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CONTROL OF THE ORIENTATION OF CILIA BY ATP AND DIVALENT CATIONS IN TRITON-GLYCEROL-EXTRACTED *PARAMECIUM CAUDATUM*

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SUMMARY

The ciliary responses of *Paramecium caudatum*, extracted in Triton X-100 and glycerol, to the external application of ATP and various divalent cations were examined. Ciliary beating could not be reactivated, but changes in the pointing direction of the cilia (the reorientation response) could be reactivated. The free Ca^{2+} concentration determined the final orientation of the cilia, which was towards the front when the Ca^{2+} concentration was above 10^{-6} mol l^{-1}, and towards the rear when below 10^{-7} mol l^{-1}. The reorientation response was inhibited by vanadate. These results indicate that the mechanism for the reorientation response is separable into two components. One is the movement of cilia to change their pointing direction, which, like normal ciliary beating, is energized by Mg-ATP^{2-}. The other is the determination of the final pointing direction of the cilia, which is Ca^{2+}-dependent. Divalent cations can be classified into two groups according to their mode of action on the Ca^{2+}-dependent component. Sr^{2+} is an agonist and Ba^{2+}, Zn^{2+} and Co^{2+} are antagonists to Ca^{2+} for the component.

INTRODUCTION

The locomotor behaviour of *Paramecium* largely depends on ciliary movement, which is controlled by Ca^{2+}-mediated membrane excitation. The external Ca^{2+} ions flow into the cilia through the activated voltage-sensitive Ca channels in the ciliary membrane in association with a Ca action potential. The resulting transient increase in the intraciliary Ca^{2+} concentration causes a transient reversal in the effective power stroke of cilia (ciliary reversal) (Eckert, 1972; Eckert & Naitoh, 1972; Naitoh & Eckert, 1974; Eckert, Naitoh & Machemer, 1976). Naitoh (1969) demonstrated that the predominant orientation of cilia of glycerol-extracted models of *Paramecium* was towards the posterior of the specimens in a reference solution, while it shifted towards the anterior in a solution containing ATP, Ca^{2+} and Zn^{2+}. Naitoh & Kaneko (1972, 1973) demonstrated that the ATP-Mg-reactivated cilia of Triton-extracted models of *Paramecium* beat in the normal direction when the Ca^{2+} concentration was below 10^{-7} mol l^{-1}, whereas they beat in the reversed direction when the

Key words: calcium, cilia, *Paramecium*.

concentration was above 10^{-6} mol l^{-1}. Furthermore, they found that non-beating cilia of the extracted models pointed towards the anterior when Ca^{2+} and ATP (without Mg^{2+}) were applied into the external solution. They proposed that the ciliary system of *Paramecium* has at least two kinds of motile components; one is concerned with the cyclic bending of the cilium and the other regulates the orientation of the effective power stroke in beating cilia, or the pointing direction in non-beating cilia. The former requires Mg^{2+}, and the latter Ca^{2+}, as co-factors for the ATP-energized reactivation.

It should be noted that Ca^{2+} sensitivity in evoking the reorientation response is 10^2–10^3 times higher in the Triton-extracted models than the glycerol-extracted models. There are also some differences in the effectiveness in evoking the response by divalent cations other than Ca^{2+} between the two models. We therefore made the models of *Paramecium caudatum* extracted by both Triton and glycerol to examine in detail the effects of ATP, Mg^{2+}, Ca^{2+} and other divalent cations on the ciliary motile mechanisms. Our results suggest that the change in the orientation of the non-beating cilia of the Triton-glycerol models is mediated by the Mg-ATP^{2-}-energized interaction between dynein and tubulin in the outer doublets of the cilia, while the final pointing direction of the cilia is determined by a Ca^{2+}-sensitive mechanism unidentified yet, and is a function of the free Ca^{2+} concentration.

MATERIALS AND METHODS

Extraction of the specimens of Paramecium

Specimens of *Paramecium caudatum* (syngen 3, strain Kyk 201) were extracted first with Triton X-100 (0·01 % by volume) for 25–35 min at 0 °C according to Naitoh & Kaneko (1973). The extracted specimens were then washed gently three times with an ice-cold washing medium, which consisted of (in mmol l^{-1}) KCl, 50; EDTA, 2; and Tris-maleate buffer, 10 (pH 7·0), and kept in the medium for 15 min to remove Triton. The specimens were centrifuged gently to make a loose pellet. The pellet was resuspended in an ice-cold glycerol medium which consisted of 50 mmol l^{-1} KCl, 30 % (by volume) glycerol, 2 mmol l^{-1} EDTA and 10 mmol l^{-1} Tris-maleate buffer (pH 7·0). The suspension was kept in an ice bath (0 °C) for 1 h. The specimens were then washed three times with an ice-cold glycerol-KCl equilibration medium which consisted of 50 mmol l^{-1} KCl, 30 % glycerol (by volume) and 10 mmol l^{-1} Tris-maleate buffer (pH 7·0) to remove the EDTA. The washed specimens were equilibrated in the medium for at least 1 h at 0 °C prior to experimentation.

The test solutions

All the test solutions contained 50 mmol l^{-1} KCl, 30 % (by volume) glycerol and 10 mmol l^{-1} Tris-maleate buffer (pH 7·0 at 20 °C) as basic components. Ca^{2+} concentration in the test solution was controlled by a Ca-EGTA buffer with 1 mmol l^{-1} EGTA. With the aid of a microcomputer, concentrations of free Ca^{2+}, free ATP, Ca-ATP^{2-}, free Mg^{2+}, Mg-ATP^{2-}, free EGTA, Ca-EGTA and Mg-EGTA in the test solutions were calculated from the stability constants of both

EGTA and ATP for H^+, Ca^{2+} and Mg^{2+} (Martell, 1964) and the apparent equilibrium constants between ATP and Ca^{2+}, between ATP and Mg^{2+}, between EGTA and Ca^{2+}, and between EGTA and Mg^{2+} (Portzehl, Caldwell & Rüegg, 1964).

Reorientation response of cilia

About 500 Triton-glycerol-extracted models with a minute amount (10 μl) of the equilibration medium were pipetted into a large amount (1 ml) of a test solution at room temperature (20–23 °C). The models transferred into the test solution were photographed by a single xenon flash through a phase contrast objective (×20). The angle between the ciliary axis and the cell surface at the right anterior edge of the extracted models was measured to determine the degree of the reorientation response (Naitoh, 1969). The measurements were made on 4–16 specimens, and a mean and its standard error were calculated.

RESULTS

Reactivation of cilia in the Triton-glycerol-extracted Paramecium *models*

The specimens of *Paramecium* pre-extracted in Triton X-100 disintegrated upon transfer into the glycerol extraction medium when the glycerol concentration was less than 30 % (by volume). The presence of glycerol in the test solution in a concentration range from 12 to 30 % (by volume) was also essential to avoid disintegration of the Triton-glycerol-extracted models upon transfer into the test solution. Therefore all the experiments were performed in the presence of 30 % (by volume) glycerol unless otherwise stated.

When the models were transferred into a Ca-ATP test solution (1 mmol l^{-1} $CaCl_2$+1 mmol l^{-1} ATP), or into a Ca-Mg-ATP test solution (10 μmol l^{-1} $CaCl_2$+1 mmol l^{-1} $MgCl_2$+1 mmol l^{-1} ATP), the cilia, which pointed perpendicular to the cell surface in the reference glycerol-KCl solution (Fig. 1A), swung once forward to point towards the front (Fig. 1B). This reorientation of cilia will hereafter be referred to as the anterior response. In contrast, the cilia swung backward so as to point towards the rear in an EGTA-Mg-ATP test solution (1 mmol l^{-1} EGTA+1 mmol l^{-1} $MgCl_2$+1 mmol l^{-1} ATP) (Fig. 1C). This ciliary response will hereafter be referred to as the posterior response.

The normal ciliary beating was not reactivated in the present models. However, the cilia exhibited a quivering or lashing movement in some cases, when the test solution contained 1 mmol l^{-1} $MgCl_2$ and 1 mmol l^{-1} ATP irrespective of their pointing direction. The addition of 100 μmol l^{-1} vanadate, a potent inhibitor of a dynein ATPase (Gibbons *et al.* 1978; Kobayashi, Martensen, Nath & Flavin, 1978), into the test solution stopped the quivering movement.

The reorientation responses in the Ca-ATP test solutions

Only the anterior response took place in the test solutions containing Ca^{2+} and ATP (without Mg^{2+} ions). The optimum pH for the response was about 7.

The concentration ratio of ATP to Ca^{2+} for the maximum response was about 1. That is, when the ATP concentration was kept constant at 1 mmol l^{-1} (or 10 mmol l^{-1}), the degree of the response increased with increasing Ca^{2+} concentration up to 1 mmol l^{-1} (or 10 mmol l^{-1}), and decreased with further increase in the Ca^{2+} concentration. ATP was the nucleotide triphosphate that was most effective in inducing the response. These results are identical with those obtained in the glycerol-extracted *Paramecium* models by Naitoh (1969).

The reorientation responses in the Mg-ATP test solutions

When a test solution contained a minute amount of Ca^{2+} (less than 10 μmol l^{-1}) together with 1 mmol l^{-1} ATP, cilia of the model did not show the anterior response, continuing to point perpendicular to the cell surface as they did in the equilibration medium. When Mg^{2+} was added to the test solution, the cilia showed the anterior response. The response intensity increased with increasing Mg^{2+} concentration, reaching its plateau level at about 50 μmol l^{-1} (Fig. 2, closed circles; Ca^{2+} concentration, 1 μmol l^{-1}). On the contrary, when the Ca^{2+} concentration in the test solution was less than 10^{-7} mol l^{-1} because of the presence of 1 mmol l^{-1} EGTA in the solution, the addition of Mg^{2+} produced the posterior response. The degree of the response reached its maximum plateau level at 50 μmol l^{-1} Mg^{2+} (Fig. 2, open circles).

Both the anterior and the posterior responses produced by Mg^{2+} were inhibited by vanadate. As shown in Fig. 3, the degree of both responses produced by 50 μmol l^{-1}

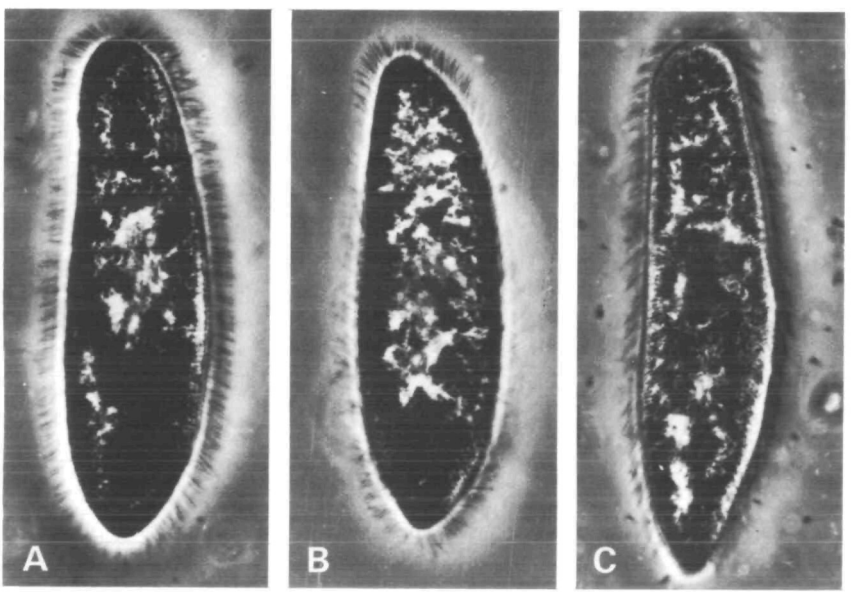

Fig. 1. Photographs of the Triton-glycerol-extracted models of *Paramecium caudatum* in three different solutions taken through a phase-contrast objective (×20) (magnification about ×360). The solutions are: (A) the equilibration medium, (B) a Ca-Mg-ATP solution and (C) an EGTA-Mg-ATP solution. See text for the details.

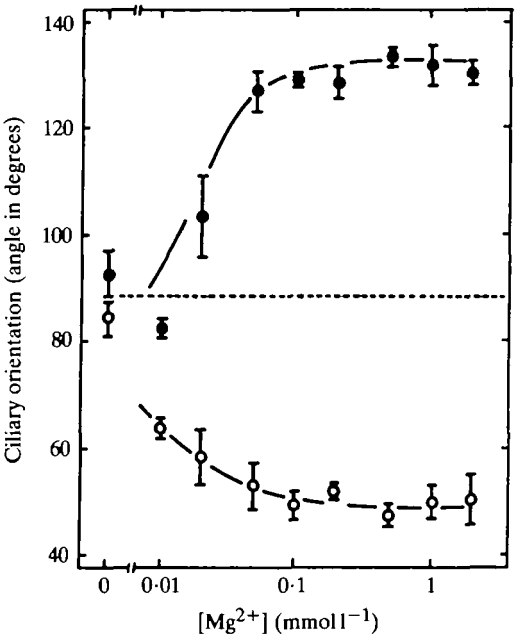

Fig. 2. Effect of Mg^{2+} concentration in the test solution on the pointing direction of cilia of the Triton-glycerol models of *Paramecium*. The dotted line corresponds to the pointing direction in the equilibration medium. The Mg^{2+} concentration was varied in the presence of 1 μmol l^{-1} Ca^{2+} in one series (solid circles), and in the absence of Ca^{2+} (less than 10^{-7} mol l^{-1} due to addition of 1 mmol l^{-1} EGTA into the test solution) in another series (open circles). ATP concentration was 1 mmol l^{-1} in all the test solutions. Bars indicate ± 1 S.E.M.

Mg^{2+} decreased with increasing vanadate concentration in the test solution. The concentration of vanadate producing 50% of the maximum inhibition was about 20 μmol l^{-1} for both responses. The vanadate concentration for 50% inhibition was higher when Mg^{2+} concentration in the test solution was higher.

The effect of Ca^{2+} concentration on the orientation of cilia of the Triton-glycerol-extracted models was examined in the presence of 1 mmol l^{-1} Mg^{2+}. The free Ca^{2+} concentration in the test solutions was controlled in a range from 10^{-8} to 10^{-5} mol l^{-1}. Four different series of experiments with different ATP concentration were performed, and the results are shown in Fig. 4 (ATP concentration; 1 mmol l^{-1} in A, 2 mmol l^{-1} in B, 5 mmol l^{-1} in C, 10 mmol l^{-1} in D). The calculated concentration of Ca-ATP^{2-} was about 0·9 times as high as that of free Ca^{2+} in the presence of 1 mmol l^{-1} ATP (Fig. 4A), 3·3 times in the presence of 2 mmol l^{-1} ATP (Fig. 4B), 12 times in the presence of 5 mmol l^{-1} ATP (Fig. 4C) and 27 times in the presence of 10 mmol l^{-1} ATP (Fig. 4D). The figure clearly shows that the cilia of the models showed the anterior response when the free Ca^{2+} concentration was above 10^{-6} mmol l^{-1}, while they showed the posterior response when the free Ca^{2+} concentration was below 10^{-7} mol l^{-1}, independently of the Ca-ATP^{2-} concentration in the test solution.

Successive treatment of the models with different test solutions

The Triton-glycerol-extracted models of *Paramecium* were first transferred into a Ca-Mg-ATP test solution, which contained 10 μmol l^{-1} Ca^{2+}, 1 mmol l^{-1} Mg^{2+} and 1 mmol l^{-1} ATP. The cilia showed the anterior response in the test solution as described in the previous section (Fig. 5; 1→2). The models were then washed gently with the equilibration medium to remove Ca^{2+}, Mg^{2+} and ATP. The washing did not affect the orientation of the anteriorly-oriented cilia of the models (Fig. 5; 2→3). Subsequent treatment of the washed models with an EGTA test solution, which consisted of 1 mmol l^{-1} EGTA and the basic components of the test solution, showed no effect on the pointing direction of the anteriorly-oriented cilia (Fig. 5; 3→4). When the models were then treated with an EGTA-Mg-ATP test solution, which consisted of 1 mmol l^{-1} EGTA, 1 mmol l^{-1} Mg^{2+} and 1 mmol l^{-1} ATP, the anteriorly-oriented cilia swung once to point towards the posterior (the posterior response) (Fig. 5; 4→5). Subsequent washing of the models with the equilibration medium did not affect the posteriorly-oriented cilia (Fig. 5; 5→6). The posteriorly-oriented cilia of the washed models were not affected by the subsequent washing of the models with an equilibration medium containing 10 μmol l^{-1} Ca^{2+}, whereas the washed models showed the anterior response when they were transferred into the first Ca-Mg-ATP test solution again (Fig. 5; 6→7). This anterior-posterior response cycle could be repeated at least twice.

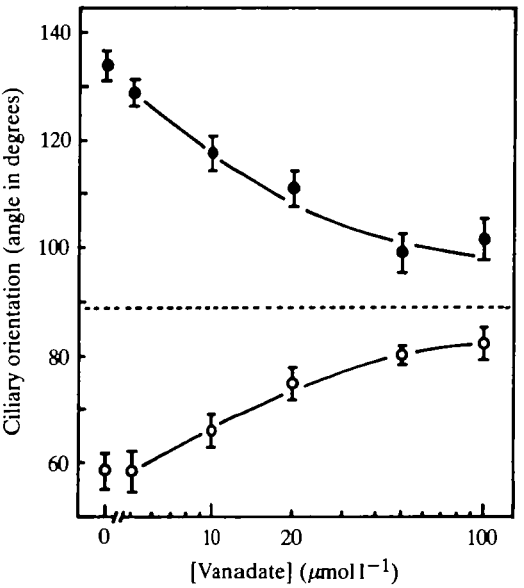

Fig. 3. Effect of vanadate concentration on the reorientation response of cilia on the Triton-glycerol models of *Paramecium*. The dotted line corresponds to the pointing direction of the cilia in the equilibration medium. The test solutions consist of 10 μmol l^{-1} Ca^{2+}, 50 μmol l^{-1} Mg^{2+}, 1 mmol l^{-1} ATP and vanadate (metavanadate) of varied concentrations (0–100 μmol l^{-1}) in one series (solid circles), and of 1 mmol l^{-1} EGTA, 50 μmol l^{-1} Mg^{2+}, 1 mmol l^{-1} ATP and vanadate of varied concentrations in another series of experiments (open circles). Bars indicate ±1 S.E.M.

Effects of divalent cations on the orientation of cilia

The effects of various divalent cations on the orientation of cilia of the Triton-glycerol-extracted models were examined in two series of test solutions. The test solutions in the first series of experiments contained 1 mmol l^{-1} divalent cations and 1 mmol l^{-1} ATP (without Mg^{2+}) and those in the second series of experiments contained 0·5 mmol l^{-1} divalent cations, 1 mmol l^{-1} Mg^{2+} and 1 mmol l^{-1} ATP.

The first series of experiments (Fig. 6A) showed that there was a similar anterior response in the Ca-ATP solution and in the Mg-ATP and Mn-ATP solutions. Sr^{2+}, Ni^{2+}, Ba^{2+}, Co^{2+} and Zn^{2+} showed almost no effects on ciliary orientation. In the second series of experiments (Fig. 6B) Mg^{2+}, Mn^{2+} and Sr^{2+} were found to be as effective as Ca^{2+} in inducing the anterior response. Ba^{2+} and Co^{2+} produced a conspicuous posterior response in the presence of 1 mmol l^{-1} Mg^{2+}.

Competition between Ca^{2+} and other divalent cations in inducing the reorientation responses

The results shown in the previous section indicate that Ba^{2+} and Co^{2+} inhibit the anterior response and in fact produce the posterior response (Fig. 6B). The test

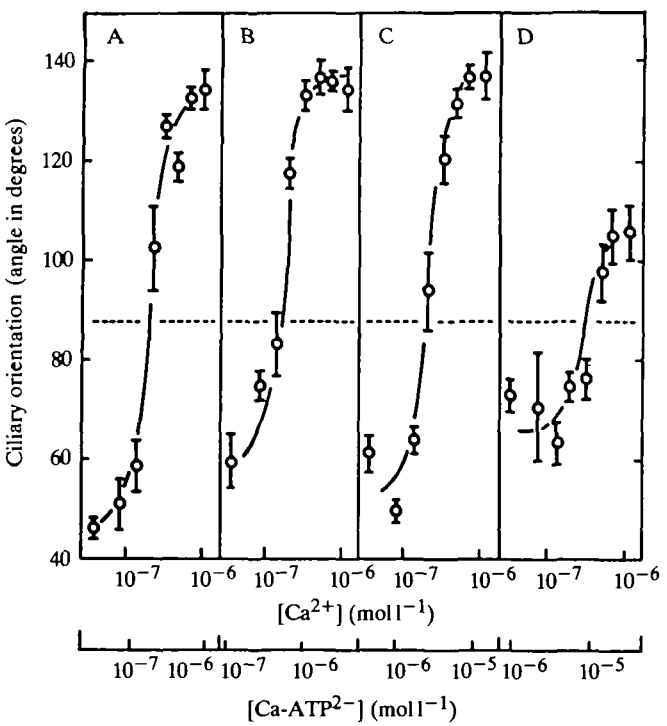

Fig. 4. Effect of Ca^{2+} concentration on the ciliary orientation of the Triton-glycerol models of *Paramecium*. The dotted line corresponds to the pointing direction of the cilia in the equilibration medium. The Ca^{2+} concentration was controlled by Ca-EGTA buffer (EGTA concentration, 1 mmol l^{-1}). Concentrations of both Ca^{2+} and Ca-ATP^{2-} are shown on the abscissa. ATP concentration in the test solutions was 1 mmol l^{-1} in A, 2 mmol l^{-1} in B, 5 mmol l^{-1} in C and 10 mmol l^{-1} in D. Mg^{2+} concentration was 1 mmol l^{-1} in all the test solutions. Bars indicate ±1 S.E.M.

solutions employed did not contain any chelating reagents. Therefore, a few μmol l^{-1} Ca^{2+} is expected to be present in all the test solutions. It is, therefore, highly probable that the inhibition of the anterior response by these ions is due to their competition with Ca^{2+} for the reorientation mechanism. In order to examine this competition, ciliary orientation was examined in two series of test solutions. In series A, Ca^{2+} concentration was varied from 0 to 0·5 mmol l^{-1} while the concentration of other divalent cations was kept constant at 0·5 mmol l^{-1}. In series B, the divalent cation concentration was varied from 0 to 2 mmol l^{-1}, while the Ca^{2+} concentration was kept constant at 10 μmol l^{-1}. As shown in Fig. 7A, in the presence of Ba^{2+} (0·5 mmol l^{-1}) the models showed the anterior response when the Ca^{2+} concentration was above 50 μmol l^{-1}, while they showed the posterior response when the Ca^{2+} concentration was below 50 μmol l^{-1}. On the other hand, when the Ca^{2+} concentration was kept constant (10 μmol l^{-1}), Ba^{2+} concentrations above 50 μmol l^{-1} always produced the posterior response (Fig. 7B). Competition of Co^{2+} with Ca^{2+} occurred in a manner similar to that of Ba^{2+}. A similar type of competition was also observed between Ca^{2+} and Zn^{2+}.

There was no competition between Sr^{2+} and Ca^{2+} in producing the anterior response. Examination of the ciliary orientation of the models in a series of test solutions with different Sr^{2+} concentrations, which was controlled by Sr-EGTA

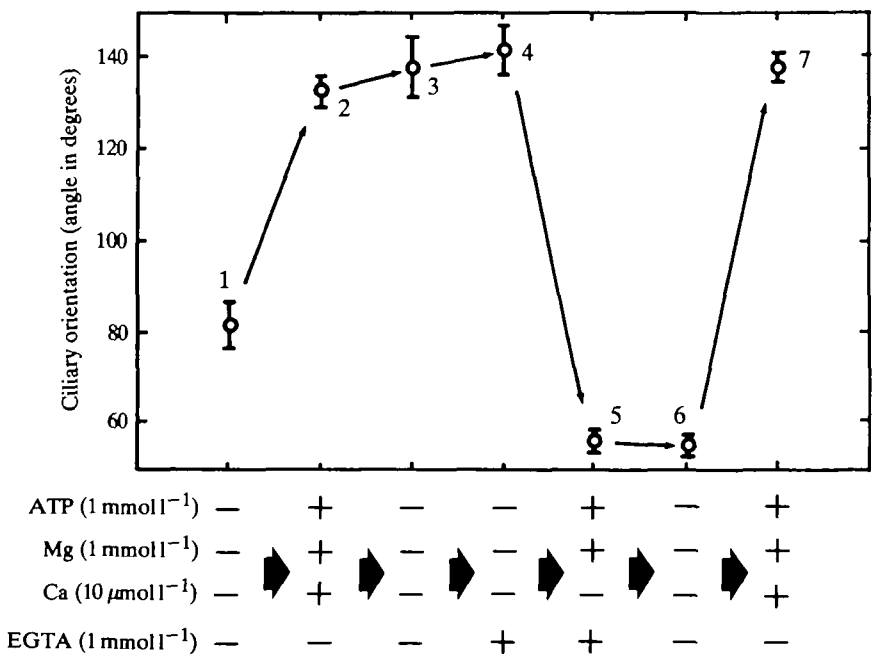

Fig. 5. Effects on the ciliary orientation of the successive treatments of the Triton-glycerol models of *Paramecium* with various test solutions. Numbers and arrows in the figure show the sequence of treatments. The symbols − and + shown under the abscissa indicate the absence and presence of the substances indicated on the left of the symbols in the test solutions. Bars indicate ±1 S.E.M.

buffer, proved that the effect of Sr^{2+} on the ciliary orientation was similar in its characteristics to that of Ca^{2+}, although its effectiveness was about one-tenth of that of Ca^{2+} (Fig. 8).

DISCUSSION

Triton-glycerol-extracted models of *Paramecium* did not show ciliary beating in a mixture of ATP and Mg^{2+}, while they showed the ciliary reorientation. Terashita, Kato & Sato (1983) reported that glycerol inhibited the 21 S dynein ATPase activity of sea urchin sperm flagella. Therefore, the absence of ciliary beating in the present models might be, at least in part, due to inhibition of the ATPase activity by glycerol in the test solution.

The Triton-glycerol-extracted models showed a similar anterior response to that of the glycerol-extracted models in the ATP-Ca test solutions without Mg^{2+} (Naitoh, 1969). The concentration ratio of ATP to Ca^{2+} for the maximum response was

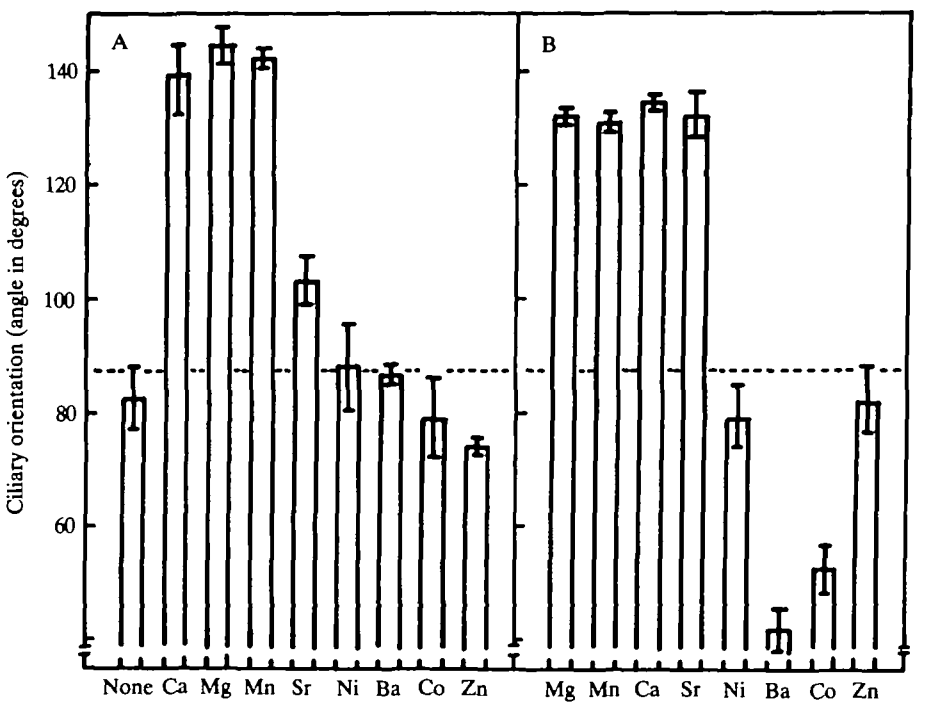

Fig. 6. Effects of various concentrations of divalent cations in the test solution on the ciliary orientation of the Triton-glycerol models of *Paramecium*. The dotted line corresponds to the pointing direction of the cilia in the equilibration medium. Each test solution employed in a series of experiments shown in A contained 1 mmol l^{-1} ATP and 1 mmol l^{-1} divalent cations in chloride form. The test solutions employed in the series B experiments contained 1 mmol l^{-1} ATP, 1 mmol l^{-1} Mg^{2+} and 0·5 mmol l^{-1} divalent cations. Bars indicate ±1 S.E.M.

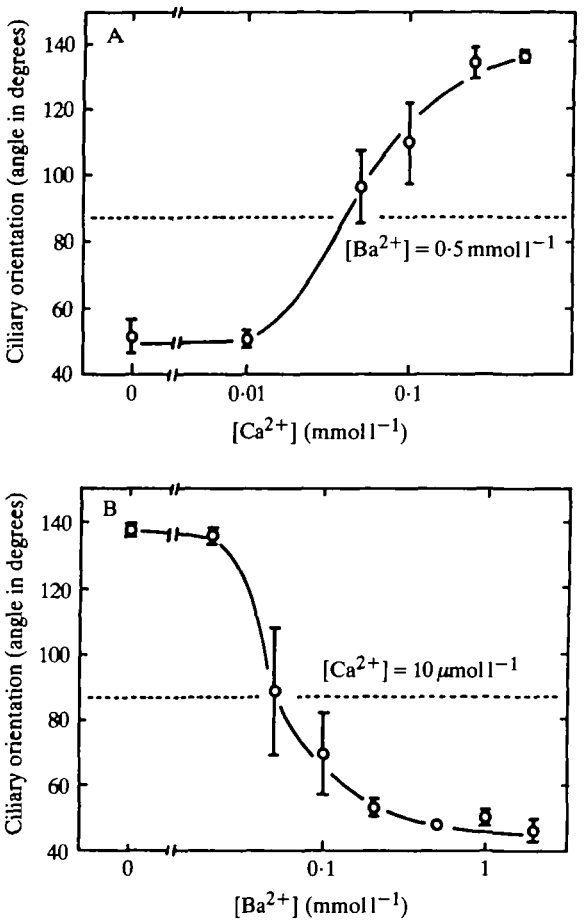

Fig. 7. Competition between Ba^{2+} and Ca^{2+} in producing the reorientation responses of cilia on the Triton-glycerol models of *Paramecium*. The dotted line corresponds to the pointing direction of the cilia in the equilibration medium. (A) Ca^{2+} concentration in the test solution was changed while Ba^{2+} concentration was kept constant at 0·5 mmol l^{-1}. (B) Ba^{2+} concentration was changed while Ca^{2+} concentration was kept constant at 10 μmol l^{-1}. The test solutions contained 1 mmol l^{-1} ATP and 1 mmol l^{-1} Mg^{2+}. Bars indicate ±1 S.E.M.

about 1. This suggests that the Ca-ATP^{2-} complex energizes the anterior response in the absence of Mg^{2+}, and free ATP seems to inhibit the response.

The anterior response of the Triton-glycerol-extracted models was inconspicuous when Ca^{2+} concentration in the ATP-Ca test solution was as low as 10 μmol l^{-1}. Addition of Mg^{2+} (more than 50 μmol l^{-1}) into the test solution produced a marked anterior response of the models (Fig. 2, solid circles). In contrast, when the Ca^{2+} concentration in the test solution was less than 10^{-7} mol l^{-1}, due to the presence of EGTA, addition of Mg^{2+} produced the posterior response (Fig. 2, open circles). As clearly shown in Fig. 4, the free Ca^{2+} concentration in the test solution determines the pointing direction of cilia after they have shown the Mg^{2+}-induced reorientation

response. The Ca-ATP^{2-} complex is not directly related to the determination of the orientation of cilia.

Cilia once reoriented anteriorly in Ca-Mg-ATP test solution kept the orientation after removal of the nucleotide and the divalent cations from the test solution (Fig. 5; 2→3). Further addition of EGTA to chelate residual Ca^{2+} did not affect the orientation (Fig. 5; 3→4). Subsequent addition of ATP and Mg^{2+} to the EGTA-containing solution produced the posterior response (Fig. 5; 4→5). These results clearly indicate that the change in the ciliary orientation of the Triton-glycerol models requires not only the change in Ca^{2+} concentration but also the presence of Mg-ATP^{2-} in the test solution.

Both the anterior and the posterior responses were inhibited by vanadate (Fig. 3). Vanadate is a potent inhibitor of the dynein ATPase activity (Gibbons *et al.* 1978; Kobayashi *et al.* 1978) and inhibits the cross-bridge reaction between dynein and tubulin (Sale & Gibbons, 1979). Therefore, it is conjectured that the reorientation responses are caused by Mg-ATP^{2-}-energized interactions between dynein and tubulin in the outer doublets of the cilia (Naitoh & Sugino, 1984). Ca^{2+} determines

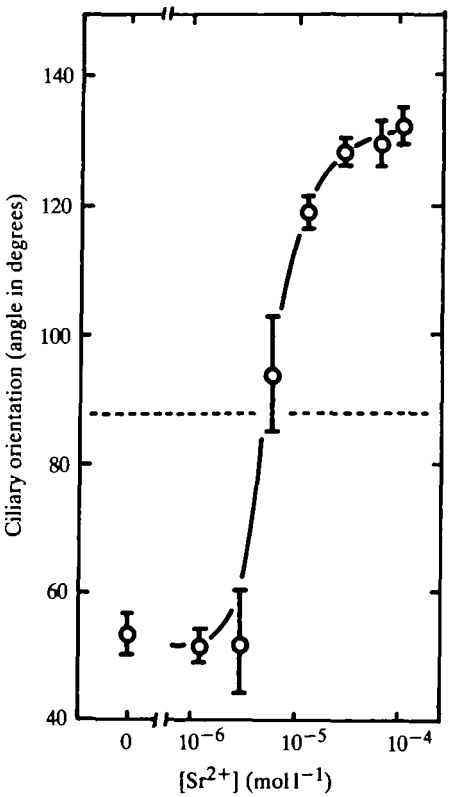

Fig. 8. Effect of Sr^{2+} concentration on the ciliary orientation of the Triton-glycerol models of *Paramecium*. The dotted line corresponds to the pointing direction of the cilia in the equilibration medium. Sr^{2+} concentration in the test solutions was controlled by Sr-EGTA buffer (EGTA concentration, 1 mmol l^{-1}) in an otherwise EGTA-Mg-ATP solution. Bars indicate ±1 S.E.M.

the direction in which the cilia point after they swing once, by mechanisms that are not yet understood. It is important to note that the direction of the effective power stroke of the ATP-Mg-reactivated cilia of Triton-extracted models of *Paramecium* is a function of Ca^{2+} concentration in the reactivation medium (Naitoh & Kaneko, 1972, 1973). The direction was reversed when the Ca^{2+} concentration was above 10^{-6} mol l^{-1} and the models swam backwards. The Ca^{2+}-controlled pointing direction of the present Triton-glycerol models seems to correspond to the Ca^{2+}-controlled direction of the effective power stroke of the Triton models (Naitoh & Sugino, 1984).

Our findings about the effects of various divalent cations on ciliary orientation (Fig. 6A) indicate that Ca-ATP^{2-} and Mn-ATP^{2-} can energize the reorientation responses as Mg-ATP^{2-} does, whereas Sr-ATP^{2-} and Ba-ATP^{2-} cannot. The anterior response in the Mg-ATP^{2-} test solution is due to the presence of residual Ca^{2+} in the test solution, because no chelating reagent was added in the test solutions. Sr^{2+} acts as an agonist of Ca^{2+} for the unknown mechanism(s) by which the pointing direction of the cilia is controlled (Figs 6, 8), whereas Ba^{2+} and Co^{2+} antagonize the action of Ca^{2+} (Figs 6, 7). Mn^{2+} does not antagonize Ca^{2+} for the mechanism.

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