Research Overview

Purinoceptors: Ontogeny and Phylogeny

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Strategy, Management and Health Policy				
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ABSTRACT   The majority of studies of the functional distribution of purinoceptors have been carried out with mammalian preparations. The objective of this article is to review the disparate literature describing purinoceptor-mediated effects in invertebrates and lower vertebrates and, in view of the concept that ontogeny repeats phylogeny, to review also the evidence for purinoceptor involvement in the complex signaling involved in embryonic development. Even with the limited information currently available, it is clear that purinoceptors are involved in early signalling in vertebrate embryos; one novel G protein-coupled P2Y receptor has already been cloned and characterized in frog embryo and hopefully more will follow. It is also clear that purinoceptors for both adenosine and ATP are present in early evolution and play a number of different roles in most, if not all, invertebrate and lower species. However, until selective agonists and antagonists are identified for the recently cloned purinoceptors subtypes in vertebrates, it will not be possible to resolve questions concerned with the evolution of these subtypes. Molecular cloning of genes encoding receptors for purines and pyrimidines from invertebrates and lower vertebrates represents an alternative approach to advancing knowledge in the area. Drug Dev. Res. 39:204–242, 1996.   © 1997 Wiley-Liss, Inc.

Key words: P2 purinoceptors; ATP; ontology; phylogeny

INTRODUCTION

Early Evolutionary Appearance of Purine Nucleotides

ATP was identified in muscle cells independently by Fiske and Subbarow and by Lohmann in 1929 [see Schlenk, 1987; Maruyama, 1991]. 5′-AMP (myoadenylic acid) was described by Embden and Zimmerman in 1927, but it became clear later that the bulk of 5′-AMP in cells occurred as ATP with less than 2% as 5′-AMP per se [Lohmann and Schuster, 1934].

It has been speculated that the first organisms consisted of RNA and that as these organisms evolved, they learned to synthesise proteins which could help them to replicate more efficiently; later, RNA-based organisms gave rise to DNA, a molecule more reliable for storing the genetic information [Waldrop, 1989; Horgan, 1991]. Phosphorylation of nucleosides and the formation of pyrophosphate bonds may have occurred on the early earth by purely thermal processes [Sawai and Orgel, 1975], the resulting compounds becoming important starting materials for further syntheses in aqueous solutions or on

surfaces [Cairns-Smith, 1985]. In this way, the formation of ATP from AMP would have paved the way for the formation of oligonucleotides and finally RNA. ATP appears to have been particularly suitable for early development because of its propensity to bond with the metal ion, Mg^{2+} that promotes dephosphorylation and generation of energy; ITP and GTP also show a metal ion-promoted dephosphorylation but their reactive rates are lower. The pyrimidines were also likely to be available in the primitive earth, but it is suggested that they were incorporated into living systems in a more passive way, possibly even directed by the purines [Sigel, 1992]. Thus, ATP appears to have played a crucial and active role in early evolution. In contemporary biochemistry, ATP is still the most important energy-rich intermediate in nucleotide processes. The enzyme-catalyzed hydrolysis of ATP to

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ADP and PO_4 is the main source of energy. It has been estimated that ATP participates in more chemical reactions than any other compound on the earth's surface, except water.

Compelling arguments have been presented for the prominent role of ATP and ADP in intracellular energy metabolism very early in evolution, including the availability of adenine compounds in the biosphere and the development of complementary binding sites on cellular proteins [see Wilson, 1984]. However, until recently, less attention has been directed towards the early evolutionary appearance of ATP and adenosine as extracellular messengers in cell communication and signalling.

In the evolution of neurochemical transmitters it has been suggested that purine derivatives may well have been the primordial transmitter substances [Trams, 1981]. There are reports of extracellular actions of ATP in very primitive organisms, including bacteria, diatoms, algae, and slime moulds. For example, ATP inhibits prodigiosin formation in *Serratia marcescens*, while adenosine does not [Lawanson and Sholeye, 1976]. Exogenous ATP stimulates generative nuclear division in pollen tubes of *Lilium longiflorum* [Kamizyo and Tanaka, 1982]. ATP regulation of oscillating torsional movement in strands of the slime mould *Physarum polycephalum* has also been described [Ogihara, 1982]. Caffeine, an adenosine antagonist, can block cell plate formation in meristem cells of onion root tips and adenosine antagonises this action [Gonzalez-Fernandez and Lopez-Saez, 1980]. Changes in membrane potential and excitability of *Chara* cells and cytoplasmic streaming in response to ATP have been demonstrated [Williamson, 1975; Shimmen and Tazawa, 1977]. Both adenosine and 2′-deoxyadenosine at very low concentrations (10^{-13} M) promoted growth in the diatom *Phaeodactylum tricornutum* [Komada et al., 1983]. A high affinity binding site for ATP has been localised on membrane-bound chloroplast ATP synthase isolated from leaves of spinach [Abbott et al., 1984]. The F_1-ATPase component of ATP synthase from chloroplasts (as well as mitochondria and microorganisms) contain six sites that can be occupied by adenine nucleotides [Senior, 1988]. Adenosine has been shown to inhibit the growth of various bacteria including *Crithidia fasciculata* [Dewey et al., 1978], *Micrococcus sodonensis* [Shobe and Campbell, 1973a,b], and *Staphylococcus aureus* [Matheiu et al., 1969]. Adenosine polyphosphates have been found in bacilli [Rhaese et al., 1972] and in *Streptomyces* [Muraio et al., 1978] and have been considered to be involved in the initiation of sporulation in *Baccillis subtilis* [Rhaese et al., 1972]. Some purine and pyrimidine nucleotides and nucleosides inhibit spore germination in *Streptomyces galilaeus* [Hamagishi et al., 1980]. Exogenous adenosine 5′-triphosphate 3′-diphosphate (pppApp) reduces growth rate and increases sporulation frequency by 100

times or more in *Bacillus subtilis* [Murao et al., 1980; Kameda et al., 1983] and *Streptomyces galilaeus* [Hamagishi et al., 1980]. Extracellular ATP and other 5′-nucleotides are broken down by the high activity of membrane-bound 5′-nucleotidase in the halophilic bacterium, *Vibrio costicole* [Sakai et al., 1987]. UTP, ATP, and pyrophosphate are metabolized by alkylsulfatase-producing bacteria [Stewart and Fitzgerald, 1981]. Membrane-associated ATPases from halophilic archaebacterium including *Holobacterium salinarium* and *Methanosarcina barkeri* have been isolated, purified, cloned, and sequenced [Ihara and Mukohata, 1991]. TmrB protein, responsible for tunicamycin resistance of *Bacillus subtilis*, is a novel ATP-binding membrane protein [Noda et al., 1992].

History and Current Perception of Extracellular Purinergic Signalling

Since the early recognition of the potent extracellular actions of ATP and adenosine by Drury and Szent-Györgyi [1929] there has been an escalating development of knowledge of the receptors involved. In 1978, Burnstock established the existence of separate receptors for adenosine (P1) and for ATP/ADP (P2) and subclasses of P1 receptors (A_1 and A_2) [Londos et al., 1980] and for P2 purinoceptors (P2X and P2Y) [Burnstock and Kennedy, 1985] followed. P1 purinoceptors have been long known to act via adenylate cyclase second messenger systems [Sattin and Rall, 1970]. On the basis of the transduction mechanisms involved in P2-purinoceptor activation [Dubyak, 1991] and cloning of the receptors [Webb et al., 1993; Lustig et al., 1993; Valera et al., 1994; Brake et al., 1994], Abbracchio and Burnstock [1994] outlined the basis for the currently accepted subclassification of P2 purinoceptors, namely the subdivision into a P2X family of ligand-gated ion channel receptors and a P2Y family of G protein-coupled receptors. To date, seven members of the P2X purinoceptor family and eight members of the P2Y purinoceptor family have been recognized [see Burnstock, 1996a; Burnstock and King, 1996].

The majority of studies of purinoceptor functional distribution have been carried out in mammalian preparations and it is one objective of this article to review the disparate literature describing purinoceptors in invertebrates and lower vertebrates firstly to establish the primitive and long-standing utilization of these receptors during evolution and secondly to see if any pattern of receptor subtype development can be recognized.

In view of the extraordinarily widespread distribution of extracellular receptors for purines during phylogeny, the second objective of this article is to review the beginnings of exploration into possible roles for purines during the complex sequences of signalling involved in embryonic development. The article will not include descriptions of the postnatal development of purinocep-

tors or changes occurring in aging, but some accounts are available [see Swedin, 1972; MacDonald and McGrath, 1984; Furukawa and Nomoto, 1989; Matherne et al., 1990; Koga et al., 1992; Nicholls et al., 1992; Zagorodnyuk et al., 1993; Peachey et al., 1996].

It is important to recognize that both *short-term signalling*, such as that occurring in neurotransmission and secretion [see Burnstock, 1972, 1996b] and *long-term signalling* involved in cell division, proliferation, differentiation, regeneration, and death and in plasticity of expression in pathological states including wound healing [see Fraser et al., 1979; Ziada et al., 1984; Teuscher and Weidlich, 1985; Dusseau et al., 1986; Meininger et al., 1988; Adair et al., 1989; Huang et al., 1989; Kubo, 1991c; Rathbone et al., 1992; Erlinge et al., 1993; Henning et al., 1993a,b; Burnstock, 1993; Abbracchio et al., 1994; Boarder et al., 1995; Neary et al., 1996; Abbracchio, 1996] should be taken into consideration when examining both the ontogeny and phylogeny of purinoceptors.

ONTOGENY OF PURINOCEPTORS

In the past, a role of ATP in early development has been interpreted merely in terms of its use as a source of energy. However, since it is now generally accepted that ATP and adenosine have potent extracellular actions mediated by the activation of specific membrane receptors, a number of these previous studies can now be reinterpreted. ATP and adenosine play key roles from the very beginnings of life, i.e., the moment of conception. ATP is obligatory for sperm movement [Yeung, 1986] and is a trigger for capacitation, the acrosome reaction necessary to fertilize the egg [Foresta et al., 1992]. Extracellular ATP also promotes a rapid increase in Na^+ permeability of the fertilized egg membrane through the activation of a specific ATP receptor [Kupitz and Atlas, 1993]. Together with the demonstration that ATP-activated spermatozoa show very high success rates in fertilization tests [Foresta et al., 1992], this strongly suggests that ATP is a key sperm-to-egg signal in the process of fertilization.

Purinoceptors in Frog Embryos

The nicotinic channels in myotomal muscle cells cultured from *Xenopus* embryos at stages 19–22 were shown to be opened by micromolar concentrations of exogenous ATP [Igusa, 1988], following the earlier demonstration that ATP increases the sensitivity of receptors in adult frog skeletal muscles without increasing the affinity of acetylcholine (ACh) for the receptor or inhibitory acetylcholinesterase [Akasu et al., 1981]. Since then, there have been a number of studies of the actions of ATP in developing *Xenopus* neuromuscular synapses [see Fu, 1995]. Extracellular applications of ATP to develop-

ing *Xenopus* neuromuscular synapses in culture potentiate ACh responses of developing muscle cells during the early phase of synaptogenesis [Fu and Poo, 1991; Fu, 1994; Fu and Huang, 1994]. The possibility that extracellular ATP co-released with ACh, may serve as a positive trophic factor at developing neuromuscular synapses has also been raised [Fu and Poo, 1991; Fu; 1995]. It is further suggested that calcitonin gene-related peptide (CGRP) and ATP co-released with ACh from the nerve terminal may act together to potentiate postsynaptic ACh channel activity during the early phase of synaptogenesis [Lu and Fu, 1995]; it is claimed that CGRP actions are mediated by cAMP-dependent protein kinase (PKA), while ATP exerts its effects via protein kinase C (PKC).

In a recent study of the regulation of rhythmic movements by purinergic transmitters in frog embryos [Dale and Gildry, 1996], it has been shown that ATP is released during swimming that activates P2Y-receptors to reduce voltage-gated K^+ currents and cause an increase in the excitability of the spinal motor circuits. It was also shown that adenosine, resulting from the breakdown of ATP, acts on P1 receptors to reduce the voltage-gated Ca^{2+} currents to lower excitability of the motor circuits thereby opposing the actions of ATP. The authors suggest that a gradually changing balance between ATP and adenosine underlies the run-down of the motor pattern for swimming in *Xenopus*.

We have recently cloned and sequenced in my laboratory a novel P2Y-purinoceptor (X1P2Y) that is expressed (as seen by Northern blots and in situ hybridization) in the neural plate of *Xenopus* embryos from stages 13 to 18 and again at stage 28 when secondary neurulation occurs in the tail bud [Bogdanov et al., 1997]. It differs from other members of the P2Y-purinoceptor family in that it has an intracellular C terminus with 216 amino acid residues (compared to 16 to 67 in $P2Y_{1-7}$). When expressed as a recombinant receptor in *Xenopus* oocytes, it shows equipotent responses to the triphosphates ATP, UTP, ITP, CTP, and GTP and smaller responses to diphosphates and tetraphosphates, but is not responsive to inorganic phosphates. Responses to activation of the X1P2Y receptor have a long duration (40–60 min). These data suggest that this novel P2Y-receptor may be involved in the early formation of the nervous system.

Purinoceptors in Chick Embryos

Together with muscarinic cholinergic receptors, extracellular receptors to ATP were shown to be the first functionally active membrane receptors in chick embryo cells at the time of germ layer formation [Laasberg, 1990]. In gastrulating chick embryo, ATP causes rapid accumulation of inositol-phosphate and Ca^{2+} mobilization in a similar way and to the same extent as ACh, whereas other neuroendocrine substances such as insulin and norad-

renaline (NA) have much weaker effects [Laasberg, 1990]. This suggests that, alongside ACh, other phylogenetically old and universal regulators of cell metabolism such as ATP (and perhaps nitric oxide) might play a leading role in the functional regulation of gastrulation via the activation of specific receptors triggering Ca^{2+} mobilization.

ATP has been shown to induce precocious evagination of the embryonic chick thyroid, an event which has been hypothesized to be involved in the formation of the thyroid gland from the thyroid primordium [Hilfer et al., 1977]. The requirement for ATP was very precise, since it could not be replaced by pyrophosphate, AMP, or ADP nor by GTP, suggesting a high degree of specificity of the ATP-induced effect.

ATP acts on embryonic and developing cells of both nervous and non-nervous systems by increasing intracellular Ca^{2+} concentrations. Release of Ca^{2+} from intracellular stores is evoked in the otocyst epithelium of the early embryonic chick, incubated for 3 days (stage 18 to 19) [Nakaoka and Yamashita, 1995] (Fig. 1), in developing chick myotubes [Häggblad and Heilbronn, 1988] and in dissociated cells from whole early embryonic chicks [Laasberg, 1990; Lohmann et al., 1991].

A recent study of embryonic chick neural retina [Sugioka et al., 1996] has shown that the ATP-induced rise in intracellular Ca^{2+} is mediated by P_{2U} (= $P2Y_2$)-

Fig. 1. Interaction between acetylcholine and ATP recorded in an otocyst from chick embryo. a: The response to 10 µM acetylcholine. b: The response to 100 µM ATP. c: The response to the co-application of 10 µM acetylcholine and 100 µM ATP. The records in a–c were taken in this order at 5 min intervals. The bath solutions contained 25 mM Ca^{2+}. Reproduced from Nakaoka and Yamashita, 1995.

purinoceptors and that there is a dramatic decline of the ATP-induced rise in intracellular Ca^{2+} just before synaptogenesis. Suramin and Reactive Blue 2 almost completely block these responses (Fig. 2). These authors also reported unpublished data that injection of Reactive Blue 2 into early embryonic chicks produced severe effects in embryogenesis.

A transmitter-like action of ATP on patched membranes of myoblasts and myotubes cultured from 12-day-old chicken embryos was first demonstrated by Kolb and Wakelam in 1983. Using biochemical methods, ATP-induced cation influx was later demonstrated in myotubes prepared from 11-day-old chick embryos and shown to be additive to cholinergic agonist action [Häggblad et al., 1985]. Later papers from this group claimed that the myotube P2-purinoceptor triggers phosphoinositide turnover [Häggblad and Heilbronn, 1988] and alters Ca^{2+} influx through dihydropyridine-sensitive channels [Erikson and Heilbronn, 1989]. ATP has a potent depolarizing action on myotubes derived from pectoral muscle cultured from 11 day chick embryos [Hume and Hönig, 1986] and its physiological and pharmacological properties have been described in a series of papers [Hume and Thomas, 1988; Thomas and Hume, 1990a,b, 1993; Thomas et al., 1991]. The myotube P2-purinoceptor is not activated by ADP, AMP, adenosine or the non-hydrolyzable ATP analogues α,β-methylene ATP (α,β-meATP) or β,γ-methylene ATP (β,γ-meATP) [Hume and Hönig, 1986]. A single class of ATP-activated ion channel conducts both cations and anions in the myotube [Thomas and Hume, 1990a] and the P2-purinoceptors involved showed marked desensitization [Thomas and Hume, 1990b]. The sensitivity of extracellular ATP has been tested at various stages of development of different muscles [Wells et al., 1995]. At embryonic day 6 [stage 30 of Hamberger and Hamilton, 1951] ATP (50–100 µM) elicits vigorous contractions in all the muscles tested, but by embryonic day 17 (stage 43) none of the muscles contract in response to ATP (Fig. 3). However, denervation of muscles in newly hatched chicks leads to the reappearance of sensitivity to ATP, suggesting that the expression of ATP receptors is regulated by motor neurons. An immunohistochemical study of the distribution of 5'-nucleotidase during the development of chick striated muscle shows that the adult exhibits a more restricted distribution compared to the embryo [Mehul et al., 1992].

Studies of the development of pharmacological sensitivity to adenosine analogs in embryonic chick heart [Hatae et al., 1989; Blair et al., 1989] show that pharmacological sensitivity to A_1 agonists begins at embryonic day 7 and then increases continuously to day 12, when the atria became fully responsive. Ligand binding shows that A_1 receptors are present at days 5 and 6, but are not responsive to adenosine, and the author concluded that
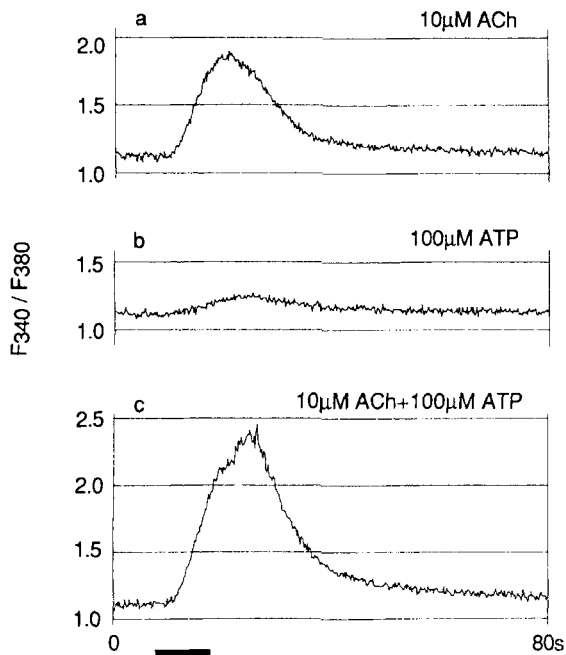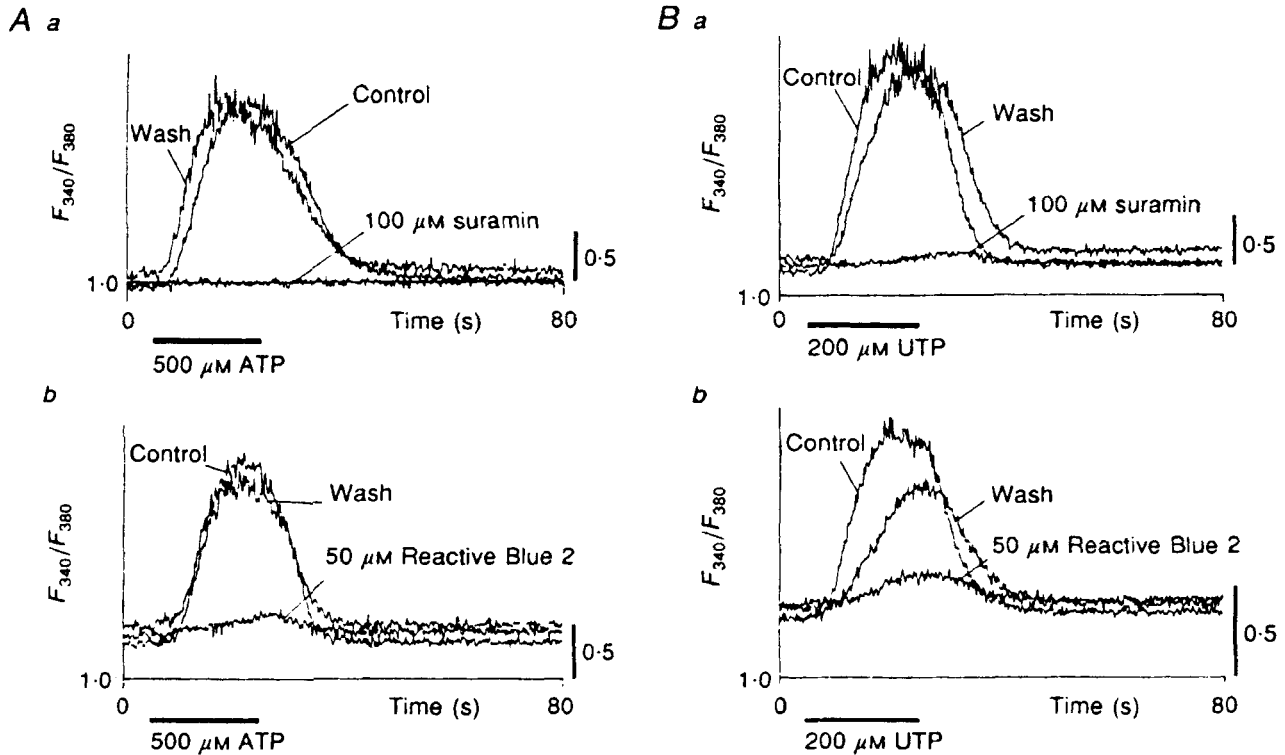
Fig. 2. Effects of P2 purinoceptor antagonists on the Ca^{2+} responses to ATP and UTP in E3 retinas. **A**: The effects of suramin (100 μM; A,a) and reactive blue 2 (50 μM; A,b) on the response to 500 μM ATP. The records in the presence of suramin or Reactive Blue 2 were taken 7 min after changing the bath solutions to the antagonist-containing medium. The recovery controls were taken after washing suramin for 7 min or reactive blue 2 for 25 min. The duration of ATP application (20 s) is indicated by the bars. All records were taken in the bath solutions containing 2.5 mM Ca^{2+}. **B**: The effects of suramin (100 μM; B,a) and Reactive Blue 2 (50 μM; B,b) on the response to 200 μM UTP. The records in the presence of suramin or Reactive Blue were taken 7 min after changing the bath solutions to the antagonist-containing medium. The recovery controls were taken after washing suramin for 7 min or reactive blue 2 for 15 min. The duration of UTP application (20 s) is indicated by the bars. All records were taken in the bath solutions containing 2.5 mM Ca^{2+}. Reproduced from Sugioka et al., 1996.

the development of sensitivity to A_1 adenosine receptor-mediated negative chronotropic responses was not paralleled by developmental changes in adenosine inhibition of adenylyl cyclase, or by the development of sympathetic and parasympathetic innervation. Chronic exposure of the embryonic chick heart (15–17-days-old) to R-PIA produces down-regulation of A_1 adenosine receptor and desensitization of the negative inotropic response to adenosine [Shryock et al., 1989].

Adenosine has been implicated in growth regulation of the vascular system in the chick embryo [Adair et al., 1989], in common with a similar role claimed for experimental angiogenesis in the chorio-allantoic membrane [Fraser et al., 1979; Teusher and Weidlich, 1985; Dusseau et al., 1986].

Responses to ATP have been described in ciliary neurons acutely dissociated from embryonic chick ciliary ganglia taken at day 14 [Abe et al., 1995]. The relative potency of agonists in producing transient inward currents with patch recording is ATP>2meSATP>ADP; neither adenosine, AMP or α,β-meATP are effective, but suramin is an

antagonist (Fig. 4). The authors suggest that the P2 receptor subtype involved might be P2Y, but in view of more recent knowledge about the functional properties of cloned subtypes of the P2 receptor family, it seems more likely to belong to the P2X receptor family.

Adenosine inhibits neurite outgrowth of chick sympathetic neurons taken from 11 day chick embryos and kills by apoptosis about 80% of sympathetic nerves supported by growth factor over the next 2 days in culture [Wakade et al., 1995]. Specific A_1 or A_2 agonists are not neurotoxic. The toxic effects of adenosine are not antagonized by aminophylline, but are prevented by an adenosine transporter or adenosine deaminase inhibitor, suggesting an intracellular site of action for the toxic effects of adenosine. The authors conclude that adenosine and its breakdown enzymes play an important role in the regulation of growth and development of sympathetic neurons.

Purinoceptors in Mammalian Embryos

Puff-applied ATP has been shown to have two main effects on a mouse mesodermal stem cell line: an increase
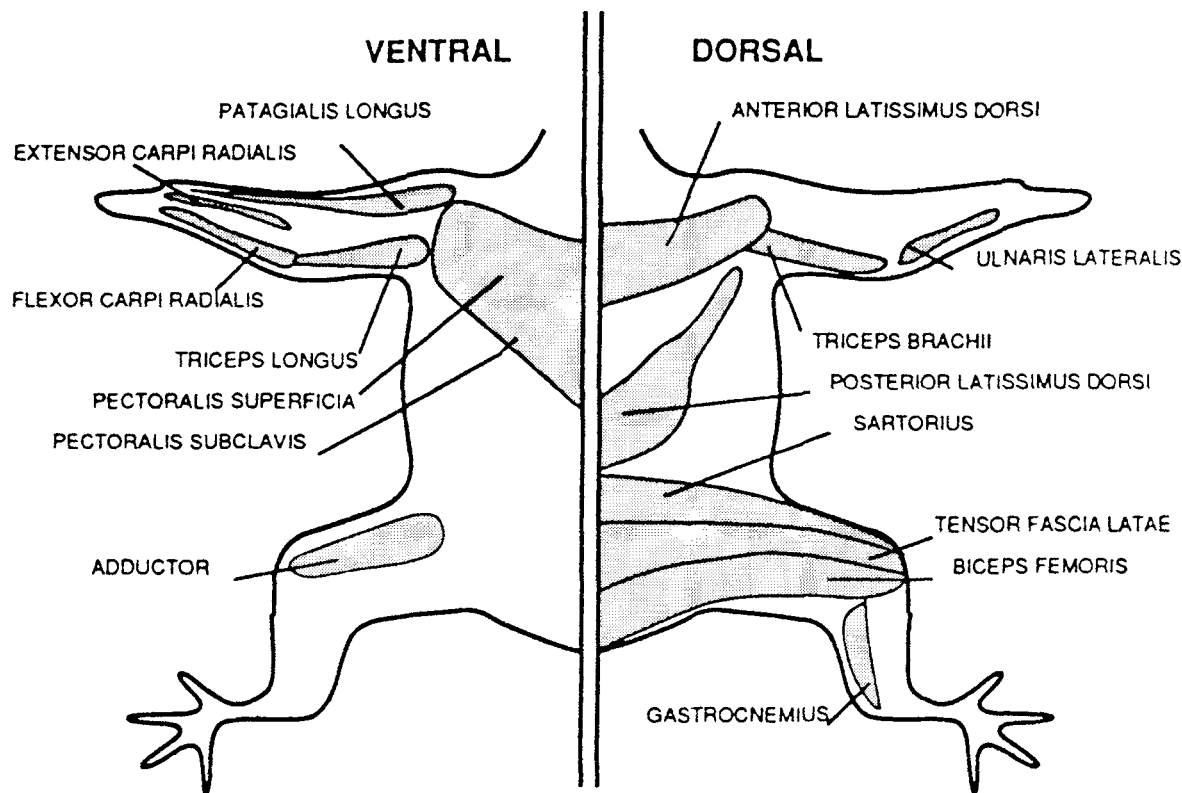
Fig. 3. Location of the muscles of the chick embryo that were responsive to ATP. Three chick embryos from stages 35–37 were sacrificed, and each muscle was identified and tested in at least two of the three embryos. All muscles tested in embryos of these ages contracted in response to ATP. By embryo day 17 (stage 43) none of the muscles contracted in response to ATP. Reproduced from Wells et al., 1995.

in intracellular Ca^{2+} concentrations and a subsequent hyperpolarization due to Ca^{2+}-activated K^+ conductance [Kubo, 1991a] (Fig. 5). The author speculates that the transient increase in intracellular Ca^{2+} may influence mesodermal cell differentiation, particularly in relation to muscle differentiation. In a later paper [Kubo, 1991b], two myoblastic cell lines, one from rat, the other from mouse, showed similar properties to those of the myogenic clonal cells derived from the mouse mesodermal stem cell line described above.

ATP and ADP have been shown to enhance, reduce, or have no effect (depending on the dose used) on the incidence of trypan blue-induced teratogenic malformations in the rat foetus at day 20 [Beaudoin, 1976]. Concomitant administration of ATP and cortisone in mice either decrease the teratogenic effect of cortisone (50 μg ATP) or enhance its teratogenic effect (>100 μg ATP) [Gordon et al., 1963].

Mouse heads of embryos from 14 to 24 pairs of body somites exposed to an ATP-containing medium have been demonstrated to undergo rapid epithelial thickening and invagination, a process that appears to take part in the shaping of nasal pits and formation of primary palate [Smuts, 1981].

Besides ATP, a number of reports implicate adenos-

ine as one of the endogenous effectors that can selectively modulate cell growth during embryonic development. For example, adenosine is shown to potentiate the delaying effect of dibutyryl cyclic adenosine monophosphate (a membrane-permeable analog of cAMP) on meiosis resumption in denuded mouse oocytes [Petrungaro et al., 1986]. The role of adenosine has been particularly well characterized in the morphogenetic outgrowth of vertebrate limb buds [Knudsen and Elmer, 1987]. Embryonic limb development in the mouse is driven by rapid mesenchymal cell proliferation induced by trophic substances secreted by the apical ectodermal ridge. This interaction can be restricted experimentally by pharmacological agents that elevate intracellular cAMP levels, or physiologically by the onset of programmed cell death triggered by naturally occurring negative regulators of growth. Mutations that affect the pattern of limb/bud outgrowth provide invaluable experimental means to investigate these growth-regulatory processes. Knudsen and Elmer [1987] studied the regulation of polydactylous out growth (an expression of the Hemimelia-extra toe (HmX/+) mutant phenotype) in hind-limb buds explanted into a serum-free in vitro system at stage 18 of gestation. Its expression was promoted by exposure to exogenous adenosine-deaminase, the enzyme which cata-
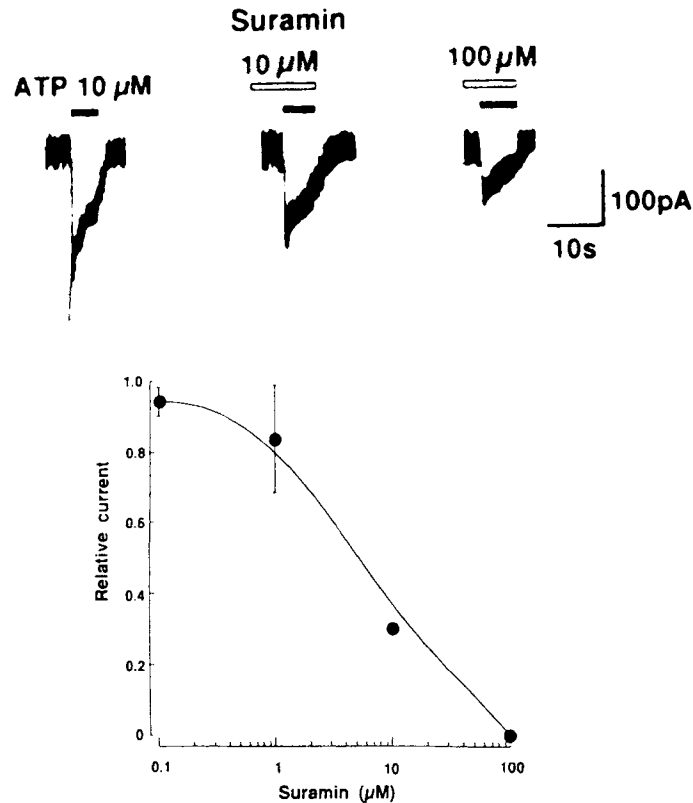
Fig. 4. Chick embryo (day 14) ciliary ganglion cells: the inhibition of ATP-induced inward current by suramin. The neurons were pretreated with suramin of various concentrations for 2 min. In the **upper panel**, the filled and open horizontal bars indicate the periods of application of ATP and suramin, respectively. In the **lower panel**, the responses in the presence of suramin are normalised to the peak current amplitude induced by 10 μM alone. Each point is the average of four neurons, and the vertical bars indicate S.E.M. Reproduced from Abe et al., 1995.

lyzes the inactivation of endogenous adenosine, and conversely suppressed by co-exposure to hydrolysis-resistant adenosine analogues. Adenosine-induced effects were mediated by activation of specific extracellular receptors, since the P1-purinoceptor antagonist, caffeine, could completely prevent suppression of polydactylous outgrowth. Measurement of both adenosine and adenosine deaminase levels in embryonal plasma and whole embryos argued against an endocrine mechanism of adenosine secretion, in favour of an autocrine (self-regulatory) or paracrine (proximate-regulatory mechanisms). These results suggest that the in vitro outgrowth of the prospective polydactylous region is induced upon escape from the local growth-inhibitory influence of extracellular adenosine.

Micromolar concentrations of adenosine, inosine, and hypoxanthine, but not guanosine block the second or third cleavage of mouse embryos developing in vitro [Nureddin et al., 1990]. Zygotes or early two-cell embryos, cultured in a purine-containing medium for 24 h, resume development following transfer to purine-free conditions. The precise mechanism of the purine-sensitive process is not known, but embryos conceived in vivo are sensitive until approximately 28–30 h after fertiliza-

tion and are no longer sensitive by 34 h [Loutradis et al., 1989]. However, a later study by this group has shown that the purine-induced block can be reversed by compounds that elevate cAMP [Fissore et al., 1992].

In a study of human fibroblasts, differential sensitivity to adenosine was demonstrated in donors of different ages [Bynum, 1980]. Fetal fibroblasts were the most sensitive to adenosine, which produced inhibition of growth and RNA synthesis; in contrast, fibroblasts taken from 4-year-old donors showed growth stimulation to adenosine.

Taken together, these results point to a role for purines in both physiological fertilization and normal development and also underline that alterations of the purinergic regulation of embryonal growth might be involved in the onset of morphological malformations. Depending upon the purine derivative, and probably upon the purinoceptor involved as well, ATP and adenosine can act as both positive and negative regulators of growth. This is also consistent with data obtained from in vitro cell lines which implicates purines in both cell proliferation and apoptosis. Further studies are needed to better characterize the receptor subtypes involved and
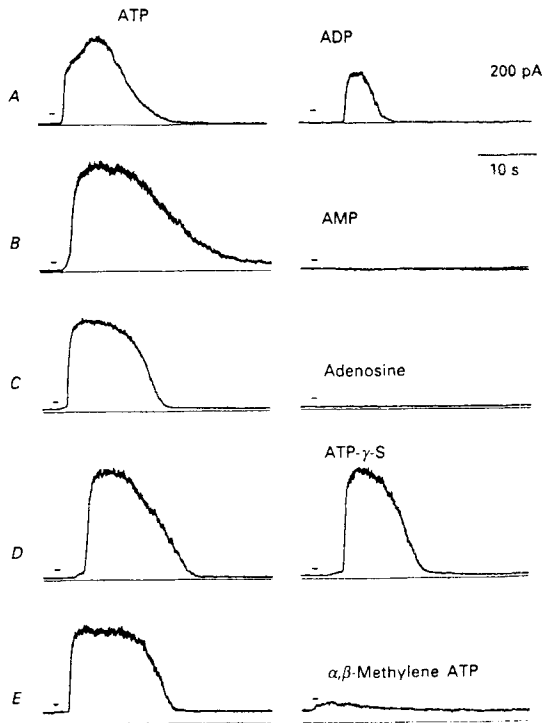
Fig. 5. K$^+$ responses to ATP analogues of a mouse mesodermal cell line. Each of the two traces was obtained from the same cell (A–E). The responses induced by ATP (left traces) and ATP analogues (right traces) are shown. The names of the analogues are shown near the traces. Each drug was applied at 20 μM, and the holding potentials were 0 mV. Reproduced from Kubo, 1991a.

also to identify more precisely the developmental events specifically controlled by purines.

Radioligand binding studies have provided information about the development of A$_1$ receptors in guinea-pig and rat brain, in particular the forebrain and cerebellum [Morgan et al., 1987, 1990]. In guinea-pig forebrain it appears that A$_1$ receptors are present from embryonic day 19, with adult binding levels achieved about 25 days postpartum. In guinea-pig cerebellum, however, A$_1$ receptor binding is low until just prior to birth, when a dramatic increase in binding is observed which then continues to increase up to adulthood. A similar development is seen in rat forebrain and cerebellum with A$_1$ receptor binding changing very gradually in the forebrain, whereas binding in the cerebellum increases markedly after birth [Marangos et al., 1982; Geiger et al., 1984].

There are a number of reports about changes in the distribution of the ectoenzymes involved in the breakdown of ATP and adenosine in the brain during foetal and neonatal development. 5′-Nucleotidase shows a marked redistribution during development of the cat visual cortex and is thought to be involved in the remodelling of ocular dominance columns [Schoen et al., 1990].

A later electron microscopic study by the same group has suggested that synapse-bound 5′-nucleotidase activity plays a role in synaptic malleability during development; its later association with glial cell profiles may reflect other functions for this enzyme [Schoen et al., 1993]. Complex changes in the activity of adenosine deaminase in the different regions of the developing rat brain suggest that there are important roles for purines in very early stages of development from 15 days of gestation, as well as in the adult in specific regions of the brain [Geiger and Nagy, 1987; Senba et al., 1987]. A histochemical study of Ca^{2+}-ATPase in the rat spinal cord during embryonic development demonstrated intense activity in the roof and floor plates, rather than in the basal and lateral plates at embryonic day 12, indicating a possible role for Ca^{2+}-ATPase in early differentiation of neuroepithelial cells [Yoshioka et al., 1987]. ATP induces rises in intracellular Ca^{2+} in embryonic spinal cord astrocytes [Salter and Hicks, 1995].

In foetal sheep, centrally administered adenosine influences cardiac function [Egerman et al., 1993]. The ontogeny of A$_1$ adenosine receptors was studied in rats with binding assays (using [^{3}H]DPCPX, an A$_1$ antagonist), and by in situ hybridization of mRNA [Rivkees, 1995]. At gestational days 8–11, mRNA expression for A$_1$ receptor was detected in the atrium (one of the earliest G protein-coupled receptor genes to be expressed in the heart), but not in other foetal structures, while at gestational day 14, A$_1$ mRNA was present in the CNS (thalamus, ventral horn of spinal cord) as well as the atrium; by gestational age 17, patterns of A$_1$ receptor expression in the brain were similar to those observed in adults [Weber et al., 1990; Reppert et al., 1991]. Determination of A$_1$ receptor density in developing rat heart using [^{3}H]DPCPX, showed that functional A$_1$ receptors are present in greater numbers in the immature perinatal heart than in the adult heart [Cothran et al., 1995].

Intravenous infusion of adenosine analogs into foetal lambs produced dose-dependent bradycardia and hypotension [Yoneyama and Power, 1992; Konduri et al., 1992; Koos et al., 1993]. In contrast, in the newborn, NECA produced dose-dependent tachycardia, while PIA and CHA produced dose-dependent bradycardia. Foetal breathing movements were interrupted by all analogs, but they did not produce apnea in the newborn [Toubas et al., 1990].

In the gastrointestinal tract, responses to ATP have been observed in rat duodenum the day after birth, suggesting that functional P2-purinoceptors are present at this time. Low concentrations of ATP were inhibitory at every age studied and its potency increased with age, while higher concentrations were excitatory but only until 15 days after birth [Nicholls et al., 1992]. It has been proposed that ATP is a non-adrenergic, non-cholinergic

(NANC) neurotransmitter in many areas of the gastrointestinal tract [Burnstock, 1972] and NANC nerve-mediated effects have been observed before birth in rat stomach [Ito et al., 1988] and in mouse and rabbit small intestine [Gershon and Thompson, 1973]. Also, quinacrine fluorescence, which indicates the presence of high levels of bound ATP in nerves, is observed before birth in rabbit ileum and stomach [Crowe and Burnstock, 1981].

ATP has also been proposed as the non-cholinergic excitatory transmitter in urinary bladder [Burnstock et al., 1978a,b] and purinergic responses are apparent at birth [Keating et al., 1990]. Purinergic innervation and responsiveness to ATP is greater in 1-day-old urinary bladder than in adult tissue [Keating et al., 1990; Zderic et al., 1990; Sneddon and McLees, 1992]. The importance of ATP in neonatal tissue has also been demonstrated in rat vas deferens, where ATP has been proposed as the cotransmitter with NA in sympathetic nerves [Meldrum and Burnstock, 1983].

Purinoceptors have been characterized in mouse C2C12 myotubes [Henning et al., 1992, 1993a,b]. Adenosine-sensitive P1-purinoceptors activating cyclic AMP formation were identified and a novel P2-purinoceptor was also postulated, sensitive to ATP, ADP, and ATPγS, which also activates the formation of cAMP. This receptor was also sensitive to UTP, but not α,β-meATP, 2meSATP, GTP, or CTP, thus resembling the $P2Y_2$ ($=P2_U$)-purinoceptor identified in mammals. The response to ATP and UTP was biphasic, a transient hyperpolarization being followed by a slowly declining depolarization; the hyperpolarization was blocked by apamin and suramin and abolished under Ca^{2+}-free conditions. Occupation of the receptor by ATP or UTP led to formation of inositol trisphosphate and release of Ca^{2+} from internal stores as well as from the extracellular space.

PHYLOGENY OF PURINOCEPTORS

While there have been some commentaries and reviews about the evolution of transmitters (including acetylcholine, monamines, amino acids, peptides, and nitric oxide), their receptors and ion channels [Venter et al., 1988; Walker and Holden-Dye, 1989, 1991; Messenger, 1991; Arbas et al., 1991; Feelisch and Martin, 1995], few have referred to receptors for purines. There is now a wealth of information about the distribution of purinoceptors in mammalian tissues, although there have been some reports of the extracellular roles of purine nucleosides and nucleotides in invertebrate and lower vertebrate species [see Burnstock, 1975, 1977, 1979; Berlind, 1977; Siebenaller and Murray, 1986; Venter et al., 1988; Hoyle and Greenberg, 1988; Walker and Holden-Dye, 1989; Feng and Doolittle, 1990; Linden et al., 1994]. It is the aim of this section to review what is known about the actions of both adenosine and ATP on a

variety of different invertebrate and lower vertebrate tissues, to attempt to characterize purinoceptor subtypes, and to consider these findings in evolutionary terms.

Invertebrates

Protozoa

The inhibitory effects of external ATP on amoeboid movement has been recognized for many years [Zimmerman et al., 1958; Zimmerman, 1962; Nachmias, 1968]. Output from the contractile vacuole of Amoeba proteus increases in the presence of ATP and, to a lesser extent, other polyphosphates [Pothier et al., 1984, 1987; Couillard, 1986]. Amoeba behaves as an excitable cell; its membrane responds to ATP by non-propagated lasting depolarizations, probably resulting from opening of sodium channels. Cell-surface receptors for adenosine and cyclic adenosine 3',5'-monophosphate have been reported in the free-living amoeba of the slime mould, Dictyostelium discoideum; they mediate signals involved in cell aggregation [Robertson et al., 1972; Clark and Steck, 1979; Newell, 1982; Newell and Ross, 1982].

Externally applied, GTP alters the motility and elicits an oscillating membrane depolarization in Parmecium tetraaurelia, a unicellular ciliated protist [Clark et al., 1993]. More specifically, it transiently induces alternating forward and backward swimming interspersed with whirling at a concentration as low as 100 nM. ATP is 1,000-fold less active, while CTP and UTP are virtually inactive. The authors suggest that GTP released from lysed paramecia, brought on by predators or noxious chemicals, may be used as a signal to neighbouring paramecia to evacuate the local area. ATP is known to alter the rate of beat of cilia in Paramecium and it is interesting that there are lower levels of adenosine triphosphate in slow-swimming mutants [Hayashi and Takahashi, 1979]. As for Amoeba, ATP has also been shown to increase the rate of output of contractile vacuoles in Paramecium [Organ et al., 1968]. The ecto-ATPase from the ciliary membranes of Paramecium is similar to that from mammalian brain and the endothelial plasma membrane with respect to kinetics, ionic requirements, and insensitivity to vanadate [Doughty and Kaneshiro, 1985]. ATP keeps exocytosis sites in Paramecium in a primed state, but is not required for membrane fusion [Vilmart-Seuwen et al., 1986].

ATP causes herniation (blebbing) and plasmodial disruption of the myxomycete Physarum polycephalum [Mante et al., 1978]; the authors suggest that these effects may reflect acceleration of normal contractile processes in this organism. Using luciferin-luciferase bioluminescence, ATP has been shown to leak out rhythmically from Physarum and the period and phase of oscillation in ATP leakage corresponds well with those of tension production [Yoshimoto et al., 1981; Uyeda and Furuya, 1987]. Extracellular ATP leads to changes in the

cytoskeletal organisation in *Physarum* apparently causing the microtubules and microfilaments to slide apart [Uyeda and Furuya, 1987].

Certain adenosine analogs have been shown to inhibit the growth of *Giardia lamblia*, a protozoan parasite that causes diarrhoea in both man and animals; drug treatment includes the use of quinacrine, which is known to bind high levels of ATP [see Berens and Marr, 1986]. Both adenosine and 2´-deoxyadenosine are inhibitory to the growth of the trypanosome protozoon *Crithidia fasculata*; this inhibition of growth is reversed by pyrimidine nucleosides [Dewey et al., 1978]. *Trypanosoma brucei* is the causative agent of sleeping sickness and like other protozoan parasites is unable to synthesize purines and therefore depends on purine salvage from the host environment; hypoxanthine transport occurs via a high affinity, energy-dependent transporter with a substrate specificity that is markedly different from any known mammalian nucleobase transporter [de Koning and Jarvis, 1995].

Platyhelminths

ATP diphosphohydrolase has been located on the external surface of the tegument of the parasitic liver fluke, *Schistosoma mansoni*; it is suggested that this enzyme could regulate the concentration of purine nucleotides around the parasites and hence enable them to escape the host haemostasis by preventing ADP-induced platelet aggregation [Vasconcelos et al., 1993].

Coelenterates

Encompassing the jellyfish, sea anenomes, and corals, these mainly marine organisms are radially symmetrical with a two layered body wall enclosing a single cavity with a single aperture, the mouth.

The pedal disc of the sea anenome *Actinia eqina* has been shown to possess a purinoceptor that is responsive to ATP, ADP, and adenosine, all of which cause contractions, but is insensitive to AMP [Hoyle et al., 1989] (Fig. 6). A role of ATP in the maturation of nematocysts from sea anenomes has been suggested [Greenwood et al., 1989]. A purine, caissarone, extracted from the sea anemone, *Bunodosoma caissarum*, has been shown to be an adenosine receptor antagonist [de Freitas and Sawaya, 1990; Cooper et al., 1995]. ATP causes ciliary reversal in the comb plates of ctenophores, probably by increasing intracellular Ca^{2+} ions [Nakamura and Tamm, 1985; Tamm and Tamm, 1989].

Annelida

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.

Electrophysiological investigations, using both intracellular microelectrodes and whole cell patch-clamp recording on identified neurones in the central nervous system of the leech *Hirudo medicinalis* revealed that ATP and ADP depolarised selected neurons but not the neuropil glial cells [Backus et al., 1994] (Table 1). The most effective responses (up to 10 mV) were observed in the noxious and touch cells. In most neurons the stable analog of ATP, ATP-γ-S (5 μM) induced larger depolarizations than ATP, indicating that ectonucleotidases were probably present. The authors concluded from further experiments that ATP activates non-selective cation channels in medial noxious cells of the leech with an order of potency ATP ADP AMP. They claimed that the results suggest that these cells express purinoceptors of the P2 type, although suramin was not an effective antagonist of this receptor. Salivary cells of the leech *Haementeria ghilianii* exhibit a selective response to ATP producing inhibition of voltage-dependent Ca^{2+} influx [Werner et al., 1996]; ATP, but not adenosine, modulates action potential firing, thought to be via a mammalian-like P2-purinoceptor [Wuttke and Berry, 1993]. A later study showed that the P2-purinoceptor involved is suramin-insensitive and that activation by ATP inhibits Ca^{2+} influx through voltage-gated Ca^{2+} channels [Wuttke et al., 1996].

Molluscs

Molluscs do not show segmentation, the body consists of a head-foot and visceral mass extended into folds which often secrete a shell. The nervous system consists of ganglia connected by commisures. This group includes snails, bivalves, and octopuses.

Adenosine has been shown to have a modulatory effect on an excitatory ACh response on an identified neuron (F_1) of the suboesophageal ganglion of the snail *Helix aspersa*; it was proposed that an A_1-receptor mediated inhibition, while an A_2-receptor mediated enhancement of the response to ACh [Cox and Walker, 1987]. ATP and α,β-meATP also enhance the response, suggesting that a P2X-purinoceptor is also present. Nanomolar concentrations of extracellular ATP and its stable analog AMP-PNP were also shown to activate calcium channels in these neurons [Yatani et al., 1982] (Fig. 7). Single neurons in ganglia of the marine mollusc *Aplysia californica* contain highly variable levels of ATP and lower levels of related purines, including ADP, AMP, adenosine, and inosine [Stein and Weinreich, 1984; McCaman, 1986], in keeping with the possibility that some are involved in purinergic transmission.

Adenosine modulates monoamine release from neurons in the pedal ganglion of the marine bivalve *Mytilus*
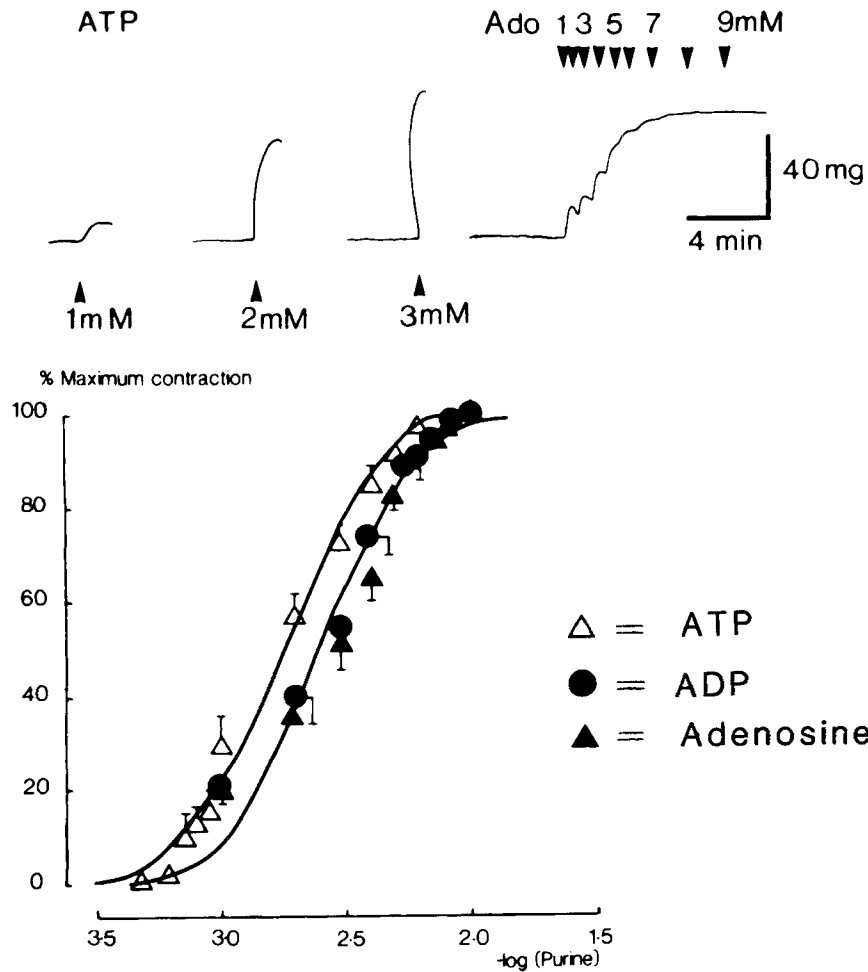
Fig. 6. Actions of adenylyl compounds on isolated circular muscle of the pedal disc of *Actinia equina*. **Upper panels**: Examples of contractile responses to ATP and an example of a cumulative concentration-response relationship for adenosine (Ado). **Lower panel**: Concentration-response curves for ATP ($\triangle$, n = 12), ADP ($\bullet$, n = 10), and adenosine ($\blacktriangle$, n = 7).

Symbols show mean percentage maximum contraction due to the adenyl compound $\pm$ S.E.M. (unless occluded by symbol). Solid lines are the curves fitted following probit transformation and horizontal averaging. For clarity the curve for adenosine has not been drawn, as it overlies the curve for ADP. Reproduced from Hoyle et al., 1989.

edulis; analogues of adenosine are also capable of inhibiting transmitter release, the receptor, being highly specific for NECA, exhibits the characteristics of the mammalian A_2-receptor [Barraco and Stefano, 1995]. Adenosine deaminase has been identified in *Mytilus edulis* [Aikawa and Aikawa, 1984] as well as in the adductor muscle and midgut of the scallop, *Patinopecten yassoensis* [Sato and Aikawa, 1991; Yoshida and Aikawa, 1993], which is consistent with a role for adenosine as an extracellular modulator.

Studies of the systemic heart of the cephalopod *Octopus vulgaris* have shown that adenosine has an opposite effect to that known for the mammalian heart, in that adenosine and AMP produced positive chronotropic and inotropic effects [Agnisola et al., 1987]. The heart of the Venus clam, *Katelysia rhyctiphora*, is stimulated

by ATP and electron microscopy revealed different types of nerve profiles, some containing large opaque vesicles and staining for Mg-ATPase and 5′-nucleotidase which resembled NANC purinergic nerves observed in mammalian gut [Sathananthan and Burnstock, 1976]. Adenosine has also been shown to cause systemic arrest of pulsations in the isolated heart auricle of the oyster *Crassostrea nippona*, but only in alkaline conditions, because the specific enzyme involved has a low optimum pH as a protective device [Aikawa and Ishida, 1966; Aikawa et al., 1967]. The hearts of *Busycon contrarium* and *Melongena corona* responded in a complex way to purine compounds, ATP causing both positive and negative inotropy [Hoyle and Greenberg, 1988]. The same is true for the hearts of the snail *Helix aspersa* and the slug *Arion ater* where the responses to ATP, ADP, AMP, and ad-

TABLE 1. Quantitative Effects of Extracellular Nucleotides on Leech Neurons and Neuropil Glial Cells*

	ATP	ADP	AMP	Adenosine	ATP-γ-S
Annulus erector cell	2.8 ± 1.6 (20)	6.3 (2)	2.5 (2)	1.5 ± 1.3 (3)	−4.2 ± 2.3 (3)
Anterior pagoda cell	1.7 ± 2.7 (6)	2.0 ± 2.9 (6)	0.9 ± 2.0 (5)	2.0 ± 2.0 (3)	3.5 ± 1.8 (10)
					−2.8 ± 1.3 (5)
Retzuis cell	2.1 ± 0.9 (7)	1.3 ± 0.8 (6)	1.3 ± 0.8 (6)	1.3 ± 0.5 (4)	10.4 ± 2.2 (10)
Medial noxious cell	4.5 ± 5.9 (35)	4.0 ± 1.7 (7)	2.7 ± 2.4 (7)	1.3 ± 1.0 (8)	6.4 ± 2.0 (32)
Lateral noxious cell	6.9 ± 3.5 (8)	9.6 ± 0.9 (7)	5.2 ± 1.8 (6)	5.4 ± 1.5 (8)	Not tested
Medial pressure cell	3.2 ± 2.2 (8)	2.4 ± 1.0 (7)	0.4 ± 0.8 (7)	Not tested	3.7 ± 2.3 (5)
Lateral pressure cell	1.2 ± 2.0 (3)	1.8 ± 1.8 (3)	0.7 ± 1.2 (3)	1.5 (2)	1.3 ± 0.5 (5)
Touch cell	6.4 ± 4.6 (4)	4.5 ± 2.8 (5)	2.9 ± 1.7 (5)	4.0 ± 3.0 (4)	3.0 ± 0.9 (3)
Neuropil glioal cell	0 (6)	0 (6)	0 (6)	0 (6)	0 (3)

*All agonists were applied at a concentration of 100 μM except ATP-γ-S (5 μM). The numbers give the mean depolarisation in mV ± the standard deviation. The number of experiments is given in parenthesis. Negative values indicate hyperpolarisations. Reproduced from Bakus et al., 1994.

enosine caused either cardioexcitation or cardioinhibition in any given preparation [Knight et al., 1992c].

Probiscis smooth muscles of *Buccinum undatum* respond to GTP and GTPγS (but not to ATP or adenosine) in the 10^{-7}-10^{-4} M range with a moderately fast twitch activity which was unaccompanied by action potentials [Nelson and Huddart, 1994]. Both the oesophagus and the rectum of the snail, *Helix aspersa*, produced contractions to ATP, ADP, and AMP, but the synthetic analogs 2-chloroadenosine, α,β-meATP and 2meSATP were inactive [Knight et al., 1992a].

The body wall of the pulmonate slug, *Ariolimax columbianus*, secretes mucous packaged in granules; newly secreted granules rupture in the presence of ATP,

Fig. 7. Activation of Ca^{2+} channels on Snail neurones: Dose-response curves for the effects of extracellular ATP (○) and AMP-PNP (●) on I_{Ca} (elicited by a 70 ms long pulse to +20 mV from a holding potential of -50 mV). Each point represents the mean of six measurements for ATP and seven for AMP-PNP obtained from different neurones (curves were drawn by eye). Reproduced from Yatani et al., 1982.

aparently via a specific ATP/ADP receptor [Deyrup-Olsen et al., 1992]. In general, nerves in the heart and gut of the pulmonate gastropod molluscs show a high affinity for quinacrine [Cardot, 1981; Knight et al., 1992b] suggesting that they contain high levels of granule-bound ATP.

Arthropods

The phylum arthropoda includes crustaceans, centipedes, millipedes, insects, and arachnids. They are characterized by their bilateral symmetry and typically each segment has a pair of jointed appendages at least one pair being modified as jaws, although the number of segments included in the head is variable. There is a strong and well-developed exoskeleton. The CNS consists of cerebral ganglia and a ventral nerve cord made up of separate paired ganglia connected by commisures. There is a contractile heart lying in a haemocoelic pericardial cavity.

Crustacea

There is considerable information about the effects of ATP and adenosine in crustaceans. The olfactory organ of the spiny lobsters *Panulirus argus* and *Panulirus interruptus* have different populations of purinergic chemoreceptors that are excited by AMP, ADP, or ATP [see Carr et al., 1986, 1987; Zimmer-Faust et al., 1988; Trapido-Rosenthal et al., 1989] (Fig. 8). These receptors reside on chemosensitive neurons that are contained within aesthetasc sensilla on the lateral filaments of the antennules. 5′-AMP odorent receptor sites have recently been localized ultrastructurally, utilizing 5′AMP-biotin, along the entire dendritic region, including the transitional zone between inner and outer dendritic segments, the region which also contains 5′-ectonuclotidase/phosphatase [Blaustein et al., 1993].

The potency order for some sensilla indicates a P1-purinoceptor [Derby et al., 1984, 1987; Carr et al., 1986, 1987]. In addition, there are chemoreceptors stimulated by ATP exhibiting properties similar to P2-purinocep-
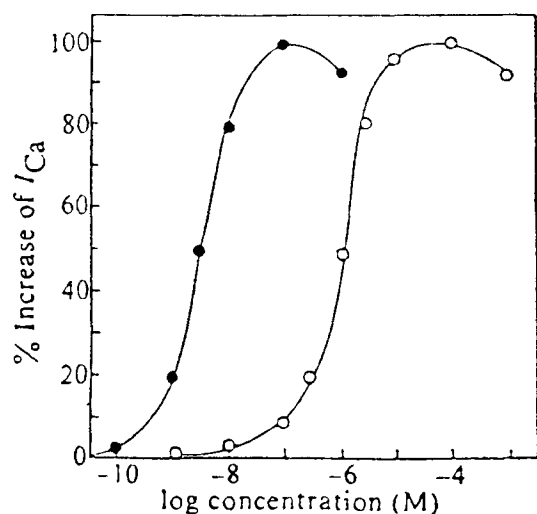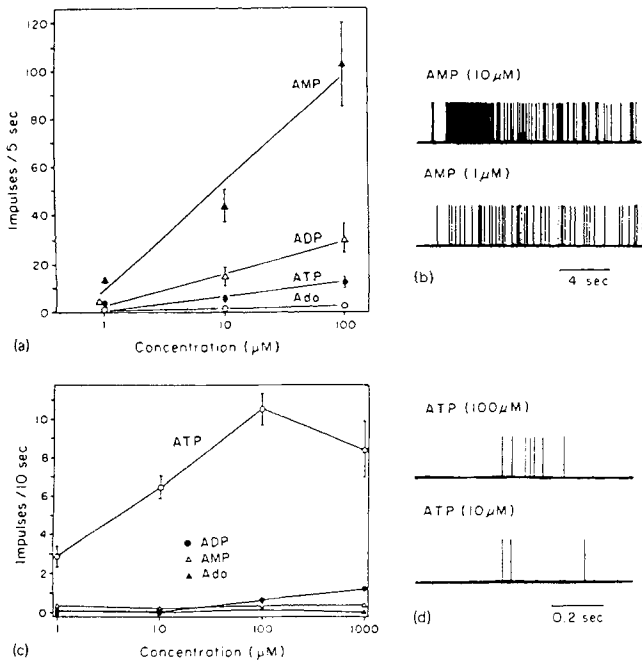
Fig. 8. Comparisons of response characteristics of AMP-sensitive and ATP-sensitive cells in the antennule of the spiny lobster. **a**: Response of AMP-best cells to the indicated compounds. **b**: Series of action potentials produced by an AMP-best cell to the indicated concentrations of AMP. **c**: Response of ATP-best cells to the indicated compounds. **d**: Series of action potentials produced by an ATP-best cell to the indicated concentrations of ATP. Note the differences in time scale in (b) and (d). Reproduced from Trapido-Rosenthal et al., 1989.

tors [Carr et al., 1986]. Since these receptors are more sensitive to the slowly degradable analogues of ATP, α,β-mcATP and β,γ-mcATP [Carr et al., 1987], they appear to be comparable to the P2X family of purinoceptors. Similarities between the P1- and P2-purinoceptors of these crustaceans and mammalian purinoceptor subtypes are extended further, in that the chemosensory sensilla inactivate excitatory nucleotides by a two-step process. Ectonucleotidases dephosphorylate adenine nucleotides to yield a nucleoside which is internalised by an uptake system [Trapido-Rosenthal et al., 1987, 1990], which is similar to mammalian systems [Burnstock, 1975].

Activation of olfactory (smell) and gustatory (taste) P2-purinoceptors in lobsters is thought to induce a feeding behavioural response [Fine-Levy et al., 1987; Zimmer-Faust et al., 1988; Daniel and Derby, 1989; Gleeson et al., 1989; Zimmer-Faust, 1993]. ATP is an ideal stimulus for such animals that feed on wounded or recently killed animals, since ATP occurs at high concentrations in fresh animal flesh but decays rapidly as cells die [Sikorski et al., 1990]. Since predators, such as lobsters, often inhabit crevices and only emerge to feed at night, foraging is directed principally by chemical stimuli, rather than visual or mechanical stimuli. ATP is detected in prey organ-

isms, such as mussels and oysters, which contain high concentrations of nucleotides and are released when the animal dies [Carr and Derby, 1986]. ATP acts as an effective signal molecule in seawater, since mechanisms exist in seawater that minimise the presence of background ATP levels that might represent a false indication of the presence of food [Zimmer-Faust et al., 1988]. These mechanisms include dephosphorylating enzymes present on the outer surfaces of many planktonic organisms which quickly degrade nucleotides released into the sea [Ammerman and Azam, 1985] in addition to nucleotidases in tissues that rapidly dephosphorylate ATP after death [Zimmer-Faust, 1987]. Hence the presence of appreciable concentrations of ATP in seawater may provide a reliable indicator that an injured or freshly killed prey is nearby. While ATP is a potent attractant, AMP has an inhibitory effect on some lobsters [Gleeson et al., 1989; Zimmer-Faust, 1993] and may therefore act to direct the predator towards only fresh prey. For those predators in which AMP acts as the attractant [Carr and Thompson, 1983; Derby et al., 1984; Carr et al., 1987], the rapid breakdown of ATP to AMP may account for this. AMP is found to be the most potent chemoattractant of *Octopus vulgaris*, initiating a locomotor response. The arms are believed to carry the sensory organs, chemoreceptors having been morphologically identified in the suckers [Chase and Wells, 1986] which would direct the arms towards the meal. Modulatory actions of AMP and adenosine were recorded in brain cells of the spiny lobster [Derby et al., 1987; Derby, 1987]; AMP was the most potent of the purines examined and its effect was antagonized by theophylline. Olfactory purinoceptors have also been identified in the shrimp *Palaemonetes pugio* [Carr and Thompson, 1983; Carr and Derby, 1986] and blue crab *Callinectes sapidus* [Buch and Rechnitz, 1989].

In lobsters and other decapod crustaceans, the sites of olfaction and gustation are anatomically distinct, the former in the antennules, the latter on the walking legs, maxillipeds and mouthparts; the sensilla on the walking legs of the spiny lobster, *Panulirus argus*, have also been shown to possess ATP- and AMP-sensitive cells as well as enzymes that dephosphorylate purine nucleotides [Gleeson et al., 1989].

Extracellular ATP has been shown to modulate calcium uptake and transmitter release from neuromuscular junctions in the walking leg of the crayfish, *Procambarus clarkii* [Lindgren and Smith, 1987], reminiscent of purinergic modulation of transmitter release at the skeletal neuromuscular junction of vertebrates [Ribeiro and Walker, 1975; Redman and Silinsky, 1993].

The inhibitory effects of ATP on the heart of the spiny spider crab, *Maia*, were reported many years ago [Welsh, 1939]. ATP potentiates the effects of electrical field stimulation of neurons in the terminal intestine of

the lobster *Panulirus argus* via a P2-like purinoceptor [Hoyle and Greenberg, 1988].

Artemia is a crustacean whose embryos become encapsulated at the gastrula stage. The cysts are viable for years in a dry environment. When placed in a suitable saline medium, the eggs resume their development and differentiate into free swimming larvae at about 24 h. During this time, *Artemia* uses the maternal stored diguanosine tetraphosphate (Gp_4G) as a source of guanine and adenine nucleotides during development from encysted gastrulae to free swimming larvae [Finamore and Clegg, 1969; Sillero and Sillero, 1987; Warner, 1992]. *Artemia* cysts contain the appropriate enzymatic machinery for the conversion of Gp_4G into AMP and GMP [Prescott et al., 1989]. However, *Artemia* is apparently unable to synthesize purines de novo [Clegg et al, 1967] and the larvae depend on a balanced dietary source of purines (nucleotides, nucleosides, or bases) for growth and survival to adulthood [Hernandarena, 1985, 1990; Sillero et al., 1993].

Insects

ATP released from erythrocytes stimulates the gorging response in a variety of blood-feeding insects such as the mosquitoes *Aedes aegypti* and *caspius*, *Culex pipiens univittatus* and *quinquefasciatus* and *Culiseta inornata*, the blackfly, *Simulium venustum* [Sutcliffe and McIver, 1979], the horse fly, *Tabanus nigrovittatus* [Friend and Stoffalano, 1984], the stable fly, *Stomoxys calcitrans*, the tsetse fly, *Glossina austeni morsitans, tachinoides*, and *palpalis*, the bug, *Rhodnius prolixus* and the haematophagous ticks, *Ixodesdammini* and *Boophilus microplus* [Hosoi, 1959; Galun et al., 1963, 1985, 1988; Galun and Margalit, 1969; Friend and Smith, 1975, 1977; Smith, 1979; Ribeiro et al., 1985; Willadsen et al., 1987; Ellgaard et al., 1987; Ascoli-Christensen et al., 1991; Liscia et al., 1993; Moskalyk and Friend, 1994].

Electrophysiological methods have been used to demonstrate that the apical sensilla of the labrum of *Culex pipiens* house the ATP receptors involved in blood feeding [Liscia et al., 1993]. Novobiocin, which blocks ATP access to its binding site on ATPase, inhibits the gorging response [Galun et al., 1985]. The ED_{50} of ATP for *Glossina tachinoides* females is 13 nM, while for males it is 140 nM; this level of sensitivity for detecting ATP is the highest recorded for an insect [Galun and Kabayo, 1988].

Other chemosensory P2-purinoceptors have been identified that are involved in the recognition of a blood meal in haematophagous insects. These represent a heterogeneous group. Many blood-feeding insects recognize ATP and related compounds as phagostimulants. In mosquitoes and tsetse flies, ATP is found to be more potent than ADP at stimulating feeding while AMP is a very poor phagostimulant, indicating an ATP-selective P2-

purinoceptor [Galun et al., 1963, 1985; Galun and Margalit, 1969; Mitchell, 1976; Galun and Zacharia, 1984]. A similar ATP-selective receptor mediates the phagostimulatory response of *Glossina tachinoides* [Galun, 1988] and *Rhodnius prolixus* larvae, suggesting that this response is not limited to the adult form [Smith, 1979; Friend and Smith, 1982]. Further investigations have revealed that the receptor can be subclassified; α,β-meATP and β,γ-meATP are less potent than ATP as phagostimulants in *G. palpalis palpalis* suggesting that the receptor may be tentatively classified as a P2Y-purinoceptor [Galun and Kabayo, 1988]. A similar order of potency is found for *Rhodnius prolixus* [Friend and Smith, 1982]. However, since 2meSATP, which is a more selective P2Y-purinoceptor agonist, has not been investigated, the classification of this receptor is still uncertain.

A P2-purinoceptor has also been identified that inititates feeding of the culicine mosquitoes *Culex pipiens* and *Culiseta inornata*. The potency order was found to be ADP $>$ATP$=$AMP$>\beta,\gamma$-meATP for *C. pipiens* and ADP$>$ATP$>\beta,\gamma$-meATP$>>$AMP for *C. inornata* [Galun et al., 1988]. ADP is also found to be the most potent phagostimulant of the horsefly *Tabanus nigrovittatus* [Friend and Stoffolano, 1983, 1984]. ADP-selective receptors, termed P_{2T}-purinoceptors, have been identified in mammals [Gordon, 1986]. However, the ADP-selective receptor of the haematophagous insects differs from the mammalian receptor where ATP acts as a competitive antagonist [Macfarlane and Mills, 1975]. ATP behaves as an agonist at the insect receptor. The potency orders of the purine nucleotides vary considerably between different groups of haematophagous insects; however, closely related species usually show a similar structure-activity relationship. It has been suggested that a pre-existing pool of nucleotide-binding proteins, present in all living cells, served as a source of the receptor proteins for the gustatory receptors involved in blood detection and that the selection of any such nucleotide bindng protein was random [Galun, 1987], perhaps accounting for the variety of receptor profiles found among the haematophagous insects.

The potency order of various adenine nucleotides suggests the receptor involved is of the P2-subtype [see Galun, 1987, 1988; Galun et al., 1988] (Table 2). In the tsetse fly *Glossina palpalis*, P2-purinoceptor stimulating gorging has been identified as being of the P2Y-subtype [Galun and Kabayo, 1988]; in contrast, in the stable fly the ATP-mediated response is antagonised by $ANAPP_3$, suggesting a receptor that resembles the P2X-purinoceptor subtype [Ascoli-Christensen et al., 1991]. The P2-purinoceptor of *G. morsitans morsitans* was not classified further, although it was noted that the phosphate chain was of importance [Mitchell, 1976]. Both AMP and ATP were also found to be potent chemoattractants, with equal

TABLE 2. Gorging Response of the Tsetse Fly, *Glossia tachinoides*, to Adenine Nucleotides*

Compound	No. flies	ED$_{50}$ (µM)	ED$_{85}$ (µM)
Females			
ATP	240	0.013 (0.007-0.024)	0.20 (0.10-0.42)
ADP	210	0.063 (0.034-0.15)	1.30 (0.50-3.8)
AMP	135	69.5 (36.4-133)	1060 (308-3640)
None	105	27% fed	
Males			
ATP	180	0.14 (0.12-0.19)	0.56 (0.31-1.02)
None	30	7% fed	

*Figures in parenthesis are 95% confidence limits. Reproduced from Galun, 1988.

potency, for the larvae of *Culex quinquefasciatus*, although unlike the adult stage, the phosphate chain is not as important, since adenosine was found to be a moderate chemoattractant [Ellgaard et al., 1987]. It is fascinating that apyrase (ATP diphosphohydrolase), a general designation for enzymes that hydrolyze ATP and ADP, has been reported to have exceptionally high activity in the salivary glands or saliva of blood sucking insects, including the bug *Rhodnius prolixus* [Ribeiro and Garcia, 1980; Smith et al., 1980; Sarkis et al., 1986], tsetse fly [Mant and Parker, 1981], mosquito [Ribeiro et al., 1984], and sandfly [Ribeiro et al., 1986]. In all cases, since ADP induces platelet aggregation, breakdown of ADP by apyrase leads to enhanced haemorrhage and more effective blood sucking [see Ribeiro, 1995]. In ticks, where each engorgement generally extends over several days, the saliva has antiinflammatory and immunosuppressive properties as well as platelet anti-aggregative apyrase and Mg^{2+}/ATPase [Ribeiro et al., 1985; Willadsen et al., 1987].

The involvement of purines in the recognition of a blood meal in haematophagous insects is interesting. Both platelets and red blood cells (RBC) release ATP and ADP as a result of wounding [Mills and Thomas, 1969; Born, 1977]; which source offers the stimulus to feed depends on the insect in question, some species are more sensitive to ATP, while others are more sensitive to ADP. For instance, ATP released from RBC preferentially induces *Rhodnius prolixus* to gorge, since plasma, which contains little ATP [Bishop et al., 1959], is considerably less effective than a suspension of washed erythrocytes [Smith, 1979]. The potency of the erythrocyte suspension is associated with RBC contents rather than the erythrocyte membrane, since RBC ghosts are ineffective as gorging stimulants [Smith, 1979]. It is suggested that the release of ATP from RBC close to the mouth sense organs act as the feeding stimulant. Platelets also contain quantities of ATP, both ATP and ADP are released into the plasma by appropriate triggers such as wounding [Holmsen, 1972]

and platelets have been implicated as the gorging stimulus of *Aedes aegypti* [Galun and Rice, 1971].

Taste chemosensilla sensitive to nucleotides have been identified in some non-haematophagous insects, for example, in the omnivorous common blowfly, *Pharmia regina* [Daley and Van de Berg, 1976; Liscia, 1985; Liscia et al., 1987]. In this species, ATP does not have a direct stimulatory action, but rather modulates the responses of the labilla sensilla; it reduces the responses to NaCl and fructose, but enhances responses to sucrose and glucose [Liscia, 1985]. Cyclic AMP inhibits neuronal firing of the labellar sugar sensitive receptor of the blowfly when applied in conjunction with the stimulant sucrose [Daley and Van de Berg, 1976]. ATP has also been reported to be a feeding stimulant in a flea, *Xenopsylla cheopis* [Galun, 1966] and a tick [Galun and Kindler, 1968].

Adenosine stimulates feeding in the African armyworm *Spodoptera exempta*; this larva of an owlmoth exclusively feeds on grasses [Ma, 1977]. Other purines and pyrimidines have no such phagostimulatory activity indicating an adenosine-selective receptor. The function of the styloconic sensilla in determining the chemosensitivity to adenosine in this animal was examined by Ma, who found that addition of a ribose group to the N6 position of the adenine molecule greatly enhances its effectiveness as a stimulus. D-ribose itself failed to excite any receptor cell in the lateral sensilla, but did stimulate some neurons in the medial sensilla.

Adenosine inhibits adenylate cyclase in the pupal fat body of the silkworm, *Bombyx mori* with characteristics comparable to those known for neuromuscular adenosine receptors, A$_1$ subtype [Morishima, 1980].

Echinodermata

This phylum includes the starfishes, sea urchins, brittle stars, feather stars, and sea cucumbers. The adults have acquired a large measure of radial symmetry (usually five-rayed) and often an ectoskeleton develops. There is a water vascular system which is linked to the tube feet assisting in locomotion as well as a 'perihaemal' blood vascular system which is less extensive. The nervous system consists of a nerve ring around the oral part of the gut with projections along the radii. Many echinoderms have a deeper-lying motor nerve component.

There are several reports of the effects of purine compounds on echinoderms. In a review of several marine species, Hoyle and Greenberg [1988] found that adenosine, AMP, ADP, and ATP all relaxed the gastric ligament of the starfish, *Asterias forbesi* with ATP being the most potent of the purines examined.

In a later study, Knight et al. [1990] showed that, in the precontracted gastric ligament in the starfish, *Asterias rubens*, the relaxation to ATP was antagonized by glib-

enclamide; in contrast, relaxation to adenosine, which was equipotent to ATP in this species, was not blocked by glibenclamide, supporting the view that separate receptors for adenosine and ATP, comparable to P1- and P2- purinoceptors of vertebrates, exist in the echinoderms. However, the antagonists for P1- and P2-purinoceptors, effective in most mammalian preparations, 8-phenyltheophylline (8-PT; for P1-purinoceptors), Reactive Blue 2 (for P2-purinoceptors) and desensitization of the P2-purinoceptors with α,β-meATP were ineffective in the starfish preparations [Hoyle and Greenberg, 1988; Knight et al., 1990]. It is not clear why glibenclamide, which is a potent inhibitor of ATP regulated-K^+ channels in mammalian preparations, is effective in antagonizing responses of the starfish gastric ligament to ATP, but it does suggest that glibenclamide may be a useful tool for examining purinoceptor subtypes in invertebrates. The circular and longitudinal muscles from the polian vesicle of the sea cucumber, *Thyone briareus* relaxed to ATP, but not to adenosine, AMP and ADP, suggesting an ATP-specific receptor [Hoyle and Greenberg, 1988]; however, the rectum of the sea urchin, *Lytechinus variegatus*, contracted equipotently to all four purine compounds which may indicate a non-selective receptor or the presence of both P1 and P2 receptors which can only be confirmed by the use of selective antagonists. ATP produces tonic contractions of the spine muscle of the sea urchin, *Anthocidaris crassispina* [Shingyoji and Yamaguchi, 1995].

Other diverse effects of purine compounds have also been identified in echinoderms. For instance, adenosine inhibits the growth of fertilized eggs of the starfish, *Asterina pectinifera*, at the early blastula stage, specifically at the 256-cell stage; adenosine causes more than a 95% reduction in the rate of protein, DNA and RNA synthesis [Tsuchimori et al., 1988]. Effects are not limited to the blastula stage; muscular activity of sea urchin *Psammechinus miliaris* larva is stimulated by adenosine [Gustafson, 1991].

ATP and its stable analog AMP-PNP modulate flagellar motility of the sea urchin *Lytechinus pictus*, [Brokaw, 1975; Penningroth and Witman, 1978; Omoto and Brokaw, 1989]. This effect has been interpreted largely in terms of the intracellular actions of ATP, the possibility that extracellular P2-purinoceptors are involved needs to be considered.

Lower Vertebrates

The vertebrates, which are a sub-phylum of Chordata, are characterized by the presence of a notochord at some time during their life-history and a high degree of cephalisation so that a proper head region is recognisable, with a definite brain enclosed by a cranium. The group contains two superclasses: Agnatha, of which the cyclostomes comprise one class, and Gnathostomata, encompassing all the more familiar vertebrates, including elasmobranchs, teleosts, amphibians, reptiles, birds, and mammals.

Cyclostome Fish

These are primitive cartilaginous fish and include the lampreys and hagfish. In the hagfish *Myxine gluitinosa* adenosine has been observed to dilate the isolated brachial vasculature, although it had no effect on the heart [Axelsson et al., 1990]. Specific binding of the P1 (A_1) adenosine receptor ligand [^{3}H]cyclohexyladenosine (CHA) to membrane fractions from the brain of the hagfish, *Eptatretus deani*, was demonstrated as well as in elasmobranch and teleost fish, but not from the brains of arthropods or molluscs [Siebenaller and Murray, 1986].

Elasmobranch Fish

There are various reports of the effect of purine compounds within this group of cartilaginous fish, including reactivity in both the gastrointestinal and cardiovascular systems. The spontaneous activity in various preparations of elasmobranch gut is inhibited by ATP, such as the stomach and spiral intestine of the ray *Raja clavata* and the dogfish, *Scyliorhinus canicula*, and rectum of *Raja* [Young, 1983, 1988]. ATP was reported to cause contraction or relaxation of the stomach of the dogfish [Young, 1980], contraction of the stomach of the ray [Young, 1983] and relaxation of the rectum of the skate [Young, 1988]. Both A_1- and A_2-adenosine P1-purinoceptor subtypes have been identified in the rectal gland of the shark, *Squalus acanthias*, which modulate hormone stimulated chloride transport [Kelley et al., 1990, 1991; Forrest and Kelley, 1995; Forrest, 1996].

An inhibitory P1-purinoceptor has been identified in the atria of the dogfish *S. canicula* [Meghji and Burnstock, 1984a] and the possibility of purinergic modulation of vagal control of the heart of *S. stellaris* has been investigated [Taylor et al., 1993]; in another species of dogfish, *Squalus acanthias*, both A_1- and A_2-subtypes of receptor have been characterized in the aorta [Evans, 1992]. In the coronary artery of the skate *Raja nasuta*, adenosine causes vasoconstriction, while ADP and ATP cause vasoconstriction at lower concentrations, but vasodilatation at higher concentrations [Farrell and Davie, 1991b]; in the dogfish 10 μM ATP produced contraction followed by relaxation [Farrell and Johanson, 1995]. In contrast, in the coronary artery of the mako shark, *Isurus oxyrinchus*, adenosine is a dilator, as in the dogfish, and ADP a vasoconstrictor; theophylline inhibited both the adenosine-mediated relaxation and the ADP-mediated contraction [Farrell and Davie, 1991a].

The electric organ of electric elasmobranch fish, which is phylogenetically derived from neuromuscular

junctions, consists of motor nerves and electrocyte cells forming electroplaques that are derived from myoblasts. Synchronous discharge of the electrocytes by motor nerve stimulation produces a total discharge of about 40 V. It has been shown that ACh and ATP are co-stored (in a ratio of about 5:1) and co-released during synaptic activity of the electric organ of the electric eel, *Electrophorus* and the electric ray, *Torpedo* [Meunier et al., 1975; Zimmermann & Denston, 1976; Dowdall et al., 1974, 1976; Tashiro and Stadler, 1978; Stadler and Fuldner, 1981). Release of ATP from synaptosomes isolated from the electric organ of *Torpedo* by either depolarisation with KCl or after the action of venom extracted from the annelid *Glycera*, exhibited closely similar kinetics to that of ACh release [Morel and Meunier, 1981] (Fig. 9). Both ACh and ATP release are inhibited by the removal of extracellular Ca^{2+} or by the addition of the calmodulin antagonist, trifluoperazine, suggesting that ACh and ATP are both released by exocytosis from synaptic vesicles [Schweitzer, 1987; see also Unsworth and Johnson, 1990, and Solsona et al., 1991], although it is interesting that ATP release (in contrast to ACh) is not blocked by teta-
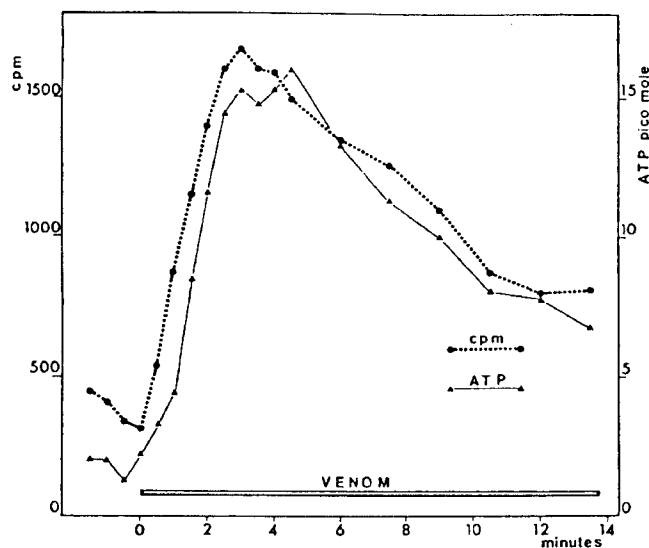

Fig. 9. *Torpedo* electric organ: ACh and ATP release from synaptosomes triggered by venom extracted from the annelid *Glycera convoluta*. Synaptosomal ACh was labelled using [1-^{14}C]-acetate. Concentrated synaptosomes deriving from 1.8 g electric organ were perfused with the physiological medium for 15 min. When a constant background radioactivity was obtained, the perfusion was switched to KCl solutions (where KCl replaces equivalent amounts of NaCl). Venom was used at a final concentration of 0.5 glands/ml physiological medium. The perfusate was collected by 200 μl aliquots and the efflux of radioactivity and of ATP determined in each aliquot. The specific radioactivity of synaptosomal ACh was 700 cpm/nmol and the ACh/ATP ratio in the synaptosomes was 6.9. Sixty-five percent of injected synaptosomes was retained in the perfusion chamber at the end of the experiment. Reproduced from Morel and Meunier, 1981.

nus toxin [Rabasseda et al., 1987], and omega-conotoxin differentially blocks ACh and ATP release [Farias et al., 1992]. A high affinity adenosine uptake system has been demonstrated in the synaptosomes for reconstitution of stored ATP [Meunier and Morel, 1978; Zimmermann et al., 1979; Tomas et al., 1982]. Isolated synapatic vesicles from *Torpedo* electric organ contain about 200,000 molecules of ACh and about 24,000 molecules of ATP; small amounts of ADP are also present (10% of ATP content) and traces of AMP [Zimmermann, 1982]. The diadenosine polyphosphates, Ap_4A and Ap_5A are both present in synaptic vesicles of *Torpedo marmorata* and binding of Ap_4A to P2-purinoceptors has been demonstrated in *Torpedo* synaptosomes [Pintor et al., 1994]. Vesicles from the closely related *Narcine* electric organ contain considerable amounts of GTP (17% of ATP content). One function for the ATP is that it increases receptor sensitivity to ACh [Akasu et al., 1981; Schrattenholz et al., 1994], i.e., it acts as a postjunctional modulator. A further role is that adenosine resulting from hydrolysis of ATP by ectoenzymes acts as a prejunctional modulator of ACh release [Ginsborg and Hirst, 1972; Israël et al., 1977; Keller and Zimmermann, 1983; Grondal and Zimmermann, 1986; Grondal et al., 1988; Sarkis et al., 1991]. The ability of bound ectoenzymes, obtained from *Torpedo* electric organ synaptosomes to dephosphorylate ATP to adenosine supported this hypothesis [Grondal and Zimmermann, 1986]. This was later further substantiated as a result of chemiluminescent investigations [Solsona et al., 1990] and studies showing that adenosine can inhibit ACh release [Israël et al., 1980]. A cDNA encoding 5′-nucleotidase was identified by screening a cDNA library from the electric lobe of the electric ray, *Discopyge ommata* using a cDNA probe for the rat liver enzyme [Volknandt et al., 1991]; the possible phylogenetic origins of vertebrate 5′-nucleotidase from multi-functional nucleotide hydrolases is described in this paper.

Teleost Fish

ATP and adenosine both produced relaxation of the intestine of the Atlantic cod, *Gadus morhua* [Jensen and Holmgren, 1985], and the circular muscle of the stomach of the rainbow trout, *Salmo gairdneri* [Holmgren, 1983]. However, ATP contracts both the longitudinal and circular muscle layers of the intestinal bulb of the carp, *Cyprinus carpio* [Kitazawa et al., 1990], the intestine of the angler fish *Lophius* [Young, 1983] and the intestine of the goldfish (*Carassius auratus*) [Burnstock et al., 1972]. Adenosine relaxed the stomach and intestine of the stickleback, *Gasterosteus aculeatus*, and this response was antagonized by 8-PT, indicating the presence of a P1-purinoceptor; ATP and its analogs, 2meSATP, and α,β-meATP caused contractions of the stomach and intestine, indicating a P2-purinoceptor [Knight and Burnstock,

1993]. ATP has been found to closely mimic the NANC responses to vagal stimulation of the pyloric caeci and duodenum of *Lophius*, even at very low concentrations, producing an inhibition followed by a rebound contraction [Young, 1980]. The possibility that there is a NANC inhibitory innervation of the gut of the brown trout *Salmo trutta* was hinted at, although the concept of NANC innervation was unknown at the time of the investigation [Burnstock, 1958, 1959]. The ileum and rectum of the flounder, *Pleuronectes*, both possess excitatory P2X-purinoceptors and inhibitory P1-purinoceptors [Grove and Campbell, 1979; Lennard and Huddart, 1989a].

Examples of the presence of various types of purinoceptors within the cardiovascular system include a P1-purinoceptor in the gill vasculature of the rainbow trout, *Salmo gairdneri*, and of the tropical cichlid, *Oreochromas niloticus*, that mediates vasoconstriction [Colin and Leray, 1979, 1981; Colin et al., 1979; Okafor and Oduleye, 1986]. A P2-purinoceptor is also likely to be present in the gill vessels since the contraction potency order of purine compounds in the rainbow trout was ATP=ADP>AMP=adenosine [Colin and Leray, 1979], while ATP produced vasodilatation in cichlid [Okafor and Oduleye, 1986]. ATP constricts the systemic vasculature of the rainbow trout [Wood, 1977]. ATP, ADP and adenosine contract the coronary artery of both the rainbow and steelhead trout probably via P1-purinoceptors [Small and Farrell, 1990; Small et al., 1990; Farrell and Johansen, 1995].

The action of adenosine on the heart of the carp, *Cyprinus carpio*, mimics that observed in elasmobranchs, acting via a P1-purinoceptor [Cohen et al., 1981; Rotmensch et al., 1981]. Similarly, in the flounder, *Platichthys flesus*, adenosine causes a positive inotropic effect [Lennard and Huddart, 1989b]; the trout is somewhat different in that adenosine and ATP are equipotent both producing negative inotropic and positive chronotropic effects [Meghji and Burnstock, 1984b].

Many fish are capable of spectacular colour changes due to the motile activities of chromatophores, controlled both by nerves and by hormones. These include melanophore-stimulating hormone (MSH) secreted from the intermediate lobe of the pituitary giving rise to darkening, often antagonized by melanin-concentrating hormone (MCH) which causes blanching by aggregation of pigments [see Fujii and Oshima, 1986]. A role for purines in the neural control of fish chematophores was first suggested by Fujii and Miyashita [1976], in a study of dispersion of melanophore inclusions in the guppy, *Lebistes reticulatus*. This was confirmed later with cultured goldfish erythrophores [Ozato, 1977]. Since methylxanthines antagonize the darkening reaction, it was concluded that an adenosine receptor was involved in the responses of melanosomes in the siluroid catfish, *Parasilurus*

(Miyashita et al., 1984), of both melanophores and iridophores in the blue damselfish, *Chrysiptera cyanea* [Kasukawa et al., 1985, 1986; Oshima et al., 1986a] and of leucophores in the medaka [Oshima et al., 1986b]. In more recent studies of denervated melanophores in the medaka, *Oryzias latipes*, the potency series for melanophore dispersion was: NECA>adenosine>ATP >2-chloroadenosine (2-CADO)>R-PIA>CHA>cAMP; this effect was antagonized by 8-PT and by adenosine deaminase and the action of adenosine was mimicked by forskolin, a potent activator of adenylate cyclase [Namoto, 1987, 1992]. It was concluded that the P1-purinoceptor involved was of the A_2 subtype. Evidence that ATP is liberated as a cotransmitter together with noradrenaline from melanosome aggregating sympathetic nerves in the tilapian fish, *Sarotherodon niloticus*, has been presented [Kumazawa et al., 1984; Kumazawa and Fujii, 1984, 1986]. It seems likely that ATP released from sympathetic nerves is broken down by ectoenzymes to adenosine which then acts on P1-purinoceptors both on chromatophore membranes leading to dispersion of pigment, and also on prejunctional sites leading to modulation of sympathetic transmitter release [Oshima, 1989]. In a more recent study, Fujii and his colleagues [Hayashi et al., 1993] found that the circadian motile activity of erythrophores in the red abdominal skin of the tetra tropical fish *Paracheirodon innesi* and *axelrodi* are controlled partly by ATP and adenosine.

Within the brain of the goldfish, *Carassius auratus*, the presence of adenosine binding sites has been demonstrated with the characteristics of the A_1, but not the A_{2A} P1-purinoceptor subtype [Lucchi et al., 1992; Rosati et al., 1995], which is claimed to inhibit glutamate release from the cerebellum [Lucchi et al., 1994]. In two congeneric marine fish, it has been found that the binding properties of the A_1-receptors are different, the receptor of the shallow-living *Sebastolobus alascanus* exhibiting a high affinity for the A_1 adenosine ligand, whereas the A_1-receptor in the deeper-living *S. altivelis*, exhibits a significantly lower binding affinity [Murray and Siebenaller, 1987]. Low temperatures and high hydrostatic pressures are typical of the deep sea; however, signal transduction by the A_1 purinoceptor system of the bathyal deep living fish *Antimora rostrata* is not disrupted by deep sea conditions [Siebenaller and Murray, 1990]. In goldfish exposed to warmth, the increase in locomotor activity is associated with increased uptake and release of adenosine from cerebellar slices, suggesting a compensatory role for adenosine in excitatory control of motor centres [Poli et al., 1995].

A NANC inhibitory response to electrical stimulation has been observed in the urinary bladder of the cod *Gadus morhua*; ATP has an excitatory effect on about half of the bladder preparations examined and was included

as a putative candidate for the NANC transmitter [Lundin and Holmgren, 1986].

There is evidence that some fish are attracted to purine compounds in a manner similar to that of carnivorous crustaceans. Chemoreceptors on the lip of the puffer fish *Fogo pandalis* exhibit an especially high sensitivity for ADP, and are thought to direct the fish to food sources [Kiyohara et al., 1975].

Amphibia

Evidence was presented in the early 1970's that ATP was a transmitter in the NANC nerves supplying the toad stomach [Burnstock et al., 1970; Satchell and Burnstock, 1971], duodenum and ileum [Burnstock et al., 1972]. ATP, ADP, and AMP were shown to be released upon stimulation of vagal NANC fibres and ATP mimicked the relaxation in response to nerve stimulation. Evidence that ATP is the transmitter substance released from NANC excitatory fibres in the splanchnic nerves supplying the small intestine of the toad was also presented, where again responses to nerve stimulation were mimicked by ATP [Sneddon et al., 1973]. Cultures of ciliated cells from the frog oesophageal epithelium and palate have been used as a model for studying the role of ATP in control of mucociliary activity. ATP in micromolar concentrations increases the ciliary activity by 3–4-fold in frequency and 4–5-fold in the rate of transport, as well as stimulating mucin release [Ovadiahu et al., 1988; Gheber and Priel, 1994]. ATP hyperpolarizes these cells [Tarasiuk et al., 1995]. Studies using 3,-0-(4-benzoyl)benzoylATP (BzATP) as a photoaffinity label for the ATP receptor involved were claimed to suggest the participation of two labelled proteins with molecular masses of 46 and 96 KDa (P46 and P96) in the stimulatory effect of ATP on the ciliary beat [Gheber et al., 1995]. Another study suggested that the extracellular ATP-induced changes in both ciliary beat frequency and membrane fluidity are triggered by similar signal transduction pathways [Alfahel et al., 1996].

Adenosine exerts effects on the amphibian heart in a manner similar to its effect upon the mammalian heart, having negative chronotropic and inotropic effects, mimicking the response to ACh by slowing the heart. This has been observed in the hearts of the frogs, *Rana ridibunda* [Lazou and Beis, 1987], *R. pipiens* [Hartzell, 1979], *R. temporaria* [Burnstock and Meghji, 1981], and *R. catesbiana* [Yatani et al., 1978; Goto et al., 1981]. In contrast, ATP has excitatory effects, increasing the force and rate of the heart beat [Cook et al., 1958; Goto et al., 1977; Burnstock and Meghji, 1981; Hoyle and Burnstock, 1986; Bramich et al., 1990]. Currents activated by extracellular ATP were studied on single voltage clamped bullfrog atrial cells; two ATP-activated conductances were demonstrated [Friel and Bean, 1988]. In frog ventricular cells,

P2-purinoceptors stimulate increases in Ca current by a pathway that might involve phosphoinositide turnover [Alvarez et al., 1990]. It has been shown that under certain conditions of physiological stress, such as hypoxia, ATP is released from the heart [Paddle and Burnstock, 1974; Doyle and Forrester, 1985]. Thus, ATP is available to directly modulate activity, and indirectly after degradation to adenosine, which in itself has been found to mediate a protective influence on the frog heart against periods of reduced calcium availability [Touraki and Lazou, 1992]. It has been shown that frog atria receive a NANC excitatory innervation [Donald, 1985]. ATP has a biphasic action, initial excitation followed by inhibition [Flitney et al., 1977], the excitatory effects being mediated by P2-purinoceptors, while the inhibitory effects are mediated by P1-purinoceptors following the degradation of ATP to adenosine [Burnstock and Meghji, 1981]. The excitatory responses to ATP partially mimics NANC stimulation of the frog and toad heart where it is believed that ATP is a cotransmitter with adrenaline [Hoyle and Burnstock, 1986; Bramich et al., 1990] acting on P2X-purinoceptors. ATP also has a biphasic action on the heart of the axolotl *Ambystoma mexicanum* [Meghji and Burnstock, 1983b]. Unlike fish, the amphibian ventricle is sensitive to adenosine and ATP. For instance, adenosine excites ventricular muscle of the toad *Xenopus laevis* [Meghji and Burnstock, 1983c] but is inhibitory in the axolotl [Meghji and Burnstock, 1983b] whereas in the frog, ATP is excitatory [Flitney and Singh, 1980; Burnstock and Meghji, 1981]. ATP in the micromolar range had two types of effect on isolated myocytes from the frog ventricle: it acted through P1-purinoceptors after breakdown to adenosine to antagonize the increase in I_{Ca} elicited by β-adrenoceptor stimulation; and directly through P2-purinoceptors (probably the P2Y-subtype) to increase I_{Ca} [Alvarez et al., 1990].

Descriptions of the effect of purine compounds on amphibian vascular preparations are somewhat limited. However, a prejunctional adenosine receptor revealed to be an A_1-adenosine receptor has been identified, which inhibits sympathetic nerve activity to the frog cutaneous muscle arterioles resulting in vasodilatation [Fuglsang and Crone, 1988; Fuglsang et al., 1989]. In addition, stimulation of vagal NANC fibres in the toad *Bufo marinus* mediates a fall in vascular resistance, although the transmitter is as yet unidentified [Campbell, 1971]. In a recent study of purinoceptors in the aorta of the frog, *Rana temporaria*, Knight and Burnstock [1995b] concluded that there appears to be a novel subclass of P1-purinoceptor mediating vasodilatation which, like the rat A_3 subclass, is not blocked by methylxanthines; they also identified a P2-purinoceptor that mediates vasoconstriction that resembles a P2X subtype in terms of agonist potencies and is antagonized by

PPADS. However, no evidence for a P2Y-purinoceptor mediating vasodilatation was found.

Purine compounds also have effects on preparations from amphibian tissues other than those of the gastrointestinal and cardiovascular systems. Vagal stimulation of the visceral muscle of the lung of Bufo marinus is purely inhibitory and the transmitter unknown, although ATP was proposed as a candidate [Campbell, 1971] and it had been shown that ATP caused relaxations of the lung preparation [Meves, 1953]. ATP has recently been shown to activate membrane current in frog Schwann cells [Vinogradova et al., 1994], perhaps playing a role in neuroglial interactions, since perisynaptic Schwann cells at the frog neuromuscular junction showed increases in intracellular calcium during motor nerve stimulation, an effect mimicked by local application of ATP [Jahromi et al., 1992; Robitaille, 1995]. Injections of ATP into the third ventricle of the mud puppy, Necturus maculosus, elicited dose-related increases in thermal tolerance [Ritchart and Hutchison, 1986]. Adenosine inhibits α-melanocyte-stimulating hormone (α-MSH) from frog pituitary melanotrophs, suggesting that A_1-purinoceptors may play a physiological role in the regulation of hormone release from the intermediate lobe of the pituitary [Chartrel et al., 1991]. An inhibitory action of adenosine on electrical activity of frog pituitary melanotrophs mediated via A_1-purinoceptors has been reported [Mei et al., 1994]. ATP regulates a quinidine-sensitive K^+ conductance in single proximal tubule cells isolated from frog kidney [Lynch and Hunter, 1994; Robson and Hunter, 1995]. P2Y-receptors on A6 epithelial cells derived from renal distal tubules of Xenopus laevis mediate increases in intracellular Ca^{2+} concentrations by releasing Ca^{2+} from intracellular stores [Mori et al., 1996].

Many years ago, Buchthal and Folkow [1944] injected ATP into the sciatic artery supplying the gastrocnemius muscle of the frog and reported tetanus-like contractions; they also observed that the sensitivity of the preparation to ACh was greatly increased by previous application of ATP. This postjunctional potentiation of ACh responses by ATP was later confirmed in bullfrog sympathetic ganglia [Akasu et al., 1981, 1983a,b] and in developing Xenopus neuromuscular synapses in culture [Igusa, 1988; Fu and Poo, 1991; Fu et al., 1993; Fu, 1994; Fu and Huang, 1994]. The effect of ATP is apparently mediated by the activation of cytosolic protein kinases and requires the influx of Ca^{2+} through the plasma membrane. In addition, a further purinergic involvement in transmission at the frog neuromuscular junction was recognized, namely modulation of ACh release via prejunctional P1-purinoceptors. Both end plate potentials (epp's) in response to nerve stimulation and spontaneous miniature epp's were reduced by adenosine [Ginsborg and Hirst, 1972; Ribeiro and Walker, 1975;

Silinsky, 1980; Ribeiro and Sebastião, 1987; Branisteanu et al., 1989; Hirsh et al., 1990; Sebastião and Ribeiro, 1990; Bennett et al., 1991; Silinsky and Solsona, 1992; Hunt and Silinsky, 1993]. Examination of the actions of adenosine analogs suggested that this presynaptic P1-purinoceptor may be of the A_1-subtype [Barry, 1990], although this has been debated [Sebastião and Ribeiro, 1989]. Later it was shown that ATP was released together with ACh from motor endings [Silinsky and Hubbard, 1973; Silinsky, 1975] where it could then act as a postjunctional potentiator of ACh action, and following breakdown by ectoenzymes to adenosine [see Cunha and Sebastião, 1991; Cascelheira and Sebastião, 1992], to act prejunctionally to inhibit ACh release [Silinksky and Redman, 1996].

ATP has been implicated as a synaptic transmitter in both sympathetic and sensory ganglia. After an early paper where high concentrations of adenine nucleosides and nucleotides were shown to have depressant and hyperpolarising actions on both dorsal and ventral root neurons in isolated hemisected perfused toad spinal cords [Phillis and Kirkpatrick, 1978], ATP was shown to depolarize bullfrog sympathetic ganglion cells [Nakamura et al., 1974; Siggins et al., 1977]. ATP probably acts by decreasing K^+ conductance, including the M-current; ATP also depressed the maximum amplitude of action potential after-hyperpolarizations and it was suggested that ATP released with ACh from presynaptic nerve terminals may act as a modulator of nicotinic transmission [Akasu et al., 1983b]. ATP inhibits calcium current in frog sympathetic neurons [Elmslie, 1992]. Silinsky and Ginsborg [1983] claimed that inhibition of ACh release from preganglionic nerves supplying neurons in the ninth lumbar sympathetic chain ganglion of the frog, Rana pipiens, was largely by ATP itself rather than by adenosine after breakdown of ATP. However, in keeping with the evidence for synaptic transmission at the skeletal neuromuscular junction, ATP was later shown to increase the sensitivity of the nicotinic ACh receptor in bullfrog ganglia cells and it was suggested that ATP, perhaps via a P2-purinoceptor, had this effect by acting on an allosteric site of the ACh-receptor-ionic channel complex [Akasu and Koketsu, 1985].

The concentration-dependence and kinetics of ionic currents activated by ATP were studied in voltage-clamped dorsal root ganglion cells from bullfrogs [Bean, 1990; Bean et al., 1990]. About 40% of the neurons responded with an increase in membrane conductance, but showed rapid desensitization, typical of the $P2X_3$-purinoceptor recently described in rat dorsal root nerves [Chen et al., 1995; Lewis et al., 1995]. Ethanol was shown to inhibit the ATP-activated current in dorsal root ganglion cells, perhaps by increasing the apparent dissociation constant of the ATP receptor [Li et al., 1993]. In a later study, Akasu and his colleagues [Tokimasa et al.,

1993] showed with dissociated bullfrog dorsal root ganglion cells that, whereas in small C-cells ATP (1–10 μM) activated a sodium-potassium current, in large A-cells (approx. 65 μm in diameter) ATP inhibited M-current. In some bullfrog primary afferent neurons, ATP reversibly augmented GABA-induced depolarizations [Morita et al., 1984].

Since spontaneous ACh release is known to regulate the development of contractile properties of the postsynaptic muscle cell [Kidokoro and Saito, 1988], the authors suggest that ATP coreleased with ACh may serve as a positive trophic factor at developing neuromuscular synapses.

Xenopus oocyte has been used in expression cloning of purinoceptors [Lotan et al., 1982, 1985; Webb et al., 1993; Chen et al., 1995; Lewis et al., 1995; Bo et al., 1995], but the follicle cells contain endogenous purinoceptors [King et al., 1996a,b]. A receptor for adenosine was the first to be reported [Lotan et al., 1985; Dascal et al., 1985] which appears to be a novel subtype [King et al., 1996a]. P2-purinoceptor activated inward currents are also present [King et al., 1996b]. The actions of purines at these receptors may be involved in the sequence of cellular events that occur in early development [see Jessus et al., 1989]. Low concentrations of extracellular ATP, present in the perilymphatic compartment of the semicircular canal of the frog, *Rana pipiens*, appears to play a role in vestibular physiology; a P2Y subtype of purinoceptor seems to be involved and Reactive Blue 2 and suramin antagonize the responses [Aubert et al., 1994, 1995].

Reptilia

An excitatory NANC innervation has been identified in the ileum of the lizard *Tiliqua rugosa*, stimulation of which can be mimicked by ATP [Burnstock et al., 1972; Sneddon et al., 1973]; however the subtype of P2-purinoceptor is not known. An excitatory effect of ATP has also been noted in the rectum of the lizard *Agama agama* [Ojewole, 1983a; Savage and Atanga, 1985] but again the subtype of the purinoceptor has not been identified.

There have been few studies of purinoceptors in the cardiovascular system of reptiles. Wedd and Fenn [1933] reported variable responses to adenosine in the heart of the turtle *Pseudomys elegans*, whereas all the purine analogues tested, including adenosine, ATP, α,β-meATP and β,γ-meATP proved to be inactive on either the atrium or ventricle of the turtle *Emys orbicularis* [Meghji and Burnstock, 1983a]. The ionic basis of the hyperpolarizing action of adenyl compounds on sinus venosus of the tortoise heart has been examined [Hutter and Rankin, 1984].

In a recent study of purinoceptors in the aorta of the garter snake, *Thamnophis sirtilis*, Knight and Burnstock [1995a] concluded that both P1-purinoceptors me-

diating vasodilatation and P2-purinoceptors mediating vasoconstriction are present. However, in contrast to mammalian aorta, both P2X- and P2Y-subtypes mediate vasoconstriction; there was no evidence for vasodilatation by ATP or its analogues. In contrast, the portal vein of the rainbow lizard, *Agama agama*, dilated in the presence of ATP [Ojewole, 1983b]. Occupation of the P2-purinoceptor led to synthesis of prostanoids as in mammals [Knight and Burnstock, 1995a].

The visceral smooth muscle of the lung of the snake, *Thamnophis sp.*, is described as having NANC/purinergic innervation [Smith and Macintyre, 1979], although there is no evidence of the involvement of ATP or adenosine in the response. In the bladder of the sleepy lizard, *Trachysaurus rugosa*, an atropine-resistant contraction in response to nerve stimulation has been found, athough the transmitter substance has not been identified [Burnstock and Wood, 1967].

Birds

An adenosine receptor has been identified in the embryonic chick heart [Hatae et al., 1989; Blair et al., 1989]; it appears to be an A_1-receptor, which can be downregulated by exposure to R-PIA [Shryock et al., 1989].

ATP is a potent dilator of vessels in the duck foot, where doses of 1.9–19 nmol produces falls in perfusion pressure comparable to those produced by stimulation of dorsal metatarsal nerves [McGregor, 1979; Bell and Rome, 1984]. ATP has also been shown to cause selective dilatation of arterio-venous shunts in the foot of the chicken [Hillman et al., 1982]. Since the feet of birds form an area of skin devoid of insulative feathers, change in blood flow is used to regulate body heat as well as preventing freezing of the foot, so that purinergic receptors may play a part in this mechanism.

There is evidence for P2-purinoceptors in different preparations of bird gut. The oesophagus of the chicken contracts to ATP via a P2-purinoceptor [Bartlet, 1974], as does the rectum [Bartlet, 1974; Meldrum and Burnstock, 1985]. α,β-MeATP also causes a contraction of the chicken rectum and is able to desensitize the excitatory response to stimulation of Remak's nerve, indicating that P2X-purinoceptors may be involved in purinergic excitatory transmission [Meldrum and Burnstock, 1985; Komuri et al., 1988] which has long been recognized in the rectum of birds [Bartlet and Hassan, 1971; Burnstock, 1972; Bartlet, 1974, 1992; Ahmad et al., 1978; Komori and Ohashi, 1982, 1988; Meldrum and Burnstock, 1985]. Recent studies have examined the effects of different ATP analogs on membrane currents and transduction mechanisms in voltage clamped smooth muscle cells from the chick rectum [Matsuoka et al., 1993]. Inhibitory NANC innervation of the bird stomach has also been demonstrated [Bennet, 1969a,b, 1970].

The involvement of ATP as a purinergic cotransmitter with ACh was first described in cultured chick myotubes and micromolar concentrations of ATP were shown to activate cation channels [Kolb and Wakelam, 1983]; this was confirmed in later studies [Häggblad et al., 1985; Hume and Hönig, 1986]. The disappearance of ATP-responsiveness of developing chick skeletal muscle shortly after muscles become innervated and the reappearance of ATP responsiveness following denervation suggest that the expression of ATP responsiveness is regulated by motor neurones [Wells et al., 1995]. 5'-Nucleotidase activity appeared during the development of chick striated muscle and increased markedly post hatching; in adult muscle it showed a more restricted distribution [Mehul et al., 1992].

Interestingly, ATP has also been shown to trigger phosphoinositide turnover in chick myotubes [Häggblad and Heilbronn, 1987, 1988], perhaps suggesting that P2Y- as well as P2X-purinoceptors are present. The later demonstration of multiple responses in chick myotubes [Hume and Thomas, 1988; Eriksson and Heilbronn, 1989] is consistent with this possibility. The responses to ATP show rapid desensitization [Thomas and Hume, 1990a] which is typical of the $P2X_1$ and $P2X_3$ subclasses of the P2X-ionotropic purinoceptor family [see North, 1996]. In more recent analyses of the ion channels involved in ATP-mediated responses of chick skeletal muscle, excitation has been shown to be due to a simultaneous increase in membrane permeability to sodium, potassium, and chloride ions, and that only a single class of excitatory ATP-activated channels are involved which do not select by charge, i.e., they conduct both cations and anions [Thomas and Hume, 1990b; 1993]. The order of potency for agonists at this receptor was ATP=ATPγS=2meSATP >2'-deoxy-ATP=3'-deoxy-ATP>ATP-OPO$_3$ =ADP and both 2',3'dialdehyde-ATP and 4,4'-diisocyanatostilbene-2,2'-disulphonic acid (DIDS) were potent irreversible inhibitors; 8-Br-ATP was a weak antagonist, while 2',3'-dialdehyde-ATP and DIDS were potent irreversible inhibitors [Thomas et al., 1991].

It has been suggested that extracellular adenine nucleotides may be involved in the PO_2-dependent regulation of red cell metabolism in late chick embryos [Koller et al., 1994]. $P1(A_2)$ purinoceptor mediated stimulation of cyclic AMP in cultured chicken pineal cells has been reported [Falcon et al., 1995]. Adenosine induced apoptosis in chick embryonic sympathetic neurones [Wakade et al., 1995]. Adenosine has been shown to modulate calcium currents in postganglionic neurones of cultured avian ciliary ganglia [Bennett and Ho, 1991; Bennett et al., 1992].

Speculations About the Evolution of Purinoceptor Subtypes

While it must be recognized that it is dangerous to look for an evolutionary pattern in the development of purinoceptor subtypes from studies of modern animals, it seems clear that two receptor types can be distinguished in divergent invertebrate and lower vertebrate groups that correspond to mammalian P1- and P2-purinoceptors. There are exceptions, where no distinction is made between the responses to adenosine and ATP, but in general there is sufficient evidence in the existing literature to support the view that two distinct subclasses, one selective for adenosine and one selective for ATP, exist in invertebrates and lower vertebrates. Tables 3–6 illustrate the distribution of P1- and P2-purinoceptors that have been identified in invertebrates and lower vertebrates.

Identification of P1-purinoceptors relies on a potency order of adenosine >AMP>ADP=ATP, supplemented with the use of selective antagonists, such as 8-PT. However, there are few studies of P1-purinoceptor subtypes in invertebrate groups partly because identification of purinoceptor subtypes has been hindered by the lack of activity of the available agonists and antagonists. Despite these limitations, the first record of subclasses of P1-purinoceptors into A_1 and A_2 subtypes has been claimed in molluscs. Adenosine agonists such as NECA, PIA, CGS 21680, and CPA, which are selective for subclasses of mammalian adenosine receptors, are found to be inert in most invertebrates. Hopefully, some of the new selective agonists and antagonists for A_1, A_{2A}, A_{2B}, and A_3 subtypes [Jacobson et al., 1996] will be applied to invertebrate and lower vertebrate preparations. Binding studies of the A_1-selective ligand [^{3}H]cyclohexyladenosine (CHA) in brain membranes were not positive in molluscs and arthropods, although they were in hagfish [Siebenaller and Murray, 1986].

There are many examples of the presence of P2-purinoceptors in a variety of tissues from many invertebrate groups. Identification relies predominantly on the selective or more potent effect of ATP compared with adenosine and the use of P1-purinoceptor antagonists to rule out responses to ATP due to adenosine resulting from extracellular breakdown of ATP. Identification of subtypes is again hindered since P2-receptor antagonists developed for use in mammalian systems [see Fredholm et al., 1994] sometimes lack activity in invertebrate tissues. For instance, in the sea anemone, Reactive Blue 2 has agonist properties [Hoyle et al., 1989] and in identified neurons of the leech, suramin failed to block the activity of ATP and behaved as an agonist [Backus et al., 1994] although it has been shown recently that suramin is not an antagonist at $P2X_4$- and $P2X_6$-purinoceptor subtypes [Bo et al., 1995; Buell et al., 1996]. Another problem in trying to distinguish P2X and P2Y subtypes is that the relative potencies of agonists depends to some extent on the presence or absence of powerful ectoATPases [see Kennedy and Leff, 1995; Kennedy et al., 1996]. Thus, in the presence of Ca/Mg ATPase inhibitors or in single cell preparations, the classical P2X- and P2Y-

TABLE 3. Summary of Reports of P1 and P2 Purinoceptors and, in Some Preparations, Their Subtypes in Various Tissues From Bacteria, Protozoans, Coelenterates, and Molluscs

Phylum	Tissue/activity	P1 (not subtyped)	P1 Purinoceptors			P2 (not subtyped)	P2 Purinoceptors	
			A_1	A_2	A_3		P2X	P2Y
Bacteria	Inhibition of growth	✓						
	Initiation of sporulation					✓		
	Regulation of binding of enterotoxin					✓		
Protozoa	Inhibition of amoeboid movement					✓		
	Increase of contractile vacuole output					✓		
	Cell aggregation	✓				✓		
	Motility increase of ciliated paramecium					✓		
	Herniation					✓		
	Cytoskeletal organization	✓						
	Inhibition of growth of pedal disc					✓		
Coelenterates	Contraction of anemone pedal disc	✓				✓		
	Maturation of nematocysts					✓		
	Ciliary reversal in ctenophores					✓		
Molluscs	Activation of snail suboesophageal neurons		✓ (i)	✓ (e)			✓	
	Neuromodulation of transmitter release from pedal ganglion of *Mytilus*			✓				
	Excitation of heart	✓				✓		
	Contraction of snail rectum and oesophagus					✓		
	Rupture of secretory glands of slug					✓		

(i) = inhibition; (e) = excitation.

purinoceptor potency series [Burnstock and Kennedy, 1985] of α,β-meATP>ATP> 2meSATP and 2meSATP>>ATP > α,β-meATP, respectively, need to be modified for P2X-purinoceptors: ATP=2meSATP>α,β-meATP and for P2Y-purinoceptors: 2meSATP>ATP>α,βmATP. Since little is known about ecto-ATPases in invertebrates and lower ver-

tebrates [see Ziganshin et al., 1994], this must be taken into account in the interpretation of P2 agonist potency series in the lower animals. Despite these problems, there are records of fast P2X-purinoceptors involving ion channels in the nervous system of molluscs, annelids, and arthropods.

One of the complications is the emerging evidence

TABLE 4. Summary of Reports of P1 and P2 Purinoceptors and, in Some Preparations, Their Subtypes in Various Tissues From Annelids, Arthropods, and Echinoderms

Phylum	Tissue/activity	P1 (not subtyped)	P1 Purinoceptors			P2 (not subtyped)	P2 Purinoceptors	
			A_1	A_2	A_3		P2X	P2Y
Annelids	Depolarization of leech neurons						✓	
	Stimulation of salivary cells					✓		
Arthropods								
Crustaceans	Stimulation of lobster olfactory and gustatory chemoreceptors	✓					✓	
	Feeding behaviour	✓ (i)				✓ (e)		
	Inhibition of crab heart					✓ (i)		
	Stimulation of lobster intestinal neurons					✓		
Insects	Initiation of the gorging reflex in blood suckers:							
	tsetse fly							✓
	stable fly						✓	
	Feeding stimulation in flea and tick					✓		
	Stimulation of fat body of silkworm				✓			
Echinoderms	Relaxation of gastric ligament of starfish	✓				✓		
	Relaxation of body muscles of sea cucumber					✓		
	Contraction of rectum of sea urchin	✓				✓		
	Contraction of spine muscles of sea urchin					✓		
	Inhibition of growth of fertilized starfish eggs	✓				✓		
	Modulation of sea urchin flagellar motility					✓		

(i) = inhibition; (e) = excitation.

TABLE 5. Summary of Reports of P1 and P2 Purinoceptors and, in Some Preparations, Their Subtypes in Various Tissues From Cyclostomes, Elasmobranch, Coelenterate, and Teleost Fishes

Vertebrates	Tissue/activity	P1 (not subtyped)	A₁	A₂	A₃	P2 (not subtyped)	P2X	P2Y
Fish								
Cyclostomes	Dilatation of brachial vessels of hagfish	✔						
Elasmobranchs	Inhibition of intestinal motility in dogfish and ray					✔		
	Contraction of stomach					✔		
	Stimulation of rectal gland of shark		✔	✔				
	Dogfish aorta		✔	✔				
	Coronary artery of:							
	Skate	✔ (c)				✔ (c,d)		
	Mako shark	✔ (d)				✔ (c)		
	Electric organ	✔				✔		
Teleosts	Cod, stickleback and flounder intestine	✔ (d)				✔ (c)		
	Carp and goldfish intestine	✔ (d)				✔ (c)		
	Rectum of flounder	✔					✔ (c)	
	Stomach of stickleback	✔ (d)					✔ (c)	
	Gill vessels of:							
	Trout	✔ (c)				✔ (c)		
	Cichlid							
	Systemic vessels of trout	✔ (c)				✔ (d)		
	Heart of carp and flounder and trout	✔				✔		
	Melanophore dispersion in medaka				✔			
	Brain of goldfish (temperature control and locomotor activity)			✔				
	Excitation of bladder					✔		

(c) = constrict/contract; (d) = dilate/relax.

for a P2-purinoceptor subtype, both in lower animals, and in certain mammalian cell lines (e.g., C6 glioma cells [Boyer et al., 1993]; DDT₁-MF2-smooth muscle cells [Sipma et al., 1994] and in mouse C2C12 myotubes [Henning et al., 1993a,b], which operates via an adenylate cyclase second messenger system, since one of the original criteria for distinguishing P1- from P2-purinoceptors was adenosine action through adenylate cyclase [Burnstock, 1978b]. It is possible that the primitive P2-purinoceptor acted via an adenylate cyclase transduction system in parallel with P1-purinoceptors and only later diverged to act via inositol trisphosphate second messenger systems or ligand-gated ion channels.

Adenosine has potent cardiovascular actions on several fish groups, including vasodilator and vasoconstrictor actions [see Nilsson and Holmgren, 1992], with many striking similarities to the effect of adenosine on mammalian cardiovascular systems [see Collis, 1989; Olsson and Pearson, 1990]. A₁ and A₂ subclasses of P1-purinoceptor first appear in elasmobranch fish; A₃ receptors have been described in amphibians. Studies of the action of adenosine on the gill vasculature of teleost fish, all report vasoconstriction, e.g., as in the trout *Salmo gairdneri* [Colin and Leray, 1979; Colin et al., 1979] and tropical cichlid *Oreochromas niloticus* [Okafor and Oduleye, 1986]. Fish ventral aorta and brachial vessels are the evo-

lutionary precursors of the pulmonary vasculature; although in mammalian pulmonary systems, such as the rabbit, adenosine is a potent vasodilator, acting via an A₂-receptor [Pearl, 1994], there are also reports of vasoconstrictor actions of adenosine in the pulmonary bed [Biaggioni et al., 1989; Neely et al., 1991].

P2X-purinoceptors have been described in teleost fish; separate P2X- and P2Y-purinoceptor subtypes are well represented in amphibians, reptiles, and birds. Cardiovascular P2-purinoceptors have also been identified in lower vertebrates which parallel those described in mammals [Ralevic and Burnstock, 1991] For example, ATP causes vasoconstriction of the systemic vasculature of the trout *Salmo gairdneri* [Wood, 1977]. ATP constricts the coronary artery of the trout *Oncorhynchus mykiss*, being equipotent with ADP, but more potent than adenosine, indicating a P2-purinoceptor [Small and Farrell, 1990]. The vascular rings are described as being endothelium-free and may therefore correspond to the vasoconstrictor P2X-purinoceptor found on mammalian vascular smooth muscle.

With the exception of the mussel and the turtle, virtually every examined species of invertebrate and lower vertebrate heart responded to adenosine or ATP, and often both, frequently mimicking the effect of adenosine and ATP on the mammalian heart. There is considerable

TABLE 6. Summary of Reports of P1 and P2 Purinoceptors and, in Some Preparations, Their Subtypes in Various Tissues From Amphibians, Reptiles, and Birds

	Tissue/activity	P1 (not subtyped)	P1 Purinoceptors A₁	A₂	A₃	P2 (not subtyped)	P2 Purinoceptors P2X	P2Y
Amphibians	Toad stomach					✔ (i)		
	Frog heart	✔ (i)				✔ (e)	✔ (e)	
	Frog isolated ventricular cells							✔
	Frog ventricle	✔						✔
	Prejunctional modulation of sympathetic nerve activity		✔					
	Frog aorta				✔ (i)		✔ (e)	
	Toad lung					✔		
	Increases in beat frequency of ciliary cells from frog palate and oesophagus					✔		
	Activation of membrane currents in Schwann cells					✔		
	Increase in thermal tolerance after brain injections					✔		
	Regulation of hormone release from pituitary		✔			✔		
	Proximal tubules of frog kidney					✔		
	Modulation in sympathetic ganglia and neuromuscular junction		✔ (i)				✔ (e)	
	Toad spinal cord					✔		
	Bullfrog dorsal root ganglia						✔ (P2X₃)	
	Xenopus oocyte follicular cells	✔				✔		
	Semicircular canal of frog							✔
Reptiles	Lizard ileum and rectum					✔		
	Snake aorta	✔ (i)					✔ (e)	✔ (e)
	Lizard portal vein					✔ (i)		
Birds	Embryonic chick heart		✔					
	Vessels of duck foot					✔ (i)		
	Chicken rectum						✔	
	Chick myotubes					✔		✔
	Stimulation of chicken pineal cells			✔				
	Ciliary ganglia	✔						

(i) = inhibition/relaxation/dilatation; (e) = excitation/contraction.

evidence for P2-purinoceptors on the amphibian atria and ventricle. ATP causes positive inotropic effects on frog atrial [Goto et al., 1977; Burnstock and Meghji, 1981] and ventricular muscle [Flitney et al., 1977; Flitney and Singh, 1980; Burnstock and Meghji, 1981], the excitatory ATP responses in the atria of the frog is inhibited by α,β-meATP, suggesting the presence of P2X-purinoceptors [Hoyle and Burnstock, 1986].

Amphibian bladders studied so far would appear to possess a contractile P2X-purinoceptor with similar characteristics to those of mammalian urinary bladders [see Hoyle and Burnstock, 1985, 1992]. These studies also provide evidence for purinergic neurotransmission in the urinary bladders of amphibians.

There is evidence that purines other than adenosine and ATP and also pyrimidines have activity on some invertebrate and lower vertebrate tissues. The purine guanosine 5′-monophosphate (GMP) acts in a similar manner as ATP by modulating calcium conductance in leech salivary glands [Wuttke and Berry, 1993]; it is less potent than ATP but may be acting via a P2-purinoceptor. GTP has been found to modulate ACh-induced depolarization of *Buccinum undatum* proboscis smooth muscles, whereas ATP and adenosine are without activity [Nelson and Huddart, 1994]. Various other purine or pyrimidine triphosphates including cytidine, inosine, and xanthosine (CTP, ITP, and XTP) stimulate chemoreceptors of crustaceans [Carr et al., 1986, 1987]; similarly, the monophosphates of inosine, guanosine, and cytidine (IMP, GMP, and CMP) together with the triphosphates ITP, GTP, CTP, and UTP, all stimulate gorging of blood-feeding insects [Galun and Margalit, 1969; Friend and Smith, 1982; Dadd and Kleinjan, 1985], as does the tetraphosphate of adenosine [Galun et al., 1963, 1988; Smith and Friend, 1976]. ATP and adenosine stimulate the hearts of two molluscs, as does uridine, UDP, and UTP, cytidine, CMP, CDP, and CTP. In contrast, GMP and GDP are inhibitory implying separate receptors [S.-Rózsa, 1968]. Actions of pyrimidines and purines other than adenosine and ATP have also been described in lower vertebrates. For instance,

guanosine 5'-diphosphate (GDP), GMP, and GTP, together with IMP hyperpolarize isolated toad ventral and dorsal root neurons, whereas ITP, TTP, XTP, CTP, UTP, and uridine 5'-diphosphate (UDP) depolarize the spinal cord [Phillis and Kirkpatrick, 1978], implying the existence of separate receptors which have yet to be identified. UDP and UTP have also been shown to depolarize explanted frog sympathetic ganglia [Siggins et al., 1977], being more potent than TTP, GTP, GDP, ITP, or IDP. High concentrations of inosine and guanosine stimulate the toad ventricle [Meghji and Burnstock, 1983c], although both are less potent than ATP or adenosine. An investigation of the snake aorta [Knight and Burnstock, 1995a] revealed a sensitivity to UTP, although its action was similar to ATP typical of P2Y$_2$ (= P$_{2U}$)-purinoceptors. Whether there are separate receptors for the pyrimidines or whether they act on P2-purinoceptors is not always clear from the studies performed. This problem has recently been recognized by the IUPHAR Nomenclature Committee, who propose that the P2-purinoceptors be replaced by P2-receptors to incorporate both purines and pyrimidines [Fredholm et al., 1997].

SUMMARY AND FUTURE DIRECTIONS

It is clear from this review of currently available information that few conclusions can be made yet, either about the roles of purinoceptors in ontogeny or about the pattern of evolution of purinoceptor subtypes. However, it is clear that purinoceptors are involved in early signalling in vertebrate embryos; one receptor has already been cloned and characterized and, hopefully, more will follow. It is also clear that purinoceptors for both adenosine and ATP are present early in evolution and play a role in most, if not all, invertebrate and lower vertebrate species. However, until selective agonists and antagonists for the recently cloned purinoceptor subtypes become available, there is little possibility of resolving questions concerned with the evolution of purinoceptor subtypes, although a phylogenetic tree has been constructed for the evolutionary relationship between the known subtypes of adenosine receptors [Feng and Doolittle, 1990; Linden et al., 1994]. The molecular cloning of genes encoding receptors for adenosine and ATP from invertebrates and lower vertebrates will be a good way forward.

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