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

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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 (M_r = 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_p)-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 Ca^{2+} and are unmasked by 10 mM Ca^{2+}. However, the fraction of receptors which are modified in response to cAMP is equal in the absence or presence of Ca^{2+}.

(R_p)-cAMPS induces down-regulation (50%) but not modification. Addition of cAMP, following down-regulation by (R_p)-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 reduction of the number of detectable cAMP-binding sites (12–16). Adaptation is a form of desensitization by which cells lose responsiveness to constant stimuli, but remain responsive to further stimulus increments (17). Half-maximal effects for down-regulation were observed at 50 nM cAMP and for adaptation at 5 nM cAMP (15, 16). Adaptation and down-regulation occur at a similar time scale of about 1–3 min (15–17). Adaptation is reversible at 20 °C with a half-time of 5 min (17), while down-regulation is reversible with a half-time of 1 h (12–14). Down-regulated receptors are not degraded, but merely unable to bind cAMP, because all binding sites remain detectable in saturated ammonium sulfate (15).

A doublet (M_r = 40,000 and 43,000) has been identified by photoaffinity labeling with [^{32}P]8-N_3-cAMP[1] as the cAMP receptor of *D. discoideum* (18–21). Adaptation of adenylate cyclase has been correlated with receptor modification. Extracellular cAMP induces a reversible modification from a form designated R (M_r = 40,000) to one designated D (M_r = 43,000). The D form has been purified to homogeneity by hydroxylapatite chromatography followed by preparative SDS-PAGE (22). The purification, monitored as ^{32}P incorporation by photoaffinity labeling or by *in vivo* labeling with $^{32}P_i$, suggests that the receptor modification is associated with phosphorylation[2] (23, 24). A monospecific, polyclonal antiserum to the receptor has been developed (23). The antibody detects both the R and D mobility forms of the receptor with similar affinity, as well as down-regulated receptors.

In many eucaryotic systems, hormonal stimulation results in the phosphorylation of the hormone receptor (25–29). Phosphorylation of the β-adrenergic receptor is correlated with desensitization of adenylate cyclase due to receptor-effector uncoupling (28). Recently, a novel cAMP-independent β-adrenergic receptor kinase, which phosphorylates preferentially the agonist-occupied form of the receptor was identified and partially purified from Kin^- cells, a mutant of S49 lymphoma cells that lacks a functional cAMP-dependent protein kinase (28, 29). This may suggest that only the agonist-occupied receptors are phosphorylated *in vivo* and provide a molecular basis for homologous desensitization. Furthermore, the preferential phosphorylation of agonist-occu-

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[1] The abbreviations used are: 8-N_3-cAMP, 8-azidoadenosine 3',5'-cyclic monophosphate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DTT, dithiothreitol; Mes, 4-morpholinoethanesulfonic acid; (R_p)-cAMPS, adenosine 3',5'-monophosphorothioate, R_p isomer.

[2] Modification of the cAMP-receptor is defined operationally as a cAMP-induced reversible alteration of the electrophoretic mobility of a polypeptide from M_r = 40,000 to 43,000. This protein is identified by photoaffinity labeling of cells with 8-N_3-cAMP (19–21), by phosphorylation of cells with $^{32}PO_4$ (22), or by a polyclonal antibody directed against the purified protein (23).

pied receptors omits the requirement for a receptor-mediated activation of a kinase.

In this report we ask whether only the agonist-occupied form of the surface cAMP receptor from *D. discoideum* can be phosphorylated *in vivo*. All receptor proteins were detected, even if they did not bind cAMP, with the receptor-specific serum raised against the purified receptor (23). Modification of the receptor was monitored as the transition in the electrophoretic mobility of the receptor. The number of receptors which bind cAMP was increased to 260% by the addition of 10 mM Ca^{2+} (30), or decreased to 57% by down-regulation with a derivative of cAMP which does not induce other cellular responses (16, 31), including receptor modification. The results show that both exposed and down-regulated receptors are modified after cAMP addition. Since down-regulated receptors are not occupied with cAMP this indicates that both occupied and unoccupied receptors can be modified. Therefore, it is likely that a kinase must be activated or a phosphatase inhibited by the agonist occupation of the receptor.

EXPERIMENTAL PROCEDURES

Materials—[2,8-^{3}H]cAMP (1.5 TBq/mmol was obtained from Amersham 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_p)-cAMPS was a generous gift of Drs. Jastorff (University of Bremen), Baraniak, and Stec (Polish Academy of Sciences, Lodz), (32). Traces of cAMP in (R_p)-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 KH_2PO_4/Na_2HPO_4, pH 6.5 (PB buffer), washed, and starved in suspension in PB buffer at a density of 10^7 cells/ml. After 4–5 h, cells were washed twice in PB buffer, one time with 15 mM Mes/NaOH, pH 6.5, and the final pellet was resuspended in Mes to a density of 10^8 cells/ml and used for all experiments.

Down-regulation—*D. discoideum* cells were incubated for 15 min at 20 °C with 100 μM (R_p)-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 [^{3}H]cAMP with 1 μM cAMP, 10 mM DTT, and 80 μl of the cell suspension. DTT, an inhibitor of cyclic nucleotide phosphodiesterase, was used to protect extracellular cAMP (34). Cell-associated [^{3}H]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 = 252 ± 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 Ca^{2+}, down-regulated). Cells (100 μl of 10^8 cells/ml) were incubated for 15 min by shaking at 20 °C with 0.5 μM cAMP and 10 mM DTT. Incubation was terminated by addition of 1 ml of ice-cold 95% saturated ammonium sulfate. After 5 min of incubation at 0 °C, cells were centrifuged at 4 °C for 10 min at 10,000 × *g*. Pellets were resuspended in 1 ml of receptor buffer (19), shaken, and centrifuged at 4 °C for 15 min at 10,000 × *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 gently shaking. Staining for peroxidase activity with 3,3'-diaminobenzidine tetrahydrochloride was done as indicated by Straus (38).

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 [^{3}H]cAMP binding to the cells, under these different conditions are presented in Fig. 1 as a Scatchard plot (14, 39). Ca^{2+} induces a 2.5-fold increase of the total number of binding sites as previously reported (30, 39). The exposure of the cells to 1 μM cAMP for 15 min results in a 80% loss of the binding sites (12–16). The number of binding sites is decreased about 50% in cells down-regulated with the cAMP derivative (R_p)-cAMPS (Fig. 1).

cAMP Binding and Modification of the Receptor in the Presence of Ca^{2+}—Cells were resuspended in Mes/NaOH buffer to a density of 10^8 in the absence or presence of 10 mM Ca^{2+}. Samples of control and Ca^{2+}-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 Ca^{2+} was increased to 260%. The incubation of cells with 0.5 μM cAMP and 10 mM DTT induced a transition from the R (M_r = 40,000) to the D (M_r = 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 Ca^{2+} (*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 Ca^{2+}. The cryptic receptors, which were not able to bind cAMP, were detectable by staining with specific antiserum to

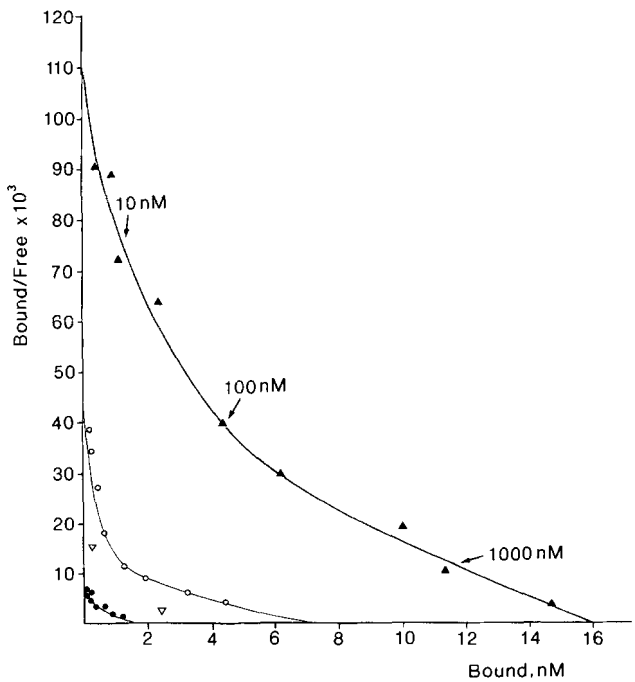

FIG. 1. **Effect of Ca^{2+} and down-regulation on the Scatchard plots of cAMP-binding sites.** Control cells (○), cells in the presence of 10 mM Ca^{2+} (▲), and down-regulated cells in 1 mM cAMP (●) were incubated with different concentrations of [^{3}H]cAMP. After an incubation period of 45 s cells were centrifuged through silicone oil. Insufficient amounts of highly purified (R_p)-cAMPS were available to perform a complete Scatchard analysis. [^{3}H]cAMP binding in the cells down-regulated by 100 μM (R_p)-cAMPS (▽) was measured with two different [^{3}H]cAMP concentrations (10 and 1000 nM).

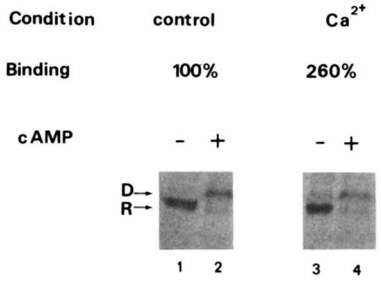

FIG. 2. **Effect of Ca^{2+} on receptor occupancy and receptor modification.** Cells were incubated in the absence (control) or presence of 10 mM $CaCl_2$ for 10 min. Receptor modification was induced by a 15-min incubation with 0.5 μM cAMP + DTT; proteins were separated by SDS-PAGE and Western blot analysis with an antiserum directed against the purified receptor. *Lanes 1* and *2* are samples from control cells; *lanes 3* and *4* from cells incubated with 10 mM Ca^{2+}. *Lanes 1* and *3* are from unstimulated cells (R, unmodified form of the receptor); *lanes 2* and *4* are from cells stimulated with cAMP + DTT (D, modified form). [^{3}H]cAMP binding was determined as described under "Experimental Procedures." 100 ± 13 represents binding in control cells, and 260 ± 11 binding in the presence of Ca^{2+}.

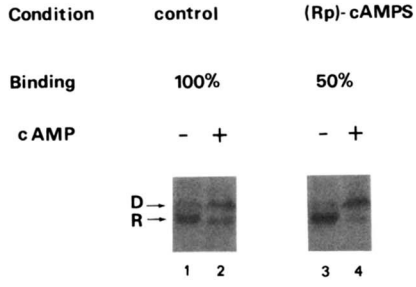

FIG. 3. **Effect of (R_p)-cAMPS on down-regulation and receptor modification.** cAMP receptors were down-regulated by incubating cells for 15 min with 100 μM (R_p)-cAMPS. Control cells were not exposed to (R_p)-cAMPS. Cells were washed with ice-cold Mes, and incubated for 15 min with 0.5 μM cAMP + DTT. *Lanes 1* and *2*, control cells; *lanes 3* and *4*, cells with down-regulated receptors; *lanes 1* and *3*, cells immediately after washing in Mes buffer; *lanes 2* and *4*, cells after a subsequent incubation with 0.5 μM cAMP + DTT. cAMP binding was measured immediately after washing of cells with Mes buffer. Cells with down-regulated receptors by (R_p)-cAMPS show 50% ± 8 of [^{3}H]cAMP binding.

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 (R_p)-cAMPS—Previously it has been shown that (R_p)-cAMPS 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 (R_p)-cAMPS 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 (R_p)-cAMPS, 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 (R_p)-cAMPS 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 (R_p)-cAMPS induces down-regulation of receptors without inducing receptor modification. In addition, the receptors which have been down-regulated by (R_p)-cAMPS still undergo cAMP-induced modification.

Down-regulation and Modification by cAMP—The effect of cAMP on down-regulation and receptor modification is presented in Fig. 4. cAMP receptors were down-regulated by incubating cells for 15 min with 1 μM cAMP in the presence of 10 mM DTT. Cells were washed at 0 °C, resuspended in buffer at 20 °C, and allowed to recover for 0, 15, and 30 min. The cAMP binding in the control cells, which were not exposed to cAMP, was defined as 100%. Treatment of the cells with 1 μM cAMP and 10 mM DTT for 15 min resulted in a 80% inhibition of the cAMP binding; little recovery was observed within the initial 15 min. At 30 min after removal of cAMP still about 50% of the receptors were down-regulated.

Control cells showed the expected pattern of cAMP receptor distribution: more than 90% of the receptors were in the R form (*lane 1*). Stimulation with 0.5 μM cAMP and 10 mM DTT maximizes the fraction of the D form (*lane 2*). The receptor of down-regulated cells was found in the D form immediately after removal of cAMP (*lane 3*), or after a subsequent incubation with cAMP (*lane 4*). At 15 or 30 min after removal of cAMP a substantial fraction of the receptors had recovered to the R form (*lanes 5* and *7*), even though the majority of the receptors remained down-regulated (76 and 57%, respectively). Exposure of these cells to a new cAMP stimulus induced the nearly complete transition to the D form.

These results indicate that cAMP induces receptor modification and down-regulation. Receptors recover from modification while they are down-regulated, and these receptors can be induced to undergo modification while they remain down-regulated.

Down-regulated Receptors Are Not Occupied with cAMP—The previous results indicate that down-regulated receptors respond to a newly applied cAMP stimulus, although they do not detect this stimulus. Are down-regulated receptors still occupied with ligand? Therefore, down-regulation was induced by [^{3}H]cAMP, cells were extensively washed, and the location of radioactivity was analyzed (Table I). Down-regulation by cAMP amounts to a loss of 0.36 pmol of binding sites/10^7 cells. Down-regulation by [^{3}H]cAMP results in the tight association of 0.24 pmol of tritium per 10^7 cells. These data are similar to those reported by Klein (13), suggesting that down-regulated receptors may have [^{3}H]cAMP bound. However, the following observations suggest that the radioactivity which becomes tightly associated with cells during

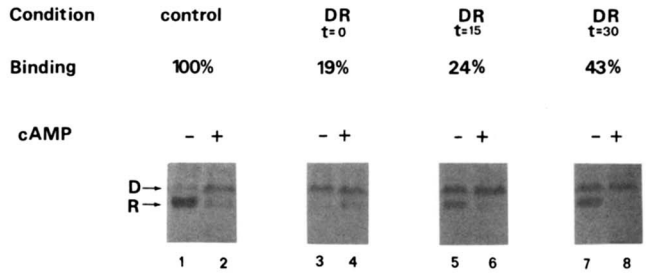

FIG. 4. **Effect of cAMP on down-regulation and shifting.** *D. discoideum* cells were incubated for 15 min in the absence or presence of 1 μM cAMP and 10 mM DTT, washed (at 0 °C), resuspended (at 20 °C) in Mes buffer. Cells were divided into three portions, which were used after 0, 15, and 30 min for the determination of cAMP binding or a subsequent incubation with 0.5 μM cAMP + DTT. *Lanes 1* and *2*, control cells; *lanes 3* and *4*, cells with down-regulated receptors at $t = 0$; *lanes 5* and *6*, *7*, and *8*, cells with down-regulated receptors, respectively, at 15 and 30 min after removal of the stimulus. *Lanes 1*, *3*, *5*, and *7* before and *lanes 2*, *4*, *6*, and *8* after the subsequent incubation with 0.5 μM cAMP + DTT. Binding data represent cAMP binding to the down-regulated receptors at $t_0 = 19 \pm 4\%$, $t_{15} = 24 \pm 5\%$, and $t_{30} = 43 \pm 8\%$.

TABLE I

Analysis of [^{3}H]cAMP that is associated with down-regulated cells

Cells (10^8/ml) were incubated for 15 min with 2 mM caffeine to inhibit cAMP oscillations and the cAMP-induced activation of adenylate cyclase. One portion of the cells (1 ml) was incubated with 1 μM cAMP + 10 mM DTT, and another portion with 1 μM [^{3}H]cAMP + DTT. After 15 min at 22 °C 14 ml of ice-cold phosphate buffer was added, cells were washed three times and resuspended in this buffer. Control cells were treated in parallel, but not incubated with cAMP + DTT. A portion of the cells was lysed through a Nuclepore filter, and the lysate was centrifuged for 5 min at 10,000 × *g*. cAMP-binding was measured in control cells and in cells down-regulated with cAMP. Tightly bound [^{3}H]cAMP was determined in cells down-regulated with [^{3}H]cAMP. The binding mixture contained 1 μM [^{3}H]cAMP + 10 mM DTT and 9×10^6 cells in 100 μl. After 1 min at 0 °C cells were centrifuged through silicone oil (binding in phosphate buffer), or 1 ml of saturated ammonium sulfate was added and cell-associated radioactivity was measured after 5 min.

	Binding in		Tightly bound [^{3}H] cAMP in	
	Phosphate	Ammonium sulfate	Phosphate	Ammonium sulfate
	pmol/10^7 cells		*pmol/10^7 cells*	
Cells, control	0.51	1.20		
Cells, down-regulated	0.15	1.15	0.24	0.24
Supernatant, control	ND[a]	ND		
Pellet, control	0.55	1.00		
Supernatant, down-regulated	ND	ND	0.22	ND
Pellet, down-regulated	0.20	0.95	0.02	ND

[a] 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_r = 40,000 to 43,000 in the electrophoretic mobility of a polypeptide identified by photoaffinity labeling with [^{32}P]8-N_3-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 vivo* labeling with $^{32}P_i$ (22, 23).

Phosphorylation of the β-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 β-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 temporally 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_p)-cAMPS 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_p)-cAMPS 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_p)-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.

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