Regulation of Development in *Dictyostelium discoideum* IV. Effects of Ions on the Rate of Differentiation and Cellular Response to Cyclic AMP

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ABSTRACT The effects of ionic environment on both the intrinsic rate of differentiation and the response to exogenous cyclic AMP in *Dictyostelium discoideum* have been examined. K^+ specifically inhibits the rate of early development when present at concentrations >15 mM. Na^+ does not inhibit at concentrations up to 25 mM, and can partially overcome K^+ inhibition. The maximum rate of development also depends upon the presence of adequate levels of extracellular Ca^{++}.

The effect of exogenous cyclic AMP on the rate of development depends on the ionic environment. Stimulation is observed only when the rate of development is inhibited by the absence of Ca^{++}, and/or the presence of high concentrations of K^+. Under optimal ionic conditions, the only effect of exogenous cyclic AMP on early development is a specific inhibition. The implications of these results for current models of early developmental regulation are discussed.

6–8 h after their developmental cycle is initiated by starvation, myxamoebae of the cellular slime mold *Dictyostelium discoideum* collect into multicellular aggregates. Cell aggregation is mediated by the chemotactic attractant adenosine 3',5'-cyclic monophosphate (cAMP). Cells, or groups of cells, making up a small fraction of the total population serve as aggregation centers by periodically releasing cAMP. Neighboring cells detect the chemotactic signal and respond to it by directed movement toward the source, and by synthesizing and releasing cAMP, thereby relaying the aggregation signal. The molecular components of the aggregation system are either absent in vegetative amoebae, or are present at low levels. These components include adenylate cyclase, membrane-bound cAMP phosphodiesterase, extracellular cAMP phosphodiesterase and its specific inhibitor, and cell surface cAMP binding proteins that act as receptors (8, 14).

Before aggregation, vegetative cells differentiate to a state of aggregation competence in which they express high levels of the components required for chemotaxis, as well as of new membrane components, called contact sites A (2), that mediate the end-to-end intercellular cohesion of aggregating cells. The expression of contact sites A, like the components of the chemotactic aggregation system, is developmentally regulated.

Addition of cAMP during early development can affect the rate of cell differentiation. Marin (11) found that addition of cAMP in final concentrations as low as 10^{-8} M shortly after the initiation of development delayed the expression of contact sites A. On the other hand, Darmon, Gerisch, Gross, and their colleagues (6, 7, 18) have found that periodic addition of cAMP at concentrations as low as 10^{-8} M or single addition of a high concentration of cAMP 1 h after the initiation of development advanced the time at which contact sites A are expressed. These two opposing effects, delay and advance, were obtained in experiments in which the cells developed under different ionic conditions. Specifically, stimulation by cAMP was observed in experiments carried out in potassium phosphate buffers;[1] inhibition by cAMP was found in a more complex phosphate buffer containing 10 mM Na^+, 5 mM K^+, and 0.34 mM Ca^{++}, as well as 7.5 mM NH_4^+ and 0.4 mM Mg^{++}. The present report describes the effects of alterations in the ionic environment both on the rate of differentiation and on modulation of that rate by addition of cAMP. The time of expression of contact sites A is shown to be significantly delayed by the absence of Ca^{++}, and by the presence of K^+ at concentrations ≥15 mM, conditions similar to those used in other laboratories. The most rapid expression of contact sites A is observed in Ca^{++}-containing, low K^+ buffers similar to those used in our previous studies. The responses of cells to exogenous cAMP under these two ionic conditions are strikingly different. Stimulation of the rate of differentiation is observed only in ionic environments in which the rate of development is suboptimal. Under more favorable ionic conditions, the only effect of added cAMP is the previously reported delay in differentiation.

MATERIALS AND METHODS

Growth

D. discoideum A3 (10) was obtained from M. Sussman (University of Pittsburgh) in 1970. Axenic stock cultures were initiated every 2 or 3 mo from spores stored on silica. Amoebae were grown as previously described (11,12) as sub-

[1] The compositions of the development buffers used in these studies were: *a*, 17 mM potassium phosphate, pH 6.2, which we calculate to contain 20 mM K^+ (3, 6); *b*, 17 mM Sorenson's phosphate buffer, pH 6.0, which contains 4.2 mM Na^+ and 15 mM K^+ (7); and *c*, 20 mM potassium phosphate buffer, pH 6.2, 2 mM $MgSO_4$, which contains 23.5 mM K^+ (18). In addition, these investigators used strain Ax-2, at 10-fold higher cell densities than the densities of *D. discoideum* A3 used in our studies.

merged monolayers in 15- × 150-mm plastic bacteriological dishes, containing 25 ml of the tryptone-based broth.

Development

The compositions of the buffers used for development are listed in Table I.

Amoebae were harvested in the exponential phase of growth (1–3 × 10^7 cells/plate) by pouring off the growth medium and resuspending the cells by vigorous pipetting in 10 ml of the appropriate development buffer. The cells were then centrifuged at room temperature for 3 min at 270 *g*, washed once, and resuspended in buffer. The cell titer was determined by hemocytometer counts and adjusted to 1 × 10^6/ml by diluting with buffer. These manipulations took 20–30 min. Time 0 is defined as the time the cells were first removed from the growth plate. Three alternative protocols were used for further development: (*a*) 6-ml aliquots were plated in 15- × 60-mm tissue culture dishes (11). (*b*) 2-ml aliquots were distributed to siliconized 18- × 150-mm test tubes, and rotated at a speed of 20 rpm about an axis 8° from horizontal (12). (*c*) For "pulsing" experiments, 15- to 20-ml suspensions were shaken in siliconized 125-ml Erlenmeyer flasks at 70–80 rpm. For periodic addition of cAMP, 8-μl drops of 2.5 × 10^{-3} M cAMP were delivered to the culture by a peristaltic pump activated by an interval timer. Because 1-ml samples were removed each hour for contact site A assay, the concentration after a pulse increased ~7% at hourly intervals during the experiment.

Assay of Contact Sites A (EDTA-resistant Cohesion)

The expression of contact sites A was measured as previously described (12), by determining the percent of cells that remain single or double in the presence of 0.01 M EDTA.

Measurement of Intracellular [Na^+] and [K^+]

Vegetative amoebae were resuspended in their overlying growth medium, centrifuged, and resuspended in ice-cold distilled water. Cell titer was determined by hemocytometer count. Aliquots were centrifuged, washed once with ice-cold distilled water, resuspended in 15 mM LiCl and lysed by heating at 55°C for 3 min. The Na^+ and K^+ concentrations in the supernates were determined in a flame photometer (model 143, Instrumentation Laboratory, Inc., Lexington, Mass.) with the Li^+ serving as an internal standard. The intracellular Na^+ and K^+ concentrations were calculated using the volume of 0.055 × 10^{-8} ml per cell. This value for cells grown under our conditions was measured by the inulin exclusion method (4).

RESULTS

Assays of the Rate of Early Development

In the experiments described below, we have used the expression of contact sites A as an easily quantified measure of the rate of early development. In some experiments we also monitored development by observing aggregation morphology in submerged monolayer cultures. These two assay procedures gave parallel results for all effects of ionic environment on the rate of early development reported (data not shown). The same is true for all inhibitory effects of cAMP observed. We therefore conclude that the expression of contact sites A provides a valid assay for the overall process of early development.

Effect of Ca^{++}

Marin (11) previously reported that the omission of Ca^{++} from Na/KPCa buffer delayed contact site A expression by ~1 h. The omission of Ca^{++} from KPCa buffer also resulted in a delay (Fig. 1 *a*). The 500 μM Ca^{++} present in Na/KPCa and KPCa was in considerable excess of the amount needed to prevent a delay because of insufficient Ca^{++} in either buffer: 10 μM Ca^{++} was found to be saturating, or nearly so, and even 1 μM could reduce the delay (data not shown).

Effect of [Na^+] and [K^+]

The rate of early development was found to be dependent not only on an adequate level of extracellular Ca^{++}, but also on the concentrations of Na^+ and K^+ in the development buffer. Cells developing in KPCa expressed contact sites A ~1 h later than cells developing in NaPCa (Fig. 1 *b*). The effect of K^+ in delaying contact site A expression became greater as K^+ was increased to 25 mM, whereas no delay was observed when Na^+ was increased to 25 mM (Fig. 2 *a*). The slower rate of development in KPCa was not simply attributable to the absence of Na^+ (Fig. 2 *b*). However, a sufficiently high level of Na^+ was able to at least partially compensate for the inhibition of development by high levels of K^+ (Fig. 2 *c*).

Intracellular Concentration of Na^+ and K^+

The effects of Na^+ and K^+ on the rate of early development (Fig. 2) prompted us to measure intracellular concentrations of Na^+ and K^+. Measurements in vegetative amoebae gave values of 8.2 ± 0.6 mM for Na^+ and 46.5 ± 2.9 mM for K^+ (mean and SD of two independent experiments). The growth medium contained 26.9 ± 0.8 mM Na^+ and 1.9 ± 0.1 mM K^+. These results demonstrate that *D. discoideum* amoebae, like mammalian cells, can regulate intracellular Na^+ and K^+ against substantial transmembrane gradients.

Effects of cAMP: Stimulation[2]

The effects of periodic addition of 10^{-6} M cAMP on the rate of contact site A expression differed in sodium and potassium phosphate buffers. The slow rate in KP buffer was enhanced by cAMP pulses to a rate equal to, or slightly faster than, the rate in KPCa (Fig. 1 *a*). Pulses of cAMP slightly enhanced the rate in KPCa but had no detectable effect on the rate in NaPCa (Fig. 1 *b*). Moreover, the cAMP-enhanced rates in KP with or without added Ca^{++} equaled but did not exceed the rate in NaPCa.

The rate of expression of contact sites A in KP buffer was also stimulated by a single addition of cAMP to a concentration of 5 × 10^{-4} M, 1.5 h after the initiation of development (data not shown).

Effects of cAMP: Inhibition

In contrast to the stimulatory effect of cAMP in KP buffer described in the previous section is the report by Marin (11) that in DMB (Defined Medium Buffer; see Table I) the expression of contact sites A was delayed by addition of cAMP. When added shortly after the initiation of development, concentrations of cAMP as low as 10^{-8} M caused a significant delay; 3 h later, 1,000-fold higher concentrations of cAMP were required for equivalent levels of inhibition. In additional experiments in which cAMP at 10^{-3} and 10^{-4} M concentrations was added after 1 or 2 h of development, a delay in contact site A expression was consistently observed (data not shown).

The results presented in Table II show the structural specificity of the inhibitory effect of cAMP when added shortly after the initiation of development to cells developing in DMB: adenine, adenosine, and 5′-adenylic acid (5′-AMP) have no inhibitory effect, and guanosine 3′,5′-cyclic monophosphate (cGMP) produces a slight delay only at 10^{-4} M.

Effects of cAMP: Influence of Buffer Composition

The results presented above demonstrate that cAMP can have two distinctly different effects on the rate of early devel-

[2] Aggregation morphology was not monitored in these experiments.

TABLE I

Composition of Development Buffers

Buffer abbreviation	Salt						
	K_2HPO_4	KH_2PO_4	Na_2HPO_4	NaH_2PO_4	$CaCl_2$	$MgSO_4$	NH_4Cl
DMB	—	5.0	5.0	—	0.34	0.41	7.5
KP	5.0	5.0	—	—	—	—	—
KPCa	5.0	5.0	—	—	0.5	—	—
NaPCa	—	—	5.0	5.0	0.5	—	—
Na/KP	—	5.0	5.0	—	—	—	—
Na/KPCa	—	5.0	5.0	—	0.5	—	—

All concentrations are millimolar. All buffers are pH 6.8.

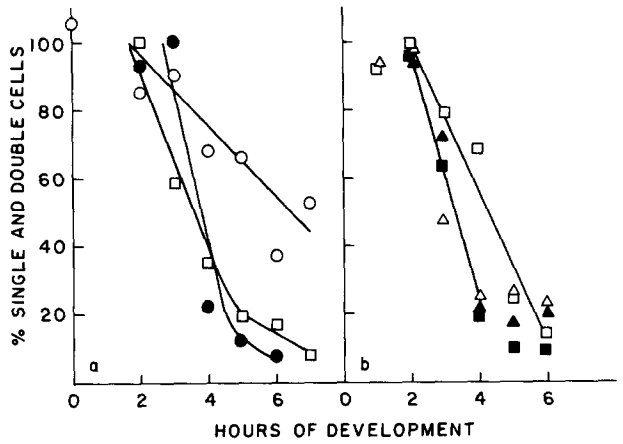

FIGURE 1 Effects of cAMP pulses and Ca^{++} on the rate of contact site A expression. Development in Erlenmeyer flasks, shaken at 78–80 rpm. *a*: Exp. 1. ○, KP buffer; ●, KP buffer plus cAMP pulses every 5.4 min beginning 2 h after starvation, [cAMP] at each pulse: 0.9–1.5 × 10^{-6} M; □, KPCa. *b*: Exp. 2. □, KPCa; ■, KPCa plus cAMP pulses; △, NaPCa; ▲, NaPCa plus cAMP pulses. Pulses every 6 min beginning at 1.5 h after starvation; [cAMP] at each pulse, 1.1–1.5 × 10^{-6} M. These data are from one of two duplicate experiments that gave similar results.

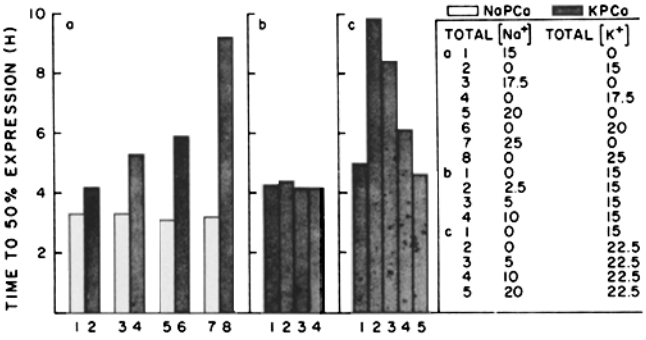

FIGURE 2 Na^+ and K^+ dependence of rate of contact site A expression. Development in roller tubes in buffer indicated, supplemented with either NaCl or KCl as necessary to give the final monovalent cation concentrations (millimolar) indicated.

opment, depending on the ionic environment. Stimulation by cAMP is observed when the rate is slowed because of the absence of Ca^{++} and/or the presence of a high level of K^+. Inhibition of the rate by cAMP is observed in buffers in which the rate of early development is not limited by these ionic conditions.

This difference in response must depend on a difference in the buffer components. The possibility that Ca^{++} is the component responsible for the difference in response was investigated by examining the effect of adding 10^{-5} M or 10^{-6} M cAMP at the beginning of development in Na/KP buffer with and without 0.5 mM Ca^{++}. A similar delay in contact site A formation was observed whether or not Ca^{++} was added, indicating that it is not the critical component (data not shown).

We then examined the effect of Na^+ and K^+ concentration on the inhibition of development by cAMP. The effect of 10^{-6} M cAMP on the rates of early development of cells in NaPCa and KPCa is illustrated in Table III. The presence of cAMP delayed the expression of contact sites A by ~2 h in NaPCa and only 1 h in KPCa, suggesting less inhibition by cAMP in KPCa. However, the absolute time of contact site A expression in the presence of cAMP is the same in the two buffers. The slower rate in the KPCa control gives rise to the impression that less inhibition has occurred. The inhibition by K^+ and the inhibition by cAMP were not additive. As the $[K^+]$ was increased, the difference between the $t_{50\%}$ of the cAMP-inhibited and control populations decreased until finally the inhibition by K^+ became equal to or greater than the inhibition by cAMP. At this concentration of K^+ (~20 mM), the inhibitory effect of cAMP became undetectable. In sodium buffer, on the other hand, increasing the $[Na^+]$ resulted in a small increase in the length of the delay induced by cAMP.

Effects of cAMP: Change in Cell Response during Development

As noted by Marin (11), cells developing in Na/KPCa buffers are sensitive to inhibition by cAMP over the range 10^{-8}–10^{-6} M during the first 2 h of development but become relatively resistant after that time. Some insight into the basis for this phenomenon was gained by examining the effect of cAMP on the rate of development of mixtures of sensitive and resistant cells. Shown in Fig. 3*a* is a control experiment that reproduces the previously reported sensitivity of freshly starved cells and resistance of 3-h developed cells to the presence of 10^{-6} M exogenous cAMP. The results presented in Fig. 3*b* demonstrate that a 1:1 mixture of a resistant (3-h developed) cell suspension with a sensitive (freshly starved) cell suspension developed with the kinetics expected for two noninteracting populations in either the presence or absence of 10^{-6} M cAMP. These results show that sensitivity and resistance arise from differences in the cellular responses per se to cAMP, and not from alterations in the extracellular environment, e.g., in levels of cAMP phosphodiesterase or putative intercellular cAMP signals.

DISCUSSION

The experiments presented in this report examine the effect of ionic environment on the rate of early development in *D. discoideum*, and the possible role of extracellular cAMP as a

rate determining factor in differentiation. As pointed out in an earlier report (11), the apparent significance of cellular regulatory processes may depend upon the conditions under which they are studied in the laboratory. Reactions that might normally be rate limiting can become masked by cellular responses to the experimentally imposed environment. The results contained in this report illustrate this caveat.

It is difficult to assess the natural ionic environment of the early developmental phase of the life cycle of *D. discoideum*, as this process occurs in the decomposing organic layers of the soil (5). In the absence of accurate information, appropriate conditions for developmental studies can be empirically determined by examining the effects of ionic environment on the rate of differentiation. The results presented in this report demonstrate that the presence of K^+ at concentrations $\geq$15 mM, or the absence of Ca^{++}, can both inhibit the rate of early differentiation.

The importance of Ca^{++} for *D. discoideum* development was first reported in 1971 (13). Mason et al. demonstrated that development could be completely inhibited by maintaining the extracellular Ca^{++} concentration at $<10^{-6}$ M. Over the range of 10^{-6}–10^{-4} M, the rate of development was proportional to the extracellular Ca^{++} concentration. The results presented in this report confirm the importance of extracellular Ca^{++}. The fact that development can occur in buffers nominally lacking Ca^{++} suggests that cellular conditioning of the extracellular environment may become a rate-limiting process for cell differentiation.

TABLE II

Specificity of cAMP Inhibition of Contact Site A Expression

Compound added	Concentration	% Single plus double cells
	M	
None	—	10 (5.5 h)
cAMP	10^{-8}	79
	10^{-7}	92
	10^{-6}	97
5′-AMP	10^{-6}	11
	10^{-5}	12
	10^{-4}	15
Adenosine	10^{-6}	10
	10^{-5}	11
	10^{-4}	17
Adenine	10^{-6}	11
	10^{-5}	9
	10^{-4}	18
None	—	12 (6.75 h)
cAMP	10^{-7}	67
	10^{-6}	84
	10^{-5}	93
	10^{-4}	96
cGMP	10^{-7}	5
	10^{-6}	5
	10^{-5}	7
	10^{-4}	63

Development was carried out in DMB buffer in tissue culture dishes. Compounds were added within 20 min after the initiation of starvation. EDTA-resistant cohesion was assayed at time of maximum expression by untreated control cultures. Numbers in parenthesis indicate actual time of assay in each experiment.

TABLE III

Influence of [Na^+] and [K^+] on Inhibition by cAMP

Buffer	Added NaCl	Added KCl	[Na^+]	[K^+]	$t_{50\%}$* No cAMP	$t_{50\%}$* 10^{-6} M cAMP
	mM	*mM*	*mM*	*mM*	*h*	
A. KPCa	0	0	0	15	4.5	5.6
	0	2.5	0	17.5	5.8	5.8
	0	5	0	20	7.5	7.5
B. KPCa	0	0	0	15	4.0	5.8
	0	2.5	0	17.5	4.7	6.0
	0	5	0	20	5.9	6.3
C. KPCa	0	0	0	15	4.0	5.4
	0	7.5	0	22.5	>9	>9
D. NaPCa	0	0	15	0	3.4	5.6
	7.5	0	22.5	0	3.9	6.8

Development in rotated tubes. A single addition of cAMP was made after resuspension and counting of cells (~20 min after initiation of starvation). Each section, *A–D*, reports data from a single experiment. Sections *A* and *B* illustrate variation in $t_{50\%}$ values, and reproducibility of trends in duplicate experiments.

* $t_{50\%}$ is the number of hours of development required to achieve 50% single and double cells in experiments of the type shown in Fig. 1.

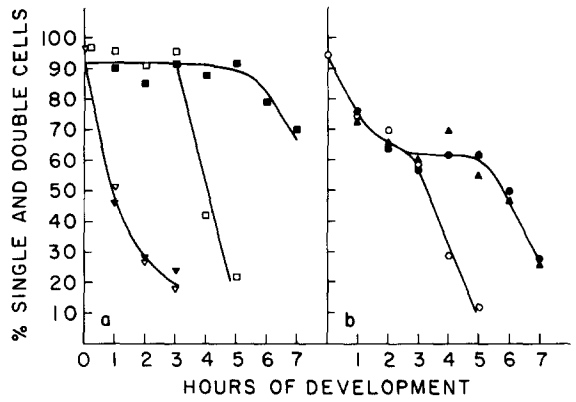

FIGURE 3 Effect of cAMP on the rate of contact site A expression in a mixture of vegetative and 3-h prestarved cells. Development in slowly rotated tubes in DMB. (*a*) Controls: □, vegetative cells, starved beginning at time 0 (t_0); ■, vegetative cells plus 10^{-6} M cAMP added at t_0; ▽, cells prestarved for 3 h before t_0; ▼, 3-h prestarved cells plus 10^{-6} M cAMP added at t_0. (*b*) At t_0, equal volumes of a culture that had developed for 3 h and of a washed, resuspended culture of vegetative cells were mixed: ○, mixed culture control; ●, mixed culture with a final concentration of 10^{-6} M cAMP added to each culture 1 min before mixing; ▲, mixed culture with 10^{-6} M cAMP added 1 min after mixing.

Concentrations of K^+ $\geq$15 mM also exert a significant inhibitory effect on the rate of early development (Fig. 2*a*). The mechanism underlying K^+ inhibition remains to be elucidated. Measurements of intracellular and extracellular Na^+ and K^+ concentrations during the growth phase, reported in this article, show that *D. discoideum* is capable of establishing transmembrane concentration gradients of these ions comparable to those found with other cell types. If, as in many other cells, the transmembrane resting potential depends primarily on the K^+ gradient, then the observed inhibition of development by extracellular K^+ may be the result of deleterious effects of membrane depolarization. Further studies are needed to evaluate possible mechanisms for the substantial Na^+ reversal of the K^+ inhibition (Fig. 2*c*).

A number of investigators have reported that the addition of cAMP to populations of cells during early development enhances the rate of cell differentiation. In some cases, repeated additions of low concentrations of cAMP were used, to mimic the putative endogenous intercellular cAMP signals (6, 7). Alternatively, single additions of much higher concentrations have proved to be equally effective (18). Pulses of low concentrations of cAMP resulted not only in stimulating differentiation of wild-type cells, but also brought about the phenotypic reversion of some differentiation-negative mutants (6).

It has been suggested that these results demonstrate a primary inductive role for endogenous intercellular cAMP signaling in the regulation of early development (9). However, all of the experiments in which stimulation by cAMP was observed were carried out in Ca^{++}-free, high-K^+ buffers. It is clear from the results presented in this report that the stimulatory effect of cAMP may simply result from compensation for the slower rate of development which results from these two ionic conditions. Analysis of how cAMP reverses the inhibition by K^+ must await further studies on the mechanism of the inhibition itself. An explanation for the cAMP stimulation of Ca^{++}-limited rates of differentiation is possible. Increased rates of Ca^{++} efflux upon exposure to cAMP, which is observed in other cell types (15), could enhance cellular conditioning of the medium. This suggestion is strongly supported by the fact that the stimulatory effects of cAMP on wild-type and mutant cells can be mimicked by addition of the Ca^{++} ionophore A23187 instead of cAMP (3).

The only observed effect of cAMP on early development of *D. discoideum* under optimal ionic conditions is a specific inhibition of the rate of cell differentiation. That inhibition is elicited by the addition of as little as 10^{-8} M cAMP shortly after the initiation of development demonstrates the extreme sensitivity of cells at this stage. Cells that have developed for 3 h are no longer inhibited by 10^{-6} M cAMP, but are inhibited by 10^{-4} or 10^{-3} M cAMP. The observation that sensitive cells are not protected from inhibition by mixing with resistant cells demonstrates that sensitivity and resistance are fundamental cellular phenotypes, and not simply manifestations of alterations in the intercellular environment, e.g., in levels of cAMP phosphodiesterase or putative intercellular cAMP signals.

In most previous investigations the strong inhibition by cAMP during early development was not detected. This may reflect the fact that under the ionic conditions generally used, stimulatory effects of cAMP predominate. Even under conditions in which cAMP does not stimulate, its inhibitory effect may be masked by the independent, and greater, inhibition resulting from high K^+ (Table III). Several observations reported in the literature probably reflect the cAMP inhibition reported here. The slowly hydrolyzable cAMP analogue adenosine-3′,5′-cyclic phosphorothioate (cAMP-S) inhibits the development of aggregation competence in strain Ax-2 when added at the onset of development, but fails to do so if added 2 h later (17). In the same investigation, a slight inhibition of development was observed when 10^{-5} M cAMP was added at the initiation of development. Continuous addition of cAMP has also been reported to delay the expression of contact sites A (7). Finally, the rate of differentiation of cell populations starved at suboptimal densities is enhanced by the presence of cAMP phosphodiesterase during the first 2 h of development (1), suggesting that endogenous extracellular cAMP delays differentiation under these conditions.

In previous work (11) we showed that even under ionic conditions that support rapid rates of early development, intercellular interactions are necessary for differentiation to take place at the maximal rate. Although the results in this report do not rigorously disprove the currently held hypothesis that these interactions are mediated by intercellular pulsatile cAMP signaling, they do indicate that the interpretations of the experiments that led to that hypothesis must be reevaluated. Our recent data (12) indicate that direct intercellular contact, mediated by two distinct carbohydrate-specific receptor-ligand interactions, regulates the rate of cell differentiation during early *D. discoideum* development. One of these interactions involves contact sites B, which mediate EDTA-sensitive intercellular cohesion (2), and are competitively blocked by glucose analogues. The other interaction is inhibited by galactose and may be mediated by the carbohydrate-binding protein discoidin (16). These intercellular inductive interactions take place during the first few hours of development and are necessary for the subsequent expression of contact sites A and of chemotactic sensitivity toward cAMP. It will be of interest to determine whether the acquisition of resistance to exogenous cAMP and the increase in intracellular cAMP concentration (4) associated with early development are also subject to regulation by these contact-mediated interactions.

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