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Haastert, Peter J.M. van; Wang, Mei; Bominaar, Anthony A.; Devreotes, Peter N.; Schaap, Pauline

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cAMP-Induced Desensitization of Surface cAMP Receptors in *Dictyostelium:* Different Second Messengers Mediate Receptor Phosphorylation, Loss of Ligand Binding, Degradation of Receptor, and Reduction of Receptor mRNA Levels

Peter J.M. Van Haastert,*† Mei Wang,‡ Anthony A. Bominaar,* Peter N. Devreotes,§ and Pauline Schaap‡

*Department of Biochemistry, University of Groningen, The Netherlands; ‡Department of Cell Biology and Genetics, University of Leiden, The Netherlands; and §Department of Biological Chemistry, Johns Hopkins University Medical School, Baltimore, Maryland 21218

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Surface cAMP receptors on *Dictyostelium* cells are linked to several second messenger systems and mediate multiple physiological responses, including chemotaxis and differentiation. Activation of the receptor also triggers events which desensitize signal transduction. These events include the following: 1) loss of ligand binding without loss of receptor protein; 2) phosphorylation of the receptor protein, which may lead to impaired signal transduction; 3) redistribution and degradation of the receptor protein; and 4) decrease of cyclic AMP (cAMP) receptor mRNA levels. These mechanisms of desensitization were investigated with the use of mutant *synag7*, with no activation of adenylyl cyclase; *fgd*C, with no activation of phospholipase C; and *fgd*A, with defects in both pathways. cAMP-induced receptor phosphorylation and loss of ligand binding activity was normal in all mutants. In contrast, cAMP-induced degradation of the receptor was absent in all mutants. The cAMP-induced decrease of cAMP-receptor mRNA levels was normal in mutant *synag7*, but absent in mutant *fgd*C. Finally, the cAMP analogue (Rp)-cAMPS induced loss of ligand binding without inducing second messenger responses or phosphorylation, redistribution, and degradation of the receptor. We conclude that 1) loss of ligand binding can occur in the absence of receptor phosphorylation; 2) loss of ligand binding and receptor phosphorylation do not require the activation of second messenger systems; 3) cAMP-induced degradation of the receptor may require the phosphorylation of the receptor as well as the activation of at least the *synag7* and *fgd*C gene products; and 4) cAMP-induced decrease of receptor mRNA levels requires the activation of the *fgd*C gene product and not the *synag7* gene product. These results imply that desensitization is composed of multiple components that are regulated by different but partly overlapping sensory transduction pathways.

INTRODUCTION

Extracellular cyclic AMP (cAMP) induces chemotaxis and differentiation in the cellular slime mold *Dictyostelium discoideum* (for reviews see Devreotes, 1989; Firtel *et al.*, 1989; Gerisch, 1987; Janssens and Van Haastert, 1987; Schaap, 1986). Cells express surface receptors that bind cAMP with high affinity and specificity. The receptors interact with G-proteins to activate several second messenger systems, including adenylyl cyclase, guanylyl cyclase, and phospholipase C (Van Haastert, 1984, 1989; Theibert and Devreotes, 1986; Van Haastert

† Corresponding author.

et al., 1987, 1989; Europe-Finner *et al.*, 1989; Janssens *et al.*, 1989). The deduced primary structure of the cAMP receptor is typical for a G-protein coupled receptor showing seven putative membrane spanning domains and a serine rich hydrophilic tail (Klein *et al.*, 1988).

Activation of the receptor leads to several alterations of the protein. Within 1 min after stimulation the receptor becomes phosphorylated at multiple serine residues (Klein *et al.*, 1985, 1987; Vaughan and Devreotes, 1988). It has been suggested that receptor phosphorylation leads to receptor-effector uncoupling, which might be part of a desensitization process (Devreotes and Sherring, 1985). After ~5 min of stimulation with a saturating cAMP concentration the majority of the receptors no longer bind cAMP (Klein and Juliani, 1977; Van Haastert, 1987b). Receptors are not degraded because they still can bind cAMP in saturated ammonium sulfate and the protein is still detectable on Western blots. Obviously, the loss of binding activity of receptors leads to desensitization of signal transduction.[1,2]

In this study we have further analyzed the processes associated with desensitization of surface cAMP receptors. Besides the loss of ligand binding and receptor phosphorylation as described previously, prolonged incubation of *Dictyostelium* cells with cAMP also induces the redistribution of receptors and their final degradation. Furthermore, cAMP induces the rapid loss of cAMP receptor mRNA. Finally, a variety of mutants and a cAMP analogue were used to characterize the molecular mechanisms of desensitization. The results show that loss of ligand binding to receptors does not require phosphorylation of the receptor or the activation of any known second messenger system. In contrast, degradation of the receptor and loss of receptor mRNA requires the activation of different second messenger systems.

METHODS

Materials

[2,8-^{3}H]cAMP and [α-^{32}P]dATP were obtained from Amersham (Buckinghamshire, UK). cAMP and dithiothreitol were from Boehringer Mannheim (Mannheim, FRG). The Sp- and Rp-isomer of adenosine 3',5'-monophosphorothioate [(Sp)-cAMPS and (Rp)-cAMPS, respectively] were generous gifts of Dr. Jastorff, University of Bremen or were provided by Biolog (Bremen, FRG). (3-[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate (CHAPS) was obtained from Sigma (St Louis, MO). Fluorescein-isothiocyanate-conjugated goat anti-rabbit IgG (GARFITC) was obtained from Nordic Laboratories (Tilburg, The Netherlands) and peroxidase-conjugated swine anti-rabbit IgG (SARPO) was from DAKO (Denmark).

Dictyostelium Discoideum Strains and Culture Conditions

Strains used were the following: the wild-type NC4; mutant *fgd*A strain HC33 with parent HC6, mutant *fgd*A strain HC213 with parent HC91; mutant *fgd*C strain HC317 with parent XP55 (Coukell *et al.*, 1983); and mutant *synag7* with parent NC4 (Frantz, 1980). Cells were grown in association with *Escherichia coli* as described (Van Haastert and Van der Heijden, 1983). Cells were harvested in the late logarithmic phase in 10 mM KH_2PO_4/Na_2HPO_4, pH 6.5 (PB), freed from bacteria by repeated centrifugations and starved on nonnutrient agar for 16 h at 6°C to induce aggregation competence.

cAMP Binding Assays

Cells were harvested from the nonnutrient agar plates and shaken in PB at a density of 10^7 cells/ml. Drugs and cAMP were added to the suspension as described in the table and figure legends. At the end of the incubation period, cells were washed three times with PB and resuspended in PB to a density of 2×10^8 cells/ml. To measure cAMP binding in PB, 1.8×10^7 cells were incubated with 10 nM [^{3}H]cAMP and 10 mM dithiothreitol in a total volume of 100 μl PB. After 1 min of incubation at 0°C, cells were centrifuged through silicon oil, and the radioactivity of the pellet was measured (Van Haastert and De Wit, 1984). To measure cAMP binding in ammonium sulfate, 1.8×10^7 cells were incubated with 1 nM [^{3}H]cAMP, 1 mM dithiothreitol, and 50 μg bovine serum albumin in a total volume of 1 ml 85% saturated ammonium sulfate. After 5 min of incubation at 0°C, the cells were pelleted by centrifugation for 3 min at $10\,000 \times g$ and radioactivity of the pellet was measured (Van Haastert and Kien, 1983). Nonspecific binding was determined by including 0.1 mM cAMP in the incubation mixture.

Immunocytochemical Methods

Aggregation competent cells were incubated with cAMP or (Rp)-cAMPS as indicated in the figure legends. Five-microliter droplets containing 5×10^6 cells/ml were deposited on a glass slide, and cells were allowed to adhere for 5 min at 20°C. The cells were overlayed with a 50–100-μm layer of 1.5% agarose in PB (Yunura and Fukui, 1985) and fixed for 20 min in ice-cold methanol. Subsequently the cells were stained with a rabbit antiserum against the purified *Dictyostelium* cAMP receptor (Klein *et al.*, 1987) and GARFITC as previously described (Wang *et al.*, 1988). The receptor antiserum was preadsorbed to methanol-fixed vegetative cells.

Western Transfer Analysis of Membrane Proteins

Cells (2×10^8) were lysed in 1 ml receptor buffer containing 1.5% CHAPS. The membranes were pelleted, washed once with receptor buffer, and resuspended in 50 μl sample buffer (Klein *et al.*, 1987). Membrane proteins were size-fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose, which was incubated for 1 h with 1:500 diluted rabbit antiserum against the purified cAMP receptor and for 1 h with 1:3000 diluted SARPO. Specific bands were visualized by means of a peroxidase reaction (Snaar-Jagalska *et al.*, 1988a).

[1] We use the operational term "loss of ligand binding" rather than "sequestration", because the data do not demonstrate that the receptor is converted to another compartment; i.e., (Rp)-cAMPS induces loss of ligand binding but no detectable redistribution of immunofluorescent staining.

[2] Definitions used: loss of ligand binding, reduction of the number of cAMP binding sites as measured in phosphate buffer, which is not accompanied by a reduction of the receptor protein as measured in ammonium sulfate; sequestration, translocation of receptors to a domain inaccessible for hydrophilic ligands; degradation, receptors are no longer detectable by binding in ammonium sulfate or on Western blots; down-regulation, all processes leading to a reduction of cAMP binding activity (this includes "loss of ligand binding", "degradation" as well as reduced *de novo* synthesis); desensitization, all processes leading to reduced cAMP-induced responses.

RNA Isolation and Analysis

Cells (2×10^7) were lysed in 800 μl 1.5% SDS, in 50 mM tris(hydroxymethyl)amino methane (Tris, pH 8.4), containing 5 μl diethylpyrocarbonate. RNA was purified by phenol extraction, and two ethanol and one LiCl precipitation steps (Mann and Firtell, 1987). RNA (15 μg) was size-fractionated on formaldehyde containing agarose gels, transferred to Gene Screen and hybridized to the full-length cAMP receptor cDNA probe 6B (Klein *et al.*, 1988), which was labeled with [α-^{32}P]-dATP by means of random primer extension.

RESULTS

Localization of cAMP Receptors During cAMP Stimulation

cAMP receptors were localized by immunofluorescence utilizing a specific rabbit antiserum against purified cAMP receptor (Wang *et al.*, 1988). Before cAMP stimulation, immunofluorescent staining appeared as a uniform layer at the cell periphery (Figure 1). After 10 min of stimulation with 100 μM cAMP, staining became heterogeneous, and after 15 min staining appeared in distinct patches suggestive of intracellular vescicles. At this stage, the homogeneous staining at the cell periphery had disappeared. After 25 min, the total intensity of staining had decreased and the remaining staining appeared to be localized at or close to the nucleus. The latter phenomenon was not always observed.[3] After 60 min of cAMP stimulation, no immunofluorescent staining remained (data not shown). Previously, it was demonstrated that preimmune serum or receptor serum that was preadsorbed to purified cAMP receptor showed no staining of any cellular component (Wang *et al.*, 1988).

To test whether the cAMP-induced disappearance of cAMP receptors was reversible, cells were treated for 30 min with 100 μM cAMP, thoroughly washed and incubated in buffer (Figure 2). After 1 h, weakly stained patches began to reappear, and staining was again evident at the cell periphery after 2 h. The intensity of peripheral staining had completely recovered after 4 h (Figure 2).

Binding Activity, Receptor Protein and Receptor mRNA During cAMP Stimulation

In *Dictyostelium* not all chemotactic cAMP receptors bind cAMP on intact cells. A portion of the receptors are exposed on the surface (and are detectable in phosphate buffer), a portion are cryptic (and can be exposed by bivalent cations), and a portion have lost cAMP binding activity because of cAMP stimulation. In nearly saturated ammonium sulfate all these receptor forms bind cAMP with similar affinity (Van Haastert, 1985). We assume that ammonium sulfate uncouples interactions of the receptor with proteins, which may mask binding activity. A short 15-min incubation of *Dictyostelium* cells with 100 μM cAMP resulted in a 70–90% loss of binding activity when measured in phosphate buffer. These receptors were not degraded, because binding in ammonium sulfate was not reduced. Fur-

[3] Experiments aimed to demonstrate the presence of cAMP receptor protein in isolated nuclei showed that some receptor protein could be detected in Western blots of nuclear fractions, but the intensity of the receptor bands did not increase after 25 min of cAMP stimulation (unpublished data).

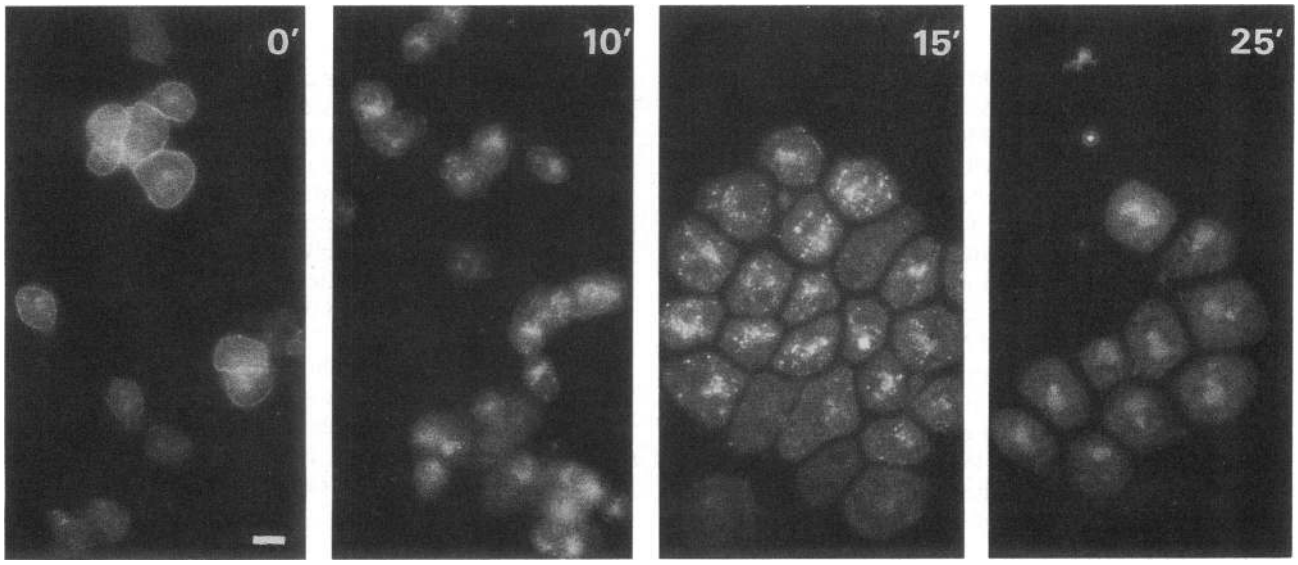

Figure 1. Receptor localization during cAMP stimulation. Aggregation competent NC4 cells at a density of 5×10^6 cells/ml were incubated with 100 μM cAMP. After the indicated time periods, 5 μl-aliquots of the cell suspension were fixed in methanol and stained with a rabbit antiserum prepared against purified cAMP receptor and GARFITC. Bar length, 1 μM.

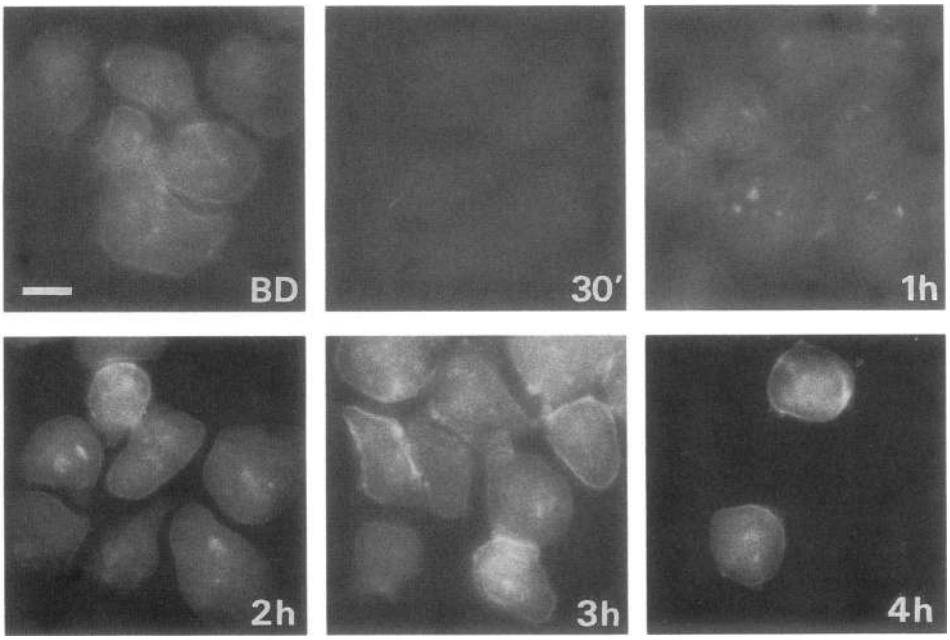

Figure 2. Recovery of receptor specific immunofluorescence after cAMP stimulation. An aliquot of aggregation competent cells were fixed in methanol (BD, before down-regulation) and remaining cells were treated for 30 min with 100 μM cAMP, washed thoroughly to remove cAMP, and resuspended in phosphate buffer at t = 0. Aliquots of cell suspension were fixed in methanol at 0.5, 1, 2, 3, and 4 h after removal of cAMP and stained with cAMP receptor antiserum and GARFITC. Bar length, 1 μM.

thermore, after removal of cAMP, binding in phosphate buffer reappeared rapidly and did not require protein synthesis (Klein and Juliani, 1977; Van Haastert, 1987b).

The results of Figures 1 and 2 indicate that during prolonged stimulation cAMP induced the redistribution and finally disappearance of the surface cAMP receptor. To analyze the molecular mechanisms of these events, the effects of persistent cAMP stimulation on exposed ligand binding activity (measured in phosphate buffer), total ligand binding activity (measured in 85% saturated ammonium sulfate), cAMP receptor protein (detected in Western blots), and cAMP-receptor mRNA (detected in Northern blots) were monitored in parallel sets of experiments. Figure 3A shows that when aggregation competent cells were incubated with 100 μM cAMP, exposed cAMP binding activity decreased with a half-life of <5 min. (■). Total cAMP binding activity (●) decreased more slowly with a half-life of ~45 min. In the absence of cAMP stimulation, total and functional ligand binding activity remained at the same level or increased (this varied somewhat between individual experiments, which is probably related to the increase of cAMP receptors during normal development).

Receptor protein levels, as detected on Western blots (Figure 3B), decreased slowly during cAMP stimulation at about the same rate as the loss of total cAMP binding activity, measured in ammonium sulfate. Note that cAMP stimulation induced a shift of the receptor protein to a conformation with lower electrophoretic mobility. This was shown earlier to be correlated with phosphorylation of the cAMP receptor (Devreotes and Sherring, 1985; Klein *et al.*, 1987; Vaughan and Devreotes, 1988).

Receptor mRNA had nearly disappeared after 1 h of cAMP stimulation (Figure 3C). A slow decrease of receptor mRNA levels was also observed in control cells and is probably related to the development of the cells (Peters *et al.*, 1991). mRNA was isolated at short intervals after addition of cAMP to determine more accurately the kinetics of the cAMP-induced loss of mRNA. Figure 4 shows that the decrease of mRNA levels on cAMP stimulation was extremely rapid with a half-life of ~5 min.

Putative Causes for cAMP-Induced Loss of Receptor Proteins

The cAMP-induced loss of cAMP-receptor protein can be due to decreased synthesis and/or to enhanced degradation. To distinguish between these possibilities, total cAMP-binding activity was measured after cells had been stimulated for 2 h with 100 μM cAMP in the absence or presence of the protein synthesis inhibitor cycloheximide (Table 1). Incubation of control cells with cycloheximide led to a 20% reduction of cAMP-binding activity in 2 h. In contrast, cAMP-binding activity declined ≥70% during stimulation with cAMP, and this decrease was only slightly affected by cycloheximide. These results suggest that receptor turnover is slow in the absence of cAMP (half-life of several hours) and that it is accelerated by cAMP stimulation. Thus cAMP induces both the degradation of existing receptor protein and, by decreasing mRNA levels, the cessation of de novo receptor protein synthesis.

Signals for Phosphorylation, Loss of Ligand Binding, Degradation of cAMP Receptors, and Reduction of mRNA Levels

The next series of experiments were designed to determine whether different transduction pathways are in-

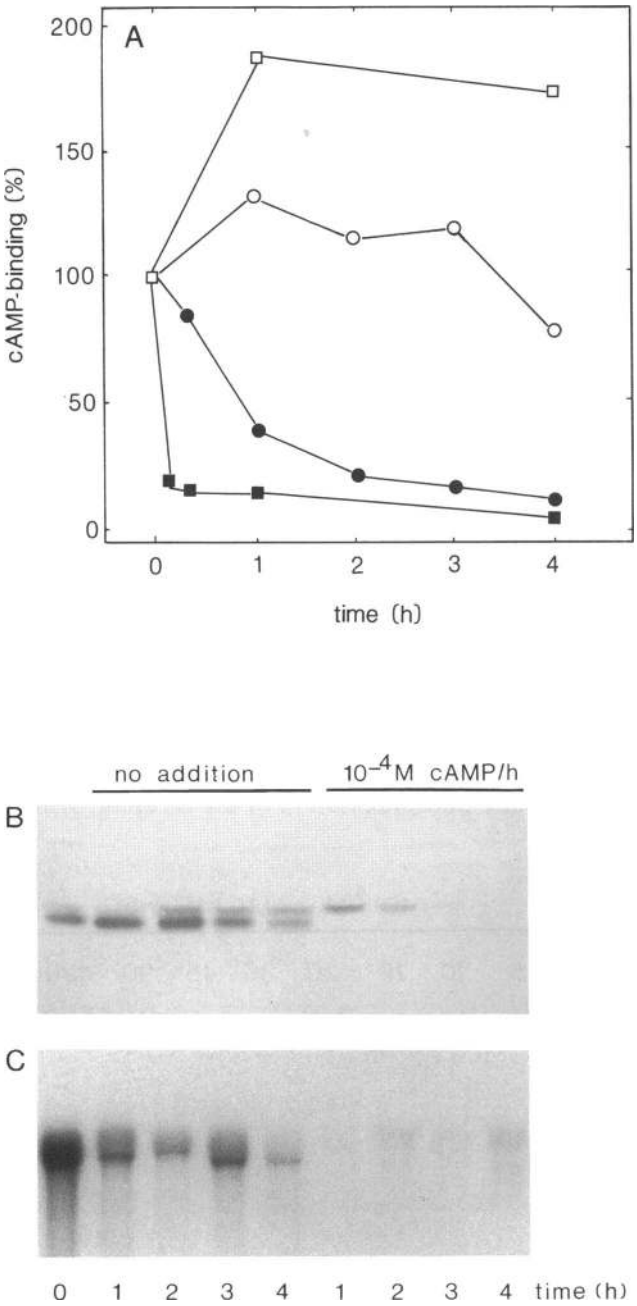

Figure 3. Effects of prolonged cAMP stimulation on cAMP binding, receptor protein and receptor mRNA levels. Aggregation competent cells were incubated during 4 h in the absence (open symbols) or presence of 100 μM cAMP added each hour (closed symbols). At the indicated time periods, cells were removed from the incubation mixture and assayed. (A) Assay for functional ^{3}H-cAMP-binding activity, measured in phosphate buffer (□, ■) and total ^{3}H-cAMP-binding activity, measured in ammonium sulfate (○, ●); (B) western transfer analysis of cAMP receptor protein levels; (C) northern transfer analysis of cAMP receptor mRNA levels. cAMP/h indicates that cAMP was added each hour at the indicated concentration. Binding data are expressed as percentage of the binding levels present at the start of the incubation period.

volved in the various aspects of cAMP-induced desensitization of cAMP receptors. For this purpose a variety of signal transduction mutants and a cAMP analogue were used. The results are presented in Figures 5–7 and summarized in Table 2.

The cAMP analogue (Rp)-cAMPS binds to surface receptors but does not induce cellular responses such as receptor phosphorylation, adenylyl or guanylyl cylase activation, or chemotaxis (Van Haastert, 1983, 1987b; Van Haastert and Kien, 1983; Van Haastert *et al.*, 1986; Snaar-Jagalska *et al.*, 1988a). As shown in Figure 5, this compound induced the rapid loss of exposed cAMP binding sites. However, prolonged stimulation with (Rp)-cAMPS induced neither the degradation (Figure 5), nor the redistribution of receptors (Figure 6). In contrast, the agonist (Sp)-cAMPS induced phosphorylation (Snaar-Jagalska *et al.*, 1988a) loss of functional binding and degradation of the receptor (Figure 5). The affinity of the surface receptor for (Rp)-cAMPS is about threefold lower than for (Sp)-cAMPS (Van Haastert and Kien, 1983). These results (Table 2) indicate that loss of ligand binding can occur in the absence of receptor phosphorylation and activation of second messenger enzymes.

Mutant *fgd*A (strains HC33 and HC213) is defective in a G-protein alpha-subunit and is characterized by the absence of signal transduction, except receptor phosphorylation (Coukell *et al.*, 1983; Kesbeke *et al.*, 1988; Snaar-Jagalska *et al.*, 1988b; Kumagai *et al.*, 1989; unpublished observations). cAMP induced the loss of ligand binding in this mutant but not the degradation of cAMP receptors (Figure 5). In mutant *fgd*C (strain HC 317), cAMP induces the normal activation of guanylyl cyclase, adenylyl cyclase, and phosphorylation of the receptor, but the activation of phospholipase C is abnormal; the defective gene has not been identified (Lappano and Coukell, 1982; Coukell *et al.*, 1983; Bominaar *et al.*, 1991). In this mutant cAMP-induced loss of binding was normal, but cAMP-induced degradation of the receptor was strongly reduced (Figure 5). Receptor mRNA levels are low in *fgd* C; stimulation of mutant *fgd* C did not result in a decrease of receptor mRNA levels (Figure 7). In mutant *fgd* A this could not be examined, because receptor mRNA levels are virtually undetectable.[4]

Mutant *synag*7 (strain N7) is defective in cAMP-stimulation of adenylyl cyclase; other responses such as activation of guanylyl cyclase, phospholipase C, and receptor phosphorylation are normal (Frantz, 1980;

[4] In mutant *fgd*A the low levels of receptor mRNA levels (<10% of wild type) seem to contrast with the relatively normal receptor protein levels (25–50% of wild type). However, cAMP does not induce degradation of the receptor protein, and receptor protein turnover is very slow. Therefore, low levels of mRNA may be sufficient for moderate levels of receptor protein.

Schaap *et al.*, 1986; Theibert and Devreotes, 1986; Van Haastert *et al.*, 1987; Snaar-Jagalska and Van Haastert, 1988; unpublished observations). In this mutant, cAMP induced the rapid loss of ligand binding as in wild-type cells, but cAMP-induced degradation of receptor was strongly reduced. Interestingly, the rapid cAMP-induced loss of receptor mRNA is normal in mutant *synag7* (Figure 4). These results suggests that the *synag7* gene product (presumably leading to the activation of adenylyl cyclase) is required for cAMP-induced degradation of the receptor but not for inhibition of de novo synthesis of the receptor. Caffeine specifically inhibits receptor-mediated activation of adenylyl cyclase in *Dictyostelium;* activation of guanylyl cyclase or phospholipase C are unaffected (Brenner and Thoms, 1984 and unpublished observations). Caffeine had no effect on the cAMP-induced loss of ligand binding, but inhibited cAMP-induced receptor degradation (Figure 5). These results suggest that activation of adenylyl cyclase is required for cAMP-induced receptor degradation.

Table 1. Effect of cycloheximide and cAMP on cAMP receptor levels

Condition	cAMP-binding, % of control
Control	100
250 μg/ml Cycloheximide	78 ± 6
100 μM cAMP	30 ± 5
Cycloheximide + cAMP	33 ± 3

Aggregation competent cells were incubated for 2 h in the absence or presence of 250 μg/ml cyclohexamide and/or 100 μM cAMP (the cAMP was added each hour). Cells were washed extensively and [^{3}H] cAMP-binding to cells was measured in nearly saturated ammonium sulfate. Data shown are the means and SD of three determinations.

DISCUSSION

The activation of surface receptors by hormones leads to the production of intracellular second messengers, which is generally followed by a process of desensitization. Previous data and the experiments presented here suggest that in *Dictyostelium* signal transduction and desensitization consist of multiple parallel responses. In this organism, extracellular cAMP activates a surface receptor that interacts with one or more G-proteins. Subsequent activation of target enzymes results in the intracellular accumulation of cAMP, cGMP, Ins(1,4,5)P_3, and Ca^{2+}. Desensitization has been demonstrated physiologically; a brief incubation of cells with constant stimulus concentration induces the attenuation of signal transduction (see Janssens and Van Haastert, 1987). At a molecular level several putative mechanisms for this desensitization process have been described. These include phosphorylation of the receptor, which

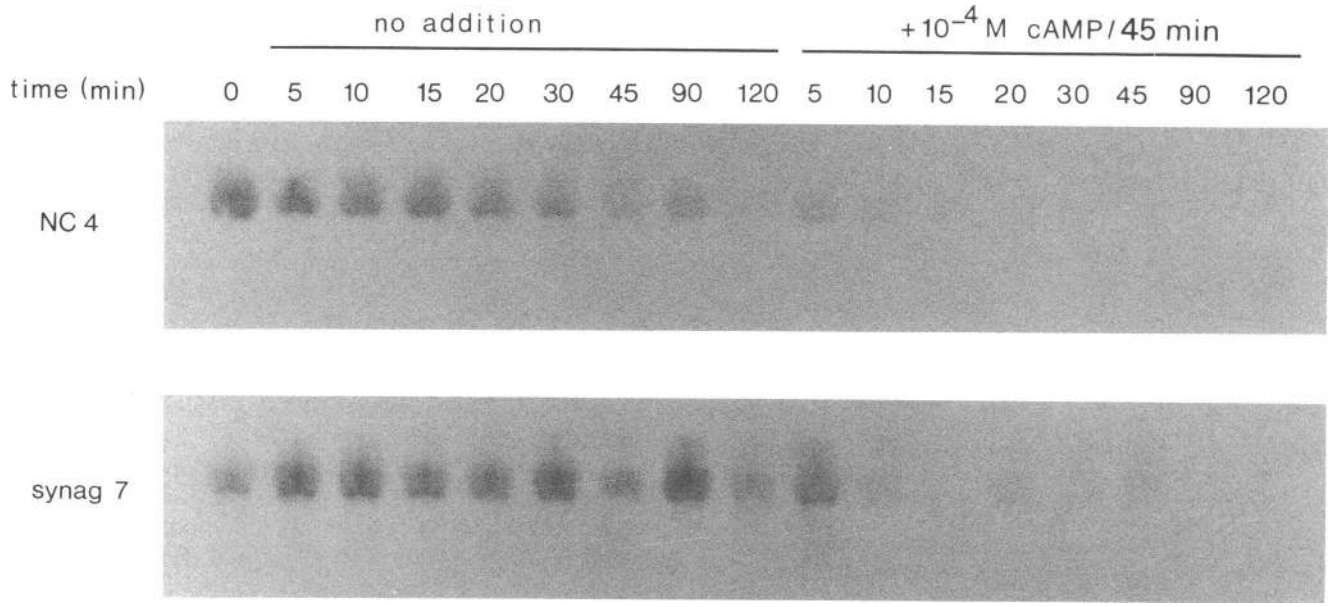

Figure 4. Kinetics of cAMP induced mRNA degradation. Vegetative NC4 (wild type) and *synag7* cells were resuspended to 10^7 cells/ml in PB and pulsed during 4 h with 30 nM cAMP at 6-min intervals to induce aggregation-competence. Cells were washed once and incubated for 30 min at 10^7 cells/ml. Subsequently cells were incubated for an additional 2 h in the presence or absence of 100 μM cAMP added at t = 0, 45, and 90 min. At the indicated time periods, 1.5-ml samples were centrifuged during 3 s at 10 000 × *g* and 0°C. Supernatants were rapidly removed and cell pellets frozen in liquid nitrogen. Total RNA was isolated, size fractionated on agarose gels, transferred to Gene Screen, and probed with ^{32}P-labeled cAMP receptor cDNA.

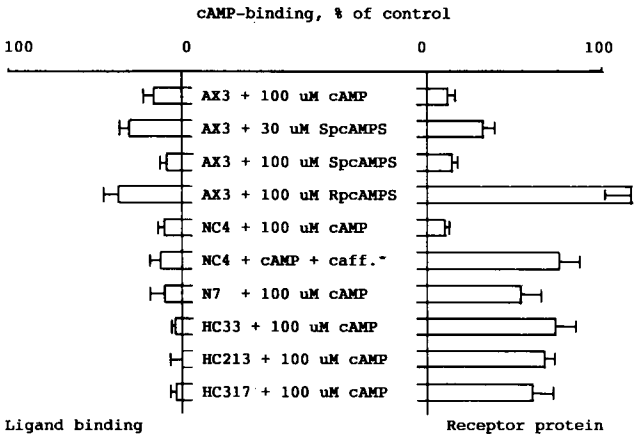

Figure 5. Signals for loss of ligand binding and receptor degradation. The wild-type cells (AX3 and NC4), *synag7* mutant (N7), *fgd*A mutant (HC33 and HC213), and *fgd*C mutant (HC 317) were pulsed with 100 nM cAMP at 6-min intervals during starvation for 6 h. Cells were then incubated in the absence or presence of 100 μM cAMP or cAMP analogues. Loss of ligand binding: cells were incubated with cAMP for 15 min, washed extensively, and [^{3}H]cAMP-binding to surface receptors was measured in phosphate buffer. Receptor protein: cells were incubated with cAMP for 3 h, washed extensively, and [^{3}H]cAMP-binding to total receptors was measured in ammonium sulfate. The control represents binding to cells that were incubated in parallel, but without cAMP. *Incubation contained 100 μM cAMP and 10 mM caffeine. The results show the means and SD of at least two experiments with triplicate determinations.

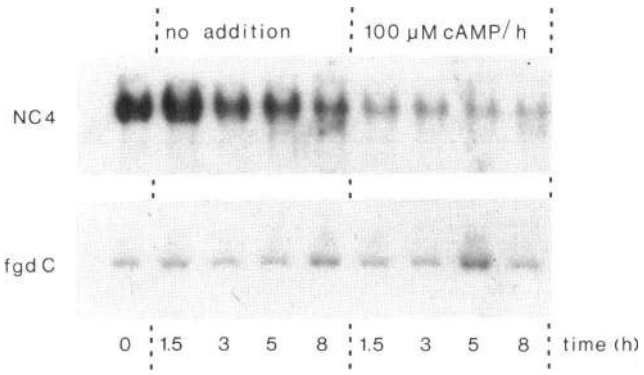

Figure 7. Regulation of cAMP receptor mRNA levels in mutant *fgd*C. Mutant *fgd*C cells (strain HC317) were treated as described in the legend of Figure 4, harvested, and incubated with 100 μM cAMP added each hour. Total mRNA was isolated at the times indicated, size-fractionated, transferred to nylon and hybridized with receptor cDNA.

is thought to impair activation of G-proteins (Devreotes and Sherring, 1985; Theibert and Devreotes, 1986; Klein *et al.*, 1987; Van Haastert *et al.*, 1987; Vaughan and Devreotes, 1988), and a rapid loss of functional binding activity of the receptor (Klein and Juliani, 1977; Van Haastert, 1987a).

We show here that two additional modes of desensitization may exist; cAMP induces a very rapid loss of receptor mRNA, and cAMP induces redistribution of receptors into patches and degradation of the receptor (Figures 1 and 3). It is possible that the receptor is degraded in an intracellular compartment and that the localization in patches represents the internalization of the receptor into endocytotic vescicles. In the absence of cAMP stimulation, the receptor protein appears to be fairly stable with a half-life of several hours, since receptor levels are not strongly affected by the protein synthesis inhibitor cycloheximide. In the presence of cAMP, the half-life of the receptor is reduced to 30–45 min. This implies that the loss of receptor protein during cAMP stimulation is not solely due to the reduced receptor mRNA levels but involves active cAMP-induced degradation of the protein. Summarizing these results, desensitization of cAMP-induced signal transduction is composed of at least four components: 1) loss of ligand binding without a loss of receptor protein; 2) receptor-effector uncoupling, presumably due to receptor phosphorylation; 3) redistribution and degradation of the cAMP receptor; and 4) loss of receptor mRNA.

On the basis of the kinetics and concentration dependencies of activation and desensitization of the receptor in wild-type cells, we deduce that the following series of events may occur. On binding of cAMP to the

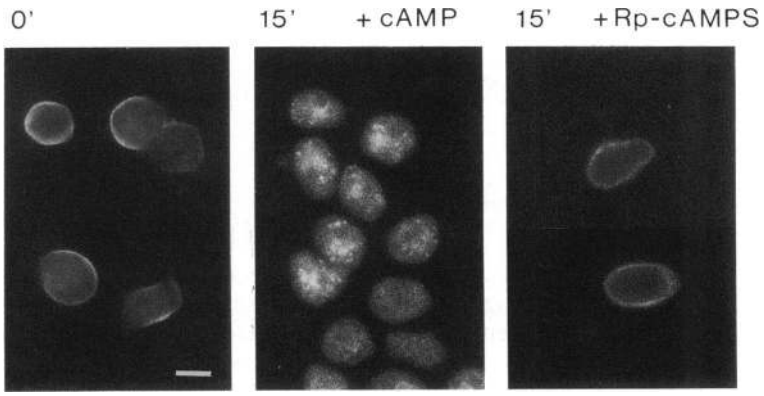

Figure 6. Effect of (Rp)-cAMPS on receptor translocation. Aggregation competent NC4 cells were incubated during 15 min with 10 μM cAMP or 100 μM (Rp)-cAMPS. Aliquots of cell suspension were fixed in methanol before and 15 min after cyclic nucleotide addition. Cells were stained with cAMP receptor specific antiserum and GARFITC. Bar length represents 1 μM.

Table 2. Signal transduction and desensitization of surface receptors

Activities	*synag7*	*fgd*C	*fgd*A	Rp-cAMPS
Activation adenylyl cyclase	−	+	−	−
Activation guanylyl cyclase	+	+	−	−
Activation phospholipase C	+	−	−	ND
Phosphorylation receptor	+	+	+	−
Loss of ligand binding	+	+	+	+
Degradation of receptor	−	−	−	−
Loss of receptor mRNA	+	−	ND	ND

+, present/normal; −, absent/strongly reduced; ND, not determined. Activation of second messengers and receptor phosphorylation have been described previously (Lappano and Coukell, 1982; Coukell et al., 1983; Van Haastert and Kien, 1983; Schaap et al., 1986; Van Haastert, 1987b; Jagalska and Van Haastert, 1988c; Kesbeke et al., 1988; Bominaar et al., 1991). Data for loss of ligand binding, degradation of receptor, and loss of receptor mRNA are from Figures 4, 5, and 7.

surface receptor, G-proteins and effector enzymes are activated within the first minute; half-maximal activation occurs at ~5 nM cAMP (Van Haastert and Van der Heijden, 1983; Van Haastert, 1987a). During this period receptors become phosphorylated, which occurs with the same sensitivity for cAMP as activation of the receptors (Vaughan and Devreotes, 1988). After ~1–5 min, loss of ligand binding occurs with a half-maximal effect at 50 nM cAMP (Van Haastert, 1987a). If cAMP is removed at this moment, cAMP-binding activity reappears at the cell surface, even if no protein synthesis takes place (Klein and Juliani, 1977). Prolonged incubation of cells with cAMP for 10–20 min leads to a redistribution of the receptor into patches (Wang *et al.*, 1988; Figure 1) and to degradation of the protein after 15–30 min (Figure 3). If cAMP is removed after 30 min, the receptor reappears at the cell surface only after several hours (Figure 2), and resynthesis is prevented by the protein synthesis inhibitor cyclohexamide (data not shown). In parallel to receptor degradation, receptor mRNA levels decrease with a half-life of ~5 min, which means that de novo synthesis of receptors is blocked (Figure 4).

Pharmacologic and genetic approaches suggest that these different routes of desensitization are largely independent (Table 2).

Phosphorylation of the Receptor and Loss of Ligand Binding

In wild-type cells, phosphorylation of the receptor occurs faster than loss of ligand binding and requires ~10-fold lower cAMP concentrations, indicating that essentially all receptors that lose binding activity were first phosphorylated. However, receptor phosphorylation is not essential for loss of ligand binding, because the analogue (Rp)-cAMPS induces the loss of binding (Figure 5) but not phosphorylation (Snaar-Jagalska *et al.*, 1988a). Loss of ligand binding is not due to the formation of receptor patches and internalization of the receptor, because (Rp)-cAMPS cannot induce these patches (Figure 6). Furthermore, this analogue in wild-type cells and cAMP in mutant *fgd*A reveal that loss of ligand binding can occur in the absence of second messenger responses. Thus activation of the receptor is not required to induce loss of ligand binding, and receptor occupancy with the agonist cAMP or the antagonist (Rp)-cAMPS may be sufficient. Receptor phosphorylation, however, may require some activation of the receptor and possibly minimal sensory transduction, because it occurs in mutant *fgd*A but is not induced by the antagonist (Rp)-cAMPS.

Receptor Degradation and Loss of Receptor mRNA

Experiments with mutants *synag7*, *fgd*A, and *fgd*C suggest that phosphorylation of the receptor and loss of ligand binding are not sufficient to induce receptor degradation. Receptor degradation requires the activation of at least two pathways that are defective in mutants *synag7* and *fgd*C, respectively. The cAMP-induced loss of receptor mRNA, however, does not require the pathway defective in *synag7*, whereas the pathway defective in *fgd*C is still required. Thus cAMP-induced inhibition of de novo receptor protein synthesis and stimulation of receptor protein degradation are mediated by partly overlapping but distinct sensory transduction pathways. In summary, these results suggest increasing requirements of sensory transduction pathways for 1) loss of ligand binding, 2) receptor phosphorylation, 3) loss of receptor mRNA, and 4) degradation of the receptor.

Mutants may allow the identification of the second messenger pathways that must be activated to induce these different desensitization processes. The role of adenylyl cyclase in receptor degradation as suggested by mutant *synag7* is confirmed by the adenylyl cyclase inhibitor caffeine; activation of this pathway is not required for the other desensitization processes. Activation of the *fgd*C gene-product is essential for both receptor degradation and loss of receptor mRNA. Although mutant *fgd*C is specifically altered in the activation of phospholipase C (as opposed to the activation of adenylyl and guanylyl cyclase), the connection of the *fgd*C gene-product with phospholipase C activation could be indirect. The defect of *fgd*C has been localized between cAMP receptor and G2, the G-protein that activates phospholipase C (Bominaar *et al.*, 1991). Because expression of G2 is also essential for the activation of adenylyl and guanylyl cyclase (and possibly other unknown second messenger pathways) (Kesbeke *et al.*, 1988), the *fgd*C gene product could be involved in the activation of the unknown second messenger pathway

as well. Therefore, a role of phospholipase C in receptor degradation and loss of receptor mRNA has to be confirmed by other mutants, preferentially in the phospholipase C gene.

Prolonged treatment of cells with high cAMP concentrations leads to a rapid decrease of receptor mRNA levels. In contrast, stimulation of cells with cAMP pulses enhances cAMP-binding, possibly due to enhanced transcription. Contact sites A are regulated by cAMP in a way similar to the cAMP receptor: increased mRNA levels after cAMP pulses and decreased mRNA levels after high cAMP (see Peters *et al.*, 1991). It will be interesting to investigate whether mRNAs of other aggregation-associated proteins are regulated in a similar way.

Desensitization of the β-adrenergic receptor adenylyl cyclase system has been thoroughly investigated (Lefkowitz and Caron, 1988). At least two components were identified: sequestration of receptors away from the cell surface and uncoupling of receptors from their effector systems. The uncoupling is thought to be mediated by phosphorylation of the receptor by cAMP-dependent protein kinase and by a β-adrenergic receptor kinase. Specific inhibitors of these processes suggest that each of these mechanisms of desensitization can occur independently but that the quantitive contribution of each of them is not additive (Lohse *et al.*, 1990).

We have demonstrated that the molecular mechanism of receptor phosphorylation, redistribution, degradation, and synthesis are regulated by different components of the signal transduction system. It is likely that these processes depend on different structural entities of the surface cAMP receptor and not only on the phosphorylation domain. Expression of mutant receptor genes may reveal the structural requirements for the different mechanisms of desensitization as well as their function during signal transduction and development.

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