Development and localization of reverse-polarity mechanotransducer channels in cochlear hair cells

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Cochlear hair cells normally detect positive deflections of their hair bundles, rotating toward their tallest edge, which opens mechanotransducer (MT) channels by increased tension in intercalary tip links. After tip-link destruction, the normal polarity of MT current is replaced by a mechanically sensitive current evoked by negative bundle deflections. The “reverse-polarity” current was investigated in cochlear hair cells after tip-link destruction with BAPTA, in transmembrane channel-like protein isoforms 1/2 (*Tmc1:Tmc2*) double mutants, and during perinatal development. This current is a natural adjunct of embryonic development, present in all wild-type hair cells but declining after birth with emergence of the normal-polarity current. Evidence indicated the reverse-polarity current seen developmentally was a manifestation of the same ion channel as that evident under abnormal conditions in *Tmc* mutants or after tip-link destruction. In all cases, sinusoidal fluid-jet stimuli from different orientations suggested the underlying channels were opened not directly by deflections of the hair bundle but by deformation of the apical plasma membrane. Cell-attached patch recording on the hair-cell apical membrane revealed, after BAPTA treatment or during perinatal development, 90-pS stretch-activated cation channels that could be blocked by Ca^{2+} and by FM1-43. High-speed Ca^{2+} imaging, using swept-field confocal microscopy, showed the Ca^{2+} influx through the reverse-polarity channels was not localized to the hair bundle, but distributed across the apical plasma membrane. These reverse-polarity channels, which we propose to be renamed “unconventional” mechanically sensitive channels, have some properties similar to the normal MT channels, but the relationship between the two types is still not well defined.

hair cells | mechanotransducer channels | calcium imaging | cochlea | transmembrane channel-like protein

Ion channels sensitive to mechanical deformation of the cell membrane are widely distributed in vertebrates and are integral to the function of specialized mechanoreceptors, such as those in the sensory neurons of the skin, vasculature, or inner ear. The molecular structure of these mechanically gated channels has been the subject of much recent research and speculation (1–4) because they are the last category of vertebrate ion-channel (after voltage-gated and ligand-activated channels) evading characterization. Although success was achieved by assigning piezo2 as a transduction channel in many cutaneous touch receptors (3), uncertainty persists about the composition of the mechanotransducer (MT) channel in hair cells of the inner ear (2, 4). There, the MT channels reside in the stereociliary (hair) bundle, where they are activated by deflections of the bundle toward the taller edge of the staircase, and so increasing tension in the oblique extracellular tip links connecting adjacent stereocilia. Transmembrane channel-like protein isoforms 1 and 2, *TMC1* and *TMC2* (5), have been suggested as candidates for the hair-cell transducer channel (6, 7) because mutations of each alters ion conduction through the MT channels (7–9). Nevertheless, it is unclear whether deleting both *Tmc1* and *Tmc2* completely abolishes mechanotransduction (7), or merely prevents the MT channels being

targeted to the stereociliary tips, so they cannot be gated by tip-link tension (10). In *Tmc1:Tmc2* double mutants, or after destruction of the tip links, the normal MT current is lost, only to be replaced by a mechanically sensitive current evoked by stimuli of reverse polarity (10–12). Whether the channels underlying these reverse-polarity currents are the same as those in unperturbed cells is uncertain (2, 13). Two questions arise about them: where are they located on the hair cell, and do they only appear under pathological circumstances, as in destruction of tip links or mutations causing loss of transduction? Here we show that during late embryonic development of the inner ear, reverse-polarity MT currents occur in both cochlear and vestibular hair cells and precede the acquisition of the normal-polarity MT current, as was described in zebrafish hair cells (14). We also show that under conditions that generate “reverse-polarity” currents, stretch-sensitive channels emerge on the apical membrane of the hair cells with properties akin to the reverse-polarity channels.

Results

Reverse-Polarity MT Currents. Following a variety of manipulations culminating in loss of normal hair-cell transduction, a mechanically sensitive current emerges that is apparently activated by hair bundle deflections of polarity opposite to those opening MT channels in normal cells (10–12, 15, 16). Because the reverse-polarity currents are insensitive to tip-link destruction by submicromolar Ca^{2+} buffered with BAPTA [1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid] (10, 12), they must be activated by an

Significance

Cochlear hair cells are the receptors of the inner ear, detecting sound stimuli by to-and-fro motion of their sensory hair bundle to open mechano-electrical transduction (MT) channels at the tips of the hairs. During embryonic development, mechanically sensitive ion channels appear transiently in mouse cochlear hair cells before acquisition of the normal adult MT channel. These channels are located on the hair-cell apical plasma membrane, are stretch-activated by membrane deformation, and have similar but not identical properties to normal MT channels. It will be important to establish whether the two types of channel possess the same protein core or are structurally distinct, as this may provide a clue to the functional significance of these supernumerary mechanically sensitive channels.

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Fig. 2. Stretch-activated channels on the OHC apical membrane. (A) Location of cell-attached patches on apical plasma membrane near insertion of kinocilium. (B) Cell-attached records at various times after exposure to BAPTA saline when stretch-sensitive channels appeared, with channel number increasing over 20 min. All records from the same cell. Amplitude histograms for 10, 15, and 20 min; holding potential, $V_m = -60$ mV, 1.5 mM Ca^{2+} in electrode solution. (C, Left) Voltage dependence of stretch-activated channels, records shown; (Right) amplitude histogram for $V_m = -90$ mV and current-voltage relationship for this and two other channels. Mean slope conductance, $\gamma = 63$ pS fitted to all points; 1.5 mM Ca^{2+} in electrode solution. (D, Left) Voltage dependence of stretch-activated channels, records shown; (Right) amplitude histogram for $V_m = -100$ mV and current-voltage relationship for this and two other channels. Mean slope conductance, $\gamma = 93$ pS; 0.07 mM Ca^{2+} in electrode solution. The upper trace shows pressure monitor, 40-mmHg suction, which relates to the top records; the lower trace records on a faster time scale were taken during the applied suction. In C and D, channels were recorded 8 and 10 min, respectively, after BAPTA application.

and was half-maximal at 0.1 d in basal OHCs, 2.5 d in apical OHCs, and 2.0 d in apical IHCs (Fig. 4 B, C, and F); the trend, in which transduction at the base of the cochlea develops sooner than at the apex, has been previously reported in both mice and rats (8, 18, 19). Decline in the reverse-polarity current had half-maximal times similar to growth of the normal MT current: 1 d in basal OHCs, 2.4 d in apical OHCs, and 1.6 d in apical IHCs. Reverse-polarity currents were also present in saccular hair cells at embryonic day (E) 16 and E17, but had virtually disappeared by P0 (Fig. S2), by which time normal transduction is fully developed (20). The reverse-polarity current was still present in *Tmc1:Tmc2* double mutants when the normal-polarity current was absent (Fig. 4 D and E); it was initially large but declined to a half-maximum at 3.3 d and disappeared by P7 (10).

Despite their constitutive nature, the reverse-polarity currents observed developmentally had several properties akin to those mechanically sensitive currents seen in *Tmc1:Tmc2* mutants. First, they were insensitive to tip-link destruction with BAPTA, perfusion of which, in five different OHCs, converted a biphasic current into a single component (Fig. S1). Second, stretch-sensitive channels recorded on the top of the hair cell in neonatal animals had a unitary conductance of 64.1 ± 3.6 pS (mean $\pm$ SD, $n = 3$ OHCs) in 1.5 mM Ca^{2+} . This conductance, measured in P0 to P1 wild-type mice at an age where the reverse-polarity current predominates, is not significantly different from stretch-sensitive channels after BAPTA ($P = 0.52$). Both single channels and reverse-polarity currents were unaffected by 50 μM PPADS (pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid Na_4), a concentration that fully blocks P2X₂ receptor channels (Fig. S3). Third, the reverse-polarity MT currents, like the normal polarity currents, were blocked by FM1-43 (Fig. 5 A–C). Normal polarity currents in P5 to P6 wild-type OHCs were blocked at -84 mV by FM1-43 with an IC_{50} of 2.8 ± 0.4 μM and Hill coefficient of 1.9 (Fig. 5D), values similar to those previously reported (17). The reverse-polarity currents in *Tmc1^{-/-}:Tmc2^{-/-}* OHCs were about 10-fold less sensitive to FM1-43 block, with an IC_{50} of 26 ± 2.3 μM and a Hill coefficient of 1.9, whereas the reverse-polarity currents in P1 wild-type had an IC_{50} of 29 ± 1.0 μM and a Hill coefficient of 2.0. Because FM1-43 is an open channel blocker, the difference in IC_{50} between normal- and reverse-polarity channels may partly stem from a difference in their resting open probability as argued for dihydropyridine, another open

MT-channel blocker (21). The clearest manifestation of this difference was seen on puffing FM1-43 onto hair bundles (Fig. 5E). In wild-type, all OHCs rapidly fluoresced because of influx of the dye through partially open MT channels. However, in the *Tmc1:Tmc2* double mutant, no intracellular fluorescence was visible until the reverse-polarity channels were opened by deflecting the hair bundle.

Calcium Imaging. Because the reverse-polarity MT channels, like the normal MT channels, are permeable to Ca^{2+} (10), fast confocal imaging of Ca^{2+} influx during mechanical stimulation was used to obtain more information about their location. This approach had previously shown the conventional MT channels to be at the tips of the shorter rows of stereocilia (22). In the present experiments on early neonates (P0–P2), diphasic steps to the hair bundle generated both normal-polarity and reverse-polarity currents on the positive and negative transitions of the stimulus, respectively (Fig. S1). Fluorescence images of hair cells filled with

Fig. 3. Stretch-activated channels in *Tmc1:Tmc2* double knockouts. (A and B) Cell-attached recordings of stretch-activated channels in apical OHC membrane of *Tmc1:Tmc2* mutants at (A) -80 mV and (B) $+120$ mV. (C) Current amplitude histograms at -80 mV and $+120$ mV. (D) Current-voltage relationships, mean slope conductance = 60 ± 8 pS ($n = 6$) in 1.5 mM Ca^{2+} in electrode solution. All are P5 apical OHCs.

Fig. 4. Reverse-polarity currents during cochlear development. (A) MT currents for sinusoidal fluid jet stimuli for P0, P2, and P5, wild-type apical OHCs. Note transformation from reverse polarity to biphasic to normal polarity with age. (B) Apical OHCs, reverse-polarity (crosses) and normal-polarity (filled circles) currents with age; mean ± 1 SD, number of cells above points. (C) Basal OHCs, reverse-polarity current (crosses) and normal-polarity currents (filled circles) with age. (D) Representative MT current traces for sinusoidal fluid jet stimuli for P0, P2, and P5, *Tmc1*^{-/-}:*Tmc2*^{-/-}, apical OHCs. Note only reverse-polarity responses at all ages. (E) *Tmc1*^{-/-}:*Tmc2*^{-/-} apical OHCs, reverse-polarity and normal-polarity currents with age. (F) Apical IHCs, reverse-polarity and normal-polarity currents with postnatal age; in all experiments depicted, holding potential = -84 mV.

the Ca^{2+} indicator Fluo-5F were acquired with confocal sections through the middle of the hair bundle or at the cell apex (Fig. 6*A* and *C*). Following a positive stimulus that generated a normal-polarity MT current (Fig. 6*A*), there was a rapid increase in fluorescence if the region of interest (ROI) was drawn over the hair bundle (Fig. 6*B*, position 1, red), influx of Ca^{2+} being triggered by hair-cell hyperpolarization to augment the electrical driving force on the ion. If the ROI was drawn outside the bundle (Fig. 6*B*, position 2, black), there was only a small delayed increase in fluorescence (ΔF), probably reflecting diffusion from the primary source in the bundle. In response to a negative stimulus evoking a reverse-polarity MT current (Fig. 6*C* and *D*), an increase was observed across the apical membrane (Fig. 6*C*, position 2); there was no ΔF in the hair bundle (Fig. 6*C*, position 1) during the reverse-polarity current, only at the end of the stimulus when the positive-going displacement back to the resting position elicited a small normal-polarity current (Fig. 6*C*, arrow). The ratio of cell-apex fluorescence for the positive and negative stimuli in Fig. 6*C* was 2.5. Similar distributions of Ca^{2+} response were observed in one other IHC and two OHCs in which the ratio of cell-apex

ΔF for the positive to negative stimuli was 6.5, 9.3, and 3.2 respectively. In another OHC, there was no normal-polarity MT current because of the young age P1, but a reverse-polarity current was present and was associated with a fluorescence increase across the top of the cell. These measurements suggest that the reverse-polarity MT channels are in the apical plasma membrane rather than confined to the hair bundle.

The small size of the Ca^{2+} response linked to the reverse-polarity current makes detailed analysis difficult but is to be expected. To a first approximation, the Ca^{2+} signal will depend on the total charge transfer, which is proportional to the product of the time-integral of the MT current and the Ca^{2+} permeability of the MT channel. If Q_R is the ratio of the charge transfers for the normal- and reverse-polarity MT currents, and F_R is the ΔF ratio for the normal and reverse-polarity MT currents, then the ratio of Ca^{2+} permeabilities, P_{CaR} , for the two channel types is given by $P_{\text{CaR}} = F_R/Q_R$, assuming no change in the channel's Cs^+ permeability. For the cell of Fig. 6C, the predicted P_{CaR} was $2.52/0.7 = 3.6$; the mean P_{CaR} value for four cells (two IHCs and two OHCs) was 3.8 ± 0.5 . Ca^{2+} selectivity values have been directly determined from measurements of reversal potentials, which gave $P_{\text{Ca}}/P_{\text{Cs}} = 6.1$ for apical wild-type OHCs and $P_{\text{Ca}}/P_{\text{Cs}} = 1.8$ for the reverse-polarity channels (8, 9), their ratio being 3.4.

Discussion

Location of Reverse-Polarity MT Channels. Three lines of evidence indicate that the reverse-polarity MT channels (10) occur mainly in the apical plasma membrane of the OHC: stimulating the bundle from different orientations, recording stretch-activated

Fig. 5. Effects of FM1-43 on normal- and reverse-polarity MT currents. (A) Normal-polarity MT currents in P6 wild-type OHC in the absence and presence of 3 μM FM1-43. (B) Reverse-polarity currents in P1 wild-type OHC in the absence and presence of 30 μM FM1-43. (C) Reverse-polarity MT currents in an OHC of P5 *Tmc1:Tmc2* mutant in the absence and presence of 30 μM FM1-43. (D) Dose-response plots under the three conditions: normal (open circle), reverse-polarity wild-type (filled circles), and *Tmc1:Tmc2* mutant (crosses) all fit with the Hill equation. Each point is the mean $\pm$ SD of 2–5 measurements. Normal-polarity points, $\text{IC}_{50} = 2.8 \mu\text{M}$, Hill coefficient $n_H = 1.9$; reverse-polarity points, $\text{IC}_{50} = 26 \mu\text{M}$, $n_H = 1.8$; holding potential = -84 mV in A–D. (E) Images of isolated organ of Corti after puff of FM1-43 in *Tmc1:Tmc2* double mutant in unperturbed condition (*Upper*) and after bundle stimulation (*Lower*). (Magnification: 500 $\times$) (F) Stretch-activated channels after BAPTA with 5 μM FM1-43 in the patch electrode are blocked at hyperpolarized holding potential (-80 , -100 mV) but block is relieved at $+150 \text{ mV}$.

Fig. 6. Ca^{2+} imaging during of normal- and reverse-polarity MT currents. (A) Positive fluid jet step plus diphasic change in membrane potential from HP = -50 mV elicits a normal-polarity current and a fast fluorescence increase, ΔF , on hyperpolarization at ROI-1 (region of interest 1) but not at ROI-2; plane of focus at the middle of bundle. (B) Bright-field images of the IHC bundle (arrow) focused at midbundle (Left) and the base of bundle (Right); FJ, fluid jet; schematic shows top view of IHC defining ROI-1 over the hair bundle and ROI-2 over apex membrane behind the bundle. (C) Negative fluid jet step plus diphasic change in membrane potential from HP = -50 mV elicits a reverse-polarity current and fast fluorescence increase on hyperpolarization at ROI-2 but not at ROI-1. A delayed fluorescence increase at ROI-1 occurs in response to the small normal-polarity current (arrow) at the positive-going offset of the fluid jet; plane of focus at the base of bundle. (D) Pseudocolor fluorescence intensities before (a) and during the reverse-polarity current (b), and during the normal-polarity current (c), each being the average of three images taken at the times indicated in C. Note the ΔF for reverse-polarity current occurs over much of cell apex (b), whereas ΔF for normal-polarity current (b) is confined to the hair bundle. All records from same apical IHC of P1 wild-type.

channels and imaging Ca^{2+} influx. The results of varying the orientation of the fluid-jet stimulator (Fig. 1) argue that the mechanically sensitive channels seen after BAPTA treatment and in *Tmc1:Tmc2* double mutants, unlike normal MT channels, do not retain sensitivity to a specific axis of hair bundle stimulation. This can be most simply explained if they are stretch-sensitive channels gated by deformation of the plasma membrane overlying the hair bundle or the cell apex around the base of the bundle. These channels should not, therefore, be referred to as “reverse-polarity” channels—which is a misnomer—but as “unconventional” mechanically sensitive channels. Ca^{2+} influx measurements through these unconventional mechanically sensitive channels confirmed they were in the apical membrane and not confined to the tips of the stereocilia. This notion was endorsed by recording stretch-activated channels in the apical membrane with properties similar to those of *Tmc1:Tmc2* double mutants inferred from whole-cell recordings. Thus, both are cation channels with a conductance in 1.5 mM Ca^{2+} of ~ 60 pS (9, 10), they are increased about 1.5-fold to 90 pS on reducing external Ca^{2+} to a level comparable to that found in endolymph (10), and they are blocked by FM1-43. Although stretch-activated cation channels have been recorded from OHCs, none exactly match the attributes seen here. Those previously reported in the OHC basolateral membrane were 150 pS K^{+} -selective channels (23, 24), 30 pS voltage-dependent cation channels (24), and channels

passing both anions and cations (25). There has been a report of ion channels on the OHC apical membrane recorded using a scanning ion conductance probe (26). OHCs possess purinergic P2X receptors on their apical and stereociliary membranes (27), but the standard P2X receptor inhibitor, PPADS (28), had no effect on the unconventional currents or channels (Fig. S3).

Unconventional MT Currents During Development. Although the existence of unconventional MT channels after tip-link destruction or in *Tmc1:Tmc2* double mutants is indisputable (10–12, 29), their physiological role and relationship to the normal MT channels gated by tension in the tip-links, is more uncertain. A possible clue is that they are present in all hair-cell classes—cochlear inner and outer, and vestibular—during maturation of the transduction machinery. We found the unconventional channels exhibit an inverse relationship to the normal MT channels: they appear in OHCs and IHCs during the late embryonic period, their density peaks around birth, and then declines with development of the normal MT current. To our knowledge, this is the first report of a systematic acquisition then disappearance of the reverse-polarity channels in wild-type animals without any genetic or chemical manipulation. Based on their properties (single-channel conductance, block by FM1-43 and Ca^{2+} selectivity), the unconventional MT currents recorded during development in wild-type mice and those seen under abnormal conditions (in mutants or after tip-link destruction) are manifestations of the same ion channel. Reverse-polarity MT currents were previously found to occur embryonically (12), but no subsequent pattern of development or relationship to the normal MT current was evident in that work, and it was further claimed that the reverse-polarity current persisted until at least P10, which we never saw. One hypothesis is that the unconventional channels are associated with the small microvilli that carpet the top surface of hair cells behind the hair bundle, and their transient neonatal appearances are linked. However, the currents are gone by P4, well before the microvilli disappear at P5–P7 (see supplemental figure 4B in ref. 6).

The ion channels underlying the developmental reverse-polarity MT current are unidentified, but their persistence in *Tmc1:Tmc2* mutants (Fig. 4F) suggest they are not TMC isoforms. The channels’ developmental role may be to sense membrane forces distributed across the top of the hair cell, and Ca^{2+} influx through them is perhaps linked to proper shaping and maturation of the hair bundle, which ensues over this perinatal period (30). However, the relationship of the unconventional to the normal MT channels is unclear and it is instructive to compare their properties. In apical OHCs, both are cation channels that pass Ca^{2+} with $P_{\text{Ca}}/P_{\text{Cs}} = \sim 5.8$ in normal and ~ 1.5 for reverse-polarity channels; both are blocked by external FM1-43 with IC_{50} of ~ 3 μM in normal and 26 μM in unconventional channels; the unitary conductances are similar in apical OHCs, with values in 1.5 mM Ca^{2+} of 63 pS in normal and 61 pS for unconventional channels. However, in basal OHCs the normal channels are about twofold larger than the reverse-polarity channels, a difference that depends on the presence of TMC1, which may contribute to an external vestibule (9, 31). In both cases the channels are blocked by extracellular Ca^{2+} , lowering its concentration to a few tens of micromolar, increasing channel conductance by 50%. Thus, biophysical attributes of the normal-polarity and unconventional channels are similar but not identical. If the channels had the same pore, such differences could reflect the presence of other subunits in the normal channel, such as LHFPL5 and TMIE (9, 32–34). It will be important to establish whether both have the same protein core or are structurally distinct, as this may provide a clue to the functional significance of the unconventional channels.

Materials and Methods

Preparation. MT currents were recorded from hair cells in the isolated organ of Corti and saccular macula of mice between E17 to P8 (where P0 = E19 is the

birth date) using methods previously described (9, 35). Mutation in the *Tmc1* gene was achieved with *Tmc1* Δ /Δ (B6.129-*Tmc1*^{tm1.1Aig/J}; Jackson Lab (6). The *Tmc2* mutant (B6.12955-*Tmc2*^{tm1Lex}/Mmudc) from the Mutant Mouse Regional Resource Center, University of California, Davis, CA, was used to generate the double-mutants *Tmc1*^{-/-};*Tmc2*^{-/-}. Mice were genotyped from tail clips taken after dissection for the electrophysiology recordings, the experimenter being blind to the genotype. Mice were killed by decapitation using methods approved by the Institutional Animal Care and Use Committees of the University of Wisconsin–Madison and Stanford University, according to current National Institutes of Health guidelines. Excised cochlear turns, low-frequency apex, or high-frequency base (~0.2 or 0.75 of the distance along the cochlea from the apex), were immobilized under strands of dental floss in a recording chamber on a Leica DLF5 fixed-stage microscope and viewed through a 63 $\times$, 0.9 NA, long working-distance water-immersion objective. The chamber was perfused with artificial perilymph of composition: 152 mM NaCl, 6 mM KCl, 1.5 mM CaCl₂, 2 mM Na-pyruvate, 8 mM D-glucose, and 10 mM Na-Hepes, pH 7.4, osmolarity ~320 mOsm/L at room temperature, 21–23 °C. Flow through a local puffer pipette (tip diameter, 8 μ m) driven by a Picospritzer (General Valve) was used to control the solution around the hair bundle. In some experiments, PPADS (Sigma-Aldrich) was added to block P2X receptor channels (28).

Electrophysiology and Stimulation. Most whole-cell recordings were made with patch electrodes filled with: 130 mM CsCl, 3 mM MgATP, 10 mM Tris phosphocreatine, 1 mM EGTA, 0.5 mM GTP, 0.5 mM cAMP, 10 mM Cs-Hepes, pH 7.2, ~295 mOsm/L, and connected to an Axopatch 200B amplifier with 5-kHz output filter. Hair bundles were deflected with a fluid jet (8, 36), driven by a 40-Hz sinusoidal voltage or voltage step to elicit a maximal MT current. In some experiments, the resulting bundle motion was determined by projecting an image of the bundle onto a pair of photodiodes (8, 37). Single stretch-sensitive channels were obtained from cell-attached patch recordings on the hair-cell apical membrane. For these recordings, patch electrodes (resistances ~4 M Ω) were filled with an extracellular Na-based saline containing either 1.5 mM or 0.07 mM CaCl₂ and just before forming a

seal, the preparation was perfused with a high K⁺ solution (140 mM KCl, 0.2 mM CaCl₂, 8 mM D-glucose, 10 mM K-Hepes, pH 7.4) to zero the hair-cell resting potential. The resting potential after exposure to the high K⁺ was measured by attaining whole-cell at the end of a channel recording yielding 1.3 ± 4 mV ($n = 4$), confirming that the high K⁺ solution did zero the membrane potential. Suction was applied via the patch pipette and calibrated with a 3460 Pressure Gauge (Control Company). It was not possible to apply multiple controlled stimuli to derive the channel kinetics and therefore ascertain the presence of channel adaptation or inactivation in ensemble averages.

Ca²⁺ Imaging. Changes in intracellular Ca²⁺ concentration were measured with the low-affinity indicator Fluo-5F (Life Technologies). The K⁺ salt of Fluo-5F was introduced at 0.5–1 mM via the patch pipette with ascorbic acid added as an antioxidant to minimize cell damage by free radicals generated during illumination. The pipette solution was: 95 mM CsCl, 40 mM Cs-ascorbate, 3.5 mM MgATP, 2 mM Cs-pyruvate, 5 mM Tris creatine phosphate, 1 mM EGTA, 10 mM Hepes, pH 7.2. Fluorescence emission was measured with a swept-field confocal (Prairie Technologies) illuminated with a 200-mW argon laser at 488 nm and viewed with a 60 $\times$ 1.0 numerical aperture water-immersion Olympus objective, scanned at 500 Hz (22). Images were collected and analyzed using Neuroplex software (RedShirtImaging) and ImageJ, the average intensity change ΔF being calculated for small areas overlying the bundle. To eliminate pseudofluorescence changes because of movement artifacts, the mechanical stimulus was preceded by depolarization to a positive potential near the Ca²⁺ equilibrium potential, which was then switched negative to initiate the Ca²⁺ influx and associated fluorescence change while the bundle remained displaced (22). Unless otherwise stated, values are quoted as mean ± 1 SD, each recording used to construct a mean being from a different animal. Statistical significance was assessed with a two-tailed Student's *t* test.

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