Published in final edited form as:

J Eukaryot Microbiol. 2015 ; 62(1): 144–148. doi:10.1111/jeu.12153.

Use of a Novel Cell Adhesion Method and Digital Measurement to Show Stimulus-dependent Variation in Somatic and Oral Ciliary Beat Frequency in Paramecium

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Abstract

When Paramecium encounters positive stimuli, the membrane hyperpolarizes and ciliary beat frequency increases. We adapted an established immobilization protocol using a biological adhesive and a novel digital analysis system to quantify beat frequency in immobilized Paramecium. Cells showed low mortality and demonstrated beat frequencies consistent with previous studies. Chemoattractant molecules, reduction in external potassium, and posterior stimulation all increased somatic beat frequency. In all cases, the oral groove cilia maintained a higher beat frequency than mid-body cilia, but only oral cilia from cells stimulated with chemoattractants showed an increase from basal levels.

Keywords

Cell immobilization; chemoattraction; ciliates; hyperpolarization; membrane potential; potassium

Introduction

Paramecium are single-celled Eukaryotes that are propelled through water by cilia that cover the entire cell. Ciliary beat frequency (CBF) in Paramecium is dependent on membrane potential. When the cell is hyperpolarized, CBF increases with an associated increase in swimming speed. When the cell is depolarized, direction of the beat reverses and CBF increases as a function of the degree of depolarization (Brehm and Eckert 1978). Various mechanisms exist that regulate membrane potential. Chemical stimulation, mechanical stimulation to either anterior or posterior parts of the cell, or the manipulation of external ion

concentrations all lead to alterations of CBF (Machemer 1989; Naitoh and Eckert 1973). Chemical stimulants generally are associated with food cues such as bacterial metabolites or compounds that indicate the likelihood of prey presence (Bell et al. 2007; Van Houten 1998). Mechanical stimulants, such as the attack of a predator or an encounter with a barrier, result in membrane potential changes that alter swimming behavior appropriate for that specific challenge (Naitoh and Eckert 1973).

Recent studies on *Paramecium* in high viscosity solutions confirm numerous anecdotal observations that the beat frequency of cilia found in the oral groove show little variation under changing solution viscosity while the somatic cilia respond in order to change swimming speed and direction (Jung et al. 2014). The underlying structures of both the somatic and oral ciliature have been studied in great detail revealing structural differences that should allow for this unique variable ciliary beat in a single-celled organism (Clerot et al. 2001; Ishida et al. 2001).

One of the great challenges of doing live cell studies on *Paramecium* is immobilization of these quick swimming organisms. Traditionally, most study on live Ciliates has involved immobilization at the end of a suction pipette or through coverslip compression (Naitoh and Eckert 1969; Yan et al. 2014). Alternatively, cells have been placed in small droplets of buffer (Klauke and Plattner 1997) or slowed through the use of viscous solutions (Jung et al. 2014). We utilized the bioadhesive Cell-TakTM, a secretion from the mussel *Mytilus edulis*, to adhere *Paramecium* to coverslips. Under these conditions, we were able to capture beat frequencies consistent with studies utilizing other techniques (Iwadata and Nakaoka 2008; Jung et al. 2014; Machemer 1976).

In this study, we ask how exposure to a variety of naturally occurring stimuli alters the beat frequency in both the somatic and oral cilia of *Paramecium*. We have employed an automated digital analysis system that yields data similar to analog systems at beat frequencies less than 15 Hz, but is significantly more accurate at faster beat rates (Sisson et al. 2003). This digital system also reduces data analysis time from hours to minutes and allows for subset analysis of specific regions. These ciliary regions show distinctive differences in response to various stimuli tested.

MATERIALS AND METHODS

Cultures and solutions

Paramecium tetraurelia (stock 51s) were grown in wheat grass media (Pines International, Lawrence, KS) inoculated with *Klebsiella pneumonia* for 24 h prior to addition of cells and grown to late log phase at 23 °C. Prior to use in beat frequency assays, *Paramecium* were centrifuged at 800 g for 2 min and the pellet transferred to a resting buffer containing 1 mM Ca(OH)₂, 1 mM citric acid, 1.3 mM Tris Base and 5 mM KCl (all chemicals from Sigma-Aldrich, St. Louis, MO) with a pH of 7.0.

Immobilization

Paramecium were adhered to 35 mm glass culture dishes (Fisher Scientific, Waltham, MA) via Cell-TakTM (BD Biosciences, San Jose, CA). Approximately, 10 ng of adhesive was

applied to the glass and allowed to air dry with occasional spreading by pipette tip. When visibly dried, the dishes were washed with 500 μ l of resting buffer twice and cells applied via 10 μ l droplets. Dishes were observed under a Nikon SMZ 1500 Stereo Microscope to assess if adequate numbers of cells were immobilized. After confirming a good population of adhered cells, 500 μ l of resting buffer was added to provide an appropriate experimental volume and to prevent drying of the samples.

Beat frequency measurements

Culture dishes were placed on a Nikon TE-300 (Nikon Imaging, Tokyo, Japan) inverted microscope and imaged with a Nikon 20X – 0.75 NA multiimmersion objective under oil. Video was captured with a Basler 602f high speed video camera controlled by the Sisson-Ammons Video Analysis System (SAVA), software specifically engineered for CBF analysis (Sisson et al. 2003). Under these conditions a wide variety of whole field or region of interest (ROI) measurements can be made and analyzed in a short period of time (Sisson et al. 2003; Wyatt et al. 2005). We took multiple ROI measurements of mid-body somatic readings on each cell and also ROI readings from the oral ciliature. Chemical stimulation of cells was accomplished by the addition of either betaine (1 mM) or ammonium chloride (5 mM) while maintaining the ionic balances of the other components of the resting buffer. Paramecium were mechanically stimulated on the posterior end of the cell by gently prodding with a pulled glass pipette controlled by a Narishige micromanipulator system. Potassium levels were manipulated by adding resting buffer with no potassium to the appropriate final dilution.

RESULTS AND DISCUSSION

Cell adhesion and stability

Paramecium adhered to coverslips were healthy and maintained a steady state CBF similar to frequencies recorded by others in free-swimming or partially immobilized cells (Iwadate and Nakaoka 2008; Jung et al. 2014; Machemer 1976). Cells immobilized in this fashion can often be observed for several hours (Movie S1) before visible signs of poor health such as slowed ciliary beating, increased contractile vacuole size, and membrane blebbing occur. In our experience, we observe an initial mortality rate of approximately 20% when cells first contact the adhesive probably due to membrane tearing related to the initial escape reaction of the trapped cell. Use of this technique dramatically expands the range of observations and manipulations one can perform on Paramecium. Although the droplet meniscus method of immobilization has been used with great success in imaging secretory processes in Paramecium (Klauke and Plattner 1997), it does not allow for perfusion and exchange of different solutions. Coverslip compression methods are quite effective at immobilization and newer devices allow for some level of perfusion (Yan et al. 2014). The cells in these devices are compressed however, which could activate mechanically gated ion channels involved in escape and avoidance responses. In addition, cell compressors distort the shape of the cell, which to some extent enhances imaging by reducing the depth of field, but can disrupt cell cyclosis and other cellular mechanisms (Aufderheide 2008). It should be noted that our laboratories have had little success immobilizing Tetrahymena with the CellTakTM technique. The elongated length of Paramecium allows for significant surface contact with

the adhesive. Tetrahymena's more rounded shape allows for only limited contact with the surface of the coverslip at any given time. Tetrahymena that adhere do so only transiently and are not stable enough for imaging. Conversely, the stability of adhered Paramecium allows for long-term studies that include confocal applications and calcium imaging (Movie S2). This stability also allows for the use of high magnification objectives providing resolution for the assessment of individual cilia or organelles (Fig. S1, Movie S3). Our experiences imaging various ions and cellular structures show normal cytoplasmic streaming, food vacuole creation, and elimination and appropriate processing of water through contractile vacuoles. Importantly, calcium and potassium levels maintain a steady state when the cell is not perturbed, thus indicating that the adhesive technique does not disrupt these critical ionic balances (W. E. Bell, unpubl. data).

Ciliary beat frequency variation between regions under different stimuli

We tested the beat frequency of both somatic and oral cilia under several different conditions. By increasing the volume of the bath solution with a potassium-free solution, we lowered the potassium concentration from 5 mM to 0.5 mM to hyperpolarize the cell membrane. Under free-swimming conditions, Paramecium demonstrate a significant increase in swimming speed when hyperpolarized via a reduction in external potassium concentration (Machemer 1989). Accordingly, we observed a significant increase in somatic CBF under these conditions (Fig. 1A). Under these conditions, the beat frequency of the oral ciliature was not significantly different (Fig 1A).

Mechanical stimulation of the posterior end of the cell activates K⁺ channels resulting in hyperpolarization of the membrane and the accompanying increase in swimming speed (Naitoh and Eckert 1973). When we used a glass pipette controlled by a micromanipulator to gently tap the posterior end of adhered cells, we observed a small, but significant increase in somatic CBF (Fig. 1B). Similar to ionically hyperpolarizing the cell, the oral CBF showed no changes in frequency (Fig 1B).

For our initial experiments with chemoattractants, we chose a strong stimulant so that we might assure measurable changes in beat frequency. We utilized ammonium chloride, an attractant that drives a robust response from Paramecium in a multitude of behavioral assays (de Ondarza et al. 2003; Van Houten 1978; Van Houten 1998). As expected, somatic CBF in the NH₄Cl treated cells rose accordingly (Fig. 1C). Surprisingly, the oral groove cilia showed a small, but significant increase above baseline (Fig 1C). NH₄Cl may work by changing intracellular pH (Davis et al. 1998), which could affect beat frequency globally. We decided to use another class of chemoattractant, betaine, which is effective at micromolar amounts for Paramecium and also functions as an attractant of many crustaceans (W. E. Bell, unpubl. data; Tolomei et al. 2003). This compound will not diffuse across the cell membrane, thus intracellular changes must be due to a receptor-mediated event, as predicted for most other stimulants of Paramecium (Van Houten 1998). Betaine produced a similar profile to NH₄Cl, an increase in both somatic and oral CBF (Fig. 1D).

Paramecium swimming behavior is directly linked to membrane potential. Hyperpolarized cells swim faster and turn less frequently. Depolarized cells exhibit more frequent turns and, under significant depolarizing events; will swim backward for extended periods (Machemer

1989). Differential regulation of somatic vs. oral beat frequency in hyperpolarized cells suggests that more complex control systems exist beyond a voltage sensitive mechanism. In addition to differences between the somatic and oral beat frequencies at rest, the ability to vary oral beat frequency based on stimulus suggests a separate transduction mechanism related to food cues.

Studies on somatic ciliature in the area of the oral groove indicate that these cilia are essential to deliver food to the oral apparatus (Ishida et al. 2001). It is possible that the increase in somatic beat frequency observed with chemical stimulants requires a matching increase in oral beat frequency to continue to efficiently capture food at higher swimming speeds. The evidence that increased particle impact in the cytopharyngeal area causes an increase in food vesicle formation suggests that a chemical or mechanically induced transduction system exists to respond to the presence of food (Ishida et al. 2001).

Signal transduction networks regulating chemical responses in ciliates have been difficult to decipher. A number of receptors that elicit appropriate membrane and behavioral responses have been identified and studied in detail (Lampert et al. 2011; Van Houten 1998). In some cases, downstream second messengers have been identified (Bonini et al. 1986; Hennessey et al. 1985), but effectors that directly interact with cilia and result in changes in beat frequency and direction are yet to be identified. Studies of ciliary dynein in both *Paramecium* and *Tetrahymena* demonstrate that these proteins could be targets for second messengers such as cAMP (Bonini and Nelson 1990; Kutomi et al. 2012; Wood et al. 2007). This study demonstrates that the differential beat frequency displayed by the oral and somatic ciliature shown by Jung et al. (2014) is not hard-wired and that responses are stimulus dependent. Clearly, changes in membrane potential allow for global changes in ciliary beat, as does change in solution viscosity. Importantly, chemical cues allow for activation of some regulatory subset of the oral cilia that increases beat frequency above the already high baseline not responsive to ionic or other stimuli. Detailed study of the differences between somatic and oral cilia could provide the insight necessary to identify effectors involved in chemical attractant pathways. The differential regulation of regional subsets of cilia establishes *Paramecium* as an ideal model organism to study ciliary beat dynamics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

This study was supported by research funding from the VMI Grants in Aid fund and the VMI Department of Biology (WEB) and by NIH grant AA008769 (JHS & TAW). We thank Dr. Emily Lilly for her insightful comments on the manuscript.

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Figure 1. Beat frequency of somatic and oral cilia in *Paramecium tetraurelia* under different stimuli

A. Stimulation by reduction in external KCl concentration. R = cells resting in 5 mM KCl buffer. S = same cells in 0.5 mM KCl. **B.** Mechanically stimulated cells. R = cells resting in 5 mM KCl buffer. S = cells recorded immediately after tapping of the posterior end with a glass micropipette. **C.** Stimulation with 5 mM NH₄Cl. R = cells resting in 5 mM KCl buffer. S = cells perfused with 5 mM NH₄Cl. **D.** Stimulation with betaine. R = cells resting in 1 mM KCl buffer. S = cells perfused with 1 mM betaine. * $p < 0.01$ using two tailed t-test.