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Cyclic AMP–phosphodiesterase induces dedifferentiation of prespore cells in *Dictyostelium discoideum* slugs: evidence that cyclic AMP is the morphogenetic signal for prespore differentiation

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Summary

We investigated whether cyclic AMP is an essential extracellular stimulus for the differentiation of prespore cells in slugs of *D. discoideum*. A local reduction of the extracellular cAMP level inside the slug was induced by implantation of cAMP–phosphodiesterase (cAMP–PDE)-coated spheres in intact slugs. This treatment caused the disappearance of prespore antigen in the vicinity of the sphere. A general reduction of extracellular cAMP levels in slugs, induced by submerging slugs in 0·25 i.u. ml^{-1} cAMP–PDE, reduced the proportion of prespore cells from 66 % to 15 %, without affecting slug morphology. The cAMP–PDE-induced dedifferentiation of prespore cells was counteracted by cAMP and was not due to the production of the hydrolysis product 5′AMP, but to the reduction of extracellular cAMP levels. We conclude that extracellular cAMP is the major morphogenetic signal for the differentiation of prespore cells in the multicellular stages of *D. discoideum* development and we present a working hypothesis for the generation of the prestalk/prespore pattern during multicellular development.

Key words: *Dictyostelium discoideum*, pattern formation, prespore differentiation, extracellular cAMP.

Introduction

During *Dictyostelium discoideum* development, unicellular amoebae aggregate by means of chemotaxis to cAMP pulses to form a multicellular organism. This aggregate transforms into a slug-shaped structure and ultimately culminates to form a fruiting body consisting of stalk cells and spores. In the slug stage of development, the presumptive stalk and spore cells can already be recognized and the two cell types are arranged in a simple anteroposterior pattern. A Differentiation Inducing Factor (DIF) has been isolated from *Dictyostelium* cells and was recently identified (Town *et al.* 1976; Morris *et al.* 1987). This factor is considered to be the morphogen responsible for the induction of stalk cell differentiation, because it can induce stalk cell differentiation in single cells *in vitro* (Kay & Jermyn, 1983).

Spore-specific gene products are first evident after aggregates have formed (Hayashi & Takeuchi, 1976; Morrissey *et al.* 1984; Cardelli *et al.* 1985) and disappear again when aggregates are being dissociated (Takeuchi & Sakai, 1971; Okamoto & Takeuchi, 1976). This suggests that the formation of short-range intercellular interactions or cell–cell contacts is responsible for the induction of prespore differentiation. Cyclic AMP can counteract the dissociation-induced loss of prespore gene products (Okamoto, 1981; Barklis & Lodish, 1983; Mehdy & Firtel, 1985) and it was furthermore shown that cAMP can induce prespore differentiation in preaggregative cells (Kay, 1982; Mehdy *et al.* 1983; Schaap *et al.* 1986). This suggests that cAMP may be the morphogen responsible for the induction of prespore differentiation. However, it is also possible that cAMP functions as a bypass for the real stimulus, which could be cell–cell contacts or other short-range intercellular interactions (Kaleko & Rothman, 1982).

In this study, we have approached this problem in the following manner. If cAMP is the major extracellular stimulus for prespore differentiation in slugs, then the reduction of extracellular cAMP levels in slugs should cause dedifferentiation of prespore cells. To induce a local reduction in cAMP levels, we

implanted Sepharose spheres linked to cAMP–PDE in slugs and, to induce a general reduction of cAMP levels, we submerged slugs in a buffer which contains cAMP–PDE. The results of our experiments indicate that extracellular cAMP is a major morphogenetic signal for prespore differentiation in slugs.

Materials and methods

Materials

Prespore-specific rabbit IgG was prepared as described by Takeuchi (1963). FITC-conjugated swine anti-rabbit IgG was obtained from Dakopatts (Denmark), 4',6-diamidino-2-phenyl-indole (DAPI) was from Sigma (USA). Cellulase Onozuka and bovine serum albumin were from Serva (FRG), beef heart phosphodiesterase was from Boehringer (FRG), CNBr-activated Sepharose was from Pharmacia (Sweden) and screw-cap septum vials (1·5 ml) were from Pierce Chemical Company (USA).

Culture and incubation conditions

D. discoideum NC4 was grown in association with *Escherichia coli* 281 on glucose–peptone agar (Schaap & Spek, 1984). To obtain migrating slugs, vegetative cells were freed from bacteria and distributed over NN agar (1·5 % agar in 10 mM-sodium/potassium phosphate, pH 6·5 (PB)) at about 3×10^6 cells cm^{-2}. The agar plates were incubated at 21 °C in the dark until migrating slugs had formed.

For those experiments in which Sepharose spheres were to be implanted into slugs, 10 μl droplets of 5×10^8 cells ml^{-1} were placed on NN agar until migrating slugs had formed. After implantation, the slugs were allowed to migrate further on the same plates.

In experiments with submerged slugs, 10–15 slugs were transferred from NN agar to a 1·5 ml screw-cap septum vial which contained 300 μl 10 mM-PB and various additions as indicated in the figure legends. The tubes were flushed for 30 s with oxygen and subsequently rotated at 21 °C and 10 revs min^{-1}. Every hour the oxygen was replenished.

Preparation of cAMP–PDE Sepharose spheres

Extracellular *D. discoideum* cAMP–PDE, partially purified by ammonium sulphate precipitation (Van Haastert & Van der Heijden, 1983), or commercially available beef-heart cAMP–PDE were coupled to CNBr-activated Sepharose according to standard procedures. After coupling, remaining active groups were blocked by reaction with ethanolamine, and the spheres were extensively washed with alternately high- and low-salt buffers at pH 8 and pH 4 to remove unbound enzyme. The spheres were again washed immediately before implantation. The PDE activity of the spheres was determined according to Thompson *et al.* (1974). Control Sepharose spheres were made by using bovine serum albumin (BSA) instead of cAMP–PDE. The spheres were stained with a 1 % solution of the vital dye Nile blue sulphate to follow their localization in the slugs.

Immunohistology and immunocytology

Slug patterns were studied by fixing intact slugs in ice-cold methanol, followed by embedding in Paraplast and serial sectioning (Schaap *et al.* 1985). The sections were first incubated with prespore-specific rabbit IgG (PSRI) and subsequently with FITC-conjugated swine anti-rabbit IgG (SARFITC).

To measure the proportion of prespore cells in slugs, slugs were dissociated into single cells by a 5–10 min treatment with 10 mg ml^{-1} cellulase and 2 mM-EDTA in PB. Droplets (10 μl) of 5×10^6 cells ml^{-1} were allowed to adhere to glass slides, fixed in methanol and incubated with PSRI and SARFITC as described above. The cells were subsequently stained with 0·2 μg ml^{-1} DAPI, a fluorescent dye that stains DNA and therefore the nuclei of all the cells in the preparation. The preparations were observed with a Leitz Laborlux fluorescence microscope equipped with a Leitz filterblock A (excitation 340–380 nm, emission > 430 nm), to discriminate between the DAPI and FITC fluorescence. The proportion of prespore cells was determined by first counting the DAPI-stained cells in the field of view and subsequently the number of cells which contain three or more FITC-stained vacuoles.

Results

Effect of local hydrolysis of extracellular cAMP on prespore differentiation in migrating slugs

As an initial experiment to establish whether cAMP is required for prespore differentiation in intact slugs, we investigated whether a local reduction of cAMP levels in the slug induces a local disappearance of prespore differentiation markers. Sepharose spheres, covalently linked to *Dictyostelium* or beef-heart cAMP–PDE, or control spheres, linked to bovine serum albumin instead of cAMP–PDE, were implanted into migrating slugs with the aid of a fine needle. The behaviour of the slug on agar was followed and after 2 h of migration, the slugs that had retained the sphere were fixed in methanol, embedded in paraffin and sectioned. Serial sections were stained with prespore-specific rabbit IgG (PSRI) and FITC-conjugated swine anti-rabbit IgG (SARFITC).

It was found that after implantation of control spheres, the slugs continued to migrate normally and left the sphere behind in the slime trail after some time (Fig. 1). After implantation of PDE spheres, migration generally slowed down and slugs became shorter and thicker. Roughly 50 % of the slugs stopped migrating completely and assumed a hemispherical shape. Two to four tips then started to sprout at the periphery, with the sphere positioned in the centre of the cell mass. As many new slugs as there had been tips were formed from the cell mass; these migrated away and left the sphere behind (Fig. 1).

Only those slugs that had retained the PDE sphere were used for immunohistology. Serial sections of slugs treated with control spheres showed no signifi-

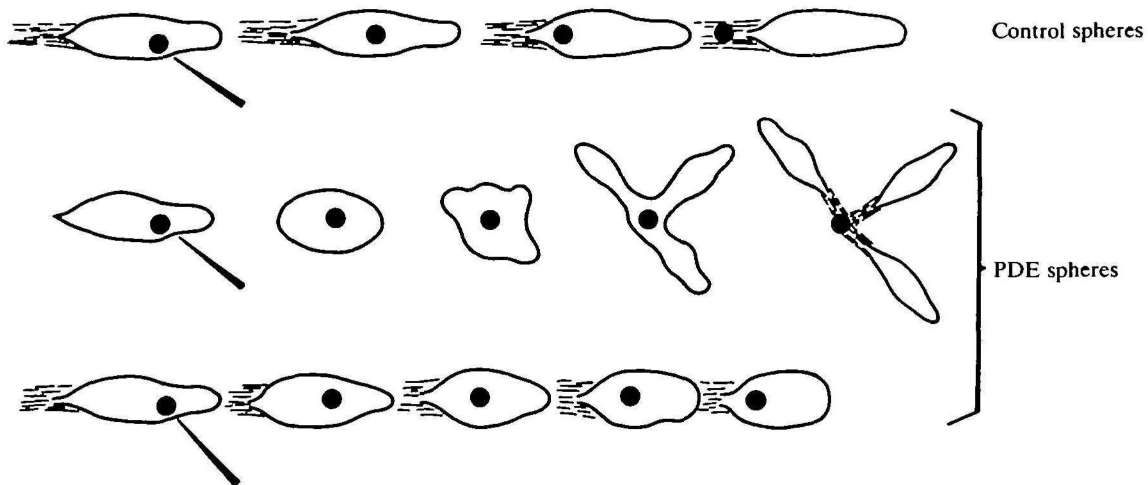

Fig. 1. Effects of cAMP–PDE Sepharose spheres on slug morphology. Sepharose spheres were implanted into migrating slugs with the aid of a fine needle. The behaviour of the slugs on agar was followed during 2 h.

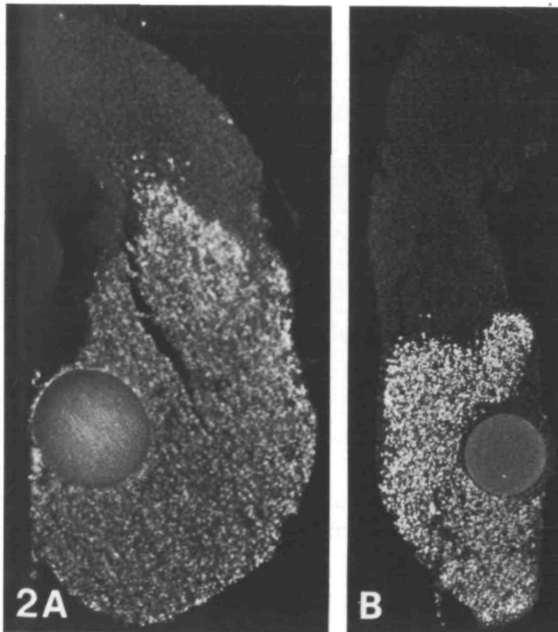

Fig. 2. Effects of cAMP–PDE spheres on slug pattern. Sepharose spheres were implanted into migrating slugs and, after 2 h, slugs that had not lost the sphere (five slugs for each treatment) were prepared for immunohistology and stained with prespore-specific antiserum. (A) Control slug with a BSA-coated sphere. ×190. (B) Slug with a sphere linked to *Dictyostelium* cAMP–PDE (about 3×10^{-9} i.u. per sphere). ×150. The five slugs treated with PDE spheres all showed disappearance of prespore-specific staining around the sphere, while the slugs treated with BSA spheres did not.

cant alterations in the prespore staining pattern of the slugs (Fig. 2A). However, in slugs treated with PDE spheres, prespore antigen had disappeared from the region of tissue that was in contact with the sphere (Fig. 2B). This suggests that local hydrolysis of extracellular cAMP has caused the prespore cells to dedifferentiate. However, considering the gross changes in slug morphology that the PDE spheres can induce (Fig. 1), it remains possible that the presence of nonprespore tissue around the PDE sphere is not due to dedifferentiation but to a redistribution of prespore and non-prespore cells.

Effect of a general reduction of cAMP levels on prespore differentiation in slugs

As a second approach to investigate whether cAMP is essential for prespore differentiation, we incubated intact slugs under submerged conditions with different amounts of beef-heart cAMP–PDE. When slugs were incubated for 6 h with 0·25 i.u. ml^{-1} PDE, which had been inactivated by boiling, neither the overall morphology of the slug nor the prespore staining pattern were significantly altered, compared to slugs migrating on agar (Fig. 3A). However, when slugs were incubated with 0·25 i.u. ml^{-1} active PDE, the amount of prespore antigen was strongly reduced; the original prespore staining pattern was not, or only barely, visible (Fig. 3B). Slug shape was not affected by the treatment. To quantify the effects of cAMP–PDE on prespore differentiation, the slugs were dissociated after PDE treatment and the proportion of prespore cells to total cells was determined (Fig. 4). The proportion of prespore cells in submerged control slugs is 66 %, which is about 5–10 % lower than the proportion of prespore cells in migrating slugs (unpublished data). The proportion of prespore cells decreases progressively when increasing amounts of PDE are added to the incubation mixture to about 15 % at 0·25 i.u. ml^{-1} PDE. Addition of the same amount of boiled enzyme does not significantly alter the proportion of prespore cells. The effect of cAMP–PDE can be completely abol-

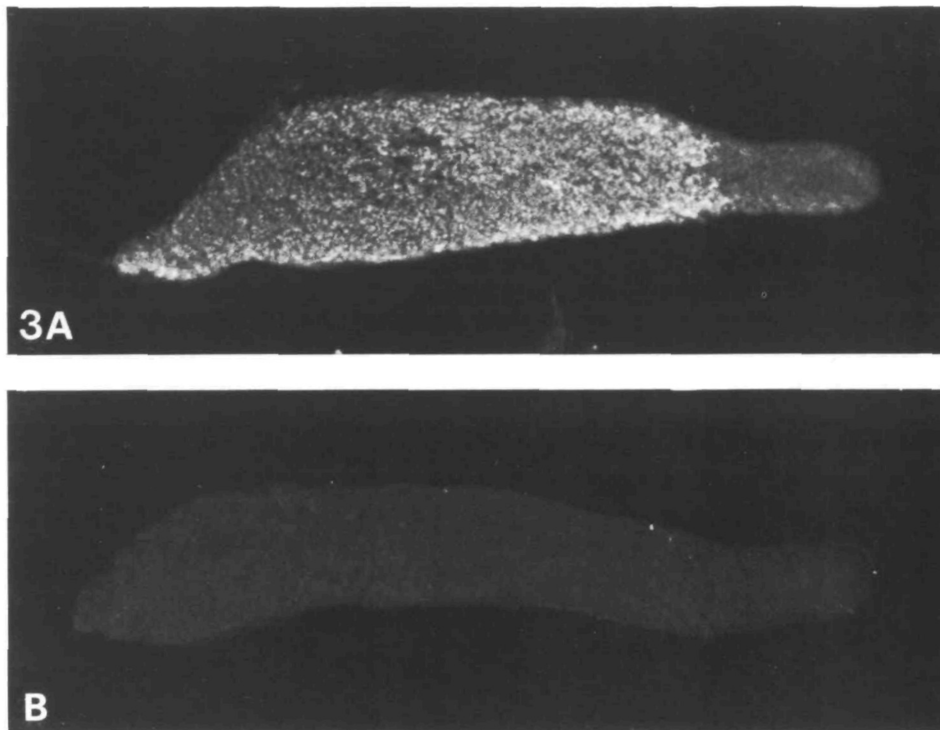

Fig. 3. Effects of cAMP–PDE on pattern in submerged slugs. Slugs were incubated in roller tubes in PB containing 0·25 i.u. ml^{-1} beef heart cAMP–PDE (B) or the same amount of enzyme, which had been inactivated by boiling for 3 min (A). After 6 h the cells were prepared for immunohistology and stained with prespore antiserum. For each treatment, ten slugs were sectioned. In all cases, the PDE-treated slugs stained only very slightly with prespore antiserum, while slugs treated with boiled PDE showed strong staining and a clearly defined anteroposterior pattern. (A) ×100; (B) ×80.

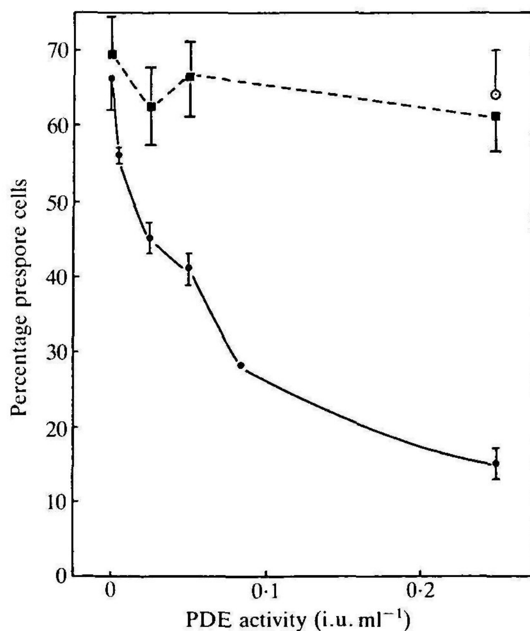

Fig. 4. Effect of cAMP–PDE on prespore proportions in slugs. Intact slugs were incubated in roller tubes with different amounts of beef-heart cAMP–PDE (●), beef-heart PDE with 10 mM-cAMP (■), and boiled beef-heart PDE (○). After 6 h of incubation, the slugs were dissociated into single cells and stained with DAPI and prespore antiserum. The percentage of prespore cells *versus* the total number of cells was determined. The data represent the means and S.D. of two experiments, in which 600 cells were counted for each data point.

ished by including 10 mM-cAMP in the incubation mixture. These experiments demonstrate that treatment of intact slugs with an enzyme that hydrolyses extracellular cAMP, which is produced by the slugs, induces a general dedifferentiation of prespore cells.

Effects of cAMP hydrolysis products on prespore proportions in slugs

The cAMP–PDE-induced dedifferentiation of prespore cells in intact slugs can be attributed to the reduction of extracellular cAMP levels in the slug, but it is also possible that the production of the hydrolysis product 5′AMP or the secondary hydrolysis product adenosine is responsible for the observed effects. To test this possibility, intact slugs were incubated during 6 h in 5 mM-5′AMP or 5 mM-adenosine and the effects of these treatments on prespore proportion were determined. Fig. 5 shows that 5′AMP does not significantly affect prespore proportions in slugs, while adenosine induces a 40 % reduction in the proportion of prespore cells, which agrees with earlier reported inhibitory effects of adenosine on prespore differentiation in slugs (Weijer & Durston, 1985; Schaap & Wang, 1986). The effects of neither 5′AMP nor adenosine are as pronounced as those of cAMP–PDE, which indicates that the reduction of cAMP levels rather than the production of hydrolysis products is responsible for the observed dedifferentiation of prespore cells.

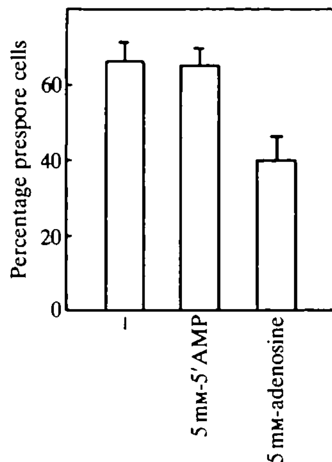

Fig. 5. Effects of 5'AMP and adenosine on prespore proportions. Intact slugs were incubated in PB in roller tubes to which alternatively 5 mM-5'AMP or 5 mM-adenosine was added. After 6 h the percentage of prespore cells was determined.

Discussion

Implantation of Sepharose spheres linked to cAMP–PDE into migrating slugs, induces the disappearance of prespore antigen in the vicinity of the sphere (Fig. 2B). When whole slugs are submerged in buffer containing cAMP–PDE, a general loss of prespore antigen takes place (Figs 3, 4). Two cAMP–PDE preparations from entirely different sources (*Dictyostelium* and beef heart) have the same effect and the effects of cAMP–PDE can be counteracted by high cAMP concentrations (Fig. 4). This indicates that the observed effects are caused by the cAMP hydrolysing activity and not by a contamination of the enzyme preparation. The hydrolysis product 5'AMP does not affect the proportion of prespore cells significantly (Fig. 5) and it therefore appears that the PDE-induced reduction of extracellular cAMP levels in the slug is the cause of dedifferentiation of prespore cells. Since it is unlikely that cell–cell contacts or other intercellular interactions besides those mediated by cAMP are affected by the cAMP–PDE treatment, we conclude that extracellular cAMP is a major requirement for the differentiation of prespore cells in slugs of *D. discoideum*.

Besides affecting prespore differentiation, the implantation of PDE spheres also affected the movement of migrating slugs. Slug migration slowed down and, in the more severe cases, the slug rounded off and formed new tips at the most-distal location from the sphere. This type of behaviour was never observed when control spheres were implanted. These effects of PDE spheres indicate that extracellular cAMP also plays a role in the control of slug movement. This agrees with earlier evidence that cell movement in the multicellular stages of *Dictyostelium* development is coordinated by cAMP oscillations, which are emitted by the tip and transmitted through the slug by a relay mechanism (Durston & Vork, 1979; Schaap *et al.* 1984; Schaap & Wang, 1984; Otte *et al.* 1986). Curiously, treatment with millimolar cAMP concentrations also induced slugs to break up into several smaller cell clumps (Wang & Schaap, 1985). Apparently, this type of interference with cAMP signalling also results in the loss of coherent cell movement.

Our present experiments indicate that in the slug stage extracellular cAMP is a primary requirement to maintain the state of prespore differentiation. Cyclic AMP can also induce prespore differentiation in preaggregative cells and studies with cAMP analogues have shown that the effect of cAMP on prespore induction is most likely mediated by the chemotactic cell surface cAMP receptor (Schaap & Van Driel, 1985; Oyama & Blumberg, 1986), indicating that cAMP acts as an extracellular stimulus. This statement is also supported by the observation that adenosine, a compound which inhibits the induction of prespore gene expression by cAMP (Schaap & Wang, 1986; Spek *et al.* 1988) exerts this function because it inhibits the binding of cAMP to the surface cAMP receptor (Newell & Ross, 1982; Van Lookeren Campagne *et al.* 1986).

It remains possible that additional factors besides cAMP are required for prespore differentiation. However, the observation that the loss of prespore gene products induced by disaggregation of slugs can be counteracted by cAMP (Okamoto, 1981; Mehdy *et al.* 1983; Barklis & Lodish, 1983) indicates that these factors can only be of minor importance. Spore maturation during the culmination stage of development may require a signal other than cAMP.

A working hypothesis for prespore induction in late aggregates

Early studies report that millimolar cAMP concentrations are required to induce or maintain prespore gene expression. This is in contrast to cAMP-induced responses such as chemotaxis and cAMP relay, which are induced by nanomolar cAMP pulses (see Devreotes, 1983). One reason for this discrepancy is the fact that unlike the latter two responses, the induction of prespore gene expression requires stimulation with elevated cAMP levels for several hours, during which cAMP is degraded by cell surface and extracellular phosphodiesterases. Recent estimates of dose dependency, which incorporate cAMP degradation by cAMP–PDE, indicate that half-maximal induction of prespore gene expression occurs between 0·1 and 1 μM-cAMP (Schaap & Van Driel, 1985; Oyama & Blumberg, 1986). Slugs contain about 10 fmol

cAMP μg^{-1} of slug tissue or $10\,\mu M$ (Merkle *et al.* 1984), which would be sufficient to induce prespore differentiation. It is, however, not known which proportion of this cAMP is intra- or extracellularly located. During cAMP oscillations at the aggregation stage, about $1\,\mu M$-cAMP is produced at the peak of a passing cAMP wave (Tomchik & Devreotes, 1981), and it is not unlikely that after aggregation even higher cAMP concentrations are accumulated by cAMP signalling in the narrow interstices between the cells. Therefore, we propose as a working hypothesis that continuation of cAMP signalling after aggregation results in the accumulation of sufficiently high cAMP levels to induce the expression of prespore genes.

Generation of the prestalk/prespore pattern

In *D. discoideum* slugs, prespore cells remain confined to the posterior region of the slug; no prespore differentiation is evident at the anterior region, which comprises about 25 % of the slug tissue. If cAMP signalling is responsible for the induction and maintenance of prespore differentiation, why is this type of differentiation then restricted to the posterior part? Two cell surface enzymes, cAMP–PDE and 5'nucleotidase, which degrade cAMP to its antagonist adenosine are preferentially associated with prestalk cells (Bonner *et al.* 1955; Armant *et al.* 1980; Armant & Rutherford, 1981; Otte *et al.* 1986; Schaap & Spek, 1984). We propose that continued oscillatory signalling in slugs, combined with the preferential conversion of cAMP to adenosine at the anterior prestalk region, creates an extracellular cAMP gradient, which increases from front to rear, and an adenosine gradient, which decreases from front to rear (Fig. 6). At the posterior region, relatively high cAMP levels induce prespore differentiation, while at the anterior region, low-time-average cAMP levels, combined with high adenosine levels are not permissive for prespore differentiation. This model is validated by the observation that reduction of adenosine levels in the slug results in the differentiation of prespore cells in the anterior prestalk region (Schaap & Wang, 1986) and by the recent observation that the anterior prestalk region is virtually absent in a 5'-nucleotidase-defective mutant, which cannot produce sufficient amounts of adenosine (M. Wang & P. Schaap, in preparation). Earlier data indicate that intracellular cAMP levels are somewhat higher in anterior than in posterior regions of slug (Garrod & Malkinson, 1973; Pan *et al.* 1974; Brenner, 1977). However, intracellular cAMP levels are irrelevant for the induction of prespore gene expression, which requires elevated levels of extracellular cAMP. A crucial, but rather difficult, experiment to perform

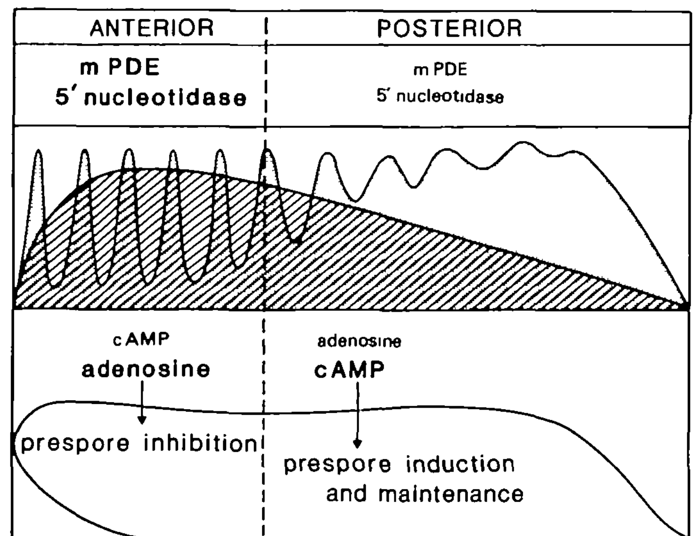

Fig. 6. Regulation of anteroposterior pattern by opposing cAMP and adenosine gradients. The anterior tip emits cAMP pulses, which are transmitted by a relay mechanism through the whole slug. Cell surface PDE and 5'nucleotidase are preferentially active at the anterior. In this region, cAMP is effectively degraded between subsequent pulses and adenosine levels build up (hatched area). In the posterior region, cAMP is not (completely) degraded between subsequent pulses, which results in an increase in average cAMP levels, while adenosine levels remain low. High ambient cAMP levels also cause partial quenching of cAMP pulses, because the cells cannot sufficiently deadapt between subsequent pulses. High cAMP levels induce and sustain prespore-specific gene expression at the posterior. At the anterior region the combination of low cAMP levels combined with high adenosine levels is not permissive for prespore differentiation.

would be to measure extracellular cAMP and adenosine levels in slugs.

Our model assumes that a prepattern of cAMP–PDE and 5'-nucleotidase activity is present before the prestalk/prespore pattern is generated. Some data indicate that this prepattern may be the result of preferential sorting of a subpopulation of cells which display relatively high cAMP–PDE activity. It is known for some time that cells that enter starvation while in early cell cycle phase (E-cells) sort preferentially to the anterior region, compared to their late cell cycle counterparts (L-cells) (McDonald & Durston, 1984; Weijer *et al.* 1984). Recent experiments show that E-cells synthesize gene products involved in the aggregation process, such as cAMP surface receptors and cAMP–PDE activity several hours earlier and to much higher levels than L-cells (Wang *et al.* 1988). E-cells initiate aggregation centres (McDonald, 1986) and probably sort to the anterior because they respond more readily to the chemotactic signal (Wang *et al.* 1988). Simultaneously, the sorting

process creates the initial prepattern of E-cells with high PDE activity at the anterior and L-cells with low PDE activity at the posterior, this being the starting point for the subsequent formation of the prestalk/prespore pattern.

The present model describes the establishment of the prestalk/prespore pattern by opposing cAMP and adenosine gradients, but it is likely that additional regulatory mechanisms are also active in the slug stage. The stalk-inducing-factor, DIF, which accumulates after aggregation (Brookman *et al.* 1982) and is preferentially present in the prespore region of slugs (Brookman *et al.* 1987) is also a potent inhibitor of cAMP-induced prespore differentiation (Kay & Jermyn, 1983; Wang *et al.* 1986). The function of this compound in the establishment of pattern requires further investigation. This study shows that cAMP is most likely the morphogen responsible for prespore differentiation and it is of major importance to analyse how this morphogenetic signal is processed by the cells to induce the expression of specific genes.

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