Biochimica et Biophysica Acta 862 (1986) 39–48
Elsevier

BBA 73159

Characterization of Ca^{2+}- or Mg^{2+}-ATPase of the excitable ciliary membrane from *Paramecium tetraurelia*: comparison with a soluble Ca^{2+}-dependent ATPase

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(Received 1 May 1986)

Key words: ATPase; Ciliary membrane; (*Paramecium*)

We have characterized divalent-cation-stimulated nucleoside triphosphate hydrolase activity of the excitable ciliary membrane and compared it with a soluble Ca^{2+}-ATPase released upon deciliation of *Paramecium*. The membrane-bound activity is strongly dependent on a divalent cation; calcium stimulates the basal activity of this enzyme at least 10-fold; magnesium and manganese stimulate less well, and strontium and barium, although less effective, also give measurable stimulation. This membrane-bound activity prefers ATP and GTP as substrates but also hydrolyzes UTP and CTP at measurable rates. The maximum velocity at saturating ATP concentrations and optimal calcium concentrations is 0.3 μmol/min per mg. The pH optimum for the membrane-bound activity is broad and centers around pH 7. From the temperature dependence of ATP hydrolysis, we calculate activation energies of 14 and 11 kcal/mol for the Ca^{2+}- and Mg^{2+}-stimulated activities, respectively. The Arrhenius plot is linear over the temperature range of 4 to 25°C. The membrane ATPase is relatively insensitive to ouabain, oligomycin, *N,N'*-dicyclohexylcarbodiimide, vanadate, Ruthenium red and two calmodulin antagonists. Polyclonal antisera raised against the purified soluble ATPase from the deciliation supernatant show low reactivity with the membrane-bound ATPase. We conclude from the comparison of properties of the two activities that the ciliary membrane-bound ATPase is distinct from the soluble ATPase released by deciliation.

Introduction

In the ciliated protozoan *Paramecium tetraurelia* ciliary motility is regulated by intracellular (or intraciliary) calcium concentrations. Voltage-sensitive calcium channels in the ciliary membrane open in response to depolarizing stimuli, allowing the entry of extraciliary calcium into the ciliary space. At calcium levels above micromolar, the direction of the power stroke is reversed, and the cell swims backward. This ciliary reversal is transient; the voltage-dependent calcium channels

Abbreviations: Triton X-100, octylphenoxypolyethoxyethanol; EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; DTPA, diethylenetriaminepentaacetic acid; Tween 80, polyoxyethylene sorbitan monooleate; CHAPS, (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate); Mes, 4-morpholineethanesulfonic acid; Mops, 4-morpholinepropanesulfonic acid; DCCD, *N,N'*-dicyclohexylcarbodiimide; EHNA, erythro-9-[3-(2-hydroxynonyl)]adenine; PCMB, *p*-chloromercuribenzoic acid.

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quickly inactivate [1,2] and calcium is believed to be pumped out of the cell against a concentration gradient [3]. Ciliary reversal requires both calcium and ATP [4]. The reversal mechanism has been localized near the base of the cilium [5], but the molecular nature of the ciliary reversal mechanism is unknown. Nothing is known about the molecular nature or cellular location of the calcium extrusion mechanism; it seems likely that the system is localized within the ciliary membrane. It is possible that the source of energy for ciliary reversal and/or calcium extrusion is hydrolysis of ATP.

The isolated ciliary membrane contains calcium-stimulated ATPase activity [6], and deciliation of paramecia releases a potent calcium-dependent ATPase activity [6,7]. We have compared several properties of the ciliary membrane-bound enzyme with those of the soluble ATPase to determine whether the two are the same enzyme in different states. Although the enzymes are similar in substrate specificity, and in pH- and temperature-dependence, they differ in other properties including cation stimulation and immunological reactivity, leading us to conclude that they are two distinct enzymes.

Materials and Methods

Growth and deciliation of cells. *P. tetraurelia* (strain 51S) was grown at 28°C in Soldo's crude axenic medium [8] or in phosphate-buffered Cerophyl medium bacterized with *Enterobacter aerogenes*.

Cells were harvested in early stationary phase by centrifugation at $200 \times g$ for 2 min in an oil-testing centrifuge (IEC Model HN-S) with pear-shaped centrifuge tubes. The cells were washed once at room temperature 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)) [9], then twice in the same solution at 4°C to remove bacteria (in the case of bacterized cultures) and extruded trichocysts.

Washed cells were immobilized in a 1 : 1 mixture of Dryl's solution and STEN (0.5 M sucrose/20 mM Tris-HCl/2 mM EDTA/6 mM NaCl (pH 7.5)). The cilia were detached by the addition of $CaCl_2$ and KCl to final concentrations of 10 mM $CaCl_2$ and 30 mM KCl. Deciliation, monitored by phase-contrast microscopy, was 80–90% complete in 10 min.

Cell bodies were removed by centrifugation at $850 \times g$ for 3 min in the HNS centrifuge, and the supernatant was recentrifuged under the same conditions to remove any remaining cell bodies. The supernatant was then centrifuged at $27\,000 \times g$ for 20 min in a Beckman JA-14 rotor to yield the deciliation supernatant and the cilia pellet. Deciliation supernatant was stored at 4°C in the presence of 3 mM NaN_3. This was used, without further purification except where stated, as the source of the soluble ATPase. Cilia were frozen at −20°C or used immediately for the preparation of ciliary membranes.

Isolation of ciliary membranes. Procedures for the isolation of ciliary membrane vesicles and an assessment of their purity have been described [10]. All procedures were performed at 4°C, and all solutions contained 3 mM NaN_3. Cilia were washed in 10 mM Tris-HCl (pH 8.0), and centrifuged at $27\,000 \times g$ for 30 min in a Beckman JA-20 rotor.

Ciliary membranes were detached from the axonemes by vortexing the ciliary pellet in 1 mM Tris-HCl/0.1 mM EDTA (pH 8.0) for 2–3 min. The suspension was centrifuged at $44\,000 \times g$ for 30 min to recovery particulate material. The pellet was resuspended in 10 mM Tris-HCl (pH 8.0) to a protein concentration of 3–10 mg/ml, then layered on a 20/45/55/60% (w/w) discontinuous sucrose gradient. After centrifugation of the gradient for 2 h at 40 000 rpm (Beckman SW 50.1 rotor), the membrane fraction was recovered from the 45% sucrose layer and washed in 10 mM Tris-HCl (pH 8.0). This membrane preparation was used as the source of the membrane ATPase activity.

Solubilized ciliary membranes were prepared by incubating membranes in 10 mM Tris-HCl (pH 8.0) plus 10 ml/l Triton X-100 for 30 min on ice. Insoluble material was removed by centrifugation for 30 min at $100\,000 \times g$ in a Beckman Airfuge.

Protein determination. Protein concentrations were measured by the method of Lowry et al. [11], using bovine serum albumin as the standard. When necessary to eliminate interference by Triton X-100, 1% sodium dodecyl sulfate was included in the Lowry reagent [12].

ATPase assay. Inorganic phosphate was mea-

sured by the method of Lanzetta et al. [13]. The standard assay medium contained 3 mM $CaCl_2$ or $MgCl_2$, 1 mM ATP, 3 mM NaN_3, 50 μg/ml bovine serum albumin, 20 mM Tris-HCl (pH 7.5) and enzyme in a total volume of 100 μl. After incubation at 20°C, the reaction was terminated by the addition of 800 μl of color reagent (0.34 g/l Malachite green oxalate, 10 g/l ammonium molybdate in 1 M HCl), followed by the addition of 200 μl of citrate solution (170 g/l sodium citrate, 1.6 ml/l Flaminox in 0.6 M HCl). Absorbance was measured at 660 nm. The amount of enzyme and the reaction length were adjusted to ensure less than 10% substrate hydrolysis. Under these conditions, the amount of phosphate released was proportional to the length of the reaction and the amount of enzyme used. One unit of enzyme activity was defined as 1 μmol P_i/min. Nucleotide concentrations were determined spectrophotometrically.

In some experiments, 2 mM EGTA or DTPA was used to buffer free cation concentrations. The total concentrations of $CaCl_2$ and $MgCl_2$ needed to obtain the desired free cation concentrations were calculated using a computer program. The association constants of EGTA, DTPA and ATP with H^+, Ca^{2+} and Mg^{2+} were used in the calculations [14,15]. In these experiments, 100 mM NaCl was added to increase the ionic strength of the assay medium to a level resembling that at which the association constants were determined.

Immunological experiments. The preparation and characteristics of antiserum raised against purified deciliation supernatant Ca^{2+}-ATPase have been described [7]. The effects of antibodies on ATPase activities were tested by including 8 μg of antibody, purified by protein A-Sepharose chromatography, in the assay medium. In immunoprecipitation experiments, antiserum was incubated with formalin-fixed *Staphylococcus aureas* cells for 30 min at room temperature in a buffer containing 40 mM Tris, 100 mM NaCl, 2 mM $CaCl_2$ (pH 7.5) and 1 ml/l Triton X-100. The cells were recovered by centrifugation at $10000 \times g$ for 2 min in a Beckman Microfuge then suspended in the same buffer containing approximately 0.3 mU of enzyme. After incubation for 30 min at room temperature, the samples were centrifuged at $10000 \times g$ for 5 min to remove the cells. ATPase activity in the supernatant was measured.

Reagents. Cerophyll powder was purchased from Agri-Tech, Inc., Kansas City, MO; scintillation grade Triton X-100 from Research Products International Laboratories, Elk Grove, IL; Na_2 ATP, substantially vanadium-free, from Sigma Chemical Co., St. Louis, MO; Flaminox from Fisher Scientific Co., Pittsburgh, PA; Malachite green oxalate from Allied Chemical, Morristown, NJ; Zwittergent 3-14 from Calbiochem-Behring Corp., La Jolla, CA; formalin-fixed *S. aureus* cells from Bethesda Research Laboratories Inc., Gaithersburg, MD. Partially purified deciliation supernatant ATPase and antibodies raised against the purified enzyme were gifts of A.E. Levin, University of Wisconsin.

Results

Yield and stability of ATPase

10 liters of cultured cells (1600 mg cell protein) generally yielded approx. 80 U of deciliation supernatant Ca^{2+}-ATPase (60 mg protein) and 1 U ciliary membrane Ca^{2+}-ATPase (4 mg protein).

Deciliation supernatant Ca^{2+}-ATPase activity was stable for weeks at 4°C; purified enzyme could be stored at −20°C for months without loss of activity. There was no significant difference in levels of ATPase activity between freshly prepared membranes and in membranes stored several months at −20°C. Therefore, in most of these studies, the frozen membrane preparations were used.

Solubility of membrane ATPase

Unlike deciliation supernatant Ca^{2+}-ATPase, which is isolated as a soluble protein, the ATPase of purified ciliary membranes is firmly bound to the membrane. Less than 5% of membrane Ca^{2+}-ATPase was released by agents capable of removing extrinsic membrane proteins: freeze-thaw cycles, 0.5 M KCl, 0.5 M LiCl, 1 mM EDTA, 5 ml/l ethanol, or 10 ml/l Tween 80. The detergents CHAPS and Zwittergent 3-14 were unsuitable for solubilizing ATPase activity from the membrane; when used at 3 mg/l, these detergents inhibited ATPase activity by more than 50%. Triton X-100 solubilized ciliary membrane ATPase in a concentration-dependent manner. At a protein

concentration of 1.5 mg/ml, up to 90% of the Ca^{2+}-ATPase was released by 10 ml/l Triton X-100.

Cation specificity

ATPase activity was strongly dependent on divalent cations in membrane-bound or Triton-solubilized ciliary membranes and in deciliation supernatant (Table I). In all preparations, maximum activity was seen when calcium was present; 3 mM $CaCl_2$ gave greater than 10-fold stimulation of membrane ATPase, and a 50-fold enhancement of the soluble ATPase.

Membrane ATPase differed from the soluble activity in its lower stimulation by strontium and barium and in its higher stimulation by manganese and magnesium. The effects of calcium and magnesium were not additive; activity in a medium containing Ca^{2+} and Mg^{2+} was lower than in the presence of Ca^{2+} alone. Triton-solubilized ciliary membrane ATPase activity was less specific for divalent cation than was the membrane-bound activity.

Monovalent cations did not stimulate ATP hydrolysis in either deciliation supernatant or ciliary membranes.

Calcium stimulation

The ATPase of ciliary membranes was activated by lower free calcium concentrations than was the deciliation supernatant ATPase (Fig. 1). Ciliary membrane ATPase showed a gradual rise in activity as the free calcium concentration was increased from 10^{-7} M to 10^{-4} M, with a steeper rise in activity as the free calcium concentration was raised to 1 mM. This stimulation by calcium was suppressed when 50 μM free magnesium was included in the assay medium. The calcium-activation profiles were similar in freshly prepared and frozen membranes and were altered less than 5% by the addition of 0.02 mg/ml saponin or 10 μM sodium vanadate.

Only at free calcium concentrations above 30 μM was a significant activity evident in partially purified deciliation supernatant. The rate of ATP hydrolysis rose sharply as the free calcium concentration was increased to 1 mM. Identical results were obtained with crude enzyme preparations.

TABLE I

CATION STIMULATION OF CILIARY MEMBRANE AND DECILIATION SUPERNATANT ATPases

Cations were present at 3 mM (chloride salts) in 1 mM ATP. ATPase activity was determined as described in Materials and Methods. The data represent the mean ± S.D. of activity measured in quadruplicate.

Addition	Relative activity (%)		
	ciliary membrane ATPase (membrane-bound)	ciliary membrane ATPase (Triton-solubilized)	deciliation supernatant ATPase
Ca^{2+}	100 ± 14	100 ± 3	100 ± 3
Sr^{2+}	10 ± 2	15 ± 1	26 ± 3
Ba^{2+}	8 ± 1	16 ± 2	21 ± 2
Mn^{2+}	27 ± 1	15 ± 1	4 ± 1
Mg^{2+}	31 ± 1	15 ± 1	4 ± 1
$Ca^{2+} + Mg^{2+}$	65 ± 2	56 ± 3	48 ± 3
Na^+	7 ± 1	6 ± 2	1 ± 2
K^+	7 ± 1	7 ± 1	1 ± 1
$Na^+ + K^+$	7 ± 1	6 ± 1	1 ± 1
EGTA	3 ± 1	2 ± 1	4 ± 1
None	7 ± 1	7 ± 1	2 ± 1

Magnesium stimulation

Ciliary membrane and deciliation supernatant ATPases were markedly different in their ability to be stimulated by magnesium. Whereas the soluble enzyme was not activated by free magnesium concentrations of up to 1 mM (data not shown), the membrane ATPase showed measurable activity at 10 μM free magnesium (Fig. 2). Membrane ATPase activity increased as the free magnesium concentration was raised to 1 mM. Addition of 10 μM free calcium to the assay medium increased the rate of ATP hydrolysis in ciliary membranes over the entire range of magnesium concentrations tested, however the effects of calcium and magnesium were not additive.

Nucleotide specificity

The membrane and soluble ATPases were similar in their ability to use several nucleotides as substrates (Table II). Purine nucleoside triphosphates were hydrolyzed more rapidly than pyrimidine nucleotides or ADP, and AMP was a poor substrate. In membranes, the Mg^{2+}-stimulated activity was less selective toward nucleotide sub-

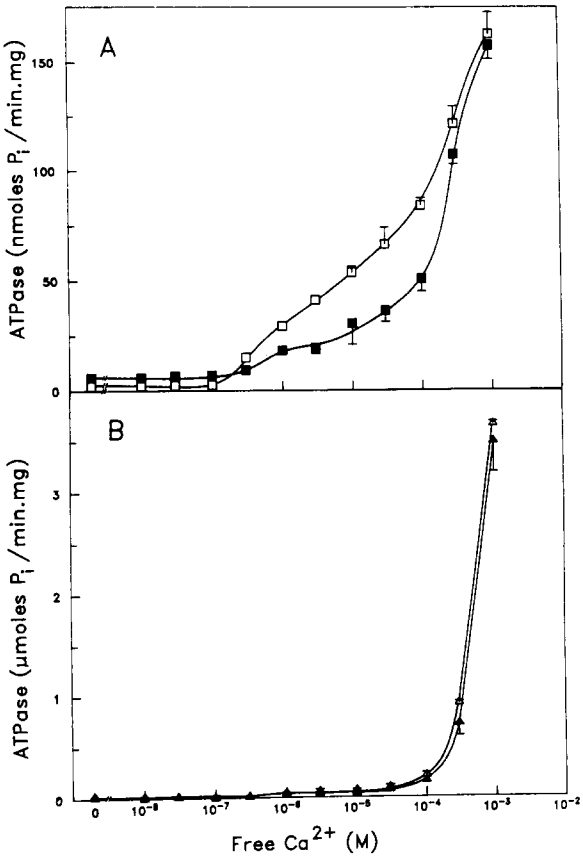

Fig. 1. Dependence of ATPase activities on free calcium concentration in the presence of 0.5 mM total ATP, 2 mM EGTA, 100 mM NaCl. Ciliary membrane ATPase (A) and partially purified deciliation supernatant ATPase (B) activities were measured in the absence (open symbols) or the presence (closed symbols) of 50 μM free magnesium. Each point represents the average ± S.D. of activity measured in quadruplicate. When S.D. is not indicated, it is within the area covered by the symbol.

strate than the Ca^{2+}-stimulated activity. It is possible that the observed ADP hydrolysis was due to the presence of Mg^{2+}-stimulated adenylate kinase.

Dependence of activity on temperature and pH

Membrane Ca^{2+}- and Mg^{2+}-ATPases and the deciliation supernatant Ca^{2+}-ATPase were qualitatively similar in the dependence of activity on temperature in the range from 4°C to 25°C; the activities increased as the temperature was increased. Arrhenius plots show a break at 25°C for each activity (Fig. 3). The activation energies below the breaks are 15, 14 and 11 kcal/mol for the

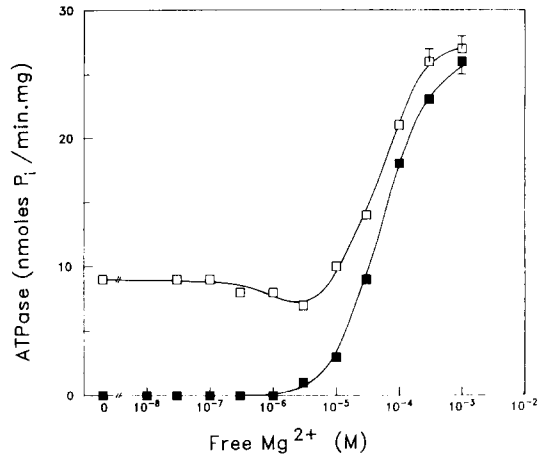

Fig. 2. Dependence of ciliary membrane ATPase activity on free magnesium concentration in the presence of 0.5 mM total ATP, 2 mM DTPA. Ciliary membrane ATPase activity was measured in the absence (□) or the presence (■) of 10 μM free calcium. Each point represents the average ± S.D. of activity measured in quadruplicate. When S.D. is not indicated, it is within the area covered by the symbol.

deciliation supernatant Ca^{2+}-ATPase, membrane-bound Ca^{2+}-ATPase and membrane-bound Mg^{2+}-ATPase, respectively. A phase-transition temperature has not been detected in *Paramecium* ciliary membranes [16,17]. This suggests that the temperature-sensitivity of the enzymes is due to the properties of the proteins themselves, not to

TABLE II

NUCLEOTIDE SPECIFICITY OF THE CILIARY MEMBRANE AND DECILIATION SUPERNATANT ATPases

Nucleotides were present at 0.5 mM concentration, cations at 3 mM. ATPase activity was determined as described in Materials and Methods. The data represent the mean ± S.D. of activity measured in quadruplicate.

Nucleotide	Relative activity (%)		
	ciliary membrane Ca^{2+}-ATPase	ciliary membrane Mg^{2+}-ATPase	deciliation supernatant Ca^{2+}-ATPase
AMP	0.4 ± 1.5	0.6 ± 0.3	1.0 ± 0.4
ADP	19 ± 3	66 ± 8	24 ± 6
ATP	100 ± 2	100 ± 11	100 ± 5
ADP + ATP	90 ± 5	140 ± 19	62 ± 10
GTP	67 ± 4	133 ± 47	81 ± 8
UTP	41 ± 4	69 ± 14	59 ± 4
CTP	12 ± 2	34 ± 4	16 ± 2

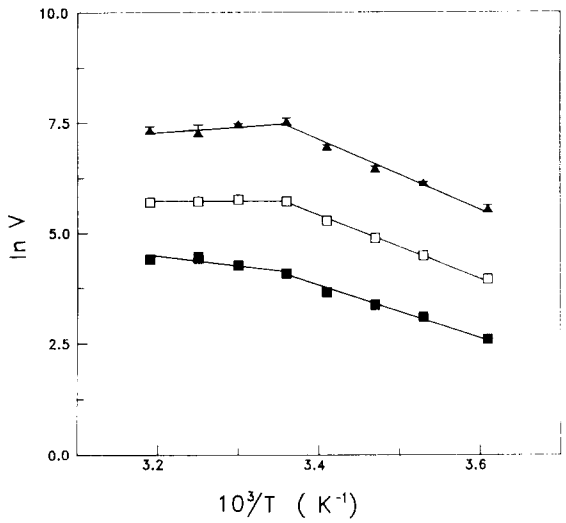

Fig. 3. Arrhenius plots of ATPase activities. Ciliary membrane Ca^{2+}-ATPase (□), ciliary membrane Mg^{2+}-ATPase (■), and deciliation supernatant Ca^{2+}-ATPase (▲) activities were measured under the standard assay conditions except that the pH was buffered with 20 mM Mops. Velocity was measured in units of nmol P_i/min per mg. Each value represents the average ± S.D. of activity measured in quadruplicate. When S.D. is not indicated, it is within the area covered by the symbol.

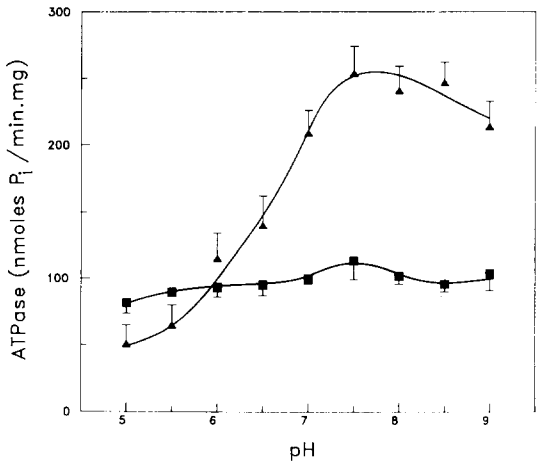

Fig. 4. pH dependence of Ca^{2+}-ATPase activities. Ciliary membrane Ca^{2+}-ATPase (■) and deciliation supernatant Ca^{2+}-ATPase (▲) activities were measured under the standard assay conditions except that the pH was buffered with 20 mM Mes-Mops-Tris adjusted to the appropriate pH with HCl or NaOH. Each value represents the average ± S.D. of activity measured in quadruplicate. When S.D. is not indicated, it is within the area covered by the symbol.

the interaction between these proteins and the lipids of the ciliary membrane.

Membrane and deciliation supernatant Ca^{2+}-ATPases showed broad pH activity curves with optima at approx. pH 7.5 (Fig. 4).

Effects of various agents

The effects of various compounds on ATP hydrolysis by membrane and deciliation supernatant ATPases was studied in order to compare the enzymes to each other and to other ATPases (Table III). The activity measured was azide-insensitive, as 3 mM NaN_3 was present in all enzyme assay solutions.

Ouabain, a potent inhibitor of $(Na^+ + K^+)$-ATPase, did not inhibit the soluble or membrane ATPases. Neither DCCD nor oligomycin, inhibitors of mitochondrial ATPase, nor EHNA, an inhibitor of dynein ATPase, had any significant inhibitory effect on the *Paramecium* enzymes.

Vanadate, a potent inhibitor of several ATPases [18], had little effect on the soluble or membrane Ca^{2+}-ATPase when present at a concentration of

TABLE III

EFFECTS OF VARIOUS COMPOUNDS ON THE CILIARY MEMBRANE AND DECILIATION SUPERNATANT ATPases

Enzyme was preincubated with inhibitor for 30 min at 20°C before the addition of ATP. Cations were present at 3 mM. ATPase activity was determined as described in Materials and Methods. The data represent the mean ± S.D. of activity measured in quadruplicate.

Addition	Concn. (mM)	Relative activity (%)		
		ciliary membrane Ca^{2+}-ATPase	ciliary membrane Mg^{2+}-ATPase	deciliation supernatant Ca^{2+}-ATPase
None		100 ± 9	100 ± 13	100 ± 7
Ouabain	0.1	91 ± 4	112 ± 16	104 ± 20
DCCD	0.1	88 ± 5	93 ± 4	91 ± 10
Oligomycin	0.2	91 ± 5	104 ± 7	89 ± 15
EHNA	1.0	82 ± 10	91 ± 19	100 ± 9
Sodium vanadate	0.1	89 ± 6	74 ± 5	96 ± 8
β-Mercaptoethanol	5.0	99 ± 10	100 ± 5	93 ± 14
Dithiothreitol	5.0	64 ± 6	88 ± 10	4 ± 3
N-Ethylmaleimide	0.1	94 ± 9	93 ± 10	17 ± 9
PCMB	0.1	27 ± 1	99 ± 4	98 ± 10
$LaCl_3$	0.2	44 ± 7	102 ± 13	20 ± 2

100 μM. The inability of vanadate to inhibit a Ca^{2+}-ATPase activity is not surprising in view of reports that magnesium potentiates and calcium attenuates vanadate inhibition of erythrocyte ($Ca^{2+} + Mg^{2+}$)-ATPase [19]. The Mg^{2+}-ATPase activity of ciliary membranes was relatively insensitive to vanadate; addition of 150 mM KCl did not enhance inhibition, as has been observed with erythrocyte ($Ca^{2+} + Mg^{2+}$)-ATPase [20] and ($Na^{+} + K^{+}$)-ATPase [21,22].

The ATPase activities were insensitive to the sulfhydryl reducing agent, β-mercaptoethanol, and the sulfhydryl alkylating reagent, *N*-ethylmaleimide.

The effects of other compounds on the ATPases indicate that the deciliation supernatant and ciliary membrane ATPases are distinct enzymes. Dithiothreitol had little effect on the membrane Mg^{2+}-ATPase; it inhibited membrane Ca^{2+}-ATPase about 40%, but almost completely inhibited deciliation supernatant Ca^{2+}-ATPase. PCMB did not alter ATP hydrolysis by deciliation supernatant Ca^{2+}-ATPase or membrane Mg^{2+}-ATPase, but it had a marked inhibitory effect on membrane Ca^{2+}-ATPase activity. Lanthanum had no effect on membrane Mg^{2+}-ATPase, but it significantly reduced the soluble and membrane Ca^{2+}-ATPase activities.

Effects of calmodulin antagonists and Ruthenium red

The Ca^{2+}-ATPase activities of the soluble and membrane enzymes differ in their sensitivities to Ruthenium red and to trifluoperazine and calmidazolium (R 24571), two potent inhibitors of calmodulin-stimulated enzymes (Fig. 5).

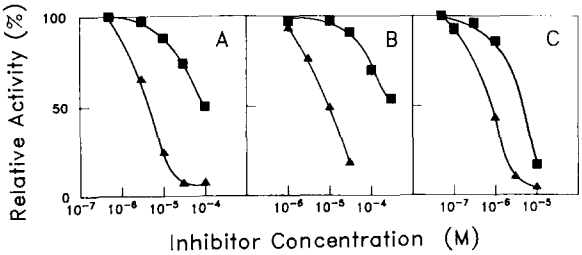

Fig. 5. Inhibition of deciliation supernatant and ciliary membrane Ca^{2+}-ATPase activities by Ruthenium red (A), trifluoperazine (B), and calmidazolium (C). Ciliary membrane Ca^{2+}-ATPase (□) and deciliation supernatant Ca^{2+}-ATPase (▲) activities were measured under the standard assay conditions. Each point represents the average of activity measured in duplicate.

Deciliation supernatant Ca^{2+}-ATPase activity was inhibited by low concentrations of trifluoperazine and calmidazolium; the concentrations required for half-maximal inhibition were 16 μM and 0.6 μM, respectively. Ciliary membrane Ca^{2+}-ATPase was less sensitive to these compounds; half-maximal inhibition occurred with 300 μM trifluoperazine and 0.6 μM calmidazolium.

The deciliation supernatant Ca^{2+}-ATPase was strongly inhibited by Ruthenium red, in agreement with the findings of Riddle et al. [6]; 4 μM Ruthenium red reduced activity 50%, 30 μM inhibited 95%. In contrast, the membrane Ca^{2+}-ATPase was inhibited only 30% by 30 μM Ruthenium red.

Immunological relationship between the ATPases

Although the enzymatic characteristics of the membrane and soluble ATPases differed, the possibility remained that the activities were derived from a single protein. To test this possibility, we explored the antigenic relationship between the soluble and membrane ATPases using polyclonal antiserum directed against purified deciliation supernatant Ca^{2+}-ATPase.

Antibodies inhibited 50% of deciliation supernatant Ca^{2+}-ATPase activity, but less than 5% of membrane Ca^{2+}-ATPase. When bound to formalin-fixed *S. aureus* cells, the antibodies precipitated more than 95% of deciliation supernatant Ca^{2+}-ATPase, but only 10–20% of solubilized membrane ATPase.

Discussion

The ciliary membrane of *Paramecium* contains Ca^{2+}- or Mg^{2+}-stimulated ATPase activity that is firmly bound to the membrane. This membrane activity is distinct from the soluble Ca^{2+}-dependent ATPase of the deciliation supernatant. Whereas both ATPase activities are maximally stimulated by Ca^{2+}, and are qualitatively similar in their dependence on nucleotide substrate, pH and temperature, the membrane ATPase differs from the soluble enzyme in several respects. It is stimulated to a greater extent by Mg^{2+} and Mn^{2+}

and by lower concentrations of free Ca^{2+}. It is significantly less sensitive to inhibition by dithiothreitol, Ruthenium red, trifluoperazine and calmidazolium. The lack of immunological cross-reactivity between the membrane ATPase and antibodies raised against the soluble ATPase indicates that the ATPases are distinct proteins.

The ciliary membrane of *Paramecium* may contain more than one ATPase. This hypothesis is supported by the results reported here. The Mg^{2+}-ATPase of the ciliary membrane differs from the Ca^{2+}-ATPase activity in its lower specificity toward nucleotide substrates and in its lower sensitivity to PCMB; these differences in properties of the Mg^{2+}-stimulated and Ca^{2+}-stimulated activities may be a result of selective stimulation of distinct membrane ATPases. The changes in cation activation of ATPase activity upon solubilization of the membranes may be due to selective solubilization of distinct ATPases or simply to a change in the properties of a single ATPase upon solubilization. The complex Ca^{2+}-activation profile suggests the presence of multiple Ca^{2+}-stimulated ATPase activities in the ciliary membrane.

Other laboratories have reported that on non-denaturing acrylamide gels, ciliary membrane proteins are resolved into two bands of Ca^{2+}- or Mg^{2+}-stimulated ATPase [23,24].

Andrivon et al. [23] purified a single ATPase from ciliary membranes; this suggests that either there is a single ATPase in the membrane, or only one ATPase whose activity survives the purification procedure. The ATPase activity catalyzed by that enzyme more closely resembles that of the deciliation supernatant Ca^{2+}-ATPase than that of the ciliary membrane ATPase which we report. Like the deciliation supernatant ATPase, the enzyme described by Andrivon et al. is easily obtained in a soluble form with a high specific activity, and it is inhibited by micromolar concentrations of Ruthenium red. This is in contrast to the membrane ATPase activity we have described, which is firmly bound to the membrane, has a lower specific activity and is resistant to micromolar concentrations of Ruthenium red. The absence of such an activity in their membrane preparations may be caused by differences in the methods used to isolate the ciliary membranes. Andrivon et al. deciliated cells with $MnCl_2$ rather than by calcium shock; the ciliary membranes were removed by extensive dialysis, not vortexing, and were purified on a sucrose gradient containing $MgCl_2$ instead of EDTA. It is possible that ATPase firmly bound to the membrane is altered or inactivated by these procedures. The absence of the weakly-bound ATPase, described by Andrivon et al., in our membrane preparations may also be due to differences in methods. Our use of the calcium shock procedure could be responsible for the release of weakly-bound ATPase into the deciliation supernatant. This release might occur through the action of calcium-stimulated proteinase; recent experiments suggest that the deciliation supernatant ATPase is isolated as a proteolytic fragment of a larger polypeptide (Ann, K. and Nelson, D.L., unpublished results).

Doughty and Kaneshiro [24,25] described a *Paramecium* ciliary membrane ATPase activity which, like the membrane ATPase reported here, is maximally stimulated by Ca^{2+} and is able to hydrolyze ATP, GTP, and UTP. Although these ATPase activities differ in their stimulation by Mg^{2+}, and in their response to PCMB and Ruthenium red, they may reflect the activity of a single ATPase. Establishment of the relationship between the membrane ATPase reported in this paper and that described by Doughty and Kaneshiro will require further purification of the enzymes.

The function of the ATPase of the ciliary membrane is unknown. ATPase activities in other systems have been shown to be associated with movement of ions and proteins, and it is possible that a ciliary membrane ATPase may serve one of these functions.

Calcium-transporting ATPases have been shown to exist in the plasma membranes of many cell types, and the presence of an ATP-dependent calcium pump in the ciliary membrane of *Paramecium* has been postulated [3,26]. However, the enzymatic characteristics of the ciliary membrane ATPase reported here differ from those of Ca^{2+}-pumping ATPases. Whereas an ATPase involved in the maintenance of low intracellular Ca^{2+} concentrations is expected to be activated by Ca^{2+} concentrations in the submicromolar range, the ciliary membrane ATPase is maximally stimulated

by millimolar concentrations of Ca^{2+}. This low-affinity Ca^{2+}-stimulated ATPase is unaffected by calmodulin antagonists at concentrations that inhibit Ca^{2+}-pumping ATPases. There is measurable ATP hydrolysis in ciliary membranes at 0.3 μM Ca^{2+}; this could reflect the activity of a Ca^{2+}-transporting ATPase present in low levels. However this activity is unaffected by vanadate at concentrations that abolish ATPase activity of Ca^{2+}-pumping enzymes. The possibility remains that the ciliary membranes contain a Ca^{2+}-transporting ATPase whose specific activity is too low to be detected over the high specific activity of the Ca^{2+}- or Mg^{2+}-ATPase.

We have not been able to detect ATP-dependent calcium accumulation in freshly prepared ciliary membrane vesicles, even though these vesicles are not leaky to internal calcium (Travis, S.M. and Nelson, D.L., unpublished results). It is possible that a Ca^{2+}-pumping ATPase is present, but is in the wrong orientation in the membrane. Or it may have been inactivated during preparation of the vesicles.

Mg^{2+}-ATPase activity is coupled to proton transport in the F_0F_1-ATPases in membranes of chloroplasts, mitochondria, and bacteria. Each of these ATPases is inhibited by DCCD, and the F_1 portion can be released from the membrane by mild procedures. In contrast, the ciliary membrane ATPase is resistant to inhibition by DCCD, and it is firmly bound to the membrane. These results suggest that the ciliary membrane ATPase is not of the F_0F_1 type.

Cell surface motility associated with cilia and flagella has been described (for review, see Ref. 27). The molecular components responsible for this force transduction are unknown, but an ATPase may be involved. This phenomenon of surface motility has not been investigated in *Paramecium*.

The dynein ATPases are involved in converting the chemical energy of ATP to mechanical work in cilia and flagella. The ciliary membrane ATPase is distinguishable from dynein by its relative insensitivity to vanadate and EHNA, and by its lower specificity toward nucleotide substrates and divalent cations.

We have shown that the enzymatic characteristics of this ATPase are distinct from those of the deciliation supernatant Ca^{2+}-ATPase. However, the physiological role of the enzyme remains to be established. The unique properties of the enzyme and the ease with which activity can be solubilized from the membrane should make possible further purification and study of the ciliary membrane ATPase.

Acknowledgements

This work was supported by NIH grants GM 31098, GM 32514, and GM 34096. S.M.T. was supported by NIH Cellular and Molecular Biology Training Grant GM 07215, and by a Steenbock Predoctoral Fellowship.

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