Binding of cAMP and Adenosine Derivatives to Dictyostelium discoideum Cells

RELATIONSHIPS OF BINDING, CHEMOTACTIC, AND ANTAGONISTIC ACTIVITIES*

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Dictyostelium discoideum cells contain one class of cAMP receptors and two classes of adenosine receptors (respectively, adenosine α- and β-receptors). A cell has $3.5 \times 10^4$ adenosine α-receptors with a $K_d = 0.8\ \mu M$ and $8 \times 10^6$ adenosine β-receptors with a $K_d = 350\ \mu M$. Binding of adenosine to the β-receptors inhibits up to 90% of the binding of cAMP to the cAMP receptors in a noncompetitive way.

Measurement of the chemotactic and antagonistic activity of 18 cAMP and adenosine derivatives for aggressive D. discoideum cells resulted in four functional groups. 1) Nine compounds are full agonists; they are chemotactic but have no antagonistic effects on the chemotactic activity of cAMP. 2) Five compounds are partial antagonists; they can be both agonists as well as antagonists, depending on the concentration used. 3) Two compounds are competitive full antagonists, and 4) three compounds are noncompetitive full antagonists.

Comparison of the quantitative data on the chemotactic and antagonistic activities of all compounds with their binding data for cAMP and adenosine cell surface receptors leads to the following conclusions on the mechanism of action of the antagonists.

The two competitive full antagonists bind to the cAMP receptor, but they do not activate the receptor; therefore, they do not induce a response, and at the same time prevent the detection of cAMP. The three noncompetitive antagonists bind to the adenosine β-receptor which inhibits the binding of cAMP to the cAMP receptor; also these compounds prevent the detection of cAMP. The five competitive partial antagonists bind to the cAMP receptor and induce a normal cGMP response. Also cAMP induces a normal cGMP response in the presence of partial antagonists. This indicates that partial antagonists do not prevent the detection of cAMP, but extinguish the intracellular response to cAMP. A model is presented for the mechanism of action of these partial antagonists which is based on false reading of chemotactic signals in terms of excitation and adaptation processes.

Chemotaxis is a prerequisite during the development of the cellular slime molds. In the vegetative stage, the amoebae are chemotactic to folic acid and pterin (1, 2), both of which are excreted by their food source, the bacteria. When the food source is exhausted, the amoebae aggregate to form a multicellular slug which then differentiates into a fruiting body. Cell aggregation in Dictyostelium discoideum is mediated by chemotaxis to cAMP (3).

The detection, and particularly the analysis of chemotactic signals, is far from understood. Mato et al. (4) presented evidence that the primary input signal for a chemotactic response is a spatial gradient of cAMP. The threshold spatial gradient of cAMP between the ends of a cell is about 1% of the mean concentration (4). Approximately the same value was found for folic acid-induced chemotaxis in D. discoideum (5) and for chemotaxis in leukocytes (6). Thus, a cell is able to discriminate between a concentration of 100 at its front and 99 at its back. Adaptation is important during the analysis of chemotactic signals (7, 8, 9). By adaptation, a cell accommodates to, and thus ignores, the mean concentration of chemoattractant. The difference between 100 and 99 is simplified by adaptation to the difference between 1 and 0.

Extracellular cAMP is probably detected by cell surface receptors (10, 11) and is degraded by cell surface phosphodiesterase (12, 13). The specificity of the chemoreceptor has been determined by measuring the chemotactic response of aggressive D. discoideum cells to cAMP derivatives (14).

Recently, we have observed that some derivatives are not only chemotactic, but that they antagonize a chemotactic response to cAMP (15, 16). The mechanism of action of antagonists may contribute to the knowledge of chemotaxis and signal transduction. Therefore, we have investigated the interactions of cAMP derivatives with cell surface phosphodiesterase (17) and with the cell surface receptor (18). The phosphodiesterase appears not to be involved in the action of the antagonists (16, 17).

Antagonists have been observed for many stimuli in a large variety of organisms (19–26). Their mechanism of action has been correlated with the nature of chemical modification of the stimulus molecule (25), with an increased off-rate of the antagonist (20), with altered binding to two receptor states (one of them is coupled to an effector (22, 24)), with differential binding to two binding sites of one receptor (26), and with altered cluster formation of receptors occupied with antagonists relative to agonists (21).

In this paper, the relationship of the binding, chemotactic, and antagonistic activity of cAMP and adenosine derivatives in D. discoideum has been analyzed. The antagonists act by at least three different mechanisms: (i) noncompetitive inhibition of the binding of cAMP with an adenosine receptor as intermediate, (ii) competitive inhibition of the binding of

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cAMP by derivatives which occupy but do not activate the cAMP receptor, and (iii) extinction of the intracellular response to cAMP.

EXPERIMENTAL PROCEDURES

Materials—[8-3H]cAMP (0.9 TBq/mmol), [2,5,8-3H]adenosine (1.5 TBq/mmol), and the cGMP radioimmunoassay kit were purchased from Amersham Corp. The cAMP derivatives (compounds 1-17, Table I; see Fig. 1 of Ref. 18 for chemical structures) were obtained as described (18). Compounds 18-20 were from Sigma. Compounds 10 and 12 were purified as described (18).

Culture Conditions—D. discoideum NC1 (H) was grown as described (18). Cells were harvested with 1% salt solution (27) for the chemotactic assay or with 10 mM KH₂PO₄/Na₂HPO₄, pH 6.5, for binding assays and for cGMP stimulations. Bacteria were removed by repeated centrifugations at 100,000 g for 4 min.

Chemotactic Assay—Chemotaxis was tested with the small population assay (28). Briefly, small droplets (0.1 µl) containing about 500 amoebae were deposited on hydrophobic agar (0.8% agar, washed with deionized water, in salt solution). Amoebae were incubated at 22 °C for 2 h and afterwards at 15 °C for 6-8 h. Cells were then placed back at 22 °C, and after 30 min, test solutions were deposited twice, close to the small populations at a 5-min interval. The distribution of amoebae within the amoeba population was observed at a 7-min interval. A reaction was considered positive if at least twice as many amoebae were pressed against the edge close to the test solution as against the opposite edge. About 20 populations were observed for each test solution. Maximal responses were observed at about 20-30 min after deposition of the test solution. Cell aggregation starts at about 1.5 h after the transfer from 15 to 22 °C. The temperature treatment synchronizes the cells by which they are optimally sensitive to cAMP.

Binding Assays—[3H]cAMP binding was measured by three assay methods as described in Ref. (18). [3H]Adenosine binding was measured by the ammonium sulfate stabilization assay as described for the binding of [3H]cAMP, except that the incubation mixture contained 1.25 × 10⁵ cells.

RESULTS

Mechanism of Action of Adenosine—In the accompanying paper (18), the binding of cAMP derivatives to the cell surface cAMP receptor was investigated. In contrast to all cAMP derivatives, adenosine is a noncompetitive inhibitor of the binding of [3H]cAMP (Fig. 1). The concentration of adenosine which induces 50% inhibition of the binding of [3H]cAMP is not affected by the concentration of [3H]cAMP. Furthermore, adenosine, and more clearly 2′-O-methyladenosine (V, W), or adenosine (III, I). The binding of 10⁻⁶ or 10⁻⁷ M [3H]cAMP in the absence of competitor was set at 100%; the binding in the presence of 10⁻⁴ M cAMP was set at 0%. Binding was determined with the ammonium sulfate stabilization assay (18). Data shown are the means of duplicate determinations.

Adenosine binds to the adenosine β-receptor (Rβ), and the adenosine β-receptor complex inhibits the binding of cAMP to the cAMP receptor.

Binding of cAMP to the cAMP receptor has been correlated with the induction of a transient intracellular cGMP accumulation (18). The inhibition of cAMP binding by adenosine predicts that adenosine also inhibits the cAMP-mediated cGMP response. Adenosine does not inhibit a cGMP response if adenosine and cAMP are added simultaneously to a cell suspension (Fig. 2). However, the cAMP-mediated cGMP response is partly inhibited if cAMP is added 30 s after adenosine addition. Previous experiments have revealed that the cAMP signal for a cGMP response is detected by D. discoideum cells within 1-2 s after cAMP addition (9). Apparently, transduction of the cAMP signal is faster than the inhibition of this transduction by adenosine.

Adenosine + Rβ + cAMP + R → cAMP + Rβ

The specificity of the α-site, the β-site, and the inhibition of cAMP binding was determined using about 20 adenosine derivatives and methylxanthines. The α- and β-sites resemble but are not identical with, respectively, the Rβ and P-site which have been identified in higher organisms (33) (P. J. M. Van Haaster, W. F. Van Woerden, and J. G. Smits, manuscript in preparation).
Chemotaxis and Antagonism of cAMP and Adenosine Derivatives

Chemotaxis and Antagonism—The chemotactic activity of 18 cAMP and adenosine derivatives (Table I; see Ref. 18 for chemical structures) was measured with the small population assay (28). The threshold concentration for chemotaxis is defined as the concentration of derivative which induces a positive response in 50% of the populations. Adenosine and adenosine derivatives do not induce a chemotactic response (Fig. 4A). The majority of the cAMP derivatives are chemotactic, although different concentrations are required to induce a threshold response.

Recently, we have observed that cAMP derivatives may antagonize the chemotactic response to cAMP (15, 16). Such derivatives inhibit a chemotactic response to cAMP at a 0.1 × 10⁻⁸ M cAMP concentration.

Fig. 3. Effect of adenosine on a cAMP-mediated cGMP response. Aggregative D. discoideum cells (100 μl, containing 10⁷ cells) were stimulated with 5 mM adenosine at t = 0 (●), with 10⁻⁸ M cAMP at 0 s (□), with 5 mM adenosine and 10⁻⁷ M cAMP at 0 s (▲), or with 5 mM adenosine at −30 s and 10⁻⁷ M cAMP at 0 s (▲). Cells were lysed at the indicated times; cGMP levels were measured radioimmunologically as described (18). Data shown are the means of three experiments.

Table I

<table>
<thead>
<tr>
<th>No.</th>
<th>Derivative</th>
<th>Binding*</th>
<th>Chemotaxis*</th>
<th>Antagonism*</th>
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<tr>
<td>1</td>
<td>Adenosine 3′,5′-monophosphate</td>
<td>0.03 μM</td>
<td>0.003 μM</td>
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<td>2</td>
<td>Adenosine-N'-oxide 3′,5′-monophosphate</td>
<td>1.23 μM</td>
<td>0.08 μM</td>
<td>d</td>
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<td>3</td>
<td>6-Chloropurineriboside 3′,5′-monophosphate</td>
<td>11.8 μM</td>
<td>3.0 μM</td>
<td>1.2</td>
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<td>4</td>
<td>7-Deazaadenosine 3′,5′-monophosphate</td>
<td>8.7 μM</td>
<td>0.8 μM</td>
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<tr>
<td>5</td>
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<td>16.0 μM</td>
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<tr>
<td>6</td>
<td>2′-Deoxyadenosine 3′,5′-monophosphate</td>
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<td>0.04 μM</td>
<td>d</td>
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<td>3′-Deoxo-3′-aminoadenosine 3′,5′-monophosphate</td>
<td>14.7 μM</td>
<td>40 μM</td>
<td>1.0</td>
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<tr>
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<td>0.01 μM</td>
<td>d</td>
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<td>Adenosine 3′,5′-monophosphorothioate S₆ isomer</td>
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<td>0.27 μM</td>
<td>d</td>
</tr>
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<td>2.4 μM</td>
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<td>9</td>
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<tr>
<td>11</td>
<td>Adenosine 3′,5′-monophosphodimethylamidate S₆ isomer</td>
<td>3.7 μM</td>
<td>0.29 μM</td>
<td>d</td>
</tr>
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<td>12</td>
<td>Adenosine 3′,5′-monophosphodimethylamidate R₆ isomer</td>
<td>6.3 μM</td>
<td>c</td>
<td>40</td>
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<td>13</td>
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<td>19.6 μM</td>
<td>100 μM</td>
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<td>Inosine 2′,5′-monophosphate</td>
<td>226 μM</td>
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<td>d</td>
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<td>c</td>
<td>c</td>
</tr>
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<td>310 μM</td>
<td>c</td>
<td>1000</td>
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<td>20</td>
<td>Adenosine 2′-O-methyladenosine</td>
<td>11.2 μM</td>
<td>c</td>
<td>20</td>
</tr>
</tbody>
</table>

*The concentration is given which results in a 50% inhibition of the binding of 10⁻⁸ M [³H]cAMP.

*The concentration is given which induces a positive response in 50% of the populations.

*The concentration is given which reduces the activity of 10⁻⁸ M cAMP to a response which would be induced by 3 × 10⁻⁸ M cAMP.

*No antagonistic effect observed up to the concentration listed under chemotaxis.

*Inactive up to 1 mM.
derivative concentration which does not induce a chemotactic response. The threshold concentration for an antagonistic reaction is defined as the concentration of derivative which reduces the chemotactic response of 10^-5 M cAMP (about 70-80% attraction) to a response as would be induced by 3 x 10^-9 M cAMP (40-50% attraction). Several derivatives are antagonistic (Fig. 4B). Comparison with chemotaxis (Fig. 4A) demonstrates that some derivatives are full agonists (1, 6, and 11 are only chemotactic), that others are full antagonists (10 and 18 are only antagonistic), while still others are partial antagonists (3 and 7 have both chemotactic and antagonistic activity). The chemotactic and antagonistic activity of all derivatives was measured in an identical way; the results are listed in Table I.

Competitive and Noncompetitive Antagonists—The chemotactic response of D. discoideum cells to different cAMP concentrations in the presence of different concentrations of the partial antagonist 7 and the full antagonists 10 and 2'-O-methyladenosine (20) are shown in Fig. 5. The cAMP derivatives 7 and 10 appear to be competitive antagonists of cAMP, while 2'-O-methyladenosine is a noncompetitive antagonist. This figure also shows that 2'-O-methyladenosine can only antagonize low cAMP concentrations. This result is compatible with the binding data (Fig. 1) where it was shown that a saturating 2'-O-methyladenosine concentration cannot completely inhibit the binding of cAMP to the cAMP receptor. Still, 10% of the cAMP receptors are available to detect cAMP, which might be sufficient to detect steep gradients of the cAMP concentration. This hypothesis is confirmed by the observation that compound 10 (10^-4 M) prevents cell aggregation, while adenosine (10^-2 M) only slightly delays cell aggregation (determined with the small population assay; data not shown).

The Relationship of Binding, Chemotactic, and Antagonistic Activities of cAMP and Adenosine Derivatives—In Fig. 6 the concentration of cAMP or adenosine derivatives which inhibits the binding of 10^-5 M [3H]cAMP for 50% is presented against the threshold concentrations for chemotaxis and antagonism. This demonstrates that: 1) a close correlation exists between binding affinity and chemotactic activity of full agonists (\( r = 0.993, p < 0.01 \)). 2) A close correlation exists between binding affinity and antagonistic activity of full antagonists (\( r = 0.97, p < 1% \)). 3) There is no correlation between binding affinity and chemotactic activity of partial antagonists (\( r = 0.98, p < 5% \)). 4) A correlation exists between binding affinity and antagonistic activity of partial antagonists (\( r = 0.88, p < 5% \)). 5) The antagonistic activity of the partial antagonists are not significantly different from the chemotactic activity of the full agonists if related to their binding data (\( r = 0.97, p < 1% \)). 6) The antagonistic activity of the partial antagonist takes place at about 10 times lower concentration than required for 50% inhibition of the binding of 10^-5 M [3H]cAMP, while the antagonistic activity of the full agonists takes place at about 3-fold higher concentrations than required for 50% inhibition of the binding of 10^-5 M [3H]cAMP.

**FIG. 6. Relationships of binding, chemotactic, and antagonistic activities of cAMP and adenosine derivatives.** Abscissa, the concentrations of compounds 1-20 which inhibit the binding of 10^-5 M [3H]cAMP by 50% are indicated. Ordinate, the threshold concentrations for chemotactic and antagonistic activities are indicated. From Table I, linear regression analysis yields for chemotactic activity of full agonists (\( r = 0.993, p < 0.01 \)) and for antagonistic activity of full antagonists (\( r = 0.97, p < 1% \)).

**DISCUSSION**

The relationships of binding, chemotactic, and antagonistic activity of cAMP and adenosine derivatives show that a distinction can be made between full agonists, competitive full antagonists, noncompetitive full antagonists, and partial antagonists.

**Full Agonists**—These compounds bind to and activate the cAMP receptor; they induce an intracellular cGMP response (18). The chemotactic activity is closely correlated with their binding affinity. The threshold concentration for chemotaxis is about 10 times lower than the binding affinity of the cAMP receptor, which indicates that at a threshold reaction only a
few per cent of the cAMP receptors are occupied with agonist.\(^2\)
In all aspects full agonists behave as cAMP, except that higher concentrations are required.

**Competitive Full Antagonists**—These compounds (10 and 12) bind to the cAMP receptor, but this does not result in activation of the receptor (18). Consequently, these compounds are chemotactically inactive, and they prevent the detection of CAMP. The threshold concentration for an antagonistic effect is about three times higher than their binding affinity, which indicates that almost 50% of the receptors should be blocked to observe a threshold antagonistic effect.\(^2\)

**Noncompetitive Full Antagonists**—These compounds (adenosine and adenosine derivatives) bind to the adenosine \(\beta\)-receptor. The adenosine \(\beta\)-receptor complex inhibits the binding of cAMP to the cAMP receptor. Also for these full antagonists almost 50% of the cAMP receptors should be blocked to induce threshold antagonistic effects.\(^2\) Recently, Newell (29) and Newell and Ross (30) have investigated the effects of adenosine on cell aggregation and on the activity of CAMP in *D. discoideum*. Their results on the adenosine receptors and on the noncompetitive inhibition of the binding of cAMP by adenosine are essentially identical with those reported presently in Figs. 1 and 2. The antagonistic effect of adenosine on CAMP chemotaxis has not been observed previously. Adenosine does not inhibit more than 90% of the cAMP receptors. The remaining 10% active receptors are sufficient to detect steep CAMP gradients. Therefore, an antagonistic effect of adenosine on CAMP chemotaxis can only be observed if low CAMP concentrations are used. The chemotactic assay used presently was more sensitive than the one used by Newell and Ross (30).

The failure to obtain more than 90% inhibition of CAMP binding by adenosine may suggest that the remaining 10% belong to a subpopulation of CAMP receptors not associated with the adenosine \(\beta\)-receptor. However, Newell and Ross (30) have shown that CAMP binding in the presence or absence of 0.77 or 5 mM adenosine results in identical curvilinear Scatchard plots with respect to apparent affinities. Adenosine reduces only the total number of binding sites. This suggests that the 10% remaining CAMP receptors in the presence of adenosine are identical with the other 90% receptors in the absence of adenosine.

The cGMP response is reduced by only 50% in the presence of previously added adenosine, which inhibits 90% of the CAMP receptors. This points to the presence of spare CAMP receptors; i.e. only a small portion (20%) of the CAMP receptors have to be present to obtain a full cGMP response. Similar experiments by Newell and Ross show no effect of adenosine on the cGMP response. This may be due to other developmental stages in which cells were used. Their 8-h starved cells may contain more CAMP receptors than the 4.5-h starved cells used in the present report. Due to spare CAMP receptors, inhibition of 90% of the CAMP receptors may leave available sufficient receptors to achieve a normal cGMP response in these cells.

**Partial Antagonists**—Depending on the concentration used, these compounds may induce a chemotactic response, or they may prevent a chemotactic response to CAMP. They are antagonistic at very low concentrations, if compared to their binding affinity for the CAMP receptor. At antagonistic threshold concentrations only a few per cent of the CAMP receptors are occupied with antagonist. This would leave sufficient receptors available for the detection of CAMP. Partial antagonists bind to and activate the receptor, which results in a normal cGMP response (18). Furthermore, preincubation of *D. discoideum* cells with low concentrations of a partial antagonist (e.g. 10\(^{-4}\) M of compound 7) does not prevent the induction of a cGMP response thereafter by high CAMP concentrations (10\(^{-4}\) M).\(^3\) This suggests that the partial antagonists do not prevent the transduction of the CAMP signal, but that they erase the response evoked by CAMP.

The data on partial antagonists combined with the detection mechanism of chemotactic signals may elucidate the mechanism of action of partial antagonists. 1) Three chemotactants are known in the cellular slime molds: CAMP, folic acid, and pterin, which are detected by separate cell surface receptors (16). Partial antagonists are known for each chemotactrant, not only in *D. discoideum*, but also in other slime mold species (16, 31, 32). This may indicate that partial antagonists interfere with a general principle for the detection of chemotactic signals. 2) Partial antagonists are receptor-specific, which means that an antagonist of CAMP does not antagonize folic acid or pterin (16). 3) Partial antagonists erase the activity of agonists at equimolar concentrations relative to their binding activity. Thus, one receptor occupied with antagonist extinguishes the response of one receptor occupied by CAMP. 4) Partial antagonists become agonistic at high concentrations, which implies a steep spatial gradient of the partial antagonist. 5) Detection of chemotactic signals implies the detection of the spatial distribution of chemotactant around the cell (4). 6) Adaptation is involved in the detection of chemotactic signals (8, 9). A cell responds to positive deviations from the mean concentration of chemotactant. The mean concentration is detected by an adaptation process (see Fig. 7a). 7) Adaptation is a receptor-specific process; cells adapted to CAMP are not adapted to folic acid or pterin (8).

I propose that the mechanism of action of the partial

\(^2\) The test solution was deposited twice at a distance of about 0.5–0.7 mm from the responding amoebae. Due to diffusion, the maximal local concentration around the cells is about 5–10-fold less than the applied concentration in the test solution (for mathematics see Ref. 4).

\(^3\) F. Kesbeke and P. J. M. Van Haaster, unpublished observations.
antagonists is based on the induction of a too high level of adaptation (Fig. 7e). This explains why steep gradients of partial antagonists induce a chemotactic response (Fig. 7d). This also explains why the response to cAMP is extinguished, although cAMP still binds to the receptor and induces excitation and adaptation (Fig. 7e).

In summary, the antagonists appear to have different molecular mechanisms of action. One class of antagonists competitively blocks the cAMP receptor, a second class blocks the cAMP receptor in a noncompetitive manner, while a third class does not block the cAMP receptor, but erases the intracellular response evoked by cAMP.

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REFERENCES