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Review Article (Invited)

RNA interference reveals the escape response mechanism of *Paramecium* to mechanical stimulation

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In *Paramecium*, a mechanical stimulus applied to the posterior portion of the cell causes a transient increase in membrane permeability to potassium ions, transiently rendering the membrane in a hyperpolarized state. Hyperpolarization causes a transient increase in Cyclic adenosine monophosphate (cAMP) concentration in the cilia, resulting in a transient fast-forward swimming of the cell. Schultz and coworkers (1992) reported that a unique adenylate cyclase (AC)-coupled potassium channel is involved in the reaction underlying this response, which is known as the "escape response." However, the AC responsible for this reaction remains to be identified. Moreover, the molecular linkage between mechanoreception and AC activation has not been elucidated adequately. Currently, we can perform an efficient and simple gene-knockdown technique in *Paramecium* using RNA interference (RNAi). *Paramecium* is one of the several model organisms for which whole-genome sequences have been elucidated. The RNAi technique can be applied to whole genome sequences derived from the *Paramecium* database (ParameciumDB) to investigate the types of proteins that elicit specific biological responses and compare them with those of other model organisms. In this review, we describe the applications of the RNAi technique in elucidating the molecular mechanism underlying the escape response and identifying the AC involved in this reaction. The findings of this study highlight the advantages of the RNAi technique and ParameciumDB.

Key words: adenylate cyclase, cAMP, hyperpolarization

Significance

The behavioral response of *Paramecium* to mechanical stimulation has been analyzed for more than 60 years. Physiological studies have demonstrated that hyperpolarization and increased cAMP concentration cause the escape response to mechanical stimuli; however, the molecular mechanism underlying this response remains unclear. This review describes our current understanding of the molecular mechanism of escape response based on gene-specific knockdown studies using RNAi.

Introduction

Paramecium, a freshwater living protist, has several thousand cilia on their cell surface, which enables them to swim freely using ciliary movement. Ciliary movement can generate propulsion forces of varying magnitude according to changes in the beat frequency and direction. *Paramecium* exhibits characteristic behaviors against various chemical, mechanical, and thermal stimuli [1]. Its behavioral responses to various stimuli can be roughly classified into the

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"avoiding reaction" and the "escape response." The avoiding reaction is a behavioral response in which the *Paramecium* spontaneously changes its swimming direction to avoid the stimulus source [1]. Conversely, the escape response is a behavioral response in which *Paramecium* temporarily rapidly swims forward to avoid the stimulus source [2]. These two distinct behavioral responses correspond to the reversal and augmentation of cilia that occur simultaneously throughout the entire cell surface. Electrophysiological experiments, taking advantage of the large size of *Paramecium* cells, have revealed that the behavior of *Paramecium* controlled by ciliary beating is regulated by membrane potential [3]. From the 1970s to the 1990s, the simplified linkage between membrane potential and behavioral responses through ciliary beat control attracted the attention of many researchers in the field. In particular, "behavioral genetics," led by Ching Kung's group, took advantage of the short lifespan of *Paramecium* to reveal many molecular linkages from sensory channels to the regulation of ciliary beating [4,5]. However, as pointed out by Preer et al. (1985), the application of modern molecular biology methods in this field has been relatively slow, owing to differences in codon usage among protists [6]. In this review, we introduce a new technique using RNA interference (RNAi) to reveal the molecular mechanisms underlying various phenomena [7]. Using RNAi, molecule-targeted knockdown cells can be generated within several days. This is a major advance over the random point mutations and behavioral screening used in "behavioral genetics." Specifically, we introduce the different roles of adenylate cyclases (ACs) in *Paramecium tetraurelia* as revealed by molecular physiological analysis, and their relationship with mechanical stimulus reception [8].

Gene-specific Knockdown in *Paramecium* Using RNAi

The principle of RNA interference (RNAi) used for studying the cellular function of genes [9], experimentally introduces double-stranded RNA (dsRNA) causing sequence-specific silencing of the target mRNA. The RNAi knockdown of selected genes can be easily performed by feeding paramecia with bacteria expressing double-stranded RNA complementary to the *Paramecium* DNA sequence [7,10]. Competitive RT-PCR showed that RNAi significantly reduced the amount of target gene mRNA, but not completely [11]. However, the product of the target gene is reduced to the autofluorescence level of untransformed cells, as shown by the immunofluorescence assay [12]. In *Paramecium*, gene-specific knockdown can be induced using dsRNA constructed with 50-100 bps sequence of the target mRNA sequence. The knockdown cells were prepared 48 h after feeding. Furthermore, multiple gene knockdown can be performed by simultaneously providing multiple dsRNAs. Therefore, RNAi is a simple and rapid method for suppressing gene expression and performing efficient molecular and functional analyses. Recently, we have used RNAi methods to elucidate the molecular mechanisms of several previously unknown phenomena [11,13].

Avoiding Reaction and Escape Response of *Paramecium*

For *Paramecium*, detailed analyses using electrophysiological experiments have elucidated changes in ciliary movement and behavioral responses against mechanical stimulation [14]. A mechanical stimulus applied to the anterior portion of *the Paramecium* causes a transient increase in the membrane permeability to calcium ions, resulting in transient membrane depolarization (Fig. 1). Depolarization activates voltage-sensitive Calcium ion channels in the ciliary membrane, causing a reversal of the cilia beating direction (ciliary reversal), which in turn causes transient backward swimming of the cells [15,16]. In addition, avoiding reactions are caused by these physiological responses. However, a mechanical stimulus applied to the posterior portion of the cell causes a transient increase in the membrane permeability to potassium ions, thereby producing transient membrane hyperpolarization. Hyperpolarization produces a transient increase in the beat frequency of cilia in the normal direction (ciliary augmentation), resulting in cells swimming transiently faster in the forward direction [17]. These physiological responses result in the escape response. With respect to the responses to mechanical stimuli, an earlier report described that the threshold for the avoiding response is higher than that for the escape response [14]. Therefore, a light tap in the culture vessel induces a sudden increase in the forward swimming speed of all cells in the culture but not in the backward-swimming speed. Tominaga and Naitoh (1994) demonstrated that anterior mechanoreceptors differ from posterior mechanoreceptors because the thresholds of these mechanoreceptors are not the same in electrophysiological experiments [18]. Schultz and coworkers (1992) found that membrane hyperpolarization in *P. tetraurelia* stimulates cAMP formation within the cilia [19]. Moreover, an increase in the intraciliary cAMP levels causes ciliary augmentation and phosphorylation of the axonemal protein p29 [20].

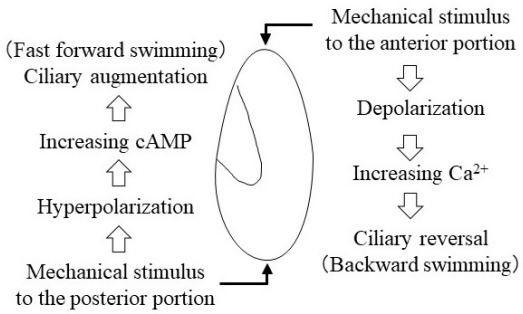

Figure 1 Schematic diagram of mechanical stimulus-response.

AC1 and AC2 are Potassium Channels Activated by Mechanical Stimulation

Weber and coworkers (2004) demonstrated in Ciliophora and Apicomplexa that the membrane-bound adenylate cyclases (ACs) correspond to the K^+ channels with a classical voltage sensor unit and a class III AC catalytic domain [21]. ParameciumDB showed that *P. tetraurelia* has at least four K^+ channel-type ACs: AC1–AC4. However, it remains unclear whether *Paramecium* uses all these ACs for hyperpolarization in response to mechanical stimuli or whether it uses different ACs for each stimulus.

To ascertain which ACs were involved in the stimulus-mediated outflow of K^+ through the ciliary membrane, AC1–AC4 knockdown cells were constructed using the RNAi method [8,22]. As presented in Fig. 2, *ac1* or *ac2* gene knockdown cells showed no rapid forward swimming in response to mechanical stimulation, whereas *ac3* and *ac4* gene knockdown cells showed rapid forward swimming, similar to normal cells. The knockdown of AC1 or AC2 in *Paramecium* impaired the escape response to mechanical stimulation, indicating that AC1 and AC2 are involved in the mechanostimulation-ciliary augmentation process. Whereas, when AC1 or AC2 knockdown cells were transferred into a solution containing 8-Br-cAMP, a membrane-permeable cAMP analog, the swimming speed of *Paramecium* increased, suggesting that AC1 and AC2 are involved in cAMP production, which in turn increases swimming speed in response to mechanical stimulation. Therefore, AC1 and AC2 play crucial roles in cAMP production and subsequent hyperpolarization via K^+ outflow during mechano-stimulation.

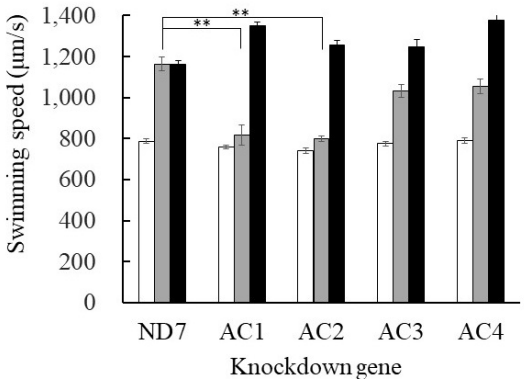

Figure 2 Swimming speed of each knockdown cell: White and gray bars respectively show swimming speed before and after mechanical stimulation. Black bars represent the swimming speed of knockdown cells treated with 8-Br-cAMP. ND7KD is a control cell. Asterisks show that a significant difference (P <0.001) from controls was found using the unpaired t-test. Modified from [8].

The current-voltage (I-V) relation, as measured using a constant current injection, revealed that cells with AC1 and AC2 knockdown displayed a more pronounced membrane potential shift at 100 ms after the start of constant current during negative currents (-0.5 to -3.0 nA) injection (Fig. 3). Control cells exhibited a sigmoidal response, characterized by minute membrane potential shift and a lower membrane resistance, in contrast to the experimental cells. This response attributed the presence of hyperpolarization-activated conductance, leading to significant hyperpolarization, particularly when subjected to large negative currents (> -5nA). The increasing linear I–V relationship and reduced conductance (larger membrane potential shift) during hyperpolarization in AC knockdown cells supports the hypothesis that AC is associated with hyperpolarization-activated potassium channels.

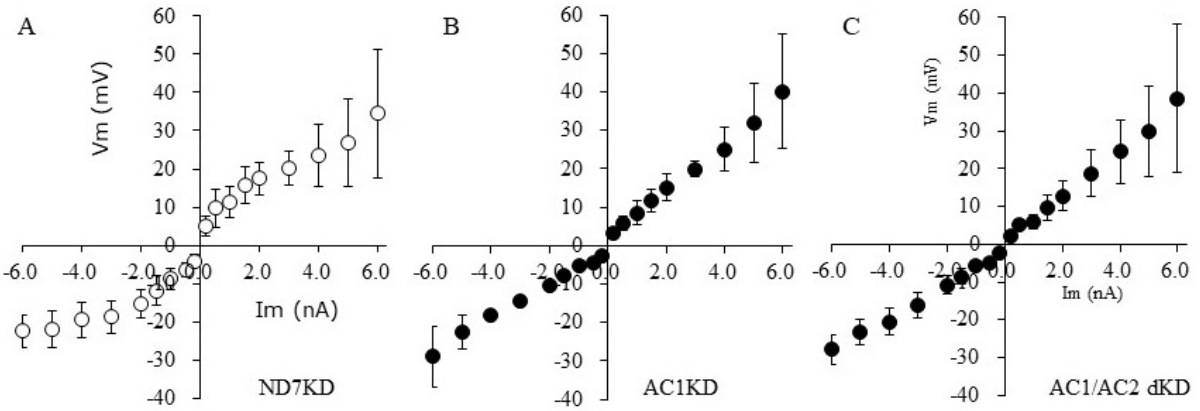

Figure 3 Injected current–membrane potential relations in gene knockdown cells: A, ND7KD; B, AC1 KD; C, AC1/AC2 double KD. Modified from [8].

The fluorescence of AC1-GFP and AC2-GFP was detected throughout the cell at the base of the cilia, whereas that of AC3 and AC4 was detected in the cortical region of the cell (Fig. 4). In each RNAi experiment, GFP fluorescent signals were used to confirm the effectiveness of gene knockdown. Within 72 h of gene knockdown, the fluorescence of each AC-GFP decreased to the auto-fluorescence level of untransformed cells when knocked down with their respective RNAi vectors. Moreover, no reduction caused by off-target effects of RNAi was observed in the fluorescence of any AC-GFP within 72 h after gene knockdown. These findings suggest that RNAi can effectively achieve gene-specific knockdown.

Results suggest that AC1 and AC2 differ from AC3 and AC4 in their localization and mechanical responses; therefore differ in function. It has been demonstrated that potassium channels are formed as heteromultimers and that the pore domain works in pairs of four proteins [23,24]. As the AC1 and AC2 have the same phenotype and localization, they might form a K^+ channel as a heteromultimer.

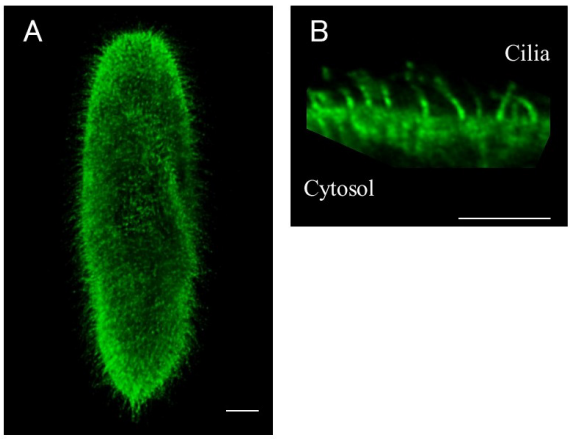

Figure 4 Localization of AC1-GFP in *Paramecium*: A, AC1-GFP; B, magnified image of an optical transverse section. Scale bar = 10 μm. Modified from [8].

Conflict of Interest

The authors have no conflicts of interest related to this report or description of the study.

Author Contributions

M.H., T.T., M.I., and K.M. wrote the manuscript.

Data Availability

The evidence data generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

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