GUANYLATE CYCLASE IN THE EXCITABLE CILIARY MEMBRANE OF *PARAMECIUM*

J. E. SCHULTZ and S. KLUMPP

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

Received 23 October 1980

1. Introduction

The ciliate *Paramecium* has a cell membrane which responds to environmental stimuli with altered conductances and potentials, the ciliary apparatus being regulated by the rate at which calcium enters the cell [1,2]. As demonstrated in deciliation experiments, the calcium inward current during excitation is carried through voltage-sensitive ion channels in the surface membrane covering the cilia [3,4]. The behavioral correlate of the Ca^{2+}/K^+ action potential is the known avoiding reaction of *Paramecium*. The excitable ciliary membrane resembles that of neurons, the Ca^{2+}/K^+ action potential being comparable to the Na^+/K^+ action potential in higher nervous systems. Cilia of *Paramecium* can be isolated and biochemical processes involved in motility and membrane excitability can be investigated. There is convincing evidence that cyclic nucleotides and Ca^{2+} serve as interrelated second messengers in a number of cellular systems [5]. Here we report the presence of a particulate guanylate cyclase (EC 4.6.1.2, GC) localized in the excitable ciliary membrane of *Paramecium*.

2. Materials and methods

Paramecium tetraurelia, wild-type strain 51s were grown axenically as in [6]. Stationary cells were deciliated by a calcium shock [7] and cilia were purified by repeated differential centrifugation. This resulted in cilia uncontaminated by cells or other organelles as evidenced by electron microscopy [8].

GC activity was determined for 8 min at 37°C in 90 μl containing 20 mM Tris–HCl buffer (pH 7.8), 3 mM $MgCl_2$, 3 mM creatine phosphate disodium salt (Serva, Heidelberg), 40 IU creatine phosphokinase (Sigma), 1 mM cGMP sodium salt (Sigma), 0.44 mM [α-^{32}P]-GTP (1 μCi, from Amersham) and 50–100 μg protein. Formation of cGMP was determined by liquid scintillation counting with correction for recovery of c[^{3}H]GMP (from Amersham) after chromatographic separation of GTP and cGMP on alumina columns as in [9]. Production of cGMP was linear in respect to time and protein. cGMP identity was verified by thin-layer chromatography and radioautography. Protein was estimated by the Lowry method using bovine serum albumin as standard.

3. Results and discussion

3.1. *Detection of particulate guanylate cyclase in cilia from* P. tetraurelia

A GC of high specific activity was identified in the cilia (table 1). On repeated freezing and thawing, ~15% of the ciliary protein remained in the supernatant after centrifugation, whereas all GC was found in the pellet (table 1). After disruption of the cilia

Table 1
Distribution of guanylate cyclase in fractions of cilia from *P. tetraurelia* wild-type strain 51s

	Protein (mg)	Specific activity (pmol cGMP . mg^{-1} . min^{-1})
A. Cilia	7.3	122
Supernatant	0.9	33
Pellet	6.3	210
B. French Press lysate	7.3	180
Supernatant	2.2	38
Pellet	4.5	310

Cilia suspended in Tris–HCl buffer (pH 7.8), 7.3 mg/ml, were repeatedly frozen and thawed (A), or disintegrated with a French Press (B) and centrifuged at 48 000 × *g* for 30 min

with a French Press, 30% of the total protein was in the supernatant after centrifugation, yet 95% of the GC remained in the sediment (table 1). The increase in specific activity in the particulate fractions is much higher than expected by the removal of the soluble proteins. Most likely this is due, at least in part, to the mechanical disruption of the fragile cilia by the procedures employed, the active site of the enzyme being more accessible for the added substrate.

3.2. *Localization of guanylate cyclase in the excitable ciliary membrane*

Two major components of the cilia are particulate in nature: the excitable membrane and the 9 + 2 complex of the motility apparatus. Since the GC is completely bound to particular structures, a search for its subciliary localization was undertaken. To fractionate ciliary components a discontinuous sucrose gradient was employed which has been shown to separate ciliary membranes from incompletely demembranated cilia and axonemes [8]. Cilia were disintegrated with a French Press as above and centrifuged to recover all particulate material. The pellet was resuspended in 10 mM MOPS buffer (pH 7.2) to 6–10 mg protein/ml. The suspension was layered on a sucrose gradient as detailed in [8] (fig.1). The ciliary membranes banded within the 45% sucrose layer. The purity of the isolated membrane fraction was controlled by SDS–polyacrylamide gel electrophoresis. As described in [8], tubulins and dyneins, the major axonemal constituents, were almost completely absent. This purified membrane fraction contained ~20% of the applied protein and exhibited GC activity of very high specific activity (fig.1), actually a 10-fold purification of the enzyme was usually obtained compared to the ciliary activity. The bands of incompletely demembranated cilia and axonemes at 55% and 65% sucrose, respectively, contained the remaining protein. However, only negligable quantities of GC were found in these fractions (fig.1). Identical results were obtained when cilia were disrupted by extended vortexing as in [8], instead of using the French Press for disintegration. Thus it can be concluded that all ciliary GC activity is localized in the excitable membrane.

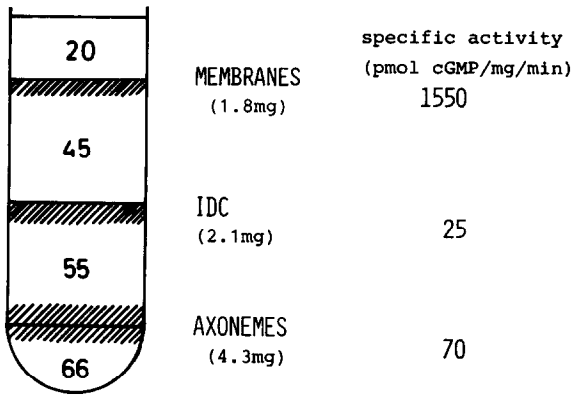

Fig.1. Guanylate cyclase activity in ciliary fragments as separated by discontinuous sucrose gradient centrifugation. The gradient consisted of 1.4 ml 66% (w/w) sucrose overlaid with 3.4 ml each of 55% and 45% sucrose and 1.4 ml 20% sucrose in 10 mM MOPS (pH 7.2). 8.3 mg protein (0.4 ml) of particulate material from French Press disintegrated cilia were layered on top. The gradient was centrifuged in a SW 40 rotor at 35 000 rev./min for 150 min (details in [8]). Diagrammatic scheme of gradient at left, GC activity was measured in the indicated fractions as described in the text. Sucrose in the assay (up to 30%) had no effect on enzyme activity as determined in control expteriments. (IDC, incompletely demembranated cilia).

3.3. *Michaelis constant pH optimum and ion requirements*

K_m values were determined in the presence of either 0.6 mM Mn^{2+} or 3 mM Mg^{2+} (see below) with 14–500 μM GTP. K_m values were 150 μM and 108 μM, respectively, well within the range of physiological GTP concentrations. The pH optimum for GC was determined using acetate (pH 4.5–6), MES (pH 5.5–6.7), Hepes (pH 6.9–8.1) and Tris (pH 7.2–9.0) as buffer systems. A rather sharp pH optimum was found around pH 8. To explore the possibility that divalent cations, in particular Ca^{2+}, may influence GC activity, the effect of metals at various concentrations was studied (fig.2). Mg^{2+} at

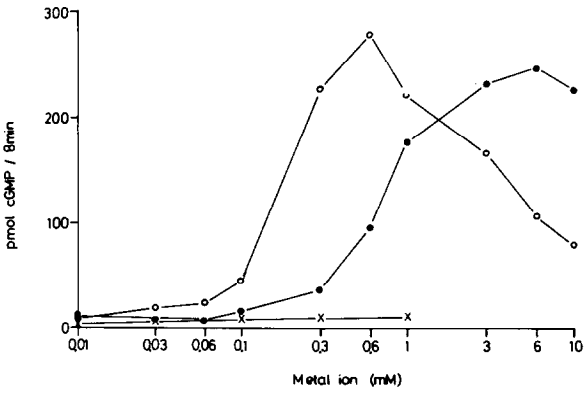

Fig.2. Effects of Mg^{2+} (•), Mn^{2+} (○) and Ca^{2+} (×) on guanylate cyclase from cilia of *P. tetraurelia*. The standard assay mixture was employed except the variations in divalent cation concentrations.

3 mM and Mn^{2+} at 0.6 mM were about equipotent to serve as cofactors. With Ca^{2+} as divalent cation no GC activity could be detected. The effect of Mg^{2+} contrasts data with many GCs from microorganisms and mammalian tissues. Most of these enzymes require Mn^{2+} as metal cofactor rather than Mg^{2+} or Ca^{2+} [10]. In *Tetrahymena pyriformis* a GC has been described where Mg^{2+} was the prevalent metal cofactor being twice as potent as Mn^{2+} [11]. Since intracellular levels of Mg^{2+} are $\geqslant$100-times those of Mn^{2+} it is likely that the GC activity with Mg^{2+} in vitro represents its behavior in vivo. Variable data have been reported on the effect of Ca^{2+} on GC. In some instances Ca^{2+} was enhancing, in others it was inhibiting GC [10]. In the presence of Mg^{2+}, the ciliary enzyme of *Paramecium* was inhibited by calcium ions (not shown). However, the $[Ca^{2+}]$ necessary for 50% inhibition of GC (300 μM in the presence of 1 mM Mg^{2+}) may not be in the physiological concentration range in order to account for a direct regulatory influence of Ca^{2+} on GC in the cilia [2].

The ciliary GC exists as a particular enzyme and, above all, is localized exclusively in the excitable membrane, which carries the voltage-sensitive calcium channels. To our knowledge, this is the first demonstration of a GC in an electrically excitable membrane. A discrete physiological role for cGMP in any cell type has not been established yet. cGMP supposedly modulates membrane permeability and membrane potential in rod outer segments [12,13]. Indeed, rod outer segments originate as modified cilia as do several other vertebrate sensory receptors [14]. The extraordinary high level of GC in the ciliary membrane makes it very tempting to speculate that some unique role for this cyclic nucleotide exists in the cilia, e.g., in ion gating or in signal transmission to the axonemal structures. In addition, the presence of cGMP-dependent protein kinase [15,16], cGMP degrading phosphodiesterase and calmodulin in cilia [17] has been shown. Hopefully, the power of the genetic approach, which has so successfully been used in *Paramecium* for dissection of electrogenesis [18,19] will aid in the understanding of the functional role of cGMP in excitable organelles as exemplified by the cilia of *Paramecium*.

Acknowledgements

We thank J. Thiele for assistance. This work was supported by the Deutsche Forschungsgemeinschaft (SFB 76).

References

[1] Kung, C., Chang. S.-Y., Van Houten, J. and Hansma, H. (1975) Science 188, 898–994.
[2] Eckert, R. and Brehm, P. (1979) Annu. Rev. Biophys. Bioeng. 8, 353–383.
[3] Dunlap, K. (1977) J. Physiol. 271, 119–133.
[4] Ogura, A. and Takahshi, K. (1976) Nature 264, 170–172.
[5] Rapp, P. E. and Berridge, M. J. (1977) J. Theor. Biol. 66, 497–525.
[6] Thiele, J., Honer-Schmid, O., Wahl, J., Kleefeld, G. and Schultz, J. (1980) J. Protozool. 27, 118–121.
[7] Gibbons, J. R. (1965) Arch. Biol. (Liege) 76, 317–353.
[8] Adoutte, A., Ramanathan, R., Lewis, R. W., Dute, R. R., Lin, K.-Y., Kung, C. and Nelson, D. L. (1980) J. Cell. Biol. 84, 711–738.
[9] Jakobs, K. H., Saur, W. and Schultz, G. (1976) J. Cycl. Nucl. Res. 2, 381–392.
[10] Murad, F., Arnold, W. P., Mittal, C. K. and Braughler, J. M. (1979) Adv. Cycl. Nucl. Res. 11, 175–204.
[11] Nakazawa, K., Shiminaka, H., Nagao, S., Kudo, S. and Nozawa, Y. (1979) J. Biochem. 86, 321–324.
[12] Caretta, A., Cavaggioni, A. and Sorbi, R. T. (1979) J. Gen. Physiol. 295, 171–178.
[13] Miller, W. H. and Nicol, G. D. (1979) Nature 280, 64–66.
[14] Wiederhold, M. L. (1976) Annu. Rev. Biophys. Bioeng. 5, 39–62.
[15] Schultz, J. E. and Jantzen, H. M. (1980) FEBS Lett. 116, 75–79.
[16] Lewis, R. M. and Nelson, D. L. (1980) Biochim. Biophys. Acta in press.
[17] Walter, M. F. and Schultz, J. E. (1981) submitted.
[18] Oertel, D., Schein, S. J. and Kung. C. (1978) Nature 268, 120–124.
[19] Satow, Y. and Kung, C. (1974) Nature 247, 69–71.