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PHYSIOLOGICAL REQUIREMENTS FOR CILIARY REACTIVATION OF BRACKEN FERN SPERMATOZOIDS

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SUMMARY

Physiological parameters affecting reactivated ciliary beat in spermatozoids of bracken fern (*Pteridium aquilinum*) were studied using a Triton/glycerol permeabilized cell model system. Reactivation frequencies of polylysine-tethered cells equalled *in vivo* rates at neutral pH. Frequency was dependent on ATP and Mg^{2+} concentration, and reactivation was inhibited by millimolar or greater free calcium. Reactivation was reversibly inhibited by micromolar concentrations of sodium ortho-vanadate, while intact cells were not affected by millimolar levels of the inhibitor. This is the first characterization of *in vitro* ciliary beat in a non-algal plant cell and demonstrates that the nucleotide and ionic requirements for reactivation of bracken cilia are similar to those of other systems.

INTRODUCTION

In the past decade, considerable effort has been directed toward understanding the mechanism of ciliary motility (see review by I. R. Gibbons, 1975). Precise descriptions of the ultrastructural organization of the axoneme during beat cycles have led to the idea that ciliary beat is caused by microtubule sliding interactions (Satir, 1968) mediated through the accessory arms, links and spokes (Summers & Gibbons, 1971; Warner & Satir, 1974; Sale & Satir, 1977; Warner & Mitchell, 1978).

Our understanding of the physiological requirements for ciliary beat has been derived largely from studies of demembranated ciliary models, using detergents or glycerol to alter the permeability properties of the ciliary membrane. Under proper conditions, beat can be restored to the naked axonemes. Reactivated beat *in vitro* has been found to be an ATP- and magnesium-dependent process (Hoffmann-Berling, 1955; Gibbons & Gibbons, 1972; Tsuchiya, 1977; Torres, Renaud & Portocarrero, 1977). Calcium has been found to have variable effects on ciliary beat *in vitro*.

The ubiquity of '9+2' axonemal organization suggests that the molecular mechanism of beat and its physiological requirements and controls may be similar, if not identical, throughout the eucaryotic realm. In testing this hypothesis, we describe here nucleotide and ionic requirements for ciliary reactivation in the morphologically complex, multiciliate male gametes of bracken fern.

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Spermatozoids of bracken fern swim in a helical path, making it impossible to monitor beat cycles of individual cilia for extended periods of time. Tethering of cells to microscope slides with phosphate-buffered polylysine permits observation of a single cilium or adjacent cilia for several minutes, making beat frequency, waveshape, and beat coordination determinations possible. Based on observations of tethered cells, this study shows a marked similarity for ionic and nucleotide reactivation requirements between the non-algal plant cilium and distantly related axonemal systems.

Bracken spermatozoids exhibit classically defined chemotactic behaviour (Pfeffer, 1884; Brokaw, 1974). Since the gametes require calcium to elicit changes in tactic motility (Brokaw, 1974), and change their swimming pattern under varying conditions of chemoattraction (Wolniak, 1978), we have attempted to correlate these behavioural modifications in swimming with *in vitro* ciliary physiology (Wolniak & Cande, manuscript in preparation). Preliminary reports of this work have appeared in abstract form (Wolniak & Cande, 1978*a*, *b*).

MATERIALS AND METHODS

Cell culturing

Approximately 10 mg of spores of bracken fern (*Pteridium aquilinum*) were grown on 50–150 ml of sterile Hoagland's solution (Machlis & Torrey, 1956) with a 16-h photoperiod at 20 °C. Spermatozoids were harvested from 4–12-week-old gametophytes by hypotonic shock. The majority of mature gametes were released in the first 15 min, and remained active for up to 90 min in distilled water.

Preparation of cells and light microscopy

For microscopic observation, swimming spermatozoids were attached to glass slides which had been thinly coated with 0·33 mg/ml poly DL-lysine buffered with 0·067 M potassium phosphate (pH 7·0). Cells tethered by the proximal portion of the cell body usually had cilia whose beat was unhindered by attachment to the substratum. Beat in these cilia was monitored with Zeiss dark-field optics, through a 25 × planopochromatic objective lens (N.A. = 0·65) at a final magnification of 250 times. The dark-field microscope was illuminated by a 150-W Leitz xenon arc lamp, outfitted with a Zeiss wide band-pass green interference filter, a Leitz infrared filter and two 430-nm barrier filters (Leitz), providing uniform yellow-green light. Intense blue light inhibited reactivation of bracken cilia, necessitating the use of this filter combination.

Partial demembranation and subsequent beat reactivation of cilia was induced by passing approximately 50 μl of solution beneath the coverslip past the cells using a bibulous paper wick to facilitate flow. The demembranation solution consisted of: 0·1 M KCl, 1 mM EDTA (1 mM EGTA in experiments with calcium), 4 mM $MgSO_4$, 0·5 mM 2-mercaptoethanol, 5 % (v/v) glycerol, 0·02 % (w/v) Triton X-100, and 20 mM PIPES (piperazine-*N*-*N*′-bis(2-ethanesulphonic acid) (pH 6·94). Contact with this solution caused almost immediate cessation of ciliary beat and blebbing of membranes. The beat reactivation solution consisted of: 0·1 M KCl, 1 mM EDTA (1 mM EGTA in experiments with calcium), 4 mM $MgSO_4$, 0·5 mM 2-mercaptoethanol, 2 % carbowax 20 M (polyethylene glycol, 20000 mol. wt), 1 mM ATP, and 20 mM PIPES (pH 6·94). All experiments were run at 20 °C.

Ciliary activity was recorded microcinematographically, and beat frequencies were determined by sequential stop frame analysis of the films. Beating cilia were filmed at a rate of 24 frames/s with an Opti-Quip 16-mm cine aparatus (Opti-Quip, Inc., Highland Mills, N.Y.), using Kodak RAR no. 2498 film. The film was developed in Diafine and had an effective ASA of 1600. Beat shape was determined by measuring arc displacement at 2 points along the length

of the ciliary shaft. The measurements were made from sequential frame tracings of the movie film and were accurate to 5 degrees in the plane of focus. Comparison of the 2 arcs in a ratio (distal value/proximal value) permits a description of beat shape: equal displacement indicates rod-like beat; distal exceeding proximal displacement indicates whip-like beat; and proximal exceeding distal displacement indicates bow-shaped beat.

Precise levels of free calcium were maintained with a Ca/EGTA buffering system (Table 1), designed to compensate for the presence of Mg^{2+} and 1 mM ATP at a pH of 6·94 (cf. Portzehl, Caldwell & Ruegg, 1964; Brokaw, Josslin & Bobrow, 1974; Zucker & Steinhardt, 1978). Over the effective range of the buffer (10^{-8}–10^{-5} M free Ca^{2+}), the free Mg^{2+} concentration was maintained at 1–1·6 mM (Table 1). Higher Ca^{2+} concentrations were obtained by adding an appropriate excess of $CaCl_2$ over EGTA concentration in the reactivation medium.

For phase-contrast microscopy, spermatozoids were fixed according to the method of Parducz (1967) using OsO_4 and $HgCl_2$. Micrographs were taken on either Kodak Panatomic-X (no. 5060) film and developed in D-76, or on Kodak SO-115 and processed in HC-110.

Table 1. *Recipes for the Ca-EGTA buffer system*

[EGTA], mM	Total [Mg^{2+}] (excess over [ATP]), mM	[Mg^{2+}]free, mM	Total [Ca^{2+}], M	[Ca^{2+}]free, M
1	1·65	1·6	$5·89 \times 10^{-5}$	$3·6 \times 10^{-8}$
1	1·43	1·4	$3·86 \times 10^{-4}$	$3·3 \times 10^{-7}$
1	1·10	1·1	$8·72 \times 10^{-4}$	$3·7 \times 10^{-6}$

Electron microscopy

Spermatozoids were fixed with 2 % glutaraldehyde with 4 mM $MgSO_4$ buffered in 0·1 M sodium cacodylate (pH 7·0). Following a wash with the same buffer, cells were postfixed in 1 % OsO_4, then dehydrated in a graded ethanol series and embedded in Epon-812 (Shell Chemical Co.). Thin sections were stained with 1 % uranyl acetate and 1 % lead citrate and observed with a Siemens Elmiskop 1A electron microscope operated at 80 kV.

RESULTS

Spermatozoids of *Pteridium aquilinum* are spiral-shaped cells with approximately 32 cilia arranged in a single row along the dorsal edge of the cell body (Figs. 1, 2; Duckett, 1975). There is no wall present surrounding the cell (Duckett, 1975).

Morphologically, bracken axonemes appeared to be identical with most other eucaryotic cilia (Fig. 3), except for the apparent absence of outer dynein arms (Fig. 3, arrow) which are present in cilia and flagella of other cell types (Gibbons & Gibbons, 1973). Outer dynein arms were not observed in bracken axonemes prepared under several different conditions of fixation.

Treatment of bracken spermatozoids with the Triton-glycerol extraction solution resulted in at least partial disruption of the ciliary membrane (Fig. 4), and in a few cases, total loss of the membrane was observed (Fig. 5). Under the extraction conditions used, virtually all cilia demonstrated some membrane disruption.

When freely swimming, intact spermatozoids had ciliary beat frequencies of approximately 6–8 Hz, which appeared to be unaffected by partial tethering. This slow

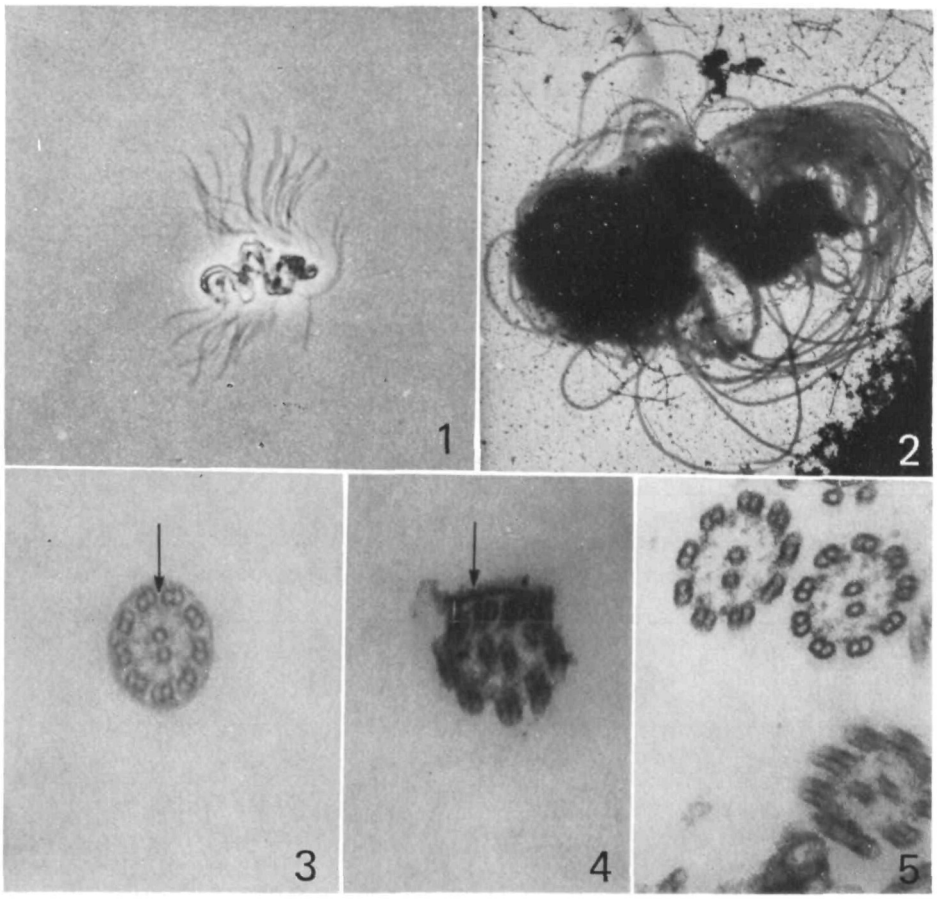

Fig. 1. $HgCl_2$-OsO_4 fixed spermatozoid of *Pteridium aquilinum*. × 1500.

Fig. 2. Whole mount of bracken spermatozoid fixed with 1 % uranyl acetate. × 3000.

Fig. 3. Transverse section of bracken cilium: intact cell. Arrow indicates apparent absence of dynein arm. × 80000.

Fig. 4. Oblique-transverse section of bracken cilium: demembranated cell. Partial disruption of ciliary membrane after exposure to Triton X-100/glycerol demembranating solution is apparent (arrow). Virtually all cilia have some membrane disruption subsequent to this treatment. × 80000.

Fig. 5. Occasionally, in approximately 10 % of cilia examined, total loss of the ciliary membrane has occurred after Triton/glycerol extraction. × 80000.

beat rate was confirmed with high-speed cinematography (50–100 frames/s). Beat shape in freely swimming cells was more like that of a cilium than a flagellum (Fig. 6). The beat cycle displayed a single effective stroke along the entire length of the shaft, followed by a recovery stroke initiated at the proximal end of the axoneme and propagated distally. Tethered intact spermatozoids exhibited a similar beat pattern. The tracings depicting this beat represent a 2-dimensional projection of a motion which is probably 3-dimensional but has not been further analysed.

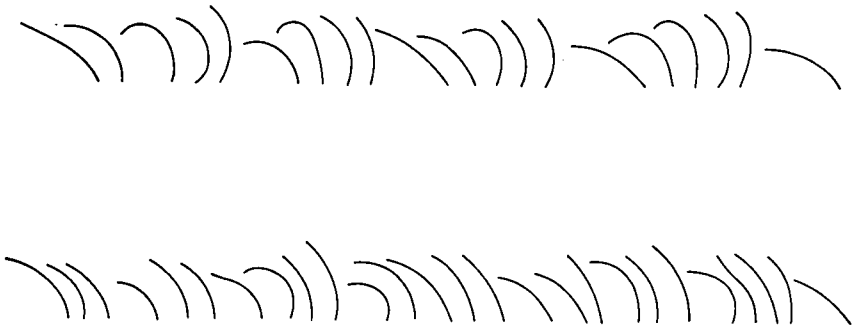

Fig. 6. Sequential frame tracings from a microcinematographic record (24 frames/s) of the same cilium prior to detergent extraction (above) and subsequent to reactivation (below) approximately 2 min later. The spermatozoid was tethered to the microscope slide with phosphate-buffered polylysine as described in the methods section. The cell anterior is on the right.

pH

Reactivation of bracken cilia by ATP and Mg^{2+} was optimal at neutral pH. In 20 mM PIPES buffer (pH 6·94) beat frequency was similar to that of intact cilia, and 50–80 % of the cells were reactivated. Beating of cilia reactivated in potassium phosphate (pH 7·0) or PIPES (pH 6·7) was also approximately equal to *in vivo* beat frequency, although the percentage of cells reactivated and the duration of beat were smaller. At lower or higher pH, the few cells reactivated had lower beat frequencies (1–4 Hz, by visual estimate). Bowlike beat shape was not altered by pH in the reactivation medium.

ATP

Within 15 s after exposure to detergent, ciliary beat ceased, and the cilia remained in a variety of waveforms. Reactivation occurred upon addition of ATP and beat rates approached *in vivo* frequencies at millimolar levels of the nucleotide (Fig. 7). In response to increasing ATP, the cilia demonstrated a minimum threshold concentration of 10 μM for reactivation with this nucleotide.

Reactivated beat was not sustained in permeabilized axonemes in the absence of ATP. Other nucleotides (e.g., ADP, GTP, and the non-hydrolysable ATP analogue, AMPPCP) could not be substituted for ATP in this system. Upon addition of the reactivation solution lacking ATP, there was a very brief burst (1–2 s) of ciliary activity. Addition of ATP to the detergent extraction solution caused a significant increase in beat frequency, to approximately 10 Hz. This increase in beat rate subsided to 6–8 Hz after 3–4 min. No increase in frequency was observed if the metabolic uncouplers DNP (3×10^{-6} M) or CCCP (carbonyl cyanide *m*-chlorophenyl hydrazone) (10^{-6} M) were present with ATP in the detergent extraction solution; beat remained constant at approximately 6–8 Hz. Treatment of intact cells with metabolic uncoupler reversibly inhibited ciliary beat. Attempts at reactivation of cilia previously inactivated

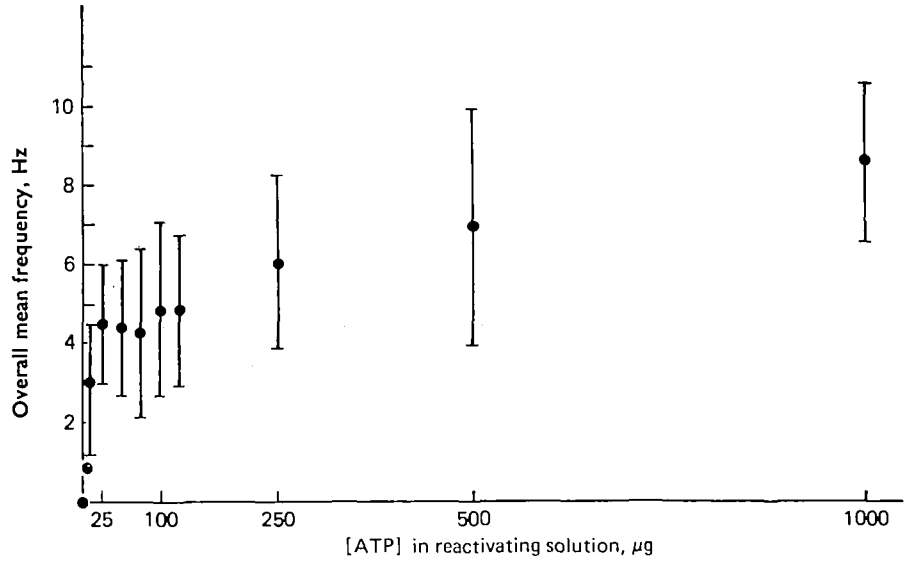

Fig. 7. Effect of changing concentration of ATP on reactivated beat frequency in detergent-permeabilized cilia of bracken fern. Bars represent a 95% confidence interval on either side of the mean. Each mean value represents at least 10 cells.

by uncoupler pretreatment were unsuccessful, even with 3 mM ATP. Cilia beating at the time of detergent extraction were the only cilia we could reactivate.

Divalent cations

Reactivation of bracken fern cilia was magnesium-dependent. There was no beat in the absence of Mg^{2+}. *In vivo* beat rates were approached with Mg^{2+} concentrations in approximately a millimolar excess over ATP (Fig. 8). In response to increasing Mg^{2+} concentration, the cilia demonstrated a minimum threshold concentration for reactivation of approximately 250 μM with this cation (data not shown).

With Mg^{2+} and ATP present, ciliary reactivation occurred within a limited concentration range of free calcium. Between 0·2 and 100 μM free calcium, reactivated beat frequency equalled *in vivo* rates (Fig. 9). Reactivation was not observed if the free calcium concentration exceeded 1 mM (Fig. 9). The effect of free calcium concentration on beat maintained by EDTA was similar to that of EGTA. EGTA was not used in other reaction experiments because the percentage of cells reactivated was reduced to approximately 50% with this chelator.

Beat shape of reactivated cilia did not appear to be under control of Ca^{2+} concentration (Fig. 10); beat shape as assessed by arc displacement ratios was predominantly bowlike under all conditions of reactivation. Although the overall beat cycle was similar in detergent-treated and intact tethered cilia (Fig. 6), intact cilia displayed more beat arc displacement than detergent-treated cilia in any reactivation experiment (Fig. 10).

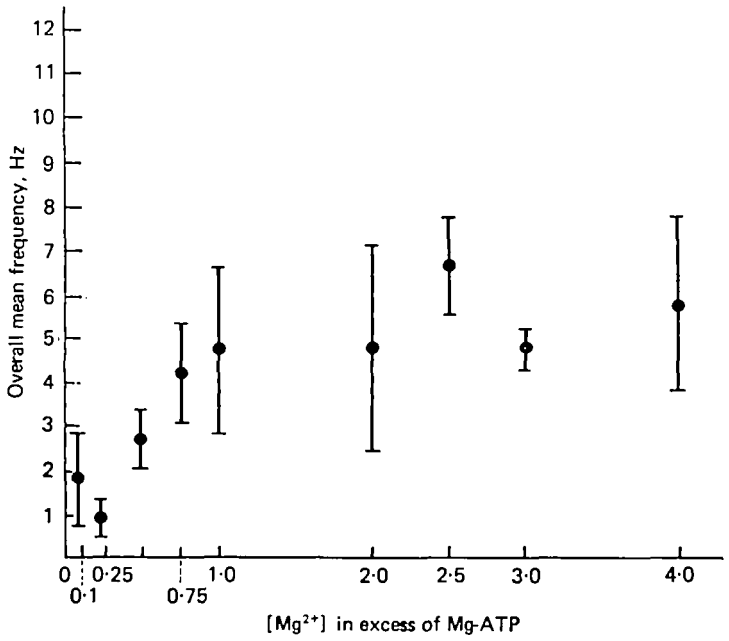

Fig. 8. Effect of changing concentration of magnesium, in excess of [Mg-ATP^{-2}] on reactivation frequency of bracken fern cilia. Bars represent a 95 % confidence interval on either side of the mean. Each value represents at least 10 cells.

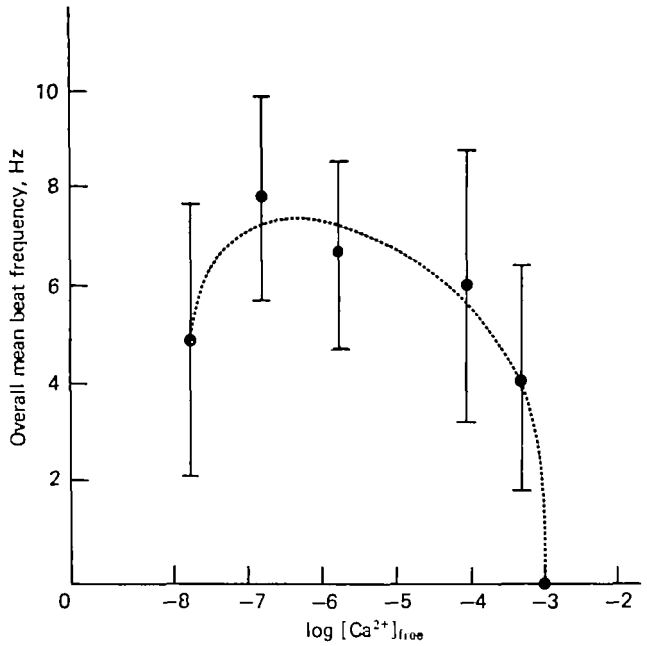

Fig. 9. Effect of changing free calcium concentrations on reactivated beat frequency in detergent-permeabilized cilia of bracken fern. Free calcium levels were maintained in a Ca/EGTA buffer system (see Table 1 and Methods section). Bars represent a 95 % confidence interval on either side of the mean. Each mean value represents at least 10 cells.

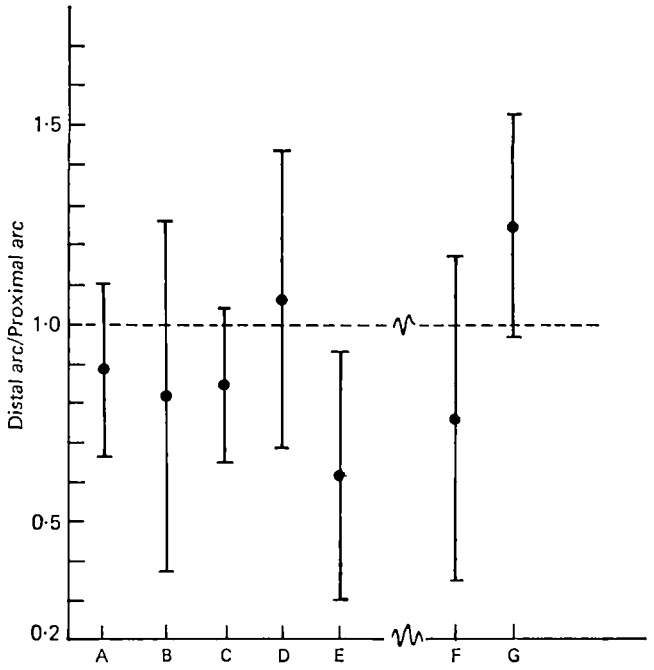

Fig. 10. Comparison of distal/proximal arc displacement by reactivated bracken cilia in the presence of varying $[Ca^{2+}]_{free}$. Ratios of 1·0 reflect rod-like beat shape; values of less than 1·0 reflect bow-shaped beat; values greater than 1·0 reflect whip-like beat shape. Intact cells (far right) have whip-like beat while reactivated cilia typically exhibit bow-shaped beat. Bars represent 95 % confidence intervals on either side of the mean. Each mean determined from at least 4 cells. Treatments: A, reactivation in $3{\cdot}6 \times 10^{-8}$ M free Ca^{2+}; B, reactivation in $3{\cdot}3 \times 10^{-7}$ M free Ca^{2+}; C, reactivation in $3{\cdot}7 \times 10^{-6}$ M free Ca^{2+}; D, reactivation in 10^{-4} M free Ca^{2+}; E, reactivation in 5×10^{-4} M free Ca^{2+}; F, reactivation in the absence of added Ca^{2+}; G, ciliary beat shape of intact cells.

Vanadium inhibition of reactivated beat

The addition of micromolar sodium ortho-vanadate (Vanadium, V^+) to reactivated ciliary models of bracken caused cessation of beat within 1 min, even in the presence of 2 mM ATP. Dilutions with reactivating buffer that lowered the vanadate concentration to levels well below 1 μM, caused restoration of reactivated beat. The beat rates obtained after reversal were equal to those observed prior to vanadate inhibition (6–8 Hz). Reversal of vanadate inhibition also occurred if 1 mM norepinephrine was added to the medium.

Ciliary beat in intact cells was totally unaffected by vanadate, even when the inhibitor was present in the external medium at millimolar concentrations.

DISCUSSION

Demembranation and reactivation

This study represents the first report on *in vitro* ciliary physiology in a non-algal plant cell. Light- and electron-microscopic observations demonstrated that bracken

cilia are extremely sensitive to the detergent extraction solution used in these experiments. Detergent treatment produced at least partial membrane disruption in virtually all cilia, as assessed by serial thin-sectioning techniques. At the light-microscopic level, as the detergent solution diffused beneath the coverslip, there was a wave of ciliary beat cessation accompanied by blebbing of ciliary (and cell) membranes, and the cilia appeared less dense in phase-contrast after extraction. The waveshapes of the arrested cilia are analogous to the 'rigor waves' in permeabilized sea-urchin flagella produced by diluting ATP from the medium (Gibbons & Gibbons, 1974). As in the sea-urchin flagellum, beat could be restored to bracken cilia in rigor by the addition of ATP.

Vanadium in the V^+ oxidation state has been shown to be a potent inhibitor of reactivated beat in sea-urchin flagella and cilia and of ATPase activity in flagellar dynein (Kobayashi, Martensen, Nath & Flavin, 1978; Gibbons *et al.* 1978; Cande & Wolniak, 1978). We find that micromolar vanadate inhibits the beat of demembranated but not intact cilia. The difference in vanadate sensitivity in intact and detergent-treated cells, coupled with the ultrastructural confirmation of partial membrane removal indicates that we have generated a functionally demembranated ciliary model system.

Tethering

In an effort to avoid beat artifacts induced by tethering, measurements were made only on freely beating cilia, whose shafts were completely unattached to polylysine. Beat rates of intact, tethered cells are not significantly different from those of tethered, reactivated cells, and wave-shape measurements of intact tethered cells appear to resemble wave-shape patterns observed in intact non-tethered cells. Direct comparisons of beat shape or frequency between tethered and non-tethered cells could not be made due to rapid cell rotation in the latter, but these parameters appear to be qualitatively similar, suggesting that no major beat artifact is induced by partial tethering. Additional causes of variability in beat rate may arise from suboptimal reactivation conditions, or inherent physiological differences in the cilia themselves, depending on their position in the ciliary row (i.e., posterior cilia may beat differently from anterior cilia; cf. Brokaw, 1963; Parducz, 1967).

The bowshaped pattern of beat in the detergent-permeabilized bracken cilia under conditions optimal for reactivation (Wolniak & Cande, 1978*a*, *b*) was unlike the whip-like pattern generated by intact cilia. Bow-shaped beat in reactivated cilia of bracken could represent apolar beat (Parducz, 1967), caused by either suboptimal reactivation conditions, and/or irreparable damage to the axoneme during extraction. Since the beat shape in intact cells appears to be similar under both tethered and non-tethered conditions, it is unlikely that bowshaped beat is exclusively an artifact of tethering in permeabilized cells.

ATP

The nucleotide specificity, ATP dependency and minimum concentration threshhold for reactivation of bracken cilia are similar to those in other reactivated flagellar

and ciliary models (Allen & Borisy, 1974; Gibbons & Gibbons, 1972; Naitoh, 1969; Tsuchiya, 1977). However, bracken cilia demonstrate half-maximal beat frequency at a relatively low concentration of ATP (25 μm). Whereas other model systems show a pronounced ATP concentration dependence through the range of 25–250 μm ATP, reactivation frequencies in bracken increase only slightly. This may be related to the observed maximal beat rates (6–8 Hz) that are substantially lower than beat rates in other systems (Gibbons & Gibbons, 1972; Naitoh & Kaneko, 1973; Tsuchiya, 1976, 1977) and lower than estimates previously made for bracken (Metzner, 1923). Ultrastructural observations of both intact and permeabilized bracken cilia have failed to demonstrate outer dynein arms on the A-tubules of the axoneme. While there is the possibility of suboptimal fixation procedures, several different techniques produced similar results, and outer dynein arms have not been observed in axonemes of a related organism, the water fern, *Marsilea* (P. K. Hepler, personal communication). Gibbons & Gibbons (1973) demonstrated a positive correlation between reactivated beat frequency and the presence of this component in the axoneme. Reduced beat frequency has also been observed in flagellar mutants of *Chlamydomonas*, which have been shown to lack specific axonemal proteins (Witman, Carlson, Berliner & Rosenbaum, 1972; Luck, Piperno, Ramanis & Huang, 1977; Piperno, Huang & Luck, 1977; Witman, Plummer & Sander, 1978). While it is possible that gametes in this population of plants lack outer dynein arms, polyacrylamide gel electrophoretic analyses of the polypeptides in isolated bracken axonemes failed to show obvious deficiencies of structural components (Wolniak, unpublished observations).

Temporal changes in reactivated beat frequency

The 1–2-s burst of activity by detergent-extracted bracken cilia which occurred with a buffer wash in the absence of ATP was probably caused by hydrolysis of endogenous ATP pools. This view is supported by the absence of the burst when intact cells were pretreated with metabolic uncouplers. Since reactivation could not be achieved in cells which had been pretreated with DNP or CCCP, it is possible that the endogenous pool of ATP, perhaps that which is bound to axonemal proteins, may be required for the successful initiation of beat reactivation. However, uncoupler pretreatment may prevent reactivation through other mechanisms inhibitory to ciliary activity (e.g., release of high concentrations of calcium, protons or mitochondrial release of inorganic phosphate and ADP).

The increase in beat frequency observed during detergent treatment when ATP was added to the extraction solution was probably the result of increased ATP pool size from both exogenous and endogenous sources. The fact that there was a gradual reduction of the beat rate after several minutes of extraction, combined with the observed frequency reduction when metabolic uncouplers were added to the extraction solution with ATP tends to support this hypothesis. These data suggest that beat frequency of bracken cilia both *in vitro* and in intact cells may be limited by the availability of ATP, and may possibly explain the low frequencies observed.

Magnesium

The magnesium requirement for beat reactivation in bracken fern resembles that in other *in vitro* ciliary and flagellar systems (e.g., Gibbons & Gibbons, 1972; Naitoh & Kaneko, 1973; Tsuchiya, 1976). In *Paramecium* reactivation frequency is proportional to Mg^{2+} concentration (Naitoh & Kaneko, 1973) while the frequency curve for bracken fern reactivation tended to level out above a minimal concentration of 0·75–1 mM Mg^{2+} in excess of [Mg-ATP^{-2}]. At very low Mg^{2+} concentrations, reactivation frequency is probably limited by the concentration of Mg-ATP^{-2} rather than the cation itself.

Calcium

Our studies on bracken fern have not demonstrated a clear regulatory function of calcium on reactivated beat frequency. Ciliary reactivation of bracken occurred when free calcium concentrations were below millimolar levels, and in low free calcium, beat frequencies equalled *in vivo* rates. Similarly, reactivated beat of sea-urchin flagella is inhibited by free calcium concentrations in excess of millimolar levels (Gibbons & Gibbons, 1972), but at lower concentrations, changes in the free calcium concentration generally do not affect reactivated beat frequency (Brokaw *et al.* 1974).

Wave shape has been found to be regulated by the concentration of free calcium in the reactivation solution for flagella of sea-urchin sperm (Brokaw *et al.* 1974; Brokaw, 1979) and *Chlamydomonas* (Besson, Fay & Witman, 1978; Hyams & Borisy, 1978). Calcium concentration had no significant effect on reactivated beat shape of bracken fern cilia.

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