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Extracellular Nucleotide Signaling in the Inner Ear

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Abstract

Extracellular nucleotides, particularly adenosine 5′-triphosphate (ATP), act as signaling molecules in the inner ear. Roles as neurotransmitters, neuromodulators, and as autocrine or paracrine humoral factors are evident. The diversity of the signaling pathways for nucleotides, which include a variety of ATP-gated ion channels (assembled from different subtypes of P2X-receptor subunit) and also different subtypes of G protein-coupled nucleotide receptors (P2Y receptors) supports a major physiological role for ATP in the regulation of hearing and balance. Almost invariably both P2X and P2Y receptor expression is apparent in the complex tissue structures associated with the inner-ear labyrinth. However P2X-receptor expression, commonly associated with fast neurotransmission, is apparent not only with the cochlear and vestibular primary afferent neurons, but also appears to mediate humoral signaling via ATP-gated ion channel localization to the endolymphatic surface of the cochlear sensory epithelium (organ of Corti). This is the site of the sound-transduction process and recent data, including both electrophysiological, imaging, and immunocytochemistry, has shown that the ATP-gated ion channels are colocalized here with the mechano-electrical transduction channels of the cochlear hair cells. In contrast to this direct action of extracellular ATP on the sound-transduction process, an indirect effect is apparent via P2Y-receptor expression, prevalent on the marginal cells of the stria vascularis, a tissue that generates the standing ionic and electrical gradients across the cochlear partition. The site of generation of these gradients, including the dark-cell epithelium of the vestibular labyrinth, may be under autocrine or paracrine regulation mediated by P2Y receptors sensitive to both purines (ATP) and pyrimidines such as UTP. There is also emerging evidence that the nucleoside adenosine, formed as a breakdown product of ATP by the action of ectonucleotidases and acting via P1 receptors, is also physiologically significant in the inner ear. P1-receptor expression (including A_1, A_2, and A_3 subtypes) appear to have roles associated with stress, acting alongside P2Y receptors to enhance cochlear blood flow and to protect against the action of free radicals and to modulate the activity of membrane conductances. Given the positioning of a diverse range of purinergic-signaling pathways within the inner ear, elevations of nucleotides and nucleosides are clearly positioned to affect hearing and balance. Recent data clearly supports endogenous ATP- and adenosine-mediated changes in sensory transduction via a regulation of the electrochemical gradients in the cochlea, alterations in the active and passive mechanical properties of the cells of the sensory epithelium, effects on primary afferent neurons, and control of the blood supply. The field now awaits conclusive evidence linking a physiologically-induced modulation of extracellular nucleotide and nucleoside levels to altered inner ear function.

Index Entries: Adenosine 5′-triphosphate; P2 receptors; P2X receptors; P2Y receptors; ATP-gated ion channels; adenosine; adenosine receptors; ectonucleotidase; cochlea; vestibular labyrinth.

Introduction

It is now well established that extracellular nucleotides can influence cellular function in a diverse group of tissues where storage and release of ATP has been detected along with the other prerequisites for signaling, such as signal termination via ectonucleotidases (analogous to acetylcholinesterase action at the neuromuscular junction) and purine reuptake via adenosine-transport pathways (for reviews *see* Burnstock, 1981, 1986; Gordon, 1986; Dubyak and El-Moatassim, 1993; Zimmerman, 1994; Burnstock, 1995; Gibb and Halliday, 1996). Through interaction with specific surface nucleotide (P2) receptors, previously termed purinoceptors (Fredholm et al., 1997), purines and pyrimidines such as ATP, ADP, UTP, and adenine dinucleotide polyphosphates (Ap_nA), act as neurotransmitters or neuromodulators and as humoral and trophic factors in the central, peripheral, and autonomic nervous systems, muscle, blood, exocrine and endocrine glands, indeed, throughout the body (Burnstock, 1995, 1993; Pintor et al., 1992; Abbracchio and Burnstock, 1994; Fredholm et al., 1994).

From the early report by Drury and Szent-Gyorgyi (1929) that extracellular ATP affected cardiovascular function, and Burnstock's proposal (1972, 1978) that extracellular ATP acted via a P_2 purinoceptor as a neurotransmitter in the enteric nervous system, the field of purinergic signaling has recently burgeoned to the stage where it is difficult to establish a tissue in the body that is not in some way directly or indirectly influenced by P2-receptor-mediated mechanisms. However, a lack of specific agonists, antagonists, and the confounding action of ectonucleotidases has made classification of the signaling pathways for ATP problematic (Dubyak and El-Moatassim, 1993). Recently, molecular cloning studies have clearly established that in common with other neurotransmitter systems, both ionotropic and metabotropic nucleotide receptor mechanisms exist. This has led to the rationalization of the previous pharmacologically based P_2 purinoceptor nomenclature which included P_{2X}, P_{2Y}, P_{2U}, P_{2Z}, P_{2D}, and P_{2t} subtypes to a structure-based P2-receptor nomenclature currently represented by $P2X_1$–$P2X_7$ ionotropic and $P2Y_1$–$P2Y_7$ metabotropic (G protein-coupled) receptor subtypes (Fredholm et al., 1997; Abbracchio and Burnstock, 1994).

In the central and peripheral nervous systems, roles for extracellular ATP as a neurotransmitter and neuromodulator are evident (Gibb and Halliday, 1996; Edwards et al., 1992; Evans et al., 1992; Edwards and Gibb, 1993; Illes and Norenberg, 1993; Edwards, 1994, 1995), along with considerable trophic influence mediated by the neuroglia (Salter and Hicks, 1994, 1995; Neary et al., 1996). ATP is coreleased with both noradrenaline, acetylcholine, VIP and neuropeptides at central, autonomic, and enteric synapses where it acts as a cotransmitter. P2 receptors mediate the rapid or phasic component of sympathetic vasoconstriction in many arteries and contraction of vas deferens (Sneddon et al., 1996); parasympathetic and enteric cotransmission in urinary bladder and intestine are contractile, whereas in some vascular beds, in association with NO release, ATP causes vasodilatation (Hoyle, 1996; Boader et al., 1995).

Operating via a variety of receptor-effector mechanisms, nucleotides and nucleosides have also been shown to underpin the physiology of many of the body's sensory modalities, *see* Thorne and Housley (1996) for a review. Sensory systems with purinergic regulation include: sensory-motor reflex loops within the enteric nervous system; nociceptive input via the dorsal root ganglia; cutaneous sensation; epicardial mechano- and chemosensory fibers, olfactory and gustatory (taste bud) systems; adenosine-

and ATP-mediated influence on neurotransmission; and tissue homeostasis in the eye (Thorne and Housley, 1996; Greenwood et al., 1997).

There is also substantial evidence that ATP plays a major role in regulation of mechanoelectrical transduction processes and neural control of cochlear sensitivity via P2 purinoceptors (Thorne et al., 1997). In the inner ear, both neural and humoral influences are evident with some tissues demonstrating a clear demarcation between ionotropic and metabotropic receptor mechanisms, whereas in other cases both signaling systems have been demonstrated on the same cells (Thorne and Housley, 1996; Heilbronn et al., 1995; Housley and Ryan, 1997). This review seeks to describe the considerable evidence for nucleotide signaling in the inner ear; including electrophysiological, imaging, biochemical, and molecular studies. The diversity and extent of the research activity focusing on the physiological significance of extracellular nucleotides in the inner ear makes this sensory system preeminent in terms of the elucidation of nucleotide signaling; revealing a considerable complexity of neural and humoral actions.

Background to Extracellular Nucleotide Signaling Mechanisms

P2X Receptors

ATP-gated ion channels, like other ionotropic receptors such as the nicotinic acetylcholine receptor, exist as a pentameric assembly of (P2X receptor) subunits (Nicke et al., 1996). Following the independent isolation of the first two subunits, now designated $P2X_1R$ (Valera et al., 1994) and $P2X_2R$ (formerly $P2XR_1$) (Brake et al., 1994), using expression screening of vas-deferens and PC12: cell mRNA, respectively, an additional five subunits $P2X_{3..7}R$ have been isolated from a number of different species. The subunits form a new major class of the ligand-gated ion channel superfamily, distinguished by the two transmembrane domains, with intracellular N- and C- termini and a single extensive extracellular domain (Fig. 1) that shows conservation of 10 cysteine residues conferring structure via disulfide bonds (Surprenant et al., 1995; North, 1996a,b). These subunits have been expressed in *Xenopus* oocytes as well as mammalian cell lines; indicating that both homo- and heteromultimeric assembly of subunits is possible (North, 1996b; Lewis et al., 1995). The multimeric assembly of P2XR subunits provides the foundation for the variability in the pharmacokinetics and ion selectivity of the native receptors. A number of these subunits are widely expressed ($P2X_{2,4,6}R$) in neurons both centrally and in the peripheral nervous system (Bo et al., 1995; Collo et al., 1996; Kanjhan et al., 1996), with homomultimeric expression indicating varied pharmacological profiles highlighted by a preference for the agonist 2-methylthioATP (2MeSATP), ineffectiveness of the agonist α,β–methylene ATP (α,βMeATP), and variable sensitivity to the competitive antagonist suramin and pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS) (North, 1996a). ATP-gated ion channels assembled from $P2X_2R$ are susceptible to both suramin and PPADS block, whereas both $P2X_4R$ and $P2X_6R$ lack this antagonist sensitivity (Bo et al., 1995; Collo et al., 1996). It therefore appears likely that the commonly observed sensitivity to these antagonists is conferred by heteromultimeric subunit assembly that includes the $P2X_2R$ subunit (Gibb and Halliday, 1996; Kanjhan et al., 1996). The $P2X_1R$ has a more limited distribution in the central nervous system (Kidd et al., 1995), with expression also occurring commonly in vascular and urogenital smooth muscle (Vulchanova et al., 1996). $P2X_3R$ is expressed in the nociceptive neurons of the dorsal-root ganglia (Chen et al., 1995c) where coassembly with $P2X_2R$ subunit is evident (Lewis et al., 1995). This heteromultimer combination may also be common to other afferent systems. The $P2X_1R$- and $P2X_3R$-derived ATP-gated ion channels are both sensitive to α,βMeATP and exhibit desensitisation. The $P2X_5R$ subunit is thought to contribute to heteromultimeric ATP-gated ion channels expressed in the mesencephalic trigeminal

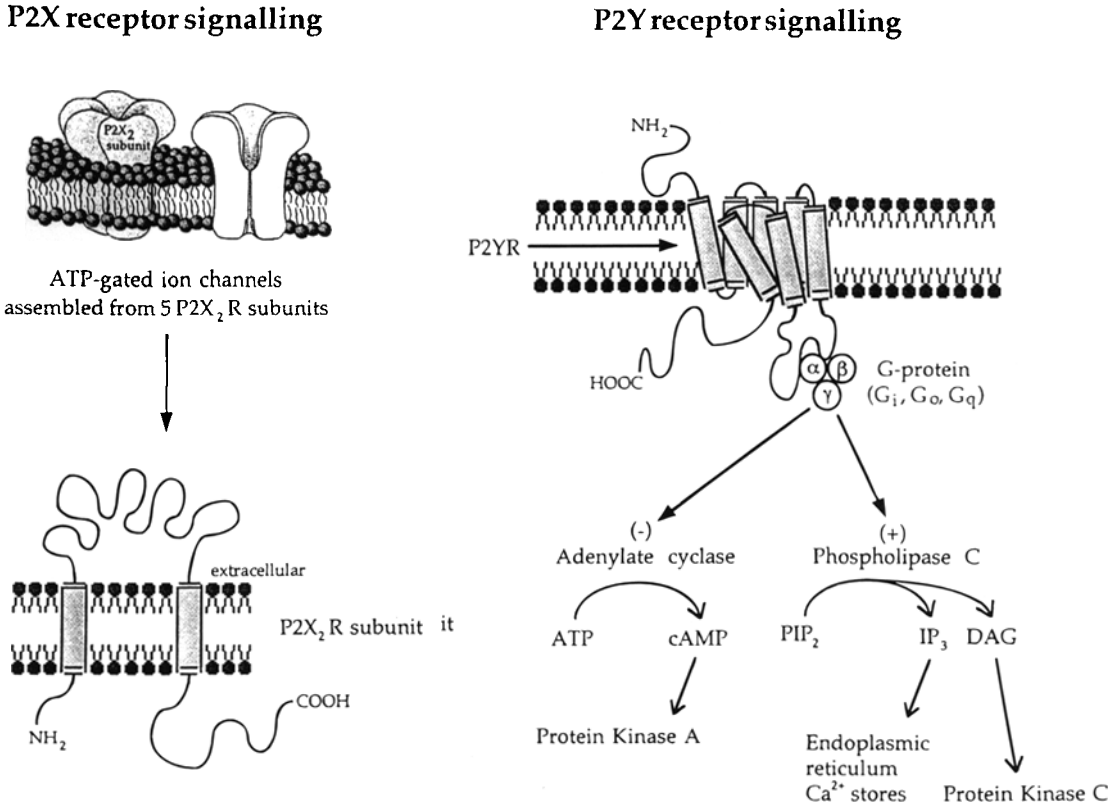

Fig. 1. Model of P2X receptor and P2Y-receptor-signaling mechanisms. The ATP-gated ion channel assembled as a pentamer of P2X-receptor subunits is modeled after (Nicke et al., 1996; Brake et al., 1994; Surprenant, 1995). The P2YR G protein-coupled receptor-signaling pathways are modeled after (Boader et al., 1995; Barnard et al., 1994; Webb et al., 1996a).

nucleus of the brain, whereas the homomultimer is sensitive to block by PPADS but not by suramin (Collo et al., 1996). The most recently cloned subunit, $P2X_7R$, is thought to form an ATP-gated ion channel by homomultimeric assembly to generate a large, nonselective ATP-gated pore with a permeability to substances approaching 1 kDa in size (Surprenant et al., 1996). This receptor, previously classified as the P_{2z} purinoceptor, has been implicated in immunological cytolysis (Surprenant et al., 1996).

P2Y Receptors

Currently nine subtypes of P2YR have been identified at the molecular level (Purine Club Webb site URL: http://mgddk1.niddknih.gov:8000/). As indicated, these receptors are members of the seven-transmembrane spanning domain receptor family (Fig. 1). The degree of homology with other members of this G protein-coupled receptor family, such as the muscarinic receptors and the β-adrenergic receptors is very low (<25%) (Barnard et al., 1994). Binding of ATP activates, via pertussis toxin sensitive (G_o, G_i) or pertussis toxin-insensitive (G_q) G proteins, the phospholipase C_β (PLC)-inositol (1,4,5) trisphosphate (IP_3) pathway that stimulates release of stored Ca^{2+} with subsequent regulation of protein phosphorylation and direct modulation of Ca^{2+}-dependent membrane conductances. Alternative P2Y-receptor pathways involving the PLC-diacylglycerol (DAG; formed with IP_3 by PCL hydrolysis of phosphatidylinositol 4,5-bisphosphate) activation of protein kinase C, or P2Y receptor-G_i inhibition

of adenylyl cyclase, have also been reported (Boader et al., 1995; Ogawa and Schacht, 1994; Webb et al., 1996a).

Mechanisms of Extracellular Nucleotide Signal Termination: Ectonucleotidases

Ectonucleotidases such as ecto-ATPase and ecto-5′ nucleotidase terminate the P2-receptor signaling mechanisms via hydrolysis of ATP through a cascade of intermediates (ADP and AMP) into adenosine and inosine; all purines with variable functional activity (Zimmerman, 1992). The membrane-bound ectonucleotidase family of enzymes are widespread in tissue (Plesner, 1995) and have been linked to the termination of P2 purinoceptor neurohumoral signaling and adenosine recovery in association with adenosine transporters (Plesner, 1995; Lin and Guidotti, 1989; Kennedy et al., 1996). The distribution and kinetics of ectonucleotidase activity makes it unlikely that ATP would remain unhydrolyzed systemically, rather ATP and other nucleotides act as transmitters or have local autocrine or paracrine function. Originally characterized in terms of their ligand specificity and divalent-cation dependency (Manery et al., 1984; James and Richardson, 1993; Ziganshin et al., 1995), the molecular characterization of these enzymes is limited and convoluted. An ecto-ATPase was first cloned from rat liver (Lin and Guidotti, 1989). This ecto-ATPase was later shown to be a cell-adhesion molecule (cell-CAM105) (Lin et al., 1991) and a member of the carcinoembryonic-antigen (CEA) gene family (Knowles et al., 1995). Subsequently, alternately spliced isoforms of what is known as the "rat liver ectoATPase/C-CAM/pp120/bile acid transporter" gene (Knowles et al., 1995) were identified (Culic et al., 1992; Najjar et al., 1993). However, more recent cloning experiments have questioned the putative ecto-ATPase function (Knowles et al., 1995). The molecular structure of an ecto-ATPase was identified in 1996. A report of homology between a newly isolated potato tuber ATP-diphosphohydrolase and human CD39, a lymphoid-cell-activation antigen (Handa and Guidotti, 1996) was followed by the demonstration that the human CD39 gene expressed Ca^{2+}-Mg^{2+}-dependent ectoATPase activity in COS-7 cells (Wang and Guidotti, 1996). Sequence information for both human and murine CD39 (ectoATPase) proteins are available. CD39 (ecto-ATPase) is a 510-amino acid glycoprotein (1.8 kb cDNA) containing transmembrane regions at the N- and C-termini and a hydrophobic folding within the extracellular region (Maliszewski et al., 1994).

The ecto-5′-nucleotidase, which dephosphorylates 5′-AMP to adenosine, has also been cloned. Protein sequences from rat liver, human placenta, and electric ray brain show considerable homology and the 548 amino acid proteins (2.8 kb cDNA) are believed to form anchored dimers in the cell membrane (Zimmerman, 1992; Misumi et al., 1990).

Most recently, a novel signal termination mechanism has also been proposed for nucleotide neurotransmission involving the corelease of soluble ectonucleotidases with ATP (Todorov et al., 1997). Such a system may also coexist alongside the membrane-bound ectonucleotidases in inner-ear tissue.

Extracellular Purines and Pyrimidines Affect Inner Ear Function

Extracellular ATP was first reported to influence inner-ear function during screening experiments for neurotransmitter substances by Bobbin and Thompson in 1978 (Bobbin and Thompson, 1978). Superfusion of the guinea-pig cochlear perilymphatic compartment with ATP caused an inhibition of the auditory-nerve compound action potential (CAP) and the cochlear microphonic (CM). The CM is an index of the sound transduction current flowing across the apical surface of the sensory hair cells. No further analysis of extracellular nucleotide activity in the inner ear was under-

taken until a decade later when ATP was reported to increase firing in the *Xenopus* lateral line bioassay preparation (Mroz and Sewell, 1989). At a cellular level, evidence for a direct activation by ATP of a Ca^{2+}-permeable nonselective cation conductance (P2X receptor) and P2Y-receptor-mediated release of intracellular Ca^{2+} in isolated guinea-pig and chick sensory hair cells was obtained by whole-cell voltage-clamp and Fura-2 ratiometric Ca^{2+} imaging (Ashmore and Ohmori, 1990; Nakagawa et al., 1990; Shigemoto and Ohmori, 1990). These studies mark the onset of intense interest in extracellular nucleotide signaling in the inner ear, with the consequence that our knowledge of the influences of extracellular ATP on cellular physiology in the cochlea currently rivals any other system. The major features of this system are outlined in Fig. 2 and are reviewed in detail below.

Extending Bobbin and Thompson's preliminary guinea-pig cochlear perfusion studies (1978), Kujawa et al. (1994a) applied P2-receptor agonists in perilymph at low to moderate sound levels and observed an inhibition of CAP, CM, and the cubic (2f1-f2 = 8 kHz) distortion product otoacoustic emission (DPOAE) (considered a selective measure of OHC function, *see* later). The effects were most pronounced at low to moderate levels of sound stimulation with ATP-γ–S (30–300 μ*M* range) being approx 10-fold more effective than ATP. Whole-cell voltage-clamp recordings of isolated guinea-pig outer hair cells (OHC) showed an opposite effect, with ATP-γ–S being less potent than ATP at eliciting inward currents (via P2X receptors). Overall, the inhibitory effects of perilymphatic perfusion of ATP and related analogs on electrocochleography were attributed largely to a P2Y-receptor mechanism acting at a variety of sites including sensory hair cells and auditory (spiral ganglion) neurons. The endocochlear potential (EP), a positive biopotential between the endolymph in scala media and the perilymph-containing scalae, increased transiently during perfusion with 100 μ*M* ATP-γ–S (Kujawa et al., 1994a). This is in keeping with the reported preferential action of ATP-γ–S on P2Y receptors in the sensory epithelium of the cochlea to stimulate inositol phosphate production (Niedzielski and Schacht, 1992; Ogawa and Schacht, 1993; Ogawa et al., 1994). An independent study by Thorne et al. (1993; 1995) involving perilymphatic perfusion of the guinea-pig cochlea with ATP (100 μ*M*) produced a 12 dB decline in CAP threshold without affecting CM at frequencies above 4 kHz.

Application of P2-receptor antagonists to the perilymphatic compartment of the guinea-pig cochlea support an endogenous role for extracellular nucleotides in the cochlea (Kujawa et al., 1994b). The antagonists, including suramin, cibacron blue, and basilen blue (the latter two are isoforms of reactive blue 2; RB2), produced variable effects on the electrophysiological and mechanical coupling (DPOAE) of the organ of Corti, whereas the CM was not affected. This may be in keeping with the likely compartmentalization of the P2-receptor expression on the hair cells. Considerable evidence detailed later suggests that the ATP-gated ion channels are localized to the stereocilia of the hair cells and therefore face the endolymph in scala media. Whereas Ca^{2+}-imaging studies suggest P2Y receptors are localized to the basolateral membrane, at least for guinea-pig cochlear OHC (Ashmore and Ohmori, 1990).

Application of suramin to scala tympani in the perfused guinea-pig cochlea reversibly blocked the decline in the quadratic (f2-f1 = 1.25 kHz) DPOAE that occurs with continuous sound stimulation (Bobbin et al., 1997). This suggests that sound increases endogenous ATP levels and activation of P2-receptor mechanisms on OHC and/or Deiters cells that inhibit the "cochlear amplifier" (*see* later section on the effect of ATP on OHC electromotility). This study also reported that cibacron blue, but not suramin, affected OHC and Deiters-cell K^+ conductances independent of any purinergic action, suggesting that earlier suppression of cubic-distortion products (Kujawa et al., 1994b) may have been via direct action on these conductances and not via block of endogenous extracellular nucleotides.

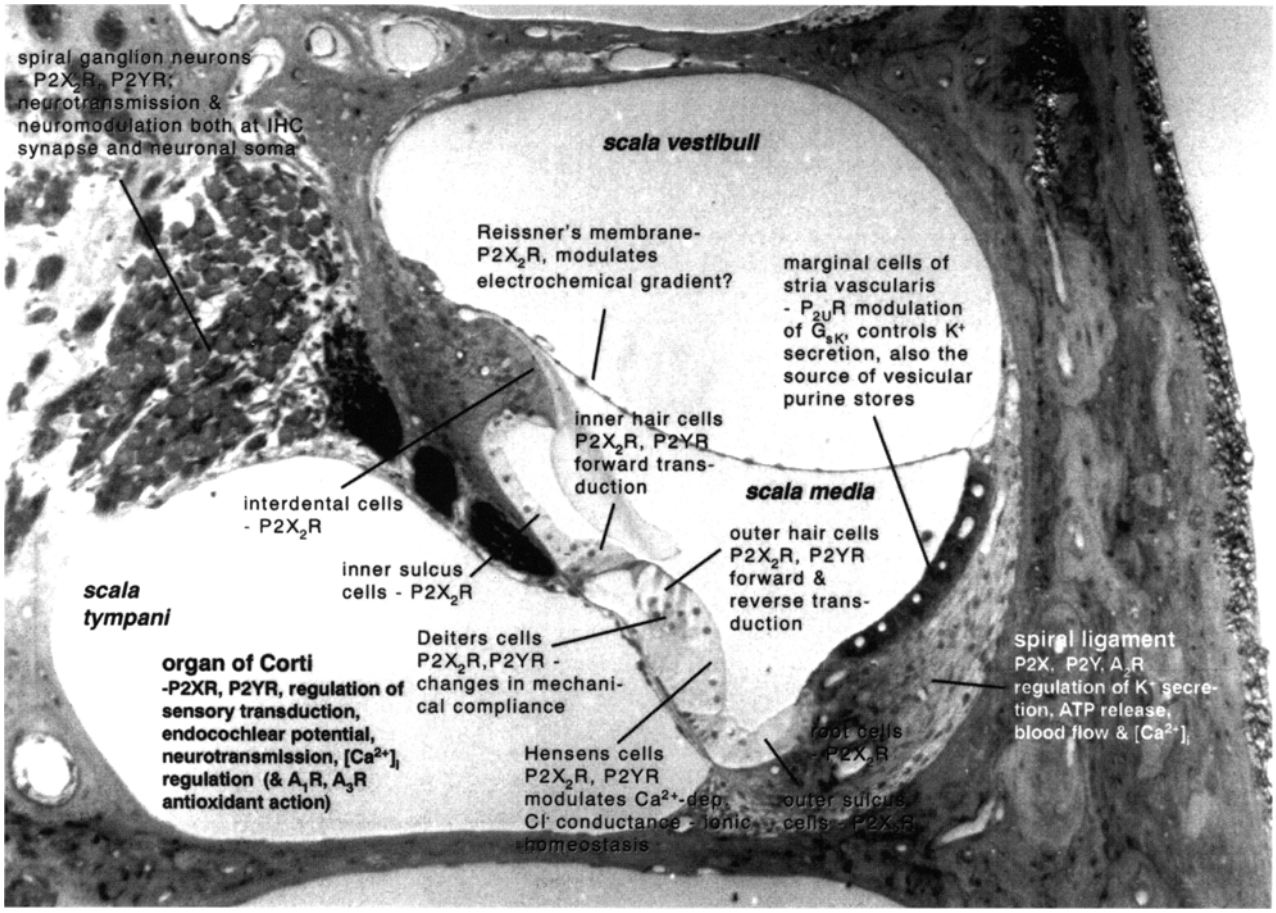

Fig. 2. Summary of signaling pathways for extracellular nucleotides and nucleosides in the cochlea.

No significant effects on CAP, brain stem auditory evoked potential or endocochlear potential were apparent with perilymphatic perfusion or round window application of the nucleoside adenosine in the cochlea (Kujawa et al., 1994a; Ramkumar et al., 1995a) although biochemical and RT-PCR evidence for P1 receptor expression (A_1 and A_3 receptors) in cochlear tissues has been linked with an increased activity of antioxidant enzymes in cochlear and vestibular tissues in response to stressors (Ramkumar et al., 1994, 1995a,b; Ford et al., 1997).

Application of ATP into cochlear endolymph (scala media) has a profound inhibitory effect on CM, as well as reducing the endocochlear potential (EP) (Muñoz et al., 1995). These effects were blocked by suramin and RB2, suggesting that P2 receptors mediate a humoral action of extracellular ATP in scala media. The EP within scala media (up to +80 mV), believed to be generated by a combination of ion transport enzymes located at the interface between the marginal and intermediate cells of the stria vascularis (Fernendez and Hinojosa, 1974; Wangemann, 1995), provides the electrical gradient for the sound-transduction current (predominantly K^+) entering via the mechano-electrical transduction channels on the tips of the cochlear hair-cell stereocilia (Hudspeth, 1989; Pickles and Corey, 1992). The ATP-induced drop in EP may reflect a direct electrical shunt across the cochlear partition produced by the ATP-activated ionotropic conductance

in hair cells and other cells lining scala media, and include action by ATP on the mechanism generating EP in scala media.

A role for purinergic signaling is also apparent in the vestibular system based on studies utilizing the isolated semicircular-canal preparation. Adenosine has a small neuromodulatory effect in the frog vestibular system, decreasing spontaneous discharge of the ampullary nerve when applied in m*M* concentrations to the perilymph (Bryant et al., 1987). The P1-receptor antagonist theophylline (50 μ*M*) and adenosine deaminase had the opposite effect, whereas dipyridamole (5 μ*M*), an adenosine-uptake inhibitor, reduced firing rate, in support of an endogenous action of adenosine. Opposing the putative inhibitory action of adenosine, application of ATP and related agonists to the perilymphatic surface of the isolated semicircular canal has revealed one of the most sensitive P2-receptor responses yet reported (Aubert et al., 1994, 1995). ATP and 2MeSATP increased spontaneous vestibular afferent nerve discharge at perilymphatic concentrations as low as 10^{-12} *M*, and the dynamic range of the mechanically evoked discharge was compressed in the presence of ATP. In contrast, the P2X agonists α,βMeATP and β,γMeATP were ineffective, even at 1 m*M*. Superfusion of the isolated semicircular canal with perilymph containing the P2-receptor antagonists suramin and RB2 provide evidence for endogenous extracellular nucleotide signaling, mediated by release of ATP. Both spontaneous and mechanically evoked afferent nerve activity was inhibited by the P2-receptor antagonists with K_d values in the range 10–100 μ*M* (Aubert et al., 1995). Decreases in spontaneous activity of 25% were reported with as low as 1 μM concentration, also antagonizing the ATP-evoked potentiation of spontaneous afferent discharge in a dose-dependent manner. The nucleotide pyrophosphatase, which would hydrolyze extracellular ATP to AMP, had comparable antagonistic actions both to putative endogenous ATP and to exogenously applied ATP. These data are compatible with a proposed P2Y receptor-mediated effect in vestibular sensory epithelium, where a pertussis-toxin insensitive G protein is coupled to a PLC pathway to stimulate IP_3 production in vestibular-sensory epithelium (Ogawa and Schacht, 1993, 1994). Whereas the P2X-selective agonist α,βMeATP produced an increase in inositol phosphate production in vestibular-sensory epithelium, adenosine did not, suggesting a lack of involvement of P1 receptors. ATP-activated inward currents and secondarily activated K^+ conductances have been recorded from both type I and type II hair cells isolated from guinea-pig (Rennie and Ashmore, 1993) and rat (Taylor and Housley, 1992; Housley et al., 1994) crista ampullaris.

Source of Extracellular Nucleosides and Nucleotides

The presence of endogenous extracellular nucleosides and nucleotides have been detected in inner-ear tissues. Electrical stimulation of the isolated frog semicircular canal, known to stimulate release of neurotransmitter, resulted in adenosine accumulation in the perfusion solution (Bryant et al., 1987). An analogous result was also reported for K^+-depolarized guinea-pig organ of Corti. Whereas vesicular release of adenosine cannot be ruled out, production of adenosine may have arisen from the breakdown of ATP by ectonucleotidases as reported in the guinea-pig cochlea (Vlajkovic et al., 1997).

Using the acridine compound quinacrine dihydrochloride, which enters cells and fluoresces in the presence of purine compounds, White et al. (1995) demonstrated the presence of putative purine-rich vesicles within marginal cells of the guinea-pig stria vascularis. Confocal and transmission electron microscopy demonstrated that these 100-nm diameter electron-dense vesicles were localized within 4 μm of the luminal surface. In support of release of ATP into endolymph, endogenous ATP has been measured at 13 n*M* in endolymph and 10 n*M* in perilymph of the guinea-pig cochlea using the sensitive luciferin luciferase bioluminescence assay (Muñoz et al., 1995).

These measurements, in the face of demonstrated ectonucleotidase activity (Vlajkovic et al., 1997, 1996, 1998), likely reflect endogenous extracellular nucleotide levels and are close to the threshold sensitivity for gating by ATP of an amiloride-sensitive nonselective cation conductance reported for isolated guinea-pig cochlear OHC (Housley et al., 1992). Brief hypoxia caused a doubling of perilymphatic ATP concentration in vivo (Muñoz et al., 1995). The concentration of ATP at the site of release may transiently reach considerably higher levels given the m*M* concentration of ATP typically stored within vesicles (Dubyak and El-Moatassim, 1993). Consistent with the vesicular storage and release of ATP, Ca^{2+}-dependent release of ATP, independent of changes in K^+ concentration, have been recorded from isolated gerbil organ of Corti (Wangemann, 1996).

Purinoceptor Signaling in Sensory and Secretory Epithelia of the Inner Ear

The inner ear sensory epithelium includes the sensory hair cells and supporting cells. The vestibular labyrinth contains the sacculus, utriculus, and orthogonally opposed cristae ampullarii, whereas in the cochlea, the organ of Corti with its sensory hair cells lies across the partition between scala media and scala tympani. The sensory transduction processes for hearing and balance rely upon the maintained electrochemical gradient across the hair cells that is generated by the dark-cell vestibular secretory epithelium and the marginal cells of the stria vascularis in the lateral wall of the cochlea. There is considerable evidence to support expression of both P2X and P2Y receptors within these tissues.

P2 Receptor Expression in Cochlear Outer Hair Cells

The mammalian cochlear outer hair cells (OHC) are the most extensively investigated element of nucleotide signaling in the inner ear. The OHC lie radially as three rows within the sensory epithelium (organ of Corti). When considering the functional significance of purinergic influence on these cells it is important to note the unique role the OHC play in the sound-transduction process. Receptor potential-driven OHC electromotility generates an active mechanical feedback to the cochlear partition (reverse transduction) that confers sensitivity and frequency selectivity to the sound-transduction process (Brownell et al., 1985; Mountain, 1986; Ashmore, 1994, 1987; Santos-Sacchi, 1992; Ashmore and Kolston, 1994). This electromotility arises from the voltage-dependent conformation changes of a unique integral membrane protein that assembles in an array resembling gap-junction plaques in the lateral cell membrane (Ashmore, 1994; Kalinec et al., 1992). The electromotility is independent of $[Ca^{2+}]_i$ or $[ATP]_i$ and follows the sound-induced oscillations of the transduction potential and the inductive changes in OHC membrane potential produced at high acoustic frequencies (Dallos and Evans, 1995). The adjacent inner hair cells (IHC), which lie medially, are the primary (forward) transducers, releasing neurotransmitter (presumed to be glutamate [Kataoka and Ohmori, 1994; Ohmori, 1995] at the IHC-spiral ganglion neuron synapse). ATP-induced changes in OHC membrane potential are therefore likely to directly affect the OHC electromotility, which because of direct mechanical coupling via the reticular lamina, influences the sensory transduction process at the IHC and thus the neural outflow to the auditory nerve. The cholinergic olivocochlear efferent innervation to the OHC provides a central control of hearing sensitivity (Desmedt and Lagrutta, 1963; Norris and Guth, 1974; Housley and Ashmore, 1991; Bobbin, 1996; Guth and Norris, 1996). There is unequivocal data supporting purinergic modulation of the OHC membrane potential, interestingly this evidence supports both a direct action mediated by ATP-gated ion channels (P2X receptors) and an indirect action via P2X- and P2Y-receptor-mediated alterations in

$[Ca^{2+}]_i$, affecting Ca^{2+}-dependent membrane conductances.

Prompted by the possible role of extracellular ATP as a cotransmitter of the cholinergic efferent innervation of the OHC, Nakagawa et al. (1990) demonstrated an ATP-activated nonselective cation conductance using whole-cell voltage-clamp of isolated guinea-pig cochlear OHC. This conductance was relatively nondesensitizing, demonstrated inward rectification and had a high Ca^{2+} permeability (based on a right shift in E_{rev} with elevated $[Ca^{2+}]_o$. Whereas the K_d for ATP was 12 μM, α,βMeATP was ineffective at activating this conductance. The lack of desensitisation (Ashmore and Ohmori, 1990; Nakagawa et al., 1990; Housley et al., 1992; Kakehata et al., 1993); and the pharmacological profile of the OHC ATP-activated conductance, including insensitivity to α,βMeATP and adenosine (Nakagawa et al., 1990) are compatible with a $P2X_2R$ classification.

Both an immediate entry of Ca^{2+} into the OHC via ATP-gated ion channels and a slower release of stored Ca^{2+} (rise time, 50s) were evident in a combined whole-cell voltage-clamp-Fura-2 Ca^{2+}-imaging study by Ashmore and Ohmori (1990). The $Ca^{2+}{}_o$-dependent rapid rise in intracellular Ca^{2+} occurred in the apical region of the OHC, whereas the slower $Ca^{2+}{}_o$-independent rise attributable to release of stored Ca^{2+} was more prevalent in the basal region. Whereas 25 μM ATP was sufficient to depolarize OHC by 20 mV, the contribution of voltage-dependent Ca^{2+} channels to the rapid Ca^{2+} influx was shown to be negligible. A subsequent analysis of cholinergic and purinergic Ca^{2+} signaling in OHC has confirmed the apical bias to the ATP-induced Ca^{2+} influx, whereas the acetylcholine (ACh)-mediated Ca^{2+} influx was localized to the basal pole of these cells (Ashmore et al., 1992; Xi et al., 1995), the location of the cholinergic membrane conductance (Housley and Ashmore, 1991). Other Ca^{2+}-imaging studies of guinea-pig OHC have reported only the $[Ca^{2+}]_o$-dependent (Ikeda et al., 1991) and $[Ca^{2+}]_o$-independent (Nilles et al., 1994) ATP-induced increases in $[Ca^{2+}]_i$. Both extracellular and intracellular-derived Ca^{2+} responses were also found in chick-papilla hair cells presented with extracellular ATP along with respective rapid inward current and delayed outward current responses (Shigemoto and Ohmori, 1990).

Focal application of acetylcholine to the base of guinea-pig OHC induces a rapidly desensitizing outward current attributable to the Ca^{2+}-dependent activation of a K^+ conductance (Housley and Ashmore, 1991). This arises from the significant Ca^{2+} permeability of the OHC $\alpha 9$ nicotinic acetylcholine receptor (Elgoyhen et al., 1994; Glowatzki et al., 1995). In contrast, the ATP-activated inward current in guinea-pig cochlear OHC has minimum latency (<10 ms) and maximum amplitude when ATP is focally applied in the region of the stereocilia (Thorne and Housley, 1996; Housley et al., 1992, 1993, 1995a). This observation that the ionotropic P2X receptors are localized to the apical surface of the OHC (Fig. 3), and therefore not associated with the efferent cotransmission, has been further supported by the analysis of the binding of the ATP analog trinitrophenol-ATP (TNP-ATP) (Housley et al., 1993; Mockett et al., 1994). TNP-ATP binds competitively to the ATP binding sites on guinea-pig cochlear hair cells. Under UV excitation, TNP-ATP fluorescences upon binding to OHC and IHC, including the stereocilia, cuticular plate and the lateral cell surface. In the presence of suramin, a competitive antagonist of the P2X receptors, TNP-ATP binding to the stereocilia of OHC was blocked, supporting the localization of the ATP-gated ion channels to this site. In contrast, removal of the divalent cations, which enhanced the ATP-activated inward current, quenched the TNP-ATP labeling of the lateral wall of the hair cells, consistent with the existence of ectonucleotidase binding sites on the perilymphatic surface (Mockett et al., 1994); although the data does not exclude similar binding sites on the apical surface of the OHC. These data are also supported by the detection of an ATP-induced fast onset Ca^{2+} signal in the guinea-pig OHC stereocilia (Ashmore, 1994; Ashmore et al., 1993) and by the imaging of a

nels expressed in cells originating from the more basal (high frequency) region (Housley et al., 1995a; Raybould and Housley, 1997). Based on the average ATP-activated conductance recorded in guinea-pig OHC using a saturating concentration of ATP (2 m*M*), and an estimated unitary conductance of 10 pS at –70mV; derived from the noise analysis of OHC neomycin-blocked ATP-activated nonselective cation conductance ($G_{C,ATP}$) by Lin et al. (1993), an estimate of 16,500 ATP-gated ion channels in basal turn OHC and fewer than 6000 ATP-gated ion channels in apical-turn OHC has been determined by Raybould and Housley (1997). Given the position-dependent variation in expression of ATP-gated ion channels in the guinea-pig cochlea, it is conceivable that expression in the adult rat is confined to the high-frequency encoding OHC, cells difficult to isolate and record from. Alternatively, the location of the ATP-gated ion channels on the tips of the stereocilia may make them more susceptible to damage during isolation in the rat.

The intriguing question is: What is the significance of a basal bias in ATP-gated ion-channel expression in the cochlea? Whereas the answer is not apparent at present, it is important to note that the increase in ATP-gated cation conductance ($G_{C,ATP}$) towards the base of the cochlea is one of a number of reported variations in the expression of OHC proteins. For example calcium-binding proteins such as calbindin and calmodulin are preferentially expressed in OHC from the more apical region of the cochlea (Slepecky, 1995), whereas electrophysiological analysis suggests that OHC electromotile protein density increases towards the base of the cochlea (Kakehata and Santos-Sacchi, 1996). The cholinergic efferent innervation to the OHC that regulates OHC activity is also largely confined to the basal half of the cochlea (Spoendlin, 1985) and OHC isolated from the basal region exhibit the most pronounced nicotinic acetylcholine receptor-mediated (Ca^{2+}-activated) K^+ conductance activation (Housley and Ashmore, 1991). The resting K^+ conductance of the guinea-pig cochlear OHC also increases towards the basal turn region; attributable to increased expression of a negatively activated voltage-dependent K^+ conductance (G_{Kn}), whose activation is also modulated by $[Ca^{2+}]_i$ (Housley and Ashmore, 1992; Mammano and Ashmore, 1995; Preyer et al., 1996; Raybould and Housley, 1997). Thus, G_{Kn} confers a considerable reduction in the OHC membrane-time constant in the basal-turn OHC, reflecting the high-frequency response characteristics of these cells. A by-product of this conductance is a considerable membrane-potential-buffering capacity. Any ATP-mediated reduction in OHC membrane potential in these cells would therefore be in the face of the opposing G_{Kn} conductance. The greater expression of $G_{C,ATP}$ in the basal OHC would therefore overcome this K^+ conductance-mediated membrane-potential (V_m) buffering and generate equivalent depolarization in basal-turn OHC as seen in the apical-turn OHC that have fewer ATP-gated ion channels. These data support a putative humoral action of extracellular ATP, acting via ATP-gated ion channels localized to the apical surface of OHC throughout the cochlea, depolarizing the OHC. In addition to G_{Kn}, a second K^+ conductance whose activation is increased by increased $[Ca^{2+}]_i$ is also expressed in OHC. This conductance, designated G_K by Mammano and Ashmore (1996), is characterized by tetraethyl ammonium (TEA) and 4-aminopyridine (4AP) block and appears to also be highly expressed in basal-turn OHC although proportionally its expression becomes more significant in the more apical (low frequency responding) OHC (Raybould and Housley, 1997; Mammano and Ashmore, 1996; Housley and Ashmore, 1992; Nenov et al., 1997). Whereas half activated at approx –25mV (Mammano and Ashmore, 1996), ATP-mediated increases in $[Ca^{2+}]_i$ may result in additional K^+ buffering via G_K at the resting potential of the OHC. The possible secondary activation of these K^+ conductances via Ca^{2+} entering through the apical ATP-gated ion channels may be limited given the high affinity but low conductance of the OHC ATP-gated ion channel for Ca^{2+} (Mockett

progressive diffusion of ATP-induced Na^+ influx from the stereocilia into the bulk of the OHC (and IHC) cytoplasm (Housley et al., 1997). Most recently immunoperoxidase and immunofluorescence labeling using an antiserum specific for an 18-amino acid segment of the extracellular domain of the $P2X_2$-receptor subunit of the ATP-gated ion channel has confirmed the presence of this protein specifically on the tips of guinea-pig and rat cochlear hair cells, supporting both the imaging and electrophysiological localization data and also supporting the molecular characterization of the receptor (Housley et al., 1996).

Molecular characterization of the ATP-gated ion channels supports expression of the $P2X_2$ receptor and a number of related splice variants, a result that matches well with the pharmacological and biophysical profile of the ATP-activated conductance in guinea-pig cochlear hair cells. Preliminary RT-PCR experiments using isolated rat organ of Corti cDNA libraries indicated that the mRNA for this subunit was present, particularly in the neonatal rat cochlea (Housley and Ryan, 1997; Housley et al., 1995b). Subsequently, riboprobe *in situ* hybridization labeling has localized the $P2X_2R$ mRNA to the adult rat OHC (Housley and Ryan, 1997; Housley et al., 1997) and single-cell RT-PCR using cDNA reverse transcribed form from neonatal rat OHC has provided evidence for expression of different splice variants of the $P2X_2R$ mRNA within individual cells (Brändle et al., 1995; Brändle, 1996; Glowatzki et al., 1997). The splice variant designated $P2X_{2-2}R$ by Brändle et al. (1997) has a 207 bp deletion corresponding to a loss of 69 amino acids in the intracellular C-terminal domain of the translated protein (Brändle et al., 1997). When $P2X_{2-2}R$ was expressed as a homo-oligomer in *Xenopus* oocytes, a more rapid desensitization was detected than that seen in the homooligomeric assembly of $P2X_2R$ (Brändle et al., 1997). The alternate splicing of the $P2X_2R$ may confer functional diversity in the ATP-gated ion channels in OHC via heteromultimeric assembly of the $P2X_{2-n}$ receptor subunits, and indeed other P2XR subunits, into

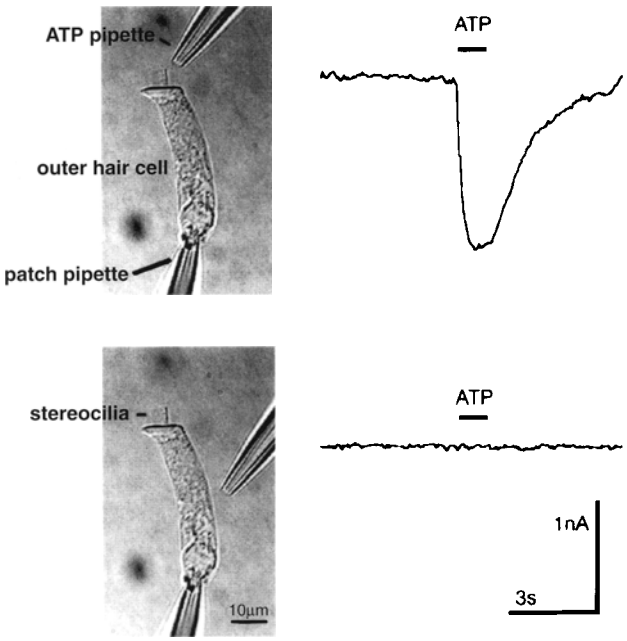

Fig. 3. Electrophysiological localization of ATP-gated ion channels to the region of the guinea pig cochlear OHC stereocilia. The OHC was voltage-clamped at –60 mV and pressure pulses of 10 μM ATP were applied to various regions of the OHC membrane. Only application of ATP at the level of the stereocilia elicited an inward current response (1.5 nA).

ATP-gated ion channels. The extent to which this occurs requires investigation across species.

While a molecular analysis of gene expression provides some indication of the sites and extent of involvement of ATP-gated ion channels (P2X receptors) in cochlear physiology, a molecular physiological approach has been advocated (Housley and Ryan, 1997). Essentially, a multidisciplinary approach adds confidence and functionality to the data. For instance, apparently contrary to the molecular-biological evidence for P2XR expression, application of ATP to isolated adult rat OHC does not affect membrane conductance (Chen et al., 1997). One potential explanation for this may be a differential expression of ATP-gated ion channels on OHC in different regions of the cochlea. In guinea-pig OHC there is a considerable increase in the number of ATP-gated ion chan-

et al., 1994) and the low Ca^{2+} concentration present in endolymph (approx 30 μ*M*; Bosher and Warren, 1978). ATP-induced increases in OHC Ca^{2+}-concentration may also arise indirectly via voltage-gated Ca^{2+} channels, as demonstrated by nifedipine block (Nilles et al., 1994). L-type Ca^{2+} channels have been characterized in OHC (Nakagawa et al., 1991) and recent evidence has shown that ATP variably modulates Ca^{2+} influx through these channels (Chen et al., 1995b). Guinea-pig cochlear OHC, which were studied under voltage-clamp, with a selection criterion that they exhibited minimal ATP-activated conductance (via P2X receptor), in some cases had an enhanced L-type Ca^{2+} current and in other cases a reduced L-type Ca^{2+} current in the presence of ATP (Chen et al., 1995b). This supports indirect ATP action, possibly via a P2Y-receptor pathway, to cause intracellular regulation (phosphorylation) of L-type Ca^{2+} channels. This P2Y-receptor pathway in OHC is clearly indicated on the basis of the slow rise in Ca^{2+} in calcium-imaging studies, some of which have been performed under simultaneous voltage-clamp (Ashmore and Ohmori, 1990; Ashmore et al., 1992; Dulon et al., 1991). The G proteins coupling P2Y receptors to PLC in the inner ear have yet to be established. The OHC P2Y-PCL-IP_3 receptor-signaling pathway, which is insensitive to UTP, appears to act via a pertussis toxin-sensitive G protein (Nilles et al., 1994). Interestingly this contrasts with the apparent pertussis toxin-(PTX) and cholera toxin-insensitive G protein associated more generally with the P2 receptor and mAChR-mediated release of inositol phosphates in the sensory and secretory epithelia of the inner ear (Ogawa and Schacht, 1994). However, the PTX-block of the OHC P2Y-receptor pathway was established with a relatively low concentration of ATP (50 μ*M*) (Nilles et al., 1994), whereas the sensitivity of the P2Y-receptor G protein to PTX may decrease with increasing agonist concentration (Ogawa and Schacht, 1994).

The molecular characterization of OHC P2Y-receptor expression is currently unavailable although RT-PCR experiments in guinea-pig and rat support expression of both $P2Y_1R$ (Young, 1997) and $P2Y_2R$ (Järlebark et al., 1995, 1996) in the organ of Corti.

Guinea-pig cochlear OHC possess a voltage-insensitive nonselective cation conductance that is modulated by the intracellular Ca^{2+} concentration (Housley and Ashmore, 1992). In an analogous fashion to the indirect modulation by ATP of OHC Ca^{2+}-activated K^+ conductances, the opening of a 25pS nonselective cation channel ($G_{C(Ca)}$) detected in cell-attached and cell-detached patch-clamp recordings from OHC basolateral wall, was enhanced by P2X-receptor agonists β,γMeATP and 2MeSATP (Abbeele et al., 1996). Given the antagonism of this slow-onset ATP-enhanced $G_{C(Ca)}$ activity by suramin but not by the P2Y-selective antagonist Reactive Blue 2 and the K_d of approx 10 μ*M* ATP, the enhanced $G_{C(Ca)}$ activity may be attributable to a second messenger action of Ca^{2+} accumulating following either entry via ATP-gated ion channels or P2Y-receptor-mediated release of stored Ca^{2+}, although the delayed onset matches that latter. Interestingly, the indirect activation by extracellular ATP of $G_{C(Ca)}$ was reduced in the presence of adenosine and the adenosine A_2 agonist 5′-*N*-ethylcarboxyamidoadenosine (NECA) and 8-Br-cAMP, but not affected by the A_1 agonist N^6-cyclohexyladenosine (CHA) in cell-attached patches. Using inside-out patches, the catalytic subunit of protein kinase similarly inhibited ATP-activated $G_{C(Ca)}$ activity, supporting A_2-receptor-induced downregulation of the $G_{C(Ca)}$ channels by an adenylyl cyclase-mediated cAMP-dependent phosphorylation. Comparable regulation of the other OHC basolateral membrane conductances by P1- or P2-receptor-mediated protein phosphorylation mechanisms may well emerge in the future.

In summary, the action of extracellular ATP on OHC is highly complex (Fig. 4). Direct gating of cation channels (P2X receptors) localized to the stereocilia leads to depolarization via a K^+ influx from scala media. The high Ca^{2+} permeability of this channel leads directly to increased $[Ca^{2+}]_i$, despite the low Ca^{2+} concentration in endolymph. P2Y-receptor activation on the basolateral surface also leads to a rise in intra-

cellular Ca^{2+} via a phospholipase C-IP_3 pathway. Ca^{2+} acts as a second messenger at three different conductances, G_{Kn}, G_K, and $G_{C(Ca)}$. P2Y-receptor-mediated changes in $Ca^{2+}{}_i$ also affect Ca^{2+}-dependent protein kinases, altering $G_{Ca,L}$ phosphorylation, mimicked by A_2-receptor-mediated downregulation of $G_{C(Ca)}$. P2 receptor-induced activation of the Ca^{2+}-dependent membrane conductances leads to a reduction in input resistance, impacting significantly on the electrical filter properties of the OHC membrane. The resulting membrane potential will depend upon the balance of expression levels and activation of the various basolateral membrane conductances, all impacting on the OHC electromotility (*see* below). Finally, sufficient depolarization may lead to additional voltage-dependent Ca^{2+}-channel activation precipitating further Ca^{2+}-mediated intracellular signaling cascades. Ectonucleotidase enzymes, likely to be present on both the endolymphatic and perilymphatic surfaces of the OHC, offer a signal-termination mechanism as well as producing adenosine that will act on the A_2 receptor and provide a source for purine recovery by the cells.

Evidence that Extracellular ATP Modulates OHC Motility

Analysis of changes in OHC shape in vitro in response to alterations in membrane potential and intracellular Ca^{2+} have revealed both Ca^{2+}-dependent and Ca^{2+}-independent shape changes are possible (*see* Schacht et al., 1995 for a review). Depolarization-induced osmotic changes have been reported (Zenner et al., 1985; Dulon et al., 1987, 1988; Brownell, 1990) that shorten the OHC. In contrast, elevation of $[Ca^{2+}]_i$, produced by permeabilizing the cells using Ca^{2+}-ionophores or Ca^{2+} influx through voltage-sensitive Ca^{2+} channels, has elicited elongation, shortening, or tilting of the cells, depending upon the different experimental circumstances; with little evidence that neurotransmitter-induced alterations in $[Ca^{2+}]_i$, including those produced by acetylcholine and ATP, are sufficient to activate the hair-cell contractile proteins (Schacht et al., 1995; Bobbin et al., 1990).

Given the propensity for guinea-pig OHC to rapidly depolarize after isolation (Housley and Ashmore, 1992) and the inward rectification of $G_{C,ATP}$, the ability to record ATP-induced changes in OHC shape in vitro are compromised. However, as indicated, increasing $[K^+]_o$ can induce such changes in OHC shape via a K^+ influx because of the alteration in the E_K, thus the mechanism is valid. In vivo, a sudden activation of $G_{C,ATP}$ by a rise of extracellular ATP in scala media would result in K^+ influx via the ATP-gated ion channels and hence at least a temporary electro-osmotic imbalance in these cells. Before we can estimate the extent to which $G_{C,ATP}$ will be activated, in particular OHC, we clearly need to measure the physiologically relevant changes in extracellular ATP concentration that occur in scala media, including the kinetics of such changes, under physiological and pathophysiological stimuli such as noise and ischemia.

Extracellular ATP-induced changes in OHC shape (volume) have been detected using both current-clamp and voltage-clamp in isolated OHC (Housley et al., 1995a). The voltage-clamp is electro-osmotically neutral, thus cation entry via ligand-gated ion channels, driven by the electrochemical gradient, causes a local imbalance in ion distribution that can be compensated by membrane transport mechanisms, standing membrane conductances, and slow dialysis via the recording pipet. Nevertheless, the extent of the ATP-activated conductance in OHC is so extensive, particularly in the smaller OHC isolated from the basal region of the cochlea, where $G_{C,ATP}$ may be in excess of 200 nS (Raybould and Housley, 1997) that temporary osmotic gradients are established. This leads to an influx of H_2O into the OHC over a period of seconds and a volume-loading that; in many cases, causes cell rupture (Housley et al., 1995a). The resulting shortening of the OHC could be extrapolated to a mechanical bias in the basilar membrane, which would alter the mechanical tuning of the cochlea (Dallos, 1996), whereas the ATP-

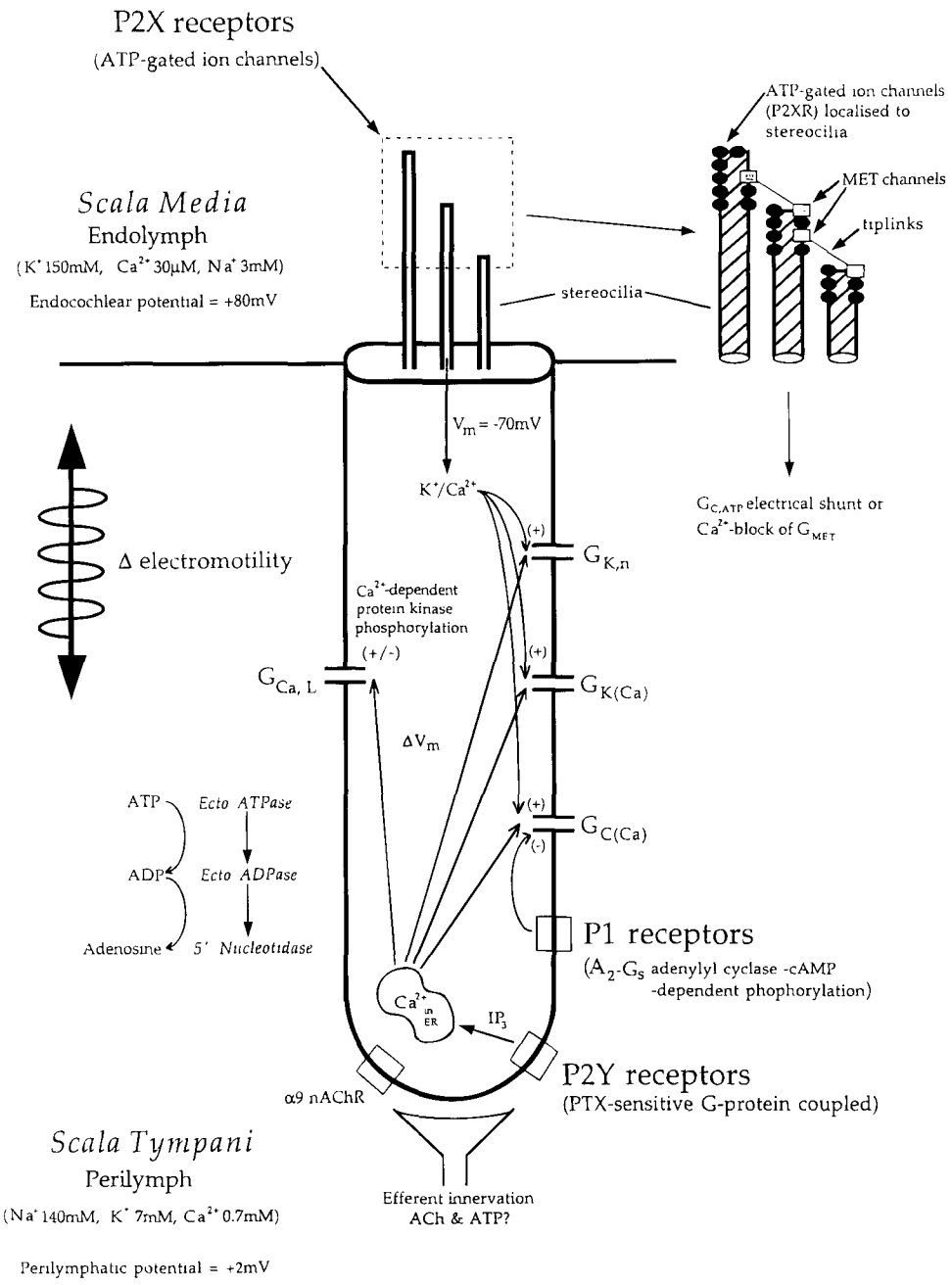

Fig. 4. Summary of nucleotide-signaling pathways in the cochlear outer-hair cell. ATP-gated ion channels are colocalized but outnumber the mechanoelectrical transduction channels on the stereocilia by up to 150:1. Changes in membrane currents affect the membrane potential that directly modulates the electromechanical feedback (reverse transduction) of the OHC. The membrane potential and input resistance of the OHC is also determined by the activation of a number of types of ion channel present on the lateral and basal regions of the cells. These channels are sensitive to changes in $[Ca^{2+}]_i$ that may arise from Ca^{2+} entry via the apically located P2X receptors or the P2Y receptor-mediated release of stored $[Ca^{2+}]$. The P2Y receptors are likely to be located at the basal pole, in association with the cholinergic efferent innervation. Thus P2X receptors may directly influence the sound transduction process, whereas P2Y receptors may mediate either efferent neurotransmission or respond to changes in ATP levels in the space of Nuel that surrounds the lateral wall of these cells. Ectonucleotidases would rapidly hydrolyse ATP to adenosine for reuptake into the OHC and also for possible action at adenosine receptors.

induced increase in OHC turgor pressure has been shown increase the efficiency of coupling of the electromotile proteins, producing an increase in gain of the voltage-length converter (Housley et al., 1995a). This turgor-pressure coupling of the OHC electromotile proteins to the underlying cytoskeletal elements required to vector the force into a longitudinal direction within the OHC has been previously directly demonstrated (Brownell and Shehata, 1991; Santos-Sacchi, 1991).

The in vitro analysis of ATP-induced OHC volume-loading has recently been extended to consider the question of whether extracellular ATP may contribute to sensory-cell loss in the cochlea under stresses such as loud noise. A combination of in vivo perilymphatic perfusions of high (m*M*) concentrations of ATP and in vitro studies of isolated OHC was performed that demonstrated electrophysiologically and by DPOAE that sensory cells were nonfunctional 2–3 wk after superfusion of ATP (Chu et al., 1997). In vitro, μ*M* ATP concentrations caused cell shortening, whereas m*M* ATP concentrations produced rapid cell lysis (Chu et al., 1997). In guinea-pigs exposed to moderate sound levels (65 dB SPL, 1.1–2 kHz, for up to 11 d), subsequent whole-cell voltage-clamp analysis of isolated OHC demonstrated a decline in ATP-activated inward currents in cells smaller than 70 μ*M* (Chen et al., 1995a). The largest OHC, presumably from the apical (low frequency) region of the cochlea demonstrated an enhanced $G_{C,ATP}$. These data are consistent with either changes in expression of the ATP-gated ion channels in OHC, physical damage, or an as yet undetermined change in the cytoplasmic regulation of ATP-gated ion channel activity.

Putting aside the question of Ca^{2+}-induced slow motility, and electro-osmotically derived cell shape changes, there is evidence for a physiologically significant influence on fast motility produced by extracellular ATP (Housley et al., 1995a). In current-clamp experiments in isolated OHC, extracellular ATP depolarized membrane potential by more than 30 mV from approx –60mV (Ashmore and Ohmori, 1990; Housley et al., 1992). This would have the effect of shifting the OHC electromotility, or reverse transduction (Ashmore, 1994), to a higher gain since the nonlinearity of the OHC voltage/electromotile gain relationship shows rectification and doubles (to 15 nm/mV; Santos-Sacchi, 1989) at the depolarized membrane potentials generated by application of ATP; cholinergic hyperpolarization of the OHC would have the opposite effect (Ashmore, 1994). There are, however, a number of confounding issues associated with this analysis. Firstly the OHC electromotility is dependent upon the sound-induced mechanoelectrical transduction (MET) potential derived from the mechanical gating of ion channels believed to be associated with both ends of the tip-links of the hair-cell stereocilia (Hudspeth, 1989; Pickles and Corey, 1992; Denk et al., 1995). These MET channels, of which there are only approx 100 per OHC (Torre et al., 1995), appear to have a similar conductance to the OHC nonselective ATP-gated ion channels, both are blocked by amiloride and aminoglycosides, albeit with different K_d values (Housley et al., 1992; Lin et al., 1993); although there is no evidence for an inward rectification of the MET channels. Extracellular ATP has been shown to reduce the OHC MET current under voltage-clamp in vitro, an effect that may possibly be associated with observed ATP-gated Ca^{2+} entry at this site (Ashmore et al., 1993). The driving force for ion entry through both these ion channels is determined by the electrochemical gradient from the endolymph to the OHC cytoplasm. The principal cation in both is K^+, estimated at 155 m*M* in endolymph (Sterkers et al., 1988), thus as defined by Davis (1965), both G_{MET} and $G_{C,ATP}$ depend principally upon the 150 mV electrical gradient (Torre et al., 1995) across the apical surface of the OHC. Thus an ATP-induced decrease of OHC membrane potential to –30 mV represents a 20% reduction in the driving force for OHC electromotility driven by the MET conductance. The physiological relevance of such analysis again depends upon establishing the dynamic range of extracellular ATP concentration in cochlear endolymph (scala media)

and a thorough understanding of the secondary actions of changes in membrane conductance resulting from activation of $G_{C,ATP}$. In addition, as indicated previously, exogenous ATP in scala media reduces EP (Muñoz et al., 1995). This effect would further limit both forward and reverse transduction in the OHC by reducing G_{MET}.

Extracellular ATP may also, via alteration in membrane conductance, alter the phase relationship of the OHC in an analogous fashion to that of the cholinergic action on OHC (Dallos, 1996; Sziklai and Dallos, 1993), affecting the active micromechanics of the cochlea. Interestingly, despite the prediction that a cholinergic hyperpolarization of the OHC membrane would shift the OHC voltage-length relationship to a lower gain, experimental evidence actually showed an ACh-induced enhancement of electromotility (Dallos, 1996). This was attributed to a second messenger-mediated action possibly involving a Ca^{2+}-dependent change in phosphorylation of the electromotile proteins. Thus, by analogy, direct or indirect P2-receptor-mediated changes may also alter OHC electromotility. Given the observation that the slower ATP-induced increase in intracellular Ca^{2+} occurred preferentially in the basal region of the OHC (Ashmore and Ohmori, 1990) via the putative P2Y-receptor mechanism, it is possible that ATP could act as a neuromodulator of OHC motility at the efferent synapse in concert with acetylcholine, and independent of any nucleotide signaling process occurring within scala media.

Extracellular Nucleotide Signaling on Inner Hair Cells and Supporting Cells of the Cochlear Sensory Epithelium

Extracellular ATP responses have also been characterized in isolated IHC and sensory-epithelium supporting cells by Ca^{2+}-imaging and electrophysiology. An early study using dual-emission ratiometric microspectrofluorometry with Indo-1 (Dulon et al., 1991) demonstrated a faster (1–5 s) Ca^{2+} response to ATP in isolated guinea-pig IHC compared with OHC where the Ca^{2+} response peaked at 60 s. Partial desensitization of the Ca^{2+} response was observed, along with a persistence in Ca^{2+}-free media, supporting release of stored Ca^{2+}. This study was not performed under voltage clamp and in the absence of extracellular Ca^{2+} a rapid run-down of the Ca^{2+} response was detected. Thus ATP-dependent entry of Ca^{2+} contributed to the Ca^{2+} response, either via the ATP-activated ion channels or via secondary activation of voltage-dependent Ca^{2+} channels. However, the latter is unlikely given a later study that showed comparable ATP-induced changes in $[Ca^{2+}]_i$ using the fluoro-3 Ca^{2+} indicator during simultaneous voltage-clamp (Dulon et al., 1995). It appears that the recycling of Ca^{2+} into storage in IHC is dependent upon availability of extracellular Ca^{2+}, loss of Ca^{2+} prior to reuptake by the endoplasmic reticulum likely reverses Ca^{2+}-Na^+ exchangers under $Ca^{2+}{}_0$-free conditions, exhausting Ca^{2+} storage. The amplitudes of the Ca^{2+} responses were comparable in IHC isolated from either the apical or basal regions of the cochlea. In these experiments the agonist profile was ATP-α-S > ATP ≫ adenosine. Neither ADP or α,βMeATP were effective.

Comparison of ATP-activated membrane conductances in isolated guinea-pig cochlear IHC with the OHC $G_{C,ATP}$ indicates that the net effect of activation of P2X-and P2Y-receptor pathways depends upon the expression profile of the Ca^{2+}-dependent membrane conductances expressed by these different cell types. Under modest intracellular Ca^{2+}-buffering (0.5 m*M* EDTA), OHC exhibit sustained inward current responses caused by the P2XR, whereas under identical buffering, IHC respond to extracellular ATP by exhibiting a transient inward current caused by the ATP-gated nonselective cation conductance rapidly becoming masked by a Ca^{2+}-activated K^+ conductance. This is because of the considerable expression in IHC of TEA-sensitive Ca^{2+}-dependent K^+ channels (Housley et al., 1993; Dulon et al.,

1995). ATP-mediated elevation of intracellular Ca^{2+} causes the rapid secondary activation of this $G_{K(Ca)}$ conductance, effectively masking the ATP-gated inward current. The net effect of extracellular ATP on IHC V_m is negligible compared with that seen for the OHC (Housley et al., 1993). The ATP-mediated increase in $[Ca^{2+}]_i$ measured using indo-1 fluorescence, has an EC_{50} of 1.8 μ*M* and a Hill coefficient of 1, whereas for the same cell population the EC_{50} for the ATP-gated inward current was 13.7 μ*M* and the Hill coefficient 2 (Sugasawa et al., 1996b). The difference in EC_{50} values and Hill coefficients, and the fact that the ATP-mediated Ca^{2+} responses could be elicited at ATP levels below activation threshold for the inward current suggest that the secondary activation of $G_{K(Ca)}$ in IHC is largely because of activation of P2Y receptors. Whereas the ATP-gated inward current was robust, the activation of the $G_{K(Ca)}$ conductance diminished with repetitive exposure of the isolated IHC to ATP, suggesting that the intracellular stores of Ca^{2+} were depleted so that the contribution of the P2Y-receptor pathway declined. Focal application of ATP to isolated IHC localized the ATP-gated inward current to the apical surface of the IHC, as has previously been demonstrated for OHC (Housley et al., 1993; Sugasawa et al., 1996b). This localization is supported by TNP-ATP fluorescence labeling that showed suramin-blocked binding on IHC stereocilia (Mockett et al., 1994).

Expression of $P2X_2R$ mRNA has been confirmed in adult rat IHC (Housley et al., 1998a) by *in situ* hybridization. As for the OHC, the $P2X_2R$-subunit protein localized to the tips of the stereocilia, based on subunit-specific immunoreactivity (Housley et al., 1996). Given the comparable pharmacology of the OHC and IHC ATP-activated nonselective action conductances (discounting secondary Ca^{2+}-dependent changes in membrane conductances), and the observation that for both IHC and OHC $G_{C,ATP}$ are partially blocked by $Ca^{2+}{}_0$ (Housley et al., 1993; Sugasawa et al., 1996b), a feature of $P2X_2R$ homomultimeric ATP-gated ion channels but not $P2X_1R$ homomultimeric expressed channels (Evans et al., 1996); it seems parsimonious to consider that the $P2X_2R$ subunit isoforms are the principal elements forming the cochlear hair-cell ATP-gated ion channels. This may well also be the case for the vestibular-system hair cells (Housley et al., 1997).

ATP also elicits P2X- and P2Y-receptor-mediated responses in Deiters cells. These cells simultaneously support the base and apex of the OHC, leaving free the space of Nuel surrounding the OHC-lateral membrane, associated with the mechanical and ionic processes of the active OHC. Electrophysiological experiments have localized the ATP-activated inward current (P2X receptors) to the base of the phalangeal process of the Deiters cell where it supports the base of the OHC (Dulon, 1995). In addition, an IP_3-mediated Ca^{2+}-release mechanism activated by P2Y receptors is also proposed based on Ca^{2+}-imaging evidence (Ashmore and Ohmori, 1990; Dulon, 1995; Dulon et al., 1993; Sugasawa et al., 1996c). Interestingly, it has been suggested that autocrine or paracrine ATP signaling may occur between these elements of the organ of Corti (Dulon, 1995) and that as the Deiters cell P2Y-receptor Kd is 0.35 μ*M* (Moataz et al., 1992), compared with approx 10-fold higher Kd values for the P2Y receptors of OHC and IHC, Deiters' cells may be relatively more sensitive to changes in extracellular ATP concentrations in the space of Nuel (Dulon, 1995). At 7.3 μ*M*, the Kd for ATP response via P2X receptors on Dieters cells is similar to that of the P2X receptors on the sensory hair cells and Hensens cells (Sugasawa et al., 1996c). The observation that the phalangeal process of the Deiters cell, which supports the cuticular plate of the OHC, undergoes $[Ca^{2+}]_i$-dependent motility (Dulon et al., 1994), suggests that a P2Y receptor-mediated release of stored Ca^{2+} would also contribute to a change in the mechanical compliance of the cochlea and therefore cochlear tuning (Dulon, 1994). Both single-cell RT-PCR experiments (Glowatzki et al., 1995) and riboprobe *in situ* hybridization confirm the expression of $P2X_2R$ subunit mRNA by these cells (Housley and Ryan, 1998a; Housley et al., 1997).

Immediately lateral to the Deiters cells in the organ of Corti are the Hensens cells. These cells functionally express both P2XR and P2YR, but in this case ATP-induced increases in $[Ca^{2+}]_i$ appear principally caused by Ca^{2+} influx via ATP-gated ion channels (P2XR) rather than release of Ca^{2+} from intracellular stores mediated by the P2YR intracellular-signaling pathway. *In situ* hybridization data indicates that $P2X_2R$ mRNA expression in Hensens cells is comparable to the high levels seen in neighboring supporting cell types (Housley and Ryan, 1997; Housley et al., 1997). These Hensens cells exhibit an ATP-gated inward current that has a rapid onset (< 50 ms) arising from ATP-gated ion channels localized to the apical (endolymphatic) surface. In addition, a Cl^- conductance, dependent upon the presence of extracellular Ca^{2+}, is also expressed by these cells. This conductance has a slow activation onset that was blocked by dialyzing the cells with 10 m*M* BAPTA through the whole-cell recording pipet (Sugasawa et al., 1996a). Comparison with the chloride conductance reported to occur on the endolymphatic surface of the strial marginal cells suggests a similar localization of this Ca^{2+}-activated Cl^- conductance, in association with the ATP-gated ion channels, and a likely role in the regulation of ionic and fluid homeostasis via Cl^- efflux into scala media (Sugasawa et al., 1996a,c). P2 receptor-mediated Ca^{2+} responses may be coupled between Hensens cells because of the presence of gap junctions (Ashmore and Ohmori, 1990; Mammano et al., 1996).

Spiral ganglion neurons are an additional major site of P2-receptor expression and are a probable site of the extracellular ATP-mediated alteration in cochlear biopotentials elicited by perilymphatic ATP perfusion (Kujawa et al., 1994a,b; Muñoz et al., 1995). Autoradiographic labeling has shown binding of ^{3}[H]-α,βMeATP and ^{35}[S]dATP in the spiral ganglion region (Mockett et al., 1995). The block of this binding by ^{3}[H]-β,γMeATP and 2MeSATP, respectively, is compatible with both P2XR and P2YR expression by these bipolar primary afferent auditory neurons located in Rosenthal's canal in the spiral limbus. With the cloning of the ionotropic and metabotropic P2 receptors, it has been possible to commence a molecular characterization of the expression pattern and both *in situ* hybridization and *in situ* RT-PCR have demonstrated expression of isoforms of the $P2X_2R$ by these neurons (Housley and Ryan, 1998a; Housley et al., 1997; Salih et al., 1998). Functional evidence for P2YR expression arises from a Fura-2 Ca^{2+} imaging study that has demonstrated that in the absence of $Ca^{2+}{}_0$, extracellular ATP elevates $[Ca^{2+}]_i$ both in the neurites and in the soma of guinea-pig spiral ganglion neurons (Cho et al., 1996). This P2 receptor-mediated response was blocked using the P2-purinoceptor antagonists, suramin and RB2. Given that glutamate-evoked Ca^{2+} responses could only be elicited from the neurites, this result suggests that P2Y-receptor expression is in both the region of the synapse with the IHC and also distributed on the soma; compatible with ATP having a role as a neurotransmitter or neuromodulator both at the IHC-spiral ganglion cell synapse and also local neuromodulation within Rosenthal's canal. Both of these sites would be exposed to perilymphatic perfusions.

Evidence for Nucleotide Signaling in the Secretory Epithelium (Spiral Ligament) and Reissner's Membrane

The detection of sound and motion by sensory cells in the inner ear is dependent upon the ionic and electrical characteristics of the endolymphatic compartments of the cochlear and vestibular systems. Endolymph is a highly specialized extracellular milieu with a high K^+ concentration almost equivalent to intracellular fluid and, in the case of the cochlear duct, with a positive (+ 80 mV) potential, the endocochlear potential (Pickles, 1988). In the cochlea, regulation of both the endocochlear potential and the generation of the K^+ concentration are dependent upon the ion transport mechanisms of the stria vascularis including the marginal cells that line the lumen; a tissue now identified

as a potential source of extracellular ATP in scala media, and possessing both ionotropic and metabotropic P2-purinoceptor expression.

In the cochlear lateral-wall tissues, extracellular ATP causes an increase in $[Ca^{2+}]_i$ (Suzuki et al., 1994, 1995; Ikeda et al., 1995) that is likely to be mediated via a P2Y-receptor coupled to a phosphoinositide pathway involving the PLC-β1 isozyme and the G_q protein that is insensitive to cholera and pertussis toxin (Ogawa et al., 1995; Ogawa and Schacht, 1995; Oba et al., 1996). The marginal cells of the stria vascularis exhibit the weakest ATP-mediated increase in $[Ca^{2+}]_i$ whereas the spiral prominence region and external sulcus cells had the greatest response (Ikeda et al., 1995). An initial transient Ca^{2+} response was attributed to both extracellular Ca^{2+} entry and release of Ca^{2+} from internal stores, whereas a delayed sustained response was entirely attributable to external Ca^{2+}. The pharmacological profile of the Ca^{2+} responses was 2MeSATP = ATP. UTP and adenosine were ineffective, consistent with P2X and P_{2y} receptor, but not P_{2u} or P1 receptor, action. Here P_{2y}- and P_{2u}-receptor designations are used to delineate between subtypes of P2Y receptor that have not been characterized at the molecular level; $P2Y_1$, $P2Y_5$, and $P2Y_7$ receptors are insensitive to UTP (Webb et al., 1993, 1996b; Akbar et al., 1996).

Application of ATP and UTP to whole gerbil stria vascularis and vestibular dark-cell epithelial preparations affects ion conductance through these tissues (Wangemann, 1995; Liu et al., 1995), consistent with P_{2y} and P_{2u}-purinoceptor-mediated modulation of transepithelial K^+ transport (Liu et al., 1995). These studies are significant in providing a putative mechanism for regulation of the electrical and ionic characteristics of the endolymph and hence the driving force for mechanoelectrical sound transduction. P_{2u} (putative $P2Y_2$) receptors, where UTP = ATP ≫ 2MeSATP, occur on the apical and basal surfaces of marginal cells of the stria vascularis and their vestibular equivalent, the dark cells, whereas P_{2y} receptors (insensitive to UTP) appear to be expressed on the perilymphatic (basal) surface of these tissues (Liu et al., 1995; Marcus et al., 1996c). Based on the data from microUsing-chamber experiments, P_{2u} receptors act at the luminal surface of the marginal cells of the stria vascularis to reduce the transepithelial conductance, forming part of a putative feedback autocrine mechanism controlling ion transport (Liu et al., 1995). Application of 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) to the luminal surface of the gerbil stria vascularis and dark-cell epithelium antagonized this reduction in transepithelial conductance. Evidence for endogenous ATP action comes from the fact that DIDS on its own caused an increase in the transepithelial conductance (Liu et al., 1995). It is therefore possible that the direct action of ATP on these secretory tissues reduces the pumping of K^+ into endolymph, whereas release of nucleotides stored in the strial marginal cells (White et al., 1995) could be feedback-regulated by these P_{2u} receptors (Wangemann, 1995; Liu et al, 1995), as part of autocrine and paracrine signaling pathways within scala media.

Protein kinase C regulates I_{sK} channels (small conductance K^+ channels) which have been shown to provide the path for K^+ secretion from the strial marginal cells into the endolymphatic lumen (Shen and Marcus, 1995; Marcus et al., 1996d; Shen et al., 1997). RT-PCR experiments with gerbil stria vascularis mRNA have confirmed the expression of an I_{sK} channel with a PKC phosphorylation site (Marcus et al., 1996a). On-cell macropatch recording of the apical surface of marginal cells with ATP (10–100 μ*M*) led to a (P_{2u}R-mediated) decrease in conductance attributable to the I_{sK} channels as predicted from the micro-Ussing-chamber recordings. Stimulation of the PKC pathway with the phorbol ester PMA (20 n*M*) mimicked this reduction in conductance, whereas stimulation of intracellular Ca^{2+} with A23187 (mimicking IP_3-pathway stimulation) caused an increase in conductance (Marcus et al., 1996b). These data suggest that the P_{2u} receptor acts on ion transport primarily via a diacylglycerol-protein kinase C (PKC) pathway rather than via the IP_3 mediated release of stored Ca^{2+}. Taken with the previously described Ca^{2+}-imaging

experiments that demonstrated only weak ATP-mediated Ca^{2+} responses, the PLC-DAG-PKC pathway appears to be the major P2Y-receptor signaling mechanism in strial marginal cells.

It is unlikely that the observed drop in endocochlear potential produced by ATP in endolymph (Muñoz et al., 1995) involves an interaction with the strial marginal cell P_{2u} receptor-G_{sK} conductance pathway because of the lack of effect of UTP on the EP response (Thorne et al., 1995). Additionally, the endolymphatic ATP-induced fall in EP was suramin- and PPADS-sensitive (Muñoz et al., 1995; Thorne et al., 1995), whereas the strial marginal cell P_{2u}R-mediated fall in I_{sK} was suramin insensitive (Liu et al., 1995) but antagonized by PPADS (Marcus et al., 1996c). PPADS is thought to be a P2X-receptor-selective antagonist, but variable P2YR-antagonist action has also been observed in other tissues; for example PPADS preferentially blocked P2y- but not P_{2u}-receptor-mediated vascular vasodilatation in rat mesenteric arterial bed (Ralevic and Burnstock, 1996a). As indicated earlier, perilymphatic perfusion with ATP-α-S produced a transient increase in EP (Kujawa et al., 1994a), presumably acting via P2 receptors on the basement-membrane side of the tight junctions within the stria vascularis.

RT-PCR and *in situ* hybridization data support the expression of $P2Y_2R$ mRNA in guinea pig and rat lateral-wall tissues (Järlebark et al., 1995, 1996); compatible with a $P2Y_2$-receptor designation for the P_{2u}R-mediated reduction in G_{sk} described above.

$P2X_2R$ mRNA expression has been localized to tissues of the rat cochlear lateral wall. Cloning of RT-PCR products indicated that the rat lateral-wall tissue expressed a splice variant that has a truncated C-terminus domain (Housley et al., 1995b,c), one of a number of isoforms of the $P2X_2R$ subunit that have now been found expressed in cochlear and vestibular tissues that confer different functional properties to the ATP-gated ion channels that assemble from these subunits (Housley and Ryan, 1997; Housley et al., 1998a; Brändle, 1996; Brändle et al., 1997). *In situ* hybridization in rat cochlea indicates that $P2X_2R$ subunit mRNA is highly expressed in lateral wall, particularly in the external sulcus cells and root cells (Fig. 5), whereas only minimal expression was detected in the fully differentiated stria vascularis (Housley and Ryan, 1997; Housley et al., 1998a). These data are compatible with the previously described variable contribution of $Ca^{2+}{}_0$ entry (via ATP-gated ion channels) to the ATP-mediated Ca^{2+}-responses of lateral wall tissues.

Reissner's membrane also expresses ATP-gated ion channels, based on *in situ* hybridization detection of $P2X_2R$ subunit mRNA (Housley and Ryan, 1997; Housley et al., 1998a). This membrane forms an impermeable barrier maintaining the ionic and electrical partition between scala media and scala vestibuli. Whereas no functional data is yet available to confirm an ATP-gated conductance across Reissner's membrane, this possible shunt pathway would impact upon the electrochemical driving force for the sound transduction process.

Purinergic Regulation of Cochlear Blood Flow

Given the close association between P2X- and P2Y-receptor expression and regulation of vascular tone (Ralevic and Burnstock, 1996b), it is not surprising that extracellular ATP exerts a role in the regulation of cochlear blood flow (CBF). The action of extracellular ATP in the cochlear vascular bed is associated with both P1- and P2-receptor mechanisms. Blood flow to the cochlea is predominantly to the stria vascularis, although vessels also run within the spiral ligament, basilar membrane of the organ of Corti, and within the modiolus. Both K^+ secretion into scala media and the maintenance of EP are believed to be associated with the secretory stria vascularis (Fernendez and Hinojosa, 1974; Wangemann, 1995). Transient ischemia rapidly produces a fall in endocochlear potential (EP) (Woolf et al., 1986), whereas the maintenance of CBF and EP is, at least in the guinea pig, resistant to a compromised blood supply

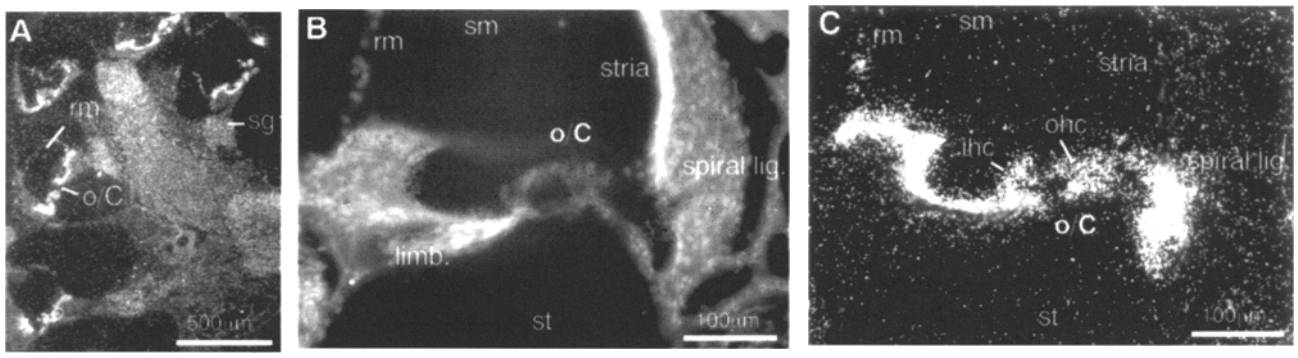

Fig. 5. ^{35}S-riboprobe *in situ* hybridization showing $P2X_2R$-mRNA expression in the adult rat cochlea. **(A)** Low-power mid-modiolar section showing autoradiographic labeling of the organ of Corti (o/C), spiral ganglion (sg), and Reissner's membrane (rm). **(B)** Bis-benzimide fluorescence showing a detailed view of the spiral limbus (limb.), organ of Corti, spiral ligament (spiral lig.), stria vascularis (stria), scala media (sm), and scala tympani (st). **(C)** Corresponding dark-field view of the section in **(B)** showing the $P2X_2R$-mRNA labeling. Note the high level of expression in the cells lining scala media, including the region containing the inner-hair cells (ihc) and the outer-hair cells (ohc). In contrast, little labeling of the stria vascularis is apparent.

produced by ligation of the common carotid and vertebral arteries (Hu et al., 1995). However, data on the effects of elevated ATP in blood on CBF are contradictory.

Intravenous infusion of ATP produced a rapid fall in guinea pig CBF although EP was relatively unaffected (Hu et al., 1995), whereas perfusion of 10 m*M* ATP (5–60 µL/min) into the guinea pig anterior inferior cerebellar artery produced dose-dependent transient increases in CBF (Ren et al., 1996). Introduction of N^w-nitro-L-arginine methyl ester significantly reduced the ATP-induced increases in CBF consistent with a P2YR-mediated stimulation of the NO (endothelial relaxing factor) pathway. Perilymphatic perfusion of the guinea pig cochlea with ATP (10–100 µ*M*) and adenosine (10–100 µ*M*) both produced increases in CBF (Muñoz et al., 1996), measured using laser Doppler flow measurement in vivo. The P2-receptor effect was totally blocked by RB2 (1 µ*M*), whereas PPADS (1 µ*M*) produced only a partial block. The P1-receptor response was antagonized by the A_2-receptor antagonist 3,7-dimethyl-1-propargylxanthine (DMPX, 10 µ*M*). It therefore seems likely that ATP and adenosine produced by ectonucleotidase hydrolysis of ATP, act via P2Y receptors and A_2 receptors, respectively to dilate the blood vessels in the spiral ligament and stria vascularis.

Molecular Analysis of P2-Receptor Expression During Inner Ear Development

Both mitogenesis and apoptosis, key elements in tissue development, appear to be regulated by P2X-receptor (Surprenant et al., 1995, 1996; DiVirgilio, 1995) and P2Y-receptor (Boader et al., 1995) mechanisms in some tissues. The variation in expression of P2 receptors during the ontogeny of the inner ear supports a role for nucleotide signaling during development in this tissue.

Many of the rat cochlear cell types are still differentiating after birth. Hearing onset occurs with maturity of the sensory hair cells and development of the EP at around P8 (Schmidt and Fernandez, 1963). Both whole-tissue and single-cell RT-PCR studies indicate expression of $P2X_1R$ and $P2X_2R$ mRNA isoforms in sensory and supporting cells of the rat organ of Corti and vestibular sensory epithelia in the early-postnatal period (P4–P10) (Housley and Ryan, 1997; Glowatzki et al., 1997, 1995; Housley et al.,

1995b; Brändle et al., 1995). From RT-PCR experiments it is apparent that $P2X_1R$ mRNA expression is lower than for $P2X_2R$, declining to undetectable levels in the adult inner ear. In contrast, $P2X_2R$ mRNA levels were greatest in the neonatal inner ear tissues but sustained, albeit at lower levels, in adult sensory/supporting, neural, and secretory tissues (Housley and Ryan, 1997; Housley et al., 1995b).

Utilizing riboprobe *in situ* hybridization based on a cloned $P2X_2R$ cDNA fragment, expression of the $P2X_2R$ subunit has been followed during development of the inner ear labyrinth (Housley et al., 1998a). Expression of the $P2X_2R$ subunit is evident as early as E12–14 in the otocyst, and cochlear duct, with levels of mRNA increasing during ontogeny of the vestibular and cochlear structures including the developing cochlear and vestibular ganglia. From birth $P2X_2R$-mRNA expression extends rapidly from the lateral aspects of the developing organ of Corti and the lateral wall, including the presumptive stria vascularis, to include the medial aspects of the organ of Corti, inner sulcus cells and interdental cells. $P2X_2R$-mRNA expression in the sensory hair cells only becomes detectable by this technique from approx P8. In agreement with the RT-PCR data, $P2X_2R$ mRNA levels are generally lower in P14–adult inner ear tissues (Fig. 5) than at the early-postnatal stage. With the rapid expansion in the number of P2-receptor cDNAs cloned from the inner ear, analysis of the expression patterns of all the P2X- and P2Y-receptor subtypes likely to be involved in inner ear ontogeny is a challenging future task.

A Model of the Regulation of Hearing Sensitivity via P2-Purinoceptor Regulation of the Endolymphatic Electrochemical Gradient

A predominance of P2X-receptor expression has been clearly demonstrated in the sensory and secretory tissues that line the endolymphatic compartment of the inner ear. This highlights the multiplicity of signaling functions of ATP in the inner ear.

Regulation of endogenous ATP in endolymph, via the proposed autocrine mechanism involving the stria vascularis, coupled with the sink produced by ecto-ATPases, may underlie the homeostatic control of hair-cell sensitivity. The mechanism for this would involve P2X receptors located on the apical surface of the hair cells and supporting cells of the organ of Corti. These receptors may regulate the standing K^+ current (silent current) (Brownell, 1982; Brownell et al., 1986) that maintains the sensitivity of the hair-cell mechano-electrical tansduction process. The observed fall in endocochlear potential (and hence driving force for the MET process) produced by exogenous ATP (Muñoz et al., 1995) provides functional support for this model. Elevation of ATP levels in cochlear endolymph, such as may occur during exposure to high sound levels or ischemia, would open the P2X-receptor conductance ($G_{C,ATP}$) on the stereocilia of the hair cells and increase the standing K^+ current flowing into the hair cells and out through the K^+-selective channels that appear localized to the basolateral surface of these cells (Santos-Sacchi and Huang, 1996). This electrical shunt would effectively desensitize the hair cells and, in the case of the OHC, shut down their active mechanical-amplication process (Housley et al., 1992), thus protecting these terminally differentiated cells. The discovery of $P2X_2R$ expression in Reissner's membrane (Housley and Ryan, 1997; Housley et al., 1996, 1998a) provides an additional short-circuit pathway for suppressing forward and reverse transduction in the sensory hair cells in the face of elevated endolymphatic ATP.

Whereas the direct action by extracellular ATP in scala media evident by the decline in EP and CM, likely involves P2X-receptor signaling as outlined; clearly secondary mechanisms affecting the electrochemical milieu are also probable. These would include the Ca^{2+}-mediated secondary conductance changes affecting membrane conductances on the perilymphatic surfaces of the cells lining scala media and on Hensen cell $G_{Cl(Ca)}$ and on strial marginal G_{sK} conductances. A detailed molecular characteri-

zation of the ionotropic and metabotropic P2-receptor signaling pathways, and associated ectonucleotidase purine "sink" regulating the sound transduction process in the ear are essential. Whereas this review highlights the progress that has been achieved regarding nucleotide signaling in the sound and balance organs to-date, considerable further investigation is required before the complexities of what are clearly fundamental processes controlling sensory transduction, neurotransmission, and electrochemical and fluid homeostasis in the inner ear are resolved.

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