

## Effect of serotonin on ciliary beating and intracellular calcium concentration in identified populations of embryonic ciliary cells

Shandra A. Doran, Ron Koss, Cam Ha Tran, Kimberly J. Christopher, Warren J. Gallin and Jeffrey I. Goldberg\*

*Department of Biological Sciences, University of Alberta, Edmonton, Alberta, Canada, T6G 2E9*

\*Author for correspondence (e-mail: jeff.goldberg@ualberta.ca)

*Accepted 12 January 2004*

### Summary

Embryos of the pond snail *Helisoma trivolvis* express three known subtypes of ciliary cells on the surface of the embryo early in development: pedal, dorsolateral and scattered single ciliary cells (SSCCs). The pedal and dorsolateral ciliary cells are innervated by a pair of serotonergic sensory-motor neurons and are responsible for generating the earliest whole-animal behavior, rotation within the egg capsule. Previous cell culture studies on unidentified ciliary cells revealed that serotonin (5-hydroxytryptamine; 5-HT) produces a significant increase in the ciliary beat frequency (CBF) in a large proportion of ciliary cells. Both  $\text{Ca}^{2+}$  influx and a unique isoform of protein kinase C (PKC) were implicated in the signal transduction pathway underlying the cilio-excitatory response to 5-HT. The goal of the present study was to characterize the anatomical and physiological differences between the three known populations of superficial ciliary cells. The pedal and dorsolateral ciliary cells shared common structural characteristics, including flat morphology, dense cilia and lateral accessory ciliary rootlets. By contrast, the SSCCs had a cuboidal morphology, reduced number of cilia, increased ciliary length and absence of lateral accessory rootlets. In cultures containing unidentified ciliary cells, the calcium/calmodulin-dependent enzyme inhibitor calmidazolium ( $2 \mu\text{mol l}^{-1}$ ) blocked the stimulatory effect of 5-HT

( $100 \mu\text{mol l}^{-1}$ ) on CBF. In addition, 50% of unidentified cultured cells responded to 5-HT ( $100 \mu\text{mol l}^{-1}$ ) with an increase in  $[\text{Ca}^{2+}]_i$ . To facilitate the functional analyses of the individual populations, we developed a method to culture identified ciliary subtypes and characterized their ciliary and calcium responses to 5-HT. In cultures containing either pedal or dorsolateral ciliary cells, 5-HT ( $100 \mu\text{mol l}^{-1}$ ) produced a rapid increase in CBF and a slower increase in  $[\text{Ca}^{2+}]_i$  in all cells examined. By contrast, the CBF and  $[\text{Ca}^{2+}]_i$  of SSCCs were not affected by  $100 \mu\text{mol l}^{-1}$  5-HT. Immunohistochemistry for two putative 5-HT receptors recently cloned from *Helisoma* revealed that pedal and dorsolateral ciliary cells consistently express the 5-HT<sub>1Hel</sub> protein. Intense 5-HT<sub>7Hel</sub> immunoreactivity was observed in only a subset of pedal and dorsolateral ciliary cells. Cells neighboring the SSCCs, but not the ciliary cells themselves, expressed 5-HT<sub>1Hel</sub> and 5-HT<sub>7Hel</sub> immunoreactivity. These data suggest that the pedal and dorsolateral ciliary cells, but not the SSCCs are a homogeneous physiological subtype that will be useful for elucidating the signal transduction mechanisms underlying 5-HT induced cilio-excitation.

Key words: serotonin, 5-hydroxytryptamine, pond snail, *Helisoma trivolvis*, intracellular calcium, ciliary beating, cilia, serotonin receptor.

### Introduction

Cilia are hair-like cellular extensions that function to generate fluid currents over the cell surface (reviewed in Satir and Sleight, 1990). This current flow is important in a number of biological activities, including unicellular movement (Chia et al., 1984), filter feeding (Murakami and Machemer, 1982), particle clearance in host defense (Salathe and Bookman, 1995) and the determination of left–right asymmetry (Essner et al., 2002). A wide variety of organisms have been used to study ciliary regulation, and, not surprisingly, the intracellular regulation of ciliary activity appears to be diverse. The most commonly identified regulatory mechanisms are

phosphorylation events acting through cyclic adenosine monophosphate–protein kinase A (cAMP–PKA), seen in gill cilia of *Mytilus edulis* (Murakami, 1987), frog esophagus (Briman et al., 1998) and human nasal epithelium (Di Benedetto et al., 1991), and changes in the intracellular calcium concentration ( $[\text{Ca}^{2+}]_i$ ), as examined in *Paramecium* (Bonini et al., 1991), frog esophagus (Zagoory et al., 2001) and ovine tracheal epithelium (Salathe and Bookman, 1999). Other regulatory elements include protein kinase C (PKC), which increases ciliary beat frequency (CBF) in frog esophagus (Levin et al., 1997) and decreases CBF in sheep trachea

(Salathe et al., 1993), and nitric oxide (NO), which increases CBF in rabbit and human airway epithelium (Tamoki et al., 1995; Yang et al., 1996).

An aspect of ciliary regulation that requires more attention is the identification of the endogenous neurotransmitters, neuromodulators and hormones that stimulate the aforementioned intracellular events. Serotonin (5-hydroxytryptamine; 5-HT), one such neurotransmitter that has been shown to be cilio-excitatory, was first identified to increase CBF in bivalve gill cilia (Gosselin et al., 1962). Other neurotransmitters and modulators that regulate ciliary activity include dopamine (Wada et al., 1997), acetylcholine, substance P (Aiello et al., 1991) and adenosine triphosphate (ATP; Morales et al., 2000). In order to develop an integrated understanding of ciliary activity, studies on the endogenous extracellular regulators and their associated signal transduction pathways from the cell membrane to the axoneme must be complimented by analysis of ciliary cell function at the whole-animal level.

The direct-developing embryos of the gastropod mollusc *Helisoma trivolvis* present an opportunity to study neuro-ciliary interactions during development. Three subpopulations of ciliary cells are evident on the surface of the embryo throughout early development: a single pedal band, paired dorsolateral bands and numerous scattered single ciliary cells (SSCCs; Kuang and Goldberg, 2001). The pedal ciliary cells and the most medial of the four cells composing each dorsolateral band are innervated by a bilateral pair of serotonergic sensory-motor neurons, termed embryonic neurons C1 (ENC1s; Diefenbach et al., 1991; Koss et al., 2003). ENC1s develop prior to the central nervous system and are the first neurons detected within the embryo. The ENC1-ciliary neural circuits generate cilia-driven rotational movements within the egg capsule in response to egg capsule oxygen content, with hypoxia stimulating an increase in embryo rotation (Kuang et al., 2002). In *Helisoma* ciliary cells, stimulation of a diacylglycerol-sensitive, phorbol ester-insensitive PKC isoform and calcium influx are known to produce an increase in CBF (Christopher et al., 1996, 1999). Additionally, the presence of nitric oxide synthase (NOS), the enzyme responsible for the generation of NO, was detected in ENC1s and ciliary cells (Cole et al., 2002). Recent studies have revealed that pharmacological manipulations of NO alter embryo rotation by directly affecting ENC1s and the postsynaptic ciliary bands (Cole et al., 2002; Doran et al., 2003). It appears that NO has a novel constitutive excitatory action in cilia that is permissive to the cilio-excitatory activity of 5-HT. Thus, the use of the *Helisoma* model system facilitates an examination of the neural control of ciliary activity as well as the signal transduction pathways and potential 'cross-talk' between pathways.

In the present study, we begin to characterize some of the individual subpopulations of *Helisoma* ciliary cells. We developed a technique to culture identified ciliary cells to enable study of the pedal ciliary cells, dorsolateral ciliary cells and SSCCs individually. Given that previous attempts to visualize intracellular  $\text{Ca}^{2+}$  in these embryonic cells using

membrane-permeable indicators were unsuccessful, ciliary cells were microinjected with impermeable Fura dextran for calcium imaging experiments. Here, we report that 5-HT stimulates a calmodulin-mediated rapid increase in the CBF of pedal and dorsolateral cilia and a slower increase in  $[\text{Ca}^{2+}]_i$ . By contrast, the SSCCs do not show a reliable change in CBF or in  $[\text{Ca}^{2+}]_i$  in response to 5-HT. These results are supported by immunohistochemical data that suggest that the pedal and dorsolateral ciliary cells, but not the SSCCs, express the 5-HT<sub>1Hel</sub> and 5-HT<sub>7Hel</sub> serotonin receptor proteins (Mapara et al., 2001). Furthermore, the SSCCs are anatomically distinct from pedal and dorsolateral ciliary cells. These results suggest that the pedal and the dorsolateral ciliary populations share many physiological and anatomical characteristics, whereas the SSCCs represent a distinct ciliary subtype.

## Materials and methods

### Animals

*Helisoma trivolvis* Say 1816 embryos were collected from a laboratory-reared albino colony raised at the University of Alberta. Snails were housed in flow-through glass aquaria containing an oyster shell substratum and de-chlorinated water (~25°C). They were maintained on a 12 h:12 h light:dark cycle and fed Romaine lettuce and trout pellets (NU-WAY; United Feeds, Calgary, Canada). Egg masses were collected, as previously described, from large plastic Petri plates placed in the aquaria (Diefenbach et al., 1991). After collection, egg masses were maintained at room temperature in artificial pond water (APW; 0.025% Instant Ocean; Aquarium Systems, Mentor, OH, USA). Embryos between stages E20 and E35, which represents 20–35% of intracapsular development (Diefenbach et al., 1998; Goldberg, 1995), were used in this study.

### Chemicals and solutions

Embryonic cells were cultured in *Helisoma* defined medium [HDM: 50% Liebovitz-15 (Gibco, Burlington, ON, Canada), 40 mmol l<sup>-1</sup> NaCl, 1.7 mmol l<sup>-1</sup> KCl, 4.1 mmol l<sup>-1</sup> CaCl<sub>2</sub>, 1.5 mmol l<sup>-1</sup> MgCl<sub>2</sub>, 5.0 mmol l<sup>-1</sup> Hepes, 50 µg ml<sup>-1</sup> gentamicin, 0.015% L-glutamine (Sigma, St Louis, MO, USA); pH 7.30–7.35). All imaging and CBF experiments were performed in *Helisoma* saline (HS: 51.3 mmol l<sup>-1</sup> NaCl, 1.7 mmol l<sup>-1</sup> KCl, 4.1 mmol l<sup>-1</sup> CaCl<sub>2</sub>, 1.5 mmol l<sup>-1</sup> MgCl<sub>2</sub>, 5.0 mmol l<sup>-1</sup> Hepes; pH 7.33–7.35). Calmidazolium chloride (Cal; Sigma) and ionomycin (free acid; Calbiochem, La Jolla, CA, USA) were dissolved in dimethyl sulfoxide (DMSO; Sigma) and then diluted to working concentration in HS so that the DMSO level did not exceed 0.1%. 5-HT (creatine sulfate complex; Sigma) was dissolved in HS. Fura dextran potassium salt (fura; 10 000 M<sub>r</sub>; Molecular Probes, Eugene, OR, USA) was dissolved in filtered distilled water to a concentration of 2 mmol l<sup>-1</sup>. All drugs were prepared on the day of use.

### Mixed population ciliary cell culture

Embryonic ciliary cells were cultured as previously described

(Christopher et al., 1996, 1999). Briefly, egg masses were disinfected with 35% ethanol and the embryos were removed. Isolated embryos were treated with 0.2% trypsin (Sigma) for 30 min and then mass dissociated by repeatedly passing them through a 63- $\mu\text{m}$  nylon mesh (Small Parts Inc., Miami, FL, USA). The resulting cell suspension was plated on poly-L-lysine-coated (hydrobromide;  $M_r$  4000–15 000; 1  $\mu\text{g ml}^{-1}$ ; Sigma) culture dishes (Falcon 3001) for ciliary beat experiments and on poly-L-lysine-coated glass bottom dishes for imaging experiments and immunohistochemistry. The cultures were maintained in the dark at room temperature (20–22°C) for 18–24 h to enable cells to adhere to the substrate.

#### Cell culture of identified ciliary cell populations

Egg masses were incubated in antibiotic-containing HS (gentamicin sulfate; Sigma) for 15 min prior to removal of embryos. Embryos were immersed in HS and examined with Normarski differential interference contrast (DIC) optics on an inverted compound microscope (Nikon, Diaphot) for identification of the pedal and dorsolateral ciliary cells and SSCs. An identified cluster of ciliary cells (see Fig. 1) was gently sucked into the 30- $\mu\text{m}$  tip of a glass micropipette (World Precision Instruments, Sarasota, FL, USA) using a micrometer syringe (Gilmont Instruments, Barrington, IL, USA), and the remainder of the embryo was surgically detached with a 30-gauge needle (Becton-Dickinson, Mississauga, ON, Canada). The tissue explants containing identified ciliary cells were then expelled onto poly-L-lysine-coated culture dishes (see above) containing HDM. The culture dishes were maintained in the dark at room temperature (20–22°C) for 18–24 h to enable the explants to adhere to the substrate.

#### Electron microscopy

For scanning electron microscopy (SEM), isolated embryos

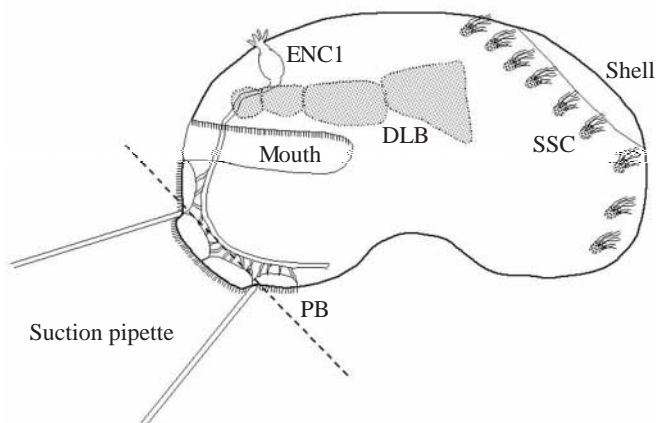


Fig. 1. Isolation of identified ciliary cells from *Helisoma trivolvis* embryos. Schematic of a side view of a stage E30 *Helisoma trivolvis* embryo reveals the location of the various ciliary cells and embryonic neuron C1 (ENC1). A suction pipette was used to remove identified pieces of tissue from the surface of the embryo. DLB, dorsolateral ciliary band; PB, pedal ciliary band; SSC, scattered single ciliary cell.

were transferred to glass vials and fixed for 30–60 min in 2% OsO<sub>4</sub> in 0.01 mol l<sup>-1</sup> phosphate-buffered saline (PBS), pH 7.5. Specimens were then rinsed for 2×10 min in PBS and dehydrated in an ascending ethanol series of 30%, 50%, 70% and 100%. After three changes of 100% ethanol, the ethanol was replaced by adding increments of 30% isoamyl acetate to reach saturation. Subsequently, specimens were dried *via* CO<sub>2</sub> critical point drying, sputter-coated with gold–palladium and viewed with a Cambridge Stereocan S250 scanning electron microscope.

For transmission electron microscopy (TEM), embryos were fixed for 1 h in 2.5% glutaraldehyde in 0.01 mol l<sup>-1</sup> PBS (pH 7.5), followed by a 1 h post-fixation in 2% OsO<sub>4</sub> in 1.25% sodium bicarbonate (pH 7.2; Wood and Luft, 1965). They were then dehydrated through an ethanol series and directly embedded in Spurr's (Ted Pella Inc., Redding, CA, USA). After a polymerization period of 48 h, silver–gold sections were cut, collected on copper grids and stained with uranyl acetate and Reynold's lead citrate (Reynolds, 1963) for 20 min and 5 min, respectively. TEM sections were then observed with an FEI transmission electron microscope (Morgagni model).

#### Ciliary beat frequency analysis

For the calmodulin inhibitor experiment, ciliary beating was monitored with a CCD video camera (JVC, TK-860U) mounted on an inverted compound microscope set up for phase-contrast optics through either a 20× or 40× objective (Nikon, Diaphot). Using a time-lapse video recorder (VCR; Panasonic AG-6720), ciliary activity was recorded over a 5–10 s interval immediately prior to and 10 min after application of the vehicle control or drug solution. Off-line analysis involved slowing the playback speed to 1/24 normal and manually counting ciliary beats over a 1-min interval. For this experiment, CBF was presented as a percentage of the pre-treatment measurement.

For the quantification of ciliary beating in identified ciliary populations, ciliary cells were viewed on an inverted microscope (Zeiss Axiovert 135; Zeiss, ON, Canada) with DIC optics and a 100× objective. Ciliary beating was recorded using either a high-speed digital videocamera (Vitana) linked to a Macintosh Powerbook containing Pixelink software (generously provided by Improvision, Inc., Quorum Technologies, Inc., Guelph, ON, Canada) or a Retiga Ex digital CCD camera (Q-Imaging; Burnaby, BC, Canada) linked to a Pentium 4 PC containing Northern Eclipse software (Empix Imaging Inc., Mississauga, ON, Canada). With both cameras, videos were collected at >50 frames s<sup>-1</sup> for 1 s segments, once every minute over the course of the experiment. Off-line analysis was performed on a Macintosh G4 computer using the public-domain NIH Image program (written by Wayne Rasband at the US National Institutes of Health and available from the Internet by anonymous FTP from zippy.nimh.nih.gov) and Particle Analysis (a user-contributed macro, written by C. J. H. Wong at the University of Alberta). The displacement of the cilia from an initial position was calculated in both *X* and *Y* coordinates. The CBF was then

determined by performing an autocorrelation on the resulting displacement waveform. The CBF for each time point was the result of an average of three measurements made in approximately the same location on the cell over the course of the experiment. For each cell type, a few experiments were performed at a higher temporal resolution to determine if there were undetected changes in CBF in response to drug treatments. Given that the CBF experiments with a collection rate of once every 15 s revealed no additional events, the data are presented with the collection rate at once every minute.

#### *Ratiometric Ca<sup>2+</sup> imaging and Ca<sup>2+</sup> calibration*

Fura dextran (Molecular Probes) was dissolved in filtered distilled water to a concentration of 2 mmol l<sup>-1</sup>. Fura dextran was utilized instead of the free acid form of the indicator because it is considered to be less toxic to the cell and more resistant to subcellular compartmentalization (Tombal et al., 1999). The dye was backloaded into custom-made pulled micropipettes (1.0 mm glass with filament; World Precision Instruments, Inc.). An Eppendorf Femtojet Rapid injection system (Brinkmann, Mississauga, ON, Canada) mounted on an inverted Nikon Eclipse microscope was used to microinject cultured ciliated cells under 100× DIC optics. Loaded ciliary cells were imaged with a 100× oil-immersion objective (1.3 N.A. Fluor) on an inverted microscope (Axiovert 135; Zeiss) with excitation at 340 nm and 380 nm from an Hg–Xe arc lamp (Hamamatsu, Hamamatsu, Japan). Emission fluorescence at 510 nm was collected using an intensified charge-coupled device (ICCD) video camera (Paultek Imaging, Grass Valley, CA, USA). Neutral density filters (Omega Optical, Brattleboro, VT, USA) were used to ensure that fluorescent images were within the sensitivity range of the camera. Data were collected as 8-bit images using custom software kindly provided by Dr. S. Kater (University of Utah). Captured images were digitized through a QuickCapture frame grabber board (Data Translation, Mississauga, ON, Canada) and saved to a computer (Macintosh Quadra 950) for off-line analysis. Vehicle or drug-containing solutions were perfused into the culture dish using a gravity-driven perfusion set-up at approximately 1 ml min<sup>-1</sup> with a latency of 15 s (Warner Instruments Corp., Holliston, MA, USA). Images were analyzed for whole-cell fluorescence intensity on a Macintosh G4 computer using the public-domain NIH Image program and Ca<sup>2+</sup>Ratiometrics (a user-contributed macro, written by C. J. H. Wong at the University of Alberta).

The 340/380 ratios, which provide a relative measure of cytoplasmic free calcium concentration, were converted to estimates of [Ca<sup>2+</sup>]<sub>i</sub> using the equation  $[Ca^{2+}]_i = K_D(R - R_{min}) / (R_{max} - R)(F_o / F_s)$ , where  $R$  is the 340/380 ratio,  $K_D$  is the dissociation constant and  $F_o$  and  $F_s$  are the fluorescence values obtained at minimal and saturating [Ca<sup>2+</sup>], respectively, at the 380 nm excitation (Gryniewicz et al., 1985). The  $K_D$  was determined to be 0.586 μmol l<sup>-1</sup> using thin-wall glass capillary tubes (20 nm in width; VitroCom Inc., Mt. Lks., NJ, USA) filled with one of 11 different Ca<sup>2+</sup> buffer solutions (Ca<sup>2+</sup> concentration ranged from 0 μmol l<sup>-1</sup> to 39 μmol l<sup>-1</sup>; calcium calibration buffer kit with magnesium II; Molecular Probes)

and 50 μmol l<sup>-1</sup> fura dextran. Fluorescence values acquired from the images taken with excitation at both 340 nm and 380 nm for all 11 Ca<sup>2+</sup> buffer solutions were entered into a computer program available on the Molecular Probes website ([www.probes.com/resources/calc/kd.html](http://www.probes.com/resources/calc/kd.html)) to determine the value of  $K_D$ . This value for  $K_D$  is consistent with other *in vitro* estimates for fura dextran (Konishi and Watanabe, 1995; Tombal et al., 1999). The values for  $R_{min}$ ,  $R_{max}$ ,  $F_o$  and  $F_s$  were determined *in situ* using ciliary cells loaded with fura dextran. Cells were permeabilized with 50 μmol l<sup>-1</sup> ionomycin in the presence of either Ca<sup>2+</sup>-free HS with 1.0 mmol l<sup>-1</sup> ethylene glycol-bis(β-aminoethyl ether) *N,N,N,N*-tetraacetic acid (EGTA) for the determination of  $R_{min}$  and  $F_o$  or unaltered HS for the determination of  $R_{max}$  and  $F_s$ .

#### *Immunohistochemistry*

Identified populations of ciliary cells cultured on glass-bottom Petri dishes were fixed in 4% paraformaldehyde in 0.01 mol l<sup>-1</sup> phosphate-buffered saline (PBS) at 4°C for 1 h. The cells were washed for 3×10 min in PBS. This and subsequent steps were performed at room temperature with agitation unless otherwise stated. Embryos were washed in PBS containing 3% horse serum (Sigma) and 0.3% Triton X-100 for 1 h. This was followed by incubation in rabbit anti-5-HT<sub>1Hel</sub> or anti-5-HT<sub>7Hel</sub> antiserum diluted 1:1000 in PBS containing 1% horse serum and 0.4% Triton X-100 for 12 h at 4°C. The cells were then washed for 4×7 min with 0.4% Triton X-100 in PBS and then incubated for 1 h with goat anti-rabbit immunoglobulin G conjugated to Alexa 488 (Molecular Probes) that was diluted in a solution of 1% horse serum and 0.3% Triton X-100 in PBS. This was followed by 4×5 min washes in 0.3% Triton X-100 in PBS and 2×10 min washes in PBS. The cells were examined in 80% glycerol in PBS. In control experiments, pre-immune serum from the same rabbit as the one used to generate the primary antibody was used in place of the primary antibody. In addition, control experiments in which the primary antibody was excluded were also performed.

#### *Preparation of antibodies to 5-HT<sub>1Hel</sub> and 5-HT<sub>7Hel</sub>*

Antibodies to 5-HT<sub>1Hel</sub> and 5-HT<sub>7Hel</sub> were raised against peptide derived from intracellular loop sequence that had a high antigenicity. Peptide 1Hel (Residues 409–423; YSRTREKLELKRERK) and Peptide 7Hel (Residues 246–261; YFKIWRVSSKIAKAEA) were prepared by Washington Biotechnology (Baltimore, MD, USA). Peptides were synthesized, coupled to keyhole limpet hemocyanin (KLH) and used to immunize rabbits. Sera were collected when the antibody gave a positive reaction against the antigen at a titer of >100 000 in an ELISA.

#### *Data analysis*

Results are presented as means ± standard error (S.E.M.) unless otherwise stated. The significance of differences among groups in the calmodulin inhibitor experiment was evaluated using analysis of variance (ANOVA) followed by a Fisher's

Protected Least Significant Difference (PLSD) test. A Student's paired *t*-test was used to determine if 5-HT stimulated a significant increase in CBF in pedal and dorsolateral ciliary cells, where the mean CBF during the 2 min prior to 5-HT perfusion was compared with the mean CBF during minutes 1–4 after the start of the 5-HT perfusion.

## Results

### *Anatomical characterization of ciliary cell subtypes in vivo*

Embryos were processed for SEM and TEM to compare the morphology and fine structure of pedal ciliary cells, dorsolateral ciliary cells and SSCCs. Fig. 2A shows the location of ciliary populations in a stage E30 embryo. Pedal and dorsolateral ciliary cells occurred in distinct, anterior ciliary bands, whereas the SSCCs were most prevalent in the posterior region of the embryo.

Structurally, pedal and dorsolateral ciliary cells appeared identical in profile and contents. Cilia were arranged in an organized fashion and ranged in number from 600 to 800 per cell (Fig. 2B,C). In sectional profile, pedal and dorsolateral cells were long, thin, flattened cells with an elliptical nucleus, containing a large nucleolus (Fig. 2D; see Koss et al., 2003). Microvilli surrounded the base of each cilium (Fig. 2B,C). The cilia possessed a typical (9×2)+2 arrangement of microtubules (data not shown), and a basal foot arose from one side of the basal body. A lengthy primary rootlet extended into the cell from the base of the cilium, and an accessory rootlet arose from the opposite side of the basal body; both rootlets were striated (Fig. 2D). Each ciliary cell contained many prominent mitochondria that were localized beneath the ciliary basal bodies (Fig. 2D). Granular endoplasmic reticulum was abundant and tended to be concentrated towards the cell periphery. Pedal and dorsolateral ciliary cells consistently displayed extensive patches of cytoplasm that appeared to be devoid of organelles, except for some small electron-translucent vesicles ranging in size from 0.03 μm to 0.06 μm in diameter (Fig. 2D). A previous study has revealed that these regions contain vesicles that display 5-HT immunoreactivity (Koss et al., 2003).

In comparison with the cells of the pedal and dorsolateral ciliary bands, SSCCs differed in profile and content. Apically, only 35–50 cilia emerged from the cell surface. The SSCC cilia displayed a more random orientation and a greater length than the pedal or dorsolateral cilia (Fig. 2E). Furthermore, SSCCs were smaller, had a wedge or cuboidal shape and a centrally located nucleus. The fine structure of individual cilia was similar to pedal and dorsolateral cilia, including a (9×2)+2 arrangement of microtubules (Fig. 2F, inset). However, there were no accessory ciliary rootlets arising laterally from the basal body (Fig. 2F). The cytoplasm of the SSCCs contained less prominent mitochondria and an absence of electron-translucent regions. Finally, the SSCCs had no basal cellular extensions such as were found on innervated pedal and dorsolateral ciliary cells, as previously described (Koss et al., 2003). Thus, the different morphology and fine structure of the

SSCCs, in comparison to the pedal and dorsolateral ciliary cells, suggest that ciliary subtypes may exhibit different physiological profiles.

### *Examination of unidentified ciliary cells in vitro*

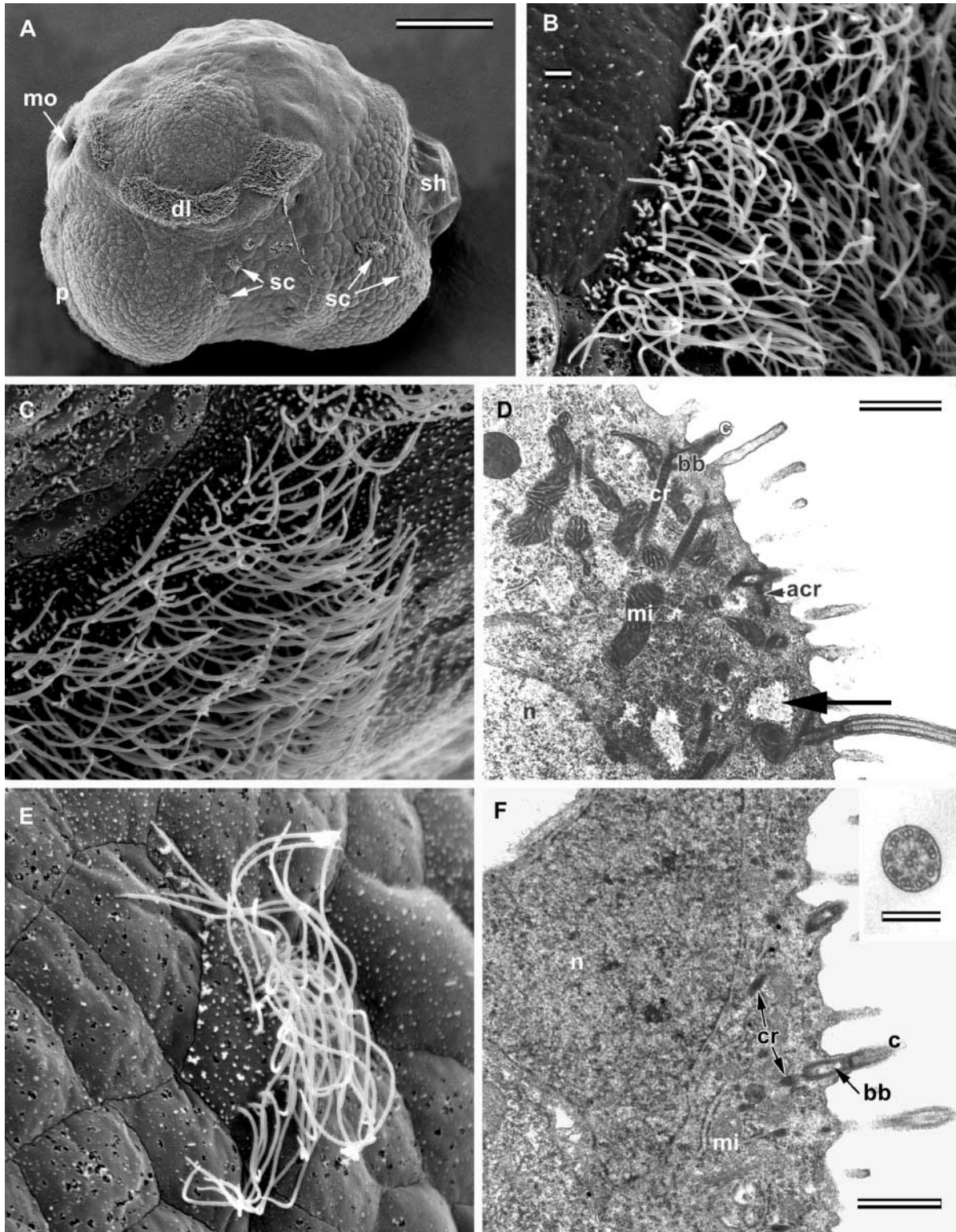
Earlier studies revealed that Ca<sup>2+</sup> influx is required to stimulate an increase in CBF in *Helisoma* cilia (Christopher et al., 1996). This prompted us to examine whether alterations in [Ca<sup>2+</sup>]<sub>i</sub> act through the calcium-binding protein calmodulin. A 10 min application of 100 μmol l<sup>-1</sup> 5-HT, a dose known to maximally stimulate *Helisoma* cilia (Christopher et al., 1996), stimulated a significant increase in CBF to 125.3±4.6% of the control (*P*<0.05, *N*=7; Fig. 3). Co-application of the calcium/calmodulin-dependent enzyme inhibitor calmidazolium (Cal; 2 μmol l<sup>-1</sup>) with 100 μmol l<sup>-1</sup> 5-HT blocked the stimulatory effect of 5-HT (Fig. 3). Application of 2 μmol l<sup>-1</sup> Cal alone or the DMSO vehicle did not produce a change in CBF (Fig. 3). These data suggest that calmodulin mediates the cilio-excitatory action of 5-HT in *Helisoma* ciliary cells.

The necessity of Ca<sup>2+</sup> influx in cilio-excitation (Christopher et al., 1996), taken together with the results of the calmodulin inhibitor experiment, prompted us to examine whether 5-HT, the primary cilio-excitatory neurotransmitter in *Helisoma* embryos, induces a change in [Ca<sup>2+</sup>]<sub>i</sub>. Using fast microinjection, unidentified ciliary cells in culture were loaded with fura-2 dextran. Whereas the calcium ionophore ionomycin (10 μmol l<sup>-1</sup>) caused an increase in the 340/380 ratio in all cells examined, only four of eight cells responded to 100 μmol l<sup>-1</sup> 5-HT with an increase in [Ca<sup>2+</sup>]<sub>i</sub> (Fig. 4). This finding prompted the hypothesis that one or more of the subpopulations of ciliary cells in mass-dissociated cultures does not respond to 5-HT with a change in [Ca<sup>2+</sup>]<sub>i</sub>.

### *Examination of identified ciliary cells in vitro*

Both the calcium imaging results described above and previous *in vivo* measurements of CBF (Kuang and Goldberg, 2001) suggest that the different ciliary populations display distinct physiological properties. To test this, we first examined the effect of exogenous 5-HT on CBF in identified ciliary cells in culture. In pedal ciliary cells, perfusion with 100 μmol l<sup>-1</sup> 5-HT produced a rapid, statistically significant increase in CBF (Fig. 5A). The pedal ciliary cells roughly doubled the rate of ciliary beating within 1 min of the start of 5-HT perfusion. This high CBF was maintained for 10 min, with only marginal desensitization (Fig. 5A). A 5-min washout with HS produced a partial recovery in CBF, and a challenge with 10 μmol l<sup>-1</sup> ionomycin increased CBF to values similar to those induced by 100 μmol l<sup>-1</sup> 5-HT.

To ensure that microinjection with fura dextran did not alter the physiological responses of these cells, the ciliary response to 5-HT and ionomycin was examined in pedal ciliary cells that had been loaded with fura dextran. These cells exhibited a nearly identical CBF response to 5-HT and ionomycin as did pedal ciliary cells that were not loaded with fura dextran (Fig. 5B). Perfusion with 100 μmol l<sup>-1</sup> 5-HT produced a rapid, statistically significant increase in CBF, followed by a partial



recovery in response to HS washout. Perfusion of  $10 \mu\text{mol l}^{-1}$  ionomycin produced an increase in CBF to approximately the same amplitude as 5-HT. The results of this experiment suggest that microinjection of fura dextran does not impair the cell's ability to respond to 5-HT or ionomycin with changes in CBF.

A similar effect was observed in dorsolateral ciliary cells, as  $100 \mu\text{mol l}^{-1}$  5-HT produced a rapid, statistically significant increase in the rate of ciliary beating within 1 min of 5-HT perfusion (Fig. 5C). These observations are consistent with *in vivo* results on the effect of 5-HT on dorsolateral and pedal ciliary cells (Kuang and Goldberg, 2001). Washout with HS

Fig. 2. Whole-mount scanning electron micrographs (SEM) and transmission electron micrographs (TEM) showing the overall external and internal anatomy of the three ciliary subtypes of a stage E28 embryo. (A) Left lateral view SEM showing the position of the scattered single ciliary cells (sc) relative to the dorsolateral ciliary bands (dl) and pedal ciliary band (p). The shell (sh) and mouth (mo) are also shown. Scale bar, 25  $\mu\text{m}$ . (B) SEM showing the configuration and density of cilia of a dorsolateral ciliary cell. Scale bar, 1  $\mu\text{m}$ . (C) SEM showing the configuration and density of cilia of a pedal ciliary cell. Scale bar, 1  $\mu\text{m}$ . (D) TEM of a dorsolateral ciliary cell showing numerous mitochondria (mi) and the nucleus (n). Electron-translucent regions are designated by an arrow. The cilia (c) with basal body (bb), ciliary rootlet (cr) and accessory ciliary rootlet (acr) are also shown. Scale bar, 1  $\mu\text{m}$ . (E) SEM showing the configuration and density of cilia of one of the scattered single ciliated cells. Scale bar, 1  $\mu\text{m}$ . (F) TEM of a scattered single ciliated cell showing the cytoplasm. Note the cilia (c) with basal body (bb), ciliary rootlet (cr), less prominent mitochondria (mi) and the nucleus (n). Scale bar, 1  $\mu\text{m}$ . Inset: cross section through a cilium showing the  $(9 \times 2) + 2$  doublets of microtubules. Scale bar, 0.5  $\mu\text{m}$ .

produced a partial recovery in CBF over 5 min, and 10  $\mu\text{mol l}^{-1}$  ionomycin again produced an increase in CBF that was similar to the 5-HT response. As with pedal cilia, ionomycin produced a change in the ciliary beat mechanics in dorsolateral cilia, and in one of the cells the ionophore treatment killed the cell (data not shown).

The SSCCs are small, cuboidal cells with longer cilia (Fig. 2E; Figs 9C, 10E) and different ciliary beat profiles compared with the pedal and dorsolateral ciliary cells. These cells exhibited higher basal rates of ciliary activity, an unstable ciliary beat profile and no discernible response to 100  $\mu\text{mol l}^{-1}$  5-HT (Fig. 5D). Furthermore, 10  $\mu\text{mol l}^{-1}$  ionomycin did not appear to produce a change in CBF (Fig. 5D). These data

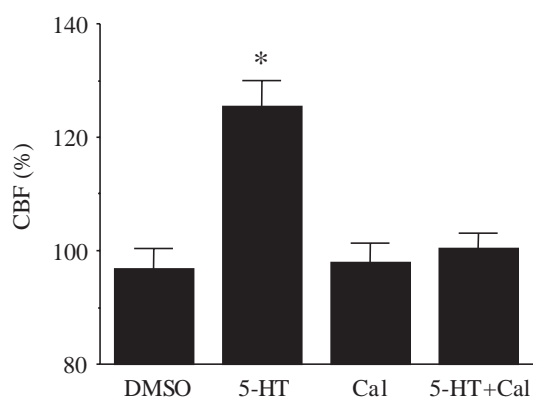


Fig. 3. Effect of the calmodulin inhibitor calmidazolium on ciliary beat frequency (CBF) in unidentified ciliary cells in culture. Application of 100  $\text{mmol l}^{-1}$  5-hydroxytryptamine (5-HT) produced a significant increase in CBF versus the saline control (asterisk:  $P < 0.05$ ,  $N = 7$  cells). Application of either 0.1% DMSO ( $N = 7$  cells) or 2  $\text{mmol l}^{-1}$  calmidazolium (Cal;  $N = 12$  cells) caused no change in CBF. When 2  $\text{mmol l}^{-1}$  Cal was co-applied with 100  $\text{mmol l}^{-1}$  5-HT, the calmodulin inhibitor blocked the stimulatory effect of 5-HT ( $N = 9$  cells).

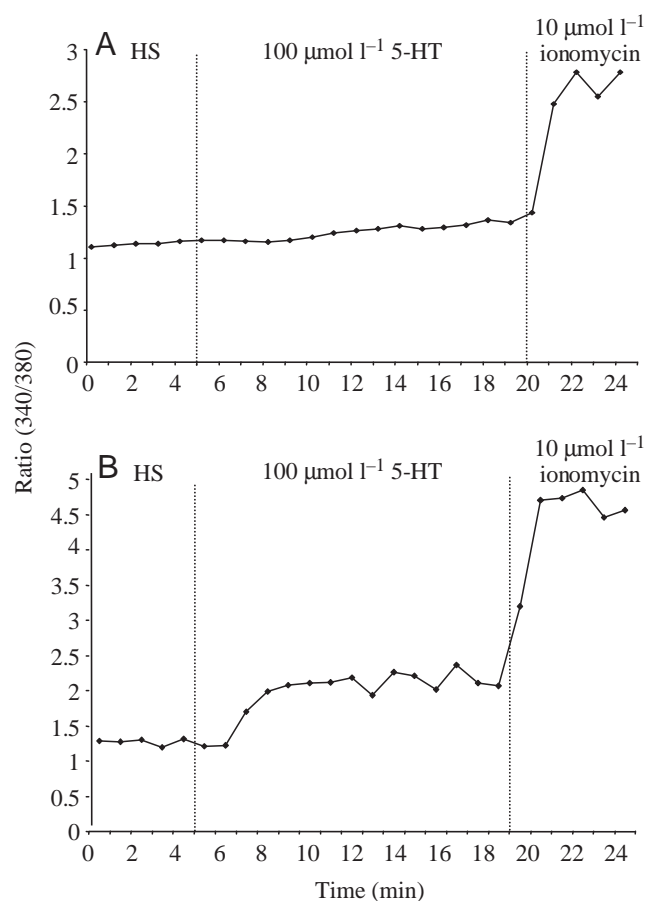


Fig. 4. Effect of 5-hydroxytryptamine (5-HT) and ionomycin on the intracellular  $\text{Ca}^{2+}$  concentration in unidentified ciliary cells in culture. Ciliary cells microinjected with fura-2 dextran were imaged at 1-min intervals in the presence of saline (HS), 5-HT or ionomycin. (A) Representative trace of a ciliary cell that was not affected by a 15 min application of 100  $\text{mmol l}^{-1}$  5-HT but displayed a rapid rise in the 340/380 ratio in response to 10  $\mu\text{mol l}^{-1}$  ionomycin. (B) Representative trace of an unidentified ciliary cell responding to both 100  $\text{mmol l}^{-1}$  5-HT application and 10  $\text{mmol l}^{-1}$  ionomycin with an increase in the 340/380 ratio. Dotted lines represent the time at which drug treatments were initiated.

suggest that ciliary beating in SSCCs may be regulated differently from that in pedal and dorsolateral ciliary cells.

To further examine the physiological differences in the subpopulations of *Helisoma* ciliary cells and to determine why only a percentage of mass-dissociated cells in culture respond to 5-HT with an increase in  $[\text{Ca}^{2+}]_i$ , we imaged intracellular  $\text{Ca}^{2+}$  in identified ciliary cells *in vitro*. For both pedal and dorsolateral ciliary cells, the average baseline  $[\text{Ca}^{2+}]_i$  was between 50  $\text{nmol l}^{-1}$  and 150  $\text{nmol l}^{-1}$ . In pedal ciliary cells, two different types of  $\text{Ca}^{2+}$  responses were evident during perfusion with 100  $\mu\text{mol l}^{-1}$  5-HT. These included an initial peak, with an average increase of approximately 350  $\text{nmol l}^{-1}$ , followed by a sustained rise in  $[\text{Ca}^{2+}]_i$  of 150  $\text{nmol l}^{-1}$  above baseline ( $N = 5$  cells; Fig. 6A) or a gradual increase in  $[\text{Ca}^{2+}]_i$  to 150  $\text{nmol l}^{-1}$  above baseline ( $N = 5$  cells; Fig. 6B). In both

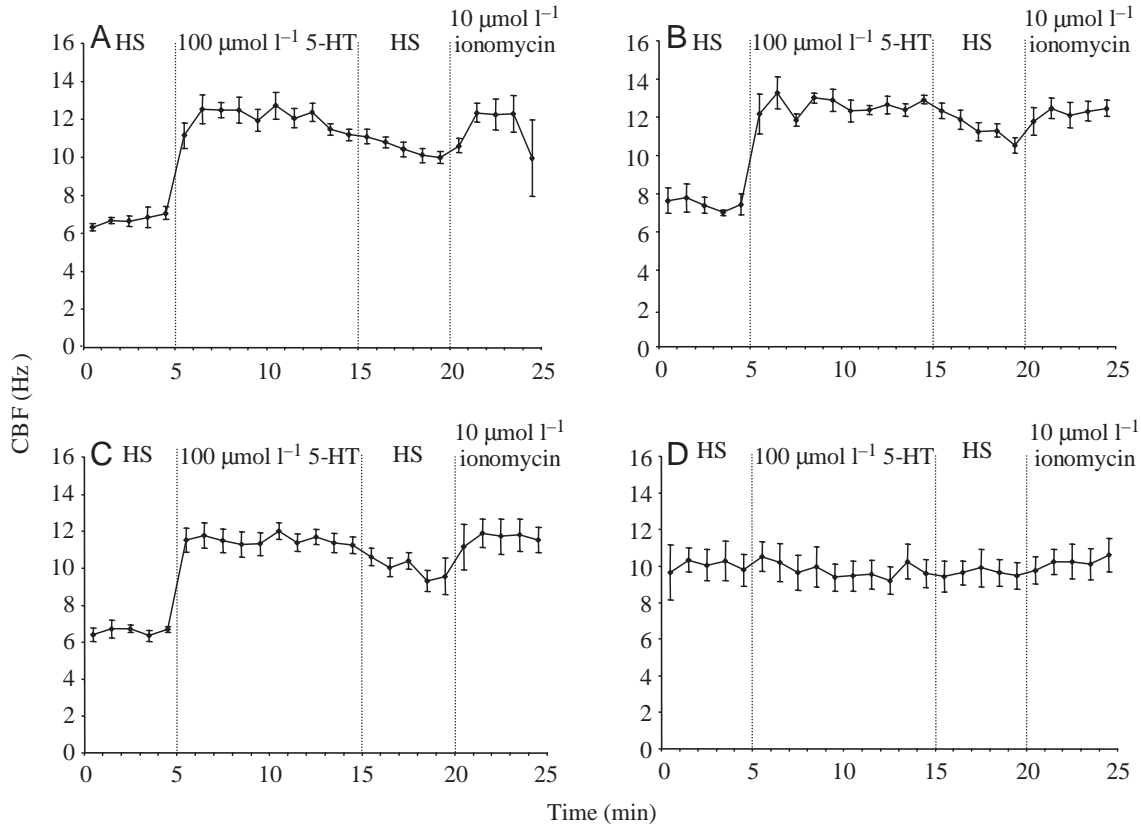


Fig. 5. Effect of 5-hydroxytryptamine (5-HT) and ionomycin on ciliary beat frequency (CBF) in pedal, dorsolateral and scattered single ciliary cells. Perfusion with  $100 \mu\text{mol l}^{-1}$  5-HT resulted in an approximate doubling in the rate of ciliary beating in pedal (A), fura dextran-loaded pedal (B) and dorsolateral (C) ciliary cells. This 5-HT-stimulated increase in CBF was statistically significant in all cases. The CBF partially recovered when cells were perfused for 5 min with *Helisoma* saline (HS), whereas perfusion with  $10 \text{ mmol l}^{-1}$  ionomycin further stimulated CBF in pedal (A), fura dextran-loaded pedal (B) and dorsolateral (C) ciliary cells. The scattered single ciliary cells did not display cilio-excitatory responses to 5-HT or ionomycin (D). Measurements were taken once every minute,  $N=6$  cells for A, C and D and  $N=4$  cells for B. Dotted lines represent the time when the specified treatments were initiated.

cases, perfusion with  $10 \mu\text{mol l}^{-1}$  ionomycin produced a relatively rapid increase in  $[\text{Ca}^{2+}]_i$  that was highly variable in amplitude, ranging from  $300 \text{ nmol l}^{-1}$  to  $1300 \text{ nmol l}^{-1}$  (Fig. 6A,B). A similar result was observed with the dorsolateral cells, as they exhibited either an initial peak followed by a plateau ( $N=4$  cells; Fig. 7A) or a gradual increase in  $[\text{Ca}^{2+}]_i$  ( $N=6$  cells; Fig. 7B). The magnitude of these 5-HT responses was also similar to that observed in pedal ciliary cells. Dorsolateral ciliary cells displayed a variable ionomycin-induced increase in  $[\text{Ca}^{2+}]_i$  ranging in amplitude from  $250 \text{ nmol l}^{-1}$  to  $600 \text{ nmol l}^{-1}$ . In both pedal and dorsolateral cells, little or no recovery of  $[\text{Ca}^{2+}]_i$  was observed after washout of 5-HT over the time frame examined. These data indicate that 5-HT does elicit an increase in  $[\text{Ca}^{2+}]_i$  within pedal and dorsolateral ciliary cells, but with a slower time course than observed during the CBF measurements.

Consistent with the CBF results, intracellular calcium measurements revealed large differences between the SSCCs and the other ciliary subtypes. In four of nine SSCCs examined, the resting  $[\text{Ca}^{2+}]_i$  was stable at approximately

$100 \text{ nmol l}^{-1}$ , 5-HT perfusion did not stimulate a notable change in  $[\text{Ca}^{2+}]_i$ , and  $10 \mu\text{mol l}^{-1}$  ionomycin produced a relatively small rise in  $[\text{Ca}^{2+}]_i$  (Fig. 8A). By contrast, five of nine SSCCs exhibited irregular spikes in  $[\text{Ca}^{2+}]_i$ , both in HS and during 5-HT perfusion (Fig. 8B). The timing, duration and amplitude of these events were highly variable depending on the cell. In most of these cells,  $10 \mu\text{mol l}^{-1}$  ionomycin only produced a transient increase in  $[\text{Ca}^{2+}]_i$ . These data reinforce the notion that the SSCCs display different physiological characteristics from the pedal and dorsolateral cells, including insensitivity to 5-HT.

Given that some of the ciliary subtypes responded to 5-HT with changes in CBF and  $[\text{Ca}^{2+}]_i$ , we tested whether identified ciliary cells express the two 5-HT receptors whose genes were recently cloned from *Helisoma trivolvis*. Isolated ciliary tissues were exposed to antibodies raised against the cloned 5-HT receptors, 5-HT<sub>1Hel</sub> and 5-HT<sub>7Hel</sub> (Mapara et al., 2001). All pedal ciliary cells expressed 5-HT<sub>1Hel</sub> immunoreactivity strongly over the apical surface of the cells, with weaker immunoreactivity throughout the remainder of the cell ( $N=15$  cells; Fig. 9A). A majority of dorsolateral ciliary cells also



expressed 5-HT<sub>1Hel</sub> immunoreactivity, with 76% of cells ( $N=25$  cells) exhibiting strong surface expression and weaker distribution throughout the cell (Fig. 9B). In the SSCCs, 0% of cells exhibited 5-HT<sub>1Hel</sub> immunoreactivity within the cell ( $N=23$  cells; Fig. 9C). However, neighboring nonciliary cells in the surrounding epithelia were consistently immunoreactive (Fig. 9C). Rabbit pre-immune serum, used in parallel controls to examine specificity of the antibody staining, did not exhibit any immunoreactivity (Fig. 9D).

5-HT<sub>7Hel</sub> immunoreactivity was more inconsistent than that of 5-HT<sub>1Hel</sub> for pedal and dorsolateral ciliary cells. 5-HT<sub>7Hel</sub> was expressed in only 40% of pedal cells examined ( $N=25$  cells; Fig. 10A). In most of these cells, there was strong expression on the apical surface of the cell, with a small percentage displaying a punctate distribution on the basal surface (data not shown). The majority of pedal cells examined (60%) did not demonstrate any 5-HT<sub>7Hel</sub> expression (Fig. 10B). Similarly, only 46% of dorsolateral ciliary cells expressed 5-HT<sub>7Hel</sub> strongly on the apical surface and weakly throughout the remainder of the cell ( $N=24$  cells; Fig. 10C), while the majority (54%) did not exhibit any 5-HT<sub>7Hel</sub> expression (Fig. 10D). Finally, there was no expression of 5-HT<sub>7Hel</sub> within any of the SSCCs, but there was expression in the surrounding epithelial cells ( $N=22$  cells; Fig. 10E). Taken together, these data implicate the 5-HT<sub>1Hel</sub> receptors, and possibly 5-HT<sub>7Hel</sub> receptors, in the signal transduction pathway for 5-HT-induced cilio-excitation in both pedal and dorsolateral cells.

### Discussion

Early in development, *Helisoma* embryos exhibit cilia-driven rotation within the egg capsule that is augmented by hypoxia (Diefenbach et al., 1991; Kuang et al., 2002). It is believed that embryo rotation facilitates mixing of intracapsular fluid to enhance oxygen diffusion into the egg capsule (Kuang et al., 2002; Hunter and Vogel, 1985). The surface of the embryo contains three known subpopulations of ciliary cells: pedal, dorsolateral and scattered single ciliary cells (SSCCs). Furthermore, ciliary cells are also expressed on the roof of the mouth and further down the gut. The activity of the pedal and dorsolateral ciliary bands is stimulated by 5-HT release from ENC1s, a bilateral pair of sensory-motor neurons (Kuang and Goldberg, 2001). Previous studies on unidentified ciliary cells in culture demonstrated that Ca<sup>2+</sup> influx and the activity of a unique PKC isoform are signal transduction elements in the cilio-excitatory response to 5-HT (Christopher et al., 1996; 1999). While the present study on

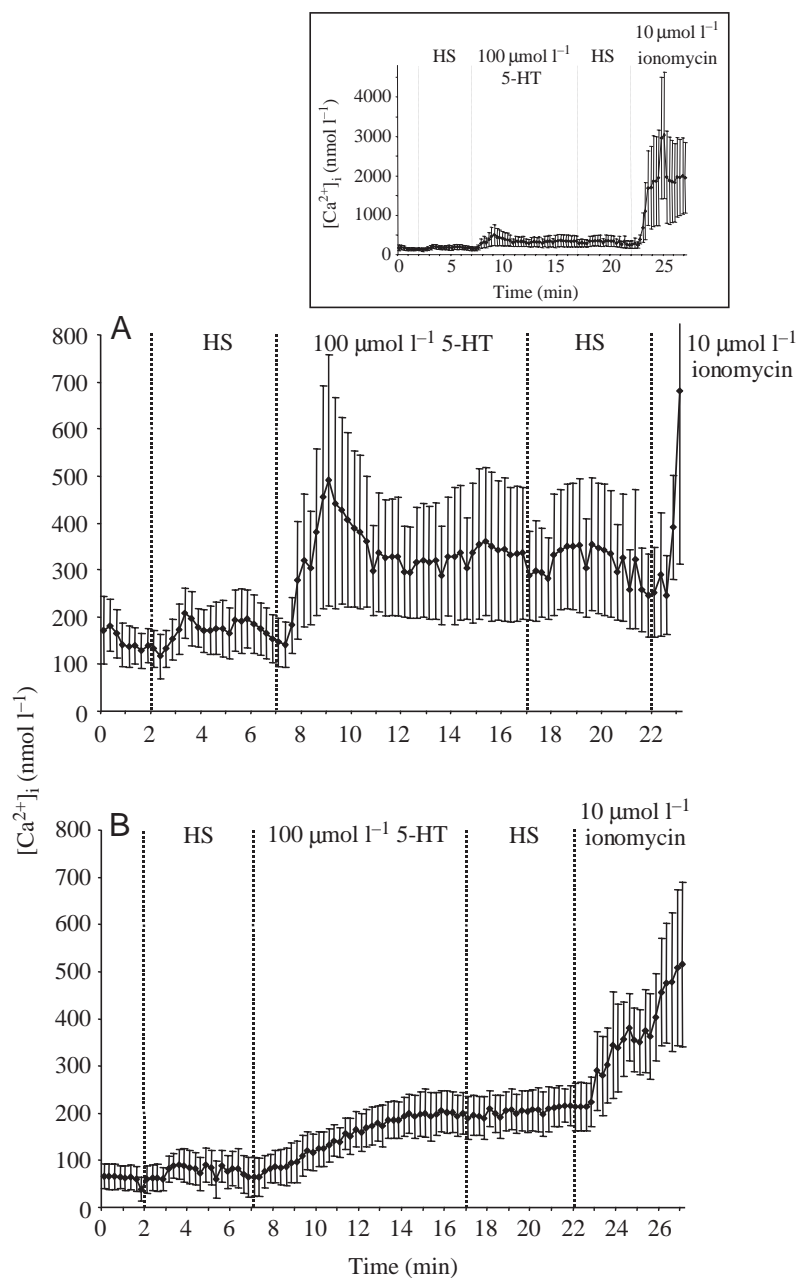


Fig. 6. Effect of 5-hydroxytryptamine (5-HT) and ionomycin on intracellular Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>i</sub>) in pedal ciliary cells. Pedal ciliary cells were microinjected with fura-2 dextran and imaged once every 15 s. Application of 100 μmol l<sup>-1</sup> 5-HT generated two different [Ca<sup>2+</sup>]<sub>i</sub> profiles – an early peak in [Ca<sup>2+</sup>]<sub>i</sub> followed by a sustained plateau (A;  $N=5$  cells) or a gradual rise in [Ca<sup>2+</sup>]<sub>i</sub> over the course of drug application (B;  $N=5$  cells). In both cases, the [Ca<sup>2+</sup>]<sub>i</sub> did not recover within 5 min of exposure to *Helisoma* saline (HS), whereas 10 μmol l<sup>-1</sup> ionomycin stimulated a large increase in the [Ca<sup>2+</sup>]<sub>i</sub>. The inset in A shows the experiment on a different scale to illustrate the magnitude of the increase in [Ca<sup>2+</sup>]<sub>i</sub> in response to ionomycin application. Dotted lines represent the time when the specified treatments were initiated.

unidentified ciliary cells in culture demonstrated that calmodulin is also involved in mediating the 5-HT response, it showed that only some ciliary cells respond to 5-HT with

an elevation in cytoplasmic calcium. These heterogeneous cellular responses, as well as structural differences between the subtypes, prompted us to develop a new microdissection technique for harvesting and culturing surface ciliary cells of known subtype. Pedal, dorsolateral and SSCCs were removed from the surface of the embryo using a suction micropipette. We found that pedal and dorsolateral ciliary cells were similar in terms of their CBF and cytoplasmic calcium responses to 5-HT and the distribution of 5-HT receptor proteins. By contrast, the SSCCs neither responded to 5-HT in CBF and calcium imaging experiments nor expressed 5-HT receptor proteins.

#### Mixed populations of *Helisoma* ciliary cells in culture

A wide variety of signal transduction elements have been

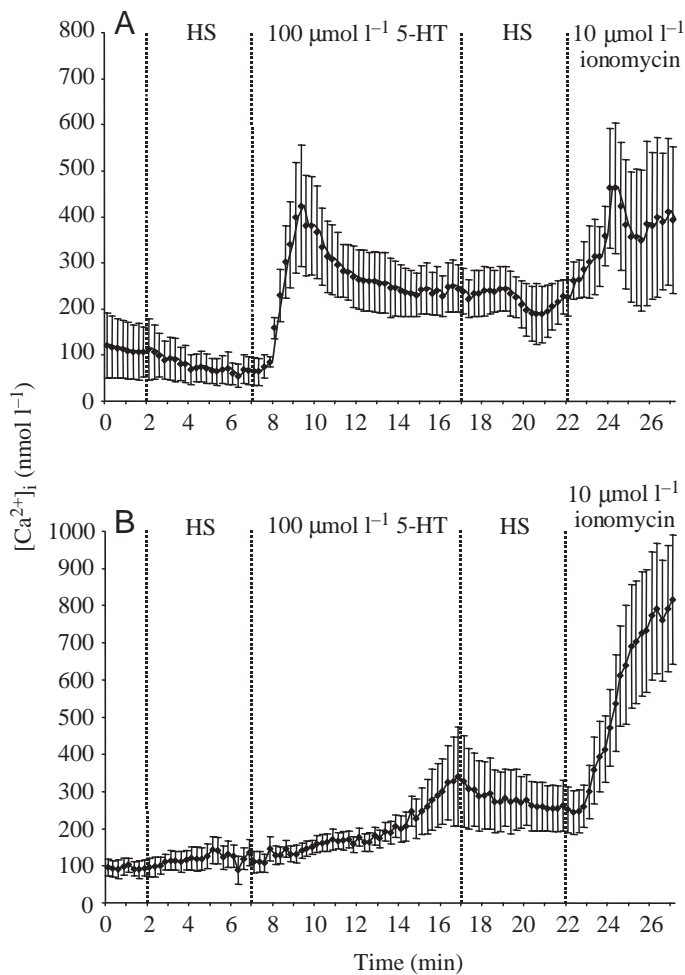


Fig. 7. Effect of 5-hydroxytryptamine (5-HT) and ionomycin on  $[Ca^{2+}]_i$  in dorsolateral ciliary cells. Dorsolateral ciliary cells were microinjected with fura-2 dextran and imaged once every 15 s. Application of 100  $\mu mol\ l^{-1}$  5-HT generated two different types of changes in  $[Ca^{2+}]_i$ , an early peak followed by a sustained plateau (A;  $N=4$  cells) or a gradual rise in  $[Ca^{2+}]_i$  over the course of 5-HT perfusion (B;  $N=6$  cells). In all cells, regardless of the response, 10  $\mu mol\ l^{-1}$  ionomycin produced a variable increase in  $[Ca^{2+}]_i$ . Dotted lines represent the time when the specified treatments were initiated.

shown to participate in regulating ciliary beating in the array of organisms examined. However, changes in  $[Ca^{2+}]_i$  appear to be a possible universal mechanism in altering CBF.  $Ca^{2+}$  regulates ciliary activity in invertebrate systems, such as ctenophore larvae (Tamm and Terasaki, 1994), *Paramecium* (Eckert, 1972) and *Mytilus edulis* (Murakami and Machemer, 1982), and in almost all vertebrate systems examined, including the salamander *Necturus maculosus* (Murakami and Eckert, 1971), the frog esophagus (Levin et al., 1997), the rabbit trachea (Lansley and Sanderson, 1999) and human respiratory epithelium (Di Benedetto et al., 1991). It appears that  $Ca^{2+}$  is either required to bind to a  $Ca^{2+}$ -binding protein at the level of the cilium or is necessary in the activation of other signal transduction cascades that result in phosphorylation of a target protein within the cilium. The  $Ca^{2+}$ -binding protein calmodulin has been shown in both invertebrates (Nakaoka et al., 1984) and vertebrates (Di

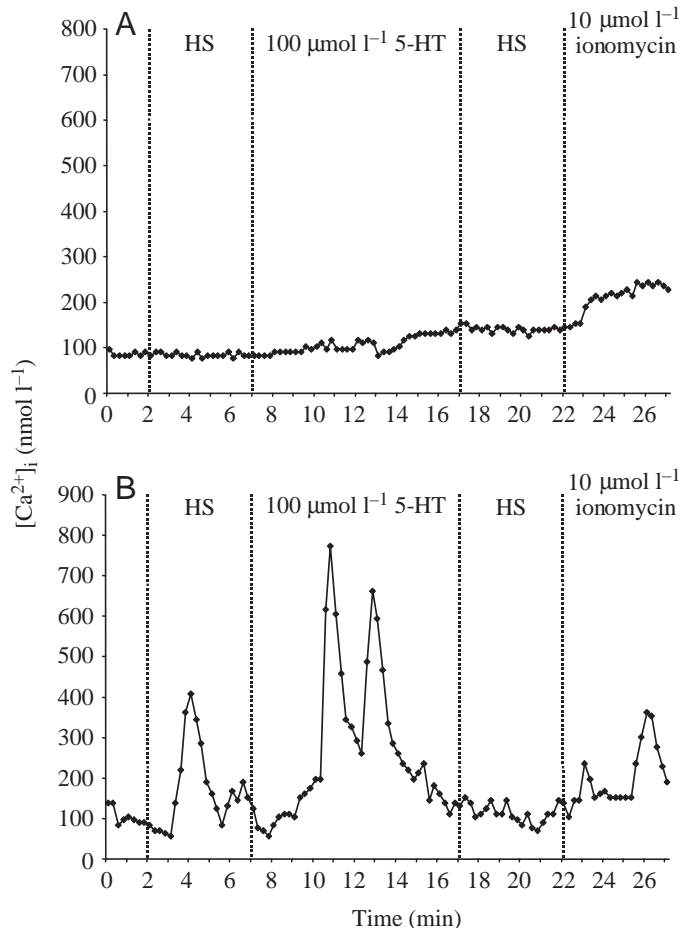


Fig. 8. Effect of 5-hydroxytryptamine (5-HT) and ionomycin on  $[Ca^{2+}]_i$  in scattered single ciliary cells. Scattered single ciliary cells were microinjected with fura-2 dextran and imaged once every 15 s. (A) A representative trace of an isolated tuft cell that did not respond to 100  $\mu mol\ l^{-1}$  5-HT and exhibited only a weak increase in  $[Ca^{2+}]_i$  in response to 10  $\mu mol\ l^{-1}$  ionomycin. (B) A representative trace of an isolated tuft cell that displayed oscillations in  $[Ca^{2+}]_i$ . Dotted lines represent the time when the specified treatments were initiated.

Benedetto et al., 1991) to participate in mediating cilio-excitation. Likewise, in *Helisoma* ciliary cells, calmodulin appears to be a necessary component in the cilio-excitatory response to 5-HT, as the calcium/calmodulin-dependent enzyme inhibitor calmidazolium inhibited this response in unidentified ciliary cells. As application of the inhibitor alone had no effect on basal CBF, it appears that calmodulin is recruited in the 5-HT response but does not contribute to basal ciliary beating. This is consistent with experiments on tracheal ciliary cells from rabbit suggesting that basal ciliary activity was independent of  $[Ca^{2+}]_i$  (Ma et al., 2002). This profile of calmodulin activity may also provide clues about the NOS isoform thought to be expressed in embryonic cilia (Cole et al., 2002). Since NO has been demonstrated to have a constitutive cilio-excitatory action in *Helisoma* ciliary cells (Doran et al., 2003), the ineffectiveness of calmidazolium on basal ciliary beating suggests that embryonic ciliary cells contain a  $Ca^{2+}$ /calmodulin-independent isoform of NOS. Whether this isoform is a unique member of the inducible (i) NOS family, or is a novel isoform, remains to be determined.

Although it has been demonstrated that  $Ca^{2+}$  is required to stimulate an increase in CBF in *Helisoma* ciliary cells (Christopher et al., 1996, 1999), direct imaging of intracellular  $Ca^{2+}$  had not been previously performed because embryonic *Helisoma* cells do not readily accept or retain the acetoxymethyl ester indicators. In the present study, we loaded embryonic tissue using pressure microinjection of fura-2 dextran. An interesting finding from these initial  $Ca^{2+}$  imaging experiments on unidentified ciliary cells was that half of those cells examined did not exhibit an increase in  $Ca^{2+}$  in response to 5-HT. Previous experiments on unidentified ciliary cells in culture revealed that a smaller percentage of cells did not exhibit an increase in CBF in response to 5-HT (Christopher et al., 1996). This finding, as well as results from CBF experiments *in vivo* (Kuang and Goldberg, 2001), suggests that the observed variations in the CBF and  $Ca^{2+}$  responses from cells in culture may be the result of population differences within the mass-dissociated cells in culture. This conclusion was further supported by the electron microscopy analysis of ciliary populations in the present study. Thus, in some ciliary subtypes, 5-HT may stimulate ciliary beating without producing a rise in intracellular  $Ca^{2+}$ , whereas other subtypes may show no response at all.

#### *Innervated Helisoma ciliary cells*

The pedal and dorsolateral ciliary bands on the *Helisoma* embryo are innervated by the serotonergic ENC1s (Kuang and Goldberg, 2001; Koss et al., 2003). These postsynaptic ciliary cells exhibited the typical morphology exhibited by cells with motile cilia (Sleigh, 1962). This includes a  $(9 \times 2) + 2$  microtubule arrangement and an extensive primary and accessory rootlet structure in close proximity to numerous mitochondria. These cells also exhibited regions of cytoplasm that were electron translucent. In an earlier study, these regions were shown to contain 5-HT-immunoreactive vesicles that were proposed to be products of endocytotic 5-HT uptake by

postsynaptic ciliary cells (Koss et al., 2003). Taken together, these morphological data support the conclusion that the pedal and dorsolateral ciliary cells have the necessary machinery to exhibit physiological responses to a chemical regulator.

With the development of the novel cell isolation techniques described in the present study, we gained the ability to examine the effects of 5-HT on identified ciliary cells in culture. Both pedal and dorsolateral cilia respond to 5-HT perfusion with a significant increase in CBF, which reaches a maximum within 45 s. Thus, there appears to be a functional 5-HT receptor on the surface of both these cell types. Prompted by previous evidence that  $Ca^{2+}$  is required for the 5-HT-stimulated increases in CBF and the fact that ionomycin was able to increase the rate of ciliary beating, we imaged intracellular  $Ca^{2+}$  in response to exogenous 5-HT. Both pedal and dorsolateral cells exhibited two different types of  $Ca^{2+}$  responses to 5-HT. Whereas the CBF consistently increased rapidly following 5-HT application, imaging experiments revealed that detectable changes in  $Ca^{2+}$  lagged behind the changes in CBF. The peak and plateau response is suggestive of the profile seen in other ciliary cells where there is an initial release from intracellular  $Ca^{2+}$  stores followed by a sustained  $Ca^{2+}$  influx (Salathe et al., 1997; Korngreen and Priel, 1994). By contrast, the slower rising change in  $[Ca^{2+}]_i$  is less typical. All of these results are consistent with an early rise in calcium occurring in a restricted cellular compartment, possibly immediately below the cilia (Lansley and Sanderson, 1999), which is not detectable in our whole-cell analysis. Through diffusion and  $Ca^{2+}$ -buffering mechanisms, the localized early signal may be transformed into the delayed peak and plateau signal or slow-rising signal that we observed in whole cell measurements. An alternative, albeit less likely, explanation is that the initial ciliary response to 5-HT is not dependent on a rise in  $[Ca^{2+}]_i$ . We hope to distinguish between these possibilities in a detailed spatial analysis of calcium signals and a study on the interaction of  $Ca^{2+}$  and PKC (Christopher et al., 1999), both of which are currently underway.

An interesting finding from this study is the slow decay of both the CBF and  $[Ca^{2+}]_i$  following washout of the 5-HT. In contrast to these experimentally induced responses, the ciliary responses to endogenously released 5-HT in intact embryos are transient, producing periodic increases in the embryo rotation (Kuang and Goldberg, 2001; Cole et al., 2002; Diefenbach et al., 1991). Thus, maintained exposure to elevated concentrations of 5-HT causes long-term changes in ciliary activity, the function of which is not fully understood. Similar maintained increases in CBF were observed after prolonged laser stimulation of ENC1 in intact embryos (Kuang and Goldberg, 2001). Furthermore, repeated exposure of embryos to environmental hypoxia caused a facilitation of the embryonic rotation response, a form of plasticity that is likely to be related to the slow response decay observed herein (Kuang et al., 2002). Perhaps the slowly rising  $Ca^{2+}$  signal observed in this study functions primarily to produce a maintained state of elevated CBF, rather than mediating the initial increases in CBF observed during transient exposure to 5-HT.

The pedal and dorsolateral ciliary cells showed similar immunoreactivity to the antibodies raised against the recently

cloned *Helisoma* 5-HT receptors. The 5-HT<sub>1Hel</sub> protein was strongly expressed on the apical surface, with weaker expression throughout the other regions of the cells. The surface expression may indicate the presence of 5-HT in the

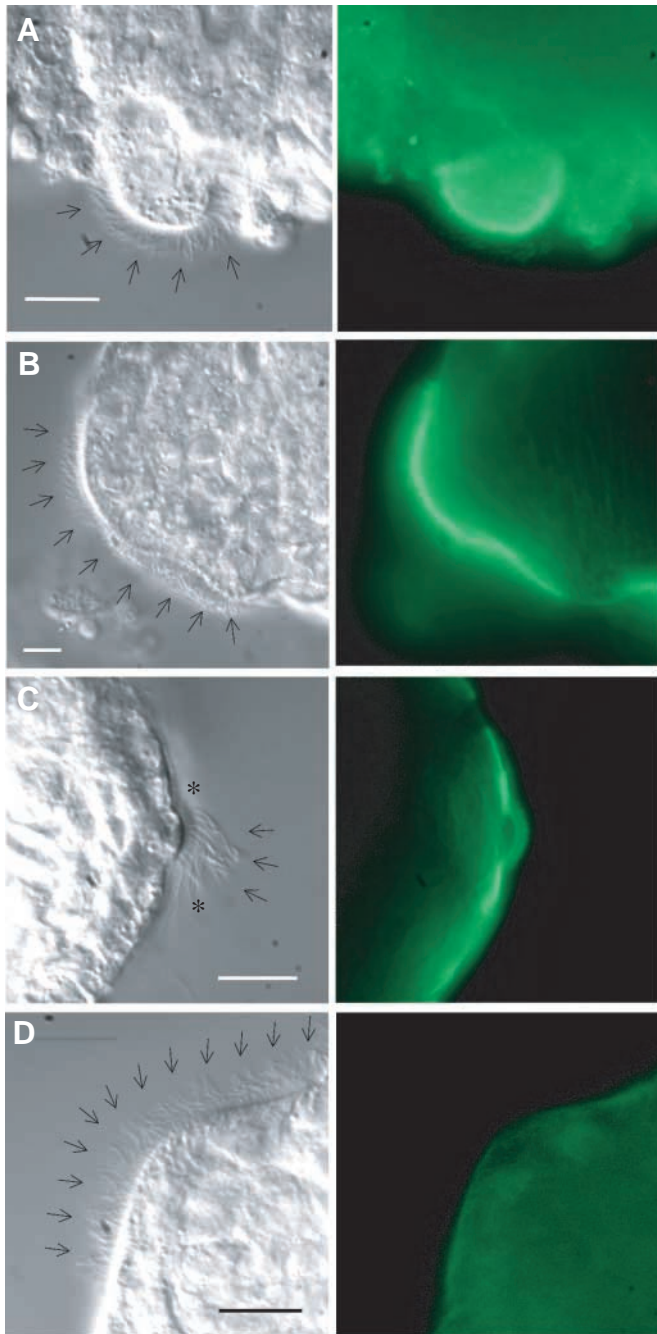
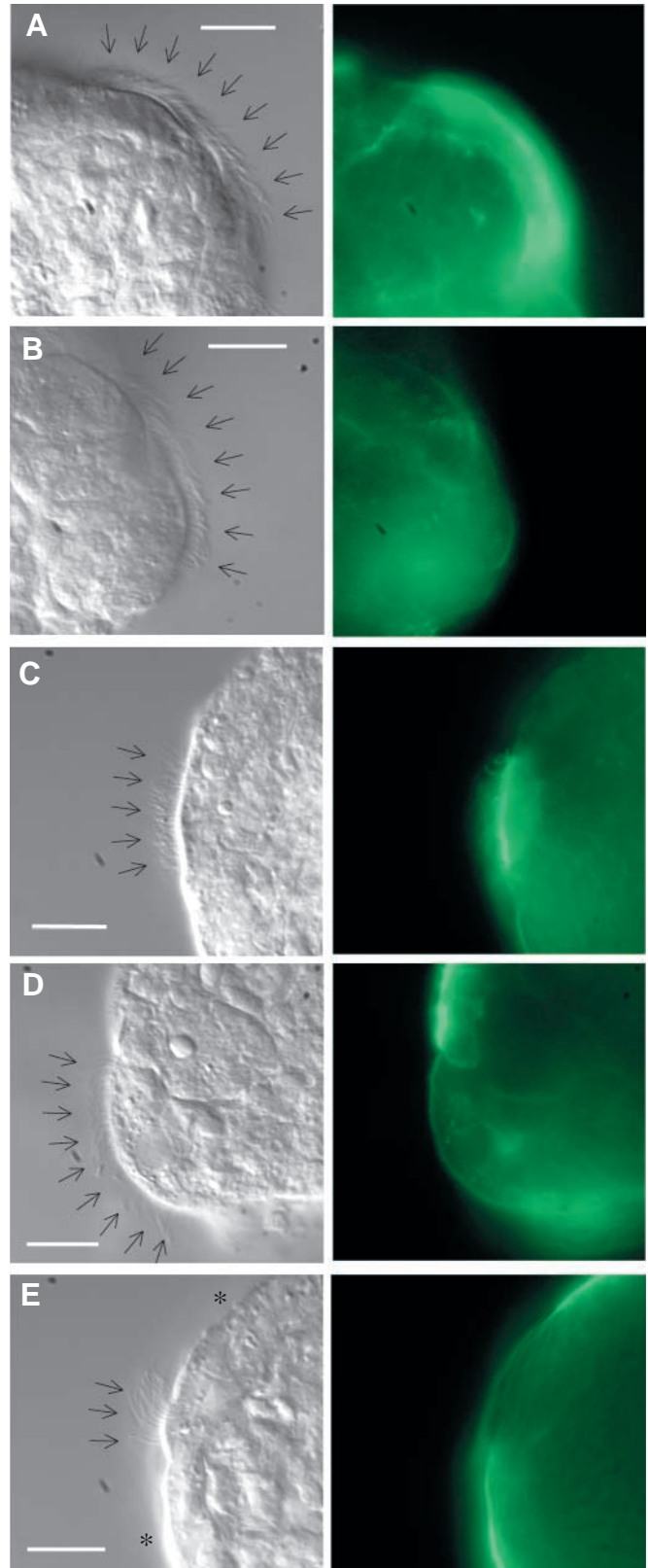


Fig. 9. 5-HT<sub>1Hel</sub> receptor immunoreactivity in identified *Helisoma* ciliary cells. Nomarski differential interference contrast (DIC) images (left panels) and corresponding fluorescence micrographs (right panels) of tissue explants containing identified ciliary cells that were processed for 5-HT<sub>1Hel</sub> immunoreactivity. Arrows indicate the apical surface of the ciliary cells. (A) Immunopositive pedal ciliary cell. (B) Immunoreactivity in multiple dorsolateral ciliary cells. (C) Immunoreactivity restricted to the non-ciliary epithelial cells (asterisks) surrounding an unstained single ciliary cell. (D) A control explant containing pedal ciliary cells was exposed to preimmune serum instead of primary antibody. Scale bars, 10  $\mu$ m.



intracapsular fluid that may contribute to the tonic rotation displayed by the embryos. Pulsatile release of 5-HT from ENC1 is believed to be responsible for the generation of periodic surges in rotation that are superimposed upon a slow basal rate of spinning (Kuang and Goldberg, 2001; Diefenbach et al., 1991). Thus, either a different 5-HT receptor or a lower concentration of the same receptor is expressed on the basal surface of the pedal and dorsolateral ciliary cells to mediate ENC1–ciliary cell communication. Since punctate 5-HT<sub>1Hel</sub>-like immunoreactivity was sometimes observed at the basal ciliary surfaces, it seems likely that the 5-HT<sub>1Hel</sub> receptor is the primary cilio-excitatory receptor in *Helisoma* embryos.

In contrast to the consistent ciliary expression of the 5-HT<sub>1Hel</sub> receptor, the 5-HT<sub>7Hel</sub> protein was expressed in only a minority of pedal and dorsolateral cells. There was either strong, specific expression that resembled the 5-HT<sub>1Hel</sub> pattern or no expression at all. This inconsistent expression, together with significant differences in the molecular structure between the 5-HT<sub>1Hel</sub>-encoding and 5-HT<sub>7Hel</sub>-encoding genes (Mapara et al., 2001) suggest that the 5-HT<sub>7Hel</sub> receptors play a different role from the 5-HT<sub>1Hel</sub> receptors. Furthermore, the appearance of the 5-HT<sub>7Hel</sub> immunoreactivity in only some cells may suggest that this protein only begins to be expressed around stage E25, with a more complete expression pattern occurring at later stages. In order to fully investigate this hypothesis, more studies are required to examine embryos at different stages of embryonic and postembryonic development.

#### *Non-innervated Helisoma ciliary cells*

In stage E25 *Helisoma* embryos, at least three groups of ciliary cells are not directly innervated by ENC1 or other identified neurons: the lateral cells of the dorsolateral bands, the SSCCs and the ciliary cells lining the gut. Since the gut cells are not accessible to the microdissection techniques introduced in the present study, their physiological characteristics remain unclear.

The dorsolateral ciliary bands each contain four cells, with only the most medial one in each band receiving innervation (Koss et al., 2003). Despite this arrangement, all dorsolateral band cells display similar 5-HT receptor immunoreactivity and responsiveness to 5-HT. Thus, paracrine actions of 5-HT and electrical or chemical signals passing through gap junctions (Koss et al., 2003) likely contribute to the ENC1-induced

stimulation of these non-innervated cells (Kuang and Goldberg, 2001).

Kuang and Goldberg (2001) identified the SSCCs (previously referred to as isolated tufts of cilia) as a third subtype of ciliary cells expressed on the surface of the embryo during development. These non-innervated cells have a different morphology from either the pedal or dorsolateral ciliary cells and do not respond to 5-HT *in vivo*. While the SSCCs do exhibit the (9×2)+2 microtubular arrangement indicative of motile cilia (Sleigh, 1962), these cells differ from the pedal and dorsolateral cilia in their less organized ciliary arrangement, a simpler rootlet structure, less prominent mitochondria and absence of electron-translucent regions. The SSCCs are not innervated by ENC1 and do not show any anatomical evidence of neurite–ciliary cell apposition sites. These morphological differences support the findings that the SSCCs are physiologically distinct from the pedal and dorsolateral ciliary cells. The present study confirmed that 5-HT does not stimulate a change in CBF in these cells *in vitro*. Thus, it is likely that these SSCCs and the uncharacterized gut ciliary cells contributed to the populations of non-responsive cells observed in mass-dissociated cultures (Christopher et al., 1996).

The SSCCs have been observed to exhibit two states of activity: a quiescent phase, with slow ciliary beating and abbreviated ciliary beat strokes, and an active beating phase, with very rapid ciliary beating and full ciliary beat strokes (data not shown). Neither the pedal nor the dorsolateral ciliary cells exhibited either of these states under basal conditions. The mechanics of the ciliary beating in the SSCCs was also different from that exhibited by the other two populations; the SSCCs beat in a flagellar fashion whereas the other two subtypes exhibited more typical ciliary wave-like beating. This observation is consistent with the finding that the SSCCs exhibited longer cilia with a different rootlet structure. The Ca<sup>2+</sup> profiles in these cells also support the idea of two different states of activity, with some cells demonstrating a relatively flat baseline with no response to 5-HT and other cells showing an unstable baseline and intermittent calcium spikes, both in the presence and absence of 5-HT. It may be that the fluctuations in [Ca<sup>2+</sup>]<sub>i</sub> are responsible for maintaining the high rate of ciliary beating demonstrated by these cells. Oscillations in [Ca<sup>2+</sup>]<sub>i</sub> have been shown to regulate the CBF in rabbit airway epithelium (Evans and Sanderson, 1999) and ovine tracheal epithelial cells (Salathe and Bookman, 1995). Simultaneous CBF and Ca<sup>2+</sup> imaging experiments need to be performed to determine if the type of Ca<sup>2+</sup> activity correlates to the ciliary beat state of the cell. In some SSCCs examined, ionomycin perfusion produced a small amplitude change in intracellular Ca<sup>2+</sup> that was often transient. This response to calcium ionophore further suggests that the SSCCs regulate intracellular Ca<sup>2+</sup> through different mechanisms than the pedal and dorsolateral ciliary cells.

The absence of CBF and Ca<sup>2+</sup> responses to 5-HT and 5-HT<sub>1Hel</sub> and 5-HT<sub>7Hel</sub> immunoreactivity suggests that the

Fig. 10. 5-HT<sub>7Hel</sub> receptor immunoreactivity in identified *Helisoma* ciliary cells. Nomarski differential interference contrast (DIC) images (left panels) and corresponding fluorescence micrographs (right panels) of tissue explants containing identified ciliary cells that were processed for 5-HT<sub>7Hel</sub> immunoreactivity. Arrows indicate the apical surface of the ciliary cells. (A) Immunopositive pedal ciliary cells. (B) Immunonegative pedal ciliary cells. (C) Immunopositive dorsolateral ciliary cells. (D) Immunonegative dorsolateral ciliary cells. (E) As with 5-HT<sub>1Hel</sub> immunoreactivity, 5-HT<sub>7Hel</sub> immunoreactivity was restricted to non-ciliary epithelial cells (asterisks) surrounding the scattered single ciliary cell. Scale bars, 10 μm.

SSCCs may be responsive to a non-neuronal cue. One possibility is that these cells are activated by mechanical stimuli. This is suggested by the finding that the start of saline perfusion triggered some SSCCs to switch from the quiescent to active phase of ciliary beating, a result not observed in the other ciliary cell types. A possible role for mechanosensitive ciliary cells in the *Helisoma* embryo would be to keep the surface of the animal free of debris during development. This may be especially important on the posterior aspect of the embryo to ensure that particulate matter does not interfere with the deposition of shell matrix. Mechanical stimulation of the cell membrane has been identified to regulate ciliary activity in *Paramecium* and the lateral gill cilia of *Mytilus edulis* (Eckert, 1972; Murakami and Takahashi, 1975; Murakami and Machemer, 1982). In these systems, mechanical stimuli generate a change in membrane potential that leads to alterations in ion conductances, specifically enabling Ca<sup>2+</sup> entry, which in turn modifies ciliary activity. Alternatively, the SSCCs may be immature at the embryonic stages examined in the present study, differentiating at later stages under the influence of posterior embryonic neurons, such as those described by Croll and Voronezhskaya (1996) in other gastropod species. Although similar dopamine- and FMRFamide-containing neurons have yet to be found in stage E25 *Helisoma* embryos (Goldberg, 1995), further studies are necessary to determine whether the SSCCs eventually become innervated in *Helisoma* embryos.

This research was supported by an NSERC Canada operating grant to J.I.G. The authors wish to thank Drs Shihuan Kuang and Calvin Wong for helpful discussions.

### References

- Aiello, E., Kennedy, J. and Hernandez, C. (1991). Stimulation of frog ciliated cells in culture by acetylcholine and substance P. *Comp. Biochem. Physiol. C* **99**, 497-506.
- Bonini, N. M., Evans, T. C., Miglietta, L. A. and Nelson, D. L. (1991). The regulation of ciliary motility in *Paramecium* by Ca<sup>2+</sup> and cyclic nucleotides. *Adv. Second Messenger Phosphoprotein Res.* **23**, 227-272.
- Braiman, A., Zagoory, O. and Priel, Z. (1998). PKA induces Ca<sup>2+</sup> release and enhances ciliary beat frequency in a Ca<sup>2+</sup>-dependent and -independent manner. *Am. J. Physiol.* **275**, C790-C797.
- Chia, F. S., Buckland-Nicks, J. and Young, C. M. (1984). Locomotion of marine invertebrate larvae: a review. *Can. J. Zool.* **62**, 1205-1222.
- Christopher, K., Chang, J. P. and Goldberg, J. I. (1996). Stimulation of cilia beat frequency by serotonin is mediated by a Ca<sup>2+</sup> influx in ciliated cells of *Helisoma trivolvis* embryos. *J. Exp. Biol.* **199**, 1105-1113.
- Christopher, K. J., Young, K. G., Chang, J. P. and Goldberg, J. I. (1999). Involvement of protein kinase C in 5-HT-stimulated ciliary activity in *Helisoma trivolvis* embryos. *J. Physiol.* **515**, 511-522.
- Cole, A. G., Mashikournia, A., Parries, S. C. and Goldberg, J. I. (2002). Regulation of early embryonic behavior by nitric oxide in the pond snail, *Helisoma trivolvis*. *J. Exp. Biol.* **205**, 3143-3152.
- Croll, R. P. and Voronezhskaya, E. E. (1996). Early elements in gastropod neurogenesis. *Dev. Biol.* **173**, 344-377.
- Di Benedetto, G., Magnus, C. J., Gray, P. T. and Mehta, A. (1991). Calcium regulation of ciliary beat frequency in human respiratory epithelium in vitro. *J. Physiol.* **439**, 103-113.
- Diefenbach, T. J., Koehncke, N. K. and Goldberg, J. I. (1991). Characterization and development of rotational behavior in *Helisoma* embryos: role of endogenous serotonin. *J. Neurobiol.* **22**, 922-934.
- Diefenbach, T., Koss, R. and Goldberg, J. I. (1998). Early development of an identified serotonergic neuron in *Helisoma trivolvis* embryos: serotonin expression, de-expression and uptake. *J. Neurobiol.* **34**, 361-376.
- Doran, S. A., Tran, N., Eskicioglu, C., Stachniak, T., Ahn, K. C. and Goldberg, J. I. (2003). Constitutive and permissive roles of nitric oxide activity in embryonic ciliary cells. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **285**, R348-R355.
- Eckert, R. (1972). Bioelectric control of ciliary activity. *Science* **176**, 473-481.
- Essner, J. J., Vogan, K. J., Wagner, M. K., Tabin, C. J., Yost, H. J. and Brueckner, M. (2002). Conserved function for embryonic nodal cilia. *Nature* **418**, 37-38.
- Evans, J. H. and Sanderson, M. J. (1999). Intracellular calcium oscillations regulate ciliary beat frequency of airway epithelial cells. *Cell Calcium* **26**, 103-110.
- Goldberg, J. I. (1995). Neuronal development in embryos of the mollusk, *Helisoma trivolvis*: multiple roles of serotonin. *Adv. Neural Sci.* **2**, 67-87.
- Gosselin, R. E., Moore, K. E. and Milton, A. S. (1962). Physiological control of molluscan gill cilia by 5-hydroxytryptamine. *J. Gen. Physiol.* **46**, 277-296.
- Grynkiewicz, D., Neufeld, G. and Schweigner, L. (1985). A new generation of Ca<sup>2+</sup> indicators with greatly improved fluorescence properties. *J. Biol. Chem.* **260**, 3440-3450.
- Hunter, T. and Vogel, S. (1985). Spinning embryos enhance diffusion through gelatinous egg masses. *J. Exp. Mar. Biol. Ecol.* **96**, 303-308.
- Konishi, M. and Watanabe, M. (1995). Resting cytoplasmic free Ca<sup>2+</sup> concentration in frog skeletal muscle measured with fura-2 conjugated to high molecular weight dextran. *J. Gen. Physiol.* **106**, 1123-1150.
- Korngreen, A. and Priel, Z. (1994). Simultaneous measurements of ciliary beating and intracellular calcium. *Biophys. J.* **67**, 377-380.
- Koss, R., Diefenbach, T. J., Kuang, S., Doran, S. A. and Goldberg, J. I. (2003). Coordinated development of identified serotonergic neurons and their target ciliary cells in *Helisoma trivolvis* embryos. *J. Comp. Neurol.* **457**, 313-325.
- Kuang, S. and Goldberg, J. I. (2001). Laser ablation reveals regulation of ciliary activity by serotonergic neurons in molluscan embryos. *J. Neurobiol.* **47**, 1-15.
- Kuang, S., Doran, S. A., Wilson, R. J. A., Goss, G. G. and Goldberg, J. I. (2002). Early developing neurons mediate oxygen sensing in pond snail embryos. *J. Neurobiol.* **52**, 73-83.
- Lansley, A. B. and Sanderson, M. J. (1999). Regulation of airway ciliary activity by Ca<sup>2+</sup>: simultaneous measurement of beat frequency and intracellular Ca<sup>2+</sup>. *Biophys. J.* **77**, 629-638.
- Levin, R., Braiman, A. and Priel, Z. (1997). Protein kinase C induced calcium influx and sustained enhancement of ciliary beating by extracellular ATP. *Cell Calcium* **21**, 103-113.
- Ma, W., Silberberg, S. D. and Priel, Z. (2002). Distinct axonemal processes underlie spontaneous and stimulated airway ciliary activity. *J. Gen. Physiol.* **120**, 875-885.
- Mapara, S., Goldberg, J. I. and Gallin, W. J. (2001). Cloning, phylogenetic analysis and localization of two serotonin receptors from the pond snail *Helisoma trivolvis*. *Soc. Neurosci. Abstr.* **786**.
- Morales, B., Barrera, N., Uribe, P., Mora, C. and Villalon, M. (2000). Functional cross talk after activation of P2 and P1 receptors in oviductal ciliated cells. *Am. J. Physiol. Cell Physiol.* **279**, C658-C669.
- Murakami, A. (1987). Control of ciliary beat frequency in the gill of *Mytilus*. I. Activation of the lateral cilia by cyclic AMP. *Comp. Biochem. Physiol. C* **86**, 273-279.
- Murakami, A. and Machemer, H. (1982). Mechanoreception and signal transmission in the lateral ciliated cells on the gill of *Mytilus*. *J. Comp. Physiol.* **145**, 351-362.
- Murakami, A. and Takahashi, K. (1975). Correlation of electrical and mechanical responses in nervous control of cilia. *Nature* **257**, 48-49.
- Murakami, A. and Eckert, R. (1971). Cilia: activation coupled to mechanical stimulation by calcium influx. *Science* **175**, 1375-1377.
- Nakaoka, Y., Tanaka, H. and Oosawa, F. (1984). Ca<sup>2+</sup>-dependent regulation of beat frequency of cilia in *Paramecium*. *J. Cell Sci.* **65**, 223-231.
- Reynolds, E. S. (1963). The use of lead citrate at high pH as an electron opaque stain in electron microscopy. *J. Cell Biol.* **17**, 208-212.
- Salathe, M. and Bookman, R. J. (1999). Mode of Ca<sup>2+</sup> action on ciliary beat frequency in single ovine airway epithelial cells. *J. Physiol.* **520**, 851-865.
- Salathe, M., Lipson, E. J., Ivonnet, P. I. and Bookman, R. J. (1997). Muscarinic signaling in ciliated tracheal epithelial cells: dual effects on Ca<sup>2+</sup> and ciliary beating. *Am. J. Physiol.* **272**, L301-L310.

- Salathe, M. and Bookman, R. J.** (1995). Coupling of  $[Ca^{2+}]_i$  and ciliary beating in cultured tracheal epithelial cells. *J. Cell Sci.* **108**, 431-440.
- Salathe, M., Pratt, M. M. and Wanner, A.** (1993). Protein kinase C-dependent phosphorylation of a ciliary membrane protein and inhibition of ciliary beating. *J. Cell Sci.* **106**, 1211-1220.
- Satir, P. and Sleigh, M. A.** (1990). The physiology of cilia and mucociliary interactions. *Annu. Rev. Physiol.* **52**, 137-155.
- Sleigh, M. A.** (1962). *The Biology of Cilia and Flagella*. Oxford, UK: Pergamon Press.
- Tamm, S. L. and Terasaki, M.** (1994). Visualization of calcium transients controlling orientation of ciliary beat. *J. Cell Biol.* **125**, 1127-1153.
- Tamoki, J., Chiyotani, A., Kondo, M. and Konno, K.** (1995). Role of NO generation in  $\beta$ -adrenoceptor-mediated stimulation of rabbit airway ciliary motility. *Am. J. Physiol.* **268**, C1342-C1347.
- Tombal, B., Denmeade, S. R. and Isaacs, J. T.** (1999). Assessment and validation of a microinjection method for kinetic analysis of  $[Ca^{2+}]_i$  in individual cells undergoing apoptosis. *Cell Calcium* **25**, 19-28.
- Wada, Y., Mogami, Y. and Baba, S. A.** (1997). Modification of ciliary beating in sea urchin larvae induced by neurotransmitters: beat-plane rotation and control of frequency fluctuation. *J. Exp. Biol.* **200**, 9-18.
- Wood, R. L. and Luft, J. H.** (1965). The influence of buffer systems on fixation with osmium tetroxide. *J. Ultrastruct. Res.* **12**, 22-45.
- Yang, B., Schlosser, R. J. and McCaffrey, T. V.** (1996). Dual signal transduction mechanisms modulate ciliary beat frequency in upper airway epithelium. *Am. J. Physiol.* **270**, L745-L751.
- Zagoory, O., Braiman, A., Gheber, L. and Priel, Z.** (2001). Role of calcium and calmodulin in ciliary stimulation induced by acetylcholine. *Am. J. Physiol.* **280**, C100-C109.