

**ATP as an excitatory cotransmitter in the autonomic nervous system**

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## Abstract

The role of adenosine 5'-triphosphate (ATP) as a major intracellular energy source is well-established. In addition, ATP and related nucleotides have widespread extracellular actions via the ionotropic P2X receptors (ligand-gated cation channels) and metabotropic P2Y receptors (G protein-coupled receptors). Numerous experimental techniques, including myography, electrophysiology and biochemical measurement of neurotransmitter release, have been used to show that ATP has two major roles as an excitatory cotransmitter from autonomic nerves; 1) It is coreleased with noradrenaline in synaptic vesicles in postganglionic sympathetic nerves innervating smooth muscle preparations, such as the vas deferens and most arteries. When coreleased with noradrenaline, ATP acts at postjunctional P2X1 receptors to evoke depolarisation,  $\text{Ca}^{2+}$  influx,  $\text{Ca}^{2+}$  sensitisation and contraction. 2) ATP is also coreleased with acetylcholine from postganglionic parasympathetic nerves innervating the urinary bladder and again acts at postjunctional P2X1 receptors, and possibly also a P2X1+4 heteromer, to elicit smooth muscle contraction. In both systems the neurotransmitter actions of ATP are terminated by dephosphorylation by extracellular, membrane-bound enzymes and soluble nucleotidases released from postganglionic nerves. There are indications of an increased contribution of ATP to control of blood pressure in hypertension, but further research is needed to clarify this possibility. More promising is the upregulation of P2X receptors in dysfunctional bladder, including interstitial cystitis, idiopathic detrusor instability and overactive bladder syndrome. Consequently, these roles of ATP are of great therapeutic interest and are increasingly being targeted by pharmaceutical companies.

## Highlights

- ATP is an excitatory cotransmitter in sympathetic and parasympathetic nerves
- ATP evokes depolarisation,  $\text{Ca}^{2+}$  influx,  $\text{Ca}^{2+}$  sensitisation and contraction
- Postjunctional P2X1 receptors are its main site of action in smooth muscle tissues
- ATP may also act at P2X1+4 heteromers in urinary bladder to elicit contraction
- ATP's action is ended by ecto- nucleotidases and coreleased soluble nucleotidases

## Key words

ATP, cotransmission, sympathetic, parasympathetic, vas deferens, urinary bladder, nucleotidase

## Abbreviations

Ach - acetylcholine	ATP - adenosine 5'-triphosphate	NTPDase - ecto-nucleoside triphosphatase
diphosphohydrolase	eJPs - excitatory junction potentials	IP <sub>3</sub> - inositol 1,4,5-trisphosphate
NA - noradrenaline	SHR - spontaneously hypertensive rats	

## 1. Introduction

The role of the endogenous nucleotide, adenosine 5'-triphosphate (ATP), as a major intracellular energy source is a well-established phenomenon. In addition, many studies have shown that ATP and related nucleotides have widespread extracellular actions via the ionotropic P2X receptors, which are ligand-gated cation channels, and metabotropic P2Y receptors, which are G protein-coupled receptors ([Burnstock and Kennedy, 1985, 2011; Khakh et al., 2001; Abbracchio et al., 2005, 2006; Kennedy et al., 2013](#)). These receptors are distributed widely throughout the body and are involved in many physiological and pathophysiological processes ([Burnstock and Kennedy, 2011](#)). Indeed, it is very likely that all cell types express at least one P2X and/or P2Y receptor subtype ([Burnstock and Knight, 2004](#)). Furthermore, the receptors present in the central, peripheral and sensory nervous systems, cardiac, skeletal and smooth muscle, the vascular system and immune and inflammatory cells are of great therapeutic interest and are increasingly being targeted by pharmaceutical companies ([Guile et al., 2009; Gunosewojo and Kassiou, 2010; Bartlett et al., 2014](#)).

The pioneering work on neurotransmission in the peripheral nervous system carried out early in the 20<sup>th</sup> century, led to the widely accepted idea that noradrenaline (NA) was the sole neurotransmitter released by postganglionic sympathetic nerves and acetylcholine (ACh) was the sole neurotransmitter released by postganglionic parasympathetic nerves. By the early 1970s, however, evidence was accumulating to support the idea that ATP was also a neurotransmitter from "purinergic" nerves in some parts of the autonomic nervous system, ([Burnstock, 1972](#)). Several years later [Burnstock, \(1976\)](#) suggested that nerves could in fact release more than one substance as a neurotransmitter. Since then, cotransmission has become accepted as the rule rather than the exception NA, ACh, ATP, nitric oxide and several peptides have all been shown to act as cotransmitters in a wide range of peripheral tissues ([Burnstock, 2013](#)).

Numerous techniques, such as myography, electrophysiology and biochemical measurement of neurotransmitter release, have been used to show that ATP has two major roles as an excitatory cotransmitter from autonomic nerves; 1) It is coreleased with NA from postganglionic sympathetic nerves innervating smooth muscle preparations, such as the vas deferens and most arteries (Figure 1). 2) On the other hand, the cotransmitter actions of ATP with ACh from postganglionic parasympathetic nerves have been most extensively characterised in the urinary bladder (see section 3). Previous reviews describe the historical development of this field in detail ([Burnstock and Kennedy, 1986; Von Kügelgen and Starke, 1991; Sneddon et al., 1996, 1999; Kennedy et., 1997a](#)). Similarly, the influence of retrograde release of ATP from sensory nerves on vascular tone has been reviewed by [Rubino and Burnstock, \(1996\)](#). In this article I will, therefore, provide an overview of the evidence that supports these cotransmitter roles and discuss more recent developments.

## 2. ATP and NA as sympathetic neurotransmitters

### 2.1 ATP storage in and release from sympathetic nerves.

The earliest evidence that ATP could function as a neurotransmitter from sympathetic nerves came from biochemical studies that showed that ATP was co-stored with NA in sympathetic synaptic vesicles (see [Burnstock and Kennedy, 1986](#)). Neuropeptide Y is also present in and released from sympathetic nerves and its main effect appears to be to modulate the effects of ATP and NA, which is discussed elsewhere (see [Burnstock, 2013](#) and references therein). In addition, numerous studies, using a variety of detection techniques, have clearly demonstrated that sympathetic nerve stimulation evokes release of ATP in the vas deferens and blood vessels of many species (see [Burnstock, 2014](#); [Mutafova-Yambolieva and Durnin, 2014](#)).

In early studies NA was always present in synaptic vesicles in much higher amounts than ATP. Subsequent evidence suggested, however, that there may well also be further populations of vesicles that store ATP and NA separately. In the most detailed study done to date, [Todorov et al., \(1996\)](#) used HPLC to monitor the release of ATP and NA simultaneously during stimulation of sympathetic nerves innervating the guinea-pig vas deferens. They found that NA release peaked 40 seconds after the start of stimulation and then remained constant. In contrast, ATP release peaked more quickly, 20 seconds after the start of stimulation, and then decreased, even though the nerves were still being stimulated. The ATP/NA ratio was initially substantially greater than 1, peaked at 20 seconds then fell rapidly throughout the remainder of the stimulation period such that NA became the predominant neurotransmitter released. This is consistent with the phasic nature of the purinergic component and the maintained nature of the noradrenergic component of neurogenic contractions in this tissue (see below). These data are also consistent with earlier reports that the release of ATP and NA are differentially modulated by both angiotensin ([Ellis and Burnstock, 1989](#)) and prostaglandin E<sub>2</sub> ([Ellis and Burnstock, 1990](#)), indicating differential storage and release of the two cotransmitters.

Action potential-evoked release of neurotransmitters is dependent on influx of extracellular Ca<sup>2+</sup> into the presynaptic neuronal terminal via voltage-dependent Ca<sup>2+</sup> channels and the role of individual channel subtypes in the release of ATP and NA during sympathetic nerve stimulation has been studied. In guinea-pig vas deferens, [Westfall et al., \(1996b\)](#) found that during 8 Hz sympathetic nerve stimulation, ATP release was inhibited more than NA by blockade of the P/Q-type (Ca<sub>v</sub>2.1) Ca<sup>2+</sup> channels, whereas blockade of the N-type (Ca<sub>v</sub>2.2) Ca<sup>2+</sup> channels inhibited NA release more than ATP. These results support this group's previous claim that in the sympathetic nerves of the guinea-pig vas deferens at least, ATP and NA originate from different vesicles and release sites (see above). A similar profile was seen in dog mesenteric artery and vein during nerve

stimulation at 4 and 16 Hz, where N-type  $\text{Ca}^{2+}$  channels mediated release of NA mainly, along with some ATP, whilst P/Q-type  $\text{Ca}^{2+}$  channels mediated release of ATP mainly, along with some NA (Smyth et al., 2009). Contrary to these data, Smith and Cunnane, (1996a) reported that when ATP release was measured electrophysiologically during nerve stimulation at 1 Hz in guinea-pig vas deferens, it was abolished by blockade of the N-type  $\text{Ca}^{2+}$  channels. Increasing the frequency of stimulation led to ATP release that was independent of N- and P/Q-type  $\text{Ca}^{2+}$  channels, but involved ryanodine-sensitive, intracellular  $\text{Ca}^{2+}$  stores (Smith and Cunnane, 1996b). The reason for the difference in results is not clear, but may be due to differences in the voltage-dependence of opening of the different  $\text{Ca}^{2+}$  channel subtypes.

Brock and Cunnane (1999) studied the release of ATP and NA from sympathetic nerves innervating rat tail artery, where the release of ATP and NA were modified in similar ways by blockers of N- and P/Q-types  $\text{Ca}^{2+}$  channels. Regardless of which channels are involved in  $\text{Ca}^{2+}$  entry into sympathetic nerve varicosities, ATP release as a neurotransmitter is highly intermittent. The use of extracellular electrophysiological recording techniques revealed that in the vas deferens this intermittence is not due to failure of the neuronal action potential to invade varicosities, but is instead due to a low probability of release once the varicosity is depolarised (Cunnane and Stjärne, 1984; Brock and Cunnane, 1987, 1988).

A controversial issue is that of non-neuronal release of ATP during sympathetic nerve stimulation that led some to question the neurotransmitter role of ATP. Several groups suggested that ATP released in guinea-pig vas deferens came from a non-neuronal source (Katsuragi et al., 1991; Vizi et al., 1992; Von Kügelgen and Starke, 1994), but in contrast, Todorov et al., (1996) found no evidence for postjunctional release of ATP in the same tissue. The reasons for this discrepancy have not been resolved, but may reflect the methods used. The luciferin-luciferase system was used in the studies where a postsynaptic component of ATP release was seen. Although very sensitive, this system can only measure intact ATP, not its breakdown products, which greatly underestimates the total release of ATP, since ATP is rapidly broken down upon release (see below). In contrast, Todorov et al., (1996) used an HPLC system that monitored ATP and all of its metabolites simultaneously. Although less sensitive than luciferin-luciferase for ATP *per se*, it provides a more accurate measure of the total amount of ATP released. Consequently, the amount of ATP measured by Todorov et al., (1996) was much greater than measured in other studies under the same stimulation conditions. It may be that the luciferin-luciferase method requires greater stimulation intensities in order to produce high enough levels of intact ATP in the superfusate and these greater intensities cause postjunctional release of ATP. Whatever the reason for these differences, it is unlikely that the postjunctional release of ATP contributes to its neurotransmitter role, as this release of ATP peaks after 2-6 minutes (Vizi et al., 1992), whereas contractions evoked

by endogenous or exogenous ATP reach a peak within 5 seconds ([Sneddon et al., 1996](#)).

## *2.2. Postjunctional P2X1 receptors*

**2.2.1. P2X subunits** Following release from sympathetic nerves, ATP diffuses across the synaptic cleft and binds to ionotropic P2X1 receptors expressed in the plasma membrane of the smooth muscle cells. Seven P2X subunits, P2X1-7, have been cloned and hydropathy analysis of their amino acid sequence indicates that each subunit possesses two hydrophobic, transmembrane spanning regions (TMR) that span the cell plasma membrane ([Khakh et al., 2001](#); [Burnstock, and Kennedy, 2011](#); [Syed and Kennedy, 2012](#); [Kennedy et al., 2013](#)). Furthermore, it was predicted that the NH<sub>2</sub> and COOH termini are intracellular and that the bulk of the protein, about 280-300 amino acids, forms an extracellular loop. When expressed alone, all P2X subunits form functional receptors, except the P2X6, which usually only assembles in a heteromeric form. Subunits can also interact with each other and to date seven functional heteromultimers formed by two different subunits have been identified: P2X2/3, P2X4/6, P2X1/5, P2X2/6, P2X1/4, P2X1/2 and possibly P2X4/7 with pharmacological and/or biophysical properties that differ from the individual homomultimers (see [Burnstock, and Kennedy, 2011](#); [Syed and Kennedy, 2012](#)). Recently atomic force microscopy identified a P2X2/4/6 heterotrimer ([Antonio et al., 2014](#)). Regardless of the subunit composition, P2X multimers are permeable to Na<sup>+</sup>, K<sup>+</sup> and Ca<sup>2+</sup> and on activation cause depolarisation and excite cells. As P2X subunits have only two TMR, a single subunit on its own cannot form a functional receptor. Recent reports of the three-dimensional crystal structure of a P2X receptor, in both drug-free ([Gonzales et al., 2009](#); [Kawate et al., 2009](#)) and ATP-bound states ([Hattori et al., 2012](#)) confirms earlier predictions that three subunits form a functional P2X receptor and that three agonist molecules must bind to a single receptor in order to activate it.

**2.2.2. P2X subunit expression** Numerous studies in which the expression of P2X subunit mRNA and protein were measured have shown that the P2X1 receptor is predominant P2X subtype present in most smooth muscle tissues, including the vas deferens and blood vessels ([Burnstock and Knight, 2004](#)), although some studies have also identified expression of other subtypes. For example, in rat pulmonary artery smooth muscle, single PCR products of the predicted size for all seven P2X receptors were seen, except the P2X2 receptor, where 2 bands were present and identified as the P2X2a and P2X2b splice variants (Figure 2a) ([Syed et al., 2010](#)). When expression was studied at the protein level using anti-P2X subunit antibodies, strong, punctate P2X1 receptor-like immunoreactivity (lir) was observed in the majority of cells (Figure 2b). In contrast, fainter, punctate staining was seen with the anti-P2X2 and anti-P2X4 antibodies, whilst P2X5-lir was barely detectable and no P2X3, P2X6, and P2X7 receptor-lir was apparent (not shown). Western blot

analysis revealed a strong immunoreactive polypeptide band for the P2X1 receptor of about 60 kDa, two bands each for the P2X2 and P2X4 receptors of about 45 and 65 kDa and about 46 and 70 kDa respectively and a single band of 45 kDa for the P2X5 receptor (Figure 2c). These values are similar to the predicted molecular weights of the receptors. Hence, in rat pulmonary smooth muscle cells, the P2X1 appears to be the predominant P2X subunit expressed at the protein level, but P2X2, P2X4 and P2X5 subunits are also present. The functional relevance of these findings is, however, unclear, as the pharmacological properties of the contractions evoked by ATP were consistent with it acting solely at P2X1 receptors.

Lipid rafts are specialised cholesterol-rich lipid microdomains present in the plasma membrane, which are involved in receptor signalling and trafficking. P2X1 receptors are present in lipid rafts in several smooth muscle tissues, including rat tail artery, vas deferens and urinary bladder (Vial and Evans, 2005) and human vas deferens (Donoso et al., 2014). Chemical disruption of the lipid rafts selectively inhibited P2X1 receptor signalling, showing that the rafts play a very important role in maintaining the functional activity of P2X1 receptors.

**2.2.3. *P2X1 receptor pharmacology*** The P2X1 receptor corresponds to the P2X receptor initially characterised in pharmacological studies of native receptors in smooth muscle tissues by Burnstock and Kennedy (1985). In the absence of agonist breakdown by ecto-enzymes (see Kennedy and Leff, 1995) it is activated by 2-methylthioATP = ATP >  $\alpha,\beta$ -methyleneATP ( $\alpha,\beta$ -meATP). None of these agonists is selective for the P2X1 receptor and all activate numerous other P2X and P2Y receptor subtypes.  $\alpha,\beta$ -meATP was initially thought to act only at homomeric P2X1 and P2X3 receptors and heteromeric P2X2/3 receptors, but was subsequently shown to also activate homomeric P2X5, P2X6 and P2X4 (though in a species-dependent manner) receptors and heteromeric P2X4/6 (Lê et al., 1998) and P2X1/5 receptors. Despite much endeavour by synthetic chemists, no selective P2X1 agonist has been produced (see Khakh et al., 2001; Syed and Kennedy, 2012).

No antagonists were available when P2X receptors were first characterised in 1985. Shortly afterwards, suramin and PPADS were reported to be effective, but with low potency and little subtype selectivity and both are also antagonists at some of the P2Y receptor subtypes (Abbracchio et al., 2006). PPADS tends to have a similar potency or be slightly more potent than suramin at P2X receptors. Its antagonistic actions tend to develop and reverse slowly and antagonism is generally non-competitive. Its ability to inhibit ATP breakdown by ecto-nucleotidases is less than suramin. Subsequently, antagonists that are more potent and which have some degree of selectivity for individual P2X receptor subtypes have been developed (see Syed and Kennedy, 2012). Suramin and PPADS are both large compounds and modifying their structures generated a number of antagonists with increased P2X1 selectivity and/or potency, including NF023, NF279 and NF449 from suramin

and the PPADS-related derivatives, PPNDS and MRS2220 and MRS2159. The introduction of these compounds was a major breakthrough that allowed the physiological functions of P2X receptors, including the role of ATP as a cotransmitter in sympathetic and parasympathetic nerves, to be studied in detail (Burnstock, 2013; Burnstock and Kennedy, 2011).

### *2.3. Functional effects of ATP release: excitatory junction potentials (ejps)*

The first functional postjunctional response that can be measured in smooth muscle cells of the vas deferens and arteries during sympathetic nerve stimulation is the ejp, a rapid, transient depolarisation. Ejps are resistant to  $\alpha$ -adrenoceptor blockade, but abolished by tetrodotoxin and guanethidine, indicating that they are due to stimulation of sympathetic nerves. In most smooth muscle tissues exogenous NA and NA released from sympathetic nerves evoke contraction without altering the membrane potential of smooth muscle cells. In contrast, ATP evokes rapid transient depolarisations or inward currents when cells are held under voltage-clamp, with a time-course that mimics that of ejps (see Evans and Kennedy, 1994; Sneddon et al., 1996; McLaren et al., 1998b and references therein). Although several explanations were proposed to explain how NA could nonetheless mediate ejps, the debate ended when ejps in the guinea-pig vas deferens were clearly demonstrated to be inhibited by desensitisation of the P2X1 receptor by  $\alpha,\beta$ -meATP (Sneddon and Burnstock, 1984), then by suramin (Sneddon, 1992) and PPADS (McLaren et al., 1994).

Subsequently, Sneddon et al., (2000) showed that the selective P2X1 antagonist, NF023, depressed ejps in a rapid, concentration-dependent and reversible manner (Figure 3). The absence of ejps in the vas deferens of P2X1 receptor knock-out mice confirmed that they are mediated by P2X1 receptors (Mulryan et al., 2000). Similar studies using desensitisation of the P2 receptor by  $\alpha,\beta$ -methyleneATP or suramin clearly demonstrated that ejps in arteries are also mediated by ATP (see Von Kügelgen et al., 1991; McLaren et al., 1995).

### *2.4. Functional effects of ATP release: contraction*

When nerve stimulation frequency is high enough, multiple ejps summate and the membrane depolarises sufficiently to trigger the opening of voltage-dependent L-type ( $\text{Ca}_{\text{v}}1.2$ )  $\text{Ca}^{2+}$  channels.  $\text{Ca}^{2+}$  influx also occurs through the P2X<sub>1</sub> receptor ion channel, as it permeable to  $\text{Ca}^{2+}$  ions.

Concomitantly, NA stimulates  $\alpha_1$ -adrenoceptors to elicit release of  $\text{Ca}^{2+}$  stores, via inositol 1,4,5-trisphosphate ( $\text{IP}_3$ ), and the combined rise in cytoplasmic  $[\text{Ca}^{2+}]$ , together with  $\text{Ca}^{2+}$  sensitisation (see below), induces contraction. Numerous studies in a variety of species using desensitisation of the P2X1 receptor by  $\alpha,\beta$ -meATP or antagonists, such as suramin, PPADS and NF023, combined with  $\alpha_1$ -adrenoceptor blockade, show that the initial phasic component of neurogenic contractions of the vas deferens is predominantly purinergic, whilst the secondary, tonic phase is predominantly

noradrenergic (see [Sneddon et al., 1996, 1999](#)). PPADS has also been used to identify a purinergic component in neurogenic contractions of human vas deferens ([Banks et al., 2006](#)). At rest the vas deferens stores sperm and when stimulated by sympathetic nerves they contract to propel sperm into the ejaculate. Knockout of P2X1 receptors caused a substantial decrease in the peak amplitude of neurogenic contractions of mouse vas deferens, which was associated with a 90% decrease in fertility ([Mulryan et al., 2000](#)). Simultaneous knockout of  $\alpha_{1A}$ -adrenoceptors and P2X1 receptors produced total infertility ([White et al., 2013](#)). Thus P2X1 receptors clearly play a crucial role in male reproductive function.

Neurogenic contractions in most blood vessels tend to be monophasic rather than biphasic, so the involvement of a non-adrenergic neurotransmitter was not seriously considered for many years. [Von Kugelegen and Starke, \(1985\)](#) and [Kennedy et al., \(1986\)](#) reported, however, that a component of sympathetic contractions of rabbit ileo-colic and central ear artery respectively, was resistant to  $\alpha$ -adrenoceptor blockade and mediated by ATP. Since then ATP has been identified as a cotransmitter in most blood vessels studied. For example, sympathetic nerve-mediated vasoconstriction of mouse mesenteric artery was inhibited by ~50% by PPADS and co-application of the  $\alpha_1$ -adrenoceptor antagonist, prazosin, abolished the response ([Vial and Evans, 2002](#)). Neurogenic contractions in vessels from P2X1 knockout mice were about 50% of the amplitude of those in wild-type animals, unaffected by PPADS and abolished by prazosin. ATP can also act at P2Y receptors to elicit vasoconstriction in some arteries (see for example [McLaren et al., 1998a, Chootip et al., 2002, 2005; Mitchell et al., 2012](#)), but there is no evidence that they play any role in neurogenic responses.

The contribution of ATP as a vascular cotransmitter is vessel-dependent. In rat tail artery neurotransmission is predominantly noradrenergic, with ATP mediating only about 10% of the peak response ([Bao et al., 1993](#)). This increases to 20-60% in rabbit central ear artery ([Kennedy et al., 1986](#)) and to 100% in rabbit mesenteric artery ([Ramme et al., 1987](#)). The contribution of ATP is also frequency-dependent, being greater at low than high frequencies and stimulus durations ([Kennedy et al., 1986; Evans and Cunnane, 1992; Bao et al., 1993](#)). Artery diameter also appears to be an important factor in the relative contribution of ATP and NA. In rat small mesenteric arteries ATP and P2X1 receptors predominate in neurogenic contractions, whereas NA and  $\alpha_1$ -adrenoceptors mediate the response of large mesenteric arteries ([Gitterman et al., 2001](#)). In addition, the purinergic component of neurogenic vasoconstriction increases at higher arterial pressure ([Pakdeechote et al., 2007; Rummery et al., 2007](#)). Thus many factors influence the relative contributions of NA and ATP to sympathetic nerve-mediated vasoconstriction.

## *2.5. Signalling mechanisms underlying P2X1 receptor-mediated contractions*

As noted above, P2X1 receptors are ligand-gated cation channels permeable to  $\text{Na}^+$ ,  $\text{K}^+$  and  $\text{Ca}^{2+}$ , so when activated they mediate depolarisation, which in turn causes an increase in cytoplasmic  $[\text{Ca}^{2+}]$  by influx through the receptor pore and/or  $\text{Ca}_v1.2$  voltage-sensitive  $\text{Ca}^{2+}$  channels. The relative contribution of these mechanisms is, however, tissue-specific. For example, P2X<sub>1</sub> receptor-mediated vasoconstriction of rat mesenteric artery is independent of  $\text{Ca}_v1.2$  channels and mediated entirely via the receptor pore (Gitterman and Evans, 2001). Consistent with these data, knockout of the P2X1 receptor abolished the localised, transient rises in  $[\text{Ca}^{2+}]$  evoked by sympathetic nerve stimulation in mouse mesenteric artery (Lamont et al., 2006). Similarly, in small arteries of guinea pig ileum mucosa  $\text{Ca}_v1.2$  channel blockade did not affect contractions evoked by  $\alpha,\beta\text{-meATP}$  (Galligan et al., 1995). In contrast, the  $\text{Ca}_v1.2$  channel blocker, nifedipine, inhibited P2X1 receptor-induced vasoconstriction (Omote et al., 1989; Bulloch et al., 1991) in large mesenteric, ileocolic and saphenous arteries. Another level of complexity is apparent in rat renal vasculature (Inscho et al., 1999, 2001), aorta (Kitajima et al., 1994) and portal vein (Grégoire et al., 1993; Pacaud et al., 1994), where  $\text{Ca}^{2+}$  influx via  $\text{Ca}_v1.2$  channels triggers  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release from ryanodine-sensitive intracellular stores. Thus the mechanisms by which cytoplasmic  $[\text{Ca}^{2+}]$  rises in smooth muscle cells following P2X receptor activation varies in a tissue-specific manner.

$\text{Ca}^{2+}$  initiates smooth muscle contraction by stimulating phosphorylation of myosin light chain (MLC). Dephosphorylation of MLC by MLC phosphatase (MLCP) then reverses the contraction. The ability of many G protein-coupled receptors to inhibit MLCP and so increase the force/ $[\text{Ca}^{2+}]$  ratio of the contractile proteins ( $\text{Ca}^{2+}$ -sensitisation) is well characterised (Somlyo and Somlyo, 2003). This occurs mainly via the  $\text{G}\alpha_{12/13}$  G protein stimulating RhoA, a small GTPase that activates Rho kinase, which in turn phosphorylates the myosin binding subunit (MYPT) of MLCP to depress its phosphatase activity. Intriguingly, the P2X1 receptor, which does not couple directly to G proteins, does nonetheless stimulate RhoA (Lecut et al., 2009). The mechanism is unknown, but may involve depolarisation and  $\text{Ca}^{2+}$  influx (Sakurada et al., 2003). Furthermore, a Rho kinase inhibitor, Y27632, depressed the amplitude of contractions evoked by sympathetic nerve stimulation,  $\alpha,\beta\text{-meATP}$  and  $\alpha$ -adrenoceptor agonists in mouse vas deferens (Büyüksar at al., 2003) and rat tail artery (Yeoh and Brock, 2005). Y27632 also inhibited contractions of rat (Amobi et al., 2006) and human (Amobi et al., 2012) vas deferens and rat kidney afferent arterioles elicited by  $\alpha,\beta\text{-meATP}$  (Inscho et al., 2009). Thus  $\text{Ca}^{2+}$ -sensitisation via Rho kinase is activated downstream of P2X1 receptor stimulation and contributes to the neurotransmitter actions of ATP.

## 2.6. Purinergic neurotransmission in hypertension

Hypertension is recognised as a major health issue, which if uncorrected leads to cardiovascular complications such as increased probability of stroke, myocardial infarction and heart failure. Apart

from a few cases, the underlying cause of hypertension is unknown and although many factors have been suggested to contribute to the genesis of the disease it is, in general, difficult to determine if these factors are caused by, rather than the cause of hypertension. In most cases of essential hypertension, elevated arterial blood pressure is associated with increased peripheral vascular resistance and it has been suggested that an enhanced activity of sympathetic nerves and adaptive structural changes in the vessel wall are important components in this change.

If sympathetic nerve activity is increased in hypertension then it would be reasonable to expect that ATP's cotransmitter role would be increased and this has been investigated in a small number of studies using spontaneously hypertensive rats (SHR). Initial reports indicated an increased contribution by neuronally-released ATP to neurogenic contractions of SHR tail artery compared with control animals ([Vidal et al., 1986](#)), but this was not confirmed in subsequent studies ([Dalziel et al., 1989](#); [Muir and Wardle, 1989](#)). More recently, [Goonetilleke et al., \(2013\)](#) reported that the membrane potential of mesenteric artery smooth muscle cells was similar in normotensive and SHR animals, but sympathetic nerve stimulation evoked larger ejps, with greater facilitation in the latter. A greater purinergic component of neurotransmission has also been seen in vessels from a hypertensive rabbit model ([Bulloch and McGrath, 1992](#)). Thus there are indications of an increased contribution of ATP to control of blood pressure in hypertension, but more research is needed.

## *2.7. Termination of the neurotransmitter actions of ATP: ecto-nucleotidases*

The neurotransmitter actions of NA are terminated by uptake back into sympathetic nerves via a well-characterised transporter. In contrast, ATP is rapidly broken down in the synapse ([Evans and Kennedy, 1994](#); [Kennedy and Leff, 1995](#)) and the majority of ATP released is detected as adenosine ([Todorov et al., 1996](#)). Four extracellular, membrane-bound ecto-nucleoside triphosphate diphosphohydrolases (NTPDase1,2,3 and 8), two ecto-nucleotide pyrophosphatases/phosphodiesterases (NPP1 and 3) and an ecto-5'-nucleotidase have been identified, which dephosphorylate ATP to ADP, AMP and adenosine ([Robson et al., 2006](#)). At present, the only useful inhibitor of these enzymes is ARL 67156, which was developed before they were cloned (see [Sneddon et al., 1999](#) and references therein). ARL 67156 competitively inhibits human NTPDase1 and 3 and NPP1 with moderate potency and is less effective against NTPDase2 and 8, NPP3 and ecto-5'-nucleotidase ([Lévesque et al., 2007](#)).

These ecto-enzymes are ubiquitous in smooth muscle tissues and have long been thought to limit the contractile activity of ATP. Indeed, knockout of NTPDase1 greatly increased both the potency of ATP in mouse aorta and the maximum contraction amplitude ([Kauffenstein et al., 2010](#)). In guinea-pig vas deferens ARL 67156 rapidly and reversibly doubled the amplitude of the initial phasic sympathetic contraction, both in the absence and presence of the  $\alpha_1$ -adrenoceptor antagonist

prazosin (Westfall et al., 1996a) (Figure 4a). In the presence of ARL 67156 the peak response to exogenous ATP was increased by over 60%, but that to  $\alpha,\beta$ -meATP was unchanged (Figure 4b), consistent with selective inhibition of ATP breakdown. Furthermore, ARL 67156 enhanced ATP overflow during sympathetic nerve stimulation by nearly 700% compared to control and increased ejp amplitude, such that ejps often reached threshold for initiation of action potentials and muscle contraction (Sneddon et al., 2000). After the P2X1 antagonist, NF023, had suppressed ejps, co-addition of ARL 67156 significantly increased their magnitude. Thus ecto-enzymes clearly terminate the neurotransmitter actions of ATP, but the contribution of the individual enzyme subtypes remains to be determined.

### *2.8. Termination of the neurotransmitter actions of ATP: soluble nucleotidases*

An exciting development in this area is the discovery that in addition to the established ecto-enzymes on the smooth muscle membrane, stimulation of the sympathetic nerves releases soluble nucleotidases into the extracellular space (Kennedy et al., 1997b; Todorov et al., 1997). When guinea-pig vas deferens was superfused, superfusate collected before nerve stimulation did not contain nucleotidase activity. In contrast, samples of superfusate taken during nerve stimulation had substantial nucleotidase activity that broke down about 90% of exogenously-added ATP. This was inhibited by adding Cd<sup>2+</sup> to, or omitting Ca<sup>2+</sup> from, the superfusate during nerve stimulation, consistent with vesicular release of a factor that promotes ATP breakdown. Exogenous NA, ATP and  $\alpha,\beta$ -meATP, each caused large contractions of the vas deferens, but did not release nucleotidases into the superfusate. The nucleotidase activity was abolished by incubation at 80°C or pH 4.0, greatly reduced by incubation at 4°C, but unaffected by inhibitors of intracellular ATPases, suggesting that “leakage” of these enzymes into the superfusate during stimulation does not contribute to the nucleotidase activity in the superfusate. ARL 67156 reduced ATP breakdown in the superfusate samples from stimulated tissues by about 50%.

The nucleotidase released from guinea-pig vas deferens metabolised ATP at similar rates at 37°C and 20°C (Westfall et al., 2000b). Lineweaver-Burke analysis of the initial rates of ATP hydrolysis gave a K<sub>M</sub> of 39  $\mu$ M and Vmax of 1039 pmol ATP metabolised min<sup>-1</sup>.ml<sup>-1</sup> superfusate. ARL 67156 inhibited ATP breakdown in a concentration-dependent manner with 50% inhibition produced by ~30  $\mu$ M. Similar nucleotidase activity was also released during sympathetic nerve stimulation of rat, mouse (Westfall et al., 2000b) and rabbit (Westfall et al., 2000a) vas deferens. Thus the enzyme has pharmacological and kinetic characteristics that are similar to NTPDases.

As yet, the molecular identities of the nucleotidases released by nerve stimulation that promoted hydrolysis of ATP to ADP, AMP and adenosine have not been determined. Indeed, it is not known if ATP metabolism is mediated by a single enzyme or by several enzymes with different

specificities. The physiological significance of the releasable nucleotidase(s) also remains to be established and a number of questions are raised by such a mechanism. How is the nucleotidase stored and released? Does it come only from the nerve, or also from other sites? If it is stored in neurotransmitter vesicles in the nerve terminal, how could ATP be stored in the same site? What is the relative importance of the releasable nucleotidase and the membrane bound ecto- nucleotidases located on the smooth muscle surface in inactivation of ATP during purinergic neurotransmission? If potent, selective inhibitors of the releasable ATPase can be identified then the physiological significance of these enzymes in modulating purinergic neurotransmission could be determined.

### **3. ATP and ACh as parasympathetic neurotransmitters**

#### *3.1 ATP storage in and release from parasympathetic nerves.*

Parasympathetic nerves provide the major excitatory innervation to the urinary bladder and mediate contraction of the detrusor smooth muscle, which causes voiding of urine (de Groat and Yoshimura, 2001; Andersson and Wein, 2004, Fry et al., 2005; Burnstock, 2014). The classical neurotransmitter released by these nerves is ACh, which acts at postjunctional muscarinic receptors and these are the main target for drugs currently used to treat dysfunctional bladder. As long ago as the 19<sup>th</sup> century, however, a large component of parasympathetic contractions of the urinary bladder of many species was recognised to be resistant to the muscarinic receptor antagonist atropine (Langley and Anderson, 1895) and substantial evidence supports a neurotransmitter role for ATP (Figure 5).

Nerve stimulation-evoked release of ATP occurs in the urinary bladder of many species (see Burnstock, 2014; Mutafova-Yambolieva and Durnin, 2014). Multiple subtypes of voltage-dependent Ca<sup>2+</sup> channels mediate release of ATP and ACh from parasympathetic nerves and in a species-dependent manner. In mouse urinary bladder, ATP release involves predominantly P/Q-type Ca<sup>2+</sup> channels, whilst ACh release depends primarily on N-type and to a lesser extent on P/Q-type Ca<sup>2+</sup> channels (Waterman, 1996). Interestingly, P/Q-type Ca<sup>2+</sup> channels appear to have a negligible role in either ATP or ACh release in rat (Frew and Lundy, 1995). Consistent with this, ACh release depends more on N-type Ca<sup>2+</sup> channels than does ATP release in both rat (Maggi, 1991) and rabbit (Zygmunt et al., 1993) urinary bladder. Also, in guinea-pig, both purinergic ejps and contractions were substantially inhibited by N-type Ca<sup>2+</sup> channel blockade (Maggi et al., 1988; Bramich and Brading, 1996). Which type of Ca<sup>2+</sup> channel mediates the remainder of the responses remains to be determined, but presynaptic L-type Ca<sup>2+</sup> channels are not involved (Hashitani and Suzuki, 1995).

#### *3.2. Functional effects of ATP release: ejps*

The initial response seen on stimulation of parasympathetic nerves is ejps in the postjunctional smooth muscle cells of the urinary bladder. In the guinea-pig these are resistant to atropine, but

blocked by desensitisation of the P2X1 receptor by  $\alpha,\beta$ -meATP (Fujii, 1988; Brading and Mostwin, 1989; Creed et al., 1994; Bramich and Brading, 1996) and by suramin (Hashitani and Suzuki, 1995). Similar effects are seen in the urinary bladder of rabbit (Hoyle and Burnstock, 1985; Fujii, 1988; Creed et al., 1994), pig (Fujii, 1988) and sheep (Creed et al., 1994).

### *3.3. Functional effects of ATP release: contractions*

Contractions of urinary bladder smooth muscle evoked by parasympathetic nerve stimulation are biphasic, although the two phases are not as well defined temporally as in the vas deferens or as well maintained as in the vas deferens or blood vessels. A rapid transient contraction is followed by an irregular contraction, which tends to decay rather than reach a steady level. In most species the ejps summate to evoke depolarisation (Young et al., 2008),  $\text{Ca}^{2+}$  influx (Heppner et al., 2005, 2009),  $\text{Ca}^{2+}$ -sensitisation via Rho kinase (Wibberley et al., 2003) and contraction, similar to as described above in the vas deferens. In 1983, Kasakov and Burnstock showed that the atropine-resistant component of neurogenic contractions in guinea-pig was abolished by desensitisation of P2X1 receptors by  $\alpha,\beta$ -meATP. This was confirmed by Brading and Mostwin, (1989) and Kennedy et al., (2007) (Figure 6) and subsequently, the non-cholinergic component was also shown to be depressed by suramin and PPADS (Hoyle et al., 1990; Creed et al., 1994; Ziganshin et al., 2002). Similar inhibitory effects were seen in the urinary bladder of rat (Brading and Williams, 1990; Luheshi and Zar, 1990; Parija et al., 1991), rabbit (Longhurst et al., 1995; Sneddon and McLees, 1992; Ziganshin et al., 1993; Creed et al., 1994), mouse (Waterman, 1996; Heppner et al., 2009) and ferret (Moss and Burnstock, 1985). These studies were all carried out on bladder muscle strips *in vitro*. Several studies have confirmed that ATP and ACh also function as excitatory cotransmitters *in vivo*, in pithed rats (Hegde et al., 1998), in anaesthetised guinea-pigs (Peterson and Noronha-Blob, 1989) and rabbits (Levin et al., 1986) and even in unanaesthetised rats (Igawa et al., 1993).

It was noticeable in these studies that PPADS and suramin tended to be less effective than  $\alpha,\beta$ -meATP, so we studied their actions in guinea-pig urinary bladder in more detail (Kennedy et al., 2007). Initially we confirmed that atropine-resistant contractions induced by parasympathetic nerve stimulation were abolished by prolonged administration of  $\alpha,\beta$ -meATP. In contrast, although PPADS and suramin inhibited the contractions in a concentration-dependent manner, the peak was only depressed by 50-60% (Figure 7). The remaining responses were abolished by tetrodotoxin, confirming their neurogenic origin. Contractions evoked by  $\alpha,\beta$ -meATP were abolished by both antagonists (Figures 7, 8a), confirming that PPADS and suramin fully blocked P2X1 receptors at the concentrations used. In contrast, the peak response to ATP was reduced by only 40-50% (Figure 7, 8b). Finally, in the presence of atropine, PPADS and suramin also failed to abolish neurogenic contractions of mouse urinary bladder, showing that these effects were not species-dependent.

That PPADS and suramin did not abolish the non-cholinergic component of neurotransmission and contractions evoked by exogenous ATP was unexpected. ATP is much less selective than  $\alpha,\beta$ -meATP for P2X1 receptors and so these data indicate the presence of a further contractile P2 receptor that is activated by ATP, but not  $\alpha,\beta$ -meATP and which is insensitive to PPADS and suramin. We, therefore, investigated the effects of other P2 receptor antagonists. At concentrations that abolished contractions evoked by  $\alpha,\beta$ -meATP, so indicating complete blockade of P2X1 receptors, reactive blue 2, NF279, MRS2159 and NF449, like PPADS and suramin reduced, but did not abolish the atropine-resistant component of neurogenic contractions (Figure 9a). The contractions evoked by ATP were also partially resistant to each of the antagonists (Figure 9b). Notably, all of the antagonists used abolished the non-adrenergic component of neurogenic contractions of the guinea-pig vas deferens ([Kennedy et al., 2007](#)).

That the non-cholinergic neurogenic contractions were not abolished by P2X1 receptor antagonists was initially a surprise, but re-examination of earlier studies shows that the data reported therein are, in fact, consistent with our findings. High concentrations of PPADS ([Ziganshin et al., 2002](#)) and suramin ([Hoyle et al., 1990](#)) inhibited contractions in guinea-pig by 30-40%. Similar effects were seen in rabbit ([Ziganshin et al., 1993; Creed et al., 1994](#)), rat ([Tong et al., 1997; Hegde et al., 1998; Benkó et al., 2003; Knight and Burnstock, 2004](#)) and sheep ([Creed et al., 1994](#)). The inability of P2X1 receptor antagonists to abolish contractions evoked by ATP was also reported in rat ([Bolego et al., 1995; Benkó et al., 2003](#)). The atropine- and P2X1 receptor antagonist-resistant component was more apparent in our study because we constructed full concentration-inhibition curves for each antagonist, whereas the previous studies used fewer concentrations of antagonist.

### *3.4. Functional effects of ATP release: a 2<sup>nd</sup> site of action of ATP?*

These results confirm that ATP, acting via P2X1 receptors, and ACh acting through muscarinic receptors, are excitatory cotransmitters in guinea-pig and mouse urinary bladder. They also identify a third component of the neurogenic contraction, which is resistant to both atropine and P2X1 antagonists. This response was not due to direct electrical stimulation of smooth muscle, as tetrodotoxin abolished all contractions, neuropeptides released from sensory nerves, as they play no role in neurogenic contractions in guinea-pig urinary bladder, or adenosine, produced by breakdown of ATP, as adenosine induces relaxation of detrusor smooth muscle and inhibits contractions elicited by ATP (see [Kennedy et al., 2007](#)). Contractions evoked by exogenous ATP are also partly resistant to P2X1 antagonists, indicating that two functional P2 receptor subtypes are expressed in detrusor smooth muscle. Consistent with this, the concentration-response curve to ATP in this tissue ([Welford et al., 1987; Kennedy, unpublished data](#)) and in humans ([Hoyle et al., 1989](#)) is biphasic.

The identity of the P2X1 antagonist-resistant receptor is unclear, but all other known P2X

receptors, except for the P2X4, are sensitive to at least one of the antagonists used in this study (Khakh et al., 2001; Burnstock and Kennedy, 2011; Kennedy et al., 2013). The P2X4 homomer is probably not involved, as suramin abolished the purinergic ejps in guinea-pig (Hashitani and Suzuki, 1995) and knockout of the P2X1 subunit abolished non-cholinergic, neurogenic contractions and responses to exogenous ATP in the mouse (Vial and Evans, 2000) (although a crucial role of P2X1 subunits in trafficking of P2X4 subunits to the plasma membrane cannot be ruled out). A role for a P2X1+4 heteromer can, however, be considered. P2X1 and P2X4 subunits co-injected into *Xenopus* oocytes copurify and form a novel functional phenotype, at which  $\alpha,\beta$ -meATP is a weak partial agonist (Nicke et al., 2005). This could explain the ability of  $\alpha,\beta$ -meATP to inhibit the P2X1 antagonist-insensitive component of contractions to EFS and ATP. The sensitivity of the P2X1+4 heteromer to suramin was much greater, however, than that of the P2X4 homomer, although a full concentration-inhibition curve was not constructed. Thus more studies are required to determine the involvement of P2X4 subunits in parasympathetic neurotransmission.

It is unlikely that P2Y receptors mediate the P2X antagonist-resistant responses, even though P2Y receptor agonists induce contraction of guinea-pig (Bailey and Hourani, 1994) and rat (Bolego et al., 1995; Naramatsu et al., 1997) bladder. Of the eight cloned P2Y receptors, ATP is an agonist at the P2Y<sub>1</sub>, P2Y<sub>2</sub>, P2Y<sub>4</sub> and P2Y<sub>11</sub> subtypes, but suramin and/or PPADS antagonise each of these (Abbracchio et al., 2006). Furthermore, suramin abolished contractions in the guinea-pig evoked by several agonists active at a range of P2Y receptors, but only partially inhibited those to ATP (Bailey and Hourani, 1994). Additionally, contractions of rat detrusor were evoked by UTP, which acts at several ATP-sensitive P2Y receptors, were unaffected by prolonged exposure to  $\alpha,\beta$ -meATP (Bolego et al., 1995). Further experiments on guinea-pig recombinant P2Y receptors, once cloned, are needed to confirm that P2Y receptors are not involved in parasympathetic neurotransmission.

### *3.5. Termination of the neurotransmitter actions of ATP*

ACh and ATP are both inactivated by breakdown by ecto-enzymes in the synapse. ACh is degraded by acetylcholinesterase. Similar to the vas deferens, ATP can be sequentially dephosphorylated to adenosine by ecto- and soluble nucleotidases released during parasympathetic nerve stimulation (Westfall et al., 1997, 2000b). ARL 67156 potentiated the purinergic component of neurogenic contractions and increased the amplitude of contractions to exogenous ATP, but had no effect on the response to  $\alpha,\beta$ -meATP (Westfall et al., 1997).

### *3.6. Functional effects of ATP release: human urinary bladder*

As discussed above, in most species tested, neurogenic contractions of the urinary bladder comprise of cholinergic and non-cholinergic components. In humans, baboons and rhesus monkeys (i.e. new

world primates), however, neurogenic contractions are abolished or virtually abolished by blockade of muscarinic receptors (Burnstock, 2014; Andersson and Wein, 2004, Fry et al., 2005). The M<sub>3</sub>-muscarinic receptor mediates the neurotransmitter action of ACh via IP<sub>3</sub>, Ca<sup>2+</sup> release and Ca<sup>2+</sup>-sensitisation and these receptors are the main target for drugs currently used to treat dysfunctional bladder (de Groat and Yoshimura, 2001; Andersson and Wein, 2004, Fry et al., 2005). The lack of function for ATP in human urinary bladder is not due to the absence of P2X receptors. The human P2X1 receptor was first isolated from this tissue and exogenous α,β-meATP and ATP cause contraction of bladder muscle strips (Husted et al., 1983; Hoyle et al., 1989; Palea et al., 1994).

### *3.7. Parasympathetic neurotransmission in the human urinary bladder: pathological conditions*

Given that ACh is the sole excitatory neurotransmitter in the healthy human urinary bladder, what is the relevance of ATP and P2X receptors to humans? The potential importance is due to the appearance of atropine-resistant contractions in unhealthy human bladder conditions, such as interstitial cystitis (IC) and idiopathic detrusor instability (IDI) or overactive bladder syndrome (see Fry et al., 2005; Burnstock, 2014). These are chronic bladder disorders, which involve pain in the bladder and pelvic musculature and bladder motor dysfunction. Their aetiology is unknown, but they are probably multi-factorial. Purinergic neurotransmission is discussed in detail elsewhere in this issue, so several important findings will be highlighted here.

In muscle strips prepared from patients suffering from IC, more than 50% of the electrically-evoked contraction was atropine-resistant (Palea et al., 1993). These contractions were abolished by prolonged exposure to α,β-meATP and so assumed to be mediated by ATP and P2X1 receptors, but this has not been confirmed using P2X1 receptor antagonists. Both exogenous ACh and histamine had a lower contractile potency in the affected tissue compared with controls. α,β-meATP was, however, more potent in the tissues from IC patients. Similarly, α,β-meATP inhibited atropine-resistant contractions in muscle strips obtained from patients with unstable bladders, IDI, neurogenic detrusor overactivity or bladder outlet obstruction, though in these tissues there was no change in the potency of ATP (Bayliss et al., 1999; O'Reilly et al., 2002; Fry et al., 2011). Whilst P2X1 is the main P2X subunit present in human detrusor smooth muscle, its expression is unchanged in dysfunctional bladder (Moore et al., 2001; O'Reilly et al., 2002; Chua et al., 2007), although an increase in P2X1 mRNA was apparent in samples from patients with symptomatic outlet obstruction (O'Reilly et al., 2001). It is notable that the P2X4 subunit is also expressed and its colocalisation with nerve varicosities more than doubles in tissue from adults with IDI (Moore et al., 2001; O'Reilly et al., 2002). Interestingly, pregnancy in rats is also associated with an increase in P2X4 subunit junctional clustering (Yunaev et al., 2000) and the PPADS-sensitive component of neurogenic contractions is smaller in pregnant rats (Knight and Burnstock, 2004).

In humans the atropine-resistant component of neurogenic contractions increases with age as does the nerve stimulation-evoked release of ATP ([Yoshida et al., 2001, 2004](#)) (but see [Fry et al., 2011](#)). The change in bladder function in ageing has also been studied in the rat urinary bladder, where contractions to ACh and several other contractile agents were found to be similar in young and old rats ([Saito et al., 1991](#)). The potency of ATP, NA and 5-HT was, however, significantly greater in the older rats. Thus it was suggested that this might contribute to the development of an unstable bladder in elderly people.

Alternative explanations for the appearance of a purinergic component of parasympathetic neurotransmission in human urinary bladder include that more ATP is released from motor nerves and that ATP is broken down less effectively in the synapse ([Fry et al., 2005](#)). To these must be added the possibility that the purinergic component is not mediated by the P2X1 receptor alone and the novel P2X1+4 component postulated by [Kennedy et al \(2007\)](#) plays a role. Dysfunction of the urinary bladder is a major and ever expanding condition for which highly effective therapeutic options are still limited ([de Groat and Yoshimura, 2001; Andersson and Wein, 2004, Fry et al., 2005](#)), so this has important implications for the search for new drugs to treat dysfunctional bladder as it identifies a new therapeutic target for what is a major and expanding therapeutic problem. Furthermore, a receptor that is only functional in dysfunctional urinary bladder is an attractive target for drug development.

#### **4. Conclusion**

The concept of purinergic cotransmission was controversial when first proposed in the 1970s, but it is now accepted to be the norm rather than the exception for nerves in the peripheral nervous system. ATP clearly has a major role as an excitatory cotransmitter in both sympathetic and parasympathetic nerves, but the clinical implications of these roles have not yet been resolved. In part, this is due to a lack of highly selective and potent antagonists for the P2 receptors which mediate the actions of ATP in visceral and vascular smooth muscle. Currently, the most promising possibility is an involvement of ATP and P2 receptors in pathological dysfunctions of the urinary bladder. Clearly, more work is necessary to confirm such an involvement, but the studies discussed above do suggest that this may be a rewarding area of research.

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## Figure legends

### Figure 1. Sympathetic cotransmission.

Schematic representation of cotransmission by ATP and noradrenaline in sympathetic nerves. NA = noradrenaline, P2X1 = P2X1 receptor.

### Figure 2. P2X receptor subunit expression in vascular smooth muscle.

a) Agarose gel electrophoresis of RT-PCR products from rat intrapulmonary artery using specific oligonucleotide primers for rat P2X1-7 subunits is shown. All seven pairs of primers yielded bands in the presence (+) of reverse transcriptase (RT), but there was no signal in its absence (-). The markers on the left hand side show the band size (base pairs). b) A wide-field fluorescence image of rat intrapulmonary artery dissociated smooth muscle cells exposed to an anti-P2X1 receptor primary antibody and the secondary, fluorescent antibody. Scale bar = 100  $\mu$ m. c) shows immunoblots of membrane preparations exposed to the anti-P2X1, P2X2, P2X4 or P2X5 receptor antibodies. Markers on the left hand side show band size. From Syed et al., (2010).

### Figure 3. Sympathetic ejps are purinergic

Ejps in a smooth muscle cell of guinea-pig vas deferens evoked by 1 Hz sympathetic nerve stimulation are shown. Control ejps were ~18 mV amplitude and inhibited in a concentration-dependent manner by NF023. This was reversible on washout of the drug. There was no significant change in the resting membrane potential of the. From Sneddon et al., (2000).

### Figure 4. Modulation of purinergic cotransmission by metabolism of ATP

a) shows the effect of ARL 67156 on neurogenic contractions of the guinea-pig isolated vas deferens evoked by sympathetic nerve stimulation at 4Hz for 20s (start indicated by filled triangle). After a control response (first panel) prazosin (0.1  $\mu$ M) was added, and was present for the remainder of the experiment. 20 min later, nerve stimulation was repeated (second panel). ARL 67156 (100  $\mu$ M) was added and another neurogenic contraction obtained 10 min later (third panel). ARL 67156 was washed out and 10 min later the potentiation was rapidly reversed (fourth panel). PPADS (100  $\mu$ M) was added 20min before the final stimulation (fifth panel).  
 b) shows the effect of ARL 67156 (100  $\mu$ M) on contractions to ATP (100  $\mu$ M) (upper panel) and  $\alpha,\beta$ -meATP (0.5  $\mu$ M) (lower panel). From Westfall et al., (1996a).

### Figure 5. Parasympathetic cotransmission.

Schematic representation of cotransmission by ATP and acetylcholine in parasympathetic nerves. ACh = acetylcholine, P2X1 = P2X1 receptor, P2X1+4 = P2X1+4 heteromer, M3 = M3 receptor.

**Figure 6. Inhibition of non-muscarinic, neurogenic contractions of guinea-pig urinary bladder by  $\alpha,\beta$ -meATP.**

The inhibitory effects of prolonged administration of  $\alpha,\beta$ -meATP on contractions of guinea-pig isolated urinary bladder muscle strips evoked by a) EFS (4 Hz, 20 s) and b) ATP (300  $\mu$ M) are shown. The left hand traces show a control response to EFS or ATP. In the middle panels  $\alpha,\beta$ -meATP (50  $\mu$ M) was added for 10 min and then another 50  $\mu$ M was added for a further 5 min. It was then washed out and the right hand panels show that 5 min later responses to EFS and ATP were abolished. Atropine (1  $\mu$ M) and prazosin (100 nM) were present throughout. Note that the response to  $\alpha,\beta$ -meATP (middle panels) are on a slower time-scale than those to EFS and ATP (left and right panels) due to the very different durations of the responses evoked by these stimuli. From Kennedy et al., (2007).

**Figure 7. Inhibition of non-muscarinic, neurogenic contractions of guinea-pig urinary bladder by PPADS and suramin.**

The inhibitory effects of PPADS and suramin on contractions of guinea-pig isolated urinary bladder muscle strips evoked by EFS, ATP and  $\alpha,\beta$ -meATP are shown. a) The left-hand trace shows a control, noncholinergic contraction evoked by EFS (4 Hz, 20 s), the middle trace shows the response remaining after incubation with PPADS (100  $\mu$ M) for 40 min and the right-hand trace shows that the contractions were abolished by tetrodotoxin (TTX) (1  $\mu$ M). The graphs shows the mean effects of b) PPADS and c) suramin on contractions evoked by EFS ( $\triangle$ ), ATP ( $\blacktriangle$ ) and  $\alpha,\beta$ -meATP ( $\blacksquare$ ). n=5-6. Vertical bars indicate s.e. mean. Atropine (1  $\mu$ M) and prazosin (100 nM) were present throughout. From Kennedy et al., (2007).

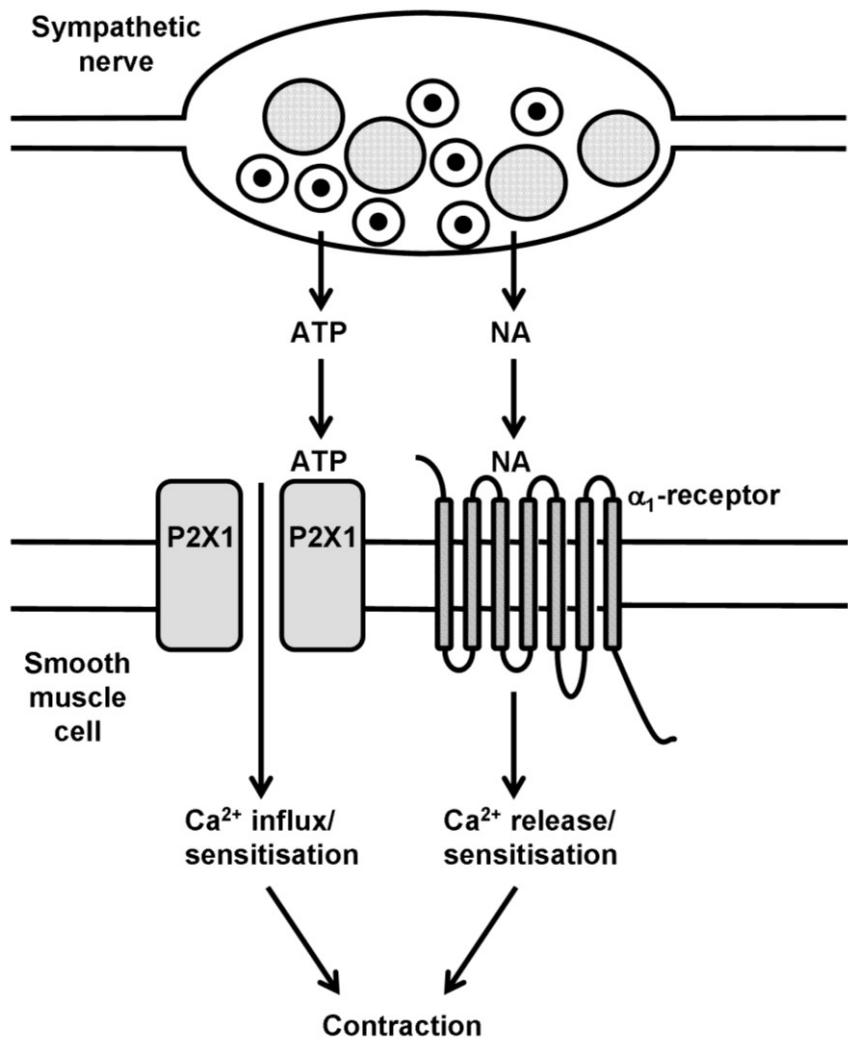
**Figure 8. Inhibition of purinergic contractions of guinea-pig urinary bladder by PPADS and suramin.**

The inhibitory effects of a) PPADS (100  $\mu$ M) on contractions evoked by  $\alpha,\beta$ -meATP (1  $\mu$ M) and b) suramin (300  $\mu$ M) on contractions evoked by ATP (300  $\mu$ M), in guinea-pig isolated urinary bladder muscle strips are shown. In both panels the left-hand trace shows a control contraction and the right-hand trace shows the response in the presence of antagonist. Atropine (1  $\mu$ M) and prazosin (100 nM) were present throughout. From Kennedy et al., (2007).

**Figure 9. Inhibition of non-muscarinic, neurogenic contractions of guinea-pig urinary bladder by P2X receptor antagonists.**

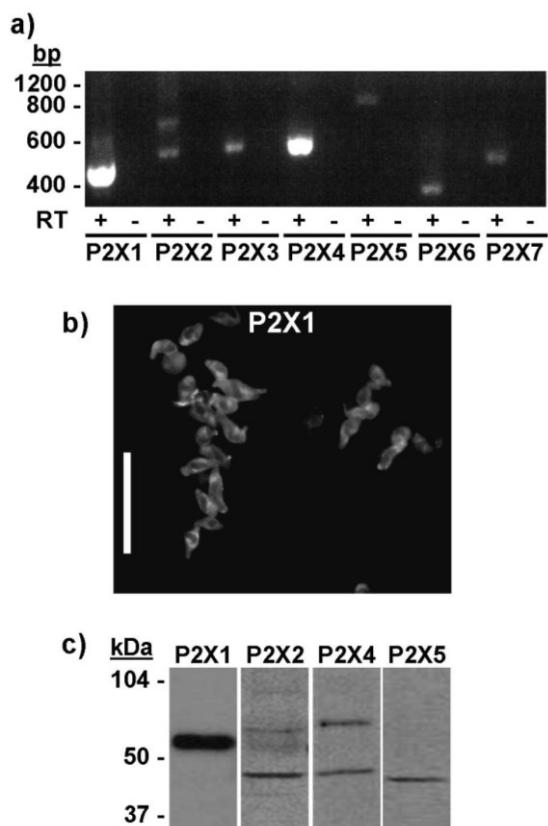
The graphs show the mean effects of PPADS (100  $\mu$ M), suramin (300  $\mu$ M), reactive blue 2 (100

$\mu\text{M}$ ), NF279 (100  $\mu\text{M}$ ), MRS2159 (100  $\mu\text{M}$ ) and NF449 (3  $\mu\text{M}$ ) on contractions of guinea-pig isolated urinary bladder muscle strips evoked by a) EFS (4 Hz, 20 s) ( $n=4-6$ ) and b) ATP (300  $\mu\text{M}$ ) ( $n=4-8$ ). Vertical bars indicate s.e. mean. Atropine (1  $\mu\text{M}$ ) and prazosin (100 nM) were present throughout. From Kennedy et al., (2007).



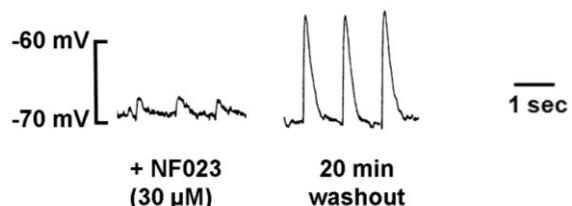
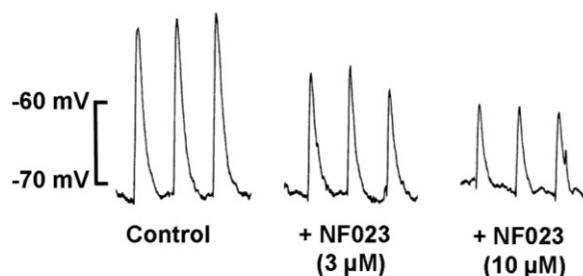
**Figure 1. Sympathetic cotransmission.**

Schematic representation of cotransmission by ATP and noradrenaline in sympathetic nerves. NA = noradrenaline, P2X1 = P2X1 receptor.



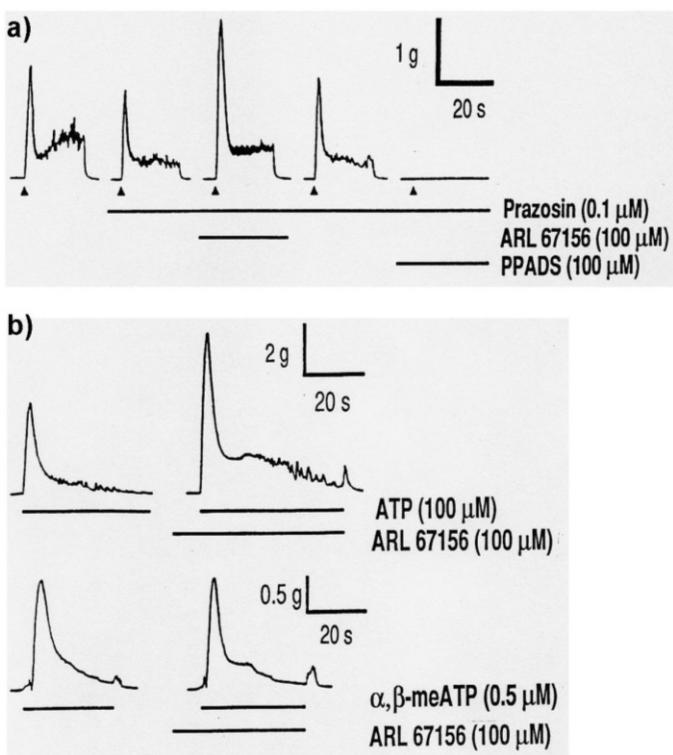
**Figure 2. P2X receptor subunit expression in vascular smooth muscle.**

a) Agarose gel electrophoresis of RT-PCR products from rat intrapulmonary artery using specific oligonucleotide primers for rat P2X1-7 subunits is shown. All seven pairs of primers yielded bands in the presence (+) of reverse transcriptase (RT), but there was no signal in its absence (-). The markers on the left hand side show the band size (base pairs). b) A wide-field fluorescence image of rat intrapulmonary artery dissociated smooth muscle cells exposed to an anti-P2X1 receptor primary antibody and the secondary, fluorescent antibody. Scale bar = 100  $\mu\text{m}$ . c) shows immunoblots of membrane preparations exposed to the anti-P2X1, P2X2, P2X4 or P2X5 receptor antibodies. Markers on the left hand side show band size. From Syed et al., (2010).



**Figure 3. Sympathetic ejps are purinergic**

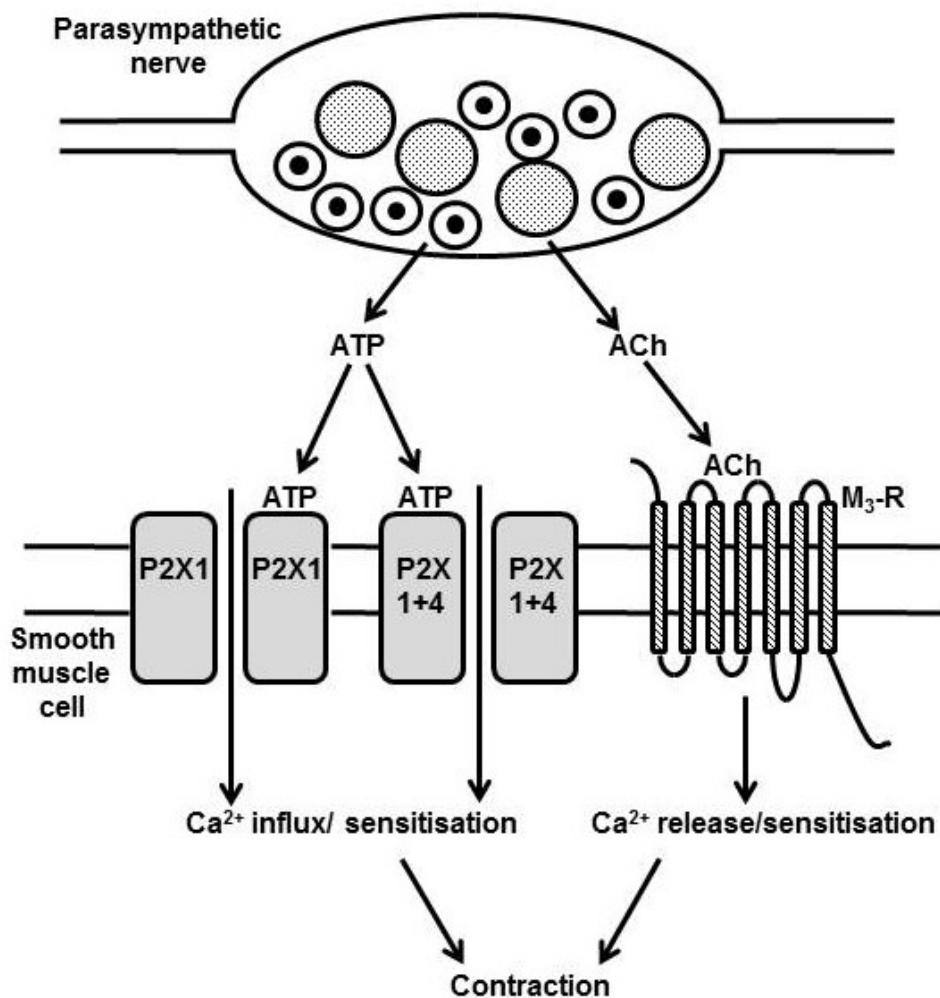
Ejps in a smooth muscle cell of guinea-pig vas deferens evoked by 1 Hz sympathetic nerve stimulation are shown. Control ejps were ~18 mV amplitude and inhibited in a concentration-dependent manner by NF023. This was reversible on washout of the drug. There was no significant change in the resting membrane potential of the. From Sneddon et al., (2000).



**Figure 4. Modulation of purinergic cotransmission by metabolism of ATP**

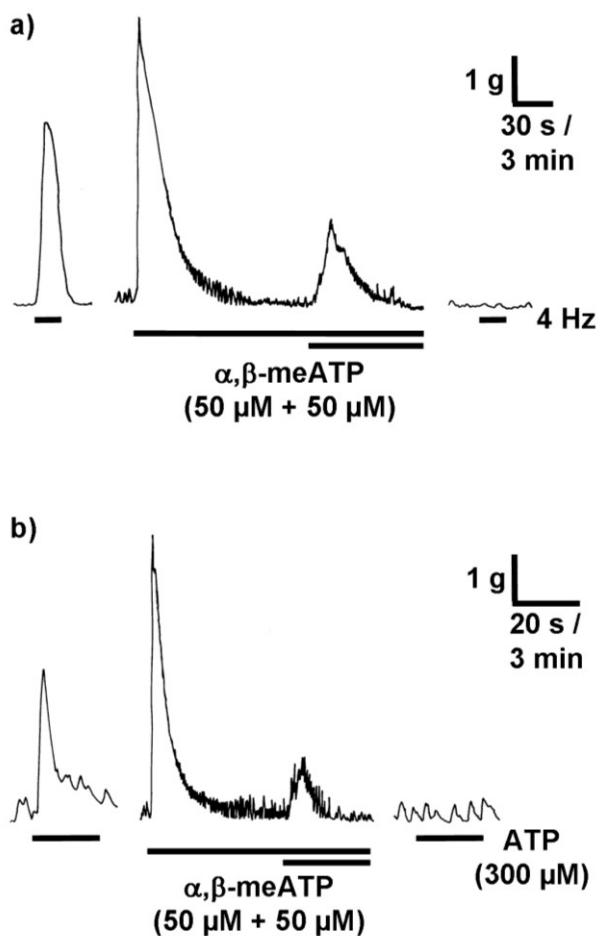
a) shows the effect of ARL 67156 on neurogenic contractions of the guinea-pig isolated vas deferens evoked by sympathetic nerve stimulation at 4Hz for 20s (start indicated by filled triangle). After a control response (first panel) prazosin (0.1 μM) was added, and was present for the remainder of the experiment. 20 min later, nerve stimulation was repeated (second panel). ARL 67156 (100 μM) was added and another neurogenic contraction obtained 10 min later (third panel). ARL 67156 was washed out and 10 min later the potentiation was rapidly reversed (fourth panel). PPADS (100 μM) was added 20min before the final stimulation (fifth panel).

b) shows the effect of ARL 67156 (100 μM) on contractions to ATP (100 μM) (upper panel) and α,β-meATP (0.5 μM) (lower panel). From Westfall et al., (1996a).



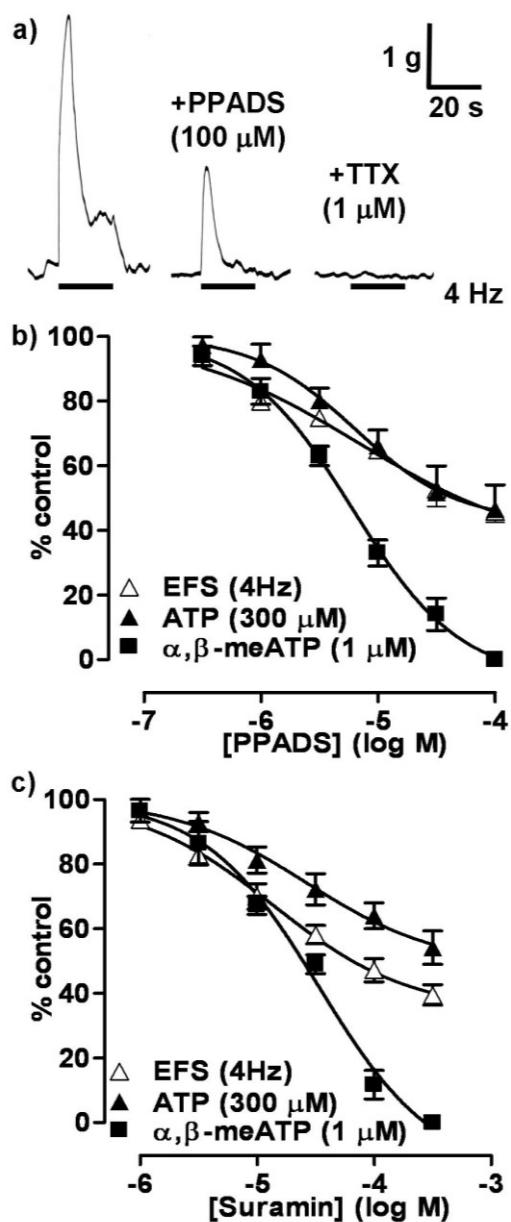
**Figure 5. Parasympathetic cotransmission.**

Schematic representation of cotransmission by ATP and acetylcholine in parasympathetic nerves.  
 ACh = acetylcholine, P2X1 = P2X1 receptor, P2X1+4 = P2X1+4 heteromer, M<sub>3</sub> = M<sub>3</sub> muscarinic receptor.



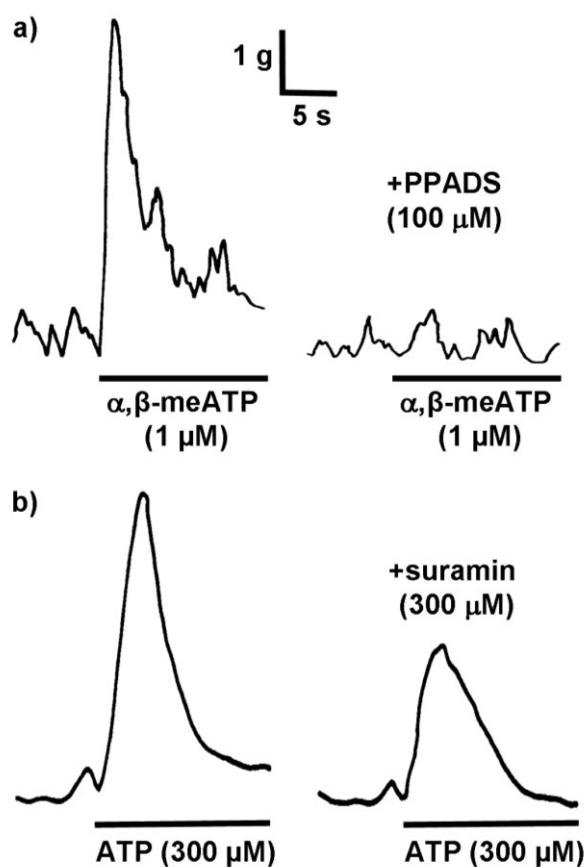
**Figure 6. Inhibition of non-muscarinic, neurogenic contractions of guinea-pig urinary bladder by  $\alpha,\beta$ -meATP.**

The inhibitory effects of prolonged administration of  $\alpha,\beta$ -meATP on contractions of guinea-pig isolated urinary bladder muscle strips evoked by a) EFS (4 Hz, 20 s) and b) ATP (300  $\mu$ M) are shown. The left hand traces show a control response to EFS or ATP. In the middle panels  $\alpha,\beta$ -meATP (50  $\mu$ M) was added for 10 min and then another 50  $\mu$ M was added for a further 5 min. It was then washed out and the right hand panels show that 5 min later responses to EFS and ATP were abolished. Atropine (1  $\mu$ M) and prazosin (100 nM) were present throughout. Note that the response to  $\alpha,\beta$ -meATP (middle panels) are on a slower time-scale than those to EFS and ATP (left and right panels) due to the very different durations of the responses evoked by these stimuli. From Kennedy et al., (2007).



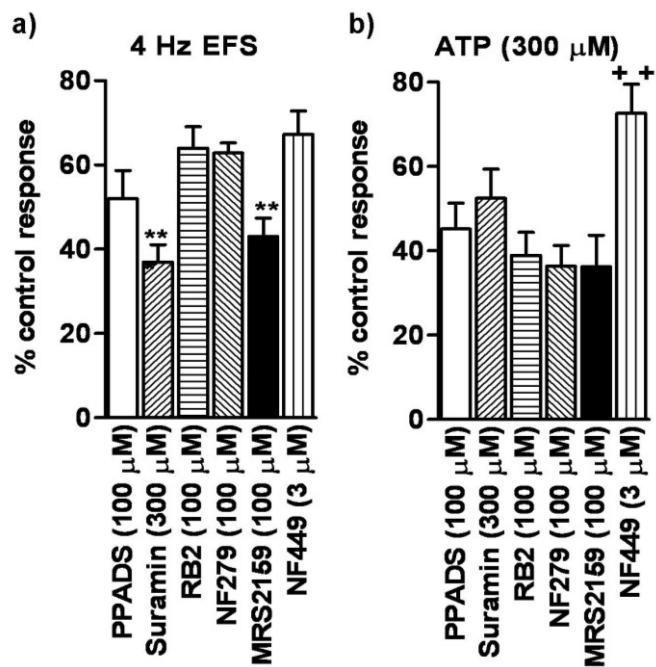
**Figure 7. Inhibition of non-muscarinic, neurogenic contractions of guinea-pig urinary bladder by PPADS and suramin.**

The inhibitory effects of PPADS and suramin on contractions of guinea-pig isolated urinary bladder muscle strips evoked by EFS, ATP and  $\alpha$ , $\beta$ -meATP are shown. a) The left-hand trace shows a control, noncholinergic contraction evoked by EFS (4 Hz, 20 s), the middle trace shows the response remaining after incubation with PPADS (100  $\mu$ M) for 40 min and the right-hand trace shows that the contractions were abolished by tetrodotoxin (TTX) (1  $\mu$ M). The graphs show the mean effects of b) PPADS and c) suramin on contractions evoked by EFS ( $\triangle$ ), ATP ( $\blacktriangle$ ) and  $\alpha$ , $\beta$ -meATP ( $\blacksquare$ ). n=5-6. Vertical bars indicate s.e. mean. Atropine (1  $\mu$ M) and prazosin (100 nM) were present throughout. From Kennedy et al., (2007).



**Figure 8. Inhibition of purinergic contractions of guinea-pig urinary bladder by PPADS and suramin.**

The inhibitory effects of a) PPADS (100  $\mu\text{M}$ ) on contractions evoked by  $\alpha,\beta\text{-meATP}$  (1  $\mu\text{M}$ ) and b) suramin (300  $\mu\text{M}$ ) on contractions evoked by ATP (300  $\mu\text{M}$ ), in guinea-pig isolated urinary bladder muscle strips are shown. In both panels the left-hand trace shows a control contraction and the right-hand trace shows the response in the presence of antagonist. Atropine (1  $\mu\text{M}$ ) and prazosin (100 nM) were present throughout. From Kennedy et al., (2007).



**Figure 9. Inhibition of non-muscarinic, neurogenic contractions of guinea-pig urinary bladder by P2X receptor antagonists.**

The graphs show the mean effects of PPADS (100  $\mu\text{M}$ ), suramin (300  $\mu\text{M}$ ), reactive blue 2 (100  $\mu\text{M}$ ), NF279 (100  $\mu\text{M}$ ), MRS2159 (100  $\mu\text{M}$ ) and NF449 (3  $\mu\text{M}$ ) on contractions of guinea-pig isolated urinary bladder muscle strips evoked by a) EFS (4 Hz, 20 s) (n=4-6) and b) ATP (300  $\mu\text{M}$ ) (n=4-8). Vertical bars indicate s.e. mean. Atropine (1  $\mu\text{M}$ ) and prazosin (100 nM) were present throughout. From Kennedy et al., (2007).