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STIMULATION OF CILIARY BEAT FREQUENCY BY SEROTONIN IS MEDIATED BY A Ca^{2+} INFLUX IN CILIATED CELLS OF *HELISOMA TRIVOLVIS* EMBRYOS

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Summary

Serotonin (5-HT) has been established as a regulator of ciliary beating in numerous systems. In early embryos of *Helisoma trivolvis*, a cilia-driven rotational movement is modulated by the release of endogenous serotonin from a pair of embryonic neurons, ENC1s, directly onto the ciliated epithelium. The present study was undertaken to examine the signal transduction mechanisms underlying serotonin-mediated cilio-excitation in *Helisoma trivolvis* embryos. Using time-lapse videomicroscopy, the ciliary beat frequency (CBF) of cultured embryonic ciliated cells was measured in response to various pharmacological manipulations. Serotonin increased CBF in a dose-dependent manner. Addition of 8-bromo-cyclic AMP, isobutylmethylxanthine (IBMX) or a combination of forskolin and IBMX, treatments that elevate the concentration of intracellular cyclic AMP, did not mimic the serotonin-induced increase in CBF. Thus, cyclic AMP does not appear to be involved in the regulation of CBF in this system. In contrast, depolarizing the cells with KCl or veratridine, and artificially raising the intracellular Ca^{2+} concentration with thapsigargin or A23187, caused a serotonin-like increase in CBF. Furthermore, the serotonin response was abolished in a Ca^{2+}-depleted medium or in a medium containing the L-type Ca^{2+} channel blockers verapamil or nifedipine. These results suggest that serotonin-stimulated cilio-excitation in cultured *Helisoma trivolvis* cells involves an influx of Ca^{2+} to increase intracellular Ca^{2+} concentration. The link between serotonin–receptor binding and Ca^{2+} influx in these cells has yet to be determined.

Key words: calcium, cyclic AMP, serotonin, ciliary beating, mollusc, gastropod, pulmonate, *Helisoma trivolvis*, embryo, cell culture, beat frequency.

Introduction

During early embryonic development, embryos of *Helisoma trivolvis* display a characteristic rotational movement within their individual egg capsules (Diefenbach *et al.* 1991). These rotations are driven by the coordinated beating of two bands of ciliated epithelium, the dorsolateral prototrochal band and the ventromedial pedal band, which is innervated by a pair of identified serotonergic neurons. Pharmacological experiments on intact embryos suggested that endogenous serotonin is released onto the ciliated cells to produce transient periods of accelerated rotation (Diefenbach *et al.* 1991; Goldberg *et al.* 1994). Furthermore, examination of ciliated cells under cell culture conditions suggested that serotonin acts directly upon these cells, producing cilio-excitation *via* a serotonin receptor with novel pharmacological properties (Goldberg *et al.* 1994). Given its suitability for analysis at both the behavioral and cellular levels, the embryonic *H. trivolvis* model system provides an opportunity to gain a comprehensive multi-level understanding of the regulation of ciliary activity by serotonin.

The neurotransmitter serotonin has been shown to be a common regulator of ciliary beating in numerous species. The lateral gill cilia of *Mytilus edulis* (Saimi *et al.* 1983), the pedal cilia of *Tritonia diomedea* (Audesirk *et al.* 1979) and *Lymnaea stagnalis* (Syed and Winlow, 1989), the palatine mucosal cilia in the frog (Maruyama *et al.* 1984) and the cilia of sea urchin plutei (Mogami *et al.* 1992) all display excitatory responses to serotonin. There are few systems, however, in which the signal-transduction mechanisms underlying serotonin-induced cilio-excitation are well understood. This question has been addressed mostly in studies on *M. edulis*. In the lateral gill cilia of this bivalve, the serotonin-induced increase in ciliary beat frequency (CBF) can be mimicked by raising the intracellular concentration of cyclic AMP (Murakami, 1983, 1987). Although cyclic AMP has also been implicated in the regulation of other ciliary systems, not all of these actions are part of a serotonin response. For example, cyclic AMP has cilio-excitatory actions in the ascidian *Ciona intestinalis*

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(Bergles and Tamm, 1992), in human nasal epithelium (Di Benedetto *et al.* 1990) and in rabbit tracheal epithelium (Tamaoki *et al.* 1989; Lansley *et al.* 1992). Furthermore, the ciliary beating underlying forward locomotion in *Paramecium tetraurelia* is stimulated by cyclic AMP (Bonini *et al.* 1986). However, these actions of cyclic AMP have not been linked to stimulation by serotonin or other neurotransmitters.

Ca^{2+} is another intracellular regulator that has been implicated in the control of ciliary activity. Like cyclic AMP, however, only a few studies have established a role for Ca^{2+} in mediating the ciliary response to specific neurotransmitters (Paparo and Murphy, 1975). For instance, the dopamine-induced inhibition of ciliary beating in the lateral gill of *M. edulis* may involve an increase in intracellular Ca^{2+} concentration (Paparo and Murphy, 1975). Likewise, Ca^{2+} has cilio-inhibitory actions in the gill cilia of the bay scallop *Aequipecten irradians* (Stommel *et al.* 1982) and the branchial basket cilia of *Ciona intestinalis* (Bergles and Tamm, 1992). Furthermore, Ca^{2+} has been implicated in the ciliary arrest that underlies the metamorphic settlement response of *Calliostoma ligatum* veligers (Arkett *et al.* 1987). In contrast to these inhibitory actions, cilio-excitation occurs in response to increased intracellular Ca^{2+} concentrations in several ciliary systems, including the abfrontal gill cilia of *M. edulis* (Stommel, 1984*a*,*b*), the macrocilia of the ctenophore *Beroë cucumis* (Tamm, 1988), respiratory cilia in humans (Di Benedetto *et al.* 1991) and rabbit (Lansley *et al.* 1992) and the oviduct cells of *Necturus maculosus* (Eckert and Murakami, 1972). In addition, ciliary reversal is triggered by Ca^{2+} in *Paramecium caudatum* (Nakaoka *et al.* 1984) and the combplates of *Mnemiopsis leidyi* (Nakamura and Tamm, 1985). As these studies suggest, multiple regulatory pathways have evolved to control ciliary beating in different organisms and in different populations of ciliated cells within the same organism. In only a few instances, however, are the neurotransmitters that are linked to these regulatory pathways known.

In the present study, time-lapse videomicroscopy was employed to study the signal-transduction mechanism underlying serotonin-mediated cilio-excitation in cultured embryonic ciliated cells of *H. trivolvis*. Our results show that the response to serotonin was not mimicked by experimentally induced increases in cyclic AMP levels. In contrast, the response was mimicked by treatments that increase the intracellular Ca^{2+} concentration and blocked by treatments that prevent Ca^{2+} influx. The link between serotonin–receptor binding and Ca^{2+} influx remains to be determined.

Materials and methods

Animals and drugs

An inbred, laboratory-reared albino colony of *Helisoma trivolvis* was raised in filtered, flow-through glass aquaria (45 l) with an oyster shell substratum (Diefenbach and Goldberg, 1990). The snails were maintained at 25 °C on a 12 h:12 h light:dark cycle. Their diet consisted of trout chow (Vextra) and romaine lettuce. Embryos are contained within individual egg capsules housed in a single egg mass. Egg masses, containing approximately 25 sibling embryos (Goldberg *et al.* 1988), were collected from the glass walls of the aquaria or from Petri dishes placed at the bottom of the tanks. Egg masses were removed with a razor blade and transferred to a 150 mm Petri dish (Falcon) containing artificial pond water (APW; 0.025 % Instant Ocean, Aquarium Systems, Mentor, OH, USA). Embryos were viewed through a dissection microscope and their development staged according to the system of McKenney and Goldberg (1989). All embryos used were of embryonic stage E25–E30, which represents 25–30 % of intracapsular developmental time.

Solutions of serotonin (5-HT creatine sulfate, Sigma), verapamil (Sigma), nifedipine (Sigma), potassium chloride (KCl; BDH), sodium chloride (NaCl; BDH) and 8-bromo-cyclic 3′,5′-adenosine monophosphate (8-br-cAMP; Sigma) were made by dissolving the compound in *Helisoma* saline (HS: 51.3 mmol l^{-1} NaCl, 1.7 mmol l^{-1} KCl, 4.1 mmol l^{-1} $CaCl_2$, 1.5 mmol l^{-1} $MgCl_2$, 5.0 mmol l^{-1} Hepes; pH 7.3). Forskolin (Calbiochem) and A23187 (Sigma) were dissolved in dimethyl sulfoxide (DMSO; BDH). Thapsigargin and veratridine (both from Research Biochemicals Incorporated) were dissolved in 100 % ethanol, while 3-isobutyl-1-methylxanthine (IBMX; Sigma) was dissolved in equal amounts of 100 % ethanol and HS. Concentrated drug solutions were added to cultures in volumes ranging from 2 to 40 μl in order to produce the desired final concentration upon dilution with the 2 ml of culture medium. The dish was gently agitated to facilitate complete mixing of the drug throughout the culture dish. Complementary culture dishes were used to examine the effects of each dissolving solution (vehicle controls) in the absence of drug. The concentrations of vehicle employed had no effect on CBF. In experiments testing the dose-dependent effects of serotonin, all cells were included in the analysis. This is in contrast to the method of Goldberg *et al.* (1994), where only cells that demonstrated robust responses were included.

Isolation and culture of ciliated cells

Embryonic ciliated cells were cultured as described previously (Goldberg *et al.* 1994). Briefly, intact egg masses were rinsed in 35 % ethanol, followed by three washes in antibiotic-containing *Helisoma* saline (AHS: HS + 150 μg ml^{-1} gentamycin; Sigma). Embryos were isolated from their egg capsules and transferred to 0.2 % trypsin in AHS for 20–25 min. Embryos were transferred to 0.1 % trypsin inhibitor in AHS for 15 min and subsequently washed in *Helisoma*-defined medium [HDM: 50 % Liebovitz-15 (Gibco), 40.0 mmol l^{-1} NaCl, 1.7 mmol l^{-1} KCl, 4.1 mmol l^{-1} $CaCl_2$, 1.5 mmol l^{-1} $MgCl_2$, 5.0 mmol l^{-1} Hepes, 50 μg ml^{-1} gentamycin, 0.015 % L-glutamine; pH 7.3] for 10 min. The embryos were mechanically dissociated by passing them repeatedly through a 63 μm nylon mesh (Nytex). The cell suspension was plated in 0.5 ml droplets onto poly-L-lysine-coated (hydrobromide; M_r 4×10^3 to 15×10^3; 1 mg ml^{-1}; Sigma) plastic 35 mm tissue culture dishes (Falcon 3001) that had been inscribed with circles to contain the droplet. The

plates were covered and kept at room temperature (22 °C) for 20–24 h to allow for cell adhesion. The following day, 1.5 ml of HDM was added to the culture dishes and the cultured cells were used for experiments on this same day.

For experiments involving Ca^{2+}-depleted defined medium (50 % Liebovitz-15, 40.0 mmol l^{-1} NaCl, 1.7 mmol l^{-1} KCl, 5.6 mmol l^{-1} $MgCl_2$, 5.0 mmol l^{-1} Hepes, 1.0 mmol l^{-1} EGTA, 50 μg ml^{-1} gentamycin, 0.015 % L-glutamine; pH 7.3), the following procedure was employed. On the day after plating, the defined medium was replaced with 2.0 ml of either 'normal Ca^{2+} medium' or Ca^{2+}-depleted medium.

Identification of ciliated cells in culture

The *H. trivolvis* embryonic cell culture system contains a variety of isolated cell types, including neurons, myocytes and ciliated cells (Goldberg *et al.* 1988). Of the latter, various easily distinguishable subtypes were observed. Flame cells derived from the protonephridia had a unique morphology, with a few long cilia that beat rapidly in a very distinct waveform. As these cells are not involved in the embryonic locomotory behavior, they were not examined in this study. In contrast, the remaining ciliated cells were usually large (diameter >20 μm), round and had a distinct dense granular appearance under phase-contrast optics. Furthermore, they were bordered by actively beating cilia that were often restricted to distinct regions of the cell margin. Most of these cells had rather long cilia, similar to those on the pedal and prototrochal ciliary bands seen in a scanning electron microscope analysis of *H. trivolvis* embryos (McKenney and Goldberg, 1989). A smaller number of ciliated cells had distinctly shorter cilia that were difficult to analyze in the CBF assay. These latter cells, which may be derived from the ciliated gut epithelium, were generally not recorded from in the present study.

Experimental protocol and data analysis

Ciliated cells, identified by their rapidly beating cilia, were viewed through phase-contrast optics on an inverted microscope (Nikon Diaphot TMD) connected to a CCD video camera (JVC, model TK-860U). The output of the camera was fed to a time-lapse video cassette recorder (Panasonic, model AG-6720) and ciliary beating was recorded on video tape for later analysis. The time-lapse recorder enabled the analysis of rapidly beating cilia at a reduced playback speed of 1/24 normal. This allowed clear resolution of single beats. In control experiments, ciliary beating was analyzed at different playback speeds to ensure that ciliary beat frequency (CBF) was not underestimated due to frequency-matching errors. In each culture dish, 3–6 cells were mapped out on an *x*,*y*-array and recorded for 10 s. Drug or vehicle solutions were added and, at 5 and 10 min post-addition, the identified cells were recorded for another 10 s. Off-line analysis consisted of slowing the playback speed down to 1/24 normal and recording the number of ciliary beats in a 1 min interval. Each culture dish was used to examine the effect of either a vehicle solution or a single concentration of the drug. Data were expressed as a percentage of the basal beat frequency observed prior to vehicle or drug application. The measurements taken 10 min post-treatment were used for analysis in all experiments (Diefenbach *et al.* 1991; Goldberg *et al.* 1994). Statistical significance was determined by analysis of variance (ANOVA) followed by Fisher's protected least significant difference (PLSD) test. In each graph, error bars represent the standard error of the mean (S.E.M.).

Results

Effects of exogenous serotonin on ciliary beat frequency

Cultured embryonic ciliated cells were used to examine the direct effects of exogenous serotonin on ciliary beat frequency (CBF). In the absence of serotonin, cilia beat at a constant rate of 8.5±0.1 beats s^{-1} (mean ± S.E.M.; N=388). Addition of serotonin produced a dose-dependent increase in CBF (Fig. 1). While a small percentage of cells displayed little or no response to the addition of 10 μmol l^{-1} serotonin, most responded with a 20–30 % increase in CBF. The threshold concentration was approximately 0.1 μmol l^{-1} serotonin, with a maximal response observed between 10 and 100 μmol l^{-1} serotonin.

Role of cyclic AMP

It has been suggested in another molluscan ciliary system, the extensively studied lateral gill cilia of *Mytilus edulis* (Murakami, 1983, 1987), that serotonin-induced cilio-excitatory responses are mediated by the elevation of cytoplasmic cyclic AMP levels. Furthermore, several neuronal responses to serotonin in molluscs, including the serotonin-induced depolarization in buccal neuron 19 of *H. trivolvis* (Price and Goldberg, 1993), involve the actions of cyclic AMP.

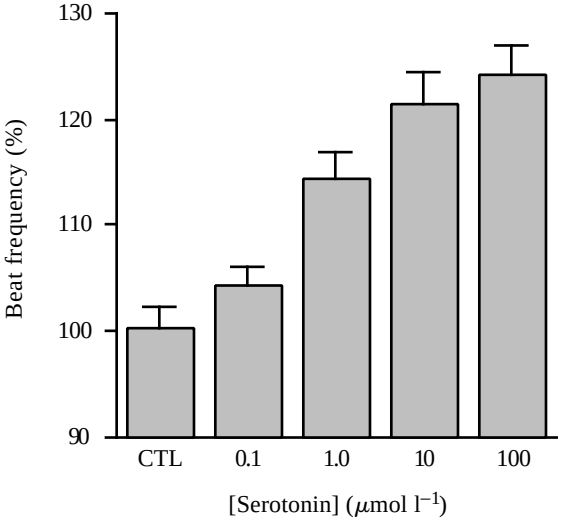

Fig. 1. Dose-dependent effects of serotonin on ciliary beat frequency (CBF). Serotonin increased the frequency of ciliary beating in a dose-dependent manner. Values are expressed as a percentage increase in CBF compared with basal levels (CTL). Each bar represents the mean response (+S.E.M.) of 28–47 cells.

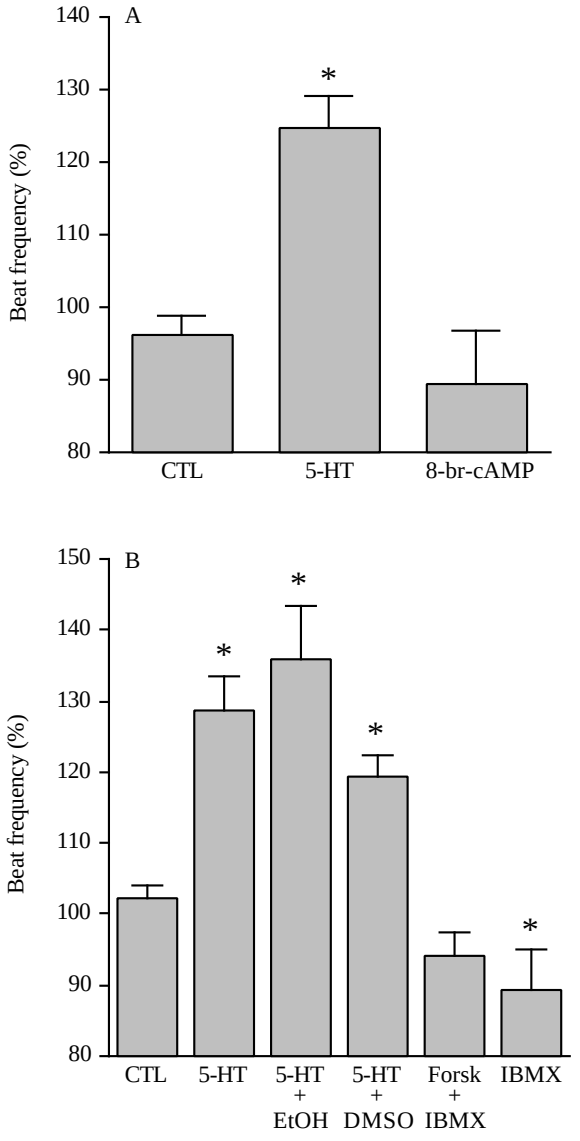

Fig. 2. Effects on CBF of treatments known to increase intracellular cyclic AMP concentration. (A) Addition of the membrane-permeable analog 8-bromo-cyclic AMP (2 mmol l^{-1}; 8-br-cAMP) resulted in a beat frequency that was not significantly different from control value (P>0.05), whereas addition of 100 μmol l^{-1} serotonin (5-HT) significantly increased CBF (P<0.01). Each bar represents the mean response (+S.E.M.) of 12–16 cells. (B) Co-addition of 10 μmol l^{-1} forskolin (Forsk) and 100 μmol l^{-1} IBMX did not increase CBF (P>0.05), whereas IBMX alone (100 μmol l^{-1}) had a weak inhibitory effect (P<0.05). The addition of 5-HT alone or in combination with the vehicles ethanol (EtOH; 0.05 %) or DMSO (0.1 %) significantly increased CBF (P<0.01). Each bar represents the mean response (+S.E.M.) of 4–8 cells. Asterisks denote a significant difference compared with the control (CTL) value.

We therefore tested the role of the cyclic AMP second messenger system in mediating serotonin-induced cilio-excitation of *H. trivolvis*. The addition of 8-bromo-cyclic AMP (2 mmol l^{-1}), a membrane-permeable analog of cyclic AMP, did not mimic the increase in CBF induced by 100 μmol l^{-1} serotonin (Fig. 2A).

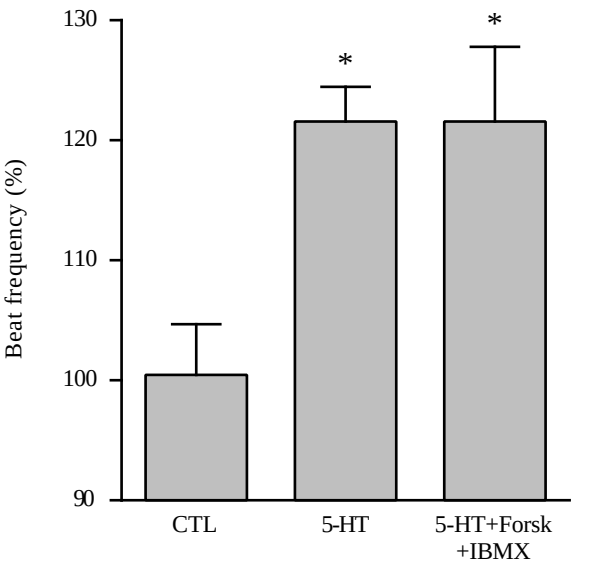

Fig. 3. Increase in cyclic AMP concentration does not affect the serotonin-induced increase in CBF. Co-addition of 10 μmol l^{-1} forskolin (Forsk), 100 μmol l^{-1} IBMX and 100 μmol l^{-1} serotonin (5-HT) resulted in an increase in CBF that was not significantly different from the increase observed in response to addition of 5-HT alone (P>0.05). Each bar represents the mean response (+S.E.M.) of 7–15 cells. Asterisks denote a significant difference compared with the control (CTL) value.

Since membrane-permeable cyclic AMP analogs can vary in effectiveness, we further tested the cyclic AMP hypothesis in another manner. Co-application of forskolin (10 μmol l^{-1}), an activator of adenylate cyclase, and IBMX (100 μmol l^{-1}), an inhibitor of cellular phosphodiesterase, should effectively raise the intracellular cyclic AMP concentration of cultured cells. Fig. 2B demonstrates that the co-addition of these agents did not mimic the serotonin-induced increase in CBF. In fact, 100 μmol l^{-1} IBMX induced a weak inhibition of basal CBF when presented alone (P<0.05). To ensure that the lack of excitatory response to forskolin and IBMX was not related to the solvents, ethanol (0.05 %) and DMSO (0.1 %), control experiments were performed to test the direct actions of these vehicle solutions. DMSO and ethanol had no significant effect on the basal CBF (not shown) nor on the serotonin-stimulated CBF (Fig. 2B). Taken together, these experiments suggest that the elevation of CBF induced by serotonin is not mediated through a stimulation of the cyclic AMP second messenger system.

Since forskolin and IBMX, or IBMX alone, appeared to have weak inhibitory actions on basal CBF (Fig. 2B), the cyclic AMP system may play an inhibitory role in the regulation of CBF. To test this hypothesis, 10 μmol l^{-1} forskolin and 100 μmol l^{-1} IBMX were added in conjunction with 100 μmol l^{-1} serotonin (Fig. 3). The stimulation of CBF induced by serotonin was not affected by these compounds that elevate the intracellular cyclic AMP concentration, suggesting that, in embryonic *H. trivolvis* ciliated cells, cyclic AMP is not involved in the regulation of CBF.

Role of Ca²⁺

Like cyclic AMP, Ca^{2+} has been implicated as a cilio-excitatory intracellular messenger in many systems (see Introduction). Therefore, we tested the hypothesis that Ca^{2+} plays such a role in embryonic *H. trivolvis* ciliated cells by experimentally manipulating intracellular Ca^{2+} concentrations. Initially, cells were exposed to agents which would depolarize membrane potential and thereby activate voltage-gated Ca^{2+} channels. Low concentrations of KCl (5–10 mmol l^{-1}) induced a significant stimulation of CBF, whereas higher concentrations (20–25 mmol l^{-1}) induced a significant inhibition of CBF (Fig. 4A). Since addition of 10 mmol l^{-1} NaCl had no effect on CBF (Fig. 4B), the responses to KCl were probably a result of its depolarizing action, rather than of the small increase in osmolarity that occurs with the addition of these compounds. The Na^+ channel agonist veratridine is another depolarizing agent that should indirectly increase the intracellular Ca^{2+} concentration. At 100 μmol l^{-1}, veratridine mimicked the cilio-excitatory action of 10 mmol l^{-1} KCl (Fig. 4B). It is not known whether the increase in CBF induced by KCl or veratridine results directly from depolarization or indirectly through a rise in intracellular Ca^{2+} concentration.

Pharmacological manipulations of intracellular Ca^{2+} concentration were carried out to test more directly the role of this intracellular messenger on CBF. The addition of the calcium ionophore A23187 (10 μmol l^{-1}) produced a significant increase in CBF that was similar to that induced by 100 μmol l^{-1} serotonin (Fig. 5). Cilio-excitation was also observed in response to 0.1 μmol l^{-1} thapsigargin (Fig. 5), a compound that increases cytoplasmic Ca^{2+} concentration by preventing the re-uptake of Ca^{2+} into intracellular storage compartments (Thastrup *et al.* 1990). These results suggest that the serotonin-induced increase in CBF may be mediated through an increase in intracellular Ca^{2+} concentration.

In order to establish a clear role for Ca^{2+} in serotonin-induced cilio-excitation, serotonin was applied in combination with manipulations that prevent the influx of Ca^{2+}. In a normal Ca^{2+}-containing medium, serotonin induced a robust increase in CBF (Fig. 6A). When cells were transferred to a medium containing no Ca^{2+}, there was no change in the basal CBF. Furthermore, the serotonin response was completely eliminated in the Ca^{2+}-free medium (Fig. 6A). Similar results were obtained when blockers of L-type voltage-dependent Ca^{2+} channels were used to reduce Ca^{2+} influx (Fig. 6B). Neither 1 μmol l^{-1} verapamil nor 1 μmol l^{-1} nifedipine caused a significant change in the basal CBF. In contrast, both compounds significantly inhibited the effect of serotonin on

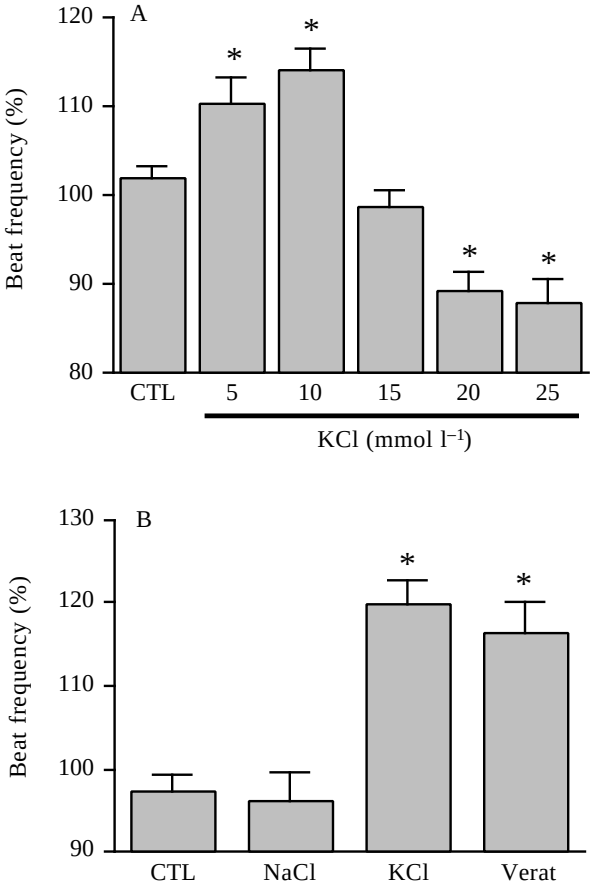

Fig. 4. Effects of depolarizing treatments on CBF. (A) The addition of KCl affected CBF in a biphasic dose-dependent manner. Addition of 5 (P<0.05) or 10 mmol l^{-1} KCl (P<0.001) resulted in significant increases in CBF relative to the control value, whereas higher concentrations of KCl (20 or 25 mmol l^{-1}; P<0.001) significantly reduced CBF. Each bar represents the mean response (+S.E.M.) of 11–15 cells. (B) Addition of 10 mmol l^{-1} KCl or 100 μmol l^{-1} veratridine (Verat) significantly increased CBF (P<0.001), whereas addition of 10 mmol l^{-1} NaCl had no effect on CBF (P>0.05). Each bar represents the mean response (+S.E.M.) of 6–22 cells. Asterisks denote a significant difference compared with the control (CTL) value.

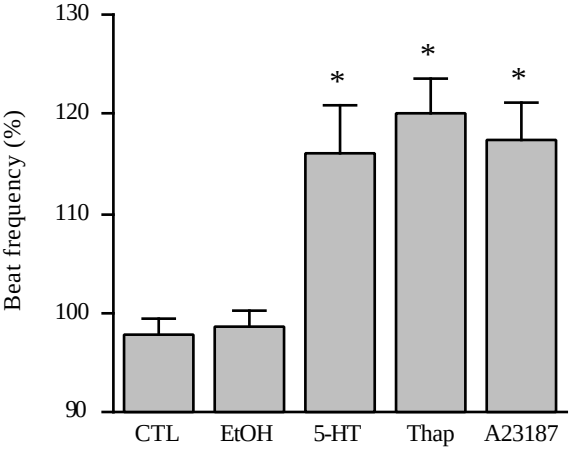

Fig. 5. Effects of increasing intracellular Ca^{2+} concentration on CBF. Addition of 0.1 μmol l^{-1} thapsigargin (Thap; P<0.001) or 10 μmol l^{-1} A23187 (P<0.001) mimicked the increase in CBF observed in response to addition of 100 μmol l^{-1} serotonin (5-HT; P<0.001). In contrast, addition of the ethanol (EtOH; 0.05 %) vehicle alone did not alter CBF compared with the control (CTL). Each bar represents the mean response (+S.E.M.) of 13–23 cells. Asterisks denote a significant difference compared with the control value.

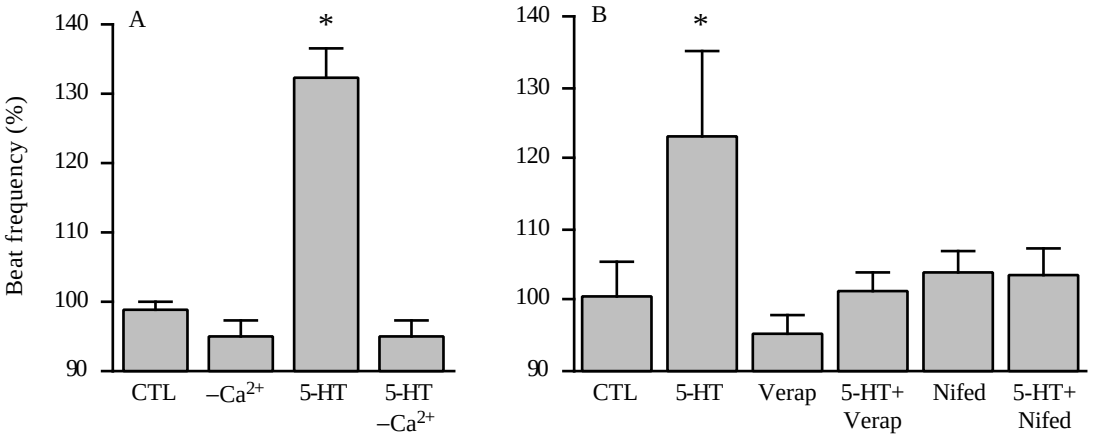

Fig. 6. Effects of inhibiting Ca^{2+} influx on CBF. (A) Ciliated cells bathed in a Ca^{2+}-depleted medium ($-Ca^{2+}$) beat at a normal frequency compared with control (CTL) cells. The addition of 100 μmol l^{-1} serotonin (5-HT) in a Ca^{2+}-depleted medium caused no change in CBF, whereas serotonin induced a significant increase in CBF (P<0.001) when cells were bathed in normal, Ca^{2+}-containing medium. Each bar represents the mean value (+S.E.M) for 14–18 cells. (B) Addition of 1 μmol l^{-1} verapamil (Verap) or 1 μmol l^{-1} nifedipine (Nifed) alone did not alter CBF compared with control (CTL) cells. Co-incubation with 100 μmol l^{-1} 5-HT and either verapimil or nifedipine abolished the increase in CBF that was observed when 5-HT was presented alone (P<0.05). All values are means + S.E.M. for 7–8 cells with the exception of the control value, which is for three cells. Asterisks denote a significant difference compared with the control value.

CBF (Fig. 6B). Taken together, these results suggest that the serotonin response involves the influx of extracellular Ca^{2+} through L-type channels, which then acts in the cytoplasm to stimulate ciliary beating.

Discussion

Embryos and veligers of gastropod molluscs locomote primarily by cilia-driven swimming (Chia *et al.* 1984; Diefenbach *et al.* 1991). Given the small size of these organisms and their poorly developed nervous systems at these early stages, little is known about the physiological mechanisms regulating these movements. In recent years, the pond snail *Helisoma trivolvis* has emerged as a valuable model system to address this question. *H. trivolvis* undergoes direct development from single cell to juvenile stages within a transparent egg mass over a period of 9–12 days, depending on the temperature of the system. Differentiation of a pair of giant serotonergic neurons, ENC1s, is one of the earliest events in the development of the *H. trivolvis* nervous system. It involves the extension of primary neurites and their extensive branching in the region of the ciliated epithelium of the primordial foot. During this period of neuronal development (between 20 and 30 % of the developmental time course; stages E20–E30), embryos also display a tonic slow cilia-driven rotation movement that is interrupted by brief periods of accelerated rotation. Whole-embryo pharmacological experiments suggested that these surges of activity result from the enhancement of ciliary beating in response to serotonin released from ENC1 terminals. Since ENC1 forms both a descending projection to the pedal cilia and an ascending projection that is tipped with a chemosensory-like apparatus at the dorsal embryonic surface (Goldberg and Diefenbach, 1992; R. Koss and J. I. Goldberg, unpublished results), it is likely that this serotonergic neuron plays both a sensory and an effector role in regulating ciliary activity.

An important complement to the whole-embryo studies described above is the analysis of embryonic ciliated cells in primary cell culture. The greater experimental accessibility and analytical precision afforded by cell culture facilitates the investigation of cellular mechanisms. Previous experiments on cultured ciliated cells suggest that the cilio-excitatory actions of serotonin are exerted directly on these cells and not through an intermediary pathway (Goldberg *et al.* 1994). Furthermore, a pharmacological analysis of the serotonin receptor mediating this response was completed through complementary whole-embryo and cell culture experiments. The results of this study suggested that the *in vivo* response was accurately recapitulated under cell culture conditions. Since the goal of the present study was to study the signal-transduction pathway underlying the serotonin response, which necessarily involves manipulation of the intracellular environment, an *in vitro* approach was essential.

Comparative analysis of ciliary control

Studies on the regulation of ciliary beating in a variety of species have led to the conclusion that multiple signal-transduction pathways have evolved to mediate cilio-excitation and cilio-inhibition. Since cyclic AMP and Ca^{2+} appear to be relatively ubiquitous intracellular messengers in ciliary control, sometimes having antagonistic actions (Bergles and Tamm, 1988; Stommel and Stephen, 1985), they served as the starting point for investigating the cilio-excitatory actions of serotonin in *H. trivolvis* embryos. An unexpected finding of the present study was that cyclic AMP does not appear to be involved in regulating ciliary beat frequency. Treatments

known to be effective indicators of cyclic-AMP-dependent responses (Price and Goldberg, 1993), such as the application of membrane-permeable cyclic AMP analogs or combined treatment with an adenylate cyclase activator and a phosphodiesterase inhibitor, had no effect on the CBF. However, addition of IBMX tended to have a weak inhibitory effect on basal CBF. Since the serotonin-mediated increases in CBF were not affected by increased cyclic AMP concentrations, the IBMX effect suggests that cyclic AMP may play a minor role in the regulation of basal CBF, but not in serotonin-mediated cilio-excitation. It is possible that a neurotransmitter other than serotonin exerts an inhibitory influence on CBF through a cyclic-AMP-dependent pathway. Dopamine and octopamine, the only other neurotransmitters tested, do not affect embryo rotation rate (Diefenbach *et al.* 1991). In contrast to the results on *H. trivolvis*, cyclic AMP is thought to mediate serotonin-induced cilio-excitation in the well-studied lateral gill cilia of the marine bivalve *Mytilus edulis* (Murakami, 1983, 1987). Similarly, in *Paramecium tetraurelia*, an elevated intracellular cyclic AMP concentration has cilio-excitatory actions on CBF, which underlies forward swimming behavior (Bonini *et al.* 1986).

Consistent with many of the previous studies on the regulation of ciliary activity, Ca^{2+} plays a central role in controlling CBF in *H. trivolvis* embryos. The cilio-excitatory effect of serotonin was mimicked by treatments that increased intracellular Ca^{2+} concentration and blocked by treatments that prevented the influx of extracellular Ca^{2+}. Thus, Ca^{2+} entry from the extracellular environment appears to be a crucial step in the signal transduction pathway underlying the serotonin response.

Ciliary beating appears to have two different modes of regulation in different cell types. In the first, basal frequency can be either up-regulated or down-regulated to increase or decrease CBF. In the second, ciliary beating is primarily under excitatory regulatory control. In most invertebrate and protozoan ciliary systems studied to date, the first mode of beating occurs; that is, both cilio-excitation and cilio-inhibition are observed. In these cases, Ca^{2+} is normally involved in mediating the inhibitory or reversal responses (Nakaoka *et al.* 1984; Bergles and Tamm, 1992; Nakamura and Tamm, 1985; Paparo and Murphy, 1975; Murakami and Machemer, 1982). In contrast, cilio-excitatory actions of Ca^{2+} are normally observed in systems that show the second mode of regulation; that is, they are primarily under only excitatory control. For example, the abfrontal gill cilia of *M. edulis* differ from the lateral gill cilia in that they do not appear to be under positive and negative control by neurotransmitters released from the branchial nerve (Stommel, 1984*a*). Rather, they respond primarily to mechanostimulation by increasing or initiating ciliary activity in a Ca^{2+}-dependent fashion (Stommel, 1984*b*). Likewise, cilia found in the respiratory and reproductive tracts of vertebrates are predominantly regulated by mechanical or hormonal excitatory stimuli (Chiyotani *et al.* 1992; Murakami and Eckert, 1972), with Ca^{2+} playing a role in the cilio-excitatory responses. The cilia examined in the present study conform to this latter type of regulation. The cilia-driven rotation movement of *H. trivolvis* embryos does not undergo arrests (Diefenbach *et al.* 1991), nor is there any known inhibitory control pathway impinging upon the cilia (Diefenbach *et al.* 1991, 1995). Thus, the precise role of Ca^{2+} in regulating ciliary activity appears to be determined by functional, rather than evolutionary, constraints.

It is highly likely that excitatory ciliary responses occur only within a specific range of intracellular Ca^{2+} concentrations. In the present study, excitation was observed at lower doses of KCl, while inhibition was observed at higher depolarizing doses. Similarly, the abfrontal ciliated cells of *M. edulis* gill were activated in response to moderate rises in Ca^{2+} concentration and arrested in response to large increases (Stommel, 1984*b*). It is not yet known how higher doses of KCl induce inhibition of ciliary activity. The involvement of Ca^{2+}-dependent Ca^{2+} channel inactivation, Ca^{2+}-activated K^+ channels or other Ca^{2+}-dependent mechanisms has yet to be examined. An analysis of changes in intracellular Ca^{2+} concentration induced by serotonin and various concentrations of KCl is required to determine the physiological range of intracellular Ca^{2+} concentrations for the excitatory and inhibitory responses.

Mechanism of serotonin-induced Ca^{2+} influx

In gastropod molluscs, a number of serotonin-mediated physiological responses have been examined in considerable detail. A unifying principle that has emerged is that the responses normally involve activation of the cyclic AMP second messenger system. However, the mode of cyclic AMP action and the type of ion channel that is affected are not uniform among different serotonin responses. Given this trend, it is somewhat surprising that the cyclic AMP system does not appear to be involved in the serotonin-induced excitation of ciliary activity. How might serotonin induce the Ca^{2+} influx that appears to be necessary for ciliary activation in these *H. trivolvis* embryonic cells?

Since the results of the present study directly implicate voltage-gated Ca^{2+} channels in the ciliary response, it follows that Ca^{2+} influx may occur as a consequence of serotonin-induced depolarization. However, in preliminary electrophysiological experiments on ciliated cells from *H. trivolvis* embryos, application of serotonin did not cause a significant change in membrane potential (D. Collins, K. G. Young and J. I. Goldberg, unpublished observations). Likewise, the stimulation of *M. edulis* lateral gill cilia by serotonin does not involve membrane depolarization (Saimi *et al.* 1983). In *H. trivolvis* cells, the increased conductance of voltage-gated Ca^{2+} channels caused by serotonin may produce a sufficient rise in intracellular Ca^{2+} concentration for ciliary activation, but may be too small to affect membrane potential significantly. In this case, a signal other than membrane depolarization would be acting on the voltage-sensitive Ca^{2+} channels to link serotonin receptor activation with channel activation. For example, protein kinase C (Doerner and Alger, 1992) and specific GTP-binding proteins (Hartzell and

Fischmeister, 1992) have been shown to regulate Ca^{2+} channel activity. In addition, there are several other intracellular messengers that could mediate the cilio-excitatory actions of serotonin. Diacylglycerol (Chiyotani *et al.* 1992), cyclic GMP (Schultz *et al.* 1983), arachidonic acid metabolites (Chiyotani *et al.* 1992) and nitric oxide (Jain *et al.* 1993) have been shown to regulate ciliary beating in various systems. Moreover, in the red abalone *Haliotis rufescens*, mRNA sequences that apparently code for two Gα signal-transducing proteins have been obtained from isolated cilia (Wodicka and Morse, 1991). Further experiments are required to test the relative roles of these second messenger systems in linking serotonin-receptor activation to the influx of extracellular Ca^{2+}.

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