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Down-regulation of Cell Surface Cyclic AMP Receptors and Desensitization of Cyclic AMP-stimulated Adenylate Cyclase by Cyclic AMP in Dictyostelium discoideum

KINETICS AND CONCENTRATION DEPENDENCE*

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Cyclic AMP binds to Dictyostelium discoideum surface receptors and induces a transient activation of adenylate cyclase, which is followed by desensitization. CAMP also induces a loss of detectable surface receptors (down-regulation). Cells were incubated with constant CAMP concentrations, washed free of CAMP, and CAMP binding to surface receptors and CAMP-induced activation of adenylate cyclase were measured. 1) CAMP could induce maximally 65% loss of binding activity and complete desensitization of CAMP-stimulated adenylate cyclase activity. 2) Half-maximal effects for down-regulation were observed at 50 nM CAMP and for desensitization at 5 nM CAMP. 3) Down-regulation was rapid with half-times of 4, 2.5, and 1 min at 0.1, 1, and 10 μM CAMP, respectively. Similar kinetic data have been reported for desensitization (Dinuuer, M. C., Steck, T. L., and Devreotes, P. N. (1980) J. Cell Biol. 86, 554–561). 4) Down-regulation and desensitization were not reversible at 0 °C. 5) Down-regulation reversed slowly at 20 °C with a half-time of about 1 h. 6) Resensitization of adenylate cyclase was biphasic showing half-times of 4 min and about 1 h, respectively; the contribution of the rapidly resensitizing component was diminished when down-regulation of receptors was enhanced.

These results suggest that CAMP-induced down-regulation of receptors and desensitization of adenylate cyclase stimulation proceed by at least two steps. One step is rapidly reversible, occurs at low CAMP concentrations, and induces desensitization without down-regulation, while the second step is slowly reversible, requires higher CAMP concentrations, and also induces down-regulation.

Extracellular CAMP functions as a signal molecule in Dictyostelium discoideum during chemotaxis (1), morphogenesis (2), and cell differentiation (3). CAMP is detected by highly specific surface receptors, which result in several cellular responses such as the activation of adenylate and guanylate cyclase (these and other responses have been reviewed (4–6)). The stimulation of guanylate and adenylate cyclase terminate within a few seconds and a few minutes, respectively, even when CAMP remains present at constant levels (7–10). Desensitization of adenylate cyclase stimulation has been studied extensively by Dinuuer et al. (11, 12).

Recent results suggest the presence of two subpopulations of surface CAMP receptors, A and B sites, that have been implicated in the stimulation of adenylate and guanylate cyclase (13, 14), respectively. Binding of CAMP to both subpopulations is complex, showing interconversions of binding states in vitro (15, 16) which are promoted by guanine nucleotide in vitro (16–19). This may suggest the involvement of guanine nucleotide regulatory proteins in the transduction pathways to adenylate and guanylate cyclase.

Desensitization of CAMP-stimulated guanylate cyclase occurs within a few seconds after CAMP addition (9) and may be caused by an impairment of receptor G-protein interaction (16). Desensitization of adenylate cyclase is much slower and requires the presence of constant CAMP concentrations during several minutes (12), which may be due to receptor modification, presumably by phosphorylation (20–22).

CAMP induces a third desensitization process, which is the loss of CAMP binding to cell surface receptors (23). In wild-type cells relatively high CAMP concentrations (100 μM) are required to induce this down-regulation. However, in a mutant which lacks cell surface and extracellular cyclic nucleotide phosphodiesterase activity, down-regulation could be induced by low CAMP concentrations (10 nM) and did occur within 5–10 min after CAMP addition (24). Cells with down-regulated surface receptors show a strongly diminished stimulation of adenylate cyclase (14).

These data suggest that CAMP-induced down-regulation of surface receptors and desensitization of adenylate cyclase stimulation may occur simultaneously. Therefore, the kinetics and concentration dependences of these processes have been investigated. The results are interpreted as a two-step model; one step induces desensitization without a loss of CAMP-binding activity, while the second step also induces down-regulation. The two steps show similar kinetics but differ in the CAMP dose dependence and reversibility after removal of CAMP.

EXPERIMENTAL PROCEDURES

Materials—[2,8-3H]CAMP was obtained from Amersham Corp., CAMP was from Boehringer Mannheim, dithiothreitol and dcAMP were from Sigma, and caffeine was purchased from the British Drug House. (Sp)-cAMPS was a kind gift of Drs. Jastorff, Baraniak, and Stce (25).

1 The abbreviations used are: dcAMP, 2’deoxyadenosine 3’,5’-monophosphate; (Sp)-cAMPS, adenosine 3’,5’-monophosphorothioate, Sp-isomer; (Rp)-cAMPS, adenosine 3’,5’-monophosphorothioate, Rp-isomer.
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Culture Conditions and Cell Treatment—D. discoideum cells (Strain NC-4) were grown on a buffered glucose-peptone medium (9). Cells were harvested in 10 mM KH2PO4/Na2HPO4, pH 6.5 (PB buffer), freed from bacteria by repeated centrifugations at 100 × g for 2 min, and resuspended in PB buffer at a density of 107 cells/ml. After 5 h cells were collected by centrifugation, washed twice, and resuspended in PB buffer at a density of 108 cells/ml.

Cells were treated by two methods. In the first method, cAMP or (Sp)-cAMPS was added to the cell suspension at the concentration and for the time period as indicated in the figure legends. In the second method, cells were incubated with 5 mM caffeine for 10 min, which was followed by an incubation with 10 mM dithiothreitol and cAMP or (Sp)-cAMPS. At the end of both treatments the cell suspensions were diluted 50-fold with ice-cold PB buffer. Cells were washed twice with PB buffer at 0 °C and resuspended in this buffer at a density of 109 cells/ml.

**cAMP Binding.**—The binding of [3H]cAMP to D. discoideum cells was routinely detected in a volume of 100 μl containing PB buffer, 10 mM dithiothreitol, 5 mM [3H]cAMP, and 8 × 106 cells. The incubation period was 60 min at 0 °C, and centrifugation was performed at 10,000 × g. The supernatant was aspirated, and the radioactivity in the cell pellet was determined.

Nonspecific binding was determined by including 0.1 mM cAMP in the incubation mixture and was subtracted from all data shown; nonspecific binding is about 0.4 and 1.4% of the input radioactivity for the silicon oil and ammonium sulfate method, respectively.

**cAMP-mediated cAMP Accumulation.**—Cells were incubated for 5 min at 20 °C in a mixture (100 μl) containing PB buffer, 10 mM dithiothreitol, 10 μM d-cAMP, and 5 × 106 cells. The reaction was stopped by addition of 100 μl of 3.5% (v/v) perchloric acid. The lysate was neutralized with 50 μl of KHCO3, 50% saturated at 20 °C, centrifuged at 10,000 × g for 2 min, and the cAMP content was determined in the supernatant by isotope dilution assay (27). d-cAMP is a potent agonist of cAMP for receptor-mediated activation of adenylate cyclase (KC = 25 nM) while it is a poor agonist of cAMP for the binding protein used in the cAMP assay (28).

**RESULTS**

Dose-response Curve of Loss of Binding Activity.—Cells were incubated with different cAMP concentrations for 15 min at 20 °C, washed extensively at 0 °C, and binding of 5 nM [3H]cAMP was determined (Fig. 1A). cAMP induced a dose-dependent loss of binding activity with a half-maximal effect at 15 μM cAMP which is in agreement with previous results (33). D. discoideum cells contain high activities of surface cyclic nucleotide phosphodiesterase which hydrolyzes 1 μM cAMP by 90% within 1 min (9). Therefore, this dose-response curve does probably not reflect the minimal cAMP concentration that can induce a receptor loss.

A nonhydrolyzable cAMP analog, (Sp)-cAMPS, can be used to bypass phosphodiesterase activity (29, 30). Alternatively, extracellular cAMP can be clamped in the presence of 5 mM caffeine and 10 mM dithiothreitol (21); caffeine blocks the stimulation of adenylate cyclase (31), and dithiothreitol blocks phosphodiesterase activity (32). The dose-response curve for loss of binding was shifted to much lower cAMP concentrations when the cAMP concentration was kept constant (Fig. 1A); a half-maximal effect was observed at 50 nM cAMP. The nonhydrolyzable analog (Sp)-cAMPS has about the same activity in the absence or presence of caffeine plus dithiothreitol, suggesting that the clamp method may be used to study cAMP-induced loss of binding. (Sp)-cAMPS is about 100-fold less active than clamped cAMP, which corresponds well with its about 75-fold lower binding affinity for the surface cAMP receptor (33).

Dose-response Curve for Desensitization.—The binding of cAMP to cell surface receptors results in a transient activation of adenylate cyclase which is followed by desensitization. Half-maximal stimulation of adenylate cyclase is induced by about 5 μM cAMP (34). The cells that were treated with cAMP or (Sp)-cAMPS for the study of receptor loss were also used to analyze the dose dependence of desensitization (Fig. 1B). Half-maximal desensitization of adenylate cyclase stimulation was induced by pretreatment of 5 μM clamped cAMP or 0.4 μM (Sp)-cAMPS. These values are about 10-fold lower than those which induce a half-maximal loss of binding.

Kinetics of Receptor Loss and Desensitization.—Cells were incubated in the presence of caffeine and dithiothreitol with 0.1, 1, and 10 μM cAMP for various time periods, washed extensively, and used for the detection of cAMP binding activity (Fig. 2). At 20 °C 10 μM cAMP induced a 80% loss of cAMP-binding activity with a half-maximal loss after about 1 min. The loss of cAMP binding induced by 1 μM cAMP was about the same but occurred more slowly (t1/2 = 2.5 min). 0.1 μM cAMP induced a 50% loss of cAMP binding with a half-life of 4 min. The loss of binding can also be induced at 0 °C, although it is about 4-fold slower and less pronounced (Fig. 2, inset).

The kinetics of desensitization of adenylate cyclase has been investigated extensively by Dinauer et al. (12). It occurred at 20 °C with a half-time of about 2.5–3 min at 1 μM cAMP and was somewhat faster at lower cAMP concentrations. In addition, desensitization of adenylate cyclase did occur at 0 °C (28), although it is about 3-fold slower than at 20 °C.2 These results indicate that loss of cAMP binding and desensitization of adenylate cyclase stimulation proceed on a similar time scale.

Reversibility of Receptor Loss and Desensitization.—Cells were incubated in the presence of caffeine and dithiothreitol

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Number of cAMP-binding Sites—In the previous experiments, cAMP binding was detected at subsaturating concentrations of $[^3H]cAMP$. The loss of cAMP binding that was observed after treatment of cells with cAMP could be due to a reduction of affinity and/or a reduction of the number of binding sites. Therefore, cells were incubated in the presence of caffeine and dithiothreitol with 1 $\mu M$ cAMP for 15 min, washed, and binding was detected at various concentrations of $[^3H]cAMP$. A Scatchard plot of these data (Fig. 4A) clearly reveals a reduction of the number of binding sites in treated cells. The data also indicate a slight reduction of the apparent affinity, since the loss of cAMP binding is 80% at 5 $nm$ $[^3H]cAMP$ and 65% at 1 $\mu M$ $[^3H]cAMP$. This has also been observed previously and is probably due to the reduction of affinity of a small subclass of the cAMP binding activity (14).

Since the loss of cAMP binding activity is predominantly a reduction of the number of binding sites it will be designated as down-regulation.

Cryptic Receptors in Down-regulated Cells—Exposure of D. discoideum cells to millimolar concentrations of divalent cations results in a 2-3-fold increase in the number of detectable binding sites, indicating that a major portion of the cAMP receptors is cryptic (35). The hypothesis that down-regulation represents the transfer of available binding sites to cryptic binding sites that can be exposed by divalent cations was investigated.

cAMP binding to control cells and cells treated with 1 $\mu M$ cAMP was detected in the absence and presence of 10 $mm$ Ca$^{2+}$ (which exposes cryptic receptors). In the absence of Ca$^{2+}$ (Fig. 4A) a reduction of 37,000 binding sites/cell was detected. Although Ca$^{2+}$ increased the binding of cAMP to control and down-regulated cells (Fig. 4B), the same loss of binding sites was detected. Thus the hypothesis that down-regulated receptors become cryptic (and exposable by Ca$^{2+}$) must be rejected.

Recently, it was observed that high concentrations of ammonium sulfate had multiple effects on cAMP binding activity (26). It not only exposed cryptic receptors but also altered the distribution of the various receptor forms and strongly retarded the rate of dissociation. Fig. 4A reveals only a slight reduction of binding sites when cAMP binding to down-regulated cells is measured in saturated ammonium sulfate. In addition, the apparent affinity is reduced about 2-fold. These results indicate that down-regulated receptors are not destroyed. Binding sites are not exposed by Ca$^{2+}$. However, binding activity recovers in saturated ammonium sulfate.

**DISCUSSION**

The kinetics and concentration dependences of cAMP-induced down-regulation of surface receptors and of cAMP-induced desensitization of cAMP-stimulated adenylate cyclase have been analyzed in D. discoideum cells in suspension. Two methods were used to provide stable stimulus concentrations. First, the analog (Sp)-cAMPS is hydrolyzed very slowly (29, 30); operationally it is called nonhydrolyzable. Second, the cAMP concentration is clamped in the presence of caffeine and dithiothreitol (21). Caffeine inhibits cAMP-mediated stimulation of adenylate cyclase (31), but its desensitization (30), while dithiothreitol inhibits cyclic nucleotide phosphodiesterase activity (32). Both methods yield essentially identical results, indicating that down-regulation occurs independent of adenylate cyclase activation. About 50-100-fold higher concentrations of (Sp)-cAMPS than cAMP were required to induce desensitization and down-regulation, which is in close agreement with the reduced affinity of this analog for surface cAMP receptors (33).

The major findings of the present study indicate that: 1) cAMP induces maximally 70% down-regulation of the number of binding sites detectable in phosphate buffer and complete desensitization of cAMP-mediated activation of adenylate cyclase. 2) Half-maximal down-regulation occurs at 50 $nm$ cAMP. 3) Half-maximal desensitization of adenylate cyclase activation occurs at 5 $nm$ cAMP; this value is identical to the half-maximal concentration for the activation of adenylate cyclase (34). 4) Down-regulation and desensitization proceed with similar rates and have a half-time of about 2.5 min at 1.
which are exposed by divalent cations (35). Down-regulated partly with a half-time of about adenylate cyclase is at least biphasic; desensitization reverts ble at 0 °C. 6) Down-regulation reverses slowly at 20 °C with CAMP-induced desensitization of adenylate cyclase. Dinauer down-regulated receptors are exposed at high concentrations receptors are not exposed in the presence of Ca2+. In contrast,ing part reverts more slowly with a half-time of about 1 h (14). 7) Resensitization of adenylate cyclase is at least biphasic; desensitization reverts partly with a half-time of about 4 min (11), while the remaining part reverts more slowly with a half-time of about 1 h (14). 8) D. discoideum cells contain cryptic cAMP receptors which are exposed by divalent cations (35). Down-regulated receptors are not exposed in the presence of Ca2+. In contrast, down-regulated receptors are exposed at high concentrations of ammonium sulfate. Thus, down-regulated receptors are cryptic (as opposed to being destroyed), but they are not the cryptic receptors that are exposed by Ca2+. The present results are combined with published data on cAMP-induced desensitization of adenylate cyclase. Dinauer et al. (11) have shown that a part of the desensitization occurs rapidly with a half-time of about 4 min, while the remaining part does not recover within 30 min. The slowly reversible component increased when desensitization was induced by higher cAMP concentrations and amounted to 10% at 10 nM CAMP, 40% at 100 nM CAMP, 60% at 10 μM CAMP (11), and 80% at 1 mM CAMP (14). These values are similar to the extent of receptor down-regulation induced by these concentrations of cAMP. Furthermore, it has been shown that cells which are down-regulated by high CAMP concentration (1 mM) recover CAMP-binding activity and adenylate cyclase stimulation in parallel with a half-time of about 1 h (14). These results suggest that desensitization of adenylate cyclase stimulation is composed of at least two components which show different affinities for cAMP and different reversible rates and can be combined in Scheme I. cAMP binds to receptors and transduces the signal to adenylate cyclase which occurs half-maximally at about 5 nM cAMP. Simultaneously an alteration in the signal transduction pathway takes place by which transduction to adenylate cyclase is impaired while CAMP binding is retained. This step is induced half-maximally by about 5 nM CAMP, proceeds with a half-time of 2–3 min, and reverses with a half-time of 4 min upon removal of cAMP. Furthermore, a portion of the receptors lose their binding activity. This step is equally rapid but requires about 10-fold higher CAMP concentrations. In addition, this step reverses very slowly with a half-time of about 1 h. It should be noted that desensitization of adenylate cyclase stimulation is associated with both steps; the first step which is rapidly reversible has been called adaptation (7). The second step, which predominates at higher CAMP concentrations, induces desensitization because of down-regulation of cell surface receptors. Presently, two criteria are available to discriminate between desensitization due to adaptation and desensitization due to down-regulation, which are the concentration dependence and especially the reversibility at 20 °C. Their rate of occurrence is similar, but not identical, and can hardly be used to discriminate between adaptation and down-regulation. Another criterion such as the reversibility at 0 °C is also not discriminatory. These criteria have been used to reveal that an analog of cAMP, (Rp)-CAMPS, induces down-regulation of surface cAMP receptors without inducing adaptation or excitation of adenylate cyclase (34). These observations suggest that the first step in the scheme described above is not a prerequisite for the second step. It is presently not resolved whether this is also possible for cAMP. Recently, it has been shown that cAMP induces an alteration in the electrophoretic mobility of a protein that was photoaffinity labeled with [32P]8-azido-cAMP, probably due to phosphorylation of the cell surface cAMP receptor (20–22). This covalent modification of the receptor is induced half-maximally by 27 nM cAMP, occurs with a half-time of 1.5–2.5 min, and is reversible with a half-time of about 4 min. It has been proposed that covalent modification could be the mechanism of adaptation of adenylate cyclase stimulation (21). Indeed, its properties agree better with adaptation than with down-regulation. The regulation by cAMP of excitation and desensitization of adenylate cyclase and the regulation of cell surface cAMP receptors in D. discoideum parallels the situation in higher

![Fig. 4. Scatchard analysis of [3H]cAMP binding to control and treated cells. Cells were incubated in the absence (○) or presence (■) of 5 mM caffeine, 10 mM dithiothreitol, and 1 μM cAMP for 15 min at 20 °C, washed extensively, and resuspended in phosphate buffer. cAMP binding was detected in phosphate buffer (A), in the presence of 10 mM Ca2+ (B), or 3.4 M ammonium sulfate (C). The abscissae have the same scale, but the scales at the ordinate differ considerably. The results shown are means of triplicate determinations. The experiment was reproduced once with similar results. The arrows show the binding at the indicated concentration of cAMP.](image-url)
organisms such as the \( \beta \)-adrenergic system. Agonists of the \( \beta \)-adrenergic receptor induce activation of adenylate cyclase which is followed by desensitization and receptor down-regulation (37, 38). Phosphorylation of the receptor has been associated with desensitization of adenylate cyclase stimulation and probably reflects an altered interaction between receptor and guanine nucleotide regulatory protein (G protein) (38, 39).

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