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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 (pH_i) changes mediate the induction of prespore gene expression by cAMP. It was found that cAMP induces a 0·15 unit increase in pH_i within 45 min after stimulation. The cAMP-induced pH_i increase precedes the induction of prespore gene expression, measured by *in vitro* transcription, by about 15–30 min. Cyclic-AMP-induced pH_i changes can be bypassed or clamped by addition of, respectively, the weak base methylamine, which increases pH_i, or the weak acid 5,5-dimethyl-2,4-oxazolidinedione (DMO), which decreases pH_i. Bypass of the cAMP-induced increase of pH_i with methylamine does not induce the expression of prespore genes, while inhibition of the pH_i 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 pH_i increase. These results show that although a morphogen-induced pH_i 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 (pH_i) 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, pH_i 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 alkalinization is a necessary step for the initiation of DNA synthesis and for growth (Pouyssegur *et al.* 1984). In *Dictyostelium discoideum*, pH_i 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, pH_i 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 pH_i. Low extracellular pH, addition of weak acids and addition of the H^+-ATPase inhibitor diethylstilbestrol, which supposedly decrease pH_i, favour stalk and inhibit spore differentiation, while high extracellular pH and addition of weak bases, which may increase pH_i, favour spore and inhibit stalk differentiation (Gross *et al.* 1983; Town, 1984; Dominov & Town, 1986). It was hypothesized that morphogen-induced pH_i changes might determine the choice between alternative pathways of cell differentiation, with high pH_i favouring the spore pathway and low pH_i 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_i, 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; Mehdy & Firtel, 1985; Schaap & Van Driel, 1985; Gomer *et al.* 1986; Oyama & Blumberg, 1986*a*) 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_i 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_i. We furthermore determined whether artificially induced pH_i 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-^{32}P]-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 was *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 (pH_i) by means of the 'null point' titration method

The 'null point' titration method is based on the determination of the external pH (pH_e) at which permeabilization of the plasma membrane no longer causes a shift of pH_e (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 (2×10^8) 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 bufer 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 horseradish peroxidase. After extensive washing, a peroxidase assay was carried out using *o*-phenylenediamine and H_2O_2 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_i 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 (pH_i). Aggregation-competent cells were incubated in suspension with 1 mM-cAMP and the pH_i 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 pH_i ($P < 0{\cdot}001$), 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 pH_i were observed.

At incubation periods longer than about 90 min, measurement of pH_i 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 pH_i actually remained high for at least 2 h were obtained from measurements of pH in frozen–thawed cell lysates. The pH of lysates obtained from cells that were

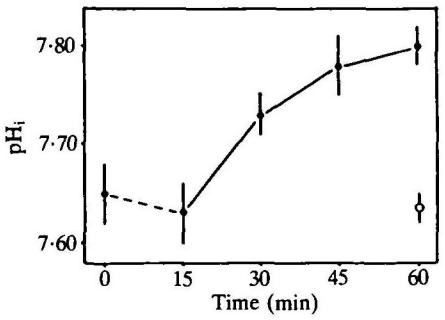

Fig. 1. Effect of cAMP stimulation on intracellular pH. Aggregation-competent cells were harvested and resuspended to 10^8 cells ml^{-1} in 3·5 mM-phosphate buffer pH 6·5 and incubated in the presence (●) or absence (○) of 1 mM-cAMP. The cells were aerated by bubbling air through the suspension. At the indicated time points, the pH$_i$ 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.

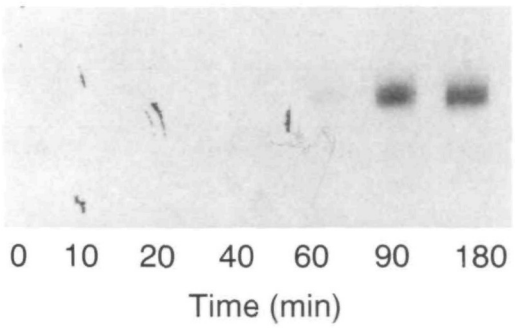

Fig. 2. Time course of the induction of D19 prespore mRNA expression. Aggregation-competent cells were resuspended at 5×10^6 cells ml^{-1} in 10 mM-phosphate buffer, pH 6·5, and shaken at 150 revs min^{-1} and 21 °C in the presence of 300 μM-cAMP, added every hour. At the indicated time points, the nuclei were isolated and incubated in the presence of [α-^{32}P]-UTP. The radioactively labelled RNAs were hybridized to strips of Biodyne membrane containing D19 cDNA insert. The amount of hybridization was visualized by autoradiography.

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$_i$ 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-oxazolidineodione (DMO) as a weak acid

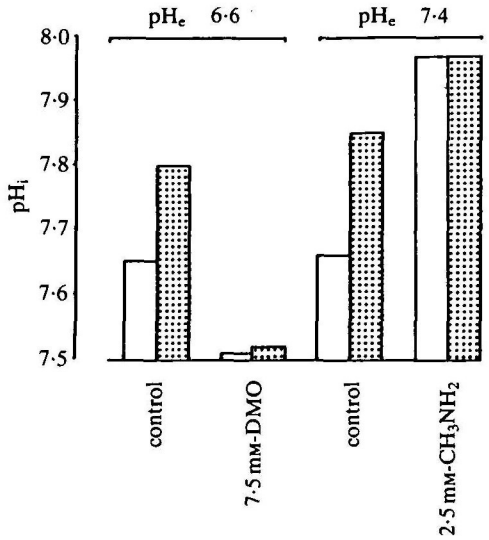

Fig. 3. Effects of pH$_e$, DMO and methylamine on cAMP induced pH$_i$ changes. Aggregation-competent cells were resuspended to 10^8 cells ml^{-1} and incubated in 3·5 mM phosphate buffer pH 6·6, to which 7·5 mM-DMO and/or 1 mM-cAMP (dotted bars) was added. Alternatively, the cells were incubated in 3·5 mM-phosphate buffer pH 7·4, to which 2·5 mM-methylamine and/or 1 mM-cAMP (dotted bars) was added. The cell suspension was aerated and after 60 min of incubation the pH$_i$ of the cells was measured. The data represent the means of 4 to 7 independent determinations.

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 (pH$_e$) 6·6) lowers the pH$_i$ from 7·66 to 7·51 and prevents the cAMP-

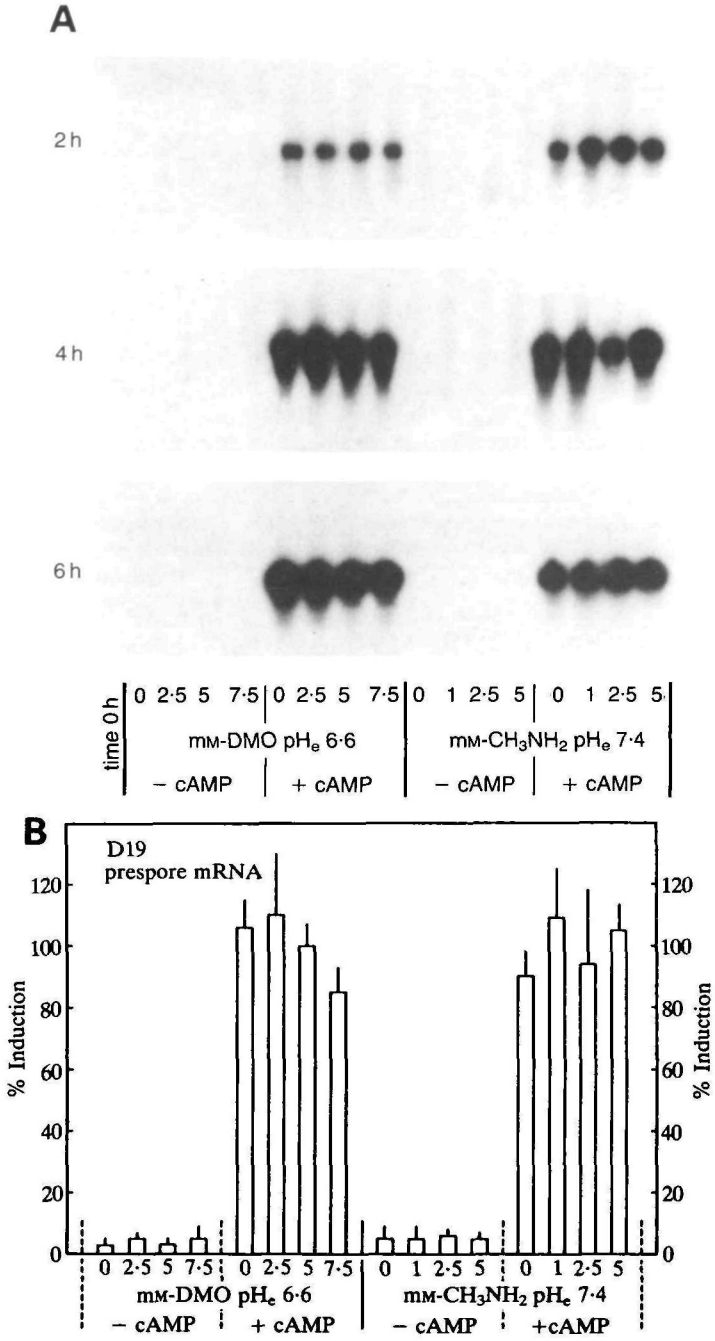

Fig. 4. Effects of cAMP, pH_e, DMO and methylamine on prespore gene expression. Aggregation-competent cells were resuspended to 5×10^6 cells ml^{-1} in 10 mM-phosphate buffer, pH 6·6 or pH 7·4. Aliquots of 15 ml were shaken at 150 revs min^{-1} and 21 °C in the presence or absence of 30 μM-cAMP and of different concentrations of DMO or methylamine set at pH 6·6 or pH 7·4, respectively. Cyclic AMP was added every hour and the cells were washed every 2 h and resuspended in fresh incubation medium. Samples for the assay of prespore mRNA were taken after 0, 2, 4 and 6 h of incubation. A shows somewhat overexposed autoradiographs of Northern transfers of size fractionated mRNA, hybridized to the ^{32}P-labelled prespore-specific cDNA D19. B represents a quantification of D19 mRNA levels, obtained by scanning the optical density of the specific mRNA bands of appropriately exposed autoradiographs. The data are expressed as percentage of the maximal value reached at the specific time point and represent the means and S.D. of two separate experiments, each consisting of three different time points.

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 mimicks 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 mimicks 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_i 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_i 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 8 h 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 8 h 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 8 h 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

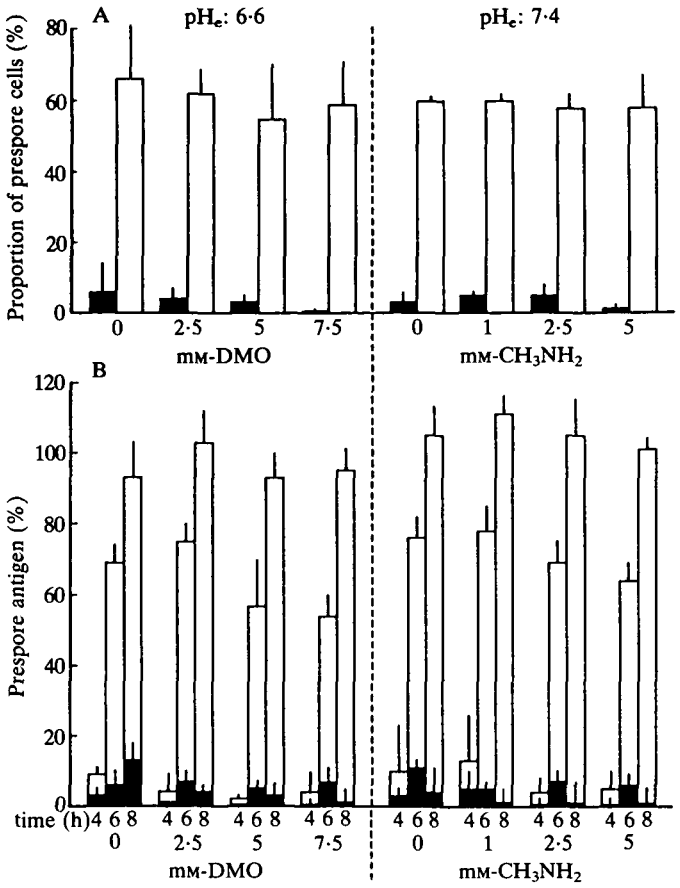

Fig. 5. Effects of cAMP, pH$_e$, DMO and methylamine on the proportion of prespore cells and the levels of prespore proteins. Aggregation-competent cells were incubated at 5×10^6 cells ml^{-1} in 10 mM-phosphate buffer pH 6·6 or pH 7·4, in the presence (open bars) or absence (black bars) of 30 μM-cAMP and of different concentrations of DMO or methylamine as indicated. After 2, 4, 6 and 8 h of starvation, 1 ml samples were withdrawn to determine the level of prespore antigens, measured by means of a prespore-specific rabbit IgG in an ELISA assay. The obtained data are expressed as the percentage of the maximum level of induction, reached after 8 h of incubation in the presence of cAMP (B). After 8 h of incubation, another 1 ml sample was taken, the cell clumps, which had formed by this time were dissociated into single cells, transferred to glass slides, fixed in methanol and stained with prespore-specific rabbit IgG and FITC-conjugated goat anti-rabbit IgG according to standard procedures and post-stained with DAPI, a dye that stains the nuclei of all the cells. The percentage of cells containing more than three FITC-stained vacuoles *versus* the total amount of (DAPI-stained) cells was determined. In each experiment, 200–300 cells were counted for each data point (A). A and B represent the means and S.D. of data derived from three separate experiments.

prespore proteins seemed about 5–10% lower at pH$_e$ 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, 1986*a*). 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$_i$, 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$_i$ (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$_i$ 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$_i$ 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$_i$, this pH$_i$ 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$_i$ in *Dictyostelium*. They measured pH$_i$ values by means of the ^{31}P-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$_i$ 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$_e$ 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_e 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 pH 5·5 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*b*).

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 alkalinization 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^{2+} antagonists (Schaap *et al.* 1986) suggest that Ca^+ may be the involved cations.

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