

# THE ROLE OF $\text{Ca}^{2+}$ IN DEFLECTION-INDUCED EXCITATION OF MOTILE, MECHANORESPONSIVE BALANCER CILIA IN THE CTENOPHORE STATOCYST

BRIAN LOWE\*

*Boston University Marine Program, Woods Hole, MA 02543, USA*

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## Summary

Motile, mechanoresponsive cilia (balancers) in ctenophore statocysts, like vertebrate hair cells, are excited or inhibited depending upon the direction in which they are deflected. Balancers, however, may become either excited (beat rapidly) or inhibited (beat slowly) by deflection in the same direction, depending on the sign of ctenophore geotaxis (positive or negative). The beat frequency of many cilia is controlled by concentrations of  $\text{Ca}^{2+}$ , membrane potential and neural input. How these factors affect deflection-induced ciliary beating in balancers was investigated. Deflection-induced excitation of balancers in whole *Mnemiopsis leidyi* larvae and dissected adult (*Mnemiopsis leidyi*, *Pleurobrachia pileus*) statocysts was reversibly inhibited by the  $\text{Ca}^{2+}$  channel inhibitors  $\text{Co}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Ni}^{2+}$  and  $\text{Mn}^{2+}$ . Deflection-induced excitation in balancers of isolated adult *M. leidyi* balancer groups was also inhibited by  $\text{Co}^{2+}$  or by  $\text{Ca}^{2+}$ -free medium. Isolated

balancer group cilia, like balancer cilia of intact ctenophores, exhibited responses to either sign of geotaxis and graded responses to deflection. Isolated balancers that were chemically depolarized in high- $[\text{K}^+]$ ,  $\text{Ca}^{2+}$ -free medium were excited by local application of  $\text{Ca}^{2+}$  onto the ciliary bases, but not onto the cell bases or the ciliary tips. It is proposed that deflection-induced excitation of balancers is due to influx of  $\text{Ca}^{2+}$  through stretch- and voltage-activated channel activity. The sign of geotaxis of whole larvae and dissected adult statocysts was switched by electrical stimulation. Thus, neural input may participate in reversing the directional sensitivity of balancer cells.

Key words: ctenophore, statocyst, cilium, mechanoresponse,  $\text{Ca}^{2+}$ , geotaxis, *Mnemiopsis leidyi*, *Pleurobrachia pileus*, *Bolinopsis infundibulum*,  $\text{Ca}^{2+}$  channel inhibitors,  $\text{Mn}^{2+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Co}^{2+}$ .

## Introduction

The beat frequency of many cilia is controlled by external mechanical stimuli (Thurm, 1968; Machemer and Eckert, 1975; Stommel, 1984; Sanderson and Dirksen, 1989). Mechanical stimulation of *Paramecium caudatum* causes stretch-activated channels on the soma membrane to open, and the ensuing transmembrane ion flux leads to a change in ciliary beat frequency or pattern (Eckert *et al.* 1976; Machemer, 1977). Beat frequency may also be regulated by intracellular  $\text{Ca}^{2+}$  (Nakaoka *et al.* 1984; Tamm, 1988*a,b*; Korngreen and Priel, 1994; Salathe and Bookman, 1995), by cyclic AMP (Hamasaki *et al.* 1995), by changes in membrane potential (Eckert *et al.* 1976; Machemer, 1977; Nakaoka and Iwatsuki, 1992; Shingyoji and Takahashi, 1995; Tarasiuk *et al.* 1995) or by synaptic input (Sanderson and Dirksen, 1989; Stephens and Stommel, 1989; Aiello *et al.* 1991). The beating of mechanically coordinated comb-plate cilia of ctenophores (Tamm, 1973) and vibrated sea urchin spermatozoa (Shingyoji *et al.* 1995) may be increased so rapidly that there is apparently no second-messenger mechanism. The motile, mechanoresponsive balancer cilia of the ctenophore statocyst

were exploited to investigate how mechanical deflection, intracellular  $[\text{Ca}^{2+}]$  and membrane potential control ciliary beat frequency.

A unique feature of balancer cilia is that they can reverse their excitatory and inhibitory responses to bending in a particular direction, i.e. deflection of a balancer in a given direction may either increase or decrease its beat frequency depending on the ctenophore's sign of geotaxis (positive or negative) (Horridge, 1965; Tamm, 1982). Balancer cilia increase or decrease their beat frequency when deflected in opposite directions by the gravitational loading of the ctenophore statolith, i.e. they have a mechanical output (Horridge, 1965; Tamm, 1982). Vertebrate hair cells have an analogous, directional sensitivity to mechanical displacement (Hudspeth and Corey, 1977; Lumpkin and Hudspeth, 1995), but their directional sensitivity is fixed and their output is neurotransmitter release. On the basis of preliminary experiments, Tamm (1982) proposed that the beat frequency of balancer cilia is regulated by changes in cell membrane potential, intracellular  $[\text{Ca}^{2+}]$  and synaptic input.

\*e-mail :blowe@codon.nih.gov.

In this study, the possible roles of transmembrane  $\text{Ca}^{2+}$  influx, membrane potential and neural input in the mechanoreponse of ctenophore balancer cilia were directly investigated. Balancer beat frequency of intact larvae, dissected statocysts and isolated groups of balancer cells was recorded while the balancer preparations were exposed to various ionic solutions. The balancer cilia were sometimes mechanically deflected during exposure to experimental solutions to determine the ionic basis of deflection-induced excitation. Inorganic  $\text{Ca}^{2+}$  channel inhibitors and  $\text{Ca}^{2+}$ -free media abolished deflection-induced excitation of balancer cilia, and high extracellular  $[\text{K}^+]$  excited cilia without mechanical stimulation. Local application of  $\text{Ca}^{2+}$  demonstrated that  $\text{Ca}^{2+}$  influx at the ciliary base may be required for deflection-induced excitation. Lastly, electrical stimulation of intact larval ctenophores and dissected adult statocysts induced switches in geotactic sign, suggesting that neural input underlies changes in geotactic sign.

## Materials and methods

### *Ctenophores/larvae*

Adult *Mnemiopsis leidyi* Agassiz, *Bolinopsis infundibulum* (Mueller) and *Pleurobrachia pileus* (Mueller) were carefully dipped from the sea near Woods Hole, MA, USA. Ripe *M. leidyi* were spawned to obtain embryos (Tamm and Tamm, 1981). Free-swimming cydippid larvae 3–5 days old were collected by pipette.

### *Dissected adult statocysts*

Adult ctenophores were relaxed in 6.75%  $\text{MgCl}_2$ :sea water (1:1) before their aboral ends were cut off. Apical organs, including the statocyst, were obtained by removing a slice of tissue (approximately 3 mm thick and approximately 7 mm along the aboral–oral axis and approximately 5 mm along the stomodeal plane) using fine iridectomy scissors (4 mm blade).

### *Isolated balancer groups*

*M. leidyi* or *B. infundibulum* statocysts from slices of tissue (see above) were teased away from the underlying mesoglea using minuten pins (Carolina Biological Supply, Burlington, NC, USA). Groups of balancer cells/cilia were isolated using one of two methods. (1) Enzyme-dissociation method: intact statocysts were immersed in a dish of  $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free artificial sea water (CMFASW; 462 NaCl, 10 KCl, 7  $\text{NaSO}_4$ , 10 Tris, 5 EGTA, in  $\text{mmol l}^{-1}$ , pH 7.7) for 10 min, and then in CMFASW containing 3  $\text{mg ml}^{-1}$  hyaluronidase and 1  $\text{mg ml}^{-1}$  trypsin (Sigma Chemical Company, St Louis, MO, USA) at 23 °C for 30 min. The statocysts were transferred to a microcentrifuge tube containing 1 ml of filtered (no. 1 Whatman paper) natural sea water (NSW) at ambient temperature, pH 8.2. The tube contents were vigorously sucked in and out with a Gilman pipette (polyethylene tip, 500  $\mu\text{m}$  o.d.) and then centrifuged (10 000 g for 20 s). Dissociated statocysts were then transferred to a Lucite slide-well filled with 1.5 ml of filtered NSW. (2) Microdissection method: the intact statocysts

teased away from the dissected tissue slices were dissociated in sea water by placing the tissue into a slide-well on an inverted microscope and dissecting out various-sized groups of balancer cells/cilia using a scissor-like movement of two micromanipulated microneedles or by vacuuming surrounding tissue/cells away from groups of balancer cells/cilia using a suction-micropipette (2–10  $\mu\text{m}$  tip o.d.).

### *Photography*

Adult *M. leidyi* were photographed in an aquarium. Slide preparations of larvae, dissected adult statocysts and isolated balancer groups were photographed through a Zeiss microscope using differential-interference-contrast or phase optics on Kodak Tech Pan film or Kodak Gold Plus color film using an Olympus OM-2N camera with electronic flash.

### *Solutions*

NSW (31‰) was filtered through Whatman no.1 paper before use. Artificial sea water (ASW) consisted of (in  $\text{mmol l}^{-1}$ ): 423 NaCl, 9 KCl, 9  $\text{CaCl}_2$ , 23  $\text{MgCl}_2$ , 25  $\text{MgSO}_4$  and 2  $\text{NaHCO}_3$ , pH 8;  $\text{Ca}^{2+}$ -free ASW (CFASW) consisted of 437 NaCl, 9 KCl, 23  $\text{MgCl}_2$ , 25  $\text{MgSO}_4$ , 2  $\text{NaHCO}_3$  and 2 EGTA, pH 8. NSW solutions containing  $\text{Co}^{2+}$  (5, 10 or 20  $\text{mmol l}^{-1}$ ),  $\text{Ni}^{2+}$  (20 or 50  $\text{mmol l}^{-1}$ ) or  $\text{Mn}^{2+}$  (10  $\text{mmol l}^{-1}$ ) were prepared by addition of  $\text{CoCl}_2$ ,  $\text{NiCl}_2$  or  $\text{MnCl}_2$  (1  $\text{mol l}^{-1}$  stock solution in distilled water), adjusted to pH 8. NSW containing excess  $\text{Mg}^{2+}$  (332  $\text{mmol l}^{-1}$ ) consisted of isotonic 6.75%  $\text{MgCl}_2$ :NSW, 1:1, pH 8.

### *Horizontal microscopy*

Slide chambers containing *M. leidyi* larvae or adult statocysts were prepared according to the method of Tamm (1982) and placed on the vertically rotating stage of a horizontal microscope (Universal, Zeiss, Thornwood, NY, USA). Balancer cilia of larvae and adult statocysts were alternately deflected towards, and then away from, the center of the statocyst by rotating the vertical stage. Each slide chamber was successively rotated to two positions so that the oral–aboral axis of the specimen was horizontal (termed 90° or 270°) for 5–20 s at each position; this rotation procedure was repeated twice in various test solutions.

### *Electrical stimulation*

Slide chambers containing a *M. leidyi* larva or an adult *B. infundibulum* statocyst were prepared for use in the horizontal microscope. Two platinum wires (36 gauge) connected to a stimulator (SD-9, Grass Instruments, Quincy, MA, USA) were inserted under opposite sides of the coverslip of the slide chamber, approximately 5 mm from the specimen. Each slide chamber with a larva or adult statocyst was placed on the horizontal microscope and rotated first to 90° and then to 270°, pausing for 30 s at each orientation to note the sign of geotaxis. Horizontally oriented specimens were then electrically stimulated with bipolar pulse trains.

Some larvae that switched sign of geotaxis after a stimulation were given additional pulse-train stimulation

before their electrically switched sign reverted back to the original sign to compare the pulse-train strength required to switch from positive to negative and from negative to positive geotaxis. Other larvae and three *B. infundibulum* adults were not given additional pulse trains until their electrically switched sign reverted back to the original sign. This stimulation strategy may determine the duration of an electrically switched geotactic sign.

The larvae and adult statocysts were rotated and positioned (for 5–10 s) at both horizontal orientations each time their sign of geotaxis was electrically switched, unless the electrically switched sign reverted in less than 5 s.

#### *Adult geotaxis*

The geotaxis of several whole adult ctenophores was examined using a Lucite cteno-tilter (Tamm, 1980). Adult ctenophores (*M. leidy* and *P. pileus*) were continuously observed for 7–50 min, while their horizontal orientation was changed from 90° to 270° every 3–5 min by rotating the Sylgard disk to which they were pinned.

#### *Deflection of cilia of isolated balancer groups*

The cell bodies of isolated balancer groups lying in profile in Lucite slide-wells (1.5 ml) on the inverted microscope were secured to the bottom of the well with a microneedle. Balancer cilia were then deflected by one of two methods. (1) Cilia of enzyme-isolated *M. leidy* groups were deflected by water currents created by a suction-micropipette (10 µm tip o.d.) positioned near the ciliary tips. The sickle-shaped cilia were deflected towards their concave side until they became maximally excited or inhibited; they were then attracted away from their concave side until they exhibited the opposite maximal response. (2) Cilia of microdissection-isolated balancer groups (*M. leidy* or *B. infundibulum*) were deflected using a suction-micropipette (1 µm tip o.d.) attached directly to the ciliary tips or to lithocytes adhering to the ciliary tips. These cilia were deflected by different amounts towards their concave side and sometimes to one position towards their convex side. Cilia were deflected three times and held (3–10 s) at each different position in various solutions. Rare exceptions included those cilia that became detached from the suction-attached pipette (1 µm tip o.d.) at maximally deflected positions. When balancer cilia were being perfused with Co<sup>2+</sup> or CFASW, they were successively deflected after 3–4 min to test their mechanoresponse. If the mechanoresponse became noticeably inhibited, the cilia were deflected twice more in succession to determine the reproducibility of the inhibition and were then perfused with sea water.

#### *Perfusion*

Slide-chambers containing larvae or dissected adult statocysts were perfused (5–10 slide-chamber volumes) with various solutions by pipetting solution onto one open edge of the slide-chamber and then drawing it through the chamber by applying Whatman no.1 filter paper to the opposite open edge. The slide-chambers were removed from the vertical stage of

the horizontal microscope, and perfusion, requiring approximately 3 min, was carried out on the stage of an upright dissecting microscope.

Slide-wells (1.5 ml) containing isolated balancer groups were treated with various solutions by perfusing (2 ml min<sup>-1</sup>) solution into the wells through polyethylene tubing from a reservoir above the microscope stage.

#### *Local application of Ca<sup>2+</sup> solutions onto isolated balancer groups in Ca<sup>2+</sup>-free ASW containing high-[K<sup>+</sup>]*

Solutions either with or without Ca<sup>2+</sup> were applied locally by ionophoresis or pressure-application at the ciliary tips, ciliary bases or cell bases. Ionophoresis (50 V, d.c., 0.1–4 s) was from a pipette (1 µm tip o.d.) positioned approximately 5 µm from cilia/cells, according to methods described by Tamm (1988b). Pressure-application was by ejection of a solution from micropipettes (2.5–4 µm tip o.d.) positioned approximately 5 µm away from the cilia/cells. Solutions were ejected from the micropipette by blowing into a polyethylene tube connected to the Luer-port of the micropipette holder (ME2HS, WPI, Sarasota, FL, USA). A continuous stream of solution was ejected onto each site until an increase in ciliary beat frequency occurred or for 8–15 s. The force (i.e. pressure) of blowing by mouth, and therefore the ejected volume, was fairly uniform but was not quantified. Micropipettes were positioned using a hydraulic micromanipulator.

#### *Videomicroscopy*

Balancer cilia of larvae and adult statocysts in slide chambers on the horizontal microscope were imaged using a Zeiss phase-contrast 40× objective. Balancer cilia of isolated balancer groups in slide-wells on the inverted microscope were imaged using Zeiss differential-interference-contrast optics, with 16× or 40× objectives. Images were recorded with a CCD camera (BD-400, Panasonic, Japan) onto a video cassette recorder (VCR) using still-field playback mode (AG-7355, Panasonic, Japan; or TLC 2051, Gyr, Anaheim, CA, USA), and video fields were numbered (QSI-VFF 6030; QSI systems, Newton, MA, USA). Video monitor (model PVM-122, Sony, Japan) images were traced onto matt acetate sheets to compare the degree of balancer cilia deflection during experiments.

#### *Frequency analysis*

Balancer beat frequencies of 1 Hz or lower were measured with a manual counter from video tapes in real time, whereas higher frequencies were measured from video tapes in still-field playback mode. Positive geotaxis was scored if the upper balancers beat more rapidly than the lower ones; negative geotaxis was scored when the lower balancers beat more rapidly. In experiments where frequencies were not quantified, such as during most electrical stimulation experiments, the upper and lower balancer beat frequencies were scored as either + (greater than approximately 1–2 Hz) or – (less than approximately 1–2 Hz). For testing geotaxis of intact adults, the upper and lower comb rows were scored as + or – using similar criteria.

Beat frequency and the extent of deflection were measured for each deflection of the cilia in the various solutions by analyzing video tapes of the deflection experiments. Beat frequencies at the most excited position in identical solutions were averaged to obtain mean deflection-induced excitation for each solution. Mean deflection-induced frequency at the least excited position was also measured.

For perfusion experiments, the mean beat frequency in each solution was obtained by counting the number of balancer beats for each isolated group in each solution, then averaging the frequencies obtained in identical solutions. Results are presented as means  $\pm$  S.D.

## Results

### General morphology of larvae and dissected adult statocysts

Free-swimming *M. leidy* cydippid larvae resemble adult ctenophores of the order Cydippida, e.g. *Pleurobrachia pileus* (Tamm and Tamm, 1981). Larvae and adults have an aboral statocyst containing a ball-like statolith supported on the tips of four compound balancer cilia (Horridge, 1965; Tamm, 1980). Each balancer group in adult animals consists of several hundred cilia which beat as a unit. Each balancer is mechanically linked *via* two ciliary grooves to a pair of locomotory comb rows composed of ciliary comb plates (Tamm, 1973; Fig. 1). Three- to five-day-old larvae are approximately 0.5–1 mm in length and have a ciliary locomotory system that is proportionately large relative to body size (Fig. 2A,B).

Dissected adult statocysts (*M. leidy* and *P. pileus*) included the epithelial floor, adjacent mesoglea with muscle and nerve as well as remnants of the gastrovascular canal system (Fig. 2C,D).

### Geotaxis

When a ctenophore changes orientation with respect to gravity, the load of the statolith shifts and deflects the balancer cilia. Deflection is often towards or away from the center of the statocyst and may excite or inhibit the beating of balancers. The balancers act as pacemakers for the comb rows; deflection-induced excitatory and inhibitory frequency responses of balancer cilia on different sides of the statocyst are mechanically transmitted down the ciliary grooves to the comb rows, resulting in steering and geotaxis (Horridge, 1965; Tamm, 1973). The normal direction of the effective stroke of all the cilia in the system is towards the center of the statocyst, so ctenophores swim mouth first.

Ctenophore geotaxis (reviewed by Tamm, 1982) is the temporary tendency to orient vertically and swim upwards (negative geotaxis) or downwards (positive geotaxis). Horizontally oriented ctenophores with positive geotaxis exhibit faster beating of upper balancers and comb rows than of lower balancers and comb rows, so they turn downwards; conversely, ctenophores with negative geotaxis exhibit faster beating of lower balancers and comb rows than of upper ones, so they turn upwards. The difference in beat frequency between

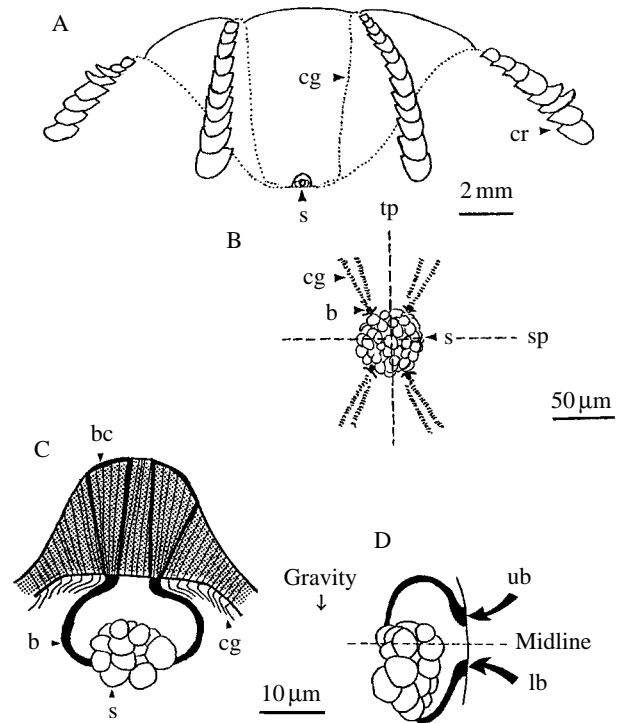


Fig. 1. Anatomy of the ciliary locomotory system of ctenophores. (A) The aboral end (side view) shows how the locomotory comb rows (cr, only four of the eight are shown) of an adult *Mnemiopsis leidy* are connected *via* ciliary grooves (cg) to the apical statocyst (s). (B) The adult statocyst (top view; modified from Tamm, 1982) showing the statolith (s), four balancers (b) and eight ciliary grooves (cg), two connected to each balancer. Dashed lines indicate the tentacular plane (tp) and the sagittal plane (sp). (C,D) *M. leidy* larvae statocyst shown at vertical orientation (mouth up, C) and horizontal orientation (D) within a slide chamber on the vertical stage of a horizontal microscope. (C) Larval statocyst (side view) shows balancer cilia groups (bc, only two shown) supporting a ball of lithocytes (statolith; s). The groups of balancer cilia are mechanically coupled to the ciliary grooves (cg). Four groups of compound balancer cilia, termed balancers (b), arise from four groups of 150–200 epithelial cells (one cilium per cell) that reside in the epithelial floor of the statocyst (bordered by heavy lines). (D) The aboral–oral axis (midline) is drawn to show how upper balancer cilia (ub) are pulled by the gravitational load of the statolith (s) towards the statocyst center (midline) whereas lower balancer cilia (lb) are pulled away from the midline. The direction of gravity is shown by an arrow. Balancers and statolith in C and D were traced (using matt acetate sheets) from video images displayed on a video monitor; epithelial floor and ciliary grooves in C were drawn afterwards approximately to scale.

upper and lower balancers becomes greater as the oral–aboral axis is rotated from vertical to horizontal (Tamm, 1980). All balancers and comb rows beat at the same frequency when the ctenophore is in a vertical position.

Whole *M. leidy* larvae and dissected adult statocysts (*M. leidy* and *P. pileus*) exhibited geotaxis when oriented horizontally on the vertical stage of the horizontal microscope. The gravitational load of the statolith bent the upper balancers

towards, and the lower balancers away from, the statocyst center, causing excitation of either the upper or lower balancers (Fig. 1D). Balancer image tracings were compared and indicated that gravity-induced balancer deflection was similar at 90° and at 270° (not shown). The difference between upper and lower balancer frequency responses was also similar at 90° and at 270°. The difference in mean beat frequency between excited and non-excited balancers of larvae was approximately 15 Hz (Fig. 3).

The sign of geotaxis was positive for 95% of 3- to 5-day-old *M. leidy* larvae tested in NSW in the horizontal microscope ( $N=92$ ), but positive for only 27% of dissected adult statocysts (28 *P. pileus* and 8 *M. leidy*) tested in NSW or ASW in the horizontal microscope. The sign of geotaxis was positive for only 11% (4 of 35) of intact adult *P. pileus* and 19% (3 of 16)

of intact adult *M. leidy* tested in NSW or ASW using the cteno-tilter apparatus.

*Influx of Ca<sup>2+</sup> is required for deflection-induced excitation of balancer cilia*

*Effect of inorganic Ca<sup>2+</sup> channel inhibitors on gravity-induced excitation of balancer cilia*

The gravity-induced excitatory responses of balancers, regardless of geotactic sign, were reversibly inhibited in *M. leidy* larvae by treatment with Co<sup>2+</sup> (5 mmol l<sup>-1</sup>, 5 of 5 larvae; 20 mmol l<sup>-1</sup>, 21 of 24 larvae; 3–6 min of treatment; Fig. 3), Mn<sup>2+</sup> (10 mmol l<sup>-1</sup>, 10 of 10 larvae; 3–8 min of treatment), Ni<sup>2+</sup> (20 mmol l<sup>-1</sup>, 7 of 7 larvae; 50 mmol l<sup>-1</sup>, 3 of 3 larvae; 10–15 min of treatment) and Mg<sup>2+</sup> (332 mmol l<sup>-1</sup>, 36 of 46 larvae; 3–8 min of treatment). The upper and lower balancers beat slowly

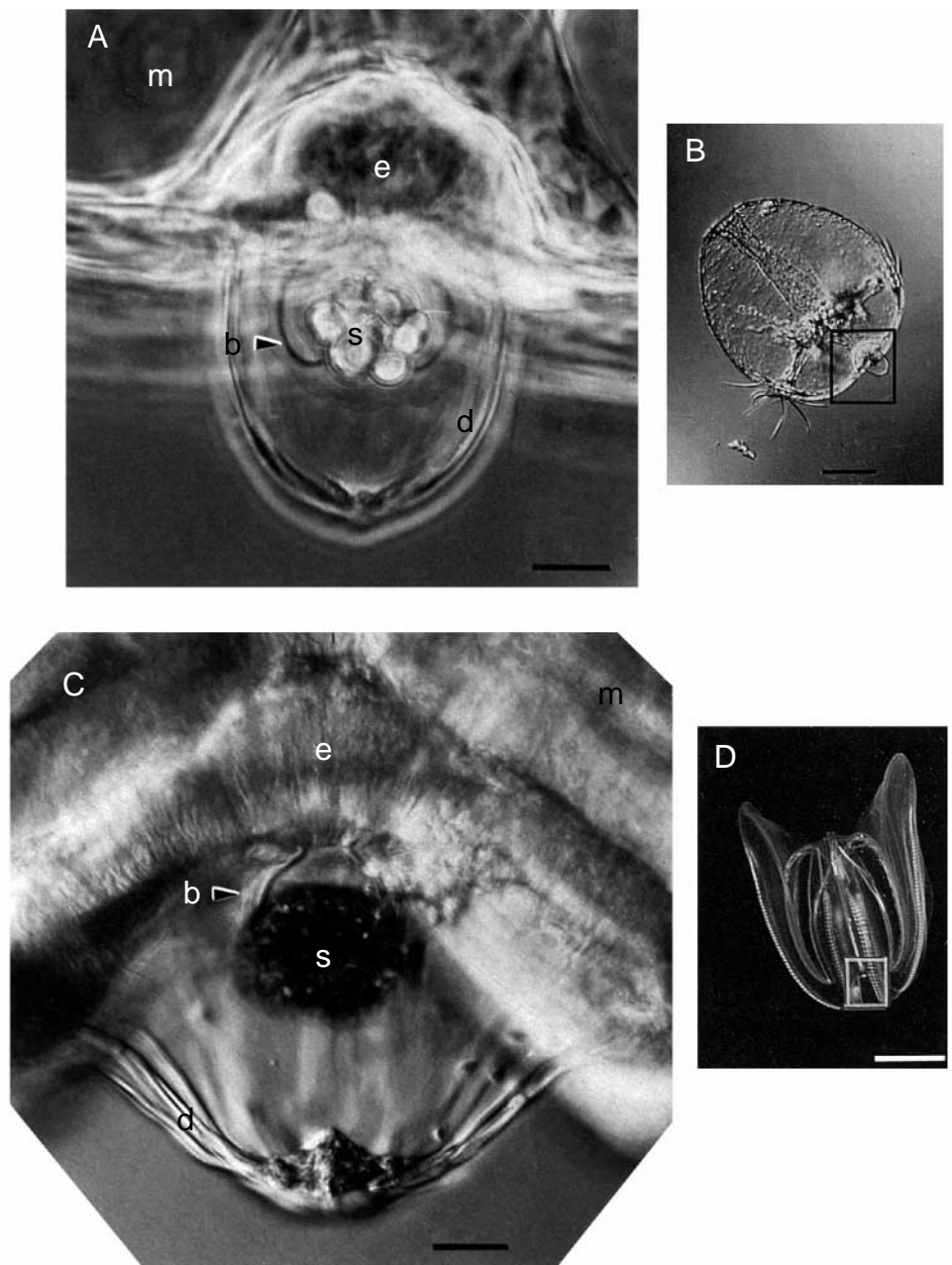


Fig. 2. Photomicrographs of statocysts from (A) a whole *Mnemiopsis leidy* larva (40× objective, phase contrast) and (C) a dissected adult *M. leidy*. Larva (3–5 days old) usually possessed approximately 10 lithocytes and balancers approximately 15 μm long. Adult statocysts possessed hundreds of lithocytes and balancers approximately 50 μm long. The muscle and gastrovascular system of the adult dissected statocysts are not shown. (B) Photomicrograph of a whole *M. leidy* larva. (D) Photograph of an adult *M. leidy*. The statocysts of in B and D are enclosed in a box. b, balancer cilia; s, statolith; m, mesoglea; d, dome cilia; e, epithelial floor. Scale bars, A, 10 μm; B, 250 μm; C, 25 μm; D, 1 cm.

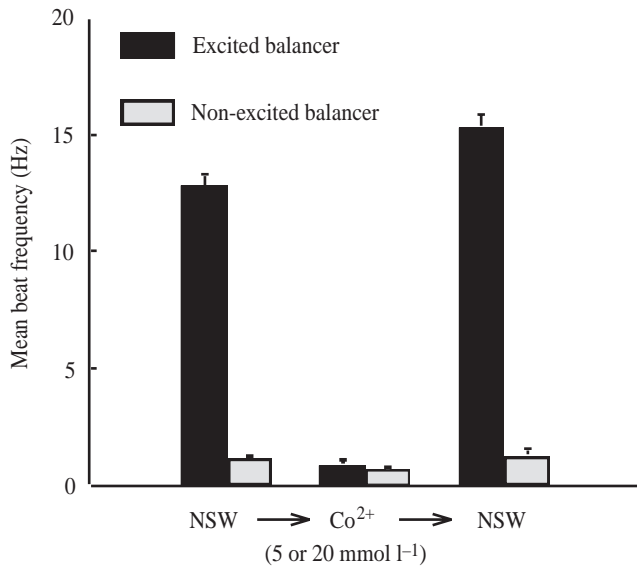


Fig. 3. Effect of a Ca<sup>2+</sup> channel inhibitor (Co<sup>2+</sup>) on gravity-induced excitation of balancer cilia in *Mnemiopsis leidyi* larvae. Bars indicate the mean excited (black bars) and non-excited (grey bars) balancer ciliary beat frequency, regardless of geotactic sign, during gravity-induced deflection in different solutions on the vertical stage of the horizontal microscope. Beat frequencies were determined for upper and lower balancer cilia of horizontally oriented larvae exposed to a succession of solutions starting with natural sea water (NSW), then to 5 mmol l<sup>-1</sup> Co<sup>2+</sup> ( $N=5$ ) or 20 mmol l<sup>-1</sup> Co<sup>2+</sup> in NSW ( $N=1$ ) (results combined) and finally to NSW again (see labels on x-axis). Error bars indicate standard deviation.

(1–2 Hz) at both 90° and 270° in these solutions. The slow beat frequency of upper and lower balancers in inhibitor solution matched the slow beat frequency of unexcited balancers in NSW or ASW. Deflection-induced excitation of larvae was reduced by 90% in Co<sup>2+</sup> (5 mmol l<sup>-1</sup>;  $N=5$ ), by 80% in Mg<sup>2+</sup> (332 mmol l<sup>-1</sup>;  $N=4$ ), by 92% in Mn<sup>2+</sup> (10 mmol l<sup>-1</sup>;  $N=10$ ) and by 97% in Ni<sup>2+</sup> (20 mmol l<sup>-1</sup>;  $N=7$ ) in NSW or ASW. The sign of geotaxis remained the same after recovery from inhibition, except for three larvae tested with 20 mmol l<sup>-1</sup> Co<sup>2+</sup> and 10 larvae tested in 332 mmol l<sup>-1</sup> Mg<sup>2+</sup>. Paired *t*-tests indicate that beat frequencies in sea water before and after inhibitor treatment were not significantly different ( $P<0.05$ ). Only three of the first 26 larvae tested switched signs of geotaxis during initial perfusion with NSW. Therefore, testing the effects of NSW perfusion on geotaxis of larvae was discontinued after this twenty-sixth larva.

The upper and lower balancer cilia of horizontally oriented larvae beat at 1–2 Hz after perfusion of Ca<sup>2+</sup>-free ASW (CFASW) (two larvae tested), but then after less than 1 min the larvae began to shrink and dissociate into detached cells. Profile views of the balancer cilia were lost when shrinkage occurred.

The deflection-induced excitation of balancers in dissected adult *M. leidyi* and *P. pileus* statocysts was reversibly inhibited by treatment for 3–10 min with Co<sup>2+</sup> (5 mmol l<sup>-1</sup>, 6 of 9 *P. pileus*; 10 mmol l<sup>-1</sup>, 9 of 11 *P. pileus* and 5 of 7 *M. leidyi*) and

Ni<sup>2+</sup> (50 mmol l<sup>-1</sup>, 2 of 6 *P. pileus*) in NSW, regardless of geotactic sign. Deflection-induced excitation was reduced by 85% in 10 mmol l<sup>-1</sup> Co<sup>2+</sup> (*M. leidyi*;  $N=6$ ) and 90% in 20 mmol l<sup>-1</sup> Co<sup>2+</sup> (*P. pileus*;  $N=6$ ). The balancer beat frequency of dissected adult statocysts was slow (1–2 Hz) in the inhibitor solution, regardless of the balancer position.

Adult balancer cilia in *M. leidyi* and *P. pileus* usually recovered their geotactic response within 10–15 min after the inhibitor solution had been replaced with NSW, except for two *P. pileus* treated with 10 mmol l<sup>-1</sup> Co<sup>2+</sup> and one treated with 50 mmol l<sup>-1</sup> Ni<sup>2+</sup>, which failed to recover a geotactic response. Geotactic responses were still present after testing for 15–20 min in inhibitors for 22% of *P. pileus* (2 of 9; 5 mmol l<sup>-1</sup> Co<sup>2+</sup>), 50% of *P. pileus* (3 of 6; 50 mmol l<sup>-1</sup> Ni<sup>2+</sup>) and 29% of *M. leidyi* (2 of 7; 10 mmol l<sup>-1</sup> Co<sup>2+</sup>).

Perfusion of NSW control solution was tested on each adult dissected statocyst prior to testing the inhibitors. The beat frequency and geotactic response of the adult statocysts were not affected by NSW perfusion, except that two *P. pileus* statocysts switched sign of geotaxis from negative to positive after perfusion of the NSW control and before perfusion of the Co<sup>2+</sup> solution.

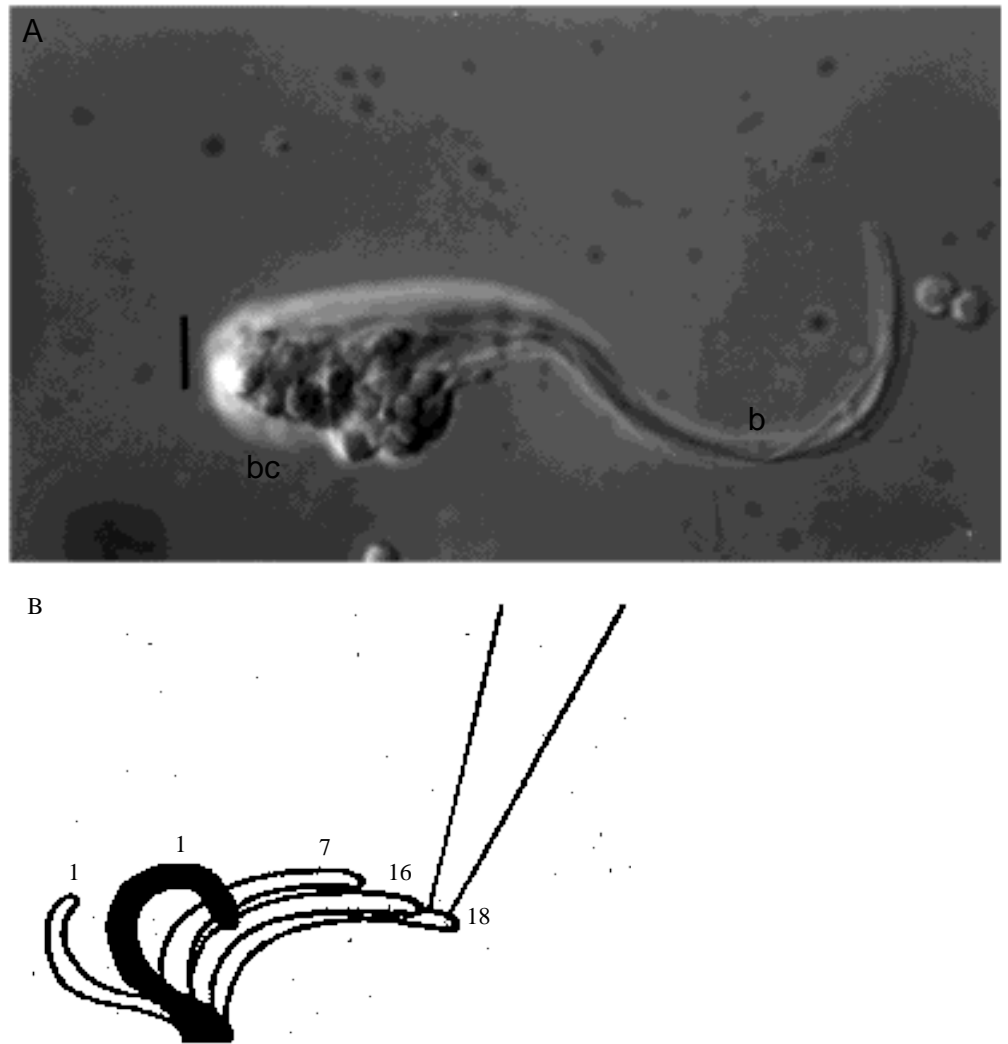
#### General morphology of isolated balancer groups

The apparent influx of Ca<sup>2+</sup> required for gravity-induced excitation of balancer cilia of whole larvae or dissected adult statocysts may occur at locations on the balancer cells or cilia or at synaptic terminals of putative regulatory neurons that synapse onto the balancer cells. Groups of balancer cells bearing balancer cilia were isolated from the body and nervous system and used to test whether deflection-induced excitation could still be inhibited by Ca<sup>2+</sup> channel inhibitors.

*M. leidyi* and *B. infundibulum* balancer groups consisted of numerous long thin cell bodies (approximately 50 μm long) that were tapered towards the apical cilia (Fig. 4A). The groups were 20–60 μm wide at the basal end and included only a subset of the complete balancer group of cells/cilia. The compound balancer cilia retained their sickle-shape and were approximately 50 μm long. Larger balancer groups often possessed ciliary groove cilia (15 μm long) and short balancer cilia that overlapped the groove cilia. The balancer and ciliary groove cilia beat with the same frequency. The balancer cilia of all enzyme-isolated groups were free of lithocytes, but microdissection-isolated groups had two or more lithocytes adhering to their tips.

The cilia of isolated balancer groups, like intact balancer cilia, increased their beat frequency (were excited) when deflected either towards or away from their concave side and did not increase their beat frequency when deflected in the opposite direction. The cilia of isolated groups beat with the effective stroke towards the concave side of the cilia. Microdissection-isolated balancer cilia of *B. infundibulum* (Fig. 4B) or *M. leidyi* (Fig. 5G,J) exhibited graded excitatory responses to deflection in NSW. Beat frequency of balancer groups in NSW increased in a linear fashion for increasing degrees of deflection, except that two *Mnemiopsis* balancer

Fig. 4. (A) Enzyme-isolated balancer group from *Mnemiopsis leidyi*. The cells of these balancer groups held tightly together and had rounded, grape-like basal ends (bc). Most balancer cilia (b) retained their sickle-shape, but some were frayed from the compound group. There were no ciliary groove cilia or lithocytes on these small groups. Scale bar, 10  $\mu\text{m}$ . (B) A composite of superimposed images of a microdissection-isolated group of *Bolinopsis infundibulum* balancer cilia at different positions of deflection. The shaded cilium is undeflected and unshaded cilia are being deflected by a suction-micropipette attached to the ciliary tips; the micropipette is shown attached to the most deflected cilium. Cilia exhibited a graded increase in deflection-induced excitation. Ciliary beat frequency (in Hz) for each position of deflection is given above each cilium.



groups (Fig. 5G,J) exhibited a reduced beat frequency at maximum deflection in the excitatory direction. Some balancer groups were positively geotactic and increased ciliary frequency when cilia were deflected towards their concave side (eight of 19 enzyme-isolated; six of eight microdissection-isolated); the remainder increased their beat frequency for convex-directed deflection, i.e. exhibited negative geotaxis. No isolated balancer group changed sign of directional sensitivity.

#### *Effect of $\text{Co}^{2+}$ and CFASW on deflection-induced excitation of isolated balancer group cilia*

Deflection-induced excitation of *M. leidyi* balancer groups (five enzyme-isolated and two microdissection-isolated) was reversibly inhibited after 6–20 min in 10 or 20  $\text{mmol l}^{-1} \text{Co}^{2+}$  in NSW (Fig. 5B,H,K). The mean beat frequency during deflection (9.8 Hz) was reduced by 95% in  $\text{Co}^{2+}$  solution among reversibly inhibited groups ( $N=7$ ). The mean time of  $\text{Co}^{2+}$  treatment was  $13 \pm 5.3$  min (mean  $\pm$  s.d.,  $N=7$ ). Deflection-induced excitation of cilia of seven other balancer groups was irreversibly inhibited by 20  $\text{mmol l}^{-1} \text{Co}^{2+}$  treatment (10–20 min) even after a 40–60 min NSW wash-out perfusion. The deflection-induced

excitation of balancer cilia of three other microdissected groups was not inhibited by 20  $\text{mmol l}^{-1} \text{Co}^{2+}$  treatment for 45 min; they responded to deflection as they did in NSW.

Deflection-induced excitation of four balancer groups (enzyme-isolated) was reversibly inhibited by treatment with CFASW for 11–35 min (mean exposure time  $24 \pm 9.9$  min; Fig. 5E). The mean beat frequency during deflection (8.1 Hz) among reversibly inhibited groups was reduced by 97% ( $N=4$  *M. leidyi*) in CFASW. Deflection-induced excitation of three other balancer groups (enzyme-isolated) was irreversibly inhibited by CFASW treatment (15–35 min), even after a 50–60 min ASW wash-out perfusion. Deflection-induced excitation of five other balancer groups (enzyme-isolated) was not inhibited by CFASW treatment (30–45 min).

The deflection-induced excitation of isolated groups that were inhibited by  $\text{Co}^{2+}$  or CFASW was gradually reduced (not quantified) with successive test deflections until excitation was greatly attenuated or abolished. The inhibition in  $\text{Co}^{2+}$  or CFASW occurred at all positions of deflection tested. The excitatory responses gradually resumed in NSW or ASW for many groups (paired *t*-tests indicate that beat frequencies in sea

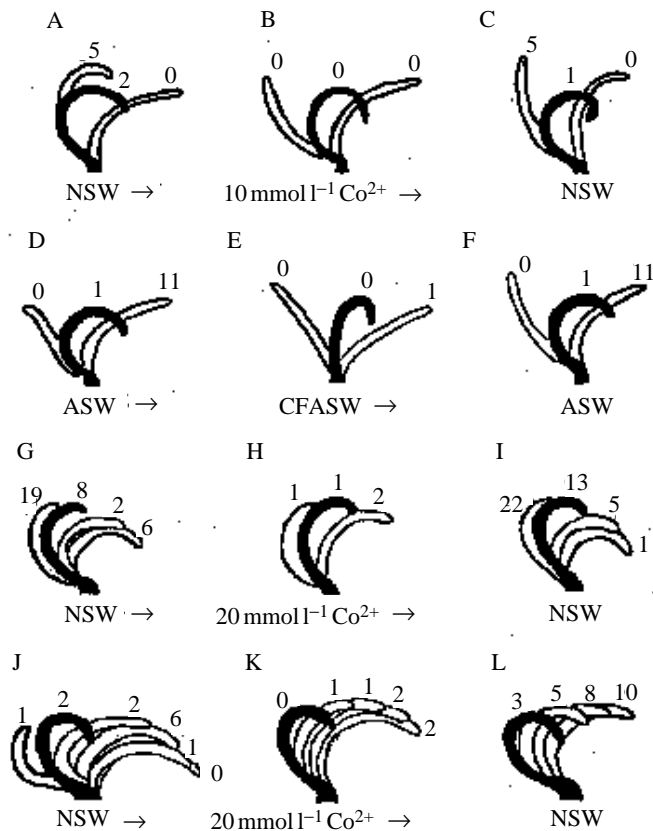


Fig. 5. (A–L) Composites of superimposed tracings of an isolated group of balancer cilia at different positions. Shaded cilia are undeflected and unshaded cilia are deflected. Balancer groups in the same row represent the same group after they had been perfused with various solutions (given below each composite). *Mnemiopsis leidyi* balancer cilia, enzyme-isolated (A, D), were deflected to positions on each side of the undeflected balancer position by suction from a suction-micropipette near the ciliary tips. A–C correspond to negative geotaxis, and D–F correspond to positive geotaxis. Cilia of *M. leidyi* (G–I and J–L) balancer groups, microdissected, were positioned with a micropipette attached to their lithocytes or cilia tips (lithocytes not shown). G–I correspond to negative geotaxis and J–L correspond to positive geotaxis. Balancer beat frequencies were determined for each deflected position in the different solutions and are given above each position (in Hz). Composite figures of balancer cilia were traced from a monitor display onto matt acetate sheets. CFASW,  $\text{Ca}^{2+}$ -free artificial sea water; ASW, artificial sea water;  $\text{Co}^{2+}$  was applied in natural sea water (NSW).

water before and after inhibitor treatments were not significantly different;  $P < 0.05$ ). The irreversibly inhibited groups continued to beat slowly, or not at all, during deflection. Each balancer group exhibited the same sign of directional sensitivity in sea water before and after treatment with  $\text{Co}^{2+}$  in NSW or ASW or with CFASW (only groups exhibiting reversible inhibition are considered). Some balancer groups not inhibited by  $\text{Co}^{2+}$  or CFASW nonetheless exhibited slightly diminished excitation (not quantified), but most non-inhibited groups exhibited similar excitation in NSW and in  $\text{Co}^{2+}$  or CFASW.

Eleven enzyme-isolated and two microdissection-isolated *M. leidyi* balancer groups beat in NSW (1–10 Hz) but did not change their ciliary beat frequency when deflected in either direction. Several other isolated groups (not quantified) did not beat either with or without ciliary deflection. These isolated balancer groups were not used for experiments.

#### *Ca<sup>2+</sup>-dependent depolarization activates balancer cilia without deflection*

##### *Effect of high-[K<sup>+</sup>] sea water on beat frequency of isolated, undeflected balancer cilia*

The effects of chemical depolarization treatment with high  $[\text{K}^+]$  sea water, with and without  $\text{Ca}^{2+}$ , were tested on the beat frequency of balancer cilia that were not mechanically stimulated.

Microdissection-isolated balancer cilia groups from *M. leidyi* and *B. infundibulum* in slide-wells were perfused with one of two series of solutions: with NSW, ASW, CFASW, CFASW- $\text{K}^+$  (50  $\text{mmol l}^{-1}$   $\text{K}^+$  in CFASW), ASW, ASW- $\text{K}^+$  (50  $\text{mmol l}^{-1}$   $\text{K}^+$  in ASW) and ASW, or with NSW, NSW- $\text{K}^+$  (50  $\text{mmol l}^{-1}$   $\text{K}^+$  in NSW), NSW, NSW- $\text{Co}^{2+}$  (20  $\text{mmol l}^{-1}$   $\text{Co}^{2+}$  in NSW), NSW- $\text{K}^+$ - $\text{Co}^{2+}$  (50  $\text{mmol l}^{-1}$   $\text{K}^+$ , 20  $\text{mmol l}^{-1}$   $\text{Co}^{2+}$  in NSW) and NSW. Each solution was perfused into the slide-well for 1–2 min and allowed to remain for 1–2 min for beat frequency recording before perfusing the next solution. Exceptions occurred when some activated balancer cilia groups required an extra 2–3 min of seawater perfusion before slowing down. The average beat frequency of undeflected balancer cilia from five *M. leidyi* and seven *B. infundibulum* balancer groups increased from a mean of  $1.2 \pm 0.7$  Hz in ASW (or NSW) to  $15.4 \pm 2.8$  Hz after perfusion with ASW- $\text{K}^+$  (or NSW- $\text{K}^+$ ) for a mean period of  $52 \pm 20$  s (means  $\pm$  s.d., Fig. 6). The activation continued for  $2.8 \pm 1.9$  min (mean  $\pm$  s.d.). The isolated groups activated by NSW- $\text{K}^+$  or ASW- $\text{K}^+$  usually slowed to their pre-treatment frequency within 2 min after reperfusion with ASW or NSW. However, four activated groups remained activated for 3–5 min in ASW before slowing to their original pretreatment frequency.

Cilia of balancer groups were usually not activated by exposure to NSW, ASW, CFASW or CFASW- $\text{K}^+$  (five *M. leidyi*, four *B. infundibulum*; Fig. 6A), or by exposure to NSW- $\text{Co}^{2+}$  or NSW- $\text{K}^+$ - $\text{Co}^{2+}$  (three *B. infundibulum* groups; Fig. 6B). Balancer cilia of three *M. leidyi* groups were twice activated by ASW- $\text{K}^+$  but not by other solutions when treated twice with the series NSW, ASW, CFASW, CFASW- $\text{K}^+$ , ASW, ASW- $\text{K}^+$  and ASW. Unexpectedly, one isolated *B. infundibulum* group became spontaneously activated in CFASW- $\text{K}^+$ , another in NSW- $\text{Co}^{2+}$  and a third in ASW (after CFASW- $\text{K}^+$  treatment). The beat frequency of these spontaneously activated balancer cilia increased from  $0.9 \pm 0.8$  Hz to  $12.6 \pm 0.3$  Hz (means  $\pm$  s.d.) and the activation lasted for 30–60 s before they slowed to their pretreatment frequency.

##### *Effect of local application of Ca<sup>2+</sup> onto isolated, undeflected ciliary groups bathed in high-[K<sup>+</sup>] CFASW*

$\text{Ca}^{2+}$  was applied locally onto depolarized, isolated balancer groups to determine the location of the putative  $\text{Ca}^{2+}$  influx.



*M. leidyi* balancer groups (microdissection-isolated) in slide-wells were sequentially perfused with ASW, CFASW and then CFASW-K<sup>+</sup> (50 mmol l<sup>-1</sup> K<sup>+</sup>) for 5 min each. Balancer groups were not excited by depolarization in the absence of Ca<sup>2+</sup> and beat at 1–2 Hz. The groups were arranged with microneedles so that the long axes of their cilia were perpendicular to the perfusion flow and so that locally applied solutions flowed away from the balancer group. Beat frequency was measured for 3 s just before and for 3 s just after termination of the local application.

Balancer cilia of four isolated groups increased their beat frequency by an average of 11.1 Hz (from an average of 0.7 Hz to an average of 11.8 Hz) when given local pressure-applications of ASW-K<sup>+</sup> containing 10 mmol l<sup>-1</sup> Ca<sup>2+</sup> (balancers 1–4, Table 1; Fig. 7) onto the bases of their cilia. In contrast, cilia from these groups (balancers 1–4) increased their beat frequency by an average of only 0.2 Hz when given pressure-application on their cell bases. Cilia from three of the above balancer groups (balancers 2–4, Table 1) increased their beat frequency by an average of only 1.1 Hz when given

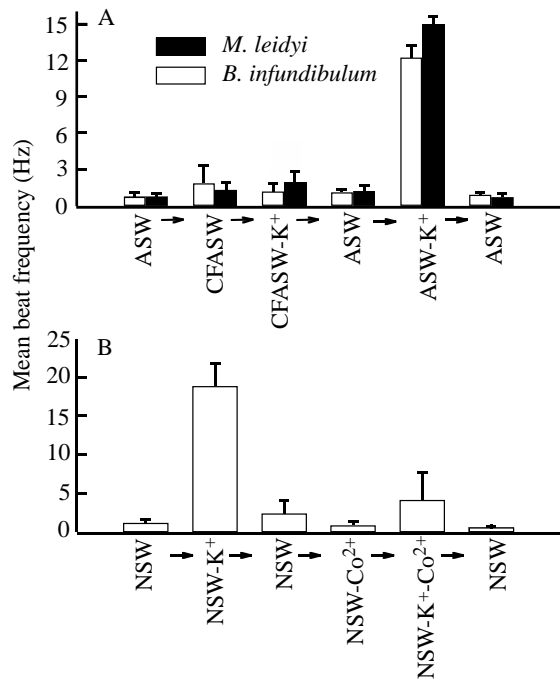


Fig. 6. Depolarization-activated beating in microdissection-isolated *Mnemiopsis leidy* and *Bolinopsis infundibulum* balancer cilia requires Ca<sup>2+</sup>. (A,B) Each set of bars shows mean beat frequencies of balancer groups perfused with a sequence of solutions. The solutions are listed below each bar (see text for abbreviations). Each solution was perfused for 1–2 min (see text for exceptions) and perfusion was then turned off for 1–2 min to determine beat frequency before starting perfusion of the next solution. The total number of balancer beats was counted for each balancer group in each solution while the perfusion was off. Each bar represents the mean frequency of all the balancer groups tested in a particular solution. (A) Black bars represent data from five *M. leidy*, isolated samples, white bars represent data from four *B. infundibulum*. (B) Columns represent three *B. infundibulum*. Error bars represent standard deviation. See text for details of solutions.

pressure-application on their ciliary tips. The lack of activation after application of ASW-K<sup>+</sup> (10 mmol l<sup>-1</sup> Ca<sup>2+</sup>) to the ciliary tips and cell bases occurred even though the solution was applied for longer at these two locations than at the ciliary bases. For one other isolated balancer group, the differences in beat frequency between balancer cilia before and after pressure-application of ASW onto the ciliary tips, ciliary bases and cell bases were 0, 13.1 and 0.6 Hz, respectively.

The balancer cilia of three groups (balancers 5–7, Table 1) increased their beat frequency by an average of 9.8 Hz (from an average of 1.8 Hz to an average of 11.6 Hz) when stimulated by ionophoresis with 1 mol l<sup>-1</sup> Ca<sup>2+</sup> onto their ciliary bases. The beat frequency of the same three groups of cilia increased by an average of only 0.1 Hz when stimulated by ionophoresis

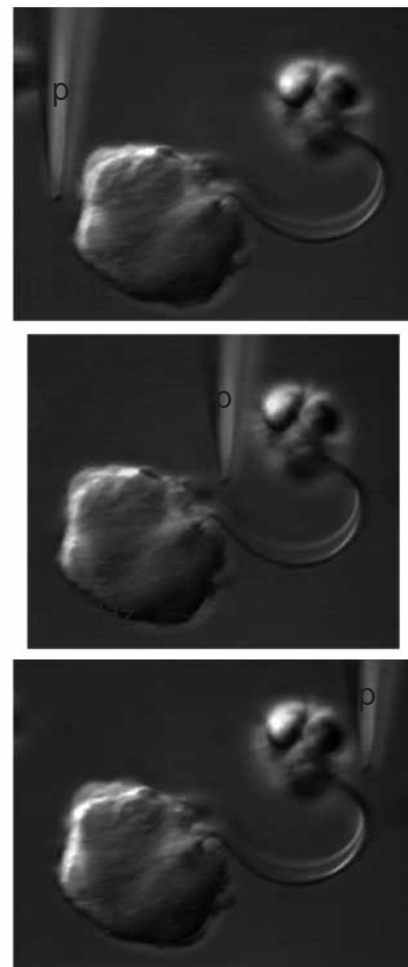


Fig. 7. Local application of ASW-K<sup>+</sup> onto cell bases (A), ciliary bases (B) and ciliary tips (C) of a *Mnemiopsis leidy* balancer group. The solutions were pressure-applied using a micropipette (P) filled with ASW-K<sup>+</sup> (containing 10 mmol l<sup>-1</sup> Ca<sup>2+</sup>, see text for other components). The resulting frequencies after terminating application of ASW-K<sup>+</sup> were 0.2 Hz (A), 10.7 Hz (B) and 1.0 Hz (C). These balancer group images (balancer 3, Table 1) were obtained using differential interference contrast (40× objective), recorded on VCR, contrast-enhanced digitally using Image-1 software (Image1, WestChester, PA, USA) and printed on a video printer (courtesy of MBL Graphic Arts, Woods Hole, MA, USA). Scale bar, 6 μm.

Table 1. Differences in beat frequency between balancer cilia before and after local application of test solutions

Pipette solution	Target	Balancer cell group beat frequency (Hz)						
		Pressure-application				Ionophoresis		
		1	2	3	4	5	6	7
CFASW-K <sup>+</sup>	Ciliary base	0.6 (5)	–	–0.3 (15)	–0.2 (10)	–	–	–
	Cell base	0.0 (5)	–	0.5 (11)	0.0 (8)	–	–	–
	Ciliary tip	–	0.3 (8)	1.0 (10)	2.1 (8)	–	–	–
ASW-K <sup>+</sup>	Ciliary base	11.8 (5)	14.6 (8)	10.7 (4)	7.4 (2)	–	0.1 (0.1)	–
	Cell base	–0.3 (5)	0.4 (9)	0.2 (10)	0.6 (6)	–	0.0 (0.1)	–
1 mol l <sup>–1</sup> Ca <sup>2+</sup>	Ciliary base	–	–	–	–	13.1 (2)	7.3 (0.1)	9.2 (0.1)
	Cell base	–	–	–	–	–0.2 (4)	–0.2 (0.1)	0.5 (0.1)

Values in parentheses are the duration of application (in s).

Groups of balancer cells with cilia were isolated by microdissection and then bathed in CFASW-K<sup>+</sup> (see Materials and methods). The groups were then given local applications of various solutions using either pressure-application or ionophoresis (50 V d.c., normal polarity). The targets of local application are shown in Fig. 7.

Beat frequencies were measured just before initiation of the application and just after termination of the application. The frequency before application was subtracted from the frequency after application. The combined mean balancer beat frequency for all groups before application was 1.2±1.1 Hz (mean ± s.d.; N=7; range 0–5 Hz).

Dashes indicate that experiments were not carried out.

on their cell bases. The beat frequency did not increase greatly when the ciliary base of one group (balancer 6, Table 1) was given a brief ionophoretic pulse (0.1 ms) of ASW-K<sup>+</sup> (which contains only 10 mmol l<sup>–1</sup> Ca<sup>2+</sup>).

The increase in beat frequency of each balancer usually occurred after applications ranging from 0.1 ms of ionophoresis (balancers 6 and 7, Table 1) to 15 s of pressure-application (balancer 3, Table 1). These increases were often preceded by quivering (low-amplitude, high-frequency vibrations) of the cilia at the base for a brief period before high-frequency beating began. Maximum beat frequency was attained within approximately 3 s after activation began. Local application was terminated when a frequency greater than approximately 5 Hz was reached. The activation (high-frequency beating) continued for an average of 6.2±5.4 s (mean ± s.d., range 2.6–17 s, N=6) for balancer groups treated with 1 mol l<sup>–1</sup> Ca<sup>2+</sup> solution and for an average of 2.9±1.0 s (mean ± s.d., range 2.1–4.0 s, N=4) for groups treated with ASW-K<sup>+</sup>.

No noticeable changes in ciliary beat frequency were seen for groups that were given pressure-applications of CFASW (balancers 1, 3, 4; Table 1) onto the ciliary tips or ciliary bases. Ionophoresis of 1 mol l<sup>–1</sup> Na<sup>+</sup> solution onto the ciliary tips, ciliary bases or cell bases did not cause noticeable excitation (results not shown). Na<sup>+</sup> and CFASW were used as control solutions to test whether the ciliary activation depended on Ca<sup>2+</sup>. Balancer cilia beat frequency did not change noticeably when groups (balancer 5–7) were given applications on their ciliary bases or cell bases by ionophoresis, in which the inside of the pipette was negative (not ejecting cations, including Ca<sup>2+</sup>; results not shown).

#### Electrical stimulation switches the sign of geotaxis

Effect of electrical stimulation on switching geotactic sign in *M. leidy* larvae and *B. infundibulum* adult statocysts

Electrical pulse-train stimulation was used to switch the sign

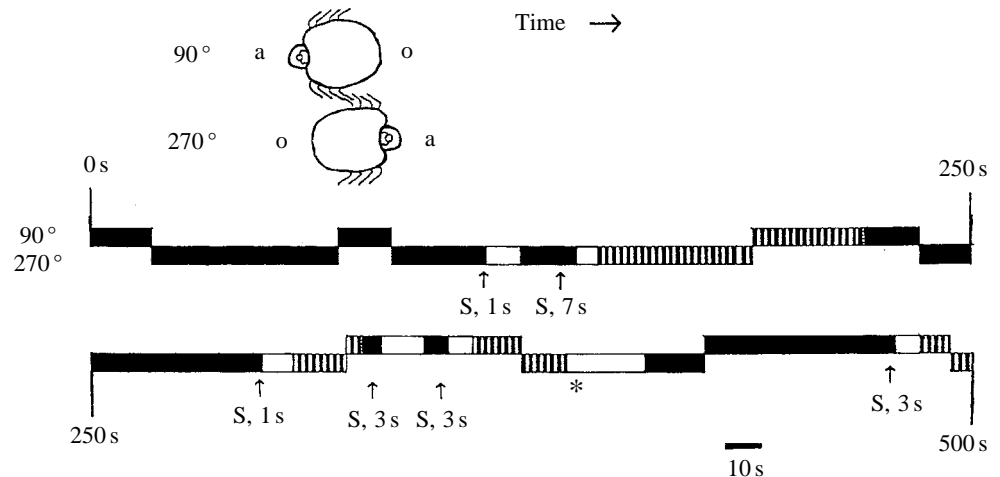
of geotaxis in whole larvae and in dissected adult statocysts. The characteristics of the stimulating bipolar-pulse trains were varied by changing the voltage (10, 20, 40 or 60 V), the frequency (5, 10 or 20 Hz) or the pulse-train duration (0.1–13 s). Pulse duration was constant (10 ms). Pulse trains were given 4–60 s apart. Pulse trains of electrical stimulation switched the sign of geotaxis in 18 of 25 *M. leidy* larvae that were horizontally oriented in slide chambers on the horizontal microscope. Pulse trains of 20–40 V (10 or 20 Hz, 10 ms, for 0.10–13 s) were commonly used to switch the geotactic sign because application of a higher voltage resulted in large muscular contractions that interfered with observation (see below). Geotactic sign was switched by 10 or 20 V pulse trains (10 or 20 Hz, for 0.1–8 s) in one of three attempts, and by 40 or 60 V pulse trains (10 Hz, for 0.1 s) in two of three attempts. One larva switched the sign of geotaxis four times after each of four pulse trains of 40 V, 10 Hz, 10 ms (Fig. 8).

Twenty-three of 25 larvae initially showed positive geotaxis in the horizontal microscope. The two larvae that initially showed negative geotaxis switched sign upon pulse-train stimulation. Geotactic sign was electrically switched back from negative to positive in six larvae that exhibited electrically switched negative geotaxis. The pulse-train strength required to switch from positive to negative or from negative to positive geotaxis was similar.

The mean beat frequency of the excited balancers of three representative larvae was 17±1.6 Hz (mean ± s.d.) and the mean frequency of non-excited balancers was 2±1.6 Hz before electrical stimulation. A few seconds after electrical stimulation (40 V, 10 Hz, 10 ms, 0.1–3 s), the mean beat frequency of the excited balancers was 2±0.4 Hz and the mean frequency of the non-excited balancers was 18±2.9 Hz. The other 15 larvae that switched the sign of geotaxis after electrical stimulation showed qualitatively similar results detected visually.

Electrically induced switches in sign began 0–21 s after the

Fig. 8. This time-line (bar) shows the geotactic sign of a *Mnemiopsis leidyi* larva on the vertical stage of a horizontal microscope while it was stimulated with electric pulse-trains. Beat frequencies of the upper and lower balancers were noted during the experiment. Periods of positive geotaxis are indicated by filled bars, periods of negative geotaxis by hatched bars, and periods without geotaxis (both balancers either fast or slow) by open bars. The raised portions of the time-line indicate periods when the larva was oriented horizontally at 90° (see drawings of larvae above the bar), and the lowered parts of the time-line indicate periods at 270° orientation. The onset of pulse-train stimulation is marked by arrows (S) and pulse-train duration (in s) is given. All pulse-train parameters were 40 V, 10 Hz, 10 ms for 0.1 s. The asterisk marks a spontaneous period of no geotaxis. a, aboral; o, oral.



termination of a pulse-train ( $7.2 \pm 6$  s, mean  $\pm$  s.d.,  $N=37$ ). Geotactic responses were abolished during this pre-switch period. The upper and lower balancers of each larva beat at the same frequency (fast or slow) or were both arrested during the pre-switch period. These non-geotactic beating behaviors were not analyzed further. The electrically induced switches in sign lasted 3–128 s ( $40 \pm 29$  s, mean  $\pm$  s.d.,  $N=37$ ) and occurred at both horizontal orientations (90 and 270°) before the larvae reverted back to their original sign. Exceptions were three larvae that reverted within 5 s after switching sign.

Seven larvae did not switch their sign after any pulse-train stimulation (40–80 V, 10–20 Hz, 10 ms pulses, 0.1–13 s pulse-train duration). Instead, the upper and lower balancers usually beat at the same frequency (fast or slow) or were both arrested for a few seconds after the stimulus and then reverted back to their original sign before stimulation.

Spontaneous switches in geotactic sign were rare because the larvae selected for pulse-train tests exhibited a consistent geotactic sign for 1–5 min before stimulation. Nonetheless, four larvae spontaneously switched signs either two or four times during testing. These spontaneous switches in sign from positive to negative and then back occurred either once or twice during testing and did not correlate with stimulation.

Brief muscular retraction (<1 s) of the larval statocyst usually occurred after pulse trains of 40 V (or higher), 10 Hz, 10 ms, for 0.1–8 s. Retraction, a common response to touch, causes temporary invagination of the statocyst floor into the body and slightly deforms the statocyst. Pulse trains of lower strength were accompanied only by muscular twitches and no retraction, but nevertheless induced switches in geotactic sign. There was no correlation between muscular retraction and switches in geotactic sign for larvae given lower-strength pulse trains.

Three dissected adult *B. infundibulum* statocysts switched geotactic sign when stimulated with pulse trains (50 V or 100 V, 5 Hz, 10 ms, for 0.1 s). The sign of geotaxis was switched by pulse trains in two statocysts only once out of

10 or 12 pulse trains and twice for 15 pulse trains in the third statocyst. Muscular retraction of the statocyst accompanied the stimulation. The pulse trains that did not switch the sign abolished the geotactic response for a few seconds, i.e. both balancers beat fast or slow or were arrested, then the statocyst reverted back to the original geotactic sign (not analyzed).

Electrically induced switches in the sign of geotaxis of adult *B. infundibulum* statocysts began 10–15 s after termination of a pulse train and lasted for 13–87 s, then the statocyst reverted to its original geotactic sign. Statocysts retained their electrically induced sign of geotaxis at both horizontal orientations. All switches were from positive to negative. There were no spontaneous switches in sign.

## Discussion

### *Ca<sup>2+</sup> and deflection-induced beat frequency*

Transmembrane influx of  $Ca^{2+}$  may be necessary for the deflection-induced increase in beat frequency exhibited during balancer cilia geotactic responses. The evidence suggesting a role for  $Ca^{2+}$  influx is that gravity-induced excitation in horizontally oriented *M. leidyi* larvae and dissected adult statocysts was inhibited by the inorganic  $Ca^{2+}$  channel inhibitors  $Mg^{2+}$ ,  $Co^{2+}$  (Fig. 3),  $Mn^{2+}$  and  $Ni^{2+}$ . Ciliary reversal in ctenophore comb-plate cilia (Moss and Tamm, 1987) and activation of ctenophore macrocilia (Tamm, 1988a) depend on  $Ca^{2+}$  and are also abolished by  $Co^{2+}$  or  $Mn^{2+}$ .  $Ca^{2+}$  conductance in synaptic terminals of various neuronal preparations is also inhibited by  $Co^{2+}$ ,  $Ni^{2+}$ ,  $Mn^{2+}$  and  $Mg^{2+}$  (Augustine, 1990; Barish, 1991).

A drawback of using whole larvae or dissected adult statocysts is that the site(s) of  $Ca^{2+}$  influx cannot be located. Influx may occur at the balancer cells or cilia or at synaptic terminals of neurons that synapse onto balancer cells and regulate balancer cilia. Neuronal control of beat frequency is common in *Mytilus edulis* lateral cilia (Stephens and Stommel,

1989), frog palate cilia (Aiello *et al.* 1991) and rabbit tracheal cilia (Sanderson and Dirksen, 1989). A continuous neural input, inhibited by  $\text{Ca}^{2+}$  channel inhibitors, may control deflection-induced excitation in balancer cells. However, this is unlikely because isolated groups of balancer cells exhibit deflection-induced excitation (see below).

The isolated balancer groups possessed cilia that exhibited graded, deflection-induced excitation and either sign of directional sensitivity (geotaxis), as do intact balancer cilia. The isolated groups may, therefore, be valuable for investigating balancer cilia function. The inhibition of deflection-induced excitation of isolated balancer group cilia by CFASW and  $\text{Co}^{2+}$  in NSW (Fig. 5) indicates that a transmembrane  $\text{Ca}^{2+}$  influx into the balancer cells or cilia is necessary for deflection-induced excitation.  $\text{Co}^{2+}$  and CFASW inhibited the graded, deflection-induced excitation in isolated groups, regardless of their sign of geotaxis. This suggests that  $\text{Ca}^{2+}$  influx into balancer cells or cilia is required for deflection-induced excitation and for the graded responses involved in either sign of geotaxis. The influx may be through voltage- or stretch-activated channels, as occurs in other organisms (Preston and Saimi, 1990). Isolated balancer group cilia did not change their sign of geotaxis, so synaptic input may be necessary to switch geotactic sign. The isolated groups were free of neural input from the body, but some responses of the groups may be due to modulatory effects of presynaptic remnants remaining on the isolated groups. Balancer groups not inhibited by  $\text{Co}^{2+}$  or CFASW may indicate that  $\text{Ca}^{2+}$  was still present at appropriate sites for activation or that other  $\text{Ca}^{2+}$ -independent activation pathways exist, e.g. cyclic-AMP-dependent pathways.

$\text{Mg}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Mn}^{2+}$  and  $\text{Ni}^{2+}$  probably do not inhibit deflection-induced excitation of balancer cilia by non-specific cytotoxic effects or by directly inhibiting dynein. Cytotoxic effects are ruled out because the deflection-induced excitation was usually inhibited rapidly and reversibly within minutes of exposure to the inhibitors. The concentrations used to inhibit deflection-induced excitation were similar to those used to inhibit  $\text{Ca}^{2+}$  conductance in other cells (Augustine, 1990; Barish, 1991). In contrast, cytotoxic effects of  $10\text{ mmol l}^{-1}$   $\text{Mn}^{2+}$  were thought to arrest rabbit tracheal cilia after a relatively long exposure of 60 min (Freedman *et al.* 1983). The failure of seven groups of isolated balancer cilia to recover deflection-induced responses after 10–20 min may be due to a long-lasting blockage by the inhibitors.

$\text{Ni}^{2+}$  is a potent inhibitor of dynein, causing arrest of beating in living *Paramecium* cilia after 5 min and arresting reactivated *Paramecium* cilia and reactivated bull sperm flagella after seconds (Larsen and Satir, 1991; Lindemann *et al.* 1995). *Paramecium* 12S dynein, but not 22S dynein, failed to translocate microtubules when exposed to  $\text{Ni}^{2+}$  (Larsen and Satir, 1991).  $\text{Ni}^{2+}$  is thought to arrest beating in demembrated sperm flagella by inhibiting dynein arms on only one side of the axoneme (Lindemann *et al.* 1995).  $\text{Ni}^{2+}$ -arrested demembrated bull spermatozoa were mechanically reactivated by deflection towards their resting curvature, but not by deflection in the opposite direction (Lindemann *et al.* 1995). Deflection-induced

excitation of balancer cilia was inhibited by  $\text{Ni}^{2+}$ , but cilia were not arrested; they continued to beat slowly with a waveform similar to that in NSW. The  $\text{Ni}^{2+}$  inhibition of balancer cilia occurred during deflection both towards and away from their concave side. Therefore, the inhibition of deflection-induced excitation by  $\text{Ni}^{2+}$  may occur because of a lack of transmembrane  $\text{Ca}^{2+}$  influx rather than because of dynein inhibition.

The beat frequency of some cilia or flagella may be dependent solely on external mechanical stimulation, without a correlating second messenger such as  $\text{Ca}^{2+}$  (Tamm, 1973; Lindemann and Kanous, 1995; Shingyoji *et al.* 1995). Lindemann and Kanous (1995) propose that mechanical deflection may increase the probability of dynein crossbridge interaction, which results in an increase in dynein–microtubule sliding and an increased beat frequency. Deflection of balancer cilia may cause changes in beat frequency by the combined effect of both mechanical and  $\text{Ca}^{2+}$ -induced deformation of axoneme proteins, but not by mechanical stimulation alone. Deflection alone may not suffice to control beat frequency because balancer cilia commonly exhibit deflection-induced excitation or inhibition depending upon their directional sensitivity. Furthermore, deflection does not induce excitation of balancer cilia that are exposed to  $\text{Ca}^{2+}$  channel inhibitors. Lindemann and Kanous (1995) and Satir (1985) propose several ways in which  $\text{Ca}^{2+}$ -induced conformational changes to key axonemal proteins (dynein arms, radial spokes, interdoublet links and dynein regulatory complex) may change the geometry of the axoneme and lead to changes in doublet sliding and an increase in beat frequency. For example, *Chlamydomonas* centrin/caltractin (a  $\text{Ca}^{2+}$ -binding contractile protein) is a dynein inner arm light-chain that interacts with a polypeptide of the dynein regulatory complex (Piperno *et al.* 1994). Binding of  $\text{Ca}^{2+}$  to centrin/caltractin may allow an association between dynein and the dynein regulatory complex that results in increased sliding and an increased beat frequency.

#### *Ca<sup>2+</sup> depolarization and beat frequency*

The proposed influx of  $\text{Ca}^{2+}$  necessary for deflection-induced excitation of balancer cilia may not occur directly as a result of deflection because the beat frequency of isolated balancer group cilia is increased by  $\text{Ca}^{2+}$ -dependent depolarization without mechanical stimulation (Fig. 6). An increase in beat frequency requires both  $\text{Ca}^{2+}$  influx and membrane depolarization because no ciliary activation occurs in a high- $[\text{K}^+]$  ASW if  $\text{Ca}^{2+}$  is removed or if  $\text{Co}^{2+}$  is added to the high- $[\text{K}^+]$  external ASW. Therefore, mechanically activated and voltage-activated ion channels may be involved in the increase in beat frequency.

The proposed influx of  $\text{Ca}^{2+}$  necessary for deflection-induced excitation of balancer cilia may occur exclusively at the ciliary base because isolated balancer group cilia that were depolarized in CFASW- $\text{K}^+$  were activated by local application of  $\text{Ca}^{2+}$  solution (ASW- $\text{K}^+$  or  $1\text{ mol l}^{-1}$   $\text{CaCl}_2$ ) onto the ciliary base, but not the ciliary tip or cell base (Table 1; Fig. 7). If remnant presynaptic terminals were responsible for excitation, then application of  $\text{Ca}^{2+}$  at the cell base would be expected to cause excitation. Exposure to a high external  $[\text{K}^+]$  is a common method

of depolarizing excitable cells. For example, the  $\text{Ca}^{2+}$ -dependent depolarization that induces ciliary reversal in ctenophore comb-plate cilia (Moss and Tamm, 1986, 1987) and the increase in beat frequency in ctenophore macrocilia (Tamm, 1988a) were detected by adding excess  $\text{K}^+$ , both with and without  $\text{Ca}^{2+}$ , to the external medium. The lack of activation by ionophoresis (0.1 ms) of ASW- $\text{K}^+$  ( $10\text{mmol l}^{-1}$   $\text{Ca}^{2+}$ ; Table 1) may result from an insufficient concentration of  $\text{Ca}^{2+}$  at the ciliary base.

The role of hyperpolarization in deflection-induced excitation of balancer cilia may be important because  $\text{Ca}^{2+}$ -induced hyperpolarization correlates with beat frequency increases in frog palate cilia (Tarasiuk *et al.* 1995) and in *Paramecium* cilia (Preston and Saimi, 1990; Preston *et al.* 1992a,b). The possibility exists that deflection-induced excitation in balancers is a result of  $\text{Ca}^{2+}$ -dependent hyperpolarization, not depolarization. The activation of balancer cilia by  $\text{Ca}^{2+}$ -dependent depolarization in the present study may, therefore, result from short-circuiting an excitatory mechanism unrelated to geotaxis. Direct evidence correlating balancer ciliary deflection and increases in beat frequency with changes in membrane potential may be obtained using whole-cell patch-clamp recordings of balancer cells (experiments in progress).

An influx of  $\text{Ca}^{2+}$  at the balancer ciliary base fits Tamm's (1994) hypothesis that the sites of entry of  $\text{Ca}^{2+}$  into cilia are distributed in specific regions, depending on how the cilia respond to stimulation, i.e. whether the cilia respond to  $\text{Ca}^{2+}$  influx by changing their beat frequency or by changing their waveform. Entry of  $\text{Ca}^{2+}$  into ctenophore comb-plate cilia occurs all along the length of the cilia and induces ciliary reversal (Moss and Tamm, 1987; Tamm and Terasaki, 1994). *Chlamydomonas* flagellae undergo waveform changes, and whole-cell patch-clamp investigation of *Chlamydomonas* cells shows that the strength of flagellar currents correlates with flagellar length, indicating that  $\text{Ca}^{2+}$  channels occur along the entire length of the flagella (Beck and Uhl, 1994). Conversely, entry of  $\text{Ca}^{2+}$  into ctenophore macrocilia occurs primarily at the ciliary base and results not in waveform alteration but in activation of beat frequency (Tamm, 1988a). In rabbit tracheal and oviduct cilia, there is no evidence that entry of  $\text{Ca}^{2+}$  occurs through ion channels on the cilia; instead, intracellular  $[\text{Ca}^{2+}]$  rises (from intracellular  $\text{Ca}^{2+}$  stores in the cell bodies) and  $\text{Ca}^{2+}$  diffuses to the axoneme and increases the beat frequency (Sanderson *et al.* 1990; Boitano *et al.* 1992). Tamm (1994) proposes that ciliary waveform repatterning (e.g. ciliary reversal) is controlled by entry of  $\text{Ca}^{2+}$  along the entire length of the cilium and that increases in beat frequency are correlated with entry of  $\text{Ca}^{2+}$  at the ciliary base (either from the external medium or from intracellular stores). The site of  $\text{Ca}^{2+}$  influx into the cilia may indicate the location of the intracellular  $\text{Ca}^{2+}$  sensor.

#### Geotaxis

Why most larvae (95%) tested in the horizontal microscope showed positive geotaxis and dissected adult statocysts showed negative geotaxis (73%) is unclear. The preferred negative geotaxis of dissected adult statocysts is not an artifact of dissection because intact adult ctenophores also preferentially

showed negative geotaxis when tested in the cteno-tilter apparatus. Adult ctenophores, and presumably larvae, have numerous sensory cells located around the body surface and especially within the apical organ (Horridge, 1965; Tamm, 1982; Hernandez-Nicaise, 1991). Information from the sensory cells may be integrated into balancer cilia regulation *via* the diffuse nerve net, but the neural circuitry of ctenophores is unfortunately not well understood (Tamm, 1982; Hernandez-Nicaise, 1991). Environmental factors such as light, temperature and pressure (Tamm, 1982) as well as internal factors such as circadian rhythm or metabolic state may regulate the sign of geotaxis, but the effects of these factors on geotaxis have not been thoroughly tested. Thus, larvae may be sensitive to the microscope light or to contact with the slide and coverslip and react by exhibiting positive geotaxis.

The horizontal microscope was used to demonstrate that the deflection-induced responses (excitation or inhibition) of balancer cilia are electrically switchable in horizontally oriented larvae (Fig. 8) and adult dissected statocysts. The electrical stimulation may excite part of the nervous system that signals a switch in sign of geotaxis. Geotactic sign was switched from negative to positive and from positive to negative by pulse-train stimulation, but not all pulse trains switched their sign. It is not clear why electrical stimulation of whole larvae or dissected adult statocysts does not directly depolarize and excite all four balancer groups. There may be two or more neural input pathways, perhaps from other sensory systems, that regulate balancer cilia, although it is possible that electrical stimulation may excite the balancers themselves. The larvae used for electrical stimulation experiments mostly showed positive geotaxis, as did those used in  $\text{Ca}^{2+}$  channel inhibitor experiments. The electrically induced switch from positive to negative geotaxis suggests that the larvae have the capability for negative geotaxis, but demonstrate a positive geotaxis for unknown reasons.

The opposite response of balancer cilia to a particular direction of mechanical deflection, depending on geotactic sign, is unique. A detailed model of the underlying mechanisms has been presented (Lowe, 1997) and will serve as the basis for future investigations.

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