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Calcium sensitivity extends the length of ATP-reactivated ciliary axonemes

(Ca-sensitive sites/*Beroë* macrocilia)

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ABSTRACT **We use the Ca-dependent activation response of macrocilia of the ctenophore *Beroë* to map the distribution of Ca sensitivity along axonemes of detergent-extracted ATP-reactivated models. Local iontophoretic application of Ca (or Sr or Ba) to any site along the length of demembranated macrocilia in ATP–Mg solution elicits oscillatory bending. Bending responses are localized to the site of application of these cations and do not propagate. Ca sensitivity for initiating bends is, therefore, distributed along the entire length of the axonemes. Since Ca triggers ATP-dependent microtubule sliding disintegration of macrociliary axonemes, a Ca-sensitive mechanism for activating microtubule sliding extends the length of the axonemes. In contrast, local application of Ca to living dissociated macrociliary cells elicits beating only when applied to the base of the macrocilium, indicating that the effective site of Ca entry is localized to the membrane at the ciliary base. Therefore, the spatial distributions of membrane Ca permeability and axonemal Ca sensors do not coincide.**

Calcium ions (Ca) play a key role in signaling various modifications of ciliary and flagellar motility in response to environmental stimuli. Ca-triggered behavioral responses include: reorientation of ciliary beat direction (1–3), reversal of flagellar wave direction (4), alteration of flagellar waveform (5–8), arrest of beating (9–11), and activation of beating (12, 13).

Stimulus-evoked increase in intraciliary Ca concentration is mediated by voltage-gated Ca channels in the ciliary membrane (14–16). Electrophysiological recording from ctenophore comb plate cilia showed that the Ca conductance controlling reversal of beat direction is distributed over most of the length of the ciliary membrane (17).

Macrocilia of the ctenophore *Beroë* are compound organelles containing hundreds (or thousands, depending on species) of "9+2" ciliary axonemes enclosed by a single common membrane (18), except at the base (19). Macrocilia are usually quiescent but are activated to beat rapidly and continuously by depolarization-gated Ca influx (13). Extracellular application of Ca to various sites along macrocilia of heat-dissociated cells indicated that Ca permeability for triggering beating is localized to the basal region of the macrociliary membrane, corresponding to a rete of unfused ciliary membranes (19).

Little is known about the site(s) and mechanism(s) of action of Ca within the axoneme. Detergent-permeabilized models of macrocilia that require micromolar levels of free Ca, Ba, or Sr for ATP reactivation of beating and/or microtubule sliding disintegration have been devised (20). In this report we iontophoretically apply these cations at various regions along demembranated macrocilia in ATP to map the distribution of the Ca-sensitive activation mechanism along the axonemes.

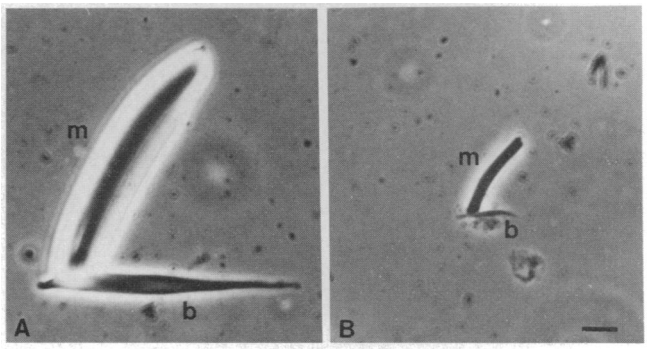

FIG. 1. Phase-contrast micrographs (at same magnification) of detergent-extracted macrocilia (m) with attached actin bundles (b) isolated from *B. mitrata* (*A*) and *B. ovata* (*B*). Macrocilia of *B. mitrata* are considerably larger than those of *B. ovata*. The plane of potential bending is parallel to the page and the effective stroke direction is to the right. Macrocilia lie at rest at the end of the effective stroke. (Bar = 10 μm.)

MATERIALS AND METHODS

Organisms and Macrocilia. Macrocilia are found inside the lips of beroid ctenophores (18). *Beroë ovata* and *Beroë mitrata* were collected at Villefranche-sur-Mer, France, in the spring of 1988. Identical results were obtained with both species, but *B. mitrata* was more suitable because of the larger size of its macrocilia (Fig. 1).

Permeabilized Models. Lip pieces bearing macrocilia were washed in Ca-free artificial seawater for 30 min. Tissue was then extracted in 0.05% or 0.1% saponin (*B. ovata*) or 0.05% Brij 58 (*B. mitrata*) with 150 mM KCl or potassium acetate/2.5 mM $MgCl_2$/1 mM EGTA/30 mM Pipes, pH 7.0 (extraction solution, ES) for 10 min at room temperature (20).

Iontophoretic Application of Ca. Detergent-extracted tissue was washed briefly in reactivation solution (RS; 2 mM ATP/2.5 mM $MgCl_2$/1 mM dithiothreitol/150 mM KCl or potassium acetate/1 mM EGTA/30 mM Pipes, pH 6.9). Tissue was then transferred to one drop of RS on a microiontophoresis slide (20) and agitated vigorously to dislodge macrocilia. Alternatively, tissue was transferred directly from ES to RS on a microinotophoresis slide and agitated. Residual lip tissue was discarded, leaving a large number of isolated permeabilized macrocilia.

Iontophoresis pipettes were pulled from microfilament glass capillaries and backfilled with 1 M solutions of $CaCl_2$, $BaCl_2$, $SrCl_2$, $CoCl_2$, $Cd(NO_3)_2$, or NaCl. Pipettes were inserted under the coverslip of the microiontophoresis slide.

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Ca, Ba, Sr, Co, Cd, or Na was electrophoretically applied to various regions of permeabilized macrocilia in RS by positive current pulses (100 V, 200 msec) passed between the pipette and the RS bath. The pipette opening was positioned 2–5 μm from the surface of the macrocilium. Only positive current pulses (which ejected cations) elicited motile re-

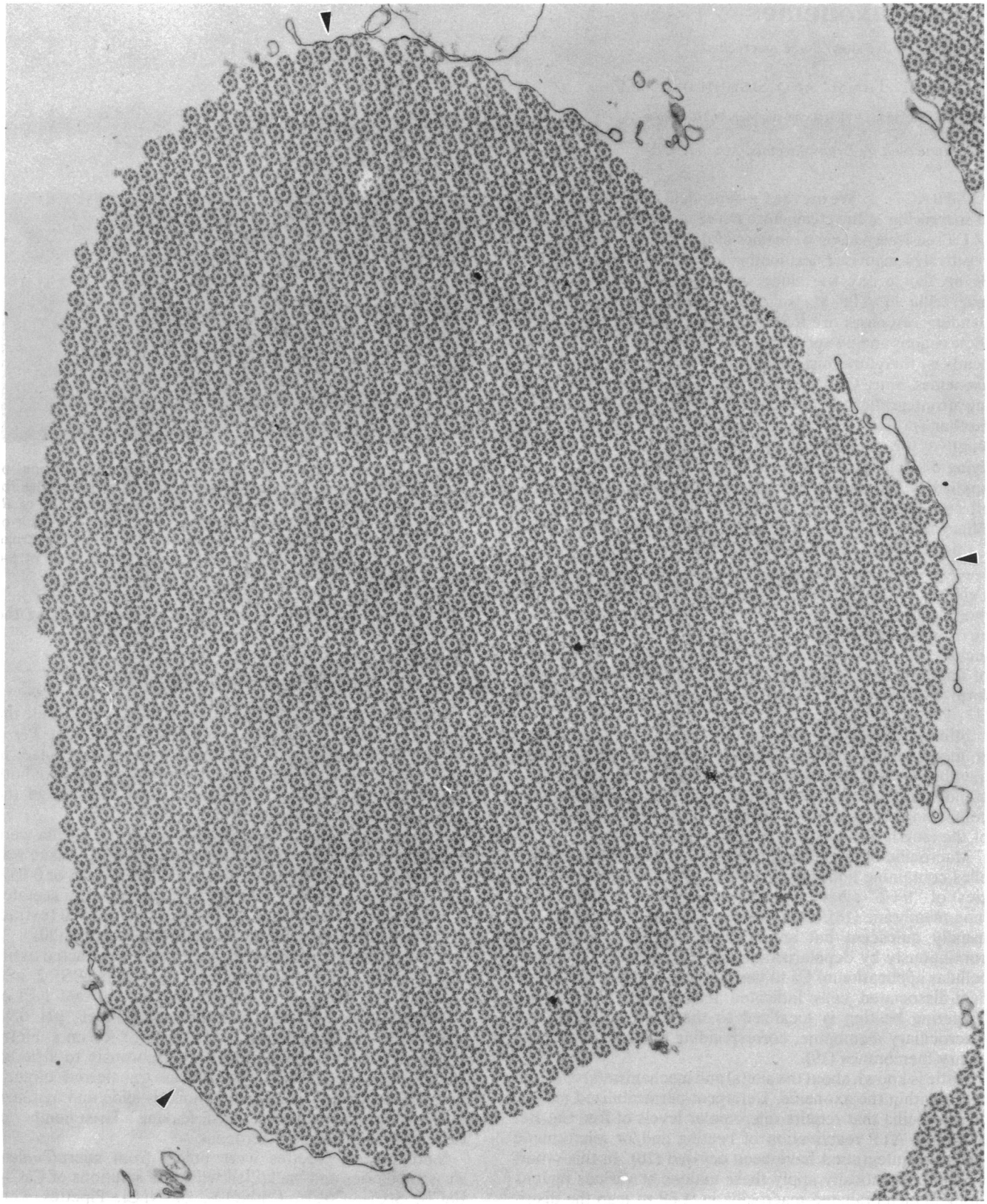

FIG. 2. Electron microscopic thin section through the shaft of a detergent-extracted macrocilium of *B. mitrata* in RS. Several thousand hexagonally packed axonemes are cross-linked into parallel rows running normal to the plane of beat (left to right). Remnants of the common membrane are visible at the periphery of the shaft (arrowheads). Effective stroke direction is to the right; recovery stroke direction is to the left.

sponses; negative current pulses had no effect. Of more than 125 macrocilia tested, those that initially responded to Ca, Ba, or Sr by bending instead of sliding apart (20) were studied here.

RESULTS

Detergent-treated macrocilia freed from the tissue typically remain anchored to their large actin bundles (20, 21). Macrocilia of *B. mitrata* are 80–100 μm long and 12–15 μm in diameter, considerably larger than those of other beroids (Fig. 1). In RS lacking Ca, Ba, or Sr, macrocilia rest at the end of the effective stroke, pointing ≈60° to the long axis of the wedge-shaped actin bundle (Fig. 1) (13, 20, 22). Macrocilia lie on the microscope slide with their actin bundles parallel to the surface—i.e., with their potential bend plane parallel to the slide.

Thin sections through detergent-extracted macrocilia show that the common peripheral membrane around the shaft (18, 22) is greatly disrupted or entirely missing (Fig. 2). Macrocilia of *B. mitrata* contain ≈10 times more axonemes (≈3000; Fig. 2) than macrocilia of other species (≈300; ref. 22).

Motile Responses of Models to Uniform Increase in Ca, Ba, or Sr. As reported (20), addition of 10^{-5} M Ca, Ba, or Sr to RS reactivates beating of detergent-extracted macrocilia. Isolated macrocilia with actin bundles beat vigorously and "swim" in circles initially, and then undergo microtubule sliding disintegration (20). The form of the reactivated beat cycle (Fig. 3) resembles that of living macrocilia (13, 22).

Motile Responses of Models to Local Iontophoretic Application of Ca, Ba, or Sr. We tested motile responses of isolated models in ATP to local iontophoretic application of Ca, Ba, or Sr at the base, midway along the shaft, and at the tip of the macrocilium. Iontophoretic application of Ca, Ba, or Sr to any site along the length of detergent-extracted, effective-pointing macrocilia in RS elicits a bending response (Figs. 4–6). The bends occur in the normal beat plane and in the direction of the recovery stroke. Most strikingly, the bends are localized to the vicinity of the pipette opening, showing the local nature of the applied cations.

For example, single positive current pulses of Ca, Ba, or Sr delivered to the base of a macrocilium induce a sudden increase in bend angle between the macrocilium and the actin bundle, causing a rapid flexion of the entire shaft in the recovery stroke direction (Figs. 4–6). The bend does not propagate distally, and the shaft remains fairly straight during its excursion, relaxing slowly to the rest position. Single stimuli evoke single bends or sometimes a series of rapid oscillations of similar nature, the first being the strongest and the others decreasing in amplitude (Fig. 6).

Ca, Ba, or Sr pulses applied midway along the shaft trigger local flexures with the concave side of the bend facing the recovery stroke direction and the center of curvature near the pipette opening (Figs. 4–6). The bends do not propagate distally. Single stimuli often elicit a series of three to five rapid oscillations of progressively smaller amplitude (Fig. 6). The total duration of the response is typically 0.5–1 sec, and maximum frequency is 10–15 Hz.

Application of Ca, Ba, or Sr to the tip of a macrocilium causes the distal part of the shaft to lift in the recovery stroke direction, with the proximal part remaining inactive (Figs. 4–6). Relaxation is often followed by several rapid repeated bends of similar nature but diminishing intensity, the total response lasting 0.5–1 sec.

In all cases, additional Ca, Ba, or Sr pulses elicit repeated bending responses, showing that ejected cations are rapidly chelated and that the continual presence of Ca, Ba, or Sr is required to maintain motility.

Repeated Ca, Ba, or Sr pulses to the tip of the macrocilium cause the tip to curl toward the effective stroke side, then to straighten slowly, and relax to the rest position. Sr and Ba elicit "tip curling" more readily than does Ca. Application of

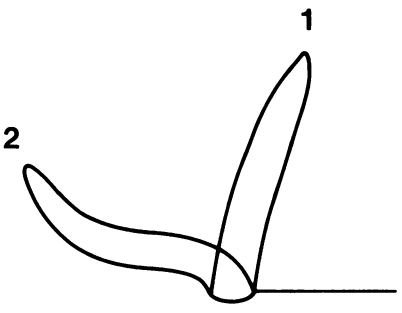

FIG. 3. Form of the beat cycle of an isolated detergent-extracted macrocilium of *B. mitrata* reactivated in an RS/10^{-4} M Sr bath. Positions at the ends of the effective stroke (position 1) and recovery stroke (position 2) are shown. The macrocilium beat at 20–30 Hz, causing the cilium/actin bundle complex to "swim" in circles.

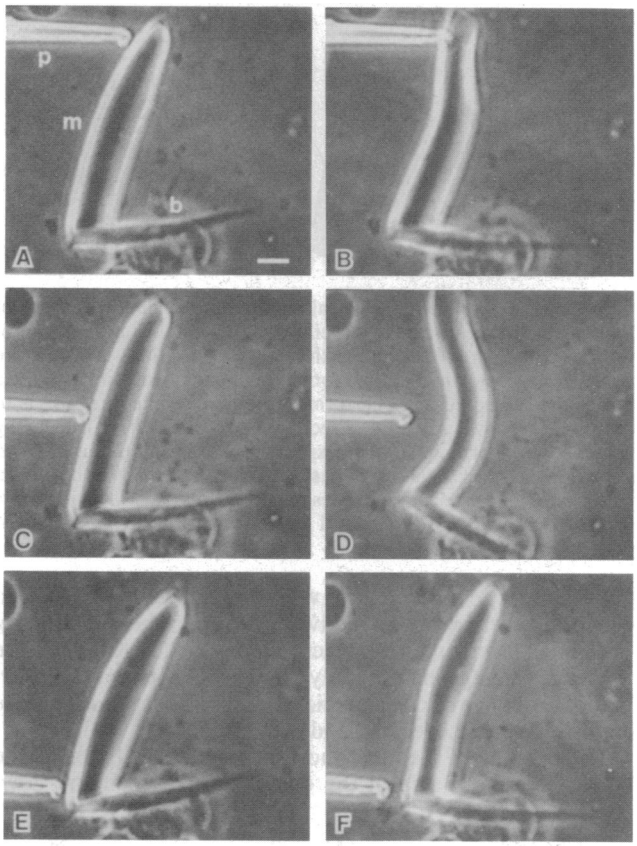

FIG. 4. Video fields showing reactivated bending responses of an isolated detergent-extracted macrocilium (m) of *B. mitrata* to iontophoresis of Ca at the tip (*A* and *B*), midway (*C* and *D*), and at the base (*E* and *F*). (*A*, *C*, and *E*) Rest positions at the end of the effective stroke in RS before current was pulsed through the Ca pipette (p). (*B*, *D*, and *F*) Maximum bending excursions after Ca pulses. Effective stroke direction is to the right; recovery stroke direction is to the left. The wedge-shaped actin bundle (b) is attached to the base of the macrocilium. A Ca pulse to the tip elicits flexure of the distal part of the macrocilium in the recovery stroke direction (*B*), followed by five rapid oscillations of similar nature but diminishing amplitude (data not shown). A Ca pulse delivered midway along the shaft triggers a strong recovery bend centered near the pipette opening, plunging the actin bundle downward (*D*). This was followed by three or four rapid bends of progressively weaker amplitude at the same location (data not shown). Total duration of bending responses in *B* and *D* was about 1 sec. A Ca pulse at the base induces a sudden increase in bend angle between the entire shaft and the actin bundle (*F*). This macrocilium is example 1 in Fig. 5. (Bar = 10 μm.)

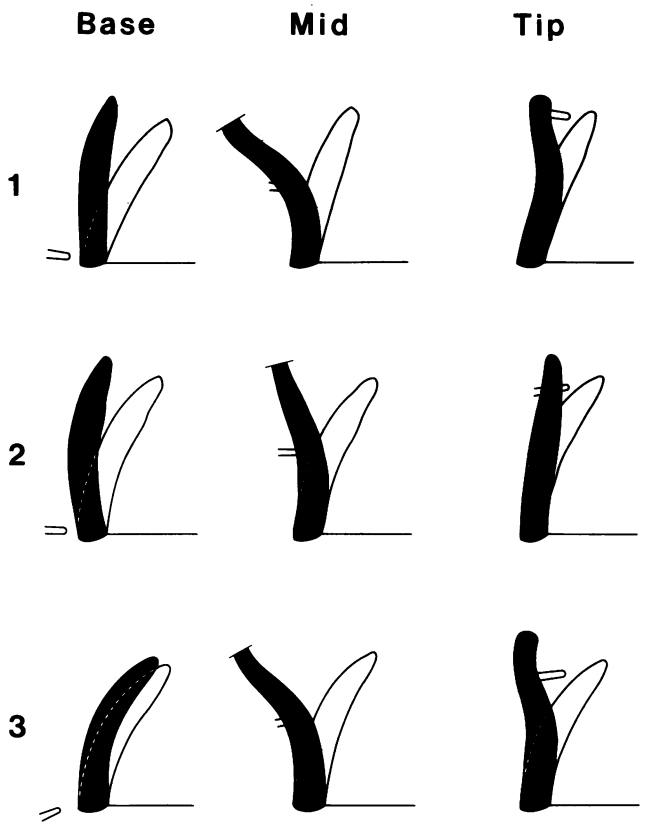

FIG. 5. Video profiles of local reactivated bending responses of three detergent-extracted macrocilia of *B. mitrata* to iontophoresis of Ca at the base, midway, and at the tip. Rest positions in RS before Ca pulses are shown in outline forms; maximum excursions after Ca pulses are indicated by solid forms. Effective stroke direction is to the right; recovery stroke direction is to the left. All bending responses are normalized with respect to the long axis of the attached actin bundle (horizontal line at base). Each column shows the typical pattern of local bending responses to application of Ca at a given site (pipettes shown). Macrocilium 1 is shown in Fig. 4. (See text for description.)

Ca, Ba, or Sr to macrocilia in wash solution lacking ATP also causes tip curling (but not active bending, see below). Curling of the distal region of the macrocilium is, therefore, not due to active sliding but probably represents a passive conformational change of doublet microtubules in response to these cations (unpublished data and ref. 23).

The opening of the iontophoresis pipette usually faced the convex recovery side of the stimulated macrocilium; however, the same pattern of bending responses was obtained when the pipette faced the opposite concave side of the shaft (data not shown). The form of the motile responses is, therefore, independent of the direction in which the cations are applied.

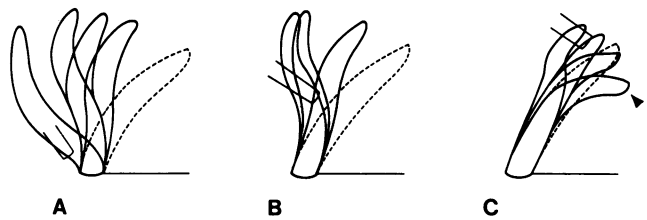

FIG. 6. Localized oscillations of a detergent-extracted macrocilium of *B. mitrata* after iontophoresis of Sr to the base (*A*), midway (*B*), and at the tip (*C*). Dashed profile shows resting position in RS before Sr pulses. Continuous line profiles show successive rapid oscillations of diminishing amplitude after single Sr pulses. Bends are localized in the vicinity of the Sr pipet position. Bending responses to Sr are similar to those induced by Ca (cf. Figs. 4 and 5); the (ATP independent) inward curling of the tip is also shown (arrowhead) (*C*). Effective stroke direction is to the right; recovery stroke direction is to the left. Motile responses are normalized with respect to the actin bundle (horizontal line at base).

Detached Macrocilia. Motile responses of macrocilia broken off from the actin bundle were also tested. Ca pulses delivered to the tip or midway along the shaft of detached macrocilia cause local oscillatory flexures similar to those described above. However, application of Ca to the base of detached macrocilia does not initiate bending. Evidently, attachment of the macrocilium to the actin bundle is necessary for manifestation of an active basal response, perhaps by providing shear resistance needed to convert sliding into bending (24).

Controls. The focal nature of iotophoretically applied Ca, Ba, or Sr was shown in several ways. (*i*) If the pipette opening is backed a short distance away from the surface of the macrocilium, positive current pulses no longer elicit bending responses; moving the pipette tip back to the macrocilium once again elicits motility. (*ii*) Application of Ca to one macrocilium of a side-by-side pair triggers motility only of the macrocilium next to the pipette opening. (*iii*) In macrocilia that are split longitudinally into several fragments (20), only the fragment nearest the pipette opening responds to Ca, Ba, or Sr pulses. (*iv*) And most conclusively, cations applied at various sites along the shaft elicit regional bends that are localized to the vicinity of the pipette opening.

Iontophoretic application of Ca, Ba, or Sr to detergent-extracted macrocilia in wash solution lacking ATP does not elicit bending responses, confirming that ATP is required for Ca, Ba, or Sr-induced reactivation of motility (20).

Iontophoretic application of Na, Co, or Cd to any region along macrocilia in RS did not elicit bending responses. Therefore, the current or voltage associated with iontophoresis is not responsible for activation of motility.

DISCUSSION

In many cilia and flagella some parameter of motility is regulated by membrane-mediated Ca influx (1–13). Ca activates macrocilia of *Beroë* to beat rapidly and continuously, as shown in living macrociliary cells (13) and detergent-permeabilized ATP-reactivated models (20).

In this report we found that local iontophoretic application of Ca, Sr, or Ba to any site along detergent-permeabilized macrocilia in Mg–ATP can elicit a bending response. The motile responses are localized to the vicinity of application of these cations. Ca, Sr, or Ba sensitivity for initiating bending of macrocilia, therefore, occurs along the entire length of the axonemes.

In contrast, brief reports or abstracts of similar experiments performed on detergent-extracted *Paramecium* cilia (25) and sperm flagella (26, 27) indicate that Ca sensitivity for inducing ciliary reorientation, flagellar arrest, and/or change in waveform is localized to specific regions of the axoneme. However, Ca sensitivity for activating tremulous beating of *Paramecium* ciliary models appears to be distributed almost equally between the base and the tip of the short axonemes (25).

Different types of Ca-induced motor responses of cilia and flagella may, therefore, require different distributions of Ca-regulatory molecules along the axoneme. In this regard, it will be informative to compare the distribution of axonemal Ca sensitivity in macrocilia, which show an activation response, to that in ctenophore comb plates, which undergo Ca-dependent ciliary reversal (17).

Distribution of Membrane Ca Channels vs. Axonemal Ca Sensors. Heat-dissociated macrociliary cells are permeable to external Ca without depolarizing stimuli and beat spontane-

ously in seawater (13). Local iontophoresis of Ca to quiescent macrocilia of isolated cells in Ca-free artificial seawater elicited beating only when applied to the base of the macrocilium (19). This region coincides with a reticulum of unfused ciliary membranes, the ciliary rete, which is continuous with the external seawater (19). These findings indicated that the effective site of Ca entry in dissociated macrociliary cells is localized to the basal ciliary rete. However, we could not rule out the possibility that the Ca conductance is uniformly distributed along the macrociliary membrane but that the Ca-sensitive activation mechanism is restricted to the base of the axonemes.

Our demonstration that Ca sensitivity for eliciting bending extends the entire length of demembranated axonemes eliminates this alternative. Instead, the basal sensitivity of macrocilia of dissociated cells to external Ca reflects localization of Ca permeability to this region of the membrane.

One may then ask why axonemal Ca sensitivity is distributed from base to tip. One explanation is that heat-dissociated cells do not reveal the actual Ca conductance of macrocilia *in situ*. For example, voltage-dependent Ca channels may exist along the entire membrane of the shaft but not be "opened" in heat-dissociated cells. Nevertheless, Ca influx solely at the base of macrocilia of dissociated cells is sufficient to trigger cycles of normal beating (19), arguing that this may also be true for macrocilia on the intact epithelium. If so, does Ca, which enters at the base, move rapidly toward the tip, binding to Ca sensors along the shaft? Or does Ca remain and act at the base, making the distal Ca sensitivity of axonemes redundant? Whatever the explanation, our findings clearly show that the spatial distribution of ciliary membrane Ca permeability and axonemal Ca sensors need not coincide to elicit a motor response.

Mechanism of Ca Action. The finding (20) that Ca, Ba, or Sr can trigger ATP-dependent microtubule sliding disintegration indicates that Ca-induced local bending described here is also caused by active microtubule sliding. However, the form of our bends (single curvature close to the Ca source) differs from those of demembranated "rigor" sea urchin sperm flagella responding to local iontophoretic application of ATP (oppositely directed bends on either side of a straight region next to the ATP pipette) (28).

Nevertheless, our results confirm that *in vivo* Ca acts to turn on the dynein-powered sliding process responsible for ciliary motility. The mechanism(s) by which Ca triggers other types of ciliary and flagellar motor responses, such as reorientation of beat direction or modification of waveform, is less clear.

Studies using calmodulin antagonists with reactivated models indicate that calmodulin plays a role in triggering Ca-dependent ciliary activation (12, 29), as well as other Ca-mediated axonemal responses (12, 30–34). In addition, fluorescence and immunoelectron microscopy show that calmodulin is distributed along the entire length of whole cilia (35) and outer-doublet microtubules (36).

Calmodulin is, therefore, a strong candidate for the axonemal Ca sensor in macrocilia. How it may act to switch on dynein-generated microtubule sliding remains an important problem.

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