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BIOCHEMICAL STUDIES OF THE EXCITABLE MEMBRANE OF *PARAMECIUM*

IV. PROTEIN KINASE ACTIVITIES OF CILIA AND CILIARY MEMBRANE

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Summary

Two protein kinases (ATP: protein phosphotransferase, EC 2.7.1.37) were detected in disrupted cilia of *Paramecium tetraurelia*. One of the enzymes exhibited maximum activity at pH 6.0, required 4 mM Mg^{2+} for its maximum activity and was stimulated by cyclic AMP and cyclic GMP. Histone was a good exogenous protein substrate for this enzyme, but protamine sulfate was not. The other protein kinase showed a peak of activity at pH 8.0, required 10 mM Mg^{2+} for its maximum activity and was slightly inhibited by cyclic AMP and cyclic GMP. Protamine sulfate was a good exogenous substrate for this enzyme. The pH 8.0 activity partitioned preferentially with the axonemes, but the pH 6.0 activity was divided almost equally between the axonemes and the membranes.

We also found indirect evidence for the presence in cilia of phosphoprotein phosphatase (phosphoprotein phosphohydrolase, EC 3.1.3.16) and adenyl cyclase (ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1) activity.

Introduction

Protein phosphorylation has been suggested as a necessary intermediate mechanism for a number of biological functions involving cyclic nucleotides and calcium. A good correlation between cyclic nucleotide effects, flux of Ca^{2+} and changes in protein kinase activity or phosphoproteins has been estab-

Abbreviations: Cyclic GMP, guanosine 3′:5′-cyclic monophosphoric acid; Mops, morpholinopropanesulfonic acid.

lished in several systems, but the existing evidence does not prove that protein phosphorylation occurs in vivo as a necessary step rather than merely a secondary effect [for reviews see Ref. 1–4]. Part of the problem with these systems lies in the difficulty of identifying the function of the phosphoproteins. This problem might be circumvented if mutants, defective in the gating of calcium or insensitive to the effects of calcium observed in wild-type cells, were available. Such mutants do exist in *P. tetraurelia*, a ciliated protozoan which exhibits a Ca^{2+}-dependent action potential. The action potential is concurrent with an influx of Ca^{2+} which causes the cilia briefly to reverse their direction of beat; the cell then swims backwards. Several mutants of *P. tetraurelia* which are defective in ion gating or ciliary reversal have been isolated and characterized by electrophysiology [5,6] and ion-flux studies [7–9].

Cyclic AMP has been shown to stimulate flagellar motility in mammalian sperm [10–12] and inhibit the motility of *Chlamydomonas* flagella [13] and *Tetrahymena* cilia [14]. Cyclic AMP-dependent protein kinase activity has been observed in deciliated bodies of *P. tetraurelia* [15] and in cytosol [16] and cilia [17,18] preparations of *Tetrahymena pyriformis*, a ciliated protozoan which is related to *Paramecium*.

If the mutations affecting ciliary regulation in *Paramecium* could be shown to affect protein kinase activity or phosphoproteins in the cilia, it would be possible to identify the role of protein phosphorylation in membrane excitation or the regulation of ciliary beating.

As a first step in determining the role of protein phosphorylation in membrane excitation and ciliary reversal in *P. tetraurelia*, we have identified at least two protein kinases in the cilia of wild-type paramecia. We concentrated our study on the cilia because they contain the calcium-gating mechanism [19, 20]. Rather than attempting to isolate the protein kinases from the cilia, we decided first to describe their properties as part of a system of enzymes (i.e., kinase, phosphatases, cyclases and phosphodiesterases) in the cilia. One of the protein kinases is cyclic nucleotide dependent and occurs in both the ciliary membrane and axonemes; the other is cyclic nucleotide independent and occurs mainly in the axonemes.

Materials and Methods

[γ-^{32}P]ATP was synthesized by the method described by Schendel and Wells [21]. Cerophyl powder was obtained from Cerophyl Laboratories, Inc., Kansas City, MO. Calf-thymus histone Type B was obtained from Calbiochem. Histone (Type IIA), phosvitin, bovine serum albumin, protamine sulfate (Grade II), ATP, Mops, Tris, 3-isobutyl-1-methylxanthine, indomethacin, cyclic AMP, cyclic GMP and caffeine were obtained from Sigma. All other reagents were of the highest grade available commercially.

Stocks and cultures

P. tetraurelia, wild-type stock 51S (supplied by Dr. Ching Kung), was grown at 28°C in phosphate-buffered Cerophyl medium bacterized with *Enterobacter aerogenes* as previously described [22]. Similar results were obtained if the cells were grown axenically in the Soldo crude medium [23].

Preparation of cilia and subciliary fractions

Cilia were isolated as previously described [22]. The cells were washed five times by centrifugation (200 × *g* for 2 min in an HNS oil testing-centrifuge (IEC))in Dryl's solution (1 mM NaH_2PO_4/1 mM Na_2HPO_4/2 mM sodium citrate/1.5 mM $CaCl_2$ (pH 6.8)) at 4°C. This procedure caused the extrusion of the trichocysts which, along with almost all of the bacteria, were removed during the washes [22]. The cells were then suspended for 15 min in a 1 : 1 mixture of Dryl's solution and STEN (STEN = 0.5 M sucrose/20 mM Tris/2 mM EDTA/6 mM NaCl (pH 7.5)). The cilia were detached by the addition of 0.20 vols. of 60 mM $CaCl_2$/180 mM KCl. This suspension was centrifuged at 850 × *g* for 2 min in the oil centrifuge to pellet the cell bodies. The supernatant was recentrifuged under the same conditions to eliminate any remaining bodies. Practically no cell lysis occurred during this procedure, and the supernatant was a suspension of cilia almost completely free of bacteria and trichocysts [22]. The cilia were removed by centrifuging the supernatant at 28 000 × *g* for 20 min.

If 'whole' cilia were to be assayed, the pellet was washed by centrifugation in 10 mM Mops buffer adjusted to the desired pH of the assay with Tris. The pellet was then vortexed in a small volume of buffer to produce a heterogeneous mixture of broken cilia, membrane vesicles and axonemes. This step was necessary to allow maximum access of the non-permeable reagents used in the assay without adding any solubilizing agents.

For assays of protein kinase in subciliary fractions, membrane vesicles, axonemes and incompletely demembranated cilia were isolated as previously described [22] with a few slight modifications. The initial 28 000 × *g* pellet was vortexed in 1 mM Tris/0.1 mM EDTA (pH 8.3) and centrifuged at 48 000 × *g* for 30 min. This pellet was resuspended in 10 mM Mops/5.5 mM Tris, pH 7.0, at a concentration of 3—6 mg protein · ml^{-1} and was layered on a 20/45/55/65% (w/w) sucrose step gradient in 10 mM Mops, 5.5 mM Tris (pH 7.0). The gradient was centrifuged in a Beckman SW50.1 rotor at 45 000 rev./min for 1.5 h at 4°C. Membranes were collected from the 45% layer; incompletely demembranated cilia were collected from the 55% layer; axonemes were collected from the interface of the 55 and 66% layers of sucrose. The fractions were washed by centrifugation in 10 mM Mops adjusted to the desired pH of the assay with Tris.

Protein determinations were by the method of Lowry et al. [24] using bovine serum albumin as a standard.

Protein kinase assay

The standard assay mixture contained 45—520 μg of cilia protein in a final volume of 0.2 ml of 10 mM Mops (adjusted to the desired pH with Tris), 4 or 10 mM $MgCl_2$ and any additional substrates or reagents required for the assay. The reaction was initiated by the addition of 0.1 μCi of [γ-^{32}P]ATP adjusted to a specific activity of 0.1 μCi/200 nmol. The final concentration of ATP was 1 mM unless otherwise noted. After a 15 min incubation at 28°C, the reaction was terminated by the addition of 0.2 ml 20% trichloroacetic acid at 4°C. The precipitate was trapped on a Whatman GF/A glass fiber filter (which had been presoaked in 1 M NaH_2PO_4) and washed three times with 20

ml 10% trichloroacetic acid at 4°C. The filters were counted in water by Cerenkov radiation [25] in a liquid scintillation counter. Background counts (usually 300—600 counts) were determined by using boiled cilia in a concurrent assay and were subtracted from the experimental counts. 1 unit of enzyme activity was defined as the amount of enzyme which catalyzed the transfer of 1 nmol of phosphate from ATP to substrate protein in 1 min at 28°C. The specific activity of the protein kinase was expressed as units of activity per mg of protein.

Unless otherwise indicated, the figures show the results of a typical experiment. The extremes of the error bars are the values of identical tubes in the assay; the curve is drawn through the average of the two values.

Results

General properties of the ciliary protein kinase

The pH at which maximum protein kinase activity was obtained was first determined using only endogenous proteins as substrates for phosphorylation. Within the pH range tested (pH 6.0—8.5) the highest activity was observed at pH 6.0, but a second, smaller peak was often seen at pH 7.5 (Fig. 1).

Although our major interest was centered on the endogenous substrates of the kinases, we found that incorporation of counts was limited by the small amount of protein substrate available in cilia. We tried to avoid this limitation by using exogenous protein substrates which would still allow us to distinguish between the activities at different pH values. Of several proteins tested, calf-

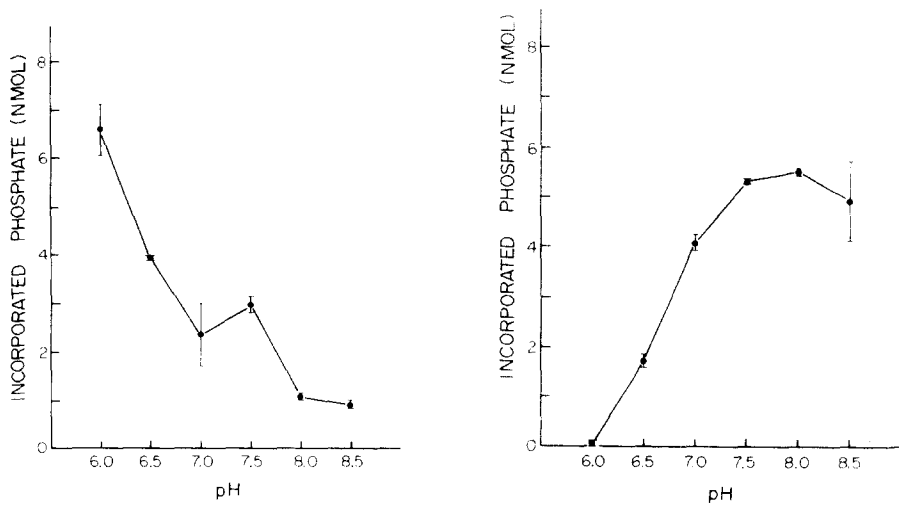

Fig. 1. pH optimum of ciliary protein kinase with cilia protein substrate. The enzyme activity was measured with 520 μg cilia protein in 0.2 ml of 10 mM Mops (titrated to the desired pH with Tris) containing 4 mM $MgCl_2$, 1 mM ATP (1 μCi/200 nmol) and 5 mM NaF.

Fig. 2. pH optimum of ciliary protein kinase with protamine sulfate as the protein substrate. The enzyme activity was measured with 220 μg cilia protein in 0.2 ml of 10 mM Mops (titrated to the desired pH with Tris) containing 4 mM $MgCl_2$, 1 mM ATP (0.1 μCi/200 nmol), 5 mM NaF and 0.5 mg protamine sulfate.

TABLE I

SUBSTRATE SPECIFICITY OF THE pH 6.0 PROTEIN KINASE IN CILIA

The enzyme activity was measured with 350 μg cilia protein in 0.2 ml 10 mM Mops, 1.5 mM Tris (pH 6.0) containing 4 mM $MgCl_2$, 1 mM ATP (0.1 μCi/200 nmol) and 0.5 mg substrate protein. No additional protein was added to the assay for endogenous ciliary proteins.

Substrate	Relative activity (%)
Histone (Type B)	485
Histone (Type IIA)	167
Endogenous cilia proteins	(100)
Phosvitin	93
Bovine serum albumin	63
Protamine sulfate	19

thymus histone Type B was the best exogenous substrate for the protein kinase at pH 6.0 (Table I). Protamine sulfate was not a good substrate at pH 6.0 and in fact inhibited phosphorylation of endogenous substrate, but protamine sulfate was a good substrate at more basic pH values. If the pH optimum was determined with protamine sulfate as the substrate, a single peak of activity occurred at pH 8.0 (Fig. 2). Since the level of activity with protamine sulphate at pH 7.5 was similar to that at pH 8.0, we assumed that it was the same enzyme observed as the small peak of activity at pH 7.5 with endogenous substrates.

Thus it appeared that there were at least two protein kinases in cilia: one which operated maximally at pH 6.0, with histone as an exogenous substrate and one which operated maximally at pH 8.0, with protamine sulfate as an exogenous substrate.

The specific activity of both the pH 8.0 and pH 6.0 protein kinases increased with increasing concentrations of ATP (Table II). (Concentrations of ATP greater than $1 \cdot 10^{-3}$ M were not tested because a precipitate formed). No measurable pH 6.0 protein kinase activity was detected within 15 min with less than $1 \cdot 10^{-5}$ M ATP. We made no attempt to derive kinetic parameters from these experiments because the cilia contain an ATPase (ATP phosphohydrolase,

TABLE II

EFFECT OF [ATP] ON THE CILIARY PROTEIN KINASE ACTIVITIES

The enzyme activity at pH 8.0 was measured with 210 μg cilia protein in 0.2 ml of 10 mM Mops 17.5 mM Tris (pH 8.0) containing 10 mM $MgCl_2$, 0.5 mg of protamine sulfate, 0.1 μCi [γ-^{32}P] ATP (0.1 μCi/0.017 nmol). The enzyme activity at pH 6.0 was measured with 200 μg cilia protein in 0.2 ml of 10 mM Mops 1.5 mM Tris (pH 6.0) containing 4 mM $MgCl_2$, 0.5 mg of histone Type B and 0.1 μCi [γ-^{32}P] ATP (0.1 μCi/0.017 mol). The reaction mixtures were adjusted to the desired [ATP] with unlabeled ATP. The specific activity is expressed as nmol phosphate transferred per mg protein per min.

[ATP] (M)	Specific activity	
	pH 8.0	pH 6.0
$1.9 \cdot 10^{-7}$	$1.5 \cdot 10^{-4}$	0
$1.1 \cdot 10^{-6}$	$1.0 \cdot 10^{-3}$	0
$1.0 \cdot 10^{-5}$	$3.6 \cdot 10^{-2}$	$5.7 \cdot 10^{-3}$
$1.0 \cdot 10^{-4}$	0.95	0.15
$1.0 \cdot 10^{-3}$	7.5	0.98

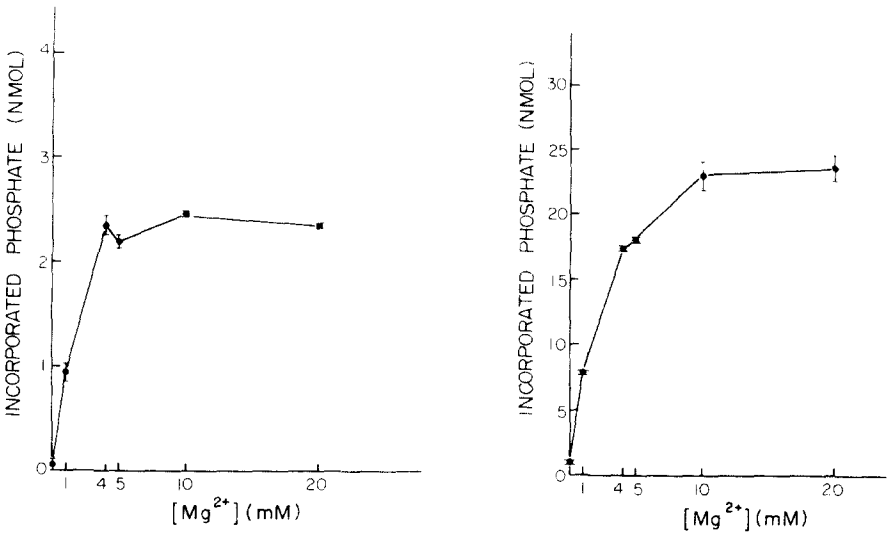

Fig. 3. Mg^{2+} requirement of the pH 6.0 ciliary protein kinase. The enzyme activity was measured with 300 μg cilia in 0.2 ml of 10 mM Mops/1.5 mM Tris (pH 6.0) containing 0, 1, 4, 5, 10 or 20 mM $MgCl_2$, 1 mM ATP (0.1 μCi/200 nmol) and 0.5 mg histone Type B.

Fig. 4. Mg^{2+} requirement of the pH 8.0 ciliary protein kinase. The enzyme activity was measured with 320 μg cilia protein in 0.2 ml of 10 mM Mops/17.5 mM Tris (pH 8.0) containing 0, 1, 4, 5, 10 or 20 mM $MgCl_2$, 1 mM ATP (0.1 μCi/200 nmol) and 0.5 mg protamine sulfate.

EC 3.6.1.3) (Riddle, L., Levin, A., Rauh, J. and Nelson, D.L. unpublished data and Andrivon, C. personal communication) and possibly an adenyl cyclase (see below) which would reduce the amount of ATP available to protein kinase. Therefore, we used $1 \cdot 10^{-3}$ M ATP, the highest concentration of ATP that would stay in solution, for the assays.

Both the pH 6.0 and pH 8.0 protein kinases required Mg^{2+} for optimum activity. At pH 6.0, activity increased with increasing concentrations of Mg^{2+} up to 4 mM (Fig. 3), and at pH 8.0, activity increased with up to 10 mM Mg^{2+} (Fig. 4). Ca^{2+} at concentrations of $1 \cdot 10^{-7}$—$1 \cdot 10^{-4}$ M (added as a $CaCl_2$/EGTA (ethyleneglycol-bis(β-aminoethyl ether)-*N*, *N'*-tetraacetic acid) buffer) had no effect on either the pH 6.0 or pH 8.0 protein kinase (data not shown).

The conversion of ^{32}P from [γ-^{32}P]ATP to trichloroacetic acid-precipitable ^{32}P at pH 8.0 continued for approx. 30 min, then decreased over the next 90 min (Fig. 5). No protease activity has been detected in this preparation of cilia [22]. If 5 mM NaF, an inhibitor of phosphoprotein phosphatase [26], was added to the reaction mixture, the decrease in counts was not observed until after 60 min and was not as rapid (Fig. 5). Higher concentrations of NaF began to decrease protein kinase activity. Sodium molybdate (which is also a phosphoprotein phosphatase inhibitor [26]) was a less effective inhibitor of this decrease, and NaCl at concentrations up to 15 mM had no effect (data not shown). No decrease in counts was observed at pH 6.0, but the initial rate of ^{32}P incorporation was higher if 5 mM NaF was present (Fig. 6). This evidence suggested that the cilia contained a phosphoprotein phosphatase. It is possible that the counts incorporated at pH 6.0 did not decrease by 2 h because the

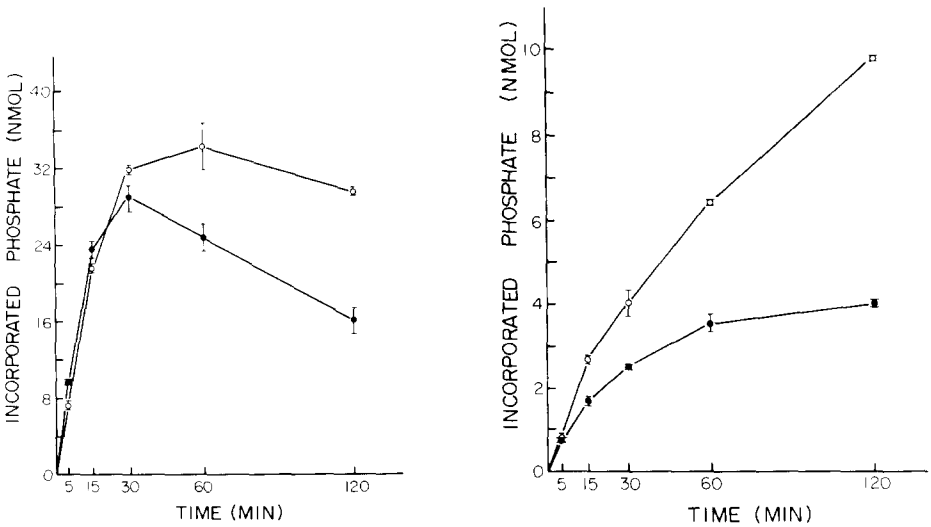

Fig. 5. Time course of pH 8.0 ciliary protein kinase. The enzyme activity was measured with 170 μg cilia protein in 0.2 ml of 10 mM Mops/17.5 mM Tris (pH 8.0) containing 10 mM $MgCl_2$, 1 mM ATP (0.1 μCi/200 nmol) and 0.5 mg protamine sulfate, ●——●, without NaF; ○——○, with 5 mM NaF.

Fig. 6. Time course of pH 6.0 ciliary protein kinase. The enzyme activity was measured with 170 μg cilia protein in 0.2 ml of 10 mM Mops/1.5 mM Tris (pH 6.0) containing 4 mM $MgCl_2$, 1 mM ATP (0.1 μCi/200 nmol) and 0.5 mg histone Type B. ●——●, without NaF; ○——○, with 5 mM NaF.

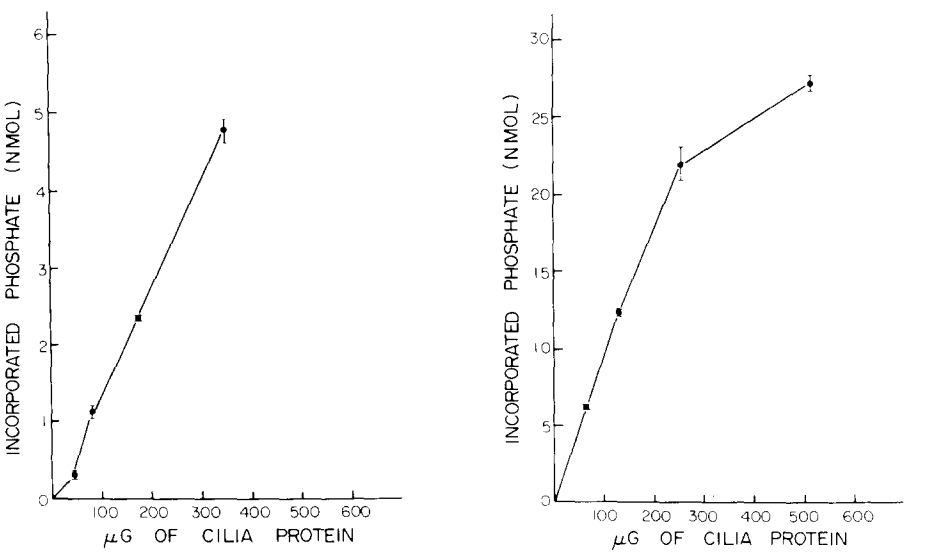

Fig. 7. Relationship of pH 6.0 ciliary protein kinase activity to amount of cilia protein. The enzyme activity was measured with the indicated amount of cilia protein in 0.2 ml of 10 mM Mops/1.5 mM Tris (pH 6.0) containing 4 mM $MgCl_2$, 1 mM ATP (0.1 μCi/200 nmol), 5 mM NaF and 0.5 mg histone Type B.

Fig. 8. Relationship of pH 8.0 ciliary protein kinase activity to amount of cilia protein. The enzyme activity was measured with the indicated amount of cilia protein in 0.2 ml of 10 mM Mops/17.5 mM Tris (pH 8.0) containing 10 mM $MgCl_2$, 1 mM ATP (0.1 μCi/200 nmol), 5 mM NaF and 0.5 mg protamine sulfate.

protein kinase phosphorylated sites on histone which were not accessible to the phosphatase.

In 15 min assays in the presence of 5 mM NaF, the incorporation of ^{32}P at both pH 6.0 and pH 8.0 was nearly linear with amount of added ciliary protein over the range of 50 to 300 μg protein (Figs. 7 and 8). The non-linearity at the highest protein concentrations at pH 8.0 may reflect the activity of phosphoprotein phosphatase which is not completely inhibited by NaF, or it may be a result of the exhaustion of the exogenous substrate, protamine sulfate.

Since our major emphasis in this study was on protein kinase as part of a regulatory system in the cilia, we did not attempt to minimize phosphoprotein phosphatase activity. The changes in phosphorylating activity observed were, therefore, the result of a steady state between protein kinase and phosphoprotein phosphatase.

Effect of cyclic AMP and cyclic GMP on the ciliary protein kinases

The activity of the pH 6.0 protein kinase was stimulated by $1 \cdot 10^{-7}$—$1 \cdot 10^{-4}$ cyclic AMP or cyclic GMP; stimulation by cyclic GMP was slightly higher (Tables III and IV). Cyclic AMP ($1 \cdot 10^{-7}$—$1 \cdot 10^{-4}$ M) caused no further increase in activity when the assay was run in the presence of $1 \cdot 10^{-5}$ M cyclic GMP (data not shown). We concluded that the same enzyme was sensitive to both cyclic nucleotides. To inhibit a cyclic nucleotide phosphodiesterase (3′ : 5′-cyclic nucleotide 5′-nucleotidohydrolase EC 3.1.4.17) in this preparation (Lewis, R.M. and Nelson, D.L., unpublished data), we added 1 mM 3-isobutyl-1-methylxanthine and 1 mM caffeine. The 10—20% increase in enzyme activity seen with 3-isobutyl-1-methylxanthine and caffeine in the absence of added cyclic nucleotides (Tables III and IV) might have been due to production of cyclic AMP by an adenyl cyclase in the cilia or to a pool of cyclic AMP or cyclic GMP in the cilia which had not been completely degraded by phosphodiesterase. Indomethacin, which inhibits a cyclic AMP-dependent protein

TABLE III

EFFECTS OF CYCLIC AMP ON THE pH 6.0 AND pH 8.0 PROTEIN KINASES IN CILIA

The enzyme activity at pH 6.0 was measured with 260—350 μg of cilia protein in 0.2 ml of 10 mM Mops/1.5 mM Tris (pH 6.0) containing 4 mM $MgCl_2$, 1 mM ATP (0.1 μCi/200 nmol, 1 mM caffeine, 1 mM 3-isobutyl-1-methylxanthine and 0.5 mg histone Type B. The enzyme activity at pH 8.0 was measured with 120 μg of cilia protein in 0.2 ml of 10 mM Mops/17.5 mM Tris (pH 8.0) containing 4 mM $MgCl_2$, 1 mM ATP (0.1 μCi/200 nmol, 1 mM caffeine, 1 mM 3-isobutyl-1-methylxanthine and 0.5 mg of protamine sulfate. The control assay contained no caffeine or 3-isobutyl-1-methyl-xanthine and was set as 100% activity. The data for the pH 6.0 protein kinase are expressed as the mean +S.E. (n = 4). The data for the pH 8.0 protein kinase are from a typical experiment. All experiments included duplicate tubes for each assay.

[Cyclic AMP] (M)	Relative activity (%)	
	pH 6.0	pH 8.0
Control	(100)	(100)
0	116 ± 3	91
$1 \cdot 10^{-7}$	123 ± 5	93
$1 \cdot 10^{-6}$	136 ± 11	84
$1 \cdot 10^{-5}$	189 ± 15	87
$1 \cdot 10^{-4}$	189 ± 20	87

TABLE IV

EFFECTS OF CYCLIC GMP ON THE pH 6.0 AND pH 8.0 PROTEIN KINASES IN CILIA

The enzyme activity was measured as described in Table III. The pH 6.0 assay contained 320—400 μg cilia protein. The data are expressed as mean ± S.E. (n = 3). The pH 8.0 assay contained 130 μg cilia protein.

Cyclic GMP (M)	Relative activity (%)	
	pH 6.0	pH 8.0
Control	(100)	(100)
0	120 ± 9	94
$1 \cdot 10^{-7}$	124 ± 7	94
$1 \cdot 10^{-6}$	171 ± 37	85
$1 \cdot 10^{-5}$	239 ± 17	90
$1 \cdot 10^{-4}$	229 ± 18	90

kinase of rabbit ileal mucosa [27], had no effect at concentrations of $1 \cdot 10^{-9}$—$1 \cdot 10^{-6}$ M on the pH 6.0 protein kinase either in the presence or absence of $1 \cdot 10^{-5}$ M cyclic AMP (data not shown).

Cyclic AMP or cyclic GMP at concentrations of $1 \cdot 10^{-7}$—$1 \cdot 10^{-4}$ M caused a slight decrease in the pH 8.0 protein kinase activity (Table III and IV). Indomethacin had no effect on the pH 8.0 protein kinase (data not shown).

Subciliary distribution of the protein kinases

Whole cilia were fractionated into membrane vesicles, axonemes and incompletely demembranated cilia; the latter consist of a broken axoneme coiled inside a membrane [22]. Protein kinase activity was found in all three fractions. Differences in specific activities from preparation to preparation were

TABLE V

DISTRIBUTION OF THE pH 6.0 PROTEIN KINASE IN SUBCILIARY FRACTIONS. EFFECTS OF CYCLIC NUCLEOTIDES

Cilia (4—7 mg) were fractionated into membranes, axonemes, and incompletely demembranated cilia (IDC) as described in the Materials and Methods section. Aliquots of each fraction were assayed for protein kinase activity in 0.2 ml of 10 mM Mops/1.5 mM Tris (pH 6.0) containing 4 mM $MgCl_2$, 1 mM ATP (0.1 μCi/200 nmol) and 0.5 mg histone Type B. If $1 \cdot 10^{-5}$ M cyclic AMP was added, the reaction mixture also contained 1 mM caffeine and 1 mM 3-isobutyl-1-methylxanthine. The values for cilia were for cilia vortexed in 1 mM Tris 0.1 mM EDTA (pH 8.3), and washed as described in the Materials and Methods section. The values for percentage of total activity were calculated from the specific activity and total protein yield for each fraction; the value for the total activity in the cilia before fractionation was set as 100%. Specific activity is expressed as nmol of phosphate transferred per mg of protein per min. Data are expressed as the mean ± S.E. The total number of experiments (n) is given at the top of each column; each experiment included duplicate tubes for each assay.

Fraction	I. % of total protein (n = 12)	II. No addition (n = 10)		III. $1 \cdot 10^{-5}$ M cyclic GMP (n = 3)		IV. $1 \cdot 10^{-5}$ M cyclic AMP (n = 5)	
		% of total activity	Specific activity	% of total activity	Specific activity	% of total activity	Specific activity
		a	b	a	b	a	b
Cilia	(100)	(100)	0.70 ± 0.14	(100)	1.02 ± 0.13	(100)	1.31 ± 0.28
Membranes	31 ± 2	24 ± 2	0.64 ± 0.12	17 ± 4	0.56 ± 0.14	19 ± 4	0.86 ± 0.19
Axonemes	8 ± 1	4 ± 1	0.51 ± 0.19	4 ± 1	0.49 ± 0.17	5 ± 2	0.71 ± 0.09
IDC	44 ± 4	33 ± 4	0.57 ± 0.10	34 ± 8	0.73 ± 0.11	25 ± 3	0.95 ± 0.20

TABLE VI

DISTRIBUTION OF THE pH 8.0 PROTEIN KINASE IN SUBCILIARY FRACTIONS

Cilia were fractionated into membranes, axonemes, and incompletely demembranated cilia (IDC) as described in the Materials and Methods section. Protein yields for each fraction were similar to those in Table V. Aliquots of each fraction were assayed for protein kinase activity in 0.2 ml of 10 mM Mops/17.5 mM Tris (pH 8.0) containing 10 mM $MgCl_2$, 1 mM ATP (0.1 μCi/200 nmol) and 0.5 mg protamine sulfate. The values for cilia were for cilia vortexed in 1 mM Tris/0.1 mM EDTA (pH 8.3) and washed as described in the Materials and Methods section. The values for percentage of total activity were calculated from the specific activity and total protein yield for each fraction; the value for the total activity in the cilia before fractionation was set as 100%. Specific activity is expressed as nmol phosphate transferred per mg protein per min. The data given are from two different experiments; each experiment included duplicate tubes for each assay.

Fraction	% of total activity [a]	Specific activity [b]
Cilia	(100)	3.40, 6.38
Membranes	4, 8	0.39, 1.49
Axonemes	8, 6	2.28, 3.82
IDC	33, 53	2.07, 5.24

great enough to make comparisons between the fractions difficult (see Discussion), but the relative amounts of total activity in each fraction were reproducible (Tables V and VI).

Although 31% of the total protein was recovered in the membranes (Table V, column I), only4—8% of the total pH 8.0 protein kinase activity was found in the membranes (Table VI, column a). In contrast, 24% of the total pH 6.0 protein kinase activity was recovered in the membranes (Table V, column IIa). Axonemes contained 8% of the total protein (Table V, column I), 6—8% of the total pH 8.0 protein kinase activity (Table VI, column a) and 4% of the total pH 6.0 protein kinase activity (Table V, column IIa). Thus, it appeared that the pH 8.0 protein kinase activity partitioned preferentially with the axonemes and

TABLE VII

RELATIVE STIMULATION BY CYCLIC NUCLEOTIDES OF THE pH 6.0 PROTEIN KINASE IN SUBCILIARY FRACTIONS

The relative stimulation $\left(\frac{\text{specific activity in fraction + cyclic nucleotide}}{\text{specific activity in fraction − cyclic nucleotide}}\right)$ of the pH 6.0 protein kinase in each fraction was calculated for each experiment described in Table V. If $1 \cdot 10^{-5}$ M cyclic GMP or $1 \cdot 10^{-5}$ M cyclic AMP was added, the reaction mixture also contained 1 mM caffeine and 1 mM 3-isobutyl-1-methylxanthine; the control values used for comparison did not. Data are expressed as the mean ± S.E. of the relative stimulation. The total number of experiments (n) is given at the top of each column. IDC, incompletely demembranated cilia.

Fraction	Relative stimulation	
	$1 \cdot 10^{-5}$ M cyclic GMP ($n = 3$)	$1 \cdot 10^{-5}$ cyclic AMP ($n = 5$)
Cilia	2.67 ± 0.10	1.60 ± 0.13
Membranes	1.31 ± 0.10	1.17 ± 0.06
Axonemes	4.06 ± 1.40	1.81 ± 0.43
IDC	2.44 ± 0.03	1.33 ± 0.11

the pH 6.0 protein kinase activity was divided almost equally between the two fractions.

The pH 6.0 protein kinase activity in each fraction was stimulated by cyclic nucleotides. The relative amounts of activity with cyclic nucleotides in each fraction (Table V, column IIIa and IVa) were similar to the relative amounts in the absence of cyclic nucleotides (Table V, column IIa). This would indicate that selective loss of cyclic nucleotide receptors did not occur during fractionation. Differences in specific activities from experiment to experiment tended to obscure the relative stimulation by cyclic nucleotides in each fraction in the pooled data of Table V (columns IIb, IIIb and IVb). However, by comparing the relative stimulation by cyclic nucleotides of each fraction from individual experiments, we found that the relative stimulation was higher in axonemes than in membranes (Table VII).

Protein kinases activity in axenically-grown cells

To eliminate the possibility that the protein kinase activities described above originated from bacteria present in the growth medium and not removed during cell washing, we prepared cilia from paramecia grown axenically. The specific activity of 'axenic' protein kinase at pH 6.0 was 0.79 nmol · min^{-1} · mg^{-1} using histone as substrate, and at pH 8, the specific activity was 6.1 nmol · min^{-1} · mg^{-1} using protamine sulfate as substrate. Both values fall within the range of specific activities normally observed with cilia from bacterized cultures (cf. Tables V, VI). The pH 6.0 activity was stimulated 2-fold by 10 μM cyclic AMP, and 3-fold by 10 μM cyclic GMP. The pH 8.0 activity was slightly inhibited by cyclic AMP or cyclic GMP. The addition of NaF to the assay increased net incorporation at pH 6.0, suggesting that the axenic preparation also contained a phosphatase inhibited by NaF.

Discussion

The cilia of *P. tetraurelia* contain at least two protein kinases. One of the kinases is stimulated by cyclic AMP and cyclic GMP and occurs in both the membranes and axonemes of cilia. It operates maximally at pH 6.0 in 4 mM Mg^{2+} and prefers histone to protamine sulfate as an exogenous protein substrate. The other kinase is slightly inhibited by cyclic AMP and cyclic GMP and partitions preferentially with the axonemes. It operates maximally at pH 8.0 in 10 mM Mg^{2+} and works well with protamine sulfate as an exogenous substrate. The presence of more than one protein kinase in an organelle is not unprecedented. Cilia of *T. pyriformis*, a protozoan closely related to *Paramecium*, contain a cyclic AMP-independent protein kinase in the axoneme [17] and one cyclic AMP-dependent and three cyclic GMP-dependent protein kinases believed to be in the soluble fraction or membrane of the cilia [18].

We chose to characterize the protein kinases of cells grown in bacterized Cerophyl so that our biochemical studies would be directly comparable with the electrophysiological and genetic studies done by others. However, we have also found that cilia from axenically-grown cells have the same types and amounts of protein kinase as those from cells grown in bacterized medium, ruling out the trivial possibility that the kinases we study are of bacterial origin.

The exact distribution of the protein kinases in the membranes and axonemes of *Paramecium* cilia is difficult to determine from the existing data. Some loss of activity occurs during fractionation which cannot be attributed completely to loss of protein (Tables V and VI). The kinases may exist on structures which link membranes to axonemes [e.g. 28] and may therefore partition differently from preparation to preparation. Since ATP, cyclic AMP and cyclic GMP cannot penetrate membranes, only those vesicles which are open or form with the protein kinase on the outside can be measured for activity. We do not yet know what the orientation or leakiness is of the vesicles formed by this technique. The subciliary distribution of the phosphoprotein phosphatase, which could affect the apparent specific activities of the protein kinases, is also not known.

Recent reviews [1—4] have suggested several possible roles for membrane-bound, cyclic nucleotide-dependent protein kinases in regulating ion flux. The effects of cyclic nucleotides on *Paramecium* have not yet been intensively studied, but a correlation between Ca^{2+} flux and changes in cyclic AMP levels has been established in *Tetrahymena* [29].

It also been suggested that phosphorylation of tubulin affects microtubule function [30] and that tubulin, or a closely associated protein, is a protein kinase [31]. These ideas, however, have been challenged [32]. Axonemal tubulin from the flagella of *Chlamydomonas reinhardtii* consists of more components, of which at least two are phosphorylated [33]. The state of tubulin phosphorylation and the polypeptides associated with the protein kinase activities in the axonemes of *Paramecium* cilia have not yet been established.

Paramecium cilia may prove to be a useful system for studying regulation by protein phosphorylation. In addition to the cyclic nucleotide-dependent and independent protein kinases, the cilia also appear to contain a phosphoprotein phosphatase and an adenyl cyclase. The voltage-dependent Ca^{2+} channel of the excitable membranes is also present in the cilia [19,20]. Although we found no measurable effect of Ca^{2+} on the overall activity of the protein kinases, we are beginning to look for Ca^{2+} effects on the phosphorylation of specific proteins in the cilia. We are also examining the possibility that cyclic GMP and cyclic AMP affect the phosphorylation of specific proteins, and we have begun screening *Paramecium* mutants to determine if any are defective in protein kinase activity.

Note added in proof (Received July 17th, 1980)

After the submission of this manuscript, we learned that J.E. Schultz and H.M. Jantzen have also detected and studied cyclic nucleotide-dependent protein kinases in *Paramecium* cilia (Schultz, J.E. and Jantzen, H.M. (1980) FEBS Lett. 116, 75—78).

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