The Cyclic Adenosine 3':5'-Monophosphate Receptor of Dictyostelium discoideum

BINDING CHARACTERISTICS OF AGGREGATION-COMPETENT CELLS AND VARIATION OF BINDING LEVELS DURING THE LIFE CYCLE*

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Both cyclic guanosine 3':5'-monophosphate and dithiothreitol stimulate binding of cyclic adenosine 3':5'-monophosphate (cAMP) to aggregation competent amoebae. Both compounds appear to function solely by preventing the hydrolysis of cAMP by the cell-bound phosphodiesterase. The dissociation constant for binding of cAMP is 36 nM.

Both cAMP binding and membrane-bound phosphodiesterase activities increase dramatically as cells develop aggregation competence, reach a maximum at about 11 hours, and remain at high levels for up to 48 hours if cells are maintained in shaken suspension. When amoebae are allowed to aggregate and develop naturally, binding of cAMP increases during aggregation, decreases during tip formation, and disappears during culmination. Phosphodiesterase activity parallels binding activity except that the decreased level after tip formation is retained throughout culmination.

Two N\(^\text{4}\)-modified cAMP derivatives compete with cAMP for binding sites. One derivative is fluorescent (1,\(N\text{\textsuperscript{4}}\)-etheno-cAMP); the other is photolyzable [\(N\text{\textsuperscript{4}}\)-(ethyl-2-diazomalonyl)cAMP]. This result opens the possibilities of using fluorescence quenching for assay of in vitro binding and of affinity labeling of binding sites. Competition by the derivatives is only partial, indicating possible heterogeneity of binding sites. Both compounds inhibit hydrolysis of cAMP by the membrane-bound phosphodiesterase.

When amoebae of the cellular slime mold Dictyostelium discoideum enter a period of starvation, they develop the competence to aggregate chemotactically into multicellular masses. Amoebae will develop and retain aggregation competence in shaken suspensions but will proceed no further with development (1). When amoebae are allowed to aggregate naturally on a surface, the cell mass develops a morphologically distinct tip region which appears to direct subsequent development. Movements within the cell mass raise the tip from the substratum forming a column of cells. The cell column can culminate directly by further morphogenetic movements and cellular differentiation to form a fruiting body consisting of a spore mass supported on a cellulose stalk. Alternatively, the cell column can form a sluglike pseudoplasmodium capable of migrating for environmentally determined periods of time prior to culmination. The cells of the tip or anterior region eventually form the stalk; the posterior cells become spores (for reviews, see Refs. 1 and 2).

The chemotactic factor during aggregation is generally accepted to be cAMP\(^1\) (3, 4). Aggregation is initiated by center or pacemaker cells which emit periodic pulses of cAMP. Following detection of the cAMP, responding cells move toward the original cAMP source, become briefly refractory to further stimulation, and produce a pulse of cAMP to which outer neighboring cells respond. Aggregation-competent cells produce an extracellular and a membrane-bound phosphodiesterase (cyclic adenosine 3':5'-monophosphate 5'-nucleotidohydrolase, EC 3.1.4.17) which can rapidly destroy extracellular cAMP. The balance of these factors permits directed, pulsatile signaling from the center across an aggregation territory (for recent accounts, see Refs. 5 and 6). Development of aggregation competence is accompanied by the appearance of cAMP binding sites on cells (7). Binding appears to be to cell surface receptors since cAMP is not taken up by the cells (8).

During postaggregation development there is evidence that

\(^1\) The abbreviations used are: cAMP, cyclic adenosine 3':5'-monophosphate; ecAMP, \(1, N\text{\textsuperscript{4}}\)-etheno-cAMP; diaa-cAMP, \(N\text{\textsuperscript{4}}\)-(ethyl-2-diazomalonyl)-cAMP; cGMP, cyclic guanosine 3':5'-monophosphate; Mes, 2-(N-morpholino)ethanesulfonic acid; MOPS, 4-morpholinopropanesulfonic acid; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene.
cAMP signaling may be involved in direction of morphogenetic cell movements (9, 10) and that high levels of cAMP induce stalk cell differentiation (11, 12).

This paper describes: (a) the effects of both cGMP and dithiothreitol on cAMP binding and on phosphodiesterase activity, (b) the dissociation constant of the cAMP binding sites, (c) temporal variation of binding and phosphodiesterase activities during development, and (d) interaction of fluorescent and photolysable cAMP derivatives with the phosphodiesterase and the binding sites.

**EXPERIMENTAL PROCEDURE**

**Strains and Growth Conditions**

Dictyostelium discoideum strain V12/M2 was obtained from G. Gerisch (Max Planck Institute, Tubingen) and strain Ax-2 from J. Ashworth (Essex University, Essex). Strain Ax-2 was grown axenically in HL5 broth (13). Strain M2 was grown on a gyrotary shaker in suspensions of Escherichia coli B/r (1 x 10^10/ml) in Buffer A (0.0017 M potassium phosphate (pH 6.1) containing 2 mM MgSO₄). For agar plate cultures, both strains were grown at 22°C on plates of Aerobacter aerogenes on a medium containing peptone, yeast extract, MgSO₄, potassium phosphate (pH 6.1), and agar (14).

**Differentiation**

Cells of strain M2 grown in shaken suspension with bacteria will develop aggregation competence after following exhaustion of the bacterial food supply (1). When it was critical to know the precise time of exhaustion (tₑ), amoebae were harvested in mid to late log phase and freed from bacteria by two differential centrifugations (2 min at 500 x g) with washing and final resuspension in Buffer A. Shaking then was resumed at a density of 1 x 10^7 amoebae/ml. If shaking was to continue for more than 12 hours, 100 µg/ml of streptomycin sulfate were included in the resuspension buffer. Strain Ax-2 and its parent NC-4 will not develop aggregation competence unless washed free of the growth medium (13) as described for M2. Cells for use in binding assays were harvested between 8 and 28 hours after removal of the bacterial associate or growth medium.

The amoebae will undergo the complete developmental program if, after harvesting and washing, they are placed on a moist, solid surface which can be a 1% non-nutrient agar in Buffer A or Millipore filters resting on moist, buffered pads (15). Cells harvested from suspension, agar, or filters were washed twice with Mes buffer (50 mM, pH 6.1) before assays.

Tests for aggregation competence and chemotactic activity of cAMP and analogs have been described (16).

**Assay of cAMP Phosphodiesterase Activity**

The assay is a modification of the method of Brooker et al. (17). The assay contains 5.2 nmol of [3H]cAMP (about 2600 cpm/nmol), 1.26 µmol of MgSO₄, 5.0 µmol of Tris-HCl (pH 7.5), and 0.1 unit (1 unit = 1 pmol/min, 25°C) of alkaline phosphatase together with sample to be assayed. The assay is at room temperature and is begun by mixing equal volumes of a 2-fold concentrated assay mixture with cells in buffer. The mixture is centrifuged for 30 s in a high speed microcentrifuge (Quickfit Instrumentation, England; maximum speed, 24,000 x g) within 40 s of adding the cells. A sample of the supernatant (50 µl) is removed and the unbound counts are determined in a dioxane-naphthalene-PPO scintillant. The control for each determination is the standard assay containing additionally 5 nmol of unlabeled cAMP, in which case binding of radioactivity is negligible due to isotope dilution. Calculation of the amount of cAMP bound to the cells requires a correction for (a) volume of the total supernatant and (b) the change in concentration of free counts when there is no binding (the competed case) due to displacement of the extracellular solution by the cell pellet. Total counts bound can be calculated as follows:

\[
\text{total cpm/assay} = \frac{1}{1 - \frac{x}{y}}
\]

where \(x\) = counts per min in 50 µl without competing cAMP and \(y\) = counts per min in 50 µl with competing cAMP. All assays were performed by this method unless otherwise specified and were in duplicate.

**Pellet Assay—**After centrifugation to separate cells and unbound cAMP as described above, the supernatant was removed by aspiration using a disposable glass pipette drawn out to a microtip. The pellet surface was gently rinsed with 2 to 3 drops (0.1 to 0.15 ml) of Mes buffer, aspiration was repeated, and 15 µl of water were added to each tube. The samples were digested overnight with 0.3 ml of NCS and radioactivity was determined by counting in 2 ml of a toluene-PPO-PPOP scintillant after addition of 10 µl of glacial acetic acid. Binding was determined by comparing the competed and un竞争ated cases. All assays were in duplicate.

No correction is required for the volume of free solution trapped in the cell pellet. Counts trapped in the pellet after sedimentation of cells from a solution of [3H]linulin were only 0.7% of the total counts in solution (per 10⁷ cells). Inulin has been used as an extracellular marker for D. discoideum (18, 19) although this may not be valid for strain Ax-2 (20). In cAMP binding assays, cells usually removed 30 to 40% of the total counts (at 1 x 10⁵ M cAMP and 10³ total cells).

**Assay of Bovine Adrenal Cortex CAMP Binding Protein**

The assay was as described in the British Drug House supplement with the protein preparation and is based on the procedure of Brown et al. (21).

**Preparation of Escherichia coli cAMP Receptor Protein**

The protein was purified from E. coli strain PP 81 which is diploid for the receptor structural gene. It was purified 160-fold as described by Anderson et al. (22) through the phosphocellulose and ammonium sulfate steps and had a specific binding activity similar to their preparation at that stage. There was no detectable phosphodiesterase activity in 1 mg of the purified protein. The binding assay was as described (22) using ammonium sulfate precipitation.

**Protein Determination**

Measurement of protein was by the Lowry method as described by Layne (23).

**Preparation of Dam-cAMP**

Ethyl-2-diazomalonyl chloride was prepared according to Vaughan and Westheimer (24) and was purified by vacuum distillation (20, 25) and crystallization. The infrared spectrum of the product was consistent with formation of the acid chloride. Dam-cAMP was purified and prepared as described by Brown and Cooperman (26). The following properties of the purified product were identical to those reported (26): ultraviolet absorption spectrum and dependence of spectrum on pH, photolytic spectral changes, and mobility on both cellulose and polyethyleneimine thin layer chromatography. The product was chromatographically homogeneous.

**Preparation of sCAMP**

The compound was prepared and purified as described by Barrio et al. (27). The purified product had the expected absorption and fluorescence emission spectra (28) and was chromatographically homogeneous.

**Thin Layer Chromatography**

Cellulose thin layer chromatograms (Eastman) were developed with a solvent of ethanol-1 M ammonium acetate (70:30). Polyethyleneimine chromatograms (Machery-Nagel) were developed with 1% LiCl in water.
Mixture for 30 s before centrifugation. A sample of the supernatant was transferred to 0.2 M formic acid (final concentration) and subsequently analyzed for cAMP and 5'-AMP by thin layer chromatography on cellulose. Based on the location of carrier spots, the cellulose was scraped into vials, 0.5 ml of water was added, and after thorough mixing the samples were counted in a dioxane-naphthalene-PPO scintillant. Very little of the original CAMP is converted to adenosine; over 90% of the original cAMP is recovered as cAMP plus 5'-AMP. The loss of 8% of the counts reflects primarily losses in recovery from the thin layer plates.

**Materials**

Tritiated cAMP, tritiated inulin, and NCS were from Amersham. cAMP, 5'-AMP, cGMP, papaverine, dithiothreitol, Mes, MOPS, and beef heart phosphodiesterase were from Sigma. Theophylline, bovine adrenal cortex CAMP binding protein, and phosgene (12.5% in toluene) were from British Drug House. Other suppliers were: ethyl diazoacetate, Aldrich; chloroacetaldehyde, Fluka, Switzerland; and alkaline phosphatase, Boehringer-Mannheim.

**RESULTS**

Effects of cGMP and Dithiothreitol on CAMP Binding and Phosphodiesterase Activities—The large amounts of cell-bound phosphodiesterase in aggregation-competent amoebae has made demonstration of CAMP binding very difficult. Malchow and Gerisch (29) reported an assay of CAMP binding which is dependent upon the presence of a large excess of cGMP. The assay was based on the observations that cGMP is a good substrate for the phosphodiesterase but is a poor chemotactic agent (30). It should, therefore, be hydrolyzed preferentially by the phosphodiesterase when in large excess over cAMP but interact only weakly with the receptor for chemotaxis.

Fig. 1 shows that cGMP prevents hydrolysis of cAMP during a brief exposure to cells. Under these conditions the cAMP is completely protected by 0.1 mM cGMP, and the standard assay used below contains twice this amount. The assay procedure used is a modification of the Malchow and Gerisch method and defines binding as radioactivity originating from tritiated cAMP which is bound to cells and can be competed away by excess unlabeled cAMP but not by 5'-AMP. This assay is reliable unless the cell-bound phosphodiesterase is greater than 100 nmol/min/ml (hydrolysis of cAMP determined in the standard phosphodiesterase assay). At maximum phosphodiesterase activity (see below), this corresponds to a limit of about 8 x 10⁶ cells per assay. Above this value, apparent binding decreases presumably due to hydrolysis of the protecting cGMP and subsequent hydrolysis of tritiated cAMP.

Since the cGMP-dependent assay has the undesirable features of limitation of time and phosphodiesterase activity, a search was made for true phosphodiesterase inhibitors. Pannbacker and Bravard (31) have reported that the soluble, extracellular form of Dictyostelium discoideum phosphodiesterase is inhibited by dithiothreitol and that 1 mM dithiothreitol potentiates the cellular chemotactic response to cAMP. Malchow et al. (32) reported that the extracellular phosphodiesterase is inhibited by glutathione but the enzyme in whole cells is insensitive. Fig. 9 shows that both mercaptoethanol and dithiothreitol are good inhibitors of the enzyme in whole cells. Theophylline, papaverine, and ICI 63,197 give very poor inhibition (20% maximum). Dithiothreitol was tested as a replacement for cGMP in the binding assay. Table I shows

![Fig. 1](http://www.jbc.org/)  
**Fig. 1.** The effect of cGMP on stability of [3H]cAMP in binding assays. The assays were as described under "Experimental Procedure." Surviving [3H]cAMP was determined as follows. Cells were added to the assay mixture (2 x 10⁶/ml) and removed from the mixture in a microcentrifuge. In each case cells were incubated in the assay mixture for 30 s before centrifugation. A sample of the supernatant was transferred to 0.2 M formic acid (final concentration) and subsequently analyzed for cAMP and 5'-AMP by thin layer chromatography on cellulose. Based on the location of carrier spots, the cellulose was scraped into vials, 0.5 ml of water was added, and after thorough mixing the samples were counted in a dioxane-naphthalene-PPO scintillant. Very little of the original CAMP is converted to adenosine; over 90% of the original cAMP is recovered as cAMP plus 5'-AMP. The loss of 8% of the counts reflects primarily losses in recovery from the thin layer plates.

![Fig. 2](http://www.jbc.org/)  
**Fig. 2.** The effect of sulphydryl compounds on activity of membrane-bound phosphodiesterase. The assays were as described under "Experimental Procedure" except for a 5-min preincubation of the cells in the presence of dithiothreitol (●) or mercaptoethanol (○) before assay.

**TABLE I**

| Dithiothreitol concentration (mM) | 0.2 mM cGMP | No cGMP |
|----------------------------------|-------------|---------|
| Experiment A                     |             |         |
| 0                                | 100         | 30      |
| 5                                | 87          | 82      |
| 10                               | 84          |         |
| Experiment B                     |             |         |
| 0                                | 100         | 77      |
| 5                                | 87          |         |
| 10                               | 83          | 96      |

binding decreases presumably due to hydrolysis of the protecting cGMP and subsequent hydrolysis of tritiated cAMP.

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that dithiothreitol gives very little (7 to 16%) reduction of binding in the cGMP-dependent assay. In the absence of both cGMP and dithiothreitol, binding is always low and variable, but addition of 10 mM dithiothreitol alone stimulates binding to the levels observed in the presence of both dithiothreitol and cGMP (Table I). Therefore, dithiothreitol appears to be well suited for replacement of cGMP in the binding assay. It, of course, remains to be shown that both methods detect the same set of binding sites, and this is under investigation.

Binding was constant between pH 6 and 8 and decreased below pH 6 (data not shown).

Dissociation Constant for cAMP Binding—The standard assay contains 10 nM cAMP. When cAMP was titrated, extrapolation of a double reciprocal plot indicated that the dissociation constant was 34 nM (Fig. 3). In other experiments this value has varied from 25 to 100 nM with an average of 36 nM (seven experiments). In some of these experiments, double reciprocal plots break downward at high cAMP concentrations suggestive of negative cooperative interactions between binding sites (33) or of a mixture of sites with different affinities. In those cases the dissociation constant was estimated from the high affinity binding, and the phenomenon has not been further investigated. However, additional evidence for a heterogeneous population of sites is presented below.

The maximum number of binding sites per cell in the experiment shown in Fig. 3 is approximately $9 \times 10^4$. The standard assay (10 nM cAMP) detects 15 to 20% of these sites. However, the sensitivity of the assay is not improved at higher cAMP concentrations since the backgrounds become elevated. Maximum binding per cell varies about 2-fold between experiments for cells harvested from shaken suspensions.

Variation in Levels of cAMP Binding and Phosphodiesterase Activity during Development—The time course of appearance of binding and phosphodiesterase activities with development of aggregation competence of cells in shaken suspension is shown in Fig. 4. Binding per cell reaches a maximum about 12 hours after removal of the food supply ($t_0$). The binding per mg of protein increases more dramatically since cells maintained in suspension beyond their normal time of aggregation (6 to 8 hours after $t_0$) begin to decrease in size and protein content. In this experiment the cells had become aggregation-competent at about 5 hours and retained this competence up to 48 hours (the last point tested). During the development of aggregation competence the cell-bound phosphodiesterase activity increases dramatically reaching a maximum at 10 to 12 hours and thereafter declines to about half the maximum level (Fig. 4).

Fig. 5 shows binding and phosphodiesterase activities for cells dispersed on a solid support at $t_0$ and allowed to undergo the normal developmental cycle. In agreement with the data of Fig. 4, both binding and phosphodiesterase activities increase as cells begin aggregation. Binding reaches a maximum at 8 to 9 hours and decreases thereafter (Fig. 5). The phosphodiesterase activity peaks and declines similarly. The stage of development at the times of harvesting for assay is indicated below the abscissa. Obvious chemotactic aggregation had been completed by 9 to 10 hours. By 11 hours the assayable binding had dropped to 10% of the maximum. However, by this time the cell contacts were very tight, and the binding assay was performed on small cell clumps rather than individual cells.

**Fig. 3.** Saturation plot (A) and double reciprocal plot (B) of cAMP titration. The procedure was as described under "Experimental Procedure" for the pellet assay. The specific radioactivity of the [3H]cAMP (in the uncompeted cases) varied from about 10,000 to 400 cpm/nmol for cAMP concentrations from 1 to 100 x 10$^{-8}$ M.

**Fig. 4.** Appearance of binding and phosphodiesterase activities during development of aggregation competence in shaken suspension. Cells were strain M2. The points are all averages of duplicate determinations. Binding assays were performed immediately after harvesting the cells. The remaining cell suspension then was frozen ($-30^\circ$C) and later thawed for determination of protein and phosphodiesterase activity. The symbols are: O, picomoles of cAMP bound/10$^7$ cells; , picomoles of cAMP bound/mg of protein; and A, phosphodiesterase activity.

**Fig. 5.** Time course of cAMP binding and phosphodiesterase activities during normal development. Cells of strain Ax-2 were grown in broth to 5 x 10$^9$/ml (mid to late log phase). A portion of the culture was harvested (A), washed and dispersed on Millipore filters (5 x 10$^7$ cells/filter). Another portion of the same culture was diluted in broth and allowed to grow to 5 x 10$^9$/ml before harvesting and dispersing on filters (Set B). Cells were harvested from filters and washed in Mes buffer prior to assay. The final time point for Set B and the earliest time point for Set A were at 11 hours (for the binding assay). Both samples gave the same value for binding. Binding assays were performed immediately after harvesting the cells. The remaining cell suspension then was frozen ($-30^\circ$C) and later thawed for determination of protein and phosphodiesterase activity. The symbols are: (O), binding; (O), phosphodiesterase.
Therefore, it can only be stated that cells at 11 to 16 hours did bind cAMP, but the data are not quantitative. At 19 and 23 hours the structures dissociated easily into pre-spore- and sporelike cells and a material of low density (probably cellulose which is made in large quantities in maturing stalk cells) which was decanted with the supernatants after low speed centrifugations in washing the cells. The absence of binding at these times suggests that spore and prespore cells do not bind cAMP.

The experiments shown in the last two figures were done with different strains of *Dictyostelium discoideum* (v12/M2 and Ax-2), and the maximum level of binding in these experiments differs by a factor of 4.5. This does not appear to be due to strain differences though Ax-2 cells usually bind somewhat less cAMP (30 to 40%) than M2 cells. More generally, this difference reflects the observation that cells of either strain harvested from filters bind less cAMP and the maximum binding occurs earlier than for cells maintained in nutrient-free suspensions.

**Effects of cAMP Analogs on Binding and Phosphodiesterase Activities**—Many cAMP analogs have been tested by other workers in attempts to distinguish between binding properties of the phosphodiesterase and the chemotactic receptor (30). I have prepared two analogs which were previously untested in *Dictyostelium* and which may be useful in study of the receptor (as discussed below).

*cAMP* is a fluorescent derivative first described by Secrist et al. (28). I have observed that *cAMP* binds with an affinity similar to that of cAMP to the *Escherichia coli* cAMP receptor protein and the bovine adrenal cortex cAMP binding protein but is a poor substrate for bovine heart phosphodiesterase. The efficiencies of *cAMP* and cAMP as chemotactic agents for *D. discoideum* are very similar. The membrane-bound phosphodiesterase of *D. discoideum* hydrolyzes both *cAMP* and cAMP with the same maximal velocity and with a similar binding affinity. The last two observations indicate that the slime mold chemotactic system and, therefore, the receptor respond to *cAMP*.

Dam-cAMP was first described by Brunswick and Cooperman (34). This photolyzable derivative has been used for covalent affinity labeling of the cAMP binding site of the erythrocyte membrane (35), and the *O*'-[(ethyldiazomalonyl)cAMP derivative has been used to affinity label the cAMP binding site of phosphofructokinase (34, 36).

Dam-cAMP binds efficiently to the *D. discoideum* membrane-bound phosphodiesterase. Unlabeled cAMP reduces the hydrolysis of [*H]*cAMP by isotope dilution, and dam-cAMP also reduces this hydrolysis as shown in Fig. 6. At the lower concentrations (100 pM added nucleotide) it appeared that dam-cAMP was less efficient than cAMP. However, the photolyzable diazo form of this compound exists in a pH-dependent equilibrium with a nonphotolyzable triazole form (26). The *pK* of the shift is about pH 6, and the phosphodiesterase assay is done at pH 7.5 where the compound exists as the triazole. Fig. 7 shows inhibition of [*H]*cAMP hydrolysis by 100 pM cAMP by dam-cAMP as a function of pH. At the lowest pH both were equally effective. Therefore, both the diazo and the triazole forms of dam-cAMP bind to the enzyme, the diazo form most efficiently.

To investigate the interaction of these compounds with the receptor, they were used in competition with [*H]*cAMP for binding to cells. Fig. 8 shows that both derivatives do compete though only for 50 to 60% of the cAMP binding sites, indicating a possible heterogeneity of binding sites (see "Discussion").
Both analogs were free of unsubstituted cAMP by thin layer chromatography. The data of Fig. 8 support this conclusion since 200 μM concentrations of either analog competed less efficiently than 0.5 μM pure cAMP, indicating greater than 99.75% nucleotide purity. The possibility that competition is due to such trace contamination by cAMP cannot be excluded until a direct demonstration of interaction between receptor and analog can be made. However, the purification procedures for both analogs give good separation from unsubstituted cAMP, and both compounds are stable under their storage conditions; that is, they do not degrade to cAMP.

**DISCUSSION**

This paper describes several modifications of a previously reported assay of cAMP binding to whole cells of *D. discoideum*. The definition of binding is more rigorous in that it excludes the possibility that bound radioactivity represents a phosphodiesterase-mediated breakdown product of cAMP.

The assay requires some means of protecting the cAMP from hydrolysis by the phosphodiesterase. Addition of cGMP to binding assays prevented hydrolysis of cAMP by the membrane-bound phosphodiesterase and also stimulated binding of cAMP as previously reported (7, 29). Potentially complex control loops between cGMP and cAMP have been reported in other systems (37, 38). Consequently, it was important to demonstrate that the cGMP-mediated stimulation of binding is due solely to its protection of cAMP in the assay. Also, the cGMP-dependent assay is far from satisfactory since the cGMP is a substrate for the phosphodiesterase, and the assay is based on the balance between cGMP and phosphodiesterase concentrations and thus contains a critical time factor. Consequently, a search was made for a phosphodiesterase inhibitor which could replace cGMP in the assay. Dithiothreitol proved to be a good inhibitor of the phosphodiesterase and to stimulate cAMP binding to cells to approximately the same level as cGMP. Dithiothreitol is, therefore, a suitable replacement for cGMP since it is a true inhibitor rather than a competitive substrate. Additionally, the ability of such structurally diverse compounds as cGMP and dithiothreitol to mimic each other in the assay suggests that their stimulation of binding is due only to effects on the phosphodiesterase.

The dissociation constant for binding of cAMP to aggregation-competent cells is approximately 36 nM in the presence of 0.2 mM cGMP. Malchow and Gerisch have reported a dissociation constant of 100 to 200 nM in the presence of 0.5 mM cGMP (7). Although cGMP is a very weak chemotactic agent, it will attract amoebae at the concentrations used in the binding assays (30) and thus may be a weak competitive inhibitor of binding. The difference between the dissociation constants may reflect the difference in the concentrations of cGMP. This point is currently under investigation.

The value of 36 nM for the dissociation constant is consistent with the chemotactic responsiveness of amoebae to cAMP. In spot tests we find that the amoebae respond at a minimum concentration of 0.1 μM cAMP at a distance of 0.3 mm and become paralyzed at about 100 μM. Considering diffusion over the distance between the cAMP and cell spots and the high activity of the phosphodiesterase, the effective concentration near the amoebae will be considerably less and could easily fall into the range of the binding curve where binding is proportional to cAMP concentration (about 1 to 100 nM).

Both cAMP binding and phosphodiesterase activities were determined at varying stages of development for cells in shaken suspension and for cells developing naturally on surfaces. In both cases the development of aggregation competence is accompanied by dramatic increases in the two activities. Cells maintained in suspension retain these high levels for extended periods. In contrast, following aggregation the assayable binding activity and the phosphodiesterase activity decrease to a fraction of their maximum level, suggesting that aggregation or a consequence of aggregation regulates the level of these activities (rather than control by a direct developmental clock). Finally, the absence of binding to prespore and spore cells also indicates that postaggregation factors regulate the levels of binding sites in cell membranes or the distribution of the receptor between prespore and prestalk cells, or both. In this connection it is of interest to note that the concentration of bound cAMP has been shown to be significantly higher in the prestalk regions of slugs and early culminating structures than in the prespore regions (12) and that high cAMP concentrations are able to induce stalk cell differentiation (11). It will be of great interest to determine the precise distribution of the cAMP binding sites during these stages.

Malchow and Gerisch followed binding and phosphodiesterase activities during the first 8 hours of development in suspensions (7). They observed a sharp rise in both activities during the first 4 to 5 hours. In contrast, the data of Fig. 4 show a rise in both activities over the first 10 to 12 hours. This difference could be due to variations in the binding assay and to the temporal rise of the phosphodiesterase. For example, consideration of only the first 8 hours of Fig. 4 suggests a constant amount of cell-associated phosphodiesterase between 5 and 8 hours.

Two N6-modified cAMP analogs previously untested in *D. discoideum* have been shown to inhibit hydrolysis of cAMP by the membrane-bound phosphodiesterase and to compete for cAMP binding sites on whole cells. The analogs were able to compete for only 50 to 60% of the cAMP binding sites, suggesting heterogeneity in the population of sites. There could be several explanations for the partial competition. It is possible that there is more than one binding protein in the membranes and that the derivatives do not compete with cAMP for all binding proteins. It is not likely that any of the binding of cAMP is to the phosphodiesterase since these assays were performed in the presence of cGMP. An alternate possibility is that the receptor exists in more than one conformational state and that one conformation can tolerate N6 substitutions while another (or others) cannot. Studies to distinguish between these possibilities are in progress.

Both analogs have great potential value for studies of the cAMP receptor. For example, binding of cAMP to the cAMP sites opens the possibility of developing a very informative and useful *in vitro* assay of binding based on fluorescence quenching (or enhancement). The photolyzable diazo derivative may provide a means of attaching a covalent affinity label to binding sites both in vivo and in vitro.

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