Ligand-induced Modification of a Surface cAMP Receptor of Dictyostelium discoideum Does Not Require Its Occupancy*

B. Ewa Snaar-Jagalska, Peter N. Devreotes, and Peter J. M. Van Haastert

From the Cell Biology and Genetics Unit, Zoological Laboratory, University of Leiden, Kaiserstraat 63, P. O. Box 9516, 2300 RA Leiden, The Netherlands and the § Department of Biological Chemistry, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

In Dictyostelium discoideum amoebae, cAMP-induced phosphorylation of the surface cAMP receptor is associated with a discrete transition in its electrophoretic mobility. The native and modified forms of the receptor are designated R and D (Mr = 40,000 and 43,000). The relationship of the number of receptors which are modified as a function of the receptors which bind cAMP was investigated. Modification was assessed by determining the amounts of R and D forms in Western blots which detect all receptors whether or not they are exposed on the surface. Cyclic AMP or the analog, adenosine 3',5'-monophosphorothioate (R,R)-cAMPS, induced a loss of cAMP-binding activity (down-regulation), which was not accompanied by a loss of the receptor protein.

About 60% of the receptors do not bind cAMP in the absence of Ca2+ and are unmasked by 10 mM Ca2+. However, the fraction of receptors which are modified in response to cAMP is equal in the presence or absence of Ca2+.

(R,R)-cAMPS induces down-regulation (50%) but no modification. Addition of cAMP, following down-regulation by (R,R)-cAMPS, causes all receptors to be modified.

cAMP induces both down-regulation (80%) and modification. Modification is more readily reversed than down-regulation; 30 min after removal of cAMP, receptors remain down-regulated (57%) but are found in the R form. All receptors shift to the D form when cAMP is readded to the cells.

These results indicate that exposed, as well as cryptic and down-regulated receptors, are modified in response to the cAMP stimulus.

In Dictyostelium discoideum extracellular cAMP functions as a signal molecule during chemotaxis (1), morphogenesis (2), and cell differentiation (3). cAMP binding by highly specific surface receptors (4) results in several intracellular responses such as the activation of guanylate and adenylate cyclase (5, 6). Cellular cGMP levels peak at 10 s after stimulation, and cAMP levels reach a maximal concentration after about 1 min (7, 8). The stimulation of guanylate and adenylate cyclase terminates within a few seconds and a few minutes, respectively, even when the stimulus remains present at constant levels (9-11). Ligand-induced desensitization of adenylate cyclase is composed of two components: down-regulation and adaptation. Down-regulation is any cAMP-induced re-

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1 The abbreviations used are: 8-N3-cAMP, 8-azidoadenosine 3',5'-cyclic monophosphate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DTT, dithiothreitol; Mes, 4-morpholinoethanesulfonic acid; (R,R)-cAMPS, adenosine 3',5'-monophosphorothioate, R,R isomer.

Modification of the cAMP receptor is defined operationally as a cAMP-induced reversible alteration of the electrophoretic mobility of a polypeptide from Mr = 40,000 to 43,000. This protein is identified by photoaffinity labeling of cells with 8-N3-cAMP (19-21), by phosphorylation of cells with 32P0, (22), or by a polyclonal antibody directed against the purified protein (23).
diaminobenzidine tetrahydrochloride was done as indicated by Straus and 0.05% bisacrylamide (20). Proteins were transferred to nitrocellulose (36). Western blots were done essentially as described (37).

RESULTS

To study the relationship between the number of CAMP receptors which can bind CAMP and the number of receptors which undergo ligand-induced modification, we chose special experimental conditions to alter the number of exposed binding sites. The combined data of [3H]cAMP binding to the cells, under these different conditions are presented in Fig. 1 as a Scatchard plot (14, 39). Ca2+ induces a 2.5-fold increase of the total number of binding sites as previously reported (8, 39). The exposure of the cells to 1 μM CAMP for 15 min results in a 80% loss of the higher affinity binding sites. The number of binding sites is decreased about 50% in cells down-regulated with the CAMP derivative (R)-cAMPS (Fig. 1).

cAMP Binding and Modification of the Receptor in the Presence of Ca2+—Cells were resuspended in Mes/NaOH buffer to a density of 106 in the absence or presence of 10 mM Ca2+. Samples of control and Ca2+-treated cells were used after about 10 min for the detection of CAMP binding and receptor modification (Fig. 2). Binding of CAMP in the presence of Ca2+ was increased to 260%. The incubation of cells with 0.5 μM CAMP and 10 mM DTT induced a transition from the R (Mr = 40,000) to the D (Mr = 43,000) form of the receptor.

Prior to CAMP stimulation, more than 90% of the receptors were in the R form, both in the absence and presence of Ca2+ (lanes 1 and 3). The amount of receptor in the R form is reduced to about 20% after 15 min of stimulation with CAMP (lanes 2 and 4). (In some experiments, a quantitative increase in the D form was not apparent because actin partly overlaps this protein and induces a negative stain.) The results suggest that a fraction of the receptors are cryptic and can be exposed by Ca2+. The cryptic receptors, which were not able to bind CAMP, were detectable by staining with specific antiserum to

FIG. 1. Effect of Ca2+ and down-regulation on the Scatchard plots of CAMP-binding sites. Control cells (A), cells in the presence of 10 mM Ca2+ (B), and down-regulated cells in 1 mM CAMP (○) were incubated with different concentrations of [3H]cAMP. After an incubation period of 45 s cells were centrifuged through silicone oil. Insufficient amounts of highly purified (R)-cAMPS were available to perform a complete Scatchard analysis. [3H]cAMP binding in the cells down-regulated by 100 μM (R)-cAMPS (△) was measured with two different [3H]cAMP concentrations (10 and 1000 nM).

EXPERIMENTAL PROCEDURES

Materials—[2,8-3H]cAMP (1.5 TBq/mmol) was obtained from Amer sham Corp.; cAMP and Mes were from Boehringer Mannheim. DTT and 3,3'-diaminobenzidine tetrahydrochloride, grade II, were purchased from Sigma. Cyclic nucleotide phosphodiesterase from D. discoideum was isolated as described (10). (R)-cAMPS was a generous gift of Drs. Jastorf (University of Bremen), Baramiak, and Stoc (Polish Academy of Sciences, Lodz), (32). Traces of cAMP in (R)-cAMPS were removed by degradation with cyclic nucleotide phosphodiesterase as described (33). Peroxidase-conjugated swine immunoglobulins to rabbit immunoglobulins were from Dakopatts.

Culture Conditions—D. discoideum cells (NC-4(H)) were grown as described (10), harvested in the late logarithmic phase with 10 mM KH2PO4/Na2HPO4, pH 6.5 (PB buffer), washed, and starved in suspension in PB buffer at a density of 106 cells/ml and used for all experiments.

Down-regulation—D. discoideum cells were incubated for 15 min at 20°C with 100 μM (R)-cAMPS or 1 μM cAMP and 10 mM DTT. During the experiment, the cell suspension was aerated at a flow rate of about 15 ml of air/ml of suspension. Cells were washed three times with ice-cold Mes and resuspended at 0°C to the original volume of Mes.

Assay for CAMP Binding—CAMP binding was measured at 0°C in a total volume of 100 μl containing 10 nM [3H]cAMP with 1 μM cAMP, 10 μM DTT, and 80 μl of the cell suspension. DTT, an inhibitor of cyclic nucleotide phosphodiesterase, was used to protect extracellular cAMP (34). Cell-associated [3H]cAMP was determined by centrifugation of the cells through a layer of silicone oil. Blank values were determined in the presence of 0.1 mM unlabeled cAMP. Typical binding data for control cells at 1 μM cAMP are: input = 43,158 cpm, specific binding = 403 ± 47 cpm, and nonspecific binding = 352 ± 27 cpm (both n = 3).

Modification of the Receptor—The procedure which induced receptor modification was identical for all preparations of cells (control, incubated with Ca2+, down-regulated). Cells (100 μl of 106 cells/ml) were incubated for 15 min by shaking at 20°C with 0.5 μM cAMP and 10 μM DTT. Incubation was terminated by addition of 1 ml of ice-cold 56% saturated ammonium sulfate. After 5 min of incubation at 0°C, cells were centrifuged at 4°C for 10 min at 10,000 χ g. Pellets were resuspended in 1 ml of receptor buffer (19), shaken, and centrifuged at 4°C for 15 min at 10,000 χ g. Pellets were resuspended in 100 μl of sample buffer (19), and shaken for 30 min at 2°C. 20-50 μl of samples were analyzed by SDS-PAGE (35) using 10% acrylamide and 0.05% bisacrylamide (20). Proteins were transferred to nitrocellulose (36). Western blots were done essentially as described (37). Primary antibody was diluted 1:500. Secondary peroxidase-labeled swine anti-rabbit immunoglobulin antibody was diluted 1:3,000. Primary and secondary antibody were incubated with blots for 1 h at 20°C by gentle shaking. Staining for peroxidase activity with 3,3'-diaminobenzidine tetrahydrochloride was done as indicated by Straus (38).
the surface cAMP receptor (lane 1 = lane 3). Both cryptic and exposed receptors display the cAMP-dependent receptor modification.

Down-regulation and Modification of the Receptor Induced by (Rp)-cAMP—Previously it has been shown that (Rp)-cAMP binds to cell surface cAMP receptors and effectively induces down-regulation of the receptor. However, this analog does not induce the activation or adaptation of adenylate cyclase (16). The incubation of cells with 100 μM (Rp)-cAMP leads to a 50% reduction of the cAMP-binding activity (Fig. 1). The electrophoretic mobility of the receptor, as observed on Western blots (Fig. 3), was not altered (lane 3) if compared to unstimulated control cells (lane 1).

The cells, in which the receptors had been down-regulated by (Rp)-cAMP, were stimulated with 0.5 μM cAMP and 10 mM DTT (lane 4). The R form of the receptor was reduced to the same extent in (Rp)-cAMP down-regulated cells (lane 4) as in control cells (lane 2). The D form appeared concomitant with the loss of the R form.

These results indicate that (Rp)-cAMP induces down-regulation of receptors without inducing receptor modification. In addition, the receptors which have been down-regulated by (Rp)-cAMP still undergo cAMP-induced modification.
TABLE I

Analysis of \(^{32}\)PiCAMP that is associated with down-regulated cells

<table>
<thead>
<tr>
<th>Binding in</th>
<th>Tightly bound (^{32})Pi CAMP in</th>
<th>Phosphate sulfate</th>
<th>Ammonium sulfate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells, control</td>
<td></td>
<td>0.51</td>
<td>1.20</td>
</tr>
<tr>
<td>Cells, down-regulated</td>
<td></td>
<td>0.15</td>
<td>1.15</td>
</tr>
<tr>
<td>Supernatant, control</td>
<td>ND*</td>
<td>0.24</td>
<td>0.24</td>
</tr>
<tr>
<td>Pellet, control</td>
<td>0.55</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>Supernatant, down-regulated</td>
<td>ND</td>
<td>0.22</td>
<td>ND</td>
</tr>
<tr>
<td>Pellet, down-regulated</td>
<td>0.20</td>
<td>0.95</td>
<td>0.02</td>
</tr>
</tbody>
</table>

*ND, not determined.

Down-regulation is not bound to down-regulated receptors. First, while down-regulated receptors are exposed in saturated ammonium sulfate, ammonium sulfate does not release the tightly bound radioactivity (Table I). Second, when down-regulated cells are lysed, essentially all radioactivity appears in a soluble fraction of the lysate. No receptors are observed in the soluble fraction by Western blot analysis. Furthermore, membranes from down-regulated cells, however, still show reduced CAMP-binding activity and all binding activity is recovered in ammonium sulfate.

DISCUSSION

Prolonged stimulation of D. discoideum cells with a constant CAMP concentration induces desensitization by at least two mechanisms: down-regulation defined as a loss of CAMP binding sites and adaptation. The kinetics and CAMP dose dependence of adaptation are closely correlated with the kinetics and CAMP dose dependence of a reversible modification of the receptor (20). Extracellular CAMP induces the transition from M, = 40,000 to 43,000 in the electrophoretic mobility of a polypeptide identified by photoaffinity labeling with \(^32\)Pi-8-N-cAMP as the CAMP receptor of D. discoideum (18). This modification is most likely due to the phosphorylation of the receptor, since the receptor or subunit of it was co-purified with \(^32\)P incorporation by photoaffinity labeling or by in vitro labeling with \(^32\)P (22, 23).

Phosphorylation of the \(\beta\)-adrenergic receptor is thought to play a role in desensitization (25–29). A novel CAMP-independent kinase, which preferentially phosphorylates the agonist-occupied form of the \(\beta\)-adrenergic receptor in vitro has been described. We report here that in D. discoideum in vivo, ligand-induced receptor modification is not restricted to the agonist occupied receptor. The strategy was to prepare cells with a variable number of exposed CAMP receptors and examine the relationship between the number of exposed versus modified receptors.

About 60% of the surface CAMP receptors are cryptic: they do not bind CAMP, but are exposed by Ca\(^{2+}\) (30, 39). The present results indicate that both exposed and cryptic receptors are modified in response to CAMP (Fig. 2). This suggests that unoccupied receptors can be modified. However, the dynamics of the exchange between exposed and cryptic population of receptors is not known. Therefore, it cannot be excluded that cryptic receptors have been exposed temporarily during the 15-min incubation with CAMP.

The incubation of cells with CAMP induces both down-regulation and modification of receptors. The differences in the reversibility after removal of CAMP make it possible to discriminate between these processes. The reversibility of receptor modification shows a \(t_{1/2} = 5\) min (20), which is 10 times faster than the reversibility of receptor down-regulation (14). Thus at 15–30 min after removal of CAMP, the receptors remain down-regulated but no longer modified. Exposure to a new stimulus at this time induced the modification of all receptors. This suggests that down-regulated receptors, which do not bind the new stimulus, nevertheless become modified in response to it. The same conclusion was reached by using a derivative of CAMP (R\(_2\)) which binds to surface receptors, induces down-regulation (16), but not the modification of the receptor from the R to the D form. Receptors which have been down-regulated by (R\(_2\)) are modified after addition of CAMP. A role of CAMP-dependent protein kinase in down-regulation as well as modification of the receptor is unlikely, because the cyclic nucleotide specificities of both processes is similar to that of the surface receptor, but distinct from the specificity of protein kinase (16, 19).

There exists ample evidence that modification of the receptor is mediated by its phosphorylation (21–24). This would imply that the receptor kinase phosphorylates down-regulated receptors. Since down-regulated receptors by definition do not detect a new CAMP stimulus, this indicates that the kinase is activated (or a phosphatase inhibited) or translocated by the new CAMP stimulus. The results of Table I suggest that down-regulated receptors are not occupied with CAMP, indicating that occupied as well as unoccupied receptors are substrates of the Kinase.

\(R_1\) cAMPs induces down-regulation of the receptor without modifying its apparent molecular weight, suggesting that the modification of the receptor is not a prerequisite for down-regulation. The receptor possesses multiple phosphorylation sites, and both the lower and higher mobility form of the receptor are phosphorylated. Therefore it cannot be excluded that down-regulation of the receptor is mediated by the phosphorylation of a site which does not alter the mobility of the receptor in gel electrophoresis.

The main finding of the present study is that the apparent electrophoretic mobility of down-regulated receptors decreases after CAMP stimulation. Down-regulated receptors do not bind CAMP and are unable to activate adenylate cyclase (14). However, they are apparently not completely removed from the system. The observation that down-regulated receptors respond to CAMP evoke the hypothesis that down-regulated receptors may actively participate in transmembrane signal transduction.

REFERENCES

Occupancy and Modification of Dictyostelium cAMP Receptors