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Localization of Chemoattractant Receptors on *Dictyostelium discoideum* Cells during Aggregation and Down-regulation

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cAMP chemoattractant receptors on the surface of *Dictyostelium discoideum* cells are visualized by means of immunocytochemistry. Receptor antigen is virtually absent from growing cells and begins to accumulate after about 6 hr of starvation, concomitant with the increase in surface cAMP binding activity. In aggregating cells, the antigen is uniformly distributed over the cell surface. Persistent cAMP stimulation, which leads to down-regulation of cAMP binding activity, induces a striking rearrangement of receptor antigen into patches or internal vesicles. A similar patching of receptor antigen is observed during tight aggregate formation, when surface cAMP binding activity decreases. These observations indicate that receptor down-regulation involves receptor agglomeration and suggest that receptor down-regulation takes place *in vivo*, when tight aggregates are being formed. © 1988 Academic Press, Inc.

INTRODUCTION

Upon starvation *Dictyostelium discoideum* cells form multicellular aggregates by means of chemotaxis. The chemotactic signal is extracellular cAMP, which is secreted by the cells in a pulsatile manner and is detected by cell surface cAMP receptors. The receptor has been purified to homogeneity, cloned, and sequenced. Its structure appears to fall into a family of receptors which interact with G-proteins and which bear seven transmembrane domains (P. Klein *et al.*, in preparation). Evidence has been presented that *D. discoideum* chemoattractant receptors interact directly with one or more G-proteins (Van Haastert, 1984; Janssens *et al.*, 1985; Theibert and Devreotes, 1986; Van Haastert *et al.*, 1986).

The chemoattractant-receptor interaction evokes a number of transient intracellular responses, such as actin polymerization (McRobbie and Newell, 1983), and the activation of adenylate and guanylate cyclases. These responses are terminated in spite of persistent stimulation by desensitization mechanisms: adaptation and down-regulation (Devreotes and Steck, 1979; Van Haastert and Van der Heijden, 1983; Klein and Juliani, 1977). It has been proposed that adaptation of adenylate cyclase involves a reversible chemoattractant-induced phosphorylation of the receptor, which causes a transition in electrophoretic mobility from M_r 40,000 to M_r 43,000. (Klein *et al.*, 1985; 1987b; Devreotes and Sherring, 1985). During down-regulation, a second mode of desensitization, constant cAMP stimuli induce a rapid loss of cAMP binding activity (Klein and Juliani, 1977; Kesbeke and Van Haastert, 1985). The mechanism of receptor down-regulation is not yet known, but the kinetics and dose dependence of this process differ from adaptation and cAMP-induced phosphorylation of the cAMP receptor (Van Haastert, 1987).

A polyclonal antiserum was prepared against the *D. discoideum* surface cAMP receptor, purified from membrane preparations by hydroxyapatite chromatography and two-dimensional SDS-PAGE electrophoresis (Klein *et al.*, 1987a,b). In Western blots of *Dictyostelium* membrane proteins these antisera specifically recognize the two bands of M_r 40,000 and M_r 43,000. In the present study, the antiserum was used to study the localization of the cAMP receptor on intact cells during development and during receptor down-regulation by cAMP. In aggregation stage cells, receptors appear to be uniformly distributed on the cell surface; however, ligand-induced receptor down-regulation is accompanied by an apparent agglomeration of cAMP receptors.

MATERIALS AND METHODS

Materials

Fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (GARFITC) was obtained from Nordic Laboratories (Tilburg, The Netherlands), peroxidase-conjugated swine anti-rabbit IgG was from DAKO (Denmark), and [2,8-^{3}H]cAMP (40 Ci/mmole) was from Amersham (UK).

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Culture and Incubation Conditions

D. discoideum NC-4 cells were grown in association with *Escherichia coli* 281 on glucose–peptone agar (Schaap and Spek, 1984). Cells were freed from bacteria by repeated washing with 10 m*M* phosphate buffer, pH 6.5 (PB). The cells were either incubated at 21°C on nonnutrient agar (1.5% agar in PB) at a density of 2.5 $\times 10^6$ cells/cm^2 or shaken at 150 rpm and 22°C in PB at a density of 10^7 cells/ml. Shaken cells were stimulated with 2×10^{-8} *M* cAMP pulses at 6-min intervals for 6 hr.

Immunocytochemical Methods

Cells were collected and resuspended to 5×10^5 cells/ml in PB. Five microliter droplets of cell suspension were placed on a glass slide and allowed to adhere for 5 min at room temperature. Excess PB was removed and the cells were fixed for 20 min in methanol at 4°C. In some experiments, the cells were covered with a 50- to 100-μm thick layer of 1.5% agarose in PB, before methanol fixation. This technique causes the cells to spread to a very thin layer and strongly enhances the visualization of cellular structures (Yunura and Fukui, 1985). The agarose layer is rinsed off during fixation. After fixation, the slides were washed three times in 0.7% NaCl in 10 m*M* phosphate buffer, pH 7.4 (PBS), and incubated overnight at 4°C with an antiserum which was evoked in rabbits against the purified *D. discoideum* cAMP receptor (Klein *et al.*, 1987b). The serum was either affinity-purified or preadsorbed to growing cells. The slides were washed four times with PBS and incubated for 1.5 hr with GARFITC. Subsequently, the slides were washed five times with PBS and mounted with 50% glycerol in PBS. Slides were observed and photographed on Kodak Tri-X Pan film, using a Leitz Laborlux 12 fluorescence microscope.

Purification of cAMP Receptor Antiserum

In methanol-fixed cells, the cAMP receptor antiserum shows some nonspecific reactivity. This background reactivity was almost completely removed when 20 μl of antiserum was adsorbed overnight with a 50-μl pellet of methanol-fixed vegetative cells, which had been washed with PBS and centrifuged at 8000*g* for 2 min. Background reactivity was also effectively removed by affinity purification of the antiserum: Membrane proteins from 2×10^9 aggregation competent *D. discoideum* cells were size fractionated by preparative slab SDS–PAGE and transferred to nitrocellulose. The blots were incubated for 18 hr with a 1:200 dilution of cAMP receptor antiserum and washed, and the receptor band was identified by staining narrow vertical strips of the nitrocellulose filter with peroxidase-conjugated swine anti-rabbit IgG. A horizontal strip of filter containing the receptor band was cut off and vortexed for 30 sec in 0.2 *M* glycine, pH 2.5. The released receptor IgG was neutralized, passed over a 10-ml Sephadex G-50 column, and concentrated by lyophilization. This method yielded a highly specific receptor antiserum, but also induced a considerable loss of specific reactivity.

Adsorption of Antiserum to Purified cAMP Receptor

The cAMP receptor was purified to homogeneity by hydroxyapatite chromatography and two-dimensional SDS–PAGE (Klein *et al.*, 1987a). One microliter of 200 ng/ml purified receptor in SDS (final concentration 0.03%) was incubated for 16 hr at 4°C with 25 μl of receptor antiserum, which had been preadsorbed to vegetative cells (see above). Control antiserum was incubated in 0.03% SDS in the absence of cAMP receptor.

Assay for Cell Surface cAMP Binding Activity

Cells from different stages of development were resuspended in PB at 10^8 cells/ml. Aliquots (45 μl) of cell suspension were incubated for 45 sec at 0°C with 5 μl of 10^{-7} *M* [^{3}H]cAMP (20,000 cpm) in 50 m*M* dithiothreitol. The cells were subsequently separated from the incubation mixture by centrifugation through silicone oil, and the radioactivity of the pellet was measured (Van Haastert and DeWit, 1984). Assay blanks, obtained by including 10^{-4} *M* cAMP in the incubation mixture, were subtracted.

RESULTS AND DISCUSSION

Localization of Receptors in Aggregation Competent Cells

We first investigated the localization of the chemotactic cAMP receptor in cells which had been stimulated for 6 hr at 6-min intervals with 20 n*M* cAMP to induce high levels of cAMP receptors. Figure 1A demonstrates that these cells exhibit a diffuse distribution of fluorescence over the entire cell with a relatively high fluorescence intensity at the cell periphery. Such a staining pattern would be expected if the antigen were equally distributed over the cell surface; the tangential view of the plasma membrane at the edge of the cell results in apparently intense fluorescence.

As a crucial control experiment, we preadsorbed the antiserum to cAMP receptor, which had been purified to homogeneity. Cells with high levels of cAMP receptors do not react to this antiserum anymore (Fig. 1B). This indicates that the antiserum, which was not preadsorbed to cAMP receptor (Fig. 1A), reacts very

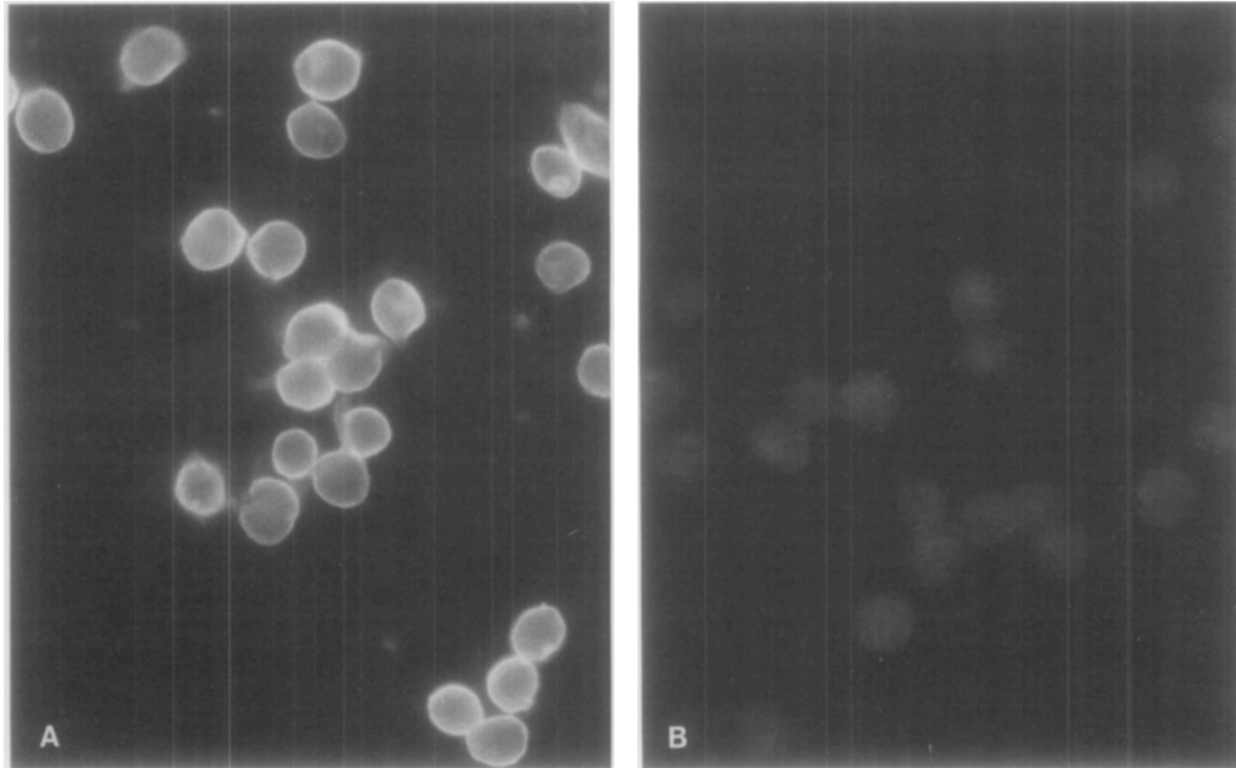

FIG. 1. Localization of cAMP receptors in aggregation competent cells. Growing cells were incubated for 6 hr at 10^7 cells/ml in PB; pulses of 2×10^{-8} *M* cAMP were added at 6-min intervals. The cells were collected and resuspended to 5×10^5 cells/ml; 5 μl droplets were placed on glass slides, covered with agarose, and fixed in 100% cold methanol. The slides were subsequently incubated with rabbit antiserum against the cAMP receptor, which was adsorbed to vegetative cells (A) or similarly treated receptor antiserum additionally adsorbed to purified cAMP receptor (B). Cell-associated anti-receptor IgG was visualized by incubation with FITC-conjugated goat anti-rabbit IgG (GARFITC). (A) 600×. (B) 600×.

specifically with the cAMP receptor in methanol-fixed cells.

Developmental Regulation of Receptor Antigen and Surface cAMP Binding Activity

As has been previously demonstrated, cell surface cAMP binding activity is developmentally regulated. It is low in growing cells, increases after a few hours of starvation to reach a maximum during early aggregation, and then decreases as the aggregates are forming tips (Fig. 2) (Malchow and Gerisch, 1974; Henderson, 1975; Green and Newell, 1975; Schaap and Spek, 1984).

This time course was compared to the expression of cAMP receptor antigen (Fig. 3). The intensity of fluorescent staining is low from 0 to 6 hr of starvation but increases strikingly during the next 4 hr. During aggregation (8 to 10 hr of starvation) fluorescent staining is specifically associated with the cell periphery. At 12 hr, when tight aggregates have been formed, the peripheral fluorescence seems to decrease, while some of the antigenic activity becomes localized in patches.

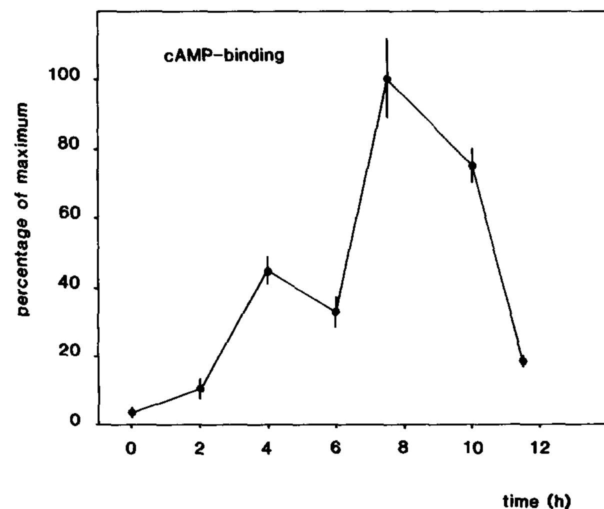

FIG. 2. Developmental regulation of cAMP binding activity. Growing cells were freed from bacteria and distributed over nonnutrient agar plates at a density of 2.5×10^6 cells/cm^2. Every 2 hr, cells were harvested for the assay of cAMP binding activity and prepared for immunocytochemistry. Binding data were expressed as percentages of the maximal level reached during 12 hr of development.

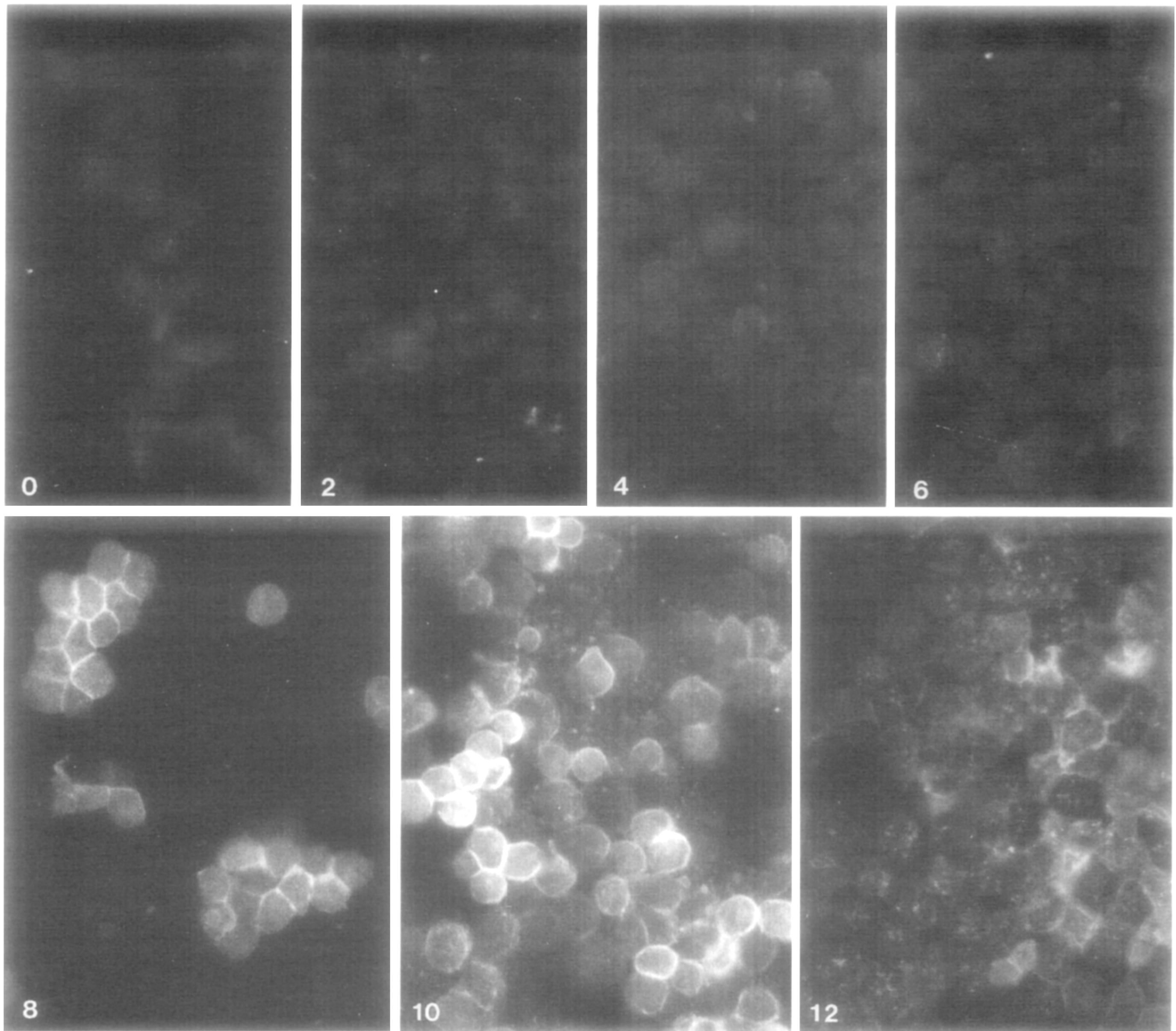

FIG. 3. Developmental regulation of receptor antigen. Cells, developing as described in the legend to Fig. 2, were harvested at 0, 2, 4, 6, 8, 10, and 12 hr of starvation, fixed in methanol, and stained with affinity-purified cAMP receptor antiserum and GARFITC. 600×.

Distribution of cAMP-Receptor Antigen during Receptor Down-Regulation

It was previously shown that the continued presence of cAMP induces a loss of surface cAMP binding activity (Klein and Juliani, 1977; Kesbeke and Van Haastert, 1985). At saturating cAMP concentrations (10 *μM*) surface cAMP binding activity decreases to 30% of the original activity after about 5 min of incubation. Within the next 20 min no further decrease takes place (Van Haastert, 1987). Little loss of receptor proteins occurs during down-regulation, since down-regulated receptors can be exposed with ammonium sulfate (Van Haastert, 1987), and receptor antigen, detected in Western blots, is not lost (Snaar-Jagalska *et al.*, 1988).

To determine whether cAMP-induced receptor down-regulation affects the distribution of receptors, the localization of the antigen was investigated after treatment of cells with 3 *μM* cAMP in the presence of 10 m*M* DTT. After 15 min of cAMP treatment, the antigen was dramatically rearranged. As shown in Figs. 4A and 4B, it appears that the antigen has accumulated into

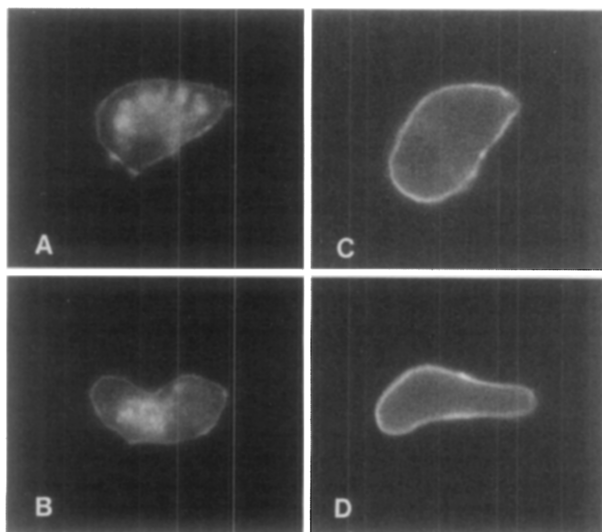

FIG. 4. cAMP receptor antigen in down-regulated cells. Vegetative cells were treated during 6 hr with 20 n*M* cAMP pulses at 6-min intervals. One batch of cells was subsequently incubated with 3 μ*M* cAMP in the presence of 10 m*M* DTT (A,B). Another batch of cells was pulsed for another 15 min (C,D). Both preparations were then covered with agarose, fixed in methanol, and stained with affinity-purified receptor antiserum and GARFITC. 1100×.

patches, while at the same time the amount of antigen at the periphery seems to be reduced. In control cells, which were not stimulated with cAMP, the antigen remained uniformly distributed over the cell periphery (Figs. 4C and 4D).

The concomitant decrease in cAMP binding activity and agglomeration of antigen, which occurs during stimulation of cells with constant cAMP stimuli, also seem to occur during tight aggregate formation *in vivo* (Figs. 2 and 3). As is the case during receptor down-regulation, a pronounced decrease in cAMP binding activity occurs during tight aggregate formation, which is accompanied by only a moderate loss of receptor antigen (compare Figs. 2 and 3, t = 12 hr). These observations suggest that during tight aggregate formation sufficiently high cAMP levels accumulate to induce receptor down-regulation.

In many systems, the down-regulation of surface receptors is accompanied by internalization of the receptor in clathrin-coated pits (Pastan and Willingham, 1981). This occurs for instance during down-regulation of the EGF receptor in KB cells and fibroblasts (Beguinot *et al.*, 1984; Stoscheck and Carpenter, 1984), during down-regulation of the α-factor pheromone receptor by its ligand in yeast (Jenness and Spatrick, 1986), and during down-regulation of β-adrenergic receptors in frog erythrocytes (De-Maw *et al.*, 1980).

In this study we demonstrate that the chemotactic cAMP receptor is uniformly distributed over the cell surface during the period of chemotactic cell movement and that a dramatic agglomeration of receptors occurs during ligand-induced receptor down-regulation. Further immunoelectronmicroscopic studies will be performed to identify the structural components which are involved in receptor patching and to follow the fate of the internalized receptor.

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