Visualization of Calcium Transients Controlling Orientation of Ciliary Beat

Sidney L. Tamm and Mark Terasaki

Boston University Marine Program, Marine Biological Laboratory, Woods Hole, Massachusetts 02543; and Laboratory of Neurobiology, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, Maryland 20892

Abstract. To image changes in intraciliary Ca controlling ciliary motility, we microinjected Ca Green dextran, a visible wavelength fluorescent Ca indicator, into eggs or two cell stages of the ctenophore *Mnemiopsis leidyi*. The embryos developed normally into free-swimming, ~0.5 mm cydippid larvae with cells and ciliary comb plates (~100 μm long) loaded with the dye. Comb plates of larvae, like those of adult ctenophores, undergo spontaneous or electrically stimulated reversal of beat direction, triggered by Ca influx through voltage-sensitive Ca channels. Comb plates of larvae loaded with Ca Green dextran emit spontaneous or electrically stimulated fluorescent flashes along the entire length of their cilia, correlated with ciliary reversal. Fluorescence intensity peaks rapidly (34–50 ms), then slowly falls to resting level in ~1 s. Electrically stimulated Ca Green emissions often increase in steps to a maximum value near the end of the stimulus pulse train, and slowly decline in 1–2 s. In both spontaneous and electrically stimulated flashes, measurements at multiple sites along a single comb plate show that Ca Green fluorescence rises within 17 ms (1 video field) and to a similar relative extent above resting level from base to tip of the cilia. The decline of fluorescence intensity also begins simultaneously and proceeds at similar rates along the ciliary length. Ca-free sea water reversibly abolishes spontaneous and electrically stimulated Ca Green ciliary emissions as well as reversed beating. Calculations of Ca diffusion from the ciliary base show that Ca must enter the comb plate along the entire length of the ciliary membranes. The voltage-dependent Ca channels mediating changes in beat direction are therefore distributed over the length of the comb plate cilia. The observed rapid and virtually instantaneous Ca signal throughout the intraciliary space may be necessary for reprogramming the pattern of dynein activity responsible for reorientation of the ciliary beat cycle.

BELIEVING often leads to seeing, particularly with the advent of new fluorescent probes and optical systems for imaging physiological processes inside living cells. Here we provide an example dealing with the role of Ca in regulation of ciliary motility.

Knowing the site(s) of Ca entry into cilia is important for understanding where and how Ca acts to trigger specific types of changes in axonemal motility (Eckert & Brehm 1979; Otter 1989; Preston & Saimi, 1990). Freeze-etch electron microscopy and EM cytochemistry revealed ciliary necklace particle arrays and divalent cation binding sites at the base of cilia (Gilula & Satir, 1972; Fisher et al., 1976; Good et al., 1990), suggesting that all or part of the Ca conductance may reside at the ciliary base. Previous deciliation/regrowth experiments on *Paramecium* showed that the depolarization-activated Ca channels allowing Ca influx for ciliary reversal are located in the ciliary membrane; however a direct determination of their distribution was not possible (Ogura and Takahashi, 1976; Dunlap, 1977). Suction electrode measurements of photo-induced currents causing flagella-type undulations and backward swimming of *Chlamydomonas* indicated that voltage-dependent Ca channels reside in the flagellar membrane (Harz and Hegemann, 1991); more recent experiments on regenerating flagella showed that the Ca channels are evenly distributed along the flagellar length (Beck and Uhl, 1994). Direct electrical recording from the giant comb plate cilia of ctenophores demonstrated that the voltage-dependent Ca conductance controlling ciliary reversal is distributed over most of the length of the ciliary membranes (Moss and Tamm, 1987). In contrast to these findings on Ca regulation of beat direction and waveform, the voltage-sensitive Ca conductance controlling activation of beating is apparently restricted to a membranous rete at the base of macrocilia in the ctenophore *Beroë* (Tamm, 1988*b*).

We wanted to confirm and extend the electrophysiological findings on comb plates by directly visualizing the sites of Ca entry, and hence the distribution of voltage-sensitive Ca channels along the ciliary membranes. To do this we used a developmental strategy to load a visible wavelength fluo-

Address all correspondence to Sidney L. Tamm, Boston University Marine Program, Marine Biological Laboratory, Woods Hole, MA 02543.

rescent Ca indicator into comb plate cilia of ctenophore larvae. This allowed us to see for the first time spatial and temporal changes in intraciliary Ca that regulate ciliary motility. A videotape of this work was presented at the ASCB meeting in New Orleans, LA, December, 1993.

Materials and Methods

Organism

Sexually mature *Mnemiopsis leidyi* were dipped from the National Marine Fisheries Service granite jetty, Woods Hole, MA, from July to October, 1992 and 1993. This is the best site for consistently and reliably obtaining ctenophores in Woods Hole. Animals were placed in large glass bowls of sea water and kept overnight on a light-dark cycle that gave freshly spawned fertilized eggs at 9–10 AM the next day.

Loading with Ca Green-1 Dextran

Fertilized eggs (~170 μm in diameter) were pipetted into a microinjection wedge chamber. Uncleaved eggs or one blastomere of two cell stages were injected through the egg envelope, using a pipette with a small droplet of mercury in back of the injected solution to provide better control of the pressure (Hiramoto, 1962). 1 mM Ca Green-1 dextran (10,000 mol wt, 1.3 mol Ca Green/mol dextran; Molecular Probes, Eugene, OR) in buffer (100 mM potassium glutamate and 10 mM Hepes, pH 7) was injected into the central yolky cytoplasm at 0.5–1% of the egg volume. Calcium Green indicator concentration in the whole egg was 6–13 μM, though the cytosolic concentration may be up to two times greater because of the volume occupied by yolk. After injection, eggs were removed from the chamber and allowed to develop for 1–2 d into free-swimming ciliated cydippid larvae.

Microscopic Stimulation and Perfusion Slides

1–2-d-old cydippid larvae in sea water (from Ca Green-injected or noninjected eggs) were pipetted onto microscope slides and prevented from swimming by light pressure of a 22 × 22 mm coverslip supported by two parallel ridges of vaseline. Two $Ag/AgCl_2$ or Pt wire electrodes were inserted under opposite open edges of the coverslip, and connected to a stimulator (Grass SD-9). Bipolar pulse trains (30–60 V, 10 Hz, 15 ms) were applied to Ca Green-loaded larvae for 2–4 s through the sea water surrounding the immobilized larvae. Nonloaded larvae were generally stimulated for longer times (~10–15 s) to obtain longer video records of motor responses (see Fig. 2). Test solutions were perfused under the coverslip by adding drops to one open edge and withdrawing fluid with a twisted Kim-wipe from the opposite side. Complete replacement of solutions under the entire area of the coverslip was not immediate, particularly for Ca-free sea water, but required at least several perfusions.

Microscopy and Recording

Larvae on electrical stimulation slides were imaged with a Zeiss RA microscope (Carl Zeiss, Inc., Thornwood, NY) using phase contrast or fluorescence (FITC) optics (40/0.75 objective) and a SIT (C2400-08; Hamamatsu Corp., Bridgewater, NJ) or intensified CCD (C2400-97; Hamamatsu Corp.) video camera. Images were recorded on a S-VHS recorder (AG-7355; Panasonic) with numbered fields (QSI-VFF 6030; QSI Systems, Newton, MA). S-VHS tapes were copied onto an optical memory disk recorder (2028F; Panasonic) to analyze the data. Video frames were captured using an Image 1/AT image processor (Universal Imaging, West Chester, PA). The deinterlace function was used to extract video fields. The average brightness in a 12 × 12 or 16 × 16 pixel square in a comb plate was measured. Average resting fluorescence intensity for 0.4 s was used to normalize the data. Data were plotted using Kaleidagraph (Synergy Software, Reading, PA) on a MacIntosh computer.

Results

Description of Cydippid Larvae and Ciliary System

Free-swimming cydippid larvae of *Mnemiopsis* resemble adult ctenophores of the order Cydippida (i.e., *Pleurobrachia*). The mouth defines the oral end of the body and a prominent statocyst is located at the broader aboral end (Figs. 1 and 2). Two tentacles emerge from opposite sides of the body, marking the tentacular plane. 1–2-d-old *Mnemiopsis* larvae are ~0.5 mm long.

The ciliary system of cydippid larvae (Figs. 1 and 2) is disproportionately large relative to body size, compared with adult ctenophores. The eight rows of ciliary comb plates are arranged in four pairs running in an aboral–oral direction. Each comb row contains 5–6 comb plates at this stage. Larval comb plates are ~100 μm long; each plate consists of a tapered, blade-shaped group of several hundred cilia that beat together as a unit. The cilia of a comb plate arise from a ridge of 3–5 elongated epithelial cells.

Electron microscopy of cydippid larvae shows synapses onto comb plate cells (Tamm and Tamm, 1981), indicating that ciliary motility in larval stages, as in adults (Moss and Tamm, 1986), is under nervous control.

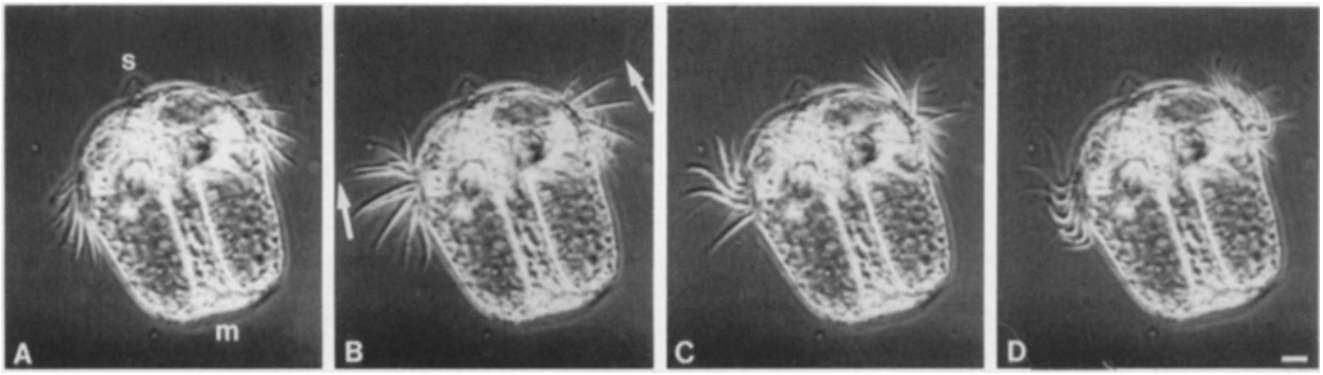

Figure 1. Normal beat cycle of comb plates of a non-Ca Green-loaded *Mnemiopsis* larva immobilized on a sea water slide by gentle pressure of the coverslip. Statocyst (*s*) and mouth (*m*) mark aboral and oral ends, respectively. A pair of comb rows is seen in profile on each side, beating slightly out of phase. The in-focus rows on either side are followed here. (*A*) The beat cycle begins with plates at the end of the recovery stroke pointing toward the mouth. (*B*) The plates perform an effective stroke in the aboral direction (*arrows*), with the plates nearest the statocyst beating first. (*C* and *D*) The plates unroll toward the mouth during the recovery stroke. Prints show successive video fields (1/60-s intervals) using strobe illumination synchronized with a Newvicon video camera. Preceding fields persist as faint "ghosts" due to camera tube lag. Digital fields were photographed on Kodak Tech Pan (2415) film. Bar, 50 μm.

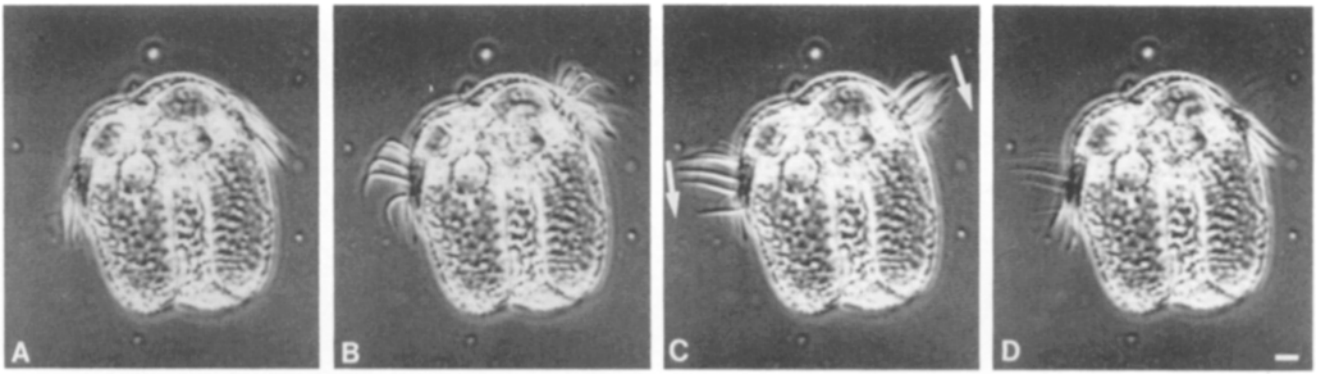

Figure 2. Reversed beat cycle of the same larva elicited by bipolar pulse train stimulation applied to the sea water bath. (*A*) The beat cycle begins with plates at the end of the effective stroke pointing toward the mouth. (*B*) The plates unroll toward the statocyst during the recovery stroke. (*C* and *D*) the effective stroke is reversed 180° toward the mouth (*arrows*). Prints show successive video fields as in Fig. 1. Bar, 50 μm.

Ciliary Activity and Ion-stimulated Ciliary Reversal

Comb plates do not beat unless stimulated, mechanically or electrically (Tamm 1982; Moss and Tamm, 1986). At rest, plates are bent at the base and point orally, lying close to the body surface. The normal beat cycle begins with a rapid swing of the plate in the aboral direction (effective stroke; Fig. 1 *B*), propelling the animal mouth foremost. The plates then unroll in the oral direction by propagating a bend distally (recovery stroke; Fig. 1, *C* and *D*). Comb plates beat in a metachronal sequence, starting with the aboralmost plate and proceeding orally along the comb row (Fig. 1; Tamm, 1982).

Previous ion substitution experiments showed that high KCl (50–100 mM) sea water causes *Pleurobrachia* larvae to swim backward due to 180° reversal in beat direction of the comb plates (Tamm and Tamm, 1981). Reversed beating occurs at high frequency (20–25 Hz) and begins with unrolling of the plates in the aboral direction (reversed recovery stroke), followed by a rapid swing toward the mouth (reversed effective stroke) (Tamm and Tamm, 1981). Ciliary reversal of *Mnemiopsis* larvae is not triggered by high KCl sea water, but by elevated Ca levels (Tamm and Tamm, 1981). The reason for this difference between larvae of *Pleurobrachia* and *Mnemiopsis* in ion dependence of ciliary reversal is not known.

Ion-stimulated ciliary reversal in *Pleurobrachia* and *Mnemiopsis* larvae is Ca-dependent, being inhibited by Ca-free ASW or inorganic Ca blockers (Tamm and Tamm, 1981). We previously used detergent-extracted ATP-reactivated models of comb plates from *Mnemiopsis* larvae to show that Ca at μM levels directly causes reversed beating of the cilia (Nakamura and Tamm, 1985).

Electrical Stimulation of Ciliary Reversal

For the present work we needed a faster way to switch ciliary reversal on and off than changing ion concentrations by perfusion. In our previous work on comb plates of adult cydippid ctenophores (*Pleurobrachia*) we found that trains of bipolar pulses delivered by a suction electrode attached to a tentacle or the adjacent body surface triggered synaptically driven volleys of Ca-dependent ciliary action potentials and reversed beating of comb plates (Moss and Tamm, 1986, 1987).

We adapted this method to slide preparations of *Mnemiopsis* larvae. We found that similar pulse train stimuli applied by $Ag/AgCl_2$ or Pt wires to the sea water bathing nonloaded larvae elicited brief muscular retractions of the comb rows, followed by high frequency (15–20 Hz) reversed beating of all comb plates for the duration of the stimulus (Fig. 2) (and backward swimming if the larvae were not immobilized by the coverslip). Ciliary reversal was accompanied by muscular bending and opening of the mouth. The beat pattern and frequency of electrically stimulated ciliary reversal (Fig. 2) is similar to that of KCl or Ca-induced ciliary reversal of *Pleurobrachia* or *Mnemiopsis* larvae (Tamm and Tamm, 1981).

Immobilized *Mnemiopsis* larvae sometimes underwent brief episodes of ciliary reversal and muscular contractions without electrical stimulation. These spontaneous motor responses are probably triggered by synaptic excitation due to mechanical stimulation of the tentacles and/or body surface by the coverslip. Mechanical stimulation of the tentacles of adult cydippid ctenophores elicits nervously mediated ciliary reversal and backward swimming (Tamm, 1982). The presumed sensory receptors are ectodermal cells bearing actin-filled projections and onion root cilia (Tamm and Tamm, 1991). Such cells are also present on the body surface of *Mnemiopsis* larvae (Tamm, S. L., unpublished observations).

Electrically Stimulated Ciliary Reversal Requires Ca Influx

To test whether electrically stimulated ciliary responses of *Mnemiopsis* larvae depend on Ca influx, we tested the effects of Ca-free sea water and inorganic Ca blockers. Perfusion of larvae with Ca-free sea water, with or without 1 mM EGTA, blocked reversal responses to pulse train stimulation within 5–10 min of perfusion. The delay in blockage probably reflects the time required to wash out most of the Ca from the bath, and possibly to exchange sea water trapped between the closely packed cilia within each comb plate. The larvae responded instead to electrical stimuli by high fre-

quency beating (12–20 Hz) in the normal direction. Treatment with Ca-free sea water also weakened and reduced muscular responses to electrical stimulation, particularly the initial retraction of rows at the onset of the pulse train. Ca-free sea water effects on larvae were reversible: after return to normal sea water electrical stimuli caused the usual ciliary reversal and muscular responses.

Ca-free sea water also blocks KCl-induced ciliary reversal of *Pleurobrachia* larvae, but blocks an increase in beat frequency as well (Tamm and Tamm, 1981). However, raising the Mg concentration of high KCl sea water blocks ciliary reversal of *Pleurobrachia* larvae without preventing high frequency normal beating (Tamm and Tamm, 1981), similar to the effects of Ca-free sea water on electrically stimulated responses of *Mnemiopsis* larvae.

The uncoupling of directional and frequency responses of comb plates in certain cases indicates that these two parameters of ciliary activity may have different thresholds and/or sensitivities to Ca. However, the previous ionic experiments on *Pleurobrachia* larvae could not distinguish between roles of Ca in axonemal responses vs. synaptic triggering of these responses.

Since Ca-free treatment of *Mnemiopsis* larvae permits an increase in beat frequency in the normal direction during pulse train stimulation, ciliary activity is still nervously controlled under these conditions. Electrically stimulated ciliary reversal therefore requires influx of Ca, presumably into the comb plate cilia, independent of synaptic triggering of the response.

Ca Green Dextran-loaded Larvae

Because a ctenophore comb plate arises from many cells, making microinjection impracticable, and the cells do not load with AM esters of Ca indicators (Tamm, S. L., unpublished observations), we used a different strategy for dye loading. We injected a dextran conjugate of Ca Green-1, a visible wavelength (FITC) fluorescent Ca indicator, into one or two cell stage ctenophore embryos. The dextran conjugate is preferable to the free acid form which compartmentalizes or leaks out of cells over a period of a few hours.

Ca Green dextran-injected eggs developed into normal free-swimming cydippid larvae with cells and comb plate cilia faintly fluorescent under resting conditions, indicating successful loading and retention of the indicator. Injection of Ca Green dextran into one blastomere of a two cell stage produced larvae with cells and cilia of one sagittal half fluorescent (Fig. 3). The boundary between the loaded and nonloaded sagittal halves was so sharp that it bisected the cup-shaped epithelial floor of the aboral statocyst. This is consistent with earlier embryological evidence that the first cleavage plane defines the future sagittal plane of the ctenophore (Chun, 1892).

Ca Green dextran-loaded, unrestrained comb plates underwent both spontaneous and electrically stimulated reversed beating at high frequency for the duration of the pulse train, showing that Ca buffering by the dye has no apparent effect on Ca-dependent ciliary motor responses.

Spontaneous Ciliary Flashes

Since relative changes in fluorescence could not be determined in rapidly moving cilia, we examined plates which were restricted or prevented from beating by contact with the coverslip.

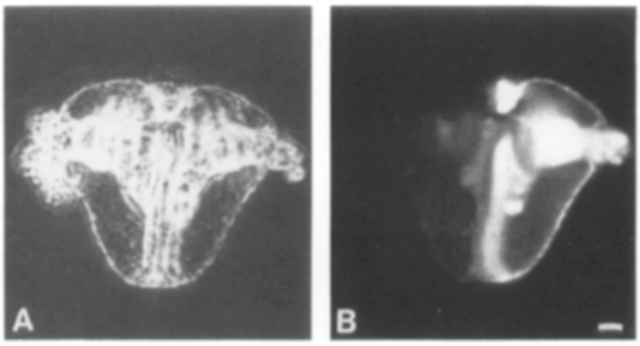

Figure 3. Ca green dextran labeling of a *Mnemiopsis* larva that was injected at the two cell stage. (*A*) Phase contrast; (*B*) fluorescence (FITC). The larva is immobilized under a coverslip in the tentacular plane (contracted tentacles emerge from either side), with the aboral statocyst facing up and the mouth down. Focus is midway through the body; two pairs of flattened comb rows are visible running over the tentacle pouches. Basal fluorescence of the resting larva (*B*) shows that only the right sagittal half is loaded with Ca Green dextran (fluorescence of comb plates is too faint to detect at this magnification and focus). Digital frames photographed on Kodak Tech Pan (2415) film. Bar, 50 μm.

Immobilized comb plates sometimes emitted spontaneous volleys of brief increases in fluorescence intensity along their entire length. Typically, 4–6 fluorescent flashes, each about 1 s in duration, were emitted over 10 s. These spontaneous flashes arose from all comb plates in a row, as well as from the ridge of cell bodies at the base of each comb plate. If the comb plates were not completely immobilized, they made brief quivering movements at the time of the fluorescent flashes.

Intensity measurements at a site midway along a comb plate typically showed a rapid rise of fluorescence emission to a maximum value within 2–3 video fields (34–50 ms), followed by a slower decline to resting level in ~1 sec (Fig. 4 *A*). However, fluorescence intensity sometimes rose in several steps which summed, reaching a plateau which was maintained for several seconds before slowly declining to baseline (Fig. 4 *B*). Spontaneous emissions averaged 64% above resting level (n = 8, SD = 23%).

Electrically Stimulated Ciliary Flashes

Bipolar pulse trains of 2–4-s duration elicited an increase in fluorescence along comb plates and in the cell bodies. The initial rise of fluorescence emission was as rapid as in spontaneous flashes, but intensity typically increased in steps (as in some spontaneous flashes), reaching a maximum value near the end of the stimulus train (Fig. 4 *C*). Upon termination of the pulse train, fluorescence declined slowly to resting level in 1–2 s. In some cases stimulated emission rose directly to a maximum value (Fig. 4 *D*). Maximum intensity of stimulated flashes averaged 77% above resting level (n = 10, SD = 26%). The similar kinetics of spontaneous and electrically stimulated fluorescence emissions confirms that bipolar pulse trains act via nervous pathways, not by opening ciliary Ca channels directly.

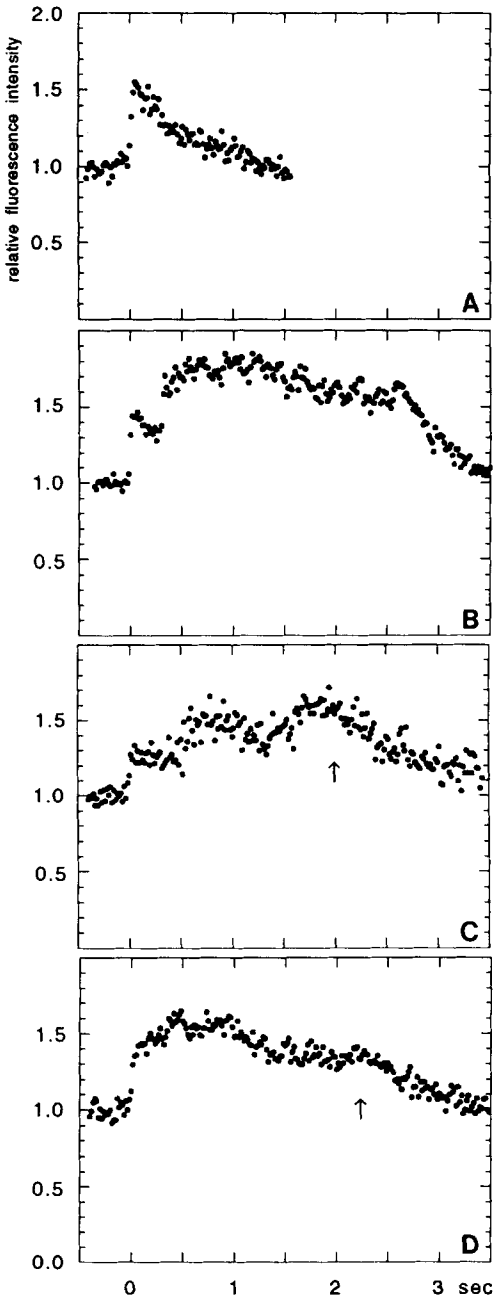

Figure 4. Time course of spontaneous and electrically stimulated changes in intraciliary free Ca concentration. Average Ca Green dextran fluorescence intensity was measured in a small area halfway along the length of a comb plate, normalized to the resting fluorescence level, and is plotted at 17-ms intervals (every video field). (*A*) Spontaneous flash. Note spike-like rise and slower decay to resting level over 1.5 s. (*B*) Spontaneous flash with a stepwise increase to a value that is maintained for 2 s and then declines to resting level. (*C* and *D*) Electrically stimulated flashes. Stimulus onset (~0 s) and termination (*arrow*) were recorded on audio track. (*C*) Staircase increase in fluorescence. (*D*) Direct rise to maximum intensity.

In partially restrained comb plates, electrically evoked fluorescence was accompanied by rapid vibration of the cilia in the reverse direction. These attempts at reversed beating began within two video fields (34 ms) after the rise of fluorescence and lasted for the duration of the flash. The decline in ciliary free Ca thus occurs well after the onset of the motor response, ruling out binding of Ca to the axonemal Ca sensor as the basis for the fluorescence decline.

Pulse trains of longer duration (more than 5 s) often elicited periodic flashes in the cilia and cell bodies, accompanied by brief movements of partially restrained plates, similar to spontaneous volleys. Higher voltage pulses induced brighter Ca Green emissions from both cilia and cell bodies.

Multiple Site Recordings

We next measured Ca Green fluorescence changes at three or four sites along a single comb plate (Fig. 5). Resting intensity levels are greater near the base and smaller near the tip of the cilia (Fig. 5). Since Ca Green is not a ratiometric dye, this proximal-distal gradient in fluorescence intensity does not represent an intraciliary Ca gradient. Rather, the resting fluorescence gradient undoubtedly reflects the decreasing number of cilia from base to tip of the tapered comb plate. We plan to confirm this by loading comb plates with rhodamine dextran as well as Ca Green dextran in order to relate total ciliary volume to comb plate length; this will allow us to normalize absolute Ca Green intensity at different sites along the comb plate.

In both spontaneous (n = 4) and stimulated (n = 4) flashes, fluorescence rises within 17 ms (1 video field) and to a similar relative extent above resting level from near the base to near the tip of the cilia (Fig. 5, *C* and *D*). The total relative increase in Ca Green intensity within a given comb plate ranges from 120 to 135%, and is complete within 50 ms at all locations. We could not detect any lag in the onset of Ca Green emission at different sites along a comb plate (Fig. 5 *E*). The decline of fluorescence after a spontaneous flash begins within 17 ms and proceeds at similar rates from base to tip of the cilia (Fig. 5, *C* and *D*).

In rare cases where fluorescence in the cell bodies was not saturating, emission increased a smaller percentage (relative to preflash levels) than in the cilia, peaked 34–50 ms after the cilia, and then decayed at a slower rate than did ciliary fluorescence. However, fluorescence of both cell bodies and cilia returned to resting levels at about the same time.

Effects of Ca-free Sea Water on Ca Green Flashes

In Ca-free sea water, no spontaneous or electrically stimulated Ca Green emissions occurred in cilia or in their cell bodies. As in nonloaded larva (above), free comb plates underwent high frequency beating in the normal direction in response to electrical stimulation. The effects of Ca-free sea water were reversible: upon return to normal sea water, both spontaneous and stimulated Ca Green flashes from cilia and cell bodies reappeared, as did reversed beating of free comb plates.

Discussion

Strategies

Our success in visualizing Ca transients in cilia for the first time depended on the choice of biological material. The large size, compound nature, and rapid development of cili-

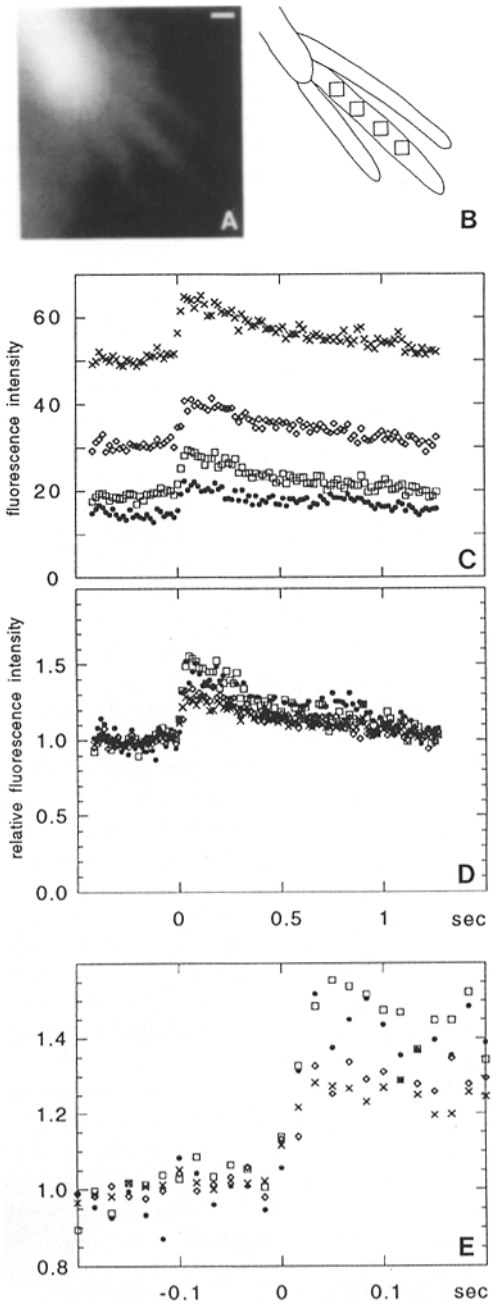

Figure 5. Intraciliary calcium measurements at four sites along the length of a comb plate during a spontaneous flash. (*A*) Fluorescence image of comb plates. (*B*) Tracing showing location and size of sites along one comb plate where average fluorescence intensity was measured by an image processor. The comb plate is 77 μm long; measurements were made in a 12 × 12 pixel box at the indicated locations. (*C*) Average Ca Green dextran fluorescence intensity plotted against time (at 17-ms intervals) at the four sites along the cilia: 11 (*cross*), 26 (*diamond*), 40 (*square*), and 57 μm (*dot*) from the base. (*D*) Same data but normalized to the average fluorescence intensity during the 0.4 s before the increase in order to compare the percent increases in intensity at the different sites. (*E*) Expanded time scale for D to show that the increase in fluorescence starts at the same video field at all four locations along the comb plate. This sequence was imaged with an intensified CCD camera. Bar, 10 μm.

ary comb plates in ctenophores provide unique experimental advantages for this and other studies on cilia (Tamm, 1982). By microinjecting ctenophore eggs with Ca Green-1 dextran and letting the embryos develop for 1–2 d into free-swimming cydippid larvae, we successfully loaded comb plate cilia with the Ca indicator, thereby avoiding the technical problems associated with introducing the dye into differentiated ciliated cells. Dextran conjugates of Ca indicators, unlike free acid forms, are retained in the cytoplasm without compartmentalization or leaking out of the cells. To our knowledge this is the first time that this developmental strategy has been used to load Ca indicators into target cells (and organelles) to study Ca physiology.

An additional requirement for our work was a method for rapidly switching ciliary motor responses on and off while imaging Ca transients by video microscopy. Fortunately, extracellular electrical stimuli that trigger nervously mediated reversed beating of comb plates in adult cydippid ctenophores (Moss and Tamm, 1986) also excite ciliary reversal of cydippid larvae when applied under a coverslip. This is the first physiological evidence that cydippid larvae of ctenophores and adult ctenophores of the order Cydippida share a similar nervous control of their comb plates, and demonstrates a functional as well as morphological resemblance between these larval and adult forms.

Source of Ca for Ciliary Ca Transients

The role of Ca in regulation of ciliary and flagellar motility is well-established (Eckert and Brehm, 1979; Otter, 1989; Preston and Saimi, 1990). Previous experiments showed that ciliary reversal in ctenophores and protozoa requires influx of extracellular Ca through voltage-gated Ca channels (Eckert and Brehm, 1979; Nakamura and Tamm, 1985; Moss and Tamm, 1986, 1987). In the absence of external Ca or in the presence of Ca channel blockers, ciliary reversal cannot be elicited. Moreover, defects in Ca channel function prevent ciliary reversal in *Paramecium* (Preston and Saimi, 1990) and inward Ca current has been recorded directly from comb plate ciliary membranes of *Pleurobrachia* (Moss and Tamm, 1987).

Although cilia and flagella contain no membrane-bound compartments that could serve as internal Ca stores, it has been suggested that calmodulin or other calcium-binding proteins present in cilia and flagella (Maihle et al., 1981; Otter 1989; Stommel et al., 1982) could act to bind and release Ca (Suarez et al., 1993). Hyperosmolality-dependent initiation of motility of teleost sperm is mediated by a rise in intracellular Ca which evidently is released from internal stores located somewhere in whole sperm (Oda and Morisawa, 1993).

It was therefore important for us to confirm by Ca-free sea water experiments that the electrically stimulated Ca Green flashes we observed in comb plates requires influx of Ca from the sea water. This Ca influx probably does not trigger release of additional Ca from internal stores (see below).

Ciliary Ca Flashes

The time course of the rise and fall of single Ca Green flashes in comb plates is similar to the kinetics of depolarization-evoked Ca transients in other cells (Cannell et al., 1987; Ahmed and Connor, 1988; Becker et al, 1989; Berlin and

Konishi, 1993). The precise shape of Ca transients depends on a number of factors, including relative contributions of Ca influx vs. release from internal stores (i.e., Ca-induced Ca release), the effects of fixed and diffusible Ca buffers (including Ca-measuring dyes and physiological Ca sensors), and the mechanisms of Ca removal (i.e., surface and/or intracellular pumps, sequestration, binding) (see Nowycky and Pinter, 1993 for models). The single rapid rise of Ca Green emission in many spontaneous ciliary flashes, together with absence of Ca Green signals in Ca-free sea water, indicates that Ca influx through voltage-gated channels is the sole source of the ciliary Ca transient.

The stepwise increase in ciliary fluorescence observed during some spontaneous and stimulated flashes may be due to variations between cells of the same comb plate in the timing and amplitude of the regenerative responses of their cilia, as found by intracellular recording from comb plates of adult *Pleurobrachia* (Moss and Tamm, 1986). This would result in progressive recruitment of Ca emissions from different groups of cilia within a single comb plate. So far we have not been able to test this possibility by seeing whether the fluorescent steps are correlated with emissions from discrete "slivers" within a plate. Since the cilia within a comb plate are mechanically coupled to one another (Tamm, 1984), the beat pattern of the plate probably reflects the activity of the majority of its cilia. Alternatively, the staircase emission profiles may reflect facilitation and the graded nature of the Ca response (Moss and Tamm, 1986) over the entire comb plate.

Several reports of intracellular Ca increases associated with sperm motility have recently appeared. Oda and Morisawa (1993) used quin-2 loaded puffer (teleost) sperm to show that hyperosmolality-induced initiation of motility is mediated by a rise of intracellular Ca, as measured spectrophotometrically in suspensions of whole sperm. Intracellular Ca increased under Ca-free conditions, suggesting that Ca was released from internal stores. Suarez et al. (1993) used indo-1 loaded hamster sperm, together with strobe fluorescence of individual moving sperm, to show that intracellular Ca levels increased in head and midpiece regions (the principal piece or sperm tail proper could not be measured) in hyperactivated vs. activated sperm. Ca oscillations, particularly in the proximal midpiece region, were coupled to flagellar beat frequency. However, the Ca oscillations evidently are the result of flagellar bending rather than the cause, and may not be physiologically significant.

Since these studies did not follow possible Ca transients within the flagellar space itself or at different sites along the flagellum, they could not report spatial or temporal patterns of Ca increase along the flagellum.

Ca Measurements along the Comb Plate

We found no detectable lag, with a time resolution of 17 ms (1 video field), in onset of Ca Green emission at different sites from near the base to near the tip (up to about 75% of the length) of the comb plate. Can these results be explained by diffusion of Ca from channels located exclusively near the base of the cilia? We can model this problem using macroscopic theory of diffusion of an initial sharp rise in solute concentration at one end of a ~100 μm long pipe with reflecting boundaries (sides) (Berg, 1983). The diffusion coefficient (D) of Ca in cytoplasm, like that of most solutes in water (Crank, 1956), is reported to increase with concentration, reaching a maximum value to 2×10^{-6} cm^2s^{-1}, or 200 μm^2 s^{-1} at more than 100 μM free Ca (Connor et al., 1981; Allbritton et al., 1992). For Ca in cytoplasm, the concentration-dependence of its D is largely due to binding (Allbritton et al., 1992). Assuming a constant D of Ca of 200 μm^2 s^{-1} (see also Nowycky and Pinter, 1992), and using a numerical solution (Berg, 1983), computation shows that ~10 s are required to relax an initial step gradient of Ca concentration, y_o, occupying 30% of the length of a 100 μm long pipe, to an almost uniform concentration, 0.3 y, that spans the full length of the pipe (or cilium) (Berg, 1983, Appendix B, Fig. B.3). Of course, if Ca entry sites are restricted to less than the proximal 30% of the comb plate cilia, then the time required to reach a more-or-less uniform concentration along the cilia would be considerably longer than 10 s. These estimates also ignore bound Ca-binding proteins in the axoneme (e.g., calmodulin; Otter, 1989) that would be expected to further slow Ca diffusion along the cilia.

Therefore, our findings cannot be explained by diffusive transport of Ca from entry sites near the base of the comb plate. Instead, our results indicate that Ca enters along the entire length of the ciliary membranes. The similar relative increase above resting level in Ca Green intensity at different sites along the comb plate indicates that the Ca channels are evenly distributed over the length of the cilia.

Our direct visualization of Ca fluxes into larval comb plates largely confirms electrical recordings from adult comb plates showing that a voltage-dependent Ca conductance is distributed over most of the length of the cilia (Moss and Tamm, 1987). The failure to record Ca action potentials near the base of adult comb plates may therefore not be due to absence of Ca channels in the proximal ciliary membrane, but to masking and/or shunting of the inward current in this region (Moss and Tamm, 1987).

In adult comb plates the duration of the ciliary action potential is about 20 ms, and its propagation velocity is ~27 mm/s. The region of inward Ca current therefore occupies almost half the length of the 1 mm long cilia (Moss and Tamm, 1987). If larval comb plates have similar membrane properties, their shorter length (~100 μm) and the poorer time resolution of video recording (17 ms) explains why we did not detect a propagated Ca Green fluorescent signal along the cilia.

With regard to the mechanism(s) for reducing intraciliary Ca following an influx, the virtually synchronous fall and similar rate of decline of Ca Green fluorescence at different sites along a comb plate are too uniform and too fast for Ca to be removed solely by diffusion from the cilia into the cell bodies. Instead, Ca efflux must occur along the entire cilia, presumably by Ca pumps distributed over the length of the ciliary membranes. However, Ca-ATPase activities with properties similar to those of known Ca pumps in other cells have not yet been detected in ciliary membranes of *Paramecium* (Wright and Van Houton, 1990; Wright et al., 1993). The possibility that a Na^+/Ca^{2+} exchanger contributes to removal of ciliary Ca following depolarization seems unlikely, since the Na^+/Ca^{2+} exchanger is electrogenic and would serve to drive Ca into the cilia rather than remove it when the membrane is depolarized (Miller, 1988; Lagnado and McNaughton, 1990; Kargacin and Fay, 1991).

The Ca signal in the cell bodies of comb plates peaked slightly after the cilia and decayed at a slower rate. Cell body Ca transients require extracellular Ca and probably depend on Ca-induced Ca release from internal stores. The initial rise in cell body Ca could result from influx of extracellular Ca and/or leakage from the proximal part of the cilia. The subsequent decline in cell body Ca may reflect extrusion and/or sequestration mechanisms that are different from those effecting Ca removal from the cilia.

Conclusions

Ciliary reversal, the Ca-dependent motor response studied here, is a 180° reorientation of the entire beat cycle. The normal asymmetric form of ciliary beat is presumed to be due to a specific pattern of asynchronous activity of dynein cross-bridges (and hence active sliding) that is coordinated from base to tip of the axoneme as well as around its circumference (Sugino and Naitoh, 1982; Satir, 1985). The uniform ciliary Ca transient we observed, reflecting an even distribution of voltage-sensitive Ca channels over the comb plate cilia, indicates that reorientation of the pattern of dynein activity requires a rapid and simultaneous Ca signal throughout the intraciliary space.

These findings are consistent with recent work on the Ca-dependent axonemal motor response of *Chlamydomonas*. Strong light induces a Ca-mediated switch from ciliary beating to flagellar-like undulations propagated from base to tip of both flagella. Electrophysiological assay of photo-stimulated flagellar Ca currents during flagellar regeneration of *Chlamydomonas* indicates that the voltage-sensitive Ca channels are evenly distributed over the total length of the flagella (Beck and Uhl, 1994). As in comb plates, a uniform Ca conductance would provide a virtually instantaneous Ca transient throughout the flagella for reprogramming the pattern of active sliding.

In this regard, calmodulin, the likely axonemal Ca sensor (Reed et al., 1982; Otter et al., 1984; Stommel, 1984; Brokaw and Nagayama, 1985; Izumi and Nakaoka, 1987), is distributed along the entire length of *Paramecium* and *Tetrahymena* cilia (Maihle et al., 1981; Ohnishi et al., 1982). These protozoan cilia also undergo Ca-dependent ciliary reversal. The second messengers, Ca and cAMP, are thought to regulate axonemal motility by phosphorylation/dephosphorylation of ciliary proteins, particularly dynein subunits (Brokaw, 1987; Bonini et al., 1991; Hamasaki et al., 1991; Stephens and Prior, 1992; Salathe et al., 1993; Walczak and Nelson, 1994). The molecular mechanisms of Ca or cAMP control are still unknown.

An interesting exception to the findings on comb plate cilia and *Chlamydomonas* flagella is macrocilia of the ctenophore *Beroë*. Macrocilia are finger-shaped organelles, 25–30 μm or 80–100 μm long, with a common fused membrane surrounding a shaft of hundreds or thousands of axonemes, depending on the species (Tamm and Tamm, 1993). However, a rete of unfused, individual ciliary membranes is present at the base of all macrocilia (Tamm, 1988*b*). Depolarization-gated Ca influx activates rapid beating of usually quiescent macrocilia without altering beat direction (Tamm, 1988*a*). Local iontophoretic application of Ca at different sites along Ca-permeable macrocilia of heat-dissociated cells showed that Ca does not enter along the entire ciliary shaft (as in comb plates), but only at the base of the macrocilium where the membranous rete is located (Tamm, 1988*b*). Similar Ca iontophoresis experiments on demembranated ATP-reactivated macrocilia showed, surprisingly, that Ca-sensitivity for activating bending is not restricted to the base of the axonemes but occurs along their entire length (Tamm and Tamm, 1989).

Does Ca normally enter macrocilia only at the base, and act there to trigger beating? If so, activation of beating may require a different distribution of axonemal Ca sensors than does change in beat orientation. Or does Ca diffuse from the rete to the tip of the macrocilium to induce beating, thereby explaining the base-to-tip Ca sensitivity of reactivated macrocilia? Or does Ca influx occur along the entire length of stimulated macrocilia on cells of the intact tissue? Further studies on macrocilia using our Ca-Green dextran method should resolve these questions. Nevertheless, these findings suggest that the spatial distribution of ciliary membrane Ca channels and axonemal Ca sensors need not coincide to elicit a motor response.

Finally, it should be noted that increases in beat frequency of vertebrate tracheal and oviduct cilia in response to mechanical, hormonal or transmitter stimulation are mediated by release of Ca from intracellular stores (Verdugo, 1980; Sanderson et al., 1990; Boitano et al., 1992). Voltage-dependent ciliary Ca channels are apparently lacking in these cells. However, given the short length of the cilia (~6 μm) and their slow response time (Sanderson and Dirksen, 1986), a second messenger-mediated increase in cytosolic Ca apparently delivers sufficient Ca to the cilia to augment beat frequency without the need for rapid Ca influx across the ciliary membrane.

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