Conditions that alter intracellular cAMP levels affect expression of the cAMP phosphodiesterase gene in Dictyostelium
(gene regulation/differentiation/8-bromoadenosine 3',5'-cyclic monophosphate/ammonia/caffeine)

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ABSTRACT We examined expression of the Dictyostelium cAMP phosphodiesterase (PDE) gene under conditions that alter intracellular cAMP levels during in vitro differentiation of wild-type strain V12M2 and a sporogenous derivative, HB200. In control cultures, cellular PDE activity peaked at 6 hr and declined by 8 hr, while secreted PDE activity continued to increase through 8 hr. Lowering intracellular cAMP levels with caffeine or progesterone increased cellular and secreted PDE activities 2-fold, increased stalk cell differentiation, and inhibited spore differentiation. In contrast, exposure to 8-bromoadenosine 3',5'-cyclic monophosphate (8-Br-cAMP; a membrane-permeable cAMP analog) or ammonia (which promotes intracellular cAMP accumulation in V12M2 and HB200 cells) lowered PDE activities by as much as 45%, decreased stalk cell differentiation, and increased spore differentiation. Simultaneous exposure to 8-Br-cAMP and caffeine gave intermediate PDE activities as would be expected if 8-Br-cAMP entered the cell and bypassed the caffeine-mediated block to adenylyl cyclase activation. In all cases, we observed commensurate changes in developmental PDE transcript levels. The developmental time course of expression was not significantly altered by these treatments. These results suggest that the magnitude of PDE gene expression is negatively regulated by intracellular cAMP levels and provide evidence for one of the earliest changes in gene expression that is consistent with cell-type specificity. These results are discussed in terms of a bistable switch employing intracellular cAMP as a regulator of cell fate.

Diffentiation of Dictyostelium amoebae culminates with the formation of fruiting bodies composed of stalk cells and spores. Simplified in vitro culture conditions permit analysis of signals that regulate the choice between stalk cell and spore differentiation. Stalk cell formation by wild-type V12M2 cells during in vitro differentiation requires two known secreted factors: cAMP and differentiation-inducing factor (DIF) (1–3). V12M2 cells cannot complete spore differentiation under these conditions, but mutants of V12M2 can be isolated that form both stalks and spores in vitro (3, 4). Such mutants are termed "sporogenous." All cells require exogenous cAMP to initiate differentiation. Later in development, high exogenous cAMP levels inhibit terminal stalk cell differentiation and promote spore differentiation (5–7). DIF antagonizes the effects of exogenous cAMP and induces several genes expressed only in prestalk and stalk cells (8, 9).

We are investigating the role of cAMP in differentiation. Extracellular cAMP binds to cell surface cAMP receptors, causing rapid accumulation of several intracellular second messengers, including intracellular cAMP (10, 11). There is growing evidence that intracellular cAMP influences the decision between stalk cell and spore differentiation. Caffeine and progesterone, which inhibit intracellular cAMP accumulation by different mechanisms (12, 13), inhibit spore and promote stalk cell differentiation in concentration-dependent manners (7). Conversely, conditions that raise intracellular cAMP levels by hyper-inducing adenylyl cyclase activity increase spore and decrease stalk cell differentiation (14). Ammonia, which promotes intracellular cAMP accumulation by inhibiting its secretion (15), and 8-bromoadenosine 3',5'-cyclic monophosphate (8-Br-cAMP), a membrane-permeable cAMP analog with low affinity for the cell surface cAMP receptor (16), also increase spore and decrease stalk cell differentiation in cultures of sporogenous mutants (2, 14). Finally, high levels of 8-Br-cAMP induce wild-type V12M2 cells to form spores in vitro (17).

To investigate how intracellular cAMP levels might regulate differentiation, we are identifying and studying genes that regulate or are regulated by intracellular cAMP levels. In this report, we investigate regulation of cAMP phosphodiesterase (PDE). A single PDE gene in Dictyostelium encodes at least two distinct transcripts: a 1.8-kilobase (kb) mRNA expressed constitutively and a 2.2-kb mRNA expressed only during development (18). Starvation and extracellular cAMP are known to regulate developmental transcript levels (18, 19), but the intracellular second messenger(s) involved has not been identified. We provide evidence that changes in intracellular cAMP levels affect the magnitude, but not the developmental time course, of PDE gene expression.

MATERIALS AND METHODS

Cells of strains V12M2 and HB200 were grown on nutrient agar in association with Klebsiella pneumoniae at 22°C and harvested when cell density reached ~8 × 10³ cells per cm². Cells were prepared for in vitro differentiation as described (14) except that the buffer contained 5 mM cAMP, and cells were developed at 2.5 × 10⁵ cells per cm². HB200 is a spontaneous sporogenous mutant of wild-type strain V12M2 (7). V12M2 and HB200 aggregate to form mounds by 6 hr and form standing slugs by 10–12 hr of development on agar. However, HB200 cells terminally differentiate by 12–14 hr (compared to 20–22 hr for V12M2) and never form migrating slugs.

The PDE assay has been previously described (20). Minor modifications are noted in the legend to Fig. 1. Total RNA was isolated and purified by lysing cells with guanidinium thiocyanate and spinning lysates over CsCl. For Northern analysis, 10 μg of total RNA was size fractionated on 1.3% agarose/6% formaldehyde gels, electroblotted onto GeneScreenPlus (NEN), and hybridized with the cloned inserts of p1.2 (PDE cDNA; ref. 19) or pLK326 (a constitutive cDNA generously provided by H. L. Ennis, ...
Roche Institute of Molecular Biology) labeled with an oligolabeling kit (Pharmacia). Hybridization and washing of blots were performed according to procedures described elsewhere (21). Washed blots were autoradiographed using Kodak XAR-5 film with intensifying screens at $-80^\circ$C.

RESULTS

Quantitation of PDE Enzyme Activities and Cell Fate. Experimental manipulation of gene expression during differentiation of Dictyostelium amoebae is difficult under normal conditions because the cells are not in direct contact with the medium and are enclosed by a slug sheath whose permeability to exogenous agents is unknown. Differentiation of sporogenous amoebae in tissue culture dishes has the advantage that cells remain in contact with the surrounding medium and complete terminal differentiation as stalk cells and spores. For this study, we used sporogenous strain HB200 because a thorough study showed that its regulation of cAMP metabolism is indistinguishable from that of its wild-type parent, V12M2 (14). During in vitro differentiation of HB200 or V12M2 cells, levels of cellular PDE enzyme activity began to rise by 4 hr, peaked at 6 hr, and declined by 8 hr (Fig. 1). This time course is considerably faster than that reported for AX3 cells, which complete aggregation and reach peak PDE transcript levels by 12–14 hr (18, 19). However, V12M2 and HB200 cells complete aggregation on nonnutrient agar by 6 hr (see Materials and Methods), and the time course of PDE accumulation is the same during in vitro differentiation and development on agar (B.B.R., unpublished data). Thus, the accelerated time course of PDE gene expression seen here reflects the generally rapid development of V12M2-derived strains.

In the following experiments, we tested whether conditions previously shown to alter intracellular cAMP levels and cell fate also alter PDE gene expression. First, we used caffeine to inhibit activation of adenylate cyclase (12). Five millimolar caffeine lowers intracellular cAMP levels, inhibits spore formation, and stimulates stalk cell formation during in vitro differentiation of HB200 cells (refs. 7 and 14; Table 1). HB200 or V12M2 cells exposed to 5 mM caffeine accumulated twice as much cellular PDE activity as control cells (Fig. 1 A and B). These changes could be mediated by low intracellular cAMP levels in caffeine-treated cells or, alternatively, caffeine could have pleiotropic effects on other second messenger systems (12). To address this, we added 8-Br-cAMP to caffeine-treated cultures. 8-Br-cAMP is a membrane-permeable cAMP analog that has high affinity for the regulatory subunit of cAMP-dependent protein kinase (23) but not for the cell surface cAMP receptor (16). This allows 8-Br-cAMP to enter the cell, mimic endogenous cAMP, and bypass the caffeine-mediated block to adenylate cyclase activation. Exposure to 1 mM 8-Br-cAMP restores spore formation to near normal levels in caffeine-treated cultures of HB200 (ref. 14; Table 1). Similarly, 1 mM 8-Br-cAMP returned cellular PDE activity to near normal levels in caffeine-treated cultures of HB200 and V12M2 (Fig. 1 A and B). Without caffeine, 1 mM 8-Br-cAMP nearly doubled spore formation in HB200 cultures (Table 1) and reduced cellular PDE activity by as much as 40% in HB200 and V12M2 cells (Fig. 1 A and B). Thus, changing intracellular cAMP levels is sufficient to alter cellular PDE accumulation, with low cAMP levels stimulating maximal PDE accumulation.

To test this further, we altered cAMP levels with agents that inhibit or stimulate cAMP secretion. First, progesterone lowers intracellular cAMP levels by stimulating its secretion and, like caffeine, inhibits spore and promotes stalk cell formation (7, 13, 14). Continuous exposure to 100 $\mu$M progesterone increased cellular PDE activity by 50% (relative to peak activity in the control) but delayed the onset of PDE accumulation by 4–6 hr (not shown), making these results difficult to interpret. We were able to eliminate this delay by removing the progesterone after 1 hr. Exposing HB200 cells to 100 $\mu$M progesterone for only the first hour in in vitro differentiation blocked spore formation, stimulated stalk cell formation, and increased cellular PDE activity by 2-fold (Table 1, Fig. 1C). Second, we have recently found that ammonia raises intracellular cAMP levels in wild-type and sporogenous cells by inhibiting its secretion (ref. 15; Table 1). HB200 cells treated with 15 mM NH$_4$Cl formed twice as many spores and accumulated 45% less cellular PDE activity than control cells (Table 1, Fig. 1C). The same results were obtained with V12M2 cells (B.B.R., unpublished data). In summary, agents that elevate intracellular cAMP levels (8-Br-cAMP, progesterone) reduce cellular PDE accumulation, whereas agents that lower intracellular cAMP levels (caffeine, progesterone) increase cellular PDE accumulation.
Table 1. Effects of various treatments on intracellular cAMP accumulation and cell fate in HB200 cultures

<table>
<thead>
<tr>
<th>Culture conditions</th>
<th>Intracellular cAMP, pmol/mg (peak levels)</th>
<th>Final spore/stalk ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>33 ± 3</td>
<td>32/64</td>
</tr>
<tr>
<td>5 mM caffeine</td>
<td>13 ± 2</td>
<td>1/94</td>
</tr>
<tr>
<td>+ 1 mM 8-Br-CAMP</td>
<td>Indeterminate</td>
<td>25/75</td>
</tr>
<tr>
<td>1 mM 8-Br-cAMP</td>
<td>Indeterminate</td>
<td>58/42</td>
</tr>
<tr>
<td>100 µM progesterone</td>
<td>11 ± 1</td>
<td>1/95</td>
</tr>
<tr>
<td>15 mM NH4Cl</td>
<td>43 ± 3</td>
<td>61/32</td>
</tr>
</tbody>
</table>

Intracellular cAMP levels (pmol/mg of protein) were measured by radioimmunoassay as described (14). Effective cAMP levels in cells treated with 8-Br-CAMP cannot be determined because cellular cAMP-binding proteins differ in their affinities for the analog relative to endogenous cAMP (16, 23). To quantitate changes in cell fate, cells were cultured under each set of conditions for 24 hr at 22°C and scored by phase-contrast microscopy for the percentage of cells forming spores, stalk cells, and amoebae. Phase bright spores and highly vacuolated stalk cells are easily distinguishable and, unlike amoebae, are resistant to 0.5% Triton X-100. The ratios shown are percent spore/percnet stalk cell.

None of the conditions used here altered the time course of cellular PDE accumulation (Fig. 1A–C).

The relative contributions of membrane-bound and cytoplasmic PDE activities to total cellular PDE activity are shown in Table 2. At 6 hr of in vitro differentiation, when cellular PDE activities were maximal, 41–45% of total cellular activity was present on the surface of intact cells (membrane-bound) in all cultures. The remainder, by inference and by direct measurement, comprised cytoplasmic PDE activity. Thus, treatments that change total cellular PDE activity affect both cellular compartments to roughly the same degree.

These treatments also affected accumulation of secreted PDE activity. Caffeine increased secreted PDE activity by 2-fold, 8-Br-CAMP decreased activity by 25–30%, and simultaneous exposure to caffeine and 8-Br-cAMP gave intermediate activities (Fig. 1D). These data suggest that activity levels of cytoplasmic, membrane-bound, and secreted PDE correlate inversely with intracellular cAMP levels.

Quantification of PDE Transcript Levels. During in vitro differentiation of HB200 cells, levels of the developmentally regulated 2.2-kb PDE transcript began to rise by 2 hr (not shown), peaked by 4 hr, and declined sharply by 8–12 hr (Fig. 2). Note that cellular PDE enzyme activities peak several hours after 2.2-kb transcript levels (compare Figs. 1 and 2), a lag that agrees with previous observations (19). Because peak levels of 2.2-kb transcript in control cultures were 15–20 times higher than 1.8-kb constitutive transcript levels, hybridization to constitutive transcript is not visible in the exposures used in Fig. 2A. Overexposures showed that 1.8-kb transcript levels were relatively constant at all times and in all cultures (not shown), confirming previous reports that its expression is constitutive (18).

Drugs that alter intracellular cAMP levels affected accumulation of 2.2-kb PDE transcript in the same manner that they affected PDE enzyme activity. Relative to the control, 5 mM caffeine increased 2.2-kb transcript levels 3-fold (Fig. 2). Addition of 1 mM 8-Br-CAMP to caffeine-treated cultures returned 2.2-kb transcript levels to near normal levels, and 8-Br-cAMP by itself reduced transcript levels by nearly half. Treating cells with 100 µM progesterone for the first hour of development increased 2.2-kb transcript levels by >2 fold, whereas 15 mM NH4Cl reduced transcript levels by more than half. Reprobing Northern blots with constitutive probe pLK326 (Fig. 2A) showed that the observed changes in 2.2-kb PDE transcript levels were not due to loading errors. These data suggest that the magnitude of developmental PDE transcript accumulation correlates inversely with intracellular cAMP levels, as does cellular and secreted PDE enzyme activities (Fig. 1).

In contrast to PDE, the treatments used here did not affect expression of developmental genes D14, D18, D19, or PL1 (B.B.R., unpublished data). This suggests that intracellular cAMP regulates PDE gene expression by a selective process, rather than by globally affecting all developmental genes.

### DISCUSSION

We have quantitated PDE enzyme and transcript levels during in vitro differentiation. Under these conditions, cells are continuously exposed to 5 mM exogenous cAMP. This causes receptor adaptation and down-regulation in derivatives of wild-type strain NC4 but not in derivatives of wild-type strain V12M2 (5, 25, 26). Thus, it is possible to manipulate intracellular cAMP levels (or other second messengers) under the conditions used here. Furthermore, exogenous cAMP levels are super-saturating, so raising or lowering intracellular cAMP levels cannot affect gene expression by altering cAMP relay.

Using this system, we determined whether conditions that alter intracellular cAMP levels affect PDE gene expression. Drugs that lower intracellular cAMP levels (caffeine and progesterone) elevated PDE enzyme activity and transcript levels. Conversely, drugs that elevate intracellular cAMP levels (ammonia, 8-Br-cAMP) lowered PDE enzyme activity and transcript levels. 8-Br-cAMP reversed the stimulating effect of caffeine on PDE gene expression, indicating that caffeine-dependent stimulation occurs only if intracellular cAMP levels remain low.

From these results, we propose that synthesis or stability of the 2.2-kb transcript of the PDE gene (or expression of factors that regulate these functions) is sensitive to intracellular cAMP levels during development and is maximal when those levels are low. Accumulation of 2.2-kb PDE transcript varies 6- to 7-fold between conditions of high and low concentrations of intracellular cAMP. While this manuscript was in preparation, another report showed that PDE gene expression increases 3-fold or more when Dictyostelium amoebae overexpress a mutant mammalian regulatory subunit of cAMP-dependent protein kinase (27). This regulatory subunit fails to bind cAMP and constitutively blocks kinase activation. Taken together, both experiments suggest that synthesis or stability of the 2.2-kb PDE transcript is negatively regulated by cAMP-dependent phosphorylation. However, this cannot be the only mechanism controlling PDE gene expression because the overall time course of expression was the same in all cultures, regardless of intracellular cAMP levels.
cAMP levels. Clearly, some other mechanism regulates developmental timing of this gene.

We have also shown that conditions that alter intracellular cAMP levels affect cell fate, such that spore differentiation is favored when intracellular cAMP levels are high and stalk cell differentiation occurs when cAMP levels are low (refs. 7 and 14; Table 1). The relationship between intracellular cAMP levels, cell fate, and PDE gene expression during in vitro differentiation suggests the working hypothesis summarized in Fig. 3. PDE and cAMP are viewed as two components of a bistable switch that controls two metastable states. In state I, intracellular cAMP levels are low and PDE activity is high as a result of active transcription of the PDE gene. This state is self-sustaining because high cytoplasmic PDE activity will erase transient increases in intracellular cAMP levels, thereby permitting continued high-level expression of the PDE gene. Increased membrane-bound or secreted PDE activity will also lower intracellular cAMP levels by reducing signal relay between cells. Our results suggest that cells fated to become stalk cells pass through this state. In state II, intracellular cAMP levels are high and PDE activity is low because expression of the PDE gene is limited. In this context, the yeast ADH2 gene, which is active unless its transcription factor (ADR1) is phosphorylated by a cAMP-dependent kinase, provides an example of transcription inhibition by cAMP (28). In our model, PDE gene expression could be limited by transcriptional or posttranscriptional processes, or both. Cells fated to become spores pass through state II, which is stable as long as cAMP levels remain high. State transitions can occur, but at differing rates. Transition from state II to state I will be rapid if intracellular cAMP levels are lowered by inhibition of adenylyl cyclase. Transition from state I to state II will result from a sustained high rate of cAMP synthesis, but the transition will be slow because the rate of cAMP degradation is initially high. Thus, the rate of transition depends upon relative rates of PDE synthesis and decay.

The model is useful for organizing many other observations. First, prestalk cells of migrating slugs contain 2- to 3-fold higher levels of cellular PDE activity than do prespore cells (24, 29). Second, Dictyostelium differentiation occurs without determination and prespore and prestalk cell types interconvert during slug migration and after bisection of slugs (30, 31). Prestalk to prespore transitions are slow (usually requiring several hours) but prespore to prestalk transitions can occur within minutes (30, 31). Third, several secreted morphogens affect developing cells in ways that could stabilize one state or the other. For example, DIF, which induces stalk cell differentiation, binds to the cell surface cAMP receptor and reduces cAMP signaling (8). Similarly, the cAMP hydrolysing product adenosine inhibits cAMP synthesis (32) and, when added to agar, increases the percentage of prestalk cells in migrating slugs (33). In contrast, ammonia increases intracellular cAMP levels in wild-type and sporogenous cells (ref. 15; Table 1) and favors spore differentiation (2, 15). Changing levels of any of these morphogens could induce a transition from one state to the other.

The model predicts that prestalk cells or terminally differentiating stalk cells should have lower intracellular cAMP levels than prespore cells or terminally differentiating spores. In apparent contradiction, several studies have shown that total cAMP levels are slightly higher in the prestalk region of

![Figure 2](image-url)
by more than half (B.B.R., unpublished data). Conversely, as noted earlier, high-level expression of a mutant regulatory subunit of cAMP-dependent protein kinase causes higher expression of the 2.2-kb PDE transcript (27). In this case, however, changes in PDE gene expression cannot be correlated with changes in cAMP because overexpression of the mutant regulatory subunit blocks development prior to differentiation of prestalk and prespore cells (27). Likewise, overexpression of wild-type Dictostelium regulatory subunit by 5- to 10-fold also blocks differentiation (38), as does overexpression of secreted PDE by 30- to 100-fold (39). If more modest increases in these proteins were achieved such that cells were able to complete development, we would expect stalk cell differentiation to be favored. Genetic studies with conditional alleles and refined analysis of regulation of genes encoding adenylate cyclase, PDE, cAMP-dependent protein kinase, and regulatory genes will provide further tests of the model.

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