CYCLIC NUCLEOTIDE-DEPENDENT PROTEIN KINASES FROM CILIA OF *PARAMECIUM TETRAURELIA*

Partial purification and characterization

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1. Introduction

The cilia from the protozoon *Paramecium tetraurelia* constitute a well suited model for a detailed biochemical study of the excitable membrane. When stimulated chemically or mechanically *Paramecium* shows an avoiding reaction while firing trains of calcium/potassium action potentials comparable to the sodium/potassium action potentials in higher nervous systems [1]. The voltage-sensitive calcium inward current is exclusively carried through the ciliary membrane as demonstrated in deciliation experiments [2]. Also, a number of behavioral mutants of *P. tetraurelia* are available which have various defects in their excitable membrane, e.g., lack of a calcium inward current (pawns). With the development of axenic mass culture [3] of *P. tetraurelia* enough uniform cell mass has become available to complement the extended electrophysiological work on *Paramecium* with biochemical studies and thus use the advantage offered by genetics in neurobiology.

In nervous tissue, the cyclic nucleotide system and calcium are considered important components in signal transduction. For example, cAMP-stimulated phosphorylation of membrane proteins from synaptic structures has been described [4]. Here we present evidence for two types of cAMP-dependent and one cGMP-dependent protein kinase (EC 2.7.1.37, ATP:protein phosphotransferase) of high specific activity in the cilia from *P. tetraurelia*. Their partial purification and characterization is described.

2. Materials and methods

Paramecium tetraurelia 51s wild-type was grown axenically in 20 l bioreactors as in [3]. Deciliation of stationary cells was carried out by a calcium shock [5] and the cilia were purified by several centrifugation steps. The purity of the cilia was checked by electron microscopy.

Protein kinase (PK) activity was measured for 6 min at 25°C in 0.2 ml containing 50 mM MOPS (pH 6.5), 10 mM $MgCl_2$, 7 mM 2-mercaptoethanol, 60 μg mixed histones, 50 μM [γ-^{32}P]ATP (20 Ci/mol). Phosphorylated histones were precipitated by 10% trichloroacetic acid, collected on Whatman GF/C filters, washed, dried and radioactivity was determined. cAMP-binding activity was determined according to [6] with 16 nM c[^{3}H] AMP. Protein was estimated by the Lowry method or with the micromodification of the Bradford technique [7] using bovine serum albumin as standard.

Radioactive ATP, GTP and cAMP were from Amersham, ATP and cAMP from Boehringer, GTP from Serva, cGMP, mixed histones (calf thymus, type II), histone H2B (type VII), protamine (salmon sperm, grade IV) from Sigma.

3. Results and discussion

3.1. *Detection and purification of protein kinases in cilia from* P. tetraurelia

A PK activity was identified in cilia from *P. tetraurelia* which could be stimulated 5–6-fold by 1 μM cAMP and somewhat less by 1 μM cGMP. On repeated freezing and thawing of the cilia about 50% of the total PK activity appeared in a 48 000 × *g*, 30 min supernatant. The specific activity in the supernatant was 5-fold higher than in the pellet containing ciliary

Table 1
Protein kinase activity in cilia from *P. tetraurelia* wild-type 51s

	Protein (mg)	Spec. act. (nmol P_i transferred . min^{-1} . mg^{-1})		
			+ 1 μM cAMP	+ 1 μM cGMP
Cilia	157.4	0.6	3.5	1.6
Supernatant	24.5	1.7	12.3	5.1
Pellet	125.3	0.4	2.7	1.5

Purified cilia were treated as described and centrifuged at 48 000 × *g* for 30 min

membranes and axonemal fragments (table 1). Treatment of the cilia with several detergents (1% Triton X-100, -X-405, Lubrol PX, Brij-30,-38,-56,-58) or sonication failed completely to release more PK activity from the pellet into the supernatant.

On DEAE-cellulose chromatography 3 peaks with PK activity were detected (fig.1). Initially, a PK was eluted which was mainly stimulated by cGMP (34-fold) and to a lesser extent by cAMP (20-fold, designated cG-PK). The specific activity of this peak was ⩽85 nmol P_i transferred . min^{-1} . mg protein^{-1}. On development of the column with a linear salt gradient 2 cAMP-dependent PK could be separated. These enzymes are similar to those isolated from most tissues of higher organisms [8,9], therefore they were designated cA-PK I and II. cA-PK I eluted around 50 mM NaCl, maximal stimulation with cAMP was 17-fold, spec. act. ~80 nmol P_i transferred . min^{-1}. mg protein^{-1}. cA-PK II was obtained at ~130 mM NaCl. This peak was stimulated 20-fold by cAMP and had spec. act. 120 nmol P_i transferred . min^{-1} . mg protein^{-1}. Compared to the ciliary extract a 10–20-fold purification of the PK activities was obtained. Rechromatography of the combined fractions of the

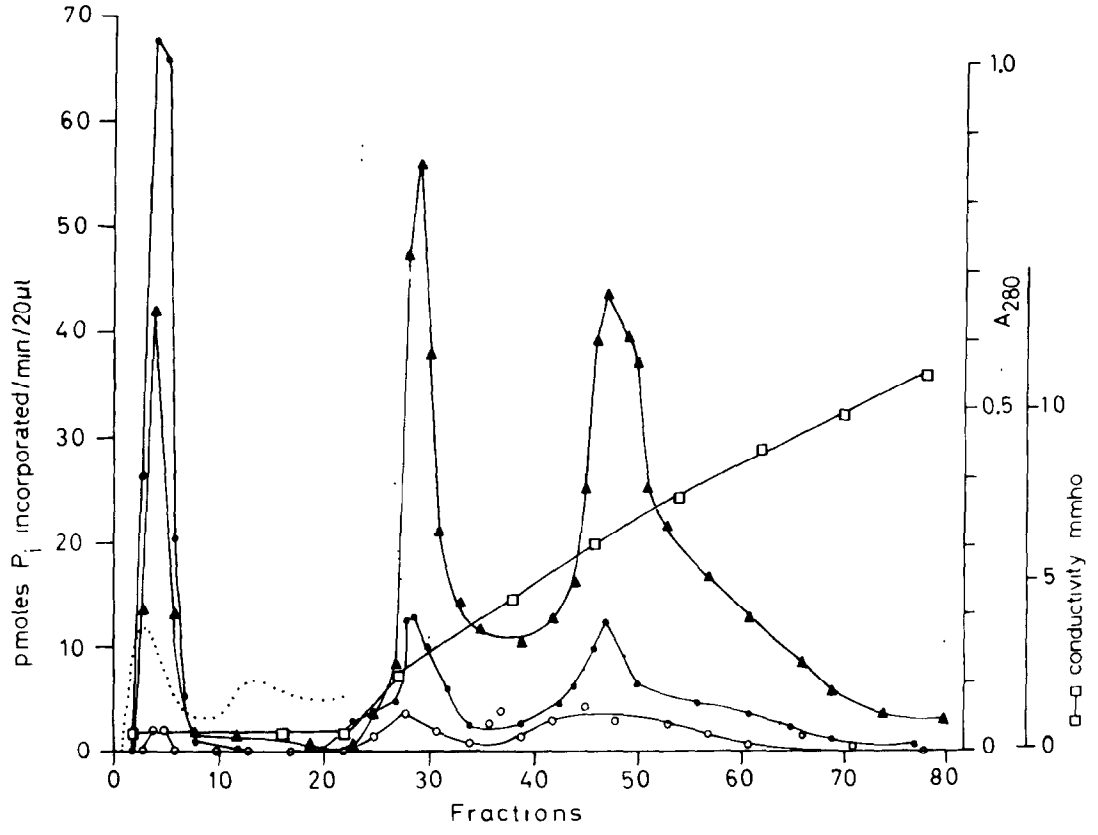

Fig.1. Separation of cAMP- and cGMP-dependent protein kinase activities from ciliary extracts by DEAE-cellulose chromatography. The dialyzed extract was applied onto the column equilibrated with 5 mM MOPS (pH 6.5), 15 mM 2-mercaptoethanol, 9 mM NaCl. A NaCl gradient up to 300 mM was used. (○——○) basal enzyme activity; (●——●) + 1 μM cGMP; (▲——▲) + 1 μM cAMP.

cG-PK on a second DEAE-cellulose column showed that stimulation of this peak by cAMP was not due to contamination with soluble cA-PK I or II.

The relative amounts of the enzymes were estimated by peak integration of the elution patterns: cG-PK and cA-PK I eluted in comparable amounts whereas cA-PK II activity was always > 3-fold higher than cA-PK I or cG-PK. When the PK-fractions from the DEAE column were assayed for cAMP binding, it was only found associated with catalytic activity. Also, no cyclic nucleotide-independent PK were found in the DEAE-cellulose eluate. Thus it appears that no significant amounts of free regulatory or catalytic subunits from PK were present.

3.2. *pH optima and ion requirements*

pH optima for the 3 PK were determined using MES (pH 5.5–6.8), MOPS (pH 6.5–7.9) and Tris (pH 7.0–9.0) as buffer systems. cA-PK I and II exhibited an optimum activity at pH 6.0–6.2, while the cG-PK had a rather broad pH optimum between pH 6.0–7.5. All 3 enzyme fractions strictly require Mg^{2+} for activity, 10 mM being optimal. The cilia of *Paramecium* are the site of the transient calcium inward current, the calcium channel being also permeable to barium ions. Therefore, it was of particular interest to study the effects of Ca^{2+} and Ba^{2+} on PK activity. Ca^{2+} at 2mM or ~20 mM Ba^{2+} inhibit cA-PK I and II by ~50% (table 2). The cG-PK, however, is much more sensitive to Ca^{2+}, with 17% inhibition at 100 μM and half-maximal inhibition at 630 μM $CaCl_2$. The latter data may justify further investigations as to a regulatory function of Ca^{2+} in conjunction with cG-PK.

Table 2
Effect of divalent cations on protein kinase activities

Enzyme fraction	Half-maximal inhibition	
	$CaCl_2$ (μM)	$BaCl_2$ (mM)
cA-PK I	2	20
cA-PK II	2	25
cG-PK	0.6	20

Enzymes stimulated by 1 μM cAMP (cA-PK I and II) or 1 μM cGMP (cG-PK) were tested under standard conditions with addition of $CaCl_2$ and $BaCl_2$ in the range 1 μM–50 mM

3.3. *Activation by cAMP and cGMP*

In fig.2, the cyclic nucleotide dependence of the 3 enzyme fractions is shown. The apparent activation constants for cA-PK I were 70 nM and for cA-PK II 40 nM with cAMP, the affinities for cGMP being ~2 orders of magnitude less. With the cG-PK fraction a non-parallel half-logarithmic plot was obtained for cAMP and cGMP stimulation (fig.2). Half-maximal activation ocurred at 100 nM cGMP and 1 μM cAMP.

3.4. *Specifities for proteins and nucleotides as substrates*

The ciliary PK accept mixed histones and histone H2B equally well as substrates in contrast to many

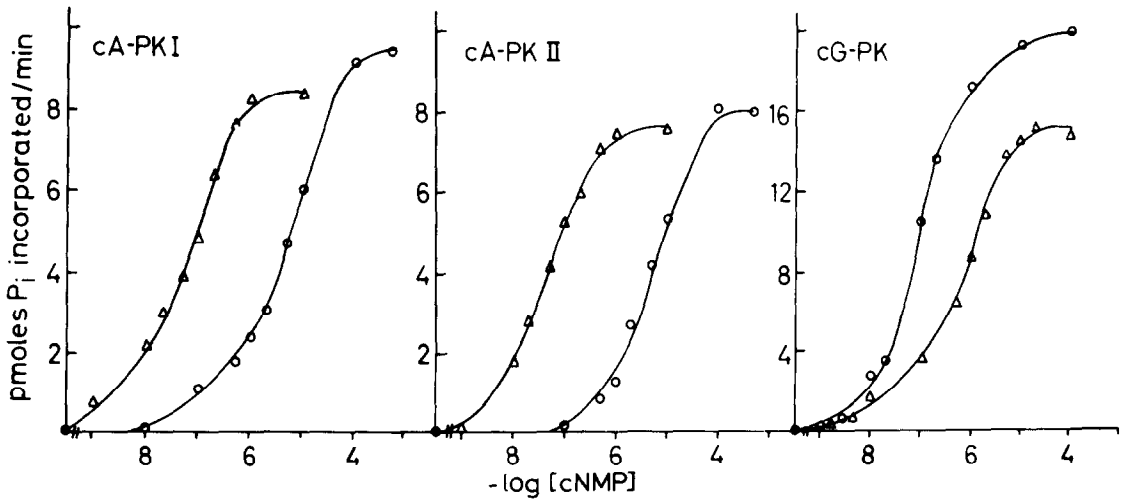

Fig.2. Effect of cyclic nucleotides (cNMP) on protein kinase activities. Combined fractions of the DEAE chromatography peaks were tested with the cNMP concentrations indicated. Activity in the absence of cNMP was subtracted from all values. (△——△) + cAMP; (○——○) + cGMP.

other PK which phosphorylate histone H2B much better than mixed histones. Protamine is barely phosphorylated, and casein is only partially accepted as substrate (~5% and ~40% compared to histones, respectively).

Using GTP instead of ATP as substrate cA-PK I and II show almost no enzymic activity (<0.5%). This finding is in agreement with data on other cAMP-dependent PK [10]. However, using cG-PK 30% of enzyme activity is measured with 50 μM GTP instead of ATP as substrate. For this unusual property the presence of a phosphotransferase activity [11] can be excluded since all of the endogenous nucleotides have been removed by the DEAE chromatography and extensive dialysis.

The presence of PK in cilia from *Paramecium* may indicate that these enzymes participate in the function of the excitable membrane. The specific activity of the soluble extract is very high compared to other tissues, e.g., 10–20-fold higher than rabbit skeletal muscle extracts [12]. The characteristics of the 2 cAMP-dependent PK seem to be similar to those of type I and II cAMP-dependent PK isolated and characterized from other tissues [12,13]. The cG-PK, however, exhibits a quite unusual behavior in respect to pH dependence, stimulation by cyclic nucleotides and apparent affinity for GTP as substrate. In *Tetrahymena*, a ciliary PK has been identified which is stimulated by cAMP and cGMP in a similar manner [14].

At present, nothing can be said about the functional role of PK in connection with the excitable ciliary membrane. The search for the individual endogenous substrates and the investigation of behavioral mutants of *Paramecium tetraurelia* should help toward this goal.

Acknowledgements

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