Motile Statocyst Cilia Transmit Rather Than Directly Transduce Mechanical Stimuli

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ABSTRACT We have investigated the role of motile cilia in mechanotransduction by statocysts of the nudibranch mollusk *Hermissenda crassicornis*. Movement of the cilia that experience the weight of statoconia causes increased variance of voltage noise and membrane depolarization of the statocyst hair cell. Two complementary approaches were used to immobilize the cilia. Vanadate anion was iontophoretically injected into hair cells. This reversible inhibitor of dynein cross-bridge cycling initially caused the cilia to lose their normally upright, rigid, vibratile form and to assume a more classic, pliable beat pattern. Voltage noise decreased as the cilia slowed and bent more extremely, nearly disappearing as motility was lost. When the intracellular vanadate concentration approached 10^{-5} M, the cilia were arrested in an effective stroke against the cell membrane. The cell no longer depolarized upon gravitational or local mechanical stimulation. Rapid reversal of ciliary inhibition by norepinephrine or slow reversal with time restored both the voltage noise and depolarization response. Cilia were rendered rigid and upright by covalent cross-linkage of their membrane "sleeve" to the 9 + 2 axoneme, using the photoactivated, lipophilic, bifunctional agent 4,4′-dithiobisphenyl azide. In the initial stages of cross-linkage, the cilia remained vibratile but slowed and moved through wider excursions. Voltage noise decreased in frequency but increased in amplitude. When the cilia were fully arrested, voltage noise was minimized while the resting potential and membrane resistance remained essentially constant. Mechanical stimulation of the rigid cilia, normal to the cell membrane, elicited a generator potential of the same amplitude but of greater duration than before treatment. Because cilia that are partially arrested by vanadate undergo increased bending, although the hair cell shows decreased noise, neither the axoneme nor the ciliary membrane proper would appear to be sites of direct transduction. In cells with beating but stiffened cilia, however, the voltage noise becomes amplified, implying an increased efficiency of transduction. We suggest that active but rigid flexure of the axoneme is involved in amplification and continuous signal detection. The basal insertion area is the most likely transduction site, being the terminal leverage point through which force is applied to the plasma membrane via the flexing ciliary shaft.

The precise role of sensory cilia in the process of mechanoreception is unclear. Gray and Pumphrey (15), investigating the ultrastructure of the locust ear, speculated that a ciliated effector, when transformed into a receptor, could be run in reverse, transducing mechanical events into chemical ones. Thurm (35) demonstrated that mechanical distortion of a resting molluscan gill cilium at the base could initiate an active, full stroke of the organelle. Moran and his colleagues (24) correlated the mechanical distortion at the constrained tip of a cilium within the grasshopper femoral chordotonal organ with the formation of a pronounced bend at the base of the cilium. These workers suggested that outer doublets at the tip actively slide in response to the distortion, forming a bend at the base because of the constancy of their length. Unable to propagate further, this

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bend would distort the surrounding membrane, open ion channels, and thus trigger a train of spikes whose frequency is a function of the degree of initial ciliary tip displacement.

The presence of microtubules in many kinds of sensory cells has led to models in which the tubules themselves are thought to play some active role in transduction. Atema (4) theorized that the binding of ligands to chemosensory cilia or the direct distortion of the 9 + 2 structure in mechanosensitive cilia is sufficient to bring about transduction events through the propagation of induced conformational states along the constituent microtubules. As a specific example, the campaniform sensilla of cockroaches are mechanoreceptors that employ a bundle of microtubules at the tip of a modified cilium to interface the sensory cell with the insect's deformable cuticle (22). Moran and Varela (23) suggest that this bundle itself may be the transducer, as its abolition with colchicine abolishes the receptor function of the sensillum. However, these authors offer the alternative possibility that the microtubule bundle may act simply as a passive rod, transmitting force from the cuticle back to the dendrite from which it arises.

The role of the ciliary membrane in the transduction process has been evaluated mainly from work on the mechanosensitive ciliate *Paramecium*. In this organism, anterior stimulation is thought to increase Ca^{++} permeability, causing depolarization, further Ca^{++} influx, and eventual ciliary reversal attributable to the direct effect of increased Ca^{++} concentration on the ciliary motor. Posterior stimulation causes K^+ efflux, hyperpolarization, and increased ciliary beat frequency (26). Deciliation with chloral hydrate reversibly eliminates the calcium action potential, implying that voltage-sensitive Ca^{++} channels occur in the ciliary membrane and that a cilium is regulated by its surrounding membrane (9, 10, 27). The lack of a measurable depolarizing receptor potential in the chloral hydrate–deciliated cells led early workers to the conclusion that the mechanoreceptors also reside in the ciliary membrane, but a recent study, using ethanol-deciliated cells and more refined stimulation techniques, has detected a depolarizing receptor potential, clearly demonstrating that the voltage-sensitive Ca^{++} channels are indeed in the ciliary membrane but that the mechanoreceptor channels are localized in the somatic membrane (20). Whether metazoan cilia use similar or related control mechanisms has not been established. It is clear, however, that increased Ca^{++} levels can cause arrest of molluscan gill cilia (32), most likely acting directly on the axoneme (36).

In certain vertebrate hair cells, a single true cilium (kinocilium) is surrounded by many large microvilli (stereocilia). Hudspeth and Jacobs (18) have shown that stimulation of the cilium alone produces no receptor potential, whereas direct stimulation of the microvilli will produce a normal response. They suggest that the kinocilium serves to convey displacements to the mechanically sensitive stereocilia.

Further insight into the role of cilia in the transduction process has been gained through studies of the molluscan statocyst, an internally ciliated, spherical organ containing suspended crystals, the statoconia. These hair cells lack stereocilia but have hundreds of kinocilia (19). The motile cilia of the statocyst hair cells interact physically with the statoconia in response to mechanical or gravitational stimuli to elicit depolarizing generator potentials (1, 2, 37). Recent work on *Hermissenda* statocysts (7, 16, 17) shows that voltage noise (a function of ciliary beat) and the generator potential (a function of applied force) have a common origin, resulting from the exertion of force on the beating cilia by the gravitationally influenced statoconia. These results suggest that the cilium serves as a more or less rigid plunger whose bending merely serves to bring about a distortion at the point where the organelle inserts into the (mechanically sensitive?) plasma membrane.

In this report, we use two related approaches to better separate the potentially important steps of the cilia-mediated transduction process in statocysts. Vanadate anion (VO_4^{-3}; V in +5 state), a potent and reversible inhibitor of dynein cross-bridging (cf. reference 30), is iontophoretically injected into a hair cell to render its cilia nonmotile, arrested against the plasma membrane. During the initial stages of arrest, and later in recovery, the cilia are pliable and beat with markedly increased bending. Photoactivated cross-linkage of the ciliary membrane to the axoneme with 4,4′-dithiobisphenyl azide (8, 21) is also used to render the cilia nonmotile but, in this case, upright and rigid. During early stages of arrest, the cilia stiffen and move through wider excursions but at a much reduced frequency. The voltage noise and generator potential of the cell are recorded and then correlated with the motile state and relative flexibility of its sensory cilia. We conclude that motile statocyst cilia transmit mechanical stimuli to the contiguous plasma membrane rather than transduce stimuli directly through bending.

MATERIALS AND METHODS

Statocyst Preparation

Hermissenda crassicornis (Peninsula Marine Biological Supply Co., Monterey, Calif.) were maintained in flowing-seawater aquaria at 12°–14°C with a 12-h daily light cycle. The circumesophageal nervous system was dissected and mounted as described in detail elsewhere (1, 2), using a Vaseline ring chamber to retain fluid and stainless steel pins to keep the preparation immobilized.

Normally, to ease penetration with microelectrodes, connective tissue was partially digested by incubation with protease (Subtilisin BPN′, no. P-5255; Sigma Chemical Co., St. Louis, Mo.) at a concentration of 0.7 mg/ml in seawater at 21°C for 6–8 min. Such treatment had no detrimental effect on ciliary motility or on the electrical properties of the statocyst hair cells. Before digestion, when it was desirable to monitor only the voltage noise and generator potentials arising from the hair cell body, eliminating hair cell impulses and synaptic interactions, axons from the statocyst hair cells were cut at their point of entry into the cerebropleural ganglion (cf. reference 1).

For microscopy and for tilting or rotation experiments where microelectrodes were not used, a cover slip was placed atop the preparation, generally leaving two opposite edges open. According to experimental design, fluids surrounding the preparation were exchanged by perfusion.

Intracellular Recording

Microelectrodes, utilizing microfilament glass (GCF-100-4, A-M Systems, Inc., Everett, Wash.), were drawn on a MI-micropipette puller (Industrial Science Associates, Inc., Ridgewood, N. Y.) to a resistance of ~400 MΩ when filled with 100 mM potassium acetate/10 mM sodium vanadate solution or 80 MΩ when filled with 4 M potassium acetate. The microelectrodes, mounted on a Narishige micromanipulator (Labtron Scientific Corp., Farmingdale, N. Y.), were connected via chlorided silver wire to a high impedance amplifier (model AM-1; Biodyne Electronics Laboratory, Santa Monica, Calif.). The reference electrode was chlorided silver wire immersed directly in the bath. Monitoring was carried out with a dual-trace oscilloscope (model 5111 storage oscilloscope; 5A22N differential amplifier; 5A18N dual-trace amplifier; 5B12N time-base; Tektronix, Inc., Beaverton, Oreg.). Recordings were made with a Brush 220 pen recorder (Gould, Inc., Instruments Div., Cleveland, Ohio). Simultaneous voltage records were made on an FM tape recorder (model 3960A; Hewlett-Packard Co., Palo Alto, Calif.) for later noise analysis. An Anapulse stimulator (model 301A; W-P Instruments, Inc., New Haven, Conn.) was used to pass current through the electrode. Membrane resistance was monitored by injection of square current pulses. Recording during rotational simulation of gravity was carried out with the apparatus described previously (1, 16).

For mechanical stimulation, a piezoelectric transducer (Bimorph type PZT-5HN; Vernitron Piezoelectric Division, Bedford, Ohio), driven by a second

Anapulse stimulator at 5–10 V for 0.1 ms, was fitted with a fire-polished microelectrode, creating a mechanical probe. The resulting 10- to 20-μm displacement was used to directly stimulate a hair cell, from which recordings were being made, by tapping the cell's external surface membrane. Alternatively, the entire statocyst was mechanically displaced by driving a short, blunt probe placed in an adjacent ganglion (cf. reference 2), here using applied voltages in the 5- to 25-V range.

Vanadate Iontophoresis

Specific amounts of vanadate anion were introduced into the statocyst hair cells from a microelectrode filled with 10 mM sodium vanadate (no. S-455, Fisher Scientific Co., Pittsburgh, Pa.) and 100 mM potassium acetate. Because of an observed deterioration of vanadate with time, the mixed electrode buffer was usually prepared daily from concentrated stocks. An average hair cell volume of 6.4×10^{-10} liter was estimated from simple geometry, approximating the cell as a disk 33 μm in diameter and 7.5 μm thick (2). The amount of time required to deliver sufficient vanadate into this volume to achieve a concentration of 5–10 μM at a current of 0.1 nA was calculated from Faraday's Law to be 90–180 s. A transference number of 0.035, determined experimentally, was used for vanadate anion in the presence of a 10-fold excess of acetate (Stommel and Stephens, manuscript in preparation).

The 5- to 10-μM concentration was chosen because vanadate in this range was shown to completely inhibit ciliary movement in reactivated "cell models" of *Paramecium*, as does this same concentration when iontophoretically introduced in vivo (Stommel and Stephens, manuscript in preparation). The electrolyte composition was chosen to optimize iontophoretic injection time and to minimize passive diffusion from the electrode tip.

Vanadate inhibition was rapidly reversed by perfusion with 10 mM norepinephrine in seawater. The reagent was prepared fresh before use.

Membrane Cross-linkage

Statocyst preparations were incubated in semidarkness for at least 10 min in seawater containing 10^{-4} M 4,4′-dithiobisphenyl azide (Pierce Chemical Co., Rockford, Ill), introduced as a 20-mM solution in ethanol (21). The preparation was then transferred to normal seawater. To photoactivate this lipophilic, bifunctional cross-linking agent, the preparation was irradiated with near ultraviolet light (366 nm) by use of either a Zeiss IV-Fl Epifluorescence illuminator (Carl Zeiss, Inc., New York) when simultaneous high magnification observations were desired or a Wild 200-W mercury arc illuminator (Wild Heerbrugg Instruments, Inc., Farmingdale, N. Y.) at a distance of 20 cm, directed downward with a mirror and a second quartz condensing lens when electrical recording was done. This latter configuration was necessary because the microelectrodes are introduced from above the preparation. Later experiments were performed with the epifluorescence unit arranged beneath the preparation. A Zeiss Neofluar × 40 phase objective served as a condenser when mounted on the illuminator with a suitably machined adapter. Under our conditions, ciliary arrest generally took place within 8–10 min when the epifluorescence unit was used and 15–30 min with the less concentrated light of the Wild illuminator.

Calcium Exchange

The possible role of calcium ions in transduction was tested through the use of EGTA and ionophore A23187. Calcium-free seawater (Moore Formula[1]) containing 5 mM EGTA and buffered to pH 8 with HCO_3^- was used to bathe the preparation and reduce Ca^{++} levels to below 1 μM. Intracellular Ca^{++} was reduced by iontophoretic injection of EGTA to 1–5 mM. Normal seawater containing the ionophore A23187 (Calbiochem-Behring Corp., American Hoechst Corp., La Jolla, Calif.) at a concentration of 10 μM was used to render the cells permeable to Ca^{++} ions. The reagent was introduced as a 10 mM solution in dimethyl sulfoxide. This solvent, at the dilution used, did not affect the motility or electrical properties of the preparation. The calcium concentration in normal seawater is ~10 mM. To competitively block calcium channels, artificial seawater containing 5 mM $CaCl_2$ and 20 mM $CoCl_2$ was perfused through the statocyst preparations (cf. reference 3)

Photodocumentation

Microscopy was carried out with a Zeiss RA microscope equipped with phase and Nomarski differential interference contrast optics. The microscope was mounted on a hinged stand so that it could be tilted 90°, making its rotating stage vertical and thus allowing the statocyst preparation to be rotated, viewed, and photographed with an equatorial gravity vector. Still photographs were taken on Kodak Panatomic-X 35-mm film with an Olympus PM 10-A automated camera (Olympus Optical Co., New Hyde Park, N. Y.). Continuous filming was done on Kodak Plus-X 16-mm negative film with a Sage 501/Bolex H16 M microcinematographic unit (Orion Research, Inc., Cambridge, Mass.), typically at 18 frames/s. To assure short exposure times for both still and motion pictures, a Wild 200-W mercury arc illuminator was normally used.

Voltage Noise Analysis

60-s samples were taken from representative segments of taped records, recorded at 15/16 ips at a band width of direct current to 500 Hz, for analysis by a fast Fourier transform program using a PDP 11/60 computer (Digital Equipment Corp., Maynard, Mass.). Sampling of the digitized data was done at 1-ms intervals after filtering at 0.1–500 Hz. Frequencies in the range of 0.1–50 Hz were analyzed. Graphical data displays were reproduced by electrostatic copy (Tektronix 4631 hard-copy unit). The system and program were calibrated by analysis of externally generated random noise of known amplitude, using a Hewlett-Packard 3722A noise generator.

RESULTS

Vanadate Inhibition of Ciliary Movement

Iontophoretic injection of vanadate anion into hair cells with a 0.1-nA current causes complete cessation of ciliary beat within 2 min. This is immediately evident in most horizontal preparations as a movement of the normally well-centered mass of statoconia toward the injected equatorial cell (Fig. 1 *a* and *b*). No movement of individual, small statoconia is evident adjacent to the inhibited cell. After 4–6 min, however, movement returns and the mass of statoconia begins to move back toward the center of the statocyst (Fig. 1 *c*). After 8–12 min, the statoconia are fully repositioned within the statocyst (Fig. 1 *d*) and their "jiggling" movement appears qualitatively normal. Movement can be restored rapidly (<1 min) by direct addition of seawater containing 10 mM norepinephrine, an agent particularly effective in reducing vanadium +5 to the +4 valence state, and hence reversing dynein inhibition.

The consequences of ciliary inhibition are more dramatic when the microscope and preparation are tilted 90°, such that the full mass of the statoconia, under the influence of gravity, rests directly upon the equator. The normally beating cilia support the statoconia on their vibratile tips (Fig. 2 *a* and *c*), but the cilia of the inhibited cell are flaccid and generally lie parallel to the cell's plasma membrane, allowing some statoconia to fall into the "notch" created by ciliary inactivity (Fig. 2 *b*). As the cilia recover from vanadate inhibition, the statoconia are ejected from the "notch" and the mass is again equally supported (Fig. 2 *d*).

It was shown previously that the cilia of the statocyst hair cells beat in an upright, vibratile fashion, with their tips moving through about ±10°. Free cilia move at ~10 Hz whereas cilia loaded with the weight of the statoconia move at ~7 Hz, still retaining their rodlike beat form (16). During vanadate inhibition, however, the cilia take on a more classic beat pattern, using a normal effective stroke and propagating full, near-90° bends along the entire length of the shaft during the recovery stroke. Ultimately, most cilia come to rest against the plasma membrane (Fig. 3 *a*). For geometrical reasons, it is not possible to photograph at oil-immersion magnifications during iontophoretic injection because the microelectrode is inserted from a near-vertical position, but the recovery from vanadate inhibition appears to be the reverse of the arrest and may be observed after removal of the microelectrode. Early in recovery, cilia of the inhibited cell may be seen in various positions characteristic of typical somatic ciliary beat (Fig. 3 *b*). In full

[1] Formulae and Methods V, Marine Biological Laboratory, Woods Hole, Massachusetts. 55.

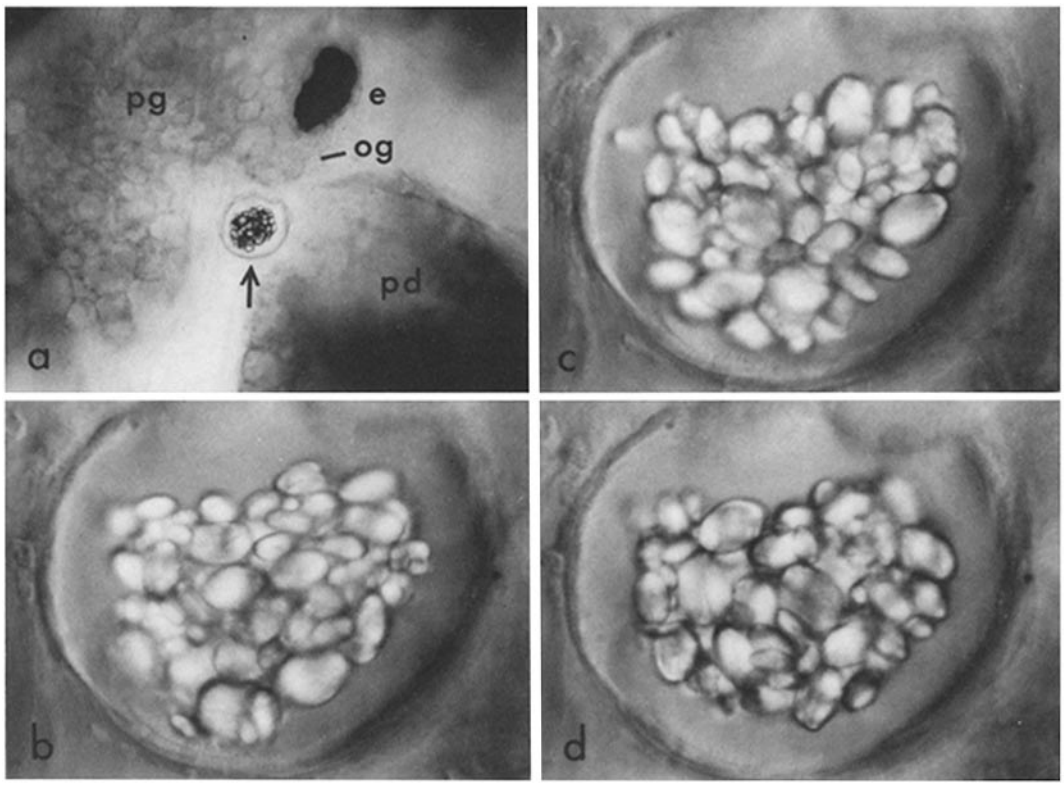

FIGURE 1 Vanadate injection in a statocyst preparation, viewed from above. (*a*) The normally well-centered statoconia move toward the injected cell (arrow). *e*, eye; *pd*, pedal ganglion; *og*, optic ganglion; *pg*, pleural ganglion. (*b*) Same as *a*. (*c*) The cilia begin to move again after ~5 min, displacing the statoconia toward the center. (*d*) After 10 min, the cilia recover fully and the statoconia are returned to their original position. *a*, Nomarski optics, × 80; *b–d*, × 500.

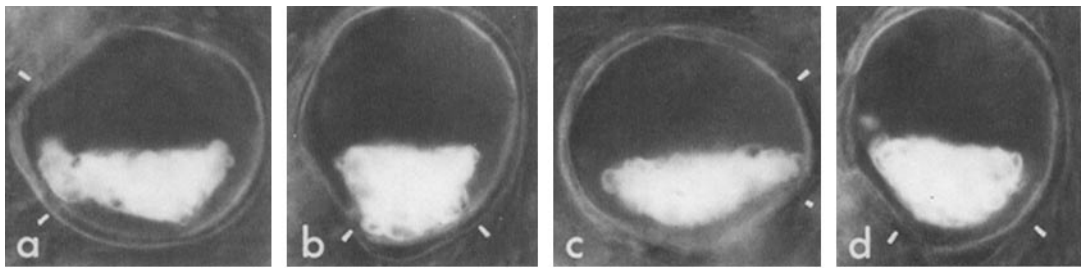

FIGURE 2 Vanadate injection in a statocyst preparation, turned 90° and viewed from the side during rotation. (*a* and *c*) At ±90°, the normally beating cilia support the statoconia on their vibratile tips. (*b*) The cilia of the injected cell (between the two white markers) are flaccid and lie parallel to the plasma membrane, allowing statoconia to fall into the "notch" created by ciliary inactivity. (*d*) As the cilia recover, the "notch" disappears as a consequence of the statoconia being propelled upward. Phase-contrast optics, from a 16-mm motion picture film; × 300.

recovery, the beat pattern again becomes vibratile and rapid (Fig. 3*c*).

Electrical recordings may be made from the hair cell before and after vanadate inhibition while the cell is subjected to simulated gravity through rotation of the statocyst preparation, off-center, on a turntable (1). The statoconia are moved centrifugally against the experimental cell at will by simple acceleration or deceleration of the apparatus. A typical experiment is illustrated in Fig. 4. As reported previously (16), application of force increases voltage noise variance and depolarizes the cell (Fig. 4*a*). After vanadate treatment, however, the noise is markedly reduced and does not change with application of force, and depolarization does not occur (Fig. 4*b*). During rotation, the inhibited cell from which the recording was made is in a state strictly analogous to that seen in Fig. 2*b*; the cilia are in the same state as those in Fig. 3*a*. During recovery, the voltage noise returns and the cell again depolarizes with rotation (Fig. 4*c*). Here, the cilia of the treated cell have nearly regained their normal beat pattern, as in Fig. 3*c*. There is no systematic change in resting potential or in membrane resistance as a consequence of the vanadate treatment; values obtained before and during treatment and after recovery differ by no more than 20%.

During vanadate arrest, and also during recovery, the cilia undergo increased bending, but the voltage noise, a direct consequence of ciliary beat, is considerably diminished. Quantitative noise analysis illustrates this point. In Fig. 5, the power spectrum of a hair cell before vanadate treatment (*a*) is contrasted with that obtained immediately after inhibition (*b*) and during recovery (*c*). It is evident that the noise is reduced nearly sevenfold after inhibition and that it recovers with essentially the same broad energy distribution as before treat-

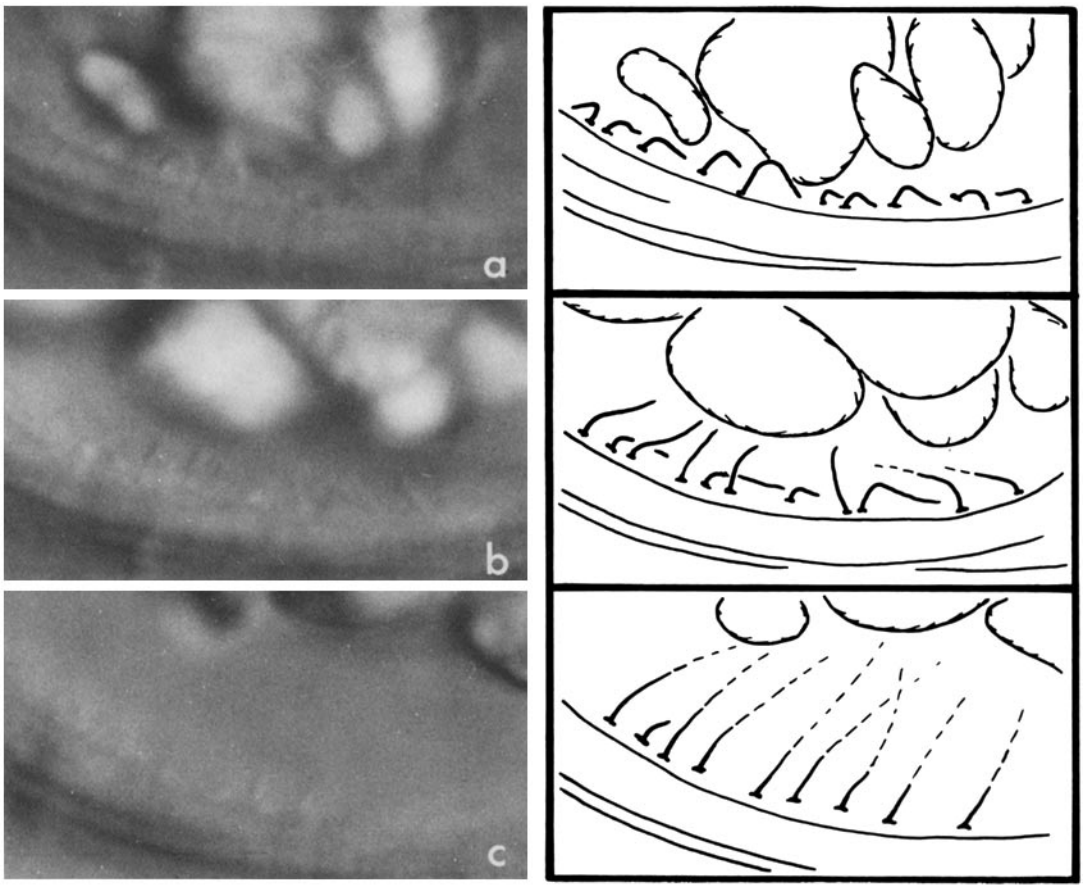

FIGURE 3 Vanadate inhibition and recovery of ciliary movement (*a*) A hair cell injected with vanadate has bent cilia, lying approximately parallel to the plasma membrane. (*b*) The beginning of recovery is characterized by movement through complete effective and recovery strokes. (*c*) When the cilia recover fully, they are upright and vibratile, blurred because of motion. 0.1-s exposure, same preparation as in Fig. 1. The accompanying drawings are to aid in interpreting the photographs, the depth of focus being small and the movement rapid. Nomarski optics, × 1,700.

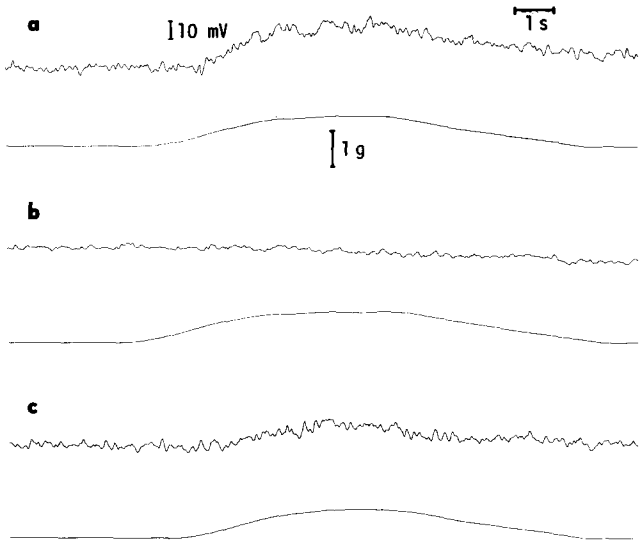

FIGURE 4 Rotational simulation of gravity. (*a*) Initiation of a generator potential and an increase in voltage noise produced by the rotation of the statocyst preparation off-center on a turntable. Upper trace: voltage vs. time; lower trace: gravity monitor. Note that the cell depolarizes in direct response to the increasing force applied to it. (*b*) The cilia have been arrested by vanadate injection, suppressing the voltage noise and eliminating the generator potential. (*c*) After partial recovery, the voltage noise and gravity-induced generator potential return.

ment (*a*) or after complete recovery with norepinephrine (*d*). In the example given, the statoconia remain in contact with the equatorial region throughout the recording, as in Fig. 1.

Of the 17 vanadate-injected cells analyzed, all underwent a reduction of voltage noise variance upon stoppage of the cilia, ranging from 2.3 to 6.8 times (average, 4.0 ± 1.2 SD). Not analyzed were cases where stoppage was incomplete after 2 min of injection, an effect that could be traced consistently to "aging" of the vanadate/acetate electrolyte.

Using a piezoelectric probe to produce a rapid (0.1-ms) mechanical pulse external to the statocyst but directly against the cell under study, it is possible to drive the ciliated cell membrane radially inward. When the preparation slide is tilted so that the statoconia are in contact with the cilia of the cell under study, a strong generator potential can be observed upon mechanical stimulation (Fig. 6*a*). This generator potential is directly proportional to the magnitude of the applied stimulus, over a voltage range of 5–25 V (cf. reference 2). After vanadate inhibition (Figure 6*a*, right portion), a considerably diminished generator potential is observed. In all cases where a strong generator potential could be elicited before vanadate inhibition, some observable generator potential remained after inhibition, though its magnitude was not readily quantifiable. When the preparation slide containing an "uncut" preparation (able to produce action potentials when sufficiently depolarized) is tilted so that the statoconia are moved away from the hair cell, the same mechanical stimulus elicits no generator

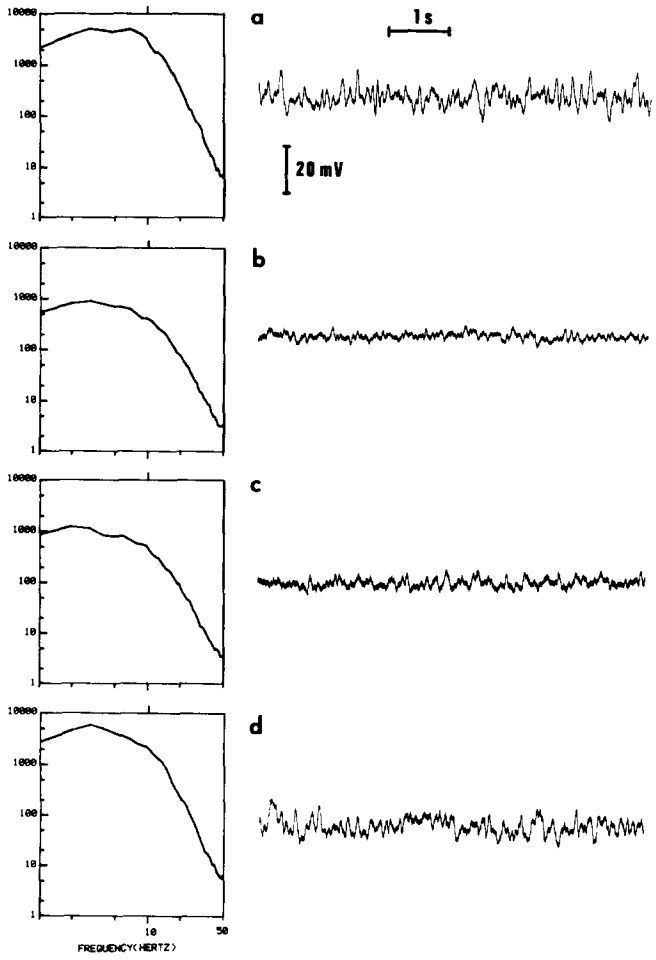

FIGURE 5 Voltage noise analysis of vanadate inhibition. (*a*) A power spectrum (left) of a 60-s voltage noise recording (right, representative sample) from a hair cell with normally beating cilia. (*b*) After injection of vanadate to a level $>5 \times 10^{-6}$ M, the maximum voltage noise variance has decreased nearly sevenfold. (*c*) After 9 min, partial recovery has occurred. (*d*) After the addition of 0.01 M norepinephrine, the voltage noise level has increased dramatically. The ordinates of the power spectra are given in arbitrary units that are proportional to V^2 (voltage noise variance; 1,000 U = 260 V^2).

potential, even when the cell is firing spontaneously (Figure 6*b*, upper trace). Conversely, when a control preparation is tilted so that the statoconia contact the cilia, the same magnitude of stimulus produces a generator potential, generally followed by an action potential (Fig. 6*b*, lower trace). The remaining permutation, that of a vanadate inhibited cell whose cilia do not contact statoconia, results in no detectable generator potential (data not shown). Thus, a mechanically evoked generator potential requires that the inner, ciliated cell surface contact the statoconia, because local stimulation of a cell out of contact with statoconia elicits no response. When loaded with statoconia, a cell with inactive cilia rendered flexible by vanadate is still capable of producing a generator potential, albeit a barely detectable one.

The fact that an unloaded cell produces no generator potential serves as a control for the above experiments and those to follow, eliminating the possibility of an artifactual tip potential resulting from simple mechanical displacement of the microelectrode at the impalement site. This argument is documented more fully in previous work with the same system (2).

Membrane-Axoneme Cross-linkage

When statocyst preparations are incubated for 10 min or more with 10^{-4} M 4,4′-dithiobisphenyl azide, the cilia of the hair cells are susceptible to light-induced, covalent cross-linkage of the membrane "sleeve" to the axoneme, rendering the organelle rigid and perpendicular to the plasma membrane. The general phenomenon is illustrated in Fig. 7. The preparation and microscope are tilted 90° so that the statoconia rest upon the equator. As cross-linkage takes place, the cilia slow and beat at 2–3 Hz, moving through wider than normal excursions, often as much as ±30°. Eventually, the cilia are arrested in a straight, upright position, bearing the full weight of the statoconia, with little evidence of bending. A few cilia are always arrested in the extreme excursion position, showing a uniform curvature (Fig. 7*a*). Even when the statocyst "collapses" (from age or injury), the cilia are so rigid that the hair cells are deformed outward (Fig. 7*b*), in contrast to an unirradiated statocyst wherein the cilia are simply forced down against the plasma membrane (Fig. 7*c*) upon collapse of the structure (cf. reference 16).

In striking contrast to the vanadate treatment, where slower movement and increased bending is reflected in decreased noise, membrane cross-linkage also slows the cilia and increases the angle through which they move, but it markedly increases voltage noise. This is illustrated qualitatively in Fig. 8, in which recordings were made from a cell through the light-induced cross-linkage process. Noise and motility appear normal when the irradiation is initiated (*a*, right) but as the cilia begin to slow, the noise level increases (*b*, right). As radiation progresses further, the amplitude of the voltage noise increases even more (*c*, right), while it appears from inspection that the average frequency decreases. In time, movement is nearly arrested and the noise decreases to an erratic level (*d*, right). The initial voltage noise amplitude increase is not attributed to hyperpo-

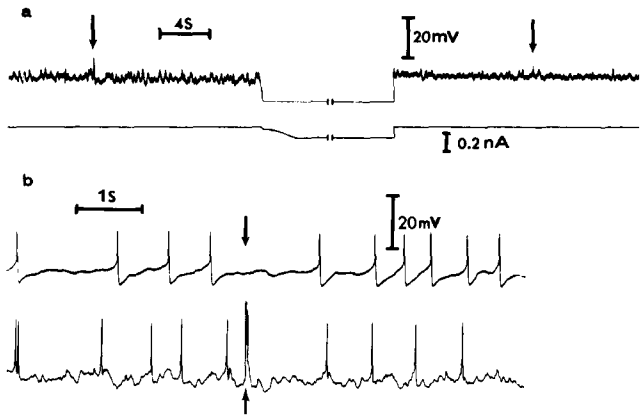

FIGURE 6 Local piezoelectric stimulation of hair cells. (*a*) When the preparation is tilted such that the statoconia are in contact with the moving cilia of the cell being monitored, a 10-mV generator potential is elicited in response to a mechanical stimulus (at arrow) applied to the external surface of the cell. After 90 s of a 0.1-nA current to inject vanadate, the noise level of the cell is reduced and the generator potential is nearly absent (stimulus at second arrow). "Cut" preparation to eliminate impulses and synaptic interactions. (*b*) Upper trace; Recording from an "uncut" preparation in which the statoconia are not contacting the cilia. With a stimulus (arrow) applied directly to the cell, it is not possible to observe a response, in spite of the fact that the cell is sufficiently depolarized to fire. Lower trace: Same, but the statoconia are in contact with the cilia. Here, a stimulus (arrow) elicits a response, causing the cell to fire.

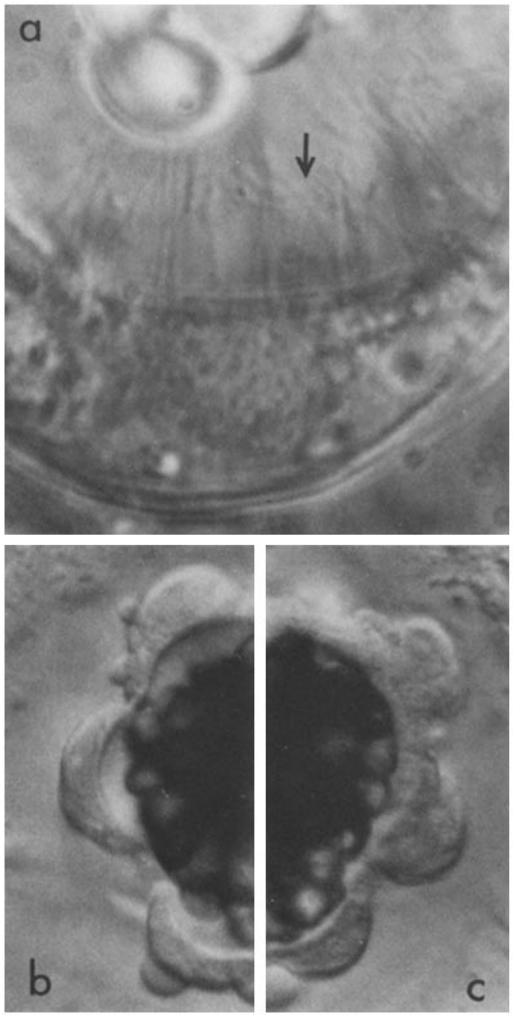

FIGURE 7 Light-induced cross-linkage of statocyst cilia. (*a*) After incubation with 4,4′-dithiobisphenyl azide, the ciliary membrane is cross-linked to the axoneme by exposure to 366-nm light. The cilia are rendered rigid, supporting the statoconial mass. The cilia are either absolutely straight or else display a uniform curvature (arrow). (*b*) Even when the statocyst "collapses" (loses fluid), the cilia of an irradiated cell are so rigid that the hair cells are deformed outward. (*c*) In the nonirradiated control statocyst from the same preparation, the pliable cilia allow the statoconia to approach the inner cell membrane. Nomarski optics: *a*, × 2,000; *b* and *c*, × 500.

larization of the cell. In fact, the resting potential sometimes decreases during irradiation; it does so in discrete decrements, indicating that the microelectrode may be pulling out of the increasingly rigid membrane. Supporting this explanation is the near-impenetrability of the hair cell membrane after complete stoppage of the cilia by cross-linkage. Within an error of ±20%, there is no systematic change in membrane resistance during the irradiation process. If the experiment is carried out without the reagent, or if the statocyst is masked by intervening tissue, no change in motility, voltage noise, or resting potential takes place, indicating that neither the presence of the reagent nor the intense near-ultraviolet light is detrimental to the preparation.

The change in the character of the noise with irradiation was analyzed quantitatively for comparison with that seen in vanadate inhibition. Fig. 8 illustrates the power spectra corresponding to the voltage traces before (*a*), during (*b* and *c*) and after (*d*) the irradiation just discussed. The phenomenon is characterized by a marked overall increase in the voltage noise variance, accompanied by a weighted shift toward lower frequencies. As the cilia stiffen further and nearly stop, the power spectrum (*d*) reflects some diminution at all frequencies, but a peak at 2–3 Hz remains prominent. The moving, stiffer cilia are thus initially more effective in producing transduction events but become less effective as movement ceases because of further stiffening restraint.

The efficiency of stoppage was a function of "masking" by tissue overlying the statocyst or portions thereof, with cells receiving full irradiation stopping first while others in the statocyst, partially masked, showed varying degrees of arrest. One could not always guarantee that a cell being recorded would be affected in a reasonable period of time, nor could

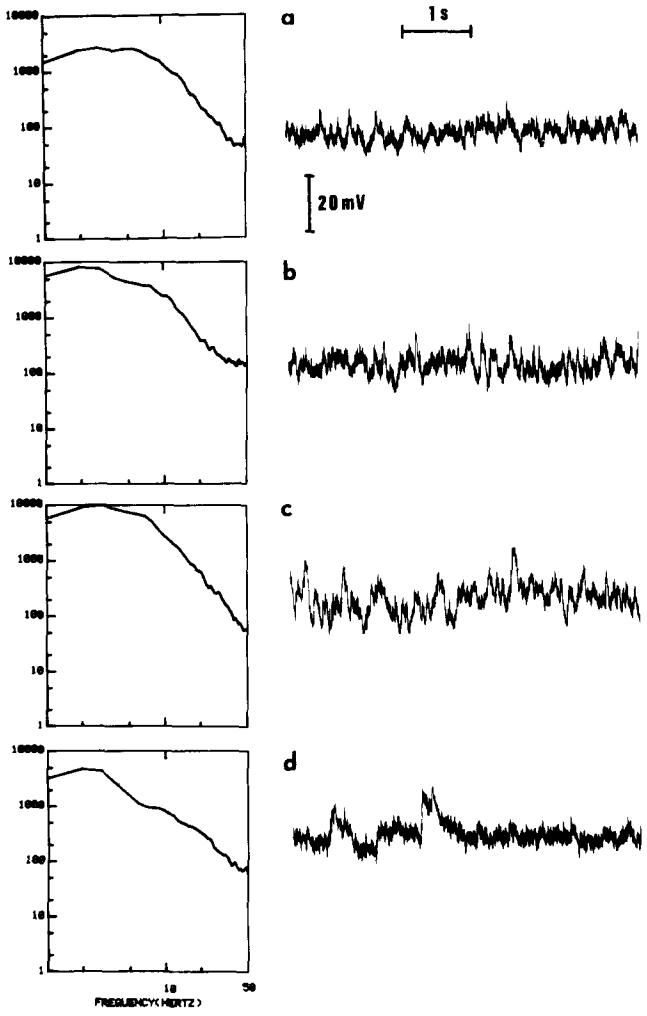

FIGURE 8 Voltage noise analysis during cross-linkage. (*a*) A power spectrum (left) of a 60-s voltage noise recording (right, representative segment) from a hair cell when irradiation begins. (*b*) After 4 min of 366-nm irradiation, the amplitude of the voltage noise increases and the power spectrum likewise increases, particularly at lower frequencies. (*c*) After 15 min, the voltage noise amplitude increases further and the power spectrum reflects a threefold increase in voltage noise variance, centered at ~3 Hz. (*d*) After 25 min of irradiation, noise at frequencies between 5 and 12 Hz have decreased but a peak at 2–3 Hz remains. At this stage, the cilia are seen beating at this same frequency. The abrupt depolarization on the voltage noise trace may be caused by groups of stiffened cilia moving in concert. Ordinates for power spectra are in arbitrary units; 1,000 U = 260 V^2.

one guarantee that an electrode would remain in place throughout the course of the experiment. In five successful irradiations of "cut" preparations, in which recording was carried out at least through the time at which the cilia were appreciably arrested (i.e., the voltage noise decreased after reaching a maximum), the cells showed a twofold to ninefold enhancement of voltage noise variance (average, 3.5 ± 0.8 SD). Only six preparations ("cut" and "uncut") were carried through complete stoppage, the difficulty being that the electrode could not be retained in the increasingly cross-linked membrane.

When a general mechanical stimulus is applied to the entire statocyst before cross-linkage, a generator potential is elicited (Fig. 9*a*). It is readily distinguishable from action potentials in such "uncut" preparations. As the cilia are cross-linked, the noise initially increases and then decreases but the amplitude of the generator potential remains essentially constant (Fig. 9*b* and *c*). Firing is often diminished as irradiation progresses. The resting potential is unchanged and the generator potential not only persists after the cilia have ceased to beat but has increased in relative duration (Fig. 9*d*). The membrane resistance in such experiments (~130 MΩ) either remains unchanged or decreases by up to 25% upon full ciliary arrest. Thus cross-linkage results in stoppage of ciliary beat but leaves the mechanism of receptor potential generation functionally intact. If sufficiently depolarized by application of current, such cells are still capable of producing action potentials, implying that sodium channels remain functional.

Calcium Ion Effects

Because calcium ions appear to be involved in mechanically sensitive ciliary movement in ciliates and in metachrony of molluscan gill cilia, we chose to investigate the possible involvement of calcium ions in hair cell motility and transduction. Piezoelectric stimulation of the entire statocyst was employed to evoke a generator potential while voltage noise was recorded. Ciliary movement was observed in parallel preparations under oil-immersion optics.

The ionophore A23187 has no measurable effect on ciliary movement, voltage noise, or the magnitude of the generator potential in a concentration range of 10^{-5}–10^{-4} M, with extensive perfusion, over a period of 20–40 min. These are conditions that will cause arrest of somatic cilia in other organisms (cf. references 25 and 32). The hair cell membrane should be accessible to this reagent, because it is obviously accessible to various ions and chloral hydrate (16) and also to 4,4′-dithiobisphenyl azide (this study).

Treatment with artificial seawater containing 20 mM $CoCl_2$ (either with or without 5 mM $CaCl_2$ to maintain tissue integrity) likewise had no effect on motility, voltage noise, or the generator potential over a period of up to 1 h. Presumably competing with calcium, cobalt under these conditions has been shown to block synaptic transmission very effectively in *Hermissenda*, without affecting the ability to generate impulses (3).

Extensive treatment with calcium-free seawater containing 5 mM EGTA was also without effect on the three parameters studied. Motility of cilia in statocysts opened to the environment by dissection appeared to be quite normal for at least 1 h after the statoconia were totally dissolved by EGTA. Lacking statoconia, such statocysts would produce only minimal noise and no generator potential because the cilia cannot be loaded. Because of this and the fragility of the preparation, piezoelectric stimulation is not feasible. Consequently, the converse experiment, to steepen any calcium gradient through ionotophoretic injection of EGTA, was undertaken. Introduction of EGTA, calculated to be in the millimolar range, was also without effect.

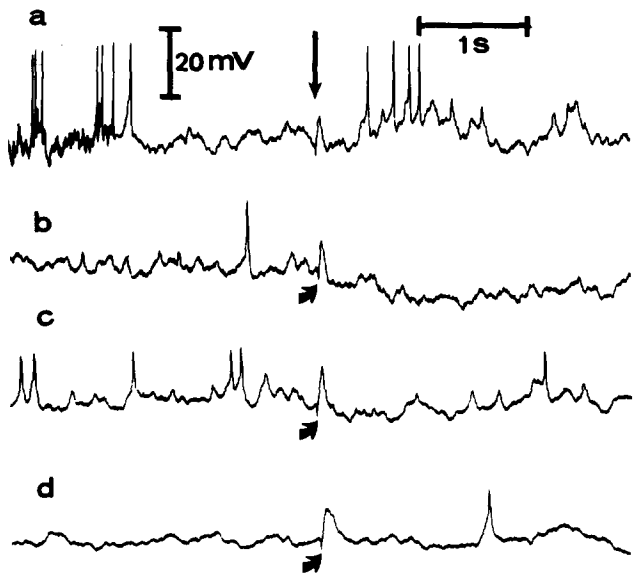

FIGURE 9 Generator potential persistence after light-induced ciliary stoppage. (*a*) In an "uncut" preparation, a mechanical stimulus (arrow) is applied before cross-linkage, eliciting a generator potential, but one not large enough, however, to evoke an action potential. (*b* and *c*) As the cilia are cross-linked, the voltage noise initially increases and then decreases, but the amplitude of the mechanically induced generator potential (arrows) remains essentially the same. (*d*) The generator potential (arrow) increases in relative duration after 12 min of irradiation, conditions sufficient to stop the cilia in an upright position. At this point, spontaneous firing has almost ceased, presumably because there is insufficient noise to reach threshold.

DISCUSSION

These data confirm and considerably extend the conclusions of earlier workers (17, 38) that voltage noise in statocyst hair cells is a direct consequence of ciliary movement against statoconia and that mechanically elicited generator potentials result from transmission of force via the cilia to the hair cell plasma membrane. Rendering the cilia flaccid by vanadate inhibition of dynein interdoublet cross-bridging or rendering them rigid by doublet-membrane cross-linkage yields a complementary result consistent with the hypothesis that motile cilia serve to modulate and transmit mechanical stimuli to transduction sites at or near their basal insertion points.

Vanadate inhibition of ciliary movement differs from the rigor mortis state seen in muscle, in that it leaves the outer doublets free to passively slide in response to stress (28, 30). Early in inhibition, or later in recovery, the normally vibratile statocyst cilia move much like somatic cilia through complete effective and recovery strokes, but in spite of this increased bending, voltage noise variance is considerably diminished. Such an observation is inconsistent with location of the transduction site in the axoneme itself or in the adjacent ciliary membrane, because both of these flex to a far greater extent during inhibition or recovery from vanadate. However, one could argue that the vanadate has coincidently inhibited some independent transduction system in addition to directly inhibiting motility.

When the cilia are totally inhibited, they lie approximately

parallel to the plasma membrane and are bent at the base. Continual application of force to such inhibited cilia through gravitational simulation causes no measurable depolarization of the hair cell, and a direct, rapid mechanical stimulation applied to the cell body, when the prostrate cilia are not in contact with the statoconia, will likewise not elicit a generator potential. However, when a cell having inhibited, basally bent cilia is rapidly driven against the statoconia, a small generator potential is observed. Conversely, when statoconia do not contact the cilia, inhibited or otherwise, no generator potential can be elicited. Taken together, these observations argue against the general deformation of the plasma membrane being involved in transduction and suggest that application of force to pliable cilia, bent at the base, can elicit transduction events only if that force is sufficiently rapid to override the plasticity of the vanadate-inhibited cilia and cause local deformation of the plasma membrane at or near the basal region.

Vanadate (V^V) anion appears to be a benign and specific agent. Membrane resistance and resting potential are constant throughout experiments conducted in the micromolar range and the voltage noise variance, generator potential, and motility are fully recoverable. The reversibility is nearly instantaneous with application of norepinephrine (because of the reduction of vanadium to the +4 valence state) and slow with time (presumably because of intracellular reduction of vanadium). Iontophoretic injection of reduced vanadate (V^{IV}) or of V^V in the presence of exogenous norepinephrine, has no measurable effect on the cell's motility or electrical properties. V^V is now known to affect other ATPases in addition to dynein, for example, muscle myosin (14), the Ca^{++}-ATPase of sarcoplasmic reticulum (29), and the membrane Na,K-ATPase (6). Considering the time-course and concentration requirements for these inhibitions, it is very unlikely that a vanadate effect on any homologous system in the hair cell could explain our results. Certainly the most obvious mode of action is directly on the dynein-based motile system of the sensory cilia.

Photochemical cross-linkage of the ciliary membrane results in a situation wherein the outer doublets are tethered to the surrounding membrane and evidently unable to slide, at least in the two quite distant species on which correlated electron microscope and biochemical studies were carried out (8). We are assuming that a similar process takes place in the statocyst of *Hermissenda*. During the initial stages of cross-linkage, the cilia continue to beat in a vibratile fashion but do so more slowly and with an increased, uniformly curved stroke. Voltage noise variance increases considerably during this process, whereas the mean beat frequency decreases to several hertz. These effects are directly measurable as changes in the power spectrum. As the cilia stop, voltage noise decreases and becomes erratic, presumably reflecting those few cilia still moving against adjacent statoconia. Complete stoppage is coincident with minimal voltage noise.

Most significantly, cells with rigid, upright, and totally nonmotile cilia are fully capable of producing a generator potential of equal or sometimes greater amplitude than that elicited before treatment. The mechanically induced generator potential increases in duration as the cross-linking proceeds, apparently reflecting the increasingly longer time required for the cilia to return to a fully upright position after deformation by the statoconia driven against them by piezoelectric stimulation. If depolarization is attributable to the opening of channels as a direct consequence of membrane deformation, the longer the deformation persists the longer the generator potential should remain. In the case of these rigid cilia, the most likely deformation point is at the basal insertion.

The cross-linkage process does not change the basic electrical properties of the statocyst hair cell, other than to diminish firing in uncut preparations. Membrane resistance and the resting potential are essentially constant throughout the course of the experiment, except where resting potential is decrementally lost as the electrode pulls out of the stiffened membrane. The action of 4,4′-dithiobisphenyl azide is reversible upon application of 2-mercaptoethanol to cleave the reagent's disulfide bond. The cilia begin to beat within seconds but the wave form is very abnormal (sinusoidal) and hence such cells were not analyzed for purposes of this study.

In summary, the data presented here are consistent with a mechanical transduction site at or near the base of the motile cilium. Under this hypothesis, making cilia more pliable should minimize membrane deformation at the basal region, minimizing voltage noise variance, whereas rendering cilia uniformly more rigid should maximize deformation, maximizing voltage noise variance. Pressure against a totally rigid cilium, normal to the plasma membrane, should be transmitted fully to the insertion point, resulting in an undiminished generator potential. Tympanic stimulation of a cell whose cilia "hang loose" because they are not loaded should elicit little or no generator potential. All of these expectations have been realized.

Available evidence suggests that voltage noise variance and generator potential in molluscan statocysts are associated with changes in sodium conductance (12, 16). Application of a hyperpolarizing current increases the voltage noise variance and generator potential, implying a conductance increase for an ion whose equilibrium potential is more positive than the resting level (2, 16). Zero external sodium hyperpolarizes the membrane and essentially abolishes the voltage noise variance and depolarizing response to rotation but has no observable effect on ciliary or statoconial movement (16). Because of the postulated involvement of an initiating calcium current in mechanoreception by ciliates (26), we manipulated the calcium concentration, externally and internally, to determine whether calcium also might be involved in statocyst mechanoreception. Removal of calcium in the bathing fluid by perfusion with calcium-free seawater containing EGTA or reduction of internal calcium by iontophoretic injection of EGTA has no observable effect on motility, voltage noise variance, or generator potential. Competitive blockage of presumptive calcium channels with cobalt or increasing calcium permeability with ionophore are likewise without measurable effect. Considering the very high levels of calcium normally present in and around the tissue, one might argue that these treatments are inadequate. However, because the role of calcium ions in ciliates and molluscan gills appears to be one of a mediator of ciliary reversal and because molluscan statocyst cilia do not show reversal, one would not necessarily expect calcium to be involved. Although we cannot unequivocally eliminate calcium ions in the mechanoreception process of the statocyst hair cell, our evidence does not suggest a major role for calcium.

Given that one accepts the assertions that statocyst cilia transmit rather than directly transduce mechanical stimuli and that the transduction site is at or near the basal region, one is still left with the basic question of why the cilia are motile when rigid rods might serve as well. The generator potential elicited from cross-linked, nonmotile cilia is, in fact, proportional to the force applied. Beating cilia produce a "carrier signal" whose mean frequency is ~10 Hz. Loading the cilia

changes the frequency only slightly, probably through simple damping, but produces a marked increase in signal amplitude. The situation is analogous to amplitude modulation in radio transmission and thus the hair cell might be said to produce an AM signal characteristic of its momentary position. Of what advantage is such a signal for information processing in the organism? Probably the most important advantage is to amplify the primary gravitational information. It was shown previously that an unloaded cell, oriented opposite the gravity vector, produces minimal voltage noise and is relatively more hyperpolarized, compared with a cell subjected to the mass of the statoconia (16). The cilia of the former cell have nothing to resist their beating. In the loaded cell, however, not only is 1 *g* of force pressing against the field of cilia but the cilia beat in such a manner as to propel the stones away from them, i.e., the force at the base of a cilium would be the sum of the gravitational vector from the statoconial mass plus the reaction vector resulting from motility. Put another way, cilia moving to or from a vertical position would serve as active, semirigid levers with respect to a point at the base, producing repetitive on-off signals, the magnitude of which would be a direct function of loading and force of beat. This is in direct contrast to loading a nonmotile field of cilia where a single deformation would result in a single pulse of ions, followed by an elastic return to the normal, undeformed state. Thus not only would motile cilia serve to amplify the initial gravitational signal but they would further serve continuously to detect whether the field of cilia is still loaded. Taking the beat of each loaded cilium as a single, depolarizing event, the summation of all of these events will approach a threshold, causing the cell to fire, and the cell will continue to fire at approximately the same rate as long as it remains loaded to the same extent. This is precisely what is observed in the functioning statocyst (1). Thus an amplitude-modulated signal (voltage noise) is converted into a frequency-modulated signal (firing rate) whose frequency and duration, respectively, reflect the magnitude and duration of the applied force.

What is the nature of the mechanoreceptor site that appears to be located at or near the base of the cilium? One can speculate on two morphological possibilities: the ciliary necklace region and the plasma membrane adjacent to the perpendicular ciliary rootlets.

The ciliary necklace was first defined and described in detail by Gilula and Satir (13). This array of membrane particles and membrane–outer doublet connections has been postulated to be the site of ion channels involved in transduction in ciliates and regulation of beat in gill cilia (11, 31). As mentioned earlier, mechanoreceptor channels of *Paramecium* are left beind in the somatic membrane upon deciliation (20). Cilia are isolated by breakage at the basal plate, leaving the necklace behind (5, 13). Lateral shear during active sliding of outer doublets, when resisted by mechanical constraint or loading, would be transmitted back to the basal plate region. The resultant distortion might deform the membrane of the necklace, attached by fine linkages to the outer doublets immediately below the basal plate.

The hair cells of *Hermissenda* have an unusual ciliary rootlet system wherein many fine rootlets radiate from the circumference of the basal body, subjacent to the plasma membrane and apparently attached to it (19). This is in direct contrast to the typical single or bifurcating rootlet that grows from the proximal end of the basal body toward the interior of most other ciliated cells. Movement of a cilium anchored normal to the plasma membrane by such perpendicular rootlets might serve to locally distort the contiguous, mechanically sensitive plasma membrane.

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