Adenylate cyclase and guanylate cyclase in the excitable ciliary membrane from *Paramecium:* Separation and regulation

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Particulate adenylate cyclase (AC) and guanylate cyclase (GC) activities localized in the ciliary membrane from *Paramecium* were solubilized by a two-step procedure using the detergents Brij 56 and Lubrol PX. The enzymes remained in the supernatant after a 100000 × *g* centrifugation. Upon gel chromatography, AC and GC were almost completely separated proving that each enzyme is a distinct molecular entity. Solubilization of GC was achieved with the calmodulin subunit remaining firmly attached to the catalytic part. Antibodies against calmodulin inhibited the enzyme as did La^{3+} and EGTA. AC activity appeared to be regulated specifically by K^+, enzyme activity being enhanced up to 100% by 15 mM K^+. Na^+ and Li^+ were inactive.

Adenylate cyclase *Guanylate cyclase* *Excitable membrane* *Calcium* *Potassium* Paramecium

1. INTRODUCTION

Adenylate cyclase (AC) and guanylate cyclase (GC) are generally thought of as intracellular amplifiers for extracellularly arriving signals. Transmembrane signal transmission is accomplished via receptors and/or ion fluxes across the membrane. The fundamental biochemical reactions involved in these processes may well be similar in all organisms. The protozoon *Paramecium* is used as a model to study the basic chemistry involved in signal transmission and amplification and their attachment to physiological responses [1,2]. The swimming behavior of *Paramecium* is under ionic control and ion fluxes across the membrane are induced by a variety of stimuli [1,2]. The cilia of *Paramecium* are of particular interest in this respect since voltage-operated calcium channels responsible for the upstroke of a Ca/K action potential are localized in their membrane [3]. In the cilia, we have identified biochemically all components of the cyclic nucleotide cascade including AC and GC [4,5]. The regulation of AC and GC is of special interest since these enzymes may constitute the initial intracellular target molecules for excitatory ionic signals which finally are connected to processes like alteration in swimming speed and direction, regulation of ion fluxes, desensitization and adaptation. The physical separation and partial purification of AC and GC from the ciliary membrane and their possible regulation by ionic factors are described in this report.

2. EXPERIMENTAL

Paramecium tetraurelia, wild-type 51s, were grown axenically as in [6]. Stationary cells were deciliated by a calcium shock [7] and purified by differential centrifugation [8]. AC and GC were assayed with α-^{32}P-labeled nucleotide triphosphates as in [4,5,9]. Solubilization of the cyclases was accomplished by a two-step procedure. Purified ciliary membrane vesicles [8] were suspended in 2.5 mM Tris–HCl buffer (pH 7.5), containing 1% Brij 56. The preparation was mixed, left on ice for 10 min, and centrifuged at 50000 × *g* for 1 h. The pellet was resuspended in buffer containing 2.5 mM Tris–HCl (pH 7.5), 20% glycerol (v/v),

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and 10 mM 2-mercaptoethanol. Lubrol PX was then added to 0.5% final concentration. The suspension was sonicated at 0°C in 700-μl aliquots for 15 s using a Branson sonifier with a microtip at setting 5. The mixture was centrifuged at 100 000 × g for 1 h; the supernatant contained both cyclase activities. Antiserum against calmodulin from *Tetrahymena* was a gift from Dr T.C. Vanaman, Duke University, antiserum against calmodulin from *Paramecium* was raised in rabbits with the help of Dr H. Plattner, University of Konstanz. Protein was determined by the Lowry method as modified in [10] using bovine serum albumin with appropriate buffer constituents as a standard.

3. RESULTS

3.1. Solubilization and separation of adenylate and guanylate cyclase

Both AC and GC are localized in the excitable ciliary from *Paramecium,* though differentially distributed as shown earlier [4]. To probe further into the mechanism of regulation and physiological function of these enzymes solubilization, separation and purification are needed. The nonionic detergent Brij 56 at 1% had almost no effect on both enzyme activities nor did it solubilize them (table 1). However, since about 60% of the total membrane protein was brought into the supernatant of a 50 000 × g centrifugation (table 1) the specific activities of AC and GC increased correspondingly by about 2.5-fold in the pellet fraction. The pellet suspended in buffer containing glycerol, mercaptoethanol and Lubrol PX was sonicated as indicated above. By this procedure, most of the protein was solubilized including both cyclases. At this step a small loss of total activity and a slight decrease in specific activity had to be accepted for the sake of solubilization. Actually, the presence of glycerol and mercaptoethanol was necessary to avoid further inactivation. AC and GC remained in the supernatant of a 100 000 × g centrifugation which was used for further purification.

So far, our conclusion of AC and GC being separate enzyme moieties was based on some rather clear-cut observations, e.g., GC activity is strongly inhibited by La^{3+}, EGTA, and antibodies against calmodulin while AC activity is unaffected under these circumstances [4,12]. Both enzymes could now be partially resolved by chromatography on Ultrogel AcA 22 equilibrated and developed with 50 mM Tris–HCl (pH 7.5), 30 mM NaCl, 10 mM mercaptoethanol, 20% glycerol (v/v) and 0.1% Lubrol PX (fig.1). About 40% of the protein applied was in the void volume and another 40% in the late fractions. AC and GC peak activities were separated by 8.9 ml and only a minor contamination with AC activity was seen in the GC peak eluting first (fig.1). Purification of AC and GC was about 3.5-fold over the input or about 20-fold with respect to the specific activity in ciliary membrane vesicles. Although the enzymes are far from being separated from all other impurities as seen by SDS-polyacrylamide gel electro-

Table 1

Solubilization of adenylate and guanylate cyclase in the ciliary membrane from *Paramecium*

	Protein (mg)	Specific activity (pmol · min · mg^{-1})		Yield (%)	
		AC	GC	AC	GC
(A) Vesicles in 1% Brij 56 (input)	5.2	392	778	100	100
Supernatant (50 000 × g)	3.3	60	95	10	8
Pellet	1.8	926	1907	85	85
(B) Brij-pellet with 0.5% Lubrol PX (input)	1.8	866	1285	77	57
Supernatant (100 000 × g)	1.4	1020	1329	70	46
Pellet	0.3	594	1224	9	9

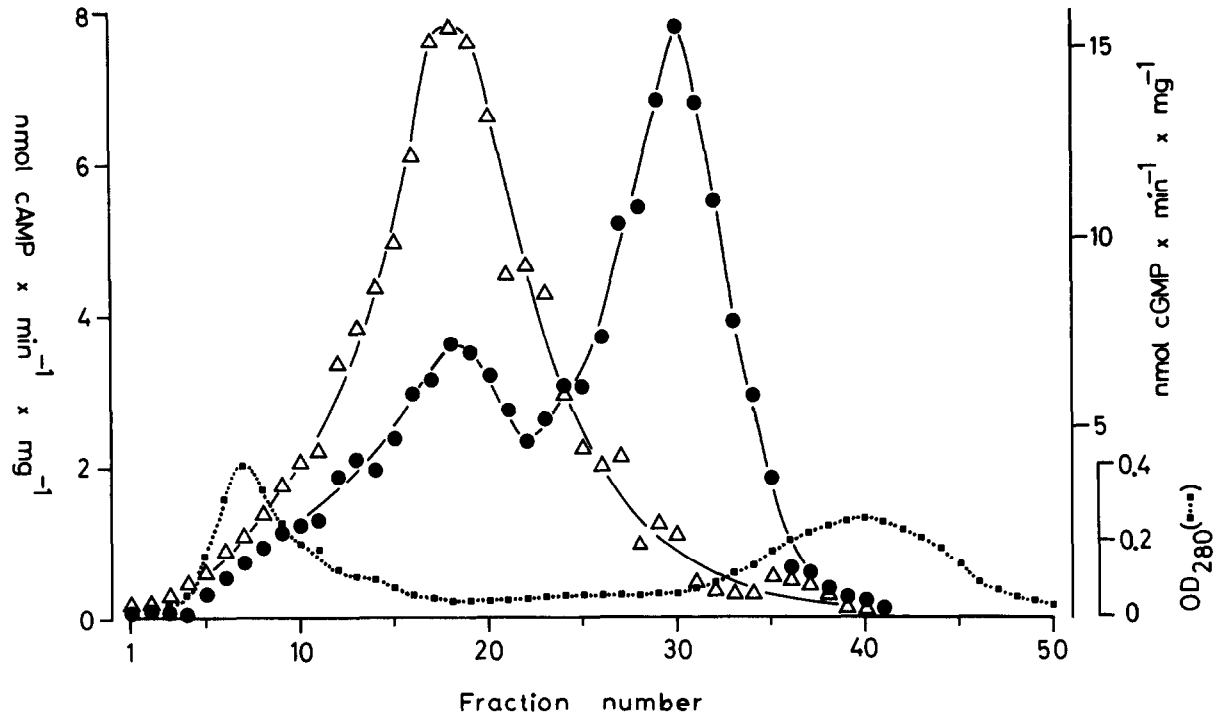

Fig.1. Separation and partial purification of solubilized AC (●) and GC (△) of the excitable ciliary membrane from *Paramecium* by Ultrogel AcA 22 chromatography. Column size was 86 × 1 cm. For further experimental details see section 3.1.

phoresis (not shown), the physical separation of AC and GC is final proof that we are dealing with two individual cyclases both contained in the cilia of *Paramecium*. This supports further experimental efforts to determine the regulation and physiological functions of the cAMP and cGMP regulatory systems in as small an organelle as the cilia of this unicellular protozoon.

3.2. *Calcium/calmodulin regulation of solubilized guanylate cyclase*

Previously, we showed that the membrane-bound GC of *Paramecium* is regulated by Ca^{2+} via a seemingly tightly bound calmodulin [11,12]. An ED_{50} value for Ca^{2+} of 8 μM was found, well within a physiologically meaningful concentration range [13]. During solubilization and chromatography, calmodulin was obviously completely retained by the enzyme as evident from several experimental observations (table 2).

Table 2

Calmodulin-dependence of solubilized guanylate cyclase from *Paramecium*

	Guanylate cyclase activity (%)
Control	100
EGTA, 50 μM	18
La^{3+}-treated GC	
no addition	9
+ *Paramecium* calmodulin (3.2 μg)	88
Paramecium calmodulin (3.2 μg)	119
Antiserum against	
Paramecium calmodulin (10 μl)	30
Tetrahymena calmodulin (10 μl)	34

100% corresponds to a specific activity of 4.1 nmol cGMP · min^{-1} · mg^{-1}. La^{3+} treatment was as follows: solubilized GC was incubated at 0°C for 10 min with 13.6 μM $LaCl_3$. The mixture was then passed through a Sephadex G-25 column to remove La^{3+} and calmodulin

(i) The Ca-chelating agent EGTA inhibited activity and Ca^{2+} was absolutely required for optimal activity.
(ii) Addition of exogenous calmodulin enhanced GC activity only slightly.
(iii) Antiserum against calmodulin from *Paramecium* or the related protozoon *Tetrahymena* inhibited soluble GC by about 70%.
(iv) La^{3+} dissociated calmodulin from the solubilized enzyme as observed earlier with the membrane-bound GC resulting in inactivation. Activity was restored upon addition of calmodulin.

The remarkable tight association between calmodulin and the catalytic part of GC throughout the detergent solubilization and subsequent purification are further support to our earlier claim that in vivo calmodulin constitutes an integral subunit of GC which renders this enzyme subject to an immediate regulation by Ca^{2+} [12]. Regulation of GC by association/dissociation of calmodulin, as discussed for other calmodulin-dependent enzymes, seems to be but a remote possibility for ciliary GC of *Paramecium* where the calmodulin portion immediately confers sensitivity toward Ca^{2+}.

3.3. *Effect of K^+ on adenylate cyclase*

In all likelihood, the physiological regulation of ciliary AC activity from *Paramecium* is decidedly distinct from the hormonally regulated AC of higher cells. The latter enzyme has been shown to consist of several polypeptides mediating the actions of hormones, GTP, NaF, forskolin, cholera toxin, and pertussis toxin. None of these compounds had any effect on AC activity from *Paramecium* [4]. Most recently, we reported that cyclic AMP levels in *Paramecium* in vivo are regulated by membrane surface charge as established very specifically by the $[K]/[Ca]^{-1/2}$ Donnan ratio [14]. Rb^+ could partially substitute for K^+, while Na^+, Li^+ or tetraethylammonium could not. To investigate the effect of monovalent cations on AC in vitro, we carried out AC assays in a buffer completely devoid of Na^+ and K^+ using Mops–Tris buffer and ATP Tris-salt as substrate. K^+ and Na^+ were added as indicated (fig.2). K^+, even at 1 mM, stimulated AC activity in whole cilia and in the detergent-solubilized enzyme (fig.2). A half-maximal effect was already seen with as little as 3 mM K^+; maximal stimulation was always observed in the range 15–25 mM. The action of K^+ was not an unspecific ionic effect, since Na^+ and Li^+ were completely devoid of stimulatory activity on AC (fig.2). Rb^+, which in many ways resembles K^+ in molecular properties, could replace K^+ although with a lower intrinsic activity. These observations parallel those made in studies of the regulation of cyclic AMP levels in *Paramecium* in vivo. It is felt that the low K^+ concentration needed for and the apparent ionic selectivity of the stimulatory effect clearly indicate a physiologically significant regulatory role.

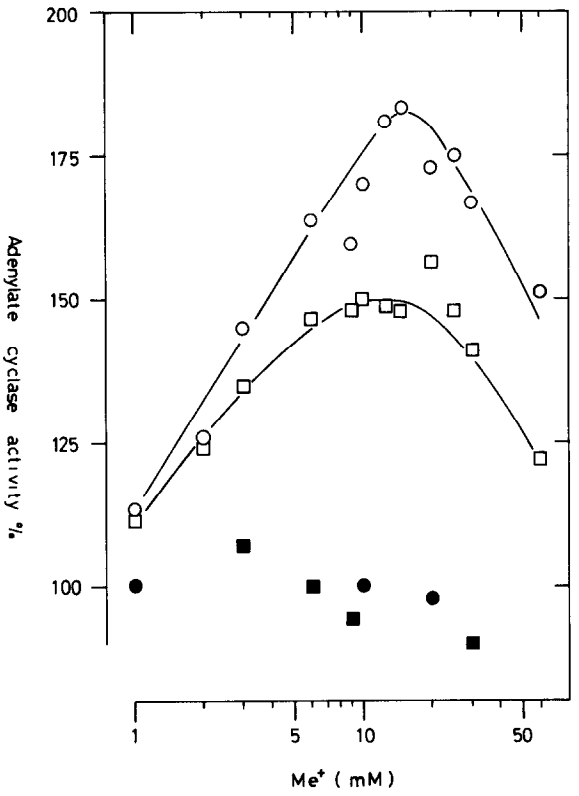

Fig.2. Effect of monovalent cations on adenylate cyclase activity from *Paramecium*. Cilia (○,●) or detergent-solubilized AC (□,■) were incubated for 8 min with the indicated metal ion concentration. Incubations: (○,□) with K^+, (●,■) with Na^+. 100% activity corresponded to 30 and 2500 pmol cAMP · min^{-1} · mg^{-1} for cilia and solubilized AC, respectively. The assay (100 μl) contained: 50 mM Mops–Tris buffer (pH 7.6), 1 mM [α-^{32}P]ATP (0.5 μCi), 2 mM [^{3}H]cyclic AMP (5 nCi), 0.5 mM of the phosphodiesterase inhibitor SQ 66007, 8 mM $MgSO_4$, and 20% gycerol (v/v): 110 and 8 μg protein was used for cilia and solubilized AC, respectively.

4. DISCUSSION

AC and GC from *Paramecium* are unique enzymes in many respects. So far, a GC with calmodulin as subunit for Ca regulation has been detected in *Paramecium* and the related protozoon *Tetrahymena* [12,15,16]. Despite intensive search in our and other laboratories, it has not yet been identified in bacteria, vertebrates or several other protozoons (unpublished). It will be most interesting to see whether this is due to deficiencies in experimental design or rather to a unique evolutionary trait expressed in *Paramecium* and *Tetrahymena* but not in other organisms. In this context our preliminary observations using antibodies directed against soluble GC from rat brain, kindly provided by Dr J. Zwiller, Strasbourg [17], are most interesting. Solubilized Ca/calmodulin dependent GC from the ciliary membrane of *Paramecium* was inhibited by up to 70% depending on the antibody used, while solubilized AC was not affected. This strongly indicates considerable antigenic conservation of GC from protozoa to vertebrates possibly independent of a particular regulatory mechanism.

To our knowledge, this is the first report of an AC which seems to be regulated by inorganic cations as K^+ in a physiologically relevant concentration range. No attempts have been made to establish the presence of similar adenylate cyclases in other species. Looking at electronic signal transmission via gap junctions in brain [18] one may well envisage enzymes of second messenger systems being regulated by membrane potential and surface charge as determined by Ca and K.

AC and GC are unique in that both are part of the excitable ciliary membrane of *Paramecium*, the site of the depolarizing Ca influx [3,4]. The successful solubilization and the now possible purification will not only allow us to learn more about the mechanism of regulation but may also be of considerable help in elucidation of a possible physiological role of cyclic AMP and cyclic GMP amplifier systems in *Paramecium* in particular and, more generally, in electrical information transfer. In the central nervous system the characterization of receptor-mediated AC regulation has been far advanced, yet, a commonly accepted physiological function for cyclic AMP and cyclic GMP in excitable tissues has not been established. In view of the numerous available behavioral mutants of *Paramecium* this neurobiological model should be useful for studies designed to delineate the main principles of a sequential interaction between ionic environment, electrical activity, chemical signal processing and physiological behavior.

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