Cyclic-AMP-induced elevation of intracellular pH precedes, but does not mediate, the induction of prespore differentiation in *Dictyostelium discoideum*

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Summary

Prespore gene expression in *Dictyostelium* is induced by the interaction of cAMP with cell surface cAMP receptors. We investigated whether intracellular pH (pHi) changes mediate the induction of prespore gene expression by cAMP. It was found that cAMP induces a 0.15 unit increase in pHi within 45 min after stimulation. The cAMP-induced pHi increase precedes the induction of prespore gene expression, measured by *in vitro* transcription, by about 15–30 min. Cyclic-AMP-induced pHi changes can be bypassed or clamped by addition of, respectively, the weak base methylamine, which increases pHi, or the weak acid 5,5-dimethyl-2,4-oxazolidinedione (DMO), which decreases pHi. Bypass of the cAMP-induced increase of pHi with methylamine does not induce the expression of prespore genes, while inhibition of the pHi increase with DMO does not inhibit the induction of prespore gene expression. Cyclic-AMP-induced prespore protein synthesis and the proportion of prespore cells in multicellular aggregates are also not affected by bypassing or inhibiting the cAMP-induced pHi increase. These results show that although a morphogen-induced pHi increase precedes the induction of prespore gene expression, this increase does not mediate the effects of the extracellular cAMP signal on the transcription or translation of prespore genes in *Dictyostelium discoideum*.

Key words: *Dictyostelium*, differentiation, intracellular pH, cAMP stimulation.

Introduction

Elevation of intracellular pH (pHi) in response to a number of mitogens and hormones is thought to play an important role in the regulation of cell growth and other cellular responses (Busa & Nuccitelli, 1984). In animal cells, pHi is regulated mainly by a Na⁺/H⁺ antiporter which can be activated in response to extracellular stimuli (Vicentini & Villereal, 1986; Ives & Daniel, 1987). Studies with fibroblast mutants lacking the antiporter have shown that growth-factor-induced alkalization is a necessary step for the initiation of DNA synthesis and for growth (Fouyssegur et al. 1984). In *Dictyostelium discoideum*, pHi has been proposed to regulate the differentiation of its two cell types, the stalk cells and spores. In this organism, as in fungi and plants, pHi is probably not regulated by the Na⁺/H⁺ antiporter but rather by a H⁺-ATPase (Pogge-von Strandmann et al. 1975).

The developmental fate of *D. discoideum* cells developing in monolayers submerged in buffer was found to be profoundly affected by conditions which were considered to affect pHi. Low extracellular pH, addition of weak acids and addition of the H⁺-ATPase inhibitor diethylstilbestrol, which supposedly decrease pHi, favour stalk and inhibit spore differentiation, while high extracellular pH and addition of weak bases, which may increase pHi, favour spore and inhibit stalk differentiation (Gross et al. 1983; Town, 1984; Dominov & Town, 1986). It was hypothesized that morphogen-induced pHi changes might determine the choice between alternative pathways of cell differentiation, with high pHi favouring the spore pathway and low pHi favouring the stalk pathway (Gross et al. 1983). Recent
reports indicate that DIF, the morphogen that is probably responsible for the induction of stalk cell differentiation, does not induce the expected decrease in pH, which would according to the above described hypothesis mediate its effect on stalk gene expression (Kay et al. 1986).

In this study, we focus our attention on cAMP; this compound is secreted by *D. discoideum* cells and acts both as a chemoattractant and as a morphogen. Cyclic AMP can induce the synthesis of spore-specific gene products in preaggregative cells through interaction with its cell surface receptor (Kay, 1982; Mehdly & Firtel, 1985; Schaap & Van Driel, 1985; Gomer et al. 1986; Oyama & Blumberg, 1986a) and was recently shown to be an absolute requirement for the induction and maintenance of prespore differentiation in the multicellular stages of *Dictyostelium* development (Wang et al. 1988). The intracellular responses mediating gene regulation by cAMP are still obscure. Previous studies made it unlikely that cAMP and cGMP act as intracellular messengers for the induction of prespore gene expression by extracellular cAMP (Schaap et al. 1986).

In this study, we investigate whether a cAMP-induced change in pH mediates the effect of cAMP on prespore gene expression. We measured whether the induction of prespore gene expression by cAMP is preceded by changes in pH. We furthermore determined whether artificially induced pH changes elevate or decrease the synthesis of prespore-specific gene products in the absence of cAMP or affect prespore gene expression in the presence of extracellular cAMP.

**Materials and methods**

**Materials**

Methylamine and 5,5-dimethyl-2,4-oxazolidinedione (DMO) were from Sigma (USA). [α-32P]UTP 600 Ci mmol⁻¹ was from New England Nuclear, and Biodyne membrane was from ICN, Irvine, USA. Prespore-specific rabbit IgG was prepared as described by Takeuchi (1963). FITC-conjugated swine anti-rabbit IgG was obtained from Dakopatts (Denmark) and peroxidase-conjugated goat anti-rabbit IgG was from Nordic (The Netherlands).

**Organisms and culture conditions**

*Dictyostelium discoideum* strain NC-4(H) was grown in association with *Escherichia coli* 281 on glucose peptone agar, harvested and starved on non-nutrient agar for 16 h at 6°C to induce full aggregation competence as described previously (Van Lookeren Campagne et al. 1988).

**Determinations of intracellular pH (pHi)** by means of the 'null point' titration method

The ‘null point’ titration method is based on the determination of the external pH (pHe) at which permeabilization of the plasma membrane no longer causes a shift of pHi (Rink et al. 1982; Aerts et al. 1985). The validation of this assay has recently been discussed and experiments were performed as described previously (Aerts et al. 1987).

**Determinations of pH of frozen–thawed cell lysates**

Cells (2 × 10⁸) were collected from incubation media, washed two times in distilled water and the cell pellets were frozen in liquid nitrogen. The pellets were thawed by shaking in a waterbath at 37°C and the pH of the resulting homogenates was measured 90 s after the onset of thawing (Aerts et al. 1985).

**Determination of D19 prespore mRNA levels and synthesis**

D19 prespore mRNA levels were determined by Northern transfer analysis as described previously (Van Lookeren Campagne et al. 1988). In vitro mRNA synthesis was assayed in isolated nuclei as described previously (Nellen et al. 1987).

**Determination of the proportion of prespore cells**

Aggregates were dissociated into single cells, fixed in methanol and stained with DAPI, and with prespore-specific rabbit IgG and FITC-conjugated swine anti-rabbit IgG (Wang & Schaap, 1988). The proportion of prespore cells (cells that contain at least three FITC-stained vacuoles) versus total cells (DAPI-stained cells) was determined by counting.

**Semiquantitative assay of prespore antigen by means of an enzyme-linked immunosorbent assay (ELISA)**

Cells were lysed by freeze–thawing and diluted in 0.1 M-phosphate/0.2 M-citrate buffer pH 5.0 to contain 1 μg protein per 100 μl. Aliquots (100 μl) were pipetted into the wells of a 96-well ELISA plate and incubated for 1 h at 37°C. Unoccupied adhesive sites of the plastic were saturated with bovine serum albumin and the wells were subsequently incubated with 150 μl of prespore-specific rabbit IgG, which had been preadsorbed to vegetative cells. The wells were washed with phosphate-buffered saline containing 0.1% Tween-20 and incubated with goat anti-rabbit IgG conjugated to hors eradish peroxidase. After extensive washing, a peroxidase assay was carried out using o-phenylenediamine and H₂O₂ as substrates. The absorbance of the reaction product was measured at 492 nm and taken as a measure for the amount of prespore antigen per μg of protein in the lysate. Reaction blanks were obtained by omitting the prespore-specific rabbit IgG.

**Results**

Are pH changes correlated with the induction of cell-type-specific gene expression?

We first determined whether the induction of gene expression by cAMP is accompanied by sustained changes in intracellular pH (pHi). Aggregation-competent cells were incubated in suspension with 1 mM-cAMP and the pHi during the course of incubation was measured by means of the digitonin null point method. Fig. 1 shows that cAMP induces a significant increase of pHi (P < 0.001), which reaches its maximum after 45 to 60 min; this increase encompasses about 0.15 pH units. In the absence of cAMP, no significant changes in pHi were observed.

At incubation periods longer than about 90 min, measurement of pHi with the null point method was no longer possible. This was probably due to the formation of tight cell clumps, which led to non-synchronous cell permeabilization by digitonin. Indications that the pHi actually remained high for at least 2 h were obtained from measurements of pHi in frozen–thawed cell lysates. The pHi of lysates obtained from cells that were
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Fig. 1. Effect of cAMP stimulation on intracellular pH. Aggregation-competent cells were harvested and resuspended to $10^8$ cells ml$^{-1}$ in 3.5 mM phosphate buffer pH 6.5 and incubated in the presence (●) or absence (○) of 1 mM-cAMP. The cells were aerated by bubbling air through the suspension. At the indicated time points, the pH of the cells was measured by means of the null-point titration method. Means and s.e. of 4 to 7 independent measurements are presented.

incubated during two hours with 100 μM-cAMP was 6.95 ± 0.03, while lysates of cells incubated in the absence of cAMP showed a pH of 6.76 ± 0.02. The values obtained by this method do not reflect cytoplasmic pH, since all intracellular organelles are disrupted, but it was previously shown that this method detects cell-cycle-dependent changes in pH of similar magnitude to those found by the null point method (Aerts et al. 1985).

Does the cAMP-induced pH$_i$ increase precede prespore gene expression?
The cAMP-induced elevation of pH$_i$ may either be the cause or the consequence of the differentiation of prespore cells. If the cAMP-induced pH$_i$ increase is an essential step in the transduction of the cAMP signal to prespore-specific gene expression, it should precede the onset of prespore mRNA synthesis. We measured the onset of the cAMP-induced expression of the prespore gene D19 (Barklis & Lodish, 1983) by means of in vitro transcription in isolated nuclei (Fig. 2). D19 mRNA synthesis can at first be detected about 40 min after the addition of cAMP and reaches a maximal rate between 60 and 90 min. D19 mRNA synthesis then remains constant up to at least 180 min. Comparison of this time course with the cAMP-induced increase in pH$_i$ (Fig. 1), shows a 15 to 30 min lag period between the cAMP-induced pH$_i$ increase and the onset of D19 mRNA synthesis. This indicates that the cAMP-induced cytoplasmic alkalinization could, at least in theory, mediate the induction of prespore gene expression by cAMP.

Are pH$_i$ changes the cause of prespore gene expression?
In order to establish a causal relationship between prespore gene expression and the cAMP-induced increase of pH$_i$, we manipulated pH$_i$ by treating the cells with either a weak acid or a weak base. We chose 5,5-dimethyl-2,4-oxazolidinedione (DMO) as a weak acid because it is not metabolized by the cells and has a suitable pK$_a$ of 6.32. Methylamine was chosen as a weak base because it is structurally different from ammonia (which may act as a natural morphogen in Dictyostelium (Sternfeld & David, 1979; Gross et al. 1983)) and has a pK$_a$ of 10.64. Fig. 3 shows that DMO and methylamine have considerable effects on the pH$_i$. DMO (7.5 mM) at extracellular pH (pHe) 6.6 lowers the pH$_i$ from 7.66 to 7.51 and prevents the cAMP-
induced increase in pH$_i$ by clamping the pH$_i$ at a low value. Methylamine (2.5 mM, at pH$_e$ 7.4) raises the pH$_i$ to 7.97, and therefore mimics the effects of cAMP on pH$_i$. Simultaneous addition of cAMP and methylamine does not induce a further increase of pH$_i$. Changing the pH$_e$, in the absence of DMO or methylamine, from 6.6 to 7.4 has no significant effect on pH$_i$.

These results demonstrate that DMO and methylamine effectively clamp the pH$_i$, at respectively, a low or a high value. If prespore gene expression is a consequence of the cAMP-induced pH$_i$ elevation, artificial elevation of the pH$_i$, which would bypass the interaction of cAMP with its receptor, should cause the induction of prespore gene expression in the absence of cAMP, whereas clamping the pH$_i$ at low levels should prevent cAMP-induced prespore gene expression. Fig. 4 shows the effect of DMO and methylamine added in the presence and absence of cAMP on the levels of prespore mRNA D19. It is clear that methylamine, which mimics the cAMP-induced increase in pH$_i$, cannot induce D19 mRNA synthesis in the absence of cAMP. Furthermore, 7.5 mM-DMO, which prevents the cAMP-induced increase in pH$_i$ completely, inhibits the cAMP-induced D19 mRNA synthesis only slightly. This indicates that the cAMP-induced increase in pH$_i$ does not mediate the effect of cAMP on prespore gene expression.

Effects of pH$_e$ on prespore differentiation

The above-described experiments are restricted to the effect of pH$_i$ on the synthesis of a single prespore mRNA, D19. We also investigated the effect of artificial elevation of pH$_e$ on a more general marker of prespore differentiation, the prespore vacuole. This organelle contains spore coat (glyco)proteins and can be detected by means of an antibody raised against intact spores (Takeuchi, 1963), which detects a large number of spore coat antigens. Aggregation-competent cells were incubated for 8h with different concentrations of DMO and methylamine in the presence and absence of cAMP and prespore differentiation was determined at two different levels: the proportion of cells containing prespore vacuoles was measured by means of immunocytochemistry (Fig. 5A), and the total amount of spore coat proteins synthesized during the 8h incubation period was determined by means of an ELISA assay using the antispore rabbit IgG (Fig. 5B).

We found that, in the absence of cAMP, no significant synthesis of prespore antigens was induced by either methylamine or DMO. After 8h of incubation in the presence of cAMP, about 60% of the cells contained prespore vacuoles and this percentage was not significantly altered by incubating the cells at either pH 6.6 or 7.4 or by the respective addition of DMO or methylamine at these pH$_e$ values. The total level of
prespore proteins seemed about 5–10% lower at pH 6.6 than at pH 7.4. A further 15% reduction is induced by addition of 7.5 mM-DMO, but no increase occurred by addition of methylamine. These experiments show that (i) cAMP is an absolute requirement for the differentiation of prespore cells in shaken suspension, (ii) the effect of cAMP on prespore protein synthesis is not mediated by an increase in intracellular pH and (iii) intracellular pH changes are not involved in regulating the proportion of cells that synthesize prespore vacuoles.

Discussion

The interaction of cAMP with cell surface cAMP receptors induces the synthesis of spore-specific gene products (Kay, 1982; Schaap & Van Driel, 1985; Gomer et al. 1986; Oyama & Blumberg, 1986a). In contrast to other surface-receptor-mediated responses such as chemotaxis and cAMP relay, prespore induction by cAMP does not adapt to constant stimulation, but instead requires a continuous elevation of extracellular cAMP levels. Earlier studies have shown that cAMP-induced responses such as adenylate and guanylate cyclase activation, which are both subjected to adaptation, are most likely not involved in the transduction from cAMP to prespore gene expression (Schaap et al. 1986).

In our search for possible intracellular messengers that control the expression of prespore genes, we investigated whether the effects of cAMP may be mediated by an alteration in intracellular pH. We found that sustained stimulation of aggregation-competent cells with cAMP induces a 0.15 unit increase of pH,, which precedes the cAMP-induced transcription of prespore genes by about 15 to 30 min (Figs 1 and 2). Extracellular cAMP also induces a rapid transient elevation in pH,, (Aerts et al. 1987), however, since this rapid increase adapts to constant cAMP stimulation, it is probably not involved in the transduction of cAMP to prespore gene expression.

The cAMP-induced increase in pH, can be mimicked by treatment of cells with the weak base methylamine and can be abolished by treatment with the weak acid DMO (Fig. 3). However, it was found that methylamine cannot induce the synthesis of spore-specific gene products, while complete inhibition of the cAMP-induced pH, increase by DMO does not prevent the cAMP-induced synthesis of spore-specific gene products (Figs 4 and 5). We conclude from these data that, although cAMP induces an sustained elevation of pH, this pH, increase is not an essential component in the transduction of the cAMP signal to spore-specific gene expression.

Contrary to our results, Town et al. (1987) reported that weak acids and bases did not affect pH, in Dictyostelium. They measured pH, values by means of the 31P-NMR method, which was recently shown to be subject to misinterpretations due to unusually high phytate concentrations in Dictyostelium cells (Martin et al. 1987). The latter investigators measured pH, by accumulating 3-phosphoglycerate, which has a pH-sensitive NMR signal, and found similar values to those reported here.

It was previously shown that weak acids, administered at a pH of 4.7, strongly inhibit prespore and
spore differentiation in *D. discoideum* strain V12M2 and derivatives (Gross et al. 1983; Town, 1984; Dominov & Town, 1986; Town et al. 1987), supposedly via a decrease in pH$i$ (Gross et al. 1983). We found that pH$i$ was optimally reduced by treatment with the weak acid DMO at pH$0$ of 6-6, while only minor effects of this pH$i$ decrease on prespore differentiation were evident. We did observe that cell viability is considerably reduced when cells are incubated in buffers of pH5.5 and lower.

A pronounced stimulatory effect of weak bases on prespore differentiation in V12M2 cells developing in monolayers have also been reported (Gross et al. 1983), but were not found by us after incubating aggregation-competent NC4 cells in shaken suspension. The discrepancies between the effect of weak bases on V12M2 cells developing in monolayers and NC4 cells incubated under conditions that allow aggregate formation may be due to the use of different strains but may also result from a possible requirement for cell-cell contacts for prespore gene expression. It was recently shown that ammonia can replace this requirement for multicellularity, when NC-4 cells are rapidly shaken in a medium containing glucose, albumin and EDTA (Oyama & Blumberg, 1986).

Our present experiments show that cAMP-induced prespore gene expression is preceded by an increase in pH$i$, but that this increase does not by itself mediate the effects of cAMP. It is possible that the observed cytoplasmic alkalization is a secondary effect of the real transduction event, which may involve the consumption of protons, caused by, for example, a cation/H$^+$ antiporter, in which the cation acts as an intracellular messenger. The fact that the induction of prespore gene expression by cAMP is strongly inhibited by Ca$^+$ antagonists (Schaap et al. 1986) suggest that Ca$^+$ may be involved cations.

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References


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