A CALCIUM-DEPENDENT PROTEIN ACTIVATOR OF MAMMALIAN CYCLIC NUCLEOTIDE PHOSPHODIESTERASE FROM *BLASTOCLADIELLA EMERSONII*

Suely Lopes GOMES, Lélia MENNUCCI and José Carlos da Costa MAIA
Departamento de Bioquìmica, Instituto de Química, Universidade de São Paulo, Caixa Postal 20780, São Paulo 05508, Brasil

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1. Introduction

A heat-stable, acidic calcium-binding protein (pI 4.3), with mol. wt 18 920 and originally described as an activator of cyclic nucleotide phosphodiesterase [1], has been purified and characterized from a variety of mammalian tissues [2–5], as well as from electroplax of the electric eel [6]. This protein has been shown to exhibit multiple calcium-dependent regulatory activities, including activation of brain adenylate cyclase [7], human erythrocyte membrane (Mg^{2+} + Ca^{2+})-dependent ATPase [8], protein kinase [9] and other calcium-sensitive reactions [10,11]. More recently, crude extracts of > 10 representative invertebrate species were examined and all were found to have the activator protein [12], referred to as calmodulin [1].

Thus, calmodulin appears to be ubiquitous in the animal kingdom. Moreover, it lacks tissue and species specificity, suggesting that it may be a very primitive protein. However, the occurrence of such a protein in bacteria or lower eukaryotes has not been described.

Here we report the occurrence, in cells of the phycomycete *Blastocladiella emersonii*, of a protein with physico-chemical properties similar to those of calmodulin. The lack of correlation between the levels of cyclic nucleotide phosphodiesterases and of the calcium-binding protein throughout the life cycle of the fungus, as well as our failure to demonstrate the existence of a Ca^{2+}-activatable phosphodiesterase in this organism, suggest that the activator has another function(s) in primitive eukaryotic cells.

2. Experimental

Stocks of *B. emersonii* were kept at 26°C on Difco agar plates. In order to obtain cells at different stages of the life cycle, zoospores obtained as in [13] were inoculated into DM_4 growth medium [14] and the cultures kept at 27°C in a gyratory shaker. In the sporulation experiments, the growth medium was replaced by buffered $CaCl_2$ [13,15] after 6 h growth, when all the cells are in the sporangial stage, and incubated until completion of sporulation (~4 h).

At different times in the life cycle, cells were harvested by centrifugation (1000 × *g*, 3 min) at 4°C, resuspended in 3 vol. buffer containing 40 mM Tris–HCl (pH 7.5), 1 mM magnesium acetate and 0.15 mg/ml phenylmethane-sulphonyl fluoride and broken in a French press cell at 1000 p.s.i. The extracts were centrifuged at 10 000 × *g* for 30 min and the supernatant fractions were then heated at 95–100°C for 6 min; precipitated proteins were removed by centrifugation and analysis of the activator was performed on the supernatant.

Activator-deficient cyclic nucleotide phosphodiesterase was prepared from bovine heart as in [2]. The protein activator from beef heart was purified by the method in [2] up to the first DEAE-cellulose column chromatography.

The activator protein was assayed on the basis of its ability to stimulate a fixed amount of the activator-deficient bovine heart phosphodiesterase. The enzyme activity was measured by the radiochemical, two-step assay [16], as in [14]. Standard reaction mixtures

contained 50 mM Tris–HCl buffer (pH 7.5), 1 mM magnesium acetate, 0.1 mM $CaCl_2$ and 1 mM cAMP (40 000 cpm, c[^{3}H]AMP) in 0.1 ml final vol. Incubation was at 30°C for 30 min. One unit of activator is defined as the amount which is required to give 50% stimulation of the standard amount of enzyme. The specific activity of the activator in fig.4 represents units/mg protein in the extract following homogenization in the French press.

The cyclic nucleotide phosphodiesterase activities of *Blastocladiella* were determined in the crude homogenates as in [17].

Analytical disc gel electrophoresis was done as in [18], using 12% polyacrylamide gels.

Protein was measured by the Lowry method [19], using bovine serum albumin as standard.

3. Results and discussion

The possible occurrence of a protein activator in cells of *Blastocladiella* was investigated using an activator-deficient phosphodiesterase from bovine heart. Figure 1 shows that, as in the case of purified bovine heart protein-activator, the activation produced by *Blastocladiella* extracts assumes the form of a

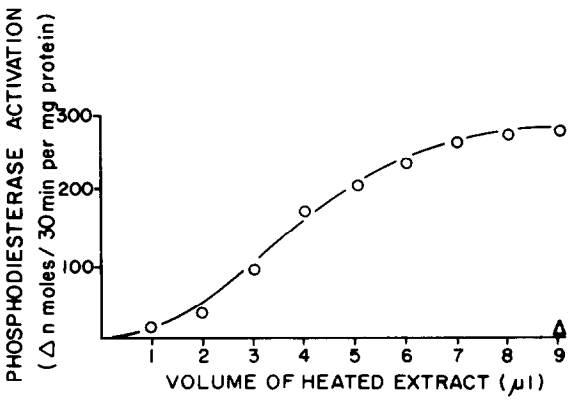

Fig.1. Dose–response curve for the activation of activator-deficient bovine heart phosphodiesterase by extracts of *Blastocladiella*. The enzyme was assayed in the presence (△–△) or absence (○–○) of 300 μm EGTA with various amounts of the heat-treated *Blastocladiella* homogenate supernatant. Enzyme activation is expressed as the difference in activity between the activated and the control (with no added homogenate supernatant) samples.

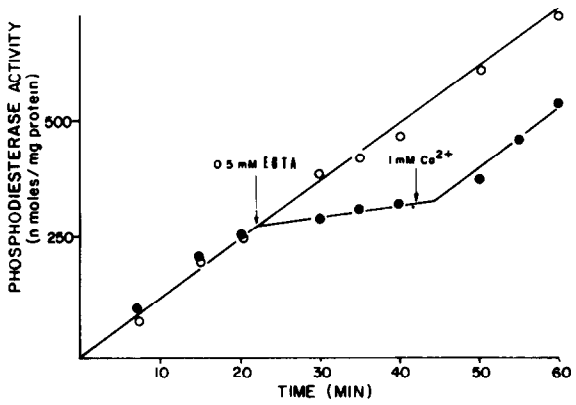

Fig.2. Reversibility of the phosphodiesterase activation: Time course of activator-deficient bovine heart phosphodiesterase reaction in the presence of 100 μM Ca^{2+} and 10 μl *Blastocladiella* heated-supernatant extract. Two identical incubation mixtures were prepared; one of them was retained throughout the course of the experiment as a control (○–○), and the other one (●–●) was adjusted to 0.5 mM EGTA at 22 min and to 1 mM Ca^{2+} at 42 min. Aliquots were removed at indicated times and analysed for cyclic AMP degradation.

sigmoidal ligand saturation curve and can be completely abolished by ethylene glycol bis(β-aminoethyl ether)*N*,*N*′-tetracetic acid (EGTA). The extent of enzyme activation by the *Blastocladiella* extracts is about 4-fold, approximately the same level as that achieved with bovine heart activator [2].

Figure 2 depicts the results of experiments which demonstrate the reversibility of phosphodiesterase activation by the factor. The activation of the enzyme by the extract can be effectively suppressed by addition of EGTA. Subsequent addition of Ca^{2+} in excess of EGTA restores the initial rate of product formation. This result excludes the possibility that activation of bovine heart phosphodiesterase by the *Blastocladiella* extracts might be due to limited proteolysis [20].

The activation of mammalian phosphodiesterase by extracts from lower animals was interpreted as suggesting that the structure of the protein activator has been conserved during evolution [12]. As is shown in fig.3, when heat-treated, dialysed *Blastocladiella* extracts were subjected to electrophoresis on 12% polyacrylamide gels, the material capable of activating the phosphodiesterase migrated as a single band on the gel at a position identical to that of the protein

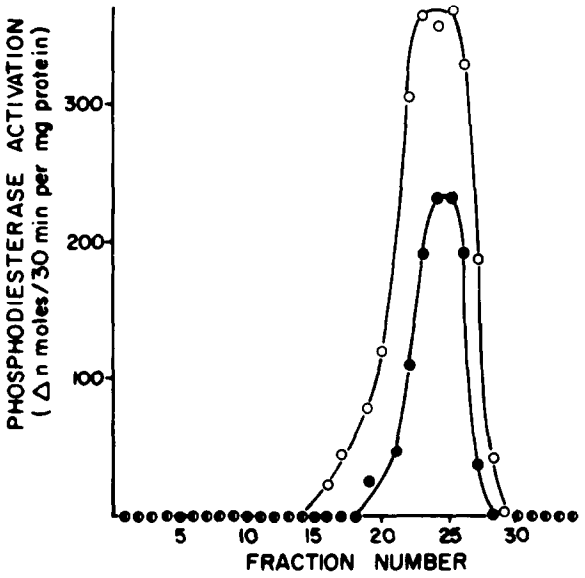

Fig.3. Gel electrophoresis of crude *Blastocladiella* extracts and purified protein activator from beef heart, as in [18] on 12% polyacrylamide gels. Heat-treated, dialysed homogenate supernatants of *Blastocladiella* (~200 units) (○–○) and bovine heart (~50 units) (●–●) were applied to the gels. After the run, the gels were fractionated in a gel fractionator (Savant Instruments Inc.), using 50 mM Tris–HCl buffer (pH 7.5) in 0.2 ml fractions. Aliquots (100 μl) were then assayed for Ca^{2+}-dependent bovine heart phosphodiesterase activation. Enzyme activation is expressed as the difference between the activated and the basal levels of enzyme activity.

activator from beef heart. These results strongly suggest that the activity of *Blastocladiella* extracts is attributable specifically to the protein activator. To confirm this, we determined the susceptibility of the activity to proteolysis. As expected for a protein activator, the activity of the extract is destroyed after 1 h incubation with trypsin (0.2 mg/ml) at 30°C (pH 7.5) in the presence of 0.1 mM EGTA [21].

Blastocladiella cells contain distinct enzymes which exhibit marked specificity for the hydrolysis of either cAMP or cGMP [17]. In addition, the level of these enzyme activities vary in a characteristic manner during the life cycle of the organism [14,22]. In order to establish whether the protein activator plays a role in the regulation of these variations, experiments designed to reveal the presence of a Ca^{2+}-activatable cyclic nucleotide phosphodiesterase in *Blastocladiella* were performed. Table 1 shows that the enzymes are

Table 1
Cyclic nucleotide phosphodiesterase of *B. emersonii*

Substrate	Activity (nmol/min.mg)	
	$+Ca^{2+}$ (100 μM)	+EGTA (500 μM)
cAMP 10 μM	1.4	1.7
cAMP 200 μM	3.0	4.0
cGMP 10 μM	1.0	1.0
cGMP 200 μM	5.0	5.0

not inhibited by EGTA under any of the conditions tested. In a further attempt to clarify the possible relationship between phosphodiesterases and protein activator, the relative amounts of activator activity were determined across the life cycle of the fungus. Figure 4 shows that, in contrast to the variation in cAMP phosphodiesterase activity, the protein activator level remains practically constant, both during the two phases of morphogenic transition (germination and sporulation) and during the growth phase. Although not shown in fig.4, the cGMP phosphodiesterase activity shows a pattern of variation very similar to that of the cAMP hydrolytic enzyme [22].

In summary, we have demonstrated the existence, in a fungal cell, of a heat-stable, non-dialysable, low molecular weight protein, which is capable of Ca^{2+}-

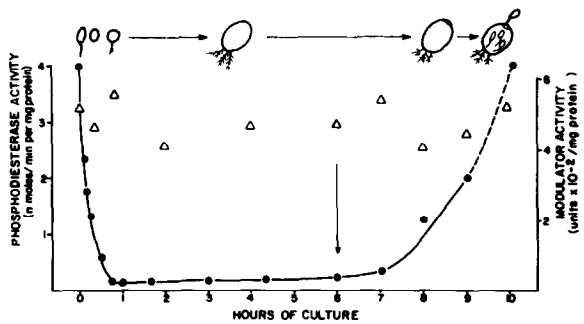

Fig.4. Comparison of cAMP phosphodiesterase (●–●) and protein activator activities (△–△) during growth and differentiation of *B. emersonii*. Measurements were as in the text. The drawings at the top represent the sequential appearance of different cell types during the life cycle, identified by examination under phase contrast microscope [13,15]; they are from left to right: zoospore, round cell, germling, vegetative cell and sporulating cell. The arrow indicates the time when growth medium was exchanged for buffered $CaCl_2$ to induce sporulation.

dependent activation of mammalian phosphodiesterase. Furthermore, the protein activators of both *Blastocladiella* and beef heart appear to have similar physical and chemical properties, the extent of activation of the phosphodiesterase and the mobility of the proteins on analytical disc-gel electrophoresis being identical.

The absence of a Ca^{2+}-activatable cyclic nucleotide phosphodiesterase in *Blastocladiella*, together with the lack of correlation between the levels of cyclic nucleotide phosphodiesterases and the activator during the life cycle, may indicate that the activator has another function(s), unrelated to cyclic nucleotide metabolism, in the fungus. These results would also imply that phosphodiesterases and protein activator are probably regulated separately at the gene level.

The relationship of this protein to the calcium transport observed during zoospore germination (S. L. G., L. M., J. C. C. M., in preparation), as well as to the phenomenon of vesiculation of the gamma particle, a zoospore organelle rich in Ca^{2+} and implicated in the initiation of cell wall synthesis [23], is presently being investigated.

Acknowledgments

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