THE PRECOCIOUS APPEARANCE AND ACTIVATION OF AN ADENYLATE CYCLASE IN A RAPID DEVELOPING MUTANT OF *DICTYOSTELIUM DISCOIDEUM*

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1. Introduction

Dictyostelium discoideum strain FR17 is a rapid developing mutant which appears to be altered at a single genetic locus [1]. This mutant commences aggregation in ~1/2 the time of its wild-type parent and it produces heat-resistant spores in about 3/4 the time [1,2]. Although the terminal structures of FR17 development are aberrant, the order of many biochemical and enzymatic changes during development is the same as in wild-type strains [2-4]. These observations suggest that the mutation(s) in FR17 alters the function of a single, early process which regulates the rate of expression of the developmental programme.

During development of *D. discoideum*, a variety of processes are regulated, at least in part, by cAMP. These include early cell differentiation [5,6], cell aggregation [7,8], stalk cell differentiation [9,10] and perhaps spore differentiation [11]. In addition, the time when the cells become aggregation competent can be accelerated by treating the cells with pulses of cAMP [12,13]. In an attempt to identify the primary biochemical defect in FR17, we have examined early cAMP metabolism in this mutant. We found that the concentration of cAMP in the mutant cells is elevated significantly during both vegetative growth and early development. Furthermore, the elevated cAMP pools in these cells can be explained by the precocious appearance and activation of an adenylate cyclase.

2. Materials and methods

Wild-type *D. discoideum* strain NC4 and a rapid developing mutant of this strain, HH201 (a temperature-sensitive derivative of FR17 [1]) were used. The amoebae were grown with *Enterobacter aerogenes* at 22°C either on SM agar plates [14] or in bacterial suspension [5]. Amoebae were harvested by centrifugation, washed twice in 17 mM phosphate buffer (pH 6.0) resuspended in the same buffer at $1-2 \times 10^7$ cells/ml and shaken at 22°C. At the times indicated, amoebae were collected by centrifugation, washed once in phosphate buffer and used in the experiments.

The average concentration of cAMP in the cells at various times during early development was determined as in [15]. Oscillations in the concentration of intracellular cAMP were detected by a modification of the method in [16]. A mixture of silicone oils AR20 and AR200, 800 μl (2:3, v/v), was layered over 500 μl of a solution of 5% glucose–5% trichloroacetic acid in a series of Eppendorf microcentrifuge tubes. At 1 min intervals, 200 μl cell suspension (2×10^8 cells/ml in phosphate buffer) was layered on top of the silicone oil in a tube and immediately spun at maximum speed in an Eppendorf centrifuge (model 3200) for 30 s. The buffer and silicone oil layers were removed by aspiration and the tubes was heated at 95°C for 10 min. The precipitate was then removed by centrifugation and the supernatant was frozen at −20°C until it was to be assayed for cAMP.

Cell lysates to be assayed for adenylate cyclase activity were prepared as in [17]. Each adenylate cyclase assay system contained in 100 μl total vol: 25 mM Tris–HCl (pH 7.5); 10 mM $MgCl_2$; 20 mM dithiothreitol; 1 mM cAMP; 10 mM creatine phosphate; 4 units creatine phosphokinase; 0.5 mM ATP; 1 μCi [α-^{32}P]ATP (Amersham); 100–300 μg cell protein; and, except where indicated, 10 mM $MnCl_2$.

The reaction was run at 25°C for 2 min and stopped by the addition of 100 μl 4% SDS in 50 mM Tris–HCl (pH 7.5). The cAMP product was separated by the method in [18] as modified [19]. Recovery of cAMP was 70–90% and the assay blanks were <0.0008% of total radioactivity.

Cell suspensions were treated with artificial cAMP pulses as in [15]. The time of appearance of EDTA-stable contacts (cs-A) on the cell surface was determined as in [12].

3. Results

Figure 1 shows the average concentration of cAMP in amoebae of strains NC4 and HH201 at various times during early development. For strain NC4, the cAMP pool was 2–3 pmol/10^7 cells at t_0 and it rose to a maximum of 10–12 pmol/10^7 cells at t_6 before decreasing to a level ~2-times the initial concentration. When the cells were pulsed with exogenous cAMP, the pools increased abruptly after t_3 reaching a peak of ~20 pmol/10^7 cells at t_4. The artificial cAMP pulses also accelerated the appearance of cs-A by ~4 h. These results are comparable to those obtained with other wild-type strains [15].

Under the same conditions, amoebae of strain HH201 at t_0 had cAMP pools of ~13 pmol/10^7 cells (fig.1B). After starvation, the pools increased to ~22 pmol/10^7 cells by t_3 and then decreased slowly after t_6. When the amoebae were pulsed with

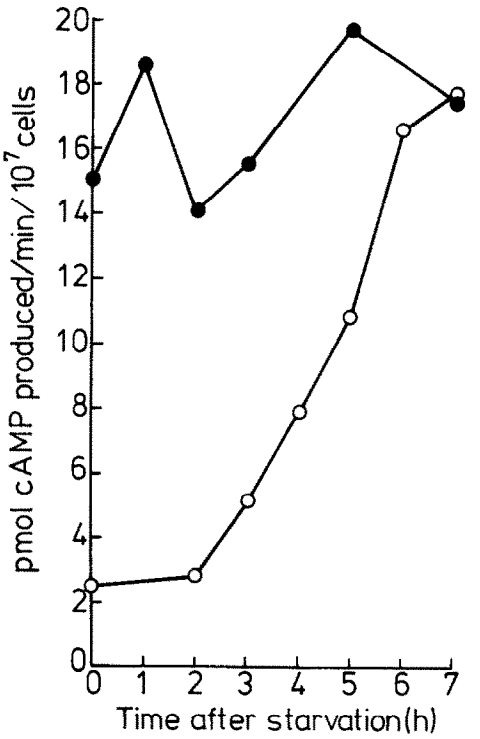

Fig.2. Adenylate cyclase activity in strains NC4 (○) and HH201 (●) during early development. The amoebae were grown in bacterial suspension to mid-log phase, washed and shaken in phosphate buffer. Each value is an average of duplicate or triplicate determinations.

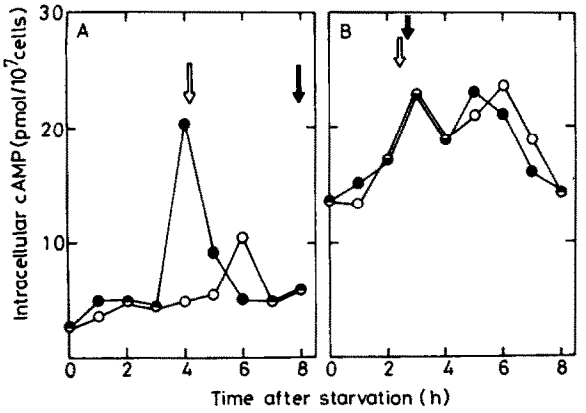

Fig.1. Changes in intracellular cAMP during early development. Amoebae of strains NC4 (A) and HH201 (B) were grown with bacteria on plates, washed and shaken in phosphate buffer. The suspensions were either incubated without treatment (○) or pulsed with 100 nM cAMP at 5 min intervals from t_0 (●). Each point is an average of 4 samples taken 90 s apart. Arrows indicate the time of appearance of cs-A on the pulsed (⇩) and on the unpulsed cells (↓).

Table 1
Effect of Ca^{2+} and Mn^{2+} on the adenylate cyclase activity of strains NC4 and HH201

Additions	% Activity		
	NC4(t_0)	NC4(t_6)	HH201(t_0)
None[a]	100	100	100
+ 1 mM Ca^{2+}	75	61	69
+ 10 mM Mn^{2+}	229[b]	233[b]	212[b]
+ 1 mM Ca^{2+}, 10 mM Mn^{2+}	215	253	204

[a] Complete assay system in section 2 minus Mn^{2+}

[b] The specific activities of the NC4(t_0), NC4(t_6) and HH201(t_0) lysates under optimal conditions were 2.6, 11.9 and 16.9 pmol/min/10^7 cells, respectively

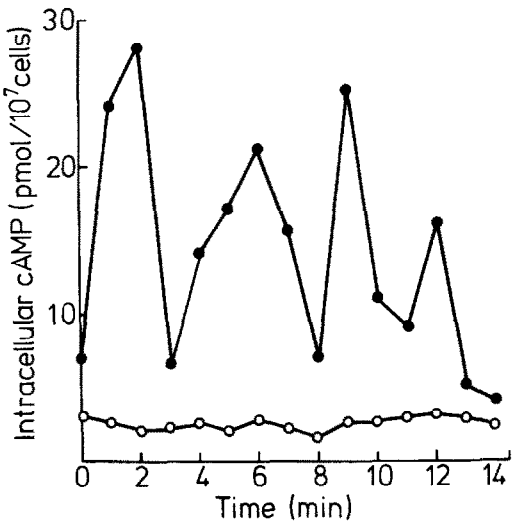

Fig.3. Concentration of cAMP in amoebae of strains NC4 (○) and HH201 (●) after 2 h starvation. The amoebae were grown with bacteria on plates, washed and shaken in phosphate buffer. After 2 h, the cells were washed once, resuspended in phosphate buffer at 2×10^8 cells/ml, shaken vigorously and assayed at 1 min intervals for intracellular cAMP.

exogenous cAMP, the intracellular cAMP profile was unaltered. In addition, cs-A appeared after 2–3 h starvation on both the cAMP pulsed and the unpulsed cells.

Since these experiments were performed with amoebae grown with bacteria on SM agar plates, we were concerned that the t_0 values might be inaccurate due to uneven growth on the bacterial lawns. Therefore, the cAMP pools in these strains were also measured in cells growing exponentially in bacterial suspension. Under these conditions, the average values obtained for NC4 and HH201 in 6 experiments were 1.5 ± 0.8 and 8.6 ± 2.5 pmol/10^7 cells, respectively.

Since the cAMP pools in HH201 cells were 5–6-fold higher than the pools in NC4 cells during vegetative growth but comparable to the maximum level detected in cAMP-treated NC4 cells after starvation, the difference in the two strains could be due to the premature appearance and activation of an adenylate cyclase in the mutant. The adenylate cyclase activities of strains NC4 and HH201 during early development are shown in fig.2. The adenylate cyclase activity in vegetative NC4 cells was low. After starvation, this activity remained low for ~2 h and then increased sharply during the next 4–5 h. A similar pattern has been reported for other wild-type strains [20]. In contrast, the adenylate cyclase activity in vegetative HH201 cells was high and remained relatively constant during the first 7 h development. From a number of experiments, the average adenylate cyclase activities for vegetative amoebae of strains NC4 and HH201 were 2.9 ± 0.8 (4 expt) and 14.3 ± 2.8 (5 expt) pmol/min/10^7 cells, respectively. A 5-fold difference was also observed when the specific activities were based on cellular protein, therefore, the higher value for HH201 is not due to a larger cell size. Since the sonicates were left on ice for ~10 min before they were assayed, these values probably represent the basal adenylate cyclase activities in these strains [17,20].

It has been reported [21] that the developmentally regulated adenylate cyclase of wild-type *D. discoideum* is inhibited by Ca^{2+} at mM levels and that this inhibition is antagonized by Mn^{2+}. Therefore, to determine whether the high adenylate cyclase activity in vegetative HH201 cells is associated with the same enzyme present in vegetative and in developing NC4 cells, the various crude activities were assayed in the presence of Ca^{2+} and/or Mn^{2+}. As shown in table 1, all 3 enzyme preparations exhibited a comparable degree of inhibition by Ca^{2+} and stimulation by Mn^{2+}, and the inhibitory effect of Ca^{2+} was overcome by the presence of Mn^{2+}. These results suggest that the adenylate cyclase present in vegetative HH201 cells and in vegetative and developing NC4 cells is the same enzyme.

Mixing experiments using t_0 lysates of NC4 and HH201 cells indicated that the low adenylate cyclase activity in vegetative NC4 cells is not due to the presence of a soluble inhibitor of the enzyme.

Signal generation in *D. discoideum* involves the periodic activation and deactivation of an adenylate cyclase [17]. This process results in sharp oscillations in intracellular cAMP as the cyclic nucleotide is rapidly synthesized and secreted [16,22]. To determine whether the precociously appearing adenylate cyclase in strain HH201 is also activated prematurely, we examined the cAMP pools of strains NC4 and HH201 for spontaneous oscillations. In vegetative cells, the cAMP pools of both strains showed little variation (data not shown). However, after 2 h starvation, the cAMP pools in HH201 cells were oscillating dramatically while the pools in NC4 cells remained static (fig.3). Spontaneous oscillations in the cAMP pools of NC4 cells were generally not observed until after 5–6 h starvation. The pool oscillations in HH201

cells were rapid (and often erratic) with a period of ~4 min. Periodic activation of the adenylate cyclase in the mutant after only 1-2 h starvation probably accounts for the sudden increase in the size of the cAMP pools at this time and the relative insensitivity of the cells to artificial cAMP pulses (fig.1B).

4. Discussion

The results obtained in this study imply that the genetic lesion in strain HH201 (FR17) causes the constitutive synthesis of an adenylate cyclase and that the aberrant temporal and morphological development of this mutant is due to the overproduction of cAMP. A variety of observations suggest that early exposure to cAMP can stimulate the rate of *D. discoideum* development. For example, cAMP at mM levels has been reported to accelerate both the onset of aggregation competence [6] and the synthesis of certain post-aggregative enzymes [23]. In addition, cAMP pulses in the nM range have been shown to accelerate early cell differentiation [12,13,15] and cell aggregation [8] and HH201 cells appear to secrete cAMP in a pulsatile fashion after only 1–2 h starvation. This latter observation might also be related to the abnormal, early signalling which has been reported for this mutant [24].

There is also evidence that elevated levels of cAMP can perturb normal patterning and morphogenesis during *D. discoideum* development. Under conditions where development is restricted, cAMP has been found to induce wild-type cells to differentiate into stalk cells [9,10] or stalk and spore cells [11]. In terminal cell aggregates of FR17, disorganized arrays of stalk and spore cells have frequently been observed, especially at high cell densities [2,10]. At low cell densities, FR17 forms aberrant fruiting bodies with short, thick stalks (particularly in the basal region) and small spore caps. As noted [1], virtually identical structures are formed when NC4 cells are exposed to cAMP at mM levels at 16–18 h development (compare fig.4 in [2] with fig.2A in [25]). These observations suggest that many, if not all, of the abnormal characteristics of FR17 development could be the result of a primary defect in the regulation of an adenylate cyclase in this strain.

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