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Competitive cAMP Antagonists for cAMP-Receptor Proteins*

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The two exocyclic oxygen atoms at phosphorus of cAMP have been replaced by a sulfur atom or by a dimethylamino group. These substitutions introduce chirality at the phosphorus atom; therefore, two diastereomers are known for each derivative: (S)-cAMPS, (R)-cAMPS, (S)-cAMPN(CH3)2, and (R)-cAMPN(CH3)2. We have investigated the agonistic and antagonistic activities of these compounds in four cAMP-dependent reactions: activation of the cellular slime mold Dictyostelium discoideum via its cell surface cAMP receptor, and phosphorylation by cAMP-dependent protein kinases type I, II (both mammalian enzymes), and type D (derived from D. discoideum). The results show that 1) the compounds (S)-cAMPS and (S)-cAMPN(CH3)2 are (mostly full) agonists for the four proteins. Half-maximal activation is at micromolar concentrations (0.8–7 μM). 2) (R)-cAMPS is a full antagonist for the cell surface receptor and protein kinases type I and II, with apparent inhibition constants between 0.8 and 8 μM. This compound is a partial agonist for protein kinase type D, where it induces maximally 50% activation of the enzyme if compared with cAMP. 3) (R)-cAMPN(CH3)2 is a full antagonist for the cell surface receptor, and for protein kinase type II. This compound is a partial agonist for protein kinase type I (at least 50% activation if compared with cAMP), and inactive for protein kinase type D. This derivative is at least 25-fold less active as an antagonist than (R)-cAMPS. 4) The activity of mixtures of different concentrations of the antagonist (R)-cAMPS with different concentrations of cAMP reveals that the compound is a competitive antagonist of cAMP at micromolar concentrations.

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The abbreviations used are: cAMPS, adenosine 3',5'-monophosphorothioate; cAMPN(CH3)2, adenosine 3',5'-monophosphodimethylamidate; MES, 4-morpholineethanesulfonic acid.
observed that the Rp isomers of cAMPS and cAMPN(CH$_3$)$_2$ no longer have activating properties. Since these compounds bind to the cAMP receptor in a competitive manner with respect to cAMP it was suggested that these compounds are antagonists of cAMP.

In the present work we have investigated the agonistic and antagonistic activities of the highly purified preparations of the Sp and Rp isomers of cAMPS and cAMPN(CH$_3$)$_2$ for four cAMP-dependent proteins: the cell surface cAMP receptor from D. discoideum, cAMP-dependent protein kinase type I from beef heart, type II from rabbit muscle, and type D from D. discoideum. The results show that antagonists are present among these four derivatives.

**EXPERIMENTAL PROCEDURES**

**Materials**—[$\gamma$-32P]ATP was obtained from New England Nuclear and the radioimmunoassay kit from the Radiochemical Centre (Buckinghamshire, United Kingdom). Sephacryl S-300 was purchased from Pharmacia (Uppsala, Sweden); Leu-Arg-Arg-Ala-Ser-Leu-Gly (kemptide) was from Sigma.

The stereoisomers of cAMPS were synthesized as described in Ref. 7. The synthesis of the isomers of cAMPN(CH$_3$)$_2$ will be described elsewhere. The compounds were purified by high performance liquid chromatography (6). After purification, the Rp isomers are free of detectable levels of the Sp isomers or cAMP (< 0.1%).

**Methods**—The cAMP-mediated cGMP response was measured in aggregative D. discoideum, NC-4 (H), cells (10). Briefly, 50-μl cell suspensions were stimulated with 10 μl of cAMP or derivative. The reaction was terminated after 10 s by the addition of 50 μl of perchloric acid. cGMP was measured in the neutralized lysates by means of a radioimmunoassay.

cAMP-dependent protein kinase type D (holoenzyme) was isolated from aggregative D. discoideum, AX-2, by gel filtration of a cytosolic cell fraction on Sephacryl S-300 as described in Ref. 11.

cAMP-dependent protein kinase type II from beef heart was isolated as described in Ref. 12 using one DEAE-chromatography step. cAMP-dependent protein kinase type II from rabbit muscle was obtained from Sigma. Protein kinase activity was measured (13) in a reaction mixture (60 μl) containing 50 mM MES buffer (pH 6.5), 5 mM MgCl$_2$, 0.5 mM EGTA, 10 mM NaF, 2.5 mM dithiothreitol, 20 μM kemptide, 0.2 mM [$\gamma$-32P]ATP (2 Bq/μmol), enzyme, and cyclic nucleotides. The reaction (30°C) was started by the addition of 30 μl of enzyme, and terminated after 10 min by the addition of 800 μl of a 50% slurry of Dowex 1 X2 in 30% acetic acid. After equilibration for 1 h, samples were centrifuged for 2 min at 10,000 × g. The radioactivity in 175 μl of the supernatant was determined.

**RESULTS**

The Cell Surface cAMP Receptor from D. discoideum—Binding of cAMP to the cell surface cAMP receptor of the cellular slime mold D. discoideum induces several responses, of which an intracellular accumulation of cGMP is the first response observed (for review on signal transduction in D. discoideum see Refs. 14 and 15). cGMP levels reach a peak at 10 s after stimulation and prestimulated levels are recovered within about 30 s. The pace of the cGMP response is the same for different cAMP analogs (10).

The cGMP response induced by cAMP and the four derivatives with modified exocyclic oxygen atoms are shown in Fig. 2A; three compounds are active, and two are inactive. Linear curves arise when these data are replotted according to Eadie and Hofstee (Fig. 2, C and D). The intersections with the ordinate indicate that cAMP, (Sp)-cAMPS, and (Sp)-cAMPN(CH$_3$)$_2$ induce about the same maximal response. The slopes of these curves represent the $K_r$ (concentration which induces half-maximal stimulation); this yields 14 nM for

**FIG. 1. Structures of the cAMP analogs with modified exocyclic oxygen atoms.**

**FIG. 2. Agonistic and antagonistic activities of exocyclic oxygen-modified cAMP analogs for cAMP-mediated cGMP response in D. discoideum.** A. D. discoideum cells were stimulated with different concentrations of cAMP (+), (Sp)-cAMPS (O), (Sp)-cAMPN(CH$_3$)$_2$ (A), or (Rp)-cAMPN(CH$_3$)$_2$ (△). Cells were lysed at 10 s after stimulation and cGMP levels were measured radioimmunoassayically. B. D. discoideum cells were preincubated for 30 s with different concentrations of (Rp)-cAMPS (O), (Rp)-cAMPN(CH$_3$)$_2$ (△). Then 50 nM cAMP was added; cells were lysed 10 s later, and cGMP levels were measured. C and D. Eadie-Hofstee plots of the data of A. $ΔV$ is the increase of cGMP levels over basal levels, and $A$ is the concentration of the nucleotides. The intersections with the ordinates are the maximal response; the slopes equal $-K_r$. Symbols are described in A. E and $F$, inspection for competitive antagonism of (Rp)-cAMPS. D. discoideum cells were preincubated for 30 s with three concentrations of (Rp)-cAMPS (0, 2.5, 8.33 μM) and then cells were stimulated with different cAMP concentrations (0, 10, 20, 30, 40, and 100 nM); cells were lysed 10 s later and cGMP levels were measured. A Lineweaver-Burk plot of the data is shown in E; preincubation without (O), or with 2.5 μM (△), or with 8.33 μM (△) (Rp)-cAMPS. A Dixon plot of the same data is shown in F; response to 100 nM (O), 40 nM (△), 30 nM (△), or 20 nM (△) cAMP.
cAMP, 0.78 μM for (Sp)-cAMPS, and 2.8 μM for (Sp)-cAMPN(CH₃)₂.

The compounds (Rp)-cAMPS and (Rp)-cAMPN(CH₃)₂ do not induce a cGMP response, although it has been shown that they bind to the cell surface cAMP receptors at micromolar concentrations (5). To test the compounds for antagonistic properties, the cells were mixed with the Rp stereoisomers and then stimulated with 50 nM CAMP. This reveals (Fig. 2B) that the analogs antagonize the stimulating activity of cAMP. Half-maximal inhibition (IC₅₀) occurs at 4 μM (Rp)-cAMPS and at about 100 μM (Rp)-cAMPN(CH₃)₂.

Since these compounds are investigated with whole cells they may inhibit the cAMP-mediated cGMP response at a site distinct from the cAMP receptor. Therefore, it had to be established that the Rp compounds are competitive antagonists of cAMP. Cells were stimulated by different cAMP concentrations in the presence of different concentrations of (Rp)-cAMPS. The Lineweaver-Burk plot (Fig. 2E) and the Dixon plot (Fig. 2F) indicate that (Rp)-cAMPS is a competitive antagonist of cAMP. The Kᵢ values for the Rp isomers can be calculated from the IC₅₀ values obtained in Fig. 2B by using the equation

\[ Kᵢ = IC₅₀ \times \frac{Kᵢ}{A + Kᵢ} \]

where Kᵢ is the activation constant of cAMP (14 nM) and A is the concentration of cAMP (50 nM). This yields Kᵢ = 0.9 μM for (Rp)-cAMPS and Kᵢ ≈ 22 μM for (Rp)-cAMPN(CH₃)₂. Kᵢ values can also be derived from a Dixon plot (Fig. 2F) which yields Kᵢ = 0.8 μM for (Rp)-cAMPS.

These data demonstrate (Table I) that (Sp)-cAMPS and (Sp)-cAMPN(CH₃)₂ are full agonists of cAMP for the induction of a cGMP response in D. discoideum. (Rp)-cAMPS and (Rp)-cAMPN(CH₃)₂ are competitive full antagonists of cAMP. The effects of the derivatives with the cAMP-dependent protein kinases type I, II, and D were investigated in a similar manner.

**Table 1** Properties of cAMP and cAMP derivatives for four cAMP receptor proteins

<table>
<thead>
<tr>
<th>Affinity, Kₑₐ (μM)</th>
<th>CSR D⁻</th>
<th>CAK I⁺</th>
<th>CAK II⁺</th>
<th>CAK D⁺</th>
</tr>
</thead>
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<tr>
<td>cAMP</td>
<td>0.014</td>
<td>0.028</td>
<td>0.4</td>
<td>0.15</td>
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<tr>
<td>(Sp)-cAMPS</td>
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<td>1.78</td>
<td>1.9</td>
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<td>Inactive</td>
<td>Inactive</td>
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<td>(Sp)-cAMPN(CH₃)₂</td>
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<td>2.24</td>
<td>7.0</td>
<td>3.2</td>
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<td>(Rp)-cAMPN(CH₃)₂</td>
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<td>100</td>
<td>Inactive</td>
<td>Inactive</td>
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<tr>
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<td>1.0</td>
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<td>Maximal response (cAMP = 100)</td>
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<td></td>
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<tr>
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<td>100</td>
<td>85</td>
<td>100</td>
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<td>&lt;5</td>
<td>&lt;5</td>
<td>52</td>
</tr>
<tr>
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<td>100</td>
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<tr>
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<td>&gt;50</td>
<td>&lt;5</td>
<td>&lt;5</td>
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<td>Antagonism Kᵢ (μM)</td>
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<td>3.7</td>
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<tr>
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<td>Classification</td>
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<td>Agonist</td>
<td>Part. agonist</td>
<td>Agonist</td>
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<td>Antagonist</td>
<td>Antagonist</td>
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<td>Agonist</td>
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<tr>
<td>(Sp)-cAMPN(CH₃)₂</td>
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<td>Part. agonist</td>
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<tr>
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<td>Agonist</td>
<td>Antagonist</td>
<td>Inactive</td>
</tr>
</tbody>
</table>

* Cell surface cAMP receptor for D. discoideum.
  * cAMP-dependent protein kinase.
partial agonist, (Rp)-cAMPN(CH₃)₂ is an antagonist, and to protein kinase type I from beef heart, which yields qualitatively identical slopes (Hill coefficient, n = 0.8). This strongly suggests that the compound (Rp)-cAMPN(CH₃)₂ is a competitive antagonist of cAMP for protein kinase type I. The data show (Table I) that (Sp)-cAMPN(CH₃)₂ is a full agonist of cAMP, (Sp)-cAMPS is a competitive antagonist, (Sp)-cAMPN(CH₃)₂ is a partial agonist, and (Rp)-cAMPS is a competitive full agonist.

The same experiments were done with cAMP-dependent protein kinase type I from beef heart, which yields qualitatively similar, but quantitatively different results (data not shown). The compounds cAMP, (Sp)-cAMPS, and (Sp)-cAMPN(CH₃)₂ activate the enzyme with apparent activation constants Kₐ = 28 nM for cAMP, Kₐ = 1.0 µM for (Sp)-cAMPS, and Kₐ = 2.2 µM for (Sp)-cAMPN(CH₃)₂. In contrast to protein kinase type II, the compound (Rp)-cAMPN(CH₃)₂ is also stimulatory in protein kinase type I with an estimated Kₐ = 1 nM. Eadie-Hofstee plots are nonlinear (conceivably a CAMP-dependent protein kinase has been isolated from D. discoideum (type D) (11, 16, 17). cAMP, (Sp)-cAMPS, and (Sp)-cAMPN(CH₃)₂ activate this enzyme (Fig. 4A). Eadie-Hofstee plots show that these compounds induce approximately the same maximal activation of the enzyme (Fig. 4B). The compound (Rp)-cAMPS is also stimulatory, but its maximal activation is only 52% of the maximum induced by cAMP. Eadie-Hofstee plots show that these compounds induce approximately the same maximal activation of the enzyme (Fig. 4B). The compound (Rp)-cAMPN(CH₃)₂ does not activate the enzyme up to 100 µM (Fig. 4A), and it also does not inhibit the stimulation by 0.5 µM cAMP (data not shown). It has been shown previously (18) that this compound competes very poorly with the binding of [3H]cAMP to the regulatory subunit of the enzyme. This suggests that (Rp)-cAMPN(CH₃)₂ is inactive.

The results show (Table I) that (Sp)-cAMPS and (Sp)-cAMPN(CH₃)₂ are full agonists of cAMP for protein kinase type D, (Sp)-cAMPS is a partial agonist, and (Rp)-cAMPN(CH₃)₂ is inactive.

**DISCUSSION**

In previous experiments on the interaction of cAMP derivatives with cAMP receptor proteins it was shown that essen-
ially all derivatives that bind to the receptors also activate these receptors (4, 5). Studies on the action of the derivatives with modified exocyclic oxygen atoms revealed that these compounds bind to the receptors, but that some of them do not fully activate the receptors (4–6). This suggests that binding of cAMP to the receptor and activation of the receptor are two distinct processes in a concerted reaction. Binding of cAMP to a receptor involves atoms or atom groups distributed all over the cAMP structure, whereas activation requires an additional interaction between the receptor protein and the phosphate moiety of cAMP (4, 5). At least three atomic interactions are possible between the receptor and the phosphate moiety: (i) a charge-charge interaction between a positively charged amino acid side chain and the negatively charged phosphate moiety; (ii) a polar interaction (hydrogen bond) between one or both of the exocyclic oxygen atoms of cAMP and the receptor protein; and (iii) a covalent bond between the phosphorus atom and the receptor protein (19). A charge-charge interaction is excluded by the results with the noncharged analog (Sp)-cAMPN(CH3)2.

The proposed activation mechanisms led us to hypothesize that an analog with a modified exocyclic oxygen atom may still bind to the receptor, but is no longer able to provide the activating interaction. Such a derivative would be the much sought after antagonist of cAMP acting specifically at cAMP receptor proteins.

In this study we have investigated the antagonistic activities of four derivatives in which one of the exocyclic oxygen atoms is replaced by either a sulfur atom or a dimethylamino atom group, (Sp)-cAMPS, (Rp)-cAMPS, (Sp)-cAMPN(CH3)2 and (Rp)-cAMPN(CH3)2. These analogs were tested with four cAMP-dependent proteins: the cell surface receptor on intact D. discoideum cells which binds and activates CAMP-dependent proteins; the cell surface receptor on intact D. discoideum cells which binds and activates CAMP-dependent proteins; the cell surface receptor on intact D. discoideum cells which binds and activates CAMP-dependent proteins; and the analog is the most potent activator of cAMP-dependent protein kinase from D. discoideum.

In the present work we show that (Sp)-CAMPS and (Sp)-CAMPN(CH3)2 are (mostly) full antagonists for the receptor proteins. (Rp)-cAMPS does not activate cell surface receptors, protein kinase type I and type II. Previously a small activation (about 10%) of type I and type II was observed (4, 6), which now appears to be due to impurities of (Sp)-cAMPS (about 2%) and cAMP (less than 0.1%). These impurities (as well as impurities in (Rp)-cAMPS, (S)-cAMPS, (S)-cAMPN(CH3)2 and (Rp)-cAMPN(CH3)2) were removed in the preparations used in the present study, and in previous reports on the cell surface cAMP receptor (4, 5).

In the present work we show that (Rp)-cAMPS is completely inactive and that it inhibits the stimulating effect of cAMP in a competitive way. The inhibition constants of (Rp)-cAMPS for the receptor proteins are similar to respective binding affinities of the analog for the receptors (4, 5, 8). Therefore, we conclude that (Rp)-cAMPS binds to the cAMP-binding sites, but that the analog is not able to activate the receptor; (Rp)-cAMPS is a competitive full antagonist of cAMP at cAMP-binding sites from the cell surface cAMP receptor, and protein kinase type I and type II. Interestingly, (Rp)-cAMPS partially activates protein kinase type D. This protein kinase from D. discoideum differs from mammalian protein kinases by molecular weight, subunit composition, and kinetic properties of CAMP-binding (11, 18). Nevertheless, the regulatory subunit can combine with the purified catalytic subunit from protein kinase type I or type II (21).

The analog (Rp)-cAMPN(CH3)2 is an antagonist for the cell surface cAMP receptor and for protein kinase type II. In contrast, the analog is an agonist for protein kinase type I, and inactive for protein kinase type D. We have not investigated the competitive nature of this antagonist, because the compound acts only at high concentrations.

Thus, we have shown that (Rp)-cAMPS is a competitive antagonist at micromolar concentrations, acting specifically at cAMP-binding sites. However, the compound cannot be used without any precautions. First, (Rp)-cAMPS does not antagonize all cAMP-dependent proteins. cAMP-dependent protein kinase from D. discoideum is activated up to 50% by the analog, and the analog is the most potent activator of β-galactosidase synthesis in Escherichia coli (22). Second, the analog binds to mammalian phosphodiesterase, but is not hydrolyzed by the enzyme (8). Therefore, phosphodiesterase is partially inhibited, which allows endogenous cAMP to accumulate, and to compete with (Rp)-cAMPS for binding to protein kinase. Despite these complicating properties, recent reports on cellular slime mold chemotaxis (20) and on glycogenolysis (25) show the usefulness of the compound.

Acknowledgment—We greatly acknowledge Theo Konijn for stimulating discussions.

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