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Hair Cells – Beyond the Transducer

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Overview This review considers the ''tween twixt and twain'' of hair cell physiology, specifically the signaling elements and membrane conductances which underpin forward and reverse transduction at the input stage of hair cell function and neurotransmitter release at the output stage. Other sections of this review series outline the advances which have been made in understanding the molecular physiology of mechanoelectrical transduction and outer hair cell electromotility. Here we outline the contributions of a considerable array of ion channels and receptor signaling pathways that define the biophysical status of the sensory hair cells, contributing to hair cell development and subsequently defining the operational condition of the hair cells across the broad dynamic range of physiological function.

Key words: Inner hair cell — Outer hair cell — Vestibular — Potassium channels — Efferent — KCa — BK channel — P2X receptor — P2Y receptor — Cochlea — Ontogeny — Noise — Neurotransmission — Hair cell tuning — Acetylcholine — Dopamine — Olivocochlear bundle — KCNQ4 — Calcium channels — Review

Development of Biophysical Properties of the Mammalian Cochlear Hair Cells

In rodents, the onset of hearing occurs between postnatal day 10 (P10) and P14 (Ehret, 1977; Uziel, Romand & Marot, 1981; Romand, 1983). However, before sound-induced responses begin, hair cells have to follow a developmental program that consists of the acquisition and/or elimination of different ion channels and other membrane proteins such as those associated with the exocytotic machinery. In this section we examine what is currently known about the developmental maturation of the hair cells' biophysical properties.

After terminal mitosis, at about embryonic day 14–15 (E14–E15) in mice (Anniko, 1983; Pujol, Lavigne-Rebillard & Lenoir, 1998), inner and outer hair cells are already recognizable by scanning electron microscopy (Lim & Anniko, 1985). These hair cell precursors start to differentiate electrophysiologically by acquiring a variety of voltage- and time-dependent ion channels, although at this early stage of development their repertoire of ion channels is very similar (Marcotti et al., 2003a, b; Helyer et al., 2005). During the following two-three weeks, both hair cell types undergo extensive biophysical changes that transform these immature cells into fully functional sensory receptors at the onset of hearing. These changes also generate exquisitely sensitive cells with properties characteristic of their position along the tonotopic axis of the mammalian organ of Corti. The maturation of these electrophysiological properties occurs simultaneously with major refinements in the neuronal connections within the mammalian cochlea (reviewed by Pujol et al., 1998).

Inner Hair Cells — the Primary Sensory Receptors

Inner hair cells (IHCs) are the primary sensory receptors of the mammalian cochlea and are responsible for signaling the reception of sound to the central nervous system. Two main critical

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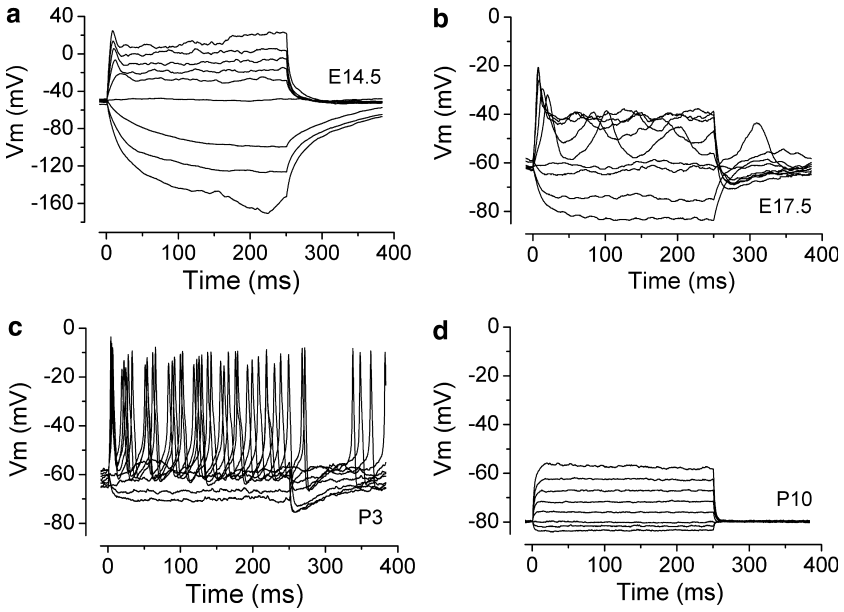

Fig. 1. Developmental maturation of voltage responses in inner hair cells. Voltage responses (modified from Marcotti et al. 2003a) from basal-coil IHCs (*a*) just after terminal mitosis (E14.5), (*b*) later in embryonic development (E17.5), (*c*) early postnatally (P3) and (*d*) just before the onset of hearing (P10). Recordings were obtained using 1.3 mM Ca^{2+} in the extracellular solution and at body temperature. Hyperpolarizing and depolarizing current injections (between −30 pA and +100 pA in 10 pA increments) were applied from the resting potential and for clarity only responses to every other stimulus are shown. Note that the resting membrane potential becomes more negative with development, mainly due to the increase in the size of the inward K^+ current I_{K1}.

developmental changes can be identified during the maturation of mammalian IHCs: the acquisition of the action potential activity just before the day of birth in mice and the disappearance of this activity at the onset of cell functional maturation about P12. The first ion channel type expressed in embryonic cochlear hair cells is an outwardly rectifying K^+ channel. These channels have a very low open probability around the resting membrane potential (~ -50 mV) and when opened by membrane depolarization, a small and slowly activating classical delayed-rectifier K^+ current named $I_{K,emb}$ can be recorded from these embryonic cells (Marcotti et al., 2003a). As a consequence, IHCs at this stage of development ($\sim$E14.5) respond to current injections with slow and large voltage responses and with no sign of action potential activity (Fig. 1*A*). This would seem to be appropriate as the newly differentiated IHCs are still functionally isolated from the brain (Pujol et al., 1998) and receive no sensory input. Within the next four embryonic days the radial afferent endings, which invade the cochlear epithelium just before terminal mitosis (Sher, 1971), tend to concentrate around the base of the IHCs (Sobkowicz, 1992) and form the first synaptic-like contacts (Pujol et al., 1998). At about the same time IHCs gradually begin to express a variety of additional currents (*see* Fig. 5), including the inward rectifier K^+ current (I_{K1}; Marcotti et al., 2003a), the Ca^{2+} current (I_{Ca}, carried by $Ca_v1.3$ voltage-gated Ca^{2+} channels: Platzer et al., 2000; Dou et al., 2004) and the TTX-sensitive Na^+ current (I_{Na}, possibly carried by $Na_v1.7$ channels; Marcotti et al., 2003b). The mechano-electrical transducer current also appears at around E18.5 in mouse cochlear IHCs (Bryant et al., 2003). I_{K1} has faster activation kinetics than $I_{K,emb}$ and is the major contributor to the resting conductance, and therefore the resting membrane potential, of these immature cells. The simultaneous presence of a large variety of voltage-gated inward and outward currents during late embryonic development causes IHCs to elicit slow and repetitive spontaneous action potentials (Fig. 1*B*). These immature (E17.5) slow action potentials have been shown to trigger exocytosis (Johnson, Marcotti & Kros, 2005), which is generally interpreted as a sign of neurotransmitter release from presynaptic cells (Neher & Marty, 1982; Parsons et al., 1994). These results suggest that embryonic IHCs can already relay information to the developing neuronal connections within the cochlear neuroepithelium.

With postnatal development the size of all the embryonic-type currents increases gradually, causing a continuous change in the cells' voltage responses. Neonatal IHCs can generate rapid spontaneous Ca^{2+} action potentials (Fig. 1*C*) up to about P6–P7 in the mouse (Beutner & Moser, 2001; Marcotti et al., 2003a, b). After the first postnatal week spikes can only be elicited following depolarizing current injections (Glowatzki & Fuchs, 2000; Marcotti et al., 2003a, b) until about the onset of hearing (P10–P12; Shnerson & Pujol, 1981) (Fig. 1*D*). The complete disappearance of action potential activity is mainly due to the expression of ion channels (K,f and K,n, *see* below) characteristic of mature cells. During the period in which spontaneous spiking activity is recorded in immature IHCs, the highly branched auditory afferent fibres, initially present during late embryonic and early postnatal stages, undergo an extensive pruning that finally results in the one-to-one axosomatic configuration characteristic of mature IHCs (Echteler, 1992; Pujol et al., 1998).

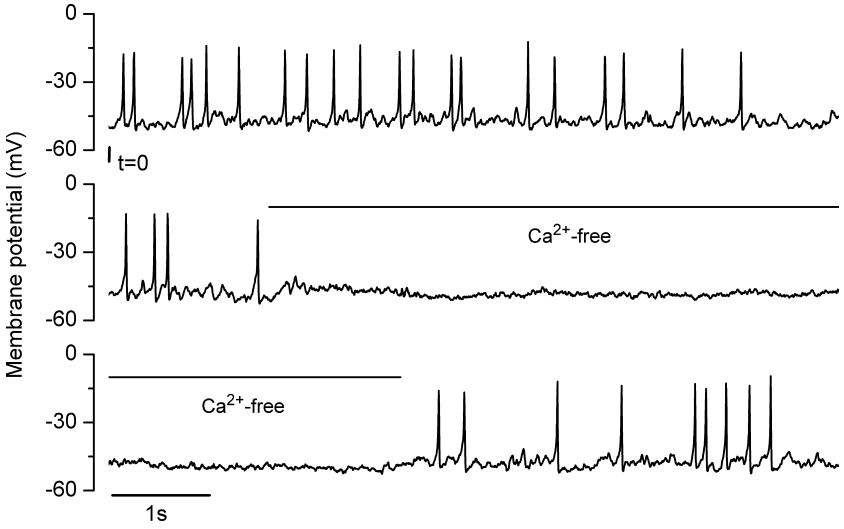

Fig. 2. Action potentials in immature inner hair cells are mainly Ca^{2+} dependent. Spontaneous action potentials recorded from an immature IHC from the apical coil of the cochlea (modified from Marcotti et al. 2003b). Continuous whole-cell voltage responses were obtained using 1.3 mM Ca^{2+} in the extracellular solution. Note that spontaneous action potentials are reversibly abolished when a Ca^{2+}-free solution was extracellularly applied.

While the role of spiking activity in immature IHCs is currently unknown, the correlation between electrophysiological and morphological development (Marcotti et al., 2003a) suggests that it could potentially contribute to the post-synaptic refinement of synaptic connections within the cochlear neuroepithelium. A similar role for regenerative spiking behavior has previously been suggested for the maturation and refinement of synaptic connections in the developing nervous system, including the visual system (reviewed by Zhang & Poo, 2001). Spontaneous activity in mammals has also been recorded in vivo from the auditory nerve and brainstem nuclei prior to the onset of hearing (Gummer & Mark, 1994; Kotak & Sanes, 1995). This activity is likely to originate from the cochlea since deafferentation, caused by surgical removal of the cochlea before hearing begins, results in substantial loss of cochlear nucleus neurons (Tierney, Russell & Moore, 1997; Mostafapour et al., 2000), possibly because of the removal of the spontaneous spiking activity originating from IHCs. Since the main architecture of the afferent auditory pathway is already in place during the second postnatal week (Mostafapour et al., 2000), the auditory afferents are likely to be less dependent on IHC activity and may thus explain why spontaneous activity is no longer needed from about P7–P8 onwards. Since the expression of the large-conductance Ca^{2+}-activated K^+ current $I_{K,f}$ (*see* below) in IHCs at the onset of hearing is likely to be induced by elevations in intracellular Ca^{2+} resulting from immature action potentials (Brandt, Striessnig & Moser, 2003), spontaneous activity could also serve an intrinsic (ion-channel expression) developmental role, as previously found in other systems (reviewed by Moody, 1998; Spitzer, 2002; Moody & Bosma, 2005).

Although immature IHCs express both Ca^{2+} and Na^+ currents, only the former is required for the generation of action potentials since they are reversibly abolished during superfusion of a Ca^{2+}-free solution (Fig. 2) but not by TTX (Marcotti et al., 2003b). In addition to I_{Ca}, other immature currents are involved in modulating the shape and frequency of action potentials. While the delayed rectifier $I_{K,neo}$ (which develops from $I_{K,emb}$; Marcotti et al., 2003a), together with I_{Ca}, determines the rate of rise and fall and the amplitude of the spike (Fig. 3), I_{Na} reduces the inter-spike interval by speeding up the time required for the membrane potential to reach spike threshold (Marcotti et al., 2003b).

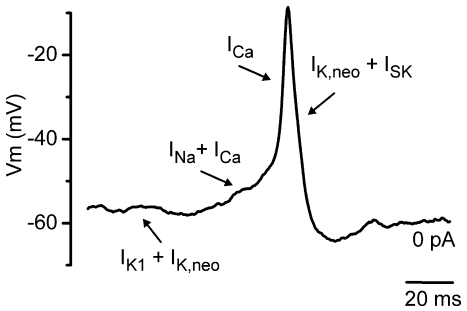

Fig. 3. Contribution of the different ionic currents expressed in immature inner hair cells to the modulation of action potential activity. A single action potential recorded from a P3 apical-coil IHC (reproduced from Marcotti et al., 2004b with permission of Blackwell Publishing). Note that some of these currents (I_{K1}, I_{Na}, I_{SK},) are only transiently expressed during the period when IHCs exhibit action potential activity (*see* text for details).

Two additional currents are required for boosting the regenerative behavior of the action potential in immature IHCs: I_{K1} and the apamin-sensitive small-conductance (SK) Ca^{2+}-activated K^+ current I_{SK} (Fig. 3). I_{K1} is the main determinant of the IHC resting membrane potential and its block by extracellular Cs^+ or Ba^{2+} eliminates action potential activity in response to depolarizing current injections (Marcotti et al., 1999). The increase in size of I_{K1}

during the second postnatal week (Marcotti et al., 1999), together with the reduced amplitude of I_{Ca} (Beutner & Moser, 2001; Marcotti et al., 2003b; Johnson et al., 2005), are also the main reasons for the disappearance of spontaneous, but not induced, action potentials from about P7–P8, since IHCs become more hyperpolarized (Marcotti et al., 2003a). SK channels are not intrinsically voltage-dependent and are activated, via the Ca^{2+}-binding protein calmodulin (Xia et al., 1998b), by local elevation of intracellular Ca^{2+}. Recent studies have shown an unexpected role for SK channels in modulating the intrinsic firing properties of different neuronal-type cells (reviewed by Bond, Maylie & Adelman, 2005). In immature IHCs I_{SK} can be activated by Ca^{2+} flowing into the cells through either the nicotinic ACh receptors (nAChRs) (Elgoyhen et al., 2001; Glowatzki & Fuchs, 2000; Katz et al., 2004; Marcotti, Johnson & Kros, 2004b; Nie et al., 2004) or Ca^{2+} channels (Marcotti et al., 2004b). When activated via Ca^{2+} channels I_{SK} is responsible for making the repolarization phase of the action potentials more robust and thus playing a crucial role for sustaining repetitive spiking (Marcotti et al., 2004b). This mechanism could potentially be boosted by Ca^{2+} release from intracellular Ca^{2+} stores known to be present in immature IHCs (Kennedy & Meech, 2002). Indeed, a role for the intracellular Ca^{2+} stores in the activation of I_{SK} has been recently demonstrated in mature OHCs (Lioudyno et al., 2004).

Most of the immature currents are only transiently expressed during a period when spontaneous or evoked action potentials occur in IHCs (*see* Fig. 5), suggesting a specific role in the modulation of spiking activity. Finally, action potentials in IHCs may also be modulated by the central nervous system via the release of the neurotransmitter ACh from inhibitory olivocochlear efferent fibres. These cholinergic fibres form transient axosomatic connections with IHCs P0–P12, (Shnerson, Devigne & Pujol, 1981; Sobkowicz, 1992; Bruce et al., 1997) before finally targeting OHCs at later developmental stages (Simmons, Mansdorf & Kim, 1996; Simmons, 2002).

ACh is the main efferent neurotransmitter in the mammalian cochlea (Eybalin, 1993) that in hair cells causes the activation of α9α10 nAChRs (Elgoyhen et al., 2001; Marcotti et al., 2004b; Gómez-Casati et al., 2005), which most likely consist of two α9 and three α10 subunits (Plazas et al., 2005). The opening of the heteromeric α9α10 nAChRs, a non-selective cation channel, by ACh, causes Ca^{2+} to flow into the cell that consequently activates the functionally coupled SK channels, resulting in an outward K^+ current. Since the K^+ current is much larger than the inward current, the dominant effect of efferent stimulation on hair cells is inhibitory, resulting in the reduction of the spike frequency (Glowatzki & Fuchs, 2000; Marcotti et al., 2004b; Goutman, Fuchs & Glowatzki, 2005). Interestingly, the period during which mRNA for α9α10 nAChRs is detected in IHCs (Morley & Simmons, 2002) coincides with the transient presence of efferent innervation of these cells (*see* also later section: Efferent Modulation of Hair Cells).

Ca^{2+} action potentials are clearly essential for the normal immature development of the cochlea but they are too slow to encode the frequency range and sound intensity levels that are characteristic of mammalian hearing. IHCs undergo crucial biophysical adaptations just before the onset of hearing, which eliminate spiking activity and transform immature cells in fully functional high-frequency sound transducers. This final transition is due to both the disappearance of some of the immature ion channels and the expression of new K^+ channels that are characteristic of adult IHCs (*see* Fig. 5). The acquisition of the BK-current $I_{K,f}$ (Kros, Ruppersberg & Rusch, 1998; Marcotti et al., 2003a) and the delayed rectifier K^+ current $I_{K,n}$ (Marcotti et al., 2003a; Oliver et al., 2003) around P12 represents the first sign of IHC functional maturation. Three additional K^+ currents are present in mature IHCs: the delayed rectifier $I_{K,s}$, which derives from the immature $I_{K,emb}$ and $I_{K,neo}$ (Marcotti et al., 2003a), an additional BK-type current named $I_{K(Ca)}$ (Marcotti, Johnson & Kros, 2004a) and a small I_{Ca} (about 100 pA near −20 mV in 1.3 mM extracellular Ca^{2+}: (Johnson et al., 2005; Marcotti et al., 2003a). The onset of IHC functional maturation is also accompanied by changes in the morphology of the synaptic machinery (Sobkowicz et al., 1982) and in the exocytotic properties (Beutner & Moser, 2001; Johnson et al., 2005). Recent evidence (Marcotti et al., 2005) suggests that most of the above biophysical changes, which occur at the onset of hearing, require the newly discovered gene *TMC1* (trans-membrane cochlear-expressed gene 1) that encodes a transmembrane protein expressed in both IHCs and OHCs from just before the onset of hearing (Kurima et al., 2002; Vreugde et al., 2002).

$I_{K,f}$ is larger (about 10 nA at 0 mV) and shows more rapid activation kinetics (activation time constant 0.4 ms at −25 mV at room temperature) than the other major repolarizing current $I_{K,s}$ (Marcotti et al., 2004a). Moreover, $I_{K,f}$ activates about 15 mV more negative (−85 mV) than $I_{K,s}$. This is because, in contrast to most BK-currents, $I_{K,f}$ is insensitive to Ca^{2+} flowing through Ca^{2+} channels but is instead directly modulated by the voltage-dependent release of Ca^{2+} from intracellular Ca^{2+} stores (Marcotti et al., 2004; Beurg et al., 2005), which is possibly mediated by ryanodine receptor type 1 (RyR1). Indeed, recent evidence has suggested that RyR1, in addition to RyR2, are present in the IHCs below their cuticular plate (Beurg et al., 2005; Morton-Jones et al., 2006) where K,f channels are likely to be present

(Pyott et al., 2004; Hafidi, Beurg & Dulon, 2005). The combination of the above characteristics allows $I_{K,f}$ to interfere with the generation of action potentials at any level of current injection and enable IHCs to respond to sustained depolarization with graded receptor potentials that are essential for accurate sound encoding. In contrast to lower vertebrates (Fettiplace & Fuchs, 1999), the absence of colocalization between BK and Ca^{2+} channels prevents mammalian IHCs from exhibiting electrical resonance (Kros & Crawford, 1990; Kros et al., 1998; Marcotti et al., 2004a), which is conceivable because electrical resonance would become progressively less efficient for frequencies above a few kHz (Wu et al., 1995). The role of the additional slower and smaller BK-current $I_{K(Ca)}$ is at present not clear. However, the colocalization between K(Ca) and Ca^{2+} channels (Marcotti et al., 2004a) at the presynaptic active zone, where the afferent nerve endings synapse onto the IHCs, suggests that $I_{K(Ca)}$ might regulate neurotransmitter release.

The linopirdine-sensitive current $I_{K,n}$ is an unusual delayed rectifier K^+ current with a negative activation range and was originally described in guinea-pig OHCs (Housley & Ashmore, 1992). Since its first discovery in 1992, $I_{K,n}$ has always been known as the K^+ current characteristic of mature OHCs. However, recently electrophysiological (Marcotti et al., 2003a; Oliver et al., 2003) and both in situ hybridization and immunofluorescence findings (Beisel et al., 2000; Kharkovets et al., 2000; Beisel et al., 2005) have demonstrated that $I_{K,n}$ is also expressed in mature IHCs from around P12 (Marcotti et al., 2003a). However, in the mouse cochlea the size of $I_{K,n}$ in IHCs (Marcotti et al., 2003a) is about 45% of that of OHCs (Marcotti & Kros, 1999). In IHCs, although the size of $I_{K,n}$ is much smaller compared to the other two major currents expressed in these cells ($I_{K,f}$ and $I_{K,s}$), its contribution is relatively important around the resting membrane potential (about −70 mV), since it is largely active at rest. Therefore, $I_{K,n}$ exerts a similar role to that of I_{K1} in immature IHCs in setting the resting potential. A role in setting the membrane potential is also carried out by $I_{K,f}$ (Marcotti et al., 2004a) and $I_{K,s}$ (Marcotti et al., 2003a), although the contribution of the latter is relatively small.

Outer Hair Cells — the Cochlear Amplifier

The functional maturation in OHCs is achieved at about P8, well before the onset of hearing, and is characterized by the acquisition of $I_{K,n}$ (Marcotti & Kros, 1999), the electromotile activity (He, Evans & Dallos, 1994; Marcotti & Kros, 1999) and the sensitivity to the efferent neurotransmitter ACh (Dulon & Lenoir, 1996; He & Dallos, 1999; Marcotti et al., 2004b). Before P8 OHCs express a similar repertoire of membrane currents to immature IHCs (*see* Fig. 5) that include a small outward ($I_{K,emb}$ and $I_{K,neo}$) and inward (I_{K1}) K^+ current (Marcotti & Kros, 1999; Helyer et al., 2005), a Ca^{2+} current (Michna et al., 2003) and a Na^+ current (Oliver et al., 1997). The size of these currents in OHCs is considerably smaller than those in IHCs. It is also worth mentioning that the positional expression along the cochlea of the K^+ currents is different between the two cell types. Basal coil OHCs exhibit significantly larger currents compared to those recorded in the apical coil (I_{K1}: Marcotti et al., 1999); $I_{K,emb}$-$I_{K,neo}$: Marcotti, Johnson and Kros, *unpublished observations*), whereas in IHCs they appear to be homogeneously expressed along the cochlea. The most distinctive characteristic between immature OHCs and IHCs is observed when recording voltage responses under current-clamp conditions, since OHCs do not fire spontaneous action potentials (Fig. 4). Depolarizing current injections, from the resting potential, trigger a single action potential followed by membrane potential oscillations (Marcotti & Kros, 1999). Since IHCs and OHCs exhibit otherwise qualitatively similar basolateral membrane currents, the lack of repetitive action potential activity in OHCs is somewhat surprising. However, this may be explained, at least in part, by the absence of the SK current in immature OHCs that has recently been shown to play a crucial role in sustaining repetitive action potential activity in immature IHCs (Marcotti et al., 2004b) and/or different concentrations of Ca^{2+}-buffering proteins between the two cell types (Hackney et al., 2005). If present in vivo, action potentials in developing OHCs could serve a similar role to those in IHCs. Indeed the lack of $Ca_v1.3$ Ca^{2+} channels in knockout mice causes the degeneration of OHCs as well as IHCs (Platzer et al., 2000; Dou et al., 2004), pointing to a possible role of Ca^{2+} action potentials in the normal maturation of both immature hair cell types.

The first sign of functional maturation in mouse OHCs occurs between P6 and P8 with the down-regulation of $I_{K,neo}$ accompanied by the expression of $I_{K,n}$ from P8 (Marcotti & Kros, 1999). $I_{K,n}$ is thought to be carried by KCNQ4 channels (Marcotti & Kros, 1999; Jentsch, 2000; Beisel et al., 2005) and is suppressed by linopirdine (K_D: 0.7 μM in OHCs and 0.6 μM in IHCs), a potent blocker of the M-type current family (Costa & Brown, 1997; Schnee & Brown, 1998). Mutation of KCNQ4 leads to a form of non-syndromic dominant deafness found in humans (DFNA2; Kubisch et al., 1999), suggesting that $I_{K,n}$ may be important for maintaining the viability of hair cells, and neuronal connections within the cochlea. Since $I_{K,n}$ is largely active around the cell's resting membrane potential (Marcotti & Kros, 1999; Marcotti et al., 2003a; Oliver et al., 2003) it could provide an efficient exit route for K^+ ions entering through the mechano-electrical transducer

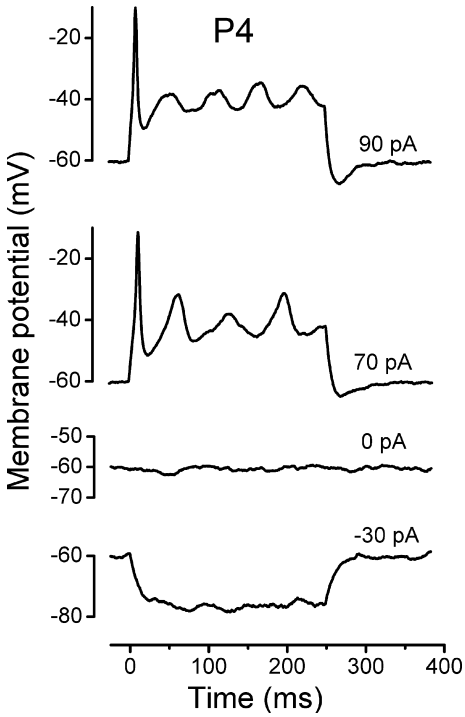

Fig. 4. Voltage responses in immature outer hair cells. Voltage responses from a P4 apical-coil OHC (modified from Marcotti & Kros, 1999). A single action potential was only elicited following depolarizing current injections. Recordings were obtained at body temperature.

channels. The functional absence of these channels in DFNA2 patients might lead to K^+ accumulation into the cells, although the hearing loss has a delayed onset. Moreover, the lack of $I_{K,n}$ will also cause hair cells to remain depolarized. In IHCs, the increased activation of the Ca^{2+} channels at rest due to the cells' depolarization will lead to an increased resting discharge activity of the afferent nerve fibres, which may lead to excitotoxicity (Pujol, 1985).

The expression of $I_{K,n}$ in mouse OHCs at P8 coincides with the onset of electromotility (He et al., 1994; Marcotti & Kros, 1999). Therefore, another possible role for $I_{K,n}$ in OHCs is that of reducing the cell's membrane time constant that could optimize the frequency response of the electromotile activity (Housley & Ashmore, 1992; Mammano & Ashmore, 1996), which is a characteristic feature of mature OHCs (Brownell et al., 1985; Ashmore, 1987; Santos-Sacchi & Dilger, 1988). This electromotile activity allows OHCs to expand or contract rapidly in response to changes in the receptor potential (Brownell et al., 1985), which is set up by the transducer current. This fast electromotility, in combination with force generated by the hair bundle of OHCs (Chan & Hudspeth, 2005; Kennedy, Crawford & Fettiplace, 2005), is likely to provide the feedback that drives cochlear amplification in mammals. In rats, the non-linear capacitance associated with electromotility increases steeply between P5 and P10 (Oliver & Fakler, 1999). These changes are driven by the motor protein prestin, an anion transporter family member, highly expressed in the membrane of OHCs (Zheng et al., 2000; Liberman et al., 2002). Finally, OHCs become responsive to the efferent neurotransmitter ACh around the time when both $I_{K,n}$ and the electromotile activity are acquired (Dulon & Lenoir, 1996; He & Dallos, 1999; Marcotti et al., 2004b). The temporal discrepancy in the responsiveness to ACh between IHCs (P0–P2) and OHCs (P6–P8) is likely to be related to the later formation of efferent synaptic connections with OHCs (Lenoir, Shnerson & Pujol, 1980; Pujol et al., 1998), which also corresponds to the expression of the heteromeric $\alpha9\alpha10$ nAChRs in these cells (Morley & Simmons, 2002). Mature OHCs also express purinergic receptors (P2X) that are activated by ATP. These receptors are localized in the stereocilia and are responsible for regulating the sensory transduction in the inner ear (*see* later section: Purinergic Regulation of Hair Cell Function).

Efferent Modulation of Hair Cells

The central auditory system plays an important role in controlling cochlear function through the efferent system. The efferent system, projecting to the inner ear or its homolog, exists in an extremely wide range of organisms, ranging from terrestrial mammals to deep-sea cephalopods (Clarke & Fitch, 1975; Dilly, 1976), which raise the intriguing possibility that evolutionary preservation of the system is of vital importance to the biological process of inner ear function. Yet, the exact mechanisms by which the system accomplishes its task have remained obscure since its discovery several decades ago (Rasmussen, 1942). The efferent innervation of the cochlea and vestibular end organs exerts control over the basic mechanisms and physiology of the cochlea and the vestibule. Several possible functions of the efferent system have been postulated. They may influence the dynamic range of hearing, sound localization, improve neural coding and signal processing with background noise, protect the ear against acoustic trauma, and may be involved with selective attention.

All hair cell systems, including lateral-line organs, vestibular organs, and the cochlea, contain an efferent innervation, originating in the brainstem and projecting to the hair cells and/or neural elements in the sensory epithelium. In the mammalian cochlea, this efferent system originates bilaterally from brainstem regions in and around the superior olivary complex and has been called the olivocochlear (OC) pathway (Rasmussen, 1946; Warr, 1975; Warr & Guinan, 1979; Guinan, Warr & Norris, 1983;

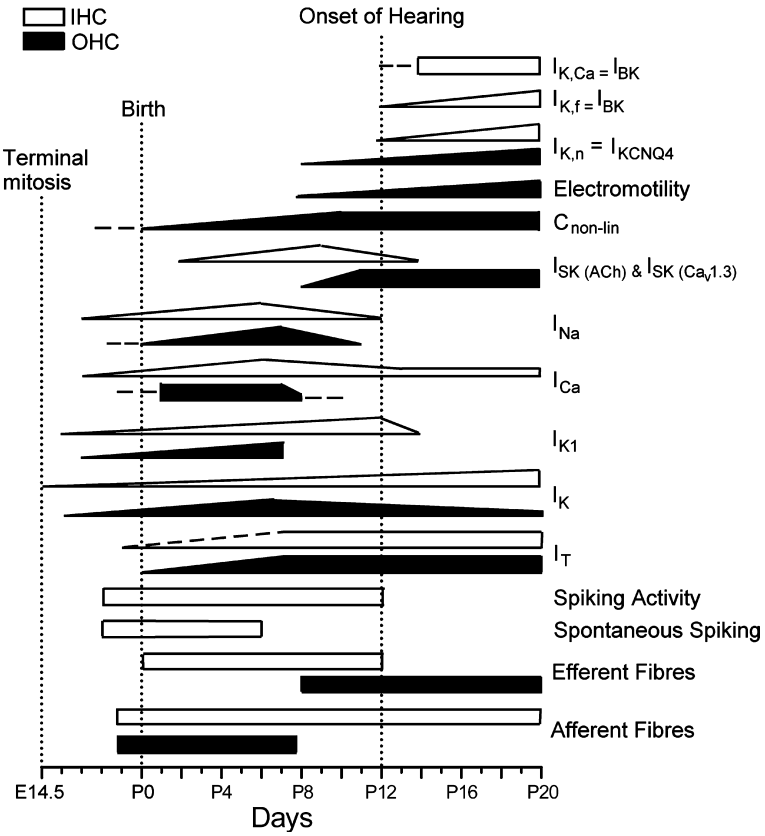

Fig. 5. Developmental changes in the expression of voltage- and Ca^{2+}-gated currents and other functional characteristics of hair cells in the mammalian cochlea. The timings of appearance and disappearance are mainly taken from the mouse. Data from other small rodents (rat, gerbil), which have a similar time course of auditory development, are also included. Changes in width of the horizontal bars give an approximate indication of developmental maturation in the size of the currents. The timing mainly refers to apical-coil cells. The day of birth is E19.5. Dashed lines are used when experimental data about the timing of expression are not available. Afferent fibres refer to radial afferent innervations. For more information about the development of both afferent and efferent fibres *see* Pujol et al. 1998. The timing of expression of the mechano-electrical transducer current (I_T) is from Marcotti, W., Richardson, G.P. and Kros, C.J. (onset of I_T: *unpublished observations*) and from Kennedy et al., (2003). I_K represents the classical delayed rectifier K^+ current (OHCs: $I_{K,emb}$, $I_{K,neo}$ and I_K; IHCs: $I_{K,emb}$, $I_{K,neo}$ and $I_{K,s}$). $I_{SK(ACh)}$ and $I_{SK(Cav1.3)}$ represent the development of the SK current activated by Ca^{2+} influx into the cells following the activation of either nAChRs or $Ca_V1.3$ Ca^{2+} channels, respectively. $C_{non\text{-}lin}$ indicates the non-linear capacitance. The development of I_{K1} in postnatal OHCs is from Marcotti, Johnson & Kros, *unpublished observations*.

Robertson, 1985; Brown, 1989). The OC system consists of two components (Warr & Guinan, 1979): the lateral (L)OC and the medial (M)OC systems. The lateral system originates in the lateral aspects of the olivary complex which gives rise to the small diameter, unmyelinated neurons that project primarily to the ipsilateral afferent nerve fibers under the inner hair cells (IHCs), with some contacting the IHCs. The LOC neurons originate from intrinsic neurons within the lateral superior olivary complex (LSO) and large "shell" neurons around the LSO (Vetter & Mugnaini, 1992). The LOC fibers are divided into two types based on unidirectional and bi-directional innervation patterns (Brown, 1987; Warr, Boche & Neely, 1997). The uni-directional LOC fibers enter the cochlea and turn either apically or basally for a short distance before terminating in discrete patches on the afferent dendrites. Moreover, unidirectional fibers account for ~85% of the LOC fibers. The bi-directional LOC fibers (15%) travel extensive distances throughout the cochlea. These fibers have many axonal swellings that were described by Warr et al. (1997) as possible synaptic boutons *en passant*. Warr et al. (1997) further characterized the bi-directional LOC fibers as originating from the shell neurons located around the LSO, while the unidirectional fibers originate from the intrinsic neurons within the LSO.

Several neurotransmitters have been identified and co-localized in cell bodies in the LSO (Altschuler, Parakkal & Fex, 1983; Altschuler et al., 1984; Altschuler, Hoffman & Wenthold, 1986; Abou-Madi et al., 1987; Altschuler et al., 1988; Safieddine, Prior & Eybalin, 1997) and in LOC terminals in the cochlea (Altschuler et al., 1985; Safieddine & Eybalin, 1992). Early immunocytochemical evidence (Altschuler et al., 1983; Vetter, Adams & Mugnaini, 1991; Safieddine & Eybalin, 1992) suggested that there may be fundamentally different subpopulations within the LOC system, based on the pattern of neuroactive substances that were co-localized (Satake & Liberman, 1996). However, subsequent results have led to the suggestion that ~90% or more of LOC efferents contain all six (acetylcholine, ACh; γ-aminobutyric acid, GABA; dopamine, DA; dynorphin, dyn; enkephalin, enk; and calcitonin-gene-related peptide, CGRP) of the putative LOC neurotransmitters (Safieddine et al., 1997). These transmitters can be further divided according to their likely effect on the afferent dendrite. ACh, dyn, and CGRP are likely to have an excitatory effect resulting in increased depolarization of hair cells and the ensuing enhancement of the glutamate released from the inner hair cells (Felix & Ehrenberger, 1992; Sahley & Nodar, 1994; Maison et al., 2003). On the other hand, the transmitters DA, Enk, and GABA are likely to be inhibitory and reduce the effect of

glutamate release from the IHC (Felix & Ehrenberger, 1992; Burki, Felix & Ehrenberger, 1993; d'Aldin et al., 1995; Malgrange et al., 1997; Oestreicher et al., 1997; Arnold et al., 1998; Sun & Salvi, 2001). Opposite effects of LOCS activation further suggest dual actions of LOC neurons (Groff & Liberman, 2003). Long-lasting suppression or enhancement of neural activity implies two classes of LOC neurons. However, co-localization of neurotransmitters suggests some other means of differentiating LOCS effects. Groff and Liberman (2003) postulated different driving circuitry and end targets, e.g., the afferent dendrite and the IHC, as well as differential innervation of low vs. high spontaneous firing rate fibers (Liberman, 1990). It is conceivable that differential effects of LOCS activation may involve the effects of dichotic stimulation that result in differences in adaptation between the two ears. Dual origins of LOCS fibers and the division of neurotransmitters into net excitatory or inhibitory effects may provide insight into differing functional effects of LOCS activation (Groff & Liberman, 2003).

The MOCS originates in the medial region of the SOC and gives rise to the large diameter, myelinated fibers directly innervating the OHCs. The efferent synaptic region of the OHC is large, and the innervation decreases in density from base to apex (Spoendlin, 1966; Liberman, 1990). The OHC plays a major role in determining the active micromechanical response of the cochlea. The afferent fibers (type II) from the OHC to the cochlear nucleus may converge with olivocochlear inputs (Brown, 1988; Brown & Ledwith, 1990; Benson & Brown, 2004). Most studies of the efferent system have attempted manipulations of the MOCS. Electrical stimulation of the olivary cochlear bundles at the floor of the 4^{th} ventricle causes a reduction of the compound action potential (CAP) amplitude (Galambos, 1956), reduction of 8^{th} nerve rate-level discharge responses (Wiederhold & Peake, 1966), augmentation of the cochlear microphonic (Fex, 1959), inhibition of the IHC receptor potential (Brown, Smith & Nuttall, 1983; Brown & Nuttall, 1984), reduction of basilar membrane velocity (Dolan & Nuttall, 1994; Murugasu & Russell, 1996), and improved the signal-to-noise ratio. Moreover, olivocochlear bundle activation can also increase basilar membrane velocity in certain stimulus conditions (Dolan, Guo & Nuttall, 1997). Thus, olivocochlear bundle stimulation affects the gain of the cochlear amplifier and the effect is typically greatest near threshold and at frequencies within the gain of the amplifier.

Acetylcholine (ACh) is the neurotransmitter released by the medial efferent fibers (Eybalin, 1993; LePrell et al., 2001). As indicated, analogous synaptic feedback has been observed in hair cells of fish lateral line, amphibian, reptilian and avian inner ears. In systems where equivalent measurements have been made, the cellular mechanisms appear similar among vertebrates. Although the mechanisms underlying the effects of ACh on the OHCs are not completely understood, ACh produces a surprising hyperpolarization of OHC (Housley & Ashmore, 1991; Doi & Ohmori, 1993; Kakehata et al., 1993; Erostegui, Norris & Bobbin, 1994). As it turns out, ACh released from efferent nerve terminals hyperpolarizes hair cells by activating calcium-dependent small conductance (SK) potassium channels.

The hair cell ACh receptor (AChR) is itself a ligand-gated cation channel, consistent with its genetic homology to nicotinic AChRs in neurons and muscle. In rat organ of Corti (OC) preparations, presynaptic depolarizations were shown to induce an inhibitory hyperpolarization of the OHC (Oliver et al., 2000). This response was blocked by 100 nM strychnine, a blocker of the $\alpha 9$ neuronal nicotinic receptors, and by 1 μM dequalinium, a blocker of the SK channel (Oliver et al., 2000; Strobaek et al., 2000). The nicotinic receptors of the OHC are thought to be heteroligomers composed of $\alpha 9$ and $\alpha 10$ nACh subunits (Elgoyhen et al., 2001). Although no homomeric ACh-activated channels were expressed in *Xenopus* oocytes when injected with $\alpha 10$ in vitro-transcribed RNA alone, co-injection of the $\alpha 9$ and $\alpha 10$ nACh subunits resulted in the expression of ACh-sensitive channels that differ from $\alpha 9$ homomeric receptors. Co-expression with the $\alpha 10$-subunit conferred a 100-fold increase in the current amplitude and indicated a direct interaction between the subunits. Furthermore, immunoprecipitation studies of cochlear protein extracts using $\alpha 9$- and $\alpha 10$-specific antibodies also showed interaction between these subunits. Additionally, when the extracts were prepared from transgenic mice engineered to overexpress the ACh $\alpha 9$, as expected, larger quantities of the $\alpha 9$ subunit were recovered in the immunoprecipitated complexes than in extracts from wild-type mice (Oliver et al., 2000). The $\alpha 9$ was then proposed to serve the function of recruiting and stabilizing the $\alpha 10$ subunits to the plasma membrane in vivo (Maison et al., 2003). ACh $\alpha 9$ receptor subunits have been cloned from the chick basilar papilla (Hiel, Luebke & Fuchs, 2000), and from rat, human and guinea pig tissues (Elgoyhen et al., 1994; Maison et al., 2003). Moreover, transcripts have been detected using in situ hybridization in the basilar papilla of the chick (Hiel et al., 2000), and in rat and guinea pig cochlear hair cells (Elgoyhen et al., 1994; Park, Niedzielski & Wenthold, 1997; Simmons & Morley, 1998). The $\alpha 10$ nACh subunit was cloned from a rat cochlea cDNA library and transcripts were detected using in situ hybridization to the hair cell in the rat cochlea and cristae (Elgoyhen et al., 2001). Thus, it is reasonable to surmise that the auditory and vestibular end organs employ AChR as an efferent target for the feedback modulation of hair cells.

Calcium entry through the hair cell AChR activates the calcium-dependent SK channels (Fuchs & Murrow, 1992). This ionotropic mechanism of cholinergic inhibition accounts for a number of important features of the cholinergic and synaptic responses of the hair cell. Most notably, the combined current is relatively fast, with the outward SK component delayed by only a few milliseconds after AChR activation (Glowatzki & Fuchs, 2000; Oliver et al., 2000), consistent with a direct, ionotropic gating process. A second latent feature is the apparent voltage dependence of the SK current. The voltage dependence of the current is a reflection of driving force of Ca^{2+} entry and has a 'bell shaped' current-voltage relationship, (Martin & Fuchs, 1992; Blanchet et al., 1996). As expected, the SK channel activation declines at positive membrane potentials where the driving force on Ca^{2+} entry is reduced, a feature which is uncharacteristic of a typical K^+ current. Furthermore, the rate of activation of the SK current is slower at positive membrane potentials, raising the intriguing possibility that Ca^{2+} must accumulate in a microdomain diffusion space to trigger the activation of SK channels. Incidentally, in HEK-293 cells co-transfected with cochlear SK2 plus $\alpha9/\alpha10$ receptors, acetylcholine induced an inward current followed by a robust outward current. The results indicate that SK2 and the $\alpha9/\alpha10$ acetylcholine receptors are sufficient to partly recapitulate the native hair cell efferent synaptic response (Nie et al., 2004). However, the time course of activation of the SK current was slower than in native cells, suggesting in OHCs there is a tight crosstalk between the SK and AChRs.

The SK channels form heteromeric complexes that contain pore-forming α-subunits and the Ca^{2+}-binding protein calmodulin (CaM). CaM binds to the SK channel through the CaM-binding domain (CaMBD), which is located in an intracellular region of the α-subunit immediately carboxy-terminal to the pore (Keen et al., 1999). Channel opening is triggered when Ca^{2+} binds the EF hands in the N-lobe of CaM (Schumacher et al., 2001). Recently, the structural basis of the ligand gating in a Ca^{2+}-activated K^+ channel has been determined using the crystal structure of a K^+ channel (MthK) from *M. thermoautotrophicum* in the Ca^{2+}-bound, opened state (Jiang et al., 2002). It was shown that eight intracellular domains form a gating ring at the intracellular surface, and that the gating ring uses the free energy of Ca^{2+} binding to perform mechanical work to open the pore of the channel.

Despite the evidence for a 'two-channel' model of cholinergic inhibition, a number of other observations argue that internal Ca^{2+} stores also might play a role in this process. First and most compellingly, efferent synapses are mirrored by an endoplasmic reticulum, or synaptic cistern, that lies within the hair cell, very near to the postsynaptic plasma membrane (Takasaka & Smith, 1971; Saito, 1980). Similar near-membrane cisterns in cerebellar neurons were suggested to be synaptically activated Ca^{2+} stores (Henkart, Landis & Reese, 1976). The fact that efferent synaptic cisterns appear to be ubiquitous among hair cells argues that these may serve some obligatory function. Evidence of a functional role for Ca^{2+} stores was obtained by pharmacological manipulation of efferent inhibition in the guinea pig cochlea (Sridhar et al., 1995; Sridhar, Brown & Sewell, 1997). These studies demonstrated that cochlear inhibition (measured by recording a variety of extracellular waveforms) rose along both fast and slow time courses during repetitive efferent shock trains. Both fast and slow effects were made larger by ryanodine, an agonist of Ca^{2+}-induced Ca^{2+} release (CICR) from internal stores. Blockers of the calcium ATPase (SERCA) enhanced the slow component. Other studies on isolated cochlear hair cells have provided additional evidence that cholinergic Ca^{2+} signaling might involve internal stores (Dallos et al., 1997; Evans et al., 2000).

There are two main store-release mechanisms, defined by the activator of the endoplasmic calcium channels. Ryanodine receptors (RyRs) form Ca^{2+} channels in endoplasmic membranes, and open in response to a rise of cytoplasmic calcium, thus providing CICR (Rossi & Sorrentino, 2002). Inositol triphosphate (IP3) receptors are equally large endoplasmic calcium channels, but activated by IP3 that in turn is synthesized by phospholipase C (Patel, Joseph & Thomas, 1999). In order to incorporate Ca^{2+} stores into a hypothetical cholinergic mechanism, their participation also must rely on Ca^{2+} influx. This would occur if Ca^{2+} influx through hair cell AChRs triggers CICR. However, the close apposition of the synaptic cistern makes it likely that such CICR could be extremely rapid, rendering it kinetically indistinguishable from the 'two-channel' hypothesis. For example, in skeletal muscles, ryanodine receptors can be activated within submillisecond time scale (Zahradnikova et al., 1999). Such tight coupling of CICR to AChR gating would be consistent with the evidence that SK channel kinetics determine the time course of cholinergic synaptic currents in OHCs (Oliver et al., 2000). This putative link between RyR and nACh-SK channel coupling is further reinforced by the recent localization of RyR1 mRNA to OHC, and supporting electrophysiological data (Lioudyno et al., 2004). Conversely, results from the guinea pig have indicated that IP3-dependent activation of SK currents by ACh may be present in OHCs (Kakehata et al., 1993; Dallos et al., 1997). The IP3 receptors can be activated by a rise of cytoplasmic calcium, albeit in the presence of 'basal' levels of IP3 (Taylor & Laude, 2002). These considerations suggest that the participation of IP3Rs should not rule out a priori,

particularly in the context of 'slow' cholinergic effects that might alter electromotility.

Finally, it may be germane to consider a third feature of calcium store signaling, the interplay between voltage-gated Ca^{2+} channels (VGCCs) and RyR (O'Brien et al., 2002). Cytoplasmic loops of the VGCC interact directly with RyR to trigger release from the sarcoplasmic reticulum. Furthermore, the gating of the VGCC is itself modulated by this interaction with RyR (Avila & Dirksen, 2000). Although there is no suggestive evidence that such cross talk occurs in hair cells, the near apposition of the synaptic cistern, like that of sarcoplasmic reticulum to muscle fiber membrane, supports the possibility and requires investigation. These suggestions also point to the scarcity of data to explain the tight relationship between the SK and AChRs in hair cells. Thus, future investigations will be centered around these fundamental questions regarding the molecular mechanisms of hair cell inhibition. How does Ca^{2+} influx through AChR confer inhibition? How does the activity of cytoplasmic calcium stores affect the mechanism of inhibition? If stores contribute Ca^{2+}, which class of Ca^{2+} release channels is involved? Are different mechanisms found in hair cells of different regions of the cochlea?

BK Channels are Required for Hearing in Hair Cells

Large-conductance Ca^{2+}-activated potassium channels are expressed by hair cells in many vertebrates. These channels have been described in both inner and outer hair cells of mammals (Ashmore & Meech, 1986; Kros & Crawford, 1990; Housley & Ashmore, 1992; Dulon et al., 1995; Langer, Grunder & Rusch, 2003; Skinner et al., 2003; Pyott et al., 2004; Hafidi et al., 2005). In mice these channels are detectable in inner hair cells co-incident with the onset of hearing at 10–12 days after birth (Kros et al., 1998). As described in the opening section of this review, the BK channels are thought to contribute significantly to the fast activating potassium current that both prevents action potentials in these cells and concurrently decreases its membrane time constant, important for its role as a high-frequency signal transducer (Kros et al., 1998). Knowledge of the BK ion channel subunit structure and subunit assembly has provided the opportunity to study the expression of these channels at a molecular level. For example, in the rat cochlea, use of antisense riboprobes directed to the transcript, and immunocytochemistry against the alpha subunit protein have shown that the inner hair cells (analogous to the turtle and chick basilar papilla hair cells) have a higher level of expression than the outer hair cells (Skinner et al., 2003; Pyott et al., 2004; Hafidi et al., 2005). However, the functional significance of the BK channels on gross measures of cochlear function is equivocal. Perfusion of the guinea-pig cochlea with the BK channel blockers charybdotoxin and iberiotoxin failed to affect either the cochlear microphonic (CM), which is an index of the hair cell receptor potential during sound stimulation, or the two-tone CM distortion product, an index of outer hair cell electromotility (Skinner et al., 2003). The exact role of these channels in mammals, however, is not known, and their significance to hearing has become controversial since knock-outs of the alpha subunit of BK channels in mice have produced quite different effects. However, knock-outs of the alpha subunits of BK channels had progressive deafness from age 4 months onwards (Ruttiger et al., 2004), although this was attributable to consequent inhibition of KCNQ4 channel expression. In contrast, a more recent study of BK channel alpha subunit knockout found no effects on auditory thresholds (Oliver et al., 2005). Confocal microscopy has demonstrated that in mouse cochlear inner hair cells, the BK channels have pronounced punctate localization in the sub-cuticular plate region (Pyott et al., 2004), with only limited additional expression in the basal synaptic pole (Hafidi et al., 2005). As noted in the preceding discussion, BK channel expression shows pronounced inactivation (Pyott et al., 2004), has a voltage dependency that indicates that it is uncoupled from VDCC (Kros & Crawford, 1990), but may be modulated by internal Ca^{2+} stores (Marcotti et al., 2004a).

In contrast to the ambiguous role of BK channels in mammalian hair cells, in the auditory system of lower vertebrates BK channels have been extremely well studied, and broad agreement on their roles has emerged. In a number of non-mammalian vertebrates, BK channels play a critical role in electrical tuning, a mechanism of frequency discrimination (Fettiplace & Fuchs, 1999). In addition, these channels are clustered and co-localized with voltage-gated Ca^{2+} channels (VGCCs) along the basolateral surface of these cells in close proximity to synaptic release sites where they likely modulate synaptic transmission (Roberts, Jacobs & Hudspeth, 1990; Issa & Hudspeth, 1994; Martinez-Dunst, Michaels & Fuchs, 1997; Rodriguez-Contreras & Yamoah, 2001, 2003; Samaranayake et al., 2004). Here we provide a background to the molecular physiology of the BK channels and indicate how variation of channel structure and function contributes to the regulation of hair cell membrane biophysics.

BK Channels — Structure, Function, and Associations

Key features of the BK channel are its large conductance (300 pS), high potassium selectivity, and (importantly for hair cells) synergistic activation by voltage and Ca^{2+} (Magleby, 2003). The gene

responsible for this current was first cloned from the *Drosophila* mutant Slowpoke (hence its name *slo*) (Atkinson, Robertson & Ganetzky, 1991). The Slo protein can form a channel when expressed alone (Adelman et al., 1992). After the discovery of other associated units, the Slo protein was named the alpha subunit (KCNMA1 in the new nomenclature, although here the term Slo will be used to refer to the alpha subunit). Four alpha subunits of the protein are needed to form a single channel (Shen et al., 1994).

The Slo primary structure has many similarities to voltage-gated potassium channels, including a "core" region of 6 transmembrane regions and a "P" loop containing the potassium selectivity filter (Butler et al., 1993; Magleby, 2003). It differs from the voltage-gated potassium channels in that it contains a large intracellular C-terminus, and an "extra" N-terminal transmembrane region (S0) that places the N-terminus on the outside of the cell (Butler et al., 1993; Meera et al., 1997). The C-terminus also contains several hydrophobic regions, named S7–S10, that could potentially span the membrane (Butler et al., 1993). Several sites responsible for physiological Ca^{2+} sensing (the so-called high-affinity sites) have been identified, including the Ca^{2+} bowl (a stretch of negatively charged amino acids in the intracellular C-terminus) and the residues M513 and D362/367 (Schreiber & Salkoff, 1997; Bao et al., 2002; Xia, Zeng & Lingle, 2002). The C-terminus of the protein contains two RCK (regulating the conductance of potassium) domains that have homology to a pair of domains in TrkA, a bacterial potassium transporter complex (Jiang et al., 2001). These RCK domains possibly function as a spring, linking the Ca^{2+} sensor in the intracellular C-terminus with the S6 transmembrane region, allowing Ca^{2+} to open the channel (Niu, Qian & Magleby, 2004).

The Slo gene is extensively spliced, and these splice variants affect the kinetics of the channel (Atkinson et al., 1991; Adelman et al., 1992; Butler et al., 1993). Most of the splice sites are located within the intracellular C-terminus (Atkinson et al., 1991; Adelman et al., 1992; Butler et al., 1993). The actual splice sites vary in different species, making correlations between structure and function in different species challenging (Atkinson et al., 1991; Adelman et al., 1992; Butler et al., 1993; Lagrutta et al., 1994). The splice sites within vertebrates appear to be conserved and in this review we will use the nomenclature used by Fettiplace and Fuchs in their recent review to enable a consistent narrative (Fettiplace & Fuchs, 1999).

The Slo protein interacts with several other protein subunits that can modulate its kinetics. These include the beta subunits 1 through 4 (Brenner et al., 2000), dSLIP (Xia et al., 1998a), dSLOB (Schopperle et al., 1998), beta-catenin (Lesage, Hibino & Hudspeth, 2004), syntaxin (Ling et al., 2003; Cibulsky, Fei & Levitan, 2005), cereblon (Jo et al., 2005), MAP1A (Park et al., 2004), and ANKRA (Lim & Park, 2005). All of the beta subunits increase sensitivity to both voltage and Ca^{2+}, but do so to a variable degree (Brenner et al., 2000). Beta-catenin was identified as a Slo-interacting protein by the Hudspeth group (Lesage et al., 2004), although a functional effect on the Slo protein has yet to be described. Syntaxin 1A was identified as a Slo-interacting protein by co-immunoprecipitation and has been shown to decrease both voltage and Ca^{2+} sensitivity of the BK channel (Ling et al., 2003; Cibulsky et al., 2005). Although syntaxin 1A also binds to the voltage-gated Ca^{2+} channel CaV 2.2 (Cibulsky et al., 2005), the authors were unable to show a complex containing Slo, Syntaxin 1A, and CaV 2.2. In fact, CaV 2.2 appeared to compete with Slo for binding with Syntaxin 1A (Schopperle et al., 1998; Cibulsky et al., 2005) and dSLIP (Xia et al., 1998a). It should be noted that synataxin 1A has been shown to interact with CaV1.3 (Song et al., 2003). However, the biochemical evidence is still lacking that BK channels interact directly with CaV1.3.

Electrical Tuning

Electrical tuning is a mechanism of frequency discrimination (Fettiplace & Fuchs, 1999). Initially described in turtle auditory hair cells (Crawford & Fettiplace, 1981), the phenomenon has been documented in other species and in hair cells of the vestibular epithelium (Ashmore, 1983; Art & Fettiplace, 1987; Fuchs & Evans, 1988; Fuchs, Nagai & Evans, 1988). Turtle auditory hair cells are arranged in a tonotopic manner, and their membrane potential oscillates in response to a small injected current (Crawford & Fettiplace, 1981; Lewis & Hudspeth, 1983). The frequency of this oscillation changes from cell to cell along the tonotopic axis and corresponds to the frequency of sound that the cell responds to best, known as the characteristic frequency (Crawford & Fettiplace, 1981). The currents responsible for this oscillation in membrane potential were first identified in frog vestibular hair cells as an interplay between an inward Ca^{2+} current and a consequent outward Ca^{2+}-activated potassium current, carried by the BK channel (Lewis & Hudspeth, 1983; Hudspeth & Lewis, 1988a, b). These currents have also been shown to cause oscillations in membrane potential in other species (Art & Fettiplace, 1987), where the inward Ca^{2+} current was determined to be carried largely by a dihydropyridine-sensitive L-type Ca^{2+} channel (Fuchs, Evans & Murrow, 1990; Rodriguez-Contreras & Yamoah, 2001). Factors responsible for the changing membrane oscillation frequency along the tonotopic axis include decreasing cell capacitance, increasing channel density in higher-frequency cells, degree of

co-localization between Ca^{2+} and BK channels (Hudspeth & Lewis, 1988b; Roberts et al., 1990), local Ca^{2+} buffering kinetics (Ricci, Gray-Keller & Fettiplace, 2000) and, most importantly, the changing kinetic properties of the BK channel (Art & Fettiplace, 1987; Wu et al., 1995). In contrast, the properties of the VGCC are invariant along the tonotopic axis (Art & Fettiplace, 1987).

Electrical tuning has been most extensively studied in the turtle and chick. In trying to explain the changing oscillation frequency of hair cells, Art et al. looked at the macroscopic current properties of the inward Ca^{2+} current and the outward K^+ current in individual hair cells from the turtle (Art & Fettiplace, 1987). They found no difference in the kinetics of the Ca^{2+} currents, but found a systematic change in the deactivation properties of the outward BK current. The deactivation time constant varied from less than 1 ms to over 150 ms in response to a step voltage pulse (+50 to −50 mV in 4 μM Ca^{2+}, thought to reflect conditions in the native hair cell), and was related to the tonotopic location of the cell (Art, Crawford & Fettiplace, 1986). In contrast, the activation of these currents was fast, noninactivating, and invariant. In a single-channel analysis of excised inside-out and cell-attached patches, these authors noted a weaker correlation between mean open times and tonotopic location (Art, Wu & Fettiplace, 1995). They also found that several properties of the BK channel were invariant along the tonotopic axis, including the single-channel conductance (320 pS in symmetric K^+ solutions) and Ca^{2+} sensitivities (K_d 2 μM at +50 mV and 12 μM at −50 mV). These results raise a fundamental question — how do single-channel kinetics relate to macroscopic currents, and why is there a discordance between the implied relationship between mean open channel times and deactivation times (the former has a weak correlation with characteristic frequency and the latter has an exact relationship)? One possible explanation for this discrepancy could be the inherent wide variation in single-channel data from BK channels. For instance, Horrigan and Aldrich (2002) report a 20–30 mV difference in open probability – voltage relationship of different patches of these channels under identical conditions.

Similar macroscopic current properties have been demonstrated in the chick basilar papilla hair cells. Earlier work from the Fuchs lab demonstrated that electrical resonance occurred in the chick hair cells and that the fluctuation in membrane potential occurred as a result of an interplay between the BK current and the L-type Ca^{2+} channel (Fuchs & Evans, 1988, 1990; Fuchs et al., 1990). More recently, Duncan and Fuchs have shown that, as for turtle hair hair cells, the non-inactivating BK currents in the chick have fast activation, with cells along the tonotopic axis of the papilla showing similar rates of activation (Duncan & Fuchs, 2003). Similarly to turtle, the rates of deactivation of the chick hair cell BK channels following a step hyperpolarization (+100 to −100) of these hair cells correlated with tonotopic location, with a linear relationship between the characteristic frequency and the inverse of the square root of the time constant of deactivation (Duncan & Fuchs, 2003). In these studies (at room temperature) time constants for deactivation varied from 0.1 ms to a few milliseconds (Fuchs & Evans, 1990).

Duncan and Fuchs also noted differences between BK channels in chick and turtle (Duncan & Fuchs, 2003). These results on the one hand support and on the other hand negate the possible role of the beta-1 subunit in electrical tuning in the papilla. They observed that in the chick, in contrast to the turtle, the Ca^{2+} sensitivity of the BK channel varied with tonotopic location, so that cells from the low-frequency region of the papilla had BK channels that were more Ca^{2+}-sensitive than those from the high-frequency region. This correlation was more marked at +50 mV than at −50mV (Duncan & Fuchs, 2003). Furthermore, the Ca^{2+} sensitivities of individual BK channels from the low-frequency region were spread over a smaller range of Ca^{2+} concentrations than channels from cells in the high-frequency region (Duncan & Fuchs, 2003). These effects may be due to the presence of the beta-1 subunit of the BK channel in the low-frequency end of the papilla in the quail and chick (Ramanathan et al., 1999). The co-expression of the avian beta-1 subunit has two effects on the Ca^{2+} sensitivity of individual splice variants expressed in HEK cells, increasing apparent Ca^{2+} sensitivity and decreasing the spread in Ca^{2+} sensitivity between different splice variants (Ramanathan et al., 1999; Ramanathan, Michael & Fuchs, 2000). However, Duncan and Fuchs also observed that the open probability–voltage relationship of hair cell BK channels at the low-frequency end of the chick papilla is steeper than at the high-frequency end of the papilla, which would argue against a role for the beta-1 subunit (Duncan & Fuchs, 2003). Bovine beta subunits cause a decrease in open probability per unit change in voltage of the mouse alpha forms evidenced by a decrease in the slope of the Po–V plot (Cox & Aldrich, 2000). Thus it is likely that additional mechanisms modulate BK channel function.

Molecular Basis of Changing BK Channel Function Along the Tonotopic Axis

Three mechanisms could influence the properties of the BK current: the changing primary structure of the alpha subunit (Slo), post-translational modifications of Slo, and association of Slo with other proteins. Although phosphorylation of the Slo protein has been shown to affect its kinetics (Reinhart & Levitan,

1995; Alioua et al., 1998; Schubert & Nelson, 2001), little else is known about this post-translational modification of hair cell BK channels. The effect of the primary structure of Slo and its associations with the beta-1 subunit as it applies to hair cell BK channel function is reviewed below.

The Role of Primary Structure (Splicing) in Electrical Tuning

There is extensive alternative splicing of the Slo channel in non-mammalian vertebrate hair cells. The pattern of alternative splicing changes tonotopically, with single hair cells expressing more than one splice variant. Although alternative splicing alters the kinetic properties of the channel, the range of deactivation time constants in heterologously expressed channels is less than that of native channels.

In the chick, alternative splicing has been explored at 3 separate positions in the Slo transcript (Navaratnam et al., 1997; Rosenblatt et al., 1997), X3, X4, and X5 in the nomenclature of Fettiplace and Fuchs (Fettiplace & Fuchs, 1999). At X3, 2 splice variants are created by alternative splicing, resulting in proteins that differ in length by 4 amino acids (Navaratnam et al., 1997; Rosenblatt et al., 1997). Data from Rosenblatt et al. suggest that the splice variant with the extra 4 amino acids (SRKR) is less abundant at the high-frequency end of the papilla, with the shorter form having the opposite distribution (Rosenblatt et al., 1997). At X4, three splice variants have been found, including a readthrough form (no extra amino acids), a form with 3 extra amino acids (IYF), and a form with 61 extra amino acids (Navaratnam et al., 1997; Ramanathan et al., 1999, 2000). Of these, the readthrough form is present in greater abundance in the middle two quadrants of the papilla than the 3-amino-acid form (Navaratnam et al., 1997). The 61-amino-acid variant has been isolated from the cochlea, but its distribution along the tonotopic axis has yet to be determined (Ramanathan et al., 2000). Two splice variants at site X5 have been isolated, including a readthrough form and an exon containing an extra 28 amino acids (Navaratnam et al., 1997). The latter is more prevalent in the high-frequency quadrants (Navaratnam et al., 1997). Finally, of two C-termini created by alternative splicing, one (KEDRL) is more abundant in the high-frequency half of the papilla (Navaratnam et al., 1997). While these experiments established the expression profile of alternate splicing isoforms along the tonotopic axis, we do not yet know with certainty if particular isoforms are coupled with other specific isoforms or if the distribution is random. A limited set of data suggested that the individual alternative spliced forms at a particular splice site were coupled randomly with other alternatively spliced variants at other sites (Navaratnam et al., 1997).

In the turtle, Jones et al., found eight isoforms expressed in the basilar papilla (Jones, Laus & Fettiplace, 1998; Jones, Gray-Keller & Fettiplace, 1999). All the splice variants were the result of splicing in 2 regions: X3 and X4 (Jones et al., 1998, 1999). X3 splice variants containing 0, 4, or 31 amino acids were variably coupled with X4 isoforms containing −26, 0, 3, or 61 extra amino acids. Thus the splice variants were 0,0; 0,3; 4, −26; 4,0; 4,3; 4,61; 31,0; and 31,3 (Jones et al., 1998, 1999). Of these 4, −26; 4,0; and 31,3 were localized in the 3 lowest-frequency quadrants, while 4,61 was found primarily in the high-frequency half of the papilla (Jones et al., 1998, 1999). The distribution of the remaining 4 variants was indeterminate (Jones et al., 1998, 1999).

The kinetic properties of the BK channel splice variants seen in the turtle were established by expressing these proteins in oocytes. The different BK channel isoforms had varying Ca^{2+} sensitivities, and there was a relationship between the Ca^{2+} sensitivity and splice insert length on the one hand and deactivation kinetics on the other (Jones et al., 1999). Channels with higher Ca^{2+} sensitivities demonstrated slower deactivation (Jones et al., 1999). However, since native channels with the fastest deactivation times are found at the high-frequency encoding end of the basilar papilla, the actual tonotopic distribution of the splice variants was the reverse of that expected (Jones et al., 1998, 1999). Ca^{2+} sensitivities varied by almost two orders of magnitude (Jones et al., 1999), in contrast to the more uniform distribution of Ca^{2+} sensitivities in the native hair cell channel (Art et al., 1995). One feature that was consistent between the expressed and native BK channels was the single-channel conductance (286 ± 16 pS in expressed vs. 290–340 pS in native channels) (Art et al., 1995; Jones et al., 1999). The turtle data suggest the following two important facts: 1) Ca^{2+} sensitivity is a key determinant of deactivation kinetics of heterologously expressed BK channels, and 2) there is a discrepancy between the tonotopic distribution of isoforms and the kinetics of native hair cell BK channels.

Similarly, several splice variants of the chick hair cell BK channels have been isolated and expressed in HEK cells, and overall these data agree with those from the turtle, with a few minor differences. As in the turtle, the chick isoforms have variable Ca^{2+} sensitivities that relate to splicing isoforms and deactivation kinetics. Ramanathan et al. (1999, 2000) describe four different splice variants at sites X3, X4, and X6: 0,0,0; 4,0,0; 0,61,0; and 4,61,28. Of these isoforms, the splice variants 0,0,0 and 4,61,28 have been shown to occur naturally in the chick cochlea. Consistent with the data in the turtle (Jones et al., 1999), variants with an insert encoding the extra 61 amino acids had significantly slower kinetics than the variants lacking these amino acids (Ramanathan

et al., 1999, 2000). Also similar to the turtle, the isoforms with the slowest deactivation had the larger inserts and the greatest Ca^{2+} sensitivity. Furthermore, these results agree with other data from the human channel, where the addition of the 4 amino acids at X3 results in a 5-fold reduction in Ca^{2+} sensitivity (Tseng-Crank et al., 1994). Rosenblatt et al. (1997) found that the isoform with the extra 4 amino acids at X3 was less abundant in the 2 quadrants with the highest frequency. Thus, there appears to be a contradiction between the expected kinetic properties of BK currents based on the tonotopic location of the splice variant (decreased Ca^{2+} sensitivity of RSRS variants in low-frequency cells) and the observed kinetics of the native channels (increased Ca^{2+} sensitivity of low-frequency cells).

The Role of Accessory Subunits in Electrical Tuning

Once it was apparent that primary structure alone could not explain the changing kinetic properties of the channel, other mechanisms that could explain the discrepancy between native and reconstituted channels were sought. The beta-1 subunit of the channel had been shown to increase the Ca^{2+} sensitivity of Slo (McManus et al., 1995). Since the heterologously expressed channels had less apparent sensitivity to Ca^{2+} than native channels, the beta-1 subunit was a good candidate for a protein that interacts with Slo in hair cells. The expression of the Slo protein with the beta-1 subunit could explain some aspects of the native channel kinetics, although it is clear that this interaction is modulated by other mechanisms (Ramanathan et al., 1999, 2000). For instance, the Fuchs group found that the beta-1 subunit increased apparent Ca^{2+} sensitivity of the different splice forms (Ramanathan et al., 1999, 2000). Thus, isoforms containing the extra 61 amino acids had a 2-fold increase in apparent Ca^{2+} sensitivity (Ramanathan et al., 1999, 2000). In contrast, forms lacking the 61 amino acids (that when expressed alone, had proportionally much lower apparent Ca^{2+} sensitivity) conferred on Slo a 5-fold increase in apparent Ca^{2+} sensitivity. These results are consistent with the findings that the beta-1 subunit is present in the low-frequency half of the chick papilla and native channels of hair cells in the low-frequency end have a lesser spread in Ca^{2+} sensitivity, suggesting a significant role for the beta-1 subunit in electrical tuning (Ramanathan et al., 1999, 2000). However, the beta-1 subunit's effects on deactivation kinetics were to prolong deactivation time constants by orders of magnitude to a range not found in native channels except at the extreme low-frequency end. In addition, the beta-1 subunit *increased* the spread between the deactivation times of the different splice forms. For example, forms having the extra 61 amino acids (that alone had slower deactivation times) showed a 20-fold slowing of deactivation time when expressed with beta-1 compared to the forms without the extra 61 amino acids, which had a 10-fold slowing in deactivation time (Ramanathan et al., 1999, 2000). Thus, it seemed that the beta-1 subunit produced two effects on the chick hair cell BK channel splice variants, increasing the apparent sensitivity to Ca^{2+} and the deactivation times between the different splice forms (Ramanathan et al., 1999, 2000).

Other BK channel subunits have been shown to affect the channel kinetics. The dSlob (Schopperle et al., 1998) and dSLIP (Xia et al., 1998a) subunits in *Drosophila* have no vertebrate counterparts, but beta-2 and -4 subunits have been described in chick hair cells (Ortega et al., 2005). These proteins both increase apparent Ca^{2+} sensitivity (Wallner, Meera & Toro, 1999; Brenner et al., 2000; Ortega et al., 2005).

Since the beta-1 subunit was found to be expressed only in the low-frequency half of the papilla with a gradient in expression levels, Ramanathan et al. made theoretical models of hair-cell tuning (membrane oscillation frequency), varying the amount of beta and alpha subunits (Ramanathan & Fuchs, 2002). Their model titrated beta subunit expression with alpha subunit expression and also increased amounts of alpha subunit expression along the tonotopic axis. Two conclusions were drawn: "1) a hair cell expressing a mixture of channel types can [have membrane voltages that] oscillate with a single frequency and 2) its tuning frequency is intermediate to those produced by each channel type expressed on its own". For instance, in their model the membrane potential oscillated at 968 Hz when one third of the alpha-0 form was bound to beta-1 sub-units while the remainder was not. Increasing the proportion of alpha0 subunits bound to beta-1 to two thirds resulted in an oscillation frequency of 717 Hz. Since individual splice variants of the alpha sub-units alone have different deactivation times, these modeling experiments would additionally suggest that varying the ratio of alpha subunit splice forms can give rise to a changing oscillation in membrane potential. This is a real possibility because individual chick hair cells can contain more than one splice variant. Furthermore, results showing varying tonotopic gradients of beta subunits that also suggest varying ratios of these proteins in individual hair cells (Ortega et al., 2005) adds to the complexity of this model.

Post Translational Modification (Phosphorylation) and its Intersection with Splicing

While important, it is clear from the review of experimental data above that alternative splicing and association with the beta-1 subunit are insufficient to

account for the wide range in kinetics BK channels display in hair cells. Post-translational modification of BK channel subunits, particularly phosphorylation, has received wide attention in other systems, and closer analysis suggests that these effects can be extrapolated to hair cells where they likely play an important role. In many tissues, phosphorylation of BK channels by protein kinases A, C and G affect activity with considerable complexity (Schubert & Nelson, 2001; Zhou et al., 2001; Zhu et al., 2005), including dependence upon alternative splicing (e.g., Tian et al., 2004; Zhang et al., 2004; Chen et al., 2005). Multiplex signaling is evident, where for example, phosphorylation of airway smooth muscle by PKC acts as a switch, allowing BK channels to subsequently respond to PKG, while inhibiting their activation by PKA (Zhou et al., 2001). Moreover, the response to kinases is dependent on alternative splicing. For example, inclusion of the C-terminal splice variants that contain phosphorylation motifs (Ser 1151 and Ser 1154) that can be phosphorylated by PKC, makes the channel responsive to PKC (and subsequently PKG), while those lacking this C terminal splice variant are unresponsive to PKC but responsive to PKA (Zhou et al., 2001). Similarly, the response to PKA is also dependent on alternative splicing, with Slo isoforms including the STREX exon inhibited by PKA and those lacking this exon activated by PKA (Tian et al., 2004; Zhang et al., 2004; Chen et al., 2005). Specific phosphatases counter the regulation of BK channel activity produced by the protein kinases (Widmer, Rowe & Shipston, 2003).

The influence of the accessory subunits on BK channel activity is also influenced by phosphorylation. For instance, the increased open probability of Slo that occurs with the addition of the beta subunit can be partially reversed with the addition of the catalytic subunit of protein kinase A (Dworetzky et al., 1996). These results have important implications to hair cell BK channels. For example, native BK channels from the low-frequency end of the papilla, where the beta-1 unit is most prevalent, do not show the prolonged deactivation times seen when the alpha and beta subunits are expressed alone (Ramanathan et al., 1999, 2000). Is it therefore possible that PKA modulates the interaction between Slo alpha and beta subunits in native hair cells?

It is evident from the data reviewed that our understanding of BK channel behavior at the molecular level is incomplete as it relates to electrical tuning. The initial hope that alternative splicing would explain the changing kinetic properties of the channel is likely to be an incomplete explanation. Alternative splicing, interaction with an ever increasing number of protein subunits, and phosphorylation with its dependence on the aforementioned mechanisms are all likely to play an important role in explaining the kinetics of this channel in native hair cells. However, it is also possible that hitherto unexplored mechanisms — other post-translational modifications (myristoylation for example) — may also play a critical role in determining native hair cell BK channel kinetics.

Purinergic Regulation of Hair Cell Function

Cochlear hair cells possess several signal transduction mechanisms which enable the cells to respond to neural and humoral stimuli. The efferent feedback regulation of outer hair cell function via the medial olivocochlear bundle is the most thoroughly studied of the neurohumoral signaling pathways. This system, along with the complementary lateral olivocochlear efferent bundle input to the inner hair cell afferent synapses, is described earlier. The focus here is on the interplay of the neural and humoral factors which likely enable the hair cells to operate over a wide dynamic range, sustaining cochlear function by regulating the sensitivity of both outer and inner hair cell mechanoelectrical transduction under stressors, mechanical vibration and increasing sound intensity (*see* Fig. 6). Under such conditions, adenosine 5′ triphosphate (ATP) is released from cochlear tissues and affects hair cell function, both directly and indirectly.

Purines and pyrimidines (for example, ATP and UTP) provide key signaling molecules for extracellular nucleotide signal transduction (Lazarowski, Boucher & Harden, 2003). The physiological significance of ATP, acting as an extracellular signaling molecule, was initially demonstrated with regard to cardiovascular blood flow by Drury and Szent-Gyorgyi (Drury & Szent-Gyorgyi, 1929). The ground-breaking studies in this burgeoning field are attributed to Professor Geoffrey Burnstock and collaborators. In the 1970's the Burnstock group established ATP as the mediator of non-adrenergic non-cholinergic (NANC) transmission in the enteric nervous system (Burnstock et al., 1970). Purinergic signaling mechanisms feature prominently in the molecular and cellular physiology of sensory systems (Thorne & Housley, 1996; Housley, 1998, 2000, 2001), including vision (Wheeler-Schilling et al., 2001; Puthussery & Fletcher, 2004), pain, touch and pressure sensors (Burnstock, 1996; North, 2003), taste (Bo et al., 1999), olfaction (Kanjhan et al., 1999; Hegg et al., 2003), and peripheral and central chemoreceptors controlling respiration (Rong et al., 2003; Spyer, Dale & Gourine, 2004; Gourine et al., 2005a, b). The elements of purinergic signaling include: (1) nucleotide release from tissues (Burnstock et al., 1978; Bodin & Burnstock, 1996; Hazama, Hayashi & Okada, 1998; Beigi et al., 1999; Vlaskovska et al., 2001; Leybaert et al., 2003; Schwiebert & Zsembery, 2003; Gale et al., 2004; Katsuragi & Migita, 2004); (2) transduction via P2X receptors (which are ATP-gated

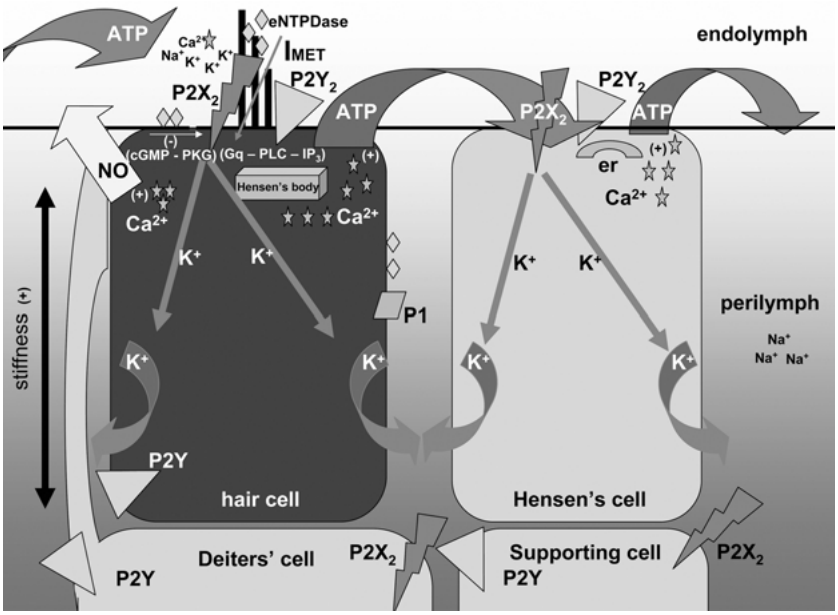

Fig. 6. Summary of purinergic signaling in cochlear hair cells showing an integrative action of extracellular nucleotides and nucleosides on cochlear partition electrochemical homeostasis and organ of Corti micromechanics. P2X and P2Y receptors for extracellular nucleotides, particularly ATP, provide autocrine and paracrine signaling pathways in hair cells and associated supporting cells in the cochlear partition. P1 receptors represent the adenosine receptor family. P2Y2 receptor activation is thought to trigger extracellular ATP-induced ATP release from a pathway which may include connexon hemichannels. Ectonucleotidases hydrolyze the nucleotides to nucleosides, which are then transported back into the cells. The P2X receptors localized to the apical surface of the hair cells and epithelial cells provide a shunt pathway for K^+ efflux out of the endolymphatic compartment — reducing the endocochlear potential. Feedback regulation by second messengers influences purinergic signaling, particularly Ca^{2+} and NO. For clarity, the basolateral prestin lattice underlying OHC electromotility is not shown. Further, the hair cell is stylized due to the relevance of this model to both inner and outer hair cells.

non-selective cation channels assembled from homo- or heteromultimers of P2X receptor subunits) and P2Y receptors, which are metabotropic G protein-coupled receptors (North & Barnard, 1997; North, 2002; Inbe et al., 2004); (3) signal termination via ectonucleotidediphosphodyrolases (eNTPDases) which successively hydrolyze extracellular nucleotide tri-and di-phosphates towards nucleosides (adenosine) (Vlajkovic et al., 1998; Zimmermann et al., 1998); (4) P1 receptors (G protein-coupled adenosine receptors; A1–A3) (Fredholm et al., 2001); (5) transporters for reuptake of nucleosides (Baldwin et al., 1999). Within sensory systems, these elements of purinergic signal transduction are arguably best characterized within the cochlea and have to a large extent been associated directly with the cochlear hair cells.

The evidence for the functional significance of purinergic signaling on hearing developed from the early demonstration by Bobbin and Thompson (1978) that perfusion of the perilymphatic compartment of the guinea pig cochlea with ATP affected auditory nerve activity. Bryant et al. (1987) demonstrated inhibition of spontaneous activity from the frog crista with adenosine perfusion, which could be antagonized by theophylline and was mimicked by application of dipyrimidole, which inhibits adenosine transport. In this preparation, application of ATP and ATP agonists had an opposing effect (Aubert, Norris & Guth, 1994). Cellular physiology complemented these studies of purinergic action in the inner ear, when Nakagawa (Nakagawa et al., 1990) showed that ATP activated inward currents in isolated cochlear outer hair cells and Ashmore and Ohmori (1990) demonstrated co-incident increase in intracellular Ca^{2+} along with ATP-gated inward currents in these cells. This is consistent with Ca^{2+} entry via ATP-gated ion channels, which have substantial Ca^{2+} permeability, and activation of P2Y receptors, which invoke Ca^{2+} release from IP_3 receptor-gated stores. While it was initially considered that the P2X receptor-mediated currents may have been associated with the cholinergic efferent regulation of OHC function, as evident in the parasympathetic nervous system (Burnstock, 2003), a comparable involvement of P2X receptors at the post-synaptic efferent synaptic membrane of the OHC was deemed unlikely when electrophysiological experiments demonstrated that the ATP-gated currents were confined to the apical region of the guinea pig OHC (Housley, Greenwood & Ashmore, 1992). Subsequent studies have resolved a complex purinergic signalplex, from accumulating evidence for wide-spread expression of P2X receptors in a wide variety of cochlear tissues, including the prominence of $P2X_2R$ transcripts in

epithelial cells lining scala media (Housley, Luo & Ryan, 1998a). These data implicate purinergic signaling in regulation of cochlear electrochemical homeostasis, auditory neurotransmission, cochlear micromechanics and cochlear blood flow (*see* Housley et al., 2002; Housley & Thorne, 2000 for reviews). Pertinent to the consideration of hair cell function are the direct and indirect mechanisms of action on the cochlear outer and inner hair cells. In particular, in the later section considering the functional significance of purinergic signaling pertaining to the hair cells, we address the interaction of P2X, P2Y, and P1 (adenosine) receptor expression, ecto-NTPDase action, and consideration of mechanisms of release of ATP, the signal molecule.

Hair Cell P2X Receptor Expression

As noted above, the ATP-gated inward currents in outer hair cells originally identified by Nakagawa et al. (1990) likely arise from what have subsequently been characterized as $P2X_2$ receptors. The first members of the P2X family of ionotropic receptors were cloned in 1994 (Brake, Wagenbach & Julius, 1994; Valera et al., 1994), now designated $P2X_1$ and $P2X_2$. The P2X family of channels has seven members, with a structure resembling the inwardly rectifying class of K^+ channel subunits which have two membrane-spanning domain subunits. However, KIR channels assemble from 4 subunits, whereas P2X receptors assemble as trimers with multiple conformation states (Khakh & Egan, 2005). The P2X receptors form ATP-gated ion channels, which are non-selective cation channels that have considerable phenotypic diversity arising from homo- and heteromeric subunit assembly (for reviews *see*: Khakh, 2001; North, 2002). Thus, the likelihood that the ATP-gated ion channels expressed by the cochlear hair cells arise from homomeric assembly of $P2X_2$ receptor subunits is based on the immunolocalization of the $P2X_2$ receptor to guinea-pig OHC stereocilia and cuticular plate, including the high spatial resolution provided by immunogold electronmicroscopy (Housley et al., 1999). This is functionally supported by comparison of the pharmacology of the OHC ATP-activated currents with those of recombinant $P2X_2$ receptors (for example, signature allosteric enhancement of the ATP-gated currents by low pH, Cu^{2+} and Zn^{2+} (Kanjhan et al., 2003). $P2X_2$ transcript has been localized to the neonatal and adult rat OHC (Housley et al., 1998a; Brandle, Zenner & Ruppersberg, 1999) and localization of ATP-gated currents and $P2X_2$ receptor protein to the hair cell stereocilia has also been confirmed in rat and mouse OHC (Järlebark, Housley & Thorne, 2000; Järlebark et al., 2002).

In the rat cochlea, $P2X_2$ expression was considerably up-regulated by exposure of the animal to moderate sound levels (90 dB white noise for 3 days), with increased intensity of the immunolabeling of the OHC stereocilia and also an increase of > 300% in the magnitude of the ATP-activated inward currents under voltage clamp (Wang et al., 2003). There is some variation in $P2X_2$ receptor expression between species, in that rat inner hair cell stereocilia were not immunolabeled in the Järlebark study. A semiquantitative analysis of ATP-gated currents, and hence $P2X_2$ receptor expression, has been performed in the guinea-pig inner and outer hair cells (Raybould & Housley, 1997; Raybould, Jagger & Housley, 2001). In the case of the OHCs, a tonotopic increase in the P2X receptor - mediated currents was detected in cells originating from more basal (high-frequency-coding) regions—with currents large enough to saturate the patch-clamp amplifier! This likely corresponds to the highest reported P2X receptor expression level in any native cell type, with an estimate of > 8,000 channels (averaging > 165 nS conductance). In contrast, the OHC from the apical-turn region had considerably smaller ATP-gated inward currents, corresponding to < 3,000 channels. A key feature of this is that the OHCs also exhibit an equivalent tonotopic gradient in K^+ conductances, and the ratio of maximum evoked ATP-activated conductance to the standing K^+ conductance of these cells (including a dominant $I_{K,n}$; *see* previous discussion of guinea-pig hair cell K^+ conductances) was constant at around 3:1, irrespective of location. It seems likely therefore that the P2X receptor conductance has the capacity to dominate cation entry from the endolymphatic compartment of the hair cells (where, interestingly, K^+ would provide the major cation for this non-selective cation conductance) to an extent that may invoke sustained depolarization of the cells against the background basolateral K^+ conductance which provides the current egress pathway. However, given that both OHCs and IHCs have considerable Ca^{2+}-regulated K^+ conductance, it is not surprising that the significant Ca^{2+} permeability of the P2X receptor conductance leads to rapid secondary recruitment of K_{Ca}. Thus the physiological significance of ATP activation of P2X receptors on cochlear hair cells is undoubtedly complex, with both depolarization and hyperpolarization evident — depolarization tending to dominate in OHCs, while hyperpolarization is invoked in IHC (Housley et al., 1993; Sugasawa et al., 1996). This is via an increased conductance through the apical membrane in parallel with the mechanotransducer current, and potentially outstripping it. It is likely that Ca^{2+} entry via the P2X channels also results in recruitment of the basolateral and apical K_{Ca} conductances. It is interesting to note that in the case of the guinea-pig IHC, the P2X receptor-mediated conductance did not exhibit such a tonotopic variation (31 nS, representing about 1500

ATP-gated ion channels), but the K^+ conductances were also largely constant in IHCs isolated from different regions of the cochlea (except for the apical-most region), and again the ratio P2X conductance to conductance around the resting potential of the cells was approximately 3:1. This finding raised the possibility that the IHC basolateral membrane conductance is tuned for a mechanoelectrical stimulus largely defined by the OHC-derived cochlear amplifier electromechanical coupling though the basilar membrane, and that release of ATP into scala media, which would recruit the P2X conductance, may substantially alter the cochlear amplifier. As we observe later, it is not tenable to consider the action of the P2X receptor activation of the hair cells in isolation, because of the contributions of the activation of these receptors on the other epithelial cells lining scala media, and also the effects of P2X receptor activation on supporting cells such as Deiters' cells. Given the localization of the $P2X_2$ receptors to the endolymphatic surface of the cochlear hair cells, and the concurrent expression of the receptors by adjacent cell types in both perilymphatic and endolymphatic compartments, experimental approaches to consider the physiological significance of $P2X_2$ receptor expression by cochlear hair cells are problematic. The current hypotheses are addressed following consideration of additional hair cell purinergic signaling elements.

There is evidence that P2X receptor subunits in addition to $P2X_2$ are expressed by cochlear hair cells (Szucs et al., 2004). $P2X_7$ receptors have been immunolocalized to the rat IHCs and OHCs in the first postnatal week (Nikolic, Housley & Thorne, 2003), and recently, Huang et al. (2006) showed that in the mouse cochlea, $P2X_3$ was transiently expressed by hair cells around this period. $P2X_3$ receptor immunolocalization occurred within the cytosol of the basal and mid-turn region hair cells prior to birth, progressing as a wave of expression towards the apex of the cochlea, ending around postnatal day 3 (P3) with residual expression in the most apical OHCs. This was not evident in their preceding analysis of rat cochlear $P2X_3$ receptor expression (Huang et al., 2005). Neonatal mouse cochlear OHCs (P0–P4) exhibit sustained ATP-gated currents that are blocked by d-tubocurarine at a comparable K_d to that of recombinant $P2X_2$ receptors (Glowatzki et al., 1997). Therefore, the significance of the transient diversity in hair cell expression of some of the other P2X receptor subtypes just prior to the onset of hearing in these animals remains to be resolved. Based on the contributions of $P2X_3$ to neurotrophic signaling (Cheung, Chan & Burnstock, 2005), these other P2X receptor elements may be associated with the maturation of the hair cells and the reorganization of the cochlear innervation pattern prior to the onset of hearing.

As noted throughout this review, intracellular Ca^{2+} plays a central role in defining hair cell physiology. P2X receptors have some of the highest Ca^{2+} permeabilities of any ionotropic receptors, including the NMDA receptor (Evans et al., 1996; Burnstock & Knight, 2004). Ca^{2+} is also involved in the feedback regulation of P2X receptor signal transduction. Using a combination of Ca^{2+} imaging and NO imaging, Shen et al. (2005) have demonstrated that application of ATP to guinea pig IHCs results in rapid production of NO, presumably via the ATP-induced increase in intracellular Ca^{2+}. This in turn leads to a cGMP - protein kinase G - dependent inhibition of the ATP-induced Ca^{2+} elevation in these cells. The effect is presumed to depend on the hair-cell P2X-receptor conductance, although P2Y receptor-mediated Ca^{2+} activation cannot be eliminated. It is interesting to note that a similar interaction between purinergic and nitric oxide signaling has been reported in spiral ganglion neurons; however, in this instance, the feedback pathway leads to enhanced ATP-induced Ca^{2+} rise in the cells (Yukawa et al., 2005). Here, activation of glucocorticoid receptors by dexamethasone increased the P2 receptor-mediated NO signal, which has a dependence on extracellular Ca^{2+}. The synergism provided by the glucocorticoid receptor appears very relevant to the likely role of P2 receptor signaling in the hair cell response to stress.

Hair Cell P2Y Receptor Expression

Evidence for P2Y receptor-mediated action on cochlear hair cells is clear with respect to cellular physiology, but the molecular characterization of the signaling pathways is less advanced than that for P2X receptors. Ashmore and Ohmori (1990) resolved ATP-induced rises in guinea pig OHC intracellular Ca^{2+} signals over two time courses. A rapid rise in the apical region of the cells is likely to be attributed to Ca^{2+} influx via the P2X receptors on the stereocilia and cuticular plate — as was subsequently resolved for Na^+ influx (Housley, Raybould & Thorne, 1998b). A delayed Ca^{2+} response distributed throughout the cytosol may be attributed to P2Y receptor-mediated release of Ca^{2+} from stores. The pharmacology of the ATP-induced Ca^{2+} response is consistent with activation of a P2Y receptor (Nilles et al., 1994). The OHC P2X and P2Y receptor Ca^{2+} signals were resolved at higher spatial and temporal resolution by Mammano et al. (1999). Here, Ca^{2+} imaging provided functional evidence that P2Y receptors were co-localized with P2X receptors to the apical pole of the OHC. Further, application of ATP resulted in the diffusion of a novel G protein to a specialized IP_3 receptor-gated Ca^{2+} store present beneath the cuticular plate — Hensen's body. These authors proposed that the regulation of Ca^{2+} at this site is likely to affect the MET conductance, particularly the adaptation rate. More recently, Gale et

al. (2004) have demonstrated that the hair cells are coupled via a propagating Ca^{2+} wave to adjacent cells in the organ of Corti, via a mechanism that involves ATP-induced ATP release signalled via P2Y receptors, which in turn invoke the phospholipase C – IP_3 pathway to induce Ca^{2+} release from IP_3 receptor-gated stores. This effect was antagonized by P2Y receptor antagonists. This group suggested that the ATP signal may provide a queue for invoking reparative responses following hair cell damage.

Development of specific antibodies to the P2Y receptors has lagged behind the P2X receptor field; however, several commercially available antibodies are now available for P2Y receptors expressed in the cochlea and details of the expression profiles of the metabotropic purinoceptors are emerging. These seven membrane-spanning G protein-coupled receptors are more diverse than the P2X receptors, with nine mammalian homologs: $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, which are mediated by Gq/11–PLC signaling; $P2Y_{12}$ – $P2Y_{14}$ which signal via Gi–adenylyl cyclase (Abbracchio et al., 2003; Burnstock & Knight, 2004), and the newly identified $P2Y_{15}$, which is activated by both AMP and adenosine and likely signals via Gq and Gs (Inbe et al., 2004). P2Y receptors generally have lower EC_{50} values for ATP than do P2X receptors — low micromolar range; hence threshold activation is seen in the high nM range. Many of the P2Y receptors can also be distinguished based on their greater sensitivity to UTP, UDP and UDP-glucose or ADP compared with ATP, and activation by diadenosine polyphosphates, all of which act as endogenous agonists (Barnard et al., 1996; Steinmetz et al., 2000; Lazarowski et al., 2003; Jacobson et al., 2004).

RT-PCR transcript analysis has detected several of the P2Y receptor mRNA transcripts in the organ of Corti, and some data are now available on protein expression via immunolabelling (Housley et al., 2004; Szucs et al., 2004). The latter indicates polarity of expression in OHC, with $P2Y_2$ expression biased to the apical pole, and $P2Y_4$ expression occurring around the neural pole; $P2Y_1$ receptor immunolabelling was reported to be distributed throughout the hair cells. The functional significance of this polarization of receptor types is unclear. The P2Y receptor-mediated increase in OHC $Ca^{2+}{}_i$ observed by Nilles et al. (1994) was insensitive to UTP, and therefore unlikely to involve the apical P2Y(2) receptor population.

Ectonucleotidase Expression by Hair Cells

The ecto-nucleoside triphosphate diphosphohydrolase (E-NTPDase) family is also genetically and phenotypically diverse (Plesner, 1995; Zimmermann, 2001). With regard to extracellular nucleotide signaling in the cochlea, Vlajkovic et al. (2002) have shown that eNTPDase1 and 2 are differentially expressed in cochlear tissues. eNTPDase2 (ectoATPase), which preferentially hydrolyzes ATP to ADP, is densely expressed at the apical pole of the hair cells, particularly the stereocilia; eNTPDase 1 (ectoATPDase), which is particularly effective at converting ADP to AMP, was also detected here. Thus, the apical pole of the hair cells colocalizes eNTPDases with P2X and P2Y receptors. Their likely function is to provide a sink for ATP-mediated paracrine signaling, hydrolyzing the nucleotides towards adenosine. Expression of these ectoNTPDases was up-regulated following protracted exposure of the cochlea to noise (Vlajkovic et al., 2004), suggesting that the ability of the cochlear hair cells to respond to stress-induced ATP signaling is dynamically controlled.

Adenosine Receptors and Adenosine Transporters

It has been known for some time that hair cells express adenosine receptors. Indeed, it has been suggested that these receptors may be otoprotective, given the production of adenosine during stress, coupling of adenosine receptors (AR) to antioxidant pathways, and AR up-regulation in response to oxidative stress from cisplatin administration and prolonged exposure to loud sound (Ford et al., 1997a, b; Ramkumar et al., 2004). AR1 in particular has been implicated, based on observed protection of chinchilla hair cells from cisplatin-induced oxidative damage when A1 receptor agonists were administered via the round window (Whitworth et al., 2004). Adenosine transporters have been localized to the hair cells and adjacent cell types, providing a pathway for local recyling of the nucleoside.

Purinergic Signaling in Vestibular Hair Cells

The resolution of purinergic signaling, and P2X receptor expression in particular, is less advanced for vestibular hair cells. Rennie and Ashmore (1993) demonstrated robust inward current responses in type I and type II guinea pig crista hair cells in response to ATP application. In many of these cells, secondary KCa currents were observed. As for the mammalian cochlea, there is a high density of ecto-ATPase expression localized to the stereocilia and cuticular plate region of the frog crista hair cells (Gioglio et al., 2003).

ATP Release and Physiological Significance of ATP Signaling in the Cochlea

It appears highly likely that ATP is released into the endolymphatic compartment of the cochlea as a local autocrine or paracrine factor to regulate the electrochemical gradients across the cochlear

partition that sustain sound transduction. Under stressors such as noise and hypoxia, ATP levels increase in the perilymph and endolymph (Muñoz et al., 1995, 2001). The basal level of ATP within the bulk fluid phase of cochlear scalae is in the low nM range. Sampling of cochlear fluids following a stressor stimulus elevates this by an order of magnitude, approaching the threshold for activation of P2X and P2Y receptors. These measurements — based on the luciferin-luciferase assay — sample the bulk fluid phase in the face of ectonucleotidase activity. The concentration of ATP released from cells and sampled close to the sites of release are certain to be higher. In other epithelial tissues, bulk phase levels of ATP lie around 10 nm, but local release from cells elevates ATP concentrations into the micromolar range, even with benign mechanical stimulation (Lazarowski et al., 2003). ATP release has also been detected in isolated organ of Corti following depolarizing stimulation with K^+ (Wangemann, 1996). Histochemical and biochemical data indicate that the marginal cells of the stria vascularis are also a source of ATP release into endolymph, via vesiculated stores (White et al., 1995; Muñoz et al., 2001). As noted previously, Gale et al. (2004) recently demonstrated propagated ATP release from the hair cells and adjacent Hensen cells which could be blocked by addition of apyrase, to hydrolyze the endogenous extracellular ATP. Thus it appears that ATP, acting via P2Y receptors, stimulates ATP release, which then acts as a paracrine signal to trigger further ATP release from adjacent cells. The P2Y receptor signaling drives the release of stored Ca^{2+} in a manner analogous to the propagation of Ca^{2+} waves via IP_3 signaling through gap junctions. Indeed, connexon hemichannels — uncoupled from adjacent cells — are a principal candidate for the ATP release channel (Goodenough & Paul, 2003; Gale et al., 2004; Katsuragi & Migita, 2004; Zhao et al., 2005). Extracellular ATP is therefore available as a humoral factor to activate the P2 receptors. Thus, while P2Y receptor activation at the apical surface of the hair cells has been implicated in propagation of ATP signaling across the surface of the reticular lamina, co-localized P2X(2) receptors are likely to provide the homeostatic regulation of the electrochemical gradient across the cochlear partition.

ATP-gated ion channels assembled from the P2X receptors on the stereocilia of the hair cells, along with the collateral expression of these ion channels on the rest of the epithelial cells lining scala media (Housley et al., 1992, 1998a, 1999; Mockett et al., 1995; Housley & Ryan, 1997; King et al., 1998; Järlebark et al., 2000; Lee, Chiba & Marcus, 2001; Järlebark et al., 2002; Wang et al., 2003), provide a current path for flux of K^+ out of scala media. This shunt has been measured in the guinea-pig cochlea with injection of ATP into the endolymphatic compartment (Thorne, Munoz & Housley, 2004), demonstrating a dose-dependent reduction in the endocochlear potential which was shown to arise from increased (K^+) conductance across the cochlear partition (fall in cochlear partition resistance). The loss of K^+ from scala media, via this P2X receptor-mediated shunt pathway, is complemented by a purinergic regulation of the electrochemical gradient mediated by P2Y(4) receptors expressed by the marginal cells of the stria vascularis (Marcus et al., 1998; Sage & Marcus, 2002). The synergism is clearly evident: while P2X receptor activation opens a shunt pathway for K^+ efflux, P2Y receptor activation, mediated by a PLC–DAG–PKC phosphorylation, inhibits the regulatory KCNE1 K^+ channel subunit on the luminal surface of these cells, hence blocking the K^+ current entry into scala media which supports EP (Marcus et al., 1998; Jentsch, 2000). Thus the P2X receptors on the hair cells contribute significantly to the global shunt across the partition, which impacts on the driving force for sound transduction, but in addition, the hair cells also suffer direct actions as a result of the parallel shunt across the transducer pole of the cells. This $P2X_2$ receptor-mediated shunt would reduce the receptor potential, and in the case of the OHC, affect the cell's electromotility via a depolarizing shift in membrane potential and increased Ca^{2+} signaling (Ca^{2+} entry and Ca^{2+}-store activation). Thus, at the apical pole of the hair cells, responding to stress-induced endolymphatic ATP release, P2X and P2Y receptor activation likely regulates forward and reverse transduction. Such a finding is also supported by the observed modulation of electrically evoked otoacoustic emissions following endolymphatic ATP injection (Kirk & Yates, 1998).

While the apical pole of the hair cells, particularly the OHCs, is a primary region for purinergic influence, the action of P2Y receptors on the perilymphatic surface, increasing [$Ca^{2+}{}_i$], is also likely. In addition, indirect effects on the cellular processes occur due to diffusion of second messengers such as IP_3 and Ca^{2+} and NO away from the P2 receptor sites. It is therefore likely that both mechanoelectrical transduction at the apex, basolateral K^+ conductances which define the membrane filter properties of the hair cells, and the basal Ca^{2+}-dependent release of neurotransmitters, are all targets of purinergic signal transduction. Outside of the hair cells, mechanical coupling with adjacent cells, particularly Deiters' cells with OHCs, will alter the micromechanics of the organ of Corti and affect hearing function. Evidence for this at the cellular level arises from experiments where application of ATP to Deiters' cells elevates $Ca^{2+}{}_i$ and causes increased stiffness in the Deiters' cell process. This

extends as coupled responses via IP_3 second messenger diffusion between adjacent Deiters' cells. (Dulon, Moataz & Mollard, 1993; Dulon, 1995; Bobbin, 2001; Lagostena & Mammano, 2001). At an integrated level, perilymphatic application of an ATP analog elicits changes in distortion product otoacoustic emissions consistent with a movement of the reticular lamina towards the scala tympani, presumably mediated by P2Y receptors (Bobbin & Salt, 2005). Overall, it appears likely that activation of the purinergic signaling pathway in the hair cells and other complementary cellular elements of the cochlear partition, reduces sensitivity of the transduction process to sound stimulation. This would effectively extend the dynamic range of these transducer cells and is therefore likely to confer some protection from noise trauma. Figure 6 provides a synthesis of many of the elements of purinergic signal transduction outlined here. Clearly, much remains to be learned about the physiological processes that cause ATP release, and the effect this has on sound transduction and auditory neurotransmission.

Conclusion

This review has highlighted the major impact K^+ channels, ionotropic and metabotropic receptors and second messenger signaling have on the input and output functions of the sensory hair cells. A feature of this is the compartmentalization of the signaling elements and the dynamic regulation of transcription (particularly via selective expression of splice variants of ion channel mRNA transcripts), translation and trafficking of the expressed proteins which provide this diverse range of intra- and transcellular communication. The spatiotemporal regulation of Na^+, Ca^{2+} and K^+ channel expression is clearly central to the maturation of the hair cells, establishment of long-term synaptic connection, and development of the hair cell biophysical envelope. The latter provides optimal conversion of receptor currents gated by the mechanoelectrical transducer channels into temporal and amplitude-coded gating of neurotransmitter release. Feedback via the efferent system, acting via a host of direct and indirect mechanisms linked to Ca^{2+}, regulates OHC sensitivity. Complementing the efferent neural feedback is a purinergic signalplex which engages as sound levels increase, mechanical forces develop and cell second messengers instigate release of ATP, UTP and related agents into the extracellular domains around the hair cells. P2 receptor activation then invokes a range of signaling pathways which likely safely extend the dynamic range of hair cell function.

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