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cAMP induction of prespore and prestalk gene expression in *Dictyostelium* is mediated by the cell-surface cAMP receptor

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ABSTRACT **Extracellular adenosine 3′,5′-cyclic monophosphate (cAMP) is required for cell-type-specific gene expression in developing *Dictyostelium discoideum*. We have developed a microassay for the expression of these genes, using antibodies directed against their protein products. To characterize the transduction mechanism, we have used in this assay cAMP analogues that preferentially activate either the cell-surface cAMP receptor or the internal cAMP-dependent protein kinase. N^6-(aminohexyl) cAMP activates the *Dictyostelium* cAMP-dependent protein kinase but does not bind to the cell-surface cAMP receptor and does not cause cell-type-specific gene expression. 2′-Deoxy-cAMP does not activate the cAMP-dependent protein kinase but binds to the receptor and causes cell-type-specific gene expression. Cyclic AMP-induced accumulation of prestalk mRNA in shaking cultures still occurs in the presence of caffeine, which blocks the receptor-coupled activation of adenyl cyclase. This suggests that the extracellular cAMP induction of cell-type-specific gene expression in developing *Dictyostelium* cells is mediated by the cell-surface cAMP receptor and that activating adenyl cyclase by this receptor is not essential. Using the N^6-(aminohexyl) cAMP to competitively inhibit phosphodiesterase, we show that 30 nM cAMP is sufficient to induce prestalk or prespore gene expression.**

Dictyostelium discoideum is an excellent system to study cell differentiation. Vegetative amoebae aggregate after removal of their food source. The aggregated cells differentiate into either prespore or prestalk cells, which in turn become the spore or stalk cells of the fruiting body (see refs. 1 and 2). The development of *Dictyostelium* is mediated in part by extracellular adenosine 3′,5′-cyclic monophosphate (cAMP). The aggregation of cells upon starvation is caused by chemotaxis toward the source of pulses of cAMP (3). Chemotaxis of up to 10^5 cells is mediated by relayed pulses of cAMP: an arriving pulse causes both chemotaxis of a cell to the source and the secretion of a pulse of cAMP (4–8). This relaying causes concentric waves of cAMP to propagate outward from the center of the aggregate. A second role of cAMP early in development is involved with inducing cellular differentiation (see refs. 9 and 10; S. Mann and R.A.F., unpublished data). Pulses of cAMP induce a number of early developmentally regulated genes (11–13), while treatment of early developing cells with a continuous level of cAMP blocks normal development (refs. 2 and 10–15; S. Mann and R.A.F., unpublished results). A third role of extracellular cAMP in *Dictyostelium* development is the induction of prespore and prestalk genes (16, 17). Conditions have been developed in which starved cells, in the absence of cell–cell contact, will express prespore- and prestalk-specific mRNAs if and only if cAMP is added to the culture medium several hours after starvation (15, 16).

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The mechanism whereby the extracellular cAMP induces cell-type-specific gene expression is unknown. One possible mechanism is that cAMP binds to a cell-surface receptor, which then mediates the gene induction. A cAMP-dependent protein kinase has been shown to exist in *Dictyostelium* (18–22). A second possible mechanism would then be that cAMP enters the cells and directly activates this kinase. A third possibility would involve a yet unidentified cAMP-binding protein. As an approach to these questions, we have used cAMP analogues that preferentially activate either the cell-surface cAMP receptor or cAMP-dependent protein kinase *in vitro*. We have established a microassay by using antibodies made against regions of the protein products of several of these cAMP-regulated cell-type-specific genes. This assay allows us to examine the ability of the analogues to activate cell-type-specific gene expression in low-density cultures measured by an immunofluorescence assay and to examine questions concerning the differential expression of these genes. We find that cAMP analogues that activate the cell-surface receptor and have very limited ability to activate the cAMP-dependent protein kinase can induce cell-type-specific gene expression, while other analogues that activate cAMP-dependent protein kinase but not the cell-surface receptor are unable to cause cell-type-specific gene expression. These low-density cultures also allow the examination of the levels of cAMP necessary for cell-type-specific gene induction.

MATERIALS AND METHODS

Enzyme Assay. The ability of cAMP or cAMP analogues to activate cAMP-dependent protein kinase isolated from developing *Dictyostelium* cells was measured by the phosphorylation of Kemptide (19).

Sources of Chemicals. cAMP, 2′-deoxyadenosine 3′,5′-cyclic monophosphate (2′-dcAMP), N^6, $O^{2'}$-dibutyryl-adenosine 3′,5′-cyclic monophosphate (Bt_2cAMP), guanosine 3′,5′-cyclic monophosphate (cGMP), and N^2,$O^{2'}$-dibutyrylguanosine 3′,5′-cyclic monophosphate (Bt_2cGMP) were from Sigma. N^6-(aminohexyl)adenosine 3′,5′-cyclic monophosphate (N^6AH-cAMP) was synthesized from 6-chloropurineriboside 3′,5′-cyclic monophosphate and 1,6-hexanediamine by a modification of the procedure of Juliani *et al.* (21) and was purified by anion-exchange chromatography on Bio-Rad AG-1-X8 in the acetate form, followed by paper chromatography on acid-washed Whatman 3MM paper using 70% (vol/vol) isopropanol as the chromatographic solvent. The isolated product gave a single UV-absorbing and ninhydrin-positive spot in several thin-layer chromatography systems and had a λ_{max} at 265 nm in distilled water.

Abbreviations: 2′-dcAMP, 2′-deoxyadenosine 3′,5′-cyclic monophosphate; N^6AH-cAMP, N^6-(aminohexyl)adenosine 3′,5′-cyclic monophosphate; Bt_2, dibutyryl.

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Immunofluorescence and RNA Assays. All experiments used midlogarithmic phase axenically grown KAx-3 cells. Fresh medium and conditioned medium were prepared as described by using medium from cells plated on Petri dishes or "developed" in slow-shake cultures (15). For the gene expression assays, cells were collected by centrifugation and washed (15). Cells were resuspended and diluted in fresh or conditioned medium to a final density of 1.8×10^4 cells per ml; 200 μl of such a cell suspension was then added to a well of a Lab-Tek type 4818 eight-chamber plastic slide (Miles). Alternatively, 100 μl of the cell suspension was plated in a well of a 96-well microtiter plate (Falcon, type 3040). When the cells settled, this gave a surface density of 4.5×10^3 cells per cm^2 on the bottom of either type of chamber. Immediately upon plating and/or 6 hr after plating, water, cAMP, or cAMP analogues were added to the medium as described in the results. Twenty hours after plating, the slides or plates were immersed in 100% methanol at room temperature for 10 min and then air-dried for 1 hr to fix the cells. Production of rabbit polyclonal antibodies against selected regions of prestalk- and prespore-specific gene products and immunofluorescence is described elsewhere (10, 23, 24).

Fast shaking suspensions of starving *Dictyostelium* amoebae and the procedures used for RNA isolation and blotting have been described by Mehdy *et al.* (16).

RESULTS

As mentioned above, cAMP binds to a cell-surface cAMP receptor and elicits chemotaxis of *Dictyostelium* cells. Substitutions at the N^6 position of cAMP block binding to the receptor (25). N^6AH-cAMP binds to the *Dictyostelium* cell-surface cAMP receptor with less affinity (by a factor of $\approx 10^4$) than cAMP (21). 2′-dcAMP binds to the receptor with only 1/8th the affinity of cAMP and has $\approx$1/13th the chemotactic activity (26, 27). Recently, it has been shown that 2′-dcAMP has $\approx$2500-fold lower activation of *Dictyostelium* cAMP-dependent protein kinase (28, 29). We assayed the ability of N^6AH-cAMP to activate the *Dictyostelium* cAMP-dependent protein kinase. Fig. 1 shows the activation of cAMP-dependent protein kinase *in vitro* by various concentrations of cAMP, 2′-dcAMP, and N^6AH-cAMP. The enzyme is activated by cAMP and perhaps even more strongly by N^6AH-cAMP, but it is activated only very weakly by 2′-dcAMP.

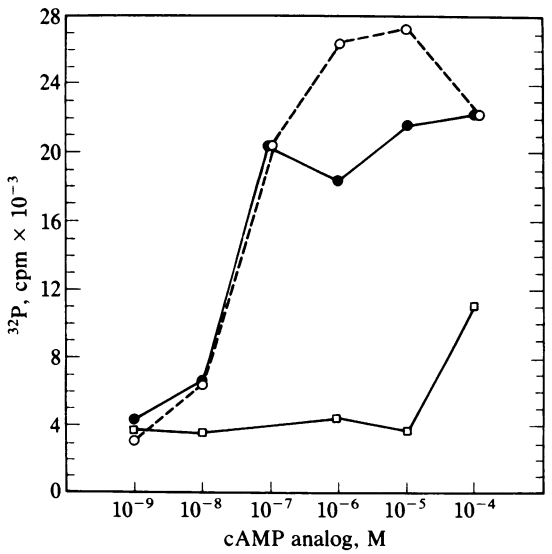

FIG. 1. Protein phosphorylation *in vitro* by purified *Dictyostelium* cAMP-dependent protein kinase. Ordinate, relative amount of phosphorylation; abscissa, amount of cAMP or cAMP analogue added to the reaction mixture. ○, N^6AH-cAMP; ●, cAMP; □, 2′-dcAMP.

Cells plated on a cell surface at a low density in conditioned medium will express prestalk- and prespore-specific mRNAs when treated with extracellular cAMP several hours after starvation, but not when treated with cAMP at the time of starvation (15). When cells grown and treated in such a manner are fixed and stained for the protein products of prestalk- or prespore-specific genes, immunofluorescence reveals that the proteins are detected in cell populations known to be expressing prespore- or prestalk-specific mRNAs and are not detected in cell populations developed in conditions in which cells do not express prespore- or prestalk-specific mRNAs.

Fig. 2 shows cells fixed and stained by immunofluorescence for a prestalk-specific protein. In Fig. 2 *B* and *D*, cells were washed out of growth medium and plated at low density in conditioned medium (CM). Six hours after plating in CM, cAMP was added to the cells shown in Fig. 2*D* to a final concentration of 300 μM, and an equal volume of water was added to the cells shown in Fig. 2*B*. The cells in Fig. 2*D* were thus treated in a manner that induces the expression of prestalk- and prespore-specific mRNAs (15). A subset of the cells can be seen to be stained for the prestalk-specific antigen. The cells in Fig. 2 *A* and *C* were plated in fresh medium; the cells in Fig. 2*C* had cAMP added as in Fig. 2*D*. Plating in unconditioned (fresh) medium with or without cAMP does not result in the expression of prespore- or prestalk-specific mRNAs (15) and no stained cells can be observed. A similar experiment using antibodies against a prespore-specific protein gave similar results.

We were unable to find any set of conditions that caused all the cells in a field to positively stain for prespore or prestalk antigens: either no cells were stained or only a subset of the cells was stained. No cells were seen that stained for both prestalk and prespore markers by double-label immunofluorescence (data not shown). The immunofluorescence staining can thus be used as an assay for prespore or

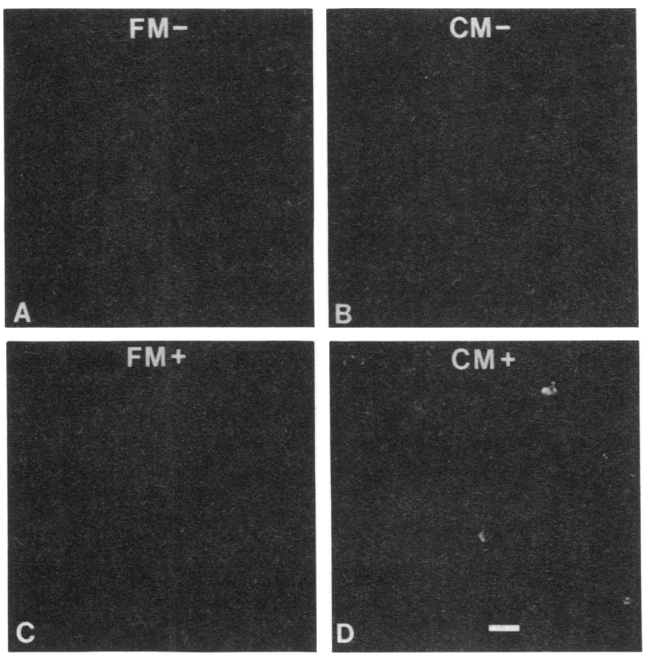

FIG. 2. Indirect immunofluorescence staining of starved low-density plated *Dictyostelium* cells. Cells were plated in a starvation buffer; after 20 hr, they were fixed and stained for a prestalk-specific antigen. Cells in *A* and *C* were starved in fresh medium (FM); cells in *B* and *D* were starved in conditioned medium (CM). Six hours after starvation, cAMP was added to the cells in *C* and *D*. (Bar = 20 μm.)

prestalk gene expression. For each set of conditions, we examined typically ≈300 cells. If three or fewer cells were stained, we considered the conditions to give no prespore- or prestalk-specific gene expression. When >10% positively stained cells could be observed, we considered the conditions to give prespore- or prestalk-specific gene expression. We were able to very easily distinguish positive from negative cell populations that had no or very few positive cells (<1%). Generally, 10% of the cells stained for prestalk antigen. These values are similar to the estimated 15% and 75% of the cells staining for prestalk and prespore antigens during normal development (R.H.G. and R.A.F., unpublished observation). For most of the experiments, the scoring was done by a researcher who was not told in advance which treatments were given in which wells. Each time this type of experiment was performed, one or more positive (CM; cAMP added at 6 h) and negative (CM; no cAMP added) controls were done.

Our first set of experiments was to examine a group of cAMP analogues for the ability to mimic the effect of cAMP. When added to cells that have been starved in conditioned medium for 6 hr, cAMP induces prespore and prestalk gene expression. Of the compounds tested, only 2′-dcAMP could mimic the effect of cAMP at concentrations of 150 μM or less when added at 6 hr. Bt_2cAMP could mimic cAMP only when added at very high concentrations (300 μM). N^6AH-cAMP, cGMP, and Bt_2cGMP failed to cause either prespore or prestalk gene expression when added at 6 hr (Table 1, rows 3 and 4). Both cAMP and 2′-dcAMP could induce prespore and prestalk gene expression at 30, 150, or 300 μM; in this range, there was no discernible effect of cAMP or analogue concentration on the percentage of cells induced to express prespore or prestalk genes.

No prespore or prestalk gene expression could be observed when cAMP and/or any of the cAMP analogues tested were added to plated cells in conditioned medium at the time of starvation (0 hr). These experiments were done by using the compounds at final concentrations of 150 μM and 300 μM, and not adding cAMP at 6 hr after plating (Table 1, rows 5 and 6). A similar set of experiments was performed, adding the compounds at 0 hr and then adding cAMP at 6 hr. cAMP, N^6AH-cAMP, 2′-dcAMP, and Bt_2cAMP all prevented both prespore and prestalk gene expression when added at 0 hr, even when cAMP was added to the medium at 6 hr (rows 7 and 8). cGMP and Bt_2cGMP when added at 0 hr did not prevent prespore or prestalk gene expression caused by adding cAMP at 6 hr.

The inhibition of prespore and prestalk gene expression by addition of a cAMP analogue at 0 hr could arise by two general methods. The first would be a direct effect at 0 hr, and the second would be by the analogue still present in the medium interfering with the extracellular cAMP-receptor pathway at 6 hr. To differentiate between the two, we plated cells in conditioned medium. At 6 hr, we added either cAMP alone or the analogue and cAMP. None of the analogues prevented prespore or prestalk gene expression induced by adding cAMP at 6 hr (Table 1).

N^6AH-cAMP, when added at 6 hr, does not induce the expression of prespore or prestalk proteins (Table 1). The possibility exists that this compound causes prespore and prestalk mRNA synthesis but interferes with protein synthesis. To examine this, cells were starved in fast shaking conditions (16), and either cAMP, 2′-dcAMP, or N^6AH-cAMP was added to 300 μM after 6 hr. At 20 hr, cells were harvested and the RNA was extracted and analyzed on gels followed by blotting to nylon membranes and hybridization of the filter with cell-type-specific probes. Fig. 3 shows an RNA blot probed for the prestalk-specific 16-G1 (pst-cathepsin) mRNA. The cells treated with cAMP or Bt_2cAMP contain pst-cathepsin mRNA, while the cells treated with N^6AH-cAMP do not contain this.

Caffeine blocks the activation of adenyl cyclase by the cell-surface cAMP receptor in *Dictyostelium* (30, 33). If the induction of prespore- and prestalk-specific gene expression by extracellular cAMP is mediated by the extracellular cAMP receptor, the receptor could conveivably be coupled to the transduction machinery via cAMP receptor-activated adenyl cyclase. To examine this, cells in fast shaking culture were incubated either in buffer alone or in buffer containing 1.5 mM or 3 mM caffeine, which was supplemented every 2 hr. After 5 hr, cAMP was added to 150 μM to the appropriate flasks, and the cAMP was supplemented at 7 and 9 hr. At 10 hr, cells were harvested and RNA was extracted and analyzed with the 16-G1 (prestalk) probe (see Fig. 3*B*). [Note: prespore genes are not induced in fast shaking culture because of the absence of cell–cell or cell–surface interactions (15, 16)]. cAMP induces prestalk gene expression in the caffeine-treated cells as well as in the control cells, although the level of expression is reduced by approximately one-half. Since the higher caffeine concentration allows the same or a higher level of prestalk gene expression compared to the lower caffeine level, the absence of a complete shutoff of prestalk gene expression is probably not due to an insufficient level of caffeine. Cells not receiving cAMP do not express

Table 1. Effect of cAMP analogues on gene expression

Time of addition of cAMP and analogues	cAMP	N^6AH-cAMP	2′-dcAMP	Bt_2AMP	cGMP	Bt_2GMP
No addition	−	−	−	−	−	−
Addition of compound at						
6 hr to 30 μM	+	−	+	−	−	−
6 hr to 150 μM	+	−	+	−	−	−
6 hr to 300 μM	+	−	+	+	−	−
0 hr to 150 μM	−	−	−	−	−	−
0 hr to 300 μM	−	−	−	−	−	−
0 hr to 150 μM; then addition of cAMP at 6 hr to 150 μM	−	−	−	−	+	+
0 hr to 300 μM; then addition of cAMP at 6 hr to 300 μM	−	−	−	−	+	+
6 hr to 300 μM followed by addition of cAMP to 30 μM	+	+	+	+	+	+

Starved axenically grown KAx-3 cells were plated at 4.5×10^3 cells per cm^2 in conditioned medium. cAMP or analogues were added as indicated. Twenty hours after starvation, the cells were fixed and stained for either a prespore- or a prestalk-specific antigen by immunofluorescence. −, Essentially no staining observed with either antibody; +, both antibodies stained cells. No reproducible conditions were found in which only one of the two antibodies showed staining (see text for details). Times given are time after starvation and plating in conditioned medium.

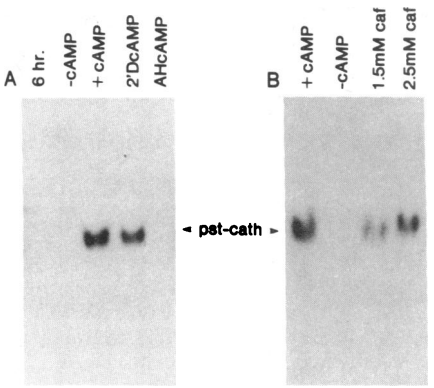

FIG. 3. Induction of cell-type-specific mRNAs by cAMP analogues. (*A*) Cells were shaken at 230 rpm to prevent lasting cell–cell contacts in starvation buffer (17). Six hours after starvation, cAMP, 2′-dcAMP (2′DcAMP), N^6AH-cAMP (AHcAMP), or water (control) was added to the cells. Twenty hours after starvation, cells were harvested and RNA was extracted. RNA blots were probed for prespore (2-H3) and prestalk (16-G1) message (15). (*B*) Cells were starved in fast-shake cultures similar to those described above (see also *Results*). RNA isolated from cells at 10 hr was probed for 16-G1 (prestalk) mRNA. Cells treated with cAMP at 5, 7, and 9 hr (+ cAMP) express 16-G1 mRNA; cells not treated with cAMP (− cAMP) do not. Cultures treated with cAMP as in lane (+ cAMP) and with caffeine (caf) at two different concentrations (see *Results*) express 16-G1 mRNA at a somewhat reduced level.

pst-cathepsin mRNA. These results indicate that activating the adenylate cyclase via the receptor is not essential for prestalk gene expression.

To determine the approximate lowest concentration of cAMP and 2′-dcAMP necessary to induce the late genes under these conditions, we did a concentration curve. cAMP (30 μM and 3 μM), added 6 hr after starvation, induced both prespore and prestalk genes. Neither 300 nM nor 30 nM cAMP induced the genes. 2′-dcAMP (300 μM or 30 μM) could induce both prespore and prestalk genes but 3 μM, 300 nM, or 30 nM 2′-dcAMP could not (Table 2). The difference in the levels of cAMP and 2′-dcAMP required to induce late gene expression agree with their relative affinity for the receptor (26, 27).

The levels of cAMP used by a number of groups to examine cell-type-specific gene expression are not necessarily within the physiological range but are necessary because of degradation of the compound by extracellular cAMP-dependent phosphodiesterase (15). N^6AH-cAMP does not induce cell-type-specific genes but apparently effectively competes with cAMP for the phosphodiesterase (14). N^6AH-cAMP was added to 300 μM to low-density cell cultures containing conditioned medium at 6 hr along with either no cAMP or 2′-dcAMP (negative control), or with cAMP or 2′-dcAMP ranging from 30 nM to 300 μM. After 20 hr of starvation, cells were fixed and stained with anti-prestalk or anti-prespore antibodies. Positive results were obtained for all concentrations of cAMP and 2′-dcAMP down to 30 nM but not for the negative control (Table 2). Moreover, the level of positive-staining cells was the same for all cAMP concentrations. These results indicate that 30 nM extracellular cAMP is sufficient to induce cell-type-specific gene expression when the phosphodiesterase is essentially blocked.

DISCUSSION

We have developed a microassay for prestalk or prespore gene expression. The assay can be done with $\approx 10^3$ cells in 0.1 ml. Using a compound at 300 μM would then require only 3×10^{-8} mol of the compound. None of the compounds tested gave differential activation or inhibition of prespore as opposed to prestalk gene expression, suggesting that these factors are not involved in cell-type choice in differentiation. If this is indeed the case, cell-type choice in *Dictyostelium* differentiation is mediated by other substances or else by factors other than extracellular conditions. The fact that only a subset of cells in any of the conditions examined express either prespore or prestalk cells suggests that cells are responding differently to the conditions and could indicate that there are differences within cells that result in the induction of either prestalk or prespore cells and that the expression of prestalk or prespore genes within a cell precludes the induction of the other. It is possible that some event earlier than 6 hr of development causes cells to choose to differentiate into a given cell type. This could be conceivably a stochastic process or dependent on the phase of the cell cycle at the time of starvation (31). The secretion of a product from cells differentiating down one pathway could regulate the differentiation of other cells, although this seems less likely at the cell density used in these experiments. It is also possible that the level of expression of the prestalk and prespore proteins is insufficient in many of the cells to give a definitively positive signal in our assay.

The addition of cAMP, N^6AH-cAMP, 2′-cAMP or Bt_2cAMP at the time of starvation blocks both prespore and prestalk gene expression even when cAMP is added at 6 hr (Table 1). This suggests that the high steady-state levels of the above compounds block some early event in the *Dictyostelium* developmental pathway that is required for prespore and prestalk gene expression (refs. 10, 14, and 15; S. Mann and R.A.F., unpublished data). A similar blockage is not observed when cGMP or Bt_2cGMP is added at 0 hr, suggesting a specificity of the blocking factor for cAMP derivatives (ref. 26; Table 1). Interestingly, we see an inhibition of *Dictyostelium* development (as measured by cell-type-specific gene expression) by addition of N^6AH-cAMP at the time of starvation. When added at 6 hr, this analogue does not induce prespore or prestalk gene expression at the level of transcription (Table 1 and Fig. 2). Since N^6AH-cAMP added at 6 hr does not inhibit the effect of cAMP added at the same time, we can conclude that N^6AH-cAMP is simply not activating the 6-hr cAMP pathway. The inhibition by N^6AH-cAMP of late gene expression

Table 2. cAMP analogue dosage requirements

Addition of cAMP or cAMP analogue at 6 hr to concentration shown	300 μM	30 μM	3 μM	300 nM	30 nM	0
cAMP to indicated concentration	+	+	+	−	−	−
2′-dcAMP to indicated concentration	+	+	−	−	−	−
N^6AH-cAMP to 300 μM followed by addition of cAMP to indicated concentration	+	+	+	+	+	−
2′-dcAMP to 300 μM followed by addition of cAMP to indicated concentration	+	+	+	+	+	−

Assay conditions and symbols used are as described in Table 1.

when the analogue is given at 0 hr is probably due to an inhibition of the *Dictyostelium* phosphodiesterase, resulting in increase in the extracellular cAMP levels (14). From other results, we know that this results in an inhibition of cAMP pulse-induced activated early gene expression (S. Mann and R.A.F., unpublished observations) and in late gene expression (15).

N^6AH-cAMP and cAMP itself both activate the *Dictyostelium* cAMP-dependent protein kinase, while 2'-dcAMP essentially does not (ref. 29; Fig. 1). Since 2'-dcAMP mimics the effect of cAMP on prespore and prestalk gene expression (Table 1; ref. 29), the effect of cAMP added at 6 hr on prespore and prestalk gene expression is probably not mediated via a direct (as opposed to an indirect) activation of the cAMP-dependent protein kinase. Moreover, the ≈10 times higher level of 2'-dcAMP compared to cAMP that is needed to activate prespore and prestalk gene expression (Table 2) is in rough agreement with its ≈10 times lower affinity for the cAMP receptor (26, 27). We assume that if cAMP can enter *Dictyostelium* cells, the N^6AH-cAMP would also enter the cells. However, it is unlikely that cAMP entering the cells is the mechanism by which cAMP mediates its effects, since concentrations of cAMP as low as 30 nM can induce prestalk and prespore gene expression. It is possible that cAMP interacts with a cell-surface receptor and causes an increase in intracellular cAMP, and then activates the cAMP-dependent protein kinase (18, 19, 30). However, our experiments using caffeine to block this event indicate that activation of adenylate cyclase is not necessary for induction of late genes. A second alternative is that there may be an intracellular cAMP-binding protein with a similar affinity toward cAMP analogues as the *Dictyostelium* cell-surface cAMP receptor present during aggregation. Such a protein could be involved in the direct activation of gene expression. *Escherichia coli* cyclic AMP receptor protein has a binding affinity to cAMP comparable to that which induces late gene expression in *Dictyostelium* (32). When all the results are taken together, they strongly suggest that the cAMP activation of cell-type-specific genes in *Dictyostelium* is mediated by the cell-surface cAMP receptor activating a pathway other than adenylate cyclase.

Finally, we have shown that cAMP given to cells at a concentration as low as 30 nM is capable of inducing prestalk and prespore gene expression in the presence of N^6AH-cAMP, a competitive inhibitor of the phosphodiesterase. Addition of the N^6AH-cAMP alone does not induce late gene expression. This level of cAMP is comparable to the level of cAMP pulsing capable of inducing early gene mRNA (S. Mann and R.A.F., unpublished data) and further supports cAMP as the intracellular factor controlling prestalk and prespore gene expression.

Note Added in Proof. After submission of this manuscript, three studies with conclusions similar to ours were published (34–36).

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