THE ROLE OF PHOSPHODIESTERASE IN AGGREGATION OF DICTYOSTELIUM DISCOIDEUM

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SUMMARY

The role of cAMP phosphodiesterase in the cAMP-mediated aggregation of the cellular slime mould Dictyostelium discoideum was investigated with a morphogenetic mutant defective in phosphodiesterase production. Mutant cells become capable of aggregating normally when incubated in the presence of exogenous phosphodiesterase isolated from Dictyostelium or rat brain. Direct contact between enzyme and the cell membrane is not required for this phenotypic suppression. The aggregateless character of this strain presumably results from an over-accumulation of cAMP in the extracellular medium since aggregation can be induced in the absence of added phosphodiesterase under conditions facilitating diffusion of the nucleotide. This suggests that phosphodiesterase is not involved in the generation or recognition of cAMP signals, but that the enzyme is essential in the control of the cAMP signal-to-noise ratio.

INTRODUCTION

The developmental programme of the cellular slime mould Dictyostelium discoideum is induced by food deprivation. After a lag period of a few hours, starved cells aggregate and form a multicellular organism. During this phase of aggregation the cells move in a defined pattern and acquire a differentiated phenotype. Both cell movement (Konijn, Van de Meene, Bonner & Barkley, 1967; Robertson, Drage & Cohen, 1972) and cell differentiation (Darmon, Brachet & Pereira da Silva, 1975; Gerisch, Fromm, Huesgen & Wick, 1975) are under the control of periodic cAMP pulses. The cellular responses to these signals appear to be mediated by plasma membrane receptors (Malchow & Gerisch, 1974). The genesis of cAMP pulses is due to periodic oscillations of adenylate cyclase activity (Roos, Scheidegger & Gerisch, 1977; Klein, Brachet & Darmon, 1977). cAMP accumulates transitorily within the cells (Gerisch & Wick, 1975) and is subsequently excreted into the extracellular medium. The degradation of the nucleotide in the environment is effected by a cAMP-phosphodiesterase (PDE). The enzyme associated with the cells is found both in a soluble and in a membrane-bound form (Malchow, Nägele, Schwartz & Gerisch, 1972; Pannbacker & Bravard, 1972). PDE is also released into the extracellular medium (Chang, 1968; Chassy, Love & Krichevsky, 1969).

PDE activity is developmentally regulated during aggregation (Malchow et al., 1972). The synthesis of the enzyme is induced by cAMP pulses (Klein & Darmon,
so that the levels of activity increase in aggregating cells. The cells also excrete a specific inhibitor, to which the extracellular PDE released early during starvation is sensitive (Riedel & Gerisch, 1971; Gerisch, Malchow, Riedel, Müller & Every, 1972). In contrast, the membrane-bound PDE and a fraction of extracellular PDE excreted during aggregation are characterized by a high level of resistance to the specific inhibitor (Malchow et al. 1972; Brachet, Barra, Darmon & Barrand, 1977). The production of this latter macromolecule is regulated, being inhibited by cAMP pulses (Klein & Darmon, 1977). It has been suggested that PDE prevents the cAMP signals from being masked by 'noise' (Shaffer, 1962; Bonner et al. 1969). Malchow, Fuchila & Nanjundiah (1975) have presented evidence that the membrane-bound enzyme plays this role. However, the extracellular PDE also appears to be important, since addition of enzyme to sparse AX2 cells accelerates their differentiation and promotes their aggregation (Alcantara & Bazill, 1976). So far, little information on the regulation and the role of PDE has been obtained from the study of morphogenetic mutants since they are usually characterized by pleiotropic defects, including impaired PDE production (Darmon et al. 1977).

An aggregateless mutant of D. discoideum has recently been isolated, which can be induced to aggregate if cells are incubated in the buffer in which wild-type D. discoideum amoebae were previously starved (Barra, 1977). The mutation responsible for this behaviour has been mapped to linkage group IV (Brachet et al. 1977). Aggregates of the mutant amoebae cannot develop further. However, this defect depends possibly on additional mutations which do not interfere with aggregation, but which affect the further transformation of aggregates into fruiting bodies (Brachet et al. 1977). Studies reported in this communication have demonstrated that the mutant cells are defective in PDE production, and that the factor able to reverse their aggregateless character is PDE. The strain appears useful, therefore, for the understanding of the role of PDE during aggregation.

MATERIALS AND METHODS

Strains and culture conditions

HP X200 is a spontaneous methanol-resistant mutant of strain X2 (Kessin, Williams & Newell, 1974). HP X235, formerly referred to as Agip 235, is an aggregateless mutant of strain HP X200 isolated by Barra (1977). HP X200 and HP X235 were grown in association with Enterobacter aerogenes on SM agar (Sussman, 1966). The axenic strain AX2 (Watts & Ashworth, 1970) and its aggregateless derivative Agip 53 (Darmon et al. 1975) were grown in HL 5-glu medium (Watts & Ashworth, 1970). Aggregation of HP X200 and HP X235 was monitored as follows: amoebae were harvested from plates which had just been cleared of bacteria; they were then washed with 17 mM potassium phosphate buffer pH 6.2 (KPB) (Beug, Katz & Gerisch, 1973) and starved on 60-mm Corning tissue culture dishes at a cell density of 1-2 x 10⁶ amoebae/cm² according to Lee (1972) in 5 ml KPB; aliquots of the buffer to be tested for aggregation-stimulating activity on HP X235 amoebae were added to the dishes at the beginning of starvation, unless otherwise indicated.

Preparation of supernatant media from AX2 or Agip 53

Amoebae which had been grown axenically were washed and starved in shaken suspension at a density of 10⁵ cells/ml. At the desired times samples were removed, the amoebae were
pellet and the supernatant was sterilized by filtration through Millipore membranes. Such solutions were kept at 4 °C.

Treatment of amoebae with cAMP pulses during starvation in KPB was performed as described by Darmon et al. (1975). Each pulse resulted in a final concentration of $10^{-7}$ M cAMP.

Preparation of D. discoideum inhibitor

Inhibitor of PDE was obtained from AX2 amoebae starved for 16 h at $10^7$ cells/ml in KPB. After centrifugation, the supernatant was heated for 10 min at 80 °C (Gerisch et al. 1972). These preparations have little or no PDE activity and may contain up to 40 units inhibitor/ml.

Preparation of PDE

Rat brain PDE was extracted and partially purified as described by Alcantara & Bazill (1976). D. purpureum PDE and inhibitor were generous gifts of Dr Gerisch. The PDE activity used was the third peak of activity obtained after filtration of the total extracellular activity through Sephadex G200.

Assay of PDE and its inhibitor

PDE activity was assayed as described by Brooker, Thomas & Appleman (1968). Titration of inhibitor was as described by Gerisch et al. (1972). Titration of D. discoideum inhibitor was carried out by using PDE produced by Agip 53 amoebae starved for 6 h in the presence of cAMP pulses (Darmon et al. 1977).

Gel electrophoresis

Gel electrophoresis was carried out in 20 mM Tris-glycine buffer, pH 8.3, in 6 % polyacrylamide gels according to Davis (1964). Supernatants were concentrated 10 times on Amicon XL50 membranes by pressure filtration before being analysed on 0.4 x 8 cm gels. Electrophoresis was carried out at room temperature at 2 mA/gel during 3-4 h. When the tracking dye was about 1 cm from the bottom of the tube gels were removed, frozen at —70 °C and sliced in 2-mm sections; each section was eluted for 24 h in 1 ml KPB.

RESULTS

Biological observations

HP X235 was isolated as an aggregateless clone following growth on SM agar. When starved under submerged conditions on tissue culture dishes, mutant amoebae of this strain remain unable to aggregate even after prolonged incubation periods (Fig. 1). At high cell density, above $3-4 \times 10^6$ cells/cm², a few aggregates form without cell streaming.

HP X235 amoebae were stimulated to aggregate when they were incubated in the presence of buffer in which wild-type AX2 cells had been previously starved. The efficiency of aggregation of cells of HP X235 appeared to be dependent on the amount of supernatant buffer added; that is, it was a function of the concentration of some biologically active factor(s). In contrast, the response of HP X235 amoebae was independent of cell density, being identical over the range of $2 \times 10^4$ to $4 \times 10^5$ mutant amoebae per cm².

The response of HP X235 amoebae to increasing amounts of buffer in which the wild type axenic strain AX2 had been starved for 7 h is shown in Fig. 1. Low doses caused the cells to form a few aggregates, in a way which resembles the spontaneous
Fig. 1. Phenotypic suppression of the aggregateless character of HP X235. Cells of strain HP X235 at a density of $1 \times 10^5$ cells/cm$^2$ were starved on tissue culture dishes for 18 h. A–F had 0, 50, 100, 200, 500 and 1000 μl, respectively, of supernatant buffer prepared from the wild-type strain Ax2 (starved 7 h) added to the buffer. (×30.) Cells were also starved with 1.5 units per ml of *D. purpureum* PDE in the absence (H) or in the presence (I) of 10 units per ml of *D. purpureum* inhibitor. (×75.) Cells of HP X235 (×75) starved in the absence of added supernatant are also represented (c).
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process seen at high cell density (Fig. 1 B). Upon addition of larger amounts of buffer an identical pattern was observed, except that all the mutant amoebae migrated into aggregates (Fig. 1 C). At higher doses the size of the territories became larger, and the population formed aggregates in a way identical to that of the parental strain HP X200 (Fig. 1 D, E). However, the kinetics of this aggregation process remained dependent on the dose of supernatant added, since increasing amounts of active buffer caused the mutant cells to aggregate more rapidly (Fig. 1 F). In no case was an inhibition of this stimulatory effect observed, even for the highest doses checked. Time-lapse cinematographic studies have demonstrated that HP X235 cells respond by migrating in a rhythmic fashion. Aggregates could never be induced to transform into fruiting bodies following transfer on to appropriate solid-air supports. However, genetic studies have shown that strain HP X235 carries secondary mutations which affect specifically the morphogenesis of the fruiting bodies.

Since the dose response of the cells to a given supernatant appeared reproducible from one experiment to another, the biological potency of supernatants could be measured in a quantitative way by serial dilutions. One unit of activity is defined as the minimal dose required per ml of starvation buffer to cause the migration of all HP X235 cells into aggregates.

The factor responsible for the aggregation of HP X235 amoebae is a heat-sensitive macromolecule being destroyed within 10 min at 80 °C. The molecular weight is apparently higher than 50,000 since it is retained on Amicon XM50 membranes. In order to elucidate its relationship to PDE, the medium from AX2 cells starved during 7 h was fractionated by electrophoresis on neutral acrylamide gels. The gels were sliced, the proteins eluted, and the biological and the PDE activities of each fraction were determined. It can be seen in Fig. 2 that one single peak of PDE was found,
which migrated at the same rate as the biological activity of the preparation. These data suggest that the PDE activity is responsible for the response of HP X235 cells.

Response of HP X235 amoebae to PDE

Assay of the cellular and of the extracellular PDE activity following the initiation of development of HP X235 amoebae revealed that this strain synthesizes much less PDE activity than the parental strain HP X200 (Fig. 3). HP X235 cells excreted variable amounts of PDE inhibitor during starvation. In most experiments, a significant amount of inhibitor was detected during starvation, while in other ones this compound was lacking in the extracellular medium.

Study of the response of HP X235 amoebae to exogenous enzyme was undertaken with partially purified rat brain PDE. This enzyme caused HP X235 amoebae to

![Graph showing phosphodiesterase production in HP X200 and HP X235](image)

Fig. 3. Phosphodiesterase production in HP X200 and HP X235. $1.5 \times 10^7$ cells were starved in 10-cm tissue culture dishes in 5 ml KPB. Results are expressed as amount of enzyme produced per $10^7$ cells. Cellular PDE of HP X200 (●) and HP X235 (▲). Extracellular PDE of HP X200 (○) and HP X235 (△). Aggregation of HP X200 began after 7 h of starvation and was completed at 12 h. HP X235 cells remained undifferentiated.
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aggregate efficiently. In the presence of 5–10 units/ml mutant cells aggregate as well as the parental strain HP X200.

The requirement for an exogenous PDE activity during aggregation of HP X235 cells was further demonstrated by addition of *D. purpureum* enzyme. During starvation this organism excretes a PDE species which is sensitive to the homospecific inhibitor of *D. purpureum* PDE but is fairly resistant to the heterospecific inhibitor of *D. discoideum* PDE (Gerisch et al. 1972). *D. purpureum* PDE caused an extensive stimulation of the ability of HP X235 cells to aggregate; optimal stimulation was obtained in the presence of about 2 units per ml. That PDE is the specifically active compound was indicated by the fact that concomitant addition of *D. purpureum* inhibitor to the mutant cells could totally abolish their ability to aggregate. Data reproduced in Fig. 1H and 1 show that the stimulation of cell aggregation observed when HP X235 amoebae were incubated with 1.5 units per ml of *D. purpureum* PDE was abolished by addition of 10 units per ml of homospecific inhibitor to the starvation buffer. This amount of inhibitor is sufficient to inhibit totally the catalytic activity of the enzyme (data not shown). In contrast, addition of the same amount of the heterospecific inhibitor from *D. discoideum* only poorly suppressed the ability of *D. purpureum* PDE to induce aggregation. To study this further we have examined the stimulating activity of buffer prepared from starved *D. discoideum* amoebae. Such cells excrete during starvation inhibitor-sensitive and inhibitor-resistant enzymes. Both PDE species are found in variable amount, depending on the strain and the starvation conditions (Brachet et al. 1977; Brachet and Darmon, in preparation).

Careful investigation of various preparations led to the conclusion that their biological activity was not proportional to their total PDE content, but was related to their content of the inhibitor-resistant species.

Possible mode of action of the extracellular PDE

With the exception of the first 90 min of starvation, PDE was required throughout for aggregation of HP X235 amoebae. If aliquots of a biologically active preparation were added to the mutant amoebae at the beginning of starvation or after the first 90 min, aggregation started at the same time. If cells were starved for longer than 90 min before the buffer was added then initiation of aggregation was delayed. However, cells starved without PDE for up to 48 h could still carry out aggregation in response to the enzyme. Conversely, replacement of the buffer by fresh PDE-free buffer inhibited aggregation of HP X235 amoebae. If cells were still in the interphase period no aggregation was observed. If aggregation was already proceeding before removal of active buffer the streams fragmented into numerous aggregates. This behaviour presumably results from over-accumulation of cAMP. It would indicate that unlike wild type amoebae, aggregating HP X235 amoebae cannot be induced to produce PDE. This conclusion was verified by direct measurements of the PDE levels. The activity of PDE around the cells was initially high after addition of supernatant from Agip 53 cells which had been stimulated to produce PDE by pulses of cAMP for 6 h (Fig. 4). The activity fell during the following 7 h presumably due to the production of PDE-inhibitor by the HP X235 cells. All of the activity could be
found in the supernatant after removal of the cells indicating that little PDE became bound to the cells. No observable activity was found associated with washed cells which demonstrates that no induction of endogenous PDE occurred while the cells aggregated.

![Figure 4: Extracellular and cellular phosphodiesterase levels during differentiation to aggregation-competence of HP X235. Aggregation of HP X235 was induced by adding 40 units/ml PDE (prepared from strain Agip 53 treated with cAMP pulses). Conditions of starvation are the same as those described in Fig. 3. Assays of cellular PDE were carried out after the cells had been washed once with KPB. Extracellular PDE (●) is expressed in units/ml. Cellular PDE of PDE-treated cells (○) and control HP X235 without PDE (△) are expressed in units/10⁷ cells. Aggregation of PDE-treated HP X235 was actively proceeding at t 7.

The requirement for PDE for aggregation of HP X235 cells could be eliminated if the supernatant containing PDE could be separated from the cells of HP X235 by dialysis membrane and still gave rise to aggregation of the mutant. The supernatant was held in a 4-cm diameter cylindrical chamber with a bottom made of dialysis membrane which was suspended over the buffer in which the mutant cells were aggregating. Under these conditions the enzyme could not directly contact the cells but could remove cAMP which diffused across the membrane.
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the volume of surrounding fluid was greatly increased such that diffusible molecules would be diluted. This was achieved by placing cells of HP X235 on top of dialysis membranes supported over a large reservoir containing 200 ml of buffer. Under these conditions long streams were observed as the cells entered aggregates (Fig. 5B). In contrast, aggregation was inhibited if the membrane rested over little liquid, such as 2 ml, soaked in a filter paper (Fig. 5A).

Fig. 5. Aggregation of HP X235 on dialysis drums in the absence of phosphodiesterase. A, HP X235 amoebae remained isolated after 15 h of starvation if they rested on little liquid (1 ml KPB). (×30.) B, aggregating HP X235 amoebae after 15 h of starvation on a dialysis membrane stretched over a large reservoir (200 ml KPB). (×30.)

DISCUSSION

Previous studies have shown that certain aggregateless mutants of D. discoideum differentiate to aggregation-competence in response to externally imposed pulses of cAMP (Darmon et al. 1975, 1977). We have now demonstrated that a mutant strain HP X235, will aggregate in the presence of exogenous PDE from slime mould or rat brain. Aggregation of this strain can also occur in the absence of added PDE but only if the cells are in contact with a large volume of buffer which can dilute excreted molecules. Since aggregation in limited volumes of buffer is specifically dependent on cAMP PDE, it appears that the critical compound diluted when the cells are in contact with large volumes of buffer is cAMP. Strain HP X235 produces very little PDE of its own during starvation, and secreted cAMP may be expected to accumulate to abnormal levels. The lack of aggregation of strain HP X235 under normal developmental conditions, in which wild-type cells aggregate well, is most likely a consequence of an over-accumulation of cAMP which does not permit recognizable gradients of the nucleotide to be formed. Therefore in wild type cells PDE appears to play the sole role of keeping extracellular cAMP at a low level so that the signal following a pulse of released cAMP can be recognized and elicit a cellular response. Although it has been suggested (Malchow et al. 1975) that membrane-bound PDE may be essential for this purpose, the observations that strain HP X235 can be induced to aggregate either by exogenous PDE separated from the cells by dialysis membrane or without enzyme under conditions facilitating diffusion, indicate that little or no membrane-bound PDE is required. It appears, therefore, that repeated functioning
of the cAMP-binding sites, which should involve the release of bound cAMP does not require hydrolysis of the nucleotide, unless trace amounts of enzyme are sufficient to ensure that function. The cAMP receptors of *D. discoideum* could resemble in that respect other systems of higher eukaryotes, such as the cholinergic receptor (Changeux et al. 1976; Nachmansohn & Neumann, 1975). Although exogenous PDE permitted aggregation of HP X235 cells, subsequent morphogenesis did not occur. However, genetic analysis of strain HP X235 has shown that it carries several morphological mutations which can be separated (Brachet et al. 1977). Therefore strain HP X235 appears inadequate for the study of the role of PDE during morphogenesis of the fruiting body. This will be performed in strains in which the mutation causing impaired PDE production is placed in the genetic background of strain HP X200.

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REFERENCES


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