THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 260, No. 3, Issue of February 10, pp. 1757-1764, 1985
Printed in U.S.A.

Identification and Cyclic AMP-induced Modification of the Cyclic AMP Receptor in *Dictyostelium discoideum**

(Received for publication, August 14, 1984)

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We have recently identified a cell surface cAMP-binding protein by specific photoaffinity labeling of intact *Dictyostelium discoideum* cells with 8-N_3-[^{32}P] cAMP. The major photolabeled protein appears as a doublet (M_r = 40,000–43,000) in sodium dodecyl sulfate-polyacrylamide gel electrophoresis autoradiography. In this study, the doublet is shown to have the characteristics of the cAMP receptor responsible for chemotaxis and cAMP signaling. Both specific photoaffinity labeling of the doublet and binding of 8-N_3-[^{32}P]cAMP are saturable (K_D = 0.3 μM), the levels of both peak at 5 h, and both are inhibited by cAMP and several cAMP analogs in the same order of potency and with K_I values similar to those measured for inhibition of [^{3}H]cAMP binding. When cAMP-binding activity was partially purified (40-fold) and then photoaffinity labeled, the same bands (M_r = 40,000–43,000) were observed.

The relative intensities of the upper and lower bands of the doublet alternated at the same frequency as the spontaneous oscillations in cAMP synthesis. When oscillations were suppressed, the lower band of the doublet predominated. Following addition of cAMP, the relative intensity gradually shifted to the upper band. When cAMP was removed, there was a gradual restoration of the lower band form. We propose that the lower band form of the receptor activates chemotaxis and cAMP signaling and that the upper band form does not. This reversible receptor modification may then be the mechanism of adaptation, the process by which the physiological responses cease to be stimulated by persistent cAMP. Several developmentally regulated genes in *D. discoideum* have been reported to be induced or suppressed by pulses of cAMP (adaptive regulation) and others by continuous cAMP (nonadaptive regulation). These observations may be explained by the receptor modification reported here if the two forms of the receptor, which bind cAMP with the same affinity, independently influence gene expression.

Upon starvation, *Dictyostelium discoideum* undergoes a well-characterized developmental program, an early phase of which is the aggregation of itinerant amoebae (1). The chemotactic mediator for this aggregation is extracellular cAMP which is secreted periodically by central cells (2, 3). Proximal cells bind cAMP, move toward the central cells, and simultaneously secrete additional cAMP to relay the chemotactic signal (4–6). The cells then become refractory to cAMP and, for several minutes, show no response to additional cAMP at physiological levels (7). As the cAMP level declines, this process of adaptation resolves spontaneously, and cAMP can again stimulate chemotaxis and cAMP signaling (cAMP stimulated secretion of cAMP) (8). Adaptation of cellular responses to extracellular signals is a fundamental process seen in wide variety of signal transducing systems (9–12). However, the biochemical mechanism of adaptation has not been elucidated for any of these systems, except the chemotactic system of some flagellar bacteria in which chemoreceptors become methylated in the adapted state following stimulation with a chemoattractant (13).

A central component of cAMP-mediated reactions in *D. discoideum* is a surface cAMP receptor. A recent study suggests that chemotaxis and cAMP signaling are mediated by the same receptor.[1] This receptor has been characterized by [^{3}H]cAMP binding studies which demonstrate saturable binding, strict specificity for cAMP analogs, and developmental regulation (14–17). We have recently reported specific photoaffinity labeling of a cAMP-binding protein (M_r = 40,000–43,000) on the surface of *D. discoideum* using 8-azido-[^{32}P] cAMP (18). We show here that this photolabeled doublet is the cAMP receptor, based on the previously established criteria of saturable binding, specific inhibition by cAMP and cAMP analogs, and developmental regulation. Furthermore, the doublet appears to be involved in adaptation since the ratio of the intensity of the two bands changes during the adaptive process. In cells undergoing spontaneous oscillations in cAMP synthesis, the relative intensity of the doublet alternates between the upper and lower bands at the same frequency as the oscillation. This shift can be reversibly induced by addition (or removal) of exogenous cAMP when endogenous cAMP synthesis is suppressed.

EXPERIMENTAL PROCEDURES

Methods

Cell Growth and Development—Ax-3 cells were grown in HL-5 medium (19) and harvested at a density of approximately 5×10^6/ml. To initiate development, cells were washed in development buffer (5 mM Na_2HPO_4, 5 mM KH_2PO_4, 2 mM $MgSO_4$, 0.2 mM $CaCl_2$, pH 6.8), resuspended in development buffer at 2×10^7 cells/ml, and shaken at 130 rpm at 22 °C (20).

Saturation—8-N_3-[^{32}P]cAMP in methanol (specific activity = 58–68 Ci/mmol) was dried under an N_2 stream and resuspended in 10 mM phosphate (5 mM Na_2HPO_4, 5 mM KH_2PO_4) with 10 mM DTT[2]

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‡ Supported by National Institutes of Health Grant GM 28007. American Heart Association Established Investigator.

[1] A. Theibert, M. Palmisano, B. Jastorff, and P. N. Devreotes, in preparation.

[2] The abbreviations used are: DTT, dithiothreitol; DB, development buffer; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate.

to a final concentration of 3.6 μM, and 3-fold serial dilutions were done. Binding and photolabeling were carried out at 0 °C as described previously (18) by both the "standard assay" and the "miniassay" with 8-N_3-[^{32}P]cAMP at 18.5 nM to 1.8 μM (values based on the specific activity given by ICN). Specific and nonspecific (inclusion of 100 μM cAMP) binding were determined by Cerenkov counting of duplicate 10–15-μl samples prior to irradiation, and specific binding was normalized as a fraction of maximal binding. Nonspecific binding was always less than 20% of specific binding. After irradiation and isolation of membranes, samples were run on 10% SDS-PAGE (as described in Ref. 18) and autoradiographed for 15–48 h. The optical density of the doublet band was normalized as a fraction of maximal optical density. When photolabeled cells were lysed in TED (20 mM Tris, pH 7.5, 5 mM EDTA, 5 mM DTT) without protease inhibitors present, significant degradation to smaller fragments (M_r = 30,000–33,000) occurred. When these autoradiographs were scanned, the optical densities from the doublet (M_r = 40,000–43,000) and from the lower molecular weight bands were summed and normalized as a fraction of total incorporation of radioactivity. Since inclusion of protease inhibitors (as described in Ref. 18) yielded the doublet band (M_r = 40,000-43,000) as the only major species in subsequent experiments, no correction of optical density was necessary.

Nonradioactive 8-N_3-cAMP was used to compete [^{3}H]cAMP binding. [^{3}H]cAMP binding at 10 nM plus 8-N_3-cAMP from 1 mM to 100 nM was carried out exactly as described for the miniassay, except that the final pellets were resuspended in 0.3 ml of H_2O and counted in 3 ml of Liquiscint. Binding of [^{3}H]cAMP was normalized as a fraction of binding in the absence of 8-N_3-cAMP.

Inhibition of 8-N_3-[^{32}P]cAMP Binding and Photolabeling—8-N_3-[^{32}P]cAMP at 0.4–0.6 μM was incubated with an equal volume of cells (developed for 5 h) at 2 × 10^8/ml in both the standard assay (final volume = 0.6 ml) and the miniassay (final volume = 0.2 ml). cAMP or cAMP analogs were present at the following concentrations: cAMP at 10 nM to 1 μM; 2′-deoxy-cAMP at 50 nM to 10 μM; 6-chloro-cAMP at 2 μM to 200 μM; 8-bromo-cAMP at 5 μM to 1 mM; and N^6-monobutyryl-cAMP at 10 μM to 1 mM. Binding and photolabeling were carried out as described above and in Ref. 18 for each concentration of cAMP or cAMP analog and were normalized to maximal binding or photolabeling (in the absence of cAMP or cAMP analogs).

Developmental Regulation—Development was initiated at 1–1.5-h intervals by washing and resuspending cells at 2 × 10^7/ml in development buffer (20). At 9–10 h, all cells were collected by centrifugation at 700 × *g* for 4 min, washed twice in 10 mM phosphate with 10 mM DTT (as above), and resuspended in the same buffer at 2 × 10^8/ml. Each sample was then photolabeled using the miniassay described in Ref. 18 with 8-N_3-[^{32}P]cAMP at 0.6–0.8 μM. Nonspecific binding was determined by the inclusion of nonradioactive cAMP at 100 μM and was constant throughout development.

Oscillations—Cells were developed for 3.5 h in DB and washed once in the same buffer. Oscillations were monitored by measuring light scattering, using a modification of the method described by Gerisch and Hess (21). Samples (1 ml) were taken at 1-min intervals for 15 min and added to 3 ml of 10 mM phosphate at 0 °C. These cells were collected by centrifugation at 700 × *g* for 4 min and resuspended in 200 μl of 10 mM phosphate, 10 mM DTT with 0.8 μM 8-N_3-[^{32}P]cAMP. Photolabeling was then carried out as described in Ref. 18. Photolabeled membranes in sample buffer were run on SDS-PAGE, using 10% acrylamide and 0.05% bisacrylamide. This low concentration of cross-linker allowed a greater separation of the two bands of the doublet. Autoradiograms were scanned for optical density, and the fraction of optical density in the upper band was plotted as a fraction of total optical density in both bands of the doublet.

To suppress oscillations in cAMP signaling, cells developed for 4.5 h were washed in DB and placed in DB with 5 mM caffeine (22). After 20 min, during which no oscillations were seen by light scattering, cAMP at 10 pM was added. (This pretreatment had no physiological effect on the cells but appeared to give more constant measurements of 8-N_3-[^{32}P]cAMP binding.) A 1-ml prestimulus sample was taken at 30 min and added to 3 ml of 10 mM phosphate at 0 °C (for eventual photolabeling). The remainder of the cells were stimulated with cAMP by adjusting them to 10 mM DTT and 1 μM cAMP. Samples (1 ml) were taken from 30 s to 25 min following addition of cAMP and added to 3 ml of 10 mM phosphate at 0 °C (for eventual photolabeling). The remainder of the cAMP-stimulated cells were washed free of cAMP by washing twice in DB at 0 °C. These cAMP-free cells were diluted into DB (with 5 mM caffeine) at 22 °C. Samples (1 ml) were taken from 30 s to 32 min and placed in 10 mM phosphate at 0 °C. All samples were then washed in 10 mM phosphate with 10 mM DTT as described above, resuspended in 200 μl of 8-N_3-[^{32}P]cAMP at 0.8 μM, and photolabeled as described previously. Samples were run on low bisacrylamide gels as described above.

Partial Purification of cAMP-binding Activity—Cells developed for 5 h were washed in DB and then in lysis buffer (200 mM sucrose, 10 mM Tris-HCl, pH 8.5, 0.2 mM EDTA) and resuspended in lysis buffer at 5 × 10^7/ml with numerous protease inhibitors as described in Ref. 18 and kept at 0 °C. Cells were lysed by passage through two nucleopore polycarbonate filters (5-μm pore size). 8 ml of lysate were added to 10 ml of HEG (10 mM Hepes, pH 7.5, 1 mM EDTA, 10% w/v glycerol) and centrifuged at 4,500 × *g* for 20 min. The resulting pellet consisted of a firmly packed "button" and a soft overlying layer which was easily separated from the button and resuspended by gentle agitation in HEG (8 ml). An aliquot of this "soft pellet" suspsension was saved for [^{3}H]cAMP binding assay. The remainder was combined 1:1 with extraction buffer (24 mM CHAPS, 20 mM Hepes, pH 7.5, 2 mM DTT, 2 mM EDTA, 20% w/v glycerol) and 0.6 mM NaCl, incubated at 0 °C for 15 min, filtered through a 1.2-μm Millipore RA filter, and centrifuged at 30,000 × *g* for 45 min, and resuspended in 8 ml of HEG. To determine specific binding of [^{3}H]cAMP to cells, the soft pellet ("membranes" in Table I) and the CHAPS-extracted membrane pellet, 50 μl of sample, were added to 50 μl of 200 nM [^{3}H]cAMP (with 10 mM DTT and 0.2 mM 5′-AMP in HEG buffer) at 0 °C. After 10 s, 2 ml of 98% saturated ammonium sulfate was added. Nonspecific binding was determined by adding nonradioactive cAMP (at 10 μM) 10 s after the [^{3}H]cAMP, followed by ammonium sulfate 30 s later. The whole cell samples were centrifuged at 3,000 × *g* for 10 min, resuspended in ammonium sulfate, centrifuged again at 3,000 × *g* for 10 min, resuspended in 0.3 ml of water, and counted in Liquiscint. 100 μl of 1% bovine serum albumin was added to membranes and CHAPS pellet as a carrier. Samples were then centrifuged at 30,000 × *g* for 30 min, resuspended in ammonium sulfate, centrifuged again at 30,000 × *g* for 30 min, resuspended in 0.3 ml of water, and counted in Liquiscint. Protein concentrations for each fraction were measured using the Bio-Rad protein determination assay.

To photolabel the partially purified receptor preparation, 200 μl of the CHAPS-extracted membranes were added to 20 μl of 8-N_3-[^{32}P]cAMP at 0.15 μM in HEG buffer with 10 mM DTT at 0 °C. Nonspecific labeling was determined by inclusion of cAMP at 100 μM. After 30 s, 1 ml of 70% saturated ammonium sulfate was added, and after 10 min, the samples were centrifuged at 30,000 × *g* for 30 min. The pellets were resuspended in 200 μl of ammonium sulfate and irradiated as described previously. Irradiated membranes were then washed in 1.3 ml TED with protease inhibitors, centrifuged at 30,000 × *g* for 30 min, resuspended in sample buffer, and run on SDS-PAGE as described in Ref. 18.

In order to partially purify the doublet photolabeled on intact cells, cells photolabeled by the miniassay (18) were lysed in TED with protease inhibitors and centrifuged at 30,000 × *g* for 30 min. The membrane pellet was then extracted with CHAPS as described above, spun at 30,000 × *g* for 30 min, and resuspended in sample buffer for SDS-PAGE as described (18).

Materials

8-N_3-[^{32}P]cAMP (specific activity = 58–68 Ci/mmol) was from ICN. Caffeine, CHAPS, DTT, cAMP and cAMP analogs, and protease inhibitors (18) were from Sigma. Liquiscint was from National Diagnostics. Centrifugation was done in a Sorvall RC-5B centrifuge using SS-34 and HS-4 rotors. Counting of radioactive samples was done in a Beckman LS-7000 scintillation counter. Optical density of autoradiographs was determined using a Hoefer GS-300 optical scanner.

RESULTS

Binding of 8-N_3-[^{32}P]cAMP—Intact cells were incubated with 8-N_3-[^{32}P]cAMP at concentrations ranging from 18.5 nM to 1.8 μM and prepared for photolabeling as described under "Experimental Procedures." Specific binding, plotted as a fraction of maximal binding, is shown in Fig. 1*A*. Saturation occurred at approximately 1 μM (K_D = 0.3 μM as determined by Scatchard analysis), indicating that the affinity of the cAMP receptor for 8-N_3-cAMP is about 10-fold lower than for cAMP (K_D = 30–50 nM (15, 16, 23)) and higher than the affinity for other 8-substituted analogs (16). Cells were then

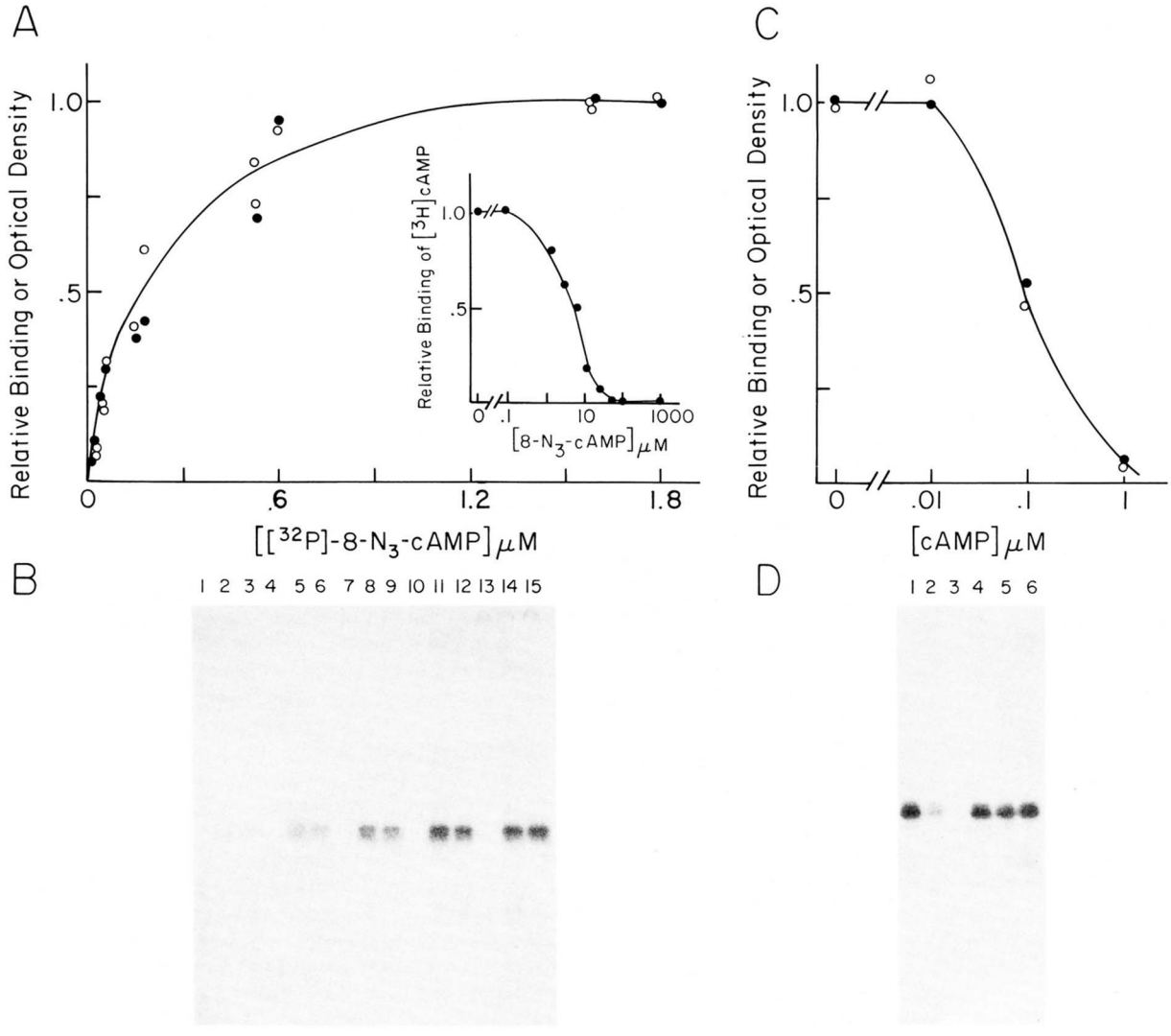

FIG. 1. **Saturation curve.** *A*, cells were incubated with 8-N_3-[^{32}P]cAMP at 18.5 nM to 1.8 μM. Binding was determined as described under "Experimental Procedures" and in Ref. 18 and was normalized to maximal binding (*closed circles*). Maximal binding was typically about 10^5 sites/cell (80,000 cpm/10^7 cells). *B*, cells were then irradiated and membranes were isolated and run on 10% SDS-PAGE. A representative autoradiogram is shown with ligand present at 20 nM (*lanes 1–3*), 55.6 nM (*lanes 4–6*), 0.167 μM (*lanes 7–9*), 0.5 μM (*lanes 10–12*) and 1.5 μM (*lanes 13–15*). The major band migrates as a doublet with an apparent M_r = 40,000–43,000 (18). *Lanes 1, 4, 7, 10,* and *13* show photolabeling when 100 μM cAMP was included in the binding assay. Autoradiograms were scanned, and the optical density in the doublet (M_r = 40,000–43,000) was normalized as a fraction of maximal optical density (*open circles* in *A*). *C*, cells were incubated with 8-N_3-[^{32}P]cAMP at 0.2 μM and binding was determined and plotted as described above (*closed circles*). *D*, cells were irradiated and membranes were analyzed on SDS-PAGE as above. The autoradiogram shows photolabeling with cAMP present at 10 nM (*lane 1*), 0.1 μM (*lane 2*), and 1 μM (*lane 3*). *Lanes 4–6* are controls in the absence of cAMP. The autoradiograms were scanned and plotted in *C* (*open circles*) as the fraction of binding in the absence of cAMP. *Inset* shows the competition of [^{3}H] cAMP binding by 8-N_3-cAMP determined as described under "Experimental Procedures."

irradiated, and the membrane pellet was analyzed by SDS-PAGE. As previously reported, about 90% of the covalently bound radioactivity migrated as a closely spaced doublet (M_r = 40,000–43,000) as the only major species. The intensity of the doublet increased with increasing concentrations of 8-N_3-[^{32}P]cAMP, saturating at approximately 1 μM (Fig. 1*B*, *lanes 2, 3, 5, 6, 8, 9, 11, 12, 14,* and *15*). Addition of 100 μM cAMP to the binding assay completely inhibited photolabeling of the doublet (Fig. 1*B*, *lanes 1, 4, 7, 10,* and *13*). Autoradiographs were scanned, and incorporation of radioactivity into the doublet was plotted in Fig. 1*A*. The saturation curves for noncovalent binding and photoaffinity labeling of the doublet are identical.

Noncovalent binding of 8-N_3-[^{32}P]cAMP and photolabeling of the doublet were measured in the presence of increasing concentrations of nonradioactive cAMP. Noncovalent binding of 8-N_3-[^{32}P]cAMP was competed by cAMP in the 10 nM to 1 μM range (Fig. 1*C*, *open symbols*). Cells were irradiated and membranes were prepared and analyzed by SDS-PAGE. Fig. 1*D* illustrates that cAMP inhibited incorporation of radioactivity into the doublet in the same concentration range (*lane 1* = 10 nM, *lane 2* = 100 nM, *lane 3* = 1 μM). Autoradiographs were scanned, and incorporation of radioactivity into the doublet band was plotted in Fig. 1*C*. The cAMP competition curves for noncovalent binding and photoaffinity labeling are identical.

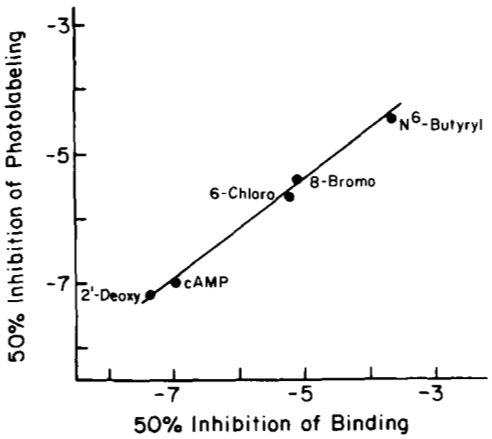

FIG. 2. **cAMP analogs.** Binding and photolabeling were carried out as described under "Experimental Procedures" with cAMP analogs present at the following concentrations: 2′-deoxy-cAMP at 10 nM to 10 μM, 6-chloro-cAMP at 2 μM to 200 μM, 8-bromo-cAMP at 5 μM to 1 mM, and N^6-monobutyryl-cAMP at 10 μM to 1 mM. The concentration that gave 50% inhibition of 8-N_3-[^{32}P]cAMP binding was plotted against 50% inhibition of photolabeling as determined by optical density on autoradiograms. Both axes are in log scale. The K_I values were 26 nM, 2.3 μM, 2.6 μM, and 150 μM for 2′-deoxy-cAMP, 6-chloro-cAMP, 8-bromo-cAMP, and N^6-monobutyryl-cAMP, respectively.

The K_I for cAMP inhibition of both photolabeling of the doublet band and noncovalent binding was about 70 nM (Fig. 1*C*), which is similar to the K_D reported for [^{3}H]cAMP binding (30–50 nM, see Refs. 15, 16, and 23). Nonradioactive 8-N_3-cAMP competes the binding of [^{3}H]cAMP (Fig. 1, *inset*) in the same concentration range as 8-N_3-[^{32}P]cAMP binds (K_I of about 1 μM). These K_I values are within the reported concentration ranges over which cAMP (1 nM to 1 μM) and 8-N_3-cAMP (approximately 1 μM to 10 μM) stimulate chemotaxis and cAMP signaling (16, 24).[3] The total number of sites (about 10^5/cell) was the same when determined by [^{3}H]cAMP or 8-N_3-[^{32}P]cAMP binding. In all of these determinations, the optical density of the upper and lower bands of the doublet were considered together. However, note that there is no apparent difference in the relative intensities of the two members of the doublet at any level of saturation or competition.

cAMP Analogs—A receptor molecule can be pharmacologically characterized by the relative potency with which structural analogs inhibit ligand binding or activate a defined physiological response. [^{3}H]cAMP binding to the cAMP receptor is inhibited by cAMP analogs in a characteristic order of potency (16). This order is consistent with the order of potency of these analogs in eliciting chemotaxis and cAMP signaling and is distinct from the order of binding to either the surface phosphodiesterase or the intracellular regulatory subunit of cAMP-dependent protein kinase (16). We determined whether this characteristic order of potency would be seen for inhibition of 8-N_3-[^{32}P]cAMP binding and photolabeling of the doublet by testing the cAMP analogs 2′-deoxy-cAMP, 6-chloro-cAMP, 8-bromo-cAMP, and N^6-monobutyryl-cAMP. The concentration of analog that gave 50% inhibition of the binding was plotted against the concentration of analog that reduced by 50% the incorporation of radioactivity into the doublet. The analogs inhibited binding of 8-azido-[^{32}P]cAMP and photolabeling of the doublet with the same order of effectiveness that had been described previously (16). The K_I values calculated from the data in Fig. 2, for 2′-deoxy-cAMP, 6-chloro-cAMP, 8-bromo-cAMP, and N^6-monobutyryl-cAMP were 26 nM, 2.3 μM, 2.6 μM, and 150 μM, respectively, which are compatible with the K_{50} values reported for stimulation of cAMP signaling, chemotaxis, and inhibition of [^{3}H]cAMP binding (30–85 nM, 1.7–2.4 μM, 2.3–47 μM for 2′-deoxy-cAMP, 6-chloro-cAMP, and 8-bromo-cAMP, respectively, (see Ref. 16 and Footnote 1). These data suggest that 8-N_3-[^{32}P]cAMP and [^{3}H]cAMP bind to pharmacologically equivalent receptors and that this receptor is the doublet.

Developmental Regulation—Axenically grown cells were suspended in nonnutrient buffer to initiate development. Aliquots were taken at various intervals from 0–11 h and photoaffinity labeled with 8-N_3-[^{32}P]cAMP, as described under "Experimental Procedures." As seen in Fig. 3, *A* and *B*, binding activity increased during development, reached a peak at 5 h, and declined sharply thereafter to less than 10% of the peak value at 9 h of development. Following irradiation, membrane pellets were isolated and analyzed by SDS-PAGE. As seen in Fig. 3*C*, the majority of the covalently bound radioactivity was incorporated into the doublet band at all stages of development, although the relative intensities of the upper and lower bands of the doublet varied in a manner not related to the developmental stage. (The origin of this variation is revealed below.) The autoradiographs were scanned, and radioactivity incorporated into the doublet was plotted in Fig. 3, *A* and *B*. The developmental pattern of photoaffinity labeling of the doublet band paralleled that of the binding activity. [^{3}H]cAMP binding during development (as previously reported, see Refs. 14 and 15) was also assayed on separate days and was found to parallel closely the developmental regulation of binding and photolabeling by 8-N_3-[^{32}P]cAMP. Photolabeling of the cytosolic regulatory subunit of cAMP-dependent protein kinase during development was carried out in parallel on two occasions. The developmental time course agreed with the previously reported developmental regulation of the kinase with a plateau at 9–10 h of development (25).

Correlation of Ligand Binding with Photolabeling and Partial Purification—Throughout these experiments, the noncovalent binding of 8-N_3-[^{32}P]cAMP correlated well with covalent binding of the photolabel (Figs. 1–3), indicating that the 8-N_3-[^{32}P]cAMP photolabels the same protein that it binds to noncovalently. Furthermore, binding and photolabeling were examined in a partially purified membrane fraction. As previously reported, when photolabeled cells are lysed and membranes collected by centrifugation, about 60–70% of the labeled doublet is recovered with significant removal of cytosolic proteins (18). The doublet is also present in membranes treated with the zwitterionic detergent CHAPS. When CHAPS-extracted membranes were prepared without prior photolabeling, the specific activity of [^{3}H]cAMP binding was increased about 40-fold as shown in Table I. Photolabeling of this fraction also identifies a doublet (M_r = 40,000–43,000) as the major cAMP-binding protein.

cAMP-induced Receptor Modification—The relative intensities of the bands composing the doublet varied in independent cultures of cells (see Figs. 1 and 3). We wondered whether this variable appearance of the receptor doublet depended on the physiological state of the cells at the moment of sampling. It is well known that *D. discoideum* undergoes spontaneous oscillations in cAMP synthesis (3). The physiological state of the cells is expected to vary periodically at the same frequency. Cells undergoing spontaneous oscillations were monitored by continuous measurement of light scattering (Fig. 4*A*), and

[3] P. Klein, A. Theibert, D. Fontana, and P. Devreotes, unpublished data.

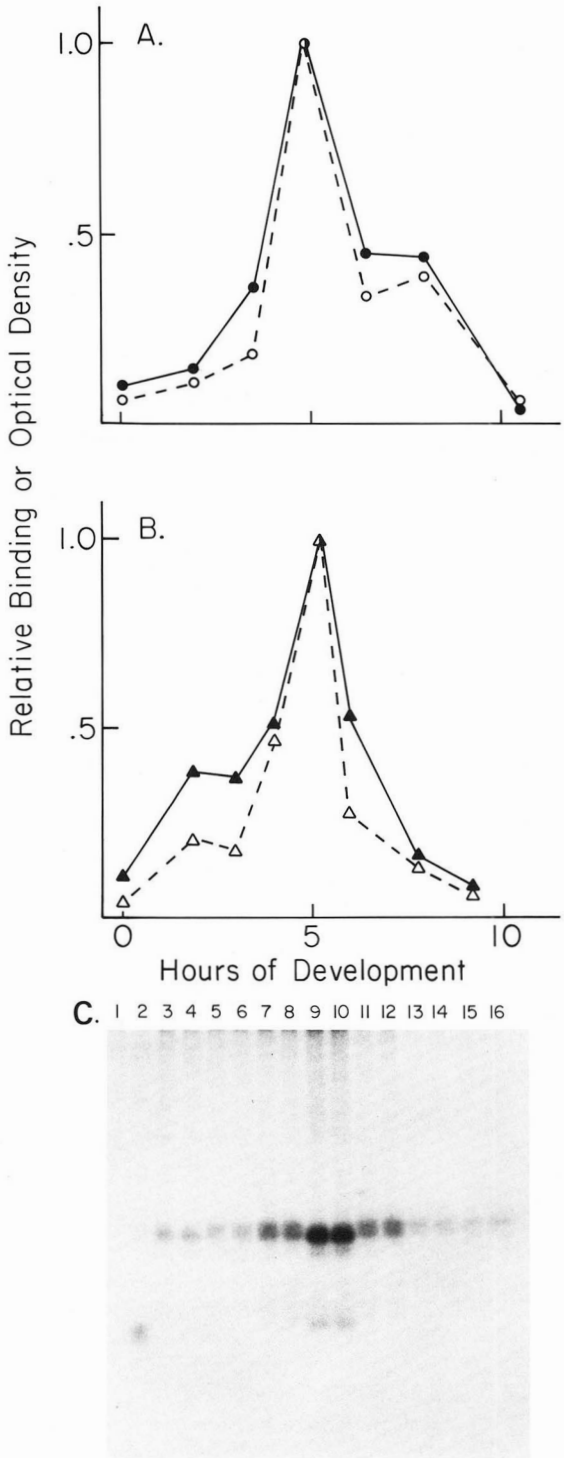

FIG. 3. **Developmental regulation.** Development was initiated by transfering cells to DB at 1–1.5-h intervals. After 9–11 h, all cells were harvested, and binding and photolabeling were carried out as described under "Experimental Procedures." *A* and *B* show a comparison of noncovalent binding with photolabeling (as determined by optical scanning of autoradiograms) as a function of developmental age in two different experiments. *C* shows a representative autoradiogram (corresponding to the data shown in *B*) with each time point run in duplicate, for cells at 0–9.5 h of development. The origin of the variation in electrophoretic mobility is discussed in the text.

samples were taken at 1-min intervals, washed at 0 °C, and surface receptors photoaffinity labeled with 8-N_3-[^{32}P]cAMP. Membranes were prepared and analyzed by SDS-PAGE. As

TABLE I

Partial purification of the cAMP receptor

Fraction	Specific activity	Recovery
	sites × 10^{-12}/*mg protein*	%
Cells	1.4	100
Membranes[a]	6.7	96
CHAPS-extracted membranes[b]	43.3	19

[a] Cells were lysed by passage through a 5-μm pore size nucleopore filter. The lysate was centrifuged at 4500 × *g* for 20 min. The pellet consisted of a tightly packed button and a soft overlying layer. This soft pellet, designated membranes, was recovered for [^{3}H]cAMP binding assays as described under "Experimental Procedures."

[b] Membranes extracted with 12 mM CHAPS as described under "Experimental Procedures" were spun at 30,000 × *g* for 45 min and the pellet ("CHAPS-extracted membranes") was recovered for [^{3}H] cAMP binding assays as described under "Experimental Procedures."

shown in Fig. 4*B*, the relative distribution of receptors between the upper and lower bands of the doublet varied with the same frequency as the periodic changes in light scattering. Two and one-half cycles are illustrated. Just before the phase of active cAMP synthesis (at about −2, 4, 10, and 16 min in Fig. 4*A*), the lower band of the doublet predominates (Fig. 4*B*). During the active phase of cAMP synthesis (4–6 min and 10–12 min in Fig. 4*A*), there is a time-dependent shift in the distribution (Fig. 4*B*). The upper band of the doublet increases in relative intensity as cAMP synthesis ceases (6 and 12 min in Fig. 4*B*). The samples were analyzed under gel conditions that more clearly separate the upper and lower bands of the doublet (Fig. 4*C*). The autoradiographs were scanned, and the fractional intensity of the upper band of the doublet was calculated. This quantitation shows that the intensity rapidly shifts to the upper band of the doublet during the time cells are secreting cAMP. The fractional intensity of the upper band reaches a peak of about 65%, then gradually returns to a value of about 30% just before the cycle repeats.

To demonstrate that extracellular cAMP causes the shift in electrophoretic mobility of the surface receptor (from M_r = 40,000–43,000), cells were pretreated with caffeine for 20 min at 22 °C. This treatment blocks spontaneous oscillations in cAMP synthesis (activation of adenylate cyclase is blocked, the remaining cAMP is degraded by endogenous phosphodiesterases, and the signaling system is expected to attain a basal state (22)). When an aliquot of these cells was photolabeled at 0 °C, 90% of the radioactivity was incorporated into the lower band of the doublet (Fig. 5, *lane 1*). These caffeine-treated cells were then stimulated with 1 μM cAMP at 22 °C (in the presence of 10 mM DTT to inhibit phosphodiesterase), and aliquots were photolabeled as above. Continuous stimulation with 1 μM cAMP (a saturating dose) caused a gradual shift until 80% of the radioactivity was in the upper band (Fig. 5, *lanes 2–7*). Cells were then washed free of the cAMP stimulus and incubated at 22 °C. Photolabeling of these cells showed a return of intensity to the lower band (Fig. 5, *lanes 8–13*).

These observations suggest that the upper and lower bands of the doublet represent two interchangeable forms of the receptor. Upon re-examination of Fig. 1, it appears that the two forms of the receptor have similar affinity for cAMP or 8-N_3-[^{32}P]cAMP. In the saturation curve for which the gel is shown (Fig. 1*B*), the upper band of the doublet predominated. Approximately the same ratio of upper band to lower band is apparent at each concentration when samples were photoaffinity labeled at concentrations from 10 nM to 1.8 μM. In another experiment (data included in graph, gel not shown),

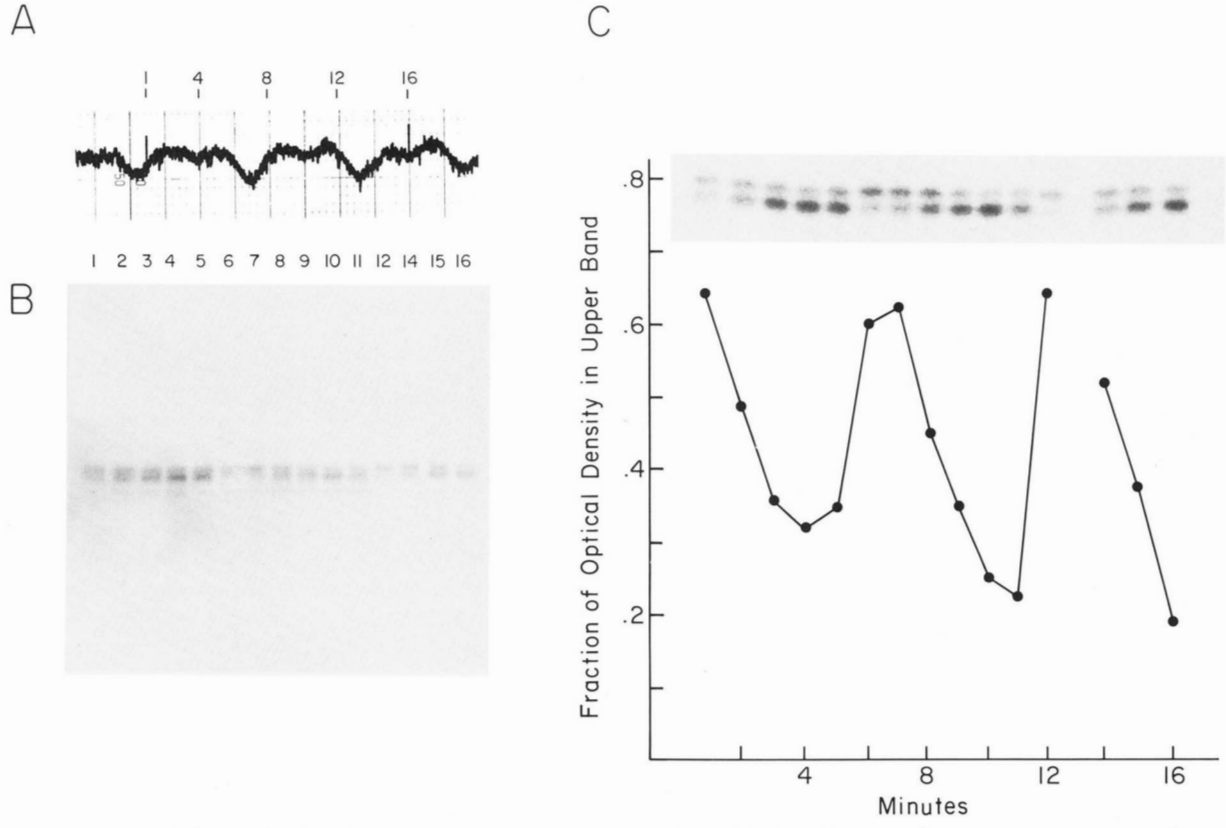

FIG. 4. **Photolabeling during spontaneous oscillations in cAMP synthesis.** Cells were developed for 3.5 h as described under "Experimental Procedures." *A*, oscillations in cAMP synthesis were monitored by measuring light scattering using a modification of the procedure described by Gerisch and Hess (21). *B*, samples were taken at 1-min intervals (sample 13 was lost) during the oscillations and photolabeled as described (18). Isolated membranes were run on 10% SDS-PAGE (10% acrylamide and 0.8% bisacrylamide), and autoradiograms were exposed without intensifying screens. *C*, the same samples were run on SDS-PAGE with 10% acrylamide and 0.05% bisacrylamide which enhanced the separation of the two bands of the doublet. The autoradiogram was scanned, and the optical density of the upper band was plotted as a fraction of total optical density in the doublet.

the two bands of the doublet were about equal, and their relative intensities did not vary across the concentration curve. In the cells taken for the cAMP competition curve, it happened that the lower band of the doublet was predominant (Fig. 1*D*). This ratio of upper to lower band did not vary at 50% (100 nM cAMP) or 95% (1 μM cAMP) competition. In other competition experiments (data included in graph, gel not shown), approximately equal amounts of upper and lower bands were apparent and did not vary as a function of cAMP in the binding assay. The affinity of both forms of the receptor, the lower band and the upper band, were also measured directly. [^{3}H]cAMP binding curves were done for caffeine-treated cells (lower band predominant, as in Fig. 5, *lane 1*) and cAMP-stimulated cells (upper band predominant, as in Fig. 5, *lanes 6* and *7*). Scatchard analysis showed that following cAMP stimulation, there were about 15% fewer cAMP-binding sites, but the two forms of the receptor had identical binding affinities. This result is consistent with previously reported binding studies that show a negligible change in the affinity for cAMP as sensitive cells become adapted (26).

DISCUSSION

The cAMP receptor of *D. discoideum* has been previously characterized by specific, saturable binding of cAMP, pharmacological specificity for cAMP analogs, and developmental regulation (14–17). The doublet identified by specific labeling of the cell surface with 8-N_3-[^{32}P]cAMP has the same properties as the receptor. 1) Both noncovalent binding of 8-N_3-[^{32}P]cAMP and incorporation of radioactivity into the doublet are specifically inhibited by cAMP with a K_I that is close to the K_D for [^{3}H]cAMP binding, and the apparent K_{50} values for cAMP activation of chemotaxis and cAMP signaling. 2) Both are saturable (with a K_D near the K_I for 8-N_3-cAMP inhibition of [^{3}H]cAMP binding). Also, binding of 8-N_3-[^{32}P]cAMP occurs in approximately the same concentration range as for 8-N_3-cAMP activation of chemotaxis and cAMP signaling (24).[3] 3) Both are stringently regulated during development, increasing to a maximum level at 5 h (when cells begin to form aggregates) and declining to low levels by 9 h as tight aggregates form. 4) Both are inhibited by cAMP analogs with the same pharmacological specificity as [^{3}H]cAMP binding. Based on these criteria, we propose that this doublet contains the external binding site of the cAMP receptor that mediates chemotaxis and cAMP signaling.

These criteria distinguish the doublet from other cAMP-binding proteins such as phosphodiesterase (M_r = 48,000) and the regulatory subunit of cAMP-dependent protein kinase (M_r = 41,000). Phosphodiesterase has a lower apparent affinity for cAMP (K_M = 1.7–30 μM, see Ref. 27). Furthermore, each of these proteins has a different and characteristic order of affinity for cAMP analogs (16). The doublet paralleled the order of affinity known for the surface receptor and was clearly distinct from the phosphodiesterase and the regulatory subunit of cAMP-dependent protein kinase. The receptor

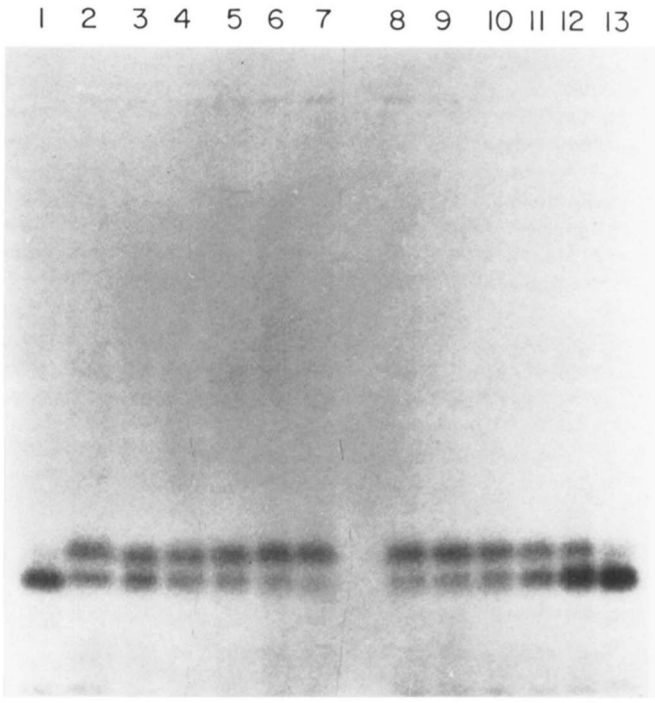

FIG. 5. **The effect of exogenous cAMP on electrophoretic mobility.** Cells were developed for 4.5 h and then incubated for 30 min in 5 mM caffeine at 22 °C to suppress spontaneous oscillations in cAMP synthesis. Cells were pretreated with 10 pM cAMP. A portion of these caffeine-treated cells was removed for photolabeling (*lane 1*), and the remainder was stimulated with cAMP at 1 μM with 10 mM DTT to inhibit phosphodiesterase. Samples were removed at 0.5, 1, 2, 4, 10, and 25 min and then photolabeled (*lanes 2–7*). At 12 min, a portion of the cells was washed free of cAMP at 0 °C and then incubated at 22 °C. Samples were taken at 0.5, 2, 4, 8, 16, and 32 min (*lanes 8–13*) and photolabeled.

doublet could also be distinguished from the regulatory subunit of cAMP-dependent protein kinase by its pattern of developmental regulation since the protein kinase reaches a plateau at 10 h of development (25) when the receptor doublet has fallen off to low levels. Furthermore, the protein kinase does not shift its position in SDS-PAGE after stimulation with cAMP and does not run as a doublet (18, 25).

Several authors have reported photoaffinity labelling of intact *D. discoideum* using 8-N_3-[^{32}P]cAMP (24, 28, 29). In each case, radioactivity was incorporated into many bands on SDS-PAGE. cAMP competition of specific bands was reported by Wallace and Frazier (24) (M_r = 40,000), Hahn *et al.* (29) (M_r = 36,000 and 33,000), and Juliani and Klein (28) (M_r = 45,000). The latter shifted (to M_r = 47,000) in down-regulated cells (*i.e.* following addition of 10 μM cAMP), similar to the shift we have observed. Each of these previously reported bands may be related to the doublet we have identified.[4] However, in addition to the large degree of nonspecific binding in those studies, the bands were not distinguished from the regulatory subunit of protein kinase. Our results differ in that the doublet was specifically labeled with high efficiency and was clearly distinguished from the protein kinase. Our techniques have allowed for unambiguous identification of the doublet as the receptor and for quantitative measurements of the cAMP-induced shift. Meyers-Hutchins and Frazier (30) have purified a cAMP-binding protein (M_r = 70,000) that can be labeled with 8-N_3-[^{32}P]cAMP. They were unable to label this band on intact cells. We were able to photolabel a band (M_r = 70,000) in intact cells, which may represent cell-surface labeling of the cAMP-binding protein described by Meyers-Hutchins and Frazier (30). Although less than 3% of the total radioactivity is incorporated into this band which precluded a detailed characterization, labeling of this band did appear to parallel receptor binding activity, and in one experiment, cAMP caused a shift in its apparent molecular weight. We cannot rule out the possibility that the major polypeptide (M_r = 40,000–43,000) that we have identified is a fragment of a larger polypeptide, despite the inclusion of protease inhibitors.

The relative intensities of the upper and lower bands of the receptor doublet varied with the physiological state of the cells at the moment of sampling (Figs. 4 and 5). The simplest explanation of these observations is that the labeling of cAMP induces a reversible modification of the surface receptor which decreases its mobility in SDS-PAGE. An exciting possibility is that this modification is the biochemical mechanism of adaptation, the process that gradually extinguishes the cAMP-induced activation of adenylate cyclase, and is the basis for the spontaneous oscillations in cAMP synthesis. We have shown here that the relative intensities of the upper and lower bands of the doublet alternate at the same frequency as the oscillations. The fraction of receptors in the upper band reached a peak value of about 65% just *after* the active phase of cAMP synthesis, suggesting a causal relationship between the deactivation of adenylate cyclase and the modification of the receptor.

When oscillations were suppressed, the receptor migrated predominantly in the position of the lower band of the doublet (Fig. 5, *lane 1*). When a cAMP stimulus was continuously applied, 80% of the doublet shifted to the upper band (Fig. 5, *lanes 2–7*). This distribution remained until removal of the cAMP stimulus, which initiated a return to the basal state where only 10% of the receptors were again found in the position of the upper band (Fig. 5, *lanes 8–13*). This is the pattern of modulation to be expected if receptor modification is the mechanism of adaptation (7, 8). We propose that the lower band is an unmodified form of the receptor which effectively activates chemotaxis and cAMP signaling. The upper band would represent the modified form. It binds cAMP with the same affinity, but chemotaxis and signaling are not activated when the majority of the receptor is in this form. An alternate possibility which cannot be excluded is that the lower band is the modified form and the upper band is unmodified.

Caffeine, which blocks the activation of adenylate cyclase (22), appears to have little effect on the cAMP-induced modification of the receptor. (The experiment shown in Fig. 5 was carried out in the presence of caffeine and is consistent with the experiment shown in Fig. 4). This result is consistent with our previous report that cells can adapt independently of adenylate cyclase activation when an exogenous cAMP stimulus is applied (31) and supports the hypothesis that the receptor modification is related to adaptation.

The doublet contains the cAMP-binding site of the receptor since it is photolabeled by 8-N_3-[^{32}P]cAMP both in intact cells and in a partially purified membrane fraction. This polypeptide also appears to contain a modification site since its position in SDS-PAGE shifts upon stimulation with cAMP. However, the two forms of the receptor have the same affinity for cAMP and 8-N_3-[^{32}P]cAMP, suggesting that the modification site is independent of the cAMP-binding site. It seems likely that this modification requires an internal metabolite and that the modification site is on the cytoplasmic

[4] We have noted that the apparent molecular weight of the polypeptide depends markedly on the percentage of bisacrylamide in SDS-PAGE. The doublet also has a tendency to break down to fragments (M_r = 33,000 to 36,000) in the absence of protease inhibitors.

face of the membrane. This would imply that the doublet is a membrane-spanning polypeptide containing a hydrophobic region. The methylated chemotaxis proteins of flagelated bacteria contain at least three functional domains on one polypeptide:an external ligand binding site, a transmembrane sequence, and a cytoplasmic domain which is methylated during the adaptation process (32, 33). By analogy, the cAMP receptor may also be a transmembrane protein with an extracellular cAMP-binding site, a hydrophobic transmembrane sequence, and a cytoplasmic domain that is modified during adaptation to a cAMP stimulus. Receptors in higher organisms, such as the insulin receptor, the epidermal growth factor receptor, and β-adrenergic receptors, can also be modified following ligand binding (34–36). While some of these receptors, such as the epidermal growth factor receptor (35), appear to carry the ligand-binding site and the modification site on the same polypeptide, others, such as the insulin receptor (34), have a complex subunit structure with a ligand-binding site and a modification site on different subunits. The native structure of the cAMP receptor could be composed of multiple, noncovalently bound subunits not identified by photolabeling. These subunits might mediate the physiological processes that follow cAMP binding. However, it is clear that the same polypeptide binds cAMP and is modified.

The receptor can also serve as a model for developmental regulation of other surface receptors since its expression is tightly regulated through development. Furthermore, the receptor itself influences the developmental regulation of several gene products. For example, the induction of the cAMP receptor, adenylate cyclase, and the membrane phosphodiesterase (as reviewed in Ref. 37) and the suppression of M4-1 RNAs[5] require pulses of cAMP every 6 min from near the onset of development. This regulation by the cAMP receptor is adaptive since cAMP supplied at a constant level, which maintains the receptor in the upper band (proposed to be the adapted form), suppresses the expression of the receptor, adenylate cyclase, and the surface phosphodiesterase, but not the expression of the M4-1 RNAs. However, the developmental regulation of other proteins, such as the soluble phosphodiesterase and its inhibitor, shows nonadaptive regulation by cAMP. Their induction (soluble phosphodiesterase) or suppression (phosphodiesterase inhibitor) is enhanced by the continuous presence of cAMP. Our observation that a modification of the receptor occurs during adaptation may offer an explanation for these observations. The activities under adaptive control may be triggered by cAMP binding to the receptor only in the lower band form. Those under nonadaptive control may be stimulated by the receptor in the upper band form or in both forms.

Acknowledgments—We wish to thank Drs. Joel Shaper, Susan Craig, and Albert Mildvan for their thoughtful reading and criticism of this manuscript. We also wish to thank Drs. Barry Knox and Cathy Berlot for helpful discussions and Jane Sherring for her expert technical assistance.

[5] A. Kimmel, personal communication.

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