in gene-deleted mice (Huang et al., 2011). Obviously, P2Y1 may release calcium from intracellular stores and P2X2 may depolarise cells and permit calcium influx; but the primary effect of activation of P2Y1 and P2X2 does not appear to involve calcium-dependent vesicular ATP release. Instead P2Y1 and P2X2 cause the opening of calcium sensitive pannexin-1 channels (Panx1) and voltage-gated CALHM1 channels, each of which are permeable to cytosolic ATP (Huang et al., 2011; Taruno et al., 2013). $CALHM1^{-/-}$ mice not only consume appreciably less food and weigh less as a result, but also extend their life-span by as much as 1 year compared to wild-type mice (Hellekant et al., 2015). Although CALHM1 regulates the electrical activity and firing

patterns of murine cortical neurons, particularly under conditions of low extracellular calcium (Ma et al., 2012), there is no information as yet on its role in the ENS.

9. Concluding remarks

Purinergic signalling in the Enteric Nervous System has developed considerably, since the seminal findings Burnstock and colleagues in 1970 and their proposal that ATP is an inhibitory transmitter in the muscularis externa of the gastrointestinal tract. Now 45 years later, a broader perspective has emerged on the role for ATP or related nucleotides in purinergic transmission in the ENS. New problems have surfaced in the intervening time and this review has addressed many of these issues. The zenith of research into purinergic signalling in the ENS still has some way to go, and the future offers the promise of more intriguing revelations. Often the role of review articles is to look backwards and define only the achievements in a field of endeavour. I have taken a different approach and focussed instead on the problems which now face researchers in this field. This was done more as a stimulus to research, but also to highlight the inquisitiveness of researchers who continue to explore and redefine concepts of purinergic signalling in the ENS. It will be interesting to see how collectively we make progress over the next few years.

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