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Purinergic signalling: past, present and future

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The discovery of non-adrenergic, non-cholinergic neurotransmission in the gut and bladder in the early 1960's is described as well as the identification of adenosine 5'-triphosphate (ATP) as a transmitter in these nerves in the early 1970's. The concept of purinergic cotransmission was formulated in 1976 and it is now recognized that ATP is a cotransmitter in all nerves in the peripheral and central nervous systems. Two families of receptors to purines were recognized in 1978, P1 (adenosine) receptors and P2 receptors sensitive to ATP and adenosine diphosphate (ADP). Cloning of these receptors in the early 1990's was a turning point in the acceptance of the purinergic signalling hypothesis and there are currently 4 subtypes of P1 receptors, 7 subtypes of P2X ion channel receptors and 8 subtypes of G protein-coupled receptors. Both short-term purinergic signalling in neurotransmission, neuromodulation and neurosecretion and long-term (trophic) purinergic signalling of cell proliferation, differentiation, motility, death in development and regeneration are recognized. There is now much known about the mechanisms underlying ATP release and extracellular breakdown by ecto-nucleotidases. The recent emphasis on purinergic neuropathology is discussed, including changes in purinergic cotransmission in development and ageing and in bladder diseases and hypertension. The involvement of neuron-glial cell interactions in various diseases of the central nervous system, including neuropathic pain, trauma and ischemia, neurodegenerative diseases, neuropsychiatric disorders and epilepsy are also considered.

Key words: Purinoceptors, P1, P2X, P2Y; ATP release and breakdown; Purinergic neuropathology; Pain; Neurodegenerative diseases

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Introduction

The story really started when I took up my first postdoctoral post in Feldberg's Department of Physiology at the National Institute for Medical Research. There I learned electrophysiological techniques and, together with Ralph Straub (who had worked with Stämpfli in Switzerland), developed the sucrose-gap technique to record correlated mechanical and electrical activity in smooth muscle (1). When Edith Bülbring, who led the leading smooth muscle laboratory in the UK, saw how useful this method was compared to the technical difficulties her group was facing with microelectrode recording from spontaneous smooth muscle of the guinea-pig taenia coli, her favourite preparation, she invited me to take up a postdoctoral position in the Department of Pharmacology, Oxford University. There I studied the actions of the classical neurotransmitters, acetylcholine (ACh) and noradrenaline (NA) using the sucrose-gap technique (2,3). Then, after a year in Ladd Prosser's laboratory in Champaign-Urbana, IL, supported by a Rockefeller fellowship, I decided to take up a Senior Lectureship in the Department of Zoology in Melbourne in 1960, where after a short time I set up the sucrose-gap technique and began to build a research group.

Non-adrenergic, non-cholinergic nerves

One day, together with my young colleagues, Max Bennett, who was a part-time electronics technician completing an Engineering degree, and Graham Campbell, a

PhD student, we decided to stimulate the nerves supplying the smooth muscle of the guinea-pig taenia coli in the presence of atropine and bretylium to block cholinergic and adrenergic neurotransmission and expected to see depolarisation and contraction in response to direct stimulation of the muscle. However, to our surprise the responses to single stimuli were rapid hyperpolarizations and relaxation. This was a moment of excitement (4) for us because we felt that we were on to something important. Interpretation of our results was discussed internationally for a while and then I was fortunate to have a Japanese postdoctoral fellow working with me whose friend in Japan had just discovered tetrodotoxin (from the puffer fish), which was shown to block nerve conduction, but not smooth muscle activity. Tetrodotoxin abolished the hyperpolarizations, so we realized that they were inhibitory junction potentials in response to non-adrenergic, non-cholinergic (NANC) neurotransmission. I then spent 6 months with Mike Rand at the School of Pharmacy in London to study details of the NANC inhibitory responses, for example, showing that they were present in intrinsic enteric neurons controlled by vagal or sacral parasympathetic nerves (5).

ATP as a transmitter in NANC nerves

The next step was to try to identify the transmitter released during NANC inhibitory transmission in the gut and by NANC excitatory transmission, which we later identified in the urinary bladder. From the work of Jack Eccles and others, we knew that several criteria needed to be satisfied to establish a neurotransmitter: synthesis and storage in nerve terminals; release by a Ca^{2+}-dependent mechanism; mimicry of the nerve-mediated responses by the exogenously applied transmitter; inactivation by ecto-enzymes and/or neuronal uptake, and parallel block or potentiation of responses to stimulation by nerves and exogenously applied transmitter. We examined many different substances in the late 1960's, including amino acids, monoamines, neuropeptides, but none satisfied the criteria. However, in reading the literature, I discovered a seminal paper by Drury and Szent-Györgyi (6) showing powerful extracellular actions of purines on heart and blood vessels, papers by Feldberg showing extracellular actions of adenosine 5'-triphosphate (ATP) on autonomic ganglia (e.g., 7) and a paper by Pamela Holton in 1959, which showed release of ATP during antidromic stimulation of sensory nerves supplying the rabbit ear artery (8). So we tried ATP and to our surprise it satisfied all the criteria needed to beautifully establish it as a transmitter involved in NANC neurotransmission (9). In 1972, I published an article in *Pharmacological Reviews* (10) formulating the purinergic neurotransmission hypothesis. Sadly, few believed this hypothesis over the next 25 years and it was often ridiculed at meetings and workshops. When I left to take up the Chair of Anatomy and Embryology at University College London in 1975, Professor Austin Doyle, said at my farewell Reception, "Geoff Burnstock is the discoverer of the pure-imagine hypothesis". Resistance to this concept was perhaps understandable because ATP was well established as an intracellular energy source involved in the Krebs cycle and other biochemical pathways and it seemed unlikely that such a ubiquitous molecule would also act as an extracellular messenger. My own view is that ATP, recognized as an early biological molecule, evolved both as an intracellular energy source and an extracellular signalling molecule.

Purinergic cotransmission

During a sabbatical leave visiting the laboratory of Che Su and John Bevan at UCLA, we were disconcerted to find ATP release not only from NANC intrinsic inhibitory enteric neurons, but also from sympathetic nerves supplying the taenia coli (11). However, this raised the question in my mind that ATP might be released as a cotransmitter from sympathetic nerves and after discovering many hints in the literature, I formulated the cotransmitter hypothesis in 1976 in a Commentary to *Neuroscience* (12), which unfortunately also raised controversy because of the widely held concept called 'Dales Principle', although actually defined by Eccles, that one nerve only releases one transmitter. The electrical recordings that Mollie Holman and I made during sympathetic neurotransmission in the guinea-pig vas deferens in the early 1960s showed excitatory junction potentials (EJPs) in response to single pulses that summed and facilitated until at a critical depolarisation, a spike was generated leading to contraction (13). However, what was puzzling was that receptor antagonists to NA as the transmitter recognized at that time in sympathetic nerves did not block the EJPs, although bretylium, that prevents release of transmitter from sympathetic nerves, did reduce them. It was not until over 20 years later, when Peter Sneddon joined my laboratory in London, that we showed that a,b-methyleneATP, a slowly degradable analog of ATP that acts as a selective desensitiser of the ATP receptor (14), abolished the EJPs and spritzed ATP mimicked the EJP, but NA did not (15). Purinergic cotransmission is now well established, not only in sympathetic nerves, but also in parasympathetic, sensory-motor and enteric nerves and more recently ATP has been shown to be co-released with glutamate, GABA, dopamine, NA, 5-hydroxytryptamine and ACh in different populations of nerve fibbers in the

central nervous system (CNS) (see Ref. 16).

Important landmark papers in the early 1990's described ATP mediation of fast synaptic transmission in both peripheral ganglia (17,18) and in the CNS (19).

Receptors to purines and pyrimidines

Implicit in purinergic transmission is the existence of specific receptors. In 1978, I proposed a basis for distinguishing two types of purinergic receptors, one selective to adenosine (called P1), which was antagonized by methylxanthines and the other selective for ATP/adenosine diphosphate (ADP; called P2) (20). This was a useful step forward, explaining some of the early confusion in the literature resulting from the rapid extracellular breakdown of ATP to adenosine and extended our concept of purinergic neurotransmission, by identifying post-junctional receptors as P2, while pre-junctional P1 receptors mediated neuromodulatory negative feedback responses or autoregulation of transmitter release. A pharmacological basis for distinguishing two types of P2-purinoceptors, defined as P2X and P2Y, was proposed in 1985 (21) and we were lucky that when P2 receptors were cloned in the early 1990's (22-25) and second messenger mechanisms examined, this subclassification was consistent with P2X ion channel receptors and P2Y G protein-coupled receptors. Currently, 4 subtypes of P1 receptors are recognized, 7 subtypes of P2X receptors and 8 subtypes of P2Y receptors, including some responsive to the pyrimidines, UTP and UDP (uridine tri- and diphosphate, respectively; see Refs. 26,27). It was shown that three of the P2X receptor subtypes combine to form cation pores (28) either as homomultimers and heteromultimers, and more recently heterodimerization has been shown between P2Y receptor subtypes. Many non-neural as well as neuronal cells express multiple receptors (29) and this poses problems about how they mediate interacting physiological events. It is becoming clear that the purinergic signalling system has an early evolutionary basis with fascinating recent studies showing cloned receptors in two primitive invertebrates, *Dictyostelium* and *Schistosoma* that resemble mammalian P2X receptors (30,31) and ATP signalling in plants has also been described (32-34).

Physiology of purinergic signalling

While early studies were largely focused on short-term signalling in such events as neurotransmission, neuromodulation, secretion, chemoattraction and acute inflammation, there has been increasing interest in long-term (trophic) signalling involving cell proliferation, differentiation, motility and death during development, regeneration, wound healing, restenosis, epithelial cell turnover, cancer and ageing (35). For example, in blood vessels, there is dual short-term control of vascular tone by ATP released as an excitatory cotransmitter from perivascular sympathetic nerves to act on P2X receptors in smooth muscle, while ATP released from endothelial cells during changes in blood flow (shear stress) and hypoxia acts on P2X and P2Y receptors on endothelial cells leading to production of nitric oxide and relaxation (36). In addition, there is long-term control of cell proliferation and differentiation, migration and death-involved neovascularization, restenosis following angioplasty and atherosclerosis (37).

For many years, the source of ATP acting on receptors was considered to be damaged or dying cells, except for exocytotic vesicular release from nerves. However, it is now known that many cell types release ATP physiologically in response to mechanical distortion, hypoxia or to some agents (38). The mechanism of ATP transport is currently being debated and includes in addition to vesicular release, ABC transporters, connexin or pannexin hemichannels, maxi-ion channels and even $P2X_7$ receptors (16).

There is now much known about the extracellular breakdown of released ATP by various types of ecto-nucleotidases including ectonucleoside triphosphate diphosphohydrolases, ecto-nucleotide pyrophosphatases/phosphodiesterases, alkaline phosphatase and ecto-5'-nucleotidase (39).

Purinergic neuropathology and therapeutic potential

It is well known that the autonomic nervous system shows high plasticity compared to the CNS. For example, substantial changes in cotransmitter and receptor expression occur during development and ageing in the nerves that remain following trauma or surgery and in disease situations (5). For example, a P2Y-like receptor was identified in *Xenopus* that was transiently expressed in the neural plate and again later in secondary neuralation in the tail bud, suggesting involvement of purinergic signalling in the development of the nervous system (40). There is transient expression of $P2X_5$ and $P2X_6$ receptors during development of myotubules and of $P2X_2$ receptors during development of the neuromuscular junction (41). In the rat brain, $P2X_3$ receptors are expressed first at E11, $P2X_2$ and $P2X_7$ receptors appear at E14, $P2X_4$, $P2X_5$ and $P2X_6$ receptors at P1, and $P2X_1$ receptors at P16 (42).

Primitive sprouting of central neurons was shown in experiments in which the enteric nervous system was

transplanted into the striatum of the brain (43). It was later shown that a growth factor released from enteric glial cell acting synergistically with ATP (and its breakdown product, adenosine) and nitric oxide were involved (44). It is suggested that similar synergistic activity of purines and growth factors might be involved in stem cell activity.

It was established early that ATP was a major cotransmitter with ACh in parasympathetic nerves mediating contraction of the urinary bladder of rodents (45). In healthy human bladder, the role of ATP as a cotransmitter is minor. However, in pathological conditions, such as interstitial cystitis, outflow obstruction and most types of neurogenic bladder, the purinergic component is increased to about 40% (5,46). Similarly, in spontaneously hypertensive rats, there is a significantly greater cotransmitter role for ATP in sympathetic nerves (47).

$P2X_3$ receptors were cloned in 1995 and shown to be largely located in small nociceptive sensory nerves that label with isolectin B4 (48,49). Central projections are located in inner lamina 2 of the dorsal horn of the spinal cord and peripheral extension in skin, tongue and visceral organs. A unifying purinergic hypothesis for the initiation of pain was published (50) and a hypothesis describing purinergic mechanosensory transduction in visceral organs in 1999, where ATP, released from lining epithelial cells during distension, acts on $P2X_3$ and $P2X_{2/3}$ receptors in subepithelial sensory nerve endings to send nociceptive messengers via sensory ganglia to the pain centres in the brain (51). Supporting evidence including epithelial release of ATP, immuno-localization of $P2X_3$ receptors on subepithelial nerves and activity recorded in sensory nerves during distension that is mimicked by ATP and reduced by $P2X_3$ receptor antagonists has been reported in the bladder (52), ureter (53) and gut (54). Purinergic mechanosensory transduction is also involved in urine voiding as evidenced in $P2X_3$ knockout mice (55). For neuropathic and inflammatory pain $P2X_4$, $P2X_7$ and $P2Y_{12}$ receptors on microglia have been implicated and antagonists to these receptors are very effective in abolishing allodynia (56,57). There is much interest in neuron-glial cell interactions in the CNS (58) and there is also strong interest in the potential roles of purinergic signalling in trauma and ischemia, neurodegenerative conditions including Alzheimer's, Parkinson's and Huntington's diseases and in multiple sclerosis and amyotrophic lateral sclerosis. There are also studies in progress of purinergic signalling in neuropsychiatric diseases, including depression, anxiety and schizophrenia and in epileptic seizures (see Ref. 57).

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