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Detection of Ca^{2+} entry through mechanosensitive channels localizes the site of mechanoelectrical transduction in hair cells

(auditory system/confocal microscopy/fluo-3/hair bundle/vestibular system)

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ABSTRACT **A hair cell, the sensory receptor of the internal ear, transduces mechanical stimuli into electrical responses. Transduction results from displacement of the hair bundle, a cluster of rod-shaped stereocilia extending from the cell's apical surface. Biophysical experiments indicate that, by producing shear between abutting stereocilia, a bundle displacement directly opens cation-selective transduction channels. Specific models of gating depend on the location of these channels, which has been controversial: although some physiological and immunocytochemical experiments have situated the transduction channels at the hair bundle's top, monitoring of fluorescence signals from the Ca^{2+} indicator fura-2 has instead suggested that Ca^{2+} traverses channels at the bundle's base. To examine the site of Ca^{2+} entry through transduction channels, we used laser-scanning confocal microscopy, with a spatial resolution of <1 μm and a temporal resolution of <2 ms, to observe hair cells filled with the indicator fluo-3. An unstimulated hair cell showed a "tip blush" of enhanced fluorescence at the hair bundle's top, which we attribute to Ca^{2+} permeation through transduction channels open at rest. Upon mechanical stimulation, individual stereocilia displayed increased fluorescence that originated near their tips, then spread toward their bases. Our results confirm that mechanoelectrical transduction occurs near stereociliary tips.**

Hair cells are specialized epithelial cells that serve as the sensory receptors of the acoustical, vestibular, and lateral-line organs. By transducing mechanical stimuli such as sounds and accelerations into electrical signals, these cells encode sensory information for analysis by the central nervous system (for review, see ref. 1). Mechanoelectrical transduction is mediated by the hair bundle, an organelle that protrudes from the cuticular plate at the apical surface of a hair cell's soma. A hair bundle comprises dozens of hexagonally packed, actin-filled processes called stereocilia, which are arranged in stepped ranks.

When a hair bundle is displaced in the positive direction, toward its tall edge, the transduction channels' open probability increases, allowing an influx of cations such as K^+, Na^+, and Ca^{2+}. This inward current depolarizes the hair cell's membrane, increasing the rate of neurotransmitter release at the cell's basolateral surface. Deflecting the hair bundle in the negative direction, toward its short edge, conversely causes the transduction channels' open probability to decrease, hyperpolarizing the membrane and decreasing the rate of neurotransmission. To maintain sensitivity to transient displacements in the presence of sustained stimulation, a hair cell adapts to both positive and negative stimuli. This adaptation, which occurs over tens of milliseconds, allows each transduction channel's open probability to return toward a resting level of ≈0.15.

The transduction channels of hair cells are directly gated by mechanical stimulation. The prevailing model of transduction supposes that each channel's open probability is set by the tension in an elastic element attached to the channel's gate (for review, see ref. 1). This gating spring's tension is determined in part by the hair bundle's position, but is adjusted by a molecular motor that mediates adaptation (for review, see ref. 2).

The tip link, a filament that stretches from the tip of one stereocilium to an insertional plaque on the side of its tallest neighbor, may be the morphological correlate of the gating spring (3). If this hypothesis is correct, transduction channels must be located at or near the stereociliary tips, at one or both ends of each tip link. Two lines of physiological evidence support the idea that transduction channels in fact occur atop the hair bundle: measurement of the extracellular potential drop created by transduction current (4) and channel blockage by iontophoretically applied gentamicin (5). Immunocytochemical studies with antibodies against pharmacologically similar channels also suggest that transduction channels occur at stereociliary tips or at the sites of stereociliary abutment (for review, see ref. 6).

Attempts to localize transduction channels by visualizing fluorescence in hair cells loaded with Ca^{2+}-sensitive fluorophores have produced conflicting results. Using the indicator fura-2, Ohmori (7) provided evidence that Ca^{2+} enters through transduction channels at a hair bundle's base. In a preliminary report, Huang and Corey (8) instead observed a "wave" of increased fluorescence traveling from the tops to the bottoms of hair bundles containing fluo-3, a Ca^{2+} indicator whose fluorescence emission increases some 80-fold upon binding Ca^{2+} (9). To resolve the discrepancies between these investigations, we have used laser-scanning confocal microscopy to examine the Ca^{2+} concentration in stimulated hair bundles. We have been able to detect changes in stereociliary Ca^{2+} concentration with submicrometer spatial resolution and millisecond temporal resolution.

MATERIALS AND METHODS

Hair-Cell Isolation. Experiments were performed at room temperature on hair cells isolated from saccular maculæ of the bullfrog, *Rana catesbeiana*. Each internal ear was dissected in 100 μM Ca^{2+} saline solution (110 mM Na^+/2 mM K^+/100 μM Ca^{2+}/110 mM Cl^-/3 mM D-glucose/5 mM Hepes, pH 7.25). After removal of the tissue overlying the saccular nerve, the ear was incubated for 15 min in a similar solution supplemented with 1 mM EGTA and 1 mM $MgCl_2$. The saccular macula was then dissected and digested for ≈20 min with subtilisin Carlsberg (protease type XXIV; Sigma) at 50 $\mu g \cdot ml^{-1}$ and for 5 min with deoxyribonuclease I (type II; Sigma) at 50 $\mu g \cdot ml^{-1}$. In a recording chamber, the otolithic membrane was peeled away, and hair cells were teased from the epithelium with an eyelash. After the cells had settled on a concanavalin A-coated coverslip at the chamber's bottom,

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the medium was replaced with standard saline solution (110 mM Na^+/2 mM K^+/4 mM Ca^{2+}/118 mM Cl^-/3 mM D-glucose/5 mM Hepes, pH 7.25).

Electrophysiological Recording. While the membrane potential of each hair cell was held at −70 mV with a voltage-clamp amplifier (EPC-7, List Electronics, Darmstadt, Germany), transduction current was measured by the tight-seal, whole-cell recording technique. The recording electrodes had resistances of 3–5 MΩ when filled with internal solution containing 102 mM Cs^+, 4 mM Na^+, 2 mM Mg^{2+}, 1 mM ATP, 104 mM Cl^-, 5 mM Hepes (pH 7.3), and 200 μM fluo-3 (pentapotassium salt; Molecular Probes). When noted below, the internal solution included 1 mM of the Ca^{2+} chelator EGTA to spatially restrict the fluorescence increase due to Ca^{2+} entry.

A hair bundle was stimulated by displacement of a glass micropipette attached by gentle suction to the bulb at the kinocilium's tip. The pipette's shank was affixed to a piezoelectrical stimulator (P-835.10, Physik Instrumente, Waldbronn, Germany) connected to a wide-band power amplifier (P-870, Physik Instrumente), which was used to deliver calibrated displacements of 150–700 nm. To prevent excitation of the stimulator's mechanical resonance, the driving signal was filtered with an eight-pole Bessel filter whose half-power frequency was 500 Hz. The rise time of the stimulus was <1 ms.

The control signals for the voltage-clamp amplifier, piezoelectrical stimulator, and confocal-scanning system were supplied by a computer programmed in LABVIEW (version 3.1, National Instruments, Austin, TX). Data were low-pass-filtered with an eight-pole Bessel filter set at 1 kHz, digitized, and recorded with the same computer system at a sampling frequency of 5 kHz.

Confocal Microscopy. Fluo-3-loaded hair cells were simultaneously visualized through epifluorescence and transmitted differential-interference-contrast optics with a laser-scanning confocal-imaging system (LSM-410UV, Zeiss) equipped with a ×63 Plan-neofluar oil-immersion objective lens of numerical aperture 1.25. Fluo-3 fluorescence was excited by the 488-nm line of an Ar/Kr laser, delivered to the specimen through a 510-nm dichroic beam splitter. Epifluorescence was detected by a photomultiplier equipped with a 515-nm long-pass filter.

The frame-scan mode of the confocal microscope was used to follow fluorescence changes in an optical section of the hair bundle and cuticular plate. Although the images were acquired at 1.1-s intervals, the actual duration of an individual frame scan was 0.7 s. During frame-scan acquisition, a 1.5-s positive displacement was delivered to the hair bundle. Because stimulation and image acquisition were not synchronized, displacements were sometimes captured in two images, sometimes in only one.

To achieve greater temporal resolution, we utilized the confocal system's line-scan mode. While a diffraction-limited point of illumination was repeatedly swept along a single stereocilium at 1.4-ms intervals, the hair bundle was displaced for 100–500 ms.

By use of NIH IMAGE software (version 1.49; National Institutes of Health), confocal images were smoothed with a 3 × 3 filter, which introduced a spread in the time domain of <0.5 ms. Contrast was enhanced for optimal reproduction of images during publication. Although this procedure saturated the brightness of the cuticular plates in some line-scan images, in no instance were >16% of the pixels saturated in the original images.

When calibrated with a 100-nm fluorescent latex bead, the axial resolution of the fluorescence imaging system, measured as the distance between half-maximal points on the bead's intensity profile, was 800 nm. The corresponding lateral resolution was 400 nm. In a bullfrog's saccular hair bundle, the centers of adjacent stereocilia are spaced ≈950 nm apart at the bundle's base and ≈500 nm apart at its top (10). When a stereocilium was examined by the line-scan technique, the fluorescence signal was therefore derived primarily from that single process, but included some contribution from contiguous stereocilia.

Three geometrical factors influenced the fluorescence pattern in a hair bundle. First, because a bundle comprises thin

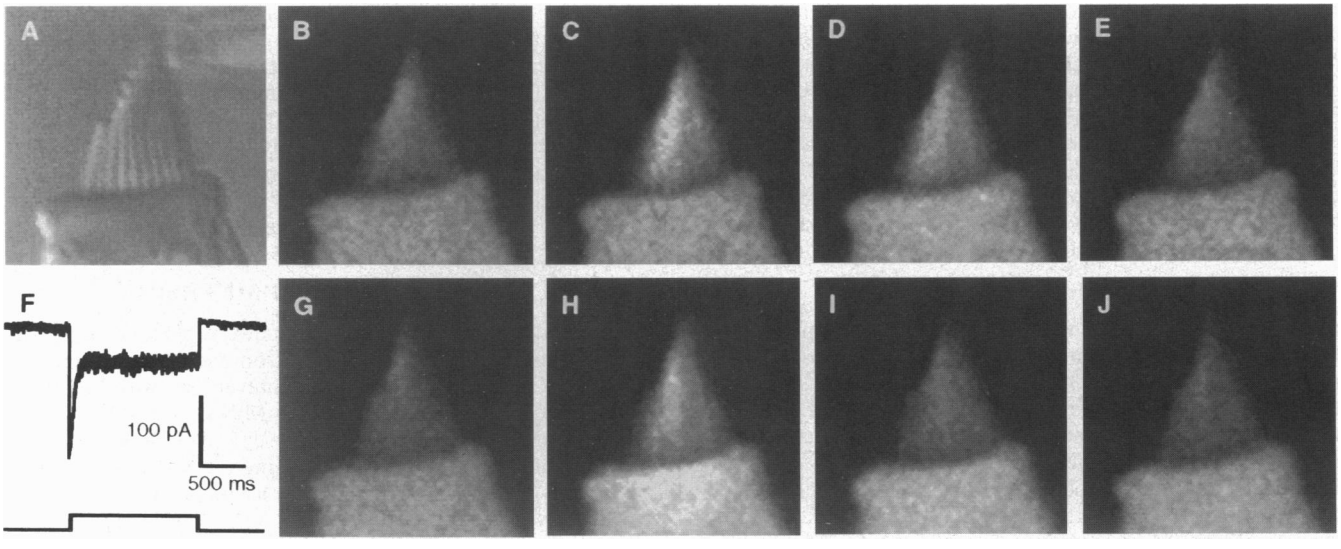

FIG. 1. Fluorescence increase accompanying mechanoelectrical transduction in a saccular hair cell loaded with 200 μM fluo-3 and 1 mM EGTA. (*A*) A differential-interference-contrast confocal image of a hair bundle shows nine of the ≈60 constituent stereocilia and the single kinocilium, to whose bulbous tip the stimulus pipette (upper right corner) was attached. (*B*) While at rest, the hair bundle exhibited a tip blush (enhanced fluorescence along its beveled top surface). (*C*) During a 1.5-s, 500-nm deflection of the hair bundle, the fluorescence increased in at least five stereocilia. This stimulus elicited a transduction current of −220 pA, which adapted completely within 100 ms. (*D*) After adaptation to the continuing stimulus, the bundle fluorescence decreased; the cuticular plate meanwhile displayed a slight, delayed fluorescence increase. (*E*) Restoration of the bundle to its resting position reduced the fluorescence to its initial level. (*F*) The whole-cell current recorded in response to a stimulus delivered to the bundle 1 min after that in *C* and *D* (and contemporaneously with acquisition of the following images) included an inward transduction current that adapted incompletely. (*G*) The tip blush was less pronounced in this image than in *B* because the resting position of the bundle was moved slightly in the negative direction. (*H*) Bundle displacement caused a fluorescence increase in the hair bundle and cuticular plate. (*I* and *J*) The fluo-3 signal in the hair bundle decreased after the stimulus's cessation.

cytoplasmic processes extending into extracellular space, an optical section of a resting bundle displayed less fluorescence than the corresponding section of a cuticular plate. Second, because stereocilia taper at their insertions into the cuticular plate, the fluo-3 signal declined at a bundle's base. Finally, because stereocilia converge near a hair bundle's top, a greater volume of cytoplasm contributed fluorescence to an image of stereociliary tips than to a comparable view farther down the stereociliary shafts. As a consequence, we occasionally observed a zone of increased fluo-3 signal spanning the entire upper surface of a hair bundle. Unlike the signals discussed below, the enhanced fluorescence due to stereociliary convergence did not change during hair-bundle deflection in either direction and did not diminish when transduction was abolished by repeated stimulation.

RESULTS

We sought the site of mechanoelectrical transduction in hair cells by using laser-scanning confocal microscopy to determine where Ca^{2+} entered mechanically stimulated hair bundles. Experiments were performed on voltage-clamped hair cells isolated from the bullfrog's sacculus and loaded with the Ca^{2+} indicator fluo-3. We first used the microscope's frame-scan mode to examine hair bundles before, during, and after stimulation (Fig. 1). Even in the absence of mechanical stimulation, a hair cell often displayed increased fluorescence, which we term the "tip blush," along the beveled top surface of its hair bundle (Fig. 1 *B* and *G*). Protracted displacement of the hair bundle in the positive direction caused the fluo-3 signal from the stereocilia to increase along their lengths (Fig. 1*C*). When a robust transduction current persisted because adaptation was incomplete, both the hair bundle and the cuticular plate fluoresced brightly (Fig. 1*H*). After the bundle was returned to its resting position, the fluo-3 signal decreased to near its original level (Fig. 1 *E, I,* and *J*).

To pinpoint the site of Ca^{2+} influx into hair bundles with a higher temporal resolution, we employed the confocal microscope's line-scan function. While a spot of illumination was repeatedly swept along the length of a single stereocilium, the hair bundle was subjected to a displacement pulse. Upon positive hair-bundle deflection, fluorescence increased near the tip of the stereocilium; the brightening then propagated toward the stereociliary base (Fig. 2). Individual stereocilia imaged in a total of 14 cells displayed a similar pattern of fluorescence increase, in which the augmented fluo-3 signals evoked by hair-bundle displacements began near the stereociliary tips (Fig. 3). In six hair cells whose transduction currents rapidly diminished during adaptation, the initial region of increased fluorescence was clearly located within 1 μm of the stereociliary tips (Fig. 3 *B* and *D*). In four cells, the fluo-3 signal appeared to spread in both directions, up and down the stereocilia, from the initial sites of fluorescence increase (Figs. 2*C* and 4). We never observed fluorescence increases originating from stereociliary bases or cuticular plates.

The tip blush could often be seen in a line-scan image of an undisturbed hair bundle (Fig. 4). When three such bundles were displaced in the negative direction, the resting transduction currents decreased and the heightened fluorescence disappeared. Upon the bundles' return to their resting positions, the transduction currents showed transients indicative of adaptation to negative stimuli, which were accompanied by restoration of the fluorescence near the stereociliary tips.

DISCUSSION

In confocal line-scan images of mechanically stimulated hair cells containing the Ca^{2+} indicator fluo-3, we consistently observed augmented fluorescence that originated near the tips of stereocilia and then progressed toward their bases. The simplest interpretation of the observed pattern of fluorescence is that Ca^{2+} enters stereocilia through transduction channels located near their tips and then diffuses down the stereocilia toward a region of lower Ca^{2+} concentration in the soma.

The position at which fluorescence initially increased upon positive bundle stimulation implies that functional transduc-

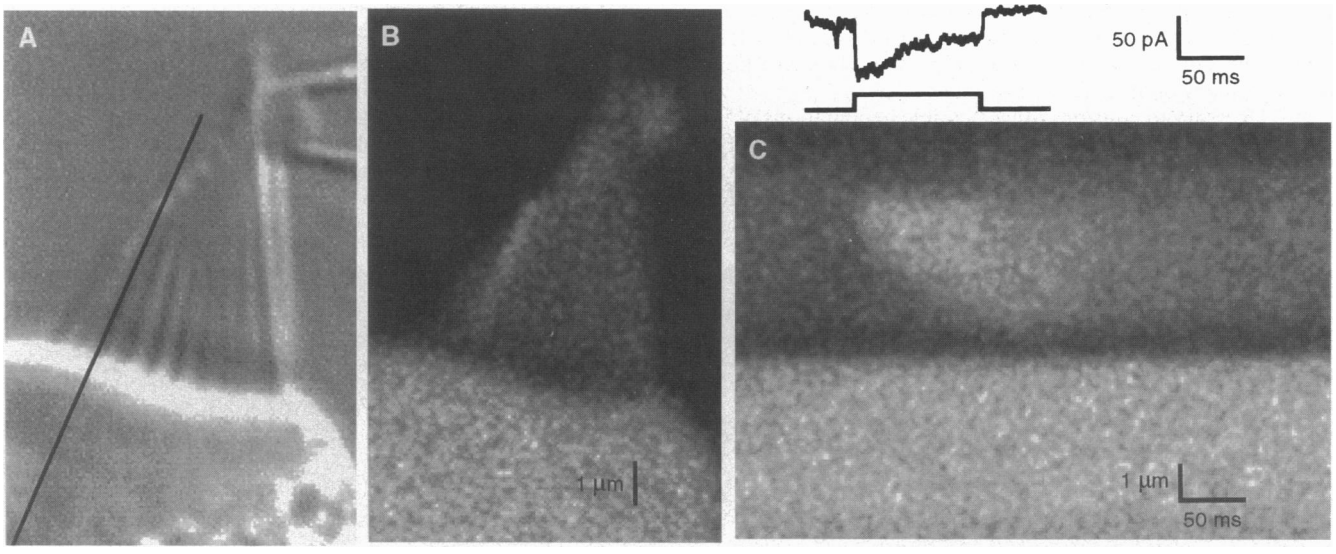

FIG. 2. Line-scan protocol. (*A*) A differential-interference-contrast and (*B*) a fluorescence image, collected 1 min prior to the acquisition of the following line-scan image, portray the undisturbed hair bundle of a cell loaded with 200 μM fluo-3 and 1 mM EGTA. The black line overlying the former image marks the transect of illumination during line-scan imaging. In this optical section, only one stereocilium displayed a strong tip blush. (*C*) In a line-scan image, the ordinate represents distance from just above the stereociliary tip (top) to the cuticular plate (bottom); time runs along the abscissa. During acquisition of the line-scan image, the hair bundle was deflected 200 nm in the positive direction to elicit Ca^{2+} influx through transduction channels. The first position of discernible brightening was slightly below the stereociliary tip. The fluorescence increase reached the tip within 20 ms and progressed toward the base within 100 ms. After the displacement ended, the fluo-3 signal in the taller neighboring stereocilium returned to its baseline level in $\approx$200 ms. The displacement step and resulting whole-cell transduction current are shown above *C* in temporal register with the line-scan image. The magnification in *B* corresponds to that in *A*; the magnification in *C* is adjusted so that the ordinate corresponds to the projection of the scanned transect onto a vertical axis.

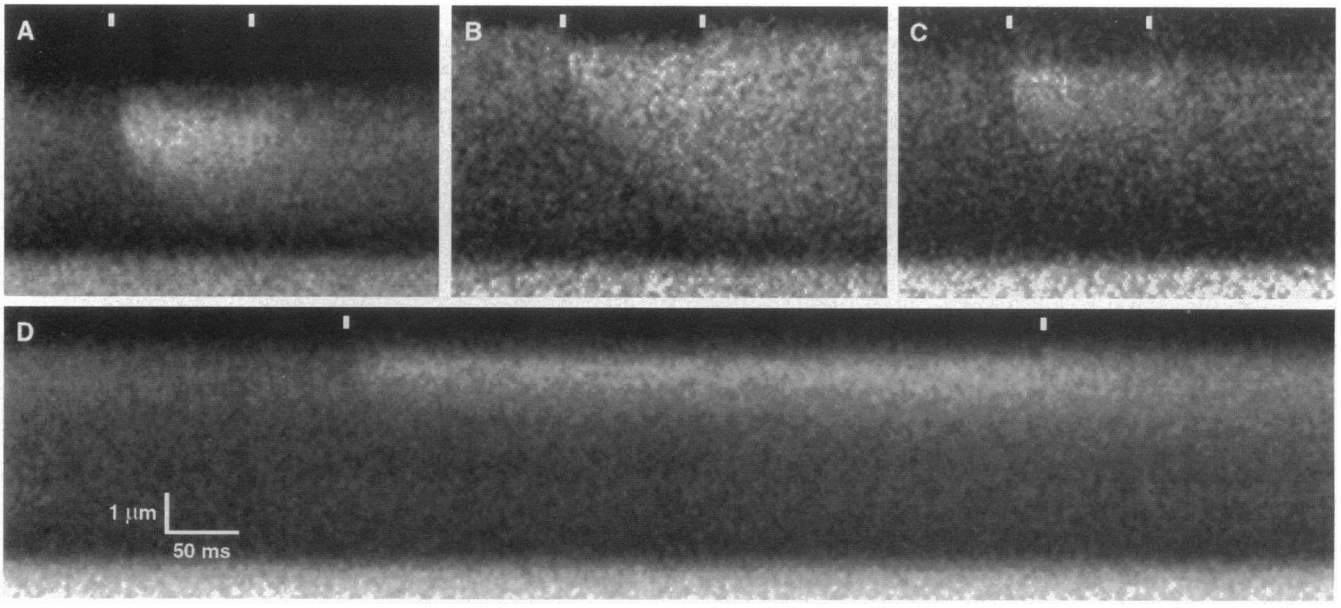

FIG. 3. Line-scan images from four different hair bundles during positive deflections. (*A*) Within 50 ms of the onset of bundle displacement, the fluorescence increase reached its full extent along a stereocilium. After stimulation ceased, the fluorescence returned to baseline in ≈120 ms. The peak transduction current measured during this deflection was −80 pA. (*B*) The time course of Ca^{2+} entry in this cell, whose internal solution included EGTA, was similar to that of Fig. 2*C*. The peak transduction current was −30 pA. (*C*) In a line-scan image of a hair cell whose transduction current of −120 pA adapted almost completely in 35 ms, the fluo-3 signal near a stereociliary tip initially increased and then declined after adaptation. Similar fluorescence patterns were observed in this and two other cells loaded with EGTA. (*D*) This deflection, which elicited a transduction current of −50 pA that adapted extensively, was accompanied by a fluorescence increase restricted to the top third of the stereocilium. The beginning and end of each stimulus are indicated by white ticks at the top of each panel; the calibration bars apply throughout.

tion channels occur within 1 μm of a hair bundle's top. Our data are not able to demonstrate conclusively whether these channels are situated at stereociliary tips, at insertional plaques, at the points of contact between adjacent stereocilia, or at other nearby sites. It is unlikely, however, that all transduction channels lie precisely at the stereociliary tips: within 20 ms of the onset of bundle displacement, we sometimes observed fluorescence spreading—and thus Ca^{2+} diffusing—toward the tip as well as the base of a stereocilium. This observation is consistent with the occurrence of at least some transduction channels at the insertional plaque or point of stereociliary abutment.

It is necessary to consider other possible explanations of the observed pattern of stimulus-induced fluorescence. It might be supposed, for example, that Ca^{2+} enters a stereocilium at its base but that fluo-3 is unevenly distributed so that fluorescence is most intense near the stereociliary tip. Above its basal taper, however, a stereocilium is a cylinder of uniform internal structure (10); there is no apparent reason that an indicator might be excluded from the base or accumulate near the tip. Moreover, the lower portion of a stereocilium yields strong fluorescence during protracted influx of Ca^{2+} through transduction channels (Fig. 1*C*) or when the Ca^{2+} concentration rises throughout a hair cell, confirming the presence of fluo-3 throughout the stereocilium.

Advantages of the Experimental Protocol. The line-scan procedure utilized in this study afforded several advantages for detecting transduction-associated Ca^{2+} influx into the hair bundle. The temporal resolution of <2 ms readily sufficed to detect the profile of Ca^{2+} diffusion along the stereocilia. At the same time, the spatial resolution of the imaging system allowed us to pinpoint the site of Ca^{2+} influx to within 1 μm.

By using voltage-clamp recording, we held the membrane potential of each cell at −70 mV throughout stimulation.

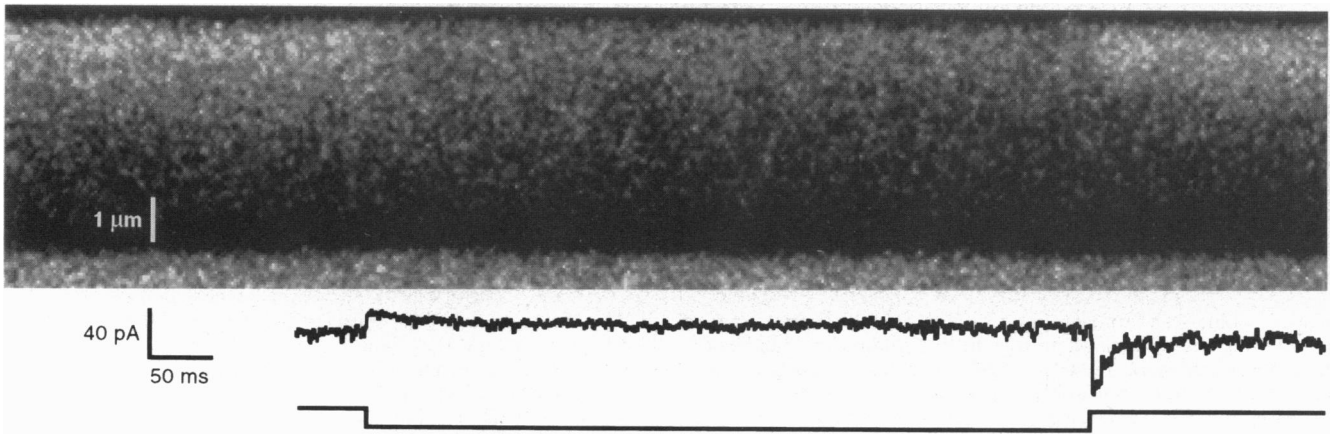

FIG. 4. Line-scan image of a stereocilium during a negative hair-bundle deflection. At the bundle's resting position, the tip blush could be seen as enhanced fluorescence near the stereociliary tip. Within 35 ms of the onset of a 200-nm negative deflection, the tip blush largely disappeared. After the 500-ms deflection concluded, the tip's fluorescence increased to its original level. This cell's internal solution included EGTA. The displacement step and resulting whole-cell transduction current are temporally aligned with the line-scan image.

Maintaining the potential at a more negative voltage than that at which voltage-gated Ca^{2+} channels activate (11) precluded Ca^{2+} influx through those channels. By recording the whole-cell current elicited by a hair-bundle displacement, we also ensured that Ca^{2+} influx occurred through transduction channels.

By displacing a hair bundle by a distance smaller than the diameter of a stereocilium, we were usually able to scan the same stereocilium before, during, and after bundle displacement. The transect of confocal scanning was occasionally misaligned; in Fig. 2, for example, the stereocilium scanned during the displacement was shorter than that imaged following the bundle's return to rest. Because the increase in fluorescence clearly progressed down the short stereocilium, however, the motion artifact in such an image does not alter the conclusion that Ca^{2+} influx originates near the stereociliary tips.

The Tip Blush. In a resting hair bundle we often detected a tip blush (augmented fluorescence near the stereociliary tips) (Fig. 1*B*). During selection of stereocilia for imaging with the line-scan protocol, this phenomenon provided a useful indicator of intact transduction (Fig. 2*B*). The tip blush likely resulted from Ca^{2+} entry through transduction channels open at rest. Consistent with that hypothesis, the tip blush disappeared when a bundle was pushed in the negative direction to close all of the transduction channels (Fig. 4). We could conversely increase the tip blush by slightly shifting the bundle's resting position in the positive direction (compare Figs. 1 *B* and *G*). When transduction vanished, the tip blush disappeared.

During a prolonged negative displacement, one might expect the tip blush first to vanish, then to reappear with the time course of adaptation. In three hair cells, we observed a diminution, but not a rebound, of stereociliary tip fluorescence. This result may be explained by the observation that, at the offset of positive or onset of negative displacements, the fluorescence decreased more slowly than adaptation reopened the channels. An initial decline in fluorescence due to channel closure was therefore concealed by the tip blush caused by Ca^{2+} that had not yet left the stereocilia.

Profile of Fluorescence Along Stereocilia. In all of the line-scan images collected, the increase in fluorescence due to Ca^{2+} influx through transduction channels did not extend into the cuticular plate. By contrast, the fluorescence increase in frame-scan images, during which the bundle was given a larger and more protracted displacement, was observed in the hair bundle and cuticular plate (Fig. 1). During the small, brief deflections used in line-scan imaging, the increase in cytoplasmic free Ca^{2+} concentration was probably insufficient to increase the fluorescence above the background level a few micrometers away from a channel. This explanation is supported by the observation that adapted currents, which carried little Ca^{2+} into the cytoplasm, corresponded to more spatially restricted fluorescence increases than sustained transduction currents bearing more Ca^{2+} (Figs. 1, 2, and 3*C*).

Within most hair bundles, all stereocilia did not show increased fluorescence during stimulation (Fig. 1). We believe that this behavior resulted from damage that inactivated some transduction channels during cellular isolation. This hypothesis is supported by the observation that cells from which small transduction currents were recorded generally displayed fluorescence increases in fewer stereocilia than cells with larger currents. These data strengthen the evidence (12) that individual stereocilia, or more probably pairs of stereocilia, are autonomous units of mechanoelectrical transduction.

Conclusion. The results presented here are consistent with previous evidence localizing transduction channels to the hair bundle's top (4–6, 8). Our conclusion differs, however, from that of an earlier study of Ca^{2+} influx into chick hair cells loaded with fura-2, in which it was inferred that transduction channels occur at the bundle's base (7). Although it is possible that our contradictory results reflect differences between the transduction mechanisms of amphibians and birds, technical difficulties with the earlier study may have led to a spurious conclusion (for review, see refs. 1 and 5).

By localizing the site of Ca^{2+} influx to the top 1 μm of the stereocilia, our results lend support to the gating-spring model for mechanoelectrical transduction. The experimental protocol developed in this study can be used to investigate other issues: with careful quantitation of fluorescence signals, we should be able to study the diffusional profile of Ca^{2+} in stereocilia and its dependence upon adaptation and to delineate the homeostatic mechanisms that regulate Ca^{2+} concentration in the hair bundle.

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