THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 263, No. 28, Issue of October 5, pp. 14538-14543, 1988
Printed in U.S.A.

Ligand-induced Phosphorylation of the cAMP Receptor from *Dictyostelium discoideum**

(Received for publication, June 6, 1988)

Roxanne A. Vaughan and Peter N. Devreotes

From the Department of Biological Chemistry, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

The cell surface cAMP receptor of *Dictyostelium discoideum* exists as a doublet of low (D) and high (R) electrophoretic mobility forms, both of which are phosphorylated *in vivo*. The R form is phosphorylated in a ligand-independent manner, while conversion of the R to D forms, induced by the chemoattractant, is accompanied by at least a 4-fold increase in the level of phosphorylation. When cells are stimulated with saturating levels of cAMP, increased phosphorylation is detectable within 5 s and reaches maximum levels by 5 min with a $t_{1/2}$ of 45 s. Dephosphorylation of receptor, initiated by removal of the stimulus, is detectable within 30 s, has a half-time of 2 min, and reaches a plateau by 20 min. At half-maximal occupancy, phosphorylation occurred more slowly than at saturation, $t_{1/2}$ = 1.5 min, and remained at intermediate levels until the cAMP concentration was increased. Accompanying electrophoretic mobility shifts occurred in all cases with similar, though not identical, kinetics. Both phosphorylation and mobility shift were half-maximal at 5 nM cAMP and saturated at 100 nM. Estimation of the specific activity of each receptor form indicates that not all sites are phosphorylated during the R to D transition; at least half of the sites are phosphorylated after the transition is completed. The rate of incorporation of phosphates into the receptor, held in the D form by cAMP, was less than one-third the rate of ligand-induced incorporation starting with the R form and was approximately twice the basal rate of incorporation. These results are compatible with ligand-induced receptor phosphorylation being an early event in the adaptation of other cAMP-induced responses.

During the developmental cycle of *Dictyostelium discoideum* many thousands of amoebae aggregate to form a multicellular structure. Aggregation is mediated by cAMP which is propagated at 6-min intervals in the form of waves throughout the cell population (1). Binding of cAMP to the cell surface elicits a variety of responses including activation of adenylate cyclase, phosphorylation of myosin heavy and light chains, and cell shape changes (2–5). After characteristic periods of time these processes spontaneously return to basal levels even in the presence of cAMP, a process termed adaptation (5, 6). Removal of cAMP results in the resensitization, or deadaptation, of these events (7). Activation and adaptation of these processes are presumed to underlie the oscillatory propagation of cAMP waves and chemotaxis of cells during aggregation. The cAMP oscillations control the expression of developmentally regulated genes. If cAMP is absent or present at fixed levels, gene expression is abnormal and cells do not differentiate (8). Thus both activation and adaptation mediated by the cell surface cAMP receptor are required for chemotaxis and normal development.

The cell surface receptor which binds extracellular cAMP has been identified (9, 10) and purified to homogeneity (11). It exists as a doublet with the higher electrophoretic mobility form or R form (M_r = 40,000) predominating in nonstimulated cells and the lower electrophoretic mobility form or D form (M_r = 43,000) predominating in cAMP-stimulated cells. The modification producing the change in forms is reversible and occurs endogenously as cells undergo spontaneous cAMP oscillations (12). In contrast to the transient cAMP-activated responses listed above, the electrophoretic mobility shift persists in the presence of constant cAMP (13) and may therefore be involved in adaptation. The kinetics and dose dependence of this shift have previously been analyzed by 8-N_3-[^{32}P]cAMP[1] photoaffinity labeling and correlate well with the kinetics and dose dependence of adaptation of adenylate cyclase and cell shape change (13).

Metabolic labeling studies have shown that the cAMP receptor is phosphorylated *in vivo* and that the level of phosphorylation is increased severalfold after cells are stimulated with cAMP (14, 15). Since both receptor shift and phosphorylation are induced by chemoattractant, phosphorylation may be the biochemical event producing the shift and may also be an early event leading to adaptation. The cAMP receptor thus resembles many other cell surface receptors such as those for insulin, epidermal growth factor, rhodopsin, and adrenergic agonists which undergo phosphorylation in response to ligand binding (16, 17). For rhodopsin and the β-adrenergic receptor, ligand-induced phosphorylation has been correlated with adaptation of physiological responses (17) although definitive proof of this relationship is still lacking. This study investigates phosphorylation properties of the cAMP chemoattractant receptor including kinetics, dose dependence, dephosphorylation, relationship to mobility shift, and compatibility with adaptation processes.

MATERIALS AND METHODS

Cell Growth and Development—AX3 cells were grown to a density of 5 × 10^6/ml in HL-5 medium and developed at 2 × 10^7/ml in development buffer as described (11). Cells were pulsed with 50 nM cAMP every 6 min throughout development.

Preparation of Membranes and in Vivo ^{32}P Labeling—After development at 21 °C for 5 h, cells were washed twice with and resuspended at 10^8 cells/ml in Mes buffer (20 mM Mes, pH 6.2, 2 mM $MgSO_4$, 0.2 mM $CaCl_2$). The cells were shaken at 100 rpm and treated with 5 mM caffeine for 15 min to prevent activation of adenylate cyclase and produce the R form of receptor (12). Where indicated $^{32}P_i$ was included

* This work was supported by Grant GM 34933 from the United States Public Health Service (to P. N. D.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] The abbreviations used are: 8-N_3-[^{32}P]cAMP, 8-azido-[^{32}P]cyclic AMP; Mes, 2-(*N*-morpholino)ethanesulfonic acid.

at the same time as the caffeine at a final concentration of 1.0 mCi/ml. When stimulated, cells were then given 10 mM dithiothreitol and the indicated concentrations of cAMP for the indicated time periods. $^{32}P_i$ labeling times prior to stimulation were 15 min unless otherwise indicated and were kept constant within groups of experiments in order to produce similar basal phosphorylation levels. These experiments were performed under conditions where [^{32}P]ATP pools were not equilibrated. However, using the conditions described the specific activity of the [^{32}P]ATP does not change appreciably during the time course of these experiments or in response to cAMP (data not shown (18)). All labelings and cAMP stimulations were performed at 22 °C.

After stimulation cells were withdrawn to a 5-fold volume of ice-cold saturated ammonium sulfate and centrifuged in a Beckman Microfuge 12 at 10,000 rpm for 8 min. The pellets were resuspended with 1 ml of ammonium sulfate and recentrifuged. Cells were lysed by the addition of 1 ml of lysis buffer (11) and membranes prepared and solubilized as described (11). For all experiments, parallel sets of $^{32}P_i$-labeled and unlabeled cells were prepared in order to directly compare changes in phosphorylation (analyzed by immunoprecipitation) and electrophoretic mobility shift (analyzed by immunoblotting).

Experimental Protocols—For phosphorylation kinetics, cells were washed, treated with caffeine, and labeled with $^{32}P_i$ as described above. For 0-min time points, aliquots were removed to ammonium sulfate before cAMP stimulation. After addition of cAMP, aliquots of cells were removed to ammonium sulfate at the indicated time points. Control experiments showed that, once in ammonium sulfate, cells could be held without further processing for at least 30 min with no change in receptor shift or level of phosphorylation.

For dephosphorylation experiments, cells at a density of 10^8/ml were labeled with $^{32}P_i$ and stimulated with 3×10^{-7} M cAMP for 15 min to produce the D form. The cells were then washed twice with and resuspended at 2×10^8/ml in 0 °C Mes buffer. To initiate the D to R transition, cells were diluted 4-fold into 22 °C Mes buffer and shaken at 100 rpm. Aliquots were removed to ammonium sulfate for processing before dilution and at intervals thereafter. Since the washes for removal of cAMP also removed some of the $^{32}P_i$ from the suspension, additional assays were performed to distinguish between $^{32}P_i$ loss from receptor due to decreasing [^{32}P]ATP pools and loss due to receptor occupancy changes. For all experimental time points, control samples were taken from $^{32}P_i$-labeled cells which were never stimulated but were carried through the washes and dilutions to assess R form phosphorylation changes and from cells which were stimulated, washed, and diluted into 22 °C buffer containing 3×10^{-7} M cAMP to determine the changes in phosphorylation of the D form. By determining the extent of these changes it was possible to determine the phosphorylation changes due only to the removal of the cAMP.

To determine the steady-state turnover rate for phosphates on the D form, cells were treated with 10^{-6} M cAMP for 5 min to produce the phosphorylated D form, and $^{32}P_i$ was then added to the suspension. A parallel set of cells was pretreated with $^{32}P_i$ and then given cAMP to determine the stimulus-induced phosphorylation rate. The protocol allowed sufficient time after cAMP stimulation for receptor to reach steady-state phosphorylation and electrophoretic mobility shift levels before the addition of $^{32}P_i$ (for the experimental regimen) or removal of cells to ammonium sulfate (for the control regimen). Two other sets of cells were analyzed for R form phosphorylation utilizing the same protocol with the omission of the cAMP stimulus. In all cases aliquots of cells were removed to ammonium sulfate for processing after 10, 20, and 40 min of labeling with $^{32}P_i$. All procedures were also performed on unlabeled cells, and receptor shift was analyzed by immunoblotting.

Immunoprecipitation and Immunoblots—$^{32}P_i$-labeled sodium dodecyl sulfate-solubilized membranes were centrifuged in a Beckman Airfuge for 15 min to remove insoluble material. Immunoblotting showed that no receptor was lost by this treatment. The supernatants were then immunoprecipitated and subjected to electrophoresis on 10% polyacrylamide gels as described (14) with the exception that the receptor was eluted from the final pellet of protein A-Sepharose CL-4B beads at 22 °C for 10 min instead of at 95 °C for 5 min. Immunoblots of unlabeled membranes were performed as described (14).

Analysis of Data—Autoradiographs of immunoblots and immunoprecipitations were scanned with an LKB densitometer, and the heights of R and D peaks were measured. For immunoblots, data are presented as the fraction of receptor in the D form, which equals the (height of D)/(height of D + height of R). For quantitation of phosphorylation, the maximal total phosphorylation (summing peak heights of R and D forms) in each experiment is considered to be 100%, and other data points are expressed as percentages of the maximum value. Unless otherwise indicated, data points represent the fractional amount of phosphorylation of the sum of R and D forms.

RESULTS

Kinetics of Phosphorylation—The kinetics of receptor redistribution determined by immunoblotting in response to saturating cAMP are shown in Fig. 1*A*. As previously demonstrated by photoaffinity labeling (13), about 90% of receptor protein from unstimulated cells migrates as the R form. When cells were stimulated with 10^{-7} M cAMP there was a rapid redistribution of receptor protein from the R to the D form with a final distribution of 80–90% in the D form. The half-time for this process was 30 s, and a steady state was reached by 5 min. No change in electrophoretic mobility was detected during the remainder of the experiment. The kinetics of *in vivo* phosphorylation of the cAMP receptor are shown in Fig.

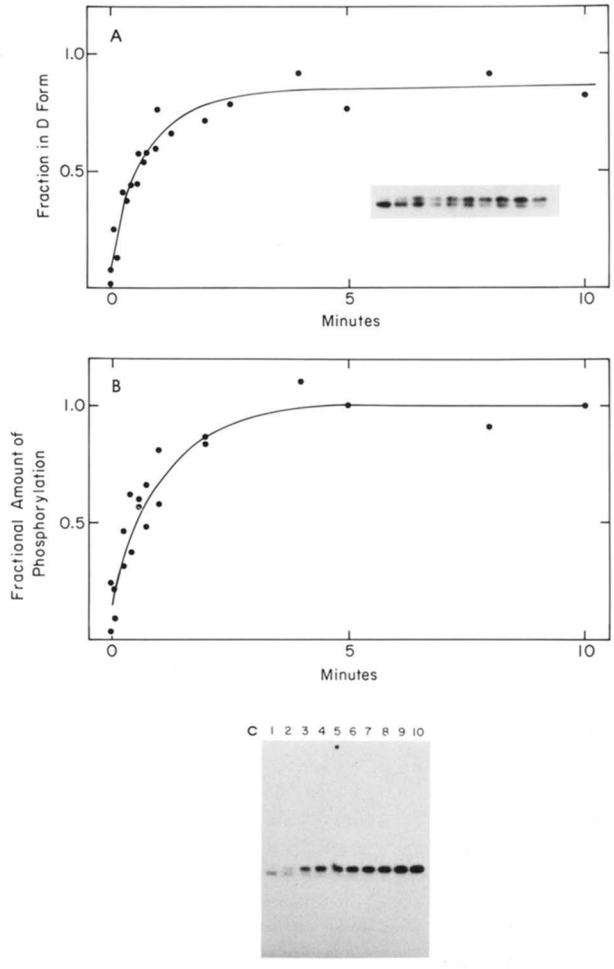

FIG. 1. **Kinetics of receptor modification induced by saturating cAMP.** Parallel sets of cells were treated with caffeine, with or without $^{32}P_i$ labeling, for 45 min prior to stimulation. Each set was then stimulated with 10^{-7} M cAMP, and aliquots were removed at the indicated times. *A*, kinetics of receptor shift determined by immunoblotting. The graph shows the combined results of two independent experiments. The *inset* shows the Western blot of one of these experiments in which the lanes contain samples taken at times 0, 5, 15, 25, 35, 45, 60, 120, 300, and 600 s after addition of cAMP. *B*, kinetics of receptor phosphorylation. The graph shows the combined results of two independent experiments. *C*, autoradiograph of immunoprecipitated $^{32}P_i$-labeled receptor taken after cAMP stimulation at the times listed in *panel A*.

1*B*. As previously reported (14), there is a basal amount of receptor phosphorylation in unstimulated cells. After stimulation of cells by cAMP there was a dramatic increase in the amount of receptor phosphorylation concomitant with the shift to the D form. Ligand-induced receptor phosphorylation was detectable within 5 s after stimulation, was complete by 5 min, and occurred with a half-time of 45 s. The process was biphasic with a rapid component occurring early (60–70% maximum phosphorylation by 1 min) followed by a slower component. No increase or decrease in phosphorylation was detectable after the steady state was achieved. Fig. 1*C* shows one of the autoradiographs of immunoprecipitated $^{32}P_i$-labeled receptor used to generate Fig. 1*B* and demonstrates the specificity of the antiserum. The region shown includes proteins of M_r 200,000–10,000. The autoradiographs of immunoprecipitations and immunoblots of all experiments described here showed similar high specificity, and therefore only the region from 38 to 45 kDa is shown in other figures.

Kinetics of Dephosphorylation—To examine the effects of removal of cAMP, cells were first stimulated with cAMP for 10 min, washed at 0 °C, and warmed rapidly by dilution into 22 °C Mes buffer without cAMP. (Immunoblotting, as well as previous photoaffinity labeling (13), showed that no D to R transition occurs when cells are held at 0 °C even in the absence of cAMP.) The redistribution from the D to the R form is shown in Fig. 2*A*. It was detectable by 30 s, occurred with a half-time of 2.5 min, and was complete by 10 min. The concurrent dephosphorylation of the receptor is shown in Fig. 2*B*. As described under "Materials and Methods," control assays were performed to determine the changes in R and D form phosphorylation levels due solely to the reduction in the specific activity of the [^{32}P]ATP pools caused by the cell washes needed to remove the cAMP. In four separate experiments, reduction in phosphorylation levels of R and D forms due to the washes and dilution were not detectable until 10 min after the cells were diluted into the 22 °C buffer. At 10 min the average reduction was 10% of the initial phosphorylation level, at 15 min it was 19%, and at 20 min it was 30%. The data points shown in Fig. 2*B* have been corrected for this $^{32}P_i$ loss and thus represent dephosphorylation due only to removal of cAMP. Dephosphorylation of receptor induced by removal of the ligand was detectable by 30 s, had a half-time of 2 min, and reached a plateau by 20 min. The process was biphasic with a rapid component occurring between 0 and 3 min and a slower component occurring thereafter. Residual amounts of phosphorylated D form were still present at 30 and 45 min after removal of cAMP (not shown).

Dose Response to cAMP—The dependence of receptor shift and phosphorylation on cAMP concentration are shown in Fig. 3. Half-maximal shift occurred at 5nM cAMP, and saturation occurred at 100 nM. Little or no receptor shift occurred below 1 nM. Phosphorylation was also half-maximal at approximately 5 nM and saturated at 100 nM. The experiments shown in Fig. 4 demonstrate that the 10-min stimulation time used was adequate for receptor shift and phosphorylation to reach steady state at subsaturating cAMP concentrations.

Kinetics at a Subsaturating cAMP Concentration—During

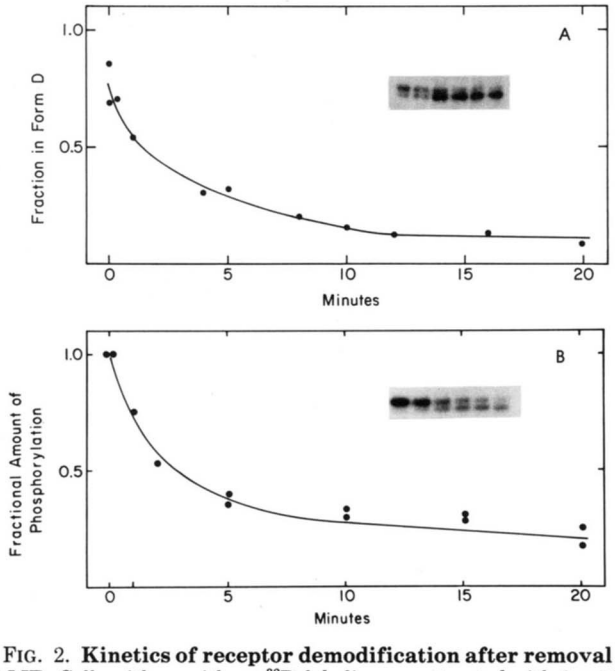

FIG. 2. **Kinetics of receptor demodification after removal of cAMP.** Cells with or without $^{32}P_i$ labeling were treated with 3×10^{-7} M cAMP for 10 min to produce the D form of the receptor and then washed by centrifugation at 0 °C to remove the cAMP. Cells were then diluted into 22 °C Mes buffer to initiate demodification, and aliquots were taken at the indicated times. *A*, kinetics of D to R shift determined by immunoblotting. The *inset* shows the Western blot of an experiment in which samples were taken at 0, 2, 5, 10, 15, and 20 min after dilution into 22 °C Mes. The results from two experiments are shown. *B*, kinetics of dephosphorylation. The *inset* shows the autoradiograph of immunoprecipitated $^{32}P_i$-labeled receptor taken at the times listed for *panel A*. $^{32}P_i$ loss from both R and D forms due to the washes was estimated as described under "Materials and Methods," and those values have been incorporated as correction factors to the actual data. The results shown are from two independent experiments.

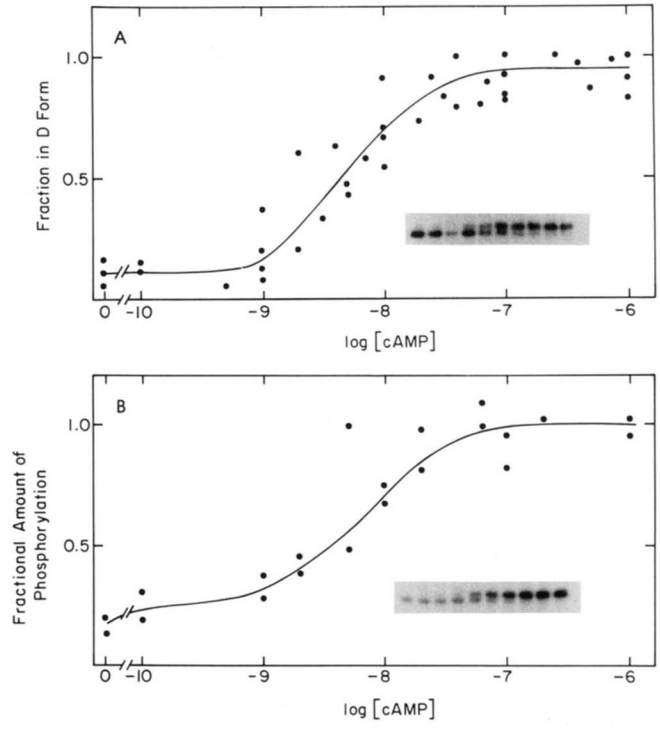

FIG. 3. **Effect of cAMP concentration on receptor modification.** Parallel sets of cells were prepared with or without $^{32}P_i$ labeling for 45 min. At that time cells were stimulated with the indicated concentrations of cAMP for 10 min and then removed for processing. *A*, dose response of receptor shift determined by immunoblotting. The graph shows the results from four independent experiments. The *inset* shows the immunoblot of one experiment in which the lanes contain samples from cells stimulated with the following concentrations of cAMP: 0, 0.1, 1, 2, 5, 10, 20, 50, 100, and 1000 nM. *B*, dose response of receptor phosphorylation. The graph shows the results of two experiments. The *inset* shows the autoradiograph of immunoprecipitated $^{32}P_i$-labeled receptors prepared from cells stimulated with the doses shown in *panel A*.

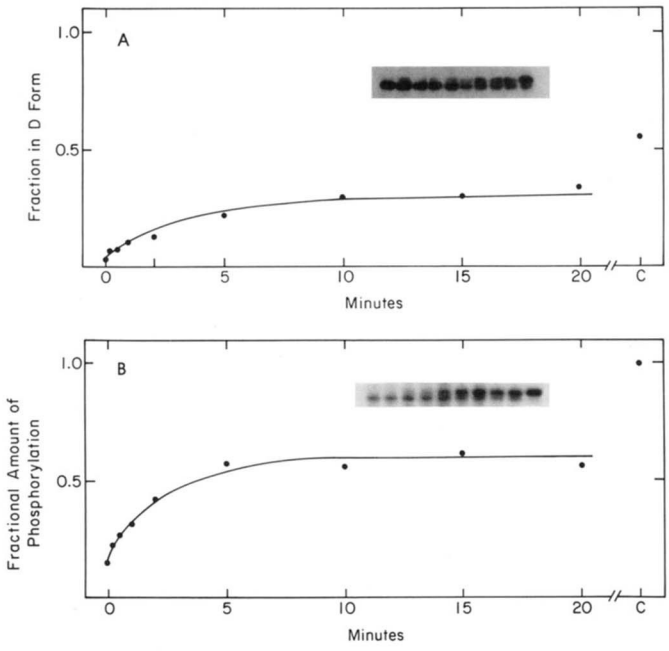

FIG. 4. **Kinetics of receptor modification at a subsaturating dose.** Parallel sets of cells were prepared with or without $^{32}P_i$ labeling for 45 min. Each set was then stimulated with 5 nM cAMP, and aliquots were removed at the indicated times. After the last time point was taken the remaining cells were stimulated with 10^{-7} M cAMP for 3 additional minutes. The graphs show the averages of two independent experiments. *A*, kinetics of receptor shift determined by immunoblotting. The *inset* shows one of the immunoblots used to generate the graph. The lanes contain samples taken at times 0, 0.17, 0.5, 1, 2, 5, 10, 15, and 20 min after stimulation. The final lane corresponding to the point labeled *C* on the graph is the sample prepared from cells stimulated with the saturating dose. *B*, kinetics of receptor phosphorylation. The *inset* shows the autoradiograph of immunoprecipitated $^{32}P_i$-labeled receptors taken at the same time points shown in *panel A*. The final lane is the sample prepared from cells stimulated with the saturating dose.

adaptation, responsiveness to subsaturating concentrations of stimuli is lost, but cells remain responsive to higher stimulus concentrations. Therefore, phosphorylation was examined at 5 nM cAMP, a concentration which produces an intermediate level of receptor shift and phosphorylation. Fig. 4*A* shows that at 5 nM cAMP, the transition occurs with a half-time of 1.5 min, reaches a steady state of 30% D form between 5 and 10 min, and remains at that level for up to 20 min. Increasing the cAMP concentration to 10^{-7} M for 3 min at that time resulted in an increase in D form to 55%. Phosphorylation also reached an intermediate steady-state level at 5 nM cAMP (Fig. 4*B*). This occurred with a half-time of 1.5 min, reached a plateau level of 60% maximum by 5 min, and remained constant for 15 additional min. Increasing the cAMP concentration to 10^{-7} M for 3 min at that time resulted in a further increase in receptor phosphorylation.

Turnover of Phosphates on Receptor—The rate of incorporation of phosphates into the pre-existing D form of receptor was investigated. Cells were treated with cAMP for 5 min to allow receptor phosphorylation to reach steady-state levels as shown in Fig. 1. $^{32}P_i$ was then added to the cells, and aliquots were removed for processing after 10, 20, and 40 min of total labeling time. The standard stimulus-induced rate of phosphorylation was determined in parallel by preincubating cells with $^{32}P_i$ for 5 min, adding cAMP, and removing aliquots at 10, 20, and 40 min after addition of $^{32}P_i$. The rate of R form phosphorylation was determined under the same conditions with the exception of the addition of cAMP. Data are expressed as the amount of phosphate in the D or R form relative to the maximum level of total phosphorylation in the stimulus-induced sample. Because the $^{32}P_i$ labeling times in this experiment are short, the data obtained reflect relative rates of phosphate incorporation into D or R forms under the various conditions. The differences observed between the samples are not due to ligand-induced variations in [^{32}P]ATP pools because cAMP does not affect [^{32}P]ATP pools over the duration of the stimulus used here (data not shown) (18).

Panel A of Fig. 5 shows the relative rates of $^{32}P_i$ incorporation into the D form induced by stimulus (*closed circles*) and when the receptor pre-exists in the D form (*open circles*). The results indicate that when receptor is held in the D form, the rate of phosphorylation is about 3 times less than the stimulus-induced rate of phosphorylation beginning with the R form. Fig. 5*B* shows that phosphorylation of the R form was linear with respect to time and was not affected by the addition of the other reagents ($^{32}P_i$, dithiothreitol, and caffeine). The rate of R form phosphorylation is about one-fifth the stimulus-induced rate and about one-half the rate of incorporation into pre-existing D form.

To assess the turnover of ligand-induced phosphorylation sites, R form phosphorylation should be subtracted from that

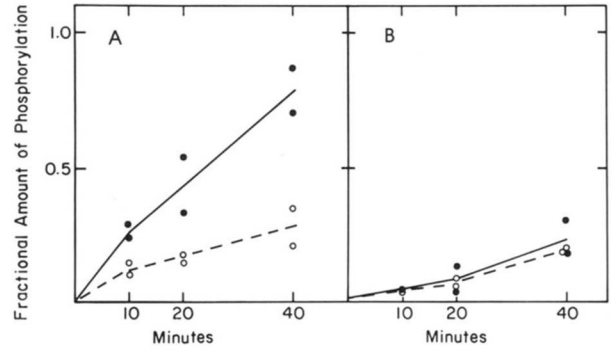

FIG. 5. **Turnover of phosphate on receptor.** *A*, cells were treated with caffeine for 10 min and labeled with $^{32}P_i$ for the times indicated. Control cells (●——●) were labeled with $^{32}P_i$ for 5 min before stimulation with 10^{-7} M cAMP while experimental cells (○——○) were stimulated with 10^{-7} McAMP for 5 min before labeling with $^{32}P_i$. Data points are expressed as percent of maximum control value and are obtained from two independent experiments. *B*, cells were treated with the same regimen as in *panel A* except that no cAMP was given. ●——●, control regimen; ○——○, experimental regimen.

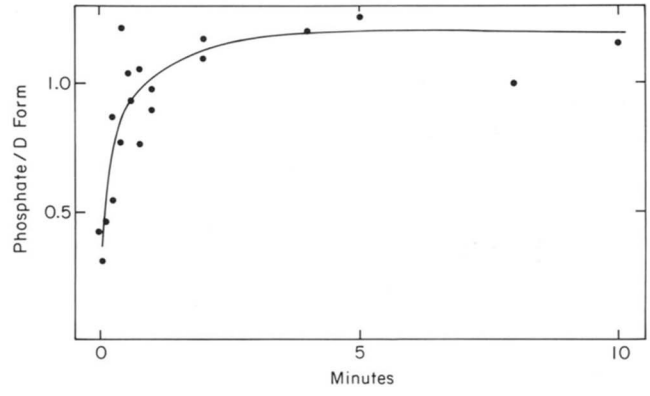

FIG. 6. **Specific activity of D form after cAMP stimulation.** The data shown in Fig. 1 were used to calculate the specific activity of $^{32}P_i$-labeled D form. The amount of $^{32}P_i$ in the D form at each time point (relative to 100% as described under "Materials and Methods") was determined by densitometric scanning. This amount (as a percent) was divided by the fraction of receptor in D form at that time point (also a percent) as determined by immunoblotting.

of the D form since it is assumed to proceed independently of the stimulus. When this correction is made, the incorporation rate of ligand-induced phosphorylation sites on pre-existing D form is approximately one-fifth the stimulus-induced rate of phosphate incorporation.

Specific Activity of D Form—The data from the experiments shown in Fig. 1 were used to estimate the changes in specific activity of receptor D form following cAMP stimulation. Specific activity is defined as the fractional amount of phosphorylation of the D form at each time point divided by the fraction of receptor protein in the D form at each time point (Fig. 6). At 5–15 s after addition of stimulus, the specific activity of the D form is about 40% of the maximum value. This value increases rapidly to about 80% of maximum by 1 min and reaches a plateau at the maximum level between 3 and 5 min. (The curve levels off above a value of 1.00 because the fractional value of phosphorylation of D form at the later time points was very close to 1.00, while receptor protein in the D form equilibrated at 0.80–0.90.)

DISCUSSION

Receptors isolated from resting cells migrate with an electrophoretic mobility on sodium dodecyl sulfate gels of M_r 40,000 and show ligand-independent incorporation of $^{32}P_i$ which is linear for at least 1 h. Stimulation of cells with cAMP results in a rapid and dramatic increase in $^{32}P_i$ incorporation into the receptor concomitant with a change in its electrophoretic mobility to M_r 43,000. The cAMP dependence of these processes is a result of receptor occupancy rather than a process activated by intracellular cAMP because phosphorylation and shift occur in the presence of caffeine which prevents activation of adenylate cyclase (19). Dephosphorylation of receptor was also highly sensitive to receptor occupancy, occurring rapidly when cAMP was removed. The cAMP receptor thus resembles rhodopsin and β-adrenergic receptors which are also phosphorylated and dephosphorylated in response to receptor occupancy (20).

Several lines of evidence support the hypothesis that cAMP-induced receptor phosphorylation is an early step leading to adaptation of one or more cAMP-stimulated events. The kinetics of phosphorylation and dephosphorylation are comparable with the kinetics of adaptation and deadaptation of several processes. Ligand-induced receptor phosphorylation is rapid, with detectable levels present at 5 s after stimulation and steady state being reached by 5 min. Adaptation of adenylate cyclase, myosin phosphorylation, and cell shape changes begin within seconds of application of chemoattractant and are completed within 3–5 min (5, 6, 15). The half-time for phosphorylation, 1.5 min, is also comparable to the half-times for adaptation of these processes (2–3 min). Dephosphorylation of receptor upon removal of cAMP had a half-time of 2 min and reached a plateau by 20 min, while adenylate cyclase deadapts with a half-time of 2–4 min and is completed at about 12 min (6, 7, 21). In addition, adaptation and deadaptation of these processes as well as phosphorylation and dephosphorylation of receptor occur in caffeine-treated cells (5, 22), indicating that none of these processes is dependent upon increases in intracellular cAMP.

The dose responses for these processes are very similar. Receptor phosphorylation was half-maximal at 5 nM cAMP and saturated at 100 nM cAMP. Many cAMP-induced processes are activated by nanomolar cAMP, including adenylate cyclase (K_d = 5 nM) and myosin phosphorylation (K_d = 5 nM), while desensitization of adenylate cyclase also occurs with a K_d of 5 nM (21).

The fractional amount of steady-state receptor phosphorylation is a function of receptor occupancy. At 5 nM cAMP, a concentration at which receptors are incompletely occupied, receptor phosphorylation reaches only a fraction of the maximal level. Steady state was achieved between 5 and 10 min after stimulation, and no further change in phosphorylation level occurred as long as the stimulus was held constant. A subsequent increase in the proportion of occupied receptors produced further receptor phosphorylation. Both adenylate cyclase and myosin phosphorylation respond to step increases in cAMP in the same manner, *i.e.* a subsaturating dose of cAMP produces a submaximal response (which in these cases then subsides), and a further increase in cAMP stimulus produces a further response (5, 7).

The kinetics of shift and phosphorylation also depended on the stimulus dose. At 5 nM cAMP, the $t_{1/2}$ for shift and phosphorylation were 2.5 and 1.5 min, respectively, compared to 30 and 45 s at 10^{-7} M. Receptor shift at 10^{-6} and 10^{-5} M cAMP was even more rapid than at 10^{-7} M (data not shown). This agrees with experiments showing that the rate of rise of adaptation is faster with higher doses of cAMP (6). In all of these respects, receptor phosphorylation characteristics are similar to properties ascribed to a theoretical adaptation mechanism proposed in several models (6, 23).

Receptor shift, analyzed by immunoblotting, was assessed in parallel with phosphorylation changes. The kinetics of both processes upon addition and removal of cAMP were similar but not identical, while the dose dependence of each was essentially the same. At subsaturating cAMP levels both receptor shift and phosphorylation were maintained at a fractional level until the stimulus concentration was increased. The two processes thus have very similar characteristics, but a causal relationship between the two remains to be unequivocably established.

Analysis of the specific activity of receptor phosphorylation revealed that the specific activity of the D form increased during the course of the experiment (Fig. 6). At early time points after stimulation the specific activity of the D form was 30–40% of the maximum value, and maximum levels of phosphorylation were not obtained until 3–5 min after stimulation. This result suggests that phosphorylation of receptor continues after the R to D transition is complete. We have estimated the molar ratio of phosphates to protein in the R and D forms to be 0.2 ± 0.1 and 4.0 ± 0.8 (14), suggesting that numerous sites on the D form are phosphorylated. It is possible that one or two sites are phosphorylated during the R to D transition, and the remainder is phosphorylated only after the receptor is in the D form. Experiments are in progress to identify the multiple ligand-dependent and -independent phosphorylation sites and to determine their relationship to the mobility shift and their contributions to the overall kinetic properties. It will be interesting to learn whether all, some, or one of the phosphorylation sites are involved in adaptation or if phosphorylation of different sites is involved with adaptation of different processes.

Incorporation of phosphate into receptor is most rapid during the R to D transition. Steady-state turnover of phosphate on the D form is estimated to be one-fifth to one-third the stimulus-induced rate. Nevertheless, the presence of detectable phosphate incorporation into the D form at steady state indicates that it undergoes cycles of dephosphorylation-rephosphorylation and that there is a phosphatase capable of dephosphorylating occupied receptors. It is not known whether or not this phosphatase activity is the same as the one which dephosphorylates receptor after removal of stimulus or if there is a stimulus-dependent phosphatase.

Several properties of ligand-induced phosphorylation of the

cAMP receptor from *D. discoideum* have been investigated. In all respects examined in this study, *i.e.* events induced by the addition of both saturating and subsaturating stimuli, events induced by the removal of stimulus, and changes as a function of stimulus dose, receptor modification resulting in electrophoretic mobility shift and receptor phosphorylation occurred with similar properties. No conditions have been found which dissociate receptor shift from phosphorylation, although a causal relationship between the two remains to be established. The similarities in kinetics and dose response between receptor phosphorylation and adaptation of adenylate cyclase and myosin heavy and light chain phosphorylation suggest that ligand-induced receptor phosphorylation is an early event in the causal mechanism of one or more of these processes.

Recent work has shown that transmembrane signal transduction processes in *Dictyostelium* are similar to those of vertebrate cells. The deduced amino acid sequence of the receptor shows significant homology to rhodopsin, and the structural model based on hydrophobicity analysis indicates that it is a member of the family of receptors with seven membrane-spanning domains.[2] The proposed cytoplasmic C terminus contains 18 serines and 9 threonines which are potential phosphorylation sites. Light-induced phosphorylation of rhodopsin occurs primarily on a cluster of serines and threonines near the carboxyl terminus (25), and the proposed cytoplasmic tail of the β-adrenergic receptor is also serine-threonine-rich (26). Ligand-induced phosphorylation of these receptors has also been correlated with functional adaptation (16). In addition to these structural and functional similarities, activation of effector enzymes in *Dictyostelium* by extracellular cAMP occurs through the action of a G-protein (3, 24). These properties in conjunction with the ease of mutant isolation and the recent advent of transformation by homologous recombination make *Dictyostelium* a powerful model system for the investigation of signal transduction processes.

Acknowledgments—We thank Dr. Peter Van Haastert for helpful discussions and suggestions during portions of this work, Drs. Peter Klein, Maureen Pupillo, and Robert Gundersen for reading the manuscript, and Peggy Ford for excellent secretarial assistance.

[2] P. Klein, C. L. Saxe, III, T. J. Sun, A. R. Kimmel, and P. N. Devreotes, submitted for publication.

REFERENCES

1. Tomchik, K., and Devreotes, P. (1981) *Science* **212,** 443–446
2. Konijn, T. (1970) *Experientia* **26,** 367–369
3. Theibert, A., and Devreotes, P. (1986) *J. Biol. Chem.* **261,** 15121–15125
4. Mato, J., Krens, F., Van Haastert, P., and Konijn, T. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74,** 2348–2351
5. Berlot, C., Spudich, J., and Devreotes, P. (1985) *Cell* **43,** 307–314
6. Dinauer, M., Steck, T., and Devreotes, P. (1980) *J. Cell Biol.* **86,** 554–561
7. Dinauer, M., Steck, T., and Devreotes, P. (1980) *J. Cell Biol.* **86,** 545–553
8. Devreotes, P. (1982) in *The Development of Dictyostelium discoideum* (Loomis, W. F., ed) pp. 117–168, Academic Press, Orlando, FL
9. Juliani, M. H., and Klein, C. (1981) *J. Biol. Chem.* **256,** 613–619
10. Theibert, A., Klein, P., and Devreotes, P. (1984) *J. Biol. Chem.* **259,** 12318–12321
11. Klein, P., Knox, B., Borleis, J., and Devreotes, P. (1987) *J. Biol. Chem.* **262,** 352–357
12. Klein, P., Theibert, A., Fontana, D., and Devreotes, P. (1985) *J. Biol. Chem.* **260,** 1757–1763
13. Devreotes, P., and Sherring, J. (1985) *J. Biol. Chem.* **260,** 6378–6384
14. Klein, P., Vaughan, R., Borleis, J., and Devreotes, P. (1987) *J. Biol. Chem.* **262,** 358–364
15. Klein, P., Fontana, D., Knox, B., Theibert, A., and Devreotes, P. (1985) *Cold Spring Harbor Symp. Quant. Biol.* **50,** 787–799
16. Ullrich, A., Bell, J., Chen, E., Herrera, R., Petruzzelli, T., Dull, T., Gray, A., Coussens, L., Liao, Y-C., Tsubokawa, M., Mason, P., Seberg, P., Grunfeld, G., Rosen, O., and Ramachandran, J. (1985) *Nature* **313,** 756–761
17. Sibley, D., and Lefkowitz, R. (1985) *Nature* **317,** 124–129
18. Berlot, C. (1987) *Methods Cell Biol.* **28,** 333–345
19. Brenner, M., and Thoms, S. (1984) *Dev. Biol.* **101,** 136–146
20. Benovic, J. L., Strasser, R. H., Caron, M. G., and Lefkowitz, R. J. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 2797–2801
21. Van Haastert, P. (1987) *J. Biol. Chem.* **262,** 7700–7704
22. Theibert, A., and Devreotes, P. N. (1983) *J. Cell Biol.* **97,** 173–177
23. Knox, B., Devreotes, P. N., Goldbeter, A., and Segel, L. A. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 2345–2349
24. Van Haastert, P. J. M., Snaar-Jagalska, B. E., and Janssens, P. M. W. (1987) *Eur. J. Biochem.* **162,** 251–258
25. Thompson, P., and Findlay, J. B. C. (1984) *Biochem. J.* **220,** 773–780
26. Dixon, R. A. F., Kobilka, B. K., Strader, D. J., Benovic, J. L., Dohlman, H. G., Frielle, T., Bolanowski, M. A., Bennet, C. D., Rands, E., Diehl, R. E., Mumford, R. A., Slater, E. E., Sigal, I. S., Caron, M. G., Lefkowitz, R. J., and Strader, C. D. (1986) *Nature* **321,** 75–79