Published in final edited form as:

Nat Neurosci. 2009 May ; 12(5): 553–558. doi:10.1038/nn.2295.

Localization of inner hair cell mechanotransducer channels using high speed calcium imaging

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Abstract

Hair cells detect vibrations of their stereociliary bundle by activation of mechanically-sensitive transducer (MT) channels. Although evidence suggests the MT channels are near the stereociliary tops and are opened by force imparted by tip links connecting contiguous stereocilia, the exact channel site remains controversial. Fast confocal imaging of fluorescence changes reflecting calcium entry during bundle stimulation was used to localize MT channels. Calcium signals were visible in single stereocilia of rat cochlear hair cells and were up to ten times larger and faster in the second and third stereociliary rows than in the tallest first row. The number of functional stereocilia was proportional to MT current amplitude indicating about two channels/stereocilium. Comparable results were obtained in outer hair cells. The observations, supported by theoretical simulations, suggest there are no functional MT channels in first row stereocilia and imply the channels are present only at the bottom of the tip links.

Introduction

The biophysics of mechanotransduction in hair cells of the inner ear that underlie hearing and balance has been extensively studied in both mammals and non-mammals and many properties are well established¹. Hair cells detect mechanical stimuli by sub-micron displacements of their stereociliary bundles, a tightly packed array of apical microvillar protrusions interconnected by extracellular filaments. All bundles have a beveled shape attributable to an increase in stereociliary height across the bundle, deflection towards the tallest stereociliary row being excitatory, opening mechanically-sensitive transducer channels. MT channels are located near the tops of the stereocilia²⁻⁵ and are activated by force applied via extracellular tip links extending from the apex of one stereocilium to the side wall of its taller neighbor⁶⁻⁸. The orientation of tip links parallel to the axis of bundle symmetry has been proposed to account for the polarization in response⁶. Tip links are composed of dimers of cadherin23 and of protocadherin15⁹. MT channels are large conductance (100 – 300 pS) cation channels preferentially permeable to Ca²⁺ (refs. 10 – 12) yet their molecular structure has so far defied identification and their precise relationship to the tip link is unclear.

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Since MT channels are highly permeable to calcium, monitoring calcium entry has been used to localize the channels to the tops of the stereocilia⁴. Two-photon imaging of calcium influx into single stereocilia of bullfrog bundles showed that, during bundle stimulation, calcium signals occurred in both the tallest and shortest rows of stereocilia, prompting the conclusion that MT channels were present at both upper and lower ends of each tip link⁵. This work was limited in both spatial and temporal resolution in that the localization experiments⁵ were performed at the hair bundle base in order to visualize individual stereocilia without the temporal resolution to determine the origin of the calcium signal. In the present work, a similar calcium imaging approach was used but the spatial resolution was enhanced by using mammalian cochlear inner hair cells where the step in height between rows of large-diameter stereocilia allowed individual stereocilia to be imaged at the apical portion of the bundle. Additionally, indicator dyes with a range of affinities were employed to limit saturation and detection to regions closer to the source of calcium while temporal resolution was improved by using a high-speed camera coupled to a swept-field confocal system. Measurements indicated that calcium signals arising in the first row of stereocilia were smaller in amplitude and slower to develop than in either the second or third rows supporting the conclusion that MT channels are not located in the first row.

Results

Stereociliary bundles of rat inner hair cells at the cochlear apex consist of about 70 stereocilia, most of which are $\sim 0.5\ \mu\text{m}$ in diameter and are arranged in three rows of heights $4\ \mu\text{m}$, $2\ \mu\text{m}$ and $1.5\ \mu\text{m}$ ^{12,13} that will be denoted as row 1 (R1), row 2 (R2) and row 3 (R3) respectively. To measure the calcium transients occurring during transduction, whole-cell recordings were made from hair cells in isolated coils of neonatal rat cochleas and calcium indicator dyes of the Fluo4 family were introduced via the patch pipette. When the bundle was deflected with a water jet, a rapid increase in stereociliary calcium could be monitored using a fast swept-field confocal microscope that allowed acquisition at 500 frames per second. This technique enabled full images of the bundle to be taken every 2 ms and showed that an increase in fluorescence could be clearly discerned in individual stereocilia (Fig. 1a; Supplementary video 1). The stereociliary fluorescence changes were eliminated by treatment with MT channel blockers, including the aminoglycoside antibiotic, streptomycin (1 mM, $N = 4$) and curare (0.2 mM, $N = 2$) which largely abolished the MT currents at $-80\ \text{mV}$ (Supplementary Fig. 1). The fluorescent signal indicative of calcium influx through the MT channels also depended on membrane potential and was abolished by depolarization from the normal holding potential ($-80\ \text{mV}$) to $+100\ \text{mV}$ near the calcium equilibrium potential. To circumvent contamination by motion artifacts, an experimental protocol was adopted⁵ in which bundle displacement was combined with a depolarizing voltage step, so no calcium influx would occur until the cell was repolarized to its normal holding potential (Fig. 1b). Upon repolarization, with the bundle still held in a displaced position, a burst of fluorescence was observed in the second row (R2) stereocilia with little change in the first row (R1). In this experiment, using Fluo-4FF dye, the bundle was viewed vertically and the microscope focused halfway down the bundle just below the top of R2. The responses were quantal in that the fluorescence change in each of the bright stereocilia was similar (Fig. 1b). This uniformity in size implies the responses in all active stereocilia were similar and justified averaging the signals. A smaller signal was seen in the two dark (inactive) R2 stereocilia with amplitude and time course similar to the R1 response. The average change in R2 fluorescence developed with a time constant of 25 ms. By comparison the change in the signal in R1 was seven-fold smaller and slower with a time constant of 77 ms. For R3 the onset time constant was 15 ms, more similar to R2 than R1. Although in some cells a double exponential was needed to fit the response onsets, the fast component was dominant and had a mean value of $18 \pm 4\ \text{ms}$ (R2, $N = 6$), $15 \pm 7\ \text{ms}$, (R3, $N = 5$) and $40 \pm 30\ \text{ms}$, (R1, $N = 6$). R2 and R3 were thus similar and both faster than R1.

Measurements on other cells yielded a ratio of peak fluorescence in R2 to that in R1, F_{R2}/F_{R1} , of 10.2 ± 0.8 in 15 inner hair cells using the low affinity dye Fluo-4FF which has a calcium dissociation constant, K_D , of 10 μM . In comparison, when the dye Fluo-4 with a K_D of 0.3 μM was used as the indicator, the fluorescence ratio, F_{R2}/F_{R1} , was 6.5 ± 0.8 in 12 other inner hair cells. The results suggest that use of a high affinity indicator may underestimate the ratio, probably because the R2 signals are saturated. Fluorescent signals were also visible in R3 stereocilia on focusing down towards the cell body or when the bundle was slightly tilted (Fig. 2a). Analysis of such images showed the R3 signals were also large and fast (Fig. 2b). A noteworthy feature of the images is that some R3 stereocilia were active even though the adjacent R2 stereocilium was not. This suggests that tension in a given tip link can open channels at its lower attachment point (on R3) without evoking a signal at its upper attachment point (on R2). The signal in R3 with an associated active R2 (black) was brighter than in R3 with an inactive R2 (green) probably because the confocal volume included some of the signal from the shaft of the underlying active R2 stereocilium. Thus the signal measured in R3 coupled to an active R2 is an over-estimate and a comparison of R2 (red trace) with R3, inactive R2 (purple trace) is likely to be a more accurate measure of the relative calcium signals in the two rows. This shows that the signal in R3 was comparable to that in R2. In nine cells, the mean ratio F_{R2}/F_{R3} was 1.47 ± 0.5 . R2 and R3 therefore behaved similarly with large fast changes while R1 responded more slowly and weakly.

It might be argued that no large stimulus-related calcium signal was ever seen in R1 because the tip links from the first to second rows were absent or were damaged during isolation. Tip links between R2 and R1 stereocilia have been reported in inner hair cells of neonatal (postnatal day 3) mice¹⁴ and are present in rats of the age used experimentally in both inner (Fig 6A of ref. 12) and outer hair cells (Fig 4 of ref. 15). In recordings from more than 50 inner hair cells with MT currents up to 1 nA, no convincing evidence of signals in the first row was ever seen. An MT current of 1 nA probably represents two-thirds of the theoretical maximum, assuming a single channel of 15 pA and 50 tip links (see ref. 12). It seems unlikely that any damage to the bundle would be restricted to R1, and the simplest conclusion from the small R1 compared to R2 signal is that there are MT channels in the second row of stereocilia but not the first row of inner hair cell bundles.

In some experiments, to visualize all stereociliary rows including those with low calcium signals, the cells were also filled with 50 μM AlexaFluor 488. This addition facilitated clear quantification of the changes in fluorescence at different bundle heights (Fig. 3a,b), at the top, at the middle and at the base of the bundle just above the cuticular plate. At the top, only the tallest row was visible whereas with the focus at the middle or base, R1, R2, and sometimes R3 could be seen. Large fluorescence signals were seen at both levels in R2, though those at the base were smaller. In contrast in R1, small signals were measured at all three positions but paradoxically the amplitude increased towards the base (Fig. 3c). These trends were observed in five cells, in which the mean fluorescence changes (in arbitrary units) in R1 were 26 ± 7 (top), 33 ± 5 (middle) and 64 ± 18 (base) and in R2 were 210 ± 24 (middle) and 167 ± 21 (base). The mean signal in R3 in these cells was 200 ± 32 (base). One explanation for these trends is that they signify the direction of the calcium gradient. Thus for R2, calcium diffuses from top to bottom of the stereocilia but for R1 it diffuses from bottom to top. Consistent with this notion, F_{R2}/F_{R1} was smaller at the base. Such a result would occur if calcium enters through MT channels at the top of R2 and diffuses down into the cell apex and then diffuses back up R1.

If the fluorescent signal mirrors calcium influx through the MT channels, the numbers of active cilia should be proportional to the amplitude of the MT current. Here only bright cilia were counted, so the behavior is independent of dye saturation and results with dyes of different affinities were pooled. To ensure R3 was also counted, images were examined at slightly lower focal planes and in some experiments the bundle were tilted to visualize the entire array (Figs.

2a and 4a). The number of active cilia was proportional to the MT current amplitude for currents up to 1 nA (Fig. 4b) with a mean slope of 35.4 ± 1.3 pA per stereocilium at a holding potential of -80 mV. This slope represents the MT current per stereocilium and may be compared with the single-channel current inferred from measurements of unitary events which in apical inner hair cells has a mean amplitude of 15.0 ± 1.6 pA at -80 mV¹². The ratio of the slope to the single channel amplitude is 2.36 ± 0.34 , implying that there are approximately two channels per tip link as previously concluded^{5,12}.

A few measurements were made on outer hair cells which have thinner stereocilia (~ 0.25 μ m in diameter) than the inner hair cells. Although this size is similar to the pixel dimensions, it was sometimes possible to distinguish the different rows (Fig. 5a; Supplementary video 2) and therefore compare the average fluorescence intensities in R1, R2 and R3. The fluorescence change in R2 was about a four-fold larger than R1 ($F_{R2}/F_{R1} = 3.8 \pm 0.5$ for a mean MT current of 580 pA; N=3). One possible reason for the smaller ratio compared to the inner hair cells is that during channel opening the calcium concentration in the stereocilia may be higher due to their smaller diameter and volume producing greater dye saturation. This would lead to an underestimate in F_{R2}/F_{R1} . The calcium response in R2 was also much faster with an onset time constant of 19 ms compared to 116 ms for R1. As with the inner hair cell measurements the intensity in R1 increased as the focus was moved towards the base of the bundle whereas in R2 it showed a small decrease (Fig. 5b), consistent with opposite directions of calcium diffusion. These results support the idea that both inner and outer hair cells have only a small calcium signal in the first row stereocilia.

Discussion

The results indicate that during opening of the MT channels by bundle deflection, the change in calcium concentration estimated from the fluorescence intensity of indicator dyes in the middle of the bundle is at least ten fold larger in the inner hair cell stereocilia of row 2 than in row 1. Because of dye saturation at some region within the confocal volume, this number should be regarded as a lower limit. Saturation of calcium binding sites on the available dye is an important experimental problem which is exacerbated by use of high affinity indicators such as Fluo-4. The simplest explanation of the results is that there are MT channels in rows 2 and 3 but not in row 1 stereocilia. Consistent with this notion, the small signals in R1 were also slowed and they became larger towards the base of the bundle consistent with back diffusion of calcium. The conclusion is supported by the observation that it was possible to see active R3 stereocilia without there being a corresponding signal in the neighboring R2 stereocilia to which the tip link from the active R3 stereocilia would have attached (Fig. 2b). A similar inference has been made from confocal imaging of bundle calcium signals in guinea pig isolated outer hair cells though no information was provided about the size of the MT current (Harasztosi & Gummer, *Assoc. Res. Otolaryngol. Abstracts* 31.654, 2008).

Reverse diffusion of calcium from the cell body to the stereocilia is the most straightforward explanation of the small but measurable calcium signal in R1 stereocilia and in inactive R2 stereocilia (Figs. 1b, 2b). A theoretical simulation of calcium diffusion in the stereocilia and cell apex was made to confirm the plausibility of this explanation. Results could be reproduced by the model (Fig. 6) provided that two MT channels were located only at the tips of R2 and R3 stereocilia at the lower ends of the tip links. The model incorporated calcium entry through the MT channels, diffusion and binding to buffers including the fluorescent indicator, and extrusion through plasma membrane CaATPase pumps. In the simulations, the dye fluorescence was integrated over a longitudinal volume set by the axial resolution of the confocal microscope (measured as a full width at half maximum, FWHM of 1.1 μ m). If simulations were performed with both channels at the lower ends of the tip links (Fig. 6a), the fluorescence ratio F_{R2}/F_{R1} was 15 when the focal plane was at the top of R2. When the focal

plane was at the base of the bundle, the amplitude of the signal in R2 was reduced but in R1 it was increased as observed experimentally, consistent with the reverse diffusion hypothesis. The time courses of the response onsets in the model were also similar to those measured for both inner and outer hair cells. Fits to the fluorescence onsets in the simulations had fast time constants of 10 ms (R2), 8 ms (R3) and 120 ms (R1). For comparison, the corresponding experimental values were 17 ms (R2), 15 ms (R3) and 40 ms (R1) for inner hair cells and 19 ms (R2), 16 ms (R3) and 116 ms (R1) for outer hair cells.

If the bundle was modeled with MT channels at both ends of the tip link (Fig. 6b), and therefore present on the side wall of R1, the integrated fluorescence signal in R1 was much larger and the fluorescence intensities in both R1 and R2 were reduced in shifting the focus from the middle to the base. If the MT channels were placed only at the upper end of the tip link (not shown), the signal in R1 was even larger relative to R2. The experimental observations are best matched by simulations in which both MT channels are sited at the lower ends of the tip links. The possibility that there are mechanically-gated channels with smaller calcium conductance at the upper end of the tip links contributing to the R1 signal was also considered. However, even when such channels have only one-tenth the conductance of those at the lower end, the reverse calcium gradient in R1 was not reproduced by the model. An unexpected conclusion from the simulations was that the calcium near the tips of R2 and R3 stereocilia achieved a concentration of more than 100 μM during the stimulus. It is therefore not surprising that high-affinity calcium indicators such as Fluo-4 would rapidly become saturated during channel opening, again consistent with the results.

Previous evidence about channel location⁵ came from two-photon imaging of calcium influx into bullfrog stereociliary bundles in which fluorescent signals could occur in both the tallest and shortest rows of stereocilia suggesting MT channels were present in all stereocilia. Although the experiments were a significant technical advance, a drawback was that calcium signals in different rows were not compared quantitatively. Furthermore, the magnitude and time course of the signals may have been distorted by use of a high affinity calcium indicator dye, Calcium Green-1 ($K_D = 0.2 \mu\text{M}$) that became saturated by a large elevation in stereociliary calcium. Evidence for dye saturation is that the fluorescent signal rapidly plateaued (Fig 4 of ref 5) even though the channels remained open. The present results agree with another confocal study⁴ in which line-scan measurements suggested the site of calcium entry was within 1 μm of the tip of the stereocilium, a resolution that did not allow discriminating between top or side of the stereocilia. A few cases were reported there of calcium diffusion both up and down the stereocilium but the reason for this observation is unclear. It is worth considering whether inner hair cells are special cases due to their unique morphology with long row 1 stereocilia to act only as a sail to move the rest of the bundle. Preliminary results suggest that outer hair cells show qualitatively similar behavior though the greater difficulty of seeing and measuring the smaller stereocilia constituted a practical limitation.

An attractive feature of the arrangement in which the MT channels are present only at one end of the tip link is that it conforms to the known asymmetry in the tip link structure. The current view is that the tip link is comprised of two intertwined molecules of cadherin23 at the upper end and two of protocadherin15 at the lower end⁹. The present results would require each channel to interact directly or indirectly with the carboxy-terminus of one of the protocadherin15 molecules. In contrast, channels at opposite ends of the tip link would need to interact with different molecular components of the link. Another advantage of the two channels at the bottom end of the link is that it avoids the negative cooperativity that might arise with channels at each end of the tip link where the opening of one channel relieves the force on the other¹⁶. However, this type of assembly has implications for the activation of myosin-1c which has been proposed as the mediator of both slow and fast adaptation^{17,18}. The prevailing view is that, during bundle deflection, calcium enters stereocilia through

channels at the upper end of the tip link, interacts with myosin-1 causing the upper attachment point of the tip link to slip down the side wall, thereby reducing tension in the link and relieving stress on the channel. If channels are present only at the lower end of the link this scenario seems less attractive. Either the slippage is driven entirely by the increased tension in the link, or the calcium-induced action of a myosin takes place at the top of the stereocilium, which abrogates some of the beauty of the model. Further understanding of the molecular structure of the MT channel and its anchoring at the tip of the stereocilium will be needed to resolve this problem.

Methods

Preparation and recordings

Recordings were made from inner and outer hair cells in the isolated organ of Corti of Sprague-Dawley rats between 6 and 9 days after birth as previously described^{12,15,18}. Animals were killed by decapitation using methods approved by the institutionally approved animal study protocols using standards set out by the National Institutes of Health. Excised apical turns were fixed in the experimental chamber with strands of dental floss and viewed through a 100× LWD water-immersion objective (numerical aperture = 1.0) and a Hamamatsu CCD camera on an Olympus BX51 microscope. One of the ties was placed across the modiolus and its position was adjusted to ensure the inner hair cell bundles were oriented vertically. The chamber was perfused with artificial perilymph of composition (in mM): 154 NaCl, 6 KCl, 2 CaCl₂, 0.5 MgCl₂, 2 Na-pyruvate, 8 glucose and 10 Na-HEPES, pH 7.4. The apical surface of the organ of Corti was separately superfused through a 100-μm pipette with artificial perilymph which in some experiments contained 1 mM streptomycin or 0.2 mM curare (Sigma Chemical Company, St Louis, MO) to block the MT channels. Experiments were performed at room temperature, 20 to 23°C. Measurements averaged are quoted as mean ± 1 standard deviation.

Borosilicate patch electrodes connected to an Axopatch 200A amplifier were introduced through a small hole in the reticular lamina. Recordings were made from inner hair cells or occasionally first row outer hair cells at a low-frequency position about 0.8 of the distance along the basilar membrane from the base. Patch pipettes were filled with an intracellular solution of composition (in mM): 135 CsCl, 2.5 MgCl₂, 1 EGTA, 2.5 Na₂ATP, 5 Creatine phosphate, 2 NaAscorbate, 10 CsHEPES, pH 7.2. The patch solution also contained 1 mM of a calcium indicator dye of the Fluo4 family (Fluo4, Fluo-5F or Fluo-4FF; Invitrogen Life Sciences, Carlsbad CA) and in a few experiments 50 μM AlexaFluo488. The three calcium indicators had different calcium affinities: 0.3 μM (Fluo4), 2 μM (Fluo-5F) and 10 μM (Fluo-4FF). Patch pipettes had starting resistances of 3 – 6 MΩ. MT currents were low-pass filtered at the output of the Axopatch 200A amplifier at 10 kHz. Hair bundles were deflected by a pressure-driven fluid jet from a Picospritzer emanating from a pipette, tip diameter ~5 μm, placed ~10 μm from the bundle so as not to interfere with its image. Rise time of hair bundle displacement with this stimulation system were measured as 5 to 10 ms. The pressure and pipette location were adjusted to elicit a maximal MT current.

Imaging

Stereociliary bundles were imaged with a swept-field confocal (Prairie Technologies, Middleton, WI) illuminated with a 200 mW argon laser at 488 nm. Use of the swept-field confocal allowed the changes in fluorescence intensity in restricted regions of single stereocilia to be tracked and quantified on a millisecond time scale and the fast time constant of calcium accumulation to be determined. Confocal images were captured with a NeuroCCD-SMQ camera (Redshirt Imaging, Decatur GA) at an image size of 80 × 80 pixels and an acquisition rate of 500 frames per second. The swept-field confocal utilizes a stationary slit scanned with a galvanometer-driven beam also at 500 Hz and synchronized to the camera acquisition speed.

The normally-used 35 μm slit was diffraction limited in the object plane. Intermediate magnification lens were inserted to achieve a resolution of 0.12 μm per pixel, about half the lateral resolution of the 100 $\times$ objective. The axial resolution of the confocal system with the 35 μm slit was determined from the axial intensity variation for 0.1 μm fluorescent beads, focused with a stepping motor at 0.5 μm intervals. The results were fitted with a Gaussian with standard deviation 0.5 μm , corresponding to a full width at half maximum (FWHM) of 1.1 μm . The confocal was controlled and the images acquired with IDL v5.6 software (Research Systems, customized by Redshirt). This software also generated the voltage and pressure pulses which were fed through a Digidata 1320 DA converter to run the experiment. Images were analyzed using the IDL v5.6 software and Image-J. The average intensity was calculated for small areas overlying the stereociliary tips and, in many cases, the fluorescence change was expressed as $\Delta F/F$, where F is the background fluorescence measured in the bundle at a depolarized potential.

Theory

Simulations of calcium diffusion in the stereocilia were implemented using a set of differential equations similar to those described previously²⁰⁻²². The hair cell was represented by a two dimensional array of three 0.5 μm diameter stereocilia of heights 4, 2 and 1.4 μm protruding from the apex of an inner hair cell 6 μm wide and 20 μm long. Bundle geometry was based on electron micrographs of rat apical inner hair cell bundles¹². Calcium was assumed to enter through two MT channels each carrying 2.5 pA of calcium current (inner hair cell MT channels 15 pA of which 15 per cent is carried by calcium¹²). Calcium diffused in the cytoplasm (diffusion coefficient, $D = 0.4 \mu\text{m}^2 \text{ms}^{-1}$), was buffered by two diffusible buffers, 1 mM EGTA (calcium dissociation constant $K_D = 0.2 \mu\text{M}$; $D = 0.2 \mu\text{m}^2 \cdot \text{ms}^{-1}$) and 1 mM Fluo-4FF ($K_D = 10 \mu\text{M}$; $D = 0.2 \mu\text{m}^2 \cdot \text{ms}^{-1}$), and by 4 mM of fixed buffer ($K_D = 20 \mu\text{M}$). Calcium was extruded by plasma membrane CaATPase pumps ($K_D = 0.5 \mu\text{M}$) at a density of 2000 μm^{-2} equal in all three rows of stereocilia. Values for other parameters and their justification are given in refs. 20,21. The precise pump density, when varied between 200 μm^{-2} and 8000 pumps μm^{-2} , had little effect on the time course and distribution of the stereociliary calcium signals for the stimulation periods used experimentally. The model calculated the fluorescence of the calcium-bound dye integrated over a confocal volume with lateral and axial resolutions having Gaussian profiles with standard deviations of 0.1 μm and 0.5 μm respectively. Differential equations were integrated in Matlab v7.3 (Mathworks, Natick, MA) with a time interval of 1 μs over a spatial grid of 0.1 μm for the stereocilia and 0.2 μm for the cell body.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by National Institutes on Deafness and other Communication Disorders Grants RO1 DC03896 to Tony Ricci and DC01362 to Robert Fettiplace. We thank Redshirt Imaging and Prairie Technologies for help in optimizing the confocal system.

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Figure 1.

Amplitude and time course of stereociliary calcium signals. **(a)** Images of an inner hair cell stereociliary bundle in fluorescence at +100 mV and -80 mV holding potential and in brightfield illumination (bottom) with the focus at the top of the middle row stereocilia. Each bright spot at -80 mV corresponds to the fluorescence of the calcium indicator in a single stereocilium in row 2 (R2) or row 1 (R1). Images are averages of 10 individual frames taken at times denoted by the thick bars in **(b)**. **(b)** Average changes in fluorescence in row 2 (R2) and row 1 (R1) in response to the pair of stimuli shown above. These correspond to a depolarization from -80 to +100 mV followed by a bundle deflection evoked by a water jet. In this and subsequent figures, the mechanical stimulus denotes the voltage pulse producing the water jet and the fluorescence traces have been zeroed, removing small difference in offset, to facilitate comparison of the traces. The calcium fluorescence developed after repolarization to -80 mV when the MT current is inward. R2 and R1 onsets have been fitted with single exponentials with time constants of 25 ms and 77 ms respectively. At the bottom are superimposed traces of the time course of the fluorescence changes in 14 individual bright R2 stereocilia (R2 bright) and two dark stereocilia (R2 dark) that are indicated by white lines in fluorescence images and dark lines in the brightfield image. Fluorescence intensity expressed in arbitrary units. Calcium indicator, 1mM Fluo-4FF; peak MT current, 760 pA.

Figure 2.

Amplitude and time course of stereociliary calcium signals of an inner hair cell bundle tilted to show all three stereociliary rows. **(a)** Average fluorescence and Nomarski images. The bright R3 stereocilia (black) have neighboring bright R2 stereocilia (red) but two bright R3 stereocilia (purple) have no corresponding bright R2 stereocilia. **(b)** Time course of the fluorescence changes (bottom) for the stereociliary groups in (a): R1 (pink), active R2 (red), non-active R2 (blue), active R3 with a contiguous active R2 (black), active R3 without an active R2 (purple). Fluorescence intensity expressed in arbitrary units. Calcium indicator, 1mM Fluo-4FF; peak MT current 900 pA (c, d).

Figure 3.

Variation in calcium signal along the stereocilia. **(a)** Transverse images of an inner hair cell bundle at the top, middle and base at +100 mV (left) and -80 mV (right). Each image is the average of ten frames taken at times indicated by black bars in (c). **(b)** Schematic of the bundle showing three focal planes at which images in (a) were taken. **(c)** Time course of calcium signals in row 2 and row 1 stereocilia in response to the pair of stimuli shown above. These correspond to a depolarization from -80 to +100 mV followed by a bundle deflection elicited by a water jet that produces a membrane current depicted in third trace. The three focal planes were taken in three consecutive presentations and the current responses in each are superimposed. Note that for row 2, the fluorescence change is larger and faster for the middle (red) than for the bottom (blue) of the bundle. For row 1, the fluorescence change is largest at the bottom (blue) and smaller and slower at the middle (red) and top (black) of the bundle. 1mM Fluo4 calcium indicator plus 50 μM AlexaFluor 488 to counter stain all stereocilia. Peak MT current, 740 pA.

Figure 4.

Relationship between number of active stereocilia and MT current amplitude. **(a)** Images of three cells showing different numbers of bright fluorescent (active) stereocilia during mechanical stimulation that evoked different MT current amplitudes which are given on the images. In all examples it is possible to see both row 2 and row 3 stereocilia as well as row 1 in the top two images. **(b)** Plot of MT current against number of (bright) active stereocilia measured in 52 different inner hair cells. Line is a fit through the origin with slope 35.4 ± 1.3 pA/stereocilium, regression coefficient 0.87.

Figure 5.

Amplitude and time course of stereociliary calcium signals in an outer hair cell. **(a)** Transverse images of an outer hair cell bundle at the middle and base of the bundle, each image being the average of ten frames taken at times indicated by black bars in **(b)**. The positions of the three rows, R1, R2 and R3 are indicated on the image. **(b)** Time course of calcium signals in row 1, row 2 and row 3 stereocilia in response to the stimuli shown above, a depolarization from -80 to $+80$ mV followed by a bundle deflection elicited by a water jet. The membrane current responses for each of the two focal planes are superimposed in third trace. Note that for row 2 (R2) and row 3 (R3), the fluorescence change is larger at the middle than at the base of the bundle whereas for row 1 (R1), the fluorescence change is largest at the base and smaller at the middle. Dashed lines superimposed on the fluorescence onsets at the middle are single exponential fits with time constants of 116 ms (R1), 19 ms (R2) and 16 ms (R3). 1 mM Fluo4 calcium indicator plus 50 μM AlexaFluor 488 to counter stain all stereocilia. Peak MT current, 520 pA.

Figure 6.

Theoretical distributions of calcium and Fluo-4FF fluorescence for two different channel locations. (a) Two MT channels (triangles) located at the tops of R2 and R3 stereocilia. The cartoon of the inner hair cell bundle shows the gradients in calcium concentration 80 ms after opening of the MT channels as in the experiments. In the middle traces, the distributions of Fluo-4FF fluorescence within the confocal volume are shown for the two focal planes indicated as dashed lines in cartoon, at the middle and at the base of the bundle. The simulations incorporated the axial and lateral resolutions of the confocal system indicated as FWHM by the green ellipse superimposed on the R3 stereocilium. Although the middle focal plane is above the top of the R3 stereocilia, there is still an R3 signal because the axial resolution is 1.1 μm FWHM, but this is smaller than at the base because much of the confocal volume is outside the R3 stereocilium. At the middle focal plane, the R2 calcium fluorescence is about 15 times larger than R1 and 3 times larger than R3. The time courses of the changes in fluorescence, scaled to the maximum occurring in the bundle are shown at the bottom for R1, R2 and R3. Dashed lines double exponential fits to onsets with time constants: $\tau_1 = 120$ ms (R1); $\tau_1 = 10$ ms, $\tau_2 = 70$ ms (R2; fast component 0.6); and $\tau_1 = 8$ ms, $\tau_2 = 35$ ms (R3, fast component 0.55). (b) Two MT channels placed at either end of the tip link. The fluorescence in R1 is larger than in R2 which is larger than in R3. Time course of fluorescence change for channels at either end of tip link. The distributions of fluorescence for channels at lower end of tip links (a) most closely resemble those observed experimentally. See Methods for details of the model.

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Figure 7.