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REVIEW

Evolutionary origins of the purinergic signalling system

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Abstract

Purines appear to be the most primitive and widespread chemical messengers in the animal and plant kingdoms. The evidence for purinergic signalling in plants, invertebrates and lower vertebrates is reviewed. Much is based on pharmacological studies, but important recent studies have utilized the techniques of molecular biology and receptors have been cloned and characterized in primitive invertebrates, including the social amoeba *Dictyostelium* and the platyhelminth *Schistosoma*, as well as the green algae *Ostreococcus*, which resemble P2X receptors identified in mammals. This suggests that contrary to earlier speculations, P2X ion channel receptors appeared early in evolution, while G protein-coupled P1 and P2Y receptors were introduced either at the same time or perhaps even later. The absence of gene coding for P2X receptors in some animal groups [e.g. in some insects, roundworms (*Caenorhabditis elegans*) and the plant *Arabidopsis*] in contrast to the potent pharmacological actions of nucleotides in the same species, suggests that novel receptors are still to be discovered.

Keywords adenosine, ATP, invertebrate, lower vertebrate, P2 receptor, plants.

A thorough review of the early literature about purinergic signalling in invertebrates and lower vertebrates was published in 1996 and speculations made about the evolution of receptors for purines and pyrimidines (Burnstock 1996). In this article we will overview the considerable advances made about the comparative physiology and the evolutionary tree of purinergic signalling since 1996 aiming to explore whether indeed the purines act as the most basic molecules for intercellular communications throughout the animal and plant kingdoms.

Purinergic signalling system

The purinergic signalling system employs extracellular purines (most notably ATP and adenosine) and pyrimidines as signalling molecules. Both purine and pyrimidine nucleotides are released from living cells via several physiologically relevant mechanisms (Fig. 1), which include exocytosis, diffusion through membrane channels and via transporters (North & Verkhratsky 2006, Pankratov *et al.* 2006, Burnstock 2007, Abbracchio *et al.* 2009). Furthermore purines and pyrimidines are released from dying cells, being early and universal indicators of cell damage (Burnstock 2007, 2008a). Immediately after release ATP and other nucleotides are enzymatically degraded by an extended family of ectonucleotidases (Zimmermann 2006); this process is physiologically relevant as ATP metabolites also act as purinergic signalling molecules. This multitude of transmitters act upon target cells through activation of three classes of the receptors (Fig. 2), the metabotropic P1 receptors to adenosine, and nucleotide receptors of the P2 family, which is further subdivided into P2Y metabotropic and P2X ionotropic sub-classes (Burnstock & Kennedy 1985, Ralevic & Burnstock 1998, Abbracchio *et al.* 2006, 2009, Burnstock 2007). The P1 class comprises four types of G protein-coupled adenosine

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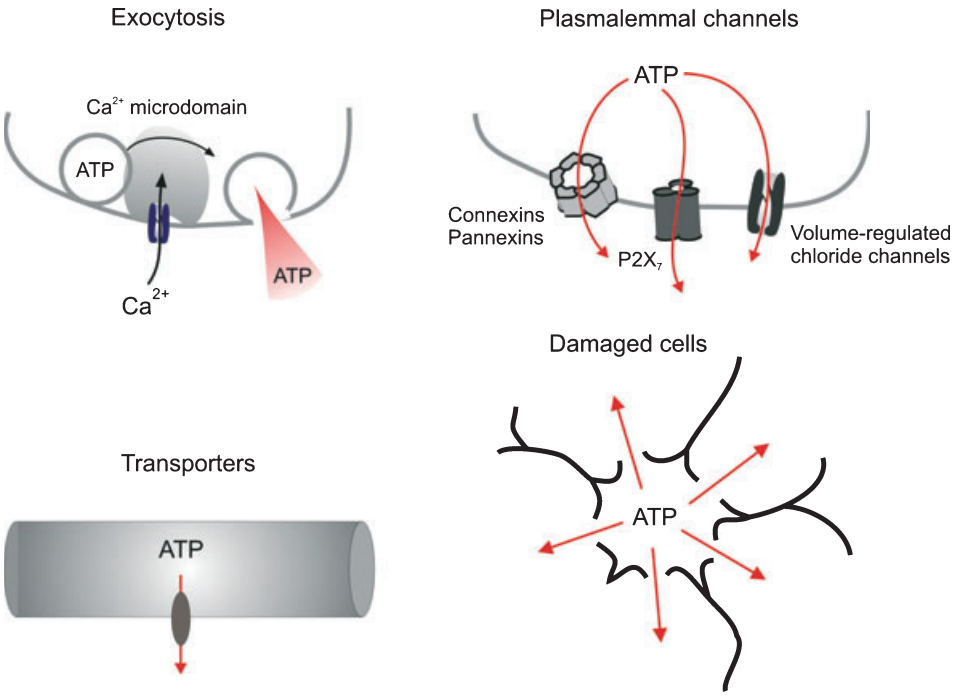

Figure 1 Pathways for ATP release from cells.

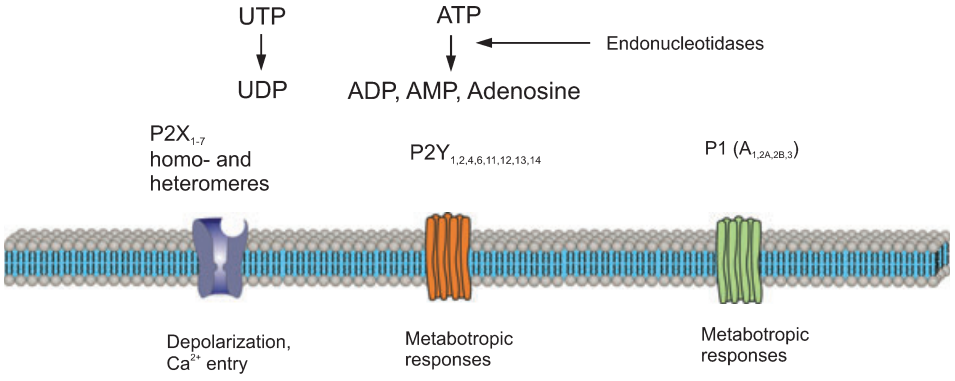

Figure 2 Purinergic receptors.

receptors A_1, A_{2A}, A_{2B} and A_3. These receptors are generally coupled to adenylate cyclase, activation of the A_1 and A_3 receptors have an inhibitory effect, whereas A_{2A} and A_{2B} stimulate production of cyclic AMP (cAMP) (Fredholm *et al.* 2001, Abbracchio *et al.* 2009). The P2X class represents ionotropic receptors, which are classic cationic (Na^+, K^+ and Ca^{2+}) ATP-gated channels composed of seven major subunits $P2X_1$ to $P2X_7$ (North 2002, Burnstock 2007, Surprenant & North 2008, Abbracchio *et al.* 2009). The P2Y receptors are nucleotide-sensitive G protein-coupled receptors, which act through second messenger systems of cAMP or inositol triphosphate ($InsP_3$) (Abbracchio *et al.* 2006).

The omnipresence of purinergic signalling

The brilliant pioneers of chemical neurotransmission, including Langley, Elliot, Loewi, von Euler and Dale, focused on acetylcholine (ACh) and noradrenaline (NA), and it was not until 1970 that non-adrenergic, non-cholinergic neurotransmission was recognized and ATP proposed as a neurotransmitter (see Burnstock 1972). Later 'Dales Principle' which, erroneously, came to present the view that one nerve only utilized one transmitter was challenged (Burnstock 1976, 2009a) and it is now clear that ATP is a cotransmitter in most, if not all, nerves in the peripheral nervous system (PNS) and central nervous system (CNS) (see Table 1;

Table 1 ATP as a ubiquitous co-transmitter

	Cotransmitters	References
Peripheral nervous system		
Sympathetic nerves	ATP + NA + NPY	Westfall *et al.* (1978)
Parasympathetic nerves	ATP + ACh + VIP	Hoyle (1996)
Sensory-motor	ATP + CGRP + SP	Burnstock (1993)
NANC enteric nerves	ATP + NO + VIP	Belai & Burnstock (1994)
Motor nerves (in early development)	ATP + ACh	Silinsky & Hubbard (1973)
Central nervous system		
Cortex, caudate nucleus	ATP + ACh	Richardson & Brown (1987)
Hypothalamus, locus coeruleus	ATP + NA	Sperlagh *et al.* (1998)
Hypothalamus, dorsal horn, retina	ATP + GABA	Jo & Role (2002)
Mesolimbic system	ATP + DA	Krugel *et al.* (2003)
Hippocampus, dorsal horn, cortex	ATP + glutamate	Mori *et al.* (2001), Pankratov *et al.* (2002, 2003)

ACh, acetylcholine; ATP, adenosine 5′-triphosphate; CGRP, calcitonin gene-related peptide; DA, dopamine; GABA, γ-aminobutyric acid; NA, noradrenaline; NANC, non-adrenergic, non-cholinergic; NO, nitric oxide; NPY, neuropeptide Y; SP, substance P; VIP, vasoactive intestinal polypeptide. Compiled from Burnstock (2007).

Burnstock 2007, 2008b, 2009b). Furthermore, purines and/or pyrimidines act as signalling molecules in virtually all non-neuronal tissues (see Tables 2–4).

Several extracellular signalling systems are present in the human body, these systems being divided into classic transmitters (which mediate neuronal signal transmission), paracrine and autocrine transmission and hormones, which exert their action through blood flow. As a rule, transmitter systems are anatomically and functionally segregated. For example: glutamate acts as an excitatory neurotransmitter in the CNS; cholinergic transmission is prominent at somatic and autonomic neuroeffector junctions and in some brain areas; γ-aminobutyric acid acts largely as a transmitter of inhibitory responses in the brain and spinal cord; NA is a major transmitter in the sympathetic nervous system and some parts of the brain; glycine is localized as an inhibitory transmitter largely in the spinal cord; and 5-hydroxytryptamine, while diffusely distributed, is limited in its transmission activities. Even stricter segregation applies to other neurotransmitter systems, such as domapinergic or peptidergic. Some of these transmitters are also released from non-neuronal cells. ATP, however, is unique, as it has virtually no anatomical segregation. Indeed, in the nervous system ATP acts as a co-transmitter in nerves in both CNS and PNS. ATP appears to be released as the principal neurotransmitter in some terminals in the medial habenula (Robertson & Edwards 1998). In the cortex, ATP is released from a separate pool of vesicles, which share the same terminals with glutamate (Pankratov *et al.* 2007). In the PNS ATP is released as the only transmitter from sympathetic nerves supplying submucosal arterioles in the intestine, while NA released from these nerves acts only as a pre-junctional neuromodulator (Evans & Surprenant 1992). ATP also acts as a major gliotransmitter, and all types of glia studied so far express various subtypes of purinoceptors (Farber & Kettenmann 2006, Fields & Burnstock 2006).

However, the role of ATP as a signalling molecule is not limited to the nervous system as indeed ATP sensitivity and ATP-mediated signalling has been identified in virtually all tissues and cell types (Tables 2 and 3). Finally, the ATP signalling system shows another unique feature, namely, the multitude of release pathways. Indeed ATP can be released by exocytosis, via transmembrane channels, via transporters or through damaged membranes (Fig. 1). Therefore, ATP appears to be the most widespread and omnipresent of all known extracellular signalling molecules, which appeared very early in evolution (see Table 4), as we shall overview below.

Invertebrates

Bacteria

Although our knowledge of the chemical sensitivity of bacteria is quite fragmentary, there is substantial evidence demonstrating that purines and pyrimidines exert a wide range of actions on bacteria (for a detailed overview of earlier work, see Burnstock 1996). Adenosine inhibits growth of several bacteria species, including *Crithidia fasciculate* (Dewey *et al.* 1978), *Staphylococcus aureus* (Mathieu *et al.* 1969) and *Micrococcus sodonensis* (Shobe & Campbell 1973). Methyl-adenosine similarly arrests the proliferation of *Mycobacterium tuberculosis* (Parker & Long 2007).

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Table 2 Tissue presence of principal P1 and P2 receptors

Neurones	
Sympathetic neurones	$P2X_{1–7}$, $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, A_1
Parasympathetic neurones	$P2X_2$, $P2X_3$, $P2X_4$, $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_{11}$, A_1
Sensory neurones	$P2X_{1–7}$, $P2Y_1$, $P2Y_2$, $P2Y_4$, A_{2A}
Enteric neurones	$P2X_3$, $P2X_4$, $P2X_7$, $P2Y_1$, $P2Y_6$, $P2Y_{12}$, A_1, A_{2A}, A_{2B}
CNS neurones	$P2X_4$, $P2X_6$, $P2Y_1$, $P2Y_6$, $P2Y_{12}$, A_1, A_{2A}, $A_{2B,}$ A_3
Glia	
Astrocytes	$P2X_{1/5}$, $P2X_7$ (reactive astroglia), P2Y, A_1
Oligodendrocytes	$P2X_7$, $P2Y_1$, $P2Y_{11}$
Microglia	$P2Y_4$, $P2Y_7$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, A_1, A_2
Special senses	
Inner ear	$P2X_1$, $P2X_2$, $P2X_3$, $P2X_7$, $P2Y_2$, $P2Y_4$, A_1
Eye	$P2X_2$, $P2X_7$, $P2Y_2$, A_1, A_2, A_3,
Tongue	$P2X_2$, $P2X_3$, $P2Y_1$, A_1
Olfactory organ	$P2X_2$, $P2X_4$, $P2Y_1$, $P2Y_2$, A_{2A}, A_3
Muscle cells	
Smooth muscle	$P2X_{1–7}$, $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, A_1, A_{2A}, A_{2B}, A_3
Skeletal muscle	
Developing	$P2X_2$, $P2X_5$, $P2X_6$, $P2Y_1$, $P2Y_2$
Adult	$P2X_{1–7}$, $P2Y_2$, A_{2A}
Cardiac muscle	$P2X_{1–6}$, $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, A_1, A_3
Non-neuronal cells	
Osteoblasts	$P2X_7$, $P2Y_1$, $P2Y_2$
Cartilage	$P2X_2$, $P2Y_1$, $P2Y_2$, A_{2A}, A_{2B}
Keratinocytes	$P2X_5$, $P2X_2$, $P2X_3$, $P2X_7$, $P2Y_1$, $P2Y_2$, $P2Y_4$, A_{2B}
Fibroblasts	$P2X_7$, $P2Y_1$, $P2Y_2$, A_{2A}
Adipocytes	$P2X_1$, $P2Y_1$, $P2Y_2$, $P2Y_4$, A_1
Epithelial cells	$P2X_5$, $P2X_6$, $P2X_4$, $P2X_7$, $P2Y_1$, $P2Y_2$, $P2Y_{11}$, A_1, A_{2A}, A_3
Hepatocytes	$P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{13}$, A_{2A}, A_3
Sperm	$P2X_2$, $P2X_7$, $P2Y_2$, A_1
Endothelial cells	$P2X_1$, $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, A_1, A_{2A}
Erythrocytes	$P2X_2$, $P2X_4$, $P2X_7$, $P2Y_1$
Platelets	$P2X_1$, $P2Y_1$, $P2Y_{12}$, A_{2A}
Immune cells	$P2X_4$, $P2X_7$, $P2Y_1$, $P2Y_2$, A_{2A}, A_3
Exocrine secretary cells	$P2X_1$, $P2X_4$, $P2X_7$, $P2Y_1$, $P2Y_2$, $P2Y_4$, A_1, A_{2A}
Endocrine secretory cells	$P2X_{1–7}$, $P2Y_2$, $P2Y_4$, A_1, A_{2A}, A_{2B}, A_3

Modified from Knight (2009), with permission from Elsevier.

Purines and pyrimidines initiate sporulation in *Bacillus subtilis* (Rhaese *et al.* 1972, Pun & Pennington 1981) and inhibit spore germination in *Streptomyces galilaeus* (Hamagishi *et al.* 1980). Extracellular ATP and its analogues stimulate growth, differentiation and protein synthesis in *Streptomyces coelicolor* A3 at 10 μM and inhibits them at 100 μM (Li *et al.* 2008). A survey of 86 heterotrophic bacteria showed that certain genera produce high levels of extracellular ATP (Ivanova *et al.* 2006).

The molecular nature of purinergic signalling systems in bacteria remains to be elucidated; ATP binding, however, was demonstrated for the epsilon subunit of F(1) ATPase from thermophilic *Bacillus* PS3, which might be a candidate for an ancient ATP receptor site (Kato *et al.* 2007b). Ecto-nucleoside triphosphate diphosphohydrolase (NTPDase) was identified from *Legionella pneumophila*, but kinetic studies showed differences from mammalian NTPDase (Sansom *et al.* 2008). A high-affinity adenine binding site has been identified in *Achromobacter xylosoxidans* suggestive of an adenine receptor, although it differs from adenine receptors found in mammals (Schiedel *et al.* 2008).

Protozoa

The inhibitory effects of extracellular ATP on amoeboid movement (Zimmerman *et al.* 1958) and on the output from contractile vacuoles (Pothier *et al.* 1987) have been recognized for many years (for details, see Burnstock 1996). Importantly, ATP triggers depolarization of amoeba, which involves increases in sodium permeability (Burnstock 1996). *Dictyostelium discoideum*, a social amoeba, is a protist that emerged in evolution after plants and from an ancestor common to fungi and animals (Baldauf *et al.* 2000). ATP was shown to be present extracellularly in suspensions of *D. discoideum* at concentrations of 0.1–0.8 μM and that these amoebae were in possession of Mg^{2+}-dependent ecto-ATPases (Parish & Weibel 1980). They also found that addition of ATP in micromolar concentrations stimulated Ca^{2+}influx into *D. discoideum*; this influx was blocked by suramin, which prompted the authors to consider the role for ecto-ATPase in regulating Ca^{2+}transport; in reality they were, in all probability, observing activation of Ca^{2+}-permeable P2X-like receptors. Recently, the expression of P2X-like receptors was directly identified in the vacuolar membranes of *D. discoideum* (Ludlow & Ennion 2006, Fountain *et al.* 2007). The gene encoding this receptor showed some resemblance to the human P2X genes. Expression of the receptor (codenamed *Dd2PX*) in the heterologous HEK293 system revealed an ATP-gated channel, activated by ATP and some of its analogues, $\beta\gamma$-imido-ATP (which was 10 times more potent than ATP), and α,β-methylene ATP (α,β-meATP). The amoebaean ATP-receptor was not sensitive to other nucleotides (e.g. UTP, CTP, ADP, cyclic AMP, etc.) and was not inhibited by P2X

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Table 3 Physiological role of purinergic signalling in living tissues

Tissue	Functional role	References
CNS	Fast excitatory co-transmission in CNS, modulation of synaptic plasticity, metabotropic transmission, regulation of growth and development, chemical transmission in neuronal-astroglial networks; signalling between axons and oligodendrocytes, CO_2 chemosensitivity, control of microglial motility and activation	Kirischuk *et al.* (1995a,b), Gourine *et al.* (2005), Farber & Kettenmann (2006), Fields & Burnstock (2006), North & Verkhratsky (2006), Burnstock (2007), Hamilton *et al.* (2008), Lalo *et al.* (2008), Abbracchio *et al.* (2009), Pankratov *et al.* (2009)
PNS	Nociception, thermal sensitivity, mechanosensitivity, chemosensitivity, neuronal-effector transmission	Burnstock & Wood (1996), Cook *et al.* (1997), Souslova *et al.* (2000), Rong *et al.* (2003), Burnstock & Knight (2004), North (2004), Khakh & North (2006), Khmyz *et al.* (2008)
Cardiovascular system: heart	Negative chronotripic and ionotropic effects in atria, positive chronotropic and ionotropic effect in ventricles, regulation of cardiomyocytes Ca^{2+} signalling, control of excitation of intrinsic cardiac neurones	Burnstock & Knight (2004), Erlinge & Burnstock (2008)
Cardiovascular system: blood vessels	Vasodilation (P2Y-mediated) and vasoconstriction (P2X-mediated)	Burnstock & Knight (2004), Harrington & Mitchell (2004), Harrington *et al.* (2007), Erlinge & Burnstock (2008)
Exocrine glands	Regulation of ionic permeability and Ca^{2+} signalling in salivary and lachrymal gland cells, induction of sweat production by sweat gland epithelial cells	Burnstock & Knight (2004)
Endocrine glands	Regulation of Ca^{2+} signalling in pituitary and thyroid cells, regulation of cathecholamine release from adrenal chromaffin cells, stimulation of insulin, glucagons and somatostatin secretion from endocrine pancreas	Burnstock & Knight (2004)
Immune system	Regulation of mitogenesis and DNA synthesis in thymocytes, regulation of activation and death of macrophages, aggregation of neutrophiles, regulation of secretory response in basophiles, and chemotactic response in eosynophiles, modulation of proliferative response in lympocytes, release of histamine and degranulation of mast cells, mediation of intercellular Ca^{2+} waves in mast cells, regulation of release of proinflammatory factors	Osipchuk & Cahalan (1992), Brough *et al.* (2003), Burnstock & Knight (2004), Coutinho-Silva *et al.* (2005), Chen & Brosnan (2006), Vaughan *et al.* (2007), Pelegrin *et al.* (2008)
Lung	Bronchodilation, stimulation of surfactant release from airway epithelial cells; stimulation of mucin secereton from goblet cells; increase in ciliary beat frequency of ciliated epithelial cells, activation of lung myeloid dendritic cells; modulation of O_2 chemotransmission in cells of neuroepithelial bodies; contraction/relaxation of tracheal ring	Burnstock & Knight (2004), Fu *et al.* (2004), Hayashi *et al.* (2005), Mounkaila *et al.* (2005), Idzko *et al.* (2007)
Gastrointestinal tract	Control of mucociliary activity of oesophageal epithelial cells, regulation of acid secretion in gastric mucosa, regulation of contraction/relaxation of small intestine, inhibition of ACh release from enteric neurones, regulation of peristaltic activity of ileum and duodenum, inhibition of amino acid, sugar and ion transport in epithelial cells of small intestine, relaxation of taenia coli, control of contraction/relaxation of colon and rectum, relaxation of internal anal sphincter	Cooke *et al.* (2003), Burnstock & Knight (2004), Furuzono *et al.* (2005), Giaroni *et al.* (2006), Van Crombruggen *et al.* (2007), Burnstock (2009b)

Table 3 *(Continued)*

Tissue	Functional role	References
Liver	Stimulation of glycogenolysis, inhibition of glycolysis, regulation of bile formation and secretion via stimulation of Cl^- efflux, mediate chemosensitivity of cholangiocyte cilia	Roman *et al.* (1999), Burnstock & Knight (2004), Doctor *et al.* (2005), Dutta *et al.* (2008), Masyuk *et al.* (2008)
Kidney	Regulation of renal blood flow, microvascular function and glomerular filtration rate, generation of prostanoids, regulation of Cl^- secretion, regulation of renal Na^+, glucose and water transport, possible involvement in biosensing activity of kidney macula densa cells	Liu *et al.* (2002), Bell *et al.* (2003), Lee *et al.* (2005), Guan *et al.* (2007), Vallon (2008), Wildman & King (2008)
Bladder and urethra	Control of contraction/relaxation of mammalian bladder, relaxation of mammalian urethra	Burnstock & Knight (2004), Werkstrom & Andersson (2005), Ford *et al.* (2006), Ruggieri (2006), Chopra *et al.* (2008)
Male genital system	Regulation of penile erection, contraction of prostate smooth muscle and seminal vesicles, micturition, peristalsis of the male excurrent duct system and thus sperm transport and ejaculation; control of steroid production by testis leydig cells, inhibition of sperm motility, initiation of acrosome reaction	Banks *et al.* (2006), Poletto Chaves *et al.* (2006), Gur *et al.* (2007), Lau *et al.* (2007)
Female genital system	Regulation of myometrium contraction, modulation of ovarian function, control of blood flow in placenta, relaxation of vaginal smooth muscle, stimulation of vaginal moisture production, stimulation of Cl^- and mucus secretion from endocervical epithelial cells	Piper & Hollingsworth (1996), Bardini *et al.* (2000), Min *et al.* (2003), Burnstock & Knight (2004), Katugampola & Burnstock (2004), Papka *et al.* (2005), Ziganshin *et al.* (2006)
Bone and cartilage	Regulation of osteoclast/bone formation and resorption, formation of multinucleated osteoclasts, stimulation of resorption in cartilage, regulation of chondrocalcinosis	Burnstock & Knight (2004), Gallagher (2004)
Skeletal muscle	Regulation of proliferation and differentiation of developing myoblasts, modulation of contractile response of myocytes	Burnstock & Knight (2004)

receptors antagonists. This channel was equally permeable for $Ca^{2+}$and Na^+ and the single-channel conductance was ~8.2 pS. *In situ*, the *Dd2PX* receptors are mostly present in the membranes of contractile vacuoles and are directly involved in osmoregulation (Fountain *et al.* 2007).

In addition to P2X receptors with intracellular functions, *Dictyostelium* also possesses plasmalemmal ionotropic ATP receptors. In apoaequorin-expressing strains of amoeba, application of ATP and ADP triggered elevation in intracellular Ca^{2+} with an EC_{50} of 7.5 and 6.1 μM respectively (Ludlow *et al.* 2008). These Ca^{2+} responses required extracellular Ca^{2+} and were completely blocked by Gd^{3+} thus indicating Ca^{2+} influx through ATP-gated channels (although the authors could not completely exclude a small metabotropic component mediated through P2Y-like receptors; Ludlow *et al.* 2008). In fact *Dictyostelium* is endowed with five P2X genes, (also labelled as P2XA–E, P2XA being homologous to the *Dd2PX* gene) (Kreppel *et al.* 2004, Ludlow *et al.* 2008). Which one is responsible for plasmalemmal purinoceptors remains unknown, although disruption of P2XA and P2XE genes did not affect ATP-induced Ca^{2+} signals (Ludlow *et al.* 2008). *Dictyostelium* also expresses metabotropic cAMP receptors, which may represent some ancestors of P2Y purinoceptors (Ludlow *et al.* 2008).

Amoebas also are in possession of ATP-degrading systems; as ecto-nucleotide triphosphatase activity has been identified in *Entamoeba*, this enzyme could regulate extracellular ATP-dependent processes and perhaps protection from the cytotoxic effects of ATP (Cerbon & Olguin 1997).

The ciliates *Paramecium* and *Tetrahymena* show high sensitivity to nucleotides, ATP and GTP by producing avoiding reactions in response to micromolar concentrations of both agents (Clark *et al.* 1993, Kim *et al.* 1999). These avoiding reactions are triggered by ATP/GTP-induced membrane depolarization (and this ATP and GTP belong to the so-called depolarizing chemorepellents) of ciliates. ATP and GTP produce measurable receptor potentials and activate Na^+ and Mg^{2+} currents;

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Table 4 Purinergic signalling system in invertebrates

Phylum	P1 receptors (Adenosine)	P2 Receptors			Ectonucleotidase	Function
		P2 (ATP/ADP)	P2Y	P2X		
Bacteria	?					Inhibits growth and proliferation
		?				Stimulates growth, differentiation and protein synthesis
					NTPDase	
Protozoa Amoeba		✔				Depolarization; inhibition of amoeboid movement; increase in Na^+ permeability
			✔?			?Initiation of Ca^{2+}influx
				✔		Osmoregulation; regulation of movement; initiation of Ca^{2+} influx
					Ecto-ATPase	
Ciliates		✔ (and GTP)				Avoiding reactions; control ciliary beat and swimming; increase output of contractile vacuole
			✔			?
					Ecto-ATPase	
Parasites	✔					Immunomodulatory properties; inhibits growth
		✔				Induction of parasitosis; proinflammatory activity; motility
		(UTP,UDP,UMP)				Activate apical exocytosis and increase infectivity of sporozoites;
					5′-nucleotidase NTPDase	
Algae				✔		Inward currents
Fungi		✔				Regulate sporulation of yeast
					EctoATPase	

Table 4 *(Continued)*

Phylum	P1 receptors (Adenosine)	P2 Receptors			Ectonucleotidase	Function
		P2 (ATP/ADP)	P2Y	P2X		
Coelenterates						
Anemones	✔	✔				Contract pedal disc
		✔				Repair of hair bundle mechanoreceptors
Ctenophores		✔				Ciliary reversal in comb plates
					Ca^{2+}-ATPase	
Platyhelminthes				✔		Pore dilation; Ca^{2+}regulation
					NTPDase	
Nematodes		✔?				?Disease pathogenesis
		Ap_3A		(Not found in *C. elegans*)	NDPK Apyrase 5′-nucleotidase	
Arthropods						
Crustacea	✔					Increase ventilation rate, cardiac performance and haemolymph velocity; presynaptic modulation
		✔				Transmitter release from neuromuscular junction
	✔	✔				Olfactory and gustatory roles
			?	?		
Insects	✔A_1 ✔A_2					Modulates neuromuscular transmission
		✔				Stimulation of gorging responses of blood feeding insects; control of salivary secretion; regulation of taste receptors
					Adenosine deaminase	Role in larval development
					Apyrase	

Table 4 *(Continued)*

Phylum	P1 receptors (Adenosine)	P2 Receptors			Ectonucleotidase	Function
		P2 (ATP/ADP)	P2Y	P2X		
Molluscs	✔					Regulates haemocyte adhesion
	✔A_1 ✔A_2					Modulation of neurotransmission
		✔				Contracts gut smooth muscle
	✔	✔				Modulates heart contractility
		GTP				Contraction of proboscis muscle
				✔		Increase in $[Ca^{2+}]_i$; depolarization of ganglia;
	(Inosine)					(Neuronal reorganization after CNS injury)
					Ca^{2+}-activated ATPase	
					Adenosine deaminase	
Annelids	✔					Cell growth; hyperpolarization
		✔				Activation of microglia leading to axon sprouting and regeneration
			✔			Development of glial cells; glial cell hyperpolarization
				✔		Noxious and touch cells; increase in Ca^{2+}permeability
Echinoderms	✔					Prevention of precocity before oocyte maturation
		ADP				Activation of the flagella of sea urchin sperm; depolarizes neurons
		ATP				Control of activity of embryonic cilia; depolarizes neurons; relaxes muscle
	✔		✔	✔		Control of luminescence
					Apyrase Ca^{2+}-ATPase	

✔Indicates presence of receptor and, where known, its function in the final column opposite the tick. ? Indicates indirect evidence of receptor.

these, in turn, trigger opening of ciliary voltage-gated Ca^{2+} currents (Hennessey & Kuruvilla 2000) resulting in graded Ca^{2+} action potentials and $[Ca^{2+}]_i$ oscillations which drive avoiding reactions (Naito & Kaneko 1972, Sehring & Plattner 2004, Hennessey 2005). Interestingly, both Na^+ and Mg^{2+} currents also depend on cytosolic Ca^{2+} concentrations and can be regulated by $[Ca^{2+}]_i$ oscillations (Hennessey 2005).

Both ATP and GTP have been shown to alter the rate of beat of cilia and swimming in ciliates and to increase output from their contractile vacuoles (see Burnstock 1996). GTP is also very potent and induces oscillations in the swimming behaviour, with the cell swimming backwards and forwards repeatedly, involving periodic activation of inward Mg^{2+}- and Na^+-specific currents (Mimikakis *et al.* 1998, Sehring & Plattner 2004). β,γ-Methylene ATP (β,γ-meATP) and the P2X receptor antagonist in mammalian systems, pyridoxal-phosphate naphthylazo-nitro-disulphate, are agonists of the ATP receptor in *Paramecium* (Wood & Hennessey 2003). In the mutant strain *gin A* of *Paramecium,* the GTP-induced avoidance reaction and GTP-induced receptor potentials are absent; although Na^+ and Mg^{2+} currents are preserved (Mimikakis *et al.* 1998), thus suggesting the separation between GTP receptor and membrane channels. The GTP receptors in *Paramecium* are also connected with intracellular Ca^{2+} sources, as at least part of GTP-induced $[Ca^{2+}]_i$ oscillations required Ca^{2+} release from the intracellular stores (Sehring & Plattner 2004). A recent study has shown that GTP-mediated avoidance by ciliary reversal of *Tetrahymena thermophila* requires tyrosine kinase activity, intracellular Ca^{2+}, nitric oxide (NO) synthase and guanylyl cyclase (Bartholomew *et al.* 2008). Furthermore, GTP, released by mechanical stimulation, also exerts trophic effects as it induces cell division in starved *Tetrahymena* (Iwamoto & Nakaoka 2002). All these hint at the expression of metabotropic-like purinoceptors, and indeed biochemical evidence has been presented which suggests that a P2Y-like $G_{i/o}$-protein coupled receptor linked to phospholipase C (PLC), NO synthase and adenylyl cyclase is involved (Rosner *et al.* 2003).

The ciliates are also endowed with nucleotide-degrading systems, as soluble ecto-ATPase has been purified from *T. thermophila* and shown to be similar to the membrane-bound ecto-ATPase of chicken gizzard smooth muscle (Smith *et al.* 1997).

For ciliates, the ATP/GTP signalling appears to play a defensive role, being a method of perceiving nearby cell lysis, which results in massive release of nucleotides (Hennessey 2005). Most likely the same remains true for all single-cell organisms, and here the defensive/avoidance reaction may be considered as a prototype of nociception in higher animals, in which purinoceptors are also intimately involved.

The parasitic protozoan *Trichomonas vaginalis* lives in the human urogenital tract causing a sexually transmitted disease trichomoniasis. Ecto-ATPases and purine nucleoside kinases have been identified in this parasite controlling levels of ATP and adenosine, which may be involved in mechanisms related to host–parasite interactions (Munagala & Wang 2003, Tasca *et al.* 2003). Ecto-5′-nucleotidase has been identified in *Trichomonas gallinae*, which parasitises birds, and it is claimed that the adenosine generated is essential for its survival (Borges *et al.* 2007).

Another parasitic protozoan *Toxoplasma gondii* is a major health problem for immunocompromised individuals, such as AIDS patients and organ transplant recipients, and adenosine has been identified as a therapeutic target. *Toxoplasma gondii*, like most parasites studied, lacks the ability to synthesize purines *de novo* and depends on the salvage of purines from their host to satisfy their requirements of purines. In this respect, the salvage of adenosine is the major source of purines in *T. gondii*. Therefore, interference with adenosine uptake and metabolism in *T. gondii* can be selectively detrimental to the parasite (el Kouni 2007). In *Leishmania*, another parasitic protozoan, the conversion of ATP, a molecule with pro-inflammatory activity, into adenosine, which possesses immunomodulatory properties, may contribute to the establishment of infection (Marques-da-Silva *et al.* 2008).

Two types of receptors for ATP with different affinities were identified by radioligand binding assay in *Trypanosoma cruzi*; these receptors were suggested to play a role in the induction of parasitosis (Inverso *et al.* 1995). NTPDase activity has been characterized in *Trypanosoma* (Fonseca *et al.* 2006, de Souza Leite *et al.* 2007). *Leishmania* releases nucleoside diphosphate kinase (NDPK), which prevents ATP-mediated cytolysis of macrophages (Kolli *et al.* 2008). Ecto-nucleotide triphosphate diphosphohydrolase activity has been characterized in *Leishmania* (Pinheiro *et al.* 2006, Coimbra *et al.* 2008) and the human enteric parasite *Giardia lamblia* (de Sa Pinheiro *et al.* 2008). Paralysed flagella mutants of *Chlamydomonas* can be reactivated to become motile by low concentrations of ATP and speculations were made about the involvement of dynein in the underlying mechanisms (Frey *et al.* 1997). Uracil and its derived nucleosides and nucleotides (UMP, UDP and UTP) activate apical exocytosis and increase the infectivity of the sporozoites of *Plasmodium*, a protozoan parasite involved in malaria (Ono *et al.* 2008).

Algae

Ostreococcus tauri is the smallest free-living eukaryote known, a primitive green algae that is close to the evolutionary origin of photosynthetic plants (Derelle

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et al. 2006). The genome of *O. tauri*, which appeared about 1 billion years ago, contains a gene for an ionotropic ATP receptor. This gene, named *OtP2X*, encoded a protein of 387 amino acid residues with a molecular weight of ~42 kDa (Fountain *et al.* 2008). The *OtP2X* receptor had 23% of homology with P2X receptor cloned from the *Dictyostelium* amoeba described above and 28% of homology with human P2X receptors. Expression of *OtP2X* protein in HEK293 cells resulted in appearance of a functional ATP-gated channel. In outside-out patches obtained from HEK293 cells, 100 μM ATP triggered flickery openings of cationic channels, whereas in the whole-cell mode ATP triggered inward currents in a concentration-dependent manner (Fountain *et al.* 2008). The threshold ATP concentration was 30 μM and $EC_{50} \sim 247$ μM. The inward currents can be also elicited by very high (5 mM) concentrations of α,β-meATP, but not by ADP, UTP, GTP, ITP, CTP, 2′3′-*O*-(4-benzoyl)benzoyl-ATP (BzATP), $\beta\gamma$-imido-ATP, NAD and FAD. Suramin and pyridoxal-phosphate-6-azophenyl-2′,4′-disulphonate (PPADS), the non-selective P2 receptor antagonists, did not affect *OtP2X*-mediated currents. Finally, in contrast to all other known P2X receptors *OtP2X* showed a very low Ca^{2+}permeability ($P_{Ca}/P_{Na} \sim 0.39$). However, experiments designed to identify functional expression of *OtP2X* in native *O. tauri* have failed. In these experiments sodium green was used as a reporter for ATP-induced Na flux; applications of ATP in concentrations up to 3 mM did not induce any measurable signals. Therefore, the functional role of *OtP2X* remains unclear; it may be confined to intracellular membranes similar to *Dictyostelium*. It has to be noted also that the genome of *O. tauri* contains four sequences that encode proteins similar to P2X receptors; similar sequences are also found in the genome of *Ostreococcus lucimarinus* (Palenik *et al.* 2007).

The gene for ionotropic P2X-like receptor was identified in the genome of the choanoflagellate *Monosiga brevicollis*; further experiments using heterologous expression demonstrated that this gene encodes a functional receptor (Fountain *et al.* 2008).

Sponges

Iso-iantheron A, 8-carboxy-iso-iantheron and iso-iantheron B have been isolated from the marine sponge *Ianthella quadrangulata*, which have been identified as novel agonists for the $P2Y_{11}$ receptor (Greve *et al.* 2007).

Fungi

Various purine derivatives, including ATP, are known to regulate the sporulation of yeasts (Jakubowski & Goldman 1988), by an as yet unidentified signalling pathway. Although purinoceptors have not yet been found in fungi, some (for example *Candida albicans*) developed a mechanism for ATP release, which most likely involves plasmalemmal channels (Koshlukova *et al.* 1999). The ATP released in this pathway can exert cytotoxic actions on neighbouring cells (Koshlukova *et al.* 1999). In another yeast species, *Saccharomyces cerevisiae*, ATP was released in a cAMP-regulated plasmalemmal pathway (Boyum & Guidotti 1997). UDP-glucose, as well as ATP, was released by *S. cerevisiae* (Esther *et al.* 2008). Ecto-ATPase activity has been identified in the fungus, *Fonsecaea pedrosoi* (Collopy-Junior *et al.* 2006).

Coelenterates

Coelenterates are comproed of jellyfish, sea anemones and corals; these are mainly marine organisms exhibiting radial symmetry with a two-layered body wall enclosing a single cavity with a single aperture, the mouth.

ATP has been shown to enhance repair of hair bundle mechanoreceptors of sea anemones, these mechanoreceptors being generally similar to those of the acousticolateralis system of vertebrates (Watson *et al.* 1999). ATP enhances the rate by which repair proteins restore the structural integrity and vibration sensitivity of anemone hair bundles. Quinacrine cytochemistry localizes stores of ATP in the apical cytoplasm of sensory neurones in the centre of the hair bundle and it was suggested that ATP is released from the sensory neurone after the hair bundle loses its structural integrity.

Platyhelminthes

The flatworms are a phylum of relatively simple, unsegmented soft-bodied invertebrate animals, more than half of which are parasitic.

An ionotropic ATP receptor was cloned from the trematode, *Schistosoma mansoni* (Agboh *et al.* 2004, Raouf *et al.* 2005). This receptor, named *SchP2X* by Agboh *et al.* or *SmP2X* by Raouf *et al.*, showed 25–36% homology with human P2X receptors, being the most similar to $P2X_4$ and $P2X_5$ receptors (Agboh *et al.* 2004, Raouf *et al.* 2005). When recombinant *SchP2X* receptors were expressed in *Xenopus* oocytes, extracellular administration of ATP evoked inward current in a concentration-dependent manner with an $EC_{50} \sim 22$ μM. Inward currents of slightly smaller amplitude were also evoked by Bz-ATP (EC_{50} 3.6 μM), whereas AMP-CPP, ADP, UTP, UDP, GTP and ITP were ineffective. The *SchP2X*-mediated currents were effectively blocked by PPADS and

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suramin and trinitrophenyl-ATP (Agboh *et al.* 2004, Raouf *et al.* 2005) and potentiated by 10 μM ivermectin (Agboh *et al.* 2004), the positive modulator of mammalian $P2X_4$ receptors (Priel & Silberberg 2004, Lalo *et al.* 2007). There is also evidence that activation of SchP2X receptors may lead to pore dilation, similar to that described for $P2X_2$, $P2X_4$ and $P2X_7$ receptors; formation of this pore was completely inhibited by 20 μM Zn^{2+} (Raouf *et al.* 2005).

The *SchP2X* receptors demonstrated significant Ca^{2+} permeability ($P_{Ca}/P_{Na} \sim 3.8$) and therefore may be involved in cytosolic Ca^{2+} regulation of *Schistosoma*. Calcium homeostasis plays a critical role for trematode survival; initiation of Ca^{2+} influx (by, for example, the specific drug praziquantel, which modulates the Ca^{2+} channel β-subunit) triggers muscle contraction and paralysis of the parasite (Kohn *et al.* 2001). Consequently, specific drugs aimed at *SchP2X* receptors may have important therapeutic potential. Another series of schistosomicide drugs, the alkylaminoalkanethiosulphuric acids, have been shown to partially inhibit the activity of ATP diphosphohydrolase in *S. mansoni* (Luiz Oliveira Penido *et al.* 2007). *Schistosoma mansoni* ATP diphosphohydrolases have been identified (Vasconcelos *et al.* 1996, Levano-Garcia *et al.* 2007) and show cross-immunoreactivity with potato apyrase antibodies (Faria-Pinto *et al.* 2006, 2008).

Adenosine triphosphatases have been described in the cestode (or tapeworm), *Bothriocephalus scorpii*, which can hydrolyse GTP, CTP and UTP as well as ATP (Burenina 2007). Tapeworms, when infecting the small intestine, interact with intestinal smooth muscle by inducing contraction. Cyclic guanosine monophosphate has been identified as a signalling molecule secreted by the tapeworm, *Hymenolepis diminuta*, to trigger such contractions (Kroening *et al.* 2003).

Nematodes

A protostome superphylum *Ecdysozoa* includes nematodes, arthropods (which are further represented by insects, chelicerata, crustaceans and myriapods), tardigrades and some other smaller phyla. Nematodes or roundworms are diverse and the species are very difficult to distinguish, many being parasitic.

Nucleotidase cascades are catalysed by enzymes secreted by the parasitic nematode, *Trichinella spiralis*; these include NDPK, apyrase, 5′-nucleotidase and adenosine deaminase. These enzymes can affect purinergic signalling in immune and inflammatory responses of the host to its own advantage (Gounaris 2002, Gounaris & Selkirk 2005). The fact that bacterial pathogens and haematophagous insects also secrete similar enzymes raises the possibility that this may be a conserved feature of several organisms with consequences for pathogenicity (Gounaris & Selkirk 2005). A diadenosine triphosphate, Ap3A, has been identified in the parasitic nematode, *Brugia malayi*, that may be involved in disease pathogenesis (Kron *et al.* 2007). In a study of the roundworm *Caenorhabditis elegans* protein sequences for P2X receptors were not found (Agboh *et al.* 2004).

Tardigrades

The tardigrades ('slow walkers' – the name given to them by Lazzaro Spallanzani in 1777, also known as 'water bears' as initially named by their discoverer Johann August Ephraim Goeze in 1773) are microscopic animals (0.2–1 mm in length), which live in the sea and in fresh water. Very recently the P2X-like receptor (*Hd*P2X) encoding sequence was identified in the genome of the tardograde *Hypsibius dujardini* (Bavan *et al.* 2009). The *Hd*P2X sequence comprised 480 amino acids and showed ~36–38% homology with some vertebrate P2X receptors. When expressed in HEK293 cells the *Hd*P2X receptors formed functional channels activated by ATP, Bz-ATP and α,β-meATP at concentrations of about 10–100 μM. The *Hd*P2X receptors were inhibited by copper, zinc, suramin and PPADS (Bavan *et al.* 2009).

Crustacea

The subphylum *Crustacea* is a large group of the phylum arthropods; they include crabs, lobsters, crayfish, shrimp, krill and barnacles. The majority of them are aquatic, living in either marine or fresh water environments, but a few groups have adapted to life on land.

There was much early information about the effects of ATP and adenosine in crustaceans, in particular about their olfactory and gustatory roles in the spiny lobster (see Carr *et al.* 1986, Burnstock 1996). In more recent studies, adenosine has been shown to increase ventilation rate, cardiac performance and haemolymph velocity in the lobster *Homarus americanus* (Stegen & Grieshaber 2001) and modulatory effects of adenosine, ATP, ADP and AMP have been described in the heart of the lobster (Maurer *et al.* 2008). The effects of temperature on growth, adenosine phosphates, ATPase and its relation to the cellular defense response of juvenile shrimp *Macrobrachium nipponense* have been described (Wang *et al.* 2006). In contrast to vertebrate neuromuscular junctions, caffeine had a depressant action at neuromuscular synapses in the crayfish *Procambarus clarkii*, perhaps indicating an excitatory effect mediated by pre-synaptic adenosine receptors (Celenza *et al.* 2007).

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Insects

The class *Insecta* are the other major group of arthropods and the most diverse group of animals, found in nearly all environments on the planet, although only a small number of species occur in the oceans.

There were exciting early studies of the role of ATP and ADP released from mammalian erythrocytes and platelets stimulating the gorging response of a variety of blood-feeding mosquitoes and flies and the intriguing high activity of apyrase in the saliva of the insects causing breakdown of ADP leading to enhanced haemorrhage and more effective blood sucking (see Burnstock 1996, Ribeiro *et al.* 2001). The laboratory of Galun continues to fine-tune this story and has shown that chemoreceptor cells in the labral apical sensilla of the yellow fever mosquito *Aedes aegypti* responded to ATP in the gorging state, but not in the non-gorging state (Werner-Reiss *et al.* 1999a) and that ATP acts together with other stimulants such as NaCl and $NaHCO_3$ in initiating the gorging response (Werner-Reiss *et al.* 1999b). Regulation of gap junctions in Malpighian tubules of *A. aegypti* by ATP has been proposed to influence secretion (Weng *et al.* 2008). Multiple receptor sites for nucleotide reception have been identified in the labellar taste receptor cells of the fleshfly *Boettcherisca peregrina* showing that the specificity of the receptor site reacting with nucleotide in the sugar receptor cell is different from that of the salt receptor cell (Furuyama *et al.* 1999). Apyrase has been cloned from the bed bug *Cimex lectularius* (Valenzuela *et al.* 1998) and characterized in the salivary glands of the cat flea *Ctenocephalides felis* (Cheeseman 1998).

The insect-derived growth factor demonstrates an adenosine deaminase activity, the latter being critically important for regulation of growth of embryonic fly cells (Homma *et al.* 2001) and it has been implicated in *Drosophila* metamorphosis and in protection from the toxic effects of adenosine (Dolezelova *et al.* 2005). The first characterization of an insect P1 (adenosine) receptor, encoded by *Drosophila* gene CG9753, has been reported. These P1 receptors control cAMP synthesis and cytosolic Ca^{2+} mobilization (Dolezelova *et al.* 2007). There are several similarities between mammalian and insect adenosine receptor functions: extracellular adenosine influences immune responses in both; adenosine agonists and antagonists modulate the sleep and waking cycle in *Drosophila* (Hendricks *et al.* 2000), perhaps associated with the endogenous expression of the adenosine receptors in the insect brain. There is conservation of the receptor and proteins involved in adenosine transport and metabolism and elevated adenosine concentrations in fly haemolymph are similar to adenosine concentrations in human blood.

Adenosine diminished the amplitude of nerve-evoked post-synaptic currents (EPSCs) and somewhat reduced the frequency of spontaneous miniature EPSCs in a neuromuscular preparation from the larvae of the blowfly *Calliphora vicina* (Magazanik & Fedorova 2003). An A_2 adenosine receptor subtype agonist potentiated EPSCs, while the A_2 antagonist, 8-cyclopentyl-1,3-dipropylxanthine, competitively inhibited the pre-synaptic inhibitory action of adenosine in a manner similar to the pre-synaptic actions of adenosine at the vertebrate neuromuscular junction. EPSCs were slightly reduced by ATP and this effect was prevented by concanavalin A (Magazanik & Fedorova 2003), which inhibits ecto-5′-nucleotidase, suggesting that ATP is acting through P1 receptors. This is in contrast to the vertebrate motor nerve terminals where ATP and adenosine affect transmitter release through distinct P1 and P2 receptors. It has been suggested that the altered ATP synthesis pathways that occur in response to mosquitoes injected with malarial parasites could be responsible for behavioural modifications by purinergic neuromodulatory actions (Lefevre *et al.* 2007). Adenosine deaminase activity has been identified in the saliva of the sand fly *Phlebotomus duboscqi* (Kato *et al.* 2007a), of *Lutzomyia longipalpis* (Charlab *et al.* 2000) and of mosquitoes (Ribeiro *et al.* 2001). Adenosine deaminase-related growth factors stimulate cell proliferation in *Drosophila* (Zurovec *et al.* 2002). A role for adenosine deaminase in larval development of *Drosophila* has been identified (Dolezal *et al.* 2005).

Molluscs

Molluscs are divided into cephalopods, such as squid, cuttlefish and octopus, and gastropods (snails and slugs). They do not show segmentation and the body consists of a head-foot and the visceral mass extended into folds, which often secrete a shell. The nervous system consists of ganglia connected by commisures.

Early studies showed adenosine to have a modulatory action via A_1 and A_2 receptors in responses of neurones in the suboesophageal ganglion of the snail *Helix aspersa*, while nanomolar concentrations of ATP and α,β-meATP activated calcium channels in these neurones, suggesting that P2X receptors were also present (see Cox & Walker 1987). Inhibition of monoamine transmitter release by adenosine acting on pedal ganglion neurones via A_2 receptors in the marine bivalve *Mytilus edulis* was also reported. Furthermore, actions of purine nucleotides and nucleosides on the hearts of *Octopus vulgaris*, venus clam *Katelysia rhytiphora* and oyster *Crassostrea nippona* were also described and the action of GTP (but not ATP) on proboscis smooth muscle of *Buccinium undatum* (for references to early studies, see Burnstock 1996). The cephalopod mollusc *Watasenia*

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scintillans is a small deep sea squid, which exhibits bioluminescence, via an ATP-dependent reaction involving luciferin-luciferase (Teranishi & Shimomura 2008).

There have been some further studies about purinergic signalling in molluscs since 1996. A unique Ca^{2+}-activated ATPase has been identified on the nervous ganglia of the terrestrial slug *Phyllocaulis soleiformis* (Da Silva *et al.* 2002) and nucleotidase activities in membrane preparations of ganglia and digestive gland of the snail *H. aspersa* have been described (Borges *et al.* 2004). Mollusc-derived growth factor, characterized in *Aplysia*, stimulates cell proliferation in the developing CNS and inosine was shown to be involved in neuronal reorganization after CNS injury (Akalal *et al.* 2003).

Real-time release of ATP from ganglia of the CNS of the freshwater snail *Lymnaea stagnalis* has been described (Gruenhagen *et al.* 2004). The release of ATP was stimulated by depolarization of ganglia with high K^+ solutions and serotonin. The release was quantified and found to vary spatially from ganglion to ganglion and within individual ganglia. RT-PCR with degenerate oligonucleotides was used to identify a P2X receptor fragment expressed in the CNS of *L. stagnalis* and the full-length sequence was obtained by RACE-PCR and the cloned receptor expressed in *Xenopus* oocytes to facilitate electrophysiological characterization (Bavan *et al.* 2008). BzATP is a partial agonist at the *Lymnea* P2X receptor, but ADP and UTP are inactive. Heavy metals, zinc, cadmium and copper, were shown to affect ATPases in the digestive gland of *H. aspersa*, but not cholinesterase, suggesting that the purinergic system may be a target related to the toxicity induced by these metals and a possible indicator of biological impact of exposure to heavy metal contaminants (de Souza Dahm *et al.* 2006). Adenosine receptor-like molecules and related signalling transduction pathways regulate haemocyte adhesion in abalone *Haliotis diversicolor* (Chen & Chen 2007).

Annelids

This phylum comprises the segmented worms, including the polychaetes, oligochaetes and hirudines. The worms possess both circular and longitudinal body muscles. The nervous system consists of dorsal cerebral ganglia and ventral nerve chord, with nerve cells along the length of the chord not necessarily confined within ganglia and with peripheral nerves from each segment.

Early electrophysiological investigations showed that ATP, ADP, AMP and adenosine depolarized selected neurones via P2X-like receptors (especially the noxious and touch cells), but not neuropil glial cells in the CNS of the leech *Hirudo medicinalis* (Backus *et al.* 1994). These P2X-like receptors had measurable Ca^{2+} permeability. In a more recent publication, ATP was shown to produce either depolarization of the leech neuropil glial cells involving activation of Na^+-permeable channels, or produced hyperpolarizations involving activation of K^+ channels via P2Y-like, as well as via P1 receptors, although leech neurones are more sensitive to ATP than glial cells (Muller *et al.* 2000). In the same preparation ATP was shown to trigger cytosolic Ca^{2+} mobilization resulting from Ca^{2+} release from intracellular stores. In addition, extracellular purines regulate transepithelial Cl^- secretion and Na^+ absorption across the integument of *H. medicinalis* (Schnizler *et al.* 2002). Apical and basolateral application of ATP stimulated Na^+ uptake, while adenosine upregulated non-Na^+ currents and acted only from the basolateral side. Mechanosensitive cation channels in the growth cones of identified leech neurones are activated by ATP and adenosine most likely through metabotropic receptors, and the authors suggest that this is consistent with a role in the Ca^{2+} oscillations associated with cell growth (Barsanti *et al.* 2006). ATP released by nerve injury is a key activator of microglia in the leech; ATP-activated microglia promoting axon sprouting and regeneration, although at the same time ATP reduces microglial migration directed to lesions by NO (Ngu *et al.* 2007).

Echinoderms

Regulation of on/off switching of dynein motile activity of the flagella of sea urchin sperm has been shown to involve ADP-induced activation and ATP-induced inhibition, probably through phosphorylation/dephosphorylation of outer arm-linked proteins (Yoshimura *et al.* 2007). ATP also appears to be involved in the activities of cilia in sea urchin embryos (Kinukawa & Vacquier 2007). Ecto-ATP diphosphohydrolase (apyrase) is present in ovarian follicle cells of the starfish *Asterina pectinifera* (Mita *et al.* 1998), and it has been suggested that the AMP and adenosine produced may play a role in prevention of precocity before the oocyte maturation stage (Mita *et al.* 2001). A Ca^{2+}-ATPase was identified from the microsomal fraction obtained from the sea cucumber (*Ludwigothurea grisea*) longitudinal body wall smooth muscle, which was found to be regulated both by K^+ and by ATP (Landeira-Fernandez *et al.* 2000a). Like the isoforms found in skeletal muscle, the sea cucumber Ca^{2+}-ATPase can convert osmotic energy into heat (Landeira-Fernandez *et al.* 2000b). Control of luminescence in the brittlestar *Amphipholis squamata* may involve both P1- and P2Y- and P2X-like receptors acting in synergy with ACh (De Bremaeker *et al.* 2000).

Lower vertebrates

Early studies describing the involvement of purinergic signalling in cyclostomes, elasmobranch and teleost fish,

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amphibians, reptiles and birds have been thoroughly covered (Burnstock 1996). Studies published since 1996 will now be reviewed.

Elasmobranch fish

The electric organ of electric elasmobranch fish, formed by dorso-ventrally arranged voltaic columns, has been studied as a model of the neuromuscular junction consisting of motor nerves and electrocytes forming electroplaques or electroplates (Altamirano *et al.* 1953, Martin-Satue *et al.* 2007) that are derived from myoblasts. A number of early studies of the electric ray *Torpedo* and electric eel *Electrophorus* showed that ACh and ATP were co-stored and co-released at these junctions (Burnstock 1996). Studies, which have appeared since, confirm and extend the earlier studies. The synaptic transmission in electric organ is always accompanied by release of large amounts of ATP from electrocytes; ATP is then degraded to adenosine, which regulates ACh release through pre-synaptic receptors (Israel *et al.* 1976). Suramin was shown to be an efficient ecto-nucleotidase inhibitor at the synapses of the electric organ of *Torpedo* (Marti *et al.* 1996). It was suggested that by reducing the degradation of ATP in the synaptic cleft, thereby reducing the formation of adenosine, synaptic depression can be prevented. Inhibition of A_1 receptor activation acted through inhibition of N-type calcium channels leading to inhibitory modulation of ACh release, while the facilitation effects of A_2 receptor activation were mediated by potentiation of P-type calcium channels (Satoh *et al.* 1997). A binding site for ATP within the extracellular region of the *Torpedo* nicotinic ACh receptor β-subunit has been identified, which may be responsible for the well-known potentiating action of ATP on ACh-mediated contractions (Schrattenholz *et al.* 1997). Diadenosine polyphosphate hydrolase, present in pre-synaptic plasma membranes of *Torpedo* electric organ, has been characterized (Mateo *et al.* 1997). Release of ACh and ATP was measured from permeabilized cholinergic synaptic vesicles from *Torpedo marmorata* electric organ and shown to be 10 times more effective in solutions containing Ca^{2+} rather than Na^+ (Gonzalez-Sistal *et al.* 2007). Interestingly the concentration of ATP measured in synaptic vesicles of *Torpedo* was rather high, approaching 120 mM (Ahdut-Hacohen *et al.* 2006). Furthermore, these synaptic vesicles contained channels gated by intravesicular ATP. It was suggested that these channels may be involved in regulation of various exocytotic events.

Cloning, molecular characterization and expression of NTPDase-1 from *Torpedo* electric organ has been carried out; it revealed high homology of the enzyme with vertebrate analogues (Martin-Satue *et al.* 2007). ATP, acting via P2Y receptors, triggers calcium mobilization in Schwann cells in the electric organ of the skate *Raja erinacea* (Dowdall *et al.* 1997, Green *et al.* 1997). This P2Y-mediated Ca^{2+} signals comprised an initial peak, which originates solely from thapsigargin-sensitive Ca^{2+} release from the endoplasmic reticulum (ER), and a sustained plateau component produced by plasmalemmal Ca^{2+} entry (Green *et al.* 1997).

Hepatocytes from the skate *R. erinacea* have been shown to express P2 receptors, which are involved in regulation of bile secretion (Nathanson & Mariwalla 1996). Activation of these receptors triggered cytosolic Ca^{2+} signals associated with an activation of the ER-resident type I $InsP_3$ receptors. The pharmacological profile and mediation by $InsP_3$ (Nathanson *et al.* 1999) suggests the presence of multiple P2Y receptor subtypes, but not P2X or P1 receptors. In a later study from this group, a $P2Y_1$-like receptor was cloned from the skate liver (Dranoff *et al.* 2000). A 2314-base pair cDNA clone was generated that contained a 1074-base pair open reading frame encoding a 357-amino acid gene product with 61–64% similarity to $P2Y_1$ receptors and 21–37% similarity to other P2Y receptor subtypes. Phylogenetic analysis suggested that this receptor is closely related to a common ancestor of the P2Y subtypes found in mammals, avians and amphibians.

The laminid shark *Lamna nasus* is an unusual fish in that it has developed endothermy and can maintain body temperatures of up to 8–10 °C above ambient water temperature. ATP induces a reverse temperature effect, a mechanism resembling that found in the bluefin tuna *Thunnus thynnus*, an endothermic teleost, suggesting convergent evolution (Larsen *et al.* 2003).

Teleost fish

A number of early studies concerning the effects of purine nucleotides and nucleosides on the gastrointestinal tract, cardiovascular system and brain of the teleost and purinergic regulation of chromatophores have been reviewed (see Burnstock 1996).

In recent years the zebrafish has become a useful and widespread model for investigating nervous system development (Strahle & Blader 1994). Zebrafish are endowed with both ionotropic P2X and metabotropic P2Y receptors. The first ionotropic P2X-like receptors identified in the zebrafish *Danio rerio*, which belongs to the *Cyprinidae* (minnow) family genome (Boue-Grabot *et al.* 2000, Egan *et al.* 2000, Norton *et al.* 2000), were similar to mammalian $P2X_3$ receptors. The zebrafish gene encodes the protein composed of 410–416 amino acids, which was 54% identical to rat $P2X_3$ receptor (Boue-Grabot *et al.* 2000) and showed considerable homology with the human $P2X_3$ receptor (Norton *et al.* 2000). When expressed in *Xenopus* oocytes or in

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HEK293 cells the zebrafish P2X receptors assembled a homomeric receptor, which upon activation with ATP generated fast inward currents, characterized by rapid desensitization. The zebrafish P2X receptor expressed in oocytes (Boue-Grabot *et al.* 2000) showed relatively poor sensitivity to ATP ($EC_{50} \sim 350$ μM) and ADP ($EC_{50} \sim 320$ μM), although it was very sensitive to BzATP ($EC_{50} \sim 5$ μM), thus being pharmacologically distinct from human and rat $P2X_3$ receptors. Interestingly, the same receptors expressed in HEK293 cells were much more sensitive to ATP ($EC_{50} \sim 1.5$ μM; Egan *et al.* 2000). Expression of this receptor was very much restricted to sensory neurones and to Rohon-Bear cells (transient sensory neurones) in the spinal cord from early development (Norton *et al.* 2000). Subsequently, P2X expression in the spinal cord completely disappeared as the Rohon-Bear cells expired, being replaced by dorsal root ganglion (DRG) neurones. In the zebrafish mutant *narrowminded* (Artinger *et al.* 1999), which demonstrate reduced sensory responses and develop small trigeminal ganglia, the expression of P2X receptors was inhibited (Norton *et al.* 2000). Immunoreactivity for $P2X_3$ receptors was also found in some neuroepithelial cells in the respiratory lamellae of zebrafish gills. These cells were almost exclusively localized in the distal lamellae, which are exposed to the external environment (Jonz & Nurse 2003). Metabotropic $P2Y_1$ receptors were identified in zebrafish thrombocytes (Gregory & Jagadeeswaran 2002).

Subsequent experiments found that zebrafish genome also has two orthologues for mammalian $P2X_4$ and $P2X_5$ receptors (Diaz-Hernandez *et al.* 2002). Both proteins, when expressed in HEK293 cells, formed functional homomeric receptors. The zebrafish $P2X_4$ receptor had a very low affinity to ATP ($EC_{50} \sim 270$ μM) and very low affinity to other purinergic ligands. Ion currents produced by zebrafish $P2X_5$ receptors expressed in HEK293 cells were very small, thus precluding precise pharmacological investigation. Further studies, using *in silico* screening, revealed that the zebrafish genome contained, in total, nine genes encoding various P2X subunits (Kucenas *et al.* 2003). Six of these genes are orthologues of $P2X_1$, $P2X_2$, $P2X_3$, $P2X_4$, $P2X_5$ and $P2X_7$ mammalian subunits, two are paralogues of $P2X_3$ and $P2X_4$ subunits, and the ninth gene, labelled as 514, showed some similarities to $P2X_6$ and $P2X_5$ mammalian subunits. The degree of identity between zebrafish and mammalian subunits was 45–55% (Kucenas *et al.* 2003). Seven genes (both $P2X_3$, labelled as $zP2X_{3.1}$ and $zP2X_{3.2}$; both $P2X_4$, labelled $zP2X_{4.1}$ and $zP2X_{4.2}$, $zP3X_5$, $zP2X_7$ and $zP2X_{514}$) assembled into homomeric functional channels when expressed in HEK293 cells and, when activated by ATP, generated cationic currents. *In situ* hybridization showed no detectable expression of $zP2X_2$ and $zP2X_{4.1}$ in 24- to 48-h-old embryos, the $zP2X_5$ was expressed in skeletal muscle, whereas $zP2X_7$ demonstrated ubiquitous expression. The $zP2X_1$, $zP2X_{3.1}$, $zP2X_{3.2}$, $xP2X_{4.2}$ and $P2X_{514}$ subunits were expressed in the nervous system of zebrafish embryo. Using fluorescent protein (GFP or red fluorescent protein-1)-labelled transgenes of $zP2X_{3.1}$ and $zP2X_{3.2}$, the patterns of expression of these subunits in developing zebrafish nervous system were revealed. The $zP2X_{3.1}$ subunits were present in the majority of Rohon-Beard neurones and trigeminal neurones. The $zP2X_{3.2}$ subunit labelling of sensory neurones was lower, although they were also present in neurones of the epibranchial ganglia. In general all neurones fall into three groups – those expressing only one of the subunits or those expressing both (Kucenas *et al.* 2006).

Expression of NTPDase 3 (ENTPD3/NTPDase3) was found in orexin-hypoicretic neurones of zebrafish hypothalamus (Appelbaum *et al.* 2007), thus showing some similarity to mammals, in which ATP signalling is involved in the regulation of orexin/hypocretin neurones. This may indicate early conservation of an ATP signalling route in the regulation of sleep and wakefulness. Finally, the membrane fractions of the brain of zebrafish contained NTPDases (Rico *et al.* 2003) and ecto-5′-nucleotidases (Senger *et al.* 2004), which can participate in purine metabolism in the CNS and may be involved in purinergic signalling in the brain. Interestingly, both enzymes were inhibited by carbofuran and malathion, broad-spectrum pesticides (Senger *et al.* 2005), and by methanol (Rico *et al.* 2006), which may somehow account for the neurotoxicity of these compounds. Activity of NTPDases and ecto-nucleotidases in zebrafish brains was also affected by mercury chloride and lead acetate (Senger *et al.* 2006a), whereas zinc, cadmium and copper caused a significant increase in ATP hydrolysis (Senger *et al.* 2006b, Rosemberg *et al.* 2007).

Studies have been carried out recently about purinergic signalling in the respiratory tract of a primitive dipnoan bony fish, the Nile bichir *Polypterus bichir bichir* (Zaccone *et al.* 2007, 2008). Nerves expressing $P2X_2$ receptor immunoreactivity projected to the lung epithelium. There are accounts of the effects of adenosine on the contractility of heart of the trout under different temperature conditions (Aho & Vornanen 2002). The silver catfish *Rhamdia quelen* can resist cold winters and grow quickly in the summer. ATP hydrolysis by NTPDase and 5′-nucleotidase in this fish is enhanced by an increase in temperature in all tissues, except for 31 °C in the brain (Lermen *et al.* 2005). The temperature sensitivity of the enzymes affects purinergic-mediated activity, which might be involved in the seasonal responses of these animals.

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Cross-talk between adenosine and glutamate has been demonstrated in the brain of the brown trout *Salmo trutta*, comparable to that seen in mammalian brain (Poli *et al.* 1999). A_1 receptors have also been identified and pharmacologically characterized in the brain of the eel *Anguilla anguilla* (Poli *et al.* 1997). Calcium-dependent release of ACh from synaptosomes of the optic tectum of brown trout *S. trutta* is inhibited by adenosine A_1 receptors (Poli *et al.* 2001). Synaptosomal NTPDase has been identified from the brain of the pimelodid fish *R. quelen* that resemble that found in chicken and rat synaptosomes (Schetinger *et al.* 2001).

It has been suggested that adenosine modulates the circulatory levels of catecholamines in hypoxic trout and hagfish (Bernier *et al.* 1996). The sculpin *Myoxocephalus scorpius* possesses considerable tolerance to hypoxia and is able to avoid cardiac adenosine accumulation through a depression of cardiac activity and an activation of anaerobic metabolism (Maccormack & Driedzic 2004). Endothelium-dependent vasodilatation of mesenteric arteries of the hagfish *Myxine glutinosa* evoked by ADP is mediated by NO, indicating an evolutionarily conserved vascular property (Feng *et al.* 2007). Vasoconstriction of the branchial vasculature of the rainbow trout *Oncorhynchus mykiss* is mediated by A_1 adenosine receptors (Sundin & Nilsson 1996). Vasodilatation of swimbladder vessels of the eel is evoked by adenosine (Schwerte *et al.* 1999).

$P2X_7$ receptors have been cloned from the seabream *Sparus aurata*. The gene consisted of 2022 nucleotides which encoded the 576 amino acid receptor peptide; the latter showing high homology (38–73%) with other $P2X_7$ receptors (Lopez-Castejon *et al.* 2007). Activation of $P2X_7$ receptors is associated with the release of interleukin-1β, suggesting that the mechanism involved in cytokine secretion is conserved during vertebrate evolution (Lopez-Castejon *et al.* 2007). Evidence for P2 purinoceptor-mediated uptake of Ca^{2+} across the intestinal brush border membrane of tilapia *Oreochromis mossambicus* has been presented (Klaren *et al.* 1997). ATP, apart from its role in regulating intracellular Ca^{2+}, is required for the fast compensatory endocytosis that follows glutamate exocytosis in the synaptic retinal bipolar neurones prepared from dark-adapted gold fish retina (Heidelberger 2001).

Extracellular nucleotides, ATP, UTP and UDP, acting via P2Y receptors are important factors promoting regulatory volume decrease that follows hypotonic swelling of hepatocytes exposed to hypo-osmotic stress in trout (Pafundo *et al.* 2004), goldfish (Espelt *et al.* 2008) and turbot (Ollivier *et al.* 2006). The kinetics of ATP release and cell volume regulation of hypo-osmotically challenged goldfish hepatocytes have also been described recently (Pafundo *et al.* 2008). Two distinct ecto-NTPDases have been identified in the liver of the goldfish (Alleva *et al.* 2002). NTPDase 1 appears to be conserved as the main ecto-enzyme in liver and kidney of the silver catfish *R. quelen* as well as in chickens and rats (Vieira *et al.* 2004).

Spermatogonia of rainbow trout proliferate in response to adenosine and ATP via both P1 (A_2) and P2 receptors present in testicular cells (Loir 1999). N^6-Cyclohexyl-adenosine is a modulator of innate immune activities of leucocytes from the gilthead seabream *S. aurata*, suggesting that teleost fish immune cells, like their mammalian counterparts, possess receptors for purines (Salinas *et al.* 2006). Lymphocystis disease virus is the causative agent of a highly infectious disease of fish and the genes encoding the ATPase involved in this disease have been isolated in rockfish *Sebastes schlegelii* (Kim & Lee 2007).

Amphibia

Early studies of the roles of ATP in controlling motility of the gastrointestinal tract and cardiovascular system of amphibians as well as ganglionic and skeletal neuromuscular transmission have been reviewed (Burnstock 1996).

Further studies of synaptic transmission at the frog neuromuscular junction have followed. Modulation of synaptic efficacy and synaptic depression by perisynaptic Schwann cells by intracellular injections of GTPγS has been described (Robitaille 1998). Pertussis toxin-sensitive and -insensitive synaptic modulation was demonstrated at the frog neuromuscular junction, suggesting that two different P2 receptor subtypes are involved in the actions of endogenously released ATP (Sugiura & Ko 2000). The pre-junctional depressant action of ATP was also evoked by UTP, suggesting that $P2Y_2$ or $P2Y_4$ receptors were involved and that these receptors had different transduction mechanisms from depression mediated by pre-synaptic P1 receptors (Sokolova *et al.* 2003, Grishin *et al.* 2005). At least in part the ATP-mediated synaptic depression was associated with the P2Y-dependent inhibition of voltage-gated Ca^{2+} channels (Grishin *et al.* 2005). ATP decreases fast inactivating potassium currents in vesicles derived from frog skeletal muscle plasma membranes and this effect may be mediated by protein kinase (Camacho & Sanchez 2002). Adenosine affects the readily releasable neurotransmitter pool at amphibian motor nerve endings by, unlike phorbol esters, acting at a later stage in the secretory process to decrease the number of calcium-charged primed vesicles (Searl & Silinsky 2003, 2005). Adenosine has been claimed to depress a Ca^{2+}-independent step in transmitter exocytosis at frog motor nerve terminals (Huang *et al.* 2002). $P2Y_2$ receptor activation regulates the expression of acetylcholinesterase and

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ACh receptor genes at the frog, as well as bird and mammal neuromuscular junctions (Tung *et al.* 2004).

Bullfrog dorsal root and sympathetic ganglia have been employed for a number of studies. For example, ATP acting via P2 receptors has been shown to inhibit the M-current in dissociated bullfrog primary afferent neurones (Tokimasa & Akasu 1990). Extracellular protons, at physiological concentrations, can regulate the function of P2X receptors in sensory bullfrog neurones by modulating the affinity of the ATP-binding site (Li *et al.* 1997a). The same group also showed that zinc can inhibit P2X function in bullfrog DRG neurones by decreasing the affinity of the binding site for ATP (Li *et al.* 1997b). Ethanol inhibits the responses mediated by the P2X receptor by an allosteric mechanism, shifting the agonist concentration–response curve to the right in a parallel manner (Li *et al.* 1998). A novel mechanism by which the P2 receptor antagonist, PPADS, inhibited the ATP-activated current in bullfrog DRG neurones has been described (Li 2000). Adenosine inhibits high-voltage-activated N-type calcium channels via A_1 receptors on salamander retinal ganglion cells investigated in a mini-slice preparation (Sun *et al.* 2002). Adenosine, via A_2 receptors, inhibits voltage-dependent Ca^{2+} influx through L-type channels limiting thereby glutamate release in cone receptor terminals of the salamander retina (Stella *et al.* 2007).

UTP was shown to inhibit the M-current in bullfrog sympathetic neurones (Lopez & Adams 1989), probably by acting on $P2Y_2$- or $P2Y_4$-like receptors (Meng *et al.* 2003). Modulation of M-channel conductance by ATP in bullfrog sympathetic B-neurones has been also reported (Chen *et al.* 2001). ATP and ADP stimulation of bullfrog sympathetic ganglion cells appear to act via metabotropic (P2Y) receptors coupled with PLC, producing diacylglycerol, which activates protein kinase C (PKC), resulting in the closing of K^+ channels (Somei *et al.* 1996). However, ATP inhibition of M-current in frog sympathetic neurones involves PLC, but not $InsP_3$, Ca^{2+}, PKC or Ras (Stemkowski *et al.* 2002).

Volume-regulated Cl^- channels are activated by ATP in *Xenopus* follicle-enclosed oocytes (Perez-Samartin *et al.* 2000). Diadenosine polyphosphate activated inward and outward currents in follicular oocytes of *Xenopus laevis*, by direct action on P1 and P2 receptors rather than on a specific receptor for dinucleotides (Pintor *et al.* 1996). Ectoenzymatic breakdown of diadenosine polyphosphates by *Xenopus* oocytes has been reported (Aguilar *et al.* 2001). The extracellular adenosine deaminase growth factor, ADGF/CECR1, plays a role in *Xenopus* embryogenesis via a P1 receptor (Iijima *et al.* 2008). During embryogenesis of *Xenopus*, all NTPDase genes, except for NTPDase8, are expressed and display distinct expression patterns (Masse *et al.* 2006).

Developmental changes in purinergic control of gastric and intestinal motor activity during metamorphosis of *X. laevis* have been observed (Sundqvist 2007, Sundqvist & Holmgren 2008). In particular, while A_1 receptors mediated relaxation, $P2X_1$ and $P2X_3$ receptors mediated contraction before, during and after metamorphosis, and $P2Y_2$, $P2Y_4$ and/or $P2Y_{11}$-like receptors mediated relaxation during metamorphosis. A $P2X_7$-like receptor has been claimed to be present on gastric smooth muscle cells of the toad (Ugur *et al.* 1997); this receptor had several common features with $P2X_7$-related responses, although it never formed a large pore. Adenosine, via A_1 receptors, inhibits secretion of α-melanocyte-stimulating hormone from frog pituitary melanotrophs (Mei *et al.* 1996). This action of adenosine is complex and it involves activation of voltage-independent K^+ conductance, increase in the delayer rectifier K^+ currents, activation of IA currents and inhibition of L- and N-type of voltage-gated Ca^{2+} channels (Mei *et al.* 1996).

Extracellular ATP evokes PKC calcium influx through non-voltage-operated Ca^{2+} channels of yet unidentified nature and sustained enhancement of ciliary beating in frog oesophagus epithelial cells (Levin *et al.* 1997). Ion transport across *Xenopus* alveolar epithelium is regulated by extracellular ATP, UTP and adenosine (Fronius *et al.* 2004). Activation of an apical Cl^- conductance by ATP in the gallbladder of *Necturus maculosus* is mediated by cAMP, but not by elevation of intracellular Ca^{2+} (Vank *et al.* 1999). Extracellular ATP activates a cation-selective conductance in single proximal tubule cells isolated from frog kidney (Robson 1999).

ATP causes a fast initial drop and a secondary, long-lasting increase in Na^+ absorption via P2X gated ion channels in the principal cells of frog skin epithelium (Brodin & Nielsen 2000a). Evidence was also presented for a P2Y receptor on the basolateral membranes of frog skin epithelial cells (Brodin & Nielsen 2000b). A P2X receptor has been cloned from larval amphibian skin (Jensik *et al.* 2001), which resembles a receptor (now recognized as an orthologue of the $P2X_5$ receptor) in chicken skeletal muscle (Bo *et al.* 2000). Internalization of these receptors is associated with desensitization (Jensik & Cox 2002).

P2X and P2Y receptors have been identified in ampullary epithelium from frog semicircular canal, probably involved in endolymph homeostasis (Butlen *et al.* 1998). The P2Y receptors are likely to be $P2Y_2$ or $P2Y_4$ and $P2Y_6$ subtypes as UTP and UDP were potent agonists (Teixeira *et al.* 2000). Calcium-ATPase has been localized in frog crista ampullaris (Gioglio *et al.* 1998).

ATP activates a P2X receptor in erythrocytes of *Necturus* during hypotonic swelling (Light *et al.* 2001). ATP raises intracellular Ca^{2+} and activates basolateral

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Cl^- conductance in the proximal tubule of *Necturus* via P2Y receptors (Bouyer *et al.* 1998). ATP modulates the firing rate of pacemaker cells isolated from the sinus venosus of the cane toad via a $P2Y_1$ receptor (Ju *et al.* 2003), activation of which modulated Ca^{2+} release from the sarcoplasmic reticulum.

Early expression of a nucleotide receptor, $P2Y_8$, was found in the neural plate of *Xenopus* embryos and reappeared transiently later during secondary neuralation in the tail bud, suggesting that it may play a role in the early development of neural tissue (Bogdanov *et al.* 1997). Subsequently, another P2Y receptor, an orthologue of the $P2Y_{11}$ receptor, has been described in *Xenopus* embryos beginning at gastrulation and is later expressed in the brain, eye, lens, otic vesicle, brachial arches, spinal cord, notochord, somites and pronephric kidney (Devader *et al.* 2007).

Reptiles

Snake envenomation employs three strategies: prey immobilization via hypotension, prey immobilization by paralysis and prey digestion. Adenosine, guanosine and inosine play a central role in this process in most advanced snakes (see Aird 2002). There is no clear correlation between quantities of venom nucleosides and 5′-nucleotidase, phosphodiesterase and alkaline phosphomonoesterase (Aird 2002). The prey of *Bitis* snakes are subjected to massive hypotension upon envenomation, which appears to be due largely to the high levels of adenosine present, which produce vasodilation by direct action on vascular smooth muscle via A_2 receptors, reduces sympathetic vasoconstriction via pre-junctional inhibition of excitatory transmitters released via A_1 receptors, and by triggering mast cell degradation via A_3 receptors releasing dilators such as histamine and 5-hydroxytrytamine (Graham *et al.* 2005).

ATP leads to rapid and transient increase in intracellular Ca^{2+} in the nucleated red blood cells from the lizards *Ameiva ameiva* and *Tupinambis merianae*, probably via a P2Y receptor as mobilization of Ca^{2+} is mostly from ER and acidic internal Ca^{2+} stores (Beraldo *et al.* 2001). A later study suggested that this was a $P2Y_4$-like receptor as UTP was a potent agonist (Sartorello & Garcia 2005). Activation of this receptor resulted in $InsP_3$ production and Ca^{2+} mobilization from the ER. In red blood cells from *Iguana iguana* and *Turdus torquatus*, ATP, UTP and also GTP elicit calcium responses, but only in the presence of extracellular Ca^{2+}, thus suggesting the involvement of P2X-like receptors (Bagnaresi *et al.* 2007). A_1 and $P2X_1$ receptors are present in the smooth muscle of the aorta of the Agama lizard and garter snake (Knight & Burnstock 2001).

Freshwater turtles, as well as some fish, are extremely anoxia-tolerant, capable of surviving hours of oxygen-deprivation at high temperatures and weeks to months at low temperatures and the possible involvement of adenosine in the underlying mechanism was explored (Lutz & Kabler 1997, Pek & Lutz 1997, Petersen *et al.* 2003, Buck 2004). Adenosine appeared to be a primary mediator responsible for anoxic survival of the turtle. In particular A_1 receptor activation mediates channel arrest of NMDA receptor activity during normoxia, but not in anoxia in turtle cortical neurones (Pamenter *et al.* 2008).

Birds

Early studies concerned with purinergic signalling in the cardiovascular, gastrointestinal systems as well as in peripheral ganglia and developing skeletal muscle have been reviewed (Burnstock 1996).

A separate P2X receptor subtype, tentatively named as the chick $P2X_8$ receptor, was cloned and characterized from embryonic chick skeletal muscle (Bo *et al.* 2000). This chick receptor comprised 402 amino acids and had a molecular weight of 45.1 kDa. The amino acid sequence showed some homology with other P2X family members, being 43% for $P2X_1$, 39% for $P2X_2$, 43% for $P2X_3$, 53% for $P2X_4$, 59% for $P2X_5$, 47% for $P2X_6$ and 24% for $P2X_7$. A later study suggested that the chick $P2X_8$ receptor probably corresponds to the $P2X_5$ receptor (North 2002). After expression of $P2X_8$ cRNA in Xenopus oocytes, application of 10 μM ATP triggered fast and large inward currents, which showed full desensitization; the recovery from desensitization was extremely slow with 31% of recovery after 30 min and 65% after 1 h (Bo *et al.* 2000). This very slow recovery has not been identified for any other known types of P2X receptors. $P2Y_1$ and $P2Y_2$ receptors are also expressed in chick skeletal muscle, and they are involved in the regulation of the ACh receptor and of acetylcholinesterase (Choi *et al.* 2001, 2003, Tung *et al.* 2004). Ectoenzymes involved in the degradation of ATP in chick skeletal muscle have been described (Delgado *et al.* 1997).

Data have been presented that suggest that ATP is the main non-adrenergic, non-cholinergic transmitter controlling the contractile activity of the quail rectum (Shiina *et al.* 2007). Ecto-ATPase and ecto-apyrase have been immunolocalized in the smooth muscle layers of chicken gizzard and stomach (Lewis-Carl & Kirley 1997) and oviduct (Strobel *et al.* 1996). P2X receptors have been identified functionally on cholinergic presynaptic terminals in the chicken ciliary ganglion (Sun & Stanley 1996); the single-channel conductance of these receptors was about 17 pS.

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ATP-activated receptors have been shown on chicken ovarian granulosa cells, which trigger an oscillatory $[Ca^{2+}]_i$ response and appear to be mediated by P2Y receptors, regulated by PKC (Morley *et al.* 1996). Excitatory purinergic neuromuscular transmission probably mediated by P2Y receptors coupled to PLC via pertussis toxin-sensitive G proteins has been demonstrated in the longitudinal muscle of chicken anterior mesenteric artery (Khalifa *et al.* 2005). $P2Y_1$ receptors have been implicated in the development of the chick embryo, including limb buds, mesonephros, brain, somites and facial primordia (Meyer *et al.* 1999).

Plants

There has been strong recent interest in the roles of extracellular ATP in plant growth and regeneration, following earlier reports of cytoplasmic streaming induced by ATP in Chara cells (Williamson 1975) and other studies about the roles of purines reviewed by Burnstock (1996). Excellent recent reviews about purinergic signalling in plants are also available (Demidchik *et al.* 2003, Jeter & Roux 2006, Roux & Steinebrunner 2007).

Auxins are plant growth regulators that increase the rate of cell enlargement in plant stems. The activity of an auxin-stimulated nicotinamide adenine dinucleotide H^+ oxidase activity from soybean hypocotyls was inhibited by nanomolar concentrations of ATP, but not by other nucleotides or nucleosides (Morre 1998). ATP, perhaps via P2-like receptors, induces accumulation of superoxide via NADPH oxidases in the leaves of *Arabidopsis* (Song *et al.* 2006). A Ca^{2+} channel blocker or chelator and calmodulin antagonist reduced ATP-induced superoxide accumulation; further ATP treatment enhanced the expression of genes that are induced by wounds and other stresses. Extracellular ATP inhibits gravitropism in both maize and *Arabidopsis* roots in concentrations that inhibit polar auxin transport (Tang *et al.* 2003). Regions of growth in *Arabidopsis* show the highest release of ATP and the highest expression of ectonucleotidases AtAPY1 and AtAPY2 (Roux *et al.* 2008). Adenosine triphosphatase activity has been identified in the salt glands of *Sporobolus virginicus* (Naidoo & Naidoo 1999). A family of apyrase genes were shown to play a role early in the modulation response before the involvement of root cortical cell division in *Medicago truncatula* leading to nodule structure (Cohn *et al.* 2001). Transgenic expression of the soybean apyrase in *Lotus japonicus* enhances modulation (McAlvin & Stacey 2005). ATP and ADP depolarize the membrane potential of growing root hairs of *Arabidopsis thaliana* and it was suggested that ADP might serve as a signal during cellular wounding or as a sensor of bacterial or fungal activity near the root surface (Lew & Dearnaley 2000). A recent study has shown ATP release induced by a fungal elicitor from yeast and its involvement in the elicitor-induced responses in *Salvia miltiorrhiza* hairy root cultures (Wu *et al.* 2008). Salt stress is a major environmental factor influencing plant growth and development. Pyridoxal kinase is involved in the biosynthesis of pyridoxal-5-phosphate, an active form of vitamin B6. It has been proposed that pyridoxal-5-phosphate, a ligand for P2X receptor channels, regulates Na^+ and K^+ homeostasis by modulating the activities of ion transporters involved in salt tolerance in *Arabidopsis* (Shi *et al.* 2002). Despite these pharmacological indicators for the presence of P2X receptors in *Arabidopsis*, no protein sequences for P2X receptors were found in this species (Kim *et al.* 2006).

Exogenously applied ATPγS and ADPβS increase intracellular free Ca^{2+} in *Arabidopsis* seedlings, as well as increasing the level of transcripts encoding mitogen-activated protein kinases and proteins involved in ethylene biosynthesis and signal transduction (Jeter *et al.* 2004). The authors suggest that ATP may play a physiological role in transducing stress and wound responses.

ATP, alone or in combination with antioxidants, is a growth regulator for the micropropagation of cucumber from nodal explants and has commercial potential (Matakiadis & Kintzios 2005). ATP is released by plant cells and acts to suppress a default death pathway, and some forms of pathogen-induced cell death are mediated by the depletion of extracellular ATP (Chivasa *et al.* 2005).

Plants can perceive a wide range of biotic attackers and respond with target-induced defences. ATP synthase γ regulatory regions mediate plant perception of herbivore through the induction of volatile, phenylpropanoid and protease inhibitor defences in cowpea *Vigna unguiculata*, attacked by armyworm (Schmelz *et al.* 2006).

Apyrases play a key role in the regulation of growth in *Arabidopsis* (Wu *et al.* 2007). The substrate specificity, affinity labelling and proteolytic susceptibility of potato tuber isoapyrases have been described (Kettlun *et al.* 2005). Levels of ATP increased in wounded potato tuber slices as well as adenosine nucleosidase and a role of adenosine salvage in wound-induced adenylate biosyntheses was suggested (Katahira & Ashihara 2006).

Extracellular ATP induces NO production in tomato cell suspensions via P2-like receptors (Foresi *et al.* 2007). To investigate whether extracellular ATP is present on root surface of the plant *M. truncatula*, a special reporter protein was constructed by fusing a

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cellulose-binding domain peptide with luciferase, an ATP-requiring enzyme and in this way it was shown that extracellular ATP is present in growing plant cells (Kim *et al.* 2006).

ATP and a chitin mixture were shown to increase reactive oxygen species activity in root hairs, which is essential for their growth, but no changes were observed in response to adenosine, AMP, ADP and β,γ-meATP. However, potato apyrase decreased reactive oxygen species activity (Kim *et al.* 2006).

Discussion

Chemical transmission, which utilizes small molecules for cell-to-cell information transfer, was an essential evolutionary step, which allowed continuous progression of life forms. Our knowledge of the initial appearance and early forms of chemical transmission is virtually nonexistent (Trams 1981), and yet some generalization can be drawn from observations of phylogenetic development and from evidence of distribution of different signalling systems in the higher life forms. Our conjectures of the modus operandi and habits of the first life forms lie entirely in the realm of speculation, and yet we may assume that some of these nascent living creatures were born and existed in the ocean, and thus the intercellular communication called for a diffusible messenger. Choices for these diffusible messengers were only a few; they can be ions or small diffusible molecules. Ions can be excluded from extracellular communication pathways because of their high background concentrations in the primordial seas, and thus only the relatively small soluble molecules existing in abundance within the cells can be employed. These could be some amino acids, or some forms of gaseous transmitters (for example NO), or protons, which may accumulate in the cells following metabolism or indeed purines, and especially purines endowed with pyrophosphate bonds.

As usual during evolution, most possibilities were explored. Indeed the most ancient receptors discovered in prokaryotes are those for glutamate (in a form of a potassium-selective glutamate receptor identified in *Synechocystis* (Chen *et al.* 1999) and further development resulted in appearance of glutamate receptors in early eukaryotes (Chiu *et al.* 1999, 2002) and for protons, recently cloned from cyanobacterium *Gloeobacter violaceus* (Bocquet *et al.* 2007). The early evolution of gaseous extracellular signalling molecules remains quite obscure; we know that NO was present already in primitive nervous systems (Garthwaite 2008). However, there is little evidence to date of gaseous transmitters in primitive unicellular life forms.

The purines were also the part of very early signalling evolution. Indeed the latter in a form of adenine and guanine phosphates occurred in pre-biotic period (as a result of purely thermal synthetic processes (Ponnamperuma *et al.* 1963, Waldrop 1989) and rapidly acquired high importance. For example, ATP participates in more chemical reactions than any other compound on the earth's surface, except water. Purines and pyrimidines were essential for construction of both RNA and DNA, and hence their intracellular concentration was high. Furthermore, ATP was selected very early as the main source of biological energy, and thus became an indispensable feature of life. The prominent roles of adenine-phosphates in energy metabolism in the most primitive life forms and ancient origin of adenine-binding sites are widely recognized (Wilson 1984). This was a critical evolutionary choice as it immediately resulted in birth of the universal intracellular signalling system based on calcium ions (Case *et al.* 2007) as keeping cytosolic Ca^{2+} extremely low became vitally important, as otherwise insoluble Ca^{2+}-phosphates would preclude the cell energetics. Thus even the most primitive ancient cell had high cytosolic concentrations of ATP (or GTP) and upon cell disintegration gradients of both would be present in the surrounding water. This may have been the initial form of chemical transmission, which remained throughout evolution. Indeed every known cell or single cell organism does display some form of sensitivity to ATP, and moreover the ATP transmission is unique in a sense that it is not confined to a particular tissue or organ of higher organisms – it exists everywhere. Indeed it is difficult to find a cell type which does not show sensitivity to ATP.

It is possible that the first function of purines was an avoidance reaction, perhaps a forerunner of purinergic nociception? In other words, perhaps ATP represents a universal signalling molecule first related to survival, i.e. sensitivity of cells to ATP maybe was initially utilized as a danger signal. It is interesting that in plants a major role of ATP signalling is to initiate avoidance reactions in roots.

It should be noted that P2X-like protein sequences are absent in some invertebrates (*Anopheles gambiae*, *C. elegans* and *Drosophila melanogaster*) (Agboh *et al.* 2004), although the P2X receptors exist in more primitive life forms. It should not be assumed, however, that receptors for purines and pyrimidines are not present, especially where there is compelling pharmacological evidence for the potent effects of agonists. It may simply be that the receptors involved have not been identified yet.

In the 1996 review, it was speculated that G protein-coupled receptors, P1 and P2Y, were expressed first during evolution and that P2X ion channel receptors appeared later with the development of the nervous system associated with the need for fast signalling.

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However, it is now clear from the elegant recent cloning of P2X receptors in *Dictyostelium*, *Schistosoma* and algae that P2X receptors appeared very early, and perhaps initially were expressed largely on intracellular organelle membranes. However, many more studies will be needed before the evolution of purinergic signalling is unravelled.

Conflict of interest

There is no conflict of interest.

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