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Calcium-binding sites on sensory processes in vertebrate hair cells

(mechanoreceptor/sensory cilia/sensory transduction/lateral line/inner ear)

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ABSTRACT **Vertebrate lateral line and vestibular systems center their function on highly mechanosensitive hair cells. Each hair cell is equipped with one kinocilium (which resembles a motile cilium) and 50–100 actin-containing stereocilia (which resemble microvilli) at the site of stimulus reception. This report describes electron-microscopic localization of calcium-binding sites on the sensory processes of vertebrate hair cells. Using the Oschman–Wall technique for calcium localization [Oschman, J. L. & Wall, B. J. (1972) *J. Cell Biol.* 55, 58–73] together with electron-probe x-ray microanalysis of thin sections, we observed: (*i*) calcium- and iron-containing deposits in the region of the ciliary necklace in goldfish lateral line hair cells, (*ii*) calcium deposits upon the surface of stereocilia of hair cells of the bullfrog inner ear, and (*iii*) calcium deposits upon stereocilia of hair cells of the guinea pig vestibular system.**

Vertebrate lateral lines and vestibular systems are equipped with hair cells of exquisite mechanosensitivity (1). Hair cells, capable of responding to a 0.5-nm stimulus (2) in less than 40 μsec (3), are fitted with a single kinocilium and 50–100 microvillous "stereocilia" at the site of stimulus reception. It is interesting to note that the kinocilium, which resembles a "9+2" motile cilium, and the microvillous stereocilia, which contain core filaments of actin (4, 5), are both derived from primitive motile systems.

Throughout the course of evolution, locomotor and sensory systems have been closely allied. Because ciliary and actin-based motile systems are, in part, activated and regulated by calcium ions, we ask: are calcium-binding sites associated with the kinocilia or stereocilia of vertebrate hair cells?

Using the Oschman–Wall technique of calcium localization (6) in concert with electron-probe microanalysis of thin sections, we have observed calcium-binding sites on the sensory cilia of hair cells of the goldfish lateral line, the bullfrog vestibular system, and the guinea pig vestibular system.

MATERIALS AND METHODS

Source of Tissues. Sense organs from the acousticolateralis system of animals from three vertebrate classes—a teleost fish, an amphibian, and a mammal—were studied. Mechanoreceptive hair cells within the following sense organs were investigated: (*i*) hair cells from the supraorbital canal of the lateral line system of the goldfish, *Carassius auratus;* (*ii*) hair cells from the macula sacculi of the vestibular system of the bullfrog, *Rana catesbeiana;* and (*iii*) hair cells from the macula sacculi and macula utriculi of the vestibular system of the guinea pig, *Cavia porcellus*.

Electron Microscopy. Tissues were fixed by following the method of Oschman and Wall (6), a preparative procedure that localizes sites on cell membranes that have a high affinity for calcium ions. Experimental tissues were fixed overnight at 4°C in a solution containing 2.7% (vol/vol) glutaraldehyde, 5% sucrose, and 5 mM $CaCl_2$ buffered to pH 7.2 with 0.08 M *s*-collidine buffer. After fixation, tissues were rinsed in the same solution without glutaraldehyde, postfixed in buffered 2% OsO_4, dehydrated in a graded series of acetones, and embedded in Spurr's (7) low-viscosity epoxy resin. Control tissues were processed in the same manner with one exception; $CaCl_2$ was omitted from both the fixative and the rinse. Thin sections were cut on a Porter–Blum MT-2B ultramicrotome fitted with a diamond knife and collected on copper "slot" grids, following the method of Rowley and Moran (8). Unstained and stained (uranyl acetate and lead citrate) sections were photographed with Philips EM 300 and JEOL JEM-100 CX electron microscopes.

X-Ray Microprobe Analysis. The elemental content of electron-dense deposits in thin sections was analyzed in the laboratory of JEOL U.S.A. (Medford, MA), using a JEM-100 CX analytical electron microscope equipped with an accessory scanning image device and a Kevex-7000 energy-dispersive x-ray analyzer. Samples were previewed under a transmission electron microscope at 80 kV. L-α and K-β calcium peaks were noted after 100 sec at 20 kV. The beam spot size was confined to the area of the analyzed deposit.

RESULTS

Morphology. The sites of stimulus reception of hair cells of vertebrate lateral line and of vestibular systems are structurally similar (1). Fig. 1, for example, is an electron image of a longitudinal section through several "typical" hair cells within the macula sacculi of the guinea pig. The apex of each cell, where the stimulus is received, is fitted with a single kinocilium and about 50 stereocilia. The kinocilium, equipped with a 9+2 axoneme, is similar in structure to motile cilia. The stereocilia, however, are not cilia at all; instead, they resemble long microvilli and are supported by a core of actin microfilaments (4, 5). The hair cells, which are modified epithelial cells, form functional synapses with afferent and efferent nerves (1). In mammalian vestibular systems (Fig. 1), neurons either envelop the hair cells in a "chalice" (type I cells) or communicate with the hair cells at the base (type II cells). Type I cells, present in mammals, are absent from fish and frog.

In the following sections, we describe the electron-microscopic localization of calcium affinity sites on sensory cilia of hair cells from the goldfish lateral line, bullfrog vestibular system, and guinea pig vestibular system.

Hair Cells of the Goldfish Lateral Line. Oschman and Wall (6) have shown that calcium-binding sites on cell membranes can be localized in thin sections as readily detectable electron-dense deposits by fixing tissues in the presence of 5 mM $CaCl_2$. We have prepared hair cells from goldfish lateral-line canal organs according to their method. In all cases, we have observed electron-dense deposits associated with the ciliary membrane at the base of the kinocilium. In Fig. 2, an unstained longitu-

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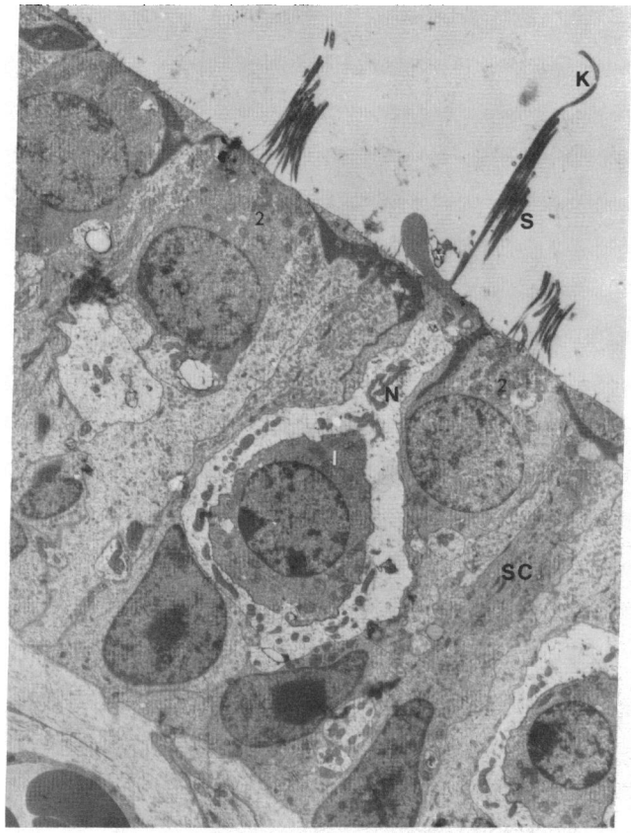


FIG. 1. Longitudinal section through the sensory epithelium of the macula sacculi of the guinea pig vestibular system, showing cell bodies of types I (1) and II (2) hair cells, an efferent nerve "chalice" (N), a supporting cell (SC), parts of several stereocilia (S), and a single bent kinocilium (K) projecting from the hair cell surface. (×3300.)

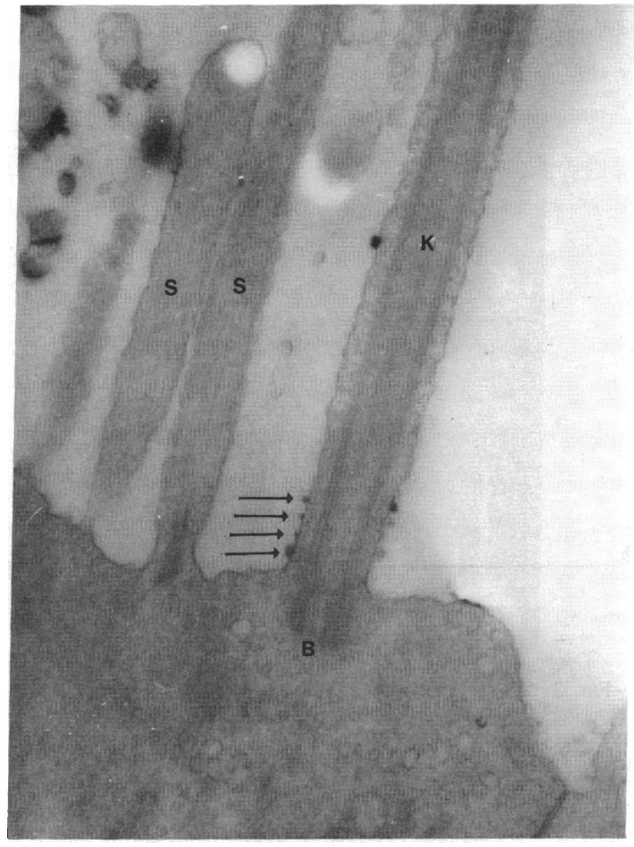


FIG. 2. Unstained longitudinal section through the basal body (B), kinociliary shaft (K), and several stereocilia (S) of a hair cell from a goldfish lateral line canal organ. Arrows indicate electron-dense calcium deposits near the base of the kinocilium evident when tissue is fixed in the presence of 5 mM $CaCl_2$. (×55,200.)

dinal section through the kinocilium and several stereocilia of a lateral-line canal organ hair cell, an orderly array of electron densities (arrows) decorates the base of the kinocilium. The dense deposits, some 16 nm in diameter, are separated in the vertical plane by an intercenter distance of 45 nm. Cross-sectional images suggest the particles occur in horizontal rows that span the circumference of the cilium. The uppermost row of particles is positioned 180 nm from the ciliary base. This arrangement matches the position and distribution of particles detected by freeze-cleaving the ciliary necklace (see figure 6 of ref. 9). Although occasional electron-dense deposits may appear at various points along the membrane of the kinociliary shaft, very few deposits have been observed on stereocilia. Control tissues fixed with $CaCl_2$ contained no densities.

X-ray spectra produced by electron-probe microanalysis of an electron-dense deposit at the base of the kinocilium are shown in Fig. 3 *Upper*. Electron-probe microanalysis data from an adjacent control area within the same ciliary shaft are shown in Fig. 3 *Lower*. The two spectra have peaks in common: osmium from the fixative, chlorine from the epoxy embedment, and copper from the grid. Two distinct peaks, calcium and iron, are evident in Fig. 3 *Upper* (microprobed deposit) that are absent from Fig. 3 *Lower* (microprobed axoneme). This shows the electron-dense deposits at the base of the kinocilium contain both calcium and iron. Although we anticipated the deposits would contain calcium, we did not expect to find iron. We cannot comment on its significance; at this point, we can only report its presence. It is unlikely that iron entered as a contaminant in the solutions used in specimen preparation, because the same reagents were used throughout our investigations, and iron—as will be seen below—was not detected in bullfrog or guinea pig hair cells.

Hair Cells of the Bullfrog Vestibular System. In contrast to the lateral line, when hair cells from the macula sacculi of the bullfrog vestibular system are fixed in the presence of 5 mM $CaCl_2$ the kinocilia do not display electron-dense deposits. The stereocilia, however, do display numerous electron densities along the external surface of their outer membrane. Control tissues fixed without $CaCl_2$ contain no densities whatsoever. Fig. 4 *Upper* is an unstained cross section through the apex of a hair cell fixed in the presence of calcium ion. Small (20 nm), round electron-dense deposits are associated with the outer surface of most of the stereocilia in the field. Electron-probe microanalysis shows that these deposits contain calcium (Fig. 4 *Lower*). X-ray spectra of adjacent control areas within the stereociliary core are virtually identical to the spectrum in Fig. 3 *Lower*; they contain no detectable peaks for calcium, iron, or other cations save copper and osmium.

Hair Cells of the Guinea Pig Vestibular System. We have examined a number of hair cells from the macula sacculi and macula utriculi of the guinea pig, and we find that the pattern of calcium deposition in both cases matches that of the bullfrog inner ear hair cells. Few deposits are seen on the kinociliary shaft; no orderly arrangement of particles is evident at the base of the kinocilium in the region of the ciliary necklace. Fig. 5 *Upper* is an electron micrograph of a stained longitudinal section through the apex of a hair cell from the guinea pig macula sacculi. Numerous electron-dense deposits stud the outer membrane of the stereocilia (arrows). When these dense deposits are

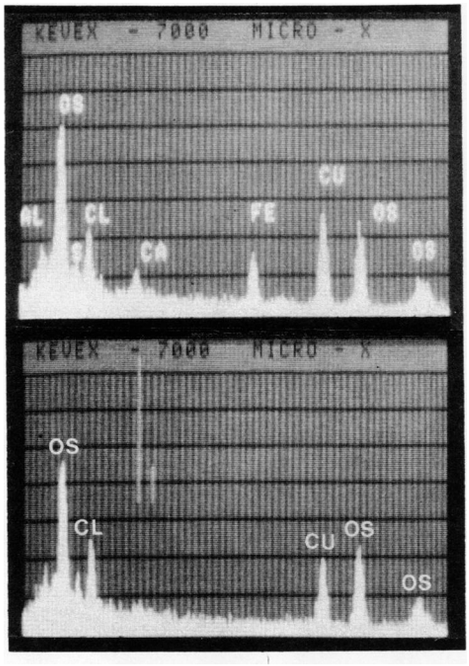


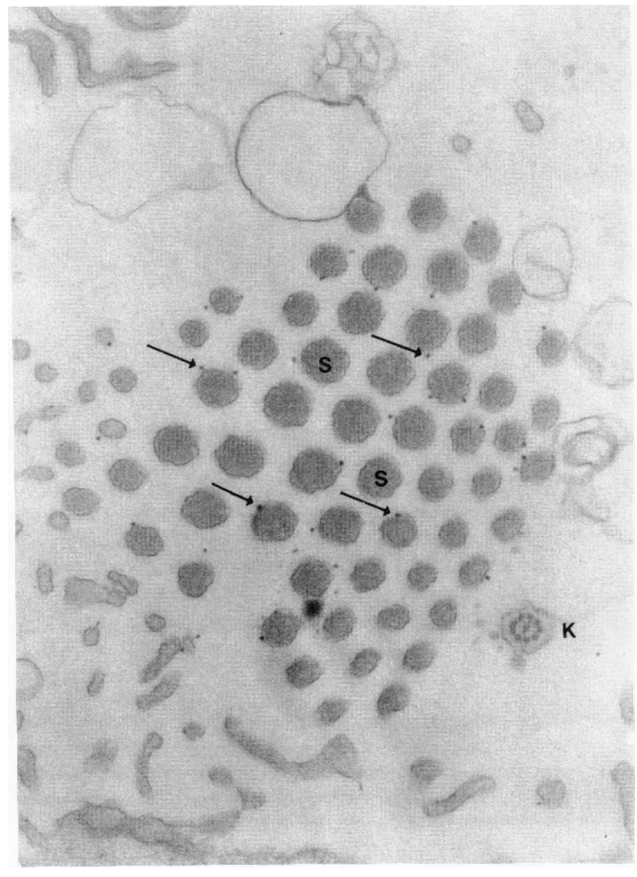
FIG. 3. (*Upper*) Emission spectrum of x-rays generated by the electron bombardment of an electron-dense deposit at the base of the kinocilium of a goldfish lateral line hair cell. Calcium and iron clearly are present in the electron-dense deposits; compare with control spectrum below. (*Lower*) Emission spectrum of the x-rays generated by the electron bombardment of a point *within* the kinocilium *away* from the electron-dense deposits analyzed in *Upper*. Although no signals for calcium or iron are recorded, strong peaks remain for osmium (from the fixative), chlorine (from the embedment), and copper (from the grid).

subjected to electron-probe microanalysis, the resultant x-ray spectra show them to be rich in calcium (Fig. 5 *Lower*). Microprobe analysis of control areas show that calcium is limited to the electron-dense deposits and is not present in detectable concentrations within the stereociliary core. We have found similar results for the hair cells of the guinea pig macula utriculi.

DISCUSSION

Two primitive motile systems, cilia and actin microfilaments, are both present in the form of one kinocilium and many stereocilia at the site of stimulus reception in each highly mechanosensitive hair cell of vertebrate lateral line and vestibular systems. Because it is well known that calcium is an important cation in the regulation of motility in cilia and actin-based systems—and because there seems to be a close relationship between motility and mechanoreception—we set out to look for calcium-binding sites on sensory cilia. Using the technique of Oschman and Wall (6) in concert with electronprobe x-ray microanalysis (10), we have found several distinct patterns of calcium binding sites on the sensory cilia of hair cells of the goldfish lateral line, the bullfrog vestibular system, and the guinea pig vestibular system.

In the goldfish lateral line, calcium and iron deposits were consistently arranged in an orderly array on the membrane in the region of the ciliary necklace at the base of the kinocilium. Electron-dense deposits were seldom observed on stereocilia. In both frog and guinea pig vestibular system hair cells, however, a different pattern was observed; calcium-binding sites occurred frequently on stereocilia but were rarely seen on the kinocilium. These observations raise the question: do calcium-binding sites on sensory cilia participate in mechanoreception?



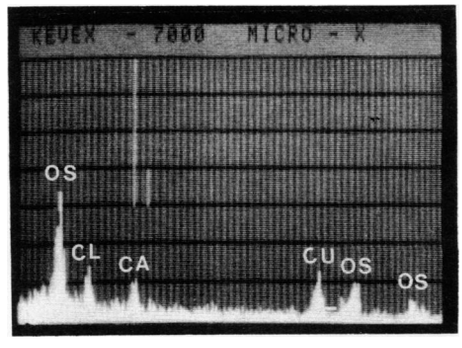


FIG. 4. (*Upper*) Cross section through the stereocilia (S) and kinocilium (K) of a hair cell in the macula sacculi of the bullfrog vestibular system fixed in the presence of 5 mM $CaCl_2$. Although the membrane of the stereocilia is labeled with electron-dense deposits (arrows), that of the kinocilium is not. X-ray microprobe analysis (*Lower*) shows that these deposits contain calcium. (×24,400.) (*Lower*) Emission spectra of the x-rays generated by the electron bombardment of electron-dense deposits on the bullfrog hair cell stereocilia shown in *Upper*.

The Kinocilium and Mechanoreception. Motile cilia can double as mechanoreceptors (11, 12). In *Paramecium*, for example, mechanical stimulation of motile cilia causes calcium "gates" to open; calcium flows into the cell, the membrane is depolarized, and the cilia reverse their direction of beating (12). *Pawn* mutants of *Paramecium* that lack calcium gates do not exhibit ciliary reversal (13). This suggests that calcium ions are involved in mechanosensory transduction and ciliary reversal in *Paramecium*. It is tempting to extend this mechanism to the 9+2 kinocilia of hair cells—especially because there is a striking

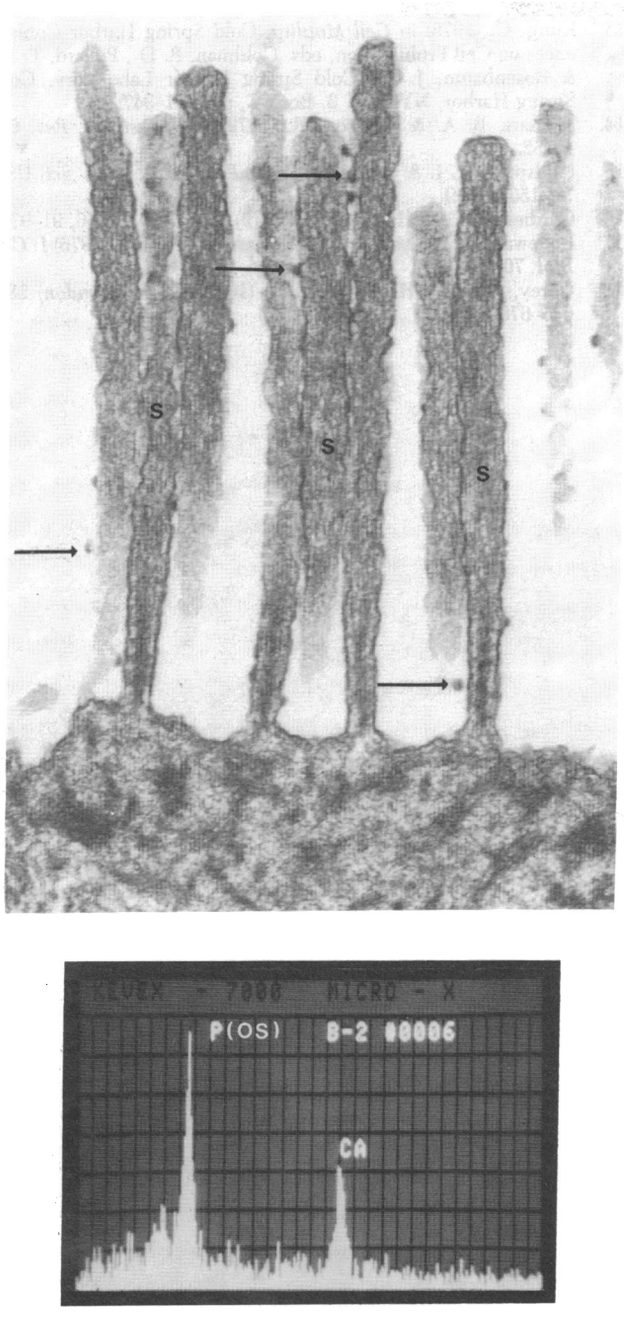


FIG. 5. (*Upper*) Longitudinal section through the stereocilia (S) of hair cells from a macula sacculi of the guinea pig vestibular system fixed in the presence of 5 mM $CaCl_2$. Arrows indicate electron-dense deposits that are shown (*Lower*) to contain calcium by x-ray microprobe analysis. (×27,000.) (*Lower*) Emission spectra of the x-rays generated by the electron bombardment of electron-dense deposits on the guinea pig hair cell stereocilia shown in *Upper*.

correspondence between the axonemal plane of the active (and recovery) stroke of motile cilia and the axonemal plane of the stimulatory (and inhibitory) displacements of the kinocilium in the hair bundle of hair cells (1). Two pieces of evidence, however, indicate hair cell kinocilia are not necessary elements for mechanoreceptive function in hair cells. First, patients with Kartagener syndrome—the so-called "immotile cilia syndrome"—have not been reported to show evidence of vestibular dysfunctions. Because many of these patients present a congenital lack of one or both dynein arms on the outer doublets of somatic cilia (14) (they have chronic bronchitis due to immotile airway cilia) and germ cell cilia (they are sterile due to immotile spermatozoa), we can conclude that kinociliary motility is not necessary for proper function of human vestibular system hair cells. Second—and more conclusive—the elegant experiments of Hudspeth and Jacobs (15) clearly show that bullfrog vestibular hair cells can function quite well *in vitro* after physical removal of the kinocilium. Removal of the kinocilium does not diminish their mechanosensitivity or their directional sensitivity. Although these experiments have not yet been done on lateral line receptors, it is probably safe to extend these findings to fish. Why, then, is the kinocilium there? And why is there such a precise arrangement of calcium-binding sites at the ciliary base? Although the answers to these questions are unknown, we agree with Hudspeth and Jacobs (15) that the role of the kinocilium in vertebrate hair cells is most probably developmental. The calcium-binding sites on goldfish kinocilia correspond precisely to the position of the membrane particles in the ciliary necklace described by Gilula and Satir (9). Establishment of the ciliary necklace particles seems to be the primary event in the development of the cilium (9, 16). Because lateral line and vestibular hair cells are directionally sensitive (1, 2, 15)—they function as vector analyzers—it is likely that some sort of structural polarity is established in the hair cell apex during development. The elaboration of the ciliary necklace at a specific site on the hair cell surface could determine the position of the kinocilium, and the development of the kinocilium, along with its basal body and attached basal foot, could influence the establishment of a structural polarity within the apical pole of the cell.

Stereocilia and Mechanoreception. Hair cells fitted with stereocilia alone can function as highly sensitive mechanoreceptors. Human cochlear hair cells, for example, have stereocilia but no kinocilium. The kinocilium, present during development, is lost at birth, leaving its basal body behind. The experiments of Hudspeth and Jacobs (15) show that hair cells can function not only after removal of the kinocilium but also after removal of half the stereocilia.

Stereocilia are not cilia at all, but rather resemble microvilli. Microvilli have core filaments of actin and can actively shorten in length (17). Stereocilia, too, have a core of 6-nm actin microfilaments (4, 5). Because calcium ions are important to the regulation of actin-based motile systems, because calcium appears to be a necessary cofactor for the mechanoreceptive response in hair cells (18), and because stereocilia have actin cores and have calcium-binding sites in their membrane, it is tempting to speculate that calcium-mediated motile mechanisms participate in mechanoreception. Recent findings by Tilney *et al.* (5), however, indicate stereocilia of lizard ear hair cells, unlike microvilli, have an arrangement of actin microfilaments that is *not* designed to shorten. They conclude, "the stereocilium is a large, rigid structure designed to move as a lever." Thus the relationship between calcium, motility, and mechanosensitivity in stereocilia, though intriguing, remains obscure.

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