Control of the Orientation of Cilia by Adenosinetriphosphate, Calcium, and Zinc in Glycerol-Extracted *Paramecium caudatum*

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ABSTRACT The predominant orientation of cilia in glycerol-extracted *Paramecium* is toward the posterior of the specimen in a KCl solution. The cilia became reoriented toward the anterior shortly after transfer of the extracted cell to a mixture of ATP, calcium, and zinc. The degree of response was graded as a function of the concentration of each of the three essential factors. Minimum concentrations for the maximum response were 0.2 mM in ATP, 0.8 mM in calcium, and 0.0002 mM in zinc. The observations support the hypothesis that cation-induced ciliary reversal in live specimens is initiated by calcium ions which become displaced from an inferred cellular cation exchanger system.

INTRODUCTION

Reversal of the effective beat direction of cilia (ciliary reversal) of *Paramecium* in response to a cationic stimulus is closely related to calcium ions initially bound and subsequently displaced from the anionic-binding sites of a cation exchanger system on the specimen (Naitoh, 1968). It was proposed that calcium ions displaced from these sites by exchange reaction with other cations activate a contractile system, and that the activation of the system in turn results in the reversal of the ciliary beat. Furthermore, Naitoh and Eckert (1968 *a,b*) demonstrated that bound calcium is a major factor influencing electrical properties of the membrane thought to be involved in the bioelectric control of ciliary reversal.

Present experiments were designed to verify the calcium hypothesis by examining the effects of calcium ions on the orientation of cilia in glycerol-extracted *Paramecium*. The inferred contractile system is expected to remain functional in extracted specimens while cell membranes and their electrical properties are believed to be disrupted, or modified. The results show that in glycerinated specimens reorientation of cilia, which is thought to be homologous with ciliary reversal in the live specimen, is caused by the addition of calcium, ATP, and zinc.

MATERIALS AND METHODS

Specimens of *Paramecium caudatum* (mating type I, Syngen 1) reared in hay infusion were washed thoroughly with 2 mM $CaCl_2$ solution buffered to pH 7.4 by 10 mM Tris-HCl buffer. Concentrated suspensions of specimens were cooled to 0°C and centrifuged gently to make a loose pellet. The pellet was then resuspended in a cold (0°C) glycerol medium which consisted of 50 % (v/v) of glycerol, 50 mM KCl, 10 mM EDTA (4K salt), and 10 mM Tris-HCl buffer (pH 7.4). The suspension was then stored at −15°C for 10–15 days.

The glycerinated *Paramecium* thus obtained were washed gently three times by cold (0°C) 50 mM KCl solution buffered to pH 7.4 by 10 mM Tris-HCl buffer, and kept in this solution for at least 15 min at 0°C before experimentation.

Several specimens together with a minute amount of the KCl solution (about 10^{-4} ml) were pipetted into a large amount of test solution (about 1 ml) which consisted of test substances plus the basic 50 mM KCl solution buffered by 10 mM Tris-HCl buffer (pH 9.0) under room temperatures of 20° to 23°C. The models transferred into the test solutions were photographed through a phase contrast objective (× 20). The angle[1] between the ciliary axis and the cell surface at the right[2] anterior edge of the glycerinated specimen was measured in the photographs (× 400). 10 measurements in 10 specimens were made, and a mean and a standard error calculated.

Various glycerol media with different ionic compositions were tested in order to select an optimum extraction medium for these experiments. The presence of EDTA (5–10 mM) in the extraction medium was essential to the present results, because in its absence, the extracted cells shrank excessively and became badly distorted and lysed. The cilia on such cells failed to show responses. A change in the KCl concentration in the extraction medium within a range from 20 to 100 mM did not affect general features of the cells or the responsiveness of their cilia. The optimum pH of the extraction medium ranged from 7.0 to 8.0. The cilia of cells extracted in acidic media did not show responses in spite of their good general appearance. Difference in the ionic composition of the suspension medium for the specimens prior to transfer into the extraction medium was without effect on the responsiveness of cilia after the extraction.

RESULTS

1. *Reorientation Response*

Fig. 1 A is a photograph of a glycerinated *Paramecium* in 50 mM KCl solution (pH 9.0 by 10 mM Tris-HCl buffer), showing the cilia pointing posteriorly,

[1] According to Párducz (1967), cilia of *Paramecium* beat in three dimensions. Presently we do not know in what phase of a beating cycle the cilia stop in the glycerol media. The angle between ciliary axis and cell surface measured in photographs therefore does not necessarily show the actual angle to the cell surface.

[2] The "right side" is at the observer's right hand when the side bearing the oral groove is down and the anterior end points away from the observer.

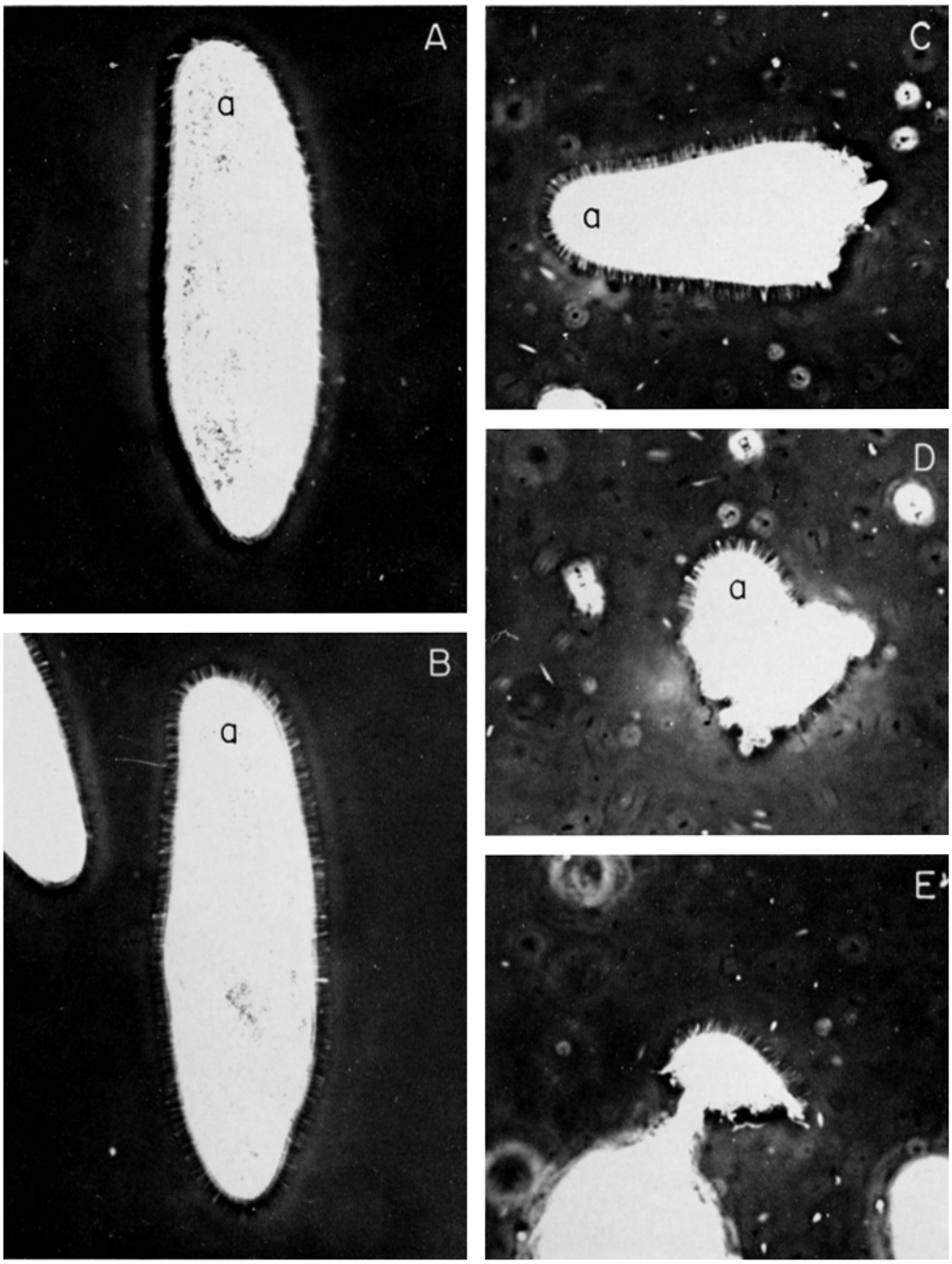

FIGURE 1. Photographs of glycerinated models of *Paramecium caudatum*. A, an extracted specimen suspended in 50 mM KCl solution. The predominant orientation of cilia is toward the posterior of the organism. B, a model after transfer into a mixture of ATP, calcium, and zinc. The cilia have become reoriented perpendicular to the cell surface or toward the anterior. C–E, Fragments of extracted cells in the ATP-Ca-Zn mixture. Extracted specimens were fragmented with a sharp glass needle in a KCl solution, and then transferred into the mixture. Reorientation of ciliary axis can be seen in each small fragment. Anterior end is marked *a*. × 290.

which approximates the orientation of beating cilia in the forward locomotion of a normally behaving live specimen (Naitoh, 1966). Preliminary experiments showed that with the addition of ATP (10 mM), calcium (10 mM), and a small amount of zinc (0.1 mM) the cilia changed orientation so as to point more anteriorly. In this position the cilia approximate the general orientation of beating cilia in a live specimen during backward locomotion (Naitoh, 1966,

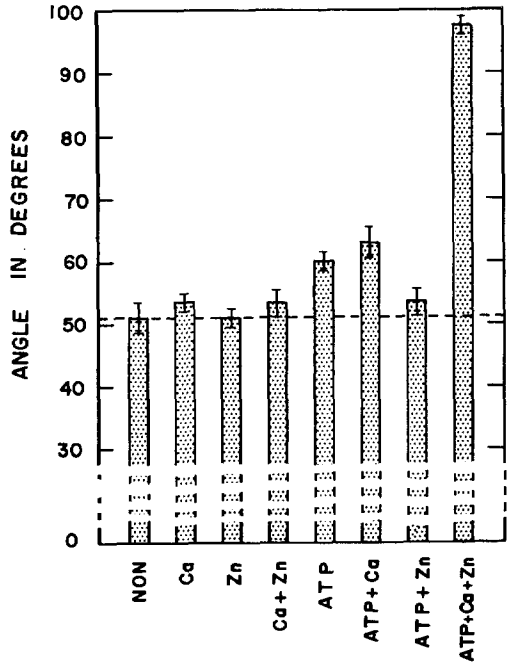

FIGURE 2. Reorientation of cilia in glycerinated paramecia exposed to test media containing various combinations of 10 mM ATP, 10 mM calcium, and 0.1 mM zinc in 50 mM solution of KCl. The orientation is expressed as the angle in degrees between the ciliary axis and the cell surface at the right anterior edge of the extracted cell, which is measured in photomicrographs. The most pronounced increases in the angle (from 50° to 98°) occurred in a mixture of ATP, calcium, and zinc. Fig. 1 B illustrates the appearance of cilia in that state of orientation. The column marked NON represents the ciliary orientation in a plain solution of KCl. This is illustrated in Fig. 1 A.

1968). This is shown in Fig. 1 B. Cilia on fragments of extracted specimens behaved similarly (Fig. 1 C–E). The reorientation response of cilia was reduced or absent in the absence of any one of the three factors (Ca^{++}, ATP Zn^{++}) in the test solution (Fig. 2).

2. *The Influence of pH*

The optimum pH for the reorientation response was determined by measuring the angular change of the ciliary axis in a series of test solutions with pH values

ranging from 6 to 10. The pH was controlled with 10 mM Tris-maleate, while concentrations of ATP, calcium, and zinc were kept constant at 10 mM, 10 mM, and 0.1 mM, respectively. Fig. 3 shows the angular changes plotted as percentages of the maximum angular reorientation, which was obtained at pH 8.5–9.0. The degree of shift from the original orientation of the cilia to a new position decreased gently with the decrease in pH below 8.5, and dropped sharply with increasing pH above 9.

The remaining experiments were all performed with the pH adjusted to 9.0 with 10 mM Tris-HCl buffer.

3. *Effect of* $[Na^+]$

Sodium ions were an unavoidable contaminant of both the ATP reagent and the Tris-maleate buffer. Therefore, the reorientation reaction was examined

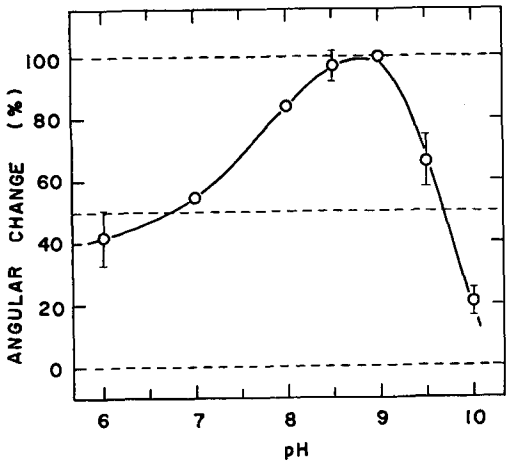

FIGURE 3. Effect of pH in a mixture of ATP, calcium, and zinc on the reorientation of cilia. Angular changes of ciliary axis in each test solution are plotted as percentage of the maximum change at pH 9.0.

in a series of solutions in which the concentration of added sodium ranged from 0 to 50 mM. The response was unaffected by sodium ions in this range of concentrations.

4. *Effect of ATP Concentration*

The reorientation response was examined in two series of test solutions with varying ATP concentration. The concentration of Ca^{++} was 1 mM in one series of the test solutions and 10 mM in the other. The concentration ratio of ATP to zinc was kept at 100:1 in all the test solutions (see section 6).

As shown in Fig. 4, the maximum response occurred at an ATP concentration which was dependent on the calcium concentration. The response in-

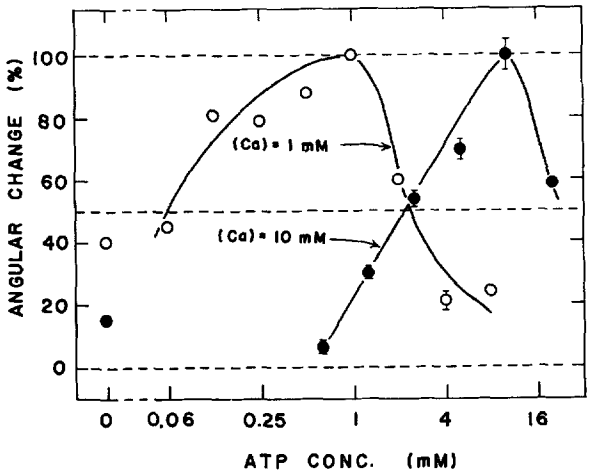

FIGURE 4. Effect of ATP concentration on the reorientation of cilia. Calcium concentration is 10 mM in one series (solid circles) and 1 mM in the other (open circles). The response is plotted as % of the maximum change at 10 mM ATP in the 10 mM calcium series and that at 1 mM ATP in the 1 mM calcium series.

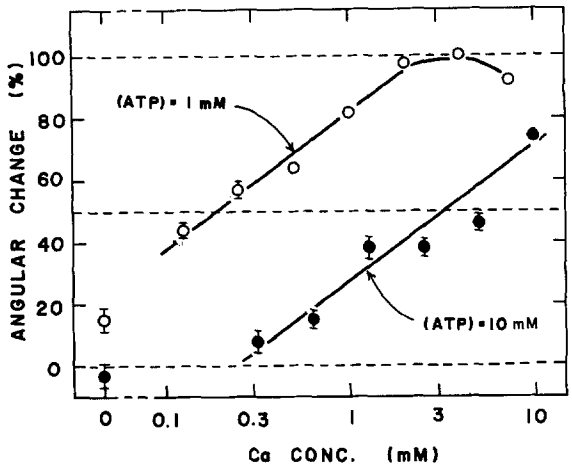

FIGURE 5. Effect of $[Ca^{++}]$ on the reorientation of cilia. ATP concentration is 10 mM in one series (solid circles) and 1 mM in the other (open circles). The response is plotted as % of the maximum angular change at 4 mM calcium in the 1 mM ATP series.

creased with ATP concentration until the latter equaled $[Ca^{++}]$, and showed a decline with further increase in ATP concentration.

5. *Effect of* $[Ca^{++}]$

The degree of the angular change was examined in two series of test solutions with varying $[Ca^{++}]$. ATP concentration was 1 mM in one series and 10 mM in

the other series. The concentration ratio of ATP to zinc was kept at 100:1 in all test solutions (see next section).

Fig. 5 indicates that the degree of the angular change increased with increased [Ca^{++}] reaching a plateau in the 1 mM ATP series at a calcium concentration of approximately 4 mM.

6. *Effect of* [Zn^{++}]

The concentration effect of zinc and its relation to ATP and calcium concentrations in the reorientation response were examined by varying [Zn^{++}] from

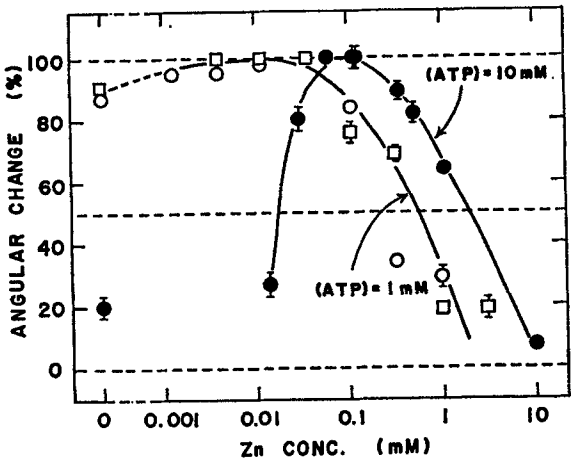

FIGURE 6. Effect of [Zn^{++}] on the reorientation response of cilia. Concentrations of ATP and calcium in the test solutions are as follows: Solid circles, ATP, 10 mM; Ca, 10 mM. Open circles, ATP, 1 mM; Ca, 1 mM. Open squares, ATP, 1 mM; Ca, 10 mM. The response is plotted as % of the maximum angular changes in each series of test solutions.

0 to 10 mM in three series of test solutions, one with an ATP concentration of 10 mM and two containing 1 mM ATP. Calcium concentration was 10 mM in the 10 mM ATP series and one of the 1 mM ATP series. In the other 1 mM ATP series [Ca^{++}] was adjusted to 1 mM.

As shown in Fig. 6, the optimum zinc concentration was 0.1 mM in the 10 mM ATP series and 0.01 mM in both 1 mM ATP series with differing [Ca^{++}]. Thus, an ATP to zinc ratio of 100 is required for a maximum response in varied concentrations of Ca^{++} and ATP. The [Zn^{++}]-dependence of the response was far more prominent in 10 mM ATP than in 1 mM ATP.

7. *Effectiveness of Nucleotide Phosphates Other Than ATP*

The effectiveness of various nucleotide phosphates on the orientation of cilia in the extracted cell was compared with that of ATP. Concentrations of the

nucleotide phosphates, calcium, and zinc were 10 mM, 10 mM, and 0.1 mM, respectively. As shown in Fig. 7, the degree of the angular change in the ADP solution was 50 % of that in the ATP solution, while almost no response occurred in AMP solution. These results suggest that the energy-rich phosphate bond in ATP is essential for the response, and that there is an adenylate kinase

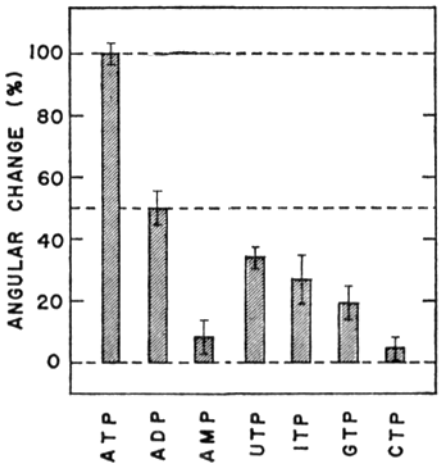

FIGURE 7. Comparison between the effects of various nucleotide phosphates on the ciliary reorientation response. Concentrations of each nucleotide phosphate are 10 mM. Responses are graphed as percentages of the maximum change in the ATP-containing medium.

TABLE I

IONIC REQUIREMENTS FOR THE REORIENTATION RESPONSE OF CILIA IN GLYCERINATED *PARAMECIUM*

Ion species		
5 mM	0.05 mM	Degree of the response
		%
Ca	Zn	100 ± 4
Ba	Zn	114 ± 7
Sr	Zn	100 ± 7
Mg	Zn	57 ± 4
Ca	Ni	71 ± 5
Ca	Co	71 ± 5
Ca	Cu	67 ± 3
Ca	Mn	54 ± 7
Ca	Cd	38 ± 7
Ca	Mg	29 ± 4
Ca	Sr	13 ± 2
Ca	Ba	13 ± 2
Ca	Sn	8 ± 2

The degree of response is represented in % of the maximum response in a mixture of ATP (5 mM), Ca (5 mM), and zinc (0.05 mM).

system present in the extracted *Paramecium*. UTP, ITP, GTP, and CTP were found to have limited effectiveness on the response.

8. *Effects of Metallic Ions Substituted for Calcium or Zinc*

The effects on the reorientation response of various cation species substituted for calcium or zinc were examined in optimum test solutions (5 mM ATP, 5 mM $CaCl_2$, and 0.05 mM $ZnCl_2$). As shown in Table I, strontium and barium effectively substituted for calcium, whereas the response in the presence of magnesium was reduced by almost half of that obtained in the presence of calcium. None of the divalent ions tested was a fully adequate substitute for zinc. Ni, Co, and Cu were most effective, but their action did not exceed 70 % of that of zinc.

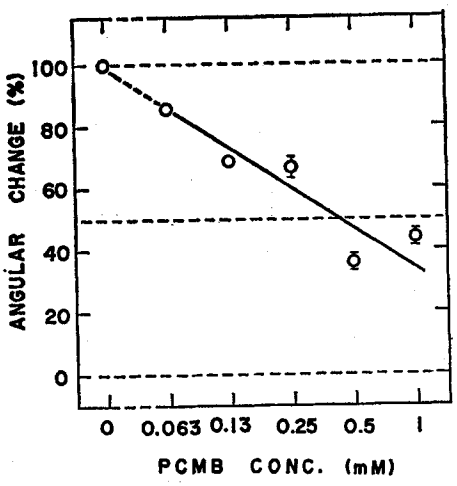

FIGURE 8. Effect of PCMB added to an ATP-Ca-Zn mixture on the reorientation response. The response is plotted as percentage of the angular change in the absence of PCMB.

9. *The Effect of Parachloromercuric Benzoate (PCMB)*

The ciliary reorientation response was tested in the presence of PCMB concentrations ranging from 0 to 1 mM in a standard test solution (1 mM ATP, 4 mM $CaCl_2$, and 0.01 mM $ZnCl_2$). At PCMB concentrations above 0.5 mM the magnitude of response was less than 50 % of maximum (Fig. 8).

10. *Effect of EDTA*

The degree of the reorientation response in test solutions with EDTA (5 mM or more) was always less prominent than that in the standard test solution without EDTA. The effect of EDTA may be due to its chelation of calcium and/or zinc to reduce their concentrations in the test solution. Most interesting was the observation that cilia previously reoriented by application of the standard test solution (1 mM ATP, 4 mM $CaCl_2$, and 0.01 mM $ZnCl_2$) resumed

their original posteriorly pointing direction when the extracted cell was washed with an EDTA solution (Fig. 9). When the ATP-treated extracted cells were washed with 50 mM KCl solution, there was no effect on the orientation of their anteriorly pointing cilia. Furthermore, an extracted cell once treated with an EDTA solution, exhibited reorientation in response to the Ca-ATP-Zn test solution. Induction of the response and its subsequent reversal with EDTA could be repeated several times.

11. *Dilution of the Test Solution*

A series of test solutions was made by diluting the standard test solution (1 mM ATP, 4 mM $CaCl_2$, and 0.01 mM $ZnCl_2$) with 50 mM KCl solution. As shown in

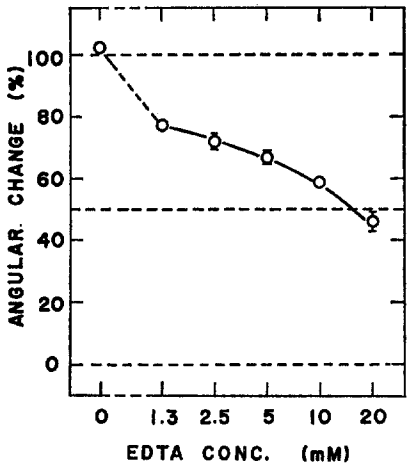

FIGURE 9. Effect of exposure to EDTA on cilia which had been previously reoriented by treatment with an ATP-Ca-Zn mixture. The second reorientation of cilia after exposure to EDTA is represented as % of the maximum first reorientation in the angle elicited by previous exposure to the standard ATP-Ca-Zn mixture.

Fig. 10, dilutions down to one-tenth the strength of the standard solution elicited reorientation responses which were depressed by 20 % or less.

DISCUSSION

The present finding that calcium is essential for ATP-induced reorientation of cilia in glycerol-extracted *Paramecium* lends support to the proposal (Naitoh, 1968) that calcium ions released from cellular binding sites are instrumental in the induction of reversal in beating cilia. According to this hypothesis, externally applied cations displace surface-bound calcium ions which then activate a contractile system controlling the orientation of beating cilia.

As shown in Fig. 5, the angular reorientation of cilia increases with increasing calcium concentration at constant levels of ATP. The degree of ciliary reversal in response to a cationic stimulus in live specimens is also graded (Kamada, 1940), being greater under conditions that cause a larger amount of calcium to be released from cellular binding sites (Naitoh and Yasumasu, 1967). Local concentration of released Ca^{++} near the inferred contractile system of live specimens is assumed to depend, at least in part, on the rate of calcium release from the binding sites which are assumed to be located near

the contractile system (Naitoh, 1968). Thus, according to the hypothesis, the general orientation of cilia in live specimens is controlled by the rate of calcium release from the cellular cation exchanger system.

Cilia of glycerinated *Paramecium* treated with an ATP-Ca-Zn mixture continued to point anteriorly (reversed direction) even after complete removal of calcium, ATP, and zinc from the external medium, whereas the cilia resumed their original direction after treatment with an EDTA solution (Fig. 9). In live specimens ciliary reversal elicited by a cationic or electric stimulus is always temporary (Kinosita, 1936). This suggests the presence of a relaxing system in live specimens which acts to return the reversed cilia to the normal

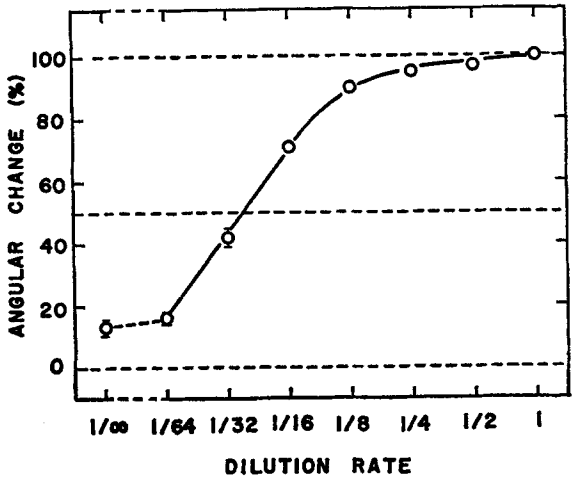

FIGURE 10. Orientation of cilia in extracted specimens exposed to various dilutions of the standard ATP-Ca-Zn mixtures. A series of test solutions is made by diluting a mixture of ATP (1 mM), calcium (4 mM), and zinc (0.01 mM) with 50 mM KCl solution. The change in the angle of the ciliary axis is plotted as percentage of the maximum change elicited in the undiluted standard mixture.

posteriorly pointing direction. This may be accomplished by chelation of calcium (and/or zinc) in a manner analogous to the effect of EDTA on the glycerinated specimen.

It had been proposed (Jahn, 1962; Grebecki, 1965; Kuznicki, 1966) that ciliary reversal results from the removal of calcium from anionic binding sites in the cell (and/or concomitant configurational changes in the binding sites). However, as pointed out by Jahn (1967), these hypotheses did not adequately explain certain details of the reversal response such as its absence upon addition of EDTA, and the transient nature of the response in the face of continued cationic stimulation. The calcium hypothesis of Naitoh (1968) is consistent with the behavior of living paramecia as well as of extracted models.

The requirement of ATP for the reorientation of the cilia of the glycerinated specimen and the inhibition of the response by PCMB, which is an

effective inhibitor of ATPase activity in various tissues, strongly suggest that the reorientation of cilia is energized by ATP. Recently Yasumasu and Naitoh (1969)[3] demonstrated calcium-activated ATPase activity in the glycerol-extracted specimen. Significantly, this ATPase activity has the same optimum pH as the reorientation response (near 9.0). These authors demonstrated that in close association with the occurrence of the reorientation response there was a splitting of labeled terminal phosphorus from ATP.

The role of zinc in the reorientation response remains unclear. The occurrence of an optimum concentration ratio of ATP to zinc for the maximum response suggests an important role for zinc in the interaction of ATP with the contractile system. In this connection it is noteworthy that zinc influences the mechanical behavior of glycerol-extracted muscle fibers. Edman (1958) reported that in the presence of ATP, zinc in small concentrations (0.001 mM) enhances isometric tension, but in large concentrations (0.25 mM) it depresses the tension. Edman (1960) later stressed the role of zinc as a relaxing agent in the contractile system of muscle. Although zinc in larger concentrations inhibits the reorientation response in glycerol-extracted *Paramecium* (Fig. 6), there is no other evidence to support a view of zinc as a relaxing agent in the ciliary system.

Many investigators (Hoffmann-Berling, 1955; Bishop and Hoffmann-Berling, 1959; Brokaw, 1961; Seravin, 1961; Satir and Child, 1963; Gibbons, 1965) have proposed that essential factors for the reactivation of the ciliary beat in glycerinated or saponified cilia are magnesium and ATP, to the exclusion of calcium. In the present extracted specimens, however, ciliary beat could not be observed with any mixtures of ATP and magnesium. According to Gibbons (1965), glycerinated, isolated cilia of *Tetrahymena* rapidly lose their ATP- and magnesium-induced motility during the course of extraction. Seravin (1961) reported that cilia of saponified ciliates lost ATP- and magnesium-induced motility after approximately 1 hr of extraction. It is likely that the longer extraction in the present experiments (over 10 days) resulted in the loss of the ability of the cilia to be reactivated by ATP and magnesium.

In view of this there appear to be two different contractile systems in the ciliary apparatus of protozoa, one located within the cilium (Gibbons, 1965), responsible for the beating motion of the organelle, and another (either internal or external to the cilium) which determines the orientation of the beating motion. Both systems are energized by ATP, but the former has magnesium as a cofactor and the latter employs calcium. Separation of these two mechanisms in the cilia of live paramecia was demonstrated (Naitoh, 1966) by the finding that nickel-inhibited (nonbeating) cilia of *Paramecium* reverse their

[3] Yasumasu, I., and Y. Naitoh 1969. ATP-splitting associated with the reorientation of cilia in glycerol-extracted *Paramecium caudatum*. Data to be published.

orientation in response to cationic as well as electric stimuli just as normally beating cilia do.

It is unresolved whether the contractile component concerned with ciliary reversal is located within the ciliary shaft itself or in the cortical complex associated with the ciliary system.

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