Centrin controls the activity of the ciliary reversal-coupled voltage-gated Ca^{2+} channels Ca^{2+}-dependently

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Abbreviations: Pccentrin1p, *Paramecium caudatum* centrin 1 protein; VGCCs, voltage-gated Ca^{2+} channels

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Abstract

In *Paramecium,* ciliary reversal is coupled with voltage-gated Ca^{2+} channels on the ciliary membrane. We previously isolated a *P. caudatum* mutant, *cnrC*, with a malfunction of the Ca^{2+} channels and discovered that the channel activity of *cnrC* was restored by transfection of the *P. caudatum* centrin (Pccentrin1p) gene, which encodes a member of the Ca^{2+}-binding EF-hand protein family. In this study, we injected various mutated Pccentrin1p genes into *cnrC* and investigated whether these genes restore the Ca^{2+} channel activity of *cnrC*. A Pccentrin1p mutant gene lacking Ca^{2+} sensitivity of the third and fourth EF-hands lost the ability to restore the channel function of *cnrC,* and mutation of the fourth EF-hand caused more serious impairment than mutation of the third EF-hand. Moreover, a Pccentrin1p gene lacking the N-terminal 34-amino acid sequence also lost the ability to restore the channel activity. Native-PAGE analysis demonstrated that the N-terminal sequence is important for the Ca^{2+}-dependent structural change of Pccentrin1p. These results demonstrate that Pccentrin1p Ca^{2+}-dependently regulates the Ca^{2+} channel activity *in vivo*.

Introduction

Voltage-gated Ca^{2+} channels (VGCCs) control cellular Ca^{2+} entry in response to a change in membrane potential. The Ca^{2+} ions regulate processes as diverse as cell proliferation, neuronal transmitter release, muscle construction, and conversion of ciliary and flagella waveforms [1-3]. To effectively control Ca^{2+} signalling, VGCCs are rapidly inactivated by voltage-dependent and Ca^{2+}-dependent inactivation [4,5]. In the case of Ca^{2+}-dependent inactivation, it is thought that the α1-subunit, which forms the channel pore, is directly inactivated [5].

Recently, calmodulin, a member of the Ca^{2+} binding EF-hand protein superfamily, was identified as an important Ca^{2+} sensor that mediates the Ca^{2+}-dependent inactivation of L-type and P/Q-type VGCCs [6-8].

The ciliated protozoan *Paramecium* is one of the best experimental materials for studying the regulatory mechanisms of VGCCs for two reasons. First, the activation of the Ca^{2+} channel is easily detected as ciliary reversal. Free-swimming *Paramecium* shows ciliary reversal and transient backward swimming when stimulated by certain chemicals, heat, or touch at the anterior. The mechanical stimulation of the front end induces activation of the mechano-sensitive Ca^{2+} channels localized at the anterior, resulting in the generation of depolarizing receptor potentials. These depolarizing stimuli activate voltage-gated Ca^{2+} channels located on the ciliary membrane, producing Ca^{2+} action potentials and the simultaneous entry of Ca^{2+} into cilia. The consequent increase of intraciliary Ca^{2+} causes the reversal of the direction of the ciliary beat [3]. Although the VGCCs on ciliary membranes have been well characterized from the physiological viewpoint [3,4], the primary structure and regulatory mechanisms of the VGCCs are not yet fully understood. Second, there are many mutants that have a serious defect in VGCC activity but are at no disadvantage for survival in the laboratory and thus are suitable for analysis. Seven different behavioral mutants with malfunctions of the VGCCs have been described in *Paramecium*. The mutants are called *cnrA, cnrB, cnrC,* and *cnrD* in *P. caudatum* [9-11] and *pwA, pwB,* and *pwC* in *P. tetraurelia* [12], and all of them are controlled by independent gene loci [10-13]. Their common phenotype is that they do not respond to depolarizing stimuli and continue to swim forward even when facing such stimuli.

P. caudatum has a transcriptionally inert and mitotically dividing micronucleus (germ nucleus) and a transcriptionally active and amitotically dividing macronucleus (vegetative nucleus). The amitotic division and high telomerase activity in the macronucleus allow *Paramecium* to be transformed by microinjection of DNA

fragments, and complementation cloning has been successfully used to identify several mutant genes in *Paramecium* [14,15]. Using this method, we previously showed that the *cnrC*$^{+}$ gene product is *P. caudatum* centrin (Pccentrin1p), a member of the Ca^{2+} binding EF-hand protein superfamily [15]. Centrin is well known to localize to microtubule organizing centers (MTOCs) and control the cell cycle-dependent duplication and segregation of MTOCs [16]. Our previous results demonstrated that Pccentrin1p is essential for the activity of the VGCCs that control ciliary reversal in *Paramecium* [15]. However, how Pccentrin1p regulates the activity of VGCCs has remained to be explored. Here, we report that the Ca^{2+} sensitivity of Pccentrin1p is important for the VGCC activity *in vivo*.

Materials and methods

Stocks and culture

The *Paramecium caudatum* wild-type strain, G3, and the *cnrC* strain, R16D 305s-27, were grown at 25˚C, as previously described [17].

Gene silencing

Pccentrin1p gene-silencing by feeding on bacteria was done as previously described [15].

Electrophysiology

Conventional electrophysiological techniques were employed for examining the activities of VGCCs in *Paramecium* [18]. The responses of the membrane potential to stimulation with an outward current were recorded as described previously [19]. The resting potential of all cells ranged from -25 to -30 mV. Voltage-

clamping was performed by inserting two glass microelectrodes filled with 3 M KCl, one for recording the membrane potential and the other for passing the electric current, into the cell and using a conventional feedback system [19,20]. Voltage-gated Ca^{2+} currents were elicited by step depolarization more positive than -25mV from a holding potential of -30mV. The VGCC activities of the *cnrC* cells were examined mainly under current clamp conditions, since *cnrC* cells were seriously damaged under voltage clamp conditions.

Preparation of the mutated Pccentrin1p genomic DNA

To make the Pccentrin1p gene mutated in the EF-hand Ca^{2+}-binding loop, the all-acidic amino acids in each Ca^{2+}-binding loop, i.e., aspartic acid and glutamic acid, were replaced by alanine and valine, respectively. These replacements were generated by PCR mutagenesis using the wild-type 1213-bp Pccentrin1p genomic DNA cloned into pUC18 as the template. The amino acid sequence of the Ca^{2+}-binding loop in each EF-hand was changed as follows: EF-hand 1 mutation: DTDKSGSIDESE→ATAKSGSIAVSV; EF-hand 2 mutation: DRDGSGTIEFQE→ARAGSGTIVFQV; EF-hand 3 mutation: DDDNEGAIDLEK→AAANVGAIALVK; and EF-hand 4 mutation: DLDQDGKVSKDE→ALAQAGKVSKAV. To make the N-terminal sequence-deleted Pccentrin1p gene, the nucleotide sequence encoding Leu^{2}-Lys^{35} was deleted from the wild-type 1213-bp Pccentrin1p genomic DNA by PCR mutagenesis.

Microinjection

The microinjection of DNA and protein into *cnrC* cells was carried out as previously described [15,17].

Test of response to K^+ stimulation

In order to estimate the activity of VGCCs induced by K^+ stimulation [21], the cells were transferred into a high-K^+ solution (20 mM KCl in modified Dryl's solution, saline solution for *Paramecium*), and the duration of continuous backward swimming of each cell was measured. When wild-type cells were transferred to this high-

K^+ solution, the cells swam backwards for 30-50 sec due to the activation of VGCCs on the ciliary membrane [22].

Purification of recombinant Pccentrin1p protein

Paramecium translates universal stop codons, TAA and TAG, as glutamine codons. To express Pccentrin1p protein in *E.coli.*, we changed nine TAA codons and one TAG codon in the Pccentrin1p cDNA to universal glutamine codons, CAA and CAG, by PCR mutagenesis. The Pccentrin1p cDNA fragment with the mutagenesis was cloned into the *Bam*HI and *Pst*I sites of pQE80L vector (QIAGEN). Expression and purification of the recombinant protein were performed as described in the manufacturer's instructions. To check the activity of purified 6×His-tagged Pccentrin1p protein, the protein was dialyzed against an injection buffer (2.43 mM Na_2HPO_4, 0.57 mM NaH_2PO_4, 57 mM KCl, and 20 mM NaCl), and was then injected into the *cnrC* cells.

Examination of Ca^{2+}-dependent structural change

Purified recombinant proteins (6 μM) were incubated with 5% glycerol and 37 mM Tris (pH 6.8) containing 0.1 mM Ca^{2+} or 0.1 mM EGTA for 12 h at 4°C, and then analysed by 15% SDS-PAGE and by 12.5% native-PAGE without SDS and 2-mercaptoethanol.

Results

Pccentrin1p is essential for the voltage-dependent activity of VGCCs

We previously reported that *cnrC* transformed with the wild-type Pccentrin1p gene completely recovered reactivity to the depolarizing constant current stimulation and produced clear Ca^{2+} action potentials. Moreover, the activity of VGCCs in wild-type cells was impaired by Pccentrin1p gene silencing, leading to the loss of current-evoked Ca^{2+} action potentials [15]. These electrophysiological data demonstrate that Pccentrin1p plays an important role in the activity of the VGCCs that control ciliary reversal in *Paramecium*. In the present study, we directly examined the Ca^{2+} channel activities by recording the voltage-gated transient inward currents elicited upon step depolarization of the membrane under voltage clamp conditions in normal compared to Pccentrin1p gene-silenced cells. In the normal control cells, transient Ca^{2+} currents were elicited by membrane step depolarization, which appeared as an inward peak during step depolarization (Fig. 1A). The current-voltage relationship of the inward current exhibited N-shaped characteristics typical of excitable membranes (Fig. 1B). These normal control cells exhibited backward swimming in the high K^+ solution. On the other hand, in the Pccentrin1p gene-silenced cells, the voltage-activated Ca^{2+} currents were not detected upon membrane step depolarization (Fig. 1C and D). These cells also lost the ability to respond to the high K^+ solution. These results indicate that Pccentrtin1p is necessary for the voltage-dependent activation of VGCCs.

Ca^{2+} sensitivity and N-terminal sequence of Pccentrin1p are indispensable for VGCC activity

Pccentrin1p is a member of the Ca^{2+} binding EF-hand protein superfamily. To examine whether Pccentrin1p regulates the VGCC activity Ca^{2+}-dependently, we prepared Pccentrin1p genes that had mutations in the Ca^{2+}-sensitivity-determining EF-hands. It is known that the acidic amino acids of the Ca^{2+}-binding loop

(Fig. 2) is important for the Ca^{2+} sensitivity of calmodulin and centrins and that both proteins lose Ca^{2+} sensitivity upon the respective substitution of alanine and valine for aspartic acid and glutamic acid of the Ca^{2+}-binding loop [23]. Like calmodulin, centrin is thought to comprise two globular domains connected by a central linker, and each domain contains a pair of EF-hands (EF-hand 1 and 2 or EF-hand 3 and 4) that has the potential to bind two Ca^{2+} ions. Based on these data, we prepared six kinds of constructs in which the 1213-bp wild-type Pccentrin1p genomic DNA was mutated (EF-hand 1 mutation, EF-hand 2 mutation, EF-hand 3 mutation, EF-hand 4 mutation, EF-hand 1 and 2 mutation, and EF-hand 3 and 4 mutation, respectively). Each of the six constructs contained mutations causing base substitution of alanine and valine for aspartic acid and glutamic acid of the indicated Ca^{2+} binding loop. To investigate the effects of these mutations on VGCC activity, we injected the mutated gene into the *cnrC* macronucleus and examined whether these genes restored the VGCC activity of *cnrC* using reactivity to high-K^+ stimulation and to depolarizing current stimulation.

First, we checked for the reactivity of injected cells to high-K^+ stimulation. When the wild-type Pccentrin1p genomic DNA fragment was microinjected into *cnrC* cells, the reactivity of the cells to high-K^+ stimulation was restored, in agreement with the findings in our previous study (Table 1). In the *cnrC* transformed with the constructs of the EF-hand 1 mutation, EF-hand 2 mutation, and EF-hand 3 mutation, more than 50 % of the *cnrC* cells were cured (Table 1). In these transformants, the mean duration of backward swimming as a result of high-K^+ stimulation was not significantly different from that obtained with transfection of the wild-type gene. On the other hand, in the *cnrC* transformed with the constructs of the EF-hand 4 mutation and EF-hand 1 and 2 double mutation, although over 40 % of the *cnrC* cells were rescued, the mean duration of backward swimming was significantly shorter (Table 1). Moreover, most of the *cnrC* cells microinjected with EF-hand 3 and 4 double-mutation constructs did not respond to high-K^+ stimulation (Table I). These results

show that the Ca^{2+}-induced conformational change of EF-hands 3 and 4 is important for VGCC activity. Thus, next we examined the importance of the Ca^{2+} sensitivity of EF-hands 3 and 4 using the reactivity to depolarizing current stimulation. In an electrophysiological examination, the uninjected *cnrC* cells did not produce the Ca^{2+} action potential and showed only a passive voltage change (Fig. 3A). By contrast, *cnrC* transformed by the 1213-bp wild-type genomic DNA fragment completely recovered reactivity to the depolarizing current and produced clear Ca^{2+} action potentials (Fig. 3B). In *cnrC* transformed with constructs of the EF-hand 3 mutation, the transformants produced a normal Ca^{2+} action potential in response to a depolarizing current (Fig. 3C). On the other hand, in *cnrC* transformed with the constructs of the EF-hand 4 mutation, the current-evoked Ca^{2+} action potentials were small (Fig. 3D). Moreover, most of the *cnrC* cells microinjected with EF-hand 3 and 4 double-mutation constructs did not produce a current-evoked Ca^{2+} action potential (Fig. 3E). Electrophysiological data also show that the cooperative actions of EF-hand 3 and 4 in the presence of Ca^{2+} may be indispensable for VGCC activity *in vivo*. Moreover, it was suggested that, for the cooperative regulation of EF-hand 3 and 4, EF-hand 4 plays a more pivotal role than EF-hand 3. Pccentrin1p of *cnrC* has a single amino acid substitution at amino acid position 156 (D to T) and a loss of the C-terminal 28-amino acid sequence containing EF-hand 4 [15]. The data showing that Pccentrin1p of *cnrC* may possibly lose EF-hand 4 may support the importance of the globular domain formed by EF-hands 3 and 4 in the VGCC regulatory mechanisms.

Pccentrin1p possesses a unique N-terminal 34-amino acid sequence compared with other centrin sequences, shown in a box in Figure 2. To investigate the importance of the N-terminal sequence, the 1213-bp Pccentrin1p genomic DNA with deletion of the nucleotide sequence encoding Leu^{2}-Lys^{35} was prepared, and then this DNA was microinjected into *cnrC*. Surprisingly, most of the *cnrC* cells microinjected with N-terminal

sequence-deleted constructs neither responded to high-K^+ stimulation (Table 1) nor produced a current-evoked Ca^{2+} action potential (Fig. 3F), indicating that the N-terminal sequence of Pccentrin1p is indispensable for its VGCC activity *in vivo*.

Unique N-terminal sequence is involved in the Ca^{2+}-dependent structural change of Pccentrin1p

In order to investigate how the functional domain of Pccentrin1p functions in its Ca^{2+}-dependent structural change, we prepared the wild-type Pccentrin1p protein using an *E. coli.* protein synthesis system (Fig. 4A, lane W). To check whether the purified wild-type Pccentrin1p protein is functional *in vivo*, the protein was injected into the *cnrC* cells and their reactivity to high-K^+ stimulation was investigated. The results showed that all the injected *cnrC* cells recovered reactivity to the stimulation, indicating that the purified wild-type protein is functional *in vivo* (Table 2).

Next, we prepared N-terminal 34-amino acid sequence-deleted (Leu^2-Lys^{35}) Pccentrin1p (N-deleted Pccentrin1p, Fig. 4A lane N) and *cnrC*-type Pccentrin1p, which contains the substitution of tyrosine for Asp^{156} and the deletion of Met^{157}-Ile^{184} (*cnrC* Pccentrin1p, Fig. 4A lane C). To examine the effects of N-terminal deletion (N-deleted Pccentrin1p) and C-terminal deletion (including loss of EF-hand 4 (*cnrC* Pccentrin1p)) on the Ca^{2+}-dependent structural change of wild- type Pccentrin1p, SDS-PAGE and native-PAGE were carried out. The SDS-PAGE patterns of these three proteins showed a single band in each case, and no mobility change of these proteins was seen in the presence versus the absence of Ca^{2+}, indicating that proteolysis did not occur during the incubation and there is no difference between the Ca^{2+}-dependent and –independent structures under the conditions of denaturing with SDS and 2-mercaptoethanol (Fig. 4A). On the other hand, in the native-PAGE patterns of wild-type Pccentrin1p, the apparent molecular weight of the Ca^{2+}-dependent structure was larger

than that of the Ca^{2+}-free structure, indicating that the structure of Pccentrin1p appeared to become large in a Ca^{2+}-dependent manner (Fig. 4B), as is true for calmodulin. The native-PAGE patterns of *cnrC* Pccentrin1p which had lost EF-hand 4 showed that the structure of *cnrC* Pccentrin1p changed Ca^{2+}-dependently (Fig. 4B). However, as shown in Table I and Figure 3, we clarified that EF-hand 4 plays the most pivotal role among the four EF-hands. Therefore, although *cnrC* Pccentrin1p can change its structure Ca^{2+}-dependently, the change of the structure without the Ca^{2+} sensitivity of EF-hand 4 may not be sufficient for the regulation of VGCCs. The native-PAGE patterns of N-deleted Pccentrin1p showed that the Ca^{2+}-dependent structural change of this protein was very slight in comparison to that of wild-type or *cnrC* Pccentrin1p (Fig. 4B). Since N-deleted Pccentrin1p possesses the intact four EF-hands identical to wild-type Pccentrin1p, it is thought that the N-terminal sequence of Pccentrin1p is essential for its Ca^{2+}-dependent structural change.

Discussion

Brehm and Eckert showed that, in *Paramecium*, an inactivation of the VGCCs takes place as a consequence of Ca^{2+} entry during depolarization rather than in response to depolarization [4]. Here, we demonstrated that Pccentrin1p regulates the VGCCs in a Ca^{2+}-dependent manner *in vivo* (Table 1 and Fig. 3). From this evidence, we deduced that Pccentrin1p participates in the regulation of VGCCs as follows. The Ca^{2+} sensitivity of centrin has K_d of more than about 1 X 10^{-6} M [24], and the Ca^{2+} concentration in the cilia (10^{-7} M or less) reaches 10^{-6} M or higher upon activation of the VGCCs on the ciliary membrane [3,4]. This information suggests that Pccentrin1p Ca^{2+}-dependently controls the VGCC activity after its activation. One possibility

might be that the Ca^{2+} sensitivity of Pccentrin1p is indispensable for release of the VGCCs from the inactivation by some unknown Ca^{2+} signalling. In *cnrC* and Pccentrin1p-silenced cells, this deinactivation system controlled by Ca^{2+}/Pccentrin1p might be impaired, and, consequently, the VGCCs of *cnrC* and Pccentrin1p-silenced cells may stay in the inactivated state and thus not be activated.

The VGCC activity of *cnrC* cells was partially restored by injection of EF-hand 1 and 2 double-mutation constructs, but not by injection of the EF-hand 3 and 4 double-mutation constructs (Table 1 and Fig. 3). Therefore, the Ca^{2+}-induced conformational change of EF-hands 3 and 4 is essential for the regulatory system of VGCCs. Moreover, we showed that the Ca^{2+} sensitivity of EF-hand 4 plays a particularly pivotal role in the conformational change. For this reason, it is supposed that Pccentrin1p of *cnrC* which loses EF-hand 4 is not able to control the activity of VGCCs.

Here, we reported that the N-terminal region play a pivotal role in the Ca^{2+}-dependent structural change of Pccentrin1p *in vivo* (Table 1 and Figs. 3 and 4). In a previous study, the N-terminal region of centrin was shown to be involved in the self-assembly of centrin [25]. In addition to the self-assembly function, the N-terminal region of other centrins might also be involved in Ca^{2+}-dependent structural changes of the centrins. Moreover, the diversity of the N-terminal region of centrins might result in part in the variety of centrin structures caused by each Ca^{2+}-dependent structural change. Our discoveries about the structure and function of centrin may offer new insights into centrin's molecular dynamics and voltage-gated Ca^{2+} channel regulatory systems.

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Table Legends

Table 1

Numbers in parentheses: microinjected cells. The duration of backward swimming of the cells cured in response to high-K^+ solution was measured.

Table 2

The duration of backward swimming of the cells cured in response to high-K^+ solution was measured.

Figure Legends

Fig. 1. Ca^{2+} currents in normal control and Pccentrin1p gene-silenced cells examined under two-electrode voltage clamp conditions. Currents exhibited by a control cell (A) and Pccentrin1p gene-silenced cell (C) were elicited by 40-ms voltage steps to -10 mV from a holding potential of -30mV. The voltage-activated Ca^{2+} current was apparent as an inward peak (arrowhead) during step depolarization in the control cell but not in the gene-silenced cell. Vm is the membrane potential, and Im represents inward Ca^{2+} current. (B) and (D) show the current-voltage relationship for the membrane current in the control cell and silenced cell, respectively. Open circles, peak value of the inward currents. Open triangles, current levels 40 ms after onset of the step change in membrane potential.

Fig. 2. Sequence comparison of Pccentrin1p with representative known centrin sequences. Black-shaded regions indicate the identity of an amino acid among the four sequences. The identity of an amino acid in at least two of four sequences is shown as a gray-shaded region. The potential Ca^{2+} binding loops of the EF-hand are underlined. Asterisks show the acidic amino acids of the Ca^{2+}-binding loops. The box represents the unique N-terminal 34-amino acid sequence (Leu^2-Lys^{35}) compared with those of other centrin sequences. Hscentrin1p, *Homo sapiens* centrin 1; Crcentrin, *Chlamydomonas reinhardtii* centrin. ICLα is an other *P. caudatum* centrin.

Fig. 3. Membrane potential responses of *cnrC* and transformed *cnrC* cells to stimulation with current with varying intensities. The non-injected *cnrC* cell did not produce the Ca^{2+} action potential in response to a depolarizing current and showed only a passive potential change (A). *cnrC* transformed by the 1213-bp wild-type Pccentrin1p genomic DNA fragments recovered reactivity to the depolarizing current and produced clear Ca^{2+} action potentials (B). (C)-(F) show the membrane electric responses in *cnrC* cells transformed by mutated wild-type Pccentrin1p genes. Arrowheads show the evoked Ca^{2+} action potential. Three records were superimposed for each cell. Vm is the membrane potential, and Is represents the intensity of the stimulatory current.

Fig. 4. Ca^{2+}-dependent structural change of wild-type, N-deleted, and *cnrC* Pccentrin1p. The three kinds of purified recombinant proteins were incubated with Tris buffer (pH6.8) containing 0.1 mM Ca^{2+} or 0.1 mM EGTA for 12 h at 4^{o}C. A portion of the sample was analyzed by 15% SDS-PAGE (A) and the remainder of the sample was analyzed by 12.5% native-PAGE without SDS and 2-mercaptoethanol (B). Lane W, wild-type Pccentrin1p; lane N, N-deleted Pccentrin1p; lane C, *cnrC* Pccentrin1p. (+) and (-) indicate the condition in the presence and absence of Ca^{2+}, respectively. Asterisks in (B) show protein bands.

Table 1
Effects of mutation in EF-hand Ca^{2+}-binding loop or N-terminal region on the transformation ability of 1213-bp wild-type genomic DNA

Type of DNA injected	No. of cells rescued	Percentage of cells rescued	Backward swimming (sec) Mean ± S.D.	Reference
Wild type	45 (69)	65.2 %	38.1 ± 28.1	Gonda, et al. [15]
cnrC	0 (28)	0	0	Gonda, et al. [15]
EF-hand 1 mutation	16 (25)	64 %	30.1 ± 21.6	This study
EF-hand 2 mutation	24 (44)	54.5 %	34.2 ± 27.6	This study
EF-hand 1 & 2 mutation	43 (98)	43.9 %	11.6 ± 8.6	This study
EF-hand 3 mutation	44 (89)	49.4 %	28.6 ± 23.5	This study
EF-hand 4 mutation	45 (106)	42.5 %	7.3 ± 3.8	This study
EF-hand 3 & 4 mutation	2 (110)	1.8 %	8.0 ± 1.0	This study
N-terminal deletion	4 (81)	4.9 %	8.3 ± 2.2	This study

Table 2
Effect of wild-type Pccentrin1p injected into the cytoplasm of *cnrC* cells

No. of cells injected	No. of cells rescued	Backward swimming (sec) Mean ± S.D.
17	17	21.5 ± 8.3