cAMP Induces a Rapid and Reversible Modification of the Chemotactic Receptor in *Dictyostelium discoideum*

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ABSTRACT Stimulation, within 1 min after cAMP stimulation, of aggregation-competent *Dictyostelium discoideum* amebae was found to cause a rapid (within 1 min) modification of the cell's surface cAMP receptor. The modified receptor migrated on SDS PAGE as a 47,000-mol-wt protein, as opposed to a 45,000-mol-wt protein labeled on unstimulated cells. The length of time this modified receptor could be detected depended upon the strength of the cAMP stimulus: 3–4 min after treatment with 10^{-7} M cAMP, cells no longer possessed the 47,000-mol-wt form of the cAMP receptor. Instead, the 45,000-mol-wt form was present. Stimulation of cells with 10^{-5} M cAMP, however, resulted in the persistent (over 15 min) expression of the modified receptor. The time course, concentration dependence, and specificity of stimulus for this cAMP-induced shift in the cAMP receptor were found to parallel the cAMP-stimulated phosphorylation of a 47,000-mol-wt protein. In addition, both phenomena were shown to occur in the absence of endogenous cAMP synthesis. The possibility that the cAMP receptor is phosphorylated in response to cAMP stimulation, and the role of this event in cell desensitization, are discussed.

The events involved in the chemotaxis of *Dictyostelium discoideum* amebae to cAMP are slowly being elucidated. One aspect concerns the regulation of the chemotactic receptor and its interaction with adenylate cyclase. Stimulation of cells with cAMP causes an immediate, transitory activation of adenylate cyclase and a consequential increase in cAMP production (1, 2). After the initial activation event, cells are refractory to further stimulation by the same magnitude stimulus: Resensitization requires a period of cell incubation in the absence of added cAMP. The length of the recovery period seems to depend upon the concentration of cAMP initially used to stimulate cells (3). The event involved in desensitizing cells to a constant level of cAMP stimulation must be a rapid and readily reversible one. Simplistically, this may involve a modification of adenylate cyclase itself or of the cell surface cAMP receptor that appears to mediate the cell's responses to cAMP (4). Our photoaffinity labeling experiments have shown that cAMP can induce a new or modified receptor. Binding of 8-N_3-[^{32}P]cAMP to a 45,000-mol-wt protein (P45)[1] exhibits all the known properties of the cAMP receptor (5). If cells, however, are stimulated with high concentrations of cAMP such that receptor down-regulation occurs (6), their residual receptor migrates as a 47,000-mol-wt protein (P47). Recently we have observed the cAMP stimulation also results in the immediate phosphorylation of a 47,000-mol-wt protein (pP47) (7). It is an intriguing possibility that this phosphorylated protein is the cAMP-receptor. For this to be feasible requires that (*a*) the altered form of the cAMP receptor be induced rapidly and reversibly upon cAMP stimulation, (*b*) low concentrations of cAMP are effective inducers, (*c*) the conditions leading to such changes parallel those affecting the phosphorylation-dephosphorylation of pP47, and (*d*) the phosphorylated protein and cAMP receptor co-purify. Experiments demonstrating such correlations are reported here.

MATERIALS AND METHODS

Ax-2 amebae were grown in HL-5 medium (8). Differentiation to aggregation competence occurred in spinner suspensions (9) of 10^7 cells/ml of 20 mM 2-(*N*-morpholino)-ethanesulfonic acid buffer, pH 6.4, 1 mM $MgCl_2$. The developmental state of cells was determined microscopically as previously described (10). For phosphorylation experiments, cells were washed twice with 20 mM 2-(*N*-morpholino)-ethanesulfonic acid buffer pH 6.4 and resuspended at a density of 2×10^7 cells/ml in buffer containing 10^{-6}M phosphate and 100 μCi/ml [^{32}P]orthophosphate (Amersham Corp., Arlington Heights, IL). After 30 min, cells were stimulated with cAMP. At specified times, aliquots for SDS PAGE were boiled in sample buffer (20 mM sodium carbonate, 25 mM

[1] *Abbreviations used in this paper*: ConA, Concanavalin A; P45, 45,000-mol-wt protein; P47, 47,000-mol-wt protein; pP47, phosphorylated 47,000 mol-wt-protein.

dithiothreitol, 1% SDS, 10% sucrose). Photoaffinity labeling using 8-N_3-[^{32}P]-cAMP was performed at 0°C as previously described (5). cAMP-induced changes in the cAMP receptor were demonstrated by pretreating cells at room temperature with the indicated concentrations of cAMP, and extensively washing them before photolabeling. Specificity of incorporation was determined by performing the photolabeling experiments in the presence of 10^{-4}M nonradioactive cAMP. In all cases, the radiolabeled products were separated on SDS PAGE according to Chua and Bennoun (11). Molecular weights were estimated using standard curves obtained from a set of known polypeptides (Bio-Rad Laboratories, Richmond, CA). Labeled components were visualized by autoradiography on XAR-5 Kodak film exposed at −70°C on DuPont lightning-plus screens (DuPont Instruments, Wilmington, DE). Densitometer tracings of autoradiograms were obtained using a laser densitometer (LKB Instruments, Inc., Gaithersburg, MD). Plasma membranes were isolated on polylysine-coated acrylamide beads according to Cohen et al. (12) and modified by Jacobson (13). Characterization of such preparations in terms of purity and cAMP-receptor isolation has been reported elsewhere (5). For Concanavalin A (ConA) treatment, amebae were incubated with 100 μg/ml ConA for periods ranging from 5 to 30 min. After several washings, cells were photolabeled with 8-N_3-[^{32}P]cAMP. If phosphorylation was to be monitored, ConA was added to ^{32}P-labeled cells. After various incubation times aliquots were taken for analysis on SDS PAGE. For caffeine experiments, cells at a density of 2×10^6 cells/ml were drug-treated either simultaneously with cAMP or several minutes before stimulation. Added cAMP ranged from 10^{-7} to 10^{-5}M. That cells treated with caffeine did not synthesize cAMP in response to exogenous stimulation was verified by radioimmune assay (14, 15). All experiments were performed a minimum of three times.

RESULTS

Demonstration of Rapid Changes in cAMP Receptor

Figure 1*A* depicts the pattern of 8-N_3-[^{32}P]cAMP incorporation on cells stimulated with two concentrations of cAMP. Untreated cells (lane *6*) incorporated the photoaffinity analogue into a broad band of 45,000 mol wt (P45). Previous experiments have suggested that this protein represents the cAMP receptor (5). Criteria used for such an identification included developmental changes in P45 labeling, its affinity constant for 8-N_3-cAMP, and specific inhibition of labeling in the presence of 10^{-7}–10^{-6}M cAMP (see reference 5 for other criteria). Lane *7* of Fig. 1*A* shows the proteins labeled in the presence of 10^{-4}M cAMP. When cells were pretreated with 10^{-5} M cAMP for 1 min, they no longer incorporated 8-N_3-[^{32}P]cAMP into P45: Instead, a protein of 47,000 mol wt (P47) was labeled (lane *1*). As in the case of P45, little or no incorporation of 8-N_3-[^{32}P]cAMP in P47 occurred in photolabeling was performed in the presence of nonradioactive cAMP (Fig. 4*A*, lanes *c* and *d*). The length of time during which P47 was present on the cell surface depended upon the concentration of cAMP used to stimulated cells. P47 was identifiable on 10^{-5}M cAMP-treated cells within 1 min of stimulation and remained for the 15-min duration of the experiment (Fig. 1*A*, lanes *1* and *2*). With 10^{-7}M cAMP, P47 was also detectable 1 min after stimulation but was present only for 2–3 min (Fig. 1*A*, lanes *3* and *4*). At 6 min, no photolabel was incorporated into this protein. Correspondingly, P45 was again present on cells (Fig. 1*A*, lane *5*). The rapidity of these changes suggest that they do not reflect the synthesis and degradation of a new receptor but rather a modification of the existing receptor.

A clearer resolution of these two molecular weight forms of the cAMP receptor is shown by the densitometer tracings presented in Fig. 2. Tracing *a* was obtained from a preparation in which cells that had not been stimulated were mixed with cells that had been stimulated with cAMP to induce the higher molecular-weight form of the receptor before photolabeling. Under such conditions, both P45 and P47 were readily discernible. As seen in Fig. 1*A*, cells stimulated with 10^{-5}M cAMP showed labeling of the P47 form of the cAMP receptor. This tracing is representative of that obtained using cells treated for 1, 6, or 15 min. In contrast, cells stimulated with 10^{-7}M cAMP showed the higher molecular weight form of the receptor only during the first few minutes, after which time, the lower molecular weight form was present on the cell surface (Fig. 2, *c* and *d*, respectively).

Fig. 1*B* shows the results of an experiment similar to that described above but in which protein phosphorylation in response to cAMP was monitored. We have previously shown that cAMP specifically increases the phosphorylation of a

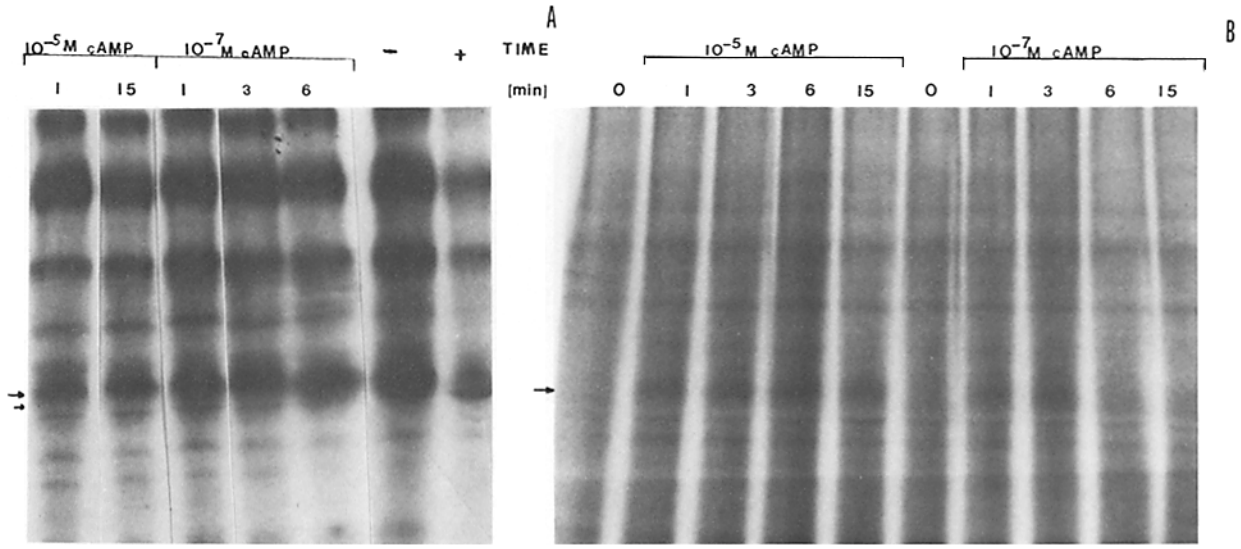

FIGURE 1 (*A*) Aggregation-competent cells were preincubated with cAMP for the indicated times, washed extensively, and then photolabeled. The last two lanes show the patterns of 8-N_3-[^{32}P]cAMP incorporated by untreated cells when photoactivation was done in the absence (−) or presence (+) of 10^{-4} M nonradioactive cAMP. The positions of 47,000- and 45,000-mol-wt proteins are indicated respectively by the long and short arrows. (*B*) ^{32}P-Labeled aggregation-competent amebae were stimulated with cAMP for the indicated times and the phosphorylated proteins analyzed by SDS PAGE. Time 0 refers to untreated cells.

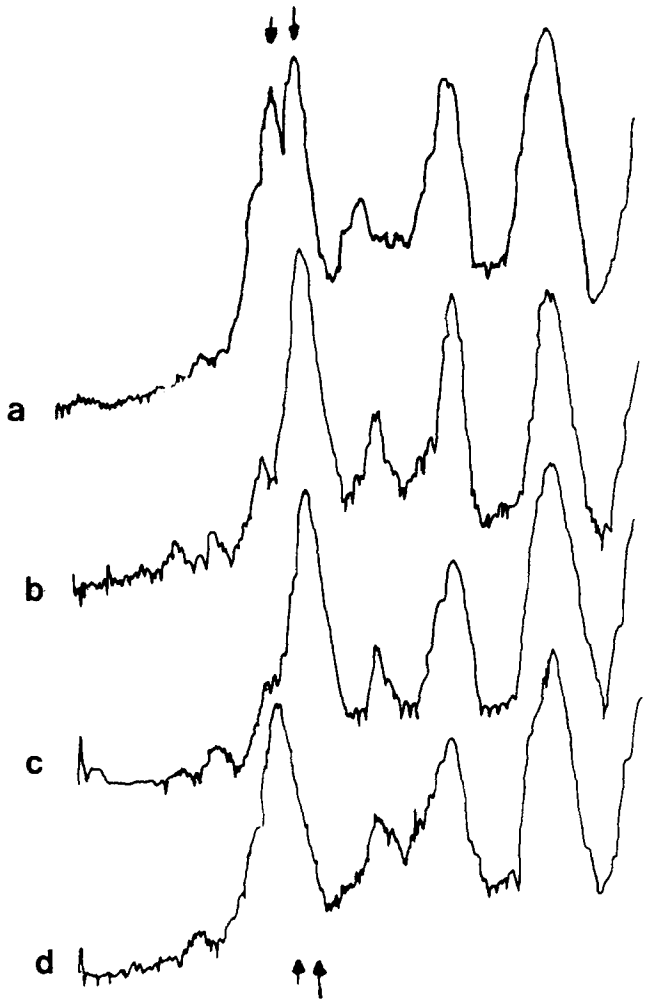

FIGURE 2 Densitometer tracing of an autoradiogram obtained (*a*) when cells that had not been stimulated were mixed with those that had been stimulated with 10^{-5}M cAMP before photoaffinity labeling. Tracings *b*, *c*, and *d* are from cells that had been stimulated with 10^{-5}M cAMP for 6 min, 10^{-7}M cAMP for 1 min, or 10^{-7}M cAMP for 6 min, respectively. The top of the gel is on the right. The positions of 47,000- and 45,000-mol-wt proteins are indicated, respectively, by the long and short arrows.

47,000-mol-wt protein, pP47 (see reference 7). ^{32}P-labeled aggregation-competent amebae showed an increased phosphorylation of this protein within 1 min of cAMP stimulation. With 10^{-5}M cAMP, this protein remained phosphorylated for the 15-min duration of the experiment. Lower stimulus concentrations resulted in more transitory labeling of pP47. With 10^{-7}M cAMP, this protein was phosphorylated for only 1–3 min. At 6 min, little or no radioactivity remained in pP47. The time course and cAMP concentration dependence of pP47 phosphorylation show striking similarities to that observed for the induction of the P47 form of the cAMP receptor.

Increased Phosphorylation and Receptor Shifts Occur Under Similar Conditions

Various stimuli were examined to determine their ability to induce P47 phosphorylation and/or the altered form of the cAMP receptor. At 10^{-4}M, cGMP is a chemoattractant for cells and can also stimulate cell differentiation to aggregation competence, as does external cAMP. At such high concentrations, cGMP would bind to the cAMP receptor (16). Previously, we have reported that cGMP concentrations of 10^{-4}M or higher elicit pP47 phosphorylation (7). The same was found to be true of P47 induction. Cells pretreated with 10^{-3} or 10^{-4}M cGMP no longer incorporated 8-N_3-[^{32}P]cAMP into P45. Correspondingly, P47 was labeled. Lower concentrations, which do not bind to the receptor, did not alter the photoaffinity-labeling pattern and did not elicit pP47 phosphorylation (data not shown). 10^{-5}–10^{-6} folic acid, 10^{-4}M Ca^{++}, or 7×10^{-7}M Ca^{++} ionophore A23187 (tested in the absence or presence of Ca^{++}) did not increase the phosphorylation of pP47 or induce the P47 form of the receptor. Folic acid, a chemoattractant for undifferentiated cells appears to function via its own surface receptor, whereas Ca^{++} and its ionophore may affect development by increasing the number of cAMP binding sites on the cell surface (17–19). ConA has been shown to cause patching, capping, and endocytosis of ConA receptors on *D. discoideum* (20). A secondary effect of such membrane perturbations is changes in cAMP receptors on cells (21). We examined if such redistribution of membrane protein could also induce pP47 phosphorylation and/or the higher molecular weight form of the cAMP receptor. Neither event was elicited when cells were treated with ConA for up to 30 min (data not shown). Both pP47 phosphorylation and the induction of the P47 form of the cAMP receptor were found to be temperature-sensitive events: Neither occurred when cells were stimulated with cAMP at 4°C (data not shown).

Receptor Shift and Phosphorylation Do Not Require cAMP Synthesis

It should be recalled that an immediate consequence of cAMP binding to its receptor is the stimulation of adenylate cyclase and an increase in cAMP production. Thus, an important question to be resolved is the degree to which cellular cAMP is involved in either or both of these phenomena. This question was investigated using caffeine-treated cells. It has been shown that amebae treated with 5 mM caffeine cannot synthesize cAMP when stimulated with cAMP levels as high as 10^{-4}M (22). Although the mechanism by which this inhibition of adenylate cyclase activation occurs is unknown, it is not related to an effect on cAMP binding to the cell surface (22). We observed that caffeine-treated cells stimulated with cAMP could still phosphorylate pP47 and alter the molecular weight form of the cAMP receptor. Neither the time course nor cAMP-sensitivity of either pP47 phosphorylation or the induction of the higher molecular weight form of the cAMP receptor was altered by the presence of caffeine. An example of the results obtained is shown in Fig. 3. Densitometer tracings *a* and *b* are obtained from ^{32}P-labeled cells that were treated with caffeine or caffeine plus 10^{-7}M cAMP for 1 min. Tracings *c* and *d* were from 8-N_3-[^{32}P]cAMP labeled cells that had been incubated with caffeine only or caffeine plus 10^{-7}M cAMP for 1 min.

Biochemical Similarities of pP47 and P47

The relationship of the P47 form of the cAMP receptor and the protein phosphorylated in response to cAMP stimulation will become clearer as these proteins are purified. Although this is proving to be elusive, some advances have been made. Fig. 4*B* shows that both the modified cAMP receptor (P47)

and the phosphorylated protein (pP47) were present in preparations of plasma membranes isolated on polylysine-coated beads. Such preparations have been shown previously to contain both the unmodified form of the cAMP receptor (P45) and an active cAMP binding activity (5, 23). Neither pP47 nor P47 was isolated on polylysine-coated beads when the cells' cytoplasmic fraction was similarly incubated with the beads. Both proteins also co-purified when plasma membranes were isolated according to Luna et al. (24) (data not shown).

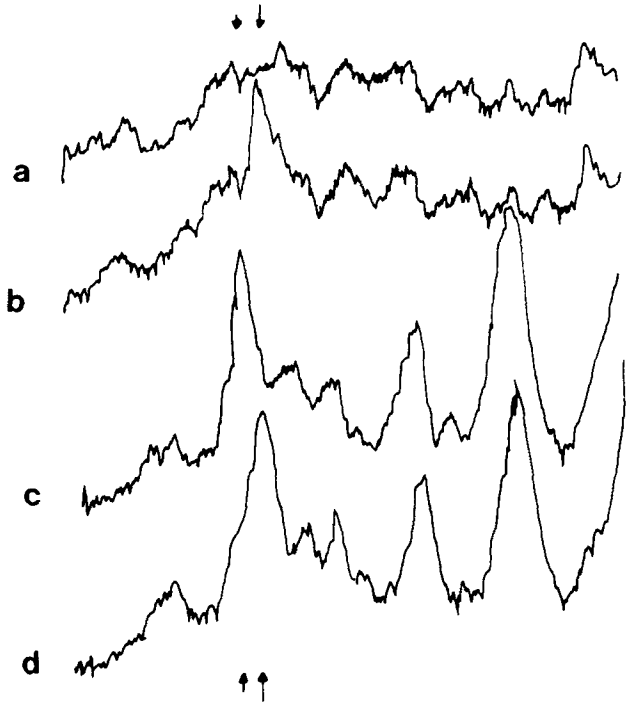

FIGURE 3 ^{32}P-labeled aggregation-competent cells were incubated with 5 mM caffeine (a) or 5 mM caffeine plus 10^{-7}M cAMP (*b*) for 1 min. Correspondingly, cells were incubated with caffeine (c) or caffeine plus 10^{-7}M cAMP (*d*) for 1 min and then photolabeled with 8-N_3-[^{32}P]cAMP. Samples were analyzed on SDS PAGE. The figure shows the densitometer tracings of the autoradiogram, the top being on the right. Positions of 47,000- and 45,000-mol-wt proteins are indicated by the long and short arrows.

Fig. 4*A* shows that P47, identified by 8-N_3-[^{32}P]cAMP labeling (lane *c*), co-migrated with the ^{32}P-labeled 47,000-mol-wt protein (pP47) on SDS PAGE (lanes *b* and *e*) (also see Fig. 3). With the exception of the bands of 47,000 mol wt, the pattern of radioactive bands obtained using ^{32}P-labeled cells and 8-N_3-[^{32}P]-labeled cells is quite different. The proteins phosphorylated by cells that had not been stimulated with cAMP are shown in lanes *a* and *f*, and those proteins photolabeled in the presence of 10^{-4}M nonradioactive cAMP are shown in lanes *d* and *h*. Fig. 4*A* also shows that P45, the protein photolabeled on unstimulated cells (lane *g*), does not co-migrate with pP47. Ideally, other analytical tools such as IEF or Cleveland digests would be performed to substantiate the co-identity of these components. However, because of the difficulties with solubilization, the low levels of radioactivity incorporated, the the extensive periods of autoradiographic exposure necessary to visualize the appropriate bands, these approaches have proven to be technically unfeasible. We have used other SDS-acrylamide gel systems such as that described by Laemmli (25) and found that pP47 and P47 still co-migrate. In other systems, the migration of receptor subunits on SDS PAGE is dramatically altered by the presence of reducing agents in the sample buffer (26). In our system, the absence of dithiothreitol in our sample buffer did not alter the apparent molecular weight of either the P45 or P47 form of the cAMP receptor nor of the phosphorylated pP47 protein (data not shown).

DISCUSSION

The results represented in this paper indicate that a cAMP receptor of 47,000 mol wt appears on cells within 1 min of

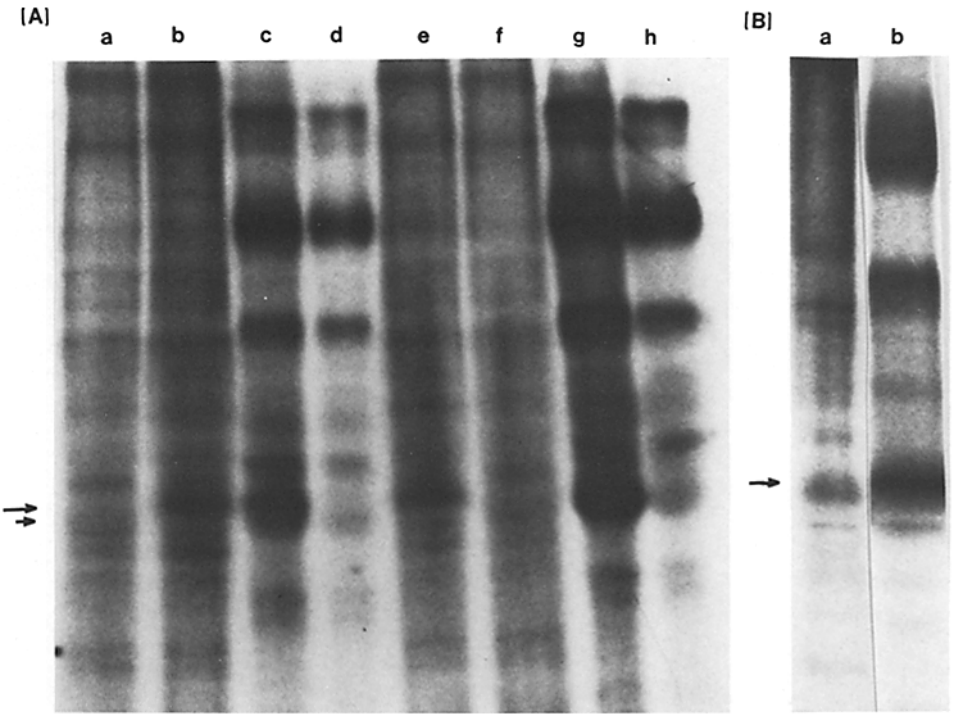

FIGURE 4 (*A*) Proteins phosphorylated by ^{32}P-labeled cells that had been stimulated with 10^{-5}M cAMP for 1 min (lanes *b* and *e*) and untreated cells (lanes *a* and *f*). Proteins photolabeled on cells that had been pretreated with cAMP (lanes *c* and *d*) and untreated controls (lanes *g* and *h*). Lanes *d* and *h* show the proteins labeled when photolabeling was performed in the presence of nonradioactive 10^{-4}M cAMP. (*B*) Phosphorylated proteins (lane *a*) and photolabeled proteins (lane *b*) of the plasma membranes isolated from cAMP-treated cells. Large and small arrows indicate 47,000- and 45,000-mol-wt.

stimulation with cAMP. The length of time this modified form of the cAMP receptor is present depends upon the concentration of stimulus used. At low concentrations (10^{-8}–10^{-7}M), there is a rapid (within a few minutes) return to the 45,000-mol-wt form. At higher concentrations, longer times are required. The time course of this phenomenon and its cAMP dependence allow for its role in desensitization. (The latter refers to the decrease in adenylate cyclase activity that occurs even if the stimulus concentration of cAMP remains constant.) Several other findings are consistent with the hypothesis that modification of the cAMP receptor is related to desensitization. The data of Dinauer et al. (27) suggest that desensitization occurs at low concentrations of cAMP and in the absence of any significant down-regulation of the cAMP receptor. The same is true of the appearance of the P47 form of the cAMP receptor. Within 1 min of stimulation with 10^{-7}M cAMP, this form was present on the cell surface. Under our experimental conditions, such stimulation does not cause a detectable decrease (down-regulation) in cAMP binding to the cell surface (reference 6; C. Klein, unpublished observations). These findings indicate that cAMP receptor modification itself is not sufficient for down-regulation to occur. If receptor modification is involved in desensitization, then its initial effect may be to alter the coupling of the receptor to adenylate cyclase. Higher stimulus concentrations, which lead to down-regulation (6) and to prolonged periods of desensitization (27), also result in the prolonged presence of P47 on the cell surface. Perhaps the higher concentrations of cAMP elicit an event(s), in addition to receptor modification, which results in receptor down-regulation and prolonged desensitization. The situation in *D. discoideum* shares an interesting parallel to that seen in higher eucaryotes. In astrocytoma cells, the degree to which down-regulation of the β-adrenergic receptor correlates with desensitization is also a function of time. To a large extent desensitization occurs within a few minutes, with little or no loss of receptor activity. Decreases in adenylate cyclase activity continue to occur with longer periods of incubation with isoproterenol and this correlates with receptor down-regulation (28).

By using cAMP analogues and other compounds that act at the cell surface to effect cell development, we have shown that perturbation of the plasma membrane was not sufficient to induce P47. Another event, the binding of cAMP or its analogues to the receptor, was required. However, at least one step subsequent to ligand binding, the activation of adenylate cyclase, was not required. Thus, the mechanism by which cells modify their receptor in response to cAMP stimulation is independent of endogenous cAMP synthesis. In terms of our hypothesis that this modification is important in the desensitization response, it is of interest to note that the experiments of Thiebert and Devreotes (22) showed that desensitization also does not require the activation of adenylate cyclase.

The nature of the modification that results in the altered mobility of the cAMP receptor on SDS PAGE is a subject of interest. In many eucaryotic systems, hormonal stimulation results in the phosphorylation of the hormone receptor. More recently, Stadel et al (29) have shown that the modified form of the β-adrenergic receptor seen on desensitized turkey erythrocytes is associated with receptor phosphorylation. The experiments presented here provide strong correlative arguments that the modification of the cAMP receptor also involves a phosphorylation event. Phosphorylation-dephosphorylation of a 47,000-mol-wt protein (pP47) occurs with the same time course as the appearance and loss of the 47,000-mol-wt form of the cAMP receptor (P47). Stimuli that did not elicit the higher molecular weight form of the receptor did not induce phosphorylation of pP47 and vice versa. In addition, both events were also found to occur in the absence of adenylate cyclase activation. This latter observation suggests that the protein kinase involved in pP47 phosphorylation may be intimately associated with the cAMP receptor and activated as a direct result of ligand binding to its receptor (as in the case of epidermal growth factor, nerve growth factor, and insulin receptors). Alternatively, a reaction subsequent to cAMP binding to its receptor may induce a cAMP-independent protein kinase. We are currently undertaking investigations to determine which of these alternatives is correct.

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