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ATP-REGENERATING SYSTEM IN THE CILIA OF *PARAMECIUM CAUDATUM*

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Summary

The energy supply for eukaryotic ciliary and flagellar movement is thought to be maintained by ATP-regenerating enzymes such as adenylate kinase, creatine kinase and arginine kinase. In this study, the energy-supplying system for the ciliary movement of *Paramecium caudatum* was examined. Arginine kinase and adenylate kinase activities were detected in the cilia. To demonstrate that phosphoarginine satisfactorily supplies high-energy phosphate compounds into the narrow ciliary space, we prepared an intact ciliated cortical sheet from live *Paramecium caudatum*. These cortical sheets, with an intact ciliary membrane, produced a half-closed system in which each cilium was covered with a ciliary membrane with an opening to the cell body. Ciliary beating on the intact cortical sheets was induced by perfusing not only ATP but also ADP. Addition of phosphoarginine (0.2 mmol l^{-1}) increased the beat frequency. A further increase in beat frequency was observed in 0.4 mmol l^{-1} phosphoarginine, and this was enhanced when the cilia were reactivated with relatively low concentrations of ATP. We have demonstrated that phosphoarginine supplies energy as a 'phosphagen' for ciliary beating in *Paramecium caudatum*, suggesting that phosphoarginine functions not only as a reservoir of energy but also as a transporter of energy in these continuously energy-consuming circumstances.

Movies available on-line:
http://www.biologists.com/JEB/movies/jeb3123.html

Key words: phosphocreatine, flagellum, muscle, arginine kinase, creatine kinase, dynein, ATPase, ciliary movement, *Paramecium caudatum.*

Introduction

Intraciliary ATP concentration is a limiting factor in the movement of eukaryotic cilia and flagella. The beat frequency (Gibbons and Gibbons, 1972; Brokaw and Simonick, 1977), the sliding velocity of the outer doublet microtubule (Yano and Miki-Noumura, 1980), the high-frequency vibration of flagella (Kamimura and Kamiya, 1989) and the oscillation frequency (by only a few dynein molecules) (Shingyoji et al., 1998) all depend on ATP concentration.

In cellular systems with high energy requirements, the limitation of ATP diffusion may be particularly critical. For vertebrate muscle and other cells, a phosphocreatine shuttle or circuit has been proposed to direct energy from the mitochondria to sites of ATP consumption (Wallimann et al., 1992). This hypothesis requires the existence of discretely located mitochondrial and cytosolic creatine kinase isoforms (Watts, 1973). ATP generated in the mitochondria would be immediately transphosphorylated to phosphocreatine, which in turn could be utilized only by an appropriate target that contains creatine kinase. Although phosphocreatine is the sole phosphagen in vertebrate and some invertebrate species, phosphoarginine is the most primitive alternative phosphagen in invertebrates (Ellington, 1989). For example, in lobsters *Homarus vulgaris* and *H. americanus*, muscle phosphoarginine maintains the free-energy balance at an appropriate level, as does phosphocreatine in vertebrate muscle (Morrison, 1973).

In cilia and flagella, subcellular compartmentation is conspicuous between sites of energy production (mitochondria) and sites of energy utilization (all dynein sites along the microtubules). To maintain intraflagellar ATP concentration, a phosphocreatine shuttle system and a phosphoarginine shuttle system have been postulated in sea urchin *Strongylocentrotus pupuratus* (Tombes and Shapiro, 1985) and horseshoe crab *Limulus polyphemus* (Strong and Ellington, 1993) sperm flagella, respectively. In the cilia and flagella of protists, the presence of arginine kinase activity has been reported for *Tetrahymena pyriformis* (Watts and Bannister, 1970). In contrast, neither arginine kinase nor creatine kinase activity has been detected in *Chlamydomonas reinhardtii* flagella (Watanabe and Flavin, 1976).

Hyams and Borisy (Hyams and Borisy, 1978) demonstrated, using an isolated flagellar apparatus, that ADP could reactivate the flagella and that adenylate kinase regenerated ATP. In

Chlamydomonas reinhardtii flagella, therefore, the ATP concentration was thought to be maintained only by ATP diffusion and adenylate kinase activity. However, the cilia of *Tetrahymena pyriformis* contain arginine kinase (Watts and Bannister, 1970), suggesting that the phosphoarginine shuttle system is active in ciliates. There is no concrete evidence that the phosphoarginine shuttle system is active in the supply of energy for ciliary movement.

We developed a cortical sheet from the demembranated cell model of *Paramecium caudatum* (Noguchi et al., 1991). In demembranated cilia, however, the phosphagen transport system had been destroyed by a detergent when the cilia were reactivated. In the present study, we attempted to prepare ciliated cortical sheets from live *Paramecium caudatum* without using detergents to keep the phosphagen transport system intact and eventually succeeded in preparing intact ciliated cortical sheets. We developed a half-closed system in which each cilium is covered with a ciliary membrane with an opening to the cell body. Using this subcellular model system, we have demonstrated that the phosphoarginine shuttle system is active in the cilia of *Paramecium caudatum*, supplying high-energy phosphate from the base of the cilium throughout the ciliary axonemes and, thus, regenerating ATP. ATP may be consumed rapidly in the cilium. Phosphoarginine, just like phosphocreatine in vertebrate muscle, may therefore be a relevant means of supplying energy within the narrow space of a cilium.

Materials and methods

Isolation of cilia

Paramecium caudatum (stock G3) were cultured in a hay infusion. Cells were grown to the late-logarithmic phase at 25 °C. Harvested cells were washed three times with a washing solution [2 mmol l^{-1} KCl, 2 mmol l^{-1} $CaCl_2$, 0.3 mmol l^{-1} phenylmethylsulphonyl fluoride (PMSF), 10 mmol l^{-1} Tris-maleate, pH 7.0]. Cells were deciliated by dibucaine treatment (following the method of Mogami and Takahashi, 1983) with slight modifications (Noguchi et al., 2000). Cilia were isolated from cell bodies by centrifugation, repeated twice, at 600 ***g*** for 5 min. The pellets were discarded. The supernatant was centrifuged at 7700 ***g*** for 10 min to pellet the cilia. The supernatant (deciliation solution containing dibucaine) was decanted into a bottle. The pellet was resuspended in TMKE solution (10 mmol l^{-1} Tris-maleate, pH 7.0, 5 mmol l^{-1} $MgCl_2$, 20 mmol l^{-1} potassium acetate, 1 mmol l^{-1} EGTA) containing 0.3 mmol l^{-1} PMSF and centrifuged at 7700 ***g***. The pellet was rewashed with TMKE solution. The isolation of cilia was monitored by dark-field microscopy. These cilia were then treated with a demembranation solution containing 0.1 % Triton X-100 in TMKE solution for 10 min at 0 °C. The suspension was centrifuged to pellet the axonemes. The supernatant was kept on ice as a membrane-plus-matrix fraction. The Triton X-100 was removed from the axonemes by washing the preparation twice with TMKE solution. The axoneme pellet was suspended in a small amount of TMKE solution.

Assay of arginine kinase and adenylate kinase activity

Arginine kinase activity and adenylate kinase activity were determined following the method of Schoff et al. (Schoff et al., 1989) with slight modifications. The reaction mixture consisted of 10 mmol l^{-1} glucose, 5 mmol l^{-1} $MgSO_4$, 1 mmol l^{-1} $NADP^+$, 5 units ml^{-1} hexokinase, 1 unit ml^{-1} glucose-6-phosphate dehydrogenase and 10 mmol l^{-1} Tris-maleate buffer (pH 7.0). A sample of approximately 10 μg of the preparation was added to the reaction mixture in a cuvette to give a volume of 2.5 ml, which was then preincubated at 25 °C for 10 min. The reaction was started by adding a small volume of ADP to give a concentration of 2 mmol l^{-1}. Adenylate kinase activity was calculated by measuring the increase in absorbance at 340 nm that accompanied the production of NADPH. Arginine kinase activity was estimated from the increase in absorbance after further addition of phosphoarginine to achieve a final concentration of 0.4 mmol l^{-1}.

Measurement of the intracellular concentration of phosphoarginine

The intracellular concentration of phosphoarginine was measured as total guanidino phosphate (Yanagisawa, 1967) following the method of LePage (LePage, 1957). The cells were washed in washing medium, which consisted of 50 mmol l^{-1} KCl, 2 mmol l^{-1} EDTA and 10 mmol l^{-1} Tris-maleate buffer (pH 7.0). Phosphoarginine was extracted from cells (2–5 mg of protein) by adding 3 ml of ice-cold 6 % trichloroacetic acid to the loose pellet of washed cells. The extraction procedure was performed at 0–4 °C throughout. After 20 min, the suspension was centrifuged at 720 ***g*** for 5 min. The supernatant was brought to pH 8.2 by addition of 2 mol l^{-1} NaOH. The pellet was extracted again with 2 ml of 6 % trichloroacetic acid, and the procedure was repeated once more. The extracts were combined and brought to pH 8.2 by addition of 2 mol l^{-1} NaOH to pH 8.2. To precipitate ATP and ADP, 0.5 ml of 25 % barium acetate was added (LePage, 1957), and the mixture was left overnight. The solution was centrifuged 10 min at 4000 ***g***. The supernatant was mixed with 1 ml of 1 mol l^{-1} sodium sulphate and centrifuged for 10 min at 500 ***g*** to remove barium sulphate (LePage, 1957). The supernatant was divided into three test tubes. Each sample was 3 ml, and trichloroacetic acid was added to give a final concentration of 2.3 %. Two samples were incubated at 100 °C for 1 min to hydrolyze phosphoarginine. The third sample was used as a blank without hydrolysis. Liberated inorganic phosphate was determined by the method of Anner and Moosmayer (Anner and Moosmayer, 1975). As a standard, 0–40 nmol of phosphoarginine was treated using the same procedure. Intracellular phosphoarginine concentration was estimated assuming that phosphoarginine distributes uniformly in the cell volume of 6.0×10^{-7} ml (Saiki and Hiramoto, 1975). Protein was determined using the method of Lowry et al. (Lowry et al., 1951) with bovine serum albumin as a standard.

Preparation of ciliated cortical sheets from live cells

The preparation of ciliated cortical sheets from live cells

(intact cortical sheets) and the reactivation of cilia were essentially the same as for the cortical sheets prepared from a demembranated cell model (Noguchi et al., 1991; Noguchi et al., 1993; Noguchi et al., 2000). Concentrated cells were washed with an ice-cold washing medium by centrifugation. The loose pellet of cells was washed with KCl solution consisting of 50 mmol l^{-1} KCl and 10 mmol l^{-1} Tris-maleate buffer (pH 7.0) and resuspended in 1 ml of the same solution. The cells were then pipetted a few times through a glass pipette with a small-diameter (approximately 0.2 mm) inside tip to tear or nick the cell cortex. This cell suspension was used for the reactivation experiments.

A simple perfusion chamber (see Fig. 2A) was prepared by placing the sample between a slide and a coverslip. The slide and the coverslip were separated by a thin layer of Vaseline applied to two opposite edges of the coverslip. To observe the reactivation of cilia on the sheet of cell cortex, 20 µl of the sample was gently placed on a glass slide, and a coverslip with Vaseline was placed over the sample. Solutions were then perfused through the narrow opening at one of the edges of the coverslip, while the excess fluid was drained from the opposite end with the aid of small pieces of filter paper. During the first perfusion using a reference KCl solution, some torn cell cortex adhered flat to the glass surface. The cortical sheets were then perfused successively with reactivation solutions. All the reactivation solutions contained 2 mmol l^{-1} $MgCl_2$, 50 mmol l^{-1} potassium acetate, 1 mmol l^{-1} EGTA and 10 mmol l^{-1} Tris-maleate buffer (pH 7.0) and the component(s) noted in the Results. Reactivation was carried out at 22–25 °C. The addition of a protease inhibitor cocktail had no discernible effect on the results. The cilia of well-prepared cortical sheets maintained their activity for 1 h. The movements of the cilia were observed under a dark-field microscope equipped with a 100 W mercury light source, a heat filter and a green filter and recorded on videotape using a National WV-1550 TV camera.

Beat frequency of the reactivated cilia

The frequencies of changes in light intensity due to cyclic beating of the cilia were monitored through a small hole fixed above the eyepiece of a dark-field microscope. Cyclic changes in the light intensity were passed to a phototransistor and amplified by operational amplifiers. The amplified signal was converted into a digital signal by an A/D converter (AB98-35B, ADTEC) and recorded on floppy disks using a personal computer (NEC PC-9801 VM). The signal of the light/dark cycle corresponded to the cycle of ciliary beating. The beat frequency was obtained simply by counting the peak of the light signal or the fast Fourier transform (FFT) spectra converted using FFT software (Wave Master, Canopus, Kobe, Japan).

Results

Arginine kinase activity in the cilia of Paramecium caudatum

Ciliary axonemes and ciliary membrane-plus-matrix fractions were separated from the isolated cilia of *Paramecium caudatum*. Arginine kinase activity and adenylate kinase activities were measured in both fractions (Fig. 1). As shown in Fig. 1B, high levels of arginine kinase activity was localized in the membrane-plus-matrix fraction of cilia. The cell body also contained arginine kinase activity (data not shown). High levels of arginine kinase activity were observed in the deciliation solution containing dibucaine (approximately 1 unit mg^{-1} protein). Creatine kinase activity was not observed in any fraction.

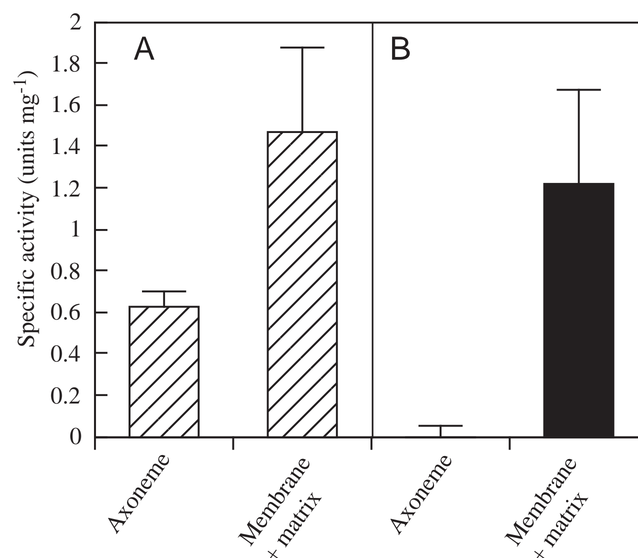


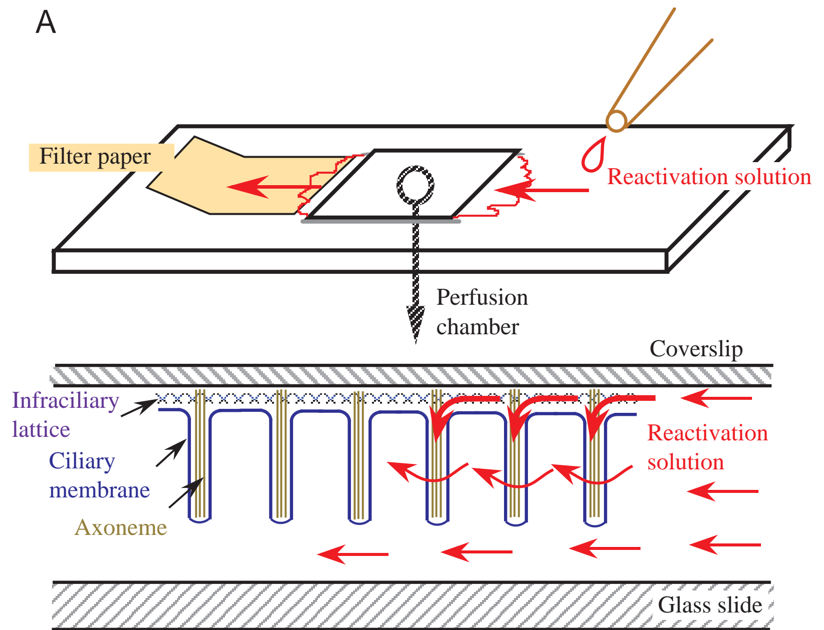
Fig. 1. Distribution of adenylate kinase and arginine kinase activity in the cilia of *Paramecium caudatum*. Adenylate kinase (A) and arginine kinase (B) activities were determined in both axonemal and membrane-plus-matrix fractions. Adenylate kinase activity (hatched columns) was detected in both fractions. These activities were measured in the presence of 2 mmol l^{-1} ADP. Arginine kinase activity (filled columns) was detected only in the ciliary membrane-plus-matrix fraction. These activities were measured in the presence of 2 mmol l^{-1} ADP and 0.4 mmol l^{-1} phosphoarginine. No creatine kinase activity was detected. 1 unit is equvalent to an initial rate of 1 µmol min^{-1}. Values are means + S.D., *N*=4.

Estimation of intracellular phosphoarginine concentration

The intracellular concentration of phosphoarginine was estimated by determining the phosphoarginine content of whole cell extracts. Intracellular phosphoarginine concentration was estimated assuming that phosphoarginine distributes uniformly in the cell volume (6.0×10^{-7} ml) (Saiki and Hiramoto, 1975). The estimated phosphoarginine content was 4.3 µmol phosphoarginine g^{-1} protein, and the average protein content of our culture of *Paramecium caudatum* was 5.3×10^{-8} g protein $cell^{-1}$. Using these values, the phosphoarginine concentration estimated from four individual preparations was 0.37 ± 0.03 mmol l^{-1} (mean ± S.D.).

Preparation of intact cortical sheets from live Paramecium caudatum

We succeeded in preparing ciliated cortical sheets from live



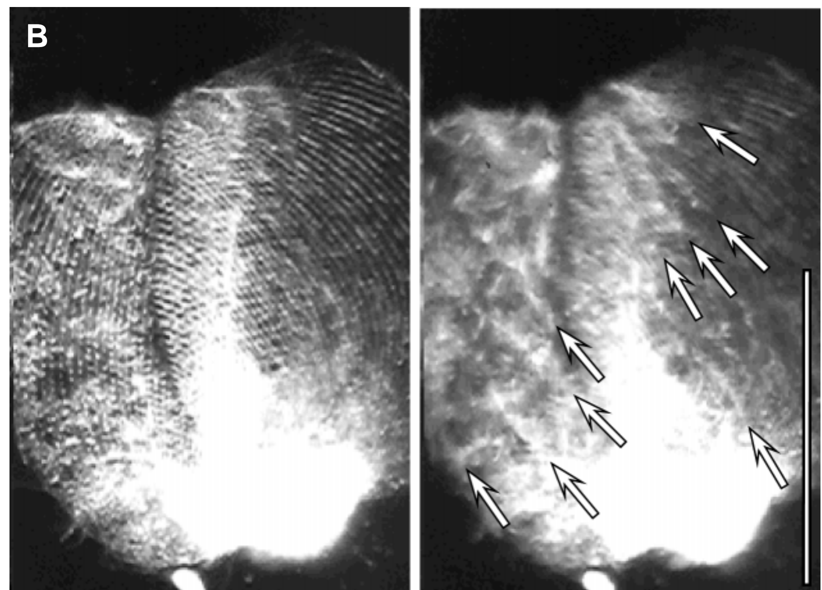


Fig. 2. Reactivation of cilia on the intact cortical sheet. (A) Ciliary reactivation on the cortical sheets. Cortical sheets adhered flat to the surface of the coverslip. Reactivation solution flowed into the space between the cortical infraciliary lattice and the coverslip and diffused into the intraciliary matrix only from the opening of the ciliary base (indicated by thick orange arrows). (B) These still frames from the video recording are images of an intact cortical sheet under dark-field microscopy reactivated with 0.05 $mmol\,l^{-1}$ Mg-ATP, 1 $mmol\,l^{-1}$ EGTA, 10 $mmol\,l^{-1}$ Tris-maleate buffer (pH 7.0) and 0.4 $mmol\,l^{-1}$ phosphoarginine. The frame is converted to a view from outside the cell. The top of the frame is the anterior direction of the cell. Left: an image focused on the cell cortex. Right: an image focused on cilia. Reactivated ciliary beating accompanying a metachronal wave (indicated by arrows). Scale bar, 100 μm.

Paramecium caudatum by pipetting the concentrated cells, as previously applied to the cell model. The cilia on the intact cortical sheets were reactivated by the same procedure as that used in the sheets from the demembranated cell model (Fig. 2A). The reactivation of cilia occurred a short time after perfusing the reactivation solutions. The cilia on the peripheral part of the sheet began to beat first, and the region of beating then spread into the centre of the sheet. Almost all the cilia on the sheet started to beat within a few seconds of perfusion (Fig. 2B and movie).

Measurement of beat frequency

Ciliary beat frequency is a good index of intraciliary ATP concentration, because the sliding velocity of the outer doublet microtubule and the resulting beat frequency both depend on ATP concentration. We equipped the detector to catch the change in light intensity produced by the beat cycle of the cilia. The estimated beat frequency from the recorded signal of cyclic changes in the light intensity and FFT spectra (Fig. 3) concurred with the direct measurement of the beat cycle using videotape (Fig. 4).

Reactivation of cilia by ADP

Both axonemes and membrane-plus-matrix fractions contain adenylate kinase activity (Fig. 1A). The cilia on the intact cortical sheet were expected to be reactivated by ADP. As shown in Fig. 5, 0.2 $mmol\,l^{-1}$ ADP reactivated ciliary beating. Beating was completely inhibited by the adenylate kinase inhibitor diadenosine pentaphosphate (diApP) (Lienhard and Secemski, 1973; Tombes and Shapiro, 1985). The cilia

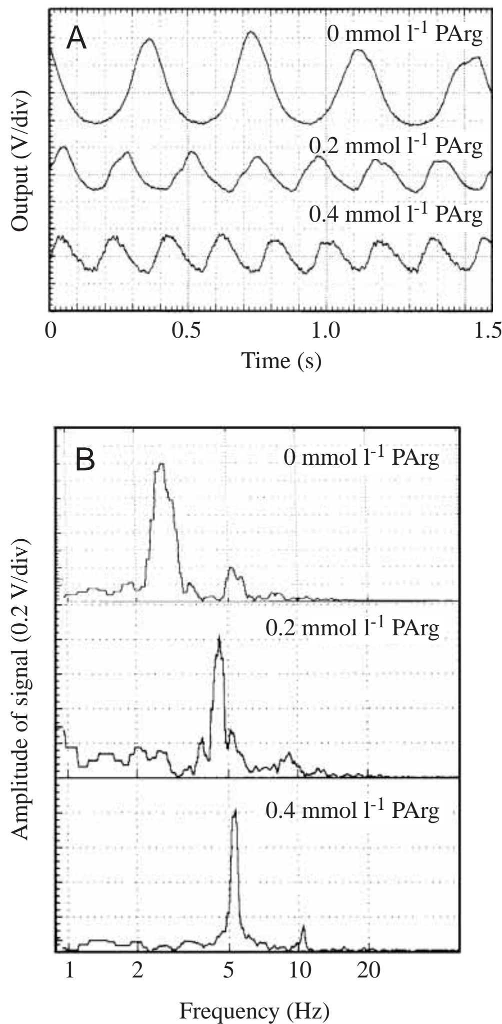


Fig. 3. Detection of the signal of beat frequency. An example of the measurement of beat frequency. (A) Output from the optical detector showing the frequency of the light/dark cycle produced by ciliary beating. The detector converted the light/dark cycle into output as a change in voltage. The frequency of ciliary beating increased with increasing phosphoarginine (PArg) concentration. (B) Fast Fourier transform (FFT) patterns of the output shown in A. Beat frequency measured by counting the peaks of the output of the optical detector and by FFT spectra coincided well. Each tick mark on the *y* axis corresponds to 1 V (A) and 0.2 V (B).

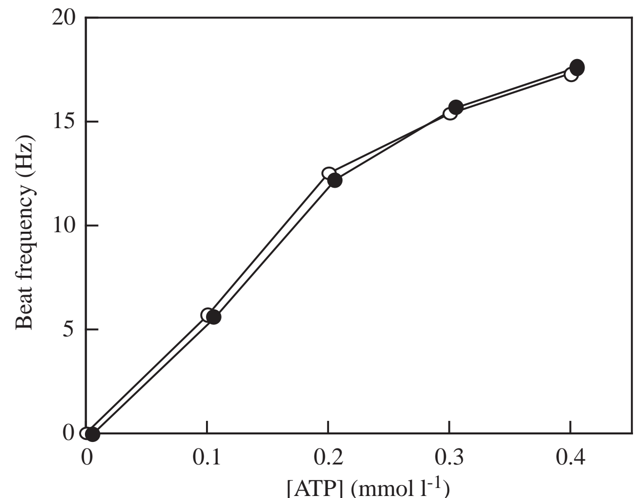


Fig. 4. Direct confirmation of estimated beat frequency. A series of beat frequencies obtained from fast Fourier transform (FFT) spectra was confirmed by directly counting the beat cycle frame by frame on videotape. Results from the two methods coincided well. ○, data from FFT; ●, values obtained from direct counting.

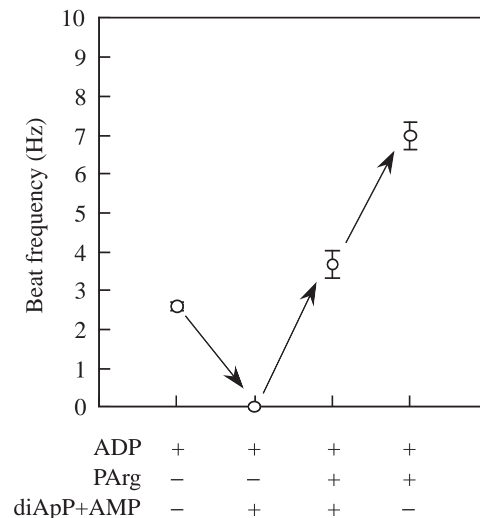


Fig. 5. Effects of an adenylate kinase inhibitor on the beat frequency of reactivated cilia. Ciliated sheets were perfused successively with reactivation solutions containing the test substances indicated. The symbols + and − indicate the presence or absence of the substance shown on the left. Arrows in the figure show the sequence of perfusion. Ciliary beating induced by 0.2 mmol l^{-1} ADP was completely inhibited by the adenylate kinase inhibitor (diApP; 10 µmol l^{-1}). Phosphoarginine (PArg; 0.4 mmol l^{-1}) restored ciliary beating in spite of the presence of diApP. Removal of the inhibitor induced a further increase in beat frequency. Values are means ± S.E.M. (N=6).

resumed beating upon further addition of phosphoarginine. Removal of the adenylate kinase inhibitor further increased the beat frequency.

Effects of demembranation on the beat frequency

Reactivation solutions containing ATP flowed directly into demembranated ciliary axonemes when the cortical sheets were prepared from the demembranated cell model (Noguchi et al., 1991; Noguchi et al., 1993; Noguchi et al., 2000; Okamoto and Nakaoka, 1994a; Okamoto and Nakaoka, 1994b). In that case, the phosphoarginine shuttle system was no longer active because arginine kinase had been extracted by Triton X-100 and had been washed out. When the cilia on the intact cortical sheet were reactivated by ATP, further addition of phosphoarginine significantly increased the beat frequency (Fig. 6A). Removal of phosphoarginine decreased the beat frequency to the control level in the presence of ATP

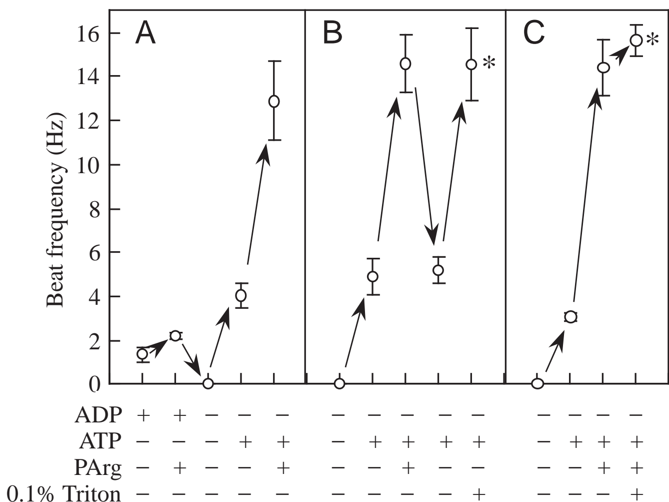


Fig. 6. Beat frequencies of reactivated cilia on the cortical sheets under various conditions. Ciliated sheets were perfused successively with reactivation solutions containing the test substances indicated. The symbols + and − indicate the presence or absence of the substance shown on the left. Arrows show the sequence of perfusion. (A) An increase in beat frequency in response the addition of phosphoarginine (PArg; 0.4 mmol l^{-1}). Both in the presence of 0.1 mmol l^{-1} ADP and in the presence of 0.1 mmol l^{-1} ATP, phosphoarginine increased the beat frequency. Phosphoarginine increased the beat frequency more effectively in the presence of ATP. (B) Effects of demembranation on the beat frequency. Beat frequency was restored to that induced by phosphoarginine after demembranation with 0.1 % Triton X-100 (indicated by an asterisk in B) in spite of the absence of phosphoarginine. (C) Effects of demembranation in the presence of phosphoarginine. In this case, the beat frequency did not increase after demembranation (indicated by an asterisk in C) and remained at the same level as that induced by phosphoarginine before demembranation. These results indicate that the intraciliary concentration of ATP was maintained at an adequate level by the ATP-regenerating system (phosphoarginine shuttle system) in the half-closed space enclosed by the ciliary membrane. Values are means ± S.E.M. (N=5).

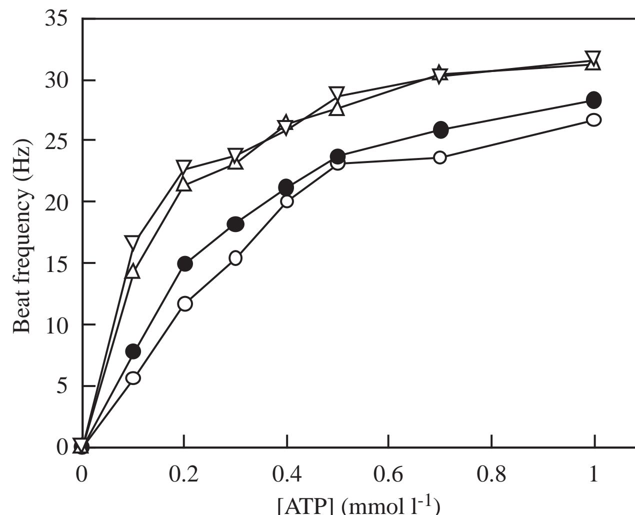


Fig. 7. Effects of phosphoarginine concentration on the beat frequency of cilia reactivated with ATP. Beat frequency was measured in the presence of various concentrations of phosphoarginine. In the presence of a relatively low concentration of ATP, phosphoarginine was more effective. ○, 0 mmol l^{-1} phosphoarginine; ●, 0.2 mmol l^{-1} phosphoarginine; △, 0.4 mmol l^{-1} phosphoarginine; ▽, 0.6 mmol l^{-1} phosphoarginine. Values are representative results of several experiments.

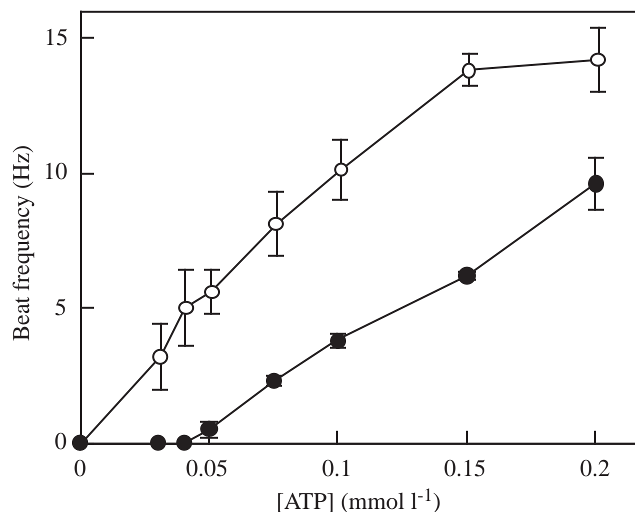


Fig. 8. Effects of phosphoarginine concentration on the beat frequency of cilia reactivated with low concentration of ATP. Beat frequency was measured in the absence (●) and in the presence (○) of 0.4 mmol l^{-1} phosphoarginine. In the presence of a low concentration of ATP, beat frequency was markedly increased by phosphoarginine . Values are means ± S.E.M. (N=5–12).

alone (Fig. 6B). Subsequent addition of Triton X-100 (indicated by an asterisk in Fig. 6B) restored the beat frequency to a level comparable with that induced by the addition of phosphoarginine. In contrast, when demembranation was performed in the presence of phosphoarginine, the beat frequency did not change after demembranation (Fig. 6C).

Effects of ATP and phosphoarginine concentration on the beat frequency

The effect of phosphoarginine concentration on the beat frequency was examined in the presence of various concentration of ATP. As shown in Fig. 7, phosphoarginine effectively increased the beat frequency, particularly in the presence of relatively low concentrations of ATP. Phosphoarginine had a measurable effect at 0.4 mmol l^{-1}. At concentrations greater than 0.4 mmol l^{-1}, phosphoarginine had no further stimulatory effect (0.6 mmol l^{-1} phosphoarginine in Fig. 7). The effect of phosphoarginine concentration in the presence of a low ATP concentration is shown in Fig. 8. In the absence of phosphoarginine, ATP did not reactivate the cilia when the concentration was below 0.05 mmol l^{-1}. Addition of 0.4 mmol l^{-1} phosphoarginine, however, induced ciliary beating in the presence of only 0.03 mmol l^{-1} ATP. Thus,

phosphoarginine was particularly effective in the presence of low ATP concentrations.

Discussion

In the cilia and flagella of protists, the presence of arginine kinase activity has been reported for *Tetrahymena pyriformis* (Watts and Bannister, 1970). However, neither arginine kinase nor creatine kinase activity has been detected in the flagella of *Chlamydomonas reinhardtii* (Watanabe and Flavin, 1976). In the present study, we detected arginine kinase activity in the cilia of *Paramecium caudatum*, and activity was localized to the membrane-plus-matrix fraction (Fig. 1B). In contrast, the more ubiquitous ATP-regenerating enzyme adenylate kinase was found in both axonemal and membrane-plus-matrix fractions (Fig. 1A). Hyams and Borisy (Hyams and Borisy, 1978), using an isolated flagellar apparatus, demonstrated that ADP could reactivate the flagella of *Chlamydomonas reinhardtii* and showed that adenylate kinase regenerates ATP.

In *Paramecium caudatum*, arginine kinase was found in the cilia (Fig. 1B). This suggests that phosphoarginine works not only as a ‘temporal energy buffer’ but also as an ‘energy transport substance’, supplying high-energy phosphate from the basal opening of the cilium to the ciliary tip through the narrow intraciliary space (Wallimann et al., 1992). Subcellular compartmentation, such as occurs in eukaryotic cilia and flagella, must require an energy transport system to direct energy from the mitochondria to sites of ATP consumption. It has been established that phosphocreatine is synthesized in the mitochondria (Wallimann et al., 1992). In *Paramecium caudatum*, phosphoarginine produced by arginine kinase in the mitochondria of the cell body is thought to diffuse into the cilia from the opening of the ciliary base, as postulated for sperm flagella (Tombes and Shapiro, 1985; Strong and Ellington, 1993). In contrast, ATP, which is the substrate of dynein ATPase and the actual energy source for ciliary beating, does not need to diffuse into the cilia. Hydrolyzed ATP must be immediately recycled by ciliary arginine kinase to be rephosphorylated by phosphoarginine. Thus, the intraciliary concentration of ATP is maintained throughout the length of the cilium.

If the phosphoarginine shuttle really works in *Paramecium caudatum* cilia, the cytosolic concentration of phosphoarginine must be sufficient to support ciliary beating. We do not know, however, the exact intraciliary and cytosolic concentrations of ATP, phosphoarginine and related substances. Only a few estimations of intracellular ATP concentration have been performed. The intracellular ATP concentration of *Paramecium caudatum* was estimated to be $0.87\,\mathrm{mmol\,l^{-1}}$ (Naitoh and Kaneko, 1973). The intracellular ATP concentration of *Paramecium tetraurelia* was estimated to be $1.25\,\mathrm{mmol\,l^{-1}}$ (Matt et al., 1978) and $1.1\,\mathrm{mmol\,l^{-1}}$ (Lumpert et al., 1990) using cell volumes of 1.2×10^{-7} ml and 10^{-7} ml, respectively. However, to our knowledge, there are no reports of phosphoarginine concentration. We estimated the intracellular phosphoarginine concentration by extracting phosphoarginine from live *Paramecium caudatum*. The estimated concentration was approximately $0.4\,\mathrm{mmol\,l^{-1}}$. If this is the case, approximately $0.4\,\mathrm{mmol\,l^{-1}}$ phosphoarginine should be sufficient to support ciliary beating.

To prove that the phosphoarginine shuttle system really works in cilia, we developed a new model system. We prepared cortical sheets (Noguchi et al., 1991; Noguchi et al., 1993; Noguchi et al., 2000) from demembranated *Paramecium caudatum* (Noguchi et al., 1986). In the demembranated cilia, however, the phosphagen transport system had been destroyed by a detergent when the cilia were reactivated. A series of studies on exocytosis has also been performed by Plattner’s group using intact cortex fragments without detergents (Vilmart-Seuwen et al., 1986; Lumpert et al., 1990). To keep the phosphagen transport system intact, we successfully prepared ciliated cortical sheets from live *Paramecium caudatum* without using detergents.

The cilia of the intact cortical sheet could be reactivated (Fig. 2). Ciliary beat frequency, an index of ATP regeneration, was measured using a custom-designed detector. The recorded signal coincided well with the ciliary beat cycle (Figs 3, 4). Using intact cortical sheets, we could observe directly the ciliary beat induced by the artificially manipulated cytosolic concentration of phosphoarginine and ATP near the basal opening of the cilia. The measured beat frequency was used to provide evidence for the phosphoarginine shuttle system, as discussed below.

The cilia on the intact sheet were reactivated by perfusing ADP instead of ATP (Fig. 5). In the case of the isolated flagellar apparatus from *Chlamydomonas reinhardtii*, ADP reactivated flagellar beating (Hyams and Borisy, 1978). *Chlamydomonas reinhardtii* flagella contain adenylate kinase, but neither arginine kinase nor creatine kinase (Watanabe and Flavin, 1976). The reactivation of isolated flagella must therefore be due solely to ATP regenerated by adenylate kinase. Adenylate kinase was also found in *Paramecium caudatum* cilia (Fig. 1A). Therefore, the reactivation of cilia by ADP was thought to be induced by ATP generated by adenylate kinase from two molecules of ADP. Complete inhibition of ciliary beating in response to the addition of diApP (an adenylate kinase inhibitor) supported this argument (Fig. 5). The resumption of ciliary beating in response to the addition of phosphoarginine indicates that the generation of ATP by arginine kinase from phosphoarginine and ADP is effective and that the ATP-regenerating system works independently from the adenylate kinase system. The additional increase in beat frequency in response to the removal of diApP indicates that the two ATP-regenerating systems work simultaneously.

The presence of adenylate kinase activity in cilia has been reported in one other ciliate, *Tetrahymena pyriformis* (Otokawa, 1974; Nakamura et al., 1999). The difference between *Chlamydomonas reinhardtii* and these ciliates indicates that there are at least two groups of energy-supplying mechanisms involved in ciliary and flagellar movement in protists. One group supplies ATP by diffusion from the

flagellar basal opening to the intraflagellar matrix and regenerates ATP using only adenylate kinase, as in *Chlamydomonas reinhardtii* flagella. The other group supplies phosphoarginine, instead of ATP, from the ciliary basal opening to the intraciliary matrix and regenerates ATP using arginine kinase. In the latter case, adenylate kinase may balance the equilibrium between ATP, AMP and ADP concentrations.

To examine the efficiency of the phosphoarginine shuttle system in supplying ATP, we demembranated a reactivated cortical sheet by perfusing a reactivation solution containing Triton X-100. When the cilia on the intact cortical sheet were reactivated with ATP, instead of ADP, a higher ciliary beat frequency was induced than that induced by ADP. In this case, phosphoarginine was more effective than in the case of ADP in increasing ciliary beat frequency (Fig. 6A). When cortical sheets were perfused with the reactivation solution containing Triton X-100 in the absence of phosphoarginine, the beat frequency increased threefold from the level before demembranation and the frequency was restored to that in the presence of phosphoarginine (indicated by an asterisk in Fig. 6B). This indicates that the ciliary axonemes obtain ATP directly from the reactivation solution around the demembranated axonemes instead of by diffusion from the opening of the ciliary base. In this case, the phosphoarginine shuttle system is no longer of use. When similar demembranation was performed in the presence of phosphoarginine, the ciliary beat frequency did not change after the demembranation (indicated by an asterisk in Fig. 6C).

Addition of phosphoarginine increased the beat frequency of the cilia on the intact cortical sheets, particulary in the presence of relatively low concentrations of ATP (Figs 7, 8). The intracellular concentration of ATP was estimated to be $0.87\,\mathrm{mmol\,l^{-1}}$ (Naitoh and Kaneko, 1973) for *Paramecium caudatum* and $1.25\,\mathrm{mmol\,l^{-1}}$ (Matt et al., 1978) or $1.1\,\mathrm{mmol\,l^{-1}}$ (Lumpert et al., 1990) for *Paramecium tetraurelia*. The intraciliary concentration of ATP, however, is thought to be lower than this and was estimated to be $0.13\,\mathrm{mmol\,l^{-1}}$ in the cilia of *Paramecium caudatum* and $0.16\,\mathrm{mmol\,l^{-1}}$ in the cilia of *Tetrahymena pyriformis* (Tanaka and Miki-Noumura, 1988). Although we do not know the exact cytosolic and intraciliary concentrations of ATP and phosphoarginine, the observation that phosphoarginine is remarkably effective when the artificially controlled cytosolic ATP concentration is low (Fig. 8) is quite reasonable. ATP may be rapidly and continuously consumed in the cilia. To avoid the depletion of energy, an efficient supply of ATP is required. Phosphoarginine, just like phosphocreatine in vertebrate muscle, may therefore be a relevant means of energy supply in the narrow space of a cilium. The estimated intracellular concentration of $0.4\,\mathrm{mmol\,l^{-1}}$ phosphoarginine supported ciliary beating well (Figs 7, 8). Thus, phosphoarginine, rather than ATP, can supply high-energy phosphate by diffusing from the cell body to the intraciliary matrix in *Paramecium caudatum* cilia.

For muscle and other cells, a phosphocreatine shuttle or circuit has been proposed to direct energy from the mitochondria to sites of ATP consumption (Bessman and Geiger, 1981; Quest and Shapiro, 1991; Quest et al., 1992; Wallimann et al., 1992; Kaldis et al., 1996). Support for the existence of such a phosphocreatine shuttle has been found in sea urchin sperm, for example, where a creatine-kinase-deficient phenotype was mimicked by specific inhibition of creatine kinase with 1-fluoro-2,4-dinitrobenzene (FDNB; Tombes and Shapiro, 1985; Tombes and Shapiro, 1987; Tombes et al., 1987). The intact cortical sheet provides an excellent system in which to study the relevance of a phosphoarginine shuttle to cellular functions and directly supports the presence of such a shuttle.

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