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Intracellular adenosine 3′,5′-phosphate formation is essential for down-regulation of surface adenosine 3′,5′-phosphate receptors in *Dictyostelium*

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*Dictyostelium discoideum* cells contain cell surface cyclic AMP (cAMP) receptors that bind cAMP as a first messenger and intracellular cAMP receptors that bind cAMP as a second messenger. Prolonged incubation of *Dictyostelium* cells with cAMP induces a sequential process of phosphorylation, sequestration and down-regulation of the surface receptors. The role of intracellular cAMP in down-regulation of surface receptors was investigated. Down-regulation of receptors does not occur under conditions that specifically inhibit the formation of intracellular cAMP (the drug caffeine or mutant cells lacking adenylyl cyclase) or conditions that inhibit the function of intracellular cAMP (mutants lacking protein kinase A activity). Cell-permeable non-hydrolysable cAMP derivatives were used to investigate further the requirement of intracellular cAMP for down-regulation. The Sp isomer of 6-thioethylpurinriboside 3′,5′-phosphorothioate (6SEth-cPuMPS) does not bind to the surface receptor, enters the cell and has relative high affinity for protein kinase A. 6SEth-cPuMPS alone has no effect on down-regulation. However, together with an agonist of the surface receptor, the analogue induces down-regulation in caffeine-treated wild-type cells and in mutant cells lacking adenylyl cyclase, but not in mutant cells lacking protein kinase A. These results indicate that intracellular cAMP formation and activation of protein kinase A are essential for down-regulation of the surface cAMP receptor.

**INTRODUCTION**

Cyclic AMP (cAMP) plays an important role in different signal-transduction pathways in *Dictyostelium discoideum*. On starvation, a group of cells starts to secrete cAMP which is recognized by surrounding cells. The cells move towards the centre of the cAMP secretion. Eventually, all the cells that join the aggregate develop into a multicellular structure, the fruiting body. This fruiting body consists of viable spore cells on top of a stalk of dead cells [see Loomis (1985)].

*Dictyostelium* cells have several receptors that interact with cAMP: cell surface receptors (cARs) to detect extracellular cAMP as a first messenger, intracellular cAMP-dependent protein kinase (PKA) to detect intracellular cAMP as a second messenger, and extracellular and intracellular cyclic nucleotide phosphodiesterase to degrade cAMP (see Van Haastert et al., 1991). The genes encoding these proteins have been identified (Lacombe et al., 1986; Mutzel et al., 1987; Klein et al., 1988; Saxe et al., 1991). The derived amino acid sequences of cARs predict proteins with seven putative spanning segments and a cytosolic C-terminal domain, indicative of G-protein-coupled receptors (Klein et al., 1988). The deduced structure of the regulatory subunit of PKA is very different from that of cARs; it shows strong homology with mammalian regulatory subunits of PKA (Mutzel et al., 1987). Using analogues of cAMP it has been shown that these receptors possess very different cyclic nucleotide-binding specificities (De Wit et al., 1982; Van Haastert and Kien, 1983; Van Ments-Cohen and Van Haastert, 1989). Cyclic nucleotide derivatives that bind specifically to cARs or to intracellular PKA have been used to demonstrate the role of each of the receptors (Van Haastert and Kien, 1983; Van Haastert, 1983; Schaap and Van Driel, 1985; Theibert et al., 1986; Haribabu and Dottin, 1986; Oyama and Blumberg, 1986; Kimmel, 1987; Mann and Firtel, 1987).

During aggregation, extracellular cAMP activates multiple second-messenger enzymes, such as adenylyl cyclase, guanylyl cyclase and phospholipase C (see Van Haastert et al., 1991). The second messengers co-ordinate at least two cellular responses, chemotaxis and induction of postaggregative gene expression (see Schaap, 1986). Besides induction of second messengers and physiological responses, prolonged activation of the receptor also leads to desensitization of sensory transduction (Devreotes and Stcek, 1977; Klein and Juliani, 1977; Van Haastert and Van der Heijden, 1983). Main components are known to contribute to desensitization at the level of cAR1 (Klein and Juliani, 1977; Klein et al., 1985, 1987; Theibert and Devreotes, 1986; Van Haastert et al., 1992): (i) phosphorylation of cAR1, which has been suggested to inhibit receptor-G-protein interaction; (ii) functional sequestration of the cAR1 protein to a state in which it no longer binds cAMP (called loss of ligand binding); (iii) physical sequestration of the cAR1 protein into patches; (iv) degradation of the receptor (called down-regulation); (v) finally, rapid degradation of cAR1 mRNA. A detailed genetic and pharmacological study has revealed that each of these components requires partly overlapping but distinct second-messenger responses (Van Haastert et al., 1992). The role of intracellular cAMP in desensitization was investigated using caffeine and the mutant *synag* 7, situations in which receptor-stimulated adenylyl cyclase is inhibited (Brenner and Thoms, 1984; Theibert and Devreotes, 1983; Schaap et al., 1986). These experiments revealed that activation of adenylyl cyclase is not required for receptor phosphorylation, loss of ligand binding and loss of cAR1 mRNA. However, the final degradation of the cAR1 protein does not occur when activation of adenylyl cyclase is prevented (Van Haastert et al., 1992).

Another approach to the study of the function of intracellular cAMP is to use cAMP derivatives that bypass the surface receptor and directly activate the intracellular receptor. In

**Abbreviations used**: cAR, cell surface cAMP receptor; PKA, cAMP-dependent protein kinase; cAMP, adenosine 3′,5′-phosphate; cAMPS, adenosine 3′,5′-phosphorothioate; 6SEth-cPuMPS, 6-thioethylpurinriboside 3′,5′-phosphorothioate; dcAMPs, 2′-deoxygenosine 3′,5′-phosphorothioate. All cAMPS derivatives have the Sp conformation, unless indicated otherwise.
Dictyostelium these cAMP derivatives require the following properties: (1) resistance to hydrolysis by phosphodiesterase to prevent side effects of metabolites; (2) cell-permeability; (3) high affinity for PKA and low affinity for cARs. Previous studies on the activity of cAMP derivatives (De Wit et al., 1982; Van Haastert and Kien, 1983) were used to select putative candidates from a series of new cAMP analogues designed to activate mammalian PKA (Genieser et al., 1988; Dostman et al., 1990). Modifications in the adenine moiety provide selectivity for PKA. Lipophilic substitutions were chosen to increase cell permeability. Finally, replacement of an exocyclic oxygen by sulphur dramatically reduces hydrolysis by phosphodiesterase (Rossier et al., 1978; Van Haastert et al., 1983; Eckstein, 1985; Braumann et al., 1989), but also introduces an asymmetric phosphorus atom. The Sp isomer of adenosine 3′,5′-phosphorothioate (cAMPS) is an agonist of PKA in mammalian and Dictyostelium cells, whereas the Rp isomer is an antagonist of mammalian PKA (Rothermel et al., 1983) but, unfortunately, not of Dictyostelium PKA (De Wit et al., 1984). The analogue that best meets the criteria for a selective cell-permeable non-hydrolysable PKA agonist is the (Sp) isomer of 6-thioethylpurineraligoside 3′,5′-monophosphorothioate (6-SEth-cPuMPs) (Schaap et al., 1993).

Mutants were recently made that either cannot make intracellular cAMP because of inactivation of the adenylate cyclase gene (Pitt et al., 1992) or cannot respond to intracellular cAMP as a result of inactivation of the catalytic subunit of PKA (Mann et al., 1992). In the present study, down-regulation of surface receptors is analysed in these mutants, demonstrating that both cell lines lack cAMP-induced degradation of the receptor protein. The cell-permeable analogue that bypasses the surface receptor and directly activates intracellular PKA is shown to overcome the inhibition of cAMP-mediated down-regulation in cells lacking adenylate cyclase, but not in cells lacking PKA activity.

**EXPERIMENTAL**

**Materials**

[8-3H]cAMP (1.92 TBq/mmol) was obtained from Amersham Corp. (Amersham, Bucks., U.K.). cAMP was obtained from Boehringer (Mannheim, Germany). Dithiothreitol and (Sp) cAMPS were from Sigma. cAMP analogues were obtained from Biolog (Bremen, Germany) and kindly provided by Dr. Jastorff and Dr. Genieser; their synthesis has been described previously (Genieser et al., 1988).

**Conditions for growth and development**

*D. discoideum* mutant cells synag 7 (strain N7) were grown in association with *Esherichia coli* 281 on a solid medium containing 3.3 g of peptone, 3.3 g of glucose, 4.5 g of KH₂PO₄, 1.5 g of Na₂HPO₄·2H₂O and 15 g of agar/litre. Wild-type cells (strain AX3) and mutant cells *aca"* and *pk3"* were grown in liquid HG5 medium containing 14.3 g of peptone, 10 g of glucose, 7.15 g of yeast extract, 0.49 g of KH₂PO₄ and 1.36 g of Na₂HPO₄·2H₂O per litre. Control transformants *aca"/ACG* and *pk3"/PK3* were grown in HG5 medium supplemented with 10 μg/ml G418. Cells were harvested, washed and resuspended in 10 mM sodium/potassium phosphate buffer, pH 6.5 (PB buffer).

**Loss of ligand binding and down-regulation of surface receptors, analysed by Scatchard plots**

Cells were starved in suspension for 5 h; mutant cells synag 7, *aca"*, *aca"/ACG* and *pk3"* were pulsed with 0.1 μM cAMP at 5 min intervals. Cells were washed, resuspended in PB buffer, and incubated at a density of 10⁷ cells/ml in the absence or presence of 300 μM cAMP for 15 min or 1.5 h.

To assess loss of ligand binding of the receptor, cells were incubated with cAMP for 15 min at 22 °C. They were subsequently collected by centrifugation at 4 °C for 2 min at 700 g, washed twice by centrifugation with ice-cold PB buffer, and resuspended in ice-cold PB buffer. cAMP binding to exposed cARs was measured in a total volume of 100 μl of PB buffer containing different concentrations of [3H]cAMP, 10 mM dithiothreitol and 80 μl of the cell suspension (10⁵ cells, unless stated otherwise). The incubation at 0 °C was terminated after 1 min by centrifugation for 2 min at 14000 g and the supernatant was aspirated.

To assess down-regulation of the receptors, cells were incubated with cAMP for 1.5 h at 22 °C. They were subsequently collected, washed and resuspended in PB buffer as described above. Unless stated otherwise, the suspension was incubated for 20 min at 22 °C, and then placed on ice. cAMP binding to all residual cARs was measured in a total volume of 1 ml of PB buffer containing 78% (NH₄)₂SO₄, different concentrations of [3H]cAMP, 1 mM dithiothreitol, 50 μg of BSA and 80 μl of cell suspension. The incubation at 0 °C was terminated after 5 min by centrifugation for 2 min at 14000 g and the supernatant was aspirated.

The pellets from both assays were dissolved in 100 μl of 0.1 M acetic acid, 1.3 ml of Scintillator (Packard 299) was added and radioactivity was determined. Non-specific binding was measured in the presence of 0.1 mM cAMP. To demonstrate that cAMP was effectively removed during the washing procedure, cells were incubated on ice with cAMP for less than 3 s and washed immediately. Binding of cAMP in phosphate buffer and in (NH₄)₂SO₄ to these cells was compared with binding to cells that were not briefly incubated with cAMP; there was no significant difference in the binding data (Van Haastert, 1987).

Binding curves were fitted to a model of one or two independent binding sites by non-linear least-squares analysis using the program Pfit. The following equation for two independent binding sites was used (Thakur et al., 1972):

\[
B/F = 0.5 \times ((B_1 - B)/K_r + (B_2 - B)/K_2) + ((B_1 - B)/K_1 + (B_2 - B)/K_2)
\]

where \(B_1\) and \(B_2\) are the maximal binding for binding sites 1 and 2 respectively, and \(K_1\) and \(K_2\) are their dissociation constants.

Model discrimination between one and two binding sites was made by calculating:

\[
F = \frac{N - p^2}{p - 1} \frac{SS_{p - 1}}{SS_2}
\]

where \(N\) is the number of data points, \(p\) is the number of free parameters and SS is the residual sum of squares; subscripts 1 and 2 denote the smaller and larger model respectively. The degrees of freedom of \(F\) are \((p_1 - p_2, N - p_2)\). In all cases the two-site model fitted the data significantly better (at \(P < 0.01\)) than the one-site model; for *aca"/ACG* cells (Figure 2b) the two-site model was preferred at \(P < 0.05\).

**Down-regulation of surface receptors induced by cAMP analogues**

Insufficient quantities of the cAMPS analogues prevent analysis of their effects on cAMP receptors using complete Scatchard plots. Cells were starved as described above. The miniature incubations contained 5 mM caffeine, 300 μM cAMP and/or 100 μM cAMPS derivatives and 0.75 × 10⁷ cells in a total volume
from an sites. Cells were exposed to cAMP binding was measured immediately to control cells (C) and cAMP-treated cells (a), or after an additional 20 min incubation at 22 °C to cAMP-treated cells (>). The binding assays were performed in phosphate buffer (total volume 100 µl; a and b) or in nearly saturated (NH₄)₂SO₄ (total volume 1 ml; c and d). The incubation mixtures contained 2 × 10⁶ cells. The results shown are means of triplicate determinations from an experiment performed twice. See Table 1 for statistical analysis of the binding curves.

Figure 1 Scatchard analysis of cAMP binding in Dictyostelium cells

Wild-type cells were incubated with 0.3 nM cAMP for 15 min (a and c) or 1.5 h (b and d). Cells were washed extensively and resuspended at 0 °C. cAMP binding was measured immediately to control cells (C) and cAMP-treated cells (a), or after an additional 20 min incubation at 22 °C to cAMP-treated cells (>). The binding assays were performed in phosphate buffer (total volume 100 µl; a and b) or in nearly saturated (NH₄)₂SO₄ (total volume 1 ml; c and d). The incubation mixtures contained 2 × 10⁶ cells. The results shown are means of triplicate determinations from an experiment performed twice. See Table 1 for statistical analysis of the binding curves.

of 250 µl. The incubation times and washing procedures were as described above, except that smaller volumes were used. Finally, cells were resuspended in 400 µl of PB buffer, incubated for 20 min at 22 °C, and assayed for cAMP binding in (NH₄)₂SO₄ using 10 nM [³H]cAMP.

RESULTS

Optimal assay conditions for loss of ligand binding and down-regulation

Dictyostelium cells possess a heterogeneous population of surface cAMP-binding sites. In phosphate buffer, exposed binding sites are detected. When the binding assay is performed in nearly saturated (NH₄)₂SO₄, the sites have a 15–20-fold higher affinity. Scatchard analysis of cAMP binding in phosphate buffer reveals two binding forms with high (60 nM) and low (750 nM) affinity (Figure 1a; see Table 1 for analysis of Scatchard plots). Treatment of these cells with cAMP for 15 min leads to a large decrease in exposed binding sites of both the high- and low-affinity class; their affinities are not changed significantly (Figure 1a and Table 1). Apparently cAMP induces the loss of exposed binding sites. These binding sites are not degraded, as they are still detectable in (NH₄)₂SO₄ (Figure 1c and Table 1), but they have an approximately 3-fold reduced affinity compared with control binding in (NH₄)₂SO₄. When cells treated with cAMP for 15 min are incubated without cAMP for 20 min, binding in phosphate buffer is partly restored (Figure 1a), whereas the reduction in affinity in (NH₄)₂SO₄ is completely reversed (Figure 1c).

Treatment of cells for 1.5 h with cAMP leads to a large decrease in binding sites detected in phosphate buffer, which only partly recovers on removal of cAMP (Figure 1b). When these cells are assayed for cAMP binding in (NH₄)₂SO₄, a 2-fold reduction in affinity is observed and a 50% decrease in the number of binding sites. This reduction in affinity is restored within 20 min on removal of cAMP, but the loss of binding sites is persistent (Figure 1d). These results suggest that a short (15 min) incubation of cells with cAMP leads to loss of about 80% of exposed binding sites. These sites are still detectable in (NH₄)₂SO₄, but have a reduced affinity, which disappears within 20 min of removal of cAMP. A long (1.5 h) incubation of cells with cAMP also leads to loss of cAMP binding in (NH₄)₂SO₄. Previous experiments have shown that the loss of binding in (NH₄)₂SO₄ is correlated with the loss of receptor protein as detected on Western blots (Van Haastert et al., 1992). Thus loss of ligand binding is detected in phosphate buffer after treatment of cells with cAMP for 15 min, whereas down-regulation of the receptor is detected in (NH₄)₂SO₄ after treatment of the cells with cAMP for 1.5 h followed by a 20 min incubation of these cells without cAMP to restore the reduced affinity of the remaining receptors.

Inhibition of intracellular cAMP formation inhibits down-regulation

Caffeine is a relatively specific inhibitor of receptor-mediated activation of adenylate cyclase in Dictyostelium. This compound completely inhibits cAMP-induced down-regulation, but has no
Table 1 Analysis of binding curves

Data from the Scatchard plot were fitted for cAMP binding to one or two independent binding sites as described in the Experimental section; in all cases the two-binding site model was preferred. The results shown are the means and 95% confidence limits. The incubation mixtures contained 2 x 10^7 cells for AX3 and pk3+ and 10^6 cells for the other strains. For calculation of the number of binding sites per cell, note that the volume of the assay was 1 ml, except for binding in PB buffer, which was 100 μl.

<table>
<thead>
<tr>
<th>Site 1</th>
<th>Site 2</th>
<th>$B_{max}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_i$ (nM)</td>
<td>$B_i$ (nM)</td>
<td>$K_i$ (nM)</td>
</tr>
</tbody>
</table>

Table 1

<table>
<thead>
<tr>
<th>AX3/PB buffer</th>
<th>AX3/(NH₄)₂SO₄</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>756 ± 143</td>
</tr>
<tr>
<td>cAMP 15 min</td>
<td>869 ± 276</td>
</tr>
<tr>
<td>cAMP 15 min, buffer 20 min</td>
<td>857 ± 187</td>
</tr>
<tr>
<td>cAMP 1.5 h</td>
<td>1227 ± 182</td>
</tr>
<tr>
<td>cAMP 1.5 h, buffer 20 min</td>
<td>1186 ± 404</td>
</tr>
</tbody>
</table>

Figure 1

<table>
<thead>
<tr>
<th>ac&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Control</th>
<th>49 ± 3</th>
<th>5.7 ± 0.2</th>
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<tbody>
<tr>
<td>cAMP</td>
<td>199 ± 50</td>
<td>5.7 ± 1.4</td>
<td>10.2 ± 1.1</td>
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<tr>
<td>cAMP 15 min, buffer 20 min</td>
<td>58 ± 6</td>
<td>4.9 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>cAMP 1.5 h</td>
<td>92 ± 13</td>
<td>2.7 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>cAMP 1.5 h, buffer 20 min</td>
<td>57 ± 13</td>
<td>2.9 ± 0.5</td>
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Figure 2

<table>
<thead>
<tr>
<th>pk3&lt;sup&gt;+&lt;/sup&gt;</th>
<th>Control</th>
<th>67 ± 24</th>
<th>0.59 ± 0.17</th>
</tr>
</thead>
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<tr>
<td>cAMP</td>
<td>45 ± 10</td>
<td>0.81 ± 0.13</td>
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<tr>
<td>pk3&lt;sup&gt;+&lt;/sup&gt;/PK3</td>
<td>38 ± 4</td>
<td>2.43 ± 0.18</td>
<td></td>
</tr>
<tr>
<td>cAMP</td>
<td>39 ± 8</td>
<td>1.19 ± 0.16</td>
<td></td>
</tr>
</tbody>
</table>

Figure 3

<table>
<thead>
<tr>
<th>Site 1</th>
<th>Site 2</th>
<th>$B_{max}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_i$ (nM)</td>
<td>$B_i$ (nM)</td>
<td>$K_i$ (nM)</td>
</tr>
</tbody>
</table>

Effect on loss of ligand binding (Van Haastert et al., 1992; see also Table 2).

Recently the gene encoding the aggregation-stage-specific adenylate cyclase (ACA) has been cloned and a cell line generated in which the gene was inactivated [aca<sup>-</sup> cells (Pitt et al., 1992)]. Mutant aca<sup>-</sup> cells have relatively normal levels of cARs, provided that the cells are pulsed with cAMP during starvation. A short incubation of these cells with cAMP results in 76 ± 7% loss of exposed binding sites, which is not significantly different from control cells (Scatchard plots not shown). In contrast, prolonged incubation of aca<sup>-</sup> cells with elevated cAMP concentrations does not lead to a significant down-regulation of the surface cAMP receptor (Table 1 and Figure 2a). A second adenylate cyclase gene has been identified, ACG, that is expressed specifically in spores (Pitt et al., 1992). This gene has been introduced into the aca<sup>-</sup> cells under the control of the actin-15 promoter which is active during growth and aggregation resulting in cells with high constitutive adenylate cyclase activity (aca<sup>-</sup> /ACG cells; Pitt et al., 1992). These aca<sup>-</sup>/ACG cells were incubated for a short and long period with cAMP, and exposed and total binding sites were measured in phosphate buffer and (NH₄)₂SO₄ respectively. The results demonstrate that cAMP induces essentially normal loss of ligand binding (Scatchard plots not shown) and down-regulation of cAMP receptors (Table 1 and Figure 2b) in aca<sup>-</sup>/ACG cells compared with wild-type cells. It should be mentioned that both aca<sup>-</sup> and aca<sup>-</sup>/ACG cells have a much larger fraction of high-affinity binding sites than wild-type cells. The precise nature of high- and low-affinity binding forms is not known for wild-type Dictyostelium cells in vivo, although it has been shown that the different receptor gene products have different affinities, and GTP changes the affinity of cAR1 in membranes.

The gene encoding the catalytic subunit of PKA has been identified in Dictyostelium (Burki et al., 1991; Mann and Firtel, 1991). A pk3<sup>+</sup> cell line was constructed, in which this gene was inactivated by homologous recombination (Mann et al., 1992). These cells have a consistently 2-4-fold reduced level of cAMP-binding sites compared with control cells. cAMP induces the normal loss of exposed binding sites during a 15 min incubation (Scatchard plot not shown), but it does not induce down-regulation of the cAR in these cells (Table 1 and Figure 3a). Statistical analysis of the Scatchard plots reveals that cAMP treatment of pk3<sup>+</sup> cells leads to a shift of high- to low-affinity binding sites and to a reduction in their affinities, but not to a reduction in the total number of binding sites (see Table 1). Down-regulation was determined in a control cell line in which PK3 was reintroduced into the pk3<sup>-</sup> strain under the control of an actin promoter (pk3<sup>-</sup>/PK3 cells); down-regulation of surface receptors was restored to control values (Table 1 and Figure 3b).

Cell-permeable PKA agonist and down-regulation of surface receptors

Recently analogues of cAMP have been identified that are cell-permeable, resistant to hydrolysis by phosphodiesterase, have high affinity for intracellular PKA and do not bind to the cAR of Dictyostelium (Schaap et al., 1993). The most potent analogue is 6Eth-cPuMPS; the analogue dcAMPS is used as a control as this compound has a similar polarity to 6Eth-cPuMPS but...
cannot activate PKA. The experiments presented in Table 2 investigated whether these analogues can restore down-regulation under conditions in which synthesis or detection of intracellular cAMP is absent. Owing to the limited availability of the analogues, the subsequent binding reactions were performed not by Scatchard analysis but at one relatively high [3H]cAMP concentration.

In wild-type AX3 cells, 6SEth-cPuMPS alone does not induce down-regulation, and also does not interfere with cAMP-induced down-regulation (Table 2). In the presence of caffeine, 6SEth-cPuMPS together with cAMP induces substantial down-regulation of the surface receptor. 6SEth-cPuMPS cannot be replaced in this response by dcAMPS, a surface-selective agonist. A dose–response curve of 6SEth-cPuMPS-contributed down-regulation reveals a half-maximal effect at about 30 μM 6SEth-cPuMPS (Table 2).

The analogue 6SEth-cPuMPS partly restores cAMP-induced down-regulation in the mutant synag 7, which shows a defect in the activation of adenylate cyclase, and in the mutant acAR-, which has no adenylate cyclase activity. The analogue cannot restore down-regulation in mutant pk3−, which is defective in PKA (Table 2).

Summarizing, the experiments demonstrate that down-regulation of cARs does not occur under conditions that specifically

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**Figure 2** Scatchard plot of cAMP binding to surface receptors in cells lacking adenylate cyclase (a; acAR-) and acAR- cells expressing a different adenylate cyclase (b; acAR-/ACG)

Cells were starved for 5 h in the presence of cAMP pulses, washed and incubated in the absence (○) or presence (●) of 0.3 mM cAMP for 1.5 h. After extensive washing of the cells, cAMP binding to 107 cells was measured in nearly saturated (NH4)2SO4. The arrows indicate cAMP concentration. cAMP is absent. Down-regulation (Table 2). PKA activation of down-regulation, by the analogues, dose-response curve (Table 2).

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**Figure 3** Scatchard plot of cAMP binding to surface receptors in cells lacking the catalytic subunit of PKA (a; pk3−) and pk3− cells expressing PK3 from the actin promoter (b; pk3−/PK3)

Cells were starved for 5 h in the presence of cAMP pulses, washed and incubated in the absence (○) or presence (●) of 0.3 mM cAMP for 1.5 h. After extensive washing of the cells, cAMP binding was measured in nearly saturated (NH4)2SO4. The binding assay contained 2 × 107 and 107 cells in (a) and (b) respectively. The arrows indicate cAMP concentration used in the experiment presented in Table 2. The results shown are means of triplicate determinations from three independent experiments. See Table 1 for statistical analysis of the binding curves.

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**Table 2** Recovery of down-regulation by cell-permeable PKA agonist

<table>
<thead>
<tr>
<th>Strain</th>
<th>Conditions</th>
<th>Down-regulation (%)</th>
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<tbody>
<tr>
<td>AX3</td>
<td>−</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>cAMP</td>
<td>58 ± 4</td>
</tr>
<tr>
<td></td>
<td>100 μM 6SEth-cPuMPS</td>
<td>6 ± 6</td>
</tr>
<tr>
<td></td>
<td>cAMP + 100 μM 6SEth-cPuMPS</td>
<td>55 ± 6</td>
</tr>
<tr>
<td></td>
<td>Caffeine + cAMP</td>
<td>2 ± 7</td>
</tr>
<tr>
<td></td>
<td>Caffeine + cAMP + 10 μM 6SEth-cPuMPS</td>
<td>9 ± 5</td>
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<tr>
<td></td>
<td>Caffeine + cAMP + 30 μM 6SEth-cPuMPS</td>
<td>23 ± 6</td>
</tr>
<tr>
<td></td>
<td>Caffeine + cAMP + 100 μM 6SEth-cPuMPS</td>
<td>31 ± 4</td>
</tr>
<tr>
<td>synag 7</td>
<td>cAMP</td>
<td>3 ± 5</td>
</tr>
<tr>
<td></td>
<td>cAMP + 100 μM 6SEth-cPuMPS</td>
<td>32 ± 6</td>
</tr>
<tr>
<td>acAR−</td>
<td>cAMP</td>
<td>10 ± 8</td>
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<td>cAMP + 100 μM 6SEth-cPuMPS</td>
<td>30 ± 7</td>
</tr>
<tr>
<td>pk3−</td>
<td>cAMP</td>
<td>6 ± 4</td>
</tr>
<tr>
<td></td>
<td>cAMP + 100 μM 6SEth-cPuMPS</td>
<td>− 3 ± 9</td>
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inhibit the formation (caffeine, aca') or function (pk3') of intracellular cAMP. A cell-permeable cAMP analogue restores down-regulation when cAMP synthesis is defective, but not in PKA mutants with defective intracellular cAMP function.

**DISCUSSION**

Down-regulation of cARs is the final irreversible step in a complex desensitization process. The kinetics of these desensitization reactions as well as mutational analysis (Van Haastert et al., 1992) suggest a scheme as summarized in Figure 4. Binding of cAMP to receptor cAR1 leads to its activation, cAR1*. The activated receptor interacts with G-proteins, leading to stimulation of adenylate cyclase, guanylate cyclase and phospholipase C. Within a few minutes cAMP also induces phosphorylation of the receptor, cAR1p, presumably leading to uncoupling of the receptor from the G-proteins. Studies with mutant fgdA reveal that activation of these second messengers is not required for receptor phosphorylation. On a similar time scale as phosphorylation, the receptor becomes functionally sequestered such that it no longer binds cAMP (loss of ligand binding, cAR1p). Loss of ligand binding is induced by the analogue (Rp)-cAMPS, which does not induce activation or phosphorylation of the receptor. Finally, after 1 h of stimulation the receptor becomes undetectable and is degraded (down-regulation, cAR1d).

In this study the role of intracellular cAMP in loss of ligand binding and down-regulation of the receptor was studied in detail. The optimum protocol for measuring loss of ligand binding is incubation of cells for 15 min with cAMP followed by removal of cAMP and detection of surface cAMP-binding activity in phosphate buffer. The results show that about 75% of the receptors have lost their binding activity after this treatment. Intracellular cAMP appears not to play an important role in this process, as loss of ligand binding is essentially normal in mutant cells that lack adenylate cyclase or the catalytic subunit of PKA.

The receptors that do not bind cAMP in phosphate buffer still bind cAMP in nearly saturated (NH4)2SO4 (Van Haastert, 1985). Thus receptors are not degraded after 15 min of incubation with cAMP. These receptors, however, have changed their affinity for cAMP (Figure 1c). This change in affinity is reversible on removal of cAMP with a half-time of several minutes (Figure 1). As mentioned above, incubation of cells with cAMP for 15 min leads to phosphorylation of the receptor and loss of ligand binding in phosphate buffer. The following experiments suggest that the reduced affinity in (NH4)2SO4 is related to phosphorylation of the receptor rather than loss of ligand binding. The analogue (Rp)-cAMPS induces loss of ligand binding but not the phosphorylation of the receptor; cells treated with (Rp)-cAMPS have receptors with a normal binding affinity in (NH4)2SO4 (Van Haastert et al., 1992). Secondly, the affinity of the receptor is restored to control values after a 20 min incubation at 22 °C (Figure 1); dephosphorylation of cAR1 occurs with similar characteristics (Klein et al., 1987), whereas loss of ligand binding reverts much more slowly at 22 °C (Van Haastert, 1987). The decrease in binding affinity hinders the rapid detection of the number of binding sites, as complete Scatchard analysis should be carried out for each experiment. The affinity of the receptor is restored relatively rapidly at 22 °C (20 min), whereas de novo synthesis of the receptor is slow [several hours (Van Haastert et al., 1992)]. Therefore the optimum protocol for measuring down-regulation is incubation of the cells with cAMP for 1.5 h, removal of cAMP and subsequent incubation for 20 min at 22 °C, followed by the binding assay in (NH4)2SO4. With this protocol, cAMP induces about 50% down-regulation of cARs. Intracellular cAMP appears to play an essential role in this process.

Previous experiments with the drug caffeine and the mutant

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**Figure 4 Role of intracellular cAMP in down-regulation of cAR**

Binding of cAMP to cARs leads to their activation (cAR1*), phosphorylation (cAR1p) and degradation (cAR1d). Conditions and mutants that inhibit specific signal-transduction components are boxed. The analogue 6SEth-cPuMPS does not bind to the cAR but can pass through the plasma membrane and activate PKA directly. Besides activation of adenylate cyclase and PKA, the results suggest additional requirements for down-regulation.
mediated down-regulation of receptors. Two observations. The derivative reaches an intracellular concentration that is sufficient to activate intracellular PKA at an applied extracellular concentration that does not activate surface receptors. The present results demonstrate that cAMP does not induce down-regulation in caffeine-treated wild-type cells, in mutant ack lacking adenylate cyclase or in mutant pk3 lacking the catalytic subunit of PKA. Down-regulation in these mutant cells is restored by expression, under the control of an actin promoter, another adenylate cyclase gene (ack/ACG) or PK3 respectively. The cell-permeable PKA agonist together with cAMP restores down-regulation of the surface receptor in wild-type cells treated with caffeine or in ack cells, but not in pk3 cells. These results imply that intracellular cAMP accumulation and activation of PKA are essential for down-regulation of surface receptors.

Although intracellular cAMP is essential for down-regulation of cARs, the present study indicates that this is not sufficient. The cell-permeable PKA agonist alone does not induce down-regulation, but must be added in combination with extracellular cAMP, suggesting two requirements for down-regulation: activation of adenylate cyclase and activation or modification of cAR. Two hypotheses could provide an explanation for these observations. Down-regulation may only occur after the receptor has been modified by either ligand-dependent phosphorylation or loss of lipid binding. As the PKA agonist does not bind to the surface receptor, it does not provide this requirement. The second hypothesis proposes the requirement of other second-messenger responses besides adenylate cyclase activation for down-regulation, i.e. activation of phospholipase C or guanylate cyclase. Additional experiments with mutants lacking other signal-transduction pathways are required to distinguish between these models.

The receptor cAR1 remains down-regulated as long as extracellular cAMP is present. The question then arises which receptor detects cAMP and how are the second-messenger systems activated in down-regulated cells? Detailed studies on the regulation of adenylate cyclase in mutant cells indicate that cAR1 is not essential for activation of this enzyme (Pupillo et al., 1992). Thus another surface receptor may transduce the signal to adenylate cyclase to keep cAR1 in the down-regulated state.

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