PIEZ02 as the anomalous mechanotransducer channel in auditory hair cells

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Abstract Throughout postnatal maturation of the mouse inner ear, cochlear hair cells display at least two types of mechanically gated ion channel: normal mechanotransducer (MT) channels at the tips of the stereocilia, activated by tension in interciliary tip links, and anomalous mechanosensitive (MS) channels on the top surface of the cells. The anomalous MS channels are responsible for the reverse-polarity current that appears in mutants in which normal transduction is lost. They are also seen in wild-type hair cells around birth, appearing 2 days earlier than normal MT channels, and being down-regulated with the emergence of the normal channels. We review the evidence that the normal and anomalous channels are distinct channel types, which includes differences in localization, susceptibility to pharmacological agents, single-channel conductance and Ca\(^{2+}\) permeability. The dichotomy is reinforced by the observation that the anomalous...
Introduction

One of the most significant recent advances in physiology has been the identification of PIEZO as a pore-forming subunit of vertebrate mechanically gated ion channels (Coste et al. 2012). The discovery was rendered more remarkable by the demonstration that one or other of the two isoforms PIEZO1 or PIEZO2 underlies transduction in most cutaneous mechanoreceptors and muscle spindles (Ranade et al. 2014; Woo et al. 2014, 2015) as well as in other tissues such as blood vessels and bone. The discovery of PIEZO marks a watershed event after a protracted search for the ion channel underpinning vertebrate mechanosensation. Until recently, knowledge about this channel type has been sparse compared to information available for the two other canonical channel types, voltage-gated and ligand-gated channels. The discovery has revealed a large set of human genetic disorders originating from mutations in PIEZO1 or PIEZO2 (Alper, 2017). One organ in which mechanosensation is central to physiological function is the inner ear, where sensory hair cells detect sound stimuli in the cochlea and head motion in the labyrinth. It is natural to enquire whether hair cell transduction also relies on PIEZO channels. The quest for the hair cell mechanotransducer channel has been long, and hindered by the paucity of tissue available for biochemical analysis (Fettiplace & Kim, 2014).

Hair cell transduction occurs in the bundle of so-called hairs or stereocilia protruding from the top of the cell in an organ pipe arrangement. Adjacent actin-filled stereocilia are connected by extracellular filaments, the most important of which is the tip link, extending from the top of one stereocilium to the side wall of its taller neighbour (Pickles et al. 1984; Furness et al. 2008). Sub-micrometre deflections of the hair bundle tension the tip links and gate mechanotransducer (MT) channels at their lower end (Howard & Hudspeth, 1988; Beurg et al. 2009). Normal transduction is functionally polarized, reflecting the morphological asymmetry of the bundle (Shotwell et al. 1981; Beurg et al. 2016). In outer hair cells (OHCs) of the mammalian cochlea, an indicator of bundle polarity is the ‘V’ shape. Deflection of the hair bundle towards its taller edge to the tip of the ‘V’ increases tension in the tip links and opens MT channels, whereas deflection towards its shorter edge relaxes the tip links, hence closing the MT channels. Some of the protein constituents of the transduction apparatus are known. The tip link is composed of dimers of cadherin 23 and of protocadherin 15, joined at their N-termini (Pickles et al. 2007). Some other components have also been identified from genetic mutations (Abstract figure panel A) (Richardson et al. 2011; Fettiplace & Kim, 2014), but controversy has persisted over the identity of the transducer channel and, as discussed later, it is unlikely to be an isoform of PIEZO. The current candidate is a transmembrane channel-like protein isoform 1 and/or 2 (TMC1, TMC2) (Kawashima et al. 2011; Kim & Fettiplace, 2013; Pan et al. 2013).

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end of the tip link where they are thought to interact with multiple membrane proteins: TMC1/2 (Maeda et al. 2014), LHFPL5 (Xiong et al. 2012), TMIE (Zhao et al. 2014) and CIB2 (Giese et al. 2017). These proteins, probably with others still unknown, are thought to form the mechanotransduction channel complex.

**Anomalous currents**

Key evidence for a role for TMC1 in hair cell transduction is the many mutations of the Tmc1 gene linked to deafness in humans and in mice. Tmc1 knockout in mice also results in loss of the hair cell MT current, though in the early postnatal period the transient expression of Tmc2 can substitute for Tmc1. Surprisingly, in Tmc1:Tmc2 double knockouts, the normal MT current is totally absent from cochlear hair cells but, paradoxically, a ‘reverse-polarity’ mechanosensitive (MS) current can still be recorded in such mutants (Kim et al. 2013). It was initially considered that the ‘reverse-polarity’ current might be a manifestation of the normal current but this turned out not to be the case. The ‘reverse-polarity’ current was so named because it could apparently be evoked by hair bundle deflections in the non-optimal direction, towards the shortest rank of stereocilia. However, use of this term is misleading, because it is now thought to be activated by deformation of the apical plasma membrane (Abstract figure panel B) rather than a specific direction of deflection of the hair bundle (Beurg et al. 2016), and it will subsequently be referred to as an anomalous MS current.

Such anomalous currents can be recorded in cells in which normal transduction has, for various reasons, been lost. The most common cause is the loss of the tip links, resulting from various manipulations or conditions including: (i) mutations in the tip link constituent protocadherin-15 (Alagramam et al. 2011); (ii) lowering Ca\(^{2+}\) in the saline bathing the hair bundle to less than 1 \(\mu\)M, usually by applying BAPTA (Kim et al. 2013; Marcotti et al. 2014), which destroys the tip links (Assad et al. 1991); (iii) some mutations of the myosin VIIa, which is part of the tip link’s upper attachment point (Kros et al. 2002; Marcotti et al. 2014); (iv) absence of the calcium and integrin binding protein CIB2, thought to be complexed with TMC1 at the tip of the stereocilia (Giese et al. 2017); and (v) mutations of other proteins linked with mechanotransduction, such as TMIE (Zhao et al. 2014). In each case, the normal MT current is absent and is replaced by the anomalous MS current. In two other mutants, anomalous currents have been recorded, along with normal MT currents. These are in the Shaker2 mutation in myosin XVa (Stepanyan & Frolenkov, 2009), and in the knockout of Vlgr1 in which the protein product is a component of the ankle links (Michalski et al. 2007). The anomalous currents in these two mutants have not been examined in detail, and it is unclear whether they are the same phenomenon as seen in other mutants where normal transduction is lost. However, there is one situation during neonatal development in wild-type animals in which both the normal MT current and anomalous MS current are present at the same time (Fig. 1) (Waguespack et al. 2007; Kim et al. 2013; Marcotti et al. 2014; Beurg et al. 2016). Here, the anomalous MS current appears first around birth, but is down-regulated as the normal MT current grows. Strikingly, the changes in the two currents are synchronized so that reduction in the anomalous current is contemporaneous with growth in the normal current (Fig. 1). This relationship between the two types of mechanosensitive current occurs in all inner ear hair cells of wild-type animals, inner hair cells (IHCs) and OHCs of the cochlea, and vestibular hair cells (Beurg et al. 2016).

Furthermore, the timing of these changes depends on the cell types. For example, the changes in both anomalous and normal currents in basal OHCs precede those changes in apical OHCs by about 2 days, reflecting a base to apex gradient in hair cell differentiation; in vestibular hair cells, changes in anomalous and normal currents both occur prenatally (Beurg et al. 2016). Whether present neonatally in wild-type animals, or initiated by loss of normal transduction, the anomalous MS current has usually been found to decline over development with a similar time course. However, it should be noted that not everyone has reported a strict temporal relationship between the normal and anomalous currents (Marcotti et al. 2014). A possible synchronization between the two currents raises the prospect that they are causally related.

**Single stretch-sensitive channels**

The prevailing view is that the anomalous current is evoked not by bundle displacement per se but rather deformation of the apical cell membrane (Beurg et al. 2016). Anomalous currents evoked with different orientations of a fluid jet indicate that the best stimulus is suction on the membrane from whatever angle, rather than polarized deflection of the hair bundle. Thus if a sinusoidal stimulus was applied, the current was activated on the negative (suction) phase of the fluid jet. Evidence derived from Ca\(^{2+}\) imaging and from single-channel recording then argued that the underlying MS channels are located on the apical cell membrane at the base of the hair bundle. They might also exist in the stereociliary membrane but the experimental assays are insufficiently sensitive to draw this conclusion. Cell-attached patches on the apical membrane, lateral to the hair bundle, showed clear synchronization of channel activity to membrane suction applied via the patch pipette with a fast pressure clamp. An example of their activity, compared with that of a normal MT channel, is shown in Fig. 2. After the onset of the stimulus, there was an initial increase in channel activity that subsequently declined, indicative of inactivation. In recordings from OHCs of
neonatal wild-type mice, the mean channel conductance was $53 \pm 3 \, \text{pS}$ ($n = 8$). Current–voltage relationships were linear with an extrapolated reversal potential close to 0 mV, consistent with a cation channel. Single stretch-activated channels were also present in $\text{Tmc1}:\text{Tmc2}$ double knockouts and had a mean conductance of $52 \pm 1 \, \text{pS}$ ($n = 5$), not significantly different from the value in wild-type animals. The ensemble averages showed an inactivating component with a time constant dependent on stimulus level, increasing from a minimum of $\sim 14$ ms for the smallest stimuli up to 30 ms for maximal saturating stimuli (Fig. 2D). The adaptation time constant was weakly voltage sensitive, but it was unaffected by external $\text{Ca}^{2+}$ concentration (Kim et al. 2013). The recordings in Fig. 2B were made in a low external $\text{Ca}^{2+}$ (0.04 mM), comparable to the concentration of $\text{Ca}^{2+}$ in the endolymph bathing the hair bundles in vivo (Gill & Salt, 1997). When an external $\text{Ca}^{2+}$ concentration of 1.5 mM,
similar to that in perilymph, was used in the patch pipette, the channel conductance was reduced to $34 \pm 1$ pS ($n = 5$), demonstrating block by external $\text{Ca}^{2+}$ similar to the block seen with normal MT channels (Fettiplace & Kim, 2014).

The anomalous MS channels were initially considered to be a possible candidate for the normal MT channel. While the properties of the anomalous channel are similar to the normal MT channel, they are not identical. The latter have been characterized principally from whole-cell recordings after destruction of the majority of tip links by exposure to sub-micromolar $\text{Ca}^{2+}$ and buffered with BAPTA, which is thought to leave a few functional MT channels. This approach is successful because the underlying channels have large conductance, 100 pS or more (Geleoc et al. 1997; Ricci et al. 2003; Beurg et al. 2006), and can be seen above the noise imposed by the series resistance of the patch pipette (Fig. 2A). Differences between the anomalous MS channels and normal MT channels include distinct single-channel conductance, $\text{Ca}^{2+}$ permeability and differential susceptibility to a range of pharmacological blocking agents (Table 1). In particular, the $\text{IC}_{50}$ for dihydrostreptomycin is one to two orders of magnitude larger for the anomalous MS currents than for the normal MT currents (Marcotti et al. 2014; Beurg et al. 2014). These differences have prompted them to be assessed as distinct channels (Marcotti et al. 2014). It should be noted, however, that the $\text{IC}_{50}$ for another blocking agent, amiloride, at $\sim 50 \mu M$, is similar between the two channel types (Rusch et al. 1994; Beurg et al. 2014). Furthermore, GsMTx4, a peptide toxin of the tarantula spider, Grammostola spatulata (Suchyna et al. 2000), blocks both normal and anomalous MS channels with similar sub-micromolar $\text{IC}_{50}$ (Beurg et al. 2014). This

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**Figure 2. Mechanosensitive channels in OHCs**

A, normal MT channels recorded under whole-cell conditions after destroying almost all tip links with BAPTA, recording and stimulation methods shown at the top. HB, hair bundle. Three records of single-channel activity for a positive hair bundle deflection, ensemble average displaying adapting and sustained components of activity, and amplitude histogram indicating a 13.5 pA channel; at the membrane potential of $-84 \text{mV}$, this current corresponds to unitary conductance of 160 pS. Mid-region of cochlea, hair bundle bathed in $\text{Na}^+$, 0.04 mM $\text{Ca}^{2+}$. B, stretch-activated channel recorded in cell-attached patch on hair cell apical membrane, method depicted above. Three records of single-channel activity for a negative pressure step imposed on membrane patch via pipette; ensemble average shows transient and sustained activity, and amplitude histogram of traces, indicating a 7.7 pA channel at $-155 \text{mV}$, equivalent to 51 pS conductance. Pipette solution $\text{Na}^+$, 0.04 mM $\text{Ca}^{2+}$. C, section of apical region of hair cell where cell-attached recordings made; note secretory vesicles (arrowheads), exocytotic pits (arrows) and microtubules (mt). D, ensemble-average currents of stretch-activated channel in B at four pressure levels at $-105 \text{mV}$. Time constant of inactivation, $\tau_{\text{inact}}$, is 14 ms for the smaller stimuli. E, dependence of current for stretch-activated channel on holding potential, linear fit gives conductance 54 pS. Results modified from Beurg et al. (2016).
Role of PIEZO2 in hair cells

Over the past 15 years, substantial information about the molecules underlying development and function of the inner ear hair cells has accrued from study of mutations in both humans and mice (Richardson et al. 2011). What role does early expression of Piezo2 play in hearing? Piezo2 knockout throughout the inner ear led to an approximately 15 dB threshold elevation of auditory brainstem response, and to a mild distortion of auditory signal, with no vestibular defect (Wu et al. 2017). OHC electromotility, as assayed by non-linear capacitance, was unaltered, suggesting no defect in prestin-mediated cochlear amplification. Since the Piezo2 null is embryonic lethal due to failure of respiration, these measurements were performed on conditional knockout mice, by crossing floxed Piezo2 with Pax2 Cre. But the role of PIEZO2 in hearing is obscure.

What is clear is that the anomalous currents, presumably reflecting PIEZO2 channels, are present during early development in all mammalian hair cell types, both auditory and vestibular. Moreover, their down-regulation is synchronized with the appearance of normal mechanotransduction (Fig. 1D). It is important to note that both types of mechanosensitive channel are permeable to Ca\(^{2+}\), but the normal MT channel is threefold more permeable, and also partially open at rest, so permitting a sustained Ca\(^{2+}\) influx (Beurg et al. 2014). The anomalous MS current has been shown to be inhibited by raising intracellular Ca\(^{2+}\) (Wu et al. 2017), but a concern with this experiment is that an unrealistically high Ca\(^{2+}\) concentration (1 mM) was needed to accomplish the block. In some cells, significant block could be achieved with 0.35 mM Ca\(^{2+}\). This high concentration may not be unrealistic, since Ca\(^{2+}\) in the stereocilia has been estimated from reversal potential measurements to be near-millimolar levels (Beurg et al. 2015). Although Ca\(^{2+}\) will be highest near the inner face of the MT channel, the concentration further down the stereocilium, and at the top of the cell, may also be substantial, as Ca\(^{2+}\) buffering and extrusion in neonates is small (Hackney et al. 2005; Chen et al. 2012). Furthermore, the Ca\(^{2+}\) concentration in endolymph bathing the hair bundle is likely to be high, millimolar, levels prior to the emergence of the.

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**Table 1. Comparison of properties of normal MT channels/currents and anomalous MS channels/currents**

| Property                  | Normal MT channels | Anomalous MS channels |
|---------------------------|--------------------|-----------------------|
| Ca\(^{2+}\) permeability \(P_{Ca}/P_{Cs}\) | 5:1                | 1.5:1                 |
| Single-channel conductance \((\text{Na}^+, \text{low Ca}^{2+})\) | $>100 \mu\text{S}$ | 50–60 \(\mu\text{S}\) |
| Dihydropyrotoxymycin IC\(_{50}\) | 11 \(\mu\text{M}\) | 169 \(\mu\text{M}\), \(\mu\text{M}\) |
| Dihydropyrotoxymycin IC\(_{50}\) | 7 \(\mu\text{M}\) | 894 \(\mu\text{M}\), \(\mu\text{M}\) |
| FM1-43 IC\(_{50}\) | 2.8 \(\mu\text{M}\) | 26 \(\mu\text{M}\) |
| Amiloride IC\(_{50}\) | 50 \(\mu\text{M}\) | 58 \(\mu\text{M}\) |
| Piezo2 knockout | Unaffected | Absent |

\(\text{IC}_{50}\) for blocking agents all given for holding potential of \(-84 \text{ mV}\). Results from Kim & Fettiplace (2013); Beurg et al. (2014, 2016); Wu et al. (2017). The two values for dihydropyrotoxymycin were: \(\text{a}^\text{Beurg et al. (2014)}\); \(\text{b}^\text{Marcotti et al. (2005, 2014)}\). Control values for amiloride from Rusch et al. (1994).
endolymphatic potential after the first postnatal week. Thus, during neonatal development, Ca\(^{2+}\) influx through the more Ca\(^{2+}\)-permeable normal MT channels may elevate Ca\(^{2+}\) under the apical membrane to a level sufficient to suppress the anomalous current. This could explain the apparent developmental relationship between the two. The Ca\(^{2+}\)-induced inhibition is unlikely to be due to direct intracellular blockade as it takes time to develop, so it may involve some other signalling cascade. Furthermore, as noted above, PIEZO channels expressed in HEK293 cells showed no inhibition by 10 mM intracellular Ca\(^{2+}\) (Wu et al. 2017). These observations argue that the PIEZO2 channels are indeed downregulated by high intracellular Ca\(^{2+}\), rather than just being blocked. Since the anomalous current also appears during damage to hair cell transduction, it is possible that PIEZO2 contributes to repair of damaged hair cells or their apoptosis during ageing. But the presence of these channels in early neonates suggests they may also play a developmental role.

**Concluding remarks**

It is puzzling that the PIEZO1 or PIEZO2 channels, which are ubiquitous in mediating transduction of mechanical forces in the majority of vertebrate mechanoreceptors, are not employed as the primary MT channel in hair cells. This is particularly perplexing when PIEZO2 is known to occur in hair cells, and to have properties comparable, in conductance, ionic selectivity and adaptation, to those of the native MT channel. A difference in Ca\(^{2+}\) selectivity may be crucial: \(P_{Ca}/P_{Cs} = 1.8\) for the anomalous PIEZO2 channels (Kim et al. 2013) but \(P_{Ca}/P_{Cs} = 5\) for the MT channel in OHCs (Kim & Fettiplace, 2013). Ca\(^{2+}\) influx through the hair cell channel is essential for generating adaptation, and has also been proposed to be necessary for regulating actin polymerization at the stereociliary tips and hair bundle organization (Velez-Ortega et al. 2017). This requirement is presumably the reason that, in order to direct bundle maturation, cochlear hair cells acquire mechanotransducer channels early in development, well before the onset of hearing, which occurs around postnatal day 12 in mice. The kinetics of activation and adaptation are fairly rapid for PIEZO channels and for the anomalous MS currents, but whether these are sufficiently fast to meet the sub-millisecond time constants needed to encode acoustic stimuli up to 70 kHz in mice is unclear. A property that may be relevant is the mode of channel activation. PIEZO1 channels reconstituted in lipid bilayers can be

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**Figure 3. Effects of Piezo2 knockout on normal and anomalous currents in cochlear hair cells**

A, normal MT and anomalous (reverse-polarity) MS currents in OHCs of Piezo2 heterozygote (top) and Piezo2 homozygote (bottom) in P0–P1 mice. B, normal MT and anomalous MS currents in IHCs of Piezo2 heterozygote (top) and Piezo2 homozygote (bottom) in P0–P1 mice. C, normal polarity MT but no anomalous MS currents seen in Piezo2 \(P2\) conditional knockout (Piezo2 cko). D, collected results of normal and anomalous currents in Piezo2 heterozygotes and homozygotes, mean ± SEM and number of cells. For anomalous currents in both OHCs (left) and IHCs (right), means were significantly reduced (*P < 0.001, Student’s t test) in homozygote relative to the heterozygote, but the mutation had no effect on normal-polarity current. E, collected results of normal and anomalous currents on Piezo2 \(P2\)–\(P4\) conditional knockouts in apical and basal OHCs.
activated by tensile forces in the bilayer in the absence of any other cellular components (Coste et al. 2012; Syeda et al. 2016). Direct force sensation, mediated via the lipid bilayer, has also been demonstrated for the bacterial MscL channel (Teng et al. 2015; Rosholm et al. 2017). However, despite modelling (Kim, 2015), there is no experimental evidence that the hair cell MT channel can be similarly activated. An alternative mechanism is for the channel to be tethered to the cytoskeleton, exemplified by NOMPC, which is thought to be anchored to cytoplasmic microtubules via an extended N-terminus comprising multiple ankyrin repeats (Zhang et al. 2015). To be ultra-sensitive with rapid activation may necessitate the MT channel to be gated by forces delivered across a protein core that is tethered on both the extracellular and intracellular faces of the membrane. Such anchors may involve special proteins such as protocadherin 15 in the tip link, and TMIE, LHFPL5 and CIB2 in the cytoplasm. Together these attachments may underlie the ‘gating spring’ that has been postulated from hair bundle mechanical measurements (Howard & Hudspeth, 1988). It is therefore conceivable that the hair cell MT channel cannot be gated by tension in the bilayer. An important implication is that activation of any candidate protein by stretching the lipid membrane of a heterologous expression system may be unsuccessful. This could be the reason that evidence for TMC1 as the MT channel pore is lacking. But at this stage, the molecular identity of the normal MT channel remains controversial, as is the functional significance of the anomalous MS channel.

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**Additional information**

**Competing interests**

None declared.

**Author contributions**

Both authors contributed to experiments, analysis of results and writing of the manuscript.

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