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Identification and partial characterization of cAMP-phosphodiesterases in the ciliate *Euplotes raikovi*

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Abstract

In the ciliate *Euplotes raikovi*, two specific isoforms of cAMP-dependent phosphodiesterases were identified, one in the soluble and the other in the particulate fraction of the cell. Their activity was shown to be stimulated by Mg^{2+}, insensitive to Ca^{2+} and cGMP, and scarcely inhibited by theophylline and 3-isobutyl-1-methyl-xanthine. They appear to be related to some phosphodiesterases of class II of other unicellular organisms in their biochemical features, and their enzymatic activity is up-regulated by elevation of intracellular cAMP level similarly to PDE-4 isoforms of mammals.

Keywords: cAMP regulation; Phosphodiesterases; Ciliate physiology

Introduction

In the ciliate *Euplotes raikovi*, intracellular cAMP levels are modulated by the interaction between cell type-specific proteins (called pheromones) and their cell surface receptors. These receptors are represented by membrane-bound isoforms of the same soluble pheromones derived from a process of alternative splicing of the primary transcripts of the pheromone genes (Miceli et al., 1992; Ortenzi et al., 2000). A low, basal cAMP level is maintained as long as the cells interact with their secreted pheromone, which promotes the vegetative (mitotic) cell growth in an autocrine fashion (Vallesi et al., 1995). When this interaction is interrupted, and this can occur either by removing the cell's own pheromone from the extracellular environment, or by competition of a different pheromone that binds to the same pheromone receptors (Apone et al., 2003), a temporary increase of cAMP concentration in the cell is observed. Cells respond to any raising of the intracellular cAMP concentration by reducing their rates of DNA synthesis and multiplication.

To acquire new insight into cAMP signaling and regulation of *E. raikovi*, we studied phosphodiesterases (PDEs) which hydrolyze cAMP to the 5′-adenosine monophosphate. Studies of PDEs in multicellular organisms (Mehats et al., 2002) and in some unicellular organisms, such as *Dictyostelium* (Franke and Kessin, 1992) and *Trypanosoma* (Zoraghi et al., 2001; Rascon et al., 2002), revealed the presence of several distinct PDE forms, each one with a specific localization and function in the regulation of cAMP signaling. In free-living protozoa such as ciliates, the biological role of PDEs remains largely unexplored. This study describes the identification in *E. raikovi* of two PDE isoforms, specific for cAMP and differently localized in the cell. They possess biochemical features similar to those reported for the PDEs of class II found in unicellular organisms; however, like PDE-4 isoforms of mammalian cells (Oki et al., 2000; Takahashi et al., 2001), they are regulated by the intracellular cAMP concentration.

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Materials and methods

Materials

Radioactive cAMP and cGMP were purchased from Amersham Biosciences (Little Chalfont, England); the phosphodiesterase inhibitors 4-3-cyclopentyloxy-4-methoxyphenyl-2-pyrrolidone (Rolipram) and 1,4-Dihydro-5-(2-propoxyphenyl)-7H-1,2,3-triazolo[4,5-d]pyrimidine-7-one (Zaprinast) from Calbiochem (La Jolla, CA); theophylline, 3-isobutyl-1-methyl-xanthine (IBMX), and additional materials for phosphodiesterase assays and chromatographic separations from Sigma Chemical Co. (St. Louis, MO).

Cells and pheromones

Cells used in this study belong to a strain derived as sexual (conjugal) offspring from the wild-type strain No. 13 of *E. raikovi* (that has been deposited, under the Accession No. 1624/19, at the Culture Collection for Algae and Protozoa, CCAP, Ambleside, UK). They secrete only pheromone E*r*-1, consistent with a determined homozygous combination at their *mat* (mating-type) locus (Luporini et al., 1986), and were grown at a density of about 10^4/ml on green algae *Dunaliella tertiolecta* under controlled conditions at 22–24°C.

Preparation of particulate and soluble fractions

Cells were concentrated by centrifugation, washed with 20 mM Tris-HCl buffer, pH 7.5, to eliminate sea salts, suspended in the same buffer containing 1 mM phenylmethylsulfonylfluoride (PMSF), 5 μg/ml leupeptin and pepstatin as protease inhibitors, and lysed by sonication. The particulate fraction was separated from the soluble fraction by centrifugation at 100,000*g* at 4°C for 30 min. Protein concentration was determined by the method described by Bradford (1976).

Measurements of phosphodiesterase activity

Phosphodiesterase activity was determined according to the method of Thompson and Appleman (1971). Aliquots of particulate or soluble fraction (0.1–0.3 mg/ml) were incubated in a 200-μl final volume, for 5 min at 20°C, with PDE buffer: 50 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 3 mM β-mercaptoethanol, 0.2 mM cAMP, 0.05 μCi (1.85 kBq) of [^{3}H]cAMP. To measure cGMP-phosphodiesterase activity, we used 0.05 mM cGMP and 0.1 μCi (3.7 kBq) of [^{3}H]cGMP instead of cAMP. The samples were then boiled for 2 min, cooled on ice, and incubated for 20 min at 34°C with 50 μg of *Crotalus atrox* snake venom, to convert [^{3}H]AMP into [^{3}H]adenosine. The reaction products were separated by anion-exchange chromatography on a Dowex 1 × 2–400 mesh resin, and the amount of [^{3}H]adenosine was determined with a β-counter. The enzymatic activity was linear with respect to time for 5 min, and total values of 7,000–13,000 c.p.m. were measured for each minute of the reaction (variations depending on protein concentration), while the blank values ranged from 4,000 to 6,000 c.p.m. This activity was expressed as nanomoles of cAMP hydrolyzed per min and mg of protein, and calculated by dividing the radioactivity values by the specific activity of [^{3}H]cAMP. To study the inhibitor effects on the PDE activity, aliquots of soluble and particulate fractions were incubated for 5 min with the inhibitor before performing the enzymatic assay.

Phosphodiesterase isoform separations

Separation of PDE isoforms by ion-exchange chromatography was performed according to the method of Russell et al. (1973). The particulate fraction was solubilized with 0.5% 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate (CHAPS) in 50 mM Tris-acetate buffer, pH 6.0, 1 mM PMSF, for 4 h at 4°C, centrifuged at 100,000*g* for 30 min, and adsorbed onto DEAE-Sephacel columns, pre-equilibrated with Tris-acetate buffer containing 4 mM β-mercaptoethanol. The soluble fraction was diluted in Tris-acetate buffer before loading onto the columns. Columns were washed with Tris-acetate buffer containing 4 mM β-mercaptoethanol (no PDE activity was recovered in the column wash) and then eluted with a linear gradient from 0 to 1 M sodium acetate, with a constant flow rate of 0.25 ml/min (for the particulate fraction, all the steps were performed in the presence of 0.5% CHAPS). Aliquots (100 μl) of each collected fraction were diluted with 100 μl of PDE buffer and directly assayed for the PDE activity.

Phosphodiesterase isoform separation by electrophoresis was performed according to the method of Goren et al. (1974). Aliquots of both particulate and soluble fractions were applied on 7.5% polyacrylamide gels, and electrophoresis was carried out in native conditions in the presence of 0.1% Triton-X 100, at 4°C for 6 h. Gels were then washed in 0.1 M Tris-maleate buffer, pH 7.0, for 30 min, and incubated with Tris-maleate buffer containing 30 mM lead nitrate, 2.5 mM magnesium sulfate, 1 U/ml bacterial alkaline phosphatase, 2 mM cAMP or cGMP, at 30°C for 4 h. Gels were then washed with water for 1 h, and treated with 3% ammonium sulfide until black bands of precipitate, corresponding to the sites of phosphodiesterase activity, appeared on the gels.

Results and discussion

Localization of PDE activity

About 70% of the total PDE activity of *E. raikovi* was found associated with the particulate fraction obtained by centrifugation of total cell lysates, and was measured to be around 100 nmol of hydrolyzed cAMP per min and per mg of protein. The other 30% of the total PDE activity was found in the soluble fraction, and was measured to be around 45 nmol of hydrolyzed cAMP per min and per mg of protein.

To assess whether this activity was due to one or more PDE isoforms differently localized in the cell, protein preparations obtained from both the soluble and the particulate fractions were separated by DEAE ion-exchange chromatography. As shown in Fig. 1, in the particulate fraction the maximum of PDE activity eluted at 0.6 M of salt, while in the soluble fraction the maximum of PDE activity was found in the material eluted with 0.4 M of salt, thus suggesting that two distinct cAMP-PDE isoforms were associated with the fractions. This result was confirmed by an alternative approach to separate PDE isoforms, in which aliquots of particulate and soluble material were separated on polyacrylamide gels in native conditions and subjected to an *in situ* enzymatic assay according to the procedure described by Goren et al. (1974). As shown in Fig. 2, only one band of cAMP hydrolyzing activity was detected in each the fraction. cAMP was then replaced by cGMP to visualize, in an analogous electrophoretic separation, cGMP hydrolyzing enzymes. In the soluble fraction, the band corresponding to cGMP-PDE activity had a different electrophoretic mobility compared with the cAMP-PDE isoform. Since the band detected in the particulate fraction by using cGMP as substrate had similar electrophoretic mobility to the band relative to the cAMP-PDE isoform, we next assessed whether the same PDE was able to hydrolyze both the nucleotides by measuring the cAMP-PDE activity in the presence of different concentrations of cGMP as competitor. This activity was found to be specific for cAMP, since it was

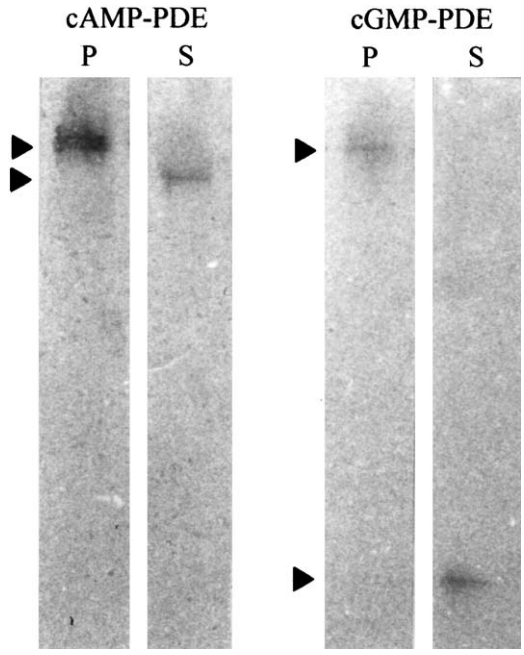


Fig. 2. Separation of the PDE isoforms by electrophoresis. Proteins of particulate (P) and soluble (S) fractions were separated by native 7.5% polyacrylamide gel electrophoresis. cAMP-PDE and cGMP-PDE activities (arrowheads) were revealed by *in situ* enzymatic assays, using cAMP and cGMP as respective substrates.

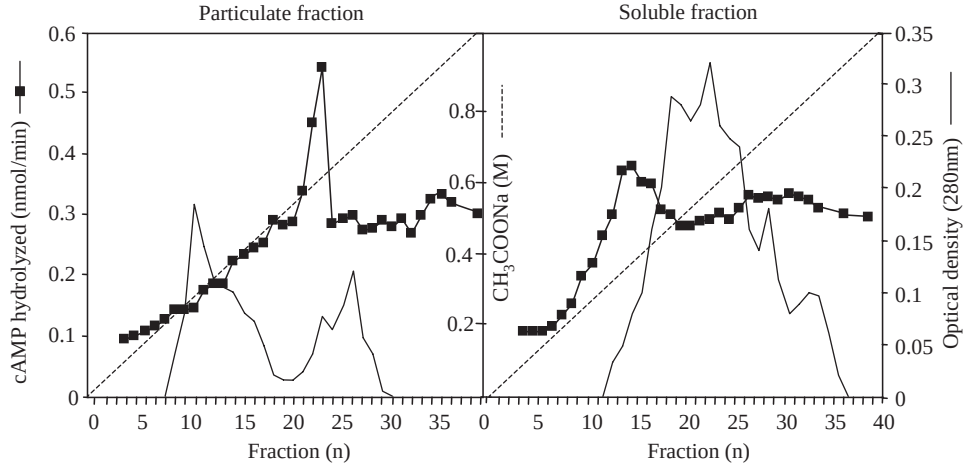


Fig. 1. Separation of the cAMP-PDE isoforms by ion-exchange chromatography. Particulate and soluble fractions, obtained by centrifugation of cell lysates, were applied to DEAE-Sephacel columns, and eluted with a linear gradient of sodium acetate, from 0 to 1 M (dotted lines); each collected fraction was directly analyzed for cAMP-PDE activity (filled squares) and protein concentration, measured as OD at 280 nm (solid lines).

Table 1. Effects of different molecules on cAMP-PDE activity of particulate and soluble fractions of *E. raikovi* cells

Added molecules	cAMP hydrolyzed (nmol/min/mg)	
	Particulate fraction	Soluble fraction
No addition	103.8	44.2
cGMP (0.02 mM)	101.9	42.2
cGMP (0.2 mM)	104.7	42.4
cGMP (2 mM)	98.7	41.2
Ca^{2+} (0.1 mM) + calmodulin (50 U/ml)	104.8	43.5
Ca^{2+} (1 mM) + calmodulin (50 U/ml)	101.9	44.3
EGTA (0.01 mM)	102.8	43.2
EGTA (0.1 mM)	99.3	44.6
Rolipram (0.02 mM)	101.9	45.6
Rolipram (0.2 mM)	99.2	38.7
Rolipram (2 mM)	93.5	34.9
Zaprinast (0.2 mM)	103.4	44.3
Zaprinast (2 mM)	105.0	43.2
PKA (5 U/ml)	102.7	44.0
PKA (50 U/ml)	100.1	41.0

Values (with a SD of about ±5 for the particulate fraction and ±3 for the soluble fraction) are means of three independent measurements obtained from one representative experiment.

not inhibited by cGMP (Table 1). Therefore, the bands visualized using cAMP and cGMP as substrates corresponded to distinct PDEs.

Effects of cations and inhibitors on PDE activity

The cAMP-PDE activity of both the particulate and the soluble fractions was stimulated by the addition of Mg^{2+} (Fig. 3), indicating that the two *E. raikovi* isoforms have a metal ion binding site. On the other hand, it was not affected by Ca^{2+} (used in presence of calmodulin alone, and in combination with Mg^{2+}), or by Ca^{2+}-chelator EGTA (Table 1), and showed low sensitivity to "non-selective" PDE inhibitors, such as IBMX and theophylline, which can inhibit almost all types of PDEs of multicellular organisms (Mehats et al., 2002). For the cAMP-PDE activity associated with the particulate fraction, IC_{50} values of IBMX and theophylline of 10 mM and 2 mM were measured, respectively. Similar values of sensitivity to methylxanthines have been reported for some PDEs of other unicellular organisms, such as those characterized in *Dictyostelium*, *Candida* and *Leishmania* (Orlow et al., 1981; Hoyer et al., 1994; Rascon et al., 2000). A weak inhibitory effect was also produced, at a 2 mM concentration, by Rolipram, a "selective" PDE inhibitor of PDE-4 isoforms of mammalian cells (Jin et al., 1998). No effect was observed when Zaprinast, i.e., a selective inhibitor of PDE-5 (Turko et al., 1999), was used in the assay instead of Rolipram (Table 1).

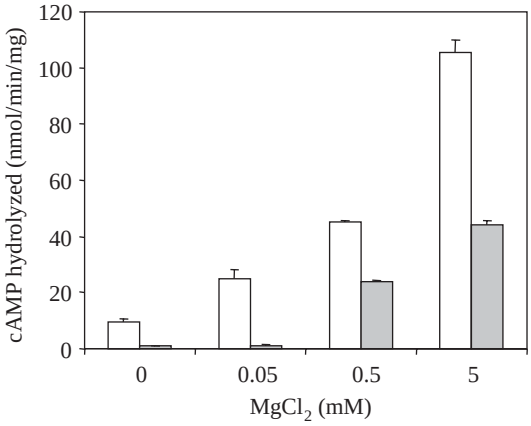
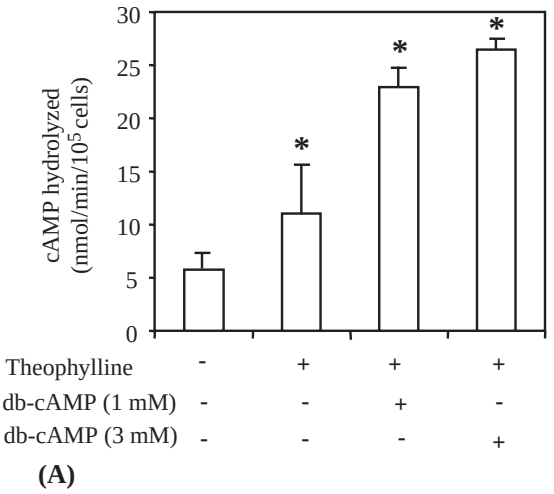


Fig. 3. Effect of Mg^{2+} on the cAMP-PDE activity. Particulate and soluble fractions (white and gray columns, respectively) were incubated with increasing concentrations of Mg^{2+} prior to the enzymatic assay. Values are means of three independent measurements from a representative experiment.

Effects of variations of the intracellular cAMP level on PDE activity

In a previous study (Apone et al., 2003), it was observed that cells conditioned to raise their cAMP level by pheromone removal, increase their cAMP-PDE activity within a few hours after resuspension in the absence of their secreted pheromone. To assess whether the PDE activity responds to variations in the intracellular cAMP concentration, cell cultures were maintained in the presence of their secreted pheromone (at a concentration of about 0.3 μg/ml), or resuspended in fresh seawater in the presence of theophylline, with or without the cell-permeable cAMP analog, dibutyryl-cAMP (db-cAMP). At 3 h from the resuspension, cells were washed free from theophylline with 3 volumes of fresh seawater, and the PDE activity was measured in total cell lysates (since the two PDE isoforms have similar biochemical features). As shown in Fig. 4A, in cells suspended with theophylline, the cAMP-PDE activity increased significantly in comparison with the activity measured in cultures maintained in the presence of their secreted pheromone. This increase was even higher when db-cAMP was also added to the cultures. When cells were resuspended in fresh seawater in the presence of theophylline with increasing concentrations of their secreted pheromone, the stimulation of the cAMP-PDE activity appeared to be completely neutralized, while no variation was detected in cGMP-PDE activity (Fig. 4B).

These results suggest that cAMP-PDE activity is up-regulated by cAMP. For the yeast PDE 1 (Ma et al.,



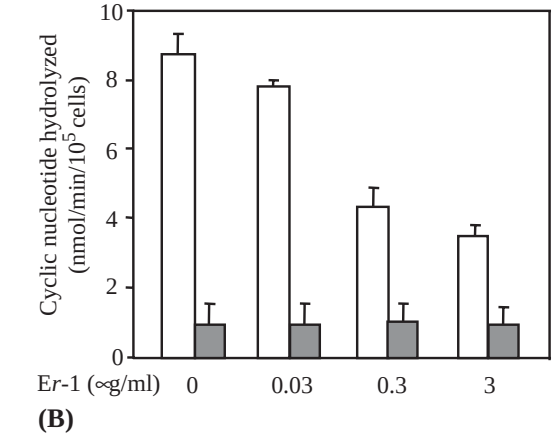


Fig. 4. Effects of variations of the intracellular cAMP concentration on the cAMP-PDE activity. (A) Cells were left unchanged or resuspended in fresh seawater in the presence of theophylline (2 mM), with and without db-cAMP, at the indicated concentrations. (B) Cells were resuspended in fresh seawater in the presence of theophylline (2 mM), with and without increasing concentrations of their secreted pheromone (E*r*-1). Cell samples were then removed at 3 h from the resuspension, washed free from theophylline with 3 volumes of fresh seawater, and lysed by sonication. The cAMP-PDE activity (white columns) and the cGMP-PDE activity (gray columns) were measured in total cell lysates. Values are means (+SD) of three independent measurements from a representative experiment. (*) Significantly different from the control by ANOVA, $P<0.05$.

1999) and for the PDE-4 isoforms of higher eukaryotes (Oki et al., 2000; Takahashi et al., 2001), that are up-regulated by increases of intracellular cAMP level, it has been shown that the mechanism of PDE activation is mediated by cAMP-dependent protein kinase (PKA), which can either phosphorylate PDE directly, or up-regulate its expression through mRNA transcription and protein synthesis control (Francis et al., 2001). To verify whether the cAMP-PDEs of *E. raikovi* were affected by phosphorylation, aliquots of particulate and soluble fractions were incubated with and without the catalytic subunit of a commercial preparation of bovine PKA in the presence of ATP, and then used in the enzymatic assay. The PDE activity measured in aliquots incubated with PKA did not appreciably change in comparison with the activity measured in aliquots not incubated with PKA (Table 1), thus suggesting that the cAMP-mediated control of the PDE activity was essentially due to a regulation at transcriptional level. However, we could not verify this hypothesis because protein synthesis in *E. raikovi* was not reduced by the tested antibiotics, even when they were used at much higher concentrations than those commonly used with other cell types.

Conclusions

We have identified two cAMP-PDE isoforms of *E. raikovi*, which are specific for cAMP since their activity is neither stimulated nor inhibited by cGMP. It was shown that their sensitivity to selective and non-selective PDE inhibitors is very low. This can be explained by considering that the sensitivity to inhibitors depends not only on the primary structure and conformation of the PDE catalytic site (which is located at the carboxyl terminus and has the highest level of conservation among the different organisms), but also on the structure of the amino-terminal region, which is the least conserved domain even among closely related organisms (Saldou et al., 1998). For the low sensitivity to PDE inhibitors and the lack of stimulation by Ca^{2+}, these isoforms appear to be different from the class I PDEs found mostly in higher eukaryotes. This large class of enzymes includes the mammalian PDEs (Soderling and Beavo, 2000), the PDEs of the freshwater sponge *Ephydatia fluviatilis* (Koyanagi et al., 1998), the product of the *dunce* gene of *Drosophila* (Chen et al., 1986), the *Caenorhabditis elegans* PDEs (Li and Baehr, 1998), the yeast PDE2 (Sass et al., 1986), RegA of *Dictyostelium* (Franke and Kessin, 1992) and two PDEs of *Trypanosoma* (Zoraghi et al., 2001; Rascon et al., 2002). *E. raikovi* PDEs thus appear to be similar to class II PDEs, such as those characterized in *Dictyostelium*, *Candida* and *Leishmania* (Orlow et al., 1981; Hoyer et al., 1994; Rascon et al., 2000), which show very little amino acid sequence identity with the class I enzymes.

We also observed that *E. raikovi* cAMP-PDE activity is up regulated by increases of intracellular cAMP level. Analogous regulation by cAMP has been reported for the PDE-4 isoforms of mammalian cells (Oki et al., 2000; Takahashi et al., 2001), as well as for yeast PDE1 (Ma et al., 1999). This type of regulation is of primary importance to keep under control any induced cAMP

variation and to restore the cAMP level required for normal cell growth.

The results presented here provide the preliminary basis to determine the structural relationships of *E. raikovi* PDEs with their counterparts in other organisms and to study, more generally, the evolution of this class of enzymes.

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