Adenylate cyclase in cilia from *Paramecium*

Localization and partial characterization

Joachim E. Schultz and Susanne Klumpp

Pharmazeutisches Institut der Universität, Morgenstelle 8, 7400 Tübingen, FRG

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A particulate adenylate cyclase was identified in the excitable ciliary membrane from *Paramecium tetraurelia*. MnATP was preferentially used as substrate, the K_m was 67 μM, V_{max} was 1 nmol cAMP . min^{-1} . mg^{-1}, a marked temperature optimum of 37°C was observed. Adenylate cyclase was not inhibited by 100 μM EGTA or 100 μM La^{3+}, whereas under these conditions guanylate cyclase activity was abolished. Fractionation of ciliary membrane vesicles by a Percoll density gradient yielded two vesicle populations with adenylate cyclase activity. In contrast, calmodulin/Ca-dependent guanylate cyclase was associated with vesicles of high buoyant density only.

Adenylate cyclase *Guanylate cyclase* *Excitable membrane* *Cilia* Paramecium

1. INTRODUCTION

The cilia of *Paramecium* represent an interesting model of an excitable organelle. The mechanical machinery, the 9 + 2 axoneme, is enclosed by an excitable membrane which carries the voltage-sensitive calcium channels [1]. These channels are triggered by receptor potentials resulting from stimulation [2]. Ca^{2+} entering the cilia during the Ca^{2+}/K^+ action potential serve as primary signals for a reversal of the ciliary beat (avoiding reaction) [3]. We have used cilia in an effort to identify possible components of the signal transfer cascade. During these studies several enzymes related to the cyclic nucleotide system have been identified. The ciliary membrane contains a Ca^{2+} – calmodulin-regulated guanylate cyclase (EC 4.6.1.2, GTP pyrophosphate-lyase-cyclizing-GC) localized in part of the ciliary membrane [4–6], soluble and particulate cAMP- and cGMP-dependent protein kinases [7,8], endogenous substrate proteins for the latter enzymes [9,10], considerable phosphodiesterase activity, and as evidenced by immunocytochemistry, calcineurin, a phosphoprotein phosphatase [11].

Now, we have identified an adenylate cyclase (EC 4.6.1.1, ATP pyrophosphate-lyase-cyclizing-AC) in the excitable ciliary membrane of *Paramecium*. Here, an initial characterization of this enzyme is given and significant differences of the properties of the AC compared to the ciliary GC are described.

2. MATERIALS AND METHODS

Paramecium tetraurelia, wild-type strain 51s, was grown axenically [12]. Stationary cells were deciliated by a Ca shock and cilia were purified by differential centrifugation [6]. Ciliary membrane vesicles were prepared by a sucrose step gradient as reported previously [13]. Percoll density gradients were run with 25% Percoll (w/w) for 15 min at 26000 rev./min in a Beckman 50.2 Ti rotor [6]. GC activity was assayed as described in [14]. AC activity was determined for 10 min at 37°C in 90 μl containing 50 mM Tris–HCl (pH 7.5), 1.1 mM cAMP disodium salt (Boehringer, Mannheim), 2.8 mM creatine phosphate disodium salt (Serva, Heidelberg), 36 IU creatine phosphokinase (Sigma), 1 mM $MnCl_2$ and 200 μM [α-^{32}P]ATP

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(0.4 μCi from NEN), 100 μM EGTA, and 10–30 μg protein. Formation of cAMP was measured by liquid scintillation counting after chromatographic separation of ATP and cAMP on alumina columns [14]. Recovery was routinely monitored by addition of c[^{3}H]AMP (Amersham). The identity of cAMP formed was verified by thin-layer chromatography and radioautography, and by phosphodiesterase digestion. Protein was estimated by the Lowry method using bovine serum albumin as a standard.

3. RESULTS AND DISCUSSION

3.1. *Detection of adenylate cyclase in cilia from* Paramecium

The actions of cAMP are probably mediated by cAMP-dependent protein kinases. Two soluble cAMP-dependent protein kinases from cilia have been characterized [7]. In addition, a considerable amount of total ciliary activity is particulate [7]. Therefore, it was obvious to expect the presence of an AC in this organelle. In initial experiments using MgATP as substrate, AC activity in cilia was low and somewhat variable. However, when Mn was used as a metal cofactor a high and consistent AC activity was found (table 1). Usually the ratio between Mn- and Mg-supported AC activity was 10:1. All further assays were therefore carried out in the presence of 1 mM $MnCl_2$. After centrifugation of a French Press lysate of cilia all AC activity was in the pellet, resembling the results obtained with GC [4]. This particulate nature made it possible to localize the AC activity in either the axonemal fraction or in the membrane of the cilia. When a sucrose step gradient known to separate membrane vesicles from incompletely demembranated cilia and axonemal fragments was used [13], about 90% of the AC was found in the membrane fraction within the 45% sucrose layer (table 1), whereas incompletely demembranated cilia (55% sucrose) and axonemal fragments (66% sucrose) contained only small amounts of AC (table 1). These findings were remarkably similar to the earlier localization of GC given in table 1 for comparison [4]. Therefore, the possibility existed that the AC may actually be a side activity of the highly active ciliary GC, using MnATP as substrate instead of MgGTP. Several lines of evidence led us to believe that this was not the case:

(i) GC is a Ca/calmodulin regulated enzyme, which exhibits only marginal activity in the presence of the Ca chelator EGTA [5,14]. However, all assays for AC activity were intentionally carried out in the presence of 100 μM EGTA which did not affect enzyme activity.

(ii) GC activity is most powerfully inhibited by lanthanum. La^{3+} dissociates the tightly bound calmodulin from the GC [7].

Table 1

Distribution of adenylate cyclase and guanylate cyclase in fractions of cilia from *Paramecium*

	Protein (mg)	Total activity (nmol cyclic nucleotide × min^{-1})	
		cAMP	cGMP
French Press			
Homogenate	28.8	5.7	7.0
10^5 g supernatant	6.2	0.0	0.15
Pellet	17.0	5.7	12.1
Sucrose gradient			
Vesicles (45%)	5.0	6.7	6.4
IDC (55%)	3.7	0.4	0.4
Axonemes (66%)	1.6	0.4	0.2

Cilia were disintegrated with a French Press and the pellet of the high-spin centrifugation was layered onto a sucrose gradient [13]. AC was assayed with 1 mM Mn, and GC with 3 mM Mg as metal cofactor. IDC = incompletely demembranated cilia

Yet, La-treated vesicle preparations as enzyme source had the same AC activity as untreated membranes. Even in the presence of 100 μM La, a concentration which completely inhibits GC, AC activity was not impaired. Although these data strongly suggest that AC and GC activity in the cilia is due to separate enzyme proteins, we cannot completely rule out the possibility that the catalytic component of the ciliary GC devoid of its regulatory calmodulin, may have lost its substrate specificity and efficiently converted MnATP to cAMP.

3.2. *Differential distribution of adenylate cyclase and guanylate cyclase in the ciliary membrane*

It has been shown, that GC activity is associated mainly with a high density vesicle population obtained when a French Press homogenate of cilia is fractionated by an isoosmotic Percoll gradient [6]. The distribution of AC and GC activity in ciliary fractions obtained by such a gradient is shown in fig.1. As expected, GC activity was particularly high in vesicles of high buoyant density. However, AC activity showed a completely different distribution. About 50% of the total AC activity was localized in membrane vesicles of low buoyant density. These vesicles have been shown to carry the bulk of the voltage-sensitive Ca channels [6]. The other half of AC activity remained associated with membrane fragments of higher density. Surprisingly, the second peak of AC reproducibly preceded the peak GC activity (4 separate experiments). Since AC and GC are membrane-bound, this differential distribution indicates a further microheterogeneity in the enzymic pattern of the excitable ciliary membrane, apart from the previous finding of a differential localization of GC and voltage-sensitive Ca channels [6].

In order to exclude any possibility of a remnant GC activity in the first part of the Percoll gradient (e.g., GC without calmodulin) the initial fractions were tested with optimal concentrations of calmodulin purified from *Paramecium* and various concentrations of Ca for GC activity. We did not obtain any indication that the low-density vesicles contained masked GC activity. Thus, it can be concluded that the AC and GC activities are due to separate protein moieties, which most likely will be subject to individual regulation.

3.3. *Characterization of adenylate cyclase*

The K_m-value was determined in the presence of 1 mM Mn with 20–800 μM ATP. Above a concentration of 400 μM ATP, substrate inhibition was apparent (fig.2). The K_m for ATP was found to be 67 μM; V_{max} was 1 nmol cAMP.min^{-1}.mg^{-1}. No cooperative interaction with the substrate was observed since a Hill coefficient of 1 was obtained. AC was measured at various temperatures. Increasing temperature greatly enhanced AC activity, regardless of the presence or absence of an ATP regenerating system; a marked optimum was observed at 37°C (fig.3). The linear Arrhenius plot (fig.3) indicates that we were probably dealing with a single enzyme entity, which, although part of the ciliary membrane, is not sensitive to possible phase

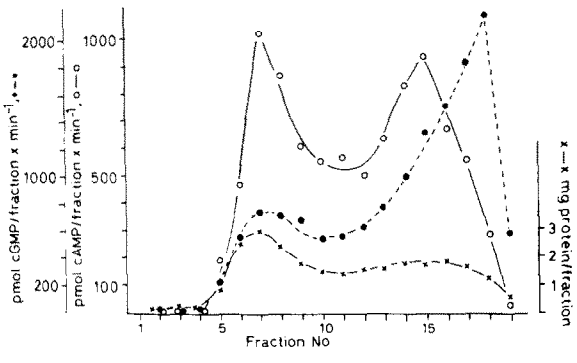

Fig. 1. Distribution of adenylate cyclase and guanylate cyclase in a Percoll gradient. Representative experiment shown (n = 4).

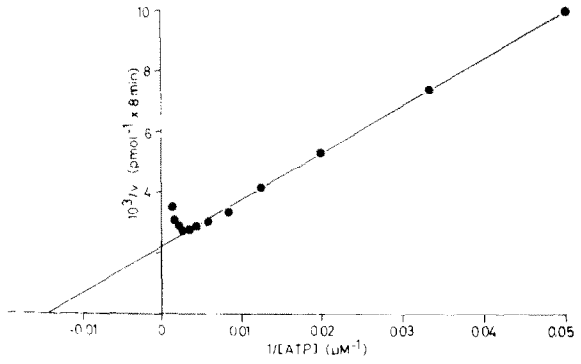

Fig.2. Effect of various ATP concentrations on the rate of cAMP formation by adenylate cyclase. Double reciprocal plot of substrate kinetics. Incubations were with 56 μg membrane protein in the presence of 1 mM $MnCl_2$.

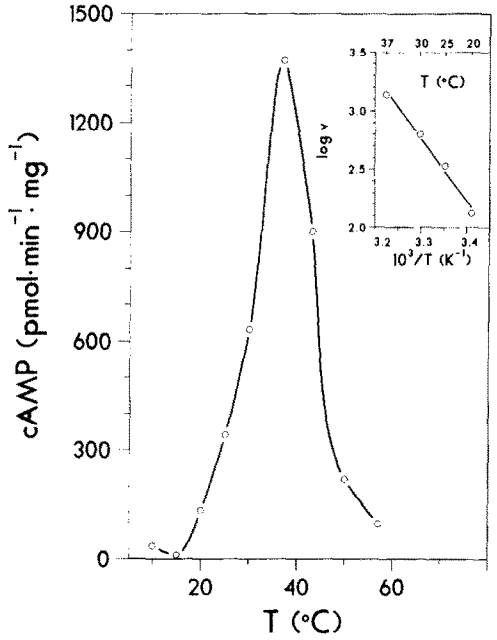

Fig.3. Temperature dependence of the activity of adenylate cyclase from *Paramecium*. Incubations were with 14 μg membrane protein. Inset: Arrhenius plot.

transitions of membrane lipids. The same was also observed with GC [14]. The activation energy for AC was 107 kJ/mol (GC = 54.5 kJ/mol [14]). Na- or K-ions up to 100 mM, Ca, Sr, and Ba up to 1 mM, and F^- up to 10 mM had no significant effect on the enzyme. The AC was also unresponsive to low concentrations of GMPPNP, GTP and forskolin, whether tested with Mn or Mg as cofactor. AC activity increased sharply from pH 6–8; no higher pH buffers were used.

The properties of the AC from *Paramecium* described here, resemble in many respects those of sperm cell AC [15] and correspond to those of the catalytic subunit of AC from many tissues [16]. There, enzyme activity is also 10–20-times higher when assayed in the presence of Mn as compared to Mg, and no effect of NaF is observed. Although we were unable to obtain comparable AC activity with MgATP and MnATP, we assume that the physiological substrate is MgATP. It is unclear at present whether a regulatory component of the AC is lost during purification of the cilia or whether the lower Mg-sustained AC activity truly represents the in vivo cAMP synthesizing capacity. Further studies will be necessary to elucidate how the AC in the excitable ciliary membrane is regulated. Considering the concomitant presence in the cilia of AC and Ca-regulated GC, albeit differentially distributed in the membrane, and the enzymic proteins of their respective amplifying cascades, it is tempting to speculate that the cAMP and cGMP systems will effect different processes in ciliary function; e.g., adaptation, ion gating or signal transfer to the axonemal structures. Toward this end measurements of cyclic nucleotide levels in *Paramecium* under a variety of stimulating conditions are currently in progress.

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