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Haastert, Peter J.M. van; Kien, Erik

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Binding of cAMP Derivatives to *Dictyostelium discoideum* Cells

ACTIVATION MECHANISM OF THE CELL SURFACE cAMP RECEPTOR*

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Peter J. M. Van Haastert and Erik Kien

From the Cell Biology and Morphogenesis Unit, Zoological Laboratory, University of Leiden, Kaiserstraat 63, NL-2311 GP Leiden, The Netherlands

The binding of 16 derivatives of cAMP to the surface of *Dictyostelium discoideum* cells was analyzed. The binding affinity is strongly reduced (more than 14.5 kJ/mol) if a cAMP derivative is no longer able to form a hydrogen bond at N^6H_2, or at $O^{3'}$. Decreasing polarity of the base moiety is closely correlated to increasing binding affinity to the cAMP receptor ($r = 0.98$, $p < 0.1\%$). Based on these results we propose that cAMP is bound to the receptor via hydrogen bonds at N^6H_2 and $O^{3'}$, and that the adenine moiety is bound in a hydrophobic cleft of the receptor. A stereospecific interaction between the receptor and the phosphate moiety of cAMP has not been observed.

The first detectable response of *D. discoideum* cells to cAMP is an increase of intracellular cGMP levels. A close correlation exists between the binding affinity of the cAMP derivatives and the potency to induce a cGMP response ($r = 0.97$, $p < 0.01\%$). There are two exceptions; both derivatives are modified in the exocyclic oxygen atoms of the phosphate moiety (sulfur in equatorial position or dimethylamino in apical position). These derivatives bind with approximately the same affinity as their stereoisomers (respectively sulfur in apical or dimethylamino in equatorial position), but in contrast to their stereoisomers, they do not induce elevations of cGMP levels. This suggests that, in addition to the binding interactions mentioned above, activation of the receptor requires a stereospecific interaction between the receptor and the phosphate moiety of cAMP. Quantum-chemical calculations by Van Ool and Buck (Van Ool, P. J. J. M., and Buck, H. M. (1982) *Eur. J. Biochem.* 121, 329–334) suggest that this activating interaction is a covalent bond between cAMP and the receptor.

cAMP acts as a first messenger in the cellular slime mold *Dictyostelium discoideum* (1). Cells of this species feed on bacteria. Exhaustion of the food supply induces cell aggregation which is mediated by chemotaxis (2) to cAMP (3). The aggregation center secretes cAMP in a pulsatile manner which is detected by individual cells in the neighborhood of the aggregation center. cAMP does not penetrate the cells (4), but is detected by cell surface receptors (5–8). In addition to these cAMP receptors, *D. discoideum* contains cell surface phosphodiesterase activity which hydrolyzes cAMP (9, 10). Destruction of cAMP probably serves to clear the cAMP receptors which enhances the detection of successive pulses of cAMP. Addition of cAMP to a suspension of *D. discoideum* cells induces several responses such as a transient increase of intracellular cGMP levels, the excretion of protons, the methylation of proteins and phospholipids, dephosphorylation of myosin, and the entrance of calcium (see Refs. 11 and 12). The transient increase of cGMP levels is the first response observed. cGMP levels change within 2 s after stimulation, and reach a peak after about 10 s (13).

Binding of cAMP to *D. discoideum* cells shows nonlinear Scatchard plots (14). This can be interpreted as one class of binding sites with negative cooperativity, or as two classes of binding sites with different affinities (20,000 binding sites/cell with a dissociation constant of 10 nM and 160,000 binding sites with a dissociation constant of 200 nM (14)). Several observations suggest the involvement of negative cooperativity rather than two classes of binding sites. (i) cAMP accelerates the dissociation of the cAMP·receptor complex (14). (ii) Photoaffinity labeling with 8-azidoadenosine 3′:5′-[^{32}P] monophosphate yields only one radioactive labeled protein (15). (iii) Only one class of high affinity sites has been observed after immobilization of *D. discoideum* plasma membranes on polylysine-coated beads (16).

cAMP derivatives have been used to elucidate the interactions between cAMP and several receptor proteins from different organisms (17–21). In *D. discoideum* the chemotactic activity of about 50 cAMP derivatives has been measured which resulted in a model of the cAMP-chemoreceptor interaction (18). Recently, we have observed that several cAMP derivatives behaved as antagonists or as partial antagonists of cAMP (22). Such derivatives inhibit a chemotactic response to cAMP at a derivative concentration which is chemotactically inactive. A more detailed study of one antagonist (23) revealed that its action takes place via the cAMP receptor. This indicates that the chemotactic activity of cAMP derivatives does not always represent the binding affinity of the cAMP receptor. To understand the ambivalent action of cAMP derivatives, we have investigated the earliest events in the signal transduction pathway: binding to the cell surface cAMP receptor and the induction of a cGMP response. The results suggest that binding of cAMP to the receptor and activation of the receptor are two distinct processes. cAMP is probably bound to the receptor by two hydrogen bonds and by a hydrophobic interaction. The receptor is activated thereafter by the formation of a covalent bond between the receptor and the phosphorus atom of cAMP. In the accompanying paper (24) the relationships between binding data, chemotactic activity, and antagonistic activity of cAMP derivatives will be presented.

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TABLE I

Properties of the cAMP derivatives

No.	Derivative	Binding data[a]			Cooperativity factor[b]	cGMP stimulation[c]	Polarity[d]
		α	β	mean			
		$\delta\Delta G$, kJ/mol				$\delta\Delta G$, kJ/mol	
1	Adenosine 3′:5′-monophosphate	0	0	0	5.5	0	0
2	Adenosine-N^1-oxide 3′:5′-monophosphate	9.1	9.2	9.2	4.9	10.1	−4.49
3	6-Chloropurineriboside 3′:5′-monophosphate	14.6	14.6	14.6	3.9	—	2.06
4	7-Deazaadenosine 3′:5′-monophosphate	13.9	12.7	13.3	5.8	12.2	0.36
5	8-Bromoadenosine 3′:5′-monophosphate	15.4	14.8	15.1	6.3	13.2	1.98
6	2′-Deoxyadenosine 3′:5′-monophosphate	5.0	6.1	5.6	4.8	6.1	−0.46
7	3′-Deoxy-3′-aminoadenosine 3′:5′-monophosphate	15.2	15.1	15.2	4.3	13.5	−0.02
8	5′-Deoxy-5′-aminoadenosine 3′:5′-monophosphate	3.8	5.2	4.5	8.0	4.0	−1.65
9	Adenosine 3′:5′-monophosphorothioate S_p isomer	10.6	10.8	10.7	4.7	10.6	1.63
10	Adenosine 3′:5′-monophosphorothioate R_p isomer	10.7	11.0	10.8	5.9	>23	0.66
11	Adenosine 3′:5′-monophosphodimethylamidate S_p isomer	11.8	11.5	11.7	5.0	14.1	2.45
12	Adenosine 3′:5′-monophosphodimethylamidate R_p isomer	13.1	13.5	13.3	6.5	>23	4.13
13	Benzimidazoleriboside 3′:5′-monophosphate	11.6	11.3	11.5	4.8	11.3	2.75
14	Purineriboside 3′:5′-monophosphate	15.9	16.1	16.0	4.2	—	−0.46
15	Inosine 3′:5′-monophosphate	21.9	21.5	21.7	≈5.4	—	−3.41
16	Guanosine 3′:5′-monophosphate	22.6	22.8	22.7	≈4.0	—	−3.21
17	Adenosine 5′-monophosphate	>30	>25	>27	—	>30	−8.60

[a] The inhibition of binding of 10^{-9} M [^{3}H]cAMP (α) or 10^{-7} M [^{3}H]cAMP (β) by the cAMP derivatives was measured with three assays. The $K_{0.5}$ of cAMP is 3×10^{-8} M for α and 2×10^{-7} M for β. The data for the cAMP derivatives were normalized using Equation 1. The three methods gave similar results, except for compounds **10** and **12** with the centrifugation assay which yielded respectively 13.9 and 16.0 kJ/mol. The standard deviation (cAMP, $n = 15$ or compound **9**, $n = 11$) is about 1 kJ/mol.

[b] The standard deviation for cAMP ($n = 15$) is about 1.5. The cooperativity factor for **15** and **16** could not be determined exactly because of their low binding affinity.

[c] The derivative concentration which induced a half-maximal accumulation of cGMP levels was determined. Data from different experiments were normalized by using Equation 1. The $K_{0.5}$ for cAMP was 1×10^{-8} M. The standard deviation is about 1.5 kJ/mol. The cGMP stimulation by derivatives **3**, **14**, **15**, and **16** could not be determined, because these derivatives show cross reaction in the cGMP radioimmunoassay.

[d] The retention of all derivatives was measured on a reversed phase column (32). Their selectivity to cAMP was normalized by using Equation 1.

EXPERIMENTAL PROCEDURES

Materials

[8-^{3}H]cAMP (0.9 TBq/mmol) and the cGMP radioimmunoassay kits were obtained from Amersham Corp.; polyethylene glycol (M_r = 8000) was purchased from Sigma. The cAMP derivatives **1**, **3**, **5**, **6**, **15**, **16**, and **17** (see Table I and Fig. 1) were obtained from Boehringer Mannheim; compound **4** was a generous gift of Dr. R. Hanze (The Upjohn Co.). The synthesis of compounds **2**, **7**, **8**, **13**, and **14** were described previously (25–28); these compounds were kindly provided by Dr. B. Jastorff, University of Bremen, Federal Republic of Germany. Compounds **9**–**12** were kindly supplied by Drs. J. Baraniak and W. Stec, Polish Academy of Science, Lodz, Poland (29).

The purity of all cAMP derivatives was analyzed by high pressure liquid chromatography using different stationary and mobile phases (30, 31). All compounds contained traces of impurities (maximally 3%). None of the impurities were degraded by cyclic nucleotide phosphodiesterase from beef heart or from *D. discoideum* (32), which suggests that the impurities are not cyclic nucleotides. Compound **10** contained about 1.5% of compound **9**; compound **12** contained about 3% of compound **11**. These impurities were removed by reversed phase high pressure liquid chromatography on LiChrosorp 10 RP18 with 1 mM Na_2HPO_4/H_3PO_4, 20% methanol, pH 6.5, as the mobile phase liquid.

Culture Conditions

D. discoideum NC4(H) was grown in association with *Escherichia coli* B/r on a solid medium containing 3.3 g of peptone, 3.3 g of glucose, 4.5 g of KH_2PO_4, 1.5 g of $Na_2HPO_4/2H_2O$ and 15 g of agar/liter. Cells were harvested in the late log phase with 10 mM sodium/potassium phosphate buffer, pH 6.5 (10 Pb 6.5), and freed from bacteria by repeated centrifugations at 100 × *g* for 4 min.

cAMP-binding Assays

Cells were starved for 5 h by shaking in 10 Pb 6.5 at a density of 10^7 cells/ml. The binding of cAMP derivatives to *D. discoideum* cells was estimated from the activity to inhibit the binding of [^{3}H]cAMP. Three methods were used to detect the binding of [^{3}H]cAMP; all incubations were at 0 °C and contained 10 Pb 6.5, 10^{-9} or 10^{-7} M [^{3}H]cAMP, 5 mM dithiothreitol, and different concentrations of cAMP or cAMP derivatives (10^{-9}–10^{-3} M). The incubations were started by the addition of 10^7 cells.

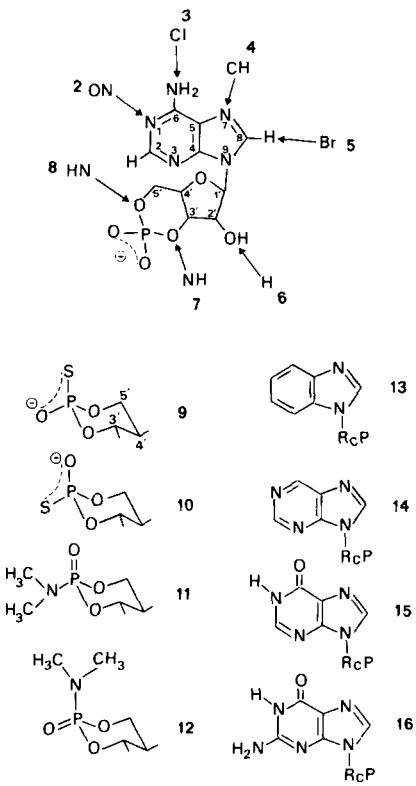

FIG. 1. **Structures of the cAMP derivatives.**

Filtration Assay (6, 14)—The incubation mixture had a volume of 1100 μl. After an incubation period of 1 min, 1 ml was filtered through Nucleopore filters (25 mm, pore size 0.6 μm). The filters were removed 15 s later, and the filter-associated radioactivity was determined.

Centrifugation Assay (33, 34)—The incubation mixture had a volume of 110 μl. After 1 min of incubation, 100 μl were carefully pipetted on top of a layer of 500 μl of 12% polyethylene glycol. Samples were centrifuged in an Eppendorf microcentrifuge at 8000 × *g* for 2 min. The liquid was aspirated and the pellet was dissolved in 110 μl of 1 M acetic acid. The radioactivity of 100 μl was determined.

Ammonium Sulfate Stabilization Assay—The volume of the incubation mixture was 100 μl. After 1 min of incubation, 1 ml of ammonium sulfate (saturated at 0 °C) was added, a few seconds later followed by the addition of 100 μl containing 1 mg of bovine serum albumin. After 5 min at 0 °C, the samples were centrifuged at 8000 × *g* for 2 min. The supernatant was removed and the pellet was dissolved in 110 μl of 1 M acetic acid. The radioactivity of 100 μl was determined.

All incubations were done in duplicate. The specificity has been determined at two concentrations of [^{3}H]cAMP, once with the filtration assay, twice with the centrifugation assay, and four times with the ammonium sulfate stabilization assay. These assays yield essentially identical results (with the exception of two analogs, see Table I).

cGMP Stimulation (35)

Cells were starved on non-nutrient agar at a density of 1.5×10^6 cells/cm^2. After 4–5 h at 22 °C, cells were harvested, washed twice, and suspended in 10 Pb 6.5 at a density of 10^8 cells/ml. One hundred-μl aliquots were stimulated with cAMP or cAMP derivatives (20 μl, final concentrations between 10^{-9} and 10^{-4} M). Ten s later, cells were lysed by the addition of 100 μl of 3.5% perchloric acid (v/v). Lysates were neutralized with 50 μl of $KHCO_3$ (50% saturated at 20 °C) and centrifuged at 8000 × *g* for 2 min. The cGMP content in 100 μl of the supernatant was measured radioimmunologically. The interaction of the cAMP derivatives with the cGMP antibody was investigated in a control experiment in which cells were lysed before the addition of the cAMP derivatives.

RESULTS AND DISCUSSION

cAMP-binding Assays—Binding of cAMP to *D. discoideum* cells is very fast; equilibrium is reached within 15 s, and the complex dissociates with a half-life of a few seconds (14). These rapid kinetics complicate the cAMP-binding assays. Separation of bound and free cAMP can be achieved either by filtration of the cells over Millipore (6, 14) or Nucleopore filters without washing the filters, or by centrifugation of the cells through a layer of silicone oil (33) or polyethylene glycol (34). Here we introduce a new procedure, which is based on the stabilization of the cAMP·receptor complex by saturated ammonium sulfate.

In the ammonium sulfate stabilization assay, cells are incubated with [^{3}H]cAMP for 1 min, followed by the dilution of the incubation mixture with saturated ammonium sulfate. After 5 min, the samples are centrifuged, and the radioactivity in the cell pellet is determined. Nonspecific binding is measured by including 10^{-4} M cAMP in the incubation mixture. Specific binding is absent if cells are boiled for 2 min prior to the incubation with [^{3}H]cAMP. Specific binding is also strongly diminished if cells are incubated with saturated ammonium sulfate prior to the incubation with [^{3}H]cAMP. Finally, the level of specific binding does not decrease if the incubation period with ammonium sulfate is varied between 1 and 15 min. This suggests that ammonium sulfate stabilizes the cAMP·receptor complex.

The three binding assays yield approximately the same results in respect to the number of cAMP receptors, their affinity, and their cyclic nucleotide specificity, except that the number of receptors measured by the centrifugation assay is about 5-fold less than with the other assays. This difference has been shown by other investigators for cAMP receptors (*cf.* Refs. 14 and 36) and folic acid receptors (*cf.* Refs. 34, 37, and 38). Possibly, centrifugation through polyethylene glycol or silicone oil is too slow, by which a major part of the cell-associated radioactivity dissociates from the receptors.

Selection of cAMP Derivatives—cAMP can form several interactions with its surrounding medium (water or receptor) such as hydrogen bonds, ionic bonds, and hydrophobic interactions. In derivatives **2–4** and **6–8** (Fig. 1, Table I), a hydrogen bond at the modified atom or atom group is no longer possible. Compounds **9–12** may reveal an ionic interaction at phosphorus, and the stereospecific involvement of the exocyclic oxygen atoms in the binding of cAMP to the receptor. cAMP has two favorable conformations, *syn* and *anti* (see Fig. 4). The distribution of these conformations, which is 1:1 in cAMP (39), is changed to 95% *syn* conformation in derivative **5** (40). Finally, the polarity of all derivatives was measured by reversed phase liquid chromatography (32) which may reveal hydrophobic interactions such as π-electron stacking by dipole-induced dipole interactions between the adenine moiety of cAMP and an aromatic group of the receptor.

The specificity of the cell surface cAMP receptor has been determined by measuring the inhibition of binding of [^{3}H]cAMP by different concentrations of the cAMP derivatives. The affinity of the cAMP derivatives for the receptor is given by $K_{0.5}$, which is defined as the concentration of derivative which results in a 50% inhibition of the binding of [^{3}H]cAMP. The results with different methods and different batches of cells are normalized by applying the following equation (19).

$$\delta\Delta G = RT \ln \frac{K_{0.5}\ \text{derivative}}{K_{0.5}\ \text{cAMP}} \qquad (1)$$

$\delta\Delta G$ values represent the reduction of binding (in kJ/mol) of a cAMP derivative if compared to the binding of cAMP. Hydrogen bond interactions have a binding energy between about 10 and 25 kJ/mol, whereas the binding energy of ionic bonds is generally above 25 kJ/mol (41).

Specificity of Binding of cAMP to D. discoideum Cells—Previously (14), it has been shown that binding of cAMP to *D. discoideum* cells resulted in nonlinear Scatchard plots. This can be interpreted as one class of binding sites with negative cooperativity, or as two classes of binding sites with different affinities, 20,000 binding sites with a K_d of 10 nM and 160,000 binding sites with a K_d of 200 nM. To determine the specificity of these hypothetically two receptors, the inhibition of binding of cAMP by the cAMP derivatives was measured at two concentrations of [^{3}H]cAMP. If two binding sites exist, then at 10^{-9} M [^{3}H]cAMP, 70% of the radioactivity is bound to the high affinity receptor, whereas at 10^{-7} M [^{3}H]cAMP, 75% of the radioactivity is bound to the low affinity receptor. Negative cooperativity can be described by a cooperativity factor *c* which we define as $c = a/b$; *a* equals the binding of 10^{-9} M [^{3}H]cAMP at a derivative concentration 10 times smaller than its $K_{0.5}$, and *b* equals the binding of [^{3}H]cAMP at a 10 times higher concentration than the $K_{0.5}$ (see Fig. 2). For noncooperative binding sites, $c = 10$; *c* is below 10 for negative cooperativity, and above 10 for positive cooperativity.[1]

The inhibition of the binding of 10^{-9} or 10^{-7} M [^{3}H]cAMP by cAMP and three derivatives is shown in Fig. 2. All inhibition curves run parallel. The results with the other deriva-

[1] The cooperativity factor is a measure for the "steepness" of the inhibition of cAMP binding by cAMP derivatives. In receptor-binding studies the cooperativity factor is often defined as the ratio of competitor concentrations yielding 10 and 90% inhibition of ligand binding. Since many cAMP derivatives have low affinities, 90% inhibition of cAMP binding cannot be always measured accurately.

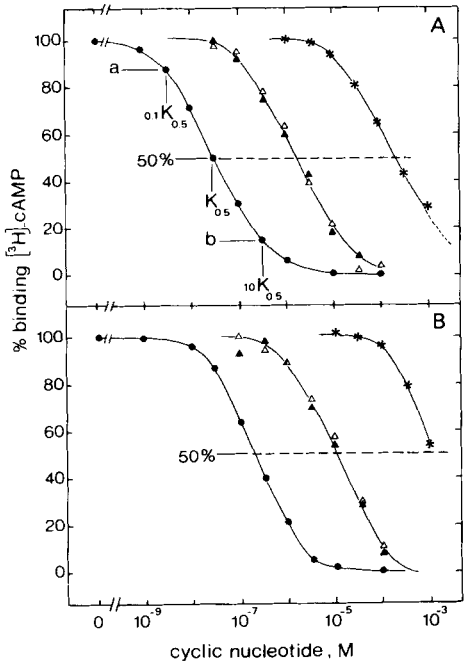

FIG. 2. **Inhibition of the binding of [^{3}H]cAMP by cAMP and three cAMP derivatives determined with the ammonium sulfate stabilization assay.** The binding of [^{3}H]cAMP in the absence of cAMP or cAMP derivatives was set at 100%. ●, cAMP; ▲, compound **9**; △, compound **10**; ⋆, compound **15**. *A*, inhibition of the binding of 10^{-9} M [^{3}H]cAMP; *B*, inhibition of the binding of 10^{-7} M [^{3}H]cAMP.

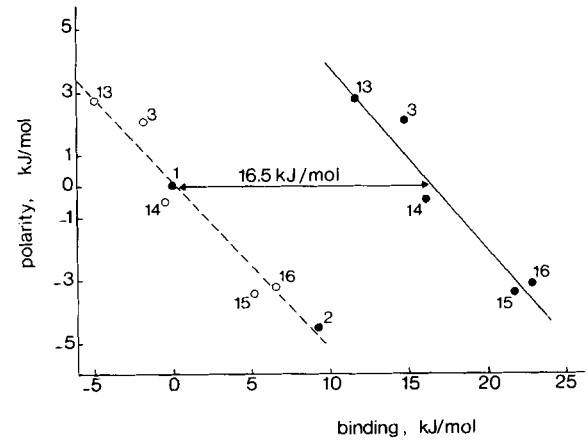

FIG. 3. **Correlation between binding data and the polarity of some cAMP derivatives.** *Filled circles* are data from Table I. Linear regression analysis of compounds **3**, and **13–16** (*solid line*) yields slope = −0.58, $r = 0.97$, $n = 5$, $p < 1\%$. These derivatives have in common the absence of the N^6-amino group. Substraction of 16.5 kJ/mol from their binding affinity yields the *open circles*. Linear regression analysis including compound **2** (*dashed line*) yields slope = −0.538, $r = 0.98$, $n = 6$, $p < 0.1\%$.

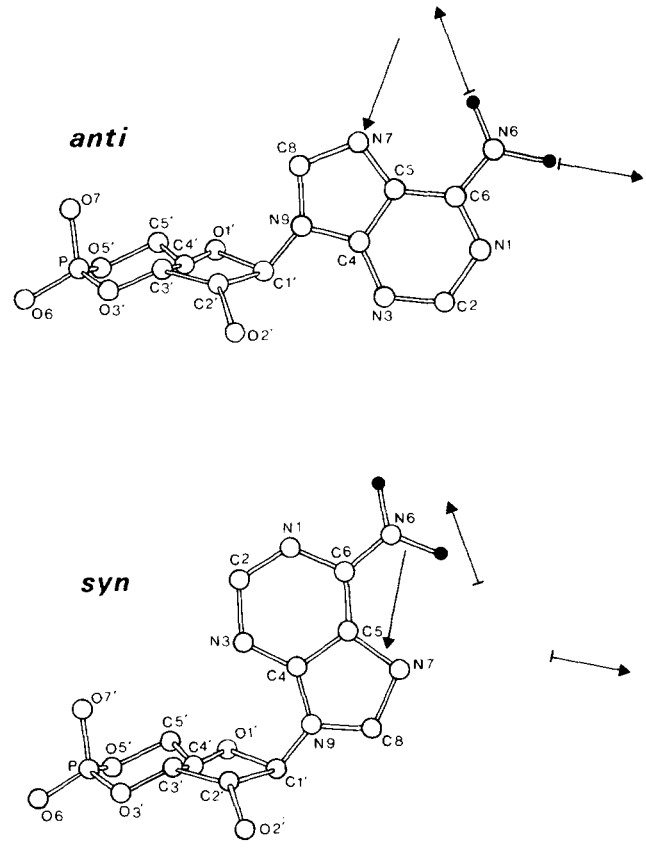

FIG. 4. **cAMP has no preference for the *syn* or *anti* conformation (39).** A bulky substituent at C^8 (*e.g.* compound **5**) changes the equilibrium to the *syn* conformation (40). The *arrows* indicate the direction of possible hydrogen bonds at N^7 and N^6H_2. The *arrows* have been drawn in the same position relative to the ribose cyclophosphate moiety in both figures.

tives are listed in Table I. This shows that all derivatives have similar potencies to compete with 10^{-9} or 10^{-7} M [^{3}H]cAMP, and that all derivatives have approximately the same cooperativity ratio. Thus, it is not possible to detect two classes of binding sites with different cyclic nucleotide specificity. This strongly supports the evidence (14–16) that cAMP binds to one class of receptors with negative cooperativity rather than to two classes of receptors with different affinities.

Table I shows that the binding affinity is strongly reduced after modifying the base moiety of cAMP. The binding affinity of compound **3**, which cannot form a hydrogen bond at N^6H_2, is reduced by about 15 kJ/mol. Fig. 3 demonstrates the close correlation between binding affinity and polarity of the derivatives which do not have this important N^6-amino function (*solid line*). The N^6H_2 atom group contributes about 16.5 kJ/mol to the binding energy of cAMP to the receptor. This high value suggests that a hydrogen bond is formed between cAMP and the receptor at N^6H_2. After correction for this binding increment, the relationship between binding and polarity indicates that the low affinity of compound **2** is probably due to its high polarity (*dashed line* in Fig. 3). Since lipophilic compounds bind better than polar ones, the adenine moiety appears to be bound in a hydrophobic cleft of the receptor. Compound **5** exists mainly in the *syn* conformation (40) and has a strongly reduced binding affinity. Since cAMP has no preference for the *syn* or *anti* conformation (39), this suggests that cAMP is bound in the *anti* conformation. The reduced binding affinity of compound **4** may point to a hydrogen bond between N^7 and the receptor. However, several recent observations do not support this hypothesis. If cAMP is bound to the receptor in the *anti* conformation at N^6H_2 and at N^7, then both interactions are impaired when cAMP has the *syn* conformation (*e.g.* in compound **5**; see Fig. 4). This is not observed; compounds **3** and **5** have similar polarity and binding affinity, which suggests that in compound **5** only the binding at N^6H_2 is impaired. Second, compounds **3** and **13–16** will have very different electron densities at N^7. The close correlation in Fig. 3 indicates that electron density at N^7 does not strongly influence the binding affinity. Finally, the pK of adenosine (42) and cAMP[2] is about 3.5; the pK of compound

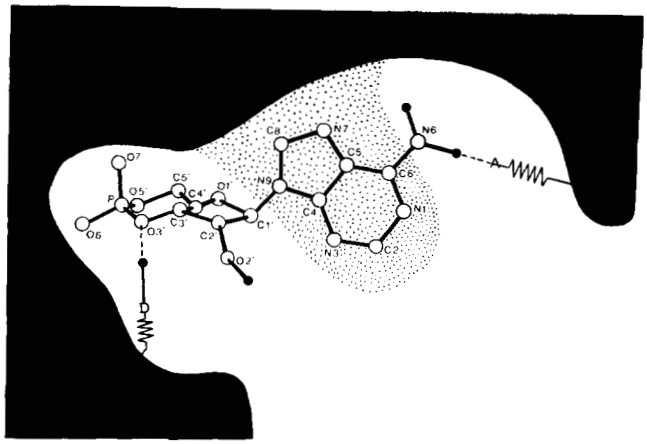

FIG. 5. **Model of the binding of cAMP by the cell surface cAMP receptor of *D. discoideum*.** The cAMP molecule binds to the receptor in the *anti* configuration by two hydrogen bonds at, respectively, N^6H_2 and $O^{3'}$. The adenine moiety is bound in a hydrophobic cleft of the receptor (⁘). There are no stereospecific interactions with the exocyclic oxygen atoms of phosphorus (O^6 and O^7). *A*, hydrogen bond acceptor; *D*, hydrogen bond donator.

4 is increased to 5.3.[2] These observations strongly suggest that the low binding affinity of compound **4** is due to a change of electron density at N^6H_2 which impairs the hydrogen bond formation at this amino group.

The binding affinity is not reduced much if the formation of a hydrogen bond is prevented in the ribose ring at $O^{2'}H$ or at $O^{5'}$ (Table I). This is in contrast to the modification at $O^{3'}$, which results in a strongly diminished binding affinity. Compounds **9–12** do not reveal a stereospecific recognition of the exocyclic oxygen atoms. The reduction of binding energy (about 12 kJ/mol) is not that much as would be expected for an ion-ion interaction, which is generally above 25 kJ/mol (41). Also dipole-dipole interactions seem to be unlikely because they require a correct orientation, and therefore, stereospecific recognition. The correlation between binding affinity and polarity is not significant ($p > 20\%$). Since a sulfur atom and a dimethylamino group are larger than an oxygen atom, the reduction of binding affinity of compounds **9–12** might be caused by the limited availability of space in the receptor site at the phosphate moiety.

Summarizing these binding specificity data, we propose that cAMP is bound to cell surface cAMP receptors in the *anti* configuration via hydrogen bonds with the receptor at N^6H_2 and $O^{3'}$ (Fig. 5). The adenine moiety is bound in a hydrophobic cleft. The phosphate moiety is not bound to the receptor by specific electrostatic forces, but is probably located in a narrow cave. cAMP is bound to one class of receptors with negative cooperativity. Binding of any derivative is sufficient to induce these receptor-receptor interactions.

Correlation of the Specificity of the Cell Surface Receptor with the Specificity of Other cAMP-binding Proteins of D. discoideum—*D. discoideum* cells contain cell surface phosphodiesterase activity. Fig. 6*A* shows the absence of any correlation between the apparent K_m values of the cAMP derivatives for cell surface phosphodiesterase (32) and the binding specificity of the cell surface receptor. Besides a cell surface cAMP receptor, *D. discoideum* cells also contain an intracellular cAMP receptor (43). Fig. 6*B* shows no correlation between the specificity of these two receptors. The intracellular cAMP receptor has a similar binding specificity as protein kinase type I from rabbit muscle (44), but seems not to be related to

[2] G. Petridis and B. Jastorff, personal communication.

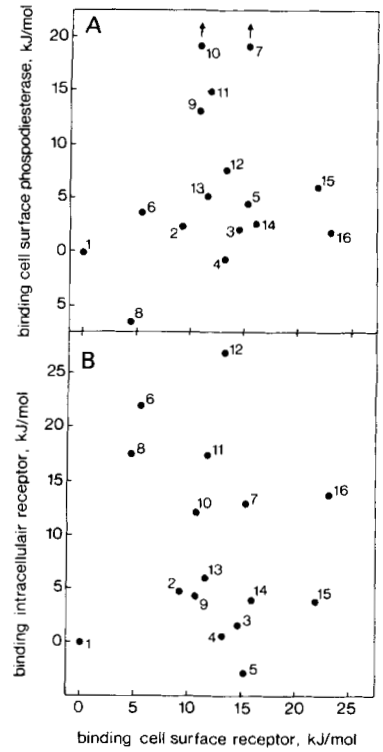

FIG. 6. **Lack of correlation between the binding affinity of cAMP derivatives for cell surface cAMP receptors with cell surface phosphodiesterase (*A*) or with an intracellular cAMP receptor (*B*).** The affinity of the derivatives for the cell surface phosphodiesterase was derived from the apparent K_m values of the cAMP derivatives for this enzyme (32). The affinity for the intracellular receptor was derived from Ref. 44. Compounds **7** and **10** do not bind to the phosphodiesterase.

the cell surface receptor of *D. discoideum*.

Specificity for the Induction of a cGMP Response—The detection of the first intracellular response after addition of the cAMP derivatives may elucidate the activation mechanism of the cell surface cAMP receptor. Recently, it was shown that the cAMP signal for an intracellular cGMP accumulation is detected within 2 s after addition of cAMP (35). Furthermore, the maximal cGMP concentration is always reached at 10 s after stimulus addition, independent of the time period that the stimulus is present (0.5 s or more than 10 min). The concentrations of the cAMP derivatives which induce a half-maximal cGMP response are shown in Table I. With the exception of two cAMP derivatives, there exists a close correlation between the affinity for the cell surface receptor and the activity to induce an intracellular cGMP response (Fig. 7). The two stereoisomers **9** and **10** have about the same binding affinity, but only the stereoisomer with sulfur located in apical position (compound **9**) induces a cGMP response. Similar results are obtained with compounds **11** and **12**; both stereoisomers bind with approximately the same affinity, but only the stereoisomer with the dimethylamino group located in equatorial position induces a cGMP response. Apparently, compounds **10** and **12** bind to the receptor, but do not activate the receptor.

Since activation of the receptor seems to be restricted to the phosphate moiety, we may consider the following mechanisms of activation after cAMP is bound to the receptor. (i) A charge-charge interaction is formed between the negatively charged oxygen atom of cAMP and a positively charged atom of the receptor. This mechanism has been proposed for the activation mechanism of cAMP-dependent protein kinase

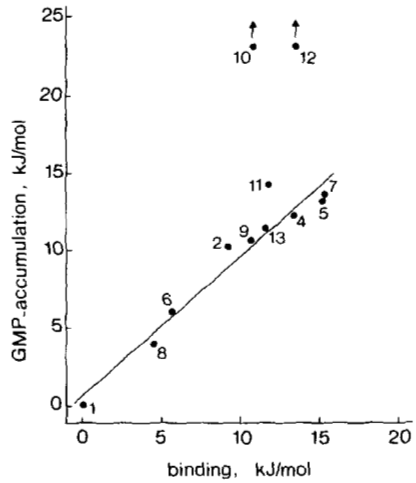

FIG. 7. **Correlation between the binding affinity of the cAMP derivatives and the potency to induce a cGMP response data derived from Table I.** Compounds **10** and **12** do not induce a cGMP response. Linear regression analysis yields (**10** and **12** not included) slope = 0.91, $r = 0.97$, $n = 10$, $p < 0.01\%$.

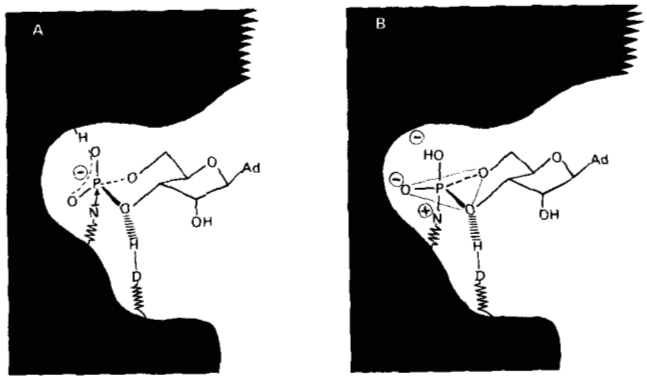

FIG. 8. **Model of the activation of the cAMP cell surface receptor.** cAMP binds first to the receptor as shown in Fig. 5, then a nucleophilic attack at the phosphorus atom takes place (*A*). This results in a pentacovalent phosphate atom with TBP configuration (*B*). The cyclophosphate ring has the favorable diequatorial position. Shielding of the apical oxygen anion, which is a prerequisite for stabilizing the TBP intermediate, is represented by protonation. *N*, nucleophile from the receptor.

(20). (ii) Another polar interaction such as charge-dipole or dipole-dipole interactions occurs between phosphorus and the receptor. (iii) A covalent bond is formed between phosphorus and the receptor. A charge-charge mechanism of activation seems unlikely since compound **11**, which does not have a negative charge, activates the receptor. Also other polar forces seem unlikely, since compound **10** suggests that they are directed to the equatorial positioned oxygen atom, while compound **12** suggests that they are directed to the apical positioned oxygen atom. The results are in agreement with the third activation mechanism: the formation of a covalent bond between the phosphorus atom of cAMP and the receptor. This will result in a pentacovalent phosphorus atom with a TBP[3] configuration. The cyclophosphate ring might be located either in diequatorial position or in equatorial-apical position. Recent quantum-chemical calculations by Van Ool and Buck (45) show that the diequatorial positioned cyclophosphate ring of cAMP in TBP configuration is about 100 kJ/mol lower in energy than the apical-equatorial positioned cyclophosphate ring. They also found that the TBP of compound **10** is about 525 kJ/mol higher in energy than the TBP of compound **9** if the cyclophosphate ring has the diequatorial position. The high energy content of the TBP of compound **10** can explain why this compound does not activate the receptor. Quantum-chemical calculations on compound **11** and **12** are not available.

We propose that, after cAMP is bound to the receptor, a nucleophilic attack of an atom or atom group of the receptor on the phosphorus atom of cAMP takes place (Fig. 8). This results in a pentacovalent phosphorus atom with the cyclophosphate ring located in the favorable diequatorial position. Due to this covalent bond, a conformational change takes place in the receptor by which the receptor is activated.

GENERAL DISCUSSION

Our results did not give evidence for more than one class of cell surface cAMP receptors in *D. discoideum* with different specificity. However, two classes of receptor with different affinities but identical specificity might be present. Furthermore, it should be kept in mind that current techniques do not allow the detection of the additional presence of a small number of receptors with low affinity. For instance, the presence of an additional 10,000 receptors with a K_d of 10^{-6} M for cAMP will increase the binding of [^{3}H]cAMP by maximally 2%, which is below the sensitivity of the binding assays.

Binding of cAMP to the main cell surface receptor and activation of this receptor are two distinct steps in a concerted reaction. The receptors show negative cooperativity. Compounds **10** and **12** bind to the receptor, but do not show activation. Interestingly, they show negative cooperativity, which indicates that activation of the receptor is not required for receptor-receptor interactions. Are there other processes induced by cAMP which do not require the activation of the cAMP receptor, or which even do not require the binding to the receptor? Investigations with compound **10** may answer these questions. First, this compound does not bind to cell surface phosphodiesterase (32); therefore, it does not interfere with the degradation of cAMP released by the cells, which otherwise may lead to artifacts (46). Second, compound **10** binds to the receptor but does not activate. Therefore, it should block the entrance of a cAMP signal if it is transduced via the cell surface receptor. We have not yet observed any response which is induced by compound **10** (phosphodiesterase induction, cGMP-accumulation, and chemotaxis).[4] Furthermore, all these responses to cAMP are blocked in the presence of a high concentration of compound **10**. This suggests that, as far as we have investigated, all responses to cAMP require the activation of the cAMP receptor.

The main extracellular function of cAMP in *D. discoideum* is the induction of a chemotactic response during cell aggregation. Probably, this requires a refined detection and analysis mechanism of temporal and spatial fluctuations of the extracellular cAMP concentration. The chemotactic response to cAMP derivatives may shed light on the mechanism of chemosensory transduction in *D. discoideum*.

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[3] The abbreviation used is: TBP, trigonal bipyramidal.

[4] Previously we reported low chemotactic activity of compounds **10** and **12** in aggregative *D. discoideum* cells (22). At that time, however, compounds **10** and **12** were still contaminated with their stereoisomers (about 1.5% of compound **9** in **10**, and about 3% of compound **11** in **12**). After further purification, compounds **10** and **12** are chemotactically inactive (24).

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