

# Immune Localization of Calmodulin in the Ciliated Cells of Hamster Tracheal Epithelium

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**ABSTRACT** Metachronal beating of cilia of epithelial surfaces of most respiratory airways moves the overlying mucous layer in a caudal direction. The molecular mechanisms controlling ciliary beat remain largely unknown. Calcium, an element in its cationic form, is ubiquitous in biological functions and its concentration is critical for ciliary beating. Calmodulin, a calcium-binding protein which regulates the activity of many enzymes and cellular processes, may regulate ciliary beating by controlling enzymes responsible for mechanochemical movement between adjacent peripheral microtubule doublets composing the ciliary axoneme. As a first step in describing a calmodulin-related controlling mechanism for ciliary beating, calmodulin was localized in the ciliated cells lining the respiratory tracts of hamsters by electron microscopy, using an indirect immunoperoxidase technique with anticalmodulin antibodies as the molecular probe. Thin-sections revealed calmodulin located on microtubules and dynein arms of the ciliary shaft, basal body, apical cytoskeletal microtubules, and plasma membranes in specimens fixed with 1 mM  $\text{Ca}^{+2}$ . Specimens fixed with less  $\text{Ca}^{+2}$  (1  $\mu\text{M}$ ),  $\text{Mn}^{+2}$ ,  $\text{Mg}^{+2}$ , and EGTA showed a diffuse pattern of calmodulin with loci of greatest densities on basal body microtubule triplets. Demembrated specimens showed a less specific localization on axonemal microtubules but only on cells fixed with  $\text{Ca}^{+2}$ . Calmodulin, by binding calcium, may function in ciliary beating in the respiratory tract of mammals either directly or indirectly through its effects on the energy-producing enzymes and by control of  $\text{Ca}^{+2}$  flux through plasma membranes.

The epithelial lining of respiratory airways contains a large number of ciliated cells responsible for mucous clearance mediated by metachronal ciliary beating. The cilia on the surface of these cells are anchored into the apical cytoplasm by basal bodies, centriole counterparts (5, 26). Microtubules and microfilaments form an integrated system linking the ciliary axoneme and basal body to the cytoskeleton (8).

The mechanism(s) controlling ciliary beat initiation, frequency, and synchronization is unknown, but changes in beat pattern of cilia and flagella are due to calcium ion concentrations, e.g., ciliary reversal in *Paramecium* (4) and *Tetrahymena* (29), ciliary arrest in molluscan gill (24), flagellar wave form change in *Chlamydomonas* (25), reversal of the flagellar wave propagation in *Critidia* (13), asymmetrical (2) and intermittent (6, 7) flagellar beating in sea urchin sperm, mammalian ciliary beat frequency change (3) and activation (21) and in vitro (14). Ohnishi et al. (23) suggested that calcium ion effect may be a two-step phenomenon: (a) an influx of  $\text{Ca}^{+2}$  into the cilia or

flagella and (b) a specific effect on the motile apparatus of the cilia or flagella. Calmodulin may be a specific intermediate target molecule for the calcium effects on cilia and flagella described above.

Calmodulin—a small, acidic calcium-binding protein that modulates numerous enzymatic reactions and the assembly and disassembly of microtubules, specifically in the mitotic spindle (20)—may participate in the mechanism of ciliary beating. A protein isolated from *Tetrahymena* cilia had characteristics similar to those of calmodulin (15–17, 27). Ohnishi et al. (23) isolated calmodulin from *Tetrahymena* cilia and in isolated axonemal preparations showed its location on interdoublet cross-bridges. Previously, calmodulin has been demonstrated in cilia of protozoa by immunofluorescence (18, 28) and in centrioles by immunofluorescence and immunoperoxidase at the electron microscopic level (21). In this paper, we present evidence that a calmodulin or a calmodulin-like molecule observed by immunoelectron microscopy using an anti-

calmodulin antibody is present on ciliary plasma membranes, axonemal components, and in the apical cytoplasm in the ciliated cells of the hamster respiratory tract.

## MATERIALS AND METHODS

For our immunological studies it was found necessary to purify and characterize the antigen, elicit the antibody and purify it, establish reactivities with its specific antigen, and apply it to properly prepared tissue under investigation. The protocols and essential steps of the various procedures are noted below.

### Calmodulin Preparation

Calmodulin was prepared from fresh chicken gizzards by an isolation procedure described previously (30, 31). Briefly, gizzards were weighed, homogenized in the appropriate buffer, and centrifuged at 10,000 g for 20 min. The supernatant was saved, made 60% saturated with ammonium sulfate, and centrifuged. The supernatant's pH was adjusted to 4.0 with 50% H<sub>2</sub>SO<sub>4</sub> and centrifuged. The pellet was resuspended, the pH was adjusted to 7.5, and the pellet was dialyzed and centrifuged at 29,000 g for 60 min. The supernatant was applied to DEAE Sephadex A-50 and washed with five-column volumes of buffer. The protein fractions eluted with 1 vol of 0.8 M KCl from the column were pooled, dialyzed, lyophilized, resuspended in a small volume, and passed through a CAPP-Sepharose column. Proteins eluted with EGTA-buffer were pooled, dialyzed, and lyophilized. Protein purity was verified on 5–15% SDS on polyacrylamide slab gel electrophoresis with samples containing Ca<sup>2+</sup> or EGTA.

### Preparation of Anticalmodulin Antibody

Anticalmodulin antiserum was prepared by the technique of Van Eldik and Watterson (32). Performic acid oxidation of calmodulin was done as described by Hirs (12) to increase its antigenicity. The performication procedure was as follows: the performic acid reagent was prepared by adding 0.5 ml of 30% (vol/vol) hydrogen peroxide to 9.5 ml of formic acid, incubating the covered solution at room temperature for 2 h, then chilling it on ice for 30 min. The purified calmodulin was dissolved in 1 ml of formic acid, incubated on ice for 30 min, 3 ml of the performic acid reagent was added to it, the reaction was allowed to proceed for 150 min on ice, and then the mixture was diluted with water and rotary-evaporated. Before injection, the residue powder was dissolved in water (2 mg/ml) and emulsified with equal volumes of either complete Freund's adjuvant (initial injection) or incomplete Freund's adjuvant (subsequent injections).

Three New Zealand white, *Pasteurella*-free female rabbits were injected twice during the first week and three times in the second. A total of 0.5–1.0 mg of vertebrate calmodulin was administered to each rabbit per injection. Affinity adsorption chromatography of the antiserum was performed on columns of calmodulin-Sepharose conjugates previously equilibrated with PBS, removal of adventitiously bound material by elution with PBS, then elution of antigen-adsorbed immunoglobulins with 2 M MgCl<sub>2</sub>. Calmodulin-Sepharose conjugates were prepared as described by Watterson and Vanaman (33). Cyanogen bromide-

activated Sepharose was obtained from Pharmacia Fine Chemicals (Div. of Pharmacia, Inc., Piscataway, NJ).

Before treatment of tissue with this antibody, the antibody was first tested for its calmodulin titer and reactivity by a modification of the ELISA procedure. Since calmodulin attached poorly to plastic dishes, the surface was precoated with a protein solution containing 10 µg/ml of clathrin-associated protein (22) that coats the polystyrene surface well and has high binding affinity for calmodulin only in the presence of calcium. An antibody (the generous gift of Dr. Watterson, Rockefeller University, New York) already shown to be specific (32) served to standardize the procedure and to compare our antibodies in tissue labeling.

### Immunohistochemical Treatment for Electron Microscopy

After removal from hamsters, 24 tracheas were sliced on cross section into 52 rings with a double-edged razor blade. 24 rings were divided into three groups, which were treated as follows: (a) with Saponin, 1%, in PBS, pH 7.2; (b) with Triton X-100, 1%, in PBS, pH 7.2; and (c) untreated. Each group was fixed in 2% paraformaldehyde with 0.037 M phosphate-buffered at pH 7.2 and with the addition of 1 mM or 1 µM of CaCl<sub>2</sub>, MgCl<sub>2</sub>, MnCl<sub>2</sub>, or CaCl<sub>2</sub> with 2 mM EDTA. After 1-h fixation, tracheal rings were washed in PBS with 10% sucrose.

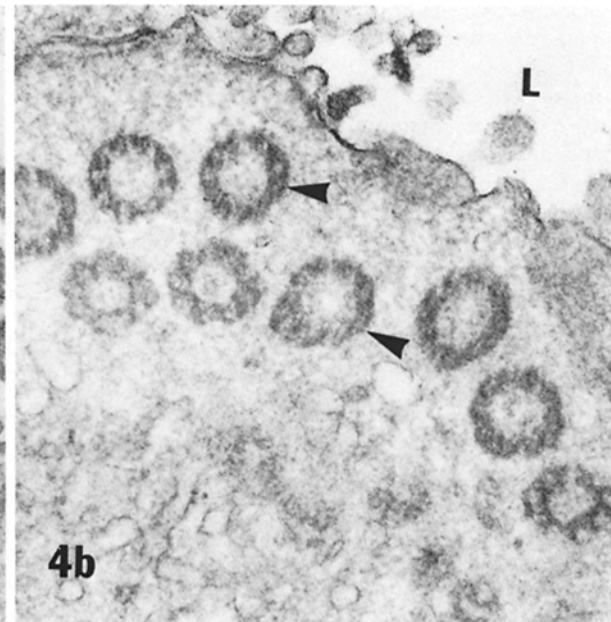
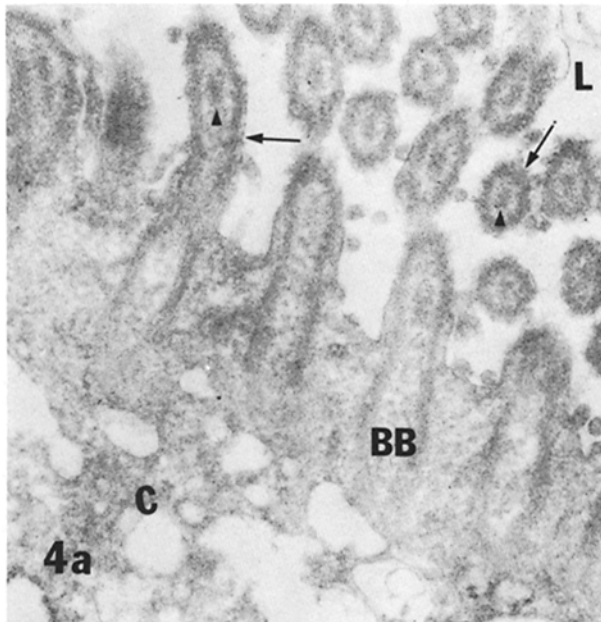
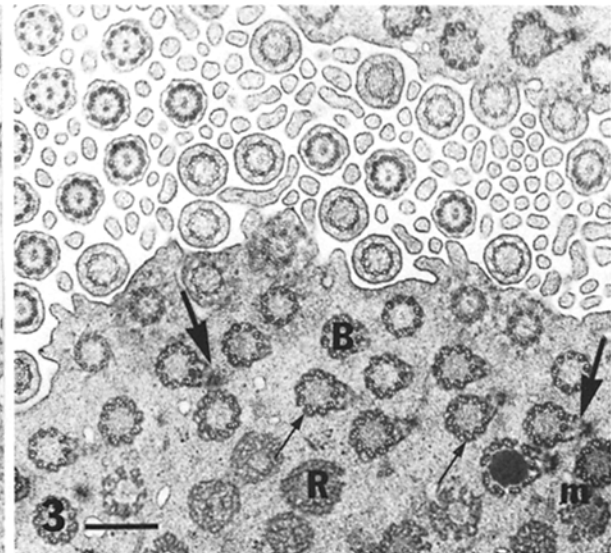
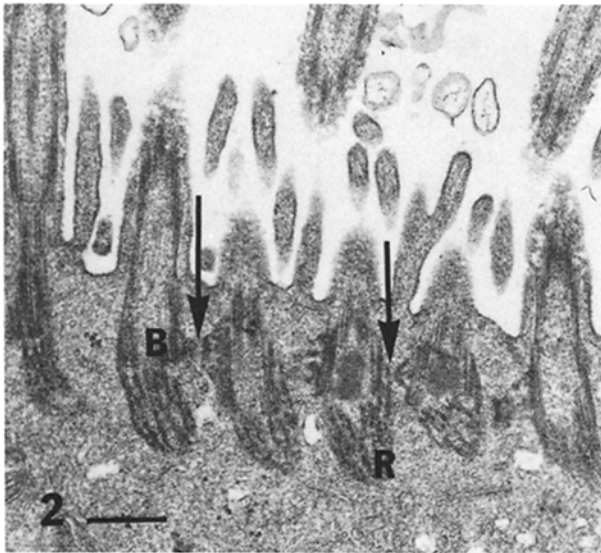
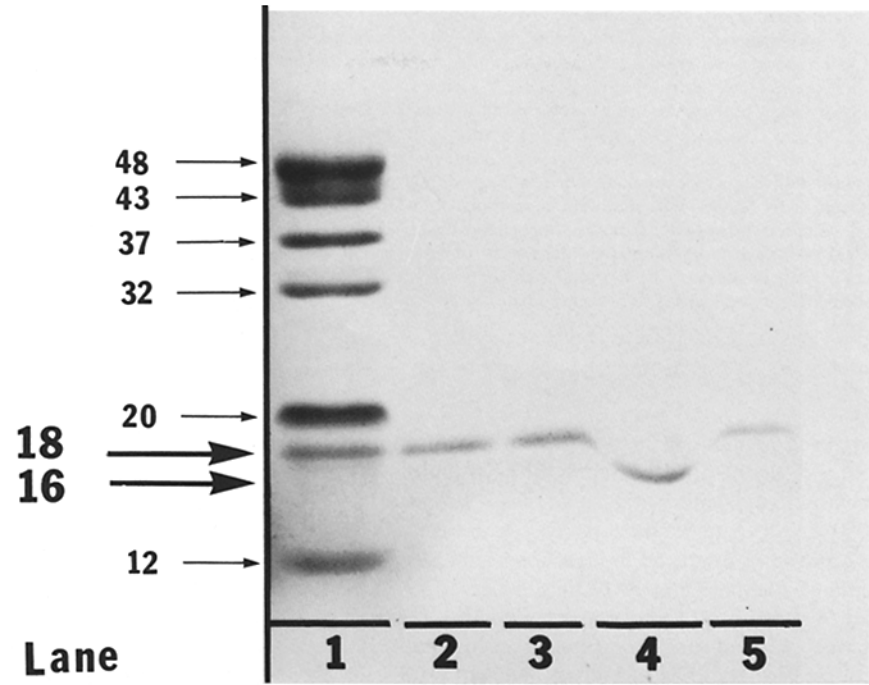
Washed tissues not treated with Saponin or Triton X-100 were frozen in liquid nitrogen and thawed to mildly disrupt the plasma membrane and to facilitate antibody penetration. Tracheal rings from each group were treated as previously described (9, 10). Briefly, the wet tissue was transferred to 1:40 PBS dilution of the rabbit anticalmodulin (9 µg/ml antibody final concentration) and incubated at 37°C for 2 h. Specimens removed from the diluted antiserum were washed for 30 min in three changes of PBS sucrose, transferred to a 1:40 dilution of sheep Fab<sub>2</sub> antirabbit IgG (SARG; 1.25 µg/ml) coupled to horseradish peroxidase (glutaraldehyde fixed), and incubated for 2 h at 37°C. After three 20-min washes in PBS–10% sucrose, the tissues were treated with 3% glutaraldehyde in PBS–10% sucrose for 30 min to fix the antibodies to their specific binding sites. The fixative was thoroughly washed out with three changes of PBS-sucrose for periods in excess of 2 h and incubated for 7–10 min in an 0.5% solution of 3,3'-diaminobenzidine tetrahydrochloride (DAB) to which H<sub>2</sub>O<sub>2</sub> had been added to make a final concentration of 0.01%. All specimens were treated with unbuffered osmium tetroxide (OsO<sub>4</sub>) to render the D reaction product electron-dense, dehydrated with ethanol, and embedded in Epon 8.

Control specimens consisted of (a) tracheas treated with normal preimmune rabbit serum (NRS) in place of the specific antiserum and sequentially treated with SARG and DAB, (b) tracheas treated with SARG followed by DAB, (c) tracheas treated with DAB alone, and (d) tracheas treated with anticalmodulin absorbed with purified calmodulin (100 µg/ml) and diluted to 1:40. After incubation with absorbed serum the tissues were treated sequentially with SARG and DAB.

Sections cut 1 µm thick from all blocks were examined with a light microscope. The presence and distribution of dense precipitates in ciliated cell were evaluated under double-blind conditions. Representative areas of labeled cells were thin-sectioned perpendicular and parallel to the plane of the epithelium to adequately

FIGURES 1–4 Fig. 1: SDS polyacrylamide gel slab showing in (lane 1) semicrude preparation before affinity chromatography through a CAPP column; calmodulin after elution from CAPP column (lane 2), 18,000 daltons; calmodulin and EDTA after elution from CAPP column (lane 3); calmodulin and calcium after elution from CAPP column (lane 4), 16,000 daltons. Gels were run at constant current for ~3 h, fixed, and stained with Coomassie Brilliant Blue. Fig. 2: Electron micrograph of ciliated cell cut so that thin sections pass through the long axis of cilia. The structural relationships between the basal bodies (B), rootlets (R), and foot processes (arrows) are demonstrated. Sections were stained with uranyl acetate and lead citrate. Bar, 0.3 µM. × 34,000. Fig. 3: Electron micrograph of a ciliated cell from tracheal epithelium sectioned parallel to the plane of the epithelial surface. This makes it possible to view the structures of the ciliary basal bodies (B) and rootlets (R) in cross section. The relationship between basal bodies and foot processes (large arrows) in the plane of the interbasal body apparatus is also observed. Microtubules (m) are seen extending from foot processes. Electron-dense material is present on the outer surface of the triple microtubules of the basal bodies at the level of the foot process (small arrows). The interbasal body cytoplasm is occupied by filaments. Sections were stained with uranyl acetate and lead citrate. Bar, 0.4 µM. × 24,000. Fig. 4: (a) Electron micrograph of a thin section cut perpendicular to the plane of the epithelium and passing through the long axis of the ciliary shaft and basal body. The tissue was fixed without calcium and then treated with rabbit anticalmodulin and peroxidase coupled to sheep anti-rabbit Fab fragments. Electron-dense material marks sites of precipitation of the peroxidase-catalyzed reaction between peroxide and DAB. The reaction product is osmiophilic and appears black on the micrograph. Calmodulin is present on the triplet microtubules forming the basal body (BB) in the central core and throughout the apical cytoplasm (C). Calmodulin is also on axonemal microtubules of the ciliary shaft which protrude into the lumen (L). They include the central pair (arrowhead) and the surrounding nine doublets (small arrow). Bar, 0.3 µM. × 34,000. (b) Electron micrograph of a thin section cut parallel to the plane of the tracheal epithelium, lining the lumen (L), and showing the axonemes in cross section. The tissue was treated as in a. Calmodulin appears on the triplet microtubules forming the basal body (arrowheads). Bar, 0.25 µM. × 40,000.

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assess the location of the precipitate by electron microscopy. The sections were examined without staining salts of heavy metals.

For comparison of immunohistochemical and conventionally fixed specimens, tracheas of four hamsters were removed and immediately immersed in a fixative solution containing 3% glutaraldehyde buffered with 0.2 M sodium cacodylate at pH 7.4. After overnight fixation, the tissue was washed in a cacodylate sucrose buffer for 2 h. During the wash period, two of the tracheas were cut into 2-mm thick rings and the other two tracheas were cut in 2-mm squares. The cut specimens were postfixed in 1% osmium tetroxide buffered with 0.2 M sodium cacodylate at pH 7.4 for 1 h, dehydrated in graded steps of ethanol, and embedded in Epon 812. Thin-sections were cut from the 2-mm rings with the knife edge perpendicular to the plane of the epithelium. Thin sections of 2-mm<sup>2</sup> specimens were cut in the plane to visualize cilia and basal bodies on cross section of the epithelium. All sections were stained with uranyl acetate and lead citrate.

## RESULTS

The calmodulin preparation used appeared as a distinct band with slightly different mobility representing the protein with and without bound calcium, molecular weight between 16,000 and 18,000 daltons, determined by SDS-gel electrophoresis (Fig. 1). Antibodies produced as described here were judged specific and of adequate titer by techniques described by Van Eldik and Watterson (22) and one adapted in our lab. The normal tissue analyzed for its morphology in longitudinal and cross sections of cilia and basal bodies is shown in Figs. 2 and 3 for comparison with tissues treated for the localization of calmodulin.

Unstained thin sections of ciliated cells treated with anticalmodulin after fixation in paraformaldehyde showed fine electron-dense deposits distributed diffusely throughout the apical cytoplasm with a preference for the triplet microtubules of basal bodies and microtubules in the axoneme, the nine doublets and central pair (Fig. 4*a* and *b*). When calcium was added to the paraformaldehyde at a concentration of 1 mM there was a different distribution seen. Electron densities were seen in close association with apical plasma membranes (Fig. 5). These deposits also were seen on a membrane system found throughout the apical cytoplasm (Fig. 5). The ciliary shaft showed deposits in cross and longitudinal sections in the regions occupied by dynein arms and spoke heads or central sheath (Fig. 6*a* and *b*). Ciliary necklace particles also showed a selective electron density (Fig. 7). Cells fixed with 1  $\mu$ M calcium added to the paraformaldehyde showed a mixture of both distributions described above. Cells treated with magnesium and manganese at both concentrations showed the same distribution as described above for tissue fixed with formaldehyde alone.

Cells treated with either saponin or Triton X-100 showed no differences. Axonemal microtubules showed electron densities throughout representing anticalmodulin binding when calcium, at both concentrations (1  $\mu$ M and 1 mM), was added to the saponin or Triton and paraformaldehyde (Fig. 8*b*). These deposits were also observed on the perimeter of basal body triplets extending onto the basal foot (Fig. 9). When calcium was not added to the Triton X-100 or saponin there were no electron-dense deposits. No deposits were seen on axonemes treated with magnesium or manganese added to the paraformaldehyde or membrane solubilizer.

When EGTA was used in the fixative along with the calcium or in the membrane solubilizers containing calcium, cells did not show any electron-dense deposits. In all cases where electron-dense deposits were seen, they were absent in tissues treated with preimmune serum, anticalmodulin serum absorbed with 100  $\mu$ g/ml purified calmodulin and all other antibody and peroxidase controls (Fig. 10).

## DISCUSSION

The calcium ion concentration appears critical to ciliary function. However, it is not known what mechanism the ciliated cell uses to control its motion. Calmodulin, a ubiquitous protein that activates many calcium-dependent processes, may be the molecule which controls calcium concentrations in the axoneme by its ability to directly bind up to 4 mol of the cation. Calmodulin may also act indirectly to activate many enzymes ultimately responsible for mechanochemical movement of axonemal and cytoskeletal elements.

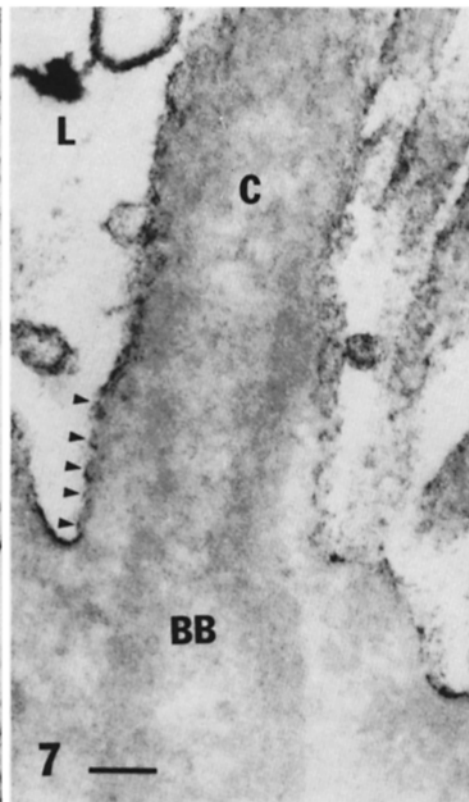
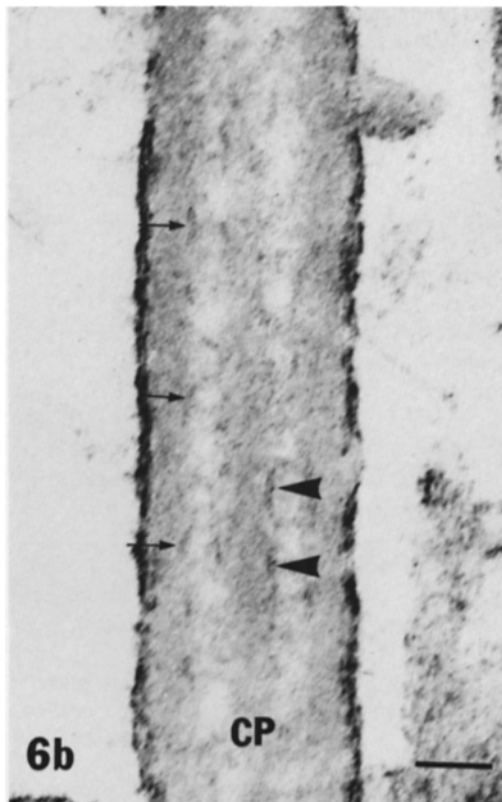
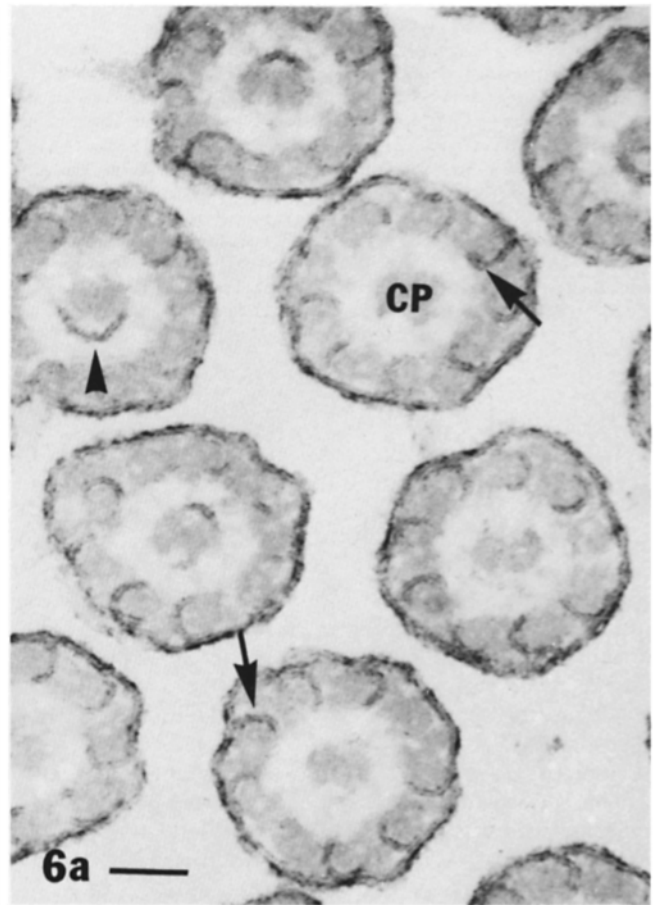
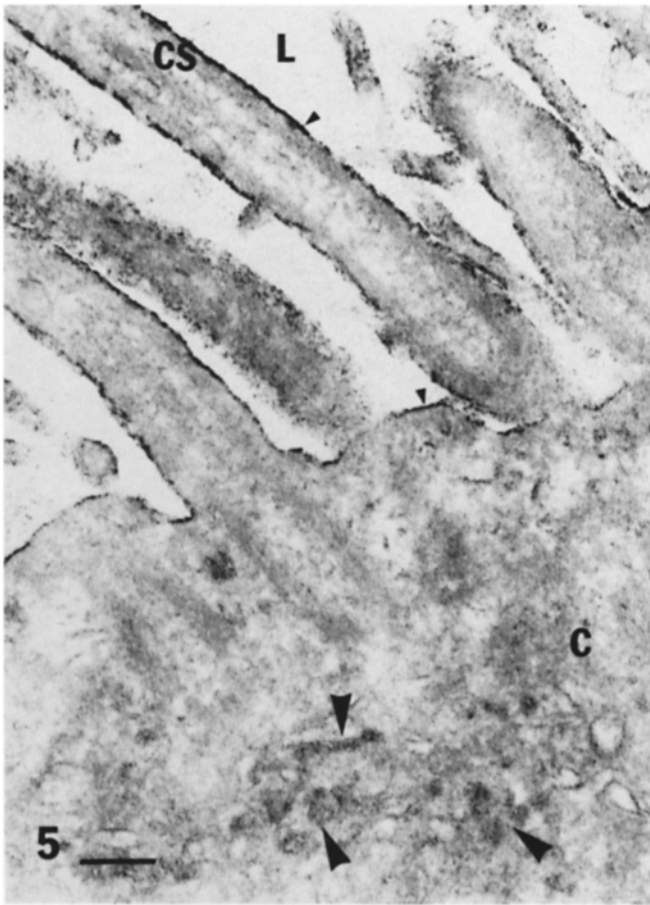
Since the calmodulin yield during isolation procedures is extremely small, it is difficult to purify this protein from the respiratory ciliated cell and cilia unless an extremely large number of animals is used. Therefore, to determine whether these cells contained calmodulin in specific locations, an indirect labeling technique with anticalmodulin antibody produced in our laboratories was used. Modification in the titrating procedure (32) showed the antigenic activities to be high and specific for calmodulin.

With purified anticalmodulin antibodies, we showed the presence of calmodulin in the apical cytoplasm and cilia of ciliated cells of airways from hamster lung. On the basis of a lack of specific calmodulin distribution in the presence of magnesium, manganese, or EGTA, with the exception of microtubules, basal bodies, and ciliary axonemes, we suggest that only in the presence of excess calcium when all binding sites are filled is the protein bound to plasma membranes, on apical cytoplasmic vesicles, on dynein arms and spoke heads or central sheath. The localizations appear specific for a calcium effect because the other two cations showed the diffuse pattern seen when cations were omitted from the fixative and when EGTA was added to the calcium-containing fixative.

Discrete localization of calmodulin in demembrated specimens on the microtubular structures and associated structures of axonemes was achieved only when calcium was present along with saponin or Triton X-100. This further suggests that calmodulin is soluble in the cytoplasm and cilia in the absence of calcium, since disruption of membranes allowed the contents to be released into the surrounding medium. When calcium is present, most probably it is bound to calmodulin. Calmodulin then undergoes a conformational change exposing hydrophobic sites which bind and activate specific enzymes.

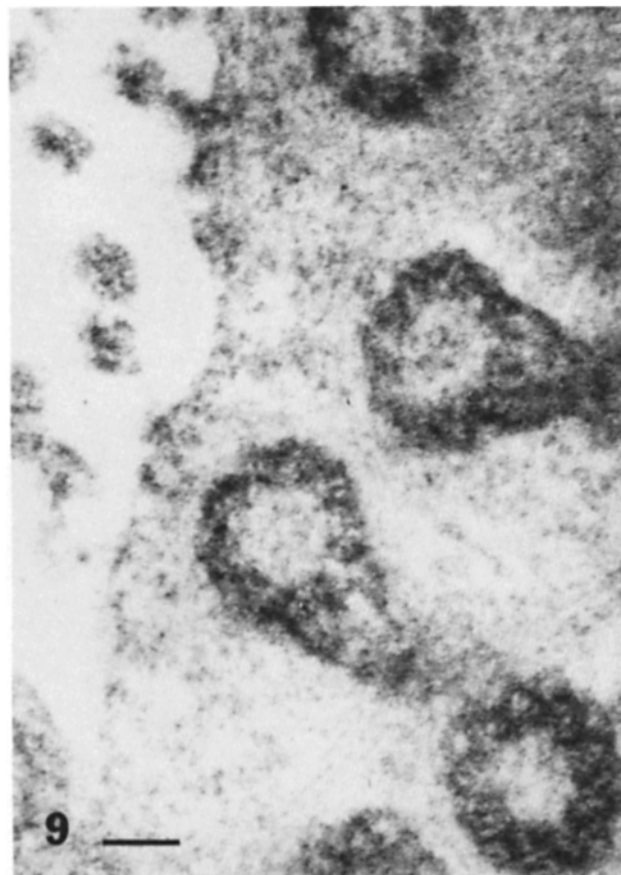
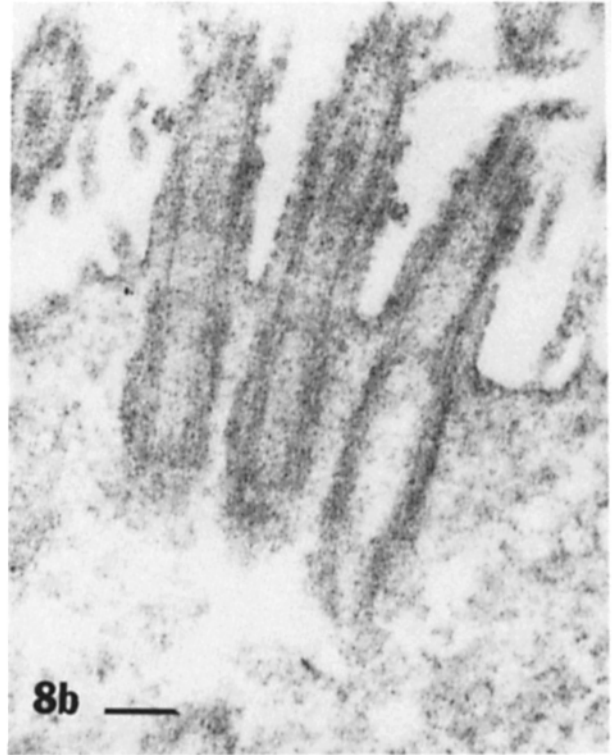
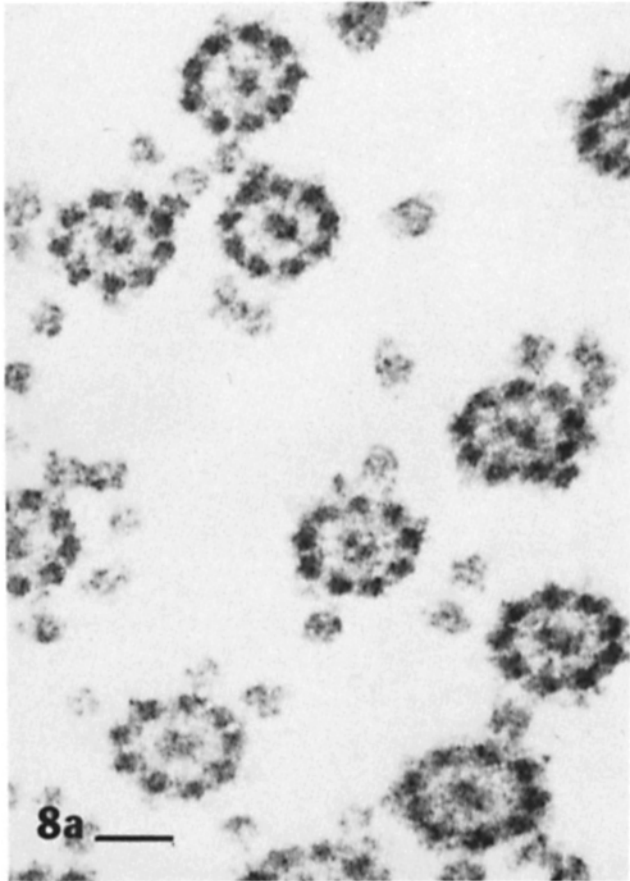
The differences in binding of anticalmodulin antibodies on isolated axonemal microtubules seen by Ohnishi et al. (23) and by us here could just be due to differences in techniques of observation, in antibodies used, or in the actual location of the calmodulin. Ohnishi et al. (23) used a direct negative staining technique on axonemal microtubules without embedment in plastic. Furthermore, the antibodies used in their study were produced by immunization with native calmodulin protein, which is known to produce low titer sera, which is not the case with the performic acid modified calmodulin. Therefore, the antibody produced, although specific for calmodulin, may be different for their antigenic site.

Based on the presence of calmodulin on or in the plasma membrane, and on axonemes, calmodulin location may reflect a multifunctional role at these two sites. On membranes there may be an excitation effect after protein phosphorylation (11) or activation of guanylate cyclase as a component of calcium channel resulting in a gating function and therefore controlling intracellular calcium levels. Intracellular levels of calcium then can be modulated by calmodulin located on or near dynein arms and spokes activating the dynein ATPase as described by Blum et al. (1).



FIGURES 5-7 Fig. 5: Electron micrograph of a thin section cut perpendicular to the epithelial plane, passing through the long axis of the ciliary shaft (CS), which projects into the lumen (L), and basal body. The tissue was fixed with calcium and then treated with rabbit anticalmodulin serum, followed by Fab fragment of SARG coupled to HRP. The electron-dense osmiophilic precipitate shows calmodulin close to the plasma membranes (small arrowheads). The apical cytoplasm (C) also showed deposits, in vesicles (large arrowheads). Bar,  $0.24 \mu\text{M}$ .  $\times 57,000$ . Fig. 6: (a) Electron micrograph of a thin section cut parallel to the plane of the tracheal epithelium, showing the axonemes in cross section. The tissue was treated as described in Fig. 5. Calmodulin is seen as deposits on the region of dynein arms (large arrows) of doublet microtubules. It also appears on the spoke heads or central sheath (large arrowhead) surrounding the central pair (CP). Calmodulin is also localized to

plasma membranes (small arrowheads). Bar,  $0.05 \mu\text{M}$ .  $\times 135,000$ . (b) Electron micrograph of a thin section cut perpendicular to the epithelial plane and longitudinally through the ciliary shaft. Tissue was treated as in Fig. 5. Dense deposits show calmodulin localization on the dynein arms of doublet microtubules (arrows), and on the spoke heads or central sheath (large arrowheads) of the central pairs (CP) of ciliary microtubules. Plasma membranes also showed calmodulin localization (small arrowheads). Bar,  $0.05 \mu\text{M}$ .  $\times 141,000$ . Fig. 7: Electron micrograph of a thin section cut through the long axis of the ciliary shaft (C), lining of lumen (L), and underlying basal body (BB). The tissue was treated as in Fig. 5. Calmodulin is located on ciliary necklace particles (small arrowheads) at the base of the ciliary shaft. Bar,  $0.05 \mu\text{M}$ .  $\times 140,000$ .



It is not known what function calmodulin might serve in the apical cytoplasmic vesicles; however, its presence may be correlated with the calcium-binding or -storing capability of the sarcoplasmic reticulum described in skeletal muscle (19).

A better understanding of the mechanisms of normal ciliary function makes it possible to explore the source of altered ciliary behavior. An alteration of clinical significance occurring in humans is a group of genetic diseases known as Immotile Cilia Syndrome where morphologic defects in cilia and basal bodies have been detected, but where the mechanism of impaired function is not understood and the functional aspects of the cilia are quite variable.

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FIGURES 8-10 Fig. 8: Electron micrograph of thin sections of ciliary axoneme cut on cross section (a) and in the plane of the long axis (b). The tissue was treated with Triton X-100 containing 1 mM  $CaCl_2$  fixed with paraformaldehyde containing 1 mM  $CaCl_2$  and immunologically reacted as described in Fig. 5. Calmodulin is seen on the peripheral doublet microtubules and close to the central sheath or central pair of microtubules. a, Bar, 0.15  $\mu M$ .  $\times 68,000$ . b, Bar, 0.18  $\mu M$ .  $\times 56,000$ . Fig. 9: Electron micrograph of cross-sectioned basal bodies from ciliated cells treated as described in Fig. 8. Calmodulin is located on the basal body triplets and extending onto the basal foot. Bar, 0.12  $\mu M$ .  $\times 83,000$ . Fig. 10: Electron micrograph of a thin section where the tracheal epithelium had been treated with anticalmodulin absorbed with the purified calmodulin protein. The tissue was treated as described for all other antisera. No precipitate is exhibited. Similarly, no precipitates were seen in the absorption controls, preimmune sera, and the routine technique controls. Bar, 1.5  $\mu M$ .  $\times 24,900$ .